

Collective Mechanics of Dynein Driven Microtubule (MT) Transport, Oscillations and Bead Motility

डायनिन द्वारा माइक्रोट्यूबूल का समुहिक परिवहन, प्रदोलन
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CERTIFICATE

Certified that the work incorporated in the thesis entitled (" Collective Mechanics of Dynein Driven Microtubule (MT) Transport, Oscillations and Bead Motility ")'

Submitted by Shivani Anand Yadav was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.



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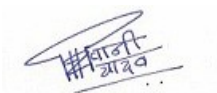
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I declare that this written submission represents my idea in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

The work reported in this thesis is the original work done by me under the guidance of Dr./ Prof. Chaitanya A. Athale

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Signature of the student

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Abstract

Dynein is a crucial ATP-driven motor protein involved in processes like spindle assembly, vesicle transport, organelle positioning, and ciliary beating. This study explores dynein's collective behavior in microtubule (MT) transport, oscillations, and organelle motility.

In *Saccharomyces cerevisiae*, mitosis is closed and requires dynein to asymmetrically position the nucleus by pulling MTs nucleated from the spindle pole body (SPB). Image analysis of MT-cortex contacts during mitosis showed that the bud cell's MTs had more frequent and longer contacts with the bud cortex than the mother cell, suggesting a role for dynein in nuclear positioning. Previous *in vitro* studies with full length cytoplasmic dynein indicated that increasing the number of dynein enhances the speed of MT, but the mechanisms behind the motor cooperativity in truncated motors remains to be explored. In this study, gliding assays with truncated yeast dynein at varying motor densities were reconstituted revealing that even minimal motor numbers influenced MT velocity. The MT velocity increases and saturates rapidly as the motor number increases prompting new models to explain motor cooperativity on the basis of single molecular behavior of the motor.

Unusual MT patterns, including oscillations and spirals, have been observed in gliding assays. Single MT oscillations were reconstituted in modified assays by clamping the MT plus ends in a dynein gliding assay. The oscillations showed dependence on MT length and motor density, highlighting the role of geometry, MT buckling, and

collective motor activity in emergence of single MT oscillations. This minimal *in vitro* set up can be used in future to study the role of MT-motor mechanics in flagellar beating. In assays with pinned MTs, MT spirals were studied, revealing that spiral radius scaled differently from acto-myosin systems. The scaling of spiral radius with the MT length was explained on the basis of length dependent persistence model of the MT. This is suggestive of anisotropic nature of MT.

This work also explored cargo transport through *in vitro* assays with purified motors and synthetic cargo. This set up can be extended to study bidirectional transport with synthetic cargoes or organelles. Collaborative efforts, taxol-stabilized MTs were tested as marker for rotational movement.

Overall, these findings reveal self-organized transport dynamics arising from the collective properties of dynein, providing a deeper understanding of motor-motor and MT-motor coordination in various cellular processes.

Synopsis

Introduction

Dyneins and kinesins are mechanochemical enzymes that convert the energy from ATP hydrolysis to mechanochemical energy as demonstrated by their movement on microtubules (MTs) [5]. Dyneins are generally minus end directed while kinesins are generally plus end directed. Dynein possesses an ATPase domain whose structure belongs to the AAA+ super-family of ATPases [104]. Cytoplasmic dyneins are two headed processive minus end directed motors which exist *in vivo* as a complex of 2 heavy chains along with several light chains and intermediate chains (IC) [133]. This motor is vital for spindle assembly [54, 95], generating cortical pulling forces acting on spindle thus determining position and movement of spindle [22, 92], retrograde trafficking of vesicles and organelles (reviewed in [123]) and positioning of organelles by cortical and cytoplasmic force generation as seen for nuclear positioning in budding and fission yeast respectively [4, 16].

Molecular motors have been studied with respect to their single molecule as well as collective properties. Both dynein and kinesin have been studied at single molecule level with respect to their velocity, run length, stepping behavior and force generation using either single molecule TIRF [120] or optical traps [148]. Since *in vivo* processes usually involve the activity of teams of motor, collective transport properties of motors are equally important as single molecule analysis. Collective behavior of high number

of motors is also involved regulating flagellar mechanics by flagellar dyneins. Both dynein and kinesin have been studied with respect to their collective transport properties by using some commonly used techniques such as (i) gliding assays where MTs are transported due to the stepping of glass immobilized motors and (ii) *in vitro* reconstitution of cargo transport as organelle transport by motors on immobilized MTs. Number dependence of motors have been studied for both dynein and kinesin using *in vitro* assays where the transport properties of the cargo changes as the motor number changes. Kinesins do not show number dependence of MT velocity in a gliding assay based set up where surface motor density of kinesin was varied [11]. Collective behavior of 1 to 7 dynein motors has been studied with the help of DNA origami based cargoes [25] where MT velocity was unaffected by the motor number (<7). However studies on full length human dynein complex and minimal yeast cytoplasmic dynein in gliding assay based studies (motor number 1 to 60) showed that the velocity of MT transport was seen to be increasing above a threshold number of dyneins transporting the MT and then saturating [65, 100]. The mechanisms by which motors are able to cooperate collective transport can be explored in details using controlled *in vitro* assays.

In the following sections collective transport properties of dynein were explored with respect to (a) *in vivo* nuclear positioning in budding yeast, (b) *in vitro* MT transport, (c) generation of single MT oscillations and (d) driving single MT spiraling and some preliminary work involving (e) organelle transport. In addition preliminary work by using MTs as a marker of rotational motion is also described in the appendix. Together, I believe these studies have helped gain insights into the role of collective dynamics in various *in vivo* and *in vitro* processes and improved our understanding of the dynein-dynein and dynein-MT mechanics better.

Dynein collective behavior in mitotic nuclear positioning of *Saccharomyces cerevisiae*¹²

During the closed mitosis of *S. cerevisiae* cortically anchored dynein generates pulling forces on the MTs which are attached to the nucleus. These pulling forces are known to position the nucleus at the bud neck which is considered important for a successful mitosis [4, 16]. The directional movement of nucleus towards the bud cell is asymmetric in nature i.e. the net movement is not at the geometric center of the system hence indicating that the net force experienced by the nucleus is asymmetric. Such asymmetry can be hypothesized to be achieved by non-uniform distribution of force generators with presence of more dynein clusters on daughter cortex. However studies of dynein anchoring protein Num1 shows presence of more Num1 clusters on mother cell cortex [107]. Hence factors other than the distribution of dynein could be responsible for driving such asymmetric positioning of the nucleus.

In order to study the underlying mechanism by which the MT-dynein contacts are optimized to cause the net movement towards the bud cortex, image analysis was performed on confocal microscopy dataset of yeast GFP-Tub1 (recorded by Dr. S. Palani). During the mitosis where the spindle and MTs can be visualized, the spindle dynamics and lateral cortical interactions were quantified manually. The spindle elongation was used as marker for identifying pre-anaphase and anaphase. The number of laterally interacting MTs as well as the lengths of interaction were measured in both the mother cell as well as bud cell cortex over the course of mitosis and were categorized into pre-anaphase and anaphase. It was observed that more number of MTs interact with the bud cell cortex as compared to the mother cell cortex during pre-anaphase as well as anaphase. The number of MT contacts were higher during anaphase. The increased

¹Yeast movies recorded by Dr. Saravanan Palani, University of Warwick.

²This chapter is published as a part of Jain *et al* 2021.

and longer contacts made by MTs on the bud cortex are suggestive of a mechanism where more motors are engaged on the bud cortex enabling asymmetric positioning of the nucleus. This helped us gain insights on the *in vivo* regulation of MT-cortical interactions that drive the nuclear positioning and the sufficient MT lengths required for nuclear positioning in pre-anaphase.

Cooperativity in motor number dependence of MT transport velocity for dynein

In *in vitro* cargo transport based assays, dynein has been observed not to show number dependence of velocity for up to 7 motors [25]. However in a gliding assay based dynein studies the velocity of MTs was observed to increase as the motor number increases [65, 100] which is unlike kinesin where the motor density did not show any effect on the MT velocity [56]. Previously, motor cooperativity has been explained on the basis of mechanical activation of full length dynein however minimal constructs which are unable to undergo mechanical activation are not studied in details. Studying collective behavior of such minimal dynein can help us gain insights into mechanisms of motor cooperativity other than linker length and mechanical activation.

To systematically study the dependence of MT transport properties on motor number and MT length, I have performed dynein gliding assays at increasing dynein densities with specific immobilization of motors via anti-GFP nanobody. With a microscopy based motor density estimation using the GFP intensity as the measure of motor density we can now study systematic effect of motor number and MT length on the MT transport properties. It was observed that both MT length and motor density affect the nature of transport of MTs. The velocity was observed to saturate rapidly as the motor number increases. By comparing the observations with existing models we propose the need of other simplified models that might be able to explain the phenomenon

of motor coordination. This has helped us gain insights into understanding the motor cooperativity across a range of motor numbers for a minimal motor.

Wave-like oscillations of clamped microtubules driven by collective dynein transport. ³ ⁴

Flagellar beating is a complex phenomenon arising from MT-motor interactions. Pinned single MTs as well as bundled MTs have been shown to perform flagella-like beating *in vitro* due to kinesin [161, 128, 50, 129]. However these studies have lacked specificity of MT immobilization, control over MT length and density and used kinesin. Theoretical studies have shown that clamped active filaments will undergo beating patterns under sufficient force and the filament length, driving force and nature of pinning will jointly determine the nature of these patterns [19]. In order to test these predictions and to better understand the mechanics of flagellar dynamics, an *in vitro* system with dynein and MTs mimicking beating could be a useful model.

Here, we report a minimal MT-motor system that drives single MT oscillations *in vitro* [169]. A modified gliding assay with yeast cytoplasmic dynein was developed where the MT plus end was clamped to the surface using biotin-streptavidin chemistry. We find, with the addition of ATP, this system begins to oscillate. The patterns observed can be classified into three types: (a) fluctuations, (b) beating and (c) looping, based on an order parameter which captures the relative space explored by the tip in X and Y. We show that MT length, motor density and the extent of MT pinning appear to jointly determine the exact nature of the patterns observed. We believe this minimal system could in future be used to further test the role of motor and MT mechanics and geometry in flagellar beating.

³Data analysis tool and frequency analysis done by Dhruv Khatri, Simulations performed by Neha Khetan and Aman Soni

⁴This chapter has been published as Yadav *et al* 2024.

Scaling of dynein-driven pinned microtubule spiraling explained by length dependent persistence length

5

In vitro gliding assay with actin and myosin have been reported to show generation of spirals by actin when the filament encounters surface defects [12]. The radius of actin spirals was seen to be inversely proportional to the force per unit length, dependent on the persistence length but independent of the filament length. These spirals have been used to estimate collective motor forces and provide insights into properties of motor-filament interactions. An *in vitro* system with MTs and dynein can be used to study the parameters affecting the formation of spirals and study MT-motor interactions.

Here I have reconstituted dynein driven single MT spirals by labeling the plus ends of MTs with biotin which in a gliding assay like set up binds to streptavidin coated surface and causes pinning of MT plus ends. We observed that dynein can drive MT spiraling at sufficient motor densities and point pinning of MTs. The scaling of radius of the MT spirals with motor densities was observed to follow a similar trend as that of myosin and kinesin but length dependence of radius was observed which has not been reported for myosin and kinesin. The spiral radius and frequency was estimated and their scaling with motor density and MT length was studied. By comparing the scaling of spiral radius and frequency to two models of MT elasticity, MT spirals were used to test models of MT elasticity. By the comparison to the models it was found that length dependent L_p explains the observed scaling, suggestive of anisotropic nature of MTs.

⁵Simulations performed by Mr. Aman Soni.

***In vitro* reconstitution of dynein-kinesin tug-of-war**

6

While collective transport using MTs was explored in previous sections, the *in vivo* transport mainly involves team of vesicle transport by team of motors. The examples of such transport are transport of golgi, ER, peroxisomes, Endolysosomes, Autophagosomes, Melanosomes, Mitochondria and lipid droplets by cytoplasmic dynein (reviewed in [119]. Transport properties of cargoes can be seen to vary with varying motor number [115]. Dynein kinesin tug of war is known to be involved in bidirectional transport of vesicles and organelles. Isolated golgi has been reported to move along the fixed MTs towards it plus ends in a preliminary *in vitro* assay confirming the role of kinesin [35, 25]. *In vivo* studies show that dynein and kinesin play a role in adhesion dependent organization of Golgi [145] suggesting a role of dynein-kinesin tug of war in Golgi organization. An *in vitro* set up with immobilized MTs and motor coated beads can help us understand number dependent motor transport as well as effects of opposing motors transporting the same cargo. Such set up can also be extended to study organelle transport properties of purified organelles *in vitro* such as Golgi.

To make an *in vitro* set up for reconstituting bead and organelle transport assay in a preliminary work a set up with purified motors and MTs was developed. A flow chamber based system with fixed Taxol stabilized rhodamine labeled MTs was prepared. To enable coating of motors on a synthetic cargo such as a polystyrene or magnetic bead, biotinylated anti-GFP nanobody was prepared to enable binding of any GFP tagged protein to streptavidin coated beads. The coating of beads with motors can be confirmed by presence of green fluorescence, both dynein and kinesin were successfully attached to the bead using this set up. Such motor coated beads

⁶In collaboration with Dr. Nagaraj Balasubramanian, Golgi and cytosol Purified by Antara Chakraborty.

were able to land and remain bound to fixed MTs and show diffusive movement upon addition of ATP. This set up was extended to reconstitute organelle gliding assay of Golgi in a preliminary assay.

MT rotors to measure the activity of DNA dependent GTPase ⁷

Previously rotational movements in F1 ATPase has been confirmed using actin as the marker^[174]. To test the possible rotational movement of a DNA dependent GTPase motor an assay with MT as the marker for rotational movement can be helpful.

In a preliminary work, in order to study the rotational ability of the DNA motor I have developed an *in vitro* set up with biotinylated MTs as the marker for rotational motion. In preliminary experiments I have been able to immobilize DNA motor complex to a glass surface using anti-His antibodies and binding of biotin MTs to the biotinylated DNA motor via streptavidin. This complex has demonstrated point pinning of biotin MTs via the complex and the ability of MTs to show rotation degree of freedom. The MTs perform incomplete back and forth rotational motion in presence of GTP, GMPPNP and in absence of GTP as well. Hence we show that MTs can be used as a marker for rotational studies of motors, however more studies should be done to confirm the rotational motion.

⁷In collaboration with Dr. Saikrishnan Kayarat, DNA enzyme modification and Purification by Aman Yadav.

Publications

Peer reviewed publications⁸

1. **Shivani A. Yadav***, Dhruv Khatri, Aman Soni, Neha Khetan and Chaitanya A. Athale (2024), Wave-like oscillations of clamped microtubules driven by collective dynein transport. Biophysical Journal. 10.1016/j.bpj.2024.01.016
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3. Kunalika Jain, Neha Khetan , **Shivani A. Yadav**, Saravanan Palani, Chaitanya A. Athale (2021) Dynein collective behavior in mitotic nuclear positioning of *Saccharomyces cerevisiae*. Yeast. doi.org/10.1002/yea.3552.
4. Kunalika Jain, **Shivani A. Yadav** and Chaitanya A. Athale (2021) Number Dependence of Microtubule Collective Transport by Kinesin and Dynein. J. Indian Inst. Sci. <https://doi.org/10.1007/s41745-020-00212-2>.
5. Dhruv Khatri, Tanvi Kale, Jashaswi Basu, **Shivani A. Yadav**, Chaitanya A. Athale. "Quantification of cell shape, intracellular flows and transport based on DIC object detection and tracking." Journal of Microscopy (2024).

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In preparation

1. **Shivani A. Yadav***, Aman Soni* and Chaitanya A. Athale. Scaling of dynein-driven pinned microtubule spiraling explained by variable persistence length. (* Joint first author)
2. **Shivani A. Yadav*** and Chaitanya A. Athale. Cooperativity in motor number dependence of MT transport velocity for dynein.

Conference abstracts

1. Self-organized spatio-temporal wave-like oscillations of clamped microtubules by dynein collective transport. Shivani A. Yadav* and Chaitanya A. Athale. NAITO foundation 49th annual meeting on "Microtubules and its related Motors" Saporro, Japan.
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3. Flagella-like beating of microtubules reconstituted *in vitro* with dynein and pinned filaments. Shivani A. Yadav* and Chaitanya A. Athale. Annual meeting Biophysical Society 2022. 10.1016/j.bpj.2021.11.1916.
4. MT motor gliding with pinning sites: Minimal flagellar beating model, **Shivani A. Yadav*** and Chaitanya A. Athale Dynein Meeting 2021.

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⁹Experiments performed by Dr. Saravanan Palani, University of Warwick.

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¹⁰Data analysis tool and frequency analysis done by Dhruv Khatri

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¹¹Sweep range parameter conceptualized by Dr. Neha Khetan to classify data from simulations

¹²Simulations performed by Dr. Neha Khetan and Mr. Aman Soni.

¹³Simulations performed by Mr. Aman Soni.

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¹⁴Golgi assays in collaboration with Dr. Nagaraj Balasubramanian, Golgi and cytosol Purified by Antara Chakraborty.

¹⁵In collaboration with Dr. Saikrishnan Kayarat and Mr. Aman Kumar Yadav, DNA enzyme modification and Purification by Aman Yadav.

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Chapter 1

Introduction

Molecular motors are molecular structures which can convert one form of energy into mechanical energy. Biological molecular motors include motor proteins which can use chemical energy such as from ATP hydrolysis to perform mechanical work [5]. One of the most important categories of motor proteins is associated with cytoskeleton of the cell. These proteins are dynein, kinesin and myosin. Dynein and kinesin are associated with microtubules while myosin is associated with actin [5].

Microtubules are dynamic polymers formed from tubulin subunits [32, 64, 149]. It has a plus and a minus end. Plus end is characterized by higher rate of tubulin polymerization than minus end [164]. Due to polarity of microtubules motors can bind to microtubules in one orientation and move in one direction upon ATP hydrolysis [122]. Kinesin is a plus end directed motor protein i.e. it can walk towards the plus end of microtubule while dynein is minus end directed motor protein [135, 23]. In the following sections I will focus on specific aspects of dynein, the minus end directed motor, its properties and role in cellular processes.

Dynein: An overview

Dynein is minus end directed motor protein associated with microtubules. It was discovered in cilia of *Tetrahymena pyriformis* [42]. Shortly after discovery of flagellar dynein, its cytoplasmic form was identified in brain [110]. It was shown to have minus end directed motility by observing direction of motion on of organelles obtained from high speed centrifugation of cytoplasmic extract, on microtubules [135].

1.1 Dynein structure and classification:

Dynein is an ATPase which belongs to the AAA+ superfamily of ATPases. These ATPases are mainly involved in unfolding of DNA, RNA and Protein Complexes [104]. While atomic structures of cytoplasmic dyneins are available, the axonemal dynein structure is less explored [62]. However due to high similarity in the amino acid sequence of both dyneins the description of dynein molecular architecture of dynein is often generalized [62]. Dynein has the characteristic hexameric ring of the ATPase domains as its core (Figure 1.1 A). The dynein heavy chain contains a stalk domain, a linker domain and the N terminal domain along with the core ATPase domain [14]. The stalk emerges between 4th and 5th ATPase domain as a coiled- coil domain with a Microtubule Binding Domain (MTBD) on the tip. MTBD interacts with the microtubule [38, 48, 77]. Amongst the six ATPase domains only 4 can bind to ATP actively [41, 76, 121] while an important role in dynein's motor activity is played by the ATPase domains 1 [98] and 3 [121, 143]. The conformational changes induced by ATP binding and cleavage shift the relative position of tail domain to the ATPase head domain. This change in position drives the power stroke of dynein [14, 75].

Dyneins are broadly classified as cytoplasmic dynein and ciliary/flagellar dyneins [167] (Figure 1.1 B). The dynein heavy chain family is classified further into 9 classes out of which 7 are associated with axoneme. They are further classified as Outer Arm

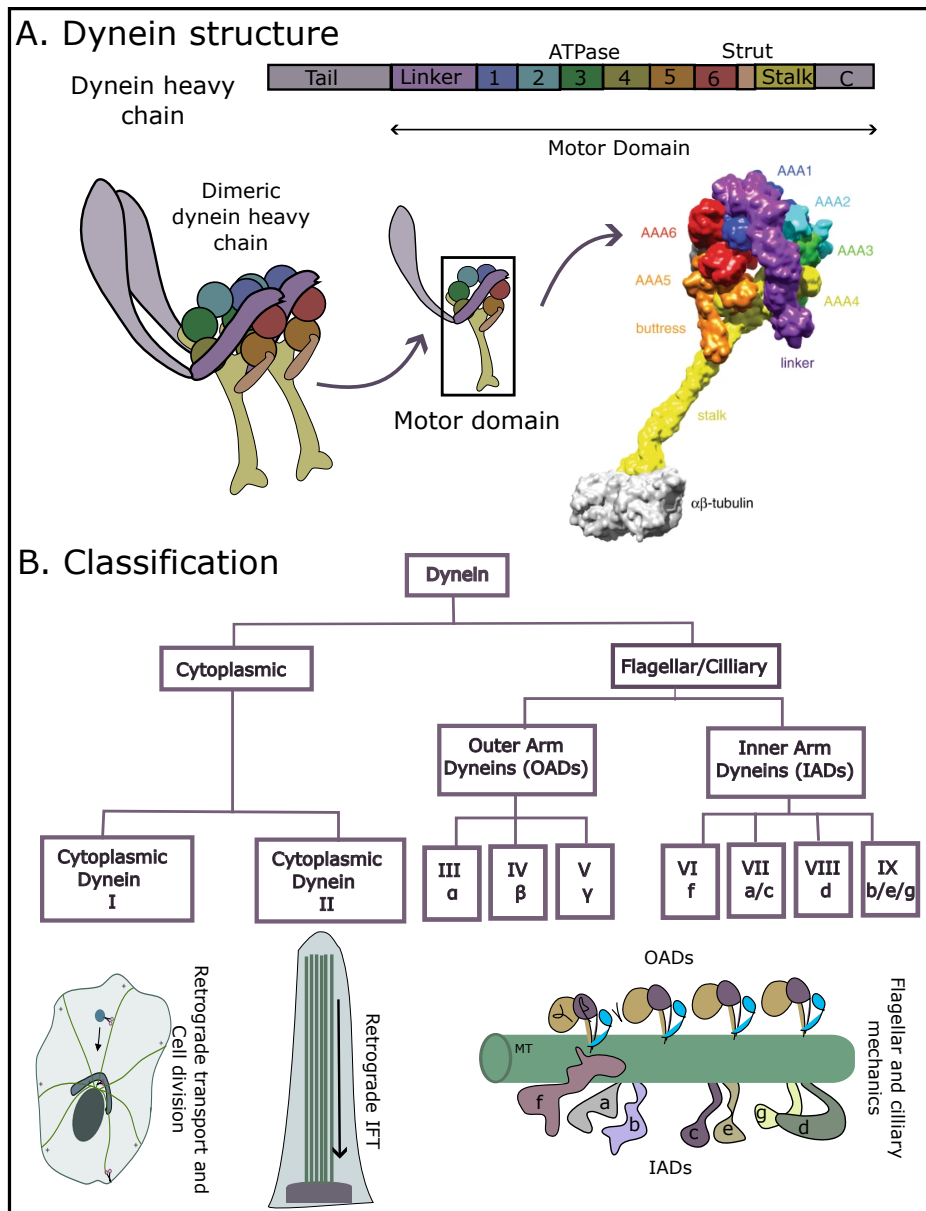


Figure 1.1: **Dynein structure and classification** (A) Dynein heavy chain has a motor domain comprising of 6 ATPase domains, linker and a Microtubule binding domain (MTBD). The linker connects the motor domain to a tail. Tail is responsible for dimerisation and binding to co-factors. The motor domain comprises of the ATPase domains arranged to form a ring like structure. MTBD emerges between 4th and 5th ATPase domains. The structural rendering of dynein motor domain bound to a tubulin dimer was taken from [118]. It was generated by combining *D. discoideum* dynein motor domain (PDB;3VKG) with mouse MTBD (PDB;3J1T). (B) Dynein is classified mainly into cytoplasmic dynein and flagellar dynein. The dynein heavy chain is further classified into 9 types. Dynein 1 is cytoplasmic dynein responsible for retrograde transport in the cell. Dynein II is cytoplasmic dynein involved in retrograde Intra Flagellar Transport. Dynein III, IV and V form OADs in the flagella while VI to IX form the IADs.

Dyneins (OAD) and Inner Arm Dyneins (IAD). The axonemal dyneins are exclusively involved in generating and regulating flagellar and ciliary beating. Amongst the remaining 2 classes of dynein heavy chains the cytoplasm while cytoplasmic heavy chain 2 is involved in Intra Flagellar Transport [167]. The cytoplasmic dynein heavy chain 1 is involved in the entire minus end directed transport. Due to its role in important cellular processes cytoplasmic dynein has been studied widely and it has become a model dynein, it is better studied at a single molecule level, therefore serving as an attractive system for collective studies etc. We will focus on the cytoplasmic heavy chain 1 (henceforth referred to as the cytoplasmic dynein) in the following sections.

Cytoplasmic dynein:

Cytoplasmic dyneins are two headed processive minus end directed motors as widely studied in *Dictyostellium* [88] and budding yeast [120]. They exist as a complex of 2 heavy chains along with several light chains and intermediate chains (IC) [133].

1.2 Functions of cytoplasmic Dynein:

Cytoplasmic dynein is involved in multiple cellular processes such as nuclear positioning, mitosis, spindle positioning, nuclear migration, spindle assembly, intra-cellular transport, organelle transport and positioning as described in detail below.

1.2.1 Spindle assembly:

Dynein helps in assembling the spindle which successively helps in chromosome segregation (Figure 1.2 A). Dynein is said to slide apart anti-parallel MTs and help in focusing minus ends of microtubules towards the spindle pole [54, 95]. Dynein also resides on the kinetochore and aids a rapid movement of chromosomes towards the pole [173]. It

also plays an important role by delaying anaphase until the chromosomes are properly attached [34, 1]. Cell division also uses pulling forces generated by dynein on the microtubule asters [22, 92].

1.2.2 Transport of cargoes:

Dynein is involved in vesicle trafficking from ER to Golgi, transport of mitochondria, phagosomes, endosomes, lipid droplets, peroxisomes, lysosomes towards the minus end of the microtubule (reviewed in [123]) (Figure 1.2 B). Certain viruses make use of dynein based transport to migrate towards the nucleus [27]. Dynein tethers various organelles such as nucleus and Golgi to microtubule network. It also helps in positioning Golgi near nucleus during inter-phase [24].

1.2.3 Organelle positioning by dynein:

Pulling forces are generated by dynein anchored to cortices of cells during both inter-phase and mitosis. These pulling forces are generated either by coupling to de-polymerizing plus end of microtubule through end-on contacts [80] or by walking towards the minus end via lateral contact [4] (Figure 1.2 B). Such pulling forces have several roles in cellular processes such as: (a) pulling of microtubules and nuclei towards the leading edge of migrating fibroblasts [28, 157], (b) MT sorting during neuronal axon development [46], (c) spindle positioning by dynein pulling forces [22], (d) oscillatory or directed movements of spindle or nuclei as seen in nuclear oscillations in *S. pombe* [152, 172], (e) mitotic nuclear positioning to the bud-neck during *Saccharomyces cerevisiae* mitosis [4] and (f) migration of the male pro nucleus towards the female pronucleus mediated by dynein dependent microtubule pulling in *C. elegans* oocytes during fertilization [71].

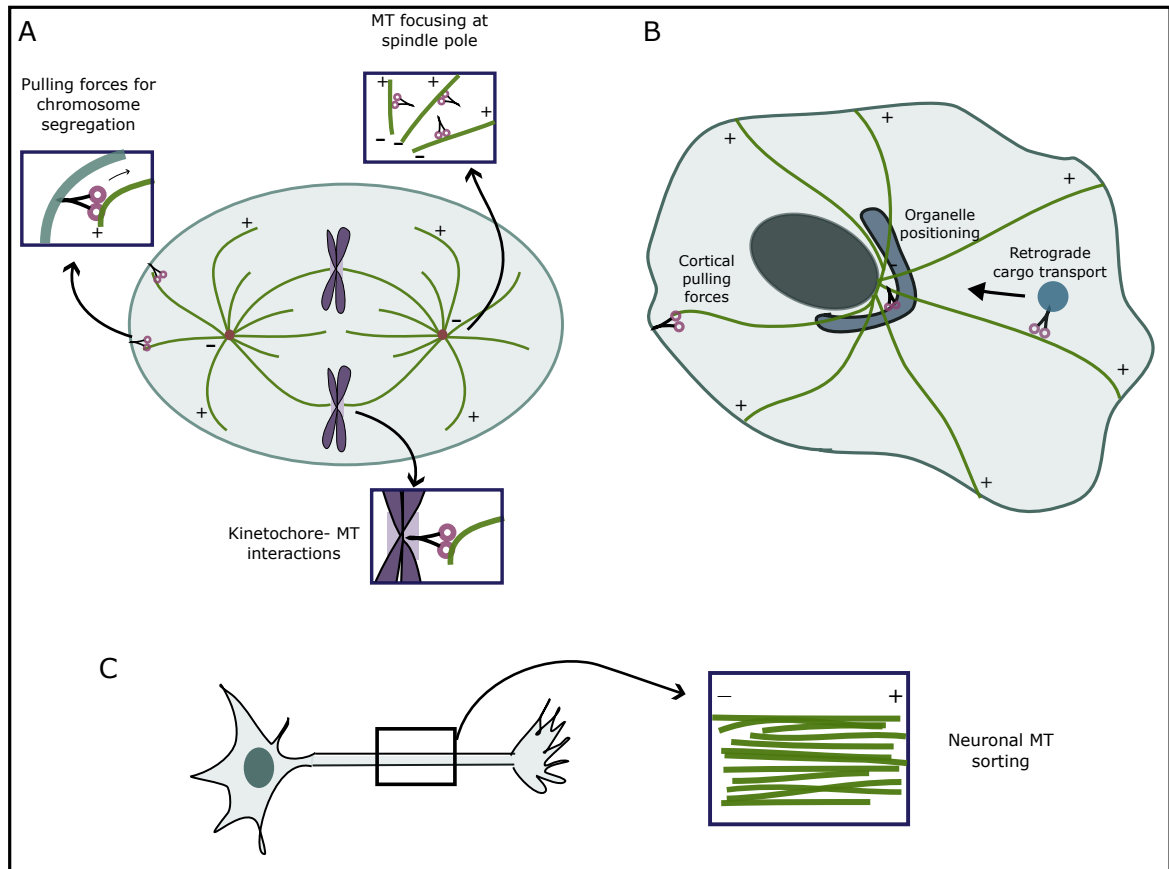


Figure 1.2: **Functions of cytoplasmic dynein** (A) Spindle assembly and chromosome segregation. Dynein participates in the assembly of spindles by focusing the MTs at the spindle pole. Cortical dynein generates pulling forces on the spindle by capturing and walking on astral MTs. Dynein situated at the kinetochores participates in chromosome segregation by capturing and attaching the MTs. (B) Dynein is involved in the entire retrograde transport in the cell. Dynein carries retrograde transport of vesicle, endosomes, organelles etc. Cortical dynein positions organelles by generating pulling forces on MTs attached to organelles such as nucleus. Dynein along with opposing motors is responsible to maintain position of organelles such as Golgi across the cytoplasm. (C) Dynein and kinesin organize MT bundles in neuronal axons.

1.2.4 Neuronal MT sorting:

Axons of neurons have polar arrangement of MT where the MT plus ends are facing away from the cell body while the dendrites have a mixed polarity of MTs [9]. This polar organization of MTs is essential for directed transport of cargoes along the axon. The mechanism by which the polar arrays of MTs is generated is called polarity sorting of MTs. Dynein and kinesin are known to play important role in the polarity sorting of axonal MTs [117, 93] (Figure 1.2 C).

1.3 Single molecular properties of Motors

Due to its role in various cellular processes dynein and its regulation has been studied widely. Motors have been studied at single molecular level as well as collectively as *in vivo* processes includes transport by a team of motors. Single molecule properties of motors includes velocity, run length and stepping behavior associated with their movement (Figure 1.3). Motors are also capable of generating force as they step on filaments which can be studied using optical traps (Figure 1.3). Due to the structural complexity of dynein, studying the single molecular properties has been challenging [106]. The mechanisms of movement of kinesin-1 and myosin V have been well studied. Both of them show a characteristic hand over hand motion with the help of conformational changes driven by nucleotide binding [159, 175]. The single molecular properties of motors can be studied either by using single molecular imaging such TIRF to directly observe the movement of the motor on a fixed MT or by attaching a motor to a bead and observing the movement of bead as moves along the MT (Figure 1.3).

While monomeric dynein processivity has been reported only for axonemal inner arm dynein C in a gliding assay based setup [125], processive single molecule motion is often seen for dimeric motors [120, 114]. Dimeric motors processively walk along the microtubule asynchronously without inter-head coordination when motor heads

are close to each other [26, 114]. The coordinated stepping important for collective behavior can be seen when inter head separation is increased by release of dynein from the microtubule caused by tension on the linker [21]. For single molecular analysis, dynein from various sources has been purified mainly bovine, murine, *Dictyostelium* and yeast cytoplasmic dynein. At room temperature and excess ATP concentration (1 mM) dynein typically moves with a velocity between 600 to 1000 nm/s. Bovine dynein typically shows run length of 700 nm which increases to 1500 nm in presence of dynactin [72, 89]. Yeast cytoplasmic dynein is slower compared to bovine dynein moving at only 50-80 nm/s [120]. Dynein is able to take steps of varying step sizes such as 8, 16, 24, 32 and 40nm [88]. Measuring stall force of dyneins has been possible due to optical trapping experiments [148]. Stall force of 1.1-1.2 pN has been observed for bovine brain dynein [160] and *Dictyostelium* dynein [147]. Yeast dynein is seen to be stalling at 5-7 pN [39].

1.3.1 Yeast cytoplasmic dynein

One of the most widely studied dynein is yeast cytoplasmic dynein. Budding yeast uses actin cytoskeleton for intracellular organelle and vesicle trafficking unlike other organisms which use microtubule system [102]. Thus, the only known role of cytoplasmic dynein in budding yeast is during mitosis [31]. The stall force of yeast dynein is 4-7 pN and the speed is 50-80 nm/s [120]. The reduced speed and increased stall force of yeast dynein as compared to other dyneins can be explained on the basis of requirement of yeast dynein for moving heavier cargoes such as nucleus during cell division. Full length as well as truncated dynein heavy chain has been shown to be processive without the need of light and intermediate chains [120]. A synthetically dimerized minimal construct of dynein, GST-Dyn1331kD has been described to show single as well as collective velocity similar to that of the full length dynein. Hence this minimal construct due to its well studied single molecular behavior makes an ideal model to

Single molecular properties

Collective transport properties

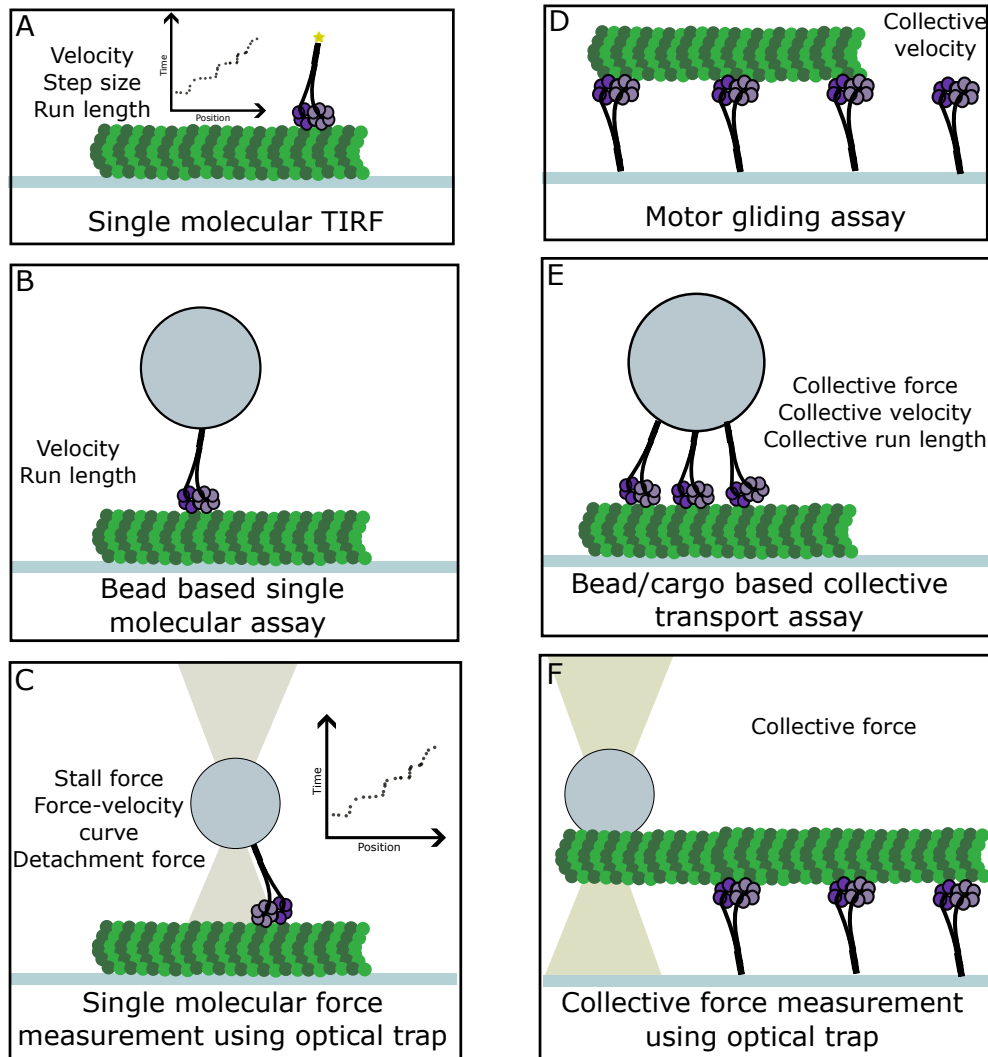


Figure 1.3: **Methods to study dynein transport properties** Transport properties of motors can either be studied as single molecule or collective properties. (A) Single molecular behavior can be studied by directly labeling the motor with quantum dots followed by their direct observation as they walk along a fixed MT using TIRF. The position vs time information data can then be used to calculate the velocity, run length and step size of the motor. (B) Another method to study single molecule behavior include a cargo based strategy where the motor is complexed to a bead. The bead is then observed as it walks along the MT. (C) Similarly, the bead based assay can be used to study the stall force and force-velocity curve of motors when combined with an optical trap. (D) Gliding assays are widely used to study collective properties such as velocity of motors. (E) A bead based strategy where a group of motors is attached to a bead can be studies as it is transported along the MT. (F) This set up can be extended to study collective force when combined with optical trap. Attaching a bead to the filament in a gliding assay like set up can be used to study collective force generation.

study collective properties *in vitro*.

1.4 Collective transport

While single molecular properties of dynein have been explored, the collective transport properties are equally important. Various processes in cell require collective transport of cargoes by a number of molecular motors. The concept of collective behavior was introduced when flagellar dyneins were studied. In flagellum dyneins are arranged in an array along the length of the microtubule. During *in vitro* studies of flagellar dyneins it was observed that the sliding velocity of microtubule filaments of flagellum was much faster than that of single molecular velocity of dynein [136]. Hence dyneins show a collective behavior by acting synergistically when working in groups [8, 127]. Vesicle transport also requires collective transport by a number of motors. The number of motors involved in vesicle transport is limited because the geometry of vesicle allows only a certain number of motors to be in contact with the microtubule simultaneously [116]. Collective transport properties of motors are velocity, run length and force generation. The collective properties have been studied either using a cargo based approach where either a bead, organelle, vesicles or DNA origami based cargo is attached to a team of motors and studied as it moves along a fixed MT (Figure 1.3). Another methods to study collective transport includes gliding assay where motors are immobilized on the surface and fluorescently labeled MTs are observed as they are transported by the motors (Figure 1.3). Collective behavior of up to 7 dynein motors has been studied with the help of DNA origami based cargos [134]. During microtubule pulling by cortical dynein the number of motors transporting a single MT can be more than what is involved in vesicle transport. *In vitro* MT sliding assays have been used to reconstitute and study MT-MT cross-linking and sliding activity of dynein [154]. In these assays dynein binds to a surface immobilized MT via one motor head while the other motor

head attached to a free MT. Due to the walking of dynein the MTs can slide against each other. Similar collective behavior where large number of motors are involved in transport is studied well for actin-myosin system [52, 68]. Such large scale collective behavior is yet to be studied in detail for dynein.

1.4.1 Number dependence of dynein and kinesin

A number dependence of transport properties has been observed for motors when the number of motors participating in the transport changes. Properties of the cargo such as velocity, run length and stepping can change when the the number of motors transporting the cargo changes. Similarly, the force exerted by team of motors can differ than that of single motor. Examples of *in vivo* number dependence include male pronuclear migration in *C. elegans* [71], centrosome centering in *Xenopus* embryo [86, 97] and nuclear positioning in fission and budding yeast which are discussed detail in later chapters. Various studies have been performed for studying interactions of kinesin and dynein with microtubule and transport of microtubules. The *in vitro* gliding assay was widely used to study collective behavior during microtubule transport by motors. These studies tried establishing the relation between transport characteristics of microtubules and the number of motors involved in the transport i.e. to study number dependence.

Kinesin: Early *in vitro* studies of bovine cytoplasmic kinesin complex showed that the velocity of MTs being transported by kinesin is independent of the concentration of kinesin immobilized on the surface when ATP is not limiting [57]. Another study of kinesin heavy chain indicates that reduction in the length of linker domain of kinesin results in decrease in velocity of MTs as the number of kinesins interacting with MT increases [11]. The extent of reduction of velocity is more for shorter linkers as compared to medium linkers [11]. Thus for kinesin to show number dependence it has to be loosely coupled (Figure 1.4). This nature of kinesin can be explained by its

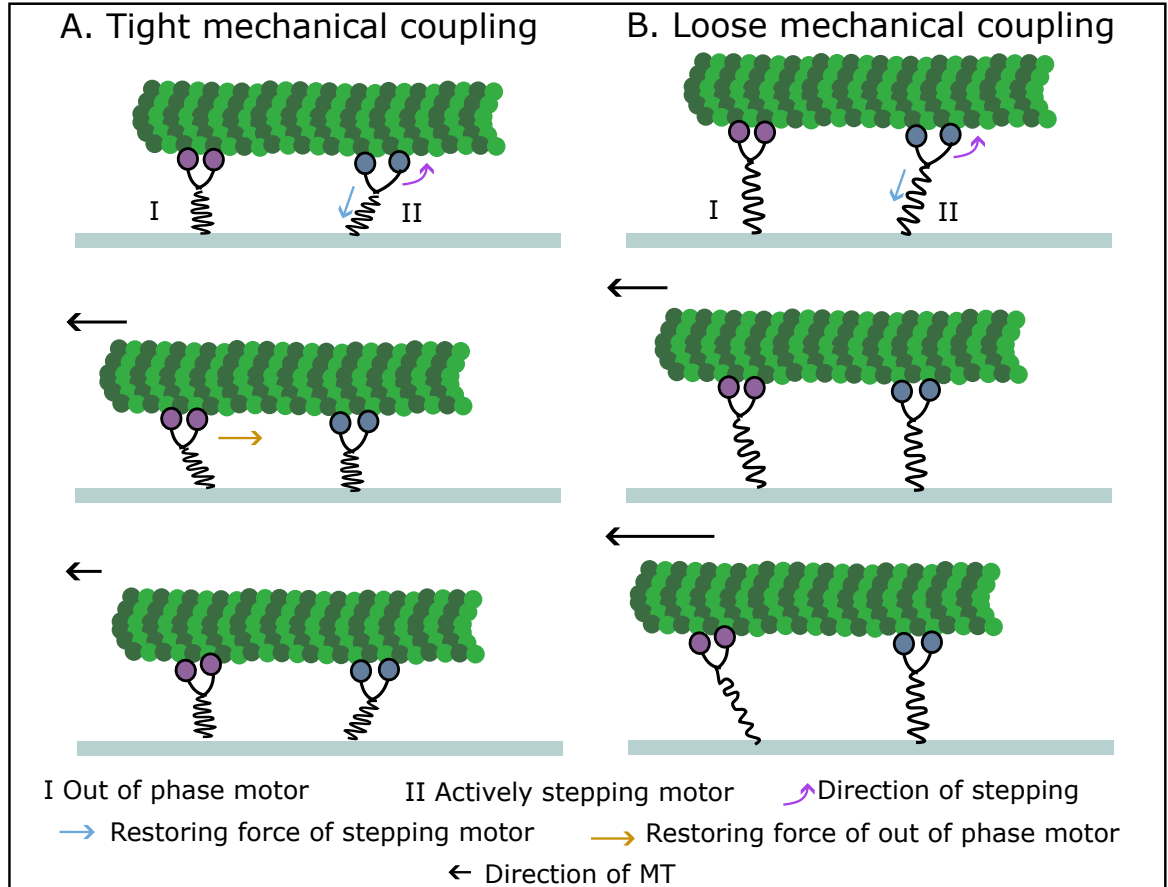


Figure 1.4: **Tight and loose mechanical coupling between motors** The schematic represents effect of mechanical coupling on collective transport of motors. Two motors I (inactive, purple) and II (active, blue) bound to a same MT (green) are attached by their stalks to a solid substrate (light blue). The motors experience either (A) 'Tight' or (B) 'Loose' mechanical coupling depending on the behavior of the individual motors. In the schematic, the active motor (I) is shown to take one step (black arrow), and depending on the nature of coupling, 'tight' or 'loose', a restoring force is produced (blue arrow) due to the inactive motor (II). The direction of MT transport is indicated by a green arrow.

inability to take coordinated steps when collectively transporting a cargo [82]. Such mechanical coupling of motors when they are transporting the same cargo can be used to explain some of the collective properties. When kinesin was anchored to a supported lipid bi-layer the velocity of MTs was seen to be increasing with increasing number of kinesins and viscosity of membrane [49]. Thus when kinesin is anchored to a solid substrate such as glass there is no number dependence [57] however kinesin anchored to a flexible substrate such as membrane shows number dependence of MT transport. This suggests that the nature of substrate to which motors are attached has influence on the collective behavior of motors.

Dynein: When the number dependence of up to 7 dyneins was studied with DNA origami based cargoes the velocity of transport did not show any number dependence. However in gliding assay based set ups where the motor number can go higher than 7 motors dynein shows number dependence of velocity. Studies on full length human dynein complex show that the velocity of MT transport was seen to be increasing with the number of dyneins transporting the MT [100]. This increase in velocity was explained to be due to mechanical activation of motors due to stretching of the the auto-inhibited ϕ conformation [100] (explained in detail in section 1.4.2). The minimal construct of yeast cytoplasmic dynein also showed number dependence in a gliding assay based study [65]. There is also sharp transition in directionality seen when number of motors changes from 8 to 10 [65]. Thus, both full length and minimal construct of dynein show number dependence even when anchored to a solid substrate such as glass. This indicates that motor domain of dynein is flexible enough to show collective behavior unlike kinesin. Dynein can be seen to take synchronous steps when transporting a cargo collectively. This behavior can be explained on the ability of dynein to take force induced bidirectional steps under load even in absence of ATP [39]. This ability of dynein to take backward steps might be able explain motor cooperativity observed for minimal dynein constructs since upon truncation they are unable to form

the ϕ conformation and hence ruling out the possibility of mechanical activation. Lipid anchored dynein is a better representative of the *in vivo* scenario such as cortical dynein. Studying collective behavior i.e. number dependence of lipid anchored dynein can help us gain insights into processes such nuclear and spindle positioning.

1.4.2 Mechanisms of cooperativity

Based on the various studies performed till now the factors or properties of motors which can contribute to the cooperativity are as follows-

Tight vs loose mechanical coupling: The study where number dependence of full length vs truncated kinesin motors was studied suggests a role of flexible neck linkers in the cooperative behavior of motors [11]. Lack of flexible linkers resulted in reduction in collective velocity. This can be explained by tight and loose mechanical coupling of motors (Figure 1.4). In a simplified scenario when two motors are attached to the same MT not all of them can be in their active stepping cycle. The out of phase motors which are tightly bound to the MTs can hinder the motion of the MT. Due to movement of the MT by active motor the out of phase motors can stretch and its restoring force which is in opposite direction can hinder the movement of the MT. The extent of restoring force will be dependent on the flexibility of the neck linkers of the motors. Absence or reduction in length of flexible linkers will result in tight mechanical coupling where the resisting force is higher leading to reduction in the velocity of the MT transport. Presence of longer flexible linkers will motors to be loosely coupled where the resisting forces are not high leading to less reduction in velocity [11] or stationary velocity [57] across increasing motor densities.

Mechanical activation: For dynein based collective transport the proposed mechanisms include mechanical activation by relieving the ϕ conformation (Figure 1.5 A). The full length human cytoplasmic dynein's cryoEM structural studies have shown how dynein is auto-inhibited [176]. The self dimerisation of the motor heads via the linker

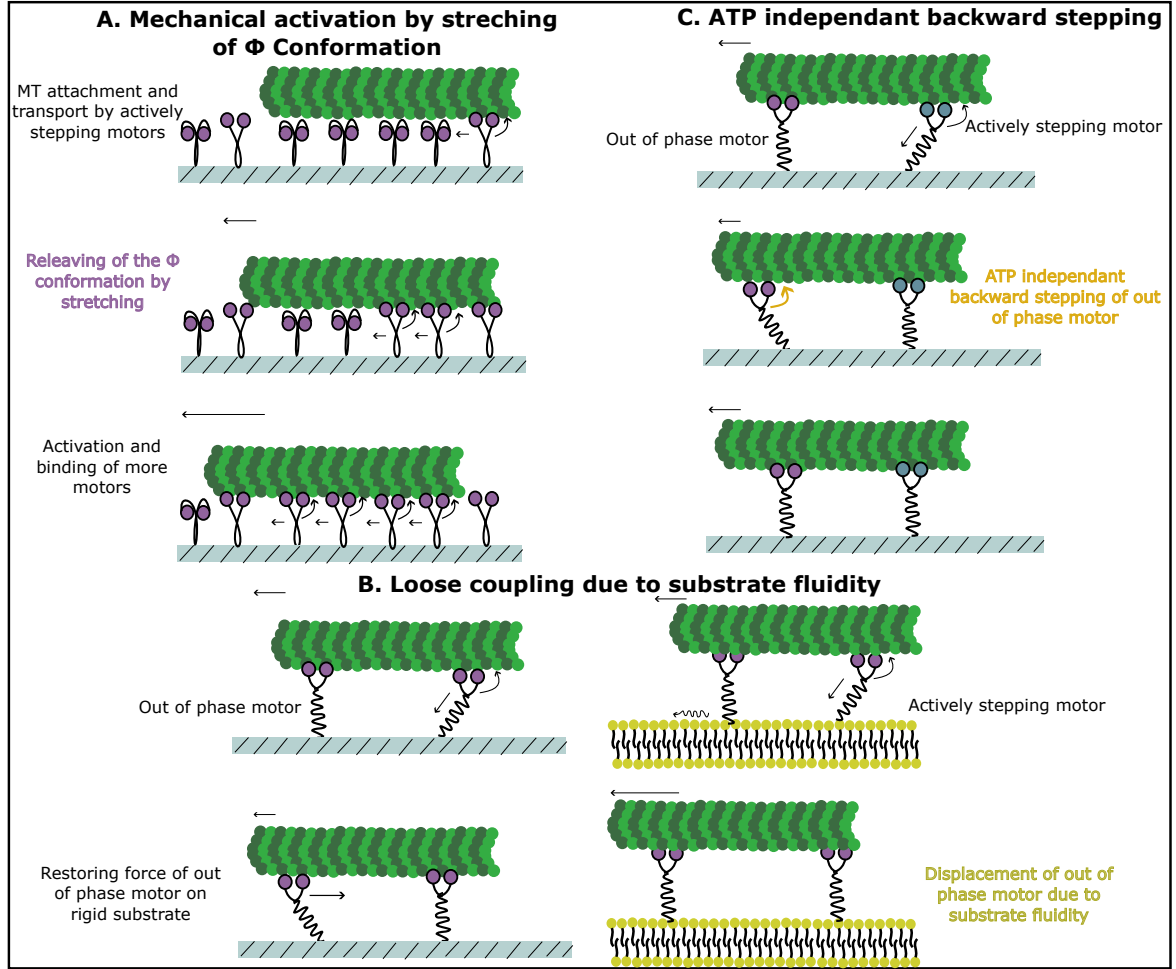


Figure 1.5: **Mechanisms of motor cooperativity during MT transport** (A) Activation of the auto-inhibited ϕ motors by binding to the MT and stretching due to transport by other motors. Due to this mechanical activation, more motors are activated as the MT is transported by other motors. This leads to availability of more motors to actively participate in the transport. Such mechanism has been used to explain the cooperativity observed in MT transport by full length human cytoplasmic dynein complex [100] (B) Upon attachment of motors to a fluid substrate such as lipid bilayer, the motors can passively move along the substrate. This degree of movement of motors can allow to minimize the resistance of the out of phase motors. This has been used to explain the cooperativity during the MT transport by truncated kinesin attached to a lipid bilayer [49] as kinesin does not show motor number dependence when attached to glass [11]. (C) Under sufficient opposing load dynein is able to take backward steps even in absence of ATP. This ability of dynein might result in less resistance during MT transport caused due to out of phase motors. This might be able to explain the cooperativity observed during MT transport by truncated dyneins.

can result in a dynein conformation (closed/ ϕ form) where the motor heads are stacked together. In this conformation the affinity of MTBDs for the MT also reduces resulting in weak diffusive binding of motors to the MT. The dynein could be forced into the open conformation by mutating the residues in the linker which are responsible for the dimerisation. However the open form of dynein still needed attachment of dynactin to orient the motor heads properly to start processively walking on MTs. The auto-inhibited state of dynein was reported to be regulated by mechanically separating the motor heads by adding rigid linkers between the motor heads [156]. Such activation and regulation of dynein's processivity by mechanically separating the motor heads has led to the proposal of the mechanical activation model for dynein cooperativity. For full length human cytoplasmic dynein mechanical activation allows motors to be activated due to stretching upon binding and transport of MT [100]. This allows more motors to actively transport the MT as the gliding continues. Mechanical activation explains increase in the MT velocity as the motor density increases based on how the number of active motors increases as the MT transport progresses. However minimal dynein constructs also show motor number dependence even though they are unable to form ϕ conformation. By studying collective transport of truncated dyneins the remaining mechanisms of cooperativity can be tested.

Backwards stepping: Studies on yeast cytoplasmic dyneins showed that dynein can step bidirectionally even in absence of ATP [39] (Figure 1.5 C). This force induced stepping of dynein can be used to explain why dynein is better at working in groups. The out of phase dynein can step along a moving microtubule passively and hence do not oppose the activity of other dyneins [39].

Asymmetric detachment: Along with the force induced stepping the asymmetric stepping behavior of dynein has also been hypothesized to drive cooperativity [65]. Asymmetric slip bond behavior of dynein is where its detachment rate increases slower in response to opposing force than assisting force. By incorporating asymmetric detach-

ment behavior in models, it is hypothesized to play an important role in cooperativity observed during collective MT transport by dynein [65].

Substrate fluidity: Kinesin shows number dependence of velocity when anchored to a fluid substrate like a lipid bilayer but not when anchored to a rigid substrate like glass. Due to the nature of bilayer the out of phase motors can diffuse across the bilayer freely. This leads to lowering of the resisting force of the out of phase motors (Figure 1.5 B). Hence fluidity of the substrate plays an important role in collective motor transport.

Mechanisms of Myosin cooperativity: Multi-motor cooperation has been extensively studied for myosin. Multiple mechanism have been described for explaining collective behavior of various myosin isoforms using *in vitro* and *in silico* methods. A study with myosin V and VI using DNA origami based cargoes showed that the flexibility of the lever arm was responsible for cooperative behavior of the motor in groups [142]. Myosins with stiffer lever arms were found to be dominating during the collective transport of the cargo. For myosin II activation status of the filament [91] and elasticity of the neck linker were found to affect collective dynamics [140]. For myosin V, lowering of velocity and run length during collective motion has been attributed to their motor stepping rates which are highly sensitive to the strain experienced by the motor [87]. Increased coordination via simultaneous binding of myosins was reported to be dependent on the geometry of the sarcomere lattice [155].

Cytoplasmic dynein has been studied at both single as well collective level. However exploring the mechanisms of cooperativity as well as role of collective transport in various cellular processes is yet to be explored in details. Another cellular function where large scale collective behavior of dynein is important is flagellar/ciliary beating. The broad overview of flagellar dyneins and their role in flagellar mechanics is explored in the next section.

1.5 Flagellar dynein and mechanics

Cilia and flagella consist of microtubule filaments including axonemal dyneins which are situated on the inner as well as outer side of microtubule doublet, which decides their identity as Inner Arm Dyneins (IADs) and Outer Arm Dyneins (OADs). These dyneins are localized to the A-tubule permanently while interacting with B-tubule during sliding in an ATP dependent manner. They can generally be classified based on the number of motor units.

Inner Arm Dyneins: The inner arms dyneins are present as monomeric proteins along with dimeric forms and are arranged in a unique 96nm repeating pattern [167]. The group of monomeric inner arm dyneins has 10 and 18 members in *Chlamydomonas* and *Tetrahymena* respectively [168]. Dimeric Inner arm dyneins have two distinct heavy chains named as 1α , 1β [113]. Dimeric IADs consist of two distinct heavy chains [113]. However *in vitro* gliding assay showed that they trans-locate microtubules very slowly as compared to OADs and other IADs [146]. Hence it can be inferred that dimeric IADs act as brake rather than the power generator to locally limit the sliding. Such restrictions to sliding are important for bending motion.

Outer Arm Dyneins: Depending upon the phylogenetic division number of OAD motors heavy chains varies [167]. In organisms with single flagellum (Unikonts), the OADs contain two heavy chains (e.g. Humans, Sea Urchin, *Leishmania*). In organisms with two flagellum the OADs have an additional heavy chain for constituting the “three-headed dynein” [167]. OADs provide up to 80% of power required for beating, as flagella lacking these structures beat significantly slower than wild type [66]. When OAD less flagellum were studied it was observed that these flagella cannot respond to changes in viscosity indicating that OADs contain a system that senses mechanical strain [96]. OADs are mostly heterodimeric or heterotrimeric rather than homo-dimeric or homotrimeric. This indicates that various HCs play different roles in motility. The

three heavy chains (α , β and γ) of *Chlamydomonas* form the three headed motor. Each chain has different roles - β provides power for stroke generation, α is involved regulation of other HCs. γ is said to be an ATP dependent brake necessary for bending [85, 126].

1.5.1 Flagellar mechanics and sliding MT theory

The first observation of microtubule filaments of flagellum sliding past each other was observed in Sea Urchin flagellum as it was broken from its base and subjected to trypsin treatment [150]. The trypsin treatment resulted in digestion of nexin links and thus freeing the microtubule doublets from each other. In presence of mg-ATP the filaments were observed to slide past each other. This study proved that the machinery required for microtubule sliding was “in build” in the flagellar structure and the sliding is result of dynein’s activity in presence of ATP which results in sliding of microtubule filaments slide past each other. As the axoneme is symmetric structure sliding across entire length would result in rigidity hence either a resistive force should be applied or only a fraction of dynein should be active at given time. This sliding can be converted to bending due to restrictions imposed by nexin links and basal anchor as proposed by Brokaw via modeling work or can be explained based on oscillatory behavior of microtubule sliding [13].

Sliding Microtubule Theory and Bend formation: Further studies on Sea Urchin flagella demonstrated that ionophoretic application of ATP enabled local activation of axoneme [141]. While a de-membranated flagella when given a bath application of ATP showed beating motion, a local ATP application in low concentration induces bending in the flagella. This suggests that bending is an effect of sliding between microtubules caused by dynein’s local activation by ATP. A sliding between the boundaries of ATP activated region and non-activated regions is said to induce bending [141]. However in the *in vivo* scenario a similar local application of ATP is not possible suggesting

that the heavy chain isoforms of dyneins respond differently to ATP inside cells. This results in heterogeneity along the microtubule length. Thus differences in amount of sliding between microtubules are responsible for bending. Dyneins are arranged to form arrays along the length of microtubules and can drive the movement of microtubules much faster than that of single motor as seen during sliding disintegration studies [136]. Hence dyneins show a collective behavior. There is a synergistic behavior when dyneins are arranged on a microtubule [8, 127].

1.5.2 Bottom up reconstitution of flagellar beating and flagella-like patterns

In vitro reconstitution of beating filament bundles

Due to the structural complexity of axoneme studying the flagellar mechanics is challenging. Simplified *in vitro* set ups with motors and filaments have emerged to study some of fundamental properties of flagellar mechanics as well as their applications in synthetic biology. Various strategies have been used to do bottom up reconstitution of flagella. In case of MTs, spontaneously formed MT bundles have been observed to perform oscillations driven by kinesin [128]. Here, MTs and kinesin tetramers were flowed into a chamber (Figure 1.6 A). Majority of MTs form asters due to binding of tetramers of kinesin to multiple MT at a given time. Some of the MTs trapped near air bubbles were spontaneously organized into MT bundles. These bundles showed oscillatory movement in presence of ATP due to the stepping of kinesin while cross linking MTs in the bundles. Hence spontaneous formations of MTs bundles by cross linking kinesin tetramers showed self organized beating of MTs similar to flagella. Similarly, MT bundle beating was demonstrated in a more controlled manner by forming MT bundles on a bead [129] instead of forming them on a spontaneous manner on water-air interface. The MT bundles performed oscillations due to the stepping of kinesin. Here

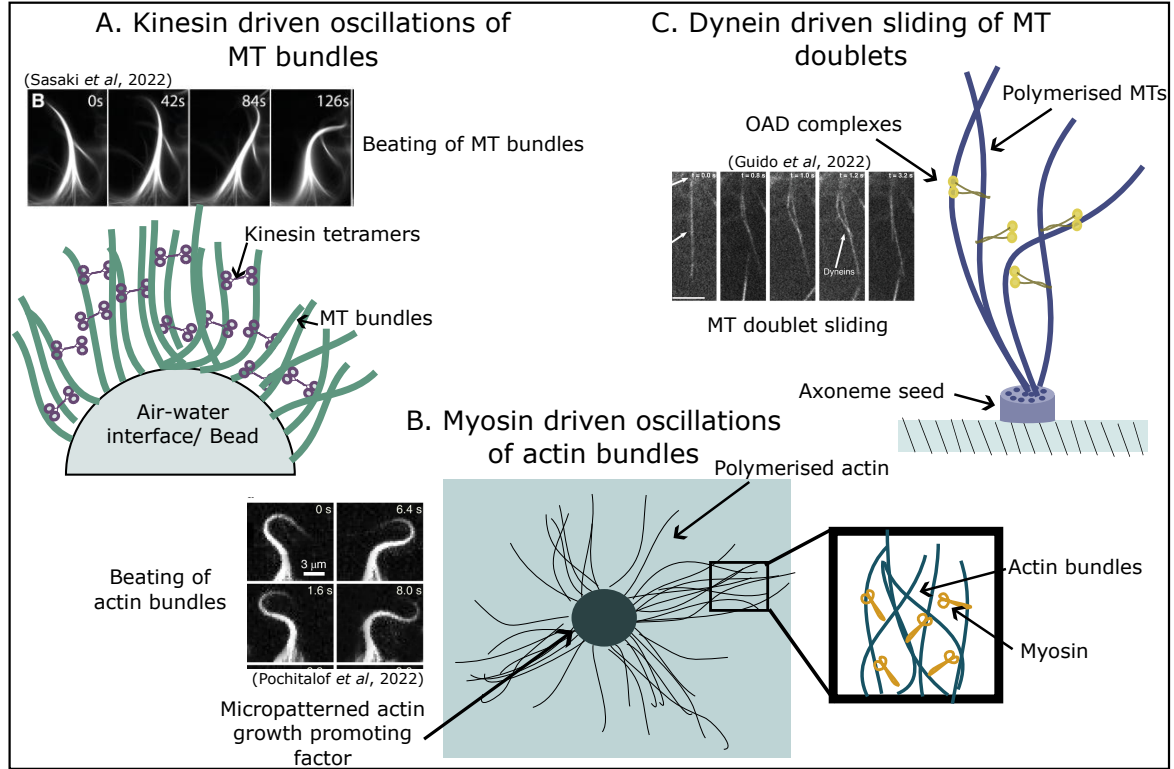


Figure 1.6: *In vitro* reconstitution of motor driven beating filament bundles (A) MT bundles organized spontaneously on air-water interface in presence of depleting agents show flagella-like waves driven by kinesin tetramers [128]. Similarly, MT bundles attached to a bead have also showed wave-like beating driven by kinesin [129] (B) Myosin driven beating of actin bundles was reconstituted by polymerizing actin on micro-patterned growth promoting factors [2] The polymerized actin filaments form bundles in presence of a depleting agent. The actin bundles perform wave-like oscillations driven by myosin. (C) Sliding of MT doublets driven by flagellar outer dynein complex (OADs) [50] MTs were nucleated by using sheared axoneme seed as the nucleator. The polymerized MTs were crosslinked by purified OAD complexes. The doublets showed sliding due to stepping of dynein.

the number of MTs, motors and geometry of the system was not finely controlled.

Similar to MT bundles, actin bundles have also been reported to perform flagella-like oscillations driven by myosins [2]. The geometry of actin bundles was controlled by using micropatterned nucleating factors along with depletion agent to polymerize the actin filaments (Figure 1.6 B). The filaments formed bundles and performed oscillations in presence of myosin. Emergence of such flagella-like patterns in systems other than MT-kinesin suggested a generality in emergence of flagella like behavior. The drawback of using filament bundles is lack of control over the number of filaments and their length.

A more controlled attempt to reconstitute flagella like beating was performed using a mixture of top down and bottom up approach. Axoneme seeds were used to nucleate MTs followed by addition of dynein-OAD complexes of *Chlamydomonas* [50] (Figure 1.6 C). The purified dynein-OADs were able to cross link MTs. The MT doublets hence formed showed buckling and looping of one of the MT in presence of ATP. With a slightly more control over the MT number. Even though this study had a better control over MT numbers and orientation it didn't report continuous flagella-like oscillations.

***In vitro* reconstitution of flagella-like beating of single filaments**

Pinned single filaments have been reported in various filament motor systems to perform oscillations [161, 112]. In a kinesin driven gliding assay, MT that are accidentally clamped to the surface have been reported to show flagella like oscillations (Figure 1.7 A). MTs have been anchored by their minus end using a biotin-streptavidin chemistry in kinesin gliding assay [43] (Figure 1.7 B). A single kinesin was seen to be able to buckle a MT. Even though buckling of MT observed, oscillations were not reported probably due to low motor density. Actin filament in a gliding assay like set up has been reported to perform myosin driven oscillations when the leading end was clamped using an optical trap (Figure 1.7 C). Spontaneous generation of oscillation in filaments due to stepping and force generation of motors is general phenomenon. However these

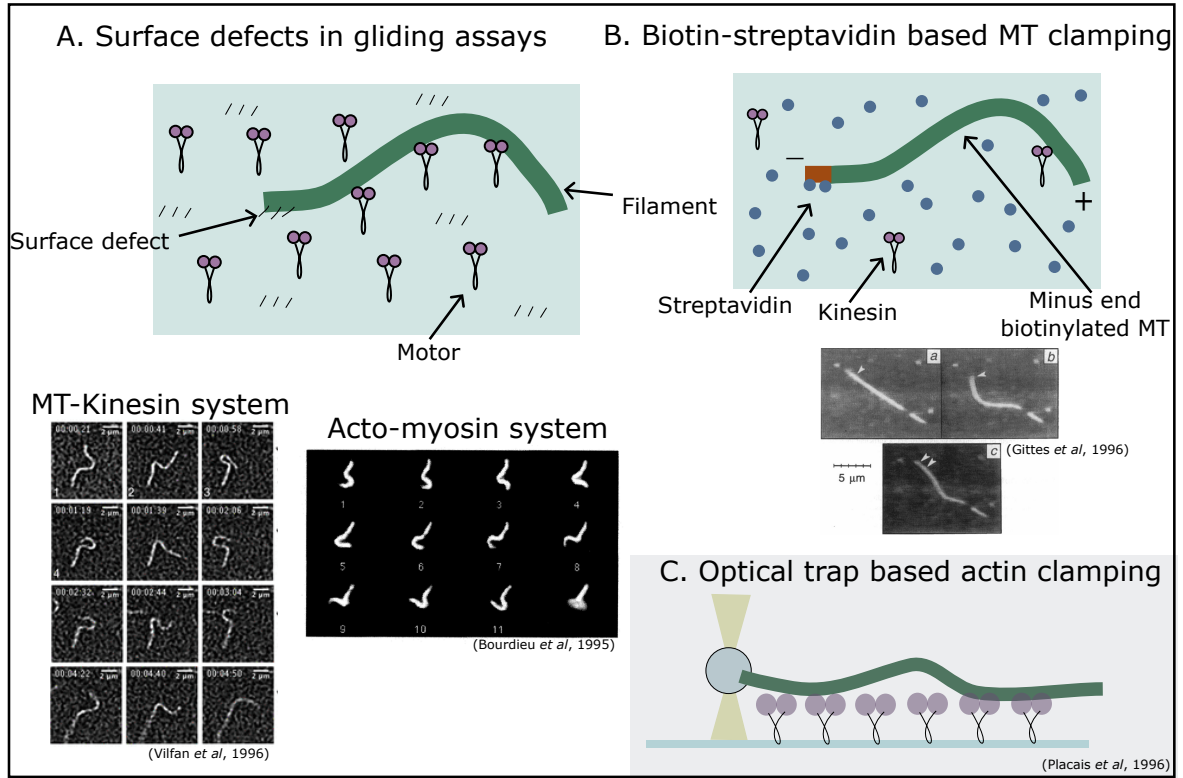


Figure 1.7: *In vitro* reconstitution of motor driven beating of single filament (A) Surface defects in gliding assays have resulted in flagella-like beating patterns. The leading filament tip is accidentally clamped to the surface upon encountering a surface defect. Due to stepping of motor towards the free end of the clamped filament drives oscillations. Such motor driven oscillations have been observed for MT-kinesin [161] as well as acto-myosin systems [12] (B) Kinesin driven bending of a single MT was observed in a gliding assay like set up when the MT minus end was clamped to the surface [43] The specific clamping of MT minus end was achieved by allowing minus end biotinylated MT to bind to the coverslip via streptavidin. Stepping of kinesin resulted in MT buckling and bending but not oscillations. (C) Reconstitution of myosin driven oscillation of single actin filament by using optical trap to clamp the leading tip of the actin filament [112].

studies have lacked specificity of MT immobilization, control over MT length, unknown motor density and used kinesin or myosin. Such oscillations driven by dynein are yet to be explored even though dynein is the main force generator in flagella.

***In silico* flagella-like oscillations**

Along with *in vitro* studies various *in silico* studies have been done to explore the emergence of similar patterns. Theoretical studies have shown that clamped chain of active self propelling particles will undergo beating patterns. The emergence and nature of patten was jointly determined by the filament length, driving force and nature of pinning [19]. In order to test these predictions and to better understand the mechanics of flagellar dynamics, an *in vitro* system with dynein and MTs mimicking beating could be a useful model. *In silico* studies where the clamp was replaced by a cargo showed that the beating of the filaments is able to generate enough forces to move the cargo, similar to a sperm [61]. Hence minimal *in vitro* systems along with theoretical studies can help us understand flagellar mechanics in detail.

1.6 Rotary motors and filaments as a marker of rotational motion

Dynein belong to the AAA+ family of proteins which comprises of several functionally diverse proteins in the P-loop type Nucleotide tri-phosphate (NTP) binding protein family [63]. The AAA+ ATPase family is a part of extended ATPases which involves F1ATPases, kinesins and myosins [29]. The AAA+ family involves proteins with diverse functions such as dynein, McrBC and RecA like proteins [29]. McrBC is a unique AAA+ protein since it hydrolyses GTP instead of ATP [29]. These are motor proteins which utilize energy from NTP hydrolysis to perform either stepping or rotational motion.

Studying the rotational dynamics of these motor is challenging as direct observation is technically limited. Hence direct observation of rotational motion is done by coupling the stator to a marker whose rotation can be visualized using the current microscopy systems. Rotation of F1-ATPase has been confirmed by using actin as a tool. The 3 α and β subunits form a barrel which rotate the γ stator upon ATP hydrolysis [105]. The biotinylated γ subunit was complexed with biotinylated actin filament via a streptavidin. By observing the rotation of fluorescently labeled actin the rotation of the F1ATPase was confirmed. Apart from actin other tools used as a marker for rotational motion involve beads and nanoparticles. Rotation of V-ATPase was confirmed using gold nanoparticles [108] while pairs of magnetic beads have also been used to mark and control the rotation of F1 ATPase *in vitro* [3].

Kinesin neck rotation has been studied by analyzing the movement of MT being transported by a single kinesin at lower ATP concentrations [59]. Since MTs have been used to study rotational flexibility of kinesin and can be complexed with fluorescent tags and biotin, they make a potential marker that can be used as a marker.

Chapter 2

Dynein collective behavior in mitotic nuclear positioning of *Saccharomyces cerevisiae*¹

2.1 Summary

In *S. cerevisiae*, cortical dynein generates pulling forces on microtubules (MTs) attached to the nucleus, helping position it at the bud neck during closed mitosis. A possible mechanism responsible for asymmetric positioning of the nucleus was explored by quantifying MT lateral interactions with the bud and mother cell cortex. It showed increased MTs interaction with the bud cortex than the mother cortex, with increased contacts during anaphase, indicating a mechanism that enhances motor engagement on the bud cortex for nucleus positioning.

¹Experiments performed by Dr. Saravanan Palani, University of Warwick.

2.2 Introduction

Budding yeast has a closed mitosis where the nuclear envelope does not breakdown. Cytoplasmic dynein is known to be important for nuclear segregation [30]. Dynein plays an important role during the mitosis by positioning the nucleus near the bud neck. The microtubule (MT) nucleating structure spindle pole body has been identified in budding yeast [138] and is known to nucleate MTs and control their numbers in the cytoplasm [74]. Both SPB (the one facing the mother cell and the other facing the bud cell) can be seen to nucleate MTs which interact with respective cell cortex [16, 4] during yeast mitosis. MTs can be seen to be interacting with the cell cortex in two ways (1) lateral contact and (2) end-on contact. In lateral interactions the MTs can contact the cortex and curl along it and slide forward [16, 4]. Stepping of cortically attached dynein on the MTs can generate a pulling force that can result in pulling of spindle/nucleus into the bud neck [16]. The end on interaction is where microtubule ends are seen to be interacting with cortex in end on manner [79]. Dynein can regulate microtubule dynamics at the plus end. By de-polymerizing the plus end of MT dynein can generate pulling forces which can position the nucleus [80]. This type of interaction is seen to be important for early positioning of nucleus near the bud neck. Thus both sliding and end on interactions are involved in nuclear and spindle positioning in budding yeast [101].

The positioning of nucleus to the bud neck can be considered as asymmetric positioning since the nucleus is not positioned at the geometric center of the system. Examples of symmetric positioning involve interphasic nuclear positioning in fission yeast while asymmetric positioning is seen in male pro-nuclear migration of *C. elegans* [6, 47] and mitotic nuclear positioning in *Saccharomyces cerevisiae*. During asymmetric positioning the net forces acting on the organelle are not uniform in all directions leading to the movement of the organelle in the direction of the net force. Budding

yeast can be used to study asymmetric organelle positioning due to simpler geometry of its astral MTs (1-2 MTs at given time) as compared to other organisms with much more complex astral geometry (*C. elegans*). Simple astral geometry along with well studied mechanics of the yeast cytoplasmic dynein at single molecular level [120] make it a good model system.

In order to study the mechanism by which the asymmetric positioning of the nucleus is coordinated the distribution of force generators can be hypothesized to be asymmetric in nature i.e. presence of more dynein on the bud cell cortex so that the net force of pulling is towards the bud. However by studying the distribution of dynein anchoring protein NUM1 it was observed that the mother cell cortex has higher number of NUM1 clusters as compared to the mother cell cortex [107]. Since MTs can be seen to be interacting with both cell cortices and dynein is also present on both cortices, the mechanism by which the asymmetry of forces is favored is yet to be explored.

Regulation of MT dynamics and MT cortical interactions could also play a role in the nuclear positioning. By studying MT dynamics during various stages of cell division it was observed that during interphase dynamic MTs generate pushing forces on nucleus [139]. Penetration of astral MTs into the newly formed bud is associated with movement of nucleus to the bud neck during S/G2 and G2/M transition [139]. MT dynamics have been measured in cell lysate from different stages of cell division *in vitro* [10]. However systematic quantification of MT dynamics according to the cell stage and SPB (mother or bud) has not been studied in as much details.

Hence studying MT cortical interactions and MT dynamics can help us gain insights into how nuclear positioning is controlled. Here, an image analysis based approach was used to study MT dynamics and cortical interactions during yeast mitosis. The spindle elongation was used as marker for identifying pre- anaphase and anaphase. The lateral interactions of MTs (sliding) were measured in both the mother as well as daughter cell cortex. This helped us gain insights on the *in vivo* regulation of MT-cortical

interactions that drive the nuclear positioning, the sufficient MT lengths required for nuclear positioning in pre-anaphase. The MT lengths were also used to calculate MT dynamic instability parameters across pre-anaphase and anaphase for each SPB.

2.3 Methods

2.3.1 Spindle Length, elongation and MT dynamics during Mitosis

A dual-labeled strain of *Saccharomyces cerevisiae* with Spc42 tagged with mCherry and Tub1 with GFP (Strain name: AKY099 ESM 356-1 mCherry:Spc42, GFP: α -tubulin as described previously [18]) was used. Time series of *Saccharomyces cerevisiae* GFP-Tub (Recorded by Dr. Saravanan Palani) were used for image analysis. The time stacks were used to measure spindle and MT lengths over time (Figure 2.1). The spindle length with time as well as MT lengths with time were measured interactively using the measure function of FIJI. Since during the anaphase the spindle elongation can contribute to the positioning of the spindle, the MT cortical interactions prior to spindle elongation were of interest. To analyze such cell stage dependent MT dynamics it was important to identify and segment the data according to the stage of cell division. This was achieved by using spindle length elongation as a marker.

2.3.2 Virtual Synchronization of Dividing Yeast Cells

In order to categorize the MT lengths according to the cell stage the spindle elongation with time was used as a marker. Since the culture grown was not synchronous each cell was dividing at different point in the given times series. To virtually synchronize the spindle elongation the spindle elongation vs time curves from all the cells were fit to a modified Hill's function (Figure 2.2 A and B) as follows.

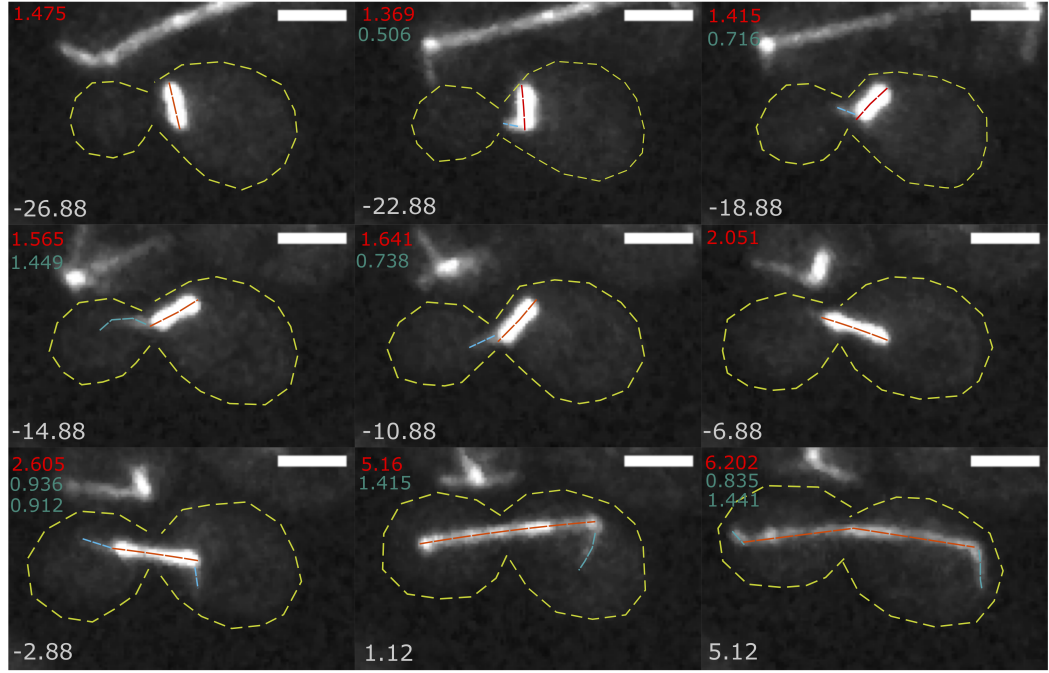


Figure 2.1: **Tracking spindle elongation and MT length dynamics.** A montage of a representative time-series of a *Saccharomyces cerevisiae* cell with GFP-Tub1 (grey) labeling both spindle and SPB nucleated astral MTs. The spindle lengths (red line) and astral MTs (cyan) were interactively tracked with the lengths in μm . The cell outline is marked by a yellow dashed line. The time in minutes is normalized with $t=0$ corresponding to the mid-phase of anaphasic spindle elongation. Scale bar: $2 \mu\text{m}$.

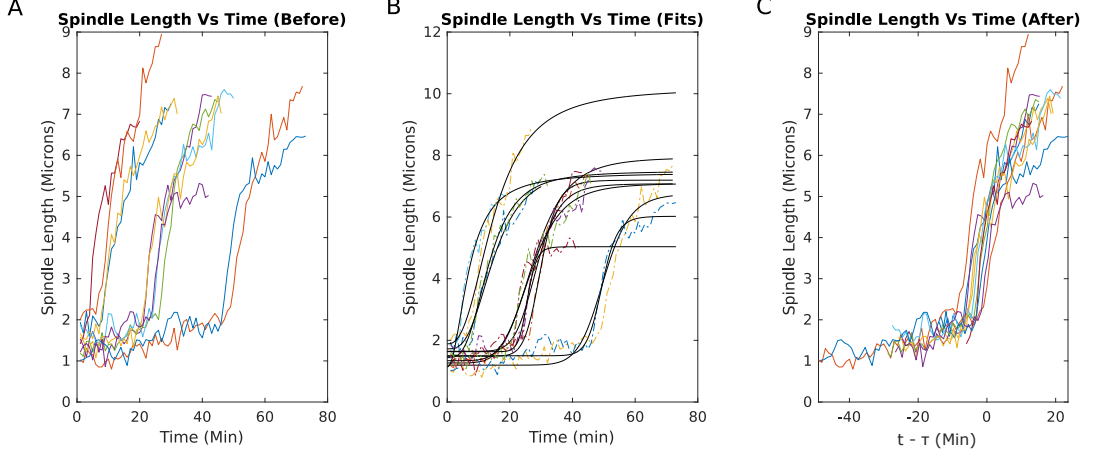


Figure 2.2: **Identifying pre-anaphase and anaphase stages in MT dynamics by spindle length analysis.** (A) The spindle lengths of each set of cells during division from un-synchronized cells were plotted as a function of time (B) The spindle length vs time data was fit to a modified Hill function (Equation 1) to estimate the half maximal time of spindle elongation τ , (C) The spindle length vs time plots with the re-scaled time axis obtained by subtracting τ from the time for each time series. After re-scaling all the plots now fall onto each other.

$$L = L_{min} + (L_{max} - L_{min})((R_{max} \cdot t)^n / (\tau^n + t^n)) \quad (2.1)$$

Where L_{max} and L_{min} are maximum and minimum spindle lengths, R_{max} is the maximum spindle elongation rate, τ is the time at which the spindle length is half maximal, and n is the Hill coefficient which was estimated from the fit for each data set and used to re-scale the x axis (Figure 2.2 C). Spindle elongation was seen to start -10 min from τ , therefore $\tau - 10$ was used to divide the data into pre-anaphase (pre-elongation) and anaphase (elongation) (Figure 2.4 A-D). Thus data fitting and re-scaling of x axis was used to achieve virtual synchronization of dividing cells and also used to classify the pre-anaphase and anaphase. The data analysis and fitting was done using MATLAB.

2.3.3 Quantification of MT dynamics

The MT lengths with time were measured for both bud and mother cell MTs. Laterally interacting MT lengths measured were divided into pre-anaphase and anaphase categories for both bud and mother cells. Frequency histograms of the MT lengths were plotted to compare the MT numbers and lengths (Figure 2.4 D). The MT lengths with time were also used to quantify the frequency of MT catastrophe and rescue events by measuring the number of event for each MTs. Similarly, MT growth and shrinkage velocities were calculated interactively for each growth and shrinkage event as change in MT length per unit time. The MT dynamics of bud cell MTs and mother cells MTs were compared for pre-anaphase and anaphase by plotting violin plots (Figure 2.5).

2.4 Results

2.4.1 Dynamics of spindle elongation and astral MTs

It was observed that as the spindle elongates and successfully divides, number of dynamic MTs are nucleated from both the daughter and mother SPBs. These MTs were dynamic and perform the ‘search and capture’ and occasionally slide along the cortex. The spindle can be observed to be positioned at the bud neck followed by its rapid elongation. Upon fitting the spindle elongation data to the modified Hill’s function it was observed that the pre-anaphase spindles is about $1.48 \mu\text{m}$ long while the anaphase spindle is $6.49 \mu\text{m}$ (Figure 2.3 A-D). The elongation takes about 15 minutes with the spindle elongation rate of $1.04 \mu\text{m}/\text{min}$. The Hill’s coefficient was observed to have variability among the data set as indicated by its high SD (Table: 2.1). The high variability is a combined effect of small dataset and complete spindle elongation not being captured for some cells due to asynchronous division during the acquisition of the data. The Hill’s coefficient is on the lower side (3) when pre-anaphase is not captured

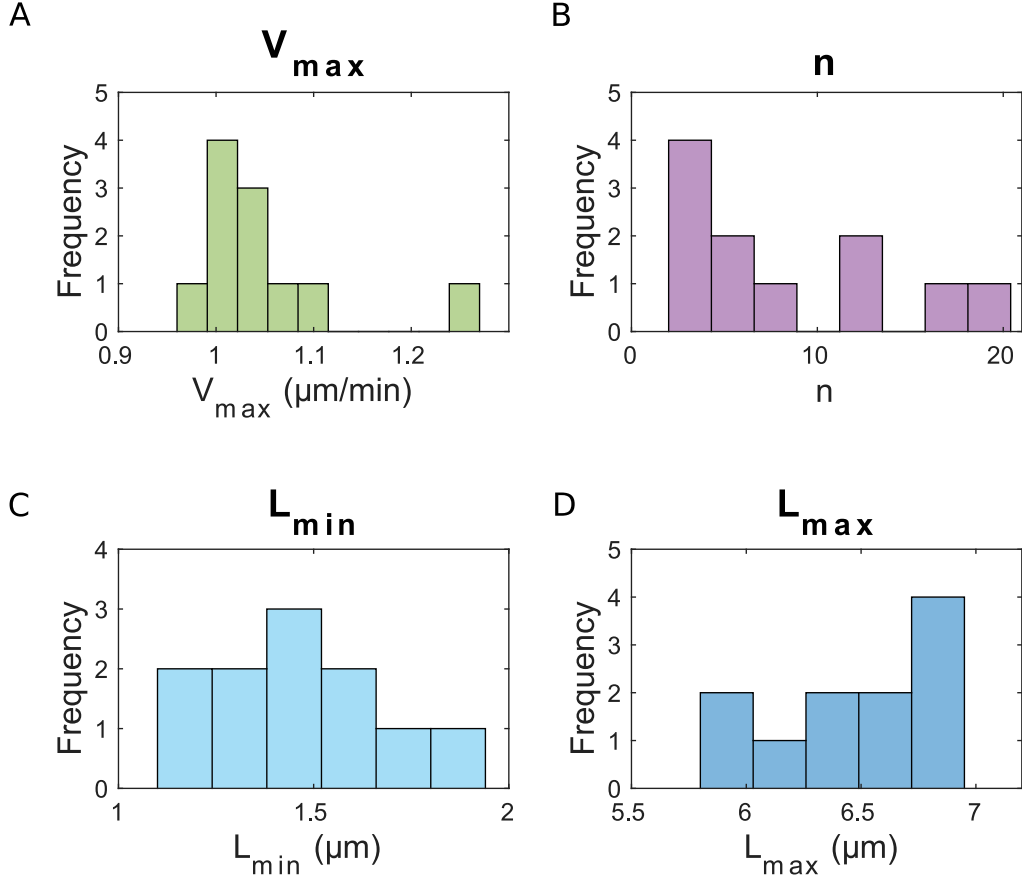


Figure 2.3: **Parameters of spindle elongation fitting.** (A-D) The distribution of spindle elongation rate (V_{max}), Hill's coefficient (n), minimal (L_{min}) and maximal (L_{max}) spindle length obtained by fitting of spindle length vs time data of 11 cells ($n = 11$) to equation [2.1](#). The mean values obtained are $V_{max} = 1.04 \pm 0.08$ ($\mu\text{m}/\text{min}$), $n = 8.18 \pm 6.28$, $L_{min} = 1.48 \pm 0.23$ μm and $L_{max} = 6.48 \pm 0.37$ μm (mean $\pm$ s.d.)

as compared to cells where the entire spindle elongation is captured (Pre-anaphase + anaphase). For a systematic study a larger dataset where the spindle elongation is completely captured for all the cells should be used in the future. Astral MTs are observed to nucleate from the both mother and daughter SPB. Number of MTs are seen to be nucleating from the SPB throughout the mitosis which are dynamic in nature. The MTs occasionally contact and slide along the cortex of both bud and mother cell. In order to distinguish Cortical interactions according to the cell division stage the data was normalized by the τ as mentioned in the methods (Figure 2.4 A).

2.4.2 Increased Lateral MT-cortical Interactions in Bud Cortex

The MTs lengths which were laterally contacting the cortex were measured for both daughter and mother cortex. The MT lengths were now plotted according to the re-scaled x axis (Figure 2.4 B and C). From the figures it can be seen that lateral interactions were more on the daughter cortex than that of mother cortex which increase further during anaphase. Both number of MTs as well as the lengths of MTs are more on the bud cell cortex during both pre-anaphase and anaphase (Figure 2.4 D). From the histogram it can be seen that length of 1-2 μm is sufficient to pull the nucleus to bud neck prior to spindle elongation. The MT lengths further increase further to 1.5 to 2 microns during spindle elongation (Figure 2.4 D). The increased lateral interactions on the bud cell cortex might be playing a role in nuclear positioning and spindle elongation.

2.4.3 Astral MT dynamics during mitosis

The astral MT dynamics were measured for bud and mother cell MTs during pre-anaphase as well as anaphase (Figure 2.5). Neither the rates of catastrophe and rescue

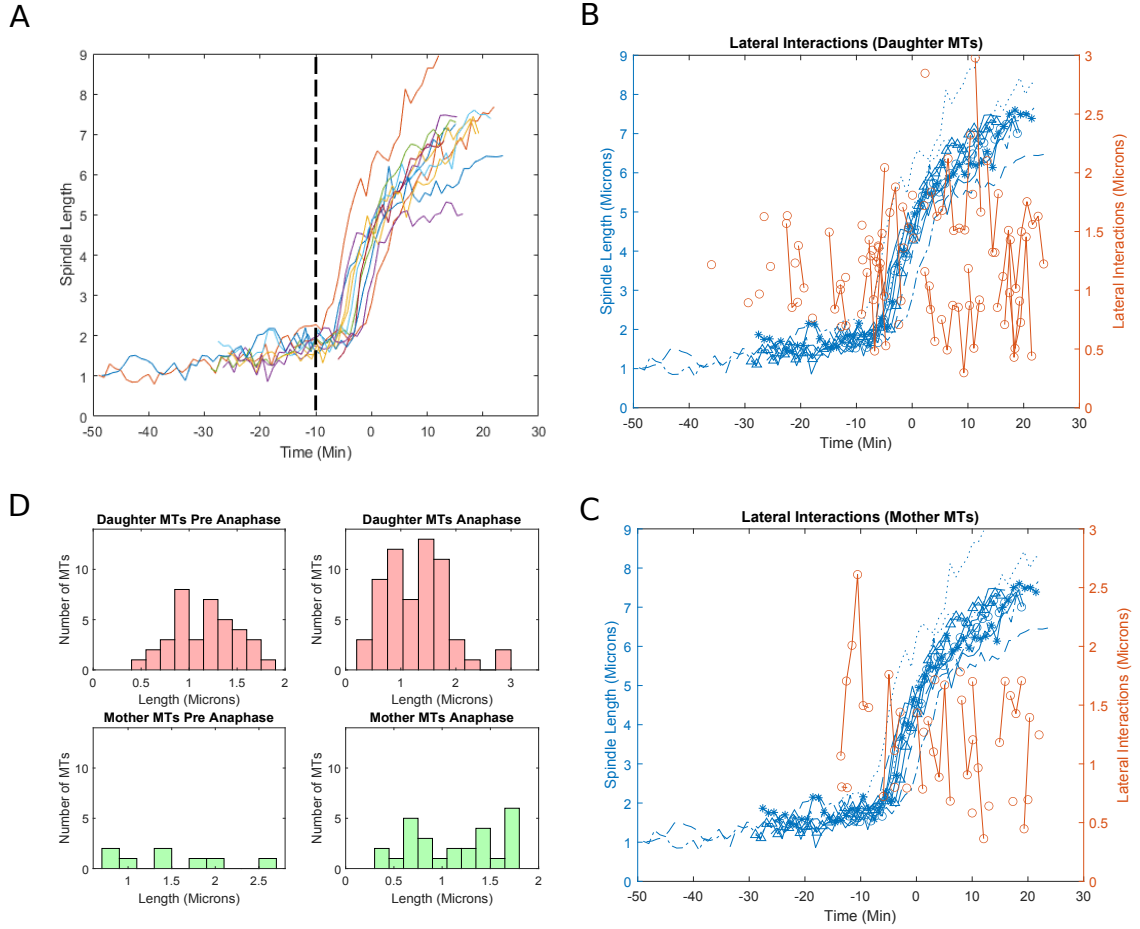
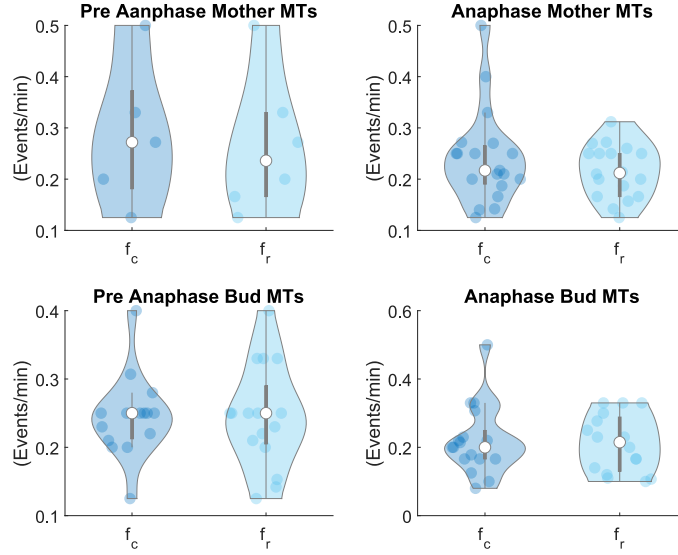


Figure 2.4: **Anaphase and pre-anaphase dynamics of spindle and astral MTs.** (A) Individual spindle lengths were re-scaled by the half-maximal time of spindle elongation by fitting a min-max scaled function and, to virtually ‘synchronize’ the cells into anaphase (spindle elongation) and pre-anaphase stages. (B, C) The spindle length dynamics (blue) are superimposed on a graph of the time-dependent lengths of lateral interaction of MTs (red) in the (B) daughter cell and (C) mother cell. (D) The frequency distributions of MT lengths laterally interacting with the cortex during PA: pre-anaphase and A: anaphase in daughter (pink) and mother (green) cells are plotted. $n=11$ cells.

A Frequency of catastrophe and rescue



B Velocity of growth and shrinkage

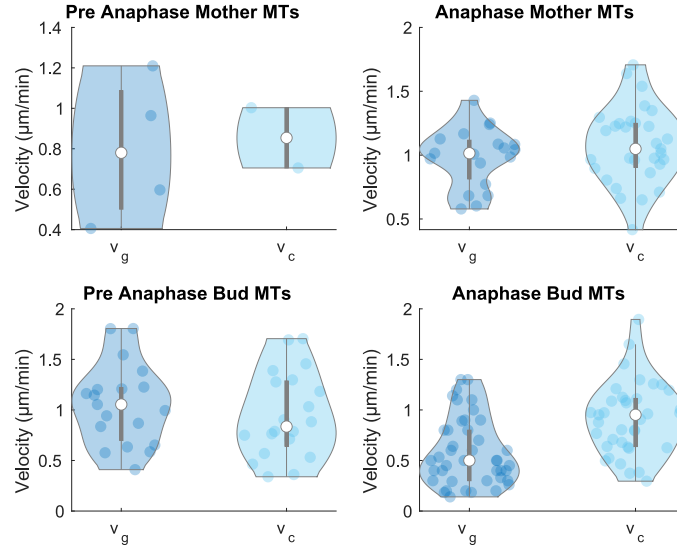


Figure 2.5: **MT dynamics of astral MT during mitosis.** (A) Frequency of catastrophe and rescue of MTs nucleating from mother and bud SPB during pre-anaphase and anaphase. (B) Velocity of MT growth and shrinkage of MTs nucleating from mother and bud SPB during pre-anaphase and anaphase.

nor the velocity of MT growth and shrinkage show any differences across cell division stage and SPB (Table 2.2 and 2.3). These suggest that MT dynamics are not regulated in order to engage more forces generators on the bud cortex and only MT number is regulated. Previously MT dynamics have been reported for various cell division stages using *in vitro* polymerization of MTs in cell extracts [10]. However *in vivo* measurement of MT dynamics according the cell division stages might vary.

Symbol	Description	Value (mean $\pm$ s.d.)
V_{max}	Spindle Elongation Rate	$1.04 \pm 0.08 \mu\text{m}/\text{min}$
L_{min}	Minimum Spindle Length	$1.47 \pm 0.23 \mu\text{m}$
L_{max}	Maximum Spindle Length	$6.48 \pm 0.36 \mu\text{m}$
n	Hill's Coefficient	8.18 ± 6.28

Table 2.1: **Values of fit parameters used for virtual synchronization of cells.** The values of parameters obtained from fitting the modified Hill's function to the data of spindle length as a function of time for 11 cells.

2.5 Discussion

Microtubule motor interactions drive a variety of cellular processes. In case of organelle and spindle positioning both MT lengths and motor number has been observed to play a role in the directional pulling. In *S. pombe* the nucleus is known to oscillate between the cell poles [20]. These oscillations are driven by MTs and motors. The directional positioning of nucleus during the oscillations is regulated by both MT lengths and number of motors [162]. The movement of nucleus is proposed to be in the direction where longer MTs and higher motor numbers are present [162]. In *C. elegans*, male pronucleus migrates towards towards the female pro nucleus from the pole to the center of the cell. Cytoplasmic dynein is known to generate pulling forces by walking on astral

MTs nucleated from the male pro-nuclear [47, 6]. These astral MTs are known to be asymmetric in lengths i.e. the MTs facing the female pronucleus are longer than rest of the MTs. Hence the directional pulling of nucleus by dynein is known to be MT length dependent [71]. Hence either MT length or motor number or both have been known to coordinate directional positioning of organelles.

During budding yeast mitosis the directional positioning of the nucleus to the bud neck is another example of asymmetric positioning. However if this type of positioning is regulated by the motor number or MT length or a combination of both was not known. From previous studies the localization of dynein is known to be favored at the mother cell cortex as compared to the bud cell cortex. By studying the MT cortex interactions we found that more MTs are nucleated by the SPB facing the bud cell. These MTs were observed to be making more lateral interactions on the bud cortex. Along with numbers the lengths of MTs interacting were also higher for bud cell cortex. Nucleating more MTs on the SPB and enabling frequent and longer interactions with the bud cell cortex could be way to engage more force generators. This type of mechanism has been proposed when yeast cytoplasmic dynein minimal construct was observed to show number dependent increase in velocity and directionality of MTs in *in vitro* gliding assay [65]. Hence, studying the number dependence of motor transport properties is considered to be important for understanding *in vivo* motor number dependence.

Along with motor number MT dynamics are also known to play a role in nuclear positioning in budding yeast. However characterization of MT dynamics across various cell stages is essential to study the exact role of MT dynamics. By quantifying the MT dynamics in pre-anaphase and anaphase we observed that there wasn't a dramatic difference in the MT dynamics parameters. Previously, yeast MT dynamics have been measured *in vivo* in budded and un-budded *Saccharomyces cerevisiae* cells [16]. The average MT growth and shrinkage observed were $0.491 \mu\text{m}/\text{min}$ and $1.350 \mu\text{m}/\text{min}$

respectively. The growth and shrinkage rates observed during the anaphase in this study are comparable to the MT dynamics reported previously for budded cells. While the growth rate for pre-anaphase is comparable to that of the un-budded cells in the previous study, the shrinkage rates vary. Here the shrinkage rate for pre-anaphasic MTs is $0.94 \mu\text{m}/\text{min}$ while the shrinkage rates for un-budded cells in the previous study was $1.784 \mu\text{m}/\text{min}$. The differences in the values could be explained on the basis of different approaches used to divide the data according to the cell stage. While previously only the onset of budding was used as a reference, in this study the timeline of spindle elongation is considered. The sample size between the two studies is also different. Hence, a more systematic study with a larger dataset along with characterization of all the cell division stages can be useful for studying the role of MT dynamics. The yeast tubulin dynamics have been explored *in vitro* as well [58]. The growth rate and shrinking rate observed *in vitro* at $0.8 \mu\text{M}$ tubulin concentration were $17.9 \pm 0.2 \mu\text{m}/\text{min}$ and $170 \pm 8 \mu\text{m}/\text{min}$ respectively. The higher *in vitro* dynamics could be due to concentrations higher than *in vivo* scenario. Along with budding yeast, *in vivo* fission yeast dynamics have been measured for various cell division stages [124]. The astral MT dynamics had growth and shrinkage rates of $3.18 \pm 0.61 \mu\text{m}/\text{min}$ and $16.99 \pm 2.39 \mu\text{m}/\text{min}$ respectively, during the interphase. Both growth and shrinkage rates were reduced during the anaphase $0.70 \pm 0.12 \mu\text{m}/\text{min}$ and $2.33 \pm 0.55 \mu\text{m}/\text{min}$. While the rates of MT shrinkage in interphase are much higher than that of the budding yeast, the reduction in rates during anaphase is common. These indicate an interesting aspect of role of MT dynamics in spindle assembly and position. Detailed cell stage dependent analysis of both mother and bud cell MTs for a larger data will be helpful to understand how MT dynamics is regulated across cell division stages.

Symbol	Description	Cell	Phase	Value (mean $\pm$ s.d.)	No.of MTs
V_g	Growth rate	Bud	Pre-anaphase	1.05 ± 0.39 $\mu\text{m}/\text{min}$	19
			Anaphase	0.58 ± 0.33 $\mu\text{m}/\text{min}$	20
		Mother	Pre-anaphase	0.79 ± 0.36 $\mu\text{m}/\text{min}$	9
			Anaphase	0.98 ± 0.23 $\mu\text{m}/\text{min}$	20
V_s	Shrinkage rate	Bud	Pre-anaphase	0.94 ± 0.41 $\mu\text{m}/\text{min}$	19
			Anaphase	0.91 ± 0.35 $\mu\text{m}/\text{min}$	20
		Mother	Pre-anaphase	0.85 ± 0.21 $\mu\text{m}/\text{min}$	9
			Anaphase	1.07 ± 0.29 $\mu\text{m}/\text{min}$	20

Table 2.2: **Values of fit parameters used for virtual synchronization of cells.** The values of parameters obtained from fitting the modified Hill's function to the data of spindle length as a function of time for 11 cells.

2.6 Conclusions

During budding yeast mitosis, the MTs nucleated by SPBs make end on as well as lateral contact with the cell cortex. The lateral contacts made by the bud cell SPB nucleated MTs to the bud cortex are longer and more frequent than that of contact made by the MTs nucleated by mother SPB. The lateral contacts by bud cell SPB increases further during the anaphase as compared to the pre-anaphase. The MTs

Symbol	Description	Cell	Phase	Value (mean $\pm$ s.d.)	No.of MTs
F_c	Frequency of Catastrophe	Bud	Pre- anaphase	0.24 ± 0.05 Seconds ⁻¹	19
			Anaphase	0.21 ± 0.10 Seconds ⁻¹	20
		Mother	Pre- naphase	0.28 ± 0.14 Seconds ⁻¹	9
			Anaphase	0.24 ± 0.09 Seconds ⁻¹	20
F_r	Frequency of Rescue	Bud	Pre- anaphase	0.24 ± 0.07 Seconds ⁻¹	19
			Anaphase	0.21 ± 0.08 Seconds ⁻¹	20
		Mother	Pre- anaphase	0.26 ± 0.13 Seconds ⁻¹	9
			Anaphase	0.21 ± 0.05 Seconds ⁻¹	20

Table 2.3: **Values of fit parameters used for virtual synchronization of cells.** The values of parameters obtained from fitting the modified Hill's function to the data of spindle length as a function of time for 11 cells.

dynamics however do not show any major differences across the cell stages and the SPB type. Hence a possible mechanism to engage more motors on the bud cortex by increasing the number of MT contacts could be involved to drive asymmetric nuclear positioning during the mitosis.

Chapter 3

Cooperativity in motor number dependence of MT transport velocity for dynein

3.1 Summary

Multi-motor cooperativity of dynein has previously been explained on the basis of multiple mechanisms. Here, gliding assays with minimal cytoplasmic dynein of yeast showed that the velocity of microtubules (MT) increases and saturates rapidly as the motor number increases. Both motor density and MT length influence MT transport properties, indicating a complex relationship between these factors and motor coordination. This suggests that motor cooperativity might involve factors beyond linker length and mechanical activation. These findings highlight the need for new models to explain motor coordination and cooperativity in minimal dynein systems.

3.2 Introduction

Dynein and kinesin are microtubule (MT) associated motor proteins involved in various cellular function. Cytoplasmic dynein is involved in vital cellular processes such nuclear positioning, mitosis, spindle positioning, nuclear migration, spindle assembly, intra-cellular transport, organelle transport and positioning [14]. Dynein plays an important role in assembling spindle by sliding apart anti-parallel microtubule filaments and help in focusing minus ends of microtubules towards the spindle pole [54, 95]. Pulling forces generated by dynein drive aster positioning and are required for segregation of chromosomes [22, 92]. Dynein is involved in vesicle trafficking from ER to Golgi, retrograde transport of mitochondria, phagosomes, endosomes, lipid droplets, peroxisomes and lysosomes (reviewed in [123]). Dynein can also sit on the cell cortex and generate pulling forces on MTs leading to positioning of organelles which are attached to the MT for e.g. nuclear positioning in *S. cerevisiae* [4, 16]. *In vivo* processes involve collective transport by team of dynein and show number dependence by working in a team of motors to overcome the shortcomings of single motor transport [90]. An example of *in vivo* number dependence of dynein includes processes such as male pronuclear migration in *C. elegans* [71]. A length dependent pulling of microtubules is seen when a dynein transporting a cargo exerts force on the microtubule in opposite direction. This viscous drag induced force is proposed to be dependent on the length of the MTs. such movement plays role in many *in vivo*. Length dependent pulling of MTs by cortically attached dyneins is also observed to play a role in nuclear positioning during *S.cerevisiae* [4, 16] mitosis and nuclear oscillations in *S.pombe* [162].

Since *in vivo* processes involve collective transport it is important to study collective behavior of motors along with the single molecular properties. Single molecular properties of both dynein and kinesin have been studied well *in vitro* [120, 51]. For studying the collective transport of motors either a cargo transport or a gliding assay

based approach is used. The minimal construct of yeast cytoplasmic heavy chain is the minimum chain of amino acids which shows single molecular processive motion similar to full length motor [120]. In a cargo transport based study where motor number on a DNA origami based cargo could be controlled, it was observed that the velocity of transport was unaffected by the number of dyneins transporting the cargo [25]. The dynein used was yeast cytoplasmic dynein truncated construct and the motor number was varied from 1 to 7 [25]. However in a gliding assay based set up where the motor number can exceed 7 motors (up to 60 [65, 100]), the velocity of the MT was observed to increase as the motor density increased. A gliding assay based study with a full length human cytoplasmic dynein complex showed that the velocity of MT transport increased with the number of dyneins transporting the MT [100]. This cooperativity of human cytoplasmic dynein has been explained on the basis of relieving of the auto-inhibitory ϕ conformation of the full length dynein due to stretching of the motor head during MT transport [100, 132] (see section 1.4.2). Full length bovine cytoplasmic kinesin complex on the other hand is known not to show motor number dependence of MT velocity [56]. However another study of *Drosophila* kinesin heavy chain showed reduction in the length of linker domain of kinesin results in decrease in velocity of MTs as the number of kinesins interacting with MT increases [11]. The extent of reduction of velocity was more for shorter linkers as compared to longer linkers. Thus for kinesin to show cooperative behavior and not show reduction in velocity at higher motor numbers it has to be loosely coupled (via linkers). This nature of kinesin can be explained by its inability to take coordinated steps when collectively transporting a cargo [82]. Hence motor number dependence for full length dynein complexes has been explained on the basis of relieving ϕ conformation and while for full length kinesins it has been explained by the loose mechanical coupling due to the flexible linkers.

In order to study the single molecular mechanism of motor cooperativity where linker flexibility and auto inhibitory conformations are not playing a role a controlled

study with a minimal dynein motor will be useful. Since truncation of motor tail domain can inhibit the formation of ϕ conformation [156], studies with minimal dynein construct can help us understand possible single molecular behavior of the motor head that is responsible for the cooperative behavior of dynein. Possible properties responsible for this cooperativity are asymmetric detachment behavior [100, 65] and force induced backward stepping of dynein [39]. Hence here dynein based gliding assays were reconstituted with a minimal yeast cytoplasmic dynein over a range of motor densities and the MT transport properties were studied over a wide range of motor numbers.

3.3 Methods

3.3.1 Protein Purification

Tubulin: Tubulin was purified from goat brain using activity cycling protocol as described for bovine brain [17] and used previously for goat brain [65]. Two freshly harvested goat brains (160 - 180 g of tissue) were homogenized in equal volume of DB (Depol buffer: 50 mM MES, 1 mM CaCl₂, pH 6.6) using a blender. The tissue homogenate was centrifuged at 20000 g for 1 hour at 4 degrees to get a clear lysate. The goat brain lysate was subjected to 2 cycles of activity cycling in presence of HMPB (1M PIPES, 10mM MgCl 20mM EGTA, pH 6.9) to ensure that active tubulin is enriched while removing MAPs [17]. The polymerization steps were performed in HMPB, 33% glycerol and 1.5 mM ATP and 0.5 mM GTP). After the second depolymerisation the tubulin was stored in monomeric form in BRB-80. Rhodamine labeled Tubulin was prepared by incubating polymerised tubulin with 5(6)-Carboxytetramethylrhodamine N-succinimidyl ester (Molecular Probes, Eugene, OR, USA) by incubating it with polymerized tubulin followed by a depolymerization-polymerization cycle similar to [17], to ensure that the activity of tubulin is maintained.

Yeast cytoplasmic dynein: Dynein construct used was GST-Dyn331 as described first by [120] was a kind gift by Dr. Andrew carter and Dr. Ron Vale. The minimal construct has protein A tag (ZZ tag) for purification and GST tag for dimerisation. It was expressed by growing a batch culture of yeast VY208 induced by 2% galactose (HiMedia, India) at 30° C. The cells were harvested after reaching OD600 of 3-5. The cell pellet was washed with MiliQ water. The cell pellet was frozen in liquid nitrogen drop by drop and crushed manually in a mortar and pestle. The frozen powder was then stored at -80° C till further use. Cell lysate was prepared by rapidly thawing the frozen power at 37° C. The lysate was clarified by spinning at 65000g. The soluble fraction was harvested and allowed to incubate with IgG sepharose beads(GE healthcare, Sweden) for 1 hour at 4° C for binding of dynein to the beads. The IgG beads were washed with buffer and incubated with TEV protease overnight to cleave dynein from the beads. Dynein was collected in the supernatant after removal of the beads and store at -80° C after flash freezing in liquid nitrogen.

Anti-GFP Nanobody: pGEX6P1-GFP-Nanobody was a gift from Kazuhisa Nakayama (Addgene plasmid 61838; http://n2t.net/addgene:61838;RRID:Addgene_61838). The plasmid was transformed in *E. coli BL 21(DE3)* and expressed using 1 mM IPTG at OD600 of 0.5 at 30° C for 4 hours. The protein was purified from the clarified lysate using glutathione sepharose (GE healthcare) and eluted using 10mM reduced glutathione. The protein was dialysed overnight to remove the reduced glutathione using a Snake Skin Dialysis Tubing with 10000 KDa cut off (Thermofischer).

EGFP Purification: The plasmid expressing EGFP was a kind gift from Dr. Thomas Pucadyil. The plasmid was transformed into *E. coli BL 21(DE3)* and expressed using 1mM IPTG at OD600 of 0.5 for 12 hours. The protein was purified from the clarified cell lysate using Co-NTA resin (Thermo Scientific, IL, USA) followed by elution using 100 mM imidazole (HiMedia, India). The protein was dialysed overnight to remove the imidazole using a Snake Skin Dialysis Tubing with 10000 KDa cut off

(Thermo Fischer Scientific).

3.3.2 Microtubule gliding assay

Rhodamine labeled MTs were prepared by polymerizing 20 μ M unlabeled tubulin with 10 μ M rhodamine labeled tubulin in BRB80 at 37° C. The MTs were stabilized by addition of Taxol (20 μ M). The monomers were removed by pelleting the MTs over a 60% glycerol cushion at 1,35,300 g in TLA 100.3 (Beckman Coulter, CA, USA) for 18 minutes. The MT pellet was re-suspended in BRB-80 containing 20 μ M taxol and used immediately.

The gliding assay was performed in a flow-cell constructed of a 1.5 cover-slip adhered to a slide by two parallel strips of double backed tape (Kapton, polyimide tape), creating a gap of 7 mm. The chamber had a dimensions of 22 mm x 7 mm x 0.01 mm. Anti GFP nanobody (1 mg/ml) was flowed in the chamber and incubated for 8 mins. The unbound antibodies were washed with Lysis Buffer (30 mM HEPES, 2 mM Mg-Acetate, 50 mM K-Acetate, 1 mM EGTA, 10 Glycerol). The surface was blocked by casein (1 mg/ml) for 8 minutes. Dynein was flowed in and allowed to incubate for 8 minutes followed by washed with lysis buffer to remove the unbound dynein. Dynein bound surface was imaged in FITC channel at 200 ms exposure for estimation of dynein density. Rhodamine labeled MTs were flowed in and allowed to boind until sufficient MTs are visible on the surface. The unbound MTs were washed using wash buffer (Lysis Buffer + 20 μ M Taxol). The gliding was observed after addition of motility buffer (Lysis Buffer + 4 mM ATP + Antifade mix[0.005 mg Glucose Oxidase, 0.0015 mg Catalase, 7.2 mM Glucose in 100 μ l 10x Phosphate buffered saline, PBS (SRL Chemicals, Mumbai, India)]). All assays were performed at 37°C. All reagents unless otherwise stated, were from Sigma-Aldrich, MO, USA.

3.3.3 Density Estimation of Motors

The surface density of dynein in motors per unit area (μm^2) was estimated based on the principle that the dynein is surface-immobilized via the anti-GFP nanobody and an image in one plane of the epifluorescence microscope can provide an estimate of the number of molecules after calibration of the PSF and intensity to molecules conversion with a GFP standard, as previously described [65]. Such an approach is based on comparable approaches used to estimate the surface-density of myosin [53, 163]. To this end, a range of concentrations of bacterially expressed and affinity purified 6xHis-eGFP molecules were introduced into the flow chamber and imaged. The number of molecules in the field of view was estimated as the product of the image volume and independently measured protein concentration. The image volume (cuboid) is the product of the PSF (height) and image area (in 2D). The PSF was estimated by measuring the fluorescence intensity (I_z) along the z-axis (z) of $0.1 \mu\text{m}$ diameter TetraSpeck beads (ThermoFisher Scientific, IL, USA). The data from multiple beads was fit to the equation $I_z = a + (b - a) \cdot e^{-\frac{(z-c)^2}{2 \cdot d^2}}$ (Figure 3.3 A-C) where a , b and c are scaling parameters and d is the standard deviation of the fit created using the Curve Fitter function in FIJI 1.54 [94]. The PSF is $1.45 \mu\text{m}$, based on the relation $PSF = 2.35 * d$ [111]. The image volume was calculated using the PSF and the image area followed by measuring the fluorescence intensities (grey values) of a known series of concentrations of purified EGFP. The fluorescence intensity of the EGFP dilution series on cover-slips was used to plot a calibration curve of intensity of the image (y-axis) as a function of protein surface density (x-axis) and fit to a straight line (Figure 3.3 D). The image intensity of dynein-GFP from the cover-slip surface was used to estimate monomer density resulting in a factor of 0.5 for the dynein dimer density. The resulting motor densities obtained from different dilutions of the stock protein ranged from 5.25 to $62.86 \text{ motors}/\mu\text{m}^2$, with control experiments performed without motors.

3.3.4 Microscopy and image analysis

The movies were recorded in Nikon Eclipse TE, 60 X Oil (NA) magnification, TRITC filter. The images were taken every 10 seconds for 10 minutes. The images were de-noised by applying a median filter and subtracting background using ImageJ. The MT plus ends were tracked using FIESTA and MT lengths were measured using the measure function of FIESTA. For figure 3.2 the MT plus ends were tracked interactively using a FIJI plug in MtrackJ.

3.3.5 Data analysis

The kymographs were generated manually using the Multikymograph feature of FIJI. All the plots were plotted using Python matplotlib. The T test were performed on the datasets using the `ttest_ind` function of the `scipy.stats` package in python.

3.4 Results

3.4.1 Effect of motor density on MT transport

In order to test the effect of motor density on MT transport gliding assays were performed with increasing surface density of dynein (Figure 3.1 A). The motor used for this study is yeast cytoplasmic dynein minimal construct mentioned previously [120], due to its well characterized single molecular properties. The motor has a GFP on its tail hence an anti-GFP nanobody was used to specifically immobilize the dynein to the cover-slip via its tail to ensure that all the bound motors are in correct orientation to be able to transport MTs. This ensures that all the motors participate in the transport of MTs as direct immobilization of motors on glass can cause denaturation and doesn't control the orientation leading to an overestimate of number of motors involved in the transport. Motor density on the surface was estimated using a previ-

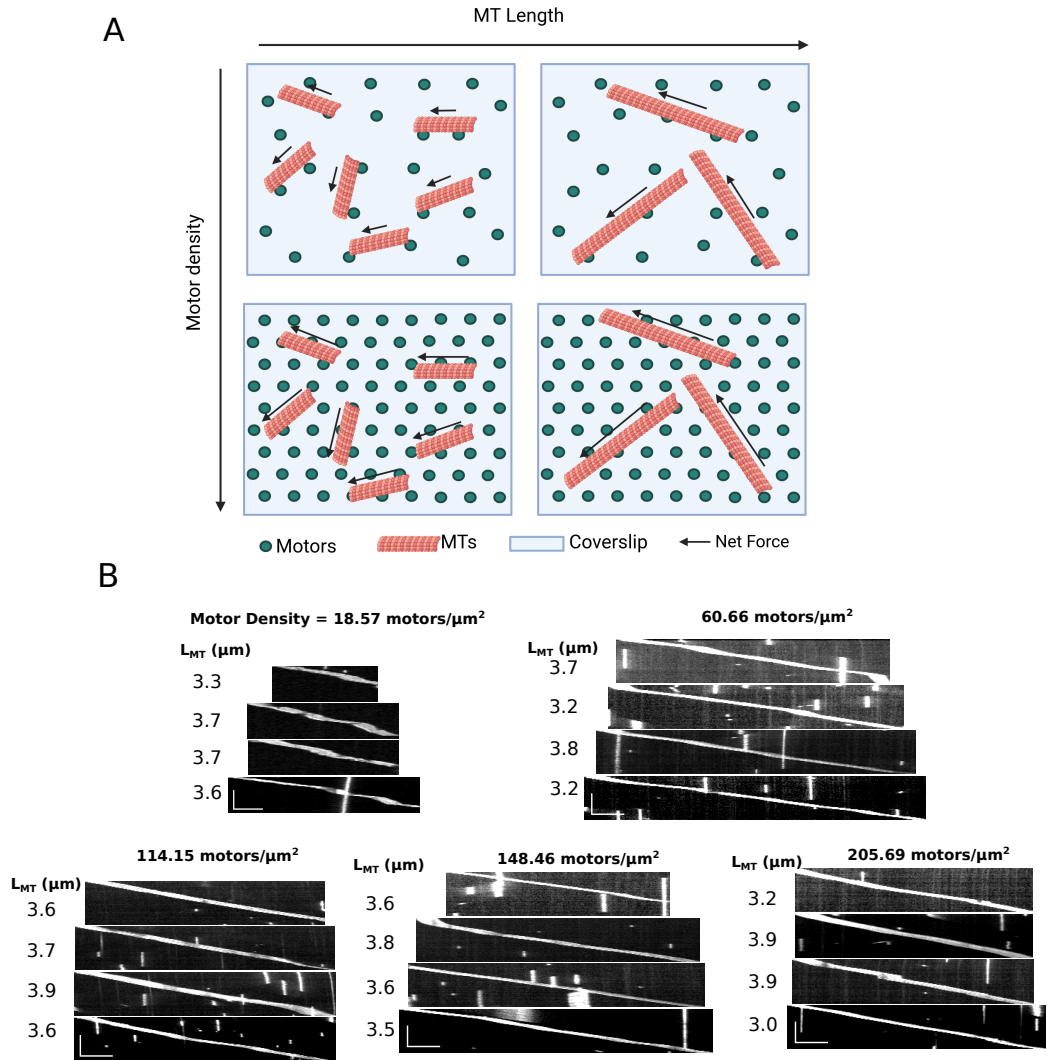


Figure 3.1: Effect of increasing dynein density on MT transport. (A) Schematic representing how the effective motor number acting on MTs can vary with change either MT length or motor density or both. Increasing motor number increases the net force (black) acting on the MTs which is represented by arrows of varying length (not to scale). (B) The figure show representative kymographs of MTs (Length = 3-4 μm) at dynein densities of 18.57, 60.66, 114.15, 148.46 and 205.69 motors/ μm^2 . [Scale bar x: 5 μm y: 5 min].

ously described microscopy based approach [65]. In presence of 2 mM ATP the motors perform MT gliding. When the kymographs of MTs of same length range ($L_{MT} = 3\text{-}4\ \mu\text{m}$) were generated and compared, it was observed that at lowest motor density ($\rho_m = 18.57\ \text{dyneins}/\mu\text{m}^2$) the MT travel for shorter distance with stalls suggesting hindered motion (Figure 3.1 B). However as the motor density increases ($\rho_m = 60.66 - 148.46\ \text{dyneins}/\mu\text{m}^2$) the MT transport appears smoother without any stalls. The MTs travel a longer distance at $\rho_m = 60.66 - 148.46\ \text{dyneins}/\mu\text{m}^2$ as compared to $\rho_m = 18.57\ \text{dyneins}/\mu\text{m}^2$. Since the motor density increases the number of motors attached to the MTs also increase. The increased path length without stalls with increasing density suggests that as the motor number increases the MT movement becomes processive. However at the highest motor density $\rho_m = 205.69\ \text{dyneins}/\mu\text{m}^2$ the MTs appear to have traveled a shorter distance but without any stall events. Motor number can vary due to either motor density or the MT length.

3.4.2 Effect of MT length and motor density on MT transport

To study the systematic effect of both MT length and the motor density on the MT transport properties, the plus ends of MTs of varying length were tracked and compared across densities (Figure 3.2). The MTs at density $18.57\ \text{dyneins}/\mu\text{m}^2$ were tracked for all the frames they glide since they detach and disappear after few frames. While for densities $60.66\ \text{dyneins}/\mu\text{m}^2$ onwards the MTs are tracked for the entire course of the movies recorded as they do not detach after a few frames due to sufficient number of motors being present on the surface. This is to ensure that the comparison between the datasets is not biased due to MTs not being tracked for a few frames. At the lowest density MT transport shows diffusive nature especially for shorter MTs ($L_{MT} = 0\text{-}5\ \mu\text{m}$) which becomes processive as the MT length increases ($L_{MT} > 5$). For $L_{MT} = 0\text{-}2$ and $2\text{-}5\ \mu\text{m}$ the MTs detach and diffuse away after a few frames. As the MT length increases the number of motors interacting with the MT increases causing

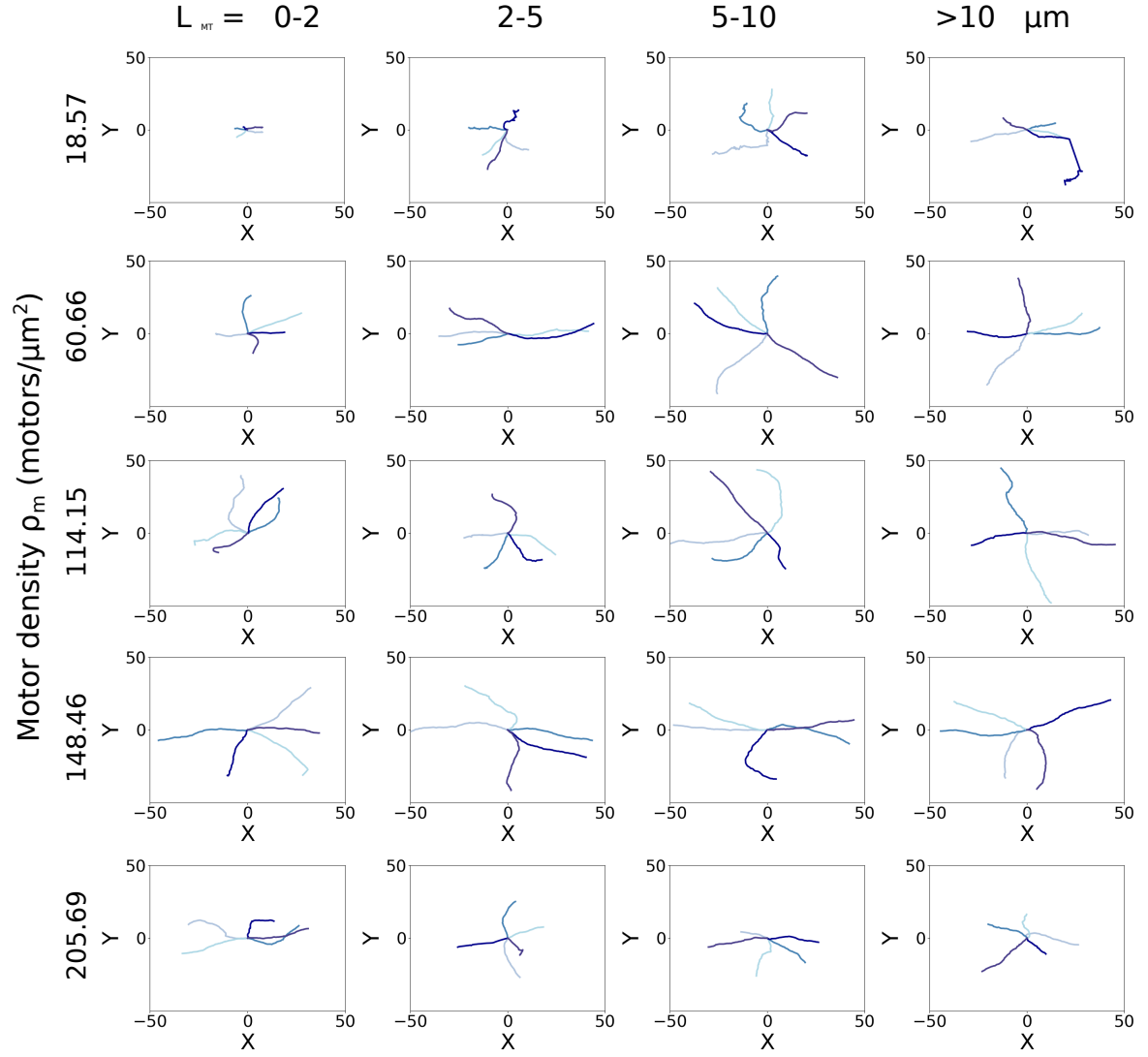


Figure 3.2: **Change in 2D trajectories of MTS based on filament length and dynein density.** The XY trajectories of representative MT (plus ends) of gliding MTs driven by dynein are plotted with their start point normalized to the origin. (*Left to right*) Increasing MT lengths (L) 0-2, 2-5, 5-10, >10 and (*top to bottom*) increasing dynein density ρ_m ranging from 18.6 to 205.7 motors/ μm^2 . X and Y-axes in units of μm . Colors represent individual trajectories.

longer transport. The directness of transport increases with length at this density. For motor densities 60.66 dyneins/ μm^2 and above, all the lengths show processive and directed movement however the distance traveled by the MTs can be seen to be affected. The distance traveled by the MTs increases as both the MT length and motor density increase for motor densities 18.57 to 114.15 dyneins/ μm^2 . At the motor density of 148.46 dyneins/ μm^2 the transport seems to be independent of length. At motor density 205.69 dyneins/ μm^2 the tracks are shorter as the MT length increases suggesting that the velocity reduces as the MT length increases at this density which in contrast to rest of the densities.

3.4.3 Length dependence of MT velocity

The average velocity of all MTs at given motor density varied (Figure 3.4) however varying MT lengths would imply varying motor numbers for the given motor density, the MT velocity would vary with the length. To study the effect of increasing motor number on the velocity of the MT the MT plus ends were tracked using FIESTA and average velocities of MTs were plotted as a function of MT length for various dynein densities (Figure 3.4 A-E). At the lowest density i.e. 18.57 dyneins/ μm^2 the MT velocity can be seen increasing with increasing MT length. At density 60.66 dyneins/ μm^2 the velocity increases with MT lengths but with a higher slope indicating that the velocity saturates at $L_{MT} > 5 \mu\text{m}$. For motor densities 114.15 and 148.46 dyneins/ μm^2 the velocity seems to be saturated and length has no effect. The highest motor density the velocity seems to be unaffected by the MT length but the average velocity is lower as compared to density 60.66-148.46 dyneins/ μm^2 . To study the effect of motor number on velocity we used a band area based assumption to calculate the number of motors along a filament using the L_{MT} , W_{MT} and ρ_m (Figure 3.4 F). Previously a square lattice based assumption has been used [65] using the L_{MT} and ρ_m . Motor number generated from the length and density was used to plot velocity vs

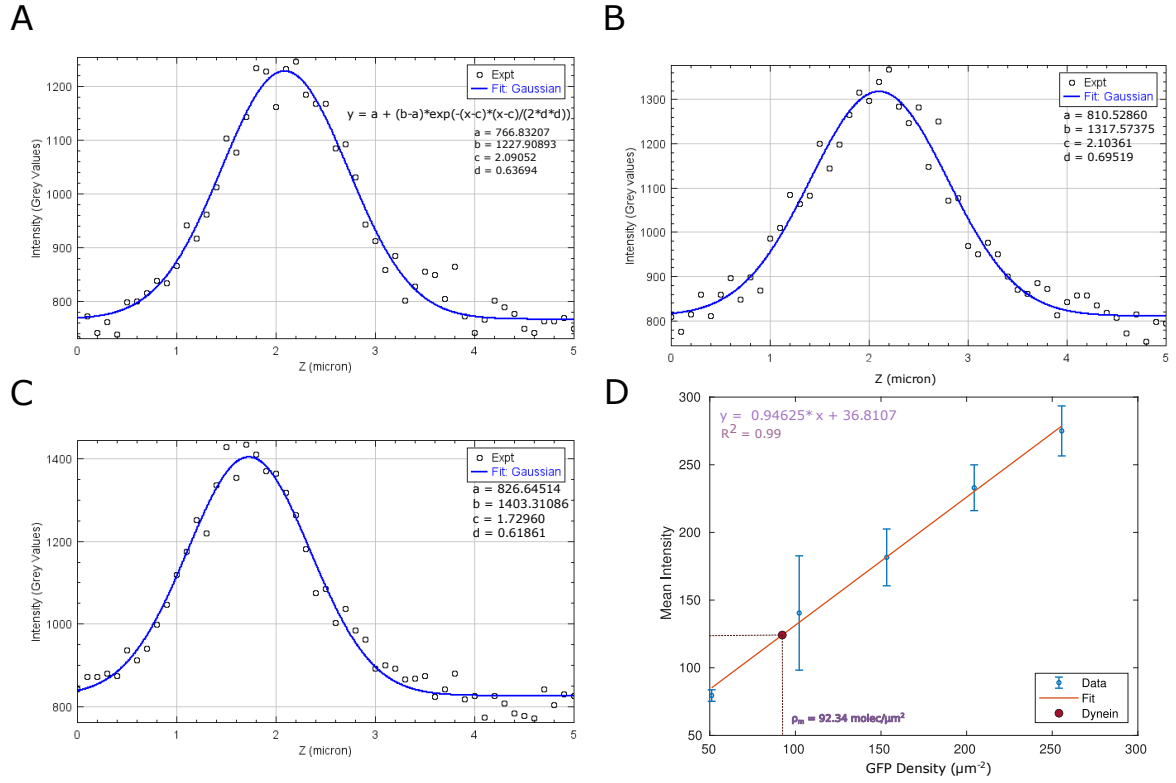


Figure 3.3: Dynein density estimation using EGFP standard calibration curve. (A-C) Representative plots of z profile of fluorescence intensity of three $0.1 \mu\text{m}$ beads (black circles) fit to a Gaussian profile (Blue) using the equation displayed on the graph (a). This plot was used to estimate the PSF as described in the Materials and Methods section. (D) The mean image fluorescence intensity in gray values (blue circles) with error bars representing s.d. (y-axis) is plotted as a function of GFP equivalents of an EGFP concentration series (x-axis). The linear fit (red line with equation) was used to calculate the number of dynein molecules (red circle) in the system. R^2 indicates the regression coefficient.

motor number plot. The plot shows that the velocity seems to be saturating rapidly as the motor number reaches 20 followed by a slight decrease for $205.69 \text{ dyneins}/\mu\text{m}^2$ motors.

3.4.4 Directed motion dominates at higher densities

In order to measure the directionality of the MT transport we calculated the tortuosity (distance/path-length) for all the MTs at each motor density. The tortuosity was plotted as a function of MT length for all the motor densities (Figure 3.5) where it was

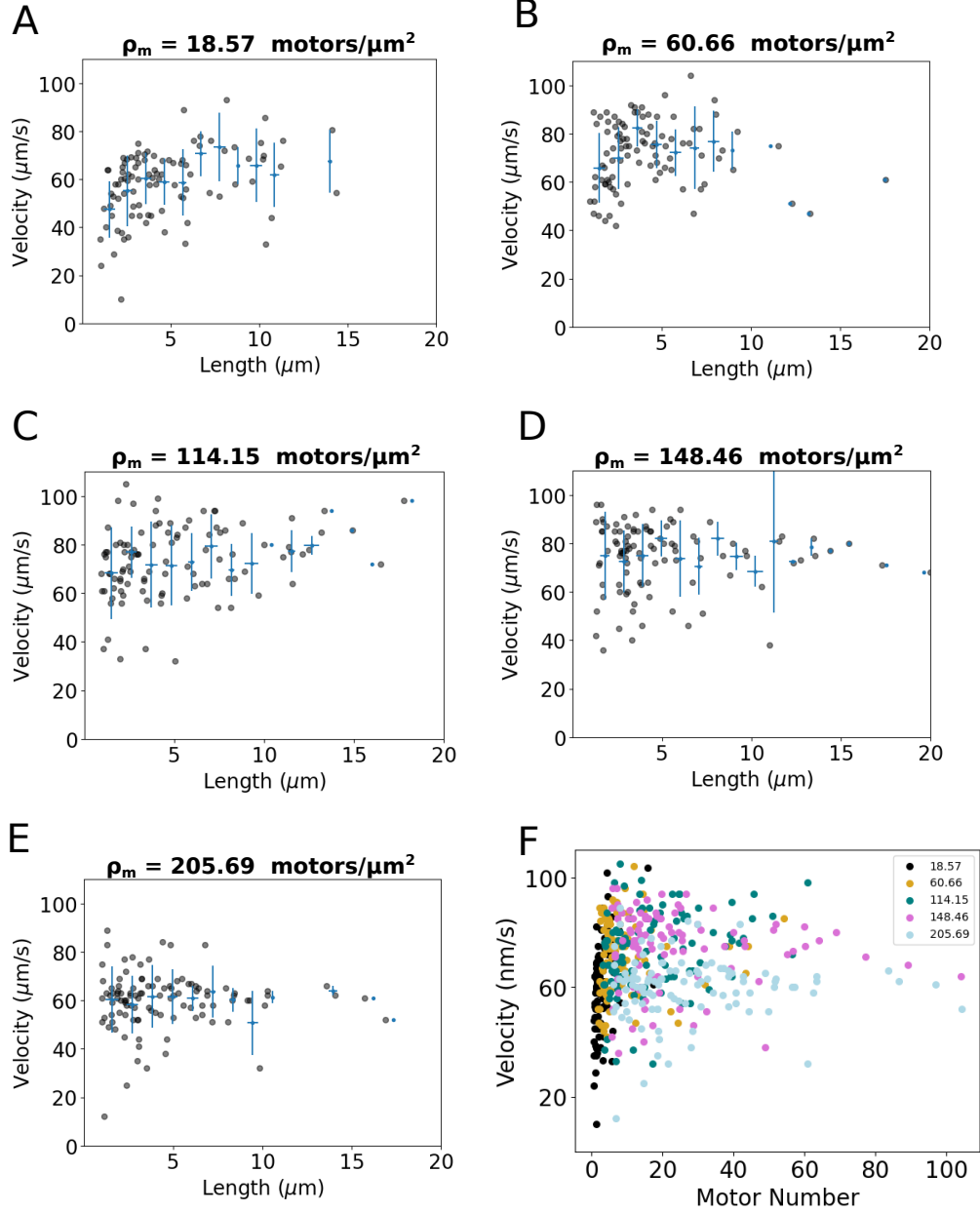


Figure 3.4: **Effect of MT length on time-averaged velocities of individual MTs for a range of motor densities.** (A-E) The time averaged velocity of individual MTs plus ends obtained from filament tracking as a function of MT length (grey circles) are plotted for each motor density. The data was further averaged for MT length (bin width = $1 \mu\text{m}$) and values are plotted as means (blue circle) with y- and x-errorbars representing $\pm$ s.d. (F) The time averaged velocity for MTs for all the motor densities are plotted as a function of motor number, calculated from the motor density, MT length and MT width ($N_{motors} = L \times W \times \rho_m$) as described in the methods.

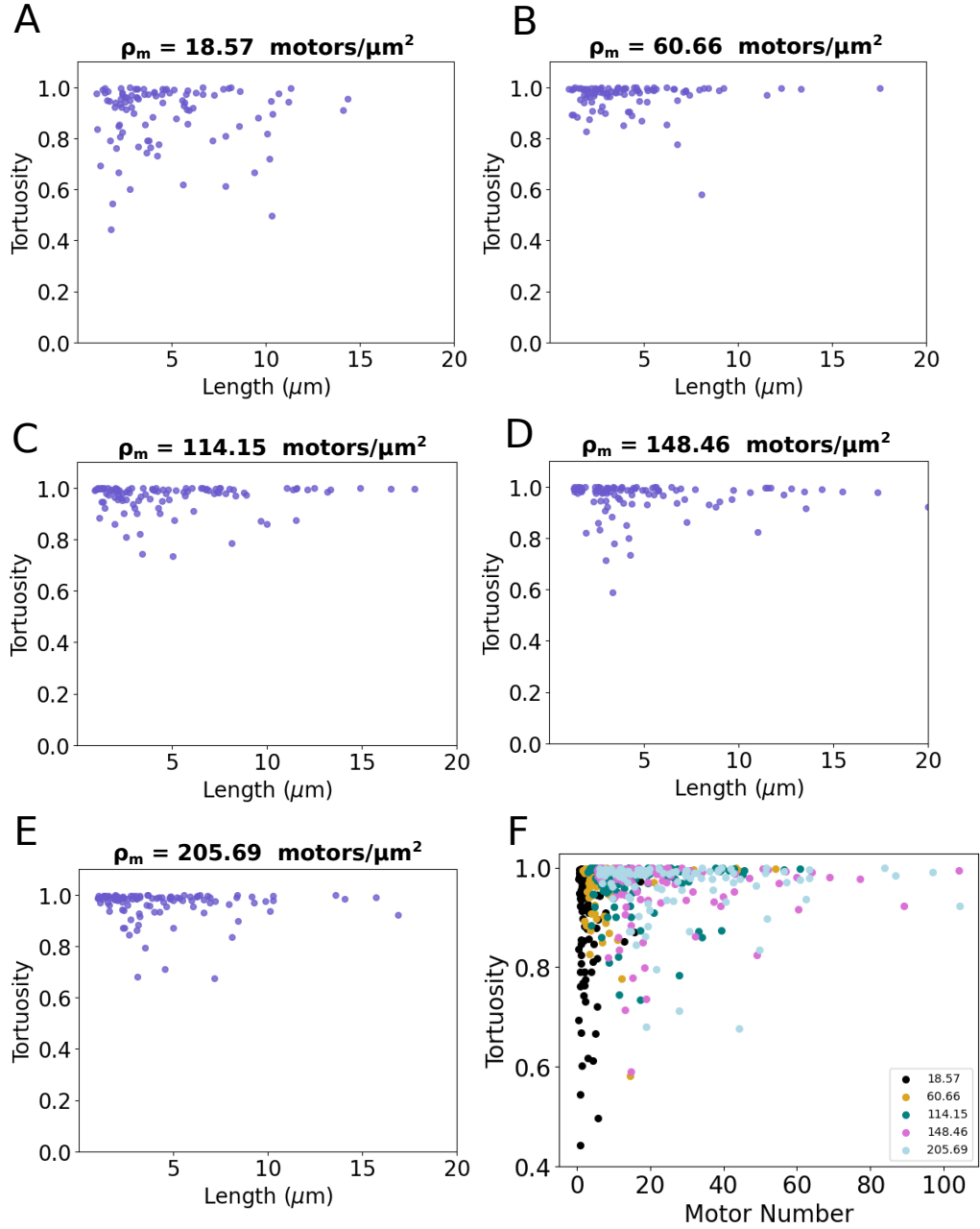


Figure 3.5: **Directionality of MT transport as a function of MT length for increasing motor density.** (A-E). Tortuosity (directionality) of individual MTs was calculated from the tracked plus ends of MTs using the formula $\text{tortuosity} = \text{distance}/\text{displacement}$ and plotted as a function of the MT lengths for all the densities. (F). Tortuosity of all the MTs plotted as a function of motor number. Colors indicate dynein density

observed that only the lowest density shows the length dependence of tortuosity while densities higher than $\rho_m = 18.57$ dyneins/ μm^2 show directed movement independent of length. Motor number can be calculated from the MT length and motor density as mentioned above. By plotting the tortuosity of all the MTs as a function of motor number it was observed that it saturates rapidly as the motor number increases from 1-10. This indicates that >10 motors are enough for directed transport of MTs.

3.4.5 MT velocity increases and rapidly saturates as the motor number increases

To study the effect of both MT length and motor density on the transport velocity of MTs, average velocity for each length category as a function of motor density was plotted (Figure 3.6 A-D). For $L_{MT} = 0-2 \mu\text{m}$ the velocity increases significantly as the motor number increases from 18.57 to 60.66 dyneins/ μm^2 . The velocity then saturates for density till 60.66- 148.46 dyneins/ μm^2 . At the highest density the velocity decreases slightly. For $L_{MT} = 2-5 \mu\text{m}$ the velocity first increases significantly with density, then saturates and then reduces significantly at the highest density similar to $L_{MT} = 0-2 \mu\text{m}$. The MT density doesn't affect the velocity for MT lengths higher than 5 except for the highest density. Each length category has a different behavior of MT velocity with increasing density (Figure 3.6 E). To test whether the effect arises from motor number alone the MT lengths were converted to the motor number. Binned average velocity for each length category was plotted as a function of average motor number for each length category (Figure 3.6 F). The behavior of average velocity with motor number follows a saturation fit. However the motor number transporting a MT can be varied either via motor density or MT length.

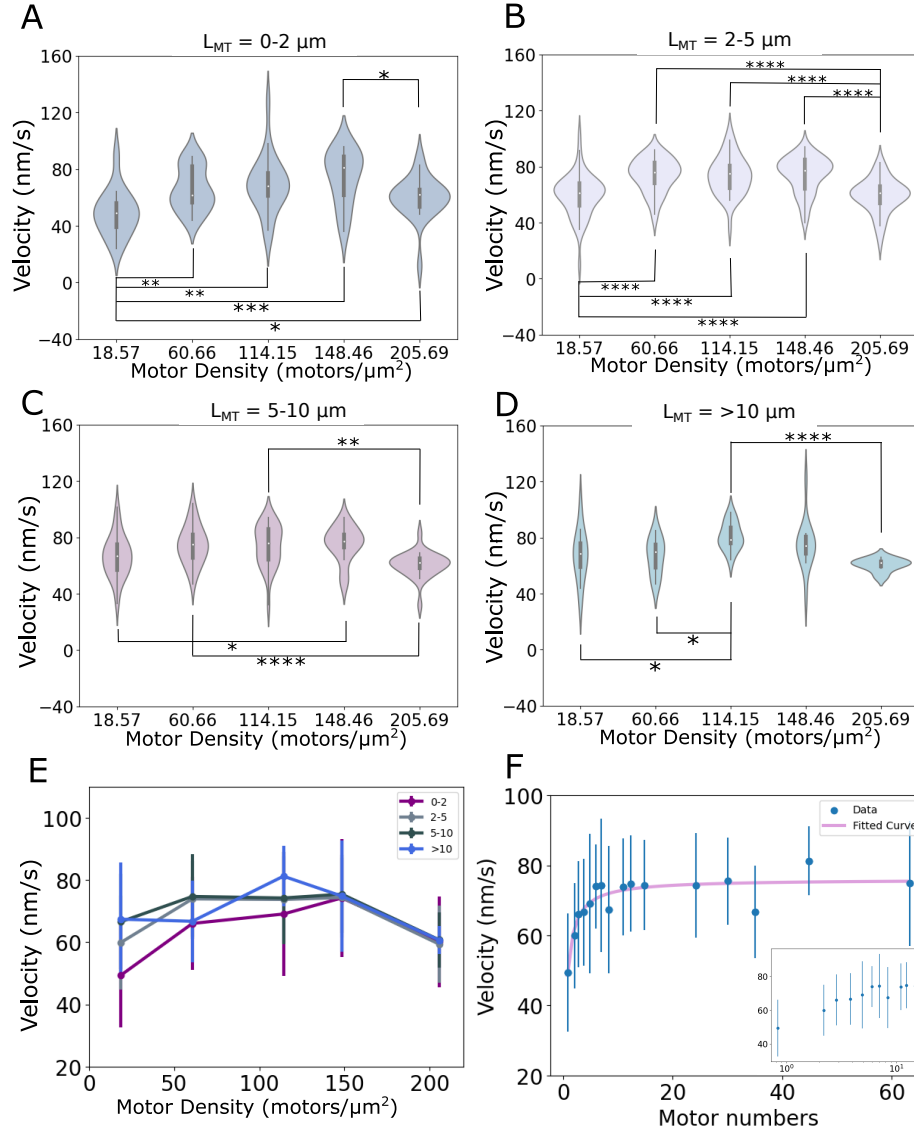


Figure 3.6: **Average velocity of MTs as a function of motor density for different lengths and motor numbers.** (A-E) The violin plots shows average velocity of MTs as a function of motor density for various MT length categories (0-2, 2-5, 5-10, $>10 \mu\text{m}$). (E) Average velocity of MTs as a function of motor density for all the length categories. (F). Average velocities of MTs were averaged further for each MT length category and plotted as a function of average motor number calculated for the respective MT length category (Blue) using the band model. The data was fit by a Saturation function (Magenta). **** = p values < 0.0001 . The inset shows velocity vs motor number on a semilogx scale till motor number 20.

3.4.6 Asymmetric detachment model qualitatively explains the motor number dependence of MT velocity

In order to test the effect of increasing motor number via either motor density or the MT length the average velocity for each length category (Figure 3.7 A) and for different motor densities (Figure 3.7 B) was plotted as a function of motor numbers. It was observed that the velocity shows the similar saturation with motor number. Hence varying the motor number via either motor density or MT length gave a similar output. These plots were further compared with existing models of motor number dependence (Figure 3.7 C-D) adapted from [100]. The study on human cytoplasmic full length dynein collective behavior was compared to computational models of gliding assay with or without the mechanical activation of motors by stretching. The collective behavior observed for the minimal yeast cytoplasmic dynein in this study shows a rapid saturation of MT velocity with the motor number as compared to the no mechanical activation model of previous study [100]. In order to understand which the single molecular behavior affect the cooperativity of dynein the details of the models used previously are helpful. The no mechanical activation model mainly has asymmetric detachment behavior of dynein implemented in the model. Hence without the mechanical activation in the picture the velocity shows a rapid saturation with the motor number and comparison to previous models suggests that asymmetric detachment behavior of the motor could be responsible for the cooperative behavior of dynein. However this can be confirmed using *in silico* gliding assay with and without the asymmetric detachment of motors. Slowing down of motors or saturation of the velocity at higher motor numbers can be explained on the basis other models such as loose coupling between the ATPase cycle and dynein movement. Dynein has been shown to reduce its step size under higher loads [165]. A theoretical model explaining the loose coupling between the chemo-mechanical cycle has been used to explain slowing down of dynein under

high external force [37]. At higher motor density the opposition due to motors which are not actively stepping can cause higher opposing forces on motors that are actively stepping. Detailed *in silico* models would be helpful in confirming the role of asymmetric detachment and loose chemo-mechanical coupling in explaining the collective behavior of dynein.

3.5 Discussion

Here gliding assays with yeast cytoplasmic dynein minimal construct were reconstituted over a wide range of motor densities and the dependence of MT transport velocities on motor number, motor density and MT length was studied.

Dynein is known to be in an auto-inhibitory conformation (ϕ conformation) where the dynein heads are stacked and hence do not produce processive movement [176, 156]. The ϕ conformation of dynein is a result of self dimerisation of the motor heads through the linker domain [176]. The affinity of the MTBDs to MTs reduces while in the ϕ conformation, resulting in weak diffusive binding of dynein to the MT. The activation of dynein from the conformation requires separation and orientation of the motor heads by attachment of dynactin [176] or by mechanically separating the motor heads by a rigid linker [156]. Based on the regulation of dynein activation and processivity by mechanical separation of the motor heads, the mechanical activation model of dynein has been used to explain the motor cooperation [100, 132]. The increase in MT gliding velocity with increasing motor density of full length human cytoplasmic dynein was proposed to be due to stretching induced mechanical activation of motors during the gliding. Which results in increasing number of motors available to actively transport the MTs as the motor density increases. Yeast cytoplasmic dynein minimal construct used here has been described as the maximally tail truncated dynein which still shows single and collective properties similar to the full length dynein [120]. In human dynein

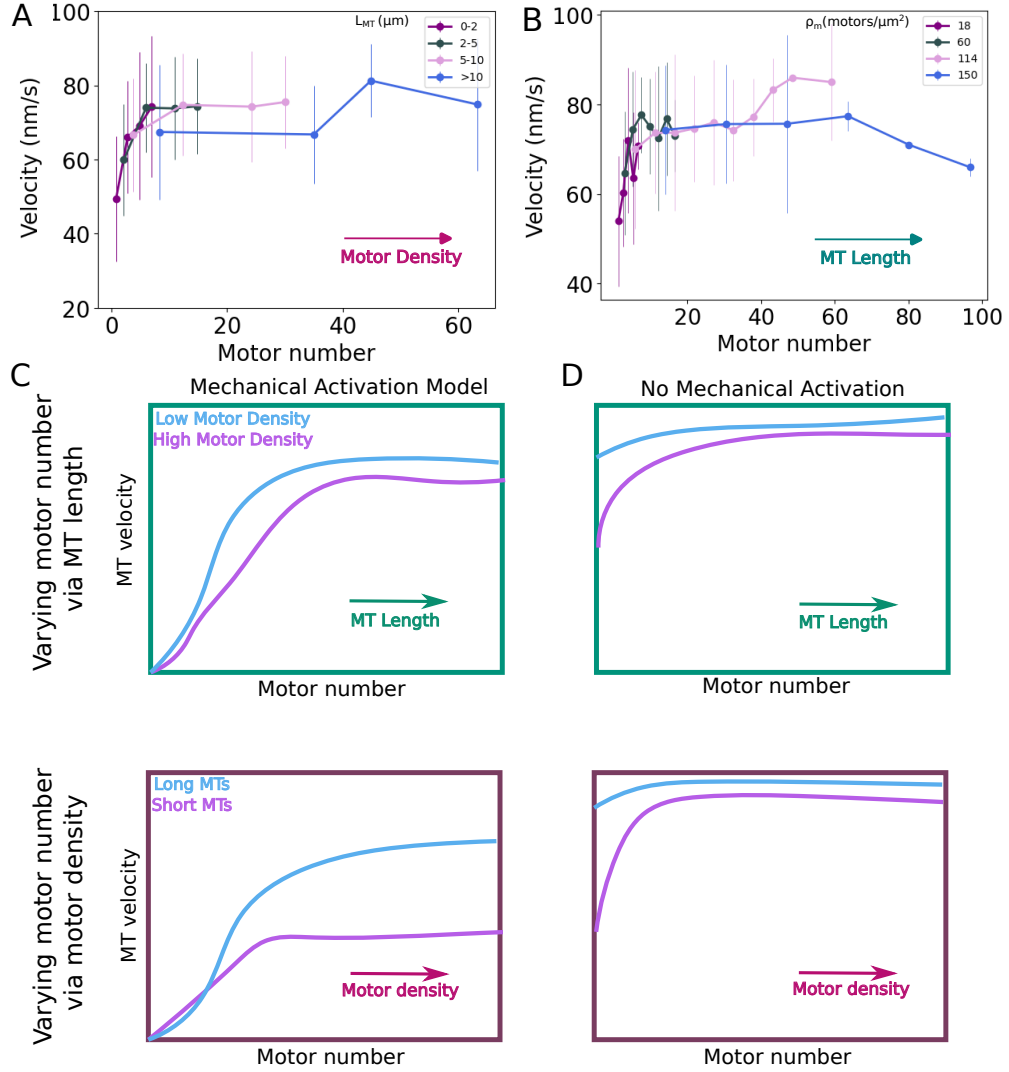


Figure 3.7: **Comparing experimental velocity dependence on motor number to model expectations.** The average filament velocity (mean $\pm$ s.d.) is plotted as a function of motor number for (A) each length category (colors: lengths) and (B) for different motor densities (colors: motor density). Effectively the motor number is varied either through (A) the MT length or (B) the motor density. (C,D) Schematic representing the expected dependence of MT velocity with increasing motor number from either the (C) mechanical activation model or (D) a passive detachment behavior as proposed by [100]. The velocities increase either by from (*top*) increasing MT length or (*bottom*) increasing motor density.

such truncation was observed to prevent folding of dynein in the ϕ conformation [156]. Hence the dynein in this study is unlikely to be in an auto inhibited state and hence the mechanical activation cannot take place. Such minimal dynein can provide a means to study motor-motor and MT-motor coordination since two step activation and presence of co-factors is not required. As hypothesized the MT velocity vs the motor number for yeast cytoplasmic dynein matches qualitatively with the no mechanical activation model (Figure 3.7 (C) upper) for both the cases where the motor number is varied via the motor density for a given MT length as well as when the motor number is varied via the MT length. Studies on yeast cytoplasmic dyneins showed that dynein shows force induced bidirectional stepping in absence of ATP [39]. This force induced stepping of dynein can be used to explain why dynein is better at working in groups. The dynein that is currently not in its stepping cycle (out of phase dynein) is tightly bound to the MT (see section 1.4.2) can hinder the movement of actively stepping motors. Such out of phase dyneins can step along a moving microtubule passively due to force induced backward stepping and hence do not oppose the activity of other dyneins [39]. Along with the force induced stepping the asymmetric stepping behavior of dynein has also been hypothesized to drive cooperativity. Since the models used for comparison also consider the asymmetric detachment behavior of dynein it is once again suggestive of cooperativity arising as a result of this single molecular property.

At the highest motor density of 208 motors/ μm^2 the MT velocity was observed to be significantly lower than motor densities of 60.66 - 148.46 motors/ μm^2 for all categories of MT lengths (Figure 3.6 A-D). Such decrease in velocity could be explained by high degree of asynchrony and tight coupling. At high motor density the proportion of motors which are not in their stepping cycle can be higher. Such out of sync motors can hinder the MT movement driven by actively stepping motors leading to more opposing force. This mechanical coupling between the motors can be relieved by having flexible neck linkers as observed for kinesin [11] or by taking passive backward steps

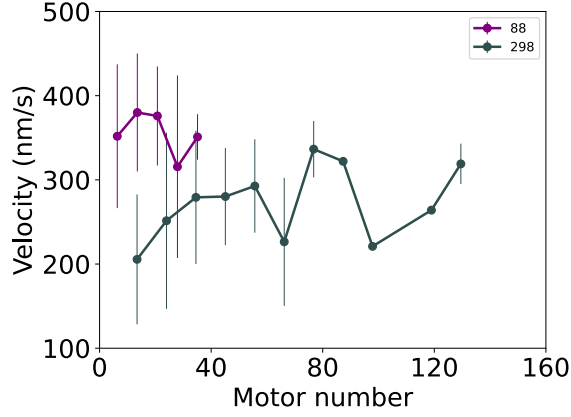


Figure 3.8: **Number dependence of velocity for kinesin.** The average filament velocity was binned average with a constant bin width and plotted (mean $\pm$ s.d.) as a function of motor number for gliding assays with kinesin (Kinesin k560) performed at motor densities 88 and 298 motors/ μm^2 . The colors represent the motor density. ($n = 50$ MTs for each density)

under opposing loads as observed for dynein [39]. Hence cooperativity can be a result of overcoming the hindrance of out of sync motors even when the number of motors involved in the system is high. However, this will have a limit and hence at very high motor densities the velocity can be seen to be reducing. Another possible explanation for such decrease in velocity can be due to dynein taking shorter steps under higher forces [88]. If force measurement methods such as optical tweezer is employed to confirm the presence of higher loads on motors in high motor density gliding assays then this gear-like behavior of dynein can be attributed to reduction in the collective velocity of dynein. The role of asynchronous stepping, tight coupling and gear-like behavior of dynein in collective MT transport can be studied with the help of *in silico* models.

There is increase in the average velocity observed as the motor number increases from 1-10. The initial rapid increase in the average velocity can be a result of reduction in the diffusive behavior of the MTs as not enough motors are able to bind to the MTs at lower motor densities. The diffusive nature of the MT transport can be seen in the MT tracks at the lowest motor density. However a systematic comparison between the average and instantaneous velocity of the MTs would be useful to test this hypothesis.

Previously in a cargo-based assay, the cargo velocity was observed to be unaffected when the motor number was varied from 1 to 7 [25]. This difference can be explained by the differences in the experimental strategy used. Since in the cargo based studies the motors do not fall off the cargo, only the run length is affected by motor number and not the velocity. In a gliding assay based set up at very low motor densities, short MTs were seen to be diffusing away after traveling shorter distance. This could be due to motors not being able to capture the MT as they are spaced apart. Hence by using a minimal dynein construct and studying the MT velocity behavior with motor number various possible mechanisms responsible for the collective behavior of dynein were explored and compared to the previous literature. Both experiments and models currently do not account for dead motors and assume that all motors are functional. The presence of dead motors can result in binding of motors to the MT but no stepping. This can hinder the motion of MTs driven by actively stepping motors and can cause jagged motion of MT. In multi-motor gliding assays molecular crowding can play an important role in pattern formation. Kinesin has been seen to drive MT vortices and loops in a gliding assay-based set-up in presence of high MT density [84]. Presence of crowding agents can affect the formation of patterns due to depletion effect [60]. High motor density on the other hand is limited by the surface area in gliding assays. In single molecular assays where MTs are fixed and labeled motors are observed, kinesin-1 has been studied at high motor density [81]. It was observed that low processivity favors the ability of motors to function in a crowded environment. Hence the effect of molecular crowding on motor-MT systems can be studied in multiple ways. For studying the effect of molecular crowding on motor coordination, single molecule TIRF assay of dynein at high motor concentration and MT gliding assays in presence of crowding agents will be useful.

Similarly, preliminary studies have also been performed to study the number dependence of kinesin on a range of motor densities. From a set of gliding assay where the

motor density was around 88 and 298 motor/ μm^2 , the velocity of MTs was plotted as a function of the motor number however no distinct dependence of velocity on the motor number was observed (Figure 3.8). This is in agreement with the previous studies of kinesin.

3.6 Conclusions

Hence yeast minimal cytoplasmic dynein which is not capable of undergoing an auto-inhibitory conformation shows a motor number dependence of velocity in gliding assays. The rapid increase in speed with the number of motors is suggestive of mechanisms other than mechanical activation that contribute to the cooperativity of the motors. By comparing the data to existing models we find that asymmetric detachment behavior of dynein can qualitatively explain the cooperativity observed.

Chapter 4

Wave-like oscillations of clamped microtubules driven by collective dynein transport

4.1 Summary

Previous studies on flagellar beating with kinesin lacked specificity in MT immobilization and control over MT length and density. A minimal *in vitro* system using yeast cytoplasmic dynein was developed to drive single MT oscillations by clamping the MT plus end to the surface, with ATP addition initiating oscillation. Three types of oscillatory patterns were observed: fluctuations, beating, and looping, with MT length, motor density, and MT pinning jointly determining the nature of these patterns. This minimal MT-motor system could be used in the future to further explore the role of motor and MT mechanics and geometry in flagellar beating.

4.2 Introduction

Flagellar beating is a complex phenomenon arising from MT-motor interactions. The flagellum consists of microtubule doublets arranged in 9+2 manner, where the 9 outer filaments are arranged cylindrically and the remaining two filaments form the central pair (CP) situated in the centre [83]. A nexin link connects each outer doublet to the next one while the outer filaments are connected to the CP via radial spokes [83]. Dynein provides the molecular stroke needed for the beating of flagella [42]. Stepping of dynein on the adjacent MT doublet is said to cause sliding of MT doublets [151]. Such sliding was observed when trypsin treated sea urchin flagella were subjected to ATP treatment [151]. The conversion of such MT sliding to MT bending and beating was attributed to the presence of cross linking factors such as nexin and spoke protein which cross link the MTs [151]. Trypsin treatment removes the proteins causing MT to slid apart due to the stepping of dyneins. The mechanism due to which MT sliding is converted to MT bending and beating is less understood due to the structural complexity of the axoneme. *In vitro* systems which can mimic flagellar beating patterns can be used to study the role of the structural components in regulation of various beating parameters.

In vitro set ups such as gliding assays with motors and filaments have been known to show various patterns such as beating and looping. The first report included formation of flagella like beating patterns arising in acto-myosin gliding assay due to hindrance of actin gliding by surface defects [12]. Kinesin is known to cause buckling of MT when the MT is clamped by its minus end [161]. Bundled MTs as well as clamped single MTs have been shown to perform flagella-like oscillations *in vitro* due to kinesin [128, 50, 129]. However these studies have lacked specificity of MT immobilization, control over MT length and density and used kinesin. Theoretical studies have predicted that clamped active filaments will undergo beating patterns under sufficient force and the

filament length, driving force and nature of pinning will jointly determine the nature of these patterns [19]. However these predictions have not been tested so far. In order to test these predictions and to better understand the mechanics of flagellar dynamics, an *in vitro* system with dynein and MTs mimicking beating could be a useful model.

Here we proceeded to study various modifications of a dynein based *in vitro* gliding assay that resulted in flagella like oscillations. The modifications included (I) addition of anti-tubulin antibodies in the dynein gliding assay to introduce random pinning of MTs during gliding, (II) clamping of MT plus end to the surface using biotin-streptavidin chemistry with either single rhodamine labeled MTs or rhodamine and Alexa-488 dual labeled MTs. We find that both the set ups showed single MT oscillations. The biotin tagged MTs provided better control and patterns were classified into three types: (a) fluctuations, (b) beating and (c) looping based on an order parameter which captures the relative space explored by the tip in X and Y. The motor density was varied from 5-65 motors and MT lengths varied between 2-25 microns. These were observed to jointly determine the qualitative nature of the patterns observed. Beating that resemble flagella like patterns can be observed above a threshold MT length and motor density. Higher motor number and long MTs show complex looping structure while pinning of 5-10 % is optimal for beating.

4.3 Methods

All the required proteins such as tubulin, rhodamine labeled tubulin, dynein, anti-GFP nanobody and EGFP were purified as mentioned in section 3.2.1.

4.3.1 Preparation of biotinylated tubulin

Biotin labeled Tubulin was prepared by incubating polymerised tubulin with (+)-Biotin N-hydroxysuccinimide ester (Sigma) by incubating it with polymerized tubulin followed

by a depolymerization-polymerization cycle similar to [17], to ensure that the activity of tubulin is maintained.

4.3.2 MT Assembly

Single labeled plus end biotinylated MT assembly: For preparation of rhodamine labeled plus end biotinylated MTs 22 μM unlabeled tubulin was polymerized with with 8 μM rhodamine labeled tubulin in BRB80 and 10% glycerol at 37°C for 40 minutes. To the reaction 18.33 μM biotin tubulin and 6.67 μM rhodamine tubulin was added and incubated further for 30 minutes to continue the polymerization of the plus end by biotinylated tubulin. Free monomers were removed by centrifuging the MTs over a 60% glycerol cushion at 135,300 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) for 18 minutes (Accel/decel 6/4). The MT pellet was re-suspended in taxol containing buffer (20 μM Taxol + BRB-80) and used immediately.

Rhodamine and Alexa-488 dual labeled plus end biotinylated MT assembly: For visualization of the plus end of MT with a different label the above mentioned protocol was varied. The MT minus end was polymerized by incubating 22 μM unlabeled tubulin was polymerized with with 8 μM rhodamine labeled tubulin in BRB80 and 10% glycerol at 37°C for 40 minutes. The polymerized MTs were separated from free monomers by centrifuging over a 60% glycerol cushion at 135,300 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) for 18 minutes (Accel/decel 6/4). The MT pellet was re-suspended in a mixture containing BRB-80, 18 μM biotin tubulin and 8 μM Alexa-488 labeled tubulin. This mixture was incubated for 30 minutes to continue the polymerization of plus end with biotin tubulin and Alexa-488 labeled tubulin to biotinylate as well as enable visualization of plus end with a different label as the rest of the MT. Free monomers were removed by centrifuging the MTs over a 60% glycerol cushion at 135,300 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) for 18 minutes (Accel/decel 6/4). The MT pellet was re-suspended in taxol containing buffer (20 μM

Taxol + BRB-80) and used immediately.

MT assembly for antibody based beating assay: Rhodamine labeled MTs were prepared by polymerizing 20 μ M unlabeled tubulin with 10 μ M rhodamine labeled tubulin in BRB80 at 37°C. The MTs were stabilized by addition of Taxol (20 μ M). Free monomers were removed by pelleting MTs at 150,000 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) over a 60 % glycerol cushion. MT pellet was re-suspended in BRB-80 with 20 μ M Taxol and used immediately.

4.3.3 Gliding assay with clamped MTs

Gliding assay with biotin-streptavidin based clamping of MTs: The beating assays were performed in a flow chambers constructed using 22 X 22 mm coverslip (No:1.5, VWR) adhered to a slide (22 mm X 60 mm, HiMedia, India) by two parallel strips of double backed Kapton polyimide tape (Ted Pella Inc., USA) spaced 7 mm apart, creating a chamber of area 22 mm X 7 mm. The flow chamber was coated with a 1:1 molar ratio mixture of nanobody and streptavidin (each 20 μ M in concentration). After blocking the surface with casein, one chamber volume of dynein of a fixed concentration was flowed in. The dynein solution was incubated for 10 minutes and unbound dynein removed by washing with Lysis Buffer (30 mM HEPES (HiMedia, India), 2 mM Mg Acetate (Amresco, OH, USA), 50 mM K Acetate (Fisher Scientific, India), 1 mM EGTA, 10 Glycerol). This was followed by flowing in plus end biotinylated MTs (either rhodamine labeled or rhodamine + Alexa-488 dual labeled MTs) and incubated for 10 mins. The unbound MTs were washed using wash buffer (Lysis Buffer plus 20 μ M Taxol). The beating was observed after addition of motility buffer (Lysis Buffer + 4 mM ATP + Antifade mix [0.005 mg Glucose Oxidase, 0.0015 mg Catalase, 7.2 mM Glucose in 100 μ l 10x Phosphate buffered saline, PBS (SRL Chemicals, Mumbai, India)]). Dynein densities were varied by flowing in purified dynein between 1.05 μ g (4.53 pmol) to 2.46 μ g (8.46 pmol) into to the chamber and incubating for 8 minutes.

All assays were performed at 37°C. All reagents unless otherwise stated, were from Sigma-Aldrich, MO, USA.

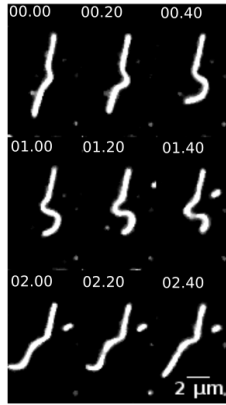
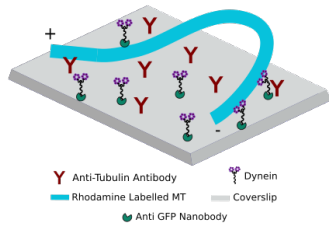
Gliding assay with antibody based clamping of MTs: The antibody based beating assay was performed in a flow chamber as mentioned before. It was similar to the gliding assay with an additional step of addition of anti-Tubulin antibodies along with the anti GFP nanobodies. The gliding assays were performed in chambers as described above. Anti-GFP nanobody (1mg/ml) and anti-tubulin antibody (Sigma Aldrich, MO, USA, Cat No.F2168) mixture (1:1 molar ratio) was flowed in the chamber and incubated for 5 mins. The unbound antibodies were washed with Lysis Buffer (30 mM HEPES, 2 mM Mg-Acetate, 50 mM K-Acetate, 1 mM EGTA, 10% Glycerol). The surface was blocked by casein (1 mg/ml). The dynein (1.18 μ g) was flowed in and allowed to incubate for 10 minutes. The unbound dynein was washed using Lysis Buffer. Rhodamine labelled MTs were flowed in and incubated for 10 mins. After the attachment of dynein, MTs were allowed to land. The unbound MTs were washed using wash buffer (Lysis Buffer + 20 μ M Taxol). Motility was recorded after addition of Motility buffer.

4.3.4 Motor density estimation and data analysis

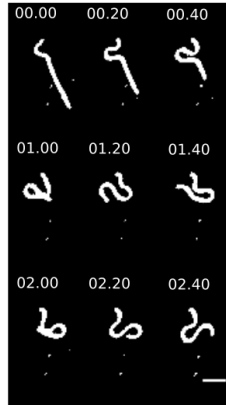
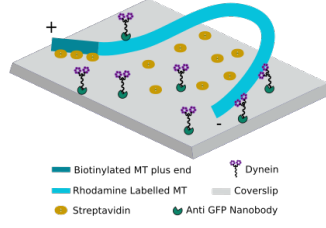
The motor density was estimated as mentioned before^[65] and described in detail in section 3.2.3. A calibration curve was made by measuring the image intensity of purified EGFP of known concentration flowed in the double back taped chambers. By calculating the image volume, the concentration of EGFP was converted into the density of molecules per unit area. This calibration curve was then used to calculate the density of GFP-dynein by measuring the intensity of GFP in the sample.

The MT contours were analyzed by a custom written MATLAB code developed in the lab by DK. The MT free tip was tracked over time using the same and used to plot the XY coordinates of the tip with time along with tangent angle.

A Antibody based pinning



B Biotin-streptavidin based clamping (Single Label)



C Biotin-streptavidin based clamping (Dual label)

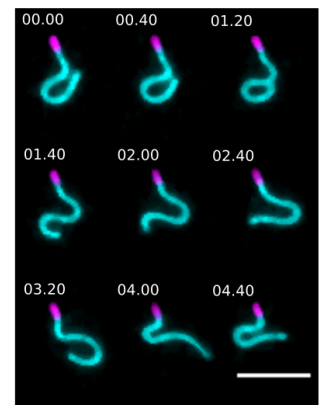
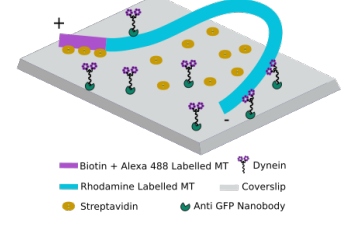


Figure 4.1: **Multiple approaches used to reconstitute dynein driven MT oscillations in a modified gliding assay.** (A) Schematic (Top) representing the modified gliding assay with dynein where MTs were clamped by introducing anti-tubulin antibodies as the pinning sites. Montage of a representative MT (Bottom) showing the oscillations of the single MT driven by dynein in presence of ATP. (B) Schematic (Top) representing the modified gliding assay where biotinylated MT plus ends were clamped to the surface via streptavidin present on the surface. Montage of a representative MT (Bottom) showing the oscillations of the single MT driven by dynein in presence of ATP. Scale bar: 2 μm . (C) Schematic (Top) representing the oscillations assay with dual labeled MTs to visualize the biotinylated MT plus end. Montage of a representative MT (Bottom) showing the dual labeled MT with the plus end (Magenta) and the minus end (Cyan) performing oscillations in presence of ATP. Scale bar: 4 μm . Time: mm:ss.

4.4 Results

4.4.1 Dynein driven single MT oscillations reconstituted in a modified gliding assay

In order to reconstitute dynein driven beating of individual MTs in a gliding assay like set up, three approaches were used to clamp MTs A) Antibody based pinning, B) Biotin-streptavidin based clamping (Single label) and C) Biotin-streptavidin based clamping (Dual label)(Figure 4.1 A). In the first approach, random pinning sites were introduced in the dynein based gliding assay by adding anti-tubulin antibodies which can bind to the MTs and act as pinning sites (Figure 4.1 A). Due to presence of tubulin binding sites throughout the surface, gliding of MTs is hindered. MTs which were clamped by their leading tips to the antibodies were seen to perform oscillations (Figure 4.1 A). The MT can be seen to buckle as the dynein stepping takes place. The buckling and recovery with time results in MT oscillations. Using antibodies implies that the position of pinning sites on the MT cannot be controlled. A more controlled method of MT clamping was required. The MT plus end was labeled with biotin tubulin by using biotin tubulin to carry out polymerization of the MT plus end. In the dynein gliding assay, streptavidin was attached to the glass surface along with the anti-GFP nanobodies (1:1 molar ratio). The dynein bind to the surface via the anti-GFP nanobodies. The biotinylated plus ends of MTs could bind to the surface bound streptavidin and hence clamping the MT plus end successfully. The MTs can be seen to be clamped by one MT end and buckles due to the stepping dynein in presence of ATP (Figure 4.1 B). The buckling MT further show oscillations with time (Figure 4.1 B). In order to confirm that the oscillations arise as a result of MT plus end clamping, beating assay with dual labeled MTs was performed. Two different dyes were used to label the minus ends and MT plus end. This was achieved by using Alexa-488 labeled tubulin along with biotin tubulin to continue the polymerization of rhodamine labeled

MT's plus end. The dual labeled MT with clamped plus end (magenta) and free minus end (cyan) were assembled as mentioned in the methods section and can be seen to perform can be seen to perform oscillations in time (Figure 4.1 C).

4.4.2 Dynein drives slow active oscillations of the free end of MTs *in vitro*¹

The oscillations of MTs were quantified by tracking the MT contour with a MATLAB Based tool developed in the lab (manuscript in review [70]). The MT contour was tracked and time projected to demonstrate the oscillatory nature of the movement (Figure 4.2 A,B). The coordinates of the MT free end (minus end) were used to plot the movement of MT tip in X and Y axis with time . From the movement of MT tip the oscillations were found to last for about 3-5 cycles (Figure 4.2 C). Since the clamped MT end defines the X axis for beating the span in Y is higher than span in X. Along with the X and Y movement the tip angle ϕ as a function of time also confirms the oscillatory nature of the beating (Figure 4.2 D). The ϕ oscillates between the range of -57° to 57° , a range of 114° (Figure 4.2 D). In order to quantify the traveling wave nature of the oscillations, the filament tangent angle ψ (radians) along the MT was measured over time. The resulting kymograph - space-time plot - of filament dynamics (Figure 4.2 E) suggests that the curvature propagates for over ~ 300 s after which the filament ‘snaps’ back and repeats the cycle. The measures of oscillations in the form of tip-angle ϕ , span in X (S_x) and Y (S_y) and local tangent angle $\psi(s, t)$ are geometric properties of the system as showed in the schematic (Figure 4.2 F). Tangent angles have been measured for whole eukaryotic flagella such as human sperm flagella [36], bull sperm flagella [166] and *Chlamydomonas* [7]. Angles range from -1.74 to +1.74 radians for bull sperm, -1.57 to + 3.14 radians for human sperm and from -3.14 to +1.74 radians for *Chlamydomonas*. The range of tangent angle is -2 to +2 rads in this

¹Data analysis tool and frequency analysis done by Dhruv Khatri

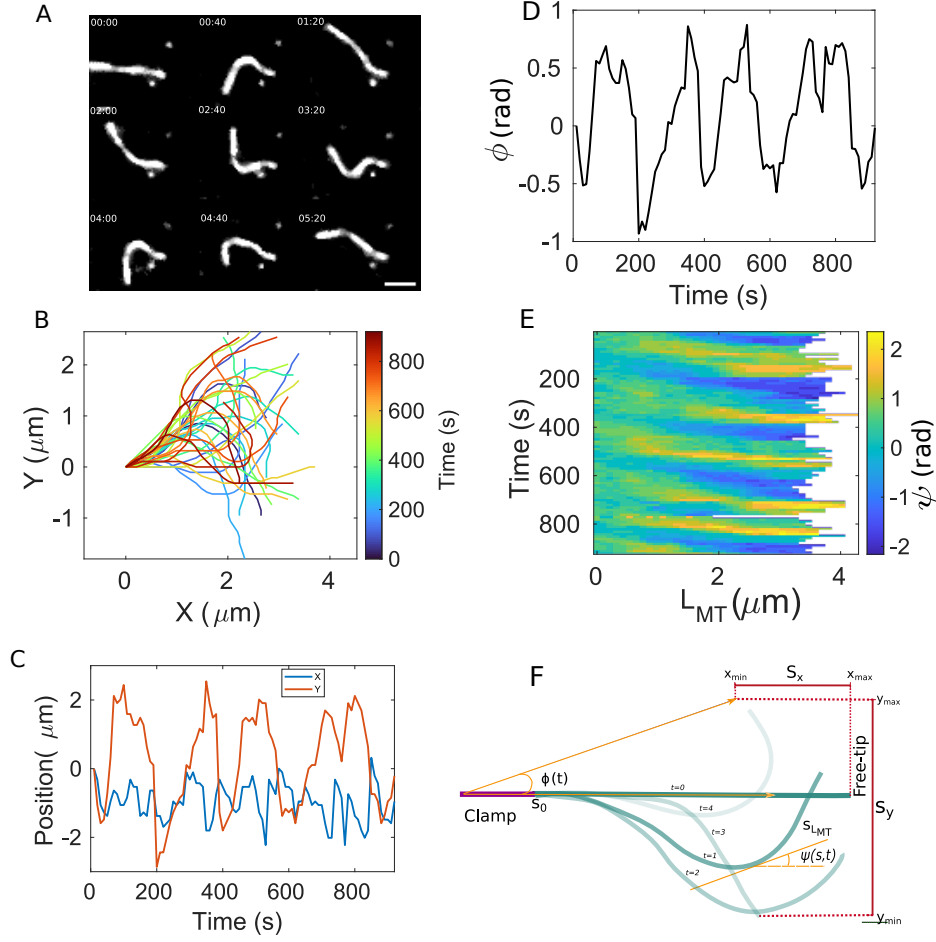


Figure 4.2: ***In vitro* reconstitution of dynein driven flagella-like MT oscillations.** (A) The montage of a representative microscopy time-series shows clamped MT transported by dynein undergoing bending dynamics. Here, $L_{MT} = 4.7 \mu\text{m}$ and dynein density: $27/\mu\text{m}^2$. Time: mm:ss. Scale bar: $4 \mu\text{m}$. (B) Filament contours from a time-series of rhodamine-tubulin labeled MT were tracked and contours projected in time. Colorbar: time (s). (C,D) The dynamics of the free filament tip (minus-end) are plotted in terms of (C) X- (blue) and Y-coordinates (red) and (D) the tip-angle ϕ in radians as a function of time in seconds. (E) the tangent angle (ψ) is plotted (colours) over time (y-axis) and MT length (x-axis) as a kymograph at 10 s intervals. Colorbar: angle in radians. (F) The schematic represents the measures used to quantify bending (dashed lines): tip-angle ϕ , span in X (S_x) and Y (S_y) and local tangent angle $\psi(s, t)$.

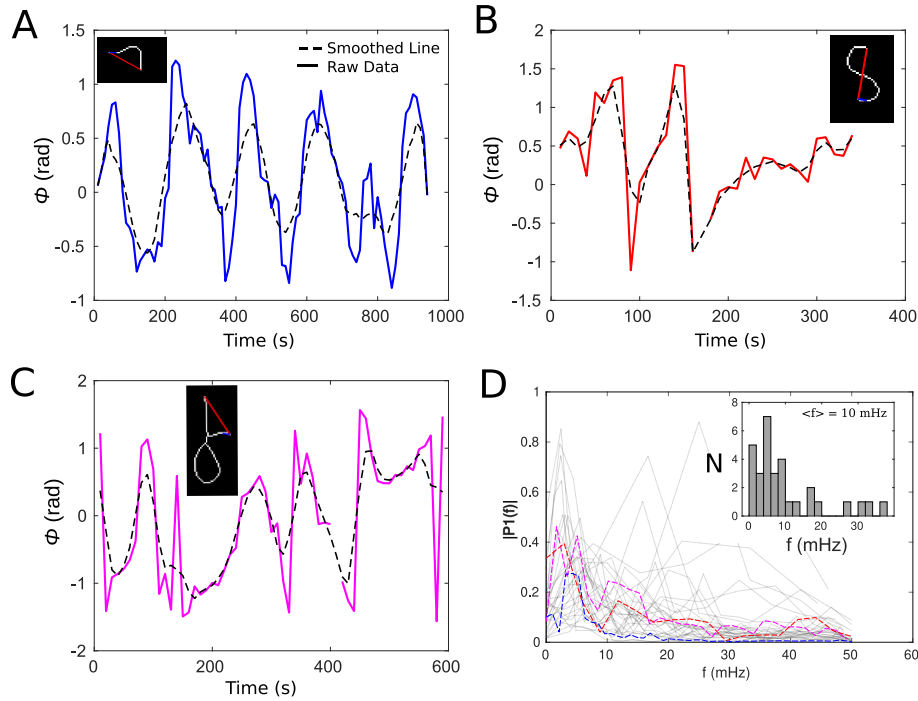


Figure 4.3: **Frequency analysis of MT tip-angle oscillation.** (A-C) The tip angle of 3 representative MTs plotted as a function of time. Raw data (Solid line) with average filter (Dashed line) (*Insets*) with Representative images of the MT segmented using the custom written tool with a red line connecting the free and fixed MT ends. (D) The amplitude spectrum of the frequencies as a function of the calculated frequency for 11 MTs. The inset shows histogram representing the dominant frequency of 10 MHz.

study, which is comparable to the tangent angles explored by beating flagella. While the tangent angles were comparable to flagella, the slow nature of the oscillations was apparent from the time dynamics of the tip. Which needed to be quantified in terms of the frequency of beating.

In order to calculate the frequency of beating the tip angles ϕ of few oscillating MTs was used. The tip angles ϕ with time was average filtered and subjected to FFT analysis to calculate the dominant frequencies (Figure 4.3 A-C). For 25 filament the frequencies ranged from 0-50 mHz while the dominant frequency was 10 mHz (Figure 4.3 D).

4.4.3 Effect of MT length and motor density on the beating patterns

Due to the intrinsic variability in MT lengths at a given motor density the MT of varying lengths showed various patterns. At a given density (30 motors/ μm^2) the smaller MTs (3 μ) were observed to perform wave like movement while MT with intermediate lengths 4-8 μm were showing flagella like beating pattern. While the longer MT of length 18 μm was showing a complex figure of eight pattern. The patterns can be seen by the color coded time projections (Figure 4.4 A). As the MT length increases the number of motors that can bind and generate force on the MT increases making the patterns complex. Since increase in motor number can also be achieved by varying the motor density on the surface, the motor density was then varied. For MTs of comparable lengths (14 to 16.5 μm) when the motor density was varied from 0 to 62.8 motors/ μm^2 various MT patterns were observed as expected (Figure 4.4 B). In absence of any motors the clamped MT performs fluctuations due to thermal motion. As the motor density increases the MTs eventually start showing the beating (16 motors/ μm^2) and eventually the complex looping (62.8 motors/ μm^2) pattern. Hence both MT length as well as motor density can affect the beating. Upon varying both motor density and MT length the MTs qualitatively showed three distinct patterns such as fluctuations, beating and looping. However to systematically study the role of MT length and motor density on the formation of patterns it is important to quantitatively distinguish the patterns from one another.

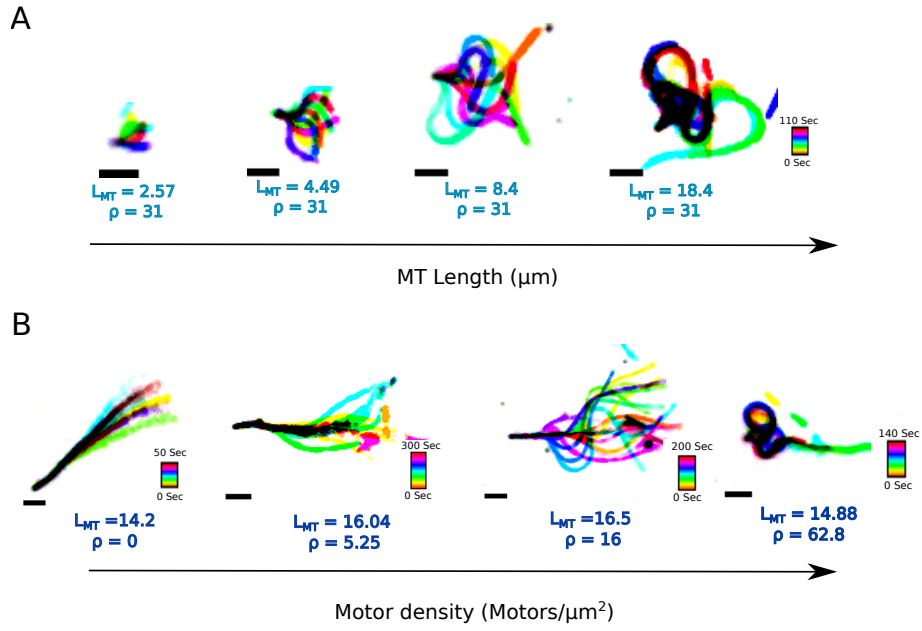


Figure 4.4: **Motor density and MT length dependent transitions of MT patterns in experiment** (A) Time projections of representative MTs of increasing length at a given motor density showing transition of MT patterns from fluctuation to beating to looping as the MT length increases. (B) Time projections of representative MTs of comparable lengths at increasing motor density. The MT show transition from fluctuations at no motors to beating and looping at higher densities. Scale bar: $2\mu\text{m}$.

4.4.4 Geometric measure to classify spatial patterns of clamped filaments driven by motor-sheet²

In order to quantitatively distinguish the observed patterns from one another a parameter called sweep range S was used. The sweep range is the binary logarithm ($\log_2$) of the ratio of distance explored by the free tip in both X and Y i.e. S_y and S_x (schematic in Figure 4.2 E). Based on the nature of movement of the free tip the span in X and Y can differ from one another 4.5. For fluctuations the span in X is about 10 times lower than span in Y due to the MT not bending during thermal fluctuations, giving the S as >3 . In case of beating due to the bending of MT the span in X is slightly lower than Y are hence giving the S value between 0 to 3. For complex looping patterns the span in X is higher than Y due to extreme bending of MT giving the S value < 0 .

$$\begin{aligned}
S &\geq 3 && \text{(I) Fluctuations,} \\
3 > S > 0 && \text{(II) Regular oscillations,} \\
0 > S && \text{(III) Looping} \\
S = 0 && \text{(IV) Swiveling}
\end{aligned} \tag{4.1}$$

$S=0$ represents the pattern called swiveling where a pinned MT is undergoing thermal fluctuations in absence of motors but due to the nature of pinning the MT has rotational freedom. Due to this the span in X and Y are equal giving the S value $=0$. Presence of all the mentioned patterns in the experiments and their characterization by the S value was confirmed (Figure 4.5).

To test if this novel order parameter, the filament sweep value S , could be used to quantitatively classify the systematic effect of MT and motor properties on the

²Sweep range parameter conceptualized by Dr. Neha Khetan to classify data from simulations

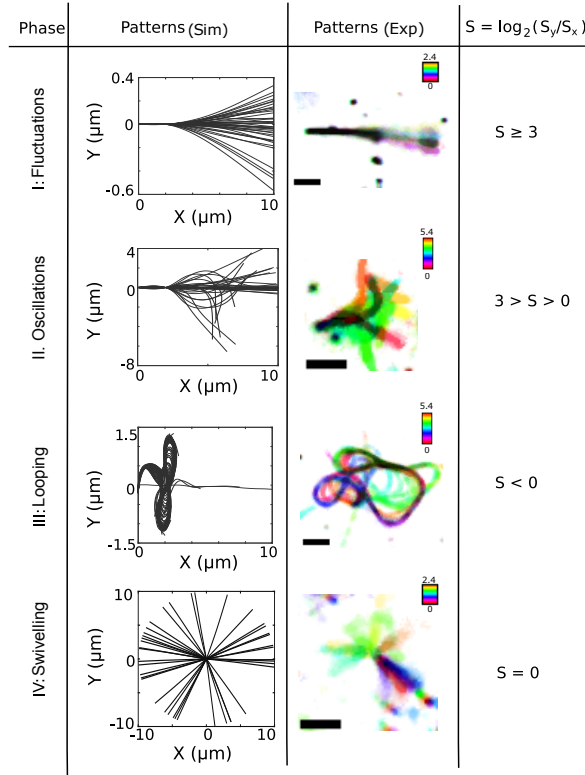


Figure 4.5: **Quantifying MT patterns in terms of space explored by the free tip in experiment and simulation.** The various MT patterns observed in experiments classified as (I) fluctuations, (II) regular oscillations, (III) looping and (IV) swivelling based on the calculated S value. In experiment, the patterns observed are fluctuations: $S = 3.8$ ($L_{MT} = 9.7\mu\text{m}$, no motors), flagella-like: $S = 0.41$ ($L_{MT} = 4.7\mu\text{m}$, $\rho_m = 27$ motors/ μm^2), looping: $S = -1$ ($L_{MT} = 24\mu\text{m}$, $\rho_m = 27$ motors/ μm^2) and swivelling $S = 0$ ($L_{MT} = 2.2\mu\text{m}$, no motors). Scale bar: $2\mu\text{m}$. Colorbar: minutes. ρ_m : motor density.

emergent dynamics of the system a phase space of beating was required to be studied.

4.4.5 Phase space of MT beating ³

In order to prepare a phase space of beating using the motor density and MT length as the parameters beating assays were performed over varying motor density of 0 (no motors), 5.25, 16.04, 31.06 and 62.86 dyneins per μm^2 . The MTs in the assay were of length 5 to 25 μm . For multiple filaments ($n = 65$) from the experiments the S value was calculated and the data was categorized into (I) fluctuations, (II) regular

³Simulations performed by Dr. Neha Khetan and Mr. Aman Soni.

oscillations, (III) looping. The categorized data can be visualized both qualitatively and quantitatively as seen in (Figure 4.6 B and C). The beating phenomenon was captured computationally by performing simulations. The phase space generated from the model predicted boundaries between the various patterns as seen in (Figure 4.6 A). To test whether the boundaries are followed by the experiments the boundaries were mapped through the MT length density space generated from experiments. The experimental data was seen to be roughly following the predicted boundaries both in quantitative and qualitative plots. The slight overlap between the boundaries in the experimental data can be explained based on several factors such as possible difference in MT elasticity measures in experiments and simulations or dyneins force dependent asymmetric detachment behavior which was not accounted in the simulations.

4.4.6 Effect of MT clamp length on beating

In experiment the length of MT varied between fields of view and so did the fraction of pinned length. While examining this variability it was observed that proportion of the MT that is clamped affected the beating patterns. The nature and proportion of the pinning can decide the degree of freedom the oscillating MT can have. For MTs where the pinning is reduced to a point pinning, the MT now has rotational degree of freedom. This can lead to formation of MT spiral rather than MT beating (See chapter 5). As the pinning length increases from point pinning to an elongated pinning the degree of rotational freedom reduces. The MTs which have upto 5% of the MTs clamped can beat while having some rotational degree of freedom. This allows the MT to explore other planes while undergoing beating. Due to combination of restricted rotational freedom and slightly elongated clamp the MTs performs slower beats (Figure 4.7). A clamping of 5-10% is optimum for beating as it restricts the MT movement to the given plane and beating is achieved faster. For MT where the clamping is more than 20% of the total length the MTs shows intermittent beating. Hence it was observed

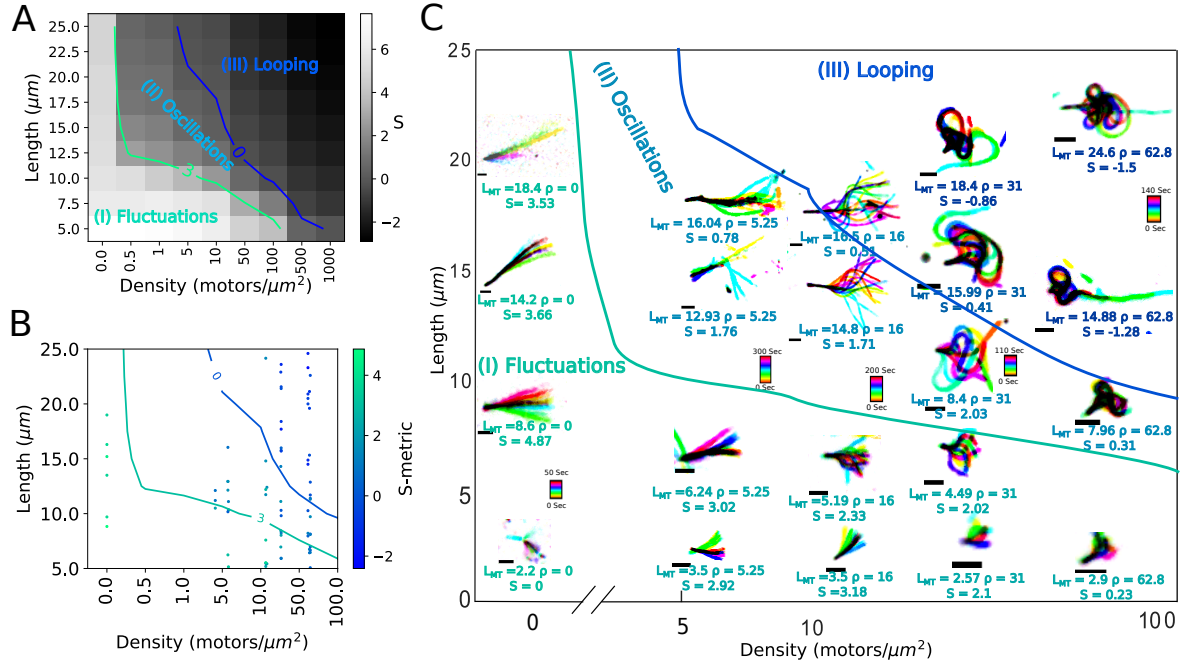


Figure 4.6: **Phase space of MT beating in experiments follows the boundaries predicted by simulations** (A, B) The parameter space of motor densities (ρ_m) and MT lengths (L_{MT}) from simulations is divided into three regions based on the S value of $\langle S \rangle = 0$ (blue) and $\langle S \rangle = 3$ (green), into (I) $\langle S \rangle > 3$ corresponds to fluctuations, (II) $3 > \langle S \rangle > 0$ to regular oscillations and (III) $\langle S \rangle < 0$ to looping. (B) Phase space from the experimental data generated for multiple filaments ($n = 65$) (circles) over varying motor density of 0, 5.25, 16.04, 31.06 and 63.02 motors/ μm^2 from 3 biological replicates and varying MT lengths. The phase space is overlapped with boundaries generated from the simulations (A). (C) Representative MTs from each density for varying MT lengths were time projected to show the qualitative changes in the MT patterns as the motor density and MT length changes. Scale bar: $2\mu\text{m}$.

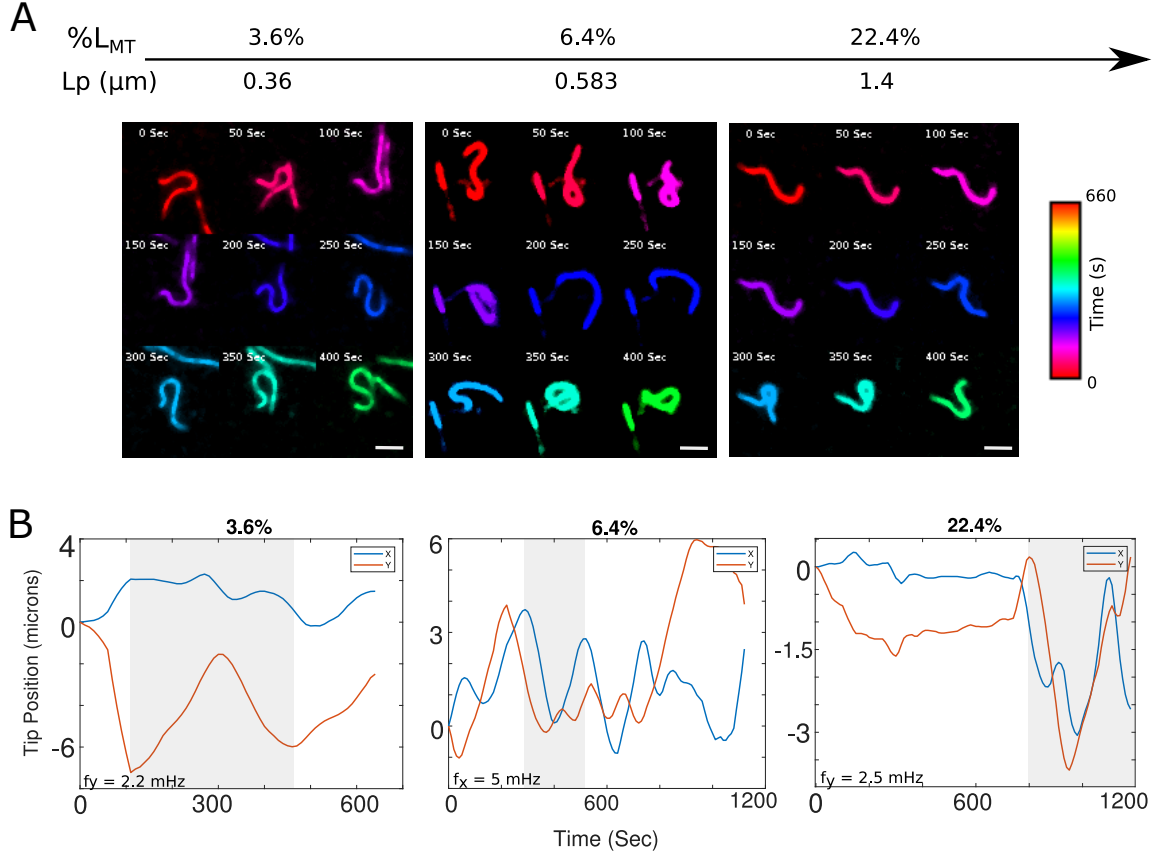


Figure 4.7: Effect of clamped MT length on beating(A)The dynamics of filaments immobilized in experiment by their biotinylated plus-ends on a streptavidin coated surface with increasing percentage of the filament length immobilized (3.6%, 6.4% and 22.4%) are seen in montages of the filament contours (A). Scale bar: 2 μm. Colors indicate time frames (time interval:50 sec).(B) X and Y coordinates of the beating MTs as a function of time for MTs with increasing pinning length (3.6%, 6.4% and 22.4%).

that 5-10% of MT clamping is required for optimum beating of the MT (Figure [4.7](#)).

4.5 Discussion

Here, a modified MT gliding assay set up was developed to reconstitute wave-like oscillations of single microtubule by dynein. In a dynein based gliding assay, MT plus ends were clamped using biotin-streptavidin chemistry. Upon addition of ATP the MTs perform flagella like beats. By quantifying the MT minus end movement it was

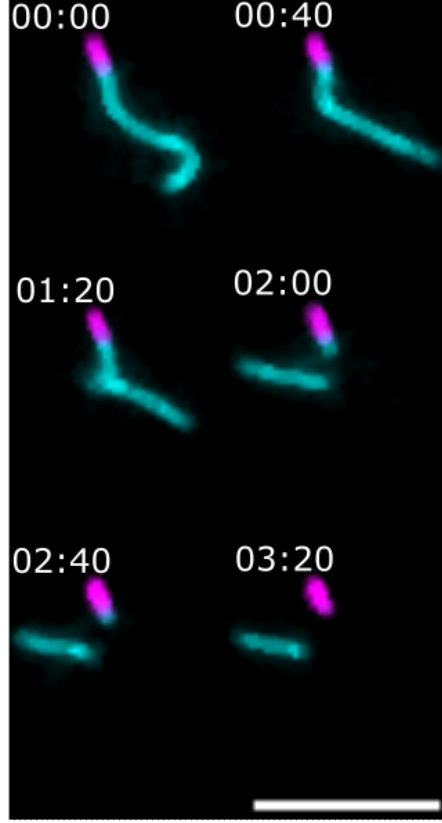


Figure 4.8: **MT breakage during beating** A montage showing MT breakage near the clamp (magenta) of a representative MT (cyan) undergoing dynein driven oscillations. Here $\rho_m = 47 \text{ motors}/\mu\text{m}^2$ and $L_{MT} = 6.7 \mu\text{m}$. Scale bar: $4 \mu\text{m}$.

observed that dynein drives slow (MHz) range oscillations of the MT. Propagation of wave from the base to tip was confirmed by quantifying the tangent angle along the length of MT with time. The observed patterns were seen to be dependent on both MT length and motor density as seen from the phase space. The various patterns observed were classified based on the space explored by the free tip in X and Y. Hence fluctuations, beating and looping were categorized according to their S value. The parameters space driving the various patterns was studied concluding that geometry, force and elastic properties can drive MT beating.

Previously beating like patterns have been reported in acto-myosin system [12]. Kinesin is known to buckle a clamped MT but was not reported to drive oscillations

[44] probably due to low motor density. Finally kinesin was reported to drive flagella like oscillations of single MT in a gliding assay where MT end was accidentally clamped due to surface defects [161]. In addition to a single MT, kinesin is known to drive oscillations in the MT bundle [128]. These use kinesin and control over MT length and motor density is not explored. Reconstitution of oscillations with dynein with systematic control over the motor density was reconstituted for the first time here.

Theoretical studies of clamped chain of active self propelled particles has been predicted to show emergence of flagella like like patterns [19]. The theory predicts that the emergence of patterns is dependent on the elastic properties of the chain, type of clamp and linear force density. *In vitro* MT-motor systems are a good way to validate these predictions. Dependence of the beating on the MT length, motor density and pinning observed in this study validate these predictions. The frequency of beating observed in this study is lower than the frequency of flagella. This can be attributed to several factors such as use of yeast cytoplasmic dynein which is about 10 times slower than flagellar dyneins and also the complex geometry of flagella (9+2) as compared to simple 1 MT + motors geometry of this study.

Occasionally MTs have been observed to move out of the imaging plane while beating. This type of movement can be explained by generation of torque by dynein on the MT. Dynein is hypothesized to generate torque on MT due to stepping of dynein in a right-handed helical path [15] in mammalian cytoplasmic dynein [69] and *Tetrahymena* axonemal dynein [171]. Enrichment of these 3D beats and exploring their mechanical properties can be used in future to study torque generation by motors. The repeated bending of MT during MT oscillations can affect the elastic properties of the MT. MTs lattices have been known to be damaged upon repeated bending and release [130, 131]. The damage to the MT lattice often results in changed altered elastic properties such as decreased MT stiffness. The MT stiffness was observed to decrease with each bending cycle [130]. In presence of tubulin monomers the damaged MT

lattice can self repair and regain its stiffness. A visible evidence of apparent lattice damage is when MTs suffer breakage. In this study, MT assays have been occasionally observed to break after a few cycles of oscillations [4.8](#). The break was seen to be near the clamped end of the MT. While MT damage visible here is via breakage, the repeated oscillations could be affecting the MT elasticity with each cycle. Such changes in elasticity induced by lattice defects are harder to measure directly. Such changes in the MT elasticity can also affect the oscillation dynamics as MT will be easier to bend as the oscillations continue. The self-repairing nature of the lattice defects can be explored by reconstituting MT oscillations in presence of low concentration of tubulin monomers. A comparison between MT oscillation dynamics in presence and absence of tubulin monomers can help us understand how lattice damage and self-repair can contribute the collective mechanics of this system.

This simple set up was used to validate some predictions from the theoretical studies confirming the generality of such patterns by observing them in dynein driven set up for the first time.

4.6 Conclusions

Hence, a minimal *in vitro* set up made up of MTs and motors along with the correct geometrical constraints can give rise to self organized patterns such as wave-like oscillations. These complex patterns resembling the beating of a flagella arise without any geometric complexity, regulation and coordination as seen in the flagella. Hence future studies for reconstituting bottom-up minimal flagella can benefit from the design principles used in this study.

Chapter 5

Scaling of dynein-driven pinned microtubule spiraling explained by length dependent persistence length

5.1 Summary

Previously, *in vitro* gliding assays with actin and myosin have shown that actin forms spirals when encountering surface defects, and the spiral radius is inversely proportional to the force per unit length. Here, dynein-driven MT spirals were reconstituted in a minimal *in vitro* setup by pinning MT plus ends in a dynein gliding assay. The radius of MT spirals followed a trend similar to that of myosin and kinesin with respect to scaling with the motor density but showed a length dependence of MT, which differed from previous studies with myosin and predictions for kinesin. The scaling of spiral radius and frequency with motor density and MT length was compared with two models of MT elasticity. The observed scaling was explained by a length-dependent persistence length (L_p) model of MT, suggesting an anisotropic nature of MTs.

5.2 Introduction

In motor and filament systems filament patterns such as bending, spiraling etc have been observed for acto-myosin as well MT-kinesin systems [12, 161, 43]. *In vitro* set ups like gliding assays have demonstrated myosin and kinesin driven actin/MT spirals arising when the filaments motion in a gliding assay is interrupted by surface defects [12]. Myosin driven actin spirals have been studied as a measure of the motor forces [12]. By balancing the moment and torque for the spiral formation it was observed that the radius of actin spirals was seen to be inversely proportional to the force per unit length $R \sim (KT\ell_p/f)^{1/3}$ [12]. While the radius was dependent on the total force i.e. motor density and persistence length, it was observed to be independent of the filament length. This is because the persistence length is considered to be a constant value for actin. Simulated data in the same study for kinesin driven MT spirals was shown to follow a similar trend. However microtubule and actin vary greatly in their elastic properties [45]. While there is great difference in the value of persistence length for actin (17.7 μm) and microtubule (5200 μm) [45], the nature of elastic properties is also different. Persistence length measurement of microtubules remains to be debated upon. In previous studies different methods have resulted in different values [109, 45, 78, 67, 33, 153]. Previously microtubules have been reported to have anisotropic material properties [73]. This was attributed to the complex lattice structure of microtubules where the longitudinal bonds i.e. bonds between two tubulin dimer is stronger than the lateral bonds i.e. between adjacent protofilaments. [137]. Such anisotropic nature of MTs can be used to explain the dependence of L_p on the total length of the MT. Length dependence of L_p has been previously measured experimentally by analyzing the thermal fluctuations shown by MTs grafted by one end in a micro-fluidic set up [109]. Other studies where the L_p was measured using different methods the L_p did not show any dependence on the MT length [45, 67]. Since conventionally MTs have been

considered as rigid rods, a constant value of L_p has been used in many studies [43]. Hence the anisotropic nature of MTs and length dependence of L_p has been debated upon. Scaling of MT spiral radius with motor density and MT length might provide some insights however MT spirals have not been studied in details.

Even though kinesin driven MT spirals have been reported previously, dynein driven single MT spirals have not been reconstituted and studied in details. While previous studies are dependent on surface defects for pinning of filaments, a method where pinning is enriched would be helpful. Such enriched pinning of filaments combined with motor density estimation can help us understand the scaling of spiral radius with motor density and filament length. Hence here dynein driven MT spirals were reconstituted using two methods and the scaling of spiral was studied with respect to the motor density and MT length.

5.3 Methods

5.3.1 Plus end biotinylated MT assembly

In order to perform a gliding assay with enhanced pinning of MT plus ends, a biotin-streptavidin based strategy was used. MTs were prepared by polymerizing 20 μ M unlabeled tubulin with 5 μ M rhodamine labeled tubulin in BRB80 and 10% glycerol at 37°C. The MTs were stabilized by addition of 20 μ M Taxol (Paclitaxel, Cytoskeleton Inc., CO, USA). Free monomers were removed by pelleting MTs at 150,000 g in TLA 100.3 rotor (Beckman Coulter, CA, USA). For plus-end biotinylation, the MT pellet was re-suspended in BRB-80 and 10% glycerol followed by addition of either 20 μ M biotinylated tubulin and 5 μ M rhodamine-labeled (single color filaments) or 10 μ M biotinylated tubulin and 10 μ M Alexa Fluor-488 labeled tubulin (dual labeled filaments). The filaments were allowed to polymerize by incubating the mixture at 37°C for 30 minutes. Free monomers were once more removed by centrifuging at 150,000 g in TLA

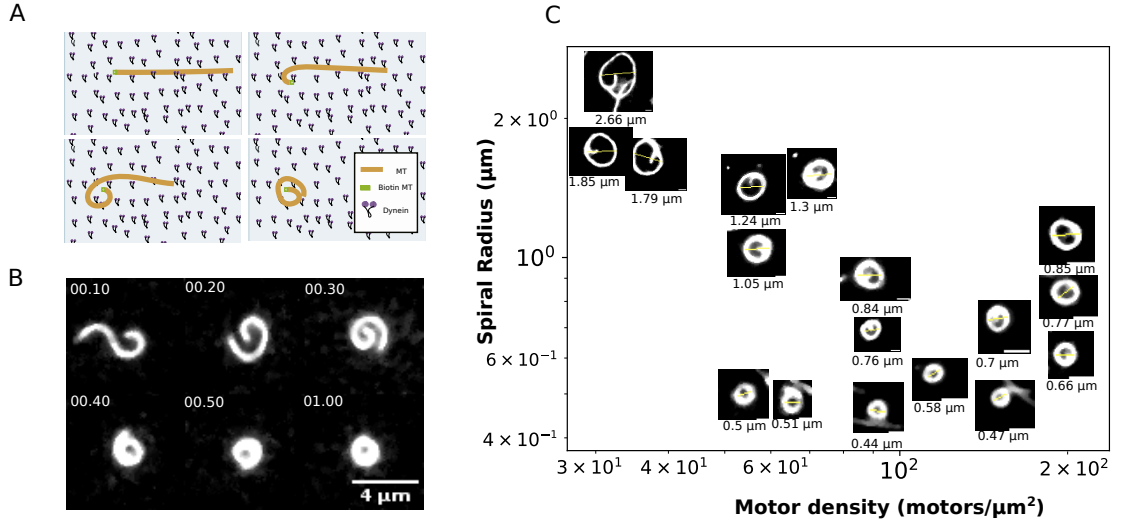


Figure 5.1: ***In vitro* reconstitution of dynein driven single MT spiral.** (A) Schematic representing the experimental set up used to reconstitute dynein driven MT spiral. In a modified gliding assay the leading end of the MT is pinned to the cover-slip surface by using either a biotin-streptavidin chemistry or random pinning. (B) Montage of a representative MT spiral being formed as a result of pinning of MT leading end and stepping of dynein in presence of ATP where $L_{MT} = 7.2 \mu\text{m}$ and dynein density was $61 \text{ motors}/\mu\text{m}^2$. Time: mm:ss. (C) Representative MT spirals of MTs of various lengths across various motor densities. The dynein density was varied from 30 to 206 $\text{motors}/\mu\text{m}^2$. The radius (μm) of each spiral is noted below each image.

100.3 rotor (Beckman Coulter, CA, USA) and MT pellet re-suspended in BRB-80 and 20 μM Taxol and used immediately.

5.3.2 MT spiral assay with biotin-streptavidin mediated pinning of MTs

The spiral assays were performed in a flow-chambers constructed using 22 X 22 mm coverslip (No 1.5, VWR) adhered to a slide (22 mm x 60 mm, HiMedia, India) by two parallel strips of double backed Kapton polyimide tape (Ted Pella Inc., USA) spaced 7 mm apart, creating a chamber of area 22 mm x 7 mm. The flow chamber was coated with a 1:1 molar ratio mixture of nanobody and streptavidin (each 20

μM in concentration). After blocking the surface with casein, one chamber volume of dynein of a fixed concentration was flowed in. In order to test the effect of motor density varying amount of dynein ($1.05\text{-}3\ \mu\text{g}$) was flowed into the chamber. The dynein solution was incubated for 10 minutes and unbound dynein removed by washing with Lysis Buffer (30 mM HEPES (HiMedia, India), 2 mM Mg-Acetate (Amresco, OH, USA), 50 mM K-Acetate (Fisher Scientific, India), 1 mM EGTA, 10% Glycerol). The density is estimated at the imaging surface, since the washes were optimized to result in no further outflow of dynein. Using a GFP-intensity calibration method (described in a later section) the surface motor density was estimated. We have assumed all the motors bound to the surface are active. This was followed by flowing in plus end biotinylated MTs and incubated for 10 mins. The unbound MTs were washed using wash buffer (Lysis Buffer + $20\ \mu\text{M}$ Taxol). The beating was observed after addition of motility buffer (Lysis Buffer + 4 mM ATP + Antifade mix [0.005 mg Glucose Oxidase, 0.0015 mg Catalase, 7.2 mM Glucose in $100\ \mu\text{l}$ 10x Phosphate buffered saline, PBS (SRL Chemicals, Mumbai, India)]). All assays were performed at 37°C . All reagents unless otherwise stated, were from Sigma-Aldrich, MO, USA.

5.3.3 MT spiral assay with enhanced surface defects

Rhodamine labeled MTs were prepared by incubating $22\ \mu$ unlabeled tubulin + $8\ \mu$ rhodamine labeled tubulin in BRB80 with 10% glycerol at 37°C for 40 minutes. The MTs were stabilized by adding $20\ \mu\text{M}$ Taxol containing BRB-80. The monomers were removed by centrifuging at 133,000 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) over a 60% glycerol cushion. The MT pellet was re suspended in BRB-80 with $20\ \mu\text{M}$ Taxol and used immediately. The assays were performed in a flow-chambers as mentioned in the previous section. The flow chamber was coated with anti-GFP nanobody($20\ \mu\text{M}$). The surface was passivated with casein but the incubation time was reduced so that the random pinning of MTs while gliding is enriched, leading to

formation of spirals. Dynein of varying concentrations was flowed in to achieve various motor densities. The dynein solution was incubated for 10 minutes and unbound dynein removed by washing with Lysis Buffer (30 mM HEPES (HiMedia, India), 2 mM Mg-Acetate (Amresco, OH, USA), 50 mM K-Acetate (Fisher Scientific, India), 1 mM EGTA, 10% Glycerol). This was followed by flowing rhodamine labeled MTs and incubated for 10 mins. The unbound MTs were washed using wash buffer (Lysis Buffer + 20 μ M Taxol). The gliding and spirals were observed after addition of motility buffer (Lysis Buffer + 4 mM ATP + Antifade mix [0.005 mg Glucose Oxidase, 0.0015 mg Catalase, 7.2 mM Glucose in 100 μ l 10x Phosphate buffered saline, PBS (SRL Chemicals, Mumbai, India)]). All assays were performed at 37°C. All reagents unless otherwise stated, were from Sigma-Aldrich, MO, USA.

5.3.4 Image and data analysis

The MT *spiral diameter* was measured from image time series manually using the measure tool of FIJI. For measuring the spiral diameter a transverse line was drawn covering the widest distance in the spiral in a single image frame. The time frame chosen to calculate the diameter was the one where the spiral formation is complete and the diameter stays constant till the final frame. The radius was calculated from the diameter (see section 9.1 for the raw data).

For *frequency* analysis the free MT tip (Minus end) was tracked in time using MTrackJ plug in of FIJI (Figure 5.2 A). From the position vs time data of the spirals the Y position with time was used to calculate the frequency of oscillations. The raw data was smoothed using cubic interpolation (Figure 5.2 B) using the `inter1pd` function of `scipy.interpolate` package. The interpolated data was subjected to FFT analysis using the `scipy.fft` package to find the dominant frequency (Figure 5.2 C) (see section 9.1 for the raw data). Motor density was estimated using a fluorescence based EGFP calibration curve as mentioned in section 3.2.3.

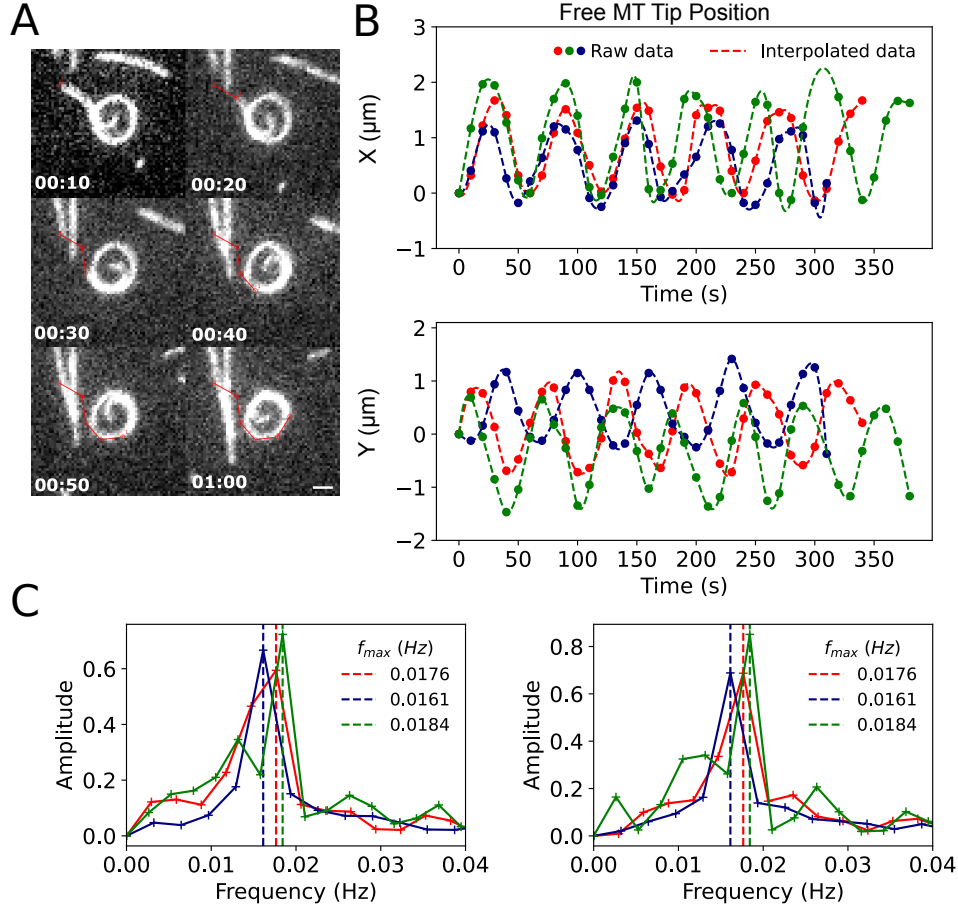


Figure 5.2: **Estimation of oscillation frequency of the spirals.**(A) A montage showing tracking of the MT free tip (minus end) of a representative MT as it undergoes dynein driven spiraling. The X and Y position with time of the free MT tip (red line) was tracked interactively using MtrackJ (see materials and methods). Scale bar: $2\mu\text{m}$. Time: mm:ss. (B) Y (Top) and X (Bottom) position as a function of time for 3 representative MTs was plotted. The raw data was smoothed using cubic interpolation. The interpolated data was further Fourier transformed to calculate the dominant frequency of the spiral. (C) The amplitude spectrum of frequencies of 3 MTs obtained from FFT analysis of both X and Y position showing the dominant frequencies f_{max} at 0.017, 0.016 and 0.018 Hz.

5.4 Results

5.4.1 Dynein driven MT spirals reconstituted in pinned MT assays

Dynein driven MT spirals were reconstituted in a modified gliding assay set up where the leading MT tip is pinned to the surface (Figure 5.1 A). A minimal yeast cytoplasmic dynein construct with GFP on its tail was used as the force generator. In the gliding assay like set up dynein was anchored to the glass via anti-GFP nanobody to maintain its orientation. The leading end of MT was clamped to the surface by using either a biotin-streptavidin strategy or enhanced surface defects (Figure 5.1 A). In the biotin-streptavidin strategy MT plus ends were biotinylated by carrying out MT plus end polymerization by biotin tubulin as described before (section 4.3.1) [170] and described briefly in the methods. Upon addition of ATP due to the stepping of dyneins MTs are propelled like a gliding assay. However upon encountering a pinning the MT buckles and then starts spiraling and gradually forms a tight spiral (Figure 5.1 B). The spiral once formed keeps rotating around the pinning site (Figure 5.1 B). By varying the density of dynein on the surface the spirals were reconstituted along a range of densities i.e. 30 - 208 motors/ μm^2 to result in differences in the sizes of the circles as measured by their diameter (Figure 5.1 C). For densities 30 - 100 motors/ μm^2 the pinning was achieved using biotin-streptavidin method while for higher motor densities (>100 motors/ μm^2) pinning was achieved by enriching surface defects (see methods) by reducing the passivation time of the gliding assay. The MT spiral diameter was observed to be decreasing as the motor density increased. In order to study the systematic effect of motor density and MT length on the spiral radius the MT lengths were quantified. The intrinsic variability of MT lengths allowed us to study the effect of MT length. The spiral radius was observed to qualitatively scale with both motor density and MT length (Figure 5.4 A).

5.4.2 Radius of spirals scales with the motor density

The spiral diameter was measured as described in the methods to analyze the scaling of spiral radius with motor density. The spiral diameter plotted as a function of motor density scales inversely with the motor density (Figure 5.3 A). This observation is qualitatively consistent with the previous predictions of myosin driven actin patterns [12]. However we observe a scaling exponent of -0.66 i.e. $-2/3$ (Figure 5.3 B) instead of previously reported value of $-1/3$ for actin as well as predicted for MT-kinesin [12]. The optimal fitting of power law function to the raw data of radius/diameter vs motor density was difficult due the high S.D in the data. This was suspected to be due to the range of MT lengths ($2\text{-}20\text{ }\mu\text{m}$) observed across the assays and because the spiral radius qualitatively varied MT lengths too. To systematically study the effect of motor density on spiral radius the MT lengths should be comparable. Hence the after measuring the spiral radius for a number of spirals across various motor densities, the data was categorized according to the MT lengths by making bins of $2\text{ }\mu\text{m}$ such as $3\text{-}5\text{ }\mu\text{m}$, $5\text{-}7\text{ }\mu\text{m}$ and so on (see 9.1). For each length category (bins) the data was now fitted with a power law function to calculate the scaling exponent (Figure 5.3 C). It was observed that not only the radius scales with the motor density but the scaling exponent varies according to the MT length. Hence not only the scaling of radius with motor density deviates from the previously predicted $-1/3$ value but also varies with the MT length. To find a generic trend a data collapse was obtained by re-scaling both radius and motor density. Spiral radius was re-scaled as $A \equiv R/L_p$ and motor density was re-scaled as $\epsilon \equiv fL_p^2/kT$ (Figure 5.3 D). Data collapse obtained by re-scaling of variables highlights that the phenomenon remains the same across ranges of motors and MT length and is governed by the same underlying principles. By fitting a power law function $A \sim \epsilon^\alpha$ to the re-scaled data we found α to be -0.33 (Figure 5.3 D). Since the scaling exponent obtained by re-scaling the data is $-1/3$. This is the predicted one, hence the spiral radius scales as $-1/3$ with the force and hence the motor density similar

to the acto-myosin system.

5.4.3 Increasing spiral radius with MT length

A length dependence of MT spiral radius was evident in our experiments, it was then explored systematically. In order to systematically study the dependence of spiral radius on the MT length the data was categorized according to the motor density (see 9.1). For each motor density the radius was plotted as a function of MT length (Figure 5.4 A). The data for each motor density was binned averaged with a constant bin width. It was observed that for each motor density the spiral radius increased with the MT length. It was also observed that as the motor density increases the scaling becomes less steep. In order to obtain a general scaling behavior of radius and length across all the motor densities the variables were re-scaled. The spiraling radius was re-scaled as $\xi \equiv Rf^{1/3}$ (Figure 5.4 B). After obtaining a data collapse, scaling analysis was performed on the data collapse by fitting a power-law function of form $\xi \sim L_{mt}^\alpha$. We found the scaling exponent α to be 0.48, hence the radius was observed to scale with the MT length as $1/2$ (Figure 5.4 B). Such dependence of radius on MT length was not observed for actin and was not predicted for MTs [12]. This MT length dependence of spiral radius can be hypothesized to be due to the differences in the elastic properties of MTs and actin. MTs can have L_p that might vary with the MT length due to their anisotropic properties [137]. To study whether the length dependence of spiral radius can be explained by length dependent L_p of MTs the experimental data was compared to simulations of pinned gliding assay with constant and variable L_p models. The simulations were performed for a dynein-based system; however, in the future they can be extended to kinesin. Since, the dependence of the spiral radius on MT length is a phenomenon explained by the anisotropic nature of the MT, it can be hypothesized to be independent of the motor properties. Hence the type of motor used might not affect the scaling relationship. However, the geometry of the setup will need to be adjusted

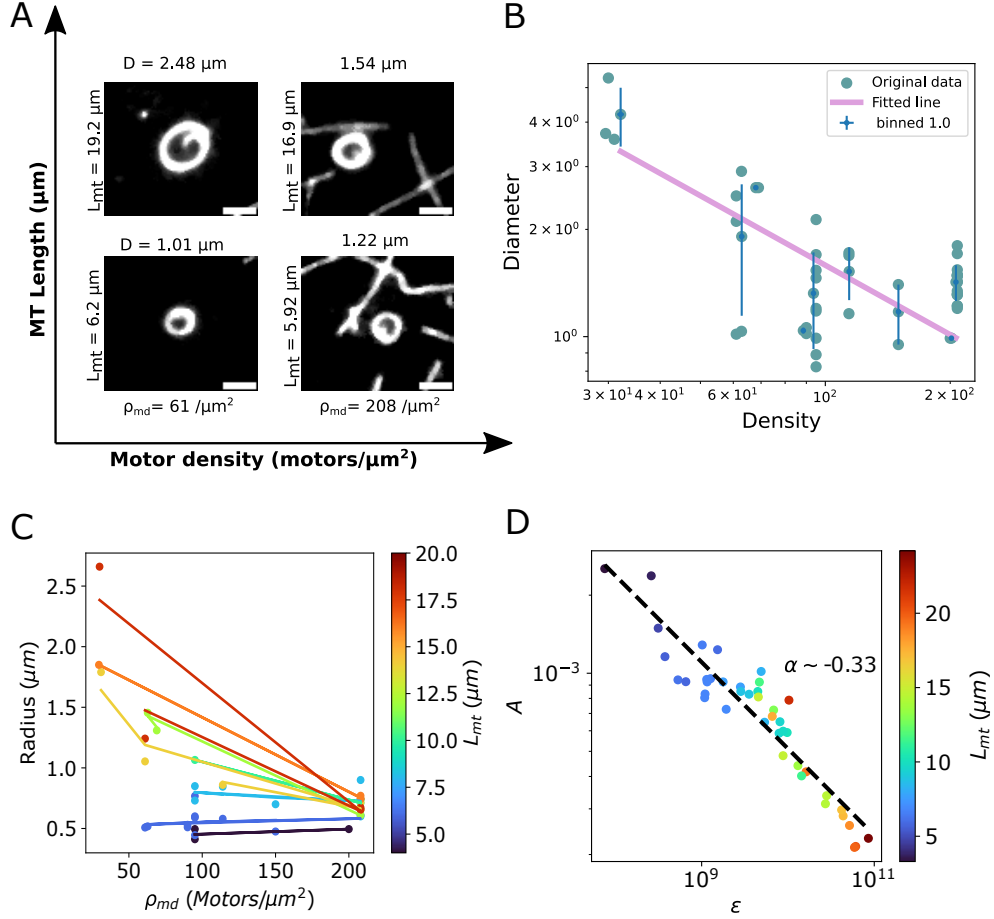


Figure 5.3: **Scaling of spiral radius with motor density**(A) The dynein density (x-axis) and MT length (y-axis) dependent changes in representative images of MT spirals show differences in spiral radius. The lengths (L_{MT}), motor density (ρ_m) and diameter (D) are listed next to the images. Scale bar: $2 \mu\text{m}$ (B) Spiral diameter plotted as function of motor density. The data for each density was binned and fitted with a straight line. The scaling exponent of the fit obtained was 0.66. (C) Radius of spirals plotted as a function of the motor density for various MT lengths. The raw data of radius vs motor density was categorized into bins according to the MT length (Bin size: $2 \mu\text{m}$) and fitted individually to a straight line. (D) To obtain a data collapse the data from (C) was re-scaled as the variables as $A \equiv R/L_p$ and $\epsilon \equiv f\ell_p^2/kT$. Scaling analysis was performed on data collapse by fitting the power law $A \sim \epsilon^a$ (dashed line).

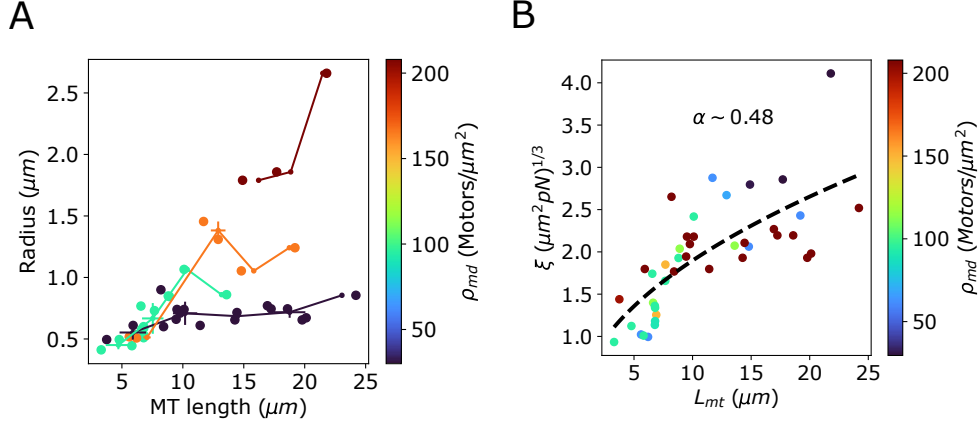


Figure 5.4: **Scaling of spiral radius with MT length**(A) Radius of spirals is plotted as a function of MT lengths with motor densities (color-coded). Data for each motor density (points) was binned averaged with constant bin width (line). (B) Data collapse from raw data of plot A was obtained by re-scaling the spiraling radius as $\xi \equiv Rf^{1/3}$. Scaling analysis is performed on data collapse by fitting a power-law function of form $\xi \sim L_{mt}^\alpha$.

based on the direction of walking of the motor. For a kinesin based study the minus ends of the MT needs to be pinned in order to facilitate spiral formation.

5.4.4 Scaling of frequency with MT length and motor density

The spiral frequency was measured based on the free tip movement in X and Y and the data was smoothed (Figure 5.2 B). This was analyzed using FFT to obtain the amplitude of frequency, which in turn was used to calculate the maximal frequency (Figure 5.2 C) (see 9.2 for data). This spiral frequency was plotted as a function of motor density for length categories of bin width 2 μm each (Figure 5.5 A). Similar to radius vs motor density spiral frequency was observed to scale with motor density with varying exponents for each length category. To obtain a general scaling behavior of frequency with motor density for all the MT lengths, the data was re-scaled as $\chi = \nu \ell_p / v$ (Figure 5.5 B). The collapsed data was then fitted to a power law function to obtain the scaling exponent. The scaling exponent α was found to be 0.35. Hence

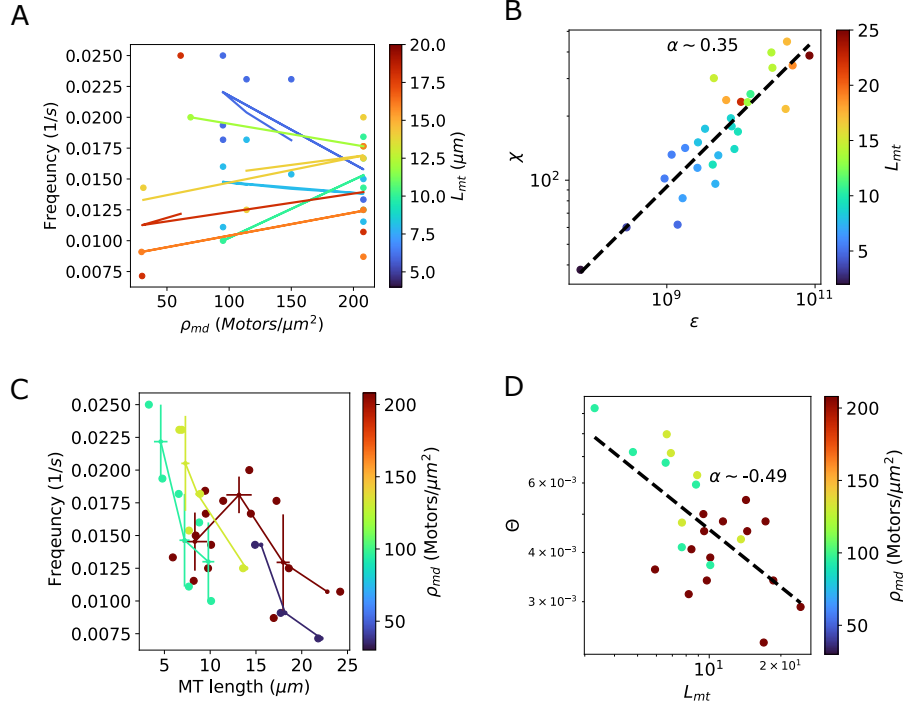


Figure 5.5: **Scaling of spiral frequency with MT length and motor density**(A) Frequency of spirals is plotted as a function of motor density for MTs of varying lengths (color-coded). The data was divided in bins of length $2 \mu\text{m}$ and each dataset was fit to a power law fit $A \sim \epsilon^a$.(B) Data collapse from raw data of (A) was obtained by re-scaling the frequency as $\chi = \nu \ell_p / v$ and the motor density as $\epsilon \equiv f \ell_p^2 / kT$. Scaling analysis was performed on data collapse by fitting the power law (dashed line). (C) Frequency of spirals is plotted as a function of MT lengths at various motor densities (color-coded). The raw data (points) for each motor density was binned averaged and plotted (Line). (D) Data collapse was obtained by re-scaling the raw data from (C) as $\Theta = \nu / f^{0.4}$. Scaling analysis was performed on data collapse by fitting the power law (dashed line).

the frequency of spirals scales with the motor density and linear force density as $1/3$ (Figure 5.5 B). The frequency was then plotted as a function of MT length for each motor density. The frequency was observed to decrease as the MT length decreased (Figure 5.5 C). To obtain a general scaling behavior the variable was resealed as $\Theta = \nu / f^{0.4}$ (Figure 5.5 D). The power law fit to the re-scaled data showed the α to be -0.49. Hence the frequency scale inversely with the MT length by a factor of $1/2$ (Figure 5.5 D).

5.4.5 Length dependent persistence length model explains the scaling behavior of spirals ¹

In order to better understand what results in the observed scaling behavior, an *in silico* model was developed. This model was a modification of a simulated gliding assay with pinned MT plus ends comparable to the previously described simulations [170]. The simulations were performed using an open-source simulator Cytosim [103]. Filament tips were immobilized where the pinning allows for rotational degree of freedom while restricting transnational motion. This resulted in formation of spiral. In this set up two alternative models of MT elastic properties were tested: (a) Constant persistence length model, where the ℓ_p is assumed to be constant due to assumption of isotropic nature of MTs [45] and (b) Length dependent ($\ell_p(\ell)$), where the ℓ_p changes as the MT length changes based on the assumption that MTs are anisotropic [73, 109]. To effectively introduce the effect of anisotropic elastic properties in coarse-grained microtubules, we approximated microtubules as isotropic materials with length-dependent bending rigidity.

$$\kappa(\ell) = K_b T \ell_p(\ell) \quad (5.1)$$

In the later model the bending rigidity of filament was varied as a function of MT length using an expression obtained by Pampaloni et al. [109]:

$$\ell_p(\ell) = l_p^\infty (1 + 441/L^2)^{-1} \quad (5.2)$$

By varying the motor densities and MT lengths in the simulations the scaling of spiral radius and frequency with MT length and motor density was studied. With alpha values for the A vs ϵ , η vs L_{MT} , χ vs e and θ vs L_{MT} as 0.325, 0.48, -0.34 and -0.41 in the simulations with length dependent L_p model vs 0.33, 0.48, -0.35 and -0.49 in the

¹Simulations performed by Mr. Aman Soni.

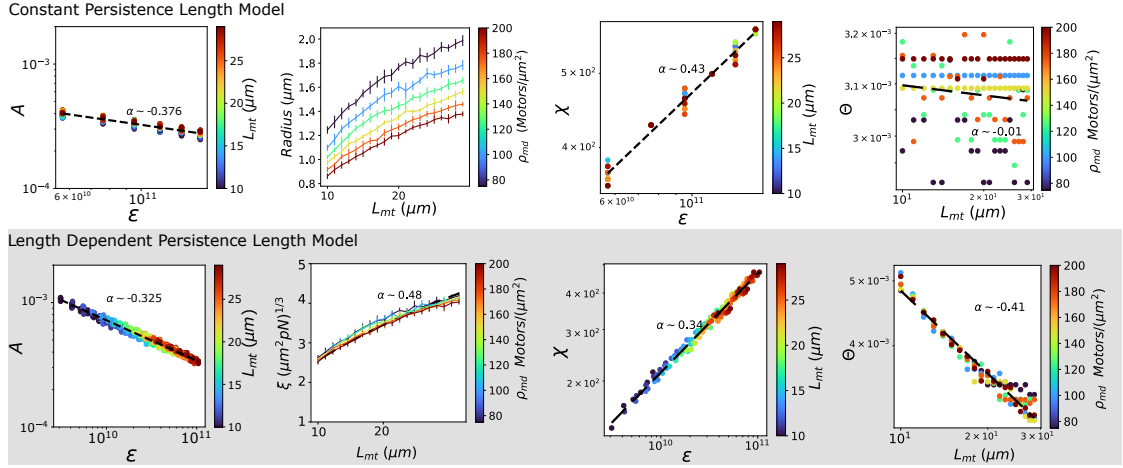


Figure 5.6: **Length dependent persistence length model explains the MT spiral scaling.** (Top) Plots of the re-scaled variables A vs ϵ , η vs L_{MT} , χ vs ϵ and θ vs L_{MT} respectively (left to right) for the spirals, obtained from the simulated pinned gliding assay for MTs with constant L_p . (bottom) Plots of the re-scaled variables A vs ϵ , η vs L_{MT} , χ vs ϵ and θ vs L_{MT} respectively (left to right) for the spirals, obtained from the simulated pinned gliding assay for MTs with length dependent L_p .

experiments (Figure 5.6). Hence in the simulations the scaling of radius and frequency with MT length and motor density observed for the experiments is best explained by the length dependent persistence length model.

5.4.6 Discussion

Dynein driven single MT spirals were reconstituted by labeling the plus ends of MTs with biotin which in a gliding assay like set up binds to streptavidin coated surface mimicking the surface defects encountered by leading filament ends. It was observed that dynein can drive MT spiraling at sufficient motor densities and point pinning of MTs. The motor density was then varied over a range of 30-208 motors/ μm^2 . For motor densities higher than 100 motors/ μm^2 pinning of MTs was achieved by enhancing the surface defects. The spiral radius and frequency was estimated and their scaling with motor density and MT length was studied. By comparing the scaling of spiral radius and frequency to two models of MT elasticity, MT spirals were used to test models of

MT elasticity. By the comparison to the models it was found that length dependent L_p explains the observed scaling, suggestive of anisotropic nature of MTs.

It was observed that the spiral radius scaled with the motor density but the scaling exponent varied with the MT length. This was suggestive of dependence of spiral radius on MT length itself. Upon re-scaling of the variables the general scaling of radius with motor density followed the previously observed scaling of $-1/3$ [12] however the dependence of radius on filament length was neither observed for actin nor predicted for MT-kinesin system [12]. In previous studies the spiral radius was observed to scale as $-1/3$ with the linear force density for actin [12]. The linear force density and the L_p were assumed to be independent of the filament length. Hence the filament length did not affect the spiral scaling only the L_p and total force i.e. motor density did [12]. The L_p is assumed to be constant for actin, however as newer studies suggested that MTs might not have a constant L_p [109]. Due to the anisotropic nature of MTs the L_p might vary with the length [73]. In order to test whether this scaling can be explained by the length dependence of MT L_p , simulations of pinned gliding assays with constant L_p and length dependent L_p were performed. By comparing all the derived variables i.e. A vs ϵ , η vs L_{MT} , χ vs e and θ vs L_{MT} plots from both the models (Figure 5.6) to the experimental data, it was found that length dependent model matches the experimentally observed scaling the best. It was observed that the length dependent L_p model matched the observed scaling behavior for both spiral radius and frequency. The variation of L_p with the change in MT length was incorporated in the model using the relationship obtained experimentally [109]. The model with constant L_p showed that the spiral radius scaled with the motor density as similar to as observed for actin but with constant L_p the radius did not scale the MT length (Figure 5.6). The length dependent L_p model also matched the scaling exponents obtained for scaling of frequency in the experiments. The constant L_p model predicted scaling of frequency with the motor density by $4/3$ however the experimentally observed scaling was by

1/3 which is predicted by the length dependent L_p model. Similarly, the constant L_p models showed no dependence of frequency on the MT length but length dependent L_p model predicted a scaling ratio of 1/2 which is closer to the experimentally obtained one.

5.5 Conclusions

Hence by using the dynein driven MT spirals as a test, two models of MT elasticity were tested. The results support the length dependent variation of L_p in MTs which is suggesting that the L_p of the MTs increases as the length of the MT increases. Such dependence of L_p on the total MT length is suggestive of the anisotropic nature of MTs.

Chapter 6

In vitro motility of kinesin or dynein bound beads ¹

6.1 Summary

Dynein-kinesin "tug of war" is critical in bidirectional transport and organelle positioning such as Golgi. Previous studies have suggested that varying motor numbers can influence cargo transport properties. A nanobody based approach was used to facilitate coating of GFP-tagged motors on a bead. An *in vitro* system was developed using fixed MTs and motor-coated beads to reconstitute bead transport. After successfully attaching dynein and kinesin to the beads, they demonstrated diffusive movement upon ATP addition. This setup shows a potential to be used as a template to reconstitute organelle motility assay.

¹Golgi assays in collaboration with Dr. Nagaraj Balasubramanian, Golgi and cytosol Purified by Antara Chakraborty.

6.2 Introduction

Motors carry out transport of multiple organelles and vesicles *in vivo* which mainly involves transport by team of motors. The examples of such transport are transport of Golgi, ER, peroxisomes, Endolysosomes, Autophagosomes, Melanosomes, Mitochondria and lipid droplets by cytoplasmic dynein (reviewed in [119]. Transport properties of cargoes can be seen to vary with varying motor number [115]. Dynein kinesin tug of war is known to be involved in bidirectional transport of vesicles and organelles [165]. Dynein moves towards the plus end of MTs and hence is responsible for the retrograde transport in the cell while plus end directed kinesin is responsible for the anterograde transport. Both the motors are simultaneously attached to the cargo [165] as seen in the case of mitochondria [40], endosomes [147], neuronal transport vesicle [55] and golgi [145]. Hence without a regulatory mechanism the directional transport of cargoes will be hindered [99] which makes studying the motor tug of war important.

In vivo studies show that dynein and kinesin play a role in adhesion dependent organization of Golgi [145] suggesting a role of dynein-kinesin tug of war in Golgi organization. Isolated Golgi has been reported to move along the fixed MTs towards its plus ends in a preliminary *in vitro* assay confirming the role of kinesin [35, 25]. While dynein played an important role in cell adhesion based Golgi organization as seen *in vivo* study [145]. Hence cell adhesion based Golgi positioning involves a tug of war between dynein and kinesin which can be studied using an *ex vivo* assay. An *in vitro* set up with immobilized MTs and motor coated beads can help us understand number dependent motor transport as well as effects of opposing motors transporting the same cargo. Such set up can also be extended to study organelle transport properties of purified organelles *in vitro* such as Golgi to study the role of motor tug of war in organelle positioning.

To make an *in vitro* set up for reconstituting bead and organelle transport assay

in a preliminary work, a set up with purified motors and MTs was developed. A flow chamber based system with fixed Taxol stabilized rhodamine labeled MTs was prepared. To enable coating of motors on a synthetic cargo such as a polystyrene or magnetic bead biotinylated anti-GFP nanobody was prepared to enable binding of any GFP tagged protein to streptavidin coated beads. The coating of beads with motors can be confirmed by presence of green fluorescence, both dynein and kinesin were separately attached to the bead using this set up. Such motor coated beads were able to land and remain bound to fixed MTs and show slight movement upon addition of ATP. This set up was then extended to reconstitute motility assay of Golgi in a preliminary assay. In order to reconstitute mixed motor assays, gliding assay based screening of various buffer conditions was performed to find conditions suitable for optimum activity of both kinesin and dynein.

6.3 Methods

6.3.1 Protein purification

Required proteins such tubulin, rhodamine labeled tubulin, dynein, anti-GFP nanobody and EGFP were purified as mentioned in details in section 3.2.1.

Kinesin K560 Purification: pET17_K560-GFP-His was a gift from Ron Vale (Addgene plasmid # 15219 ; <http://n2t.net/addgene:15219> ; RRID:Addgene_15219). The plasmid expresses GFP tagged Human neuronal kinesin heavy chain (KIF5B) truncated at amino acid 560 (K560-GFP) and has a 6x His tag for purification. The plasmid was transformed into *E.coli* BL21 DE3 and grown in enriched LB () at 22° C overnight. The secondary culture was inoculated using 1% inoculum from the overnight culture. The protein was expressed using 0.1 mM IPTG at OD600 of 0.4 for 16 hours at 22° C . The protein was purified from the clarified cell lysate using Co-NTA resin (Thermo Scientific, IL, USA) followed by elution using imidazole (HiMedia, India) in

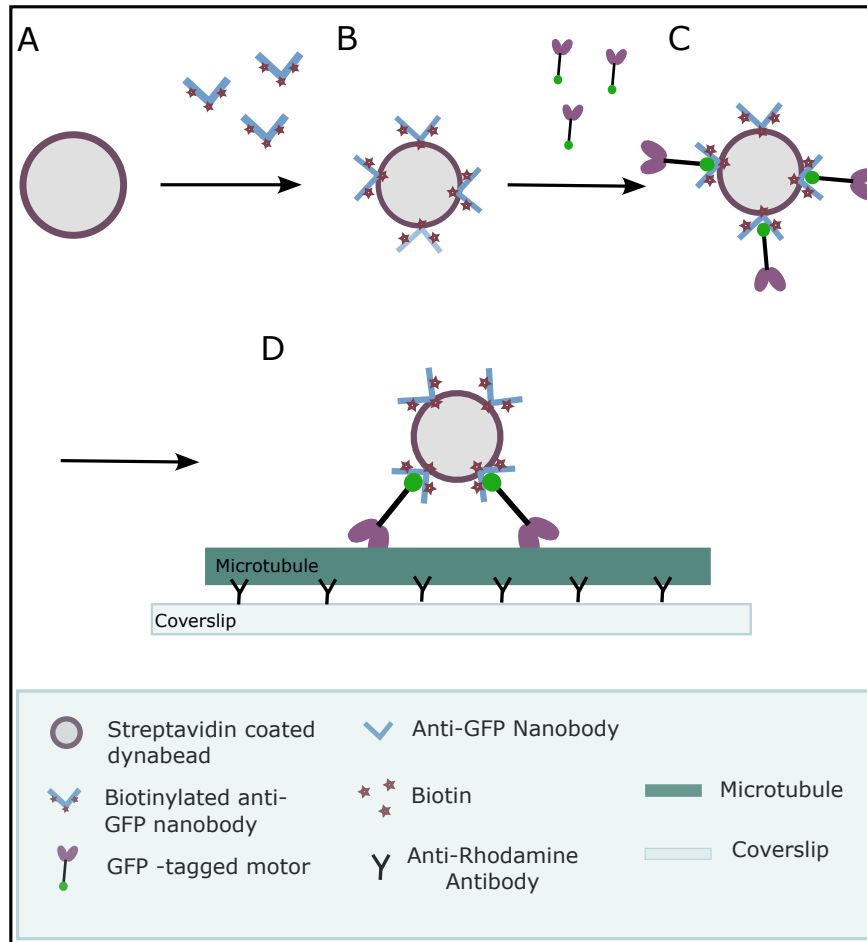


Figure 6.1: ***In vitro* reconstitution of cargo motility using biotinylated anti-GFP nanobody.** The schematic represents the strategy used for coating of GFP tagged proteins on beads to reconstitute bead based motor motility assay. (A) Anti-GFP nanobody which is biotinylated *in vitro* is complex-ed with streptavidin coated dynabeads. (B) The nanobody can now bind to any GFP or EGFP tagged protein including motors. (C) Hence GFP tagged motor can be coated on the streptavidin complexed beads via the biotinylated anti-GFP nanobody. (D) The beads are then allowed to land and walk on MTs which are fixed on a coverslip in a flow chamber like set up.

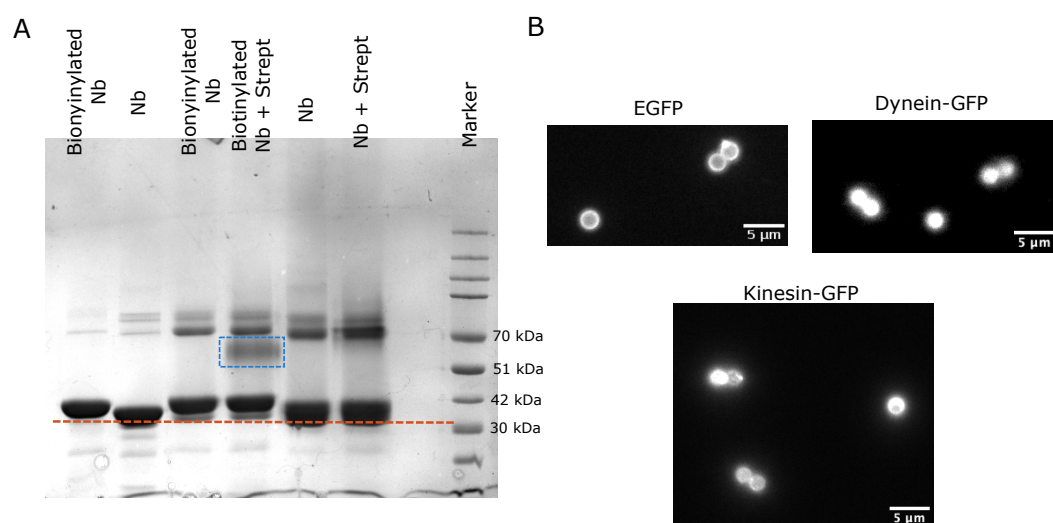


Figure 6.2: ***In vitro* biotinylation of anti-GFP nanobody and coating of dynabeads with GFP.** (A) SDS-PAGE gel shift assay performed to confirm the biotinylation of the nanobody. The molecular weight of the nanobody shifted post biotinylation by 1-2 kDa. The appearance of a higher molecular weight protein band in the gel shift assay is observed only for biotinylated nanobody upon incubating it with streptavidin (blue box) further confirming the biotinylation. (B) Representative images of the dynabeads as imaged for GFP intensity confirming the binding of EGFP, Dynein-GFP and Kinesin-GFP to the dynabeads via the biotinylated anti-GFP nanobody.

sodium phosphate buffer. The eluted protein was stored at -80°C with 20% sucrose.

6.3.2 *In vitro* biotinylation of Anti-GFP nanobody

The purified anti-GFP nanobody was biotinylated *in vitro* using NHS-Biotin. The purified nanobody (8.47 mg) was subjected to biotinylation by incubating it with NHS-Biotin (100 μg) at RT for 2 hours. The reaction was performed in PBS (10 mM Na-Phosphate buffer with 140 mM NaCl (pH 8.5)). After the biotinylation the excess biotin was removed by dialyzing the protein overnight using a Snake Skin Dialysis Tubing with 10000 KDa cut off (Thermofischer) in PBS. After dialysis the protein was flash frozen and stored at -80°C . The biotinylation was confirmed by both HABA based estimation of biotin and on SDS-PAGE by shift in molecular weight of the nanobody. The concentration of biotin was measured by using HABA/avidin reagent

based absorbance at 500 nm. The labeling ratio obtained from biotin concentration and the protein concentration (A280) was 4:1 (Biotin: Nanobody). The nanobody shows a shift in the molecular weight post biotinylation which is visible on a SDS-PAGE (Figure 6.2 A). To further confirm the biotinylation, a gel shift assay was performed. The nanobody (15 μ g) was treated with a non-reducing SDS-PAGE dye (no DTT) for 5 minutes at 95° C. It was then incubated with streptavidin (2 μ g) at RT for 15 minutes. The samples were then subjected to SDS-PAGE. The biotinylated nanobody shows a shift in the molecular weight when allowed to incubate with streptavidin. It is visible as an extra band in the gel (lane 4) (Figure 6.2 A). Hence the anti-GFP nanobody was biotinylated *in vitro*.

6.3.3 Preparation of motor coated dynabeads

The motors were coated on a bead using biotin streptavidin chemistry. A 5 μ l slurry of streptavidin coated dynabeads (M-270, Invitrogen) was washed with either BRB-80 (for kinesin) or Dynein lysis buffer (for dynein)(30 mM HEPES, 2 mM Mg-Acetate, 50 mM K-Acetate, 1 mM EGTA, 10 Glycerol) by re-suspending the bead slurry in buffer followed by precipitating the beads using a magnet. The buffer was exchanged twice for removal of the storage buffer. The beads were then coated with biotinylated anti-GFP nanobody by incubating the beads with 100 μ g nanobody at RT for 15 minutes on a rotor. The beads were then incubated with either 60 μ g kinesin k560 diluted to the total volume of 100 μ l (with BRB-80) or 3 μ g dynein (in dynein lysis buffer). The motors were allowed to bind to the beads via the anti-GFP nanobody by incubating at RT for 15 minutes on a rotor. The excess unbound motor were removed by washing the beads with twice plain buffer (BRB-80 OR Dynein lysis buffer). The washed beads were re-suspended in 20 μ l and used immediately. To confirm the binding of GFP to the beads, purified EGFP was used instead of motors and the binding was confirmed by imaging the beads in FITC (Figure 6.2 B).

6.3.4 *In vitro* reconstitution of dynein and kinesin bead transport

MT preparation: Rhodamine labeled MTs were prepared by polymerizing 20 μ M unlabeled tubulin with 10 μ M rhodamine labeled tubulin in BRB80 at 37° C. The MTs were stabilized by addition of Taxol (20 μ M). The monomers were removed by pelleting the MTs over a 60% glycerol cushion at 1,35,300 g in TLA 100.3 (Beckman Coulter, CA, USA) for 18 minutes. The MT pellet was re-suspended in BRB-80 containing 20 μ M taxol and used immediately.

The bead motility assay was performed in a flow-cell constructed of a 1.5 cover-slip adhered to a slide by two parallel strips of double backed tape, creating a gap. The chamber had a dimensions of 22 mm x 7 mm x 0.01 mm. The chamber was coated with anti-rhodamine antibodies for 8 minutes. The surface was blocked by casein (1 mg/ml) for 8 minutes. Taxol stabilized MTs were flowed in the chamber and incubated until sufficient MTs land on the surface. The unbound MTs were washed with BRB-80 with 20 μ M Taxol. The motor coated beads were flowed in and allowed to land on the MTs. Unbound beads were removed by flowing either BRB-80 or dynein lysis buffer. The motility was observed after addition of motility buffer. For kinesin BRB-80 with 1 mM ATP and Antifade (0.005 mg Glucose Oxidase, 0.0015 mg Catalase, 7.2 mM Glucose in 100 μ l BRB-80) was used while for dynein Lysis Buffer + 2 mM ATP + Antifade mix was used. The motility of beads was recorded at 60x in TRITC (for MTs) and FITC(for beads) for 2-5 minutes at every 5-10 seconds. All assays were performed at 37°C. All reagents unless otherwise stated, were from Sigma-Aldrich, MO, USA.

6.3.5 Buffer optimizations for mix motor assay

To perform mixed motor bead assays or organelle transport assays where effect of opposing motors is to be studied, it is important that the conditions of the assay

are such that both the motors perform optimally. To find optimum buffer conditions where both dynein and kinesin can function optimally a series of gliding assays were performed. Here dynein gliding assay was performed in BRB-80 based buffer which is usually used for kinesin based assay. And kinesin gliding assays were performed in HEPES based buffers which is used to study motility of dynein. The gliding assays were performed as mentioned in the previously (see section 3.2.2 for detailed methodology). The motility of MTs was recorded in the various buffer conditions as summarized in (figure 6.4). For kinesin based gliding assays a BRB-80 based buffer has been optimized in the lab (Kinesin buffer-80 mM PIPES pH 6.8, 1 mM EGTA, 2 mM Mg-Acetate, 1 mg/ml Casein, 0.5 % β -ME, 5 mM MgCl_2 , 2 mM ATP and Anti-fade mix). For dynein based motility assays HEPES based motility buffers have been used [65, 121]. To find a buffer optimum for motility of both the motors, kinesin gliding assay was performed in a HEPES based buffer (Kinesin test buffer 1). Since kinesin showed no motility in the HEPES based buffer dynein motility was tested in kinesin buffer. Dynein motility in various BRB-80 based buffers with varying salt concentrations as well as presence of β -ME and casein were also tested ((Dynein test buffers 1-4)). The detailed composition of buffers is provided in (figure 6.4).

6.3.6 *In vitro* Golgi motility assay

In order to study motility of Golgi by dynein and kinesin, a preliminary *ex vivo* assay was reconstituted. The aim of the assay was to study the movement of purified Golgi to which dynein and kinesin are attached on immobilized MTs. Cytosol and Golgi(membrane fraction) were obtained from cultured cell using differential centrifugation of the cell lysate (By Antara Chakraborty). The membrane fraction was re-suspended in Golgi buffer(10mM HEPES pH 7.1, 100 mM K-Acetate, 3 mM Mg-Acetate, 5 mM EGTA, 1 mM DTT and freshly added Protease Inhibitor Cocktail (Roche)) and either used immediately or frozen after adding 250 mM sucrose for fu-

ture use [35]. The assay was performed in a double back taped flow-chamber created using slide and coverslip. Rhodamine labeled MTs were immobilized on the coverslip using anti-Rhodamine antibodies as mentioned earlier. The surface was passivated using using 1 mg/ml casein to avoid non-specific attachment of Golgi. Golgi enriched membrane fraction was mixed with 10 mM ATP, Antifade mix, equal volume of cytosol and ATP regenerating mix [20 units/ml PK (Pyruvate Kinase) and 3 mM phosphoenolpyruvate (PEP)]. The motility fractions was then introduced in the chamber and recorded in DIC and TRITC. Dual imaging in TRITC and DIC allowed visualization of both Golgi (DIC) and the immobilized MTs (TRITC). The movies were recorded every 3 seconds in DIC while for each field of view the first frame was recorded in TRITC to visualize the MT tracks.

6.4 Results

6.4.1 Coating of GFP-tagged motors on dynabeads via biotinylated anti-GFP nanobody

The *in vitro* biotinylation of the anti-GFP nanobody was successful as confirmed by both HABA estimation and gel shift assays. The ratio of biotinylation was 4 biotin per every nanobody. Binding of EGFP as well as GFP-Dynein and GFP-kinesin to the beads was confirmed by presence of GFP signal when the beads are imaged at FITC. Hence any GFP and EGFP tagged protein can be coated on streptavidin beads using these *in vitro* biotinylated nanobodies. The binding of kinesin as well as dynein was confirmed by imaging the GFP and binding of beads to MTs.

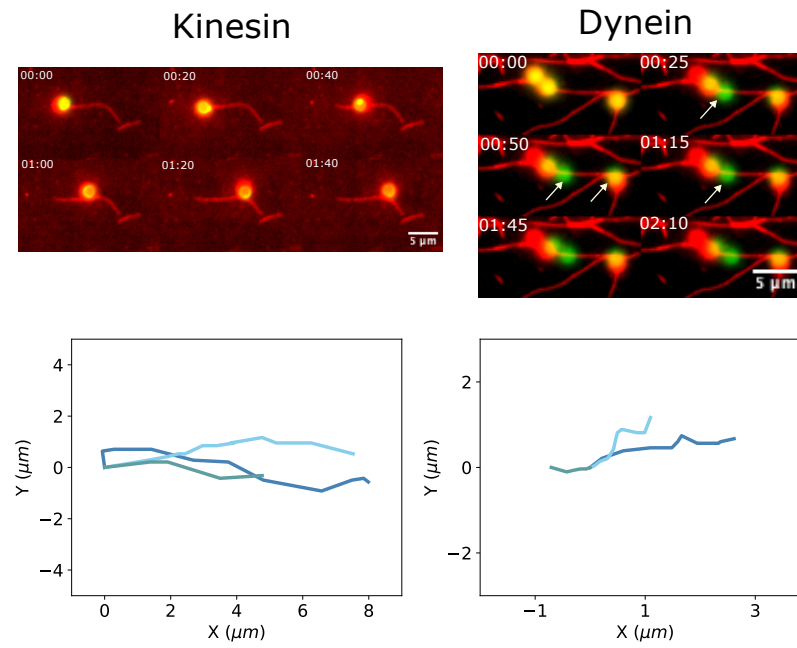


Figure 6.3: **Dynein and kinesin driven bead motility.** Representative montages showing dynein and kinesin coated dynabead moving on a fixed rhodamine labeled MT. The directed movement of beads can also be seen from the XY trajectory of 3 representative beads as plotted below.

Components	Dynein buffer	Kinesin buffer	Kinesin test buffer 1	Dynein test buffer 1	Dynein test buffer 2	Dynein test buffer 3	Dynein test buffer 4
30 mM HEPES	☒	☐	☒	☐	☐	☐	☐
10 mM PIPES pH 7	☐	☐	☐	☐	☐	☒	☒
80 mM PIPES pH 6.8	☐	☒	☐	☒	☒	☐	☐
50 mM K acetate	☒	☐	☒	☐	☐	☒	☒
4 mM MgSo4	☐	☐	☐	☐	☐	☐	☐
1mM EGTA	☒	☒	☒	☒	☒	☒	☒
2 mM ATP	☒	☒	☒	☒	☒	☒	☒
10 mM DTT	☐	☐	☐	☐	☐	☐	☐
Antifade	☒	☒	☒	☒	☒	☒	☒
2 mM Mg-Acetate	☒	☒	☒	☐	☐	☐	☐
1 mg/ml casein	☐	☒	☒	☐	☒	☐	☐
0.5 % b-ME	☐	☒	☒	☐	☐	☐	☒
5 mM MgCl2	☐	☒	☒	☒	☒	☒	☒
10% Glycerol	☒	☐	☐	☐	☐	☐	☐
Kinesin motility	–	Smooth Gliding	No Gliding	–	–	–	–
Dynein motility	Smooth Gliding	Gliding + MT depol	–	Gliding + MT bending	Gliding + MT bending	Gliding + MT bending	Hindered Gliding

Figure 6.4: **Buffer optimizations for dynein-kinesin mixed motility** Table showing composition of various buffers used to test motility of dynein and kinesin. The last two rows indicate the observation of MT gliding in kinesin or dynein based assay in the given buffers.

6.4.2 Reconstitution of bead transport

The MTs were seen to attach to the cover slip surface. The motor coated beads were seen to be landing on the MTs. The beads stayed attached to the MT even after the washing step. Upon addition of ATP a few beads showed directed movement along the MT (Figure 6.3). Only a fraction of beads occasionally showed movement along the MTs, while many beads detached and diffused away upon addition of ATP. The beads that showed the directed movement along the MTs were tracked using MtrackJ and the XY position of the bead was plotted (Figure 6.3). From the trajectory of the bead a directed movement can be seen. The average velocity (mean $\pm$ s.d.) for dynein based motility was 18.5 ± 7.5 nm/s ($n = 4$) and 80.33 ± 31.2 nm/s ($n = 3$) for kinesin. The observed velocity is lower than the single molecule velocity of both the motors. Releasing of beads upon addition of ATP and lower velocities indicate that either the motor density or the buffer conditions are not optimal for the motility. Hence this set up can be further optimized and in future used to study collective transport of motors as well as tug of war between opposing motors.

6.4.3 Buffer conditions for mixed motility assays

Kinesin shows optimum smooth MT gliding in a BRB-80 based buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 2 mM Mg-Acetate, 1 mg/ml Casein, 0.5 % β -ME, 5 mM MgCl_2 , 2 mM ATP and Anti-fade mix). However in a HEPES based buffer used for dynein gliding assay, kinesin shows no motility (stationary MTs) at all. Hence dynein gliding assay was performed in various BRB-80 based buffers. Even though dynein showed motility in most of the BRB-80 based buffer few issues were noted. MTs showed rapid depolymerisation/shrinkage in the dynein gliding assays when kinesin motility buffer was used as it is. Such MT shrinkage can be attributed to the presence of reducing agents such as β -ME in the buffer. To further optimize the system a few variations

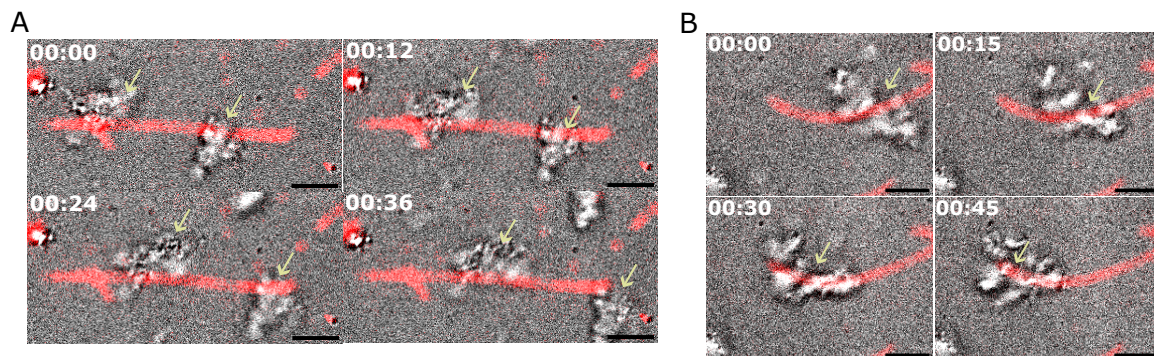


Figure 6.5: **Golgi motility on fixed MTs** Representative montages showing Golgi (gray) moving on fixed MTs (red) in presence of 10 mM ATP and cytosol. The movement of Golgi with time along the fixed MTs can be seen as marked by yellow arrows. Scale bar 5 μm . Time: mm:ss.

of PIPES based buffer (BRB-80 or BRB-10) were used (Dynein test buffer 1-4). The buffers varied in the concentration of PIPES as well as presence and absence of β -ME and casein. The optimum MT gliding by dynein in the PIPES based buffers was observed to be in dynein test buffer 1 (BRB-80, 5mM MgCl_2 , Anti-fade mix, 2 mM ATP). A few bending MTs were observed to be in the field of view however that could be due to artifacts of passivation. Hence a for mixed motility assay a BRB-80 based buffer with Anti-fade and ATP could be used. However kinesin motility in such minimal buffer should be tested and a quantitative analysis by comparing their velocities should be performed before performing mixed motor assays.

6.5 Discussion

Here we describe the optimization of bead motility driven by either dynein or kinesin in a preliminary *in vitro* assay. This assay used biotinylated anti-GFP nanobodies via which any GFP or EGFP tagged motor can be complexed to the streptavidin coated beads. Binding of the motors (kinesin or dynein) to the beads was confirmed by their fluorescence as well as by their movement on MTs. The set up can be further

optimized to improve the fraction of motile beads. Changes in terms of increasing density of functional motor on the beads, buffer conditions, and presence of crowdants for optimum motility of the motors can be done.

To be able to extend this system to perform *in vitro* mixed motor assays with purified motors it is essential to find conditions optimum for functioning of both the motors. A gliding assay based screening of various buffer compositions was performed for dynein as well as kinesin. The flow chamber based set up with immobilized MTs can be extended to be used to reconstitute organelle motility assays as done in the preliminary Golgi motility assays. This set up combined with optimized *in vitro* buffer conditions for the optimal functioning of both dynein and kinesin can be used to study the regulation of motor tug of war. With polarity marked MTs the directions of transport (plus or minus end directed) of the bead or organelle can be tracked. Hence in future such set up can be used to study positioning of organelles by motor tug of war.

In preliminary assay attempts were made to reconstitute Golgi motility *in vitro* as done previously [35]. In a flow chamber based set up MTs were immobilized on the coverslip and purified Golgi enriched membrane fractions were introduced in the chamber in presence of ATP. In a given field of view occasionally a few membranous structures were observed to move along the MTs while most of them remained diffusing away due to buffer flow. However the movement of the Golgi along the MTs was also along the flow of the buffer in the chamber. Due to non-frequent appearances of such motion and presence of flow in chamber it is hard to conclude whether this movement is purely due to motors. A setup with minimized fluid flow and thermal vibrations would be beneficial to distinguish the motility of organelle from that of the flow based artifacts. In order to minimize the fluid flow in the system use of sealed flow chambers or microfluidics based chamber can be used. Golgi has been demonstrated to show kinesin based plus end directed motility in similar *in vitro* assays. In order to distinguish the

direction of motion of the Golgi, end-labeled MTs where MT plus ends are labeled with a different fluorophore can be used in the future.

6.6 Conclusions

This simplified *in vitro* set up allows binding and visualization of any GFP-Tagged motor proteins to a streptavidin beads by using *in vitro* biotinylated anti-GFP nanobodies. Combined with the flow-chamber-based set-up with immobilized MTs, this can be used to reconstitute bead motility assays. This simple set up provides a way to attach any GFP-tagged motor to bead and hence in future can be used to study collective motor transport properties along with tug of war between opposing motors. The set-up can be extended to reconstitute organelle motility assays of Golgi to study the role of dynein-kinesin tug-of-war in Golgi organization.

Chapter 7

MT as a marker to measure the activity of DNA dependent GTPase motor¹

7.1 Summary

In preliminary assays, attempts were made to use MT as a marker to study possible rotational motion of a DNA dependent GTPase motor. MTs could be attached to the motor and undergo thermal fluctuations similar to the control assays hence suggesting that the set up requires further optimizations.

7.2 Introduction

Rotary motor proteins with a stereotypical stator-rotor geometry can utilize energy from NTP hydrolysis to perform rotational movement of the rotor [158]. Detecting rotational movement of proteins or protein complexes has been a technical challenge.

¹In collaboration with Dr. Saikrishnan Kayarat and Mr. Aman Kumar Yadav, DNA enzyme modification and Purification by Aman Yadav.

In vitro techniques in which the rotor is complexed with a marker that can be easily visualized can be used to demonstrate rotational movement. Previously single-molecule rotational movements of the F₀F₁ ATPase complex have been confirmed using biotinylated actin as a marker [174]. Biotinylated actin filaments of lengths 2.4-2.6 μ , labeled with Rhodamine were used as a visible rotor to mark the rotation of the F₀-F₁ rotor. Due to its elongated structure and ability to be complexed with biotin and fluorescent labels, actin was used as a marker of rotational motion. Apart from actin, pairs of magnetic beads have also been used to mark and control the rotation of F₁ ATPase *in vitro* [3]. In both the set ups biotin-streptavidin based chemistry was used to complex the marker (actin or bead) to the rotor. Elongated cells of *E. coli* have also been used to study rotational motion of its flagella [144]. The cells were attached to the coverslip via their single flagella causing the cells to rotate and hence using the cells as a marker to study rotational properties of the flagella. Since MTs are elongated structures which can be labeled with fluorophores as well as biotin they can be potentially used as a visible rotor that can mark the rotational motion of motors.

In order to study the rotational ability of a DNA dependent GTPase motor (DNA motor), I have developed an *in vitro* set up with biotinylated MTs as the marker for rotational motion. In preliminary experiments I have been able to immobilize the DNA motor complex to a glass surface and attach biotin MTs to the biotinylated rotor via streptavidin. This complex has demonstrated point pinning of biotin MTs via the DNA motor and the ability of MTs to show rotation degree of freedom. The MTs perform incomplete back and forth rotational motion in presence of GTP, GMPPNP and in absence of GTP as well. Occasionally the MTs pivoted around a point rotate for more than 180° and break off. Hence we show that MTs can be used as a marker for rotational studies of motors, however further optimizations of the experimental conditions is required to unambiguously demonstrate directional rotations.

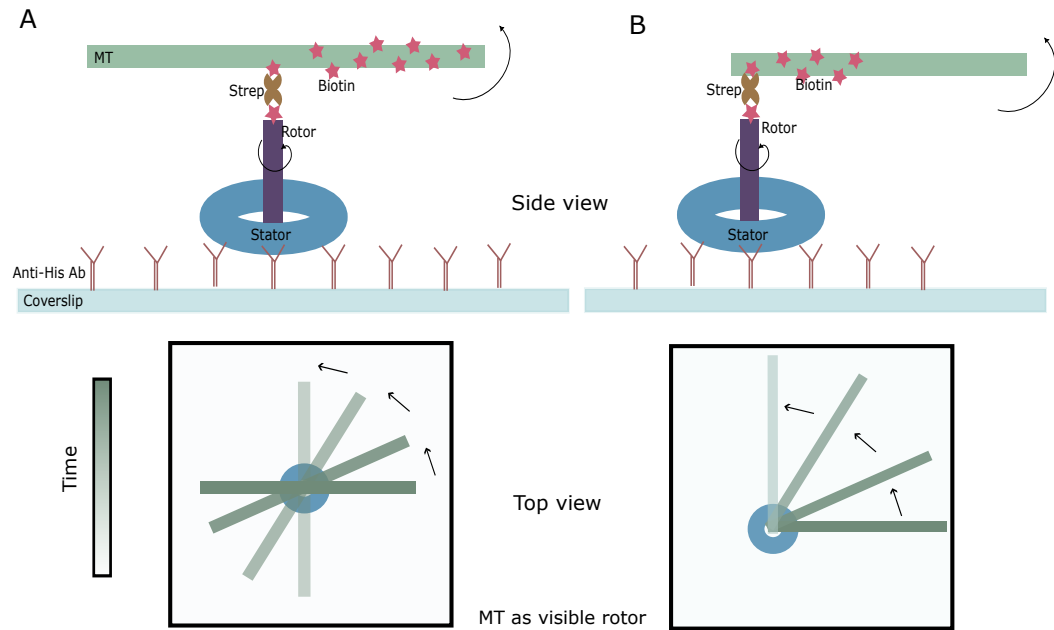


Figure 7.1: MTs as the visible rotor for studying rotational motion of DNA motor. (A) A schematic representing the set up used to perform DNA motor rotation assay with MTs as the rotational marker. The DNA motor stator (blue circle) is immobilized on the cover-slip using anti-His antibodies (red). The biotinylated (pink stars) DNA motor rotor (purple rod) binds to the stator forming the DNA motor complex. Biotinylated MTs are complexed with the rotor tip via a streptavidin (brown). The MTs are assembled in presence of rhodamine tubulin in the ratio 2:1 (unlabeled:labeled) hence allowing the visualization in fluorescence. Since the MTs are plus end biotinylated the MTs can attach to the DNA motor complex anywhere from the center (A) to the tip (B). The top view of both the scenarios show how the movement of MT would look like in time as the rotor is rotated by stator.

7.3 Methods

7.3.1 MT preparation

For preparation of rhodamine labeled plus end biotinylated MTs 22 μM unlabeled tubulin was polymerized with with 8 μM rhodamine labeled tubulin in BRB80 and 10% glycerol at 37°C for 40 minutes. To the reaction 18.33 μM biotin tubulin and 6.67 μM rhodamine tubulin was added and incubated further for 30 minutes to continue the polymerization of the plus end by biotinylated tubulin. Free monomers were removed by centrifuging the MTs over a 60% glycerol cushion at 135,300 g in TLA 100.3 rotor (Beckman Coulter, CA, USA) for 18 minutes (Accel/decel 6/4). The MT pellet was re-suspended in taxol containing buffer (20 μM Taxol + BRB-80) and used immediately.

7.3.2 DNA motor rotation assay

The DNA motor complex consisted of a rotor and a stator (mentioned as DNA motor henceforth). Both were purified and modified by Aman Yadav. The assay was performed in a chamber constructed using a slide and cover-slip where the dimensions of 22 mm X 5 mm was achieved by placing the double-back tape strips 5 mm apart. The chamber was first coated with anti-His antibodies (Rabbit, polyclonal, Sigma) for 8 minutes (Figure [7.1](#)). The remaining surface was blocked by casein (2 mg/ml) by incubating the casein for 8 minutes. Casein solution was prepared by dissolving casein in the assay buffer at 37°C for 1 hour, followed by centrifugation at 10,000 g for 5 minutes to remove aggregates. The unbound casein was washed by flowing the complex buffer 3 times. The stator (25 μg) was allowed to immobilize on the cover-slip through the anti-His antibody for 8 minutes. The rotor (20 μg) was then allowed to attach to the stator. Streptavidin (25-30 μg) was introduced in the chamber and allowed to bind to rotor for 5 minutes. The biotinylated MTs were then flowed in the chamber and incubated till enough MTs bind to the surface. After washing out the unbound

MTs movies for no GTP or no nucleotide were recorded at 60X in TRITC channel for 5-8 minutes every 3 seconds (Exposure 800 ms). Movies with 2 mM GTP (Jena Biosciences, Germany), 10 mM GTP and 2 mM GMPPNP (Jena Biosciences, Germany) were also recorded similarly after addition of the respective nucleotide. Along with the assays where the nucleotide was added at the end of the assay, trials where 1-2 mM GTP was kept throughout all the steps were also performed. This is due to the observation that the stator needed GTP for stability. However when GTP was included in all the steps, MTs were seen to be diffusing around and never landing or attaching to the DNA motor complex.

7.3.3 Control assays to distinguish MT fluctuations from rotational movement

To distinguish the thermal fluctuations of MTs from the rotational movement of the DNA motor complex, various control assays were performed. First, binding of biotinylated MTs with streptavidin was recorded. The chamber was prepared as mentioned earlier. It was first coated with streptavidin (2 mg/ml) followed by blocking with casein (2 mg/ml) to prevent non specific attachment of MTs. After washing out the casein, MTs were then introduced in the chamber and allowed to attach. After washing away the unbound MTs the attached MTs were recorded at 60 x TRITC. To check the stability of MTs they were imaged in BRB-80 (+ 20 μ M Taxol) and Complex buffer with Anti-fade mix. This is to ensure that the MTs remain stable during the imaging in the complex buffer. In order to distinguish the movement of DNA motor complex from that of rotational fluctuations by just the rotor another set of controls was performed. Here, the chamber was first coated with the rotor followed by blocking by casein. The MTs were then allowed to attach to the rotor via streptavidin as

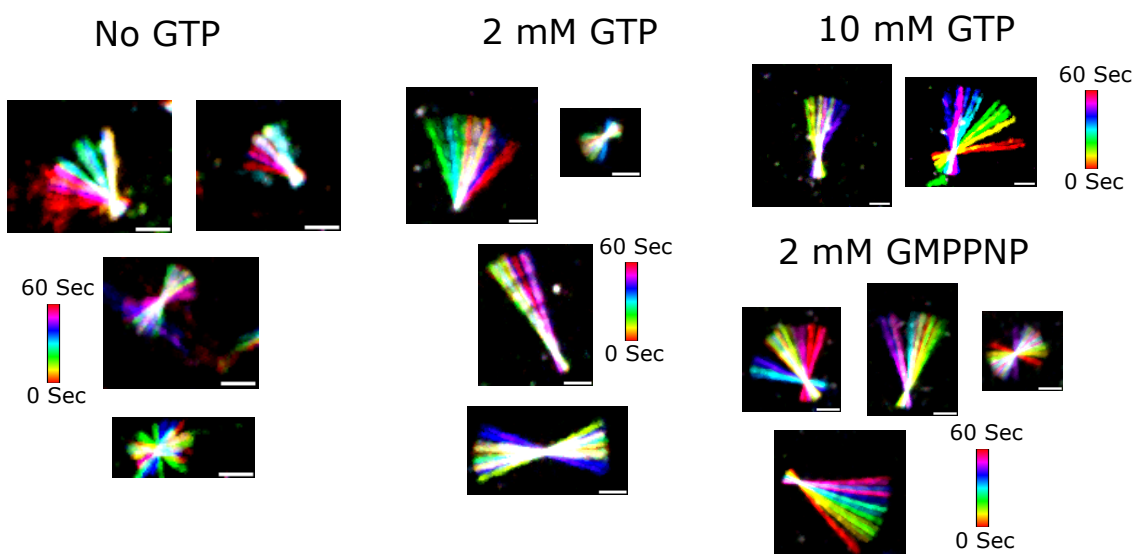


Figure 7.2: **MT pinning and rotational fluctuations in DNA motor rotation assay.** Color coded time projections of representative rhodamine labeled MTs showing pinning and rotation of MTs around the pivot in DNA motor rotation assay in presence and absence of various nucleotides. The time frames were color coded and Z projected to show the rotational fluctuations for 20 frames (60 seconds) for each MT. scale bar 2 μm .

mentioned previously. The rotation of MTs by the rotor protein was then recorded by at 60 X TRITC channel.

7.3.4 Microscopy and Image Analysis

The filaments were imaged using a 60x Oil immersion lens (NA=1.45) mounted on a Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan) using a TRITC filter (and FITC in case of dual color images) with temperature control system (Okolab, Pozzuoli, Italy). The filaments were imaged every 3 seconds for 5-8 minutes using an Andor Clara2 CCD camera (Andor Technology, Belfast, UK). The images were de-noised by applying median filter followed by background subtraction using FIJI [134]. *Zstack Depth ColorCoder 0.0.2* (<https://github.com/ekatrunkha/ZstackDepthColorCode>). The XY coordinates of the tips were generated by tracking the tip using plugin MTrackJ 1.5.1 also in FIJI.

7.4 Results

7.4.1 MT pinning and rotational fluctuations in DNA motor assay

In the DNA motor rotation assays it was observed that there were a few MTs in the field of view which landed on the cover-slip surface and a few of them remained bound even after the washing step (Figure 7.2). The MTs appear to be pinned to the surface via a point pinning and shows rotational fluctuations around this pivot. The pivot was observed to be anywhere on the MT from the center to the tip which was expected as the MTs are end-labeled. Along with the control assay with only streptavidin was on the surface (Figure 7.3), most of assays showed presence of MTs with point pinning. When the biotinylated MTs bind to the streptavidin in the control assays, the MTs can be seen to be bound to the surface with pinning as well as elongated clamps instead of point pinning. When the streptavidin binding control assay was performed in complex buffer with anti-fade instead of BRB-80, it was observed that a few MTs show attachment through point pinning. The control assay with only rotor showed the presence of similar point pinning of MTs with rotational fluctuations.

When GTP was introduced in all the steps throughout the assay, the MTs did not land and only kept diffusing. The MTs showed binding to the surface only when there was no GTP in the set up. The MTs once bound showed point pinning and rotational fluctuations in presence and absence of various nucleotides.

7.4.2 Effect of no nucleotide, GTP and GMPPNP on rotational fluctuations

After addition of GTP and GMPPNP the MTs showed rotational fluctuations around the pivot point was qualitatively similar to the fluctuations observed in absence of any

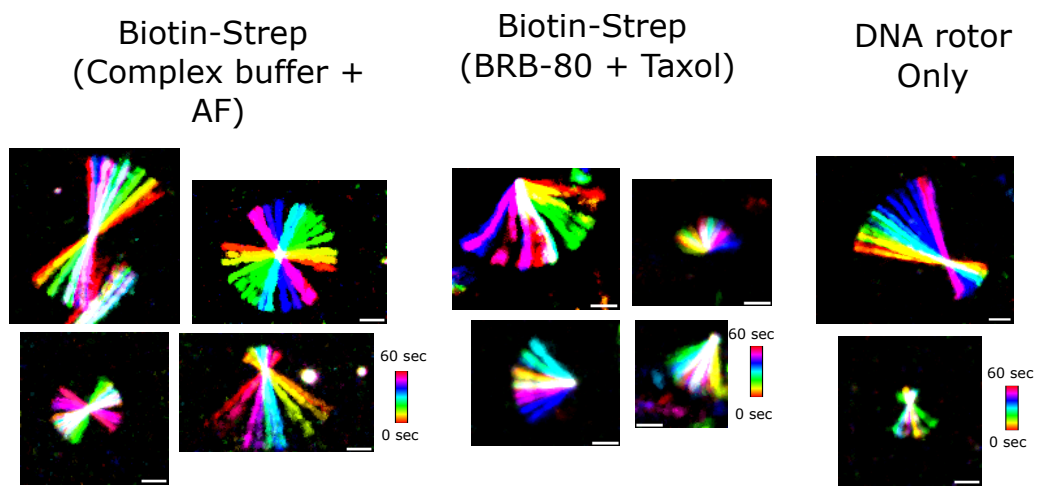


Figure 7.3: **MT pinning and rotational fluctuations in control assay.** Color coded time projections of representative MTs showing pinning and rotation of MTs around the pivot in control assays where binding of biotinylated MTs to streptavidin coated surface and to rotor were tested. The binding of MTs to streptavidin was checked in either BRB-80 with 20 μm taxol or the DNA motor complex buffer with Antifade (AF). The time frames were color coded and Z projected to show the rotational fluctuations for 20 frames (60 seconds) for each MT. scale bar 2 μm .

nucleotide (Figure 7.2). It was expected that in presence of GTP the frequency of the fluctuations would increase or a directed net movement of the MT takes place. The GMPPNP on the other hand was expected to block the movement of the DNA motor and hence show stationary MTs with very little thermal fluctuations around the pinning. Since neither of the nucleotides nor the control assays showed any qualitative differences making it hard to conclude whether the observed movement is via the DNA motor rotation or thermal fluctuations. The position of the MT tip was tracked and plotted as a function of time (Figure 7.4). However no distinct difference was observed in presence of GMPPNP, GTP or absence of any nucleotides. Movement of tip of MT bound to streptavidin or only rotor also did not show any distinct difference than that of binding of MTs to stator-rotor complex. Systematic study of number of MTs in various conditions will be required in order to study the effect of nucleotides on the MT movement. However qualitatively the movement resembles the fluctuations seen for control assays where no motor complex is present. Due to this similarity further controlled studies will be required to confirm the rotation of DNA motor.

7.4.3 MTs with more than 180° rotations

In the DNA motor rotational assay with 2 mM GTP in total 3 MTs have showed rotations more than 180° (Figure 7.5). The MTs appear to be clamped along the length of the MT instead of the pinning observed most of the times. The end of the clamped MT starts moving and performs a rotation of more than 180° before breaking off and diffusing away. Similar movement has been noted for 1 MT in the motor complex assay in presence of 2 mM GMPPNP. Since such clamping and rotation and eventual breaking off has been noted to some extent in the control assay with Biotin MT binding to streptavidin, it is hard to distinguish whether this movement is due to the DNA motor complex. Clamped MTs with free MT tip undergoing thermal fluctuations can rotate and break off similarly due to presence of flow in the chamber.

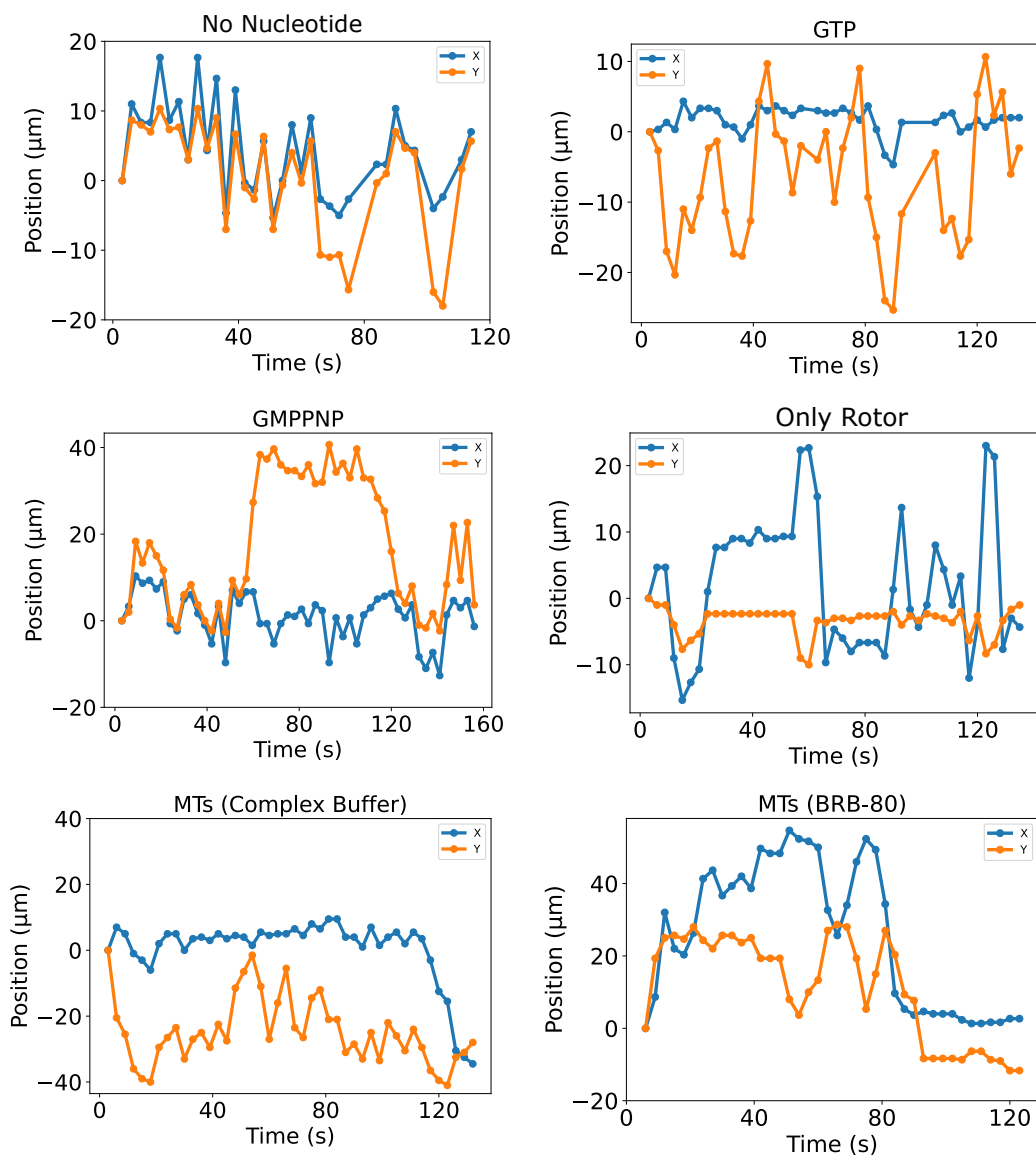


Figure 7.4: **MT tip position as a function of time in DNA motor and control assay.** The X and Y position of moving MT tip was plotted as a function of time for the DNA motor rotation assay with various nucleotides and control assays where MT binding to streptavidin or only DNA motor rotor was tested.

This kind of breakage occurs when some proportion of the MT is clamped to the surface instead of a point pinning. Since in the above mentioned cases the MTs are clamped to the surface in a similar way, the breaking off of MTs can be attributed to effect of thermal fluctuations combined with the flow. Hence in order to distinguish rotation from various artifacts arising from thermal fluctuations a microfluidic set up where flows can be avoided would be ideal along with enriching point pinning of MTs instead of clamping.

7.5 Discussion

Here we have tried to use MT as a marker for rotational motion of a DNA dependent GTPase motor. Due to the use of actin filament in previous studies, MTs were expected to be a suitable candidate to be used as a marker. In preliminary assay the DNA motor complex was immobilized on the cover-slip, followed by attachment of MTs to the rotor via a biotin streptavidin chemistry. Even though point pinning of MTs was observed along with rotational fluctuations around the pivot, no complete rotations were observed.

To confirm whether the observed pinning and rotational fluctuations are due to pinning of MT via the DNA motor complex various controls were tested. In case of control assays where MTs were immobilized on streptavidin, it was observed that occasionally MTs show a similar pinning along with the rotational fluctuations. Since the binding of plus end biotinylated MTs to the streptavidin coated coverslip was qualitatively similar to that of assays with DNA motor it cannot be concluded that the pinning is an effect of DNA motor only. The XY trajectory of the representative MTs also show no difference between the No GTP and GTP conditions.

Previously mentioned assays have reported that the observations of full rotations are rare however no full rotation was observed in these assays. Here, a few MTs were

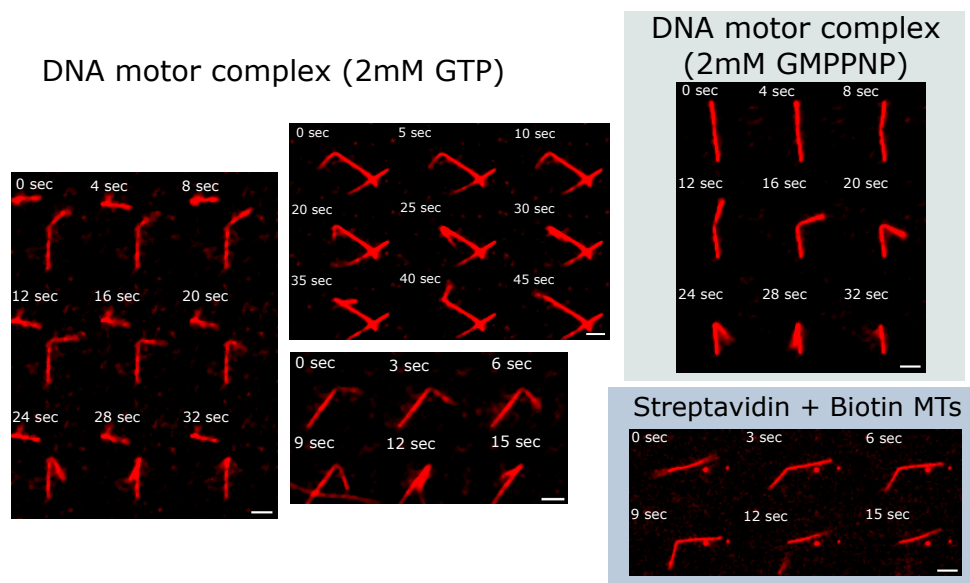


Figure 7.5: **MT Rotations with directed movement.** Montages of rhodamine labeled MTs where the MTs showed rotation of more than 180° and eventually broke off. Such rotations were observed for DNA motor rotation assay with GTP and GMPPNP and in the control assays where MT binding to streptavidin was tested. Scale bar: $2\mu\text{m}$.

observed to perform rotations more than 180° in presence of 2 mM GTP. However observation of similar rotations in the control assays is suggestive of this movement being an artifact. These rotations are accompanied by breakage of MTs in all the assays. This type of rotation and breakage of clamped MTs can be attributed to be a combined effect of thermal fluctuations of the free tip combined with the flow in the set up.

Hence further studies with the assays performed in a controlled microfluidic set up with minimal buffer flows and thermal fluctuations will be required to study the rotation of such motors. In order to avoid such breakage, MTs stabilized with GMPPNP could be used as GMMPPNP stabilized MTs have higher bending rigidity as compared to taxol. The assay set up can also be optimized to enrich point pinning of MTs and also to keep the MT lengths less than $3\mu\text{m}$ as used for actin based assays. This set up needs to be optimized in order to show unambiguous directed rotations of MTs by the DNA motor complex.

7.6 Conclusions

Hence, in a set of preliminary assays MTs show a potential to be used as a marker for rotational motion. The set up worked partly by allowing pinning of MTs to a DNA dependent GTPase motor. The pinned MTs showed thermal rotational fluctuations hinting at the possibility of capturing true rotations in future. The rotation of MTs appears for now to be random. It may either be a result of artifacts arising buffer flows or suboptimal conditions with random motion expected from point pinned filaments with rotational degree of freedom. These experiments however can be used to design optimize better experimental set up and test for rotational motion of proteins.

Chapter 8

Conclusions and Future Work

In the above mentioned set of studies I have been able to study the motor number dependence in yeast nuclear positioning and how it is regulated by enabling shorter or longer MT contacts with the cell cortex. This is suggestive of a possible mechanism by which more number of motors on the bud cell cortex are engaged to allow asymmetric positioning of the nucleus to the bud neck. The MT-cortical interactions and MT dynamics of bud and mother SPBs across various cell stages have been of interest to study the regulation of mitosis. Astral MT dynamics are of interest to study MT search based regulation of nuclear positioning and spindle orientation [139]. Detailed astral MT dynamics measurements across various cell stages can help us understand such regulations. The measurement of MT dynamics during pre-anaphase and anaphase done in this study can be used as a preliminary dataset for exploring MT dynamics across all cell division stages in future.

Using *in vitro* gliding assay as the system I have been able to study the motor number dependence of truncated dynein. The velocity of MTs was observed to increase rapidly and saturate as the motor number increases. For full length motors such increase in the velocity was explained on the basis of mechanical activation of the ϕ conformation of the motor. Since minimal motors are not capable of forming the

ϕ conformation, the motor cooperativity can be hypothesized to be driven by other mechanisms. The possible mechanisms could be asymmetric detachment behavior and backward stepping of dynein. This dataset can be used to test various theoretical models of such motor cooperativity in order to understand how dynein coordinates collective transport. Since *in vivo* dyneins can be anchored to a membrane, the collective properties of the transport can be affected [49]. Hence studying collective behavior of lipid anchored dyneins can help us understand *in vivo* regulation of collective transport properties. Comparing the two can help us gain insights into how loose coupling of dynein affects the motor coordination across varying numbers.

I have been able to reconstitute MT beating and MT spirals *in vitro* which have helped us study MT-motor interactions and motor-motor coordination and their role in emergence of patterns. The MT beating set up can be used to derive complex beating structures which can be helpful in studying the role of MT geometry on beating parameters and flagellar mechanics. Since the oscillations observed were slower than flagellar oscillations studies with a faster motor using a similar set up would be helpful to reconstitute minimal flagella like oscillations *in vitro*. MT spirals reconstituted with dynein were used to test various models of MT elasticity. It was observed that length dependent persistence length model was able to explain the observed scaling of MT spirals with MT length and motor density. This study has helped us gain insights into elastic properties of MTs suggestive of their anisotropic nature. Such simple *in vitro* systems can be used to models of elasticity and collective behavior.

Preliminary work of developing *in vitro* set ups for studying motor tug of war can be used to study organelle/cargo positioning by tug of war between opposing motors. The Anti-GFP Nanobody based set up allows coupling of any GFP-tagged motor to the bead and hence can be used to study cargo based collective transport and mixed motor motility. The systems can be optimized further by enhancing the surface motor density for efficient and processive transport. As dynein and kinesin based tug of war

has been proposed to play a role in cell adhesion based positioning of Golgi [145], the *in vitro* bead motility assay combined with *ex vivo* Golgi motility assay can be used to study the regulation of Golgi positioning. In preliminary work, MTs have were used as a marker to demonstrate the rotational movement of a DNA depenent GTPase motor. However in order to see true rotations the system needs to be optimized.

Chapter 9

Appendix

9.1 Supporting material to chapter 5

MT length (microns)	Spiral Diameter (microns)	Radius (microns)	Motor density (motors/ microns^2)
3.3	0.823	0.4115	95
3.75	0.99	0.495	200
4.78	0.99	0.495	95
5.82	0.89	0.445	95
5.92	1.22	0.61	208
6.56	1.535	0.7675	95
6.63	1.16	0.58	114
6.78	1.2	0.6	95
6.78	1.02	0.51	90
6.8	1.18	0.59	95
6.8	1.06	0.53	90
6.9	0.95	0.475	150
7.67	1.461	0.7305	95
7.68	1.4	0.7	150
8.2	1.8	0.9	208
8.42	1.2	0.6	208
8.8	1.699	0.8495	95
8.9	1.69	0.845	114
9.45	1.32	0.66	208
9.52	1.48	0.74	208
9.77	1.42	0.71	208
10.1	1.48	0.74	208
10.1	2.131	1.0655	95
11.42	1.22	0.61	208
11.7	2.91	1.455	62.8
12.9	2.619	1.3095	69
13.59	1.72	0.86	114
14.27	1.31	0.655	208
14.44	1.43	0.715	208
14.8	2.107	1.0535	61
14.9	3.58	1.79	31
16.94	1.54	0.77	208
17.24	1.49	0.745	208
17.7	3.716	1.858	29.55
18.59	1.49	0.745	208
19.2	2.483	1.2415	61
19.79	1.31	0.655	208
20.12	1.344	0.672	208
21.8	5.32	2.66	30
24.2	1.71	0.855	208

Figure 9.1: **Raw data: Spiral radius vs motor density and MT length.** The table shows raw data (MT length, spiral radius and motor density) used to plot spiral radius vs motor density and spiral radius vs MT length plots. The data here sorted according to the MT length.

MT length (microns)	Motor Density (motors/micron^2)	Frequency X (Hz)	Frequency Y (Hz)
17.7	29.55	0.01818	0.00909
21.8	30	0.00769	0.00714
14.9	31	0.01333	0.01429
19.2	61	0.025	0.025
12.9	69	0.0125	0.02
7.67	95.11	0.0111	0.0111
4.78	95.11	0.01875	0.01935
3.3	95.11	0.025	0.025
8.8	95.11	0.01739	0.016
10.1	95.11	0.0087	0.01
6.56	95.11	0.01538	0.01818
8.9	114	0.01538	0.01818
6.63	114	0.02143	0.02308
13.59	114	0.01538	0.0125
7.68	150	0.01667	0.01538
6.9	150	0.02308	0.02308
11.42	208	0.01765	0.01765
14.27	208	0.02	0.02
17.24	208	0.01765	0.01765
5.92	208	0.01333	0.01333
18.59	208	0.01053	0.0125
8.2	208	0.01154	0.01154
9.52	208	0.01667	0.01667
9.45	208	0.01842	0.01842
8.42	208	0.015	0.015
14.44	208	0.01667	0.01667
10.1	208	0.01429	0.01429
16.94	208	0.0087	0.0087
9.77	208	0.0125	0.0125
24.2	208	0.01071	0.01071

Figure 9.2: **Raw data: Spiral frequency vs motor density and MT length.** The table shows raw data (MT length, X and Y frequency of the free tip and motor density) used to plot spiral frequency vs motor density and spiral frequency vs MT length plots. The data here sorted according to the motor density.

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Publications

Wave-like oscillations of clamped microtubules driven by collective dynein transport

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ABSTRACT Microtubules (MTs) are observed to move and buckle driven by ATP-dependent molecular motors in both mitotic and interphasic eukaryotic cells as well as in specialized structures such as flagella and cilia with a stereotypical geometry. In previous work, clamped MTs driven by a few kinesin motors were seen to buckle and occasionally flap in what was referred to as flagella-like motion. Theoretical models of active-filament dynamics and a following force have predicted that, with sufficient force and binding-unbinding, such clamped filaments should spontaneously undergo periodic buckling oscillations. However, a systematic experimental test of the theory and reconciliation to a model was lacking. Here, we have engineered a minimal system of MTs clamped at their plus ends and transported by a sheet of dynein motors that demonstrate the emergence of spontaneous traveling-wave oscillations along single filaments. The frequencies of tip oscillations are in the millihertz range and are statistically indistinguishable in the onset and recovery phases. We develop a 2D computational model of clamped MTs binding and unbinding stochastically to motors in a “gliding-assay” geometry. The simulated MTs oscillate with a frequency comparable to experiment. The model predicts the effect of MT length and motor density on qualitative transitions between distinct phases of flapping, regular oscillations, and looping. We develop an effective “order parameter” based on the relative deflection along the filament and orthogonal to it. The transitions predicted in simulations are validated by experimental data. These results demonstrate a role for geometry, MT buckling, and collective molecular motor activity in the emergence of oscillatory dynamics.

SIGNIFICANCE Previous work has demonstrated buckling and flapping oscillations of end-clamped microtubules (MTs) driven by collective force generation of plus-end-directed molecular motors, kinesins. Models of such buckling instabilities have predicted general properties based on following forces, which remain to be tested. In this study, we have systematically immobilized MTs by their plus ends and allowed them to interact with a sheet of minus-end-directed motors, dyneins, in a “gliding-assay” setup. In the presence of ATP, the free MT end spontaneously oscillates with a frequency in the millihertz range. A computational model and a geometric order parameter predict the generality of such self-organized motor-driven MT oscillations. The phase space of patterns predicted in simulations is matched by experiments.

INTRODUCTION

Collective force generation by molecular motors and microtubules (MTs) is responsible for multiple vital cellular processes, including spindle assembly and segregation, intracellular cargo transport, and the dynamics of flagella and cilia. Collective mechanical effects arising from the activity of multi-motors has typically involved measurement of vesicle transport by both plus- and minus-end-directed motors (i.e., kinesins and dyneins). The presence of both antagonistic motors results in a tug of war that is resolved by multiple mechanisms, as reviewed elsewhere (1). Typically

these studies used motor-decorated vesicles or beads with diameters ranging between 10^{-2} and a few micrometers, with only a small fraction of motors encountering the MT track, due to the spherical geometry that limits the contact area (2). This limits the range of collective motor activity that can be examined. In contrast, in MT “gliding assays,” typically one type of motor—kinesin or dynein—is immobilized on a surface with MTs transported as a linear cargo. The number of motors encountered is proportional to the length of the MT. Such assays have been used to show kinesin-dependent transport is independent of density (3), whereas varied linker lengths of kinesins provided evidence for “loose coupling” in driven MT transport (4). Dynein collective transport velocity did not change with increasing numbers of dyneins, but the study was limited to < 10 motors due to the low rigidity the synthetic DNA-origami-based cargo used (5). Gliding

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assays of dyneins with up to ~ 100 motors transporting a single MT demonstrated transport velocity and directionality increasing with number of motors, potentially as a result of the cooperativity of dynein (6,7). Thus, gliding assays can examine the effect of collective motor transport over a large range, not limited by cargo geometry.

Such large numbers of dynein motors transporting MTs in a physiological context can be seen in axonemal mechanics where dynein stepping on adjacent MTs and their coordination result in bending mechanics (8). Mathematical models of such sliding forces combined with elastic coupling of adjacent filaments demonstrate the emergence of wave-like oscillations (9). The spatial regulation of dynein activity is required to generate the coordinated asymmetric forces as described in the “switch inhibition” mechanism (10,11). Theoretical models have suggested aspects of such oscillations can be self-organized (12–14). However, the connection to detailed mechanics of collective motor transport still remains unclear. A model of collective transport by multiple ($\sim 10^4$) molecular motors walking on filaments was shown reproduce oscillations that were quantitatively distinct from thermal fluctuations (15). Experimental observations of dynamics of spirals and curvature propagation in single filaments in actin gliding assays where motility was driven by a sheet of immobile myosin motors were reproduced by theoretical models of motor-driven filament-bending dynamics (16). Similar models have more recently predicted flagella-like motion of a chain of active Brownian particles anchored at one end with the pinned length, filament length, and noise levels determining the dynamics (17). The closest experimental test has been the observation of transient waves of bending observed when free MTs were bound to randomly distributed clusters of the plus-end-directed motor kinesin, nonspecifically bound to the surface (18). Although such experiments demonstrate the possibility of spontaneous onset of oscillations, they arise from defects. A systematic experimental test of this theory has not been attempted so far.

Here, we have reconstituted a minimal system of MT filaments clamped by their plus ends with the free ends interacting with immobilized dynein motors in a gliding-assay format, resulting in spontaneous, slow (millihertz) oscillations with wave-like deformations from base to tip. In contrast to previous work summarized above, where the kinesin motors were used, we demonstrate the emergence of such regular waves using a cytoplasmic yeast dynein as the force generator. We observe a qualitative change in spatio-temporal patterns with motor density and MT length in our minimal system. Compared to filaments in the absence of motors, we demonstrated a specific, dominant, millihertz frequency of oscillations. A computational model using discrete stochastic motors interacting with semi-flexible rods, MTs, predicts qualitatively comparable patterns. Using a deflection-based “order parameter,” we quantitatively classify the patterns and compare the model predictions to experiment. Our results could help in an improved understanding of the emergence of

periodic oscillations in the context of collective motor-filament interactions.

MATERIALS AND METHODS

Protein purification and labeling

Tubulin was purified from goat brain using the high-molarity PIPES-based activity cycling protocol described for porcine brain tubulin (19) and used previously for goat brain tubulin (7).

Tubulin was labeled with either 5(6)-carboxytetramethylrhodamine N-succinimidyl ester or Alexa Fluor 488 NHS ester (Molecular Probes, Eugene, OR, USA) by incubating it with polymerized tubulin followed by a depolymerization-polymerization cycle, similar to the activity-based cycling protocol (19). This ensures that the labeled tubulin is active. Biotin-labeled tubulin was prepared by the same method by incubating (+)-biotin N-hydroxysuccinimide ester with polymerized goat brain tubulin. The molar ratios of label to tubulin monomer obtained for rhodamine, Alexa 488, and biotin were 3.66, 0.275, and 2.88, respectively. Dye concentrations were measured using absorbance measurements with minimal volumes (uCuvette G1, BioSpectrophotometer, Eppendorf, Germany).

Saccharomyces cerevisiae (yeast) strain VY208 was used to express a genomic copy of a modified cytoplasmic dynein fusion construct zz-GFP-GST-Dyn1331 under the control of a galactose inducible promoter with the N-terminal ZZ-tag for purification, GFP for visualization, and GST for dimerization as described by Reck-Peterson et al. (20). Cells were grown in batch cultures and induced with 2% (w/v) galactose (HiMedia, India) followed by affinity purification using the ZZ-tag using immunoglobulin G beads (GE Healthcare, Sweden). The protein was cleaved from the beads using a 6 $\times$ -His tagged TEV which was expressed in *E. coli* BL21 DE3 and purified using Co-NTA resin (Thermo Scientific, IL, USA) followed by elution using imidazole (HiMedia, India).

To specifically immobilize dynein on glass surface, anti-GFP nanobody was used. The pGEX6P1-GFP-nanobody construct was a gift from Kazuhisa Nakayama (Addgene plasmid 61838, <http://n2t.net/addgene:61838>, RRID: Addgene_61838). The nanobody was transformed and expressed in *E. coli* BL21-DE3 cells that were induced with 1 mM isopropyl β -D-1-thiogalactopyranoside, IPTG (SRL, India) for 4.5 h at 30°C. The protein was purified from the clarified lysate using glutathione sepharose (GE Healthcare, Sweden) and eluted using reduced glutathione (21).

MT beating assay

MT filament assembly

MTs were prepared by polymerizing 20 μ M unlabeled tubulin with 5 μ M rhodamine-labeled tubulin in BRB80 and 10% glycerol at 37°C. The MTs were stabilized by addition of 20 μ M Taxol (paclitaxel, Cytoskeleton, CO, USA). Free monomers were removed by pelleting MTs at 150,000 $\times g$ in TLA-100.3 rotor (Beckman Coulter, CA, USA). For plus-end biotinylation, the MT pellet was resuspended in BRB-80 and 10% glycerol followed by addition of either 20 μ M biotinylated tubulin and 5 μ M rhodamine-labeled (single-color filaments) or 10 μ M biotinylated tubulin and 10 μ M Alexa Fluor 488-labeled tubulin (dual-labeled filaments). The filaments were allowed to polymerize by incubating the mixture at 37°C for 30 min. Free monomers were once more removed by centrifuging at 150,000 $\times g$ in TLA-100.3 rotor (Beckman Coulter, CA, USA) and MT pellet resuspended in BRB-80 and 20 μ M Taxol and used immediately.

Dynein motors and flow-chamber assembly for motility

The beating assays were performed in a flow-chambers constructed using 22 $\times$ 22-mm coverslip (no. 1.5, VWR) adhered to a slide (22 $\times$ 60 mm, HiMedia, India) by two parallel strips of double-backed Kapton polyimide tape (Ted Pella, USA) spaced 7 mm apart, creating a chamber of area 22 $\times$ 7 mm.

The flow chamber was coated with a 1:1 molar ratio mixture of nanobody and streptavidin (each 20 μM concentration). After blocking the surface with casein, one chamber volume of dynein of a fixed concentration was flowed in. To test the effect of dynein density on patterns, purified dynein ranging in mass between 1.05 μg (4.53 pmol) and 2.46 μg (8.46 pmol) were introduced into the chamber and incubated for 8 min. Dilutions were used, resulting in effectively 1.07 μg (3.23 pmol) and 1.18 μg (3.56 pmol) dynein expected to occupy the chamber. Using a GFP-intensity calibration method (described later), this resulted in densities of 27 and 46 motors/ μm^2 respectively. The dynein solution was incubated for 10 min and unbound dynein removed by washing with lysis buffer (30 mM HEPES (HiMedia, India), 2 mM Mg-Acetate (Amresco, OH, USA), 50 mM K-Acetate (Fisher Scientific, India), 1 mM EGTA, 10% glycerol). The density was estimated at the imaging surface, since the washes were optimized to result in no further outflow of dynein. We have assumed all the motors bound to the surface are active. This was followed by flowing in plus-end biotinylated MTs and incubating for 10 min. The unbound MTs were washed using wash buffer (lysis buffer + 20 μM Taxol). The beating was observed after addition of motility buffer (lysis buffer + 4 mM ATP + Antifade mix (0.005 mg glucose oxidase, 0.0015 mg catalase, 7.2 mM glucose in 100 μL 10 $\times$ PBS (SRL Chemicals, Mumbai, India))). All assays were performed at 37°C. All reagents, unless otherwise stated, were from Sigma-Aldrich, MO, USA.

Microscopy and image analysis

The filaments were imaged using a 60 $\times$ oil immersion lens (NA = 1.45) mounted on a Nikon Ti-E inverted microscope (Nikon, Tokyo, Japan) using a Tetramethylrhodamine Isothiocyanate (TRITC) filter (and fluorescein isothiocyanate in case of dual-color images) with temperature control system (Okolab, Pozzuoli, Italy). The filaments were imaged every 10 s for 10–20 min using an Andor Clara2 CCD camera (Andor Technology, Belfast, UK). The images were denoised by applying a median filter followed by background subtraction using FIJI (22). Filaments were either interactively traced using the NeuronJ plugin (23) in FIJI and when feasible using active contours and skeletonization using the MATLAB Image Processing Toolbox (Mathworks, Natick, MA, USA). Time-projected images with encoding of time was based on the FIJI plugin ZstackDepthColorCode (<https://github.com/ekatrakha/ZstackDepthColorCode>). The xy coordinates of the tips were generated by tracking the tip using plugin MTrackJ 1.5.1 also in FIJI. Drift observed in some videos was corrected by subtracting the tracked coordinates of the minus end of the MT by the position coordinates of the immobilized plus tip. All the analyses and plotting were performed in MATLAB (R2021b, Mathworks, MA, USA).

Estimating motor surface density

The surface density of dynein in motors per unit area (μm^2) was estimated based on the principle that the dynein is surface immobilized via the anti-GFP nanobody and an image in one plane of the epifluorescence microscope can provide an estimate of the number of molecules after calibration of the point spread function (PSF) and intensity to molecules conversion with a GFP standard, as previously described (7). Such an approach is based on comparable approaches used to estimate the surface density of myosin (24,25). To this end, a range of concentrations of bacterially expressed and affinity-purified 6xHis-eGFP molecules were introduced into the flow chamber and imaged. The number of molecules in the field of view was estimated as the product of the image volume and independently measured protein concentration. The image volume (cuboid) is the product of the PSF (height) and image area (in 2D). The PSF was estimated by measuring the fluorescence intensity (I_z) along the z axis (z) of 0.1 μm diameter TetraSpeck beads (Thermo Fisher Scientific, IL, USA). The data from multiple beads were fitted to the equation $I_z = a + (b - a) \cdot e^{-\frac{(z - c)^2}{2 \cdot d^2}}$ (Fig. S4 A–C) where a, b, and c are scaling parameters and d is the SD, using the function

CurveFitter in FIJI (26). The PSF is 1.45 μm , based on the relation $PSF = 2.35 \cdot d$ (27). The fluorescence intensity of the eGFP dilution series on coverslips was used to plot a calibration curve of intensity of the image (y axis) as a function of protein surface density (x axis) and fitted to a straight line (Fig. S4 D). The image intensity of dynein-GFP from the coverslip surface was used to estimate monomer density resulting in a factor of 0.5 for the dynein dimer density. The resulting motor densities obtained from different dilutions of the stock protein ranged from 5.25 to 62.86 motors/ μm^2 , with control experiments performed without motors.

Filament mobility and contour analysis

The 2D filament deflections in x and y axes were quantified in terms of the free-tip (minus-end) mobility by plotting the deflection in x and y, angle subtended at the immobile tip with the positive x axis, the tip angle ϕ , and a combined “order parameter” that we refer to as deflection or sweep range, S . The fixed end (plus end) is considered the origin and the clamped segment, approximated as a straight rod, determines the x axis. The tip position at every time, t , is calculated as Euclidean distance between the clamped filament and the free end. The tip angle ϕ is the minimal angle subtended at the fixed end by two lines, the ray connecting the free (minus) end with the immobilized end of the filament (plus) and the line connecting the rigid segment (Fig. 1 G). Due to the difficulty in detecting the exact length of the clamped segment in experiments, the calculation of the tip angle starts from the immobile end of the filament. This position of the vertex of the angle does not alter the frequency of oscillations. The time series of ϕ for multiple filaments were smoothed using a moving average with a window size of 0.1 (fraction of total length) to denoise the signal (Fig. 2 A–C (insets)). The frequency of the smoothed oscillation was estimated using the fast Fourier transform (FFT), using the function *fft* in MATLAB (Mathworks, Natick, MA, USA) (Fig. 2 D). The difference in tip angles at the consecutive time points is defined as the angular velocity, $\omega = (\phi(t + \Delta t) - \phi(t)) / \Delta t$, where Δt is the time interval between frames. The shape of filament is quantified as the local tangent angle (ψ) calculated at every discrete point along the filament contour and is given by:

$$\psi(s, t) = \tan^{-1} \left(\frac{y_{n+1} - y_n}{x_{n+1} - x_n} \right), \quad (1)$$

where (x_n, y_n) are the coordinates of the filament segment (s_n) at time, t . The beat frequency, amplitude, and the time period are inferred from time series of the angle $\phi(s, t)$, which is denoised using the function *smoothdata* and Fourier analysis performed to extract the frequency. Filament length estimates from our image analysis pipeline are subject to orientation-dependent discretization errors; to avoid such variation in length that may not have anything to do with the experimental data, a spline interpolation of the filament and running average MT length contour is plotted for the various kymographs of tangent angle. All the analyses and plotting were performed in MATLAB (R2021b, Mathworks, MA, USA).

Classification of MT motility patterns

We quantify the pattern of the free end of the filament, based on the deflection in x and y, as S_x and S_y , where $S_y = y_{\max} - y_{\min}$ and $S_x = x_{\max} - x_{\min}$ (Fig. 1 G). We combine the deflections S_x and S_y into a variable we refer to as S metric, a measure of sweep, which is the log base 2 of the ratio of y and x extent represented by Eq. 2.

$$S = \log_2 (S_y / S_x) \quad (2)$$

The ratio was transformed by $\log_2$ to obtain a range where small changes were magnified and large changes compressed to infer qualitative patterns. We use this score to quantify the spatial patterns seen in simulations and examine any systematic effect of parameter changes on the patterns, as described in the “results” sections.

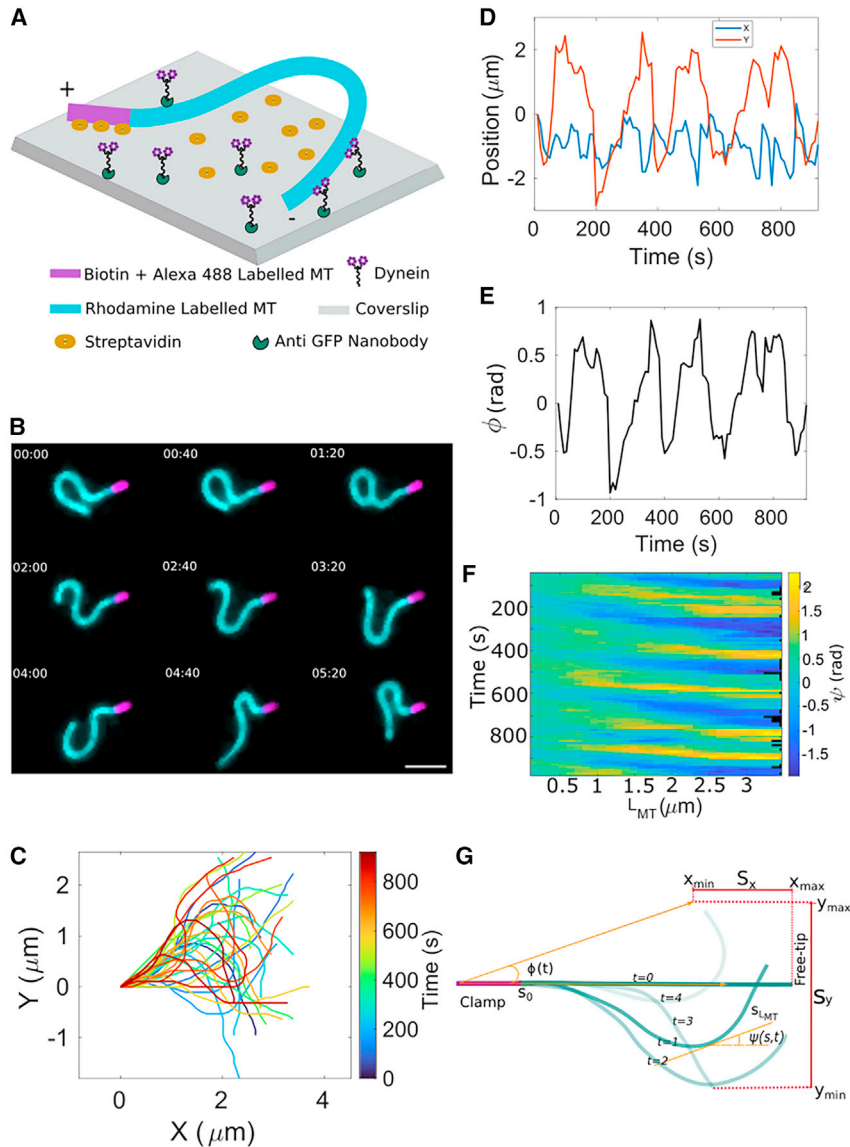


FIGURE 1 In vitro reconstitution of dynein-driven flagella-like MT oscillations. (A) The schematic represents the in vitro reconstitution setup with a single MT with a biotinylated plus-end (magenta) pinned to the surface by streptavidin, whereas the remainder of the filament is free (cyan) and interacts with surface-immobilized dynein, which can propel the MT. (B) The montage of a representative microscopy time series (Video S1) shows MT plus ends labeled with biotin- and Alexa 488-tubulin (magenta) bound to streptavidin on glass, together with free minus ends labeled with rhodamine-tubulin (cyan), transported by dynein undergoing bending dynamics. Here, $L_{MT} = 6.7 \mu\text{m}$ and $\rho_m = 47/\mu\text{m}^2$. Time, mm:ss. Scale bar, $4 \mu\text{m}$. (C) Filament contours from a time series of rhodamine-tubulin-labeled MT of length $L_{MT} = 4.7 \mu\text{m}$ similarly immobilized by a biotinylated plus end (Video S2) were tracked and contours projected in time. Dynein density, $27/\mu\text{m}^2$. Color bar, time (s). (D and E) The dynamics of the free filament tip (minus end) are plotted in terms of (D) x (blue) and y (red) coordinates and (E) the tip angle ϕ in radians as a function of time in seconds. (F) The tangent angle (ψ) is plotted (colors) over time (y axis) and MT length (x axis) as a kymograph at 10-s intervals. Color bar, angle in radians. (G) The schematic represents the measures used to quantify bending (dashed lines): tip angle ϕ , span in x (S_x) and y (S_y), and local tangent angle $\psi(s, t)$. To see this figure in color, go online.

Computational model of MT-motor mechanics

The 2D stochastic computational model consists of the mechanics of semi-flexible rods representing MTs, stochastic spring-like motors with the emergence of collective effects arising from motors being bound to the same MT. All movement is subject to both thermal noise (random) and viscous drag. The simulator solves the Langevin dynamics of deterministic and stochastic components using an agent-based approach in C++ simulation code Cytosim (28). Collective mechanical effects then emerge from this, as previously described for gliding assays (7) and aster transport in confinement (29). The dynamical behavior of the individual components is described in the following section.

MTs

MTs are modeled as semi-flexible filaments. The lengths of all filaments (L_{MT}) are constant during a simulation run. The model points along the filament are spaced regularly at intervals of $0.05 \mu\text{m}$. The bending elastic modulus of the filament (κ), thermal energy, drag experienced by the filament, and the motor forces determine the filament dynamics, with most values taken from literature (Table 1).

Motors

Motors are modeled as Hookean springs, represented as a bead with a spring (Fig. 4 A). The bead represents the motor head and the base of the spring depicts the tail, which is immobilized on the surface. The motor head can bind to the MT with a probabilistic attachment rate of r_a if it is within a certain distance to the filament, the binding range d_a . The motors are modeled as continuous steppers with a fixed load-free velocity (v_0) resulting in an instantaneous velocity v at each time step dt , following a linear force-velocity relation:

$$v = v_0 \cdot (1 - f_{\parallel} / F_s) \quad (3)$$

Here, the forces $f_{\parallel}$ and F_s are respectively the component parallel the MT and the stall force. The motor is modeled to step forward and backward depending on the ratio of the parallel to stall force since the step size is given by $\delta r = \delta v \times \delta t$ and δv can be positive or negative. The force experienced by the motors is calculated as $F_m = -k_m \cdot \Delta L_{MT}$, where ΔL_{MT} is the extension along the motor and the k_m the stiffness constant of the spring. The motor can detach probabilistically with a detachment rate (r_d), dependent on the

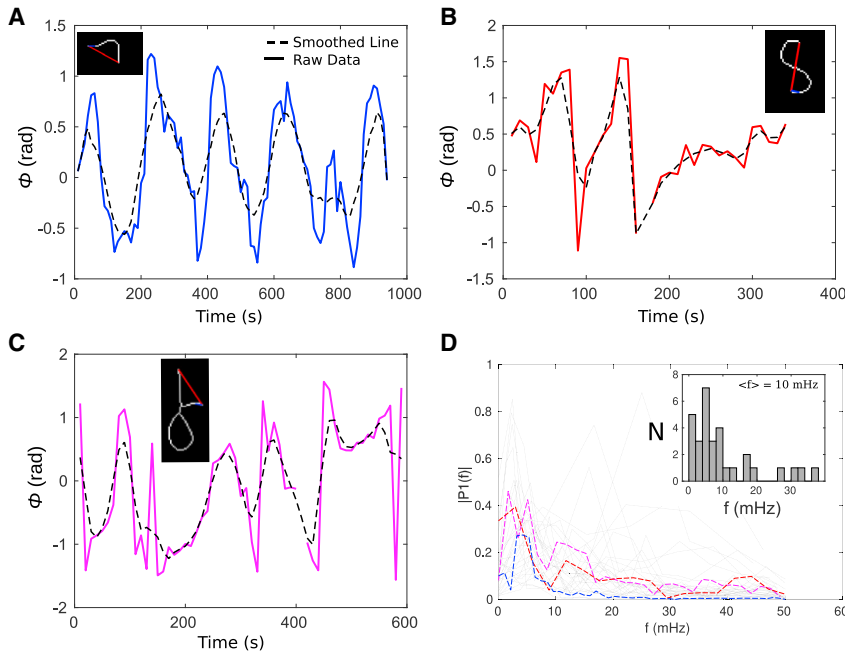


FIGURE 2 Frequency analysis of MT tip angle oscillation. (A–C) The tip angle, ϕ , in radians is plotted with time in seconds for three representative filaments (colored lines) and overlaid with a smoothed curve based on an average filter (dashed lines). (Insets) A representative frame of the time series with segmented MTs (white) are overlaid with the linear fit to pinned segment of the filament (blue line) and the line connecting the two ends of the filament (red line). ϕ is the minimal angle between the blue and red lines. (D) The amplitude spectrum, $|P1(f)|$ (y axis) of the frequencies, f (x axis) from FFT analysis of the smoothed time series of ϕ is plotted (dashed lines). Colored lines correspond to the filaments in (A)–(C), gray lines are additional data. (Inset) The dominant frequencies of multiple ($n = 25$) filament time series analyzed with the mean frequency $\langle f \rangle = 10$ mHz. Dominant frequencies of individual trajectories are (A) 5.6, (B) 5.9 and (C) 1.2 mHz. To see this figure in color, go online.

magnitude of the force experienced by the motor under the stretch (F_m) and the basal load-free detachment rate (r_0). The detachment force (F_d) is the threshold force determining single-motor force-dependent detachment kinetics governed by the Kramers law (30):

$$r_d = r_0 \cdot e^{F_m/F_d} \quad (4)$$

wherein r_d is the instantaneous and r_0 is the basal load-free detachment rate of a single motor. Motor parameters are also taken largely from experiment (Table 1).

MT pinning sites

MTs are pinned to anchors, which are similar to motors but immobile (i.e., they have no velocity). They are modeled as already bound to filaments, with extremely high detachment forces, which results in no detachment during the simulation. These pinning sites are also very stiff with a high elasticity constant k_a . The spacing between the anchors determines the length of MT pinning (L_p).

Simulations

Simulations were run in a 2D space with a periodic boundary condition using Cytosim version f7921c4 from the gitlab (<https://gitlab.com/f.nedelec/cytosim>). The effects of thermal energy and viscosity on the MT-motor interactions are also modeled, with $k_B T = 4.3$ pN nm, viscosity η of 0.826 cP based on the viscosity of the buffer that contains 10% glycerol (31). Simulations were integrated with a time step of 0.01 s. The motors are initialized randomly in the space with MT positioned at the center aligned along the x dimension. The simulation parameter file relating to the scenario described can be downloaded from <https://github.com/CyCelsLab/MT-beating> and run in Cytosim.

RESULTS

Dynein gliding assay with plus-end-clamped MTs produces traveling-wave oscillations

We have developed a minimal experimental reconstitution by modifying a typical 2D gliding assay by clamping one end of

the MT. The system has only two key components: 1) cytoplasmic dynein motors grafted to a glass coverslip surface, and 2) MTs whose plus ends are immobilized via a biotin-streptavidin linkage with the rest of the filament free to bind to motors (Fig. 1 A). The dynein has a GFP-tag in the tail domain, which is used to orient the motor with the motor head pointing away from the glass surface by immobilizing an anti-GFP nanobody on the coverglass surface. MT tip-immobilization is achieved by labeling the plus ends with biotinylated tubulin monomers combined with streptavidin layered on the glass surface. We use the yeast cytoplasmic dynein due to the high processivity, well-established methods for purification and activity (20), and unidirectional transport of filaments in gliding assays (7). The intrinsic variability of MT lengths combined with density variation allowed us to test the effect of increasing motor numbers with motor density, ρ_m , ranging from 5.25 motors/ μm^2 to 62.86 motors/ μm^2 , measured using calibrated microscopy (as described in the “materials and methods” section).

The collective force generation on MTs clamped at one end and driven by motors is akin to a cantilever beam with a uniformly distributed force along the entire length. To achieve this, the clamp involves labeling between 2% and 20% of the total length of the filament with biotinylated tubulin (described in the “materials and methods” section). Pinning at a point with rotational degrees of freedom corresponds to the simplest case and results in filament swiveling, as previously shown in MT gliding assays when one single molecule attaches to the tip of a filament (7,32). When the clamp is sufficiently long, the MT plus end remains straight (magenta), whereas the free minus end (cyan) bends and buckles in response to increasing elastic force (Fig. 1 A). Once the filament bending exceeds the energy of the MT-motor

TABLE 1 MT and Motor Parameters Used in Simulations

Symbol	Description	Value	Reference
MTs			
K	MT bending rigidity	20 pN μm^2	Gittes et al. (34)
L_{MT}	MT length	5–25 μm	this study
Dynein			
v_0	single-motor velocity	0.10 $\mu\text{m/s}$	Reck-Peterson et al. (20)
r_a	attachment rate	5 s^{-1}	Leduc et al. (52)
d_a	attachment range	0.02 μm	Estimated from kinesin-binding range (53) and molecular size of dynein
r_0	load-free detachment rate	0.04 s^{-1}	Reck-Peterson et al. and Rao et al. (20,54)
F_d	detachment force	3 pN	Nicholas et al. (38)
F_s	stall force	5 pN	Gennerich et al. (55)
k_m	linker stiffness	100 pN/ μm	Lindemann and Hunt; Burgess et al.; Kamiya et al. (56–58)

The values for mechanochemical properties of the MTs, motors, and the simulation system are taken from literature where available.

attachment, the motors detach resulting in a recovery stroke, as seen in time series of both dual-labeled (plus end, magenta; minus end, cyan) filaments (Video S1) and single-dye-labeled filaments (Video S2). Typical tip trajectories complete one cycle in ~ 200 – 400 s. Time-projected filament contours demonstrate the oscillatory nature of the movement (Fig. 1 C). The x and y coordinates of the free end of the MT describe oscillatory movement of filament tip deflections over three to five cycles (Fig. 1 D). The amplitude of the deflections in y are higher than in x because the clamped part of the filament defines the x axis, and, as expected from bending and buckling, deflections in y (orthogonal to the length) dominate. The tip angle ϕ as a function of time combines the x and y deflections and shows periodic, and somewhat regular, oscillations between -1 and 1 radians (i.e., -57° to 57° , a range of 114°) (Fig. 1 E). In ciliary motion, tip oscillations are observed to happen at 1 Hz and faster, whereas these oscillations are $\sim 10^3$ slower. To quantify the apparent traveling-wave nature of the oscillations, we also measured the filament tangent angle ψ (radians), pixel-wise, along the contour and over time, resulting in a kymograph (a space-time plot) of filament dynamics (Fig. 1 F). Such kymographs have been used to demonstrate the oscillatory and wave-like nature of minimal reconstituted filaments driven by motors, both MT-kinesin systems (18) and more recently actin-myosin systems (33). The kymograph of the filament curvature ψ of the representative filament suggests a propagation of curvature over ~ 300 s, after which the filament snaps back and repeats the cycle, suggesting a slow oscillation with a frequency in millihertz. The tangent angles (ψ) at the same position along the MT length appear to show some variability or “noise,” suggesting that, as the filament bends, local effects of binding-unbinding and thermal effects influence the propagation of the buckling along the filament length. Such noise is also seen in the tip position (Fig. 1 D) and angle dynamics (Fig. 1 E).

The measures used to quantify MT oscillation dynamics (schematically represented in Fig. 1 G) are 1) tip angle ϕ ,

angle subtended at the plus end by the straight line of the pinned segment (cyan) and the free minus-end tip (cyan); 2) tangent angle ψ , the angle made by the tangent to each point along the contour with the projection of the clamped segment (cyan), treated as the positive x axis; 3) kymographs (space-time plot) of filament bending are based on the tangent angle ψ along the length of the MT with time; 4) span S_x and S_y , the maximal deflection of the free end of the MT in x and y axes, respectively. These measures are used throughout our study to further quantify the dynamics to explore the principles driving the observed phenomenon.

Active, slow, symmetric oscillations of the free end of MTs in experiment

The tip angle ϕ was analyzed from multiple, representative filament time series showing periodic dynamics with noise and some instability (Fig. 2 A–C). We smoothed the data and used them to examine the oscillation frequency and amplitude spectrum using a FFT (as described in the section “materials and methods”). The power spectra of time-dependent ϕ dynamics from multiple pinned filaments ($n = 25$) showed frequencies ranging between 0 and 50 mHz (Fig. 2 D). The frequency with the maximal amplitude, i.e., dominant frequency, was found to be 10 mHz after averaging ($n = 25$) for multiple filaments (Fig. 2 D (inset)). This assay is robust since the filaments are seen to be either transported in a processive manner similar to a 2D gliding assay or oscillating (Video S3), with comparable statistics of 77% gliding as compared to 23% oscillating between replicates and densities (Fig. S1). The higher proportion of gliding filaments could be either because not all filaments were biotinylated equally or not all biotinylated filament ends are captured by the streptavidin on the surface, related to the streptavidin density. Our choice of the proportion of streptavidin to anti-GFP nanobody to immobilize motors was optimized to increase the proportion of oscillating filaments, since there appears to be a trade-off

between plus-end pinning and motor accessibility to the minus ends. At the same time the presence of such gliding MTs confirms that oscillations arise due to motor activity, serving as an internal control for the active nature of the process. In absence of motors, filaments undergo passive oscillations undergoing rapid and random thermal fluctuations of very small amplitude, less than $\sim 1 \mu\text{m}$ (Video S4), with filaments visualized using rhodamine-labeled tubulin. Filaments are clamped in a manner similar to the oscillating filaments, but their motion arising from thermal energy shows comparatively small amplitude bending and lacks any buckling or traveling waves.

We examined the symmetry of the oscillations in time by examining the filament time series (Fig. 3 A and B). The angle made by the free end with the clamped segment, ϕ , showed oscillations as before (Fig. 3 C). The oscillatory data were denoised and peaks and troughs detected. The instantaneous angular velocity ($d\phi/dt$), the discrete derivative of the angle between subsequent time-steps, was classified as either belonging to onset, the stage between one trough and a succeeding peak, or recovery, from a peak to the succeeding trough (Fig. 3 C (right y axis)). The goal was to use the angle to address the question of whether there was any quantifiable difference in the free end, as it oscillated from a minimum (trough) to the maximum (peak), as compared to maximum (peak) to trough, representative of the entire cycle through

onset and recovery. The values of the angular velocity distribution (ignoring the sign) of onset and recovery strokes were found to be statistically indistinguishable (Fig. 3 D).

Previous reports have included comparable cytoskeletal filament oscillations driven by other motors such as MTs with kinesin (18) as well as actin-myosin (16). This suggests such oscillations could constitute a general class of pattern-forming systems. Identifying the theoretical minimum of mechanical components and interactions could help better understand both the generality of such patterns and the limitations within which our experimental setup may function. We therefore proceeded to develop a computational model to examine the principles governing the patterns and explore the sensitivity of the system to motor density and MT length.

2D agent-based model of clamped MTs driven by collective motor activity results in regular wave-like oscillations

The computational model consists of two components, MTs and motors, with MT plus ends pinned. MTs are modeled as semi-flexible polymers with plus ends clamped to the surface and the remaining filament free to bind molecular motors, which are minus-end directed with probabilistic binding and unbinding and linear force-velocity relations (Fig. 4 A). The

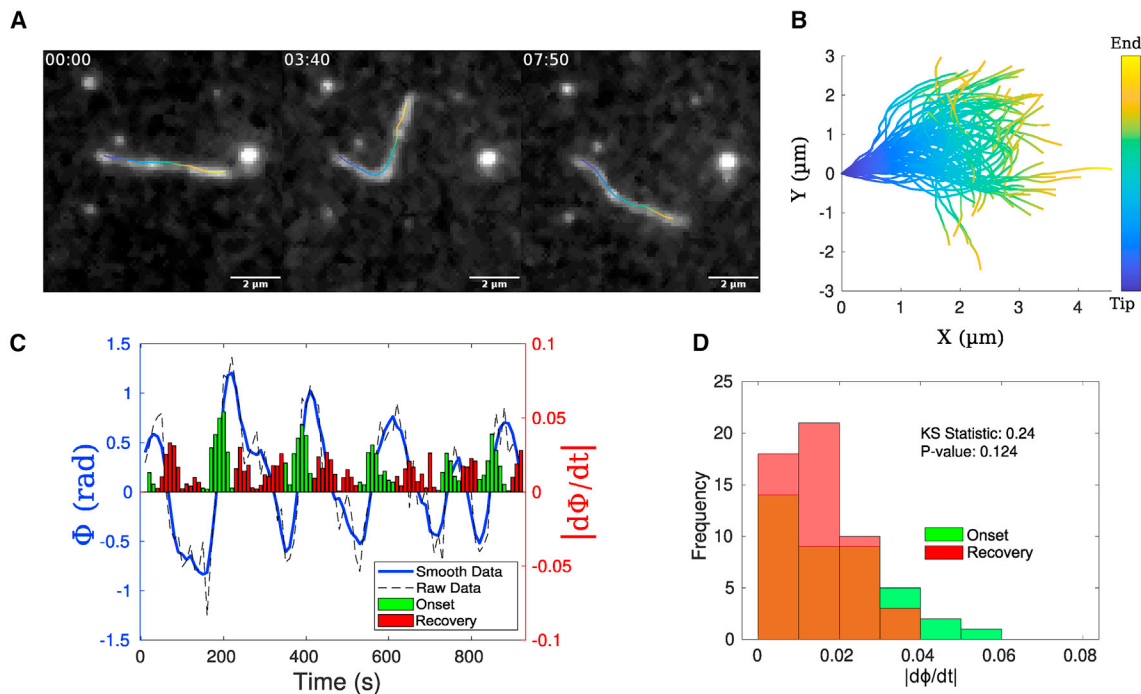


FIGURE 3 Analysis of the symmetry of free-end oscillations of MTs. (A) The representative fluorescence microscopy time series of a single plus-end-pinned MT (gray) in presence of surface-immobilized dynein. Time, mm:ss. Scale, 2 μm . Color scale, start (blue) to end (yellow) of filament contour. (B) Detected filament contours are overlaid with an interval of 10 s over a total period of 940 s. Color bar, contour length of the MT from plus-tip (blue) to minus end (yellow). (C) Tip angle is plotted for the time series in (A). The raw data (black dashed lines) is smoothed using a Savitzky-Golay filter (blue lines). Peak finding to determine the local peaks of the smoothed trajectory was used to plot the absolute instantaneous angular velocity ($|d\phi/dt|$), right y axis for the onset (green) and recovery (red) strokes of the free end of the MT. (D) Frequency distributions of the onset (green) and recovery (red) phases of the absolute value of tip angular velocities are plotted and compared using Kolmogorov-Smirnov statistics showing them to be statistically similar. Test value = 0.24, p value = 0.12. To see this figure in color, go online.

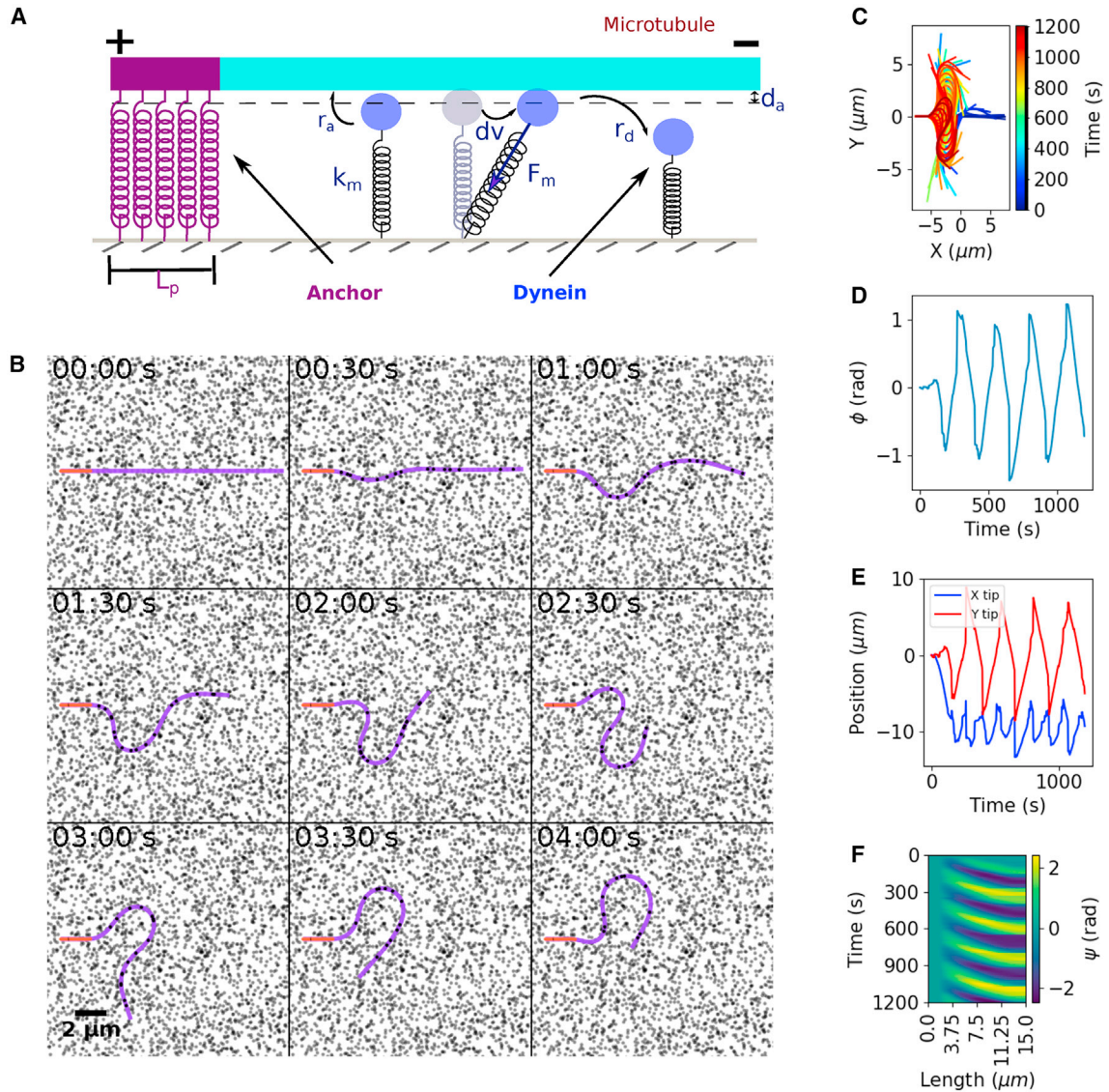


FIGURE 4 Simulating motor-driven flagellar beating of a pinned MT. (A) The schematic of the model of MTs clamped at one end from their plus ends with stiff attachment (magenta) over the length of (L_p), whereas motors (gray circles) stochastically attach to an MT (gray circle), step a distance d_v (violet circle), and detach at a force-dependent rate r_d . Motors are modeled as springs (black) with a constant k_m resulting in a force F_m when extended. d_a , attachment distance (dashed line); r_a , attachment rate. (B) Representative image time series of the 2D simulation of filament beating. Gray circles, motors; red line, clamped segment; gray line, free segment. Time in seconds. Scale bar, 2 μm . (C) Filament contours projected in time at 15-s intervals over 1200-s simulation. (D and E) The dynamics of the free plus end are plotted in terms of (D) the tip angle ϕ (radians) and (E) the x (blue) and y (red) positions. (F) The local tangent angle along the MT filament ψ (radians) is represented as a kymograph over time (y axis) and along the MT length (x axis). Here, filament length $L_{MT} = 15 \mu\text{m}$, motor density $\rho_m = 10 \text{ dyneins}/\mu\text{m}^2$, pinned MT length $L_p = 2 \mu\text{m}$. Color bar, angle in radians. To see this figure in color, go online.

model description lists the equations governing the MT-motor model (“materials and methods” section), whereas model parameters are taken from the literature wherever possible (Table 1). MT pinning is modeled by introducing multiple rigid attachments that clamp a segment of the MT plus end to the surface. The attachment is through binding elements that have a high spring constant, k_a , of $10^3 \text{ pN}/\mu\text{m}$ and do not detach from the MTs. In the 2D simulation box, MTs are initialized to be straight, with the free segment rapidly binding to a sheet of motors along its length, similar to previous models

of gliding assays. Once motors have bound to the MT, their stepping collectively produces forces, which, combined with the clamped plus end, spontaneously result in bending and buckling if the force exceeds the critical force and motors detach, resulting in a recovery stroke (Fig.s 4A and Video S5). The collective behavior at the spatial scale of micrometers emerges over time, based on the interaction rules of each component (model) and their single-parameter values at nanometer range, and is not explicitly encoded in the model formalism. The patterns of bending appear symmetric

between the onset and recovery strokes (Fig. 4 B), also seen in time-projected filament plots (Fig. 4 C). The periodic movement of the free tips can be observed in the time dependence of the tip angle φ (Fig. 4 D) as well as the x and y positions of the free end (Fig. 4 D). We also observe some stochasticity in the position-time and angle-time plots, which originates from stochastic binding-unbinding. One cycle completed in ~ 300 s, corresponding to a frequency of ~ 3 mHz and corresponding closely to experimental observations. The curvature propagates in a base-to-tip manner, as seen from the kymograph of the tangent along the contour (ψ , described in detail in the “materials and methods” section and Eq. 1) with time (Fig. 4 E). Interestingly our simulations are both qualitatively comparable to experiments in terms of base-to-tip bending, symmetry of onset and recovery strokes, as well as quantitatively in terms of amplitude and timescale of free-end oscillation angles. This is despite the lack of optimization of parameters that we believe arises from the choice of mechanical and dynamical parameters of the underlying components, taken from experimental literature. The dynamics in that sense are then emergent.

To further explore the limits of the theory, we explore parameter sensitivity of our model and compare the predictions to experiment.

Simulations predict qualitative transitions in oscillations depending on motor density and filament length

Based on a previously developed model of cytoskeletal filament bending by motors on a surface where the radius of curvature of spirals (R) formed by pinned filaments scaled as $R \sim (k_B T \xi_p / f)^{1/3}$, where ξ_p is the persistence length and f is the linear force density, i.e., motors acting per unit filament length (16). Therefore MT filament lengths (L_{MT}) were varied over a range of 5–25 μm , based on typical experimental observations, and dynein motor density (ρ_m) was varied over three orders of magnitude, based on previous experimental reports (6,7). The time-projected images of filament contours demonstrate the formation of multiple patterns, qualitatively classified as fluctuations (irregular and uncorrelated bending), oscillations (prominent curvature with changing direction), and looping (multiple segments of the filament take on different curvatures) (Fig. 5 A). Fluctuations are observed in the complete absence of motors, similar to those reported for cantilevers in a thermal bath. As the motor density increases, the patterns appear to depend on the MT length. We observe short filaments of length 5 μm subjected to increasing motor density appear to undergo bending to one side, do not recover, but higher length or density both demonstrate oscillations (Fig. 5 A). In representative kymographs, we examine the effect of constant length and constant motor density on the local tangent angle with time and length, indicative of the traveling-wave oscillations. The length dependence

is seen when a constant motor density of $\rho_m = 10^1$ motors/ μm^2 shows onset of oscillations when MT length is greater than 15 μm (Fig. 5 B). On the other hand, a constant MT length of 15 μm in presence of low motor densities ($\rho_m = 10^0$ motors/ μm^2) results in only fluctuations with a density of $\rho_m = 10^1$ motors/ μm^2 or greater required to result in regular filament buckling and oscillation (Fig. 5 C Video S6). Looping also emerge at lower motor densities for longer filaments (10–25 μm). In the combined matrix of ρ_m and L_{MT} variation, along a diagonal we observe a narrow region of patterns that resemble regular oscillations (Fig. 5 A).

Although the motor density and length suggest that there is a clear motor number dependence on patterns, we find that the number of motors bound to a filament fluctuate in time for all lengths, as observed for representative traces from simulations of filaments of increasing lengths from 5 to 25 μm interacting with motors of density $\rho_{2D} = 10$ motors/ μm^2 (Fig. S2 A). Fluctuations themselves were expected from the stochastic binding-unbinding kinetics, forces acting on individual motors, and thermal noise, and the number of bound motors is lower than the expected value $N_{expected}$ (Fig. S2 B). Here, the expected number serves as an upper limit, obtained from a square lattice approximation of the distribution of motors, i.e., $N_{expected} = L_{MT} \times \sqrt{\rho_{2D}}$. Interestingly, although tip angles oscillate in a regular manner for L_{MT} of 15 μm and longer, this correlates with more than 10 motors being bound to the MT on average. The time-dependent number fluctuations, however, do not correlate with the tip oscillations (Fig. S2 C). This could result from the fact that, although a minimal number of motors is required to generate the bending and buckling forces, filament mechanics also plays a role in the oscillatory patterns of MTs.

These results suggest the onset of regular oscillations requires a minimal MT length and motor density (motor number), with both factors determining frequency of oscillations. We test whether a specific frequency dependence can be seen in simulations by analyzing simulated filament oscillations in presence of a specific motor density (Fig. 6 A) for the free-end angle φ dynamics (Fig. 6 B) by examining the Fourier spectrum and identifying the highest-amplitude oscillations, f_{max} (Fig. 6 C). A systematic analysis of the effect of MT length and motor density suggests the oscillations of the free end show a distinct coordinated increase with both variables in amplitude (Fig. 6 D) and a decrease in corresponding frequency to ~ 10 mHz (Fig. 6 E). Data generated from multiple runs demonstrate a high degree of variability in individual runs in terms of both amplitude (Fig. 6 F) and frequency (Fig. 6 G). This further emphasizes the stochastic nature of the oscillations.

To compare experiments to theory we proceed to examine the qualitative nature of the transition in observed patterns of MT oscillations.

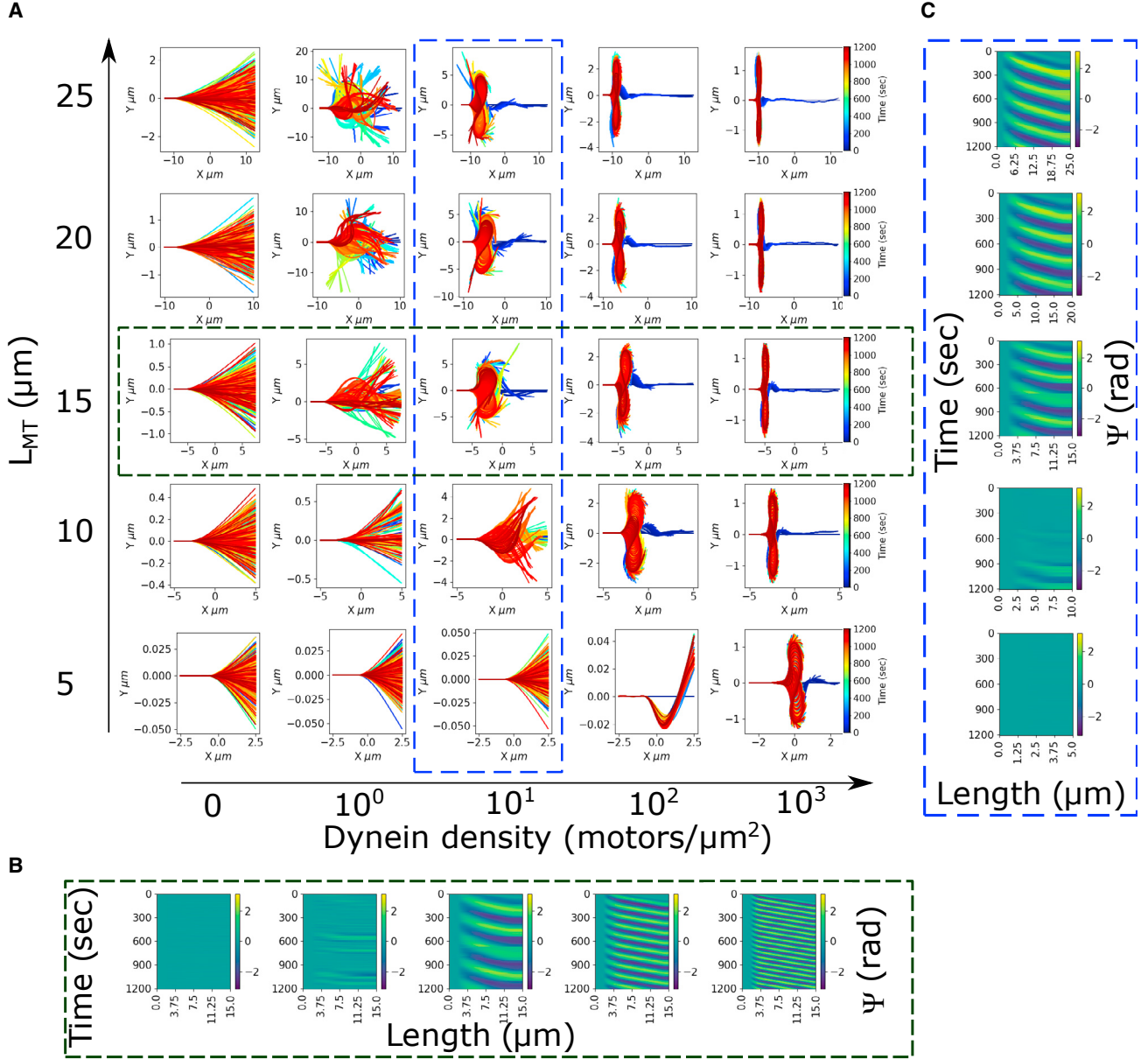


FIGURE 5 Simulating the effect of MT length and motor density on flagellar beating. (A) The dynamics of filaments are simulated with MT lengths ranging from 5 to 25 μm (rows) and motor densities ranging from 1 to 10^3 motors/ μm^2 (columns). The projected filament contours are plotted at 3-s intervals over a total time of 1200 s. (B and C) Representative kymographs of the local tangent angles, ψ (radians), plotted as a function of position along the filament (x axis) and time (y axis). Dashed lines indicate either (B) increasing motor density with MT lengths constant, here 15 μm , or (C) increasing MT lengths with motor density constant, here $\rho_m = 10$ motors/ μm^2 . To see this figure in color, go online.

Geometric measure to classify spatial patterns of clamped filaments driven by motor-sheet

Our experiments and simulations demonstrate the systematic emergence of periodic beating patterns arising from a simple 2D system that are specific to the filament length and motor density. Simulations were run for a range of filament lengths and densities and the outputs evaluated in terms of the time-projected filament profiles (Fig. 7). These demonstrate that short filaments (~ 5 μm) will only undergo (I) fluctuations even when ρ_m is varied from 0 to 10^2 motors/ μm^2 , whereas long fil-

aments (~ 20 μm) will exhibit (II) regular oscillatory motion, even at lower dynein densities of 10^{-1} motors/ μm^2 . Filaments undergo (III) looping when the MTs are long (≥ 10 μm) and motor density sufficiently high ($\geq 10^2$ motors/ μm^2).

The state (IV), swiveling, refers to movement that corresponds to very short pinned regions akin to a filament being bound at the very end and encountering a very low density of motors, or their absence. We find that no single quantitative measure of filament dynamics used to describe the dynamics in simulations can help distinguish between the visibly different scenarios, i.e., the maximal range of values

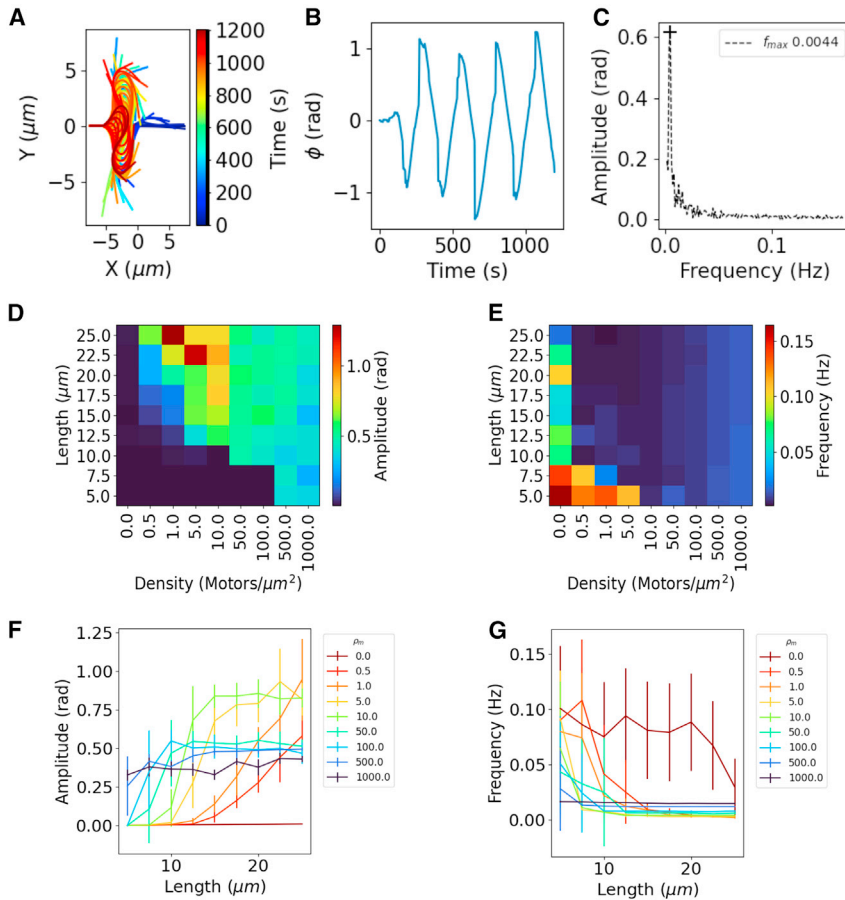


FIGURE 6 Frequency analysis of filament oscillations from simulations. (A–C) Representative simulations of motor-driven filament beating were used to plot (A) time-projected contours (color bar, time) of MTs of length 15 μm in presence of a motor density ρ_m of 10 motors/μm², (B) tip angles (ϕ) with time, and (C) the amplitude of oscillation frequency from FFT analysis. f_{max} is the frequency with highest amplitude. (D) The amplitude and (E) frequency of oscillations from simulated filaments as a function of varying lengths ($L_{MT} = 5\text{--}25\text{ }\mu\text{m}$) and motor densities ($\rho_m = 0$ (no motors) to 10^3 motors/μm²) are shown as mode of multiple simulation runs ($n = 15$). (F and G) The relationship of the (F) dominant frequency and (G) amplitude as a function of filament length (x axis) are plotted at different motor densities (color). Circles, mean $\pm$ SD. To see this figure in color, go online.

explored in x and y (S_x, S_y), oscillation frequency f , maximal tip angle ϕ_{mx} , angular velocity ω , and number of motors bound (Fig. S3). To address this, we came up with a simple metric that serves as an order parameter for this system, which we refer to as the sweep range, the S metric (Eq. 2; “materials and methods” section). This metric, S , is the binary logarithm ($\log_2$) of the ratio of the deflection along the respective y and x axes, S_y and S_x (schematic in Fig. 1 E). We arrived at this measure after testing multiple alternative order parameter measures to quantitatively identify distinct states of the filament dynamics. This captures the relative proportion of space explored by the tip along the y axis relative to the x axis (the span) and the binary logarithm scales the range. The specific values of the S metric corresponded to distinct patterns of MT dynamics (Fig. 7). As a result, the following thresholds were used to classify patterns:

$$\begin{aligned} S &\geq 3 && \text{(I) Fluctuations,} \\ 3 > S > 0 && \text{(II) Regular oscillations,} \\ 0 > S && \text{(III) Looping} \end{aligned} \quad (5)$$

$S = 0$ corresponds to the special case of swiveling that arises when the point of attachment of a filament is one molecule with universal degree of rotation. The value of $S = 0$ corresponds to the situation when the range of values covered in x and y are equal, i.e., $S_y = S_x$.

To test of the utility of our order parameter, we asked if it also matches the patterns observed in experiments. We examined the tracked coordinates of multiple experimentally measured time series of clamped MTs of different lengths showing diverse patterns, with multiple motor densities and MT lengths. (I) Fluctuations were observed in the absence of motors, whereas increasing MT lengths and motor density gave rise to (II) oscillations and (III) looping. We also observe (IV) swiveling when a very short segment of the MT end is anchored with the rest of the filament free to move. We find not just that experimentally observed filament pattern dynamics match the qualitative behavior of simulation outputs but even the quantitative measure of the sweep variable (S) thresholds match (Fig. 7).

We proceed therefore to test if this novel order parameter, the filament sweep value S , could be used to quantitatively classify the systematic effect of MT and motor properties on the emergent dynamics of the system.

Comparing simulation predictions to experiment: Effect of MT length and motor density

Using the combined x- and y-deflection measure, we classify simulation outputs into the categories 1) fluctuations, 2) regular oscillations, and 3) looping, and we find that a

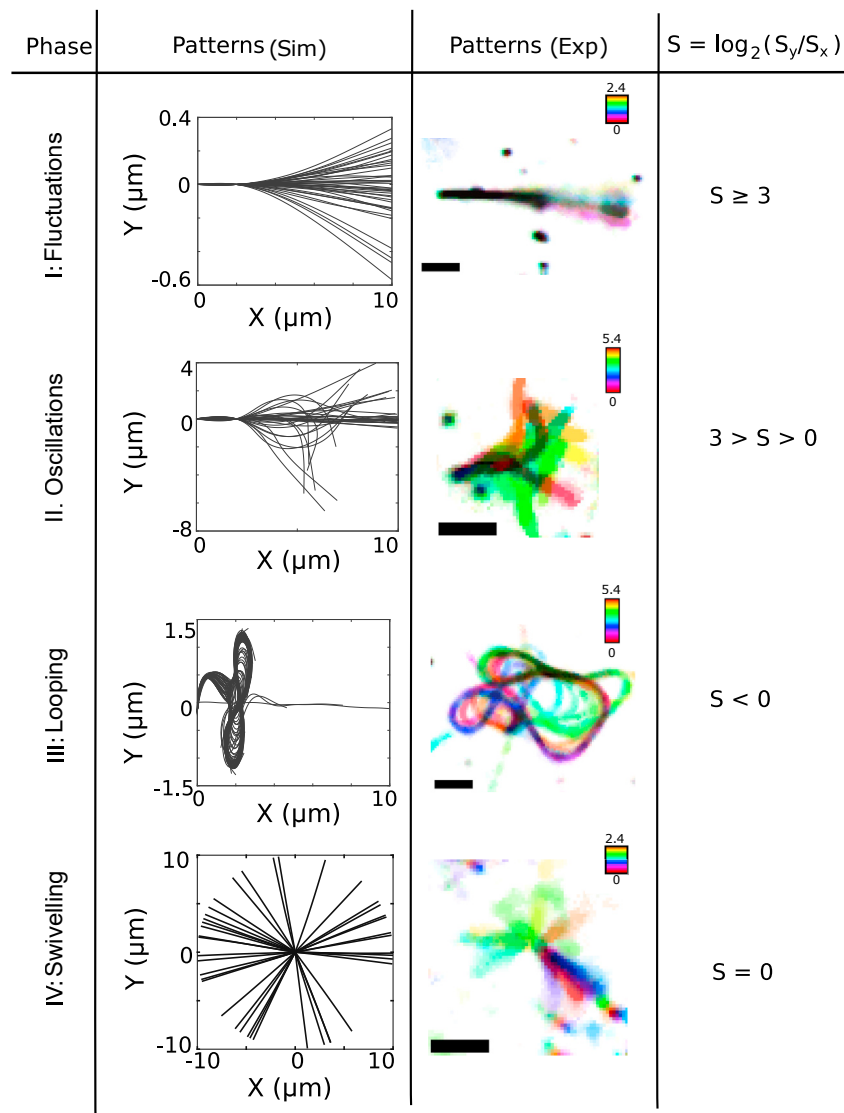


FIGURE 7 Quantifying MT patterns in terms of space explored by the free tip in experiment and simulation. The qualitative phases are distinguished based on the sweep value $S = \log_2(S_y/S_x)$, where S_x and S_y are the extent of tip movement along x and y axes as described in Fig. 1 G. The phases are as (I) fluctuations, (II) regular oscillations, (III) looping, and (IV) swivelling based on the time-projected patterns of representative simulations and experiments. In experiment, the patterns observed are fluctuations, $S = 3.8$ ($L_{MT} = 9.7 \mu\text{m}$, no motors); flagella-like, $S = 0.41$ ($L_{MT} = 4.7 \mu\text{m}$, $\rho_m = 27$ motors/ μm^2); looping, $S = -1$ ($L_{MT} = 24 \mu\text{m}$, $\rho_m = 27$ motors/ μm^2); and swivelling, $S = 0$ ($L_{MT} = 2.2 \mu\text{m}$, no motors). Scale bar, $2 \mu\text{m}$. Color bar, minutes. ρ_m , motor density. To see this figure in color, go online.

combined variation of MT length and motor density corresponds to a transition between these patterns in an objective manner (Fig. 8 A). Based on the thresholds that correspond to qualitatively distinct patterns (Fig. 7), we mapped interpolated boundaries of $\langle S \rangle = 0$ and 3 through the MT-length density space (Fig. 8 A). We proceeded to test the validity of the predicted phase-diagram boundaries by plotting experimentally analyzed $\langle S \rangle$ values (colors) from multiple filament time series ($n = 65$) for motor densities: 0 (no motors), 5.25, 16.04, 31.06, and 62.86 dyneins per μm^2 and the MT lengths from 5 to $25 \mu\text{m}$, as calculated in simulations (Fig. 8 B). Each point corresponds to one filament time series with the color based on the value of the S metric. Motor densities were estimated in three biological replicates using a GFP calibration approach as described in the “materials and methods” section and Fig. S4, with biological replicates showing small differences in the exact density obtained. We find that filaments in the absence of motors (density =

0 motors/ μm^2) indeed correspond to the $S > 3$ threshold predicted from simulations, independent of MT length. The high value of the span measure can be understood to arise from the fact that the pinned segment of filaments corresponds to the x axis in both experiments and simulations and shows very little movement (S_x in Eq. 2) compared to other patterns, whereas the y axis value emerges from the interplay of the thermal and filament bending energy scales (the numerator in in Eq. 2). Looping predicted in simulations to correspond to $S < 0$ is observed experimentally at high motor density and long filaments (upper right quadrant of Fig. 8 B), arising from the movement of the tip closer to the base of the MT along the x axis and therefore having a high value of S_x (Eq. 2). This is expected to arise due to the high motor density and resultant force. Regular wave-like oscillations were predicted in simulations to correspond to $0 < S < 3$ (between green and blue lines). Although experimental values of the S metric of short filaments ($< 10 \mu\text{m}$)

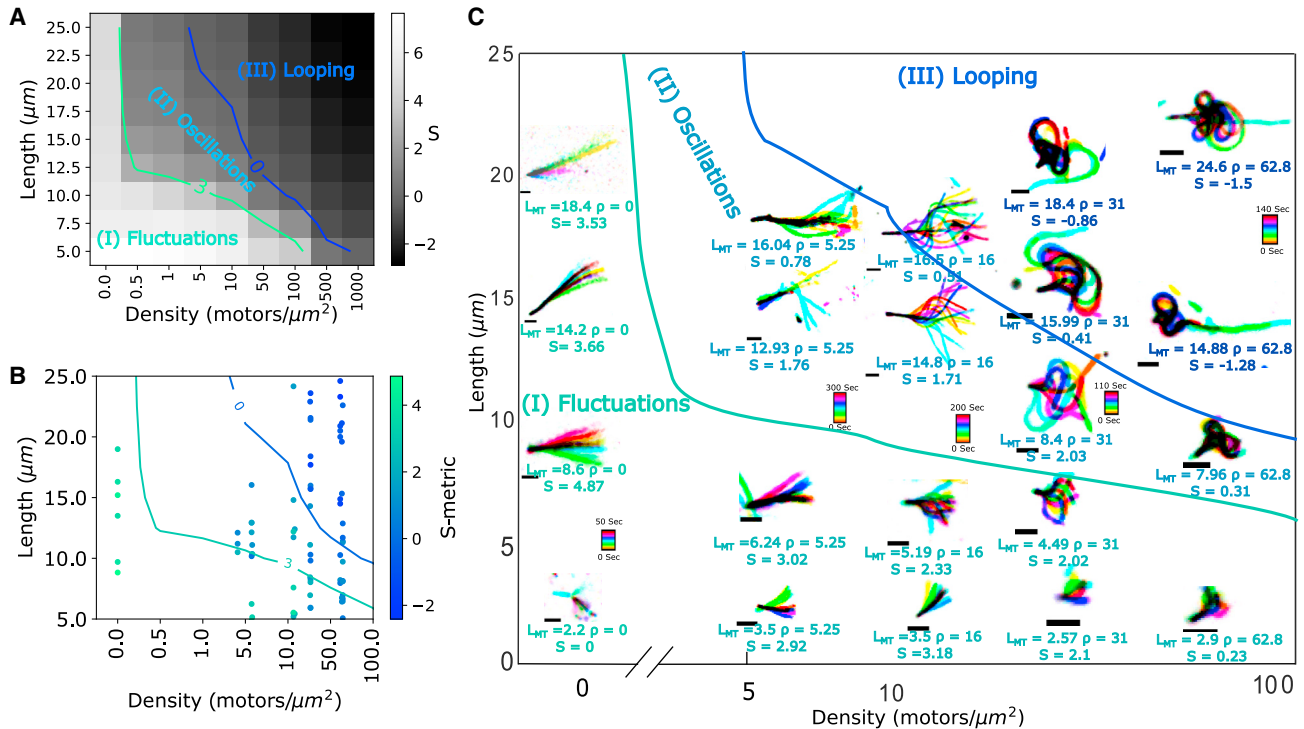


FIGURE 8 Motor density and MT-length-dependent transitions in experiment and simulation. (A and B) The parameter space for varying motor densities (ρ_m) and MT lengths (L_{MT}) from (A) simulations and (B) experiments are divided into three regions based on their S values of (S) = 0 (blue) and (S) = 3 (green), which distinguish qualitative filament tip dynamics such that (I) $\langle S \rangle > 3$ corresponds to fluctuations, (II) $3 > \langle S \rangle > 0$ to regular oscillations, and (III) $\langle S \rangle < 0$ to looping. (A) Simulated values are averaged over 10 runs for each parameter, whereas (B) experimental data are plotted for multiple filament time series analyzed (circles) where the motor density was varied from 0, 5.25, 16.04, 31.06, and 63.02 motors/ μm^2 from three biological replicates ($n = 65$). (C) Representative time-projected regions of interest of filaments taken from (B) demonstrate the qualitative change in MT dynamics with MT length and motor density. The corresponding time series sampled from multiple density and length values are seen in Video S4 (no motors) and SV7 (increasing density and length). To see this figure in color, go online.

do correspond to the predicted boundaries and show oscillations, at high motor densities, the S metric also indicates looping (Fig. 8 B). Thus, although experimental data in the absence of motors and at high motor densities and long MTs are consistent with simulations, some differences are observed between simulation predictions and experimental observations in cases of short filaments at high motor density. The S metric value predicted by simulation should indicate fluctuations ($S \geq 3$) but, in experiment, it appears to correspond to wave-like oscillations ($0 < S < 3$). This could arise either from filament bending rigidity differences in stabilized MTs (34,35) as compared to those used in simulations or the deviation from a linear force-velocity and detachment rate dynamics of the yeast dynein (36–38). A qualitative match in terms of the effect of increased motor density and lengths resulted in transitions from 1) fluctuations through 2) wave-like oscillations to 3) looping seen in experimental filament time series (Fig. 8 C and Video S7). The quantitative deviations from experiment suggest the need for further improved experimental control over MT lengths, geometry, and motor immobilization in future.

In summary, our experimental reconstitution of a two-component system of clamped MTs and a 2D sheet of

dynein motors drives low-frequency (millihertz) wave-like oscillations that are qualitatively consistent with a theoretical model with some quantitative deviations of experimental data from in predicted phase boundaries. The model predicts the self-organized emergence of regular oscillatory movement of filaments. These patterns emerge at an intermediate motor density and length of MTs in simulation. We find our in vitro reconstitution experiments validate the predicted phase space providing a quantitative match between theory and experiment. The abrupt transition between fluctuating and regular wave-like oscillatory states merits further exploration of potential phase-transition-like behavior.

DISCUSSION

We have described a minimal experimental system and the constraints that can spontaneously result in wave-like oscillatory patterns. The experiment involves a modified dynein gliding assay where MT plus ends are biotinylated and bind to surface-bound streptavidin. In the presence of ATP, this system results in regular wave-like oscillations that propagate from the clamp to the tip of the MT. We find these

oscillations are low frequency (in the millihertz range), are symmetric between peaks and troughs, and are driven by active motor activity. We combine this observation with a computational model of semi-flexible polymers driven by point models of surface-bound motors that stochastically bind and unbind from MTs and, when bound, generate forces resulting in bending and buckling due to the clamping of one end. Using a simple geometric measure of deflections, simulations show a motor density and MT length dependence of transitions between distinct patterns that are qualitatively comparable to experiments.

Comparable *in vitro* approaches have indeed been taken in the past with a minus-end-clamped MT showing kinesin-driven bending in a gliding-assay geometry (39). However, they did not report oscillations, possibly due to the low density of motors chosen. Actin filaments gliding on a sheet of myosins were reported to occasionally get pinned by their ends and move in spirals, and longer filaments were observed to oscillate (16). This is qualitatively comparable to our findings, with the notable difference that the stiffer filaments such as MTs will be expected to quantitatively differ in the forces required and filament lengths that drive the behavior. Kinesin itself was shown to result in flagella-like beating patterns when one section of MT filaments was passively immobilized and the free end was stochastically bound to kinesin complexes (18). However, that experiment lacked the control of our experiments, where we were able to control the length of the MT clamp and average surface density of the motors. Additionally, unlike previous studies, we used dynein, thus demonstrating the generality of the process. MT bundles driven by kinesin multimers were also demonstrated to self-organize into ciliary and flagellar beating waves (40). However, the study lacked information about or control over motor numbers, which we have addressed in our work. Indeed, in future, combinations of our approach and the bundles could provide a deeper insight into mechanisms. A theoretical model of a single semi-flexible chain clamped at one end by a section, driven by forces, was predicted to produce wave-like oscillations sensitive to boundary conditions, filament length, and total force (17). Although the relation between frequency and motor forces cannot be directly tested in our experiments, we do for the first time validate their predictions with the geometry of clamped filaments and forces acting along the MT lengths. Thus, our study demonstrates the self-organized emergence of smooth wave-like oscillations from a single semi-flexible filament driven by dynein motors binding and pushing it along the length, with patterns observed depending on length and motor density.

Although our observations are based on an artificial setup, comparable processes of point-clamped MTs and load exerted either along the length or at the free end of a filament have been invoked to explain *in vivo* observations of highly curved MTs that also show buckling (41). The absence of wave-like oscillations inside cells could arise from multiple causes, such as motor force, detachment, speed, density, and MT geometry.

On the other hand, when oscillatory beating patterns have been observed previously *in vitro*, based on pinned MTs and motors, they were based on nonspecific pinning of kinesin complexes and accidental clamping of MTs (18), suggesting a distinct lack of control. In addition, in case of beating patterns of MT bundles driven by kinesin complexes, both the number and lengths of MTs were unclear (40). In addition, our experiments directly address model predictions, including the length of the filament (17), that have not been reported before. Therefore, our approach demonstrates both a simpler set of components and constraints and a quantitative experimental approach that validate model prediction of oscillatory wave-like patterns in single MTs driven by motor forces.

The single-filament oscillatory patterns observed in both our experiments (Fig. 1) and simulations (Fig. 4) propagate from base to tip, appearing to be flagella-like (42). However, for the moment, this comparison is purely qualitative. In previous comparable work with clamped MTs, kinesin-driven buckling was observed (39), but not periodic oscillations. Other work has also shown waving and rotational motion (16,43) and loop formation at high motor density (44). The generality of such structures was illustrated by spirals and flagellum-like bending of actin formed when filament tips were pinned to surface defects and myosin motors generated forces (16). To test the effect of motor-specific properties on our simulations, we varied the single-molecule velocity of the motors by a factor of 20 and found the dominant frequency of oscillations increases over the range by 10-fold in a nonlinear manner (Fig. S5). This predicts that faster motors could indeed increase oscillation frequency. The yeast cytoplasmic dynein that we used in our experiments has a reported *in vitro* velocity of 0.1 $\mu\text{m/s}$ (7,20). In contrast, conventional kinesin-1 has an unloaded velocity of 0.8 $\mu\text{m/s}$ (45), whereas the outer-arm dynein from *Tetrahymena* transports MTs in a gliding assay at a velocity of 5 $\mu\text{m/s}$ (46). The simulations over one order of magnitude demonstrate an almost identical increase in frequency. However, single-motor velocities required to approach the frequency observed in more complex oscillating structures, such as flagella, require single-molecule velocities exceeding known natural MT-dependent motor velocities. It is no surprise that a single filament structure and random encounters with a sheet of motors cannot therefore achieve the high-speed oscillations, and further additions to the model, such as filament bundling, geometry mimicking the axoneme, and motor arrangement, bring us closer to understanding the principles driving *in vivo* MT-motor wave-like oscillations seen in flagella.

In our experiments, during wave-like oscillatory motion of filaments, we occasionally observe segments moving in and out of plane (Fig. S6). This effect is likely to result from torsional movement of the filament, consistent with the reported ability of cytoplasmic dynein to generate torque based on its motion in a right-handed helical path (47). Such helical movement has also been reported in cases of mammalian cytoplasmic dynein (48) and *Tetrahymena* axonemal dynein (49).

Reports of such MT rotation appear to also extend from mammalian dynein to kinesin-1 and kinesin-8 (50). At high motor density, the torque produced by motors has even been shown to result in supercoiling of filaments, as seen in the case of pinned actin filaments transported by myosin (51). In our study, we do not observe such dramatic supercoiling as often, potentially due to the low motor density used. Additionally, our simulations are, for the sake of simplicity, conducted in 2D. Extensions to our work to use both higher density of motors as well as more quantitative estimation of 3D displacement could demonstrate consistency with other work in terms of torsional motion, and improved simulations could be used to test the generality of the process.

Conclusions

The self-organized nature of the observed patterns suggests that a minimal system consisting of mechanical components of filaments and motors can drive qualitatively comparable oscillations to the more complex flagella. At the same time, the absence of the geometric complexity, regulation, and coordination seen in bona fide flagella suggests that future work designing a bottom-up minimal synthetic flagellum could benefit from some of the design principles defined in this study, along with highlighting the fundamental properties of collective mechanics that arise from combinations of components and geometric constraints.

SUPPORTING MATERIAL

Supporting material can be found online at <https://doi.org/10.1016/j.bpj.2024.01.016>.

AUTHOR CONTRIBUTIONS

S.A.Y. performed the experiments and analyzed the data. N.K. and A.S. performed simulations and wrote the analysis codes. S.A.Y., N.K., D.K., and A.S. prepared the figures. D.K. analyzed the image data. N.K., S.A.Y., D.K., and C.A.A. wrote the manuscript. C.A.A. conceptualized and supervised the work and obtained the funding.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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Bioimage informatics

KnotResolver: tracking self-intersecting filaments in microscopy using directed graphs

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Abstract

Motivation: Quantification of microscopy time series of *in vitro* reconstituted motor-driven microtubule transport in “gliding assays” is typically performed using computational object tracking tools. However, these are limited to non-intersecting and rod-like filaments.

Results: Here, we describe a novel computational image-analysis pipeline, KnotResolver, to track image time series of highly curved self-intersecting looped filaments (knots) by resolving cross-overs. The code integrates filament segmentation and cross-over or “knot” identification based on directed graph representation, where nodes represent cross-overs and edges represent the path connecting them. The graphs are mapped back to contours and the distance to a reference minimized. The accuracy of contour detection is sub-pixel with a robustness to noise. We demonstrate the utility of KnotResolver by automatically quantifying “flagella-like” curvature dynamics and wave-like oscillations of clamped microtubules in a “gliding assay.”

Availability and implementation: The MATLAB-based source code is released as OpenSource and is available at <https://github.com/CyCelsLab/MTKnotResolver>.

1 Introduction

Microtubules (MTs) are an essential component of the cell cytoskeleton, and their interaction with molecular motors serves to generate forces required for organelle positioning and the assembly of supra-molecular structures like the mitotic spindles, cilia, and flagella (Woolley 2000, Guérin *et al.* 2010). The collective properties of MT growth and interactions result in complex networks *in vivo*. However, quantifying the mechanical properties in such an intracellular environment has proven to be difficult due to the crowded nature of the intracellular space (Ellis 2001). As a result, the collective mechanical properties of both MTs and motors have been extensively studied *in vitro*. One prominent method used to examine collective transport by molecular motors is by immobilizing motors on the surface and allowing filaments to bind to them, which results in transport of MTs, referred to as “gliding assays” (Howard *et al.* 1989, Nitzsche *et al.* 2010, Sumino *et al.* 2012). Typically in such MT “gliding assays” with short filaments, i.e. of lengths 5 μm or less, are observed almost as rod-like objects in microscopy in the presence of either kinesin (Scharrel *et al.* 2014) or dynein (Monzon *et al.* 2018, Jain *et al.* 2019). Actin filament motility driven by myosin, however, shows highly curved and bent structures at similar length scales (Kron and Spudich 1986, Ishijima *et al.* 1991, Kron *et al.* 1992). This is consistent with the reported persistence length of MTs *in vitro* at thermal equilibrium of the scale of millimetres, while actin has an average persistence length of the order of

$\sim 10 \mu\text{m}$ (Gittes *et al.* 1993). Additionally, highly curved filaments are seen in “gliding assays” either due to torsional forces exerted by myosin resulting in filaments “twirling” (Beausang *et al.* 2008). Such buckled MTs have also been experimentally observed when filaments were bent using optical tweezers in order to measure their rigidity (Kurachi *et al.* 1995). Molecular motor-driven filament “gliding assays” modified such that one end was clamped also resulted in highly curved filaments, the dynamics of which were tracked manually (Gittes *et al.* 1996). We have demonstrated a comparable end-clamping of MTs in a dynein “gliding assay” results in wave-like motion of filaments, that self-intersect (Yadav *et al.* 2024). Such self-intersections proved difficult to track with existing tools. Axonemes reconstituted using a bottom-up approach have also been observed to form complex curved structures, which are challenging to track using conventional filament tracking tools (Guido *et al.* 2022). Thus, tracking curved and self-intersecting filaments, i.e. knots, from time series are of wider relevance for quantification of collective motor-driven filament transport studies.

Quantifying such “gliding assays” typically involves detecting and tracking the movement of individual filaments in microscopy time series. Some of the widely used tools include a MATLAB-based tool, FIESTA, which was shown to track MTs with sub-micron precision (Ruhnow *et al.* 2011), an active contour-based approach applied to track actin filaments, JFilament that works as a FIJI plugin (Li *et al.* 2009, Smith *et al.* 2010) and MTrack, a versatile FIJI plugin (Kapoor *et al.* 2019). While these tools are optimized for a general

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“gliding assay,” they assume non-intersecting filaments that are straight over the time series, assumptions that are violated in either complex geometries or crowded environments.

Multiple algorithmic approaches to resolving networks from microscopy have been developed, including a general algorithm for the decomposition of filamentous networks (DeFiNe) by weighted, undirected graph representation, and roughness minimization that can resolve simulated, cellular, and astronomical images of meshes of filaments (Breuer and Nikoloski 2015). At a whole cell scale, NeuriteTracer has been used to automatically detect neuronal extensions based on low-level segmentation and skeletonization (Pool et al. 2008), which has been integrated with a graph theoretical representation in simple neurite tracer (Arshadi et al. 2021). At an organismal scale, images of *Caenorhabditis elegans* worms at high density were resolved by combining skeletonization with a graph-theoretical representation of worm contours as edges and adjacencies as nodes (Raviv et al. 2010). Representing networks as open curves using B-spline surface representation of filaments optimized by gradient-descent to the image, combined with image-formation model to detect and segment unknown numbers of filaments (Xiao et al. 2016). Thus, across problem-types, the application of graph representations has been useful to resolve filaments in complex geometries. However, these have, to our knowledge, not yet been applied to single filaments with cross-overs in combination with tracking.

In this study, we describe a novel approach to segment and track single MT filaments in time-series data that undergo self-intersections or knots, which we refer to as KnotResolver. Our approach combines active contours seeded by an initial guess based on an intensity threshold and geometry, the representation of the intersecting segments as elements of a directed graph, and distance minimization between time-frames by comparing curves predicted by the directed graph with an initial reference that is not intersecting. We demonstrate the utility of our approach by applying it to resolve knots in both *in silico* and *in vitro* oscillations of clamped MTs described in a recent study (Yadav et al. 2024). We demonstrate the improvements in tracking by comparing KnotResolver to existing filament tracking tools.

2 Materials and methods

2.1 Reconstituting dynein-driven MT oscillations

The filament oscillation assays were performed in a flow chamber made by sandwiching double-backed tape between a slide and a coverslip. The chamber was coated with a 1:1 (molar ratio) mixture of anti-green fluorescent protein (anti-GFP) nanobody and streptavidin. The surface was blocked with casein to prevent non-specific attachment. Dynein was allowed to attach by incubating in the chamber followed by washes to remove unbound motors. The biotinylated MTs were added to the chamber and allowed to land followed by washes to remove the unbound MTs (Fig. 1A). Finally, a motility buffer containing anti-fade and 4 mM ATP was added to the chamber and MT movement was recorded. The setup was imaged using a 60× (NA 1.45) oil immersion lens using a motorized fluorescence microscope (Nikon TiE, Nikon Corp. Japan) with 10s time intervals for 10–20 min in a lexan enclosure with temperature maintained at 37°C (Okolab, Pozzuoli, Italy) as previously described (Jain et al. 2019, Yadav et al. 2024). Plus-end biotin-labeled MTs were prepared by first polymerizing filaments with a

1:4 molar ratio of rhodamine-labeled tubulin: unlabeled tubulin, centrifugation to remove monomers and plus labeling with a mixture of 1:4 rhodamine-labeled:biotinylated tubulin, with filaments then stabilized in taxol. The filament oscillation assays were performed in double-backed tape flow chambers made by sandwiching double-backed tape between a slide and a coverslip to form a chamber that was coated with the nanobody and streptavidin in a 1:1 molar ratio, followed by casein to block non-specific attachment. To this, the anti-GFP nanobody was added, then dynein-GFP, followed by multiple washes to remove unbound motors, biotinylated MTs, more washes, and finally motility buffer containing anti-fade and 4 mM ATP. The setup was imaged using a 60× (NA 1.45) oil immersion lens using a motorized fluorescence microscope (Nikon TiE, Nikon Corp. Japan) with 10s time intervals for 10–20 min at 3°C in a temperature-controlled Lexan enclosure (Okolab, Pozzuoli, Italy) as previously described (Yadav et al. 2024).

2.2 Simulating oscillatory filament dynamics and the effect of image “noise”

2.2.1 Simulations

We performed simulations of oscillatory wave-like dynamics of simulated MTs based on a stochastic agent-based simulation engine for motor–cytoskeleton interactions, Cytosim (Nedelec and Foethke, 2007), based on a previously described 2D dynamics model of individual plus-end clamped MTs in a gliding assay (Yadav et al. 2024). We systematically varied MT lengths from 5 to 50 μm and motor density over 12, 25, 50, and 100 motors/ μm^2 . Each simulation was performed for 5 min and 120 frames were saved. The objective was to generate skeleton contours of varying lengths, comparable to experiments.

2.2.2 Adding “noise” to simulated images

The simulated skeletons were dilated by a diamond-shaped structuring element of size 2 and the dilated skeletons were convolved with a Gaussian point spread function (size = 3, sigma = 2). We introduced noise of varying signal-to-noise ratio (SNR_{db}) to the simulated data. The noise model is Gaussian with a specified noise power level P_{noise} . The desired P_{noise} is related to SNR by the relation:

$$P_{\text{noise}} = P_{\text{signal}} / \text{SNR} \quad (1)$$

where P_{signal} is equal to the variance of intensity of the original image. SNR in linear scale is related to SNR_{db} by the following relation

$$\text{SNR} = 10^{\frac{\text{SNR}_{db}}{10}} \quad (2)$$

The SNR_{db} values used were 5, 10, 20, and 30. The generated Gaussian noise is computed as *Noise*:

$$\text{Noise} \sim \sigma_{\text{noise}} * \mathcal{N}(0, 1) \quad (3)$$

where, $\mathcal{N}$ is a normal distribution with zero mean and the σ_{noise} is equal to

$$\sigma_{\text{noise}} = \sqrt{P_{\text{noise}}} \quad (4)$$

The *Noise* is added to the original image to get a noisy image with specific P_{noise} .

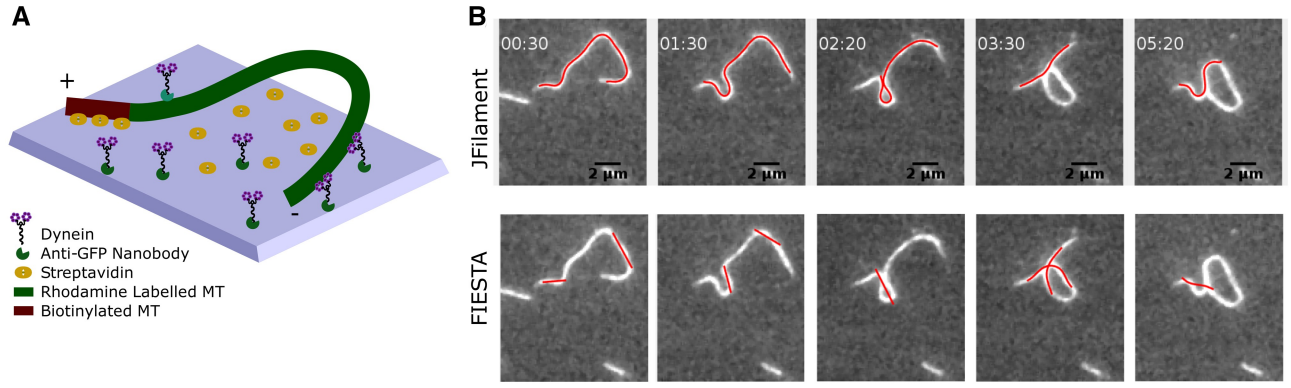


Figure 1. *In vitro* assay of filament oscillations and errors in detection using Gaussian fitting and active contours. (A) The schematic depicts the experimental setup of plus-end clamped MTs based on biotinylated tubulin bound to surface-immobilized streptavidin, combined with dynein motors immobilized by antibodies in a “gliding assay” that drives MT bending as described previously (Yadav *et al.* 2024). MTs are labeled with rhodamine-labeled tubulin for visualization in fluorescence microscopy. (B) Representative frames from a microscopy time series of the oscillations of rhodamine-labeled MTs (gray) oscillating over 5 min 20 s were segmented detected segments overlaid (red lines) based on either (top) an approach using open, active contours in JFilament (Smith *et al.* 2010) or (bottom) threshold and Gaussian fitting approach using FIESTA (Ruhnnow *et al.* 2011). Scalebar: 2 μm , Time: mm: ss

2.3 Quantifying filament detection accuracy

The simulated dataset described previously was used to create benchmarks to assess the proposed filament tracking method. We quantified detection accuracy using the simulated dataset as a “gold standard” and some typical measures used in image-analysis accuracy using two measures: (i) Sørensen–Dice Index for segmentation and (ii) Fréchet distance to compare two curves.

2.3.1 Dice index

The Dice score or Sørensen–Dice index (Dice 1945; Sorensen 1948) takes into consideration TP: true positives, FP: false positives, FN: false negatives, and TN: true negatives and combines them as follows:

$$\text{Dice index} = \frac{2 \times \text{TP}}{2 \times \text{TP} + \text{FP} + \text{FN}} \quad (5)$$

True positives are defined as segmented pixel indices that are within a threshold distance (2 pixels) from the ground truth annotations. The choice of the threshold was based on the point spread function used to convolve the filament skeletons to resemble experimental images. False positives are segmented pixels that are not within this threshold. Ground truth pixels that were not detected as TP are counted as false negatives.

2.3.2 Fréchet distance

This metric computes the minimum distance between two curves that is sufficient to traverse their separate paths (Fréchet 1906, 1924) using a MATLAB implementation of the discrete Fréchet distance. This provides the maximum separation in distance metric between the two curves. The metric is useful for comparing the overlap of two curves, while taking into account the proximity of indices between the two curves. For a pair of duplicate curves, Fréchet distance should be zero, which indicates a perfect overlap and identical ordering of pixel indices between two curves. Fréchet distance is computed between the indices of input curve $I(t) = (I_x(t), I_y(t))$ and the resolved curve $R(t) = (R_x(t), R_y(t))$ obtained from the KnotResolver pipeline. Here

t signifies the orientation or ordering of the two pixel indices in their respective curves. The Fréchet distance is computed as follows:

$$F(I, R) = \inf_{\alpha, \beta} \max_t \{\rho(t, \alpha, \beta)\} \quad (6)$$

where $\rho(t, \alpha, \beta)$ is the distance function defined as:

$$\rho(t, \alpha, \beta) = \sqrt{(I_x(\alpha(t)) - R_x(\beta(t)))^2 + (I_y(\alpha(t)) - R_y(\beta(t)))^2} \quad (7)$$

and α and β are re-parameterizations of the curves $I(t)$ and $R(t)$, respectively.

2.4 Analysis of filament contours

All analysis was performed in MATLAB R2020b (Mathworks Inc., Natick, MA, USA). We calculate the tangent, normal, and curvature vectors for each smoothed contour by using the Frenet–Serret formulas (Mate 2017). Smoothing of the x and y coordinates is performed using the Savitzky–Golay filter (Savitzky and Golay 1964). The end-to-end distance of the curve is computed as Euclidean distance between the tip and end of each filament. All results are visualized by plotting the curve with color interpolation, which indicates the arrangement of contour indices from tip to end.

2.5 Code availability

The tool was written in MATLAB2020b (Mathworks, Natick, MA, USA) and uses functions called from the “Signal Processing Toolbox,” “Image Processing Toolbox,” “Statistics and Machine Learning Toolbox,” and an optional “Parallel Computing Toolbox.” The code for the filament bending analysis has been made OpenSource and is available at the Github URL <https://github.com/CyCelsLab/MTKnotResolver>, together with an example time series with optimized segmentation parameters.

3 Results

3.1 Filament looping and cross-over detection: limits of existing tools

We have recently demonstrated MTs can undergo waves of high curvature and oscillate in a modified *vitro* “gliding assay,” where plus-ends of MTs were clamped to the glass substrate, while a surface-immobilized minus-end directed motor, dynein, generated the transport force (Yadav et al. 2024) (Fig. 1A). The filaments undergo wave-like millihertz (slow) oscillations in a motor-density and filament length-dependent manner. In order to quantify filament contours from such image time-series data, we applied multiple standard image analysis tools for segmenting filaments in “gliding assays” of which we describe the result of using the two more widely used: JFilament and FIESTA. JFilament (Li et al. 2009, Smith et al. 2010), based on a robust active contour approach, resulted in filament detections only when they were not looped, but once filaments underwent self-intersections, the method took what appeared to be the shortest path [Fig. 1B (top)]. This relates to the nature of the active contour method used. FIESTA (Ruhnow et al. 2011), on the other hand, is based on Gaussian profile fitting to improve threshold-based segmentation with filament tracking broadly based on distance minimization. The outcome of using the same dataset resulted in detection only of the straight segments of filaments, with highly curved regions of the same filament fragmented into multiple straight segments [Fig. 1B (bottom)]. While tuning multiple parameters of the code did change the results slightly, it did not lead to a qualitative improvement, due perhaps to the inherent assumption of straight rod geometry built into the method. Thus, we proceeded to address both these limitations of the existing approaches—straightness assumption and path length optimization—to develop a novel approach.

3.2 Resolving self-intersecting “knots” in filament contours by minimizing paths using directed graphs

The “KnotResolver” pipeline consists of the following steps: (i) “Segmentation” used to extract skeletons of a single MTs (Fig. 2A). Segmentation parameters are interactively set by visually inspecting the output with the optimal values saved in a CSV file. (ii) “Branch identification” is called after the segmentation optimization script that identifies filament self-intersections (Fig. 2B). (iii) “Mapping ‘knots’ to graph”: converts the elements into a graph representation, (iv) “Template matching” to reconstruct the correct filament geometry by path minimization to previous time frames (Fig. 2B and C).

i) “Segmentation”: images were first pre-processed to enhance contrast by saturating the top and bottom 1% of intensity values and median filtering using a 3×3 kernel to smooth the intensity fluctuations. The pre-processed image stack was converted to binary (black-white) by Otsu’s method of finding the threshold (Otsu 1979) and the segmented image with filament contours used as the initial seed for an energy-based active contour model based on Chan and Vese’s method (Chan and Vese, 2001). This improves the segmentation by optimizing for the filament boundary. Finally, the optimized binary contour is skeletonized using the medial axis transform (Lee et al. 1994) to obtain one-pixel width filament contours,

which are stored in a structured array for all frames in the image stack [Fig. 2A and C (left)].

- ii) “Branch identification”: Skeletons obtained in the previous step are tested for branch points in a neighborhood of 8 (Moore neighborhood) by testing whether the summation of individual pixel values (I_{nb} = either 0 or 1) satisfies $\sum_{nb}^N(I_{nb}) \geq 4$. The central pixel ($nb = 5$) has a value 1, by virtue of being on the skeleton. In cases where there are no branch points in a segmented skeleton, the correct orientation of the filament indices is determined using the geodesic distance algorithm (Soille et al. 1999) implemented using the *bwdistgeodesic* function in MATLAB (Mathworks Inc., Natick, MA, USA). Filament end points are provided as a seed to the function. When the skeleton is identified as being a “knot,” i.e. with branches, we proceed to use a graph representation, as described in the following section.
- iii) “Mapping ‘knots’ to graphs”: a skeleton containing branch points is represented as a directed graph $G = (V, E)$ consisting of vertices V and edges E , with the direction given by a chosen start point. They are labeled as either branch (V_b) or filament segment (V_f) (Fig. 2B). A branch vertex is identified by connected pixels that satisfy the branch-point criterion, while the remaining regions are labeled as filament vertices. Edges E are the connectivity between unique label groups, forming connecting pairs of V_b and V_f vertices (Fig. 2C). All edges are bidirectional, i.e. they can be $E(V_b, V_f)$ or $E(V_f, V_b)$, enabling traversal in either direction, except for start and end vertices where traversal is limited to a single direction. Instances where a filament merges with itself are represented as loops, in the graph, i.e. where heads and tails coincide (Bang-Jensen and Gutin 2001), e.g. for the pair of edges: $E(1,7)$ (Fig. 2C).
- iv) “Template matching”: a branched skeleton thus represented as a graph $G = (V_i, E_{ij})$, where V_i denotes the set of vertices and E_{ij} denotes the set of bidirectional edges, where i and j are indices of branched or filament vertices. The graph structure is determined by the number of unique branch points, which either indicate intersections with other filaments or an instance where the filament forms a loop. We generate all possible path combinations between the start and endpoint by traversing every edge connecting vertices V_f (filament vertex) and V_b (branch vertex). For graphs that lack an end point, the path ends at the last visited node, after all nodes are visited once. Since the number of paths can increase exponentially with the number of nodes, we optimized the number of possibilities generated at each step to capture filament paths correctly, based on comparison to the “ground truth,” obtained from manual analysis. Knot resolution is based on minimizing the difference to a resolved skeleton, which is the template (Fig. 3A). We have implemented a multi-step approach: (i) coarse identification of key vertices using Euclidean distance matching, (ii) path generation to output possible paths between the start and end point, and (iii) template matching of possible paths to the previous resolved contour. (i) Identifying key vertices: Euclidean distance scores of vertices compared to the template that help identify connections with minimal distance to a reference—to identify the three vertices with minimal distance to the template ($V_{m=1to3}$). These vertices are used to limit the search and

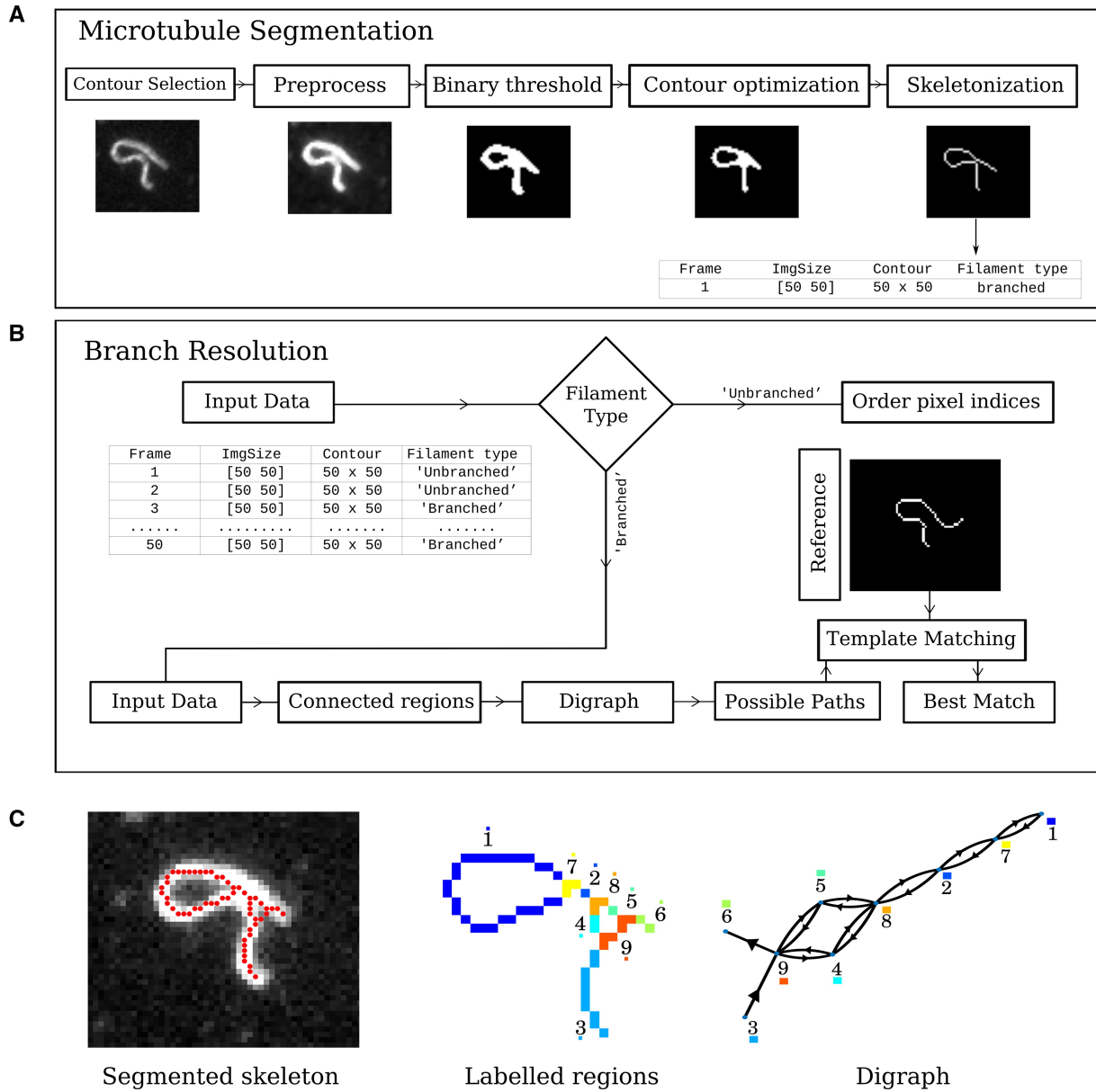


Figure 2. Algorithmic flow diagram of graph-based resolution of self-intersecting filament image time series. (A) “Segmentation and branch finding”: In a frame-wise manner, images are binarized by thresholds. These binary objects are used to seed active contours to smooth the curve. The contour is skeletonized to 1 pixel width using medial axis transform, and pixels with 4 or more neighbors are labeled as branched, suggesting a self-intersection. (B) “Branch resolution”: The skeleton with sequential pixels is used as an input labeled as either “branched” or “unbranched.” Branched contours are mapped to a directed graph (digraph) with branch points as vertices connected to each other by segments of the contour as edges. Paths are calculated using a two-step coarse and refined approach to reduce complexity. Graph-paths are mapped back to possible contours, whose distance is then minimized by comparison to the last unbranched contour (template), to find the best match. (C) (left to right) A representative grayscale image (gray) segmented resulting in a skeleton contour (single pixel wide contour), that is classified into regions (numbered) that connect branch points. These regions are mapped to edges and branches to vertices, with both directions possible based on a user-input start point, to produce a directed graph

significantly reduce the number of paths generated. (ii) Path generation: multiple paths are generated between a start node and a defined end vertex (Fig. 3B). The first combination of path is generated by taking into account the template found in the previous step. The remaining paths are generated by finding number of combinations between the fixed start point and all of the remaining vertices sequentially. The number of paths generated at every combination is limited to 10 to avoid exponential growth of possibilities. (iii) Template matching: all possible paths are converted to contours, and an alignment score is assigned using dynamic score warping, DTW

(Sakoe and Chiba 1978). The score is calculated as the cumulative cost function $D(i, j)$ between a selected contour $S(x_i, y_i)$ and $R(x_j, y_j)$ given by:

$$D(i, j) = |S(x_i, y_i) - R(x_j, y_j)| + \min(D(i-1, j-1), D(i-1, j), D(i, j-1)) \quad (8)$$

where i is the reference index and j the template index. This score is compared among the possible paths, and the path with the minimal score is selected (Fig. 3C). Once a match is

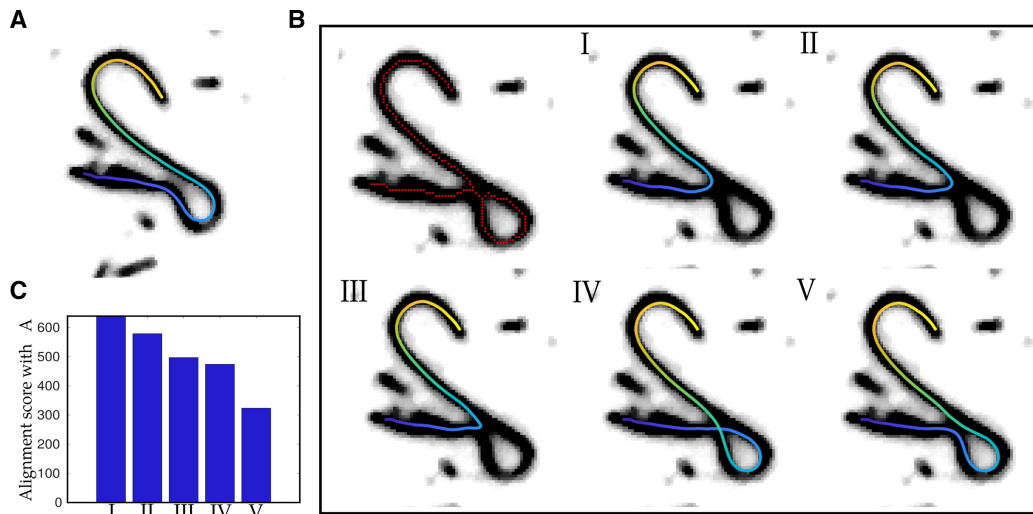


Figure 3. Resolve the knot by minimizing distances between template and paths generated by the graph. (A) A representative image of a bend microtubule at time t , when it does not have any knots (unbranched) with the segmented contour. The intensity of the contour maps the position index along the contour. (B) The microtubule in frame $t+\delta t$ showing a knot (branched) as seen from the contour (red line). (I–V) The possible paths from the start to the other end are overlaid as contours with the position-index in color. Blue: start, yellow: end. δt : interval between frames. (C) The alignment score is calculated for all paths (I–V) compared with the unbranched path from the previous time frame based on dynamic time warping. The path with the lowest score is then the optimal

found, pixel indices are iteratively ordered, along the best found path using the geodesic distance (Paliwal et al. 1982).

The pipeline of the implementation is optimized for single filaments, but the input file can be used to batch process multiple ROIs. This proceeds with the user creating a *myParams.csv* file with details of the file names and paths on each line—a new line indicates a new file. This is run as an input to *KnotSegmenter.m*, which after graphic user interface (GUI) based interactive optimization of segmentation parameters, results in an output *myParams_optimized.csv* file. This has the optimized segmentation parameters for each ROI, and is used as an input by *KnotResolver.m* to batch process the ROIs.

3.3 KnotResolver has Sub-pixel filament contour detection accuracy and is robust to image “noise”

In order to assess the segmentation accuracy of our method, we created images of simulated time series of filaments that undergo cross-overs based on plus-ends clamped and minus-end directed motors in a modified “gliding assay,” as described previously (Yadav et al. 2024) and briefly summarized in Section 2. These simulated MT coordinates in time series were plotted as binary skeletons to provide the “ground truth” and processed to resemble experimental images of filaments by dilation with a diamond-shaped structuring element of Size 2 and convolution with a Gaussian point spread function (Size: 3, σ : 2) centred around the central pixel. Salt-and-pepper noise of different intensities was added and the SNRs were calculated. The error in position detection is calculated as the minimal distance between the segmented skeleton and the ground truth pixel indices. “KnotResolver” was then used to segment the images of bent filaments, and compared to a simple intensity threshold and active contour-based approach, when the SNR increased from 5 (Fig. 4A), 10 (Fig. 4B), 20 (Fig. 4C), to 30 (Fig. 4D). We find the mean pixel error in position detection of “KnotResolver” and active contour methods remains sub-pixel for the range of SNR as seen by the Gaussian peak from fitting (μ : mean, σ : SD), but simple threshold-based methods show errors exceeding few pixels for SNR 5 and 10. In image-frames where the

filament contour self-intersect, only KnotResolver can correctly identify contours, with both segmentation and active contour methods failing (Fig. S1A). The outputs of “KnotResolver” are robust for SNR ranging from 30 to 5, in contrast to threshold-based methods and active contours that are sensitive to the same SNR range, as quantified by the Dice Score, as described in detail in Section 2 (Fig. S1B). Threshold-based methods as highlighted are sensitive to low SNR, while active contour methods are sensitive to rapidly changing geometry that throws off the initial seed and leads to filament shrinkage. The combination of these two methods, implemented in KnotResolver, is robust to both low SNR and rapidly changing filament positions, which is key for tracking single filaments showing sustained oscillations.

In order to identify possible limitations of our method and solve them, we tested the effect of multiple self-intersections that arise in simulations with increasing filament length and motor density and find Dice score decreasing after ~ 60 frames in MTs with lengths $>20 \mu\text{m}$ (Fig. S2A). As a measure of the geometric shape, we also use the Fréchet distance score between segmentation result and ground truth and find a comparable increase in distance indicating drop in accuracy of segmentation for long MTs at high densities (Fig. S2B). To address this limitation, we have implemented a manual restart, which can be used when the code encounters problematic frames using user-input to interactively map the path in the graph structure and correct the errors (Fig. S2C). In those time series where the first frame itself is self-intersecting, the user can manually initialize the correct path of the contour elements (labeled regions) by first running *KnotSegmenter.m* to obtain the skeleton, and then running *KnotResolver.m* with the entry “ManualCheck” set as [p], where $p=1$ to N , the frame number where manual intervention is required (Fig. S3A). Running the code pops up an interactive plot with the labeled regions (Fig. S3B), which the user can click on segments sequentially to provide the path of resolution (Fig. S3C), which in turn resolves the knot (Fig. S3D).

In summary while sub-pixel accuracy can be achieved by other methods alternative to the proposed KnotResolver

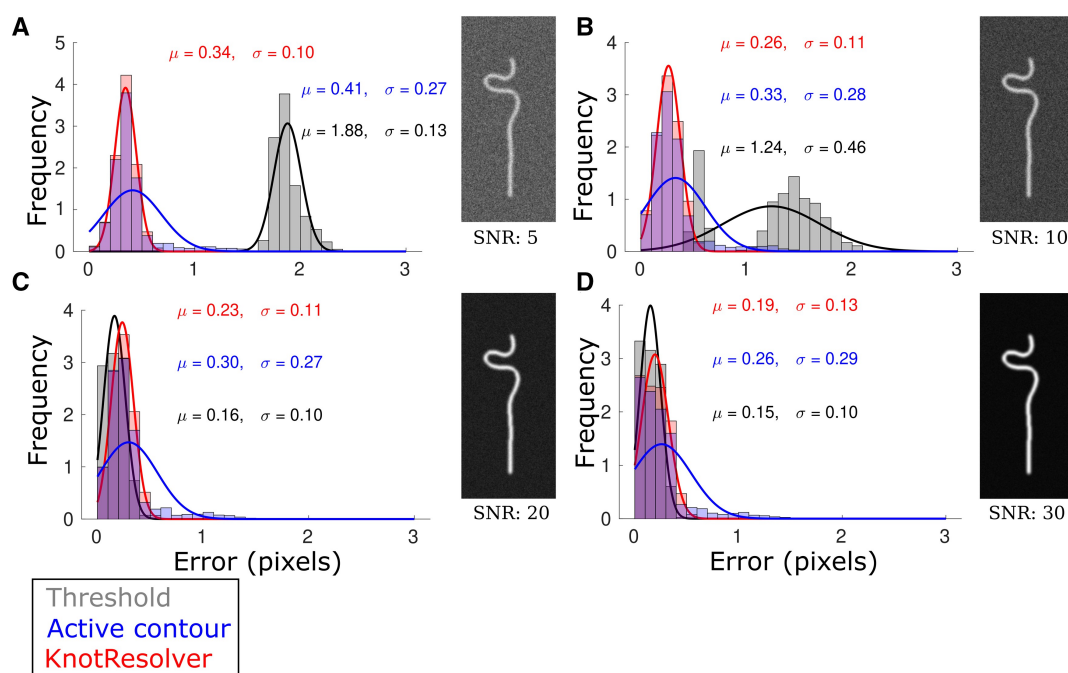


Figure 4. Position detection error compared between segmentation methods with increasing noise. (A–D) The segmentation error in pixels for each filament compared to ground truth from simulations is plotted as a frequency histogram for the following methods: (i) threshold, (ii) active contour, and (iii) KnotResolver (legend). The inputs were simulated filament images with increasing signal-to-noise ratios (SNR) (A) 5, (B) 10, (C) 20, and (D) 30. The mean (μ) and SD (σ) of a Gaussian fit to each error-histogram is noted ($n = 2880$). The images are representative filaments with increasing SNR, with salt and pepper noise resulting in low SNR

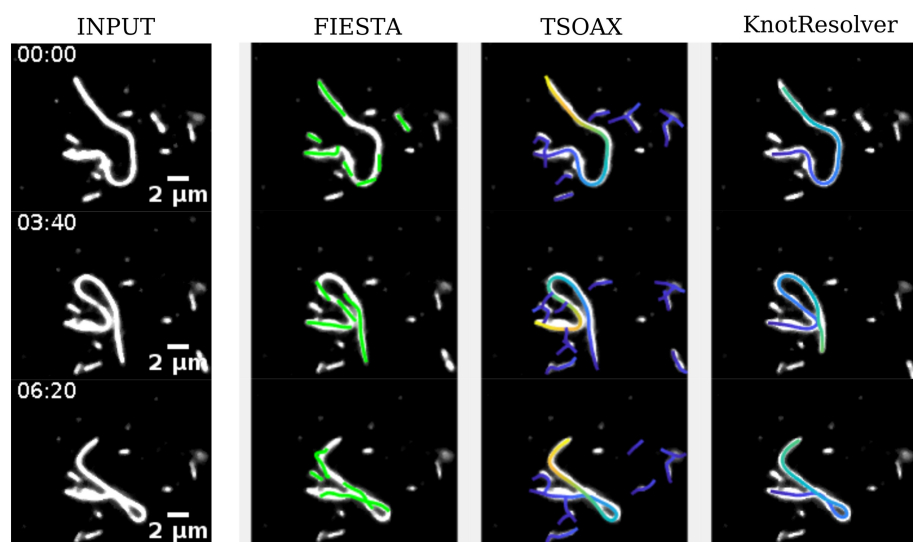


Figure 5. Comparing KnotResolver to FIESTA and TSOAX. (INPUT) A representative time series of labeled MTs undergoing bending and buckling forming a knot was used as input and tracked using FIESTA (Ruhnow *et al.* 2011), TSOAX (Xu *et al.* 2019), and KnotResolver. Parameters used for (i) FIESTA were FWHM = 1000 nm, proportion curved filaments = 25%, minimum track length = 1 frame, maximum break = 4, (ii) for TSOAX default parameters were used with manual input of the minimum and foreground intensity value and (iii) KnotResolver were intensity threshold = 0.46, number of iterations for active contours = 10, contraction bias = 0.4, and smoothing factor for active contours = 0.2, with no manual corrections. Scale bar: 2 μm . Time: mm: ss

pipeline, the higher Dice score in cases of self-intersections demonstrates both high accuracy and robustness to noise. The addition of a manual override also offers the user control over the path selection in exceptional cases.

3.4 KnotResolver outperforms existing methods for MT filament tracking

We compare of our approach to resolving self-intersecting filaments in terms of segmentation and tracking to two widely

used computational tools—FIESTA (Ruhnow *et al.* 2011) and TSOAX (Xu *et al.* 2019). FIESTA is based on intensity thresholding and Gaussian fitting of contours to achieve sub-pixel localization accuracy. Filament intersections are semi-automatically handled with cross-point identification followed by linking based on a minimum angle criterion. TSOAX, on the other hand, combines stretching open active contours based software (SOAX) (Xu *et al.* 2015) with tracking. This is achieved through automatic initialization of

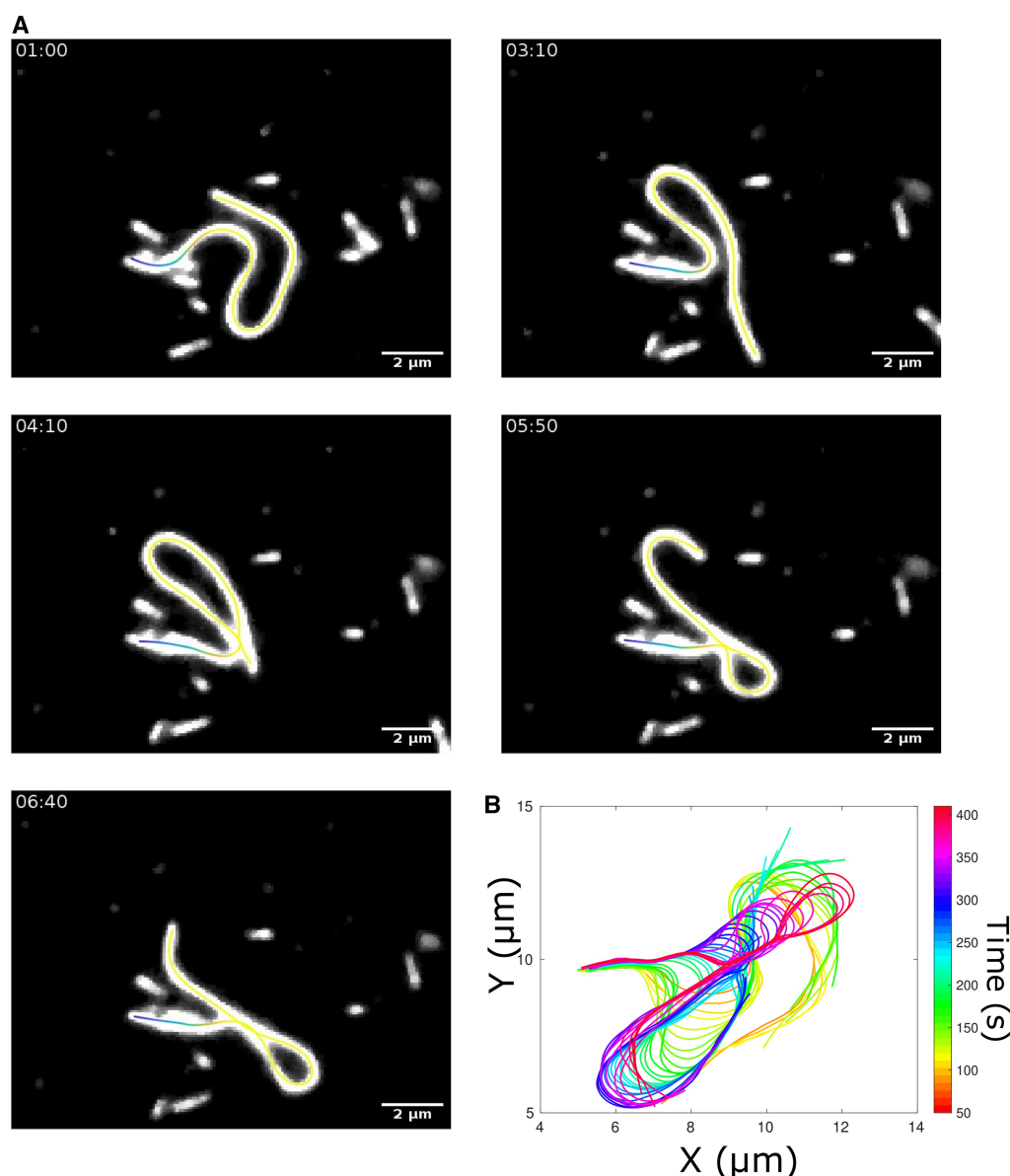
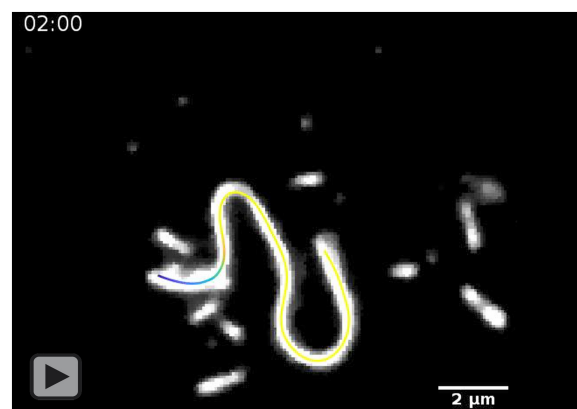


Figure 6. Representative time series of filament with tracked contours. (A) The fluorescence time series of bent and crossing over filaments are overlaid contour segmented using proposed pipeline. The skeleton is color-coded along its length (from tip to end). Scale bar: 2 μm . Time: mm: ss. (B) The filament contours from successive frames are projected showing the traveling wave of curvature. Colorbar: time

multiple open curves during the detection stage, which elongate and stop at filament intersections and tips. Intersections of curves are addressed by identifying T-junctions linked through a temporal local matching step. In order to compare the outcomes, we use an experimental microscopy time series of MT filaments in a clamped dynein-driven gliding assay. The filaments are observed to change from straight, to highly curved, to self-intersecting filaments (Fig. 5). While the output from FIESTA demonstrates only straight segments are detected, TSOAX can identify curved filaments, but cross-overs are incorrectly detected. The output of KnotResolver is the only one that allows us to correctly identify curved sections and maintain the identity of the segments at cross-over.

We therefore proceeded to apply the KnotResolver pipeline to a representative experimental time series of clamped filaments undergoing periodic wave-like oscillations and



Video 1. Representative time-series of filament knot resolution with tracked contour. The fluorescence microscopy time-series of a filament undergoing self-intersection (gray) is overlaid with the segmented and tracked contours. The parameters used to automatically track the series without manual intervention were as follows: intensity threshold = 0.46, contour iteration = 10, contraction bias = 0.4, smooth factor = 0.2. Scalebar: 2 μm , interval between frames (Δt): 10 s.

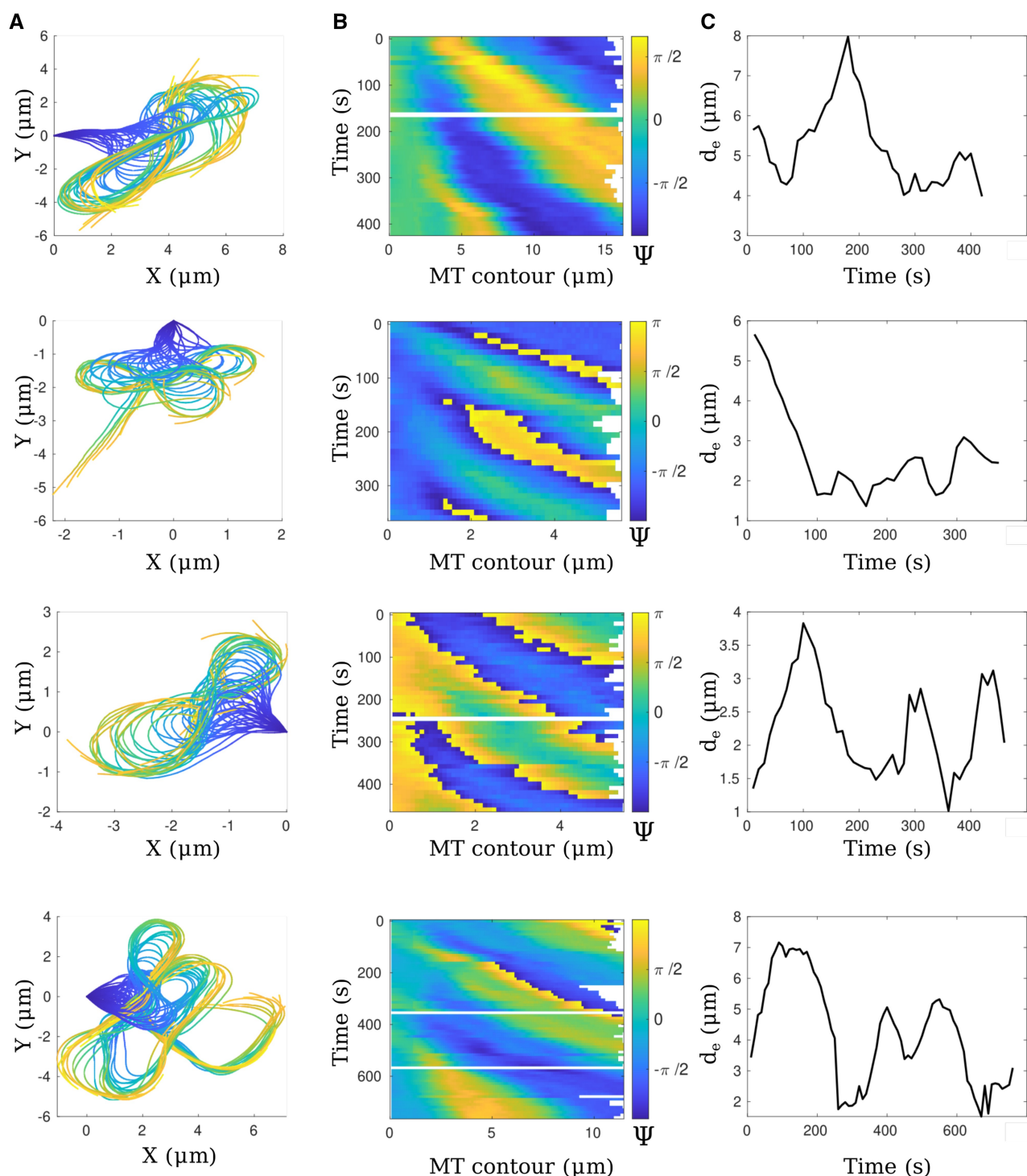


Figure 7. Contour analysis in time of representative experimental filaments. Four filaments showing beating mobility patterns are shown with (A) the montage of the skeletal line, coded along its length (fixed-tip to end), (B) a 2D plot of tangent angle along the microtubule length (μm) with colorbar representing the angle in radians and (C) the end to end distance (y -axis) plotted with time in seconds (x -axis)

demonstrate that it outputs a smooth contour (Fig. 6A, Video 1). The time-resolved data of filament dynamics projected demonstrates the wave-like oscillations (Fig. 6B). We test the tool on multiple such time series, resulting in correct segmentation of experimental inputs, as seen in time-projected contours (Fig. 7A). Since the wave-like propagation is flagella-like, we use a similar measure to quantify the dynamics in terms of the local tangent angle (Ψ) dynamics along the filament length in a kymograph and find the curvature to

propagate base-to-tip in a periodic manner (Fig. 7B). We also plot the distance between the filament ends (d_e) in order to examine whether the angular dynamics are reflected in tip position movement. The periodic change in the end-to-end distance mirrors the periodic angular movement of the filament (Fig. 7C). The oscillatory period from peak-to-peak in both angular plots and the end-to-end distance is a few millihertz, similar to what we had previously reported for clamped MTs in dynein gliding assays (Yadav *et al.* 2024). This low

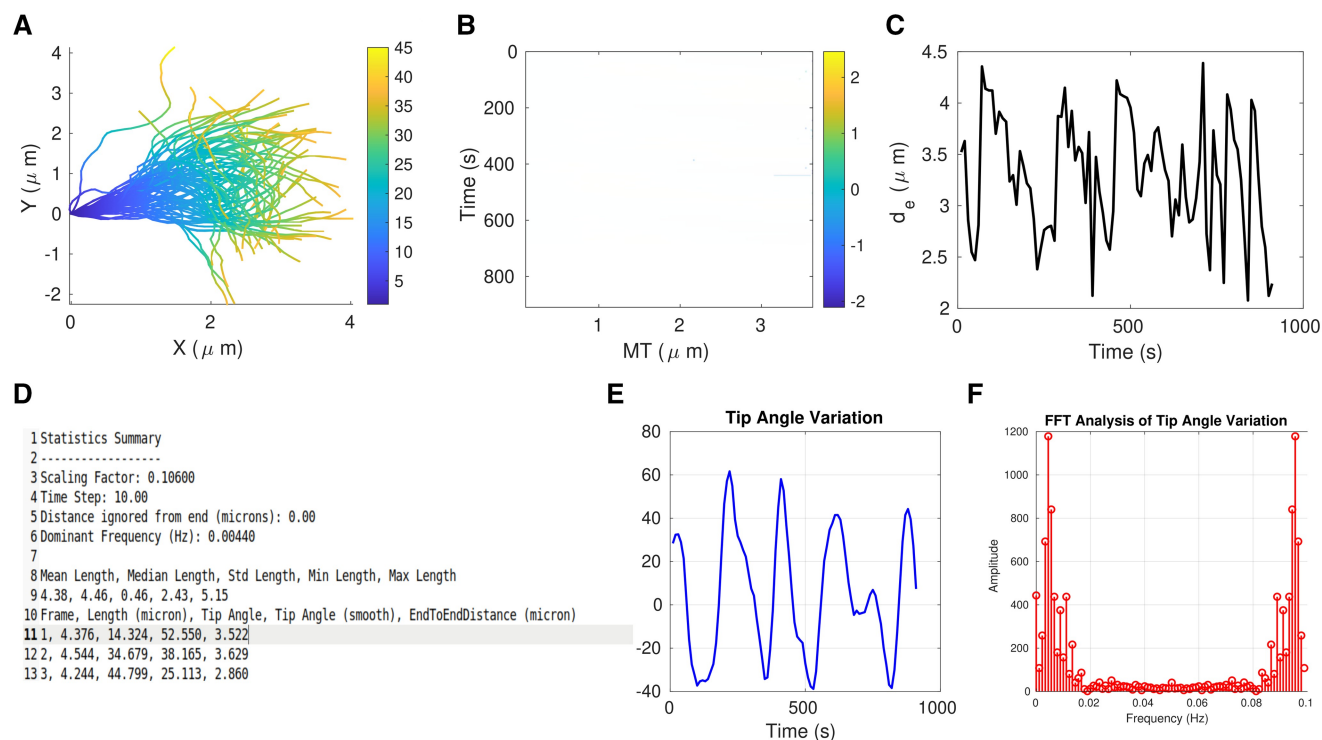


Figure 8. KnotResolver outputs. (A) The time-projected contours detected by KnotResolver of a tracked filament is plotted. Colorbar: Map of position from the immobile (blue) to the free end (yellow). (B) The kymograph of the tangent angle (color) with time (y-axis) and position along the contour (x-axis). Colorbar: angle in radians. (C) End-to-end distance between the fixed tip and the free end of the pinned filament (y-axis) plotted as a function of time (x-axis). (D) The text file output (*statsSummary.txt*) provides a summary of input parameters followed by frame-wise information about (left to right) frame number, filament length, tip angle, and the end-to-end distance. (E) The tip angle plotted with time is used to calculate (F) the power spectrum by FFT analysis (using the MATLAB command *fft*) of tip-angle dynamics. The graph represents the amplitude (y-axis) as a function of frequency (x-axis)

frequency oscillation is also consistent with theoretical expectations based on filament bending rigidity and motor mechanics. The tool results in multiple plots (Table S2) and a text file with frame-wise statistics of tracked filaments (Fig. 8). This can be used to further analyse biologically meaningful properties like filament rigidity and time-dependence of the dynamics and their relation to motor-filament interactions.

Thus, we demonstrate the utility of the directed graph based pipeline KnotResolver in automated quantification of filament time series that could be used for other systems.

4 Discussion

Here, we have demonstrated the limitations of existing methods and softwares to detect and track self-intersecting filaments in microscopy time series. We describe a computational image-processing pipeline, KnotResolver, based on intersection detection, mapping to directed graphs, contour tracing in two steps and distance minimization to a template. We demonstrate that this approach results in sub-pixel position detection and high Dice scores in identification, robust to image noise. In comparison to existing tools, we demonstrate KnotResolver overcomes their limitations and when applied to experimental fluorescence microscopy time-series images of a “gliding assay” where the filament end is clamped, can allow us to automatically quantify geometry and frequency of oscillations.

Similar to many such software developed, KnotResolver has some parameters that need to be user input (Table S1). These are optimized for MTs in a dynein gliding assay with pinned filaments. A further test of the utility of this code

could be to analyse time series in more complex backgrounds such as inside cells where compressive forces result in buckling instabilities seen in MT filaments (Brangwynne *et al.* 2006). We also expect that actin-based gliding assays with typically bent filament with complex curvature, due to the lower persistence length compared to MTs (Gittes *et al.* 1993), could also be tracked using this tool. Indeed surface-defects in myosin-driven actin gliding assays have been previously reported to also result in qualitatively comparable structures (Bourdieu *et al.* 1995). We expect the use of KnotResolver to extend to multiple cytoskeletal filament analysis.

In conclusion, our OpenSource computational image analysis tool that uses a novel directed graph-based mapping to resolve self-intersections of cytoskeletal filaments from both simulated and experimental data could be useful to automate the segmentation and tracking of “gliding assays” with complex geometries, but also flagellar dynamics and possibly filament oscillations in cells.

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Prince Sah was involved in the early stages of the project.

Author contributions

Dhruv Khatri wrote the software and performed the validation. He also wrote the initial draft and made most of the figures. Shivani A. Yadav performed the experiments, wrote parts of the methods section, and made some of the figures. CAA conceptualized the project, provided resources, wrote

and edited the manuscript, and was responsible for supervision, project administration, and funding acquisition.

Supplementary data

Supplementary data are available at *Bioinformatics* online.

Conflict of interest

None declared.

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Data availability statement

The code for the filament bending analysis has been made OpenSource and is available at the Github URL <https://github.com/CyCelsLab/MTKnotResolver>

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RESEARCH ARTICLE



Collective dynein transport of the nucleus by pulling on astral microtubules during *Saccharomyces cerevisiae* mitosis

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Abstract

Positioning the nucleus at the bud neck during *Saccharomyces cerevisiae* mitosis involves pulling forces of cytoplasmic dynein localized in the daughter cell. Although genetic analysis has revealed a complex network positioning the nucleus, quantification of the forces acting on the nucleus and the number of dyneins driving the process has remained difficult. To better understand the collective forces involved in nuclear positioning, we compare a model of dyneins-driven microtubule (MT) pulling, MT pushing, and cytoplasmic drag to experiments. During *S. cerevisiae* mitosis, MTs interacting with the cortex nucleated by the daughter spindle pole body (SPB) (SPB-D) are longer than the mother SPB (SPB-M), increasing further during spindle elongation in anaphase. Interphasic SPB mobility is effectively diffusive, while the mitotic mobility is directed. By optimizing a computational model of the mobility of the nucleus due to diffusion and MTs pushing at the cell membrane to experiment, we estimate the viscosity governing the drag force on nuclei during positioning. A force balance model of mitotic SPB mobility compared to experimental mobility suggests that even one or two dynein dimers are sufficient to move the nucleus in the bud neck. Using stochastic computer simulations of a budding cell, we find that punctate dynein localization can generate sufficient force to reel in the nucleus to the bud neck. Compared to uniform motor localization, puncta involve fewer motors suggesting a functional role for motor clustering. Stochastic simulations also suggest that a higher number of force generators than predicted by force balance may be required to ensure the robustness of spindle positioning.

Take Away

- *Saccharomyces cerevisiae* cytoplasmic viscosity is estimated by quantifying the in vivo mobility of the nucleus and comparing to simulations.
- The in vivo length dynamics of microtubules (MTs) interacting with daughter bud cortex during nuclear positioning in mitosis are measured.
- We developed a model of astral MT interactions with cortical dynein in the process of nuclear positioning during mitosis.
- A force balance model predicts the minimal force required for nuclear positioning.

- In simulations, the spatial distribution of dynein appears affects nuclear positioning.

KEYWORDSdiffusion, dynein, mechanics, microtubule, nucleus, *Saccharomyces cerevisiae*, SPB, viscosity

1 | INTRODUCTION

Centrosomes nucleate radial microtubule (MT) arrays or asters that are vital for mitotic spindles and maintaining cell geometry in interphase in animal cells. A structure comparable to centrosomes, the spindle pole body (SPB), has been identified in the model budding yeast *Saccharomyces cerevisiae* (Seybold & Schiebel, 2013) consisting of centrins and γ -tubulin (Schiebel & Bornens, 1995) and regulating nuclear and cytoplasmic MT (cMT) nucleation and number (Knop et al., 1999). During mitosis astral or cMTs play a vital role in positioning the nucleus by asymmetric pulling forces, but unlike in higher eukaryotes, the asymmetry appears to be generated by the differential transport of force generators like dynein into the daughter cell (Chen et al., 2019; Markus et al., 2012a). Once localized, dynein interacts with the astral MTs and position the spindle at the during nuclear positioning and spindle elongation in *S. cerevisiae* mitosis (Adames & Cooper, 2000; Baumgärtner & Tolić, 2014; Carminati & Stearns, 1997). Evidence has accumulated for an “offloading model” of dynein transport in an inactive form to the plus ends of astral MTs before it binds the cortex (Lee et al., 2005; Markus & Lee, 2011). In turn, a protein She1 inhibits dynein movement selectively in the mother cell by resulting in asymmetric pulling forces into the daughter cell (Markus et al., 2012b). In the daughter cell, cortical dynein both walks on MTs and destabilizes them (Estrem et al., 2017). This role of cortical dyneins pulling on cMTs to position spindles has also been well studied in the *Caenorhabditis elegans* embryos (Grill et al., 2001) and human cells (Kiyomitsu & Cheeseman, 2013), while in *Schizosaccharomyces pombe*, the combined effect of MT lengths and motor numbers results in nuclear oscillations (Ding et al., 1998; Svoboda et al., 1995; Yamamoto et al., 2001). The orientation of spindles in animal cells has been shown to be mechanically regulated by forces acting through the number and length of astral MTs interacting with dyneins localized at the cell cortex (Grill et al., 2001; Kotak et al., 2012; O'Connell & Wang, 2000). Models proposed for spindle positioning suggest that MTs interact with cortical dyneins either (i) end-on (Kozlowski et al., 2007) or (ii) laterally (Adames & Cooper, 2000). Indeed, it appears an end-on interactions transition to lateral ones and might be necessary for multiple motors to act on astral MTs and generate sufficient force to position a spindle or nucleus. Together, these form an increasingly complex picture of dynein and cMTs in spindle positioning during budding yeast mitosis. Despite the progress in imaging dynamics of the process (Fees et al., 2017; Lammers & Markus, 2015), quantification of the forces driving nuclear positioning remains incomplete. Therefore, there exists a need for a detailed mechanical model reconciled to experiments.

The *S. cerevisiae* dynein has become a model for understanding dyneins generally, due to advances from single-molecule dynamics demonstrating a high degree of processivity (Reck-Peterson et al., 2006) that has been attributed to tension dependence of coordinated stepping of pairs of heads (Qiu et al., 2012). A clear picture of single-molecule coordination has been emerging from the demonstration of force-dependent bidirectional stepping of dynein (Cleary et al., 2014) and load sharing between heads (Belyy et al., 2014), and most interestingly an asymmetry in detachment of the motor from MTs depending on the direction of the load force (Nicholas et al., 2015). The potential connection between these single-molecule properties and collective transport of MTs was recently demonstrated in our study combining experiment and simulations, with ensembles in excess of 8 to 10 motors resulting in cooperative transport, explained by the asymmetry in load dependent detachment rates of the single motor (Jain et al., 2019). However, the implications of these in vitro and in silico observations for the precise number of dynein molecules in vivo remain unclear. Thus, a mathematical model reconciled to experimental data from *S. cerevisiae* could provide a clearer picture of the nature and magnitude of the forces involved.

The mobility of organelles through the cytoplasm is expected to be dominated by viscosity, due to the low Reynolds number of organelle movement. Estimations of viscosity of budding yeast cells have ranged from 0.3 to 0.7 Pa s (Baumgärtner & Tolić, 2014). In contrast, the value of the fluid phase viscosity using electron spin resonance (ESR) spectroscopy in the fungus *Talaromyces maritimus* ranged from 2.5 to 15×10^{-3} Pa s, while in *Penicillium* sp. ranged between 3.5 and 4.8×10^{-3} Pa s, depending on the developmental stage (Dijksterhuis et al., 2007). In addition, the estimation of intracellular viscosity in cell culture cells typically estimates microviscosity or fluid phase viscosity, which for cytoplasm is reported to be only 1.2 to 1.4×10^{-3} Pa s (Fushimi & Verkman, 1991; Seksek et al., 1997). However, larger objects such as organelles or micron-scale particles that encounter intracellular structures and other organelles could experience a higher effective viscosity, which can even reach values as high as 2 Pa s (Bacher et al., 2004). This object size dependence of cytoplasmic effective viscosity has been attributed to a “sieving” effect in the cytoplasm (Mika et al., 2010; Seksek et al., 1997). It also implies a scale-specific method of estimation of the drag force acting on nuclei will be required.

MTs nucleated from centrosomes or SPBs that do not form part of the spindle are referred to as astral or cMTs. They contact the cell cortex, experience pulling forces, and determine the division plane. The first stage of this is reminiscent of “search and capture” of dynamic MTs seen during spindle assembly (Holy & Leibler, 1994). In

large cells, additional directional cues such as gradients of regulatory proteins that affect MT growth dynamics in *Xenopus* oocyte meiosis II (Athale et al., 2008, 2014) or motor gradients in mouse oocyte meiosis I (Khetan & Athale, 2016) are also thought to play a role. Once captured, cortical motors generate pulling forces on cMTs as seen in early embryonic divisions of *C. elegans* (Grill et al., 2001; Kozłowski et al., 2007). In yeast cells, in addition to these cortical interactions, the “capture” of MTs by motors is also thought to be followed by their depolymerization (Adames & Cooper, 2000). Such depolymerization while walking on the MTs could compensate for an important source of mechanics in spindle positioning based on MT pushing by polymerization seen in vitro (Dogterom et al., 2005; Dogterom & Yurke, 1997). The most recent modeling study of nuclear positioning in yeast based on MT-motor mechanics is limited by the fact of continuum assumptions about motors and MTs (Sutradhar et al., 2015), while the number of motors and MTs seen in experiment is small in number and likely to be subject to fluctuations. This suggests that a model is required that takes into account individual MTs and single motor mechanics for an improved understanding of the biophysical basis of nuclear positioning in *S. cerevisiae* mitosis.

Here, we have used a video microscopy approach to analyze time series of *S. cerevisiae* nuclei by single particle tracking (SPT) of fluorescently labeled SPBs. The mobility of pre-budding cells appears to be random, with both diffusion and MT pushing playing a role. We develop a model of nuclear mobility driven by dynamic MTs pushing on the cell membrane and diffusion and estimate the effective cytoplasmic viscosity, by reconciling theory with experiment. Based on this quantification, we then examine intracellular SPBs mobility during budding, as the nucleus is pulled into the bud neck. We model the process using a minimal geometry and based on force balance and direct simulations (Data S2), estimating the collective force driving nuclear positioning, by comparison to experiment.

2 | MATERIALS AND METHODS

2.1 | Yeast growth conditions and live imaging

Yeast strains expressing either a GFP-tagged copy of the SPB protein Spc42 or a dual-labeled strain with Spc42 tagged with mCherry and GFP-Tub1 (Caydasi & Pereira, 2009) were used (Table 1). The methods of yeast culture and growth media follow a previous study

(Sherman, 1991). Log phase cells were cultured in filter-sterilized yeast peptone dextrose medium containing 0.1-mg/L adenine (YPDA) medium at 30°C for time-lapse imaging. Time-lapse movies were acquired for 2 to 3 h at 30°C under temperature controlled incubation chamber, and yeast cells were immobilized on CellASIC microfluidic yeast plates (Y04C size) with a constant flow of YPDA medium for 3 h. Time-lapse movies were acquired using the spinning disk confocal microscope (Andor Revolution XD imaging system, equipped with a 100 times oil immersion 1.45 NA Nikon Plan Apo lambda objective (Nikon Corp., Japan), a Confocal Yokogawa CSU-X1 unit, an Andor sCMOS ZYLA detector, and Andor iQ software (Oxford Instruments, UK). Z-stacks comprised fifteen 0.5-μm spaced slices were generated for GFP-Tub1 and Spc42-3xmCherry at 1-min intervals.

2.2 | Image analysis

Image time series of yeast cells were contrast adjusted, denoised using a median filter (radius=1 pixel), and histogram normalized using Image J ver 1.45 s (Schneider et al., 2012) with the *Stackreg* plugin to align stacks of images that underwent drift during acquisition. The channel with the SPB is labeled by Spc42-3xmCherry. Spindle length and laterally interacting MT lengths were measured using measure function of ImageJ. In order to virtually synchronize MT dynamics, the spindle length (L) during elongation was fit to a modified Hill function:

$$L = L_{min} + (L_{max} - L_{min}) \cdot \frac{(R_{max} \cdot t)^n}{(\tau^n + t^n)}, \quad (1)$$

where L_{max} and L_{min} are maximum and minimum spindle lengths, R_{max} is the maximum spindle elongation rate, τ is the time at which spindle length is half maximal, and n is the Hill coefficient. The fit value of τ obtained from fitting each cells individual dynamics (Figure S1a,b) was used to rescale the time axis (Figure S1c) with -10 min the threshold time for determining pre-anaphase ($t < -10$ min) anaphase ($t > -10$ min) (Figure S1c), which ranged from 3 to 20 in the data fit. The image time series of dual labeled cells were tracked for the mCherry labeled SPBs using a SPT program FIESTA (Ruhnow et al., 2011) implemented in MATLAB R2020a (Mathworks Inc., MA, USA), with a threshold search velocity of 50 nm/s and a minimal frame persistence of 15 min. The XY coordinates of the tracks were plotted and overlaid on the DIC channel used to visualize and categorize cells into either pre-budding or budding stages. SPB movement in the budding stages was analyzed using tracks that were at least 50 min (frames) long.

2.3 | Simulations

Two-dimensional simulations were performed using Cytosim, a C++ discrete particle stochastic dynamics simulation engine (Nedelec & Foethke, 2007) that we use to model discrete filaments, their stochastic fluctuations, stochastic motor binding, Brownian diffusion, and the mechanics that generates forces originating from the

TABLE 1 Strain list: The list of yeast strains used in this study

Strain name	Genotype
ESM 616 GFP:Spc42	ESM 356-1-Spc42-GFP-KanMX6
AKY099 ESM 356-1 mCherry:Spc42, GFP:α-tubulin	Transformed with pAFS125-GFP-TUB1 to integrate into URA3 locus.
	Spc42-mCherry-with-spacer-KanMX6

Note: These strains have been described in a previous study by Caydasi and Pereira (2009)).

Hookean spring model of motors and bending mechanics of MTs. The cell is modeled as an ellipse in interphase and during mitosis a second ellipse added to represent the budding daughter cell, connected to the mother cell by a bud neck with rigid boundaries. The daughter cell size is maintained constant during the simulation because the process we simulate is shorter than the total time of budding. The nucleus is modeled as an ellipse and initialized at the center of the mother cell with the bud. SPBs are embedded on the nuclear envelope with only one SPB at interphase and two diametrically opposite SPBs at mitosis, with details of model parameters described in Table 2. Each SPB nucleates two MTs that undergo dynamic instability modeled by the two state-four parameter model (Verde et al., 1992). Dynamic instability parameters are taken from primarily two papers that quantify dynamics in a cell-cycle stage specific manner (Table 3). The system components are confined in the cellular space. The dynein motors are localized at the cortex along the arc of the ellipse as chosen arbitrarily to mimic the curvature of the bud. The integration time was chosen to be smaller than the fastest timescale (Table 2). In the pre-budding cells, the cytoplasmic viscosity was varied and the difference in mean simulation outputs compared to the same metric from experimental quantification were rank sorted, based on the minimization of deviation between experiment and simulations. This validation from experiments was used to estimate the optimal effective cytoplasmic viscosity η_{cyto} that was then used for budding calculations.

A typical simulations run required approximately 20 min on a 12 core Intel Xeon machine with 16-GB RAM for total time 20 min, time step Δt of 10^{-3} s and a configuration involving one nucleus, an SPB nucleating a pair of MTs and up to 200 motors.

2.4 | MSD and diffusivity analysis of trajectories

We have used a standard method of MSD calculations as previously described (Arcizet et al., 2008; Athale et al., 2014; Khetan & Athale, 2016) based on the 2-D positional coordinate of the filament (r) using a sliding-window approach of increasing time intervals (δt) based on the expression:

$$MSD(\delta t) = \frac{1}{N} \sum [r(t) - r(t + \delta t)]^2_{t \leq \frac{3T}{4}}, \quad (2)$$

where $N = T/\delta t$ is the number of steps of a given time interval, t is the time point, and T is the total time. The time series was analyzed only for values of δt less than or equal to $0.75 \times T$, based on previous reports (Arcizet et al., 2008; Jain et al., 2019; Michalet, 2010), and MSD was plotted as a function of δt . We fit two models of effective diffusion to the MSD data from both experiment and simulation: (a) anomalous diffusion and (b) diffusion with drift. The (a) anomalous diffusion model is described by the expression:

TABLE 2 Simulation parameters determining cell geometry, viscosity, MT rigidity, and motor density and distribution

Symbol	Description	Stage	Value	Reference
d_{nuc}	Nuclear diameter	Pre-budded	1 μm	This study
	Long axis	Budded	1.75 μm	This study
d_{cell}	Cell diameter along long and short axes	Pre-budded	6 and 5 μm	This study
		Budded	8 and 5 μm	This study
n_{SPB}	Number of SPBs per cell	Pre-budded	1	This study
		Budded	2	This study
n_{MT}	Number of MTs per SPB	Pre-budded and budded	2	This study
η_{cyto}	Yeast cytoplasmic viscosity	Optimized	$5 \cdot 10^{-3}$ to $5 \cdot 10^1$ Pa s	This study
K	MT bending modulus and measure of flexural rigidity	Pre-budded and budded	20 N/m ²	Gittes et al. (1993)
$\rho_{m(2D)}$	2-D dynein motor density at the cortex	Uniform	3, 6, 9, 12, and 15 motors per micrometer	This study
p	Number of dynein punctae per daughter cell	Localized on the daughter cell cortex	4 and 13	This study
N_m/p	Number of motors per puncta	Punctate	1, 2, and 3	This study

Abbreviations: MT, microtubule; SPB, spindle pole body.

TABLE 3 MT dynamic instability parameters used in simulations of both pre-budding and budding cells

Cell stage	v_g	v_s	f_c	f_r	Reference
Pre-bud	0.009 $\mu\text{m/s}$	0.03 ($\mu\text{m/s}$)	0.007 per second	0 per second	Carminati and Stearns (1997)
Pre-anaphase	0.03 $\mu\text{m/s}$	0.05 ($\mu\text{m/s}$)	0.023 per second	0.03 per second	Fees et al. (2017)

Abbreviation: MT, microtubule.

$$MSD = 4 \cdot D_{eff} \cdot t^\alpha + c_1, \quad (3)$$

where D_{eff} is the effective diffusion coefficient, t is time, α is the parameter of anomalous diffusion, and c_1 is the error in detection. (b) The diffusion with drift model is given by the following:

$$MSD = 4 \cdot D'_{eff} \cdot t + (v_{drift} \cdot t)^2 + c_2, \quad (4)$$

where D'_{eff} is the alternative estimate of the effective diffusion coefficient, t is time, v_{drift} is the drift velocity, and c_2 is the error in detection.

3 | RESULTS AND DISCUSSION

3.1 | Mitotic spindle positioning, elongation, and astral MT length dynamics

The dynamics of spindle growth and positioning in dividing *S. cerevisiae* during mitosis was followed by video microscopy, using a GFP labeled tubulin strain described previously (Caydasi & Pereira, 2009) (Table 1). Consistent with previous reports, we observe spindle movement toward the mother-daughter bud neck. In the initial phase, the spindle is of a fixed length, corresponding to pre-anaphase. Subsequently,

spindle elongation follows, corresponding to anaphase (Figure 1 and Video S1). The astral MTs nucleated from the SPB also show fluctuations in lengths and appear to contact the budding daughter cell cortex. Quantification of the spindle elongation dynamics suggests that it is completed in ~ 15 min (Figure 2a). By fitting the spindle elongation from multiple cells in different fields of view to a modified Hill function, we determine a half-maximal time of elongation in order to compare across samples (Figure S1a–c), as well as estimate the mean length of pre-anaphase spindles to be $1.48 \pm 0.23 \mu\text{m}$, while those in anaphase are $6.49 \pm 0.37 \mu\text{m}$ (Figure S1d). The length of astral MTs interacting with the cortex increases during and after anaphase spindle elongation an effect seen more dramatically in the daughter cell (Figure 2b and less prominently in the mother cell (Figure 2c. This is also seen in the statistics of cortical MT interaction lengths comparing pre-anaphase to anaphase stages between mother and daughter cells (Figure 2d. As a result, we expect MTs of length between 1 and $2 \mu\text{m}$ to interact with the daughter cortex pre-anaphase are sufficient to move the nucleus to the bud neck prior to spindle elongation. This raises the question about the nature and magnitude of the forces generated and whether they are sufficient in moving the nucleus. Based on previous work, we assume that this phase of nuclear movement is driven by pulling forces generated by cortical dynein binding to and pulling on astral MTs. The motion of the nucleus is expected to be opposed by cytoplasmic drag. Thus, in order to quantify the mechanics

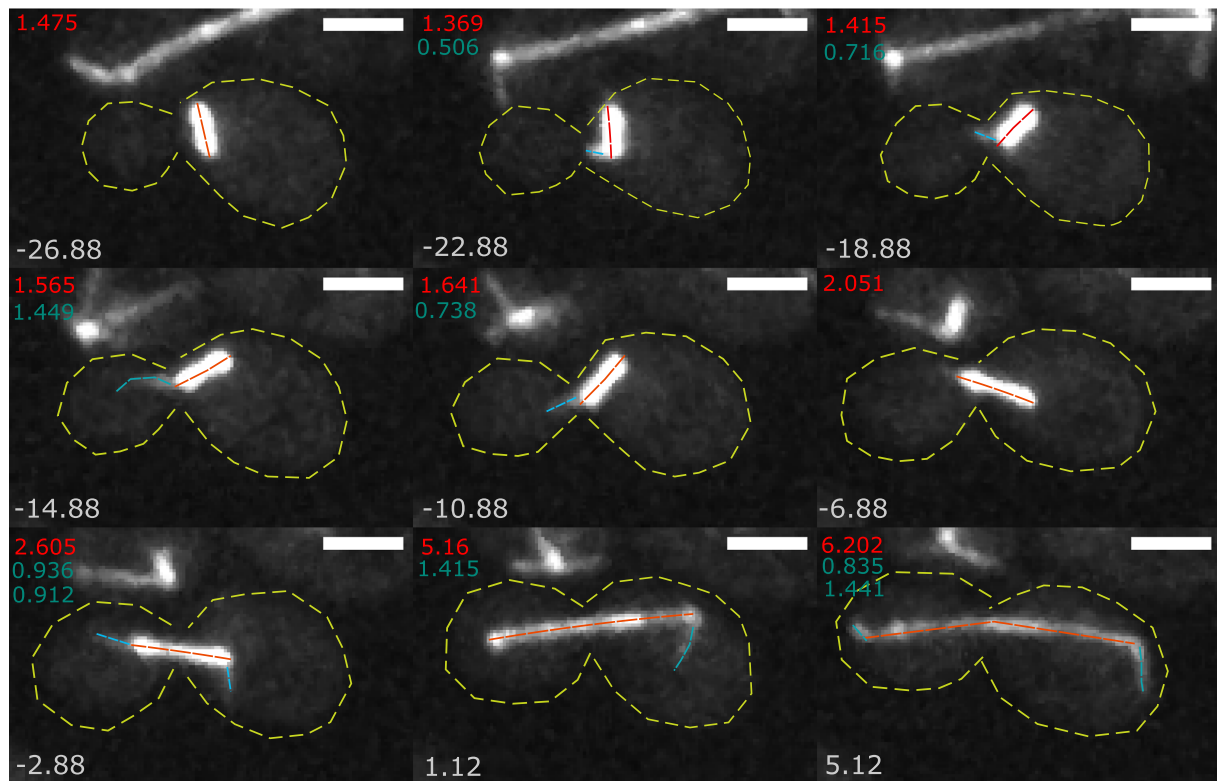


FIGURE 1 Tracking spindle elongation and microtubule (MT) length dynamics. A representative time series of a *Saccharomyces cerevisiae* cell with GFP-Tub1 (gray) labeling both spindle and spindle pole body (SPB) nucleated astral MTs corresponding to Video S1. The spindle lengths (red line) and astral MTs (cyan) were interactively tracked with the lengths in micrometers. The cell outline is marked by a yellow dashed line. The time in minutes is normalized with $t = 0$ corresponding to the mid-phase of anaphasic spindle elongation. Scale bar: $2 \mu\text{m}$

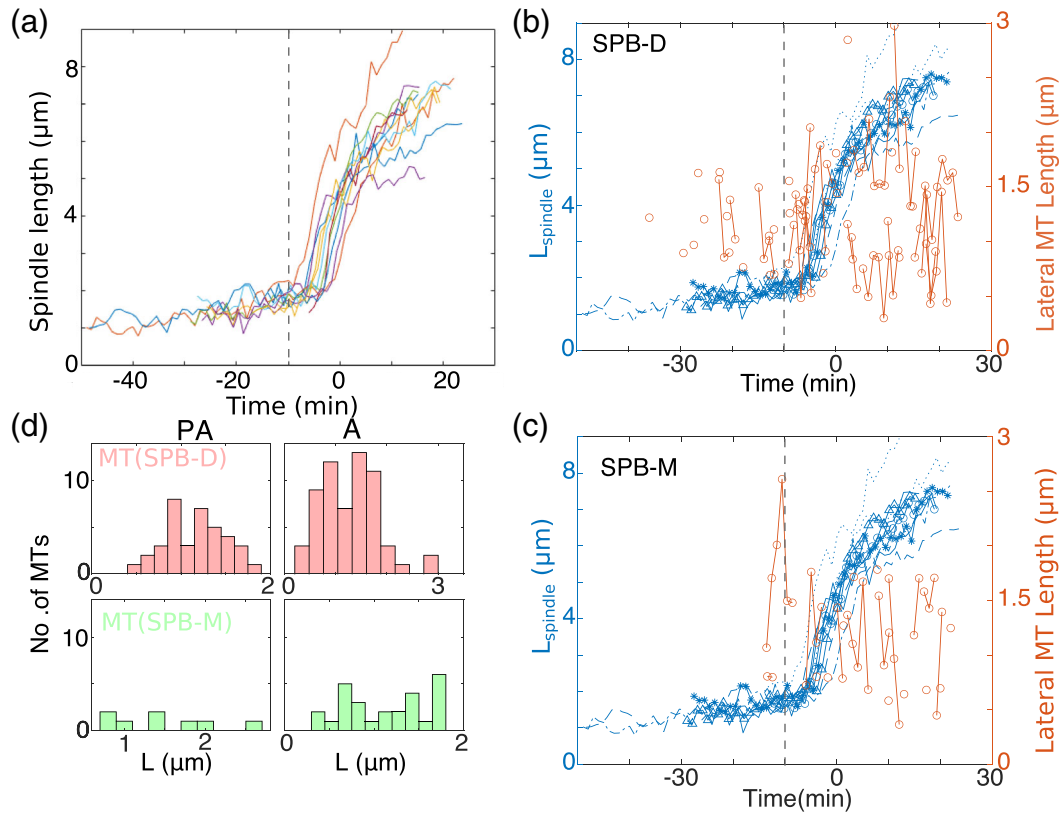


FIGURE 2 Anaphase and pre-anaphase dynamics of spindle and astral microtubules (MTs). (a) Individual spindle lengths were rescaled by the half-maximal time of spindle elongation by fitting a min–max scaled function and, to virtually “synchronize” the cells into anaphase (spindle elongation) and pre-anaphase stages. (b and c) The spindle length dynamics (blue) are superimposed on a graph of the time-dependent lengths of lateral interaction of MTs (red) in the (b) daughter cell and (c) mother cell. (d) The frequency distributions of MT lengths laterally interacting with the cortex during PA: pre-anaphase and A: anaphase in daughter (pink) and mother (green) cells are plotted. $n = 11$ cells

of movement, as a first step we proceed to measure the viscosity of the cytoplasm, that will allow us to estimate the force opposing the movement of the nucleus.

3.2 | Estimating cytoplasmic viscosity by fitting a nuclear mobility model to experiment

In order to measure nuclear mobility and use that to infer cytoplasmic viscosity, we followed the dynamics of the fluorescently tagged SPB (Spc42-3xmCherry) (Caydasi & Pereira, 2009), because it produces a point signal in time series microscopy. By SPT of the signal in the mCherry Video S2 and Figure 3a), we obtain the XY trajectories of up to 20 SPBs. A qualitative view of origin normalized SPB trajectories suggests that they resemble a random walk exploring cytoplasmic space (Figure 3b).

We estimated the MSD values for increasing time intervals from experimental trajectories and to account for potential boundary “corralling” and MT dependent pushing effects, fit the data with a model of anomalous diffusion (Equation 3) based on previous evidence of the utility of such an approach (Athale et al., 2014). The resulting fit value of effective diffusion coefficient D_{eff} was

$0.0565 \pm 0.09 \mu\text{m}^2/\text{min}$ and of the coefficient of anomaly of diffusion α was 1.172 ± 1.3 (mean $\pm$ SD) for SPBs in pre-budded cells (Figure 3c). The value of $\alpha \approx 1$ is suggestive of diffusive motility. The angular frequency distribution of the angles of SPB with the positive x axis shows a wide distribution suggesting rotational diffusion (Figure 3d). The change in angles $d\theta$ is relatively tight (Figure S2).

In previous work, drag forces have been successfully estimated by measuring the viscosity of the cytoplasm by microrheology of vesicle (Hayashi et al., 2013) and lipid granule mobility (Grill et al., 2001). However, nuclear mobility is determined not only by thermal forces opposed to drag but also forces due to MT pushing at the boundary. In order to account for both effects, we use a numerical simulation of the factors affecting nuclear mobility and compare the statistics to experimental measures.

We simulate the well parameterized model of MT mechanics, stochastic length dynamics and diffusion in Cytosim (Nedelec & Foethke, 2007) and minimize the difference between experimental SPB mobility statistics and simulation. The nucleus is modeled to have a single SPB embedded in the periphery, nucleating a pair of dynamic MTs based on the factors driving pre-budding nuclear mobility (Figure 3e). The parameters of cell and nuclear sizes, MT dynamics, and flexural rigidity of the MTs are all taken from literature (Table 2).

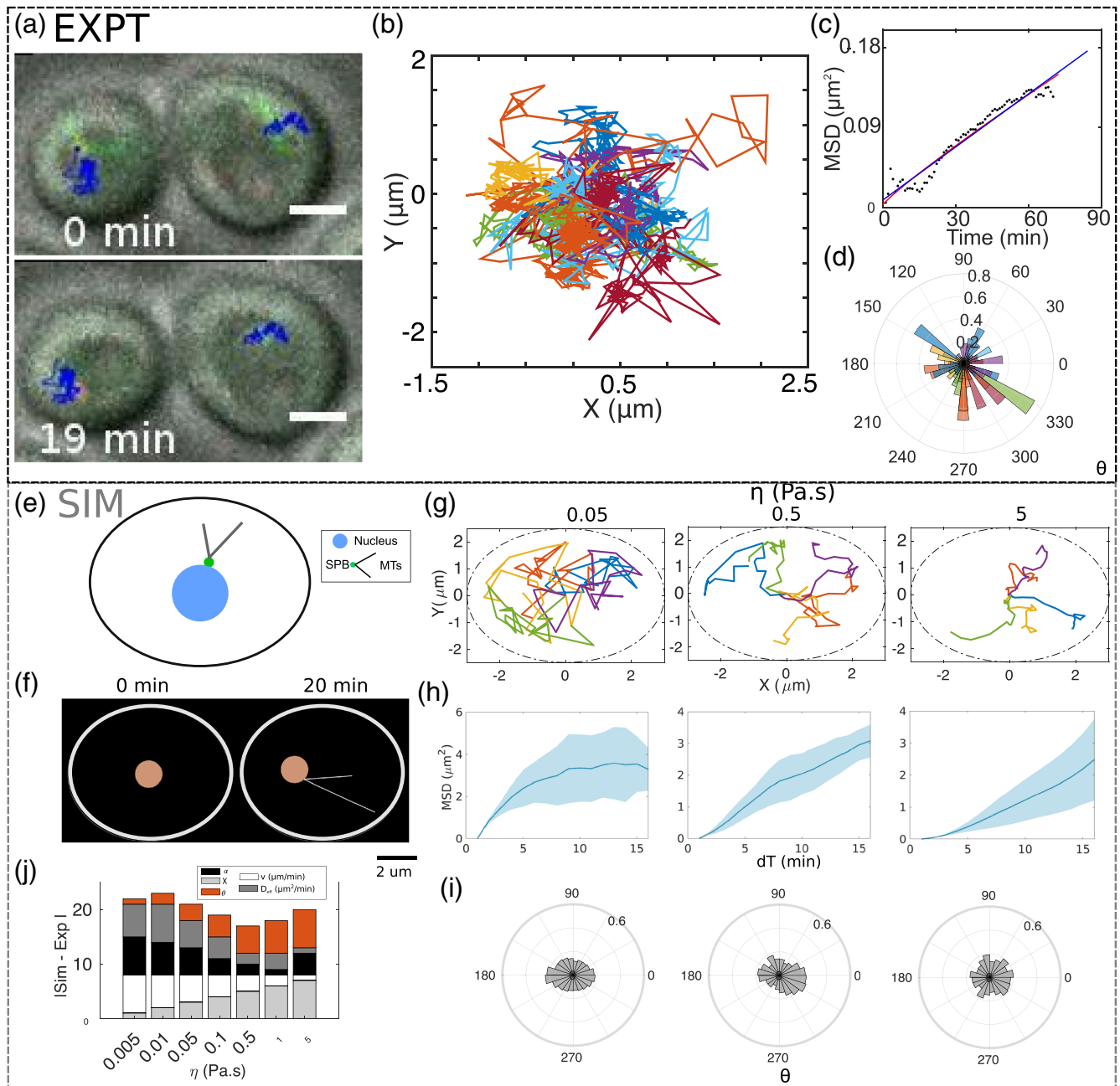


FIGURE 3 Estimating cytoplasmic viscosity from spindle pole body (SPB) mobility comparing simulations to experiments. (a) Representative image of yeast cells in the pre-budding stage in DIC (gray) are overlaid with the Spc42-3xmCherry (red) and GFP-Tub1 (green) channels (corresponding to Video S2) and trajectories of SPB mobility (blue). $t = 0$ (start), 19 min (end), and scale bar 2 μm . (b) Tracks of multiple SPBs ($n = 20$) are plotted with their origin at 0,0 by deducting the start position from all tracks. (c) The MSD (μm^2) with time (min) and (d) histogram of the angle subtended by each SPB (color) at the origin with the positive x axis are plotted. (e) We model a nucleus (blue) with an SPB (green) embedded in the nuclear periphery nucleating two MTs. (f) Representative simulation outputs at $t = 0$ and 20 min are displayed (corresponding to Video S3). Scale bar 2 μm . (g–i) The effect of increasing viscosity (η) was evaluated in terms of (g) XY trajectories ($n = 5$), (h) MSD averaged over all SPBs (blue) with $\pm\text{SD}$ (light blue area) fit to the anomalous diffusion model (Equation 3), and (i) the frequency distribution of angles subtended by the SPB with the x axis θ . (j) Varying the viscosity η affects the magnitude of the deviation between the experiment and simulation evaluated in terms of α (black), Deff (dark gray), χ (light gray), θ (red), and velocity v (white). Simulation time is 20 min, $n = 50$ runs

Simulations were run for increasing cytoplasmic viscosity (η) from 10 to 2 to 101 Pa s with the nucleus initialized at the cell center and a random orientation of the SPBs and MTs for 20 min (Figure 3f). Given the rigid nature of the cell boundary, growing MTs that contact the

boundary produce an inward force of polymerization acting on the SPBs (Video S3). Translational and rotational diffusion of the nucleus results in a random repositioning of SPBs with additional randomness due to the stochasticity of length fluctuations with the degree

exploration of cellular space by the trajectory reducing with increasing cytoplasmic viscosity η (Figure 3g). Mobility of the SPBs for a low value of viscosity appears to be restricted while that at high viscosities

appears qualitatively to be diffusive (Figure 3h). However, on close examination of the anomaly parameter, we find the movement transitions for low viscosity from restricted motion ($\alpha < 1$) that is diffusion

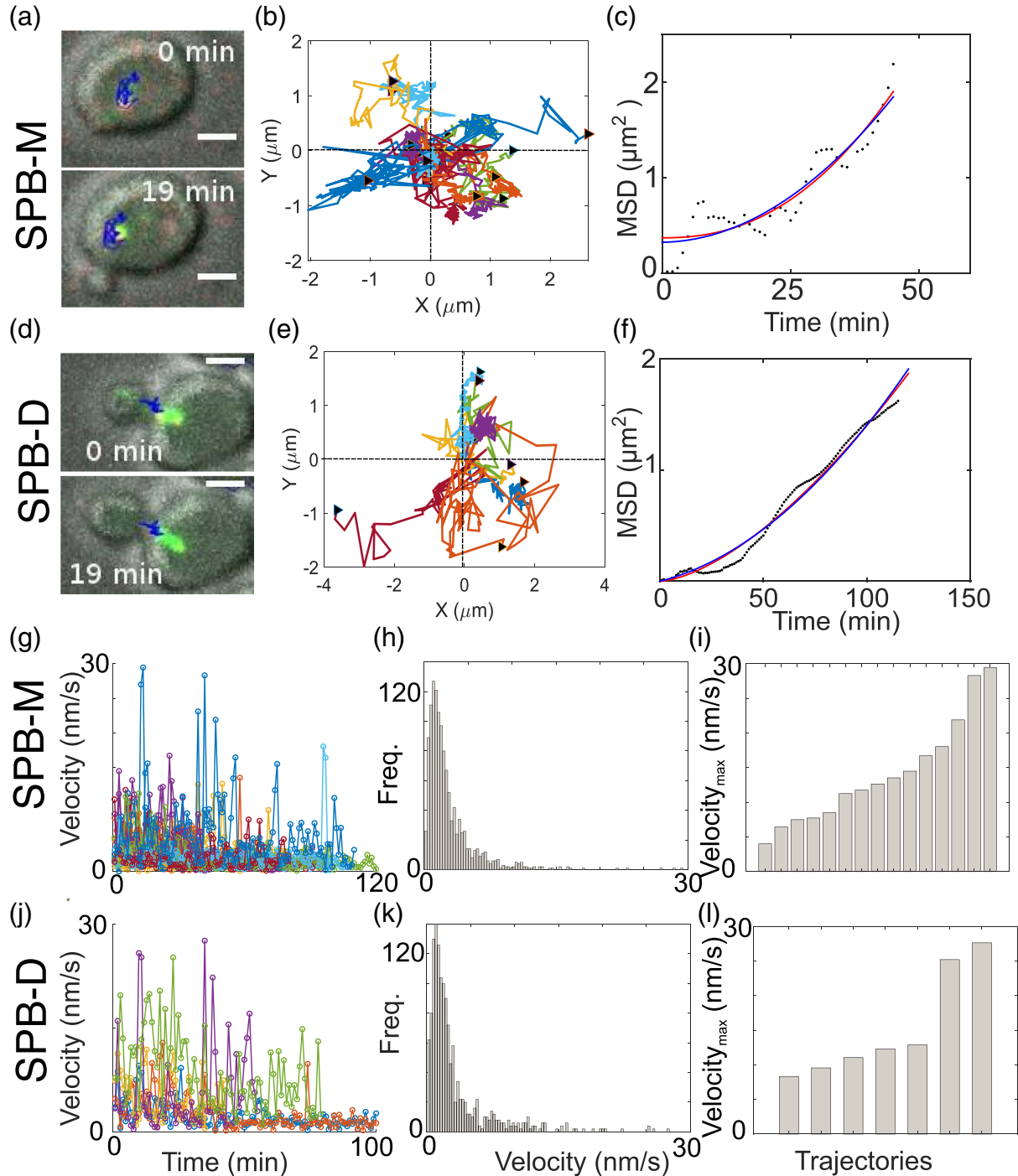


FIGURE 4 SPB mobility during mitosis. (a and d) Representative images of budding cells in DIC (gray) merged with the SPC42-3xmCherry (red) and GFP-Tub1 (green) channels are overlaid with the trajectories of the tracked spindle pole bodies (SPBs) entering the (a) mother cell (SPB-M) and (d) daughter cell (SPB-D). Scale bar 2 μm . These images correspond to Videos S4a,b. (b and e) The XY trajectories of (b) SPB-M ($n = 20$) and (e) SPB-D ($n = 16$) are plotted. Colors represent individual trajectories, Δ marks the end point of each trajectory and the origin marking the start point due to normalization by the respective initial position. (c and f) Representative MSD profiles of SPBs from experiment are plotted with time (dots) for (c) SPB-M and (f) SPB-D and fit to an anomalous diffusion model (red, Equation 3) and diffusion with drift model (blue, Equation 4). SPB-M and SPB-D movement is compared in terms of (g and j) time-dependent frame-wise velocity, (h and k) velocity (nm/s) the frequency distribution, and (i and l) the maximal velocity for each SPB

dominated, through diffusive ($\alpha \sim 1$), to MT pushing dominated for high viscosity, correlating with $\alpha > 1$ (Figure S3a-d).

The “corralling” radius for low viscosity of $\eta = 0.05$ Pa s is ≈ 2 μ m, corresponding to radius along the short axis of the cell (Table 2) and consistent with our expectations. Despite the changes in D_{eff} and α , the SPB velocity shows a consistent decrease with increasing viscosity suggesting diffusion dominated mobility is faster for exploring cellular space than MT pushing. The angle between subsequent positions of the SPB acts as a measure of rotational diffusion of the nucleus (Figure 3i). The values of η were varied and the deviation between simulation and experiment (Data S1) for each of the measured variables $d\theta$ (Figure S2), χ , velocity (Figure S3), D_{eff} , and α (Figure S4) were ranked. The ranks across these measures were summed as a function of η (Figure 3j), arrive at an estimate of 0.5 Pa s as the best match for experiments and taken to be the cytoplasmic viscosity η_{cyto} . Simulations with four instead of two MTs per nucleus also result in an optimized viscosity of 0.5 Pa s (data not shown).

The cytoplasmic viscosity serves to damp random motion of the nucleus in the pre-budding stages, but during mitosis when the nucleus is transported to the bud neck, it is expected to produce an opposing force due to drag. In order to understand the mechanics of the process, we proceed to quantify the in vivo mobility of the nucleus during the budding process, by tracking the SPB.

3.3 | In vivo SPB motility in mitotic cells and minimal force of pulling the nucleus

In order to estimate the forces driving the movement of the nucleus during mitosis, we follow the mobility of the SPBs that move to the diametrically opposite poles of the nucleus (Knop et al., 1997; Seybold & Schiebel, 2013) with one staying in the mother cell (SPB-M) and the other moves to the daughter cell (SPB-D). The time series of punctate localization of Spc42-mCherry that mark the SPBs were tracked in cells that were budding as seen in the DIC channel (Video S4a,b) typically over 15 to 20 min (Figure 4a,b,d,e). The mobility appears to be qualitatively more directional as compared to pre-budding cells due to the qualitative trend in the MSD profile (Figure 4b,e). This is confirmed by the anomaly exponent $\alpha > 1.5$

TABLE 4 Quantification of experimental measures of SPB motility during the budding process for the two SPBs that enter the daughter cell (D) and mother cell (M)

Motility measure	SPB-D	SPB-M
D_{eff}	$1.6 \pm 4 \times 10^{-4} \mu\text{m}^2/\text{s}$	$2.416 \pm 0.3 \times 10^{-4} \mu\text{m}^2/\text{s}$
α	1.96 ± 1.89	1.6 ± 1.7
c_1	$0.589 \pm 1.0983 \mu\text{m}^2$	$0.141 \pm 0.179 \mu\text{m}^2$
D_{eff}^0	$2 \pm 3.5 \times 10^{-5} \mu\text{m}^2/\text{s}$	$2.5 \pm 3.6 \times 10^{-5} \mu\text{m}^2/\text{s}$
v_{drift}	$17 \pm 18 \text{ nm}/\text{min}$	$4.1 \pm 8.4 \text{ nm}/\text{min}$
c_2	$0.5733 \pm 1 \mu\text{m}^2$	$0.252 \pm 0.169 \mu\text{m}^2$
χ	$1.16 \pm 0.55 \times 10^{-1}$	$7 \pm 4 \times 10^{-2}$

Abbreviation: SPB, spindle pole body.

obtained by fitting Equation (3) to the MSD (Figures 4c,f and S4a,b and Table 4). Additionally, the SPB-D appears to migrate with a mean drift velocity of 17 nm/min, while the SPB-M drift velocity is 4.1 nm/min based on fitting the MSD data to the diffusion and drift model (Equation 4 and Figure S4c,d). The very low drift velocity as compared to reported directional velocity result from the heterogeneity in fit values and the time dependence of SPB mobility. Instead of manually separating what might be directed stretches of movement, we attempted to identify maximal velocity values from the time course of velocities of the SPB-D and SPB-M (Figure 4g,j). We find intermittent increases in velocity, resulting in frequency distributions with long tails (Figure 4h,k). The ranked velocities of SPB-M and SPB-D both suggest velocities ranging from 4.5 to 27 nm/s, more comparable to those reported in literature (Marzo et al., 2019; Moore et al., 2009; Omer et al., 2020).

Transitioning of aster motility from random to directed in case of centrosome nucleated asters in *Xenopus* oocyte extracts involve a gradient of MT stabilization that increases length asymmetrically and results in a net force in the direction of transport driven by binding to cytoplasmic dyneins (Athale et al., 2014). In contrast, in mouse oocyte meiosis, a gradient of dynein but uniform MT lengths were seen to bias the positioning of MTOC asters (Khetan & Athale, 2016). However, the size scale of budding yeast cells is a few micrometers, compared to the diameter of vertebrate oocytes ranging between 102 and 103 μ m and involves far fewer MTs, two MTs per SPB, and two SPBs in a dividing cell. Thus, a model that takes into account dynein pulling forces, drag, and MT pushing at the cortex could account for the forces driving nuclear positioning.

We proceed to use a force balance approach to estimate the minimal number of motors required to transport the nucleus, from experimental data. The directional motility of the nucleus during budding is influenced by two broad classes of forces, (a) those that resist transport into the bud: F_M T, due to MT pushing and polymerization at the membrane and F_d , the drag force opposing the motion of the nucleus and (b) those driving the nucleus into the bud: F_m : motor pulling forces (Figure 5a). Therefore, at any stage, the combination of forces acting on the nucleus can be termed as the net force $\vec{F}_{net}$ which we define as the vector sum of the forces, $\vec{F}_{net} = \vec{F}_d + \vec{F}_m + \vec{F}_{MT}$ where $\vec{F}_d$ is the drag force opposing the motion, $\vec{F}_{MT}$ is the force of MT pushing by polymerization into the cell and $\vec{F}_m$ is the motor force pulling the nucleus to the bud. At equilibrium all forces cancel out and $\vec{F}_{net} = 0$. However, in the budding process, the directed motion of the nucleus suggests the dominance of the pulling force. In our simple model, pulling is only due to cortical dynein. The forces opposing it are the drag force due to cytoplasmic viscosity and the force of MTs opposing the movement. We can therefore infer a condition for movement of the nucleus to the daughter cell as follows:

$$F_m > F_d + F_{MT} \quad (5)$$

The movement of the nucleus and the interphase viscosity η_{cyto} allow us to estimate the drag force as follows:

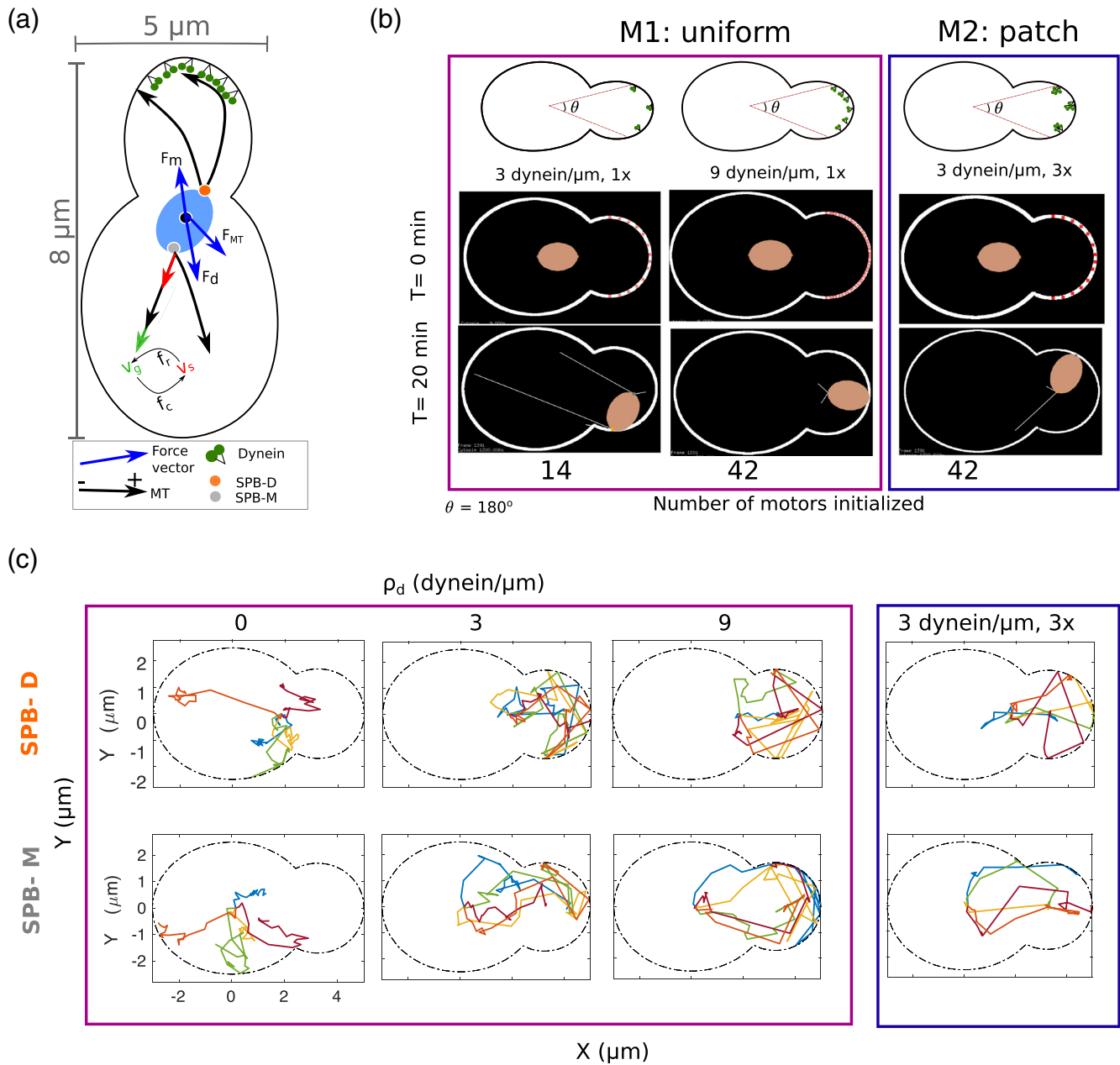


FIGURE 5 Simulating nuclear positioning by dynein localization in the bud. (a) Schematic of the geometry of a budding yeast cell with the ellipsoid mother cell and budding daughter cell. Dynein motors (green) are localized in the daughter cell and capture fluctuating microtubules (MTs), the dynamics of which are determined by f_c , f_r : frequencies of catastrophe and rescue; v_g , v_s : velocities of growth and shrinkage. Pairs of MTs are nucleated from the mother and daughter SPBs embedded in the nuclear membrane. The nucleus moves if there is a force acting it (blue arrow). (b) M1: uniform distribution of dyneins (gray zone) and M2: punctate localization of dynein are compared in simulations. The simulation outputs represent the start and end time points for the two models and different densities. (c) Representative XY trajectories of SPB-D and SPB-M are plotted for M1 and M2 for the same densities. Colors indicate individual SPB tracks. Simulation time = 20 min, $N_{\text{runs}} = 5$

$$\vec{F}_d = 6\pi\eta_{\text{cyto}} R_{\text{nuc}} v_{\text{max}}, \quad (6)$$

where R_{nuc} is the nuclear radius and v_{max} is the maximal velocity of transport. We measure the maximal velocity of nuclear translocation driven by motors to be 27.6 nm/s, considered to be the “terminal velocity.” The drag force F_d is 0.13 pN, and the pushing force of MTs F_{MT} depends on the number of MTs. Single MT pushing forces (F_{MT}^S) have been estimated to be 2.7 pN (Dogterom et al., 2005;

Laan et al., 2008), with multiple filaments resulting in an additive increase in the total force, that is $F_{MT} = n_{MT} F_{MT}^S$, where n_{MT} is the number of MTs (Laan et al., 2008). Although theoretical predictions have suggested cooperative increase in force of pushing slightly greater than additive (Das et al., 2014), the experimental measure and additive effect gives us a lower limit of force that MTs can generate. Assuming the total force exerted by the motors to be $F_m = N_m \cdot f_s$ (additive) where f_s is the single motor stall force and simplifying

Equation (5) combined with the linear dependence of force on the number of motors N_m , we can estimate the minimal number of motors $N_m(\min)$ required as follows:

$$N_m(\min) = \frac{F_d + (n_{MT} \cdot F_{MT}^s)}{f_s}. \quad (7)$$

This is the threshold number of motors, above which the opposing forces acting on the nucleus can be overcome and the nucleus positioned. Given the single motor yeast cytoplasmic dynein stall force ranges between 4 and 7 pN (Belyy et al., 2014; Cleary et al., 2014; Gennerich et al., 2007; Reck-Peterson et al., 2006) and the maximal velocity of SPB-D in experiments, we arrive at $N_m(\min)$ of one to two dynein dimers per nucleus (Figure S5). This simple arithmetic is consistent with the in vivo measurements of anything between zero and three puncta of cortical dynein molecules (Markus et al., 2009; Markus & Lee, 2011) being involved in sliding MTs in the bud cell of *S. cerevisiae* based on quantification of fluorescently tagged Dyn1 puncta (Markus et al., 2011). More recent work has assumed that Num1 dimers bind 1:1 to dynein and interact with astral MTs during capture and sliding (Omer et al., 2020). Indeed, from experiments, there is a clear indication of variation of number of puncta between bud cells, the number of dyneins anchored to the Num1 foci,

and the number of Num1 per puncta. Therefore, the comparison between our force balance calculations and experiments satisfies the range of values of dyneins based on the assumption of 1:1 stoichiometry with Num1 but leaves multiple questions unanswered whether our estimates of the minimal numbers of motors would be affected by factors such as the role of nonuniform localization of motors, stochasticity of only few (1 to 2) MTs, effect of intermittent contact of MTs with the cortex, role of the bud-mother geometry. To test this, we proceed to develop a spatially explicit model to test the role of punctate motors and their numbers required to minimally transport the nucleus.

3.4 | Model of astral MTs pulling by cortical dyneins and their localization

We extend the pre-budding model of nuclear mobility with stochastic simulations that include (a) random Brownian motion of all components, (b) stochastic MT dynamics of growth and shrinkage, (c) stochastic binding and force-dependent unbinding of motors, and (d) drag force that acts proportional to the velocity of movement. Such a model, with a 2-D geometry that represents the budding stage more realistically, with dynein motor localization at the bud cortex is

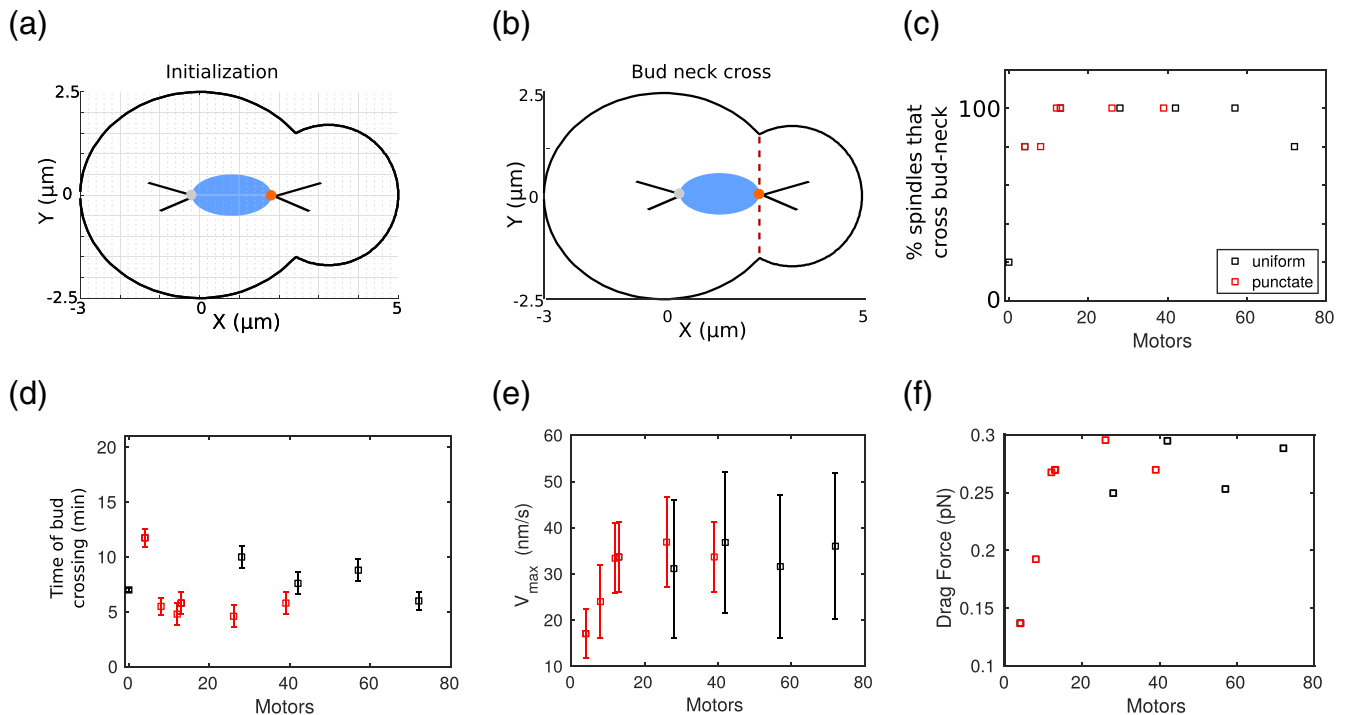


FIGURE 6 Pulling forces of dynein in the daughter cell. (a and b) The schematic represents the geometry of the minimal model of a budding cell based on experimental images with (a) the initialization of the nucleus in the center of the mother-bud cell and (b) the evaluation of successful positioning of the nucleus evaluated as the daughter spindle pole body (SPB) (SPB-D, red) crossing of the bud-neck (dashed line). (c–f) The effect of increasing the total number of dynein motors ($\rho d \times \text{perimeter}$) in the daughter-cell cortex evaluated in terms of (c) the percentage of total nuclei simulated N_{nuc} that cross the bud neck at the least once, (d) the mean time ($\pm$ SD) for an SPB to cross the bud neck, (e) the maximal velocity of nuclear movement and (f) the corresponding effective drag force. Here, uniform localization of motors (M1): black, punctate localization of motors (M2): red. Number of simulations $n = 5$. Viscosity $\eta_{cyto} = 0.5$ Pa s, $r_{nuc} = 1$ (short axis) and $1.75 \mu m$ (long axis), total time of simulation: 20 min

then used to compare the dynamics of simulation predictions to experiments (Figure 5a). We focus on the pre-anaphase stage because spindle elongation is expected to produce pronounced directed motion unrelated to astral MT interactions with the cell cortex. The two SPBs are embedded at diametrically opposite sides of the periphery of the nucleus, and as in the experiment, the SPB migrating toward the daughter cell is referred to as SPB-D and that which stays in the mother cell SPB-M, comparable to experiment. Each SPB nucleates two dynamic MTs with their plus-ends free and minus-ends embedded in the SPB. Length fluctuations of MTs are determined by dynamic instability. MT dynamics turnover is reported to be higher in pre-budding cells as compared to budded (Carminati & Stearns, 1997). We therefore choose dynamic instability parameters previously reported from pre-anaphase cells (Fees et al., 2017) (Table 3). In our model, the dynein motor is modeled as a stochastic stepper with linear

force velocity dependence and force-dependent detachment. The spring-like motors are immobilized at the cell cortex stochastically bind to, that is, “capture,” astral, or cMTs if they are within the minimal binding distance and once bound, walk to the minus-end of the MTs, embedded in the SPB. The dynein pulling forces result in MT sliding and movement of the nucleus (Figure 5a). We first set out to ask the question whether the localization of motors affected the transport dynamics of the SPB and nucleus. To this end, we assumed two models. Model 1 (M1) assumes that motors are uniformly localized on the cortex, while in Model 2 (M2), the motors are localized in a punctate manner, consistent with more recent experimental reports. We find the XY trajectories of the SPB-M and SPB-D do not show a qualitative difference between the two models (Figure 5c). We therefore set out to examine the successful positioning of the nucleus at the bud neck as indicated by location of SPB-D (Figure 6a,b). We find

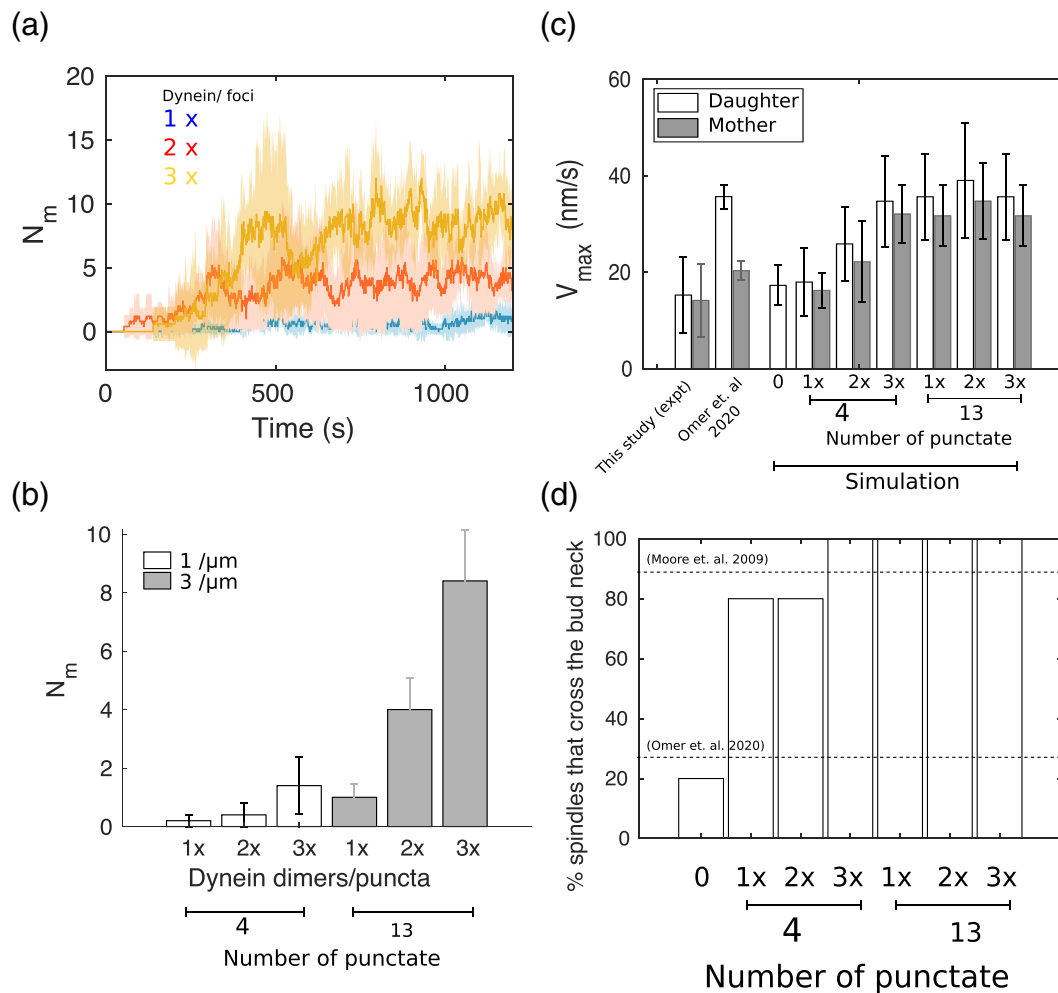


FIGURE 7 Effect of dynein number and localization on nuclear mobility. (a) The representative dynamics of the mean number of motors bound to microtubules (MTs), N_m , is plotted with time for varying numbers of dynein dimers per puncta, 1 (blue), 2 (red), or 3 (yellow), with ρ_m of 3 motors per micrometer. The faint colors correspond to the SD for the respective number of dimers/puncta. (b) The corresponding mean $\pm$ SD values at steady state are plotted. (c) The maximal velocity of spindle pole body (SPB) mobility in the absence of dynein is compared to 4 and 13 puncta with 1, 2, and 3 dynein dimers puncta as before and compared to the SPB-M (black) and SPB-D (gray) velocity reported by Omer et al. (2020). (d) The percentage of SPB-D crossing the bud-neck region are quantified for varying numbers of puncta and dyneins/puncta. The dashed lines indicate values reported in literature by Moore et al. (2009) and Omer et al. (2020) for comparison. These calculations correspond to Video S5

motors localized in a punctate manner (M2) result in nearly 100% transit efficiency even when the total number of motors is less than 20, whereas uniform density motor distributions achieves this at higher motor densities (Figure 6c). A similar timescale of ≈ 5 min for crossing of the nucleus is observed for M2, whereas for a comparable number of motors (~ 40 per bud) the uniform localization (M1) takes twice as long, 10 min (Figure 6d). The potential explanation for the higher efficiency (rapid and correct positioning with fewer motors) of punctate dynein (M2) compared to uniform motor densities (M1) could be explained by the higher maximal velocity achieved comparable motor numbers ~ 30 (Figure 6d) and a correspondingly higher force estimated from the drag equation (Figure 6e).

Given the higher efficiency of the punctate dynein localization Model (M2) to position nuclei and the evidence from experimental reports, we proceed with examining the number dynamics of motors. We find the number of motors bound to the astral MTs fluctuate due to both stochastic binding-unbinding kinetics of the motor and MT length dynamics, with a steady-state saturation approached after 5 min (Figure 7a). The steady-state values suggest that not all motors on the cortex are bound (Figure 7b), potentially due to combination of factors fluctuations and bud geometry with high curvature. Indeed, our model makes testable predictions that the number of bound motors per MT will approach the maximal binding only in presence of clustered dyneins with multiple clusters. The maximal velocity of SPB-D and SPB-M averaged from experiments across cells are comparable in our experiments as compared to previous reports (Omer et al., 2020), with magnitude and differences arising from the use of only directional stretches in the study by Omer et al. and others. Indeed, we find that simulations predict the maximal velocity of both SPBs with between zero and one puncta with one motor per puncta are minimally required to match the maximal velocity from our experiments, while three to four dimers per puncta are more comparable to v_{max} values reported in literature (Figure 7c). The difference in SPB-D and SPB-M velocities in simulations arises from the model assumption of pulling forces localized only in the bud cell. This suggests that the transient bud-directed mobility of the spindle minimally requires asymmetry in pulling forces. The experimental reports of the percentage of spindles crossing the bud neck vary between reports and while one report is more comparable to no motors, the older study is more consistent with at least four puncta with three dynein dimers per puncta (Figure 7d). A high proportion of correctly positioned spindles require multiple motors with “robustness” of positioning saturating for dynein densities exceeding four puncta with three dimers each (3 times).

Thus, simulations predict that the punctate localization of a few motors is sufficient to both capture and generate sufficient forces and produce collective forces necessary to drive nuclear positioning. Interestingly, the stochastic model predicts that four puncta of dynein 1 and 2 times dimers are required to enable 80% and more spindles to cross the bud neck. Such percentages match those reported in the study by Moore et al. (2009). This predicted number of motors is higher than the minimal dynein number of dyneins predicted by the simple force balance model. The difference in predictions can be

understood in terms of discrete and intermittent nature of interactions, which our 2-D stochastic model is likely to have captured. Despite not implementing a more detailed “offloading model” as reported in recent literature of cortical dynein in *S. cerevisiae* (Markus & Lee, 2011; Markus et al., 2012b; Lee et al., 2005), our simulations predict that convergence times of spindles to the bud neck comparable to experiments. This could be related to process being dominated by binding and force as compared to “search.” However, more calculations will be necessary to understand the precise functional role for the “offloading model.”

4 | CONCLUSIONS

In this study, we use a model of nuclear mobility in pre-budding cells of *S. cerevisiae* driven by diffusion and MT pushing and by reconciling to experimental mobility measures of the SPB, estimate the effective cytoplasmic viscosity to be 0.5 Pa s. By reconciling the mobility statistics of SPB-D in the budding stage from experiments with a simple force balance model, we estimate minimal numbers of dynein motors that must collectively bind to and transport astral MTs that are anchored in the SPB, in order to position the nucleus. Although force balance suggests one to two dynein dimers suffice to generate the force required to overcome viscous drag, discrete stochastic simulations suggest higher numbers of dimers (4–12) might be involved. The model predicts dynein clustering into puncta results in higher collective force generation with fewer motors. Stochastic simulations when compared to experimental data of the proportion of correctly positioned nuclei predict greater numbers of dynein dimers could be required for robust spindle positioning, compared to force balance predictions, to damp out fluctuations arising from the dynamics of MTs and motors.

AUTHOR CONTRIBUTIONS

Kunalika Jain designed and performed the mobility analysis of experimental data, wrote the code, and prepared the figures; Shivani A. Yadav performed image analysis and anaphase-scaling analysis, wrote the code, and prepared the figures; Saravanan Palani performed the *S. cerevisiae* in vivo time-lapse experiments; Neha Khetan performed the simulations, wrote the code, and prepared the figures; and Chaitanya A. Athale designed the research wrote the manuscript, prepared the figures, and obtained funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Number Dependence of Microtubule Collective Transport by Kinesin and Dynein

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Abstract | Translational motors that depend on cytoskeletal elements, either actin or tubulin, for their activity are critical for cellular function in eukaryotes. Microtubule (MT)-dependent motors are broadly classified as dyneins and kinesins, based on sequence similarity. Typically, dyneins walk towards the minus-ends of MTs, while kinesins walk towards the plus-ends, with some plant and animal kinesins also seen to be **minus-end** directed. While our understanding of motor mechanics at a single-molecule level has rapidly improved due to developments in force spectroscopy, in vivo motor transport often involves multiple motors acting together. Here, we review our current understanding of collective effects that emerge in motor-driven transport in vivo based on physical mechanisms inferred from in vitro reconstitution experiments involving MT transport, or '**gliding assays**'. We discuss the evidence for number dependence in cargo transport at MT cross-overs, orientation sorting of MTs during axonal regeneration in neurons, spindle bipolarization by MT transport, and nuclear positioning during mitosis in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. We discuss how minimal in vitro gliding assays have been successfully used to identify the mechanical properties of collective motor transport that produce such cooperative effects. The 'loose coupling' mechanism of Oosawa, which was developed to explain the emergence of cooperation in collective motor transport, appears to be consistent with evidence from kinesin, but not dynein. Additionally, substrate rigidity also appears to play a role in collective force generation, as seen in lipid-anchorage studies of collective transport. Thus, a deeper understanding of the intra- and inter-motor properties of **kinesins and dyneins**, as well as non-motor effects due to the substrate is required, for the emergence of a complete picture of the in vivo mechanobiology of collective motor transport.

Keywords: Microtubule, Motor, Collective transport, Kinesin, Dynein, Loose coupling, Detachment, Membrane anchorage, Reconstitution

1 Introduction

Molecular motors are molecular structures which can convert one form of energy into mechanical energy. Biological molecular motors include motor proteins which can use chemical energy such as from ATP hydrolysis to perform mechanical work. Microtubules (MTs) are dynamic

polymers formed from tubulin subunits, and the orientation of the α - and β -subunit results in a kinetic polarity of fast-growing plus-ends and slow growing or shrinking minus-ends. The motors are classified based on sequence similarity and orientation of transport into either plus-end-directed kinesins, or minus-end-directed

Microtubule plus-and minus-end: The kinetic polarity of MTs with plus indicating net growth and minus net shrinkage.

Gliding Assay: Microtubule (MT) transport by immobilized molecular motors.

Kinesin and Dynein: MT dependent molecular motors that walk predominantly towards the plus- and minus-ends of the MTs respectively.

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dyneins²⁹. However kinesin-14 variants such as that found in *Drosophila*, referred to as Ncd⁴⁵ as well as plant kinesin-14 proteins⁷⁴, belong to the kinesin superfamily based on sequence, but are found to walk towards the minus-ends of MTs. Proteins of the kinesin superfamily share structural similarities with myosin, the actin-dependent linear motor³⁴. Dyneins on the other hand are more complex and in many cases can only function as holoenzymes^{64,77}. In the following sections, we will discuss the specific role of collective motor transport in cellular processes, the in vitro results that have allowed the dissection of mechanical properties that govern cooperativity and the gaps in our understanding of collective mechanics of dynein as compared to kinesin.

2 Kinesin and Dynein Superfamilies

Kinesins or plus-end directed motors are by far the better studied of the MT-dependent motors. Based on the phylogenetic analyses, the kinesin superfamily of proteins (KIFs) has been grouped into 14 subfamilies starting from kinesin-1 to kinesin-14^{25,35}. Each of these is further sub-grouped into three types depending on the position of the motor domains in the molecule: (1) N-type KIFs, where the motor domain is located at the amino terminal with plus end-directed movement, (2) C-type KIFs, where the motor domain is located at the carboxyl terminal with minus-end-directed movement, and (3) M-type KIFs, with the motor domain is located between the amino and carboxyl terminal, and majorly causes MT depolymerization⁴⁷. In contrast, dynein, named after the unit of force (dynes)¹⁵, has been categorized as either axonemal dynein or cytoplasmic dynein based on their functions^{55,79}. While axonemal dyneins are involved in cilia and flagellar beating, cytoplasmic dyneins are involved in a wide range of cellular processes like cargo transport, mitosis, migration, and polarization (reviewed in^{9,16,25,26}). Unlike kinesins that have a single globular motor domain, dyneins have a large motor domain formed by hexameric rings of AAA+ (ATPase associated with diverse activities) superfamily³². The motor domains generate power by ATP hydrolysis for cargo trafficking. The activities of the molecular motors in vivo typically involve the action of more than one motor at a time in generating force, i.e., the process of collective transport.

3 Mechanics of Mixed Motor Transport and Models of Coordination

Molecular motor-driven cytoskeleton transport was first shown in early experiments on actin moved by myosin fixed to a glass surface, a 'gliding assay'⁷³. The invention of optical tweezers² provided the opportunity to measure the force exerted by a few myosin motors immobilized on a surface acting on an actin filament, complexed to a polystyrene bead^{48,67}. More recently quantum dots complexed with dyneins⁶⁰ and kinesins³⁶ have been used to measure the processivity of these motors, revealing similar step sizes. Insights from such single-molecule approaches have shown that kinesin velocity decreases with an increase in the opposing load⁷⁰, while in the case of dynein, increasing opposing load on motors reduces their step size from 30 nm (load-free) to 8 nm (high-load) referred to as 'gear-like' behavior^{43,56}. These highly precise measurements of single motors in vitro are now being used to examine the in vivo role of collective motor transport.

The transport of intracellular cargoes which have both kinesin and dynein attached are seen to undergo bidirectional transport. Occasionally, these then convert to uni-directional transport, and this resolution of antagonistic tendencies is thought to be resolved by either (a) a mechanical 'tug of war' or (b) biochemical regulation. In the 'tug of war', motors of opposite polarity bound to the microtubule are equally active. A stochastic detachment of motors of one polarity under the opposing force of other results in a resolution an uni-directional transport^{39,50,51,69}. In contrast, the *regulation* model predicts that both polarity motors can bind to cargo, only one is active at any point of time, and the activity reversal takes place only in the presence of some co-factor³⁸. Evidence for a 'tug-of-war' has come from endosomal transport where the detachment rate of dyneins decreases in vivo⁴³ and is thought to arise from 'catch bond' interactions of dyneins with microtubules⁵⁶ as well as direction-dependent detachment rates⁵². In contrast, protein kinase-A is seen to bias the transport of pigment granules in melanophores of fish⁶² and *Xenopus*¹⁷, by switching from MT to actin-dependent transport. However, the spherical geometry of small vesicles that have been extensively studied as

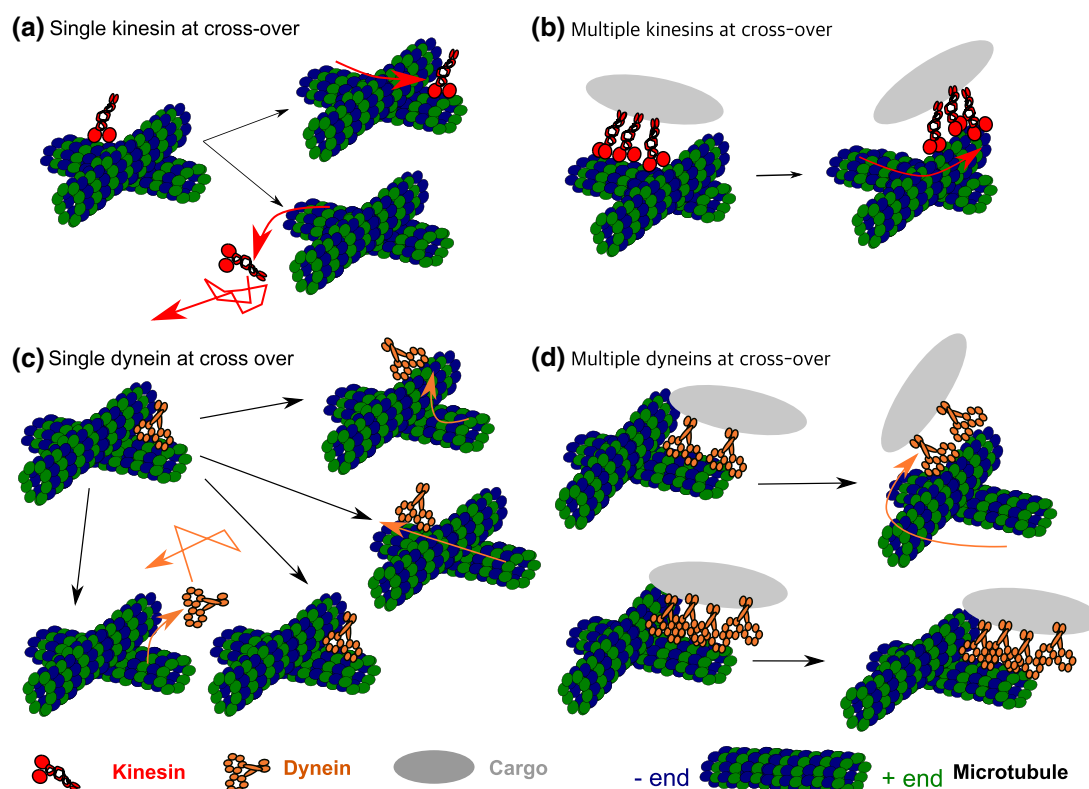


Figure 1: Effect of motor numbers in cargo transport along MT networks. **a** Kinesin walking along a microtubule intersection can either cross the protofilament at the junction or dissociate from the filament. **b** When multiple kinesins transporting a cargo arrive at MT cross-over, they switch the filament. **c** Unlike kinesin, dynein exhibits multiple stepping behavior. They either switch and follow a new MT, or pass through the same one, or halt at the junction or dissociate from the filament. **d** When multiple dyneins are transporting a cargo, they either change the MT track during cargo transport for low dynein numbers or anchor the cargo without stepping for high dynein numbers. Schematic representation based on findings of Ross et al.⁶³.

cargoes results in steric limits to the range of motor numbers that can be seen. Thus, a more extensive motor-number dependence necessarily involves transport of either larger cargoes or elongated cargoes, a prominent one of which is MT transport.

4 Motor Number Dependence of Collective Transport

Collective motor transport by multiple motors of the same orientation has been seen to produce unexpected effects that appear to depend on the number of motors acting in the process of transport at a given time point. These are seen broadly in two classes of transport processes—either motors are bound to and transport a cargo by walking on static MTs or the motors are immobilized and the MTs are transported.

4.1 Motility of Motors Attached to Cargoes

A combination of electron microscopy studies and force measurements have shown that typically vesicles⁷⁶, mitochondria²¹, and lipid droplets^{19,66} are engaged by 1–7 motors^{18,27}. When such a motor bound cargo encounters an intersection of two MTs, the cargo undergoes one of four possible outcomes: (i) detach, (ii) stall, (iii) continue along the MT it was walking along, or (4) cross-over to the other MT, based on a systematic study using flow to arrange the angle of MT cross-overs, and then compared the progress of single-molecule transport⁶³. It was found that while single kinesin-1 largely crossed the intersection, beads with teams of kinesins always switched filaments at intersections (Fig. 1a, b). In contrast, dynein–dynactin complexes had varied responses. In case of a single-molecule cases, the dynein–dynactin complex demonstrate all

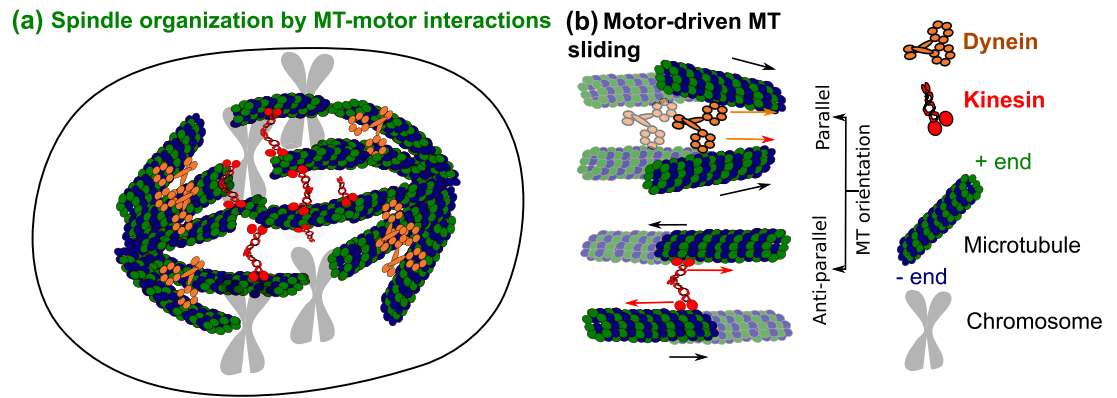


Figure 2: Microtubule transport by sliding of motor domains. **a** The schematic represents microtubule movement by molecular motors during MT reorganization to form spindles. While kinesins (red) anchor acts to push apart anti-parallel MT bundles, dynein (orange) walks to the minus-ends resulting in spindle pole focussing. **b** The activities of these antagonistic motors can be understood in terms of MT orientation combined with motor walking direction, acting on two MTs simultaneously. The forces generated by the dynein (orange arrow) and kinesin (red arrow) result in the movement of MT (black arrow) opposite to the direction of motor movement.

possible outcomes—pause, continuation, cross-over, and detachment (Fig. 1c), while teams of dynein predominantly stalled at intersections (Fig. 1d), interpreted as a feature relating to the role of dynein in anchorage. An example in case is the anchorage of the Golgi apparatus near the MTOC on MT asters at the centre of the cell that depends on dynein activity^{8,61}, while kinesin-driven outward dispersal along astral MTs is regulated by reducing the number of dyneins bound, by small GTPase signaling which in turn is controlled by cell adhesion⁶⁸. Thus, the number control of one kind of motors can determine the behavior of transport at MT intersections, and when antagonistic motors are bound to cargoes, it determines intracellular organelle topology.

4.2 MT Transport in Cells

Apart from cargo transport and anchorage, motor proteins in cells are also found to be immobilized with their motor domains free, and in such cases, they transport MTs and their tracks. Prominent examples of physiologically MT transport are observed in eukaryotic spindle assembly at the time of division, maintenance of MT polarity in axons, and organelle positioning in growth and division.

4.2.1 MT Transport During Spindle Assembly

At the onset of pro-Metaphase in M-phase of the cell cycle, spindle assembly in the presence of centrosomes results in the ‘search and capture’ of chromosomes by astral MTs, which in large cells

can involve the role of RanGTP gradients around chromosomes causing preferential MT growth seen in *Xenopus* oocyte extracts^{3,7}. In large cells, dynein appears to form an important component in spindle assembly, exerting a length-dependent pulling force on either centrosomal asters or small MTOC asters in acentrosomal spindles as seen in mouse meiosis I³¹. Kinesin tetramers acting to push apart anti-parallel MTs combined with dynein localized to spindle poles that correspond to MT minus-ends (Fig. 2) are essential for the formation of a stable bipolar spindle^{71,72,78} and confirmed by simulations⁴¹. In the complete absence of a centrosome or aster, dynein and kinesin can self-organize MT networks to form focused spindles^{14,24} as confirmed with artificial chromatinized beads¹¹ and summarized in Fig. 2. The regulation of the orientation of the MTs, parallel or anti-parallel, is in itself likely to result from a short-range chromosome-driven nucleation gradient.

4.2.2 Neuronal MT Sorting

In neurons, that are stereotypically polarized, axons and dendrites have different alignment patterns for microtubule (MT) cytoskeleton. While the MT bundles in axons are aligned with their plus-ends directed away from the cell body (‘plus end out’ alignment), dendrites have a mixed polarity of MT bundles²³. Axons achieve the distinct state by means of a polarity sorting mechanism involving many cellular players with the mechanical components primarily consisting

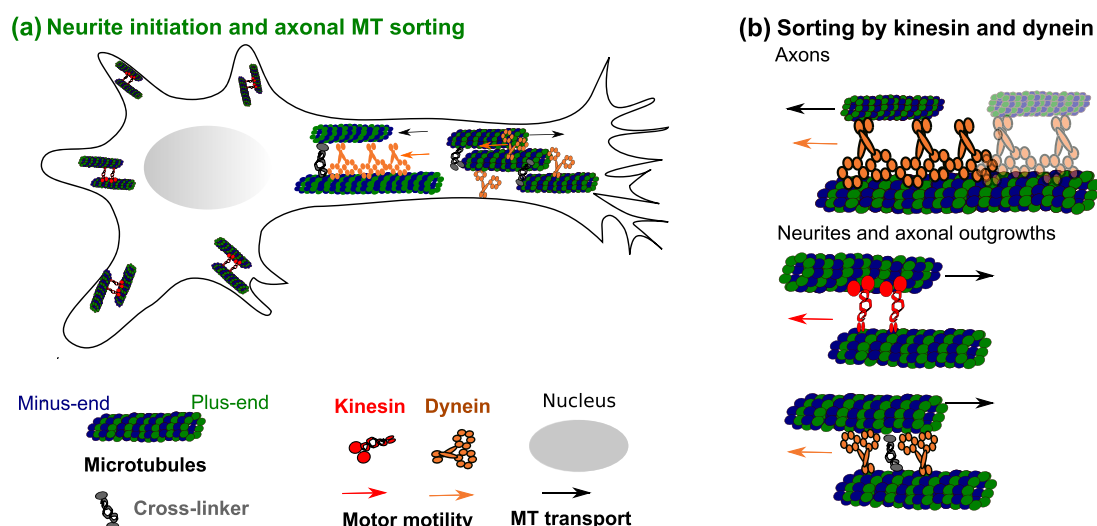


Figure 3: MT transport in neuronal axons. **a** The schematic depicts a neuron with MTs both in dendrites and the axon. The MT ends can be distinguished as plus- (green subunits) and minus-ends (blue subunits). The interaction of dynein (orange) and cross-linkers (grey) is sufficient to explain the 'polarity sorting' of MTs seen in developing axons as well as at the time of repair (based on experimental evidence⁵³ and computational predictions¹⁰). Kinesin (red) is required for neurite initiation and dendritic MT orientation. The arrow indicates the direction of movement of MTs. **b** The minimal model of such sorting assumes that a larger MT serves as an anchorage point for dyneins, which bind smaller, mis-oriented MTs, and transport them out of the axon. Additionally, in neurites at the time of initiation and growth into axons, dynein plays an important role, while in dendrites, polarity of minus-end out MTs is achieved by the activity of kinesins. The central region of the neuronal soma is occupied by the nucleus (grey).

of cytoplasmic dynein and static cross-linkers⁵⁸ and highlighted in Fig. 3. However, in addition to this minimal set of motor-MT proteins, genetic evidence also points to a role for kinesin in maintaining axonal width and filopodial lengths in the growth cone⁴⁶. An antagonistic MT-motor model has been used in the past to explain the dynamics of MT polarization in growth cones⁴², and might point to related functions in initiating MT transport and organization in the axon. A computational model of axonal MT 'polarity sorting' proposes a critical role for only cytoplasmic dynein and cross-linkers¹⁰ are yet to be experimentally tested as a sufficient explanation. Additionally, the transport and sorting of dendritic MTs is thought to be driven by the presence of local MTOC-like nucleators combined with kinesin²³. The precise role of the force generated during the collective transport in axons and the motor numbers required is still unclear.

4.2.3 Organelle Positioning by MT Pulling by Cortical Motors

In multiple cell types, dynein-dynactin complexes are found to be transported along astral MTs and cortically anchored in spots where they serve as force generators for spindle positioning and

orientation during mitosis⁶. In mammalian cells and *Caenorhabditis elegans* embryos, the complex of the nuclear mitotic apparatus protein (NuMA) is also referred to as LIN5 in nematodes, a $G\alpha$ binding protein (LGN) called GPR-1 in *C. elegans*, and the $G\alpha$ protein combined to localize dynein to the membrane and ensure spindle positioning and orientation³³. Indeed, despite correct localization, the over-expression of either of the components of the complex—NuMA or the Leu-Gly-Asn repeat enriched protein (LGN) that belongs to the Partner of Inscuteable (Pins) protein family—results in spindle oscillations in MDCK cells¹² and increased oscillatory frequency in *C. elegans*³³. This suggests motor number and by extension force generation have a critical role to play in the fundamental position of spindle segregation. Such oscillations have also been reported during meiosis in yeast *Schizosaccharomyces pombe* and mitosis in *S. cerevisiae* driven by dynein-dependent MT pulling Fig. 4a. In *S. pombe* meiosis, dynein heavy chain (DHC) localized to the cell membrane pulls on astral MTs⁸¹ and shortening of the 'leading' MT resulting in oscillatory number of motors pulling on the astral MTs, producing nuclear oscillations⁸⁰. A similar dynein dependence of transport was reported in the budding yeast *S. cerevisiae* during nuclear positioning with oscillations reported in Anaphase

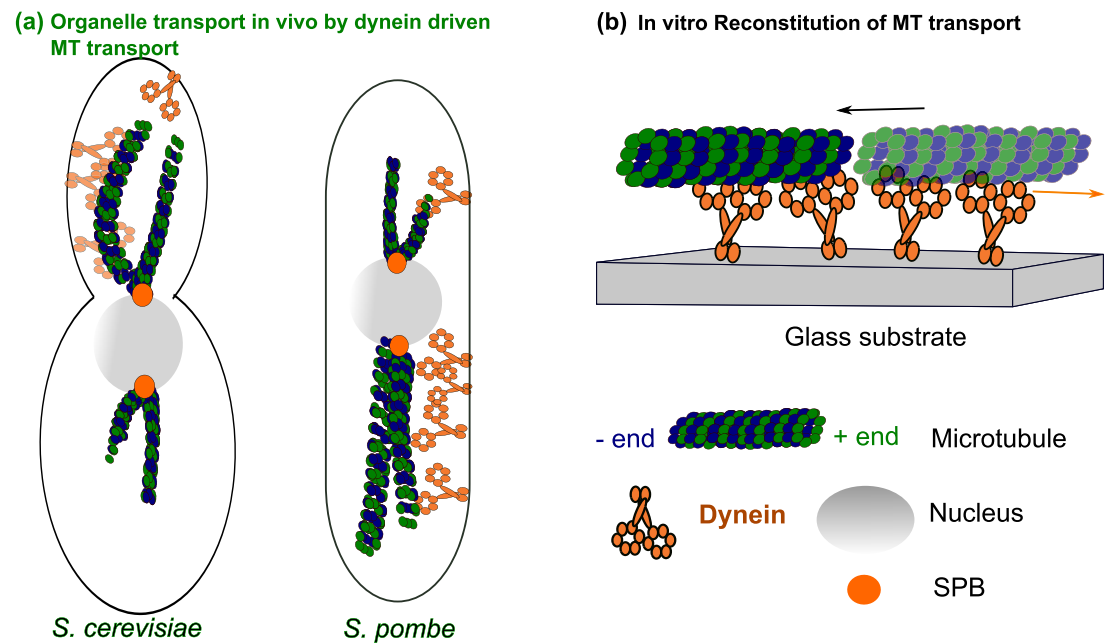


Figure 4: Microtubule transport by gliding on motor domains. **a** The in vivo transport of MTs by anchored dynein proteins is represented schematically for *Saccharomyces cerevisiae* mitosis and *Schizosaccharomyces pombe* meiosis. The resulting force on the spindle pole body (SPB) that nucleates astral MTs (orange circles) results in translocation of the nucleus (grey). **b** A minimal system to examine such MT pulling by immobilized motors based on in vitro reconstitution is depicted, referred to as the 'gliding assay'. Here, surface immobilized dyneins (orange) with their motor domains free bind to MTs and attempt to walk towards the minus-ends (orange arrow). Since the motors are immobilized, we instead observe the effect of net force generation in terms of MT translocation in a direction opposite to motor movement (black arrow).

B⁸². Indeed, a combination of MT shrinkage and dynein–dynactin pulling of cortically sliding MTs were both implicated in nuclear positioning in mitosis¹. Simulations of MT asters transported by dynein suggest the need for dynamic instability to resolve single-motor 'tug-of-wars' arising out of geometry⁴. The differences in the precise nature of the dynein motor, the complex that localizes it to the cortex, whether localization is patchy or a continuum, the density and single-molecule properties, all combined with the dynamics of MTs, paint a complex picture of the process by which vital physiological processes are regulated in terms of the collective mechanics. Thus, in vitro reconstitution approaches such as gliding assays (Fig. 4b) could improve our understanding of the specific combination of effects that have been adapted for the in vivo function.

5 In Vitro Analysis of Motor Number Dependence of MT Transport

Since both MT lengths and motor densities appear from in vivo evidence play a role in MT transport, it is expected that MT transport

properties will be affected by both. Indeed, in a now classic study, Duke–Holy–Leibler (DHL) predicted the role of spacing between motors bound to filaments and the statistics of filament swivelling with MT length in 'gliding assays' based on analytical theory and stochastic simulations¹³. Importantly, they predicted a minimal filament length required for processive motion driven by motors with both high and low duty ratios. This model predicts as expected a reduction in the mean swivelling angle (θ) with increasing filament length relative to the motor reach, and a concomitant exponential increase in the curvilinear path of the filament transport (S). However, these trends are continuous, suggesting that a more detailed model of binding–unbinding and asymmetry of force-dependent detachments could result in counter intuitive behaviour. Assuming the width of the filament to be negligible, the number of motors interacting with a given MT (N_m) depends on the motor density ρ_m and MT length, L_{MT} (Fig. 5). Assuming the motors are distributed in a regular square lattice equally spaced, we can equate these terms as:

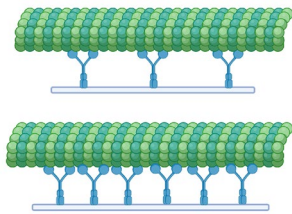
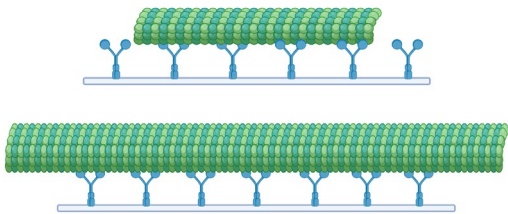
(a) Motor Density**(b) MT Length**

Figure 5: Motor density and MT length in collective transport. The schematic represents changes in number of motors attached to a MT either due to increased **a** motor density ρ_m or **b** MT length L_{MT} . Motors (green) are attached to a solid substrate (light blue) and bound to MT (green) (Created with BioRender.com).

$$N_m = L_{MT} \times \sqrt{\rho_m} \quad (1)$$

to obtain the number of motors per MT from a given surface density and MT length³⁰. This assumes that all the encountered motors are bound and that MTs are effectively 1D objects. The geometric simplification can be corrected by assuming a nearest neighbor model²² or band-model⁷⁵.

While the DHL model, interestingly, predicted the effect of density (Fig. 5a) and MT length (Fig. 5b) on filament transport statistics, it assumed that the effect due to spacing between motors would be continuous. Indeed, the mean swivelling angle decreases linearly with MT length on a log-log axis. More sparsely separated set of motors will be more subject to thermal fluctuations in the velocity, while the mean may remain the same.

The first clear measurement in vitro suggested that the velocity of microtubule transport was constant despite increasing kinesin density, which was immobilized on a glass substrates²⁸. In contrast, a more recent study that examined a similar density dependence of dynein on MT transport demonstrated a number dependence of velocity during transport⁴⁹. This was attributed to stretch based activation of dynein and resemble the effect

of co-factor binding to motors in experiments. The study demonstrated a very good match between experiments and simulations, despite the limitations inherent to bulk measurement of the motor density, using the difference between the concentration flowed in and the flow-through in a flow chamber. Given the progress made in understanding the single-molecule mechanics of the minimal yeast cytoplasmic dynein⁶⁰, we have followed an alternative approach to examine the number dependence of MT transport. By first calibrating the fluorescence in the microscope, we can calculate the surface density of tagged motor proteins. In contrast to the predictions of the DHL theory, we found a discontinuous jump in the displacement relative to mean free path (directionality) of filament transport. A model of direction-sensitive detachment in response to force could explain our directionality data, with velocity staying more or less constant as a function of motor numbers³⁰. Underlying these studies is a search for a more general answer to the question of how asynchronous, stochastically stepping molecular motors can achieve cooperativity in transport, either through passive or active mechanisms.

6 Motor Number Dependence of MT Transport and ‘Loose Coupling’

The early results from in vitro gliding assays showing the independence of MT transport velocity on motor density of kinesin²⁸ suggested an inherent property in motors that entrained otherwise random steppers into working cooperatively. In a classic study by Oosawa and Hayashi, they proposed a ‘loose coupling’ between the number of ATP molecules consumed and the shortening of muscles, suggesting that the chemical and mechanical cycles could not be mapped 1-to-1^{53,54}. Indeed, this concept taken to a multi-motor transport assay was used to explicitly answer whether such ‘loose coupling’ in a mechanical sense, i.e., multiple motors bound to the same MT filament, but their spring energies being of the scale of thermal energy could result in some passive extension in time of asynchronous stepping. A study that examined this very assumption precisely used engineered versions of the kinesin heavy chain (KHC), demonstrating that reduction in the length of linker domain of the kinesin resulted in decrease in velocity of MTs as the number of kinesins interacting with MT increases, i.e., negative number dependence⁵. The reduction of velocity was steeper with increasing motor numbers for mutants with short

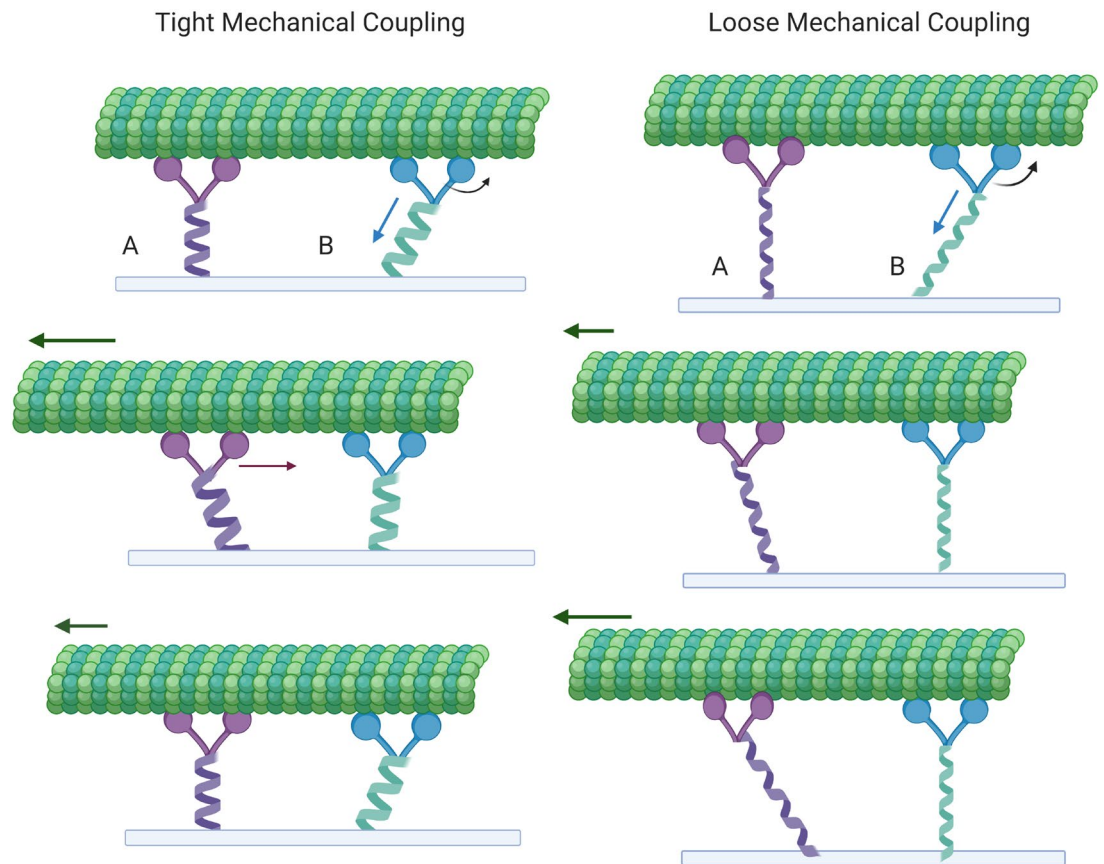


Figure 6: 'Tight' and 'loose' mechanical coupling in collective motor transport. The schematic represents effect of mechanical coupling on collective transport of motors. Two motors A (inactive, purple) and B (active, blue) bound to a same MT (green) are attached by their stalks to a solid substrate (light blue). The motors are experience either 'Tight' or 'Loose' coupling depending on the behaviour of the individual motors. In the schematic, the active motor (B) is shown to take one step (black arrow), and depending on the nature of coupling, 'tight' or 'loose', a restoring force is produced (blue arrow) due to the inactive motor (A). The direction of MT transport is indicated by a green arrow (created with BioRender.com).

linkers as compared to medium-length linkers. It was inferred that effective collective transport was enabled by the added flexibility afforded by a certain length of the linker domain, so that when the bound motors one stepped, i.e. 'active', and the others did not ('inactive'), due purely to the asynchronous nature of the process, the passive stretch in the 'inactive' motors would still allow the filament to be transported without exerting an opposing force (Fig. 6). Indeed, tightly coupled motors would then be expected to act as 'brakes' or like the rigor mutants that have been previously seen to decrease collective velocity in gliding assays⁶⁵.

More recent reports of in vitro gliding assays with dynein from mammalian⁴⁹ and yeast sources⁶⁰ have shown that motor numbers make an impact on MT transport velocity and directionality³⁰. This has been tested in silico to gain

mechanistic insights based on the single-molecule parameters. Such careful in vitro quantitations attested by in silico models are becoming a popular approach to gain insights into the ensemble behavior of cargo and MT transport by motors.

The contrast between dynein and kinesin collective behavior is thought to lie in the inability of kinesin to coordinate during multi-motor transport as seen in experiments with kinesin-1 carrying cargos in teams of 1–3³⁷, while dynein acts like a gear taking smaller steps under load with higher power⁴³ making dynein motor teams more coordinated⁴⁴.

7 Effect of Substrate Stiffness on Number Dependence

Most conventional 'gliding assays' have been performed using glass substrates. Some studies have been performed using lipid membrane

as a substrate in myosin⁸³ and kinesin^{20,40} based gliding assays. Therefore, it was somewhat surprising to see, contrary to the classic studies of number dependence of kinesin collective transport, when kinesin was anchored to a supported lipid bilayer, the velocity of MTs was seen to be increasing with increasing number of kinesins, modulated by membrane viscosity²⁰. Upon anchorage to lipid membrane, kinesin exhibits reduced binding kinetics depending upon the substrate fluidity⁴⁰. Dynein on the other hand shows number dependence even when attached to glass unlike kinesin. Given the flexibility of motor domain of dynein, it will be interesting to explore its behaviour in a flexible environment such as lipid bilayer. Lipid-anchored dynein is a better representative of the *in vivo* scenario such as cortical dynein. Studying collective behaviour, i.e., number dependence of lipid-anchored dynein can help to gain insights into processes such as nuclear and spindle positioning. Clustering of dynein with the help of lipid microdomain formation has seen to be involved in increasing efficiency of collective transport during phagosome maturation⁵⁷. Hence, studying the effect of substrate mobility on the collective behaviour properties of dynein can help to gain insights on several aspects such as regulation of collective transport of MTs by motors.

8 Discussion and Conclusion

Based on the collective behaviour studies of various kinesin and dynein constructs, the factors that appear to affect it are predominantly of three kinds. (a) 'Intrinsic': Single-motor mechanics as seen in case of 'loose coupling' and kinesin linker lengths. It could be interesting to examine whether a 'tug-of-war' of tight coupled motors might be observed, where the opposition from out of phase motors will make the MT oscillate or whether it results in uninterrupted processive translocation with reduced velocity. If such an oscillatory behavior exists, it is expected to have an amplitude comparable to the step size and frequency comparable to the stepping rate of the motor, requiring ultrafast ($\approx 1/100$ s) nanoscopy ($1/100$ μm) to detect it. Additionally, geometric frustration of forces by either anti-parallel MT doublets or radial asters with controlled MT densities (e.g., artificial asters) could also serve to answer these questions with larger transient oscillations expected. (b) 'Extrinsic': The nature of the substrate coupling decides the degree to which collective transport is cooperative as seen for kinesin immobilized in lipids as contrasted

to glass, due to the mechanical flexibility of the substrate, which damps out asynchrony in stepping. Additionally, spatial inhomogeneities could also result from the lipid diffusion and produce more complex patterns of mobility. (c) While the nature of the MT as static or dynamic is now very well understood, the degree of fluctuation of the MT length could affect average transport rates. In addition, a less well-studied aspect of MTs is the assumption about the uniformity of the mechanics of all MTs. It remains to be seen if differences along the tubulin lattice could also play a functional role *in vivo*.

The effects of numbers of motors on the collective mechanics of kinesin are dependent on linker length, while dynein provides a more complex picture of cooperativity due to changes in both force and step size. The framework of the mechanical flexibility, the role of regulators, MT dynamics, and substrate stiffness all point to a need to increase the complexity of *in vitro* reconstitutions, so as to better explain the rich diversity of *in vivo* processes governed by motor-driven MT transport.

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Quantification of cell shape, intracellular flows and transport based on DIC object detection and tracking

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Abstract

Computational image analysis combined with label-free imaging has helped maintain its relevance for cell biology, despite the rapid technical improvements in fluorescence microscopy with the molecular specificity of tags. Here, we discuss some computational tools developed in our lab and their application to quantify cell shape, intracellular organelle movement and bead transport in vitro, using differential interference contrast (DIC) microscopy data as inputs. The focus of these methods is image filtering to enhance image gradients, and combining them with segmentation and single particle tracking (SPT). We demonstrate the application of these methods to *Escherichia coli* cell length estimation and tracking of densely packed lipid granules in *Caenorhabditis elegans* one-celled embryos, diffusing beads in solutions of different viscosities and kinesin-driven transport on microtubules. These approaches demonstrate how improvements to low-level image analysis methods can help obtain insights through quantitative cellular and subcellular microscopy.

KEYWORDS

differential interference contrast (DIC), diffusion, granule, object detection, tracking, transport

1 | INTRODUCTION

Differential interference contrast (DIC) microscopy uses label-free bright-field optics to produce local contrast based on interference. It continues to be widely used in cell biology, with low photodamage particularly making it useful for time-lapse imaging. With the rapid advances in fluorescence microscopy, DIC is used in combination with molecular specific probes to correlate higher order structure, such as cells or tissues with the molecule of interest. The principle of DIC optics involves illumination with plane-polarised light, a pair of prisms and local differences in refractive index of the sample resulting in interference

producing image contrast. This method of obtaining contrast is also referred to as Nomarski contrast. The setup for DIC involves a light source that is polarised in a plane and then split by a Nomarski or Wollaston prism into two divergent beams. These in turn pass through a condenser, followed by the sample and into the objective. On reaching the second prism, the two beams are recombined and passed through an analyser, producing interference that results in the characteristic local contrast as reviewed by Yenjerla et al.¹ Dramatic improvements in fluorescence microscopy, both dye-based and using fluorescent protein variants of GFP, have made molecular tagging a very powerful tool. Yet for some applications DIC optics are still in use – either to minimise photo-toxicity or due to the difficulty in tagging due to the nature of the sample. The light intensity used to record GFP fluorescence using

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CCD cameras is for example higher than that of sunlight, resulting in toxic levels of light being used in live-cell microscopy, which has been circumvented by the use of label-free imaging as evidenced by multiple cell division cycles that can be observed in DIC.^{2,3} Quantification of such image time-series for intracellular dynamics required combining DIC optics with methods to enhance and follow cellular and subcellular structures.

Intracellular transport dynamics has been studied using DIC microscopy referred to initially video-enhanced DIC, VE-DIC that combines DIC optics with fast video camera recordings (60 frames per second, fps) and gain-offset image enhancements applied by Allen et al.⁴ to measure microtubule (MT) dependent movement of intracellular organelles in the marine foraminifer *Allogromia laticollaris* (NCBI:txid71427). VE-DIC enhancement was based on electronic adjustments to the signal, which at the time were challenging for typical cell biology labs.⁵ It was extended to follow the in vitro dynamics of molecular motors transporting beads on immobile microtubule tracks in presence of ATP with nanometre precision.⁶ Coupling receptor proteins to gold-particle linked antibodies and imaging them in time-lapse microscopy, referred to as nanovid microscopy, has allowed for the imaging of single receptor dynamics⁷ and their movement into intracellular compartments like endosomes.⁸ Single particle tracking (SPT) of such microscopy time-series provided individual traces as compared to statistical averages obtained from fluorescence photobleaching recovery (FPR) for diffusive mobility measurement of membrane receptors,⁹ which was applied to follow multiple receptors such as epidermal growth factor receptor (EGFR), transferring receptor and E-cadherin in fibroblasts in culture.¹⁰ The insights from such approaches to improved understanding of cell membrane dynamics are reviewed by Saxton and Jacobson.¹¹ Intracellular mobility measurements were demonstrated by resolving the velocity and directionality of chromosome movement during anaphase in cultured newt lung epithelial cells.¹² Progress in computing allowed the development of computer programs to semi-automatically track and quantify motion.¹³ Since then rapid improvements in computing have driven development in quantifying transport dynamics using DIC time-series.^{14,15} The development of computational frameworks has enabled quantification of transport with nanometre sensitivity as demonstrated for microtubule movement.¹⁶ Combining these approaches for intracellular object detection and tracking will require additional developments in the area of contrast enhancement, to further enhance the utility of DIC for quantifying cellular and subcellular dynamics.

In some cell types, it has been observed that the collective action of transport of organelles leads to a flow of the

surrounding cytoplasm. Such streaming has been observed to span velocities from 0.3 $\mu\text{m/s}$ in *Drosophila* oocytes driven by microtubule-motor interactions,¹⁷ 12 $\mu\text{m/s}$ during bidirectional flows in *Physarum polycephalum* cytoplasm¹⁸ and acto-myosin driven high-speed streaming at 50 $\mu\text{m/s}$ in internodal cells of *Chara* (muskgrass).¹⁹ The physical mechanisms driving cytoplasmic streaming are diverse and are reviewed by Goldstein and van de Meent.²⁰ Most of the measurements of cytoplasmic streaming used a combination of bright-field microscopy with segmentation and tracking based on image contrast. An alternative explicitly 2D and 3D tracking has been to reduce dimensions during streaming using kymograph, projection of movement in time along one spatial dimension with the second being time. Typically kymography of cytoplasmic streaming has used fluorescently tagged markers as seen in case of actin filament flows in mouse meiotic oocytes²¹ and fungal hyphae²², with our lab having developed tools to automate the analysis of kymographs²³. *Caenorhabditis elegans* embryos with high DIC contrast of intracellular structures are also observed to undergo cytoplasmic flows driven by spindle oscillations in anaphase²⁴. Indeed these oscillations were seen to be evolutionarily conserved between related species with small differences in oscillation dynamics²⁵. The movement of the spindle also produces movement in the surrounding yolk granules. In previous work we have combined DIC microscopy with single particle tracking (SPT) to measure such granule movements²⁶ and we proceed to review aspects of our work that could be of wider interest to the study of cytoplasmic flows.

Cell sizes are variable in a populations that arise from multiple causes: noise arising from variability in internal components between individuals and extrinsic as well as differences in cell stages. *Escherichia coli* cells have proved to be a model for such investigations from the perspective of integration of intra- and extracellular processes^{27,28} and tunability²⁹. Measurement of the properties of bacteria has involved image-analysis tool development to segment and follow microbial growth using fluorescence time-series for gene expression dynamics³⁰, MicrobeTracker that takes phase contrast images as inputs³¹, Oufi for tracking touching cells in confluent samples³². While multiple tools have been developed to keep with the improvements in microscopy and developments in DeepLearning-based pattern recognition that can take phase contrast, bright-field and fluorescence microscopy data as inputs³³, they are often unable to quantitatively capture data from DIC images, due to the local gradients that arise from DIC optics. In previous work, we have developed such a method based on image-gradient filtering and edge detection,³⁴ which was applied to examine population size statistics of *E. coli* cells in different growth

conditions³⁵ and allometric scaling of *E. coli*³⁶. In the following sections, we compare the advantages, limitations and perspectives on quantifying single cell morphology using DIC microscopy images.

Here we proceed to review some work done by our lab over the past few years related to quantitative DIC image analysis in the context of (A) bacterial cell shape analysis^{34,35}, (B) microrheology by tracking diffusing beads^{26,37}, (C) quantifying intracellular flows in *Caenorhabditis elegans* by tracking lipid granules²⁶ for intracellular microrheology³⁸ and (D) tracking MT-motor-driven bead motility (unpublished) as described in Figure 1.

2 | CELL SIZE ANALYSIS FROM LABEL-FREE IMAGES OF ROD-SHAPED BACTERIAL CELLS

Previously, we had examined *E. coli* cell morphology in the population using DIC microscopy³⁴ to ask how growth rate altered size distributions in populations of different lab-strains and growth rates³⁵. In order to quantify their sizes, we developed a DIC rod-shaped cell segmentation pipeline that took advantage of an apparent shadow along the long axis by adjusting the analyser angle after the objective, the image gradient. The global gradient of the image in X and Y was then thresholded to detect edges in the image, which mapped to the long axis of individual cells in the population (Poster, panel A). Skeletonisation was used to reduce these contours of multiple cells to a 1-pixel wide outline, the length of which was used as an estimate of cell lengths from the population³⁴. Comparison to manual analysis was used to confirm the error in estimation of lengths was within acceptable limits. Occasionally cells were found to on top of each other, resulting crossovers or branches in terms of the cell skeletons. We proceeded to resolve these by minimising the angle of all possible branch points, based on the assumption, that the least bend in the cells is likely to be the correct path, based on the rigidity of the rod-shaped cell. The cell length distributions was found to follow a log-normal distribution, with an increase in the growth rate resulting in very little change in the average lengths, but showing an increase in the spread of the length distribution³⁵. This initial progress in segmenting cells in DIC led us to attempt time-series analysis to understand dynamics.

3 | DIC OBJECT TRACKING (DICOT) PIPELINE FOR IN VITRO AND IN VIVO MOBILITY MEASUREMENT

The segmentation of high contrast intracellular organelles has been widely used to measure both flows¹⁸ and rheolog-

ical properties of the cytoplasm³⁹. The approach of particle tracking based microrheology has been comprehensively reviewed in terms of the utility measure mechanical properties of cells beyond simple viscosity and flow⁴⁰. The use of endogenous cellular structures that provide sufficient contrast in a label-free method like DIC provides an opportunity to perform such microrheology, provided the image-analysis can be optimised. In recent work, we had developed a pipeline where multiple modifications of Gaussian filters like Laplacian and difference (LoG and DoG) filter were compared to a novel scaling of Gaussian SoG. These were tested on both in vivo granule detection in *C. elegans* embryos and polystyrene beads in vitro. This automated detection and tracking pipeline for DIC object tracking (DICOT) was tested for positional detection error as well as precision, sensitivity and F1-score with SoG filtering showing improvements over others (Poster, panel 'Tool')²⁶. We proceeded to apply this pipeline to a few problems of motion tracking of micron-scale structures as described in the following sections.

4 | TRACKING DIFFUSIVE BEAD MOTILITY IN VITRO AS A MEASURE OF VISCOSITY

Experiments described in a previous report by us²⁶ were repeated to obtain time-series movies in DIC of 1 μ m diameter polystyrene beads³⁷. The movies were analysed to generate tracks which are overlaid on the first frame of the bead image, demonstrating random motion (Poster, panel B). The mean square displacement (MSD) shows a decrease with increasing concentration of the visco-gen, and as previously described, when fit to the relation $MSD = 4Dt^\alpha$, where D is the diffusion coefficient, t the time interval and α the anomalous diffusion exponent, as described in previous reports by both us^{26,41} and others^{42,43}. The estimate of D is combined with Stokes–Einstein relation to obtain a viscosity value, that matched other independent measurements of the same samples. Using this as a validation tool for estimating fluid properties, we proceeded to apply the method in vivo to estimate cytoplasmic viscosity.

5 | TRACKING CYTOPLASMIC GRANULE OSCILLATIONS IN *C. ELEGANS* ONE-CELLED EMBRYOS FROM DIC TIME-SERIES

Caenorhabditis elegans embryos contain a high density of birefringent inclusions, yolk granules, visible in DIC microscopy. We proceeded to examine the motion of



Quantification of cell shape, intracellular flows and in vitro transport based on DIC object detection and tracking

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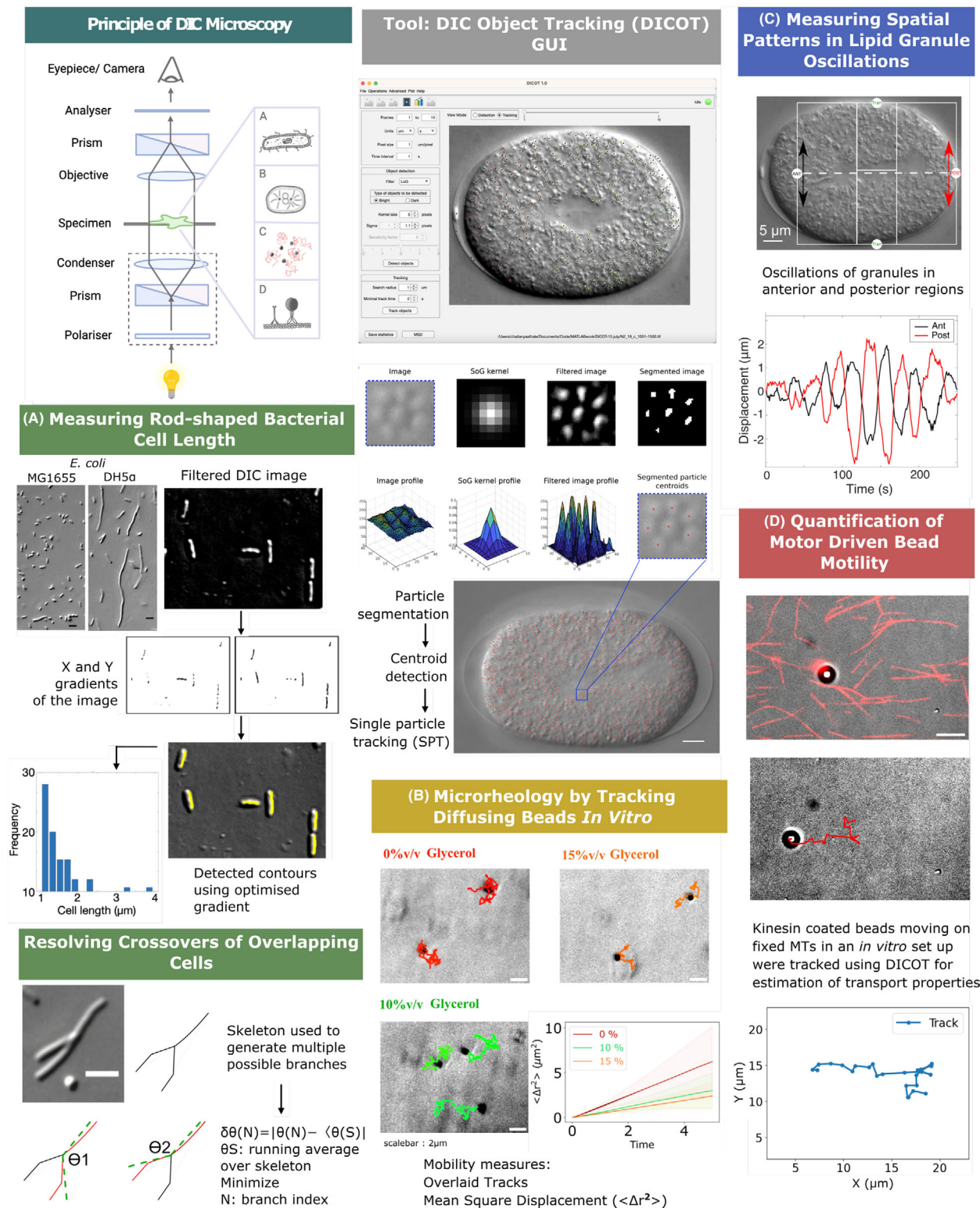


FIGURE 1 The application of DIC image analysis and tracking is illustrated in the context of (A) bacterial cell shape analysis, single particle tracking of (B) diffusing particles, (C) oscillatory movement of cytoplasmic yolk granules and (D) motor driven bead motility.

these granules as a marker of cytoplasmic mobility as described in our study describing the analysis pipeline²⁶. DIC time-series data of multiple one-celled embryos of nematodes related to *C. elegans* were taken from a previous study²⁵. The embryo geometry is identified in terms of the Ant(erior), Post(erior) and T1-, T2-transverse regions (Poster, panel C). The dashed line between the anterior–posterior (AP) regions indicates the AP axis. The DICOT pipeline resulted in yolk granules positions over time in all regions. The position of representative granules in the Ant- and Post-regions were plotted using only the coordinates on the axis orthogonal to the AP axis (Poster, panel C, colored arrows) as a function of time, resulting in regular oscillation that are offset by half a period (Poster, panel C). In our analysis, we found the frequency of oscillations of was ≈ 40 mHz, comparable to the reported frequency of spindle (centrosome) oscillations²⁶.

6 | TRACKING ACTIVE TRANSPORT OF MOLECULAR MOTOR-DRIVEN TRANSPORT OF BEADS IN VITRO ON MT TRACKS

As a further extension of the utility of DIC tracking, we examined the transport of a GFP-tagged 1-560 amino acid truncated human neuronal kinesin-1 heavy chain from Ron Vale (AddGene id: pET17_K560_GFP_His URL: <http://n2t.net/addgene:15219>). In this setup, GFP-kinesin was bound to biotinylated anti-GFP nanobody (Addgene:61838) as described previously⁴⁴ that was in turn bound to streptavidin-coated Dynabeads (M-270 Strep-tavidin, Invitrogen, Norway). Taxol stabilised MTs were assembled using a 3:1 ratio of 30 μ M goat brain tubulin with rhodamine-labelled goat brain tubulin in BRB80 and 10% glycerol, as described in our earlier work on 'gliding assays'⁴⁵. MTs were bound to cleaned glass double-backed tape chambers, passivated, the beads flowed in with ATP and imaged for every 5 min at 37

C in DIC in presence of an Antifade mix described in our previous work⁴⁵. Tracking the bead resulted in a directed trajectory (Poster, panel D, unpublished data). The measured velocity is comparable to the reported single molecule velocity of this minimal kinesin of 0.2–0.7 μ m/s⁴⁶. Using such a setup can help trace both the movement of beads as a mimic of organelle transport using a combination of correlative DIC and fluorescence microscopy combined with DIC object tracking.

7 | PERSPECTIVE

We have described some of our work that leverages DIC image intensity gradients to (A) automate the detection

of bacteria and quantify cell lengths in *E. coli* as described in our previous work^{34,35}. We go on to demonstrate the development of a modified Gaussian that we refer to as scaling of Gaussian (SoG) filter combined with tracking in the form of a GUI tool²⁶ that has been combined made OpenSource (<https://github.com/CyCelsLab/DICOT>) in the hope of allowing further improvements to the method by others in the field, along with testing the reproducibility of our methods. We apply this tool to analyse (B) DIC microscopy time-series analysis of polysytene bead diffusion³⁷, (C) *C. elegans* yolk granule mobility in vivo³⁸ and (D) in vitro transport of kinesin-bound beads on immobilised MTs (unpublished). The comparison between diffusion and MSD measurements that emerge from using the DICOT tool both on in vitro and in vivo data suggest the generality of the approach and the usability of the tool. Such morphological and dynamical measurements made with minimal sample modification suggest that even in future, DIC label-free microscopy and its analysis will continue to be a useful tool for cell biology and biophysics.

AUTHOR CONTRIBUTIONS

TK ran the cell shape analysis code and modified the poster. DK wrote parts of the DIC object tracking DICOT code and analysed the data. JB and performed the bead diffusion experiments following our previous work and made figures corresponding to them. SAY performed the unpublished kinesin-driven bead motility assay and made parts of the figure. CAA acquired funding, supervised the project, wrote the bacterial gradient filter code and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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