Measuring force generation within reconstituted microtubule bundle assemblies using optical tweezers

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Abstract
Kinesins and microtubule associated proteins (MAPs) are critical to sustain life, facilitating cargo transport, cell division, and motility. To interrogate the mechanistic underpinnings of their function, these microtubule-based motors and proteins have been studied extensively at the single molecule level. However, a long-standing issue in the single molecule biophysics field has been how to investigate motors and associated proteins within a physiologically relevant environment in vitro. While the one motor/one filament orientation of a traditional optical trapping assay has revolutionized our knowledge of motor protein mechanics, this reductionist geometry does not reflect the structural hierarchy in which many motors work within the cellular environment. Here, we review approaches that combine the precision of optical tweezers with reconstituted ensemble systems of microtubules, MAPs, and kinesins to understand how each of these unique elements work together to perform large scale cellular tasks, such as but not limited to building the mitotic spindle. Not only did these studies develop novel techniques for investigating motor proteins in vitro, but they also illuminate ensemble filament and motor synergy that helps bridge the mechanistic knowledge gap between previous single molecule and cell level studies.

KEYWORDS
in vitro reconstitution, kinesin, microtubule associated proteins, microtubules, motors, optical tweezers

1 | INTRODUCTION

Microtubule (MT)-based motors and associated binding proteins are essential players in large scale cellular tasks, such as mitosis, cargo transport, and cell motility (Hirokawa, Noda, Tanaka, & Niwa, 2009). While the kinesin family of motors has a conserved ATPase domain, their remaining structural features distinguish each motor’s unique function; these structure–function relationships have been reviewed extensively (Block, 2007; L. S. Goldstein, 2001; L. S. B. Goldstein & Philp, 1999; Gross, 2004; Hirokawa, 1998; Hirokawa et al., 2009; Hirokawa & Takemura, 2004; Jon Kull & Endow, 2013; Kolomeisky & Fisher, 2007; Marx, Hoenger, & Mandelkow, 2009; Miki, Okada, & Hirokawa, 2005; Rath & Kozielski, 2012; Vale & Fletterick, 1997; Wordeman, 2010). A defining characteristic of kinesins that is highly influenced by its structure is force generation capacity, which yields the family-specific functions of kinesins. For instance, the terms “porters” and “rowers” have been used to describe highly processive kinesin-1 and less processive mitotic kinesins, respectively, where porters carry intracellular cargo over long distances and rowers work together to remain in contact with their track to facilitate sliding (Figure 1a; Chowdhury, 2014; Leibler & Huse, 1993). However, as we continue to attain higher resolution structures and force generation profiles of motors using both experimental methods and computational approaches, we realize that the boundaries of these two motor classes can become blurred, as with mitotic kinesin-12 Kif15 and kinesin-14 HSET that exhibit a combination of nonprocessive and processive properties (Reinemann et al., 2017; Reinemann, Norris, Ohi, & Lang, 2018). Kinesins, such as these that are not true porters...
or rowers, work between bundled MTs, and warrant further study to understand the nuanced influence of their structural features on cytoskeletal function. However, the question arises regarding how to approach, execute, and analyze such studies of “nontraditional” kinesins that work in teams. Within the last decade, innovative optical trapping (OT) methodologies have emerged that have begun to address long-standing issues within the single molecule (SM) biophysics field: (1) how to study these nontraditional motors and (2) how to investigate force generation by motor teams within a physiologically relevant environment. Here, we review studies of reconstituted ensemble systems of multiple MTs, kinesins, and microtubule associated proteins (MAPs) using OT whose goal is to better understand how these proteins work together at the molecular level to perform large scale cellular tasks, such as building the mitotic spindle.

SM techniques, such as fluorescence and OT, have revolutionized the way we approach interrogating and understanding the force generating properties of motor proteins (Ashkin, 1992; Duke, 1999; Finer,
Simmons, & Spudich, 1994; Kitamura, Tokunaga, Iwane, & Yanagida, 1999; Kuo & Sheetz, 1993; Mehta et al., 1999; Molloy, Burns, Kendrick-Jones, et al., 1995; Molloy, Burns, Sparrow, et al., 1995; Palmiter, Tyska, Aeberle, & Alpert, 2000; Ruegg et al., 2002; Svoboda & Block, 1994; Svoboda, Schmidt, Schnapp, & Block, 1993; Takagi, Homsher, Goldman, & Shuman, 2006; Tyska et al., 1999, 2000; Matthew J. Tyska & Warshaw, 2002; Veigel, Molloy, Schmitz, & Kendrick-Jones, 2003). Measurements yield information overshadowed in bulk experiments, such as bond lifetimes, dissociation kinetics, step sizes, dwell times, and stall forces, among others, with piconewton force and nanometer displacement resolution.(Neuman & Block, 2004) In OT assays, a micron-sized bead functions as a handle to examine SMs and is trapped by a tightly focused laser beam which acts as a Hookean spring. Displacements are then translated into force measurements via trap stiffness calibrations. (Ashkin, 1992; Neuman & Block, 2004) OT has been used to investigate both porter and rower type motors, but certain assay orientations are limited on the mechanistic information that can be gained due to the nature of the interrogated motor’s motility. Therefore, to understand the capabilities and limitations of motor-based OT approaches, we need to review the evolution of these motor-filament assays and how they have been adapted to study single motors, motor teams, and motors across the processivity spectrum.

2 | HISTORICAL OVERVIEW OF OT MOTOR ASSAYS

2.1 | Single molecule assays

The evolution of the multi-motor, multi-filament OT experiment is found on the realization that the motility properties of motors, for instance whether they are porters or rowers, will dictate the necessary OT geometry required for productive study. Therefore, it is important to review how kinesin OT assays have transformed over the years. The earliest measurements of the classical porter kinesin-1’s force generation using optical tweezers came from the Sheetz and Block labs. Kuo and Sheetz used OT to measure force produced through a streptavidin coated bead bound to a biotinylated MT and was displaced by a single kinesin attached to a coverslip (Kuo & Sheetz, 1993). Svoboda et al. reversed the orientation, where the MT was bound to a coverslip and a kinesin motor was attached to a trapped bead (Figure 1b; Svoboda et al., 1993). In the presence of ATP, the kinesin attached to the bead would “walk” from the trap center along the MT track, concomitantly stretching the trap spring and increasing force. Both assay orientations have been adapted and customized to study kinesins and other molecular motors, especially the latter bead with bound motor orientation.

Problems arise when investigating rowers or motors that do not fit either motility category as SMs. Here, rower motors have low or no processivity, or ability to take several steps along its track without dissociating, which would make trapping measurements using the bead with bound motor geometry challenging at best. However, the concept of a motor as processive and nonprocessive should not necessarily be a rigid classification either. Some motors, such as mitotic kinesin-5, kinesin-12, and kinesin-14, exhibit hybrid motility properties. Kinesin-5 and kinesin-14 have been previously classified as nonprocessive at the SM level but are able to slide MTs within the spindle(Crevel, Lockhart, & Cross, 1997; DeCastro, Ho, & Stewart, 1999; Foster & Gilbert, 2000); yet, additional studies have demonstrated that single Eg5 and HSET motors can take multiple steps along a MT before dissociating.(Reinemann et al., 2018; Shimamoto, Firth, & Kapoor, 2015; Valentine, Fordyce, Krzyziak, Gilber, & Block, 2006) Those motility properties, including maximum generated force, attachment time, and stepping coordination, change even further when working in motor teams.(Furuta et al., 2013; Furuta & Toyoshima, 2008; Reinemann et al., 2018, 2017; Shimamoto et al., 2015) Therefore, processivity should be viewed as a spectrum, where classical porters would be on the high end, and classical rowers would be on the low end. Understanding where the motor may lie on that spectrum will aid in justifying an analytical technique or assay geometry that will thus provide informative behavioral and mechanistic data.

To combat this using OT, one approach is to bind multiple motors to a bead to interrogate whether SM versus team motility differs (Figure 1c). Motors can be bound nonspecifically using a high concentration of motor during an incubation step. Recently, more defined geometries have been implemented using nanotools like DNA origami to bind motors at user-defined intervals to investigate how this spacing affects team motility (Furuta et al., 2013). On the other hand, in order to study rower motors at the SM level, a seminal study by Finer et al. (1994) employed OT using a different assay orientation. Muscle myosin II is a classic example of a rower motor protein that works in teams to contract actin filaments (AFs) within a sarcomere but is non-processive at the SM level. Myosin II detaches from the AF after every stroke, making the conventional motor-bound bead OT assay approach ineffective. Therefore, Finer and co-works developed a new experimental geometry where a “three-bead assay” or “dumbbell assay” was utilized (Figure 1d; Finer et al., 1994). Here, two laser traps are used to suspend an AF over myosin motors sparsely distributed on a bead stuck to the coverslip surface. By immobilizing the non-processive motor on a bead, myosin will not have the opportunity to diffuse away after it has completed its power stroke, and the AF will still be within crossbridge-forming range for the next ATP cycle. Force generation by the motor is then recorded through displacements of the AF within the trap. Discrete power strokes by myosin were measured to produce around 11 nm of movement and 3–4 pN of force per ATP hydrolysis.(Finer et al., 1994).

Variations on the three-bead SM experiment have been extended to other molecular motors, including nonconventional kinesins, in order to accommodate their lack of traditional processivity (Duke, 1999; Kitamura et al., 1999; Mehta et al., 1999; Molloy, Burns, Kendrick-Jones, et al., 1995; Molloy, Burns, Sparrow, et al., 1995; Takagi et al., 2006; M. J. Tyska et al., 2000; M J Tyska et al., 1999; Veigel et al., 2003). However, regardless of orientation, most OT motor investigations to date involve a single motor interacting with a
single filament. While these studies have revolutionized our understanding of molecular motor mechanics, the reductionist single motor/single filament geometry does not necessarily recapitulate the architecture or complexity of the in vivo environment in which the motor functions. For example, mitotic kinesins work together as multi-motor teams between two MTs within the bipolar spindle. A prevalent challenge for biophysicists is reconstituting a motor-filament environment that reflects physiological function by having enough systematic components to obtain specific and meaningful data but not so many that the system is too complicated to study as one entity (Dogterom & Koenderink, 2019; Elting & Spudich, 2012). To combat this, other in vitro assay orientations, involving OT and not, have been explored. Assay alterations include adding multiple motors, multiple filaments, or a combination of both.

2.2 Multi-motor assays

An example of incorporating multiple motors in vitro is the “gliding filament” assay, which is a multi-motor variant of the Kuo and Sheetz study mentioned earlier. Here, multiple motors are bound to a coverslip surface, and addition of filaments in the presence of ATP results in their sliding, akin to crowd surfing at a concert. This orientation for kinesin and MTs has been probed by both total internal reflection fluorescence (TIRF) microscopy (standard practice in many motor studies) and OT assays (Fallesen, Roostalu, Duellberg, Pruessner, & Surrey, 2017; Reinemann et al., 2017), where the trapped bead is attached to the gliding filament, and force generation is measured (Figure 1e). In addition, multiple filaments have been assembled in the form of three-dimensional microtubule intersections using holographic OT (Figure 1f) and bundles, or filament-motor-filament “sandwiches,” using conventional OT and TIRF microscopy (Figure 1g; Bergman, Ousubayo, & Vershinin, 2015; Bergman et al., 2018). In the latter case, through staged introduction and incubation steps, filaments are adhered to the coverslip surface (the substrate filament), and subsequently, motors bind the substrate filament and crosslink the top or cargo filament. In the context of kinesin and MTs, fluorescence bundle assays have been assembled and investigated to understand the motility and crosslinking effects of both mitotic kinesins and MAPs (Braun, Drummond, Cross, & Mcainsh, 2009; Braun et al., 2011, 2017; Britto et al., 2016; Dogterom & Surrey, 2013; Drechsler & Mcainsh, 2015; Drechsler, McHugh, Singleton, Carter, & McAinsh, 2014; Fink et al., 2009; Forth & Kapoor, 2017; Furuta et al., 2013; Gerson-Gurwitz et al., 2011; Gicking, Qiu, & Hancock, 2018; Henrich & Surrey, 2010; Hepperla et al., 2014; Kapitein, Janson, et al., 2008; Kapitein, Kwok, et al., 2008; Kapitein et al., 2005; Molodtsov et al., 2016; Popchock et al., 2017; Reinemann et al., 2017; Roos, Ulmer, Gräter, Surrey, & Spatz, 2005; Roostalu et al., 2011; Sturgill et al., 2014; Sturgill, Norris, Guo, & Ohi, 2016; Su et al., 2013; Subramanian et al., 2010; Tanenbaum, Vale, & McKenney, 2013; Tao et al., 2006; van den Wildenberg et al., 2008; Weinger, Qiu, & Yang, 2011; Wijeratne & Subramanian, 2018). These TIRF assays have revealed important insight into properties of motor ensembles, such as bundling propensity, velocity, and effects of filament architecture, such as bundle polarity. In complex environments like the mitotic spindle, microtubules are in both parallel and anti-parallel orientations, and activity in such environments reveals unique location-specific roles that motors play to build the spindle and balance forces. Behaviors of kinesin-MT bundles have also been investigated computationally, especially to shed light on motor cooperativity that accomplishes force generation and thus cargo movement (Blackwell et al., 2017; Chowdhury & Ghanti, 2020; Edelmaier et al., 2020; Kapoor, Hirst, Hentschel, Preibisch, & Reber, 2019; Lera-Ramirez & Nédélec, 2019; Prelogovic, Winters, Milas, Tolic, & Pavin, 2019; Sherin, Farwa, Sohail, Li, & Bég, 2018; Uçar & Lipowsky, 2019; Winters et al., 2019; Zemel & Mogilner, 2009; Ziebert, Vershinin, Gross, & Aranson, 2009). Using this approach, theorists and experimentalists are able to work together to reconcile to what degree motors are synergistically coupled and how their structural features facilitate the experimental results obtained.

Experiments that incorporate both MT architectural hierarchy and OT have unveiled new assay approaches and mechanistic information about kinesin and MAP ensembles (Figure 1h; Bodrug et al., 2020; Fallesen et al., 2017; Forth, Hsia, Shimamoto, & Kapoor, 2014; Gaska, Armstrong, Alfieri, & Forth, 2020; Laan, Hsson, Munteanu, Kerssemakers, & Dogterom, 2008; Lansky et al., 2015; Lüdecke, Seidel, Braun, & Diez, 2018; Reinemann et al., 2018, 2017; Shimamoto et al., 2015; Shimamoto & Kapoor, 2018). These experiments push the boundaries of the motor biophysics field by combining OT, which possesses the precision and resolution to study single motor mechanics, with a more physiologically relevant environment for motors that work in complex environments, like the mitotic spindle, and may not necessarily fit entirely in the “porter” or “rower” categories. Further, it is important to understand the construction of multi-motor assay geometry in order to be able to analyze the force generation of such geometries, the number of interacting motors, and how the force propagates throughout the system. This multi-motor, multi-filament approach allows for custom-building the local cytoskeletal environment through including staged introduction and timely incubations, polarity marking, fluorescence protein labeling, probing protein ensembles of various sizes and composition, and incorporating multiple types of kinesins and MAPs. Here, we review such studies, analyze the techniques utilized to construct each OT-bundle assay, and what mechanistic information can be extracted in each case.

3 PROBING MICROTUBULE BUNDLE FORCES IN MICROFABRICATED DEVICES

MTs can generate pushing and pulling forces without the assistance of motors or MAPs due to their constant switching between growing and shrinking states, referred to as dynamic instability (Mitchison & Kirschner, 1984). Individual MTs have been analyzed in vitro to evaluate how force affects assembly dynamics, where growth velocity decreases and catastrophe rate increases as the force on the end of the MT increases (Dogterom & Yurke, 1997; Janson, De Dood,
Dogterom, 2003). However, MTs do not operate in isolation in vivo. Specifically, during mitosis, multi-filament bundles of kinetochore MTs are formed that are parallel in orientation and bind chromosomes (Walczak & Heald, 2008). Here, parallel refers to alignment of the MTs’ plus and minus ends. As the chromosomes are initially bound and then pulled toward the spindle poles during anaphase, the MT bundles experience pushing and pulling forces, respectively, where dynamic instability is thought to contribute. Therefore, Laan et al. (2008) asked how much force a growing bundle of parallel MTs can generate. The authors used OT to track the dynamics of a MT bundle, where growing MTs polymerize tubulin and push against a microfabricated, rigid barrier (Figure 2a).

The authors used a time-shared optical tweezers set up that allowed them to measure the forces and dynamics of MTs growing from an axoneme, which is naturally polarity aligned, against a wall. (Laan et al., 2008) The time-shared “keyhole” trap allows one strong trap, or point trap, to hold the bead while multiple shallow traps form a line trap to limit the axoneme’s movement. To perform the force measurements, a flow system was created that included microfabricated chambers made from clean coverslips using SU-8 negative tone photoresist to produce a 7 μm thick layer. After steps of baking and UV exposure, 7 μm high chambers of 40 x 80 μm separated by 20 μm wide walls were formed. Once incorporated into the flow system, a series of blocking steps were performed with agarose and BSA solutions. Then, axonemes and beads were added to the flow system, where a bead was trapped with the point trap, and an axoneme was captured with a line trap and stuck to the bead. The axoneme bound bead was then positioned close or pressed against one of the fabricated walls to keep the MTs short and prevent them from buckling (Figure 2b). Tubulin was added to the flow system to initiate MT growth, and force measurements by the bundle were measured by the trapped bead.

Using this approach, Laan et al. (2008) measured force generated by the growing MT bundles and did so under a variety of nucleotide (GTP vs. GMPCPP) and tubulin concentration (10 and 25 μM) conditions (Figure 2c–e). In Figure 2c, force is measured as tubulin is incorporated into the MT bundles with a tubulin concentration of 25 μM in the presence of GTP. They were able to distinguish between plus and

![FIGURE 2](image-url)  
**FIGURE 2** Force generation by growing MT bundles. (a) Assay setup of a MT bundle growing from an axoneme against a microfabricated wall. The axoneme bound bead is trapped in the strong point trap, and the time-shared line trap holds the MT bundle in place. (b) Scanning electron micrograph of a microchamber that is used as a rigid barrier. A schematic of the trapped bead and bundle is overlaid. (c) Force generation as a function of time of growing MTs in the axoneme bundle at a tubulin concentration of 25 μM in the presence of GTP. (d) Force traces of the growing MT bundle at a tubulin concentration of 10 μM. (e) Force traces at a tubulin concentration of 10 μM but in the presence of GMPCPP. Adapted from Laan et al. (2008), PNAS. Copyright 2008 National Academy of Sciences
minus end growth due to plus ends growing faster and experiencing catastrophes. Traces were obtained from the polymerizing MTs pushing against the microfabricated wall and revealed that growing parallel MT bundle force generation is a linear addition of single MT maximum forces. As shown in Figure 2c, the authors observed the force generated by a single MT polymerizing and correlated that force to the maximum force generated when all of the MTs in the bundle were polymerizing together. As bundle force rate depends on polymerization rate, lowering the free tubulin concentration to 10 μM resulted in catastrophes occurring before maximum forces were reached (Figure 2d). Using GMPCPP instead of GTP to inhibit catastrophe restored the linear addition of individual MT forces (Figure 2e). Experiments in this paper were limited to a smaller than maximum force range due to the bundle construct getting stuck to the chamber wall. However, with further optimization, this approach could be utilized in conjunction with motor ensembles, such as dynein, that drive axoneme movement in systems like cilia and flagella, as well as using the time-shared OT to control larger and more extended synthetic MT-kinesin systems.

4 | MEASURING MAP MECHANICS IN MICROTUBULE BUNDLES

During the process of cell division, the cytoskeleton goes through many conformational changes that require mechanical force to accomplish the large rearrangements necessary for each phase, including formation of the mitotic spindle (Walczak & Heald, 2008; Wordeman, 2010). There are two main categories of forces that contribute here, termed active and passive forces by Forth and Kapoor (2017). Active force is generated, in part, by assembly and disassembly mechanics of MTs with the aid of motor proteins and is essential for transporting cargos inside the cell during division, in addition to facilitating MT sliding to separate centrosomes and segregate chromosomes to form two daughter cells. For example, calculations based on the size and speed of chromosomes moving through a viscous environment suggest that it would require only approximately 0.1 pN to move chromosomes, but Nicklas showed that the spindle machinery can generate up to 700 pN before chromosome motion stalled (Forth & Kapoor, 2017; Nicklas, 1983, 1988). The active forces generated by spindle machinery and motor proteins need to be balanced and overcome by an opposite force, or passive forces, such as elastic forces, frictional resistance, and viscous drag (Forth & Kapoor, 2017). Crosslinkers are nonenzymatic molecules that diffuse between or statically connect two cytoskeletal filaments. They are capable of generating frictional resistance against active forces and help maintain the structural integrity of higher order cytoskeletal assemblies. Thus, crosslinkers are critical in describing the mechanics of MT networks as their binding can disrupt MT sliding and therefore generate resistive forces that control the direction and magnitude of sliding (Lansky et al., 2015).

Forth et al. investigated mitotic MAPs EB1, PRC1, and NuMA using a combined OT and TIRF approach to understand how active forces influence MAP behavior (Forth et al., 2014). OT were used to pull each MAP along the MT lattice in a traditional SM assay orientation. PRC1-MT binding was found to be symmetric under load with respect to filament polarity, while NuMA and EB1 MT binding were asymmetric (Forth et al., 2014). In order to test whether frictional asymmetry can lead to directional movement in MT networks, the authors needed to analyze NuMA-MT interactions in MT “sandwiches” or “sandwiches” rather than just as a single MAP acting on a single MT. To accomplish this, the authors developed a dimerized construct NuMA-Bonsai-Tail-GFP II that was capable of crosslinking MTs while retaining the frictional asymmetry found as a monomer (Figure 3a). The directionally-dependent behavior of dimerized NuMA-Bonsai-tail II-GFP in MT bundles were analyzed using optical tweezers by applying load on the polarity-marked MT system. A bead bound with rigor kinesin was attached to the end of the top, free MT, and high trap stiffness was used (0.2 pN/nm) to trap the bead while the stage and thus bottom MT underwent continuous sinusoidal oscillation (Figure 3a). By combining OT with TIRF, the authors were able to observe the movement of NuMA-Bonsai-tail II-GFP toward MT minus ends when in a parallel configuration only, and anti-parallel sandwiches did not facilitate MAP clustering (Figure 3a). Moreover, the magnitude of the oscillation was proportional to the speed of NuMA motion in parallel microtubule bundles (Forth et al., 2014). Therefore, using the combined microtubule bundle OT assay, the authors were able to determine that different nonmotor MAPs exhibit unique frictional resistance based on hierarchical MT geometry and force application.

In a similar assay setup, Lansky et al. (2015) demonstrated the role of Ase1, the S. pombe analog of PRC1, in generating directed mechanical forces as diffusible microtubule crosslinkers confined between two partially overlapping microtubules in vitro (Figure 3b). Experimental work was performed by first preparing MT overlaps by immobilizing dimly-rhodamine-labeled MTs on a coverslip termed the “template” MT. Next, Ase1 with a GFP tag was added and incubated to allow binding to the immobilized MTs. Bright rhodamine-labeled “transport” microtubules were flushed into the flow cell where immobilized template microtubules were bound to Ase1, also washing out any unbound Ase1 in solution. Hydrodynamic flow of assay buffer was then performed on the flow cell to allow the sliding of transport microtubule along the template MTs. TIRF was used to visualize the entropic expansion caused by friction forces generated by Ase1 and to determine how it would affect the sliding of partially overlapping MTs. Results from TIRF imaging revealed that the crosslinkers do not leave the regions they were initially bound to before the movement of the MTs. This is due to the high affinity of crosslinkers for the overlapping MTs. Upon MT sliding, Ase1 crosslinkers became more confined in the overlaps, and when induced sliding from hydrodynamic flow ceased, Ase1 expansion caused directed MT sliding. Further, Ase1 crosslinkers distributed themselves evenly within the overlaps (Lansky et al., 2015).

Optical tweezers were used to measure the force generated by Ase1 crosslinkers due to this “entropic expansion” (Figure 3b; Lansky et al., 2015). Template and transport MT overlaps were assembled in...
a similar manner, except the transport MT was biotinylated and a NeutrAvidin bead was added in the last Ase1-absent flow step. The bead was trapped and attached to the transport MT. A piezo translation stage moved the template MT in the same axis with respect to the transport MT held in a fixed position by the trap to compact the Ase1 proteins. After each stage step, the system was allowed to equilibrate before measuring force. Results showed that the entropic expansion of Ase1 generated forces that are in the pico-newton range, which suggests that those forces are on the same scale as forces generated by molecular motors and could be of importance for balancing forces inside the cell. Kinesin-14 molecular motors were employed with partially overlapping MTs to prove this finding. The results showed that with the addition of Ase1, the direction of MT sliding caused by the kinesin-14 motor molecule was reversed. Through using the bundled MT assay with the optical trap and piezo stage, the authors were able to demonstrate that the force generated by nonmotor Ase1 is on the same scale as the force generated by mitotic motors, better putting these crosslinkers into context within spindle force balance (Lansky et al., 2015).

PRC1, like Ase1, has a high affinity to crosslink antiparallel microtubules. Gaska et al. (2020) investigated PRC1 mechanics in MT bundles, utilizing optical tweezers and TIRF microscopy to control MT sliding motions, quantify resistive forces generated by PRC1 crosslinkers, and observe PRC1 distribution within the overlaps simultaneously (Figure 3c). First, PRC1-mediated MT bundles with various overlap lengths and concentrations of PRC1 molecules in the overlap were made by immobilizing biotinylated MTs containing HiLyte-647 on a passivated coverslip by neutravidin. Next, GFP-PRC1 and rhodamine-labeled MTs were added into the sample chamber to form a MT bundle. For OT measurements, beads were coated with truncated kinesin-1 and introduced to bind the top rhodamine-labeled microtubule. MT sliding was performed to induce force on the PRC1 bundle ensemble by trapping the bead bound to the top MT and moving the sample stage at a fixed velocity parallel to the microtubule bundle axis to control filament separation. TIRF was used to image each of the moving and the trapped microtubules and PRC1. Results revealed that PRC1-crosslinked microtubules pairs generate passive force specifically as viscous resistance that acts like a mechanical dashpot during MT sliding (Figure 3c). This viscous force showed linear dependence on MT sliding velocity and number of PRC1 crosslinkers molecules (Gaska et al., 2020). On the other hand, no change of viscous force with MT overlap length nor density of PRC1 was observed. Overall, these studies performed on nonmotor MT crosslinkers demonstrated how force generating behavior of these molecules deviates with respect to filament polarity and sliding, and these behaviors are well-captured using the MT bundle OT assay. These results have strong implications for the roles MAPs play in balancing forces and maintaining structural integrity within the MT cytoskeleton, revealing that they have unique properties outside of static crosslinking.

**FIGURE 3** Nonmotor MAPs generate force in MT bundles. (a) Assembled polarity-marked MT bundles with NuMA “sandwiched” between were probed using OT. Upon oscillating the stage, NuMA moved toward MT minus ends in parallel MTs while showing no transport preference in anti-parallel MT bundles. Adapted from Forth et al. (2014), Cell. (b) OT is used to probe the entropic forces of nonmotor MAP Ase-1 within MT overlaps. As Ase-1 is able to diffuse within the overlap, entropic expansion facilitates MT sliding, generating forces in the pN range. Adapted from Lansky et al. (2015), Cell. (c) Sliding of MTs crosslinked by mitotic PRC1 results in resistive forces and sliding within MT overlaps. Adapted from Gaska et al. (2020), Developmental Cell. Reproduced with permission from Elsevier under licenses 4958850791490, 4958850073143, and 4958850683308, respectively.
5 FORCE GENERATION BY MITOTIC KINESIN TEAMS IN MICROTUBULE BUNDLES

5.1 Kinesin-5

Molecular motors play an important role in regulating the mitotic spindle by generating pulling and pushing forces that control the sliding motion of adjacent MTs and therefore yield the proper bipolar structure for faithful segregation of chromosomes (Wordeman, 2010). One of the main contributors in the assembly of MT-based metaphase spindle is kinesin-5 (Mann & Wadsworth, 2019; Sawin, LeGuellec, Philippe, & Mitchison, 1992). Kinesin-5 is a conserved homotetrameric motor protein that has two motor domains at each end of a central stalk, giving kinesin-5 the ability to crosslink and slide two MTs (Kapitein et al., 2005). When kinesin-5 crosslinks antiparallel overlapped MTs, the stepping of kinesin-5 toward the plus ends of each MT causes them to push apart (Kapitein, Kwok, et al., 2008; Kapitein et al., 2005). On the other hand, when in parallel MT geometries, kinesin-5 is able to act as a brake, regulating the speed of sliding (Shimamoto et al., 2015). Kinesin-5 has also been classified to have both processive and nonprocessive qualities, not allowing it fit well in either the porter or rower motility categories (Crevel et al., 1997; Valentine et al., 2006). Therefore, to determine how kinesin-5 facilitates differing force generating mechanisms within MT overlaps, Shimamoto et al. analyzed kinesin-5 ensembles in crosslinked, overlapping MTs using OT combined with fluorescence (Figure 4a). To understand the capabilities of this assay setup, we will review the experimental methods used to construct each experiment. The assay

![Figure 4](image-url)
setup, termed a mini-spindle, was employed to analyze force generation by kinesin-5 crosslinking overlapping MTs (Shimamoto et al., 2015; Shimamoto & Kapoor, 2018). Force measurements were carried out using optical tweezers which controlled filament orientation, sliding speed, and overlap length to measure force generation. Nonbiotinylated and biotinylated MTs were assembled onto a glass coverslip which was precoated by polyethylene glycol to prevent subsequent nonspecific binding. The assay was made as a sandwich where the MTs were prepared on top of each other to mimic a bundled shape. Recombinant GFP-tagged full-length Xenopus kinesin-5 motor was used to crosslink the overlapped MTs. TIRF was employed to detect the regions of the overlap and kinesin-5 location, while at the same time, the optical trap controlled the overlap length and sliding speed and measured the force generated with changing these factors. A trapped bead was attached to the free end of the nonbiotinylated MT to measure sliding forces (Figure 4a). As kinesin-5 mediated bundles would preferentially form and move within the anti-parallel orientation, in order to get measurements in parallel MT bundles, the trapped bead attached to the top MT of a moving bundle was flipped around to the opposite side in order form a parallel MT bundle (Shimamoto et al., 2015; Shimamoto & Kapoor, 2018). Results revealed that a group of kinesin-5s acting between two antiparallel MTs can push those MTs apart by generating force that scales with the overlap length and number of motors (Figure 4a; Shimamoto et al., 2015). The traces have an initial force ramp that is followed by a plateau that is achieved either through crowding, maximum force generated by the system, or both, and this maximum force decreases as the known plus-end-directed motility mechanism. In doing so, force spectroscopy was used to measure both the plus and minus end forces that ensembles of purified budding yeast kinesin-5 Cin8 produce in microtubule gliding assays (Fallesen et al., 2017). Further analysis revealed that the tail has a powerful role in down-regulating force generation with wild type kinesin-5 (Figure 4a). Further analysis revealed that the tail domain of kinesin-5 acts as a “brake-like” resistance against relative microtubule sliding in both parallel and anti-parallel orientations. This behavior was observed when the template MT was moved with the piezostage at low speed (20 nm/s), and the magnitude of the generated pushing force that assisted MT sliding was proportional with the overlap length. On the other hand, faster relative sliding velocities (200 nm/s) opposed filament sliding. Thus, these kinesin-5 ensemble measurements between two MTs using OT illuminated how when working concertedly, the motors can read local structural and concentration cues that result in a specific mechanical output, which has important implications for force regulation within the mitotic spindle (Shimamoto et al., 2015).

Kinesin-5 motors were originally described as unidirectional plus-end-directed motors, whether as SMs or in ensembles (Fallesen et al., 2017). Kinesin-5 family members from budding yeast (Cin8 and Kip1) and fission yeast (Cut7) have since been found to have the ability to move toward the MT plus and minus ends (Singh, Pandey, Al-Bassam, & Gheber, 2018). Fallesen et al. (2017) elucidate whether the mechanism of minus-end-directed motility in kinesin-5 is similar to that of the known plus-end-directed motility mechanism. In doing so, force spectroscopy was used to measure both the plus and minus end forces that ensembles of purified budding yeast kinesin-5 Cin8 produce in microtubule gliding assays (Fallesen et al., 2017).

A combined optical tweezers and microtubule gliding assay (Figure 4b) was assembled by first adding motility buffer into a prepared flow chamber (Fallesen et al., 2017). After Cin8-mGFP addition and incubation, polarity-marked biotinylated microtubules were then added to the flow cell followed by an incubation and wash step. Finally, a dilute streptavidin bead was added to act as the optical handle on the gliding MT. The force spectroscopy experiments were conducted by capturing a calibrated streptavidin-coated bead and moving it into contact with the lagging end of a biotinylated polarity-marked MT so that the motors pull the bead out of the trap. Fluorescence imaging was used to determine the gliding direction of the MT. OT measurements demonstrated that the bidirectional kinesin-5 Cin8 of budding yeast is capable of producing piconewton forces that are similar in magnitude in both directions of movement. These results are similar to assay performed with Eg5 in Figure 4a minus the ability to generate force toward the MT minus-end direction. Thus, minus-end directed motion may follow a conventional mechanism similar to that of plus-end directed motion. Also, Cin8 force production was shown to have a linear relationship with motor number, as was also found with Eg5, which indicates that additive force production is a conserved property of kinesin-5 motors (Fallesen et al., Shimamoto et al., 2015). This result could imply a functional requirement for spindle assembly and elongation during cell division. However, this linearity, as well as the magnitude of SM force produced by Cin8 and Eg5, are conserved properties of kinesin 5-motors but are not seen in all members of the kinesin family.

Moreover, each kinesin-5 consists of twin tail and twin motor domains originating from two sets of antiparallel folded dimeric subunits that emerge in close proximity at each end of the bipolar homotetramer (Bodrug et al., 2020). In order to investigate the dependence of this tail domain on kinesin-5 ensemble force production, Bodrug et al. employed a similar experimental setup to Figure 4a where force generated by kinesin-5s without their tail domains is measured in motor ensembles between two MTs (Figure 4c; Bodrug et al., 2020; Shimamoto et al., 2015) MT-kinesin-5-MT sandwiches or bundles were formed in vitro, and force measurements by the motor-MT system were obtained through the top MT bound to an optically trapped bead. Upon removal of the kinesin-5 tail domain, MT sliding events generated weak forces (Figure 4c) compared to the linear dependence of motor number on force generation with wild type kinesin-5 (Figure 4a). Further analysis revealed that the tail has a powerful role in down-regulating microtubule-activated ATP hydrolysis by assisting the capture of the nucleotide-free or ADP state. Also, the tail’s presence helped stabilize a unique conformation of the motor N-terminal subdomain by facilitating the opening of its active site. Full-length kinesin-5 motors demonstrated relatively slow motility, likely to help maintain the spindle’s integrity and avoid rupture of microtubules during sliding. Kinesin-5 motors also cluster together along the MT tracks, whereas tail-less motors exhibited high speed motility and no clustering. All together, these findings revealed that the tail domain of kinesin-5 has a significant impact on kinesin-5 ensemble mechanics within anti-parallel MT overlaps through tuning the motor’s mechanochemical cycle, which in turn regulates assembly and force balance within the spindle (Bodrug et al., 2020).
5.2 | Kinesin-12

The kinesin-12 Kif15 acts as a redundant back-up mechanism for centrosome separation during spindle assembly when Eg5 has been inhibited by drugs (Reinemann et al., 2017; Sturgill et al., 2014, 2016; Sturgill & Ohi, 2013). However, Kif15 and Eg5 have different structures, where Kif15 contains motor heads, a second nonmotor MT binding site, and an inhibitory tail, and Kif15 has a smaller SM stall force than Eg5 (Reinemann et al., 2017; Sturgill et al., 2014, 2016; Sturgill & Ohi, 2013). These disparities then beg the question of how Kif15 is able to efficiently rescue spindle assembly. To answer this, Reinemann et al. devised an OT assay to investigate Kif15 as an ensemble of motors between two MTs, as they would function physiologically in the spindle (Reinemann et al., 2017). The assay was assembled using polarity marked MTs to determine MT bundle orientation. As Kif15 is plus end directed, contains a second MT binding site, as has the ability to change locations from parallel-oriented kinetochore MTs to anti-parallel interpolar MTs upon Eg5 inhibition, they hypothesized that force generation mechanisms would differ in these MT geometries (Reinemann et al., 2017). The bottom or template MT was polarity marked using fluorescently labeled tubulin, where GMPCPP seeds were brightly fluorescent and indicated the minus end, and dimmer elongations from polymerization marked the plus end (Figure 4d, top). The top or cargo MT was polarity marked using biotinylated GMPCPP seeds for the minus end and nonlabeled elongations for the plus end. Streptavidin beads were utilized to facilitate only binding the minus end of the cargo MT. Therefore, by knowing the location of the trapped streptavidin bead and visualizing the relative location of the bright minus end seed of the template MT using fluorescence, the bundle was identified as parallel or anti-parallel in orientation (Reinemann et al., 2017).

The MT bundle assay was assembled from the bottom up. First, template polarity marked MTs were added to a flow cell, incubated, and washed with casein to prevent nonspecific binding of motor. A combination of Kif15, biotin-marked cargo MTs, and streptavidin beads were then added to facilitate bundling of MTs by Kif15. A bead would then be trapped in solution, calibrated, and lowered to a MT bundle identified by differential interference contrast (DIC) imaging on the surface. Upon binding the bundle, polarity orientation was verified using fluorescence and the relative location of the trapped bead. Force generated by the Kif15 ensemble was then measured via displacement of the bead attached to the cargo MT from the trap center. The authors determined that Kif15 slides antiparallel MTs apart while parallel bundles remain stationary. This is reflective of the location-specific roles that Kif15 plays in the spindle: static MT crosslinker/force regulator under physiological conditions (parallel kinetochore MTs) and active MT slider under drugged conditions (anti-parallel interpolar MTs; Reinemann et al., 2017).

The authors wanted to further dive into the ensemble behavior of Kif15. As demonstrated by Shimamoto et al. (2015), Eg5 has an additive, linear dependence of force generation with respect to motor concentration. To determine if this was the case for Kif15, Reinemann et al. (2017) adapted a combined MT gliding/OT assay for both Eg5 and Kif15 where motor is bound to the coverslip surface and glides a biotinylated MT for attachment of a streptavidin bead. After a bead was trapped in solution, it was lowered to the flow cell surface to a gliding MT. In the case of Eg5, their results corroborated those from Shimamoto et al., indicating that as the concentration of Eg5 increased, the force generated increased accordingly. However, at even higher surface concentrations than used for Eg5, ensembles of Kif15 would not generate force above that of the SM stall force, indicating a level of force feedback within these motor-filament systems (Figure 4d, bottom). Therefore, the modularity of the MT bundle OT assay allowed the authors to determine how Kif15 motors function in different hierarchical MT environments, as they would exist within the spindle, but also allowed for comparison to its relevant partner Eg5 that it supports as a mitotic backup to differentiate how spindle assembly might occur under physiological versus chemotherapy drugged conditions.

5.3 | Kinesin-14

The kinesin-14 family also aids in regulating force within the spindle, with the distinction that kinesin-14s move toward the minus-end of MTs (Fink et al., 2009; Furuta & Toyoshima, 2008; Mountain et al., 1999; Norris et al., 2018; Pechatnikova & Taylor, 1999). Kinesin-14 motors, such as Ncd, each interact with crosslinked MTs in two ways: through the low-processive motor domains and diffusive tail domains. The tail domains' influence on the protein's performance is not yet understood. Therefore, Lüdecke et al. (2018) determine whether velocity and force produced by Ncd is governed by the tail domain-mediated diffusive anchorage of the motors to MTs. Their approach was to use a kinesin-MT bundle assay combined with OT (Figure 4e), very similar to the assay constructions previously discussed for kinesin-5 and kinesin-12. To assemble the bundle assay, anti-SNAP antibodies were bound to diphenyl(dimethoxysilane) (DDS)-functionalized coverslips in a flow cell. Using Pluronic F127, the surface was then passivated before Ncd tail motors in ATP buffer were injected into the chamber. Finally, the chamber was washed, and MTs were added. For experiments using crosslinked microtubules, anti-digoxigenin antibodies were first allowed to bind to the DDS-functionalized coverslips before the surfaces were blocked with Pluronic F127. Digoxigenated dimly-cy5-labeled microtubules were then added to the chamber and allowed to bind to the surface-immobilized antibodies. Next, Ncd in ADP buffer was added and allowed to bind MTs, followed by biotinylated, brightly-cy5-labeled MTs were then added to the chamber to form microtubule pairs. Finally, the buffer was replaced with ATP buffer. For the optical tweezer experiments, the ATP buffer was supplemented with NeutrAvidin-functionalized silica microspheres (Lüdecke et al., 2018).

For force measurements of MTs driven by diffusively anchored Ncd motors crosslinked to surface-immobilized MTs, a trapped and calibrated microsphere was lowered directly on top of the moving MT. Bead movement relative to the surface-immobilized template MT was used to measure the force-velocity relationship by setting a
constant force and monitoring the velocity or vice versa. The study showed that statically anchored Ncd motors transport microtubules faster and with greater force than diffusibly anchored Ncd motors, suggesting that anchoring of the tail domain regulates the force transmission between microtubules in motion. (Lüdecke et al., 2018) Thus, the inefficient force production of kinesin-14 may be due to tail diffusion on the MT surface, and the role of kinesin-14 in sliding MTs may be due to both sliding nucleated microtubules that are otherwise unattached to the spindle poles while also crosslinking antiparallel microtubules.

However, kinesin-14s typically do not work alone within the spindle (Norris et al., 2018; Reinemann et al., 2018). Kinesin-14 has been proposed to oppose kinesin-5 and aid in maintaining force balance during division, but how these motors are able to accomplish this task is not clear (Fink et al., 2009; Gicking et al., 2018; Hepperla et al., 2014; Norris et al., 2018; Peterman & Scholey, 2009). Kinesin-14 Ncd has been reported to be nonprocessive, and as demonstrated by Ludecke et al. and others, relies, at least in part, by the tail’s diffusion (Braun et al., 2017; Lüdecke et al., 2018). On the other hand, kinesin-5 generates a 5 nM stall force and exhibits a level of processivity (Valentine et al., 2006). Therefore, how a seemingly substantially weaker motor like kinesin-14 effectively opposes and resists force generated by kinesin-5 was not well understood. Ensembles of kinesin-14s on beads have been shown to produce processive motility and substantial force generation, which is markedly different from their SM properties (Furuta et al., 2013; Norris et al., 2018). However, how these motors work together between two MTs to resist kinesin-5 is not clear.

Reinemann et al. (2018) examine this question using the bundled MT assay and incorporate a mixed motor ensemble of kinesin-14 HSET and kinesin-5 Eg5 (Figure 4f). Bundles were assembled similarly to the Kif15 study except varying concentration ratios of Eg5 to HSET were introduced. Interestingly, even though Eg5 is notably stronger than HSET, HSET seems to dictate bundle force and velocity, serving as a force brake against Eg5 in bundles. Additionally, by noting the polarity of the bundle and the direction of cargo MT transport, the authors observed that when in bundles with Eg5, HSET's directionality is compliant, adopting the overall plus end directional movement facilitated by Eg5 while still resisting overall force production. These unique characteristics that were discovered using the multi-motor, multi-filament OT approach provide insight as to why kinesin-14 overexpression causes spindle elongation as oppose to collapse and how HSET adopts specific properties in varied conditions: weak force production and motility as SMs, very processive in small groups, and resistive in mixed motor ensembles. (Furuta et al., 2013; Norris et al., 2018; Reinemann et al., 2018).

### Outlook

Here, we review novel approaches of incorporating microtubule structural hierarchy, MAPs, and motors into OT assays in order to determine ensemble properties of motors that may not necessary fall in the porter or rower motility categories. We included many of the experimental details to convey how these assays are currently constructed and demonstrate that understanding assay geometry has implications for interpreting mechanistic results. By incorporating MT hierarchy in OT experiments, motor biophysicists are able to extract information regarding ensemble force generation dynamics of kinesins and MAPs that would otherwise be difficult to investigate using conventional methods. Using this approach, the influence of cooperativity and communication between motors or MAPs themselves, as well as through the motor-filament connection, on ensemble performance can be captured as opposed to having motors spaced out on a rigid coverslip or bead surface where that communication is likely dampened. Further, the influence of changes made at the molecular level on cytoskeletal system synergy are directly measured, and the modular nature of the assays opens exciting doors for the field. By taking advantage of the fact that individual cytoskeleton proteins and filaments are routinely isolated and/or polymerized on the bench or purchased from commercial sources, we can treat these elements like LEGOs, custom-building cytoskeletal architectures in a stepwise manner and interchanging the “building blocks” as needed, with the goal of ultimately understanding how these alterations propagate up to system level force generation.

While innovative, these approaches have room for expansion and increased complexity. The studies reviewed here are confined to the surface of the flow cell's coverslip, immobilizing one of the bundled MTs and thus lessening the potential dynamic influence from the bottom half of the system. These assays are also the foundation for approaching higher levels of complexity in in vitro reconstitution. Mixed motor and crosslinker assays would be beneficial in elucidating the force balance in higher order structures like the mitotic spindle, including the MAPs and kinesins discussed in this review. However, structural hierarchy also is not confined to the MT cytoskeleton. Actin assemblies also have critical roles in essential processes like muscle contraction, cell division, and cell motility. (Huxley, 2004; Mandato, Benink, & Bement, 2000) Further, traditionally, the MT and actin cytoskeleton have been evaluated separately in in vitro assays. Yet, it is becoming clear that actin-MT crosstalk should be evaluated more ardent with increasing evidence of direct, coordinated relationships, and OT cytoskeletal hierarchical assays provide a unique platform to investigate the synergy of the molecular linkages that connect these structurally distinct filaments (Dogterom & Koenderink, 2019; Even-Ram et al., 2007; Mandato et al., 2000; Pimm & Henty-Ridilla, 2021). MTs consist of multiple protofilaments that form a tube and have a higher persistence length than helical AFs (Sept, Baker, & McCammon, 2009; Steffen, Smith, Simmons, & Sleep, 2001). The differences in pliability of not only the filaments, but also the crosslinkers that connect them, will surely influence the tension and compression forces that propagate throughout the system and thus the resulting ensemble force generation. (Bouck, Joglekar, & Bloom, 2008; Kim, 2015; Murrell & Gardel, 2012) In addition, dynein is another MT motor protein that could be incorporated into these hierarchical OT assays to investigate their group dynamics on single and multi-filament complexes. Minus-end directed dynein works to transport...
cargo with the dynactin complex and facilitate large-scale motion in structures like cilia and flagella. (T. J. Mitchison & Mitchison, 2010; Waterman-Storer et al., 1997) These large-scale complexes and multi-motor axon models could then be constructed and interrogated using the combined MT bundle OT approach outlined above. (Mallick, Carter, Lex, King, & Gross, 2004; Sims & Xie, 2009; Waterman-Storer et al., 1997)

Further, new OT techniques and technology continue to emerge that have the ability to push these bundle assays to higher resolution. Recently, ~70 nm diameter germanium nanospheres were employed to interrogate the mechanics of a single kinesin-1 motor interacting with a microtubule in order to enhance the spatiotemporal resolution that can be limited by using traditional micron-sized beads (Sudhakar et al., 2021). Sudhakar et al. (2021) observed 4 nm center-of-mass movement with a microtubule in order to enhance the spatiotemporal resolution to interrogate the mechanics of a single kinesin-1 motor interacting with a microtubule from sliding completely apart. Nature Cell Biology, 13(10), 1259–1264. https://doi.org/10.1038/nccb232

In summary, combining OT with in vitro MT hierarchy and kinesin ensembles has yielded a novel approach for investigating higher order mechanics of the cytoskeleton. Force measurements of kinesin and MAP teams reveal that ensemble mechanics are not necessarily the sum of the SM properties, and motor/MAP/filament communication and cooperativity dictate concerted force generation. As biochemists and biophysicists work toward grand challenges like building a synthetic cell or even building minimal working subsystems like a spindle or sarcomere, they can utilize such methods to grasp the physical basis of each building block so that we can understand how they each contribute to overall system function.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

As this is a review article, there is no original data available for sharing. Interested parties should contact the corresponding authors of each article reviewed.

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