EXPERIMENTAL AND THEORETICAL INVESTIGATIONS OF KINESIN-2 MECHANOCHEMISTRY

A Dissertation in

Bioengineering

by

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**ABSTRACT**

Kinesins are biological motors with the remarkable capability of coupling chemical energy from ATP hydrolysis into structural changes that generate force and directed motion along microtubules. Serving as a model protein, findings from single molecule kinesin biophysics provide general insights into mechanobiology. Kinesin comprises of 14 families that vary in structure, function and biophysical properties. The goal of this project is to elucidate details of the chemomechanical coordination between the two heads of the Kinesin-2 motor family, during their processive stepping and relate their *in vitro* biophysical properties to their *in vivo* behavior. Kinesin-2 motors are involved in intraflagellar transport as well as cytoplasmic transport of melanosomes.

Understanding protein behavior at the single molecule level requires developing techniques to image and manipulate them. In chapter 2, development of quantum dots as fluorescent tags to enhance spatial and temporal resolution of imaging is discussed. Kinesin-1 was tagged with biotinylated quantum dots and viewed using epifluorescence and total internal reflection fluorescence microscopy (TIRFM). Both techniques exhibited similar image resolution and the velocities and run lengths obtained compared favorably with values in literature.

Optical tweezers open avenues in mechanobiology by enabling the application of mechanical loads and manipulation of single molecules. An optical tweezer was constructed with back focal plane interferometry detection, and detailed calibration
was performed. Preliminary data of motor stepping with 8 nm step sizes at low motor velocity was demonstrated. This optical trap was used to conduct bead assays at minimal loads to study the processivity and velocity of Kinesin-1 and Kinesin-2 motors. Mouse KIF3A/B was compared to homodimeric chimeras, KIF3A/A and KIF3B/B, and to conventional Kinesin-1 motors. At saturating ATP, KIF3A/B moved at 436 ± 129 nm/s and the homodimers moved at similar speeds, while Kinesin-1 moved at 703 ± 136 nm/s. The run lengths of all three KIF3 motors were approximately 600 nm, while the run length for Kinesin-1 was three-fold higher. When the ATP concentration was reduced from 1 mM down to 1 µM, Kinesin-1 run lengths were constant, consistent with previous reports. This implies that Kinesin-1 waits in the same chemomechanical state regardless of the time it takes for ATP to bind. In contrast, the run length of KIF3A/A increased nearly three-fold when the ATP was lowered from 1 mM to 1 µM. This implies that during the time the motor waits for ATP to bind at limiting ATP levels, the motor transitions to a different chemomechanical state, resulting in a lower probability of detachment following ATP binding. Stochastic simulations of motor stepping showed that at saturating concentrations, ATP binds to the motor while both heads are bound to the microtubule leading the motor to have a higher probability of detachment from the microtubule. Structural biology indicates that KIF3 has a 3-amino acid longer neck linker than Kinesin-1, which reduces the rearward strain on the leading head and causes absence of ATP gating. These results provide constraints on the Kinesin-2 mechanochemistry.
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Chapter 1

Introduction: Kinesin Mechanochemistry

1.1 Kinesin – Microtubule System

Kinesins are motor proteins that transport intracellular cargo over microtubule tracks using energy derived from ATP hydrolysis. Their functions include active transport of vesicles, proteins and organelles in cell cytoplasm, axons, cilia and flagella. During cell division, kinesins help in the maintenance of mitotic spindles, in separating the chromosomes and in depolymerizing the microtubules (Vale and Fletterick, 1997).

Microtubules are polymers of the protein tubulin, which are composed of alpha- and beta-tubulin subunits. The two subunits together are 8 nm long (Nogales, 1998). A microtubule protofilament has tubulin monomers arranged in a head to tail fashion and 12-16 protofilaments join laterally to form a hollow cylindrical microtubule with a diameter of 25 nm (Desai and Mitchison, 1997). The microtubules have a structural polarity with a fast polymerizing plus-end towards the beta-tubulin and a slow growing minus-end towards the alpha-subunit. In cells, the microtubules are marked by dynamic instability, i.e., the growth of microtubules occurs through a cycle of polymerization, followed by transition to catastrophe or the depolymerizing phase, and then is rescued back to the growing phase (Mitchison and Kirschner, 1984; Nogales, 1998). Polymerization occurs by addition of GTP-tubulin to the end of the protofilament fueled
by the hydrolysis of GTP on inner tubulin subunits. As long as the rate of addition of GTP-tubulin is faster than the hydrolysis, a GTP-cap is formed at the plus-end of the microtubule, which stabilizes the structure. At low tubulin heterodimer concentrations, this cap disappears, exposing GDP-β-tubulin, which causes fraying and depolymerization of microtubules (Mitchison and Kirschner, 1984; Nogales, 1998). Microtubules emanate from the centrosome near the nucleus, dynamically grow, and are stabilized at the cell wall. Therefore, apart from acting as tracks for transport by motors, they help maintain the integrity of the cellular structure and form the intricate spindle during cell division.

The kinesin superfamily is comprised of 14 subfamilies of molecular motors that are classified based on their sequence and structure (Lawrence et al., 2004). Structurally, conventional kinesin consists of two identical heavy chains and two light chains. The heavy chains consist of a well conserved catalytic motor domain and the microtubule binding site, followed by a flexible neck linker and a long coiled-coil stalk containing a hinge and finally the tail domain (Figure 1.1) (Scholey et al., 1989; Yang et al., 1989). The light chains interact with the tail of the heavy chains and are responsible for cargo binding (Hirokawa et al., 1989). In the kinesin superfamily, the tail is highly variable depending on the cargo they are responsible for transporting. To understand how the motor works, models of the chemomechanical pathway, have been developed based on experiments using conventional kinesin or Kinesin-1 motors, the most widely studied motor family (Gilbert et al., 1995; Hancock and Howard, 2002; Ma and Taylor, 1997). Here, we will use this motor as a control and for comparison with Kinesin-2 motors.
Since the light chains are not present in our construct, this motor will be referred to as KHC (Kinesin heavy chain).

\[ \text{Figure 1.1: Rat brain Kinesin-1 dimer crystal structure showing the motor domain, neck linker and short stretch of the coiled-coil (Kozielski et al., 1997).} \]

1.2 Biophysics of Kinesin-1

Kinesin-1 are plus-end directed processive motors: that is; one motor can take multiple steps on a microtubule filament before detaching and diffusing away (Howard et al., 1989). Processivity is important for motors to transport cargo long distances like in axons, flagella and cilia. Motor stepping is coordinated with ATP hydrolysis and conformational changes in the motor head domains. For Kinesin-1 the ATPase rate is
tightly coupled with stepping rate at one ATP per step (Coy et al., 1999; Hua et al., 1997). Step sizes of 8 nm, consistent with tubulin subunit length, were detected in single molecule bead assay using optical tweezers (Svoboda et al., 1993). The maximum forces motors generate were found to be 6-7 pN (Meyhofer and Howard, 1995; Svoboda and Block, 1994; Svoboda et al., 1993). From the motor run length of about 800 nm (Block et al., 1990) and velocity of 800 nm/s (Howard et al., 1989), it can be estimated that the off-rates of these motors from the microtubule are 1 per second. For the motor to be processive, the two heads have to coordinate their cycle, so that one head is bound to the microtubule at all times.

### 1.3 Coordination between motor heads

Models have to be developed to understand how processivity is maintained through chemomechanical coupling of ATP hydrolysis with mechanical stepping. The general consensus model of motor chemo-mechanical pathway is the asymmetric hand-over-hand model (Asbury et al., 2003). The working model in the Hancock lab, shown in Figure 1.2, is modified and refined from the model developed by Schief et. al. and by Hancock et. al. (Hancock and Howard, 2002; Schief and Howard, 2001). The model starts with both heads attached to adjacent tubulin subunits in State 1 with the front head in the no nucleotide state and the rear having hydrolyzed its ATP. The next two transitions the motor needs to make are i) ATP needs to bind to the front head and ii) the rear head needs to detach from the microtubule. The order of these two steps is still being debated. There is evidence that spontaneous detachment (SD) of rear head occurs
due to strain (State 2) (Hancock and Howard, 1999; Klumpp et al., 2004). This
detachment is also thought to be required for the ATP binding pocket in the bound head
to open, allowing ATP to bind (State 4). This model cannot entirely discard the evidence
of ATP binding when both heads are bound to the microtubule as shown by Yildiz et.
al. (State 3) (Yildiz et al., 2004). In either case, ATP binding results in neck linker
docking, which causes reorientation of the rear head towards the next tubulin binding site
towards the plus-end of the microtubule (Rice et al., 1999; Vale and Milligan, 2000).
The ATP hydrolyzes (State 5) and then most often the tethered head binds releasing its
ADP (State 1). For Kinesin-1, about 1% of the time the bound head which is in a weak
binding state of ADP.Pi detaches before tethered head can bind and the whole motor
comes off (State 6).

There are differing theories concerning the details of the intermediate steps of the
asymmetric hand-over-hand model and hence many questions are yet to be answered
(Cross et al., 2000; Hancock and Howard, 2002; Schief and Howard, 2001; Vale and
Milligan, 2000). i) In the model shown here, the state of motor (State 2 or State 3) in
which ATP binds to the motor head is speculated. Other questions include: ii) is the ATP
hydrolyzed before or after tethered head binding? iii) For the overall mechanism, which
is the rate limiting step; rear head detachment, ATP hydrolysis or binding of tethered
head? iv) Which of these steps is force dependent; the rear head detachment or it pivoting
forward and binding? v) Is the step size of 8 nm distributed among different sub-steps
and if so what is the distribution?
The flexible neck linker plays a vital role in answering these questions. Neck linker docking of the ATP bound head is shown to be responsible for directed movement of the motor (Rice et al., 1999). Recently, Hwang et. al. used molecular dynamics to argue that the N-terminal cover strand also ordered into a $\beta$-sheet when ATP was bound to the motor. The $\beta$-sheet associated with the docked neck linker and stabilized the conformational change (Hwang et al., 2008). This behavior introduces a new paradigm to the existing neck linker docking theory. Apart from directed movement, the neck linker also translates strain between the two heads to maintain processivity (Hancock and Howard, 1999; Rosenfeld et al., 2003). The magnitude of the strain depends on the nucleotide binding state of the head and consequently, the neck linker conformation. When both heads are bound, there can be rearward strain on the front head and forward strain on the rear head (Rosenfeld et al., 2003). According to Rosenfeld et. al., theoretically, the forward strain has to be minimal, or else the motor will unbind in just a few steps. Instead, they find that rearward strain of the front head slows down the ATP binding to it by closing the binding pocket (Klumpp et al., 2004; Rosenfeld et al., 2003). In other words, strain in the neck linker also behaves as an ATP gate. Slowing of the ATP binding helps maintain processivity by keeping the head bound longer until the rear head unbinds from the microtubule and is available to bind again. Therefore, the neck linker structure is extremely important in processivity studies. It must be noted that though parts of the neck linker sequence are conserved between motor families, subtle difference are present and they can change the mechanical characteristics.
To better understand the general mechanochemical cycle for kinesins and to extend our knowledge to other families of motors, we shift our focus to Kinesin-2 family. This family of motors is characterized by having two different heavy chains, as opposed to being homodimeric like Kinesin-1. The understanding of the intermotor domain coordination between the heads in Kinesin-2 will be a key tool in answering questions about Kinesin-1.

Figure 1.2: Hancock model of intermotor domain coordination.
1.4 Kinesin-2 structure

Kinesin-2 motors are heterotrimeric, containing two different heavy chains and a non-motor kinesin associated polypeptide (KAP) that binds to the tail domain of the motor and to the cargo (Figure 1.3) (Rashid et al., 1995; Wedaman et al., 1996; Yamazaki et al., 1995; Yamazaki et al., 1996). The first kinesin-2 motor identified was KRP85/95 in sea urchins (Cole et al., 1993; Yang et al., 1988). Subsequently, mouse KIF3A/B, Drosophila KLP64D/68D (Pesavento et al., 1994; Stewart et al., 1991), Osm-3 in C. elegans (Shakir et al., 1993) and Chlamydomonas FLA10 (Walther et al., 1994) were identified. The main function of this class of motors is in transporting proteins and organelles in cilia and flagella, a process called intra-flagellar transport (IFT). They are also important in cytoplasm to transport melanophores and during cell division to maintain the mitotic spindle (Haraguchi et al., 2006; Tuma et al., 1998).

Figure 1.3: Structure of KRP85/95 with a non-KRP subunit as an example of Kinesin-2 motor (The Kinesin Homepage, www.proweb.org/kinesin, A.J. Kim and S.A. Endow, April 2000).
Here we are interested in the mouse ortholog, KIF3A/B. KIF3A was first purified in 1994 by Kondo et. al. in murine brain neurons where they are associated with membrane organelles fractions (Kondo et al., 1994). KIF3B was later purified from the same murine brains and was found to associate with KIF3A. The two full length subunits are 47% identical. The heads have 69% sequence identity while they are about 44% identical with KHC. The fact that this structure of non-identical heads dimerizing has been maintained through evolution, from Chlamydomonas (a unicellular green algae) to mammals, indicates its importance in nature (Yamazaki et al., 1995). Uncovering the advantage and necessity for the two heads to have dissimilar sequences is an important goal in this field. The difference in the motor heads also makes it an excellent candidate to study kinesin biochemical and mechanical pathways, as homodimeric chimeras can be constructed and each head studied individually. Another important structural difference is the neck linker, which is only 15 amino acids long for KHC but is 18 amino acids for KIF3A/B. The additional three amino acids add 1 nm to the neck linker which will in turn affect the strain between the heads. Hence, any changes in motor processivity will require probing the neck linker and the N-terminal cover strand.

1.4.1 Cellular functions of Kinesin-2

Kinesin-2 motors play an important role in transporting intraflagellar proteinaceous particles along axonemal microtubules both in flagella and cilia in different cell types like in the Chlamydomonas flagella, the sperm flagella and cilia of
photoreceptors and in kidney cells (Henson et al., 1997; Lin et al., 2003; Marszalek et al., 2000; Walther et al., 1994). It has been shown that KIF3A knock-out in mammalian rod cells caused rapid degeneration of the receptor cells (Marszalek et al., 2000). In kidneys, Lin et. al. found that dysfunctional cilia led to polycystic kidney disease through a complex mechanism after conditionally inactivating KIF3A (Lin et al., 2003). In mouse embryonic cells, the absence of KIF3A/B in the cilia caused immotile cilia that led to abnormal nodal flow of morphogens causing loss of left-right axis symmetry and disruption of cardiac development (Nonaka et al., 1998).

In cytoplasm too, many membrane bound organelles are transported through multiple motors including Kinesin-2. For example, in *Xenopus* melanophores, the dispersion and aggregation of melanosomes is carried out by three motors which includes kinesin-2, dynein and myosin V(Rogers and Gelfand, 1998; Tuma et al., 1998). Kinesin-2 and myosin work to disperse the melanosomes in response to melanocyte-stimulating hormone and dynein works to aggregate the melanosomes in response to melatonin. Though regulation is governed primarily by chemical signals, since all three motors are present on the pigment, a combination of these signals along with mechanical forces is hypothesized (Gross et al., 2002). Recently, Haraguchi et. al., showed association of KIF3A/B with the mitotic spindle and centrosomes during mitosis (Haraguchi et al., 2006). Cells, which could not associate with KAP protein due to mutations in KIF3B, showed abnormal spindle formation leading to aneuploidy (chromosome missegregation).
1.4.2 Significance of studying Kinesin-2 motors

The implications of dysfunction Kinesin-2 motors are multifold. Primary Ciliary Dyskinesia (PCD), a genetic disease, is a generic name for immotile cilia. It is marked by recurring lung infections due to improper function of bronchial cilia. PCD is accompanied by sperm immotility, female submotility and retinal and kidney defects that are known as PCD-associated diseases (Ibanez-Tallon et al., 2003; Lin et al., 2003). Since Kinesin-2 is important in cilial function and directly affects many of the mentioned diseases, it necessitates understanding the molecular biomechanics of Kinesin-2 and using the knowledge to develop therapeutics against these diseases necessary (Ibanez-Tallon et al., 2003). Also, kinesin in general, being active in cell division, becomes an immediate target for cancer therapy. Hence, developing techniques and models to understand their molecular function is an important first step in therapeutic research involving motors.

1.5 Advanced microscopy techniques for in vitro experiments

To study the biochemistry and biophysics of molecular motors, in vitro functional assays involving the purified motor-microtubule system have been well established (Block et al., 1990; Howard et al., 1989; Vale et al., 1985). Most common experiments use multimotor system, like the microtubule gliding assay or multimotor bead assays, because the motility can be observed with simple epifluorescence or DIC microscopy (Howard et al., 1993; Vale et al., 1985). While this is sufficient to acquire preliminary characteristics of the motor, the capability to observe single motor activity
has to be developed so as to understand the mechanochemistry of kinesin and to study \textit{in vivo} movement and regulation. For imaging and detecting single molecule motor events such as motor step size, both the time and spatial resolution have to be as high as possible. Also since motors perform mechanical work, investigating their force production and load dependent behavior (imitating \textit{in vivo} cargo), is an area of interest in this field. In this thesis, two techniques are researched to apply in Kinesin-1 and Kinesin-2 motor studies.

The first technique is called Total Internal Reflection Fluorescence Microscopy (TIRFM). This key feature of this technique is elimination of background fluorescence in the sample. This is achieved by creating an evanescent wave using lasers and high numerical aperture objective to shine light on sample at angles greater than critical angle. The main advantage of this system is that only molecules at the surface (where microtubules are present and motors bind) are illuminated since the light intensity decreases exponentially to the distance of 100 nm from the surface of the sample. This technique has been widely used in cells for imaging proteins in membrane and signaling pathways without worrying about out of plane fluorescence and in \textit{in vitro} assays for receptor ligand binding and molecular motor tracking. In order to improve data precision especially for tracking motility, continuous high intensity of fluorescence is preferable. Therefore, fluorophores with high quantum yield and photostability are required. The most popular fluorophores used today to couple with kinesin are GFP (green fluorescent protein) and synthetic dyes(Pierce et al., 1999; Yildiz et al., 2004). Here, we have used
quantum dots known for its stability and intensity as fluorescent tags on truncated Kinesin-1 motors and analyzed the motor run lengths and velocities.

Optical tweezers in conjunction with bead assays forms basis of single molecule technique used to study force related characteristics. Bead assay involves binding motors non-covalently to silica or polystyrene beads (< 1 µm diameter) and tracking their motion along immobilized microtubules. Single molecule behaviors are ensured by reducing motor to bead concentrations such that about 98% of beads have one or no motors. At such low motor concentrations, waiting for an event of motor-microtubule interaction to occur can become tedious. Hence, using optical tweezers just to capture beads in solution and facilitate interactions become time and cost effective. The basic principal of optical tweezers is that a large gradient of focused light behaves like a spring with minimum potential energy at the center of gradient and linear decrease towards the edges. Therefore, when beads come within the proximity of the trap they get attracted into the center. Since force is used in trapping, this is translated to load on the motor against which it has to move on microtubule. This opens a whole range of motor behavior like run length and velocity dependence on force, stall force and force dependent steps that can be researched.

1.6 Summary of thesis

In order to improve existing imaging and detection techniques used in the laboratory, we experimented with attaching quantum-dots as fluorescent probes to
truncated kinesin-1 to improve resolution of single molecule data obtained using TIRF. In this work, described in chapter 2, we used biotin-streptavidin chemistry for linking the protein to the nanocrystal. Run length and velocity data obtained from epifluorescence and TIRF microscopy imaging were compared to each other and to bead assays. While the results were encouraging, the non-covalent linkage used with streptavidin having four binding sites was not ideal for consistency in single molecule binding, especially when it has to be extended to different motor types. Also, this technique does not allow force related studies on the motor. Hence, in chapter 3, the focus was shifted to building an optical trap with a back focal plane interferometry detection system. This involved setting up optics for the trap, developing instrumentation for quadrant photodiode photon-to-voltage converter and LabView programming for instrument control and data acquisition. To test the system and standardize the single molecule bead assays for Kinesin-1 and Kinesin-2 motors, dilution profiles of motors were performed. Finally, single molecule Kinesin-1 stepping was recorded.

In chapter 4, Kinesin-2 biophysics was investigated in-depth. First, run length and velocity at saturating ATP of KHC and KIF3 motors were compared. Relation between the biophysical and in vivo behaviors was determined. Subsequently, processivity and speeds at varying ATP concentrations (from 1 µM to 1mM) were obtained for Kinesin-1 and Kinesin-2 motors. The difference in the ATP-dependent behavior of the two motors prompted the need to extend the consensus chemomechanical model for Kinesin-2. This theoretical model was tested and verified using a stochastic simulation of kinesin stepping coded in MATLAB. The results provided insights into the
different pathways and the rate constants involved in Kinesin-2 processivity. In chapter 5, the results obtained in this thesis are elucidated in context of the current trends and the future of the field of kinesin biophysics.

1.7 References


Chapter 2
Transport of Semiconductor Nanocrystals by Kinesin Molecular Motors

2.1 Introduction

Kinesin molecular motors harness the energy of ATP hydrolysis to transport cargo such as vesicles and organelles along intracellular microtubules. Purified components of this system can be used for nanoscale transport by integrating the motors and filaments into MEMs and NEMs devices. Hence, it is important to understand the function of these proteins for biological, therapeutic, as well as nanotechnological applications. Existing techniques for studying motors include the microtubule gliding assay, optical traps, and ATPase assays. Single-molecule visualization is crucial for investigating the motor mechanism and their ability to move and assemble nanoparticles. Here, we synthesize semiconductor nanocrystals, attach them to kinesins, demonstrate that single motors can be visualized by simple epifluorescence or evanescent wave microscopy, and show that motor function is unaffected by particle functionalization.

Single kinesin motors functionalized with green fluorescent protein (GFP) or synthetic fluorophores can be imaged by total internal reflection fluorescence (TIRF)
microscopy,(Pierce and Vale, 1998) and their position resolved to within nearly one nanometer.(Yildiz et al., 2004) By tracking kinesins in which one of the two motor domains (heads) was labeled, this technique was used to show that at limiting ATP concentrations each head takes 16 nm steps along a microtubule, ruling out the “inchworm” model of kinesin motility.(Yildiz et al., 2004) However, because the spatial resolution is based on the number of photons collected, the temporal resolution using these fluorophores is limited to roughly 300 msec. Brighter fluorophores are needed to measure faster events. While fluorescent beads have higher signal intensities, their size alters the diffusion properties of the tagged molecule and complicates intracellular experiments.

Semiconductor nanocrystals (Quantum dots) have great potential in biological imaging due to their small size (~5-10 nm radius with functionalization), high quantum yield, large excitation band, and negligible photobleaching. Quantum dots with different optical properties can be synthesized with ease by growing them to different sizes,(Murray et al., 1993) and single fluorophores can be visualized by simple epifluorescence microscopy rather than the evanescent wave microscopy that is generally required for GFP and other synthetic fluorophores. In addition, they can be introduced into cell by a variety of methods.(Derfus et al., 2004) By synthesizing our own quantum dots, we have the advantage of being able to separately tune the emission wavelength and control the surface functionality.
The goal of this study is to functionalize quantum dots with active kinesin biomolecular motors and transport these dots along immobilized microtubules. This new labeling approach will open up a number of avenues of investigation. First, it will enable more precise tracking of motors in vitro to understand motor stepping and detachment under controlled conditions. Second, these bright particles should enable individual kinesins to be followed in cells, which is very difficult with current labeling procedures. Third, quantum dots can be used as models for biomotor-driven nanoparticle assembly in vitro. More and more materials are being synthesized at nanoscale geometries, which confer novel and enhanced functionality. However, despite the success of various self-assembly processes, organization of these nanoparticles into configurations far from their thermodynamic minima is a continuing hurdle. Because kinesins are specialized transport motors that have evolved to organize the intracellular environment, they provide a powerful tool for transport and assembly of synthetic nanomaterials. Harnessing these biological motors for this purpose requires a model system that can be easily visualized and quantified. At present, microtubules that have been coated with quantum dots have been shown to move along immobilized motors so long as the region of functionalization is limited. (Bachand et al., 2004) Furthermore, in a related and impressive recent study, individual myosin V motors were labeled with a different colored quantum dot on each head to definitively show that the two heads alternately step along an immobilized actin filament. (Warshaw et al., 2005) Here, we demonstrate for the first time that individual kinesin motors can be functionalized with quantum dots, and their movement along microtubules easily tracked by either TIRF or epifluorescence microscopy.
Using quantum dots for this purpose comes with a number of hurdles. Generally, quantum dot cores are synthesized in an organic phase and usually with cytotoxic compounds,(Derfus et al., 2003; Hoshino et al., 2004; Shiohara et al., 2004) so for biological applications the cores need to be protected and transferred to an aqueous phase by coating with a shell of a second semiconductor with a larger band gap and with protective ligands. Additional ligands must be added to the quantum dot surface to enable motor attachment while preventing aggregation. Direct covalent chemistry or, as shown here, biotin-avidin can be used to link the motors to quantum dots.

2.2 Materials and Methods

Protein Preparations: *Drosophila melanogaster* conventional heavy chain kinesin plasmid(Coy et al., 1999) was modified by deleting the tail and coiled-coil domains (cut at site corresponding to amino acid 401) and adding sequences for the biotin carboxyl carrier protein (BCCP)(Cronan, 1990) and hexa-histidine tag. The recombinant protein was expressed in *E. coli* BL21(DE3) cells and purified by Ni-NTA affinity chromatography using an Amersham Biosciences FPLC system following established procedures.(Hancock and Howard, 1998) Kinesin concentration was quantified by gel densitometry using a UVP BioChemi System (UVP, Inc., Upland, CA) and SyproRed protein stain (Molecular Probes, Invitrogen) using bovine serum albumin as a standard. Tubulin was purified from calf brains and labeled with Alexa Fluor® 647 carboxylic acid succinimidyl ester (Molecular Probes, Invitrogen) according to standard techniques.(Hyman et al., 1991; Williams and Lee, 1982) Microtubules were
polymerized for 20 min at 37 °C in the presence of GTP (1 mM), MgCl₂ (4 mM) and DMSO (5 %), and stabilized by diluting into a solution of paclitaxel (10 µM) in BRB80 buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8).

Quantum dot synthesis: CdSe/ZnS core/shell quantum dots were synthesized by published methods (Qu et al., 2001) and functionalized with biotin. The recently reported Successive Ion Layer Adsorption and Reaction (SILAR) technique was used to impart a 3 monolayer protective ZnS shell. (Li et al., 2003; Xie et al., 2004) CdSe/ZnS particles were transferred to an aqueous phase and biotinylated by introducing polyethylene oxide phospholipid (DSPE-PEG(2000), Avanti Polar Lipids, Inc.) which preferentially forms a protective micelle structure. (Dubertret et al., 2002) We used a mixed micelle solution of 1% biotin terminated phospholipid (DSPE-PEG(2000)Biotin), the rest being methoxy terminated phospholipid. See Supplementary Information for further details on quantum dot synthesis and functionalization.

Quantum dot-kinesin (Q-kin) conjugation: The biotinylated kinesin (7.2 nM) was first incubated with neutravidin (180 nM) (Pierce Biotechnology, Inc.) for 5 min in BRB12 buffer (12 mM PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.8) containing MgATP (1 mM). We chose BRB12 buffer for this study because at lower ionic strengths the motor-microtubule on-rate is higher, leading to more observable events. Subsequently, biotinylated quantum dots (36 nM) were added and incubated for 20 min. Finally, a ten-fold excess of free biotin was added to block any free biotin-binding sites on neutravidin,
and the complex centrifuged in a Beckman Airfuge for 10 min at 30 psi (~95000 rpm or 178000 x g) to remove any aggregates.

Q-kin motility assay: Glass coverslips (Corning® Cover Glass, 18 mm Squares, No. 1½) were treated with 2 M KOH for 30 min, rinsed in distilled water, then incubated in a 0.1% poly-L-lysine solution for 30 min, rinsed extensively in water, and dried. In contrast to aminosilane treated surfaces, which adsorbed quantum dots to the point where measurement of moving particles was nearly impossible, non-specific binding to the casein-coated poly-L-lysine treated surface was minimal (limited to 5-10 per 760 µm² screen). Flow cells were constructed from a coverslip, a microscope slide, and two-sided tape. Microtubules (0.06 µM tubulin dimer) were introduced and incubated for 5 min, and the surface was then blocked with a solution of casein (5 mg mL⁻¹) and paclitaxel (10 µM) in BRB12 buffer. Finally, a solution of Q-kin conjugates in motility buffer (10 µM paclitaxel, 0.2 mg mL⁻¹ casein, 1 mM ATP, 20 mM glucose, 0.02 mg mL⁻¹ glucose oxidase, 0.008 mg mL⁻¹ catalase, 0.5% β-mercaptaethanol in BRB12) was introduced into the flow cell (Figure 2.1).

Microtubule gliding assay: The microtubule gliding assay was performed according published procedures,(Hancock and Howard, 1998) except that instead of adsorption of full-length kinesin directly to the glass, 1 mg mL⁻¹ neutravidin was adsorbed to the glass and biotinylated motors were bound to the neutravidin. The motility of Alexa Fluor 647-labeled microtubules in BRB12 motility buffer was observed.
Bead Assay: Neutravidin coated beads were prepared by incubating 0.2 µm diameter silica beads (1 nM) (Bangs Laboratories, Inc.) with neutravidin (0.4 mg mL\(^{-1}\)) for 15 minutes in BRB80 while sonicating. Subsequently, rhodamine labeled casein (0.4 mg mL\(^{-1}\)) and regular casein (4 mg mL\(^{-1}\)) were added to the bead solution and the mixture was sonicated for one hour, and the beads were centrifuged for 5 min at 18,400 x g to remove excess neutravidin and rhodamine-casein from the solution. Beads were functionalized with biotinylated kinesin by incubating motors (1 nM or 2 nM) with beads (100 pM) for 10 min in BRB12 with casein (5 mg mL\(^{-1}\)) and ATP (1 mM). Alexa Fluor 647 labeled microtubules were immobilized on the poly-L-lysine surface and the surface blocked similar to Q-kin motility assay. For motility measurements the kinesin-coated beads were diluted to 50 pM in BRB12 motility buffer, introduced into the flow cell, and visualized by fluorescence microscopy.

Microscopic visualization: Fluorescent microtubules and quantum dots were observed both under epifluorescence and TIRF using a Nikon TE2000 inverted microscope (60X 1.45 NA CFI Plan Apo TIRF oil objective), and imaged using a Photometrics Cascade 512B CCD camera (Roper Scientific). The camera was controlled and images acquired using Meta-Vue software (Universal Imaging, PA) run on a PC. Stacks of images were captured with 50 msec or 100 msec acquisition times and analyzed for motor velocities and run lengths. Silica beads were visualized by epifluorescence on a Nikon E600 upright microscope (100X 1.3 NA objective) with a Genwac GW-902H CCD camera, recorded to VHS and analyzed off-line.
Curve fitting: Exponential run length data were fit to the cumulative probability function

\[ P(x) = 1 - \exp\left(-\frac{(x-x_0)}{\lambda}\right) \]

where \( P(x) \) is the probability of the motor detaching at or before distance \( x \), \( x_0 \) is the minimum distance measured (0.5 \( \mu \)m), and \( \lambda \) is the mean run length in \( \mu \)m. (Thorn et al., 2000) This method was chosen because it removes variability due to bin width choice. When 1 \( \mu \)m bin widths were used and the Q-kin data fit to a single exponential with no weighting, the run lengths were within 5\% of the cumulative fit. Data fitting was performed using SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA).

2.3 Results

To maximize single motor labeling and minimize aggregation, quantum dot-kinesin (Q-kin) complexes (Figure 2.1) were assembled using a 0.2:5:1 ratio of biotinylated kinesin/neutravidin/quantum dots. Kinesin motility was observed using Q-kin motility assay (see Experimental section) and in parallel experiments, motors were investigated using the microtubule gliding and bead assays. (Hancock and Howard, 1998; Hancock and Howard, 1999)
The motors were visualized by both TIRF and epifluorescence microscopy with similar results (Figure 2.2). The evanescent excitation in TIRF reduced the background fluorescence, but the tradeoff was that only binding events to microtubules that were tightly adhered to the surface, and thus within the thin evanescent wave, were visualized. However, we find that a large fraction of the motor-microtubule interactions occur on weakly bound microtubules both in this study and when motors are adsorbed to 200-nm silica beads. Epifluorescent illumination also enabled visualization of the moving
quantum dots. Because this mode is more widely used and easier to implement than TIRF, quantum dot functionalization of kinesins should lower the bar for performing single molecule kinesin observations.

Figure 2.2: Microscopic assay showing a quantum dot-kinesin complex moving along an immobilized fluorescently-labeled microtubule. Images at left are in epifluorescence mode (Elapsed time = 13 s) and images at right are in TIRF mode (Elapsed time = 20 s).
In standard kinesin assays using fluorescent microtubules, an antifade cocktail consisting of an oxygen scavenging system (20 mM glucose, 0.02 mg mL\(^{-1}\) glucose oxidase, 0.008 mg mL\(^{-1}\) catalase) and reducing agent (75 mM \(\beta\)-mercaptoethanol) is required to prevent free radical induced fluorophore bleaching and protein degradation. In a recent report, Hohng and Ha found that adding 140 mM \(\beta\)-mercaptoethanol to quantum dots in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) virtually eliminated blinking, which led to a higher time-averaged fluorescence.(Hohng and Ha, 2004) In contrast, even in our best preparations reducing agents did not eliminate blinking, and a portion of the quantum dots were quenched when added to the motility solution. To quantify this effect, we immobilized quantum dots on a glass coverslip, passed buffers with and without \(\beta\)-mercaptoethanol over them, and counted the number of emissive particles. Addition of \(\beta\)-mercaptoethanol caused a reduction in the number of observable dots to 19.7 ± 6.9 % (mean ± SD, N = 5) in BRB12 buffer and to similar levels in BRB80 and TE buffer. Blinking was observed in all cases, and the remaining dots were apparently unaffected by the reducing agent. Hence, in our hands \(\beta\)-mercaptoethanol quenches a subset of the quantum dots, presumably due to defects in the protective shell, but does not suppress blinking. Thiols are known to cause surface traps on CdSe particles, where the thiol binding site becomes a trap site, and promotes the formation of a disulfide that then dissociates and leaves a non-luminescent particle.(Aldana et al., 2001) The penetration of a small thiol molecule (such as \(\beta\)-mercaptoethanol) into a shell defect site would presumably be sufficient to cause the total quenching of a quantum dot with available shell defects. Although this phenomenon appears not to affect the
remaining quantum dots, this result has important implications for future studies that combine reducing agents and quantum dots.

To analyze motor function, the first metric measured was the motor run length – the distance motors moved along microtubules before detaching and diffusing away. This was done by monitoring all dots that bound to the microtubule surface and measuring the axial displacement of those that moved a minimum of 0.5 µm. At the ratio of 0.2 kinesin/quantum dot, the run length was exponentially distributed with an average of $2.11 \pm 0.18$ µm (exponential fit ± SE, N = 81) (Figure 2.3A), and at 2 kinesin/quantum dot, the run length was $2.46 \pm 0.15$ µm (exponential fit ± SE, N = 101). These numbers are slightly higher than some reported measurements of individual kinesin motors attached to sub-micron silica beads (Block et al., 1990; Hancock and Howard, 1999) or fused to GFP, (Romberg et al., 1998; Thorn et al., 2000) but match the $2.3 \pm 0.7$ µm run length reported for a very similar biotinylated Drosophila kinesin construct bound to silica beads. (Vugmeyster et al., 1998) When our biotinylated motors were attached to neutravidin-coated 0.2 µm diameter silica beads at 20 kinesin/bead, the measured run lengths were $2.92 \pm 0.16$ µm (exponential fit ± SE, N = 96) (see Supplementary Information). These results suggest that the movement of the Qkin complexes is driven by individual kinesin motors and that a single biochemical transition determines the detachment of the motor from the microtubule.

The second motor parameter analyzed was the transport velocity, an indicator of the stepping frequency and ATP hydrolysis rate of the motor. In standard microtubule
gliding assays in BRB12 buffer, these biotinylated kinesin motors moved microtubules at speeds of $0.17 \pm 0.03 \mu m/s$ (mean ± SD, N = 50). This speed is slower than the $0.56 \pm 0.04 \mu m/s$ (mean ± SD, N = 50) measured in BRB80 buffer, but the lower ionic strength is chosen to maximize motor-microtubule interactions in the quantum dot assays. When standard bead assays were performed, by attaching biotinylated kinesins to neutravidin-labeled 0.2 μm silica beads, the mean bead velocity was $0.33 \pm 0.08 \mu m/s$ (mean ± SD, N = 50). Kinesin-functionalized quantum dots at 0.2 kinesin/quantum dot moved at $0.28 \pm 0.12 \mu m/s$ (mean ± SD, N = 101) (Figure 2.3B), and dots with 2 kinesin/quantum dot moved at $0.26 \pm 0.11 \mu m/s$ (mean ± SD, N = 101). Hence, attaching quantum dots to these biotinylated kinesins does not compromise motor function.
Lastly, to demonstrate the potential for long term imaging, quantum dot – kinesin complexes were bound to immobilized microtubules (in the presence of the non-hydrolyzable nucleotide analog AMP-PNP) on to glass surfaces, illuminated continuously...
by evanescent excitation at 488 nm, and monitored over time. The mean time that particles could be continuously visualized was >1200 seconds, which we regard as a lower limit due to motor unbinding and eventual microtubule depolymerization. This duration is nearly two orders of magnitude longer than the ~17 sec or ~12 sec that Cy3 (Lakamper et al., 2003; Pierce et al., 1997) or GFP – labeled kinesin (Thorn et al., 2000) can be imaged before photobleaching. Quantum dot labeling should therefore eliminate the need for correcting estimates for motor run lengths due to photobleaching events (Thorn et al., 2000).

2.4 Discussion

Hence, by conjugating quantum dots to motors through biotin-avidin, these semiconductor nanocrystals can be used as bright and robust fluorophores to track conventional kinesin movement along cytoskeletal filaments. With further optimization of quantum dot conjugate preparation techniques, these labels can potentially be used to image other members of the 13 families of kinesins as well as other molecular motors. Additionally, because of their small size and bright fluorescence, the quantum dots can be used for in vitro investigations of biomotor-driven nanoparticle transport and organization along the microtubule lattice. Such motor-driven organization should be much more deterministic than simple diffusion, surface force-mediated aggregation, or other self-assembly approaches. Finally, because these particles can be synthesized with a range of emission properties and surface chemistries, they can be used to investigate bidirectional transport by motors and co-assembly of heterogeneous particles.
2.5 Supplementary Information

2.5.1 Q-kin assay at ratio of 2 kinesin/quantum dot:

In an effort to confirm that we were indeed measuring single-molecule events, the motility of kinesin-functionalized quantum dots was measured across a range of kinesin/quantum dot ratios. Figure 2.4 shows the run lengths and velocities for Q-kin complexes labeled at 2 kinesin/quantum dot. Because of uncertainties in both the active motor concentration and the fraction of biotinylated motors that bind to the neutravidin, this mean of 2 kinesin/quantum dot is an upper limit. The mean run length for these Q-kin complexes was 2.46 ± 0.15 (mean ± SE of exponential fit, N=101), and the mean velocity was 0.26 ± 0.11 µm/s (mean ± SD). These data agree well with the corresponding values at 0.2 kinesin/quantum dot of 2.11 ± 0.18 µm and 0.28 ± 0.12 µm/s, respectively.
2.5.2 Bead assay run length and velocity data:

To establish whether attachment to quantum dots had an effect on kinesin function, we measured the motility of biotinylated kinesin attached to 0.2 μm silica beads.

Figure 2.4: (a) Run lengths of Q-kin complexes at 2 kinesin/quantum dot. Data from 101 events were fit to a single exponential curve as described in Experimental Section, neglecting values below 0.5 μm. The mean run length was 2.46 μm. (b) Velocity distribution of Q-kin complexes at 2 kinesin/quantum dot, including all observed events. The mean velocity was 0.26 μm/s.
under identical experimental conditions. The kinesin motors used in this study are truncated at residue 401 and biotinylated, making them shorter than most motors studied in the bead assay.\textsuperscript{[5, 6]} We chose ratios of 10 and 20 kinesin/ bead for our assays because at lower ratios, events were exceedingly rare. Based on measurements by Rosenfeld et al using a similar biotinylated construct (in which all Cys residues were replaced in the motor domain) and 0.5 μm diameter beads,\textsuperscript{[7]} single motor events are expected at ratios below 200-400 kinesin/ bead. Data for 10 and 20 kinesin/ bead were pooled, and the run lengths and velocities are shown in Figure 2.5. The run length was 2.92 ± 0.16 μm (exponential fit ± SE, N = 94), which is somewhat higher than the corresponding value for Q-kin, and it cannot be ruled out that some of the bead movement may result from multi-motor events. The measured velocity of the beads was 0.33 ± 0.08 μm/s (mean ± SD, N = 50).
Figure 2.5: (a) Run lengths of kinesin-bead conjugates. Run lengths of 96 events were measured and the histogram was fit to an exponential curve, giving a mean run length of 2.92 µm. (b) Kinesin-bead velocity distribution. Velocity of a subset of events (N= 50) were calculated, which gave an average of 0.33 µm/s.
2.6 References


Chapter 3

Construction and Calibration of Optical Tweezers with Back Focal Plane Interferometry Detection to Observe Kinesin Processivity

3.1 Introduction

In the previous chapter, fluorophore based single molecule assay using TIRFM was discussed. Fluorescence tagging of motors has an advantage: tags can be added to specific locations and techniques like TIRFM and FRET can be used to study the binding and stepping characteristics of motors. However, motors perform mechanical work which cannot be understood from examining freely diffusing molecules. Instead, motors passively absorbed on beads of dielectric material can be used in conventional bead assays developed by Vale group (Vale et al., 1985) and simultaneously manipulated with optical tweezers as shown by Block et al. (Block et al., 1990). An optical trap added to a conventional microscope provides a tool to hold and apply forces on the bead which translates to the motor while the laser being used provides mechanism for nanometer precision detection.

3.1.1 Optical Tweezers

In 1970, Arthur Ashkin from Bell labs observed and reported optical gradient and scattering forces on micrometer beads for the first time (Ashkin, 1970). It
was only later in 1986 were they able to trap beads in a focused optical trap. In the late
80s, biophysicts like Steve Block pioneered use of the traps in biology to study molecular
motors (Block et al., 1989; Block et al., 1990).

The principal behind optical tweezers is the use of radiation pressure of
highly focused laser beam to manipulate micrometer sized particles (Ashkin, 1980). For
objects with sizes comparable to wavelength of trap light (Mie regime), the physics is
explained using ray optics (Ashkin, 1998; Ashkin et al., 1986; Block, 1992). In an
optical trap, a focused beam has a large gradient of light intensity from the center to the
edge of beam as shown in Figure 3.1. When light passes through a dielectric particle, it
refracts, causing change in direction of momentum of light. Hence, according to
Newton’s third law, the bead exerts an equal and opposite force and moves in the
opposite direction. For explanation purposes, in a particular z-plane, the direction of light
after refraction can be split into their orthogonal components which are scattering force in
axial direction and lateral gradient force. The differential intensity in the lateral direction
created by a highly focused beam dominates the force on the bead, bringing the bead to
the center of the trap. There is a gradient in light intensity in z-direction too with the
maximum at the focus. Therefore, if the bead is within a certain distance behind plane of
focus, again due to refraction of angular rays (from the gradient) upon interacting with
bead, there is net force back into the focus of the trap. Therefore, when the particle is
within the beam gradient, the lowest potential energy is at the center of the trap. The trap
force increases linearly up to a certain distance mimicking a Hookean spring. When the
object is much smaller than the wavelength of light, it can be treated as a dipole, and the
same trapping effect can be explained using electromagnetic theory (Ashkin et al., 1986).

Details of building an optical tweezers system is given in the Methods section.

Figure 3.1:  a) Optical trap explained using ray optics (Ashkin, 1997). b) Comparing bead in trap to Hookean spring.

3.1.2 Applications in biophysics

Optical traps from single stationary beams to multiple piezo-controlled traps are used presently for manipulating and studying mechanical properties of molecules and
cells. At the cellular level, uses of optical traps include cell sorting, developing non-contact in-vitro fertilization techniques, and analyzing mechanical properties of cells such as viscoelastic changes due to force (Schutze et al., 1994; Svoboda et al., 1992; Takahashi et al., 2003). At the molecular level, optical traps have been used to investigate DNA unwinding, DNA and RNA polymerase activity, flagellar rotation, myosin motility, microtubule dynamic instability, and kinesin function (Block et al., 1989; Block et al., 1991; Block et al., 1990; Shepherd et al., 1990; Wang et al., 1997).

For molecular motors, optical tweezers are used as a tool to hold particles and apply mechanical loads to them. The simple mechanism of picking dielectric particles in solution helps save valuable experimental time with motors because it eliminates the need to wait for a bead to diffuse to the microtubule and move if it has a motor in the right orientation to interact. Instead, a bead can be picked and held along a microtubule giving more interaction time. Since motors are force generating mechanoenzymes, applying loads provides a means of characterizing their stall forces, force-velocity and force-run length relationships. Finally, laser interaction with particles also provides a means of detecting their position. Changes in interference pattern of laser light when interacting with the bead can be imaged on a quadrant photodiode (QPD) which, when hooked up to a current to voltage converter, provides a novel detection mechanism for resolving bead position with nanometer scale resolution. In this work, we place the quadrant photodiode at the conjugate plane of the back focal plane of the condenser. Hence, the technique is called back focal plane interferometry.
The sophistication of optical trap and detection system has led to detailed research of the mechanics of Kinesin-1. Step size of 8 nm for Kinesin-1 was observed by Svoboda et. al. (Svoboda et al., 1993). The maximum force the motor can generate can be deduced from the stall force, i.e., the trap force sufficient to stop the motor’s movement. For Kinesin-1, stall force was shown to be around 6 - 7 pN (Svoboda and Block, 1994; Visscher et al., 1999). Similarly, for a two-headed Eg5, a Kinesin-5 motor, the maximum observed force (due to short run lengths) was 5 pN, though the estimated stall force from force velocity relationship was again 7 pN (Valentine et al., 2006). This work also showed that the resisting forces did not affect the velocity of Eg5 as much as they did Kinesin-1. Force dependent transition in the motor chemomechanical cycle is an important feature of motors that can be obtained from force-velocity curve and/or by changing nucleotide conditions to probe different motor states (Schnitzer et al., 2000; Visscher et al., 1999). For example, to model the Kinesin-1 force-velocity relationship, it was found that at least two 4 nm force dependent steps are required (Schnitzer et al., 2000).

From the force dependent behavior of different motor families, we know that the motor (head/neck linker/coiled-coil) structure affects the load dependence of motility. Therefore performing these experiments on Kinesin-2 motors is important since the similarity between Kinesin-1 and Kinesin-2 is only 44%. The parameters that can be compared are step size (are there substeps), stall force, force-velocity relationship and the number of force dependent steps. Similarly, stepping and force dependent behavior of
the individual heads KIF3A and KIF3B will give insight into coordination between heads during processive movement.

3.2 Methods

3.2.1 Optical Trap and imaging

The optical trap was built around an existing Nikon TE2000 Inverted Microscope. A 1064 nm, Nd-YAG 10W laser (Spectra-Physics) light was passed through a beam expander to fill the back aperture of the objective to obtain a highly focused trap. The beam expander consists of a plano-concave lens (ThorLabs LC1035, BK7, d = 6 mm, f = -18 mm) to expand the light and plano-convex lens (ThorLabs LA1461-C, BK7, AR Coating 1050-1580nm, d = 25.4 mm, f = 250 mm) to collimate the beam. It must be noted that the internal lenses of the microscope are not perfectly aligned, i.e., a collimated beam entering the microscope did not give a collimated output beam. Therefore, the distance between the lenses of the beam expander lenses was empirically established to ensure a collimated beam at the objective.

The laser power needed for trapping is between 10 and 100 mW (higher power is not required for experimental purposes and might damage the lenses). Therefore, attenuation of the 10 W laser was achieved in two ways. First, the laser diode current was adjusted using the controller settings to run at powers as low as 100 mW. However,
the efficiency of the diode laser pump is reduced at low power settings, resulting in a distorted beam profile. Hence, the laser power was set to approximately 2 W to improve the beam profile and a power attenuator was added.

The power attenuator consists of a half wave plate (Newport 05RP12-34, $\lambda = 1064$ nm, 12.7 mm diameter) and laser line polarizing cube beam splitter (Newport 05BC16PC.9, $\lambda = 1064$nm, 12.7mm cube). A half wave plate retards the polarization of light by one half wavelength (hence $\lambda/2$) and hence rotates the direction of polarization of light. Therefore, depending on the angle between the linearly polarized laser and the half wave plate crystal’s axis, the degree of rotation of polarized light can vary. For the power attenuator, the polarizing cube beam splitter was placed such that the axis of polarization is 90° with respect to that of incoming laser, thus not allowing light to pass through. When the half wave plate was placed in between the incoming laser and polarizing cube beam splitter, the laser polarization was rotated such that part of the beam could pass through the cube beam splitter (the rest of the beam gets reflected in the orthogonal direction in the beam splitter). Therefore, the intensity of light is also reduced. For safety, a beam dump (ThorLabs BT500 70W/cm² CW) was placed at right angles to the beam splitter to absorb the reflected beam.

From the power attenuator, the beam passes through a dichroic mirror (passes IR and reflects visible light) at the back of the microscope to the Nikon objective (Oil immersion 100X 1.3 NA). The TRITC filter cube in the path has an excitation lens modified such that both green (528-553 nm) and IR (1064 nm) wavelength light are
allowed to pass. The existing dichroic mirror reflected both green and IR. The barrier filter in the TRITC cube does not block IR wavelength sufficiently, hence an IR barrier filter was placed in the lower turret (where filters for TIRF are placed). Since green light can be reflected by the dichroic, the rhodamine-labeled microtubules were observed using epifluorescence and polystyrene beads using DIC. Both images were captured on a Roper Scientific Cascade 512B CCD camera, and saved and analyzed using MetaVue™ Imaging System (Molecular Devices).

Figure 3.2: Optical trap and detection instrumentation. HWP = half-wave plate, PBS = polarized beam splitter, ND = neutral density filter, QPD = quadrant photodiode, I-V converter = current to voltage IC circuit
3.2.2 Back Focal Plane Interferometry

The same IR laser used to build the optical tweezers along with the DIC system are used for detection of bead movement from center of trap. The principal of back focal plane interferometry is imaging the intensity pattern at the back focal plane of the condenser on the quadrant photodiode. The pattern created is due to change in angular intensity distribution of IR light when it interacts with the dielectric particle (Allersma et al., 1998; Gittes and Schmidt, 1998). While the overall intensity will not vary, the pattern created due to change in bead position changes. This pattern is imaged on the QPD which is placed in the conjugate plane as described below.

The set up is as follows. The light, after interacting with the bead, passes through the condenser. The condenser does not perfectly collimate the light from the specimen plane. A 45° dichroic mirror placed in a holder between condenser aperture and polarizer reflects the light 90°. This beam then passes through a convex lens (Newport KPX091, dia = 25.4 mm, f = 88.3 mm) and is imaged on quadrant photodiode (Spot 9 DMI UDT Instruments, San Diego). A neutral density filter of 1.0 (allows only 10% of light to pass) is placed between the holder and the QPD. The quadrant photodiode is connected to a current to voltage converter (IC circuit) built by Husam Katnani (former undergraduate, Hancock Lab). This IC also adds, subtracts and divides the light from quadrants such to give three outputs indicating position change in X, change in Y and overall voltage on four quadrants (A+B+C+D). The QPD is connected
in reverse bias mode. The circuit design is shown in Figure 3.3. The resistance and capacitance values were chosen based on maximum voltage of 10 V output.

The circuit is connected to the computer data acquisition card (DAQ) through National Instruments connector block (SCB-68 Shielded I/O Connector Block). Hence, the voltage changes from the IC due to bead movement are observed on an oscilloscope coded in LabVIEW 8.0 by Chris Lengerich (undergraduate student, Hancock Lab) (Figure 3.4). The next step is to calibrate the voltage change to the actual distance. Since the distances we are attempting to observe are in nanometer range, a stage that moves accurately at that resolution was required.

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Figure 3.3: Quadrant Photodiode circuit (image adapted from UDT catalogue Spot Speries).
3.2.3 Nanostage

A NanoView-M piezo-controlled, nano-precision stage was purchased from Mad City Labs, Madison, WI. LabView programs to control stage movement were written by Husam Katnani using the library functions provided with the stage. The first program was designed to move the stage to the position the user inputs in X, Y and Z. The second program was used to calibrate voltage observed on the oscilloscope to the distance moved by the stage. The processive movement program allowed user to define the number of steps, step size and time between steps in any one axis. The third program (Figure 3.5) was designed to move the stage with a known function (sinusoidal, triangle,
square or saw-tooth) with user input frequency and amplitude. This program records both stage position and oscilloscope output. To avoid overloading the program, the actual oscilloscope movement cannot be observed in real time. The main problem with the third program was the output for stage position was a graph showing stage movement with respect to sample number instead of time. Also, when the sampling rate and frequency were each set above 100, the actual movement duration did not follow the input command. Therefore, before performing an actual experiment, a slide with beads bound on the surface was oscillated with different input signals and the video was recorded on MetaVue. The actual velocity of the stage for given input signal was obtained from tracking these beads using the MetaVue tracking application.

Calibration of the QPD signal with respect to stage position and the stiffness of optical trap will be discussed in the Results section.

Figure 3.5: LabView program that allows input of different functions to the NanoView-M stage. The program also allows oscilloscope recording.
3.2.4 Dilution Profile

As a first step to performing single molecule bead assays, we have to develop a motor dilution profile to identify the single molecule regime where only one motor interacts with any given microtubule. Since there may be motors that are inactive in solution or are inactivated by binding to the beads, it is necessary to determine the number of functional motors per bead. The probability of motors binding to bead follows a Poisson distribution. A Poisson distribution describes events where there are many discrete states, i.e., 0,1,2,3,…n motors per bead and small number of favorable outcomes, i.e., beads that actually have 1,2,3…n motors is low (Bortkewitsch, 1898). The Poisson distribution of probability that a bead carries \( n \) functional motors is given by

\[
P(n) = \frac{e^{-\lambda} \cdot \lambda^n}{n!}
\]

where \( \lambda \) = average number of functional motors per bead.

Accordingly, the equation that determines the probability of one or more motors bound to a bead is

\[
P(n \geq 1) = 1 - P(0) = 1 - \exp(-x/k)
\]

where \( x \) = actual number of motors bound to a bead

\[
k = \text{functional parameter} = \frac{\text{actual no. of motors/bead}}{\text{functional no. of motors/bead}}
\]

\[
x/k = \lambda = \text{functional motors per bead}
\]

This probability (\( P(n \geq 1) \)) can be obtained by performing bead assay (as explained below) at different motor-to-bead dilutions and determining the number of
beads that bind and move on microtubules compared to the total number of beads brought in contact with the microtubules (Block et al., 1990).

If the average functional motor/bead ratio is 1 (i.e. \( \lambda = 1 \)), then the probability that two or more motors are bound on a bead is:

\[
P(n \geq 2) = 1 - P(n = 0) - P(n = 1) = 1 - (1 + \lambda) \cdot e^{-\lambda} = 0.264 \text{ or } 26.4\%
\]

Hence, as roughly a quarter of the beads have 2 or more motors, it is no longer a reliable single motor assay. If we want at least 98% of the beads to have one motor or less, then for \( P(n \geq 2) \) to be less than 2%, \( \lambda \) has to be 0.22. This means \( P(n \geq 1) \) should be 0.2, i.e., the motor to bead ratio should be such that 20% of beads in solution move. This also means that of the beads that move, 88.3% have only 1 motor or only 12.7% have 2 or more motors. The concentration of motors required to be added will take into account this \( \lambda \) and the k factor.

Another method of tackling the issue of ensuring single motor assay is to consider the geometry of the bead (Figure 3.6) by determining the number of motors that can be present on the bead such that when one interacts with a microtubule, the probability of other motors interacting is near zero. For a 560 nm diameter bead, assuming a motor length of 50 nm from its tail to its head, then the probability a second motor will bind within the possible surface area of interaction (for example, the hatched area) is only 5%. Hence, if only 2 motors are present, it is unlikely both will interact. Therefore, going back to the calculation of number of motors present on beads that move (for \( \lambda = 0.22 \)),
we can confidently say that 97.5% of the observations (moving beads) are due to single motor interaction.

3.2.5 Bead assay

560 nm diameter, carboxylated –polystyrene beads (146 pM) (Bangs Laboratories, Inc.) were passivated with 4 mg mL\(^{-1}\) casein in BRB80 (80 mM PIPES, 1 mM MgCl\(_2\), 1mM EGTA, pH 6.8) and sonicated for 1 hr to prevent aggregation in this high ionic strength buffer. Subsequently, the motors were incubated with the 14.6 pM casein coated beads at 4°C for 30 min. Rhodamine tubulin was purified from bovine-brain and labeled following established techniques(Hyman et al., 1991; Williams and Lee, 1982). Tubulin (40 µM) was polymerized in presence of GTP (1 mM), MgCl\(_2\) (4 mM) and DMSO (5%) at 37°C for 20 mins. This solution was then diluted 100 fold and stabilized in BRB80 solution containing 10 µM paclitaxel. Flow cells were prepared using clean glass coverslips (Corning, no. 11/2, 18 mm squares). Microtubules were
flowed in and allowed to bind to the glass coverslip. The surface was blocked with 2 mg mL⁻¹ bovine serum albumin to prevent non-specific absorption of motors. Finally, the motor-functionalized beads were diluted ten fold in motility solution containing 10 µM paclitaxel, 0.2 mg mL⁻¹ casein, 1 mM ATP and an antifade system consisting of 20 mM glucose, 0.02 mg mL⁻¹ glucose oxidase, 0.008 mg mL⁻¹ catalase, 0.5% β-mercaptoethanol in BRB80. This solution was introduced in to the flow cell and the beads and microtubules were observed simultaneously with DIC and epifluorescence on a Nikon TE2000 inverted microscope (100X, 1.3 NA objective) and imaged using a Photometrics Cascade 512B CCD camera (Roper Scientific). The images and movies were recorded using MetaVue™ software (Universal Imaging, PA).

3.3 Results

3.3.1 Laser power characterization

The power of Nd-YAG 1064 nm laser is controlled by the changing diode current. Output power, read on the power meter (Spectra Physics 407A), was plotted against the diode current. This gave a linear dependence as shown in Figure 3.7. In our standard configuration, the current is set at 16 mA, giving a steady 1.6 W output. From the power density specifications of the beam expander lenses, 2 W is the maximum allowable power before lens damage occurs.
The function of the power attenuator was assessed by measuring the output power dependence on the angle of the half wave plate. The result (Figure 3.8) showed sinusoidal dependence as expected.

Figure 3.7: Laser power at the source as function of input current.

Figure 3.8: Laser power after the polarized beam splitter as a function the half wave plate angle.
After the power attenuator, the laser beam enters the Nikon microscope and passes through internal lenses and apertures, which further attenuates the power by 63%. The input power as low as 15 mW which gives a trap stiffness of approximately 0.005 pN/nm, is sufficient to trap the beads. Finally, the power of laser entering the microscope was calibrated to the voltage A+B+C+D on the QPD (Figure 3.9). A+B+C+D is the voltage indicator converted from the light intensity on all four quadrants of the QPD. The QPD responded linearly to the power change.

![Figure 3.9: The total voltage out of the QPD as a function of power input into the microscope.](image)

\[
y = 0.0101x - 0.0897 \\
R^2 = 0.998
\]

### 3.3.2 Position calibration

Voltage changes due to IR interaction with the bead were calibrated to a known change in the bead position using the processive steps program. For the calibration,
beads (stock concentration = 1.86 pM) are first diluted 2000 fold in water (in BRB80 or with casein, beads do not bind to surfaces). A bead is trapped in solution around 1 \( \mu \)m above the surface and the QPD position is adjusted using thumb screws to zero X and Y on the oscilloscope (LabView). The bead is then brought to the surface and allowed to bind. If the bead binds off-center, the stage is moved such that X and Y are zeroed again. After binding, the stage is moved away from the center in X (or Y) about 600 nm. Then the stage is moved 70 steps in 25 nm increments allowing 1 s between steps (Figure 3.10a). The change in voltage in each direction is recorded in X and Y signals. If the bead is moved in the X axis, the mean Y signal should remain constant to ensure that calibration is through the center of QPD (linear region). Though the mean is constant, it must be noted that the noise in Y signal will increase as it steps towards the center of the QPD since the intensity of light is maximum at the center (Figure 3.10a). The data was analyzed in Excel by averaging data in each step and assumed that each step is 25 nm exact (Figure 3.10b and c).

The linear range was found to be around ± 100 nm (Figure 3.10b and c), consistent with those previously reported (Allersma et al., 1998). However, the X and Y voltage changes were not equal \( (X = 305.48 \text{ nm/V}, \ Y = 248.82 \text{ nm/V}) \). The first reason was that the light reflecting off the mirror at the back focal plane of the condenser was not perfectly circular. The source of this circular aberration could not be located; hence the aperture between condenser and mirror was closed till a symmetrical hexagon was reflected from the mirror. Nonetheless, the X and Y voltage changes were not equal.
The difference was around 20%. Because the X and Y could be calibrated independently, this was deemed acceptable.
Figure 3.10: Voltage-position calibration. a) Raw oscilloscope data of bead moving in X. b) and c) Distance calibration with voltage in X and Y respectively.
3.3.3 Stiffness calibration

Before performing mechanical studies, the stiffness of the optical trap must be calibrated. There are three ways of determining the stiffness: variance analysis, power spectrum analysis, and viscous drag application (Brouhard et al., 2003; Svoboda and Block, 1994; Svoboda and Block, 1994; Svoboda et al., 1993). The first method exploits thermal diffusion of the bead in the trap, which reduces as laser power and trap stiffness increase. According to the equipartition theorem, thermal energy is directly proportional to the variance of the bead position. This is given by $\frac{1}{2}k_bT = \frac{1}{2}\kappa \text{Var}(x)$, where $k_b$ is Boltzmann’s constant, $T$ is time in Kelvin, $\kappa$ is the trap stiffness and $x$ is the distance of bead movement in one axis. The results of trap stiffness in X and Y as a function of laser power at the back focal plane of objective are shown in Figure 3.11. The trap stiffness as a function of laser power was determined to be $7 \times 10^{-4}$ pN/nm/mW in both X and Y according to the Variance method (Brouhard et al., 2003).
The second method describes bead thermal motion in trap using Langevin equation (Brouhard et al., 2003; Kojima et al., 1997; Svoboda and Block, 1994). This includes the drag coefficient of the bead, $\gamma (3\pi \eta d$ where $\eta = \text{viscosity} \text{ and } d = \text{diameter of bead})$ along with the thermal diffusion. The viscosity is corrected for the temperature increase due to the IR laser from the equation, $\eta (\text{Pa·s}) = 2.414 \times 10^{-5} \times 10^{247.8/(T-140)}$. The temperature of the flow cell is measured using a thermocouple. The inertial component is assumed negligible because this is an overdamped system. The Langevin equation gives

$$F = \gamma \frac{dx}{dt} + \kappa x$$

This equation rewritten as power spectral density gives

$$S_x(f) = \frac{B}{f^2 + f^2} \text{, } B = \frac{k_B T}{\gamma \pi^2}$$

Figure 3.11: Variance analysis of trap stiffness.
\[ f_c = \text{corner frequency of power spectrum} \]

Here, the stiffness \( \kappa = 2\pi f_c \)

From the experiment, the stiffness varies with power as \( 1 \times 10^{-3} \text{ pN/nm/mW} \) in the X direction and \( 7 \times 10^{-4} \text{ pN/nm/mW} \) in the Y direction as shown in Figure 3.12b.

Figure 3.12: Power spectrum analysis of trap stiffness. a) Example of analysis of FFT of variance data with a Lorentzian equation fit. b) Trap stiffness as a function of laser power.
The final method is based on imposed drag forces on the bead. When the stage is moved at a constant velocity, the bead in a trap is subjected to a drag force (Brouhard et al., 2003; Svoboda and Block, 1994) from the moving solution. This resulting bead displacement is opposed by trap restoring forces and hence the bead is still held in the trap but is displaced from the trap center. Therefore, the stiffer the trap, the smaller the displacement for a constant stage velocity. The higher the stage velocity, the larger the displacement (as drag force is higher) for a constant trap stiffness. The equation governing this principal is

\[ F_{\text{Drag}} = F_{\text{Trap}} \]

\[ \gamma^c \cdot v = k^c \cdot \Delta x \]

where \( v = \) velocity of stage and \( \Delta x = \) displacement of bead from center of the trap

\[ c = \text{correction factor for wall effects of the glass slide on the moving bead (Muller et al., 1995)} \]

\[ = \left(1 - \frac{9}{32} \left(\frac{d}{T}\right) + \frac{1}{64} \left(\frac{d}{T}\right)^3 - \frac{45}{4096} \left(\frac{d}{T}\right)^4 - \frac{1}{512} \left(\frac{d}{T}\right)^5 \right)^{-1} \]

Figure 3.13a shows an example of oscilloscope reading of the bead position and corresponding stage velocity. The resulting trap stiffness as a function of increasing power is shown in Figure 3.13b. The trap stiffness is calculated as an average of the values at varying drag forces. The stiffness varies as \( 6 \times 10^{-4} \) pN/nm/mW in X and \( 7 \times 10^{-4} \) pN/nm/mW in Y. This result is similar to that obtained by variance method.
comparison of stiffness values from the different methods and those in literature will be discussed in the Discussion section.

Figure 3.13: Drag force analysis of trap stiffness. a) Example of voltage change (top section) observed from bead movement when stage is oscillated with a triangular wave (bottom section). b) Trap stiffness in X and Y as a function of power at the back of the microscope.
3.3.4 Motor dilution profile

In this work, kinesin motors are adsorbed through their tail domain to casein-coated 0.56 µm diameter-carboxylated-polystyrene beads. Different concentrations of motors are incubated with beads, and the probability of a bead moving is determined by placing about 20 trapped beads on immobilized microtubules on the surface and waiting for 30 s to check for interaction. If they do not move, they are placed on another microtubule to eliminate the possibility that the failure of movement is due to microtubule denaturation. From these experiments, the mathematical probability that a bead moves is the same as the probability that the bead has one or more functional motors. As described in Methods, this measured probability is fit to a probability distribution

\[ P(n \geq 1) = 1 - \exp(-x/k) \]

The parameter k describes ratio of the actual motor/bead ratio to the functional motor/bead ratio. Figure 3.14 shows the probability distribution graph for four motor types, KHC, KIF3A/B, and chimeric homodimers KIF3A/A and KIF3B/B. The parameter k for KHC was 9.2, for KIF3A/B, it was 5.9. This shows that KIF3A/B binding to beads is similar to conventional kinesin.
To ensure single molecule assays, as mentioned in Methods we want 98 - 99% of the beads to have only 1 motor or less. Therefore, to reduce this number to less than 2% \( \lambda \) has to be 0.22. Taking the functional parameter into account, the actual number of KHC required per bead is 1.58, for KIF3A/B it is 0.86, for KIF3A/A it is 1.2, for KIF3B/B the value is 0.75 motors/bead.

Figure 3.14: Probability of a motor-bead complex binding and moving on a microtubule versus the motor to bead ratio is fit to Poisson’s equation of \( P(n \geq 1) = 1 - \exp(-x/k) \) for a) KHC, \( k = 9.2 \), b) KIF3A/B, \( k = 5.9 \), c)KIF3A/A, \( k = 8.3 \), d) KIF3B/B, \( k = 5 \)
3.3.5 Kinesin stepping

To show the robustness of the optical trap, sample preliminary data of a single molecule bead assay of Kinesin-1 at low trap strength were collected (Figure 3.14). Kinesin-1 (KHC) was diluted to single molecule concentrations on beads and a bead assay was performed as described in Methods. Sample of results obtained from kinesin stepping are shown in Figure 3.15. The bead is seen stepping in the X direction, finishing its run (as seen by video), coming back to the center of the trap and repeating the process upon rebinding to the microtubule. This result shows that the detection system for bead tracking is operational.

Figure 3.15: Typical oscilloscope reading of motor stepping from center of trap. The motor comes back to trap center after it completes its run and diffuses with the trap distance.
In order to increase the signal to noise of the bead position, we reduced the motor velocity by increasing the trap strength to roughly 0.01 pN/nm and lowering the ATP concentration down to 10 μM. Thus, the motor spends from 100 ms to 1 s between each step. Since the trap is stationary, the force on the bead increases proportionally with distance from the center of the trap. As shown in Figure 3.16, at a distance of about 60 nm from the trap center, the trap strength is sufficiently high to identify individual steps. The steps, identified from the plateaus in the graph, were drawn by eye. The difference between average position at each plateau as shown in Figure 3.16 corresponded to 8.9 ± 1.0 nm distance. This conforms with the tubulin dimer length of 8.2 nm.
Figure 3.16: Example of motor steps observed from voltage changes in the oscilloscope. The average distance between the plateaus indicated by red lines is $8.9 \pm 1.0$ nm.
3.4 Discussion

In this chapter, the development and effectiveness of optical tweezers and back focal plane interferometry in kinesin biophysics are illustrated. The device principal is simple and the system could be integrated with the existing epifluorescence-cum-DIC-cum-TIRF Nikon system, making it a true multi-modal microscope. The time consuming aspect of the work was the construction and calibration, since they need to be accurate for reliable measurement of both piconewton forces and nanometer distances. In this section, the accuracy of the calibrations and possible improvements to the system will be discussed.

The optical tweezers construction was checked by confirming that the IR laser filled the back focal plane of the objective and the beam was collimated. The attenuation of laser power was carefully calibrated since the force applied on the bead is directly affected by these values.

The calibration of the detection was manifold. First, the sensitivity of the QPD to position changes in X and Y was determined. The difference between the two values was 20%, similar to that reported in literature (Brouhard et al., 2003). Second, the dependence of the trap stiffness on the laser power was determined using three different techniques - variance analysis, power spectral analysis and viscous drag application. In all 3 cases, the Y signal gave stiffness of $7 \times 10^{-4}$ pN/nm/mW comparable to literature values of $4 \times 10^{-4}$ - $7 \times 10^{-4}$ pN/nm/mW (Allersma et al., 1998; Brouhard et al., 2003).
the X direction, the values showed more variation (ranging between $6 \times 10^{-4}$ and $1 \times 10^{-3}$ pN/nm/mW). This could be the contribution of many errors associated with each method. In the variance analysis, the electronic noise, temperature changes and vibrations in system can contribute to extra noise and variance, underdetermining the stiffness. This noise was observed in the power spectrum analysis. In the power spectrum analysis, this noise was removed before fitting with the Lorentzian equation. Unfortunately, the fit did not always match the experimental curve resulting in higher corner frequencies. The above two methods can be improved, if the sources of noise are eliminated or, before analysis, if these frequencies are filtered out from the raw data. In the viscous drag method, the stage velocities were not accurate as mentioned in the method section. Though for the analysis here the problem was circumvent by measuring the velocity of bead stuck on the surface, this is not an ideal technique for regular use. The two sources of inaccuracy in the current program were identified as 1) the stage did not keep up with the increased sampling rate and 2) when the oscilloscope was also run at high frequency along with the stage, the computer processor could not keep up with both activities and slowed down the command signal to the stage. One method to overcome both these issues is by using the in-built data logging facility in the stage controller. The stage controller can store up to 1000 position command values and 1000 position sensor values. These can be accessed using the library functions provided by the stage. Therefore this will help eliminate errors in velocities due to sampling rate and computer processor speed.
Using the calibration values obtained, preliminary bead assay experiments were performed. The oscilloscope signal showed evidence of processive motor movement. These traces were acquired for both Kinesin-1 and Kinesin-2 motors. Step sizes of 8 nm were also observed for these motors when the motors were slowed down using low ATP concentrations and high trap strengths. However, to observe steps at normal conditions of high ATP and low load, an important hurdle to overcome with the current set up is obtaining nanometer scale displacements at high time resolution. The noise in the output signal (~ 75Hz, 220 Hz) is the main contributor to unobservable steps. While the noise can be filtered out for calibration, for kinesin, the stepping signal itself is around the 100 Hz range (motor steps at ~ 10 ms time scale or 100 Hz). Therefore filtering the 75 Hz noise will interfere with the signal.

In conclusion, the optical tweezers and the back focal plane interferometry system are currently fully functional for experiments that do not require high sensitivity. The optical tweezer system alone was used to obtain informative run length and velocity data for KHC and KIF3 motors (described in detail in Chapter 4). In the future, automation of stiffness calibration and reduction in noise will help reduce experimental time and increase the sensitivity and nanoscale precision of data. This will help answer more questions related to kinesin biophysics like are there sub 8-nm steps, what are the force dependent steps and for Kinesin-2 heterodimer, how do the forces in the two different heads coordinate?
3.5 References


Chapter 4

Kinesin-2 Processivity Dependence on ATP reveals Diminished Gating of Bound Head

4.1 Introduction

Kinesins are biological motors with the remarkable capability of coupling chemical energy from ATP hydrolysis into structural changes that generate force and produce directed motion on microtubules. Serving as a model protein, key findings from single molecule kinesin biophysics contributes to research of all mechanoenzymes. Kinesins have 14 families (Lawrence et al., 2004) that vary in structure, function and biophysical properties. Uncovering the mechanistic differences is important for understanding their in vivo function and building the motor chemomechanical pathway (Cross, 2004; Guydosh and Block, 2006; Hancock and Howard, 1998; Hancock and Howard, 1999; Svoboda et al., 1993).

Kinesin-2 family is unique since most of their proteins have two different heavy chains with one light chain, hence are heterotrimeric. This sequence dissimilarity in the heavy chains has been maintained through evolution from chlamydomonas to humans, emphasizing the importance of its presence in nature. In vivo functions of Kinesin-2 include ciliogenesis, intraflagellar transport (IFT), transport of proteins from golgi, melanosome and late endosome transport and in mitosis (Brown et al., 2005; Cole et al., 1998; Haraguchi et al., 2006; Staub et al., 2006; Tuma et al., 1998). Therefore, absence
of Kinesin-2 leads to problems ranging from left-right asymmetry in embryos, polycystic kidney disease to Bardet-Biedl syndrome (Lin et al., 2003; Pan et al., 2006; Takeda et al., 1999). Functionally, this necessitates studying Kinesin-2 \textit{in vitro} using a purified system to relate the biophysics to the \textit{in vivo} behavior. Structurally, the presence of two distinct heavy chains and ability to study them individually will give clues about coordination between the motor heads during processive movement.

In this paper, we use the mouse ortholog of Kinesin-2, KIF3A/B as the model protein (Yamazaki et al., 1995). Current \textit{in vitro} studies involve examining the processivity of KIF3A/B at saturating ATP conditions (Berezuk and Schroer, 2007; Pierce et al., 1999; Zhang and Hancock, 2004). Zhang et. al. researched KIF3A/B and chimeras KIF3A/A and KIF3B/B using low density gliding assay but did not entirely eliminate the effects of multimotor interaction. Also, data regarding processivity of the motors have been contradictory in these reports. Hence, the questions for Kinesin-2 are; first, are KIF3A/B, KIF3A/A and KIF3B/B processive or not? How does the processivity relate to Kinesin-1 and to their \textit{in vivo} function? Second, what insight do we get into the chemomechanical pathway of Kinesin-1 and Kinesin-2 by varying ATP concentrations and recording the run length and velocity changes? Finally, to explain Kinesin-1 and Kinesin-2 results, what modifications to the kinetic pathway model are required? Do stochastic simulations of the motor stepping using this model explain results obtained?

Here, we use KIF3A/B motor constructs developed previously (Zhang, 2005) in single molecule bead assays. Since, the tail is absent in the system studied here, the motor is referred to as a heterodimer.
Because Kinesin-1 motors are the best understood family of kinesins, our approach to understanding the Kinesin-2 hydrolysis cycle is to identify similarities and differences with the Kinesin-1 hydrolysis cycle. Figure 4.1 shows a consensus Kinesin-1 hydrolysis cycle, building on a body of experiments and discussed in previous publications (Hancock and Howard, 2002; Schief and Howard, 2001). Motors in solution have high affinity for ADP, and upon binding to the microtubule release one ADP (State 2) (Hackney, 1988; Hackney, 1994). The motor waits in this state until ATP binds to the front head, which results in docking of the neck linker and a displacement of the tethered head towards the next binding site (State 3) (Rice et al., 1999). While the tethered head searches for the next binding site, the ATP on the bound head is hydrolyzed (State 4). Following hydrolysis (State 4) there are two possibilities. Most of the time (~99% for Kinesin-1), the tethered head will bind to the next binding site and release its ADP (State 1), and then the rear head will detach (State 2) completing an 8 nm step. Alternatively, the bound ADP.Pi head in State 4 will unbind from the microtubule, terminating the processive run. In this kinetic cycle, the overall cycle time is simply the sum of the times for each transition, and the probability of the motor dissociating per step is equal to \( k_{\text{unbind}}/(k_{\text{attach}} + k_{\text{unbind}}) \).

While this Kinesin-1 model is sufficient to explain most aspects of Kinesin-1 mechanochemistry, it is not exclusive. For instance, while Rosenfeld has argued that ATP binding to the leading head in State 1 is prevented by rearward strain (Rosenfeld et al., 2003), other data suggest that ATP binding can precede detachment of the trailing head
(Mori et al., 2007; Yildiz et al., 2004). Here, we use this model framework to interpret single molecule motility results from Kinesin-1 and Kinesin-2 motors and propose that observed processivity differences result from differences in the kinetic cycles between these two kinesin families.

Figure 4.1: Simple model (Model 1) of kinesin chemo-mechanical pathway. Nucleotide abbreviations are T = ATP, D = ADP, DP = ADP.Pi, φ = No nucleotide.
4.2 Methods

4.2.1 Protein Purification:

Full length his-tagged *Drosophila melanogaster* kinesin heavy chain was bacterially expressed in BL21(DE3) cells and purified using standard procedures (Hancock and Howard, 1998). KIFA/B and the chimeras KIF3A/A and KIF3B/B plasmid were constructed and expressed in baculovirus system as previously described (Zhang and Hancock, 2004). All motors were purified on Ni-NTA affinity chromatography column using an Amersham Biosciences FPLC system.

Due to problems with purification and motility of KIF3 motors (Zhang, 2005), the plasmids used for experiments in previous work (Zhang and Hancock, 2004) were re-sequenced and nonsilent mutations were identified. There were five in KIF3A, with three of them on the head domain (Gly_{121} to Glu_{121}, Ala_{210} to Val_{210} and Pro_{296} to Ser_{296}), one in the coiled-coil (Lys_{404} to Arg_{404}) and one in the Myc tag. KIF3B had only two mutations, one in the head (Ala_{34} to Val_{34}) and the other in the coiled-coil (Ala_{465} to Val_{465}). The plasmids were corrected using QuikChange® Multi Site-Directed Mutagenesis (Stratagene, Inc.) (Zhang, 2005). Subsequent to protein purification, motors showed no significant velocity differences between KIF3A/B and the chimeras KIF3A/A and KIF3B/B in the functional microtubule gliding assay (Zhang, 2005).
4.2.2 Bead assay:

560 nm diameter, carboxylated –polystyrene beads (146 pM) (Bangs Laboratories, Inc.) were passivated with 4 mg mL\(^{-1}\) casein in BRB80 (80 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, pH 6.8) and sonicated for 1 hr to prevent aggregation. Subsequently, motors were incubated with the 14.6 pM casein coated beads at 4\(^\circ\)C for 30 min. Rhodamine tubulin was purified from bovine-brain and labeled following established techniques (Hyman et al., 1991; Williams and Lee, 1982). Tubulin (40 µM) was polymerized in presence of GTP (1 mM), MgCl\(_2\) (4 mM) and DMSO (5%) at 37\(^\circ\)C for 20 mins. This solution was then diluted 100-fold and stabilized in BRB80 containing 10 µM paclitaxel. Flow cells were prepared using clean glass coverslips (Corning, no. 11/2, 18 mm squares). Microtubules were flowed in and allowed to bind to the glass coverslip. The surface was then blocked with 2 mg mL\(^{-1}\) bovine serum albumin to prevent non-specific absorption of motors. Finally, the motor-functionalized beads were diluted ten fold in motility solution containing 10 µM paclitaxel, 0.2 mg mL\(^{-1}\) casein, 1 mM ATP and an antifade system consisting of 20 mM glucose, 0.02 mg mL\(^{-1}\) glucose oxidase, 0.008 mg mL\(^{-1}\) catalase, 0.5% β-mercaptoethanol in BRB80. This solution was introduced in the flow cell and the beads and microtubules were observed simultaneously with DIC and epifluorescence on a Nikon TE2000 inverted microscope (100X, 1.3 NA objective) and imaged using a Photometrics Cascade 512B CCD camera (Roper Scientific). Images and movies were recorded using Meta-Vue software (Universal Imaging, PA).
To facilitate motor microtubule interactions, an optical trap was built to grab beads and bring them in contact with microtubules. The trap system includes a 1064 nm Nd-YAG 10W laser (Spectra-Physics) operated at 2 W output power, a beam expander to expand laser 10 times and a half-wave plate and beam-splitter for power attenuation. The laser enters the rear of microscope and passes through the epifluorescence pathway and fills the back aperture of the objective. The microscope was set up to observe beads and rhodamine-labeled microtubules simultaneously in DIC and epifluorescence modes respectively. Back focal plane interferometry detection system was constructed to calibrate trap stiffness. The stiffness was set between 0.002 and 0.008 pN nm\(^{-1}\) (over a linear range of 130 nm) for all experiments. The load conditions at these stiffness are negligible considering the stall force of Kinesin-1 is 6 pN (Svoboda and Block, 1994).

To determine number of motors required to ensure single motor stepping, beads incubated with different motor concentrations were placed on microtubules for 30 s. The fraction of beads that moved was plotted against motor-to-bead ratio and was fit to the Poisson equation of probability of bead having one or more motors, i.e.,

\[
P(n \geq 1) = 1 - P(0) = 1 - \exp(-x/k), \quad \text{where } x \text{ is actual number of motors bound to a bead}
\]

and \(k = \text{functional parameter} = \frac{\text{actual no. of motors/bead}}{\text{functional no. of motors/bead}}\) (Block et al., 1990; Svoboda et al., 1993). This parameter helped control motor concentration to maximize beads to have single motor. For all subsequent experiments, motor concentration was chosen such that \(\leq 20\%\) beads moved, ensuring single motor interactions. Hence, the concentration of the four motors, KHC, KIF3A/B, KIF3A/A and KIF3B/B, added during incubation with beads were 34 pM, 22 pM, 25 pM and 22 pM respectively.
4.2.3 Analysis:

4.2.3.1 Experimental run length and velocity

Run lengths and velocities were obtained by manually tracking the bead position in Meta-Vue. Velocities were calculated by simple averaging. Run lengths were obtained by two methods: 1) calculating mean after subtracting 250 nm due to uncertainty in detecting run lengths below this value, 2) fitting an exponential decay to a histogram using Origin 7.0 (bin size = 0.5 µm for KHC and 0.25 µm for KIF3s). The first bin for run lengths was ignored in all cases because of uncertainty in detecting events below 250 nm. The plot of ATP dependence on velocity was fit to Michaelis-Menten equation, which gave $V_{\text{max}}$ and $K_M$.

4.2.3.2 Estimated KIF3A/B run length

To understand the coordination between heads of the heterodimer, a theoretical calculation of the expected run length of KIF3A/B from experimental run lengths of KIF3A/A and KIF3B/B was performed using probability. The probability of unbinding of a motor is given by inverse of average number of steps a motor takes, i.e., $p_A =$ probability of KIF3A head detaching = $1/(\text{Average Run Length of KIF3A/A} / 8 \text{ nm})$. Similarly, $p_B =$ probability of KIF3B head detaching. Let $\text{ERL}_A$ and $\text{ERL}_B$ be the expected run lengths of the motor when the motor binds first with KIF3A or KIF3B head respectively. The expected run length of KIF3A/B is a simple average of the $\text{ERL}_A$ and $\text{ERL}_B$. 
ERL_A = (probability of KIF3A not unbinding) * (first step taken by KIF3A head + run length of KIF3B) + (probability of KIF3A head unbinding in the final step) = (1 - p_A) * (1 + ERL_B) + p_A * 1

By symmetry, equation for ERL_B can be expressed in terms of p_B and ERL_A. Therefore, values of ERL_A and ERL_B can be solved from the two equations with two variables. Solving and taking a simple average for KIF3A/B gives

\[
ERL_{AB} = \frac{4 - p_A - p_B}{2 \times (p_A + p_B - p_A p_B)}
\]

4.2.3.3 Strain in the neck linker

To estimate the tension, f, in the neck linker when stretched to a distance of x, a worm-like chain model was adopted (Marko and Siggia, 1995). The governing equation is

\[
f = \frac{k_b T}{l_p} \left[ 4 \left(1 - \frac{x}{L}\right)^2 + \frac{x}{L} - \frac{1}{4} \right]
\]

where \( k_b \) = boltzmann constant

T = temperature in Kelvin

\( l_p \) = persistence length of polymer (assumed 0.4 nm (Hyeon and Onuchic, 2007))

L = contour length
4.2.4 Simulation:

We developed a stochastic model of motor stepping on MATLAB (code in the Appendix), incorporating rate constants between different chemo-mechanical states. The kinetic pathway shown in Figure 4.1 was used for Kinesin-1 and an expanded pathway described in results Figure 4.) was used for Kinesin-2. According to Gillespie algorithm, for each state, the time to transition to the next state is given by:

\[ t = \frac{1}{k} \ln \frac{1}{\text{rand#}} \]

where \( k \) is the rate constant and \( \text{rand#} \) is a random number between 0 and 1 (Gillespie, 1977). The simulation begins with motor binding to the microtubule in State 2. The transition time to go to every other state is calculated and for multiple transitions from a given state the shortest time wins. This is repeated for every state until detachment is favored over tethered head attachment in State 4 for Model 1 (Figure 4.1) and States 5 or 7 for Model 2 (Figure 4.). An 8 nm step is associated with the transition from state 4 to state 1 for Model 1 and from state 7 to State 1 for Model 2.

These simulations calculate distributions of dwell times between different steps as well as the total number of steps preceding motor detachment. From these values, the average run length and average velocity are calculated. For a given set of rate constants, 10000 motor runs were simulated at a range of ATP concentrations. To identify the set of rate constants that best match the experimental run length and velocity value, a linear plot was fit to the experimental run length versus velocity plot and this plot was then fit
to the simulated run length versus velocity graph. The curve with lowest r-squared was chosen as the best simulation fit. Because of the large parameter set, the rate constant parameter sets are not unique. Instead, the model is intended to show plausibility of the proposed mechanics while maintaining as many parameters constant between Kinesin-1 and Kinesin-2 as possible.

4.3 Results

The first goal of this work was to relate the in vitro processivity of Kinesin-2 (KIF3A/B) to the motors’ intracellular function and to compare it to Kinesin-1. The next goal was to provide insights to the Kinesin-2 chemo-mechanical pathway and uncover differences with the standard Kinesin-1 model. Finally, we also investigated biophysical differences between the heads of the Kinesin-2 heterodimer using chimera KIF3A/A and KIF3B/B. In contrast to microtubule gliding assay of KIF3A/B (Zhang and Hancock, 2004) and bead assay with native chicken Kinesin-2 (Berezuk and Schroer, 2007), here we not only analyze run lengths and velocities of recombinant mouse ortholog KIF3A/B but also that of individual heads KIF3A and KIF3B and their ATP dependence.

In the dilution profile, where the probability of beads moving is scored against the motor/bead ratio, KHC, KIF3A/B and homodimers KIF3A/A and KIF3B/B all gave good fits (Figure 4.) to the equation \( P(n \geq 1) = 1 - \exp(-x/k) \) (see Methods). This gives direct evidence for the processivity of a motor, i.e., is one motor sufficient for motility? Results confirm that the heterodimer KIF3A/B is processive as previously reported (Berezuk and
Schroer, 2007; Zhang and Hancock, 2004). Here, we also prove that individual heads in the form of their homodimers are processive as opposed to the previous report of KIF3A/A and KIF3B/B not being processive (within their detection limits) (Zhang and Hancock, 2004).

4.3.1 Kinesin-2 is less processive than Kinesin-1

The processivity and velocities of KIF3 motors were characterized at saturating ATP and compared to KHC. The velocity of the heterodimer KIF3A/B, determined from manually tracking beads, was $436 \pm 129$ nm/s (mean $\pm$ SD, $n = 90$). This was approximately half that of Kinesin-1, which was $703 \pm 136$ (n = 90). The KIF3 homodimers, KIF3A/A ($455 \pm 115$ nm/s, $n = 101$) and KIF3B/B ($458 \pm 106$, n = 102) also had similar velocities to the wild type heterodimer (Table 4.1). While KIF3B/B

![Diagram](Figure 4.2: Dilution profiles with fit to Poissons distribution: $P(n \geq 1) = 1 - \exp(-x/k)$ . a) KHC, $k = 9.19$, b) KIF3A/B, $k = 5.88$.)
velocity agreed with gliding assays performed previously, KIF3A/A velocity was about 10 times higher after mutations were corrected (Zhang and Hancock, 2004). Both KIF3A/B and KHC velocities were in the same range as those reported by Berezuk et. al. (Berezuk and Schroer, 2007).

In contrast to the velocities, the run length of wild type Kinesin-2 was approximately four fold lower than Kinesin-1. KIF3A/B had a run length of only 410 ± 35 nm (mean ± SEM, n = 85) compared to 1747 ± 199 nm (mean ± SEM, n = 57) for KHC (Figure 4.). KIF3A/B run length was also 3 fold less than that obtained by Berezuk et. al. using native chick embryo brain kinesin-2 (Berezuk and Schroer, 2007). This difference could stem from the origin of the motors and the method of analysis. We fit an exponential to the run length data, allowing it to estimate the number of runs below 250 nm. In contrast, Berezuk et. al. averaged their values without including an estimate of run lengths below 750 nm which could have skewed their data to higher values (Berezuk and Schroer, 2007). From the run lengths obtained, the probability of motor unbinding per step is 0.018 for Kinesin-2 but only 0.05 for Kinesin-1. This higher rate of motor unbinding for Kinesin-2 is attributed to a higher unbinding rate of bound head or a lower attachment rate of tethered head. The obvious next question was, how do the KIF3 homodimers differ in processivity?
4.3.2 KIF3A/A is less processive than KIF3B/B

Processivity of chimeras KIF3A/A and KIF3B/B were both lower than KHC too, but KIF3A/A (410 ± 35 nm, n = 85) had a lower processivity than KIF3B/B (704 ± 81, n = 83) (Table 4.1). KIF3A/A had about twice the probability of unbinding compared to KIF3B/B. The expected run length of KIF3A/B calculated from $p_A$ and $p_B$ is 64. Therefore, in the heterodimer, KIF3A/B, KIF3A head dominates the processivity of the motor, reducing its affinity to the microtubule. To build the model further and also to have quantitative information on ATP binding to Kinesin-2, bead assays at varying ATP concentrations were performed.

<table>
<thead>
<tr>
<th>Motor Type</th>
<th>KHC</th>
<th>KIF3A/B</th>
<th>KIF3A/A</th>
<th>KIF3B/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Length (nm)</td>
<td>1747 ± 199(57)</td>
<td>449 ± 30 (87)</td>
<td>410 ± 35 (85)</td>
<td>704 ± 81 (83)</td>
</tr>
<tr>
<td>(mean ± SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Velocity (nm/s)</td>
<td>703 ± 136 (58)</td>
<td>436 ± 129 (90)</td>
<td>455 ± 115 (101)</td>
<td>458 ± 106 (102)</td>
</tr>
<tr>
<td>(mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of steps</td>
<td>218</td>
<td>56</td>
<td>51</td>
<td>88</td>
</tr>
<tr>
<td>$P_{unbind}$</td>
<td>0.005</td>
<td>0.018</td>
<td>0.02</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Table 4.1: Run length and velocity data at saturating ATP for Kinesin-1 (KHC), wild type Kinesin-2 (KIF3A/B) and Kinesin-2 homodimers. The probability of motor unbinding is calculated from the mean run length. KIF3A/B expected run length is obtained from $p_A$ and $p_B$ (see Methods).
4.3.3 KIF3A/A run length and velocity are ATP dependent

To further investigate differences between Kinesin-1 and Kinesin-2 hydrolysis cycles we measured the dependence of motor run lengths and velocities on ATP concentration from 1 µM to 1 mM. While the motor velocity is expected to vary with ATP concentration, the influence of ATP on motor run lengths is much less clear. In previous work, Kinesin-1 run lengths were found to have very little dependence on ATP (Yajima et al., 2002). At limiting ATP concentrations, the forward head is thought to be waiting in a nucleotide-free state that has a high microtubule affinity. Hence, although the motor is associated with the microtubule for much longer times at limiting ATP, it is not surprising that the number of steps does not vary with ATP concentration.

Figure 4.3: Exponential fit of run Length of Kinesin 1 (KHC) and Kinesin-2 (KIF3A/B). The first bin (0-0.5 for Kinesin-1 and 0-0.25 for Kinesin-2) were ignored due to uncertainties in detecting events below 250nm.
While the activity of Kinesin-1 at low ATP concentrations remained consistent, no binding events were observed for KIF3A/B beads at these limiting ATP concentrations. To investigate this reduction in binding events, functional gliding assay using the reverse geometry were performed. Microtubule gliding assay involves binding motors on a casein coated glass coverslip surface and allowing microtubules to bind and move on them. In this assay, at moderate motor densities (180 molecules/µm²) and ATP concentration of 10 µM, no microtubules landed on KIF3A/B motors even after 20 minutes but binding on KHC motors were observed under similar conditions. Adding extra 10 µM ADP to the motility solution in KIF3A/B, to compensate for low nucleotide condition, did not help motility. Between the homodimers, while KIF3A/A was functional at low ATP, KIF3B/B behaved similar to KIF3A/B. This indicates some instability of KIF3B in a low nucleotide environment. Therefore, we used only KIF3A/A for our ATP studies.

![Graphs](image)

**Figure 4.4**: Velocity dependence on ATP concentration. For KHC and KIF3A/A, the data with standard deviation is fit to Michalis-Menten equation. Total number of events for KHC is 290 and for KIF3A/A is 467.
First, from velocity changes with respect to ATP concentration, the $K_{M}^{ATP}$ for KIF3A/A was ~19 µM, which was four fold less than ~ 82 µM of KHC (Figure 4.). Surprisingly, the ATP dependence on run length also differed between the motor types. For Kinesin-1, there was a weak increasing dependence of KHC processivity on ATP (Figure 4.5a). Slow detachment rate (range of 0.01) from State 2 (Figure 4.1) may explain this increase in processivity with ATP (Hancock and Howard, 1999). Calculating statistical significance between the highest and lowest run lengths using t-test gave p of 0.07 which indicated no significant dependence of run length to ATP (alpha set to 0.05).

On the other hand, KIF3A/A showed a strong inverse trend; the run length reduced from roughly 1 µm to 400 nm with increase in ATP concentration from 1 µM to 1 mM (Figure 4.5b). The paired t-test showed statistically significant difference when comparing run lengths at 1mM to that at 1 µM (p = 0.02) and 10 µM (p = 0.002). To ensure this dependence is not an artifact, experiments were conducted at lower KIF3A/A concentrations. When a 2 fold lower concentration of motors were incubated with beads, the run length at 1 µM averaged to 1260 ± 160 nm (mean ± SEM, n = 20). Lowering the concentration further by another 2 fold resulted in no binding events of beads to microtubules. The combined plot of run length versus velocity emphasizes the differences between KHC and KIF3A/A in processivity (Figure 4.5c). Hence, the increase in run length is not a result of multimotor interaction.
4.3.4 Kinetic model simulation

This interesting difference in processivity with ATP concentration must relate to the structure and chemomechanical pathway each motor follows. To identify this relation, the pathways were modeled using stochastic simulation on MATLAB (see Methods). First, simulation of Kinesin-1 stepping was performed based on the consensus
kinetic model shown in Figure 4.1 (see Introduction). Since this simulation requires input of rate constants, we chose initial rate constants from values in literature as shown in Table 4.2. The transitions that were practically not possible were set to have a rate constant of zero. Systematically rate constants were changed to match $K_{M}^{ATP}$, velocity and run length dependence on ATP for Kinesin-1. The final rate constants are indicated in Table 4.3 and the ATP dependence of run length and velocity are shown in Figure 4. The $K_{M}^{ATP}$ from the simulation was 75 µM which is similar to 81 µM obtained from experimental data. The run lengths from simulations increased over the range of ATP concentrations as expected. Therefore, this simulation method gave good prediction of experimental results.
Table 4.2: Initial parameters used in stochastic simulation of motors (Cross, 2004; Gilbert and Johnson, 1994; Hackney, 2005; Hancock and Howard, 1999; Uemura and Ishiwata, 2003). $k'$ indicates reverse rate constants.

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>Value</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{detach}}$</td>
<td>100 - 250 s$^{-1}$</td>
<td>Empirical</td>
</tr>
<tr>
<td>$k'_{\text{detach}}$</td>
<td>0.1 - 0.25 s$^{-1}$</td>
<td>1/1000 of $k_{\text{detach}}$</td>
</tr>
<tr>
<td>$k'_{\text{on}}$(ATP)</td>
<td>40 - 200 s$^{-1}$</td>
<td>Based on $K_M$(ATP)</td>
</tr>
<tr>
<td>$k'_{\text{hydrolysis}}$</td>
<td>2 - 8 s$^{-1}$</td>
<td>Hackney, D.D. (2005) PNAS 102:18339-18343</td>
</tr>
<tr>
<td>$k_{\text{attach}}$</td>
<td>100 - 450 s$^{-1}$</td>
<td>From $k_{\text{unbind}}$ &amp; run length at 1µM ATP</td>
</tr>
<tr>
<td>$k'_{\text{attach}}$</td>
<td>0.1 – 0.45 s$^{-1}$</td>
<td>1/1000 of $k_{\text{attach}}$</td>
</tr>
<tr>
<td>$k_{\text{unbind}}$</td>
<td>1 - 5 s$^{-1}$</td>
<td>Hancock, W.O., Howard, J. (1999) PNAS 96:13147-13152</td>
</tr>
</tbody>
</table>
Table 4.3: Rate constants in simulation that gave the best fit to the experimental data. Best fit was determined by maximizing R-squared value of run length versus velocity curve.

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>KHC (Figure 4.1)</th>
<th>KIF3A/A (Figure 4.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{detach}}$</td>
<td>250 s$^{-1}$</td>
<td>250 s$^{-1}$</td>
</tr>
<tr>
<td>$k'_{\text{detach}}$</td>
<td>0.1 s$^{-1}$</td>
<td>0.1 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{on}}$(ATP)</td>
<td>2 µM$^{-1}$s$^{-1}$</td>
<td>3 µM$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>$k'_{\text{on}}$(ATP)</td>
<td>200 s$^{-1}$</td>
<td>15 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{hydrolysis}}$</td>
<td>280 s$^{-1}$</td>
<td>120 s$^{-1}$</td>
</tr>
<tr>
<td>$k'_{\text{hydrolysis}}$</td>
<td>3.5 s$^{-1}$</td>
<td>3.5 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{attach}}$</td>
<td>370 s$^{-1}$</td>
<td>380 s$^{-1}$</td>
</tr>
<tr>
<td>$k'_{\text{attach}}$</td>
<td>0.1 s$^{-1}$</td>
<td>0.1 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{fhdetach}}$</td>
<td>N/A</td>
<td>11 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{unbind}}$</td>
<td>1.7 s$^{-1}$</td>
<td>2.3 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{unbind2}}$</td>
<td>0.003 s$^{-1}$</td>
<td>0.003 s$^{-1}$</td>
</tr>
</tbody>
</table>

Using the same model (Figure 4.1) for KIF3A/A required adjusting rate constants to fit both, increasing velocities as well as decreasing run lengths trend of the motor with increasing ATP concentrations. While velocity dependence on ATP could be mimicked, the run lengths remained constant with ATP concentration irrespective of changes to the rate constants. This agrees theoretically with the fact that in Model 1 the step determining the unbinding of a motor from the microtubule is independent of the ATP concentration.
The logical conclusion was to modify the existing chemomechanical pathway shown in Figure 4.6 to include a pathway for run length dependence on ATP. From the experiments, we can first say that the waiting state of the motor for ATP to bind affects the subsequent unbinding of the motor from the microtubule. Therefore there has to be two pathways for ATP attachment to the motor. Similarly, there has to be at least two pathways for the motor to unbind. The pathway taken should depend on the ATP concentration. Combining these conditions, the model we propose is shown in Figure 4.6.
According to the model in Figure 4.6, ATP binding to the front head occurs either with the rear head tethered (State 1 to 2) or while the rear head is still bound in the ADP.Pi state (State 1 to 3). From the literature, there is evidence for both of these pathways occurring in Kinesin-1 (Mori et al., 2007; Rosenfeld et al., 2002; Yildiz et al., 2004), although the bulk of the data favors rear head detachment preceding ATP binding (Rosenfeld et al., 2003). In this expanded model, at low ATP concentrations rear head detachment occurs preferentially, while at high ATP concentrations, ATP binds to the front head before the rear head has time to detach. ATP binding then sets up a race between hydrolysis and detachment of the rear head, and if hydrolysis wins (state 3 to 4) and then the leading head detaches before the trailing head (State 4 to 5), then the motor can detach from a second pathway (State 5). At high ATP concentrations, this second detachment pathway reduces the run length of the motor. At low ATP, the probability of following the standard pathway (from State 1 to 2) increases, eliminating the influence of the second detachment pathway, and resulting in longer run lengths. This combined model also causes saturation of run lengths at very low and very high ATP concentrations as mostly follows either one path or the other.

The next step was to include the extended pathway shown in Figure 4. to simulate KIF3A/A run length and velocity. The rate constants for KIF3A/A were chosen by starting with those used for KHC and changing \( k_{\text{on}}(\text{ATP}), k'_{\text{on}}(\text{ATP}), k_{\text{hydrolysis}} \) and \( k_{\text{unbind}} \) to agree with KIF3A/A experimental values of \( K_M^{\text{ATP}} \), velocity and run lengths. Using the rate constants in Table 4.3 for KIF3A/A, simulating the velocity versus ATP gave \( K_M^{\text{ATP}} \)
of 19.2 μM similar to the experimental value of 18.8 μM (Figure 4.7a). The run length versus ATP concentration also corresponded well with the experimental data (Figure 4.7b). Plotting a linear curve to experimental run length versus velocity and fitting it to the simulation data gave an R-squared of 0.96, the maximum obtained with the rate constants evaluated. Therefore the new chemomechanical pathway explained KIF3A/A results accurately, introducing a new dimension to kinetic models describing kinesin in literature.
Figure 4.7: Simulation results versus experimental data. a. Velocity vs. ATP concentration. Symbols are experimental data and the line is a Michalis-Menten fit to the simulated data. Black indicates kinesin-1 and red is kinesin-2. $K_{ATP}^M$ for KHC for simulated data was 75 and for experimental data was 81 µM. $K_{ATP}^M$ for KIF3A/A was 19.2 and for experimental data was 18.8 µM. b. Run length vs. ATP concentration. The filled triangles (blue) are experimental KHC data while open are simulation. Similarly, filled diamonds (red) are KIF3A/A experimental and open are simulations.
4.4 Discussion

Members of the kinesin superfamily perform a range of crucial transport functions in cells, making it important to understand how each family’s biophysical properties relate to their cellular role. For this reason, solving the motor’s ability of chemomechanical coupled kinetics is a necessity. Information from structural and ATPase activity studies of Kinesin-1 has led to the favored idea of asymmetric hand-over-hand model for kinesin (Asbury et al., 2003). Still questions regarding the details of this model remain unanswered as described in the introduction. Similarly, mechanochemistry of other motor families and their contribution to general kinesin mechanism are yet to be understood.

Here, we focus on the mouse Kinesin-2, KIF3A/B and the chimeras KIF3A/A and KIF3B/B, by examining their processivity and velocity in detail. We show that KIF3A/B, KIF3A/A and KIF3B/B are processive using dilution profiles obtained from single motor bead assays. At saturating ATP, velocities of KIF3A/B and homodimers were about 60% of KHC. In comparing run lengths, KIF3A/A has run lengths similar to KIF3A/B but KIF3B/B run length was about 1.5 times higher (Table 4.1). These results gave first clues to the coordination between heads. Though individual heads have different affinities towards microtubules, coordination gives rise to motor that is dominated by head with lower affinity, i.e., KIF3A head truncates KIF3A/B runs.
What are the *in vivo* implications? Whereas Kinesin-1 has to transport cargo along axons that can be centimeters in length, Kinesin-2 carries cargo mainly along flagella and cilia which are maximum of tens of microns long. Also, the shorter run length raises questions about the number of motors required in transporting cargo which will depend on the function of the cargo too, such as, IFT versus cytoplasmic melanosome transport. Evolutionarily, we can say that kinesin has modified its structure to help in these shorter more specific transport functions.

To better understand the Kinesin-2 chemomechanical cycle, we examined the influence of ATP concentration on the motor processivity. Since KIF3A/B and KIF3B/B were unstable at low ATP concentrations in standard buffer conditions, we analyzed only KIF3A/A. The velocity dependence showed KIF3A/A to have lower $K_M^{ATP}$ than KHC which from the simulations indicate increased $k_{on}(ATP)$ and decreased $k_{off}(ATP)$ and $k_{hydrolysis}$.

More interestingly, for the first time we observed run lengths of a motor change with ATP concentration. Unlike Kinesin-1 which showed a small increase in run length with ATP concentration, for KIF3A/A there was a sharp decrease in run length at high ATP levels. This inverse dependence of motor run length on ATP concentration could not be explained with current kinetic models as shown from simulations for Kinesin-1. New pathways had to be added based on the result that high ATP concentrations reduced the binding affinity of motor to microtubule indicating existence of a second detachment pathway. The only possible way this could be introduced is if ATP binds and hydrolyzes
in the front head while rear head is still attached to the microtubule in ADP.Pi state. Two modifications were included to existing model in developing this extended chemomechanical path. First, the motor in State 1 is allowed to wait in different states for ATP to bind and second, ATP hydrolysis in the front head before rear head detachment is allowed to occur in State 3. Experimentally, because the transition from State 1 to 3 is ATP dependent, it occurs predominantly at high ATP concentrations. The simulation results show that in comparing rate constants in going from State 3 to State 4 or 6, the rear head detachment is favored compared to hydrolysis, but only by two times. Therefore, chance for the front head ATP to hydrolyze, making it a weak binding state is probable. When in this weak binding state, the eventual unbinding of the whole motor is favored. The stochastic simulation using this model, predicted the experimental results accurately.

Most research with Kinesin-1 has led to a model which shows that due to an internal strain between neck linkers and ATP gating, the rear head needs to unbind before ATP binds to the front head (Klumpp et al., 2004; Rosenfeld et al., 2003; Schief et al., 2004). However, there is some speculation in literature of ATP binding to the front head of Kinesin-1 when both heads are bound to the microtubule. This was predicted from noisy 16 nm steps by Yildiz et. al. and from FRET efficiencies which may not be accurate given their time resolution by Mori et. al (Mori et al., 2007; Yildiz et al., 2004). While either method of ATP binding can explain Kinesin-1 processivity, in the case of Kinesin-2, the pathway where ATP binds before rear head detachment is a necessity to explain the chemomechanical cycle.
So what is the molecular origin of this difference between Kinesin-1 and Kinesin-2 motors? According to recent computational simulation by Hyeon and Oncuchic (Hyeon and Oncuchic, 2007), the strain in the neck linker propagates to the motor head and affects the native contacts present in the nucleotide binding pocket making it unfavorable for nucleotide binding. In Kinesin-1, release of the strain by trailing head detachment brings the binding pocket back to the native state rendering it favorable for nucleotide binding. Therefore, to analyze the strain present in Kinesin-2, we first identified the neck linker from the protein structure of KIF3B (Chen et al., 2003). KIF3B neck linker has three more amino acids (totally 18 amino acids) than KHC which increases the neck linker length from 5.7 nm for KHC to 6.8 nm for KIF3B. From sequence alignment, KIF3A neck linker is also of the same length. Assuming a worm-like-chain model for the neck linker with a 3.1 nm extension, the tension in this linker is calculated to be only 10.9 pN for KIF3A/A compared to 15.5 pN for KHC. This difference in tension is significant for motors when comparing to stall forces (6-7 pN) of KHC. In Kinesin-2, this reduction in the rearward strain allows the motor to wait for ATP with both heads bound. Since ATP binds to the front head in the two-head bound state, it leads to the inference that the nucleotide binding pocket is in its native state and rear head detachment is not required, i.e., ATP gating does not exist. Hence, the concept of ‘back seat’ driving by the rear head becomes invalid for Kinesin-2.

In conclusion, Kinesin-2 motors have shorter processive runs and lower velocities than Kinesin-1 which helps in their in vivo function of shorter transport distance. The
heads of the Kinesin-2 motor, KIF3A and KIF3B though structurally different, are similar in velocity to KIF3A/B but KIF3B has longer run lengths. Importantly, processivity and velocities of KIF3A/A depends on the ATP concentration differently from Kinesin-1. An inverse dependence of run length on the ATP concentration has been recorded for the first time for a motor protein. Modeling the recorded data and running simulations have given insight into a new pathway in the kinesin mechanochemistry, a secondary detachment pathway stemming from two head bound ATP waiting state at high ATP concentrations. From structural biology analysis, this two-head bound state is allowed primarily due to longer neck linker for Kinesin-2 which reduces tension between heads. This directly leads to next step of studying differences in neck linker structure and function between kinesin families in detail. Also within the Kinesin-2 family, next questions are; does KIF3B behave similarly and can we predict KIF3A/B biophysical behavior from coordination between the heads?

4.5 References


Chapter 5

Conclusion and Future Work

Since kinesin’s discovery in 1985, its importance in the many roles it plays in normal cell function and during cell division has been realized through extensive cell biology studies (Lasek and Brady, 1985; Vale et al., 1985). Thereafter, kinesin has been extensively characterized \textit{in vitro} using a range of biophysical and biochemical techniques. Understanding the mechanochemistry of these motors is important in both the development of therapeutic targets as well as the translation of this knowledge to other similar mechanoenzymes. In this thesis, models for the chemomechanical pathway have been proposed using the rate constants and structural information obtained for the transition between different nucleotide states for wild type and mutated motors (Hackney, 1995; Hancock and Howard, 1998; Hancock and Howard, 1999; Rice et al., 1999). In the process of elucidating the different steps of motor behavior, biochemical and biophysical techniques, such as ATPase assays, cryoelectron microscopy, TIRF, FRET and optical tweezers, have been adapted and have evolved (Block et al., 1990; Hackney et al., 1989; Hirose et al., 1996; Rice et al., 1999). Until recently, most of the \textit{in vitro} work has been limited to the Kinesin-1 family. Extending this research to the other 13 kinesin families is important for relating their physical behavior to their \textit{in vivo} function and also for refining and extracting differences in the kinesin chemomechanical pathway (Hunter et al., 2003; Valentine et al., 2006; Zhang and Hancock, 2004).
5.1 Insights into Kinesin-2 Biophysics

The goal of this thesis was to build new techniques to enable better imaging and detection at the single molecule level and to elucidate details of the chemomechanical coordination between the two heads of Kinesin-2 motors during processive stepping. Quantum-dot tagged kinesin proved useful in performing single molecule imaging with a basic epifluorescence microscope. On the other hand, the single molecule bead assay using optical traps and a back focal plane interferometry system gave a more direct method for manipulating beads and detecting motor stepping position at nanometer scales. Using this trap at low loads, bead assays of KHC, KIF3A/B and homodimers KIF3A/A and KIF3B/B were performed. Run length and velocity data obtained at saturating ATP indicated that Kinesin-2 have lower velocities and run lengths than Kinesin-1. Decreasing the ATP concentration showed an increase in the KIF3A/A processivity. These results gave novel insights into the existing chemomechanical pathways. The new pathway proposed and verified using simulations indicated that at saturating ATP concentrations, ATP binds to the motor, while both heads are bound to the microtubule leading the motor to have a higher probability of detachment from the microtubule. This leads to the hypothesis that the three extra amino acids in the neck linker of the KIF3 motors compared to KHC plays a role in reducing the strain between the heads, hence allowing both heads to be bound to the microtubule for longer times. Since ATP can still bind with both heads on the microtubule, the theory of ATP gating proposed by Rosenfeld is questionable for Kinesin-2 motors (Rosenfeld et al., 2003).
5.2 Future of Kinesin-1 and Kinesin-2 Mechanochemistry

The future of the research performed here will include optimizing and refining the back focal plane interferometry system and conducting further experiments to confirm the model and the inferences developed for the Kinesin-2 chemomechanical pathway. One approach would be to increase the length of the neck linker of KHC by three amino acids and check if a similar response to KIF3A/A is obtained. The second approach would be to completely exchange the neck linkers between KIF3A/B and KHC and measure their behavior to ATP concentrations. A recent study indicating that the N-terminal residues also play a critical role in stabilizing the conformational change upon ATP binding requires comparing the N-terminal sequences between the motor types (Hwang et al., 2008).

Another important aspect to be understood is the necessity for two different heads in Kinesin-2 and the coordination between them. The first step would be to determine appropriate buffer conditions for KIF3B stability at limiting ATP concentrations and subsequently obtain KIF3A/B and KIF3B/B run length and velocity at these concentrations. Force studies including determining step size, stall forces and force-velocity relationship on all KIF3 motors will give clues to coordination between the two heads. These results will put further constraints on the kinetic cycles for Kinesin-1 and Kinesin-2 motors.
5.3 References


Appendix

Stochastic Simulation Code

1 Transition between motor states

function [NewMotorState, DwellTime] = transition(CurMotorState, ...
    RateConst1,RateConst2,RateConst3,RateConst4,RateConst5,RateConst6,...
    RateConst7,RateConst8)

% TRANSITION calculates the dwell time and resultant state of a motor transition.
%
% The function TRANSITION is a part of the kinesin program. Given the
% current state of a motor protein and the rate constants of its three
% possible transitions, TRANSITION calculates the motor's next
% chemomechanical state and the time it takes to transition to this new
% state (the dwell time).
%
% The motor protein can transition from its present state to any of the eight states.
% Rate constants for each of these transitions are input into
% the ‘kinesin’ function. Using these rate constants, the dwell time of a motor
% transition to each of eight states is calculated. The motor
% transitions in the direction corresponding to the smallest dwell time.
%
% TRANSITION outputs this dwell time and the new state of the motor.
% It is possible that two dwell times may be equal and less than the third,
% or that all three dwell times are equal. If either of these cases
% occurs, TRANSITION chooses the transition direction in the following
% order: forward (to state with higher number), backward (to state with lower number) or
% off. For example, if the dwell times
% corresponding to a backward and off transition are equal, and these dwell
% times are less than the dwell time corresponding to a forward transition,
% then TRANSITION chooses the backward transition.
%
% The dwell times are calculated using the following function:
% Dwell Time = (1 / Rate Constant) * log(1 / Random Number) where the
% random number is in the open interval (0,1). A different random number
% is generated in the calculation of each dwell time (otherwise the motor
% would always transition in the direction with the largest rate constant).
% The dwell times are in units of (1 / Rate Constant Units). If any of the
% rate constants are equal to zero, MATLAB will produce a divide-by-zero
% warning. The command 'warning off MATLAB:divideByZero' (without quotes)  
% can be used to suppress this warning.

% The current state of the motor input into TRANSITION should be an integer  
% on the closed interval [1,8]. Each of these numbers represents a  
% chemomechanical state in the motor protein's stepping cycle.  
% Motors in state 7 transition forward to  
% state 1, and motors in state 1 transition backward to state 7. If a  
% motor transitions off the microtubule, its new state is output as state 8.  
% TRANSITION only does valid calculations for motors that are attached  
% to a microtubule (states 1-7). As stated above, the current state input  
% into TRANSITION should be an integer on the closed interval [1,8].

% Define Variables:  
% Input  
% =====  
%   CurMotorState -- The current motor state (an integer from 1 to 8).  
%   RateConst  -- The rate constant for a transition.

% Output  
% ======  
%   NewMotorState -- The new motor state (an integer from 1 to 8).  
%   DwellTime -- The time the motor waits to transition to from  
%   CurMotorState to NewMotorState.

warning off MATLAB:divideByZero

DwellTime1 = (1 /RateConst1) * log(1 / rand());
DwellTime2 = (1 /RateConst2) * log(1 / rand());
DwellTime3 = (1 /RateConst3) * log(1 / rand());
DwellTime4 = (1 /RateConst4) * log(1 / rand());
DwellTime5 = (1 /RateConst5) * log(1 / rand());
DwellTime6 = (1 /RateConst6) * log(1 / rand());
DwellTime7 = (1 /RateConst7) * log(1 / rand());
DwellTime8 = (1 /RateConst8) * log(1 / rand());

DwellTime = min([DwellTime1,DwellTime2,DwellTime3,DwellTime4,DwellTime5,...  
    DwellTime6,DwellTime7,DwellTime8]);

if DwellTime == DwellTime1  
    NewMotorState = 1;  
elseif DwellTime == DwellTime2  
    NewMotorState = 2;  
elseif DwellTime == DwellTime3  
    NewMotorState = 3;
NewMotorState = 3;
elseif DwellTime == DwellTime4
    NewMotorState = 4;
elseif DwellTime == DwellTime5
    NewMotorState = 5;
elseif DwellTime == DwellTime6
    NewMotorState = 6;
elseif DwellTime == DwellTime7
    NewMotorState = 7;
elseif DwellTime == DwellTime8
    NewMotorState = 8;
end % function transition

2 One motor stepping

function [StepTimes, OffTime] = onemotor(RateConst, RunTime)
%ONEMOTOR calculates the step times and off time for a single motor.
%
% The function ONEMOTOR is part of the kinesin program. Given the run time
% of the simulation and the rate constants of the motor protein,
% ONEMOTOR outputs the step times of the motor and the time the motor takes
% to disengage from the microtubule.
%
% A step is defined as any sequence of motor states beginning at 1 and
% ending at 7.
% A forward step is defined as a sequence of states that begins at state
% 1 and transitions forward to state 7. A backward step is defined as a sequence of
% states that
% begins at state 7 and transitions backwards to state 1. Note that a motor can transition
% erratically back and forth part way through a step. For example, this
% sequence of states counts as a forward A step: 1,2,1,7,1,2, 6,3,4,5.
%
% If the motor fails to disengage from the microtubule during the run time
% of the simulation, ONEMOTOR outputs an off time of Inf.

% Define Variables:
% Input
% =====
% RateConst -- An 8 x 8 array of rate constants for 64 different
% chemomechanical transitions between 8 unique states.
% RateConst(x,y) indicates transition from State X to State Y
% RunTime -- The run time of the simulation in seconds. Note that
this is not the actual time that the function will
run, but rather the longest duration of simulated
time that the motor is allowed to travel along the
microtubule.

Output

StepTimes -- A two column array containing the step times of the
time that the motor is allowed to travel along the
number of steps. 1 is a forward step, 2 is a backward
step. The second column contains the actual step
time corresponding to the type of step in the
first column.

OffTime -- The amount of simulated time the motor takes to
disengage from the microtubule. If the motor fails
to disengage during the run time, then OffTime is set
equal to Inf.

LocalVariables

CurTime -- The amount of simulated time that has elapsed.
MotorState -- The state of the motor at CurTime.
StepTime -- The amount of time that has elapsed since the last
motor step of any type.
LastMotorState -- The previous state of the motor. The state the motor
was in before its current state (i.e. the state
before MotorState).
DwellTime -- The dwell time of the motor's most recent transition
between states.

Record of Revisions:

Date(mm/dd/yy) Programmer Description
08/04/05 James Dizikes Origional Code

Initial Conditions Of Four Local Variables

CurTime = 0;
MotorState = 1;
StepTime = 0;

Output Initialization

StepTimes = [];

Note: The following warning is turned off for function TRANSITION.

warning off MATLAB:divideByZero
while CurTime <= RunTime && MotorState ~= 8

The following block of code calculates the dwell time between the
last state of the motor and the new motor state.

LastMotorState = MotorState;
[MotorState, DwellTime] = transition(MotorState, ...
RateConst(MotorState,1),RateConst(MotorState,2),RateConst(MotorState,3),...
RateConst(MotorState,4),RateConst(MotorState,5),RateConst(MotorState,6),...
RateConst(MotorState,7), RateConst(MotorState,8));
CurTime = CurTime + DwellTime;
StepTime = StepTime + DwellTime;

% StepTimes calculator
if CurTime <= RunTime
    if MotorState == 1 && LastMotorState == 7
        StepTimes = [StepTimes; 1, StepTime];
    elseif MotorState == 7 && LastMotorState == 1
        StepTimes = [StepTimes; 2, StepTime];
    end
    StepTime = 0;
end

warning on MATLAB:divideByZero

% OffTime calculator
if CurTime <= RunTime
    OffTime = CurTime;
else
    OffTime = Inf;
end

end % function onemotor

3 Multiple kinesin simulation and output

function kinesinF(ATP, Force)
% (ATP) The ATP concentration in micromoles per liter (uM).
% (Force) The force on the motor in the simulation (pN). Ex: [0, 1.05, 3.59, 5.63]

% *************************************************************************
% ******************** Kinesin Monte-Carlo Simulation ********************
% *************************************************************************
% USER INPUT

% Simulation Parameters
ATP = 1000;
NumMotors = 10000; % The number of motors in the simulation.
Force = 0;
delta = 4; % Structural change (force-dependent)
RunTime = Inf; % The run time of the simulation in seconds. Note that
    % this is not the actual time that the program will run, but
% rather the longest duration of simulated time that the
% motors are allowed to travel along the microtubule. If
% you would like the simulation to run until all of the
% motors detach from the microtubule, enter Inf for the run
% time (i.e. RunTime = Inf;).

% ----------------- Rate Constants ---------------------
RateConst = zeros(8,8); % Do not alter this input.
% Forward Rate Constants (s-1)
RateConst(1,2) = 250; % State 1 to State 2: Rear Head detachment
RateConst(1,3) = ATP*0*exp((-Force*delta)/4); % State 1 to State 3: ATP binding
(ATP Induced Detachment)
RateConst(2,6) = ATP*2*exp((-Force*delta)/4); % State 2 to State 6: ATP binding
(Spontaneous Detachment)
RateConst(3,6) = 0; % State 3 to State 6: Rear Head Detachment
RateConst(3,4) = 0; % Forward head hydrolysis (AID model)
RateConst(4,5) = 0; % Forward head detachment (AID model)
RateConst(4,7) = 0; % Rear Head Detachment
RateConst(5,8) = 0; % Motor disengages
RateConst(6,7) = 280; % State 6 to State 7: ATP hydrolysis
RateConst(7,8) = 2; % Motor disengages
RateConst(7,1) = 370; % Rear head attachment
% Reverse Rate Constants (s-1)
RateConst(1,7) = 0.1;
RateConst(2,1) = 0.1;
RateConst(3,1) = 0;
RateConst(4,3) = 0;
RateConst(5,4) = 0;
RateConst(6,2) = 180;
RateConst(6,3) = 0;
RateConst(7,4) = 0;
RateConst(7,6) = 3.5;
% Not possible transitions
RateConst(1,1) = 0;
RateConst(1,4) = 0;
RateConst(1,5) = 0;
RateConst(1,6) = 0;
RateConst(1,7) = 0;
RateConst(1,8) = 0;
RateConst(2,2) = 0;
RateConst(2,3) = 0;
RateConst(2,4) = 0;
RateConst(2,5) = 0;
RateConst(2,7) = 0;
RateConst(2,8) = 0;
RateConst(3,2) = 0;
RateConst(3,3) = 0;
RateConst(3,5) = 0;
RateConst(3,7) = 0;
RateConst(3,8) = 0;
RateConst(4,1) = 0;
RateConst(4,2) = 0;
RateConst(4,4) = 0;
RateConst(4,6) = 0;
RateConst(4,8) = 0;
RateConst(5,1) = 0;
RateConst(5,2) = 0;
RateConst(5,3) = 0;
RateConst(5,5) = 0;
RateConst(5,6) = 0;
RateConst(5,7) = 0;
RateConst(6,1) = 0;
RateConst(6,4) = 0;
RateConst(6,5) = 0;
RateConst(6,6) = 0;
RateConst(6,8) = 0;
RateConst(7,2) = 0;
RateConst(7,3) = 0;
RateConst(7,5) = 0;
RateConst(7,7) = 0;
RateConst(8,1) = 0;
RateConst(8,2) = 0;
RateConst(8,3) = 0;
RateConst(8,4) = 0;
RateConst(8,5) = 0;
RateConst(8,6) = 0;
RateConst(8,7) = 0;
RateConst(8,8) = 0;

% ------------------------- Plotting Options ------------------------------
Hist_StepTimes = 'y';  % Entering 'y' outputs histograms of the step
% times for forward and backward A and B steps.
% The figure includes the average step time
% for each of these four types of steps.
Hist_MotorsDisengage = 'y';  % Entering 'y' outputs histograms of the run
% length and off time of each motor that
% disengages from the microtubule. The figure
% includes the average off time and the average
% run length of the motors along with the
% standard deviation from these averages.

% --------------------------- Saving Options ------------------------------
SaveData = 'y';  % Entering 'y' saves the simulation parameters
% and the data used to construct the plots
% chosen above.
FileLocation = 'D:\Research\Modeling\Model including 2 pathways
data\HKin_simulation\012808\sim_1000uMATP_10000n';
% Input the name and location of the data file
% to be saved. For instance, if you want to
% name the data file 'test' and save it to they
% C: drive, then you enter 'C:\test'. Note
% that if the file already exists it will be
% overwritten.
% Simulation Code
% Do Not Change Code Below This Line

TimeOfSim = datestr(clock); % Time stamp for the simulation.

% Initializing arrays for step time data.
if strcmp(lower(Hist_StepTimes), 'y')
    Fwd_StepTimes = [];
    Back_StepTimes = [];
end

% Initializing arrays for motor disengage data.
if strcmp(lower(Hist_MotorsDisengage), 'y')
    Data_OffTime = zeros(NumMotors,1);
    Data_RunLength = zeros(NumMotors,1);
    Data_Velocity = zeros(NumMotors,1);
end

% Main Simulation Loop
for CurMotor = 1:NumMotors
    [StepTimes, OffTime] = onemotor(RateConst, RunTime);
    if length(StepTimes) == 0
        StepTimes = [0,0]; % This value will be skipped using the masks.
    end

    % Create logic masks for vectorized calculations.
    Fwd_Mask = StepTimes(:,1) == 1;
    Back_Mask = StepTimes(:,1) == 2;

    % Step Histogram Data
    if strcmp(lower(Hist_StepTimes), 'y')
        Fwd_StepTimes = [Fwd_StepTimes; StepTimes(Fwd_Mask,2)];
        Back_StepTimes = [Back_StepTimes; StepTimes(Back_Mask,2)];
    end

    % Off Histogram Data
    if strcmp(lower(Hist_MotorsDisengage), 'y')
        Data_OffTime(CurMotor,1) = OffTime;
        Data_RunLength(CurMotor,1) = (length(StepTimes(Fwd_Mask,2)) + ... - length(StepTimes(Back_Mask,2))) * 8;
        Data_Velocity(CurMotor,1) = Data_RunLength(CurMotor,1)./
    end
end

% Step Histograms

end
if strcmp(lower(Hist_StepTimes), 'y')

% Create new figure and make it as big as the screen.
figure
Screen = get(0, 'screensize');
if Screen(3) == 800
    set(gcf, 'Units', 'normalized', 'Position', [0 0.0467 1.00 0.84])
elseif Screen(3) == 1024
    set(gcf, 'Units', 'normalized', 'Position', [0.00 0.032 1.00 0.92])
elseif Screen(3) == 1152
    set(gcf, 'Units', 'normalized', 'Position', [0.00 0.032 1.00 0.89])
elseif Screen(3) == 1280
    set(gcf, 'Units', 'normalized', 'Position', ...
        [0.00 0.032 1.00 0.895])
elseif Screen(3) == 1600
    set(gcf, 'Units', 'normalized', 'Position', ...
        [0.00 0.032 1.00 0.905])
end

% Create histogram of forward step times.
subplot(2,3,1)
if length(Fwd_StepTimes) ~= 0
    BinWidth = 3.49 * std(Fwd_StepTimes) / (length(Fwd_StepTimes) ... ^ (1/3));
    hist(Fwd_StepTimes, min(Fwd_StepTimes) + (BinWidth / 2): ... BinWidth: max(Fwd_StepTimes) + (BinWidth / 2));
    xlim([0 max(Fwd_StepTimes) + BinWidth]);
    title(['Forward Steps | BW: ', num2str(BinWidth), ' sec']);
    xlabel('Time (s)');
    ylabel('Number of Steps');
    AvgFwd_StepTime = sum(Fwd_StepTimes) / length(Fwd_StepTimes);
else
    axis off
    clear TextBox
    TextBox(1) = {' No motors in the simulation '};
    TextBox(2) = {' made a forward step. '};
    text(0,1, TextBox, 'HorizontalAlignment', 'left', ...
        'VerticalAlignment', 'top', 'BackgroundColor', 'white', ... 'EdgeColor', 'black');
    AvgFwd_StepTime = 'n/a';
end

% Create a histogram of the backward step times.
subplot(2,3,3)
if length(Back_StepTimes) ~= 0
    BinWidth = 3.49 * std(Back_StepTimes) / ...
        (length(Back_StepTimes) ^ (1/3));
    hist(Back_StepTimes, min(Back_StepTimes) + (BinWidth / 2): ... BinWidth: max(Back_StepTimes) + (BinWidth / 2));
    xlim([0 max(Back_StepTimes) + BinWidth]);
    title(['Backward Steps | BW: ', num2str(BinWidth), ' sec']);
    xlabel('Time (s)');
ylabel('Number of Steps');
AvgBack_StepTime = sum(Back_StepTimes) / length(Back_StepTimes);
else
    axis off
    clear TextBox
    TextBox(1) = {' No motors in the simulation '};
    TextBox(2) = {' made a backward A step. '};
    text(0,1, TextBox, 'HorizontalAlignment', 'left', ...
        'VerticalAlignment', 'top', 'BackgroundColor', 'white', ...
        'EdgeColor', 'black');
    AvgBack_StepTime = 'n/a';
end

% Plot Step Trace of Last Motor
StepTrace = zeros(size(StepTimes,1) + 1,2);
for ii = 1:size(StepTimes,1)
    StepTrace(ii+1,1) = StepTrace(ii,1) + StepTimes(ii,2);
    switch StepTimes(ii,1)
    case {1,2}
        StepTrace(ii+1,2) = StepTrace(ii,2) + 1;
    case {3,4}
        StepTrace(ii+1,2) = StepTrace(ii,2) - 1;
    end
end
if RunTime ~= Inf
    StepTrace = [StepTrace; RunTime, StepTrace(ii+1,2)];
else
    StepTrace = [StepTrace; OffTime, StepTrace(ii+1,2)];
end
subplot(2,3,5)
stairs(StepTrace(:,1), StepTrace(:,2))
title(['Stepping Trace']);
xlabel('Time (s)');
ylabel('Total Number of Steps');
if strcmp(lower(Hist_MotorsDisengage), 'y')

% Remove off times and run lengths for motors that fail to
% disengage from the microtubule.
Mask = Data_OffTime ~= Inf;
Data_OffTime = Data_OffTime(Mask);
Data_RunLength = Data_RunLength(Mask);

% Create new figure and make it as big as the screen.
figure
Screen = get(0, 'screensize');
if Screen(3) == 800
    set(gcf, 'Units', 'normalized', 'Position', [0 0.0467 1.00 0.84])
elseif Screen(3) == 1024
    set(gcf, 'Units', 'normalized', 'Position', [0.00 0.032 1.00 0.92])
elseif Screen(3) == 1152
    set(gcf, 'Units', 'normalized', 'Position', [0.00 0.032 1.00 0.89])
elseif Screen(3) == 1280
    set(gcf, 'Units', 'normalized', 'Position', ...
        [0.00 0.032 1.00 0.895])
elseif Screen(3) == 1600
    set(gcf, 'Units', 'normalized', 'Position', ...
        [0.00 0.032 1.00 0.905])
end

% Create a histogram of the off times of the motors.
subplot(3,2,[1 3]);
if length(Data_OffTime) ~= 0
    AvgOffTime = sum(Data_OffTime,1) / size(Data_OffTime,1);
    % Bin width calculation (see the following link):
    %http://www.fmrib.ox.ac.uk/analysis/techrep/tr00mj2/tr00mj2/node24.html
    BinWidth = 3.49 * std(Data_OffTime) / (length(Data_OffTime) ^ ...
        (1/3));
    hist(Data_OffTime, min(Data_OffTime) + (BinWidth / 2): ...
        BinWidth: max(Data_OffTime) + (BinWidth / 2));
    xlim([0 max(Data_OffTime) + BinWidth]);
    title([''Bin Width: '', num2str(BinWidth), ''. sec'']);
    xlabel('Off Time (s)');
    ylabel('Number of Motors');
else
    AvgOffTime = 'n/a';
    axis off
    clear TextBox
    TextBox(1) = {' No motors in the simulation '};
    TextBox(2) = {' disengaged from the microtubule. '};
    text(0,1, TextBox, 'HorizontalAlignment', 'left', ...
        'VerticalAlignment', 'top', 'BackgroundColor', 'white', ...
        'EdgeColor', 'black');
% Create a histogram of the run lengths of the motors.
subplot(3,2,[4 6]);
if length(Data_RunLength) ~= 0
    AvgRunLength = mean(Data_RunLength,1);
    StdevRunLength = std(Data_RunLength,1);
% Plot Data_RunLength
% Bin width calculation (see the above link):
    BinWidth = 3.49 * std(Data_RunLength) / (length(Data_RunLength) ... ^ (1/3));
    hist(Data_RunLength, min(Data_RunLength) + (BinWidth / 2): ... BinWidth: max(Data_RunLength) + (BinWidth / 2));
    xlim([min(Data_RunLength), max(Data_RunLength) + BinWidth]);
    title(['Bin Width: ', num2str(BinWidth), ' sec']);
    xlabel('Run Length (nm)');
    ylabel('Number of Motors');
else
    AvgRunLength = 'n/a';
    axis off
    clear TextBox
    TextBox(1) = {' No motors in the simulation '};
    TextBox(2) = {' disengaged from the microtubule. '};
    text(0,1, TextBox, 'HorizontalAlignment', 'left', ... 'VerticalAlignment', 'top', 'BackgroundColor', 'white', ... 'EdgeColor', 'black');
end
% Velocity from average run length and off-time
AvgVelocity = mean(Data_Velocity,1);
StdevVelocity = std(Data_Velocity,1);
% Add text box to figure with simulation parameters.
subplot(3,2,2);
axis off
clear TextBox
TextBox(1) = {'Kinesin Model | ', TimeOfSim};
TextBox(2) = {' Simulation Parameters'};
TextBox(3) = {' - ATP Concentration: ', num2str(ATP), ' uM'};
TextBox(4) = {' - Total Number of Motors: ', num2str(NumMotors)};
TextBox(6) = {' - Number that Disengaged: ', ... num2str(length(Data_RunLength))};
if Data_OffTime ~= 0
    TextBox(7) = {' - Last Motor Disengaged After: ', ... num2str(max(Data_OffTime), ' sec')};
end
text(0,1, TextBox, 'HorizontalAlignment', 'left', ... 'VerticalAlignment', 'top', 'BackgroundColor', 'white', ... 'EdgeColor', 'black');

% Add text box to figure with average off times and run lengths.
subplot(3,2,5);  
axis off  
clear TextBox  
TextBox(1) = {' Above: A histogram of the time each motor takes to'};  
TextBox(2) = {' disengage from the microtubule.'};  
TextBox(3) = {' - Average Off Time: ', num2str(AvgOffTime), ' sec'};  
TextBox(4) = {' };  
TextBox(5) = {' Right: A histogram of the distance traveled by', ...  
'each motor'};  
TextBox(6) = {' before disengaging from the microtubule.'};  
TextBox(7) = {' - Average Run Length: ', num2str(AvgRunLength), ...  
'nm'};  
TextBox(8) = {' - Velocity: ', num2str(AvgVelocity), ' nm/s'};  
text(0,1, TextBox, 'HorizontalAlignment', 'left', ...  
'VerticalAlignment', 'top', 'BackgroundColor', 'white', ...  
'EdgeColor', 'black');  
end  

%%%%%%%%%%%%%%%%%%%%%%%%%%%% Saving Plot Data  
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%  
if strcmp(lower(SaveData), 'y')  
  fid = fopen([FileLocation, '.txt'], 'wt');  
  fprintf(fid, '
');  
  fprintf(fid, [Time of Simulation: ', TimeOfSim, '
']);  
  fprintf(fid, [ATP Concentration: ', num2str(ATP), ' uM
']);  
  fprintf(fid, [Number of Motors: ', num2str(NumMotors), '
']);  
  fprintf(fid, [Run Time: ', num2str(RunTime), ' sec
']);  
  fprintf(fid, '\n');  
  fprintf(fid, 'Forward Rate Constants (s-1):\n');  
  fprintf(fid, [State 1 to State 2: ', num2str(RateConst(1,2)), '\n']);  
  fprintf(fid, [State 1 to State 3: ', num2str(RateConst(1,3)), '\n']);  
  fprintf(fid, [State 2 to State 6: ', num2str(RateConst(2,6)), '\n']);  
  fprintf(fid, [State 3 to State 6: ', num2str(RateConst(3,6)), '\n']);  
  fprintf(fid, [State 3 to State 4: ', num2str(RateConst(3,4)), '\n']);  
  fprintf(fid, [State 4 to State 5: ', num2str(RateConst(4,5)), '\n']);  
  fprintf(fid, [State 4 to State 7: ', num2str(RateConst(4,7)), '\n']);  
  fprintf(fid, [State 5 to State 8: ', num2str(RateConst(5,8)), '\n']);  
  fprintf(fid, [State 6 to State 7: ', num2str(RateConst(6,7)), '\n']);  
  fprintf(fid, [State 7 to State 8: ', num2str(RateConst(7,8)), '\n']);  
  fprintf(fid, [State 7 to State 1: ', num2str(RateConst(7,1)), '\n']);  
  fprintf(fid, '\n');  
  fprintf(fid, 'Backward Rate Constants (s-1):\n');  
  fprintf(fid, [State 1 to State 7: ', num2str(RateConst(1,7)), '\n']);  
  fprintf(fid, [State 2 to State 1: ', num2str(RateConst(2,1)), '\n']);  
  fprintf(fid, [State 3 to State 1: ', num2str(RateConst(3,1)), '\n']);  
  fprintf(fid, [State 4 to State 3: ', num2str(RateConst(4,3)), '\n']);  
  fprintf(fid, [State 5 to State 4: ', num2str(RateConst(5,4)), '\n']);  
  fprintf(fid, [State 6 to State 2: ', num2str(RateConst(6,2)), '\n']);  
  fprintf(fid, [State 6 to State 3: ', num2str(RateConst(6,3)), '\n']);  
end
fprintf(fid, ['State 7 to State 4: ', num2str(RateConst(7,4)), ' 
']);
fprintf(fid, ['State 7 to State 6: ', num2str(RateConst(7,6)), ' 
']);
fprintf(fid, ' 
');
fprintf(fid, 'Disengage (Off) Rate Constants (s-1): 
');
fprintf(fid, ['State 1 to Off: ', num2str(RateConst(1,8)), ' 
']);
fprintf(fid, ['State 2 to Off: ', num2str(RateConst(2,8)), ' 
']);
fprintf(fid, ['State 3 to Off: ', num2str(RateConst(3,8)), ' 
']);
fprintf(fid, ['State 4 to Off: ', num2str(RateConst(4,8)), ' 
']);
fprintf(fid, ['State 5 to Off: ', num2str(RateConst(5,8)), ' 
']);
fprintf(fid, ['State 6 to Off: ', num2str(RateConst(6,8)), ' 
']);
fprintf(fid, ['State 7 to Off: ', num2str(RateConst(7,8)), ' 
']);
fprintf(fid, ' 
');
if strcmp(lower(Hist_StepTimes), 'y')
    fprintf(fid, 'Average Step Times (sec): 
');
    fprintf(fid, ['Forward Steps: ', num2str(AvgFwd_StepTime), ' 
']);
    fprintf(fid, ['Backward Steps: ', num2str(AvgBack_StepTime), ' 
']);
    fprintf(fid, ' 
');
end

if strcmp(lower(Hist_MotorsDisengage), 'y')
    fprintf(fid, 'Disengaged Motor Statistics 
');
    fprintf(fid, ['- Number that Disengaged: ', num2str(length(Data_RunLength)), ' 
']);
    fprintf(fid, ['- Last Motor Disengaged After: ', num2str(max(Data_OffTime)), ' sec 
']);
    fprintf(fid, ['- Average Disengage Time: ', num2str(AvgOffTime), ' sec 
']);
    fprintf(fid, ['- Average Run Length: ', num2str(AvgRunLength), ' nm 
']);
    fprintf(fid, ['- Standard Deviation Run Length: ', num2str(StdevRunLength), ' nm 
']);
    fprintf(fid, ['- Average Velocity: ', num2str(AvgVelocity), ' nm/sec 
']);
    fprintf(fid, ['- Standard Deviation Velocity: ', num2str(StdevVelocity), ' nm/sec 
']);
    fprintf(fid, ' 
');
else
    fprintf(fid, '--> No Motors Disengaged 
');
    fprintf(fid, ' 
');
end

if strcmp(lower(Hist_StepTimes), 'y')
    fprintf(fid, '*** Step Time Data *** 
');
    fprintf(fid, '-------------------- Forward Step Times (sec): 
');
    fprintf(fid, '%f 
', Fwd_StepTimes);
    fprintf(fid, ' 
');
    fprintf(fid, '-------------------- Backward Step Times (sec): 
');
    fprintf(fid, '%f 
', Back_StepTimes);
fprintf(fid, "\n");
end

if strcmp(lower(Hist_MotorsDisengage), 'y')
    fprintf(fid, "*** Disengaged Motor Data ***\n");
    fprintf(fid, "-------------------- Disengage Times (sec)\n");
    fprintf(fid, '%f\n', Data_OffTime);
    fprintf(fid, "\n");
    fprintf(fid, "-------------------- Run Lengths (nm)\n");
    fprintf(fid, '%f\n', Data_RunLength);
    fprintf(fid, '\n');
    fclose(fid);
end

fclose(fid);

end

AvgRunLength
StdevRunLength
AvgFwd_StepTime
AvgVelocity
StdevVelocity

 clear ATP NumMotors RunTime SimType SD_RC AID_RC Plot_FracMotorsState ...
 clear Hist_StepTimes Hist_MotorsDisengage SaveData FileLocation CurMotor ...
 clear StepTimes OffTime FwdA_Mask FwdB_Mask BackA_Mask BackB_Mask Mask ...
 clear Screen BinWidth ii TextBox
VITA

Gayatri Muthukrishnan

**Education:**
Dissertation: *Experimental and theoretical investigations of Kinesin-2 mechanochemistry*
Thesis Advisor: William O. Hancock

Thesis: *Patterning Surface-bound Microtubules through Reversible DNA Hybridization*


**Publications:**

**Selected Conference Presentations:**
- American Society of Cell Biology Annual Meeting – December 2007
  *Kinesin-2 and Kinesin-1 differ in both magnitude and ATP-dependence of processivity*
- Biophysical Society Annual Conference - March 2007
  *Uncovering differences in the processivity of Kinesin-2 and Kinesin-1 motors*
- Biomedical Engineering Society Annual Meeting (Platform Session) – October 2006
  *Single molecule fluorescence measurements on Kinesin-1 and Kinesin-2 motors*
- ICAM conference on Biologically Inspired Nanomaterials – November 2005
  *Motor Protein and Microtubule Tagging with Quantum Dots/Magnetic Particles for Selective Attachment and Imaging*
- Biophysical Society Annual Conference (Platform Session) – February 2004
  *Student Travel Awardee*
  *Functionalizing microtubules with single stranded DNA for biomolecular separations and directed assembly*