EFFECT OF MOTOR PROTEINS ON MICROTUBULE GROWTH AND NUCLEAR MIGRATION

By

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To my family

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LIST OF ABBREVIATIONS

AAA	ATPase associated with various cellular activities
ADP	Adenosine 5’ Diphosphate
ATP	Adenosine 5’ Triphosphate
CCD	Charge-Coupled Device
DBS	Donor Bovine Serum
DMEM	Dulbecco’s Modified Eagle Medium
E	Elasticity
EB1	End Binding Protein 1
EGFP	Enhanced Green Fluorescent Protein
EI	Flexural Rigidity
GDP	Guanosine 5’ Diphosphate
GFP	Green Fluorescent Protein
GMPCPP	Guanosine Monophosphate– Carboxypiperazin-4-yl-Propyl-1-Phosphonic Acid
GTP	Guanosine 5' Triphosphate
I	Moment of Inertia
KHC	Kinesin Heavy Chain
KIF5	Kinesin superfamily protein 5
L_p	Persistence Length
LINC	Linker of Nucleoskeleton to Cytoskeleton
MT	Microtubule
MTOC	Microtubule Organization Center
NA	Numerical Aperture
NIH	National Institute of Health
PBS	Phosphate Buffered Saline

RNA	Ribonucleic Acid
ROI	Region of Interest
SEM	Standard Error of the Mean
+TIP	Microtubule plus end tracking proteins

Abstract of Thesis Presented to the Graduate School
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EFFECT OF MOTOR PROTEINS ON MICROTUBULE GROWTH AND NUCLEAR MIGRATION

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Microtubules are highly dynamic protein polymers that play a critical role in cell division, migration and intracellular transport. The mechanical properties of microtubules are important in enabling these diverse functions. Many *in vitro* experiments suggest that microtubules under thermal forces have a persistence length on the order of millimeters however*; in vivo* investigations show that these microtubules exhibit bends on micron length scales. This suggests that within a living cell, microtubules are exposed to large non-thermal forces mediated by motor proteins and other cytoskeletal elements. However the nature and magnitude of these forces still remain unknown.

We wished to gain a fundamental understanding of the effects of non-equilibrium forces exerted by motor proteins like dynein and kinesin on growing microtubules. By analyzing the motion of microtubule tips in control and dynein-inhibited NIH-3T3 fibroblasts expressing EGFP-EB1, a fluorescently labeled end binding protein, the trajectories of growing microtubules were reconstructed. Shape fluctuations in microtubule trajectories of dynein-inhibited cells were far smaller when compared to control cells. We employed methods rooted in Fourier analysis to quantify the fluctuations in both conditions. We found that during growth, the variances in the mode

amplitudes are much larger for microtubules in control cells compared to dynein-inhibited cells. Our results suggest that dynein can exert lateral, fluctuating forces on a growing microtubule tip and may play a role in controlling the shape of growing microtubules.

The migration and the positioning of the nucleus are physiologically critical for cellular development and structural integrity of many organisms. Two types of nuclear movement are commonly observed in cells; rotation and translation. Dynein has been shown to cause nuclear rotation in NIH-3T3 fibroblasts. We investigated if it also played a role in nuclear translation. We found that directional nuclear migration was unaffected by the inhibition of dynein. Additionally, we observed that the nucleus moved significant distances without the detachment of the trailing edge and any significant changes in cell shape in both control and dynein-inhibited cells. Collectively, our results argue against a role for dynein in nuclear translation.

CHAPTER 1
INTRODUCTION

Cytoskeleton

The cytoskeleton is a three-dimensional filamentous network that extends throughout the cell[1]. It establishes a framework for cellular organization and is the primary determinant of cell shape and polarity[2,3]. The cytoskeleton is composed of three major filament systems; actin filaments; microtubules and intermediate filaments[4]. Each of these is composed of linear filaments of thousands of identical protein molecules[4]. In addition, individual cytoskeletal elements can associate with other accessory proteins that stabilize the structure and with molecular motors that transmit and generate forces[1]. Forces mediated by motor proteins in particular are critical for transport of cargo to different parts of the cell[5].

The microtubule cytoskeleton is an integral component of the eukaryotic cytoskeleton (Figure 1-1). It is composed of individual hollow filaments that nucleate from the Microtubule Organizing Center (MTOC) near the nucleus and radiate outward towards the cell periphery[4].

Owing to their structure, microtubules have a high rigidity and hence can bear significant loads[6]. It is for this reason, they are key components in cell development and maintenance of cell shape[7]. Microtubules can also associate with other proteins to form cilia and flagella that propel a cell through the surrounding environment[8]. During mitosis, microtubules form the mitotic spindle that help in chromosome segregation between the daughter cells[9]. During the interphase period of mitosis, they function as tracks for cargo transport[10]. Molecular motors of the dynein and kinesin family facilitate the movement of cargo along these tracks.

Microtubule Cytoskeleton

Microtubule Structure

Microtubules are non-covalent linear polymers comprised of repeating units called tubulin. Tubulin is a heterodimeric protein that has two subunits α and β, each having a molecular mass of 50 kDa[4] (Figure 1-2a). The molecular structure of the α and β subunits are quite similar to each other and both subunits have the ability to bind to GTP[5]. They are linked in a way that the GTP molecule on β subunit is exposed to water while that on the α subunit is buried inside the dimer[1]. Since only the GTP on the β subunit can be hydrolyzed, the heterodimers associate in a linear fashion to form a protofilament as depicted in Figure 1-2a[4]. Around thirteen protofilaments associate laterally to form a hollow cylindrical tube of 25 nm diameter[11] (Figure 1-2b).

The monomers associate in a -$(\alpha\text{-}\beta)_n$- like configuration[1]. This imparts a polarity to the microtubule; with the plus end found on the β subunit and the minus end on the α subunit[5] (Figure 1-2b). In a typical cell, the minus ends point inwards and are anchored at centrosome or the MTOC while the plus ends radially extend outwards to explore the cell[5].

Microtubule stability and structure is regulated by the nucleotide state of the β subunit[5] (Figure 1-2c). Electron micrographs show that growing microtubules are relatively straight while depolymerizing protofilaments splay outward at the tip[11]. Experiments with GTP and GDP bound β tubulin showed that single protofilaments made with GTP- β tubulin were straight while those with GDP- β tubulin were curved[5]. In another study, microtubules grown under the presence of slowly hydrolyzed GTP analog GMPCPP were highly stable[12]. It was thus inferred that growing filaments terminate with GTP- β tubulin. The GTP bound to tubulin units down the length of a

growing microtubule could hydrolyze to GDP[4]. The energy released during hydrolysis is stored within the lattice and can be used to do work during depolymerization[5]. The GDP bound tubulin units are held in place by lateral interactions with neighboring units and by the presence of a GTP-cap[13]. The GTP cap whose exact size is not known is also thought to provide stability to sustain growth[13]. It is postulated that the loss of this cap switches the microtubule to a shrinking phase by a process termed 'catastrophe'[14].

Microtubule Associated Motor Proteins

The radial organization of microtubules makes them suitable for long-range transport within cells. Molecular motors use microtubule as tracks and energy derived from ATP hydrolysis to drive the movement of different cargoes to various parts of the cell[5]. Based on the structural features, microtubule motor proteins are divided into two categories, dynein and kinesin[15]

Dynein family

There are two main classes of dynein motors; cytoplasmic and axonemal[16]. While cytoplasmic dyneins are involved in minus-end directed cargo transport, axonemal dyneins are responsible for microtubule sliding in ciliary and flagellar movement[16]. Cytoplasmic dynein (hereafter referred to as dynein) is the largest and most complex of all the three known eukaryotic molecular motors. But in terms of diversity, the dynein family is small and well conserved compared to the kinesin family[17].

Dynein has a molecular mass of 1.2 MDa[18]. Its structure consists of two identical heavy chains, three intermediate chains and four light intermediate chains[19] (Figure 1-3a). The heavy chain houses the ATP hydrolysis and microtubule binding domains[20]. The motor domain on the C-terminal of the heavy chain is made up of six AAA domains arranged in a ring[21]. The first four AAA domains (AAA1-AAA4) are capable of binding to

ATP[21]. The hydrolysis of ATP in AA1 is crucial for movement while the other sites may have role in regulation of ATPase cycle and movement of dynein along the microtubule[21]. Emerging from the AAA4 and AAA5 is the 10-25 nm long coiled-coil stalk with the microtubule-binding domain at the tip[22]. The tail of the heavy chain is involved in the recruitment of several light chains. The light chains are proposed to have a regulatory function.

Through coordination among its motor domains, dynein walks processively along the microtubule. The motion of a motor protein is processive when it can take many consecutive steps along a filament without dissociation[23]. Dynein binds to a multisubunit protein complex called dynactin that is proposed to help dynein bind to various cargo molecules and assist in the motor's processive motion[1]. Dynein has the unique ability to take steps of varying sizes. Steps sizes between 4-32 nm have been observed till date[21]. In addition, dynein has also been observed to move to adjacent protofilaments[24].

Dynein has been implicated in many fundamental cellular processes like cargo transport, nucleus orientation, cell division, chromosome movement and centrosome positioning[25–27]. Dynein aggregates cargo molecules by carrying them towards the cell center[27].

Kinesin family

Unlike dynein, kinesins are a much more diverse family of proteins. There are over 45 types of kinesins known till date that have been divided into 15 groups[28]. Depending on the family, kinesins can have a wide variety of functions ranging from cargo transport, microtubule depolymerization, cell division, and mitotic spindle organization[28,29]. Depending on the type, kinesins have been observed to move towards both ends of the microtubule while dynein has been observed to move only to the minus

end[17]. Our study will focus on the main member of the kinesin family; kinesin-1 or KIF5 (hereafter referred to as kinesin)

Kinesin is a homodimer where each monomer contains a heavy chain (120 kDa) and an associated light chain (64 kDa)[30]. Kinesin is about 70 nm in length[31]. Each heavy chain consists of two domains joined by a coiled coil stalk[21]. While the N-terminal motor 'head' domain of the heavy chain hydrolyses ATP to ADP and binds to the microtubule; the C terminal domain interacts with the light chain, which in turn associates with linker molecules to mediate the binding of cargo molecules[32,33]**.** During organelle transport, kinesin moves an average of 8.3 nm along a microtubule for every ATP hydrolyzed at a speed of 800 nm/s[34]. Optical trap experiments have shown that kinesin stepping is hindered by the application of a force of magnitude 6 pN[35].

The contrast in directionality of motor proteins can be reflected in their functions. While kinesin disperses the cargo molecules by moving them from the cell center to peripheral regions of a cell, dynein aggregates the cargo molecules by carrying them towards the cell center[36]. In addition, kinesin has a role in organelle transport, regulation of microtubule dynamics and cell division[28,29,36].

Forces That Could Deform Microtubules

Microtubules have been thought to be important in maintaining the structural integrity of cells[37]. Indeed to perform its diverse roles, the microtubule network needs to be resistant to deformation[7]. The flexural rigidity is defined as a filaments resistance to bending due to thermal fluctuations and is commonly expressed as EI where E and I are the microtubules elastic modulus and moment of inertia[6]. The bending of filaments can also be discussed in terms of the persistence length, the natural length scale beyond which a filament exhibits bends due to random thermal fluctuations[11]. The

persistence length denoted as L_p is represented as $\frac{EI}{k_B T}$ where T is the temperature and k_B is Boltzmann's constant[11].

Since its mass is distributed along a hollow annulus, microtubules have a large second moment of area or moment of inertia. Due to this structural feature, microtubules have a higher flexural rigidity when compared to other filaments of the same mass. Using Fourier mode analysis, the flexural rigidity and thermal persistence length of microtubules have been estimated to be $2.2*10^{-23}$ Nm^2 and 5200 μm[6]. Experimental studies of mechanical properties of microtubules using methods that include optical tweezers and atomic force microscopy have also verified this[38,39].

However despite their high bending rigidity, the microtubule network in cells is highly buckled and bent[40] (refer Figure 1-1). This suggests that microtubules experience significant mechanical forces in cells that are non-thermal in nature[7]. Microtubules are embedded in the cellular cytoplasm. Hence they could experience forces from other cytoskeletal elements that include actin and intermediate filaments. Forces generated by microtubule associated motors could cause significant bending of microtubules[37,41]. Finally microtubule polymerization forces could also play a role in bending and buckling. Each of these possibilities is discussed below in detail.

Polymerization forces

Microtubules are highly dynamic structures that alternate between periods of growth and shrinkage with rare occurrences of a small rest period in between[14]. This behavior is called dynamic instability[42]. The interconversion between phases is thought to be a stochastic process[43]. Dynamic instability allows rapid reorganization of the microtubule cytoskeleton to allow them to explore the entire cellular space[7]. It also

allows the microtubule plus end to probe the local environment, attach to structures like kinetochores of chromosomes and play a role in cell signaling, polarity and migration[44].

Microtubules continue to polymerize even after reaching the cell periphery; the addition of tubulin units at this barrier can generate pushing forces[45]. The addition of tubulin units to growing ends of microtubules *in vitro* generates enough force to buckle the microtubule[46]; however whether these forces are large enough for microtubules *in vivo* to buckle is unknown. These compressive polymerization forces have been proposed to play a role in stabilizing the cell shape. It was observed that even when depolymerizing, free microtubules can increase in curvature; suggesting that forces other than polymerization can deform microtubules *in vivo*[41].

Actomyosin derived forces

Through cross linking proteins like plakins and plectins, microtubules in the cytoplasm can be physically linked to other cytoskeletal networks like actin microfilaments and intermediate filaments[47]. Forces transmitted from these structures could influence the growth and dynamics of microtubules. Actin-based motors like myosin II and myosin V have been proposed to drive the buckling of microtubules.[41]. Acto-myosin retrograde flow in newt lung epithelial cells has been reported to influence the bending and buckling of microtubules[48].

It was recently proposed that the surrounding elastic cytoskeleton can mechanically reinforce the microtubules to bear enhanced compressive loads[49]. A fraction of microtubules in cardiomyocytes were seen to buckle and unbuckle with every contraction and relaxation of the cell[49]. This suggests that only a small fraction of microtubules are coupled to the elastic elements in a cell. In LLC-PK1 cells, actomyosin contraction forces play an inconsequential role in microtubule bending since the

microtubules were observed to be bent even with the F-actin network being stationary and with the inhibition of myosin II activity with blebbistatin[41].

Molecular motor derived forces

Since motor proteins 'bind' and 'walk' on microtubules, it is plausible that they can directly exert forces that influence microtubule growth. Wu *et al.* (2011) have shown that dynein can cause bending of severed minus-ended microtubules in living cells[26]. In addition, RNA interference of kinesin-1 heavy chain in *Drosophila* S2 cells eliminated lateral movement and looping of microtubules[37]. Taxol stabilized microtubules sliding over kinesin coated surfaces were occasionally seen to bend and buckle[50]. However results from gliding assays need not necessarily compare to microtubule behavior in living cells.

Microtubules are seen to bend as they grow in cells[7]. Moreover the fluctuations in the amplitudes of the microtubule shape are roughly hundred times larger than what would be expected by thermal forces alone[7]. This suggests that a significant source of chemical energy is driving the lateral fluctuations of growing microtubule tips. Since molecular motors directly interact with microtubules, we tested the hypothesis that dynein causes fluctuations in the growing tips of microtubules.

To test this hypothesis, we analyzed the motion of microtubule tips in NIH-3T3 Fibroblasts expressing EGFP-EB1, a fluorescent +TIP protein that specifically binds to the growing ends of microtubules. We tracked the position of tips in these cells and reconstructed the trajectories using plusTipTracker, an open source software[51]. We did the same analysis in cells where dynein was inhibited using DsRed-CC1. Our results suggested that dynein contributes significantly to the bending of growing microtubules. Microtubule trajectories in dynein-inhibited cells appeared to be a lot straighter than

control cells. To further investigate the role of dynein, we performed a Fourier analysis of hundreds of reconstructed trajectories. Our results indicate that dynein makes a significant contribution to the fluctuations at higher wavelengths.

Molecular Motors and Nuclear Movement

Cell polarity and migration are essential for the proper functioning of cells. A number of functions like cell and tissue development, differentiation, would healing, cancer metastasis and immune response are dependent on the directional migration of cells[52]. Defects in cell migration and polarity can lead to a variety of diseased states[53].

Cell polarity is established by the anisotropic compartmentalization of proteins that lead to certain morphological changes. Migrating eukaryotic cells have a typical structure that predicts their direction of movement. They have a rich actin network at the leading edge that forms active ruffling lamellipodia and filipodia, a thin trailing edge, a centrosome roughly located at the cell centroid and a nucleus positioned just behind the centrosome[54]. The cell travels persistently in a given direction by continuously forming new actin-rich protrusions at the stable leading lamella while using actomyosin tension to detach and retract its trailing edge[54].

While migrating, the cell brings about coordinated motion of all its organelles. Of particular importance is the nucleus since the cell isolates all its genetic information in it. During migration, the cell moves the nucleus and maintains its position close to the cell center. On the cellular length scales, the nucleus is massive (~10-15 μm in diameter) and stiff relative to the cytoplasm[55]. Motion of such a large object in the crowded intracellular space requires a significant expenditure of energy and is an important task for the motile cell.

Nuclear rotation about an axis and directional translation of the nuclear centroid are the two typical nuclear motions observed in all cell types. The cell accomplishes nuclear motion by transferring active cytoskeletal forces onto the nuclear surface through nuclear embedded tethers called LINC complex proteins (for linker of nucleoskeleton to cytoskeleton[56,57]. The LINC complex links the various cytoskeletal elements to the nucleus[56]. Disruption of the LINC complex results in a off-center nucleus and hinders the ability of cells to move[58,59]. Precisely how the different cytoskeletal elements coordinate to bring about nuclear movement is not clear.

Early experiments showed that microtubules were essential for the establishment of cell polarity. Cells treated with nocodazole, a microtubule depolymerizing drug showed impaired forward movement[60]. Following wounding, cells migrated from the wounded edge with the centrosome positioned roughly between the nucleus and the leading edge[61]. Observation of GFP-tubulin dynamics in such cells showed that the nucleus moved away from the leading edge while the centrosome remained stationary[62].

Microtubules in wounded cells tend to be polarized along the axis of cell migration; post-translationally modified stable microtubules are oriented with the plus ends facing the leading edge[63]. Since these arrangements occur after wounding, it is possible that they might influence actin dynamics at the leading edge. The lamellipodia generally consists of a dense actin network; however at times microtubules are seen to extend to the cell edge[64]. It was observed that the protein Rac1 promoted the growth of ‘pioneer’ microtubules at the leading edge that have a decreased catastrophe frequency and spend a great time in the growth phase[48]. In addition to actin and leading edge

dynamics; microtubules can influence trailing edge detachment by interacting with actin filaments, myosin II and focal adhesions[65–67].

Inhibition of dynein is known to interfere with centrosome positioning[68]. It also impaired cell motility and the rotation of the nucleus[25,69,70]. Since it affects nuclear rotation, can dynein activity also cause translational motion? It is possible that dynein physically links the microtubules to the nucleus; thus driving nuclear movements like rotation and translation.

We explored the role of dynein activity on the directional motion of the nucleus in single polarized NIH-3T3 fibroblast cells. Our experiments showed that the nucleus moved significant distances without any detachment of the trailing edge and any significant change in cell shape. The nuclear movement correlated strongly with the movement of the cell centroid. No significant changes in either of these results were observed in dynein-inhibited cells. Thus our results argue against a role for dynein in nuclear translation.

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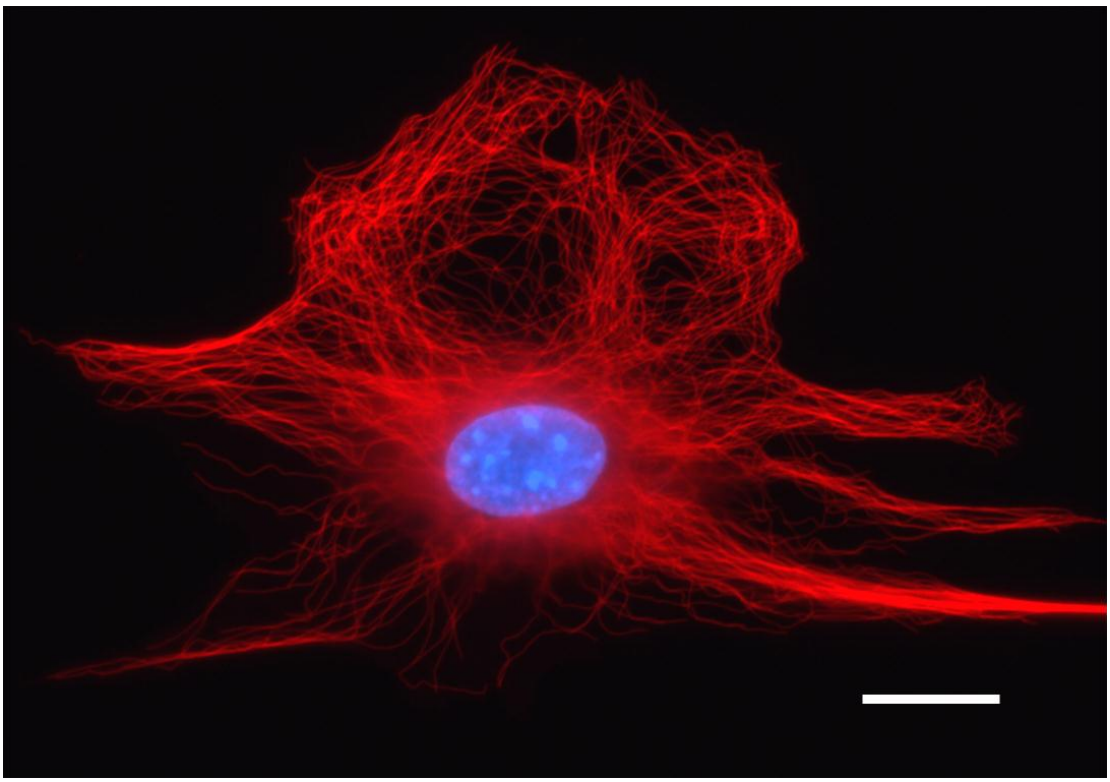

Figure 1-1. Microtubules have a wavy and buckled appearance in interphase NIH-3T3 fibroblast cells. The microtubule network was imaged by staining for tubulin (in red) and the nucleus is stained in blue. Scale bar-10 μm (Image courtesy: Jun Wu, Department of Chemical Engineering, University of Florida)

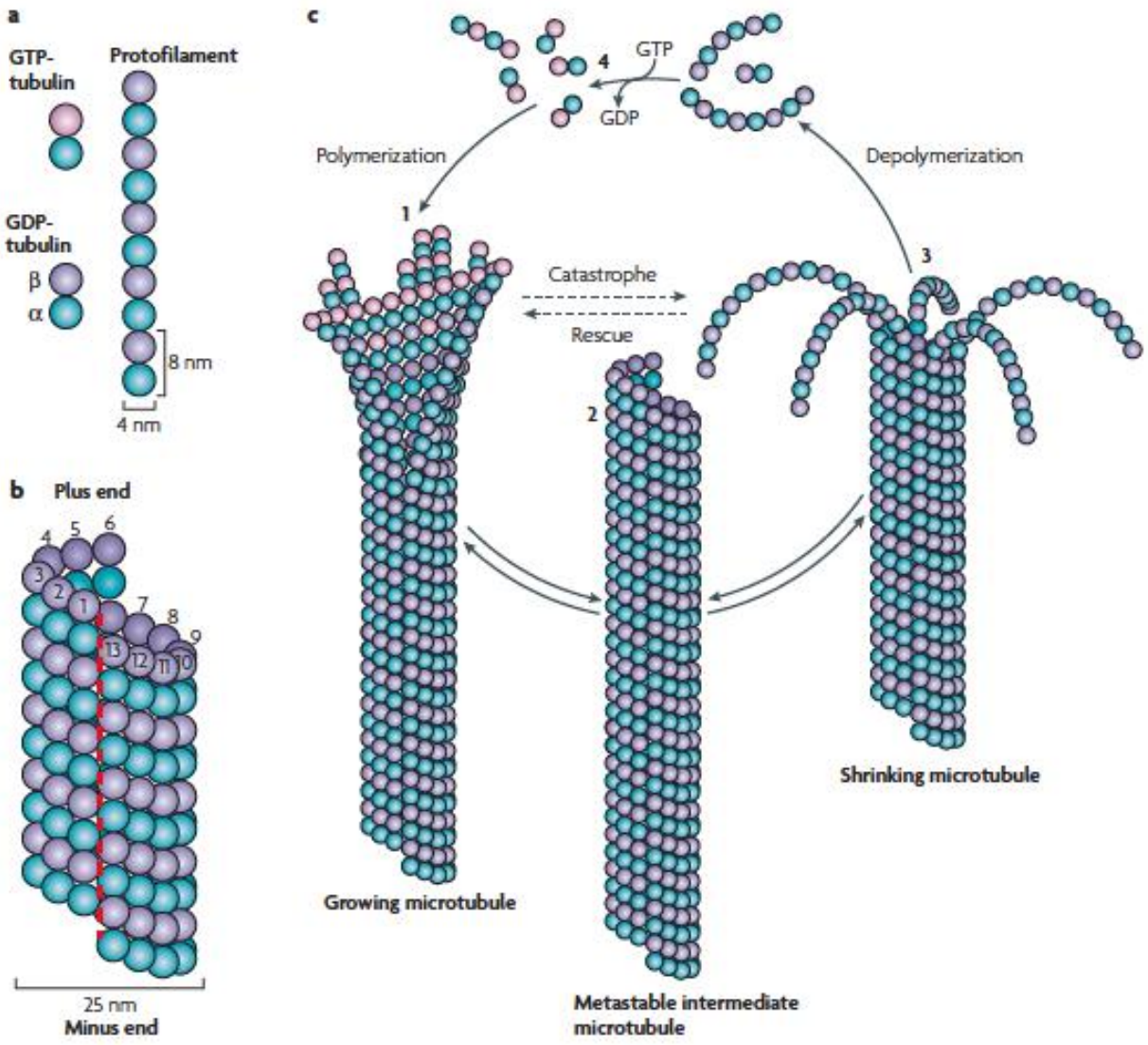

Figure 1-2. Microtubule structure and dynamic instability. (a) Microtubules are composed of tubulin heterodimers that align in a linear head to tail fashion to form a protofilament. (b) In living cells, the cylindrical microtubule wall typically comprises 13 parallel protofilaments. The 12-nm helical pitch in combination with the 8-nm longitudinal repeat between the tubulin subunits along a protofilament generates the lattice seam (red dashed line). (c) Polymerization and depolymerization of microtubules is driven by the binding, hydrolysis and exchange of a guanine nucleotide on the β-tubulin monomer (GTP bound to α-tubulin is non-exchangeable and is never hydrolyzed). GTP hydrolysis is not required for microtubule assembly per se but is necessary for switching between catastrophe and rescue. (Adapted by permission from Macmillan Publishers Ltd: *Nat. Rev. Mol. Cell Biol*., Akhmanova, A. & Steinmetz, M. O. Tracking the ends: a dynamic protein network controls the fate of microtubule tips, **9**, 309–322, © (2008))

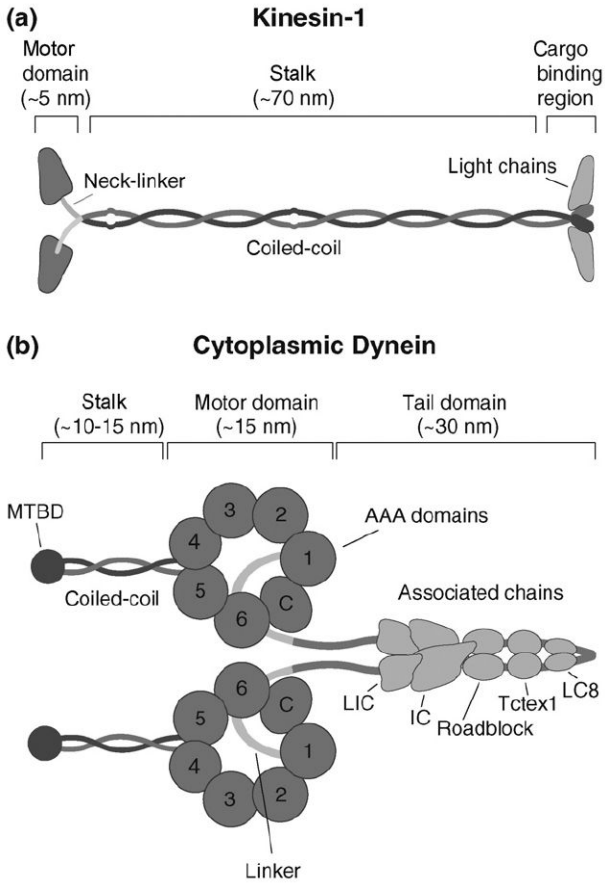

Figure 1-3. Schematic representations of kinesin and dynein and associated chains (a) Kinesin structure. Kinesin is a homodimer consisting of two identical heavy chains and two light chains. The heavy chain contains an N-terminal motor 'head' domain that possesses catalytic and MT-binding activity, a neck-linker element that connects the motor domain to the common coiled-coil dimerization domain, and a C-terminal light chain and cargo-binding region. (b) Cartoon representation of dynein. Dynein is composed of two identical heavy chains and several associated chains. The heavy chain forms a C-terminal catalytic motor domain that consists of multiple AAA ATP-binding sites (1–4) and a coiled-coil stalk with the microtubule-binding domain (MTBD) at its tip; AAA domains 5 and 6 do not contain sequences associated with nucleotide binding and the C-terminus (C) does not contain sequences characteristic for AAA proteins. The motor and dimerization domains are joined by a linker element. Multiple associated chains bind to dynein's tail domain (LIC, light intermediate chain; IC, intermediate chain; Roadblock, Tctex1 and LC8, light chains). ("Reprinted from *Curr. Opin. Cell Biol*, 21/1, Gennerich, A. & Vale, R. D., Walking the walk: how kinesin and dynein coordinate their steps, 59-67, © (2009), with permission from Elsevier")

CHAPTER 2
MOTOR FORCES ON GROWING MICROTUBULE TIPS

The microtubule cytoskeleton is characterized to be a stiff and rigid network which is straight on millimeter length scales under the action of thermal forces alone[6]. However instead of having a straight shape, microtubules in living cells have bends and buckles on micron length scales[7]. The Fourier mode amplitudes of trajectories reconstructed from tracking growing microtubule tips are much too large to be accounted for by thermal forces alone[7]. These experiments suggest that microtubules experience large non-thermal forces in cells. The potential sources of these forces have been outlined in the previous section.

Recent experiments have shown that dynein motors distributed along the microtubule filament pull on microtubules in living cells[26]. New minus ends of laser severed microtubules were seen to increase in curvature[26]. Conversely, similar experiments in dynein-inhibited cells showed that the microtubules rapidly straightened[26]. This suggests that microtubules are under tension due to pulling forces mediated by dynein motors. Could these motor proteins also cause bends in growing microtubules?

We hypothesize that molecular motor forces are required for fluctuations in growing microtubule tips. Using a MATLAB software, we have reconstructed the shapes of growing microtubules in control cells and in cells where motor activity has been inhibited. Our results suggest dynein motor activity contributes significantly to the bending in growing microtubules. In the absence of dynein, the microtubules appeared to grow straight while in the presence of dynein, there was significant variance.

Materials and Methods

Cell Culture, Plasmids and Transfection

Swiss NIH 3T3 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Manassas, VA) with 10% Donor Bovine Serum (DBS) (Gibco, Grand Island, NY). The cells were maintained at 37^{o} C. in humidified 5% CO_2. For microscopy the cells were plated on 35 mm glass-bottom dishes (WPI, Sarasota, FL) and allowed to spread overnight at 37^{o} C and 5% CO_2. The glass bottom dishes were coated with 5 µg/ml fibronection (BD Biocoat^{TM}, Franklin Lakes, NJ) and kept in 4^{o} C overnight.

For control experiments the cells were transiently transfected with EGFP-EB1 (Addgene) and incubated for 18-24 hours prior to plating. For dynein inhibition experiments, cells were additionally transfected with plasmid DsRed-CC1 (gift by Prof. Trina A. Schroer, Johns Hopkins University). For kinesin inhibition experiments, cells were transfected with mCherry-KHC (gift by Prof Kristen Verhey, University of Michigan).

The protein expressed by DsRed-CC1 renders dynein inactive by competitively binding to it. This prevents dynein from forming a complex with dynactin thereby inhibiting the binding and transport of cargo. mCherry-KHC can inhibit kinesin by either inhibiting the motor during transport, inhibiting binding of cargo or preventing the dimerization of the motors. Transient transfection of plasmids into NIH-3T3 fibroblasts was performed with Lipofectamine^{TM} 2000 transfection reagent (Life Technologies, Invitrogen, Carlsbad, CA).

For fixing samples, the cells on glass bottom dishes were treated with 4% Paraformaldehyde in Phosphate Buffered Saline (PBS) without calcium and magnesium (Mediatech, Manassas, VA). Fixed samples were stored in PBS at 4^{o} C.

Confocal Microscopy

The cells were observed on a Leica SP5 DM6000 confocal microscope equipped with a 63X oil immersion objective. During microscopy, cells were maintained at 37 ˚C in a temperature, CO_2 and humidity controlled environmental chamber. In order to image EGFP-EB1, 488 nm laser with 10% power and an appropriate GFP bandpass filter was used. For dynein and kinesin inhibition studies, expression of DsRed-CC1 and mCherry-KHC was confirmed using epifluoresence microscopy through the eyepiece and a single confocal image was recorded using 543 nm laser at 40% power. Images were taken at a resolution of 1024*1024 and with a speed of 400 Hz. Images were taken for 2-3 minutes at a rate of 3 seconds/frame. The images were exported onto Windows 7 computer using LAS AF Lite (Leica Systems) software.

Trajectory Analysis

Microtubule trajectories were constructed from the analysis of EB1 movies using plusTipTracker, a MATLAB based open source software package that combines automated tracking, data analysis and visualization tools for movies of fluorescently labeled microtubule plus end binding proteins (+TIPs)[51]. Version 1.1.1 of the software was downloaded through http://lccb.hms.harvard.edu and was run on MATLAB R2011b in a Windows operating system. The software detects EB1 comets by application of locally optimal thresholds using a watershed-based technique. The track reconstruction uses a spatially and temporally optimal framework described elsewhere[71]. Tracking is done in two steps; linking of comets in consecutive frames to create growth subtracks

and linking of collinear subtracks into compound tracks. Optimization is achieved by solving a linear assignment problem. The links that minimize the global cost among all candidate pairs of growth tracks is chosen.

Tracking Parameters

Tracking parameters were set through visual inspection of track overlays on movies. The same parameters were used for all movies in the dataset. For this study since only MT growth events were of interest parameters were chosen such that fgaps or bgaps weren't included in the trajectory. Maximum Gap Length=0 frames; Minimum Track length=4 frames; Search Radius Range=7-15 pixels; Max Shrinkage factor=1.5; Maximum Forward angle=30; Maximum Backward angle=10; Fluctuation radius=1.0.

Image Quality and ROI

Normally all the images were taken for analysis. If the effects of photobleaching were seen to be significant; the latter frames in a set were discarded. The images were loaded onto ImageJ prior to analysis by plusTipTracker. The images were smoothened using the Gaussian Blur function. For analysis in plusTipTracker, the whole cell was manually selected using the Region of Interest (ROI) tool in plusTipGetTracks. To eliminate the compression forces that a growing tip might encounter at the cell periphery, the ROI did not include the cell periphery.

Trajectory Selection

Using a code developed in MATLAB R2011b that inputted the projData file (meta folder), track ID numbers of trajectories that travelled a certain distance were loaded. These track ID numbers were then inputted in the 'Make Track Movie' tool of the plusTipSeeTracks function to visually inspect the accuracy of the tracking. Only those trajectories that successfully met the criteria were used for further analysis.

Trajectory Plots

To generate trajectory plots, the tracks were further processed using a code developed in MATLAB R2011b. To see if dynein indeed causes fluctuations in growing microtubule tips, the trajectories needed to have the same initial direction. To reduce uncertainity due to positioning error, the initial direction of the trajectory was determined by fitting a line to the first four points in the trajectory. Thus each trajectory was first translated to the origin and rotated onto the positive X axis by an angle that was determined by the slope of the fitted line. The rotated trajectories thus had the same initial direction.

Fourier Mode Analysis

To quantitatively investigate the difference between the shape fluctuations of trajectories of control and dynein-inhibited trajectories, a Fourier decomposition technique developed in previous studies was used[6]. Briefly the shape of the microtubule was characterized as a sum of cosine waves of increasing frequency.

$$\theta(s) = \sqrt{\frac{2}{L}} \sum_{n=0}^{\infty} a_n \cos\left(\frac{n\pi s}{L}\right) \tag{2-1}$$

From a set of N coordinates (x_k, y_k) of a trajectory, the length Δs_k and angles θ_k of the (N-1) segments that connect the coordinates was calculated.

$$\Delta s_k = \left[(x_{k+1} - x_k)^2 + (y_{k+1} - y_k)^2\right]^{1/2} \tag{2-2}$$

$$\theta_k = \tan^{-1}\left[\frac{(y_{k+1} - y_k)}{(x_{k+1} - x_k)}\right] \tag{2-3}$$

The amplitudes were calculated by taking an approximation of the Fourier inverse of (2-1)

$$a_n \cong \sqrt{\frac{2}{L}} \sum_{k=1}^{N} \theta_k \Delta s_k \cos\left[\frac{n\pi s_k^{mid}}{L}\right] \quad (2\text{-}4)$$

where

$$L = \sum_{k=1}^{n} \Delta s_k \quad (2\text{-}5)$$

and

$$s_{mid}^{k} = \Delta s_1 + \Delta s_2 + ... + \Delta s_{k-1} + \frac{1}{2}\Delta s_k \quad (2\text{-}6)$$

Growth Speed Analysis

The growth speeds of the trajectories were retrieved from trackData in the projData file.

Results

Dynein Causes Shape Fluctuations During Microtubule Growth

We tested the hypothesis that dynein motors influence the growth and bends of growing microtubules. We tracked the position of microtubule tips in control and dynein-inhibited NIH-3T3 cells expressing EGFP-EB1 using plusTipTracker[51]. To test the accuracy of the software for our experiments, we first measured the positional error in the measurements. Fixed NIH-3T3 cells expressing EGFP-EB1 were imaged for two minutes at three-second intervals. Since the position of the tips is expected to be fixed, the variation in the +TIP detection by the software will give an estimate of the positional error. The deviation from the mean was calculated for about eight tips. Figure 2-1 shows the plot of the frequency versus the deviation from the mean. Fitting a normal distribution to the data yielded a standard deviation of 0.06 μm. The average distance travelled by the tips in between successive frames was about 1.1 μm; thus there is a

5.5% error in the measurements. This error was deemed to be small enough to enable reliable tracking of the tips.

We tracked the position of the microtubule tips in control and dynein-inhibited NIH-3T3 cells expressing EGFP-EB1 and using plusTipTracker, reconstructed the microtubule trajectories over time. The tracking parameters have been listed in the Materials and Methods Section. To avoid the use of spurious tracks for further analysis, the correct detection of each trajectory by the software was visually confirmed using the plusTipSeeTracks feature (Figure 2-2). We visualized trajectories that had a length over 5 μm. Trajectories that weren't reconstructions of the growth of a single tip over time were discarded. About 8 cells and a total of 800 trajectories were used for future analysis for each condition.

Next the trajectories were translated to the origin. To reduce uncertainty due to positioning error, the initial direction of the trajectory was determined by a line fitted to the first four points in the trajectory. The trajectories were then rotated by an angle determined by the slope of the fitted line. All the trajectories have the same initial direction pointing towards the positive X direction. Figure 2-3 shows the trajectory plots for growing microtubules in control and dynein-inhibited cells. Control trajectories are seen to spread out considerably at early times compared to trajectories in dynein-inhibited cells, which are much tighter. Thus, dynein activity contributes significantly to the bending of growing microtubules. The shape fluctuations for dynein-inhibited trajectories are far smaller than the control cells. This can be observed in the plot of the variance of the Y-coordinate versus time in Figure 2-4 (All the trajectories are oriented initially along the X-axis, so the Y-variance is a measure of the departure from linearity).

Dynein Causes Fluctuations at Higher Wavelengths

To quantitatively investigate microtubule bending, we next performed a Fourier decomposition of the trajectories. Fourier decomposition and the amplitude of the modes resulting from the analysis are a convenient measure of the shape of the instantaneous microtubule. The variance of each mode provides information about the range of fluctuations and the length scale over which bending fluctuations occur. To compare between all the trajectories, it was necessary that all the trajectories have a similar length. For the analysis shown here, the length of the trajectories was set at 8 μm.

The wavelength ‘λ' is related to the mode number 'n' as

$$\lambda = \frac{n\pi}{L} \tag{2-7}$$

where 'L' is the length of the trajectory. The plot of variance of the Fourier amplitudes versus mode number is shown in Figure 2-5. The plot shows that amplitude variances for microtubules in control cells are almost thrice as higher than those in dynein-inhibited cells. This again shows that dynein contributes to the bends and fluctuations in growing microtubules. The maximum amplitude variance occurs at mode n=1. Using relation (2-7) it suggests that dynein causes significant fluctuations wavelength of 16 μm. As seen in Figure 2-5, the amplitude fluctuations decrease as the mode number increases or as the wavelength decreases, however, the microtubule fluctuations in control cells are comparatively higher than those in dynein-inhibited cells.

Dynein Increases the Velocity of Microtubule Growth

EB1 is a +TIP protein that binds to the plus end of growing microtubules. Hence the velocity of the EB1 comets is the same as the growth rate of individual microtubule

polymers. If forces other than those generated by polymerization influence the growth of microtubules, EB1 comets would move with a velocity equal to the growth rate plus or minus the velocity caused by the external force.

The growth speeds of microtubule tips were analyzed using plusTipTracker[51]. Figure 2-6 shows the microtubule speeds in control and dynein-inhibited cells. Around eight cells were analyzed for each condition. A statistical comparison between the means of all the cells in each group showed that the difference between dynein-inhibited and control cells was significant (p value<0.01).

In Figure 2-6, the growth rate of microtubules is higher in control cells in comparison to dynein-inhibited cells. Dynein exerts pulling forces on the microtubule while walking towards the minus end[26]. It is possible that these forces pull the microtubule segment towards the cell periphery. These forces along with polymerization forces increase the growth speed of microtubules in control cells.

Discussion

Despite their high rigidity, microtubules in living cells exhibit bends on short and long length scales[6,40]. The mechanism underlying the shape of growing microtubules remains unknown. Microtubule tips have been observed to undulate on orders much higher than what would be expected from thermal forces alone[7]. The results of this chapter bring new insights on the role of motor proteins forces in influencing the shape of a growing microtubule.

Previous reports suggested that motor proteins bound to the cortical actin network could be responsible for microtubule deformation[41]. We have provided experimental evidence that qualitatively and quantitatively elucidate the effect of a key motor protein, dynein on the shapes of microtubules.

Trajectory plots show that dynein activity significantly influences the bends in growing microtubules (Figure 2-3). Microtubules in the absence of dynein appeared to grow a lot straighter, while there is much variance in the presence of dynein. However as time and the lengths increases, microtubules in dynein-inhibited cells spread out. This suggests that other mechanisms can contribute to the fluctuations in growing microtubules. Possible sources could be kinesin motors walking along the microtubule and myosin motors walking along actin filaments cross-linked to microtubules.

To quantify the fluctuations observed in the plots, a Fourier decomposition was applied on the reconstructed trajectories. Briefly the shape of trajectories was characterized a sum of cosine modes of increasing frequency. The amplitude of the modes is a convenient measure of the shape of the microtubule. The variance of the amplitudes can give an estimate about the range of fluctuations and the length scale over which bending fluctuations is significant. As seen from Figure 2-5, the amplitude fluctuations for trajectories in control cells are almost thrice as higher than those in dynein-inhibited cells. The maximum amplitude fluctuations corresponded to a wavelength of 16 μm. This is comparable to the size of fibroblast cells. Hence, it would be interesting to investigate if dynein dependent microtubule fluctuations play a role in cell motility, intracellular transport, cell development, dynamic instability and other functions of the cell.

Previous work showed that the shape fluctuations in microtubule shapes are many orders higher than what would be expected from thermal forces alone[7]. We wished to compare the amplitude variances shown in Figure 2-5 to amplitude variances that result

only due to thermal forces. The variances due to thermal forces can be calculated using the following relation[6].

$$\mathrm{var}(a_n) = \left\langle \left(a_n - a_n^0\right)^2 \right\rangle = \frac{1}{L_p}\left(\frac{L}{n\pi}\right)^2 \qquad (2\text{-}8)$$

where a_n is the amplitude for mode number n, a_n^0 is the amplitude in the absence of thermal forces, L_p is the persistence length and L is the length of the filament.

With a persistence length of 800 μm[72], we calculated the amplitude variances due to thermal forces (Figure 2-6). It can be seen that fluctuations due to thermal forces are not very significant and it is likely that these forces play a negligible part in determining the shapes of growing microtubules. What is interesting to note is that the amplitude fluctuations in the absence of dynein are still comparatively higher than fluctuations due to thermal forces. Thus, other mechanisms can exist that influence the shape of growing microtubules. Some of these could include kinesin motors, myosin motors, and crosslinks with other cytoskeletal elements.

A discrete distribution of dynein motors walking on microtubules exerts pulling forces causing them to bend. Can these pulling forces affect the growth speed of microtubules? Preliminary investigations suggest that dynein influences the growth speed of microtubules (Figure 2-7). However, further analysis and experiments is needed to explain this observation.

We have thus proven that dynein causes fluctuations in the growing tips of microtubules, but whether it is the main contributor still remains to be explored.

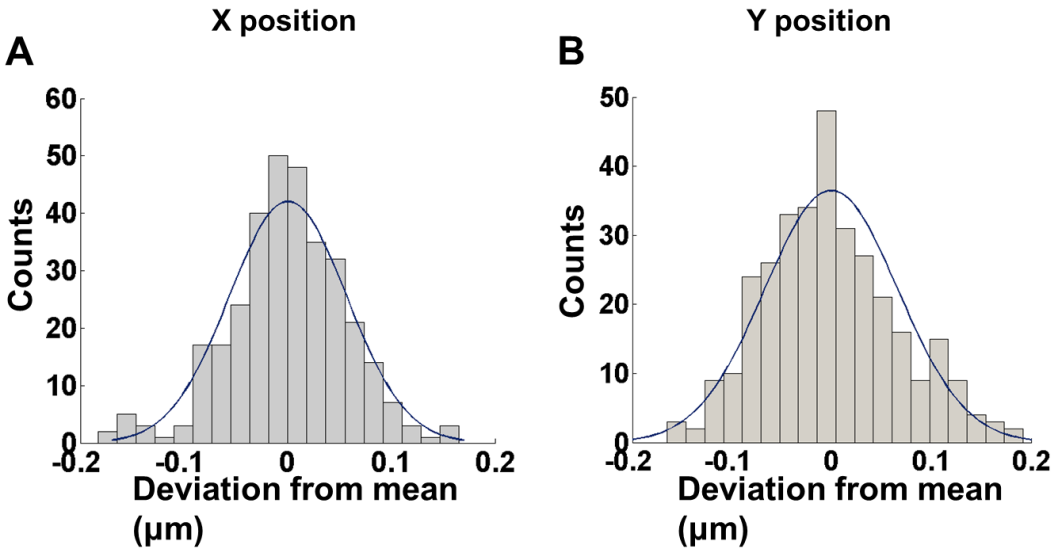

Figure 2-1. Positioning error in tip tracking. Fixed NIH-3T3 cells expressing EGFP-EB1 were imaged for two minutes at three-second intervals. The position of the tips is expected to be stationary, hence the variation in the +TIP detection by the software gives an estimate of the positioning error. The deviation from the mean was calculated for about eight tips. The plot of the frequency versus the deviation from the mean is shown here. Fitting a normal distribution to the data yielded a standard deviation of 0.06 μm.

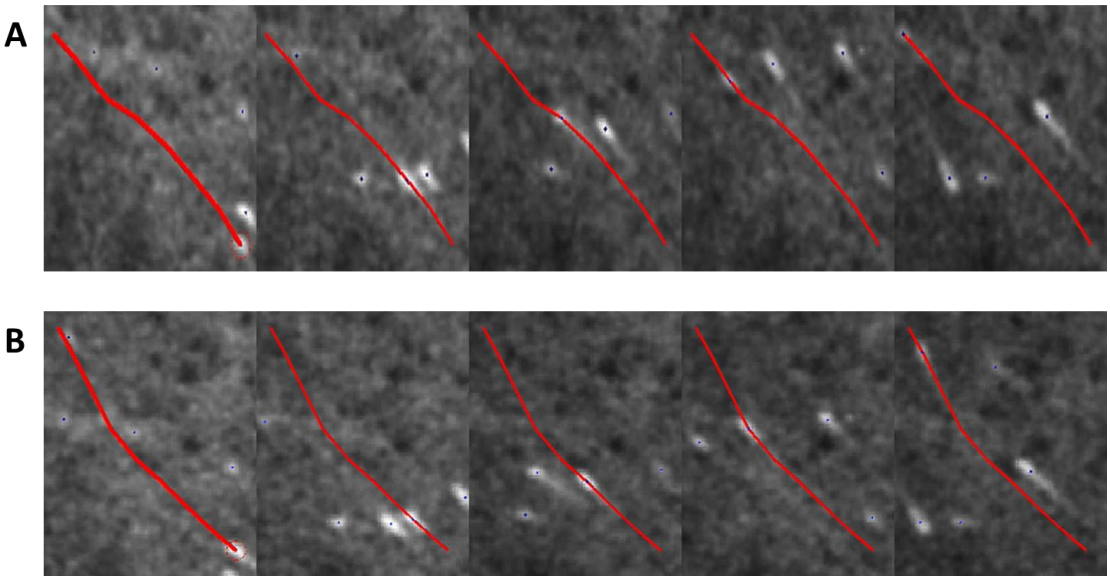

Figure 2-2. Validation of +TIP tracking by plusTipTracker. (A) and (B) Montage of trajectories reconstructed and visualized using 'Track Overlay' tool of plusTipSeeTracks function.

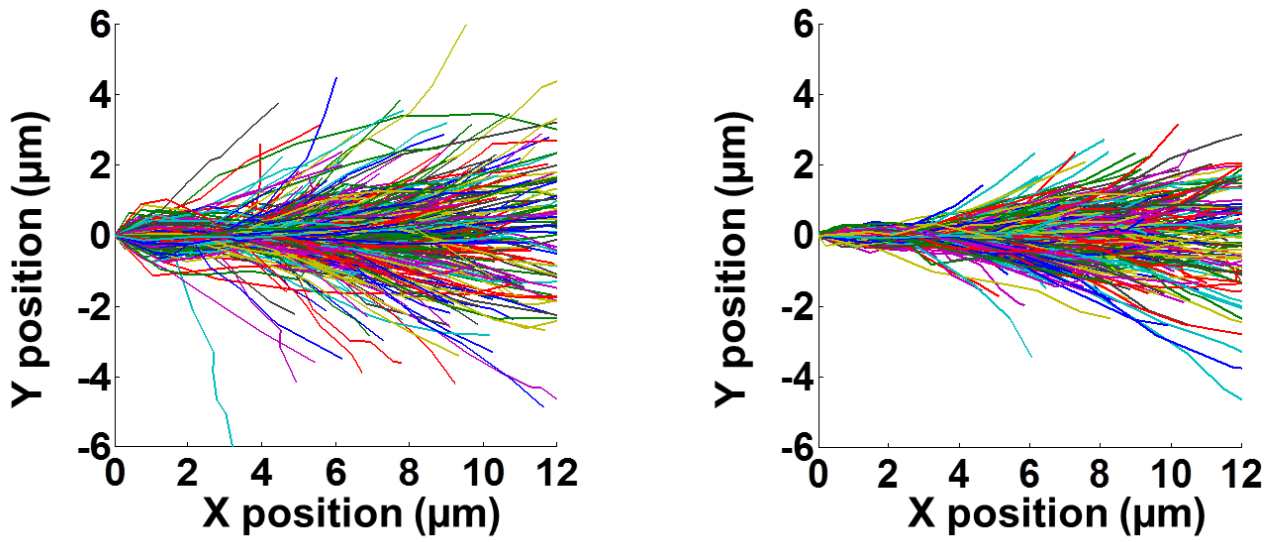

Figure 2-3. Dynein causes bending during microtubule growth. Trajectories of growing microtubules were reconstructed using plusTipTracker. The plots show that fluctuations are smaller in dynein-inhibited cells (right) compared to control cells (left). This suggests that dynein causes fluctuations in growing tips of microtubules. Around 8 cells and 800 trajectories were studied for each condition.

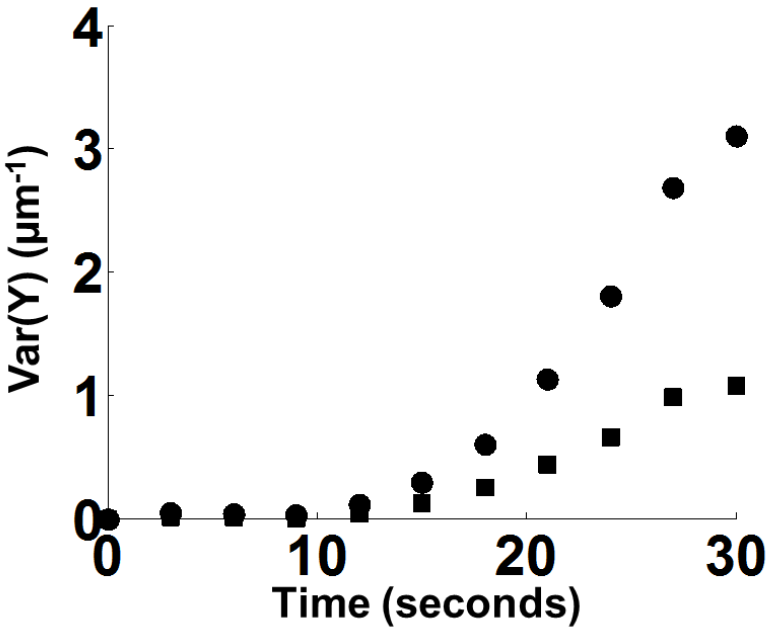

Figure 2-4. Comparison of the spread in growth trajectories in control and dynein-inhibited cells. The variance of the Y position for all the trajectories in Figure 2-3 is plotted versus time. Data for control cells (solid circles) and for dynein-inhibited cells (solid squares) is shown. The plot shows that there is significant variance in the growth of microtubules in the presence of dynein.

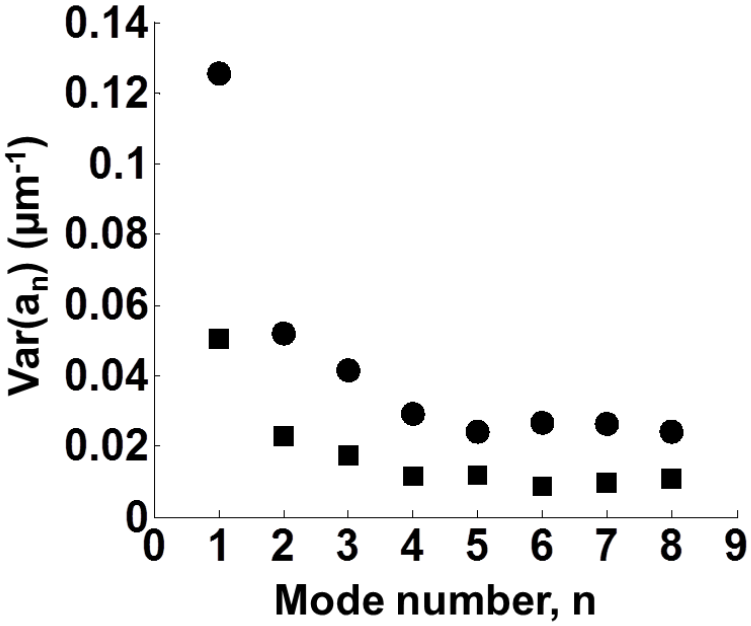

Figure 2-5. Fourier mode analysis for growing microtubule trajectories. The variance of Fourier amplitudes of each mode versus mode number for control cells (solid circles) and dynein-inhibited cells (solid squares) is shown. The fluctuations in microtubule tip trajectories in the presence of dynein are higher at smaller modes (longer wavelengths). As the mode number increases, the fluctuations decrease but those of microtubules in control cells still are higher than those in dynein-inhibited cells.

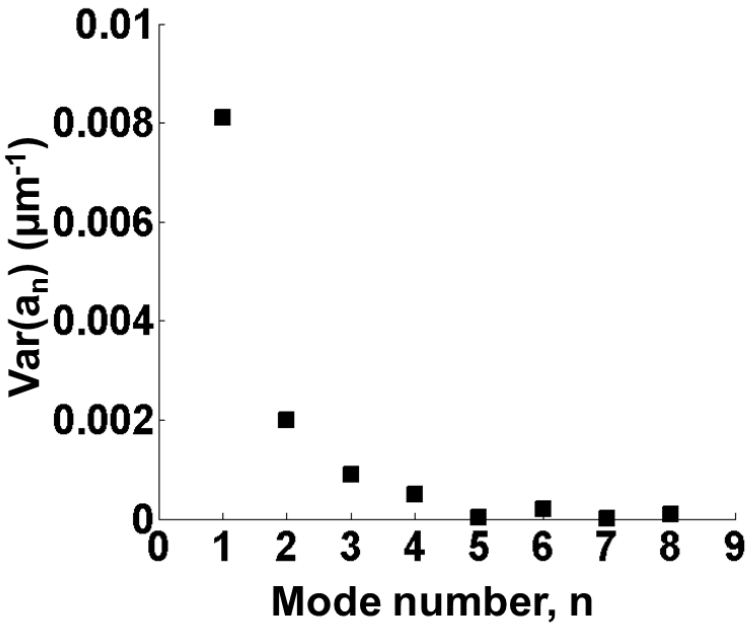

Figure 2-6. Predicted Fourier mode analysis for growing microtubule trajectories subjected to thermal forces. The variance of Fourier amplitudes of each mode versus mode number for microtubules in cells subjected to thermal forces is shown. The fluctuations for microtubules subjected to thermal forces are an order of magnitude smaller than the experimentally observed fluctuations in control cells. This suggests that thermal forces play an insignificant role in influencing the shape of microtubules.

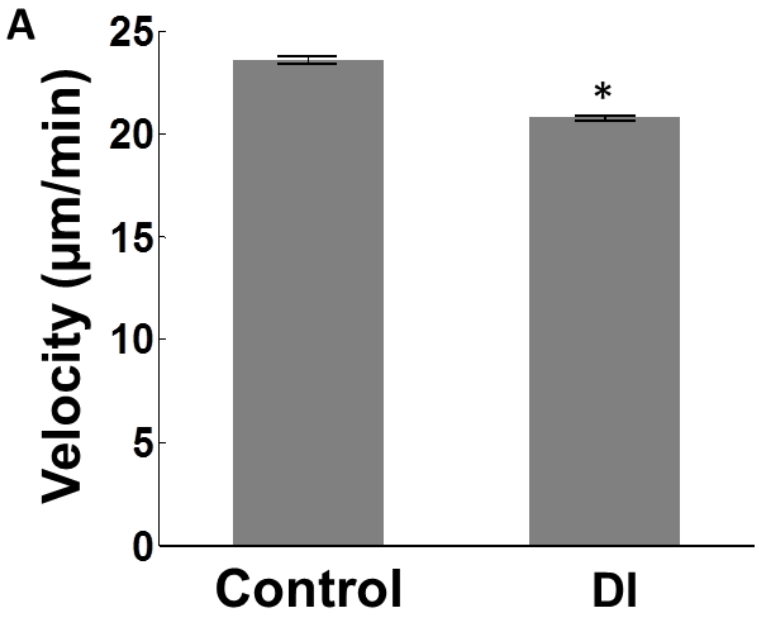

Figure 2-7. Growth speeds of microtubules reduce with the inhibition of dynein. The growth speeds of the microtubules were calculated using plusTipTracker. (A) Microtubule growth speeds in control cells are higher than dynein-inhibited cells. About 8 cells were analyzed for each condition. Error bars indicate standard error of the mean (SEM). '*' ($p<0.01$)

CHAPTER 3
NUCLEAR TRANSLATION IN MOTILE CELLS DOES NOT REQUIRE DYNEIN ACTIVITY

Two types of nuclear motion are observed in cells: rotation about an axis of symmetry and translation of the nucleus in a directional fashion. Rotation of the nucleus about an axis is observed in nearly all cell types. Wu *et al.* (2010) showed that rotation of the nucleus occurs due to dynein activity[69]. In their model, nuclear-bound dynein processively motors along microtubules toward the minus end[69]. This motion generates pulling forces on the nucleus[69]. Assuming that the resistance to translation motion is high, and summing up the torque generated by dynein pulling on randomly oriented microtubules originating from the MTOC and traversing close to the nuclear surface, it was possible to quantitatively explain the persistent random walk nature of nuclear rotation due to dynein activity[69].

The study by Wu *et al.* (2010) raises the question: does dynein activity cause directional translation of the nucleus? It is to be expected that the nucleus moves with the cell as the cell crawls on a two-dimensional surface. Nuclear motion in a single, migrating cell is thought to occur through the generation of pushing forces on the nuclear surface during detachment of the trailing edge. We explored if trailing edge detachment was necessary for motion of the nucleus in a single crawling cell. Surprisingly, we found that the nucleus moves significant distances (microns) without any detachment of the trailing edge, or any significant change in cell shape. The movement of the nucleus correlated strongly with the movement of the cell centroid. When similar studies were performed in dynein-inhibited cells, the nucleus was similarly observed to translate without requiring trailing edge detachment. No significant effects were observed on the magnitude of nuclear translation. Collectively, these results argue

against a role for dynein in nuclear translation. Together with the results on nuclear rotation by Wu *et al.* (2012), we suggest that dynein holds the nucleus in place through frictional effects, and pulls on the nucleus for reorienting it along a desired direction, but is not strong enough to generate directional translational motion of the nucleus[73].

Materials and Methods

Cell Culture, Plasmids and Transfection

Swiss NIH 3T3 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Manassas, VA) with 10% Donor Bovine Serum (DBS) (Gibco, Grand Island, NY). The cells were maintained at 37^{o} C. in humidified 5% CO_2. For microscopy the cells were plated on 35 mm glass-bottom dishes (WPI, Sarasota, FL) and allowed to spread overnight at 37^{o} C and 5% CO_2. The glass bottom dishes were coated with 5 µg/ml Fibronection (BD Biocoat^{TM}, Franklin Lakes, NJ) and kept in 4^{o} C overnight.

For dynein inhibition experiments, cells were additionally transfected with plasmid DsRed-CC1 (gift by Prof. Trina A. Schroer, Johns Hopkins University). Transient transfection of plasmids into NIH 3T3 fibroblasts was performed with Lipofectamine^{TM} 2000 transfection reagent (Life Technologies, Invitrogen, Carlsbad, CA).

Time-Lapse Imaging and Analysis

Time-lapse imaging for cell and nuclear movement was performed on a Nikon (Melville, NY) TE2000 inverted fluorescent microscope equipped with a 40X/1.3 Numerical Aperture (NA) oil immersion objective and Charge-Coupled Device (CCD) camera (CoolSNAP, HQ^2, Photometrics, Tucson, AZ). During microscopy, cells were maintained at 37 ˚C in a temperature, CO_2 and humidity controlled environmental chamber. Images from cell migration experiments were imported in ImageJ (NIH).

Images were processed in ImageJ and then imported into MATLAB R2011b (Mathworks, Natick, MA). Programs were developed in ImageJ and MATLAB R2011b to track the nuclear centroid and the contour of cells.

Results

Forward Nuclear Movement Does Not Require Trailing Edge Detachment

We observed the movement of the NIH-3T3 fibroblast cells and its nucleus for a time period of 30 minutes. We found that forward motion of the nucleus did not necessarily require detachment of the trailing edge (Figure 3-1 A and B). Quantification of the movement in many cells showed that the forward motion of the nucleus correlated with forward motion of the cell centroid, but not with the trailing edge (Figure 3-2 A, B and C). This is not to suggest that nuclear motion does not occur when the trailing edge detaches; but that significant forward motion can occur of the nucleus without large changes in the shape of the trailing edge.

Directional Motion of the Nucleus Does Not Depend on Dynein Activity.

Next, we inhibited dynein in NIH 3T3 fibroblasts by over-expressing DsRed-CC1. Similar to control cells, we found that in dynein-inhibited cells, the nucleus moved without requiring trailing edge detachment (Figure 3-3 C). The nucleus continued to track the cell centroid (Figure 3-3 A and B). These results suggest that nuclear movement in the crawling cell does not necessarily require the activity of dynein.

Discussion

Nuclear movement in migrating cells arises as a result of force generation in the cytoskeleton[74]. It is known that microtubules exert forces on the nucleus through the activity of nuclear embedded motors like dynein and kinesin. While dynein has been

shown in NIH 3T3 fibroblasts to cause rotational motion[69], its role in translational motion has remained unexplored.

When a crawling cell migrates, it undergoes changes in its shape due to formation of lamellipodia at the leading edge and detachment of the trailing edge[54]. Directional forces due to dynein activity can arise when the microtubule network itself translates in the direction of motion. Directional motion of the microtubule network would cause motion of the nucleus due to interaction either with an increased number or length of microtubules resulting in higher pulling forces due to dynein activity, or due to dynein acting as a cross linker that transmits microtubules forces to the nuclear surface. We found however that even when dynein was inhibited, nuclear translation was unaffected. Thus, either dynein is not directly involved in causing nuclear translation, or the translation of the microtubule network itself is affected by dynein inhibition resulting in a decrease in translation. Separately, Wu *et al.* (2012) have shown that directional motion of the nucleus occurs primarily due to the generation of actomyosin forces in the newly formed lamellipodium and is not dependent on microtubule activity[73]. Therefore, in combination with those studies, we have reason to conclude that nuclear translation in crawling cells does not require dynein activity; rather dynein serves to frictionally damp nuclear positional fluctuations[73].

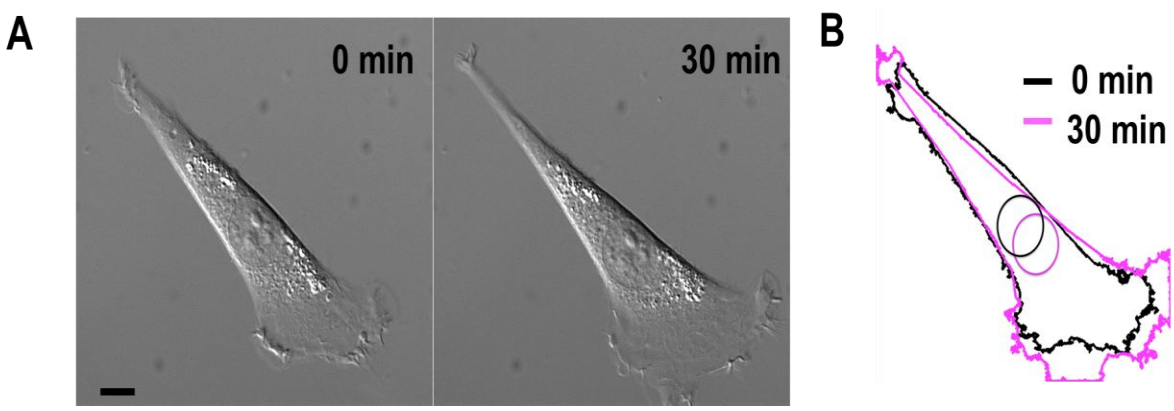

Figure 3-1. Nuclear movement in motile fibroblasts does not require detachment of the trailing edge. (A) Nucleus moved towards leading edge as the lamellipodia formed.(B) Superposition of cell outline at 0 minute (black) and 30 minute (pink). The nucleus moved forward upon the formation of lamellipodia, while the trailing edge did not retract.

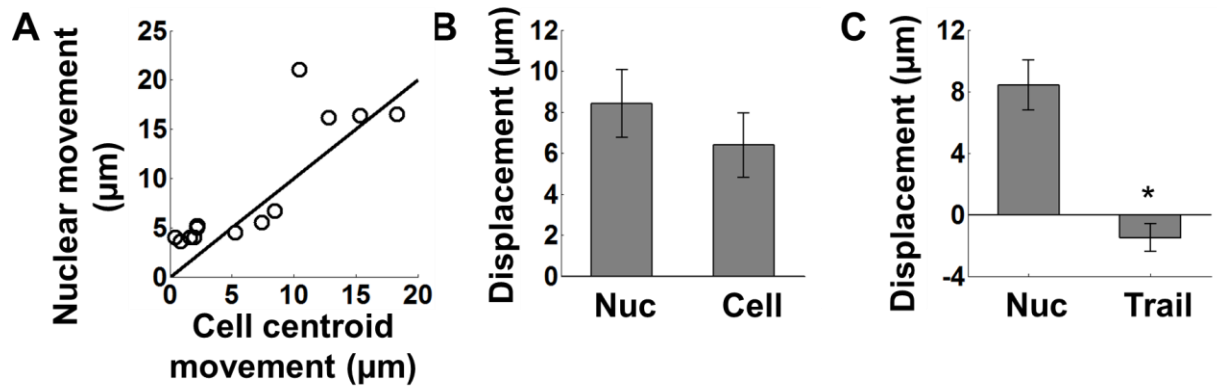

Figure 3-2. Nuclear movement correlates with cell movement. (A) Nuclear movement is linearly correlated with cell centroid movement in control (n=13) cells. Black line is y=x line. (B) Average movement of the nucleus and cell centroid in 30 minutes shows that they moved similar distances. (C) Average movement of the nucleus and trailing edge in control cells (n=13) in 30 minutes, the trailing edge did not move appreciably. Error bars indicate standard error of the mean (SEM), '*', $p < 0.01$

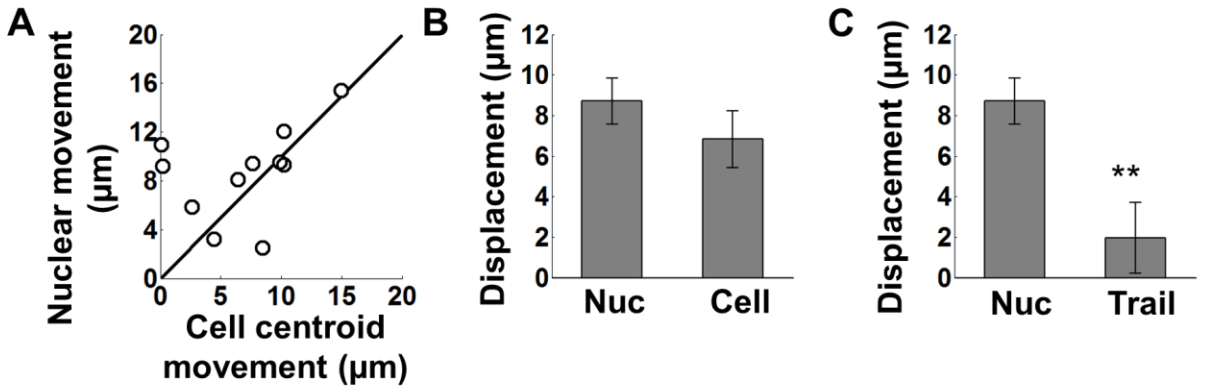

Figure 3-3. Effect of dynein inhibition on nuclear movement (A) Cells were transfected with DsRed - CC1 to inhibit dynein. The nucleus was observed to move similar to control cells (Figure 3-2), and the motion still correlated with the cell centroid. Black line is y=x line. (B) Average movement of the nucleus and cell centroid in dynein-inhibited cells in 30 minutes shows that they moved similar distances. (C) Average movement of the nucleus and trailing edge in dynein-inhibited cells (n=11) in 30 minutes, the trailing edge did not move significantly. Error bars indicate standard error of the mean (SEM), '*',$p < 0.01$

CHAPTER 4
CONCLUSIONS

Summary of Findings

Motor Forces on Growing Microtubule Tips

Microtubules are critical for a wide range of cell functions that include mitosis, organelle migration, cell shape and migration. To effectively perform these tasks, certain requirements are imposed on the mechanical properties of microtubule cytoskeleton. Microtubules need to be resistant to deformation to not break under the strong forces exerted by motor proteins and other intracellular material[7]. However despite its high rigidity, microtubules in cells are often observed bent or buckled[49]. This suggests that these microtubules experience large non-thermal forces; that they sense and respond to.

A major focus of this project was to investigate the role of molecular motor forces on the deformation of microtubules. Through trajectory reconstructions, we found that the motor protein dynein contributes significantly to the bends of growing microtubules. Using a Fourier decomposition analysis, we found that shapes of growing microtubules in control cells fluctuate almost thrice as higher than those in dynein-inhibited cells. The fluctuations of growing microtubules in dynein-inhibited cells were relatively higher than the fluctuations expected from thermal forces alone. This suggests that other mechanisms contribute to the fluctuations in growing microtubules. Possible sources of these fluctuations have been listed in the Future Work section.

Nuclear Translation in Motile Cells Does Not Require Dynein Activity

Cell polarity, migration and the positioning of the nucleus are critical for physiological processes like wound healing, tissue development and differentiation. Due

to its massive size and stiffness, the movement of the nucleus is a force and energy intensive purpose. The movement of the nucleus is a cytoskeleton dependent process. Microtubules exert forces on the nucleus through the activity of its associated molecular motors like kinesin and dynein. Dynein has been shown to cause nuclear rotation in NIH-3T3 fibroblasts. Its role in nuclear translation however remains unexplored.

We investigated the role of dynein on the directional motion of the nucleus in NIH-3T3 fibroblast cells. Our experiments showed that nuclear translation was unaffected on the inhibition of dynein. Furthermore in control and dynein-inhibited cells, the nucleus moved significant distances without requiring detachment of the trailing edge and without significant changes in cell shape. In combination with other studies[73], our results suggest that nuclear translation in migrating cells do not require dynein activity; rather dynein serves to frictionally damp the fluctuations in nuclear positioning.

Future Work

The purpose of this section is to list possible studies that could be done to expand the work done in this thesis.

Influence of Other Motor Forces on Microtubule Growth

The data in this thesis suggests that dynein is an important generator of fluctuations in microtubules. However there is some evidence that other mechanisms might play a role in influencing the growth of microtubules (Figures 2-4 and 2-5). Possible regulators of microtubule growth could be motor proteins like myosin and kinesin.

The role of myosin and kinesin can be investigated by treating cells with blebbistatin or by expressing the protein mCherry-KHC. Preliminary results for kinesin-inhibited cells is shown in Figure 4-1. The role of all motor proteins can be investigated

by the depletion of ATP in cells[75]. If kinesin and myosin activity is found to significant, laser ablation experiments could be performed in kinesin inhibited and myosin inhibited cells. If significant results are obtained, these proteins can be incorporated in the motor model given elsewhere[26].

Influence of Motor Forces on Microtubule Shapes

We have shown that dynein influences the growth of microtubule. If the motor proteins in the previous study are found to influence the growth of microtubules, we can extend the Fourier mode analysis to study the fluctuations of growing microtubules in either condition.

Influence of F-actin on Microtubule Growth

Actin filaments are another major component of the eukaryotic cytoskeleton. Microtubules can be cross-linked to the actin cytoskeleton through tethers like plakins and plectins. Dynein exerts pulling forces on microtubules by binding to the underlying F-actin network.

The highly dynamic cytomatrix could influence the growth and dynamics of the microtubules. We can investigate the role of F-actin on microtubule growth by either disrupting the network with cytochalasin-D or by inducing the flow of new actin filaments by treatment with a photoactivable Rac1[76].

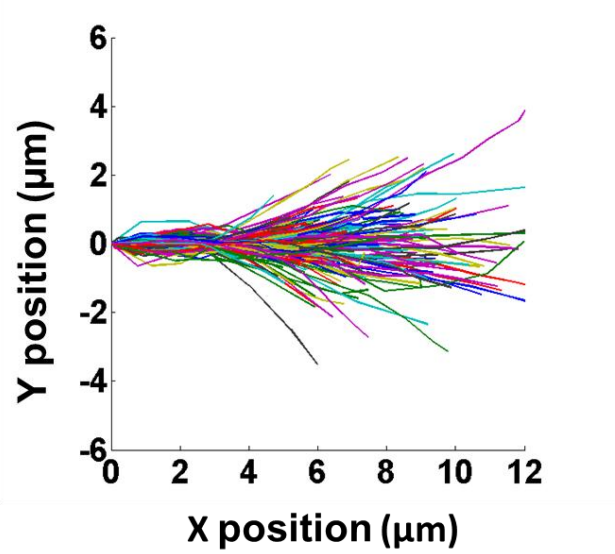

Figure 4-1. Effect of kinesin inhibition on microtubule growth. Trajectories of growing microtubules in kinesin-inhibited cells were reconstructed using plusTipTracker. Around 14 cells and 300 trajectories were studied in this condition.

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BIOGRAPHICAL SKETCH

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