Investigating the Slow Axonal Transport of Neurofilaments:
A Precursor for Optimal Neuronal Signaling

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

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April 2016
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This dissertation titled
Investigating the Slow Axonal Transport of Neurofilaments:
A Precursor for Optimal Neuronal Signaling

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Abstract

JOHNSON, CHRISTOPHER M., Ph.D., April 2016, Physics

Investigating the Slow Axonal Transport of Neurofilaments: A Precursor for Optimal Neuronal Signaling (192 pp.)

Director of Dissertation: Peter Jung

Neurofilaments are the intermediate filaments of neurons and are the most abundant structure of the neuronal cytoskeleton. Once synthesized within the cell body they are then transported throughout the axon along microtubule tracks, driven by the molecular motors kinesin and dynein. This movement is characterized by long pauses with no movement interrupted by infrequent bouts of rapid movement, resulting in an aggregate dense cytoskeletal structure, which serves to regulate an axon's shape and size. Curiously, the modulated kinetics of these polymers produces a very regular, yet non-uniform, morphology in myelinated axons which are composed of discretely spaced myelin-ensheathed segments that are separated by short constricted regions called "nodes of Ranvier". This unique design optimizes the conduction velocity of myelinated axons at minimal fiber size. Hence, neurofilaments regulate the axon caliber to optimize neuron function.

The goal of this dissertation is to investigate the motile mechanism of neurofilament transport as well as the resulting electrophysiological effects that follow. We start by examining highly time-resolved kymograph images generated from recorded neurofilament movement via epifluorescence microscopy. Using kymograph analysis, edge detection algorithms, and pixel smoothing tactics, neurofilament trajectories are extracted and used to obtain statistical distributions for the characteristics of how these filaments move within cells. The results suggest that the observed intermittent and bidirectional motions of these filaments might be explained by a model in which dynein and kinesin motors attach to a single neurofilament cargo and interact through mechanical
forces only (i.e. a "tug-of-war" model). We test this hypothesis by developing two discrete-state stochastic models for the kinetic cycles of kinesin and dynein, which are then incorporated into a separate stochastic model that represents the posed tug-of-war scenario. We then systematically vary the number of motors in the model and attempt to identify those combinations of motors that show an agreement with the motility characteristic found from the above mentioned kymographs. By pruning the modeled data in accordance with the experimental results, our model can render an estimate of how many motors are attached to the cargo during transport. The model predicts that, on average, the total number of active motors on each neurofilament is relatively small and relatively independent of polymer length, which suggests that the motors may not be distributed uniformly along the filaments. Finally, we develop a model to explore the physiological function of axon morphology sculpted by neurofilament kinetics. Specifically, nodal constrictions are generated by slowing of neurofilaments in the internodal domain (Monsma et al., 2014), but the physiological function of these constrictions is unknown. To address this, we develop a computational model to investigate the effect of nodal constrictions on the axonal conduction velocity. For a fixed number of ion channels, we find that there is an optimal extent of nodal constriction which minimizes the internodal axon caliber that is required to achieve a given target conduction velocity, and we show that this is sensitive to the precise geometry of the axon and myelin sheath in the flanking paranodal regions. Thus axonal constrictions appear to be a biological adaptation that serves to minimize axonal volume, thereby maximizing the spatial and metabolic efficiency of these processes.
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2.15 **Summary-Statistics of collective neurofilament trajectories:** Our final data set consists of 726 neurofilament traces consisting of 5,589 (28.5%) retrograde bouts of movement, 6,615 (33.8%) anterograde bouts of movement, and 7,395 (37.7%) states of no movement (pausing states). On average, the lengths of these filaments (FL) are longest during the pausing states, but not much greater than the average length during anterograde running-states (5.9 $\mu$m vs 5.6 $\mu$m, respectively). Shorter neurofilaments appear to move retrogradely more often as the average filament length recorded for retrograde runs was 14%-18% less than the average filament length during both anterograde runs and pauses, respectively. During movement, the average lengths and times of a contiguous-run are compatible to within 18% and 16%, respectively. However retrograde-directed filaments move about 50% faster than anterograde-directed filaments, on average. Note: RL, RT, V, and PT are abbreviations for the different kinetic characteristics of these filaments; run-length, run-time, run-velocity, and pause-time, respectively.

2.16 **Distributions of neurofilament lengths:** We show the distributions for polymer lengths for retrograde moving filaments (a), paused filaments (b), and anterograde moving filaments (c). Each histogram is overlaid with an exponential fit (dashed lines), which corresponds to a distribution having frequency factor $\lambda = 1/\mu$, where $\mu$ is the average polymer length for a given kinetic state.

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2.18 **Examining the correlation between the running/pausing frequency and neurofilament length:** In (a) we show the scatter plots of the frequencies for each kinetic state relative to the neurofilament length. The insets of (a) represent the same data plotted on a log-log-scale for better visualization. In (b) we plot the density profiles of the retrograde running frequencies (orange), the pausing frequencies (blue), and anterograde running frequencies.
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2.20 **Neurofilament motility characteristics binned by filament length**: We show the histograms for the average run-lengths, run-times, and run-velocities of both anterograde (green) and retrograde (orange) moving neurofilaments, as well as the average pause times (blue). In contrast to the scatter plots (see Fig. 2.19), we divide the data into unit-interval subsets relative to each run’s average filament length. That is to say, each bin in the histogram corresponds to the average motility characteristic of all filaments whose length resides within that bin. The inset panels show the same data, using a semi-log scale, where upper ’+’ ticks corresponds to the maximum value for a given bin.

3.1 **Illustration of the generic structures of kinesin and cytoplasmic dynein**: Illustration of cytoplasmic dynein (left) and kinesin (right). Each motor contains a motor domain responsible for ATP catalysis, and a stalk region that joins each motor domain with its opposing polypeptide branch, where cargo association occurs. The kinesin motor binds to a protofilament nucleotide site on its motor domain region, while Dyneins bind with a linker. The illustrations are taken from Fig. 1 of Vale (2003) with reprint permissions granted from Rightslink. Permission to crop the specific illustrations from that figure were granted by Elsevier.

3.2 **Schematic of the microtubule structure and growth**: The α and β protein monomers form into dimer structures, which are coupled via a GTP molecule. The dimers then form into protofilament tracks which are organized into a helical structure having a 25 nm outer diameter. The composite structure of 13 protofilament tracks forms the microtubule structure. Dimer polymerization occurs at the plus-end of the microtubule which is directed towards the distal end of the axon, while depolymerization occurs at the minus-end of the microtubule towards the proximal end of the cell. Depolymerization occurs upon GTP-GDP hydrolysis which reduces the binding affinity between the α/β tubulin.
3.3 **Schematic of the kinesin enzymatic cycle**: Our model represents the kinetic cycle of kinesin, which has four unique nucleotide-dependent conformations with it. The outline goes as follows in a left-to-right manner: A kinesin head that is bound to a nucleotide pocket along a protofilament (orange head) binds to an ATP molecule. Once substrate is bound, the motor domain catalyzes the ATP-hydrolysis process, which induces the primary power stroke that translates the motor head that is not bound to the microtubule (purple head) to a position now ahead of the microtubule bound head. This puts the bound head into a complex state in the presence of ADP and a phosphate ion. We assume that the release of phosphate and ADP entails transitioning through two more unique conformations that occur while the unbound head binds to a nucleotide pocket that is 8 nm in front of the previously bound motor head. The cycle then repeats.  

3.4 **Kinesin velocity relative to various ATP-concentrations/load with corresponding stalling forces**: We show the quantitative estimates for the kinesin velocity and stall force plotted against the measurements of Visscher et al. (1999). In (a) the velocity is plotted as a function of [ATP] for three different loads: 1.05 pN, 3.59 pN, and 5.63 pN. In (b) the velocity is plotted as a function of load for 2 mM [ATP] (right axis) and 5 µM [ATP] (left axis). (c) shows the corresponding stalling force measurements for a fixed clamp assay and a position clamp assay. The solid lines plotted against each data set represent the results following Eqs. 3.24 and 3.25 using the values listed in Table 3.1 with constant values for ADP and P_i concentration of 0.01 mM and 1.0 mM, respectively. The dashed line in (c) corresponds the stall-force–ATP curve with an ADP concentration of 0.1 mM, which is shown only to illustrate the sensitivity with respect to [ADP]. The experimental data points (with error bars) were extracted by hand using the GraphClick software package (Boyle et al., 2013).  

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3.6 Schematic of the Dynein enzymatic cycle: Our model represents the kinetic cycle of dynein, which has two unique nucleotide-dependent conformations for each of the four active AAA+ sites within its motor domain. The outline goes as follows in a left-to-right manner: A dynein head that is bound to a nucleotide pocket along a protofilament (orange head) binds—up to four—ATP molecules to its AAA+ domain. Once substrate is bound, the motor domain catalyzes the ATP-hydrolysis process, which induces the primary power stroke that translates the motor head that is not bound to the microtubule (purple head) to a position now ahead of the microtubule bound head. The distance of the powerstroke varies, depending on which AAA+ site underwent ATP hydrolysis and displaces the two motor-domains by a distance of either 8-, 16-, 24-, or 32-nm apart from one another. We assume that the release of the product, ADP·P_i, is instantaneous which follows by a rapid detachment of the, now trailing head, from the microtubute. The cycle then repeats.

3.7 Dynein stepping behavior under various applied loads and [ATP]: Panels (a) and (b) compare the stepping characteristics of the model (solid/dashed lines (a) and grey bins (b)) to the measurements of Rai et al. (2013) (blue circles (a)) and Mallik et al. (2004) (blue bins (b)). Rai’s measurements were taken for dynein movement within cells under various conditions of applied load, while Mallik’s measurements correspond to dynein steps at low ATP concentration (2 µM) and under no load. Data points were extracted by hand using the GraphClick software package (Boyle et al., 2013). In panels (c) and (d) we show the predicted step-size distributions under various loads between 0.0-1.0 pN for [ATP] = 2 mM (c), and [ATP]=500 µM (d). The modeled data results corresponds to those using the parameters listed in table 3.2.

3.8 Dynein velocity under various applied loads and [ATP]: We show the predicted dynein force-velocity and ATP-velocity curves in comparison with the results of Rai et al. (2013) (a), and (Ross et al., 2006) (b). The data points in (a) (i.e. blue circles) correspond to Rai’s data, which have been scaled accordingly to the values printed in Soppina et al. (2009) (see table S1 of supplement). The modeled data (solid line) is plotted as a function of load for the same ATP concentration of 1 mM ATP. The data points in (b) follow from Fig. 3c (top) and Fig 4a of Ross’ data reflecting dynein’s velocity-[ATP] dependence under ”minimal load”. As a fair comparison, we plot the corresponding modeled data against three cases of ”minimal load”: 0.0 pN (solid line), 0.05 pN (dashed-dot line), and 0.1 pN (dotted line). See table 3.2 for the specific list of parameter values used in the model. The experimental data points were extracted by hand using the GraphClick software package (Boyle et al., 2013).
3.9 **Emphasizing the effects of applying the catch-bond kinetics for dynein:**

We show the velocity curves for 1 mM [ATP], both with (dashed line) and without (solid line) the catch-bond enabled (a). (b) shows the corresponding stall-force of dynein as a function of ATP concentration for each case. For ATP concentrations below $\approx 1$ mM, the motor stalls at forces below 1-1.2 pN regardless of whether or not the catch bond is "active" (by active we mean that the catalysis rate for site 4 is load dependent–see table 3.2). When the catch bond is inactive, dynein stalls at loads $\leq 1$ pN, and decreases for decreasing ATP concentrations. When the catch bond is active, the stall force also decreases with decreasing ATP concentrations, however, at about $\approx 900$ mM [ATP], the effects of the active catch-bond become very apparent as the stall force jumps from about 1 pN to 4 pN (indicated by black arrow in (b)). For 1 mM ATP the catch-bond induces a 4-fold increase in the dynein stall force (compare the difference in applied load between the red and blue circles for each plot), giving rise to a possible explanation for the vast differences seen experimentally for dynein.

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5.7 **Analysis of the effects of an extended PARA length or a nodal bulge.** We show the contour lines of constant conduction velocity on the Cartesian plane of internodal diameter versus nodal diameter for conduction velocities of 20 m/s, 30 m/s, and 50 m/s (indicated on lines) and $1.5 \cdot 10^4$ nodal sodium channels. The nodal and internodal diameters are identical on the dotted line. The solid lines represent simulations in the presence of an extended PARA length (a) or a nodal bulge (b), while the dashed lines indicate simulations in their absence.

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5.10 Analysis of the number of additional nodal sodium channels required to match the conduction velocity gains resulting from optimal nodal constrictions. (a) The conduction velocity of a 20\(\mu\text{m}\) fiber is plotted versus the number of nodal sodium channels for an optimal cable and an unconstricted (uniform) cable. For a specified conduction velocity (vertical axis), the horizontal distance between the two curves represents the number of additional sodium channels needed for the unconstricted cable. (b) The necessary internodal diameter for a conduction speed of 25\(m/s\) is shown as a function of the number of nodal sodium channels for the optimal cable and the unconstricted cable. For a specified internodal diameter (vertical axis), the horizontal distance of the curves represents the number of additional sodium channels needed for the unconstricted cable to conduct with the same speed of 25\(m/s\).
1 INTRODUCTION

1.1 Motivation

Vertebrate nerve cells sustain their functionality by means of their cytoskeleton, which stabilizes a cell’s size and shape. All cytoskeletons of eukaryotic cells are composed of three filamentous protein structures, which conform to regulate and maintain the organization and structure of the cytoskeleton: microtubules, actin filaments, and intermediate filaments. In turn, proper organization of these cytoskeletal filaments enables a cell to transport the necessary components throughout the cytoplasm, which are requisites for cellular function. This process is known as axonal transport (or axoplasmic transport) and serves to accelerate the, otherwise diffusion limited, transportation of cellular materials to and from the cell body (e.g. mitochondria). The underpinnings of this movement follows from the enzymatic features of special proteins called molecular motors (also referred to as motor proteins). These proteins are special in the sense that they are able to haul cargo within the cell (hence the tag ”motor”) by converting excess chemical energy into mechanical work upon catalyzing the hydrolysis of adenosine triphosphate (ATP).

Curiously, one of the most abundant materials transported within the neuronal cytoskeleton is the most abundant component of the cytoskeleton itself: neurofilaments. Neurofilaments are the intermediate filaments of neurons and the primary space filling structures within axons, and their abundance determines the axon diameter (Reles and Friede, 1991). They are synthesized within the cell body and then transported out along the axon on microtubule tracks by the molecular motors kinesin and dynein. Kinesin transports neurofilaments towards the distal end of the cell (away from the cell body), while dynein transports neurofilaments towards the proximal end of the cell. While the specific mechanism guiding the transport kinetics of individual neurofilaments (aside from
being motor-based motion) is not known, it is clear that their movement stands to permit cells to a build a vast range of geometries and structures. In particular, myelinated axons possess a very unique nonuniform structure where the axon is composed of many discretely spaced myelin-ensheathed segments that are separated by narrowing constricted regions called nodes of Ranvier. These constrictions are only about 1 μm in length, un-myelinated, and contain the bulk of the voltage-gated ion channels in the axon. It has been reported that these morphologies are associated with differential transport kinetics of neurofilaments. At the nodes of Ranvier, where the axons are thin, neurofilaments move more rapidly than in regions where the axon is fatter (Monsma et al., 2014). Hence, the understanding of neurofilament transport kinetics and its modulation is key for our understanding of axon morphology and neuron function.

1.2 Outline

To gain insight on the biological strategies that neurons apply to sustain their functionality we will examine three key processes that potentially link neuron function with intracellular transport: the kinetic cycles of the enzymatic motor proteins, kinesin and cytoplasmic dynein; the slow axonal transport of neurofilaments; and the possible ramifications of these modulated kinetics in regard to axon morphology and nerve impulse conduction.

We will begin our investigation in chapter two by examining the kinetics of individual neurofilaments via in depth analysis of their motility characteristics, which are extracted from an in-vivo assay using epifluorescence microscopy. Our goal in this chapter is to establish a quantitative characterization of the transport kinetics of neurofilaments and to seek for correlations between movement characteristics and filament length. Following this, in chapter three, we investigate the enzymatic cycles of the molecular motors, kinesin and cytoplasmic dynein—the motors that are primarily
responsible for neurofilament transport. After a thorough review of their molecular structure(s) and mechanochemical behavior(s) we will introduce two discrete-state stochastic models that accurately represent the kinetic cycles of these enzymes and show excellent agreement with the recorded in-vitro measurements of their run-lengths and velocities under various conditions of load and [ATP]. In chapter four, we incorporate these two models into a third model that simulates neurofilament movement, which can be used to test hypotheses regarding their slow transport. There is a long-standing controversy over whether kinesin and dynein motors exhibit an unregulated tug-of-war, where the stronger motors win, or whether the activity of the motors is coordinated. Experimental evidence for coordination has been provided for microtubule-based transport of peroxisomes through chronic disabling of one of the types of motors with the result that the function of the other motor type(s) was reduced as well. We hypothesize that filamentous cargoes such as neurofilaments - in contrast to peroxisomes - could be a cargo where motor-motor coordination may be small or absent because of the large possible spatial separation of the motors. By comparing the kinetic features observed from experiment (chapter two) with those of our model, we introduce a method that renders an estimate for the average number of motors bound to a neurofilament during transport.

In chapter five, we investigate the role of nodal constrictions of axons, sculpted by the differential kinetics of neurofilaments in both the nodal and internodal domains. It is well known that neurons signal to each other and to other cell types via action potentials, which are waves of membrane depolarization that propagate from the cell body to the nerve terminus. The longest region these waves travel along is the axon. Hence, neuronal communication is limited by the axonal conduction velocity, and for long axons this can result in a significant delay. Two important ways in which neurons maximize axonal conduction velocity are to (1) introduce myelination, and (2) increase the axon diameter, which reduces the internal resistance to the longitudinal diffusion of ions. Collectively,
these processes allow for saltatory nerve conduction to occur at speeds much faster than in unmyelinated axons. Here we will study the effect of the non-uniform geometry found within myelinated axons via computational analysis. Following a Hodgkin-Huxley framework, we develop a computational assay which accurately models both the axon morphology and action potential signaling. In particular, by fixing the number of ion-channels that are required for sustained nerve conduction, we examine the relationship between nerve signaling and morphology by comparing the rate of propagation of the nerve impulse for axons having nodal constrictions with axon having a uniform axon diameter. Finally in chapter 6 we will discuss the implications and possible ramifications of this study in summary as well as potential future projects.
2 INVESTIGATING THE MOTILE MECHANISM BEHIND NEUROFILAMENT MOTILITY USING EPIFLUORESCENCE MICROSCOPY.

2.1 Introduction

Axons are the slender projections of nerve cells that are responsible for conducting electrical signals from the cell body. They can extend up to a meter in length and can be myelinated or unmyelinated. The essential components within the axon are generally synthesized within the nerve cell body, which are then transferred to the axon by motor proteins. Materials that are transported away from the cell body move anterogradely, while materials that move towards the cell body move retrogradely. The process associated with this movement within the axon is known as axonal transport. There are two distinct kinetic phases within axonal transport: fast and slow. Fast axonal transport refers to the patterns of movement for membranous organelles that practice rapid movement at speeds on the order of microns per second. Slow axonal transport is the transport of protein complexes that make up the cytoskeleton which move less frequently at speeds 10x-100x less than molecules associated with fast axonal transport.

Neurofilaments are associated with slow axonal transport and are of particular interest due to their role in maintaining the morphological structure of axons. In contrast to other cytoskeletal filaments such as microtubules and actin polymers, whose movement follows from "treadmilling", these structures are cargoes that move alongside microtubules at speeds on the order of 0.3-3 mm/day Brown (2000).
More specifically, neurofilaments are a class of intermediate filaments found within vertebrate nerve cells. Following neurofilament synthesis, they distribute slowly throughout the cell where they aggregate into a dense complex, making up the bulk of cytoskeleton. In mammalian cells, their structure is composed of heteropolymer chains that are formed by a co-assembly of neurofilament proteins; NF-L (light), NF-M (medium), and NF-H (heavy)—a convention used based on the apparent molecular mass of the individual proteins. Each heteropolymer contains a single NF-L protein, associated with either NF-M proteins or NF-H proteins. These medium and heavy polymer subunits form the "sidearms" of neurofilaments, which are hairlike extensions spread along a filament’s main axis and act to regulate the spacing between nearby filaments. As a whole,
these polymers are flexible and have a very high tensile strength. Their flexible nature allows them to bend and fold into various configurations, however it is thought that folding predominately occurs when these polymers are static (Taylor et al., 2012). They have an effective diameter of about 25 nm, and an inner diameter (excluding the side-arms) of about 10 nm. In contrast to the consistency in diameter(s), the lengths of these polymers varies greatly, ranging between 1-100 µm.

This broad spectrum of lengths arrises due to the dynamic process of adding and subtracting protein sub-units to/from the heteropolymers, which, in contrast to microtubules and actin, does not occur strictly at the ends of neurofilaments and can occur throughout the entire length of the heteropolymer chains (Ngai et al., 1990; Coleman and Lazarides, 1992; Vikstrom et al., 1992). Uchida et al. (2013) verified this by applying red and green fluorescent neurofilament fusion proteins to distinct populations of neurofilaments, which, over time migrated into a single population of chimeric filaments possessing both (red and green) of the fluorescent neurofilament fusion proteins. Furthermore, in their experiment, they observed an ongoing change in the composition of specific filaments. Over time, the number of red-green junctions increased, while the individual lengths of the chimeric segments decreased per filament. This suggests that the "intercalary subunit exchange" (Çolakoğlu and Brown, 2009), which occurs throughout neurofilament heteropolymers could follow a dynamic cycle of severing events and end-to-end-annealing events (Uchida et al., 2013).

As mentioned, neurofilaments move very slowly throughout the axon, with average speeds ranging on the order of 0.3-3 mm/day (Brown, 2000). Initially, it was assumed that their movement was simply a continuous flow in which the neurofilament-cytoskeleton construct migrated slowly in a uniform direction (Lasek et al., 1984). However, recent studies using fluorescence microscopy have shown that neurofilaments have distinct kinetic phases that are made up of rapid and intermittent bouts of movement, which are
interrupted by lengthy states of no movement (Wang et al., 2000; Wang and Brown, 2001; Roy et al., 2000; Yabe et al., 2001). We now know that filaments move both anterogradely as well as retrogradely throughout the axon, but anterograde movement predominates over retrograde movement—which is what lead to the initial belief that the cytoplasm moved away from the cell body in a unidirectional fashion. Furthermore, we know that during movement, neurofilaments behave similar to cargoes of fast axonal transport and can reach speeds up to $0.4 - 0.6 \mu m/sec$. The explanation of these discrepancies in apparent speed (0.3-3 mm/day vs 50-400 mm/day) is best explained by Brown’s ”stop-n-go” hypothesis (Brown et al., 2005), which suggests that, on average, neurofilaments spend about 97% of their time expressing no movement. Hence, since neurofilaments are static most of the time, they express a very slow net velocity over long periods of time.

Deciphering neurofilament motility is by no means a simple task. These structures can move both fast and slow (in different directions), or can remain static for long periods of time. They are flexible, can fold or bend, and come in a wide variety of lengths (severing and annealing). Fortunately, through the extensive works of Brown et al. (2005), Trivedi et al. (2007), Jung and Brown (2009), Li et al. (2012), Monsma et al. (2014), and Li et al. (2014) we have much better understanding of the mechanism(s) behind the slow transport of neurofilaments, for which, the current model, is outlined in summary in Fig. 2.2. Li’s model (Li et al., 2014) posits a six state kinetic scheme, which suggests that these filaments have two district kinetic states; ”on-track” and ”off-track”. The off-track state represents the phase of static behavior, where it is thought that neurofilaments are not in contact with microtubule tracks, and hence, ”pause” for prolonged periods of time. The on-track state represents the moving phase of these polymers when motor-protein junctions link a neurofilament to a microtubule. During movement, either kinesin motors and/or cytoplasmic dynein motors bind to both a microtubule and a neurofilament structure. The microtubules are used as tracks for the motors, which enables them to
translocate the neurofilaments to-and-from the cell body by means of catalyzing the ATP hydrolysis process. Since kinesin and dynein move in opposing directions along microtubules, the on-track state is characterized by two states of on-track movement and two states of on-track pausing (no movement).

Figure 2.2: The Li et al. (2014) six state model of neurofilament transport: The model depicts the kinetic scheme used to interpret the slow axonal transport of neurofilaments. States $r_o$ (retrograde off-track) and $a_o$ (anterograde off-track) represent the states when neurofilaments lack a motor-protein-junction to a microtubule track. In these states the filament can transition between off-track retrograde to/from off-track anterograde, where the probabilities of transitioning follow the rates $\gamma_{ra}$ and $\gamma_{ar}$, respectively. The states representing the on-track kinetics are $r$ (retrograde moving), $r_p$ (retrograde pausing), $a_p$ (anterograde pausing), and $a$ (anterograde). The transition probabilities of going to and/or from on-track moving to on-track pausing follow from the rates $\gamma_{01}$ and $\gamma_{10}$, respectively, while the probabilities of going to and/or from on-track pausing (retrograde) to on-track pausing (anterograde) follow from the rates $\gamma_{ra}$ and $\gamma_{ar}$, respectively. $\gamma_{on}$ and $\gamma_{off}$ dictate the probabilities of transition to and from the states of off-track pausing to on-track pausing. The cartoon is based off of the schematics within Li et al. (2012) and Li et al. (2014).

The six-state model (Li et al., 2014, 2012) (see Fig. 2.2) is able to explain the movement of neurofilaments quite well in terms of movement within the axonal cytoskeleton as a whole. However it does not incorporate the motility features of the molecular motors responsible for the kinetics of neurofilaments. It is thought that kinesin
and cytoplasmic dynein are the primary motors responsible for neurofilament motility as experiments have shown that the inhibition of these motors leads to a substantial reduction in polymer movement (Uchida et al., 2009; Wang and Brown, 2010). However, motor proteins move in nanometer step-sizes at speeds of micrometers per second, both in vivo (Ashkin et al., 1990; Soppina et al., 2009) and in vitro (Ross et al., 2006; Schnitzer et al., 2000), which suggests, upon comparison with the average speeds of neurofilaments, that microtubules, motors, and neurofilaments interact quite infrequently, or at least for very short periods of time. Fortunately, recent advances in epifluorescence microscopy, used in conjunction with time-lapse imaging stands to provide both the spatial and temporal resolutions necessary to study motor-based filament movement on such time scales.

2.2 Imaging Techniques – a Quick Review

To date, there are three different experimental assays used to study the axonal transport of neurofilaments: accumulation assays, pulse-labeling assays, and imaging assays. The accumulation assay was the first of three developed (Weiss and Hiscoe, 1948) and was used to first characterize axonal transport. This assay measures the apparent rate of directed cellular flow within an axon by means of a mechanical constriction applied to the axon. In doing so, the natural flow of cellular movement is blocked and the rate at which material accumulates about the ends of the pinched region can be measured. While this method is simple, it has the disadvantage of being non-selective in the sense that the measured accumulation rates apply to the entire cytoplasm and therefore provides limited information on the kinetics of specific molecules. The pulse-labeling technique (which led to the six-state model) is more precise than the accumulation technique because it applies radio-labeled precursors of macromolecules into specific regions of a nerve cell that are used to track the movement of specific cellular materials in vivo. To track neurofilament proteins, specific radio-labeled amino acids that can bind to neurofilament proteins are
injected into the cell body, which are then observed along an axon once the radio-labeled bound neurofilaments exit the cell body and move towards the distal end of the cell. The third approach is known as light microscopy and is used to observe the movement of molecules which have cellular granules that are able to refract and/or scatter light. This technique has evolved substantially over the years and has made it possible to observe the movement of specific molecular structures in live cells by means of using fluorescent labeling in conjunction with light microscopy (fluorescent microscopy). Upon recording the movement of the fluorescently labeled structures using time-lapse imaging techniques, one can analyze the kinetic features of moving structures within cells (Brown, 2013).

2.3 Experiment Outline

In this experiment, we use transfected primary cultures of cortical neurons taken from neonatal rat brains with GFP-tagged neurofilament protein M in parallel with epifluorescence microscopy techniques to record neurofilament movement within cells. Movies of this movement were recorded using an Andor Ultra cooled EMCCD camera with streaming acquisition using 30.3 millisecond exposures that have a linear pixel density of 160 nm²/pixel. The exposures for the movies are then mapped onto kymograph images using the ImageJ software package. The kymographs then undergo edge-detection analysis using the ImageJ Canny-Deriche subroutine package to extract the leading and trailing ends of the recorded filaments, which are then translated into position-time coordinate traces that are used to quantify the kinetic characteristics of the moving filaments. To analyze the data we had to develop a new unbiased noise-filtering algorithm that would allow us to accurately define smooth bouts of movement to within a certain degree of pixel error, relative to the original traces. By analyzing the distribution of filament speeds from the ”smoothed” traces, we were able to distinguish between phases of movement versus phases of no movement, which enabled us to obtain statistical
distributions for the motility characteristics observed for each of the respective kinetics states (i.e. distributions for the pause-times, run-times, run-lengths, and run-velocities).

2.4 Cell Culturing and Transfection

Cell cultures were prepared by applying a glial sandwich culture, which is commonly known as the "Banker” method from the researcher who developed it (Kaech and Banker, 2006). (See Wang and Brown (2010) for an outline.) Cerebral cortices are removed from P0 mice. We remove the hippocampus, hypothalamus/thalamus, and any other non-cortical tissue. The remaining cortex tissue is prepared into smaller, more suitable samples within a HBSS media. (HBSS is an isotonic saline solution that maintains pH and osmotic balance as well as provide water for the cells within the remaining cortex tissue.) We then use Trypsin (0.25%) and DNase-I (1%) and 0.27 mM EDTA to dissolve the extracellular matrix (ECM). Using light trituration we are able to isolate individual neurons for plating, for which the cells are then maintained in growth media for 7-10 days before imaging. To prepare the glia, cortical tissue is first removed from P1 rat brains. As with the neuronal cell preparation, cortical tissue is prepared into small samples with added trypsin/EDTA/DNase to help isolate individual cells, which are then isolated via trituration. The key difference between the glial and cortical preparation lies in the media. While both cortical and glial cells are grown in the presence of 0.7% glucose, 5 µg/mL gentamicin, glial cells require the addition of 10% horse serum, as these cells need horse serum to divide. Following a week or two of growing, the glial cells are then added to the cortical cultures in a horse serum free environment. At that point, the glial cells do not need the horse serum, because they have already grown to a confluent monolayer, in which case glial cell division is no longer needed.

1 The experiments discussed in this chapter were all conducted by Daniel Fenn at the Brown Lab of The Ohio State University. Daniel also created the kymograph images and extracted all of the edge-lines from the kymographs for our analysis.
Following sample preparation, neuronal cells are transfected via electroporation using a Lonza Amaza Nucleofection kit in which 4-6·10^6 cells were used per electroporation. Cells were dual transfected with 4 µg eGFP-NFM and 2 µg mCherry-EB3–fluorescent tags used to see the NFM and EB3 proteins. EB3 (end binding protein 3) is a protein that only binds to the plus-end of microtubules, which allows us to identify the proximal and distal axonal directions. Once transfection is completed, the neuronal cells are platted on glass-bottom dishes that are coated with poly-D-lysine. The previously prepared glial coverslips are then inverted and added on top of the neuronal cells, separated by wax-paraffin dots as spacers. This creates a "sandwich" with the neuronal cells on the bottom, media conditioned by the glia in the middle, and the glial feeder layer at the top. The feeder layer of glia serves to add necessary hormones, proteins and other substances to the culture media that enhance nueronal growth. However, keeping the glial layer separate allows us to remove all glial cells before imagining to reduce background noise, which improves the overall imaging conditions. For one day after plating, the media has 5% FBS (Fetal Bovine Serum), which helps the cells recover from the electroporation as this is very damaging to the cells. Following this, the FBS is removed and replaced with a serum free media. 5 µg cytosine arabinoside (AraC) is then added to assist in preventing cell division to prevent any glial overgrowth that could occur. The media is replaced every two days but in the absence of AraC - only a serum free media is used to maintain the prevention of excessive glial growth.

2.5 Imaging

To image neurofilaments/microtubules the media is replaced with Hibernate-E (a low fluorescent media more suitable for imaging). Imaging was then performed on a Nikon TiE inverted widefield epifluorescence microscope using a Lumencore SOLA LED light engine and a 100x/1.4NA Plan-Apochromat VC oil-immersion objective. To control
temperature and humidity we use a stage top incubation chamber made by Okolab. Recordings were made using an Andor iXon Ultra EMCCD camera, which is extremely sensitive to low levels of light and can provide a very high acquisition frame rate. Light from the SOLA engine is then attenuated by 98% to minimize the effects of photobleaching. While this is extremely low light, it is still visible to the camera. In doing so we are able to take thousands of frame recordings at a very fast acquisition rate. Traditionally after only a few hundred frames the fluorescence of our molecule can drop to a level that is no longer visible. Low light and a high-sensitivity camera correct for that issue. Neurofilament images were then acquired in a continuous stream-to-RAM acquisition mode using 30.3 ms exposures with no delay between exposures. For each movie recording, we stream for approximately 5-7.5 minutes, which provides between 10,000-15,000 frames for analysis, which are all acquired using the MetaMorph software package from Molecular Devices (Molecular Devices, Sunnyvale, CA). To obtain the neurofilaments directionality, we imaged the EB3 molecules via a time-lapse imaging using 200 ms exposures with a 2 second delay between each image for a total of 4 minutes. By imaging the rate of growth of the EB3 plus end of the microtubules, we could determine which direction the neurofilaments were moving.
Figure 2.3: **Data Acquisition techniques:** Postnatal rat cortical neuron cultures were transfected with eGFP-tagged NFM and imaged seven days later using a high-sensitivity Andor iXon Ultra EMCCD. For each movie recording, we stream for \(\approx 5-7.5\) minutes which provides between 10,000-15,000 frames for analysis, which are all acquired using the MetaMorph software package from Molecular Devices (Molecular Devices, Sunnyvale, CA). The cartoon was provided by our collaborators (Fenn and Brown) who performed the experiment.

### 2.6 Image Processing

Once the neurofilament movement is recorded, the trajectories of the neurofilaments are extracted using kymograph analysis. Kymograph images are 2D graphical representations of the spatial position of an imaged object over time in which one of the spatial axis represents time. To generate a kymograph we first locate the axon by generating a maximal projection image of the entire movie. A maximal projection image is an image that consists of all the maximum pixel intensity values for all pixel intensity values that are recorded throughout an experiment. For example, suppose a given movie consists of 60 movie frames that were captured over a minute’s time (i.e. the change in position of the fluorescing neurofilament within two images taken consecutively corresponds to movement that occurred over a 1 second time interval). Now stack all 60 movie frames atop one another in the same orientation. Since the neurofilament travels within the axon, the intensity profile of the entire stack provides an outline of the
encompassing axon (see upper right panel of Fig. 2.4a). After stacking the movie frames, we produce a maximal projection image of the entire movie by selecting each pixel that has the highest intensity for all pixels within the movie stack. For example, take the set of intensity values for a single pixel with coordinate \((x,y,I(t))\), where \(x\) and \(y\) represent the pixel position in the x-y plane and \(I(t)\) is the intensity profile for that pixel over the time lapsed in the movie. The pixel value corresponding to the \(\max\{(x,y,I(t))\} = (x,y,\max\{I(t)\})\) is taken as the value for that pixel in the maximal projection image of the movie. This is done for all the pixel values recorded to produce the maximal projection image. Once the maximal projection image is created we then insert a hand-drawn curve atop the location of the axon that follows the projected path of the neurofilament being tracked. This line is then used as a guide to generate a kymograph.
Figure 2.4: **Method to obtain Kymographs:** In panel (a) we outline the method to obtain each row of pixels within a kymograph. A maximal projection image is generated for the entire movie to draw a curve atop the axon, which is used to estimate the position of the neurofilament for each time frame. Kymograph lines are taken by choosing the maximum intensity value for each pixel along the axon curve that is within a ±2 pixel tolerance from the axon curve. In (b) we show the stacking of kymograph lines, frame by frame. Panel (c) shows the generation, and placement, of seven movie frames within an entire kymograph sample (yellow lines in kymograph at the bottom of panel c) of 6000 frames. The images used in (b) and (c) were provided by Fenn and Brown.
To generate a kymograph, we examine our stack of movie frames, one frame at a
time, wherein the axon curve generated from the maximal projection image is laid atop
each movie frame. Generally speaking, the pixel intensity values for each row of pixels
within a kymograph will correspond to the pixel intensity values that span the length of
the axon curve for each corresponding movie frame. However, since the neurofilaments
become less visible over time (photobleaching), we chose (through trial and error) to
maximize the pixel intensity values of kymographs by setting a ±2 pixel tolerance for
each pixel intensity value selected for our kymograph lines. First we take two duplicates
of the hand-drawn axon curve and shift them above/below the axon curve such that each
pixel of the duplicated axon curves is ±2 pixels away the original axon curve. The pixel
intensity values chosen for the kymograph lines/rows are taken as the intensity values that
corresponds to the maximum. For example, suppose we are extracting the first row of
pixels for our kymograph (i.e. frame #1). The first pixel of the kymograph row is taken as
the pixel which has the highest intensity value such that the pixel with the highest
intensity is within ±2 pixels of the hand-drawn axon curve (see Fig. 2.4a for an
illustration). This is then repeated for all pixels along the axon curve for each movie
frame. The final subset of pixels extracted using the axon curve, for each movie frame, are
then mapped into straight lines (kymograph lines). Each of these lines represents the
position of the neurofilament along the axon curve relative to the movie frame that the line
was extracted from. The kymograph is then generated by sequentially stacking all of the
kymograph lines in order of frame number (see Fig 2.4). Our kymograph analysis was
done using the ImageJ software package (Abràmoff et al., 2004).

Once the kymographs are created, the edge-lines of the neurofilaments are extracted
using the ImageJ Canny-Deriche Plugin which follows from Deriche (1987). The
extracted edges of each kymograph are then converted into position time coordinates for
analysis, wherein the directionality of each trace is defined by the direction of microtubule growth found in the kymographs from the mCherry-EB3 images (see Fig 2.5).

**Figure 2.5: Edge detection and directionality:** In panel (a) we show a kymograph obtained from the ImageJ software package. In (b) we show the same kymograph after it is analyzed using the Canny-Deriche plug-in package, which is used to extract the edge-lines of the moving neurofilament (c). These edge-lines are then used to generate a position-time coordinate trace (e). In (d) we show the kymograph generated from the EB3 images obtained using time-lapse imaging. The ends of the microtubules are growing in the positive direction, which indicates the neurofilament is moving towards the distal end of the cell (anterogradely). The images and data used for this figure were all provided by Fenn and Brown of The Brown Lab at The Ohio State University.

### 2.7 Initial Imaging Results

Our image data set consists of a collection of 117 movies that provided 301 kymographs for analysis. The Canny-Deriche Plugin detected 33,830 edge-lines for which 17,489 of these edges pertain to a neurofilament’s leading end (the filament end which
leads the directed movement) and 16,341 of these edges pertain to a neurofilament’s trailing end (the filament end which trails behind the leading end). The discrepancy between the number of leading/trailing edges arises because (1) a clear edge was not detected by the Canny algorithm, and/or (2) only part of the filament was within the field of view while recording). Collectively, these edge lines were combined to produce position-time traces for 726 different neurofilaments that contain trajectories for both the leading and trailing edges. In figure 2.6 we show nine examples our initial results.

Figure 2.6: **Image processing results:** We show nine examples of the position-time traces generated from our kymograph recording using the ImageJ software package. Neurofilament movement is viewed at three different spatial scales of (a) 30 \( \mu \text{m} \), (b) 60 \( \mu \text{m} \), and (c) 90 \( \mu \text{m} \). Anterograde movement goes upward (positive direction), while retrograde movement is downward (negative direction). The data for these plots was provided by Fenn and Brown.
2.8 Defining Movement

To quantify the kinetic characteristics of neurofilament transport, we first need to define, and distinguish between, the specific kinetic phases that these polymers practice. As mentioned, neurofilaments express bouts of movement that are interrupted by prolonged intervals of no movement, which are referred to as the ”running states” and ”pausing states”, respectively. However, upon analyzing the initial trajectories that were extracted following our kymograph/edge-detection analysis, we find a number of issues that need addressed before we can begin analyzing the transport kinetics of these polymers. The first problem arises due to the fact that the kymographs are pixelated, which upon applying the canny-deriche plugin, generates edge-lines that are irregular and jagged (Fig. 2.7a). Second, the edge lines are not always continuous and can have gaps–both spatially and temporally (Fig. 2.7b). Spatial gaps arise when multiple edge-lines overlap for a given time interval while temporal gaps are regions where no edge-line was detected. To address these issues, we developed a method to create a set of abraded traces that accurately estimates the original data to within a high level of precision. The abraded traces are free of pixel noise and have well defined, continuous bouts of movement, which are distinguishable from well defined states of no movement, and hence, allows us to obtain statistical distributions of the motility characteristics of these neurofilaments. We dub this method the abrasion algorithm.

First, we address the issues with gaps. Temporal gaps provide no information on filament behavior, and hence will be ignored for our final analysis. However, when we address the issue of pixelation a continuous trace is needed wherein every time frame of the trace has a defined position. As such, we initially connect the two edges of all temporal gaps by introducing false pixels that follow a straight line connecting the two ends of the gap. Spatial gaps are contrary to temporal gaps in which multiple edge lines overlap in time and thus, introduces a pixel-range of uncertainty as to where to true edge
of the neurofilament lies. As a best guess, we estimate the true filament edge to be the average pixel value of all pixels making up the spatial gap. Once gaps are accounted for we apply the abrasion algorithm, which is a three-step algorithm designed to generate a collective set of smoothed neurofilament trajectories that lack the pixel-noise artifacts following filament edge-detection, as well as contain smoothed regions of movement that are not pixelated. Step one is referred to as \textit{pause-filtering}, which addresses pixel noise. Step two is referred to as \textit{velocity-smoothing}, which interpolates the data (following step one) into a single, smoothed, trajectory that is absent of any pixel-plateaus. The third and final step is referred to as the \textit{pruning} step, which is used to define bouts of movement that are distinguishable from the prolonged phases of no movement.

Figure 2.7: \textbf{Example of the artifacts left behind from Image Analysis}: In (a) we have an example of a 3 $\mu$m neurofilament moving in the anterograde direction. The insets show the granulation and pixelation effects that follow from image processing. In (b) we show a 12.5 $\mu$m retrograde directed filament trace which illustrates the occurrences of both spatial gaps (inset) and temporal gaps (empty red boxes). The data for these plots was provided by Fenn and Brown.
2.8.1 Pause-Filtering

The initial trajectories created from our kymographs appear – when taking a "birds-eye view" – to express clear phases of movement that are distinguishable from phases expressing no movement. However, upon inspection, it is not clear how to distinguish between states of movement and states of no movement as the edge-lines extracted from our kymographs possess a sufficient amount of noise within them (see insets of Fig 2.7a). We have to find a method that objectively determines the true transition point that separates a pausing state from a running state. To help distinguish between these two distinct kinetic phases of movement and no movement – in an unbiased fashion – we introduce a method that we refer to as pause-filtering, which is designed to reduce the pixelated noise seen within the edge-lines extracted from the kymographs.

Intuitively, if a neurofilament is experiencing a pause then its pixel-edge position would be relatively the same over an extended period of time, whereas if the filament is undergoing a run, then the pixel deviations along the edge-line would be larger on shorter time scales. This is, in essence, of how pause filtering works. Consider a trajectory that was extracted from a kymograph and suppose you chose a random time interval \([t_i, t_j]\) such that \(t_i < t_j\). If \(x(t_i) = x(t_j)\) then, potentially, the neurofilament is in a state of no movement (i.e. a pausing state), whereas if \(x(t_i) \neq x(t_j)\), then the filament is potentially in a state of movement (i.e. a running state). To pause-filter the data, we cycle through all the possible time intervals for a given trace in which pixel deviations can occur (starting from the largest time interval – the entire trace, to the smallest time interval – three time frames). For each time interval size (\(\Delta t\)), we then randomly select a time interval \([t_i, t_i + \Delta t]\) and check if \(x(t_i) = x(t_i + \Delta t)\). If this is true, then the entire set of pixels \(\{x(t_i), x(t_i + \Delta t)\}\) is set equal to the nearest integer value of their average; \(\text{nint}(\langle x_j \rangle \mid x_j \in \{x(t_i), x(t_i + \Delta t)\})\), and if this is false, then the pixels within the time domain \([t_i + \Delta t]\) are left unmodified as they stand to represent a running state. This routine is then continued for \(N\) randomly selected
time intervals for all interval sizes, where N is chosen as an arbitrarily large number that exceeds the total number of time frames in a given trace. See Fig. 2.8.

Pause-filtering is very powerful in the sense that it lets the data decide what is a pause and what is a run. Moreover, since the method is done randomly, it is completely unbiased as well. However, there is one minor setback thus far in the routine. Suppose a random time interval is selected where \( x(t_i) = x(t_i + \Delta t) \) such that \( t_i \ll t_i + \Delta t \) and within that time the filament actually moved (say it starts moving in the retrograde direction, stops, and then starts to move in the anterograde direction). This region would appear to be a state of no movement based on the common pixel positions of the beginning/end points of the time interval, but in fact contains two successive runs wherein a reversal occurs. To account for this special case, we include an empirically chosen \( \pm 2 \) pixel error limit to constrain the pause-filtering routine. And our final pause-filtering criteria reads as follows: for a randomly selected interval having \( x(t_i) = x(t_i + \Delta t) \), if \( x(t_i) = x(t_i + \Delta t) \land \max(\{X\}) - \min(\{X\}) \leq 4 \) pixels (where \( \{X\} = \{x(t_i), x(t_i + \Delta t)\} \)), then the time interval \([t_i, t_i + \Delta t]\) is defined as interval where the neurofilament is static having a pixel position \( y = \text{nint}(\langle x_j \rangle \mid x_j \in \{x(t_i), x(t_i + \Delta t)\}) \). See Fig. 2.8.
Figure 2.8: Showing the effects of pause-filtering. In (a) we illustrate the method of pause-filtering by labeling two sets of randomly selected time intervals on a segment of an edge-line spanning 15 seconds (black line). The green dots/arrows illustrate an example when two pixels spanning a randomly selected time-interval do not share the same position (horizontal green arrows) and are interpreted as a potential run during the pause-filtering. The red dots/arrows show an example when two pixels spanning another randomly selected time interval do share the same position (horizontal red arrows) and are interpreted as a potential pausing state. Since the maximum pixel displacement for all pixel values within that time interval (see vertical red arrows) are within ±2 pixels from the randomly selected pixels (red dots), the state of the edge-line for this neurofilament is interpreted as a pause and all pixel values within this time interval (vertical red arrows) are redefined as the average pixel position of all pixels within that time interval. In (b) we show the final pause filtered edge-line (red) atop the original edge-line (black) that was discussed in (a). In (c) we show an example of an entire neurofilament trace, with both the leading and trailing edge-lines (black lines) that has undergone pause-filtering (red lines).
2.8.2 Run-Smoothing

The run-smoothing routine is the second step in the abrasion algorithm and is much simpler than pause-filtering. In the pause-filtering step, we reduced the pixel noise by averaging over pixel values that shared a common position to within a small degree of tolerance (±2 pixels). By reducing the pixel noise, pause-filtering created a set of edge-lines composed of a sequence of flat pause-plateaus that are interrupted by single time-frame pixel steps. Hence, at this stage, the pause-filtered edge-lines suggest that the neurofilaments are expressing a large amount of pauses that are interrupted by single pixel-step runs. Run-smoothing essentially reverses the effects of pause-filtering by taking every prolonged pause and changing it into a contiguous run. In doing so, we generate set of edge-lines that are interpreted as single continuous run, wherein distinct phases of movement are defined by connecting the starting points of the pause-filtered plateaus with a regression line (see the green trajectory in Fig. 2.9). Hence the name, run-smoothing.

![Figure 2.9: Run-smoothing argument](image)

Figure 2.9: **Run-smoothing argument:** We simulate random particle motion which is course grained to spatiotemporal resolution of the raw data. The red-lined trajectory represents the motion of the particle using a time step of \( dt = 10^{-5} \), while the red line shows what the particle’s path would resemble if viewed at the same spatiotemporal resolution of our recorded neurofilament trajectories following image analysis. The green lines represent the estimated path of the particle by applying the argument of run-smoothing, which interpolates the trajectories as a linear regression that connects the beginning points of successive pauses.
The argument for run-smoothing follows from simulation. In Fig. 2.9 we show the simulated movement of a particle moving randomly in time at two different resolutions. The red line represents the random motion of the particle using a time step of $dt = 10^{-5}$ seconds, while the black line shows the path after being course-grained to the same resolution of our experimental data. That is to say, if the real motion of the random particle followed the path of the red line and we recorded its movement using our Andor Ultra cooled EMCCD camera (Sec. 2.5), our data set would resemble the path of the black line, following pause-filtering. What this tells us is that if we extracted the kinetic characteristics of our neurofilaments from the pause-filtered traces alone, then our analysis would drastically under-estimate the run-lengths and run-times, as well as drastically over-estimate the running-velocities of these neurofilaments. A more accurate estimate can be seen from the green trajectories in figure 2.9, which are regression lines that follow the paths of the beginning and end points of the plateaus in the course grained simulated trace. As such, following pause-filtering, we apply run-smoothing to all the pause-filtered traces by inserting sub-pixel positions between the start- and end-times of every successive pause using a linear regression (see Fig. 2.10).

Figure 2.10: **Showing the effects of run-smoothing.** The black lines are the edge-lines obtain from image-processing, while the green lines are the resulting estimates following the run-smoothing routine.
2.8.3 Pruning

The final step of the abrasion algorithm is referred to as the pruning step. Here we use the unpixelated run-smoothed traces to provide an objective manner that allows a way to distinguish between states of movement versus no movement. To distinguish between the two, we look at the relative frequency distributions of the individual speeds for each bout of movement, defined in the unpixelated run-smoothed traces using a non-uniform log-binning method. Using a log-binning approach allows us to see if different modes of movement exist at very short time scales, and hence, allows us a way to segregate between the two different kinetic states – pausing versus running.

![Density plots for the running-speeds of the run-smoothed traces](image)

Figure 2.11: **Density plots for the running-speeds of the run-smoothed traces**: We plot the density profiles (i.e. distributions) for the speeds of the estimated running trajectories for the run-smoothed traces (see Figs. 2.9 and 2.10). To the left we show the density profile for the instantaneous speeds measured pixel-by-pixel. In the right panel we show the density profile for the speeds of each contiguous run. To estimate the threshold velocity, we draw lines atop both profiles and insert circles around the regime where an apparent change occurs amongst the profiles. The circle in (a) approximates the local minima of pixel-weighted profile, while the circle in (b) approximates the inflection point of the run-time-weighted profile. Both circles are within 0.1-0.2 $\mu m/sec$, which we interpret as the region where neurofilaments transition from pausing to moving, or vice-versa.

In figure 2.11 we show the log-log density profiles of the speeds extracted from the run-smoothed trajectories. To be thorough, we examined these data at two different time...
scales. In one case, we calculated the instantaneous speed for two consecutive pixels (i.e. the instantaneous speed occurring over a single time-frame of 30.3 msec), while in the other case, we measured the average speed of each interpolated line generated by run-smoothing. Fig. 2.11a shows the running-speed density profile weighted pixel (i.e. the instantaneous speed distribution), while Fig. 2.11b shows the running-speed density profile weighted by the run-time (i.e. the distribution of the average speeds). In both cases we see a transition between the two curves occurring around 0.1-0.2 \( \mu m/sec \) (see circles in Fig 2.11). As such, we arbitrarily choose 0.1 \( \mu m/sec \) as our velocity-threshold cutoff speed \( (v_{cut}) \) to segregate between states of movement versus states of no movement. That is to say, any bout of movement in the run-smoothed traces that has an instantaneous speed less than \( ||v|| = 0.1 \mu m/sec \) is defined to be a pausing state, while any bout of movement having a speed above \( ||v||_{cut} \) is defined as a running state. This constraint is then applied to the run-smoothed traces, wherein we prune the data such that for any run that has a speed below the velocity-threshold-cutoff, the pixel positions of those runs are replaced by the pixel positions of the paused-filtered traces. All other pixel positions that have an instantaneous speed above the velocity-threshold-cutoff are left unchanged. As such, we generate a complete set of abraded traces that have well defined running-states that are distinguishable from well defined pausing-states (see Fig. 2.12).
Figure 2.12: **Comparing abraded traces with raw Data:** In (a) we show the leading and trailing edge lines generated from the abrasion algorithm (yellow) atop the original kymograph that was used to generate the initial position-time coordinates. In (b) we show the same edge-lines (red and green) compared with the original edge-lines (black) generated by the ImageJ software package. The red lines represent the pausing states, which are interrupted by the green lines that represent the running states. The estimated edge-lines are shifted up by one micron for better visualization.

### 2.8.4 Analysis Criteria

Once the imaged edge-lines are processed and abraded we set criteria for data analysis. The abraded edge-lines estimate the raw data with a high level of precision, but cannot be used strictly for analysis as there are inconstancies between portions of leading and trailing edges, in which an asymmetry arises between the two filament edges where both ends are not in the same kinetic state. For example, one edge might tell us that the filament is in a pausing state, while the other edge implies the filament is in a running
state; or both edges appear to be in running states, but have opposing directionality (i.e. the filament appears to be moving in both the retrograde and anterograde directions). Potentially, these events could be states that represent folding and/or unfolding events, but cannot say for certain as these instances could merely be artifacts from the abrasion algorithm. Therefore, to avoid misinterpretation of the data, we exclude all events that render an asymmetry between the edge-lines.

Another problem arises from the temporal gaps of our data. Upon generating the abraded traces, temporal gaps were filled with false pixels to generate smooth continuous edge-lines. In reality these filled gaps represent artificial data and must be excluded from our analysis. Furthermore, the temporal gaps render an underestimate of the extent of each run and/or pause flanking every gap. The same reasoning applies to the first and last run and/or pause of every edge-line. Again, to avoid misinterpretation of the data, we exclude all the pixels of the abraded edge-lines that overlap a temporal gap of the raw data. All runs and pauses that flank a temporal gap are excluded, and the first and last run and/or pause of every edge-line is excluded as well (see Fig. 2.13).
Figure 2.13: **Defining Movement**: In the lower panel we show a detailed visual outline of the processes used to extract well defined pausing states and running states. The raw-data (bottom edge-lines) is obtained using the ImageJ software package. These lines are processed using the abrasion algorithm (see Secs. 2.8.1-2.8.3) to generate smooth, continuous edges that are free of pixel-noise (middle edge-lines). We then selectively exclude all artificial data to create a final data set of common running states and common pausing states. (I.e. All temporal gaps are excluded. All runs and pauses flanking the gaps as well as the first and last runs/pauses are excluded. All runs and pauses wherein opposing edges exhibit a discrepancy in the kinetic state of the neurofilament–potential folding events–are excluded.)
While we cannot say that our method(s) used to estimate the "true" trajectories of the neurofilament edge-lines obtained from our image analysis (see Sec. 2.6) are exempt from mistakes, it is noteworthy to point that the abrasion algorithm carries a high degree of accuracy and precision with it. In Fig. 2.14 we show the frequency histograms of the pixel deviations between our final collective set of neurofilament runs and pauses compared to the original image-processed edge-lines extracted via the Canny edge-detection software.

Figure 2.14: **Accuracy of the estimated edge-lines**: In (a) we show the frequency of pixel-deviations between the image-processed edge-lines and the final estimated run data. In (b) we show the frequency of the pause-block sizes for all pauses defined by the abrasion algorithm, wherein the block size is taken from the original images processed via the ImageJ software package.

The pixel error for the running states is measured as the pixel deviations between the smoothed run trajectories and the raw data for each time frame (i.e. $\Delta_{\text{pixel}}(t) = |x(t)_{\text{data}} - x(t)_{\text{filtered}}|$). For the pausing states, we measure the maximum difference that occurs between any two pixels that are within that pause-time interval. This is referred to as the block-size of a given pause (i.e for each pause interval $\delta t$,

$$\Delta_{\text{pause block-size}} = \max\{|X(\delta t)| - \min\{|X(\delta t)|\},$$

where $\{X\}$ is the set of all pixel values spanning over $\delta t$). In figure 2.14 we show the frequency plots for the run-pixel-errors and pause-block-sizes. The maximum error for both the running states and pausing is 7 pixels,
which are extremely rare occurrences. Over 99% of all continuous runs have a
$\Delta_{\text{pixel}}(t) \leq 3$ pixels, in which almost 97% of those errors are within 2 pixels. The
maximum pixel deviation for all continuous runs is 7 pixels, which only occurred once.
The errors for the pause-blocks are also all within 0-7 pixels, in which over 99% of all
pause-blocks have a $\Delta_{\text{block-size}}^{\text{pause}} \leq 5$ pixels, which is expected given our $\pm 2$ pixel cutoff that
was established in the pause-filtering criteria.

2.9 Results

Upon applying the above criteria in Sec. 2.8, our final data set consists of 726
neurofilament traces consisting of 5,589 retrograde bouts of movement, 6,615 anterograde
bouts of movement, and 7,395 states of no movement (pausing states). For each pausing
state, we record the pause-time interval and the corresponding average filament length
(FL). For both the anterograde and retrograde states, we record each trajectory’s
run-length (RL), run-time (RT), run-velocity (V), and the corresponding average filament
length (FL). For our collective data, we find that the running states represent 62.3% of all
states extracted from the kymographs and only 37.7% of the states represent pausing
states. However, the amount of time that these filaments are static greatly outnumbers the
amount of time that they are mobile. Our final data set consisted of 1010.2 minutes ($\approx 16.8$
hours) of recorded movement for all running states and all pausing states, for which 89%
of this time ($\approx 901.4$ mins) is accounted for purely from the pausing states. This value is
within the range of those reported by Wang and Brown (2001), Wang et al. (2000), Roy
et al. (2000), and Brown et al. (2005) who reported that neurofilaments remain static 67%,
73%, 80%, and 97% of the time, respectively. The remaining 11% of our recorded data is
accounted for by 61.6 mins of anterograde runs ($\approx 6\%$ of the time) and 47.6 mins of
retrograde runs ($\approx 5\%$ of the time). Upon considering only the time spent in a running
state, we find that anterograde bouts occur 56.4% of the time, which is significantly less
than the findings of Wang and Brown (2001) who reported anterograde movements make up 69-83% of all on-track movement. Our explanation for this discrepancy is best explained by the fact that our movies are recorded at a much higher frame-rate and capture movement that occurs over shorter time-scales, which gave rise to observing frequent occurrences of reversals that were not interrupted by a prolonged pause (i.e. these reversals are interpreted as instantaneous reversals).

Figure 2.15: **Summary-Statistics of collective neurofilament trajectories**: Our final data set consists of 726 neurofilament traces consisting of 5,589 (28.5%) retrograde bouts of movement, 6,615 (33.8%) anterograde bouts of movement, and 7,395 (37.7%) states of no movement (pausing states). On average, the lengths of these filaments (FL) are longest during the pausing states, but not much greater than the average length during anterograde running-states (5.9 µm vs 5.6 µm, respectively). Shorter neurofilaments appear to move retrogradely more often as the average filament length recorded for retrograde runs was 14%-18% less than the average filament length during both anterograde runs and pauses, respectively. During movement, the average lengths and times of a contiguous-run are compatible to within 18% and 16%, respectively. However retrograde-directed filaments move about 50% faster than anterograde-directed filaments, on average. Note: RL, RT, V, and PT are abbreviations for the different kinetic characteristics of these filaments; run-length, run-time, run-velocity, and pause-time, respectively.

First we examine the frequency distributions of polymer length for each of the individual kinetic states: during pause intervals, anterograde run intervals, and retrograde run intervals. To our surprise, we find that the average length of these filaments is longest during a pausing state, which is in contrast to Taylor et al. (2012). Their observations
suggest that filaments move in a completely unfolded (hence, completely extended) configuration, while paused filaments express the most fluctuations in polymer length due to folding events. When we estimated the edge-lines of these polymers, we set out to exclude the events where active folding and unfolding occurs, however we did not exclude folding events that progress for extended periods of time. That is to say, if a polymer experienced a folding event, we eliminated that portion of the folding event when a asymmetry appeared between the two edges of the filament. We did not, however, eliminate edge-lines where a filament could be folded as long as the leading and trailing edges were in the same kinetic state during that fold. As such, we would expect that if folding events generally occurred during the pausing states, then this feature would illustrate itself in the average. The fact that it does not is quite interesting and suggests that folding events do not predominately occur when neurofilaments are static.

![Figure 2.16: Distributions of neurofilament lengths](image)

Figure 2.16: **Distributions of neurofilament lengths**: We show the distributions for polymer lengths for retrograde moving filaments (a), paused filaments (b), and anterograde moving filaments (c). Each histogram is overlaid with an exponential fit (dashed lines), which corresponds to a distribution having frequency factor $\lambda = 1/\mu$, where $\mu$ is the average polymer length for a given kinetic state.
We note the deviations in the average filament lengths between the running states and pausing states: 1.1 µm between the retrograde-directed filaments and paused filaments; and 0.3 µm between the anterograde-directed filaments and paused filaments. These deviations, 0.3 µm and 1.1 µm, corresponds to pixel widths of about 1.9 pixels and 6.8 pixels, respectively. 1.9 pixels is well within the range of the calculated pixel errors between our estimated edge-lines and the raw data, implying that the discrepancy between the polymer lengths of the anterograde states and the pausing states is merely due to the accuracy of the abrasion algorithm. Most likely it follows from the ±2 pixel threshold that was applied during the pause-filtering step. However, pixel deviations between the raw traces and the abraded traces rarely exceed six pixels (see Fig. 2.14), which suggests that the deviation between the average polymer lengths for the retrograde states and the paused states is possibly significant. Two possible explanations for this could be that (i) shorter neurofilaments tend to move retrogradely more often, or (ii) filaments that are experiencing a folding-event move predominantly in the retrograde direction.

Next we analyze the movement of these polymers in more detail. The net velocities of these traces (including pauses) ranged between -3.7 and 2.3 µm/sec. The average net-anterograde-velocity was 0.3 µm/sec and the average net-retrograde-velocity was -0.4 µm/sec. For the anterograde runs, the run-lengths, run-times, and run-velocities ranged between 0.003-29.28 µm (mean=0.97 µm), 0.03-19.5 seconds (mean=1.29 sec), and 0.01-10.56 µm/sec (mean=0.98 µm/sec), respectively. For the retrograde runs, the run-lengths, run-times, and run-velocities ranged between -21.95 to -0.003 µm (mean=-1.24 µm), 0.03-13.4 seconds (mean=1.08 sec), and -1.49 to -0.02 µm/sec (mean=0.98 µm/sec), respectively. The pause-times range between 0.03-234.5 seconds with an average pause-time of 7.3 seconds. (See Fig. 2.17)
Figure 2.17: **Distributions for pause-time, run-length, run-time, and run-velocity:** In (a) we show the pause-time distribution(s). The inset of (a) shows the same data plotted as a density profile using a non-uniform log-binning approach. The lower panels show the frequency histograms for the run-lengths (b), run-times (c), and run-velocities (d) for all retrograde runs (orange histograms) and anterograde runs (green histograms). In (d) we show the distribution for the net-velocities extracted from each edge-line of each abraded trace. The average values for each motility characteristic is listed in the color boxes, inserted at the respective corners of each distribution. The run-time distribution for the retrograde runs has its x-coordinate listed at negative values to allow visual comparison with the run-times of the anterograde runs.
What is remarkable about these values are the similarities between these measurements and those found from experiments involving the fast transport of organelles within live cells. Soppina et al. (2009) observed the bi-directional cargo motions of endosomes that were transported by kinesin and dynein within cells. Their findings suggest that these molecules move at speeds around 2 µm/sec, which fits well within the range of our measured velocities for neurofilaments. Furthermore, Ma and Chisholm (2002) reported the movement of dynein directed cargos moving at speeds of 1.84 ± 0.34 µm/sec, which is comparable to the average velocity of our retrograded directed filaments. While they report that dynein can move at such speeds in either retrograde and anterograde directions, the fact that the velocity for the anterograde runs is 50% less, on average, suggests that dynein is not the motor primarily responsible for the anterograde transport of these neurofilaments. Similar to Soppina et al. (2009), Kural et al. (2005) suggests in their study of the motions of peroxosomes within cells that dynein manages retrograde transport, while kinesin manages anterograde transport. They reported motions of these molecules moving at speeds between ±1-10 µm/sec which spans the range of run-velocities of these neurofilaments (Fig. 2.17c). Whether the modulated kinetics of these filaments follows a "tug-of-war" scenario (which was observed by Soppina et al. (2009)), a coordinated type model (Kural et al., 2005), or both remains unclear.

Nevertheless, the similarities between the running speeds of these polymers and those of fast-transported molecules (ibid) is quite remarkable.

Another curious feature of these results is the fact that the distributions of run-lengths and run-times for the retrograde runs and anterograde runs are reasonably symmetric. This suggests that, while we don’t know the number of motors participating in the movement of these filaments (nor which class(es) of motors), these motors could follow similar detachment kinetics. If we assume that the asymmetry in the run-velocity distributions for retrograde and anterograde runs follows from different motors being attributed to different
directions of polymer movement, then it is quite surprising to find a reasonably symmetric pattern in the run-length/run-time distributions, especially considering the vast differences in the stalling forces for kinesin and dynein: 6 pN vs 1 pN, respectively (Soppina et al., 2009; Mallik et al., 2004; Svoboda and Block, 1994). However, if the modulated kinetics of the ”on-track” phase of neurofilament transport follows that of the fast-axonal transport of organelles and vesicles, then perhaps the symmetry in the run-length/run-time distributions follows from a greater numbers of dynein motors working together in the retrograde direction against fewer kinesin motors working towards the anterograde direction. Another finding of the Rai et al. (2013) study was that dyneins collectively work together while hauling endosome cargoes within cells, which enabled them to withstand forces well beyond the 1 pN stalling force of a single dynein motor. Presumably, this remarkable feature follows from dynein’s ability to practice ”catch-bond” type kinetics that allows them to increase their binding affinity to microtubules during times of increased strain. Presumably, this would delay dynein-microtubule detachment to allow more motors to join in and participate in hauling the strained cargo. Hence, perhaps dyneins move together to counter tug-of-war events and therefore regulate cargo movement.

While we don’t know the exact processes responsible for neurofilament transport, clearly the literature supports a model in which kinesin and dynein move these filaments as cargoes. The question is however, given the extensive range of filament lengths that we observed, is there a correlation between filament length and filament kinetics. Presumably, longer filaments can have more motors attached, which could certainly change the way they move. For instance, if a single motor class (say just kinesins) were bound to a filament then it seems logical that the filament would move more persistently along the microtubule as more motors joined in – much like a horse carriage having four horses pulling it versus one horse – and one would guess that longer filaments would then
express longer run-lengths and longer run-times. If the same filament now has both retrograde- and anterograde directed motors bound to it (i.e. let’s add some dynein motors), then the filaments would move less robustly merely by introducing an opposing load and hence pause more often (reversals would occur). Now extend the filament length by a 2-fold increase and increase the total number motors by the same factor but keep the ratio of retrograde vs anterograde motors the same. By increasing the total number of motors, we expect an increase in the overall stepping frequency—more motors implies more steps more often, collectively—which introduces more frequent occurrences where each motor experiences a higher load on average. Hence, by adding more motors, we expect more stalling events and hence, more pausing events (more reversals). To explore this, we calculate the frequencies of each of the kinetic states for every abraded trace and then compare these relative to the average filament length of each corresponding trace. To our surprise, we find no correlation between the running-frequencies, nor pausing-frequencies of these filaments with respect to the average lengths of these filaments (see Fig. 2.18a). However, we do find that the distributions of the running-frequency for these filaments to be quite different from distribution of the pausing frequency (Fig. 2.18b). On average, neurofilaments experience a pause at a rate of 0.07 sec\(^{-1}\), while runs occur at a rate of 0.04 sec\(^{-1}\) for both retrograde runs and anterograde runs, which is consistent with the fact that (1) we extracted nearly twice as many runs as we did pauses from the abraded traces, and (2) retrograde directed runs occur almost as frequent as anterograde directed runs (recall Fig. 2.15 and see below).
Figure 2.18: Examining the correlation between the running/pausing frequency and neurofilament length: In (a) we show the scatter plots of the frequencies for each kinetic state relative to the neurofilament length. The insets of (a) represent the same data plotted on a log-log-scale for better visualization. In (b) we plot the density profiles of the retrograde running frequencies (orange), the pausing frequencies (blue), and anterograde running frequencies.

While it is surprising that filament kinetics appear to have no correlation with their length, we cannot say for certain that this is true. The ratio of our average running frequency to the average pausing frequency merely implies that for every pausing event there are two running events flanking each pause \( \frac{1_{\text{run}}}{0.04_{\sec}} / \frac{1_{\text{pause}}}{0.07_{\sec}} \approx 2 \frac{\text{run}}{\text{pause}} \), which is obvious. However, while Fig. 2.18a suggests that longer filaments appear to behave similar to shorter filaments (i.e. longer polymers move/pause just as frequently as shorter polymers do), we question the possibility that filament length is related to the specific characteristics of each run and/or pause, which could render some insight on the collective dynamics of the motors responsible for polymer transport. As mentioned, a long standing controversy
still stands regarding the mechanism(s) behind axonal transport in regards to whether or not the mechanism of cargo movement follows a coordinated scheme versus a competitive scheme (i.e. a "tug-of-war"), or perhaps a combination of both. While it appears that both types of movement have been observed in vivo (Soppina et al., 2009; Kural et al., 2005), there is no evidence that would favor one over the other for neurofilaments. So far our results show that neurofilaments move very sporadically with a motion that is stochastic in nature. They appear to reverse frequently as well as experience folding events quite often (see Figs. 2.12 and 2.10). The observation of frequent reversals implies uncoordinated movement is at least partly responsible for the translocation of these polymers, however not all filaments appear to reverse (see Fig. 2.13), which could favor a coordinated-type mechanism. Nevertheless, without knowing the way in which motors distribute themselves about a polymer, it is not reasonable to claim either mechanism dominates. Since we find no correlation between filament length and the running/pausing frequencies of these polymers, it seems likely that motor number is not correlated with length either. If it were, then longer filaments would presumably practice different kinetics than shorter filaments and therefore, would exhibit differences in their motility characteristics relative to shorter polymers. However, Fig. 2.19 suggests that no correlation exists between any motility characteristic and polymer length, which either suggests that motor number does not correlate with polymer length either, or the mechanism behind polymer transport does not strictly follow a coordinated model or an uncoordinated model between the motors.
Figure 2.19: **Scatter plots for neurofilament motility characteristics:** We show the scatter plots for the run-lengths, run-times, run-velocities, and pause-times extracted from the abraded traces. The green circles represent measurements for anterograde moving polymers, while the orange circles represent measurements for retrograde moving filaments. The blue circles represent static filaments. The run-time data for the retrograde moving polymers is plotted as its negative value purely for visual comparison with the run-times of the anterograde moving filaments.

We see in Fig. 2.19 that shorter filaments appear to demonstrate a wider range of kinetics than longer filaments. For instance, the pause-times for filaments of length 5 \( \mu m \) and less range between 0.3-150 seconds which is comparable to the range of pause-times for filaments between 20-25 \( \mu m \)–there are just fewer occurrences. Furthermore, while shorter filaments can move at speeds between \( \pm 15 \mu m/sec \), there are many occurrences of them moving much slower at speeds more comparable to those of the longer filaments. This could suggest that while polymer speed does not appear to be related to polymer length, perhaps the range of speeds that polymers move could depend on its length. A
possible explanation for this is that perhaps shorter filaments merely exhibit more diverse kinetics than longer filaments, which is reasonable if one considers how densely-packed the axonal cytoskeleton is. Nevertheless, it is quite surprising that longer neurofilaments don’t express an obvious difference in behavior relative to filament length.

To expand on this, we tested whether or not different filaments behaved similarly (or differently) for a given range of filament lengths, on the average. In Fig. 2.20 we show the histograms of the average values of the pause-times, run-times, run-lengths, and run-velocities for different subsets of filament lengths. Again, there is no obvious correlation between any motility characteristic and polymer length. However, in agreement with our summary statistics (Fig. 2.15) we find, for a given subset of polymers having similar lengths, the retrograde run-lengths are longer than the anterograde run-lengths, while the run-times are about the same. Hence, on the average, retrograde directed filaments move faster than anterograde directed filaments.
Figure 2.20: **Neurofilament motility characteristics binned by filament length:** We show the histograms for the average run-lengths, run-times, and run-velocities of both anterograde (green) and retrograde (orange) moving neurofilaments, as well as the average pause times (blue). In contrast to the scatter plots (see Fig. 2.19), we divide the data into unit-interval subsets relative to each run’s average filament length. That is to say, each bin in the histogram corresponds to the average motility characteristic of all filaments whose length resides within that bin. The inset panels show the same data, using a semi-log scale, where upper '+' ticks corresponds to the maximum value for a given bin.

### 2.10 Discussion

The fact that axonal transport has been under investigation since the late 1940s (Weiss and Hiscoe, 1948) and still remains under investigation is quite staggering. While transport within the cytoplasm was an easily accepted notion considering protein synthesis for the entire neuron occurs within the cell body, the notion of transport related to components of the cytoskeleton has been less accepted. Even today, while it is well known
that the cytoskeleton is a dynamic process, the staticity of the network still remains up for
discussion. Part of this is due to the misconception that the neurofilament network
practices extensive cross-linking with adjacent filaments via the NF-H sidearm
projections. However, as pointed out by Brown and Jung (2013), this is highly implausible
when considering the studies of Price et al. (1988) and Brown and Lasek (1993), which
did not observe extensive cross-linking to occur between the side-arms of neurofilaments.
Rather, it appears that the side-arms act in a more repulsive manner and serve to generate
space between neighboring filaments (Price et al., 1988; Brown and Lasek, 1993; Brown
and Hoh, 1997; Kumar et al., 2002). The more pertinent question is the mechanism(s) that
underpin the kinetics of the neurofilaments themselves. Within the past 20 years, it has
been discovered that while the overall neurofilament construct moves very slowly on
average, individual filaments move much faster, at rates comparable to those of
cytoplasmic materials (i.e. components of fast axonal transport). During movement,
neurofilaments move along axons in a rapid intermittent and bidirectional manner that is
characterized by frequent long intervals where these filaments remain static, which are
then interrupted by infrequent, intermittent bouts of rapid movement.

To gain further insight on the motile mechanism behind neurofilament transport, we
transfected primary cultures of cortical neurons from neonatal rat brains with GFP-tagged
neurofilament protein M and then recorded neurofilament movement by epifluorescence
microscopy at 33 frames per second using an EMCCD camera. We used kymograph
analysis to track neurofilament movement, and edge detection algorithms to extract the
paths of the leading and trailing ends of the moving filaments. By applying a newly
developed, unbiased, abrasion algorithm we were able to estimate the true trajectories of
the edge-lines to within a high degree of pixel accuracy, which enabled us to extract well
defined bouts of movement that were distinguishable from longer intervals of no
movement. Upon applying this method, we were able to distinguish between the different
kinetic states of these neurofilaments (pausing versus running), which allowed us to examine the specific characteristics of each kinetic state as well as variations in polymer length over time.

First we examined the range of filament lengths for the individual kinetic states and found that the average filament length of the retrograde bouts of movement were about 17% shorter than the average filament lengths for both the anterograde bouts of movement and the pausing states. This implies that on average, longer filaments undergo either an anterograde-directed run, or a pause more than a retrograde-directed run. However, we cannot say for certain that this is an inherent feature of neurofilament transport as it could merely arise from biased sampling error. The average filament lengths between anterograde bouts of movement and the pausing states only showed a 5% difference between them, which is not considered to be significant when considering pixel error.

Remarkably, we found that the overall average velocities, the average velocities during a contiguous run, the average run-lengths, average run-times, as well as the average pause-times all to be independent of neurofilament length across a range of lengths of 1-36 µm. We also found there to be no length dependence with the running frequencies as well as the pausing frequencies of these filaments. How to interpret these results is somewhat puzzling in terms of their implications of motor interactions. Intuitively, our results suggests that the number of motors associated with a neurofilament does not change considerably with regard to filament length. However, perhaps a better interpretation would be to posit that the number of actively engaged motors does not change relative to a polymer’s length. That is to say, while longer neurofilament cargoes may have more motor interactions occurring with them, only a fixed number of motors—on average—are actively engaged with both the neurofilament and the microtubule during transport.
3 Computational Modeling of Molecular Motors

3.1 Enzymatic Motor Proteins – an Introduction

Molecular motors are responsible for maintaining the essential translocation of organelles, vesicles, and fibrous polymers within axons. Such movement is required to maintain both form and function within a cell. While dozens of different motors have been observed over the years, there are three main classes: kinesins, myosins, and dyneins. Within the axon of a nerve cell, kinesin and dynein both move along microtubules within the center of an axon while myosins migrate along actin filaments located about the peripheral edges of an axon near the membrane. These motors are all quite different (molecularly speaking), however, they possess a common ATPase functionality where they catalyze the process of ATP hydrolysis and harness the excess chemical energy released subsequently. This process underlies their ability to both move, generate force, and hence, haul cellular cargoes.

The motors responsible for the slow axonal transport of neurofilaments are kinesin and dynein. Kinesin is thought to be the primary motor responsible for guiding anterograde-directed polymer movement, and cytoplasmic dynein is thought to guide the retrograde-directed polymer movement. The generic morphology of both types of motors consists of two monomer proteins that pair into a single dimer, which has four primary regions; two binding domains that are connected by a helical stalk and a motor domain (see Fig. 3.1). The binding domains are what enable these motors to attach to both cytoskeletal fibers, as well as cellular cargoes. Generically, these domains are referred to as the nucleotide binding domain(s) (NBD)—also known as the ”heads”–and the cargo binding domain (CBD)–which we will call the ”tails”. The heads provide the means for motor movement and are responsible for microtubule binding/unbinding, while the tails are responsible for cargo attachment/detachment (hence the name). The ATP binding and
hydrolysis processes occurs within the motor domains. Kinesin’s motor domains are located within its NBDs while cytoplasmic dynein’s motor domains are about 12 nm above its NBDs at the uppermost end of a linker.

As mentioned, motor proteins bind to microtubules, which are the platforms that motors move along to carry cellular cargoes. These structures are about 25 nm in diameter and possess a cylindrical tube-like geometry composed, primarily, of polymerized α/β-tubulin dimers, as well as γ-tubulin. The α/β dimers are protein complexes that are created when an α-monomer protein binds to a β-monomer protein, which is stabilized by a GTP (Guanosine-5′-triphosphate) molecule that couples the two monomers together. During microtubule nucleation, 13 γ-tubulin molecules combine with various accessory proteins to form a ring shaped γ-tubulin complex that acts as a scaffold for the α/β-tubulin dimers (Moritz et al., 1995). The microtubules are formed when thirteen α/β dimers bind to the γ-tubulin scaffold, which begins to grow as more α/β dimers bind together in an
end-to-end fashion to form long extensions called protofilaments. Hence microtubules consist of 13 protofilament structures in parallel alongside one another, where each protofilament is slightly offset from one another, such that the microtubule appears as a left-handed, 3-start helix with a helical pitch spanning over three $\alpha/\beta$ tubulin subunits. Over time, the GTP molecules bound within the $\alpha/\beta$ dimers gets hydrolyzed to GDP, which weakens the bond between the two monomers and destabilizes the dimer structure (i.e. the binding affinity between the $\alpha/\beta$ monomers is reduced). As GTP-hydrolysis progresses, dimers at the left-most end of the microtubule (the minus-end) depolymerize and detach from the protofilament, which reduces the overall microtubule length. However, at the same time newly polymerized $\alpha/\beta$ tubulin bind at the plus-end of the microtubule, which increases the overall length of the microtubule structure. Hence, microtubules possess a dynamic instability in their growth, which allows them to vary in length. However, the rate of depolymerization is greatly outweighed by the rate of polymerization and can lead to microtubules reaching lengths up to 50 $\mu m$ – well beyond the average run-length of both kinesin and dynein.
Figure 3.2: **Schematic of the microtubule structure and growth**: The \( \alpha \) and \( \beta \) protein monomers form into dimer structures, which are coupled via a GTP molecule. The dimers then form into protofilament tracks which are organized into a helical structure having a 25 nm outer diameter. The composite structure of 13 protofilament tracks forms the microtubule structure. Dimer polymerization occurs at the plus-end of the microtubule which is directed towards the distal end of the axon, while depolymerization occurs at the minus-end of the microtubule towards the proximal end of the cell. Depolymerization occurs upon GTP-GDP hydrolysis which reduces the binding affinity between the \( \alpha/\beta \) tubulin.

The mechanism behind kinesin and dynein movement rests heavily on the mechanism governing their ability to bind to microtubules. Initially, it was thought that dimeric motor proteins (such as kinesin and dynein) moved along microtubules by a rectified diffusive mechanism wherein motor heads could diffuse along microtubules in a fashion that was guided by the processes of both nucleotide binding and ATP hydrolysis. However, while diffusion could play a role, this model cannot explain the measured forces generated by these motors, which are substantially larger than those predicted from a diffusion model (Howard, 2011). Another possible mechanism is the flashing-ratchet model (Ajdari and Prost, 1992; Magnasco, 1993; Rousselet et al., 1994; Astumian and Bier, 1994), which assumes that motor movement results from the heads alternating between a purely diffusive state and a state where they are bound within a sawtooth potential. Unfortunately this model cannot be used as it predicts that "on average the motor requires at least two switching processes per step because half of the time the net
diffusion is in the wrong direction” (Howard, 2011), which contradicts the measurements of Coy et al. (1999); Carter and Cross (2005); Svoboda et al. (1993) who report that (1) ATP hydrolysis is the process responsible for motor-stepping and (2) only a single hydrolysis occurs per step. The most widely excepted model is the powerstroke model, which was developed based on the thermal ratchet model of Huxley (1957). The power stroke model assumes that motors undergo conformational changes during their catalytic cycles which allows these protein structures to reform themselves into functional shapes that favor mechanochemical work. By cycling through a given number of conformations, which follow from a given number of chemical reactions, excess chemical energy is converted into mechanical work to produce a powerstroke that allows the motor to translocate itself. More specifically, kinesin and dynein move along the microtubule protofilament tracks by conforming into structures that enables motor-domain binding to the nucleotide junctions (or ”pockets”) of the microtubule α/β-dimer subunits. Once bound to a pocket, these motors undergo further nucleotide-dependent conformational changes during the ATP/ADP dependent enzymatic cycles that enables them to harness excess chemical energy following the hydrolysis of a single ATP molecule, which results in the translocation of the motor domains to neighboring α/β junctions. Curiously, the kinetics that underly microtubule-motor binding/unbinding are also ATP/ADP dependent. In particular, the binding/unbinding affinities of these motors with the nucleotide junctions increases substantially when ATP/ADP is present at the motor domain. Together, these two process work in unison with one another and define the mechanism behind motor-protein motility, and hence, cargo transport.

3.1.1 The Enzymatic Cycle

The movement of both kinesin and cytoplasmic dynein follows from their ability to catalyze the hydrolysis of ATP into ADP. By lowering the free energy of activation
required for ATP hydrolysis, they not only accelerate the rate of hydrolysis, but also expose themselves to an energy surplus, which they harness for work. Hence, the steps in motor-isomerization dictate the motility characteristics of these motors. For both kinesin and cytoplasmic dynein, movement occurs by completing a sequence of conformational changes that results in the translocation of a motor domain to/from the nucleotide binding sites of microtubules. As such, enzyme kinetics are modeled using chemical reaction schemes where each state of the reaction represents a different structural conformation of the motor. For dimeric mobile enzymes, the kinetic cycle is repetitive as the motor domains operate in tandem, suggesting these motors practice an ATPase pathway that follows the analog of a chemical reaction loop composed of N intermediate states,

$$\begin{align*}
S_0 &\xrightleftharpoons{\gamma_0,1} S_1 \xrightleftharpoons{\gamma_1,2} S_2 \xrightleftharpoons{\gamma_2,3} \cdots \xrightleftharpoons{\gamma_{N-1,N-2}} S_{N-1} \xrightleftharpoons{\gamma_{N-1,0}} S_0,
\end{align*}$$

where the rate at which the enzyme transitions from state $i$ to state $j$ is denoted by the coefficients, $\gamma_{i,j}$ ($j=i\pm1$), which follow an exponential form comparable to the *Arrhenius equation*, i.e.

$$\gamma_{i,j} = \gamma_{i,j}^0 \exp\left(-\frac{\Delta E_{i,j}}{k_B T}\right).$$

Generally speaking, in terms of chemical kinetics, the states $S_i$ would correspond to reactants and products while $\Delta E_{i,j}$ in Eq. 3.2 would represent the activation energy required for a reaction to occur between states $i$ and $j$. A negative energy difference favors the forward production of products ($S_i \rightarrow S_j$), while a positive energy difference implies the reverse process is favored ($S_i \leftarrow S_j$). If no state is favored, then the free energies of each state are equal such that each reaction is in equilibrium wherein

$$\gamma_{i,i+1}[S_i] = \gamma_{i+1,i}[S_{i+1}]$$

implying for the N-state reaction loop in Eq. 3.1 that

$$\prod_{i=0}^{N-1} \gamma_{i,i+1} = \prod_{i=0}^{N-1} \gamma_{i+1,i},$$

which—by definition—is the principle of detailed balance (Keener and Sneyd, 2009).
The standard approach at estimating the net reaction rate for Eq. 3.1 would be to apply the law of mass action to formulate a set of differential equations that represents the time dependence of the concentrations for all the reactants and/or products of the scheme. However, this methodology does not directly apply to enzyme kinetics as they do not directly follow the law of mass action. For example, consider the simplest case of Eq. 3.1 in terms of enzyme kinetics where we have three components that make up a reaction scheme wherein an enzyme (E) combined with [ATP] hydrolyzes to a enzyme with [ADP],

$$ E + [ATP] \rightarrow E + [ADP]. $$

(3.3)

According to the law of mass action, the forward reaction velocity should always increase with increasing [ATP]. However for reactions that include an enzyme, the reaction speed plateaus at some saturating concentration of [ATP]. That is to say, the reaction is limited by the binding/unbinding/hydrolysis processes that occur between the enzyme and the substrate/product. In other words, the rate at which the enzyme changes conformations during its catalytic cycle limits the rate of the enzymatic cycle. Michaelis and Menten first explained this in 1913 (Michaelis and Menten, 1913; Johnson and Goody, 2011) and proposed a modified version of Eq. 3.3 that introduced an intermediate enzyme-substrate complex state that occurred before ATP hydrolysis (i.e.

$$ E + [S] \rightleftharpoons [ES] \rightleftharpoons E + [P], $$

which provided a means to explain why reaction speeds plateau for enzyme based reactions.

For our motor analysis, we will follow the framework of Michaelis and Menten by assuming that the enzymatic cycles of kinesin and dynein contain at least two different conformations within them, for which one of the conformations permits ATP-hydrolysis. Each motor will follow a specific mechanochemical cycle following the analog of Eq. 3.1 where the states, $S_i$, represent the nucleotide-dependent conformations that are required for enzyme motility. The rate constants governing the enzyme kinetics will follow an
Arrhenius type mechanism using a free energy landscape model in which $\Delta E$ from 3.2 represents a potential energy barrier that separates two different enzyme conformations. We assume that the primary constraint that inhibits enzyme movement is purely mechanical, implying that the work done by the enzymes upon transitioning from one conformation to another is proportional to the applied load acting on the enzyme. Hence, our rate constants follow the general form:

$$\gamma_{i,j} = \gamma_{i,j}^0 \exp \left( - \frac{\delta_{i,j} F}{k_B T} \right),$$  \hspace{1cm} (3.4)

where $F$ represents the mechanical stress that the enzyme undergoes during a conformation change, and $\delta_{i,j}$ represents the characteristic distance that separates the two conformations (i.e. $\delta_{i,j} F = \Delta E_{i,j}$) (Schnitzer et al., 2000; Fisher and Kolomeisky, 2001; Singh et al., 2005; Kunwar et al., 2008).

3.1.2 Comparing Motors: Force Generation and Stepping Behavior.

Over the years, extensive research studies have been developed to gain insight on the specific processes associated with motor movement as well as cargo translocation (Ashkin et al., 1990; Svoboda et al., 1993; Svoboda and Block, 1994; Hackney, 1996; Schnitzer and Block, 1997; Schnitzer et al., 2000; Brown, 2000; Wang et al., 2000; Roy et al., 2000; Wang and Brown, 2001; Yabe et al., 2001; Burgess et al., 2003; Mallik et al., 2004; Wagner et al., 2004; Singh et al., 2005; Brown et al., 2005; Yan and Brown, 2005; Kural et al., 2005; Toba et al., 2006; Ross et al., 2006; Trivedi et al., 2007; Kunwar et al., 2008; Sims and Xie, 2009; Jung and Brown, 2009; Uchida et al., 2009; Soppina et al., 2009; Li et al., 2012; Rai et al., 2013; Cleary et al., 2014; DeWitt et al., 2014; Li et al., 2014).

While the mechanisms behind motor movement are becoming more and more clear, cargo movement is still not well understood in regard to motor function. Any factor that stands to enable (constrain) motor-kinetics also stands to enable (constrain) the kinetics of material within the neuronal cytoskeleton. Hence, understanding the specific details of the
mechanism(s) behind the kinetic cycles of kinesin and dynein is paramount in understanding the mechanism underpinning the axonal transport of neurofilaments – or any type of cellular transport for that matter.

Both kinesin and cytoplasmic dynein have been observed to be force-generating enzymes that participate in the translocation of neurofilaments (Wagner et al., 2004; Uchida et al., 2009). In addition to their molecular differences, they are quite different, dynamically speaking, as well. These motors move in opposing directions – kinesins moves anterogradely while dyneins move retrogradely – which is thought to follow from the polar nature of microtubules by restricting the orientation in which the kinesin and dynein motor domains can bind to the α/β junctions along protofilaments (Alberts et al., 2000). However, recent reports have shown that while both kinesin and dynein have a "preferred" direction of movement, they can both move in either retrograde or anterograde directions along microtubules (Ross et al., 2006; Carter and Cross, 2005). While the specific mechanism behind directionality is not fully understood, some reports suggest that motor directionality is linked to the specific orientation of the individual motor domains (Henningsen and Schliwa, 1997; Endow and Waligora, 1998; Rice et al., 1999). Another factor that has been seen to promote changes in motor directionality, specifically for kinesin, follows from the amount of load being applied to the motor during movement (Hyeon et al., 2009). In addition to their differences in directionality, these enzymes possess different stepping characteristics as well as different loading constraints. Kinesin has a consistent 8 nm step-size (Svoboda et al., 1993; Schnitzer and Block, 1997; Sims and Xie, 2009) and can generate forces up to 5-8 pN during movement (Svoboda and Block, 1994; Visscher et al., 1999; Schnitzer et al., 2000). Dynein, on the other hand, displays multiple step sizes of 8-, 16-, 24- and 32-nm (Mallik et al., 2004; Toba et al., 2006; Reck-Peterson et al., 2006; Sims and Xie, 2009; Rai et al., 2013) and does not appear to have a well defined stalling force. In some reports dynein can only withstand
forces around 1 pN during movement (Mallik et al., 2004; Rai et al., 2013; Ori-McKenney et al., 2010), while others have suggested that dynein can withstand forces that are comparable to those generated by kinesin (Toba et al., 2006; Sims and Xie, 2009; Walter et al., 2010). It is thought that some of these differences can be explained by the differences between the motor domains of these two enzymes. The kinesin motor domains are much simpler than the motor domains of dynein, in the sense that the kinesin motor domain only possesses one ATP binding site and one hydrolysis site while dynein motor domains possess four (Gennerich and Vale, 2009; Reck-Peterson et al., 2012). Each dynein motor domain is formed by a 6 AAA+ (ATPases Associated with diverse cellular Activities) protein ring construct (Sakato and King, 2004; Gleave et al., 2014) that is responsible for its ATPase functionality (Sakato and King, 2004; Gennerich and Vale, 2009). It is not clear exactly how many of the AAA+ proteins contribute to motor locomotion, however it is thought that at least four of the six (AAA1-AAA4) are able to bind to ATP (Sakato and King, 2004) and at least one (AAA1) can hydrolyze ATP (Sakato and King, 2004). However, experimental studies (Kon et al., 2004; Cho et al., 2008) using knockout proteins to inhibit the individual ATPase functions of each AAA+ domain (ATP binding and hydrolysis), suggests that sites AAA1-4 all contribute to dynein processivity, and hence possibly contribute to its variability in stepping behavior (see section 8.5.1 of Reck-Peterson et al. (2012) for a discussion of the dynein AAA+ domains).

Regardless of differences between directionality and stepping characteristics, both kinesin and dynein are susceptible to the same biological constraints: ATP and ADP. The presence of ADP increases the microtubule binding affinities for both motors. In turn, microtubules increase the motor-domain binding affinity to ATP. Mechanical load is also a common constraint (Coppin et al., 1997), which affects the rate at which ATP can be hydrolyzed (i.e. results in a reduction in motor speed (Schnitzer et al., 2000; Rai et al., 2013)) as well as the binding affinity to microtubules (Coppin et al., 1997). However,
while load generally impairs motor movement for both motors, dynein has exhibited catch-bond like kinetics (Rai et al., 2013) where the load enhances motor movement. This suggests that dynein may act as a regulating protein during bi-directional cargo transport. Furthermore, it has been observed (Mallik et al., 2004; Rai et al., 2013), that dynein motors take larger steps on average (24-32 nm) in the absence of load – independent of [ATP]. As load increases its average step size reduces to that comparable to the kinesin step size (8-16 nm).

### 3.2 General Theory

To model single motor motility, we treat the enzymes as particles that have the ability to transition between different discrete states. Each state will represent a specific conformation that occurs during an enzymatic cycle. Upon successfully completing one cycle in the forward direction (left to right), a motor head is translocated to the next nucleotide junction. Following the notation of Eq. 3.1, we assume that each state, $S_i$, has an occupation probability, $P_i(t)$, for any given time. Assuming the system follows a Markov process, the probability of being in state $S_i$ at time $t + \Delta t$ follows (García-Ojalvo, 2014)

$$P_i(t + \Delta t) = P_i(t)(1 - \gamma_{i,i-1}\Delta t - \gamma_{i,i+1}\Delta t) + P_{i-1}(t)(\gamma_{i-1,i}\Delta t) + P_{i+1}(t)(\gamma_{i,i+1}\Delta t) + O(\Delta t^2),$$

(3.5)

where the terms $O(\Delta t^2)$ (and greater) represent the probabilities in which multiple transitions occur over $\Delta t$. By solving for $(P_i(t + \Delta t) - P_i(t))/\Delta t$ in Eq. 3.5, and taking the limit for $\Delta t \to 0$, we compute the matrix form of the master equations that represents Eq. 3.1.
\[
\begin{pmatrix}
\dot{P}_0(t) \\
\dot{P}_1(t) \\
\dot{P}_2(t) \\
\vdots \\
\dot{P}_{N-1}(t)
\end{pmatrix} =
\begin{pmatrix}
-(\gamma_{0,0} + \gamma_{0,N-1}) & \gamma_{1,0} & 0 & \ldots & \gamma_{N-1,0} \\
\gamma_{0,1} & -(\gamma_{1,2} + \gamma_{1,0}) & \gamma_{2,1} & \ldots & 0 \\
0 & \gamma_{1,2} & -(\gamma_{2,1} + \gamma_{2,2}) & \ldots & 0 \\
\vdots & \vdots & \vdots & \ddots & \vdots \\
\gamma_{0,N-1} & 0 & 0 & \ldots & -(\gamma_{N-1,N-2} + \gamma_{N-1,0})
\end{pmatrix}
\begin{pmatrix}
P_0(t) \\
P_1(t) \\
P_2(t) \\
\vdots \\
P_{N-1}(t)
\end{pmatrix},
\]

which in shorthand notation reads as \( \dot{\vec{P}}(t) = T \vec{P}(t) \) where the matrix \( T \) is referred to as the transition matrix. The product \( T \vec{P}(t) \) constitutes the probability current \( \vec{J}(t) \) (García-Ojalvo, 2014).

\[
J_i(t) = -(\gamma_{i,i-1} + \gamma_{i,i+1}) \cdot P_i(t) + \gamma_{i-1,i} \cdot P_{i-1}(t) + \gamma_{i+1,i} \cdot P_{i+1}(t).
\] (3.7)

It's clear that \( \sum \dot{P}_i(t) = 0 \) for any given time, implying that (under the proper set of initial conditions) \( \sum \dot{P}_i(t) = 1 \), as it should. As the rate coefficients in \( T \) have no time-dependence (Eq. 3.4), the general solution to Eq. 3.6 reads as

\[
\vec{P}(t) = \sum_{i=0}^{N-1} c_i \cdot \exp(\lambda_i t) \cdot \vec{v}_i
\] (3.8)

where the \( \lambda_i \)s are the eigenvalues of \( T \) with corresponding eigenvectors \( \vec{v}_i \), and the \( c_i \)s are all constants. By taking the limit for \( t \to \infty \) and using the initial condition that \( P_0(0) + P_1(0) + \ldots + P_{N-1}(0) = 1 \), we can obtain the stationary probabilities for each conformational state.

As a short example, consider the simple 2-state enzymatic cycle first posed by Michaelis and Menten: \( E + [S] \Rightarrow [ES] \Rightarrow E + [P] \). The two states are defined by the state of the enzyme. Either the enzyme is in a free state having no substrate bound to it (E), or it is in an enzyme-substrate complex (ES). In terms of our state model this reads as \( S_0 \Rightarrow S_1 \Rightarrow S_0 \), which seems to possess a redundancy in its structure by having two different pathways for \( S_1 \) to transition to \( S_0 \) as well as two different starting points for \( S_0 \).
However, in terms of reaction kinetics, this enzymatic cycle consists of four possible pathways that can lead to the two different conformational states, implying the schematic is correct and reads as

\[ S_0 \xrightleftharpoons[\gamma_{0,1}^\gamma_{1,0}]{\gamma_{1,0}^\gamma_{0,1}} S_1 \xrightleftharpoons[\gamma_{0,1}^\gamma_{1,0}]{\gamma_{1,0}^\gamma_{0,1}} S_0 \]  

(3.9)

which, to avoid confusion, we have included the ± superscripts to clarify the directionality of the enzyme cycle. Solving the master equations, we compute the transition matrix and the corresponding eigenvalues and eigenvectors.

\[
T = \begin{pmatrix}
-(\gamma_{0,1}^\gamma_{1,0} + \gamma_{0,1}^\gamma_{1,0}) & \gamma_{1,0}^\gamma_{0,1} + \gamma_{1,0}^\gamma_{0,1} \\
\gamma_{0,1}^\gamma_{1,0} + \gamma_{0,1}^\gamma_{1,0} & -(\gamma_{1,0}^\gamma_{0,1} + \gamma_{1,0}^\gamma_{0,1})
\end{pmatrix}
\]  

(3.10)

\[
\lambda_0 = 0, \quad \lambda_1 = -(\gamma_{0,1}^\gamma_{1,0} + \gamma_{0,1}^\gamma_{1,0} + \gamma_{1,0}^\gamma_{0,1} + \gamma_{1,0}^\gamma_{0,1})
\]  

(3.11)

By applying the initial condition, \( P_0(t = 0) + P_1(t = 0) = 1 \), we obtain the coefficients \( c_0 \) and \( c_1 \),

\[
c_0 = \frac{1}{1 + K_d} \quad c_1 = -\left( P_0(t = 0) - \frac{K_d}{1 + K_d} \right), \quad \text{where} \quad K_d = \frac{\gamma_{0,1}^\gamma_{1,0} + \gamma_{1,0}^\gamma_{0,1}}{\gamma_{0,1}^\gamma_{0,1} + \gamma_{0,1}^\gamma_{1,0}}
\]  

(3.12)

which, in terms of the disassociation constant, \( K_d \), leads to the solution of Eq. 3.9 being

\[
P_0(t) = \frac{K_d}{1 + K_d} + \left( P_0(t = 0) - \frac{K_d}{1 + K_d} \right) \cdot \exp(\lambda_1 \cdot t)
\]

\[
P_1(t) = \frac{1}{1 + K_d} + \left( P_1(t = 0) - \frac{1}{1 + K_d} \right) \cdot \exp(\lambda_1 \cdot t)
\]  

(3.13)

In steady state, Eq. 3.13 reduces to two simple expressions for the occupational probabilities of this two-state system.

\[
P_{0}^{ss} = \frac{K_d}{1 + K_d}, \quad P_{1}^{ss} = \frac{1}{1 + K_d}
\]  

(3.14)

While the solutions to Eq. 3.9 results in the occupation probabilities for the two different conformational states in this simple two-state enzyme cycle, they tell us nothing about the rate at which the enzyme moves during cargo transport. Nor, do they provide
information about motor robustness (i.e. the number of enzyme cycles that occur in series before the motor ceases to bind with a protofilament track). Fortunately, the biochemical nature of these motors provides insight on this. Both kinesin and dynein possess a stoichiometry of one (Schnitzer and Block, 1997; Coy et al., 1999; Reck-Peterson et al., 2006), meaning for every successful completion of their enzymatic cycle a single step is taken (one step for every ATP hydrolyzed). Experimental studies have shown that the step sizes of these motors are remarkably consistent (on average), however this can vary (specifically for dynein) depending on the applied load (Mallik et al., 2004; Rai et al., 2013). Similar studies have also shown that, under certain circumstances, these motors lose their ability to process along the microtubule, either by stalling and/or detaching (Kunwar et al., 2008; Sims and Xie, 2009; Ori-McKenney et al., 2010; Rai et al., 2013).

To quantify these characteristics, we continue with our 2-state example for which the occupation probabilities are already known. For clarification, we will rewrite the kinetic scheme in an equivalent form that properly illustrates the sequence of biochemical reactions that occur. An enzyme (E) binds to a substrate (S) and forms a complex (ES). Upon catalyzing the substate-hydrolysis process, the enzyme produces a product (P), i.e.

\[
E + [S] \overset{\gamma_{0,1}}{\rightleftarrows} [E \cdot S] \overset{\gamma_{1,0}}{\rightleftarrows} E + [P]
\]

(3.15)

### 3.2.1 Motor Velocity

The rate at which the enzyme completes an entire cycle, is equivalent to the rate of product production \(v_{\text{reaction}} = \frac{d[P]}{dt}\), which is proportional to the actual velocity of the enzyme moving along a protofilament, \(v_E = \bar{d}_E v_{\text{reaction}}\), where \(\bar{d}_E\) is the average displacement between the two motor domains of the enzyme (i.e. its center-of-mass displacement). Combining this with the steady state occupation probabilities \(P_{0s}^s\) and \(P_{1s}^s\), we quantify the average enzyme velocity, for this 2-state system, to be

\[
\bar{v}_{E,(N=2)} = \bar{d}_E \cdot \frac{d[P]}{dt} = \bar{d}_E \cdot \left(\gamma_{1,0} \cdot P_{1s}^s - \gamma_{0,1} \cdot [P] \cdot P_{0s}^s\right),
\]

(3.16)
which we extrapolate to its general form for the enzymatic cycle composed of N-states in Eq. 3.1

\[
\bar{v}_E = d_E \cdot \left( \gamma_{N-1,0} \cdot P_{N-1}^{ss} - \gamma_{0,N-1} \cdot P_0^{ss} \right).
\]  
(3.17)

### 3.2.2 Stall Force

Conveniently, upon setting Eq. 3.17 equal to zero and expressing the steady state occupancy probabilities in terms of the rate coefficients (Eq. 3.2) leads to an expression for the enzyme’s stall force (Fisher and Kolomeisky, 2001),

\[
F_{\text{stall}} = \frac{k_B T}{\sum_{i=0}^{N-1} (\delta_{i,i+1} + \delta_{i+1,i})} \cdot \prod_{j=0}^{N-1} \left( \frac{\gamma_{i,j+1}^0}{\gamma_{j+1,j}^0} \right),
\]  
(3.18)

where the \( \gamma_{i,j}^0 \) values correspond to pre-exponential factors of our load-dependent rate functions.

### 3.2.3 Processivity

To quantify the processivity (i.e. the run-length) we assume that the average run-length is related to the average motor velocity and the average run-time,

\[
\bar{x}_E = \bar{v}_E \cdot \bar{t}_{\text{run}}.
\]  
(3.19)

where the run-time is defined as the time interval for which a motor remains bound to a protofilament track, before it detaches. During a single processive run, an enzyme takes, on average, \( \bar{N}^+ \) steps forward and \( \bar{N}^- \) steps backward. By definition, a processive run is a single run composed of \( \bar{N}_{\text{tot}} = \bar{N}^+ + \bar{N}^- \) steps taken before the motor detaches from the microtubule. Hence the probability that the motor detaches (\( P_{\text{det}} \)) goes as the reciprocal of the total number of steps taken over time, \( \bar{t}_{\text{run}} \) (i.e. \( P_{\text{det}} = \frac{1}{\bar{N}_{\text{tot}}} \)). The rate of detachment, \( (k_{\text{det}} = 1/\bar{t}_{\text{run}}) \) then follows as the rate at which stepping events occur, multiplied by the
probability $P_{\text{det}}$ (Kunwar et al., 2008). By examination of Eq. 3.17, the rates for which both forward and backward steps are taken follow as $\gamma_{N-1,0} \cdot P_{N-1}^{ss}$ and $\gamma_{0,N-1} \cdot P_0^{ss}$, respectively. Hence, the run-time follows as

$$\bar{t}_{\text{run}} = \frac{1}{k_{\text{det}}} = \frac{1}{P_{\text{det}} \cdot \gamma_{N-1,0} \cdot P_{N-1}^{ss} + P_{\text{det}} \cdot \gamma_{0,N-1} \cdot P_0^{ss}}.$$ (3.20)

which is then used to compute the average run-length via Eq. 3.19. Upon expanding Eq. 3.19, we find—as expected—the run-length to be equal to the average step-size of the motor, multiplied by the total number of steps displaced before detachment.

$$\bar{x}_E = \tilde{v}_E \cdot \bar{t}_{\text{run}}$$

$$= \tilde{d}_E \cdot \left( \gamma_{N-1,0} \cdot P_{N-1}^{ss} - \gamma_{0,N-1} \cdot P_0^{ss} \right) \cdot \bar{t}_{\text{run}}$$

$$= \tilde{d}_E \cdot \left( \gamma_{N-1,0} \cdot P_{N-1}^{ss} \cdot \bar{t}_{\text{run}} - \gamma_{0,N-1} \cdot P_0^{ss} \cdot \bar{t}_{\text{run}} \right)$$

$$= \tilde{d}_E \cdot \left( \bar{N}_{\text{tot}} \cdot \gamma_{N-1,0} \cdot P_{N-1}^{ss} + \gamma_{0,N-1} \cdot P_0^{ss} \right)$$

$$= \tilde{d}_E \cdot \left( \bar{N}_{\text{tot}} \right)$$

$$= \tilde{d}_E \cdot \left( \text{“Total plus-end steps”} - \text{“Total minus-end steps”} \right) \equiv \tilde{d}_E \cdot \bar{N},$$ (3.21)

where $\bar{N}$ is the defined as the average number of steps displaced during a continuous run.

We note that defining the quantity $P_{\text{det}} = \frac{1}{\bar{N}_{\text{tot}}}$ may seem somewhat redundant in our approach for quantifying motor processivity—why not just use $\bar{N}_{\text{tot}}$ in our expression for the run-time (Eq. 3.19). However, as we will see in the upcoming sections, 3.3 and 3.4, $P_{\text{det}}$ is in fact a function of both ATP and applied load, which follows from the reports of Kunwar et al. (2008) (see supplement) who quantified the detachment probability (specifically for kinesin) based off of the work of Schnitzer et al. (2000). While Kunwar does not explicitly define a relationship between the detachment probability and the total number of steps taken as $P_{\text{det}} = \frac{1}{\bar{N}_{\text{tot}}}$, we wanted to include it in our general motor analysis.
to illustrate the connection. We will address the specific form of $P_{det}$ in the upcoming sections 3.3 and 3.4.

3.3 The Kinesin Model

3.3.1 Motivation

We model the dynamics of kinesin using a 4-state enzymatic cycle, which resembles the methods discussed by Fisher and Kolomeisky (2001) and Kunwar et al. (2008). In both cases, simple, discrete-state, sequential stochastic models were developed to study enzyme motility. Both models can reasonably explain the measurements of Schnitzer et al. (2000) and Visscher et al. (1999) who measured single kinesin molecule movement using a molecular force clamp (Visscher and Block, 1998). Kunwar’s model uses a 2-state kinetic cycle that follows a Michaelis-Menten scenario. However, in their model, they neglect ATP-synthesis events (i.e. $\gamma_{0,1} \rightarrow 0$ in Eq. 3.16) under the assumption that these events are rare and can be ignored. While this assumption is reasonable (Hackney, 1996), it restricts the model’s ability to accurately represent the motors behavior under high load and cannot explain the stalling kinetics of kinesin (see Eq. 3.18 for $N=2$ and set $\gamma_{0,1} = 0$). Kunwar recognized this and, instead, introduced an efficiency factor to represent the load-dependent coupling efficiency between ATP hydrolysis and mechanical stepping. This allowed them to neglect the load dependence of the catalysis rate which was presumed to be constant. While their approach is reasonable, the model predicts that the stalling kinetics follow a process that is independent of ATP, which is in contradiction to findings of Visscher et al. (1999), who reported that the stalling force of kinesin reduces for lower ATP concentrations.

In Fisher’s study, which was an early study, they examine the mechanochemical cycle of kinesin with two distinct models. The simplest model uses a two state representation of the enzyme cycle, which is then compared with a more sophisticated four state model. In
both schemes they were able to account for the measurements of Schnitzer et al. (2000) and Visscher et al. (1999). However, while their approach is quite sound, one feature of their model that enabled them to explain these data was the implementation of a final reversal rate that adopted a phenomenological form not consistent with a reaction scheme that strictly follows Arrhenius kinetics. Even though this approach is justified in their discussion on the analysis of Visscher’s data, their model does not provide insight on the explicit dependence of [ADP] and ADP-binding events, which has been reported, more recently, to promote release from the microtubule during movement (Uemura et al., 2002; Dogan et al., 2015)

3.3.2 Model Outline

In our model, we follow a kinetic scheme similar to the 4-state model of Fisher and Kolomeisky (2001), which follows as the analog of the enzymatic cycle of Svoboda and Block (1994). In this model, a free kinesin motor (K), first binds to an ATP molecule and forms the enzyme-molecule complex, K·ATP (KT for short). Once ATP is bound, kinesin catalyzes the ATP hydrolysis process, which results in a second complex composed of the hydrolyzed products of ADP and a phosphate ion, K·ADP·P (KDP for short). Upon release of the phosphate (which we assume is a rapid process), the third enzyme complex is formed in which the motor is now bound to ADP only, K·ADP (KD for short). Upon entering the KD state, we assume that a full working stroke has been completed, in which the trailing motor domain has been translated a distance of 16 nm in the anterograde direction along the microtubule, putting it 8 nm in front of the now trailing motor head. Once the translated–now leading–head binds to the microtubule and enters the (K) state, the KD bound head unbinds from the microtubule and releases the ADP molecule, for which then, the cycle repeats itself (see Eq. 3.22 and Fig. 3.3).

\[
K + [\text{ATP}] \xrightleftharpoons{\gamma_{0,1}} K \cdot \text{ATP} \xrightleftharpoons{\gamma_{1,2}} [K \cdot \text{ADP} \cdot P_i] \xrightleftharpoons{\gamma_{2,3}} [K \cdot \text{ADP}] + P_i \xrightleftharpoons{\gamma_{3,0}} K + \text{ADP} \quad (3.22)
\]
Figure 3.3: **Schematic of the kinesin enzymatic cycle**: Our model represents the kinetic cycle of kinesin, which has four unique nucleotide-dependent conformations with it. The outline goes as follows in a left-to-right manner: A kinesin head that is bound to a nucleotide pocket along a protofilament (orange head) binds to an ATP molecule. Once substrate is bound, the motor domain catalyzes the ATP-hydrolysis process, which induces the primary power stroke that translates the motor head that is not bound to the microtubule (purple head) to a position now ahead of the microtubule bound head. This puts the bound head into a complex state in the presence of ADP and a phosphate ion. We assume that the release of phosphate and ADP entails transitioning through two more unique conformations that occur while the unbound head binds to a nucleotide pocket that is 8nm in front of the previously bound motor head. The cycle then repeats.

### 3.3.3 Implementation and Results

In agreement with the power-stroke model, we assume that all transition rates can potentially possess a load dependence. The rates $\gamma_{0,1}$ and $\gamma_{1,0}$ represent the rates of ATP binding and unbinding, respectively. $\gamma_{1,2}$ and $\gamma_{2,1}$ represent the catalyzed rates of ATP hydrolysis and synthesis, respectively. The unbinding and re-binding of phosphate ions, $P_i$, follows from $\gamma_{2,3}$ and $\gamma_{3,2}$, respectively. The rates guiding the unbinding and re-binding of ADP follow from $\gamma_{3,0}$ and $\gamma_{0,3}$, respectively. Note that in this kinetic scheme each forward transition can be undone by a countering reverse transition. Generally speaking, the reverse transitions leading to ATP synthesis (ie. $\text{KS} \leftarrow \text{KDP} \leftarrow \text{KD}$) are thought to be extremely rare and are generally neglected, however to account for the stalling-force characteristics we include them in our model under the assumptions that the release of both $P_i$ and ADP are rapid, relative to ATP synthesis and phosphate rebinding (Hackney, 1988; Howard et al., 2001).
Upon applying the methods outlined in section 3.2 we compute the steady-state occupation probabilities of each state in Eq. 3.22,

\[
P_{ss}^K = P_{ss}^{KD} \cdot \frac{\tilde{\gamma}_3 \gamma_{2, 1} \gamma_{1, 0} + \gamma_{3, 0} \gamma_{2, 1} \gamma_{1, 0} + \gamma_{3, 0} \tilde{\gamma}_3 \gamma_{1, 0} + \gamma_{3, 0} \tilde{\gamma}_3 \gamma_{2, 3} \gamma_{1, 2} + \tilde{\gamma}_3 \gamma_{1, 2} \gamma_{2, 3}}{\gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 2} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 2} + \gamma_{0, 3} \gamma_{1, 2} \gamma_{2, 3}} \equiv P_{ss}^{KD} \cdot \Gamma
\]

\[
P_{ss}^{KT} = P_{ss}^{KD} \cdot \frac{\tilde{\gamma}_3 \gamma_{2, 1} \gamma_{1, 4, 3} + \gamma_{2, 1} \gamma_{1, 0} \tilde{\gamma}_3 \gamma_{2, 2} + \gamma_{2, 1} \gamma_{1, 0} \tilde{\gamma}_3 \gamma_{1, 3, 0} + \gamma_{2, 1} \gamma_{1, 2} \gamma_{2, 3} \gamma_{1, 0} + \gamma_{2, 1} \gamma_{1, 2} \gamma_{2, 3} \gamma_{1, 2} + \gamma_{2, 1} \gamma_{1, 2} \gamma_{2, 3}}{\gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 2} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 2} + \gamma_{0, 3} \gamma_{1, 2} \gamma_{2, 3}} \equiv P_{ss}^{KD} \cdot \Delta
\]

\[
P_{ss}^{KDP} = P_{ss}^{KD} \cdot \frac{\tilde{\gamma}_3 \gamma_{1, 0} \tilde{\gamma} \gamma_{0, 3} + \gamma_{0, 3} \gamma_{1, 2} \tilde{\gamma}_3 \gamma_{2, 2} + \gamma_{0, 3} \gamma_{1, 2} \tilde{\gamma}_3 \gamma_{1, 2} + \gamma_{0, 1} \gamma_{1, 2} \gamma_{2, 3}}{\gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 2} \gamma_{1, 0} + \gamma_{0, 3} \gamma_{2, 1} \gamma_{1, 2} + \gamma_{0, 3} \gamma_{1, 2} \gamma_{2, 3}} \equiv P_{ss}^{KD} \cdot \Lambda
\]

\[
P_{ss}^{ss} = \left(1 - P_{ss}^K - P_{ss}^{KT} - P_{ss}^{KDP} \right) = \frac{1}{\gamma + \Delta + \Lambda}
\]

wherein the rates having a “tilde” are of second order and, hence, are concentration dependent (e.g. \(\tilde{\gamma}_{0, 3} = \gamma_{0, 3} \cdot [ADP]\)). Furthermore, the corresponding motor velocity, follows from Eq. 3.17 and reads as (Fisher and Kolomeisky, 2001)

\[
\bar{v}_K = d_k \cdot \left(\gamma_{3, 0} \cdot P_{ss}^{KD} - \tilde{\gamma}_{0, 3} \cdot P_{ss}^K \right),
\]

which upon setting equal to zero provides the expression for the motor’s stalling force (Fisher and Kolomeisky, 2001),

\[
F_{stall} = \frac{k_b T}{\sum_{i=0}^{N=1} (\delta_{i+1} + \delta_{i+1, j})} \cdot \prod_{j=0}^{N=1} \left(\frac{\gamma_{j+1}^{0}}{\gamma_{j+1}^{0}} \right)
\]

where \(N=4\). The step-size \(d_k\) is taken as 8nm (Coy et al., 1999). The choice of values for the transition rates and working stroke parameters (Table 3.1) are chosen to model the data presented by Visscher et al. (1999) and Schnitzer et al. (2000), which were used as a benchmark for our model to follow. Eqs 3.24 and 3.25 show excellent agreement with their measurements of both motor velocity as well as its stalling force (Fig. 3.4).
Table 3.1: Choice of transition rates and working stroke parameters for the Kinesin 4-state model:

<table>
<thead>
<tr>
<th></th>
<th>( \gamma )</th>
<th>( \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_{0,1} )</td>
<td>( 1.7 \cdot 10^6/(M \cdot sec) )</td>
<td>( 0.8 ) nm</td>
</tr>
<tr>
<td>( \gamma_{1,2} )</td>
<td>( 480/sec )</td>
<td>( 0.1 ) nm</td>
</tr>
<tr>
<td>( \gamma_{2,3} )</td>
<td>( 750/sec )</td>
<td>( 0.0 ) nm</td>
</tr>
<tr>
<td>( \gamma_{3,0} )</td>
<td>( 170/sec )</td>
<td>( 0.3 ) nm</td>
</tr>
</tbody>
</table>

\( \gamma_{1,0} = 8.0/sec \)
\( \gamma_{2,1} = 4.0/sec \)
\( \gamma_{3,2} = 3.0 \cdot 10^2/(M \cdot sec) \)
\( \gamma_{0,3} = 8 \cdot 10^3/(M \cdot sec) \)

\( \delta_{1,0} = 2.8 \) nm
\( \delta_{2,1} = 4.0 \) nm
\( \delta_{3,2} = 4.4 \) nm
\( \delta_{3,0} = 1.3 \) nm

Figure 3.4: **Kinesin velocity relative to various ATP-concentrations/load with corresponding stalling forces:** We show the quantitative estimates for the kinesin velocity and stall force plotted against the measurements of Visscher et al. (1999). In (a) the velocity is plotted as a function of [ATP] for three different loads: 1.05 pN, 3.59 pN, and 5.63 pN. In (b) the velocity is plotted as a function of load for 2mM [ATP] (right axis) and 5 \( \mu \)M [ATP] (left axis). (c) shows the corresponding stalling force measurements for a fixed clamp assay and a position clamp assay. The solid lines plotted against each data set represent the results following Eqs. 3.24 and 3.25 using the values listed in Table 3.1 with constant values for ADP and P\textsubscript{i} concentration of 0.01mM and 1.0mM, respectively. The dashed line in (c) corresponds the stall-force–ATP curve with an ADP concentration of 0.1mM, which is shown only to illustrate the sensitivity with respect to [ADP]. The experimental data points (with error bars) were extracted by hand using the GraphClick software package (Boyle et al., 2013).

To model the running lengths of kinesin we implemented the detachment kinetics following the work of Kunwar et al. (2008). In their two-state model, they assume that motor-microtubule detachment occurs at two different conformational states: before ATP-binding (i.e. the free enzyme state (K)) and after binding to ATP (i.e. the enzyme-ATP complex (KS)). When in the K state, detachment occurs due to a lack of ATP
and follows an inverse relation to the substrate concentration: $P_{\text{det}}^{\text{ATP}} = B/\text{[ATP]}$ where B is a fitted parameter. When the motor is in the KS state, detachment is caused from the applied load acting on the motor, which follows an exponential function:

$$P_{\text{det}}^{\text{load}} = \left(\frac{1}{A}\right) \exp\left(\frac{F\delta_{\text{det}}}{k_BT}\right)$$

where A represents the maximum number of catalytic cycles completed during a run and $\delta_{\text{det}}$ is the characteristic distance associated with the load-dependence (Schnitzer et al., 2000). For our model we implement their logic by assuming the total detachment probability for the system to be

$$P_{\text{det}} = P_{\text{det}}^{\text{ATP}} + P_{\text{det}}^{\text{load}} - P_{\text{det}}^{\text{ATP}} \cdot P_{\text{det}}^{\text{load}}$$

which provides us with an expression for total number of steps taken per run, $P_{\text{det}} = \frac{1}{N_{\text{run}}}$. Following Eq. 3.19 we compute the average run-time before a detachment occurs as $\bar{t}_{\text{run}} = 1/(P_{\text{det}} \cdot (\gamma_{\text{ss}} P_{\text{ss}}^{\text{KD}} + \gamma_{0.3} P_{\text{ss}}^{\text{K}}))$, which enables us to compute the average running length: $\bar{x} = \bar{v}_K \cdot \bar{t}_{\text{run}}$.

Figure 3.5: **Kinesin processivity relative to various ATP-concentrations and/or applied loads:** We show the quantitative results for the kinesin run-lengths plotted agains the measurements of Schnitzer et al. (2000). In (a) run-lengths are plotted as a function of [ATP] for three different applied loads of 1.1 pN, 3.6 pN, and 5.6 pN. In (b) the run-lengths are plotted as a function of load for 2mM [ATP], and 5 µM [ATP]. The lines plotted against each data set represent the modeled results using the detachment probabilities following Kunwar et al. (2008) with the detachment parameters taken as: B=0.02 µM, A=120, and $\delta_{\text{det}}$=1.4 nm. The ADP and Pi concentrations are 0.01 mM and 1.0 mM, respectively. See table 3.1 for all other parameter values. The experimental data points (with error bars) were extracted by hand using the GraphClick software package (Boyle et al., 2013).
3.3.4 Summary

Here we investigated the enzymatic cycle of the kinesin motor protein by developing a stochastic model that follows Arrhenius type kinetics, which is in agreement with the power-stroke model of enzyme motor proteins. Our model is based off the frame-work of previous models presented by Fisher and Kolomeisky (2001) and Kunwar et al. (2008). In agreement with Fisher and Kolomeisky (2001), our model requires 4-discrete nucleotide-dependent states to explain the enzymatic cycle of kinesin, which follows the analog of the kinetic scheme outline by Svoboda and Block (1994). The difference between our model and those mentioned above is that our model explicitly defines the specific conformations that kinesin undergoes following ATP hydrolysis. More specifically, we included two enzyme-complex states that represent the motor conformations undergone during the release of both phosphate, as well as ADP. In doing so we were able to accurately model the data of both Visscher et al. (1999) and Schnitzer et al. (2000) with a model that (1) does not include an external process associated with futile hydrolysis (Kunwar et al., 2008), and (2) explicitly models the kinetics governing phosphate binding as well as ADP binding using second order rate coefficients that follow an Arrhenius type mechanism. In doing so, we were able to investigate the dependence between ADP-binding and phosphate-binding with the stalling kinetics of kinesin. In our model, the processes that carry the most load dependence are all related to reverse transitions that favor retrograde movement, where ATP synthesis and phosphate binding carry the most load dependence, which we found to be a characteristic requirement upon choosing these values to fit both Visscher’s, and Schnitzer’s data. The dependence of ADP on stalling kinetics appears to a key parameter as well. While the values chosen for both the concentrations of ADP and phosphate are within reasonable values of those found typically within cells (Howard et al., 2001), a 10-fold increase in [ADP] (while keeping all
other parameters the same) reduced the stalling force of kinesin by as much as 16% over a range of ATP concentrations (see dashed line in Fig. 3.4c).

3.4 The Dynein Model

3.4.1 Motivation

To date, few theoretical models have been developed to study the dynamics of cytoplasmic dynein, which is likely due to its structural complexity and the fact that this motor appears to be much more versatile than other motors. In particular, the distance of the dynein power-stroke following hydrolysis does not appear to be an easily predictable characteristic of its mechanochemical cycle. In 2004, Mallik and his co-workers studied the motions of optically trapped polystyrene beads driven along microtubules by single cytoplasmic dynein motors (Mallik et al., 2004). Through examination of the recordings of the bead motions, they found that cytoplasmic dynein practices a mixture of step-sizes which appeared to have a dependence on the amount of load being applied to the motor. At low loads (< 0.4 pN), dynein takes a majority of larger steps (24-32 nm), which reduces to mixture of mid-sized steps (with an average of 16 nm) at intermediate load (0.4-0.8 pN). At high loads (> 0.8 pN), dynein takes a majority of 8 nm steps before stalling at loads of about 1 pN. Their conclusion was that dynein has the ability to change its stepping behavior in response to load, which implied that its mechanochemical cycle possessed a molecular gear mechanism. Singh et al. (2005) attempted to explain this behavior with a model that assumed that the dynein step-size was regulated by the number of ATP bound to its motor domain. Recall that the dynein motor domain structure is made up of a six AAA+ ring complex that is responsible for both ATP binding and hydrolysis. At the time, it was thought that at least four of the six AAA+ domains had the ability to bind to ATP, for which one of the four domains could catalyze ATP hydrolysis (Sakato and King, 2004).
The stepping characteristics of Singh’s model thus followed under the assumption that the overall ATP binding affinity of its motor domain reduced as the number of bound ATP molecules increased. By further assuming that the binding affinities of the secondary sites (AAA2-AAA4) were all load dependent, the average step-size of dynein varied under different loading constraints. At the time, their approach, which was quite novel, proved very convincing in its ability to explain the data of Mallik et al. (2004) in regards to both stepping and motor stalling. However, only few experimental studies had been done at the time, which provided minimal data to test the model further. Fortunately, within the past decade, dynein has become a topic of key interest because of its role in axonal transport, which has prompted many experimental studies to gain insight on its structure and behavior. While these studies have not verified, nor disproved Singh’s “molecular gear” model, there are many discrepancies between the results of Mallik et al. (2004) and others (Kural et al., 2005; Toba et al., 2006; Reck-Peterson et al., 2006; Walter et al., 2010) which has prompted dynein to be a topic of controversy in terms of both stepping characteristics as well as the motor’s stalling force.

Shortly following the reports of Mallik et al. (2004), Toba et al. (2006) reported values for dynein’s step size and forces produced by single cytoplasmic dynein molecules that were obtained via an optical trap and fluorescence imaging. Their results were inconsistent with those of Mallik, in showing that dynein exhibited a single 8 nm step-size (consistent with the kinesin step) and could generate forces up to 7 pN before stalling (also consistent with kinesin). However, the step size reported by Toba was consistent with the previous reports of Kural et al. (2005) who also reported a consistent 8 nm step size for dynein motors that were observed using fluorescence imaging techniques to analyze organelle movement driven by both kinesin and dynein motors. To add more confusion, Walter et al. (2010) who used a three-bead optical trap assay on mammalian brain dynein, reported that dynein exhibited 8 nm steps under saturating ATP levels and generated
forces of up to $\approx 5$ pN (comparable to the findings of Toba). Furthermore their study also revealed that the dynein stall force reduced to about 1 pN at 50 $\mu$M ATP (comparable to findings of Mallik), but still took 8 nm steps. Most recently, however, dynein has been shown to express a broader range of step sizes comparable to the values reported by Mallik (Belyy et al., 2014; Cleary et al., 2014; Rai et al., 2013), for which among them, Rai et al. (2013) appears to be the most consistent. Both Rai et al. (2013) and Belyy et al. (2014) report that dynein stalls at loads between 1-2 pN, while Rai et al. (2013) and Cleary et al. (2014) also report stepping behaviors similar to those of Mallik et al. (2004).

Assuming that these experiments are equally sound in their methods of both application and data acquisition techniques, one begins to wonder what type of mechanism(s) guide the mechanochemical cycle of cytoplasmic dynein. While Singh et al. (2005) demonstrated that the dynein step-size could follow a gearing mechanism that followed the dependence of the number of ATP molecules bound to its motor domains, it cannot account for the vast differences between the reported stalling kinetics of the motor. Furthermore, in their study, they assumed while all four of the active AAA+ sites (AAA1-4) could bind to ATP, only one site (AAA1) could undergo ATP hydrolysis. This assumption is a reasonable one given the fact that the observed dwell-time distribution of dynein follows a single exponential curve reasonably well (Reck-Peterson et al., 2006). However, as mentioned before, the studies of Kon et al. (2004) and Cho et al. (2008), suggests that each of the active AAA+ sites within the dynein motor domain all contribute to the overall ATPase functionality of the motor and hence possibly contribute to its variability in stepping behavior as well. That is to say, perhaps the dynein step-size is regulated by the overall turnover rates of each of the AAA+ sites, which would depend on each sites’ ATP-binding affinity as well as ATP-hydrolysis. If this is the case, such a scheme would closely resemble the dynein model of Mallik et al. (2004) with an additional assumption that all four of the active AAA+ could undergo ATP-hydrolysis as
well. In a sense, Mallik implemented this into his model by downgrading the overall ATPase rate of site AAA1 as more ATP molecules bound to the AAA+ domain (see Assumption 5 of Singh et al. (2005)). This constraint within their model was required to reduce the excessive number of larger (32 nm) steps at no load for low ATP concentrations. While this assumption is consistent with the reports of Kon et al. (2004), it leads to a predicted average step size of \( \approx 18 \) nm for saturated ATP levels under no load (see Fig. 4b of Mallik et al. (2004)) , which is not consistent with the experimental reports of Mallik et al. (2004) and Rai et al. (2013) that show the dynein step size to be between 24-32 nm under the same conditions. Furthermore, Rai et al. (2013) reports that dyneins express catch-bond kinetics which enables them to tenaciously bond to microtubules for increasing loads, which enables them to withstand forces comparable to those produced by kinesin motors. While we don’t know if such a mechanism exists within the mechanochemical cycle of dynein, it does suggests that perhaps dyneins practice two different flavors of bond kinetics.

### 3.4.2 Model Outline

In an attempt to explain the vast differences reported for the observed behavior of cytoplasmic dynein we introduce a new model to study the mechanism(s) behind dynein motility such that the kinetics governing its mechanochemical cycle fall under two distinct categories, which stand to maximize the motor’s processivity over various ranges of both load and [ATP]. Our hypothesis is that, in general, dyneins move in a manner similar to that of kinesin (i.e. the power-stroke model) but has the ability to increase its microtubule binding affinity when under load, which enhances its ability to process while stalling. More specifically, we propose that dynein employs all four active AAA+ sites in each of its motor heads, in which each site can undergo both ATP binding and ATP hydrolysis independent of one another and that each successive ATP hydrolysis results in a step-size
that is unique to the site undergoing catalysis. In accord with the literature, we assume that one site (AAA1) is the most active and responsible for the most ATPase activity, while at least one of the secondary sites has the ability to increase its ATPase activity in response to applied load.

For simplicity, we model each of the active AAA+ domains (AAA1-4) to have two nucleotide-dependent conformational states (i.e. four independent 2-state systems per motor head) in which each active domain follows the kinetic scheme outlined in Eq. 3.15 (i.e. Michaelis-Menten kinetics). Thus, each AAA+ site has an enzyme-substrate-free state, $D_j$, and an enzyme-substrate complex, $D_j \cdot ATP$ ($DT_j$ for short). The transition rates follow the same generic form outlined in Eq. 3.4 which incorporates the load dependence, for which we assume each AAA+ has unique set of rate coefficients and load parameters.

To take a step, a single ATP molecule undergoes hydrolysis by any of the four AAA+ sites, which translates the motor-domain forward by a step of either 8-, 16-, 24-, or 32-nm, where the step-size is unique to the AAA+ domain that underwent ATP hydrolysis. That is to say, only one AAA+ site can induce a 32 nm step, only one AAA+ site can induce a 24 nm step, and so on...

$$
D_j + [ATP] \xrightleftharpoons[\gamma_{0,1}]{\gamma_{1,0}} [D_j \cdot ATP] \xrightleftharpoons[\gamma_{j,-i}]{\gamma_{j,i}} D_j + [ADP] + [P_i] \quad j \in \{1, 2, 3, 4\} \quad (3.26)
$$
Figure 3.6: **Schematic of the Dynein enzymatic cycle**: Our model represents the kinetic cycle of dynein, which has two unique nucleotide-dependent conformations for each of the four active AAA+ sites within its motor domain. The outline goes as follows in a left-to-right manner: A dynein head that is bound to a nucleotide pocket along a protofilament (orange head) binds—up to four—ATP molecules to its AAA+ domain. Once substrate is bound, the motor domain catalyzes the ATP-hydrolysis process, which induces the primary power stroke that translates the motor head that is not bound to the microtubule (purple head) to a position now ahead of the microtubule bound head. The distance of the powerstroke varies, depending on which AAA+ site underwent ATP hydrolysis and displaces the two motor-domains by a distance of either 8-, 16-, 24-, or 32-nm apart from one another. We assume that the release of the product, ADP·P_i, is instantaneous which follows by a rapid detachment of the, now trailing head, from the microtubule. The cycle then repeats.

### 3.4.3 Implementation and Results

As each AAA+ site is mutually exclusive from the others, they all possess a unique set of steady-state occupation probabilities that all resemble the form of Eq. 3.14,

\[
P_{Dj}^{ss} = \frac{K_{d}^{j}}{1 + K_{d}^{j}} \quad P_{DTj}^{ss} = \frac{1}{1 + K_{d}^{j}}, \quad \text{where} \quad K_{d}^{j} = \gamma_{1,0}^{j+} + \gamma_{1,0}^{j-} \gamma_{0,1}^{j+} + \gamma_{0,1}^{j-}
\]

(3.27)

where \( j = 1, 2, 3, 4 \), and each \( P_{Dj}^{ss} + P_{DTj}^{ss} = 1 \), accordingly.

To compute the average velocities and average running-lengths we make use of the fact that these sites are acting independently from one another. Recall from section 3.2, the relationship between the average run-length and average velocity was given as

\[
\bar{x} = \bar{v} \cdot \bar{t}_{run}
\]

where \( \bar{t}_{run} \) was defined as the average time elapsed during a continuous run before the motor detaches from the microtubule. Under the notion that one detachment occurs per continuous run, we defined a detachment probability that goes as \( P_{det} = \frac{1}{\bar{N}_{tot}} \),

where \( \bar{N}_{tot} \) is taken as the average number of total steps taken per run. For our dynein...
model we implemented a detachment probability that follows the functional form used for our kinesin model. I.e. \( P_{\text{det}} = P_{\text{det}}^{\text{ATP}} + P_{\text{det}}^{\text{load}} - P_{\text{det}}^{\text{ATP}} \cdot P_{\text{det}}^{\text{load}} \), where \( P_{\text{det}}^{\text{ATP}} = B/[ATP] \) and \( P_{\text{det}}^{\text{load}} = (1/A) \exp(F\delta_{\text{det}}/k_BT) \). The parameters A and B where chosen as 70.0 and 0.02 \( \mu \text{M} \), respectively, and \( \delta_{\text{det}} \) was chosen as 4.3 nm. The average run-time is then taken as one over the rate of detachment, \( k_{\text{det}} \), which is the net rate at which steps are taken (both forward and backward), multiplied by a detachment probability. Since each AAA site acts mutually exclusive to the other sites, the net rate of stepping then follows as the combined sum of each AAA site’s rate of stepping. Hence,

\[
t_{\text{run}} = \frac{1}{P_{\text{det}} \cdot \sum_{j=1}^{4} (\gamma_{j,0}^{-1} \cdot P_{DT}^{ss} + \gamma_{j,0}^{+} \cdot P_{DT}^{ss})} 
\]

which can be used to compute the total number of steps displaced during a single run (see Eq. 3.21).

\[
\tilde{N} = \frac{d[P]}{dt} \cdot \tilde{t}_{\text{run}}
\]

\[
= (\gamma_{1,0}^{+} \cdot P_{DT_1}^{ss} - \gamma_{0,1}^{-1} \cdot P_{DT_1}^{ss}) \cdot \tilde{t}_{\text{run}} + (\gamma_{1,0}^{+} \cdot P_{DT_2}^{ss} - \gamma_{0,1}^{-1} \cdot P_{DT_2}^{ss}) \cdot \tilde{t}_{\text{run}}
\]

\[
+ (\gamma_{3,0}^{+} \cdot P_{DT_3}^{ss} - \gamma_{0,1}^{-1} \cdot P_{DT_3}^{ss}) \cdot \tilde{t}_{\text{run}} + (\gamma_{4,0}^{+} \cdot P_{DT_4}^{ss} - \gamma_{0,1}^{-1} \cdot P_{DT_4}^{ss}) \cdot \tilde{t}_{\text{run}}
\]

\[
= (\tilde{N}_{1,1} + \tilde{N}_{2,1} + \tilde{N}_{3,1} + \tilde{N}_{4,1}) - (\tilde{N}_{1,1} + \tilde{N}_{2,1} + \tilde{N}_{3,1} + \tilde{N}_{4,1})
\]

\[
= \tilde{N}_{1,1}^{+} - \tilde{N}_{1,1}^{-} \equiv \text{”Total retrograde steps” - ”Total anterograde steps”}
\]

Since we assign specific step-sizes to each AAA+ site, we can compute the average step-sizes for both retrograde (\( \tilde{d}_{\text{ret}} \)) and anterograde (\( \tilde{d}_{\text{ant}} \)) movement

\[
\tilde{d}_{\text{ret}} = \frac{d_1\tilde{N}_{1,1} + d_2\tilde{N}_{2,1} + d_3\tilde{N}_{3,1} + d_4\tilde{N}_{4,1}}{\tilde{N}_{1,1} + \tilde{N}_{2,1} + \tilde{N}_{3,1} + \tilde{N}_{4,1}}
\]

\[
\tilde{d}_{\text{ant}} = \frac{\tilde{d}_1\tilde{N}_{1,1} + \tilde{d}_2\tilde{N}_{2,1} + \tilde{d}_3\tilde{N}_{3,1} + \tilde{d}_4\tilde{N}_{4,1}}{\tilde{N}_{1,1} + \tilde{N}_{2,1} + \tilde{N}_{3,1} + \tilde{N}_{4,1}}
\]

\[\text{Eq. 3.30}\]
for which then, the total running-length is taken as the effective net displacement that the motor travels during the elapsed time, $\bar{t}_{\text{run}}$.

$$\bar{x} = \bar{d}^{\text{ret}} \cdot \bar{N}^+ - \bar{d}^{\text{ant}} \cdot \bar{N}^-$$

\[
= \frac{\bar{d}^{\text{ret}} \cdot \bar{N}^+ - \bar{d}^{\text{ant}} \cdot \bar{N}^-}{\bar{N}^+ - \bar{N}^-} \cdot (\bar{N}^+ - \bar{N}^-) \tag{3.31}
\]

$$= \bar{d} \cdot \bar{N}$$

and the average motor velocity follows as $\bar{v} = \bar{x}/\bar{t}_{\text{run}}$.

At first glance, the above proposed dynein scheme appears to contradict a key feature of the experimental implications of the dynein enzymatic cycle. Specifically, by modeling each AAA+ site independently, we have permitted the possibility of multiple hydrolysis events to occur at the same time. The measured dwell times (the time lapsed between successive steps) for cytoplasmic dynein have been shown to fit well to a single exponential function (Reck-Peterson et al., 2012), implying that a single hydrolysis rate dictates the overall dynein mechanochemical cycle, which would imply that our hypothesis is incorrect. However, as pointed out by Cho et al. (2008), this could be explained by assuming that a single nucleotide binding site dominates the ATPase functionality of the motor, which suggests that the primary site would have a ATP binding affinity that greatly outweighs the ATP binding affinities of the other three binding sites. In such a scenario, the ATPase activity of the secondary sites would be heavily constrained by their ability to bind ATP, and therefore, regardless of their ability to undergo hydrolysis, would be the rate-limiting step for the enzymatic cycles of the secondary AAA+ sites.

While this suggests that the secondary sites may not play a significant role under saturated levels of [ATP], we cannot discard them entirely as each secondary site has been shown to contribute to dynein’s overall turnover rate (Reck-Peterson et al., 2012). As such, we constrain our model by the first assumption that there exists one AAA+ site which
dominates the other three sites in terms of ATP binding affinity (Mocz et al., 1998) (i.e. 
\[ K_{\text{ATP}}^{\text{AAA1}} = \max\{K_{\text{ATP}}^{\text{AAA1}}, K_{\text{ATP}}^{\text{AAA2}}, K_{\text{ATP}}^{\text{AAA3}}, K_{\text{ATP}}^{\text{AAA4}}\} \] where \( K_{\text{ATP}}^{\text{AAAi}} = \gamma_{0}^{i}/\gamma_{1}^{i}; i = 1, 2, 3, 4 \).

The next constraints are in regards to the ATP catalysis and the stepping behavior of dynein. As mentioned, dynein is quite versatile in its ability to vary its step-size (between 8-32 nm) and there appears to be a controversy as to which step-size is the most preferred. For example, in both of the studies of Mallik et al. (2004) and Rai et al. (2013) dynein is observed to predominately take larger step-sizes of 32 nm and 24 nm, independent of the concentration of ATP, wherein smaller step-sizes become more frequent upon increasing the applied load. However, in the study of Toba et al. (2006), dynein appears to only exhibit 8 nm and 16 nm steps in which the step-size distribution peaks sharply at 8 nm, regardless of both [ATP] and load. In addition to this, these assays observed quite different stalling kinetics as well. Both Mallik et al. (2004) and Rai et al. (2013) observed that dynein stalls at loads of about 1-1.2 pN ([ATP] = 1 mM) while Toba et al. (2006) observed dyneins generating forces up to 7 pN ([ATP]=1 mM) before stalling. In an attempt to explain these differences observed in dynein’s behavior against load, we examined the measurements reported from Rai and Toba, specifically with regard to the force-velocity curves produced from these two studies. In the Rai et al. (2013) study, dynein’s velocity was measured for different applied loads that ranged between 0-1.2 pN while Toba measured dynein’s velocity for different loads between 0-7 pN. However, upon comparing the results of these two experiments, we found that these data do not overlap in terms of the specific loads that were tested against dyneins behavior (compare Fig. 4b of Rai et al. (2013) with Fig. 2c of Toba et al. (2006)). In fact, aside from their measurements for dynein’s behavior at \( F=0 \) pN and \( F \approx 1 \) pN, these two data sets are completely disjoint from one another. More specifically, the only real controversy between the observed behavior of dynein under load arrises for the measured step-size at \( F=0 \) pN (Mallik reported a 24 nm or 32 nm step-size, while Toba reported an 8 nm step-size) and the
measured velocity at $F \approx 1$ pN (Mallik claims dynein approaches its stalling force with $V \to 0$, while Toba claims $V \approx 700$ nm/s). Hence, the combined measurements of the two curves suggest that dynein’s force velocity curve possesses a bi-modal feature wherein the motor reduces its speed as load approaches the stalling force of $\approx 1$ pN, but has the ability to employ a mechanism that allows it to enhance its processivity to go beyond the 1 pN stalling regime. One way to explain this mechanism is through the means of a catch-bond, wherein dynein increases its microtubule binding affinity as load increases (Rai et al., 2013). Furthermore, DeWitt et al. (2014) suggest that the AAA3 site of the motor domain acts as a switch to regulate microtubule release. In their study, they claim that while the AAA3 site hydrolyzes nucleotides an order of magnitude slower than the AAA1 site, it is required for dynein to modulate release from the microtubule, suggesting that AAA3 acts as a modulator for microtubule anchoring. While we don’t know how dynein would incorporate these mechanisms within its mechanochemical cycle directly, or if the AAA3 switching mechanism is connected with its catch-bond behavior, one could pose the hypothesis that a component of the dynein kinetic scheme acts as a switch that enhances its processivity by means of enhancing its binding affinity to microtubules. As such, we further constrain our model by assuming that (1) all secondary sites have catalysis rates considerably less (by 50% or more) than the primary site, and (2) at least one site enhances dynein’s microtubule binding affinity under load. By inspection of Eq. 3.28 we see that in our model the run-time is inversely related to the catalysis rates of each AAA+ site. Hence, we attempt to mimic the dynein catch-bond behavior by invoking that at least one AAA+ site has a catalysis rate that increases as load increases. We then assign the step-size for each site based on the stepping characteristics reported by Rai et al. (2013) and Mallik et al. (2004) who both observed a decrease in dynein’s step size with an increase in applied load. In our model, the most active site (i.e. the analog of AAA1) practices 32 nm steps and is the most sensitive to load. The second- and third-most active
sites practice 24 nm and 16 nm steps, respectively. Both sites have slower catalysis rates than the most active site, and are less sensitive to load than the most active site. The last site, which practices 8 nm steps is responsible for the catch-bond kinetics. It has the slowest catalysis rate under zero-load (making 8 nm steps infrequent, however possible). As load increases, its catalysis rate begins to dominate relative to the other three sites, making 8 nm steps more frequent.

Table 3.2: Choice of transition rates and working stroke parameters for each of the AAA+ sites of the dynein model:

<table>
<thead>
<tr>
<th></th>
<th>site 1</th>
<th>site 2</th>
<th>site 3</th>
<th>site 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma_{0,1}^+ (M^{-1} sec^{-1})$</td>
<td>$8 \cdot 10^6$</td>
<td>$1.33 \cdot 10^5$</td>
<td>$2 \cdot 10^5$</td>
<td>$4 \cdot 10^5$</td>
</tr>
<tr>
<td>$\delta_{0,1}^+ (nm)$</td>
<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
<td>8.0</td>
</tr>
<tr>
<td>$\gamma_{1,0}^- (sec^{-1})$</td>
<td>50.0</td>
<td>150.0</td>
<td>175.0</td>
<td>200.0</td>
</tr>
<tr>
<td>$\delta_{1,0}^- (nm)$</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>$\gamma_{1,0}^+ (sec^{-1})$</td>
<td>30.0</td>
<td>15.0</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>$\delta_{1,0}^+ (nm)$</td>
<td>13.0</td>
<td>10.0</td>
<td>1.0</td>
<td>5.5</td>
</tr>
<tr>
<td>$\gamma_{0,1}^- (sec^{-1})$</td>
<td>0.0</td>
<td>5.5</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>$\delta_{0,1}^- (nm)$</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>step size (nm)</td>
<td>32</td>
<td>24</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

Part of the results of Rai et al. (2013) displayed the stepping of a motor driven latex bead phagosome (LBP) within a cell against load during dynein/kinesin driven motion, wherein dynein moved retrogradely and kinesin moved anterogradely. While kinesin motors moved with a dominate 8 nm step, dynein appeared to acclimate its step-size in accord with the applied load. In the same study, they also showed that the dynein force-velocity curve exhibits a concave up characteristic that supports the observation of its step-size reducing to 8 nm (on average) upon reaching its stalling force. Their
recordings are in agreement with Mallik’s results, which was the first study to propose a *molecular gear* mechanism (Mallik et al., 2004) that suggested that dynein has the ability to acclimate its stepping behavior in response to load. While it is not fair to compare Rai’s stepping recording with those of Mallik due the substantial differences between these two assays, the fact that our model can replicate both sets of data quite well is remarkable. For instance, in Fig. 3.7 we show the recorded dynein steps of Mallik against the average step-size ($\bar{d}$, see Eq. 3.31) predicted by our model as a function of applied load for ATP concentrations comparable to those within cells (1-2 mM). In our model, the effective step size reduces in response to the way each AAA+ site is modeled, wherein under zero-load the sites responsible for smaller steps (8-16 nm) are highly inactive relative to the other two sites. As load increases, the sites responsible for larger steps (24-32 nm) become less active relative to the other two sites, especially in comparison to the site responsible for 8 nm steps, which upon reaching loads $\approx 1$ pN is highly active due to its catch-bond-like behavior.
Figure 3.7: **Dynein stepping behavior under various applied loads and [ATP]:** Panels (a) and (b) compare the stepping characteristics of the model (solid/dashed lines (a) and grey bins (b)) to the measurements of Rai et al. (2013) (blue circles (a)) and Mallik et al. (2004) (blue bins (b)). Rai’s measurements were taken for dynein movement within cells under various conditions of applied load, while Mallik’s measurements correspond to dynein steps at low ATP concentration (2 µM) and under no load. Data points were extracted by hand using the GraphClick software package (Boyle et al., 2013). In panels (c) and (d) we show the predicted step-size distributions under various loads between 0.0-1.0 pN for [ATP] = 2 mM (c), and [ATP]=500 µM (d). The modeled data results corresponds to those using the parameters listed in table 3.2.

One of the key implications of the noted studies of the dynein stepping behavior is the notion that, while step-size is highly sensitive to load, it is seemingly insensitive to ATP concentration (compare Figs 3.7a and 3.7b). While the model agrees with such implications (compare figs 3.7c and 3.7d for a given fixed load), this does not mean that the activity of the secondary sites goes unnoticed in regards to their contribution to motor movement. In Ross’ in vitro study of the dynein-dynactin complex (Ross et al., 2006) they
observed, using a TIRF assay, bidirectional motions of dynein molecules that were subject to minimal load conditions for which they recorded the relative speeds of these motors as a function of ATP concentration. Part of their analysis suggests that the ATP-dependence on dynein velocity can be explained if one includes at least two sets of Michaelis-Menten constants in the dynein velocity-ATP function. In agreement with their findings we observe similar biphasic features of the dynein velocity-ATP function under minimal load constraints, which are a direct result of the fact that we constrained the ATP-binding affinities of the secondary sites to be in reasonable accord with the findings of Mocz et al. (1998), which consequently, also gives rise to the proper force-velocity characteristics measured by Mallik (see Fig. 3.8). As load increases, the stepping frequency of the larger steps (24-32 nm) drops rapidly once load is applied causing a huge plummet in motor speed. The trend continues as the mid-sized steps (16-24 nm) become less frequent as load continues to grow, until 8 nm steps dominate as a result of the catch-bond kinetics, leading to a concave-up force-velocity curve.
3.4.4 The Catch-Bond Mechanism

One of the most interesting results of this model lies in the relation between the catch-bond kinetics and the motor stalling force. Recall, the dynein stalling force is one of the characteristics that raises the most questions in comparing the experimental findings within the literature. We already discussed the controversy between the results of Mallik et al. (2004) and Toba et al. (2006), which suggests that the dynein stall force is either $\approx 1$ pN or $\approx 7$ pN. Furthermore, we also noted the study of Walter et al. (2010) who found that dynein exhibited 8 nm steps under saturating ATP levels and generated forces of up to $\approx 5$ pN, which is comparable to the stall force reported by Toba. Walter’s study also revealed that the motor’s stall force reduced to about 1 pN at 50 $\mu$M ATP, yet still displayed 8 nm
steps. Collectively, these results are difficult to interpret in terms of the mechanism that governs dynein’s stalling kinetics. In our model, however, we find a possible way to explain these differences by examining the behavior of the model both with and without the catch-bond kinetics. We find, that upon deactivating the catch-bond mechanism within the model, that dynein stalls at loads $\approx 1$ pN for $[\text{ATP}] \leq 4$ mM, which is comparable to the measurements of Mallik et al. (2004). However, when the catch-bond is active, dynein’s stall force increases by as much as 5-fold for saturated levels of [ATP]. (see Fig. 3.9).

![Figure 3.9](image_url)

**Figure 3.9: Emphasizing the effects of applying the catch-bond kinetics for dynein:** We show the velocity curves for 1 mM [ATP], both with (dashed line) and without (solid line) the catch-bond enabled (a). (b) shows the corresponding stall-force of dynein as a function of ATP concentration for each case. For ATP concentrations below $\approx 1$ mM, the motor stalls at forces below 1-1.2 pN regardless of whether or not the catch bond is ”active” (by active we mean that the catalysis rate for site 4 is load dependent–see table 3.2). When the catch bond is inactive, dynein stalls at loads $\leq 1$ pN, and decreases for decreasing ATP concentrations. When the catch bond is active, the stall force also decreases with decreasing ATP concentrations, however, at about $\approx 900$ mM [ATP], the effects of the active catch-bond become very apparent as the stall force jumps from about 1 pN to 4 pN (indicated by black arrow in (b)). For 1 mM ATP the catch-bond induces a 4-fold increase in the dynein stall force (compare the difference in applied load between the red and blue circles for each plot), giving rise to a possible explanation for the vast differences seen experimentally for dynein.
That is two say, if dynein employs a mechanism that would allow it to enhance its processivity by means of a catch-bond, wherein upon activating said mechanism the motor predominately takes 8 nm steps, then we may have a means to justify why dynein appears to be so versatile in both its stepping behavior, as well as its stalling force.

### 3.4.5 Summary

Cytoplasmic dynein is a molecular motor that drives retrograde axonal transport within neurons. Similar to other molecular motors, such as kinesin and myosin, dynein kinetics follow a mechanochemical cycle that promotes motor movement occurring as a result of ATP catalysis. Here we studied the relationship between dynein’s stepping behavior and its ability to generate force by developing a discrete state stochastic model that follows Michaelis-Menten kinetics. Our model is similar to the one developed by Singh et al. (2005), who hypothesized that this motor’s stepping characteristics depended on the number of ATP molecules bound to its motor domain. While their model can explain the distribution of steps and stalling kinetics reported by Mallik et al. (2004) reasonably well, their model lacks some of the characteristics that have been reported in other experiments (Kural et al., 2005; Toba et al., 2006; Reck-Peterson et al., 2006; Ross et al., 2006; Walter et al., 2010). For example, the velocity-ATP curve reported by Ross et al. (2006) shows a biphasic behavior for increasing ATP (see Fig. 3.8), which suggests that dynein kinetics do not follow from a single hydrolysis process. While Mallik et al. (2004) do introduce an adjustment to the hydrolysis kinetics with respect to the number ATP molecules bound, their predicted velocity-ATP curves do not possess the biphasic features of Ross’ data. Another question arises due to the vast difference in reports regarding the distribution of steps for dynein and the motor’s stalling kinetics. Clearly, Mallik et al. (2004) suggests that dynein’s stepping behavior follows from some type of
"molecular gear" mechanism, but it is not clear if this mechanism operates solely on the number ATP molecules bound, as modeled by Singh et al. (2005).

Our model assumes that each of the active AAA+ sites (AAA1-4) of its motor domain act independently of one another and all have the ability to bind ATP, as well as undergo catalysis. The kinetics of each site follow from a simple 2-state Michaelis-Menten kinetic scheme and each site exhibits a unique step-size following ATP hydrolysis. We modeled the differences in the ATP binding affinities and catalysis rates for these sites in accordance with the literature (Mocz et al., 1998; Cho et al., 2008; DeWitt et al., 2014) and assumed that, within the dynein motor domain, one of AAA+ sites (AAA1) is responsible for the majority ATPase activity. The kinetics for each site are all load dependent such that the overall ATPase activity of the motor reduces under increasing load. However, the site responsible for 8 nm steps is modeled with a different flavor of kinetics to mimic the "catch-bond" behavior of dynein reported by Rai et al. (2013). To mimic the catch-bond we assumed that this site’s catalysis rate increased with increasing load, while its ATP binding rate decreased with increasing load. In doing so, we were able to explain the stepping characteristics reported by both Mallik et al. (2004) and Rai et al. (2013), as well as the force-velocity curve that was also reported by Rai et al. (2013). While our model cannot, for a single choice of parameters, explain the combined data of the force-velocity curves of both Rai et al. (2013) and Toba et al. (2006), it can explain the differences observed between dyneins stepping behavior and stalling kinetics. In particular, the mimicked catch-bond kinetics ensure a dominating 8 nm step-size for high loads at saturating ATP, and allows dynein to generate forces comparable to those reported by Toba et al. (2006) and Walter et al. (2010). Furthermore, by assuming the four sites within the motor domain act independently, dynein’s velocity follows as a function composed of four unique Michaelis-Menten constants, which proved
to be a characteristic necessary for our model to best explain the velocity-ATP data reported by Ross et al. (2006).

### 3.5 Monte-Carlo Simulations

One way to test our theory for the enzymatic cycles of both our kinesin and dynein models is to implement Monte-Carlo experiments to verify the validity of our solutions for each motor’s running-velocity and processivity. In each model we quantified the occupation probabilities for each of the conformational states that make up the enzymatic scheme. These occupation probabilities represent the discrete probability distribution that includes all conformations possible during movement and are all functions of the corresponding rate coefficients. In the Monte-Carlo experiment, we test the validity of our solutions by doing a random sampling of all possible events that occur within the enzyme cycle. For a given event “E_{i,j}”, in which the enzyme transitions from state ”i” to state ”j”, the conditional probability of that event occurring for a given time ”P_{E_{i,j}}” (where 0 \leq P_{E_{i,j}} \leq 1) is defined as $\gamma_{i,j} \cdot dt$. In practice, for a given time-step of order $dt$, a random number $\eta$ is sampled from a uniform distribution. If $\eta \leq P_{E_{i,j}}$, then the event occurs; if $\eta > P_{E_{i,j}}$, then the event does not occur. As an example, assume an enzyme is in state ”i”. Following our notation outlined in Sec. 3.2, the enzyme has four possible options, it can transition to state ”i+1”, transition to state ”i-1”, remain in state ”i”, or detach from the microtubule. Using our Monte-Carlo framework, just explained, the conditional probabilities for each event (or transition) to occur are: $P_{E_{i,i+1}} = \gamma_{i,i+1} \cdot dt$, $P_{E_{i,i-1}} = \gamma_{i,i-1} \cdot dt$, and $P_{det} = dt/\bar{t}_{run}$. We then map these probabilities to a unit interval of which then a uniformly distributed random number $\eta$ is taken to choose the subsequent state of the motor protein. To ensure appropriate sampling we choose the time step to be smaller than the inverse of the fastest rate constant. For both the kinesin and dynein models, the fastest
events follow from ATP binding, which for saturated ATP levels is of the order $10^{-4}$. Hence we take $dt=10^{-5}$ sec (Kunwar et al., 2008).

Figure 3.10: **Implementation of Monte-Carlo modeling:** In (a) we illustrate the method used for random sampling of events. We draw a number from a uniform distribution of random numbers. We then see where that random number lies on a unit interval that has the conditional probabilities of the possible events that can occur at that time. For example the circled state in (b) represents the conformation in which kinesin is bound to ATP, which is defined by the state variable, $s=1$. In this state the motor head can detach, or unbind from ATP ($s=1 \rightarrow 0$), or undergo ATP hydrolysis ($s=1 \rightarrow 2$), or remain in its current state ($s=1$). During our simulations, the event that occurs corresponds to where the random number lies within unit interval comprised of the conditional probabilities. The lower panels illustrate the conformations of the motors during simulation for kinesin (b) and dynein (c), where each conformation is defined by a state variable $s_i$. For simplicity, we only illustrate one state variable in (c) as in a generic form $s_i$, where in reality, the dynein model implements four state variables, one for each AAA+ site of the motor domain.

During our simulations, we monitor which state the enzyme is in by defining a state variable "$s$", which changes over time as events occur. In our kinesin model, $s$ has four
possible values: 0, 1, 2, and 3. The $s=0$ state represents the enzyme-free state (K); the $s=1$ state represents the enzyme-substrate complex (KS); the $s=2$ state represents the first enzyme complex following hydrolysis (KDP); and the $s=3$ state represents the second complex state following phosphate release (KD) (see Fig. 3.10). For the dynein model, the methodology is the exact same, however each AAA+ has its own state variable, $s_1, s_2, s_3, s_4$, where each $s_i = 0$ (the free enzyme state; $D_i$) or 1 (the dynein-ATP complex; $DT_i$). Each simulation starts with $t=0$, $s=0$, and $x=0$. For each time-step, we check the state that $s$ is in. Given the current value of the state variable, the appropriate conditional transition probabilities are computed and distributed across a unit interval as described above. We then randomly select a number, $\eta$, from a uniform random number distribution and see where it falls within the interval and update the state variable accordingly, in which the cycle is then repeated. For example, for some time "$t$" within a simulation, if kinesin is in the $s=1$ state, the transition options are (1) ATP-unbinding, (2) ATP-catalysis, or (3) microtubule detachment. If ATP-unbinding occurs, then $s=1 \rightarrow s=0$. If catalysis occurs then $s=1 \rightarrow s=2$. If microtubule detachment occurs, the run is terminated. If nothing happens then the state variable remains the same. In the event that a detachment occurs, the simulated run is terminated and we compute the corresponding run-length and run-velocity for that simulation. The run-lengths for a given run correspond to the number of completed forward/backward steps that occur during a simulation. In accord with our theory, stepping events can occur when the enzyme is in either the first or last state of an enzyme cycle (see Fig. 3.10). For kinesin, forward steps occur when $s=3$ and ADP-unbinding occurs while reverse steps occur if $s=0$ and ADP binding occurs. For dynein, forward steps occur when any of the four AAA+ sites are in the $s_i = 1$ state, and catalysis occurs, while reverse steps occur when $s_i = 0$ followed by ATP synthesis. (See table 3.2 for a list showing which AAA+ is responsible for a given step-size.)
3.5.1 The Kinesin Monte-Carlo Algorithm

Using the state variable "s" as described above, we start a run with the initial condition that the leading motor head is in the s=0 state—the free enzyme state (K), and $t=0$. We define the conditional probabilities for each time step as $P_{s}^{f}, P_{s}^{r}, P_{s}^{det}$ (see table 3.3) where the superscripts indicate the directionality of the event with respect to the enzymatic scheme ("f" for "forward transition event"; "r" for "reverse transition event", "det" for a "detachment event"). The probabilities are then distributed across the unit interval for random sampling. For each time-step we invoke the following algorithm (see below) to update the state of the enzyme as well as its position (where $d_{step} = 8$nm) (see Kunwar et al. (2008) for a very similar approach). A TRUE value indicates the event occurred (e.g. If $P_{s}^{f}=$TRUE, then the event occurred), while a FALSE value indicates the event did not occur. The results of the Monte-Carlo simulations (averaged over 5000 simulations) are plotted against the theoretical values in Fig. 3.12

Figure 3.11: Motor trajectories generated from Monte-Carlo simulation: We show four examples of simulated runs for a single kinesin motor. Each trajectory corresponds to a simulation where the motor experiences a constant amount of applied load acting on it (see labels above/below each trajectory). In panel (a) we show the trajectories simulated for a constant 2 mM ATP concentration. In (b) we show the trajectories simulated for a constant 5 $\mu$M ATP concentration.
Table 3.3: Conditional probabilities for the Monte Carlo simulations of the kinesin model

<table>
<thead>
<tr>
<th>$s$</th>
<th>$P_f^s$</th>
<th>$P_r^s$</th>
<th>$P_{det}^s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$\gamma_{0,1} \cdot [ATP] \cdot dt$</td>
<td>$\gamma_{0,3} \cdot [ADP] \cdot dt$</td>
<td>$dt/\bar{t}_{run}$</td>
</tr>
<tr>
<td>1</td>
<td>$\gamma_{1,1} \cdot dt$</td>
<td>$\gamma_{1,0} \cdot dt$</td>
<td>$dt/\bar{t}_{run}$</td>
</tr>
<tr>
<td>2</td>
<td>$\gamma_{2,2} \cdot dt$</td>
<td>$\gamma_{2,1} \cdot dt$</td>
<td>$dt/\bar{t}_{run}$</td>
</tr>
<tr>
<td>3</td>
<td>$\gamma_{3,3} \cdot dt$</td>
<td>$\gamma_{3,2} \cdot [P_i] dt$</td>
<td>$dt/\bar{t}_{run}$</td>
</tr>
</tbody>
</table>

*Monte-Carlo Algorithm–Kinesin*

1. Initialize the simulation with $t=0$, $s=0$, $x=0$.

2. For each time-step, draw a uniform random number on the unit interval and check if an event occurs.

   If $P_f^s = \text{TRUE}$, then $s \rightarrow s + 1$
   
   if $s = 3 \rightarrow s = 0$, then update the position with $x \rightarrow x + d_{\text{step}}$

   else-if $P_r^s = \text{TRUE}$, then $s \rightarrow s - 1$

   if $s = 0 \rightarrow s = 3$, then update the position with $x \rightarrow x - d_{\text{step}}$

   else-if $P_{det}^s = \text{TRUE}$, then terminate simulation.

   The run-length is taken as $x_{\text{run}} = x$, and the run-time is taken as $t_{\text{run}} = t$.

   The run-velocity is taken as $x_{\text{run}}/t_{\text{run}}$.

end-if
Figure 3.12: **Comparing the kinesin model to Monte Carlo simulations:** We show the simulation results of our Monte Carlo experiment against the theoretical values for the kinesin processivity and velocity under various loads and ATP concentrations. The lines in each plot correspond to the theory, which are overlayed against the corresponding simulated results (symbols). Note that the lines correspond to the same data plotted against the experiment data points of Figs. 3.4 and 3.5.

### 3.5.2 The Dynein Monte-Carlo Algorithm

The procedure for the dynein simulations is identical to that of the kinesin simulations, with the exceptions that for each time step, there are four active sites that can undergo ATP-binding/unbinding or ATP-hydrolysis/synthesis. There are four state variable, $s_j$ (j=1,2,3, or 4), which uniquely represent the current state of the $j^{th}$ AAA+ site. We define the specific conditional probabilities for each time step as $P^f_{s_j}$, $P^r_{s_j}$, and $P^{det}_{s_j}$ (see table 3.4), where the step sizes for each binding site are taken in agreement with our theory, and the run times for each site follow from Eq. 3.20:
\[ \bar{t}_i = \left( P_{det}(\gamma_{1,0}^{i,0} P_{ss}^{DT_i} + \gamma_{1,0}^{i,1} P_{ss}^{DT_i}) \right)^{-1}. \]

The results of the Monte Carlo simulations (averaged over 5000 simulations) are plotted against the theoretical values in Fig. 3.14

Figure 3.13: **Motor trajectories generated from Monte Carlo simulation:** We show eight examples of simulated runs for dynein under different conditions of constant applied load at a constant 2 mM ATP concentration. In (a) we see in a reduction in the overall distance traveled, and hence, overall speed as the applied load approaches the 1 pN stalling threshold reported by Rai et al. (2013). In (b) we show what happens as a result of the mimicked catch-bond mechanism. Dynein’s processivity increases as load increases up to about 4 pN. The eventual failure of the catch-bond can be seen in the simulated run having a constant applied load of 6 pN (lower trajectory of panel b), which is comparable to stalling forces reported by Toba et al. (2006).

Table 3.4: Conditional probabilities for the Monte Carlo simulations of the dynein model

<table>
<thead>
<tr>
<th></th>
<th>site 1</th>
<th>site 2</th>
<th>site 3</th>
<th>site 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>( s_i = 0 )</td>
<td>( \gamma_{0,1}^{i,0} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,0} \cdot dt )</td>
<td>( \gamma_{0,1}^{i,1} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,1} \cdot dt )</td>
</tr>
<tr>
<td>( s_i = 1 )</td>
<td>( \gamma_{0,1}^{i,0} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,0} \cdot dt )</td>
<td>( \gamma_{0,1}^{i,1} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,1} \cdot dt )</td>
</tr>
<tr>
<td>( s_i = 0 )</td>
<td>( \gamma_{0,1}^{i,0} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,0} \cdot dt )</td>
<td>( \gamma_{0,1}^{i,1} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,1} \cdot dt )</td>
</tr>
<tr>
<td>( s_i = 1 )</td>
<td>( \gamma_{0,1}^{i,0} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,0} \cdot dt )</td>
<td>( \gamma_{0,1}^{i,1} \cdot \text{[ATP]} \cdot dt )</td>
<td>( \gamma_{1,0}^{i,1} \cdot dt )</td>
</tr>
</tbody>
</table>

Note: The leftmost superscript values of the rate coefficients indicate the site number. (See table 3.2 for the actual parameter values used during simulation)
Monte-Carlo Algorithm–Dynein

(1) Initialize the simulation with $t=0$, $x=0$, and $s_1 = s_2 = s_3 = s_4 = 0$.

(2) For each time-step, draw four random numbers and check which events occur.

If $j = 1$ (i.e. Site 1, $d_{step} = 32$ nm)

- If $P_{s_1}^f = \text{TRUE}$, then $s_1 \rightarrow s_1 + 1$
  - if $s_1 = 1 \rightarrow s_1 = 0$ then update the position with $x \rightarrow x + d_{step}$
- else-if $P_{s_1}^r = \text{TRUE}$, then $s_1 \rightarrow s_1 - 1$
  - if $s_1 = 0 \rightarrow s_1 = 1$, then update the position with $x \rightarrow x - d_{step}$
- else-if $P_{s_1}^{det} = \text{TRUE}$, then terminate simulation.

  The run-length is taken as $x_{run} = x$, and the run-time is taken as $t_{run} = t$.

  The run-velocity is taken as $x_{run}/t_{run}$.

end-if

else-if $j = 2$ (i.e. Site 2, $d_{step} = 24$ nm)

...same logic as for $j = 1$, with the appropriate probabilities.

else-if $j = 3$ (i.e. Site 3, $d_{step} = 16$ nm)

...same logic as for $j = 1$, with the appropriate probabilities.

else-if $j = 4$ (i.e. Site 4, $d_{step} = 8$ nm)

...same logic as for $j = 1$, with the appropriate probabilities.

endif
3.5.3 Simulating an Optical Trap

One characteristic of motor proteins that is not evident within the current framework of our models is how they behave upon undergoing a stall. Rai et al. (2013) and Schroeder et al. (2012) found that motors bound within an optical trap assay tend to act quite tenacious upon reaching their stalling limit. In other words, they can withstand substantial load for extended periods of time before they detach from the microtuble track. Rai found that the stall time of a single kinesin motor is generally longer than the stall time of a single dynein motor: $T_{\text{kinesin \, stall}}^{\text{stall}} \approx 1 \text{sec} \, \text{vs} \, T_{\text{dynein \, stall}}^{\text{stall}} \approx 0.2 \text{sec}$ (Rai et al., 2013). Schroeder on the
other hand (who only tested kinesin and kinesin-2 motors) observed stalling times that are more compatible to those reported by Rai for dynein.

To further test our model, we simulate an optical trap assay following from the methods of Kunwar et al. (2008) who investigated the behaviors of multiple kinesin motors moving spherical-bead-cargoes in the presence of thermal noise and viscosity. In their model, each motor acts independently of all the others and experiences a load unique to its contribution of action being applied to the trapped bead. As the bead gets transported, the tension on the cargo increases in a linear fashion, which is proportional to the trap stiffness, $k_{\text{trap}}$. The motors then counter this load by generating a force which is proportional to the extension length of their stalk-linkage, which we model as springs. Eventually, as with a typical optical trap assay, the motors stall due to the magnitude of the load acting on them, which is followed by a detachment event. For our simulation, we follow this framework with the same assumptions as Kunwar. In particular, we treat the springs of the motors as ”special springs” that only ”exert a restoring force when their linkage is stretched”. Bead movement follows from external loads as well as thermal noise. The brownian motions are modeled by drawing from a normal distribution with mean square displacement, $\text{MSD} = 2D\Delta t$, where the diffusion coefficient is related to the friction constant associated with viscous drag acting on the bead when experiencing an external load, $\zeta = k_B T / D$, where $\zeta = 3\pi \eta_d$. Hence, for each time-step the position of the bead is $\mathbf{x}(t + \Delta t) = \mathbf{x}(t) + \mathbf{r}_{\text{random}} + \mathbf{F}_{\text{net}} \cdot \Delta t / \zeta$. (See supplement of Kunwar et al. (2008).) The external load acting on the bead is generated when a single motor (kinesin or dynein), pulls the cargo in the direction opposing the optical trap, where the movement of the enzymes follow from the Monte Carlo simulations explained above.
Figure 3.15: **Sample runs of single kinesins and single dyneins in a simulated optical trap.** We show the force-time plots for optical trap simulations of kinesin or dynein pulling a spherical bead within a 5 centipoise solution (Haak et al., 1976) (to mimic that of the axoplasm). In panels (a) and (b) we show multiple simulations stacked sequentially alongside one another for kinesin (a) and dynein (b) spanning a total time of 10 seconds. In panels (c) and (d) we show a snapshot spanning 1 second to exemplify the kinetic features of these motors. Kinesin shows a very ordinary behavior as it approaches stall in a very robust manner. Once the motor stalls at \( \approx 7 \) pN (or less) it remains stalled for an average time of 0.98 seconds. Dynein (c) however, shows a much more diverse type of behavior due to its catch-bond mechanism. Stall forces range between 1-4 pN and last an average time of 0.18 seconds. The average stall-time values for both kinesin and dynein models are consistent with those of Rai et al. (2013), which were obtained by averaging the time lapsed once the motor feels a load greater than or equal to its half-maximum stall force (red arrows in (c) and (d)). Our statistics follow from averaging over 1000 trap simulations where the trap stiffness was taken as 0.05 pN/nm (Schroeder et al., 2012; Rai et al., 2013).

To test our model, we estimate the stall-times (or plateau time) by measuring the time-lapsed during which the motor applies a force greater than the half-maximal force it can exert before reaching stall (consistent with Rai et al. (2013)). At saturated levels of ATP, our model’s half-maximal stall force is about 4 pN for kinesin and about 2 pN for
dynein. We note that the half-maximal force of our dynein model is about 5-fold larger than that measured by Rai, which we attribute to the catch-bond mechanism embedded within our model. However, as seen in Fig. 3.14, dynein approaches stall about 1 pN before its catch-bond becomes evident. As such, we artificially set the half-maximal value of dynein to be 0.5 pN, which is justified when inspecting Fig. 3.15(c). By averaging over 1000 optical trap runs, we find that the average stall-times for kinesin and dynein (0.98 sec and 0.18 sec, respectively) are consistent with those measured in Rai’s study.

3.6 Discussion

The study of motor proteins that work in concert with filamentous proteins, such as microtubules, has been a topic of discussion and research for the past 30 years. The existence of dynein has been known for over 50 years, however its role as a microtubule ATPase motor remained unclear until the 1980’s (Paschal et al., 1987; Lye et al., 1987), which became evident shortly after the discovery of its counter motor, kinesin (Brady, 1985; Vale et al., 1985). These milestones led to the development of assays that enabled single-molecule studies of motor-microtubule interactions to be conducted (Howard et al., 1989), which made it possible to identify the step-sizes of these motors as well as give insight on the processes underlying mechanochemical transduction.

Here, we developed a framework to model the enzymatic cycles of kinesin and dynein that is based on the power-stoke hypothesis (Howard, 2011), which assumes motor motility follows from a series of conformational changes occurring in tandem with a series of chemical events underlying the catalysis of ATP. While this approach has been proven to model the dynamics of kinesin quite well (Fisher and Kolomeisky, 2001; Schnitzer et al., 2000; Kunwar et al., 2008), few models have been developed to study the dynamic nature of cytoplasmic dynein. In the model presented by (Singh et al., 2005), it was posed that the dynein stepping mechanism followed directly from its ATP binding affinity, in
which the step size of dynein was dictated by the number ATP molecules bound to its motor domain. While their model proved effective then, recent experimental studies have suggested that their hypothesis cannot entirely explain the dynein mechanochemical cycle–specifically in regards to its stepping nature and stalling kinetics.

In our present study, we modeled the dynamics of both kinesin and dynein in a Markovian fashion, wherein motor motility occurs upon successfully completing a sequence of nucleotide-dependent conformational changes that follow the analog of a reaction kinetic scheme. We confirmed that the kinesin cycle follows closely in accord with the enzymatic scheme posed by Svoboda and Block (1994), which was previously examined using a theoretical framework by Fisher and Kolomeisky (2001). Our model, which closely resembles Fisher’s model, accounts for the experimental recordings of kinesin’s velocity, running-length, and stalling force (Visscher et al., 1999; Schnitzer et al., 2000).

To model cytoplasmic dynein, we followed the work of (Singh et al., 2005). The key difference between our model and their model is that, while ATP-binding affinity does contribute to the motor’s stepping behavior, we assumed that each active AAA+ site within the dynein motor domain acts independently of the other sites and that the step-size of dynein follows from the specific ATPase activity of each site. To account for the stepping behavior reported by Rai et al. (2013) and Mallik et al. (2004), we constrained the model in a fashion similar to that of Singh et al. (2005). The primary AAA+ site has the highest ATP binding affinity (Mocz et al., 1998) and the highest catalysis rate (DeWitt et al., 2014). The step-size for each AAA+ site is then assigned in terms of hydrolysis activity. Site 1 practices the largest (32 nm) step-size, while sites 2, 3, and 4 practice smaller steps (see Table 3.2), wherein the most active sites experience the effects of load more than the others–save one–which is the catch-bond site (see Sec. 3.4: The dynein model). Collectively, these constraints/assumptions, allowed us to explain many of the
features reported on dynein’s motility characteristics, specifically in regards to its stepping behavior and velocity as functions of both load and ATP (see Figs 3.7, and 3.8) as well as its stalling behavior(s) (see Fig. 3.15). Furthermore, by mimicking the catch-bond like mechanism, we find a way to possibly explain why dynein is so versatile in its ability to generate forces.

Finally, to verify our solutions to the kinetic cycles of these enzymes, we developed two separate Monte-Carlo experiments that simulated enzyme movement in real-time. In these experiments, the motor’s transitioned between different nucleotide-dependent conformations by simulating a random sampling of the events that outlined each motor’s mechanochemical cycle. Motor movement then followed from the completion of successive transitions that permit ATP-hydrolysis (and product release), which is the mechanism that allows these motors to take steps along microtubules. For each simulated run, a single motor (kinesin or dynein) walked for a given duration before it detached from the microtubule track, wherein the detachment kinetics were modeled in accordance with Kunwar’s model for kinesin (Kunwar et al., 2008). In the event that detachment occurred, we terminated the simulation and recorded the distance that the motor traveled (the run-length), which was then used to calculate the average speed of the motor. By fixing the applied load acting on the motor during a simulated run, we were able to take an ensemble average of each motor’s running-length and velocity for a fixed concentration of ATP. Upon taking an ensemble average over 5000 simulations, for a given fixed load and fixed [ATP], we were able to verify our solutions for each motor’s processivity, velocity, stalling kinetics, as well stepping behavior (specifically for dynein). We then used these Monte-Carlo models to simulate the cargo movement of optically trapped beads in the presence of thermal noise and viscosity, which allowed us to verify that the average stalling times for both of our motor models (kinesin and cytoplasmic dynein) were in agreement with those reported by Rai et al. (2013).
4 Computational Modeling of Neurofilament Transport

4.1 Cargo Movement Within Cells.

Cellular cargo transport is mediated by the locomotion of force-generating molecules (motor proteins) such as kinesin and cytoplasmic dynein. Transport occurs when these ATPase motors form a junction between the cellular cargo and cytoskeletal microtubule filaments that serve as the highways for these molecules. However, the specific mechanism behind axonal transport is yet to be determined as the observed motions of biological cargoes does not appear to follow a single, specific, mechanism when observed both in vivo and in vitro. In some assays, cargo movement is uni-directional and very robust, while in others, cargo trajectories rapidly change direction and act in a very stochastic fashion. Furthermore, the dynamic characteristics of different cargoes, in regards to speed and processivity are quite different as some cargoes move in a very regular fashion, while other cargoes move in a very irregular fashion (movement is intermittent). These two categories of movement have been dubbed fast-axonal transport and slow-axonal transport, respectively. In chapter 2 we briefly discussed these two variants of movement and observed that, while neurofilament cargo movement is infrequent and slow on long timescales, these polymers move quite rapidly and stochastically in a fashion similar to cargoes associated with fast cargo movement. The fact that these similarities are visible only on shorter times-scales indicates that the frequency of interaction between neurofilaments and microtubules is the rate-limiting mechanism behind their movement. When considering the complexity of neurofilament-microtubule binding kinetics, this becomes quite apparent. If neurofilament transport is solely guided by the modulated kinetics of molecular motors, which are themselves stochastic in nature, then deciphering the mechanism behind said transport requires a very precise and accurate understanding of the processes that dictate both microtubule and cargo attachment/detachment.
Unfortunately, it is still not known how molecular motors even identify cargoes, let alone how the associated binding kinetics are regulated. Notwithstanding the uncertainty in motor association, some insights have been gained on this matter in comparing hypothetical cargo-transport mechanisms with the observations of cargo movements.

To date, three flavors of motor-based cargo transport have been posited; an un-coordinated tug-of-war model, an exclusionary-presence model, and a coordinated model. In the tug-of-war model, motors of opposing polarity act upon cargoes independently and compete against one another in a mechanical fashion. The resulting cargo movement then follows from whichever transport system (retrograde motors versus anterograde motors) generates the most force. This scenario agrees with observations of bio-molecules exhibiting rapid bouts of cargo reversals that occur—presumably—due to stochastic variations in the total number of active motors acting on the cargo. However, as pointed out by Gross (2004), experimental observations suggest that the tug-of-war scheme is not sufficient to explain cellular cargo movement entirely. In particular, in vitro studies which tested cargo movement upon impairing the movement of a specific class of motors, revealed that cargo movement does not improve in either the plus-end, nor minus-end direction (Morris and Hollenbeck, 1993; Welte et al., 1998; Gross et al., 2002a,b). This is puzzling because one would intuitively expect that a reduction in the number of plus-end directed motors would lead to an increase in minus-end movement, and vice-verse. In the exclusionary-presence model, only a single transport system is actively engaged with a cargo during transport. This model supports the findings of unidirectional cargo movement, but cannot account for the observations of Rogers et al. (1997); Ma and Chisholm (2002), and Ligon et al. (2004), which revealed both motor classes to be engaged during cargo movement, regardless of its direction. This observation debunks the exclusionary-presence model entirely. The coordinated model is somewhat ill-defined in the sense that no specific coordination mechanism is favored. Here, motors
somehow acclimate their behavior(s) relative to their local environment in the sense that only a specific motor class is actively engaged at a given time. This does not mean that only a single motor class is present during cargo movement, it merely presumes that a switching mechanism exists that activates/deactivates a motor class from engaging with either the cargo, the microtubule, or both. If a motor class is active, the cargo moves in a unidirectional fashion (towards say the plus end of the microtubule) while the other motor class tethers to the cargo and is disengaged from the microtubule. While this model is favored in the sense that it offers a more efficient mechanism in terms of energy consumption (ATP), it fails to explain the rapid bidirectional motions seen on shorter time-scales.

The fact that we observe characteristics of the above mentioned cargo-based models within the observed trajectories of neurofilaments (see Chapter 2) suggests that axonal transport follows from a combination of these models. For instance, we noted before the findings of Uchida et al. (2009), which suggests that dynein is essential for neurofilament transport—regardless of directionality. This is however, not true for kinesin (Wang and Brown, 2010). If kinesin function is inhibited, retrograde motion still persists, but there is a reduction in the overall frequency and flux of the observed neurofilament motions. Furthermore, kinesin inhibition actually leads to an increase in neurofilament velocity (ibid), which raises some difficulties in trying to explain these data mechanistically—as pointed out by Wang. Some of these features imply neurofilament movement is coordinated, while other features support an opposing scheme guided by a competitive mechanism, which is curious when considering the trajectories of the edge-lines that were examined in chapter two. (see Fig. 2.6). In some cases we see neurofilaments moving in a uni-directional fashion and very robustly, while other motions appear quite jagged, indicating a competitive strategy is at work. The fact that dynein appears to be a precursor to transport suggests that it may act as a coordinator, while kinesin may act as a
competitor. However, since each motor displays a preference in directionality, the combination of coordination overlapped with competition could be interpreted as sheer competition.

In chapter 3 we investigated the enzymatic cycles of kinesin and cytoplasmic dynein in detail. We developed two models that both accurately represent the motility characteristics of these motors observed in in-vitro studies in regards to substrate-dependence, force-generation, stepping behavior, and stall-times. In terms of competition, kinesin dominates dynein. It is more resilient to applied load and moves in a very robust fashion, while dynein acts less robustly. However, as noted in our optical trap simulations, dynein can overcome substantial load when invoking its catch-bond. Perhaps dynein’s catch-bond acts as a coordinator (while it technically acts in competitive nature) to regulate cargo movement within cells. That is to say, perhaps dynein plays the role of the parent motor, who is—in an effortful manner—continuously trying to coordinate cargo movement by managing the kinesin child motors. When need be, the parent motors employ their catch-bond in an effort to manage cargo transportation. This analogy is somewhat in tune with the findings of Rai et al. (2013), who not only put emphasis on dynein’s catch-bond like behavior, but also show that dyneins collectively work together, which enables to them to withstand the larger forces generated by kinesin motors.

4.2 An Asymmetric Tug-of-War Model for Neurofilaments

To model neurofilament movement, we invoke a stochastic tug-of-war representation of cargo transport in which variants of opposing motor teams comprised of kinesin and dynein molecular motors attach to a single neurofilament cargo and interact solely through mechanical forces. Each motor acts independently of the other motors and functions in accord with the enzymatic cycles discussed in chapter three. Since each motor acts separately, it experiences load specific to its relative contribution in transporting the cargo
at a given time. To implement this we treat the stalk domains of each motor as springs that respond to load in a linear fashion (Hooke’s Law), wherein each motor then experiences an applied load relative to the extent of which its linker domain is extended beyond its natural length (Kunwar et al., 2008). The amount of force generated by each motor is felt by the neurofilament cargo, which responds accordingly to balance the total amount of load acting on the cargo during transport. While in reality neurofilaments are long and flexible polymers, we treat them as massless point particles in our simulation, which is a fair simplification to make based on our findings of chapter two, which suggest neurofilament kinetics follow from a mechanism independent of its length. While our interpretation of how the motors are distributed along the neurofilament may be inaccurate, the fact we model them to act independently from one another makes this detail irrelevant.

Note that the only binding kinetics incorporated into the enzymatic cycles of these motors are in regards to substrate binding/rebinding and microtubule detachment. However, to simulate actual cargo-movement, we should include the binding kinetics that correspond to both cargo attachment/detachment as well as microtubule attachment. Unfortunately, little is known about motor-cargo binding, aside from the fact that it does occur. In previous computational studies of cargo motions, it was assumed that the motors always remain bound to the cargo but can detach/reattach from the microtubule throughout a simulation. These models assumed microtubule attachment rates between 1-5 sec$^{-1}$ Kunwar et al. (2008, 2011), which are taken from in-vitro measurements of lipid droplet transport. However, while these rates were used to model the protofilament attachment kinetics of the same types of motors that we are modeling, we cannot say for certain that these values are accurate for modeling neurofilament transport. For example, in the previously discussed six-state neurofilament model (Li et al., 2014), the rate at which neurofilaments engage with microtubules is 100x less than Kunwar’s values. To be fair, in Li’s model, their ”on-track” rate corresponds to the average rate in which
individual neurofilaments engage with microtubules relative to a population of neurofilaments, which were observed via a pulse escape assay. Hence, this rate, if correct, is an effective rate that accounts for both microtubule binding as well as cargo binding. Presumably, this rate is related to the average on-track pause time of the neurofilaments analyzed in that assay. For our simulations we will assume that all motors remain attached to the neurofilament cargo throughout a simulation, but can detach and reattach to the microtubule (if a motor is detached, then it is pulled along with the cargo). To account for the neglected motor-cargo binding/rebinding events, we chose to reduce the rate at which motors bind to microtubules. This rate is interpreted as an effective rate to account for both microtubule binding as well as cargo binding and is chosen in accordance with the average pause-time of our results from chapter 2 (see Fig 2.15), which corresponds to a cargo-microtubule binding rate ($\gamma_{C-M}$) of about 0.1-0.2 sec$^{-1}$.

Figure 4.1: Example of motor attachment/detachment kinetics: The illustration depicts two different arrangements of motors for a simulation that has three dyneins moving retrogradely and five kinesins moving anterogradely. Due to the fact that motors can attach/detach from the microtubule during our simulations, the number of actively engaged motors can vary over time.

For our simulations we start by defining a maximum number of allowable motors that can engage with both the neurofilament cargo and the microtubule, which equals the total number of kinesin motors ($N_k$) plus the total number of dynein motors ($M_d$). Initially, each motor is attached to both the cargo as well as the microtubule where the motors are
randomly distributed about the microtubule at nucleotide binding sites that are within a
distance of $\pm L_{\text{stalk}}$ from the cargo (where $L_{\text{stalk}}$ corresponds to the stalk lengths of the
motors: 16 nm for dynein and 50 nm for kinesin). At this stage, each motor is within a
distance from the neurofilament cargo that is less than or equal to its stalk length, and
therefore experiencing no applied load. For every time step, we simulate motor movement
using the Monte Carlo simulations used in chapter 3, for which we include an additional
event that can occur, corresponding to the cargo-microtubule binding rate. While each
motor follows the same Monte Carlo simulation scheme, the instantaneous behavior of
each motor depends on the amount of load it feels. Motors that are not constrained under
large applied loads are more likely to take steps, while motors that are experiencing large
applied loads are more likely to stall and/or detach from the microtubule. If any motors
detach/reattach from/to the microtubule, we update the number of engaged motors. For all
the motors that did not detach, we compute individual displacements of each motor,
relative to the current position of the cargo. For any motor that is now a distance
$L_{\text{stalk}} + \Delta x_i$ away from the cargo, it generates a force $F_i = \Delta x_i \cdot k_i$, which acts upon the
neurofilament. The net displacement of the cargo $\Delta x_{NF}$ then follows from the net force
generated from all the motors acting on it,

$$\Delta x_{NF} = \frac{\vec{F}_{\text{net}}^k + \vec{F}_{\text{net}}^d}{N_{\text{active}}^k \cdot k_{\text{spring}}^k + M_{\text{active}}^d \cdot k_{\text{spring}}^d}$$

(4.1)

where the superscripts indicate the motor type (k: kinesin and d: dynein), and N and M
correspond to the total number of actively engaged motors that contribute an applied load
upon the cargo at time t. We then update the position of the cargo for the next time step as
$x(t + dt) = x(t) + \Delta x_{NF}$, which then requires us to update the amount of load being applied
to each motor for the next time step. The cycle is then repeated. (See Fig. 4.2.)
Table 4.1: Parameters for the "tug-of-war" simulations

<table>
<thead>
<tr>
<th></th>
<th>$L_{stalk} (nm)$</th>
<th>$k_{stalk} (pN/nm)$</th>
<th>$\gamma_{C-M} (sec)^{-1}$</th>
<th>$N_{motors}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinesin</td>
<td>50.0§</td>
<td>0.3§§</td>
<td>0.2</td>
<td>0-30</td>
</tr>
<tr>
<td>Dynein</td>
<td>16.0†</td>
<td>0.07††</td>
<td>0.1</td>
<td>0-30</td>
</tr>
</tbody>
</table>


Figure 4.2: Illustration of the tug-of-war model: For each time step, the motors are in a given configuration (a) at some distance away from the neurofilament point particle (purple circle). We then update the positions of all engaged motors (relative to the neurofilament), which follows from each motor’s individual enzyme cycle. In the case of (b), kinesin has taken an 8 nm step in the anterograde direction, while dynein has remained in its current position. We then update the position of the neurofilament under the condition that the net force acting on the neurofilament in both the retrograde and anterograde directions is zero. In (c) the neurofilament has displaced a distance $\Delta x_{NF}$ (see Eq. 4.1)
4.3 Analysis Criteria

For the purposes of this dissertation, our analysis is focused on the implications that our model has in regards to the way neurofilaments move on short time scales. In particular we are interested in comparing our model with the results obtained from our analysis of neurofilament movement done in chapter two. Recall that our analysis on neurofilament transport entailed an extensive strategy to estimate the true trajectories of these polymers that were extracted from kymographs obtained from movies via time-lapsed imaging techniques interlaced with epifluorescence microscopy. That is to say, our analysis results were highly dependent on both the spatial resolution as well as the maximal frame-rate of the camera used. Hence, to have a fair comparison, our simulated "tug-of-war” trajectories must be course-grained to meet the same spatiotemporal resolution of our experimental data, as well as undergo the same strategies that were applied to differentiate between running-states and pausing-states.

For course-graining, we take snap shots of the modeled trajectories to correspond with the 30.3 ms temporal resolution from chapter 2. That is to say, the only position-time coordinates that are taken for our tug-of-war analysis, are those that occur every 30.3 ms of simulated experimental time. We then adjust the positions of the modeled neurofilament particle to be in agreement with the spatial resolution (160 nm\(^2\) per pixel) of the kymographs from chapter 2. For example, suppose at time \(t=303\) ms (i.e. ten time-frames), the net displacement of the particle is 312 nm away from the origin. This corresponds to a pixel position of one (312 nm displaced/(160 nm/pixel) = 1.95 pixels → 1 pixel displaced) and is taken as the particle position for timeframe #10. As time evolves within the simulation, the particle moves in accordance with our tug-of-war framework,

\(^2\) Note: Lindemann and Hunt estimated the maximal stalk stiffness from the data of Burgess et al. (2003) to be 0.1 pN/nm. We adjusted this value to 0.07 pN/nm such that our dynein model produced stall-times in accord with Rai et al. (2013) (See Fig. 3.15). If you assume that the B-link domain (stiffness 0.46 pN/nm (Lindemann and Hunt, 2003)) runs in series with the stalk domain, dynein’s effective stiffness is of the order 0.08 pN/nm.
until the next time-frame \((t=333.3 \text{ ms})\) passes for which then we update the particle’s course-grained position relative to its previous ”pixel” position. Each simulation represents 50.5 mins of experimental time, which corresponds to \(10^6\) timeframes and hence, \(10^6\) pixel positions. Following this, each course-grained trace then undergoes the processes within the abrasion algorithm (see chapter two–defining movement) where we apply the same threshold cut-off velocity of, \(v_{cut} = 0.1 \mu m/sec\) to define both the running-states and the pausing states. From here, we compute the run-length, run-time, and run-velocity for each bout of movement, as well as the time lapsed for each pausing event, wherein each event is flagged appropriately with the number of motors that can engage in the transport of the cargo for a given simulation.

4.4 Initial Results

We first examine the tug-of-war model by computing the distributions for each motility feature that was examined for our neurofilaments (save filament length since our model does not incorporate a length for the cargo). Our sample data set consists of 259k (46%) anterograde bouts of movement, 240k retrograde bouts of movement (43%), and 60k pausing events (11%). The fact that number of pausing states does not make up 33% of all of the extracted events of movement and pauses seems puzzling (every pausing state should be flanked by two runs). However, upon further examination we found that this is due to the fact that a significant number of reversal events occur over the course of a single time-frame (30.3 ms), especially in the cases when there are large numbers of both motor types acting upon the cargo (i.e. rapid ”tug-of-war” events).

In our analysis, we find that the tug-of-war pause-time is 2.6 fold less that the average pause-time of the neurofilaments examined in chapter 2 (2.8 secs vs. 7.3 secs) (see Fig. 4.3). However, we do still observe pausing events that exceed the average pause-time of our neurofilament data. Furthermore, upon comparing the characteristics of the
modeled running states with those observed for neurofilaments, we find that the average values for both the running-lengths and running-times exceeds the values reported for neurofilaments—especially for the extracted anterograde bouts of movement. However, on average the bout velocities for both anterograde and retrograde motions are within the range of values for the bout velocities reported for neurofilaments in chapter 2, indicating that the longer running lengths extracted from our modeled results occur with a longer run-time as well (see Fig. 4.3).
Figure 4.3: Distributions of the run-lengths, run-times, run-velocities, and pause-times for the tug-of-war model: We show the initial results for the kinetic characteristics generated from the tug-of-war model. The orange histograms represent the distributions for all retrograde bouts of movement, while the green histograms represent the distributions for all anterograde bouts of movement. The pause-time distribution is in blue. For each distribution, the average value for each characteristic is printed in the above right/left corner of each histogram with the appropriate color scheme. On average we see that anterograde directed movements exceed retrograde movements in terms of the run-lengths and run-times. However, on average the bout velocities for both anterograde and retrograde motions are equivalent in magnitude, indicating that the longer running lengths observed for anterograde bouts occur with a longer run-time as well.

At first glance, our model appears to express motility features that are related more with the kinetics of fast axonal transport than slow axonal transport. In particular, our model cannot produce the extensive periods of time that neurofilaments remain in a static
state. For example, regarding our analysis of neurofilaments from chapter 2, we found that while neurofilaments are expressing bouts of movement, they can pause for as much as 5 minutes before they continue to move, which is about five times longer than our tug-of-war model can reproduce. However, notwithstanding this, given the fact that our model produces run-lengths, run-times, and bout velocities that go beyond the range of values reported for neurofilaments (chapter 2) suggests that our tug-of-war model could explain the intermittent motions of neurofilaments (i.e. the kinetics during a running-state). For instance, in Fig. 4.4, we show four examples of our tug-of-war simulations that are compared with the trajectories of actual neurofilaments (chapter 2), for which the similarities are quite remarkable. For example in Fig. 4.4c we compare the trajectory of a 1.2 µm neurofilament with a simulated trajectory that had three kinesins and three dyneins (at most) practicing a tug-of-war. In both cases, the overall distance traveled in about 100-110 seconds is about 20 µm. Both trajectories express rapid bouts of movement in both the retrograde and anterograde directions. These observations suggests that while our collective set of run-lengths and run-times from the tug-of-war model exceeds those seen for neurofilaments, perhaps a portion of our simulated data can be of use. In particular, if a portion of our simulated data can reproduce the same motility characteristics of real neurofilaments, then perhaps we can gain some insight on how many motors engage with a neurofilament when it is being transported. That is to say, for a given subset of our modeled data, can we create a sample data set that can reproduce the same distributions for the motility characteristics of neurofilaments, and if so, what is the corresponding distribution of motors? Furthermore, while we know that our model cannot explain extensive pausing states, the range of pause-times seen within our model go well beyond the average pause-time of our neurofilament data. Hence, it is reasonable to compare the variability in motor distributions for runs and pauses that are in agreement with those of real neurofilaments.
Figure 4.4: **Visual comparison of ”tug-of-war” versus real neurofilament movement:** We show four samples of comparing the observed motions generated by the tug-of-war model (lower row) with four sample trajectories of neurofilaments taken from our collective data analyzed in chapter two (upper row). Our point here is to merely observe the similarities in movement between our simulation and experiment. The fact that the simulated traces in the lower panel have two edge-lines should not confuse the reader in how our model is implemented and is purely for visual purposes. These edge-lines are merely the same modeled data that has been offset by a length comparable to the average lengths of filaments whose trajectories they resemble.

### 4.4.1 Comparing the ”Tug-of-War” Model with the Movement of Neurofilaments

To correlate the number of motors with our neurofilament data, we start by taking the collective set of all the run-lengths, run-times, run-velocities, and pause-times that were extracted from the modeled traces. We then compare the characteristics of each run and each pause with the compiled data from our neurofilament analysis from chapter 2. By inspection, we then eliminate all runs and pauses that do not have a run-length, run-time, or pause-time that matches those within our experimental data. Hence, we generate a ”compatible” subset of modeled data that only contains the runs and pauses that are in agreement with the motility characteristics observed for neurofilaments. We then use this subset to create ”ideal” data sets that represent the neurofilament data. That is to say, for a
given motility characteristic, for example the running-length, we generate an "ideal" data set from our "compatible" modeled data that provides us with a histogram comparable to the run-length histogram that agrees with the run-length distribution found for neurofilaments (see Fig. 4.5). In doing so, we can predict which combinations of motors are necessary to reproduce the same statistics, in terms of the kinetics, as neurofilaments.

Figure 4.5: Distributions of the "mined" data from the tug-of-war simulations: We compare the distributions for the run-lengths, run-times, run-velocities, and pause-times created from the tug-of-war model (red bins) with the distributions of neurofilaments (blue bins) whose movement was recorded by epifluorescence time-lapsed imaging techniques (see chapter 2). The distributions for the modeled data correspond to a unique subset that was generated by pairing runs and pauses that show an agreement with experimental data of neurofilaments that were examined via epifluorescence microscopy (chapter two).

Our "ideal" data sets are created by pairing the data from our model to experiment in a random fashion. Recall in our our tug-of-war model that we allow motors to both detach and reattach to/from the microtubule, meaning that the total number of motors varies over time. Hence, simulations that have a larger number of maximum motors that can engage with the microtubule can express bouts of movement (and pauses) that could be generated
from simulations having a smaller number of maximum motors that can engage. By pairing the modeled data to the experimental data in a random fashion, we are ensuring that we are choosing the runs and pauses that follow from motor combinations that are most likely to practice them. For instance, suppose 50 runs from our experimental data set have the same run-length of 2 µm, and upon comparing this run-length to our modeled data we find that there are 2 different combinations of motors that have runs with the same run-length. However, within those two combinations, combination ”A” has 20 runs with a 2 µm run length while combination ”B” has only 2 runs with a 2 µm run-length. To create a data set that matches the experimental data, we need to include 50 runs that have a run-length of 2 µm. Hence we need to select the runs from our simulation that have 2 µm run length, 50 times. But how many runs do we include from the simulation with combination ”A” verses combination ”B”? Intuitively, combination ”A” is the correct choice since a 2 µm run occurs more often. However, since the simulation with motor combination ”B” can also have a run of 2 µm we cannot neglect it. However, if we choose 50 2 µm runs randomly from our modeled data we would select the runs from combination ”A” ten times more often than combination ”B”, and hence our data set that is created to match the run-length distribution from our experiment has 50 2 µm runs where most of the runs follow from motor combination ”A” and only a few runs follow from motor combination ”B”. By applying this methodology, we find that, on average, the total number of motors engaged during neurofilament transport is between 13-16 motors, where the average number of engaged kinesins is between 4-5 motors and the average number of engaged dyneins is between 8-12 motors. (See Figs. 4.6 and 4.7.)
Figure 4.6: **Predicted motor distributions for neurofilaments (2D)**: We show the distributions for the maximum total number of the motors engaged during neurofilament transport. These distributions correspond to a subset of data generated by the tug-of-war model that best explains the motility features of real neurofilaments that were recorded by epifluorescence time-lapsed imaging techniques (see chapter 2). Each distribution of motors corresponds to the distribution of a given motility characteristic (see Fig. 4.5). For example, the upper right distribution is the distribution of motors that corresponds to the subset of modeled data that best explains the run-length distribution for the recorded neurofilaments (see Fig. 4.5, upper right panel).

It is curious to note the similarities between the predicted motor distributions, especially when considering the fact that they each come from a different subset of the modeled tug-of-war data. It is even more curious to note that, on average, the total number of motors that contribute to anterograde bouts of movement is identical to the total number of motors that contribute to pauses. Intuitively, we expected that the total number of motors engaged during the pausing states would be greater than the total number of motors engaged during the running states. Contrary to our intuition, we find that, on average, retrograde runs have a larger number of motors engaged than both the pausing states.
states and the anterograde running states. Our interpretation of this is that, if the tug-of-war model is an accurate representation of "real" neurofilament transport, retrograde bouts of movement occur when either (1) no kinesins are bound, or (2) enough dyneins are available to engage with both the microtubule as well as the neurofilament cargo. This can be seen when considering the three-dimensional density profiles corresponding to the different combinations of motors predicted from our model (see Fig. 4.7). We find that the maximum number of kinesins needed to match the run-lengths, run-times, and run-velocities rarely exceeds 15 motors, while the maximum number of dynein motors can go as high as 30 motors total. However, as seen in Fig. 4.7 the majority of runs that have a maximum number of dyneins greater than 20 motors total corresponded to retrograde bouts of movement. Upon further analysis, we found that this occurs due the fact that simulations having a larger number of dynein motors leads to more frequent occurrences of sustained reversals, which leads to retrograde runs that have the same characteristics as our experimental data.
Figure 4.7: **Density profiles for the predicted maximum number of engaged motors (3D)**: We show the three dimension density profiles for the maximum total number of the motors engaged for each subset of modeled data that reproduces the distributions for the different running characteristics measured for real neurofilaments (see Fig. 4.5). The top row corresponds to anterograde directed movement, while the bottom row corresponds to retrograde directed movement. Panel (a) is the density profiles for modeled data whose run-length distribution matches that of the experiment (see Figs. 4.5, and 2.17.) for both anterograde (top) and retrograde (bottom). Panels (b) and (c) correspond to the same profiles, but with respect to the experimental distributions for run-time and run-velocity, respectively.

### 4.5 Discussion

Over the past two decades, extensive studies have been done to explore both the characteristics of neurofilament movement as well as the underpinning mechanism that leads to their transport (Jung and Brown, 2009; Uchida et al., 2013; Wang et al., 2000; Wang and Brown, 2001; Roy et al., 2000; Yabe et al., 2001; Li et al., 2014; Brown et al., 2005; Li et al., 2012; Yan and Brown, 2005; Trivedi et al., 2007; Taylor et al., 2012; Monsma et al., 2014; Wang and Brown, 2010; Uchida et al., 2009; Lasek et al., 1992;
Price et al., 1988; Brown and Lasek, 1993; Kumar et al., 2002; Li, 2013; Wagner et al., 2004). While much is known about the characteristics of the motors responsible for transporting these cargoes, little is known about the kinetics that guide these polymers during translocation. For instance, it is clear that neurofilaments move along axons in a rapid intermittent and bidirectional manner, which is characterized by frequent long intervals of pauses where these filaments remain static, which are then interrupted by infrequent, intermittent bouts of rapid movement. While it is known that their movement is driven by the molecular motors, kinesin and dynein, it is still unclear how these motors coordinate (if at all) with one another during transport. In the "un-coordinated" scenario, motors compete against one another in a mechanical fashion such that cargo movement follows a "tug-of-war" mechanism where the cargo moves with the strongest team of motors. In the "coordinated" scenario, motors somehow regulate their catalytic activity such that the cargo motions follow a less competitive mechanism. While both models have been shown to explain the intermittent motions of biological cargoes (Soppina et al., 2009; Gross et al., 2002b; Hendricks et al., 2010) associated with fast axonal transport, the fact that neurofilaments can extend up to 10-100 µm in length suggests that coordination between dissimilar motors is unlikely.

In this chapter, we developed a computational model to investigate whether the observed motile behavior of neurofilaments can be explained by a model in which dynein and kinesin motors attach to a single neurofilament cargo and interact through mechanical forces only (i.e. a "tug-of-war"). To test our model, we simulated 960 different cases of motor combinations in which the total number of motors that can engage in neurofilament transport varied between 0-30 motors for both dynein and kinesin. We then extracted the individual bouts of movement and pauses by applying the same analysis criteria that had been applied to neurofilament trajectories that were extracted from kymographs generated from recorded neurofilament movement (see chapter 2). By systematically varying the
number of kinesin and dynein motors in the model, we were able to identify those combinations of motors that matched the global distributions of the recorded neurofilament velocities, run-times, and run-lengths. We find that, on average, the total number of motors that engage with a neurofilament during movement ranges between 13-16 motors, where, on average, 4-5 kinesins are competing against 8-12 dyneins. While our model cannot explain the range of pause-times observed from the recorded neurofilament movement, it can explain the shorter pausing events that last about a minute in time. To our surprise, we find that the distribution of motors responsible for the shorter pausing events closely resembles the distribution of motors corresponding to anterograde directed movement. Our model suggests that the tug-of-war hypothesis is plausible in explaining the intermittent motions of neurofilaments within cells and suggests that the number of active motors attached to a neurofilament cargo during movement is relatively small in comparison with its length.
5 Minimizing the Caliber of Myelinated Axons by Means of Nodal Constrictions.

5.1 Introduction

3Neurons signal to each other and to other cell types via action potentials, which are waves of membrane depolarization that propagate along long slender projections called axons. Thus the speed of neuronal communication is limited by the axonal conduction velocity, and for long axons this can result in a significant delay. Two important ways in which animals maximize axonal conduction velocity are to increase axonal cross-sectional area, which reduces the internal resistance to the longitudinal diffusion of ions, and to myelinate axons, which allows for saltatory nerve conduction. Each of these strategies comes with metabolic costs (Hartline and Colman, 2007). Large caliber axons require more metabolic investment for their growth and maintenance because of their larger volume and surface area, and they also increase body size. Myelination allows fast conduction velocities with much thinner axons, requiring less axonal metabolic investment and smaller body size, but this also requires metabolic investment in the growth and maintenance of the myelinating glia (Harris et al., 2012). Thus there are evolutionary constraints on the strategies that animals adopt to maximize axonal conduction velocity.

The myelin sheath of myelinated axons is discontinuous, consisting of discrete myelinated segments called internodes that are separated by short gaps called nodes of Ranvier, where the axon has direct access to the extracellular space (Swärd et al., 1995; Arroyo and Scherer, 2000; Poliak and Peles, 2003; Salzer et al., 2008). The voltage-gated sodium channels, which are responsible for the large inward current that generates the action potential, are largely confined to the nodes, where they are present at very high density. Each internode is myelinated by a single myelinating cell (Schwann cell), which

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3 All of the sections throughout chapter 5 have been published in Johnson et al. (2015)
extends a sheet-like protrusion that wraps the axon spirally. Immediately flanking each node are paranodal regions, where the myelin lamellae terminate in the form of paranodal loops that are linked to the axon membrane by specialized axo-glial junctions. Beyond the paranodal regions are the juxtaparanodal regions, which represent the edges of the compact myelin. The length of the internode increases with increasing internodal caliber and can exceed 1 mm for large axons, but the length of the nodal gap is generally about 1 µm regardless of internodal caliber. Saltatory conduction arises because myelination decreases the membrane capacitance in the internode, allowing the transient depolarization of one node to trigger the transient depolarization of the next, thereby propagating the nerve impulse in a discontinuous manner. This results in a rapid propagation of action potentials at velocities of up to 100 m/s or more.

While the relationship between the electrophysiological function of myelinated axons and the spatial organization of their ion channels is understood fairly well, these axons exhibit additional anatomical features whose electrophysiological functions are not well understood. One of the most striking anatomical features of axons in the peripheral nervous system, which has been known for more than a century (Cajal, 1899; Hess and Young, 1952), is the narrowing of the axon at the nodes and the paranodes. The extent of these constrictions is minimal for axons below 2 µm in diameter but increases as axons expand, reaching 3-fold for the largest axons, which corresponds to a 9-fold reduction in cross-sectional area (Berthold, 1968; Dun, 1970; Rydmark, 1981; Berthold and Rydmark, 1983a,b; Rydmark and Berthold, 1983; Bertram and Schröder, 1993; Swärd et al., 1995).

Given the known dependence of the cable properties of axons on axonal cross-sectional area, it is interesting to ask what effect the constriction of axons at nodes of Ranvier may have on the propagation of the nerve impulse. This question has been addressed by Halter and Clark (1993) using computational modeling. A key result of their study was that constriction of axons at nodes of Ranvier increases axonal conduction
velocity and that there is an optimal extent of constriction at which the conduction velocity is maximal (see Fig 5.3 in Halter and Clark (1993)). Nodal radius was varied from 0.1 µm to about 6 µm, which corresponds to a 60-fold increase in nodal membrane surface area (if assumed to be cylindrical). However, the density of sodium channels was kept constant, leading to a corresponding 60-fold increase in the number of sodium channels. Moreover, the finding of an optimum extent of nodal constriction was dependent on the width of the periaxonal space between the axon and the myelin sheath and occurred only if this space was large enough. Hence, from this study we do not know what the sole influence of nodal morphology is on conduction velocity. To address this, we have used computational modeling to investigate the influence of nodal morphology on axonal conduction velocity assuming a fixed number of sodium channels.

5.2 Methods

We used a cable-equation based representation of a myelinated axon to study the dependence of conduction velocity on nodal, paranodal and juxtaparanodal axonal morphology. Berthold and Rydmark (1983a) compiled detailed information on the anatomy of nodes of Ranvier and their surrounding paranodal elements in feline spinal nerve root fibers. They divided the myelinated axon into four distinct regions termed NODE, MYSA ("myelin sheath attachment"), FLUT ("fluted region") and STIN ("stereotyped internode"). In modern terminology, the STIN corresponds to the internode where the axon is tightly ensheathed by myelin. The FLUT corresponds to the juxtaparanodal region where the voltage-gated potassium channels are localized, and the MYSA corresponds to the paranodal regions, where the myelin lamellae terminate in paranodal loops. Along the paranode, the axon diameter drops sharply to about 30%-50% of its diameter in internode (Berthold and Rydmark, 1983a). The node of Ranvier is a 1 µm bare segment between the paranodes. The axon in this region has a barrel-like shape,
bulging slightly in the center relative to its proximal and distal ends (Rydmark and Berthold, 1983). While the axon’s juxtaparanodal regions were originally described to have a tapering diameter, more recent studies using freeze-substitution electron microscopy reveal a constant juxtaparanodal diameter, with tapering occurring in the paranodal regions (Okamura and Tsukita, 1986; Sosinsky et al., 2005) In this paper we will refer to these regions as INTER (internode), JUXTA (juxtaparanode), PARA (paranode) and NODE (node itself) as shown in Fig. 5.1.

Figure 5.1: The single-cable model: We simulated a generic axon fiber, consisting of 30 repeating INTER-JUXTA-PARA-NODE-PARA-JUXTA-INTER sections. Nodes contained fixed numbers of sodium channels ($5 \cdot 10^3$, $1.5 \cdot 10^4$, $2.5 \cdot 10^4$), which have fast $Na^+$ membrane dynamics and a linear leak conductance. JUXTA sections contained fast-gated potassium channels. While all sections contained a membrane capacitance in parallel with their respective conductance, non-nodal regions contained two capacitors in series, one representing the myelin capacitance $C_{myelin}$, and the other representing the membrane capacitance $C_{mem}$. The figure is not to scale.

Previous studies of electrical phenomena in myelinated nerve fibers used geometric representations that explicitly defined sections of the axon corresponding to the nodal,
paranodal, juxtaparanodal and internodal compartments (Okamura and Tsukita, 1986; Halter and Clark, 1993; McIntyre et al., 2002; Sosinsky et al., 2005). The geometry of the models in Halter and Clark (1993) and Nygren and Halter (1999) is based on the anatomical data provided in Berthold and Rydmark (1983a). Their models are multi-cable models, which consider that current flows radially across the axon membrane and the myelin, and longitudinally down the axon, the periaxonal space, and the extracellular space. McIntyre et al. (2002) used a similar double-cable model but without the longitudinal current flow in the extracellular space. Given the large resistance of the myelin, little transmembrane potassium current is conducted across the myelin. Consequently we replaced the double-cable model in McIntyre et al. (2002)(see their Fig. 5.1) by a single-cable model with the axon and myelin capacitance in series (Fig. 5.2) and we accounted for the resistance of the periaxonal space with a reduced potassium conductance per channel.

The resulting single-cable model was implemented using the NEURON software package, version 7.2 (Hines and Carnevale, 1997; Carnevale and Hines, 2006). NEURON's default implicit integration method was applied with a time-step of 0.5 $\mu$s.

We designed an axon model consisting of 30 identical axon sections. Each fiber section was comprised of a node (NODE), a paranodal region (PARA), a juxtanodal region (JUXTA), and a central internodal region (INTER). These regions were organized in a sequential INTER-JUXTA-PARA-NODE-PARA-JUXTA-INTER structure (Fig. 5.1). The length of PARA was fixed at 4 $\mu$m, JUXTA at 75 $\mu$m and the NODE at 1 $\mu$m.

For the tapering of the myelin and the fiber/axonal diameters along the PARA from JUXTA to NODE, we used three different geometries (see Fig. 5.2a). The first geometry was a linear taper, where the axon diameter and fiber diameter decreased linearly. The second geometry was a nonlinear taper in which the fiber diameter decreased slowly initially and then more rapidly near the node, whereas the axonal diameter decreased
rapidly initially and then more slowly near the node. In this case, the fiber diameter in the PARA region was modeled as a sinusoid

\[ D_{\text{fiber}}^{\text{PARA}}(x) = (D_{\text{fiber}} - D_{\text{node}}) \cdot \sin(\pi x / 2L_{\text{PARA}}) + D_{\text{node}}, \]

where \( x = 0 \) at the node, \( x = L_{\text{PARA}} \) at the JUXTA-side of PARA, \( L_{\text{PARA}} = 4\mu m \). \( D_{\text{fiber}} \) is the fiber diameter in the internode and \( D_{\text{node}} \) is the nodal diameter. The corresponding axon diameter was modeled as an exponential function

\[ D_{\text{axon}}^{\text{PARA}}(x) = D_{\text{min}} \exp(x / L_{\text{PARA}}) \cdot \ln(D_{\text{axon}} / D_{\text{min}}) \]

where \( D_{\text{min}} \) is the smallest diameter of the axon next to the nodes \( (x = 0) \) and \( D_{\text{axon}} \) is the axon diameter in the internode \( (x = L_{\text{PARA}}) \). The third geometry was an abrupt step-like narrowing of the axon diameter at the JUXTA:PARA interface. In each of the three different geometries, the myelin thickness along the PARA was taken as the difference between the corresponding fiber diameter and axon diameter,

\[ 2\Delta_{\text{myelin}}^{\text{PARA}}(x) = D_{\text{fiber}}^{\text{PARA}} - D_{\text{axon}}^{\text{PARA}} \]

The internodal region’s capacitance was modeled as an equivalent capacitance for two capacitors in series: one representing the intracellular-to-membrane boundary, and the other representing the membrane-to-myelin boundary (see Fig. 5.2b). Each region was treated as a cylindrical conductor, whose capacitance per unit length was given by

\[
\frac{C}{L} = \frac{2\pi \varepsilon_0 \varepsilon_r}{\ln(D_{\text{out}} / D_{\text{in}})}
\]

(5.1)

where \( D_{\text{out}} \) is the diameter of the outer cylinder, \( D_{\text{in}} \) is the diameter of the inner cylinder, \( \varepsilon_0 \) is the permittivity of empty space, and \( \varepsilon_r \) is the relative dielectric constant (Griffiths and College, 1999).
Figure 5.2: **Representation of an internode (INTER region).** (a) The three tapering designs of the axons and the myelin around the nodes of Ranvier used in this study. (b) The cylindrical capacitance model for the axon: The axon fiber consists of the axon (the inner core) with diameter $D_{axon}$, the membrane with thickness $\Delta_{mem}$ and a layer of myelin of thickness $\Delta_{myelin}$ generating the outer cylinder. The fiber diameter $D_{fiber}$ is then determined by $D_{fiber} = D_{axon} + 2\Delta_{mem} + 2\Delta_{myelin}$.

For the capacitor representing the plasma membrane of the axon, the diameters of the outside and inside cylinders are much larger than their difference, i.e. the membrane thickness $\Delta_{mem}$. Expanding the logarithm, i.e.

$$\ln(D_{out}/D_{in}) = \ln((D_{axon} + 2\Delta_{mem})/D_{axon}) \approx (2\Delta_{mem})/D_{axon},$$

yields then, for the capacitance per length,

$$\bar{C}_{mem} \equiv C_{mem}/L = \pi \epsilon_0 \epsilon_{mem} D_{axon}/\Delta_{mem}, \quad (5.2)$$

and for the specific capacitance of the membrane (capacitance per unit area)

$$c_{mem} = \bar{C}_{mem}/\pi D_{axon} = (\epsilon_{mem} \epsilon_0)/D_{mem}$$, which for a typical value of $\epsilon_{mem}$ gives the commonly used value $1 \mu F/cm^2$. We used this value for the specific capacitance of the plasma membrane at the NODE and INTER.

In the INTER, the additional capacitor in series with the membrane capacitor has a capacitance per length given by
\[ \tilde{C}_{\text{myelin}} \equiv \frac{C_{\text{myelin}}}{L} = \frac{2\pi \varepsilon_0 \varepsilon_{\text{mem}}}{\ln(D_{\text{fiber}}/D_{\text{axon}})} \]  
(5.3)

where \( D_{\text{fiber}} \) is the diameter of the entire fiber (axon and myelin sheath), \( D_{\text{axon}} \) is the diameter of the axon (we neglected the membrane thickness), and \( \varepsilon_{\text{myelin}} \) is the relative dielectric constant of the myelin. Reported values for the relative dielectric constant of the myelin range from 5 to 15 (Basser and Roth, 1991; Min et al., 2009). In this study we choose \( \varepsilon_{\text{myelin}} = 10 \). The specific capacitance of the myelin, \( c_{\text{myelin}} \), is then given by \( c_{\text{myelin}} = \tilde{C}_{\text{myelin}}/(\pi D_{\text{fiber}}) \).

To determine the diameter of the fiber \( D_{\text{fiber}} \) for a given axon diameter, we generated a linear regression of the reported data (Berthold et al., 1983), i.e.

\[ D_{\text{axon}} = 0.666 \cdot D_{\text{fiber}} - 0.429 \mu m \]  
(5.4)

which we used as a lookup table. The corresponding myelin capacitance, \( \tilde{C}_{\text{myelin}} \), then follows from Eq. 5.3

For the computational modeling we needed the capacitance per length for our double capacitor model Fig. 5.2. Combining Eq. 5.2 with Eq. 5.3, and dividing by the circumference of the fiber, the specific equivalent capacitance is obtained as

\[ c_{eq} = \frac{\tilde{C}_{eq}}{\pi D_{\text{fiber}}} = \frac{c_{\text{myelin}}}{1 + \left( \frac{C_{\text{myelin}}}{C_{\text{mem}}} \right) L} = \frac{c_{\text{myelin}}}{1 + \frac{c_{\text{myelin}} D_{\text{fiber}}}{c_{\text{mem}} D_{\text{axon}}}} \]  
(5.5)

where \( c_{\text{myelin}} \) and \( c_{\text{mem}} \) are the specific myelin capacitances and membrane capacitance. Replacing \( D_{\text{fiber}}/D_{\text{axon}} \) using Eq. 5.1, we finally find

\[ c_{eq} = \frac{c_{\text{myelin}}}{1 + \frac{c_{\text{myelin}}}{c_{\text{node}}} \cdot \exp \left( \frac{2\varepsilon_0 \varepsilon_{\text{myelin}}}{D_{\text{fiber}} c_{\text{myelin}}} \right)} \]  
(5.6)

where we have assumed that the specific capacitance of the NODE compartment \( c_{\text{node}} \) is equal to the specific capacitance of the membrane capacitor in the internode. Given the fiber diameter, we used this expression for the specific capacitance of the fiber.
We defined the number of sodium channels as $N_{Na}$ and the number of potassium channels as $N_K$. Then $N_{Na}$ sodium ion channels with a conductance of $g_{Na} = 20pS$ each were placed in the NODE region, and $N_K$ potassium ion channels with a conductance of $g_K = 20pS$ were placed in the JUXTA region (Rasband and Shrager, 2000). The conductance and voltage-dependent dynamics were modeled using the Hodgkin-Huxley framework (Hodgkin and Huxley, 1952). The sodium and potassium currents are given by

$$J_{Na} = N_{Na} \cdot g_{Na} \cdot n_1^3 n_2 (V - V_{Na})$$
$$J_K = N_K \cdot g_K \cdot n_3^4 (V - V_K)$$

where $V_{Na}$ and $V_K$ denote the sodium and potassium reversal potentials, $V$ the membrane potential, and $n_1, n_2, n_3$ gating variables satisfying the set of differential equations

$$\frac{d n_i}{dt} = \alpha_i (V) (1 - n_i) - \beta_i (V) n_i$$

where the $\alpha_i(V)$ and $\beta_i(V)$ are defined as in McIntyre et al. (2002).

$$\alpha_1 = \frac{6.57(V + 20.4)}{1 - \exp \left( -\frac{V + 20.4}{10.3} \right)} \quad \alpha_2 = \frac{-0.34(V + 114)}{1 - \exp \left( -\frac{V + 114}{11} \right)} \quad \alpha_3 = \frac{0.0426(V + 83.2)}{1 - \exp \left( -\frac{V + 83.2}{1.1} \right)}$$

$$\beta_1 = \frac{-0.304(V + 25.7)}{1 - \exp \left( -\frac{V + 25.7}{9.16} \right)} \quad \beta_2 = \frac{12.6}{1 - \exp \left( -\frac{V + 31.8}{13.4} \right)} \quad \beta_3 = \frac{-0.0824(V + 66)}{1 - \exp \left( -\frac{V + 66}{10.5} \right)}$$

A leak current density of the form

$$j_{leak} = g_{leak} \cdot (V - V_{leak})$$

was added to the nodal compartment, with $g_{leak} = 0.007S/cm^2$ (McIntyre et al., 2002).

5.3 Results

We first examined the role of nodal diameter on conduction velocity with the number of ion channels and all other parameters held constant. We used the above mentioned
single-channel conductances and took the number of potassium channels in the JUXTA region to be 250, which is within the range of numbers extracted from previous work (McIntyre et al., 2002). The NODE specific capacitance was chosen to be $1 \mu F/cm^2$. A current stimulus, between 1 nA and 5 nA, was then applied in the first NODE region, triggering a healthy action potential, and average conduction velocities were calculated by measuring time for an action potential to pass from node 5 to node 25. Conduction velocity as a function of nodal diameter is shown in Fig 5.3 for $5 \cdot 10^3$ (a), $1.5 \cdot 10^4$ (b), and $2.5 \cdot 10^4$ (c) nodal sodium ion channels, each for a range of fiber diameters (diameter of internodal axon plus myelin sheath) with linear tapering of both the axon and myelin sheath in the paranodes. For a given fiber diameter, the conduction velocity increases with increasing nodal diameter until it reaches a maximum velocity and then decreases with increasing nodal diameter. At a fixed nodal diameter, the conduction velocity is invariably larger for larger fiber diameters. The dashed curves in Fig 5.3a-c denote the conduction velocities for unconstricted axons, i.e. when the internodal and nodal diameters are identical. The thick solid lines are the curves for the maximal velocities, which vary linearly with respect to fiber diameter. This indicates that the optimal nodal diameter increases with increasing fiber diameter. For comparison, we show in Fig 5.3d the conduction velocity versus nodal diameter for three different fiber diameters, keeping the NODE sodium channel density and JUXTA potassium channel density constant so as to yield conductances of $g_{Na} = 3.0 S/cm^2$ and $g_K = 1.64 \cdot 10^{-4} S/cm^2$ respectively. The potassium channel conductance was based on the value of $g_K = 0.08 S/cm^2$ used in McIntyre et al. (2002) but adjusted to account for the fact that in our model the potassium channels are distributed over a longer and fatter JUXTA (i.e. at a lower density) compared to the NODE. In agreement with the findings in Halter and Clark (1993) for a vanishingly small periaxonal space, we found a monotonic increase in conduction velocity and no optimal nodal constriction.
Figure 5.3: **Analysis of velocity gain using a linear paranodal taper.** The conduction velocity is plotted versus nodal diameter for values of fiber diameter (internode plus twice the myelin thickness) ranging from 4$\mu$m to 20$\mu$m (indicated in figure) and (a) $5 \cdot 10^3$ nodal sodium channels, (b) $1.5 \cdot 10^4$ nodal sodium channels, and (c) $2.5 \cdot 10^4$ nodal sodium channels. The numbers above the curves indicate fiber diameters. The dashed lines indicate velocities for unconstricted axons, i.e. where the nodal and internodal axon diameters are identical. The thick solid lines indicate the maximum conduction velocities for each fiber diameter. The insets in (a)-(c) indicate the percentage gain of conduction velocity compared to an unconstricted axon. In (d) we show the conduction velocity as a function of the nodal diameter for three values of the fiber diameter with a constant specific sodium conductance of $g_{Na} = 3.0S/cm^2$, i.e. when the numbers of nodal sodium channels increase linearly with the nodal diameter. The inset displays the linear tapering of the axon and myelin sheath in the PARA region.
The emergence of a maximal conduction velocity at an optimal nodal diameter can be explained as follows. For decreasing and vanishingly small nodal diameters, the conduction velocity will decrease to zero because of the increasing intra-axonal resistance. Conversely, for increasing nodal diameters, the total nodal capacitance will also increase, resulting in a slower action potential upstroke and hence a smaller conduction velocity. These two opposing trends result in the observed peak for the conduction velocity. In support of this interpretation, we did not observe optimal nodal diameters if we kept the nodal capacitance artificially constant as we increased the nodal diameter (data not shown).

To better quantify the gain in conduction velocity due to axonal constriction at nodes of Ranvier, we calculated the increase in the conduction velocity for a fiber with an optimal nodal constriction compared to an equivalent fiber with no nodal constriction (i.e. when $D_{node} = D_{internode}$ (dashed lines)) and then plotted this increase versus the fiber diameter (see insets in Fig 5.3a-c). We found that larger fibers exhibit a larger gain in conduction velocity than smaller fibers. For fibers of 20$\mu m$ diameter, the gain was 60% in the case of $5 \cdot 10^3$ nodal sodium channels (see inset in Fig 5.3a). Generally, the smaller the number of nodal sodium channels, the larger the velocity gain.

From Fig 5.3 we observe that a given conduction velocity can be obtained generally with two different nodal diameters given a specific value of the fiber diameter. This raises the question of what cable morphologies are associated with a given target conduction velocity. To address this, we calculated the iso-conduction velocity contour lines in the two-dimensional Cartesian plane of internodal versus nodal diameters. Such curves are shown in Fig 5.4 for target conduction velocities ranging from $10 m/s$ to $80 m/s$ and $5 \cdot 10^3$ (a), $1.5 \cdot 10^4$ (b), $2.5 \cdot 10^4$ (c) nodal sodium channels. Starting with an unconstricted axon (dashed lines), i.e. when the nodal diameter is equal to internodal diameter, a decrease in the nodal diameter also allows a decrease in the internodal diameter, although by a smaller
amount, until a minimum is reached. The minima of the curves in Fig 5.4 represent the fiber morphologies that yield a given target conduction velocity with the smallest internodal diameter and hence with the smallest possible fiber diameter. With further decreases in nodal diameter, the required internodal diameter increases again. Thus, nodal constrictions allow axons, with a constant number of ion channels, to reduce their volume without any loss in electrical performance (specifically conduction velocity). To quantify the space-savings, we plot in the insets of Fig 5.4 the percentage increase in the fiber volume (axon plus myelin sheath), i.e. the spatial cost, that is necessary for an unconstricted axon to achieve the same conduction velocity as an axon with optimal nodal constrictions. The largest benefit of nodal constriction (i.e. the highest spatial cost for an unconstricted axon) is found for the smallest number of nodal sodium channels (compare the insets in Fig 5.4a-c).

Figure 5.4: **Analysis of space cost using a linear paranodal taper.** We show the contour lines of constant conduction velocity on the Cartesian plane of internodal axon diameter versus nodal diameter for a range of conduction velocities (indicated on lines) and (a) $5 \cdot 10^3$ nodal sodium channels, (b) $1.5 \cdot 10^4$ nodal sodium channels, and (c) $2.5 \cdot 10^4$ nodal sodium channels. The nodal and internodal diameters are identical on the dashed line. The thick solid lines indicate fiber morphologies with the minimal internodal axon diameter for the given target conduction velocities. The insets indicate the percentage increase in volume (i.e. spatial cost) for an unconstricted axon (internodal diameter = nodal diameter) compared to an axon with optimal nodal constrictions.
The thick solid lines connecting the minima in Fig 5.4 indicate optimal fiber morphologies in terms of space usage. Since a minimal internodal diameter in Figs 5.4 is the smallest possible internodal diameter for the axons to conduct at a given velocity, this conduction velocity must also be the maximum of the graph in Fig 5.3 if plotted for that minimal internodal diameter. In more mathematical terms, if the conduction velocity as a function of nodal diameter and internodal diameter is denoted by \( v(x, y) \), the contour lines in Fig 5.4 are characterized by \( dv = (\partial v / \partial x) dx + (\partial v / \partial y) dy \) or equivalently \( y'(x) = - (\partial v / \partial x) / (\partial v / \partial y) \). The maximum of the conduction velocity \( v \) as a function of the nodal diameter \( x \) at a fixed internodal diameter \( y \) occurs when \( \partial v / \partial x = 0 \), i.e. exactly at the same nodal diameter where the contour lines in Fig. 5.4 exhibit a minimum. For example, from Fig 5.4c we find for a target velocity of 55 m/s an optimal morphology with an internodal diameter of 9 \( \mu m \) and a nodal diameter of 1.5 \( \mu m \). The corresponding fiber diameter can be obtained from Eq. 5.4 as \( D_{fiber} = 14.2 \, \mu m \). Consistent with this, we find in Fig 5.3c that the maximum conduction velocity of a 14 \( \mu m \) fiber is 55 m/s for a nodal diameter of 1.5 \( \mu m \). In conclusion, the fiber morphologies that maximize the conduction velocities also minimize the space cost.

The above simulations were obtained assuming an idealized nodal morphology in which the axon and myelin sheath were assumed to taper linearly in the PARA region as described in section 5.2. To explore the effect of deviations from this idealized morphology on the optimal nodal constrictions identified above, we repeated our simulations with a nonlinear tapering in which the axonal diameter in the PARA region decreases according to an exponential function and the fiber diameter in the PARA decreases according to a sine-function (see Sec. 5.2). Such tapering better resembles the micrographs in (Sosinsky et al., 2005). In Fig. 5.5a, we show the conduction velocity versus nodal diameter for a range of fiber sizes and a fixed number of \( 5 \cdot 10^3 \) nodal sodium channels. While the general behavior is the same as reported in Fig 5.4a, the conduction
velocities are larger and the increase in conduction velocity generated by the nodal constriction is smaller. This is due to differences in the capacitance of the myelin in the PARA region. In Fig. 5.5b we show contour lines of constant conduction velocity. As in Fig 5.4, we find that there is one minimal internodal diameter for each target conduction velocity. The extent of constriction at the nodes is, however, smaller. For example, for an axon with an internodal diameter of 9µm and a fixed number of 5 · 10³ nodal sodium channels, the optimal nodal diameter is about 1.4µm (i.e. a 6.4-fold constriction) with linear tapering of the axon and sheath in the paranode, and about 1.7µm (i.e. a 5.3-fold constriction) with nonlinear tapering.
Figure 5.5: **Analysis of velocity gain and space cost for a nonlinear paranodal taper.**

In (a), the conduction velocity is plotted against the nodal diameters for values of fiber diameter ranging from $4\mu m$ to $20\mu m$ (indicated in figure) and $5 \cdot 10^3$ nodal sodium channels. The dashed lines in (a) indicate velocities for an unconstricted fiber, i.e. an axon with identical nodal and internodal diameters. The thick solid line indicates the velocities of fibers optimized for maximum conduction velocities. The inset in (a) indicates the percentage gain in conduction velocity for a range of fiber diameters compared to a fiber with an unconstricted axon. In (b) we show contour lines of constant conduction velocity on the Cartesian plane of nodal diameter versus internodal diameter for conduction velocities ranging from 10 m/s to 40 m/s (indicated on lines) and $5 \cdot 10^3$ nodal sodium channels. The nodal and internodal axon diameters are identical on the dashed line. The thick solid lines indicate fiber morphologies with the minimal internodal axon diameter for the given target conduction velocities. The insets indicate the percentage increase in volume (i.e. spatial cost) for an unconstricted axon (internodal diameter = nodal diameter) compared to an axon with optimal nodal constrictions. In (c) we show the conduction velocity as a function of the nodal diameter for three values of the fiber diameter when the sodium channel density is constant, i.e. when the numbers of nodal sodium channels increase linearly with the nodal diameter. The inset displays the non-linear tapering of the axon and myelin in the PARA region.

Finally, we considered the case of a step-taper, where the axon diameter decreases abruptly from the internodal diameter to the nodal diameter at the JUXTA-PARA interface and the myelin ends abruptly at the PARA:NODE interface (Fig. 5.6. The results are qualitatively similar to those for the other tapers. The maximum velocities occur at larger nodal diameters and the optimal fibers have a larger nodal diameter than for the linear and nonlinear tapers. For the same example as above (i.e., a $9\mu m$ internodal axon diameter and
5 · 10^3 sodium channels), the optimal nodal axon diameter is about 2.5µm, i.e. a 3.6-fold constriction.

Figure 5.6: **Analysis of velocity gain and space cost for a step-like taper.** In (a), the conduction velocity is plotted against the nodal diameters for values of fiber diameter ranging from 4µm to 20µm (indicated in figure) and 5 · 10^3 nodal sodium channels. The dashed lines in (a) indicate velocities for an unconstricted fiber, i.e. an axon with identical nodal and internodal diameters. The thick solid line indicates the velocities of fibers optimized for maximum conduction velocities. The inset in (a) indicates the percentage gain in conduction velocity for a range of fiber diameters compared to a fiber with an unconstricted axon. In (b) we show contour lines of constant conduction velocity on the Cartesian plane of nodal diameter versus internodal diameter for conduction velocities ranging from 10 m/s to 40 m/s (indicated on lines) and 5 · 10^3 nodal sodium channels. The nodal and internodal axon diameters are identical on the dashed line. The thick solid lines indicate fiber morphologies with the minimal internodal axon diameter for the given target conduction velocities. The inset indicates the percentage increase in volume (i.e. spatial cost) for an unconstricted axon (internodal diameter = nodal diameter) compared to an axon with optimal nodal constrictions. In (c) we show the conduction velocity as a function of the nodal diameter for three values of the fiber diameter when the sodium channel density is constant, i.e. when the numbers of nodal sodium channels increase linearly with the nodal diameter. The inset displays the non-linear tapering of the axon and myelin in the PARA region.

What is remarkable about these findings is the sensitivity of the conduction velocity to details of the nodal ultra-structure. For a 20µm fiber with 5 · 10^3 nodal sodium channels, a linear taper promotes a conduction velocity gain of 60% in comparison to an unconstricted axon, whereas for a nonlinear taper the gain is approximately 35% (compare
Fig. 5.3a and Fig. 5.4a). The corresponding conduction velocities for the nonlinear taper are between 5%-20% higher than for the linear taper, depending on the extent of the nodal constriction.

When the density of nodal sodium channels, instead of the number of nodal sodium channels, is held constant, we again find a monotonic increase in conduction velocity with increasing nodal diameter (Fig. 5.6c). But here again the nodal morphology is an important influence on the conduction velocity. For example, for a 20µm fiber with a nodal diameter of 12.9µm, the conduction velocity is about 15% larger for a nonlinear taper compared to a linear taper. Thus the precise morphology of the axon and myelin sheath in the paranodes has a significant effect on the conduction velocity of myelinated axons.

In light of these findings on the sensitivity of the conduction velocity to the shape of the paranodal taper, we investigated if there was also any significant effect of altering the length of these regions. Generally speaking, the PARA region, where the myelin terminates, is considered to be on the order of a few microns, but in fact this varies (e.g. Okamura and Tsukita (1986)). Thus we repeated our simulations with an extended PARA region length of 8µm, while still maintaining the same node-node separation (i.e. internode length) of 1 mm. The behavior was qualitatively similar to that seen in Figs. 5.3 5.4 5.5 and 5.6 but for a given fiber diameter, the corresponding optimal nodal diameters were about 15%-20% higher. In Fig. 5.7a we show the conduction velocity contours at three different conduction velocities for fibers with our standard 4µm paranodal length compared with fibers having an extended 8µm paranodal length. The percent increase in the optimal nodal diameters is higher for larger fibers.
Figure 5.7: **Analysis of the effects of an extended PARA length or a nodal bulge.** We show the contour lines of constant conduction velocity on the Cartesian plane of internodal diameter versus nodal diameter for conduction velocities of 20 m/s, 30 m/s, and 50 m/s (indicated on lines) and $1.5 \cdot 10^4$ nodal sodium channels. The nodal and internodal diameters are identical on the dotted line. The solid lines represent simulations in the presence of an extended PARA length (a) or a nodal bulge (b), while the dashed lines indicate simulations in their absence.

Given the above findings for the axon and sheath morphology in the PARA region, we also investigated the effect of the slight axonal bulging that has been reported at nodes of Ranvier (Rydmark and Berthold, 1983). In Fig. 5.7b, we compare the conduction velocity contour lines, now with and without a nodal bulge, and find that nodal bulging has an effect similar to that seen when extending the length of the PARA regions, though smaller in magnitude. The same effect is observed if the channel density is kept constant (data not shown).

In the above simulations we held the number of sodium channels at the nodes constant when we varied nodal and internodal diameter, but in reality we fully expect that the number will increase with increasing nodal surface area. Therefore, we investigated how the optimal node designs discussed above depend on the number of nodal sodium channels. In Fig 5.8, we show the lines of optimal design for $5 \cdot 10^3$ sodium channels.
(dot-dashed line), \(1.5 \cdot 10^4\) sodium channels (long dashed line) and \(2.5 \cdot 10^4\) sodium channels (solid line) for a nonlinear-tapering arrangement. The similarity in the slopes of these lines indicates that the optimal extent of nodal constriction is not very sensitive to the number of sodium channels. This suggests that an increase in nodal sodium channel number with increasing axonal caliber would not be expected to change the optimal morphological design of these fibers significantly.

![Graph](image)

**Figure 5.8:** *Analysis of the effect of sodium channel number on the optimal fiber morphology.* We show the computed lines of optimal fiber morphologies for a nonlinear tapering of the axon and myelin sheath in PARA (see inset Fig. 5.5c) and \(5 \cdot 10^3\) (dashed-dotted line), \(1.5 \cdot 10^4\) (dashed line), and \(2.5 \cdot 10^4\) (solid line) nodal sodium channels.

We then compared our optimal designs with "real" myelinated fibers. We used the experimental data of Rydmark (1981) for nodal and internodal diameters in axons of cat spinal nerve roots, plotted in Fig. 5.9a. While there is scatter in the data and some difference between dorsal and ventral roots, in both cases the data can be fitted well with a linear regression line. This shows that the linear relationship between nodal and internodal diameter seen for the optimal fiber morphologies in our simulations is also observed in real axons.
We note that the slope of the lines relating nodal and internodal diameter in Fig. 5.9a are different in dorsal nerve roots (which are comprised of sensory axons) and ventral nerve roots (which are comprised of motor axons). In view of the insensitivity of the optimal fiber designs with respect to numbers of ion channels and the sensitivity of these designs to the nodal and paranodal morphology, we investigated whether these differences could be accounted for by differences in the morphology of the paranodal regions. The results are shown in Fig. 5.9b. The slope of the line representing the optimal fiber design is largest for a linear taper (left-most solid/dashed lines), and smallest for an abrupt step-like taper (right-most solid/dashed lines), with the slopes for a nonlinear tapering design somewhere in between (middle solid/dashed lines). As shown above, for each tapering morphology the slope was lower for 8 $\mu$m-long paranodes (solid lines) than for 4$\mu$m long paranodes (dashed lines). Moreover, the range of these slopes (about 2.6-fold) is more than the difference between the dorsal and ventral nerve root axons (about 1.4-fold). Thus, the difference in the slope of the linear relation between nodal and internodal diameter for dorsal and ventral nerve roots may be explained by differences in local structure of the axons and their myelin sheaths near the nodes of Ranvier without changing the number of required nodal sodium channels.
Figure 5.9: **Comparison of actual and predicted fiber morphologies.** In (a), we show morphologic data for feline dorsal spinal nerve root (squares) and ventral spinal nerve root (circles) extracted from Figs. 1 and 2 of Rydmark (1981). In (b), we show the computed lines of optimal fiber morphologies for a linear tapering of the axon and myelin sheath in the PARA region (leftmost solid/dashed lines), for a nonlinear tapering (middle solid/dashed lines), and for an abrupt step-like narrowing of the axon and myelin sheath at the JUXTA:PARA interface (rightmost solid/dashed lines). The dashed lines represent fibers having a 4µm PARA region length and the solid lines represent fibers having an extended 8µm PARA region length. The arrows indicate the corresponding tapering method used.

While the above analysis indicates that the optimal fiber morphology at nodal constrictions is relatively insensitive to changes in the number of sodium channels, increasing the number of sodium channels does increase the axonal conduction velocity. Thus we investigated how many additional nodal sodium channels would be required to match the effect of nodal constrictions. In the lower panel of Fig. 5.10, we plot the internodal axon diameter required to generate a conduction velocity of 25m/s versus nodal sodium channel number for axons with no nodal constrictions ("uniform cable"; upper curve) and for axons with optimal nodal constrictions ("constricted cable"; lower curve). The horizontal difference between the two curves represents the number of additional channels needed for a given axon diameter (vertical axis). For example, for an
internodal diameter of $7\mu m$, an axon with no nodal constrictions would require 3900 nodal sodium channels in addition to the 5000 channels for the optimal cable, an increase of 76%. For smaller fibers the horizontal distance between the curves increases, indicating that more additional channels are required to attain the same conduction velocity. In order to address how the required number of additional channels depends on the target conduction velocity, we plot in the upper panel of Fig. 5.10 the conduction velocity versus the number of nodal sodium channels for an axon with an internodal diameter of $12.9\mu m$ and optimal nodal constrictions ("constricted cable"; upper curve) or no nodal constrictions ("uniform cable"; lower curve). As above, the horizontal distance between the curves depicts the numbers of additional channels required for an axon with no nodal constrictions to generate the same conduction velocity. For example 6000 additional channels are needed for a conduction velocity of about $45m/s$, an increase of 120%. At larger conduction velocities the horizontal distance between the curves increases, indicating that even more sodium channels are required to attain the same conduction velocity. Thus, in addition to increasing the conduction velocity for a given internodal axon diameter, nodal constrictions also reduce substantially the number of required sodium channels for a target conduction speed.
Figure 5.10: **Analysis of the number of additional nodal sodium channels required to match the conduction velocity gains resulting from optimal nodal constrictions.**

(a) The conduction velocity of a $20\mu m$ fiber is plotted versus the number of nodal sodium channels for an optimal cable and an unconstricted (uniform) cable. For a specified conduction velocity (vertical axis), the horizontal distance between the two curves represents the number of additional sodium channels needed for the unconstricted cable. (b) The necessary internodal diameter for a conduction speed of $25m/s$ is shown as a function of the number of nodal sodium channels for the optimal cable and the unconstricted cable. For a specified internodal diameter (vertical axis), the horizontal distance of the curves represents the number of additional sodium channels needed for the unconstricted cable to conduct with the same speed of $25m/s$.

### 5.4 Discussion

The fact that myelinated axons in the peripheral nervous system are constricted at nodes of Ranvier has been recognized for more than a century (Cajal, 1899; Hess and Young, 1952) but only a few studies have addressed its physiological significance. Dun (1970) appears to have been the first to speculate that these constrictions may influence nerve conduction. Moore et al. (1978) investigated this using computational modeling and concluded that nodal conduction is insensitive to nodal constriction, but Halter and Clark (1993) showed that when the detailed ultrastructure of nodes and their flanking regions is taken into account, nodal constrictions are predicted to increase axonal conduction.
velocity for a given fiber diameter and there is an optimal extent of constriction at which the conduction velocity is maximal. However, as we noted in the introduction, the latter study did not reveal the sole influence of nodal morphology on conduction velocity because the number of nodal sodium channels was varied linearly with nodal diameter.

In the present study we extended the analysis of Halter and Clark by investigating the influence of nodal morphology on conduction velocity for a fixed number of sodium channels per node. We confirmed that for any given axon there is an optimal extent of nodal constriction that minimizes the internodal caliber required to achieve a given target velocity, and we found that this is relatively insensitive to the number of sodium channels. However, in contrast to Halter and Clark (1993), these findings were independent of assumptions about the size and the organization of the periaxonal space. For nodal diameters larger or smaller than the optimal value, the conduction velocity decreased. While linear cable theory (Koch, 2004) does not apply directly to heterogeneous cables, it suggests that the cause of this decrease may be an increase in nodal capacitance because conduction velocity is inversely proportional to membrane capacitance (for a recent review on axon physiology, see Debanne et al. (2011). We verified this assertion through computer simulations for a nodal capacitance held artificially constant as we increased the nodal diameter (data not shown).

To explore the benefit of nodal constrictions for axonal physiology, we compared the conduction velocities of fibers with optimal nodal constrictions to the conduction velocities of fibers with the same internodal diameter but without nodal constrictions. The difference between these two conduction velocities represents the conduction velocity "gain" attributable to the nodal constriction. We found that the gain in conduction velocity increased with increasing fiber diameter and could be very substantial. The magnitude of the gain in conduction velocity depended on the number of nodal sodium channels, i.e. for small numbers of sodium channels we found a larger gain in conduction velocity. Hence,
nodal constrictions have the greatest benefit for the largest axons with low numbers of nodal sodium channels.

The longest axons in the peripheral nervous system can represent more than 99.9% of the neuronal cytoplasmic volume (Cleveland, 1996) and thus axons can represent a major metabolic drain on neuronal metabolism. While one effect of axonal constriction at nodes of Ranvier is to increase the axonal conduction velocity, our analyses highlight another and perhaps even more important benefit, which is to minimize the fiber volume. In this way, nodal constrictions reduce the metabolic investment required to form and maintain their processes. Our analyses indicate that this cost reduction is realized in multiple ways including a decrease in the volume of axonal cytoplasm (due to a decrease in the internodal axon caliber required to attain a given target conduction velocity), a corresponding decrease in myelin sheath thickness (due to the constant ratio between axon diameter and the myelin sheath thickness; see Eq. 5.4), and a decrease in the number of nodal sodium channels (due to the decrease in nodal volume).

Remarkably, the curves of all optimal fiber morphologies corresponding to a fixed given number of sodium and potassium channels showed a linear relationship within the accuracy of our computations (see solid lines in Fig. 5.4a-c and Fig. 5.5b). Each point on these straight lines represents an optimal fiber morphology, but for a different target conduction velocity. While the linearity held over a wide range of numbers of nodal sodium channels (as far as tested) the slopes of the lines changed only slightly (see Fig. 5.8). Such a linear relationship between internodal and nodal diameter has also been shown in experimental studies (Rydmark, 1981), as shown in Fig. 5.9a. It is not valid to compare our simulations directly with this experimental data because we do not know that our model faithfully describes the precise morphology and physiology in those axons, but it is nonetheless noteworthy that the slopes are comparable (compare Figs. 5.9a and 5.9b). In other words, the extent of nodal constriction in real axons (approximately 2-4 fold,
based on the slopes in Fig. 5.9a) is similar to the optimum constrictions predicted by our model (approximately 2-6 fold depending on the paranodal geometry). Thus, while we cannot say that the nodal morphology in real axons is optimal, we can say that real axons appear to follow the general design principles predicted by our model.

We noted in the results that the slope of the linear relationship between the internodal and nodal diameter in the data of Rydmark (1981) was different for axons in dorsal roots compared to ventral roots. While such differences in slope could in principle result from (large) differences in the numbers of nodal sodium channels in these axons, there could be other factors. We thus investigated factors that influence the slopes of the lines of optimal fiber morphology in our computational model. The optimal morphologies were sensitive to the choice of nodal capacitance (data not shown), but this was determined by the specific capacitance of the membrane only, which is not expected to vary much. However, the optimal morphologies were also very sensitive to the precise geometry of the axon and myelin taper in the paranodal region, which could well vary considerably. For example, while the relationship between the nodal and internodal diameters of the optimal fiber designs was linear for all tapering morphologies examined, the slopes were approximately 100-130% greater for the linear tapers compared to the step-like tapers, and approximately 50% greater for the non-linear tapers (Fig. 5.9b). This sensitivity results from the fact that the characteristic time constant associated with the effective paranodal capacitance is determined by the geometry of the entire paranodal structure, and this influences the voltage experienced by the sodium channels concentrated in the node. Thus it is possible that the difference in the slope of internodal versus nodal diameter between axons in dorsal and ventral roots noted above was due to differences in the morphology of the axon and sheath flanking the node. A detailed ultrastructural mapping of the morphology and electrical parameters of nodes and paranodes in dorsal and ventral nerve roots would be required to test this hypothesis.
To summarize, we conclude that the constriction of myelinated axons at nodes of Ranvier is a biological adaptation to minimize fiber caliber for a given target conduction velocity. The fact that these constrictions can influence fiber caliber so dramatically is quite remarkable in view of the short length of the nodal and paranodal regions (a few \( \mu m \)) compared to the long length of internodes, which can exceed 1000 \( \mu m \) for large axons. The significance of reducing fiber caliber is that it reduces the metabolic cost to the neuron for axonal growth and maintenance, in addition to reducing overall body volume. The metabolic cost is realized not just by a reduction in the number of channels and pumps required to maintain the membrane potential and excitability of the axon, but also by a reduction in the myriad cellular processes that are required to support axonal cytoplasm and their myelinating cells. Thus nodal constrictions can have significant selective benefits for organisms that myelinate their axons.
6 GENERAL DISCUSSION

6.1 Summary

Axons of myelinated neurons are constricted at the nodes of Ranvier, where they are directly exposed to the extracellular space and where the vast majority of the ion channels are located. These constrictions are generated by a reduction in the local abundance of the neurofilaments polymers, relative to the myelinated regions of the axon, which are much fatter than the nodes. In chapter five, we explored the importance of this unique design by studying the relationship between nodal constrictions and action potential signaling via computational analysis. While the nodes are short (≈ 1 µm) in comparison to the distance between nodes (hundreds of µm) they have a substantial influence on the conduction velocity of neurons. In particular, our analysis showed that for a fixed number of ion-channels nodal constrictions allow axons to (1) maximize the rate of propagation of the nerve impulse, and (2) minimize the required fiber diameter for a given target conduction velocity. We further found that the relationship between the nodal-diameters and internodal diameters that correspond to these “optimal morphologies” closely match the same relationship reported for real axons. Thus the axonal morphology at the node of Ranvier is optimized to minimize the total axonal volume occupancy within the extracellular matrix, thereby minimizing the spatial requirements and metabolic demands of these processes.

As mentioned, the above-described nodal constrictions follow from the local abundance of neurofilaments within them, which suggests that the physiological function of axon morphology is related to the transport kinetics of neurofilaments. In chapter two we investigated these kinetics by examining recorded neurofilament movement within cultures of cortical neurons from neonatal rat brains having GFP-tagged neurofilament protein M. Using epifluorescence microscopy and time-lapsed imaging techniques,
kymograph images were generated and analyzed to provide trajectories for the leading and trailing ends of the moving filaments. For our analysis, we developed an unbiased noise-filtering algorithm to extract well defined bouts of movement and pauses, which allowed us to obtain statistical distributions of pause times, run times, run lengths, and velocities. Remarkably, we found that the overall average velocities, the average velocities during a contiguous run, the run length distributions and the pause time distributions were all independent of neurofilament length across a range of lengths from 1-36 µm. We also found, that on average, retrograde directed filaments move ≈ 50% faster than anterograde directed filaments, which suggests that different families of motor proteins are responsible for the different phases of movement.

Given the observed motions seen from our experiment, we then decided to see if the intermittent motions observed for these neurofilaments could be explained by a "tug-of-war" model where different families of motors interact with one another solely through mechanical interactions. To do this we constructed a model for bidirectional cargo transport with kinesin and dynein motors (chapter 4), wherein the dynamics of each motor family are modeled in a Markovian fashion that is based off of the power-stroke model (chapter three). To test the tug-of-war hypothesis, we systematically varied the number of kinesin and dynein motors in the model and attempted to identify those combinations of motors that matched the neurofilament velocity, pause time, run time, and run length distributions in each kymograph trace. While our model fails to reproduce the extensive pause-time intervals that go beyond the order of about a minute in time, it can account for the kinetics observed when neurofilaments are moving. Upon comparing the distributions of motors that best explain the neurofilament data, we find that, on average, the total number of motors engaged during cargo transport is between 13-16 motors total. Given our average length measured for neurofilaments (chapter 2) is between 5-6 µm, our model suggests that the total number of motors that contribute to neurofilament transport
is much less than the number of motors that could potentially bind to the neurofilament. For instance, a five micron segment along a single protofilament track contains over 1200 $\alpha/\beta$ nucleotide junctions that motors could bind to while transporting their cargo, which implies that there could be, potentially, up to hundreds of motors distributed along the neurofilament. Hence, if the tug-of-war model is an accurate description of neurofilament transport, then the model predicts that the total number of engaged motors on each neurofilament is relatively small and relatively independent of polymer length. Thus the motors may not be distributed uniformly along the filaments.

### 6.2 Future work

The works discussed in this dissertation, throughout, are considered (by the author) as works in progress. There are many important questions to consider, for example the role of dynamic neurofilament structure, motor-localization along the filaments, motor-motor interactions, dynamic microtubule organizations and much more. In particular, the dynamics governing the interactions between both neurofilaments and microtubules are of particular interest. In our current study of motor-based transport of neurofilaments (chapter 4), we assumed that the motors were constantly attached to the neurofilament cargo during our simulations and accounted for the neurofilament-motor attachment kinetics by introducing an effective binding rate for the motor-microtubule interactions that was based on the average pause-time measured for neurofilaments that were analyzed via kymograph images. However, there is no reason to assume that the kinetics governing the interactions between molecular motors and microtubules is the same (or even similar) to the kinetics governing the interactions between molecular motors and neurofilaments. The fact that neurofilaments move at speeds much less than cargoes associated with fast axonal transport implies that the kinetics associated with motor-cargo binding do not follow from a single mechanism.
Another question lies in the relationship between motor-motor interactions. One of the implications of Rai’s study (Rai et al., 2013), in addition to dynein’s ability to catch-bond, was that dyneins work in a somewhat coordinated fashion when moving peroxosomes within cells. In particular, Rai observed that the average time a team of dynein motors could withstand sufficient load increased as more dynein motors participated in transporting the cargo, which was in contrast with the behavior observed for teams of kinesin motors that were moving the cargo in the opposite direction. While both dyneins and kinesins were reported to generate large forces, collectively, dynein motors appear to be much more tenacious than kinesin motors in regards to the amount of time that each team can withstand an opposing load. While our dynein model (chapter 2) mimics the catch-bond kinetics, it cannot account for the extensive stall times reported by Rai. This suggests that dynein motor teams practice a type of coordinated movement where they acclimate their behavior with respect to their local environment. In our motor models we tested the stalling kinetics for both kinesin and dynein by simulating an optical trap assay, which verified that our model could reproduce the stalling times (for individual motors) reported by Rai et al. (2013). However, in our tug-of-war model, each motor’s enzymatic cycle is modeled separately from one another and motor-motor interactions follow strictly from each motor’s respective force-generation, which is then felt by all of the other motors. Hence, there is no coordination between these motors meaning they all practice the same stalling kinetics as a single dynein motor. Hence, each motor can withstand sufficient loads for about the same time. While it is true that teams of motors acting on a cargo can withstand sufficient loads for longer times by merely distributing the load across more motors, the fact that our tug-of-war model does not account for the observed pause-time intervals observed for neurofilaments that go beyond about a minute in time, suggests that an additional mechanism might be needed to account for such behavior. However, since the running kinetics for neurofilaments can be explained by a
tug-of-war mechanism, it is unclear how such a mechanism would be incorporated into our model in such a way to not impair its ability to explain the running kinetics of these filaments. As a suggestion, one could consider a model that follows the tug-of-war framework that we have captured in our study, which further incorporates a mechanism that modulates the overall turnover rate of the dynein motors relative to the number of dyneins participating in the tug-of-war. In doing so, one could–potentially–alter the stalling kinetics for groups of motors such that their stalling behavior followed the reports of Rai et al. (2013).
BIBLIOGRAPHY


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