INVESTIGATING THE CELLULAR FUNCTIONS OF KINESIN,
MICROTUBULES AND +TIPS USING IN VITRO RECONSTITUTION,
MICROSCALE ENGINEERING AND NOVEL STATISTICAL
TECHNIQUES

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Abstract

Microtubules are cytoskeletal filaments that self-assemble from αβ tubulin dimers and are essential for cellular mechanics, cell division and intracellular cargo transport. Carrying out these functions requires numerous accessory proteins that bind to and interact with microtubules. The study of microtubules and microtubule associated proteins (MAPs) is relevant for understanding aging, developing cancer therapies, and providing approaches to treating neurodegenerative diseases. This thesis is devoted to using bioengineering tools and biophysical reconstitution methods to elucidate molecular mechanisms by which MAPs carry out their functional roles in mitosis, in organizing the neural cytoskeleton, and other processes.

The mitotic motor kinesin-5 is best known as a homotetramer, however, motor properties independent of its homotetrameric configuration are not well understood. From in vitro assays, an engineered kinesin-5 dimer was found to stabilize microtubules by slowing GMPCPP microtubule depolymerization and promote dynamic microtubule growth by inhibiting catastrophe. Single-molecule experiments showed that kinesin-5 remains associated with microtubule plus-ends for a duration of 7 seconds. These results demonstrate that in addition to its role in sliding apart antiparallel microtubules, kinesin-5 is a microtubule stabilizer, polymerase and end-tracker.

To maintain microtubule uniformity in dendrites of Drosophila neurons, microtubules growing into branches need to be guided towards the cell body. To investigate the mechanism of microtubule guidance, purified EB1 and kinesin-2 were dimerized to form
an EB1-kinesin complex, and this complex was shown to be sufficient to steer the growth of one microtubule towards plus-end of another microtubule, providing a mechanism for maintaining uniform microtubule orientation, not only in neurons but also in other cells. Also, this work demonstrates that the dynamic EB1-microtubule interaction is sufficiently strong to carry out mechanical functions in cells.

*In vitro* reconstitution can be used to investigate microtubules and MAPs in well-defined environments, but it is usually limited to single microtubules. To eliminate this constraint, micro-patterned electrodes were fabricated on elevated pedestals and high frequency AC fields were used to align microtubules on the opposite electrodes, thus forming a bipolar artificial mitosis spindle in 3D with microtubule plus-ends oriented towards the overlap zone. These aligned microtubules provide a platform for investigating MAPs in a spindle-like geometry.

Cellulose synthase complex (CSC) is a membrane bound multi-subunit complex that synthesizes cellulose microfibrils and has great meaning in plant cell morphologies and commercial applications. To estimate the copy number and stoichiometry of CSC in a nondestructive way, GFP-CESA3 in *Arabidopsis* was photobleached under total internal reflection (TIRF) microscopy. A step detection algorithm was developed and the smooth bleaching traces were analyzed by to identify discrete bleaching steps. The resulting broad distribution of step sizes was analyzed by Gaussian Mixture Model to determine the unitary step size, thus allowing estimation of the copy number. These analyses can be applied both to other photobleaching studies and to molecular motor stepping.
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\[
\left( \frac{\text{DOM}}{\text{multiplier}(L)\sigma} \right)
\]

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Preface

Most of Work described in Chapter 4 was accomplished in close collaboration with Raymond Fok from Dr. Thomas Jackson’s lab in PSU Electrical Engineering.

Work presented in Chapter 5 is collaboration with Dr. Charlie Anderson in PSU Plant Biology and was published in the following paper:


Work in Chapter 2 is comprised of the following paper:


A paper describing research in Chapter 3 is currently under preparation with tentative title as following:

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Chapter 1  Introduction

Dynamic microtubules form various structures, from bipolar spindles during cell division to uniform bundles in axons, to carry out specific cellular functions, including chromosome segregation, intracellular transport and cell migration. The successful achievement of these functions requires additional proteins, including kinesin family members and plus-tip tracking proteins that can interact with microtubules and regulate microtubule assembly. Defects in these microtubule associated proteins can lead the failure of corresponding processes and are the major cause of microtubule related diseases, like cancer and Alzheimer's diseases (Goldstein and Yang 2000; Goldstein 2001; Chevalier-Larsen and Holzbaur 2006; Falnikar and Baas 2009). Molecular studies of the interplay between microtubule and microtubule associated proteins can provide mechanistic insights into those cellular processes. This thesis is devoted to using interdisciplinary methods to study impact of EB1 and kinesin on microtubule dynamics and organization. Engineering platforms and statistical algorithms are developed to provide novel ways of exploring cellular processes in vitro.

1.1  Microtubule Structure and Dynamic Instability

Microtubules are a class of cytoskeletal filaments that are protein polymers assembled by αβ tubulin dimers (Figure 1.1A). The sequential alignment of αβ tubulins leads to formation of a protofilament (Figure 1.1B) and 13 parallel protofilaments (Evans,
Mitchison et al. 1985) bind laterally to form the hollow tube, the microtubule, with an outer diameter of 25 nm (Figure 1.1C). The 8 nm length of tubulin dimers leads to the 8 nm longitudinal repeat of α and β tubulins. The polarity of αβ tubulin heterodimers causes the intrinsic polarity of microtubules with α tubulin end termed the “minus end” and β tubulin end termed the “plus end”. The plus ends of microtubules polymerize faster and are more dynamic than minus ends (Desai and Mitchison 1997; Dammermann, Desai et al. 2003; Jiang and Akhmanova 2011).

During the polymerization phase, GTP tubulins bind to the end of microtubules causing the extension of slight curled protofilaments (Figure 1.1D) (Chrétiens, Fuller et al. 1995; Downing and Nogales 1998). Adjacent protofilaments are stabilized by the lateral interactions, and form a GTP tubulin sheet, whose enclosure leads to the formation of microtubules (Figure 1.1D). Once added to the end of microtubules, the hydrolysis of GTP tubulin is catalyzed by interaction with adjacent tubulins (Carlier and Pantaloni 1981; Nogales, Downing et al. 1998). The hydrolysis of GTP is accompanied by a slight conformational change causing the weakly curled GTP tubulin to switch to straight GDP tubulin in microtubule lattice (Buey, Diaz et al. 2006; Nogales and Wang 2006; Rice, Montabana et al. 2008). This conformational change causes the intrinsic stress of microtubules that is stabilized by GTP cap, layers of GTP tubulins at the end (Desai and Mitchison 1997; Seetapun, Castle et al. 2012). The loss of GTP cap can lead to stall of growth or to depolymerization, a rapid shortening of microtubules. During the depolymerization phase, protofilaments disassemble to generate a characteristic “banana-peel” structure (Figure 1.1E) (Chrétiens, Fuller et al. 1995). Microtubules
commonly switch between polymerization-depolymerization state and this dynamic instability enables microtubules to be rearranged into different structures to fulfill different functions in the cell (Gelfand and Bershadsky 1991; Desai and Mitchison 1997).
1.2 Successful Fulfilment of Microtubule Functions Requires Specific Assembly of Microtubules

In most cells, microtubules are nucleated from the centrosomes (Mitchison and Kirschner 1984; Moritz, Braunfeld et al. 1995), γ –tubulin ring complex (γ-TuSC) (Moritz, Braunfeld et al. 2000; Kollman, Merdes et al. 2011), or self-nucleated at high tubulin concentration (Johnson and Borisy 1977). Dynamic microtubules are constantly regulated by various microtubule associated proteins (MAPs). For instance, Xmap215 can strongly increase microtubule turnover rate by enhancing both polymerization and depolymerization (Gard and Kirschner 1987; Shirasu-Hiza, Coughlin et al. 2003), and the motor protein MCAK can depolymerize microtubules from both ends (Hunter, Caplow et al. 2003; Helenius, Brouhard et al. 2006). Spatial organization of dynamic microtubules is required to fulfill specific cellular functions. The bipolar assembly of microtubules in dividing cells is the platform for chromosome segregation and the uniform bundles of axonal microtubules are critical to neuronal polarity.

In actively dividing cells, microtubules form a bipolar spindle structure with microtubule minus-ends focused in two poles and plus-ends overlapping in the middle, resulting to three major microtubule populations, defined by their location and function (Figure 1.2A). Microtubules that are attached to chromosomes at the kinetochore, a multiple subunit protein complex that bridges microtubule and DNA (Cheeseman and Desai 2008), are termed kinetochore microtubule or K-fibers. Interpolar microtubules are microtubules that extend to the middle of spindle and form antiparallel bundles with microtubules from the other pole. These antiparallel bundles are active regions that
many mitotic MAPs act on to generate the outward-directed forces required to maintain the bipolar structure of the spindle. Kinetochore dynein that localizes to kinetochore in prometaphase generates poleward pulling forces to promote chromosome congression and alignment (Li, Yu et al. 2007). The Drosophila kinesin-5, KLP61F, cross-links Interpolar microtubules (Sharp, McDonald et al. 1999) and generates poleward forces to antagonize forces generated by NCD on microtubule minus-ends (Tao, Mogilner et al. 2006; Brust-Mascher, Sommi et al. 2009). The third class of microtubules, which radiate away from the spindle, is called aster microtubules. Aster microtubules can interact with the plasma membrane of the cell and are important for spindle position and length (Dumont and Mitchison 2009; Goshima and Scholey 2010).

During cell division, chromosomes attach to k-fibers, congress to the equator following spindle formation and nuclear breakdown, oscillate around the equator due to poleward forces from opposing poles, and are separated by microtubules depolymerization and motor pulling towards the pole. This division process is precisely controlled (Compton 2000; Goshima and Scholey 2010), and mistakes during the process can cause unequal separation that can lead to diseases like Down syndrome, or uncontrolled cell division, a characteristic of cancer cells (Jordan and Wilson 2004; Stanton, Gernert et al. 2011). Research on mitotic MAPs can provide insights into molecular mechanisms of mitosis, help to improve understanding proliferation of stem cells, and seek clues to early diagnosis and cure of diseases.
Following cell division, microtubules are rearranged to accommodate cell specific functions (Bartolini and Gundersen 2006). Epithelial cells adopt an asymmetric microtubule system with minus end anchored at the apical side and plus ends pointing towards the basal region (Figure 1.2B) (Meads and Schroer 1995; Musch 2004; Bartolini and Gundersen 2006). Cortical microtubules in plant cells are aligned perpendicular to the longitudinal axis of the cell, and lack uniform polarity (Figure 1.2D) (Dixit and Cyr 2004). The microtubules beneath the cell membrane in plant cells are responsible for organizing cellulose microfibrils by serving as tracks for the cellulose synthase complex (McFarlane 2014).

In neuronal axons, microtubules are uniformly aligned with plus-ends oriented away from the cell body towards the synapse (Figure 1.2C) (Conde and Caceres 2009). Dendritic microtubules are thought to have mixed orientation in cultured mammalian neurons (Baas, Deitch et al. 1988) but were found to be uniformly minus-end out in *Drosophila* larvae (Stone, Roegiers et al. 2008; Rolls 2011). These uniformly-oriented microtubule arrays are bundled and stabilized by tau and other MAPs, and serve as tracks for bidirectional axonal transport that is carried out by molecular motors (Craig and Banker 1994; Horton and Ehlers 2003). The disruption of microtubules and neuronal MAPs can lead to defects in the bidirectional transport, thus causing various neuronal degenerative diseases, like Alzheimer’s disease (Charrin, Saudou et al. 2005; Chevalier-Larsen and Holzbaur 2006; Vos, Grierson et al. 2008). The microtubules are also involved in axonal response to external clues and neuronal repair after injury (Franze, Janmey et
Despite its importance, it is still not well understood how microtubule uniformity is initiated during neuron generation and maintained through life of neurons.

1.3 How are Microtubule-structures Formed and Maintained?

The essential structural role that microtubules play in diverse cell functions makes it challenging to understand how these microtubule assemblies are formed and maintained. In cells, microtubules are polymerized from MTOC and can form asters. Certain microtubule structures can be achieved by positioning MTOC (Baas 1999). Microtubule organization in cells without MTOC is more complicated. The dynamics of microtubules means that any microtubule structures are inherently unstable and require constant stabilization and maintenance.

In an early stage of mitosis, when microtubules are polymerizing from two duplicated centrosomes, kinesin-5 family motor proteins slide the antiparallel microtubules apart, thus separating two centrosomes (Blangy, Lane et al. 1995; Tanenbaum, Macurek et al. 2008). Inhibition of kinesin-5 during mitosis leads to the formation of monopolar spindles (Sawin, Leguellec et al. 1992). After formation of the bipolar spindle, forces generated by kinesin-5 are important to antagonize inwards forces by NCD and dynein from poles (Tao, Mogilner et al. 2006; Ferenz, Paul et al. 2009). Other proteins are also targeted to the overlap region and serve to maintain the spindle structures. For instance, PRC1 preferentially binds to antiparallel microtubules and can increase the friction of microtubule sliding (Mollinari, Kleman et al. 2002; Zhu and Jiang 2005; Braun, Lansky et
al. 2011; Watanabe and Goshima 2014). The recruitment of kinesin-4 by PRC1 to the midzone results to the size of antiparallel region being actively controlled (Bieling, Telley et al. 2010; Subramanian, Ti et al. 2013).

The position of the centrosome has previously been shown to be crucial to axon specification and neuronal microtubule organization (de Anda, Pollarolo et al. 2005). However, more recent work suggests that in mature neurons the centrosome may not play a major role in microtubule organization (Zolessi, Poggi et al. 2006; Nguyen, Stone...
et al. 2011), suggesting that other factors must control microtubule organization far from the cell body. Polarized transport of microtubules has been suggested to be important for microtubule organization in axons (Baas and Lin 2011), but this may also be most relevant in developing neurons. In mature neurons, local microtubule nucleation seems to play a role in dendritic microtubule organization (Ori-McKenney, Jan et al. 2012). Nevertheless, whichever mechanisms contribute to initial establishment of microtubule organization, microtubules are dynamic polymers that frequently switch between phases of growth and shortening. This continued microtubule growth has the potential to disrupt polarity, particularly in branched regions of neuronal processes. For example, in the branched minus-end-out dendrites of *Drosophila* neurons, uncontrolled microtubule growth through branch points would lead to mixing of polarity over time. The solution to this paradox seems to be that the direction of microtubule growth is, in fact, controlled.

### 1.4 Microtubule Associated Proteins

MAPs are functionally defined as proteins that interact with microtubules, containing motor and non-motor proteins. In general, MAPs can function by walking on microtubules to transport cargo (Hirokawa, Noda et al. 2009), regulate microtubule assembly dynamics (Howard and Hyman 2007), and modulate microtubule mechanic properties (Felgner, Frank et al. 1997; Lopez and Valentine 2013). This thesis is focused on kinesin motors and plus-tip tracking proteins.
1.4.1 Kinesin Superfamily Members

Kinesin is molecular motor that takes discrete 8 nm steps on microtubule tracks by utilizing energy from ATP, and carries out diverse functions in cells. The majority of kinesins form dimers with motor heads on one end, which is followed by the neck linker region and the coiled–coil dimerization domain (Figure 1.3, A and B). The short neck linker contains 14 to 18 amino acids in different kinesins (Hariharan and Hancock 2009) and is responsible for kinesin processivity (Yildiz, Tomishige et al. 2008; Shastry and Hancock 2010; Shastry and Hancock 2011). The tail domain of kinesin is responsible for interaction with cargos and can inhibit motor activity to prevent futile ATP hydrolysis in conditions without cargo binding (Coy, Hancock et al. 1999). The location of the motor domain correlates with the direction that a kinesin moves along microtubules: N-terminal motors towards microtubule plus-ends and C-terminal motors towards minus-ends (Vinogradova, Reddy et al. 2004; Jana, Hyeon et al. 2012). When moving along microtubules, kinesin binds, hydrolyzes and release ATP (Figure 1.3C), and takes an 8 nm-step per nucleotide hydrolyzed (Svoboda, Schmidt et al. 1993; Schnitzer and Block 1997; Coy, Wagenbach et al. 1999). While most kinesins are dimers, some kinesins have different configurations for specific functions. Native kinesin-5 is a homotetramer that can slide microtubule apart (Kapitein, Peterman et al. 2005) and some kinesin-3 family members are processive monomers (Tomishige, Klopfenstein et al. 2002; Al-Bassam, Cui et al. 2003; Okada, Higuchi et al. 2003). Motors from different kinesin families differ in their kinetic cycles and force generating properties. Kinesin-1 has fast kinetics and can
sustain loads up to ~ 5 pN, while kinesin-2 is slower and detaches more frequently before reaching the maximum load (Meyhöfer and Howard 1995; Coppin, Pierce et al. 1997; Visscher, Schnitzer et al. 1999; Schroeder, Hendricks et al. 2012; Andreasson 2013). Notably, the response to load is asymmetric – kinesin generally resists hindering loads, but detaches rapidly when pulled by an assisting load (Kawaguchi and Ishiwata 2001; Milic, Andreasson et al. 2014). This asymmetry has great relevance to bidirectional cargo transport, where motors will be under either assisting load or hindering load depending on cargo movement (Muller, Klumpp et al. 2010).

While kinesin proteins are depicted classically as motors that walk on microtubule lattice to transport cargos and generate mechanical forces, emerging studies have shown that motors in the kinesin superfamily can also regulate microtubule dynamics by accumulating at the ends of both static and dynamic microtubules. Super-processive yeast kinesin-8, kip3p, accumulates at microtubule plus-ends and depolymerizes microtubules by taking one tubulin dimer per kinesin (Varga, Helenius et al. 2006; Varga, Leduc et al. 2009). The processivity and plus-end accumulation of kinesin-8 results in greater depolymerase activity for long microtubules than short ones, which is thought to enhance uniformity of spindle microtubules, and provide a way to read-out microtubule length (Varga, Leduc et al. 2009). However, the mechanism by which kinesin-8 pauses at microtubule plus-ends remains unknown. Chromokinesin kinesin-4 can localize to microtubule plus-ends either by itself or with help of PRC1, and there
suppress microtubule growth and thereby control microtubule length, especially antiparallel microtubule length (Bieling, Telley et al. 2010; Subramanian, Ti et al. 2013; van der Vaart, van Riel et al. 2013; He, Subramanian et al. 2014). Kinesin-13 family member, MCAK was shown to depolymerize microtubules from both ends despite lacking processivity (Desai, Verma et al. 1999; Hunter, Caplow et al. 2003; Friel and Howard 2011).

Besides controlling microtubule length by depolymerization or inhibiting growth, some kinesin that accumulate at ends also processes other functions. Kinesin-2, which functions primarily in intracellular transport, can be targeted to growing microtubule plus-ends by hitchhiking on the plus-tip tracking protein, EB1, and forming a complex that steers microtubule growth (Jaulin and Kreitzer 2010; Mattie, Stackpole et al. 2010; Doodhi, Katrukha et al. 2014). CENP-E, a kinesin-7 family member, is a kinetochore-binding motor that bridges kinetochores and microtubules as well as promoting microtubule elongation (Sardar, Luczak et al. 2010). It tracks both growing and shrinking microtubule plus-ends with assistance of its tail domain (Gudimchuk, Vitre et al. 2013). It has been shown that kinesin-5 can also highlight microtubule plus-ends (Fridman, Gerson-Gurwitz et al. 2013) but the relevance to its cellular functions is not clear at present.

1.4.2 Plus-tip Tracking Proteins
Plus-tip tracking proteins (+TIPs) are proteins that accumulate preferentially at growing microtubule plus-ends (Akhmanova and Hoogenraad 2005; Akhmanova and Steinmetz 2008). They can modulate microtubule dynamics and interact with other filaments (Akhmanova and Hoogenraad 2005; Akhmanova and Steinmetz 2008). The canonical +TIP is the end binding (EB) family of proteins that can independently tracks growing microtubule plus-ends dynamically and recruit other proteins to the end of microtubules (Akhmanova and Steinmetz 2008). EBs are dimers due to a central α-helical coiled-coil (Sen, Vephtsev et al. 2013). N-terminal domains of EBs contains calponin homology (CH) domains and are found to be responsible for microtubule binding (Figure 1.3D) (Hayashi and Ikura 2003). Following the coiled-coil dimerization region, there is a unique end-binding homology domain and a conserved EEY/F motif (Figure 1.3D), which is responsible for interactions with and recruitment of other +TIPs through SxIP motif (Honnappa, Gouveia et al. 2009). EB proteins are thought to recognize growing microtubule plus-ends through preference to the GTP-cap (Zanic, Stear et al. 2009; Maurer, Fourniol et al. 2012), and they bind with higher affinity to microtubules polymerized with slow hydrolysable GTP analogs of GMPCPP or GTPγS than to standard taxol-stabilized GDP microtubules (Bieling, Kandels-Lewis et al. 2008; Dixit, Barnett et al. 2009; Zanic, Stear et al. 2009; Maurer, Bieling et al. 2011). Binding dynamics of +TIPs at growing microtubule plus-ends are characterized by the end duration, which ranges from tens to hundreds milliseconds for EB proteins (Bieling, Kandels-Lewis et al. 2008; Dixit, Barnett et al. 2009; Montenegro Gouveia, Leslie et al. 2010; Buey, Mohan et al. 2011). EB proteins are found to increase both microtubule
Another distinct +TIP that can also autonomously track growing microtubule plus-ends through a different mechanism is Xmap215 (Brouhard, Stear et al. 2008). Xmap215 contains multiple TOG domains that can bind to free tubulins, as well as microtubules (Widlund, Stear et al. 2011). The duration of Xmap215 at microtubule plus ends can last up to several seconds (Brouhard, Stear et al. 2008). In cells, Xmap215 functions to increase both catastrophe and rescue frequency, thus leading to an increased growth rate (Vitre, Coquelle et al. 2008; Komarova, De Groot et al. 2009).
microtubule turnover rate (Gard and Kirschner 1987; Popov and Karsenti 2003; Shirasu-Hiza, Coughlin et al. 2003). Synergy between EB1 and Xmap215 can speed up *in vitro* microtubule polymerization from tens nm per second to hundred (Zanic, Widlund et al. 2013), a level comparable to polymerization speed in cells.

+TIPs are also implicated in linking microtubule plus-ends to actin filaments and intracellular membranes (Gundersen, Gomes et al. 2004; Lansbergen and Akhmanova 2006; Akhmanova and Steinmetz 2008). Adenomatous polyposis coli (APC) protein that tracks growing microtubule plus-ends (Morrison, Wardleworth et al. 1998; Slep, Rogers et al. 2005) can bind to actin as well (Moseley, Bartolini et al. 2007). ACF7, a protein containing both microtubule and actin binding domains, is accumulated at microtubule plus-ends in cells (Kodama, Karakesisoglou et al. 2003).

Besides those non-motor proteins, some motor proteins are found to possess +TIP activity as well, by dwelling at end autonomously or interacting with other +TIPs. Microtubule minus-end directed motor NCD and ATK5 were found to track growing microtubule plus-ends in cells (Ambrose, Li et al. 2005; Goshima, Nedelec et al. 2005), suggesting +TIPs might involve in process of generating forces in light of motor mechanical properties. This hypothesis is further supported by observations of Bim1-Myosin complex in yeast (Bim1 is yeast homolog of EB1) (Liakopoulos, Kusch et al. 2003) and EB1-kinesin2 complex in fly neurons (Mattie, Stackpole et al. 2010). It is hypothesized that motor proteins are targeted to the growing microtubule plus-ends by interacting with +TIPs and steer growth directions along another filaments, either
microtubules or actin. However, this model has not been validated through direct reconstitution and it is not certain whether dynamic EB1-microtubule interaction is sufficient to carry out mechanical functions.

1.5 Significance of Studying Microtubule and Microtubule Associated Proteins

Extensive research has revealed that microtubules are essential to cell division, neuronal development, and cell polarity. The precise fulfilment of these cellular functions requires accurate spatial organizations of microtubules and persistent maintenance of these organizations, both of which rely on interplay between microtubule and MAPs, including molecular motors, +TIPs, microtubule stabilizing proteins and microtubule severing enzymes. Malfunction of these proteins are the primary causes to microtubule associated diseases (Jordan and Wilson 2004; Stanton, Gernert et al. 2011).

Defects in intraflagellar transport, which involves kinesin-2 and dynein, are linked to various cilia diseases (Pazour and Rosenbaum 2002). Drugs that inhibit the activity of kinesin-5 or kinesin-7 are under clinical trials for cancer treatment (Rath and Kozielski 2012). The mutant of spastin, a microtubule-severing enzyme, is the most frequent cause of hereditary spastic paraplegia, inherited neurological disorders (Fassier, Tarrade et al. 2013). The axonal transport of amyloid precursor protein, accumulation of which leads to Alzheimer’s disease, is mediated by kinesin-1 (Kamal, Stokin et al. 2000), and
disruption of this transport is related to malfunctioning of tau (Stamer, Vogel et al. 2002; O'Brien and Wong 2011), a microtubule stabilizer.

Thus, research on MAPs and microtubules will help understand the mechanistic basis of these diseases, and provide information important for early disease diagnosis and prevention, as well as providing targets for drug therapy.

1.6 Thesis Motivation and Summary

The overall goal of this thesis is to use interdisciplinary approaches including biology, engineering, physics and statistics, to investigate the impact of MAPs on microtubule dynamics and functions, and develop novel methods for investigating the role of microtubules in specific cellular processes. A number of methods, including in vitro reconstitution of dynamic microtubules, protein engineering, statistical modeling and analysis, micro fabrication and electrophoresis, are utilized in this thesis to investigate EB1 and kinesins, as well as cellulose synthase. The organization of the subsequent chapters is as follows.

Chapter 2... Work described in this chapter is published in the paper “An EB1-kinesin complex is sufficient to steer microtubule growth in vitro” by Yalei Chen, Melissa M. Rolls and William O. Hancock, Current Biology, volume 24, issue 3, 316 – 321. From live imaging in Drosophila larvae dendrites, it was proposed that EB1 tracks the plus-end of a microtubule growing into a branch and an associated kinesin-2 motor walks along a static microtubule to steer the plus-end toward the cell body (Mattie, Stackpole et al.
The number of possible accessory proteins, assisting mechanisms in cells and the fast dynamics of EB1 microtubule interactions challenge this model. Thus, to test the model, purified EB1 and kinesin were linked to form a complex and, using in vitro reconstitution with dynamic microtubules, the ability of steering microtubule growth by the complex was investigated.

Chapter 3...Tetrameric Kinesin-5 plays a crucial role in spindle formation and maintenance by sliding antiparallel microtubules apart. However, previous studies focused on the tetramer configuration and it is not known whether motor domain has any distinct properties that are essential to its cellular functions. A dimer of kinesin-5 motor and neck linker region with kinesin-1 coiled-coil was constructed to investigate kinesin-5 and microtubule interactions. Its behaviors on microtubules and impacts on microtubule dynamics were characterized by fluorescence microscopy and the new insights into kinesin-5 function were facilitated by statistical modeling.

Chapter 4...Single molecular studies have provided detailed views about motor proteins. However, the simplicity of using single microtubules in these experiments sets questions about deducing protein behaviors in cells, where microtubules are counted in hundreds or thousands, by observing them on a single filament. To eliminate this concern, researches on microtubule-MAPs interactions have to be carried out in microtubule organizations close to that in cells. By fabricating metal electrodes and selective patterning of surfaces, microtubules were aligned to electrodes to form three dimensional bipolar structures, termed an “artificial spindle”, with overlaps in the
middle. This method provides a way to assemble microtubules close to their cellular geometry and has the potential to serve as platform for studying mitotic spindle and associated MAPs.

Chapter 5... This chapter is comprised of the following paper “Molecular counting by photobleaching in protein complexes with many subunits: best practices and application to the cellulose synthesis complex”, Yalei Chen, Nathan C. Deffenbaugh, Charles T. Anderson, and William O. Hancock, accepted and in print by Molecular Biology of the Cell. Cellulose synthase complex (CSC) synthesizes cellulose microfibrils into the extracellular cell wall and is hypothesized to track microtubule filaments. The extensive potential uses of cellulose and the impact of cellulose organization on plant cell morphology have led to extensive studies on CSCs. However, the exact copy number and stoichiometry of subunits inside the CSC remain unknown. To estimate copy number of cellulose synthase in a non-destructive way, GFP protein was attached to CESA3 in Arabidopsis and bleached under total internal reflection fluorescent (TIRF) microscopy. The difficulty of identifying discrete bleaching steps and estimating the unitary step size was overcome by statistical analysis that eventually leads to a reliable estimation of copy number. The technique developed for CSC can be applied to identifying the stoichiometry of other multi-subunit complexes in cells.

1.7 References


Morrison, E. E., B. N. Wardleworth, et al. (1998). "EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle." Oncogene 17: 3471-3477.


Chapter 2

An EB1-Kinesin Complex Steers Microtubule Growth

Work presented in this chapter is previously published as:

Yalei Chen, Melissa M. Rolls, William O. Hancock, An EB1-Kinesin Complex Is Sufficient to Steer Microtubule Growth In Vitro, Current Biology, Volume 24, Issue 3, 316 - 321

2.1 Summary

Proper microtubule polarity underlies overall neuronal polarity, but mechanisms for maintaining microtubule polarity are not well understood. Previous live imaging in Drosophila dendritic arborization (da) neurons showed that, while microtubules are uniformly plus-end out in axons, dendrites possess uniformly minus-end-out microtubules (Mattie, Stackpole et al. 2010). Thus, maintaining uniform microtubule polarity in dendrites requires that growing microtubule plus-ends entering branch points must be actively directed towards the cell body. A model was proposed in which EB1 tracks the plus-ends of microtubules growing into a branches and an associated kinesin-2 motor walks along a static microtubule to steer the plus-end toward the cell body. However, the fast plus-end binding dynamics of EB1 (Bieling, Kandels-Lewis et al. 2008; Dixit, Barnett et al. 2009; Buey, Mohan et al. 2011; Maurer, Bieling et al. 2011) appear at odds with this proposed mechanical function. To test this model in vitro, we reconstituted the system by artificially dimerizing EB1 to kinesin, growing microtubules from immobilized seeds, and imaging encounters between growing microtubule plus-ends and static microtubules. Consistent with in vivo observations, the EB1-kinesin complex actively steered growing microtubules. Thus EB1 kinetics and mechanics are
sufficient to bend microtubules for several seconds. Other kinesins also demonstrated this activity, suggesting this is a general mechanism for organizing and maintaining proper microtubule polarity in cells.

2.2 Reconstructing +TIP-kinesin Complex in vitro through Chemically Induced Heterodimerization

Based on previous work (Mattie, Stackpole et al. 2010), it was hypothesized that the microtubule +-Tip Interacting Protein (+TIP) EB1 recruits the molecular motor kinesin-2 via the scaffolding protein Adenomatous polyposis coli (APC) to form a +TIP-kinesin complex at growing microtubule plus-ends. Microtubules growing into branch points are bent toward the plus-ends of stable microtubules at the junction by the motor activity of kinesin-2 (Figure 2.1A). To reconstruct the +TIP-kinesin complex in vitro, we linked kin2, a M. musculus kinesin-2 construct having similar motor properties to KIF3A/B heterodimer (Muthukrishnan, Zhang et al. 2009; Shastry and Hancock 2010), to human EB1 (Figure 2.1, B and C). EB1 and kin2 were fused at their C-termini to FKBP and FRB, respectively, which form a tight ($K_D \sim 12$ nM) ternary complex in the presence of rapamycin (Choi, Chen et al. 1996; Banaszynski, Liu et al. 2005).

To confirm that the fusion tags did not alter EB1 or kin2 functions, we assessed their activities in TIRF-based functional assays. GFP-tagged kin2$_{FRB}$ moved processively along microtubules and EB1$_{FKBP}$ linked to GFP$_{FRB}$ through rapamycin clearly accumulated at growing microtubule plus-ends (Figure 2.2A and C). Because both kin2 and EB1 are
dimers with each subunit containing a FKB or FRBP binding domain, addition of rapamycin could potentially generate a range of species beyond simple 1:1 complexes of dimers. It has been shown that linked kinesin dimers (such as kinesin-5 tetramers) can form a bridge between microtubules and slide one relative to the other (Kapitein, Peterman et al. 2005), so we particularly wanted to avoid complexes containing multiple motors and large daisy-chained aggregates. To minimize the possibility that a single EB1 dimer could bind two kinesin dimers, we combined kin2, EB1 and rapamycin in a 1:5:5 ratio, and characterized the resulting complexes by gel filtration. In the absence of rapamycin, two clear peaks were observed, corresponding to the isolated species (Figure 2.2B, blue curve). Adding rapamycin reduced the peak corresponding to free EB1, completely eliminated the kin2 peak, and led to the emergence of a new single
peak corresponding to the EB1-kinesin complex (Figure 2.2B, red curve). Gel densitometry analysis of the peak indicated a stoichiometry of 1.9 EB1 dimers per kinesin dimer, consistent with the expected 2:1 ratio. Hereafter, we refer to the kinesin-2-GFP_{FRB}\:EB1_{FKBP}\:rapamycin complex as the EB1-kinesin complex.

### 2.3 EB1 Recruits Kinesin to Growing Microtubule Plus-ends and Increases Its Processivity.

We next introduced the EB1-kinesin complex into a flow cell containing dynamic microtubules extending from surface-immobilized GMPCPP microtubule seeds. The EB1-kinesin complex consistently walked along microtubules indicating that formation of the complex did not affect kinesin motor activity. More importantly, EB1-kinesin complex also accumulated at growing microtubule plus-ends, which was not seen in the absence of rapamycin (Figure 2.2C). To ask whether EB1 interacts with the microtubule during kinesin stepping, we carried out single-molecule experiments on taxol-stabilized microtubules. Linking kin2 to EB1 increased its run length from 0.44 ± 0.02 µm to 0.80 ± 0.07 µm (mean ± SE from fit) (Figure 2.2D and 2.3), suggesting that EB1 acts as a tether to enhance kinesin-microtubule interactions.

### 2.4 EB1-kinesin Complex is Sufficient to Bend Growing Microtubules

To test the ability of the EB1-kinesin complex to steer microtubules *in vitro*, we increased the surface density of GMPCPP seeds in our reconstitution assay to increase
frequency of microtubule crossing events. If the EB1-kinesin complex is able to steer microtubule growth, then when one microtubule grows and encounters another microtubule laterally, the growing microtubule plus-end should be directed towards the plus-end of the static microtubule. Kin2GFP<sub>FRB</sub> and EB1<sub>FKBP</sub> were pre-incubated with rapamycin on ice for 20 minutes, added to the final extension solution containing 20 μM free tubulin, and the solution introduced into the flow cell. Kin2GFP<sub>FRB</sub> consistently walked along the microtubules, resulting in the entire length of the microtubules being highlighted. Microtubule plus-ends could be identified both by the direction of kinesin walking and by the accumulation of the EB1-kinesin complex at growing ends. Videos were recorded and analyzed for events in which the plus-end of a growing microtubule encounters the lattice of another microtubule. During these collision events we found that growing microtubule plus-ends, which were highlighted by the fluorescent EB1-kinesin complex, were bent and directed towards the plus-ends of the encountered microtubule (Figure 2.4, A and B; Movie 2.1 and 2.2). In the presence of rapamycin, 23 out of 60 encounters (38%) resulted in microtubule redirection, while in the absence of rapamycin, growing microtubule plus-ends all crossed over static microtubules without interacting (Movie 2.5).
Figure 2.2: EB1-kinesin complex. (A) EB1$_{FKBP}$-GFP$_{FRB}$ localizes to growing microtubule plus-ends. GMPCPP seeds were immobilized on silanized coverslips through biotin-neutravidin and free tubulin was added to generate dynamic microtubules. 150 nM GFP$_{FRB}$ was incubated with 750 nM EB1$_{FKBP}$ and 750 nM rapamycin, combined with 20 μM free tubulin, and introduced into the flow cell. +TIP tracking was observed by TIRF microscopy and is presented both as a montage (left) and a kymograph (right). (B) Hydrodynamic analysis of EB1-kinesin complex. 5 μM kin2GFP$_{FRB}$ and 25 μM EB1$_{FKBP}$ were incubated with (red) or without (blue) rapamycin on ice for 20 minutes before loading onto a gel filtration column. UV absorbance and Coomassie-stained SDS-PAGE gel of corresponding fractions are shown. (C) Localization of kin2GFP$_{FRB}$ on dynamic microtubules when incubated with EB1$_{FKBP}$ in the absence (left) and presence (right) of rapamycin. Upper panels show static views and lower panels show kymographs. (D) Run length of kin2GFP$_{FRB}$ on taxol-stabilized microtubules when incubated with EB1$_{FKBP}$ in the absence (blue, n = 201) or presence (red, n = 172) of rapamycin. Data were fit to single exponentials; mean run lengths with SE of fit are shown in legend.
This result demonstrates that EB1-kinesin complexes at growing microtubule plus-ends are sufficient to direct the growth of microtubules along existing microtubules and lends strong support that this is a viable mechanism for maintaining uniform microtubule polarity \textit{in vivo}. The entire bending process lasted up to several seconds and the microtubules eventually sprang back to their original relaxed position. In some cases, after the bent microtubule snapped back to its original position, the bright fluorescence at the plus-end continued to move along the static microtubule, suggesting that the point of failure was the link between EB1 and the growing microtubule plus-end and not the kinesin-microtubule link.

2.5 Linking to Kinesin Slows EB1 Turnover at Growing Microtubule Plus-ends

The relatively long microtubule deformations produced by the EB1-kinesin complex (bends lasting multiple seconds) appear at odds with the reported fast
Figure 2.4: Microtubule steering by EB1-kin2 complex. (A) and (B): Two independent microtubule bending events are shown, imaging the GFP-labeled kinesin. The original encounter position is indicated by a red star. Kinesin, EB1 and rapamycin were incubated at ratio of 1:10:10 with 250 nM kin2GFP<sub>FRB</sub>. Montages are made from Movie 2.1 and 2.2, respectively. (C) and (D): EB1 dwell time at growing plus-ends. In (C), EB1<sub>FKBP</sub> was visualized by linking it to a streptavidin coated quantum dot (Qdot 565, Life Technologies) through biotinylated anti-his antibody (Qiagen) with 1:4:4 ratio of EB1:antibody:qdot and 3 nM of EB1 used; while in (D), EB1 was linked to kin2GFP<sub>FRB</sub> through rapamycin and visualized by GFP fluorescence at single-molecule concentrations alone (black) or spiked into 100-fold excess of unlabeled complex (red). (E): Diagram illustrating targeting of EB1-kinesin complexes to growing microtubule plus-ends either by direct EB1 binding or by kinesin walking. (F): Kymographs of EB1<sub>FKBP</sub>-kinesinGFP<sub>FRB</sub> targeting to growing microtubule plus-ends by the two mechanisms. Scale bars for both images are 1 second and 1 micron. Table shows fraction of events for each binding mode for data in panel C.
binding/unbinding kinetics of EB1 at growing microtubule plus ends (dwell times from 0.055 s to 0.81 s (Bieling, Kandels-Lewis et al. 2008; Dixit, Barnett et al. 2009; Buey, Mohan et al. 2011; Maurer, Bieling et al. 2011)). To understand the dynamics of the system, it is important to characterize the residence time of EB1 and EB1-kinesin complexes at growing microtubule plus-ends. Using GFP fluorescence on dynamic microtubules in our assay buffer, dwell times of EB1 alone were too short for us to reliably measure. Therefore, we switched from dynamic microtubules to GTP-γ-S microtubules, which have been proposed to be faithful mimics of growing microtubule plus-ends (Maurer, Bieling et al. 2011), and labeled EB1 with quantum dots to increase our temporal resolution. The mean dwell time of individual EB1 dimers was 0.054 ± 0.007 s (mean ± SE of fit, n = 117, Figure 2.4C and Figure 2.6), corresponding to an off-rate of 18.5/s. To measure turnover rates of EB1-kinesin complexes at plus-ends, dynamic microtubules were extended from GMPCPP seeds as before, but very low concentrations (1 nM) of EB1-kinesin complex were introduced, enabling the visualization of individual complexes.

An exponential fit to the data yielded a mean of 0.50 ± 0.079 s (n = 29, Figure 2.4D). The experiment was repeated using 2 nM labeled complex in the presence of 200 nM unlabeled complex and a similar duration of 0.57 ± 0.053 s (n = 38, Figure 2.4D) was found, indicating that crowding effects or cooperative interactions do not affect dwell time at the concentrations used in the microtubule bending assays. Hence, the EB1 residence time at growing microtubule ends is considerably shorter than the seconds-long observed bending durations.
The final question was: how are EB1-kinesin complexes targeted to growing microtubule plus-ends – by direct binding or by kinesin-driven transport (Figure 2.4E)? Targeting by kinesin-based transport was easily identified on kymographs as particles that moved rapidly along the microtubule until reaching the end and then continued at the slower microtubule growth rate (Figure 2.4F, right). However, events were also seen in which complexes bound directly to the growing plus end (Figure 2.4F, left). Interestingly, in both cases individual complexes tracked the growing plus-end, consistent with the kinesin domains generating plus-end movement and the EB1 domains maintaining plus-end association.

2.6 Microtubule Steering Ability is not Restricted to Kinesin-2

It is not known whether kinesin-2 motors have particular characteristics that make them uniquely suited for this microtubule steering function or whether this ability is common to all N-terminal kinesins. Even for kinesin-2 there is a coordination issue – microtubules polymerize at rates of several microns per minute, while kinesin-2 walks along microtubules at 10s of microns per minute, suggesting that the growing microtubule would not be able to keep up with the rate of motor-induced bending.

To address this question, we chose two recombinant kinesins that were characterized previously in single-molecule motility experiments – kin1, a tail-less Drosophila kinesin-1, that moves at twice the speed of kin2, and kin5, an engineered Xenopus kinesin-5 (KSP), that moves at one fifth the speed of kin2 (Shastry and Hancock 2011). The motors were
engineered identically to kin2 (Figure 2.1B), and kin5 was additionally modified by shortening its neck linker such that it matched the processivity of kin1 (Muthukrishnan, Zhang et al. 2009; Shastry and Hancock 2010; Shastry and Hancock 2011). Hence the two motors have nearly identical run lengths but roughly 10-fold different velocities. Similar to kin2GFP\textsubscript{FRB}, both kin1GFP\textsubscript{FRB} and kin5GFP\textsubscript{FRB} accumulated at growing microtubule plus-ends when linked to EB1 (Figure 2.5A and 2.7). Strikingly, both EB1-kin1 and EB1-kin5 complex were able to direct microtubule growth in the same manner as kin2 (Figure 2.5, B and C; Movie 2.3 and 2.4). One difference between motors was the concentration of EB1-kinesin complex necessary for steering; the minimum concentration for reliable steering for kin2 was 250 nM, while kin5 required only 25 nM and kin1 was intermediate at 200 nM (Figure 2.5D). Interestingly, the concentration of motors required for bending scaled linearly with the microtubule off-rate (= speed ÷ run length), meaning that (assuming similar on-rates) the microtubule affinity and not the motor velocity is the principal determinant of microtubule bending. The fact that all three motors were able to steer growing microtubules indicates that this property is not unique to kinesin-2 and it could potentially be a general mechanism involving motors other than kinesin-2.

2.7 Microtubule Organization in Cells

By recruiting other binding partners to microtubule plus-ends, EB1 has been implicated in controlling microtubule dynamics (Tirnauer, Grego et al. 2002; Manna, Honnappa et
al. 2008), bridging microtubule ends to cellular structures (Kodama, Karakesisoglou et al. 2003; Lansbergen, Grigoriev et al. 2006; Moseley, Bartolini et al. 2007), and proper positioning of the mitotic spindle (Liakopoulos, Kusch et al. 2003; Akhmanova and Steinmetz 2008). The idea that EB1 has the ability to sustain mechanical forces at growing microtubule plus-ends has, until now, lacked direct experimental support. This question is of particular importance because a number of motor proteins capable of generating both pulling and pushing forces in microtubule networks can be targeted to growing microtubule plus-ends with the help of EB1 (Liakopoulos, Kusch et al. 2003; Manna, Honnappa et al. 2008; Cai, McEwen et al. 2009; Jaulin and Kreitzer 2010).

Neurons are not the only polarized cells whose function requires uniformly oriented microtubule bundles or arrays. In fact, many if not most differentiated cells have cell-type-specific noncentrosomal microtubule networks (Bartolini and Gundersen 2006; Luders and Stearns 2007). For instance, in epithelial cells, microtubules are aligned along the apico-basal axis with their minus-ends towards apical side and plus-ends towards the basal side (Bartolini and Gundersen 2006; Luders and Stearns 2007). The molecular mechanisms that guide microtubule remodeling during epithelial differentiation and maintain proper microtubule polarity post-differentiation are still largely unknown. A recent study showed that septin binds both EB1 and microtubules, and that growing microtubule plus-ends track existing septin-coated microtubules in epithelial cells (Bowen, Hwang et al. 2011). RNAi knockdown of septin leads to entangled microtubule plus-end trajectories, suggesting that septin and EB1 act together to co-align microtubules. In another study in epithelial cells, the homodimeric kinesin-2 motor
KIF17 was reported to co-localize with EB1 and APC at growing microtubule plus-ends and play a role in proper epithelial polarization (Jaulin and Kreitzer 2010).

In addition to microtubule-microtubule interactions, there is also evidence that EB1 maintains proper microtubule organization in cells by linking growing microtubule plus-ends to actin filaments. Knockout of the microtubule-actin cross-linking factor ACF7 in keratinocytes led to a model in which EB1 and ACF7 coordinate their activities to guide growing microtubules to focal adhesions along existing actin filaments (Wu, Kodama et al. 2008). An even better analog to the EB1-APC-kinesin complex is found in yeast, where proper mitotic spindle orientation requires a myosin V motor (Myo2) bridged through the adaptor protein Kar9 to Bim1, the yeast EB1 homolog. The Bim1-Kar9-Myo2 complex localizes to microtubule plus-ends and guides microtubules along polarized actin filaments (Liakopoulos, Kusch et al. 2003). Together, these reports suggest that +TIP-motor complexes provide a general system for controlling microtubule organization in cells by directing the growth of microtubule plus-ends using existing cues. In this context, the present work demonstrating that a minimal system of just EB1 and kinesin is competent to steer microtubule growth provides vital biophysical support for these models.

2.8 Mechanical Properties of EB1

The observed plus-end steering requires that EB1 proteins remain at the growing microtubule plus-end while kinesin walks along the lattice of an existing microtubule,
Figure 2.5: Microtubule steering by kin1 and kin5-based complexes. (A) kin1GFP<sub>FRB</sub> and kin5GFP<sub>FRB</sub> accumulated at growing microtubule plus-ends only when incubated with EB1<sub>FKBP</sub> and rapamycin. Kymographs are shown in Figure 2.7. (B) and (C): Microtubule steering by EB1-kin1 and EB1-kin5 complex, respectively. The original encounter position is indicated by red star. Kinesin, EB1 and rapamycin were incubated at ratio of 1:10:10. 200 nM kin1GFP<sub>FRB</sub> and 25 nM kin5GFP<sub>FRB</sub> (with shortened neck linker to enhance processivity) were used. Montages were made from Movie 2.3 and 2.4, respectively. (D) Table of motor properties showing that minimum motor concentration for bending scales with motor off-rate and not velocity. Probability of bending is defined as the fraction of microtubule crossing events that resulted in the growing microtubule bending toward the plus-end of the static microtubule. Ratio of tip/wall is defined as the peak fluorescence intensity at the microtubule tip divided by the peak along the microtubule wall; see the Experimental Procedures for details.

<table>
<thead>
<tr>
<th>Motor Type</th>
<th>Kin2</th>
<th>Kin1</th>
<th>Kin5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run length (nm)*</td>
<td>710 ± 30</td>
<td>2100 ± 100</td>
<td>1770 ± 200</td>
</tr>
<tr>
<td>Speed (nm/s)*</td>
<td>480 ± 98</td>
<td>990 ± 130</td>
<td>81 ± 21</td>
</tr>
<tr>
<td>Off rate (1/s)**</td>
<td>0.676</td>
<td>0.471</td>
<td>0.046</td>
</tr>
<tr>
<td>Min. conc. for bending (nM)</td>
<td>250</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>Probability of bending</td>
<td>0.38 (n=60)</td>
<td>0.39 (n=31)</td>
<td>0.41 (n=34)</td>
</tr>
<tr>
<td>Ratio of tip/wall (mean ±SD)</td>
<td>3.09 ± 1.19 (n= 37)</td>
<td>2.61 ± 1.44 (n=36)</td>
<td>2.14 ± 0.64 (n = 34)</td>
</tr>
</tbody>
</table>

* Taken from ref (13) with mean ± SE from fit for run length and mean ± SD for speed.

** Calculated from speed/run length.
meaning that EB1 must bear the mechanical forces generated by microtubule bending. While EB1-kinesin complexes had 500 msec plus-end dwell times, for a complex bridging two microtubules the upper limit for the duration of the interaction would more likely be defined by the 53 msec dwell time of isolated EB1 on GTP-γ-S microtubules. Depending on the motor type used, microtubules were bent for an average of between 3 and 11 seconds, or roughly 100-fold longer than the duration of a single EB1-microtubule interaction. Hence, this microtubule steering mechanism requires a pool of EB1-kinesin complexes (perhaps upwards of 100 based on the discrepancy in kinetics) that dynamically bind and unbind with kinetics much faster than the rate of microtubule bending.

While EB1 and kinesin were artificially dimerized in our in vitro assay, one question is whether EB1-APC and kinesin-APC interactions (neither of which has been characterized) are sufficiently strong or long-lived to sustain microtubule bending. As a first approximation, if their off-rates are slower than the $18 \, \text{s}^{-1}$ dissociation rate of EB1 from microtubules, then they should not be the weak link in the system. The fact that APC was replaceable in vitro supports the idea that APC acts as a scaffold, but because APC

![Figure 2.6: Duration of EB1\textsubscript{GFP} binding events on GTP\textgamma{}S microtubules in assay buffer without added KCl and KAc,\textsuperscript{c}.

EB1\textsubscript{GFP} concentration was 1 nM, and buffer was 80 mM K-PIPES, 1 mM EGTP, 4 mM MgCl\textsubscript{2}, pH 6.8. Data were fit to an exponential (red line) giving an average dwell time of $0.288 \pm 0.28 \, \text{s}$ (mean $\pm$ SE of fit, $n = 109$). These data can be compared to dwell times of quantum dot-functionalized EB1 off-rates in normal assay buffer, shown in Figure 2.4C.
itself binds microtubules, it could play an important role in enhancing microtubule interactions in vivo. For instance, it may enhance the affinity of EB1 to the growing microtubule and may also act as one of perhaps many microtubule crosslinking proteins that stabilize the bent conformation. The membrane will also serve as an important mechanical barrier such that the small deflection of the growing plus-end is “locked in” by the barrier and further stabilized as the microtubule continues to grow.

In conclusion, we demonstrate that a complex of EB1 and kinesin is mechanically capable of force generation at microtubule plus-ends and these forces can be used to bend microtubules. This work expands the cellular functions of both kinesin motors and +TIPs.
2.9 Movies

**Movie 2.1:** Microtubule steering by EB1_{FKBP}\-kin2GFP\_FRB complex, related to Figure 2.4A. EB1: rapamycin: kinesin were used at a ratio of 10:10:1 with a kin2GFP\_FRB concentration of 250 nM. Video was acquired using TIRF microscopy at 5 fps. The EB1_{FKBP}\-kin2GFP\_FRB complex highlights the growing microtubule plus-ends and during an encounter the plus-end of the growing microtubule is steered towards the plus-end of the immobilized microtubule.

**Movie 2.2:** Microtubule steering by EB1_{FKBP}\-kin2GFP\_FRB complex, related to Figure 2.4B. The video is from an independent experiment using conditions identical to Movie 2.1.

**Movie 2.3:** Microtubule steering by EB1_{FKBP}\-kin1GFP\_FRB complex, related to Figure 2.5B. Experimental conditions are the same as described in Movie 2.1 except 200 nM of kin1GFP\_FRB was used. Similar steering of growing microtubule plus-ends was observed, confirming that kin1 also has the ability to steer growing microtubules when complexed to EB1.

**Movie 2.4:** Microtubule steering by EB1_{FKBP}\-kin5GFP\_FRB complex, related to Figure 2.5C. Experimental conditions are the same as described in Movie 2.1, except 25 nM of kin5GFP\_FRB was used. In this movie the growing microtubule plus-end changes direction as it hits another microtubule laterally.

**Movie 2.5:** Negative control showing microtubule cross-over event in the absence of EB1. Related to Figure 2.4. Experiment was performed using conditions identical to
Movie 2.4, but without EB1-FKBP. Microtubules are labeled weakly by moving kin1GFP\textsubscript{FRB} motors (200 nM), and no accumulation is observed at growing plus-ends. Note that encounters consist of microtubules crossing over one another without observable bending. Movie is 4x real time.

The above movies can be found at


2.10 Experimental Procedures

2.10.1 Cloning and Protein Expression

To make kin1, \textit{Drosophila} conventional kinesin was truncated at position 559 and eGFP, FRB and a His\textsubscript{6} tag were added to the C-terminal sequentially. Kin2 was cloned by swapping the mouse KIF3A head and neck-linker into kin1 as previously described (Shastry and Hancock 2010). Kin5 was engineered by swapping head and neck-linker of XIKSP into kin1 and shortening the neck-linker to 14 aa as previously described (Shastry and Hancock 2011). Human EB1 was fused to FKBP and a His\textsubscript{6} tag at the C-terminal. All motors were expressed in bacteria and purified by Ni column chromatography as previously described (Hancock and Howard 1998), frozen in liquid N\textsubscript{2}, and stored at -80\textdegree C in storage buffer (50 mM K-phosphate, 300 mM NaCl, 2 mM MgCl\textsubscript{2}, 100 μM ATP, 10 mM β-mercaptoethanol, 500 mM imidazole, pH = 7.2, with 10% sucrose added). EB1 was expressed and purified similarly, except that expression was induced with 0.5 mM IPTG and grown overnight at 23\textdegree C. The cell pellet was resuspended in ice-cold buffer B.
(50 mM K-phosphate, 400 mM NaCl, 2 mM MgCl₂, 10 mM β-mercaptoethanol, pH 7.2) (Bieling, Kandels-Lewis et al. 2008). EB1 storage buffer consisted of 50 mM K-phosphate, 400 mM NaCl, 1 mM MgCl₂, 100 μM ATP, 5 mM DTT, pH = 7.0, with 10% sucrose added. At the highest concentrations used, EB1 and kinesin comprised 4% and 2.5% of the final volume, respectively; single-molecule investigations were carried out at concentrations 100-fold lower.

2.10.2 Microscopy Assays and Curve Fitting

All experiments were carried out in assay buffer (80 mM K-Pipes, 85 mM KCl, 85 mM potassium-acetate, 1 mM EGTA, 4 mM MgCl₂, pH 6.8). Flow cells were assembled by attaching OTS-coated coverslips to glass slides with double-sided tape. The flow cell was first coated with 0.5 mg/ml neutravidin and blocked by 5% Pluronic F108 at room temperature. Then, the flow cell was incubated with Cy5- and biotin-labeled GMPCPP microtubules seeds at 35°C. Microtubule polymerization was initiated by flowing in a buffer containing 20 μM free tubulin in assay buffer supplemented with 0.1% methyl cellulose, casein, 1 mM GTP, 1 mM MgCl₂, oxygen scavengers (glucose, glucose oxidase, catalase, β-mercaptoethanol) and proteins to be assayed. Flow cell temperature was maintained at 32°C through an objective heater.

Run length and dwell time data were fit to single exponentials with no offset using Origin software.
To calculate tip/wall fluorescence ratio in Figure 2.5D, two line scans were made perpendicular to the microtubules at the tip of microtubule and 1μm away from the tip, respectively. Peak intensities above the background were used to calculate the ratio.

### 2.10.3 Analytical Gel Filtration

A 300 μL sample of 5 μM kin2GFP\textsubscript{FRB} and 25 μM of EB1\textsubscript{FKBP} in assay buffer supplemented with 25 μM rapamycin was incubated on ice for 15 minutes before loading on to a Superdex 200 10/300 GL column (GE Healthcare). The fractions were eluted in assay buffer containing 1 μM rapamycin, 0.5 mL were fractions collected, and the absorbance monitored at 280 nm. SDS-PAGE gels were Coomassie stained, band intensities analyzed in ImageJ, and stoichiometries calculated by dividing each band intensity by its molecular weight.

### 2.11 References


Chapter 3
Kinesin-5 Acts as a Microtubule Stabilizer, Polymerase and Plus-tip Tracker

The work in this chapter is comprised of a paper in preparation listed as following:


3.1 Introduction

Kinesin, a motor protein that couples chemical energy into mechanical movement, conventionally functions as cargo transport by stepping along microtubules. However, a number of kinesins have been found to accumulate at microtubule plus-ends and affect microtubule dynamics, capture kinetochores or bridge to other microtubules. Yeast kinesin-8, kip3p, accumulates at microtubule plus-ends and depolymerizes microtubules (Varga, Helenius et al. 2006; Varga, Leduc et al. 2009). The kinesin-13 family member, MCAK was shown to depolymerize microtubules from both ends despite lack of processivity (Desai, Verma et al. 1999; Hunter, Caplow et al. 2003; Friel and Howard 2011). Kinesin-2, which functions primarily in intracellular transport, can also be targeted to growing microtubule plus-ends by hitchhiking on plus-tip tracking protein, EB1, and forming a complex that steers microtubule growth (Jaulin and Kreitzer 2010; Chen, Rolls et al. 2014; Doodhi, Katrukha et al. 2014). Chromokinesin kinesin-4 can localize to microtubule plus-ends either by itself or with help of PRC1, and there suppresses growth of microtubules to control microtubule length (Bieling, Telley et al.)
2010; Subramanian, Ti et al. 2013; van der Vaart, van Riel et al. 2013; He, Subramanian et al. 2014). Kinetochore kinesin CENP-E, a kinesin-7 family member that bridges kinetochores and microtubules as well as promoting microtubule elongation (Sardar, Luczak et al. 2010), tracks both growing and shrinking microtubule plus-ends with assistance of tail domain (Gudimchuk, Vitre et al. 2013).

As one of the most important proteins for cell division, homotetrameric kinesin-5 can slide apart antiparallel microtubules and is thought to be responsible for separating centrosomes, maintaining bipolar spindles, and proper segregation of chromosomes (Goulet and Moores 2013). Besides functioning during mitosis, kinesin-5 also plays a role in neuronal development (Myers and Baas 2007; Nadar, Ketschek et al. 2008). However, these cellular functions of kinesin-5 are primarily based on its ability to slide antiparallel microtubules, an ability conferred by its unique tetrameric configuration. But, replacing the kinesin-5 motor and neck linker domains with those from either kinesin-1 or kinesin-10, which preserves the tetrameric architecture and motor directionality, cannot rescue kinesin-5 loss of functions during mitosis (Cahu and Surrey 2009). Still, it stays a mystery what specific properties of kinesin-5, besides its configuration, are essential for its roles and can distinct kinesin-5 further apart from other motor proteins.

The recently found microtubule binding domain on the C-terminus of kinesin-5 enhances kinesin-5’s affinity to microtubules without affecting motility, thus improving kinesin-5’s sliding ability (Mayr, Storch et al. 2011). This finding suggests that the non-
motor region of kinesin-5 has additional functionality besides providing heterodimerization. Extensive chemical kinetic studies on recombinant kinesin-5 dimers show that kinesin-5 is a low motor with rate-limiting ATP hydrolysis (Cochran, Krzysiak et al. 2006; Krzysiak and Gilbert 2006), but the relevance of these studies to its cellular functions is not straightforward and it remains uncertain whether kinesin-5 motor domain has any other uniqueness.

Several studies showed that inhibition of kinesin-5 leads to longer microtubules in cells, leading to the hypothesis that kinesin-5 might be a microtubule depolymerase (Gardner, Bouck et al. 2008; Wang, Gao et al. 2010). A recent study found that microtubules became more dynamic in cells with loss function of kinesin-5, an indication that kinesin-5 actually stabilizes microtubules (Fridman, Gerson-Gurwitz et al. 2013). It remains controversial that how kinesin-5 interacts with microtubules and how this relates to its primary functions. Another interesting observation comes from in vitro reconstitution of microtubule sliding. When antiparallel microtubules are pulled apart by *Xenopus* kinesin-5 tetramers in vitro, microtubules will reach the end of each other eventually, and instead of just detaching, are tethered through plus-end to plus-end (Kapitein, Peterman et al. 2005). The end tethering of microtubules implies that motors might pause at the ends as reported in yeast cells (Fridman, Gerson-Gurwitz et al. 2013), and potentially should increase the sliding efficiency. However, it is not further examined whether kinesin-5 can accumulate at microtubule plus-ends independently, and if true, by which mechanism. Additionally, the relevance between end dwelling of kinesin-5 and its impact on microtubule dynamics remains uncertain.
Here, we engineered a recombinant kinesin-5 dimer to eliminate the tetrameric configuration and showed that kinesin-5 stabilizes microtubules by slowing GMPCPP microtubule depolymerization. In an environment with dynamic microtubules, kinesin-5 promotes the growth of microtubules and suppresses the catastrophe, an evidence of polymerase activity. The stabilization of microtubules in cells can prevent futile hydrolysis due microtubule catastrophe during microtubule sliding. More interestingly, inhomogeneous plus-end structures were seen by direct observation of GFP motors, presumably by binding of kinesin-5 to tubulin filaments. This extraordinary phenomenon suggests that protofilaments should be stabilized by kinesin-5 in order to be seen under fluorescent microscopy. Single molecule total internal reflection fluorescent microscopy experiments revealed that kinesin-5 dimers can dwell at static plus-ends of microtubules for 7 seconds. This evidence leads us to a model that kinesin-5 pauses at plus-end microtubules and stabilizes newly incorporated tubulin by bridging two adjacent tubulins.

3.2 Results

3.2.1 Kinesin-5 Slows the Depolymerization of GMPCPP Microtubules

Wild-type kinesin-5 is a homotetramer with a pair of catalytic motor domains on each end, and has been found to switch between diffusive and processive movement by cargo activation(Kapitein, Kwok et al. 2008). To investigate kinesin-5 motor properties and eliminate effects from tetrameric configuration, as well as potential regulation by the C-terminal tail domain, we fused the motor domain and neck-linker region of
kinesin-5 to the coiled coil of *Drosophila* conventional heavy chain (KHC) (Figure 3.1A and see Methods), resulting to stable dimers that have comparable motor properties to full-length kinesin-5 (Valentine, Fordyce et al. 2006; Shastry and Hancock 2011). The recombinant kinesin-5 dimer with its native 18 amino acids neck-linker (kin5_18) is minimally processive with a run length of 0.33 ± 0.03 µm (mean ± s.e.m) (Figure 3.2). To mimic the processive state, we made another recombinant dimer (kin5_14) having a longer run length of 1.02 ± 0.12 µm (mean ± s.e.m) (Figure 3.2) by shortening the neck-linker to 14 aa (Shastry and Hancock 2011) (Figure 3.1A).

**Figure 3.1: kinesin-5 is a microtubule stabilizer and polymerase.** (A) The construct of chimeric kinesin-5 dimers. Kinesin-5 was truncated at the end of neck linker and fused to KHC coiled coil to make kin5_18. To make kin5_14, the neck linker was shortened by 4 amino acids at the end. (B and C) kinesin-5 slows depolymerization of GMPCPP microtubules. (B) Surfaced immobilized GMPCPP microtubules were incubated with/without motors as indicated. Red color: initial microtubules; green color: microtubules after 20 min. (C) Quantitative measurement of average depolymerization rate at conditions indicated. P-values are from two sample t-tests.
To test whether kinesin-5 can depolymerize stable microtubules, we immobilized GMPCPP microtubules on glass through biotin-neutravidin interactions, and measured microtubule lengths over a 20-minute interval in the presence and absence of kinesin-5. To minimize the effects of photobleaching, the laser was shuttered for most of the recording duration (see methods). In absence of kinesin-5, the GMPCPP microtubules

Figure 3.2: Rescue frequency and catastrophe speed of dynamics microtubules. (A) Microtubules with kin5_18 showed slightly lower rescue frequency, but this difference is not statistically significant. (B) Speed of shortening for microtubules with kin5_18 is significantly faster than control without motors.
Figure 3.3: Microtubule polymerization with kinesin-5. (A) Microtubule polymerization over 5 minutes in the conditions of 10 µM free tubulin with/without kin5_18 as indicated. In experiments with kin5_18 present, microtubules grow longer. (B and C) Microtubule growth speed and catastrophe frequency in conditions with and without kin5_18. (B) The growth is significantly faster when kin5_18 was added, while the catastrophe frequency is reduced in (C). (D) Diagram to show possible end structures of growing microtubules. (E) Fit of error function to intensity profiles of growing microtubule plus-ends. The intensity from line scans along microtubules is normalized relative to the background and amplitude. (Points: normalized raw data; lines: fit from error function). (F) Standard deviation (s.t.d.) obtained from fit in (E) Kin5_18 causes a significant increase in the s.t.d. compared to control experiments. (G) Diagram of simulation on growing microtubule plus-ends. To simulate the tapering of end, the longest filament is fixed to a number of tubulins (Nmax) in each set, and number of tubulins in the rest 11 filaments are randomly selected from 0 to Nmax. The dye density is 1:20 (dye : tubulin), the same as that in experimental condition. (H) An exemplary intensity from simulation of Nmax = 150 and the corresponding fit. (I) The s.t.d. vs Nmax. Ranges of Nmax were searched to get s.t.d. The 418 nm s.t.d. correlates to Nmax ~ 140, and 222 nm s.t.d. corresponds to Nmax ~ 60.
depolymerize slowly at an average speed of 17.4 ± 1.6 nm/min (mean ± s.e.m., n = 26, Figure 3.1, B and C), similarly to speeds reported previously (Varga, Leduc et al. 2009). Surprisingly, when either kin5_14 or kin5_18 was added, the depolymerization rate was even slower (Figure 3.1B). Average depolymerization rate of 9.0 ± 0.8 nm/min (mean ± s.e.m., n= 21) for kin5_18 and 6.7 ± 0.7 nm/min (mean ± s.e.m., n= 19) for kin5_14. This decrease in the depolymerization rate was statistically significant (p-value < 0.0001) (Figure 3.1C), and argues against the hypothesis that kinesin-5 is a microtubule depolymerase. Instead, this result indicates that kinesin-5 acts as a microtubule stabilizer under these conditions.

3.2.2 Kinesin-5 Promotes Microtubule Growth and Inhibits Catastrophe

To further investigate the impact of kinesin-5 on microtubule dynamics, surface-immobilized short biotinylated GMPCPP microtubule seeds in a flow cell were extended by introducing cy5 labeled tubulin and the resulting microtubule dynamics was observed by total internal reflection (TIRF) microscopy in the presence and absence of kinesin-5 motors. The first obvious result was a clear increase in microtubule length 5 minutes after adding kin5_18 compared to control (Figure 3.3A). This result is consistent with kinesin-5’s microtubule stabilizing ability, but could result from other effects on microtubule dynamics.

To systematically understand how kinesin-5 affects microtubule growth, we quantified microtubule dynamic instability parameters under control conditions and in the
presence of kin5_18. The growth speed with kin5_18 present was 19.1 ± 1.3 nm/s (mean ± s.e.m., n = 21, Figure 3.3B), which is significantly faster than the growth speed of 8.1 ± 0.9 nm/s (mean ± s.e.m., n = 19, Figure 3.3B) without kinesin-5. Furthermore, Kin5_18 reduced the catastrophe frequency from 0.102 ± 0.034 s⁻¹ (mean ± sd., n = 9) to 0.032 ± 0.011 s⁻¹ (mean ± sd., n = 9) (Figure 3.3C), a 3-fold decrease. In contrast, there was no statistically significant change in rescue frequency (Figure 3.4A). These results clearly show that kinesin-5 promotes microtubule elongation by accelerating growth and preventing catastrophe. Notably, the shrinkage speed was also increased in presence of kin5_18, from 43.7 ± 5.5 nm/s (mean ± s.e.m., n = 9) to 79.2 ± 10.2 nm/s (mean ± s.e.m., n = 9) (Figure 3.4B). One possible explanation for enhanced shrinkage rate is that kinesin-5 stabilizes the microtubules during growth and overcomes stresses introduced by the defects that are incorporated during extension. When the stress build up exceeds the stabilizing capacity of kinesin-5, microtubules eventually catastrophe and shrink at a faster rate.

**Figure 3.4: rescue frequency and catastrophe speed of dynamics microtubules.** (A) Microtubules with kin5_18 showed slightly lower rescue frequency, but this difference is not statistically significant. (B) Speed of shortening for microtubules with kin5_18 is significantly faster than control without motors.
3.2.3 Kinesin-5 Leads to More Tapered Plus-ends of Growing Microtubules

Measuring tip tapering can provide insights on GTP cap-size and end structure (Coombes, Yamamoto et al. 2013). Due to the stochastic incorporation of tubulin dimers at the end, the extension of each protofilament will proceed at various speeds. Stochastic extension of tubulin protofilaments will show up as more tapered ends (Figure 3.3D). To investigate the tapering at the end, we performed line scans along the growing microtubule plus-ends and fit the resulting intensity drop at the edge by an error function as done previously (Demchouk, Gardner et al. 2011) (Figure 3.3E). The standard deviation from the fit represents the tapering at the end: a larger standard deviation denotes more tapered end. The standard deviation from control microtubule plus-ends was 222 ± 45 nm (mean ± s.e.m., n = 18, Figure 3.3F), which is close to the sigma (131.4 ± 4.3 nm, mean ± s.e.m., n = 17) of point spread functions of the microscope at experimental conditions. This result suggests that control microtubule plus-ends are nearly minimally tapered. However, once kin5_18 is added, the standard deviation is increased almost 2-fold to a degree of 418 ± 59 nm (mean ± s.e.m., n = 19) (Figure 3.3F). The more tapered plus-ends suggest that the discrepancy between extensions of 13 tubulin filaments is getting larger, and indicate potential existence of interactions between kinesin-5 and tubulin filaments at plus-ends.
To get a quantitative view of end tapering, we simulated microtubules ends and obtained the standard deviation by error function fitting. The microtubule backbone was simulated with 13 filaments, and when tapering starts at the end, length of 12 filaments are randomly generated. For each set of simulation (Figure 3.3G), the length of longest filaments is set to a fixed number Nmax of tubulins, and the rest 11 filaments contain tubulin numbers ranged from 0 to Nmax. The dye density is set to 1:20 (dyes : tubulins), which is the same in the experimental conditions. Intensity profiles are generated by convoluting with a point spread function of sigma = 131 nm and sequentially fit with error functions to obtain standard deviation (Figure 3.3H). By varying the Nmax, the relationship between s.t.d. obtained from fit and Nmax is
examined (Figure 3.3I). The 222 nm s.t.d corresponds to Nmax ~ 60 and the 418 nm s.t.d. correlates to Nmax ~ 140. From this simulation, it estimates that kinesin-5 causes the filaments tappers more than twice longer than the control.

3.2.4 Kinesin-5 Generates Diverse Plus-end Structures on Growing Microtubules

In order to understand mechanisms by which kinesin-5 affects microtubule dynamics, we observed the dynamics of GFP tagged kinesin-5 at the ends of growing microtubules. Kin5_18GFP is sufficiently high that, at 20 nM dimer concentration, microtubules made from unlabeled tubulin are easily visualized. Strikingly, diverse, irregular end structures were observed (Figure 3.5, A-E). These were not seen in control experiments with kinesin-1 GFP. A subset of microtubules was seen growing with curled plus-ends that occasionally formed ring structures. These microtubules kept growing while the end-structures are kept relatively constant, suggesting the structures are at pre-steady state. To achieve this pre-steady state, tubulins have to be added to the very ends and extend the curls while the curls can be straightened and form microtubules. Based on these, we think the curls are composed of several leading tubulin filaments and straightening happens when trailing filaments are catching up and form hollow microtubule tubes.

Another notable observation was that microtubules grew with two peeled filaments/filament bundles, like a ‘banana peel’ people have proposed for microtubule plus-ends during catastrophe (Chrétien, Fuller et al. 1995), and one side of filaments got
straightened first and followed by the annealing of the other half (Figure 3.5F). Based on electronic microscope pictures, it was shown that tubulin filaments at growing microtubule plus-ends are slight curved and “banana peel” structure is often thought to be the characteristic when microtubules switch to catastrophe (Chrétien, Fuller et al.).

Figure 3.6: kinesin-5 highlights taxol-stabilized microtubule plus-ends. (A and B) Kin5_18GFP and kin5_14GFP on taxol-stabilized microtubules and corresponding intensity profiles. (C and D) Single molecule kin5_14GFP on taxol-stabilized microtubules. (C) Montage of single kin5_14GFP walks along microtubules and pauses at the end. (D) Kymograph of (C), showing pausing at plus-end of microtubules. (E) The exponential fit of residence time at taxol-stabilized microtubule plus-ends. (F) Simplified cycle after kinesin reaches microtubule plus-ends. The rear head should detach in time less than regular stepping (~0.1s) and followed by ATP binding and hydrolysis. The final status can be either at no nucleotide, ATP, ADP.Pi or ADP.

(G and H) Residence time of kin5_18GFP on microtubules at ADP state. (G) Diagram and kymography of kin5_18GFP on microtubules. (H) The exponential fit to the data result to an average residence time of 1.07s. 1mM ADP was used.
1995). These structures have never been seen under fluorescent microscope, presumably due to the fast dynamics at plus-ends and insufficient signal-to-noise ratio. Directly observing kin5-18GFP will give a much higher signal-to-noise ratio because of the low concentration of kin5_18GFP compared with fluorescent tubulin, and high specificity of kin5_18GFP to microtubules. Additionally, to successfully observe those phenomena, kinesin-5 has to be able to step on the tubulin protofilaments and slow the disassembly dynamic by stabilizing them. The discrepancy of phenomena between observing kin5_18GFP and kinesin-1GFP suggests that either kinesin-1 GFP do not bind to tubulin filaments or it bind but do not stabilize the structure. Considering kinesin-5 is a slow motor on an average of 10 steps per second, while kinesin-1 steps at about 100 steps per second, the slow hydrolysis cycle means that kinesin-5 might spend longer time in two head bound state, which can serve as a mechanism of stabilizing tubulin filaments by bridging two adjacent tubulin dimers. Additionally, the two head bound state can be more favorable on curled microtubules that can reduce the tension between two motor domains, which in turn will reinforce the filaments.

### 3.2.5 Kinesin-5 Highlights Taxol-stabilized Microtubule Plus-ends with Residence Time of 7 Seconds

To further explore microtubule-kinesin-5 interactions, we immobilized taxol-stabilized microtubules and observed kinesin-5 binding by TIRF microscopy. At the moderate concentration of 5 nM kin5_18GFP dimers, streaming of motors along microtubules due
to kinesin walking was observed along with clear accumulation of motors at microtubule plus-ends (Figure 3.6A). Next, to investigate the impact of processivity on end dwelling, the experiment was repeated with the more processive kin5_14GFP. At 3 nM kin5_14GFP dimers, a similar accumulation at the plus-ends of taxol-stabilized microtubules was observed (Figure 3.6B). These observations suggest that kinesin-5
walks to the plus-ends of microtubules and pause there. To characterize the duration of end dwelling, the concentration of kin5_14GFP was lowered to single molecule level (25 pm), the duration that single kin5_14GFP dimers paused at the plus-ends of taxol-stabilized microtubules was quantified (Figure 3.6, C and D). Fitting an exponential to the lifetime distribution gave an average duration of 7.2 ± 0.6 s (mean ± s.e.m.). The mean dwell time of kin5_18GFP was 7.0 ± 0.9 s (mean ± s.e.m.) (Figure 3.6E), meaning that the duration of end-binding was independent of motor processivity. These findings indicate that when kinesin-5 walked and reached the end of microtubule track, the trailing head should detach without sensing what’s in front and ATP will bind to the front head causing the neck linker docking and motor pausing at single-head bound state, which can be at ATP, ATP-Pi, or ADP state (Figure 3.6F). To rule out the hypothesis that kinesin-5 stays at the microtubule plus end at ADP state, we carried out experiments to measure the duration of kin5_18GFP on microtubules at 1 mM ADP concentration under TIRF microscopy (Figure 3.6G). From single exponential fit, the average duration time is 1.07 ± 0.09 s (mean ± s.e.m., n = 120, Figure 3.6H), which is much less than the 7-second duration at the static end. Additionally, kinesin-5 was reported to have a rate-limiting ATP hydrolysis. Hence, the most likely state that kinesin-5 bound to the microtubule plus-ends is single-head bound ATP state.
3.2.6 Kin5-14 and Kin5_18 Differ in Growing Microtubule Plus End Tracking

Microtubule associated proteins (MAPs) that regulate microtubule dynamics often show preference for growing microtubule plus ends (Jiang and Akhmanova 2011). It is important to know whether kinesin-5 can track growing microtubule plus-ends. To address this question, we extended microtubules at higher tubulin concentration of 15 µM in order to obtain sustained growth. However, only occasional plus-end accumulation was seen for kin5_18GFP at motor concentration of 30 nM (Figure 3.7, A and B). When we replace kin5-18GFP with 20 nM processive kin5_14GFP, the growing plus-ends were constantly highlighted by GFP (Figure 3.7, A and B), suggesting that processivity is crucial for kinesin-5 tip tracking. To fully characterize the tip-tracking of kinesin-5, we lowered the concentration of kin5_14GFP to single molecule level (50 pM) to measure the residence time at growing microtubule plus-ends. By fitting an exponential to the observed dwell time (Figure 3.7C), we get an estimated mean duration of $7.04 \pm 1.01$ s (mean ± s.e.m. of fit).

How does kinesin-5 track growing microtubule plus-ends? One possible scenario is that kinesin-5 might bind to free tubulin dimers like the microtubule polymerase XMAP215. However, the discrepancy between kin5_14GFP and kin5_18GFP argues against that. To further test this hypothesis, we performed gel filtration experiments by mixing kin5_14 and free tubulin at the ratio of 3 uM motor dimers to 20 uM tubulin dimers. Only two peaks corresponding to kinesin and tubulin were seen (Figure 3.7D). The lacking of peak
containing both kin5_14 and tubulin suggest that kinesin-5 does not bind to free tubulin dimers.

3.2.7 Enhanced Processivity is Important for Plus-end Tracking Ability of Kinesin-5

One possible scenario is that kinesin-5 catches the growing plus-ends by walking and pauses at the end while waiting for the formation of the next binding site. But, it seems not straightforward how processivity of motor contribute to its pausing at the end. To
reconcile those observations, we built a model by assuming that kinesin-5 can pause that the static microtubule plus-ends for 7 seconds and polymerizing the microtubules at various rates. Kinesin-5 can step if a new tubulin is added to the end. From this modeling, it is shown that a processive motor will track the end longer if microtubule grows faster, while the duration of a less processive motor at plus-end will decrease with increasing microtubule growth speeds (Figure 3.7E). This is because that during stepping, less processive motors will have higher probability to detach that leads to a reduction in its ability to track growing microtubules. The predicted duration for kin5_14GFP is 8.1 s, close to the observed 7.04 s. The dwell time predicted for kin5_18GFP is 1.7 s, which is close to the boundary of getting reliable observation of dwelling at dynamic microtubule ends in our experimental setup. This simulation explains why we only saw consistent plus-tip tracking for kin5_14GFP while both motors dwell at static microtubule plus-ends. Based on these, we think that the processivity is responsible for the different ability of kin5_18 and kin5_14 to track growing microtubule plus-ends.

**End-attachment of Kinesin-5 is Decreased by Trailing Kinesin-5**

How does kinesin-5 interact with each other at the plus-end of microtubules? Previous findings show that the residence of end-bound kinesin-8 can be decreased by coming kinesin-8, an indication of direct interaction between motors at the end of microtubules. With a contrary function of stabilizing microtubules, how will the duration of end-bound kinesin-5 be affected by other kinesin-5? To directly address this question, we increased
Figure 3.9: Proposed mechanisms of kinesin-5 end duration and effects on MT polymerization. When kinesin pauses at the plus-ends of growing microtubules (panel 1), it will wait for a tubulin binds to the end (panel 2). The dynamics of tubulin incorporating will lead to two possibilities that the new tubulin can either detach before kinesin steps (panel 4) or kinesin steps and stabilizes the tubulin (panel 3). The (1) to (3) cycle will repeat and the longer end duration of kinesin will increase the probability that the cycle goes to (3). The stabilization of tubulins will lead to long and stable tubulin filaments which is slightly curled (panel 5). The curvature in the filament should reduce the tension between two heads, thus favoring two-head bound state that in turn increases stabilization effects. (B) Illustration of microtubule sliding with end tethering. (C) Proposed microtubule steering scheme. (D) Catastrophe of microtubules will impede the sliding efficiency.

the motor concentration in spiking experiments (Figure 3.8, A and B). At the mixing of 70 pm GFP motor with 15 nM unlabeled motor, the end duration of kin5_18GFP was reduced to 2.26 ± 0.30 s (mean ± s.e.m., Figure 3.8C). Similarly, at the similar mixing scenario, the end duration of kin5_14GFP was decreased to 2.61 ± 0.29 s (mean ± s.e.m.). These findings show that there is direct interaction between kinesin-5 motors at the plus-ends of microtubules. The similar observations across different end-bound
motors indicate that it might be universal for same motor interact with each other at microtubule ends.

3.3 Discussion

Here we demonstrated that a recombinant kinesin-5 dimer can promote microtubule growth and stabilize microtubules. The addition of kinesin-5 also leads to inhomogeneous plus-end structures of growing microtubules, presumably due to stabilization of tubulin filaments by kinesin-5. Direct observation of GFP labeled motor under TIRF microscopy reveals that kinesin-5 can pause that the plus-ends of taxol stabilized microtubules for 7 seconds, and have a processivity dependent ability of tracking growing microtubules. These observations have great implications in both kinesin-5 chemomechanics and its cellular functions.

3.3.1 Linkage Between Kinesin-5 Molecular Behaviors and Microtubule Dynamics

Native kinesin-5 is a slow motor that takes roughly 8 steps per second or equivalently spending 125 ms per step, which is about 100 times slower than kinesin-1. This slowness indicates that kinesin-5 might spend longer time at two-head bound state, which should be necessary to stabilize tubulin-tubulin interactions and prevent catastrophe. When a kinesin-5 reaches the plus-end and pause there, eventually the rear head should detach from the microtubule, leading to single-head bound state. Considering that kinesin-5 has
a rate-limiting ATP hydrolysis from kinetic studies and dwells on microtubule for about 1s at ADP state, we speculate that kinesin-5 stays that the end of microtubule at single-head bound ATP state. If this hypothesis is true, to resolve with the ~0.1s regular stepping time of kinesin-5, the ATP hydrolysis rate has to be accelerated during once the tethering head binds to the next binding site, which usually accompany with confirmation changes as well as increase in interhead tension.

When a microtubule grows, the addition of tubulin is dynamic before a stable interaction between tubulin and microtubule can be established. The quick on-off switch of tubulin binding to the end means that there is a competing between detachment of a transient tubulin and stepping of motor on the freshly incorporated tubulin (Figure 3.9). It should be easy to image that a motor that simply detaches when reaching microtubule plus-ends will have lower chance to win the competition. So, the pausing at the ends, which allows it to wait and binds to transient incorporated tubulin efficiently, will be essential for kinesin-5 to promote microtubule growth. Additionally, the previous EM studies (Chrétien, Fuller et al. 1995) shows that profilaments at growing microtubule plus-ends are slightly curved, and this curvature should reduce the tension between two heads of a bound kinesin-5 and might leads to a slower ATP hydrolysis and release from two-head bound state, which is in favor of stabilization. Overall, the slowness, unique kinetic cycle and end-pausing properties make kinesin-5 possible to stabilize microtubules and promote microtubule growth.
3.3.2 Implications for Kinesin-5 Cellular Functions

The great importance of kinesin-5 comes from its ability to slide antiparallel microtubules, which is essential to proper cell division as well as neuronal development (Sharma, Mozgova et al. 2011; Waitzman and Rice 2014). During mitosis, two centrosomes are thought to be separated by kinesin-5 sliding antiparallel microtubules and the separating forces generated by kinesin-5 are also important maintain bipolar spindle by antagonizing inward forces generated by other motors like NCD (Tao, Mogilner et al. 2006; Tanenbaum, Macurek et al. 2008; Ferenz, Paul et al. 2009). When a kinesin-5 slides two antiparallel microtubules, more often, one pair of heads will reach the end of microtubule earlier than the other pair (Figure 3.9B). Quick detachment from microtubule plus-ends that leads to loss of contact with one microtubule seems inefficient for sliding. A much more durable sliding can be achieved by maintaining the interaction with microtubule when motors reach the end. This might be part of the reason that simply replacing kinesin-5 head with kinesin-1 head cannot rescue cells with kinesin-5 loss of functions.

The ability of kinesin-5 to track slow growing microtubule plus-ends may contribute to microtubule organization. It was previously shown that a complex contains EB1 and kinesin is sufficient to steer microtubule growth (Chen, Rolls et al. 2014; Doodhi, Katrukha et al. 2014). The essential function of that complex is making a kinesin tracking growing plus-ends to allow kinesin guide the growth by walking on another microtubule. If the plus-tip tracking activity of kinesin-5 is preserved, the homotetrameric kinesin-5
will be a one-protein representation of microtubule steering complex. When one pair of head is tethering microtubule plus ends, the other pair can walk on another microtubule to steer the growth (Figure 3.9C). This hypothesized function provides alternative interpretations of previous studies of kinesin-5 in neurons (Myers and Baas 2007; Sharma, Mozgova et al. 2011; Nadar, Lin et al. 2012).

Also, the intrinsic dynamic instability of microtubules will cut the productivity of sliding. The autonomous shrinkage of microtubules will eliminate an established antiparallel microtubule pair. Additionally, it is hard to image a stable bipolar spindle if underlying microtubules catastrophe frequently. Similarly, a depolymerase activity of kinesin-5 would be disastrous: the shortening of microtubules will be counterproductive of sliding by limiting microtubule length (Figure 3.9D). To have stable microtubules in spindle, many microtubule associated proteins are found to dampen microtubule dynamics during mitosis (Bieling, Telley et al. 2010; Sardar, Luczak et al. 2010; Stumpff, Du et al. 2011; Gudimchuk, Vitre et al. 2013; Subramanian, Ti et al. 2013; He, Subramanian et al. 2014). The stabilization effects of kinesin-5 can be just adding another layer of redundancy, or it might have its own uniqueness.

The microtubule polymerase activity of kinesin-5 is also notable. Xmap215 is a polymerase that is found to increase microtubule growth rate comparable to physiological level, a speed that is over 100 nm/s (Brouhard, Stear et al. 2008). However, the microtubule growth rate that kinesin-5 can promote will be topped at its moving speed in order for the motor to catch growing plus-ends. So, the regime that kinesin-5
demonstrate its effect is complementary to that of Xmap215, allowing cells regulate microtubule growth at wider ranges.

3.4 Methods

3.4.1 Molecular Cloning

Kinesin-5 dimers were made by swapping the Xenopus XlKSP head and neck-linker into Drosophila conventional kinesin which is truncated at position 559 as previously described (Shastry and Hancock 2011). eGFP and a His$_6$ tag were added to the C-terminus. All motors were expressed in BL21DE3 cells, induced with Isopropyl β-D-1-thiogalactopyranoside overnight and purified by Ni column chromatography as previously described (Hancock and Howard 1998). Purified proteins are flash freeze in liquid N2, and stored at -80°C in storage buffer (50 mM K-phosphate, 300 mM NaCl, 2 mM MgCl$_2$, 100 μM ATP, 10 mM β-mercaptoethanol, 500 mM imidazole, pH = 7.2, with 10% sucrose added). Protein concentrations are determined by GFP absorbance at 488 nm for GFP-tagged motors, or by overall absorbance at 280 nm for non-GFP motors.

3.4.2 TIRF Microscopy Assay

Flow cell assembly is similar as previously described (Chen, Rolls et al. 2014) except that all experiments are using buffer BRB80. Bitinylated tubulin and Cy-5 labeled tubulin were mixed at ratio of 1:3 and polymerized at final concentration of total 20 μM tubulin and 0.25 mM GMPCPP at 37°C for 30 minutes. Coverslips were clean in piranha, treated
with 0.5% Octadecyltrichlorosilane (OTS) in toluene for 1 hour, and used to assemble flow cells after another cleaning with toluene. Flow cells were first incubated with 0.5 mg/ml neutravidin and followed by 5% Pluronic 108 in ddH2O to block the surface. GMPCPP Cy5-biotin microtubules were introduced together with 1 mg/ml casein for 5 minutes and microtubule extension is induced by free tubulin. To observe Cy5 microtubule growth, 0.5 µM Cy5 tubulin was mixed with unlabeled tubulin and antifade to get desired final tubulin concentration. For experiments of GMPCPP microtubule depolymerization, the laser was shutter for most of time during recording and focus was checked every 3~4 minutes.

3.4.3 Gel Filtration

A 500 µL sample of 3 µM kin5_14 and 20 µM of unlabeled tubulin in BRB80 supplemented with 1 mM ATP was incubated at room temperature for 5 minutes before loading on to a Superdex 200 10/300 GL column (GE Healthcare). The absorbance is monitored at 280 nm. The fractions were eluted in BRB80 containing 1 mM ATP, and collected at 0.5 mL fraction. SDS-PAGE gels using Coomassie staining were performed to examine proteins in fractions.

3.4.4 Error Function Fitting and Point Spread Function

The intensity drop at the end of microtubules is analyzed by doing a line scan along microtubules in ImageJ and the resulting intensity profile is fitted to the error function (equation 1) by nonlinear least square fit in R®3.1.1.
\[
I = \frac{1}{2} I_{MT} \text{erfc} \left( \frac{x-U_{pf}}{\sqrt{2} \sigma} \right) + I_{BG}
\]  

(1)

Where \( I_{MT} \), \( U_{pf} \), \( \sigma \) and \( I_{BG} \) are intensity without background, mean protofilament length, combined standard deviation and background intensity respectively.

The point spread function of Cy5 dye is done by immobilizing Cy5 dyes on coverslip through nonspecific binding. The images taken were fit to a 2-D Gaussian by FIESTA (Ruhnow, Zwicker et al. 2011) to obtain standard deviation of PSF.

3.5 Movies

**Movie 3.1**: Microtubule grows with bifurcates. Microtubules were polymerized at 10 µM unlabeled tubulin with 20 nM kin5_18GFP at 32\(^{\circ}\)C. The binding of kin5_18GFP to microtubule is sufficient to highlight whole microtubules. Scale bar is 2 µm.

**Movie 3.2**: Microtubule plus-end curls and breaks. The experimental condition is the same as movie 3.1. Scale bar is 1 µm.

3.6 Reference


Chapter 4

Three-Dimensional Bipolar Assembly of Microtubules

Work described in this chapter is done in close collaboration with Raymond Fok, a graduate student from Dr. Thomas Jackson’s lab in PSU Electrical Engineering.

4.1 Introduction

The mitotic spindle, which comprises a bipolar assembly of microtubules, microtubule associated proteins and other regulatory proteins, is the cellular machinery that divides chromosomes equally into two daughter cells during cell division (Compton 2000; Wittmann, Hyman et al. 2001; Goshima and Scholey 2010). The essence of the mitotic spindle in cells leads it into a target of anticancer therapy and a focus of stem cell study (Jordan and Wilson 2004; Stanton, Gernert et al. 2011). Extensive studies have focused on elucidating the molecular mechanisms of spindle formation and maintenance (Wittmann, Hyman et al. 2001; Goshima and Scholey 2010). Traditional biological methods under reductionist philosophy that harness RNAi knockdown or protein immuno-depletion in reconstitution of spindles from frog egg extracts have identified various important proteins that are essential to mitosis (Bulinski and Borisy 1979; Heald, Tournebize et al. 1996). However, the intrinsic cellular redundancy limits the ability of these methods and there are still a lot of unanswered questions (Wittmann, Hyman et al. 2001; Goshima and Vale 2003; Zhu, Zhao et al. 2005). Biophysical research using purified proteins has provided ample information of individual proteins. But, usually in these
single molecular experiments, observations were made on individual microtubules and the relevance of these studies to its cellular functions in complicated cellular environment sometimes remains unclear. To gain further understanding of mitosis, the reconstitution of close-to-cell microtubule structures is demanding.

To construct a platform for in vitro studies of mitotic proteins, it was previously shown in our lab that microtubules can be aligned into two micro-patterned electrodes under AC electrophoresis (Huang, Uppalapati et al. 2007; Uppalapati, Huang et al. 2008; Uppalapati, Huang et al. 2011), leading to a geometrical reassemble of the bipolar spindle. Despite the ability to assemble bipolar microtubule structures, termed “artificial spindle”, issues impairing the robustness and reliability of assembling need to be investigated and solved. Additionally, to closely mimic the mitotic spindle in cells that is essentially a three dimensional structure, alignment design to achieve three dimensional microtubule assembly should be developed. Here we first investigated ways for improved surface passivation to prevent non-specific protein binding, and then migrated the assemble environment into micro-fabricated microfluidic channels for the purpose of making an ultimate lab-on-chip device. After that, we redesigned the microfabrication pattern to achieve the 3-D assembly of microtubules.

4.2 Surface Patterning with PEG Polymers

The attachment of microtubules to the electrodes after turning off AC field requires the selective patterning of neutravidin to electrodes and passivation of the rest areas to
prevent non-specific protein binding (Huang, Uppalapati et al. 2008). Additionally, the reduced binding of fluorescent proteins to surface will improve the signal-to-noise of imaging. The classic way of surface passivation by casein provides an easy solution for early investigation, but lacks sufficiency in rigorous conditions, like protein concentrations at nanomolars or above. One alternative method is using Polyethylene glycol (PEG) polymers, amphiphilic molecules with hydrophobic backbones and hydrophilic ends, which can form self-assembled monolayer (SAM) to prevent protein

Figure 4.1: Surface passivation by OTS-F108 system. (A) A schematic diagram. The OTS is thought to form SAM on glass surface while covalently bound to glass. The resulting surface after OTS treatment is hydrophobic and allows Pluronic F108 to self-assemble on top to prevent proteins from binding. (B) GFP proteins attached to surface non-specifically. The adding of F108 to OTS coverslips increase the surface passivation effect significantly compared with just cleaned glass and OTS coverslips only.

We utilized a two-step passivation procedure that involves making a hydrophobic surface first and then assemble PEG polymer monolayer on the surface (Figure 4.1A). The PEG polymers serve as the primary passivation by forming SAMs on the hydrophobic surface. The secondary passivation effects come from the hydrophobic surface, which is generally repulsive to proteins that usually have hydrophilic surfaces (Zheng, Li et al. 2005). Additionally, proteins bound to hydrophobic surfaces tend to denature and the denaturation of fluorescent proteins can reduce its fluorescent ability (Saeed and Ashraf 2009).

To make hydrophobic surfaces, glass substrates are cleaned with piranha solution for 20 minutes to remove organic debris on surface. Dried glass after cleaning is incubated with 0.5% octadecyltrichlorosilane (OTS) for 1 hour to allow the formation of OTS SAMs and sequential cleaning in toluene is required to remove any unreacted chemicals. The OTS glass is used to assemble flow cells and further incubated with Pluronic F108 to form final passivation layers.

To test the passivation effects, flow cells were assembled with surface modified coverslips, and absorption of purified GFP proteins were used as measurement of surface passivation quality and observed under TIRF microscopy. A clear reduction of GFPs bound to surface in PEG passivated flow cells was seen compare to control flow cells with regular casein passivation (Figure 4.1B). This result demonstrates that OTS-
F108 is a better passivation method than casein. However, the passivation efficiency is not complete due to minimal GFPs surface can still be seen.

4.3 Microtubule Assemble in Fabricated SU-8 Channels

To develop lab-on-a-chip substrates for spindle alignment, it is important to enclose patterned electrodes within microfluidic channels. SU-8 is a commonly used material in microfabrication with low cost and easy process. To be able to use SU-8 made channels in biological systems, the compatibility of SU-8 with microtubules were examined. A layer of SU-8 was deposited on the glass substrates and used to assemble flow cells. Polymerized microtubules were locked to surface-immobilized motors using AMP-PNP, a non-hydrolysable ATP analog. Microtubules were observed under Epi-fluorescent microscopy (Figure 4.2). From these time-lapse images, microtubules stayed intact with
slight bleaching, similar to microtubules on glass surface. This test shows that SU-8 is compatible with microtubules and can be used in systems for biological usage.

Next, SU-8 channels were fabricated on glass substrate with electrodes patterned through photolithography to enclose electrodes in between and a clean coverslip was bounded on top through a hydraulic pressure laminator, leading to an enclosed ready-to-use system (Figure 4.3, A and B). To test whether enclosed SU-8 channel affects microtubule alignment, polymerized microtubules were introduced into channels using vacuum on one side after surface passivation with casein. An AC electric field with 30 V and 5 Mhz was used to align microtubules. After 2-minutes alignment, microtubules were steady accumulated to the electrode tips and formed a bipolar structures with overlaps in the middle (Figure 4.3C). These results demonstrate that microfluidic channels made by SU-8 can be successfully used in microtubule alignment and has potential applications in other biological applications.

4.4 Assemble Microtubules in Three-Dimensional Geometry

Mitotic spindles in cells are a three-dimensional assembly of microtubules. To closely reconstitute microtubule organization in vivo, fabrication designs that allow formation of 3-D microtubule alignment should be developed. To achieve that, electrodes were designed to be patterned on elevated pedestals (Figure 4.4A).
Initially, a Parylene-C layer of about 7 µm thick was deposited to the cleaned glass surface and electrodes were patterned on top of Paralyene-C layer. To pattern Parylene-C as pedestals, reactive ion etch (RIE) was used to remove undesired Parylene-C by using electrodes as masks. With oxygen at 10 mtorr and a self-bias of 250 V, an etching rate of 0.2 µm/minute was achieved. The etching profile was examined under SEM (Figure 4.4B). First, significant grass-like residuals were seen, which is presumably due to nonuniform etching by RIE. Second, an undercut of about half micron was obviously seen and the overall electrode edge profile was not very well (Figure 4.4C). Next, the attempt of aligning microtubules were made, but failed (Figure 4.4D). Instead of

**Figure 4.3: Spindle alignment on SU-8 channels.** (A) A close look-up of fabricated SU-8 Channels. (B) The final substrate sample. (C) Microtubules are aligned into bipolar shape in the substrate containing SU-8 channels. An AC filed of 30 V and 50 MHz was used. Scale bar is 10 µm in (C).
accumulating to the tips of electrodes, microtubules were targeted to the edges of electrodes significantly. Better designs were needed to improve the alignment effects.

![Figure 4.4: Microtubules fail to align on Parylene-C elevated electrodes.](image)

(A) A schematic view of the desired design. Elevated pedestals are intended to elevate electrodes above glass surface. (B) SEM image of electrodes on Parylene-C pedestals. Residuals on the glass surface were seen. Picture is captured by Raymond Fok. (C) The electrodes under the wide field microscope. The irregularity at the edge of electrodes was caused by partial etching from RIE. (D) Microtubules aligned on the substrate in (C). Most of microtubules accumulated to the edges of electrodes, suggesting that the electric field distribution is disturbed, presumably due to fuzzy edges.

To improve edge profiles of electrodes, methods using wet etching were used to generate self-aligned electrodes on SU-8. Sequential layers of SU-8, aluminum and Titanium were deposited on glass substrate, and covered by photoresist Shipley 1811
(Figure 4.5, step (1)). After patterning Shipley 1811 by photolithography, exposed Titanium was removed by RIE that led to the exposure of Aluminum (Figure 4.5, step (2)), which was etched in solution of 5% sodium hydroxide. The wet etching of Aluminum led to a critical undercut and allowed the following self-assembly of electrodes (Figure 4.5, step (3), and Figure 4.6A). The desired SU-8 was exposed to UV light (Figure 4.5, step (4)), and a uniform layer of Titanium was deposited on top (Figure 4.5, step (5)). To render the final electrodes, unexposed SU-8 was removed by SU-8 developer (Figure 4.5, step (6)). Under the SEM, intact, smooth-edging electrodes on SU-8 pedestals were seen.
(Figure 4.6, B and C). After assemble the electrodes into a flow cell, fluorescent microtubules were aligned to the tip of electrodes by an AC electrical field of 30V and 5 MHz (Figure 4.6D). These fabrication and alignment led to a successful reassembly of microtubules into three dimensional geometry that can be used to study microtubule-MAPs in a microtubule environment similar to cells but with components well-defined.

Figure 4.6: Microtubule assembling on self-aligned electrodes. (A) The SEM picture after Aluminum etched. The undercut is important to the flowing self-alignment of electrodes. (B) SEM picture of a electrode pair. The edging profile is smooth as desired. (C) Electrodes under wide field microscope shows the intact of electrodes. (D) Microtubules are aligned to the tip of electrodes successfully with minimal background noise. Panel A and B are captured by Raymond Fok.
4.5 Experimental Methods

4.5.1 Microtubule Preparation

Full-length hexaHis-tagged *Drosophila* conventional kinesin was used for all motility experiments (Hancock and Howard 1998). Motor proteins were expressed in bacteria and purified by Ni column chromatography as previously described (Hancock and Howard 1998; Coy, Hancock et al. 1999). Tubulin was purified from bovine brains and labeled with rhodamine as previously described (Williams and Lee 1982). Microtubules were polymerized by mixing 10 μM rhodamine-labeled tubulin, 30 μM unlabelled tubulin, 4 mM MgCl₂, 1 mM GTP and 5% DMSO in BRB80 buffer (80mMPIPES, 1mM EGTA, 1mM MgCl₂, pH6.9 with KOH), incubating at 37°C for 20 min, and then diluted into a BRB80 solution containing 10 μM paclitaxel.

4.5.2 Fabrication of SU-8 Channels

Cr electrode was deposited on glass substrates by sputtering and patterned by photolithography as previously described. After removing all photoresist, 30-micron of SU8 was spin-coated and baked at 60 degree C for 5 minutes and then at 90 degree C for 15 minutes on a hot plate. After patterning SU8 by lithographically, samples are baked at 60 degree for 1 minutes and 90 degree for 4 minutes. Finally, SU8 was developed by 1-Methoxy-2-propyl acetate to form final channels. The sample was heated at 65 degree C for 1 minute before it was bonded to the piranha cleaned coverslip by a hydraulic press laminator at 25psi and 90 degree C for 2 minutes.
4.6 Reference


Chapter 5

Molecular Counting by Photobleaching in Protein Complexes with Many Subunits: Best Practices and Application to the Cellulose Synthesis Complex

Research in this chapter is a result of collaboration with Dr. Charlie Anderson in PSU Plant Biology and has been previously published as:


Charlie Anderson grew Arabidopsis seedlings; Nathan Deffenbaugh designed Tdeteors and generated simulation data as stated in the figure legends.

5.1 Introduction

Cellulose is a major structural component in the plant cell wall that regulates plant cell growth and morphology and also has extensive commercial value for applications such as papermaking, textile manufacturing, and biofuel production (Carroll and Somerville 2009). However, the molecular processes involved in the biosynthesis of cellulose, which is composed of large numbers of β(1,4)-linked glucan chains that associate via hydrogen bonds to form cellulose microfibrils, remain incompletely understood despite
intensive research over the past 15 years (McFarlane 2014). It is generally believed that cellulose is synthesized at the plasma membrane and extruded into the extracellular space by a cellulose synthesis complex (CSC). Each CSC contains many GT2-family glucosyltransferases called cellulose synthases (CESAs) and is assembled into a large integral membrane complex with a membrane-spanning rosette configuration of approximately 25 nm in diameter (Haigler and Brown 1986). The complex is formed in the Golgi and transported to the plasma membrane, where it becomes active to synthesize the glucan chains that constitute the cellulose microfibril (McFarlane 2014).

Genetic and biochemical data indicate that a minimum of three different CESA isoforms are present in each CSC; in the model plant *Arabidopsis thaliana*, AtCESA1, AtCESA3, and AtCESA6-type proteins are present in CSCs that synthesize cellulose in the primary walls of growing cells, whereas AtCESA4, AtCESA7, and AtCESA8 proteins are present in CSCs during secondary wall synthesis in cells that have ceased growth (Taylor, Howells et al. 2003; Desprez, Juraniec et al. 2007; Persson, Paredez et al. 2007). Estimations based on structural studies of cellulose microfibrils (Fernandes, Thomas et al. 2011; Thomas, Forsyth et al. 2013) and molecular modeling of CESAs (Sethaphong, Haigler et al. 2013) predict that each CSC is composed of anywhere between 12 and 36 subunits (Guerriero 2010; McFarlane 2014); however, the precise stoichiometry of CESA isoforms within each CSC remains undefined. Empirically determining protein copy numbers for intact membrane-bound CSCs through nondestructive means is challenging, especially since reconstituting active, purified plant CSCs has proven to be extremely difficult (Lai-Kee-Him, Chanzy et al. 2002; Cifuentes, Bulone et al. 2010; Fujii, Hayashi et al. 2010).
One alternative method of estimating protein copy numbers in integral membrane complexes is to count bleaching steps for subunits tagged with intrinsically fluorescent proteins, such as green fluorescent protein (GFP), under total internal reflection fluorescent (TIRF) microscopy (Ulbrich and Isacoff 2007). However, the number of proteins that can be estimated using current methods is limited: higher copy numbers lead to increases in both fluctuations in the fluorescence signal and the initial rate of photobleaching, complicating the identification of discrete photobleaching steps. This issue can be addressed by using a median filter to reduce noise in the data, and constructing pairwise distance distributions to determine the unitary step size of photobleaching (Svoboda, Schmidt et al. 1993; Leake, Chandler et al. 2006). However, implementing this approach to estimate subunit number typically requires empirical selection of the optimal median filter, and still does not readily resolve the precise timing and magnitude of individual bleaching steps.

Step detection algorithms, which are frequently used to analyze the spatial steps undertaken by motor proteins, are capable of automatically detecting change points in data traces (Carter, Vershinin et al. 2008). Numerous methods have been developed to detect steps, but most of them depend heavily upon pre-selected parameters. Notably, the $\chi^2$ method developed by Kerssemakers et al. requires an input of the number of steps to be detected (Kerssemakers, Munteanu et al. 2006), which is difficult to calculate if prior information about the data is unavailable. Methods based on information criteria are objective and do not require user-defined input parameters (Kalafut and Visscher 2008). However, they have only been implemented in step
detection algorithms by assuming that the variance associated with each step is constant (Kalafut and Visscher 2008), which is adequate for single motor protein stepping but not for photobleaching. Because intensity fluctuations of individual fluorophores around their means are uncorrelated, the presence of multiple active fluorophores in a complex will result in a higher variance in the fluorescence intensity signal than the variance associated with a single fluorophore. Hence, algorithms designed to detect steps in photobleaching data need to consider these variance changes to avoid overfitting during periods of high fluorescence intensity. Another complexity in photobleaching data is that with increasing copy number, there is an increasing probability that two or more fluorophores will bleach within a short timeframe (e.g., within a single acquisition period), which can also skew the step size distribution and complicate the estimation of a unitary photobleaching step size. Thus, there also exists a need for the development of objective analytical tools to extract unitary step sizes from step-size distribution densities that improve upon current methods of data binning and fitting a user-defined number of Gaussian functions.

In the present work we developed a novel procedure that combines step detection and density estimation to determine unitary step size and copy number from experimental photobleaching data. A mathematical model was constructed to generate simulated bleaching data, and the simulated data were used to optimize the performance of the step detection and density estimation algorithms and demonstrate their ability to accurately retrieve copy numbers from simulated data with varying degrees of experimental noise. A key goal in developing these tools was to make them as objective
as possible by minimizing the number of user-defined parameters, and it is hoped that these procedures will establish best practices for analyzing photobleaching data derived from complexes with high copy numbers. We applied these analytical tools to photobleaching data collected for GFP-tagged AtCESA3 in intact cells of *Arabidopsis thaliana* seedlings and estimated the lower limit of copy number per particle to be ten.

### 5.2 Results

#### 5.2.1 Imaging CesA Complexes in *Arabidopsis* Seedlings

To estimate the copy number of GFP-AtCESA3 in membrane-localized particles in living cells of *Arabidopsis thaliana*, 5-to-6-day-old light-grown seedlings expressing GFP-AtCESA3 (Desprez, Juraniec et al. 2007) were mounted in an imaging chamber and recordings of GFP bleaching were carried out in hypocotyl cells containing low densities of GFP-AtCESA3 particles (Movie 5.1). Imaging was performed using variable-angle epifluorescence microscopy (Konopka and Bednarek 2008), which like total internal reflection fluorescence (TIRF) microscopy reduces background fluorescence but allows for the imaging of proteins farther from the coverslip, such as those in the plasma membrane of plant cells that are separated from the coverslip by the cell wall (Konopka, Backues et al. 2008; Konopka and Bednarek 2008). To quantify photobleaching rates, time lapse recordings were collected (Movie 5.1), and fluorescence intensity traces for individual GFP-containing particles were measured using ImageJ (see Materials and
Figure 5.1: In vivo photobleaching of GFP-AtCESA3. (A) Photobleaching trace of a single GFP-AtCESA3 particle in hypocotyl cells of *Arabidopsis* seedling. Video is recorded at 5 fps and total time is 100 s to allow most GFP to be photobleached. Representative movie is included in Supplementary Data. Inset: ensemble average of 77 photobleaching traces with exponential fit to the data. (B) Quantitative model describing photobleaching. The fluorescence signal is assumed to fall over time with constant step sizes, matching the quantal fluorescence of a single GFP. The GFP fluorescence and the background signal are treated as Gaussian distributions, Normal ($\mu, \sigma^2$) and Normal (0, $\delta^2$), respectively. The time before fluorophore bleaching, $T$, is assumed to be exponentially distributed with mean $\tau = 1/\lambda$ where $\lambda$ is the photobleaching rate constant. The signal to noise ratio (SNR) is defined as the step size divided by the standard deviation. (C) Simulated photobleaching trace from 12 fluorophores with $\mu = 500$ a.u., $\sigma = \delta = 250$ a.u. (D) Simulated stepping data such as a kinesin walking along a microtubule in an optical trap experiment, with $\mu = 1$, $\sigma = 1$ and 10% backward steps.

Methods). Instead of exhibiting discrete steps, the intensity changes during photobleaching for many traces appeared to be relatively smooth (Figure 5.1A, Movie 5.1), suggesting that the number of fluorophores per particle is relatively high.

The photobleaching rate constant for GFP-AtCESA3 was estimated by ensemble averaging all of the photobleaching collected traces and fitting a single exponential
function using MATLAB’s nonlinear least squares method (Figure 5.1A inset). The fitted rate of 0.0278 ± 0.0003 s\(^{-1}\) (mean ± SEM of fit, N = 77 traces) is the expected rate of photobleaching events regardless of the true number of independent photobleaching units present.

The experimental background noise was estimated by analyzing the distribution of the final plateau variance (as defined by the Tdetector2 step detection algorithm; see below) for the 77 measured traces. As expected, the distribution had more than one mode (Figure 5.2), due to the fact that complete photobleaching had not occurred in some of the traces. Therefore the lowest variance mode was defined as the background variance, while the next mode indicates the sum of the background variance plus the variance associated with one fluorophore. To allow for more precise quantitative analysis of bleaching for multiple fluorophores, we developed a statistical method of photobleaching analysis, as described below.

### 5.2.2 Generating Simulated Fluorescence Photobleaching Data

Fluorescence intensity from a single fluorophore is typically described as a Gaussian distribution (Lakowicz 2010) with mean intensity \(\mu\) and variance \(\sigma^2\) (Figure 5.1B, inset panel). While intensity fluctuations at low photon counts are better modeled as a Poisson distribution, added signal variance due to rapid fluorophore blinking events, fluctuations in the background signal, and camera read noise justify the assumption that
the signal is Gaussian. We postulate that the fluorophores are independent of one another and thus the intensity fluctuations for each fluorophore are uncorrelated with those of neighboring fluorophores. Thus, when \( n \) fluorophores are localized in a diffraction-limited spot, the overall intensity will be the sum of the mean intensities (\( I_{\text{tot}} = n \mu \)), and the overall variance will be the sum of the variances plus the variance of the background, \( \sigma^2 \) (\( \sigma_{\text{tot}}^2 = n \sigma^2 + \delta^2 \)). Notably, in photobleaching traces the variance scales with signal intensity, and if background fluctuations are low and/or signal variance is high, then variance is proportional to intensity. This situation contrasts with typical positional step detection problems (for instance, identifying step displacements for motor proteins), where the variance is independent of position and is thus constant for
each step (Svoboda, Schmidt et al. 1993). As a result of this scaled variance, with each intensity drop during a photobleaching experiment, there will be an associated decrease in the signal variance.

Another aspect of multi-fluorophore photobleaching data that complicates the identification of bleaching steps is the fact that the frequency of photobleaching events for an ensemble of fluorophores changes over time. Photobleaching is typically modeled as a first order process with rate $\lambda$ and characteristic bleach time $T$, where $\lambda = 1/T$. Thus, the time it takes for a single fluorophore in a set to bleach will follow an exponential distribution with mean of $T$. If there are $n$ fluorophores in a diffraction-limited spot, then the mean time before the first bleaching event will be much faster because any of the fluorophores can bleach. Assuming that photobleaching events are independent of one another, the time before the first bleaching event will also follow an exponential distribution, with a rate equal to $n*\lambda$, and the mean time before the first photobleaching event will be $T/n$. Thus, at the beginning of an experiment, bleaching events will be more frequent and will be associated with larger signal variance, making it difficult to identify individual events.

To assess the ability of step detection algorithms to detect photobleaching events, we simulated a photobleaching signal for a complex containing 12 GFP fluorophores (Figure 5.1C), each having a mean intensity $\mu$ and variance $\sigma^2$ that approximated the GFP-AtCESA3 intensity trace shown in Figure 5.1A. In parallel, we simulated a signal having a uniform stepping rate and a constant variance, similar to motor protein displacement
Datasets with various SNR values were generated to represent a range of possible experimental scenarios. For motor stepping data (Figure 5.1D), the SNR is defined as ratio of step size over the standard deviation ($\mu/\sigma$). Defining SNR for bleaching traces, however, is complicated by the fact that the variance changes with the intensity (a.u.) from previous step BIC value.
number of active fluorophores. Thus, the SNR for the photobleaching data was defined as the mean intensity $\mu$ of a single fluorophore divided by its standard deviation $\sigma$ ($\mu/\sigma$). The variance of the background signal, $\delta^2$, was chosen to equal the variance of a single fluorophore, $\sigma^2$. Different SNR values were achieved by setting $\mu = 500$ a.u. and varying the standard deviation. To objectively identify each bleaching event, we developed multiple step detection algorithms that use statistical analysis to detect photobleaching events and compared their performance using the simulated data.

5.2.3 Using Step Detection Algorithms to Identify Bleaching Events

To analyze our photobleaching data, we developed two step detection algorithms that use statistical tests to identify steps. For each method, approaches were developed that assumed the different plateau regions in the signal had either equal or unequal variances. The first method is based on the Bayesian Information Criterion (BIC) (Schwarz 1978) and predicts steps purely based on statistical information in the data. Kalafut and Visscher used this approach for step detection previously, but assumed that the variance within each step was constant (Kalafut and Visscher 2008). We modified this implementation to allow for changes in variance. A second algorithm was developed based on the two-sample t-test with or without assumed equal variance. These four algorithms are named Bdetector1 and Bdetector2 for the BIC-based methods assuming equal or unequal variance respectively, and Tdetector1 and Tdetector2 for the t-test based methods assuming equal or unequal variance.
Both pairs of algorithms use a conceptually similar step detection approach of iteratively searching for change points until no statistically significant step can be added (Figure 5.3, and Movie 5.3). The algorithms are summarized as follows:

(1) The data are scanned, and for each potential time at which a step may occur, the mean and variance is calculated for the time preceding the step and the time following the step.

(2) Using these means and associated variances, a BIC value (Bdetector) or the significance from a two-sample t-test (Tdetector) is calculated and used to identify the optimal step. The optimal step is the one that leads to the lowest BIC value (Bdetector) or the largest significance (Tdetector). If no step leads to a BIC value smaller than the current one or a significance value above a defined threshold then no step is chosen.

(3) The process is repeated until no additional statistically significant steps can be detected, at which point it terminates.
To validate their performance, the step detection algorithms were first tested on simulated stepping data having SNR values from 0.4 to 5 (Figure 5.4). The step times were sampled from an exponential distribution with an expected value of 100 time points per plateau, with 90% of steps being a unit step increase and 10% being a unit step decrease. At high SNR values, the mean predicted step size was close to the actual value, but with diminishing SNR, an additional peak corresponding to twice the unitary
step size emerged (Figure 5.4A, and Figure 5.5). We defined two metrics, sensitivity and precision to assess the performance of the algorithms. Sensitivity is defined as the proportion of the true steps that are identified by the step detection algorithm. Precision is defined as the proportion of identified steps that are true steps (see Materials and Methods). Overfitting will lead to high sensitivity and low precision (false positives), while underfitting results in high precision but low sensitivity (missed events). With SNR values above 2, all four algorithms performed well and had both high sensitivity and precision values (Figure 5.4, B and C). Reasonable predictions were obtained at SNR values between 1 and 2, but sensitivity and precision both fell sharply for SNR values below 1. The BIC-based algorithms displayed a tradeoff between sensitivity and precision, with Bdetector1 (constant variance) having higher sensitivity and Bdetector2 (unequal variance) having higher precision (Figure 5.4, B and C: blue and green plots). In contrast, for the two-sample t-test methods both Tdetector1 (assumed constant variance) and Tdetector2 (assumed unequal variance) performed similarly (Figure 5.4, B and C: red and black plots).

After benchmarking the step detection algorithms on the stepping data, the algorithms were used to detect unitary steps in the simulated photobleaching data. For ease of comparison, the step size was fixed at 500 a.u. for all simulated data and the variance was altered to achieve different SNR values. As seen in Figure 5.6A, both algorithms identified similar steps in the simulated photobleaching data with SNR = 1. Considering the performance at different SNR values, the methods assuming unequal variance (Bdetector2 and Tdetector2) resulted in higher precision but lower sensitivity compared
with the methods assuming equal variance (Bdetector1 and Tdetector1, Figure 5.6, B and C). For estimating subunit numbers from photobleaching data, the most important factor is properly estimating the amplitude of a quantal photobleaching event (the first mode). Hence, a loss in sensitivity corresponding to missed steps (resulting in higher modes) is acceptable. In contrast, the falsely identified steps corresponding to low precision can lead to underestimating the quantal photobleaching amplitude. Based on these considerations, the two methods assuming constant variance were inferior to the methods assuming unequal variance. The Tdetector2 algorithm performed the best overall and was chosen for the subsequent analyses described below.

5.2.4 Determining Unitary Step Size from Step Detection Results

Figure 5.5: Detecting steps in simulated stepping data with SNR = 5. (A) Step size distribution of simulated steps. (B)-(E) predicted step size distributions by Tdetector1, Tdetector2, Bdetector2, and Bdetector1 respectively. Note Tdetector1, Tdetector2 and Bdetector2 all accurately predict steps with one main peak at 1 and a small peak at -1. Extra steps predicted by Bdetector1 between 1 and -1 indicate over-fitting of the data.
After identifying steps, the next task in analyzing photobleaching data is to use the identified step amplitudes to extract the amplitude of a unitary photobleaching event. The total subunit number is subsequently estimated by dividing the initial (high) fluorescence amplitudes by this quantal unit. We initially focused on results from the simulated dataset shown in Figure 5.6A having a SNR = 1 and a GFP copy number of 12. A histogram of step amplitudes predicted by the Tdetector2 algorithm suggests the presence of at least two modes (Figure 5.7A). The simplest method of estimating the unitary step size is to fit the binned histogram data with multiple Gaussian functions corresponding to the different modes. However, estimation by this method is strongly dependent on bin size (Figure 5.7A and B), and there are no existing objective methods for identifying the optimal bin size.

Kernel Density Estimation (KDE) is a non-parametric method of density estimation that can be used to identify modes without requiring data binning. In short, each step represents a probability of $1/N$, where $N$ is total number of steps, and a Gaussian

![Figure 5.6: Detecting steps in simulated photobleaching data.](image-url)
centered at each step is used to estimate the distribution of this \( 1/N \) probability, resulting to a total of \( N \) Gaussians. The overall probability density is obtained by the sum of these \( N \) Gaussians (Silverman 1986). Although the main peak from the KDE is obvious, it is difficult to retrieve information for subsequent modes because there are poorly separated (Figure 5.7C).

Density estimation by a Gaussian Mixture Model (GMM) can provide predictions of peak position for each mode in a way that avoids the drawbacks of KDE. In this method the distribution of steps is estimated by a mixture of Gaussians and the means and variances of these Gaussians are obtained by maximizing the expected posterior probability, computationally achieved by expectation–maximization (EM) algorithms (Dempster, Laird et al. 1977). However, one uncertainty of this method is choosing the number of Gaussians (\( K \)) to be fit to the data, which can alter the fitting results. To provide an objective method for choosing the number of Gaussians, the step amplitude data were fit using the Gaussian Mixture Model by an increasing number of Gaussians and the Bayesian Information Criterion (BIC) value associated with each fit was determined. The optimal number of Gaussians was defined as the number that gave the lowest BIC value, which for the simulated photobleaching data was 5 (Figure 5.8A and B). The different peaks were assumed to be multiples of the unitary photobleaching amplitude, and the mean unitary step size was calculated as a weighted average of each peak, giving a value of 528.3 a.u. This estimate is within 6\% of the step size value of 500 a.u. that was chosen for this simulated photobleaching data.
To further assess the performance of this method in estimating copy number from diverse photobleaching data, we performed identical analyses on simulated bleaching data with copy numbers from 2 to 20 at a range of SNR values (Figure 5.8C). Strikingly, for simulated data with copy numbers below 12, the analysis method predicts the value of the unitary step within 10% even down to an SNR of 1 (Figure 5.8C). With a copy number of 20, predicted step sizes are within 7% of the true step size for SNR of 2 and above, but rise toward twice the true step size at lower SNR values. Based on these results, the ability of this method to estimate copy numbers from photobleaching data is limited for data with both very high copy numbers (20 and above) and low SNR values.

Figure 5.7: Comparing methods of fitting photobleaching step size distributions to extract unitary step size. Histograms represent step size distributions from Tdetector2 applied to simulated photobleaching data with copy number = 12 and SNR = 2. The distribution is made up of 570 detected steps. (A) Fit of two Gaussian functions to the data using a bin size of 50. Fit parameters are $\mu_1 = 510$ a.u., $\sigma_1 = 55$, $\mu_2 = 836$ a.u., and $\sigma_2 = 335$. (B) Fit of two Gaussian functions to the data using a bin size of 150. Fit parameters are $\mu_1 = 568$ a.u., $\sigma_1 = 67$, $\mu_2 = 873$ a.u., and $\sigma_2 = 342$. In both cases fits to more than two Gaussians did not converge. (C) Identifying modes by Kernel Density Estimation (KDE). A histogram with bin size 50 is plotted for the purpose of visual comparison, but not used for fitting. Smooth curve is the estimation of multiple Gaussians (kernels) by KDE.
(below 2). In these cases, the design of the photobleaching experiment should be further optimized to improve the SNR.

5.2.5 Using Unitary Step Size to Estimate Fluorophore Copy Number

The final task in estimating the number of fluorophores in a complex is to calculate the amplitude of the overall fluorescence drop by taking the difference between the initial fluorescence and the value of the final plateau and dividing by the unitary step size. Accurately estimating the total amplitude of the photobleaching signal can be challenging, however, due to uncertainties in measuring the initial fluorescence amplitude and uncertainties in whether the final plateau represents full bleaching. The first few time points of photobleaching traces have the most variability due to the fast rate of photobleaching and high signal variance associated with a large number of fluorophores. Simply averaging over the first few points reduces the noise but also leads to underestimating the true maximum fluorescence. To avoid introducing any bias, we chose to simply take the initial fluorescence value as the maximum for each trace.

The proportion of fluorophores that are expected to bleach during the finite acquisition time can be estimated by fitting an exponential to the ensemble average of the photobleaching traces (see Materials and Methods). The simulated photobleaching data had a duration of 100 s and, because it was modeled on the experimental data, was well fit by an exponential with a rate constant of 0.0278 s$^{-1}$. Thus, 93.9% of the fluorophores...
are expected to bleach (see Equation 9), and the overall intensity drop of the simulated data was corrected upward by dividing by 0.939. Dividing the total intensity drop of each trace by the unitary step size results in a distribution of copy numbers with a mean of 12.3 estimated by KDE (Figure 5.8D), within 3% of the correct copy number of 12.
Copy number errors were within 10% for SNR = 1 and above for copy numbers of below 12, and for SNR = 1.8 and above for a copy number of 20 (Figure 5.8E).

### 5.2.6 Estimating Copy Number for Kinesin-4XGFP

To validate the ability of the developed methods to estimate copy numbers from a protein with a known number of GFP subunits, we engineered a kinesin construct containing four GFPs (see Materials and Methods). Proteins were attached to the coverslip surface through non-specific interactions and imaged using TIRF microscopy (Shastry and Hancock 2010). Steps were fit to the 71 acquired photobleaching traces using the Tdetector2 algorithm (Figure 5.9A), resulting in 455 detected steps. The step size distribution was fit using the Gaussian Mixture Model and based on the calculated BIC values, the optimal number of modes was determined to be four (Figure 5.9B). When the step size distribution was fit using four modes, the corresponding unitary step size was determined to be 60.8 a.u. (Figure 5.9C). Based on this step size and the standard deviation of noise in the traces, the SNR was calculated to be 1.1 for these measurements.

The resulting copy number distribution can be influenced by several factors. First, the probability that a GFP will fluoresce is not expected to be unity, which leads to the distribution having a binomial nature. Second, the probability of observing every single bleaching event during an experiment is less than unity due to the finite acquisition time,
meaning that the number of acquired bleaching events from each sub-population of fluorescing GFPs will itself be binomially distributed. Third, due to normal intensity fluctuations, the overall intensity drop for each trace will have an associated error value simply from the fluorescence fluctuations. Fourth, it is difficult to rule out the presence of a small percentage of aggregates in the sample or pairs of complexes residing in the same diffraction-limited spot. Due to these factors, the expected copy number distribution will be a binomial distribution broadened by Gaussian noise. As a conservative approach, we chose to fit the copy number distribution using the Gaussian Mixture Model.

To estimate fluorophore copy number, the total intensity drop for each photobleaching trace was calculated by taking the difference of the initial point and the mean value of the final plateau. Each intensity drop was then divided by the estimated unitary step size of 60.8 a.u. to generate a copy number estimate. The fit to the copy number distribution shows two peaks at 3.28 and 6.65 (Figure 5.9D). Given an expected copy number of four, these peaks are consistent with the binomial nature leading to a slight shift towards lower copy number for the first mode, and the second mode corresponding to pairs of complexes either due to aggregates or to two surface-bound complexes being within the same diffraction-limited spot. These results demonstrate that the method can give an accurate prediction of minimum protein copy number even in a data set having a SNR of 1.1.
5.2.7 Estimating Copy Number for GFP-AtCESA3

After developing an objective method for estimating subunit copy number for protein complexes tagged with large numbers of fluorophores and assessing its performance on simulated photobleaching data, we applied the technique to a set of photobleaching data for GFP-AtCESA3 particles (Figure 5.10A). Based on the trend of BIC values (Figure 5.9A), we chose to fit the data with a Gaussian mixture model (GMM) and estimated the copy number distribution (Figure 5.9D). The histograms (black boxes) in Figure 5.9C were also plotted for reference but not used in the GMM fitting.

**Figure 5.9: Estimating copy number for kinesin-4XGFP.** (A) Trace of kinesin-4XGFP bleaching (black) with steps fit by Tdetector2 (red). (B) The BIC search leads to a best fit of $k = 4$ Gaussians for fitting the step size distribution. (C) Estimating the unitary step size (60.8 a.u.) from the step size distribution (455 total detected steps). The mean values of the four modes were 63.9, 109.9, 165.8, 258.1 a.u., relative weights were 0.622, 0.289, 0.062, 0.027, and the standard deviation was 19.6 a.u. (D) Copy number distribution. There were two peaks, centered at 3.28 and 6.65. These peaks are consistent with the binomial nature leading to a slight shift from four towards lower copy number, and with a double-aggregate population at roughly twice the copy number of the first peak. Histograms (black boxes) are also plotted in (C) and (D) for reference but not used in the GMM fitting.
a model consisting of six Gaussians was used to estimate the distribution of predicted step sizes, and the final estimate for a single step was calculated to be 445.4 a.u. (Figure 5.10C). This step size indicates that the SNR is roughly 2 to 2.5, within the range that our methods can reliably uncover copy number. However, in the final copy number histogram, instead of seeing a single mode as for the simulated data, two modes, one around 10 and the other around 20, are apparent (Figure 5.10D). This factor of two suggests that a subpopulation of the analyzed particles might be composed of two complexes within the focal limited spot, either because there are two populations of CSCs in cells or because pairs of CSCs occasionally exist in close proximity, especially when they are immobile as was the case for this dataset. A fit consisting of two Gaussians identifies peaks at 9.56 and 23.5 copies. Considering that protein misfolding, incomplete maturation of GFP, and bleaching events occurring before data acquisition can all potentially lead to underestimating the true number of GFPs present, we conclude that the 10 copies is a lower limit for the estimated number of GFP-AtCESA3 subunits in each CSC particle.

5.3 Discussion

Determining the stoichiometry of proteins in large multi-subunit membrane complexes by biochemical methods is challenging, and despite producing a highly abundant and useful biopolymer, the molecular makeup of the cellulose synthesis complex, one such protein complex, has remained enigmatic. The goal of this work was to quantify the
number of CESA subunits in cellulose synthesis complexes by non-destructive in vivo photobleaching. Plant seedlings expressing GFP-AtCESA3 were imaged using variable-angle epifluorescence microscopy and the fluorescence intensities of individual GFP-AtCESA3-containing particles were recorded as the signals bleached to near background levels. However, despite efforts to maximize the SNR, individual photobleaching steps...
were not easily identified by eye, preventing an objective estimate of CESA copy number. This hurdle motivated us to develop a set of statistical tools to estimate unitary step size and fluorophore copy number from photobleaching data involving many fluorophores.

Using imaging to quantify subunit copy number for intact protein complexes \textit{in vivo} provides a method to probe the quaternary structure of these complexes that circumvents the difficulty and potential disruption of the complex inherent in biochemical purification. For copy numbers under five, it is often easy to simply estimate the number of steps by eye (Ulbrich and Isacoff 2007; Nakajo, Ulbrich et al. 2010). In other cases, it is possible to estimate unitary step intensity by measuring the amplitude of the last step, but that approach ignores much of the rich information present in the data. Because small errors in the estimation of the unitary step intensity can propagate to larger errors in the copy number estimation, it is important to use as much of the available information as possible to achieve the best possible estimate for unitary photobleaching. In our photobleaching data analysis, we identified three major challenges to accurately measuring high copy numbers: 1) detecting steps in traces having non-uniform variances due to the summed fluctuations of multiple fluorophores, 2) precisely identifying the unitary step size from step size distribution densities, and 3) accurately quantifying the total intensity drop corresponding to bleaching for all of the subunits in the complex. We developed a solution for each of these challenges, and we hope that this set of tools will be adopted as “best practices” for analyzing photobleaching data in other systems with high protein copy number.
While signal variance in molecular motor stepping data is independent of the motor position, photobleaching data present the unique challenge of signal variance that scales with intensity. Previous step detection methods have used the approach of constructing pairwise distance distributions to estimate unitary step size for each step (Svoboda, Schmidt et al. 1993; Leake, Chandler et al. 2006), but assumed a constant variance. This variance is important because it is used in tests to determine statistical significance. Applying step detection algorithms that assume constant variance to photobleaching data results in overfitting of steps in early time points when both the signal and variance are high. Thus the technique developed here to estimate the time-dependent variance of the signal was a key advance that improved the performance of both the BIC-based and t-test-based step detection algorithms over those assuming constant variance.

The step detection algorithms output a step size distribution density that needs to be analyzed to extract the unitary step size. We found Kernel Density Estimation to be a vastly superior approach over the traditional technique of binning the data and fitting multiple Gaussians because it eliminated the decision of what bin size to use. However, one weakness of KDE was fitting to higher modes. The Gaussian Mixture Model proved to be the optimal tool for identifying the modes of step intensity and assigning them proper weights. The multiple modes of step sizes can be explained by at least two reasons. First, it is possible that two or more fluorophores can bleach at the same time, resulting in larger steps. This probability grows with increasing copy number. Second, a step detection algorithm might group two steps into one when fitting the two steps
separately is not statistically significant. This can happen when noise is high, which also often correlates with high copy numbers. The probability of observing single steps consisting of multiple bleaching events is represented by the proportion of each mode in the GMM density estimation.

The final technique that we developed was a best estimate of the total photobleaching amplitude, taking into account the bleaching rate. From the ensemble average, a photobleaching rate constant could be readily extracted. This parameter will vary with excitation intensity, cellular conditions, and other factors, and so needs to be measured for each experiment. If the duration of the experiment is longer than five times the photobleaching time constant, then it is expected that 99% of the signal has bleached, minimizing the need for any correction. However, long acquisition times are not always possible due to stage or sample drift, camera memory, and underlying cellular dynamics. Hence, correcting for the expected maximum amplitude is important to avoid underestimating copy number.

While the statistical analysis indicated an average copy number of 10 GFP-CESA3 in the observed complexes, we consider this to be a lower limit for the following reasons. First, the GFP-AtCESA3 transgene is present in a background of the partial-loss-of-function \textit{AtCESA3}\textsuperscript{jes} allele of \textit{AtCESA3} (Desprez, Juraniec et al. 2007), meaning that endogenous non-fluorescent \textit{AtCESA3} can potentially still be expressed and comprise a portion of each CSC. Second, the time required for microscope focus adjustments necessary to pinpoint the focal plane of the membrane means that some GFP molecules might bleach
before images are recorded. Third, it is impossible to rule out the presence of GFP molecules that are misfolded or have not matured (though the estimated 15 minute maturation time constant for eGFP is expected to be sufficiently fast for the present measurements (Iizuka, Yamagishi-Shirasaki et al. 2011)). To improve upon this initial result, we are engineering plants that contain GFP-AtCESA3 expressed in a CESA3 null background. We are also exploring the use of slow-bleaching versions of fluorescent proteins in order to minimize pre-bleaching. Slow bleaching will also improve the ability of step detection algorithms to detect early bleaching steps. An additional uncertainty is whether the two peaks in the copy number distribution indicate that some particles are aggregates of multiple complexes or that two different populations of CSCs exist. To distinguish these two hypotheses, future experiments will focus on photobleaching analysis of motile GFP-AtCESA particles, which presumably represent single CSCs.

In conclusion, we have developed a reliable method for determining copy number in multi-subunit complexes from \textit{in vivo} photobleaching data. The statistical analysis combines step detection and density estimation to accurately determine the unitary photobleaching step and takes into consideration the bleaching rate constant when determining the maximum fluorescence signal. This method is generic and can be used to estimate the stoichiometry of other membrane-bound complexes and can be applied to fluorophores other than GFP. Because the signal variance and unitary step size are calculated directly from the raw data, it is not necessary to carry out new controls for different fluorophores, but fluorophores that display more prominent and prolonged dark states such as YFP are expected to have lower SNR, which may set an upper limit
on maximum copy numbers that can be reliably estimated. These algorithms can also be adapted to analyze molecular motor stepping data. Applying this method to \textit{in vivo} photobleaching data gave a lower limit of 10 copies of GFP-AtCESA3 in cellulose synthesis complexes.

5.4 Materials and Methods

5.4.1 Photobleaching Experiments

\textit{Arabidopsis thaliana} seeds of the genotype At\textit{CESA3}^{ies} \textit{GFP-CESA3} (Desprez, Juraniec et al. 2007) were surface-sterilized for 20 min in 30\% bleach + 0.1\% SDS, washed 4X with sterile water, and stored in sterile 0.15\% agar at 4 °C for 3 days before being sown on square petri plates containing MS medium (2.2 g/L Murashige and Skoog salts (Caisson Laboratories) + 0.6 g/L 2-(N-morpholino)-ethanesulfonic acid (MES, Research Organics) + 8 g/L agar-agar (Research Organics), + 10 g/L sucrose, pH 5.6). The plates were incubated in a 22 °C growth chamber under 24h illumination for 5-6 days before use in microscopy experiments. Seedlings were mounted on glass slides between two pieces of permanent double-stick tape (3M), 30 μL of sterile water was added to the seedling, and a 24 x 40 mm #1.5 coverslip was adhered to the tape to generate an imaging chamber. Seedlings were imaged on a Nikon TE2000 microscope in variable-angle mode with a 60X 1.4 NA oil immersion objective and a 100 mW 488 nm excitation laser. Hypocotyl cells containing sparse GFP-AtCESA3-positive particles were imaged using a
Photometrics Cascade 512b camera in streaming mode using maximum gain with 200 msec exposure time for 500-600 frames, during which time many particles bleached to background levels.

As a control, *Drosophila* kinesin heavy chain truncated at residue 559 was modified to have GFP at both the N- and C-termini, generating a dimer containing four GFP fluorophores. The protein was bacterially expressed and Ni column purified as previously described (Shastry and Hancock 2010). Surface-immobilized fluorophores were imaged by TIRF illumination (Shastry and Hancock 2010) and acquired in an identical manner to the GFP-AtCESA3 data.

### 5.4.2 Image Analysis

Image stacks were processed in ImageJ ([http://imagej.nih.gov/ij/](http://imagej.nih.gov/ij/)) as follows. First, the Background Subtract tool (10 pixel radius, sliding paraboloid) was used to subtract background fluorescence from each frame in the stack. Next, an Average Projection of the stack was generated and used to select 7-pixel-radius circular regions of interest (ROI) surrounding immobile GFP-AtCESA3 particles. Finally, photobleaching traces were generated from the background-subtracted image stack by measuring the total pixel intensity of each ROI for every frame of the stack. A total of 77 particles were analyzed.
5.4.3 Tdetector1 Algorithm

The Tdetector1 algorithm carries out an iterative two-sample t-test that assumes the expected variance throughout the entire input data vector to be constant. It also assumes that the input data vector is a piecewise-constant step function hidden in normally distributed white noise. There are no user-defined variables, and the only input to the algorithm is a single vector of data, X.

To begin, the algorithm must calculate the variance of the underlying white noise, $\sigma^2$, of the input data vector. The conventional method of calculating variance ($\text{Var}(X) = E[(X - \mu)^2]$) cannot be used because the data is expected to contain steps that would result in a large overestimation of the underlying variance. Instead a pairwise difference calculation must be used (Equation 1). Pairwise differences that are significantly greater in magnitude compared to the rest (possibly due to a large step there) are discounted from the calculation (see Supplemental Methods for further details).

$$\sigma^2 \approx \frac{\sum_{i=1}^{(L-1)}(x_{i+1} - x_i)^2}{2(L-1)} \tag{1}$$

Where $X =$ data vector, $\sigma^2 =$ variance of underlying noise in $X$, $L =$ length of $X$, $i =$ index of $X$.

The first round of the step detection process iterates through every possible way of splitting $X$ into two sections and calculates the difference of means (DOM) of those two sections. Each DOM is then rated for significance based on the expected distribution of DOMs that would result from splitting a normal random vector of the same length, with
no steps, at that respective index (given in Equation 2). This process is similar to comparing to the t-distribution as in a two-sample t-test.

\[ \text{DOMs} \sim \mathcal{N}\left(0, \sigma^2 \left(\frac{1}{i} + \frac{1}{L-i}\right)\right) \tag{2} \]

Where \( \sigma^2 \) = variance of underlying noise in \( X \), \( L = \) length of current subset of \( X \) (for first round of step detection: \( L = \) length of entire \( X \) vector), \( i = \) index of splitting.

If there is a calculated DOM that is significant (see Supplemental Methods) compared to the normal distribution shown in Equation 2, then the null hypothesis (that the observed DOM is due to variations of a normal random vector without a step) is rejected, the two sections are declared as two separate plateaus, and a possible step is declared at that index. For each round of step fitting, only the most significant DOM results in a declared step. After the first round of step fitting, the process is repeated on each new plateau, and any new plateaus from a round of step fitting will go through the same process until no new plateaus are declared.

Finally, the algorithm undergoes a step-checking phase that performs DOM significance testing for all adjacent plateaus declared (see Supplemental Methods). MATLAB code for the Tdetector algorithm is included in Supplemental Materials.
5.4.4 Tdetector2 Algorithm

The Tdetector2 algorithm is very similar to Tdetector1, except that it assumes that different sections of the data have different expected variances (as found in photobleaching traces where higher numbers of unbleached fluorophores lead to higher variances). Again, it assumes the input data vector is a piecewise constant step function hidden in normally distributed white noise, and it requires only this single vector of data, \( X \), as input to the algorithm.

The first task of the algorithm is to find sections of the data that have significantly different variances from one another. To accomplish this, it first calculates the variance of underlying noise throughout all of \( X \) using the same process described for Tdetector1 (Equation 1). Next, it uses the same process that the Tdetector1 algorithm uses to test each possible DOM for significance, but instead of comparing means it tests each possible difference of variances (DOV) for significance. The expected distribution of DOVs is approximated as normal, with a variance (Equation 3, derivation in Supplemental Methods) that depends on nearly the same variables defining the variance of DOMs in Equation 2. The only difference is that \( \sigma^2 \) is always the underlying variance of the entire \( X \) vector in Equation 2, while in Equation 3 it is the underlying variance of only the subset of \( X \) that is currently being split into two sections.

\[
\text{DOVs} \sim N \left( 0, \sigma^4 \left[ \frac{i^2+i-3}{(i-1)^2} + \frac{(L-i)^2+(L-i)-3}{((L-i)-1)^2} - 2 \right] \right)
\]
Where $\sigma^2 = \text{variance of underlying noise in current subset of } X$, $L = \text{length of current subset of } X$, $i = \text{index of splitting.}$

As in the iterative step fitting process of $T_{detector1}$, this variance-sectioning continues to declare and test new plateaus until no new significant variance sections are declared. Once the algorithm has completed the variance-sectioning process, it begins the same step detection process as in the $T_{detector1}$ algorithm, with two exceptions: (1) For DOM significance testing, $T_{detector2}$ uses $\sigma^2 = \text{mean underlying variance of the current subset of } X$ in Equation 2 rather than the underlying variance of the entire $X$ vector; and (2) Once the most significant index of splitting has been determined, the resulting DOM is again tested for significance with respect to a slightly different distribution of DOMs shown by Equation 4 (similar to Welch’s t-test). This distribution takes into account the possibility of unequal variances between the two sections. If both tests have shown significance with respect to their distributions, then a step and two new plateaus are declared at that index.

$$\text{ DOMs } \sim N\left(0, \frac{\sigma_1^2}{i} + \frac{\sigma_2^2}{L-i}\right) \quad (4)$$

Where $\sigma_1^2 = \text{underlying variance of the first section}$, $\sigma_2^2 = \text{underlying variance of the second section}$, $L = \text{length of current subset of } X$, $i = \text{index of splitting.}$
5.4.5 Bdetector Algorithms

The Bdetector1 algorithm is identical to the method described in (Kalafut and Visscher 2008), with the algorithm implemented in R (http://www.r-project.org). The Bdetector2 algorithm was developed by modifying Bdetector1 to allow for changing variance, as follows:

For a data with points $x_i$ (i is from 1 to N), if k steps are fitted at position $l_1$, $l_2$, ..., $l_k$, and for notational simplicity, let $l_0=0$, and $l_{k+1} = N$, then the maximum likelihood estimator for mean and variance are:

$$u_j = \frac{1}{l_j-l_{j-1}} \sum_{i=l_{j-1}}^{l_j} x_i \text{, where } j = 1, ..., k+1$$

(5)

$$\sigma_j^2 = \frac{1}{l_j-l_{j-1}} \sum_{i=l_{j-1}}^{l_j} (x_i - u_j)^2$$

(6)

Recall that the BIC for a statistical model is calculated as

$$\text{BIC} = -2 \cdot \log L + p \cdot \ln(N)$$

(7)

Where $\log L$ is the log-likelihood of a model, and $p$ is the number of parameters to estimate.

Thus, the BIC for fitting k steps will be:

$$\text{BIC} = \sum_{j=1}^{k+1} (l_j-l_{j-1}) \cdot \ln(\sigma_j^2) + N \cdot \ln(2\pi) + N + p \cdot \log(N)$$

(8)

Where $p = 2 \cdot (k+1) = 2k+2$. 

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To add a step, Bdetector2 scans each potential step position and calculates a BIC value. If the difference between the minimal BIC value and BIC from not adding a step is greater than 5 (Kass and Raftery 1995) a new step is added at the position that leads to smallest BIC value. While holding all previous steps, this process is then repeated to detect subsequent steps. Bdetector2 terminates when no more steps that result in a lower BIC value can be added.

5.4.6 Photobleaching Rate Estimation

By ensemble averaging many photobleaching traces and fitting to an exponential, the photobleaching rate constant can be estimated with high accuracy. Because each GFP photobleaches independently of one another, the rate constant for the exponential decay of the ensemble average will be the same as the first-order bleaching rate of a single GFP.

Comparing the photobleaching rate constant to the total acquisition time also allows for a correction due to photobleaching events that are expected to be missed due to the finite acquisition time of the experiment. Based on the known acquisition time and calculated photobleaching rate, Equation 9 calculates the fraction of photobleaching events that are expected to occur during acquisition. This number is critical because the final copy number is estimated by dividing the total intensity drop for each photobleaching trace by the experimentally-determined unitary step size. If the
photobleaching trace has not fallen all the way to background, then copy number will be underestimated. Hence, to correct for missed photobleaching events, the total intensity drop for each trace is corrected by dividing by the expected fraction of observed events given by Equation 9. According to our fitted photobleaching rate \((0.0278 \pm 0.0003 \text{ s}^{-1})\) and acquisition time \((a = 100 \text{ s})\), we expect to observe \(\sim 93\%\) of the photobleaching process.

\[
\text{Fraction observed} = 1 - e^{-ak}
\]  
\[(9)\]

Where \(a = \text{acquisition time in seconds, } k = \text{fitted photobleach rate in inverse seconds}\)

### 5.4.7 Definition of Sensitivity and Precision Ratings for Step Detection Algorithms

The ability of each step detection algorithm to correctly identify steps was tested using simulated data with added white noise containing steps at known indexes. Each algorithm was given the same collection of simulated data, and then the indexes at which each algorithm declared steps were compared to the true step indexes. If a declared step index was within a certain range of a true step index, then it was regarded as a correct declared step (i.e. if Equation 10 is satisfied). The range was defined by a constant percentage multiplier (0.05) of the two true plateau lengths on either side of a true step index.
\[- \text{round}(0.05 \, p_1) \leq (i_{\text{declared}} - i_{\text{true}}) \leq \text{round}(0.05 \, p_2) \]  \hspace{1cm} (10)

Where \( p_1 = \# \text{ of data points in plateau that precedes the true step} \), \( p_2 = \# \text{ of data points in plateau that follows the true step} \), \( i_{\text{declared}} = \text{index of the declared step} \), \( i_{\text{true}} = \text{index of a true step} \).

Once a declared step is defined as correct, the true step to which it was matched is no longer allowed to be matched to again. This means that if there are multiple declared steps within a certain range of the true step, only one of those declared steps is allowed to be defined as correct.

The sensitivity of an algorithm was defined as the fraction of true steps that have a declared step within their range (detected true steps). The precision of an algorithm was defined as the fraction of declared steps that are correct (Equations 11 and 12).

Underfitting the data will result in low sensitivity and generally higher precision, while overfitting will result in low precision and generally higher sensitivity.

\[
\text{sensitivity} = \frac{\text{detected true steps}}{\text{total true steps}} \]  \hspace{1cm} (11)

\[
\text{precision} = \frac{\text{correct declared steps}}{\text{total declared steps}} \]  \hspace{1cm} (12)
5.4.8 Density Estimation

Least-squares fitting on binned histogram data was carried out in R with nonlinear least-squares fitting. Center of bins and bin height are used. For Kernel Density Estimation, bandwidth is as specified by Scott (Scott 1992). The “normalmixEM” function in the R package “mixtools” (T. Benaglia 2009) was used to implement the Gaussian Mixture Model, and the variance of each Gaussian was assumed to be the same while means were unconstrained. The BIC value, is calculated based on the log-likelihood of each fitting, and was used to objectively determine the number of Gaussians to use in the final model.

5.4.9 Pairwise Difference Calculation of Variance of Underlying Noise
(Equation 1)

Let $X$ be a vector of $L$ independent random variables with a mean of 0, and variance of $\sigma^2$. Let $Y$ be a piecewise-constant vector of $L$ values, containing a step of amplitude $d$ between index $i$ and $i + 1$. Now let the sum of these two vectors, $Z = X + Y$, represent a data vector given to our step-detection algorithm.

$$X = [x_1, x_2, ..., x_{L-1}, x_L], \ Y = [0, 0, ..., d, d], \ Z = [x_1, x_2, ..., x_{L-1} + d, x_L + d]$$
The goal is to estimate $\sigma^2 / g_{2870}$ (the variance of the underlying noise, $X$), but we are given only the vector $Z$. Using the conventional calculation of variance on $Z$ would yield an answer composed of both $\sigma^2$ and the value of $d$ (step amplitude of $Y$).

$$\text{Var}(Z) = \text{Var}(X + Y)$$

$$= \text{Var}(X) + \text{Var}(Y)$$

$$= E[(X - E[X])^2] + E[(Y - E[Y])^2]$$

$$= \sigma^2 + \frac{i(L - i)}{L^2} d^2$$

If $Z$ contained more than one step, $\text{Var}(Z)$ would be an even greater overestimation of $\sigma^2$. Therefore, a method aimed at calculating the variance of only the underlying noise – a pairwise difference calculation – should be used instead. Generally speaking, it calculates variance based on the difference between neighboring data points rather than the difference of each data point from the mean. The following demonstrates how
one-half of the expected value of squared pairwise differences of $X$ equates to the variance of $X$, $\sigma^2$.

$$\frac{\sum_{n=1}^{(L-1)} (x_{n+1} - x_n)^2}{2(L-1)} = \frac{E[(x_{n+1} - x_n)^2]}{2} = \frac{E[x_{n+1}^2 - 2x_{n+1}x_n + x_n^2]}{2}$$

Since $X$ is an independent random vector with a mean of zero:

$$= \frac{E[x_{n+1}^2] - 2E[x_{n+1}]E[x_n] + E[x_n^2]}{2} = \frac{E[x_{n+1}^2] + E[x_n^2]}{2} = \frac{\sigma^2 + \sigma^2}{2} = \sigma^2$$

This yields Equation 1 given in Materials and Methods.

$$\text{Var}(X) = \frac{\sum_{n=1}^{(L-1)} (x_{n+1} - x_n)^2}{2(L-1)}$$

This equation holds only if all values in $X$ have an expected value of zero. If it is instead applied to $Z$, a piecewise constant step function hidden in noise, then the equation does not give $\text{Var}(Z)$, but rather a value composed of the variance of underlying noise and a relatively small contribution from $d$ (step amplitude of $Y$).

$$\frac{\sum_{n=1}^{(L-1)} (z_{n+1} - z_n)^2}{2(L-1)} = \frac{1}{2(L-1)} d^2$$

As is, this approach yields a much better estimate of the variance of underlying noise than simply using the variance of $Z$ when $L \geq 4$. However, an even better estimation of $\sigma^2$ can be obtained by performing an iterative outlier analysis on the pairwise difference values of $Z$ before taking their mean. If the magnitude of any pairwise difference is
significantly greater than the rest, then we can hypothesize that it is due to a step in the
data vector, consider it an outlier, and therefore exclude it from the average. More
specifically, if its magnitude is greater than three times the standard deviation of
pairwise differences of \( X (\sqrt{2}\sigma) \) then it should be excluded. Of course we do not know
the value of \( \sigma \), so we use the current best estimate. This process is iterated until there
are no outliers remaining. Iterations are necessary because each time an outlier is
removed, the value of \( \sigma \) changes slightly. The following pseudo/MATLAB code describes
the iterative process explicitly.

```matlab
L = length(Z);

% construct pairwise differences of Z vectors
for i = 1:L-1
    pdz(i) = Z(i+1) - Z(i);
    pdz2(i) = (Z(i+1) - Z(i))^2;
end

while true
    % current estimate of sigma of X
    sigmaC = (mean(pdz2)/2)^0.5;

    % remove outlier values from pdz vectors
end
```
% new estimate of sigma of X
sigmaN = (mean(pdz2)/2)^0.5;

if sigmaN == sigmaC
    break
end

% final sigma estimate
sigma = sigmaN;

5.4.10 Difference of Variances (Equation 3) Derivation

Let X be a vector of L independent normally distributed random variables with a mean of 0, and variance of \( \sigma^2 \).

\[
X = [x_1, x_2, x_3, \ldots, x_L]
\]

Next, if X is split into two sections, \( X_A \) and \( X_B \), of length N and M respectively,

\[
X_A = [x_1, x_2, x_3, \ldots, x_N], \quad X_B = [x_{N+1}, x_{N+2}, x_{N+3}, \ldots, x_{N+M}]
\]
then both $X_A$ and $X_B$ will have their own sample variance. The difference of these two variances is referred to as the DOV.

\[
\text{DOV} = \text{Var}(X_A) - \text{Var}(X_B)
\]

If this process was repeated on many newly generated $X$ vectors of length $L$, split into two sections of lengths $N$ and $M$, and a DOV was calculated each time, then the resulting collection of DOVs would have a variance itself.

\[
\text{Var}(\text{DOV}) = \text{Var}(\text{Var}(X_A) - \text{Var}(X_B))
\]

We wish to know $\text{Var}(\text{DOV})$ in order to test for the significance of a DOV calculated from a given data vector. Even though $X_A$ and $X_B$ as we have stated in this derivation are not expected to contain steps, we still represent their variance with the pairwise difference method (Equation 1 in Materials and Methods) because that is how variance values for a DOV of a given data vector will be calculated.

\[
\text{Var}(\text{DOV}) = \text{Var}\left(\frac{\sum_{n=1}^{N-1}(x_{n+1} - x_n)^2}{2(N-1)} - \frac{\sum_{n=N+1}^{N+M-1}(x_{n+1} - x_n)^2}{2(M-1)}\right)
\]

These two terms, $\text{Var}(X_A)$ and $\text{Var}(X_B)$, are independent of one another, therefore:

\[
\text{Var}(\text{DOV}) = \text{Var}\left(\frac{\sum_{n=1}^{N-1}(x_{n+1} - x_n)^2}{2(N-1)}\right) + \text{Var}\left(\frac{\sum_{n=N+1}^{N+M-1}(x_{n+1} - x_n)^2}{2(M-1)}\right)
\]

We can simplify the variances above, $\text{Var}(\text{Var}(X_A))$ and $\text{Var}(\text{Var}(X_B))$, to functions of the population variance of $X$, $\sigma^2$, that depend on lengths $N$ and $M$ respectively, using the
conventional formula \( \text{Var}(X) = \text{E}[(X - \text{E}(X))^2] = \text{E}[X^2] - (\text{E}[X])^2 \). The simplification of \( \text{Var}(\text{Var}(X_A)) \) is as follows.

\[
\text{Var}\left( \frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N-1)} \right)
\]

\[
= \text{E}\left[ \left( \frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N-1)} \right)^2 \right] - \left( \text{E}\left[ \frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N-1)} \right] \right)^2
\]

\[
= \text{E}\left[ \left( \frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N-1)} \right)^2 \right] - (\sigma^2)^2
\]

\[
= \left( \frac{1}{2(N-1)} \right)^2 \text{E}\left[ \left( \sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2 \right)^2 \right] - \sigma^4
\]

\[
= \left( \frac{1}{2(N-1)} \right)^2 \text{E}\left[ \left( \sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2 \right)^2 \right] - \sigma^4
\]

\[
= \left( \frac{1}{4(N-1)^2} \right) \text{E}\left[ \left( \sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2 \right)^2 \right] - \sigma^4
\]

Next, we can simplify the term highlighted in blue to a multiple (defined by length, \( N \)) of the squared population variance of \( X \), \( \sigma^4 \).

\[
\text{E}\left[ \left( \sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2 \right)^2 \right] = \text{E}\left[ \left( x_1^2 - 2 \sum_{n=1}^{(N-1)} x_n x_{n+1} + 2 \sum_{n=1}^{(N-2)} x_{n+1}^2 + x_N^2 \right)^2 \right]
\]
Therefore all red terms above can be dropped.

When expanded, the expected value of each term within all red terms will be equal to zero. This is because each term will contain at least one value raised to the first power \( (x_n^1) \), which has an expected value of zero, resulting in the expected value of that entire term being equal to zero. Therefore all red terms above can be dropped.

\[
= E[x_1^4] + 2E \left[ x_1^2 \left( \sum_{n=1}^{(N-2)} x_{n+1}^2 \right) \right] + E[x_1^2 x_N^2] + 4E \left[ \left( \sum_{n=1}^{(N-1)} x_n x_{n+1} \right)^2 \right] \\
+ 2E \left[ x_1^2 \left( \sum_{n=1}^{(N-2)} x_{n+1}^2 \right) \right] + 4E \left[ \left( \sum_{n=1}^{(N-2)} x_{n+1}^2 \right)^2 \right] + 2E \left[ x_N^2 \left( \sum_{n=1}^{(N-2)} x_{n+1}^2 \right) \right] \\
+ E[x_1^2 x_N^2] + 2E \left[ x_N^2 \left( \sum_{n=1}^{(N-2)} x_{n+1}^2 \right) \right] + E[x_N^4] \\
\]

Recall: \( \sigma^2 = E[x_n^2] - E[x_n]^2 = E[x_n^2] \), and \( E[x_n^2 x_m^2] = E[x_n^2]E[x_m^2] = \sigma^4 \) since \( x_n \perp x_m \) where \( n \neq m \).
Note: the 4th central moment of a normal random variable, \( E[x_n^4] = 3\sigma^4 \)

\[
E\left[\left(\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2\right)^2\right]
\]

\[
= 3\sigma^4 + 2(N - 2)\sigma^4 + \sigma^4 + 4(N - 1)\sigma^4 + 2(N - 2)\sigma^4
+ 4[(N - 2)^2 - (N - 2) + 3(N - 2)]\sigma^4 + 2(N - 2)\sigma^4 + \sigma^4 + 2(N - 2)\sigma^4 + 3\sigma^4
\]

\[
= (4N^2 + 4N - 12)\sigma^4
\]

This term can now be plugged back in for the blue highlighted term:

\[
\text{Var}\left(\frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N - 1)}\right) = \left(\frac{1}{4(N - 1)^2}\right) E\left[\left(\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2\right)^2\right] - \sigma^4
\]

\[
= \left(\frac{1}{4(N - 1)^2}\right) (4N^2 + 4N - 12)\sigma^4 - \sigma^4
\]

\[
= \left(\frac{N^2 + N - 3}{(N - 1)^2}\right) \sigma^4 - \sigma^4
\]

\[
= \left(\frac{N^2 + N - 3}{(N - 1)^2} - 1\right) \sigma^4
\]

Now this term for \( \text{Var}(\text{Var}(X_A)) \) and its counterpart representing \( \text{Var}(\text{Var}(X_B)) \) in terms of \( M \) can be plugged into the \( \text{Var}(\text{DOV}) \) equation.

\[
\text{Var}(\text{DOV}) = \text{Var}\left(\frac{\sum_{n=1}^{(N-1)} (x_{n+1} - x_n)^2}{2(N - 1)}\right) + \text{Var}\left(\frac{\sum_{n=N+1}^{(N+M-1)} (x_{n+1} - x_n)^2}{2(M - 1)}\right)
\]
\[
= \left( \frac{N^2 + N - 3}{(N - 1)^2} - 1 \right) \sigma^4 + \left( \frac{M^2 + M - 3}{(M - 1)^2} - 1 \right) \sigma^4
\]

\[
= \left[ \frac{N^2 + N - 3}{(N - 1)^2} + \frac{M^2 + M - 3}{(M - 1)^2} - 2 \right] \sigma^4
\]

Instead of using \(N\) and \(M\), we can represent the lengths of \(X_A\) and \(X_B\) with \(i\) and \((L - i)\) respectively. This yields the variance shown in Equation 3 of Materials and Methods.

\[
\text{Var}(\text{DOV}) = \left[ \frac{i^2 + i - 3}{(i - 1)^2} + \frac{(L - i)^2 + (L - i) - 3}{((L - i) - 1)^2} - 2 \right] \sigma^4
\]

5.4.11 Tdetector Difference of Means (DOM) Significance Testing

A DOM is declared significant if its absolute value is greater than a certain value (multiplier) times the standard deviation of its respective DOM distribution (Equation 2 of Materials and Methods). The multiplier determines the frequency of false rejections of the null hypothesis (i.e. false positives). For a given data vector of length, \(L\), there are \(L-1\) ways to split the data into two sections, and hence that many DOM values being tested for significance (i.e. “chances” for a false positive).

We want the probability that a given data vector will return a false positive to be 0.05, but choosing the corresponding multiplier is analytically difficult due to the fact that DOM values are not independent of one another. If they were independent, the relation
would be simple; given L-1 chances for a false positive, the probability, p, that a single DOM should give a false positive should be:

\[
p = 1 - (0.95)^{\frac{1}{L-1}}
\]

The normal distribution standard deviation multiplier (as a function of L) that would yield this probability can be calculated using the inverse error function as follows.

\[
\text{multiplier}(L) = -\sqrt{2} \text{erfinv} \left( - (0.95)^{\frac{1}{L-1}} \right)
\]

This relation was used as guidance for estimating multiplier values empirically. Multiplier values in the range of this relation were tested on several generated random vectors of different lengths L in order to achieve a 0.05 false positive probability. The resulting empirical multiplier table is shown below (Table 5.1).

<table>
<thead>
<tr>
<th>L</th>
<th>multiplier</th>
<th>L</th>
<th>multiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0000</td>
<td>181</td>
<td>3.1207</td>
</tr>
<tr>
<td>2</td>
<td>1.9600</td>
<td>256</td>
<td>3.1500</td>
</tr>
<tr>
<td>3</td>
<td>2.1700</td>
<td>362</td>
<td>3.1975</td>
</tr>
<tr>
<td>4</td>
<td>2.3400</td>
<td>512</td>
<td>3.2400</td>
</tr>
<tr>
<td>6</td>
<td>2.4700</td>
<td>724</td>
<td>3.2801</td>
</tr>
<tr>
<td>8</td>
<td>2.6000</td>
<td>1024</td>
<td>3.3048</td>
</tr>
<tr>
<td>11</td>
<td>2.6563</td>
<td>1448</td>
<td>3.3183</td>
</tr>
<tr>
<td>16</td>
<td>2.7500</td>
<td>2048</td>
<td>3.3252</td>
</tr>
<tr>
<td>23</td>
<td>2.8156</td>
<td>2896</td>
<td>3.3295</td>
</tr>
<tr>
<td>32</td>
<td>2.9000</td>
<td>4096</td>
<td>3.3311</td>
</tr>
<tr>
<td>45</td>
<td>2.9406</td>
<td>5793</td>
<td>3.3328</td>
</tr>
<tr>
<td>64</td>
<td>3.0000</td>
<td>8192</td>
<td>3.3332</td>
</tr>
<tr>
<td>91</td>
<td>3.0422</td>
<td>10000</td>
<td>3.3333</td>
</tr>
<tr>
<td>128</td>
<td>3.1000</td>
<td>1e+10</td>
<td>3.3333</td>
</tr>
</tbody>
</table>

Table 5.1: Empirically calculated standard deviation multiplier lookup table for DOM significance testing. Data vector lengths, L, are rounded values of \(2^{(n/2)}\) where \(n = 0, 1, 2, \ldots, 26\). Multipliers between given L values can be linearly interpolated with good reliability. The last two L values in the table are untested extrapolations of the trend.
5.5 Movie Legends

**Movie 5.1: Photobleaching of GFP-AtCESA3 in *Arabidopsis* Seedlings.** Seedling was mounted in a flow cell and observed under variable-angle epifluorescence microscopy. Movie was recorded at 5 fps with a total of 500 frames. During this period, most GFP molecules were photobleached.

**Movie 5.2: Demonstration of Tdetector1 Algorithm on Simulated Stepped Data.** The movie begins with the first round of the step detection process (calculation of the variance of underlying noise occurs before the movie). The top panel shows the entire input data vector plotted in blue, the light blue shading at the bottom indicates the subset undergoing the current round of step detection (i.e. DOM significance testing). The middle panel shows only the current subset plotted in blue, and the mean of each of the two sections in magenta. The bottom panel plots the significance rating of the current DOM in green if it is greater than the green threshold line at a significance of 1 (significant), or red if it is less (not significant). The final step-checking phase is indicated by light green shading at the bottom of the top panel instead of blue. This phase ensures that each declared step is significant when only its two adjacent plateaus are considered in the DOM significance calculation.

The movie of above can be found at

http://www.molbiolcell.org/content/early/2014/09/15/mbc.E14-06-1146/suppl/DC1
5.6 References


Chapter 6

Conclusions and Future Directions

A central question in understanding the role of microtubules in cell function is how microtubule dynamics are controlled to achieve and maintain specific spatial organizations such as the mitotic spindle (Compton 2000; Wittmann, Hyman et al. 2001) and polarized microtubule bundles in axons and dendrites (Gunning and Hardham 1982; Desai and Mitchison 1997; Conde and Caceres 2009; Kollins, Bell et al. 2009; Dent and Baas 2014). The reductionist methodology of removing one component at a time and deducing their functions based on resulting phenotype of the knockout has identified numerous key proteins that control these processes (Bulinski and Borisy 1979; Heald, Tournebize et al. 1996). However, the build-in redundancy of biological systems limits the utility of reductionist methods (Wittmann, Hyman et al. 2001; Goshima and Vale 2003; Zhu, Zhao et al. 2005). The philosophy of this thesis is to investigate molecular mechanisms through bottom-up reconstitution methods. Chapters 2 and 3 focus on understanding microtubule guidance by EB1 and kinesin motors, and the regulation of microtubule dynamics by an engineered dimeric kinesin-5 motor. Chapters 4 and 5 are devoted to developing interdisciplinary research methods to study cellular complexity.
6.1 Microtubule Organization in Neurons

Neuronal microtubules are uniform plus-end out in axons (Conde and Caceres 2009). In dendrites, microtubules are found to possess mixed orientations in cultured mammalian neurons and be uniform minus-end out in *Drosophila* larvae (Baas, Deitch et al. 1988; Stone, Roegiers et al. 2008). Dendritic microtubules are more dynamic, while axonal microtubules are stable except in the growth cone (Conde and Caceres 2009; Kollins, Bell et al. 2009). The dynamics of microtubules pose a challenge to maintaining the uniform microtubule geometry in highly branched dendrites: growing microtubules that enter a branch point and exit towards the distal dendrite end would disrupt the uniformity. A model of microtubule guidance by kinesin and +TIPs was proposed, but never tested in vitro (Mattie, Stackpole et al. 2010).

In chapter 2, purified kinesin-2 and EB1 were dimerized by rapamycin induced heterodimerization of FKBP and FRB. This reconstituted complex was introduced into the *in vitro* system containing dynamic microtubules immobilized on a glass coverslip, and encounters between growing microtubules were observed. It was shown that growing microtubules that encounter a second microtubule lattice can be directed toward the encountered microtubule plus-end by end-accumulated kinesin-EB1 complex. The fact that kinesin-EB1 is sufficient to steer microtubule growth provides a direct mechanism for maintaining uniform microtubule organization in cells. This finding also reveals novel functions to both kinesin and +TIPs and has important implications for microtubule organization in other cells.
Parallel work from Anna Akhmanova’s lab in the Netherlands, which was published in the same issue of *Current Biology*, investigates a similar complex that was created by attaching a SxIP motif to kinesin to link it to EB1, and demonstrates that this kinesin-EB1 complex is also sufficient to guide microtubule growth *in vitro* (Doodhi, Katrukha et al. 2014). Studies in this work are a direct support and complement to my reconstitution work. The use of the SxIP motif suggests that the natural intracellular interaction between EB1 and kinesin is strong enough to support the microtubule bending. Binding kinetics between EB1 and proteins containing SxIP motif will not only shine lights on microtubule steering, but also other cellular processes involving proteins targeting to microtubule plus-ends.

Despite the work showing the mechanical capability of dynamic EB1-microtubule interactions for bending microtubules, the underlying mechanisms and protein interactions are not fully resolved. While the duration of the complex at the growing microtubule plus-ends is well characterized, details regarding the interactions between these two proteins at the end are not known at present. Also, the *in vitro* reconstitution systems only harness a simplified minimum of two components, and lack other proteins, such as APC, that are suggested to be involved in the process. To explore answers to these questions, future experiments are discussed below.
Mechanical properties of EB1

Interaction of EB1 with growing microtubule plus-ends is highly dynamic with a fast turnover rate of EB1. The demonstrated capability of kinesin-EB1 complex to steer microtubule growth seems incompatible with these fast dynamics. Although dimerization with kinesin slows the overall turnover rate of the complex at the end, presumably due to additional binding provided by kinesin, when bridging between two microtubules the kinesin and EB1 will be interacting with different microtubules. Furthermore, the load exerted by microtubule bending during steering would be expected to accelerate the detachment of EB1 from microtubule plus-ends. To elucidate this paradox, experiments investigating mechanical properties of EB1 are warranted. One possible experiment is to absorb EB1 onto beads and use optical tweezers to study the force-dependent properties of the beads at growing microtubule plus-ends. If the difficulty of working with polymerizing microtubule tips makes the experiments intractible, alternative experiments can be performed on static GMPCPP or GTPγS microtubules. A collaboration with Erik Schaeffer at Tubingen was initiated to investigate these force-dependent EB1 off-rates; we have sent them protein and they are currently in the process of developing the assay.

Details about proteins working at the plus end

Three different kinesins were tested in chapter 2 and all of them are capable of microtubule guidance, but with different concentration requirements. The
concentration differences presumably will lead to a different number of complexes at growing microtubule plus-ends. Despite different force-dependent properties of these three kinesins, the maximum loads of all three kinesins do not vary much (Block 2007; Schroeder, Hendricks et al. 2012). To understand how these proteins collaborate in the microtubule steering process, it is important to know the number of complexes at the end. One way is to calculate the energy stored in deformed microtubules based on microtubule curvature and stiffness, and deduce the minimum force requirement of complex. Another way is to use quantitative fluorescence to estimate number of complexes at the end. This method requires the benchmark of single complex fluorescence at the same microscopy and laser setup. The results from these experiments can be used to simulate microtubule bending and predict single EB1 force property, which in turn can be cross validated from experimental force measurement.

**APC functions and microtubule interaction with actin filaments**

One significant component that is not present in the *in vitro* complex is APC protein. APC is a giant scaffold protein that possesses multiple functional domains, including both microtubule and actin binding domain (Munemitsu, Souza et al. 1994; Moseley, Bartolini et al. 2007). One proposed function of APC, besides bridging EB1 and kinesin during cellular microtubule steering, is that APC bundles microtubules and reinforces the parallel microtubules architecture that results from steering. One prediction from this model is that the presence of APC should reduce the concentration of complexes
required for steering or increase the probability of steering at the same complex concentration. The challenge of reconstituting APC with microtubule-steering system is that functional full-length APC is difficult to purify. One alternative choice is to make truncated proteins containing all, or at least two, microtubule binding domains. Additionally, APC was shown to promote microtubule assembly (Nakamura, Zhou et al. 2001), and the interaction between APC and EB1 on microtubule dynamics will provide another view of microtubule steering and other cellular processes.

Besides APC, other actin binding proteins have been indicated in guiding microtubules along actin filaments. ACF7, an actin crosslinking factor, has been shown to be targeted to microtubule plus-ends and bind to actin filaments (Wu, Kodama et al. 2008). The yeast functional homolog of APC, Kar9, has been suggested to target Myo2, a Myosin V, into microtubule plus-ends (Lee, Tirnauer et al. 2000). Microtubule guidance along actin filaments can be investigated in a reconstitution system with both dynamic microtubules and stable actin filaments. To understand this system, complexes containing a minimal EB1 and processive myosin should be reconstructed and the mechanistic details studied.

6.2 Insights into Kinesin-5 Functions

In another organized microtubule structure, the bipolar mitotic spindle, the homotetrameric kinesin-5 is crucial to proper spindle formation and maintenance (Blangy, Lane et al. 1995; Goshima and Vale 2003; Tanenbaum, Macurek et al. 2008;
Brust-Mascher, Sommi et al. 2009). The loss of kinesin-5 function in the early stages of cell division leads to monopolar spindles (Kapoor, Mayer et al. 2000; Skoufias, DeBonis et al. 2006). Additionally, kinesin-5 is also essential in neurons (Nadar, Ketschek et al. 2008; Falnikar, Tole et al. 2011; Sharma, Mozgova et al. 2011). Extensive research has been focused on the tetrameric structure that enables kinesin-5 to slide antiparallel microtubules apart (Waitzman and Rice 2014). In only rare examples has there been an examination of the question of whether any properties independent of tetramer configuration are important to kinesin-5’s function.

To eliminate the complicating effects of the tetrameric configuration, in Chapter 3 a kinesin-5 dimer was engineered by swapping kinesin-5 motor domain and neck linker with kinesin-1. The resulting dimer can pause at static microtubule plus-ends and in some instances track growing microtubule plus-ends, a property of a plus-tip tracker. When bound to microtubules in a population, the dimeric kinesin-5 can slow microtubule depolymerization and promote microtubule growth by inhibiting catastrophe. These experiments demonstrate that kinesin-5 possesses multiple functions comprising microtubule stabilizer, microtubule polymerase and plus-tip tracker. These functions advance our understanding of the molecular mechanism of kinesin-5, and have broad implications in understanding both mitotic spindles and neuronal development.

The novel functions of kinesin5 revealed in this work demonstrate that kinesin-5 is a MAP that interacts with microtubules in various ways. The end pausing behavior at
static microtubule plus-ends is similar to yeast kinesin-8 kip3p (Gupta, Carvalho et al. 2006; Varga, Helenius et al. 2006; Varga, Leduc et al. 2009), but the underlying mechanisms of both kinesins remains unclear. The diverse end structures of growing microtubules caused by kinesin-5 indicate that kinesin-5 might bind and stabilize protofilaments. Structural evidence for this proposed kinesin-5-protofilament interaction should be obtained to provide further details. Finally, if these concerns can be addressed, a complete mechanistic picture of the enhancement of microtubule growth by kinesin-5 can be put together. Experiments to obtain answers to these questions are proposed below.

*Mechanisms of motor pausing at the end*

The most thoroughly studied kinesin, kinesin-1 will detach from static microtubule plus-ends without showing significant dwelling at physiological buffer concentrations (Varga, Leduc et al. 2009). However, other kinesins besides kinesin-5 have been shown to be able to bind to or track static or dynamic microtubule plus-ends. The most relevant example comes from kinesin-8 family. The yeast kinesin-8, kip3p, is almost as slow as kinesin-5 and stays at the end much longer than kinesin-5 (Varga, Leduc et al. 2009). However, kip3p is a microtubule depolymerase while kinesin-5 stabilizes microtubules. The end-pausing property is correlated with the motor domains based on the constructs used in those studies, suggesting that unique aspects of their hydrolysis cycles might be responsible. We hypothesize that during the normal kinesin-5 stepping cycle, ATP
hydrolysis is greatly accelerated by inter-head tension following a step, such that kinesin-5 pauses at microtubule ends in a single-head-bound ATP state due to slow ATP hydrolysis. This hypothesis requires further experimental support. One strategy is to study kinesin-5 monomers, which lack inter-head tension. However, the behavior of monomers might not necessarily represent the state of one-head bound dimers. Another strategy is to manipulate nucleotide conditions to explore the end-duration at various ADP and ATP concentrations and concentration ratios, and use this evidence to constrain possible mechanisms. Kinetic studies from both stop flow and single-molecule experiments are also valuable tools to understand key characteristics of kinesin-5 chemomechanical cycle.

Biochemical role of end-pausing

One well-studied sequence in Kinesin-5 is the 8-amino acid extended loop-5, which is the target of the majority of anti-kinesin-5 drugs. Loop-5 was suggested to be involved in ADP release and structural changes during the kinetic cycle (Waitzman, Larson et al. 2011; Goulet, Behnke-Parks et al. 2012). It will be important to identify the relationship between residues in loop-5 and end-binding duration, as well as its ability to affect microtubule dynamics. Besides loop-5, nucleotide-sensing elements, switch-I and switch-II, can also be considered as targets for mutagenesis analysis. Recently, a unique loop-8 in kinesin-8 is suggested to be responsible for its cellular functions (Kim, Fonseca et al. 2014). It will be interesting to examine whether kinesin-5 contains such uniqueness in its loop-8.
Polymerase or depolymerase: insight from a structural basis

Despite similar speed and duration at microtubule plus-ends, kinesin-5 and kip3p have contrasting activities at microtubule ends: kinesin-5 stabilizes and promote microtubule growth, while kip3p depolymerizes microtubules. To further understand how kinesin-5 enhances microtubule growth, it is important to examine motors binding to microtubules. One important observation is the diverse microtubule plus-end structures induced by kinesin-5, suggesting that kinesin-5 might be able to bind and stabilize growing protofilaments. Cryo-EM can be utilized to observe kinesin-5 binding at microtubule plus-ends, as well as the resulting microtubule end structures that can be seen in some cases by fluorescence microscopy but could be visualized in much higher detail by CryoEM. Similar experiments on kip3p should also be performed and compared to kinesin-5 to help to understand similarities and differences in their behavior.

Microtubule dynamics during kinesin-5 inhibition

The fact that inhibition of kinesin-5 leads to arrested mitosis makes kinesin-5 an ideal target for anticancer therapy (Waitzman and Rice 2014). Drugs inhibiting kinesin-5 have been tested in various clinical trials, but have showed lower than expected efficacy (Sarli and Giannis 2008). Kinesin-5 inhibitors block the kinesin-5 hydrolysis cycle, but do not cause complete detachment from microtubules (Cochran, Gatial et al. 2005; Luo, Parrish
et al. 2007). One question is whether drug-inhibited kinesin-5 can still affect microtubule dynamics. Work in Chapter 3 provides a framework for experiments to investigate microtubule dynamics with kinesin-5 present. Furthermore, it is hoped that next generation kinesin-5 inhibitors will be developed that work by different inhibition mechanisms, and it will be important to assess the relative importance of blocking the kinesin-5 hydrolysis cycle, inhibiting motility, and disrupting the effects of kinesin-5 on microtubule dynamics.

*Interplay between kinesin-5 and other MAPs*

MAPs including motor proteins kinesin-8, kinesin-4, kinesin-13 and kinesin-7 were identified to work at microtubule plus-ends during mitosis and showed effects on microtubule dynamics (Hunter, Caplow et al. 2003; Varga, Helenius et al. 2006; Varga, Leduc et al. 2009; Stumpff, Du et al. 2011; Gudimchuk, Vitre et al. 2013; Subramanian, Ti et al. 2013). Other non-motor +TIPs are also involved in mitotic microtubule dynamics. Interactions between MAPs can lead to unexpected effects, like the synergistic enhancement of microtubule polymerization between EB1 and Xmap215 (Zanic, Widlund et al. 2013). To further assess multi-motor effects on microtubule dynamics, systems of dynamic microtubules with kinesin-5 and other MAPs should be constructed *in vitro*. One example question is how kinesin-5 and kinesin-8 compete with each other at microtubule plus-ends. Interpretation of these experiments will be facilitated by the use of mathematical models based on the mechanisms of each specific component.
In summary, this research using an in vitro reconstitution system validated models that were proposed based on cellular studies and elucidated molecular mechanisms of interplay between MAPs and microtubules. The success of these methods also inspires the design and development of other novel methods to study important biological questions.

6.3 Developing Novel Research Methods Using Engineering Techniques

Single-molecule studies have provided explicit insights into the molecular mechanisms of various MAPs. However, the microtubule geometries in cells are usually assemblies of multiple microtubules, such as the mitotic spindle. To connect the results from single-molecule studies on isolated microtubules with events occurring in the cellular environment involving higher level microtubule organizations, microtubule structures that mimic cellular geometries should be reconstructed in vitro. In chapter 4, using AC electric fields, microtubules were aligned into pairs of electrodes on elevated pedestals, forming a three dimensional bipolar organization. Microtubules used in current studies are relative short, and longer microtubules should be used to generate significant antiparallel overlap regions. Also, PRC1 and kinesin-4 has been shown to control overlap length of two antiparallel microtubules, and it is important to know if this two component systems is capable to control the overlap region involving multiple
microtubules. The corresponding experiments will be to assemble short microtubule seeds on two electrodes, introduce free tubulins together with purified PRC1 and kinesin-4, and observe the establishment of antiparallel regions. Overall, the “artificial spindle” has huge potential to be used as platforms for studying MAPs in spindle-mimic microtubule environment.

6.4 Applying Statistical Analysis in Biological Studies

Membrane proteins are notorious for their difficulty of study (Sachs and Engelman 2006). Cellulose synthase in plant cells is an important membrane-bound enzyme that makes cellulose microfibrils, and it is an active topic in plant research due to its importance to cell morphologies and the great commercial value of cellulose. However, the stoichiometry of cellulose synthase complex, which contains multiple subunits of cellulose synthases, is still not clear. In chapter 5, the challenge of using non-destructive photobleaching to estimate high protein copy number is addressed by statistical techniques. Statistical step-detection algorithms were developed to predict individual steps out of noisy traces, and the resulting step distribution was further analyzed to calculate the unitary step size, which eventually makes it possible to estimate copy numbers. To obtain the final stoichiometry of a whole CSC, other subunits need to be fluorescently tagged, expressed, bleached and analyzed. One issue in the work is that the kinetics fluorescent protein maturation and the nonunitary probability that a fluorescent protein will be active limit the final copy estimation to minimum lower
bound. To improve this limitation, mechanistic studies on protein maturation and fluorescent probability should be carried out. While the motivation of these statistical analyses is to understand CSCs, techniques described in Chapter 5 can easily be adopted to determine copy number and/or stoichiometry of other multi-subunit protein complexes. This analysis method can also be used to study protein or vesicle movements that involve irregular steps, such as the bidirectional transport mediated by multiple motors.

### 6.5 Interdisciplinary Knowledge in Biological Sciences

Basic science studies over decades have built a deep knowledge framework for understanding biological systems, but further understanding demands innovative research methods and complementary input from collaborative fields of biology, engineering, mathematics and computational science. One great example is genetic studies. The invention of high throughput DNA sequencing has led to complete sequencing of many genomes, and statistical and computational analysis have been developed to accommodate the requirement of extracting information from these large data sets. Future basic science research can be composed of a similar workflow with three major procedures:

1. Studies at cellular levels or above. These studies can either lead to new questions or validate results of other studies.
(2) Extensive in vitro reconstitutions through automated devices and/or high throughput methods. These experiments will generate large amount of data on biological systems of interest.

(3) Computational application of statistical algorithms to analyze data. The extracted information can either be validated by step (1) or used to build mathematical models to give predictions for new experiments.

Conventional scientific research requires extraordinary labor input and high degrees of uncertainty. Innovative methods automated by engineering processes could generate devices, such as lab-on-a-chip devices, with great utility for basic research investigations.
Additionally, the use of multi-disciplinary methods may also provide ways of overcoming current equipment limitations.

6.6 References


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