DEVELOPMENT OF A CHROMOKINESIN-MICROTUBULE SYSTEM FOR USE IN OPTICAL TWEEZER-BASED PROCESSIVITY ASSAYS

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Abstract

Optical tweezer-based experiments on molecular motors and trackways allow the kinetics of the interactions to be measured. This information helps us to understand the motors’ motion, which could be a first step in developing nanomachines. We set out to study the interactions between the commercially available molecular motor, chromokinesin, and its associated trackway, the microtubule, through bead assays using an Optical Tweezer system. We developed an optimal protocol and concentration for microtubule reconstitution and coating of the viewing chamber surface. We subsequently developed a protocol for coating polystyrene beads with chromokinesin and for introducing the beads into a chamber with sedimented microtubules (necessary for a bead assay). A study of the alignment of the optical tweezer system was conducted in an effort to develop the ability to move the trap in the z-direction, which is crucial for these experiments and has not been achieved hitherto with our trapping system. We suggest a course of action in order to continue the study of chromokinesin-microtubule interactions using the optical tweezer system.
1 Introduction

1.0 Importance of Molecular Motors

The cell is the building block of all life. Molecular motors, or motor proteins, are a group of large molecules that perform a variety of functions inside cells, including movement of objects and transport of cargo from one place to another inside the cell. Myosin motors are involved in muscle contractions, kinesin motors play specific roles in cell division, among other things, and dynein motors are significant in the movement of vesicles (Alberts et al., 2002). Due to their importance in the cell and biological functions, molecular motors have been widely studied and analyzed. Through these studies, the interactions between the motors and the trackways along which they move are now better understood, along with the biophysical aspects of how the motors work. This insight into the mechanical nature of molecular motors holds promise for applications in nanotechnology, where nanomachines might be made to operate using similar principles if those principles could be understood.

Motors have been studied both in vivo and in vitro. In vivo refers to studies conducted within living cells, while in vitro refers to the experimentation being conducted in a simulated environment (literally, “in glass”) (Howard, 2001). The majority of studies concerning molecular motors have been conducted in vitro, since in vitro experiments yield results comparable to those conducted in vivo, and because it is easier to study the motors in a more controlled environment (Howard, 2001). There are two types of in vitro motility assays: the gliding assay and the bead assay. In the gliding assay, the motors are fixed to the bottom surface of a viewing chamber and
the trackways diffuse down to the motors, attach, and “glide” along the surface. By contrast, in the bead assay, the trackways are fixed to the base of the chamber and beads coated with motors diffuse down to the trackways, attach, and move along the trackway.

We wished to bring such a system for studying molecular motors to Ohio University for further research on specific motors and to develop a simplified system for use in graduate and undergraduate laboratory courses. In order to do this, we had to develop protocols and optimize our systems for doing bead assays. In the Introduction, the biology and physics of molecular motor systems and the methods that have been used to study them will be laid out. Since trackways are the foundation upon which the molecular motors move, we will first provide a description of the trackways before discussing molecular motors in more detail below.

1.1 The Cytoskeleton and Motor Trackways

The cytoskeleton is a system of protein filaments, responsible for maintaining cellular shape and structure, and playing important roles in cellular division and motion, and intracellular transport (Alberts et al., 2002). The three major types of protein filaments that compose the cytoskeleton are actin filaments, intermediate filaments and microtubules (Alberts et al., 2002). Kinesin and dynein motors associate with microtubules, while myosin motors associate with actin filaments (Alberts et al., 2002). Actin filaments are two-stranded helical polymers, made up of the protein actin (Alberts et al., 2002). They have a diameter of 5-9 nm. Intermediate filaments are fibers that resemble ropes, and are made up of intermediate filament proteins (Alberts
et al., 2002). They have a diameter of ~10 nm. Microtubules are hollow cylinders, made up of the protein tubulin (Alberts et al., 2002). They have an outer diameter of 25 nm. Figure 1 shows the structures of the cytoskeleton’s protein filaments and their respective subunits and scales.

![Figure 1. Structures of the three protein filaments that make up the cytoskeleton: actin filament, microtubule, and intermediate filament. The subunit of each protein filament is also shown](image)

The subunits that make up actin filaments and microtubules can add or dissociate from either end of the microtubule and actin filament, meaning that these structures can grow or shrink dynamically (Alberts et al., 2002). Microtubules and actin filaments have an inherent polarity, due to the arrangement of their subunits. This polarity means that polymerization will occur faster at one end than the other (Howard, 2001). The fast-growing end is referred to as the “plus” end and the slow growing end is the “minus” end. It is more likely that they add at the plus end of the trackway (Alberts et al., 2002). The positive and negative ends of the actin filament and microtubule are labeled in Figure 1.

### 1.1 Microtubules

The microtubule is a filamentous structure consisting of the protein subunit, tubulin (Howard, 2001). Tubulin is a heterodimer, formed from an α-tubulin and a β-
tubulin tightly bound by noncovalent bonding (See Figure 1) (Alberts et al., 2002). The microtubule is composed of 13 parallel protofilaments, made up of tubulin (Alberts et al., 2002). The tubulin is arranged in the protofilaments so that the α-tubulin and β-tubulin alternate (Alberts et al., 2002).

The tubulin subunits are all arranged in the same direction in the protofilaments, resulting in the microtubule being polar (Alberts et al., 2002). As seen in Figure 1 above, the plus end of the microtubule corresponds to the end where the β-tubulins are exposed and the minus end corresponds to the end where the α-tubulins are exposed (Alberts et al., 2002). Microtubules are very rigid and structurally straight, due to the tight packing of the tubulin subunits (Howard, 2001). The physical characteristics of the trackways will be discussed in more detail in the following section.

1.2 Physical Characterization of Trackways

Young’s modulus, $E$, is a measure of the stiffness of a material. If a force is applied to a solid of uniform cross-sectional area, the strain will be proportional to the force per area. This is represented formally by the equation:

$$\frac{F}{A} = E\frac{\Delta L}{L}$$  \hspace{1cm} (1)

where $F$ is the force applied, $A$ is the cross-sectional area, $\Delta L$ is the change in length, and $L$ is the original length of the material (Howard, 2001). The persistence length, $L_p$, is another useful way to quantify the stiffness of the protein filaments. It describes how well a filament resists thermal forces, by measuring the length of filament to the point where thermal bending becomes significant enough to notice (Howard, 2001).
The persistence length is related to Young’s modulus by

$$L_p = \frac{EI}{kT}$$

(2)

where $I$ is the second moment of inertia, $k$ is Boltzmann’s constant, and $T$ is the temperature (Howard, 2001). Table 1 contains the persistence lengths and Young’s moduli of the cytoskeleton’s protein filaments.

<table>
<thead>
<tr>
<th>Protein Filament</th>
<th>Persistence Length</th>
<th>Young's Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin Filament</td>
<td>15 µm</td>
<td>2</td>
</tr>
<tr>
<td>Intermediate Filament</td>
<td>~1 µm</td>
<td>2</td>
</tr>
<tr>
<td>Microtubule</td>
<td>6 mm</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. Values of persistence length and Young’s modulus, for the protein filaments associated with the cytoskeleton, according to Howard (2001)

From Table 1, it is apparent that all three types of protein filaments have Young’s moduli of 2 GPa, which is comparable to those of silk, collagen, and hard plastics (Howard, 2001). Howard (2001) asserts that their high level of rigidity indicates that these proteins have indeed evolved to play structural roles in cells, as hypothesized. Table 1 also shows that microtubules have the largest persistence length at 6 mm, followed by actin filaments at 15 µm and intermediate filaments at ~1 µm. Thus the three types of protein filaments are rather resistant to thermal forces, with microtubules being the most resistant. All three of the structures do not necessarily achieve lengths that are equal to their persistence lengths. They are usually a few micrometers long (about a cell’s size). The fact that their persistence lengths are greater than their actual lengths indicates that they are pretty straight. They form a mesh of straight, relatively rigid fibers within the cell.
1.3 The Molecular Motors

There are three molecular motor superfamilies that move on elements of the cytoskeleton: dynein, myosin, and kinesin (Alberts et al., 2002).

Dyneins are a family of motors that process to the minus-end of the microtubule (Alberts et al., 2002). They are made up of two or three heavy-chains, the motor domain (2-3 heads depending on the type of dynein), and a large, variable number of light-chains (Alberts et al. 2002). One major branch of dyneins, the cytoplasmic dyneins, is responsible for vesicle trafficking and localizing the Golgi apparatus of the cell (Alberts et al., 2002). The other major branch, the axonemal dyneins, is responsible for the mechanism that causes the beating of cilia (lungs) and flagella (sperm) (Alberts et al., 2002). Dyneins are the largest and fastest of molecular motors. Axonemal dynein can move microtubules at a speed of 14 µm/sec, while kinesin’s fastest speed is a mere 2-3 µm/sec (Alberts et al., 2002).

Myosin was the first molecular motor identified (Alberts et al., 2002). It is responsible for generating the force needed to contract muscle. Initially, researchers thought myosin to only be present in muscle, however, in the 1970’s, different types of myosin were discovered in non-muscle cells, including protozoan cells and freshwater amoeba (Alberts et al., 2002). Myosins are found in nearly all eukaryotes, implying that myosins showed up in the early stages of the evolution of eukaryotes (Alberts et al., 2002). Myosin generally consists of two-heavy chains, the motor domain, and four-light chains (Alberts et al., 2002). The vast majority of myosins move toward the plus-end of the actin filament (Albert et al., 2002).
Kinesin is most structurally similar to myosin, consisting of two heavy-chains, a motor domain, and two light-chains (Howard, 1996). Kinesins generally process to the plus-end of the microtubule (Howard, 2001). They play specific roles in chromosome separation and spindle formation during cell division (Alberts et al., 2002). In addition to the three motor superfamilies, there are other biological proteins that function as molecular motors, such as DNA polymerase and RNA polymerase when they replicate DNA or produce RNA from DNA (Bustamante et al., 2003).

All motors have a motor domain, typically composed of two heads, that binds and moves the motor along the trackway (Howard, 1996). At least one head must be bound to the trackway in order for the motor to stay attached. To move processively along the trackway, alternately each head must detach from the trackway, advance and then reattach itself. Thus the speed with which the motor moves, is determined by the binding and unbinding rates of the motor. The binding and unbinding rates will also affect the motor’s ability to sustain motion without getting stuck or completely dissociating itself from the trackway (Alberts et al., 2002).

Kinesin and dynein are processive motors, which means that they will undergo multiple ATP cycles while attached to a microtubule. (ATP hydrolysis is the source of energy for the molecular motors, and will be described in more detail below.) Myosin, on the other hand, is not a processive motor. It will undergo one ATP cycle while attached to an actin filament and will then detach from the filament (Howard, 2001). In order to get processive motion with myosin, there must be a lot of myosin motors
involved, so that at least one myosin motor is attached at all times. This is what happens in muscle.

The study of the motor, kinesin offers several advantages over the study of dynein or myosin. Kinesin’s processive behavior is convenient, since it only requires one motor for continuous movement, while myosin (as explained below) would require many. Kinesin has the smallest motor domain of the three, and paired with the ability to study single kinesin motors, this allows for functional and structural analysis of the motor.

1.6 General Motor Structure of Kinesin

Kinesin comes from the Greek “kinein,” which means to move (Lawrence et al., 2004). As mentioned above, kinesin consists of four chains, two heavy and two light. The light-chains do not affect the speed of kinesin’s motion, and are not essential for motility (Howard, 1996). The heavy-chains contain seven domains, responsible for performing various functions. The knowledge of these domains is based on evidence from protein chemistry, sequence analysis, and electron microscopy (Howard, 1996). Figure 2 contains a diagram of kinesin’s structure. While Figure 2 is specific to kinesin, the other motors, in particular myosin, have a lot of structural similarities to kinesin. Kinesin’s seven domains are described below:

I. The motor/head domain: is responsible for kinesin’s movement. It is globular in shape, and measures to be ~ 9nm by ~3 x 3 nm
II. The dimerization domain: forms a predicted parallel coiled-coil dimer
III. The link domain: contains a break in the predicted coiled-coil
IV. The coil 1 domain: another predicted coiled-coil
V. The kink domain: separates coil 1 and coil 2 domain
VI. The coil 2 domain: predicted coiled-coil that probably binds to the light chains

VII. The tail domain: binds to organelles, and to glass/plastic surfaces of chambers used in experiments studying kinesin. It is positively charged

![Diagram of kinesin's heavy chain domains](image)

**Figure 2. Diagram of kinesin’s heavy chain domains**

Kinesin does not have bilateral symmetry, it has approximate two-fold rotational symmetry (Howard, 1996). There are many different types of kinesin, responsible for performing a host of functions within the cytoskeleton. Kinesin has 14 recognized sub-families (Lawrence et al., 2004). These families differ in function, structure, and evolution of the motors. Most kinesin motor domains are located at the N-terminus of the motor (proteins are linear chains of amino acids; the N-terminus refers to the end that has an amino (NH$_2$) group, as opposed to the C-terminus, which ends with a carboxyl (COOH) group) (Vale & Fletterick, 1997).

Most types of kinesin process to the plus end of the microtubule (Alberts et al., 2002). However, the subfamily, Kinesin-14 has the motor domain at the C-terminal and walks towards the minus-end of the microtubule (Alberts et al., 2002). The Kinesin-5 subfamily has motor members that self-associate through the tail domain, sliding microtubules of opposite orientation past each other, similar to a myosin motor
This thesis focuses on the kinesin family member, chromokinesin, and will be discussed in more detail in the following section.

1.5 Chromokinesin

Chromokinesin is a member of the Kinesin-4 subfamily (Lawrence et al., 2004). It is responsible for organelle and chromosome movement (Vale & Fletterick, 1997). According to Vale and Fletterick (1997), chromokinesin moves at 0.2 \( \mu \text{m/second} \) towards the plus end of the microtubule. Chromokinesin has a structure very similar to the general motor structure described in the next section. It is commercially available, which is useful for developing a general lab experiment.

1.7 Energy Source for Motor-Trackway Interactions: ATP Hydrolysis

Adenosine Triphosphate, or ATP, hydrolysis provides the energy needed for the motors to move along the trackways. The hydrolysis of ATP is represented by the reaction

\[
\text{ATP} \rightleftharpoons \text{ADP} + \text{P}_i
\]  

where ADP is adenosine diphosphate, and \( \text{P}_i \) is the phosphate ion (Howard, 2001). In the case of kinesin, the chemical and mechanical cycles are coupled. The binding of ATP to head 1 (chemical step) causes the attachment of head 2 to the microtubule (mechanical step), which in turn causes the release of ADP from head 2 (chemical step) and causes the detachment of head 1 from the microtubule (mechanical step) (Howard, 2001). The cycle is completed when head 1 releases phosphate (chemical step) (Howard, 2001). For ATP hydrolysis, the change in Gibbs’ free energy, \( \Delta G \), is -7.3
kcal/mole or -30.5 kJ/mole (Nelson, 2004). For each molecule of ATP hydrolyzed, Gibbs’ free energy change is -0.32 eV. Gibbs’ free energy is the energy associated with a reaction that can be used to do work (Nelson, 2004). Next will be discussed the work that the energy of ATP hydrolysis is used for: the motion of the motors.

1.8 Physical Analysis of Motor Speed

In the study of the kinetics of motors Howard (2001) defines the following useful parameters:

I. The working distance, $\delta$, is the distance that the load-bearing region moves along the filament’s axis, while the head is attached.

II. The path distance, $d$, is the distance between two subsequent binding sites. Step size means path distance when referring to kinesin.

III. The distance per ATP, $\Delta$, is the distance the motor domain moves throughout the course of one ATP cycle.

From careful work using optical tweezers and particle tracking under the microscope, values for these parameters have been measured for a few selected motor proteins. Table 2 contains the working distance, path distance, and distance per ATP values for skeletal muscle myosin and conventional kinesin.

<table>
<thead>
<tr>
<th>Characteristic Distance</th>
<th>Skeletal muscle myosin</th>
<th>Conventional kinesin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working distance ($\delta$)</td>
<td>5 nm (Small load)</td>
<td>$\geq$8 nm</td>
</tr>
<tr>
<td>Path distance (d)</td>
<td>36 nm</td>
<td>8 nm</td>
</tr>
<tr>
<td>Distance per ATP ($\Delta$)</td>
<td>200-400 nm</td>
<td>16 nm</td>
</tr>
</tbody>
</table>

Table 2. Values for the characteristic distances of skeletal muscle myosin and conventional kinesin (Howard, 2001)
Molecular motors generate the force needed to move unidirectionally along the filaments, by using the energy from ATP binding and hydrolysis (Alberts et al., 2002). The speed with which the motors move varies greatly between the types of motors, and also within each motor family. Myosin ranges in speed from 0.2 – 60 µm/sec, while kinesin motors travel between speeds of 0.02 - 2 µm/sec (Alberts et al., 2002). The speed of a motor, $v$, is given by

$$v = k_{\text{ATPase}}\Delta$$

(4)

where $k_{\text{ATPase}}$ is rate at which ATP is hydrolyzed by each head and $\Delta$ is the distance traveled by the motor in one ATP hydrolysis cycle. From equation 4, it is evident that the speed of the motor will increase as the ATPase rate increases, or as the distance traveled in a hydrolysis cycle increases. These factors vary greatly within each motor protein family and thus explain the large range in motor speeds described above.

The duty ratio is the fraction of time that the motor is attached to the filament. The duty ratio, $r$, is given by

$$r = \frac{\tau_{\text{on}}}{\tau_{\text{on}} + \tau_{\text{off}}} = \frac{\tau_{\text{on}}}{\tau_{\text{total}}}$$

(5)

where $\tau_{\text{on}}$ is the average time the motor is attached to the filament, $\tau_{\text{off}}$ is the average time the motor is detached from the filament, and $\tau_{\text{total}}$ is the sum of $\tau_{\text{on}}$ and $\tau_{\text{off}}$, or the average time per hydrolysis cycle (Howard, 2001). The speed of the motor increases as the duty ratio decreases (Alberts et al., 2002). The duty ratio of kinesin is 0.5 per head, for a combined duty ratio of 1 (2 heads x 0.5 each = 1) (Howard, 2001). This is consistent with the fact that kinesin is a processive motor and is attached to the filament for the entire ATP hydrolysis cycle. The duty ratio of the non-processive
motor, myosin is 0.02 per head, for a combined duty ratio of 0.04 (2 heads x 0.02 each = 0.04) (Howard, 2001). Since each myosin head spends so little time attached to the filament during an ATP hydrolysis cycle, there must be many heads acting together in bundles to achieve processivity (as in muscle).

Next, the effect of the cargo load of the motor proteins on the motor speed will be considered. Low loads do not affect the stepping rates of the motors, and thus the motors are tightly coupled for low loads, which means that there is exactly one step for each ATP molecule that is hydrolyzed, and the speed is not affected (Howard, 2001). However, there is evidence that higher loads affect the coupling of the motors, and thus decrease the speed of the motors (Howard, 2001). High loads decrease kinesin’s stepping rate and thus decrease kinesin’s speed (Howard, 2001).

Svoboda and Block (1994b) measured the force-velocity curves of beads coated with conventional kinesin. Conventional kinesin is a member of the Kinesin-1 subfamily (Lawrence at al. 2004). They used optical trapping interferometry to apply forces on the pN scale to the beads and to track the motion of the bead. The kinesin is allowed to walk away from the trap’s center. As the kinesin moves further away, the load/force increases, thus decreasing the speed until eventually the motor “stalls.” The stall force refers to the maximum force the motor can withstand before all motion ceases. Svoboda and Block found the stall force of conventional kinesin to be 5-6 pN. Svoboda and Block were able to conclude that the decrease in kinesin’s velocity is possibly due to a decrease in the displacement for every hydrolyzed ATP molecule, indicating that kinesin is a loosely coupled motor under high loads.
In two separate experiments, conducted by Kuo and Sheetz (1993) and Kojima et al. (1997), it was found that conventional kinesin had stall forces of 1.9±0.4 pN and 8-9 pN, respectively (Greulich, 1999). These results, paired with Svoboda and Block’s result of 5-6 pN, show a relatively large discrepancy. This disagreement could be caused by several factors. A possible explanation is one group used an energy source other than ATP, such as GTP (Guanosine Triphosphate) (Greulich, 1999). It could be due to different calibration methods or different methods of preparing the kinesin (Greulich, 1999). Each of these factors is extremely important and greatly affects the results of the experiment. The results above were obtained through the use of optical tweezers. Optical tweezers have an invaluable role in the study of molecular motors and trackways.

1.9 Optical Tweezers

Arthur Ashkin (1997) developed optical tweezers at Bell Labs in the 1970’s and 80’s. He first became interested in the application in 1969, by performing a calculation regarding the radiation pressure of laser light on small particles, and finding that the particle acceleration due to the radiation is non-trivial and should exhibit significant dynamical effects (Ashkin, 1997). In 1986, optical cooling and trapping of atoms was observed for the first time, and in 1989, biological applications using the optical tweezers yielded the new field’s foundational results (Ashkin, 1997). Ashkin’s colleague, Steven Chu, along with Claude Cohen-Tannoudji and William D. Phillips won the Nobel Prize in Physics in 1997 “for development of methods to cool and trap atoms using laser light” (Nobel Prize, 2010).
Optical tweezers is the name given to an experimental setup that can manipulate small particles using optical traps. The traps are created by radiation pressure, which is pressure exerted on a surface due to exposure to light, and by momentum transfer from redirection of light that scatters from the particles (Svoboda et al., 1994a). In this application, the light is from a laser, and therefore optical tweezers is sometimes called “Laser Tweezers”.

Optical tweezers have been extremely useful in the study of biological systems. They have been used to create interactions between killer T-cells and target cells to study the immune response, to manipulate chromosomes as a means to study cell division, stretch plant membranes, measure the swimming force of sperm, and perform different types of microsurgery, among many, many applications (Svoboda et al. 1994a).

1.10 Optical Trapping Force

Optical forces are characterized by the equation

\[ F = \frac{Q n_m P}{c} \]  

where \( Q \) is efficiency (dimensionless), \( n_m \) is the suspending medium’s index of refraction, \( P \) is the laser power incident on the sample, and \( c \) is the speed of light (Svoboda & Block, 1994a). \( Q \) is the fraction of power that is used to exert force and is dependent on the numerical aperture (NA) of the microscope objective, laser wavelength, laser mode structure, light polarization state, relative index of refraction, and the geometry of the particle (Svoboda & Block, 1994a). \( Q \) is equal to unity for the
case of plane waves incident on a perfectly absorbent particle (Svoboda & Block, 1994a).

In order to trap stably, the radiation pressure has to establish a constant, three-dimensional equilibrium (Svoboda & Block, 1994a). The index of refraction of the suspending medium \((n_m)\) is usually that of an aqueous medium, and thus is not a parameter that is easily varied in achieving greater trapping force (Svoboda & Block, 1994a). The speed of light \((c)\) is a constant, and the incident laser power \((P)\) can only be increased so much before optical damage occurs. Thus, the determining factor for the trapping force is the efficiency \((Q)\).

The Rayleigh regime refers to when an object’s dimensions are significantly smaller than the wavelength of light \((d \ll \lambda)\) (Svoboda & Block, 1994a). Particles can be approximated by point dipoles, since the electromagnetic field is constant across the dielectric in this regime (Svoboda & Block, 1994a). The first component of the trapping force, the scattering force, \(F_{\text{scattering}}\), is given by

\[
F_{\text{scattering}} = n_m \frac{\langle S \rangle \sigma}{c}
\]

where

\[
\sigma = \frac{8}{3} \pi (kr)^4 r^2 \left( \frac{m^2 - 1}{m^2 + 2} \right)^2
\]

is the Rayleigh sphere’s scattering cross-section of radius, \(r\), \(\langle S \rangle\) is the time-averaged Poynting vector, \(m\) is the relative index and is given by \(n/n_m\), where \(n\) is the particle’s index of refraction, and \(k\) is the light’s wave number and is given by \(2\pi n_m/\lambda\) (Svoboda & Block, 1994a).
The second component of the trapping force, the gradient force, $F_{\text{gradient}}$, is given by

$$F_{\text{gradient}} = \frac{\alpha}{2} \nabla \langle E^2 \rangle$$  \hspace{1cm} (9)$$

where

$$\alpha = n_m^2 r^3 \left( \frac{m^2 - 1}{m^2 + 2} \right)$$  \hspace{1cm} (10)$$
is the particle’s polarizability and $\nabla \langle E^2 \rangle$ is the gradient of the energy density (Svoboda & Block, 1994a). In order to create a stable trap, the gradient force in the $-\hat{z}$ direction must be greater than the scattering force (Svoboda & Block, 1994a). An increase in the numerical aperture decreases the focal spot size and increases the gradient force (Svoboda & Block, 1994a).

In the Mie regime, the particle is much larger than the wavelength of light ($d \gg \lambda$) and a ray optics argument applies. Consider two rays, $a$ and $b$, of the focused laser beam seen in Figure 3. The ray’s momentum will change when the incident rays refract at the top surface of the particle. This change in momentum results in an optical pressure force, $F_{\text{at}}$, exerted perpendicular to the sphere surface in an upward direction. When ray $a$ is emitted from the bottom of the sphere, optical pressure force, $F_{\text{avo}}$, is also exerted. The sum of these two pressure forces results in force, $F_a$, due to ray $a$. By the same processes, ray $b$ contributes force, $F_b$. In Figure 3, the sum of the $F_a$ and $F_b$ forces, $F$, is shown pointing in the direction of the laser focus, $f$, from the center of the sphere. The sphere will be trapped stably at the point where the resultant optical pressure force, $F$, balances the difference between the gravity force and the buoyant force (Ukita, 2006).
As laser light, which has momentum, enters an object and is refracted, it will exert forces on the object. The centers of spherical objects, such as beads, are drawn into the focus of the laser beam. The force by which the bead is pulled to the focus can be approximated as a linear spring (Spudich et al. 2008). The trap stiffness is determined by the strength of the spring constant. The larger the displacement is from the focus of the beam, the larger the force on the particle, and thus the greater the trap stiffness.

To summarize, when light is incident on a bead with a higher index of refraction, the light bends toward the optical axis. The result is a change in the light’s momentum and by Newton’s third law of motion, the bead compensates for this change and thus a force is exerted upon the bead in the direction of the beam’s center, resulting in a “trapped” particle (Svoboda & Block, 1994a). The optical tweezers are
used in bead assays to trap a motor-coated bead and move it to a trackway on the surface. Once the motor interacts with the trackway, the optical tweezers are used to trap the bead and stall out the motion. In order to develop a bead assay, protocols for coating beads with motors and the bottom surface of the viewing chamber with microtubules must be developed. The coating of the beads and the glass used here occurs by physical adsorption, not covalent coupling. Development of a bead assay will be discussed in the following chapter.
2 Materials and Methods

2.0 Reconstitution of Microtubules

Lyophilized, or freeze-dried, microtubules (4x500μg; MT001; Cytoskeleton; Denver, CO) were reconstituted to a concentration of 1 mg/mL in a pH 7.00 buffer consisting of 15mM HEPES (H-7523; Sigma-Aldrich; St. Louis, MO), 2 mM Taxol (TXD01; Cytoskeleton; Denver, CO) and 1 mM MgCl$_2$ (Magnesium Chloride) (M-2670; Sigma-Aldrich; St. Louis, MO). See Appendix for the detailed instructions for preparation of the microtubule HEPES resuspension buffer. The reconstitution instructions from the supplier, suggested the use of PIPES (1, 4-Piperazine-diethanesulfonic acid), a buffer used in biological applications. However, HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) was readily available and is a commonly used buffer in biological applications, so it was decided to substitute PIPES with HEPES (Good & Izawa, 1972). Taxol, or paclitaxel, from the Pacific yew tree is commonly used in chemotherapy treatment to fight cancer (Foss et al. 1998). For our purposes, the taxol serves to stabilize the microtubules, by binding to them and interrupting the cell cycle progression, thus preventing further growth or decay (Foss et al. 1998).

After reconstitution the microtubules were gently mixed on a rotator for about fifteen minutes in order to allow microtubule formation. According to supplier specifications, the mean length of the microtubules is ~2 μm following reconstitution and they are stable for 2-3 days at room temperature. For long term usage, aliquots were snap frozen by immersion in liquid Nitrogen, each containing 20 μL of
reconstituted microtubules. The aliquots were then stored in a -20°C freezer, ensuring the stability of the microtubules for 3 months.

The first goal related to the microtubules was to be able to identify and observe them. In order to work with the microtubules, a frozen aliquot was rapidly thawed by incubation in a water bath at 25°C (Carter & Cross, 2001). The microtubules were then diluted by adding 180 µL of the microtubule resuspension buffer to the 20 µL of microtubule solution already in the aliquot. The concentration of microtubules decreased 10x, from 1 mg/mL to 100 µg/mL. In the Results and Discussion section, the process by which the best concentration and method of reconstitution (for optimal viewing and sedimentation of microtubules) were determined will be discussed.

In order to create a bead assay, microtubules must attach to the bottom surface of the viewing chamber. In order to accomplish this, we incubated the desired concentration of microtubules with 2.5 mg/mL casein (37532; Pierce; Rockford, IL), which works as a binding agent. We then filled the viewing chamber with the casein-incubated microtubules without sealing the ends and left it overnight, so the buffer would evaporate, leaving behind microtubules adhered to the bottom surface. This method was adapted to fit our needs from protocols in the literature (Ray et al., 1993; Yokokawa et al., 2004).

2.1 The Viewing Chamber

To observe the microtubule solution, it was necessary to prepare a viewing chamber. The chamber consists of a square glass cover slide (22 x 22 mm; 01023-AB; SPI Supplies; West Chester, PA), double-sided tape, and a round coverslip (18mm;
01025-AB; SPI Supplies; West Chester, PA). In order to build the chamber the square slide was placed on a rectangular microscope slide (3” x 1” x 1 mm; VWR Scientific; West Chester, PA) for convenience and stability, since the cover slides are very fragile. A piece of double-sided tape was cut down the middle length-wise and applied to the surface of the square slide, with about one millimeter separating the two pieces. The round coverslip was laid on top of the tape and blunt-ended tweezers were then used to press firmly on the round coverslip to ensure that the seal between the tape and the glass was tight. See Figure 4 for a schematic diagram of the chamber.

![Diagram of the chamber](image)

**Figure 4.** a) The layers used in the construction of the chamber; beginning with the rectangular slide, followed by the square cover slide, double-sided tape, and finally the round cover slip. b) top view of the chamber

Once the chamber was created, 3 µL of the microtubule solution was micropipetted and a drop was placed at the edge of the round cover slip between the two pieces of tape. Capillary action causes the bead suspension to move into the chamber. Clear nail polish was used to seal the two ends of the chamber. The average volume of the chamber was measured to be 0.806 mm$^2$ ± 0.051 mm$^2$ or 0.806 µL ± 0.051µL.
After preparing a chamber with microtubule solution, the rectangular slide with the chamber was placed on the microscope’s stage (ECLIPSE TE2000; Nikon; Melville, NY). The particular microscope used has DIC imaging capabilities. DIC stands for Differential Interference Contrast, and is useful in the study of transparent living cells and isolated organelles (Shribak et al. 2007). DIC works by separating rays of polarized light before they come into contact with the sample plane (Preza et al. 1996). The separated light then interacts with the sample, which has areas of different thickness and/or refractive index (dependent on the material studied), thus causing a phase change in the light rays relative to one another (Preza et al. 1996). The light is recombined for observation, and depending on their interference, or the difference in their optical paths, the image will be lighter or darker in areas, resulting in a monochromatic, shadowcast image of the sample (Preza et al. 1996). With an outer diameter of 25 nm, microtubules are too small to be resolved using a microscope, however due to the way the light interacts with the structures, DIC imaging allows the microtubules to be visible. The Results and Discussion section contains DIC images of the microtubules.

2.2 Reconstitution of Chromokinesin and Bead-Coating

To begin working with the Chromokinesin (2x25µg; CR01; Cytoskeleton; Denver, CO), one tube from the supplier was reconstituted to a concentration of 50 µg/mL in a pH 7.00 buffer consisting of 100 mM PIPES, 200 mM KCl (Potassium Chloride) (P-9333; Sigma-Aldrich; St. Louis, MO), 2 mM MgCl₂, 1 mM DTT (Dithiothreitol) (43817-1G; Sigma-Aldrich; St. Louis, MO), and 20 µM ATP (A3377-
500MG; Sigma-Aldrich; St. Louis, MO). See Appendix for the detailed instructions for preparation of the chromokinesin PIPES reconstitution buffer. Polystyrene beads (1.0% w/v, 1.87 µm; SVP-15-5; Spherotech; Libertyville, IL) were coated with casein and then incubated with reconstituted chromokinesin. Instructions for coating beads with chromokinesin can also be found in the Appendix. Protocols for coating beads exist in the literature (Spudich et al., 2008; Uemura et al., 2002; Kojima et al., 1997) and we adapted and optimized these for our own use. In the Results and Discussion section, the introduction of the chromokinesin-coated beads into the chambers and efforts to facilitate chromokinesin-microtubule interactions will be discussed.

2.3 Optical Trapping System at Ohio University

The optical tweezer system at Ohio University was designed and developed by Physics and Astronomy Honors undergraduate, Paul Ingram, and Dr. Ido Braslavsky and Dr. David Tees, of the Physics and Astronomy Department. The current optical tweezer set-up can be seen in Figure 5.

![Figure 5](image_url)

*Figure 5. Image (a) shows Ohio University’s optical tweezer system. Image (b) is an aerial view of the system*

The infrared diode laser (DP-1064-800; Lasermate Group, Inc.; Pomona, CA) used in the optical trapping system has a wavelength of 1064 nm and outputs power at
800 mW. While operating the optical tweezers, the collimated laser beam interacts with lens 1 ($f_1 = 35$ mm; Thorlabs Inc.; Newton, NJ) and lens 2 ($f_2 = 200$ mm; Thorlabs Inc., Newton, NJ), which together expand the beam’s diameter by a factor of 200:35. Lenses 1 and 2 are positioned on a cage system which makes lens position adjustment more convenient (lenses 3 and 4 are also situated on a cage system). Mirrors 1, 2, and 3 all manipulate the beam path and direct the beam towards the Holographic Optical Trap (HOT) Plate (Bioryx 200; Arryx Inc.; Chicago, IL), which is a liquid crystal-based Spatial Light Modulator (SLM). The HOT plate acts as a computer-controlled, addressable mirror, and has the ability to create and manipulate multiple laser traps.

Due to the beam expansion, the incident beam size is large enough to fully cover the HOT plate’s target area. The beam reflected from the HOT plate travels to lens 3 ($f_3 = 150$ mm; AC254-150-C; Thorlabs Inc.; Newton, NJ), which focuses the light. The focused light is then imaged into the sample after passing though lens 4 ($f_4 = 150$ mm; AC254-150-C; Thorlabs Inc.; Newton, NJ), a dichroic mirror, which reflects IR light and allows visible light to pass through, and the objective lens. The microscope used in the system is an inverted optical research microscope (TE200; Nikon; Melville, NY). Figure 6 contains a schematic of Ohio University’s optical tweezer set-up and the path of the laser beam.
Martín-Badosa et al. (2007) describes a common arrangement for imaging telescopes: the 4-\(f\) configuration. In a 4-\(f\) configuration, the SLM is located at the front focal plane of lens 1, in order to form the image at the back focal plane of lens 2. Between the two lenses the light rays are parallel and the total distance, \(L\), from the SLM to the objective back aperture is given by

\[
L = 2f_1(1 + M)
\]

where \(f_1\) is the focal length of the first lens and \(M\) is the magnification. High light efficiency is an advantage of this arrangement.

Figure 6. Schematic of optical trapping system at Ohio University
3 Results and Discussion

3.0 Development of a Protocol for Microtubule Reconstitution

Placing a chamber containing 100 µg/mL microtubule solution on the microscope stage, the depth of focus was adjusted until fluidic motion of particles was observed, confirming that the inside of the chamber was being observed. Figure 7 shows the inside of the chamber ~18 hours after microtubules were introduced, using the 20x objective and DIC imaging. Waiting overnight allows microtubules to settle to the bottom of the chamber.

![Figure 7. DIC images of the chamber after 18 hours. Images a) and b) are at different locations inside the chamber. The ellipses indicate structures of interest](image)

As seen in Figure 7 above, semi-linear structures that resemble microtubules and also some larger, amorphous aggregates were observed. In an effort to break up the aggregates, the aliquot was initially sonicated for ~ 5 minutes in a sonication bath (FS30; Fisher Scientific; Pittsburgh, PA). Sonication is the use of sound energy to disrupt or agitate particles. A new chamber was prepared with the sonicated solution, however there was no observable difference in the aggregate size. Sonication was tried
for increasing 5 minute intervals until the aliquot had been sonicated for ~ 25 minutes. Figure 8 shows the result of 25 minutes of sonication on the microtubules.

![Figure 8. DIC images at different positions in the chamber after ~25 minutes of sonication (100 µg/mL concentration). Circles indicate structures resembling microtubules](image)

The particle size observed after 25 minutes of sonication in Figure 8 appears to be smaller than the particle size in Figure 7. The average length of the particles was found to be 16.7 µm ± 0.6 µm before sonication and 6.6 µm ± 0.3 µm after 25 minutes of sonication, confirming our observations.

In order to observe the effect of sonication on a higher concentration of microtubules, another aliquot of microtubules was rapidly thawed in a water bath at room temperature and new microtubule resuspension buffer was prepared, by freshly adding taxol to the HEPES and MgCl₂ buffer. Adding 30 µL of the resuspension buffer to the microtubule aliquot, yielded a concentration of 400 µg/mL, or 4x more concentrated than the last dilution. Preparing a chamber with the 400 µg/mL microtubule solution, a higher density of large aggregates was observed than with the 100 µg/mL concentration. After 5 minutes of sonication, there was no observable difference in aggregate size. The aliquot was sonicated for increasing 5 minute intervals, and then 10 minute intervals (10, 15, 20, 25, 35, and 45 minutes).
Figure 9 shows the initial aggregate size and the results of sonication on the aggregate size after sonicating for 10, 20, and 45 minutes, after leaving the chambers overnight.

Figure 9. DIC images a), b), c), and d) of the aggregates initially and then with sonication for 10, 20, and 45 minutes respectively. Image a) was obtained using the 20x objective, while the rest were obtained using the 60x objective. (400 µg/mL concentration)

Figure 9 indicates that sonication works to break up the aggregates, however, in a way that seems to be destructive. Instead of large aggregates, there were small debris-like fragments dispersed throughout the chambers. Measuring the longest structures resembling microtubules (for all the sonication times) the average length was found to be 4.3 µm ± 0.10 µm.

The next method employed was a dilution of the microtubules, in an effort to determine the effect on microtubule viewing and concentration. Dilutions (2x) were
performed, starting at the initial aliquot concentration of 1 mg/mL and diluting to concentrations of 0.5, 0.25, 0.125, and 0.0625 mg/mL. Figure 10 shows DIC images of the chambers containing these dilutions after several hours, to allow for the material to settle to the bottom of the chamber.

Figure 10. DIC images a), b), c), d), and e) of chambers containing microtubule concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, respectively (60x objective)
At each concentration, to be as consistent as possible, the largest aggregates in each chamber were located and then images were taken of them, as seen above in Figure 10. To quantitatively assess the observed concentration in each image, the percentage of area occupied by the microtubule aggregates in each image was calculated. The calculation method performed for the 0.0625 mg/mL concentration (Figure 10, Image e) will be shown here as an example. The area covered by the microtubules was approximated by rectangles as shown in Figure 11.

![Figure 11. Rectangular area elements used in calculation of the area occupied by microtubules at the 0.0625 mg/mL concentration](image)

The areas of the rectangular elements were then summed and divided by the total area of the chamber shown in the image, yielding the percentage of the area occupied by microtubules. Using this method, the area occupied by the microtubules at 0.0625 mg/mL was 8.7%. The same method was used for the other concentrations. The values obtained for the 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL concentrations can be seen in Table 3.
Table 3. The percentage of the area occupied by microtubules at 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL.

The uncertainty (Δ) in the area calculation was determined by choosing other rectangular elements that could have also reasonably been chosen and finding the difference between our original rectangle choice’s area and the other reasonable choices’ areas. These results can also be found in Table 2.

In order to check the cause of the presence of the large aggregates in the viewing chamber, PIPES buffer (80635-50G; Sigma-Aldrich; St. Louis, MO) was ordered. A new microtubule resuspension buffer was prepared replacing HEPES with PIPES, as suggested by the microtubule supplier. See Appendix for instructions for the preparation of the microtubule PIPES resuspension buffer. Preparing a chamber of microtubules reconstituted with the PIPES buffer, the images seen in Figure 12 were observed, using the 100x objective.

Figure 12. Images a) and b) show the inside of the chamber containing microtubules reconstituted with PIPES buffer (100x objective, without DIC)
In Figure 12 above, spindly structures can be observed, or microtubule asters. This was an improvement from the large aggregates and we proceeded to work with these microtubule structures. Varying the concentration of the reconstituted PIPES microtubules, it was determined that 0.125 mg/mL was the optimal concentration for microtubule sedimentation and viewing.

3.1 Caveats: Microtubule Reconstitution

Figure 13 shows observations for the chamber being placed on top of a rectangular microscope slide and then being placed on the microscope stage, using the 20x objective and DIC imaging.

![Figure 13. Initial observations, using 20x objective and DIC imaging, of rectangular slide and chamber. Images a) and b) were observed at different locations of the chamber. The ellipses indicate structures resembling microtubules](image)

While these structures look remarkably similar to microtubule structure images in Carter and Cross (2001), they are in fact a visual effect caused by the contact of the microscope slide and the square slide. This is actually an observation of a surface below the chamber, rather than the inside of it. Removal of the rectangular microscope
slide, and placement of the chamber directly on the stage, will no longer result in the misleading observations in Figure 13.

The second thing to beware of when observing microtubules is that the taxol necessary for microtubule reconstitution forms crystals that resemble microtubules (Foss et al. 1998). Taxol has low solubility in solution and tends to form spindly structures, much like microtubules (Foss et al. 1998). Figure 14 shows images of the types of structures taxol can form in solution.

![Figure 14. Image a) shows a taxol aster. Image b) shows a taxol bundle. Images from Foss et al., 1998. (Obtained from http://www.plosone.org/article/info:doi/10.1371/journal.pone.0001476 (Open Access)) (Scale bars added based on original captions)](image)

Comparing our own observations with the taxol structures in Figure 14, we noticed a striking resemblance, particularly with the microtubules seen in Figure 12. As a control, we prepared a chamber containing only the freshly made PIPES reconstitution buffer. We did not observe any particles or structures in the chamber. We also prepared a chamber with one of the older HEPES reconstitution buffers (~4 days old). In this case, we observed large aggregates. We concluded from these observations that the taxol does not fall out of solution immediately, but rather after a couple of days. The observations of the microtubule chambers were conducted
promptly after taxol addition, and thus it is safe to conclude that we were not observing taxol structures, but rather, microtubule structures. Taxol does not fall out of solution immediately, but it is very important to make sure that the taxol is freshly added to the buffer used in microtubule reconstitution.

3.2 Introduction of Chromokinesin-Coated Beads into Chamber

Following the production of the chromokinesin-coated beads as described in the Materials and Methods above, both the microtubules and the chromokinesin beads were introduced into a chamber. As described in the Materials and Methods section, the casein-incubated microtubules were introduced in the chamber and left overnight without sealing the ends. This allowed the buffer to evaporate and the microtubules to sediment to the bottom of the chamber. After sufficient time had passed for the sedimentation of the microtubules to occur, the chromokinesin beads and 2 mM ATP were introduced into the chamber and the ends were sealed. The chamber was then placed on the viewing stage and observed under 100x magnification. Figure 15 shows images of the beads in the chamber.

![Figure 15. Images of the chromokinesin coated beads in the chamber. Both image a) and b) were obtained using the 100x objective. The darker, fuzzy areas are beads that are located at different depths of the chamber, and are not in the focal plane](image-url)
The beads exhibited Brownian motion (random walk caused by thermal fluctuations) and settled to the bottom of the chamber after 2-3 hours. Even though ATP was present in the chamber, the beads were not observed to interact with the trackways within a reasonable amount of time. This motivates the use of the optical tweezers to trap the beads and bring them to the microtubules on the surface. Once interacting with the trackways on the surface, we need to be able to trap the beads in order to stall out the motion. The ability to trap at the surface of the chamber is crucial for what we are trying to accomplish.

In an effort to bring the beads down to the microtubules on the chamber’s bottom surface, the laser was turned on and the optical trapping system was used. Using Labryx, beads were trapped and easily manipulated in the $x$ and $y$ directions, by clicking on the trap and slowly dragging it across the screen. In order to test the mobility of the trap in the $z$ direction, the $z$-position controller was adjusted to bring the beads lower in the chamber. Figure 16 contains images of the Labryx control window.
Whenever an attempt was made to move the $z$-position of the traps, the beads would “fall” out of the traps. The beads would stay trapped initially, but as the $z$ position continued to change, the bead would fall from the trap, consistently moving away from the trap at a $-45^\circ$ angle and once again undergoing Brownian motion. After trapping a bead and adjusting the focus knob of the microscope, the bead would also fall out of the trap, exhibiting the same behavior. In a correctly adjusted optical tweezer system, the trap will shift as the focus changes (Fällman & Axner, 1997). Due to the failure to manipulate the position of the trap in the $z$-direction, the alignment of the optical tweezer system was investigated to find the conditions needed for the trap to successfully move in the $z$-direction.
3.3 Optimization of Optical Tweezer Alignment

In order to observe the laser light’s diffraction pattern, a mirror calibration slide (Bioryx 200 Calibration Chip; Arryx Inc.; Chicago, IL) was placed on the microscope stage. Figure 17 shows an image of the focused laser beam, using the 100x objective.

![Image a) is an image of the laser beam using the 100x objective. The arrows point to extraneous points of light. Image b) is what the laser beam should look like in a well-aligned system. Image b) from Martín-Badosa et al., 2007 (Obtained from http://iopscience.iop.org/1464-4258/9/8/S22/pdf/1464-4258_9_8_S22.pdf)](image)

We expected to see a focused circle, as seen Figure 17, Image b (Martín-Badosa et al., 2007), however we observed an optical aberration, as seen in Figure 17, Image a). There were two extraneous points of light (see arrows in Figure 17, Image a), and the light was not centrally focused. In order to check the beam alignment through the two lens systems (L1 and L2 (beam expander) and L3 and L4), a cage alignment tool (VRC4CPT; Thorlabs Inc.; Newton, NJ) was used, as shown in Figure 18. The alignment tool has an infrared disk, that glows green where the laser beam hits, and the disk has a hole at the exact center of the cage that the lenses are situated on.
Figure 18. Photo of the cage alignment tool, used in checking the laser beam alignment

Placing one cage alignment tool on each cage, the cages were adjusted until the laser beam traveled as close to exactly center of the cages as possible. The laser’s diffraction pattern was observed again, however, there was no significant improvement in the aberration pattern. The next method we tried was to move the position of the cages. The cage containing lenses 1 and 2 was moved in 1 cm increments, while the cage containing lenses 3 and 4 remained stationary. At each new position of the first cage the laser beam was observed, but there were not any significant changes in the aberration. We then returned the first cage to its initial position and moved the second cage in 1 cm increments. This also failed to improve the image of the laser beam.

The next approach was to move the lenses relative to each other. Lenses 1 and 2 initially had 23.5 cm (the sum of their focal lengths) between them. We moved the lenses in 1 cm increments from having 18.5 cm between them to having 28.5 cm between them. When we did not observe any improvement, we returned lenses 1 and 2 to their initial position relative to each other, and tried the same approach with lenses 3 and 4. Lenses 3 and 4 initially had 30 cm between them (the sum of their focal
lengths), and we moved them in 1 cm increments from having 25 cm between them to having 35 cm between them. Again, there was not a marked improvement in the laser beam image, as can be seen in Figure 19.

Figure 19. Image a) shows the laser beam for a distance of 19.5 cm between lenses 1 and 2. Image b) shows the laser beam for a distance of 34 cm between lenses 3 and 4.

Several other methods used in trying to improve the alignment were the repositioning of lenses 1 and 2 in the optical train to after mirror 1, as opposed to before it (see Figure (optical setup) above), completely removing lenses 1 and 2 from the laser path, completely removing all of the lenses from the optical path, and finally, removing the HOT plate from the optical path (by placing a mirror over it). Figure 20 contains images of the laser obtained from these variations in the optical tweezer setup.
Figure 20. Image a) is the laser observed after lenses 1 and 2 have been removed from the optical train. Image b) is the laser observed after all of the lenses have been removed from the laser path. Image c) is the laser observed after the lenses were removed and a mirror was placed over the HOT plate.

From Figure 20 it is apparent that the optical aberration survives the methods employed to improve it. Reassembling the optical train, the same optical aberration observed in Figure 17 was again observed. It is possible that the laser is not working properly or the mirrors need to be replaced, and further assessment of the optical tweezer system will be necessary to improve the trapping capability.
4 Conclusion and Suggestions for Future Work

Optical tweezers is a powerful technology that is extremely useful in biophysical applications. This thesis prepared the foundation for one such application: the study of the molecular motor, chromokinesin, and the trackway, the microtubule, using the optical tweezer system at Ohio University. We have determined the optimal method of microtubule reconstitution (using the PIPES buffer), and found that a concentration of 0.125 mg/mL was the best concentration for sedimenting microtubules to the bottom surface of chambers. We developed a protocol for coating beads with chromokinesin and for introducing the beads into chambers with microtubules adhered to the surface. We also presented the work we did to improve the trapping of the optical tweezer system in the $z$-direction, crucial to creating chromokinesin-microtubule interactions. In order to fully develop this application, with hopes that it will someday be a general lab experiment for students, we propose the following action items:

1. *Develop the ability to trap reliably in the $z$-direction.* As a next step, look at the mirrors and laser more closely. Also, SLMs can be responsible for major optical aberrations due to die level bows and warps (Martín-Badosa et al., 2007). This can be corrected by finding a phase function which reverses the aberration, through a wavefront sensor or trial and error parameters (Martín-Badosa et al., 2007).

2. *Enable DIC-imaging on the microscope used for optical trapping.* At present, the microscope used for optical trapping does not have DIC imaging
capabilities. In order to better see the chamber and its contents, it would be beneficial to either move the existing DIC attachment from the other microscope to the optical trapping system’s microscope, or perhaps invest in another one, depending on the need.

3. *Get the chromokinesin-coated beads to successfully attach and move along microtubules.* Use either the optical trap or optimize the conditions so diffusion works to bring the chromokinesin-coated bead to the microtubules on the surface. If there are no observed interactions, play with the ATP concentration. The proteins, MAP2 and tau, bind to the microtubule’s tubulin subunits and create a longer and more stable microtubule, which could be useful for our purposes (Howard, 2001).

4. *Develop a gliding assay.* Develop a protocol, based on the literature, to coat the chamber’s bottom surface with chromokinesin. Use the optical trapping system to bring the trackways to the chromokinesin coated surface. Again, play with the ATP concentration to create interactions.

5. *Study the interactions between the motor and trackway in both the bead and gliding assay.* Use optical traps to stall out the motors/trackways. Measure and determine step size, velocity, stall force, duty ratio, distance per ATP, and working distance.
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References


Appendix

Preparation of Microtubule Resuspension Buffer (HEPES)

1. Decant about 70 mL of sterile water
2. Measure 0.1787 g HEPES into weigh boat
3. Wash into 50 mL volumetric flask with sterile water
4. Measure 0.0101 g MgCl₂ into weigh boat
5. Wash into same flask with sterile water
6. Fill flask to 50 mL with sterile water
7. Move to 100 mL beaker
8. Calibrate pH meter
9. Check pH of buffer
10. Add NaOH dropwise if pH is <7.00, or HCl if pH >7.00
11. Adjust pH until pH is stable at 7.00
12. Sterile filter the solution, in order to remove any impurities or contamination
13. Buffer can be stored in 4°C fridge until ready to add Taxol
14. Once ready to add Taxol, remove buffer from fridge and allow to warm to room temperature
15. Remove DMSO (Dimethyl Sulfoxide; supplied with Taxol) from -20°C freezer and allow to come to room temperature
16. Using a micropipette, resuspend one tube of Taxol in 100 µL of DMSO. This results in a 2mM Taxol solution
17. Store remaining Taxol solution in -20°C freezer (stable for six months)
18. Add 30 µL of the 2 mM Taxol solution to 3 mL of the room temperature buffer containing HEPES and MgCl₂
19. Resulting buffer is 15 mM HEPES, 1 mM MgCl₂, and 2 mM Taxol
20. Store buffer at room temperature. Taxol will remain stable for about one week

Preparation of Microtubule Resuspension Buffer (PIPES)

1. Follow the procedure used in the preparation of the microtubule HEPES buffer, however, use 0.2268 g PIPES in place of 0.1787 g HEPES
2. Resulting buffer will be 15 mM PIPES, 1 mM MgCl₂, and 2 mM Taxol

Preparation of Chromokinesin Resuspension Buffer (HEPES)

1. Decant about 70 mL of sterile water
2. Measure 0.5958 g HEPES into weigh boat
3. Wash into 50 mL volumetric flask with sterile water
4. Repeat steps 3 and 4, with 0.0203 g MgCl₂, 0.9319 g KCl, 0.0077 g DTT, and 0.0006 g ATP
5. Fill flask to 50 mL with sterile water
6. Move to 100 mL beaker
7. Calibrate pH meter
8. Check pH of buffer
9. Add NaOH dropwise if pH is <7.00, or HCl if pH >7.00
10. Adjust pH until pH is stable between 7.00 and 7.50
11. Sterile filter the buffer
12. Resulting buffer is 50 mM HEPES, 250 mM KCl, 2 mM MgCl₂, 1 mM DTT, and 20 µM ATP
13. Store in fridge until needed for use

**Preparation of Chromokinesin Resuspension Buffer (PIPES)**

1. Follow the procedure used in the preparation of the chromokinesin PIPES buffer, however, use the quantities; 1.5119 g PIPES and 0.7455 g KCl in place of those listed above
2. The resulting buffer will be 100 mM PIPES, 200 mM KCl, 2 mM MgCl₂, 1 mM DTT, and 20 µM ATP

**Coating of Polystyrene Beads with Casein and Chromokinesin**

1. Decant ~10 mL of chromokinesin PIPES buffer into 100 mL beaker
2. Decant ~10 mL of casein into 100 mL beaker
3. Cover both beakers with parafilm
4. Vortex Spherotech beads on max. for ~15 seconds
5. Micropipette 90 µL of casein into eppendorf tube
6. Micropipette 10 µL of beads into the same tube
7. Label tube with permanent market
8. Vortex tube for ~5 seconds
9. Open the microfuge lid
10. Place the tube with beads and another tube containing about the same amount of liquid, inside opposite holes, in order to balance
11. Lock in spill guard
12. Close lid of microfuge
13. Set the microfuge to 5000 rpm for 1 minute
14. Start microfuge
15. Once completed, open microfuge, and remove tube with beads
16. Remove 90 µL with micropipette, avoiding the white sediment on the bottom/side of the tube (beads)
17. Add 90 µL of fresh casein
18. Vortex tube for ~5 seconds
19. Repeat steps (8-17) 2-3 more times, for a total of 3-4 washes
20. Incubate on rotary mixer for ~1 hour
21. Remove 90 µL of solution
22. Add 90 µL of chromokinesin PIPES buffer
23. Repeat steps (8-17) 4 times, however using chromokinesin PIPES buffer instead of casein
24. Add 100 µL of chromokinesin (reconstituted to 100 µg/mL) to casein coated beads in eppendorf tube
25. Vortex ~5 seconds
26. Incubate chromokinesin and beads for ~1 hour on the rotary mixer
27. Store in fridge