kinesin-1 mechanical flexibility and motor cooperation

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ABSTRACT.

Conventional kinesin (kinesin-1) transports membrane-bounded cargos such as mitochondria and vesicles along microtubules. In vivo it is likely that several kinesins move a single organelle and it is important that they operate in a coordinated fashion so that they do not interfere with each other. Evidence for coordination comes from in vitro assays, which show that the gliding speed of a microtubule driven by many kinesins is as high as one driven by just a single kinesin molecule. Coordination is thought to be facilitated by flexible domains so that when one motor is bound another can work irrespectively of their orientations. The tail of kinesin-1 is predicted to be composed of a coiled-coil with two main breaks, the “swivel” (380-442 Dm numbering) and the hinge (560-624). The rotational Brownian motion of microtubules attached to a glass surface by single kinesin molecules was analyzed and measured the torsion elasticity constant. The deletion of the hinge and subsequent tail domains increase the stiffness of the motor (8±1 k_BT/rad) compared to the full length (0.06±0.01 k_BT/rad measured previously), but does not impair motor cooperation (700±16nm/s vs. full length 756±55nm/s - speed in high motor density motility assays). Removal of the swivel domain generates a stiff construct (7±1 k_BT/rad), which is fully functional at single molecule (657±63nm/s), but it cannot work in large numbers (151±46nm/s).

Due to the similar value of flexibility for both short construct (8±11 k_BT/rad vs 7±1 1 k_BT/rad) and their different behavior at high density (700±16 nm/s vs. 151±46 nm/s) a new hypothesis is presented, the swivel might have a strain dependent conformation. Using Circular Dichroism and Fluorescence the secondary structure of this tail region was studied. The central part of the swivel is dimeric α-helical and it is surrounded by random coils, thereby named helix-coil (HC) region. Furthermore, an experimental set-up is developed to exert a torque on individual kinesin molecules using hydrodynamic flow. The results obtained suggest for the first time the possibility that a structural element within the kinesin tail (HC region) has a force-dependent conformation and that this allows motor cooperation.
Estoy solo en esta lucha.
No tengo quién vigile mis pasos por el laberinto
o que me indique cómo salir de él
cuando llegue el momento.
Por eso hay que pensar bien;
Pese a la confusión habrá que ordenar
la concatenación de los hechos
con calma y a sangre fría,
sin exagerar, sin dramatizar,
buscando explicaciones escuetas y
palabras claras que permitan diferenciar
las cosas de los fantasmas y
los hechos de los sueños.

Deliro
Laura Restepo

Tamerlane: I shall rule destinies!
[gets stabbed from behind]
Zoar: you can’t even rule your own.

Dnevnoy Dozor
Timur Bekmambetov
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CHAPTER ONE. INTRODUCTION.

The motions of living and non-living things have interested humans since immemorial times. These motions have been described ranging from celestial to sub-atomic objects. But living things have resisted this scrutiny, at least in some sense. The movements of cells and their molecular constituents have received expanded attention only in the last century, which also brought them into proper discussion. From the cellular perspective, vesicles need to be transported inside cells and large distances have to be traveled (e.g. fast axonal transport). The molecules involved in some of these processes, nowadays known as kinesins, were discovered not so long ago, in the mid 1980’s\(^1\), identified in their molecular context and named in the last two decades [1]. But how these molecules work is still fuel for research for many years to come. Many studies, from chemical kinetics to newly applied optical techniques, have shed light in the complex mechanism of what is called mechanochemistry [2], i.e. the transformation of chemical energy into mechanical work.

In addition to the problem of intracellular transport, the molecular motor field (including myosin, dynein, ATPase, etc.) has also served to generate an enormous amount of effort on the theoretical side. The so-called “ratchets” noise-induced transport, generically known as “Brownian motors”[3, 4], are now an active branch of non-equilibrium statistical mechanics in what it is called “biological inspired physics”. The experiments are essential to prove or disprove specific models, and theory is required for the complete understanding of mechanisms and to predict new and undiscovered behaviors.

**The Problem of Intracellular Transport.** Intracellular transport poses a problem from a biological- and physical-oriented approach. Biologists tend to think in regulatory and evolutionary terms, so vesicles as well as mitochondria must be transported within the cell by means of proteins that are regulated. On their side, physicists argue that active transport is necessary since given the physical dimensions of a cell and the viscosity of its cytoplasm, diffusion would not achieve transport that would allow the fast cellular responses observed.
A neuron can have an axon of almost a meter long, with extreme polarity and quite a range of shapes. Small particles (e.g., 40nm in diameter) have a diffusion constant of 0.6μm²/sec, which would take ~26.4 thousand years to reach the synaptic terminal from the soma. Even a small protein of 3nm in radius would take 150 years to diffuse a distance of 1 meter[2].

Movement of material towards the synaptic bottom or to the neuronal soma defined what was called axonal transport. This transport can be anterograde (soma to synaptic terminal) or retrograde (towards the cell body). Currently anterograde is a more general term to classify motion from the soma to the periphery in any cell type. Either by differential interference contrast microscopy (DIC) or by radio-labelling[5, 6], it was shown at the beginning of the eighties that different motion rates existed, ranging from very fast (1.2-4.6μm/sec⁰) to very slow (0.03-0.2μm/sec), with intermediary speeds of about 0.2-0.8μm/sec. Retrograde transport was showed to have a very fast speed of ~3.5μm/sec.

Today we know that kinesin’s speed ranges from 0.06-2μm/sec depending on the family and class to which it belongs[2]. Very recently, a mean speed of ~12μm/sec has been observed for GFP-endosomes driven by kinesin-1[7] in Drosophila S2-cells, raising many questions[8] about the speeds achieved in vivo by kinesin or other motors.

**Discovery of kinesin.** How is an organelle transported along an axon? Or, how does an animal cell transport organelles within its cytoplasm? These were questions that had no answer prior to 1985.

The established properties of fast axonal transport were[5, 6]:
1) It used microtubules as its railroad to move along.
2) Translocation of the cargo was ATP dependent (either anterograde or retrograde).

Since it depends on ATP, it is inhibited by AMP-PNP (a non-hydrolyzable ATP analog), which upon addition to axonal extracts formed stable vesicle-microtubule complexes that could be purified.

---

¹ All speeds are given in μm/sec that I have substituted from the common mm/day that literature of the 80’s used.
Myosin and dynein were known in 1985, but neither of them could be responsible to axonal transport. This is because myosin interacts with actin, and dynein moves towards the minus ends of microtubules, which are located at the neuronal soma. In addition, any attempts to identify dynein in neural tissue were not very successful in the seventies.

In 1985 Ron Vale, et al. [1] observed that the ATPase activity associated with the fractions that produce motion was very low (0.1sec\(^{-1}\)). This was counterintuitive since they were looking for the enzyme that used ATP to move cargo at speeds up to 2\(\mu\)m/sec, and more energy (and a higher ATPase rate) would be required.

The discovered protein was ~600 kilodaltons (kDa) and contained two 110-120kDa and two 60-70 kDa polypeptides. The 60kDa protein co-migrated with the 110kDa peak and densitometry scans of Coomassie-stained gels gave a stoichiometry of 1.5:1 to 2.1:2, which argued for a tight 1:1 association between these two peptides. They named the protein “kinesin”, which comes from the Greek \textit{kinein}, meaning to move[1].

All of the experiments that led to the discovery of kinesin were performed in the laboratory of Scott T. Brady while he was in the Marine Biological Laboratory, Woods Hole, Massachusetts. He then moved to the Department of Cell Biology of the University of Texas Health Science Center, Dallas, Texas. The three authors of the original paper, Ronald Vale, Thomas S. Reese and Michael P. Sheetz were there for a very short time (Vale and Sheetz were actually at the University of Connecticut Health Center) and Scottt had some samples in the freezer…

One interesting thing about the paper that began the field and named the protein is that although it used motility assays to identify the protein responsible for microtubule translocation, there is not a single picture depicting motion of a microtubule or a bead in the paper. They used co-sedimentation assays to isolate the protein that would associate with microtubules in the presence of AMP-PNP and release it when replaced by ATP. By 1985 the gliding assay was a very common technique in the axonal transport field, which could explain why it does not appear in the paper. The Vale, et. al., paper appeared in the August issue of \textit{Cell} and a month later on September 5\textsuperscript{th} of 1985, Scott Brady published a letter in \textit{Nature} which, identified of a similar 130kD polypeptide and showed that AMP-PNP treatment prevented depletion, caused by ATP, of membranous material
associated with microtubules in axon preparations[9]. Moreover, the electron micrographs showed small “side arms” in the presence of AMP-PNP linking the membranous material to the microtubules. He suggested that “the binding of ATP (or AMP-PNP) facilitates interaction of the protein with a microtubule and hydrolysis of ATP promotes release. The result would be an ATP-dependent cycle of binding and release of the protein from the microtubule. With the concentration of ATP used in these studies, the ATPase activity would result in the ATPase being primarily in the release or soluble state.” This was the first statement suggesting how the molecule could have an ATP hydrolysis cycle and how this would be coupled to motion of the cargo.

An uncommented fact is that most of the measurements of fast axonal transport gave speeds of 1.1-3.5 μm/sec, which no neuronal kinesin known to date is able to achieve in vitro (the kinesin with the fastest in vitro speed is Neurospora crassa kinesin with 2μm/sec [10]).

**The first five years.** In November of 1986 Sergei Kuznetsov and Vladimir I. Gelfand [11] solved the discrepancy of the low ATPase activity measured by Vale in 1985 and the ability of the protein to translocate beads or membranous cargo along microtubules at fast speeds, which would assume an ATPase of at least an order of magnitude higher. Using purified kinesin from bovine brain, they provided direct evidence that its ATPase activity depends on whether or not it is associated with microtubules. The rate increased from 0.07μmol·min⁻¹·mg⁻¹ in absence to 4.6 μmol·min⁻¹·mg⁻¹ in the presence of microtubules. The insight that the ATPase activity might be stimulated by microtubules is not trivial given that no other ATPase known at that date had an intrinsic activity with the exception of myosin and dynein which are also activated in the presence of the filament along which they translocate. They used the similarity with myosin and dynein to infer that “the main difference between the conditions of ATPase activity and motility assays was the presence of the microtubules in the latter but not in the former case.” This led them to add taxol-stabilized microtubules to the assay and a dramatic increase in the measured ATPase activity was observed. Discovering that kinesin is a microtubule-activated ATPase. The final idea they had was that the rate-limiting step was the binding of ATP and that the active site for binding to the microtubule was formed directly by the ATP
and the kinesin molecule. Both of these have been shown to be wrong, since the ADP release is the rate-limiting step, and the active site for ATP binding is different from the one for microtubule association. Knowing that this enzyme should move cargo from one place to another along microtubules in an ATP-dependant (cyclic) manner, two questions were asked: “What is the molecular mechanism of the kinesin ATPase working cycle? and, what is the coupling mechanism of ATP hydrolysis to mechanical work?” Both have given origin to a new set of techniques and have provided most of the impetus seen until today.

Hydrolysis of ATP requires the dissociation of a water molecule. A hydroxyl ion attacks the gamma phosphate and forms a pentaphosphate transition state. The product is a HPO_4 molecule where one of the oxygen atoms comes from the water molecule. Oxygen isotope (^18O) exchange can then be used to measure the rate at which ATP is hydrolyzed and products are released. The hydrolysis reaction can be written as:

\[
E + ATP \leftrightarrow E \cdot ATP \leftrightarrow E \cdot ADP \cdot Pi \leftrightarrow E + ADP + Pi
\]

where \(E\) is the enzyme and \(k_i\) the rate constant of the step \(i\). If \(k_3 \gg k^-2\), then no reversal of step 2 occurs and only one water-derived oxygen is incorporated into the released Pi. However, if \(k^-2\) is greater than or comparable to \(k_3\), reversal of the hydrolysis step can occur and more than one water-based oxygen can be incorporated into the Pi before release. David Hackney used this approach in 1988 to show that the Pi produced by the kinesin microtubule-activated ATP hydrolysis incorporated one and only one ^18O, providing evidence that the rate limiting step was the release of ADP from the active site. Together with these results was the discovery that ADP is tightly bound to the kinesin molecule and when isolated contains stoichiometric amounts of bound ADP which is resistant to removal [12].

The first piece of direct evidence that indeed the ATPase activity kinesin was coupled to the production of force and movement came in 1987 by Stanley A. Cohn, Amie L. Ingold and Jonathan M. Scholey. They measured the inhibition of ATP hydrolysis in solution by AMP-PNP, vanadate, addition of Mg-free ATP, chelation of Mg by EDTA, and the same reagents inhibited microtubule motion in a gliding assay [13].

Soon it was recognized that only the heavy chains and in particular their globular domains were required to produce force and microtubule motility in vitro [14]. These
works also established the expression of full length and/or truncated kinesin in *E. coli*, providing the means to study it further.

![Figure 1.1. EM of full length kinesin-1. Bar represents 100nm.](image)

**Figure 1.1.** EM of full length kinesin-1. Bar represents 100nm.

![Figure 1.2. EM of a kinesin molecule attached to a vesicle along a microtubule](image)

**Figure 1.2.** EM of a kinesin molecule attached to a vesicle along a microtubule [15]

Electron microscopy (EM) studies shed light on the structure of kinesin. Figure 1.1 shows micrographs of full-length adrenal medulla kinesin [15]. Figure 1.1 also shows
the two globular or motor domains and the rod-like tail with one interruption in the middle.

It was Nobutaka Hirokawa who first photographed a kinesin molecule attached to a vesicle and a microtubule [15]. It was thanks to a quick-freeze, deep-etch EM that a cross-link between organelles and the neuronal microtubule cytoskeleton was observed (figure 1.2).

The concept of processivity was coined in 1989 by Jonathon Howard, Jim Hudspeth and Ron Vale [16] when using a kinesin-coated surface showed that one kinesin molecule was enough to move a microtubule up to its own length without detaching from the microtubule. A single kinesin molecule, therefore, is able to take many hundred of steps along the microtubule lattice without dissociating from it and hydrolyze ATP at the same time. That same paper also shows that microtubule speed is independent of the kinesin density, something we will explore further in Chapter 3. Later on, in 1990, similar results were obtained when adsorbing kinesin molecules to silica beads and observing them move several hundreds of nanometers [17].

**Kinesin structure.** The kinesin-1 molecule of *Drosophila melanogaster* is a tetramer *in vivo* [18], consisting of two heavy chains (figure 1.3) and two light chains (KLC).

![Figure 1.3. Structure of kinesin-1 [19]](image)

The two heavy chains of kinesin dimerize into a functional molecule, where each heavy chain consists of a motor domain (1-325aa)$^2$, neck linker (325-340aa) and neck (coiled-

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$^2$ *Drosophila melanogaster* kinesin numbering.
coil 345-378aa). The rest of the tail is mainly a coiled-coil with two interruptions (378-440aa and 560-624aa) the second break is also known as the Hinge or Kink (figure 1.4).

Crystallographic methods have been applied to study several motor domains from different kinesins [20]). The kinesin-3 KIF1A is the only one for which the structure has been solved in two different nucleotide states, the ATP-like AMPPCP- and the ADP-bound form [21].

![Figure 1.4. Left. Structure of monomeric kinesin (rK354) in ribbon representation. The motor domain is a cone-shaped alpha/beta structure, the tip of the cone pointing to the bottom, in this view. The central beta sheet (beta-1 to beta-8) is seen roughly face-on with helices alpha-1, alpha-2, and alpha-3 in front of the core sheet, helices alpha-4, alpha-5, and alpha-6 in the back. Helix alpha-7 of the neck region emerges at the tip of the cone and points to the left side. Right. Ribbon representation of monomeric kinesin as in Figure 1a, view after 180-degree rotation about the vertical axis. Parts involved in microtubule binding are shown in green (MT1 = loop L7 - beta5 - loop L8a, MT2 = alpha4 - loop L12 - alpha5). Structural elements painted with purple color are involved in nucleotide binding [22]).](image-url)

The motor domain is a cone-shaped alpha/beta structure (figure 1.4). The central beta sheet (beta-1 to beta-8) is seen roughly face-on with helices alpha-1, alpha-2, and alpha-3
in front of the core sheet, helices alpha-4, alpha-5, and alpha-6 in the back. Helix alpha-7 of the neck region emerges at the tip of the cone and points to the left side [22]). The folds of all known kinesin structures within the catalytic core are similar, as expected from their sequence homology (around 40%).

Figure. 1.5. Kinesin dimer with multiple neck – swivel conformations (357-386aa) determined by NMR methods [23] superimposed on the X-Ray structure of dimeric rat kinesin motor domain RnKin379 (Kozielski et al., 1997). Taken from http://www.mpasmb-hamburg.mpg.de/ (Eckhard Mandelkow Lab)

The neck has been shown to be essential in several kinesin families for properties such as directionality of motion or activity regulation. Together, the catalytic core and the neck form the motor domain. The highest deviation between kinesin structures are found in those regions which have been proposed to change conformation during the ATP hydrolysis cycle, such as switch-1, switch-2 and the microtubule-binding surface (green and purple regions in figure 1.4). The other regions that show great dissimilarity between structures are the neck and the neck linker. These are largely undefined in most structures, which indicate a highly mobile structure in this region. A well-defined neck
linker (docked onto the body of the motor domain) and $\alpha$-helical neck is visible in the rat kinesin-1 (figure 1.5).

The coiled-coil stalk of kinesin-1 is interrupted by several non-$\alpha$-helical regions which presumably operate as hinges, allowing the stalk to kink and swivel [24]). The first break in the coiled-coil (also known as the “swivel”) comprises the residues 378-440 for $Dm$ kinesin-1 and indeed the neck helix terminates at W370 in the crystal structure of $Rn$ kinesin-1 (figure 1.5). NMR structure of the peptide $Rn$ K357-D386, containing the second half of the neck helix and the first half of the break, corroborates this picture (figure 1.5). These results from NMR have also been supported by Circular Dichroism measurements [25].

Figure 1.6. Rotary-shadowed stalk protein from kinesin-1, several representatives. The molecules in the lower panels show bent conformations commonly found among each preparation (taken from [26]). Bar, 100nm.

De Cuevas, et. al., presented data in support that the coiled-coil tail is parallel [26] and able to bend approximately in the middle using peptides derived from the tail [26] (figure 1.6). Similarly, Hirokawa showed that the full length kinesin molecule could bend or fold [27] (See figure 1.2).

Coy et. al., showed that the hinge allows the kinesin molecule to fold onto itself and thereby inhibit the ATPase activity of the motor domain. David Hackney, et. al., showed that a specific set of amino acids perform this job by mutating these 3 residues the inhibition was abolished.

Howard, et al. proved that the speed of gliding microtubules is independent of the density of motors, i.e. the speed at which a microtubule is moved does not depend on the
number of kinesin molecules that drive this motion [16]. Given that there is no evidence that would make us think of synchronization (biochemical or mechanical) this result suggests that the molecule is flexible enough to allow a large number of motors to work together. The elements that could allow was though to be the coiled-coil breaks within the stalk. Although many kinesins from homodimers, kinesin-3 can move processively as monomers. Members of kinesin-2 form heterodimers, and there are even some like the members of kinesin-5 family (Eg5) that work as homotetramers.

**Microtubules.** Here I give an overview of microtubule structure and important aspects that affect kinesin motion, for other sources I refer the reader to [2, 28].

Fig. 1.7. Structure of the αβ-tubulin dimer, in red are α-helices, in blue β-turns, in yellow loops, pink extended helices and in gray unstructured elements; nucleotides are depicted by green circles. Made with VMD® from PDB code 1TUB.

Microtubules are key cytoskeletal components of every eukaryotic cell, and play important roles in cell motility and division. A microtubule is a hollow tube of approximately 25nm in outer diameter and 18nm of inner diameter. A microtubule is also a polymer and its building block is the tubulin dimer made of 1 α-tubulin and 1 β-tubulin,
the αβ-tubulin heterodimer in a head-to-tail conformation (see figure 1.7). The tubulin monomer is a GTPase, so each subunit binds and hydrolyses GTP. In a dimer, the GTP of the α-tubulin is buried and is not hydrolyzed, in contrast to the GTP of the β-tubulin, which is in close contact to the α-tubulin and is rapidly hydrolyzed.

Because of the αβ-tubulin heterodimer head-to-tail conformation each protofilament and the microtubule as a whole has a defined polarity. The β-tubulin end is called the plus (+) end and the α-tubulin side is the minus (-) end. The plus end was originally named as such because polymerization occurs much faster at this end than at the other end. The lateral association of protofilaments and its consequent closure into a tube form the microtubules. The number of protofilaments is normally 13, and due to a small offset of their lateral association (~0.92nm) after 13 protofilaments there is a total offset of 3 tubulin subunits. Microtubules grow mainly from the microtubule-organizing center (MTOC) that contains γ-tubulin as the nucleation factor. The MTOC is close to the nucleus in interphase cells, so most of the plus ends are towards the cell cortex. This array, together with the polarity of microtubules, establishes directionality for intracellular transport. In the axon and dendrites, microtubules run in a longitudinal orientation (), and serve as one way highways, with the plus end pointing away from the cell body. Moreover, microtubules in proximal dendrites are of mixed polarity (). Microtubules in neurites are usually more stable than those in most post-mitotic cells, owing to the presence of neuronal microtubule associated proteins (MAPs).

Combinations of cross-linking or footprinting experiments with mass spectrometry or Western-blot analysis confirmed that the motor domain of kinesins studied so far share a very similar tubulin-surface interaction site [29]. The microtubule-binding site of kinesin-1 has been mapped in detail biochemically using cross-linking studies and alanine scanning techniques [30-33]. Docking of the crystal structure into 3D electron density maps of kinesin-decorated microtubules confirmed the biochemical findings [34]. The switch-II cluster (α4/L11/α5) of kinesin-1 interacts with the helix H12 of β-tubulin while the region β5a/b forms partial contacts with both the β- and the α-subunit [35, 36].

Given the contact interface between the kinesin motor domain and the microtubule (figure 1.8), the interaction is very sensitive to post-translational
modifications of the tubulin c-terminus, especially in the β-subunit. It has been shown that the removal of the c-terminal part of tubulin reduces kinesin processivity [37]. Although several modifications are known to occur in vivo [38] practically none of their effects on microtubule-based motor are understood.

Figure 1.8. Docking of kinesin head structure with the microtubule. Taken from http://www.mpasmb-hamburg.mpg.de/ (Eckhard Mandelkow Lab)

In vivo functions of kinesin. The ~360-residue globular domain is the hallmark of the kinesin superfamily proteins (KIFs). The motor domain is the one signature for all families, i.e. it is well conserved, whereas the tail region is highly divergent, even within a family. Most families have the motor domain at the N-terminus and have microtubule plus-end directionality [39]. By contrast, kinesin-13’s have the motor domain in the middle of their sequence and do not seem to follow the 1-ATP-per-8nm-step mechanism common in many families. Instead, they seem to promote microtubule depolymerization
at both ends [24]. Yet another family, kinesin-14, has the motor domain at the C-terminus and move towards the microtubule minus end.

I will only consider vesicle transport in neurons as an example of a biological situation where one or more than one type of kinesin have been implicated in order to give an overview of kinesin function in vivo. For comprehensive reviews and other cellular and physiological roles I refer the reader to [20] (Hirokawa) [40].

KIF17 from the kinesin-2 family is a plus-end directed motor that is localized in dendrites and specifically involved in the transport of vesicles containing the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor from the cell body to the postsynaptic sites [41]. Transport of the NMDA receptor by KIF17 is physiologically important for learning and memory, as has been demonstrated by transgenic mice (ref). When over expressed in the postnatal forebrain, the mice showed improved performance in a delay-matching-place task and a Morris water maze task, suggesting that their spatial learning and memory, respectively, were enhanced.

It was initially assumed that transmembrane cargo proteins would bind directly to specific motors. However, it is now clear that kinesins tend to use adaptor/scaffold protein complexes for cargo recognition and binding. For example, it seems now that cargoes bind both to the C-terminal tail of kinesin-1 and to the kinesin light chains (KLC). Binding to either one depends on which direction the cargo is going to take, KLC binding for transport along axons and binding to the C-terminal tail of kinesin-1 for dendrites, like RNA granules [42].

Kinesin dysfunction can be a cause of disease. For example, it has been recently shown that a mutation in a kinesin-3 (KIF1Bβ), which transports synaptic vesicle precursors in the axon, causes a human peripheral neuropathy, Charcot-Marie-Tooth disease type 2A (CMT2A)[43-45]. Another example is Alzheimer’s disease, where the amyloid-β-peptide is deposited in the brain. Amyloid-β is produced by cleavage of a type I integral membrane protein, the amyloid precursor protein (APP), by β- and γ-secretases. The latter is a large multiprotein complex in which presenilin 1 and presinilin 2 are thought to be catalytic subunits. It has been shown that kinesin-1 mediates the anterograde transport of vesicles containing APP, β-secretase and presenilin-1 in axons [46, 47]. Proteolytic processing of APP can occur in this membrane compartment.
Kinesin-1 also transports oligomeric tubulin in a large transport complex that is distinct from those of stable polymers or other cytosolic proteins (vale). When fluorescently labelled tubulin is microinjected into axons it moves at a speed that is compatible with slow axonal transport.

The existence of distinct fast and slow components of axonal transport has been known for more than 25 years, but the mechanistic significance underlying these different rates of movement has been obscure for most of that time. The principal reason for this protracted period of uncertainty has been our inability to observe slow axonal transport directly in living cells. The recent discovery that cytoskeletal polymers conveyed by slow axonal transport actually move as fast as membranous organelles indicates that both fast and slow axonal transport may be generated by fast motors. Cargoes as diverse as vesicles, mitochondria, and neurofilaments all move at comparable speeds but they differ in the proportion of the time that they spend moving, i.e., the average speed of movement is slow because the motion is both infrequent and bidirectional [48].

Although initially just viewed as curiosity, the ‘saltatory’ motion of cellular organelles [49] – where the moving organelle starts, stops and changes direction – is now recognized as a widespread phenomenon. Cargoes as diverse as endosomes [50], secretory vesicles [50], mitochondria [51], intermediate filaments [50] and virus particles [52] have been shown to move bidirectionally as have many identified and unidentified vesicles in neuronal axons and growth cones [50]. Such cargoes employ motors of opposite polarity in rapid succession. Although these cargoes constantly reverse their direction of motion, they can achieve polarized distributions within the cell, by biasing the time they spend moving towards the plus or minus end. The problem of bidirectionality has proven to be more complex than previously thought. For example, motors of both directionalities are stably attached to some cargoes, so that regulation of the connection between motors and cargoes is not how the problem is solved. Instead, directionality and timing of motion depend on correctly tuning the activities of the opposing motors, thereby modulating their run length. As we will see in the next section, motor properties may be regulated by force, and hence, processivity or run length may depend on the forces motor exerts when moving cargo inside the cell.
Kinesin mechanochemistry. At the beginning of the chapter, we explored the structure and \textit{in vivo} functions of kinesin, along with the filament it moves on. Now, let’s dwell a little longer on how it uses the energy stored in ATP to produce motion and on the force it can generate.

In order to produce motion with ATP hydrolysis (Equation 1.1) there must be at least two states with respect to microtubule association: a bound and an unbound state, i.e., $E \cdot M$ and $E + M$. From Equation 1.1 we saw as well that there are 3 fundamental states: ATP-bound, ADP-bound and nucleotide free active sites. So, how do these enzyme states relate to microtubule binding? Let’s have a look at the structural aspects first.

Structurally the ATP-bound and ADP-bound states exhibit only a small difference between the presence and absence of the $\gamma$-phosphate at the active site, but this small difference triggers a cascade of structural alterations. These alterations are then propagated to distal parts of the kinesin motor domain through the P-loop and to the switch-1 and -2 regions. The mechanism is similar to the movements in EF-Tu or the swing of the lever arm in myosin-II [53, 54]. Distinct enzyme states would have a different affinity for the microtubule (or actin in the myosin case). In general the nucleotide pocket (see figure 1.5) is considered to be either in the OPEN or the CLOSED form, as measured by the distance between the first glycine in the P-loop (corresponding to G86 in RnKin) and the conserved glycine in the switch-2 region (corresponding to G235 in RnKin). The closed form is thought to correspond to the tight binding of ATP. Translation and amplification of the small local changes at the nucleotide sensing switch regions into large-scale effects seems to rely on a mechanism involving the switch-2 helix ($\alpha 4$). In the end, it is not rotation of a rigid lever arm as in myosin that takes place, but the modulation of surface properties which control the docking of the flexible neck linker onto the core domain [55-57].

So far, comparison of kinesin structures from different sources has given us some information about the structural rearrangements that take place during ATP hydrolysis (Chapter 12 from [20]):

1) Each kinesin structure that has been determined shows a unique structure for the switching region.
2) The length of the switch-1 helix, $\alpha_3$ is different and the helix $\alpha_3a$ shows different secondary structures: $\alpha$-helix or $\beta$-strand.

3) The “relay” helix, $\alpha_4$, is variable in length and can be tilted around 20° between different structures.

4) Loop 11 in the switch-2 region is mostly disordered except for NcKin, but the length of the disordered loop differs.

5) There are different sets of salt bridges present in different structures.

6) The neck linker region is mostly disordered but it is ‘docked’ close to the catalytic core of the molecule in RnKin [58], semi-docked in NcKin [36] and possibly docked in KIF1A-AMPPCP [59].

Processivity was first shown by [16] and [17], the latter gave a measure of how far the kinesin molecule would go without dissociating from the microtubule, that being 1µm. Several other sources have also shown that kinesin is processive by giving hundreds of 8nm steps per microtubule encounter [60-62] or by showing that each encounter resulted in the hydrolysis of ~120 ATP molecules [63].

To define the exact path of a kinesin molecule along the microtubule lattice, microtubules of different protofilament number were used. As we saw in the microtubule section, microtubules have a variable protofilament number and with it the axis of each protofilament can be either parallel, left- or right-handed compared to the microtubule axis. Knowing this, a simple trick was envisioned and using conditions to grow them in either one of the orientations, microtubules were observed to rotate in a gliding assay [64], giving proof that indeed the kinesin molecule follows one protofilament with high fidelity. Recently, with the use of quantum dots attached to the ends of microtubules, the results have been reproduced by using TIRF to observe the quantum dots go up and down in fluorescence, consistent with rotating microtubules.

The amount of ATP consumed per 8nm step was measured using kinesin-coated beads or truncated versions, given the fact that the full-length molecule can fold onto itself and inhibit ATPase activity. Dimeric kinesin has an ATPase rate of ~100 ATP/sec. Correlating this with the speed of a bead or a microtubule of ~800 nm/s, the coupling is 1 ATP molecule per 8nm step [65].
What makes the kinesin motor processive is how both heads are coordinated, and this was a bit harder to prove. Let’s start with one-headed kinesin. By means of a construct that contains as much full length structure as possible (all coiled-coil domain) but only one head Hancock and Howard proved that the one-headed kinesin was not able to move processively on the microtubule lattice, while the wild type can [50]. Kinetically the one-headed kinesin detaches from the microtubule lattice with a rate of 3 sec⁻¹, while the two-headed has a rate of 50 sec⁻¹, which accounts for the 100 steps/sec. Therefore the second head promotes ATP hydrolysis and microtubule detachment on the first head. Hence, two heads are needed to move processively and continually hydrolyze ATP while bound to the microtubule.

Here I have reviewed some of the literature and arguments for kinesin processivity and coordination between heads, for more information I suggest a couple of excellent reviews (Chapter 10 from [20], The two head review).

So far we know that kinesin takes 8nm steps per ATP that it hydrolyzes, and that both heads are required to be processive, but we have not said anything about the forces that it generates, which would require a section on its own.

With the development of optical tweezers, it became possible to measure picoNewton forces[62] exerted by single kinesin molecules [66]. Close to the trap center the tweezers behave as an elastic spring with a spring constant \( \sim Q \left( \frac{n^2 P}{\lambda c} \right) \), where \( nP/c \) is the force exerted by light on a perfect absorber and \( Q \) is a dimensionless constant called the trapping efficiency. A typical trapping efficiency is \( \sim 0.1 \) therefore the forces are \( \sim 0.5 \) pN/mW of the laser power [2, 67]. With either polystyrene or silica beads the spring constants are in the range of 0.01-0.1 pN/nm [62]. The kinesin molecule would then pull the bead out of the focal plane, and then the trap exerts a force back on the kinesin. A maximal force of \( \sim 6 \) pN has been measured. This result has been corroborated by glass-needles or buckling microtubules by single kinesin molecules, given forces of 5.4pN [68] and 6pN [69].

In general a force-dependence of a reaction rate in a chemical reaction is written as (Howard2001) (BustamanteRev):

\[
k(F) = k(F = 0) \cdot e^{\frac{\Delta G - Fdx}{k_BT}}
\]
where $k(F=0)$ is the reaction rate in the absence of force, $\Delta G$ is the Gibbs free energy of the reaction, $F$ is an external force applied by a distance $dx$, $k_B$ is the Boltzmann constant and $T$ the absolute temperature. Which of the transition states of kinesin ATP hydrolysis are force dependent? To investigate this question different ATP concentrations were used as well as ADP and/or the presence of Pi. At high ATP concentration (e.g. 1mM) the speed of kinesin was shown to have an almost linear dependence with force [60-62, 68]. At low ATP concentrations, when the rate-limiting step is ATP binding, the speed displayed a similar response to force [70].

Using a similar approach but with the nucleotide analog AMP-PNP (non-hydrolysable ATP), Kawaguchi and Ishiwata proved that the nucleotide-free and the ADP state are single-headed microtubule bound states. The bound state was measured by the amount of force that it took to unbind them from the MT-lattice. The presence of ATP in the nucleotide-binding pocket makes the kinesin dimer to bind with both heads to the microtubule [71].

One fundamental question that we are still missing is: how does kinesin actually step? Or, what can we do with two legs? Figure 1.9 show us the alternatives, each head does exactly the same every time (figure 1.9a). This mechanism would imply a 180-degree rotation with every step and it was suggested originally by Jonathon Howard [72]. One can also think of an inchworm model (figure 1.9b), where only one of the heads is active and the other head follows, something like a crouching mechanism. Finally, the most anthropocentric one figure 1.9c, we switch from right to left and left to right each time.
Figure 1.9 The three possible mechanisms of kinesin stepping in side and top view. 
a) Symmetric hand-over-hand, b) Inchworm and c) Asymmetric hand-over-hand. [73]).
CHAPTER TWO. INHIBITION BY ADP AND PHOSPHATE

INTRO.

Whether the model for stepping is an asymmetric or a symmetric hand-over-hand, their kinetic mechanism is the same (figure 2.1a). In contrast, the inchworm model, shown in figure 2.1b, is kinetically different from both hand-over-hand models. For both models every 8nm step requires 1 ATP molecule. In the inchworm model one head always leads and the other always follows, this means that only one head is hydrolyzing ATP. Both diagrams are displayed in figure 2.1 and each one of them can be written as:

Hand-over-hand

\[ E \xrightarrow{\text{ATP}} E \cdot D \cdot P_i \xrightarrow{\text{ADP}} E \cdot D \xrightarrow{\text{Pi}} E \]

Inchworm

\[ E \xrightarrow{\text{ATP}} E \cdot D \cdot P_i \xrightarrow{\text{Pi}} E \cdot P_i \xrightarrow{\text{ADP}} E \]

Figure 2.1 Kinetic schemes for both heads treated as one enzyme. a) Hand-Over-Hand; b) Inchworm model. Taken from [74]

The distinctive feature of each kinetic mechanism is the sequence of release of ADP and Pi. Taking both heads as a single enzyme, the inchworm model predicts an ATPase behavior similar to any other ATPase or GTPase, where the Pi is the first product to be released. This is because only one head is active. For either of the hand-over-hand
mechanisms, the ADP will be released first, i.e., on ATP binding and hydrolysis to one head, ADP is released off the other head before the Pi is released from the head that just hydrolyzed ATP.

In the presence of hydrolysis products, ADP or Pi, the backward reaction rates in a kinetic scheme become non-zero, inhibiting the forward reaction. Enzyme inhibition has been extensively studied and provides a framework to make predictions and interpret results [Segel]. For a three-state reaction mechanism, it is predicted that the first released product would be a so-called “mixed” inhibitor, followed by the second released product acting as a “competitive” inhibitor. In terms of models this means that in the hand-over-hand model the ADP would be a mixed inhibitor, while the Pi is only a competitive inhibitor. On the other hand, in the inchworm model the Pi would be have a mixed behavior and the ADP the competitive one. Here, the kinetic mechanism is elucidated by means of product inhibition. The speed of reaction is measured as the speed of kinesin-driven microtubules.

The work of this Chapter was a joint effort with William Schief and Rutilio Clark from the University of Washington, USA. They performed the experiments that led to figure 2.2. The author of this thesis studied the single-molecule level and is fully responsible for figures 2.3, 2.4 and table 2.1.

METHOD.

Protein and reagents: Kinesin was purified from bovine brain[75]. Full length *Drosophila* kinesin was expressed in *E.coli* and purified using a Ni-NTA column (Amersham Biosciences) as follows. Agarose-plates were prepared with 100µg/ml of Ampicillin (100mg/ml sock in H2O) and 34µg/ml of Chloramphenicol (34mg/ml sock in EtOH) using L-agar stock from the media kitchen (MPI-CBG). Similarly LB broth from the media kitchen was used. Cells were thawed and kept on ice. 0.5µl of the plasmid stock was added to 50µl of cells, mixed gently by tapping the tube with a finger and were incubated on ice for 10-20 min. Cells were heat shocked at 42°C for 60s then placed back on ice immediately. Cells recover with the addition of 950µl of fresh LB without antibiotics 2 minutes after the heat shock and shaking them for 45 min at 37°C at 300rpm in a Thermomixer. Cells were
centrifuged at 3,000g for 2 min in a Heraeus biofuge fresco. 700µl of the supernatant was discarded and the pellet was resuspended. 100µl of cells were spread on agar plates with antibiotics. The plates were incubated gel side on top overnight at 37°C, without shaking. One colony was taken from the plate using a sterile toothpick or inoculation loop and transferred it into 3 ml of LB-antibiotics in a 15ml air exchanging falcon tube with loose lid. The colony was grown over-night at 37°C shaking at 160-180 rpm. Cells were collected by centrifuging them at 3,000 rpm for 5 min. The supernatant was discarded, and the pellet resuspended in 1 ml of fresh LB-antibiotic broth. The solution was added to a 750ml of pre-warmed (at 37°C) LB-antibiotic. The re-suspension was grown and shaked at 180 rpm at 37°C in a sterile non-aerating 2.8L flask. The OD at λ=600nm was measured until it reached 0.5 (1cm pathlength cuvette, UV-Spec Agilent). 1ml sample of the cells was taken for SDS-PAGE analysis of uninduced cells. Cells were cooled to 15°C on a bed of ice, induced with 1 mM of IPTG. The flask was shaked at 180 rpm at 15°C overnight. 1ml sample of the induced cells was taken for SDS-PAGE analysis. The weight of the centrifuge tubes was recorded (Beckman Centrifuge Bottles Polycarbonate 1L, Order #366751) as the weight of the pellet was to be determined after centrifugation. The culture was centrifuged at 8000xg for 10 minutes at 4°C in a Beckman Coulter Avanti J-20 centrifuge (rotor JLA.8.1000). The supernatant was discarded and the pellet resuspended in an equal volume of phosphate buffer saline PBS (by weight-volume), and kept in cold. Cells were frozen in a 50ml falcon tube placed in the net of the N2-dewar and filled with N2. The cells were pipetted into N2(l) and frozen as little pearls. The pearls were ground to a paste in the N2(l) cooled mortar and pestle, the powdered cells were then transferred to a 50 ml falcon by using a pre-cooled spoon and kept cold. All these protein purification steps were made in a 4°C room. The powdered cells were redissolved in a volume of cold Lysis buffer (1g of pellet in 10 ml lysis buffer (50mM Na2HPO4, 300mM NaCl, 10% glycerol and 40mM imidazole at 7.5pH)). This was called "Crude Extract", and 50µl were taken for SDS-PAGE analysis. The cells and DNA were sheared by sonicating the crude extract in a 0°C ice bath using the Sonifier W-450 D, Branson Digital Sonifier, set to 40%, 6x10sec with 20sec off in between. To pellet the cellular debris, this sonicated solution was centrifuged in a Beckman Centrifuge Bottles # 355622, 70ml at 38000 rpm for 1 hour using a Type 45 Ti rotor at 4°C, in a Beckman Optima LE80K
ultracentrifuge. 50 µl of the sample were taken for SDS-PAGE analysis. The supernatant was filtered through a Millipore 0.22µm gauer lock syringe filter, with a 10ml syringe. 50µl of this filtered solution were taken for SDS-PAGE analysis. Chromatography on chelating 5 ml HiTrap was set in the 4°C room. The flow rate was set to 5 ml/min adjusted with cold water. The metal from the column was stripped off using 3 column volumes (CV) of 50 mM EDTA pH 8, then washed with 5 CV of water, re-charged with 1 CV of 100 mM NiCl₂, washed with 3 CV of water and washed with 10 CV of 3 % Buffer B plus 97% Buffer A. The extract was loaded on the column at 2 ml/min in the cold room and the flow through (FT) was collected in a separate falcon tube, and 50µl were saved for SDS-PAGE. The column was washed with 10 CV of 10 % buffer B, the whole flow through was collected in a beaker and 50µl were saved for SDS-PAGE. Then the column was washed with 10 CV of 30 % Buffer B repeating the procedure for 10%. To elute the protein, 10 CV of 100 % Buffer B were used and it was collected in 2 ml fractions with a 96-well-2ml plate. The peak was located by using a Bradford protein assay, and the peak fractions were pooled Additionally 5µl of extract flow-through (FT), 10% buffer B FT and 30% buffer B FT were taken for Bradford assay. 50 µl of the pooled fractions was saved for SDS-PAGE. The pooled fractions were desalted into storage buffer (100 imidazole, 300mM NaCl, 10µM ATP 1mM MgCl₂ and 10% sucrose) using a PD10 column.

The SDS-PAGE method was the following, to concentrate the sample it was spun at 13000 rpm for 60sec, in a Heraeus Biofuge fresco. The supernatant was discarded and the pellet was resuspended in 50µl 1xPBS (PBS stock from MPI-CBG media stocks). The pellet was treated further like all 50µl samples for SDS-PAGE, which was that for 50µl of sample 50µl of sample buffer with DTT was added to reduce all disulfide bonds. The sample was then heated to 95°C for 10-15 minutes to denature all proteins. Once denatured, the samples were run on a SDS-PAGE gel at 90V for 40-45 min. Afterwards the gel was stained with Easy Blue®, distained with tap water and stored in between layers of cellophane paper.

**Motility Assays.** Flow cells were made as previously described [16] where we have used double-sided tape as spacer. The flow cell volume was ~5 µL [74]. Glass coverslips
(Corning) for flow cells were washed with a Mucasol Soap 2% (Mucasol/Contrad 70 Soap (rm 426) Merck/Fisher) and sonicated for 20 min. Coverslips were rinsed in ddH₂O for 1 min, 10 min. in Acetone (Merk 1.00014), 10 min in Ethanol (Merck 1.00983) and rinsed again in ddH₂O. Subsequently the coverslips were soaked in 0.1M KOH (Aldrich 30,656-8, 99.99) for 30 min and rinsed in ddH₂O. Finally were dried with N₂(g) and stored between layered sheets of lens paper. The chamber was first incubated with 1mg/ml of casein, previously desalted in a PD10 column to remove inorganic phosphate. Subsequently, the kinesin was incubated for 5 min followed by motility solution with microtubules 2-6 μm long (320nM of polymerized tubulin), and a given ATP concentration. Motility assays of high and low kinesin density correspond to 200-2000/μm² and 1-10/μm² respectively. Microtubules (mean length 5mm) were visualized by fluorescence microscopy, representative fields were videotaped, and the speed was measured off-line as described [76].

RESULTS.

Because one 8-nm step consumes exactly 1ATP molecule we can use the speed of a gliding microtubule over a kinesin-coated surface as a measure of the speed of the ATP hydrolysis reaction. The speed of the reaction is well described by Michaelis-Menten kinetics:

\[
S = d \cdot k_{cat} \frac{[ATP]}{K_{eq} + [ATP]}
\]

where \(K_{eq} = [ADP] \cdot [Pi] / [ATP] = 4.9 \times 10^{11} \mu M\) is the equilibrium constant (measured at 25°C, 250mM of ionic strength, 1mM of free Mg²⁺ and pH 7). Due to the \(K_{eq}\) large value we can work with,

\[
S = d \cdot k_{cat} \frac{[ATP]}{K_M + [ATP]} \tag{2.1}
\]

where \(k_{cat}\) is the catalytic constant, \(d\) is the step size (8nm), and \(K_M\) is the Michaelis-Menten constant (i.e., the ATP concentration at which half maximal speed is obtained).
In general the maximum ATP hydrolysis at steady state (the \( k_{\text{cat}} \)) is expected to depend on the hydrolysis product concentration, ADP and Pi [77]:

\[
(2.2) \quad k_{\text{cat}} = \frac{k_{\text{cat}}^{00}}{1 + \frac{[\text{ADP}]}{K_{ii}^{\text{ADP}}} + \frac{[\text{Pi}]}{K_{ii}^{\text{Pi}}} + \frac{[\text{ADP}][\text{Pi}]}{K_{ii}^{\text{ADP-Pi}}}}
\]

and

\[
(2.3) \quad K_M = \frac{k_{\text{cat}}^{00}}{k_{\text{cat}}^{00} K_{M}^{00}} \left[ 1 + \frac{[\text{ADP}]}{K_{ii}^{\text{ADP}}} + \frac{[\text{Pi}]}{K_{ii}^{\text{Pi}}} + \frac{[\text{ADP}][\text{Pi}]}{K_{ii}^{\text{ADP-Pi}}} \right]
\]

where \( K_{M}^{00} \) represents the \( K_M \) value at zero ADP and Pi, and \( K_{ii}^{[1]} \) is the noncompetitive inhibition constant for ADP, Pi or joint ADP \cdot Pi.

Figure 2.2 Microtubule speed at high kinesin density vs. ATP concentration at several ADP and Pi concentrations. Solid lines are global fits to Equations 2.1-2.3 and including a dead-end inhibition by ADP (Figure from [74]).
Inhibition is defined as in Segel [50], i.e., ‘competitive’ inhibition is when the $K_M$ is affected but $k_{cat}$ is unaffected; ‘non-competitive’ when only $k_{cat}$ is affected; ‘mixed’ when both $K_M$ and $k_{cat}$ are affected, and a ‘dead-end’ is a trap off the main cycle.

The microtubule speed was measured at several ATP, ADP and Phosphate concentrations using bovine kinesin-1 at high surface densities, shown in figure 2.2. The black points and curve in figure 2.2 show the results in the absence of ADP and Phosphate, giving a $k_{cat}^{00} = 113 \pm 1 \text{s}^{-1}$ and a $K_M^{00} = 28 \pm 1 \mu\text{M}$ that agrees with previous measurements (refs). With the addition of 10mM of Phosphate the $k_{cat}^{00}$ was 117$\pm$2s$^{-1}$ but the $K_M$ increased to 59$\pm$3 $\mu$M, acting then as a competitive inhibitor (yellow curve). 1 or 5mM of ADP (blue and red curves) had the following effect: each raised the $K_M$ to 1000$\pm$110 and 2900$\pm$550 $\mu$M, and decreased the $k_{cat}$ to 105$\pm$4s$^{-1}$ and 92$\pm$8s$^{-1}$ respectively, which corresponds to a mixed inhibition.

One possible weak point with the previous results is that all were obtained at high kinesin densities and we are trying to make statements about single molecules. In order to support the evidence presented so far, single-molecule data was necessary and key experiments were reproduced, see figures 2.3, 2.4 and Table 2.1.

![Fig. 2.3 Bovine kinesin motility inhibited by ADP and inorganic phosphate (Pi). Blue bars are high-density measurements and red bars are single-molecule. Conditions in pairs, numbers indicate mM amount of ATP and to its right the same is used supplemented with the given mM concentration of ADP or Pi.](image)
The gliding microtubule speed measurements in the absence or presence of ADP or Pi are independent of whether they are at high (blue bar) or low (red bars) kinesin densities either for bovine (figure 2.3) or Drosophila kinesin-1 (figure 2.4). At 1mM ATP the ADP reduces the speed from 901±53nm/s to 457±54nm/s, whereas in 10mM ATP, 1mM of ADP only reduces the speed from 867±46nm/s to 757±45nm/s, demonstrating the mixed inhibition behavior. Inorganic Phosphate reduced the speed from 179±20nm/s to 91±9nm/s for bovine kinesin. The effects were similar for Drosophila kinesin and are given in Table 2.1.

![Graph showing Drosophila kinesin motility inhibited by ADP and Pi. Bars and numbers are as in previous figure.](image)

**Fig. 2.4.** *Drosophila melanogaster* kinesin motility inhibited by ADP and Pi. Bars and numbers are as in previous figure.

**DISCUSSION.**

We can now compare the predictions from the models and the results obtained. ADP affected both the the $K_M$ and the $k_{cat}$, i.e., had a mixed inhibition, while the Pi was only competitive, because it only affected the $K_M$. These results are only consistent with ADP being released first than Pi, supporting a hand-over-hand mechanism.
Table 2.1. Nucleotide and motor-density dependence of the speed of microtubules moved by *Drosophila* kinesin

<table>
<thead>
<tr>
<th>Density and nucleotide condition</th>
<th>Speed (nm/s)</th>
<th>SEM (nm/s)</th>
<th>N</th>
<th>Temp. (°C)</th>
<th>Corrected speed (nm/s) *</th>
<th>Corrected SEM (nm/s) †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High density‡</strong></td>
<td></td>
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<tr>
<td>1 mM ATP</td>
<td>616</td>
<td>23</td>
<td>55</td>
<td>23</td>
<td>702</td>
<td>48</td>
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<tr>
<td>1 Mm ATP + 1 Mm ADP</td>
<td>344</td>
<td>16</td>
<td>88</td>
<td>23</td>
<td>392</td>
<td>45</td>
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<tr>
<td>10 Mm ATP</td>
<td>639</td>
<td>24</td>
<td>88</td>
<td>25</td>
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<td>43</td>
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<tr>
<td>10 Mm ATP + 1 Mm ADP</td>
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<td>19</td>
<td>88</td>
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<tr>
<td>5 µM ATP</td>
<td>93</td>
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<tr>
<td>5 µM ATP + 10 Mm Pi</td>
<td>49</td>
<td>3</td>
<td>61</td>
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<td><strong>Low density¶</strong></td>
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<tr>
<td>1 mM ATP</td>
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<tr>
<td>1 Mm ATP + 1 Mm ADP</td>
<td>405</td>
<td>22</td>
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<tr>
<td>10 Mm ATP</td>
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<td>32</td>
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<td>10 Mm ATP + 1 Mm ADP</td>
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<tr>
<td>5 µM ATP</td>
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<td>16</td>
<td>22</td>
<td>120</td>
<td>25</td>
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<tr>
<td>5 µM ATP + 10 Mm Pi</td>
<td>20</td>
<td>0</td>
<td>1</td>
<td>22</td>
<td>24</td>
<td>0</td>
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</tbody>
</table>

*Speed and SEM were corrected to 25°C using 7%/°C.
†Systematic errors of 3% in the speed and 5% in the concentrations of ATP, ADP and Pi were assumed.
‡100 and 1000 motors/µm²
¶1-10 motors/µm²

One could also consider a model where the product release, ADP and Pi, was random, however this would lead to a scheme with too many free parameters from which a nonzero but not detectable noncompetitive inhibition by Pi would occur. Besides, since there is no evidence that would suggest such a model, it is simply discarded. The binding of ATP, ADP or Pi could cause the kinesin molecule to go to a “dead-end” inhibition state (a kinetic trap off cycle, from which the molecule must go out before it can hydrolyze ATP and produce motion). The inclusion of this state creates a stronger competitive inhibition by ADP than the simple mechanism postulated in figure 2.1. There is reported evidence that ADP can bind to the nucleotide-free pocket of kinesin [76, 78], suggesting the inclusion of this dead-end, which was done for fitting the data in figure...
2.2. The inhibition is similar between bovine and *Drosophila* kinesin, and the inhibition can be monitored in bulk or with single-molecule assays.

![Diagram of kinesin's hydrolysis cycle](image)

Figure 2.5 Kinesin’s hydrolysis cycle, defined as a three-state mechanism with an ADP dead-end, first postulated by Schief, Hancock and Howard (SHH). Below, rate constants obtained in sec$^{-1}$. Structures in brackets depict hypothetical transition states. T, ATP; D, ADP; P, Pi; and $\phi$, empty nucleotide pocket [79].

The symmetric hand-over-hand model predicts a 180-degree rotation for each step, based on the fact that both heads are identical peptides and, therefore, they could undergo the exact same cycle every time [72]. On high density gliding assays this could lead to a reduction of the speed with time, as the rotation accumulates and the molecule loses the flexibility required to generate the next step. Such behavior has never been seen,
and single molecule experiments with a short kinesin construct demonstrated that there is no rotation between the steps, ruling out the symmetric mechanism. The only plausible scheme then, is an asymmetric hand-over-hand mechanism, where each head is capable of ATP hydrolysis and ADP can bind to the nucleotide-free pocket. The resulting three-state model with a dead-end is a condensed version of the six-state model suggested by Hancock and Howard [76], and Schief and Howard [79]. It is postulated that for very strict head coordination, the release of ADP from the forward head creates strain between the heads in the double-headed state, and this strain helps the rear head to release the Pi and detach from the microtubule.

The mechanism in figure 2.5 leads to a precise mechanocoupling between the stepping and the ATP consumption by each head. The State 1, the attached head binds and hydrolyzes 1 ATP molecule, with a rate constant of $4,000s^{-1}$ and a reverse rate of $0.0004s^{-1}$. In State 2, the forward head attaches to the microtubule lattice and releases its ADP, with a forward rate of $116s^{-1}$ and a reverse rate of $2.5s^{-1}$. In State 3, the rear head detaches and releases its Pi, with a forward rate of $5,000s^{-1}$ and a reverse rate of $500s^{-1}$. At physiological nucleotide concentrations (ATP 1mM, ADP 0.01mM and Pi 1mM), ATP binding and hydrolysis is very fast and practically irreversible. The transition from state 2 to state 3 is the rate-limiting step, which agrees with previous results [12, 63], and the transition from state 3 to state 1 is very rare. All forward rates are faster than reverse rates, which explain why the motor is processive at least at low loads. Whether the motor follows the same model at high forces is still to be discovered.

Very recently, 3 different laboratories have given further evidence for an asymmetric hand-over-hand mechanism [80-82], leaving very little room for any other stepping model.
CHAPTER THREE. MOTOR COOPERATION.

INTRODUCTION.
Several motors may move a single cargo. Vesicles, mitochondria, chromosomes or cross-linked microtubules are examples where many motors working together are necessary. But, how do motors work together?

In a classic paper by Jonathon Howard in 1989, he described how one kinesin-1 molecule is processive, and showed that the microtubule gliding speed is independent of the number of kinesin molecules at the surface.

![Figure 3.1 Microtubule gliding speed dependence on surface kinesin density [16].](image)

If one looks carefully at figure 3.1 it may become clear that the result is far from obvious. For example, an increment in the number of surface myosin-II motors leads to an increase in the driven filament speed (Howard2001). Large number of motors working together occurs naturally for myosin-II, which is the responsible for muscle contraction. For myosin-II, the explanation comes from the fact that it is a low duty ratio\(^3\) motor, counterbalancing any mechanical interaction between motors [2, 83]. It is interesting to note that at very high density the speed of actin seems to be less than expected given the slope of speed vs. myosin density for lower densities [83]. Very recently, a study of single-headed kinesin-1 molecules showed a similar speed at low and medium densities

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\(^3\) Duty ratio is defined as the fraction of the time that each head spends in its attached state.
but reduced for high densities [84]. So, overall, there are a couple of hints around that maybe hindrance between the motors is present when large numbers drive filaments.

There is no evidence up to now that would indicate biochemical synchronization of motors. One way to solve this lack of biochemical or mechanical synchronization is to postulate that there is enough flexibility within the motor, so that one behaves independently of what another is doing. In this case, kinesin-1 would contain a flexible element that could allow this cooperation. This possible flexibility has been attributed to be in the “swivel” domain (the predicted coiled-coil break-1) [85](Coy99). Removal of this predicted coiled-coil break drastically reduces (>75%) the microtubule gliding speed at high densities (from 2.6 to 0.7 μm/s) [86].

In this Chapter the predicted coiled-coil break-1 region has been removed from the kinesin-1 molecule and study how the microtubule speed is affected by motor density.

**METHOD.**

All kinesin constructs were expressed in E. coli similar to Coy et al. The deletion construct plasmids for stubby and stubby delta swivel were prepared as in [87]. Tubulin was purified and microtubules polymerized as in [74]. All reagents were purchased from Sigma-Aldrich, except where otherwise noted. The deletion constructs were made by Michael Wagenbach. I expressed and purify the recombinant protein.

All enzymes were purchased from New England Biolabs, except T4 DNA ligase which was from BRL, unless noted otherwise. All DNA propagation performed in DH5a unless noted. All oligonucleotides were purchased from Operon.

**Constructs.** Protein purification was performed as in Chapter 2 for all 3 constructs. Wild Type Kinesin from pPK113 plasmid. Expressed in BL21(DE3)pLysS Novagen cells. 6-histidine tagged Kinesin motor protein. Drosophila Kinesin. Concentration was 2.235μM (250μg/ml) A280=0.0869 ε=38860M⁻¹cm⁻¹, 118μg/ml by Bradford assay. Frozen in storage buffer (H₂O pH 7.0, 200ml, 100mM imidazole, 300mM NaCl, 10μM ATP, 1mM MgCl₂, 10% sucrose). Stubby (Δtail) Kinesin from pPK124, expressed in BL21 (DE3) pLysS Novagen cells 6-histidine tagged Kinesin motor protein Conc.= 1.343μm=86.4μg/ml A280-0.0433 ε=32220M⁻¹cm⁻¹, 77μg/ml by Bradford Assay. “Stub”
Frozen in storage buffer. Stubby Δ Swivel protein prep (12/2/97) from pPK125 clone B, concentration ~0.7mg/ml, 400µg/ml by Bradford Assay.

Figure 3.2. Kinesin constructs: Full length *Dm* kinesin heavy chain (upper), stubby (medium) and stubby Δ swivel (lower). Figure adapted from [Vale].

**Motility Assays:** Flow cells were made as previously described [16] where we have used double-sided tape as spacer. The flow cell volume was ~5 µL [74]. Glass coverslips (Corning) for flow cells were washed 55 min. in acetone, 10 min. in ethanol, rinsed in water, one hour at 60°C, 70% H₂SO₄ 97% (MERCK; #1.00732.2500) plus 30% H₂O₂, thrice rinsed with H₂O, 15min. in 0.1 M KOH, rinsed twice in H₂O, dried with N₂, silanized with dichlorodimethylsilane (0.05% DDS in Trichloroethylen (MERK; #1.11872.1000)), excess of silane removed by sequentially 5, 15 and 30 min. in methanol (MERCK; #1.06009.2511), dried with N₂ and stored in layers of lens paper. The chamber was first incubated with 50µg/ml antipenta-Histidine IgG from (Qiagen) with the remaining hydrophobic surfaces blocked with F-127 (MW 12,600, PEO98-PPO67-PEO98, (Chandaroy1994)). Subsequently, the kinesin was incubated for 5 min followed by motility solution with microtubules (320 nM polymerized tubulin MTs 2-6 µm long), and 5µM ATP. Motility assays of high and medium kinesin density correspond to 200-2000/µm² and 20-100/µm² for stubby and stubby delta swivel. Single molecule swivel assays were performed with 0.01-0.1nM kinesin construct concentration.

Microtubules were visualized with an Axioplan 2 epi-fluorescence microscope. Representative fields of A) high and low motor density motility assays were digitally
captured for 40-60 seconds with IP Labs software and tiff image stacks were analyzed off-line in NIH ImageJ. And B) Single molecule experiments were videotaped with a Super VHS recorder system at a rate of 30 frames per second. Recorded frames were digitized using a PCI-1407 single-channel monochrome frame-grabber with a 8-bit Analog-to-Digital conversion and LabView 6.1 Software (National Instruments, Austin, TX, USA) – See Chapter 4 for details on Image Acquisition for single-molecules.

Data acquisition: Motility assays were digitally captured with IPLabs software and image stacks were analyzed in NIH image. Swiveling MTs were recorded to SVHS videotape and captured with a frame grabber driven by LabView. Additionally image stacks were cropped in MetaMorph® and decomposed to individual numbered tiffs. The average speed is calculated as the slope of a least squares linear fit of the displacement vs. time.

RESULTS.

Figure 3.3 shows the results for Wild Type, Stubby and StΔSw of speed versus motor density. At high densities, full-length kinesin-1 drives microtubules with a speed of 702 ± 55 nm/s (mean±SEM, n=55). This behavior is similar to and expected from previous findings [16]. Stubby shows a wild type-like behavior, with a microtubule speed of 700 ± 16 nm/s (n=60) at high densities and 810 ± 94 nm/s (n=40) at single molecule level. Removal of the first predicted coiled-coil break (StΔSw) reduces the microtubule speed to 151 ± 46 nm/s (n=50) at high densities. At single-molecule level StΔSw drives microtubules like the full length construct with a speed of 657 ± 72 nm/s (n=45), providing evidence that this truncated kinesin molecule is fully functional. It is observed that the effect of adding motors is relatively weak. Increasing the density an order of magnitude (to 10 motors per squared micron approximately) the speed has decreased to 586 ± 12 nm/s (n=52), which is only about 20%. Increasing the density further another order of magnitude, the speed is reduced to 374 ± 35 nm/s (n=50) that is about half of the maximal kinesin-1 velocity.
While functional at single-molecule level StubbyΔSwivel seems to hinder itself at larger densities. If we think of the number of kinesin molecules as $N_{kin} = A_{MT} \cdot \delta$, where $A_{MT}$ is the area covered by the microtubule (assuming a mean microtubule length of 4mm), and $\delta$ is the kinesin density, then ~14 motors are required to reduce the speed to half maximal.

DISCUSSION.

The speed at high densities for StubbyΔSwivel agrees well with the previously reported decrease after the removal of the Swivel or first break in the predicted coiled-coil [86]. Here the previous work has been extended to examine how this reduction is achieved, and it was observed that the mechanical hindrance is rather weak, requiring ~14 motors to reduce the microtubule speed to half maximal. The swivel region then is necessary and sufficient to produce maximal microtubule speed at high motor densities.
Simple average? To gain further insight to what is the molecular nature of the speed decrease by \(\text{St}\Delta\text{Sw}\), one can think of the simplest case: an average of motors. Lets assume that the speed of each individual motor depends on the torque it experiences given by the difference between the preferred kinesin orientation and the microtubule protofilament, i.e., \(s = s(T)^4\), the torque is \(T = \kappa_r \Delta \theta\) where \(\Delta \theta = |\theta_{\text{pref}} - \theta_{\text{kin}}|\) and \(\kappa_r\) is the torsion elasticity constant. The assumption that each motor is randomly orientated on the surface can be made, and then the speed at high densities for a single microtubule is given by

\[
V = \left(\frac{1}{n}\right) \sum_{i=1}^{n} s_i = \left(\frac{1}{n}\right) \sum_{i=1}^{n} -a\kappa_r \Delta \theta_i + b
\]

where we have assumed a linear torque-velocity relation \(s = -aT + b\) (See Chapter 6 for more on this issue). A very simple case would be maximal speed at \(T(\Delta \theta = 0)\) and 0 at \(T(\Delta \theta = \pi)\). Then \(a = \frac{v_{\text{max}}}{T(\pi)}\) and \(b = 0\), which would lead to \(V = \frac{v_{\text{max}}}{2}\). At lower densities, it is only a problem of sampling. A large number of microtubules would be needed in this case, and hence the mean is the same for large motor number and for a single motor. Thus, the observed decrease is not a product of a simple motor averaging with a linear torque-velocity relationship. One may think of more complex scenarios to include in an average, but since we do not have any further evidence I will restrict the analysis up to this point.

This very simple model already makes a clear point, the interference or hindrance between motors is very high. Already two motors have a very strong effect on each other, contrary to the observed results.

**Role of motor flexibility.** Diehl, et al. [88] have shown that linking motors through mechanical elements can increase the speed at which the microtubule is moved. They claim that it is because the link is flexible that the motors can cooperate and hence the

---

4. To facilitate analysis and discussion the distinction between microtubule speed \(V\) and single-motor speed \(s\) is made.

5. It is easily seen that this result would also be obtained from a Gaussian distribution.
speed can increase, but it has already been shown that just linking motors can also lead to speed increments. For example, Jorge Jose [89] simulated microtubule gliding assays using a spring constant of 0.2 pN/nm. For high densities, they obtained smaller speeds than for single molecules, suggesting interference between motors. Diehl, et al. calculated a spring constant of 0.4 pN/nm, twice as much that Jose, et al. used. In the same work by Diehl, upon stiffening of the mechanical link by temperature increase they observed a speed decrease, arguing that indeed stiffening of the link prevents motor cooperation.

A stiff mechanical link between semi-flexible motors increases the speed of the complex, and the more motors the larger the speed, up to a certain extent [2]. The stiffening of the motors on this background would lead to a decrease in velocity. One would think that there are three levels of flexibility: One being the cargo (surface in gliding assays), which links all the motors; two, the tail that links the cargo to the motor domain; and three, the neck linker.

Flexibility at the neck linker is necessary for force production, without it there is no motion generated.

The movement-producing unit (motor domain plus neck linker and neck) is the minimal unit necessary to produce force and motion. This unit is different depending on whether the motor is processive or not.

Non-processive motors do not need flexibility of the tail because they will only spend a very short time attached to the filament lattice (myosin-II for example). So, as long as the neck linker of one motor is flexible enough to perform the conformational changes within the required time it will not disturb the rest of the motors.

On the other hand, high duty ratio motors require the tail flexibility to allow the motor unit to work independently (up to certain extent) of what the other motors do.

Let’s think of a very stiff tail in processive motors. Stiffening the link between them would only reduce the overall speed of motion. Flexibility in the tail and neck linker can be treated as an effective stiffness, given the fact that they are very interrelated. Indeed, flexibility in either the link or the tail can be considered similar when measuring filament speeds. This equivalence has been used by theorists [90-92] to explore the effect of a flexible link between the motor and the cargo. An increase in the effective stiffness reduces the speed of a single motor down to a minimum [93].
An infinitely flexible link is no link at all. In bead and microtubule gliding assays, where a bead or the glass surface links the motors to each other, and this link is for practical purposes infinitely stiff. A membrane vesicle has a very low bending rigidity but it is practically unstretchable, a chromosome has a Young modulus of ~40 Pa [94], a microtubule (relevant for cross-linking motors) of 1.9 GPa [95], so even if the same motor can drive all these cargos the speeds may vary significantly.

An obvious question that comes from this digression is: what is the flexibility of a single kinesin molecule? Let’s find out in the next chapter.
CHAPTER FOUR. SINGLE-MOLECULE FLEXIBILITY

INTRODUCTION.
Intra-flagellar transport (IFT) makes use of different types of kinesin to move vesicles along the flagella. These different types of kinesin must coordinate in order to achieve vesicle movement [96]. For the transport of organelles in Xenopus melanophores a similar cooperation must occur, here between kinesin-2, dynein and myosin-V [97, 98]. Chapter 3 showed that predicted coiled-coil breaks within the kinesin tail, in particular the “swivel” (380-440aa Dm numbering), are necessary to make one motor able to cooperate with other motors. Removal of the coiled-coil break affects speed at high densities, while leaving the speed at the single molecule level unaffected. This suggests a mechanical hindrance between motors at high densities with a fully functional motor (by single molecule). Due to its flexibility an unstructured domain would be the responsible element that allows this cooperation. The flexibility of an unstructured peptide or disordered domain comes purely from an entropic origin [2]. Mechanically denatured peptides, which have lost all structure, behave as entropic springs [99]. The most common used model to analyze mechanically denatured peptides is the Worm-Like-Chain (WLC) model, which is an entropic spring [2].

The favored hypothesis is that breaks in the coiled-coil tail, specifically the “swivel”, allow one motor to work independently of what other motors do. Like springs that loosely connect the motor domain (or the force producing unit, Chapter 3) from the domain that binds the cargo. If this region is missing then one motor may physically affect (push or pull) the other ones. Thereby affecting the hydrolysis cycle and detachment rates. This results in the inhibition of the overall motility due to mechanical linking between motors. If this is so, one kinesin construct with a swivel (unstructured) region would be more flexible while another kinesin lacking this coiled-coil break would be stiffer in comparison.

Hunt and Howard [100] measured the flexibility of full length Drosophila kinesin by measuring the fluctuations of a microtubule tethered to a glass surface by a single kinesin molecule in the absence of any nucleotide (ADP or ATP). Their measured value was $117\pm19\times10^{-24}$ Nm/rad (mean ±SEM) and the drag was $6.0\pm1.3\times10^{-21}$ Nms/rad.
The single molecule conditions used in Chapter Two and Three were developed further to measure the torsion elasticity constant of an individual kinesin molecule. The approach taken was to measure the random angular fluctuations of a microtubule with respect to an arbitrary x-axis. The microtubule was driven by a single kinesin molecule in the presence of low ATP concentrations to ensure observation of functional motors. This approach is similar to the one followed by Hunt and Howard [100].

METHOD.

Single Molecule Motility Assays. Microtubules were grown from porcine tubulin at 4mg/ml for 30 min at 37°C as described in Chapter Two. Single molecule conditions were used as those presented in Chapter Three (see figure 4.1). Once a swiveling microtubule was visualized a region of interest was drawn around it and data was acquired to hard disk using the image acquisition system described below with 500-1000 frames at 30 fps (video rate). Due to the difficulty of knowing accurately what is a functional single kinesin molecule the following criteria used: 1. Very low surface kinesin density (0.1-1 molecules/mm2), which made the landing rate very low as well (~0.2 microtubules/min); 2. Freely rotating microtubule with one single pivot point; 3. Gliding microtubule (i.e., pivot point moves along the microtubule lattice) at a speed close to the expected for the ATP concentration following Michaelis-Menten kinetics (Chapter 2); and 4. Run length equal or less than microtubule length.
Figure 4.1. Single kinesin molecule microtubule gliding assay. The 6x His kinesin tail binds the kinesin molecule to the glass surface through a PentaHis Antibody, the rest of the glass has been covered with pluronic F-127 at 1%. Microtubules have been grown using 3:1 Unlabelled:labeled tubulin with rhodamine (TAMRA) for visualization.

**Image Acquisition System.** A Carl Zeiss Microscope (Axioplan 2 imaging) was used connected to a Fujitsu-Siemens PC Intel XEON (Celsius) with a Sony black and white monitor (PVM-137). The Image system was composed of an Image Intensifier Controller (M4314), an Image Processor – Argus, with a Camera Controller - C2400 and a CCD Camera - C2400 (all Hamamatsu®) illuminated with Carl Zeiss AttoArc and HAL 100 Halogen Lamp. Video tape recording was made using a Panasonic S-VHS VCR (AG-7350) and transferred to a PC via a National Instruments Frame Grabber - PCI 1409. See set-up in figure 4.2.
Figure 4.2. Image acquisition system diagram. A Zeiss microscope is illuminated by an Arc Mercury Lamp. A CCD camera is used to image the field of view of $(512 \times 512 \mu m^2)$. An NTSC signal is then sent to an Image Processor where auto enhance and contrast is adjusted. Image display (monitor) and storage (PC) happens simultaneously.

Whether the NTSC video signal to the frame-grabber comes from the Argus Image Processor or from the VCR is not important. If a BNC cable is plugged to the CCD camera directly the signal is split in two. This split requires an extra adjustment of light or image contrast that is unnecessary.

**LabView Programming.** An image acquisition VI was programmed using as template the demo VI “HL Grab in IMAQ Vision Display.vi” (C:\Program Files\National Instruments\LabVIEW 6.1\examples\IMAQ). Having this basic feature, “Save ROI as TIFF.vi” was written localizing the acquisition.vi into a while loop, and creating a saving option after acquire and before closing (figure 4.3). The “acquire” core substituted the “buffer extract” and “closure” present in previous versions. This substitution makes it simpler, easier to use and eliminates some bugs present in earlier versions. The program can save the whole image as well as only selected areas (Region of Interest – ROI), in the designated file path.
Calibration of Image Acquisition. The image acquisition system (LabView and IMAQ Vision Builder – figure 4.3) was calibrated using a caliper of 10μm. To have a good and reliable calibration ten independent measures were taken of 10, 20, 30, 40 and 50μm. The average values are, 185nm±1nm for x-axis pixels and 188±1nm for y-axis (mean±SEM).

Image Analysis for Single Microtubules. This is depicted in figure 4.4 and works as follows: 1. Import the raw image – loads the image to the working directory of MATLAB®. 2. Compute global image threshold using Otsu's method – which chooses the threshold to minimize the intraclass variance of the black and white pixels. 3. Convert the image to a binary image, based the computed threshold – Using the threshold from 2, it converts the image into black and white. 4. Label connected components in a binary image – To calculate properties of regions of interest, MATLAB® defines the number of objects present in an image. The region with the largest aspect ratio is the region defined as the microtubule and its location is passed to the next step. 5. Measure properties of image regions – Measure of center of mass, angle, etc. for each of the labeled objects. MATLAB® uses a statistical approach to this problem. The first moment is the centroid and from the second moment it calculates the lengths of the major and minor axes as well as the orientation (Appendix A).
Figure 4.4 Flow diagram of the Image Analysis algorithm used to analyze microtubules driven by single kinesin molecules (see text for details).
**Data Analysis.** The average speed was calculated as the slope of a least squares linear fit to the displacement versus time. This displacement is the distance of the pivoting point (kinesin) to the centroid of the ellipse and because it is so it may change sign at one point in time (figure 4.6). When this happens the speed can be measured in both sides and an average taken.

Data selection criteria: a) Visual similarity of angle versus time experimental data with a simulated trace for similar torsion elasticity constant (figure 4.7 and Appendix B); b) Run length equal or less than the length of the microtubule; c) Speed close to expected (for a given ATP concentration); d) Microtubule length between 2-9μm, to avoid fitting problems (when it is too small) or that the microtubule dips out of focus or bends (when it is too large).

Microtubule angular fluctuations are well described by,

\[
 \gamma \frac{d\theta}{dt} = -\kappa \theta + \eta(t)
\]

where \( \gamma \) is the drag coefficient, \( \kappa \) is the torsion elasticity constant, and \( \theta \) is the angle (in radians) of the microtubule with an arbitrary \( x \)-axis. Here the inertia has been neglected since the Reynolds number is very low \( \sim 0.001 \) [2]. \( \eta(t) \) is a noise term due to the random collision of water molecules on the microtubule lattice and is fully given by:

\[
\langle \eta(t) \rangle = 0 \quad \text{and} \quad \langle \eta(t) \eta(t') \rangle = 2\gamma k_B T \delta(t - t')
\]

where \( k_B \) is the Boltzmann constant, \( T \) the temperature and \( \delta \) the Dirac delta function. The rotational drag coefficient is per unit length for translation perpendicular to the microtubule axis,

\[
\gamma = \frac{1}{3} c_\perp (L_1^2 + L_2^2)
\]

where \( L_1 \) and \( L_2 \) are the distance of each end of the microtubule to the pivot point (kinesion location), and

\[
c_\perp = \frac{4\pi \zeta}{\cosh^{-1}(h/r)}
\]
is $c_\perp = 9.4 \, \text{mN} \cdot \text{s} \cdot \text{m}^{-2}$ [100]. $\zeta$, $h$ and $r$ are the viscosity of the surrounding fluid, the height above the surface and the radius of the microtubule respectively (Jeffrey81).

The one-sided Power Spectral Density (PSD) of the angle at the frequency $f$ is given by,

$$S(f) = \frac{k_B T}{\gamma \pi^2 (f_c^2 + f^2)}$$

where $S(f)$ is the power at the frequency $f$, $f_c$ is the corner frequency, $k_B$ is the Boltzman constant, $T$ the temperature and $\gamma$ the drag coefficient. The torsion elasticity constant is calculated as $\kappa = 2\pi f_c \gamma$, and the drag $\gamma = D/k_B T$.

The periodogram is calculated from the angle versus time trace at the sampling frequency (30Hz). A one-sided PSD is then calculated from the periodogram. To fit Equation 4.4 with the experimental PSD two different algorithms were implemented and both give the same results.

Levenberg-Marquadt. The torsion elasticity constant is measured by calculating the analytical Lorentzian fit to the power spectral density, and can be written as:

$$S(f) = \frac{1}{(a + bf^2)}.$$  

Fitting a lorentzian to the Power Spectral Density by least-squares is

$$\chi^2 = \sum_k \left( \frac{P_k^{alias} - P_k^{ex}}{\sigma_k} \right)^2$$

where $\sigma_k$ is given by any one of the following cases:

i) $\sigma(P_k^{ex})$ which has a 2n dependence.

ii) $P_k^{alias, old}$ which has no n-dependence

iii) $P_k^{alias}$ which has n-dependence
The problem is that least-squares algorithm assumes normal (Gaussian) distributed residuals and this is not the case. The residuals of a single PSD are exponential and Gamma distributed for several (convolution of n identical exponential distributions). The residuals slowly approach a Gaussian distribution only when $n \to \infty$. In order to correct this, a Maximum Likelihood Estimation has been used [30]. It turns out that maximizing the probability density for the PSD is (practically) equivalent to minimizing a $\chi^2$ with a $n$-correction: $n/(n+1)$, where $n$ is the number of PSD averaged.

All weight cases have been implemented using a Levenberg-Marquadt algorithm in a Non-linear Least Squares method. Here $n = 1$, so the correction is a factor of 2. This algorithm also provides with the calculation of the 95% confidence interval (or at any other desired confidence). A “block” replaces $n_b$ consecutive points $(f, P_{ex}(f))$ with a single new point $(\bar{f}, \bar{P}_{ex}(f))$ with coordinates that are simply block averages. When $n_b$ is so large that we can ignore terms of non-leading power in $n_b$, then $\bar{P}_{ex}(\bar{f})$ is Gaussian distributed with $\langle \bar{P}_{ex}(\bar{f}) \rangle = P(\bar{f})$ and $\sigma(\bar{P}_{ex}(\bar{f})) = P(\bar{f})/\sqrt{n_b}$. Residuals are given by $P_{ex}/P_{fit}$ so the mean is 1 and $\sigma = 1/\sqrt{n_b}$.

**Analytical Fit.** Modified from [30]. The analytical best fit to equation 4.5 is:

$$f_c = \left(\frac{a}{b}\right)^{1/2} = \left(\frac{S_{0,1}S_{2,2} - S_{1,1}S_{1,2}}{S_{1,1}S_{0,2} - S_{0,1}S_{1,2}}\right)^{1/2}$$

and

$$D = \frac{2\pi^2}{b} = \frac{2\pi^2 n S_{0,2}S_{0,2} - S_{1,2}}{n+1 S_{1,1}S_{0,2} - S_{0,1}S_{1,2}}$$

where $S_{p,q} = \sum_f f^{2p}S_{ex}(f)^q$.

Here $n_{win} = 1$ because there is not an average of several PSD; so Equation 4.5 only has a $1/2$ correction factor. I have considered explicitly the one-sided PSD and that is why the 2 from $D$ is not present here [30] The torsion elasticity constant is calculated as $\kappa = 2\pi f_c \gamma$, and the drag $\gamma = k_q T / D$.

It was verified that the code for the calculations satisfy Parseval’s theorem. The first point in the power spectra is equal to the average of the squares of the signal data.
divided by the frequency resolution \((\delta f = 1/N\delta t)\), which should not be used when fitting the experimental data.

**Errors.** The propagation errors are given by [17] 

\[
\frac{\sigma(f_c)}{f_c} = \frac{s_{f_c}(x_{\text{min}}, x_{\text{max}})}{\sqrt{\pi f_c T_{\text{msr}}}} \quad \text{and} \quad \frac{\sigma(D)}{D} = \left(\frac{1 + \pi/2}{\pi f_c T_{\text{msr}}}\right)^{1/2} s_D(x_{\text{min}}, x_{\text{max}})
\]

where \(x_{\text{min}} = f_{\text{min}}/f_c\), \(x_{\text{max}} = f_{\text{max}}/f_c\) and

\[
s_{f_c}(x_{\text{min}}, x_{\text{max}}) \equiv \left(\frac{\pi}{u(x_{\text{min}}, x_{\text{max}}) - v(x_{\text{min}}, x_{\text{max}})}\right)^{1/2},
\]

\[
s_D(x_{\text{min}}, x_{\text{max}}) \equiv \left(\frac{u(x_{\text{min}}, x_{\text{max}})}{1 + \pi/2(x_{\text{max}} - x_{\text{min}})}\right)^{1/2} s_{f_c}(x_{\text{min}}, x_{\text{max}})
\]

\[u(x_{\text{min}}, x_{\text{max}}) \equiv \frac{2x_{\text{max}} - x_{\text{min}}}{1 + x_{\text{min}}^2} + 2\arctan\left(x_{\text{max}} - x_{\text{min}}\right)
\]

\[v(x_{\text{min}}, x_{\text{max}}) \equiv \frac{4}{x_{\text{max}} - x_{\text{min}}} \arctan^2\left(x_{\text{max}} - x_{\text{min}}\right)
\]

**Swivel Simulation.** Experimental obtained traces of angular fluctuations were compared with simulated swivel data by three different methods, a Langevin time-discrete approach, an exponentially decaying function-convolving Gaussian White Noise (GWN), and by using the exact discrete sampled version of Brownian motion [30] (Appendix B).

**RESULTS.**

**Predicting Kinesin Torsional Mechanical Properties.** How can the kinesin molecule be understood in mechanical terms? One approach is to simplify the structure and consider only rods for coiled-coils and WLC for disordered regions (figure 4.5).
Coiled–Coils as Cylindrical Regions [2]. A coiled-coil region of the kinesin molecule is considered as a solid cylinder with a circular cross sectional area. The following equation can be used: \( T = \theta \cdot \frac{C_{cc}}{L_{cc}}, \) where \( T \) is the torque, \( L_{cc} \) the length of the object, \( \theta \) the twisting angle and \( C_{cc} \) is the torsional rigidity of the object. The torsional spring constant is: \( \kappa = \frac{C_{cc}}{L_{cc}}. \) For a homogeneous isotropic object with circular cross-section, the shear modulus and the torsional rigidity are related by \( C_{cc} = 2GI, \) where \( G \) is the shear modulus and \( I \) the second moment of inertia. The shear modulus \( G \) (and torsional rigidity) is related to the flexural rigidity as \( G = \frac{E}{2(1+\sigma)}, \) where \( \sigma \) is the Poisson ratio, the relative amount of sideways contraction of the material compared to the lengthwise strain, and \( E \) is the Young’s modulus. The Young’s modulus of a solid is the proportionality constant between its extension and the tensile force applied to it. The Young’s modulus does not depend on the object’s size or shape, is a material property(H01). If \( \sigma \approx 0.25, \) for rods of circular cross-section, then \( G = \frac{1}{2.5}E \) and \( C_{cc} = \frac{2}{2.5}EI. \) Using the relation of the persistence length, \( L_{p}^{c} = EI/k_{B}T, \) we then obtain for the spring constant:

\[
\kappa = \frac{2}{2.5} \frac{k_{B}T L_{p}^{c}}{L_{cc}}.
\]

Then the torsional rigidity of a coiled-coil is 660 ± 60 pNm²(Howard01), for a persistence length \( L_{p}^{c} \) of 200nm at 25°. The StΔSw kinesin construct is composed therefore of:

1. The neck = 345-378 (Dm numbering) = 33aa = 4.95nm ≈ 5nm, and
2. The coiled-coil-1 is from 440 to 560 = 120aa = 18nm, assuming an ideal helix of 0.15 nm per amino acid.
So the StΔSw would have a total length of 23 nm, which should have a spring constant of $C_{cc}/L_{cc} = 28 \pm 2 \text{ pNm/rad} \ (\sim 7 \ k_B T/\text{rad})$.

**Swivel and Hinge: Disordered Regions.** In order to calculate the disordered regions of the molecule (i.e., the swivel and hinge region), the WLC model is assumed to describe each polypeptide chain. Although assumed to be unstructured the regions are composed of two chains linked at the ends, in a very similar way to the DNA braiding geometry. Gilles Charvin and David Bensimon have addressed the braiding of DNA with theory, simulations and experiments on DNA molecules. One of the main results is that “the torsion elasticity modulus does not depend on the material properties of the chain, neither on the separation of the chains at the attachment point, and it is only of entropic origin”[101]. The value of $C_{WLC}/k_BT$ is $45 \pm 5 \text{nm/rad}$, where $C_{WLC}$ is the torsion elasticity modulus of a WLC model in $k_BT/\text{rad}$.

The swivel region contains 62aa (378-440) and the hinge 64aa (560-624). The number of segments times their length gives the total length of a chain. For a protein described by the WLC model, the segment length is 0.4nm (which is the same value of its persistence length[102, 103]). Therefore the total length of the swivel domain is: $0.4 \times 62 = 24.8 \text{nm}$. The torsion elasticity constant would be: $1.6 < \kappa_r < 2 \ k_BT/\text{rad}$. 

The theory describing the DNA braiding system is a geometrical model [101]. This theory is only valid in the range $n<|1/2|$, where $n$ is the number of turns. Using this theory in the case of the swivel region (total length and persistence length) the first twist before contact between the chains requires less than $1 \ k_B T$, which would give a peak-to-peak amplitude of fluctuations approximately of 180 degrees (90 for left and 90 for right). This amplitude can be thought of coming from a Gaussian distribution which would have as an approximation for 3 standard deviations $\sim \pi/2$. The variance then would be $\sim 0.3 \ rad$, which in turn corresponds to a spring constant of $\sim 3.6 \ k_BT/\text{rad}$. This estimation agrees well with the value obtained in the last paragraph.

Using springs in series the rest of the constructs used can be calculated:

$$1/\kappa_{eff} = \sum_{i=1}^{n} 1/\kappa_i$$

wild type is 2 coils + 2 braiding WLC. The value for the wild type can
go down to $0.45 \, k_B T$. This if one considers the small regions at the end of the tail that presumably are not coiled-coils. The results are presented in Table 4.1.

**Experimental measure of kinesin torsion elasticity constant.** A gliding and swivel microtubule is shown in figure 4.6a with the parametrization used in figure 4.6b.

---

Figure 4.6. Thermally driven rotational Brownian motion and gliding of a microtubule bound to a single Stubby kinesin molecule in the presence of 5μM ATP. **a)** Sequence of a 3.9μm long rhodamine labeled microtubule exhibiting random rotations and motion along its major axis. Yellow cross: pivoting point; blue cross: ellipse centroid; scale bar: 2μm.  

**b)** The Image Processing Toolbox from the programming language MATLAB® was used to implement a routine to fit an ellipse to the microtubule image. The fit gives an angle $\theta$, measured from an arbitrary x-axis to the major axis, the center (C - blue cross) and the length of the ellipse.  

**c)** Distance from the kinesin (K) to the center of the ellipse versus time, the speed is 78nm/s.
The distance kinesin-centroid versus time appears in 4.6c. An example of a selected trace that when compared to true Brownian motion (simulation) appears identical is shown in figure 4.7. This similarity indicates that indeed the swiveling of a microtubule tethered by a single kinesin molecule is a product of the random collision of water molecules with the microtubule lattice. The simplification implied here, i.e., a dimensionality reduction from 3D to 2D rotation of the microtubule, seems also to be supported by figure 4.6. A 2D rotation must come from the proximity of the microtubule to the surface, reducing the number of degrees of freedom available for motion.

![Figure 4.7](image)

Figure 4.7. Example of an angle vs. time trace of a) the microtubule shown in figure 4.6 and b) a simulation of Brownian motion given by equation 4.1 and 4.2 sampled at finite times (third simulation method, Appendix B).

Stubby and Stubby Delta Swivel kinesin torsion elasticity constants were then analyzed with a Student’s t-test for two samples unpaired, resulting in a $t = 0.67$ with $df = 14.24$ and $P = 0.51$. The torsion elasticity constant of the two constructs is not significantly different from each other.

The power spectral density (figure 4.8) of the angular fluctuations (figure 4.7) of a microtubule tethered by a single kinesin molecule is well described by a Lorenztian
function with a $\kappa = 11.8 \pm 0.3 \ k_B T/rad$ and $\gamma = 3.0 \pm 0.1 \times 10^{-20} \ \text{N}\cdot\text{m}\cdot\text{s}/\text{rad}$ ($\pm 95\%$ confidence interval). The torsion elasticity constant and drag coefficient values using a weighted Least-squares Levenberg-Marquadt fitting algorithm are equal to those obtained by the analytical fit.

Figure 4.8. Power Spectral Density (PSD) of figure 4.6; the solid red line is a Levenberg-Marquadt least-squares weighted fit to the data with the Lorentzian:

$$S(f) = S_0 \left[1 + \left(\frac{f^2}{f_c^2}\right)\right]^{-1},$$

where $S(f)$ is the power at the frequency $f$, $f_c$ is the corner frequency, and $S_0$ is the plateau power. The torsion elasticity constant is calculated as $\kappa = 2k_B T \left(S_0 \pi f_c\right)^{-1}$, where $k_B$ is the Boltzmann constant and $T$ the absolute temperature.

To avoid artifacts fitting was performed up to $f_{\text{max}}/2$.

The torsion elasticity constant for Stubby $\Delta$ Swivel is $7.4 \pm 1.0 \ k_B T/rad$ (Geometric Mean ±SEM), $n = 8$. The values are consistent with each other, $\chi^2 = 5.6$, df = 7, $P = 0.6$. While the value for Stubby is $8.7 \pm 1.7 \ k_B T/rad$ (Geometric Mean±SEM), $n = 14$ in this case the values are also consistent with each other, $\chi^2 = 17.7$, df = 13, $P = 0.17$. 
The measured drag coefficient was close to the bounds of expected drag based on a pivoting point at the middle or at the end of the lattice (equation 4.3 and figure 4.9). The drag seems to be closer to the expected value for a middle bound kinesin than for the end case. The only large deviation was a 5.6μm microtubule driven by StDtSw, whose drag appeared less than expected. We might expect an intrinsic variation of the measured drag because we do not know the precise height for each microtubule and this may have a rather broad distribution [104]. The torsion elasticity constant does not seem to correlate with the microtubule length nor with the gliding speed for any of the constructs (figure 4.10).

Figure 4.9. Measured microtubule drag coefficient for Stubby kinesin (green diamonds) and StΔSw kinesin (blue squares) compared to the expected drag assuming either end (black line) or middle bound (red line).
Figure 4.10. Logarithm of the Torsion Elasticity constant ($\kappa$ in $k_B T$/rad) versus microtubule lengths for Stubby (green diamonds) and StDSw (blue squares). Error bars are the 95% Confidence Interval from the Lorenztian fit to the PSD.

Figure 4.11. Logarithm of the Torsion Elasticity constant ($\kappa$ in $k_B T$/rad) versus microtubule speeds for Stubby (green diamonds) and StDSw (blue squares). Error bars are the 95% Confidence Interval from the Lorenztian fit to the PSD.
DISCUSSION.
The measured drag coefficient for more than 70% of the microtubules observed (for both kinesin constructs) falls between the expected drags for either end- or middle-lattice bound predicted by hydrodynamic theory [2]. Also, for both kinesin constructs the measured torsion elasticity constant is independent of microtubule length and kinesin speed, which suggests that the motor stiffness does not affect its speed at single molecule level. Additionally the chosen range of microtubule lengths to observe kinesin rotational thermal fluctuations does indeed reflect that the stiffness of the motor and the drag is not large enough to affect these fluctuations.

The measured torsion elasticity constant of StΔSw agrees well with the one predicted considering a coiled-coil a homogeneous isotropic rod with a circular cross-section, 7.4 ±1 versus 7 \( k_B T/\text{rad} \), assuming that coiled-coils behave as solid rods with circular cross-section and a persistence length of 200nm. Stubby has a \( \kappa \) of 8.7±1.7 \( k_B T/\text{rad} \) and does not agree with the prediction made (Table 4.1) assuming that the swivel region is a disordered domain. There is no significant difference between Stubby and StΔSw, although these two constructs behave very differently in high density assays.

Table 4.1 Predicted versus Experimentally measured Torsion Elasticity Constants (in \( k_B T/\text{rad} \))

<table>
<thead>
<tr>
<th>Construct</th>
<th>Predicted</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>StΔSw</td>
<td>7</td>
<td>7.1±1 (n=7)</td>
</tr>
<tr>
<td>Stubby</td>
<td>1.4</td>
<td>8.1±1.2 (n=14)</td>
</tr>
</tbody>
</table>

The main difference with the experimental approach followed here and the one by Hunt and Howard is the use of non-zero ATP concentrations. Similarly Hua and Gelles also used non-zero ATP concentrations and measured a \( \kappa \) of 2.0±0.9×10\(^{-20}\) Nm/\( \text{rad} \) (4.8±2.1 \( k_B T/\text{rad} \)). Here, as in [105], we found that the torsion elasticity constant is largest for the shortest construct.
The surprise comes from Stubby which contains the first predicted coiled-coil break ("swivel"), and behaves as full length (Chapter Three) but has an elasticity constant not significantly different from StΔSw that lacks the break.

Based on the results presented in this Chapter it seems that Hunt and Howard [100] measured non-specific binding of microtubules to the surface or that the motors the microtubules were attached to were non-functional (i.e., partially denatured protein that conserved the microtubule binding ability but was not able to hydrolyze ATP and/or to produce force). Hua and Gelles also reported non-specific binding of microtubules to the surface and large angular fluctuations were observed. Therefore the large measured flexibility [75] of the full length could be an artifact.

The assumption that predicted coiled-coil breaks are disordered regions may not be accurate. It may well be that there is some secondary structure in the swivel. The search for this secondary structure is the topic of the next chapter.
CHAPTER FIVE. STRUCTURE OF THE “SWIVEL” DOMAIN

INTRODUCTION.
The working hypothesis has been so far that a flexible domain within the kinesin molecule allows it to work independently of other motors. Indeed the removal of the predicted coiled-coil break-1 impairs motility (Chapter Three). Measurements of the flexibility at the single molecule level between different kinesin constructs show no significant difference. How is it possible then, that the same region (the predicted coiled-coil break-1) behaves as a flexible element in multi-motor assays but does not seem to be flexible at single molecule level? There is a contradiction between the hypothesis proposed at the beginning of this thesis and the results of the last two Chapters so a new hypothesis is needed.

The obvious difference between the two experimental conditions (Chapter 3 versus Chapter 4) is the use of large number of motors for the high-density assays and single motor for the measurements of torsion elasticity constant. A single motor does exactly the same, in terms of stepping and ATP hydrolysis, whether in high density or at single molecule. The difference between high-density assays and single molecule level is the presence of many other motors in the former case. In high-density assays the motors will push and/or pull each other, so there are forces exerted between them. A flexible element may absorb this energy, just like a spring, and allow the motor to keep moving.

A possible way of reconcile the contradictory results would be if the swivel region can switch between a folded and unfolded state that depends on strain. The folded state would explain why at the single molecule Stubby has the same torsion elasticity constant that StΔSw. The unfolded state would then provide the necessary flexibility to the kinesin molecule to work independently of what any other number of motors does. The strain-dependent conformation of the “swivel” would make possible to coordinate and exhibit full-length behavior at high densities and be stiff at single molecule. The hypothesis proposed is therefore:

1. The swivel region is a domain that can fold and unfold in a strain dependent manner.
The first obvious implication that this hypothesis makes is that there is some structure (secondary or tertiary). It is this structure in turn that can adopt a folded or unfolded conformation, which may depend on the application of an external force. Here, Bioinformatics tools, Circular Dichroism, Fluorescence and Gel Filtration techniques assess the secondary structure. It is shown that the “swivel” region consists of a dimeric α-helix flanked by random coils.

METHOD.

Polipeptides for CD and Fluorescence. In order to study the swivel region of kinesin-1 (*Drosophila m.*) by Fluorescence Spectroscopy and Circular Dichroism 30aa long peptides not too hydrophobic were designed. The whole predicted coiled-coil break-1 “swivel” was cut into 30aa of overlapping pieces (figure 5.1). The first and last contain at least 2 heptads to promote coiled-coil formation. All peptides should contain a Trp(W) residue to monitor local environment. The first one is natural to the *Dm* kinesin, the second to other kinesin species.

![Amino acid sequence of the peptides synthesized, each contains a W at the same relative position.](image)

Rationale of Substitution (Q→W). The location and environment of a Triptophan residue (W) can be monitored by Fluorescence Spectroscopy, which could give information about unfolding processes, i.e., the change of fluorescence when its involved in secondary structure formation (folded state) and when it has no intra- or inter-chain interactions (unfolded state).
After been checked with other species and it seems reasonable to change a polar for a polar aminoacid. To change glutamines (Q) for triptophan (W), where there are at least sequences of three kinesins from fungi (*N. crassa, U. maydis and S. racemosum*) and the *C elegans* one that have a W instead of a Q (at the end of the first peptide 'eeQ(->W)inme'). This gives a W at the beginning of the second one. By adding 4 aa to the N-terminus and removing 4 aa from the c-terminus the introduced W is brought to the same relative position as the native W in peptide 1. This is very important in monitoring the site up to which unfolding will occur at a given temperature. The shift will as well monitor the fluorescence in similar sites that will allow a much more convincing data evaluation. According to the domain description of figure 5.1, the peptide 2 contains only three aa from the neck (RAG) and I think it can thus still be considered a meaningful model for the onset of swivel.

**CD Spectroscopy.** A CD Spectroscope (Jasco J-810 15OS with the following settings: Nitrogen (N2) flow at 5 L/min, Scan speed at 100nm/min, Bandwidth and Data pitch 1nm, Continuous scanning mode and 1mm cuvette path length. We measured from 260 to 190nm whenever possible and reduce to 195nm when the high tension went up more than 700V. We scan from long to short wavelengths. Buffer: 10mM KCl + 5mM Na2HPO4 in ddH2O pH 8.69. Units of CD data: The Mean Residue Weight (MRW) is calculated with: \[ MRW = \frac{MW}{N-1}, \] where MW is the molecular weight of the polypeptide chain (in Da) and N is the number of amino acids in the chain; the number of peptide bonds is N-1. For most proteins the MRW is 110±5 Da[106]. The mean residue ellipticity at wavelength \( \lambda \) is given by[107], \[ \theta_{\text{mew},\lambda} = (MRW/d \cdot c) \cdot 100 \cdot \theta, \] where \( \theta \) is the observed ellipticity (degrees) at wavelength \( \lambda \), d is the path length [108] and c is the concentration (g/ml). Alternatively one can use: \[ \theta_{\text{molar},\lambda} = \left( \frac{100}{d \cdot m} \right) \cdot \theta, \] when we know the molar concentration (m). In both cases the units are deg cm² dmol⁻¹. We scanned 20 times for the measurement at 20° and 10 times for all the rest. 10 scans give a good signal-to-noise ratio, which is proportional to the square root of the number of scans [109] and is enough for a good measurement while reducing the time required for the temperature dependence experiment to half. The range of wavelengths used for our CD data yields similar results.
to those truncated at 178nm [106]. CDSSTR® was used to calculate the percentage of secondary structure present for each spectroscopic CD trace.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Absorbance $A_{280}$</th>
<th>Extinction Coefficient $(\text{M}^{-1} \text{ cm}^{-1})$</th>
<th>Concentration $(\mu\text{M})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neck_swivel</td>
<td>0.3286</td>
<td>5500</td>
<td>60</td>
</tr>
<tr>
<td>Swivel_one</td>
<td>0.25135</td>
<td>5500</td>
<td>45.7</td>
</tr>
<tr>
<td>Swivel_two</td>
<td>0.3356</td>
<td>5500</td>
<td>61</td>
</tr>
<tr>
<td>Swivel_three</td>
<td>0.20166</td>
<td>5500</td>
<td>37</td>
</tr>
<tr>
<td>Swivel_coil</td>
<td>0.35018</td>
<td>6990</td>
<td>63.6</td>
</tr>
</tbody>
</table>

The molar extinction coefficient was calculated in the ExPASy website with ProtParam Tool (http://us.expasy.org/tools/protparam.html and references therein). The prediction is based on the Edelhoch method using the corrected values for Trp and Tyr. The concentration was calculated by the Beer-Lambert Law:

$$A = \varepsilon l C$$

where $A$ is the measured absorbance, $\varepsilon$ the extinction coefficient, $l$ the path length and $C$ the molar concentration. These measurements were made three times.

Thermodynamic parameters were obtained by measurement of the stability of the $\alpha$-helix adapted from [110-112]. The observed mean residue ellipticity must be converted to fractional helicity, $f_H$, for each peptide, according to:

$$f_H = \frac{[\theta]_{\text{obs}} - [\theta]_C}{[\theta]_H - [\theta]_C},$$

where $[\theta]_H$ and $[\theta]_C$, are the $[\theta]$ values for helix and coil respectively, and are defined as:

$$[\theta]_H = H_0 \cdot (1 - 2.5/n) + 100 \cdot T \quad \text{and} \quad [\theta]_C = C_0 - bT,$$

where $T$ is the temperature in degrees Celsius and $b$ comes from results on the thermal response of denatured proteins and small peptides and has the value of 53[107]. In general the helix and the coil conformation depend on the temperature.

The observed fractional helicity at any temperature, calculated as shown in the equation below, is used to determine the Zimm-Bragg helix-coil transition theory parameters $s$ and
σ, the helix propagation and nucleation parameters, respectively. The observed fractional helicity at any temperature is given by,

$$f_H = \frac{\sigma s}{(s-1)^2} \left( \frac{ns^{n+2} - (n+2)s^{n+2} + (n+2)s - n}{n\left(1+\frac{\sigma s}{(s-1)^2}\right)\left[s^{n+1}+n-(n+1)s\right]} \right)$$

where $n$ is the number of amino acids in the chain, $s$ is the propagation parameter, and $\sigma$ is the helix nucleation parameter. $s$ is also the equilibrium constant for making a new helical unit at the end of a helical sequence and can be expressed as (following integration between $T_m$ – the temperature at the midpoint of the transition- and $T$):

$$\ln s = \frac{\Delta H}{R} \left( \frac{T - T_m}{TT_m} \right)$$

where $\Delta H$ is the enthalpy change for adding one more helical unit onto a preexisting helical section. The CD measurements were done in collaboration with Sineej Madathil.

**Gel Filtration.** A Superdex Peptide 10/300 GL column (Amersham Biosciences 17-5176-01) was used. The Buffer contained 100mM KCl and 100mM Na$_2$HPO$_4$ at pH 8.6. All liquids to used were filtered with 0.22μm filter and degassed in bottles rinsed 3 times with filtered water. As standards we have used: a) Myc2 – Exp. mw = 2731, HPLC <80%. (Neosystem, Part Nr. SP030607B, Lot AW04291F), b) Ribonuclease, (mw =13700), c) Aprotinin (mw = 6512), d) Insulin chain A (Sigma-Aldrich I1633, mw = 2531.64) and e) Insulin chain B (Sigma-Aldrich I6383, mw = 3495.89). The column was equilibrated by first cleaning all tubes by flowing at 30ml/min with water. Then were flowed only A and B with buffer and water respectively. A program was set to run at 0.25 ml/min (it was the minimum it could go) for 4CV of EtOH 20% and then 2CV of buffer. All nubs and entry device to be used was sprayed with EtOH 70%. Standards were run (myc2, ribonuclease, aprotinin, insulin chain A and insulin chain B) making sure before that the loop for injection was cleaned by flowing water at 5ml/min. Each sample was filtered before loading them into the loop. The syringe was cleaned with 3x water and 3x buffer. Before samples the column was once more cleaned with 4CV water, then 2CV EtOH, pump off for some time, 2CV of water and then 2CV of buffer. Approximate time 16 hrs. The samples were filtered and run the same method that we used for the
standards. 100μl of sample were loaded for each peptide. To store the column 4CV of water + 4 CV of EtOH 20% were applied. Stored later at room temperature.

**Fluorescence.** This was performed at the Forschungszentrum Rossendorf by Sineej Madathil under the supervision of Dr. Karim Fahmy. Five peptides from the neck to coil region where synthesised with a Tryptophane (Trp) residue at position 11 to serve as a site-specific monitor of the environmental change from aqueous to hydrophobic that occurs during thermal unfolding. Emission of the peptides was excited at 280 nm and measured from 295 to 400 nm. As control, free Trp was measured identically in the same buffer solution (50 mM phosphate buffer, 100 mM KCl, pH 8.6). The ratio: peptide 340 nm emission (Ep340(T)) / 340 nm emission of free trp (Ew340(T)) was determined and plotted as a function of temperature. Thereby, the relative fluorescence quantum yield φ(T) of Trp in each peptide is determined as multiple of the quantum yield of free Trp in aqueous buffer represented by the control,

\[
\phi_p(T) = \frac{E_{p340}(T)}{E_{w340}(T)}
\]

(5.1)

where Ep340 is the fluorescence of the Trp residue in the peptide and Ew340 is the signal of a Trp residue in water, both as function of temperature.

The same plot was generated with free Trp in buffer supplemented with different amounts of ethanol to mimic different degrees of hydrophobicity of the Trp environment

\[
\phi_{\text{EtOH}}(T) = \frac{E_{\text{EtOH340}}(T)}{E_{w340}(T)}
\]

(5.2)

where E_{EtOH340} is the fluorescence of single Trp residue in a given amount of EtOH.

**RESULTS.**

Bioinformatics analysis of the kinesin heavy chain sequence supports the notion of an ordered structure in the swivel region.

The neck-swivel-coil1 sequence was run through COILS [113] and PairCoil [114], to see prediction for coiled-coils. The results of both programs are consistent with each other (Figure 5.2). Both predict the neck as a coiled-coil, and the coil1. For the swivel region there is a coiled-coil value of less than 0.3 in COILS using a 28aa window.
The beginning of the coil 1 is not predicted as accurate as the neck. It depends on the amino acid window (14, 21 or 28) and it may reflect a not so stable coiled-coil.

Figure 5.2. Domain organization of Dm kinesin-1 sequence residues 366 to 472. Amino acid sequence alignment made with ClustalW of wild type kinesin-1 (Dm: Drosophila melanogaster; Hs: Homo sapiens; Mm: Mus musculus; Nc: Neurospora crassa). Above the sequence “a” and “d” denote the positions of these amino acids in an heptad repeat. X-ray up to W376 [115]; α-helical content by Circular Dichroism [25]; NMR measurements [23]. Coiled-coil likelihood calculated from the program PairCoil [114] and Coils [113]. The x-axis represents the amino acid position. Predicted probability (expressed as confidence) for secondary structure formation given by PSIPRED [116] and PredictProtein (PP) [117]. Below each plot the predicted secondary structure is presented; the subset (from PP) with probability higher than 80% is shown.
X-ray crystallography showed a coiled-coil up to W376 [115]; Percentage of α-helical content by Circular Dichroism of neck peptides presented evidence for an α-helix that ends approximately at W376[25]; NMR measurements from peptide derived from the rat kinesin (residues 357-386, 363-393 in Dm numbering) gave an α-helix (K357-W370), flexible (R371-G373), extended (E374-V376), bend (P377-Q381) and helical turn (I382-386), indicated with blue, red, green, pink and light blue respectively[23].

**Circular Dichroism.** 1 and 5 peptides have a low percentage of helicity, which probably comes from that two heptad repeats are not enough to form a coiled-coil. 2 is the most unstructured peptide, supporting previous findings where the 15 amino acids following the neck coiled-coil were mostly unstructured[23]. 3 and 4 are more than 70% helical (according to the linear model and signal decomposition by CDSSTR®), result that agrees with the secondary structure predictions and indicating that a substantial fragment of the Predicted Coiled-Coil Break1 is α-helical.

![Figure. 5.4. Far UV Circular Dichroism Spectra of all peptides at 20°C.](image-url)
The secondary structure present in the peptides is independent of protein concentration (FTIR uses 4mg/ml (data not shown) and CD measurements were with 0.4mg/ml and repeated with 0.1 mg/ml, Table 5.1) that is reproducible and that it is independent of the technique used. The “swivel” region is not disordered but instead it contains a large α-helical section.

Table 5.2 α-helix percentages by signal decomposition in CDSSTR®.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>α-helix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Neck_swivel</td>
<td>31.8</td>
</tr>
<tr>
<td>(2) Swivel_one</td>
<td>15.8</td>
</tr>
<tr>
<td>(3) Swivel_two</td>
<td>72.8</td>
</tr>
<tr>
<td>(4) Swivel_three</td>
<td>77.4</td>
</tr>
<tr>
<td>(5) Swivel_coil</td>
<td>37.1</td>
</tr>
</tbody>
</table>

The measured ellipticity at 222nm was used to calculate the α-helix fraction present in the peptide (3 and 4) at each temperature (Method). The fraction of α-helix versus temperature is plotted in figure 5.4 for peptides 3 and 4.

Figure 5.4 Thermal unfolding curves for the peptides a) 3 (swivel 2) and b) 4 (swivel 3). Solid lines are least squares fit to the Zimm-Bragg model (Equations 5.3-5.4).

Table 5.3 Thermodynamic values obtained from thermal unfolding data in figure 5.4.
The Zimm-Bragg model was used to calculate the enthalpy, nucleation and propagation parameters of an idealized α-helix (Method). The free energy would be the necessary to remove one single amino acid from an α-helix. The results from Table 5.3 compare well for previously measured values using alanine-based peptides in water: \(\Delta H = -960 \pm 20 \text{cal/mol}, \sigma = 0.0029 \pm 0.0003 \text{ and } s_0 = 1.35 \pm 0.02\), enthalpy, nucleation and propagation parameters respectively[111]. 1 kcal/mol is approximately 4.2 kJ and the thermal energy is 0.5921 kcal/mol, so the helix is stable at 25°C.

**Flourescence.** It has been found that: A) \(\log(\phi_{\text{EtOH}}(T))\) exhibits a linear dependence on T; and B) The slope of the \(\log(\phi_{\text{EtOH}}(T))\) versus T strongly depends on the amount of EtOH and is thus strongly correlated with hydrophobicity in a protic environment.

The temperature dependency of fluorescence has been used to determine the change in enthalpy (\(\Delta H\)) and change in entropy (\(\Delta S\)) of unfolding. A two state model is the simplest way to describe thermal unfolding, where the probability of being unfolded is given by

\[
U(T) = \frac{1}{1+K}
\]

where

\[
K = e^{-\frac{\Delta G}{kT}}
\]

is the equilibrium constant of the unfolded and native state \([U]/[N]\) (reversibility was found for all peptides in the assessed T-range 23-70 °C). For the application of the two state model the following assumptions have been made,

a) There are only two states (U and N), and

b) the slope \(d\phi_{\text{EtOH}}(T)/dT\) is determined by the hydrophobicity of the environment where the Trp residue exists, for both the unfolded and the native (folded) state.
Figure 5.3. A) Relative Trp fluorescence ($\phi_{\text{EtOH}} (T)$) versus temperature at different EtOH concentrations (black -0% to pink – 50%). B) Relative Trp fluorescence in the peptide ($\phi_p (T)$) versus temperature for the different peptides (black, 3; red, 4; green, 2; dark blue, 1; light blue, 5). Symbols in A and B represent experimental data, solid lines in B are least squares fit to Equations 5.3 and 5.4.
With the assumptions for two state model and defining $p_N = d\phi_p^N (T)/dT$ and $p_U = d\phi_p^U (T)/dT$, it is obtained

$$\frac{d\phi_p (T)}{dT} = p_U \cdot U (T) + p_N \left[1 - U (T)\right],$$

or

$$\frac{d\phi_p (T)}{dT} = U (T) \cdot \Delta p + p_N,$$

where $\Delta p = p_U - p_N$. This equation relates the fluorescence of the Trp residue with the folding state of the peptide in a given hydrophobic environment. Equation 5.5 has been used to reproduce the experimental traces by integration.

Remembering that

$$\Delta G = \Delta H - T \Delta S$$

the thermodynamic parameters $\Delta H/R$ and $\Delta S/R$ (R is the gas constant in kcal/mol) can be calculated by least squares fit of equations 5.5 and 5.6 to the data.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>neck-swivel</th>
<th>swivel 1</th>
<th>swivel 2</th>
<th>swivel 3</th>
<th>swivel-coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H/R$</td>
<td>9298</td>
<td>9000</td>
<td>11364</td>
<td>10507</td>
<td>9693</td>
</tr>
<tr>
<td>$\Delta S/R$</td>
<td>28.35</td>
<td>27.9</td>
<td>34.63</td>
<td>31.8</td>
<td>31.62</td>
</tr>
</tbody>
</table>

the ranking from lowest two highest disorder of the native peptides by fluorescent data is as follows: 3 < 5 = 2 < 4 < 1. The only discrepancy with CD data is concerning peptide 4, which was second in $\alpha$-helical structure.

**Gel Filtration.** Standards, Myc2 behaves as an unfolded peptide with a larger elution volume than it is expected to have by its size. It is because it lacks a defined structure and it is known that unfolded peptides run as if were larger proteins in gel filtration. Ribonuclease, Insulin chain A, Insulin chain B and aprotinin are very well behaved, showed only one well defined peak at their expected elution volume according to their size. 1, 2 and 5 show one single well-defined peak located close to the elution volume of myc2 and Aprotinin indicating an unstructured peptide.

3 shows three peaks each with different height. The medium one appears to be the monomer running well behind myc2 and aprotinin. The largest peak runs closer to the elution volume of myc2 and aprotinin that is 6000kDa, suggesting dimerization. And the
smallest peak is a broader peak running as ~12000kDa close to ribonuclease, which would suggest that is a tetramer or other form of large aggregation.

4 shows two peaks each with different height. The smaller one appears to be the monomer running well behind myc2 and aprotinin. The larger peak runs closer to myc2 and aprotinin, suggesting dimerization, just like for 3.

The peaks for 1, 2 and 5 agree with the CD data indicating unstructured peptides. The presence of the larger peak at an elution volume for ~6000kDa suggests dimerization in 3 and 4, 67% and 57% respectively.

**DISCUSSION.**

Figure 5.4 summarizes the results obtained in this Chapter. There is a large a-helix (~50%) flanked by random coils. The N-terminal coil is larger than the C-terminal one. The fact that in vivo the two chains are in very close proximity could favor dimerization beyond the 0.6 probability found. This would mean that *in vivo* the α-helices are forming a dimer. This dimer in addition is less stable than the coiled-coil regions in the kinesin-1 tail. The name Helix-Coil (HC) is proposed for this region instead of calling it predicted coiled-coil break-1.

![Figure 5.4 Scheme of the secondary structure for the predicted coiled-coil break-1 “swivel” of kinesin-1. It is composed of a dimer of α-helices flanked by random coils.](image)

The swivel region is important for high-density motility[86]. The first half contains locally defined structure elements (extended chain, helical loop) connected by flexible joints [23] (these results would be confirmed by the first fragment) and is sufficient for wild type-like speed in high-density assays[35].

The idea that the neck could open during the ATP hydrolysis cycle has been much dismissed by Tripet and Hodges [25], showing that capping interactions exist at
both ends of the coiled-coil. De-Cuevas and Goldstein [26] used fragments of the tail that include coiled-coil-1 and coiled-coil-2. Their results were that for coiled-coil-1 the melting temperature was lower than for coiled-coil-2, indicating a less stable coiled-coil. In contrast all the studies with the neck region have shown a very stable coiled-coil[23, 25].

A comparison between thermal and mechanical denaturation would help us understand how a single domain might respond under tension in \textit{in vivo} situations. The unfolding pathway may be very different but the energy differences should be the same.

\textbf{Proposed model.} Depicted in Figure 5.5, one motor is mechanically linked to other motors \textit{in vivo} (via cargo) or in a gliding assay (via a glass surface). In its detached state it can have any orientation with respect to the microtubule, e.g. perpendicular to it.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.5.png}
\caption{A Proposed model of how the HC region may function \textit{in vivo}. Upon force application the \(\alpha\)-helical part of the HC region (red) may unfold, allowing the neck to rotate and the attachment of the unbound head to the microtubule lattice.}
\end{figure}

One head can rotate and bind; here the rotation is on the neck linker a single peptide chain. In order to step both heads must bind, but given its orientation that cannot happen. While one head is bound the motor may experience pulling or pushing forces from other motors. This force will act on the less stable parts of the dimeric protein, hence in the HC
region. In this one-headed state, mechanical unfolding takes place with a concomitant reduction of the torsion elasticity constant of the whole molecule. Once flexible, the other head can bind and the hydrolysis cycle as the stepping process can proceed forward. An alternative model would be that the disordered N-terminal part of the HC domain might be sufficient for high-density motility. This is, that the random coil could provide the necessary flexibility for motor cooperation. Deletion of this specific element could provide insight into which are the responsible fragments for the support of motor cooperation. This alternative model would also explain why all the other kinesin-1 do not show any \( \alpha \)-helical propensity and have a higher proline content. Although, it would remain controversial why the insertion of a random coil does not rescue the microtubule low velocity at high kinesin density [86]. Presumably even with the higher proline content there is an \( \alpha \)-helix in each kinesin-1 HC region. This structure could unfold to allow motor cooperation. The degree of stability of the \( \alpha \)-helix in each kinesin-1 HC region could vary from specie to specie.

A further step is to investigate whether truly a force-unfolding event is taking place. This may affect the very short construct (St\( \Delta \)Sw) but not the one that contains the HC region. That will be the topic of the next Chapter.
CHAPTER SIX. TORQUE MEASUREMENTS

INTRODUCTION.
In Chapter 2 and 3 it was used microtubule gliding assays to extract information about kinetics and cooperation of kinesin molecules. In Chapter 4 we proceed to measure the flexibility of individual truncated kinesin molecules. Chapter 5 we provided evidence that indeed in the so-called swivel region there is an $\alpha$-helical dimeric structure flanked by random coils. Here we try to connect the idea that every kinesin has a random orientation at the surface and the velocities they show. One possibility that we explored for the decreased speed of StASw was a simple average, where we assumed that every molecule would have a different speed depending on how it was oriented with respect to the microtubule axis (figure 6.1). The data did not support such model. The question then that it is faced in this Chapter is exactly how does the speed depends on a given torque (or angular displacement) $s = s(T)$?

Figure 6.1. A) Cartoon representing several kinesin molecules bound to a surface driving the movement of a microtubule. B) Resting orientation of each kinesin that appears in A.

The idea proposed in Chapter 4 and developed in Chapter 5 is that force could open (or unfold) the swivel, just as force can open RNA hairpins (see for example figure
2 in (BustamanteScience2001)). These folding-unfolding processes are usually described by two-state kinetics, experimentally this is observed as a threshold in the force applied. Below that threshold no molecule unfolds, and above it all molecules are unfolded. With the application of torque on individual kinesin molecules we should be able to observe whether there is any threshold suggesting two-state dynamics. The torque that will be exerted is with hydrodynamic flow. The flow will align a microtubule that is swiveling freely around one point, which is the assumed kinesin position.

![Diagram of a microtubule](image)

Figure 6.2. Resting orientation ($\theta_0$) of a microtubule tethered by a single kinesin molecule (pivoting point) and angle of the external force ($\theta_F$).

Our aim here is to measure the gliding speed of a microtubule driven by a single kinesin molecule as a function of the displaced angle and the system is depicted in figure 6.2. In the case of figure 6.2 the application of flow in the $\phi_F$ direction would cause the $L_2$ to rotate clockwise until it has reached the $\phi_F$ position. The other interesting thing to measure is the microtubule arm length at the start of the flow (both $L_1$ and $L_2$), because the force applied to the microtubule and hence to the kinesin is proportional to the difference between arm lengths (see Calibration of forces in Method). So not only the effect of torque but also the forces necessary to orient a single kinesin molecule can be measured. The experimental set-up provides answers to both questions. Here it has been implemented a GeSim® flow system together with a LED flash for temporal precision to exert a torque using hydrodynamic flow.
METHOD.

Flow Calibration Measurements: GeSim Flow-Cell System

In order to apply a torque to kinesin molecules via microtubules using flow, it is important to know the velocity profile inside the flow cell, so that torque measurements can be more precise.

Fluorescent latex beads (Polystryrene, Amine modified, Sigma L-1405 Lot94H0871), mean diameter 0.046 μm, StDev 19.1%, 2.5% Solids diluted 1000-fold. Flow Cell Dimensions: 19 x 3 x .105 mm (L, W and tape thickness). Two silanized coverslips 22x22 mm. In the upper one two holes of 1cm in diameter have been drilled with a dentist drill (Hartmut). Inverted Zeiss Microscope, 100 oil objective, 1x Optovar. CoolSNAP Camera at 18 frames per second, streaming mode, binning factor 2, pixel size 0.129 μm. GeSim Flow device with a flow rate of 1μl/sec.

A picture taken at 18 frames per second where the exposure time is 0.5555 ms does not allow visualizing a bead. The image taken displays instead the path that the bead has follows during that time. It integrates the motion over time (55ms). The length of such a trace is proportional to the velocity that the bead has at that position in the Z-axis. Dividing the length of the trace by the exposure time, the speed of the bead (and the fluid) can be known.

Fig. 6.3 a) Schematic of a flow cell to be used for fluid flow experiments. b) Photograph of the GeSim device made with PDMS mounted on a metal holder to insert in a) inverted microscope. Tubing is connected to the entrance and exit of the flow cell as seen in b).
Figure 6.4. **a)** Bead trace at 33μm above the surface and **b)** At 11μm above the surface using a pumping flow rate of 1μl/s.

Figure 6.5. Definition diagram from channel flow (Laminar flow). Width of channel is shown exaggerated with respect to length.

**Data Calibration Analysis.** To fit the data, laminar flow was assumed (figure 6.5). A fluid moving between two plates obeys the so-called Poiseuille flow equation 6.1:

\[
(6.1) \quad v(h) = \frac{\Delta P}{2\eta L} \left( H^2 - h^2 \right); \quad h = \pm H \text{ and } \Delta P = (P_1 - P_2)
\]

that we can change to,

\[
(6.2) \quad v(h) = \frac{P}{2\eta} \left( H^2 - h^2 \right)
\]

using, \( P = \Delta P / L \); where \( H \) is half the height of the channel (50±2.5μm, \( n=10 \)), \( h \) is the distance from the center of the channel, \( P \) is the pressure difference and \( \eta \) is the viscosity.
of the fluid (water) was taken as 0.01 mPa. Fitting the calibration data with $y = ax^2 + c$, gives $P$ that then it is used to extrapolate to a desired height. At surface (usually one can find a stuck microtubule as guide), plus 10, 20 and 30 µm above it. Localization of the other surface and walls formed by the tape are also very important, since all measurements must be done at least 0.5µm away from the walls. For example, in one flow cell the walls were at ~81200 |----| ~83842 (nm in x-axis display MetaMorph). The closest point to surface (~2µm) was measured using beads out of focus that passed when the focus is on surface.

Figure. 6.6. Fluid Flow-Cell H-Calibration and flow profile. a) Fluid velocity as a function of the height, position from center of the channel, fitted with Equation 6.5, Possieville profile. b) Fluid velocity vs. time at 10µm height; and c) At 50µm height.
The fluid velocity at 30nm above the surface with 0.2µl/s is extrapolated from calibration to be $\nu = 1.44 \, \mu m/s$, which for 0.5µl/s become $\nu = 3.6 \, \mu m/s$ where the linearity of the fluid velocity versus pumping flow rate has been used.

The flow profile of each individual flow cell is measured and then used for force calculations of molecules observed only for that flow cell.

![Figure. 6.7. Fluid Flow-Cell Calibration, x-axis flow profile. a) Fluid velocity as a function of the channel width measured at center (height) of the channel.](image)

**LED flash.** For temporal precision we implemented a LED coupled to the TTL signal coming from the GeSim system when it switches on and off, generating a light flash as an indicator of the beginning and end of the flow, see figure 6.6b and c. This was developed by Robet Müller from the Workshop at the MPI-CBG, Dresden.

**Motility assays.** Surface-coated cover slips, single kinesin molecules and microtubules were used as described in Chapter 4. Once one molecule is visualized a region of interest is drawn around it and data is stream-acquired (500-1000 frames, 18 fps), applying a fluid flow pulse of 6 sec with a flow rate of 0.5µl/s. This experiment is technically very difficult to do.
RESULTS.

**Torque and Force Exerted by Fluid Flow - Theory.** In the presence of flow the torque experienced by a kinesin molecule (considering it as a rotational spring), neglecting fluctuations as in figure 6.2, is described by:

\[
\gamma \frac{d\theta(t)}{dt} = -\kappa \left[ \theta(t) - \theta_0 \right] + FL \sin \left[ \theta_r - \theta(t) \right]
\]

where \( \kappa \) is the torsion elasticity constant, \( F \) the external force applied on the microtubule, \( L \) is \( |L_1 - L_2| \), the absolute difference between microtubule arm lengths; \( \theta(t) - \theta_0 \) is the difference between the initial and actual MT orientation; and, \( \theta_r - \theta(t) \) is the angle between the external force and the MT orientation. The force is given by,

\[
F = \frac{1}{3} c_\perp L \nu
\]

the rotational drag coefficient is,

\[
\gamma = \frac{1}{3} c_\perp \left( L_1^2 + L_2^2 \right)
\]

where

\[
c_\perp = \frac{4\pi \zeta}{\cosh^{-1} \left( \eta/r \right)}
\]

and \( \eta, h \) and \( r \) are given in Table 6.1.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Symbol</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td>( \zeta )</td>
<td>0.851 mN m(^{-2}) s</td>
<td>[100]</td>
</tr>
<tr>
<td>Microtubule radius</td>
<td>( R )</td>
<td>15 nm</td>
<td>[2]</td>
</tr>
<tr>
<td>MT height above the surface</td>
<td>( H )</td>
<td>32 nm</td>
<td>[104]</td>
</tr>
<tr>
<td>Fluid flow speed</td>
<td>( V )</td>
<td>1 ( \mu l ) s(^{-1})</td>
<td>FFC calibration</td>
</tr>
<tr>
<td>Lever arm length</td>
<td>( L )</td>
<td>5 ( \mu m )</td>
<td>Max. arm difference</td>
</tr>
<tr>
<td>Torsion elasticity constant</td>
<td>( \kappa_r )</td>
<td>0.1-30 ( k_B T/\text{rad} )</td>
<td>Obtained values</td>
</tr>
</tbody>
</table>

The maximal force that the flow can exert on a microtubule already oriented (parallel to the direction of flow) is,

\[
c_\perp = \frac{4\pi \left( 0.851 \text{mN} \cdot \text{m}^{-2} \text{s} \right)}{\cosh^{-1} \left( 32 \text{nm}/15 \text{nm} \right)} \approx 0.0077 \text{N} \cdot \text{m}^{-2} \text{s},
\]
then

\[ F = \frac{1}{3} (0.0077)(5\mu m)(1\mu m/s) = 0.013\text{ pN} . \]

This is an important value to be considered in the course of the experiments, if this force would be 200 times lager, it would affect considerably the detachment rate of kinesin from the microtubule.

![Figure 6.8](image_url)

Figure 6.8. Solution of Eq. 6.1 when the force is 0.02 pN, the initial angle is very close to 0, the resting orientation is 0, and the force angle is \( \pi \). \( \kappa_r \) in kB/rad.

We can easily implement how to solve Equation 6.1 for a given set of initial conditions \((\theta(0), \theta_s)\), and for known parameter values (see Table 1). The steady state is reached very fast, and that the orientation in the steady state depends on the torsion elasticity constant (figure 6.8). For 0.1 kB/T/rad the flow completely orients the microtubule with the flow; for 1 kB/T/rad is almost complete and probably experimentally hard to distinguish from completeness; for 10 kB/T/rad it goes half way between the flow and its resting orientation; and for 25 kB/T/rad it goes directly into its resting configuration, i.e. it does not feel the flow. For the torsion elasticity constant of a single kinesin measured some microtubules are expected to partially align, where others will fully orient with the flow.
The steady state is given by, 
\[
\frac{1}{\tau}[\theta - \theta_o] = F \frac{L}{\Gamma} \sin[\theta_c - \theta],
\] which is plotted in figure 6.9 for three different \( \kappa \) (we have used \( \tau = \frac{\Gamma}{\kappa} \)).

The x-axis in figure 6.3 is given by flow or \( L \) variations according to the steady state. The solid black lines in figure 6.3 represent the maximal force that can be exerted with \( 1 \mu l/s \) fluid flow. \( L \), the effective lever arm subjected to flow, gives the force range. The minimum was calculated for \( L = 0.1 \mu m \).

![Figure 6.9](image)

Figure 6.9. Final angular displacement (steady state) versus force (at \( \pi/2 \)), for three different torsion elasticity constants (given in \( k_B T/\text{rad} \)): 25, blue; 10, red; and 1, black.

We should exert forces in a range of fluid flow 0.1-10\( \mu l/s \) to explore the maximum angle regime for very stiff molecules.

For Wild Type flows in the range of 0.1-1\( \mu l/s \) are sufficient to orient the microtubule with the flow. For Stubby and St\( \Delta \)Sw flows from 1-10\( \mu l/s \) are required. Knowing the microtubule length and its response to flow, we can calculate (assuming a given height) an independent measure of the torsion elasticity constant for that motor.
Table 6.2. Force values vs. height

<table>
<thead>
<tr>
<th>Height (nm)</th>
<th>Flow Speed (μm/s)</th>
<th>$C_\perp$ (Nm$^{-2}$s)</th>
<th>Force (pN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0,683</td>
<td>0,013</td>
<td>0,015</td>
</tr>
<tr>
<td>30</td>
<td>1,024</td>
<td>0,008</td>
<td>0,014</td>
</tr>
<tr>
<td>40</td>
<td>1,366</td>
<td>0,007</td>
<td>0,015</td>
</tr>
<tr>
<td>50</td>
<td>1,707</td>
<td>0,006</td>
<td>0,016</td>
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<tr>
<td>60</td>
<td>2,048</td>
<td>0,005</td>
<td>0,018</td>
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<td>70</td>
<td>2,389</td>
<td>0,005</td>
<td>0,019</td>
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<tr>
<td>80</td>
<td>2,730</td>
<td>0,005</td>
<td>0,021</td>
</tr>
<tr>
<td>90</td>
<td>3,071</td>
<td>0,004</td>
<td>0,022</td>
</tr>
<tr>
<td>100</td>
<td>3,412</td>
<td>0,004</td>
<td>0,024</td>
</tr>
</tbody>
</table>

Fig. 6.10. Force versus height. Left, The force increases linearly with height (up to ~0.5μm) and a factor of ~2 up to 100nm above the surface. Right, The force reaches a constant of 200 fN value at 4μm approximately.

Figure 6.10 shows force (exerted by flow) versus height (z-distance from the surface), so that even if the kinesin molecule gets fully extended during flow application (~70nm) it won’t create a force difference that could make the kinesin molecule detach from the microtubule (the force would be at maximum larger by a factor of 2). And it also sets an upper limit in the experimental error, because we do not where the kinesin molecule is precisely located.

Each flow cell was calibrated after the experiment to know exactly the fluid velocity acting on each observed microtubule. An observation is that with time there were less microtubules gliding and swiveling. After half an hour no moving microtubule was detected and only a few either stuck or non-motile were present. This phenomenon plus all the requirements to ensure single functional kinesin molecules made the data
acquisition and experiment very hard to perform and only a few truly measurements were made.

Figure 6.10 shows an example of a microtubule driven by a single Stubby kinesin molecule at 5μM ATP. At certain time point hydrodynamic flow was applied (on) during 6 seconds. The flow had a 90° direction with respect to the microscope field of view. The microtubule was swiveling and gliding at a speed of 75±3nm/s. The microtubule orientation followed the direction of the flow only after 5.5 seconds, when the kinesin was almost at the end of the microtubule. Then, the microtubule detached at the same moment that the flow was switched off.

![Angle versus time of a microtubule driven by a single Stubby kinesin molecule at 5μM ATP and the application of hydrodynamic flow (solid black line). Inset, orientation of the microtubule and flow direction.](image)

Figure 6.11. Angle versus time of a microtubule driven by a single Stubby kinesin molecule at 5μM ATP and the application of hydrodynamic flow (solid black line). Inset, orientation of the microtubule and flow direction.

From the microtubules that responded to the flow (n=6), half aligned immediately while the other half it took 2.9 seconds in average. There were microtubules that aligned with flow but its speed was indistinguishable from 0 (not counted for the total n). This was probably due to microtubule non-specific attachment to the surface. Approximately two thirds of the microtubules subjected to flow seem not to “feel it”, i. e., does not align and there is no change in speed. Of the microtubules that did orient parallel to the direction of flow they fell off from their attachment point at an average time of 0.3 seconds. This
detachment time did not allow for a reliable measurement of possible speed variations before and after orientation.

Partially oriented microtubules were not observed as was expected for torque application on a system with the previously found torsion elasticity constant (figure 6.8). Microtubules either aligned parallel to the direction of flow or not.

The force for each aligned microtubule was calculated according to Equation 6.4. The fraction of aligned microtubules for each force was calculated using a Cumulative Distribution function and is shown in figure 6.12.

![Figure 6.12. Probability of alignment versus force was obtained by summing a normalized histogram of microtubules aligned versus force. Solid red line least squares fit with a two-state system (Equation 6.7).](image)

The effect of force on a rate constant is given by\textsuperscript{6} [bustamante04],

\[
k(F) = A \exp^{-(\Delta G^1 - F\Delta s)/k_B T}
\]

\textsuperscript{6} Here it has been assumed that the position of the initial, final and transition state are force independent.
where \( A \) is the attempt of frequency, \( \Delta G^\ddagger \) is the transition state free energy, \( \Delta x^\ddagger \) is the distance from the initial state to the transition state, \( F \) is the force, \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature.

The equilibrium constant of the unfolded and native state \([U]/[N]\) including the presence of force is

\[
K = \exp \left( \frac{-\left(\Delta G^\ddagger - F\Delta x^\ddagger\right)}{k_B T} \right).
\]

(6.8)

Equation 6.8 can be written as,

\[
K(F) = B \exp^{F\Delta x^\ddagger / k_B T}
\]

(6.9)

where \( B = \exp^{\Delta G^\ddagger / k_B T} \).

A two-state system has a probability \( p(E) \) of

\[
p(E) = \frac{1}{1 + K(F)}
\]

(6.10)

Equations 6.9 and 6.10 have been used to fit (by least squares) the cumulative probability of alignment. The obtain values are \( \Delta G^\ddagger = 30.0 \pm 1.2 \) pNnm and \( \Delta x^\ddagger = 1619 \pm 83 \) nm (±95% confidence interval). The half force for alignment \( F_{1/2} \) equals 0.0185 pN.

**DISCUSSION.**

Theoretical investigation of the experimental set up explored in this Chapter provided with limits on the possible measurements. Due to the technical difficulty of these experiments the total number of molecules subjected to torque is very low, which constrains the discussion.

One prediction was partial orientation, something that was not observed. In the theoretical part it was only modeled a spring subject to an external force. If the HC (predicted coiled-coil break-1) region inside the kinesin molecule can open as a result of the acting of an external force, the elastic response is going to be different from a simple spring. Suggesting that there is a more complex phenomenon occurring when applying a torque to a single Stubby kinesin molecule.
A larger force required to align the microtubule explains why there is a delay in figure 6.11. As the kinesin walks along the microtubule lattice the pivot point changes and the microtubule arm difference does as well. The larger the arm difference the larger the force. When the arm difference (hence force) is larger than $F_{1/2}$ then the probability of alignment is larger than 0.5. And this probability keeps increasing as the kinesin keeps walking toward the end of the microtubule. The cumulative probability of alignment was well described by a two-state model, which is the most common description of folding-unfolding events [2] [99].

Unfortunately, here there is no straight connection between $\Delta x^9$ and the mechanical deformations of the protein in contrast to RNA unfolding experiments [118].

The force necessary to align the majority of the microtubules (and hence the motors) is 0.025pN. At 0.025 pN in figure 6.9 only the springs with a $\kappa$ of 1 kB T or less would have aligned. This indicates that what is been twisting has a torsion elasticity constant of 1 kB T or less. The measured $\kappa$ value for Stubby was $8.1 \pm 1.2 \text{kBT/rad}$. In order to respond to the hydrodynamic flow the torsion elasticity constant must change. Again, pointing out towards a mechanical-induced unfolding process in the HC region.

The fact that microtubules orient with the flow and keep moving even though for a very short period of time suggests that indeed the whole molecule has been twisted and not only one single polypeptide chain. Another possibility is that not the whole molecule has been twisted, and is just one polypeptide chain. If so the rotation of the microtubule lattice occurs in a way that the kinesin molecule cannot step forward, i.e. it stays bound with one head and the other tries to find the next binding site but because its not there it will stay in this one headed state until it detaches. Hancock and Howard [119] measured the detachment rate for one headed kinesin molecules $\sim 3 \text{s}^{-1}$, for 1mM ATP, 1mM ADP and 1mM ADP +10mM Pi. At 1\muM ATP is $\sim 1 \text{s}^{-1}$. So for 5\muM ATP would be 2.5-3 \text{s}^{-1} or a duration of 0.3 second in the experiments, which corresponds to the measured detachment time. It may be possible that larger forces are required to exert a torque on a double-headed state. In order to have longer-lived double-headed state lower ATP concentrations should be used, because it is known that the nucleotide free state is a double-headed state (Introduction).
In overall there is some evidence suggesting the plausibility of the hypothesis that the HC region may mechanically unfold to allow motor cooperation.
OUTLOOK

This project started with the hypothesis that motor cooperation was achieved through mechanical flexibility. The predicted coiled-coil break-1 "swivel" was the favored element within the kinesin-1 molecule to provide such flexibility. Its removal led to an impaired motor at high densities but fully functional at single molecule level. Then, it was expected that measuring the flexibility at the single molecule level would reveal the molecular cause of mechanical hindrance at high density, the explanation why kinesin-1 can work in groups. But it was not that easy. The torsion elasticity constant turned out to be the same whether this "swivel" region was present or not. The simplest explanation was just ruled out.

What could be flexible at high densities but stiff at single molecule level? Could it switched between two-states, one stiff one flexible? It is this two-state switching a force unfolding mechanism?

It was time to look deep into the structure of this "swivel" region. Sequence analysis put on the screen the first hint. It is not a random coil, even though it is not a coiled-coil. Traditional techniques were called out, Circular Dichroism, Fluorescence, gel filtration to give information about the secondary structure. Indeed, there was a structure. A large central dimer α-helix surrounded by random coils. The random coils are true ones we can be sure. Still, the main piece of this new idea a force dependent flexible state, needed to be proved right. A new experimental set-up was developed to twist individual kinesin molecules with flow. Then again, experiments were tough and the fantastic data did not come flying to our hands. Nonetheless, the results do suggest that we might be in the right track, and that this "swivel" (now called helix-coil, HC) is responding to force by changing its conformation. Structure to unstructured, stiff to flexible are the changes required for a motor to cooperate, to coordinate with several other motors.

In this last four years, physical theory, simulations, mutations and deletions of DNA, in vitro microscopy assays, image analysis and computer programming, and traditional biochemistry techniques were used. It is obvious then, that when trying to solve a problem we should not pay attention to the artificial borders of scientific
disciplines. Pull out any technique needed, any number of collaborations and the more scientific areas as possible to attack the problem. At the end, we still may not know the answer.
APPENDIX A.
Image moments and Ellipse fitting in MATLAB.

How to calculate the length, angle and center of a binary image? A binary image is a set of black and white pixels (a zeros and ones matrix). So, \( I(x, y) = \{a_y\} \). Matlab takes a P-by-2 matrix, where P is the number of pixels belonging to the object in that region. Each row contains the row and column coordinates of a pixel. And makes the following calculation:

\[
(x_0, y_0) = \text{mean}(P) \quad \text{centroid coordinates. Which is the center of mass of the object in that region.}
\]

To obtain the major and minor axis as well as the orientation:

\[
x = P(:,1) - x_0
\]
\[
y = -(P(:,2) - y_0)
\]

\[
Ux = \frac{1}{N} \sum x^2 + \frac{1}{12}
\]
\[
Uy = \frac{1}{N} \sum y^2 + \frac{1}{12}
\]
\[
Uxy = \frac{1}{N} \sum xy
\]

where the 1/12 is the variance of a top-hat distribution of size 1 [120]. See below more on this issue. Now, we define

\[
C = \left[ (Ux - Uy)^2 + 4Uxy^2 \right]^\frac{1}{2}
\]

then the lengths are:

\[
M = 2 \sqrt{2} \cdot (Ux + Uy + C)^\frac{1}{2} \quad \text{for the major axis and,}
\]
\[
m = 2 \sqrt{2} \cdot (Ux + Uy - C)^\frac{1}{2} \quad \text{for the minor axis.}
\]

\(^7\) remember that the y axis in Matlab is inverted.
The orientation is: \( \tan \theta = \frac{n}{d} \), where

if \( U_y > U_x \),
\[
n = U_y - U_x + \left[ \left( U_y - U_x \right)^2 + 4U_{xy}^2 \right]^{1/2}
\]
\[
d = 2U_{xy}
\]
else \( U_x > U_y \)
\[
n = 2U_{xy}
\]

In moments of an image they are defined as,
\[
\tan \theta = \frac{C + \sqrt{C^2 + 4}}{2} \quad \text{if} \quad \sigma_{xy}^2 > 0, \text{and}
\]
\[
\tan \theta = \frac{C - \sqrt{C^2 + 4}}{2} \quad \text{if} \quad \sigma_{xy}^2 < 0,
\]
where \( C = \frac{\sigma_x^2 - \sigma_y^2}{\sigma_{xy}^2} \).

The moments are, \( \mu_0 = \frac{1}{MN} \sum_{i,j} a_{i,j} \) that is the mean intensity of an image of size \( NxM \).

The coordinates of the centroid are, \( \mu_x = \frac{1}{MN} \sum_{i,j} i \cdot a_{i,j} \) for \( x \) and \( \mu_y = \frac{1}{MN} \sum_{i,j} j \cdot a_{i,j} \) for \( y \).

Finally the variances are given by,
\[
\sigma_x^2 = \frac{1}{MN} \sum_{i,j} i^2 \cdot a_{i,j} - \mu_x^2
\]
\[
\sigma_y^2 = \frac{1}{MN} \sum_{i,j} j^2 \cdot a_{i,j} - \mu_y^2
\]
\[
\sigma_{xy}^2 = \frac{1}{MN} \sum_{i,j} i \cdot j \cdot a_{i,j} - \mu_x \mu_y
\]

Making a comparison with the statistical approach (and a bit of algebra) it is easily shown that:
\[
U_x = \sigma_x^2, \quad U_y = \sigma_y^2, \quad \text{and} \quad U_{xy} = \sigma_{xy}^2.
\]
This shows that when using Matlab we are using a statistical algorithm to calculate the center of mass, the orientation and the lengths of the major and minor axis.

Adding background noise to the image and its analysis. One way to know that our algorithm is working fine is to add noise and still can calculate correctly the moments of the image.

If now we add noise to each pixel we have \( I(x, y) = \{a_{ij} + \varepsilon_{ij}\} \). The error will have the following properties:

\[
E\{\varepsilon\} = 0, \text{ the mean is zero,}
\]

\[
E\{\varepsilon^2\} = \sigma^2, \text{ it has a finite variance and}
\]

\[
E\{\varepsilon_{ij} \cdot \varepsilon_{jk}\} = 0 \quad \text{they are statistically independent or uncorrelated.}
\]

This may not be completely right since if we have a diffraction-limited object its Airy disk will spread to the neighbor pixels. Now we can calculate the moments of the image:

\[
\mu_0 = \frac{1}{MN} \sum_{ij} (a_{ij} + \varepsilon_{ij}) = \frac{1}{MN} \sum_{ij} a_{ij} + \frac{1}{MN} \sum_{ij} \varepsilon_{ij}
\]

\[
= \frac{1}{MN} \sum_{ij} a_{ij} + E\{\varepsilon_{ij}\} = \frac{1}{MN} \sum_{ij} a_{ij} = \hat{\mu}_0
\]

from where we can see that the mean intensity of the image is not affected by background noise. Now we can calculate the moments of the image:

\[
\mu_0 = \frac{1}{MN} \sum_{ij} (a_{ij} + \varepsilon_{ij}) = \frac{1}{MN} \sum_{ij} a_{ij} + \frac{1}{MN} \sum_{ij} \varepsilon_{ij}
\]

\[
= \frac{1}{MN} \sum_{ij} a_{ij} + E\{\varepsilon_{ij}\} = \frac{1}{MN} \sum_{ij} a_{ij} = \hat{\mu}_0
\]

from where we can see that the mean intensity of the image is not affected by background noise. In the same way we can calculate the total variance:

\[
\sigma_0^2 = \frac{1}{MN} \sum_{ij} \left[(a_{ij} + \varepsilon_{ij})^2 - \mu_0^2\right] = \frac{1}{MN} \sum_{ij} \left[a_{ij}^2 + 2a_{ij}\varepsilon_{ij} + \varepsilon_{ij}^2 - \mu_0^2\right]
\]

\[
= \left[\frac{1}{MN} \sum_{ij} (a_{ij}^2 - \mu_0^2)\right] + E\{\varepsilon_{ij}^2\} = \hat{\sigma}_0^2 + \sigma_{\varepsilon}^2
\]
For the $x$ and $y$ moments we have (I’ll only do for $x$, for $y$ the treatment is similar):

$$\sigma_x^2 = \frac{1}{MN} \sum_y i^2 (a_{ij} + \varepsilon_{ij}) - \mu_x^2$$

where

$$\mu_x^2 = \left[ \frac{1}{MN} \sum_y i (a_{ij} + \varepsilon_{ij}) \right]^2 = \frac{1}{M^2 N^2} \sum_y i^2 \left( a_{ij}^2 + 2a_{ij}\varepsilon_{ij} + \varepsilon_{ij}^2 \right)$$

$$= \left[ \frac{1}{MN} \sum_y i a_{ij} \right]^2 + E\{\varepsilon_{ij}^2\} = \hat{\mu}_x^2 + \sigma^2$$

which makes

$$\sigma_x^2 = \frac{1}{MN} \sum_y i^2 a_{ij} - \hat{\mu}_x^2 + \sigma^2 = \hat{\sigma}_x^2 + \sigma^2$$

And now for the cross-term:

$$\sigma_{xy}^2 = \frac{1}{MN} \sum_y ij (a_{ij} + \varepsilon_{ij}) - \mu_x \mu_y$$

where

$$\mu_x \mu_y = \frac{1}{MN} \left[ \sum_y i (a_{ij} + \varepsilon_{ij}) \times \sum_j j (a_{ij} + \varepsilon_{ij}) \right] = \frac{1}{M^2 N^2} \sum_y ij \left( a_{ij}^2 + 2a_{ij}\varepsilon_{ij} + \varepsilon_{ij}^2 \right)$$

now using

$$\frac{1}{M^2 N^2} \sum_y ij \varepsilon_{ij}^2 = \frac{1}{MN} \sum_y \varepsilon_{ij}^2 = E\{\varepsilon_{ij}^2\} = 0$$

which makes

$$\mu_x \mu_y = \hat{\mu}_x \hat{\mu}_y$$

then

$$\sigma_{xy}^2 = \hat{\sigma}_{xy}^2$$

from where we can see that the variance with noise is the same one that without it. Now using this result for $C$ of the previous part we get,

$$C = \frac{\sigma_x^2 - \sigma_y^2}{\sigma_{xy}^2} = \left( \hat{\sigma}_x^2 + \sigma^2 \right) - \left( \hat{\sigma}_y^2 + \sigma^2 \right) = \frac{\sigma_x^2 - \sigma_y^2 + \sigma_x^2 - \sigma_y^2}{\sigma_{xy}^2} = \frac{\hat{\sigma}_x^2 - \hat{\sigma}_y^2}{\sigma_{xy}^2}$$

And we notice that the angle calculation is insensitive to background noise, because it uses only its noiseless counterparts.
APPENDIX B.  
Simulation of Brownian motion.

In the swivel experiments, a single kinesin molecule moves a microtubule and the microtubule swivels due to Brownian motion. If the microtubule rotates freely the system can be described by,

\[ \gamma \frac{d\theta}{dt} = -\kappa \theta + \eta(t) \]

where \( \gamma \) is the drag coefficient, \( \kappa \) is the spring constant, and \( \theta \) is the angle of the microtubule with an arbitrary \( x \)-axis. \( \eta(t) \) is the noise due random fluctuations:

\[ \langle \eta(t) \rangle = 0 \quad \text{and} \quad \langle \eta(t) \eta(t') \rangle = 2\gamma k_B T \delta(t-t') \]

where \( k_B \) is the Boltzmann constant, \( T \) the temperature and \( \delta \) the Dirac delta. The goal in this Appendix is to simulate Brownian motion as a way of representing the single kinesin-microtubule system.

In order to obtain sample paths we used three different methods:

1) The convolution of GWN with an exponential decaying function \( e^{-\tau t} \) where \( \tau \) is the relaxation constant given by, \( \tau = \gamma / \kappa \). The GWN was provided by the MATLAB function \texttt{randn(mu,stdev)}, which gives normally distributed random numbers (the \texttt{randn} function generates arrays of random numbers whose elements are normally distributed with mean 0, variance 1, and standard deviation 1). For the convolution see Appendix. The amplitude of the convolution resultant was rescaled to fit the expected by energy equipartition theorem for a given spring constant, \( \frac{1}{2} \kappa \langle \theta^2 \rangle = \frac{1}{2} k_B T \).

2) To obtain sample paths a time discretization procedure for the Langevin equation has been used [fall],

\[ \theta(t + \Delta t) \approx \theta(t) - \frac{\kappa}{\gamma} \theta(t) \Delta t + \sqrt{2D \Delta t} \]

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where $Z$ is a standard random variable with mean 0 and variance 1. The step size was taken to be 0.03 (also a dt of 0.0001 was used and no differences were observed), the same time step that we use to acquire data. A sample path is produced by iteration of this equation.

3) The exact solution of the model equations [30]. I have produced sample paths that are solution to: \[ \dot{x}(t) + 2\pi f_c x(t) = (2D)^{1/2} \eta(t) \] where the corner frequency is given by $f_c = \kappa/(2\pi\gamma)$, and Einstein’s equation, $D = k_B T / \gamma$ has been used.

To model a microtubule with different length, the drag coefficient was changed accordingly to: \[ \gamma = 1/3 \cdot C_\perp (L_1^2 + L_2^2) \] where $C_\perp = 9.4 \text{mN} \cdot \text{s} \cdot \text{m}^{-2}$ is the drag coefficient per unit length for translation perpendicular to the microtubule axis. We assumed for simplicity that the kinesin stayed all the time at the center of the microtubule ($L_1 = L_2$).

The programs used were PSDplots.m, swivel_mt.m and swivel_mt_many.m.

Fitting a lorentzian to the Power Spectral Density by least-squares is
\[
\chi^2 = \sum_k \left( \frac{P_{\text{alias}}^k - P_{\text{ex}}^k}{\sigma_k} \right)^2
\]

where $\sigma_k$ is given by any one of the following cases:

- **i)** $\sigma\left(P_{\text{ex}}^k\right)$ which has a 2n dependence.
- **ii)** $P_{\text{alias, old}}^k$ no n-dependence
- **iii)** $P_{\text{alias}}^k$ n-dependence
- **iv)** 1 no weight

The problem is that least-squares assumes normal (Gaussian) distributed residuals and this is not the case. The residuals from a single PSD are exponential and for several are Gamma distributed (convolution of n identical, exponential distributions) and slowly approach a Gaussian only when $n \to \infty$. In order to correct this, a Maximum Likelihood Estimation has been used [30]. It turns out that maximizing the probability density fr the
PSD is (practically) equivalent to minimize $\chi^2$ with a n-correction: $n/(n+1)$, where n is the number of PSD averaged.

**RESULTS.**

Simulation by Convolution.

![Simulation by convolution](image)

Figure A.1 Simulation of Brownian motion by Convolution of GNW with an exponential function. $\kappa$ is given in Nm/rad and $\gamma$ in Nms/rad. Each unit $\{n \ n+1\}$ interval has 100 points to avoid discretization effects.
Figure A.2 Calculating back the kappa and gamma by PSD. $\kappa$ is given in Nm/rad and $\gamma$ in Nms/rad. The red line is a fit using the analytical fit described in Chapter 4.

The convolution method works for spring constants of $10^{-18}$ up to $10^{-21}$, beyond this point the number of points (10,000) was not enough to provide a complete decay for very low spring constants, nor have enough resolution for very stiff ones. Fortunately the experimental data is found within this range, which serves as a control for Brownian motion traces.

Figure A.3 Simulation by Langevin.
Ten sample paths were generated for each MT length (2, 3, 5, 8 and 10µm) of 10 and 100 seconds.

Using the power spectrum and fitting a lorentzian we can calculate back the parameters of interest. The Langevin approach, as by convolving GWN, is good enough to provide sample paths for Brownian motion.

Figure A.4 PSD and fit for Brownian motion from exact solution of the model equations sampled at a finite time. Here the sampling creates aliasing as can be observed at frequencies larger than 1. The red line is a fit using the analytical fit described in Chapter 4 from $f_{\min}$ to $f_{\max}/2$. 
DISCUSSION.

The time discretization of a Langevin, the convolution of Gaussian white noise with an exponential decay produces, and the simulation of Brownian motion sampled at finite times produce all similar results. The problems associated with the first method are well known [121], although for the purposes of this thesis are neglect able.

One feature of both simulations is the presence of high frequency motion (Langevin and Convolution methods), not present in the experimental data. The camera is averaging out these high frequencies and that is why they are no visible.

Using the analytical fit described in Chapter 4 from $f_{\text{min}}$ to $f_{\text{max}}/2$, avoids any possible problem, like aliasing. Aliasing could be present in the experimental traces due to a finite sampling of the time-continuous Brownian motion.

Simulating Brownian motion can then be used to be compared to the experimental traces of the angular fluctuations of a microtubule tethered by a single kinesin molecule. It is another way of validate the use of the power spectral density, which assumes Brownian motion to measure the torsion elasticity constant.
REFERENCES


