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**Optical Trap Based Studies of Single, Bundled, and
Cross-Linked Microtubules**

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Cross-Linked Microtubules**

by

Daniel Joseph Losowyj

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For my parents

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Optical Trap Based Studies of Single, Bundled, and Cross-Linked Microtubules

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Cytoskeletal networks within cells provide structural support and fulfill crucial biological roles for eukaryotic organisms. These networks display emergent behaviors like highly nonlinear elasticity that originate in the properties of individual filaments and the three-dimensional architecture of the network. In particular, the connectivity between filaments has major implications for the stress distribution and mechanics of the network, which, in combination with the stiffness of the constituent filaments, determine the magnitude of thermal fluctuations within the network. Beyond their structural importance, cytoskeletal networks also serve as highways for intracellular transport while simultaneously acting as obstructions that hinder diffusive motion on the nanoscopic scale. The immense difficulty associated with studying these networks from a single filament up to a collection of cross-linked filaments

means that there is a dearth of experimental data in this area. Here, we fill this gap with two novel methods of studying cytoskeletal filaments focusing specifically on microtubules, semiflexible filaments with rich behavior on the single filament level and network properties that depart from those of other cytoskeletal networks. First, we use thermal noise imaging, a high bandwidth, probe-based technique, to observe cross-linked networks of microtubules. New analysis methods allow us to localize individual filaments with 2.5 nm precision, one-tenth of the diameter of a microtubule, and additionally to track filaments' positions through intersections in the network, which have thwarted localization attempts in previous studies. This leads to the first direct measurement of a cross-link between connected filaments with a distance of just 1.4 nm found to be separating the microtubules. Furthermore, observations of filament fluctuations show that they are directly connected to the network architecture, suggesting that tensions within the network caused by cross-links may be quantified by these measurements. Diffusion of a spherical particle is also tracked throughout these experiments so that spatial variations in the diffusion constant can be mapped with a resolution of 10 nm. These measurements show that single microtubules can dramatically influence the motion of our probe particle, and, since the probe is similar in size to vesicles within cells, microtubules may play a significant role in the intracellular diffusion of such vesicles and other biologically important particles. After analyzing cross-linked networks, we then present the standing wave optical trap (SWOT) as a means of manipulating individual filaments with the potential to craft cus-

tom networks of microtubules. We show that not only can the SWOT trap a single microtubule, it can also manipulate microtubule bundles and individual filaments through the polarization of the trapping laser. This surprising degree of experimental control can only be explained if microtubules have a birefringence of at least 0.02, almost ten times larger than previously thought, opening the door to new possibilities for crafting designer networks filament by filament.

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Chapter 1

Introduction to Cytoskeletal Filaments and Networks

Multi-protein interactions are fundamental to cellular life and the cytoskeleton is a prime example of this. Starting from nanoscopic subunits, cytoskeletal biopolymers assemble into huge structures that can span tens of microns and effect mechanical and biological changes across the cell. By themselves, individual filaments can serve as molecular highways for motor proteins and produce forces through their polymerization to restructure the internal components of the cell, but they are not limited to these solitary roles. Together, cross-linked networks of filaments provide outstanding structural support to ensure that the cell maintains its shape under a range of extracellular forces and preserves its internal organization. These networks are even capable of creating cellular locomotion through the exertion of molecular motors that pull constituent filaments past each other. These various interplays between subunits within filaments and filaments within networks lead to a rich diversity of phenomena that beg for more study, beginning with the constituent elements of the cytoskeleton: microtubules, actin, and intermediate filaments.

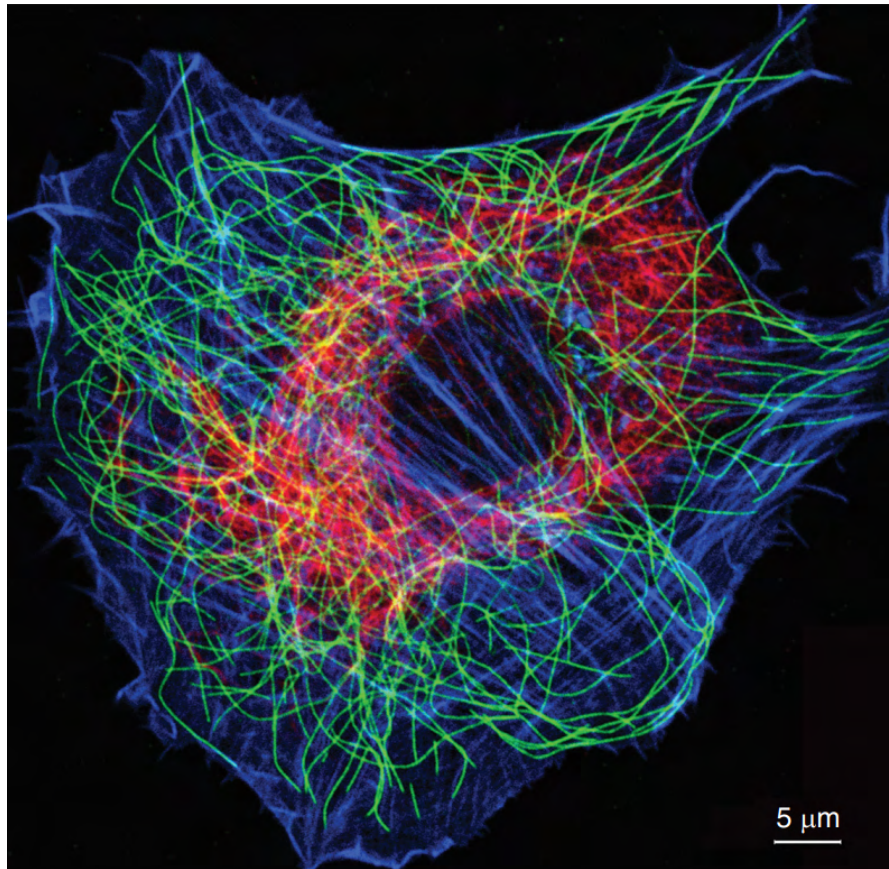


Figure 1.1: **Fluorescently labeled cytoskeleton of a fibroblast.** The cytoskeleton of a eukaryotic cell is a complex assembly of many kinds of biopolymer filaments. Here, microtubules are shown in green, actin filaments in blue, and intermediate filaments in red. (Image courtesy of Harald Herrmann, University of Heidelberg, Germany.)

1.1 Cytoskeletal Filaments

1.1.1 Microtubules

Microtubules are the stiffest biopolymers in the cytoskeleton, appearing as hollow tubes with ~ 15 nm inner diameters and ~ 25 nm outer diameters [1]. The fundamental building block of each microtubule is a pair of associated pro-

teins (α - and β -tubulin) that are chaperoned into a 4 nm by 8 nm unit called the $\alpha\beta$ -tubulin heterodimer [2]. These dimers bind head-to-tail into protofilaments, which exhibit polarization as a consequence of α -tubulin being exposed on one end and β -tubulin being exposed on the other. Tubulin can associate or disassociate from either end, but the association rate is much higher on the β -tubulin end than the α -tubulin end. This has led to the β -tubulin end being called the “plus end” and the α -tubulin end being called the “minus end” [3]. Groups of protofilaments then align laterally to form a transient sheet before rolling into a complete microtubule that can be 10 μm long [4]. Naturally occurring microtubules are controlled to have specific numbers of protofilaments (usually 13 in plant and animal cells [5]), but artificially created microtubules, like those used this dissertation, can have a variable number of protofilaments between 8 and 19 with 13–15 being the most common [6].

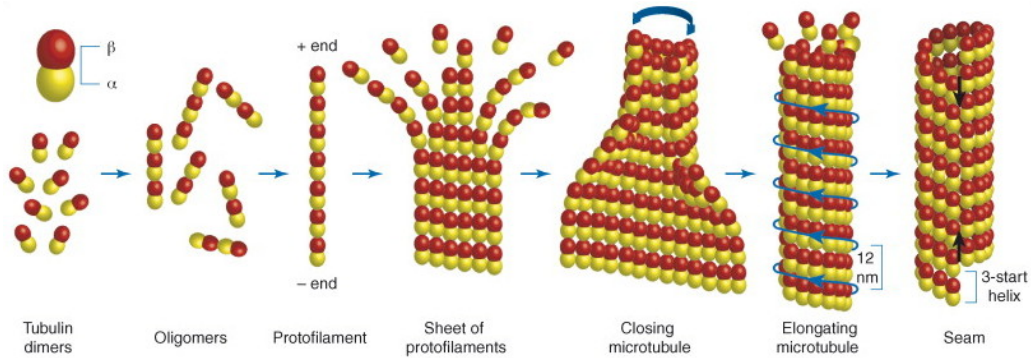


Figure 1.2: **Structure and assembly of a microtubule.** $\alpha\beta$ -tubulin heterodimers (red and yellow) bond longitudinally into protofilaments that align in parallel and then roll into microtubules. Modified with permission from [1].

Fully formed microtubules exhibit an interesting property known as

dynamic instability. On the timescale of seconds to minutes, microtubules can switch between periods of rapid assembly and rapid disassembly [7]. The origin of this instability comes from the structure of the heterodimers themselves. Both α - and β -tubulin have guanosine triphosphate (GTP) binding sites and, after being incorporated into a filament, β -tubulin will stochastically hydrolyze its bound GTP into GDP. This hydrolysis leads to a conformational change that compresses the dimer weakening its lateral interactions with adjacent protofilaments while strengthening its longitudinal interactions within the same protofilament [8]. As long as the rate of dimer association is faster than the rate of GTP hydrolysis, the microtubule maintains a “GTP cap” of newly added $\alpha\beta$ -tubulin that preserves the inter-protofilament interactions and allows for continued growth of the microtubule. Without the cap, however, protofilaments peel away from each other and the microtubule falls apart. The rapid disassembly of a microtubule due to this process is known as “catastrophe” and the growth phase is known as “rescue”. Certain microtubule-associated proteins (MAPs) like Tau are able to bind to microtubules and stabilize them to prevent repeated catastrophe while others like MCAK serve to heighten this dynamic instability by increasing the rate of depolymerization [9, 10].

The roles filled by microtubules in the cell are wide-ranging thanks to their ability to function in both static and dynamic capacities. All eukaryotic cells, for instance, leverage stable microtubules as routes for molecular motors that transport vesicles and even organelles throughout the cytoplasm [3]. Mi-

microtubules often emanate outwards from microtubule organizing centers near the cell nucleus giving them clear polarities with respect to the cell membrane. Motors harness this polarization to carry out directed transport within cells: kinesin walk towards the plus ends while dynein walk towards the minus ends of microtubules (with some exceptions) [11]. Neurons show extreme manifestations of this polarity with microtubules arranged in parallel throughout the length of their microns-long axons and those same microtubules provide mechanical support to the elongated cell bodies. Microtubules are also highly important for their interactions with other kinds of cytoskeletal filaments, particularly with actin. As cells migrate, microtubules interact with the actin network to maintain the cell's polarity and modulate the activity of actin for protrusion at the leading edge and retraction at the trailing edge [12]. Possibly the most famous part played by microtubules, however, is as dynamic force generators in cell division. During mitosis, the dynamic instability of microtubules is on full display with the filaments repeatedly undergoing catastrophe and rescue as they search for chromosomes, eventually latching onto the kinetochores and then, through a combination of motor forces and depolymerization, pulling the sister chromatids apart.

1.1.2 Actin Filaments

Actin filaments in the cell are smaller and more flexible than microtubules, having diameters of 8 nm and lengths of up to several microns [3, 13]. Polymerization starts when adenosine triphosphate (ATP) bound monomers

(G-ATP-actin) nucleate into a filament (F-ATP-actin). Once the monomers bind, their ATP hydrolyzes, but the phosphate group (Pi) remains trapped in the filament lattice (F-ADP+Pi-actin) for some time. Eventually, the phosphate group escapes and F-ADP-actin is left behind [14]. New monomers bind more readily to F-ATP-actin than its hydrolyzed counterpart so, following the same convention as microtubules, that end is called the plus end and the other end is called the minus end. Fully formed filaments have a helical nature to them with one rotation occurring every 37 nm.

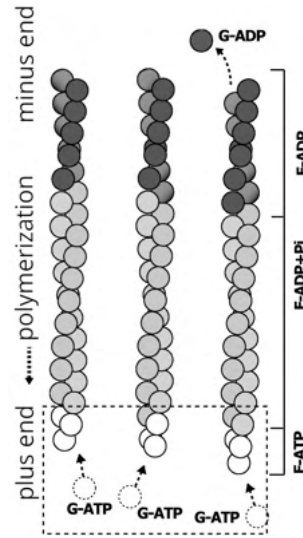


Figure 1.3: **Actin polymerization.** G-ATP-actin (white) nucleate into F-ATP-actin filaments (white) causing the bound ATP to hydrolyze. The phosphate group remains trapped in the lattice as F-ADP+Pi-actin (light grey) until it finally escapes and leaves behind F-ADP-actin (dark grey). Modified with permission from [14].

Like microtubules, actin filaments have a hand in a number of biological processes within the cell. While individual actin filaments can act as tracks

for myosin motors and apply small forces through polymerization, many of actin's functions emerge from interactions between multiple filaments. Static bundles of parallel filaments cross-linked by actin binding proteins (ABPs) form microvilli protrusions that maximize epithelial cell surface area for nutrient uptake in the intestines [15]. Other ABPs like filamin A create branched networks of perpendicular filaments that can restructure to allow cells to migrate [16] while myosin motors can also be used to cross-link filaments and create active gels. Contractions of these gels can restructure the cytoplasm and even cause macroscopic muscle contractions [3, 17].

1.1.3 Intermediate Filaments

Although they are only found in some animal cells, there are a wide variety of intermediate filaments [18]. They are commonly ~ 10 nm in diameter and grow up to a few microns long with structures built around lateral interactions between alpha helices on adjacent subunits. Consequently, their rope-like structures have bending stiffnesses that are significantly lower than those of microtubules and actin. The keratin family of intermediate filaments are found prominently in epithelial tissues where they provide mechanical strength by anchoring themselves at cell junctions [3] while other filaments like nuclear lamins are heavily involved in cellular differentiation and can influence the cell aging process [19, 20]. Like microtubules and actin, intermediate filaments commonly interface with the rest of the cytoskeleton where they help couple the cell membrane and nuclear envelope for signal transduction [21].

1.2 Mechanical Properties of Semiflexible Filaments

One of the most remarkable characteristics of cytoskeletal filaments is their ability to fulfill vastly different functions given the same basic structure. For example, microtubules are stiff enough to act as tracks for kinesin and dynein over several microns while being simultaneously so flexible that they can explore the cytoplasm for chromosomes during cell division. What are the properties of these filaments that make such varied behavior possible and how can they be quantified?

1.2.1 Persistence Length

A logical place to start in describing the mechanical properties of a polymer is with its bending stiffness. Single filaments are often still flexible enough that they exhibit significant bending as they undergo thermal fluctuations so the persistence length l_p is a commonly used metric [22]. The persistence length describes the distance over which the correlation between tangents to the polymer's contour decay (Figure 1.4). Mathematically, it is described implicitly by

$$\langle \hat{u}(s) \cdot \hat{u}(s+L) \rangle = e^{-\frac{L}{l_p}}, \quad (1.1)$$

where $\hat{u}(s)$ is the unit vector tangent to the polymer's contour at position s and L is the distance between the two tangents along the contour. Using the bending stiffness κ , the temperature of the system T , and Boltzmann's

constant k_B , one can calculate the persistence length as

$$l_p = \frac{\kappa}{k_B T}. \quad (1.2)$$

Essentially, a stiffer filament will have a larger bending stiffness and hence a longer persistence length, but this will be dependent on the magnitude of the thermal fluctuations being driven by the surrounding thermal bath. For this dissertation, the temperature will be relatively constant at 25 °C so the bending stiffness will be the dominant factor.

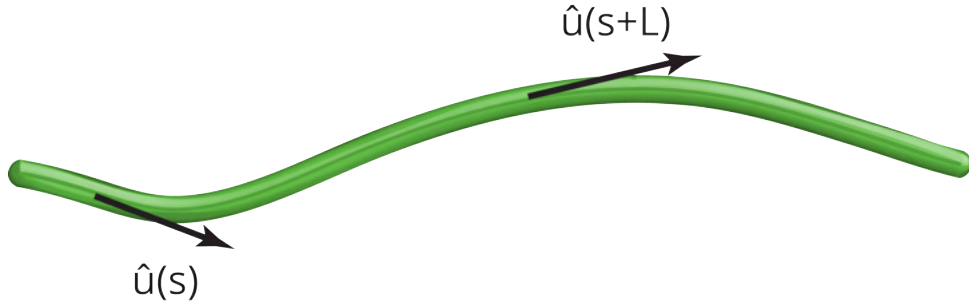


Figure 1.4: **Persistence length of a polymer.** A polymer's (green) persistence length measures the decay in the correlation between tangents to contour (black). See equation (1.1) for a mathematical definition.

Comparisons between the persistence length and the contour length (l_c) of a filament are typically used to classify it into one of three categories. When $l_c \ll l_p$, the filament has few enough undulations that it can be treated as elastic. In contrast, filaments with large fluctuations have $l_c \gg l_p$ and are considered flexible. The polymers falling in the middle range of $l_c \sim l_p$ are appropriately called semiflexible. *In vitro* measurements have been performed

on the various cytoskeletal filaments to determine their persistence lengths with intermediate filaments being the most flexible at $l_p \sim 50\text{--}200\text{ nm}$ followed by actin at $l_p \sim 10\text{ }\mu\text{m}$ [23, 24]. Microtubules are by far the stiffest with $l_p \sim 1\text{--}5\text{ mm}$, but their persistence lengths are heavily dependent on their contour lengths [25, 26].

1.2.2 Transverse Fluctuations and End-to-End Extension of a Filament Under Tension

The behavior of a filament experiencing no load is important in understanding its base properties, but a cytoskeletal filament is often under some amount of tension that alters its dynamical motion. In response to longitudinal forces, a filament stretches or compresses with contributions from entropic and elastic (or enthalpic) components. On length scales longer than the persistence length, tension applied to a polymer primarily pulls out undulations that result from thermal fluctuations. This can be thought of as reducing the number of microscopic states that a polymer is capable of exhibiting and thus represents entropic elasticity. The enthalpic contributions then take over at lengths shorter than the persistence length as the elasticity of the bonds become more important.

To quantitatively describe the average fluctuations of a filament, we follow the derivation in Broedersz’s and MacKintosh’s 2014 review [22]. We start by examining a filament that is bound on one end and has tension τ applied to the other end (Figure 1.5). The deviations of this filament from a

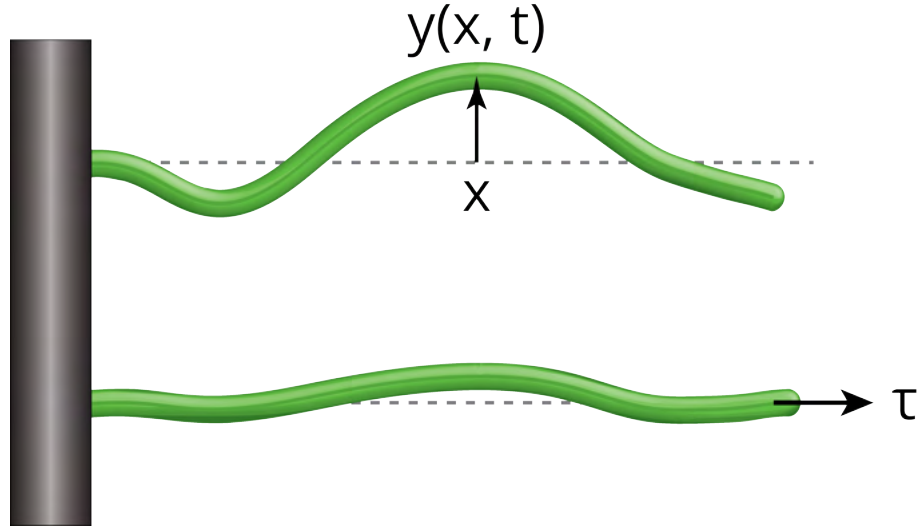


Figure 1.5: **Transverse fluctuations under tension.** A filament (green) is attached to a support on one end and is left free to fluctuate on the other. Thermal fluctuations drive the filament to deviate from a horizontal line at each point x and time t by an amount $y(x, t)$. Under no tension (top) the fluctuations are large. When a tension τ is applied (bottom) the fluctuations are suppressed.

straight line are given by $y(x, t)$ for a given position x and time t . We can represent these deviations as a Fourier series

$$y(x, t) = \sum_q y_q \sin qx \quad (1.3)$$

with the sum being over wave vectors $q = \pi/l_c, 2\pi/l_c, \dots$ for a chain with fixed ends ($y(0, t) = 0$ and $y(l_c, t) = 0$). Accounting for the energy stored by the filament bending and the energy of the extension caused by the tension, we can write the total bending energy as

$$H = \frac{1}{2} \int_0^{l_c} dx \left[\kappa \left(\frac{\partial^2 y}{\partial x^2} \right)^2 + \tau \left(\frac{\partial y}{\partial x} \right)^2 \right] \quad (1.4)$$

$$= \frac{l_c}{4} \sum_q (\kappa q^4 + \tau q^2) y_q^2. \quad (1.5)$$

We then link this energy to the filament fluctuations through the equipartition theorem. With each mode receiving $k_B T/2$ of thermal energy, the time averaged fluctuations become

$$\langle |y_q|^2 \rangle = \frac{2k_B T}{l_c (\kappa q^4 + \tau q^2)}. \quad (1.6)$$

This result gives us a lower cutoff for fluctuation modes due to the q^4 in the denominator, which strongly suppresses modes with wavelengths shorter than the persistence length. The lower order modes are thus dominant and, if their amplitudes could be extracted, would provide insight into the amount of tension applied to a filament.

The thermal fluctuations of a filament also create changes in the filament's end-to-end extension, the minimum distance between the fixed and free ends of the polymer. If the filament does not significantly stretch, then its end-to-end extension L is shorter than its contour length due to the transverse fluctuations. This shortening of the polymer can be calculated by integrating over all of the fluctuations.

$$l_c - L = \frac{1}{2} \int_0^{l_c} \left(\frac{\partial^2 y}{\partial x^2} \right)^2 dx \quad (1.7)$$

$$= k_T \sum_q \frac{1}{\kappa q^2 \tau} \quad (1.8)$$

We can then work backwards to find the tension as a function of the extension or compression in the filament. Assuming a small change in length ($\delta l = l_c - L$), we Taylor expand about τ and find

$$\tau \sim \frac{\kappa^2}{k_B T l_c^4} \delta l. \quad (1.9)$$

Importantly, this relation functionalizes the tension in terms of the filament's change in end-to-end extension. For a freely fluctuating filament, this may only appear through external forces, but for filaments within a network, these tensions can develop from interfilament connections.

1.3 Mechanics of Semiflexible Filament Networks

The properties of individual filaments play key roles in the behavior of biopolymer networks, but it is impossible to completely describe such systems without accounting for the architecture of the filament arrangements. In randomly cross-linked networks, filaments are inherently stressed by their connections throughout the matrix as they are forced into extension or compression (equation (1.9)). This creates a force distribution that in turn affects the macroscopic properties of the network [27, 28].

1.3.1 Strain-Stiffening in Biopolymer Networks

A common parameter used to characterize networks is the shear modulus G , which quantifies a material's resistance to deformation. If a material is purely viscous, then it will flow in response to shear stress and will not return to its original state even when the stress is removed. If a material is purely elastic, then it will store the energy and then release it to return back to its original state. Water is an example of a viscous material, while steel is an example of an elastic material. Biopolymer networks occupy a middle range between these extremes exhibiting both viscous and elastic properties.

Measuring a material's degree of viscous or elastic response is done by applying an oscillatory strain and tracking its stress over time. The strain γ is the ratio of the object's deformation parallel to direction of stress ΔL to the object's initial length in that direction L

$$\gamma = \frac{\Delta L}{L} \quad (1.10)$$

and the stress σ is defined as a force applied parallel to the object's surface F divided by the area A

$$\sigma = \frac{F}{A}. \quad (1.11)$$

Under an oscillatory strain $\gamma(t) = \gamma_0 \sin \omega t$, the elastic stress is in phase with the strain while the viscous stress is $\pi/2$ out of phase.

$$\sigma(t) = \gamma_0 [G'(\omega) \sin \omega t + G''(\omega) \cos \omega t] \quad (1.12)$$

Equation (1.12) shows these responses in terms of the storage modulus G' and the loss modulus G'' , which capture the elastic and viscous behavior, respectively. These two moduli are often written together as the complex-valued shear modulus G

$$G(\omega) = G'(\omega) + iG''(\omega). \quad (1.13)$$

Many kinds of biopolymer networks including those made of actin and intermediate filaments show strain-stiffening in which their storage moduli remain constant under moderate strains and then grow larger as the strain is increased past a critical point. Storm *et al.* observed this behavior in bulk rheological tests of several biopolymer networks (Figure 1.6). They placed macroscopic samples (roughly a few milliliters in volume) between two parallel plates and then rotated one while holding the other fixed. Models of this strain-stiffening behavior only assume that the networks are homogeneous and isotropic, and that they are strained uniformly. The simple cross-linking of the filaments is enough to bring about this property in biopolymers that is not easily duplicated in synthetic materials [29]. The critical strain at which stiffening occurs depends strongly on the persistence length of the filament with more flexible filaments like vimentin not stiffening until strains of nearly 100%. It is not until the thermal fluctuations are pulled out of the filaments

that the enthalpic stretching of the filament takes over and the strain-stiffening behavior becomes visible. In evolutionary terms, it is thought that biopolymer networks developed this behavior to prevent large deformations that could damage cells and tissues.

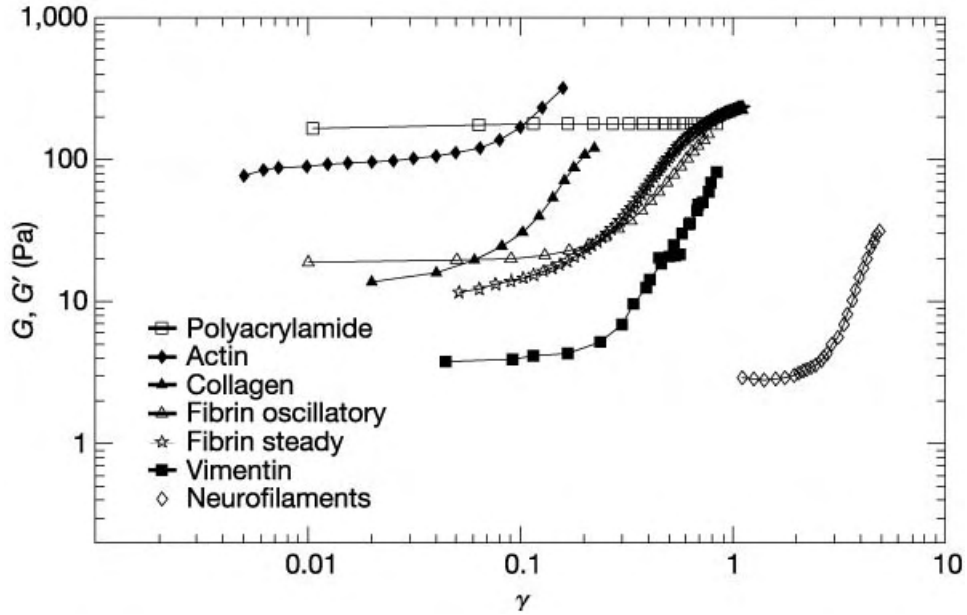


Figure 1.6: **Strain-stiffening in biopolymer networks.** The storage modulus for a variety of polymer networks remains constant at low to moderate strains, but then increases after a critical strain. This behavior is called strain-stiffening. Reprinted with permission from [30].

1.3.2 Strain-Softening in Cross-Linked Microtubule Networks

Networks of microtubules are less well-studied than networks of other biopolymers, largely due to the lack of mechanically stable cross-linking proteins. Solutions of microtubules have been studied without cross-linking agents, but the inherent stiffness of microtubules prevents entanglement that would

create more interesting behavior [31]. Thus, microtubules are commonly formed into networks by biotin-avidin bonds.

In contrast to other biopolymer networks, networks of cross-linked microtubules show strain-*softening* rather than strain-stiffening behavior. Figure 1.7 shows this response over a range of cross-linker concentrations from 1 mg/mL to 5 mg/mL [31]. Increasing the cross-linker concentration led to increased stiffness of the network as expected, but only up to a critical point at which the distance between cross-links was comparable to the distance between microtubule contact points. Models that have traditionally been successful in describing other biopolymer networks, like those made of actin, drastically overestimate the stiffness of microtubule networks at low strains by nearly four orders of magnitude [31, 32]. The authors suggested that the relatively short contour lengths of their microtubules (a few microns) were not sufficient to form many cross-links per filament.

Explaining the strain-softening behavior of these networks required Lin *et al.* to use a model that considered weak interactions between microtubules together with the cross-links. Any time two filaments cross, there is some interaction force between them. These interactions may arise from denatured tubulin in the solution that couples neighboring filaments, but the specific origins have not been thoroughly investigated. Shear stresses then shift these filaments relative to one another and break the non-specific, interfilament interactions thereby weakening the integrity of the network. The yield stress is finally reached when the biotin-avidin cross-links are ruptured and the net-

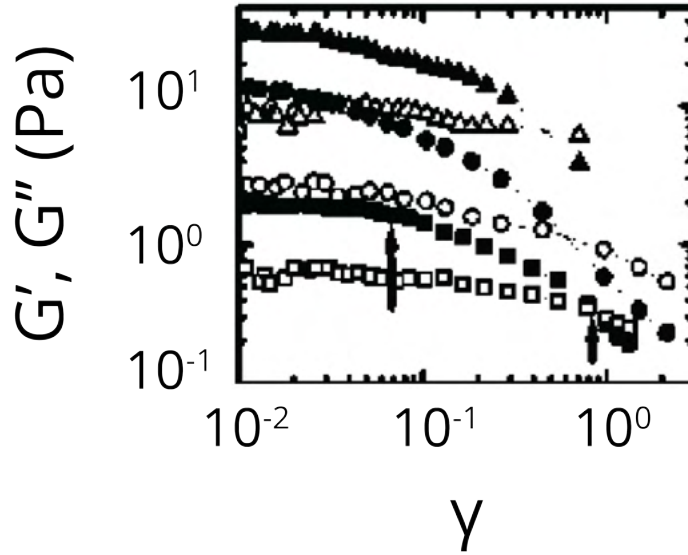


Figure 1.7: **Strain-softening in microtubule networks.** Oscillatory strain measurements of G' (solid symbols) and G'' (open symbols) are shown for cross-linked microtubule networks at cross-linker concentrations of 1 mg/mL (squares), 3 mg/mL (circles), and 5 mg/mL (triangles). The storage modulus of cross-linked microtubules decreases at larger strains, a behavior known as strain-softening. The yield strains (defined as when $G' = G''$) are shown by the arrows. Modified with permission from [31].

work loses its structure. As for why there is no strain-stiffening prior to yield in microtubule networks, the high persistence length relative to the short contour lengths means that there are few thermal fluctuations to pull out under applied shear and therefore little to no entropic stretching is expected.

To examine the microscopic origins of these behaviors, more recent studies have investigated microtubule networks through microrheology experiments [33]. These types of experiments measure the local viscoelastic response of networks, rather than the average response over large areas like in tradi-

tional rheology experiments. Microrheology follows the motion of microscopic particles embedded in between filaments to gauge the network’s response. In this case, magnetic tweezers were used to apply picoNewton forces to $4.5\text{ }\mu\text{m}$ magnetic particles within in the network while their positions were tracked. On short time scales, the networks stiffened under small forces and softened under larger loads as was observed in macroscopic studies [30]. This softening was followed by significant creep in the bead positions at longer times as the network rearranged. Both the strain-softening and the creep were attributed to force-induced unbinding of the cross-linkers. If enough bonds remained, then the network was able to preserve its elastic modulus through the formation of new connections. It is important to note that this behavior would not have been observed without an examination of the network mechanics closer to the single filament scale.

1.4 Transport in Cytoskeletal Networks

The interior of the cell is a highly complex, viscoelastic environment through which all intracellular transport must occur. Many biological processes like vesicle secretion and mitochondria movement between dividing cells are sped up through active transport by molecular motors while others like nutrient distribution make use of advection generated by other motion within the cytoplasm. Fundamental actions like intracellular signaling are even limited by diffusion itself. Together, active transport, advection, and diffusion enable the transport seen within living organisms.

1.4.1 Active Transport

As discussed in Section 1.1, cytoskeletal filaments play key roles in the cell as highways for molecular motors. These motors have been tracked moving up to $10\text{ }\mu\text{m/s}$ *in vivo* with average speeds of around $0.3\text{--}2\text{ }\mu\text{m/s}$ in animal cells, making them ideal for rapid secretion of vesicles and transport between organelles [3, 34]. Far from just being static paths for these motors, however, *in vitro* measurements have suggested that microtubules can actually contribute to the forward procession of dynein motors through their thermal fluctuations [35]. Ezber *et al.* centered an optical trap on the motor cargo and oscillated it as the motor walked to simulate thermal fluctuations of the filament. The attached dynein relied on a slip bond to resist backward motion while harnessing forward motion to move along more quickly, a process that would presumably function within cells to speed up active transport. It stands to reason, then, that the stiffness of the individual filaments and the network architecture of cytoskeleton would contribute heavily to the motor speeds within cells through their connection to the filament fluctuations.

1.4.2 Advective Transport

First observed in plant cells in the 18th century, advection is the transportation of material by flows within the cytoplasm [36]. These flows, which can reach up to $100\text{ }\mu\text{m/s}$, originate from active processes within the cell with common sources being the contraction of actin filaments by myosin motors or the active movement of organelles [34]. The low Reynolds numbers within

cells means that there is strong coupling between the large moving cargo and the surrounding fluid. This coupling is enough to explain not only nutrient transport within plant cells, but even the bidirectional movement of certain proteins within neuronal axons [37, 38]. For all this coupling between fluid and the environment, there has been little care paid to the influence of the cytoskeleton on these flows. Given the confined nature of cellular environments, the final state of advective flows are enormously predicated on their specific boundary conditions at the cell membrane and within the cytoplasm [39].

1.4.3 Diffusive Transport

Despite its random nature, diffusive motion drives signaling cascades, organization of the mitotic spindle, and cytoskeleton assembly [40]. The slow speed of diffusive motion would suggest that it is a bad candidate for any urgent biological process in the absence of other flows or concentration gradients. However, at least in the case of signaling, evolution seems to have selected for diffusion-limited reactions in order to amplify signals more quickly [41]. It has even been suggested that cell size has been specifically optimized for diffusion in both prokaryotes and eukaryotes [42]. For these reasons, it is critical to understand the fluidity of the cytoplasm in tandem with its coupling to internal structures like the cytoskeleton.

Particles within the cytoplasm have shown subdiffusive behavior in fungal, bacterial, and mammalian cells, among others [43–45]. Subdiffusion is observed when objects do not diffuse as far as Stokes-Einstein predictions would

suggest for free space due to some kind of obstruction or transient binding to immobile structures within the cytoplasm. Figure 1.8 shows this behavior by plotting the mean squared displacement of granules within a fission yeast cell versus the lag time between position measurements. A slope of less than one in this plot is a characteristic sign of subdiffusive motion. Several explanations of subdiffusion have been provided for different experimental conditions including crowding within cells, pH-induced aggregation of proteins, and cytoskeletal networks obstructing the motion of particles [43, 46, 47].

The influence of cytoskeletal networks on particle diffusion has been examined numerous times *in vitro* in an effort to precisely characterize their effects and take advantage of the tunability of their properties. Wong *et al.* showed that the MSD of a 250 nm particle in an entangled F-actin network was strongly connected to the mesh size of that network [48]. As the mesh size decreased, the slope of the MSD versus the lag time similarly decreased as particles found themselves confined to smaller regions of space. The specific nature of this dependence, however, has been challenged for two reasons: it does not account for transient interactions between filaments, and it does not consider filament rigidity. A combination of pore size and particle interactions with the network were shown to cause transient anomalous diffusion wherein the particle briefly bound to obstructions before unbinding and resuming diffusion [49]. This detail could be missed depending on the duration of the binding times relative to the time between particle observations. The effect of network stiffness was then interrogated by creating hybrid networks

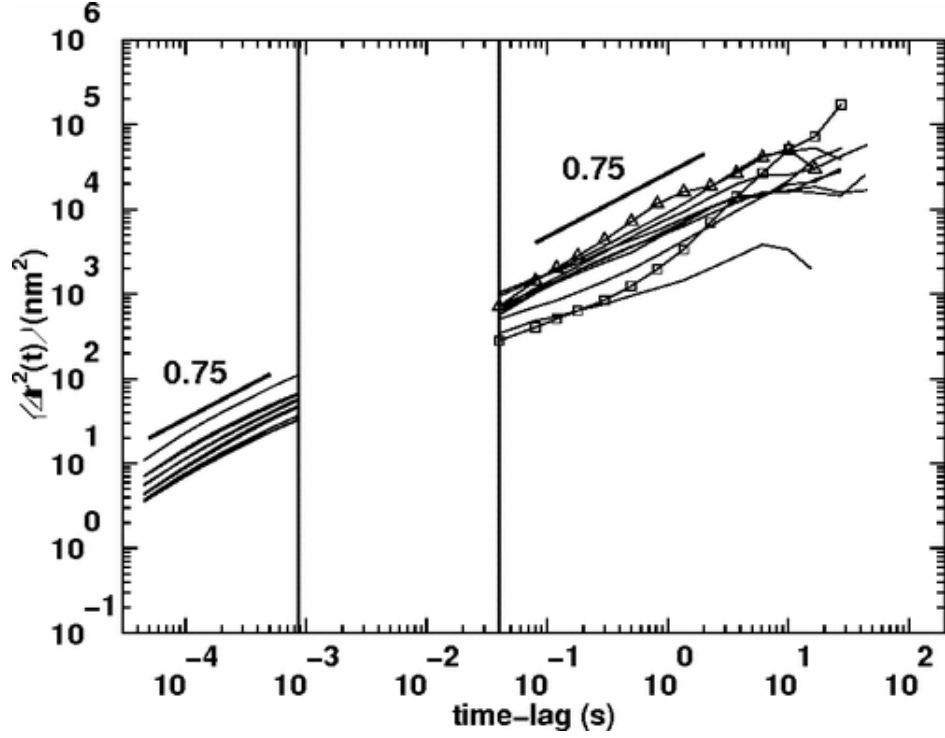


Figure 1.8: **Anomalous diffusion in yeast cells.** The mean squared displacement of yeast lipid granules is plotted versus the lag time showing anomalous diffusion. This data demonstrates subdiffusive behavior with its slope being less than one, the value predicted by free diffusion. Reprinted with permission from [43].

of microtubules and F-actin filaments, which were shown to alter the MSD relationship with mesh size as a consequence of the varying filament properties [50]. The authors used a combination of widefield fluorescence and light sheet microscopy to track particles over a wide range of timescales, analyzing the widefield fluorescence data with single particle tracking and the light sheet data with differential dynamic microscopy. Both methods were limited to ~ 1 – 10 ms time resolutions. They found that networks with higher concentra-

tions of stiff filaments hindered diffusion more than flexible networks with the moduli of networks being measured via microrheology.

1.5 Research Objectives and Dissertation Outline

The goals of this dissertation are to connect the microscopic architecture of microtubule networks to individual filament fluctuations and the diffusive environments surrounding them, and to develop a new optical manipulation method for studying isolated filaments. Microscopic studies of microtubule networks require high bandwidth and high spatial precision that will be achieved through the use of thermal noise imaging. Optical manipulation of filaments will be performed using a standing wave optical trap. Chapter 2 provides the theoretical background for the techniques and describes the experimental setups employed. Chapter 3 discusses thermal noise imaging of microtubule networks and shows results for precise filament localization and quantification of filament fluctuations. Chapter 4 leverages thermal noise imaging data to develop maps of the spatially varying diffusion constant for particles within microtubule networks. Chapter 5 demonstrates optical manipulation of microtubules via their birefringence and examines the similarities to simulations. Finally, Chapter 6 lays out future directions for both techniques.

Chapter 2

Methods

2.1 Diffusion

Diffusive motion is fundamental to the work presented in this dissertation. Both of the microscopes employed (the photonic force microscope and the standing wave optical trap) leverage Brownian motion and restriction of it in studying experimental systems so we will begin by looking at some properties of diffusing bodies.

2.1.1 The Langevin Equation

When working with experimental systems that utilize Brownian motion, it is important to get a sense of the time and distance scales over which the dynamics of interest occur. One of the principal entry points to this discussion is through the use of the Langevin equation, named after Paul Langevin [51]:

$$m\ddot{x} = -\gamma_0\dot{x} + F_{th}(t) + F(x). \quad (2.1)$$

This equation describes the position x of a particle with mass m and drag coefficient γ_0 as it experiences random thermal forces $F_{th}(t)$ from its environment and external forces $F(x)$. Here, $\gamma_0 = 6\pi\eta a$ is the Stokes drag on a sphere of

radius a immersed in a fluid with dynamic viscosity η . The external forces will be taken to be zero for now, but we will return to this later (Section 2.2.2). That leaves us with

$$m\ddot{x} = -\gamma_0\dot{x} + \sqrt{2k_B T \gamma_0} \xi(t), \quad (2.2)$$

where k_B is Boltzmann's constant, T is the temperature, and $\xi(t)$ is a stochastic quantity defined by an average over an ensemble of colliding molecules at times t and τ

$$\langle \xi(t) \rangle = 0 \quad (2.3)$$

$$\langle \xi(t) \xi(\tau) \rangle = \delta(t - \tau). \quad (2.4)$$

It represents the random thermal “kicks” that the particle will experience as a result of collisions with the surrounding fluid.

From here, we can actually make a simplification due to the small size of our particle. The timescale over which a particle's inertia is dissipated by friction is m/γ_0 , which is 10 ns for the 208 nm particles used in thermal noise imaging. This is much shorter than our experimental bandwidth of 1 MHz (Section 2.3.3) putting our measurements of the system in the thoroughly overdamped regime. We can use this to drop the inertial term $m\ddot{x}$ from our equation

$$-\gamma_0 \dot{x} + \sqrt{2k_B T \gamma_0} \xi(t) = 0. \quad (2.5)$$

At this point, we have a description of the translational diffusion of a spherical particle, but this is not the only incarnation of the Langevin equation. It can just as easily be written for the rotation of a rigid body (like a freely diffusing microtubule) as

$$I \ddot{\theta} = -\gamma_{r0} \dot{\theta} + \sqrt{2k_B T \gamma_r} \xi(t). \quad (2.6)$$

The notable changes are switching the position x to the angle θ , the mass m to the moment of inertia I , and the drag coefficient γ_0 to the rotational drag coefficient γ_{r0} . For a rod rotating about its center, the rotational drag coefficient can be written in terms of its length L and its diameter d as [52]

$$\gamma_{r0} = \frac{\pi \eta L^3}{3 \ln(L/2d)}. \quad (2.7)$$

We can similarly write an overdamped version of the Langevin equation for rotational motion, this time requiring that I/γ_{r0} is much smaller than our experimental timescales. A 10 μm long microtubule has an inertial dissipation time of approximately 132 ns, considerably longer than the $\sim 10 - 100$ Hz operation of the standing wave optical trap's camera. The overdamped rotational Langevin equation is thus

$$-\gamma_{r0} \dot{\theta} + \sqrt{2k_B T \gamma_r} \xi(t) = 0, \quad (2.8)$$

where we have dropped the moment of inertia term $I\ddot{\theta}$

2.1.2 Mean Squared Displacement

The mean squared displacement (MSD) of a particle's motion describes how the variance in a diffusing particle's displacement evolves in time. If we start with the Langevin equation for a spherical particle (equation (2.5)), we can find the MSD of a freely diffusing particle in terms of the diffusion constant using the Stokes-Einstein relation $D_0 = k_B T / \gamma_0$ [53]

$$\langle \Delta x^2 \rangle_{\text{free}}(t) = 2D_0 t. \quad (2.9)$$

This linear dependence of the MSD on time is a hallmark of unrestricted Brownian diffusion.

It is, however, important to recognize that diffusion in complex media (like within cytoskeletal networks) does not always follow the clean form laid out in equation (2.9). The mean squared displacement of a particle can take on four distinct modes of motion: normal diffusion, anomalous diffusion, directed motion with diffusion, and confined motion. They are characterized by the following relations

$$\langle \Delta x^2 \rangle = 2D_0 t \text{ Normal Diffusion} \quad (2.10)$$

$$\langle \Delta x^2 \rangle = 2D_0 t^\alpha \text{ Anomalous Diffusion} \quad (2.11)$$

$$\langle \Delta x^2 \rangle = 2D_0 t + (vt)^2 \text{ Directed Motion with Diffusion} \quad (2.12)$$

$$\langle \Delta x^2 \rangle = \langle \Delta x_c^2 \rangle [1 - A_1 \exp(-2A_2 D_0 t / \langle \Delta x_c^2 \rangle)] \quad \text{Confined Motion} \quad (2.13)$$

with examples plotted in in Figure 2.1. Anomalous diffusion can result from a number of factors, but is most often associated with crowding in a cellular environment [54]. Directed motion with diffusion is seen when there is advective transport within a system in addition to diffusive motion or when a particle is being actively transported by a molecular motor. Finally, confined motion occurs when some restriction is placed on the particle's range of motion by an external force like a dense biopolymer network or an optical trap.

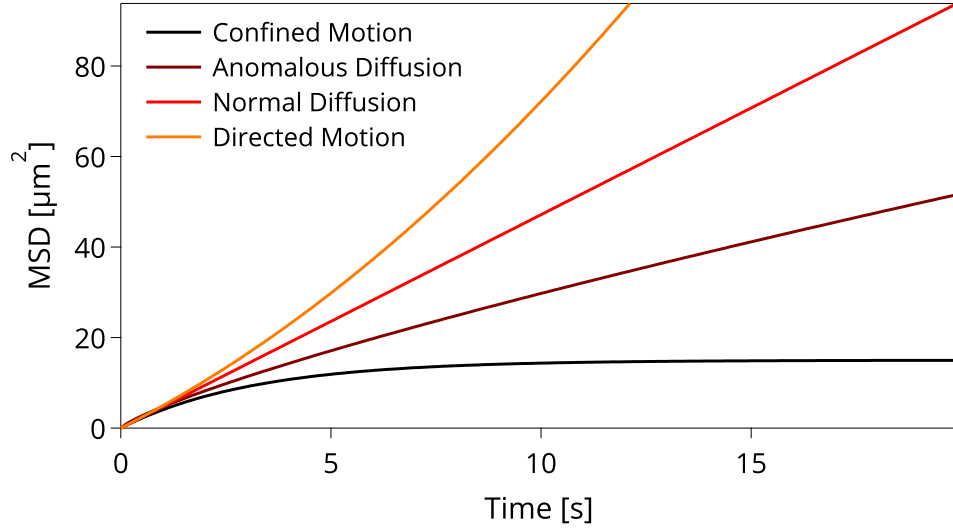


Figure 2.1: **MSD for different types of diffusion.** The MSD as a function of lag time for normal diffusion, anomalous diffusion, directed motion, and confined motion.

2.1.3 Spatially Varying Diffusivity

Previous sections have explored the properties of Brownian motion when the drag coefficient γ_0 and diffusion constant D_0 are spatially uniform, but interesting behavior results when these values are allowed to vary in response to the surrounding environment. In free space, a moving particle can displace fluid, which then flows away without serious restriction, but this situation changes when the particle begins moving near a static surface with no-slip boundary conditions. The fluid displaced by the particle now runs into the surface and cannot flow freely. This restriction on the fluid flow hydrodynamically couples the particle to the surface with the separation distance controlling the strength of the coupling. For motion perpendicular to an infinite, planar surface, Bevan and Prieve approximated the drag coefficient $\gamma_\perp$ from Goldman *et al.* as

$$\gamma_\perp \approx \gamma_0 \frac{6h^2 + 9ah + 2a^2}{6h^2 + 2ah}, \quad (2.14)$$

where the particle's radius is a and its center is a distance h above the surface [55, 56]. A related formula for the perpendicular drag coefficient can also be found for a surface that is free to fluctuate in response to flow, for instance, at a liquid-gas interface. If the surface is planar, then the tangential stresses and velocity perpendicular to it vanish and the drag coefficient for the sphere becomes

$$\gamma_{\perp, \text{fluct}} = \gamma_0 \frac{4}{3} \sinh \alpha \sum_{n=1}^{\infty} \frac{n(n+1)}{(2n-1)(2n+3)} \times \left\{ \frac{4 \cosh^2([n+1/2]\alpha) + (2n+1)^2 \sinh^2(\alpha)}{2 \sinh([2n+1]\alpha) - (2n+1) \sinh(2\alpha)} - 1 \right\}, \quad (2.15)$$

where $\alpha = h/a$ [57]. Finally, the interaction between two spheres has been studied and found to follow the form

$$\gamma_{\perp, \text{sph}} \approx \gamma_0 \left[1 + \frac{9ab}{4h^2} + \frac{3}{4} \left(-2 \frac{a^3b}{h^4} + \frac{27}{4} \frac{a^2b^2}{h^4} + 3 \frac{ab^3}{h^4} \right) \right], \quad (2.16)$$

where b is the radius of the second sphere [57]. Figure 2.2 shows how all three of these drag coefficients vary as a 104 nm radius particle approaches a planar surface or the surface of a 12.5 nm radius sphere. Both of the coefficients for diffusion near a planar surface diverge although the expression for a freely fluctuating surface does so more slowly. Meanwhile, the coefficient for diffusion near another sphere goes to approximately 1.15 times the free space value. This smaller increase in the drag stems from the finite extent of the spherical surface. Whereas the planar surfaces are infinite, the sphere only has no-slip boundary conditions over its surface and therefore has a limited impact on the hydrodynamics of nearby objects.

2.2 Optical Tweezers

Since their invention by Arthur Ashkin in 1986 [58], optical tweezers have been extraordinary tools for studies across physics and biology. From

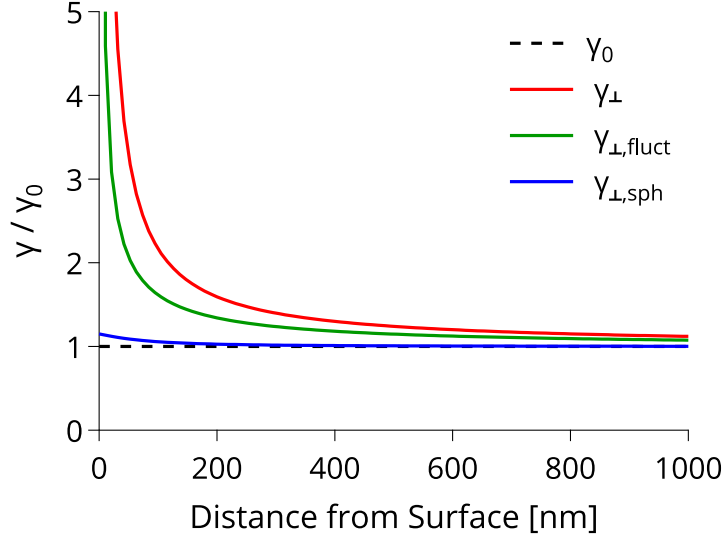


Figure 2.2: **Drag coefficients perpendicular to a surface.** The drag coefficients perpendicular to a surface are shown as a function of distance from the surface for a 104 nm radius sphere. Both drag coefficients for planar surfaces diverge (red and green) while the drag coefficient for a spherical surface that is 12.5 nm in radius goes to approximately 1.15 times the coefficient in free space (blue).

measurements of the instantaneous velocity of a Brownian particle [59, 60] to examinations of the inner ears of fish [61] and even measuring the step size of a protein [62], optical tweezers are immensely versatile instruments that, at their core, require very few components. By passing a laser through a high numerical aperture objective ($\text{NA} \gtrsim 1$), it becomes possible to manipulate single cells, nanoscopic particles, and even individual viruses with precision [63–65]. The properties of an optical tweezers setup can vary greatly from firm confinement through plasmonic resonances [66] to weak trapping at constant force [62], but they all rely on the interplay between two forces: the scattering force and the

gradient force.

2.2.1 Optical Forces

2.2.1.1 The Scattering and Gradient Forces

The scattering force is most commonly known for its dynamic impact on the tails of comets. Photons from the sun strike the comet and displace dust and debris that creates their characteristic tails [67]. This same force plays a key role in optical traps except now it is photons from a focused laser that impinge upon a sample of interest. The photon momentum is transferred to the trapped object, pushing it in the direction of the laser's propagation. Since the scattering force depends on the momentum flux, larger objects feel larger scattering forces following this relation:

$$\vec{F}_{scat}(\vec{x}) = \frac{8\pi^3\alpha^2}{3cn_m^3\epsilon_0^2\lambda^4} I(\vec{x}) \quad (2.17)$$

$$= \frac{128\pi^5 a^6 n_m}{3c\lambda^4} \left[\frac{(n_p/n_m)^2 - 1}{(n_p/n_m)^2 + 2} \right] I(\vec{x}) \hat{z}, \quad (2.18)$$

where a is the radius of the particle, n_p and n_m are the refractive indices of the particle and the surrounding medium, respectively, λ is the wavelength of the excitation light, α is the polarizability of the particle, and $I(\vec{x})$ is the intensity of the light at the position of the particle $\vec{x}$ [58]. The force is directed along the direction of light propagation, which is chosen to be $\hat{z}$ in this dissertation. Notably, the scattering force goes with the sixth power of the particle radius

(for particles smaller than the wavelength) so larger particles will feel intense forces that push them quickly out of the laser focus.

Seeing that stable trapping was very difficult to achieve using just the scattering force from a single trap, Ashkin actually utilized two counter propagating lasers to cancel out the photon momentum and hold a trapped particle in place [68]. This technique led to zero net forces along the optical axis, but he also found that there was weak stability along the perpendicular directions. This force seemed to push particles up the intensity gradient towards the highest intensity region of the laser. Consequently, it was termed the gradient force.

Ashkin's discovery of the gradient force proved critical for the future of optical trapping. Without the gradient force, scientists would have been restricted to counterpropagating beam setups, but now they could make use of traps built around single objective lenses. The high numerical aperture of these objectives created beams with large intensity gradients and hence large gradient forces. These gradient forces can be intuitively understood in multiple ways depending on the size of the trapped particle relative to the wavelength of the trapping light. For particles much larger than the wavelength (the ray optics regime), the refraction of light by the particle changes the light momentum and draws the particle to the regions of highest intensity (Figure 2.3). Particles that are much smaller than the wavelength (the Rayleigh regime) instead behave as dipoles in an electric field, feeling a force $\vec{F} = \vec{p} \cdot \nabla \vec{E}$, where $\vec{p}$ is the dipole moment induced by the electric field. Yasuhiro Harada derived

the commonly-used form of this equation for Rayleigh particles [69]:

$$\begin{aligned}\vec{F}_{grad} &= \frac{1}{4}\alpha\vec{\nabla}\left|\vec{E}(\vec{x})\right|^2 \\ &= \frac{2\pi n_m a^3}{c}\left[\frac{(n_p/n_m)^2 - 1}{(n_p/n_m)^2 + 2}\right]\vec{\nabla}I(\vec{x}),\end{aligned}\tag{2.19}$$

where we have used the definition of the intensity of an electric field

$$I(\vec{x}) = \frac{cn_m\epsilon_0}{2}\left|\vec{E}(\vec{x})\right|^2.\tag{2.20}$$

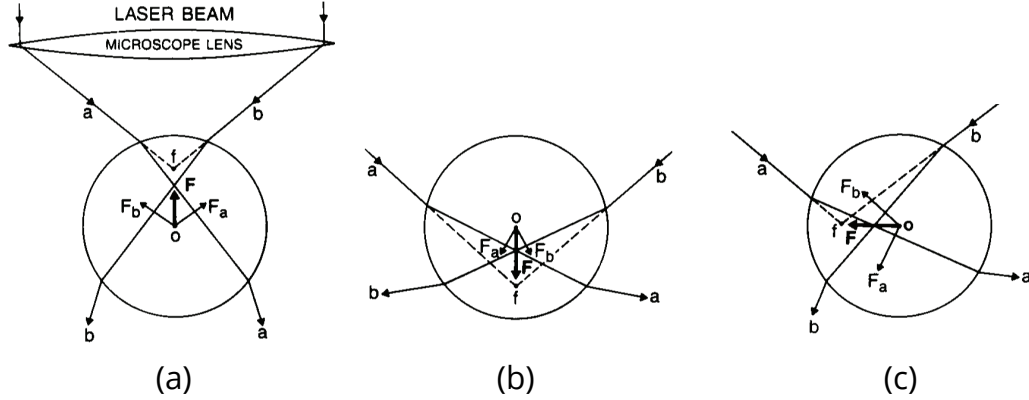


Figure 2.3: **Gradient force in the ray optics regime.** Light rays focused by the microscope lens refract as they pass through a trapped particle. The net force generated (F) is always towards the focus (f) from the center of the particle (O). Forces are shown for when the particle is displaced (a) below the focus, (b) above the focus, and (c) laterally from the focus. Adapted with permission from [70].

While Harada's result gives a clear depiction of the gradient force, it is not suitable for particles used in thermal noise imaging ($a = 208$ nm and $\lambda = 800$ nm in solution), which fall into the Mie regime. Calculations of

forces for particles of this size require intensive treatments using Mie theory to calculate the scattered and absorbed fields followed by integration of the Maxwell stress tensor over the surface of the particle [71]. However, theory by Rohrbach and Stelzer confirmed that the gradient force increases linearly with small displacements from the trap center even for particles in the Mie regime [72] allowing us to treat the particle-trap system as a three-dimensional harmonic potential

$$E_{\text{trap}}(\vec{r}) = \frac{1}{2} (k_x x^2 + k_y y^2 + k_z z^2) . \quad (2.21)$$

Above, E_{trap} is the energy of the trapped particle and k_x , k_y , and k_z are the spring constants of the trap along each direction. Surprisingly, k_x and k_y are not, in general, equal as the polarization of the laser introduces asymmetry making the trap focus wider along the direction of polarization. A wider focus lessens the intensity gradient so the trap is weaker in the direction parallel to the polarization than it is in the perpendicular direction. Lastly, the z-direction exhibits the weakest trapping due to the extended focus along the optical axis. These spring constants can be extracted with high precision and helpfully characterize the behavior of optical tweezers setups [73].

2.2.1.2 Stability of Optical Traps

Knowing that the scattering force always pushes a trapped particle in the direction of the laser propagation while the gradient force tries to hold the particle in the region of highest intensity, it is clear that the maximum

gradient force must be larger than the scattering force at the same position for stable trapping. Optical traps typically utilize Gaussian beams with intensities governed by

$$I(x, y, z) = \frac{2P}{\pi} \left[\frac{1}{w_0^2 + 4z^2} \right] e^{-\frac{2(x^2 + y^2)}{w_0^2 + 4z^2}}, \quad (2.22)$$

where the power P is related to the electric field amplitude by $P = \frac{1}{4}\pi w_0^2 n_m \epsilon_0 c E_0^2$ and the radius of the diffraction-limited beam waist w_0 is related to the numerical aperture (NA) by $w_0 = \frac{\lambda}{2NA}$. Substituting the above into equation (2.19), we find that the strongest gradient force is located on the optical axis at $(x, y, z) = (0, 0, \frac{\pi\lambda}{4\sqrt{3}NA^2})$. We can then take the ratio of the gradient force at its strongest to the scattering force at the same point to find the condition for stable trapping of a Rayleigh particle [69]:

$$\frac{F_{grad}}{F_{scat}} = \frac{3\sqrt{3}\lambda^3 NA^2}{32\pi^5 a^3} \left[\frac{(n_p/n_m)^2 + 2}{(n_p/n_m)^2 - 1} \right] \geq 1. \quad (2.23)$$

While the above result was derived only for Rayleigh particles, it is still instructive for two trends it describes. First, a high numerical aperture is crucial for a single-beam optical tweezers setup as the strong focusing drastically increases the gradient force without having as significant an effect on the scattering force. Second, the radius of the trapped particle has an enormous impact on the trapping stability. This radius-dependence is a consequence of the scattering force going as a^6 while the gradient force only goes as a^3 . Notably, this provides an upper bound on the size of particles that can be

trapped while it does not give a lower bound. To find a lower bound on the radius of stable trapping, one must consider the thermal forces present in the system.

The scaling of optical trapping forces with particle radius means that smaller particles feel the effects of the gradient force more strongly than the scattering force, but particles of all sizes experience the same magnitude of thermal forces. The environment contributes $\frac{1}{2}k_B T$ of energy per degree of freedom to particles regardless of their dimensions and drives trapped particles to undergo Brownian motion that competes against the confining gradient force. Translating the gradient force to an energy well U_{grad} that the particle must escape, we see that $U_{grad} = \int \vec{F}_{grad} \cdot \vec{s} \propto a^3$ (where $\vec{s}$ is the escape path) so smaller particles sit in smaller energy wells. Once the well is shallow enough, thermal energy can eject the particle from the trap no matter the size of F_{grad}/F_{scat} thereby setting the lower bound on the size of trappable particles.

2.2.1.3 Optically Induced Torques

Up to this point, we have only considered forces that originate from the intensity of light and have not considered torques that arise from changes in light's angular momentum. A graduate-level electricity and magnetism textbook can show that changes in the angular momentum of light lead to mechanical torques on illuminated objects [74]. While this interaction can often be neglected for linearly polarized light in optically isotropic materials,

angular momentum transfer may become a dominant force in systems with multiple indices of refraction [75].

Richard Beth was the first to measure the transfer of angular momentum from light to an object in 1936 when he measured the torque on a birefringent plate suspended on a torsional pendulum as it was illuminated with circularly polarized light [76]. His experiment was remarkable both because it confirmed the sign and magnitude of the torque predicted by theory, and also because it was one of the first examples of electromagnetic radiation being harnessed for mechanical motion. This measurement was a great experimental feat, but since then it has become a commonly exploited process in optical tweezer systems. Experimentalists often rely on Laguerre-Gaussian beams to impart angular momentum [77,78], or they exploit the physical asymmetry [79] or birefringence of the trapped particle to achieve rotation [80]. The field has come so far that it is now possible to spin nanoscale silica dumbbells at rates of up to 1.1 GHz [75], and even create colloidal assemblies that experience negative torque [81,82]. For this dissertation, we will focus on the case of light impinging on a birefringent material.

We can find a quantitative description of the optical torque on a birefringent particle by following Friese, *et al.*'s method [80]. First, an elliptically polarized beam of light given by $\vec{E} = E_0 e^{i\omega t} \cos \phi \hat{x} + i E_0 e^{i\omega t} \sin \phi \hat{y}$ (where ϕ describes the ellipticity) is incident on the sample. The light hits the sample at an angle θ between its polarization and the optic axis of the birefringent sample so we rewrite the field in this frame

$$\begin{aligned}\vec{E} &= E_0 e^{i\omega t} (\cos \phi \cos \theta - i \sin \phi \sin \theta) \hat{x} \\ &+ E_0 e^{i\omega t} (\cos \phi \sin \theta + i \sin \phi \cos \theta) \hat{y}.\end{aligned}\tag{2.24}$$

After passing through the sample of thickness d with extraordinary and ordinary indices of refraction n_e and n_o , respectively, the outgoing light has a phase shifts of kdn_e and kdn_o

$$\begin{aligned}\vec{E} &= E_0 e^{i\omega t} e^{ikdn_e} (\cos \phi \cos \theta - i \sin \phi \sin \theta) \hat{x} \\ &+ E_0 e^{i\omega t} e^{ikdn_o} (\cos \phi \sin \theta + i \sin \phi \cos \theta) \hat{y}.\end{aligned}\tag{2.25}$$

Finally, we can calculate the angular momentum for each field as $\vec{J} = (\epsilon/2i\omega) \int d^3r \vec{E}^* \times \vec{E}$ and use the difference between them to calculate the time-averaged torque per unit area

$$\begin{aligned}\tau &= -\frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \cos 2\phi \sin 2\theta \\ &+ \frac{\epsilon}{2\omega} E_0^2 \{1 - \cos[kd(n_0 - n_e)]\} \sin 2\phi.\end{aligned}\tag{2.26}$$

For the linearly polarized light we will be using in this dissertation, $\phi = 0$ and we can reduce the above to

$$\tau = -\frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \sin 2\theta.\tag{2.27}$$

This simple derivation shows that unlike for the scattering and the gradient forces, the optical torque exerted on an object does not have a simple

polynomial dependence on the size of the trap object. It is instead a sinusoidal one in which the key quantity is the phase shift of light as it passes through the material ($kd(n_0 - n_e)$). We can, however, still reduce this to a linear relation between the torque and the angular displacement for small θ just as Rorhbach did for the gradient force and the linear displacement

$$\tau \approx -\frac{\epsilon}{\omega} E_0^2 \sin[kd(n_0 - n_e)] \theta, \quad (2.28)$$

which can be useful in analyzing the important timescales in optical trapping systems (Section 2.2.2).

2.2.2 Confined Brownian Motion

Taking the main ideas of the previous two sections, we can now assemble a description of particle dynamics within an optical trap. On short timescales, many of the following results mimic those presented earlier, but the nuances introduced here will be important for properly dissecting experimental results in later chapters.

2.2.2.1 The Langevin Equation and Mean Squared Displacement Revisited

Section 2.1.1 already discussed the Langevin equation in terms of a freely diffusing particle, but we will now return to it with the addition of an optical trapping force. For a particle in the linear region of an optical trap, equation (2.5) can be rewritten as

$$-\gamma_0 \dot{x} + \sqrt{2k_B T \gamma_0} \xi(t) - kx = 0, \quad (2.29)$$

where k is the spring constant of the optical trap, and the particle's position x is measured from the trap center. This in turn leads to confined motion, which is most easily seen in the MSD of the particle. Uhlenbeck and Ornstein go explicitly through the derivation to find [83]

$$\langle \Delta x^2 \rangle(t) = \frac{k_B T}{k} [1 - \exp(-2kt/\gamma_0)], \quad (2.30)$$

where there is still an initial increase in the MSD at short timescales, but this increase slows down at longer timescales and the MSD plateaus as it does for the confined motion in Figure 2.1. The MSD cannot continue to increase linearly because the particle is simply not free to explore an infinite space as it was in equation (2.9). Rather, it is restricted to the confines of the trap. If, however, we look on shorter timescales, we can recover the MSD for free space from equation (2.30). This timescale is known as the autocorrelation time $\tau_{\text{auto}} = \gamma_0/k$ and it can be thought of as the time it takes for the particle to diffuse deep into the confining potential. At times much shorter than the autocorrelation time, we can assume that the particle does not sense the optical trap and therefore exploit equation (2.9) for things like detector calibration (Section 2.3.1). For a 208 nm particle in a trap with spring constants of $k_x = 1.5 \text{ pN}/\mu\text{m}$, $k_y = 1.5 \text{ pN}/\mu\text{m}$, and $k_z = 0.3 \text{ pN}/\mu\text{m}$, these autocorrelation times are $\tau_x = 1.1 \text{ ms}$, $\tau_y = 1.7 \text{ ms}$, and $\tau_z = 5.5 \text{ ms}$.

Again, we can just as easily write down these equations for rotational Brownian motion. Starting with equation (2.6), we just add in the optical torque (equation (2.27) multiplied by the area A) to find

$$-\gamma_r \dot{\theta} + \sqrt{2k_B T \gamma_r} \xi(t) - A \frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \sin 2\theta = 0. \quad (2.31)$$

The autocorrelation time for rotational motion can be found in this case by taking a small angle approximation as in equation (2.28) and then taking the ratio of the rotational drag coefficient to the effective rotational spring constant k_{eff}

$$-\gamma_r \dot{\theta} + \sqrt{2k_B T \gamma_r} \xi(t) - \underbrace{A \frac{\epsilon}{\omega} E_0^2 \sin[kd(n_0 - n_e)]}_{k_{\text{eff}}} \theta = 0. \quad (2.32)$$

Since γ_r varies so drastically with the aspect of trapped rods, it is difficult to give a general value for the autocorrelation time. A quick benchmark would be that a 9 μm long microtubule that is 25 nm in diameter trapped in a 50 mW standing wave optical trap has an autocorrelation time of roughly 10 s. This is considerably longer than the autocorrelation times for spherical particles in tightly focused optical traps due to the slower dynamics of rotating rods and the weaker relative strength of optical torques.

2.2.2.2 Power Spectral Density

Another useful quantity that comes out of the Langevin equation for confined Brownian motion is the power spectral density (PSD) of the particle's position. The PSD is a Fourier transform of the particle position given by

$$PSD(f) = \frac{D_0/2\pi^2}{f^2 + f_c^2}, \quad (2.33)$$

where $f_c = k/(2\pi\gamma_0)$ is the corner frequency and $f = i/T_{\text{msr}}, (i = 1, 2, \dots)$ is the frequency measured in integer multiples of the inverse measurement time T_{msr} [84]. This Lorentzian curve can be used in fitting experimental data to extract quantities like the spring constant and even the particle radius if a known, periodic force is applied to the system [85]. Furthermore, the diffusion constant from this fit is often used for detector calibration [86] although we will use a slightly different method that combines the power spectral density with measurements of particle diffusion (Section 3.1.5.3).

2.2.2.3 Full Hydrodynamic Theory

The previous sections focused heavily on the overdamped Langevin equation for confined Brownian motion, but those treatments neglected two main things: the inertia of the particle and the inertia of the fluid itself. Both of these stem from the drag forces that couple the particle and the surrounding fluid together. As the particle is slowed by the fluid, the fluid is simultaneously dragged along by the particle leading to memory effects that

cause the past motion of the particle to influence the current motion of the fluid. These memory effects ultimately change the friction between the fluid and the particle from a simple linear relation with the particle velocity to

$$F_{fr} = -\gamma_0 \dot{x} - \frac{2}{3} \pi \rho_f a^3 \ddot{x} - 6a^2 \sqrt{\pi \rho_f \eta} \int_0^t \frac{\ddot{x}(\tau)}{\sqrt{t-\tau}} d\tau, \quad (2.34)$$

where ρ_f is the density of the fluid [87]. This complete form of the fluid friction retains the Stokes drag as the first term, but adds a fluid inertia component as the second term and a memory effect as the third term. Berg-Sørensen and Flyvbjerg accounted for these effects in a hydrodynamically correct form of the Langevin equation to calculate an updated PSD [84]

$$PSD_{hydro}(f) = \frac{D_0/\pi^2 \left[1 + (f/f_\nu)^{1/2}\right]}{\left(f_c - f^{3/2}/f_\nu^{1/2} - f^2/f_m\right)^2 + \left(f + f^{3/2}/f_\nu^{1/2}\right)^2}. \quad (2.35)$$

Here, $f_m = \gamma_0/(2\pi m^*)$, with $m^* = m + 2\pi\rho_f a^3/3$, is the timescale for friction to dissipate the inertia of the fluid and a spherical particle and $f_\nu = \eta/(\pi\rho_f a^2)$ is the timescale for flow near the particle. This full hydrodynamic treatment is unnecessary for the relatively long timescales in the current work, but is of great importance for high bandwidth measurements like the observation of ballistic Brownian motion [59, 60].

2.3 Photonic Force Microscopy

We use a custom built optical trap known as a photonic force microscope (PFM) to study microtubule networks with high bandwidth and high spatial precision. Its design has been refined over several years with the current iteration having been built by previous students, Dr. Pinyu Thrasher, Dr. Martin Kochanzyk, and Dr. Tobias Bartsch [88–91].

2.3.1 Position Detection

A microscope’s spatial precision is only as good as its temporal resolution, which is why the heart of the photonic force microscope is its high bandwidth detector. Traditional video imaging relies on cameras with integration times of $\approx 1 - 100$ ms, which can lead to motion blurring as a trapped particle undergoes Brownian motion. Using an interferometric detection scheme with a quadrant photodiode (QPD) circumvents this problem and results in high precision particle tracking. With a bandwidth of 1 MHz, the QPD samples much more quickly than the autocorrelation time for a 208 nm particle (1 – 10 ms) so we can reduce equation (2.30) to (2.9)—the expression for free diffusion of a particle—and then calculate how far a trapped particle will move. The 1 MHz bandwidth translates to a 1 μ s QPD integration time so a trapped 208 nm particle will only move 2 nm during a single sampling period, based on the Einstein relation. This small amount of movement ensures that our setup is suitable for measurement down to the nanometer range.

The position measurement itself relies on back focal plane interferome-

try in which laser light scattered by the trapped particle is collected together with unscattered light by the condenser lens and focused onto the QPD. The resulting interference pattern casts different amounts of light onto each quadrant of the QPD depending on the position of the particle within the trapping laser (Figure 2.4) and allows for spatial resolution below the diffraction limit. We shall refer to the signals on each quadrant as S_I , S_{II} , S_{III} , and S_{IV} . Differentials of these signals give the x-signal (S_x) and the y-signal (S_y)

$$S_x = (S_I + S_{II}) - (S_{III} + S_{IV}) \quad (2.36)$$

$$S_y = (S_I + S_{III}) - (S_{II} + S_{IV}), \quad (2.37)$$

which are linearly proportional to the change particle position near the trap center.

Initially, this detection scheme was only developed for tracking in two dimensions, but it was discovered that the particle's position along the optical axis could be inferred from the sum of the four quadrants

$$S_z = S_I + S_{II} + S_{III} + S_{IV}, \quad (2.38)$$

where S_z is the z-signal [92, 93]. This detection along the optical axis is made possible by the Gouy phase shift in a Gaussian beam in which the phase of the light changes by π as it moves through the focus. Light scattered by the particle does not undergo the same shift as the unscattered light so the

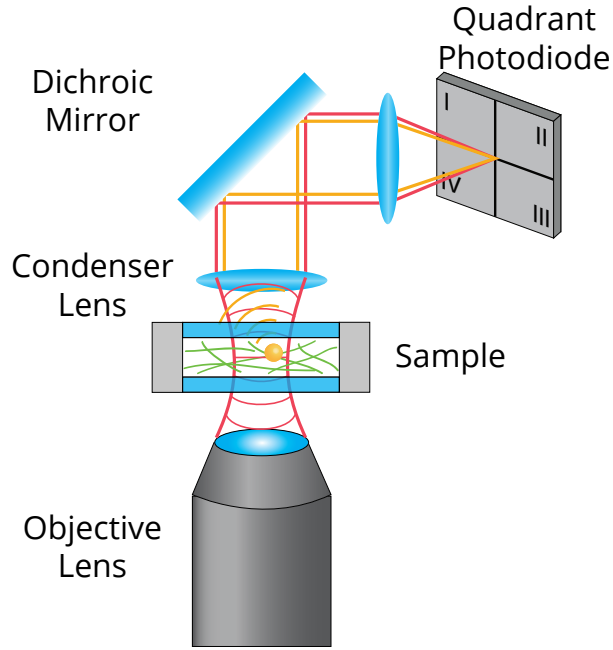


Figure 2.4: **Back focal plane interferometry.** The position of a trapped particle can be tracked by capturing the unscattered laser light together with the light scattered by the particle and focusing it onto a quadrant photodiode. The resulting interference pattern allows for tracking below the diffraction limit.

intensity of the pattern (given by the sum of all four quadrants of the QPD) varies linearly with the particle's axial position inside the focus [94].

2.3.1.1 Detector Calibration

To determine the particle's position, the QPD voltage signals need to be converted into distance measurements. This conversion can be seen most easily by attaching a spherical particle to a glass coverslip and then moving it through the laser focus. Polystyrene beads with a diameter of 208 ± 10 nm

and fluorescently labeled with Suncoast Yellow (FSSY002, Bangs Laboratories, Fishers, IN) are diluted from their initial concentration of 1% solids by 10^5 times into water and the diluted solution is evaporated onto a glass coverslip using a hotplate. This coverslip is then affixed to the bottom of the sample chamber using dental wax before the top coverslip is attached with vacuum grease and the sample is filled with water. Once the sample is mounted on the PFM, the beads are visualized with fluorescence and a single particle is positioned in the center of the trap by moving the piezo stage. Controlled steps of constant size are then taken by the piezo to move the particle through the laser focus while its voltage signals are recorded by the QPD.

Near the trap center, S_x , S_y , and S_z change approximately linearly with the particle's position in the trap (Figure 2.5) allowing for conversion between voltage and position through a simple fit of the detector response. However, this response becomes more complex as the particle moves farther from the center of the trapping beam until it is no longer possible to make a simple conversion between the voltage and position signals. Moreover, this kind of calibration that relies on a particle baked to the coverslip cannot be used for particle tracking in solution. Interference of the laser light with the coverslip along with abrupt shifts in index of refraction at the surface alter the signal offsets from what we would expect away from the coverslip. Fortunately, Christian Tischer solved this issue through a nonlinear detector calibration that can be run *in situ* with trapped particles [95,96].

Nonlinear calibration for a trapped bead starts far from any surfaces

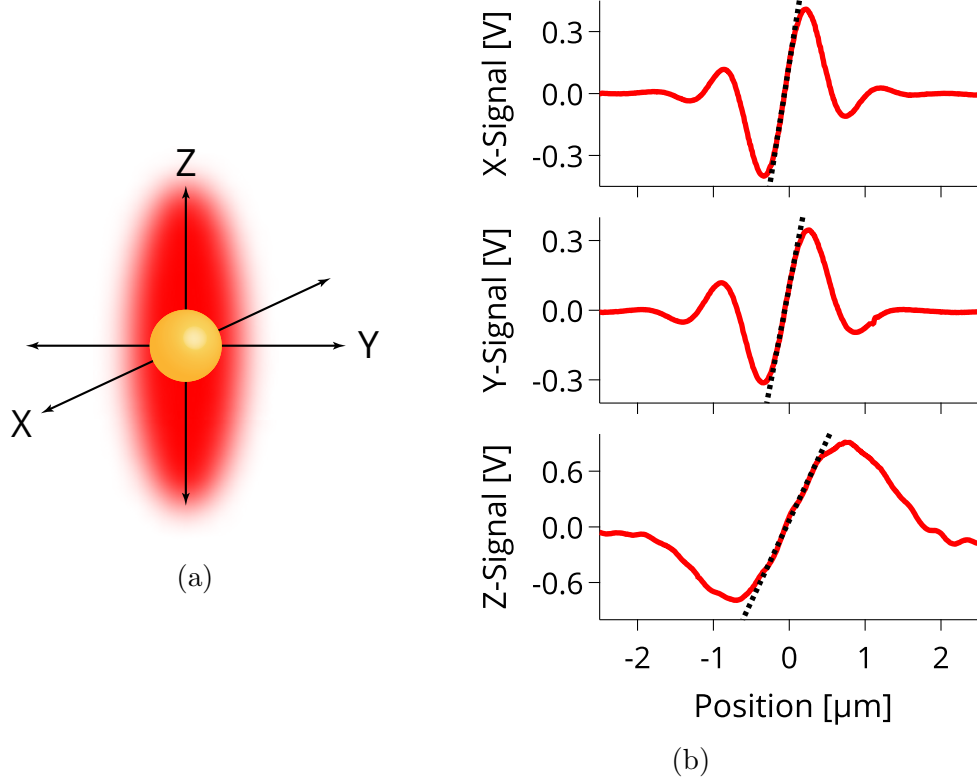


Figure 2.5: **Detector response of a spherical particle.** (a) A particle (yellow) near the center of an optical trap (red) is moved along each axis individually. (b) As the particle moves along any single axis, it produces a characteristic response on the QPD (red) with a linear region near the center of the trap (black dashed).

($\gtrsim 10\mu m$) that might affect the diffusion of the particle (Section 2.1.3). With the high bandwidth of the QPD, the rate at which the particle's position is recorded is much faster than the timescale for the particle's diffusion. As such, we expect the motion of the particle to be linearly related to the diffusion constant over a time Δt as

$$\text{Var}(\Delta x)|_{\vec{r}} = \langle \Delta x^2 \rangle|_{\vec{r}} - \langle \Delta x \rangle^2|_{\vec{r}} = 2D_0\Delta t, \quad (2.39)$$

where $\vec{r}$ is the particle's position. Since we are far from any surfaces, D_0 is a constant and we can exploit this by comparing the expected variance in particle displacements from equation (2.39) to the measured variance in detector voltages from the QPD. Writing the changes in voltage at each time step as ΔS_x and taking the detection signal at position x_0 to be $S_x(x_0) = S_{x0}$, we write the variance in voltage changes at a specific point S_{x0} as

$$\text{Var}(\Delta S_x)|_{S_{x0}} = \langle \Delta S_x^2 \rangle - \langle \Delta S_x \rangle^2. \quad (2.40)$$

Even though the detector response is nonlinear, we can make use of the fact that the response is approximately for small displacements Δx .

$$\Delta S_x \approx \frac{\partial S_x}{\partial x}|_{S_{x0}} \Delta x \quad (2.41)$$

Combining equations (2.40) and (2.41) and linking them to the variance in the particle position, we now see that

$$\text{Var}(\Delta S_x)|_{S_{x0}} \approx \left(\frac{\partial S_x}{\partial x}|_{S_{x0}} \right)^2 \text{Var}(\Delta x)|_{\vec{r}} = \left(\frac{\partial S_x}{\partial x}|_{S_{x0}} \right)^2 2D_0\Delta t. \quad (2.42)$$

After rearranging for the detector response:

$$\frac{\partial S_x}{\partial x}|_{S_{x0}} \approx \sqrt{\frac{\text{Var}(\Delta S_x)|_{S_{x0}}}{2D_0\Delta t}}. \quad (2.43)$$

With this expression for the local detector sensitivity, we can finally integrate it to find the position of the particle as a function of the detector voltage

$$dx = \frac{\partial S_x}{\partial_x S_x} \Rightarrow x(S_x) = \int \frac{1}{\partial_x S_x|_{S'_x}} dS_x|_{S'_x}. \quad (2.44)$$

This final expression is fit with a fifth-order polynomial to capture the nonlinear behavior of the detector throughout this region.

The above procedure was for the x-direction of the trap, but it can be applied identically to the y- and z-directions to obtain the detector response in 3D. This calibration is done for each particle at the start of an experiment with the detector sensitivity being calculated for $10 \times 10 \times 10 \text{ nm}^3$ voxels prior to the integration step.

2.3.2 Thermal Noise Imaging

Given enough time, an optically trapped particle will fully explore the region around the laser focus unless it is inhibited by another object. When tracked with high spatial and temporal precision, that particle's exploration can be used to learn about its environment with a technique known as thermal noise imaging [89]. The principle of this technique is that the particle's surroundings are mapped by observing where the probe particle cannot go. The space explored by the particle is divided up into smaller volumes known

as voxels and then the number of times that the particle visits each of these smaller volumes (known as the occupancy) is recorded. By checking on the occupancy of these voxels, it is possible determine which regions in the trapping volume exclude the particle and are likely to contain biopolymers or other objects of interest (Figure 2.6).

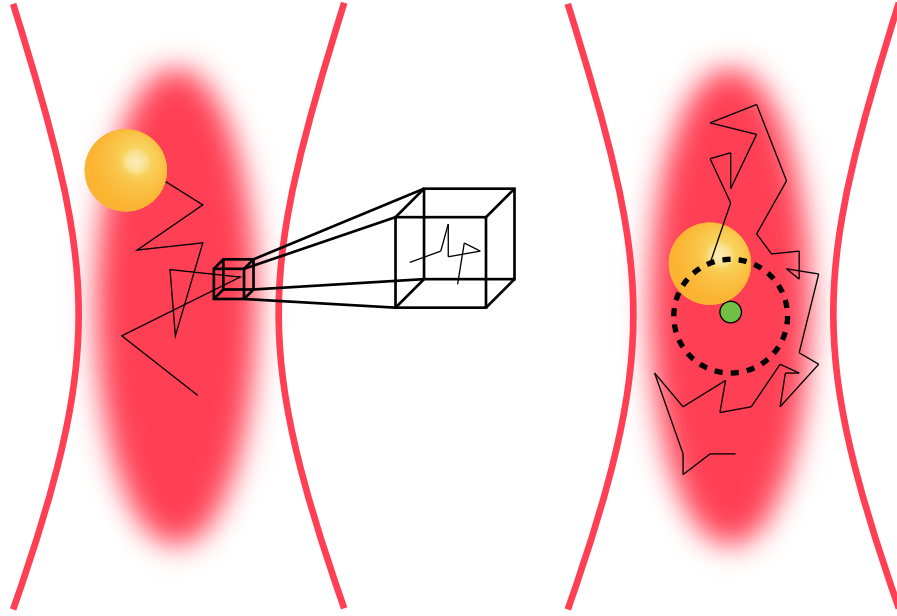


Figure 2.6: **Thermal noise imaging.** An optical trap (red) confines the movement of a probe particle (yellow) as it explores the surrounding environment. Particle positions are tracked and binned into $10 \times 10 \times 10 \text{ nm}^3$ voxels. Biopolymer filaments in the sample (green) repel the particle and create excluded volumes.

The confinement imposed by an optical trap means that a trapped particle can only explore a small region around the laser's focus. For the system in this dissertation, the laser focus is roughly $300 \times 300 \times 900 \text{ nm}^3$. This is obviously not large enough to study structures like microtubule networks

that span microns so the trap needs to be repositioned several times with each voxel's occupancy being aggregated over the scanned region. One experiment covering a $1 \times 1 \times 1 \mu\text{m}^3$ volume can take over thirty minutes so it is important to keep the sample stable to maintain the consistency of the voxel positions. If the sample moves too unpredictably during a scan, it can cause voxels to overlap in unexpected ways thereby reducing the accuracy and resolution of the data.

Another important factor in the resolution of a thermal noise imaging scan is the size of the probe particle. The probe's size determines not only how strongly it interacts with the optical trap, but also sets a lower limit on the resolution of structures that can be imaged. This applies specifically to concave structures within a thermal noise image. It is clear in Figure 2.7 that a particle may easily explore a convex structure that is smaller than its diameter, but that steric interactions inhibit exploration of concave structures at the same size scale. Such imaging artifacts need to be addressed during the analysis of thermal noise images.

A logical response to artifacts caused by the finite size of the probe particle would be to reduce the probe's size, but that leads to other problems. Reducing the size of the probe makes trapping more unstable as thermal energy is size invariant (Section 2.2.1.2). Smaller particles could be used if they were optically dense gold particles, which can be trapped with approximately six times stronger spring constants than polystyrene particles of the same size [97, 98]. The increased optical density, unfortunately, comes with strong absorption

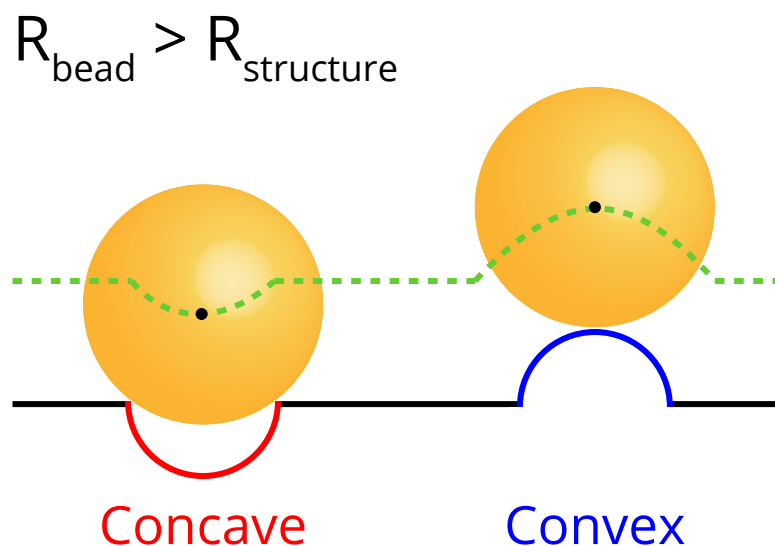


Figure 2.7: **Thermal noise imaging artifact caused by a finite probe radius.** A bead (yellow) traces out a path (green) as it moves across concave (red) and convex (blue) surfaces. The concave surface cannot be fully mapped by the bead due to its smaller radius, which restricts structural information in thermal noise images.

of laser light by gold and other metallic particles that causes intense heating and makes them unsuitable for studies of temperature-sensitive biopolymers [99]. To avoid these issues while maintaining sufficiently small particle sizes for imaging, we use 208 nm diameter polystyrene particles as probes.

With 208 nm polystyrene probe particles, thermal noise imaging has several advantages over competing techniques for studying biopolymer networks. Atomic force microscopes (AFM) are commonly employed in the study of biological filaments, but their cantilevers are considerably stiffer ($0.1 - 1$ N/m) than optical traps ($10^{-6} - 10^{-7}$ N/m), leading to increased deformation of and damage to soft biological materials. It is also not possible to study 3D

arrangements of filaments within a network using an AFM. Super resolution microscopy techniques like stimulated emission depletion and photoactivated localization microscopy avoid this pitfall while maintaining resolution beyond the diffraction limit [100, 101]. These techniques are not without their own drawbacks, however, as they require labeling that may change protein structures and long exposure times to achieve their high spatial precision. Even when dynamics are studied, they necessitate high photon fluxes from fluorescent excitation sources that can damage biological materials.

2.3.3 Setup

A diagram for the PFM used in this dissertation is shown in Figure 2.8. Since stability of the laser is of the utmost importance, a 500 mW 1064 nm, linearly-polarized laser with a neodymium-doped yttrium aluminum garnet medium is used (Mephisto, Noise Eater Option, Coherent, Santa Clara, CA). The laser is first sent through a Faraday isolator (EOT Optics, Traverse City, MI) to prevent back-reflections before entering a 10x beam expander (Sill Optics, Wendelstein, Germany) that also collimates the beam. To maintain a weak trap, the beam power is reduced 70% by a neutral density (ND) filter. After passing through a shutter that can block the laser, a dichroic mirror directs the beam upwards as it overfills the back aperture of the objective lens (UPlanSApo, 60 $\times$, water immersion, 1.2 NA, Olympus, Tokyo, Japan), which strongly focuses it through the 0.17 mm thick bottom coverslip (15CIR-1.5, Thermo Fisher, Waltham, MA) into the sample. A water-immersion objective

is used to reduce the number of index refraction changes when going from the objective to the sample. The laser light that makes it through the sample crosses the top coverslip and is then gathered by a condenser lens (oil immersion, 1.4 NA, Zeiss, Oberkochen, Germany) and turned by a dichroic mirror onto the detection pathway. A set of two lenses briefly collimate the beam so that an ND filter can reduce the power by 95% before impinging on the QPD.

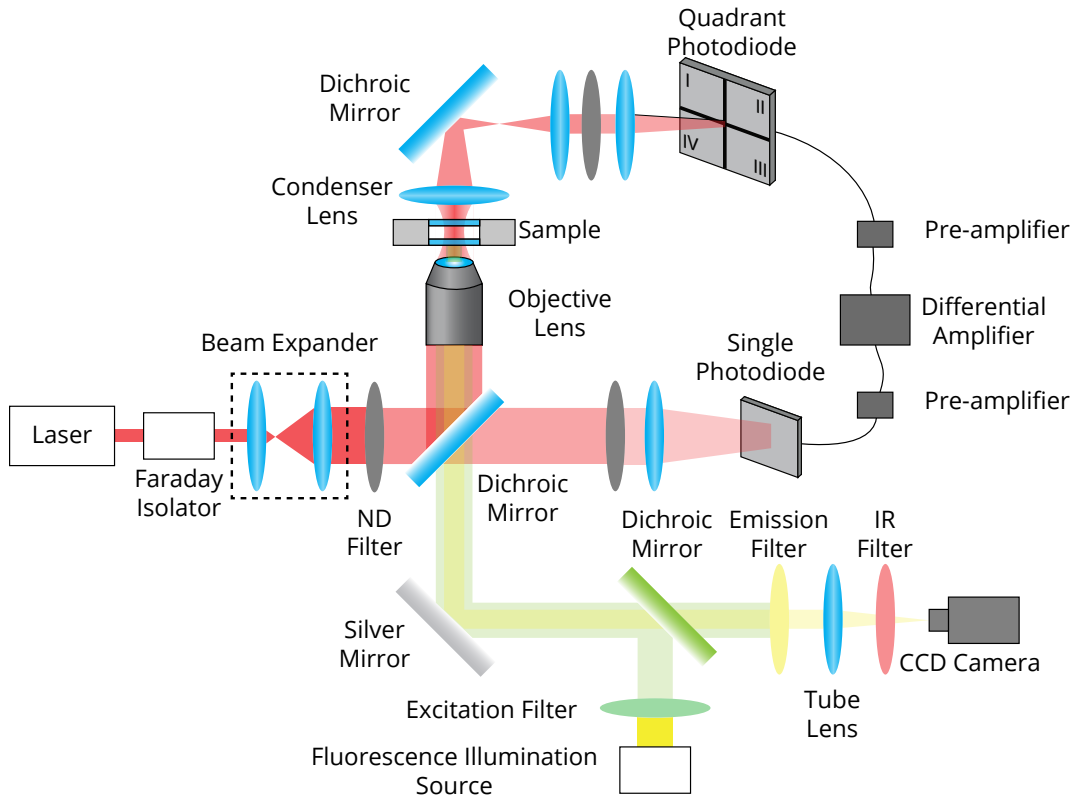


Figure 2.8: **Photonic force microscope setup.** See Section 2.3.3 for a detailed description.

The QPD itself is a 2 mm diameter device composed of 4 indium gallium arsenide quadrants separated by small gaps (G6849, Hamamatsu, Hamamatsu

City, Japan). Each section can measure intensity with a high bandwidth up to 120 MHz. To center the QPD within the beam, it is mounted on a two-axis micrometer positioning stage (FV 40-XY, OWIS, Staufen, Germany). The QPD output is passed through a preamplifier and then a differential amplifier, both of which were custom made by Oeffner Measurement Technology (Plankstadt, Germany) and have 1 MHz bandwidths. A 16-bit data acquisition board (PCI-6120, National Instruments, Austin, TX) digitizes the signals from the amplifier with each of its four channels capable of sampling at 800 kHz. It is important to note that even though its maximum sampling rate is less than the 1 MHz of the amplifiers, this board has an integration time of ~ 9 ns per data point allowing it to preserve the signal without additional averaging.

To achieve maximum stability, a custom-built chamber houses the sample and is turn held by a three-axis piezo electric translation stage (PDQ375, Mad City Labs, Madison, WI). This piezo is capable of $75\text{ }\mu\text{m}$ of movement in both the x- and y-directions (perpendicular to the optical axis) and $50\text{ }\mu\text{m}$ of movement along the z-direction (parallel to the optical axis). For larger movements across the sample, the piezo is secured to a two-axis micrometer stage that has 25 mm of travel (MCL- μ S1550, Mad City Labs, Madison, WI).

Particles and microtubules within the sample chamber are visualized using epifluorescence with broadband excitation light coming from a metal-halide lamp (X-cite 120, EXFO, Quebec City, Canada). The light then passes through a bandpass filter (HQ535/50 $\times$, Chroma, Bellow Falls, VT) to give

it the appropriate spectrum before it reflects off a dichroic mirror (Q565LP) and passes into the objective lens. Fluorescence emission is collected by the objective and goes through a longpass filter (HQ615/60m) before hitting a charge-coupled device (CCD) camera (SensiCam QE, PCO, Kelheim, Germany).

2.3.4 Stability and Position Detection Resolution

As mentioned in Section 2.3.2, stability is of the utmost importance when performing thermal noise imaging scans across large volumes. The Brownian motion of the probe particle intrinsically links the PFM's temporal resolution to the precision of its position detection. By choosing a detection system with 1 MHz bandwidth, we can track a 208 nm particle as it moves approximately 2 nm every 1 μ s, but it is important to remember that a typical experiment can take up to 30 minutes. This leaves an enormous range of timescales from 1 mHz to 1 MHz that must be examined for sources of noise to get the most out of the instrument.

Each piece of equipment from the laser through to the sample and then on to the detection path and electronics can be a source of noise in our experiment. This noise can range from mechanical vibrations in the optics and mounts at 50-1000 Hz to laser power and pointing instabilities that appear at frequencies above 1 kHz. The most direct way to characterize the noise from all of these sources is to use beads fixed to the coverslip as a measuring device. By baking a 208 nm bead to a coverslip, we can move it through the trapping

laser and determine a calibration from voltage to position (Section 2.3.1.1). Then we can move the bead so that it is centered in the beam and record its response for several seconds at 100 kHz to measure noise in our system. Since the bead is firmly fixed to the bottom coverslip, any fluctuations in the signal must come from noise in our system instead of being dominated by thermal fluctuations. Moreover, these changes in the bead's signal can be converted into positions so that the impact of any noise on our spatial precision can be easily determined.

The specific mathematical techniques employed here are the power spectral density and the cumulative relative standard deviation (CRS), with the CRS being defined over a range of frequencies f_1 to f_2 as

$$\text{CRS}(f_1, f_2) = \left[\int_{f_1}^{f_2} \text{PSD}(f) df \right]^{1/2}. \quad (2.45)$$

Kochanczyk *et al.* showed the power of this technique in identifying noise sources that may be hidden in PSD analyses [102]. Figure 2.9 shows both the PSD and CRS for a 208 nm particle with the last point on each CRS curve giving the standard deviation of the measurement in the band of 1 Hz-50 kHz. This gives 1.2 nm along the x-axis, 0.8 nm along the y-axis, and 0.9 nm along the z-axis. It is important to note that these curves can change not just due to the noise in the system, but also due to alterations in the optical path such as using a larger or smaller aperture in the condenser lens. While the CRS provides a good measurement of the noise in the system, it is limited to

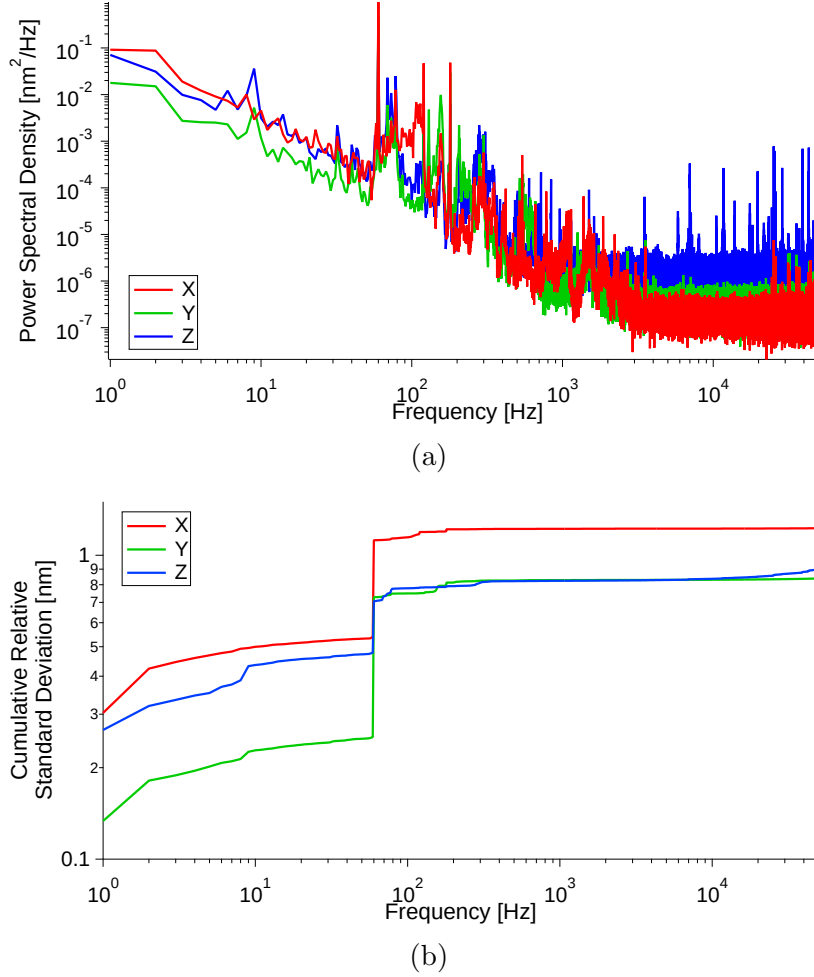


Figure 2.9: **Measuring sources of instrument noise.** A 208 nm polystyrene bead is immobilized to the bottom coverslip of the sample chamber and is centered in the trapping laser. The x, y, and z detector signals are sampled for 7 seconds at 100 kHz then they are converted into bead positions using nonlinear fitting of the bead's detector response. (a) The PSD for each axis shows the contributions of different frequencies to the total noise as peaks and shows the noise floor. (b) By integrating the PSD using equation (2.45), we can more clearly show the total noise in the instrument. The final point on each curve gives the standard deviation of the measurement along that axis for the bandwidth shown.

timescales on the order of 1 Hz and faster. For longer timescales, we need to concern ourselves not with the particle motion and instrument noise, but rather with the motion of the sample relative to the objective lens.

It takes roughly 30 minutes to scan a $1 \times 1 \times 1 \mu\text{m}^3$ volume so the sample's drift must, at a minimum, be less than 30 nm/min to keep the region of interest from moving out of the scanning volume. We tested this by baking 208 nm diameter polystyrene particles to the bottom coverslip of a sample and then tracking their movement over time. Every 5 minutes, the piezo took 25 steps upwards (50 nm at a time) while the CCD captured an image after each step. The point spread functions of the particles were fit by two-dimensional Gaussian functions that provided the particle positions in all three dimensions [103]. For each 5 minute interval, we found the average displacement of all the particles and hence tracked the sample drift relative to the objective lens over time.

Drift along the y- and z-axes was highest during the first 10 minutes after the sample has been moved, but it quickly leveled out. The drift along the x-axis was not as large and also decreased over time. We found steady-state drifts of roughly 2 nm/min along each of the axes, which can be easily accounted for in analysis of thermal noise images. The settling of the drift is thought to stem from the relaxation of the micrometer screws that move the sample over large distances. To ensure stability during experiments, the sample should be left to settle for 20 minutes before a thermal noise imaging scan is started.

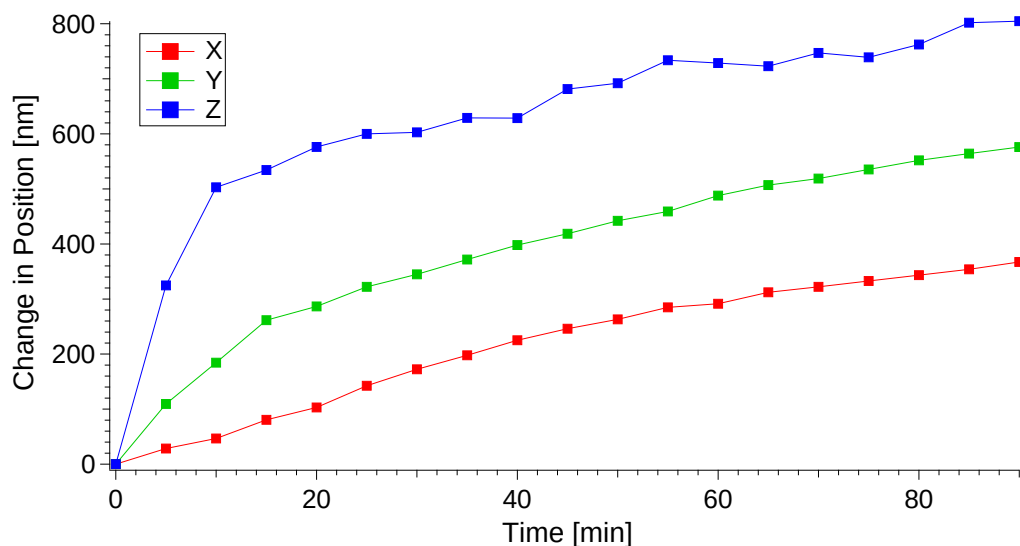


Figure 2.10: **Long-term drift measurements.** 208 nm diameter polystyrene particles were baked onto the bottom coverslip of a sample and images were taken every 5 minutes as the bottom coverslip was moved through the objective's focus in 50 nm steps. Gaussian fits to the point spread functions of each particle tracked their displacements over time. The average displacement for 21 beads is shown here.

2.4 The Standing Wave Optical Trap

Single beam optical traps have always relied on a balance of the scattering force against the gradient force in order to achieve stable trapping. This placed strict limits on the sizes of particles that could be trapped. If the particles were too large, then the gradient force could not hold the particles against the scattering force, but if the particles were too small, then the gradient force could not hold the particles against thermal motion. Then in 1998, Pavel Zemánek and his colleagues at the Academy of Sciences of the Czech Republic developed a new method of optical trapping that solved the issues with trap-

ping large particles and went a long ways towards reducing the difficulty of trapping small particles [104].

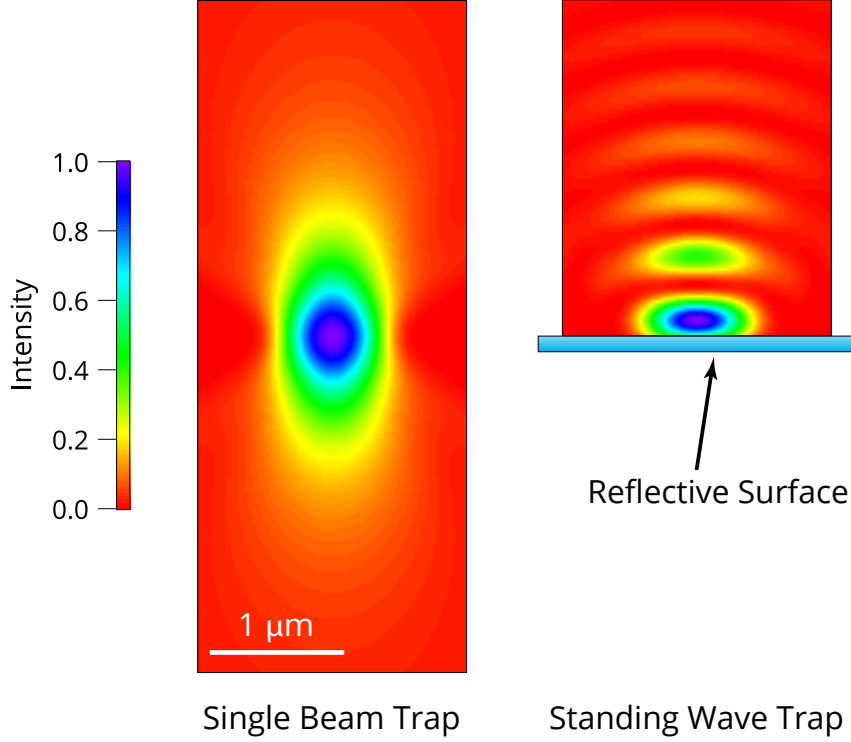


Figure 2.11: **Comparison between single beam and standing wave optical traps.** Intensity distribution for a focused beam (*left*; wavelength in medium $\lambda = 800$ nm and beam waist radius $w_0 = 350$ nm) and after a reflective surface has been inserted at the focal point (*right*; same beam parameters with a $3\pi/2$ phase shift upon reflection). Note that the peak intensity for the standing wave is 3.7 times greater than for the focused beam. Both plots were created using a fifth-order Gaussian beam description [105].

Zemánek proposed using a reflective surface in the sample to create a standing wave from the incident and reflected beams that would then trap particles. This standing wave optical trap (SWOT) has two distinct advantages

over standard optical traps. First, if the laser is focused onto a surface that is close to 100% reflective, then the scattering force is canceled out leaving only the gradient force. With no scattering force, there is not as strict an upper limit on the sizes of particles that can be trapped. Canceling out the gradient force also means that the equilibrium position for particles is closer to the focal point of the objective lens rather than displaced along the axial direction, which improves observation conditions for small objects. Second, the gradient force in a SWOT is much stronger along the optical axis compared to a single beam trap due to the increased intensity and decreased area from the standing wave. Figure 2.11 shows this clearly as the intensity of the beam is now concentrated into a much tighter region ($\sim \lambda/4$ in a SWOT instead of $\sim 2\lambda$ in a traditional optical trap). When combined with the lack of scattering force, this means that the particle spends more time in the higher intensity regions of the trap and experiences greater forces maintaining its position, which has great benefits for long term observation of trapped objects.

2.4.1 Setup

A diagram for the SWOT used in this dissertation is shown in Figure 2.12 with the system being built around an inverted microscope (Axiovert 100, Carl Zeiss, Germany). The linearly polarized 1064 nm trapping laser (CL1064-1W0-S, CrystaLaser, Reno, NV, USA) first passes through a Faraday isolator (110-21052-0002-B1, EOT, Traverse City, MI, USA) to prevent back reflections, which is particularly important in a SWOT arrangement.

Next, the laser passes through a beam expander (S6EXP0050/328, Sill Optics, Wendelstein, Germany) that increases the diameter 5 times and then a half-wave plate (WPH05M-1064, Thor Labs, Newton, NJ, USA) that allows for adjustment of the polarization. A dichroic mirror (ZT532rdc NIR-UF2, Chroma Technology Corporation, Bellows falls, VT, USA) reflects the beam up through an objective lens (Plan-Neofluar 100x/1.3 Oil Pol, Carl Zeiss, Germany) so that it is focused in the sample made of a 0.17 mm thick square coverslip on the bottom and custom-made dichroic coverslip on the top (1064 nm reflectivity > 99%, Institute of Scientific Instruments of the ASCR, Czech Republic). Visualization of the sample takes place through a darkfield condenser connected to a fluorescent source capable of red, green, and blue broadband illumination (X-cite XLED1, Excelitas, Waltham, MA, USA). Taking advantage of the darkfield illumination and the iris in the objective to eliminate the excitation light, the emitted fluorescent light is sent unfiltered to a CCD camera (Rolera-XR Fast Cooled, Q-Imaging, Surrey, BC, Canada).

Since the setup utilizes fluorescence illumination with a darkfield condenser and an objective lens with an iris, all data analysis is done through particle tracking. The darkfield optics eliminate much of the excitation light, but there is still some scattering that makes it through the system. This means that the images observed on the camera are a combination of fluorescence emission as well as darkfield scattering, but the fluorescence signal is much stronger for fluorescently-labeled objects like microtubules.

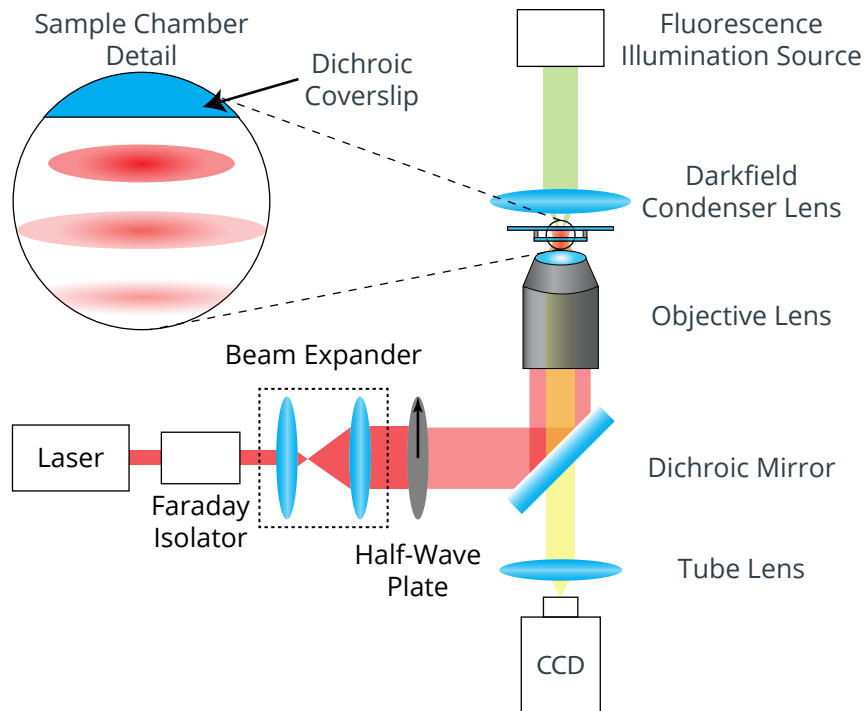


Figure 2.12: **Standing wave optical trap setup.** See Section 2.4.1 for a detailed description.

2.5 Simulating Confined Brownian Motion

While it is possible to carry out statistical mechanics calculations to determine averages, variances, and other quantities pertinent to motion in thermalized systems, computational simulations can provide direct insight into how well theory matches with experimental data. Forces can be added or removed with relative ease to determine which ones are dominant in the observed phenomena. We will make use of simulations in this dissertation to study the degree to which microtubule birefringence is responsible for the behavior of microtubules in a SWOT.

2.5.1 Discretization of the Langevin Equation

In addition to its usefulness in the derivation of statistical quantities like the MSD, the Langevin equation provides a good foundation for the simulation of confined Brownian motion. Starting with equation (2.6) for rotational Brownian motion, we add in an optical torque from equation (2.27) and use a finite difference method to calculate the derivatives

$$I \frac{\theta_i - 2\theta_{i-1} + \theta_{i-2}}{(\Delta t)^2} = -\gamma_r \frac{\theta_i - \theta_{i-1}}{\Delta t} + \sqrt{2k_B T \gamma_r} \xi_i - A \frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \sin 2\theta_{i-1}, \quad (2.46)$$

where θ_i is the angle at the i^{th} time step and ξ_i is white noise with 0 mean and variance $1/\Delta t$. We then substitute $\xi_i = w_i/\sqrt{\Delta t}$ (with w_i being chosen from a Gaussian distribution centered at 0 with a variance of 1) and rearrange for θ_i

$$\theta_i = \frac{2 + \Delta t (\gamma_r/I)}{1 + \Delta t (\gamma_r/I)} \theta_{i-1} - \frac{1}{1 + \Delta t (\gamma_r/I)} \theta_{i-2} + \frac{\sqrt{2k_B T \gamma_r}}{I [1 + \Delta t (\gamma_r/I)]} (\Delta t)^{3/2} w_i - A \frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \sin 2\theta_{i-1} \frac{(\Delta t)^2}{I [1 + \Delta t (\gamma_r/I)]}. \quad (2.47)$$

Just as we did for the continuous Langevin equation, we can simplify our discretized result if we neglect the inertial term (i.e. the left hand side of equation (2.46)). Then we find

$$\theta_i = \theta_{i-1} - \sqrt{2D\Delta t} w_i + \gamma_r A \frac{\epsilon}{2\omega} E_0^2 \sin[kd(n_0 - n_e)] \sin 2\theta_{i-1} \Delta t, \quad (2.48)$$

noting that we have simplified the square root expression by using the Stokes-Einstein relation.

2.5.2 Time Step Selection

Both equation (2.47) and equation (2.48) can be used in Brownian motion simulations, but the choice of time step is critical in choosing between the two. At times shorter than the inertial dissipation $\tau = I/\gamma_r$, the simulation cannot neglect the particle inertia represented by the second derivative term and must use equation (2.47). Figure 2.13 compares angular displacement traces for 2D simulations of rotating rods under no external torque with time steps less than the inertial dissipation time. The trace that maintains the influence of the rod's mass is smooth and continuous as the rotation of the rod shows inertial resistances to changes in direction. On the other hand, the non-inertial approach leads to a jagged and discontinuous curve as the rod instantaneously changes its orientation at each time step. For this dissertation, we do not need to observe phenomena that depend on the inertia of trapped objects so we will use time steps much larger than the inertial dissipation time and neglect the rod's mass as shown in equation (2.48).

The other timescale of note is the autocorrelation time. If the time step of the simulation is on the order of or larger than the autocorrelation time, then particle traces may show oscillatory motion and unphysical behavior [106]. This is more commonly an issue for simulations of spherical particles in optical traps where the particle may diffuse far outside the bounds of the trap, feel

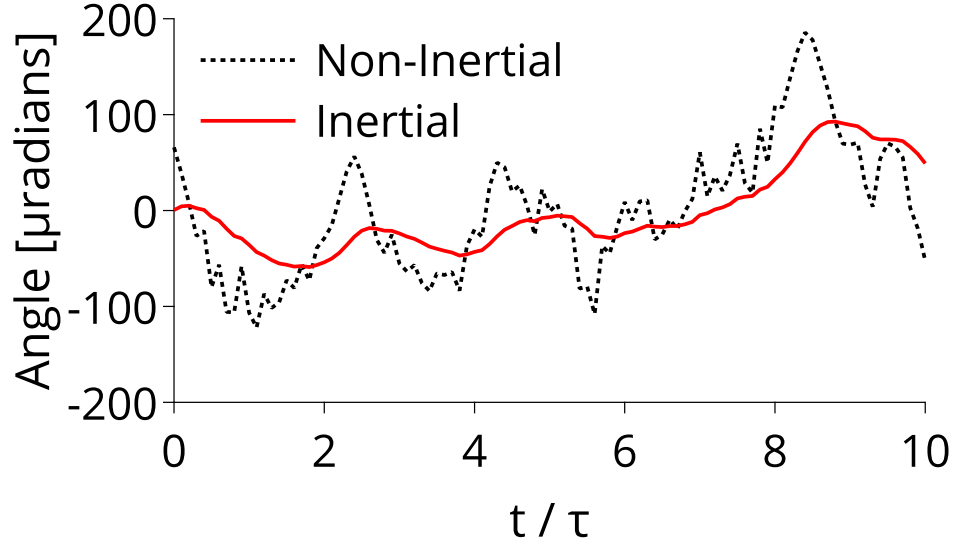


Figure 2.13: **Inertial vs non-inertial simulations.** For times comparable to or smaller than the inertial dissipation time (τ) of a Brownian object, the mass of the particle cannot be neglected. An inertial simulation (red) shows a smoothly changing particle trajectory while a non-inertial simulation (black dashed) produces a jagged, discontinuous trace. Simulations were performed for rigid rods with $\Delta t = 0.1\tau$, $L = 9\mu\text{m}$, $d = 25\mu\text{m}$, $m = 2.6\text{ pg}$, $\eta = 8.9 \times 10^{-4}\text{ Pa s}$, $T = 298\text{ K}$, and $\tau = 132\text{ ns}$.

a huge restoring force in the next time step of the simulation, and then move far outside the trap in the opposite direction to repeat the behavior. Since the restoring force for optical torques is not linearly dependent on angular displacement, a larger time step is not as likely to cause large oscillations, but it will still be avoided by staying far below the autocorrelation time of the simulated system.

Chapter 3

Thermal Noise Imaging of Microtubule Networks

A complete understanding of biofilament network mechanics requires drawing a connection between the individual filaments and the larger network architecture. The nature of the intersections between filaments dictates the behavior of the network as compressing or stretching a filament drastically changes its response to further loading. A compressed filament may straighten under applied tension as thermal fluctuations are pulled out, but a filament that is already under tension can only elastically stretch before rupturing its cross-links. Measuring both the architecture of the network to visualize these connections and the fluctuations of individual filaments to understand the forces they are under is therefore crucial to linking the system's microscopic properties to its macroscopic behavior under load. Thermal noise imaging is a powerful tool for this as its high spatial and temporal precision allow for scans of volumes containing multiple filaments while maintaining sub-filament resolution. Previous studies have applied this technique to systems of collagen filaments and cross-linked microtubules with great experimental success [107, 108], and this section furthers that work through more thorough analyses of cross-linked microtubule networks. We discuss the methods

used to prepare cross-linked microtubule networks and introduce new ways of analyzing thermal noise images to extract microtubule positions through intersections with other filaments. These analyses allow us to localize a filament intersection for the first time and show that excluded volumes encode dynamical information about microtubule fluctuations.

3.1 Materials and Methods

3.1.1 Tracer Particle Preparation

The tracer particles used in thermal noise images have to be small so that they can diffuse through the network mesh while being easily tracked, and inert so that they do not interact too strongly with the filaments. Polystyrene particles that are 208 ± 10 nm in diameter and fluorescently labeled with Suncoast Yellow (FSSY002, Bangs Laboratories, Fishers, IN) fulfill this requirement. To keep them from sticking to individual filaments, they were coated with poloxamer 407 (16758, MilliporeSigma, Burlington, MA). They were diluted 1:1000 into a solution of 20 mg/mL poloxamer in water and incubated overnight at room temperature to allow the coating to form. At the end of incubation, the particles were covered in hydrophilic polyethylene glycol (PEG) brushes that extended outward from each particle's surface. Similar methods have found the lengths of these brushes to be about 10 nm in length so the size of the probes were not increased too significantly [109].

3.1.2 Coverslip Preparation

To maintain the stability of the microtubule network over time, it was anchored to a glass coverslip that was carefully prepared to electrostatically bind to the microtubules. This started with sonication for 15 minutes in a 2 % Hellmanex III (Hellma, Müllheim, Germany) and MilliQ water (Millipore-Sigma, Burlington, MA) solution followed by a 5 minute flush with water. The coverslips were then sonicated for another 15 minutes, this time in only MilliQ water, followed by another 5 minute flush with water. This process was performed three times in total before coverslips were dried with ultra-pure nitrogen to create clean, hydrophilic surfaces. The hydrophilicity was further increased by plasma cleaning the coverslips in a tabletop plasma cleaner (PDCODP, Harrick Plasma, Ithaca, NY) for 60 seconds.

The final preparation step was to coat the coverslips in poly-L-lysine (PLL), which contained a positive amino group that attracted the negatively charged microtubules. Each PLL solution was prepared by diluting 0.1% w/v PLL in water (P8920, Sigma Aldrich) 500 times into ethanol. Coverslips were immersed in this solution for 15 minutes and then dried in an oven for 15 minutes. They were kept for up to five days at a time in a sealed glass dish filled with ultra-pure nitrogen.

3.1.3 Microtubule Preparation

Microtubules were prepared with a mixture of unlabeled, rhodamine-labeled, and biotinylated tubulin. The rhodamine tubulin allowed for fluores-

cent visualization while the biotinylated tubulin provided connection points between filaments once NeutrAvidin cross-linkers were introduced. Tubulin derived from porcine brain was purchased as a lyophilized powder from Cytoskeleton Inc. (Denver, CO). The rhodamine-labeled tubulin was left as a powder upon delivery while the unlabeled and biotinylated tubulin were suspended in a solution consisting chiefly of BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgCl_2 , pH 6.8). It was then supplemented with 1 mM GTP/BRB80 to increase the stability of the tubulin and additional MgCl_2 to increase the concentration to 5 mM since magnesium is thought to increase tubulin's affinity for nucleotides like GTP [110]. Aliquots were frozen in liquid nitrogen, 10 μL at a concentration of 10 $\mu\text{g}/\mu\text{L}$ for the unlabeled tubulin and 5 μL at a concentration of 5 $\mu\text{g}/\mu\text{L}$ for the biotinylated tubulin.

To polymerize non-biotinylated microtubules, one aliquot of unlabeled tubulin was thawed and combined with one vial of rhodamine-labeled tubulin and 15 μL of 1 mM GTP/BRB80 buffer to create a ratio of 1:6 rhodamine tubulin to unlabeled tubulin. The tubulin was then put through a cold, clarifying spin at 90,000 RPM (360,000 relative centrifugal force) for 5 minutes at 4°C in a TLA-100 rotor with a tabletop ultracentrifuge (Optima TLX-120, Beckman Coulter, Brea, CA). This spin collected misfolded proteins at the bottom of the centrifuge tube, and the supernatant containing the remaining tubulin was extracted and polymerized for 15 minutes at 37°C. After polymerization, a cut-tip pipette was used to remove the solution to avoid shearing microtubules suspended within it. The microtubule-containing solu-

tion was then placed back into the ultracentrifuge for a final spin at 40,000 RPM (71,000 relative centrifugal force) and 37 °C for 5 min to pellet them at the bottom of the centrifuge tube. The pellet was washed twice with 50 μ L of 20 μ M taxol/BRB80 (hereafter taxol buffer) and then resuspended by gentle pipetting in 50 μ L of fresh taxol buffer using a cut-tip pipette. Microtubules were stored for up to 5 days away from light to prevent photobleaching and oxidation.

Biotinylated microtubules were prepared in a nearly identical way. The only difference was that one aliquot of biotinylated tubulin was combined with one aliquot of unlabeled tubulin, one vial of rhodamine tubulin, and only 10 μ L of 1 mM GTP/BRB80 buffer to create a 1:7 ratio of biotinylated to total tubulin and a 1:7 ratio of rhodamine to total tubulin.

It should be noted that the taxol buffer needed to be prepared in a particular way to avoid formation of taxol crystals. These crystals can capture fluorescent proteins and appear similar to microtubules under the microscope [111]. Consequently, taxol was prepared from 2 μ L frozen aliquots made with 1 mg taxol (T1912, MilliporeSigma, Burlington, MA) diluted to 10 mM in dimethyl sulfoxide. Before use, an aliquot was warmed to 37 °C and diluted with BRB80 to 20 μ M concentration. It was then put into a tabletop centrifuge (5415C, Eppendorf, Hamburg, Germany) for a 5 minute spin at 8,000 RPM (6800 RCF) to separate taxol crystals from the solution. The top 600 μ L of taxol buffer was taken from the 1 mL aliquot and considered ready for use in microtubule polymerization.

3.1.4 Microtubule Network Preparation

Once fluorescently-labeled and biotinylated microtubules were prepared, they could be put into a sample chamber and then be cross-linked into a network. A PLL-coated coverslip was attached to the bottom of the sample chamber using dental wax and then 5 μL of biotinylated microtubules were added to the coverslip and left to sit for 10 minutes. This allowed some microtubules to diffuse down and stick to the positively charged coverslip. Next, 1.25 μL NeutrAvidin (hereafter referred to as avidin) at 3 μM (0.2 mg/mL) was added to cross-link the microtubules giving a ratio of 10:1 biotin to avidin. The top coverslip was attached to decrease evaporation and the sample was left for 1 hour to give the network time to form. The biotin-avidin bonds that formed the network were highly stable, but fluorescence creates oxygen radicals that can damage the microtubules [112,113]. In the past, oxidation in single molecule experiments had been counteracted by using glucose oxidase catalase, but this reagent was found to cause undesirable decreases in the pH. Instead, an oxygen scavenging system of 3 μg pyranose oxidase (P4234, MilliporeSigma), 90 μg catalase, and 44 mmol dm^{-3} glucose was employed that does not alter the pH significantly [114]. This scavenging system was added along with the tracer particles to the network and the sample was sealed.

A former student, Dr. Michael Himmelsbach, created this procedure to ensure an adequate mesh-size in the network [108]. The ratios of biotin to NeutrAvidin (10:1) and biotinylated to non-biotinylated tubulin (1:7) created enough cross-links for the network to be stable over time while leaving large

enough pores that tracer particles could diffuse through the network.

3.1.5 Thermal Noise Imaging Microtubule Networks

Microtubule networks made using the described procedure end up being 50-150 μm thick from the bottom coverslip and are highly heterogeneous in nature. There are often highly dense areas with many cross-linked filaments and then other regions that are more sparsely populated. Such variation also appears in dense actin networks cross-linked by filamin [115] so we can have confidence that our artificial network still resembles biologically-relevant systems.

3.1.5.1 Scanning Procedure

The imaging process started near the bottom coverslip. Since the sample was found to have minimal drift (Section 2.2.1.2), staying within 10–50 μm of the bottom coverslip ensured high stability since the network was firmly bound to the bottom coverslip of the sample chamber. We did, however, need to maintain a separation of at least 10 μm from the coverslip for two reasons: to prevent variations in the diffusion constant of the probe particle (Section 2.2.2.3) and to avoid position detection artifacts. The small radius of the bead made the former less of a problem even within a few microns of the surface, but optical artifacts could pose a major problem close to the coverslip. It is thought that the relatively small scattering signal from the probe particle combined with the change in index of refraction between the coverslip and the

particle interferes with position detection so we circumvented this by staying away from the coverslip surface.

Staying at least $10\text{ }\mu\text{m}$ above the surface, we first moved in large steps through the sample using the micrometers on the stage. A region of interest (typically an intersection between multiple microtubules) was identified in fluorescence and then a bead was captured in the optical trap by moving the piezo stage. Careful positioning of the bead relative to the intersection was accomplished by tracking the detector voltages and then displaying them as pairs in two-dimensional histograms. Small steps would then be taken until the centers of the histograms were occluded by the microtubules. This region became the center of the thermal noise image.

The scan itself began by moving to the edge of the volume, away from other microtubules, again to avoid variation in the particle's diffusivity during the calibration of the position detection system (Section 2.3.1). Once the calibration finished, the probe particle was moved through a raster scan of $11 \times 11 \times 3$ positions in the x-, y-, and z-directions separated by 100 nm laterally and 300 nm axially relative to the trap propagation (Figure 3.1). These distances were chosen to ensure sufficient overlap between trapping volumes that were approximately 300 nm in x and y, and 900 nm in z. Data was collected at 100 kHz with a 1 MHz electronic bandwidth for 4 seconds at each location so this portion of the scan took 24 minutes.

Next, the probe particle was released so that offset scans could be taken throughout the network to record the filaments' responses to the laser.

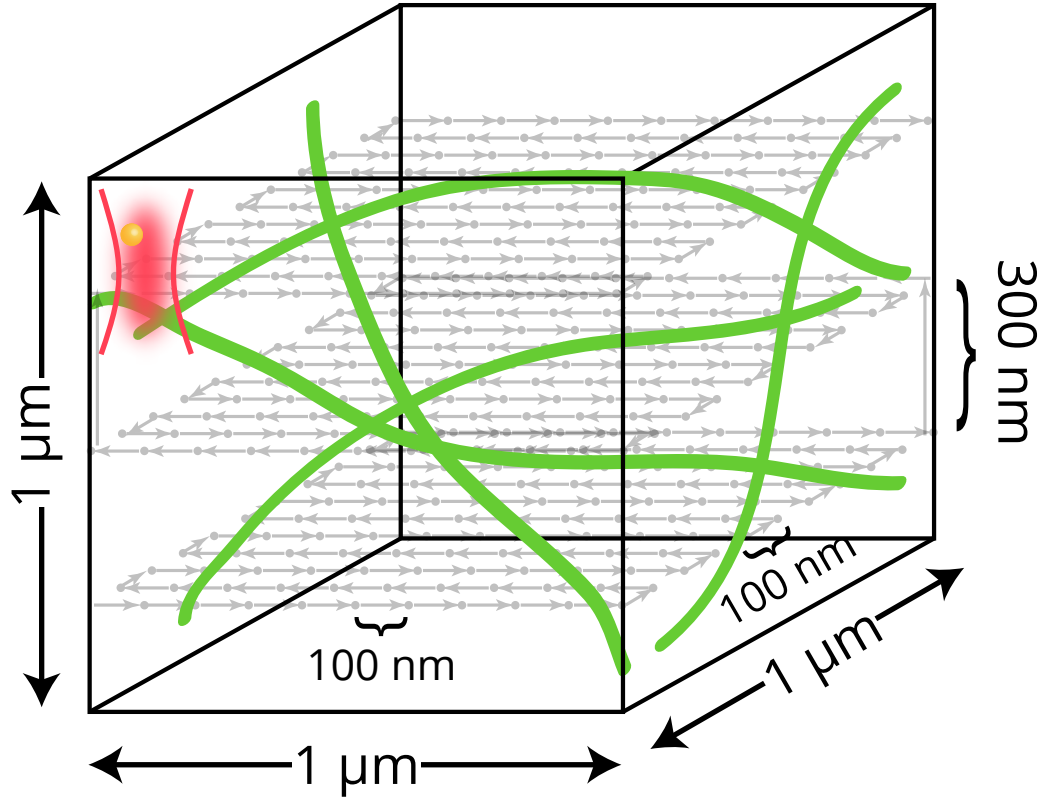


Figure 3.1: **Raster scan diagram of microtubule network.** The trapped particle (yellow) is moved through 363 total positions in the microtubule network (green), stopping at each location for 4 seconds. Data is collected at 100 kHz with a 1 MHz electronic bandwidth.

The empty trap was scanned through the same path of 363 locations staying at each one for 200 ms to record the detector voltages at 100 kHz with a 1 MHz electronic bandwidth. An average was calculated for each time trace to quantify the change in the detector voltage caused by light scattering from the filaments and surrounding environment. It was then subtracted from the trapped particle data at that same location. Offset scans are particularly important for imaging thick filaments like collagen that scatter a lot of light,

but Bartsch *et al.* did not find offsets necessary for thermal noise images of individual microtubules [107, 116]. However, Figure 3.2 shows that the offsets in a cross-linked microtubule network can vary enough throughout a scan that they need to be taken into account. For context, typical x voltages for trapped particles ranged between -1.5 V and 1.5 V for this experiment and the offset voltages spanned nearly 1 V across the scanning volume. The bundling and intersection of filaments likely created a larger offset signal than single microtubules in free space.

The offset-adjusted trapping data must then be aggregated to form a complete thermal noise image. The scanned volume was divided up into $10 \times 10 \times 10\text{ nm}^3$ voxels and the occupancy of each voxel was calculated by counting the number of times the particle visited each one. This created a three-dimensional histogram (also called a thermal noise image) that we could analyze using custom-written software in Igor Pro (Wavemetrics, Portland, OR). Figure 3.3 shows an example of a thermal noise image that displays voxels with occupancies of 30.

3.1.5.2 Drift Correction

The length of a thermal noise imaging scan leaves significant time for the sample and surrounding setup to drift from their original positions. While this drift has been minimized by the PFM's design (Section 2.3.3) and by waiting for the sample drift to relax, there is still some drift in the probe particle's signal that needs to be corrected. Dr. Michael Himmelsbach developed the

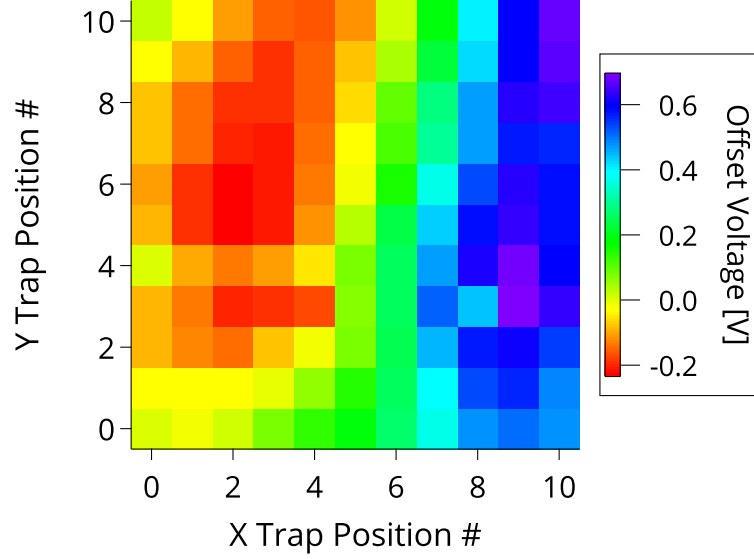


Figure 3.2: **Average x-signal voltage offsets for one xy-plane of a thermal noise image.** Offset values are collected by moving the trapping laser to the same locations that were imaged with a probe particle and then recording the detector voltages at each of these locations for 200 ms at 100 kHz with a 1 MHz bandwidth. These voltages are then averaged to obtain an offset for that location. Light scattered by filaments leads to shifts in the offsets throughout the sample. Typical x voltages from the trap were -1.5 to 1.5 V for this sample.

procedure for this, which assumes a linear drift in the particle voltage over time [108]. For each of the 363 trapping positions, the average voltage over the 4 s data collection time was calculated for the x-, y-, and z-signals. These were each fit with linear functions and the coefficients from that fit were used to correct the position of each trap.

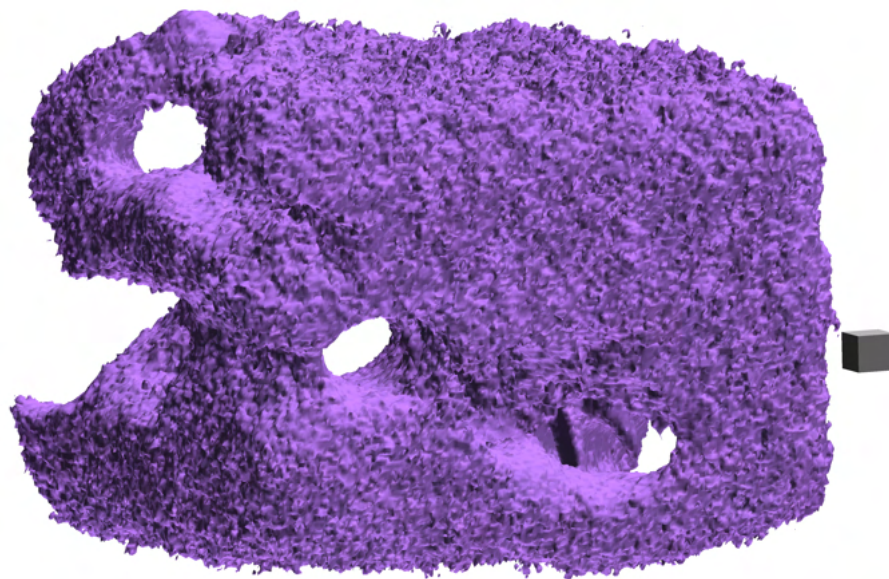


Figure 3.3: **Thermal noise image of a microtubule intersection.** Voxels shown in purple have occupancies of 30. The outer structure of the thermal noise image captures the cubic nature of the scan, but the inner regions that excluded the particle show the presence of microtubules. The scale cube in grey is $100 \times 100 \times 100 \text{ nm}^3$.

3.1.5.3 Power Spectral Density Correction of Detection Artifacts

Section 2.3.1.1 discussed Tischer’s method of calibration for a nonlinear detector response, but it does not capture the behavior with complete accuracy for the particles used in this dissertation. To test the efficacy of the calibration, 208 nm beads (not coated with poloxamer) were placed in a solution of phosphate buffered saline (PBS) and one of them was trapped with the PFM. The nonlinear detector calibration was then performed on the trapped particle free in solution. This same particle was then lowered to the coverslip surface until it adhered to the glass. Normally, the particle would not stick

to the coverslip, but the PBS screened the electrostatic repulsion between the surfaces so that they could make contact and stick together. With the particle immobilized on the surface, it became possible to measure the actual nonlinear detector response using the piezo to move the particle in controlled, 10 nm steps along each axis while recording the detector voltages. Comparing the nonlinear calibration to the detector response on the surface as in Figure 3.4, it is clear that the calibration does not completely capture the behavior of the detector and needs to be adjusted.

Since the nonlinear behavior of the detector has already been accounted for, the final correction to the response is just a linear scaling. Section 2.2.2.2 discussed the use of power spectral density in linear detector calibration so it is already well-suited for this application. Taking the raw detector voltages, the nonlinear calibration was run on them to get the particle positions. The power spectral densities of the positions were then calculated and fit using the methods outlined in Tolić-Nørrelykke *et al.* to obtain the diffusion constants along each axis [85]. Taking the square root of the ratio of this diffusion constant D_{PSD} to the one calculated from the Stokes-Einstein relation D_0 for a 208 nm diameter particle gives the scaling factor

$$\beta = \sqrt{\frac{D_{\text{PSD}}}{D_0}}. \quad (3.1)$$

Finally, we took this scaling factor and multiplied it by the positions obtained from the nonlinear calibration. If the nonlinear calibration already captures

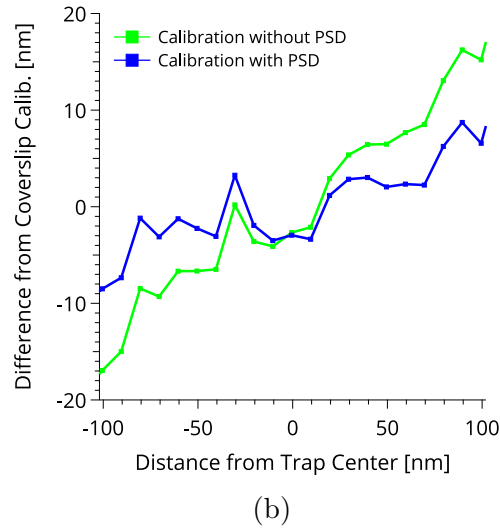
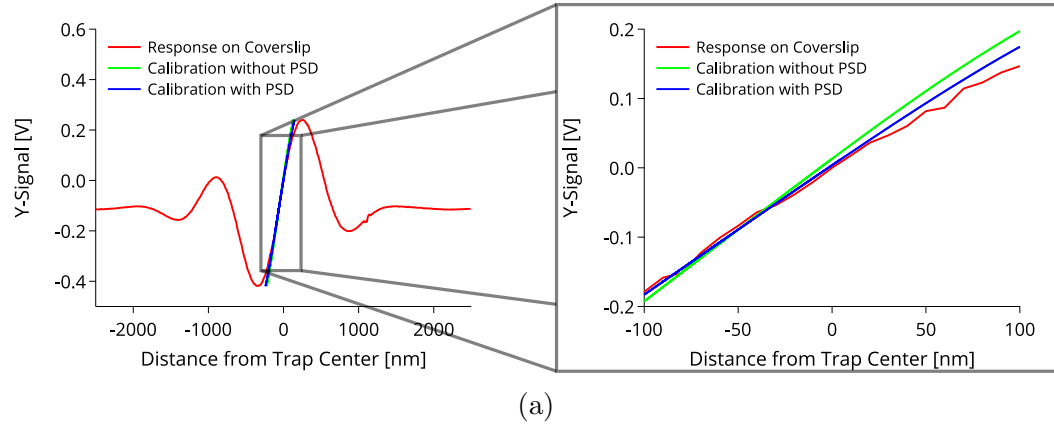


Figure 3.4: **Power spectral density correction to nonlinear detector calibration.** A 208 nm particle was trapped in solution and run through Tischer's nonlinear detector calibration method. It was then adhered to the coverslip surface so that its response could be recorded by moving the piezo. (a) The calibration without the PSD correction (green) does not capture the particle response (red) as well as the calibration with the PSD correction (blue). (b) Calibration without the PSD correction (green) does not minimize differences between the actual and measured particle positions as well as the calibration with the PSD correction (blue). $\beta = 1.10$ for the y-signal here.

the detector response perfectly, then $\beta = 1$ and the scaling does nothing. Typical values for β are $1 - 1.1$ for the x- and y-signals, and $1.05 - 1.15$ for the z-signal. In thermal noise imaging scans, the β values for each direction are obtained from the calibration trap taken at the start of each experiment.

It is not clear why the nonlinear calibration fails to completely capture the detector response nor is it clear why this power spectral density scaling ameliorates the problem. One possible cause could be that the time step chosen in calculating the voltage MSD during calibration (Section 2.3.1) leads to the appearance of confined diffusion thereby lowering the apparent diffusion constant of the particle. The power spectral density accounts for all timescales within the bandwidth of the measurement so it can avoid this time step pitfall. However, this is explored in Chapter 4 and the chosen time step for calibration should not be causing any issues. No matter the cause, it is important to maintain use of the nonlinear calibration in addition to the power spectral density scaling because of the nature of thermal noise imaging. Since the particle can be pushed by filaments to extremes of the trapping region, assuming that the detector response is entirely linear leads to underestimates of the particle positions. Obtaining accurate trapping information therefore necessitates use of this combined method.

3.2 Results and Discussion

The analysis of microtubule networks in this dissertation focuses on the negative space within thermal noise images like the one in Figure 3.3. Steric

interactions between the probe particle and microtubule prevent the particle from completely exploring the surrounding space, which leads to the elliptical channels (or excluded volumes) seen in thermal noise images. Finding the center of a channel gives the filament axis of the microtubule that created it, but each channel also encodes information about the dynamics of the network. While a static microtubule always excludes probe particles from regions equal to the radius of the particle plus the radius of the filament, a fluctuating microtubule allows the particle to explore more space as it vibrates. Carefully fitting these excluded volumes opens a window into both the static architecture and dynamic nature of these networks that is not possible with other techniques.

3.2.1 Filament Localization

Thermal noise images contain a huge amount of data (roughly 5 million points in a three-dimensional space) making them rich sources for analysis, but they also present interesting difficulties. The goal of this section is to extract microtubule positions from the excluded volume channels. This analysis relies on the occupancies of 10 nm cubic voxels that form isosurfaces centered on the microtubules.

Filament localization begins first by simplifying the problem from three dimensions to two. An isosurface (usually for occupancy 30) was calculated from the thermal noise image and rotated so that the x-axis was aligned with the channel of interest, putting the volume’s elliptical cross-section in the yz-plane. Note that throughout this dissertation the y-axis is displayed horizon-

tally and the z-axis is displayed vertically. Two-dimensional slices were taken along the length of the microtubule in 10 nm steps and contours of constant occupancy (isolines) were extracted as shown in Figure 3.5. In regions without microtubules, these contours do not convey much information and just show the homogeneity of empty space. In regions with microtubules, however, these isolines form concentric ellipses centered on each microtubule filament axis with the contour values getting smaller as they move radially closer to a microtubule. Finding the center of multiple contours within a cross-section gives the filament position for that 10 nm thick slice and these can be combined with other slices to reconstruct the three-dimensional position of the microtubule.

Previous work showed that a simple average of the y- and z-positions around the contour was sufficient to localize microtubules to 4 nm in the best cases [108], but the elliptical fitting employed in this work improves upon this in two ways: first, by extracting filament positions from open contours and second, by obtaining better localization through error-based weighting. Taking a simple average of the contour positions only works when the contour is closed. Near a microtubule intersection, contours of thermal noise images are often open as a consequence of the particle not being able to fit in the space between two filaments. The average of an open contour is biased in the direction opposite to the opening and cannot be used in filament localization, but an ellipse can still be fit to such a contour while still extracting the center position accurately. Moreover, the deviations of the data from the fit ellipse can be used to gauge the precision in finding the filament axis.

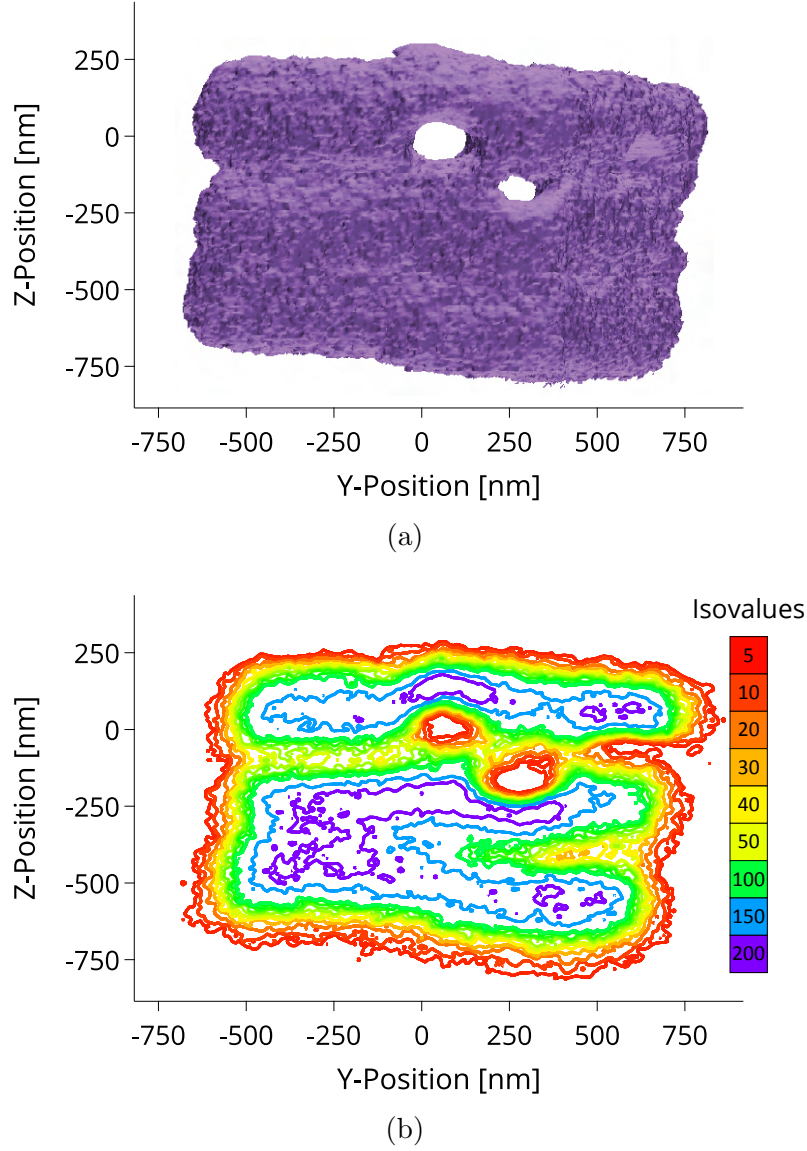


Figure 3.5: **Thermal noise image and contours of constant voxel occupancy.** (a) The thermal noise image is rotated so that the excluded volume to be fit is aligned with the x-axis. The isosurface is for an occupancy of 30. (b) Contours of constant voxel occupancy are then extracted for fitting. The color scale denotes the isovalue of each contour.

All of the thermal noise images in this dissertation have multiple isolines centered around each filament, so it was possible to take a weighted average of many contours to find the microtubule axis. First, contours of a chosen isovalue were fit with ellipses of the form

$$\frac{[(y - y_0) \cos \theta + (z - z_0) \sin \theta]^2}{a^2} + \frac{[(y - y_0) \sin \theta + (z - z_0) \cos \theta]^2}{b^2} = 1, \quad (3.2)$$

where y_0 and z_0 are the y- and z-positions of the ellipse center, a is the semi-major axis, b is the semi-minor axis, and θ is the angle the semi-major axis makes with respect to the y-axis. For each slice along the microtubule of interest, the fit coefficients and the errors in the fit (ϵ , taken as the absolute value of the difference between an isoline and the nearest point on the fit ellipse) were recorded. Each set of isolines provided a series of center positions along the microtubule given by $\vec{r}_{10}, \vec{r}_{20}$, etc, where the subscript denotes the isovalue of the contours used to find those positions. These positions were combined from different contours in each slice using a weighted average

$$\vec{r}_{\text{avg}} = \frac{\sum_i w_i \vec{r}_i}{\sum_i w_i}. \quad (3.3)$$

Here, the sum is over the isovalues of the fit contours and the weights are $w_i = \sqrt{i}/\text{med}(\epsilon)$ (i.e. the root of the isovalue divided by the median error). The standard deviation should be inversely proportional to the square root

of the number of times the bead visited a voxel while the median error is representative of how well an ellipse fits the data. These same weights were used to calculate a standard deviation as

$$\sigma_y = \sqrt{\frac{N \sum_i w_i (y_i - y_{\text{avg}})^2}{(N - 1) \sum_i w_i}}, \quad (3.4)$$

where y_i is the y-position for a contour of isovalue i , y_{avg} is the weighted average position from equation (3.3), and N is the number of contours in the average. Equation (3.4) is similarly used to calculate the uncertainty in the z-positions.

The final step in determining the filament axis was to fit a polynomial curve to the average center positions. The degree of the polynomial was chosen to be either quadratic (if the microtubule had no intersections) or cubic (if the microtubule crossed another filament inside the imaged area). An intersection could act as an inflection point in the microtubule's path and thus require a higher order fit to capture the possible changes in curvature. Igor Pro was used to perform the weighted fit by minimizing χ^2 , which for the y-positions is defined as

$$\chi^2 = \sum_i \left(\frac{y_{\text{fit}} - y_{\text{avg},i}}{\sigma_{y,i}} \right)^2, \quad (3.5)$$

where y_{fit} is the fitted value, $y_{\text{avg},i}$ is the weighted average position at the i^{th} point, and $\sigma_{y,i}$ is the weighted standard deviation at the i^{th} point. This fitting

was repeated for the z-axis while the x-axis was used to parameterize both the y- and z-polynomials. Using the standard deviation from equation (3.4) ensured that both the number of times the particle visited the area and the error in the ellipse fitting procedure were taken into account when finding the filament axis. Figure 3.6 shows the result of this procedure for several intersecting microtubules.

Precision of the fitting was estimated by measuring residuals from the average positions to the polynomial fit. Figure 3.7a shows the average positions as red spheres and the fit as a green line inside the thermal noise image. The fit itself is highly precise showing average residuals of 2.5 nm (Figure 3.7b), an improvement over previous localization methods. Furthermore, this localization method does not sacrifice the high temporal bandwidth necessary to follow the dynamics in microtubule networks (Section 3.2.4).

3.2.2 Outlier Detection Near Filament Intersections

Using elliptical fits rather than isoline averages was instrumental in the high precision of this technique, but outlier detection also played a major role. When fitting contours to find the microtubule’s axis, certain slices yielded positions that deviated significantly from neighboring areas. Stiff filaments like microtubules should not show large deviations on the scale of 10 nm (the distance between neighboring slices) so these deviations must come from overlaps between the isolines of one microtubule and a neighboring one. An example of this can be seen in Figure 3.8. The translucent plane shows the position of

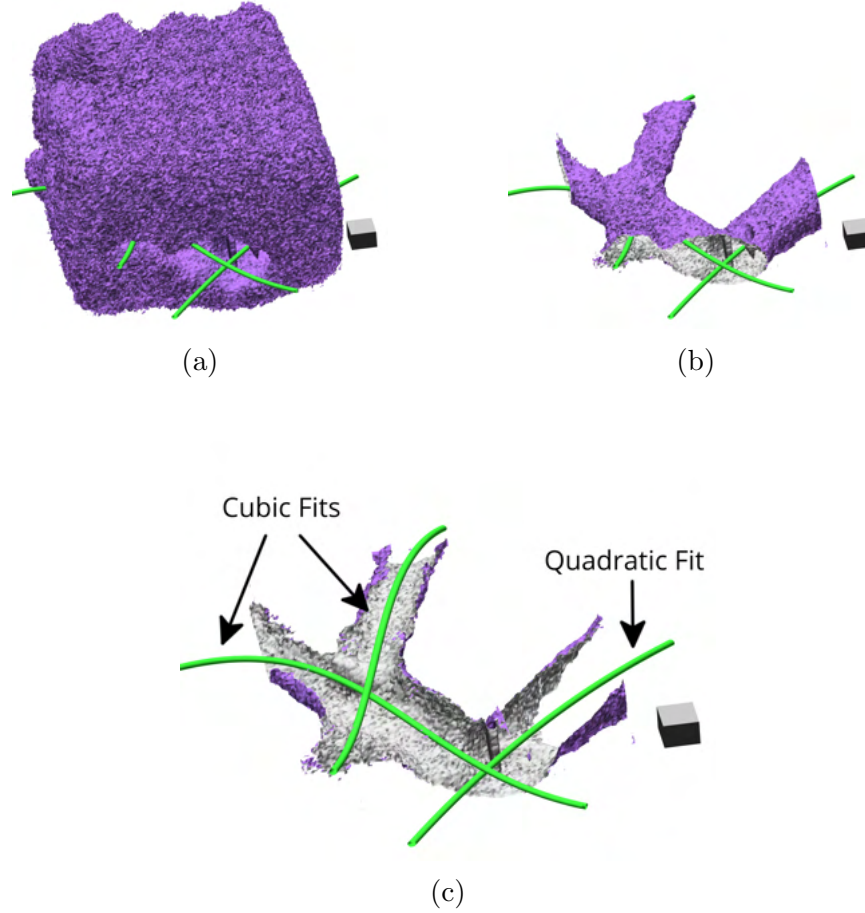


Figure 3.6: **Filament axes of intersecting microtubules.** Polynomial fits to weighted average positions (green) are shown inside thermal noise images of isovalue 30 (purple and grey). Third-degree fits are performed for microtubules that intersect inside the trapping volume and second-degree fits are performed otherwise. (a) The full thermal noise image is shown without edits. (b) The outside edges of the thermal noise image caused by the trap have been removed. (c) The thermal noise image has been edited to reveal the full path of the filament axes. All scale cubes are $100 \times 100 \times 100 \text{ nm}^3$.

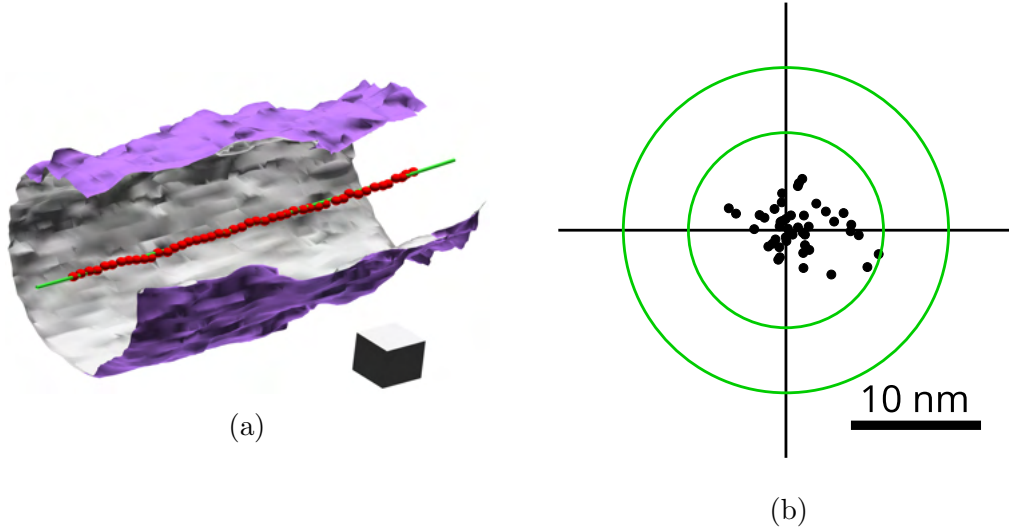


Figure 3.7: **Microtubule localization precision.** (a) An isosurface of 70 is shown (purple and grey) with the weighted averages of ellipse fits to the 30, 50, and 70 isovalue contours (red) and a second-order polynomial fit (green). (b) Residuals from the polynomial fit (black) have an average size of 2.5 nm. For context, the inner and outer diameters (15 nm and 25 nm) of a microtubule are shown (green).

the slice through the isosurface and the neighboring panel displays the contours in that region. The lower left contours are almost entirely closed except for higher isovalues which open slightly into the edge of the imaged volume. In contrast, the upper right contours come from two microtubules that are close to their intersection point. Even at lower isovalues, the contours become distorted towards the adjacent microtubule contours and they finally merge at higher isovalues. Identifying the outlier points for removal that come from separate filaments would significantly improve fitting precision so a method was developed based on the least median of squares.

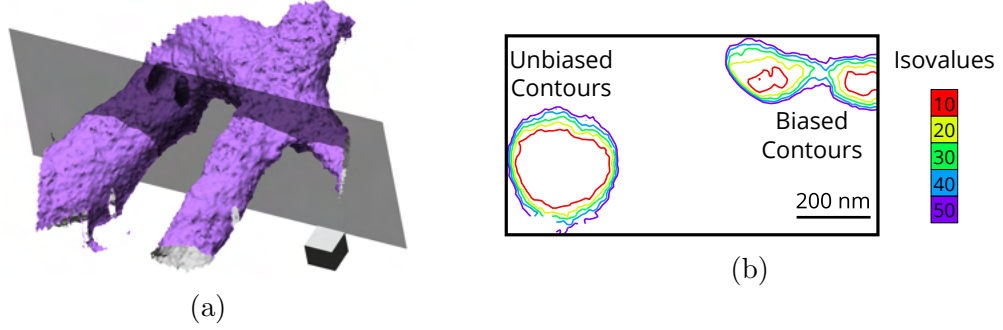


Figure 3.8: **Overlapping isolines from separate microtubules.** (a) An isosurface for 30 occupancy voxels with a 100 nm scale cube. (b) Contours from the slice of the isosurface shown in (a). The bottom left contours show an isolated microtubule with no isolines overlapping with other filaments. For the upper right contours, the isolines from separate microtubules do not overlap much at lower values. At larger isovalues, however, the isolines merge and distort the shape of the individual filaments. The far right contours are open because that microtubule is not perpendicular to the displayed cross-section.

The least median of squares method seeks to remove outliers that are far from the median fitting error, and here, it is used assuming that non-outlier points lie on an elliptical curve [117]. It starts by calculating the median absolute deviation (mad) for a fit

$$\text{mad} = \text{med}_i \left| \epsilon_i - \text{med}_j \epsilon_j \right|, \quad (3.6)$$

which finds the median of the absolute difference between all the errors in a fit and the median error (with the error defined as in Section 3.2.1). This value is then adjusted by f , a Gaussian normalization and finite-sample correction factor

$$f = 1.4826 \left(1 + \frac{5}{n-1} \right), \quad (3.7)$$

where n is the number of points in the fit. Each error's absolute value is then compared to $3 \times f_{\text{mad}}$. If the error is smaller, then the point is not likely to be an outlier and is left in the fit. However, if the error is larger, then the point is removed and the fit is repeated. This was applied to filament localization by calculating errors between the fit ellipse from the previous slice and the current slice's isoline since microtubules should not deviate much over 10 nm. More robust methods would calculate ellipses from randomly sampled points, but they are more computationally intensive and not needed for this work [117].

To properly gauge the efficacy of the least median of squares method, it was tested on data generated from an ellipse with known coefficients. First, the coefficients were used to generate data points around the perimeter of the ellipse, and then Gaussian noise was added to simulate the variation seen in experimental thermal noise imaging isolines. Several points were also replaced with outliers that extended away from the ellipse perimeter, mimicking the effect of a neighboring microtubule. The top path in Figure 3.9a shows the influence that outliers (marked in red) can have on fitting as the ellipse is distorted towards the outliers. Outlier detection and removal was accomplished by taking the original ellipse coefficients and adding Gaussian noise to them so that they could act as the ellipse of a previous slice. Finally, the least median of squares method was run using these coefficients and the biasing points were removed. Fitting the simulated data with this outlier recognition allowed

the original coefficients to be recovered as Figure 3.9b shows. Each coefficient was more accurately predicted after using the least median of squares method, particularly those coefficients in the y-direction of the fit. Non-zero differences between the coefficients without outliers and the original generating coefficients are due to the Gaussian noise added to the data points. This outlier detection is critical, not only for accurate fitting of the ellipse centers, but also for determining the volume excluded by microtubules, which is discussed in detail in Section 3.2.4.

3.2.3 Mapping Filament Intersections

With precise filament localization that could account for outliers near filament intersections, it became possible to use thermal noise imaging to find filament intersections directly for the first time. Previous work relied on extrapolation between closed contour regions of thermal noise images to determine possible intersection points [108]. This achieved an approximate precision of ± 10 nm, but using the methods described in Sections 3.2.1 and 3.2.2, these intersections could be localized to a much finer degree.

The distance between microtubules was determined by sampling from the space of possible fits when accounting for the uncertainty in the filament axes. Starting with the y- and z-center positions of the filament obtained from equation (3.3), Gaussian noise was added to each position. Parameters of the noise were a central value of 0 and a standard deviation equal to the uncertainty in the position from equation (3.4). Once noise was added, the positions were

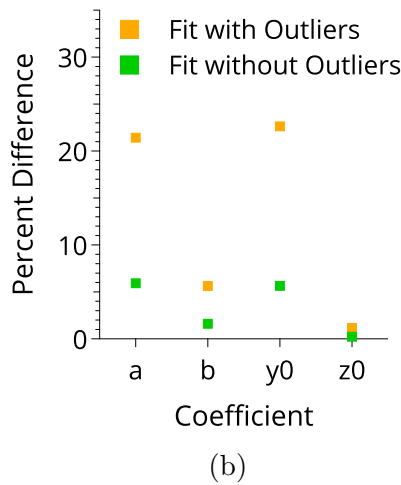
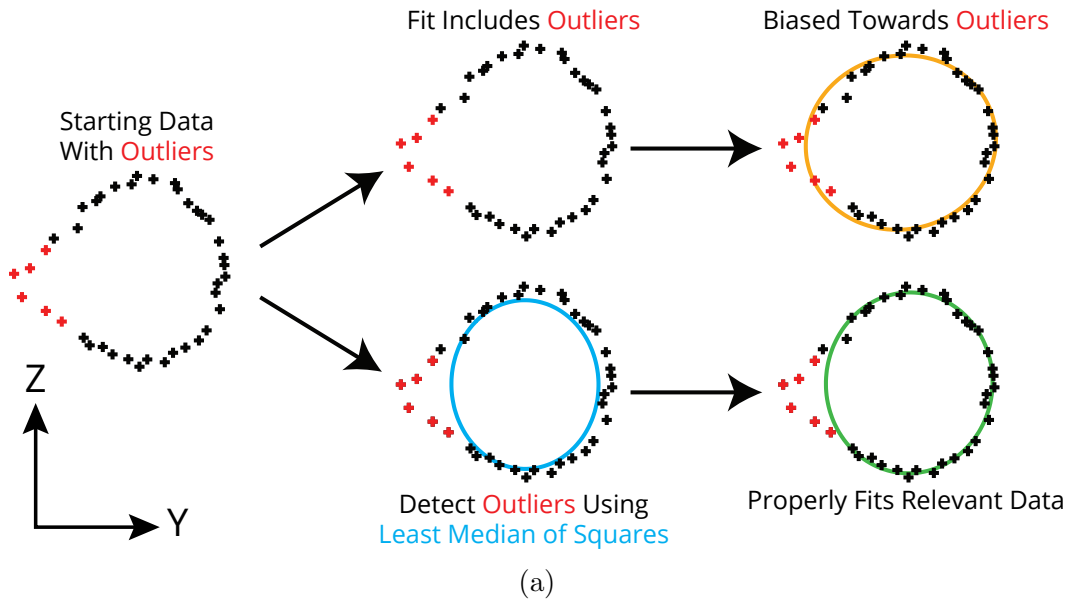


Figure 3.9: **Outlier detection for an artificial ellipse.** (a) An ellipse of known coefficients was generated with Gaussian noise added to each point (black crosses). Several outlier points were added (red) that do not conform to the ellipse. If the outliers are ignored, then the ellipse is biased by the outliers and does not properly fit the data (orange). If the outliers are removed using a similar ellipse (cyan), then the newly fit ellipse (green) captures the original coefficients. (b) Percent error between the fit ellipse coefficients and the original, generating ellipse's coefficients. Small differences in the fit without outliers stem from the Gaussian noise added to the original data.

refit with a polynomial as described in Section 3.2.1. After both microtubules in the intersection underwent this procedure, the minimum distance between the polynomial fits was recorded and the whole process was repeated from adding noise to refitting and finding the interfilament separation. After finding the minimum distance for 10,000 iterations, the data was condensed into the histogram shown in Figure 3.10. There is some variation in the minimum distance over the thousands of fits, but they are centered around a separation of 26.4 nm with a standard deviation of 1.4 nm. Given that microtubules have a diameter of 25 nm, it is highly likely that these two filaments are cross-linked by a biotin-avidin bond. The confirmation of this intersection suggests that there should be some interesting filament dynamics occurring in this region.

3.2.4 Thermal Fluctuations as Excluded Volumes

For a static microtubule, the size of the excluded volume around it would be the radius of the particle plus the radius of the microtubule, but microtubules in this work are not entirely static. Thermal energy from the surrounding fluid constantly drives fluctuations of the filaments so that they do not rest in a single position as the probe particle moves throughout the optical trap. Instead, the particle can visit voxels that are typically blocked by microtubules, moving into spaces left open as microtubules undergo their own thermal motion. The sizes of the excluded volumes around microtubules thereby encode dynamical information about the network's fluctuations. In general, the more a filament is fluctuating, the more space the probe par-

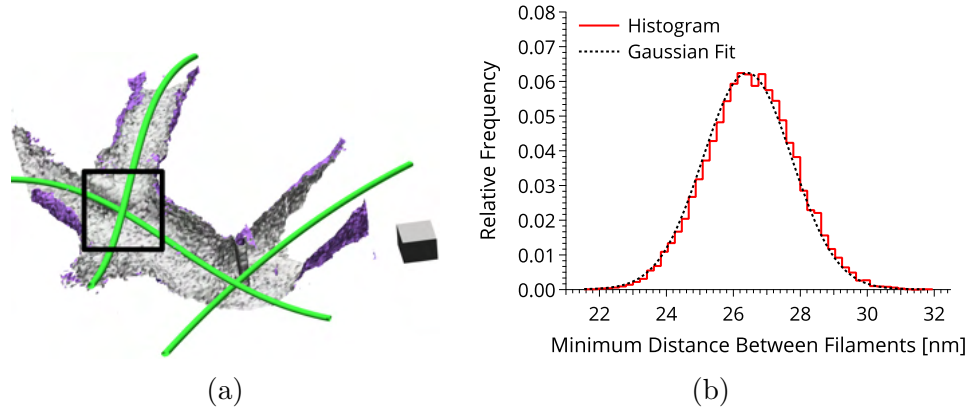


Figure 3.10: **Precision of filament intersection localization.** (a) The filament fits highlighted in the box are used to extract the minimum distance between the microtubules. (b) Center positions of the microtubule axes are varied by Gaussian noise with a mean of 0 and a standard deviation equal to the uncertainty in each center position from equation (3.4), and new fits are found for each variation. The minimum distance between each fit is recorded and summarized in a histogram with an average minimum separation coming from a Gaussian fit. For this intersection, the distance is 26.4 ± 1.4 nm. Two microtubules in direct contact would be 25 nm apart, but the biotin-NeutrAvidin link would extend this distance for cross-linked microtubules.

ticle can explore and the smaller the excluded volume will be. Figure 3.11 demonstrates this behavior for a hypothetical filament pinned to a wall. Near the wall, the filament cannot fluctuate much and the volume it excludes is large. Farther from the wall, the filament has more freedom to fluctuate and move over a larger area leading to a smaller excluded volume. The excluded volumes around a microtubule thus give insight into the fluctuations of the filament through this inverse relationship.

The ellipses fit to the thermal noise imaging isolines allowed us to investigate this behavior in the context of cross-linked microtubules. Since we

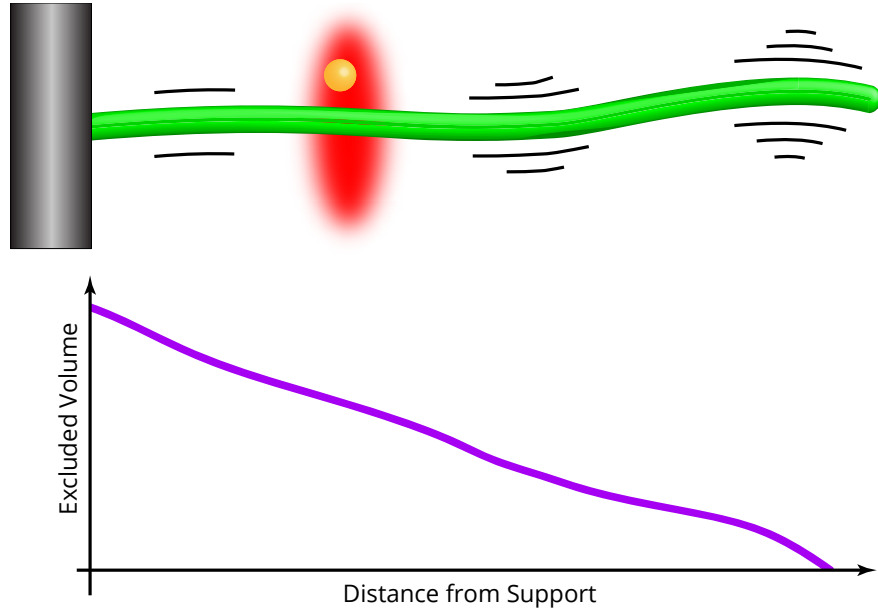


Figure 3.11: **Filament fluctuations and excluded area.** A microtubule fluctuates more the farther it is from supporting structures. As it fluctuates more, the trapped probe particle has more chances to explore space that is usually blocked. Fluctuations are thus inversely connected to the excluded volume seen by the particle.

fit the ellipses in two dimensions, we worked with excluded areas instead of volumes. These areas can be easily calculated from fit ellipses as

$$A = \pi ab \pm (\pi b \Delta a + \pi a \Delta b), \quad (3.8)$$

where A is the excluded area, a and b are the axes as defined in equation (3.2), and the Δ terms are uncertainties from the fit for each axis.

The area excluded from the particle motion shown in Figure 3.12 has a distinctive behavior that seems to follow the architecture of the network.

Moving from left to right along the dark green microtubule in the inset, the filament's fluctuations are expected to be increasingly suppressed as the filament approaches an intersection with the leftmost light green filament. In response, the excluded area for all isolines increases linearly up to a local maximum at the intersection marked by a dashed line. The excluded area for all isolines then enters a brief period of decline after the intersection. This behavior is all in line with the model presented in Figure 3.11 with the particle being able to explore more space far from the intersection and being more restricted around the intersection leading to a peak in the excluded area. Unexpectedly, however, the decrease in excluded area only extends for roughly 40 nm after the intersection before plateauing for over 100 nm. It then begins increasing again until the microtubule is out of the observed volume. While this increase initially seems to be contradictory, it actually still agrees with the model. Fits to the dark green microtubule and the farther right light green microtubule in the inset suggest that they intersect just outside of the imaged region at the position marked on the graph by a second dashed line. Such an intersection would again suppress filament fluctuations and lead to an increase in the area excluded by the microtubule just as the first intersection did. Thus, the increase in the excluded area after the first intersection can be explained by this second crossing.

Similar behavior is observed for the excluded area around the dark green microtubule in Figure 3.13. In this case, the area starts off decreasing for several hundred nanometers. It then plateaus for approximately 200 nm

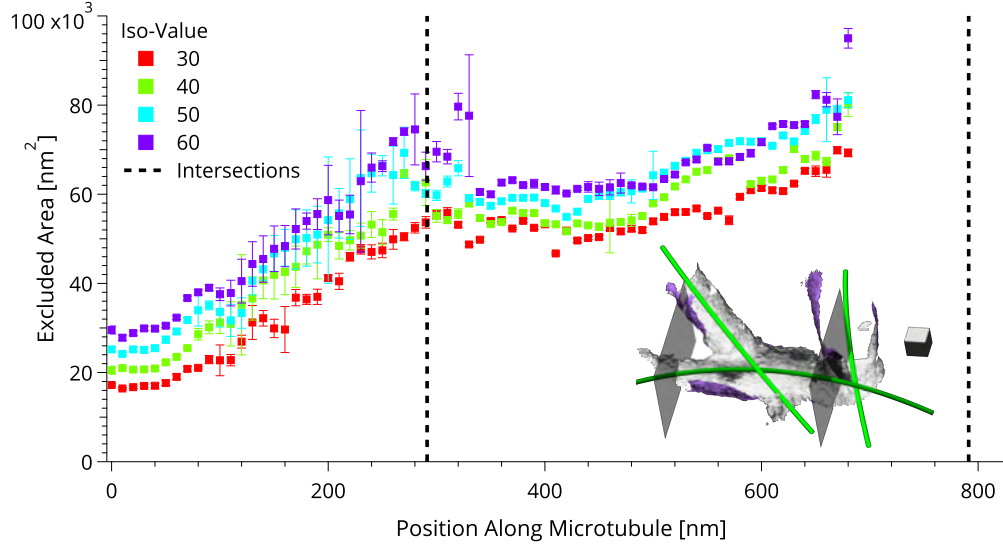


Figure 3.12: **Excluded area along a microtubule.** The excluded area for each two-dimensional slice of a thermal noise image is calculated using equation (3.8). This area increases linearly to an intersection with another microtubule (black dashed line), then briefly decreases and plateaus until increasing again towards a second intersection. This behavior is mirrored across all isovalues examined. (Inset) Isosurface of occupancy 30 (purple and grey) with filament axes (green). The dark green filament's excluded area from the left to the right grey plane is shown. Scale cube is 100 nm.

before increasing towards an intersection outside of the trapping volume. The decrease in the excluded area in the beginning suggests that there may be an intersection just before the start of the imaged region. That intersection would act as a support point causing the excluded area to decrease moving away from it as the imaged filament's fluctuations increase in magnitude. It is strange, however, that the excluded area does not decrease to a sharp point before increasing again. Previous work showed that the excluded area can go to zero around a freely fluctuating filament end so there should be some

point at which the probe particle can diffuse completely through space as the filament fluctuates [108]. One explanation is that the finite radius of the probe particle limits the region the particle can explore even when fluctuations are large. If the center of the microtubule is highly dynamic, the bead can still only explore that region if it is not inhibited by steric interactions with nearby, more static sections of the filament. These steric interactions would create a plateau region as seen in Figure 3.13.

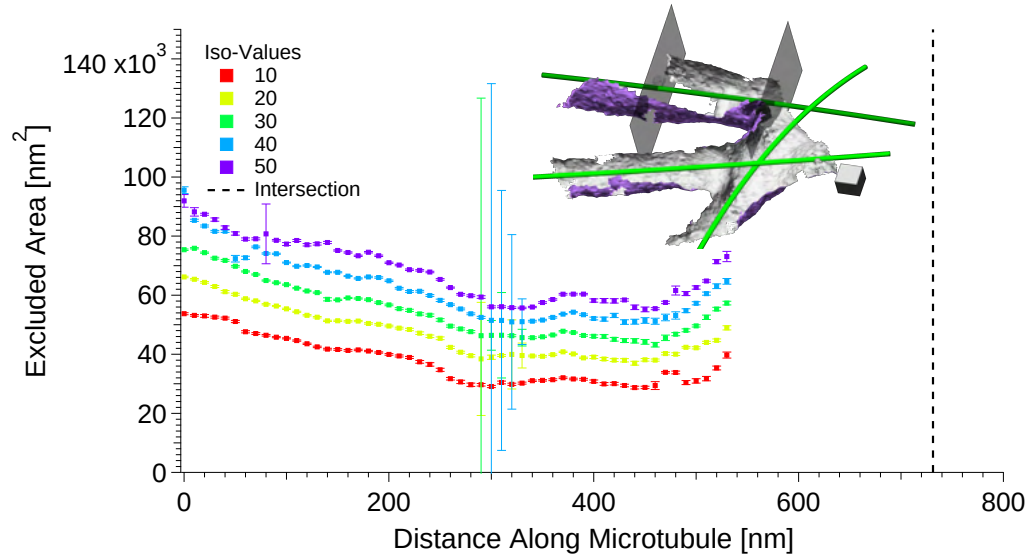


Figure 3.13: **Excluded area along a microtubule.** The excluded area for each two-dimensional slice of a thermal noise image is calculated using equation (3.8). This area linearly decreases at first suggesting an intersection outside the trapping volume, to the left of the displayed areas. The excluded area then plateaus before increasing again towards an intersection extrapolated from the polynomial fits of the microtubule positions. (Inset) Isosurface of occupancy 30 (purple and grey) with filament axes labeled in green. The dark green filament's excluded area from the left to the right grey plane is shown. Scale cube is 100 nm.

Since linear decreases and increases in area are found in both Figure 3.12 and 3.13, this behavior may be common enough that it can be used to extrapolate outside of the scanned volume. Intersections of imaged microtubules with other filaments outside the imaged region would cause the excluded area to increase as the probe moves closer to them. Thermal noise imaging scans could be greatly reduced in extent and duration if only a few sections along a microtubule needed to be imaged to discover other connections. The key to finding these intersections would be to calculate a maximum excluded area that signifies the point of intersection. For a static microtubule, the radius of this excluded area would be equal to the radius of the particle plus the radius of the microtubule. Assuming a range of microtubule diameters of 24 – 29 nm for 13-, 14-, or 15-protofilament filaments, this would lead to a combined radius of roughly 117.3 ± 7.5 nm and an excluded area of $4.3 \pm 0.6 \times 10^4$ nm² for voxels with 0 occupancy. Looking at Figure 3.12 that has a clear peak at an intersection, we find that the excluded area at that peak is $5.4 \pm 0.1 \times 10^4$ nm² for the minimum occupancy analyzed, but the excluded area continues increasing after that local maximum up to $6.9 \pm 0.1 \times 10^4$ nm². These areas are for an occupancy of 30 so it is not surprising that they would be above the theoretical maximum area for an occupancy of 0, but it is surprising that the maximum excluded area did not occur at the intersection. The continued increase in excluded area could mean that the extrapolated intersection in Figure 3.12 is with a more stable microtubule or bundle of microtubules making it more static than the intersection near the local maximum.

There are two ways that a microtubule intersection could be made more stable: one of the filaments could be under a lot of tension that suppresses fluctuations or a bundle of filaments may be involved at the intersection. The former can be explained by noting that applying tension to a filament suppresses fluctuations perpendicular to the tension. Thus, if a microtubule is stretched tightly between other filaments, it will not move much and be a nearly static connection point for other filaments. Alternatively, a bundle of filaments would be inherently stiffer than a single filament making it another stable connection point. Determining which of these is the cause of increased excluded areas would be crucial to quantifying the fluctuations and tension within a system. Unfortunately, disentangling these two effects is extremely difficult due to the dynamics between the particle and filament. Studying these filaments in a more controlled setting like a microtubule suspended across a carbon grid could allow for more precise quantification of filament fluctuations and encourage new theory on the topic [107, 108]. Other techniques such as activity microscopy may be better suited to study these fluctuations quantitatively as the absence of a probe particle simplifies the dynamics of the system [116, 118].

3.3 Summary: Thermal Noise Imaging of Microtubule Networks

Our thermal noise imaging data contains a wealth of information about the architecture and dynamics of microtubule networks. We can scan large

$1 \times 1 \times 1 \mu\text{m}^3$ volumes encompassing numerous, fluctuating microtubules while maintaining filament localization precision down to 2.5 nm in the best cases. Furthermore, novel fitting techniques allow us to track microtubule positions through regions influenced by multiple filaments for the first time and identify intersections, again with sub-filament precision. Once these intersections are identified, we can explore the fluctuations of filaments through the excluded volume of the probe particle. These excluded volume plots are thought to be inversely related to the filament fluctuations and show linear behavior approaching and departing filament intersections suggesting that we can extrapolate intersections with filaments outside of the imaged volume. Future studies may be able to link the excluded volumes directly to the filament fluctuations, but this will require extensive developments in the theory for our system.

Chapter 4

Characterizing Diffusion in Microtubule Networks

Transport within cells is carried out through a number of complex processes from the ATP-driven motion of molecular motors that carry vesicles to the advective flows that distribute nutrients. Cells must even rely on diffusion for key biological processes. Each of these mechanisms are heavily influenced by the confined nature of the cytoplasm, which alters flow boundary conditions and, in particular, restricts diffusive motion (Section 2.1.3). Up to this point, the mechanisms of these constraints have only been explored on network length scales and have failed to examine their nanoscopic origins. Thermal noise imaging provides the spatial precision and temporal resolution necessary to tackle this problem. The motion of an individual particle is tracked as it traverses a cross-linked microtubule network and the spatial variations in its diffusion constant are mapped throughout. For the first time, the direct impact of biopolymer filaments on diffusion are measured on the nanometer length scale and connected to known theories of hydrodynamic coupling between spheres and surfaces.

4.1 Materials and Methods

4.1.1 Sample Preparation and Data Collection Procedure

Diffusion constants of trapped particles can be directly calculated from thermal noise imaging data. As such, the methods of sample preparation and data collection are identical to those described in Chapter 3. All data and results presented in this chapter are taken from cross-linked microtubule networks imaged by the confined Brownian motion of 208 nm diameter polystyrene particles.

4.1.2 Calculation of Spatially Varying Diffusion Constants

The diffusion constant of a particle is usually calculated by tracking a diffusing particle's position over time and then fitting the mean squared displacement (MSD). Outside of the ballistic regime, the MSD has a linear relationship with the diffusion constant as shown in equation (2.9). Unfortunately, this averages over all particle positions and is unsuitable for resolving variations in the diffusion constant due to obstructions like surfaces and biopolymer filaments. To achieve the desired nanometer resolution, the MSD must be calculated separately for each region of interest.

Starting with equation (2.9), we rearrange for the diffusion and simultaneously rewrite the MSD in terms of the variance in particle positions

$$D_x(\vec{r}) = \frac{\text{var}(\Delta x)_{\vec{r}}}{2\Delta t} = \frac{\langle \Delta x^2 \rangle_{\vec{r}}}{2\Delta t} - \frac{\langle \Delta x \rangle_{\vec{r}}^2}{2\Delta t}, \quad (4.1)$$

where $D_x(\vec{r})$ is the diffusion constant of the particle in the x-direction at position $\vec{r}$, $\Delta x = x(t + \Delta t) - x(t)$ is the change in the particle's x-position over a time Δt , and $\langle \rangle_{\vec{r}}$ is an average taken over all values at position $\vec{r}$. In free space, the second term in equation (4.1) would vanish as Brownian motion has no preferred direction, but this changes if the particle interacts with non-thermal forces like those originating from optical traps or repulsion from nearby surfaces. The second term then becomes important as it characterizes the amount of variance in the particle's position that comes from drift rather than diffusive motion. Subtracting that term ensures that only the diffusive motion is resolved.

For experimental data, the spatially-resolved diffusion constant needs to be expanded from a single position to a small volume $\delta V(\vec{r})$ centered around $\vec{r}$ to obtain sufficient counts. We can write this out explicitly as

$$D_x(\vec{r}) = \left[\frac{1}{n_{\delta V}(\vec{r})} \sum_{x(t) \in \delta V(\vec{r})} (x(t + \Delta t) - x(t))^2 - \left(\frac{1}{n_{\delta V}(\vec{r})} \sum_{x(t) \in \delta V(\vec{r})} (x(t + \Delta t) - x(t)) \right)^2 \right] / 2\Delta t, \quad (4.2)$$

where $n_{\delta V}(\vec{r})$ is the number of times the particle is found in $\delta V(\vec{r})$. Equation (4.2) is utilized for $10 \times 10 \times 10 \text{ nm}^3$ voxels throughout a thermal noise image. Each voxel then has a diffusion constant associated with it and variations between voxels should come largely from changes in the probe's environment.

The choice of time step Δt in the equation (4.2) is a critical one in diffusion calculations. If calculated for a particle diffusing in free space without external forces, the diffusion constant would be the same regardless of the time step, however, the optical trap inherently restricts the diffusion of the particle and removes this time invariance. This can cause the MSD of a trapped particle to vary with its location in the laser. Near the trap center, the gradient force is relatively weak so the particle is not considerably restricted. Farther from the trap center, the gradient forces become stronger and heavily influence the particle's motion. Equation (2.30) shows confinement of a particle's MSD when it diffuses from the trap center, but there should also be super diffusive motion over certain time scales. When the particle starts closer to the edge of the trap, the stronger gradient forces quickly accelerate the particle leading to faster transport than free diffusion would predict. Subtracting the drift component from the variance in particle positions should mitigate these effects, but they can be further reduced by the proper choice of time step. We quantify the effect of the trap by writing an MSD that starts at some arbitrary distance from the trap center x_0 as

$$\begin{aligned} \langle (x(\Delta t) - x_0)^2 \rangle = & \frac{k_B T}{k} \left[1 - \exp\left(-\frac{2k}{\gamma_0} \Delta t\right) \right] \\ & + x_0^2 \left[1 + \exp\left(-\frac{2k}{\gamma_0} \Delta t\right) - 2 \exp\left(-\frac{k}{\gamma_0} \Delta t\right) \right], \end{aligned} \quad (4.3)$$

where k is the spring constant of the trap [119]. This reduces to equation (2.30) when $x_0 = 0$, but accounts for the stronger trapping forces that a particle

experiences near the edges of the trap when $x_0 \neq 0$. Figure 4.1 demonstrates the effect of these forces for a variety of distances from the trap center. When displacements begin near the center, there is a simple linear dependence of the MSD on the time lag before confinement. At larger initial displacements, this linear region is shorter and is followed by a super diffusive region before again showing confinement from the trap.

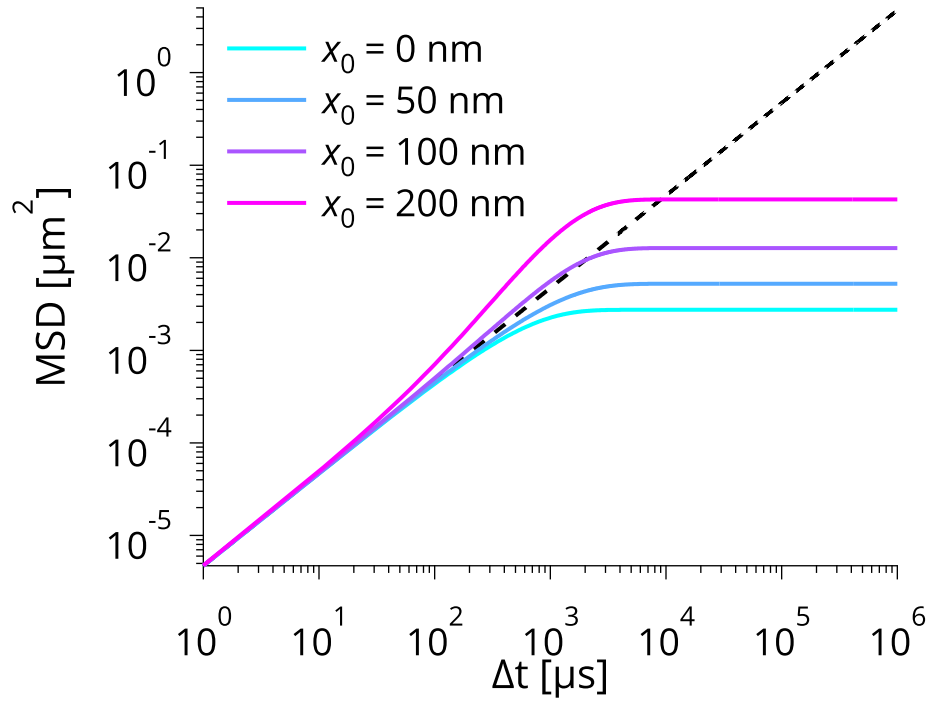


Figure 4.1: **Mean squared displacements in an optical trap.** The mean squared displacement (MSD) of a particle caught in an optical trap depends strongly on its initial position. The black dashed line shows the MSD for a freely diffusing particle while the colored lines show the MSD for particles starting 0, 50, 100, and 200 nm from the trap center. Higher initial displacements lead to superdiffusive behavior starting at shorter time lags.

Since equation (4.2) assumes that the diffusion constant is linearly proportional to the MSD, the chosen time step needs to avoid the super diffusive and confined regions in Figure 4.1. If the influence of the optical trap is ignored, then the optical trap will dominate measurements of the particle's diffusion and obscure the impact of the microtubule network we are interested in. The effect of the trap on diffusion measurements can be quantified by using equation (4.3) to calculate the MSD for a variety of x_0 . Then, the diffusion constant can be obtained assuming a naive, linear relationship with the MSD given by equation (2.9) for different time steps. At shorter time steps, this diffusion constant should match the value for a freely diffusing particle given by the Stokes-Einstein relation. Longer time steps, however, will yield diffusion constants that are larger than expected (due to the super diffusive region) and then smaller than expected (due to the confined region). Diffusion constants from all of these time steps can be compared to find the maximum lag before the trap affects the measurement of a particle's diffusion constant. Following the standard set by Bartsch *et al.*, τ_D is defined as the maximum usable time lag at which the naively calculated diffusion constant deviates by no more than 10% from the diffusion constant of a particle in free space. This should ensure that measurements of the diffusion constant from thermal noise imaging are close to the free space value despite the influence of the optical trap. Any deviations from the free space value should therefore come from interactions between the particle and its environment.

Figure 4.2 shows τ_D as a function of x_0 for several spring constants,

which have been chosen to be representative of the typical trapping strength of the PFM (Section 2.2.2). Initially, τ_D increases with x_0 as the particle can freely diffuse for a larger distance on one side of the trap before encountering significant gradient forces. This is followed by a sharp decrease in τ_D as those

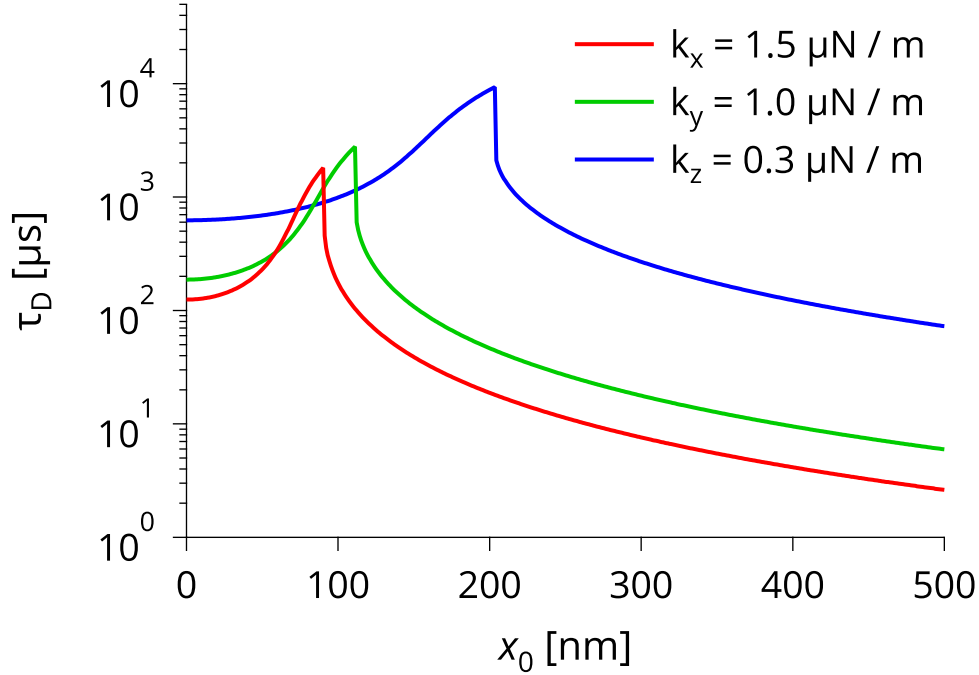


Figure 4.2: **Maximum time lag for diffusion constant measurements.** The time lag used to calculate diffusion constants has a large impact on the accuracy of the calculations. τ_D is the maximum time lag that can be used before the measured diffusion constant deviates by more than 10% from the actual diffusion constant. This lag is shown for spring constants of $1.5 \mu\text{N/m}$ (red), $1.0 \mu\text{N/m}$ (green), and $0.3 \mu\text{N/m}$ (blue), corresponding to the x-, y-, and z-spring constants of the PFM, respectively. Initially, τ_D increases as the particle can explore more space on one side before encountering the trap, but this is followed by a sharp decrease as strong gradient forces heavily impact the diffusion constant.

gradient forces increase in strength. Referring back to Figure 4.1, the particle experiences super diffusion followed by confinement, both of which drastically change the MSD from its linear relationship with the diffusion constant leading to the observed behavior at large x_0 . Fortunately, the changes in the MSD do not affect the majority of positions the particle visits within a trap. The Gaussian nature of the particle's position distribution means the particle will spend 99.7% of its time within 3 standard deviations of the trap center. For the spring constants given in Figure 4.2, these distances correspond to 150 nm in the x-direction, 190 nm in the y-direction, and 350 nm in the z-direction. Consequently, time lags of 30 μ s in the x- and y-directions and 150 μ s in the z-direction are chosen to maintain accuracy within 10% of the true diffusion constant of the trapped particle.

4.2 Results and Discussion

4.2.1 Measuring Diffusion Within a Single Trap

Before calculating the diffusion constant throughout a whole thermal noise image, it is important to gauge the accuracy of the technique over a single optical trap. For this test, a 208 nm diameter polystyrene particle was trapped 10 μ m above the bottom coverslip of a sample chamber and tracked for 4 s at a 100 kHz sampling rate. After calibrating the particle's position as described in Sections 2.3.1 and 3.1.5.3, equation (4.2) was used to determine the diffusion constant for $10 \times 10 \times 10$ nm³ voxels throughout the trapping volume.

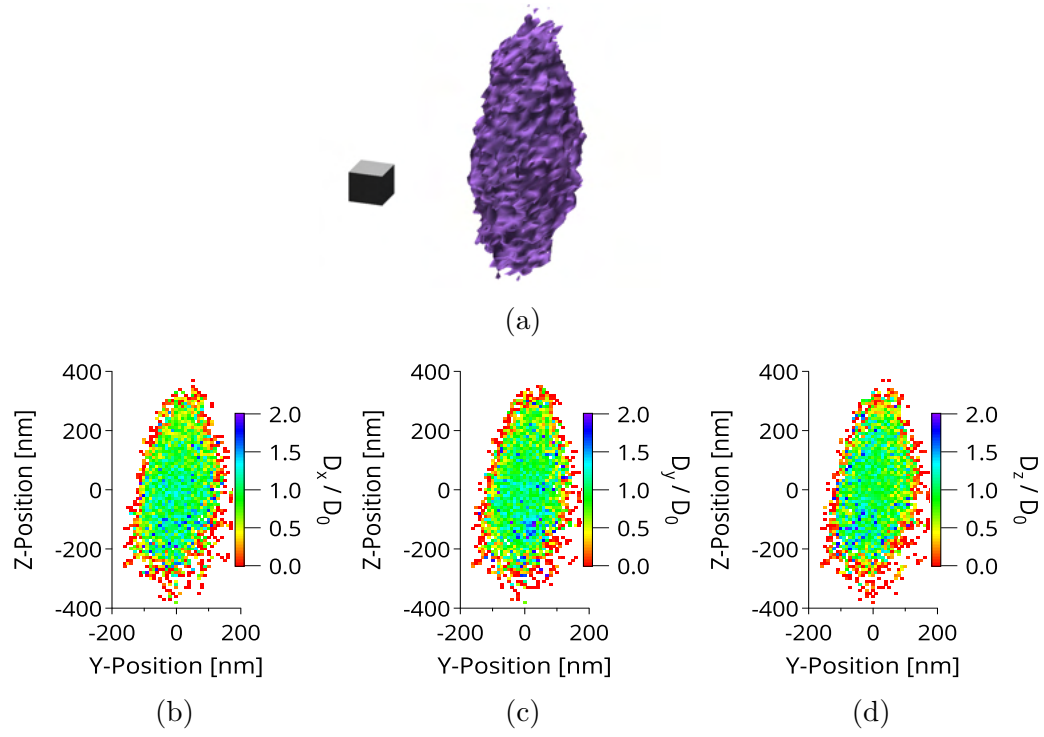


Figure 4.3: **Thermal noise image and diffusion constants for a single optical trapping volume.** (a) The isosurface of a single optical trap is shown for an isovalue of 30 next to a 50 nm scale cube. (b)-(d) Plane cuts taken through the center of the trapping volume give the x-, y-, and z-diffusion constants calculated using equation (4.2) for each voxel. All constants have been normalized to the diffusion constant for a 208 nm diameter particle in free space.

The results of these calculations are shown in Figure 4.3. A thermal noise image of the trap with an isovalue of 30 is shown with a 50 nm scale cube next to it. Below the thermal noise image are the x-, y-, and z-diffusion constants for a two-dimensional slice of voxels from the center of the trapping region. With each voxel's diffusion constant normalized to the diffusion constant of a particle in free space, it is clear that applying equation (4.2) to the thermal noise imaging data gives reasonable results. The majority of pixels are close to the expected diffusion constant with some deviations as the particle approaches the edges of the trapping volume. While the time steps chosen for the analysis were supposed to minimize these deviations (Section 4.1.2), that is only true for the majority of particle positions. Displacements far from the trap center will still lead to incorrect measurements of the diffusion constant in those regions.

Furthermore, the regions showing the largest deviations from the expected behavior occur in regions with low voxel occupancy. The low counting statistics near the edges of the trapping volume lead to less precision in those measurements as a few particle displacements dominate the entire behavior. As can be seen in Figure 4.4, the regions in which the diffusion constant drops to less than half of the expected value all occur within regions that have less than 10 total counts. Such low numbers of measurements make it difficult to accurately calculate the drift term in equation (4.2), which can be further compounded by changes in the gradient force near the edges of the trap. When combined, these effects can lead to deviations that do not necessarily originate

from interesting physics, but rather come from a lack of data.

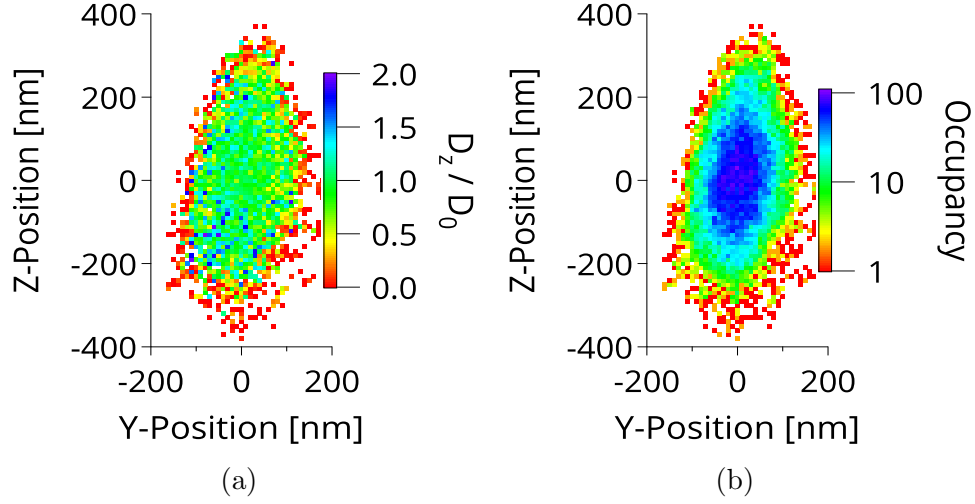


Figure 4.4: **Diffusion constant and voxel occupancy for a single optical trapping volume.** The measured diffusion constants for a particle within an optical trap can vary due to low voxel occupancy far from the center of the trapping volume. (a) For a two-dimensional slice at the center of the trapping volume, there is relatively uniform diffusion until the edges of the trap are reached. (b) Low voxel occupancy in these outer regions can lead to some voxels showing large deviations in the expected diffusion constant as they only have a few position measurements. Note the log scale on the occupancy.

4.2.2 Measuring Diffusion Across Multiple Traps

Analyzing the diffusion constant throughout a thermal noise imaging volume requires an extra step that is not present for single traps: diffusion constants in overlapping regions must be combined. Since thermal noise imaging relies on 363 overlapping traps to image a $1 \times 1 \times 1 \mu\text{m}^3$ region, there will be voxels that have contributions from multiple trapping volumes. A single voxel in the final thermal noise image might lie at the center of one trap while

simultaneously being at the edges of one or two others, and as Section 4.2.1 showed, the occupancy of a voxel can have a large impact on diffusion measurements. If diffusion constants from overlapping traps were blindly averaged, then voxels with only one or two position measurements would be treated the same as voxels with tens or hundreds of measurements.

To prevent low occupancy voxels from disproportionately impacting the diffusion constants in thermal noise images, a weighted average was employed. The diffusion constant in a thermal noise image voxel D_{avg} was calculated by weighting the diffusion constants of individual traps D_i by weights $w_i = \sqrt{n}$, where n is the occupancy of the individual trap's voxels and the sum is over voxels from overlapping traps.

$$D_{\text{avg}} = \frac{\sum_i w_i D_i}{\sum_i w_i} \quad (4.4)$$

Just as in the weighted average for the microtubule positions in equation (3.3), the weights were chosen to be proportional to the square roots of the voxel occupancy so that they would be inversely proportional to the standard deviation of the measurements.

Following this weighted average of voxels from overlapping traps, adjacent two-dimensional planes were combined in a weighted average. Planes for 50 nm on either side of a central region were used to increase the precision of measurements at each position. Figure 4.5 shows the result of this averaging for a thermal noise image of empty solution away from any surfaces. Since

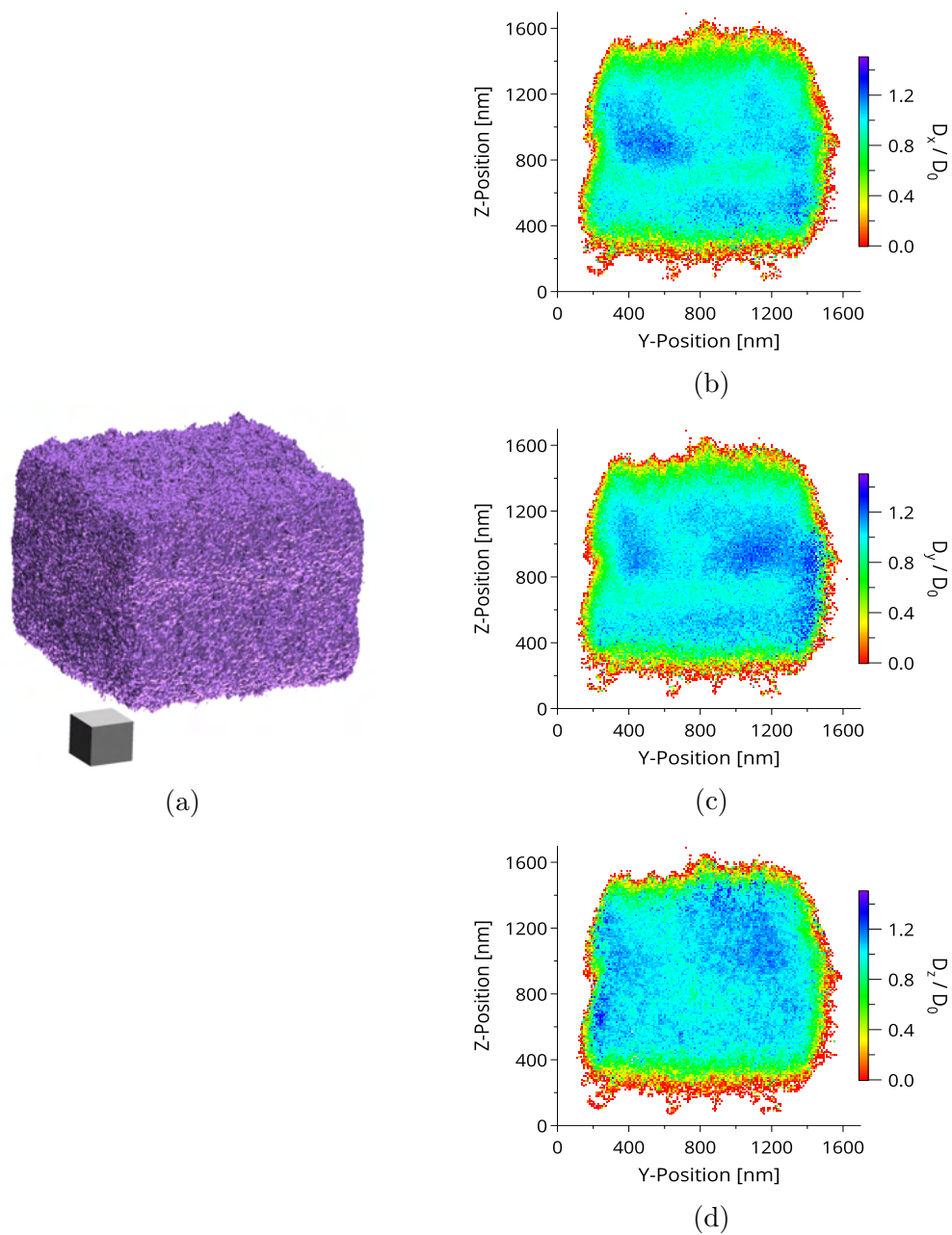


Figure 4.5: **Thermal noise image and diffusion constants in free space.** (a) A thermal noise image of empty solution is shown in purple for an isovalue of 30 next to a 100 nm grey scale cube. (b-d) Plane cuts taken through the center of the imaged region give the x-, y-, and z-diffusion constants calculated using equation (4.4) for each voxel.

there were no obstructions present in the environment, the described procedure should result in uniform diffusion constants throughout the volume. The x-, y-, and z-directions all show relatively homogeneous diffusion throughout the center of the volume with decreases along the outer edges. Such decreases are expected as the thermal noise image is still composed of individual traps. Increased gradient forces at the edges of each trap can still alter the measured diffusion constant. Regions of the imaged volume with overlapping traps, however, have smoothed out the edge effects from individual traps by incorporating data from the centers of neighboring traps.

A clearer picture of the homogeneity of the diffusion constants can be found by taking a line profile through the center of the thermal noise image. The line profile was taken along the z-axis as a weighted average over 50 nm on either side of a central value using equation (4.4). Figure 4.6 shows the results of these profiles with the error bars being weighted standard deviations

$$\sigma_D = \sqrt{\frac{N \sum_i w_i (D_i - D_{\text{avg}})^2}{(N - 1) \sum_i w_i}}. \quad (4.5)$$

Diffusion constants in the x- and y-directions show some structure, visible as two slight dips. This structure likely stems from the overlap of traps along the z-direction. While the spring constants used in the calculations for Figures 4.1 and 4.2 are assumed to be the same throughout the trap, there can be some change in stiffness as the laser focus narrows at the top and bottom of trapping volumes. Such changes could alter the diffusion constants measured

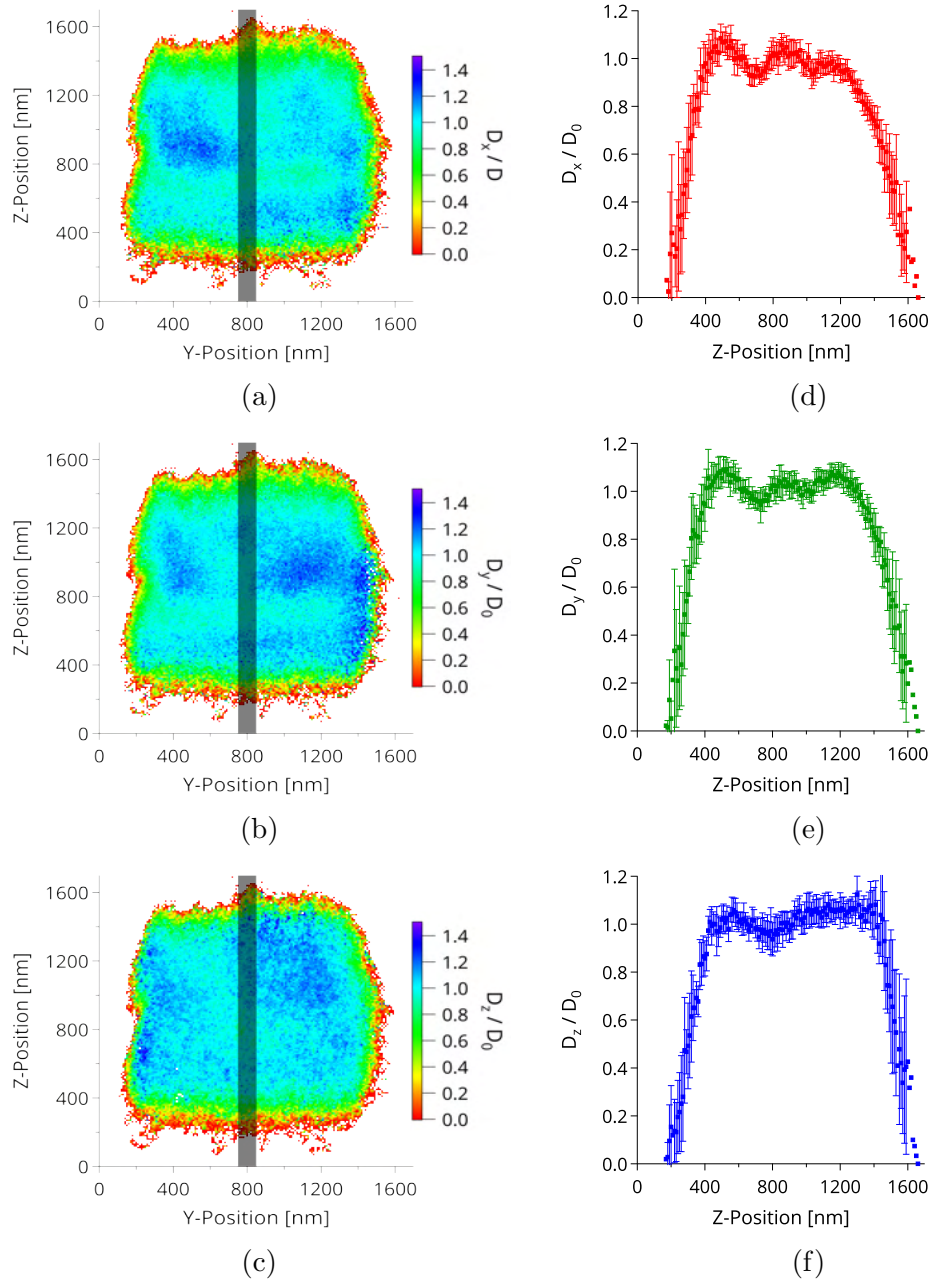


Figure 4.6: **Line profiles of diffusion constants.** (a-c) Line profiles were taken through two-dimensional slices of the diffusion constant along the grey boxes, averaging together values for 50 nm on either side of a central value. (d-f) The central region of the image yields consistent diffusion constant values with error bars coming from a weighted standard deviation.

at the overlapping regions. However, it should be noted that the diffusion constants are still quite uniform throughout the central region of the image with the maximum differences only being 10-15% and their values overlapping within the error bars. The diffusion constants in the z-direction are even more consistent with little variation until the edges of the imaged volume.

Before moving on to diffusion measurements within a microtubule network, the connection between the diffusion constant and voxel occupancy on the edges of thermal noise images needs to be explored. It is crucial to discern the difference between changes in the diffusion constant due to physical obstructions and changes due to poor counting statistics. Figure 4.7 shows a line profile through the z-diffusion constant data alongside the total voxel occupancy at each location. Again, the data is quite consistent throughout the central region and the diffusion constant only falls to below 90% of its expected value once the occupancy drops below 1000. This suggests that the diffusion data can be trusted throughout a large portion of the image volume, even when the counts are much lower than in the central region. It is important to note that the error bars in the figure only account for statistical variation in the measurements and are thus insensitive to the systematic errors that affect the extrema of the imaged volume. The combination of low voxel occupancies from the edges of individual traps with the changes in the gradient forces in those regions leads to biased estimations of the diffusion constant. Fortunately, the voxel occupancy acts as an indicator for these biases so that such regions can be avoided in analysis.

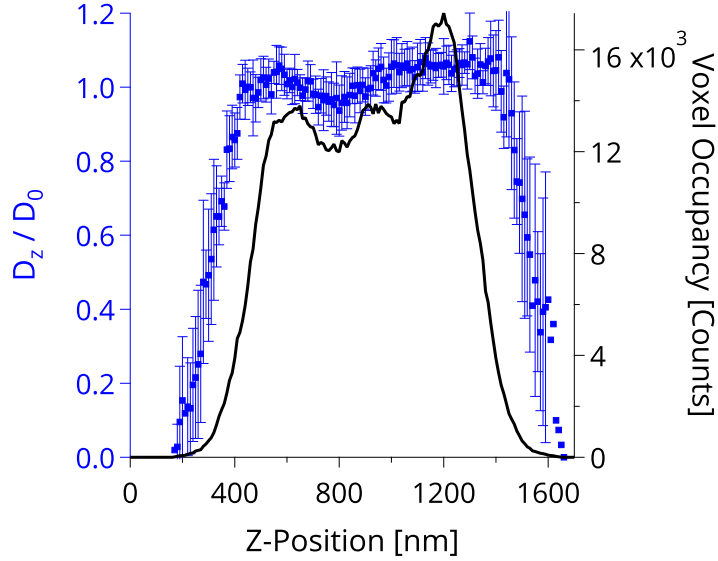


Figure 4.7: **Diffusion constant and voxel counts.** The diffusion constant of a particle along the z-direction is shown alongside the voxel occupancy in the same locations. Error bars are the weighted standard deviation calculated from equation (4.5).

4.2.3 Measuring Diffusion in a Microtubule Network

The presence of obstructions like microtubules within a diffusing particle's environment opens the door to a host of new physics. As a particle diffuses through a microtubule network, it encounters numerous filaments that may alter the properties of the surrounding environment. In particular, the fluid displaced by the particle can run into the no-slip boundary conditions at the surface of a microtubule and hydrodynamically couple the particle to the filament. Section 2.1.3 discussed various models of spherical particles coupling to surfaces and here it will be tested against thermal noise imaging data from

a cross-linked microtubule network.

Theories for the coupling between a spherical particle and another surface often assume that the surface remain stationary, so the analysis will start at the most stable part of networks: the intersection points of filaments. Starting with the thermal noise image in Figure 4.8a, it was rotated so that the axis of a microtubule was aligned with the x-axis, just as in Chapter 3. Two-dimensional slices of the diffusion constant in the yz-plane were then taken perpendicular to the axis of a microtubule as it intersected with another filament. Slices within 50 nm on either side of the intersection were combined in a weighted average using equation (4.4) with the results displayed in Figures 4.8b-4.8d.

The two-dimensional plots of diffusion constants in Figure 4.8 show much more structure than the ones taken far from other objects (Figure 4.5). Microtubules can be recognized in the lower half of each plot by the decreasing diffusion constants shown as red on the color scale (or white if there was no occupancy in the given voxel). These stand in stark contrast to the free space away from the filaments, which largely match the theoretical values. There is, however, a problem with noise in the x-diffusion constant. The x-direction shows markedly higher diffusion constants in areas away from microtubules than any other data shown so far—up to three times the expected value. Unfortunately, this was the result of a higher gain setting on the differential amplifier during the experiment that introduced electrical noise to the x-position measurements making them unsuitable for further analysis. The y-diffusion

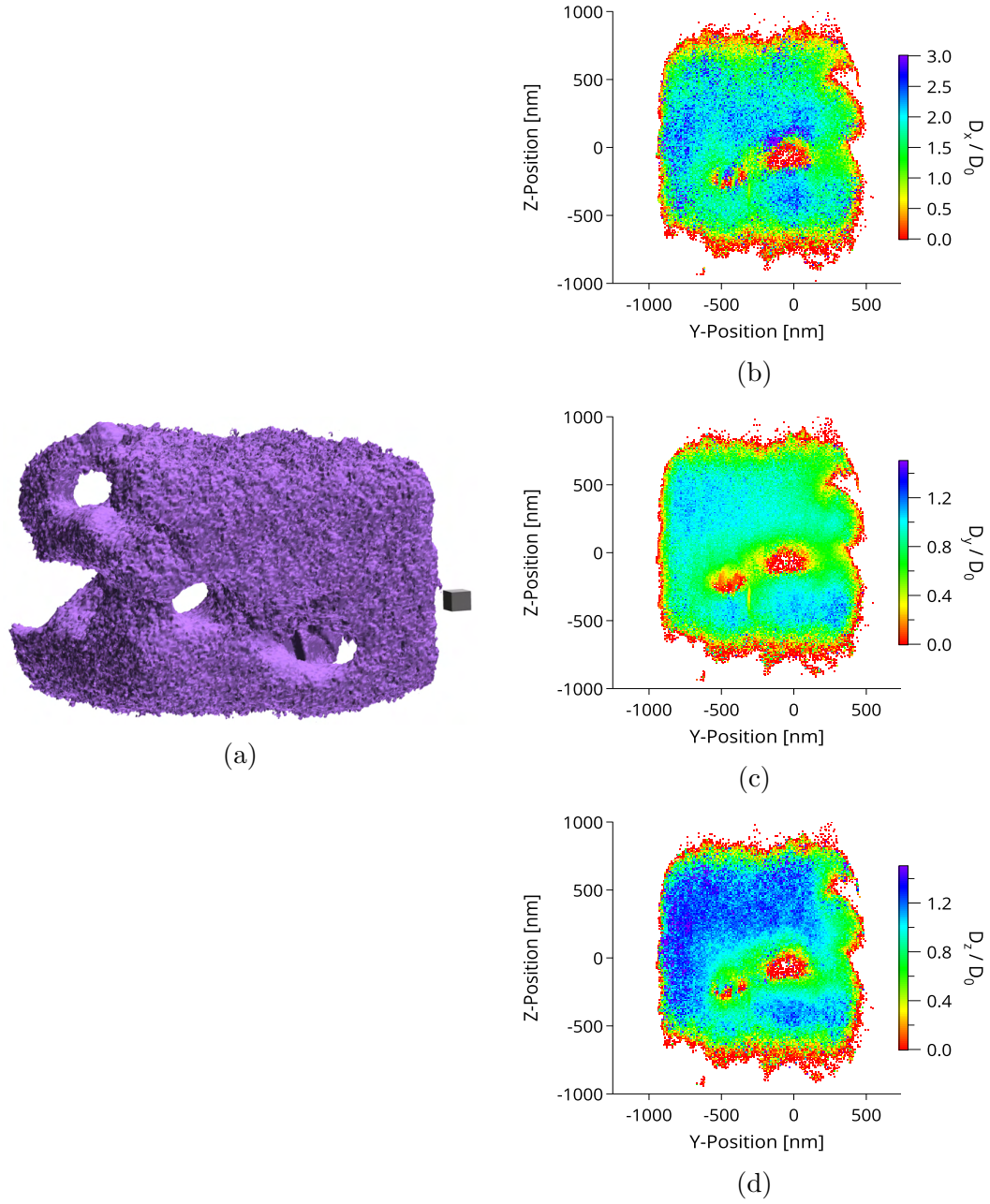


Figure 4.8: **Thermal noise image and diffusion constants in a microtubule network.** (a) A thermal noise image of a microtubule network is shown in purple for an isovalue of 30 next to a 100 nm grey scale cube. (b-d) Plane cuts taken through the center of the imaged region give the x-, y-, and z-diffusion constants calculated using equation (4.4) for each voxel.

constant did not suffer from this problem and while there is a slightly higher diffusion constant in the z-direction than expected, most values are still within 20% of the theoretical value given by the Stokes-Einstein relation.

To test this data against the diffusion theories presented in Section 2.1.3, line profiles were taken through the two-dimensional slices. The line profiles were centered on the axis of a microtubule at its intersection point with another filament. Y- and z-diffusion constants are shown in Figure 4.9 and both sets of measurements have been normalized to the average value of the diffusion constant in regions far from microtubules. The goal of this normalization was to make the microtubule's effect on the particle's motion clear relative to its motion free in solution. Values near 1 would indicate free diffusion whereas values smaller or larger than 1 would suggest that the particle is hydrodynamically coupling to another object in its environment. Near the microtubule ($z \approx 0$), the diffusion constant quickly drops off as the motion of the particle appears to be heavily influenced by the filament's presence. At positions below the microtubule ($z < 0$), there is not much space between the edge of the scanned volume and the microtubule so the diffusion constant never stabilizes around the free space value making it difficult to draw conclusions from this region. When farther above the microtubule ($z > 0$), the diffusion constant does plateau suggesting that there is free diffusion. The analysis and comparisons with existing theory will be focused on this region moving from the microtubule axis to positions above the filament.

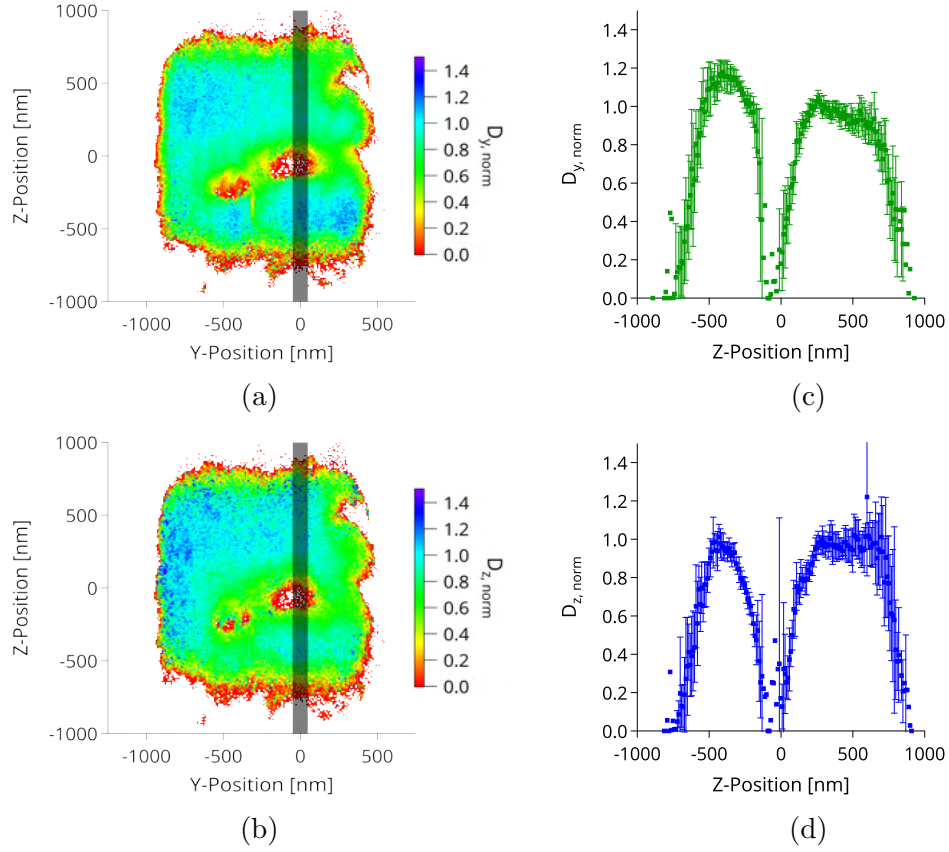


Figure 4.9: **Line profiles of the y- and z-diffusion constants in a microtubule network.** (a-b) Line profiles were taken through two-dimensional slices of the diffusion constant along the grey boxes, averaging together values for 50 nm on either side of the microtubule filament. (c-d) Below the microtubule ($z < 0$), the diffusion constant is affected both by the microtubule and then by the edge of the imaged volume. Above the microtubule ($z > 0$), the diffusion constant has enough space to freely diffuse and assume a constant value. At the microtubule itself ($z \approx 0$), the diffusion constant quickly drops off as the particle's motion is affected by the filament.

As noted earlier, the measured diffusion constant is still representative of the actual value as long as the voxel occupancy is high enough, but it can

be inaccurate beyond that point (Figure 4.7). This trend was used to select a portion of the diffusion constants for comparison to theory. Moving from the microtubule towards positive z -values, the voxel occupancy was tracked while observing the diffusion constant. Once the diffusion constant dropped to 90% of its expected value in free space, the voxel occupancy was noted and used as an indicator that the diffusion constant was no longer accurate. Only diffusion constants from voxels with greater occupancy than the noted value were used in the analysis.

Figure 4.10 shows the z -diffusion constant from this high occupancy region alongside theories for hydrodynamic coupling of spheres to other surfaces. The z -direction is perpendicular to the microtubule so equations (2.14), (2.15), and (2.16) were plotted for a 104 nm radius sphere moving near a static plane, a fluctuating plane, and a 12.5 nm radius sphere representing the microtubule, respectively. While all three theories capture the general trend of the diffusion constant increasing as the probe particle moves away from the microtubule, the equations for movement of a sphere near a plane predict much longer coupling ranges than shown by the data. Such behavior makes sense due to the physical extent of a microtubule when compared to a plane. The no-slip boundary condition at an infinite planar surface extends over all space while the same condition at a cylindrical microtubule's surface only functions over its narrow width and its length. In contrast, the line showing the theory for coupling between two spheres shows much better agreement with the observed behavior, and given the lack of theory for this experiment's specific geometry,

the sphere-sphere case is the best match to the data.

Moving forward with equation (2.16) as the model, the z-diffusion data was fit with the microtubule's position, the probe particle's radius, and the microtubule's radius as free parameters. The data and fit are shown in Figure

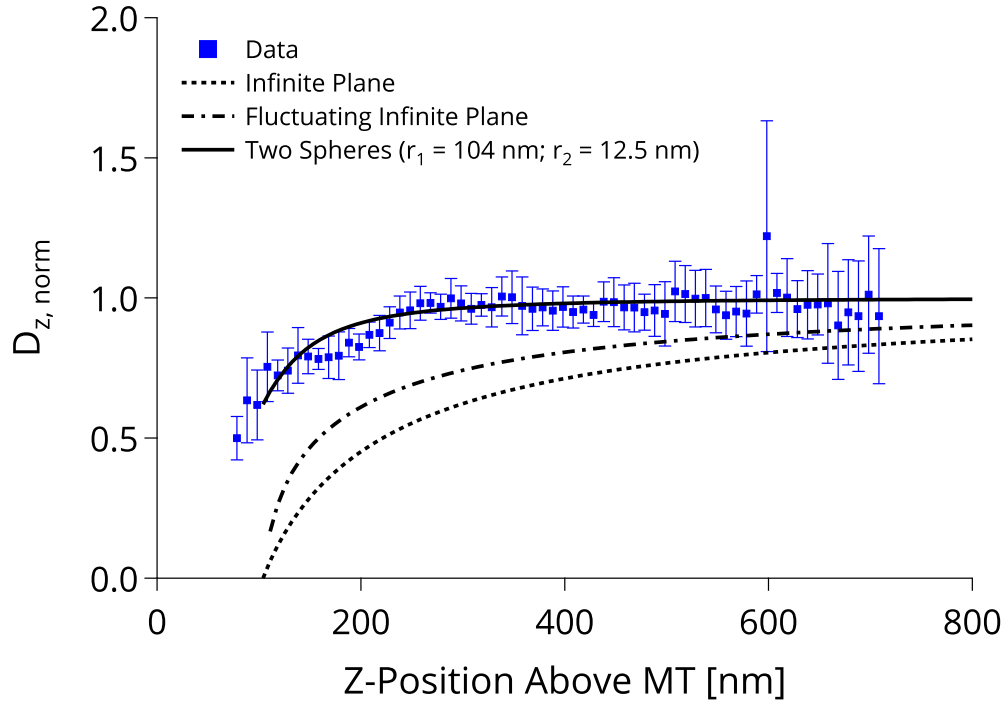


Figure 4.10: **Z-diffusion constant of a sphere near a microtubule with hindered diffusion models.** The z-diffusion constant is plotted (blue) as a function of the distance from the center of the probe particle to the microtubule axis. Theories for the diffusion of a 104 nm radius sphere near infinite planes (static and fluctuating; dashed and dashed dot) at the position of the microtubule predict hydrodynamic coupling over much greater distances than the data show. A model for the coupling between two spheres (solid), with one representing the probe and one representing the microtubule, predicts the trends in the data with much greater accuracy.

4.11 along with the parameter results. Not only is the model a good fit to the data, the microtubule position and probe radius are within uncertainty of their predicted values (0 nm and 104 nm). While the expected radius of a microtubule is 12.5 nm, it is not surprising that the fit returned a larger result because this model assumes that the microtubule is a sphere. The microtubule is a cylinder so it has greater extent along its axis of symmetry than a sphere of the same radius so a sphere would need to be larger to exhibit the same hydrodynamic effects. A similar fit was attempted for the y-diffusion constant, but it resulted in parameters with enormous uncertainties that made interpretation difficult.

One factor that hasn't been considered in this fit is the effect of the dynamics of the microtubule. Microtubules are constantly fluctuating as they undergo Brownian motion in a thermal bath, so this could affect their hydrodynamic interactions as it does for infinite planes. If microtubules follow the same trend as planes, then a freely fluctuating microtubule's coupling with a sphere would fall off more quickly than it would for a static filament's coupling. Meanwhile, this microtubule network provides a clear means of testing this prediction. Section 3.2.4 showed that microtubule fluctuations generally decrease near intersections and increase away from intersections, therefore there should be observable differences in the coupling between a microtubule and a sphere as the sphere moves away from the intersection.

Moving away from the microtubule intersection in steps of 100 nm, line profiles like the one in Figure 4.11 were taken and fit with equation (2.16).

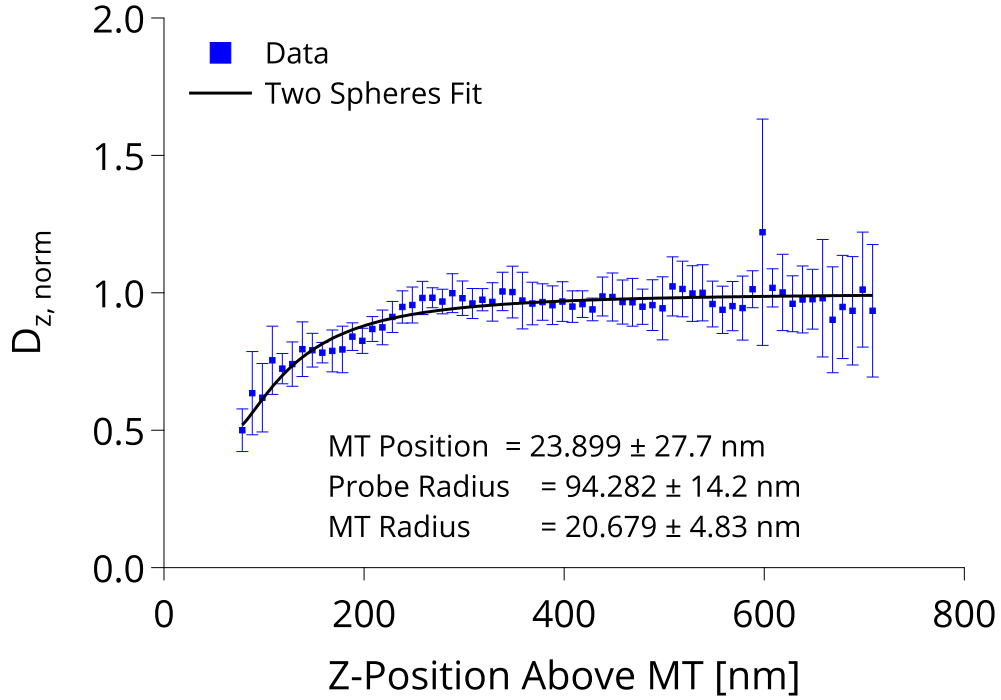


Figure 4.11: **Z-diffusion constant of a sphere near a microtubule with sphere-sphere coupling fit.** The z-diffusion constant is plotted (blue) as a function of the distance from the center of the probe particle to the microtubule axis. Equation (2.16) is used to fit the data with the microtubule's position, the probe particle's radius, and the microtubule's radius as free parameters. The microtubule position and probe radius are within uncertainty of their expected values.

At each step, the microtubule's position was fixed at the filament's expected position (0 nm) and the probe's radius was fixed at the manufacturer's specified value (104 nm) while the microtubule's radius was allowed to vary. If fluctuations weakened the coupling between the filament and the probe, then this would present as the radius of the microtubule shrinking as the distance from the intersection increased. Interestingly, this behavior was not observed.

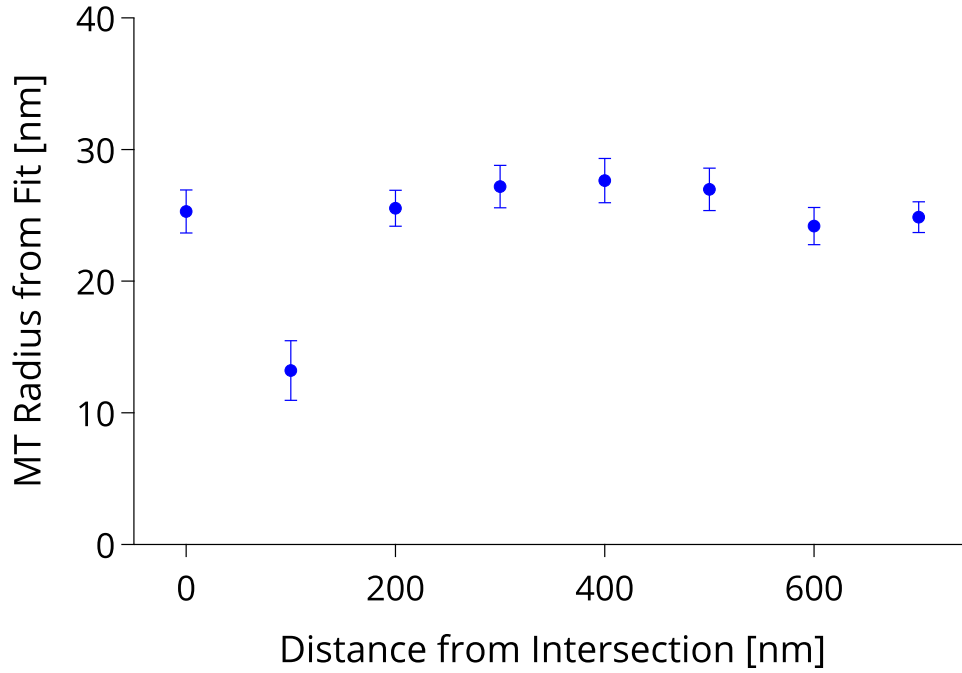


Figure 4.12: **Microtubule radius from fit versus distance from intersection.** Equation (2.16) was used to fit the diffusion constant of the probe particle at different distances from a microtubule intersection. The microtubule position and the probe radius were held constant while the microtubule radius was allowed to vary. Given the consistency of the radius fit parameter, the fluctuations of the microtubule (which increase moving away from the intersection) do not appear to impact the coupling between the filament and the probe particle.

Figure 4.12 instead shows that the fitted value of the microtubule radius is roughly constant no matter the distance from the microtubule intersection. There is an abrupt dip in the microtubule radius 100 nm from the microtubule intersection, but it returns to its original value for every position thereafter. This suggests that microtubule dynamics do not significantly impact the diffusion of surrounding particles and that the coupling is dominated by the

geometry of the system. It is possible that microtubules are simply too stiff for fluctuations to affect the hydrodynamics of their surroundings, and that only more flexible biopolymers like actin would show the described trends.

4.3 Summary: Characterizing Diffusion in Microtubule Networks

Using thermal noise imaging data, we were able to quantify the spatial variation in a spherical particle's diffusion constant on a nanoscopic scale. Particle motion was shown to yield the expected diffusion constant despite confinement by the trap, provided voxel occupancy was high enough. When combining multiple traps, it became possible to smooth out variations that were seen near the edges of individual trapping volumes and create diffusion maps on the micron scale. These combinations even allowed us to track the diffusion constant throughout a cross-linked microtubule network for the first time. Fitting a model of one sphere diffusing perpendicular to the surface of a separate, stationary sphere accurately extracted the position of the microtubule as well as the radius of the probe particle. When examining the effects of filament fluctuations on the hydrodynamic coupling between the probe and the microtubule, it appeared that the dynamics had no significant effect although this may have been a consequence of the low amplitude of the fluctuations resulting from the high persistence length of microtubules. While this sphere-sphere model did return a larger radius than would be expected for a microtubule, it only serves to show that more theoretical work needs to

be done for the geometry of this system.

Chapter 5

Optical Manipulation of Single and Bundled Microtubules

Developing an in-depth knowledge of the properties of single microtubules is crucial to a firm understanding of the mechanics of microtubule networks. As a network is subjected to load, its constituent filaments will experience differing forces, which will deform the filaments, alter their fluctuations, and lead to changes in the overall network behavior. Thus, it is imperative to study how parameters like filament length and structure affect persistence length and other microtubule properties [25,26]. Existing methods for studying microtubules often require binding them to surfaces or observing them as they fluctuate uninhibited throughout a volume [26,120]. The method described here seeks to combine the benefits of both of these techniques by using a standing wave optical trap (SWOT; Section 2.4.1) to manipulate single and bundled microtubules. A SWOT cancels out the scattering force while simultaneously increasing the strength of the gradient force compared to a single beam trap operating at the same laser power. In this way, it is possible to observe filaments over long times without restricting their motion through binding while also maintaining them in the field of view of a microscope. Enabling such long observations allows for continuous access to the

dynamics of single filaments and even opens the possibility of forming larger microtubule structures if filaments can be coerced into the same volume. This chapter shows that it is even possible to control the orientation of microtubules through the polarization of a trapping laser, which leverages the form-induced birefringence of these cytoskeletal filaments [121, 122].

5.1 Materials and Methods

5.1.1 Bundled Microtubule and Sample Preparation

For experiments in a SWOT, microtubules were prepared as either single filaments or as bundles of multiple filaments. Single filaments were prepared using the process described in Section 3.1.3 for unbiotinylated microtubules. Bundled filaments were created through the same procedure, but with an additional step. Spermine (S3256-1G, MilliporeSigma, Burlington, MA) was added after the ultracentrifuge spin at 4 °C and before the tubulin is placed in the hot water bath at 37 °C to bring the concentration up to 1 mM. The positively charged spermine serves to connect negatively charged filaments laterally to form bundles [123].

The sample chamber was designed to increase the probability of finding microtubules in solution near the dichroic coverslip for ease of trapping. Towards this end, 10 μm spheres were used to separate the top and bottom coverslips, which were then bound together with clear nail polish. Microtubules were pipetted into the sample before being sealed with additional nail polish. This thin design ensured that microtubules were likely to be found close to

the dichroic coverslip instead of far in solution.

5.1.2 SWOT Calibration

Since the SWOT uses video-based microscopy instead of a QPD, it must be calibrated using a different method than the PFM. A 208 nm diameter polystyrene particle is trapped approximately 200 nm from the dichroic coverslip in the strongest anti-node of the trap. The particle is then tracked by fitting a two-dimensional Gaussian to its point spread function as it explores the trapping volume. Note that only motion in the xy-plane is tracked, and strong confinement in the z-direction makes this a reasonable assumption. From the variance of the particle's positions, it is possible to extract the spring constants of the trap with the following relation

$$k_x = \frac{k_B T}{\text{Var}(x)}, \quad (5.1)$$

where x is the x-position of the particle, $\text{Var}(x)$ is the variance in the x-position, k_x is the spring constant in the x-direction, k_B is the Boltzmann constant, and T is the temperature of the surrounding fluid.

This calculation is trivial with high detection bandwidth, but it can cause problems with slower detection methods like the CCD camera used in the SWOT. The long exposure time of the camera leads to motion blurring of the particle and artificially lowers the measured variance in the particle's position. If not corrected for, this will lead to a measured spring constant that is larger than its true value. Fortunately, Wong and Halvorsen showed that this effect

can be corrected for and the true trapping stiffness can be extracted, assuming that the distribution is Gaussian [124]. For a given camera integration time W , the true variance in the particle position $\text{Var}(x)$ can be determined from the measured variance $\text{Var}(x_m)$ using

$$\text{Var}(x_m) = \text{Var}(x) \left[\frac{2}{\alpha} - \frac{2}{\alpha^2} (1 - e^{-\alpha}) \right], \quad (5.2)$$

where $\alpha = Wk_x/\gamma$ and γ is the drag coefficient of the particle. Equation (5.1) can be substituted into equation (5.2) and then be numerically solved for the true particle variance.

The results of particle tracking for the SWOT are shown in Figure 5.1 for a laser polarization of 90° relative to the x-axis. As expected for Brownian motion in an optical trap, a histogram of particle positions shows a Gaussian distribution. Using equation (5.1), the spring constant was extracted for the measured x-positions as $k_{x,\text{measured}}$, but this is an overestimate of the trapping stiffness. Accounting for motion blur with equation (5.2) yields the true spring constant $k_{x,\text{corrected}}$.

A spring constant for the y-direction of the trap can be calculated in the same way, which elucidates the effect of the laser polarization on the strength of the trap. Section 2.2.1.1 mentioned that the optical trap is wider in the direction of the laser's polarization, which leads to weaker spring constants along that axis. Since this work seeks to manipulate microtubules through the polarization of the laser, it is important to characterize the variation in the trapping

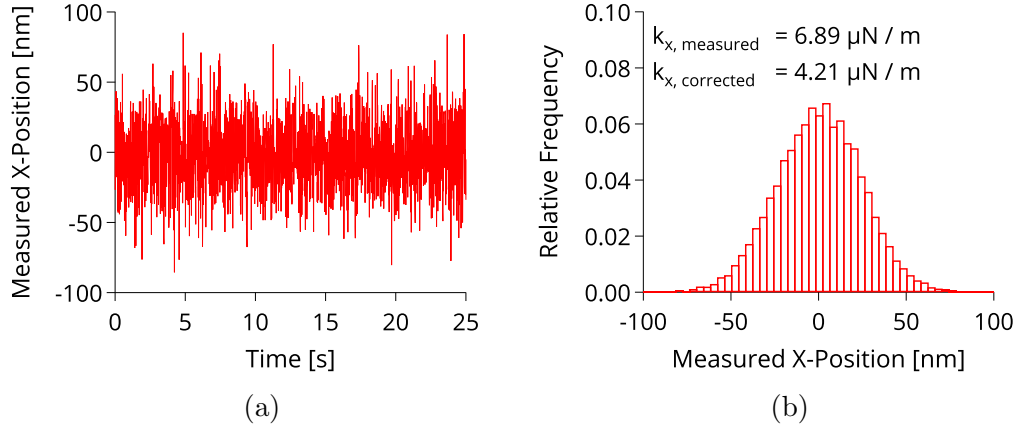


Figure 5.1: **Calibration of a standing wave optical trap.** (a) The x-position is of a 208 nm diameter trapped particle is tracked over time. (b) A histogram of the particle's positions shows a narrow distribution, but this must be corrected for motion blur caused by the finite integration time of the camera. Equation (5.2) is numerically solved to calculate the true spring constant $k_{x, \text{corrected}}$ from the measured spring constant $k_{x, \text{measured}}$. The laser power in the sample chamber was 50 mW and the integration time of the camera was 500 μs .

strength with the polarization. Table 5.1 summarizes the trapping strengths of the SWOT along the x- and y-directions for different laser polarizations. Trapping is always stronger perpendicular to the polarization, as expected, but the strength is not consistent. Naïvely, one would expect $k_{y, \text{corrected}}$ at 0° to be equal to $k_{x, \text{corrected}}$ at 90° since they are both perpendicular to the laser polarization, but this is not the case as $k_{x, \text{corrected}}$ is actually 10% larger. This arises from variation in the reflectivity of the dichroic mirror that couples the laser into the objective lens (Figure 2.12). The mirror is angled at 45° relative to the incoming laser so its reflectivity is at a maximum when the polarization is perpendicular to the slope of the mirror. For this setup, that means that

the laser power in the sample is highest when the laser polarization is at 90° relative to the x-axis and at its lowest when the polarization is at 0° . While this variation in power is small, it is important to remember when analyzing results from trapping experiments.

Polarization Angle	$k_{x,\text{corrected}}$ [pN/ μm]	$k_{y,\text{corrected}}$ [pN/ μm]
0°	3.34	3.82
45°	3.49	3.77
90°	4.21	3.65

Table 5.1: **Spring constants for a 208 nm diameter particle under different polarizations in a standing wave optical trap.** The spring constants of a standing wave optical trap for a 208 nm diameter particle are shown for different laser polarizations relative to the x-axis. All constants have been corrected for motion blur. The laser power in the sample was 50 mW

5.2 Results and Discussion

5.2.1 Counting Microtubules

To determine the effect that laser polarization has on the manipulation of bundled microtubules, it is important to have a measurement of the number of microtubules in a bundle. The microtubules in this work are fluorescently labeled so the intensity of the emitted light can act as a gauge for the number of filaments. Each individual microtubule should have roughly the same number of fluorophores, and the emission intensity should increase linearly with the number of fluorophores. Taken together, these assumptions suggest that the intensity of light emitted by a bundle should be directly related to the number of filaments in that bundle. A bundle with two filaments

would then appear twice as bright as a single filament. This would suggest that there is a linear relationship between the brightness of a bundle and the number of microtubules within the bundle, which could be tested by looking for discretization in the brightness of different filament bundles.

One difficulty that arises when comparing the brightnesses of microtubules and microtubule bundles comes from the limited dynamic range of CCD cameras. In order to visualize a single microtubule, long exposure times are required that, in turn, cause thicker bundles to saturate the camera sensor. Furthermore, strong background signals from the environment (like the dichroic coverslip) necessitate long exposure times to properly resolve trapped filaments. A saturated signal cannot accurately describe the intensity of the light emitted from the bundle so it is not possible to test if there is a linear relation between the brightness and the number of microtubules in a bundle. Therefore, the first step in counting microtubules becomes the determination of how brightness varies with exposure time. This relationship would allow for comparison between single filaments imaged at longer exposure times and bundles imaged at shorter exposure times.

Over the long, millisecond timescales of a camera exposure, the number of photons hitting the sensor (and hence the measured brightness) should increase linearly with the exposure time. This was tested by adhering single microtubules to a coverslip with poly-L-lysine and then imaging one of them at different exposure times. For each exposure, line profiles were taken at different points along the microtubule's length. The peaks in brightness were

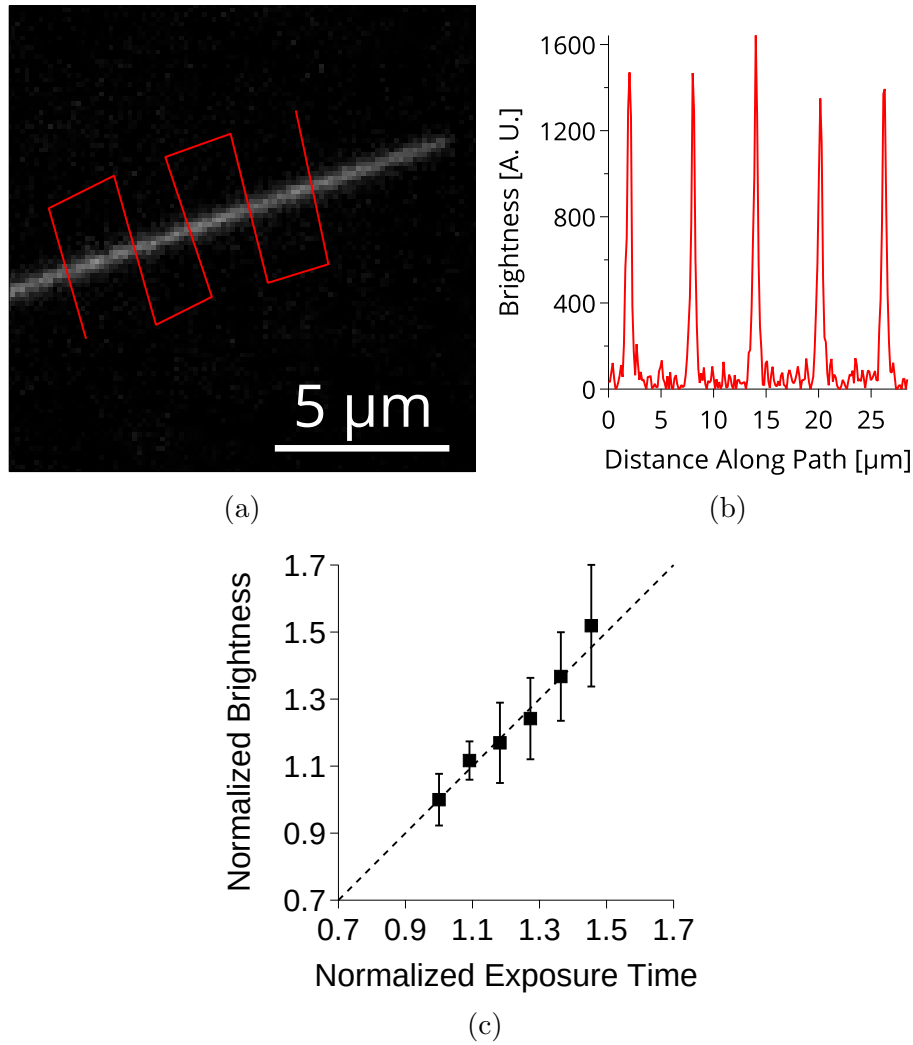


Figure 5.2: **Brightness of a microtubule at different exposure times.** (a) A line profile (red) is taken along a single microtubule. (b) The brightness along the line profile path is displayed. (c) For exposures from 55 ms to 85 ms, the brightness of the microtubule was determined by fitting gaussians to the line profile peaks, integrating over them to determine the total fluorescence emission at each location along the filament, and then averaging them together to find the microtubule brightness at a given exposure. These were normalized to the brightness at 55 ms and the exposure time of 55 ms for comparison. The black dashed line has a slope of 1, the expected trend of brightness versus exposure time. Error bars are standard deviations of the integrated peaks.

then fit with Gaussians and integrated to find the total brightness (including out of focus emissions). Finally, the integrated brightnesses from the same exposure time were taken together to find the average and standard deviation for the brightness of the microtubule. The average microtubule brightness at each exposure time was normalized to the brightness at the shortest exposure time of 55 ms to make comparisons between exposure times easier. These normalized brightnesses were plotted against exposure times normalized to 55 ms (again for ease of comparison) with the results displayed in Figure 5.2. There is a clear linear relationship between the brightness of the microtubule and the exposure time so images from different exposure times may be compared.

Moving on to counting the number of microtubules in a bundle, a sample of bundled filaments was prepared and then attached to a coverslip coated in poly-L-lysine. A bundle was found that had varying brightness along its length, and since the entire bundle was in focus at once, such variations should have come from different numbers of filaments grouped along its length. Brighter regions would have more filaments and darker regions would have fewer. This bundle was imaged with an exposure time of 35 ms and the average brightness was found using a line profile for three distinct regions along its length that showed different brightnesses. Figure 5.3 shows this bundle and the line profiles taken across it. Region #2 was the dimmest and was equal in brightness to the single microtubule from Figure 5.2, after adjusting for the difference in exposure time. Once all three regions were normalized to this brightness, it appeared that region #1 was twice as bright while region #3

was three times as bright. With this appearance of brightness discretization, it seems reasonable to conclude that differences in brightness come from increases in the number of filaments in a bundle. In this case, region #1 would have two microtubules, region #2 would have one, and region #3 would have three.

Figure 5.3 shows that the idea of discretized microtubule bundle brightness is reasonable, but the result is not perfect. Regions #1 and #3 overlap with each other within uncertainty, and both regions' averages fall below the expected values. Such variations, particularly for brighter regions of the bundle, likely stem from self-quenching of the rhodamine dye as several filaments lie in close proximity [125]. Instead of continuing to increase linearly with the number of filaments in a bundle, high concentrations of dye molecules can cause the intensity of emitted light to increase more slowly as neighboring fluorophores interact with each other. These fluorophores can transfer excitation energy to ground state molecules in lieu of releasing that energy as emitted light thereby decreasing the measured intensity. This problem would likely worsen with larger and larger bundles, but is still within reasonable limits for the filaments studied in this dissertation.

5.2.2 Optical Response of a Microtubule Bundle in a Polarized SWOT

Equation (2.19) shows that the gradient force exerted on an object depends on the volume of the trapped material as well as the object's index of

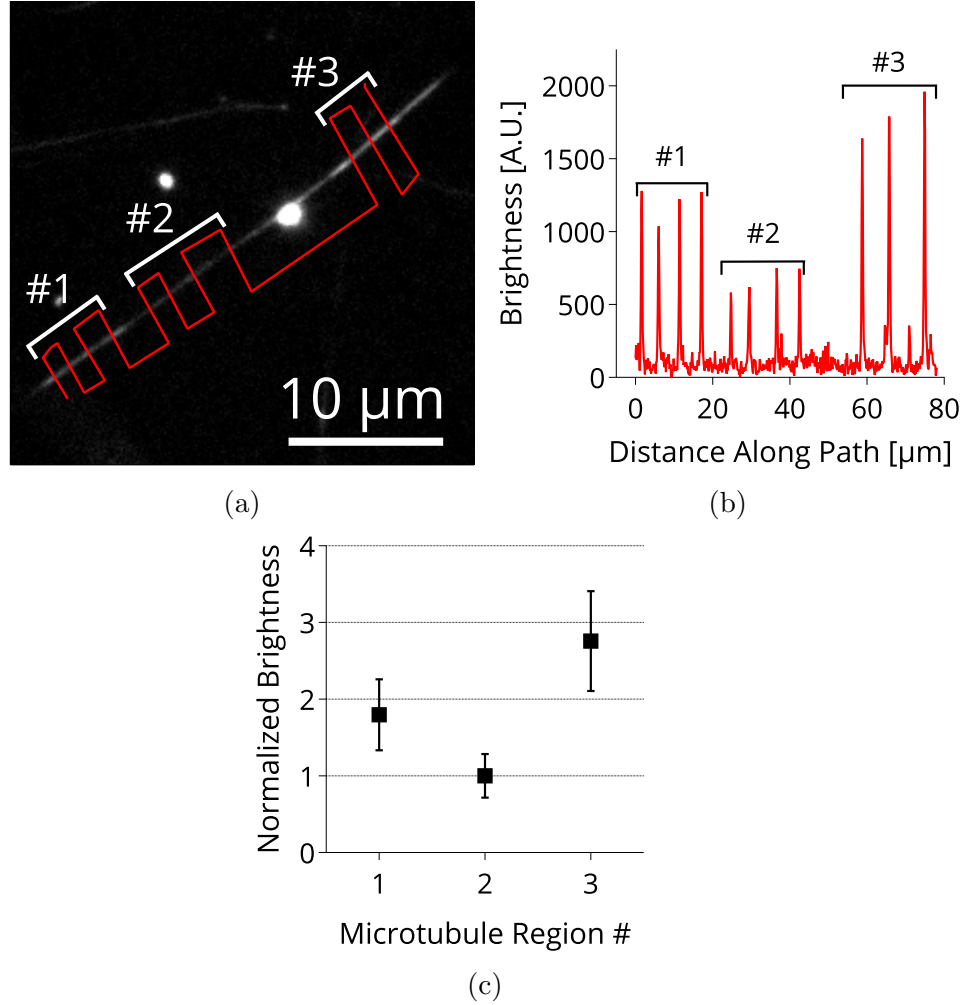


Figure 5.3: **Microtubule bundle brightness varying with number of filaments.** (a) A line profile (red) is taken along a bundle of microtubules with varying brightness coming from different numbers of filaments along its length. Exposure time is 35 ms. (b) The brightness along the line profile path is displayed. (c) The average brightness of each region normalized to the dimmest region (#2) appears to be discretized. Error bars are standard deviations of the brightnesses in each region.

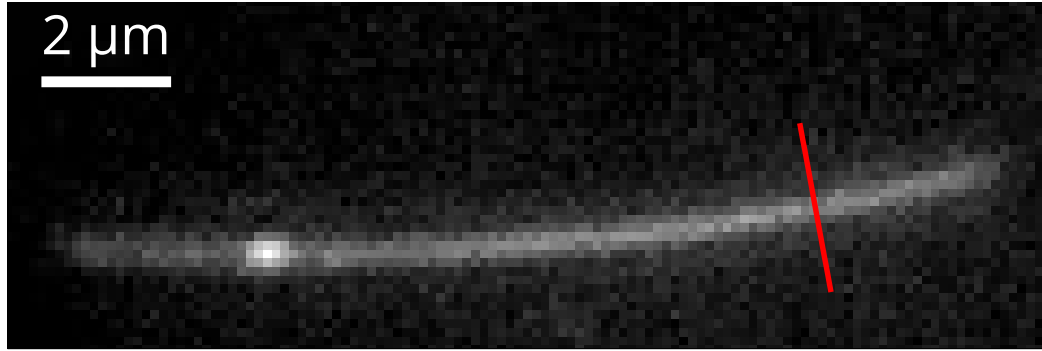
refraction. If the material is birefringent, then its response to an optical trap will be different depending on the polarization of the laser light. It should therefore be possible to determine the birefringence of a microtubule based on its motion in an optical trap and the effects of its birefringence should be more easily observed for a bundle of filaments that has several times more volume than an single filament.

When observing a sample of bundled microtubules, a bundle was found with one end attached to the surface of the dichroic coverslip while the rest of the bundle freely fluctuated in solution. Such an arrangement provided a good opportunity to study the optical response of microtubules without having to stably trap the entire filament. First, the brightness of the bundle was analyzed and used to determine that there were three microtubule filaments in the bundle. The fluctuations of the free end of the bundle were then tracked along a line perpendicular to the filament axis to ensure that it wasn't obstructed by the coverslip surface. A histogram of these fluctuations is shown in Figure 5.4. There is a slight asymmetry to the distribution that likely originates from the curvature of the bundle, but it still appears Gaussian in nature.

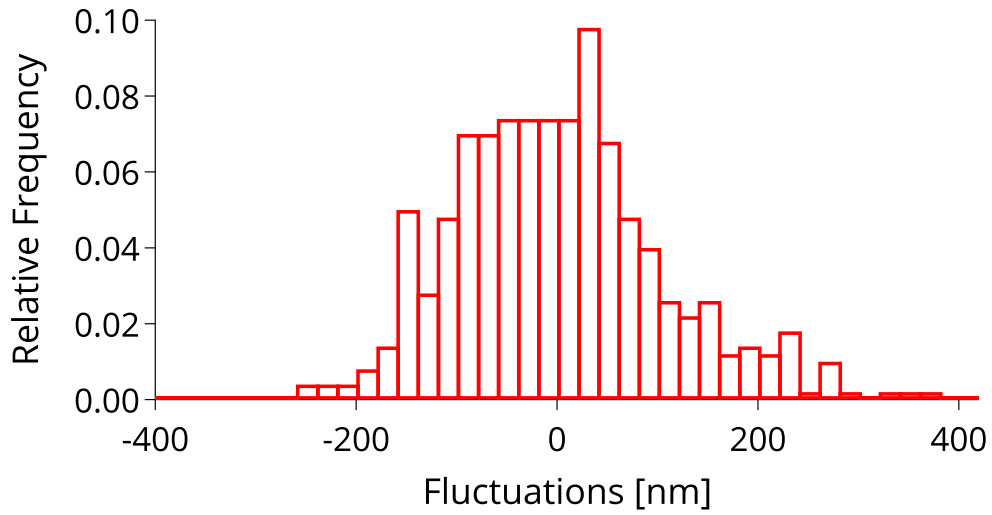
From these fluctuations, the persistence length of the filament can be extracted as

$$l_p = \frac{L_a^3}{3\text{Var}(x)}, \quad (5.3)$$

where l_p is the persistence length, L_a is the distance from the pinned section



(a)



(b)

Figure 5.4: **Fluctuations of a microtubule bundle pinned on one end.** (a) A bundle of three microtubule filaments is pinned at the brightest region on the left. Fluctuations were analyzed along the line profile (red). (b) Fluctuations of the filament are corrected for motion blur resulting from the 35 ms integration time of the camera and are displayed as a histogram.

of the microtubule to the fluctuating region, and $\text{Var}(x)$ is the variance in the microtubule's position [25, 126]. As with the variances in particle positions from Section 5.1.2, the variance in the microtubule position was corrected for motion blurring. Here, the drag coefficient of a cylinder moving perpendicular to its long axis and parallel to a nearby surface was used

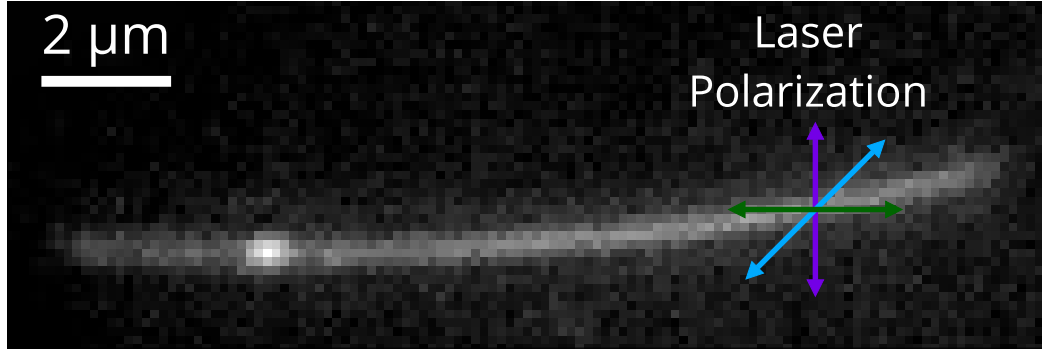
$$\gamma = \frac{4\pi\eta L}{\cosh^{-1}(h/r)}, \quad (5.4)$$

where η is the dynamic viscosity, L is the length of the cylinder, r is the radius of the cylinder, and h is the distance above the surface [127]. This yields a persistence length of 18.3 mm. While single microtubules of a similar length typically have persistence lengths of about 2 mm [25, 26], theory for bundles of polymer chains suggest there is a strong dependence of a bundle's persistence on the number of filaments [22, 128]. Specifically, the persistence length scales with the square of the number of filaments (if the filaments are tightly coupled) and this has been confirmed experimentally for actin filaments bundled by depletion forces in a polyethylene glycol solution [129]. This suggests that three filaments should have a persistence that is nine times that of a single microtubule, which aligns well with the results presented here, although this assumes that spermine tightly couples neighboring filaments together.

Having observed the mechanical properties of this bundle, the SWOT was turned on so that the bundle's optical properties could be studied. The freely fluctuating end was positioned so that it sat in the center of the laser

focus and its behavior was tracked under different polarizations while fluctuations were measured along the same line as in Figure 5.4. Predictably, the fluctuations of the filament were suppressed under any polarization (Figure 5.5) as the bundle experienced the gradient force of the SWOT with all three histograms showing a narrower range of explored positions than was seen for the freely fluctuating filament. This suppression can be thought of as a system of two springs in parallel: one spring coming from the inherent stiffness of the bundle and the other coming from the SWOT. The stiffness of the filament is effectively augmented by the trapping laser with the magnitude of the effect being dictated by the strength of the gradient force.

Unexpectedly, the filament fluctuations show a strong dependence on the polarization of the SWOT. This dependence can be quantified by calculating spring constants for the filament using equation (5.1) with corrections being made for motion blurring. Assuming that the filament's stiffness acts in parallel with the gradient forces so that its contribution can be subtracted out, the trapping stiffness for each polarization is shown in Table 5.2. The stiffness at 0° (parallel to the bundle orientation) is nearly twice that at 90° (perpendicular to the bundle orientation). The laser power in the sample only varies by a few percent under the different polarizations and the power is actually at its highest under a polarization of 90° so this result must come from the birefringence of the microtubule. If a microtubule has a higher index of refraction along one direction than another, it will feel stronger gradient forces along the higher index direction (equation (2.19)). Results here suggest that



(a)

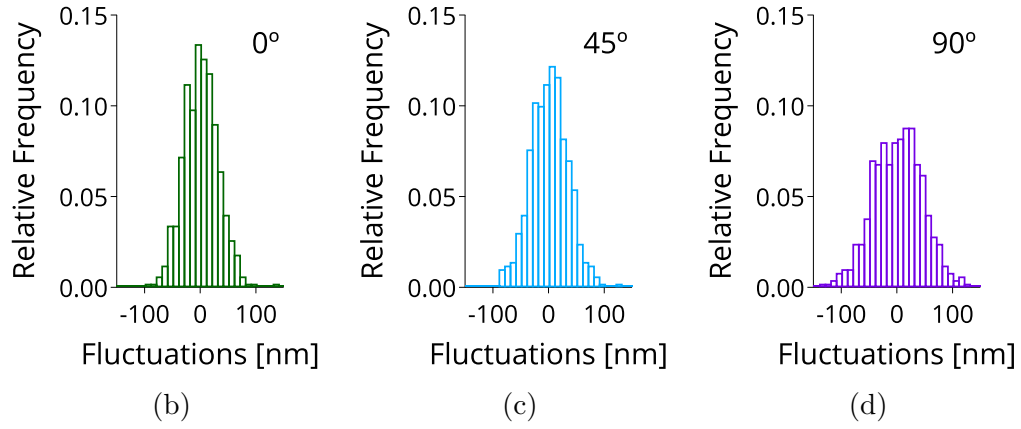


Figure 5.5: **Fluctuations of a microtubule bundle pinned on one end in a SWOT.** (a) The pinned microtubule's free end was trapped in the SWOT under polarizations of 0° (green), 45° (cyan), and 90° (purple) relative to the horizontal. Position fluctuations of the filament perpendicular to its axis are shown for polarizations of (b) 0° , (c) 45° , and (d) 90° .

the index of refraction is much higher along the filament axis than along the shorter, perpendicular direction.

Previously the birefringence of microtubules had been measured by Oldenbourg *et al.* and found to be 0.003 for a single microtubule at visible wavelengths with the higher index of refraction being parallel to the long axis

of the filament [122]. This qualitatively agrees with the findings in Table 5.2 that show stronger trapping when the laser polarization is parallel to the long axis, but it can also be examined quantitatively. Here, the birefringence is calculated by taking a ratio of two spring constants at different polarizations. Since the gradient force is proportional both to the spring constant and to the polarizability, a ratio of the spring constants is equivalent to a ratio of the polarizabilities. This can be related to the index of refraction by the Clausius-Mossotti relation for a sphere

$$\alpha = 4\pi\epsilon_m a^3 \left[\frac{(n_p/n_m)^2 - 1}{(n_p/n_m)^2 + 2} \right], \quad (5.5)$$

where α is the polarizability, ϵ_m is the permittivity of the medium, a is the particle radius, n_p is the particle's index of refraction, and n_m is the medium's index of refraction. A ratio of the spring constants can therefore be written as

$$\frac{k_1}{k_2} = \frac{\alpha_1}{\alpha_2} = \frac{((n_{p,1}/n_m)^2 - 1)((n_{p,2}/n_m)^2 + 2)}{((n_{p,1}/n_m)^2 + 2)((n_{p,2}/n_m)^2 - 1)}, \quad (5.6)$$

where k_1 is the spring constant for one polarization and k_2 is the spring constant for the other polarization, and $n_{p,1}$ and $n_{p,2}$ are the corresponding indices of refraction along each of those polarization directions. Recently, there has been some controversy concerning the index of refraction of microtubules so we will take the most recent value of 1.6 found by Krivosudský *et al.*, which is in line with theory for optical properties of proteins [130,131]. Substituting $n_{p,1} = n_{p,2} + \Delta n$ (where Δn is the birefringence) into equation (5.6) and taking

$n_{p,2} = 1.6$, a ratio of the spring constant at 0° to the spring constant at 90° leads to $\Delta n = 0.26$. This value is nearly two orders of magnitude larger than the value predicted by Oldenbourg *et al.*. Only heavy metal nanorod films like LaPO_4 and coordination polymers like $\text{Ag}\{\text{HB}(\text{C}_2\text{H}_2\text{N}_3)_3\}$ that leverage metals and aligned ring structures to achieve high polarizabilities can attain similar birefringence values ($\Delta n = 0.06 - 0.43$) [132–134]. Without such structures, it is unclear how microtubules exhibit such high birefringence.

Polarization Angle	$k_{\perp, \text{corrected}}$ [pN/ μm]
0°	2.09
45°	1.86
90°	1.11

Table 5.2: **Spring constants for a bundle of two microtubules in a standing wave optical trap.** The spring constants of a standing wave optical trap for a bundle of three microtubules are shown for different laser polarizations relative to the x-axis. All constants have been corrected for motion blur. The laser power in the sample was 50 mW

Examining this experiment more closely does not appear to provide any simple explanations for this result. Equation (5.5) is based on the polarizability for a sphere, which is clearly inappropriate for a cylindrical microtubule, but the volumetric contributions in the form of a^3 cancel out so they should not significantly affect the result. One possible origin of this larger value is that the change in polarization significantly alters the volume of the microtubule bundle that is trapped by the beam. The extension of the laser focus along the direction of the electric field means that more of the bundle is illuminated by the optical trap and therefore subject to gradient forces over a larger region

than it would be otherwise (Section 2.2.1.1). These increased forces would translate to larger spring constants that stem not from birefringence, but simply from the increased bundle volume in the trapping beam. However, the increased beam width would also decrease the strength of the gradient force at each point so it is unclear whether this can explain the observed birefringence. Finally, blocking and unblocking the trapping beam showed that the bundle was still free to fluctuate so it was not further pinned to the surface during the course of the experiment.

It is possible that microtubule birefringence may be larger at wavelengths in the near-infrared than in the optical ranges observed by Oldenbourg, but no studies were found of microtubule dispersion and birefringence in those parts of the spectrum. Changes in the refractive index of proteins have been studied and an empirical formula was put forward by Zhao *et al.* based on Perlmann and Longsworth's work [131,135]. They gave the change in index of refraction with the concentration of protein in solution $\frac{dn}{dc}$ as

$$\frac{dn}{dc} = \left(\frac{dn}{dc} \right)_{578\text{nm}} \left(0.94 + \frac{20000}{\lambda^2} \right), \quad (5.7)$$

where $\left(\frac{dn}{dc} \right)_{578\text{nm}}$ is the change in index with concentration at 578 nm and λ is the wavelength of interest in nanometers. This does suggest that the index of refraction of a microtubule solution will increase more slowly with concentration at 800 nm (the wavelength of the laser in water) than at 578 nm, however, this means that microtubules interact less strongly with near-infrared wavelengths than optical wavelengths. Furthermore, it does not give any indication

for how the birefringence of microtubules may change since that is a result of their aspect ratio [121].

5.2.3 Optical Trapping of a Single Microtubule

The strong interactions between the microtubule bundle and the SWOT laser polarization observed in the previous section were also seen in another experiment, this time with a single microtubule. Fluorescently labeled microtubules were prepared with no bundling agents and then placed inside a sample. One of the filaments was diffusing near the dichroic coverslip so it was trapped with the SWOT and, based on the brightness, was confirmed to be a single microtubule. As a result of the stiffness of the filament, it remained in a two-dimensional plane in the region near the trap, but the ends were free to fluctuate in the third dimension. By tracking the angle of the filament near the trap center where it was mostly straight, its angle relative to the horizontal axis could be observed over time as the microtubule interacted with the vertically-aligned laser polarization. Figure 5.6 shows an image of the filament along with a time trace of its angular position. Both the trap and the dichroic coverslip are visible due to the long 100 ms exposure time, but no lowpass filtering adjustments were needed because rotational diffusion is such a slow process.

Over more than ten minutes, the filament only explored a small range of orientations as it underwent rotational diffusion. If the microtubule had been free to rotate without restriction, then it should have explored a wide

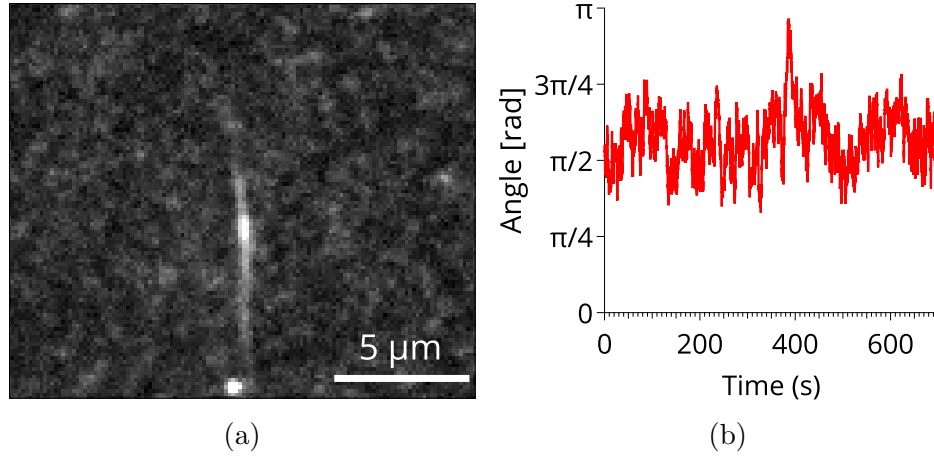


Figure 5.6: **Time trace of a single microtubule in a SWOT.** (a) A single microtubule is caught in a SWOT. The strong background comes from the dichroic coverslip at a 100 ms exposure time. (b) The microtubule diffused rotationally over time and the angle of the microtubule near the trap center is shown over time.

variety of angular positions over the course of the experiment. The apparent confinement of the microtubule suggests that there is some interaction with the polarization of the laser just as there was in the previous section. However, this interaction would be in the form of optical torques rather than gradient forces. Confinement from these torques can be quantified through a histogram of the filament's angular positions and by calculating its MSD. Figure 5.7 displays the result of these calculations. The histogram is roughly Gaussian in shape and centered at an angle slightly greater than $\pi/2$. This aligns with the laser polarization albeit at a larger angle than expected, possibly as a result of a slight misalignment of the camera relative to the trap's polarization. At short time lags, the MSD actually appears to be larger than what theory

expects for a cylinder undergoing free rotational diffusion. It is only at longer timescales after roughly 10 seconds that the filament shows confinement as the MSD plateaus. The trapped filament was only 200 nm from the surface of the sample chamber so the theory curve in Figure 5.7b uses an approximation of the drag coefficient of a cylinder rotating near a surface [52]

$$\gamma_{r,\text{surface}} = \frac{\eta\pi L^3}{4} \left(1 - \frac{9r}{16h} + \frac{r^3}{8h^3} - \frac{45r^4}{256h^4} - \frac{r^5}{16h^5} \right)^{-1}. \quad (5.8)$$

In equation (5.8), η is the dynamic viscosity, L is the length of the cylinder, r is the radius of the cylinder, and h is the height above the surface. This may underestimate the diffusion constant since the ends of the filament were fluctuating farther above the surface than the trapped region, which could have led to the initially larger MSD found in Figure 5.7b.

Based on the behavior of the bundle trapped in Section 5.2.2, it is likely that the confinement comes from the microtubule's interaction with the laser's polarization as the range of angles over which the microtubule diffuses are all roughly aligned with the polarization. Unfortunately, these measurements were taken before a half-wave plate was installed in the setup so the response of the filament to other polarizations could not be examined. It is possible that the observation time was too short for the filament to explore other orientations so the MSD only deviates from free diffusion at long timescales. Testing this without additional data requires a computational approach.

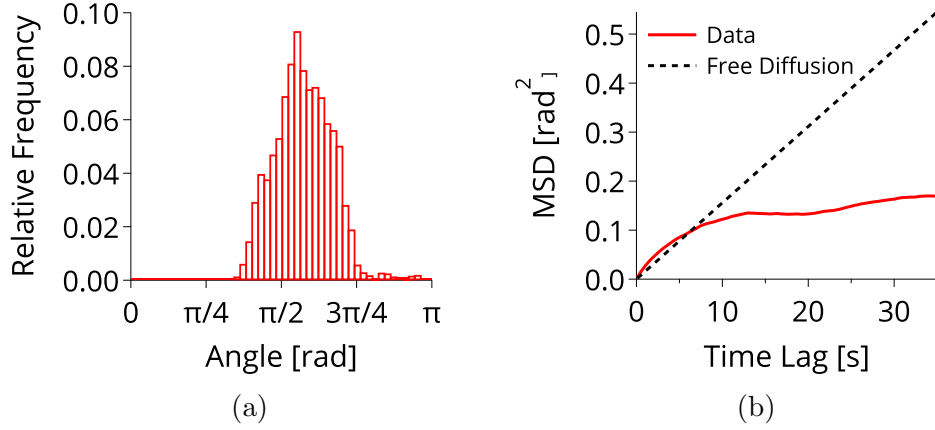


Figure 5.7: **Angular position histogram and MSD of a trapped microtubule.** (a) The histogram of a trapped microtubule’s angular position is shown. (b) The mean squared displacement (MSD) of a trapped microtubule is calculated for lag times up to 5% of the total recorded time.

5.2.4 Simulation of an Optically Trapped Microtubule

Simulations of physical systems allow hypotheses about experimental results to be explored simply without more data. While this can require simplifying assumptions, it can still provide invaluable insight into the system. In the case of the trapped microtubule in the previous section, it is unclear whether the polarization of the laser is responsible for the apparent confinement of the filament or if it is a result of the relatively short duration of the observation. Fortunately, this system can be simulated using the Langevin equation and techniques described in Section 2.5.

To start, a microtubule undergoing rotational diffusion in two dimensions is simulated in a SWOT using equation (2.48) with the birefringence chosen to match Oldenbourg’s value ($\Delta n = 0.003$). The microtubule was cho-

sen to have the same parameters as the one observed in the previous section ($n = 1.6$, $L = 9\mu\text{m}$, $r = 25\text{nm}$, and $h = 200\text{nm}$) and equation (5.8) was used to calculate the diffusion constant while the laser power was chosen to be 50 mW to match the experiment. A time step of 1 ms was selected, which is far above the inertial dissipation time and far below the position autocorrelation time. The data was then downsampled to 10 frames per second to match the experimental data. Plotting the position histogram and MSD for the simulation, the filament shows very little confinement. While there is some plateauing of the MSD at longer time lags, it is not to the same degree as seen in Figure 5.6. Given that this simulation generated the same amount of data as was collected during the experiment in the previous section, it is reasonable to see the plateau effect around the 10 second mark in Figure 5.7 as coming from confinement and not just a lack of statistics at longer time lags.

Such weak confinement in this simulation would suggest that the microtubule should not show any significant interaction with the laser polarization, but this contradicts the previously detailed observations. If, instead, the birefringence in the simulation is set to the value found from the bundled microtubules ($\Delta n = 0.26$), then much more confinement is observed. The results of this simulation show more confinement than the experimental results placing them at the other end of the spectrum from our first simulation. Figure 5.9 shows that the filament barely explores any orientations that are not aligned with the laser polarization at $\pi/2$. This can be seen as the almost immediate plateauing of the MSD far sooner than the 10 second lag that was seen in

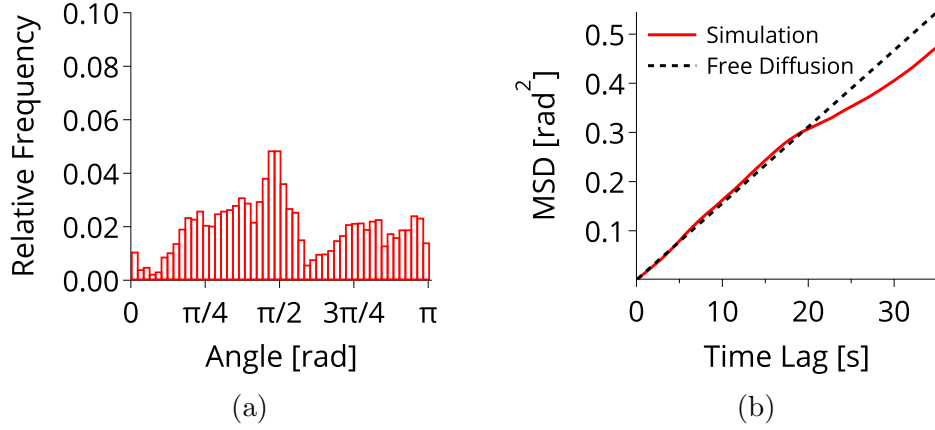


Figure 5.8: **Angular position histogram and MSD of a simulated, freely rotating microtubule.** (a) The histogram of a simulated microtubule’s angular position is shown. (b) The mean squared displacement (MSD) of the simulated microtubule is calculated for lag times up to 5% of the total simulated time. Simulation parameters are $n = 1.6$, $\Delta n = 0.003$, $L = 9\mu\text{m}$, $r = 25\text{nm}$, $h = 200\text{nm}$, $\Delta t = 1\text{ms}$, and a laser power of 50 mW.

the experiment. Large torques exerted by the trap in this simulation quickly restore the filament to a vertical orientation after almost any change in angle. Clearly, this is beyond the confinement observed in the previous section, which suggests that birefringence of the microtubule bundle in Section 5.2.2 is too large to explain the motion of the single trapped filament.

In an attempt to match the experimental results, several simulations were run for different birefringences and their histograms and MSDs were compared to those in Figure 5.6. A birefringence of $\Delta n = 0.02$ was found to reproduce the observed behavior most closely. Figure 5.10 shows the results of this simulation alongside those for the experimental data, where the simulated data has been shifted to align with the mean of the experimental

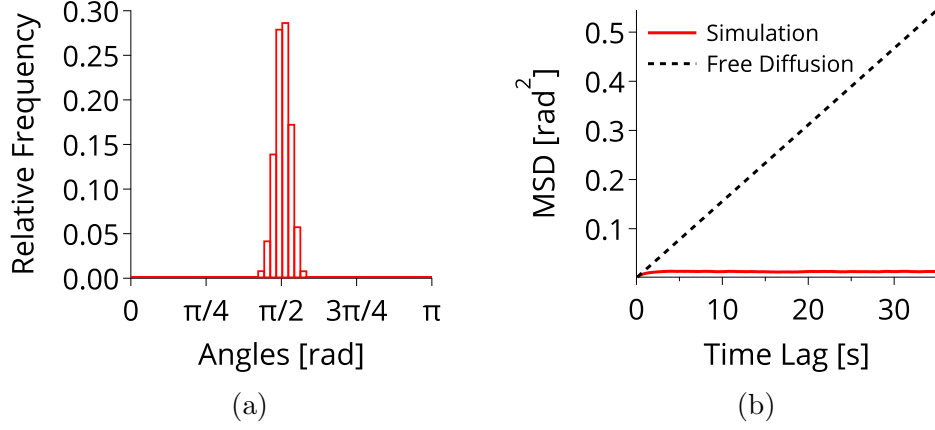


Figure 5.9: **Angular position histogram and MSD of a simulated, freely rotating microtubule with higher birefringence.** (a) The histogram of a simulated microtubule’s angular position is shown. (b) The mean squared displacement (MSD) of the simulated microtubule is calculated for lag times up to 5% of the total simulated time. Simulation parameters are $n = 1.6$, $\Delta n = 0.26$, $L = 9\mu\text{m}$, $r = 25\text{nm}$, $h = 200\text{nm}$, $\Delta t = 1\text{ms}$, and a laser power of 50 mW.

data. The ranges of the histograms appear to be quite similar although the simulated data appears to have a narrower and taller peak. Then in the MSD, the simulated and experimental data appear extremely similar with the main difference being the larger slope in the experimental data at small time lags. Despite this birefringence value simultaneously being an order of magnitude larger than Oldenbourg’s value and an order of magnitude smaller than the value found experimentally in Section 5.2.2, the simulation seems to agree well with experimental observations.

To reconcile this birefringence value with the one measured in Section 5.2.2, there should be some connection between a bundle’s geometry and its

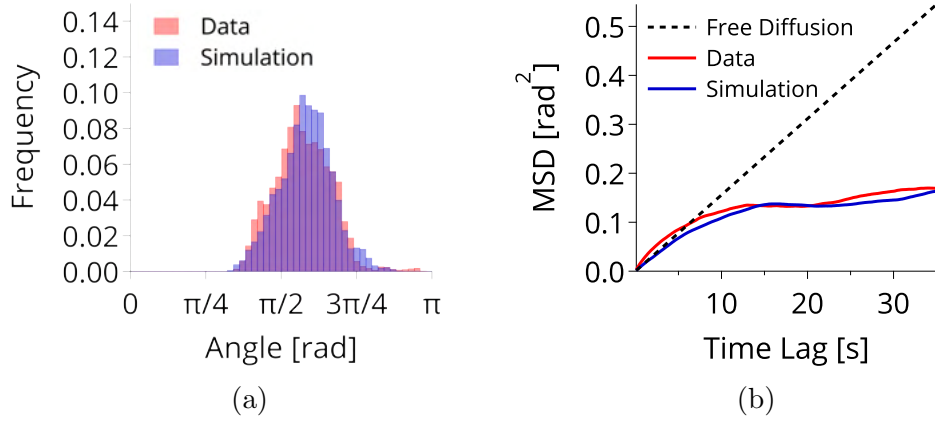


Figure 5.10: **Angular position histogram and MSD of a simulated and experimentally trapped microtubule.** (a) The histogram of a simulated microtubule’s angular position (blue) is shown alongside data from Figure 5.7 (red). (b) The mean squared displacement (MSD) of the simulated microtubule (blue) is calculated for lag times up to 5% of the total simulated time and is shown alongside data from Figure 5.7 (red). Simulation parameters are $n = 1.6$, $\Delta n = 0.02$, $L = 9\mu\text{m}$, $r = 25\text{nm}$, $h = 200\text{nm}$, $\Delta t = 1\text{ms}$, and a laser power of 50 mW.

optical response. Sato *et al.* found that individual tubulin monomers were not birefringent, but that a polymerized microtubule filament was birefringent, demonstrating that its form is the origin of its optical response [121]. The nature of the bundle arrangement could therefore have some impact on the birefringence (e.g. filaments arranged side by side may have a different birefringence than filaments stacked in a triangular configuration). If this connection to form and geometry is strong enough, then it could cause the large growth in Δn between the observed single filament and the microtubule bundle. Determining this link, however, requires further experiments on individual and bundled microtubules.

It is also important to keep in mind that these simulations made simplifications to the system that may have caused them to deviate from the actual experiment. All of the dynamics were calculated assuming that the filaments were rigid rods that did not deform whereas actual microtubules flex as they move. Energy that simply rotated the filaments in the simulations would be expended in the experiment through elastic deformations of the filament ends outside the trapping region. This rigid rod assumption also caused the simulations to ignore the out of plane flexion that was observed in the experiment. The diffusion constant in the simulation assumed that the whole filament was always near the surface while, in reality, the ends of the filament often fluctuated out of plane. These motions could have reduced the overall diffusion constant and allowed for faster rotation of the filament than these simulations would suggest. Consequently, the large birefringence for the bundle may be reasonable for single microtubules, but did not result in as much confinement in the experiment as it did in the simulation due to the larger experimental diffusion constant. Finally, the optical torque calculation used the full diameter of the microtubule as the thickness, but the filaments themselves are hollow. Thus, the proper thickness to use may be less than the diameter, which would also have led to weaker confinement.

5.3 Summary: Optical Manipulation of Single and Bundled Microtubules

Through the use of a standing wave optical trap, we demonstrated optical manipulation of bundled and single microtubules for the first time. A bundle pinned on one end provided a good model system to test the efficacy of the SWOT polarization on altering microtubule motion. In addition to exhibiting confinement at all tested polarizations, the bundle's response suggested that the birefringence of microtubules may be much higher than previously thought, at least in the near-infrared. This interaction with the polarization carried over to the first observed trapping of a single filament as it underwent rotational diffusion. Even with only a single microtubule in the trap, the laser appeared to exert large optical torques on the filament, causing it to remain in close alignment with the polarization. Brownian dynamics simulations based on birefringences from the literature and this dissertation were unable to recreate the degree of confinement observed in experiments, but they did support the finding that the birefringence in the near-infrared is larger than was found at optical wavelengths in previous studies. Ultimately, a birefringence in between the literature value and the one calculated here yielded results that agreed with experimental observations for a single filament, implying that there may be geometric factors contributing to the observed discrepancy. Future experiments could illuminate this connection further along with more detailed simulations that account for factors like microtubule flexibility.

Chapter 6

Summary and Outlook

Prior to this dissertation, little was understood about the dynamics within cross-linked microtubule networks and no studies had examined their hydrodynamic impact on the nanoscale. Rheological measurements of these networks showed rich, nonlinear behavior that could not be explained without further experimental studies on the single filament level, requiring precision that was not possible for standard microscopy techniques [31, 33]. Previous work in our group had already presented thermal noise imaging as a versatile, probe-based method that could be applied to networks of stiff biopolymer filaments like collagen as a means of mapping filament architecture [90, 91, 107, 116], making it a prime candidate for examining microtubule networks. Recently, it was extended to cross-linked networks of microtubules, showing the versatility of thermal noise imaging as a technique, and even hinting at a means of tracking filament dynamics, although this was only possible for the specific geometry of a filament confined on one end [108].

There was an even larger gap in knowledge when it came to the manipulation of individual filaments. Many studies have been devoted to examining single microtubules, showing that mechanical properties like their persistence

lengths were directly connected to their contour lengths [1, 25, 120], but little attention was paid to optical properties like their birefringence [121, 122]. While small ($\Delta n = 0.003$), this birefringence suggested that microtubules could be manipulated through optical forces, if applied in the correct way.

6.1 Summary

In this dissertation, we showed that photonic force microscopy could be leveraged to map the arrangement of filaments with unprecedented precision while simultaneously tracking the fluctuations of filaments and the hydrodynamic coupling of particles to the network with thermal noise imaging. We developed new analysis methods that allowed us to localize individual filaments with 2.5 nm precision over several hundred nanometers, a nearly two-fold improvement over previous analyses [108]. Those same methods were also used to extract microtubule positions in every part of an imaged region, even at filament intersections, which distorted thermal noise images and had defied analysis in the past. With such high fidelity imaging throughout the network, we made the first direct measurement of two cross-linked microtubules based on their small separation distance of just 1.4 nm and connected the intersection to the filament dynamics. Fluctuations of the microtubules were shown to be suppressed at filament intersections and then found to increase as they moved away from such support points, demonstrating, for the first time, the impact of the network architecture on a doubly constrained filament. These experiments open the door for force measurements within biopolymer networks that

may finally elucidate the connection between individual filament mechanics and macroscopic network behavior. We further succeeded by creating a map of the diffusivity within a network with unprecedented 10 nm precision and showed that even individual microtubules are capable of heavily influencing the diffusion of spherical particles. To our knowledge, these represent the first nanometer scale measurements of diffusivity within a cytoskeletal network, and they have important implications for intracellular transport and hydrodynamic interactions within cells.

Next, we showed that a standing wave optical trap (SWOT) could be used to manipulate bundled and single microtubules. By reflecting a trapping laser off of a dichroic coverslip, the scattering force on an object in a SWOT could be almost entirely removed while the gradient force was strongly increased [104]. Filaments could then be trapped in solution and away from surfaces for long term observation and manipulation. Characterization of the trapping force on a bundle of filaments showed unexpectedly strong interactions with the polarization of the trap, suggesting that the birefringence of microtubules may be as large as $\Delta n = 0.26$ in the near-infrared, considerably larger than the $\Delta n = 0.003$ that was found for previous measurements at optical wavelengths [122]. Additionally, the first confirmed optical trapping of a single microtubule reinforced this hypothesis as the filament remained strongly aligned with the polarization of the laser over several minutes. The confined motion of the filament could not be explained in simulations without a birefringence of $\Delta n = 0.02$, a value that was, again, much larger than the one

found in the literature, but also smaller than what was found for the bundle measurements. Geometric effects remain a likely candidate for the source of this discrepancy and will require more experiments to uncover fully. Nevertheless, these measurements show that microtubules may be freely manipulated away from surfaces using a SWOT, providing a new means of building complex arrangements of filaments. In the future, single filaments may be coerced into bundles or even into larger networks built filament-by-filament with their orientations and connections controlled by the trapping laser.

6.2 Outlook

6.2.1 Thermal Noise Imaging of Actin Networks

As previous thermal noise imaging studies of biofilament networks have progressed from stiff filaments like collagen to semi-flexible ones like microtubules, the next logical step would be to image actin networks [107, 108]. While collagen networks have been shown to be athermal, this dissertation has demonstrated that fluctuations of microtubules can provide rich information about the connectivity of biopolymer networks. A network composed of flexible actin filaments (Section 1.1) could therefore present even more dynamic system for study. Furthermore, actin filaments have been found to condense into stress fibers and other large bundles in cells so networks of such bundles could be interesting not only from a physics perspective, but also from the standpoint of biology [136]. The high concentration of actin filaments within cells also provides excellent motivation to study the diffusive properties of

spherical particles within actin networks. Since actin filaments are found so readily at the periphery of motile cells where complex signaling takes place, it is important to understand the effect that they have on diffusive motion [39,41]. Their large fluctuations could influence diffusion differently than stiffer microtubules, even to the extent of opening and closing passages for probe particles to move through the network mesh. Analyzing such complex dynamics would be extremely beneficial for theorists and biologists alike.

6.2.2 Assembly of Macromolecular Complexes

Chapter 5 showed that the standing wave optical trap is capable of both confining and manipulating large, macromolecular complexes like microtubules. This ability to restrict the motion of proteins for long-term observation makes this technique an ideal candidate for studying protein assemblies. The standing wave optical trap can be shaped into a line profile with an extended aspect ratio, which could then be used to watch the assembly of filamentous structures [137]. Such a line trap could be used to confine a small microtubule while the laser power was adjusted to modulate the concentration of tubulin in the surroundings. As the laser power was increased, it would create a deeper energy well for unpolymerized tubulin thereby increasing the concentration and encouraging the microtubule to grow in length. Similarly, the laser power could be decreased to lower the tubulin concentration and promote disassembly. In principle, this could be applied to other systems like the tobacco mosaic virus or even an artificial muscle consisting of actin bundles

and myosin motors [3].

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Vita

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