

**Regulation Mechanisms in Molecular Motors: Engineering
Bioinspired Transportation at Micron-Scale**

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Acknowledgements

Reflecting upon the journey that led me to this point, I am reminded of the time when I wasn't confident in my abilities and skillset to produce any scientifically valuable contribution. I find myself humbled and grateful for the unwavering support, guidance, and inspiration I have received from a remarkable group of individuals who have played pivotal roles in my pursuit of knowledge.

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Dedication

Dedicated to the working mothers who nurture curiosity and character, including my exceptional mother and my loving wife. Your collective dedication is an inspiration to us all.

Abstract

Intracellular cargo transport, orchestrated by teams of molecular motors on cytoskeletal tracks, is a fundamental process within eukaryotic cells. This thesis presents a comprehensive investigation into multi-motor cargo transport, employing an array of cutting-edge tools, including instrumental, computational, and DNA-based single-molecule methods. These diverse approaches converge to unveil unprecedented insights into the coordination mechanisms of molecular motors and the regulation of intracellular cargo transport.

The research explores the impact of various mechanical and biochemical factors, including cargo-motor linkage stiffness, the influence of adapter proteins like GIPC, and cargo-motor interaction kinetics, on multi-motor cargo dynamics. Through detailed analyses, the interplay of these factors is deciphered, shedding light on the intricate regulation of cargo transport, including pausing phenomena and forces acting on the actin network.

Notably, this work extends its focus beyond fundamental biology to propose novel avenues for engineering bioinspired transportation systems at the micron-scale and beyond. The insights gained from this research not only contribute to our understanding of cellular complexities but also highlight promising solutions to micro-scale transport challenges. Moreover, this thesis provides a glimpse into emerging tools and methodologies that hold the potential to revolutionize the field, opening up new horizons for realizing efficient and precise transportation at micron and sub-micron scales. As we continue to unlock the mysteries of intracellular cargo transport, the integration of these multidisciplinary approaches paves the way for groundbreaking innovations in diverse domains, from drug delivery to nanotechnology, and beyond.

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Chapter 1

Introduction

A cell, the fundamental biological unit and building block of life, is often likened to a complex clockwork system composed of interconnected mechanical elements [1]. The century-old paradigm of cells being mere reaction chambers is rapidly evolving, revealing an increasing number of dynamic processes within the cell. These processes, ranging from cell migration to cell division, rely on intricate mechanochemical mechanisms that demand exceptional spatial and temporal precision. A vital component of intracellular function is intracellular transport, a process enabled by molecular motors—nanometer-sized proteins that employ a stepping motion to 'walk' along intracellular tracks, which consist of two main types: microtubules and actin filaments, both of which are composed of proteins and serve distinct roles in cellular transport.

There are three major superfamilies of molecular motors that play prominent roles in various cellular processes: Kinesin, Dynein, and Myosin.

Kinesins are predominantly associated with the transport of cargo toward the plus-end of microtubules, which are cylindrical structures formed by polymerized tubulin proteins [2]. They are crucial for processes such as intracellular trafficking and cell division. Kinesins are often found in the cytoplasm, participating in the movement of vesicles and organelles to specific locations within the cell.

Dyneins on the other hand, move cargo in the opposite direction, toward the minus-end of microtubules. These microtubules, composed of α -tubulin and β -tubulin subunits, serve as the tracks for dynein-mediated transport [3, 4]. Dyneins are essential for processes such as retrograde axonal transport and the positioning of cellular organelles,

particularly in nerve cells. Dyneins are typically located near the cell periphery, including the axons and dendrites of neurons.

Myosins primarily function in the movement of cargo along actin filaments, which are composed of globular actin (G-actin) subunits polymerized into long, helical filaments [5]. Actin filaments are essential for processes such as muscle contraction, cytokinesis, and organelle transport. Myosins are distributed throughout the cell, with specific isoforms present in muscle cells, as well as various non-muscle cell types.

These distinct molecular motor families are integral to the proper functioning of various cellular processes, ensuring the timely and precise delivery of cargo to specific cellular destinations. Failure or dysregulation of these motors can lead to a range of cellular malfunctions and diseases, underscoring their importance in cell biology and disease pathology.

Molecular motors play critical roles in numerous intracellular processes, including chromosome migration during cell division, vesicle transport, waste elimination, and endocytosis. Often, multiple motors work in coordination to accomplish these tasks. The timely and precise delivery of cargo to its specific destination is crucial, as failures or delays are associated with various diseases, such as Huntington's and Alzheimer's [4]. Hence, motor proteins, the microtubules and actin filaments they move on, and the cargos they transport collectively form a complex transportation infrastructure within the cell. Protein-protein interactions are fundamental to this transport system, and any malfunction, akin to 'traffic jams' in this network, can have life-threatening consequences, as seen in the case of the fatal disease Amyotrophic Lateral Sclerosis (ALS) [6].

To decipher the mechanisms underlying these diseases and explore potential cures, a fundamental understanding of this intracellular transportation infrastructure is imperative. Often, the root causes of failures can be traced back to abnormalities in intracellular processes, which are, in turn, linked to mutations in molecular motors or anomalies in their interactions with each other.

Hence, a central question in the study of intracellular transport is how its processes are regulated. Studies have indicated that cargo velocities and distances covered depend on factors such as the number of motors carrying a common cargo, the presence or absence of specific adapter proteins, and more. Additionally, mutations can alter the physical parameters of motors, affecting their flexibility or load-bearing capacity

and consequently disrupting their normal functions. However, the small size of these molecular motors, typically around 100 nm, presents a significant technical challenge for accurate investigation. This challenge has prompted opportunities for substantial technical advancements, leading to key scientific discoveries. Although fluorescence microscopy has been invaluable, it alone is insufficient for thorough molecular motor investigation. Researchers worldwide have turned to optical tweezers and atomic force microscopes, employing force spectroscopy techniques to uncover essential insights into disease mechanisms.

Furthermore, biophysicists and mathematical biologists have applied probability theory to understand the stochastic nature of these molecular motors, allowing for highly accurate behavior predictions. However, significant technical challenges and gaps persist. Investigating multiple molecular motors simultaneously at high temporal and spatial resolutions remains an arduous task, and computational techniques become increasingly expensive as the number of motors under examination grows. Moreover, the coordination among multiple molecular motors implies the existence of a communication protocol among them, a phenomenon still not fully understood. This presents a fertile ground for innovation and discovery.

Yet, the implications of our investigations reach beyond the confines of the cell. The intricate choreography of molecular motors provides inspiration for engineering bioinspired transportation systems at the micron scale—an endeavor that promises transformative innovations across a spectrum of applications. As we delve deeper into the mechanisms that govern the actions of these molecular machines, we unlock the potential to create micron-scale transportation systems that can revolutionize fields such as drug delivery, nanotechnology, and micro-robotics.

Recent advancements at the intersection of biology and engineering have showcased the remarkable progress in crafting bioinspired mechanochemical systems at the micron scale. In an exciting breakthrough, Hendrik Dietz and his team harnessed the principles of DNA origami to engineer a rotary motor at the molecular level [7]. Their innovative work demonstrates the transformative power of understanding mechanochemical biomolecules and harnessing biomimetic principles to create artificial systems that mimic nature’s architectural precision.

In this thesis, we embark on a multifaceted journey. We delve into the intricacies

of novel factors responsible for intracellular transport regulation by molecular motors. Simultaneously, we embrace the possibilities that this knowledge affords us, as we seek to engineer bioinspired micron-scale transportation systems. Our three-pronged approach—comprising the development of experimental assays, predictive software tools, and cutting-edge instruments—enables us to explore uncharted territories, making critical observations that were previously hidden from view.

This thesis serves as both a testament to our curiosity about the inner workings of life’s fundamental unit and a beacon of hope for the transformative possibilities that lie ahead. It underscores the immense potential that emerges at the intersection of biology, engineering, and innovation.

The primary objective of this thesis is to identify novel factors that regulate intracellular transport, and then eventually use the insights obtained to inspire engineered transportation at micron-scale . To achieve this goal, a three-pronged approach is adopted: (1) Develop biochemical experimental assays engineered to isolate the effects of one factor at a time. (2) Create predictive software tools to explore scenarios where experiments are not feasible and to guide new experiments. (3) Design new instruments capable of probing intracellular transport with improved spatial and temporal resolutions. This thesis is organized such that each chapter presents the development of the aforementioned tools followed by their application to make critical, previously unknown observations regarding molecular motor regulation and multi-motor coordination processes. Briefly,

- Novel DNA origami based single molecule methods to decorate artificial cargoes with known number of molecular motors are presented in chapter 2. Subsequently a detailed account of how these methods can be used to understand the effect of attachment strength between the cargo and molecular motor on cargo transport properties. A future extension of this work is under progress where DNA origami is used to create an *in-vitro* tug-of-war like scenario between different kind of molecular motors to understand the intricacies of a bi-directional transport by two opposite directed motors.
- A detailed account of study elucidating the role of GIPC adapter protein on myosin VI kinetics and transport properties is presented in chapter 3.

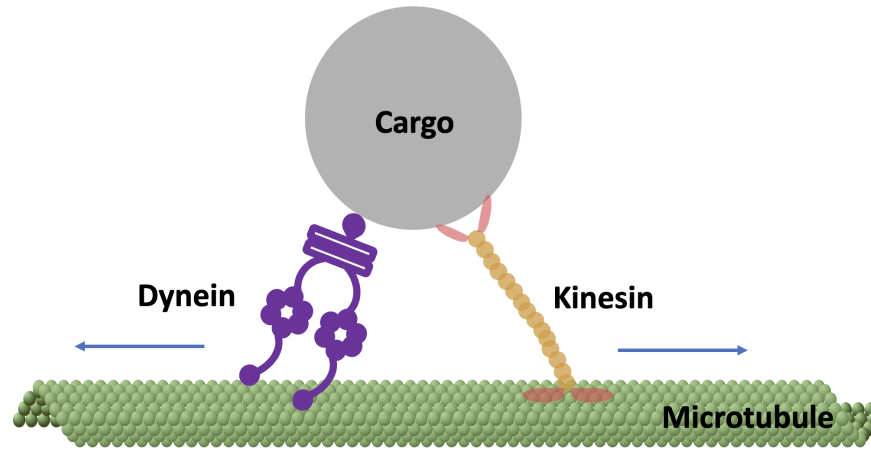


Figure 1.1: Schematic of intracellular cargo transport process by molecular motors kinesin and dynein. Figure recreated based on schematic presented in [8].

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- Chapter 4 deals with novel extensions of the existing markov chain based computational tools in order to predict the transport of cargo by multiple molecular motors while motors are weakly attached to cargo and are capable of stochastically detach from and reattach to the cargos. A possible extension of these software tools to incorporate bi-directional motors is also presented.
 - Chapter 5 covers the details of instrumentation including the development for a hybrid instrument which combines an optical tweezer with a TIRF Microscope which enables optical trapping of cargos while simultaneously imaging the molecular motors on the cargo.
 - Finally, in chapter 6 I present an exposition on how can this research direction be propelled further drawing attention to still unsolved problems, subsequently concluding the thesis.

Chapter 2

Effect of Cargo-Motor Linkage Stiffness

We examine the effect of cargo-motor linkage stiffness on the mechanobiological properties of the molecular motor Myosin VI. We use the programmability of DNA nanostructures to modulate cargo-motor linkage stiffness and combine it with high precision optical trapping measurements to measure the effect of linkage stiffness on motile properties of Myosin VI. Our results reveal that a stiff cargo-motor linkage leads to shorter step sizes and load-induced anchoring of Myosin VI, while a flexible linkage results in longer steps with frequent detachments from the actin filament under load. Our findings suggest a novel regulatory mechanism for tuning the dual cellular roles of anchor and transporter ascribed to Myosin VI.

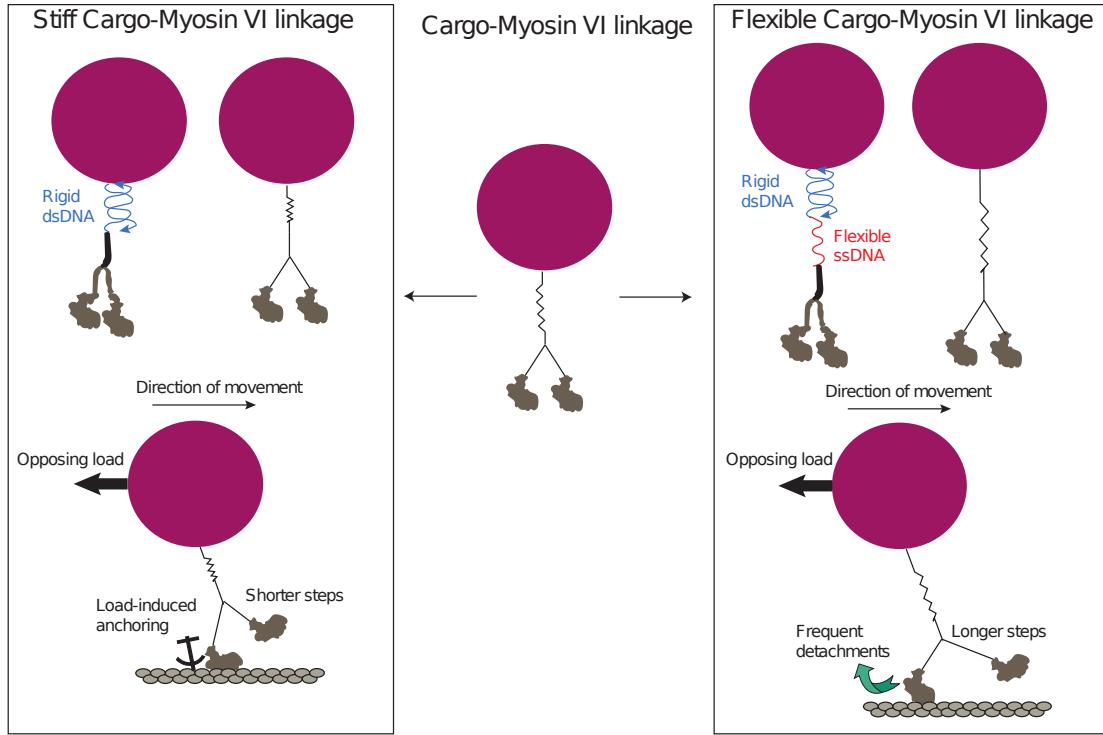


Figure 2.1: Summary Graphic

2.1 Background

Bustamante et al. describe the cell as a "clockwork" made up of multiple interconnected mechanical elements rather than a "reaction vessel", the analogy which was prevalent among biochemists more than half a century ago [1]. This emerging paradigm in cell biology emphasizes the importance of mechanical communication in cellular functions. Cellular processes such as cell adhesion, organelle transport, and migration rely on mechanical communication in macromolecular clusters. Effective force transmission in such ensembles depends on the mechanical properties of the linkage between biomolecules. In this study, we focus on the impact of linkage stiffness on the mechanical communication between groups of molecular motors driving cargo transport along cytoskeletal filaments.

Cytoskeletal motors link to their cellular cargoes through diverse mechanisms ranging from adaptor protein intermediates to lipid molecules that directly bind the motor [9,10].

The diversity in cargo-motor attachments translates into distinct linkage stiffness regimes in cellular membrane traffic. Both experimental [11–13] as well as theoretical studies [14–17] have shown that linkage stiffness alters motile properties, such as velocity and run length of cytoskeletal motors. However, the mechanistic basis of these changes, in terms of stepping behavior and force generation is not well understood and forms the focus of this study.

To investigate the impact of linkage stiffness on stepping behavior and force generation of individual motors in ensembles, we need experimental systems wherein the motor number and linkage stiffness can be controlled in concert with high precision force spectroscopy techniques. We have previously demonstrated [18] that DNA origami scaffolds offer programmability of motor number and linkage stiffness to impact motile properties. Here, we use optical trapping to measure the intersection of linkage stiffness and force sensitivity of Myosin VI. We have adapted the system to couple DNA origami to micron-sized spherical beads that are subsequently used as probes in an optical trapping setup. Our assay provides resolution of piconewton forces on the motor coupled with measurements of motor movement with nanometer precision, while varying cargo-motor linkage stiffness.

2.2 Results

The protocol for coupling DNA origami to beads and oligo-labeled Myosin VI is shown in Figure 2.2A. Briefly, origami is coupled to avidin-coated beads via a biotinylated DNA strand whereas the oligo-labeled Myosin VI is linked to the bead-origami system via complementary base pairing [19]. To modulate cargo-motor linkage stiffness, the length of the oligo coupled to Myosin VI is varied to achieve a stiff or flexible linkage of the motor to the cargo (Figure 2.2A, inset). To quantify the change in cargo-motor linkage stiffness, the motor linkage stiffness is measured through a clamping of the bead variance that occurs when a motor engages with the actin filament and exerts force (Figure 2.2B). The optical trap and the motor linkage can be modeled as springs attached in parallel to the bead (Figure 2.2C). Thus, the stiffness measured during a motor binding event is a sum of the stiffness of the trap and that of the motor linkage. The stiffness of the linkage can be estimated from the difference between stiffness measured with the

motor bound to the actin filament and the trap stiffness measured with the unbound bead. Moreover, the motor linkage is a combination of the oligo spring attaching the motor to the origami and the inherent motor spring connected in series to each other (Figure 2.2C, inset). Since Myosin VI is common for both a stiff and a flexible linkage, the change in the linkage stiffness is essentially a function of the oligo spring stiffness. Quantification of the linkage stiffness distributions measured for a stiff versus flexible linkage is shown in Figure 2.2D. We observe a significant decrease ($P < 0.001$) in cargo-motor linkage stiffness when Myosin VI is coupled to DNA origami through a flexible oligo link (Mean = 0.007 ± 0.003 pN/nm) when compared to a stiff oligo linkage (Mean = 0.012 ± 0.004 pN/nm). Since the applied load could have an impact on the stiffness of the linkage, we measured changes in linkage stiffness as a function of load (Figure 2.2E and Figure 2.2F). Indeed, we observed a load-dependent stiffening of the entire linkage at higher loads for both stiff and flexible linkage geometries. Given that the majority of events (~ 75 %) occur at low loads (< 0.5 pN), the use of single/double stranded DNA linkages to modulate stiffness is appropriate for motor ensembles under low load conditions.

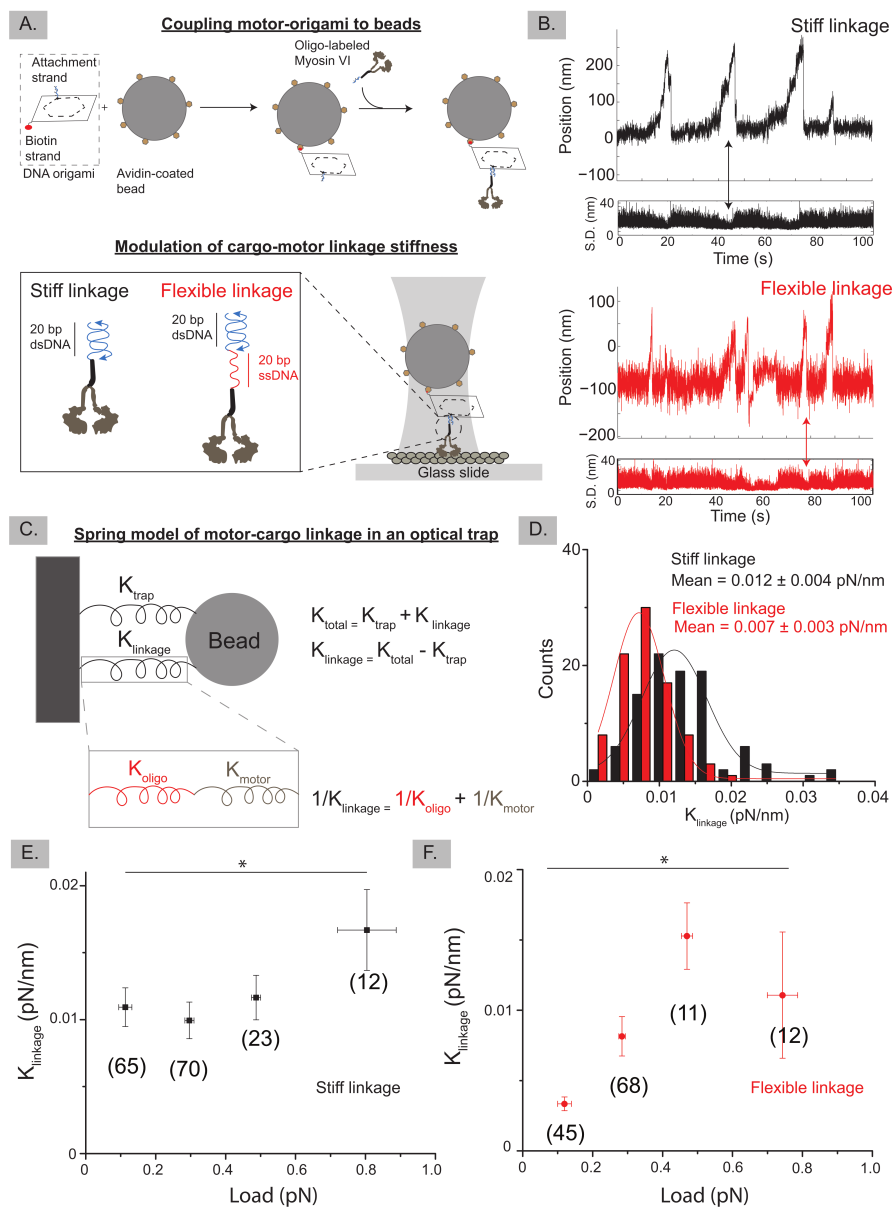


Figure 2.2: (A) Schematic illustrating the protocol for coupling DNA origami scaffolds and oligo-labeled Myosin VI to beads for optical trapping (top) and the rationale for changing linkage stiffness via the length of the ss-DNA oligo coupled to Myosin VI (inset, bottom). (B) Representative position v/s time plots of Myosin VI force generation events in an optical trap when connected via a stiff versus flexible linkage. Also shown is the standard deviation (S.D.) in position to illustrate the clamping of bead variance (double-headed arrow) that occurs when Myosin VI binds to an actin filament. (C) Model of cargo-linkage and optical trap coupled to the bead as elastic springs in parallel. The inset shows the cargo linkage spring as being a combination of the oligo and Myosin VI spring connected in series to each other.

(continued)

Figure 2.2: (D) Distribution of linkage stiffness for a stiff v/s flexible linkage. Both distributions have been fit to a single-peak Gaussian with $R^2 = 0.87$ for stiff and 0.99 for a flexible linkage. $n = 97$ and 89 variance clamping events pooled from three different protein preparations of Myosin VI were used for linkage stiffness calculation for the stiff and flexible linkage respectively. Z-test was used to estimate the statistical significance of the difference between mean stiffness with $P < 0.001$. Error values are reported as standard deviation. (E) and (F) Linkage stiffness plotted as a function of load for stiff and flexible linkage. Error bars are standard deviation. The number in parentheses below each data point indicates the number of events used to generate the values of that data point. Significance between the linkage stiffness at the lowest load and highest load points was estimated using a one way ANOVA with a post hoc Tukey's test ($P < 0.05$). The overall weighted average load experienced by a stiff linkage (0.32 pN) was found to be higher than that of a flexible linkage (0.26 pN).

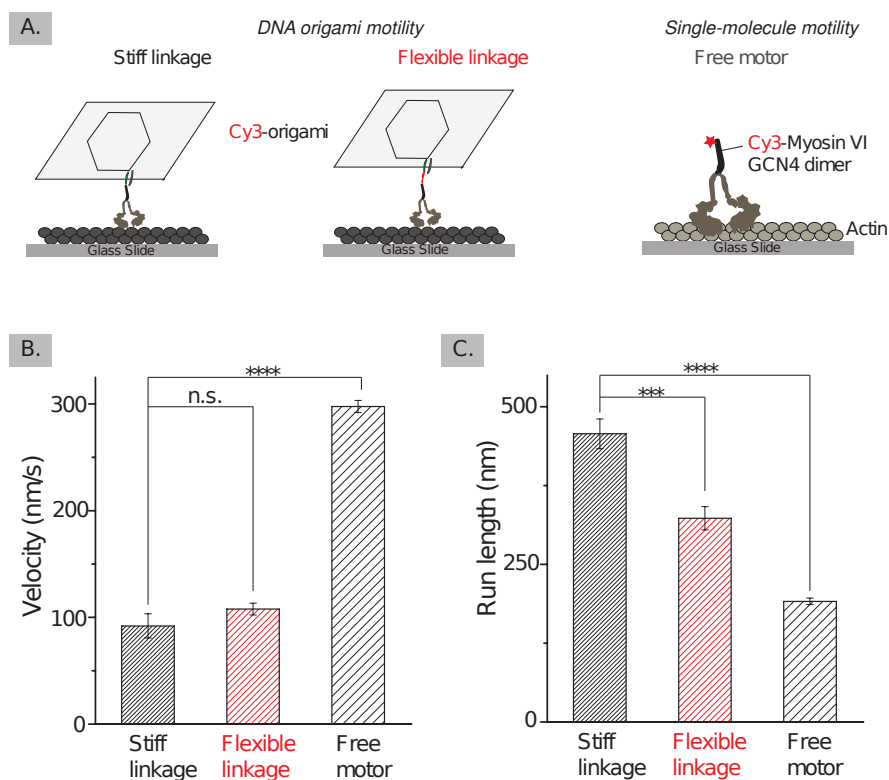


Figure 2.3: (A) Schematic for the motility assay when origami is attached to the Myosin VI via a stiff linkage (left), via a flexible linkage (middle), and in case of no origami, i.e. free motor (right). (B) Average velocities (nm/s) for stiff linkage, flexible linkage and free motor case. (C) Average run lengths (nm) for stiff linkage, flexible linkage and free motor case. Student's t-test was used to estimate the significance of the difference between mean values of velocity and run length. $N > 1000$ motility events across three different protein preparations were used to get mean values of velocity and run length. Error values are reported as standard error of mean.

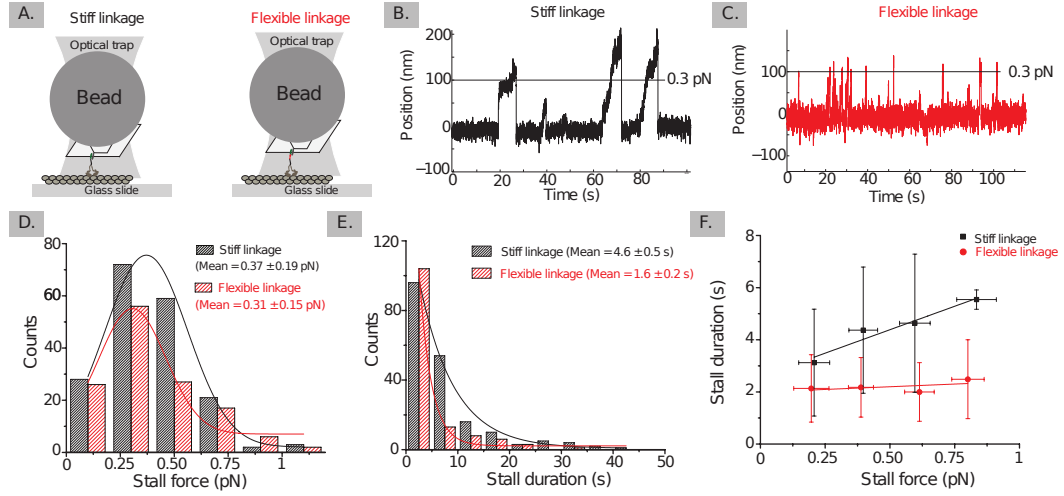


Figure 2.4: (A) Schematic illustrating the optical trapping geometries with stiff and flexible cargo-motor linkages for Myosin VI. (B) and (C) Representative force generation events with stiff and flexible linkage for Myosin VI respectively. As illustrated, stiff linkage is characterized by force generation events which last for a longer duration of time as compared to flexible linkage events. (D) Distribution of stall forces for Myosin VI with stiff (black) and flexible (red) cargo-motor linkage. $R^2 = 0.99$ and 0.87 for the Gaussian fit of stiff and flexible linkage respectively ($P < 0.01$). (E) Stall duration distributions for stiff versus flexible linkage fit to a single exponential decay with mean values indicated in the plot. $R^2 = 0.98$ and 0.99 for the single exponential fit of stiff and flexible linkage respectively. Flexible linkage exhibits significantly shorter ($P < 0.001$) stall duration as compared to a stiff linkage. (F) Correlation of stall force against stall duration as a measure of the ability of Myosin VI to sustain load. For a stiff linkage, there is a linear improvement in the stall duration with increasing stall force which does not happen for a flexible linkage of Myosin VI (smaller slope). Slope of linear fit was 3.59 s/pN for stiff and 0.41 s/pN for flexible linkage. $n = 192$ and 136 force generation events pooled from three different protein preparations of Myosin VI were used for stall force and stall duration calculation for the stiff and flexible linkage respectively. Error bars are standard deviation. Z-test was used for statistical significance in differences.

After quantifying the change in linkage stiffness using a flexible oligo link, we investigated the effect of linkage stiffness on motility of Myosin VI attached to DNA origami. The unloaded motility of stiff vs. flexible linkage was assessed as shown in Figure 2.3B. Single-molecule motility of Myosin VI without any cargo attached (free motor) is used as a control to assess the effect of cargo binding and cargo size on motility. We observe that the velocities for Myosin VI bound to DNA origami (92 ± 11 nm/s for stiff linkage and 107 ± 5 nm/s for flexible linkage) are lower as compared to velocities for Myosin VI without DNA origami (297 ± 5 nm/s). In contrast, the run lengths for Myosin VI bound to DNA origami (457 ± 23 nm for stiff linkage and 323 ± 18 nm for flexible linkage) are

higher as compared to run lengths of Myosin VI without DNA origami (191 ± 5 nm). The physical basis of the differences observed in motile properties of the free versus origami-bound Myosin VI is unclear. Nonetheless, the velocities with flexible and stiff linkages were not found to be significantly different. In contrast, there was a significant decrease ($P < 0.001$) in run length observed with a flexible linkage (Mean = 323 ± 18 nm) as compared to a stiff linkage (Mean = 457 ± 23 nm) (Figure 2.3C). The increase in run length with a stiff linkage could result from an effect of linkage stiffness on the load-dependent motor properties of Myosin VI such as step size and detachment rate. Thus, we next measured the impact of linkage stiffness on load-dependent motor properties of Myosin VI using optical trapping (Figure 2.4A).

To investigate the effect of linkage stiffness on Myosin VI behavior under load, we first quantified the force at which the motor stalls (stall force) and the time for which the motor is able to sustain the stall load (stall duration). A stall event is defined as the slowing down of motor velocity to <10 nm/s during a force generation event in the optical trap. Stall duration was the time duration from the beginning of a stall event to the point at which the motor detached and the bead fell back to the center of the trap. The force-generation profile of Myosin VI in Figure 2.4B and Figure 2.4C shows that Myosin VI connected via a stiff linkage exhibits longer stalling events, whereas Myosin VI connected via a flexible linkage displays multiple shorter stall events in a similar time window. The stalling profile of Myosin VI was quantified in terms of stall force and stall duration. The stall force distributions shown in Figure 2.4D indicate that there is an increase ($P < 0.01$) in the stall force for Myosin VI connected with a stiff linkage (Mean = 0.37 ± 0.19 pN) compared to that of a flexible linkage (Mean = 0.31 ± 0.15 pN). Also, the stall duration (see Figure 2.4E) was significantly higher ($P < 0.001$) for Myosin VI connected with a stiff linkage (Mean = 4.6 ± 0.5 s) compared to that of a flexible linkage (Mean = 1.6 ± 0.2 s). Furthermore, the stall duration showed a higher rate of increase with increasing stall force (see Figure 2.4F) for Myosin VI when it is attached via a stiff linkage (Slope = 3.59 s/pN) as compared to Myosin VI connected via a flexible linkage (Slope = 0.41 s/pN). This faster rise in stall duration with increasing load for Myosin VI attached through a stiff linkage parallels the load-induced anchoring of Myosin VI seen in earlier optical trap measurements [20, 21].

To understand the effect of linkage flexibility on stepping behavior of Myosin VI,

we analyzed the optical trapping data to obtain the step size and time interval between the subsequent steps (dwell time) of Myosin VI connected via stiff and flexible linkage to the beads (Figures 2.5A and 2.5B). Step sizes distributions (Figure 2.5C) reveal a significant increase ($P < 0.001$) in step size for Myosin VI connected via a flexible linkage (Mean = 28 ± 13 nm) as compared to a stiff linkage (Mean = 19 ± 10 nm). It was also observed that dwell time between steps changed considerably with linkage stiffness (Figure 2.5D). The mean dwell time for Myosin connected via a stiff linkage (Mean = 0.9 ± 0.5 s) was found to be significantly higher ($P < 0.001$) than that for a flexible linkage (Mean = 0.3 ± 0.1 s). Since both step size and dwell time are shown to be load-dependent for other processive motors [22, 23], we next measured step size and dwell time as a function of applied load for both stiff and flexible linkage (Figure 2.5E-H). We observed no significant trend in step size and dwell time of Myosin VI with increasing load for both stiff as well as flexible linkages. It is important to note here that our measurements are performed at physiological ATP concentrations (2 mM ATP) and dwell time has previously been shown to be dependent on ATP concentration [21]. Therefore, our observations here are consistent with previous literature of Myosin VI stepping where neither the step size nor the dwell time of Myosin VI were observed to have a load dependence at physiological ATP concentrations and loads less than 2 pN [21].

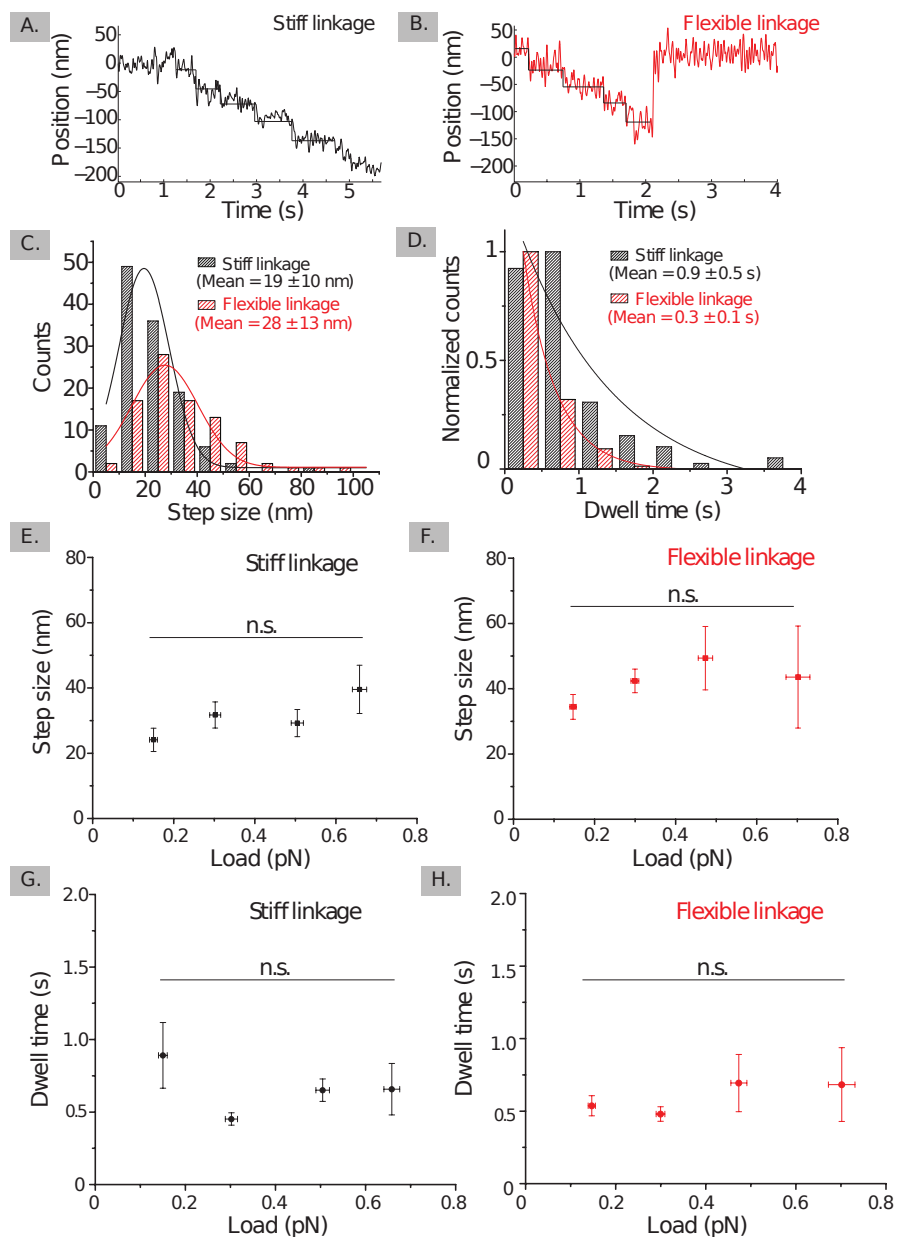


Figure 2.5: (A) and (B) Representative bead position data showing stepping profile for Myosin VI attached with a stiff and a flexible linkage. (C) Distribution of step sizes of Myosin VI with stiff (black) and flexible (red) cargo-motor linkage. The distributions are fit to a single-peak Gaussian with $R^2 = 0.93$ and 0.88 for stiff and flexible linkage respectively. There is an increase in mean step size of Myosin VI connected with a flexible linkage ($P < 0.001$).

(continued)

Figure 2.5: (D) Distribution of dwell times of Myosin VI with stiff (black) and flexible (red) cargo-motor linkage. The dwell time distributions are fitted to a single exponential with $R^2 = 0.99$ and 0.96 for stiff and flexible linkage respectively. Myosin VI attached with a stiff linkage shows a significantly higher mean dwell time ($P < 0.001$) as compared to that of a flexible linkage. $n = 124$ and 89 stepping events pooled from three different protein preparations of Myosin VI were used for step size and dwell time calculation for the stiff and flexible linkage respectively. (E) and (F) Step size as a function of load for stiff and flexible linkage. Error values are standard deviation. Significance between the linkage stiffness at the lowest load and highest load points was estimated using a one way ANOVA with a post hoc Tukey's test. (G) and (H) Dwell time between steps as a function of load for stiff and flexible linkage. Error bars are standard deviation. Significance between the linkage stiffness at the lowest load and highest load points was estimated using a one way ANOVA with a post hoc Tukey's test. Z-test was used for statistical significance in differences. Error bars are standard deviation.

2.3 Chapter Summary

In this study, we have investigated the impact of cargo-motor linkage stiffness on Myosin VI transport. A stiff cargo-Myosin VI linkage results in load-induced anchoring of Myosin VI characterized by higher stall duration, longer dwell time between steps and shorter step sizes. In contrast, a flexible cargo-Myosin VI linkage leads to frequent detachments under load resulting in shorter stall duration and dwell time between steps. Interestingly, however, a flexible linkage leads to longer step sizes for Myosin VI presumably through extension of the effective Myosin VI lever arm [24]. Theoretical modeling of motor transport has previously predicted an inverse relationship between dwell time and load-dependent detachment rate of the motor from the filament [25]. Also, Kunwar et al. have shown through Monte Carlo simulations of motor transport that a decreased linkage stiffness leads to impaired collective motor function due to stretching of the linkage and higher detachment rates [26]. Our results provide experimental validation for such a role of linkage stiffness in regulating the load-dependent behavior of the motor suggested by the aforementioned theoretical models. A flexible linkage provides greater Brownian diffusion of the two heads relative to the cargo scaffold ($\Delta x = \sqrt{K_b T} / \sqrt{K_{linkage}}$; Figure 2.5C) stochastically enabling a larger step size. In contrast, the flexible linkage yields smaller resistive loads (Figure 2.2E,F) for the same distance traveled by the myosin dimer. Consequently, the load-induced anchoring phenomenon that has been well-documented for myosin VI [21, 27] is not engaged as frequently in the presence of a flexible linkage.

Myosin VI has been ascribed dual functionality as a transporter and a tether function inside the cell [28]. It acts as a transporter to processively move organelles such as endosomes and secretory vesicles, whereas it functions as a molecular tether to maintain the structure of stereocilia in inner ear hair cells [29]. Myosin VI was shown to display load-induced anchoring at higher loads in optical trapping experiments [20, 21], which likely forms the mechanistic basis for its dual functions. However, previous studies did not explore a role for linkage stiffness in the load-induced anchoring behavior of Myosin VI. Our results here highlight the cargo-Myosin VI linkage stiffness as an additional layer of regulation for load-induced anchoring of Myosin VI whereby both transporter and anchor roles can be tuned by the mechanical properties of the motor-cargo attachment.

In conclusion, the methodology described in this study combines DNA origami with optical trapping and opens up avenues of studying the effect of cargo-motor linkage stiffness on motor transport. Enabled by the methodology, our results reveal that the mechanical properties of cargo-motor linkage play a key role in regulating cellular context-dependent function of Myosin VI. This work was presented in American Physical Society’s annual meeting [30], and published in American Chemical Society’s Biochemistry Journal, featuring as a cover article [31].

2.4 Future Direction

The above methodology can be extended to include bidirectional motors on the same cargo and recreate tug of war like scenarios *in-vitro*. For instance, Myosin V and Myosin VI were attached to same bead using a DNA origami with two ss-DNA attachment strands of different types, one having complementarity to Myosin VI strand and the other to Myosin VI. In parallel, a computational toolbox is also under development [32,33] to inform future experiments and validate existing experimental findings.

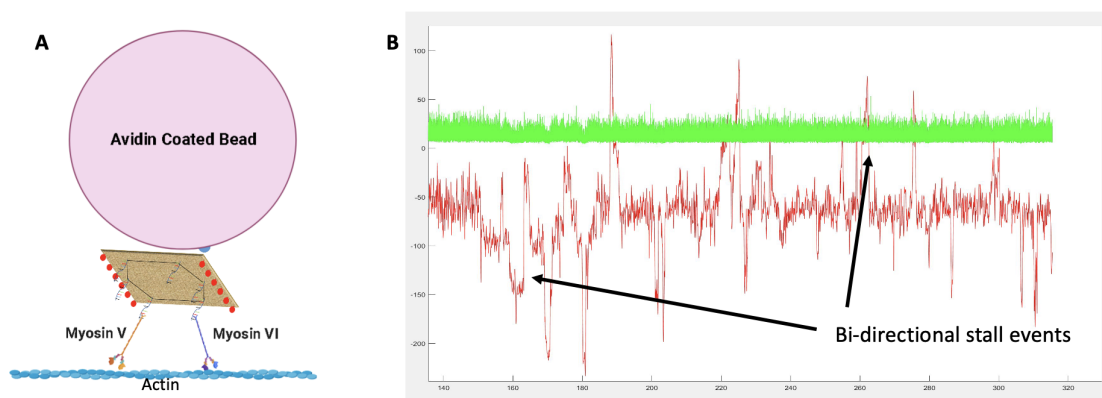


Figure 2.6: Tug Off War between myosin V and mysosin VI demonstrated using DNA Origami. (A) Schematic showing the assembly of a cargo with both myosin V and VI simultaneously attached to it using a two-site ab type DNA Origami with stiff linker. (B) Optical trapping data with bi-directional stalls.

2.5 Materials, Methods, and Supporting Information

2.5.1 Buffer and reagents

Assay Buffer (AB): 25 mM imidazole (pH 7.5), 4 mM MgCl₂, 1 mM EGTA, 25 mM KCl, 1 mM DTT; AB.BSA: AB buffer + 1 mg/ml BSA; AB.BSA.Cam: AB.BSA buffer + 9 μ M calmodulin; AB.BSA.nt: AB.BSA + 10 nM 42 nt random oligo sequences; AB.BSA.nt.Cam: AB.BSA.nt + 9 μ M calmodulin.

2.5.2 Benzyl guanine (BG) conjugation of ss-DNA attachment strand

Custom made 5' C6-amine modified oligonucleotides were covalently conjugated with benzyl-guanine NHS ester (BG GLA-NHS; NEB). Briefly, 170 μ M oligo was incubated with 3.2 mM BG GLA-NHS in 100 mM sodium borate (pH 8.5) at 37°C with rotation for 4 hours. The oligo was desalted on G-50 MicroColumns (GE Healthcare) and the oligo concentration was determined using UV absorbance at 260 nm (Nanodrop). BG-oligo was stored at 4°C and used for protein labeling.

2.5.3 Myosin VI expression, purification and conjugation with BG-oligo

The procedures for Myosin VI expression and purification from Sf9 insect cells and the labeling of the motor with BG-ssDNA attachment oligo has been described by us previously [18, 19]. The Myosin VI (UniProt Accession number Q9UM54) construct consisted of an N-terminal motor domain and lever arm followed by a GCN4 leucine zipper motif to artificially dimerize the motor followed by a C-terminal SNAP and FLAG tags for oligo labeling and affinity purification respectively. An anti-FLAG resin based affinity purification was used to bind the protein from clarified Sf9 cell lysates. Myosin VI bound to anti-FLAG resin was incubated with excess (>10 μ M) of BG oligo overnight at 4°C with rotation for oligo labeling of the protein. Excess oligo was removed by washing the resin three times with buffer containing 20 mM Imidazole pH 7.4, 3 mM DTT, 1 μ g/ml PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Myosin VI labeled with the BG-oligo attachment strand was eluted from the anti-FLAG resin with 0.2 mg/ml FLAG-peptide (Sigma) and was stored at -20°C in 55% glycerol as a cryoprotectant.

Temperature Range	Degree Change	Time Interval
90-80 °C	0.1 °C	3 sec
80-70 °C	0.1 °C	3 sec
70-60 °C	0.1 °C	6 sec
60-50 °C	0.1 °C	30 sec
50-40 °C	0.1 °C	6 sec
40-30 °C	0.1 °C	6 sec
30-20 °C	0.1 °C	6 sec
4 °C	Hold	Hold

Table 2.1: Annealing protocol for DNA nanostructures

2.5.4 DNA Origami preparation

The DNA strands used for origami preparation have been listed previously [19]. Nanostructures were prepared by mixing single-stranded M13mp18 DNA (NEB) with excess of short staple strands (IDT) in TAE-Mg₂ + buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, 12.5 mM MgCl₂) followed by the annealing protocol listed in Supporting Information Table 2.1. Each scaffold contains 23 Cy5 molecules for imaging and a biotinylated strand to facilitate conjugation of nanostructures to avidin-coated beads.

2.5.5 Purification of Myosin-Origami scaffolds for motility assay

Cy5-nanostructures were incubated for 10-15 min with streptavidin-coated magnetic beads (NEB) at 30 °C with shaking in AB.BSA.nt buffer. Resin was washed three times with AB.BSA.nt and then incubated with excess (150 nM for saturably labeling 1-site origami) oligo conjugated Myosin VI for 10-15 min at 30 °C with shaking. The origami labeled with Myosin VI was eluted off the streptavidin magnetic beads by incubation with 0.1 μ M of ss-DNA elution strand in AB.BSA.nt.Cam buffer which binds to the biotinylated DNA strand on the origami and causes a strand displacement reaction to release the biotinylated strand from the origami.

2.5.6 Motility assay with DNA origami on single actin filaments

Motility assays were performed using plasma-cleaned glass coverslips coated with 0.1% colloidin in amyl acetate (Corning, 22 X 22 mm). Flow chambers were prepared by

adhering the colloidin coated glass coverslips to glass slides (Fisher Scientific, 3" $\times$ 1" $\times$ 1 mm) using strips of double-sided tape. A biotin-neutravidin linkage was created on the surface by flowing in biotin-conjugated BSA at 1 mg/ml and neutravidin at 0.2 mg/ml. Excess biotin and neutravidin was removed by three washes with AB.BSA. Biotinylated Alexa-488 F-actin filaments were adhered to the surface via the BSA biotin-neutravidin linkage. Purified myosin-DNA origami complexes in AB.BSA.CAM.nt supplemented with motility assay reagents (2 mM ATP, 1 mM phosphocreatine, 0.1 mg/ml creatine-phospho-kinase, 25 μ g/ml glucose-oxidase, 45 μ g/ml catalase, 1% glucose, 1 μ M random library 42-nt ssDNA) were added to the flow chamber. Motility of origami scaffolds was assayed at 1 Hz and 100 $\times$ magnification on a Nikon Eclipse Ti inverted epifluorescence microscope.

2.5.7 Coupling of Myosin Origami Scaffolds to Avidin Coated beads for Optical Trapping

To prepare beads with DNA origami, 5 μ l of 1 μ m diameter (Spherotech, 1 % w/v) avidin coated beads are first spin washed twice (16000 g, 1 min) in 50 μ l AB buffer and then once with 50 μ l of AB.BSA to passivate the bead surface. Then the beads are resuspended in 50 μ l of AB.BSA and incubated with 5 μ l of biotinylated DNA Origami stock for 10 minutes at 30°C in a dry incubator shaker. Excess unbound DNA origami are then removed with two washes of 50 μ l AB.BSA.nt. The bead-origami pellet is resuspended in 50 μ l of AB.BSA.nt and purified oligo-labeled Myosin VI is added at a concentration of 150 nM and incubated for 10 minutes at 30°C in a dry incubator shaker. Excess unbound Myosin VI is removed with two washes of 50 μ l AB.BSA.nt.Cam and the origami-Myosin VI bound beads were finally resuspended in 50 μ l AB.BSA.nt.Cam. The final dilution of beads coupled to origami Myosin VI used in the optical trapping assay was made in AB.BSA.nt.Cam supplemented with motility assay reagents (2 mM ATP, 1 mM phosphocreatine, 0.1 mg/ml creatine-phospho-kinase, 25 μ g/ml glucose-oxidase, 45 μ g/ml catalase, 1% glucose).

2.5.8 Optical Trapping Assay

The sample preparation procedure for optical trapping experiments was similar to that of motility assay where in the last step, avidin beads coupled with myosin-origami scaffolds was added in the mix instead of just the myosin-origami scaffolds keeping the other reagents and concentrations the same.

2.5.9 Optical Trapping Setup

The experimental setup consists of a 1064 nm wavelength trapping laser source (Coherent Inc.) that passes through a two-axis acousto-optic-deflector (AOD, IntraAction Corp., DTD-274HA6). The beam is expanded and steered into the microscope objective (Nikon 100x, 1.4 NA, oil immersion). Detection laser (Point Source Inc., iFLEX 2000, 50 mW, 830 nm, p-polarized) is added collinear to the trapping laser using a polarizing beam splitter (PBS) cube. Intensity of the detection beam is reduced by placing a neutral density filter (ND) in its path. Intensity is adjusted such that it is less than required to trap a bead. After passing through the sample, the beams are collected by a 1.25NA condenser (obtained from a bench microscope). The trapping laser is blocked using a laser line filter (Thorlabs, FL830-10) and the back-focal-plane image of the detection laser is imaged onto a quadrant photo-diode (Pacific Silicon Sensors, QP50-6SD2) with integrated amplifier circuit. The photodiode module provides three signals V_x , V_y , and V_z that represent asymmetry of light distribution on the photodiode along x coordinate, along y co-ordinate and the total intensity of light respectively (Fig.1). The signals are captured by a Field Programmable Gate Array (FPGA) based data acquisition card (National Instruments, 7833R). Control logic and voltage to position mapping is programmed on this hardware using custom written code in LabVIEW for FPGA.

2.5.10 Custom MATLAB Software, Step Detection, and Data Analysis

Data obtained from optical trapping experiments was analyzed using a custom MATLAB software which takes in the timed bead position data from photodiode. Bead position data was first filtered by applying a 4th order low pass Butterworth filter with cut-off

frequency at 10 Hz in order to filter out the high frequency noise components. Filtered bead position data is then plotted against time. Events are identified by observing the shift in bead position outside of the trap via a series of stepping motion and then bead position suddenly falling back at the center of the trap indicating the detachment from actin filament. The variance of the bead position is also calculated in a moving window of 200 points and plotted on the same figure as bead position data. The events are also confirmed by observing the reduction in variance of bead position due to attachment of Myosin with Actin filament and increase in variance after detachment. Quantifying the reduction in variance upon filament binding of the motor gives a measure of linkage stiffness. For measuring the steps, the software prompts user to select the 4 points. The first two points determine the baseline position of the bead. The third and fourth point are chosen to be the starting and ending point of the bead at a new shifted baseline position. The difference between the mean position values of first two points and the mean position values of last two point gives the step size which is stored in an array. The difference between the time values of fourth point and third point gives the dwell time for the corresponding step which is also stored in an array along with corresponding value of step size. Steps with dwell time of less than 0.05 seconds are neglected in the analysis. Force values are obtained by multiplying the distance travelled out of the center of the trap with the corresponding stiffness values. It must be acknowledged that automated tools for stepping analysis such as [34, 35] also exist but the need for extended analysis of variance, stiffness, and forces required a custom software.

Chapter 3

Effect of Adapter Protein GIPC on Myosin VI Motility

3.1 Introduction

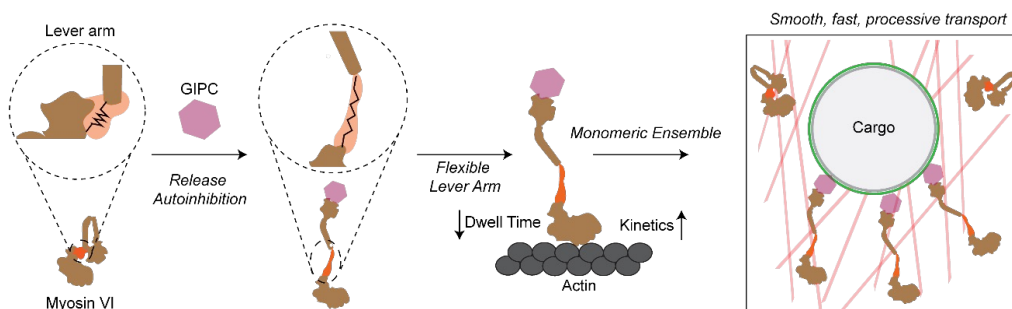


Figure 3.1: Summary graphic representing multimodal regulation of the dual transporter-anchor function of myosin VI by GIPC. Myosin VI binding to GIPC MIR releases motor autoinhibition, extends the lever arm, and increases the flexibility of the motor-cargo linkage. This results in increased speed of single motors and enhances the processivity in ensembles. GIPC-mediated regulation leads to fast, smooth, cargo transport across dense actin networks.

Cytoskeletal motors organize the cell interior through the transport of material and maintenance of cellular structures and architecture. Decades of extensive research have given us unprecedented insight into the structure, biophysical properties and cellular roles of cytoskeletal motors [36–40]. Several key themes of motor regulation in cells have

emerged, including structural autoinhibition [41, 42], motor dimerization [42, 43], and load-dependent effects on the mechanochemical cycle [43]. The functional output of a motor likely stems from a combination of these individual modes of regulation. However, the integration of multiple modes of motor regulation to achieve unique functional roles, such as cargo transporter, membrane tether, load-dependent anchor, in distinct cellular processes remains an outstanding challenge.

In this collaborative study between Sivaramakrishnan’s group and Salapaka Group at University of Minnesota, we investigated the role of the cargo adaptor linkage in cytoskeletal motor regulation. Distinct cellular processes typically employ a unique set of proteins, termed cargo adaptors, that recruit the same motor to distinct scaffolds [37, 44]. While early studies had generalized the cargo adaptor as a passive connector between motor and scaffold, more recent work has highlighted the role of adaptors in actively regulating motor structure and function [4, 45–47]. We focused on the cytoskeletal motor myosin VI, a multi-functional cytoskeletal motor with key roles in many cellular processes including endocytosis, secretion, and the maintenance of stereocilia structure [48]. Dysregulation of these processes has been implicated in numerous pathologies such as deafness and cancer [49, 50]. To perform its cellular functions, myosin VI has evolved unique molecular adaptations, exemplified by an extensible lever arm, regulated homodimerization, structural autoinhibition, and a load-dependent anchoring activity [51]. Despite extensive insights into the individual workings of these molecular mechanisms of myosin VI, their coherence to achieve a specific cellular task remains unclear. The regulatory mechanisms of myosin VI are mirrored by a diversity of adaptor proteins that link the motor to cellular scaffolds [52]. While myosin VI is capable of interacting directly with cargo through membrane phospholipids, more often specific adaptor proteins link myosin VI to a specific cellular cargo [48, 53]. For example, myosin VI gets recruited by Dab2 [54] and GIPC [55, 56] to endosomes, by optineurin to Golgi [50] and NDP52 to intranuclear structures [57]. Recent studies have shown that in addition to providing compartmental identity to myosin VI, these adaptor proteins also regulate its structure and function. The adaptor Dab2, for example, was recently shown to regulate homodimerization of myosin VI that in turn profoundly impacts the actin remodeling effects of myosin VI [45, 58, 59]. Here, we dissect the multimodal regulation of myosin VI through its interaction with GIPC.

The adaptor GIPC has been shown to recruit myosin VI to uncoated vesicles during endocytosis [55]. The PDZ domain of GIPC interacts with cargo molecules such as integrins and cell signaling receptors, while the GH2 domain has been shown to interact with myosin VI [60]. Thus, by bridging the gap between the motor and proteins expressed on the cell surface, GIPC has been implicated as a key regulator of endocytosis for cellular signaling and adhesion molecules. Further supporting this role, GIPC overexpression has been found in numerous cancers [61]. Interestingly, myosin VI has been correlatively shown to be overexpressed in the same cancers as GIPC and downregulation of both GIPC and myosin VI was shown to ameliorate the cancerous phenotype [61]. Therefore, understanding the regulation of myosin VI activity by GIPC is key to addressing these pathologies. While a recent structural study has explored the receptor-mediated activation of GIPC-myosin VI interactions, the direct impact of GIPC on myosin VI motility is unknown [60].

In this study, we use a broad combination of techniques, including motility assays, FRET-based conformational sensors, optical trapping and DNA origami-based cargo scaffolds to dissect the multifunctional regulation of myosin VI by the myosin interacting region (MIR) of GIPC. We report that GIPC MIR binding to myosin VI leads to a 2-3-fold enhancement in myosin VI speed at an ensemble level. In contrast to other myosin VI adaptors like Dab2 and optineurin, GIPC MIR binding does not stimulate processive motility. At the structural level, GIPC MIR binding releases an auto-regulatory interaction within myosin VI, leading to both extension and increased flexibility of the myosin VI lever arm. The enhanced flexibility leads to faster motor-actin interaction kinetics by reducing the dwell time of the motor on actin filaments. Taken together, these effects augment the speeds of motor ensembles. In summary, our study delineates the multimodal regulation of myosin VI by the cargo adaptor GIPC, while highlighting linkage flexibility as a novel biophysical mechanism for modulating cargo motility.

3.2 Results

The following are the key findings from the experiments conducted by Sivaramakrishnan's group [62]:

Observation 1: GIPC enhances myosin VI speed independently of motor density.

In this study, the researchers used a minimal myosin VI interaction region (MIR) of GIPC to enhance myosin VI's speed. They measured the binding affinity between the GIPC MIR and myosin VI and found a K_d of 120 nM. Regardless of motor spacing on a DNA nanotube, GIPC MIR stimulated a 2.5-fold increase in actin gliding speed, demonstrating that GIPC MIR enhanced myosin VI speed irrespective of motor density. When recruiting full-length myosin VI to GIPC MIRs patterned on DNA origami scaffolds, they observed a 2.3-fold increase in scaffold speeds on single actin filaments compared to direct attachment of myosin VI or a processive HMM dimer to the scaffold.

Observation 2: Myosin VI recruited through GIPC MIR remains non-processive. Despite previous observations of myosin VI's processive movement driven by homodimerization, recruiting myosin VI through GIPC MIR did not make it processive. Both actin landing rate assays and single-molecule TIRF motility assays showed that myosin VI remained non-processive, similar to a myosin VI HMM monomer. GIPC MIR binding did not alter the non-processive motility of myosin VI, as indicated by kymographs and run length measurements. Furthermore, GIPC MIR did not promote myosin VI oligomerization or processive behavior.

Observation 3: GIPC MIR enhances myosin VI motility by releasing motor auto-inhibition. The study revealed that GIPC MIR binding induced an open conformation of myosin VI, similar to the effect of calcium. Mutating the putative GIPC binding site, the RRL motif, in the CBD of myosin VI resulted in a decrease in FRET, indicating that this motif served as the structural interface involved in maintaining the inactive closed conformation of the motor. The RRL/AAA mutation enhanced myosin VI speeds, similar to the effects of GIPC MIR on wild-type motors. These findings suggest that GIPC MIR enhances myosin VI motility by competitively releasing an auto-inhibitory interaction within the motor.

Observation 4: GIPC MIR binding induces an open conformation in the PT domain, suggesting that GIPC MIR can modify the mechanical properties of the actomyosin cross-bridge. We also found that flexible attachment strategies, similar to GIPC MIR binding, significantly increase actin gliding speeds with myosin VI. While some variability in the effects of GIPC MIR on actin gliding speed is noted, the overall findings support the idea that reduced linkage stiffness, influenced by GIPC, enhances cargo speeds.

Observation 5: GIPC mediates smooth, processive movement of myosin VI cargo on cellular actin networks. Multiple GIPC-myosin VI complexes on DNA origami scaffolds were found to provide processive cargo movement at enhanced speeds. The presence of GIPC MIR significantly enhanced cargo speeds and run lengths on cellular actin networks. GIPC MIR reduced pauses during cargo movement and decreased the duration of pause events, indicating that the pausing behavior was related to motor coordination rather than the ability of the adaptor to recover motility after pauses. These results suggest that GIPC plays a crucial role in mediating smooth, processive movement of myosin VI cargo on cellular actin networks.

These findings collectively illustrate the multifaceted regulatory effects of GIPC on myosin VI motility, including enhancing speed, releasing auto-inhibition, increasing stroke size and activity, and promoting processive cargo movement on cellular actin networks. For complete details of these experiments and observations, reader is referred to the original article [62]. We attempt to investigate the mechanistic reasoning behind the above behaviors by employing an optical trapping assay where full length are attached to beads at a single molecule level in the presence or absence of GIPC.

3.2.1 GIPC MIR increases stroke size and activity of myosin VI

Release of autoinhibition and structural changes in the myosin VI lever arm can impact the effective stroke size and consequently motor ensemble speeds. Hence, single-molecule optical trapping was used to quantify the effect of the GIPC MIR on myosin VI stroke size (Figure 3.2A). The beads were labeled with low concentrations of motor to establish the single-molecule Poisson limit, with one to two out of 10 trapped beads showing motile events (see Materials and Methods section). Under these conditions, we observe single stroke events that were consistent with the monomeric nature of FL myosin VI (Figure 3.2 B and C). We measured a stroke size of 19.3 ± 8.5 nm (Figure 3.2D), consistent with previous optical trapping experiments for human FL myosin VI (25). The addition of saturating concentrations of GIPC MIR increases stroke size by 30% (Figure 3.2D; 25.6 ± 8.1 nm; $p < 0.0001$). This modest change in myosin VI stroke does not address the approximately twofold increase in ensemble gliding speeds (See Figure 1 in [62]) . Hence, in parallel, we examined the effect of the GIPC MIR on the kinetics of the mechanochemical cycle of FL myosin VI. We quantified the dwell time

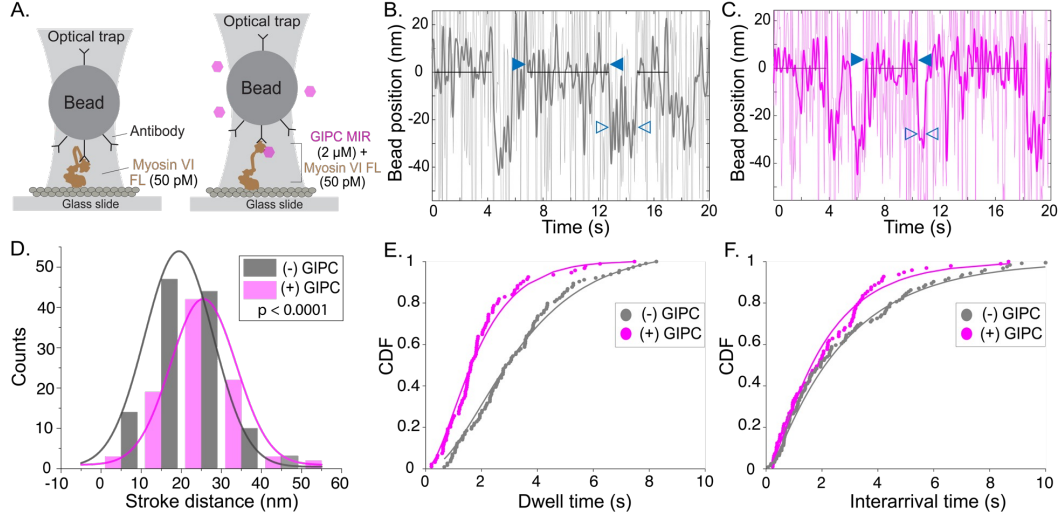


Figure 3.2: GIPC MIR increases the stroke distance of the myosin VI lever arm. A. Depiction of myosin VI labeled beads in a single molecule optical trap experiment. B,C. Example optical trap traces for full length myosin VI alone (B) and in the presence of GIPC MIR (C). Black solid lines indicate the baseline with filled in arrowheads indicating the interarrival time and hollow arrowheads indicating the dwell time. D. Trap displacement data for myosin VI-labeled beads. E,F. Cumulative distribution function (CDF) of the dwell (E) and interarrival (F) times for myosin VI in the presence and absence of GIPC MIR. Dwell times are fitted to a double exponential with $k_1 = 1.01s^{-1}$ and $k_2 = 1.02s^{-1}$ with GIPC and $k_1 = 0.61s^{-1}$ and $k_2 = 0.62s^{-1}$ without GIPC. Interarrival times are fitted to a single exponential with $\lambda = 0.09s^{-1}$ with GIPC and $\lambda = 0.06s^{-1}$ without GIPC (see methods for more details). Experiments were performed using $N \geq 3$ independent protein preparations.

and interarrival time of myosin VI-labeled beads in the optical tweezers set up (Fig. 5, E and F). Dwell time quantifies the duration of stroke events (hollow blue arrowheads; Figure 3.2B and C), whereas interarrival time quantifies the period between sequential stroke events (solid blue arrowheads; Figure 3.2B and C) in the optical trap. GIPC MIR decreases the dwell time of myosin VI on actin filaments from 0.83 to 0.5 s (Fig. 5E; $p < 0.0001$) and interarrival time between motile events from 14.8 to 11.1 s (Figure 3.2F; $p < 0.01$). For high duty ratio motors such as a myosin VI, the dwell time on actin filaments is inherently rate limiting to the actomyosin crossbridge cycle [60]. Hence, the 1.6-fold decrease in dwell time combined with a 1.3-fold increase in stroke size translates into an approximately twofold increase in speed at the actomyosin crossbridge that sufficiently captures the approximately twofold to threefold increase in gliding speeds observed in motility assays [62].

3.3 Discussion

This collaborative study highlights the integrated effects of multiple modes of adaptor-mediated regulation of the cytoskeletal motor myosin VI (Figure 3.1). GIPC MIR binding results in the release of auto-inhibitory interactions within myosin VI. This allows for conformational changes in the lever arm, in particular the proximal tail, thereby increasing both myosin stroke and flexibility of the crossbridge formed by the myosin, between the actin and the cargo. A flexible link enables faster dissociation and association of the acto-myosin crossbridge, which combined with the increased myosin stroke translates to 2-3 fold greater speeds of myosin VI ensembles. Although GIPC MIR was not observed to affect the oligomerization of the motor, ensembles of myosin VI/GIPC MIR complexes were capable of similar run lengths as ensembles of forced processive HMM dimers. The individual modes of myosin VI regulation merge to allow for fast, smooth, processive cargo transport on cellular actin networks. Taken together, our in-depth characterization of this motor/adaptor interaction and interplay among different regulatory modes, delineates motor-cargo linkage stiffness a novel mechanism of cytoskeletal motor regulation used to specifically direct traffic within the cell.

Here, we demonstrate the ability of GIPC MIR binding to relieve the autoinhibited conformation of myosin VI. Autoinhibition of cytoskeletal motors is one of the most prevalent and well-studied themes of motor regulation and is widely believed to prevent futile ATP hydrolysis when the motor is not engaged to a cellular cargo [41,42,63]. This autoinhibited conformation typically occurs through an interaction between the cargo-binding tail domain and the ATP-hydrolyzing motor domain that results in a folded conformation and a decrease in ATPase activity of the motor. Conversely, binding to a cargo and/or divalent cations has been shown to relieve the autoinhibited motor conformation, thereby activating the motor and stimulating ATPase activity. A structurally characterized unconventional myosin, myosin Va, presents a prototypical example of a head-tail autoinhibited motor, where binding to the cargo adaptor melanophilin was shown to relieve the autoinhibited conformation [64,65]. For myosin VI, Batters et al. used single particle EM analysis to show that myosin VI existed in a folded back, autoinhibited conformation and calcium binding resulted in a switch to a partially open motor conformation [66]. Similarly, using an intramolecular FRET sensor, Fili et al.

showed that binding to the cargo adaptor NDP52 relieved the folded back conformation of myosin VI [57]. In this study, we used an intramolecular FRET sensor to probe the effect of GIPC MIR binding on the autoinhibited conformation of myosin VI. We found that, much like the adaptors outlined above, GIPC MIR binding was sufficient to switch myosin VI from an autoinhibited to an open conformation. Furthermore, our results show that the GIPC-binding RRL motif on the myosin VI cargo binding domain is itself involved in maintaining the head-tail autoinhibition of myosin VI. Thus, our results highlight an elegant mechanism, wherein sequestration of the RRL motif by GIPC MIR breaks the head-tail interaction to relieve motor autoinhibition.

Myosin VI is unique among unconventional myosins in having only a single calmodulin-binding IQ motif, suggesting that it has a short lever arm and by consequence a short step size on the actin filament [67]. However, a wealth of studies have revealed that myosin VI has additional domains in its tail region, termed the proximal (PT) and medial tail (MT), which contribute to an extension of the “effective” myosin VI lever arm, thereby extending its reach to take a longer step. The myosin VI PT domain is a three-helix bundle that exists in a folded conformation in the autoinhibited, inactive myosin VI [68, 69]. While previous studies had demonstrated that the PT domain can unfold to increase myosin VI step size, they had done so in the context of a truncated, artificially homo-dimerized motor [68, 69]. Here, we show that GIPC MIR binding to the full-length myosin VI unfolds the PT domain, which in concert with an extended MT domain can increase the myosin VI stroke. Our results, therefore, provide a direct structure-function connection for adaptor-mediated conformational changes in the lever arm that enhance the motile properties of myosin VI.

Linkage stiffness has been previously stipulated as a mechanism to influence the motility of cytoskeletal motor ensembles [70, 71]. Artificially dimerized myosin VI ensembles patterned on DNA nanotubes yielded higher actin gliding velocities when the stiffness of the synthetic linkage between motor and nanostructure was decreased [72]. Likewise, artificially dimerized myosin VI motors patterned on DNA origami scaffolds moved faster on single actin filaments when the cargo-motor linkage stiffness was reduced [71]. While these studies demonstrated a biophysical mechanism to modulate cargo speed, its cellular relevance has not been established. Here, we directly demonstrate that adapter mediated modulation of motor-cargo linkage stiffness is exploited in

full-length myosin VI to drive higher ensemble speeds. GIPC MIR binding releases autoinhibition and results in the unfolding of the PT domain of full-length myosin VI. This not only increases the effective stroke length of the myosin VI lever arm but also results in a more flexible motor. We have previously shown that increased flexibility of the motor-cargo linkage decreases the motor dwell time on the actin filament (t_{dwell}) [71]. Here, we observe a similar effect of GIPC MIR binding on single molecule t_{dwell} (Figure 3.2E). Since t_{dwell} is a rate-limiting factor in the acto-myosin ATPase cycle for myosin VI, decreasing it enhances cross-bridge turnover rates, with consequent higher cargo speeds. Together, our findings dissect the biophysical basis of GIPC-mediated enhanced cargo speeds, while establishing linkage stiffness as a regulatory mode in cargo transport.

The significance of myosin VI homo-dimerization in cargo processivity is unclear. Early studies had predicted that myosin VI has an intrinsic potential for dimerization through a putative coiled-coil motif in its tail domain [73]. However, multiple subsequent studies have shown that full-length myosin VI is natively monomeric and requires binding to cargo proteins and/or lipids to assist dimerization and consequent processivity [68]. Likewise clustering of myosin VI monomers on actin filaments, presumably due to a high local concentration facilitated by adaptor/lipid membrane engagement has also been shown to induce processive movement [74]. Spudich et al. showed that binding to PIP2 phospholipids induces an increase in helicity in the myosin VI cargo-binding domain that results in motor dimerization [56]. Similarly, structural studies showed that cargo adaptor proteins such as Dab2 and optineurin were capable of dimerizing myosin VI and driving processive motility [58,75]. In contrast, multiple truncated myosin VI monomers recruited to an artificial polystyrene bead scaffold were sufficient to drive processive motility on a cellular actin network [76]. In parallel, structures of GIPC MIR in complex with the cargo binding domain (CBD) of myosin VI support a 1:1 molar ratio for this interaction [60]. However, crystal packing and analytical ultracentrifugation analysis suggests oligomerization of the GIPC MIR and CBD in a 5:5 complex [60]. Further, full length GIPC exists as a domain-swapped dimer, wherein the MIR within the GH2 domain is obscured by binding in trans with the PDZ domain. Interaction of GIPC with a signaling receptor such as plexin D1 releases the domain-swapped dimer and enables interaction with myosin VI. This activated GIPC is suggested to oligomerize through inter-molecular interactions involving a GH1 domain. Together, these data

suggest the GIPC-mediated clustering of myosin VI could facilitate membrane traffic. Accordingly, our studies with myosin VI ensembles on DNA origami scaffolds, interacting with single actin filaments and cellular actin networks, sufficiently demonstrate robust cargo motility.

Our study provides a framework to investigate the signaling-mediated regulation of myosin VI. A number of cell surface receptors, including plexin D1, β 1-adrenergic receptor (β 1-AR), megalin, Glut1, and Integrin 5a 45,46 interact with the PDZ domain of GIPC. Recruitment to these cell surface receptors can overcome GIPC auto-inhibition, revealing the myosin interacting domain (MIR) used in this study [60]. In turn, the GIPC MIR influences myosin VI conformation to enable multi-level regulation of motility by a signaling receptor-adaptor-motor complex. We propose that understanding such super-regulation of motor ensembles can inform the context-dependent regulation of signaling processes.

3.4 Materials and Methods

3.4.1 List of constructs

GIPC MIR: GH2 domain of human GIPC1 (a.a. 261-333) was used as the minimal myosin VI interaction region as defined previously 26. Myosin VI FL: The isoform of human myosin VI containing both the long insert (LI) and short insert (SI) was used in all experiments involving the full-length myosin VI.

C-terminal GFP and SNAP tagged fusion proteins of adaptor MIRs were cloned in the insect cell expression vector pBiex1 (Novagen) with a C-terminal FLAG tag incorporated to facilitate affinity-based protein purification. An N-terminal mCitrine version of adaptor MIRs with a FLAG tag was used for bimolecular FRET assays. A GFP nanobody construct with C-terminal SNAP and FLAG tags in pBiex1 vector were used to bind GFP-tagged proteins as reported by us previously [45, 70, 77].

The following versions of Myosin VI were used in this study: Myosin VI FL-GFP: A C-terminal GFP and FLAG tagged construct of full-length human myosin VI cloned in pBiex1. Myosin VI FL-FLAG: A C-terminal FLAG tagged version of full-length human myosin VI cloned in pBiex1. Myosin VI FL-SNAP: A C-terminal SNAP, FLAG and a 6X-His tagged construct of full-length human myosin VI cloned in pBiex1. Myosin VI

Δ CBD dimer: Myosin VI, residues 1–992 from *Sus scrofa*, containing both the IQ and SAH domains with a GCN4 leucine zipper (for dimerization), a SNAP tag (for DNA oligo attachment), a FLAG tag (for purification) and a 6X-His tag cloned in pBiex1. Myosin VI Δ CBD monomer: Myosin VI, residues 1–992 from *Sus scrofa*, containing both the IQ and SAH domains with a SNAP tag, FLAG tag and a 6X-His tag cloned in pBiex1. Myosin VI conformational FRET sensor: Human myosin VI FL with an N-terminal mCerulean and a C-terminal mCitrine fluorescent tags with a FLAG tag and a 6X-His tag cloned in pBiex1. Myosin VI PT domain sensor: Human myosin VI FL with tetracysteine motifs inserted after lysine 833 and lysine 921 flanking the proximal tail (PT) domain with a C-terminal FLAG tag cloned in pBiex1. A similar construct with a single tetracysteine inserted after lysine 833 was used as a control construct. Myosin VI CBD constructs: The C-terminal a.a. 1030-1284 of human myosin VI was used as the putative cargo-binding domain (CBD) of myosin VI as defined previously. C-terminal mCerulean tagged versions of CBD with a FLAG tag cloned in pBiex1 were used in this study.

3.4.2 Buffers and Reagents

Assay buffer (AB): 20 mM Imidazole (pH 7.5), 25 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 1 mM DTT. AB.BSA: AB containing 1mg/mL bovine serum albumin (BSA). AB.BSA.Cam: AB.BSA containing 10 μ M calmodulin. AB.BSA.nt: AB.BSA containing 0.1 μ M random nucleotide mix. Stop solution (for ATPase assay): 60 mM EDTA (pH 6.5), 6.6% SDS. Developing solution (for ATPase assay): 0.5% ammonium molybdate (2% stock in 4N H₂SO₄), 5 mg/mL ferrous sulfate. Lysis Buffer (for protein purification): 20 mM Imidazole (pH 7.5), 200 mM NaCl, 4 mM MgCl₂, 0.5 mM EDTA, 1 mM EGTA, 0.5% IGEPAL, 7% sucrose Wash Buffer (for protein purification): 20 mM Imidazole (pH 7.5), 150 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1mM EGTA

3.4.3 Protein expression and purification

All proteins used in this study were expressed and purified in Sf9 insect cells and purified using a FLAG-tag based affinity purification. The protocol is described briefly here. Transient transfection of constructs in Sf9 cells was achieved using the Escort IV system

(MilliporeSigma). For protein purification, transiently transfected Sf9 cells at a cell number of 60×10^6 cells were centrifuged at 350x g for 5 min to pellet the cells. The supernatant was discarded and the cell pellet was resuspended in 3 mL of ice-cold lysis buffer supplemented with 1 $\mu\text{g/mL}$ PMSF, 10 $\mu\text{g/mL}$ aprotinin and 10 $\mu\text{g/mL}$ leupeptin. Cell lysis was achieved by 20 cycles of pipetting of the resuspended cell pellet. The cell lysate was centrifuged at 176,000x g for 25 min. at 4°C in a TLA 100.4 rotor (Beckman) to pellet the cell debris. The supernatant was incubated with 50 μl of anti-FLAG M2 affinity resin for 2 hrs at 4°C with rotation. The resin-lysate mix was centrifuged at 1000g for 1 min at 4°C to pellet the resin. The resin was then washed thrice with ice-cold wash buffer supplemented with 1 $\mu\text{g/mL}$ PMSF, 10 $\mu\text{g/mL}$ aprotinin and 10 $\mu\text{g/mL}$ leupeptin by resuspending and then pelleting the resin by centrifuging at 1,000x g for 1 min at 4°C. The supernatant from the last wash was removed and the resin was resuspended in wash buffer supplemented with 0.2 mg/mL FLAG peptide (MilliporeSigma) to elute the protein. Protein estimation was done either using a NanoDrop spectrophotometer for fluorescently-tagged proteins or using BSA standards on a 10% SDS-PAGE gel for non-fluorescent proteins.

3.4.4 Surface actin gliding motility assay

Plasma cleaned glass coverslips (22 X 22 mm, Corning) were coated with 0.1% colloidin (Electron Microscopy Sciences) in amyl acetate. Flow chambers were prepared by sticking the colloidin-coated coverslips to a glass slide using strips of double-sided tape. GFP nanobody was added to the flow chamber at a final concentration of 200 nM in AB for non-specific surface adsorption by incubation for 4 min at room temperature. The unbound GFP nanobody was washed off with three washes of AB. Surface passivation was then carried out by AB.BSA incubation for 4 min. Then, the GFP-tagged protein (adaptor MIR or myosin VI FL) at a concentration of 200 nM was added to the chamber and incubated for 4 min. The unbound protein was washed by three washes with AB.BSA. For adaptor MIR based assay, myosin VI FL-FLAG was added to the chamber at a concentration of 200 nM and incubated for 4 min. The unbound myosin VI was washed with AB.BSA. Finally, a motility mix was prepared in AB.BSA.Cam buffer containing Alexa-647 phalloidin (Invitrogen) labeled F-actin at 0.5 μM , an ATP regenerating mix (1 mM phosphocreatine and 0.1 mg/ml creatine phosphokinase), an

oxygen-scavenging system (0.6% glucose and 45 μg per ml catalase, 25 μg per ml glucose oxidase), 2 mM ATP and added to the flow chamber. The actin gliding motility was assayed using a Nikon Eclipse Ti inverted epifluorescence microscope at 100 X using a 1.4 NA oil- immersion objective at a frame rate of 1 Hz for 2 min. The actin gliding data was analyzed using FIESTA software 48. For landing rate experiments, the myosin VI concentration was varied from 200 nM to the low dilution at which actin landing on the surface could no longer be observed. Surface density of motors and actin landing rates were calculated as described previously [78].

3.4.5 DNA Scaffold-myosin preparation

DNA nanostructures were prepared based on the detailed description in our previous work [70]. Single-stranded M13mp18 DNA (NEB) were mixed with fourfold excess of short stable strands (IDT, Coralville, IA), followed by 2 hr annealing as previously described [79]. Intact scaffolds were separated from excess staple strands using Amicon Ultra 100K cutoff spin columns (EMD Millipore). Purified scaffolds were mixed with excess labeled GIPC MIR, a mixture of 42-nt oligos with randomized sequences (blocking oligos), and 1–5 μM calmodulin in $1\times$ AB.BSA. After 10 min of incubation at room temperature, excess streptavidin-coated magnetic beads (NEB) were added and incubated at room temperature with shaking for 10 min. The beads were washed with AB.BSA.CAM. Finally, the beads were incubated in AB.BSA.CAM containing an imaging solution of 2 mM ATP, 1 mM phosphocreatine, 0.1 mg/ml creatine-phosphokinase, 45 $\mu\text{g}/\text{ml}$ catalase, 25 $\mu\text{g}/\text{ml}$ glucose oxidase, 1–2% glucose, myosin VI at 1 μM concentration and excess elution strand for strand displacement of origami from streptavidin magnetic beads.

3.4.6 DNA scaffold motility assay on single actin filaments

Motility assays were acquired at $100\times$ magnification on an epifluorescence microscope (Nikon TE2000). Motility assays were performed using flow chambers prepared with nitrocellulose-coated coverslips (Corning 22 mm $\times$ 22 mm). First, biotinylated, 488Alexa-phalloidin-stabilized actin filaments were immobilized to the inner surface of the capillary tube by BSA-biotin-neutravidin- linkages. Unbound actin filaments were washed

with AB.BSA. Purified GIPC-myosin VI-scaffold complexes in AB.BSA.CAM + imaging reagents (2 mM ATP, 1 mM phosphocreatine, 0.1 mg/ml creatine-phospho-kinase, 25 μ g/ml glucose-oxidase, 45 μ g/ml catalase, 1% glucose, 1 μ M random library 42-nt ssDNA) were added to the flow chamber. Movies of Cy5-scaffold motility on the actin filaments were obtained at 1 Hz for ≥ 2 min.

3.4.7 Single-molecule TIRF motility assay

Flow chambers were prepared as outlined above for surface and nanotube motility assays. BSA biotin-neutravidin interaction was assembled on the surface similar to that described under nanotube motility assay. Biotinylated F-actin (1:9 ratio of biotin-G-actin: G-actin) was then added to the flow chamber at a concentration of 0.5 μ M and incubated for 4 min. Unbound actin filaments were washed off with three flows of AB.BSA. The adaptor-MIR-GFP + Cy3-myosin VI complex or Cy3-myosin VI alone was added to the flow chamber at a concentration of 50 pM in a motility mix like that used for surface motility assay. Dual color imaging of single-molecule motility was done on a Zeiss TIRF microscope equipped with a Coherent 100 mW 488 nm and 561 nm OPSL laser and a 100x 1.4 NA oil-immersion objective with a DualView 2 for simultaneous two Channel imaging and a Photometrics QuantEM 512SC EMCCD camera for high sensitivity single-molecule detection. Single-molecule motility was assayed at a frame rate of 10 Hz. Particle tracking of single-molecule motility data was done using the Trackmate plugin [80] in FIJI [81, 82]. Kymographs of single-molecule motility were generated using the reslice tool in FIJI. Spot intensities of Cy3-myosin VI were obtained by a line intensity scan along the kymograph in the Cy3 channel. Run lengths of adaptor-Myosin VI complexes were obtained from the analysis of kymographs of motile runs in the adaptor MIR-GFP channel.

3.4.8 Optical trapping Experiments

Sample Preparation - Polystyrene beads of 1 μ m diameter, (Polysciences, 2.5% solids (w/v)) were diluted 1:100 in AB and washed twice with AB by centrifugation at 15000 rpm for 1 min and resuspending the bead pellet in 50 μ L of AB. The beads were then conjugated with penta-HIS antibody to myosin VI cargo-binding domain at a 1:10 dilution

in 50 μL of AB by incubation at 4 $^{\circ}\text{C}$ with rotation for 30 min. The antibody-conjugated beads were washed twice with AB.BSA to remove unbound myosin VI antibody. Myosin VI was added to the antibody-conjugated beads in 50 μl AB.BSA.Cam at a concentration of 5 nM and incubated for 30 min. Flow chamber and single actin filaments on the coverslip surface were prepared similarly to that described under single-molecule motility assay. Beads conjugated to myosin VI were added at a 1: 50 dilution in AB.BSA.Cam supplemented with an ATP regenerating mix (1 mM phosphocreatine and 0.1 mg/mL creatine phosphokinase), an oxygen-scavenging system (0.6% glucose (% w/v) and 45 $\mu\text{g}/\text{mL}$ catalase, 25 $\mu\text{g}/\text{mL}$ glucose oxidase) and 2 mM ATP. Beads floating in solution were optically trapped and brought close to an actin filament on the surface to begin assaying myosin VI force-generation events. For GIPC condition, GIPC was added to the final mix at a saturating concentration of 2 μM to form the GIPC-myosin VI complex.

Optical Trapping Setup

The experimental setup, as reported previously [83, 84], consists of a 1064 nm wavelength trapping laser source (Coherent Inc.) that passes through a two-axis acousto-optic-deflector (AOD, IntraAction Corp., DTD-274HA6). The beam was expanded and steered into the microscope objective (Nikon 100x, 1.4 NA, oil immersion) using standard optical components. A detection laser (Point Source Inc., iFLEX 2000, 50 mW, 830 nm, p-polarized) was added collinear to the trapping laser using a polarizing beam splitter (PBS) cube. Intensity of the detection beam was reduced by placing a neutral density filter (ND) in its path. The intensity of the laser was adjusted such that it was less than the intensity required to trap a bead. After passing through the sample, the beams are collected by a 1.25 NA condenser. The trapping laser is blocked using a laser line filter (Thorlabs, FL830-10) and the back focal plane image of the detection laser is imaged onto a quadrant photo-diode (Pacific Silicon Sensors, QP50-6SD2) with integrated amplifier circuit. The photodiode module provides three signals V_x , V_y , and V_z that represent asymmetry of light distribution on the photodiode along x coordinate, along y co-ordinate and the total intensity of light respectively). The signals were captured by a Field Programmable Gate Array (FPGA) based data acquisition card (National Instruments, 7833R). Control logic and voltage to position mapping was programmed on this hardware using custom written code in LabVIEW for FPGA.

Data Acquisition

Myosin coated beads exhibiting Brownian motion in solution were trapped using the optical trapping setup. Upon trapping a bead, the photodiode reads the bead position at 50 kHz, which was displayed on a custom LabVIEW software interface. At this point, the bead trace exhibits oscillations characteristic of Brownian motion in the trap and its mean position indicates the center of the trap. The position of the optical trap was then gradually changed to position the bead close to an actin filament. The engagement of a motor on the bead to an actin filament produces a stroke that displaces the bead trace outside the center of the trap. Detachment of the myosin from the actin filament results in the sudden return of the bead trace back to the center of the trap. Multiple such events were recorded and the data obtained was imported in MATLAB for further analysis.

Data Visualization

Data obtained from optical trapping experiments were analyzed with custom MATLAB software using timed bead position information from the photodiode. Bead position data acquired at 50 kHz were first filtered by applying a 4th order low pass Butterworth filter with a cut-off frequency at 10 Hz to filter out the high frequency noise components. Filtered bead position data were then plotted against time to manually identify the presence of displacement events in the bead position data using a similar method as used in [85]. Upon identification, unfiltered bead position data is used to quantify and analyze stroke size, dwell times and interarrival times of the events.

Data Analysis

Events were manually identified based on sudden displacement of the bead trace from the center of the trap. Baseline bead position was determined by manually selecting a segment of the bead trace in which the optically trapped bead was held around 3 μm above the coverslip surface. Displaced bead position was determined by manually selecting a segment of the bead trace where the mean of the filtered bead position data was displaced from the center of the trap beyond the Brownian noise level at baseline bead position. The difference between the mean unfiltered bead position during

a displacement event and the mean unfiltered baseline position gave the stroke size. The dwell time duration of a displacement event was obtained using the width of the bead trace at the peak of a displacement event. Following the identification of an event, the time duration to the next event constituted the interarrival time. Standard Deviation (square root of variance) of the bead was obtained using a 200 point moving window in the bead position data and was plotted against time. A significant decrease in the standard deviation (variance clamp) was visually identified and used to estimate the stiffness of the linkage connecting the bead and the actin filament.

Linkage Stiffness Estimation

The optical trap is modelled as a simple harmonic spring with stiffness k_{trap} . The stiffness of the linkage between the bead and the actin filament, $k_{linkage}$, can be estimated by quantifying the reduction in variance of the bead position trace during myosin VI motility events in the optical trap using the equipartition theorem [86]. The variance of the bead inside an optical trap is related to k_{trap} via the following relationship derived from the equipartition theorem:

$$\frac{1}{2}K_bT = \frac{1}{2}k_{trap}x^2 \quad (3.1)$$

where K_b is the Boltzman Constant, T is the ambient temperature, and x is the standard deviation of the bead position data. Thus, the lower the variance (square of the standard deviation), the higher the effective linkage stiffness. When an optically trapped bead is attached to an actin filament via a motor, it can be represented as a bead attached to two Hookean springs in parallel. One spring can be modeled as the optical trap itself and other as the linkage between the bead and the actin filament via motor(s) (Figure 5A). The variance of an optically trapped bead attached to an actin filament via myosin(s) is therefore be represented as:

$$\frac{1}{2}K_bT = \frac{1}{2}(k_{trap} + k_{linkage})x^2 \quad (3.2)$$

To measure the effective linkage stiffness of motor(s), we first obtained the baseline stiffness of the optical trap (k_{trap}) by recording the variance of the bead position when the bead is not attached to the actin filament via motors. Subsequently, this baseline optical trap stiffness was used to estimate the net acto-myosin cross-bridge stiffness

$(k_{linkage})$.

Curve Fitting to CDFs

The raw data for dwell time was fit to a model approximating the motor's mechanical cycle by 2 independent irreversible steps which is given by the PDF of the sum of two independent exponential distributions. This model, which is given by equation 3.3, has also been used previously 58 to fit the dwell times of myosin VI:

$$P(t, k_1, k_2) = \frac{k_1 k_2}{k_1 - k_2} (\exp(-k_2 t) - \exp(-k_1 t)) \quad (3.3)$$

Where $P(t, k_1, k_2)$ is the probability distribution function with rates k_1 and k_2 which are the rates of two independent steps in the motor mechanical cycle. We obtained a value of $k_1 = 1.01s^{-1}$ and $k_2 = 1.02s^{-1}$ with GIPC as compared to $k_1 = 0.61s^{-1}$ and $k_1 = 0.62s^{-1}$ in the absence of GIPC MIR.

For interarrival times, events were modelled as a Poisson process with the interarrival times fitting to an exponential distribution (equation 3.4), which is the defining property of a Poisson process.

$$P(t, \lambda) = \lambda(\exp(-\lambda t)) \quad (3.4)$$

Where $P(t, \lambda)$ is the probability distribution function, with λ as the rate parameter. This rate parameter is equal to the inverse of the mean interarrival time. We observed that the mean interarrival time for myosin VI in the presence of GIPC is 11.1 seconds as compared to 14.8 seconds in the absence of GIPC.

Chapter 4

Effect of Cargo-Motor Interaction Kinetics on Multi-Motor Cargo Transport

4.1 Background

A cell's functionality is intricately reliant on force transductions among its various components communicating via mechanochemical signals [87,88] and hence deciphering mechanical interactions inside a cell is of great scientific interest [89]. For instance, one such highly important mechanochemical process is transportation of intracellular cargo by teams of molecular motors. Numerous eukaryotic cellular processes involve transport of cargoes, such as vesicles, organelles, and chromosomes, from one location of the cell to another [90]. Cargo carrying molecular motors mechanical force generating proteins, broadly classified into three superfamilies - kinesins, myosins, and dyneins. These motors *walk* on cytoskeletal tracks such as microtubules (kinesin and dynein) and actin filaments (myosins) taking nanometer sized steps [91]. Apart from cargo transport, motor proteins working in tandem are also an integral part of cytoskeletal restructuring during cell division [92] and for maintaining the structure of cellular sub-components [93], such as stereocilia in the sensory cells of inner ears [94]. Consequently, the three families of molecular motors are studied at length where many single molecule studies have revealed

the biophysical and chemical properties of motor proteins [2, 95–97]. A vast knowledge base now exists which provides concrete evidence that irregularities in the mechanobiology of these motors lead to diseases including various types of cancers [98–100], neurodegenerative diseases such as ALS [3, 101], serious infections [102, 103], and deafness [94]. Aforementioned diseases arise from various aspects of the cellular cargo transport being compromised [91, 104, 105]. Traditionally, global environmental factors such as the average number of motors on the cargo, forces on cargo, ATP concentration, and temperature were considered to be the key regulatory factors for cargo transport by teams of molecular motors [106, 107] and the cargo transport properties evaluated in terms of velocities and runlengths. Subsequently, local parameters such as linkage stiffness of motors and presence of other adapter proteins have been found to also impact the cargo transport properties [45, 62, 108, 109] and both experimental and simulation data suggested that faster and longer cargo trajectories might not always be better, and cargo pausing can play an important part in signal transductions inside the cell. A newer paradigm of multiplexed transport has recently emerged where a single factor can impact multiple aspects of cargo transport [45, 62] and some factors such as linkage stiffness can even alter fundamental functions of molecular motors, making motors such as myosin VI more versatile and involved at a variety of cellular locations during different processes ranging from cargo transport [110] to providing structural stability [94]. Another recent insight is that cargo transport by molecular motors is not just a mode of material transport but also a signaling pathway [111] and in certain scenarios, cargo can act as a vessel making materials, chemicals, [112] or signals [111]. During such processes, robustness and versatility of the intracellular transportation infrastructure needs to be evaluated beyond the cargo runlength and velocities. For instance, regular cargo pauses might be necessary for material exchange [112] while force induced restructuring of actin network by molecular motors could lead to the entire network collapse [113–116]. While several factors can effect the robustness of transport, we investigate how dissociation of motors from the cargo can impact the multi-motor cargo transport behavior within the cells. Recent data suggests that certain conditions inside the cells, such as presence of adapter proteins [45, 62, 117] or coatings on the cargo can impact the interaction kinetics between the cargo and the motors. However, effect of such a alteration in cargo-motor interactions has not been systematically studied in detail.

Experimental tools are upcoming where different attachment methodologies such as DNA origami [71] or cargo coatings [118] have been employed to alter the interaction kinetics between cargo and motor but such experiments still remain hard to control and could have confounding effects, complicating studying the effect of cargo-motor dissociation on intracellular transportation in isolation. Moreover, large amount of experimental data using extremely time consuming experiments are needed to generate relevant statistics while dynamically observing each individual motor in an ensemble carrying a common cargo under load still remains challenging. Computational studies exist where simulations of multi-motor cargo transport and have shown great utility in elucidating novel phenomenon which are otherwise experimentally hard to observe. In prior work, a steady state model for molecular motor coordination is presented in [107] accurately predicting steady state cargo runlengths and velocities by multiple motors. Monte Carlo simulations performed in [108] model provided an alternate strategy to simulate cargo transport in a more detailed environment with thermal fluctuations, however the simulations are time consuming due to added details as a result of more complex simulation environment. [119] uses the Gillespie algorithm to reduce the time complexity and simulated bi-directional cargo transport by a teams of kinesin1, kinesin2 and dyneins motors. [120–122] provide an even faster solution by using master equation based approach obviating the need of simulating small time steps but they still need to perform numerical integrations to calculate run lengths and need supercomputers to simulate cargo transport by ensembles of more than 5 motors. However, the one assumption common to all the above studies is that they all assume motors to be permanently attached to the cargo. Recent data suggests that it might not always be the case [62] where dissociation of motors from the cargo is potentially a biophysical mechanism with which cellular cargo transport is regulated. Incorporating cargo-motor dissociation in the existing algorithms further increases the computational complexity necessitating more efficient algorithms. In light of the new data and need for a highly customizable and efficient simulation strategy, we leverage the concepts of hitting time in Markov chains and a generalized simulation framework is presented in this article which has the following novel features lacking in state-of-the-art:

1. It can accommodate variable number of motors on the cargo addressing cargo-motor dissociation and association events

2. It is highly modular and can simulate any family of molecular motors by taking motor parameters and motor type as input
3. Leveraging the hitting time properties of the markov chain, it obviates the need for monte-carlo steps as in [108, 119] and also for numerical integration as in [120, 121] that employ Master Equations, which increases its speed several folds and makes it suitable for running on regular desktop PCs
4. Using a similar strategy as [121], it can be extended to simulate multiple types of molecular motors on the same cargo, similar to [119] but additionally allowing motors to also dissociate from cargo
5. It provides exact steady state probability distributions of configurations that a multi-motors ensemble exhibits while carrying a common cargo

All these features make the simulation framework presented here novel and highly tunable fitting the use case of a large base of biophysicists investigating various aspects of multi-motor cargo transport. Since our framework allows us to use the rate of dissociation of motors from the cargo as a simulation variable, here we employ our simulation engine to analyze the effect of dissociation of motors from the cargo on the overall multi-motor intracellular cargo transport and identify scenarios where a higher dissociation of motors from the cargo can lead to a more robust cargo transport [123–125]. Leveraging the speed and efficiency of our platform, we predict and identify regimes of interests in simulation variable i.e. cargo load forces, F_{load} , and cargo-motor dissociation rate (CM-DR). We then further perform Monte-Carlo simulations for some interesting corner cases to dig deeper into the biophysical mechanisms. We find that a higher CM-DR can lead to a cargo transport scenario more suitable for material and signal exchange by enhancing pausing. Frequent detachment of motors from cargo also leads to a more robust intracellular transportation infrastructure by reducing the total force on actin network, decreasing the possibility of actin network collapse while compromising on cargo velocities and runlengths. Our key findings are presented in Section 4.2. We discuss the implications of the results in the context of regulation of multi-motor cargo transport in Section 4.3. The general simulation assumptions, algorithm, calculation of biologically relevant quantities, and simulation parameters are presented in Section

4.4. We have further implemented this framework in python and cross-verified the results using algorithms developed in the past. Validation and standardization of this methodology against existing work is presented in supporting information section along with other supplemental data. Our software is available for download as a standardized open-source solution for easy distribution and wide accessibility. It must be noted that this new framework subsumes all other past approaches by simply making the rate of dissociation of motors from the cargo zero. We demonstrate the modularity of the our toolbox by simulating transportation of cargo by multiple kinesins as well as myosins.

4.2 Results

We define the rate at which motors dis-associate from and associate to the cargo as cargo-motor dissociation rate (CM-DR) and cargo-motor association rate (CM-AR), respectively. We observe how multi-motor cargo transport is affected when we change either of these rates.

4.2.1 Velocity sensitivity to load force increases due to cargo-motor dissociation

We first analyze the variation of velocity with load force for both kinesin and myosin type motors (Figure 4.1). Each curve in Figure 4.1a,b,c,d represents the simulations performed for a fixed ensemble size which is defined as the maximum number of motors that *can* attach to the cargo. However, it must be noted that for each curve, motor number on the cargo remains fixed at the ensemble size in figures 2a and 2c because there is no dissociation of motors from the cargo. In contrast, the number of motors on the cargo varies in figures 2b and 2d but are upper bounded by the ensemble size.

We note that the magnitudes of velocities observed are similar to the typically observed cargo velocities under a load force by ensembles of kinesins [107,108] and myosins (see [62,71] cargo velocities by single motor and [72] for multi motor. The velocities also match with our unpublished data for multiple myosin VI motors pulling a cargo under an optical trap). We observe that for same ensemble size at lower load forces, the change in velocity is nominal (1.4 fold decrease at 2 pN load force) when duty ratio is changed from 100% to 80%. However, as the cargo load forces increase, the decrease in velocity

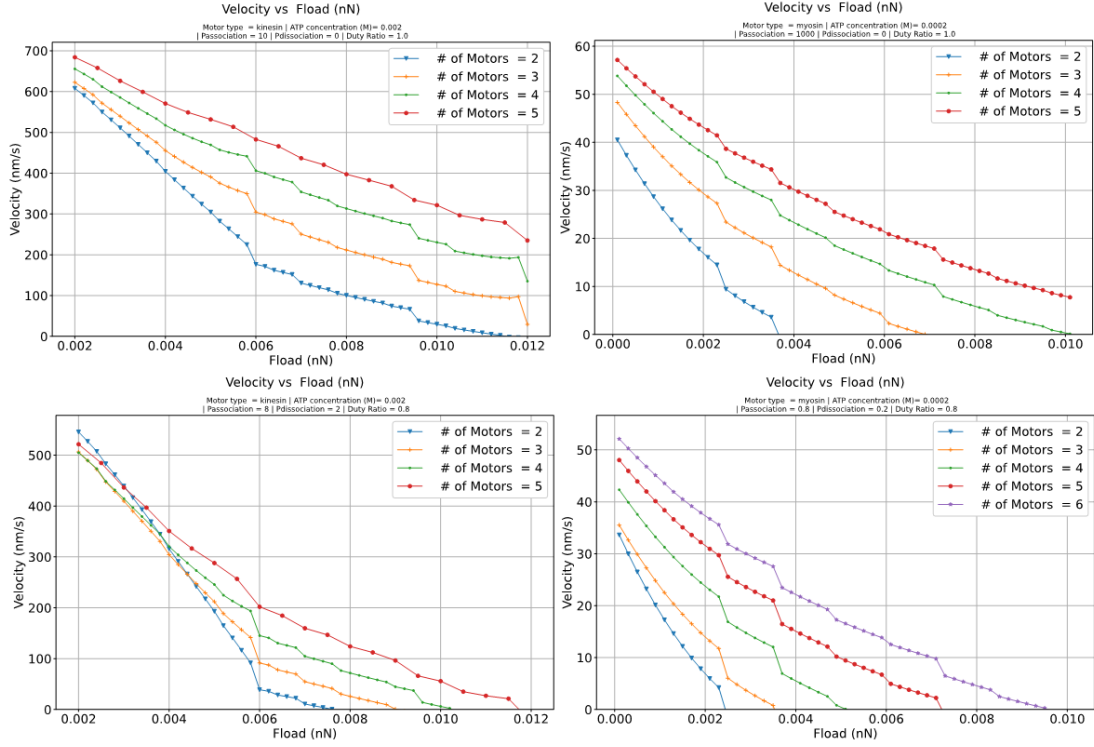


Figure 4.1: Velocity vs Load Force without dissociation (a for kinesin, c for myosin) and with dissociation (b for kinesin, d for myosin). Different curves in each subplot denote different maximal ensemble size.

is much higher (approximately five fold decrease at 10 pN load force). This translates to an increase in slope of the velocity vs load force curve indicating that the cargo velocities are more sensitive to load forces at higher cargo-motor dissociation. Another observation is, at low load forces and with dissociation of motor from the cargo, the velocity for a two motor ensemble is higher than three, four or five motor ensemble of kinesin (see Figure 4.1a vs 4.1c). A similar trend was observed by Kunwar et. al. in [108] where they observed the velocities for a single motor were higher than multi-motor ensemble. It was hypothesized that this higher velocity for single motor is due to the detachment of motors from the microtubule and the trend was reversed upon making the detachment zero. Our data confirms that similar phenomenon occurs when motors detach from the cargo with the trends reversed upon making the detachment rate of motor from the cargo zero (Figure 4.1a and c). Our results indicate higher velocities can be achieved

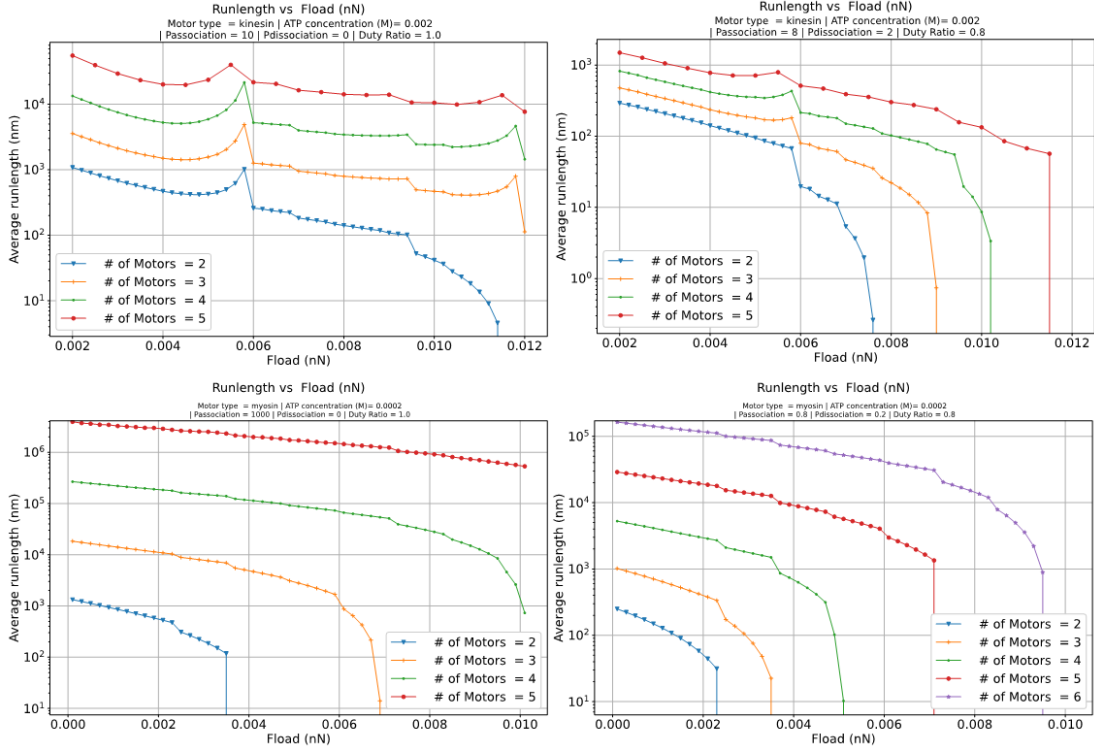


Figure 4.2: run length vs Load Force without dissociation (a for kinesin, c for myosin) and with dissociation (b for kinesin, d for myosin). Different curves in each subplot denote different maximal ensemble size.

with fewer number of motors at lower load forces when motors are allowed to dissociate from the cargo.

4.2.2 Cargo-motor dissociation leads to more robust run lengths during varying load forces

Previous studies in [107, 108, 120] have reported data similar to Figure 3a where run length decreases with increase in load force. We observe a similar trend, however, the decrease in run length is much larger when motors dissociate from the cargo. The steep cliffs in run length vs force graphs at very high load forces represent that the velocities falling to zero rendering the cargo unable to move resulting in near zero run lengths. Since myosin is a weaker motor (stall force ≈ 2 pN) compared to kinesin (stall force

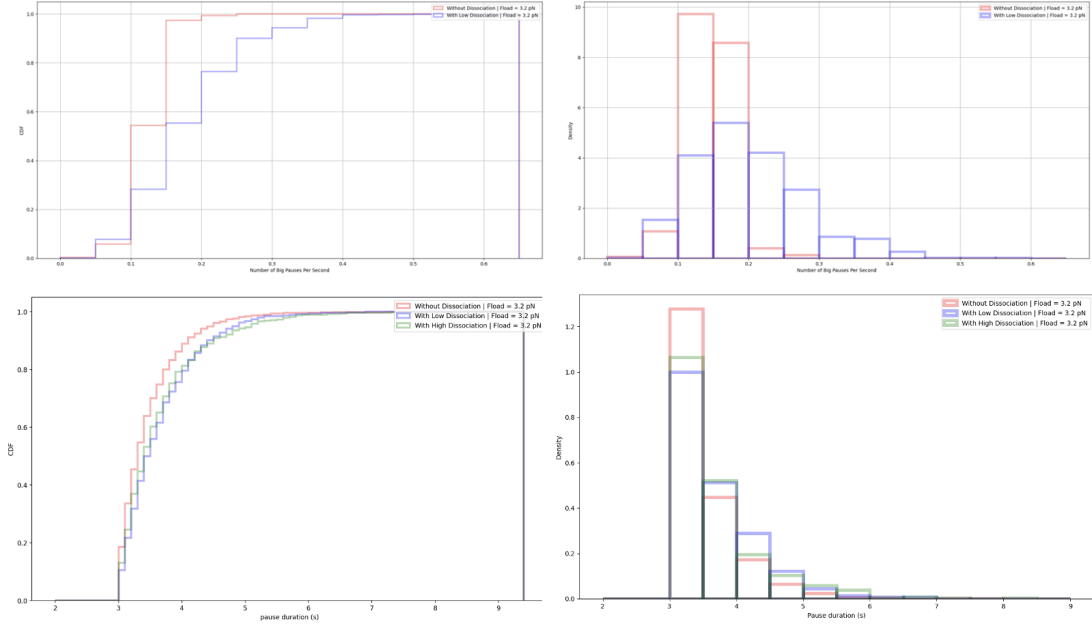


Figure 4.3: Shift in distribution of number of pauses and pause duration with and without dissociation of motors from the cargo. (a) and (b) represent the CDF and histogram of number of big pauses per second, respectively. Both distributions shift to right when motors are capable of dissociating (blue plots) compared to without dissociation (red plots). (c) and (d) show the CDF and histograms of pause duration with (blue) and without (red) dissociation of motors from the cargo. We again observe a right shift in both CDF and histograms when motors are allowed to dissociate from the cargo.

6pN), the cliffs appear at a lower load force for myosin as compared kinesin. Another key observation is that the peaks (indicated by solid arrows with 100% duty ratio and dashed arrows with 80% duty ratio in Figure 4.2a and 4.2c, respectively) in the graphs for kinesin decrease as the duty ratio is decreased from 100% to 80%. The peaks correspond to a sudden increase in run lengths when the load force is close to a single motor's stall force. The phenomenon of peaks in run length has been described in detail in [120,121]. Briefly, near the stall force, the motors tend to assume a clustered formation decreasing the probability of detachment from the tracks and ultimately leading to increased run lengths. We observe that the peaks in run length decrease with increasing cargo-motor dissociation rate. This is likely due to the fact that even though motors have a low probability of detachment from the tracks when load force is near the stall force of single motor, the clustering formation can break when the motor detach from the cargo

due to higher cargo-motor dissociation. Thus, cargo-motor dissociation results in a more consistent run length across different load forces indicating a more robust mode of transportation in presence of varying load forces while compromising on the absolute value of run lengths and velocities. We discuss this observation in more detail in the next subsection.

4.2.3 Higher cargo-motor dissociation leads to longer and more frequent pauses during cargo transport

Taken together, Figure 1 and 2 indicate that a higher CM-DR leads to lower average velocities and lower run lengths. This indicates an overall slower transport often observed when there are frequent longer pauses observed during the cargo transport. To check this hypothesis, we perform Monte-Carlo simulations and obtain the statistics of pauses during transportation. We define a pause in the trajectory as an event when there is no change in cargo position for more than a certain period of time. For thresholding purposes, we have taken that period as 3 seconds. We start the simulation with a random number of motors on the cargo below the maximum ensemble size. The simulation ends when there are no motors connecting cargo to the track, in other words, the cargo is lost. First observation from the Monte-Carlo simulations is that the trajectories have much shorter runlengths. This confirms our observations from semi-analytical model. Furthermore, we observe that the trajectories terminate much sooner in case of higher CM-DR (~ 50 seconds) vs lower CM-DR (> 250 seconds). This doesn't directly indicate that a higher CM-DR should lead to a lower average velocities. However, upon further investigating the trajectories, we indeed find that a higher CM-DR leads to overall lower average cargo velocities mainly due to the fact that cargo trajectory undergoes longer and more frequent pauses when motors frequently dissociate from the cargo. Figure 3a and 3b show the CDF and histogram of number of big pauses (> 3 seconds) per unit time. We calculate this quantity by counting the number of pauses which are bigger than 3 seconds during a trajectory and dividing it by the total trajectory time. We observe a statistically significant increase in number of pauses per unit time with a higher CM-DR. Figure 3c and 3d show the distributions of pause duration with (blue) and without (red) cargo-motor dissociation. Again, we observe a significant increase in pause duration when CM-DR is non-zero.

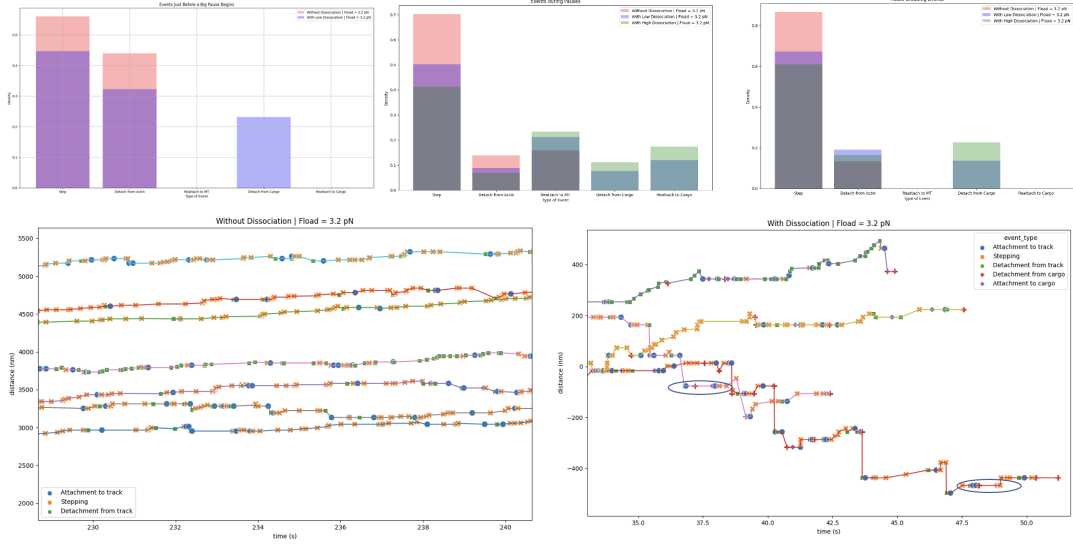


Figure 4.4: Investigating longer and higher number of pauses when motors can dissociate from the cargo. (a) shows the events occurring just before a big pause. (b) shows events during a paused cargo trajectory. Notice that even if the cargo is paused, motors can still move in a stochastic manner, eventually leading to a pause breaking event. (c) shows the events which break the pause and cause the cargo to move. (d) and (e) show some sample Monte-Carlo trajectories without and with cargo-motor dissociation, respectively. Statistics have been obtained from 10000 monte-carlo trajectories but only a few trajectories are shown for enhancing the details.

4.2.4 Investigating longer and higher number of pauses

We further investigate the reason behind the increase in pause duration and frequency when motors are capable of dissociating from the cargo. Figure 4a,b,c show the breakdown of events happening before a pause begins, during the pause, and right before a pause breaks, respectively. Figure 4a shows that when motor can dissociate from the cargo, the dissociation event can initiate a pause. Upon careful analysis of individual monte carlo trajectories (Figure 4d and e), we observe that when we allow for dissociation of motors from the cargo, then during some long pauses we see a detachment of the motor from the cargo followed by reattachment of motor to the cargo eventually leading to a pause breaking event (Figure 4e). This detachment and reattachment of motors on cargo elongates the pauses and leads to longer pauses on an average. This elucidates a possible mechanism by which a higher dissociation rate of motors from the cargo can lead to a cargo trajectory with more pauses. A similar phenomenon was observed in

Seamster et al., 2012, where the affinity of motors to the cargo was systematically altered and a higher cargo-motor detachment rate led to shorter pauses and smaller runlengths. Longer and frequent pauses has varied implications in the cellular context where on one hand, pausing leading to cargo accumulation in certain diseases [refs] and on the other hand, cargo pausing could give rise to a more efficient signaling pathways [refs]. We discuss this point in further detail in the discussion section.

4.2.5 Maximal force on actin network

We go back to Figure 1 to observe another subtle trend in the Force-Velocity curves. Note that the average cargo velocities go to zero at a lower load force with higher CM-DR. The load force value at which velocity goes to zero has been previously experimentally calculated in [refs] and is of key biological significance during cargo transport by molecular motors. Briefly, the load force value represents the maximal force that the molecular motor ensemble can translate to the tracks. Also, it has been observed that a high force on actin tracks by motors can lead to actin restructuring or a collapse of actin network. Hence, it is critical for the intracellular transport infrastructure for these forces to be properly regulated. Thus, we investigate the distribution of the total force on actin network as the CM-DR increases (Figure 4). We observe that a high proportion ($> 80\%$) forces on actin network are below 1 pN (Figure 4a). However, as the CM-DR is increased, the proportion load forces above 2 pN increases (Figure 4b). Since actin network restructuring, and eventually collapse, could initiate via rare, high force events due to cascading effects, a lower proportion of high load forces ($> 2\text{pN}$) when motors are capable of dissociating from the cargo could lead to an overall more robust cargo transport by reducing the probability of actin network collapse. We discuss the implications in more detail in the discussion section.

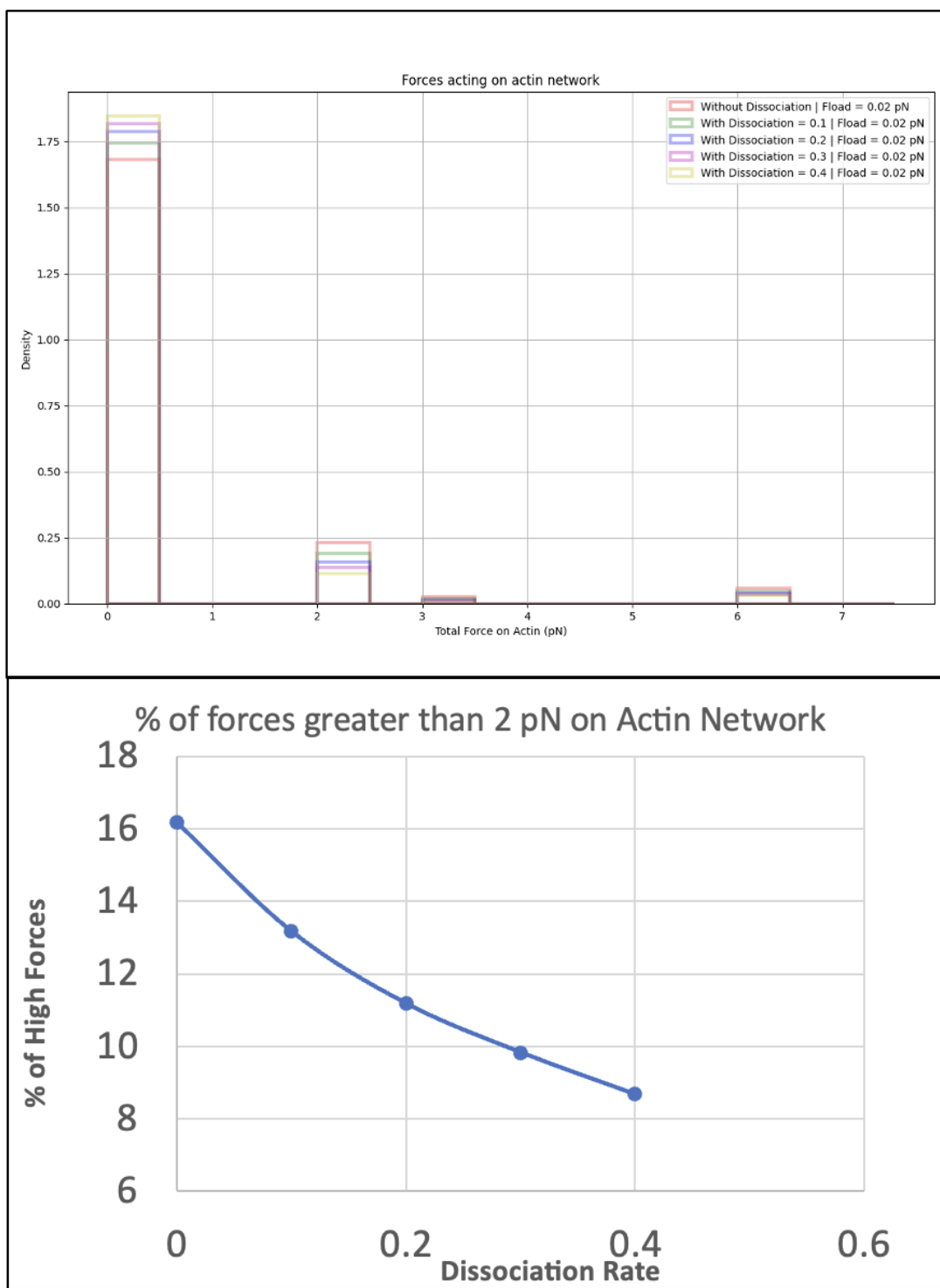


Figure 4.5: (a) Variation in distribution of forces acting on the actin network when CM-DR is varied from 0 to 0.4, with 0 representing no dissociation of motors from the cargo. (b) Change in percentage of forces greater than 2 pN with increasing CM-DR.

4.3 Discussion

4.3.1 Biological Implications of Pausing

Pauses in molecular motors driven cargo trajectories have been investigated at length. Often, cargo pauses are viewed as an anomaly in the intracellular transportation and have been linked to several diseases, especially neurodegenerative ones. Weiss and Littleton showed that During FAT of synaptic vesicles, polyQ mutation leads to a decrease in distance and duration of cargo transport and increase in duration and number of pauses [126]. Huntingtin (HTT) acts at cargo interaction sites to regulate processivity. This polyglutamine (polyQ) expansion in Huntingtin (Htt) causes Huntington’s disease. We note that changing cargo-motor dissociation rate has a similar effect on cargo transportation process with an increase in CM-DR leading to decreased cargo runlengths and cargo loss times with cargo trajectories experiencing longer and higher number of pauses. In another report, Szpankowski et al. showed that cargo pausing can lead to abnormal accumulation of proteins and vesicles during transport and is thought to be a causative event in several neurodegenerative diseases [127]. It is important to note that a direct correlation between cargo-motor interaction kinetics and cargo pauses has not been studied in the above study. We merely observe that the symptoms of an increased CM-DR are similar to those of above-mentioned diseases. However, there have been other reports of experimental investigation of impact of detachment rate of motors from cargo on pausing and cargo velocities. Seamster et. al., created artificial cargos with varied affinity towards motors, translating to different cargo-motor detachment rates [128]. It was shown that a Higher CM detachment rate leads to longer pauses and shorter run lengths. The data presented in [128] qualitatively matches our data, however a quantitative match is not feasibly due to unknown number of motors on the cargo in [128].

The above accounts in [126, 127] highlight the role of pausing, and subsequently, cargo-motor detachment in disease mechanism. However, some other studies, including more recent ones, have pointed out the significance of pausing in effective mediation of cytoskeletal signaling pathways [129]. We provide a physical insight on how these pauses could be regulated to mediate an effective material or information exchange.

4.3.2 Forces on Actin Network

An important information that could be obtained from the force velocity curves such as in Figure 1 is the maximum force that a cargo-motor ensemble can exert on the tracks. The force at which the velocity in the force-velocity curves becomes zero provides a good estimate of the maximal force on actin network [130]. Our data shows that with higher CM-DR, the maximal force on actin network decreases. We investigate this further via monte carlo simulations and confirm this finding 4.5. An extensive body of literature exists where forces on actin networks and their restructuring has been studied. Gardel group has performed measurements on the forces and elucidated several biophysical mechanisms, including forces by cargo-motor complexes, leading to the actin network collapse [131]. Moreover, There have been several force spectroscopy experiments which have confirmed the force levels at which the actin filaments disintegrate. We have shown that CM-DR plays a key role in downregulating the forces on actin network while compromising on cargo runlengths and velocities, thus preserving the overall sanctity of cytoskeleton.

Our study elucidates that cargo-motor detachment rate could be novel biophysical parameter which can impact the cargo transport as well as signaling pathways inside the cells. On one hand, a dysregulation of CM-DR can elevate cargo pausing which could potentially explain disease mechanisms such as in case of Huntington's. On the other hand, in certain cellular contexts, longer and higher number of pauses can provide opportunities for hitchhiking [132] and establishing better signaling pathways [129]. Additionally, a higher CM-DR could lead to a more robust transportation infrastructure by downregulating the forces on actin network while compromising on higher cargo velocities and run-lengths. While the role of CM-DR or its effects on cargo transport have been studied in the past, both in biophysical as well as physiological context, our study provides a comprehensive account and attempts to provide an underlying biophysical mechanism which could be at play at multiple locations in cytoskeleton and can participate in multiplexed regulation of several sub-processes during cargo transport process inside the cell.

Additionally, we also present a new paradigm for intracellular cargo transportation process. A higher number of motors on cargo can lead to higher run length and velocities might, but at a price of high forces on actin network which can be disrupted due to

larger forces. Our study provides a reconciling mechanism by which motors on cargo are regulated via a new biophysical parameter, i.e., CM-DR, which can lead to a more robust transportation process in cases where extremely high cargo runlengths and velocities are either not required or not feasible.

4.4 Online Methods

We adopt a Master Equation based approach as presented in [120] and modified for multiple species in [121] to obtain the emergent transport properties of molecular motor ensemble carrying a common cargo under an opposing load force. This method utilizes the stochastic behavior and parameters of an individual molecular motor which are obtained via various single molecule as well as bulk experiments performed by previous studies. In an ensemble carrying a common cargo, individual motors can undergo the following transitions.

1. A motor attached to the cargo and also the filament track can take a discrete step of fixed size with probability rate λ_{step}
2. A motor attached to the cargo and also the filament track can detach from the track with probability rate λ_{detach}
3. A free motor attached to the cargo but not to the track can attach to the track with probability with probability rate λ_{attach}
4. A motor attached to the cargo (but may or may not be attached to the filament track) can detach from the cargo with probability rate $\lambda_{dissociation}$
5. A free motor in the solution can attach to the cargo with probability rate $\lambda_{association}$

The transport process is modeled as a continuous time Markov process. The absolute positions of molecular motors attached to both cargo and cytoskeletal track along with the number of free motors available on the cargo form an absolute state Ω of the Markov process. An ensemble of molecular motor transitions from one absolute state Ω to another state Ω' through the above mentioned transitions with transition rates that are determined using the equations presented in the subsection 4.4.2. The collection of all

the absolute states along with the transition rate matrix forms an infinite-dimensional Markov Model which is then utilized to obtain the biologically relevant quantities.

4.4.1 Description of System and Modelling Assumptions

We consider an ensemble of $m \leq \bar{m}$ identical motors moves the cargo on the cytoskeletal track, where $\bar{m}$ is the maximal number of motors that can be present on the cargo which is dependent on the concentration of the motor in the solution and also on the geometry of cargo [133–135]. The number of motors on the cargo (m) is a stochastic variable which varies according to the cargo-motor attachment and detachment kinetics. The track is modelled as bi-infinite sequence of equally spaced locations which are one step length (d_s) apart from each other. Multiple motor can occupy the same spot on the track. A constant opposing load force, F_{load} is assumed to act on the cargo at all times.

4.4.2 Single Motor Model and Transition Rates

Each motor is assumed to be bound to the cargo via a flexible linkage of known rest length and behaves like an non-linear elastic spring with the following force-extension relationship

$$|F_m(l)| = \begin{cases} K_{el}(l - l_0) & \text{if } l \geq l_0 \\ 0 & \text{if } l < l_0 \end{cases} \quad (4.1)$$

where $F_m(l)$ is the force being experienced by the motor of linkage stiffness K_{el} and rest length l_0 stretched to length l . Each motor can undergo one of the above mentioned five transitions whose rates are obtained as follows (similar to the formulation in [108] and [120]. Note that the differences and similarities between the kinetic cycles of myosin, kinesin, and dynein are highlighted in [136].

Stepping Rate and Stalling for kinesin: While stepping, a motor converts ATP into kinetic energy and the reaction rates are given by Michaelis-Menten Dynamics which describe the force dependent stepping rate of a motor given by (4.2).

$$\lambda_{step,kinesin} = \frac{k_{cat}[ATP]}{[ATP] + k_m} \epsilon, \quad (4.2)$$

where $k_m = \frac{k_{cat} + k_{off}}{k_{on}}$ and ϵ is a force dependent quantity given by:

$$\epsilon(F) = \begin{cases} 1 & \text{if } F \leq 0, \\ 1 - \left(\frac{F}{F_d}\right)^2 & \text{if } 0 < F < F_s, \\ 0 & \text{otherwise.} \end{cases} \quad (4.3)$$

Note that the stepping rate of a motor becomes 0 if there is an opposing force larger than the stall force (F_s) acting on the motor. It is also assumed that k_{off} is a force dependent quantity given by $k_{off} = k_0 e^{F d_l / K_b T}$, where k_0 is the backward reaction rate of the hydrolysis when $F = 0$, K_b is the Boltzmann constant, T is the temperature and d_l is an experimentally determined parameter.

Detachment Rate for kinesin: When a motor is experiencing a force $F < F_s$, the detachment rate can be obtained using stepping rate via the following relationship as defined in [Schnizer et al. 2000]:

$$\frac{\lambda_{step,kinesin}(F)}{\lambda_{detach,kinesin}(F)} = \frac{[ATP] A e^{-F \delta_l / K_b T}}{[ATP] + B(1 + A) e^{-F \delta_l / K_b T}} \quad (4.4)$$

where A, B and δ_l are experimentally obtained parameters. When $F \geq F_s$, a constant detachment rate of $\lambda_{detach,kinesin}(F) = P_{back} = 2s^{-1}$ is assumed.

Stepping and Detachment Rate for Myosin: Stepping and detachment rates for myosin VI are obtained from experimental studies in [97], [85], and [137]. An empirical formula for stepping rate is given by:

$$\lambda_{step,myosin} = \tau_b + \tau_m \exp\left(\frac{F \delta}{k_b T}\right)$$

where F is the net force acting on the individual motor, τ_b, τ_m and δ are constants dependent on ATP concentration with $\tau_b = 0.3$, $\tau_m = 0.28 \times 10^{-20}$ and $\delta = 91$ at

saturating ATP concentrations and $\tau_b = 0.16$, $\tau_m = 0.3$ and $\delta = 2.9$ at sub-saturating ATP concentrations.

Detachment for myosin VI, $\lambda_{detach,myosin}$, is assumed to be independent of force on motor or ATP concentration and is given in table 4.1.

Attachment Rate and locations of reattachement: A constant attachment rate of $\lambda_{attach} = 5s^{-1}$ is assumed as found by experimental studies in [ref.]. If a free motor is available on the cargo, it is assumed that it can only attach at the locations where it doesn't have to stretch itself i.e. $\pm l_0$ distance from the cargo position. It is assumed that attachment to any of these locations is equally likely.

Motor-Cargo Dissociation and Association Rates: These rates are assumed to be constant with respect to F_m and are varied as the simulation parameters to see their effect on the emergent cargo transport properties.

4.4.3 Absolute States

The absolute locations of motors on the track carrying a common cargo on the track along with number of free motors on the cargo constitute an absolute state (Ω) of the Markov Process. For illustration consider Figure 4.6 where, there are two motors attached to both the cargo and the track whose position on the track are represented by "1" in the bi-infinite sequence array which forms the first part of the absolute state. Also, there is one free motor attached to the cargo which is represented by a "1" stacked together with the array to form the absolute state.

The cargo-motor-track system transitions from one absolute state, Ω , to another absolute state, Ω' via the above-mentioned transitions with their respective rates. Thus the probability that the system transitions from the absolute state Ω at time t to another absolute state Ω' at time $t + \Delta t$ is given by Equation 4.5:

$$P_{abs}(\Omega', t + \Delta t | \Omega, t) = \lambda_{abs}(\Omega', \Omega) \Delta t \quad (4.5)$$

where $\lambda_{abs}(\Omega', \Omega)$ is the transition rate from Ω to Ω' and depends on the type of transition. Thus, the master equation governing the time evolution of probabilities of any absolute state Ω , $P_{abs}(\Omega, t)$, is given by equation 4.6:

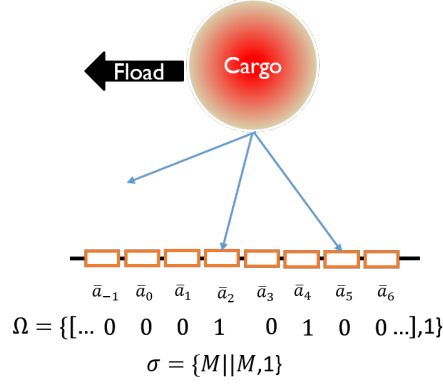


Figure 4.6: Example of an absolute configurations of motors on the track and the cargo constituting the absolute state Ω .

$$\begin{aligned}
 \frac{\partial}{\partial t} P_{abs}(\Omega, t) = & \sum_{\Omega' \in \mathcal{Z}} \lambda_{abs}(\Omega, \Omega') P_{abs}(\Omega', t) \\
 & - P_{abs}(\Omega, t) \sum_{\Omega' \in \mathcal{Z}} \lambda_{abs}(\Omega', \Omega)
 \end{aligned} \tag{4.6}$$

where $\mathcal{Z}$ is the set of all possible absolute configuration and the equation is obtained by the conservation law of total probability measure. Since the track is assumed to be a bi-infinite sequence, the number of such absolute states are infinite. These infinite states constitute an infinite dimensional system of first order differential equations which governs the time evolution of probabilities of all absolute states. However, solving the infinite dimensional system of first order differential equation is an intractable problem. To resolve this issue we obtain a projection of infinite dimensional absolute state space onto a finite dimensional relative state space by aggregating the absolute states into a finite number of "macro-states" termed as the relative states and develop a finite dimensional system of differential equation governing the time evolution of probabilities of relative "macro-states". We then show that solving such a finite dimensional system is tractable and its solution leads to the derivation of many biologically relevant quantities.

4.4.4 Relative States and Corresponding Master Equation

A finite dimensional Markov model is obtained by using a similar strategy as employed by Bhaban et al. in [121] and Materassi et al. in [120]. Briefly, any absolute configuration can be represented by using two symbols M , and $|$. First we identify the first motor from the left in any absolute configuration. This is termed as a "rearguard" motor. Starting with the rearguard motor, we start writing symbol " M " if a motor is present on the track and use symbol " $|$ " to separate distinct locations on the track. We thus form this sequence of M s and $|$ s until we encounter the last motor in the configuration from the left, termed as the "vanguard" motor. We use multiple M s to represent multiple motors at the same location on the track. The configuration thus obtained is termed as *relative configuration*. For example in Figure 4.6, the relative configuration for the absolute state shown is given by $\{M||M,1\}$, with " 1 " at the end representing the number of free motors on the cargo. Essentially, the relative configuration represents the relative "arrangement" of motors on the cytoskeletal track with respect to each other, along with the number of free motors on the cargo. It is important to note that multiple absolute configurations can have the same relative configuration. In fact, all absolute configurations which have the same relative configuration, are "translated", i.e. laterally shifted versions of their relative configurations along the track. Thus we define the set $\mathcal{Z}(\sigma) := \{Z | \Gamma(Z) = \sigma\}$ which represents the set of all absolute configurations Z whose relative configuration is σ . Here $\Gamma : Z \rightarrow \sigma$ is a projection map to obtain the relative configuration given any absolute configuration.

Observing the fact that in this study we only consider scenarios which involve a finite number of motors on the cargo, and also owing to the fact that the motors cannot take a step after they feel a force greater than or equal to the stall force, we show that the number of these relative configurations are finite. A crucial result that leads to the finiteness of number of relative configuration is that in any given relative configuration, the distance between the vanguard and rearguard motor, termed as *extent*, is upper-bounded by n which is given by (4.7) below.

$$n = \lceil \max \left\{ \frac{\overline{m}F_s - F_{load}}{K_{el}d_s} + 1, \frac{F_{load}}{K_{el}d_s} + \frac{2l_0}{d_s} \right\} \rceil \quad (4.7)$$

Here $\lceil \cdot \rceil$ represents the ceiling function. Thus the total number of relative configurations

with a maximum $\bar{m}$ number of motors possibly present on the cargo is given by:

$$N = \sum_{m=1}^{\bar{m}} \left[\left(1 + \sum_{i=1}^m \frac{(n+i-2)!}{(n-1)!(i-1)!} \right) (\bar{m} - m + 1) \right] \quad (4.8)$$

A detailed derivation of equations (4.7) and (4.8) is presented in the [120]. We then obtain the transition probability from a relative configuration σ at time t to another relative configuration σ' at time $t + \Delta t$ using the following

$$P_{rel}(\sigma', t + \Delta t | \sigma, t) = \lambda_{rel}(\sigma', \sigma) \Delta t \quad (4.9)$$

Interestingly, the transition rates in the relative configuration space can be obtained using the knowledge of transition rates in the absolute configuration states using the following result:

$$\lambda_{rel}(\sigma', \sigma) = \sum_{0 \leq \beta \leq \lceil \frac{n}{d_s} \rceil} \lambda_{abs}(\gamma^\beta \Omega', \Omega), \quad (4.10)$$

where $\Gamma(\Omega) = \sigma$, $\Gamma(\Omega') = \sigma'$, $\gamma^\beta \Omega'$ is the absolute configuration obtained by linearly shifting all the motors in Ω' by β locations to the right in the cytoskeletal track. This result is previously reported by us in [ref1] for the case with fixed number of motors on the cargo and also in [ref2] in which it is shown to hold in case when a fixed number of both healthy and mutated motors are present on the cargo. A derivation for the formula in equation 4.10 for the case when the number of motors on the cargo is stochastic but upper bounded by $\bar{m}$ is a direct extension of the formula presented in [120]. Using the transition rates obtained from each relative configuration to every other relative configuration, we form the $N \times N$ transition rate matrix, $\mathcal{A}$, with

$$\mathcal{A}_{j,i} = \begin{cases} \lambda_{rel}(\sigma_j, \sigma_i) & \text{for } j \neq i \\ -\sum_{j \neq i} \lambda_{rel}(\sigma_j, \sigma_i) & \text{for } j = i \end{cases} \quad (4.11)$$

This transition matrix and the properties of Markov chains are used to obtain the biologically relevant quantities.

4.4.5 Biologically Relevant Quantities

We now present the methods for obtaining some of the biologically relevant quantities.

Average Time Before Cargo is Lost: We consider the cargo is lost when our Markov chain hits the state 0. Thus the average cargo loss time becomes a hitting time calculation problem [138] which can be obtained by solving the following set of recursive linear equations:

$$\mathbb{E}_j[T_0] = \mathbb{E}_j[T_1] + \mathbb{E}_1[T_0] \quad (4.12)$$

and

$$\mathbb{E}_1[T_0] = \frac{1}{\mathcal{A}_{0,1}} \left[1 + \sum_{j>1} \mathbb{E}_j[T_1] \mathcal{A}_{j,1} \right] \quad (4.13)$$

where $\mathbb{E}_j[T_i]$ is the expected hitting time to state i starting from state j . The average cargo lost time can be calculated via the following steps:

1. Determine $\mathbb{E}_j[T_0]$ for all j using recursive equations 4.12 and 4.13
2. Form a vector $\mathbf{g}_0 = [\mathbb{E}_1[T_0], \mathbb{E}_2[T_0], \dots, \mathbb{E}_N[T_0]]$
3. $\mathbf{g}_0 \cdot \mathbf{p}_0$ gives the average cargo loss time where $\mathbf{p}_0$ is the initial probability mass function for all the states $1, 2, \dots, N$ determined as described in [108] and [120]

For further details on this method, refer to [139].

Average Run Length: Average run length can also be calculated via a similar set of recursive equation as above formulated for distance in place of time.

Average Velocity: Average velocity is estimated by taking the ratio of average run length with average cargo loss time.

Note that, none of the equations above require time step simulations or numerical integration, increasing the simulation speeds several folds compared to [120] and [108].

4.4.6 Parameters of Motors Used

Numerical parameters used in this study are given in Table 4.1.

Parameter	Value	Value[ref]
Motor Type	Kinesin-1	Myosin
References	[Kunwar]	[ref]
$k_{on} (M^{-1}s^{-1})$	$2 \cdot 10^6$	-
$k_{off} (s^{-1})$	55	-
$k_{cat} (s^{-1})$	105	-
$d_s (nm)$	8	30 [140]
$d_l (nm)$	1.6	-
$\delta_l (nm)$	1.3	-
A	107	-
$B (\mu M)$	0.029	-
$[ATP](mM)$	2	2
$T(K)$	300	300
$K_{el}(pN/nm)$	0.32	0.12 [85]
$F_s(pN)$	6	2 [140]
$l_0(nm)$	100	80
Motor actin reat- tachment rate ($\mu M^{-1}s^{-1}$)	5	5 [97]
Motor actin de- tachment rate ($\mu M^{-1}s^{-1}$)	-	9 [85]

Table 4.1: Numerical Parameters used in this study

4.5 Supporting Information

4.5.1 Validation

We observe how multi-motor cargo transport is affected when we change the duty ratio of motors on the cargo. We define the duty ratio as:

$$\text{duty ratio} = \frac{\text{association rate}}{\text{association rate} + \text{dissociation rate}}$$

Note that the duty ratio is decreased when the cargo-motor dissociation rate is increased.

Validation: Previous studies have performed Markov chain simulations [120, 121] and Monte Carlo simulations [108, 141] and have observed the variation of average cargo velocities and run lengths with load force acting on cargo that is being carried by an

ensemble of up to four motors. However, they do not include the possibility of association and dissociation events between the cargo and the motor. To validate our approach, we set the cargo-motor dissociation rate to zero (or equivalently duty ratio = 100%) in our simulation and observe the average cargo velocities and run lengths (Figure S4.7) while keeping all the other parameter the same as [120] and [108]. We find that our results match almost exactly with both the previous studies.

In our algorithm, we have leveraged the properties of Markov chains (see Section 4.4) and employed a hitting time calculation, obviating the need of doing numerical integrations as in [120] and [121], or time step simulations as in [108]. This makes our algorithm significantly more computationally efficient enabling us to simulate a more realistic ensemble of up to seven motors using regular desktop PCs and even more if we take advantage of supercomputing clusters. We have omitted the simulations of ensembles of more than six motors to focus on the key findings of the study which are presented in the next section. All our simulations are performed using parameters for kinesin 1 as well as processive HMM myosin VI dimer motors listed in table 1.

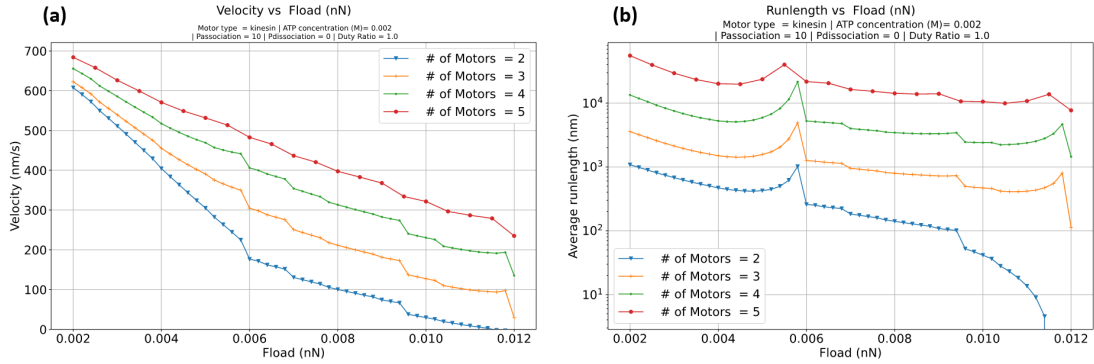


Figure 4.7: Comparison of results using Markov chains based simulation engine with previous studies. Results are nearly indistinguishable as compared to studies [120] and [108] for both velocity (a) and run lengths (b) when simulated for identical parameters. The dissociation rate is set to 0 in the Markov chain based model to match the conditions with previous studies.

cargo varies in figures 2b and 2d but are upper bounded by the ensemble size.

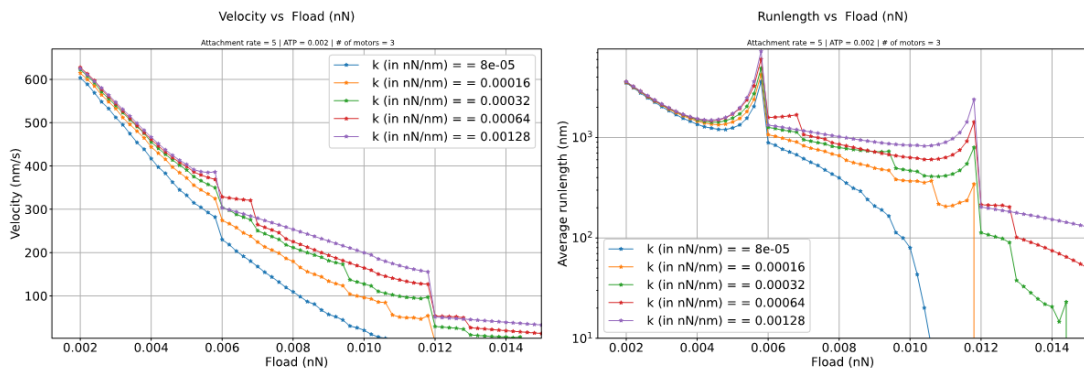


Figure 4.8: Variation of velocity and run length with respect to load force for an ensemble of 3 motors with varying linkage stiffness.

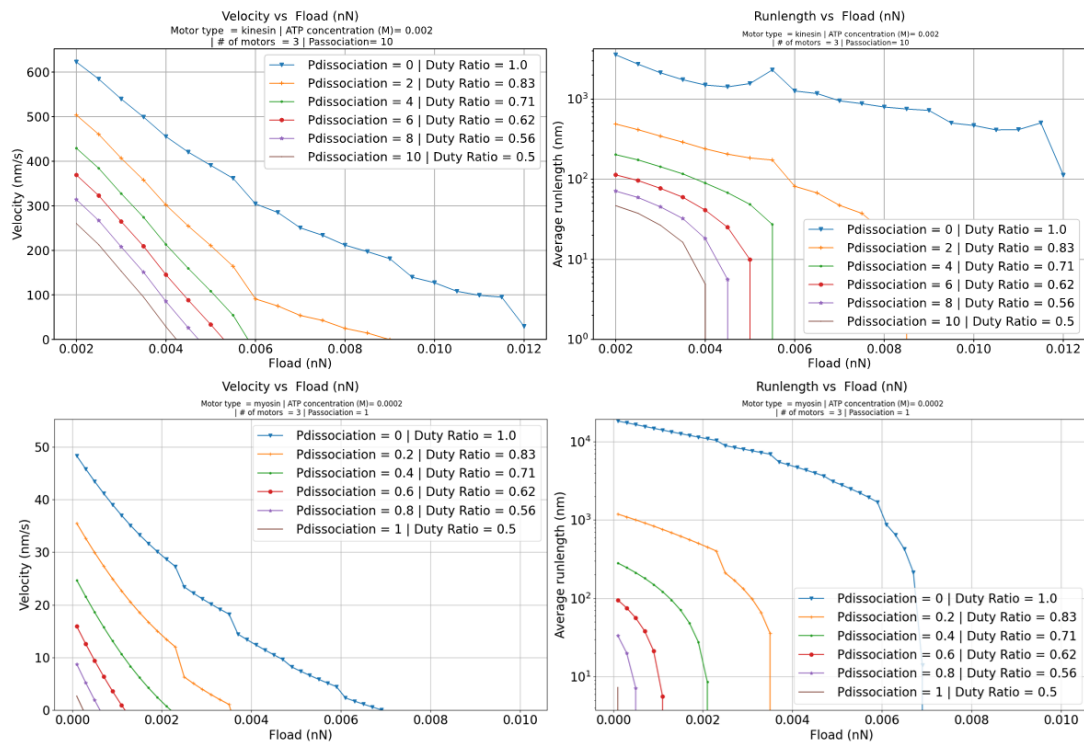


Figure 4.9: Variation of velocity and run length with respect to load force for an ensemble of 3 motors with varying cargo-motor dissociation rate

4.5.2 Supplemental Data

Decrease in effective linkage stiffness: Previous studies have shown that motor induced actin or microtubule networks restructuring can alter cargo transportation processes [142]. Such restructuring has been implicated in diseases such as Salmonella typhimurium infection [103]. Our study provides a mechanism through which this restructuring could be tuned inside a cell by modulating cargo-motor dissociation rates. We recently have shown in our previous studies [62, 71] that the cargo-motor interaction kinetics can be modulated either by artificially changing the linkage stiffness of cargo-motor cross-bridge or via certain adapter proteins such as GIPC. On the other hand, previous simulations [108] have also shown that increasing the strength of the crossbridge linkage between the cargo and the motor increases both velocity and run length during a multi-motor cargo transport. Observing a similar phenomenon when we decrease the dissociation of motor from the cargo, we hypothesize that an increase in cargo-motor dissociation rates could lead to a lower effective linkage stiffness resulting in a weaker cross-bridge between the cargo and the tracks. We test this hypothesis by performing simulations with varying linkage stiffness while setting the cargo-motor dissociation rate at 0 (Figure 4.8). We see a similar trend in the force-velocity curves (Figure 4.8a) as we saw in Figure 4.9a and 4.9b but not to the same extent. This indicates that increasing cargo-motor dissociation rate leading to a lower effective linkage stiffness may only partially cause a decrease in cargo velocities. We also see a decrease in peaks in run length vs load force with increasing stiffness (Figure 4.8b) curves but again, the fractional decrease in peaks is much lower compared to that observed in Figure 4.9c. One possible explanation for along with altering the effective linkage stiffness, a change in cargo-motor dissociation rate could modify other biophysical parameters as well which could explain the full drop in velocities and run lengths, however, the analysis of those parameters is currently out of scope of the present study.

Chapter 5

Conclusion and Future Work

5.1 Conclusion

The investigation into the complex interplay of cargo-motor interaction kinetics in multi-motor cargo transport has revealed novel insights into the mechanics of intracellular transportation. This thesis has addressed fundamental questions concerning the regulation and biophysical underpinnings of cellular cargo transport, shedding light on the intricate machinery that governs this crucial process within living cells.

In Chapter 2, we explored the impact of cargo-motor linkage stiffness on multi-motor cargo transport. This research uncovered how local parameters, such as linkage stiffness, could significantly affect cargo transport properties. We observed that variations in linkage stiffness could influence cargo runlengths and velocities, challenging the traditional understanding of global factors as the sole regulators of cargo transport. The findings from this chapter underline the importance of considering the mechanical properties of cargo-motor linkages in the broader context of intracellular transportation.

Chapter 3 delved into the role of adapter proteins, specifically GIPC, in modulating the motility of myosin VI motors. The study demonstrated how the presence of adapter proteins could impact cargo transport by altering interaction kinetics between the cargo and motors. This insight highlights the complexity of the cellular transport system and emphasizes the need to account for the diverse array of molecules and structures that influence cargo motion.

In Chapter 4, we presented the results of our investigations, which unveiled the profound effects of cargo-motor dissociation on multi-motor cargo transport. We observed that a higher rate of cargo-motor dissociation led to a range of intriguing phenomena. Specifically, we noted increased sensitivity of cargo velocity to load force, suggesting that higher dissociation rates could result in more efficient cargo transport under certain conditions. Additionally, we found that cargo-motor dissociation induced more robust run lengths during varying load forces, reducing the likelihood of actin network collapse. However, these benefits came at the cost of lower absolute velocities and run lengths, as higher dissociation rates led to longer and more frequent pauses in cargo transport. These pauses, we argue, could have biological implications, potentially mediating more efficient signaling pathways and material exchange within cells. Furthermore, we explored the role of cargo-motor dissociation in downregulating maximal forces on the actin network, which could help prevent network collapse and maintain cellular infrastructure.

5.2 Future Work

While this thesis has provided valuable insights into the role of cargo-motor interaction kinetics in cellular cargo transport, numerous avenues for future research remain unexplored. The following are potential directions for future investigations:

1. **Experimental Validation:** Some of the findings presented in this thesis are based on computational simulations. Experimental validation of the effects of cargo-motor dissociation under controlled conditions would strengthen the conclusions and bridge the gap between computational models and biological reality.
2. **Biological Context:** Extending the research to specific biological contexts and systems could uncover the role of cargo-motor dissociation, linkage stiffness, and adapter proteins in diseases and cellular processes. For example, studying the impact of these factors in neurons could yield insights into neurodegenerative diseases.
3. **Adaptation to In Vivo Models:** Applying the simulation framework developed in this thesis to in vivo models or real cellular systems could provide a more comprehensive understanding of the role of these factors in cellular processes.

4. **Cargo Types and Motor Families:** Investigating the effects of cargo-motor dissociation, linkage stiffness, and adapter proteins on various cargo types (e.g., organelles, vesicles, chromosomes) and motor families (e.g., kinesins, myosins, dyneins) would yield a more nuanced understanding of the regulatory mechanisms at play.
5. **Biophysical Mechanisms:** Further exploration of the biophysical mechanisms behind cargo pausing, the role of cargo-motor detachment and reattachment events during pauses, and the influence of cargo-motor linkage stiffness on pausing could offer insights into potential therapeutic interventions.
6. **Regulation and Signaling Pathways:** Research into how cargo-motor dissociation rates, linkage stiffness, and adapter proteins are regulated within the cell and their specific roles in cellular signaling pathways is an exciting avenue for future investigation.
7. **Clinical Implications:** Investigating the potential clinical implications of cargo-motor dissociation, cargo-motor linkage stiffness, and adapter proteins in disease states, such as neurodegenerative disorders and cancer, could open doors to novel therapeutic strategies.

5.3 Bioinspired Micron-Scale Transportation Systems

The insights gained from this research also hold promise beyond the realm of cell biology. The principles uncovered regarding the regulation of cargo transport at the micron scale, where cargo motion is mediated by molecular motors, can be applied to engineer bioinspired transportation systems. By mimicking the biophysical mechanisms observed within cells, we can potentially design and develop efficient and adaptable micron-scale transportation systems. These systems could find applications in drug delivery, microscale robotics, and other emerging fields, where precise and controllable cargo transport is of paramount importance.

In summary, this thesis has advanced our understanding of the mechanical intricacies governing intracellular cargo transport. By elucidating the effects of cargo-motor dissociation, cargo-motor linkage stiffness, and adapter proteins, we have unveiled new

dimensions of this critical biological process. Future research in this field holds the promise of unraveling further mysteries and potentially contributing to the development of therapies for a range of diseases with cellular transport dysregulation at their core, while also inspiring innovative solutions in the realm of micron-scale transportation systems.

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