MECHANOCHEMICAL BASIS OF FUNCTIONAL DIVERSITY IN THE KINESIN SUPERFAMILY

A Dissertation in
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by

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Kinesin motor proteins drive numerous active processes the cell, including vesicle transport, DNA and organelle repositioning, intraflagellar transport, and mitotic spindle organization. There are 45 kinesin genes in the human genome, each of which encodes an isoform that is optimized to drive some processes, but is incapable of driving others. Years of in vitro total internal reflection fluorescence microscopy (TIRFM) and optical trapping assays have helped to characterize biophysical metrics like velocity, run length, and stall force for various kinesin family members. Coupled with structural and cellular work, the kinesin field has produced a solid understanding of what different kinesins are capable of. What remains misunderstood, however, is how the 14 kinesin families are capable of performing such diverse jobs overall, but such specific jobs individually. For example, it is unknown why a kinesin-1 knockdown is embryonic lethal despite the fact that kinesins -1, -2, and -3 are all ubiquitously expressed transporters and are all capable of attaching to similar cargoes. Understanding the functional diversity of kinesins is important because it is fundamental to cell biology, and it provides insights into how kinesin dysfunction leads to disease states such as Charcot-Marie-Tooth disease, hereditary spastic paraplegia, hereditary sensory and autonomic neuropathy type II, Alzheimer’s disease, and various cancers. The goal of this dissertation is to determine the mechanistic underpinnings of functional diversity across the kinesin superfamily. This is done by carrying out comparative studies of motors in the kinesin-1 and kinesin-2 families to determine how critical structural differences in these transport motors tune transition rates between conformational intermediates in the mechanochemical cycle. This kinetic tuning describes exactly how the aforementioned biophysical metrics arise and provides insight into the structure-function relationship underlying family-specific emergent behaviors.
Answering cornerstone questions regarding the mechanistic differences between kinesins has to date been hampered by the resolution barriers of single-molecule techniques. Dimeric transport kinesins take discrete 8-nm steps along microtubules in a hand-over-hand fashion, hydrolyzing one ATP per step. Kinesins thus use a single ATP molecule to transition from a state with both heads bound to the microtubule, to a one-head-bound state where the rear head has been lifted off the microtubule, and back to a two-heads-bound state where the front and rear heads have now switched positions. Because kinesins take tens to hundreds of 8-nm steps per second, measuring steps under the microscope requires very high spatiotemporal resolution. Measuring the transitions between the two- and one-head-bound states (substeps) that subdivide each step requires higher resolution still. TIRFM, the ubiquitously used microscopy technique for single-molecule studies is fundamentally incapable of achieving the spatiotemporal resolution necessary to measure kinesin substeps at physiological stepping rates.

This dissertation introduces two new breakthrough superresolution microscopy techniques, total internal reflection dark-field microscopy and interferometric scattering microscopy, both of which are capable of measuring the position of active proteins with 1-nm and 1-ms resolution. These new microscopes are first described in detail, including instructions on how to build them, and then are applied to studying kinesin mechanochemistry. By tracking single kinesin motors with unprecedented spatiotemporal resolution, the first measurement of substeps are made. This exciting discovery opens up the entirely new paradigm of measuring structural kinetics from single-molecule tracking experiments.

Beyond the introduction of new microscopy techniques, this dissertation provides four major contributions to motor protein cell and molecular biology. First, the mechanochemical cycles of kinesin-1 and -2 are solved, providing a complete and quantitative description of how these motors transduce chemical energy into mechanical motion. Second, the generalized
mechanochemical basis of processivity is elucidated, providing a detailed strategy for mapping structural differences between kinesin isoforms to differences in their ability to transport cargoes long distances. Third, the mechanochemical basis of kinesin sidestepping is elucidated, providing a means of mapping structural differences between isoforms to their ability to navigate microtubules crowded with other proteins, especially in disease states like Alzheimer’s. Finally, the mechanochemical basis of motor-motor coordination in multimotor transport is uncovered, providing a clear description of why both kinesins -1 and -2 are needed for effective cargo transport. Overall, this dissertation provides engineering design principles and instructions for two new exciting superresolution microscopy techniques, and leverages those techniques to answer critical biological questions about how functional diversity is achieved in the kinesin superfamily.
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Chapter 1

Introduction

The cell is a collection of protein machines(1). The various behaviors that cells exhibit, from internal organization to motility to mitosis and beyond, are active processes driven by the coordinated actions of well-designed proteins(1, 2). Hence, to understand cell physiology in health and disease, it is necessary to think in terms of molecular mechanisms, with proteins as actors rather than as passive biochemical species. Indeed, as structural, computational, and functional tools have improved, it has become increasingly possible to break proteins down into their component subdomains and think about how the dynamical rearrangements of these parts can underlie function, and enable protein machines to be mechanistic actors.

One particularly interesting protein machine is the molecular motor kinesin. Kinesin was initially discovered in 1985 as translocating enzyme purified from squid axoplasm(3). Over the past three decades, kinesin has been vigorously studied and many exciting discoveries have been made about its action as a microtubule-based transporter that moves intracellular cargoes in the anterograde direction, i.e. towards the cell periphery(4–6). In addition, kinesin has been used as a model system for developing and characterizing various new biophysical tools including the optical trap(7–9), nanometer-precision fluorescence microscopy(10), and many others. However, despite all of this work, the exact mechanism by which kinesins work, and how that mechanism is differentially tuned between the many kinesin isoforms, has remained elusive.

This dissertation is devoted to developing and applying advanced biophysical tools for elucidating the inner workings of kinesin motor proteins. A cross-family approach is used to see how kinesins that are built differently operate as machines differently, and how these differences drive unique molecular mechanisms in the cell.
1.1 Kinesin motor proteins hydrolyze ATP to take 8-nm steps

Kinesin motor proteins exercise anterograde transport by literally walking along microtubules, taking steps towards the microtubule plus end(11). Kinesins are typically homodimers with four important domains (Figure 1-1): An N-terminal motor domain (also called a head), which interacts with the microtubule as well as binds and hydrolyzes ATP fuel; a neck-linker domain, which connects the motor domain to the stalk; a stalk, which is a coiled-coil with hinges that holds the dimer together, and a tail-domain, which interacts with cellular cargoes either directly or indirectly through adapter proteins(11, 12).

Figures

**Figure 1-1 Domain structures of common molecular motors.** Kinesin, highlighted on the left as a homodimer, walks along microtubules using its motor domains (also called heads). The neck-linkers are highlighted by red arrows. Figure adapted from (11).

Kinesin has been shown to take discrete steps as dictated by the tubulin dimer spacing in microtubules, which amounts to 8-nm if the tail domain is tracked(8, 13), and 16-nm if a single head is tracked(10, 14, 15) (Figure 1-2). Kinesin has also been shown to hydrolyze only one ATP per step, making it a highly efficient motor(16). Kinesin stepping has been shown to follow a hand-over-hand mechanism(10, 17), where one head stays planted while the other steps over it (Figure
1-2). Kinesins typically track a single protofilament along a microtubule track as they move(18), and they do not move indefinitely. Instead, each kinesin only translocates (walks) for some characteristic distance, known as the run length, which is about 800-nm, or 100 steps, for kinesin-1(19). Kinesin is often referred to as a processive enzyme, since it will hydrolyze multiple ATP molecules (one per step) for every diffusional collusion it has with a microtubule track(19). The terms run length and processivity are often used interchangeably.

![Figure 1-2 Kinesin steps hand-over-hand along microtubules.](image)

**Figure 1-2 Kinesin steps hand-over-hand along microtubules.** The spacing between each tubulin dimer in a microtubule (only one protofilament shown here) is about 8-nm. If the tail is tracked, each step appears as a 8-nm translocation. If a single head is tracked (as in the gold tracker shown here), then steps appear as alternating 0-nm and 16-nm translocations. One ATP molecule is used per step.

1.2 **Functional diversity in the kinesin superfamily**

When we say kinesin, we are typically referring to canonical kinesin-1(3). However, there are actually 45 different kinesin genes in humans, a superfamily that sub-divides into 14 families(12, 20). The kinesin superfamily tree is shown in Figure 1-3. Kinesins are known to be responsible for a huge number and diversity of essential cellular roles, and interestingly, these roles tend to group by the family(21). Kinesin-1 family members (N1 in Figure 1-3) are responsible for the intracellular anterograde transport of vesicles and organelles(3, 12). Kinesin-2 family members
(N-4 in Figure 1-3) are also responsible for intracellular transport, but extend even farther towards the cell periphery into dense microtubule networks such as flagella and cilia(12, 22). Kinesin-3 family members (N-3 in Figure 1-3) are also intracellular transporters(12). Kinesin-5 family members (N-2 in Figure 1-3) are mitotic kinesins that help drive metaphase plate alignment and pole separation during metaphase(12, 23). Kinesin-6 family members (N-6) make up part of the centralsplindlin complex and helps initiate the cleavage furrow during cytokinesis(24). Kinesin-7 family members function to couple the motion of dynamic microtubule tips to kinetochores(25, 26). Indeed, the list of cellular roles that kinesin superfamily members is very large and very diverse.

**Figure 1-3 Phylogenetic tree of the kinesin superfamily.** 45 kinesin genes in human can be put into 14 families. Figure adapted from (12).

An extremely interesting question, and indeed the driving question behind this dissertation work, is how kinesin family members carry out such diverse jobs overall, but such specific jobs
individually. As the list of cellular roles above implies, there is a surprisingly lack of functional redundancy between members of different kinesin families. For example, kinesins-1, -2, and -3 are all ubiquitously expressed transport motors, but knockout of kinesin-1 is embryonic lethal and leads to a failure to disperse mitochondria (27) despite the fact that kinesin-3 can also transport mitochondria (28). Similarly, kinesins-1 and -3 cannot rescue intraflagellar transport when kinesin-2 is inhibited (22), even though kinesin-1 and -2 can largely associate with the same cargoes (28, 29). But why? After all, each kinesin is just a motor that takes 8-nm steps towards the plus ends of microtubules. In some cases, we can answer this question with structure. For example, kinesin-5 forms homotetramers, and that particular four-headed structure is essential to its particular cellular role (23). However, especially amongst the transport kinesins, the structures are extremely similar. Hence, why kinesins-1, -2, and -3 cannot rescue one another is not due to major structural differences nor is it due to different abilities to attach to cargoes. Rather, it is due to the way that they step. And to understand the different ways that different kinesins walk, to link minor structural differences to major functional differences, we need to understand mechanochemistry.

1.3 Basics of kinesin mechanochemistry

Kinesin mechanochemistry refers to the cyclical procession of the two motor domains through a sequence of states that each have a chemical component (nucleotide state) and mechanical component (physical arrangement of the heads). The mechanochemical cycle underlies each step that kinesin takes, and explains precisely how the hydrolysis of ATP is coupled to stepping and force generation. The general mechanochemical cycle that was solved as a part of this dissertation is shown in Figure 1-4. As highlighted in Figure 1-4, the affinity of a kinesin head for the microtubule is nucleotide-state dependent, with ADP-bound being a low-affinity state, and both ATP-bound and no-nucleotide-bound being high affinity states (15, 30–35). Thus, the rear head must transition to the ADP-state before it can lift up to take a hand-over-hand step. The major
mechanical states are: (1) the two-heads-bound state, where both heads are attached to the microtubule and the neck-linker of the read head is docked forward, and (2) the one-head-bound state, where one head is bound and the other is tethered. Docking of the next-linker in the bound head of the one-head-bound state, thought to be triggered by ATP-binding, is often considered to be the force generating state transition within the mechanochemical cycle (36–38). However, this model has recently been challenged by a series of papers, some of which form part of this dissertation, and which emphasize the importance of hydrolysis in the completion of the one-head-bound to two-heads-bound transition (15, 30, 33, 39). Critical to mechanochemistry is the relative rate constants between the various mechanochemical states, as explored below.

![The general kinesin mechanochemical cycle](image)

**Figure 1-4 The general kinesin mechanochemical cycle.** The sequence of five mechanochemical states that kinesin must process through to state a single step. One ATP molecule is hydrolyzed in the process. The two heads are identical, but colored blue and green for clarity. T refers to ATP bound, D refers to ADP bound, DP refers to post-hydrolysis pre-phosphate release, and φ refers to no-nucleotide bound. This figure is adapted from (15), which comprises Chapter 4 of this dissertation.

### 1.4 Mechanochemistry links structure to function

The biophysical properties of a given kinesin isoform are critical for determining what type of jobs that kinesin can carry out in the cell. Examples of biophysical properties include velocity (proportional to the number of steps taken per second), run-length, and stall force (the applied load at which stepping ceases). But what sets the biophysical properties of a given kinesin isoform? In observing Figure 1-4, we can see that all of these biophysical parameters are set by the rate constants dictating the transition rates between the five mechanochemical states. To increase velocity, one or more of the rate constants must be increased. To increase run length, rate constants controlling exit from the vulnerable state (state 3) must be tuned (explored in detail in Chapter 5). To increase stall force, the relative duration in strongly-bound states 4-5 must be increased. Indeed,
mechanochemical kinetics underlie a given kinesin isoform’s biophysical properties. Taking this one step further, we can hypothesize that small structural differences between kinesin isoforms can tune mechanochemical kinetics. Under this hypothesis, we can see a clear mechanochemical basis for functional diversity in the kinesin superfamily – small structural changes isoform-to-isoform drive differential tuning of mechanochemical kinetics, thereby altering the biophysical properties of the kinesin and functionally specifying it for a particular job in the cell. This idea is explored in detail and gradually verified throughout this thesis.

1.5 Clinical relevance of kinesins and their mechanochemistry

Since kinesins are responsible for so many jobs inside the cell, it should come as no surprise that they are highly clinically-relevant proteins. Kinesin dysfunction leads to disease states such as Charcot-Marie-Tooth disease(40), hereditary spastic paraplegia(41, 42), hereditary sensory and autonomic neuropathy type II(43), Alzheimer’s disease(44, 45), and various cancers(46, 47). Various kinesin isoforms have therefore been identified as candidate drug targets for multiple diseases(45, 48). Studying kinesin mechanochemistry therefore is not only a basic biology endeavor, but a biomedical endeavor, as drugs that act on kinesin in different mechanochemical states end up having hugely different effects and efficacy(49).

1.6 Methods for studying mechanochemistry

Since the mechanochemical cycle underlies each step that kinesin takes, studying mechanochemical kinetics necessarily requires the identification and quantification of substep intermediates. In general, this can be done biochemically, by pre-forming a population of kinesins in a particular mechanochemical state and characterizing the exit rate, or biophysically, by observing single kinesins as they walk and quantifying the duration they spend in substep
intermediate positions. The details, strengths, and weaknesses of these different approaches are explored below.

1.6.1 Biochemical approaches

Biochemical approaches to studying kinesin mechanochemistry draw from the established field of pre-steady-state enzymology. The most popular technique used is stopped-flow spectroscopy, where two solutions are rapidly mixed and a spectroscopic signal is monitored over time (50). These assays are typically run in single-turnover conditions, where each enzyme runs through a mechanochemical transition once and only once due to limiting reagents (50). Stopped-flow spectrofluorimetry utilizing fluorescent mant-nucleotides has been a reliable tool for kinesin mechanochemistry due the fact that mant fluorescence intensity is enhanced when it is bound to kinesin, and therefore nucleotide binding and release kinetics can be directly measured (32, 34, 51–58). Major limitations of stopped-flow biochemical techniques are (1) it is not possible to pre-form every intermediate in the mechanochemical cycle, (2) the observed signal can end up being a convolution of the output from multiple, simultaneously occurring processes, leading to false conclusions about rate constants, (3) fluorescent nucleotide analogs can have different kinetics than unmodified nucleotides, (4) observed signals are often generated by multi-step kinetic processes, and therefore require a model to fit the data, (5) the experiments require large amounts of protein, meaning it is not feasible to use kinesins from mammalian sources and that recombinant truncated constructs generally must be used (6) ensemble averaging blurs out all heterogeneity and subpopulations, meaning that rare events cannot be observed.

1.6.2 Single-molecule approaches

Single-molecule techniques rely on the tracking of individual kinesin molecules. The discrete, 8-nm steps that kinesin takes can be identified from the position over time traces of single
moving kinesins. Kinetic information can then be extracted from the distributions of step sizes and step dwell times (10, 16, 59–63). Advances single-molecule techniques, as highlighted in this dissertation, can go even further and directly observe mechanical intermediates in the stepping cycle simply by looking as position over time (15, 39, 64, 65).

1.6.2.1 Total internal reflection fluorescence microscopy

The workhorse technique in single-molecule experiments is total internal reflection microscopy (TIRFM). In TIRFM, a coherent light source is focused onto the back focal plane of a high numerical aperture objective at a spot towards the periphery of the back aperture such that light comes out of the objective at an oblique angle (Figure 1-5). At a sufficiently oblique angle, total internal reflection is achieved and the incident light is reflected back into the objective. However, an evanescent wave is created on the opposite side of the glass-water boundary of the sample (66). The evanescent field intensity decays exponentially from the glass-water boundary, with a typical 1/e decay length of about 100 nm. Hence, TIRFM can be used to selectively excite fluorescent molecules that are within 100 nm of the surface. This selective excitation provides TIRFM with improved signal to noise compared to widefield fluorescence microscopy.

Figure 1-5 Basics of total internal reflection fluorescence microscopy. A coherent light source (in this example, a blue laser used for exciting GFP) is directed towards a high NA objective using a dichroic mirror.
1.6.2.2 Point spread function fitting for breaking the diffraction barrier

A typical TIRFM image of a single GFP-labeled kinesin is shown in Figure 1-6A. As can immediately be seen by comparing the size of the white spot to the 1 μm scale bar, the apparent size of the GFP-kinesin is much larger than the true size of the kinesin (each head is about 5 by 5 nm(38)). This discrepancy is due to the diffraction limit of light(67, 68). The apparent diffraction-limited spot observed is, for a point source of light, the transfer function of the microscope itself, which is often referred to as the point spread function (PSF)(67–69). The size of the PSF is approximately half a wavelength (still more than an order of magnitude larger than the kinesin step size of 8-nm), meaning that tracking the center of mass of a moving PSF is insufficient for generating position over time traces with the precision needed to see kinesin steps(10, 69–72).

Hence, the Fluorescence Imaging with One Nanometer Accuracy (FIONA) method was developed for nanometric particle localization via PSF fitting(68–70). As highlighted in Figure 1-6A, the FIONA method estimates the PSF (which is truly an Airy Disc) with a 2D Gaussian function, and the center of the Gaussian corresponds to the nanometric position. The localization precision is approximately one-nanometer as long as a sufficiently large number of photons is collected (i.e. the diffraction-limited spot appears bright enough relative to the background). As shown in Figure 1-6B, application of FIONA to TIRFM movies of translocating kinesin has successfully been used to measure the sub-diffraction-limited step size of kinesin.
1.6.2.3 Limits of fluorescence-based methods

TIRFM with FIONA is a powerful technique for single molecule analysis, but has a fundamental tradeoff between temporal resolution and spatial precision. In order to obtain the most precise fit to a 2D Gaussian in FIONA, a large number of photons need to be collected from the point emitter; like many fitting processes the localization precision goes like $1/\sqrt{N}$, where $N$ is the number of photons collected in a given camera exposure time(72). To increase the sampling frequency, or frame rate, it is necessary to decrease the exposure time of the camera. Decreasing the exposure time of the camera necessarily decreases the number of photons collected, and hence reduces the localization precision. For low light, fluorescence-based methods, FIONA begins to fail in the 10-100 frames per second regime.

The original kinesin stepping data gathered using TIRFM with FIONA, as highlighted in Figure 1-6B, was collected at 0.5 frames per second. Kinesin-1, however, typically steps at about...
100 steps per second (30). If kinesin-1 was moving at full speed, TIRFM would need to be run at 200 frames per second or higher in order to collect two data points per step on average. However, 200 frames per second is not possible for TIRFM, because the number of photons collected per frame would be far too low to properly do FIONA fitting. Hence, all FIONA data for kinesin stepping prior to this dissertation has been done at sub-physiological ATP concentration (10, 14). The steps that have been seen have therefore largely been composed of an unnaturally long ATP waiting state. The substep dynamics corresponding to individual states within the mechanochemical cycle all occur in much less than one frame in those traces. Hence, to truly use single-molecule data to get accurate information on mechanochemical kinetics, many more photons per second than TIRFM can provide is necessary.

1.7 Development of new microscopy techniques

A fundamental part of this thesis is the development and application of new microscopy techniques that enable high precision FIONA fitting at very high camera frame rates. These techniques necessarily generate a higher number of photons per second from a single emitter than TIRFM, such that the optimal precision can be achieved even at very low camera exposure times. Two microscopy techniques that have been developed and applied for this thesis work are total internal reflection dark field microscopy (TIRDFM) and interferometric scattering microscopy (iSCAT). Both of these techniques work on the basis of collecting backscattered light from the sample, but they differ in the manner that the backscattered signal is collected and measured.

TIRDFM was originally developed as an advancement upon laser dark field microscopy, which was in use as early as 1990 (73–75). TIRDFM is similar to TIRFM except backscatter, rather than fluorescence emission, is created by the incident evanescent wave and collected onto the camera (13). Since it is a single-wavelength method and backscatter is measured directly, the totally-externally reflected excitation beam must be filtered out geometrically rather than
chromatically as in TIRFM(13, 76–78). As of the time of this dissertation, TIRDFM has been successfully applied to high speed (10,000 frames per second or higher) tracking of F1-ATPase(74, 79, 80), myosin(81, 82), and kinesin(13, 39, 76, 83–85). However, TIRDFM is still a highly customized method that is not commercially available and must be constructed. Details on how to build a TIRDFM setup are outlined in chapter 2.

iSCAT was developed as an advancement upon multiple existing backscatter interference imaging modalities(86–88). In iSCAT, backscattered light from the sample plane is measured as an interferometric modulation upon a well-defined reference beam. iSCAT has been run at 50,000 frames per second for molecular motors(88), and in the case of measuring simple diffusion has been run at as high as 500,000 frames per second(89). iSCAT is also highly capable of detecting the scattered signal from unlabeled proteins, as has been shown for myosin(90) and microtubules(91). iSCAT has been applied to multiple protein systems including myosin(65, 90), dynein(64), and membrane-binding proteins(92). Like TIRDFM, iSCAT is a customized method that must be self-constructed. Details on how to build an iSCAT setup is outlined in chapter 3.

1.8 Thesis motivation and summary

The overall goal of this dissertation is to discover the mechanochemical link between kinesin structure and function, in order to understand how different kinesin isoforms are tuned for carrying out particular jobs in the cell. iSCAT and TIRDFM microscopes were constructed and applied in this dissertation work in order to obtain high-resolution single-molecule tracking data. High-resolution tracking was used to directly detect substep intermediates in the kinesin stepping cycle and this information was used to order the transitions that make up the mechanochemical cycle. A cross-family approach was used to determine how mechanochemical kinetics were differentially tuned in kinesin from different families, and thus to uncover how small structural differences between families can lead to large differences in functional properties in cells.
In Chapter 2 of this dissertation, instructions are given on how to build and use a TIRDF microscope. A list of parts, instructions for assembly, troubleshooting notes, and kinesin assay protocols are included. This work was originally written as a book chapter for a Methods in Molecular Biology book entitled Molecular Motors. This book chapter is in revision as of the time of this dissertation (May 2018). However, the chapter is still written in the style of a Methods in Molecular Biology book chapter, and will be released to press in the near future. This chapter should be of particular use to future students looking to build or tune a TIRDF microscope.

In Chapter 3 of this dissertation, instructions are given on how to build and use an iSCAT microscope. Although I was middle author on Methods in Enzymology paper that tackled this subject (64), this chapter is nonetheless all new for this thesis. It presents a detailed guide to building the precise instrument (1D scanning iSCAT) made at Penn State in 2015-18, rather than a generic building guide. Tips and knowledge are hence directly institutionalized here. Comparisons are made between iSCAT and TIRDFM to help guide future users on which is best for specific applications. This chapter should be of particular use to future students looking to build or tune an iSCAT microscope.

In Chapter 4 of this dissertation, iSCAT microscopy is used to solve the mechanochemical cycle of kinesin-1. Novel use of high-resolution tracking enabled the first published identification of a structural intermediate in the kinesin-1 mechanochemical cycle. A series of in vitro iSCAT experiments utilizing controlled nucleotide conditions were used to test the effect of kinesin’s chemical state on its sub-step mechanical transition rates. Stopped-flow experiments were used to support the single-molecule results. This chapter is a direct reprint of a first-author paper published in the Proceedings of the National Academy of Sciences in 2015 (15).

In Chapter 5 of this dissertation, TIRDF microscopy is used to uncover differences in mechanochemical tuning between kinesins -1 and -2, in order to discover how the run lengths of kinesin motors are controlled. This work presents the first unloaded high-resolution measurements
of kinesin-2 stepping, and concentrates on the roles of the neck-linker and microtubule-binding portions of the motor domain in determining processivity. Stopped-flow experiments were used to support the single-molecule results. A general model is presented in which kinesin processivity is understood as a kinetic race between tethered head attachment and bound head detachment. This chapter is direct reprint of a first-author paper published in the Biophysical Journal in 2017(39).

In Chapter 6 of this dissertation, a short, as-yet unpublished comparison of gold-tracking data from this thesis to that of concurrent paper(84) is presented. The paper from Isojima et al. came out only one month after the initial iSCAT data was published (Chapter 3), and contained two major differences: (1) that the kinesin head necessarily swung to the right during each step, and (2) that the ATP waiting state resembled an unbound state. In this Chapter, the experiments from Isojima et al. are repeated, and reasons as to why the two differences arose are explored. Ultimately, a resolution is found that properly describes all the data sets without discrediting either author’s claims about how kinesin stepping works.

In Chapter 7 of this dissertation, lessons learned from studying kinesin mechanochemistry are used to formulate and test predictions about how kinesins-1 and -2 should be able to perform different tasks in the cell. A first section explores the differing abilities of kinesin-1 and -2 to take sidesteps and navigate crowded microtubules, with the idea that kinesin-2 should be more capable of doing so due to its unique mechanochemical kinetics. This section is curated, with some text and figures replicated, from a second author paper where the first author was Dr. Gregory Hoeprich from the University of Vermont(93). A second section explores the differing abilities of kinesin-1 and -2 to coordinate in teams during multimotor transport, with the idea that kinesin-2 should again be more capable of doing so due to its unique mechanochemical kinetics. This section is curated, with some text and figures replicated, from a second author paper where the first author was Qingzhou Feng in the Lab of Dr. William Hancock(94). Except where explicitly noted, all the
experiments presented in this chapter were personally done as a part of this dissertation. This chapter should be of particular use in bridging biophysics to cell biology.

In Chapter 8 of this dissertation, an overall conclusion is drawn together that unifies the ideas and concepts from both published and unpublished work. The importance of microscopy and technology development is discussed. The generalized model for kinesin mechanochemistry is discussed, and ideas and predictions are made for various kinesin families not investigated in this dissertation. This chapter highlights outstanding issues in the kinesin field, and will be of particular work for future students focusing on kinesin.

1.9 References

15. Mickolajczyk, K. J., Deffenbaugh, N. C., Ortega Arroyo, J., Andrecka, J., Kukura, P., and


Chapter 2

High-resolution single-molecule kinesin assays at kHz frame rates

2.1 Introduction

Kinesins are molecular motors that walk processively along microtubules. Advances in force-free imaging techniques have elucidated much about how kinesins hydrolyze ATP to take steps and walk in a hand-over-hand fashion (1–4). However, the small step size and short dwell time of kinesins walking at high ATP concentrations necessitates both high spatial and temporal resolution tracking in order to measure individual steps (5–8). Detecting the conformational changes in the motor domains that underlie the steps requires higher resolution still. New methods such as total internal reflection dark field microscopy (TIRDFM) have been developed to meet these resolution needs (9–20), but have not yet pervaded into common use. TIRDFM offers 1 nm and 1 ms resolution, or better, and can be constructed relatively simply and inexpensively. TIRDFM works similarly to total internal reflection fluorescence microscopy (TIRF) (21, 22). A laser light source is expanded and focused onto the back focal plane of a high numerical aperture (NA) objective. Translating the focused spot towards the periphery of the objective aperture produces light incident on the sample at an angle greater than the critical angle, resulting in total internal reflection at the glass-water interface. In TIRDFM, the evanescent field generated from total internal reflection is used to image the positions of strongly scattering gold nanoparticles. Light scattered from gold is collected by the objective (epi configuration). Scattered light and totally internally reflected light have the same wavelength, so rather than separating them chromatically (as in TIRF), they are separated geometrically.

TIRDFM images appear very similar to TIRF images, meaning image processing and data analysis tools developed for TIRF can be directly applied to TIRDFM. The significantly stronger signal obtained from scattered light in comparison to fluorescence emission means that many more
photons are collected per diffraction-limited spot per unit time, enabling higher spatial and temporal resolution tracking.

In this chapter, we first provide a walkthrough of how to construct a TIRDFM system. This relatively easy build should be achievable even in labs that do not normally focus on constructing optical systems. We next describe single-molecule assays that are optimized for high-speed imaging. Protocols include both the standard ATP landing assay and the AMP-PNP unlocking assay, which provides a way to capture motile events at very high frame rates where the exposed region of the camera is necessarily reduced (12). By locking a kinesin with AMP-PNP and unlocking with ATP just before data acquisition (23, 24), it is possible to have a priori knowledge of where a processive run will occur rather than having to wait for rare events. The assays described here are in no way limited to TIRDFM, and are applicable to TIRF and many other imaging modalities including interferometric scattering microscopy (iSCAT), a different single-wavelength technique that has recently come to prominence as a method for very high-speed tracking of kinesin and myosin motors (9, 10, 12, 14). To conclude, we suggest image and data analysis tools for extracting mechanistic information from movies of processive runs.

2.2 Materials

The following sections list suggested materials for constructing a TIRDF microscope and carrying out kinesin assays.

2.2.1 Buffers

1. BRB80: 80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH to 6.8 with KOH (see Note 1)
2. BRB80C: BRB80, 0.5 mg/mL casein
3. BRB80T: BRB80, 10 µM taxol
4. BRB80CT: BRB80, 0.5 mg/mL casein, 10 µM taxol
No-nucleotide imaging solution (NN): BRB80, 0.5 mg/mL casein, 0.2 mg/mL bovine serum albumin, 10 µM taxol, 20 mM glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 0.5% β-mercaptoethanol (see Note 2)

2.2.2 Kinesins
1. Any kinesin can be used in the assays described here, and biotin-neutravidin chemistry is a suggestion for motor-gold conjugation. There are many ways to biotinylate a kinesin, including Avi-tag (12), BCCP tag (25), biotin-maleimide (2, 26–28), and biotinylated antibodies (15) (See Note 3).
2. A rigor mutant of full-length kinesin-1 is suggested for immobilizing microtubules. A single point mutation (R210A in Drosophila KHC) will make the motor hydrolysis-incompetent and thus perfect for microtubule immobilization (12, 29).

2.2.3 Tubulin
1. Tubulin can be extracted from bovine brain as described in detail previously (30), or purchased from Cytoskeleton, Inc.

2.2.4 Molecular probes
1. 40-nm gold nanoparticle neutravidin conjugate (Nanopartz C11-40-TN-50)
2. 150-nm gold nanoparticle neutravidin conjugate (Nanopartz C11-150-TN-50)

2.2.5 Flow cells
1. Glass slides, 75 by 25 mm, thickness 1 mm (Corning 2947-75x25)
2. Rectangular glass coverslips, 24 by 30 mm, thickness 1 1/2 (Corning 2980-243)
3. Double stick tape
2.2.6 Microscope

1. Optical table with passive floated legs

2. Microscope base (Mad City Labs Micromirror TIRF system)

3. High NA objective (Olympus 60x APO N, 1.49 NA)

4. 150 mW, 532 nm CW laser (Coherent Sapphire LP) (see Note 4)

5. Basler Ace USB 3.0 CMOS camera (acA2000-165um) (see Note 5)

6. Periscope assembly: Tall 1 inch post (e.g. Thorlabs RS12 and BE1), 2x counterbored post mounts (Thorlabs RM1A), 2x kinematic mounts (Thorlabs KM100), 2x 45-degree mirror mounts (Thorlabs H45), 2x broadband dielectric mirrors (Thorlabs BB1-E02)

7. Alignment tool: Slotted lens tube (Thorlabs SM1L30C), lens tube (Thorlabs SM1L20), 2x frosted glass disk with 1-mm hole (Thorlabs DG10-1500-H1), threading adapter (Thorlabs SM1A4)

8. Pinhole assembly: 25-µm pinhole (Thorlabs P25C), XY translator (Thorlabs ST1XY-A), Z-translation mount (Thorlabs SM1Z), 30 mm cage assembly rods (Thorlabs ER3-P4), 10x objective lens (L1 in Figure 2-1; Thorlabs RMS10x), threading adapter (Thorlabs SM1A3) (see note 6)

9. Objective coupling mirror: 45 degree mirror mount (Thorlabs H45), kinematic mount (Thorlabs KM100), broadband dielectric mirror (Thorlabs BB1-E02)

10. Aspheric doublet lenses (e.g. Thorlabs AC254-XXX-A-ML) and lens holders (e.g. Thorlabs LMR1) for L2, L3, and L4. Choose L2 focal length to optimize beam expansion. Choose L3 focal length based on geometry. A shorter focal length will achieve tighter focus onto the objective back focal plane, but may generate more and closer-spaced back reflections. Choose L4 focal length to optimize magnification.

11. 3x Irises (Thorlabs ID25)
12. Base (Thorlabs CP02), lens tube (Thorlabs SM1L20) and threading adapter (Thorlabs SM1A9) for mounting Basler Ace CMOS
13. High stability mirror mounts (Newport U100-A3K) and broadband dielectric mirrors (Thorlabs BB1-E02) for beam steering (see note 7)
14. Miscellaneous posts, bases, and mounting forks
15. Stage micrometer (Thorlabs R1L3S2P)
16. Half-wave plate suited for 532 nm (Thorlabs WPH05M-532)
17. Shearing interferometer (Thorlabs SI254)

2.3 Methods

The following sections list suggested instructions for constructing a TIRDF microscope and carrying out kinesin assays.

2.3.1 Microscope construction

The optical arrangement of the TIRDF microscope is shown in Figure 2-1. This setup can be achieved by customizing a TIRF inverted microscope (17), or constructed as described here (see note 8). The custom-built design is based off the micromirror TIRF setup published by Larson et al. and Friedman et al. (31, 32), with adjustments to simplify and optimize the setup for dark-field. The setup is based around a Mad City Labs RM21 microscope base, which is available together with translatable micromirrors that are placed under the high NA objective. The following protocol describes how to construct a simple custom TIRDF microscope that is ideal for single-molecule kinesin assays.
Figure 2-1 Diagram of a TIRDF microscope. The beam of the table-mounted laser is first brought up to the height of the micromirrors using two mirrors (M1 and M2) in a periscope assembly. Irises Ir2 and Ir3 are used to make sure that the beam remains parallel to the optical table after the height is changed. Alternatively, the periscope can be placed after L3. A short focal-length lens or objective (L1) is used to clean the mode of the laser by passing it through a pinhole (PH). Light is recollimated by a second lens (L2), which has a focal length set to select a particular expansion of the beam diameter. A half-wave plate (WP) can be placed after L2 or equally anywhere in the excitation path. Lens L3 focuses the expanded laser onto the back focal plane of the high NA objective. A first micromirror (mm1) is used to direct the beam onto the objective; translation of it along the optical axis will control the angle of incidence out of the objective. A second micromirror (mm2) picks the totally internally reflected beam and steers it onto a beam block (BB). Light scattered by particles in the sample is collimated by the objective and the majority of it passes in between the two mirrors. An objective coupling mirror (M3), placed at a 45 degree angle, steers the scattered light to the camera. Iris Ir3 blocks any spurious reflections. Lens L4 focuses the light onto the CMOS camera.

2.3.1.1 Excitation line construction

1. Secure the 532 nm laser to the optical table.

2. Secure the RM21 microscope base to the optical table, allowing enough space for excitation and emission lines to be built.

3. Set the height of the micromirrors so they sit just below the base of the objective port. Center one of the micromirrors below the objective port.

4. Make an alignment tool by placing two frosted glass alignment disks into a lens tube. Screw the alignment tool into the objective port with an appropriate threading adapter.

5. Use a periscope assembly to raise the laser height and direct it onto the micromirror. Place two irises between the periscope and the micromirror and walk the beam through them to ensure the beam is flat. Adjust the micromirror such that the beam goes through both holes in the alignment tool.
6. Place the pinhole assembly into the optical path in front of Ir1. Adjust the position of L1 to maximize the intensity that passes through the pinhole. Adjust the position of the assembly such that the center of the diverging beam passes still through Ir1 (see note 9).

7. Place L2 to recollimate the beam. Reposition L2 so the beam still goes through Ir2. Use a shearing interferometer to ensure the beam is properly collimated.

8. Place L3 approximately one focal length away from the objective. Reposition L3 such that the beam still passes through both holes of the alignment tool.

9. Remove the alignment tool and place a plain glass slide over the objective port. Look for back reflections off the glass slide, and make adjustments to the excitation path to make sure the back reflections overlap with the incident beam.

10. Remove the glass slide and place the objective in its holder. The beam exits the objective pointed directly upwards (be sure to use protective eyewear). Translate L3 along the optical path such that the diameter of the beam exiting the objective is minimized (direct it onto a distant surface like the ceiling).

11. Place a flow-cell containing 150 nm gold nanoparticles (5 pM in BRB80 buffer) on the stage. Translate the micromirror towards the edge of the objective until TIR is clearly achieved (propagation will be parallel to the coverslip). Place the second mirror to capture the TIR beam and steer it into a beam block.

### 2.3.1.2 Emission line construction

1. Secure the objective-coupling mirror to the optical table beneath the objective. If a sample with 150-nm gold nanoparticles is in place, the scattered light should be visible to the eye, making placement easy.

2. Place iris Ir3 after the 45 degree mirror.
3. Visually observe the intensity pattern along the emission path by blocking it with a piece of paper. If the objective is at its optimal Z position, then the scattered light will be collimated and the gold will not be visible. If the objective is not properly placed in Z, then gold nanoparticles will be visible at some point along the path. Adjust the Z position of the objective such that gold nanoparticles are not visible at any point along the emission path.

4. Place the imaging lens L4 after the iris. The focal length relative to the objective will set the magnification (see note 10). Attempt to place the lens such that it does not displace the beam vertically or horizontally.

5. Place a piece of paper one focal length away from the imaging lens. Adjust the Z position of the objective slightly until the 150 nm gold nanoparticles become visible, if they are not already.

6. Place the CMOS camera where the piece of paper was. The gold should be clearly visible on the camera.

7. Remove the imaging lens and increase the exposure. Steer the scattered signal if necessary, so it is centered on the camera.

8. Replace the imaging lens such that the image is centered on the camera. Minimizing displacement of the beam by the imaging lens will reduce astigmatism.

9. A half-wave plate can be placed in the excitation path to adjust the polarization state. Rotate the half-wave plate to achieve the brightest PSF. This step is not strictly necessary.

2.3.1.3 Calibration

1. Turn the laser off, remove the 150 nm gold sample, and place a stage micrometer on the stage.

2. Transmit white light (a cell phone LED or pen light is sufficient) through the micrometer and objective. Adjust the objective Z position such that the micrometer comes into focus.
3. Take images of the micrometer, and measure the distance in pixels between the lines (in ImageJ or similar) to get a nm-to-pixel conversion. The conversion obtained should be close to the physical pixel size of the camera divided by the magnification (see note 11).

2.3.1.4 Measuring and testing the point spread function

1. Place a flow cell containing 40-nm gold nanoparticles (dissolved in BRB80 or similar) on the stage and focus on particles adhered to the coverslip surface. Slightly adjust the position of the micromirror to maximize the intensity of the gold signal.

2. If there is no astigmatism, the XY point spread function (PSF) should appear as an Airy disk (Figure 2-2)

3. Use the piezo Z stage to take discrete Z steps, imaging the gold at each position.

4. Observe the XZ and YZ PSFs by taking line scans through the XY PSF at each Z position (Figure 2-2; see note 12)

5. Refocus on the 40-nm gold, and take a high frame-rate movie as the piezo is stepped in X or Y in small steps (8 nm or so) separated by 500 ms dwells.

6. Fit the PSF with a 2D Gaussian (See Image Analysis below) and plot the position versus time. Steps should be clearly distinguishable by eye (Figure 2-2). Re-align the excitation and emission paths if 8 nm steps cannot be detected.

Figure 2-2 Point spread functions and example steps. (A) Top left, an example PSF of a 40 nm gold nanoparticle in TIRDFM. This PSF should appear as an Airy disc. Scale bar is 200 nm. Top right, the same
2.3.2 Microtubule preparation

1. Combine 10 µL of 4 mg/mL tubulin in BRB80, 1 µL 100 mM MgCl$_2$, 1 µL DMSO, 1 µL 25 mM GTP, and 10 µL BRB80 (25 µL total). Flick tube to mix.

2. Incubate at 37°C for 30 minutes to polymerize.

3. Add 75 µL BRB80T to stabilize microtubules (see note 13). Dilute 10 µL of this solution into 190 µL BRB80CT (solution MT/20).

2.3.3 Flow chamber preparation

1. Wash coverslips thoroughly before use, as debris will be visible in TIRDFM. Wash first with DI water thoroughly, then incubate in 1% Hellmanex for 30 min, then wash with DI water, then wash with ethanol, and finally wash with DI water again. Blow dry (see note 14).

2. Place two pieces of double-stick tape perpendicularly across a glass slide with about 5 mm of space between them. Trim the tape to the width of the slide with a razor blade.

3. Place the coverslip across the two pieces of tape. It is important that the length of the coverslip is greater than the width of the glass slide. A short lip of coverslip should overhang on each side of the slide, which will allow for fluid exchange after the slide is mounted upside-down on the microscope stage.

4. Fluid can be exchanged through the inverted flow cell by pipetting a drop onto one coverslip lip, and placing a small wick (i.e. a 5 cm by 1 cm piece of filter paper or paper towel) on the second lip (see note 15).
2.3.4 Microtubule immobilization for single-molecule assays

1. Create a flow chamber and flow through 20 µL BRB80C. Wait five minutes. Casein will block the surface for proper binding of the rigor mutant tails to the glass surface (33, 34).

2. Dilute rigor mutant to 200 nM in casein solution plus 1 µM ATP. Add 20 µL to the flow cell and wait five minutes (see note 16).

3. Remove excess rigor mutants from the flow cell by flushing through 20 µL casein solution.

4. Add 20 µL MT/20 solution to flow cell. Wait five minutes.

5. Remove excess microtubules from the flow cell by flushing through with 20 µL NN solution

6. Microtubules can be visualized in TIRDFM if the electric field vector of incident light is perpendicular to their long axes (Figure 2-3; see note 17). Ensure proper immobilization by inspecting for persistent, weak, time-varying signals near the surface (see note 18). Alternatively, fluorescent microtubules can be visualized in TIRF simply by placing an emission filter in the emission path (Figure 2-3). Note that the rigor mutants in the flow cell should not be left without nucleotide for long periods of time.

Figure 2-3 Microtubule visualization. Left, rhodamine-labeled microtubules are visualized in TIRF by placing a 565 nm long-pass filter in the emission path. All fluorescent microtubules are visible at a 200 ms exposure time. Center, only vertically-aligned microtubules are visible when the emission filter is removed and exposure time is reduced to 20 ms, as the linear polarization state of incident light has its electric field vector aligned perpendicular to them (21). Right, switching linear polarizations by rotating the half wave plate results in no microtubules being visible (same 20 ms exposure). If the flow cell were rotated 90 degrees, different microtubules would be visible or invisible.
2.3.5 Single-molecule landing assay

1. Dilute Avi-tagged kinesin motor and neutravidin-gold nanoparticles to 100 pM of each in NN solution plus the desired ATP concentration (see note 19).

2. Invert the tube to ensure nanoparticles go into solution. Let mix on ice for at least 30 minutes.

3. Create a flow cell and immobilize microtubules on the surface as in sections 3.3 and 3.4.

4. Flow in 20 µL NN solution plus the desired ATP concentration (see note 20).

5. Flow in 20 µL of the motor-gold solution prepared in step 1. Landing events should begin occurring immediately and processive runs should be evident.

6. Adjust the Z position of the stage to optimize the signal obtained from moving particles. This focal plane may differ from the position that optimizes signal from particles nonspecifically bound to the surface.

7. Adjust the position of the micromirror to optimize the apparent signal-to-noise ratio if freely diffusing nanoparticles give too large of a background signal. Moving the mirror closer to the periphery of the objective will increase the angle of incidence and thus the initial intensity and penetration depth of the evanescent field (21).

8. Adjust the exposure time, frame rate, and exposed chip size of the camera as desired.

9. Begin taking movies. Be sure to test the analysis scheme before building large data sets (see note 21).

2.3.6 Single-molecule AMP-PNP unlocking assay

1. Dilute Avi-tagged kinesin motor and neutravidin-gold nanoparticles to 100 pM of each in NN solution plus 1 mM AMP-PNP.

2. Invert the tube to ensure nanoparticles go into solution. Let mix on ice for at least 30 minutes.
3. Create a flow cell and immobilize microtubules on the surface as in sections 3.3 and 3.4.

4. Flow in 20 µL NN solution plus 1 mM AMP-PNP (see note 22).

5. Flow in 20 µL of the motor-gold solution prepared in step 1. Landing events should begin occurring immediately, but no particles should move after landing.

6. Allow 3-5 minutes for AMP-PNP-locked motors to accumulate on the microtubules.

7. Adjust the Z position of the stage and the micromirror position to optimize the signal from locked motor-gold complexes (see note 23).

8. Adjust the position of the micromirror to optimize the total signal from a locked motor-gold complex. The bulk will be cleared before image acquisition, which minimizes the free nanoparticle background signal. Thus, the strongest possible evanescent wave should be used.

9. Adjust the exposure time, frame rate, and exposed chip size of the CMOS as desired. Center one or more locked motor-gold complexes on screen (see note 24).

10. Taking great care not to jostle the flow cell, flow in 40 µL NN solution plus 2 mM ATP (see note 25).

11. Fluid exchange will make the flow cell swell, and thus the sample will fall out of focus. Readjust the Z position of the stage as needed.

12. As soon as the chamber is cleared, motors will begin to unlock following an exponential distribution with a mean of approximately 30 sec. Begin taking movies one after another as soon as step 11 is complete.

13. Once all molecules are unlocked, the assay is over. Refill the chamber by going back to step 4 and repeating. It is not recommended to refill a chamber more than 3 times due to accumulation of dead motors and nonspecifically bound gold.

14. Make new flow cells and repeat as desired.
2.3.7 Image analysis

The PSF apparent in TIRDFM is very similar to TIRF, and will be an Airy Disk if setup properly. Thus, the many well-developed tools for sub-diffraction limited particle tracking in fluorescence imaging (such as FIONA) can be used for TIRDFM images (2, 35). These tools generally work by estimating the central order of the Airy Disk as a 2D Gaussian function, and performing nonlinear regression to find the Gaussian parameters that minimize residuals with the pixel intensity values of the PSF. The parameters returned will include nanometric estimations of X and Y position over time, as well as errors on the estimations (see note 26). A very useful free Gaussian fitting software is FIESTA, released by B CUBE Center for Molecular Bioengineering at Technische Universitaet Dresden (36).

2.3.8 Step-finding and data analysis

In order to extract steps from the time versus position (X, Y, t) traces obtained from the image analysis software, a step-finding algorithm is applied. The analysis performed depends on the design of the experiment and the parameters one wishes to determine, but in all cases it is best to try both a model-free and model-dependent step-finding algorithm. A model-free step-finding algorithm places steps based solely on statistically significant differences within the data, with no knowledge or assumption on what the step size is or how many steps are expected. Our lab has recently released free versions of two model-free algorithms, tDetector and bDetector, which have been applied to kinesins stepping as well as photobleaching data (12, 37). A model-dependent step-finding algorithm uses prior knowledge, such as the microtubule lattice spacing (12) or the approximate number of steps (38), in order to fit steps. Model-free algorithms will often overfit the data, making measurements such as dwell times difficult. Model-dependent algorithms overcome this difficulty, but it is essential to justify their use by first proving their basis with a model-free algorithm.
2.4 Notes

1. The ionic strength of BRB80 buffer is near physiological ionic strength and is highly recommended. Reducing the ionic strength can enhance motor-microtubule interactions, which can make data collection easier, but it results in nonspecific motor-microtubule interactions that can mask measurement the true kinesin mechanochemistry (39, 40).

2. Nucleotides (ATP or AMP-PNP in this protocol) should be prepared with equimolar MgCl₂. Magnesium is necessary for proper kinesin function (41).

3. Our lab has adopted Avi-tagging as the primary means of biotinylation, as cysteine-light versions of kinesin-1 have been seen to exhibit different force-velocity behavior than wild-type (42). Whatever biotinylation and molecular probe strategy is chosen, it is important to run control experiments to make sure that the tagging scheme does not alter velocity and run length.

4. This wavelength of 532 nm is set to match the surface plasmon of 40-nm gold nanoparticles, and so will produce a strong scattering signal for that probe. The scattering amplitude depends strongly on the incident wavelength, with lower wavelengths producing stronger signals. Longer wavelengths are, however, gentler on the sample. It is recommended to match the wavelength to the surface plasmon resonance peak of the nanoparticle primarily used.

5. The Basler Ace CMOS is a great camera choice since it is inexpensive, small, has suitable quantum efficiency and gain, can achieve moderate high frame rates, and utilizes USB 3.0 connection so can be used with almost any computer. It also comes with free imaging software and LabVIEW drivers. Many other CMOS and CCD cameras also work well for TIRDFM. A Camera-Link connection will in almost all cases allow for higher frame rates, but requires additional hardware. Unlike single-molecule TIRF, an expensive camera is not strictly necessary in TIRDFM.
6. The pinhole assembly is used as part of a spatial filter, which will clean the mode of the laser. This is very important for success in TIRDFM, and has been found to be helpful even with single-mode lasers. An alternative to the spatial filter is a single-mode optical fiber. This may be more convenient than a periscope and spatial filter, but will result in more laser power loss.

7. The minimal setup described here does not include additional mirrors. However, placement of mirrors allows for greater beam control and a more compact optical setup. Placement of two mirrors in series allows for one to walk the beam through two irises, as is suggested here using the two mirrors in the periscope.

8. As shown in Figure 2-1, the excitation beam is converging when it falls incident on the 45 degree mirror. Thus, the closer the 45 degree mirror is to the back focal plane of the objective, the smaller it can be. In the Ueno et al. design (17), the perforated mirror is placed in a filter cube and thus is a set distance away from the base of the objective. This distance cannot readily be controlled, and is longer than desirable, thus inherently limiting the size of the “hole” through which scattered light is captured, and reducing the NA of the setup.

9. It is critical to mount the pinhole assembly as stably as possible. Two one-inch-diameter posts are recommended.

10. Total magnification will equal the objective magnification times the imaging lens focal length divided by the manufacturer-expected tube lens focal length (180 mm for Olympus). For example, a 500 mm lens after the 60X Olympus objective will give 167x magnification. A 1,000 mm lens would give 333x magnification. The magnification should be set such that the conversion factor will be in the range of 30-70 nm/pixel for single-molecule tracking.
11. For example, the Basler Ace has a physical pixel size of 5.5 microns. Thus, with 167x magnification, it should be expected to achieve roughly 33 nm/pixel. The measured value will be close to, but not exactly the same as, the calculated value, and should be trusted as the true calibration.

12. If there is astigmatism or if the excitation beam is incident on the back aperture of the objective at an angle, then the XY PSF will appear oblong, and the XZ and YZ PSF will appear diagonal and asymmetrical.

13. For fluorescent microtubules, it is highly recommended to centrifuge down the product at this step (using Airfuge or equivalent), and to discard the supernatant and rinse the pellet in BRB80T. This procedure removes excess free dye and unpolymerized tubulin (30). For unlabelled microtubules, pelleting is not necessary.

14. Dust, debris, and indeed anything that can scatter will be visible in TIRDFM, and can distract from or confound the desired signal. Thus, thorough washing is absolutely necessary.

15. Using a small wick with crisp corners is highly recommended, since it can be gently placed onto the lip of the flow chamber to absorb fluid without applying any force that may move the coverslip. This is critical for not jostling the flow cell in the AMP-PNP unlocking assay.

16. Once immobilized in the flow chamber, the rigor mutants will have their motor domains free to tightly bind microtubules. However, they may release the microtubules if, at any point, there is not at least 1 µM MgCl₂ present in the flow cell. This condition can lead to very slow microtubule gliding that complicates the experiments. Also noteworthy, if the kinesin being tested also has the full length kinesin-1 tail, then it too can bind to the casein-treated glass surface and may lead to gliding.

17. Microtubules will be visible in TIRDFM if the electric field vector is perpendicular to their long axis. This can be taken advantage of in order to visualize the microtubules, but should
be minimized when imaging gold nanoparticles. Control the geometry of the flow cell (microtubules tend to align in flow) and the polarization state of the incident light (using the half-wave plate) to manage this.

18. In our experience, the largest problem limiting spatial resolution in kinesin tracking assays is lack of complete microtubule immobilization on the surface. Other immobilization methods, such as anti-tubulin antibodies, have been found to be insufficient in our hands, and rigor mutants have been the best solution. If microtubules wobble even with the rigor immobilization, making a new preparation of rigor mutant protein is recommended.

19. It is important to ensure that only one active kinesin binds to each gold nanoparticle. The equimolar 100 pM working concentration is recommended, but the necessary amount of motor might change depending on the relative activity and degree of biotinylation of the motor preparation. It is highly recommended to carry out control assays to identify an optimal working concentration by maintaining the gold concentration at 100 pM and varying the motor dimer concentration. Count the number of landing events that occur per second per micron of microtubule. In order to best count the microtubules, it may be easiest to use fluorescently labeled microtubules and insert a long-pass emission filter right before the camera (switching to TIRFM). According to Poisson statistics, if the number of landing events increases linearly with the motor concentration, then there is only one motor per gold nanoparticle (33). At motor concentrations where more than one motor binds to each gold nanoparticle, the landing rate versus motor concentration curve will begin to plateau (43). Choose a working concentration from the linear regime.

20. Each exchange through the flow cell may only actually remove about 80% of the fluid. Thus, to tightly control the ATP concentration, it is recommended to already have the desired ATP concentration in the flow cell before exchanging fluids to add motors.
21. It is recommended to take movies from multiple flow cells on multiple days when building up a data set. This approach averages out the effects of any errors inherent to a given preparation or flow cell. Additionally, temperature can be a significant factor in single-molecule assays (44). Illuminating a sample with laser light will heat it up over time. Significant velocity increases will occur if the sample heats up. A single flow cell should not stay on the microscope for more than 20 minutes. It is highly recommended to keep a thermometer on the microscope base and make sure all measurements are done at the same console temperature.

22. Be sure to make the AMP-PNP stocks with equimolar MgCl₂.

23. Even with good surface blocking, some degree of nonspecific binding is inevitable. Motor-gold complexes on the microtubule can be differentiated because they will colocalize with microtubules, or will appear “lined up” if the microtubules are not visible.

24. Exposing only a small portion of the CMOS chip will allow for much higher frame rates. However, it will make landing assays extremely difficult. Once a few co-aligned AMP-PNP locked motors are found, the field of view can be shrunk down to a tiny size with the locked motors centered in it. Thus, events can easily be measured even with the tiny field of view.

25. A strategy for not jostling the flow cell is as follows. Place the 40 µL in a single drop on one lip of the flow cell. After a few seconds, the liquid line on the opposite lip will reach the edge of the coverslip. Gently drop a wick at the opposite lip, and flow through will begin to occur. Start refocusing the stage as soon as the wick is dropped.

26. The error on the X and Y localization determined by the fitting software will usually be only 1-2 nm. This does not, however, mean that one truly has a 1-2 nm positional baseline in kinesin tracking assays. Additional noise will be introduced by any wobbling of the microtubule immobilization system, stage drift, the compliance of the motor-gold linkage,
and spurious signals like unbound gold nanoparticles diffusing into the area of the walking motor.

2.5 References


Chapter 3

Building and using a scanning interferometric scattering microscope

3.1 Introduction

Interferometric scattering microscopy (iSCAT), is a single-wavelength, scattering-based microscopy technique that enables high spatiotemporal resolution of labelled and unlabeled biomacromolecules(1, 2). The basis of iSCAT is very similar to total internal reflection dark field microscopy (TIRDFM, presented in Chapter 2) with signal coming from the strong scattering of single particles. The major difference is that in iSCAT, the scattered signal is collected as an interferometric modulation upon a well-defined reference, whereas it is collected raw in TIRDFM. A detailed comparison of iSCAT and TIRDFM was published by Ortega-Arroyo and Kukura(2) and some further notes are included in section 3.5 below. In general, however, both iSCAT and TIRDFM are powerful techniques that enable nanometer-level localization precision of gold-nanoparticle-labeled proteins at kHz frame rates. iSCAT has been applied to multiple protein systems, including kinesin(3), dynein(4), myosin(5, 6), unlabeled microtubules(7), supported lipid bilayers(8–10), and others. A building manual for iSCAT has already been prepared by the Kukura Lab, who largely pioneered the technique, and serves as an excellent companion to this chapter(11).

3.2 Interferometric scattering microscopy concepts

The general diagram for an iSCAT microscopy is shown in Figure 3-1A. A laser beam is shaped and focused onto a specimen using a high numerical aperture objective. For biological samples, the specimen is aqueous, meaning that when the laser beam travels from the glass coverslip to the specimen it experiences a drop in the index of refraction, and hence a portion of it is reflected back to the objective. In dark field or fluorescence methods, great care would be taken to filter out this back reflected portion of the illumination beam. In iSCAT, however, the back
reflected beam is used as a tool. The important assumption in iSCAT, which in general holds true, is that the back reflected illumination beam is time and space invariant. What this means is that if one was to scroll around the sample or wait long periods in time, there would be no change to the spatial intensity profile of the back reflected beam. Hence, it is possible to use the back reflected beam as a reference, and as shown in Figure 3-1A it can be directed and focused onto a camera. The goal is then to simultaneously collect scattered light from objects of interest, and to image the interference pattern generated between the back reflected beam and scattering signal.

The basic interference concept is shown in Figure 3-1B. The illumination beam, labeled as field $E_i$, falls incident on the sample. As it travels from glass to water, a portion of it gets reflected ($E_r$), and the rest of it transmits through the sample. Objects in the sample plane, such as 20-nm gold nanoparticles, get illuminated by the transmitted beam and scatter a portion of the light backwards ($E_s$). Both $E_r$ and $E_s$ are collected by the objective and focused on the camera. Since these fields are coherent and of the same wavelength, they can interfere, and the light intensity seen on the camera will be the sum magnitude:

$$I_{detected} = |E_r + E_s|^2 = |E_i|^2 (r^2 + |s|^2 - 2r|s| \sin \phi)$$

where $r$ and $|s|$ are the reflected and scattered amplitude coefficients, respectively, and $\phi$ is a phase that depends on the difference in optical pathlength between the $E_r$ and $E_s$ (i.e. how far in $Z$ above the surface the scatterer is) as well as on the wavelength(2). The contrast can then be stated as the relative intensity for when there is versus when there isn’t a scattering contribution:

$$Contrast = \frac{|E_r + E_s|^2}{|E_i|^2 r^2} = 1 - \frac{2|s| \sin \phi}{r}$$

Where the assumption is made that $|s|^2$ is very small and can be ignored. Notably, the contrast is linear with $|s|$ and hence goes as particle diameter to the third power rather than to the sixth power as it is for dark field(2). For this reason, iSCAT outperforms TIRDFM in detecting very small particles.
Since in iSCAT particles are seen as a signal modulation upon the well-defined reference beam, images appear “gray field”, as shown in Figure 3-1C. Notably, iSCAT images are shown as a percent contrast rather than as grayscale counts. This is because iSCAT images are normalized relative to a sample where no scatters are present. The flat-fielding concept for normalization is explored in detail in section 3.4.5.

Figure 3-1 Concept and performance of an iSCAT microscope. (A) Simplified iSCAT microscope setup. A laser beam is shaped and focused onto the specimen using an objective (OBJ). Linearly-polarized laser light first transmits through the polarizing beam splitter (PBS) and is then changed to circularly-polarized light using a quarter wave plate (QWP). Reflected and scattered light come back through the objective and go through the QWP a second time, adding a second quarter wave retardation. This light is hence reflected by the PBS and can be directed onto the camera. (B) At the glass-water interface of the sample, a portion of the illumination laser (E$_i$) is back-reflected (E$_r$), while the rest of it transmits through the sample. Small objects, like gold nanoparticles, are bathed in incident light and scatter some of it backwards (E$_s$). Both E$_r$ and E$_s$ are collected by the objective and focused onto the camera. (C) Example flat-fielded iSCAT image of two 20-nm gold nanoparticles. The distance between them can be measured nanometrically with a precision dependent on the incident laser power. Images taken at 1,000 frames per second. Figure replicated from (4).
3.3 Non-scanning versus scanning iSCAT

iSCAT illumination can be set up in either a non-scanning or a scanning modality (Figure 3-2). In the non-scanning modality, the laser light is focused onto the back focal plane of the objective at the center of the objective back aperture, as in epi-fluorescence methods. In this case, the laser is collimated by the objective, and hence bathes a wide area in the sample plane. In the scanning modality, the laser light is collimated when it reaches the back focal plane of the objective. The objective hence focuses the laser into a diffraction-limited spot at the sample-plane, similar to confocal microscopy. Since only a tiny spot is illuminated, it is necessary to raster scan the diffraction limited spot across the entire sample plane during each frame acquisition. Non-scanning iSCAT has the advantages of being extremely simple to setup and not requiring any expensive electro-optics. It has the downside, however, of bathing scatterers both in and out of the sample plane similarly, which contributes to background noise. Scanning iSCAT selectively illuminates the scatterers in the sample plane (similar to the advantage of confocal versus epi-fluorescence), but is more difficult to set up and requires expensive equipment.

A simplification of the scanning iSCAT design is the 1D scanning iSCAT (also called line scanning iSCAT), which is shown in figure 3-2C and explored in detail in this chapter. 1D scanning still has the selective illumination advantage relative to non-scanning, but requires only one direction of scanning. Instead of scanning a diffraction-limited spot in 2D, a diffraction-limited line is generated using a cylindrical lens, and the line is scanned in 1D. 1D scanning is not only easier and cheaper to build than 2D scanning, it also has the potential to work at higher frame rates. To achieve an even illumination across the sample field, multiple scans are made across the field during the time when the camera shutter is open; the time to complete these raster scans thus sets the upper limit for the frame rate. Simpler scanning means faster scanning and thus faster frame rates. In this chapter, acousto-optic deflectors (AOD) are used for scanning, but multiple means are available.
Building a 1D scanning iSCAT

The following sections outline the necessary parts and instructions for building an iSCAT with the 1D scanning excitation configuration. The complete diagram, including all pieces, is shown in Figure 3-3. The parts and instructions listed correspond directly and specifically to iSCAT built in the Hancock Lab in 2015-2018. However, multiple changes to the design are possible, and changes may lead to improvements. This particular design is a simple one, and can be put together quickly, but due to the lower electron well depth camera, the higher (650 instead of 450 nm) wavelength, and various imperfections, does not perform as well as the 2D scanning iSCAT designed by the Kukura Lab (2, 11). It should be noted that the choice of laser wavelength is extremely important. Here, 650 nm is used to be gentle and to avoid heating samples. However, 650 nm scatters much less strongly than 450 nm; using a redder wavelength can reduce the scattered field strength as much as 4.35-fold. The design described here works well for tracking unlabeled microtubule dynamics, for tracking 40-nm gold nanoparticles, and for visualizing 20-nm gold

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**Figure 3-2 Different iSCAT excitation modalities**

(A) Non-scanning iSCAT excitation. The laser is focused onto the back focal plane of the objective, leading to collimated output light. Shown to right is the surface of the sample plane, with light propagating out of the page. Here, a cylinder of light bathes the sample.

(B) A 2D-scanning iSCAT setup. Two AODs are used to scan a diffraction-limited spot in XY across the sample plane in order to make a square illumination pattern.

(C) A 1D scanning iSCAT setup. One AOD scans the beam, which is focused into a diffraction-limited line using a cylindrical lens. The width of the line is limited by the cylindrical lens, meaning the field of view cannot be as large as in the 2D scanning scenario. For all panels: Laser (LAS), lens (L), polarizing beam splitter (PBS), quarter wave plate (QWP), objective (OBJ), sample (S), acousto-optic deflector (AOD), cylindrical lens (CL).

### 3.4 Building a 1D scanning iSCAT

The following sections outline the necessary parts and instructions for building an iSCAT with the 1D scanning excitation configuration. The complete diagram, including all pieces, is shown in Figure 3-3. The parts and instructions listed correspond directly and specifically to iSCAT built in the Hancock Lab in 2015-2018. However, multiple changes to the design are possible, and changes may lead to improvements. This particular design is a simple one, and can be put together quickly, but due to the lower electron well depth camera, the higher (650 instead of 450 nm) wavelength, and various imperfections, does not perform as well as the 2D scanning iSCAT designed by the Kukura Lab (2, 11). It should be noted that the choice of laser wavelength is extremely important. Here, 650 nm is used to be gentle and to avoid heating samples. However, 650 nm scatters much less strongly than 450 nm; using a redder wavelength can reduce the scattered field strength as much as 4.35-fold. The design described here works well for tracking unlabeled microtubule dynamics, for tracking 40-nm gold nanoparticles, and for visualizing 20-nm gold...
nanoparticles. However, the Kukura design is superior for label-free single-molecule tracking, and for tracking 20-nm gold nanoparticles with high precision.

**Figure 3-3 Diagram of a 1D scanning iSCAT microscope.** The excitation path leads from the laser to the sample on the objective. The emission path tracks backwards from the objective and towards the CMOS camera. Laser (LAS), mirrors (M), lenses (L), single-mode fiber (SMF), objectives (O), acousto-optic deflector (AOD), half wave plate (HWP), irises (I), cylindrical lens (CL), polarizing beam splitter (PBS), quarter wave plate (QWP), camera (CMOS). The optical fiber is necessary for mode-cleaning. Diode lasers have multi-mode output and hence to not have a Gaussian beam profile. Only one mode can get through the optical fiber, and thus the beam profile will become Gaussian on the far side of the optical fiber.

### 3.4.1 List of parts

**Optical components:**

1. Broadband dielectric mirrors, 10x (Thorlabs BB1-E02-10)
2. High NA aspheric lens for coupling into fiber (Figure 3-3 L1; Thorlabs A110TM-A)
3. Single-mode optical fiber (Thorlabs P1-630PM-FC-2)
4. 10X Olympus Plan Achromat objective (Figure 3-3 O1; Thorlabs RMS10x)
5. Zero-order half-wave plate, 1" mounted, 633 nm (Thorlabs WPH05M-633)
6. 500 mm anti-reflection coated singlet lenses, 2x (Figure 3-3 L2-L3, Thorlabs LA1908-A)
7. 400 mm anti-reflection coated cylindrical lens (Figure 3-3 CL; Thorlabs LJ1363RM-A)
8. 30 mm cage-cube-mounted polarizing beamsplitter cube (Thorlabs CM1-PBS251)
9. Zero-order quarter-wave plate, 1” mounted, 633 nm (Thorlabs WPQ05M-633)
10. Irises (Thorlabs ID25) and cage irises (Thorlabs CP20S)
11. High NA 60x TIRF objective (Olympus 60x APO N, 1.49 NA)
12. 500 mm achromatic doublet lens, 2 inch (Figure 3-3 L4Thorlabs AC508-500-A-ML)

Mounts, adapters, and posts

1. Kinematic mirror mounts, 10x (Thorlabs KM100)
2. Static lens mount, 1 inch optics, 5 pack (Thorlabs LMR1-P5)
3. Static lens mount, 2 inch optics (Thorlabs LMR2)
4. 45-degree mirror mount (Thorlabs H45)
5. SM1-threaded 30 mm cage plate, 6x (Thorlabs CPO02)
6. SM1 to M9 x 0.5 lens cell adapter (Thorlabs S1TM09)
7. Optical fiber adapter plate with external SM1 threading, 2x (Thorlabs SM1FC)
8. Z-axis translation mount, 2x (Thorlabs SM1Z)
9. 30 mm cage XY translating lens mount for 1” optics (Thorlabs CXY1)
10. Adapter with External SM1 Threads and Internal RMS Threads (Thorlabs SM1A3)
11. 2” manual rotation stage (Thorlabs RP01)
12. Cage rotation mount, 3x (Thorlabs CRM1)
13. 1” lens tubes, various lengths (e.g. Thorlabs SM1L20)
14. 1” double-threaded lens tube, 1” length (Thorlabs SM1S10)
15. Adapter external C-mount to internal SM1 (Thorlabs SM1A9)
16. 4 pack of 30 mm cage rods, 1 inch (ER1-P4)
17. 4 pack of 30 mm cage rods, 2 inch (ER2-P4)
18. 4 pack of 30 mm cage rods, 3 inch (ER3-P4)
19. 4 pack of 30 mm cage rods, 4 inch, 2x (ER4-P4)
20. Heavy 1” diameter posts for mounting larger pieces, various lengths (e.g. Thorlabs RS2)
21. Regular 0.5” diameter posts for mounting single optics, various lengths (e.g. Thorlabs Tr2)
22. Regular 0.5” diameter post holders (i.e. Thorlabs PH2)
23. Clamping forks, many (Thorlabs CF125-P5)
24. Pedestal base adapters, many (Thorlabs BE1)

Large items, tools, and custom-machines parts:

1. Optical table top, 48”x72”x8” (TMC 784-451-02R)
2. Passive, floated legs for optical table top (TMC 14-417-34)
3. Equipment shelf for optical table (TMC 81-231-01)
4. Heavy microscope stage/base with objective holder (Mad City Labs RM21)
5. Heat sink for mounting laser (aluminum cube milled flat and tapped)
6. AOD holder (aluminum plate milled flat and tapped)
7. Heat sink for AOD driver (aluminum cube milled flat and tapped)
8. Alignment tool: Slotted lens tube (Thorlabs SM1L30C), lens tube (Thorlabs SM1L20), 2x frosted glass disk with 1 mm hole (Thorlabs DG10-1500-H1), adapter (Thorlabs SM1A4)
9. Shearing interferometer (Thorlabs SI254)
10. Stage micrometer (Thorlabs R1L3S2P)
11. Beam height ruler (Thorlabs BHM2)
12. Appropriate protective eyewear (for 650 nm, Thorlabs LG4)

Hardware:
1. Diode CW laser, 650 nm 200 mW (Odic Force OFL384)
2. Acousto-optic deflector for 633 nm (Gooch and Housego 45070-5-6.5DEG-633)
3. Driver for AOD (Gooch and Housego 97-02333-14)
4. 50 MHz function generator (Wavetek model 81)
5. DC power supplies, 3x (e.g. Dr. meter PS-305 DM, Korad KD3005D)
6. Mad City Lab 3-axis piezo controller (Nanodrive)
7. SMC coaxial cable, SMC female to BNC male, 50 ohm, 48" (Thorlabs CA2648)
8. SMA coaxial cable, SMA male to BNC male, 50 ohm, 48" (Thorlabs CA2848)
9. SMB coaxial cable, SMB female to BNC male, 50 ohm, 48” (Amazon B00YBI71YA)
10. USB 3.0 camera (Basler acA2000-165um) and cable
11. Computer with LabVIEW software, IMAQ-DX drivers, vision package

Figure 3-4 Images of a completed 1D scanning iSCAT. (A) Laser secured to the optical table and directed towards the single-mode optical fiber. (B) Outcoupling from the optical fiber and directing the laser towards the AOD. (C) The laser comes out of the small hole in the cardboard box, and is shaped and directed towards objective. The yellow line denotes the chosen row of holes for the last leg of the excitation line. The laser will travel perfectly down this yellow line. (D) View from the other side of the microscope stage. The final mirror steering the laser towards the objective can be seen under the stage. The high NA 60x objective used for imaging is secured in the stage. All abbreviations match Figure 3-3.
3.4.2 Directions for optical construction

1. Secure the laser to its heat sink on one corner of the optical table. Provide 12V to power the laser (Figure 3-4A). Use a beam height ruler to estimate the beam height (wear safety goggles at all times)

2. Secure the microscope base on the opposite side of the table. Observe Figure 3-3 and Figure 3-4 to get an idea of the relative positioning. Have an idea of where all the optics will go on the table before beginning to build

3. Use four 3” long 30 mm cage rods to make an assembly that will hold the single-mode optical fiber and the coupling lens. Use an appropriately long heavy 1” post to get the height of the assembly as close as possible to the beam height. A 30 mm cage plate can be used both to secure the rods and to attach the post (Figure 3-4A)

4. Secure the high NA coupling lens (Figure 3-3 L1) into the cage system XY-translating lens mount using a SM1-M9 threading adapter. Add the mount to the cage assembly

5. Secure the single mode fiber into a Z-translation mount using an optical fiber adapter plate. Add the mount to the cage assembly, sliding it down the cage until the fiber is approximately one focal length away from the coupling lens

6. Fork the cage assembly down to the optical table in a position where two mirrors can be used to steer the laser towards it (Figure 3-4A)

7. Use two mirrors to direct the laser towards the optical fiber. Using two irises between the second mirror and the cage assembly can be useful for ensuring the beam is parallel to the table before entering the cage assembly. Walk the beam using the two mirrors until the maximum amount of power possible is coming out of the far side of the optical fiber. Use the Z-translation stage to find the optimal distance between the coupling lens and the fiber such that the maximum amount of power gets through the fiber. This process may be cumbersome, but is absolutely essential for mode cleaning
8. Make a second 30 mm cage assembly for the out-coupling side of the optical fiber. Connect two cage plates using 4” long 30 mm cage mounts. Between the two cage plates, place a Z-translation mount housing a high-quality Olympus 10x objective. Use a RMS-SM1 threading adapter to screw the objective into the Z-translation stage.

9. Attach the remaining free end of the optical fiber to the second cage assembly using an optical fiber adapter plate (inserted into one of the cage plates). The optics should be oriented such that the nose of the objective is facing the optical fiber (Figure 3-4B). In this arrangement, collimated light will come out of the back aperture of the objective.

10. Fork the second cage assembly down to the table in the desired position (Figure 3-4B). Use heavy 1” posts for securing the assembly. Choose the length of these posts wisely, as they will set the beam height for the rest of the excitation line. An excellent total beam height to choose is the height that matches the entry port of the RM21 microscope stage (where the third 30 mm cage assembly can be seen attached in Figure 3-4C). Look ahead to instruction 19 below when making this choice.

11. Use a shearing interferometer to check if the beam is collimated coming out of the objective. Translate the Z stage until the beam is perfectly collimated. The beam intensity profile should appear Gaussian at this point.

12. Place the Acousto-optic deflector (AOD) onto the custom mount. Estimate the beam height out of the outcoupling objective using the beam height ruler, and choose an appropriate post length for the AOD to get the height of the AOD window to match the beam height. Make sure this height is comparable to the microscope base input port (look ahead to step 19). Connect the AOD to the optical table using a 2” rotation stage (Figure 3-4B).

13. Power the AOD (see section 3.4.3 below).

14. Place two mirrors in between the outcoupling assembly and the AOD, and use them to steer the beam into the AOD window. Place two irises in between the second mirror and the
AOD to ensure that the beam is traveling parallel to the plane of the table prior to entering the AOD. It is very important that the beam height is not changing while traveling through the AOD, and that the beam hits the center of the AOD crystal.

15. Rotate the AOD using the rotation stage. At some rotation angle, a maximal amount of power will be transferred from the zeroth mode (will appear as a spot that transmits through the AOD without getting deflected) to the first mode (will appear as a spot that clearly gets shot off at an angle from the AOD). This is the Bragg angle. Only the first mode will scan. Get as much power into the first mode as possible.

16. Use a mirror to select the first mode from the AOD and direct it towards to where the next set of optics will be. Block the zeroth mode with a beam block or similar.

17. At this point the beam is being scanned back and forth by the AOD. Although the beam is collimated, if the beam is observed at a distance far away from the AOD it should appear spread out more in one direction then the other. If the scanning frequency is reduced to the 1 Hz regime, it should be easy to see the beam being scanned in real time. Check that this is the case. If so, the next goal should be to steer the scanned beam towards the objective.

18. Think through carefully the exact position of all the remaining optics. Use Figures 3-3 and 3-4C to get an idea. Note that L3 needs to be exactly one focal length away from the final objective back aperture, and that L2 needs to be two focal lengths (L2 focal length plus L3 focal length) in front of L3. Place mirrors M6, M7, and M8 (Figure 3-3) such that there is enough optical path length for this to occur. Note that mirror M8 is the objective coupling mirror, and needs to be mounted at a 45-degree angle to bounce the beam up towards the objective (place a 45-degree mount into a kinematic mirror mount for maximal control).

19. Build a third 30 mm cage assembly and connect it to the input plate of the RM21 microscopy base (Figure 3-4C). The input plate has a one-inch diameter hole with internal SM1 threading. Use a double-SM1-threaded lens tube; one end will screw into the
microscope base and the other with screw into a 30 mm cage plate. Use 3” 30 mm cage rods. Screw one end of the rods into the mounted polarizing beam splitter. Slide on the quarter wave plate in a 30 mm rotation mount and a 30 mm cage iris, and screw down to secure, Finally, slide on a 30 mm cage plate and secure. The order of these pieces should match Figure 3-3 (the cage plate is not shown, it is simply for attaching the double-threaded lens tube). Attach the assembly to the microscope base using the double-threaded lens tube as noted above. Note here the height of the cage iris (matches I2 in Figure 3-3), as this will be the beam height for the second half of the excitation line.

20. Mount another iris (not in a cage assembly), and match the height to the cage-mounted iris in the previous step. It is very important that these two irises have their centers at the exact same height. Place this iris (I1) right after M7 (Figure 3-3 and Figure 3-4C). Note that the RM21 microscope stage is imperial-measured, and the entry port will be exactly in line with one of the lines of hole in the optical table (yellow line in Figure 3-4C). Hence, iris I1 can be screwed down directly to the table, rather than attached using a fork. It is extremely important that I1 and I2 are both perfectly in line with one another right along this line of holes in the optical table. Make all the adjustments necessary to make sure this is true.

21. Place the half wave plate in a rotation mount and place it shortly after M5 in the excitation line. Rotate the half wave plate, and pay attention to how light transmits or reflects at the interface of the polarizing beam splitter. Find the optimal half wave plate rotation position such that as much light as possible transmits through the polarizing beam splitter.

22. Use mirrors M6 and M7 to walk the beam as perfectly as possible through irises I1 and I2. Use the beam height ruler to convince yourself that the beam is truly perfectly straight. The beam should propagate perfectly down the chosen line of holes, and should not change height even one millimeter. Getting the beam perfectly “flat” like this is absolutely essential to getting high quality iSCAT images. Spend as much time as is necessary to get
everything perfect at this point. Note that the beam is being scanned, and so it can be somewhat difficult to choose the “best” position in the scanned direction. Reducing the scanning amplitude as much as possible at this point (at the function generator) should help here. Note that if the function generator is turned off, the beam will disappear. This is because we are using the first diffracted mode off the AOD

23. Build the alignment tool and screw it into the microscope objective slot. Place mirror M8 into a 45-degree mirror mount, and in turn place that mirror mount into a KM100 kinematic mirror mount. Place M8 directly under the objective slot (figure 3-4D). Use the M8 knobs to steer the beam directly through both holes in the alignment tool. If it is not clear how to build the alignment tool, see reference (13) figure 6.

24. Remove the alignment tool and place a glass slide over the objective slot. The laser beam will reflect backwards off the glass slide. Using an index card or similar, check that the back reflection is indeed perfectly overlapped with the input beam. Make small adjustments to mirrors M6 and M7 if this is not true

25. The beam should be hitting the ceiling (make sure you are wearing safety googles)! Place a piece of tape on the ceiling where the beam is hitting

26. Remove the glass slide and screw in the objective. Make a flow cell with water in it, and load it onto the objective using immersion oil

27. Mount lens L3 and place it in the excitation path one focal length away from the back aperture of the objective. Slide the lens around such that the laser is still hitting the piece of tape on the ceiling. Slide the lens back and forth along the direction of beam propagation (yellow line in Figure 3-4C), and note that as you do so, the size of the spot on the ceiling will change. Find the position of L3 that creates the smallest, most perfectly symmetric spot on the ceiling. Achieving the smallest spot means the lens is perfectly one focal length away from the objective back focal plane
28. Remove the objective and put back on the alignment tool. Make sure the laser is still going through the two holes. Then put back on the glass slide and make sure the back reflection is still overlapping with the input beam. Repeat steps 27 and 28 until it’s perfect.

29. At this point, although the beam is being scanned, the iSCAT is effectively in the non-scanning excitation configuration (Figure 3-2A). Surprisingly, the easiest way to find the best position for lens L2 is to use the camera. Hence, at this point we will build the emission line and then come back to finish the excitation line.

30. Load a sample with ~1 pM of 40-nm gold nanoparticles onto the microscope. We will use this easy sample to guide the emission line.

31. Trace the emission path from the objective to the polarizing beam splitter. The returned beam should get reflected by the polarizing beam splitter. Rotate the quarter wave plate to get the maximum amount of power into this reflected emission beam.

32. Place the CMOS camera using lens tubes, a c-mount to SM1 threading adapter, and a 30 mm cage mount (Figure 3-4C). Attach the cage plate to the table using a heavy 1” diameter post. Get the height of the camera as close as possible to the beam height. Place the camera such that there is enough space for mirror M9 and lens L4 (Figure 3-3 and 3-4C). There will need to be enough space for lens L4 to be one focal length away from the camera. After mounting, attached the camera to the computer using a USB 3.0 cable.

33. Use LabVIEW to access the camera. Two pre-existing codes will be present on the computer: VidCap_KJM_Tiff_BaslerAce_M8bit_v2.vi and StageControlSimple.vi. Turn on the Mad City Labs piezo controller then start up both of these codes.

34. Initially run the camera at the full chip size. Place mirror M9 and steer the beam towards the camera. When the beam is located on the camera, place lens L4 one focal length away from the camera (note it should be one focal length from the chip itself, not the lip of the camera). Adjust the position of the lens such that the light hits the same spot on the camera.
is it did before the lens was placed (steering the beam with L4 can lead to astigmatism).

Do an excellent job in making sure L4 is one focal length away

35. Find the focal plane in Z by using the stage micrometer (coarse focus) and the Z-axis piezo (fine focus). The 40-nm gold nanoparticles should appear as black spots (Figure 3-5). When you think you have found them (looking at the raw images, as no flat field has been made yet), try scrolling in X and Y using the manual stage micrometers. If you are indeed looking at the proper spot of light, and you are indeed in focus, then black spots will move in X and Y in the expected way as you move the stage. If not, then the spot of light you are looking at may not be the iSCAT signal (and is just some random back reflection), or you may just not be in focus

36. Once the gold particles have been found, check the quality of the point spread function (PSF). First, flat field the image using the “Ramp X Piezo”, “Create MF”, and :apply MF” buttons in the two pre-made LabVIEW codes. The XY-PSD (how the spots look when you are in perfect Z focus), should look like symmetrical black circles with white rings around them. If the PSF does not appear symmetrical, then there is either astigmatism, or the beam is not perfectly flat through the objective. Go back to step 22 to get this to get the PSF to a pretty good point. You can come back and get it to a perfect point after switching to scanning mode

37. Once satisfied, it is now possible to switch to scanning mode. To do this, place lens L2 two focal lengths (L2 focal length plus L3 focal lengths, 1000 mm if the suggested lenses were chosen). Place L2 such that the beam still goes through I1 and I2 properly (i.e. make sure you are not steering the beam with L2). Now look on the camera. If L2 was placed properly, you should see a very bright white line of intensity. This is the diffraction-limited spot being scanned in 1D. Notice that as you move L2 forwards and backwards along the direction of light propagation, the width of the line of intensity will get broader or narrower.
Position lens L2 such that you get the narrowest line of intensity on the camera as possible. If you do so, then L2 is perfectly place two focal lengths away from L3.

38. Using the steering knobs on mirror M9, get the line of intensity centered on the camera screen. Now place the cylindrical lens in a rotation mount and position it right before L3 in the excitation path. Rotate the cylindrical lens such that the beam is spread out in the direction perpendicular to the direction of scanning. Translate the cylindrical lens backwards and forwards along the direction of light propagation (yellow line in Figure 3-4C) such that you get as much spreading as possible. On the camera, you should be able to fill a 312x312 field entirely (Figure 3-5A). As visible in Figure 3-5A, light intensity should be even in the direction of propagation, but spread out in a Gaussian in the direction of spreading by the cylindrical lens. Flat-fielding will correct this unevenness, but you will still technically get the best signal to noise at the place where the raw image is brightest.

39. Building is now technically complete. Go back and do some slightly tuning. Tune the AOD rotation angle, the HWP angle and the QWP angle to get the brightest, least-distorted image. Note that the angle of the QWP can influence the shape of the PSF.

40. Place an index card in front of the camera. You should see a minimum of three spots (Figure 3-5B). One is the initial reflection from the polarizing beam splitter. This one should appear the smallest, and will not disappear if you block the beam just before the objective. A second will a back reflection off the objective. This one will be very hard to get rid of. It should appear dimmer and highly elongated. The third is the true iSCAT signal. If none of these three spots overlap, then the iSCAT signal to noise will be maximized. However, steering the objective back reflection off of the iSCAT signal will introduce a bit of astigmatism (notice in Figure 3-5A that the spots are slightly elliptical). Pick the best compromise between signal to noise and PSF quality.
41. Continue to tune the iSCAT microscope by getting the laser as flat as possible through the objective. This is done by steering the beam with mirrors M7 and M8. Do a perfect job to get a perfectly symmetrical PSF, keeping the points raised in step 40 in mind.

![Figure 3-5 Images from a completed 1D scanning iSCAT. (A) Image of 40-nm gold nanoparticles before and after flat-fielding using a 1D scanned iSCAT. The yellow line shows the direction of scanning, and the blue line shows the direction of spreading by the cylindrical lens. Note that flat-fielding corrects the uneven illumination. It also corrects time and space invariant imperfections in the raw image, such as the piece of dust on the camera surface shown with a red arrow. Since raw is an image (origin in top left corner) and the flat-fielded pseudo-image is actually a 2D array (origin at bottom left), the images appear rotated relative to one another. The 40-nm gold particles here are generating about 20-25% contrast. (B) The three spots of light that will reach the camera. Spot 1 is the initial reflection off the polarizing beam splitter. Spot 2 is a back reflection off the objective. Spot 3 is the iSCAT signal. Avoiding overlap between these three spots will maximize the signal to noise.]

3.4.3 Setting up the acousto-optic deflector

1. Locate the acousto-optic deflector (AOD) and the driver (Figure 3-6)

2. Machine an aluminum heat sink and mill it flat. Mount the driver on the heat sink (Figure 3-6A)

3. Hook up the four connections of the driver (Figure 3-6A). In the top left port, use a SMC to BNC cable to connect the driver to the function generator (Figure 3-6B). In the bottom left port, use a SMB to BNC cable to connect the driver to a power supply (called power supply 2 from here on). In the top right port, use jumper cables to connect the driver to another power supply (called power supply 1 from here on). The black cable in Figure 3-6A is ground (does
not attach to the pin) and the red cable (attached to pin) is the + supply in. In the bottom right port, use a SMA to BNC cable to attach the driver to the AOD itself. Note that all cables need to be 50 Ohm for proper impedance matching. Don’t turn anything on yet

4. Power supply 1 provides power to the AOD driver. It needs 28.0 V DC, and should draw about 0.72 A when running. Be careful to properly ground and to not supply a negative voltage

5. Power supply 2 is the tuning voltage. Apply 0.5-1.0 V DC. Note that the scanning homogeneity will change a bit as the voltage is tuned. Pick that value that empirically gives the best result (usually 0.5 V works fine). Never apply more than 1.0 V DC

6. The function generator controls the driver. Note that the AOD works like a diffraction grating. It is a crystal with an ultrasound transducer connected to one side. The transducer creates a standing pressure wave across the crystal. Increasing the frequency of this standing wave is equivalent to adding more lines per cm to a diffraction grating – light will get diffracted at a higher angle. For this particular AOD and driver pair, the input voltage from the function generator is proportional to the standing wave frequency. Hence, if we were to apply a positive DC voltage, light would diffract at a constant angle. Increasing the voltage would lead to a higher diffraction angle. Therefore, to scan, we want to use a triangle wave with a high DC offset. The DC offset will separate the 0\textsuperscript{th} from the 1\textsuperscript{st} diffraction mode, and will provide the zero position for scanning. The triangle wave amplitude will set the scanning amplitude. A triangle wave is ideal because it will lead to even scanning (i.e. it’s first time derivative is a constant). A sine wave would over-illuminate the edges in a time-average.

7. Now set the function generator properties. Offset 4.5 V, suggested amplitude 500 mV (increase or decrease as need be). Be careful never to provide >10V, as this will break the AOD. Set the frequency to be an integer multiple of the camera exposure time. For example, to run the camera at 100 frames per second a 9.45 ms exposure time can be used. To scan 290
times per exposure, set the function generator frequency to 30.72 kHz. More scans per exposure will lead to more even illumination. For higher frame rates, something like 21-29 scans per exposure works fine. It will be very obvious if an incorrect scanning frequency is chosen, as the live feed off the camera will look highly-time variant.

8. The AOD itself must be mounted on a rotation mount. This is because the amount of power that goes into the first diffraction mode depends on the incident laser angle (the angle perpendicular to the direction of sound propagation). The incident laser angle must come in at the Bragg angle. Figure 3 from Romer and Bechtold(12) shows this clearly, and in general provides a wealth of knowledge about AODs worth knowing. It is much easier to rotate the AOD relative to the laser than it is to rotate the laser relative to the AOD.

![Figure 3-6 Setting up the acousto-optic deflector driver. (A) The driver needs four input signals, each of which uses a unique type of cable. The driver gets very hot and must be bolted to a heat sink. (B) The hardware plugs in to the driver. SMA, SMB, SMC, and BNC are four different types of 50 Ohm coaxial cables. PS1 and PS2 refer to DC power supplies. FG stands for function generator.](image)

### 3.4.4 Calibration

The iSCAT microscope can be calibrated using a stage micrometer. Place the stage micrometer face down on the objective (still using immersion oil), and transmit light through it using a bright white light (e.g. the LED on your cell phone). Focus in Z until the micrometer bars become visible, then take some pictures. The bars are separated by 10 microns for the suggested stage micrometer. Use ImageJ to measure the number of pixels between each bar (L), then divide
10,000 nm / L pixels to get the nm to pixel conversion. The theoretical value can be easily calculated by dividing the physical pixel size of the camera by the microscope magnification. The system magnification for the microscope in Figure 3-3 (if O2 is an infinity-corrected objective) can be calculated as:

\[
M_{\text{System}} = M_{\text{Obj}} \frac{f_{\text{TL}}}{f_{\text{STL}}}
\]

where \(M_{\text{Obj}}\) is the objective magnification, \(f_{\text{TL}}\) is the focal length of the final focusing lens (Figure 3-3 L4) and \(f_{\text{STL}}\) is the standard tube lens length (a value set by the objective manufacturer, 180 mm for Olympus objectives, 160 mm for Zeiss objectives, 210 mm for Nikon objective). The system magnification here is 167x. Since the physical pixel size is 5.5 microns, the expected conversion is 33 nm/pix. If the measured value deviates far from the theoretical value, then there is an error in the emission path (likely L4 is not properly one focal length away from the detector). Always use the measured value for calibration.

### 3.4.5 Flat-fielding and taking iSCAT images

Images taken in iSCAT require a post-processing step known as flat-fielding in order to get ratiometric intensity pseudoimages. In the flat-fielding process, iSCAT images are normalized relative to an image where no scatterers are present (see equation for contrast in Section 3.2)(11). To get an image where no scatterers are present, the standard process is to first get into perfect focus in Z, and then to slowly scan (by linearly ramping the piezo stage) in X or Y and taking an image stack. The median intensity projection is then taken from this stack, and as long as scatterers are relatively sparse, this median projection will be the denominator for flat fielding. In the flat-fielded pseudo-image, each position represents the pixel-wise percent difference between the current raw image and the median projection previously made. An example image before (raw) and after flat-fielding is shown in Figure 3-5A. Note the intensity scale bar, showing a -8 to 8 percent
difference relative to the no-scatterer basis image. Also note that it is not actually an image (which is strictly integer), but just a 2D array of double numbers. To save to a TIFF file, the flat-fielded pseudo-image needs to be back-converted to positive integers between zero and the bit depth.

3.4.6 Processing iSCAT images for single-particle tracking

In order to perform single-particle tracking on iSCAT data, a second post-processing step is necessary. The goal of this post-processing step is to make the PSF of a traveling gold particle look like that of a fluorescence image, a white spot on a black background. This is done via image subtraction; subtract all the images where the gold is present from an image where the gold is not present. First, the flat-fielded iSCAT image is inverted such that the PSF appear white on a gray background (unless is already appears white). Next, a “no gold” image is made. For example, if there is a 100-frame movie where a gold-labeled kinesin lands on a microtubule at frame 16 and then steps for 50 frames, then frames 1-15 and 66-100 all have no-gold. Take the median intensity projection of about ten of the no-gold frames. Then subtract this median no-gold image from all the frames with a gold present. Now the gold will appear as a white spot on a black background, and standard PSF Gaussian fitting tools(14) can be used to find the nanometric X and Y positions. A flat-fielded image before and after the no-gold subtraction can be seen in Figure 3-1B. Note that this second post-processing step removes the scattering contribution of the microtubule, and will fail if the microtubules are moving over time.

3.4.7 Notes and troubleshooting

- Note the strengths and weaknesses of doublet versus singlet lenses. Singlets are typically a fine choice for collimating light in non-demanding scenarios. They work fine for the lens system in the 1D scanning iSCAT excitation line. However, they do no focus light down to as small a point as do doublets, and they scan induce some aberrations. Another
weakness for singlets is that their performance suffers if light is not perfectly centered on the center of the lens. It should be noted that singlets are directional, and need to be oriented the right way depending on if one is collimating or focusing light. Be very careful not to place singlets backwards. Doublets cover much of the weaknesses of singlets, and are ideal for the final focusing lens in the emission line.

- Be sure to use aspheric lenses when in-coupling or out-coupling from the optical fiber. Divergence angles are high in this scenario, and even a doublet lets would induce large spherical aberrations. In the in-coupling scenario, spherical aberrations would lead to the laser not getting focused down to a true-diffraction limited spot. Hence, a sub-optimal amount of light would get into the fiber. In the out-coupling scenario, spherical aberrations would decrease the beam quality. Microscope objects are lens systems that are specialized for reducing spherical aberrations, and thus are a really good choice for outcoupling (although a high NA ashpere would also do the trick).

- Make absolute sure that every lens used in iSCAT is anti-reflection coated. Lenses are cheap; buy new ones if you are not sure if the anti-reflection coating is working well. Back-reflections are the natural enemy of iSCAT. Every back reflection that makes its way to the detector will contribute to the interference pattern and decrease the effective signal to noise of the microscope. The best way to improve your iSCAT setup is to minimize the back reflections that reach the detector.

- Note that the laser chosen for this setup is an absolutely awful laser. Good lasers typically (1) emit in only a single mode, (2) have a long coherence length, (3) have a tiny beam diameter, (4) are truly monochromatic. For iSCAT, only point (4) is important. The single-mode fiber is used to down-select a single mode, so starting with one mode isn’t needed. It is true that selecting a single mode from a multimode laser will lead to huge power loss, but this doesn’t matter: the laser chosen is 200 mW, which is orders of
magnitude more power than would be advisable to put onto a biological sample. Losing even 90% of the laser power in mode selection is inconsequential. Since we are using a single-mode fiber, the beam diameter for the excitation line is ultimately set by the focal length of the objective used in outcoupling. Playing with the beam diameter before in-coupling may help get more power through the fiber, but as noted above we are starting with much more power than we need and thus do not care if we lose a lot. The short coherence length of the poor-quality laser chosen is surprisingly a strength for iSCAT. Any two fields that are superimposed more than one coherence length away from the detector will not have a proper interference pattern on the detector. Hence, as long as the coherence length is longer than the distance between the objective and the detector, shorter coherence lengths are better. Back reflections from the excitation line should be incoherent by the time they reach the detector. Adding more length to the excitation line helps this

- Performance from the polarizing beamsplitter cube is never perfect. Some light will always get reflected when the laser if first put through the cube (even though you expect it all to transmit). Make sure this initial reflection does not overlap with the desired reflection (the signal coming back from the objective). This initial reflection would be the worst killer of signal to noise if it was overlapping. It is easy to check for overlap: block the beam right before the objective and see if there is any signal on the camera. See also Figure 3-5B

- If there are any noticeable fringes in the raw intensity pattern, and if any of these fringes clearly change when the stage is stepped up and down in Z, then there are likely back reflections contributing to the interference pattern. This is undesirable. Take care to remove them as much as possible
• Note the black bars that appear on the edges of the scanned beam. These come from the AOD, and cannot be removed. Increase the scanning wave intensity (at the function generator) such that the black bars are not on the camera imaging area. Note that as the scanning frequency is increased, there may be some ringing around those black bars. Decrease the scanning frequency if this is the case. If there is a black bar in the center of the scanned region, then the beam is not going through the AOD flatly. Try harder to get the beam parallel to the table and transmitting straight through the AOD using the two mirrors before the AOD.

• Note that this protocol advises the use of an 8-bit camera code. Running the camera in 8-bit is faster and leads to smaller file sizes. However, the dynamic range is limited, and it is not ideal for high-precision particle tracking. To do the best single-particle tracking in iSCAT, the maximum camera bit depth should be used. This is 12-bit for the Basler Ace camera suggested. However, TIFF images can only be 8-bit or 16-bit. Hence, the 12-bit flat fielded intensity arrays either need to be stored in a non-image format such as a TDMS file, or the image data need to be back-converted to 12-bit and then dumped into the center of a 16-bit image. Dump without any scaling to avoid artifacts (i.e. only $2^{12}$ of the $2^{16}$ possible intensity values will be used, centered around the middle value such that a 0 or $2^{16}$ count can never be achieved). There is an older LabVIEW code called VidCAP_KJM_TIFF_BaslerAce_MF.vi that saves 16-bit TIFF images this way.

• Note that the pre-written LabVIEW codes only work for square regions of interest.

• Make sure that the correction dial on the microscope objective is properly set for the coverslip thickness and temperature used. Typically this is 0.17 and 23˚ C. If the dial is set incorrectly, then the PSF can be blurred.
3.5 Strengths and weaknesses of 1D scanning iSCAT versus TIRDFM

The 1D scanning iSCAT has the strengths of (1) being gentle on the sample, (2) exciting with circularly polarized light such that microtubules can be seen label-free, (3) being able to see smaller gold nanoparticles and more label-free objects than TIRDFM, (4) being able to deal with spurious back reflections. The 1D scanning iSCAT has the weaknesses of (1) requiring two rounds of post-processing for single particle tracking, (2) having a limited field of view unless a second AOD is purchased, (3) exciting two Rayleigh lengths of Z space, which is narrow, but not as narrow as an evanescent field, (4) having signal to noise constantly change in Z, (5) suffering loss of image quality if the flat-field correction begins to fail over time, (6) having a huge number of wavelength-dependent elements, making it very expensive and time-consuming to change colors.

The TIRDFM setup has the strengths of (1) being very simple to set up and modify, (2) exciting only the first ~100 nm of Z space, (3) requiring no post-processing at all, (4) having a large field of view without the need for scanning, (5) the only wavelength-dependent optic is the half wave plate, so changing color is as easy as getting a new laser and half wave plate. The TIRDFM setup has the weaknesses of (1) being unable to see microtubules off all alignments, (2) having no way to deal with scattering contributions from microtubules, (3) being hugely sensitive to spurious back reflections, (4) decreased ability to see label-free proteins. In general, TIRDFM has proven to provide higher-quality single-molecule data for 30-nm gold nanoparticle tagged proteins. This is largely due to the green wavelength (matches the 30-nm gold surface plasmon), narrower excitation light sheet, and the lack of post-processing. The 1D-scanning iSCAT has proven to be ideal for microtubule growth assays and for all types of label-free imaging.

3.6 Conclusions and future applications

Building a 1D-scanning iSCAT is an exciting endeavor, and the microscope provides a wealth of opportunity in answering biophysical questions. The iSCAT build is more difficult than
the TIRDFM build, but provides a great training opportunity in optics, electronics, and hardware. iSCAT is a highly desired technology, and simply using the instrument instantly raises the profile of any published work. The ability to measure label-free microtubule dynamics at kHz frame rates is game-changing. Large molecules like myosin has easily been measured label-free in iSCAT(5), so it is reasonable to attack other large molecules like dynein in this fashion. Label-free imaging of multimotor ensembles is another exciting future application of iSCAT.

3.7 References


Chapter 4

Kinetics of nucleotide-dependent structural transitions in the kinesin-1 hydrolysis cycle

4.1 Introduction

Kinesin-1 is a motor protein that steps processively towards microtubule plus-ends, tracking single protofilaments and hydrolyzing one ATP molecule per step (1–6). Step sizes corresponding to the tubulin dimer spacing of 8.2 nm are observed when the molecule is labeled by its C-terminal tail (7–10) and to a two-dimer spacing of 16.4 nm when a single motor domain is labeled (4, 11, 12), consistent with the motor walking in a hand-over-hand fashion. Kinesin has served as an important model system for advancing single-molecule techniques (7–10), and is clinically relevant for its role in neurodegenerative diseases (13), making dissection of its step a popular ongoing target of study.

Despite decades of work, many essential components of the mechanochemical cycle remain disputed, including (i) how much time kinesin-1 spends in a one-head bound state when stepping at physiological ATP concentrations, (ii) whether the motor waits for ATP in a one- or two-heads bound state, and (iii) whether ATP hydrolysis occurs before or after tethered head attachment (4, 11, 14–20). These questions are important because they are fundamental to the mechanism by which kinesins harness nucleotide-dependent structural changes to generate mechanical force in a manner optimized for their specific cellular tasks. Addressing these questions requires characterizing a transient one-head-bound (1HB) state in the stepping cycle in which the unattached head is located between successive binding sites on the microtubule. This 1HB intermediate is associated with the force-generating powerstroke of the motor and it underlies the detachment pathway that limits motor processivity. Optical trapping (7, 19, 21, 22) and single-molecule tracking studies (4, 8–11) have failed to detect this 1HB state during stepping. Single-molecule fluorescence approaches have detected a 1HB intermediate at limiting ATP
concentrations(11, 12, 14, 15), but apart from one study that used autocorrelation analysis to detect a 3 ms intermediate(17), the 1HB state has been undetectable at physiological ATP concentrations.

Single-molecule microscopy is a powerful tool for studying the kinetics of structural changes in macromolecules(23). Tracking steps and potential substeps for kinesin-1 at saturating ATP has until now been hampered by the high stepping rates of the motor (up to 100 s\(^{-1}\)), which necessitates high frame rates, and the small step size (8.2 nm), which necessitates high spatial resolution(7). Here we apply interferometric scattering microscopy (iSCAT), a recently established single-molecule tool with high spatiotemporal resolution(24–27) to directly visualize the structural changes underlying kinesin stepping. By labeling one motor domain in a dimeric motor, we detect a 1HB intermediate state in which the tethered head resides over the bound head for half the duration of the stepping cycle at saturating ATP(21, 22). We further show that at physiological stepping rates, ATP binding is required to enter this 1HB state and that ATP hydrolysis is required to exit it. This work leads to a significant revision of the sequence and kinetics of mechanochemical transitions that make up the kinesin-1 stepping cycle and provides a framework for understanding functional diversity across the kinesin superfamily.

4.2 Results

4.2.1 High-resolution single-molecule microscopy reveals and intermediate in the stepping cycle at saturating ATP

We directly observed the stepping cycle of kinesin-1 at saturating ATP by performing \textit{in vitro} single-molecule assays using iSCAT microscopy(25–27) (Figure 4-1A-B). Drosophila kinesin-1 (k560) was fused to an N-terminal AviTag and conjugated to a 30 nm streptavidin-coated gold nanoparticle. This conjugation strategy allowed us to maintain the wild-type sequence for the motor domain, and had no measurable effect on either the velocity or run length (Figure 4-6). Microtubules were attached to a glass coverslip, and the position of the nanoparticles carried by
single kinesin motor domains was tracked with sub-diffraction limited precision using established methods (4, 25, 28). The high spatiotemporal resolution of iSCAT enabled tracking at saturating ATP such that stepping kinetics could be recorded for kinesins moving under physiological conditions.

At 1,000 frames per second, gold nanoparticles on moving kinesins were localized with a run-wise average precision, defined as the error of the Gaussian fit to the point spread function averaged over a processive run, of 1.9±0.4 nm (SEM for N=31 processive runs; Figure 4-7G). To extract steps, the motor position along the microtubule axis was analyzed using a model-free t-test based algorithm (29), and 982 total steps were detected. The plateau standard deviation, calculated through pairwise differences with outliers removed (see methods, Figure 4-7H), was 3.8±0.3 nm. This base noise level enabled identification of substeps beyond the 16.4 nm steps that have been detected previously (Figure 4-1C-D) (4, 11, 12). Because 16.4 nm represents a complete mechanochemical cycle for one head, we interpret the substep position as a stable mechanochemical intermediate with the labeled head unbound and positioned partway between successive microtubule binding sites. Accordingly, sequential steps were seen to add up to 16.4 nm (Figure 4-7). A Gaussian mixture model was fit to the distribution of positive step sizes, and the principle component (78%) had a mean ± SD of 8.4±3.0 nm, approximately half the distance between successive binding sites for a single head (Figure 4-1E). Larger components were found with means 16.1 and 29.7 nm, likely corresponding to missed substeps.

For verification, we repeated the experiment at 200 frames per second and achieved an average precision of 3.9±0.3 nm and plateau standard deviation of 5.3±0.3 nm for N=26 processive runs. Under these conditions, the mean positive step size was 16.4±0.2 nm (SEM, 636 steps; Figure 4-8), consistent with previous tracking studies that used lower ATP concentrations to slow the stepping rate (4, 11). We carried out step-finding analysis on simulated steps generated with experimental signal-to-noise matching both this and previous studies, and validated that substeps
are only consistently detected with high frame rates and low plateau standard deviations (Figure 4-9).

**Figure 4-1 Kinesin substeps at saturating ATP.** (A) Schematic of the experimental set up. The incident field ($E_i$) is provided though an objective (O), and the fields reflected ($E_r$) off the coverslip (CS) and scattered ($E_s$) by a gold nanoparticle are collected by the same objective. (B) Raw image of a 30 nm gold nanoparticle (in focus) on a motor walking along a microtubule (out of focus) at 1,000 frames per second. Inset: the isolated point spread function after background subtraction. (C) Example traces in XY space at 1,000 frames per second and 1 mM ATP. (D) Same traces as in (C) represented as position along the microtubule axis versus time, showing clear evidence of substeps. (E) Histogram of positive step sizes at 1,000 frames per second at 1 mM ATP including a Gaussian Mixture Model fit. Individual fit components shown in color, and mixture fit (sum of components) shown in black. Step-size distributions for lower frame rates are shown in Figure 4-8.

### 4.2.2 Kinesin spends equal fractions of time with one- and two-heads bound at saturating ATP

The precision achieved when tracking at 1,000 frames per second enabled kinetic analysis of the dwell times preceding each substep. The stepping cycle for each head involves detaching from the microtubule and reattaching to the next microtubule binding site 16.4 nm away. Hence,
the mechanochemical cycle can be broken down into two mechanical states: a two-head-bound state (2HB) in which both motor domains are positioned on microtubule binding sites, and a one-head-bound state (1HB) in which one motor domain is bound to the microtubule and the other is positioned partway between adjacent microtubule binding sites. Because only one motor domain is labeled, the durations of the two measured states are asymmetric—the time that the labeled head is bound to the microtubule includes the entire stepping cycle (1HB plus 2HB) of the unlabeled head plus the 2HB duration of the labeled head, while the second state measures only the 1HB duration of the labeled head. We therefore divide the two-step cycle that generates each 16.4 nm translocation of the gold nanoparticle into $\tau_{\text{long}}$, the waiting time preceding the first substep, and $\tau_{\text{short}}$, the waiting time preceding the second substep (Figure 4-2A).

In order to preserve the phase of alternating substeps along processive runs, a 2-state Hidden Markov Model (HMM) was designed and the Viterbi algorithm was used to return the most likely sequence of states in a given run(23, 30). Each 16.4 nm translocation was thereby divided into $\tau_{\text{long}}$ and $\tau_{\text{short}}$. An example trace with $\tau_{\text{long}}$ and $\tau_{\text{short}}$ indexed by black and red coloring, respectively, is shown in Figure 4-2B (additional traces in Figure 4-10). In most cases there was no mean displacement in the off-axis position between long and short plateaus (Figure 4-10). The distributions for $N=223$ $\tau_{\text{long}}$ measurements and $N=239$ $\tau_{\text{short}}$ measurements from 27 traces are shown in Figure 4-2C. $\tau_{\text{short}}$ follows an exponential distribution (red line in Figure 4-2C), consistent with it containing one rate-limiting step, while $\tau_{\text{long}}$ follows a gamma distribution with shape parameter 3 (gray line in Figure 4-2C), consistent with it containing three similar rate-limiting steps. Using population means, we found that $\tau_{\text{1HB}} = \tau_{\text{short}}$ was $8.0\pm0.5$ ms and $\tau_{\text{2HB}} = (\tau_{\text{long}} - \tau_{\text{short}})/2$ was $7.8\pm0.5$ ms, or that kinesin spends approximately equal durations in each mechanical state. The sum of these state durations (15.8 ms) matches the total cycle time calculated from the weighted mean velocity of all the traces of $530.6\pm28.1$ nm s$^{-1}$ (Figure 4-6), or $15.5\pm0.8$ ms per 8.2 nm step.
4.2.3 Kinesin waits for ATP with 2HB

To address the question of where the rear head is positioned during the ATP waiting state, we repeated the 1,000 frames per second tracking assay at reduced ATP concentrations. We justified use of the HMM for fitting step durations by first showing that substeps are still detected in reduced ATP using the model-free step-finding algorithm (Figure 4-7). $\tau_{\text{long}}$ and $\tau_{\text{short}}$ were measured as before, with the hypothesis that both plateau durations would increase if the motor waits for ATP in the 1HB state, while only $\tau_{\text{long}}$ would increase if the motor waits for ATP in the 2HB state (see Figure 4-2A). As shown in Figure 4-3A-C, $\tau_{\text{long}}$ was significantly extended at 10 and 100 $\mu$M ATP while $\tau_{\text{short}}$ was unaffected, consistent with kinesin waiting for ATP in the 2HB state. The plateau durations were decomposed into the 1HB and 2HB durations using $\tau_{1HB} = \tau_{\text{short}}$ and...
\[ \tau_{2HB} = \frac{(\tau_{long} - \tau_{short})}{2} \] as previously (Figure 4-2A), and \(\tau_{1HB}\) and \(\tau_{2HB}\) were plotted as a function of ATP (Figure 4-3D). \(\tau_{1HB}\) showed a weak dependence on ATP concentration, while the ATP dependence of \(\tau_{2HB}\) was well fit by a Michaelis-Menten model with a fit \(K_{m}^{ATP}\) of 40.7 \(\mu\)M, in agreement with the ATP dependence of kinesin-1 velocity and ATPase reported previously (2, 31, 32).

At very low (1 \(\mu\)M) ATP concentrations, where ATP waiting dominates the step duration, a mixture of 1HB and 2HB ATP waiting states was observed (Figure 4-12A). These results were well described by a model (see SI text) in which the motor, when forced to wait long times for the arrival of ATP, transitions from a 2HB to a 1HB ATP waiting state at a rate of 1.9 s\(^{-1}\) (Figure 4-12B). The best fit to the heterogeneous \(\tau_{short}\) and \(\tau_{short}\) data was achieved by incorporating different ATP affinities in the 2HB and 1HB states, with fit values \(K_{m}^{ATP,2HB}=39\) \(\mu\)M and \(K_{m}^{ATP,1HB}=23\) \(\mu\)M. The 2HB ATP affinity closely matches the value measured at higher ATP (Figure 4-3D), while the 1HB ATP affinity is consistent with a model in which pulling the neck linker backward reduces the ATP affinity of the front head.

![Figure 4-3 Kinesin waits for ATP in the 2HB state](image)

Example traces in XY space at 100 \(\mu\)M (A) and 10 \(\mu\)M ATP (B) taken at 1,000 frames per second, with \(\tau_{long}\) indicated in black and \(\tau_{short}\) indicated in red. (C) Same traces shown as position along microtubule versus time. (D) Population measurements for the duration kinesin spends in the 1HB (red) and 2HB (blue) states at different ATP concentrations. Error bars represent SEM (N=233/239, 99/103, and 275/289 for \(\tau_{long}/\tau_{short}\) in 1 mM, 100 \(\mu\)M, and 10 \(\mu\)M, respectively) and black line is weighted fit to Michaelis-Menten equation plus offset with \(K_{m}^{ATP}=40.7\pm8.1\) \(\mu\)M (fit weighted by inverse SEM, 95% confidence intervals).
4.2.4 Kinesin hydrolyzes ATP with 1HB

After defining that the transition from the 2HB to the 1HB state principally occurs after ATP binding, we set out to determine whether completion of the 16.4 nm step, which is marked by the return to the 2HB state, occurs before or after ATP hydrolysis. Single-molecule tracking assays were repeated at 1,000 frames per second using saturating concentrations of ATPγS, a slowly-hydrolyzable ATP analogue (19, 31, 33). Motor velocity at 1 mM ATPγS was 13.6±1.0 nm s⁻¹ (SEM, N=33 runs), roughly equal to the velocity for 1 µM ATP (13.3±0.8 nm s⁻¹, N=36 runs). The localization precision in ATPγS was also comparable to 1 µM ATP (1.8±0.1 nm and 1.8±0.2 nm, respectively). If hydrolysis occurs in the 2HB state, as might be expected if nucleotide binding was sufficient to dock the neck linker (NL) and enable binding of the tethered head (33, 34), the motor should reside overwhelmingly at microtubule binding sites 16.4 nm apart, and appear very similar to the 1 µM ATP traces. Surprisingly, the opposite was observed, as exemplified in Figure 4A (time courses in Figure 4-13) – the motor took highly variable steps, changes in position along the microtubule axis were gradual rather than sudden, and movements perpendicular to the microtubule axis without a simultaneous step along the microtubule were common. This behavior suggests that during the key transition that follows ATP binding by the bound head but precedes hydrolysis, the second head is in a tethered 1HB state that is flexible and can sample multiple positions.

We next examined processivity in ATPγS. If hydrolysis can occur in either a 1HB or 2HB state, then run length should increase in ATPγS because the tethered head would have a much greater chance to bind before the bound head hydrolyzes its nucleotide and enters a vulnerable 1HB post-hydrolysis state (19). The opposite was observed – in single-molecule Qdot tracking experiments, run lengths were slightly shorter in saturating ATPγS than in saturating ATP (Figure 4-4B).
4.2.5 **Independent measurement of a long 1HB state by stopped-flow**

The results at saturating ATP (Figure 4-2) show that kinesin spends approximately half of each step in a 1HB state, starkly contrasting current models based on step measurements from optical trapping(21, 22). To confirm the single-molecule results, as well as to help constrain rate-limiting steps in the mechanochemical cycle, we carried out ensemble biochemistry experiments using stopped-flow spectrofluorometry. Half-site ADP release experiments were first performed by flushing varying ATP concentrations against microtubule-bound dimer motors incubated in 2’(3’)-O-((N-methylanthraniloyl)adenosine 5’-diphosphate (mADP). The maximal ATP half-site release rate, which includes all states from ATP binding to mADP release, was 112 s\(^{-1}\) (Figure 4-5A), in agreement with literature values(33, 35, 36). Next, to estimate the mADP release rate, we measured mADP exchange kinetics in the front head when motors are locked on to microtubules in a 2HB state with AMP-PNP(14, 15, 37, 38). By performing linear regression to the mADP dependence of \(k_{obs}\), we estimate a mADP off-rate of 367 s\(^{-1}\) (Figure 4-5B). Comparison of this mADP off-rate with the maximal ATP half-site release rate (see block diagram in Figure 4-5C).
yields a purely biochemical calculation of the 1HB state duration of 6.3 ms, in good agreement with the 8.0 ms from single-molecule measurements.

Finally, to better understand the divergent stepping behavior in ATPγS, half-site mADP release experiments were repeated using ATPγS. A maximal rate of 28.7 s$^{-1}$ was measured (Figure 4-5A), consistent with previous measurements(33). Interestingly, this rate was approximately 20-fold higher than the 1.35 s$^{-1}$ single-molecule stepping rate in ATPγS, indicating that significant off-pathway events such as side steps must be available from state 4. It was also 4-fold slower than the ATP half-site rate and 2-fold slower than the stepping rate in ATP, indicating that a 1HB exit without hydrolysis is kinetically inconsistent with the measured stepping rate.

**Figure 4-5 Ensemble biochemistry and mechanochemical cycle for kinesin.** (A) Nucleotide half-site release kinetics with Michaelis-Menten fit for ATP (circles, $K_m=90.4\pm20.6$ µM, $V_{max}=111.7\pm8.8$ s$^{-1}$) and ATPγS (triangles, $K_m=55.4\pm22.1$ µM, $V_{max}=28.7\pm2.7$ s$^{-1}$). (B) Front head mADP exchange kinetics under strain in AMP-PNP locked motor-microtubule complexes. Linear fit ($R^2=0.98$) to $k_{obs}=k_{on}[\text{mADP}]+k_{off}$ yields $k_{off}=367\pm4$ s$^{-1}$ and $k_{on}=0.46\pm0.08$ µM$^{-1}$s$^{-1}$. Inset: example stopped-flow transient at 75 µM mADP with fit to biexponential. All error bars are SEM and all fits weighted by inverse SEM. (C) Mechanochemical cycle of kinesin-1 in saturating ATP, with block diagram showing the sequence of states measured in the single-molecule and ensemble experiments. The 2HB duration involves ADP release from the front head (1 $\rightarrow$ 2) followed by a transition to the 2HB ATP waiting state (2 $\rightarrow$ 3), which we tentatively define as detachment of the rear head to a weak-binding intermediate with no associated displacement but possibly involving phosphate release. Binding of ATP (3 $\rightarrow$ 4) marks the transition to the 1HB state, and is followed by ATP hydrolysis (4 $\rightarrow$ 5) and tethered head attachment (5 $\rightarrow$ 1) to return to the 2HB state. In the half-site mADP release experiment, the motor starts in an ATP waiting state similar to 3 (see Figure 4-14 for further details), and completes the entire cycle save for the final transition back into the ATP waiting state (2 $\rightarrow$ 3). The mADP off-rate in the front head exchange experiment directly measures 1 $\rightarrow$ 2. Calculation of rates by subtraction, made visible by comparing rows in the block diagram, yields an independent estimate of 6.3 ms 1HB duration.
at saturating ATP from biochemical experiments, in agreement with the 8.0 ms estimate from the single-molecule data.

4.3 Discussion

In the standard model of the kinesin hydrolysis cycle, ATP binding drives NL docking, swinging the tethered head forward towards the next binding site (34, 39, 40). Optical trapping studies visualize discrete 8.2 nm steps, and previous single-molecule tracking studies visualized 16.4 nm steps without substeps, which together suggest that kinesin spends the vast majority of time with both heads centered at microtubule binding sites at saturating ATP (4, 11, 21, 22). The single-molecule tracking data presented here conflicts with the current consensus in three ways: 1) at saturating ATP kinesin-1 spends half of each cycle with one head unbound and positioned between microtubule binding sites, 2) ATP binding occurs when kinesin is in a state resembling a two-heads-bound state, and 3) ATP binding alone is insufficient to complete the powerstroke under normal conditions. Overall, these data motivate a new way of thinking about how kinesin-1 takes steps, and help resolve a number of conflicting reports in the literature.

4.3.1 Detection of substeps at saturating ATP

By using iSCAT and gold nanoparticle labeling, we were able to detect substeps across a range of ATP concentrations that were not detected in previous N-terminal head tracking experiments (4, 11, 12). Substep detection requires low positional variance in the plateau regions relative to the size of the substep, as well as sufficient temporal resolution to capture the fast intermediate plateau ($\tau_{short}$) that separates longer plateaus ($\tau_{long}$). We show through simulations that substeps generated using the signal-to-noise achieved in this study can be properly detected by a step-finding algorithm, whereas substeps generated with a signal-to-noise corresponding to the best available resolution from fluorescence experiments (11) or with reduced frame rates are missed and only 16.4 nm steps are detected (Figure 4-9).
Optical trapping studies with C-terminal labeled motors, which have the highest spatiotemporal resolution to date, have ruled out intermediate positions between 8 nm steps having durations longer than 30 µs\(^{(21, 22)}\). Substeps were also not detected in high-resolution C-terminal tracking experiments under zero load\(^{(8–10)}\). One way to reconcile these studies with the substep measured here is to posit that the position of the coiled-coil is determined by the bound head when the motor is in the 1HB state and the front head when the motor is in the 2HB state. Additionally, Guydosh et al. showed that forces as low as 0.4 pN applied to a single labeled head are sufficient to fully extend a tethered intermediate either forward or backward, providing an explanation as to why the substeps observed here are not seen under load\(^{(12)}\).

To support our single-molecule measurement of a 8.0 ms duration substep, we independently estimated the duration of the 1HB state by ensemble biochemistry techniques and obtained an estimate of 6.3 ms. A similar 1HB state with a duration of 3 ms at saturating ATP was identified by Verbrugge et al. using autocorrelation analysis of FRET fluctuations\(^{(17)}\). We attribute the different durations to the differing experimental approaches and different constructs used (N-terminal AviTagged *Drosophila* wildtype versus Cys-modified human kinesin-1). It was recently shown that for Cys-lite kinesin-1 constructs, the force-velocity profile and the ability to take backsteps under hindering loads differ significantly from wild-type\(^{(41)}\), suggesting possible deviations in the mechanochemical cycle.

### 4.3.2 The conformation of the ATP waiting state varies with [ATP]

There is disagreement in the literature of whether the trailing head detaches before ATP binds to the front head, or ATP binds in the 2HB state, followed by trailing head detachment. Interhead strain in the 2HB state is thought to slow binding of ATP to the front head and accelerate detachment of the trailing head\(^{(21, 42–46)}\), leading to a model in which the trailing head detaches before ATP binds to the leading head. In support of this model, single-molecule fluorescence
studies have found that the fraction of time the motor spends in a 1HB state falls with increasing ATP concentrations(14, 15). In contrast, single-molecule tracking studies with a probe on one head(4, 11) and biochemical measurements of the reversal of ATP hydrolysis in dimeric kinesin(18) suggest a model in which ATP binding occurs when the motor is predominantly in a 2HB state. This question is important because a 1HB ATP waiting state implies a ratchet-like mechanism in which the tethered head diffuses freely until its forward conformation is stabilized by ATP binding and NL docking. In contrast, a 2HB ATP waiting state implies a powerstroke-like mechanism in which detachment of the trailing head is triggered by a conformational change in the leading head that occurs when its NL is pulled backward to accommodate the 2HB state and presumably unable to dock.

We found that at ATP concentrations down to 10 µM, the ATP waiting state was clearly identified as having both motor domains positioned on or above microtubule binding sites (Figure 4-3C). Thus, under normal conditions ATP binding precedes complete detachment of the trailing head. However, at 1 µM ATP, where the motor steps at ~2 s⁻¹, we measured both short- and long-lived 1HB states (Figure 4-12). The data were well fit by a model in which kinesin can transition to a 1HB ATP waiting state at a rate of 1.9 s⁻¹. Our data provide a mechanism to explain why single-molecule FRET and fluorescence polarization studies observe a 1HB waiting state at low nucleotide concentrations but not at saturating ATP(14, 15), while it is still possible to detect 16 nm steps even at low ATP(4, 11). A noteworthy implication of a 2HB ATP waiting state is that kinesin’s first step, characterized by colliding with a microtubule while having ADP in both heads and subsequently releasing one, is unique from all steps that follow it (Figure 4-14). Thus, in any experiment where the ATP waiting state is pre-formed by incubation in low nucleotide concentrations, an ensemble of first steps alone will be measured, meaning the results may not be representative of the normal stepping cycle.
4.3.3 Hydrolysis accelerates completion of the powerstroke

Another debated issue is whether hydrolysis occurs before or after the tethered head binds to the next binding site on the microtubule. Forced unbinding, fluorescence polarization microscopy, and single-molecule FRET studies with the non-hydrolyzable ATP analog AMP-PNP all show that hydrolysis is not required for the motor to enter a 2HB state (14, 15, 33, 37, 47). However, in stopped-flow experiments, AMP-PNP and ATPγS in wild-type motors, and ATP in a hydrolysis mutant all trigger release of ADP from a tethered intermediate at a rate of about 30 s⁻¹ (Figure 4-5A) (20, 33). This rate is four-fold slower than the release rate measured using ATP (Figure 4-5A) (33), and is two-fold slower than the stepping rate (Figure 4-6), strongly suggesting that under physiological conditions hydrolysis occurs before tethered head attachment. Milic et al. recently showed that processivity under assisting load is not increased in ATPγS but is increased by adding phosphate to the media, in support of ATP hydrolysis occurring before tethered head attachment (19, 41). We extend that result by showing that in a zero-load assay the run length in ATPγS is not higher than the run length in ATP (Figure 4-4B), and we further show that the position of the motor domain while waiting to hydrolyze ATPγS is not predominately localized on microtubule binding sites like it is while waiting for ATP (Figure 4-4A).

Our results are most consistent with a 2-step model of the powerstroke in which ATP binding only partially docks the NL, and hydrolysis completes docking to accelerate tethered head attachment (19, 41, 48). This model contrasts with the conventional model of ATP binding driving full NL docking (34, 39). Structural support for the conformational changes that enable NL docking upon ATP binding, either by clamshell-like closure of the “nucleotide cleft” and the associated opening of the “docking cleft” via rotation of the N-terminal subdomain (49) or by reorientation of switch I/II and P-loop subdomains (50), come from comparisons of the no-nucleotide structure to the ADP•Al•Fx structure. If the ADP•Al•Fx structures actually represent the ADP•Pi state (48, 51, 52) rather than the ATP state (which is further supported by the low tethered head resolution from
CryoEM in AMP-PNP(53)), our data are in full agreement with these structural analyses. A two-step powerstroke is also consistent with the N-terminal strand forming a bundle with the NL that stabilizes its forward-biased state(54, 55); our data suggest that neck cover bundle formation at a rate necessary to match physiological stepping rates requires hydrolysis.

### 4.3.4 Rate limiting steps in the hydrolysis cycle

Randomness measurements from optical trapping experiments suggest that there are two rate-limiting steps in the hydrolysis cycle at saturating ATP(2). The finding here that the 1HB and 2HB states are of approximately equal duration at saturating ATP is consistent with there being one rate-limiting step in each. Our data enable us to define the sequence of mechanochemical transitions that make up the ATP hydrolysis cycle and to constrain the rate-limiting steps (Figure 4-5C). The duration the motor spends in the 1HB state is comprised of the time it takes for ATP hydrolysis plus the time it takes for tethered head attachment. As $\tau_{1HB}$ follows an exponential distribution (Figure 4-2C), one of these rates is expected to dominate. Burst hydrolysis rates ranging from 50 s$^{-1}$ to over 500 s$^{-1}$ have been reported from acid quench experiments on kinesin-1(20, 56, 57); hence this parameter is not tightly constrained by the literature. The measured 1HB time sets a lower limit of 125 s$^{-1}$ for the hydrolysis rate, but the rate of tethered head attachment is unknown. The question of whether ATP or ADP•Pi is the predominant species in the bound head during the 1HB state is important because the duration of the 1HB ADP•Pi state is expected to determine processivity(19, 41) and it is reasonable to expect that this parameter is tuned differently in noncanonical kinesins that carry out different cellular tasks than kinesin-1(45).

Candidates for the rate-limiting step in the 2HB state include ADP release by the tethered head, the transition to the ATP waiting state, and ATP binding (Figure 4-5C). Direct measurements of the mADP off-rate here (Figure 4-5B) and in previous studies(57) support this rate being very fast. ATP binding is also expected to be very fast at saturating ATP. Hence, the rate-limiting step
in the 2HB state is the transition into the 2HB ATP waiting state (State 2→3 in Figure 4-5C). Based on our data, this transition involves the trailing head entering a weakly-bound state that is centered on the trailing tubulin binding site and the front head becoming competent to bind ATP. This transition likely involves phosphate release by the trailing head and the ability of the front head to bind ATP may result from relief of inter-head tension, but these details go beyond our current data.

In the cell, the high velocity, stall force, and processivity of kinesin-1 contribute to its ability to traffic cargo towards the periphery. The mechanochemical cycle defined here by real-time observation of conformational changes in processive kinesin-1 dimers helps to explain the mechanism underlying these distinct biophysical properties. Other processive motors such as dynein and myosin-V couple their chemical and mechanical transitions in different ways than kinesin, and it is expected that kinesins having different intracellular roles will employ different mechanochemical tuning. Application of the methods established here to the diverse kinesin superfamily and other protein machines will further our understanding of how cells tune their moving parts.

4.4 Materials and methods

4.4.1 Protein preparation

The k560-AviN construct was made by adding the biotin ligase consensus sequence (AviTag) GLNDIFEAQKIEWH (Avidity) as well as two glycine residues for flexibility directly after the start codon in Drosophila kinesin-1 truncated at amino acid 560. A C-terminal 6x His tag was also inserted directly before the stop codon. For the k560-AviC construct, the Avi-tag was placed directly upstream of the 6x His tag, and the two glycine residues were placed upstream rather than downstream of the AviTag. All insertions were carried out using Q5 site-directed mutagenesis (New England Biosciences). Motors were expressed polycistronically with BirA
Biotin ligase (Avidity), in BL21(DE3) (New England Biosciences), and 0.5 mM biotin was added to the LB media at IPTG induction. Affinity chromatography and buffer exchange were carried out as described previously (46). Final motor concentration was measured by absorbance at 280 nm. Biotinylation efficiency was tested using a colorometric HABA assay (Sigma) and found to have a maximum of 0.2 biotin per kinesin head, which minimizes the probability of kinesin dimers with biotin on both heads.

4.4.2 Single-molecule experiments

Single-molecule in vitro experiments were carried out using a custom-built iSCAT microscope (25, 26). Cover slips were washed thoroughly with DI water, then incubated in 1% Hellmanex III (Helma), washed with methanol and again with DI water, and blown dry. Taxol-stabilized microtubules, prepared as in (46), were immobilized on the glass surface by first introducing 0.2 mg/ml casein, then adsorbing a rigor (Switch I) mutant of full-length Drosophila kinesin-1 (R210A) to the surface and finally introducing microtubules, which bound strongly and irreversibly to the rigor mutant (20). Imaging solution contained 0.5 mg/mL casein, 10 µM taxol, 20 mM glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 0.2 mg/mL BSA, 1:200 β-mercaptoethanol, and nucleotide in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8).

Biotinylated motors were incubated with streptavidin-coated 30 nm gold nanoparticles (BBI Solutions) on ice for 30 min at a final concentration of 300 pM of each in the imaging solution, and added directly to the flow cell. For 1 µM ATP and 1 mM ATPγS experiments, 1200 pM motor to 300 pM 30 nm gold were used. Motor concentrations by absorbance represent an extreme upper limit of the concentration of active, biotinylated motors, and the single-molecule landing rate showed a linear relationship up to 6:1 motor-to-gold ratio, justifying the use of these molar ratios with the expectation of only one motor per nanoparticle. In some cases, AMP-PNP was used to first lock the motors onto the immobilized microtubules before switching to ATP in order to
maximize the number of observable motile events. In the 200 and 400 frames per second traces, microtubules were adhered to the surface by short treatment (<30 s) with 0.5% glutaraldehyde. This method was less effective at stabilizing microtubules, and most likely explains the increase in plateau standard deviation.

For controls and run length measurements, Qdots (Q565 Qdots, Life Technologies) were used instead of gold nanoparticles. Motors and Qdots were incubated at a final concentration of 100 pM each, and flow cell construction and microtubule immobilization were identical. Qdot imaging was performed with a Nikon TE2000 inverted microscope set up for total internal reflection using an 80 mW Argon laser (Spectra Physics). Movies were recorded using a Cascade 512 EMCCD camera (Roper Scientific) and MetaVue software (Molecular Devices) at 10 frames per second. Qdot positions were determined by Gaussian fitting to moving point spread functions using FIESTA software(28). Velocity was calculated by linear fitting to the distance over time trajectories. Traces with obviously curved trajectories were not included, and a threshold of 4 points (400 ms) was set as the minimum for deciding whether or not a tracked object represented a processive run. Run length was determined by the total distance travelled. For ATPγS, run lengths below the minimum average measurement for ATP, 212 nm, were excluded. Velocity for a population was calculated as a weighted average using run lengths, as longer runs produced more confident linear fits: $\bar{V} = (\sum_{j=1}^{N} L_j)^{-1} \cdot \sum_{i=1}^{N} L_i V_i$, where L and V represent run length and velocity, respectively, and N represents the number of traces obtained. All experiments were performed at 22-23°C.

4.4.3 **Ensemble biochemistry**

All stopped flow experiments were performed on an Applied Photophysics SX20 spectrofluorometer at 22-23°C. Data acquisition and fitting were performed in Pro-Data SX software (Applied Photophysics). For the nucleotide half-site experiments, one syringe was filled
with 2 µM microtubules, 10 µM taxol, 500 nM mADP, and 200-400 nM biotinylated k560-AviN in BRB80 buffer. This mixture created a motor-microtubule complex with one head bound to the microtubule in the apo state and the second head free and containing mADP(1). The second syringe was filled with ATP or ATPγS in BRB80. All concentrations shown in Figure 4-5 show final nucleotide concentrations after mixing the contents of the two syringes. Excitation was set to 356 nm for the mADP nucleotide and 450 nm emission collected using HQ480SP emission filter. An integration time of 1 ms was set for the photomultiplier tube. The fluorescence transient generated upon mixing was fit with a double exponential in the range of 1-500 ms, and the faster rate constant was reported. Each nucleotide concentration was measured 3-5 times, and the mean and inverse SEM were used to perform a weighted fit to the equation:

\[ k_{obs} = \frac{k_{max}^{ATP/HS} [ATP]}{[ATP] + K_m^{ATP}} \]

with \( k_{max}^{ATP/HS} \) and \( K_m^{ATP} \) as free parameters.

For the front-head nucleotide exchange experiment, one syringe was filled with 7.5 µM microtubules, 10 µM taxol, 100 µM AMP-PNP, and 1 mM k406 in BRB80. The dimeric k406 construct, D. melanogaster kinesin-1 truncated at amino acid 406, was used because it could be prepared at higher concentration and also produced cleaner fluorescence transients. This mixture created a motor-microtubule complex with two heads bound, the rear head with AMP-PNP and the front head in the apo state (42). The second syringe was filled with varying mADP in BRB80. Excitation was set to 280 nm to measure FRET between tryptophan residues in the front head and mADP nucleotides(18). An integration time of 200 µs was set for the photomultiplier tube. The fluorescence transient generated upon mixing was fit with a double exponential in the range of 1.6-100 ms (lower bound was adjusted ±0.4 ms to account for the dead time on a per-trace basis, see Figure 4-5B inset for example), and the faster rate constant was reported. Each mADP
concentration was measured 4-6 times, and the mean and inverse SEM were used to perform a weighted fit to the equation:

\[ k_{obs} = k_{on}^{mADP}[mADP] + k_{off}^{mADP} \]

with \( k_{on}^{mADP} \) and \( k_{off}^{mADP} \) as free parameters.

### 4.5 Supplementary information

#### 4.5.1 Image analysis, trace preparation, and step finding for gold nanoparticle tracking

Images were collected using custom LabVIEW software (National Instruments), and a background subtraction of all static scatters was performed in MATLAB (Mathworks) as previously described (24, 25). Isolated point spread functions were fit using FIESTA (28) to return sub-diffraction limited measurements of nanoparticle X and Y position over time. Localization precision was determined by the error of this fitting. Traces were split into linear sections if microtubules were not fully linear to start, and rotated to minimize the standard deviation in the direction perpendicular to the track. The resulting position data along the microtubule axis was used for step finding. The plateau standard deviation, estimated using pair wise differences, was used as the best estimator of the inherent experimental noise in the traces:

\[
\sigma \approx \sqrt{\frac{\sum_{i=1}^{(N-1)} (x_{i+1} - x_i)^2}{2(N-1)}}
\]

Where \( \mathbf{X} \) is a vector of length \( N \) that represents the position data along the microtubule axis. To account for relatively fast steps that separated plateau regions, iterative pairwise-difference outlier removal was used (removing pairwise differences greater than 3\( \sigma \) from the mean; see Chen 2014 (29) supplemental materials for further detail).

For the model-free analysis used for building step size distributions, a t-test based step-finding algorithm was used (tDetector, described in detail in Chen 2014) (29). Given an input time
series of length N, the algorithm essentially performs N-1 (for each possible partitioning of the data) two-sample t-tests, and then declares a new step at the index yielding the most significant difference of means, thus creating two new plateaus (if none of the t-tests were significant then no step is declared). This process is repeated on each new plateau until no new statistically significant steps can be added.

For estimating durations of alternating long and short plateau regions, a 2-State hidden Markov model was used. The nanoparticle was modeled as a Gaussian emitter with true positions set at intervals of 8.2 nm and standard deviations set by the measured plateau standard deviation. An emission matrix \( E \) was constructed using these step and substep positions, and the 16.4 nm multiples were allowed to “breathe” up to 2 nm from the rigid register:

\[
E = \begin{bmatrix}
0 + L + l_1 \\
8.2 + L + l_1 \\
16.4 + L + l_2 \\
\vdots \\
n \cdot 16.4 + L + l_n \\
n \cdot 16.4 + 8.2 + L + l_n 
\end{bmatrix}
\]

Here, \( L \) represents a whole trace translation that was applied to align the register with the experimental data. This value was found by rounding each point in the experimental data to the nearest integral multiple of 16.4 and selecting the value of \( L \) that minimized root-mean-square error (RMSE) between the rounded data and the unmodified data. Each of the \( n \)-by-16.4 nm full-step positions were then individually translated up to 2 nm in either direction, \( l_i \), based off of minimizing the RMSE between the true and rounded data. This “breathing” helped account for inherent experimental error in position detection as well as any possible stage drift, and minimized any overestimation of the substep durations. A transition matrix was constructed to only allow transitions to the next 8.2 nm multiple or to remain in the same position. The emission matrix, transition matrix, and experimental data were used as input to the Viterbi algorithm \( (30) \), which returned the mostly likely sequence of hidden states. Points of state transition were used to call
boundaries of long and short phases. Not all traces used in the tDetector traces were amenable to fitting (about 30% rejected), either due to backsteps, areas of high error, or anything else that led to a loss in register of the major 16.4 nm spacing.

4.5.2 Kinetic model and simulations for 1 uM ATP data

The empirical cumulative density function of $\tau_{\text{short}}$ at 1 µM was fit using the following kinetic scheme, with $k_{\text{on},2HB}^{ATP}$, the first order rate constant for ATP binding in the 2HB state at 1 µM ATP, and $k_{RHD}^\phi$, the nucleotide-independent rear-head detachment rate as free parameters:

$$F(t) = 1 - A_1 e^{-k_1 t} - A_2 e^{-k_2 t}$$

$$A_1 = \frac{k_{\text{on},2HB}^{ATP}}{k_{\text{on},2HB}^{T} + k_{RHD}^\phi}$$

$$A_2 = \frac{k_{RHD}^\phi}{k_{\text{on},2HB}^{T} + k_{RHD}^\phi}$$

$$k_1 = k_{\text{exit},1HB}$$

$$k_2 = \frac{k_{\text{on},1HB}^{ATP} \cdot k_{\text{exit},1HB}}{k_{\text{on},1HB}^{ATP} + k_{\text{exit},1HB}}$$

Here, $k_{\text{exit},1HB} = 125 \text{ s}^{-1}$ from the mean of $\tau_{\text{short}}$ at saturating ATP (assumes $k_{RHD}^{ATP}$ is infinitely fast), $k_{\text{on},1HB}^{ATP} = \frac{k_{\text{cat}[ATP]}}{k_{m,1HB}^{ATP}}$, and $k_{\text{on},2HB}^{ATP} = \frac{k_{\text{cat}[ATP]}}{k_{m,2HB}^{ATP}}$ (here $k_{\text{cat}}$ is the maximal stepping rate of 64.6 s$^{-1}$ from single-molecule results). This model assumes that $k_{\text{on},1HB}^{ATP}$ dominates $k_2$, such that the two-step process collapses to a single exponential. Initially, $k_{m,2HB}^{ATP}$ and $k_{m,1HB}^{ATP}$ were set to be equal and the values for the free parameters that minimized RMSE with the empirical curve were chosen. Artificial steps were then simulated by drawing exponential random variables for each mechanochemical transition with means set at the experimentally measured or fit value. Summation
of the transitions into 1HB or 2HB was set as a competition between drawn values of $k_{RHD}^\phi$ and $k_{on,2HB}^{ATP}$:

$$
\tau_{2HB} = \begin{cases} 
\tau_{off}^{ADP} + \tau_{exit,2HB} + \tau_{on,2HB}^{ATP}, & \tau_{on,2HB}^{ATP} < \tau_{RHD}^\phi \\
\tau_{off}^{ADP} + \tau_{exit,2HB} + \tau_{RHD}^\phi, & \tau_{on,2HB}^{ATP} \geq \tau_{RHD}^\phi 
\end{cases}
$$

$$
\tau_{1HB} = \begin{cases} 
\tau_{exit,1HB}, & \tau_{on,2HB}^{ATP} < \tau_{RHD}^\phi \\
\tau_{on,1HB}^{ATP} + \tau_{exit,1HB}, & \tau_{on,2HB}^{ATP} \geq \tau_{RHD}^\phi 
\end{cases}
$$

$\tau_{off}^{ADP}$ was set to 1 ms and $\tau_{exit,2HB}$ was set to 6.8 ms based on single-molecule measurements of $\tau_{2HB}$ compared to the half-site release rate in ATP. 10,000 total steps were generated. Finally, $\tau_{short}$ was set to be every even-indexed value of $\tau_{1HB}$, and $\tau_{long}$ was set to be the sum of two sequential draws of $\tau_{2HB}$ plus the draw of $\tau_{1HB}$ in between them.

For the second round of fitting, the 1HB and 2HB ATP waiting times were not forced to be equal, which resulted in three free parameters: $k_{on,1HB}^{ATP}$, $k_{on,2HB}^{ATP}$, and $k_{RHD}^\phi$. An arbitrary value was assigned to $k_{on,2HB}^{ATP}$, the entire process of fitting to the analytical curve and simulation was repeated, and the RMSE between the simulated $\tau_{long}$ and the empirical data for $\tau_{long}$ was calculated. This was repeated for many values of $k_{on,2HB}^{ATP}$, and the parameter set that minimized RMSE for the $\tau_{long}$ data was chosen. All simulation and fitting work was performed in MATLAB.

4.5.3 Substep simulations and step finding

Substep simulations were performed to verify model-free step-finding results and to test the spatial and temporal resolution necessary to resolve substeps of designed duration. Values of $\tau_{short}$ were generated as exponential random numbers with a mean of 8 ms. Values of $\tau_{long}$ were generated as a sum of three exponential random values each with a mean of 8 ms for the 1 mM ATP case, and with one 8 ms and two with 8 ms plus the calculated ATP waiting time for the 10 $\mu$m ATP case. Distance values starting at 0 nm were generated at a specified frame rate, and a
Gaussian random number with mean 0 and a specified standard deviation was added at each time point. When the summed time since the last event exceeded the current random value of $\tau_{long}$, 8.2 nm was added to the distance vector. The clock then reset and distance values were generated with white noise until the sum time exceeded the current random value for $\tau_{short}$, at which point 8.2 nm was added to the distance vector. This process was repeated for 1,000,000 points in the distance vector. Step-finding with the tDetector algorithm was then performed on the distance vector, and the mean step size was reported.

### 4.5.4 Supplemental figures

![Figure 4-6 Velocity and run length controls for N-terminal biotinylation via AviTag.](image)

(A) To-scale schematic of k560-AviN motor with 30 nm gold nanoparticle attached through biotin-streptavidin. (B) Velocity measurements for k560 show negligible change for various probes and assay styles. k560 average velocity weighted by run length was 517.1±7.6 nm/s (SEM, N=299) when labeled at the N-terminus with a Qdot, 529.8±28.1 nm/s (N=27) when labeled at the N-terminus with a 30 nm gold nanoparticle, 554.2±9.7 nm/s (N=225) when labeled at the C-terminus with a Qdot, and 532.9±3.4 nm/s (N=279) when unlabeled and measured in a gliding assay. (C) Normalized histograms of run lengths measured for N- and C-terminus Qdot-labeled motors. Distributions are seen to almost perfectly overlap, save a lengthened tail for the C-terminus labeled motor, showing that N-terminal labeling does not affect processivity. Both k560-AviN (1.09±0.94 µm, mean±SD, N=299) and k560-AviC (1.34±1.47 µm, mean±SD, N=225) are well-estimated by exponential distributions.
Figure 4-7 Model-free fitting to 1,000 frames-per-second data at various [ATP]. All step finding carried out using the tDetector algorithm. Histograms of measured steps for (A) 1 mM ATP (948 steps, 31 runs) (C) 100 µM ATP (364 steps, 7 runs) and (E) 10 µM ATP (1,634 steps, 33 runs). Modes here are seen to be smaller than 16.4 nm. Sums of sequential step sizes (B), (D), and (F) for 1 mM, 100 µM, and 10 µM, respectively, show clear modes at 16.4 nm. Inset in (F) shows an example raw trace at 10 µM ATP with tDetector fit (horizontal lines at 16.4 nm spacing). The tDetector algorithm is expected to have a 5% false-positive rate, which likely explains the apparent backsteps, and the longer plateaus at low [ATP] are expected to generate more false-positives. The peak at 0 nm for the sum of sequential steps is consistent with resolution of false backsteps back to the plateau. (G) Cumulative density functions for localization precision, obtained as the error on the Gaussian fit to the point spread function at each frame and averaged over entire processive runs. A precision of 1.9±0.4 nm (SEM) was obtained for 1 mM, 1.8±0.2 nm for 100 µM, 1.8±0.1 nm for 10 µM, 1.8±0.1 nm (N=55 runs) for 1 µM, and 1.8±0.2 nm (N=32 runs) for 1 mM ATPγS. (H) Cumulative density functions for plateau standard deviation, calculated from pairwise differences in position along the microtubule axis for a processive run and corrected for outliers (>3 standard deviation units). A plateau standard deviation of 3.8±0.3 nm was obtained for 1 mM, 4.8±0.7 nm for 100 µM, 3.9±0.3 nm for 10 µM, 4.0±0.2 nm for 1 µM, and 4.0±0.3 nm for 1 mM ATPγS.
Figure 4-8 Model-free fitting of k560-AviN at saturating ATP and lower frames rates. Example traces in (A) and population histogram in (B) show data for saturating ATP and 200 frames per second. The mean of positive step sizes is 16.4±0.2 nm, showing that no substeps are detected. Here 636 steps are collected from 36 processive runs. A run-wise localization precision of 3.9±0.3 nm and a plateau standard deviation of 5.3±0.3 nm were achieved. Example traces in (C) and population histogram (D) are at saturating ATP and 400 frames per second. Here 1,155 steps were collected from 30 processive runs. A run-wise localization precision of 3.1±0.3 nm and a plateau standard deviation of 4.6±0.3 nm were achieved. Because the population mode is much less than 16.4 nm, a Gaussian mixture model was fit to the positive steps (E). 56% of steps were centered on a mode at 9.4 nm, and 40% of steps centered around 16.3 nm. Substeps are thus only measured in approximately 35% of instances here, versus approximate 67% at 1,000 frames per second (Figure 4-1E).
**Figure 4-9 Substep simulations at varying spatiotemporal resolution.** Artificial 8.2 nm steps were generated at 1 mM ATP (A) and 10 µM ATP (B) by drawing exponential random numbers using experimental values for 1HB and 2HB dwell times. The artificial steps were corrupted with Gaussian noise to produce a desired plateau standard deviation, and then were fit using the tDetector algorithm. Mean positive step sizes from experiments are plotted as a function of their plateau standard deviation at different sampling rates (single points with SEM error bars). As can be seen, at lower spatiotemporal resolution the substeps are missed and a 16 nm mean step size is detected. In contrast, at high spatiotemporal resolution most of the substeps are detected and the mean step size approaches the correct 8.2 nm. The orange data point in (B) shows experimental values reported by Toprak et al. (11) at 10 µM ATP and 500 frames per second, and comparison to the blue and orange lines explain why substeps may have been missed in that study. It is also noteworthy that the step finding algorithm used in that study requires the approximate number of steps in the given trace as an input parameter, biasing it against the detection of a mixture of steps and substeps.

**Figure 4-10 Additional HMM fitting data.** 3 example traces at 1 mM ATP with $\tau_{\text{long}}$ (black) and $\tau_{\text{short}}$ (red) indexed by color in XY space (A) and time versus position along the microtubule axis (B). The state space constructed for the HMM requires entry into a $\tau_{\text{short}}$ before entering the next $\tau_{\text{long}}$, so in instances where
substeps are missed, a single point from an adjacent plateau is taken as the $\tau_{\text{short}}$ that best fits the data. (C) Distribution of displacements perpendicular to the microtubule for each $\tau_{\text{short}}$ plateau relative to the mean position in the preceding $\tau_{\text{long}}$ plateau for every detected step at 1 mM ATP (blue) and 1 µM ATP (green).

Most substeps had no associated perpendicular displacement, mean ± SD are 1.0 ± 6.2 nm, N=239 for 1 mM and 0.6 ± 4.4 nm, N=217 for 1 µM. A rightward (positive) bias might be expected given that the NL docks on the right side of the motor domain when viewed from above, but that result is not prominent. The perpendicular displacement should vary depending on which protofilament the motor is walking on, which cannot be controlled experimentally, and thus a lateral bias of the tethered head is not rigorously ruled out by the data. (D) The cumulative density functions for the entire population of $\tau_{\text{short}}$ (pink for 100 µM, red for 10 µM) and $\tau_{\text{long}}$ (gray for 100 µM, black for 10 µM) phases, showing relatively constant $\tau_{\text{short}}$ and $\tau_{\text{long}}$ that vary with ATP.

Figure 4-11 Comparison of apparent flexibility of bound versus tethered heads at 1 mM ATP. For each $\tau_{\text{long}}$ or $\tau_{\text{short}}$ plateau called by the HMM algorithm, the standard deviation parallel and perpendicular to the microtubule axis was calculated. Increased diffusional excursions of the tethered head are expected to increase the positional standard deviation for the short plateaus. (A) Distributions of standard deviations in the dimension parallel to the microtubule for $\tau_{\text{long}}$ (black; 4.0±0.1 nm, mean±SEM, N=223) and $\tau_{\text{short}}$ (red; 4.8±0.2 nm mean±SEM, N=239) plateaus. (B) Distribution of standard deviations in the dimension perpendicular to the microtubule for $\tau_{\text{long}}$ (black; 5.5±0.2 nm, N=223) and $\tau_{\text{short}}$ (red; 4.8±0.2 nm mean±SEM, N=239) plateaus. Thus, an increase is observed for the on-axis displacements but not for the off-axis displacements, and in both cases the differences are small.
Figure 4-12 Rear-head detachment at very low ATP concentration. (A) Example traces at 1 µM ATP and 1,000 frames per second, including instances of both short and long substeps. X-axis tick marks represent 15 nm. All data shown in gray, and 5 ms median boxcar filtered data shown in black (τ_{long}) and red (τ_{short}). Downsampled data were used for HMM analysis. In some cases, multiple long substeps in series were seen (middle trace) and in other cases a series of short substeps was observed (second half of right trace), but there was no consistent behavior and questions of hysteresis were not pursued further. (B) At 1 µM ATP, the cumulative density function of N=217 measurements of τ_{short} (red circles) followed a biexponential, while N=185 measurements of τ_{long} (black circles) followed a gamma distribution with a non-integer shape parameter. A simple kinetic model, shown in (C), was constructed to describe this data. An analytical expression of the model was fit to the τ_{short} data, and full mechanochemical cycle simulations were performed utilizing the fit parameters (see SI Methods for full model and simulation details). Overlaid in (B) are CDFs of simulated steps using best fit parameters to a kinetic model using only a single K_m (pink and gray dashed lines for τ_{short} and τ_{long}, respectively) and separate K_m values for the 1HB and 2HB ATP waiting (dark red and gray solid lines for τ_{short} and τ_{long}, respectively). The parameter set that best describes the data is K^{ATP}_m,1HB=23 µM, K^{ATP}_m,2HB=39 µM, and k_{RHD}=1.9 s^{-1}. 
Figure 4-13 Tracking data in saturating ATPγS at 1,000 frames per second. Here Y corresponds to positions along the microtubule axis, and X corresponds to position perpendicular to the microtubule axis. Traces Ti and Tii are 1 µM ATP traces, shown for reference. Trace γi-iv correspond to the XY traces in Figure 4-4A. The steps in ATPγS starkly contrast steps in 1 µM, where relatively sudden 16.4 nm steps were observed. In ATPγS there are gradual movements in Y, as well as apparent steps smaller than 16.4 nm. The X position shows switching between discrete positions that do not always synchronize with movements in the Y position, which is only possible if the labeled head is free. Note difference in X-scaling between examples.
4.6 References


Chapter 5

Kinesin processivity is determined by a kinetic race from a vulnerable one-head-bound state

5.1 Introduction

Kinesin motor proteins drive many active processes the cell, including vesicle transport (1–3), DNA and organelle repositioning (4, 5), intraflagellar transport (6, 7), microtubule dynamics control (8–10), and mitotic spindle organization (11–13). There are 45 kinesin genes in the human genome, each of which encodes an isoform that is optimized to drive some processes, but is incapable of driving others (14, 15). Understanding the nature of this functional specialization is critical to elucidating the molecular bases of Charcot-Marie-Tooth disease (16), hereditary spastic paraplegia (17, 18), Alzheimer’s disease (19, 20), and the various cancers (21, 22) associated with either kinesin dysfunction or over-activity. Part of kinesin’s functional diversity can be understood by major structural differences between isoforms. For example, kinesin-5 acts as a tetramer instead of the typical dimer (23), and kinesin-14 has its motor domain at its C-terminus rather than its N-terminus (24). However, most kinesins have a similar structure, and their functional specialization thus comes from the tuning of motility parameters such as velocity, processivity, and force-sensitivity. Here we focus on processivity, or the number of steps kinesin takes per interaction with a microtubule. Processivity values for different kinesins vary over multiple orders of magnitude from just a few steps (25) to a thousand steps or higher (26, 27), in part enabling them to perform their different tasks in the cell. Despite its fundamental importance, a consensus quantitative model that explains how processivity is tuned between kinesin isoforms is absent from the literature. This is in part due to the inability to measure kinetic intermediates in the ATPase cycle from which processivity can be controlled. Here we apply new high-resolution tracking technology (25, 28–31) to fill this gap in the literature.
The simplest model for kinesin processivity is the *kinetic race model*, which posits that kinesin must proceed through a single vulnerable state each turnover of its ATPase cycle (32). In our previous work, we identified a one-head-bound post-hydrolysis state as a candidate for the vulnerable state (28, 33). This result suggested that processivity could be controlled by a kinetic race between tethered head attachment and bound head detachment from the one-head-bound intermediate. In the present study, we examine these rate constants with high-resolution single-molecule microscopy as we implement four methods to alter processivity: changing the motor domain from kinesin-1 to -2, altering the neck-linker length, changing the solvent ionic strength, and using slowly-dissociating ATP analogs. We find that any method for increasing processivity leads to either the tethered head finding the next microtubule binding site more quickly or the bound head dissociating from the current microtubule binding site more slowly. Thus, we find that the kinetic race model is sufficient for quantitatively explaining processivity under physiological conditions, and that the intracellular capabilities of a given motor in part stem from the tuning of the two rate constants in the race.

### 5.2 Results

#### 5.2.1 High-resolution single-molecule microscopy enables direct measurement of a one-head-bound intermediate in the stepping cycle of kinesin-1 and -2

To test the hypothesis that kinesin processivity is set by a kinetic race between attachment of the tethered head and detachment of the bound head when the motor is in the one-head-bound (1HB) post-hydrolysis state (Figure 5-1A state 3), we performed high-resolution single-molecule tracking to measure the 1HB duration for multiple kinesins with different processivities. The motors used were wild-type kinesin-1 (K114), kinesin-1 with the neck linker (NL) extended to 17 amino acids (K117), wild-type kinesin-2 (K217), and kinesin-2 with the NL shortened to 14 amino acids (K214). It was previously shown that extending the NL reduces processivity independent of
the motor domain (34–36). For all motors, a 30-nm diameter gold nanoparticle was bound to the motor domain using biotin-streptavidin, a technique that has been shown both experimentally (28, 30) and theoretically (37) to have little influence on stepping. The motors were observed in an *in vitro* single-molecule stepping assay under total internal reflection dark field microscopy at 1,000 frames per second in 2 mM ATP. Example position versus time traces for the four kinesin constructs, obtained by fitting point spread functions of gold nanoparticles moving along microtubules (38, 39), are shown in Figure 5-1B. Kinesin-1 with a single motor domain tagged has previously been shown to take 16.4 nm hand-over-hand steps (40–42), and we recently established that at saturating ATP a one-head-bound intermediate can be measured in which the tethered head is displaced ~8 nm from its previous binding site (28). Here we clearly observe this intermediate state for all kinesin-1 and -2 constructs tested (Figure 5-1B colored points). Because only one head is labeled, this short duration intermediate (“short state”; Figure 5-1C, D) represents a single 1HB state. The longer duration intermediate (“long state”), where the labeled motor domain is bound to the microtubule (Figure 5-1B black points) represents the two-head-bound (2HB) state of the labeled head plus the 1HB and 2HB states of the unlabeled head (Figure 5-1C). To quantify the duration the motors spent in the long and short states, the (X,Y,t) data were fit using a Hidden Markov Model (state space diagram in Figure 5-5). From the stepping traces, distributions of at least 300 determinations of each long and short state were built up for each of the four motors studied (K1d distributions shown in Figure 5-1D; see Figure 5-6 for all distributions). 1HB and 2HB durations were then calculated from long and short distribution sample means (Figure 5-1E). The key result was that for both kinesin-1 and kinesin-2, extending the NL from 14 to 17 amino acids increased the 1HB duration, while having little effect on the 2HB duration.
5.2.2 Altering the neck-linker lengths modulates the processivity of kinesin-1 and -2 by tuning the tethered head attachment rate

We next measured the run lengths (RL) of the 4 motors using GFP constructs (Figure 5-1F; distributions in Figure 5-7). Consistent with previous measurements (34–36), constructs with
the same NL length had similar RLs independent of motor domain. Importantly, a strong negative correlation was observed between the 1HB duration and the run length when comparing the 14 to the 17 amino acid NL construct of each motor type (Figure 5-1F). From the established mechanochemical cycle (Figure 5-1A), the 1HB state includes both a pre- and post-hydrolysis state with detachment occurring from the post-hydrolysis state (28, 33). Therefore, the total 1HB duration (Figure 5-1A states 2 and 3) is:

\[ \tau_{1HB} = \frac{1}{k_{\text{Hydrolysis}}} + \frac{1}{k_{\text{Attach}} + k_{\text{Detach}}} \]

Assuming that the motor rarely returns backward to the 1HB state from the 2HB state (Figure 5-1A state 4→3 transition; discussed in detail below), we can derive the probability of stepping as a simple kinetic race between attachment and detachment:

\[ P(\text{step}) = \frac{k_{\text{Attach}}}{k_{\text{Attach}} + k_{\text{Detach}}} \]

Since the probability of stepping is proportional to the run length, and the run length is greater than 50 steps for all the motors investigated here, it follows that \( k_{\text{Attach}} \gg k_{\text{Detach}} \). Moreover, the existing evidence (44) indicates that \( k_{\text{Hydrolysis}} \) is not significantly affected by NL length. Thus, the difference in the \( \tau_{1HB} \) values for kinesins with the same motor domain but different NL lengths is:

\[ \Delta \tau_{1HB} = \tau_{1HB}^{17} - \tau_{1HB}^{14} = \frac{1}{k_{\text{Attach}}^{17}} - \frac{1}{k_{\text{Attach}}^{14}} \]

Therefore, the differences in 1HB duration in Figure 5-1E,F are best understood as resulting from differences in \( k_{\text{Attach}} \). It follows that for both kinesin-1 and -2, extending the NL from 14 to 17 amino acids reduces processivity by decreasing \( k_{\text{Attach}} \). This result shows that for kinesin-1 and -2, \( k_{\text{Attach}} \) is primarily determined by the NL domain and not by the motor domain.
5.2.3 Pre-steady-state kinetics independently support 1HB duration differences

To measure the effect of NL length on 1HB duration in an independent experimental platform, we performed ATP half-site release experiments for all four motor constructs using stopped-flow. Motors were incubated with microtubules and low concentrations of fluorescent 2'(3')-O-(N-methylanthraniloyl) nucleoside disphosphate (mantNDP) in order to create a 1HB ATP waiting state with the mantNDP in the tethered head (45–47). The motor-microtubule complexes were then flushed against ATP, enabling the motors to proceed through their 1HB states, and a drop in fluorescence intensity was detected when they released their mantNDP into solution (Figure 5-2A). In the scheme of Figure 5-2A, if a high ATP concentration (fast $k_{on}^{ATP}$) and a low-affinity mantNDP (fast $k_{off}^{D^*}$) are used, the rate-limiting step in the half-site release process is $k_{1HB}$. Importantly, extending the NL was recently shown not to affect the mantNDP release rate from the front head in the 2HB state (Figure 5-2A $k_{off}^{D^*}$; see Figures 5E and 7D in reference (43)). Thus, by comparing the half-site release rate of 14 to 17 amino acid NL constructs, differences in 1HB duration ($\Delta \tau_{1HB}$) can be calculated. Results for half-site release for the four motor constructs are shown in Figure 5-2B (example transients in Figure 5-8). In these experiments mantATP was used for kinesin-1, but due to the high mantADP affinity of kinesin-2 (43), mantGDP was used instead (investigated in detail below). Consistent with previous measurements using different motor constructs (48), elongating the NL was seen to decrease the half-site release rate. The $\Delta \tau_{1HB}$ measured by half-site was in excellent agreement with the $\Delta \tau_{1HB}$ measured by single-molecule (Figure 5-2C), providing a second independent line of evidence that extending the NL reduces processivity by reducing the tethered head attachment rate.
5.2.4 Altering ionic strength modulates the processivity of kinesin-1 and -2 by tuning the bound head detachment rate

We next investigated the effect of reducing the ionic strength on the velocity and run length of all four motor constructs. Each motor was measured in BRB80 (as above in Figures 5-1 and 5-2), and in buffers containing 40, 20, and 12 mM PIPES. Consistent with previous kinesin-1 measurements (35), reducing the ionic strength was seen to slightly decrease the velocity of all motors (Figure 5-3A) and to increase the run lengths of all motors to different extents (Figure 5-3B; distributions in Figure 5-7). Under the model that processivity is controlled by a kinetic race between tethered head attachment and bound head detachment in the 1HB state, an increase in processivity due to an increase in $k_{\text{Attach}}$ must lead to a faster, not a slower velocity. Thus, the measured increase in processivity at low ionic strength more likely stems from a decrease in $k_{\text{Detach}}$.

To test this claim, we measured the microtubule dwell time of all four motors at saturating ADP as a function of ionic strength (Figure 5-3C; distributions and example kymographs in Figure 5-9).
Consistent with the prediction that low ionic strength reduces detachment rates, the dwell time increased as the ionic strength was reduced for all four motors. Strikingly, as highlighted when the run lengths (Figure 5-3D) and ADP dwell times (Figure 5-3E) were normalized to their values in BRB80, increases in both parameters were shared by each motor type independent of NL length: K1\textsubscript{14} and K1\textsubscript{17} both had an \textasciitilde{}2.5-fold increase in RL and ADP dwell time, while K2\textsubscript{14} and K2\textsubscript{17} both had an \textasciitilde{}4-6-fold increase in RL and ADP dwell time. Thus, k\textsubscript{Detach} can be adjusted by altering the electrostatic interactions between the motor domain and the microtubule, and tuning k\textsubscript{Detach} is a means of controlling RL in an approximately proportional fashion. The fact that elongating the NL did not affect the ADP dwell time bolsters the claim that changes in RL due to differences in NL length stem from changes in k\textsubscript{Attach}.

**Figure 5-3 Ionic strength alters processivity by tuning the detachment rate from the 1HB vulnerable state.** (A) Velocities in 2 mM ATP, (B) run lengths in 2 mM ATP, and (C) dwell times in 2 mM ADP for GFP motors in BRB12, BRB20, BRB40, and BRB80 buffers. Velocities are shown as mean ± SEM with a 10\% error added for \textasciitilde{}1°C variability in temperature (49). RLs and dwell times were determined by distribution fitting with error bars determined by bootstrapping (50). (D) Run lengths and (E) ADP dwell times normalized to BRB80 values (with propagated error) to emphasize the pairing by motor domain rather than by NL length. All panels color coded by legend in (B), consistent with Figures 5-1 and 5-2.
5.2.5 Slowing the ADP off-rate modulates processivity by shunting the motor backward into the 1HB state

The data in Figures 5-1, 5-2, and 5-3 establish that kinesin-1 and-2 processivity can be understood as a kinetic race between $k_{\text{Attach}}$ and $k_{\text{Detach}}$ when the motor is in the vulnerable 1HB state (Figure 5-4A state 2). However, completion of a forward step also involves ADP release to “lock in” the gains made by the attachment step. Hence, there is a second kinetic race following tethered head attachment – either ADP is released to complete the step, or the attachment step is reversed and the motor returns to the vulnerable 1HB state (Figure 5-4A state 3→4 versus 3→2 transition). Depending on the relative kinetic parameters, this process can be described in two ways. In a *first passage model*, $k_{\text{Attach}}$ is relatively slow and ADP release follows rapidly such that the motor rarely reverts to the 1HB state. In this sense tethered head attachment is the irreversible ratcheting step. Alternatively, in a *rapid equilibrium model*, both $k_{\text{Attach}}$ and the reverse rate $k_{\text{Detach}}^{FH}$ are very fast, and the equilibrium favors the 1HB state sufficiently that it is detected in single-molecule tracking (28). Here, the rare outpacing of $k_{\text{Detach}}^{FH}$ by $k_{\text{Off}}$ locks the motor into the 2HB state (Figure 5-4A equilibrium between states 2 and 3 until the 3→4 transition occurs), and ADP release is thus the irreversible ratcheting step. To test between these models, we measured the run lengths of our four GFP constructs in mantATP. Although the off-rate for mant- and cold ADP are similar for kinesin-1 (Figures 5-4B and 5-10A) (28), kinesin-2 has a 15-fold lower off-rate for mantADP than cold ADP (Figure 5-4B) (43). Thus, under the first passage model the kinesin-2 RL should drop only slightly in mantATP, whereas under the rapid equilibrium model the kinesin-2 RL should drop ~15-fold in mantATP. We found that in mantATP the RL of K2_{14} and K2_{17} only dropped roughly 2-fold, thus ruling out the rapid equilibrium model (Figure 5-4C, distributions in Figure 5-10B). As expected from the similarity in their ADP and mantADP off-rates, the K1_{14} and K1_{17} RLs were not measurably different in mantATP versus cold ATP.
To ensure quantitative consistency of the first passage model, we modeled the 6-state process shown in Figure 5-4A as a Markov chain. We solved for $k_{\text{Attach}}$ and $k_{\text{Detach}}$ using our cold ATP RL and $\Delta \tau_{1HB}$ values (Figures 5-1A and 5-2C), used literature values for $k_{\text{off}}^{\text{ADP}}$ or $k_{\text{off}}^{\text{mantADP}}$ (28, 43), and used measured values for $k_{\text{Detach}}^{\text{ADP}}$ (Figure 5-3C) (see Table 5-1 for details). Using transition probabilities calculated from the rate constants, we determined the run length by calculating the average number of times the motor entered the ATP waiting state before detaching (Figure 5-4A state 6). Comparing the modeled mantATP versus cold ATP RL (changing only $k_{\text{off}}^{\text{ADP}}$ to $k_{\text{off}}^{\text{mantADP}}$ as in Figure 5-4B) we were able to nearly perfectly replicate the experimental data (Figure 5-4C), validating the first-passage model. Hence in physiological ATP, tethered head attachment is the ratcheting step of the cycle and locking-in steps via ADP release occurs with nearly 100% probability.

**Figure 5-4 ADP release locks-in tethered head attachment.** (A) Mechnochemical model based on Figure 5-1A, showing allowed transitions. When ADP release (state $3\rightarrow 4$) is significantly slowed, front head (FH) or rear head (RH) detachment from state 3 may occur, forcing kinetic flux into vulnerable states (2 and 5). In the first passage model, $k_{\text{Detach}}^{FH}$ is small relative to $k_{\text{off}}^{ADP}$, whereas in the rapid equilibrium model $k_{\text{Detach}}^{FH}$ is very fast relative to $k_{\text{off}}^{ADP}$ (red arrows). (B) Previous determinations of $k_{\text{off}}^{ADP}$ by front head nucleotide exchange experiments for kinesin-1 (28) and kinesin-2 (43). These rates are independent of NL length (43). (C) Run lengths in mantATP relative to run lengths in unlabeled (cold) ATP for each motor. Consistency of experiment and model (black squares) supports first passage model for $k_{\text{Attach}}$. 

<table>
<thead>
<tr>
<th>Motor</th>
<th>$k_{\text{off}}^{\text{ADP}}$, s$^{-1}$</th>
<th>$k_{\text{off}}^{\text{mantADP}}$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>550</td>
<td>367</td>
</tr>
<tr>
<td>K2</td>
<td>375</td>
<td>25</td>
</tr>
</tbody>
</table>
5.3 Discussion

By changing the identity of the motor domain, the length of the neck linker domain, the ionic strength of the solvent, and the nucleotide off-rate, we demonstrate here that processivity can be quantitatively described as a kinetic race between tethered head attachment and bound head detachment. This model should generalize to N-terminal kinesins beyond kinesin-1 and -2, and it provides a quantitative framework for understanding how small structural differences between isoforms can tune rates in the ATPase cycle to yield functional differences in motor behavior.

5.3.1 Elongating the neck-linker tunes processivity by changing the tethered head attachment rate

Elongating the NL has been previously shown to reduce processivity (35, 36, 41, 48), but the underlying mechanism has remained mysterious. Using high-resolution tracking and stopped-flow, we show here that NL mutants alter the vulnerable state duration, and that independent of motor domain there is a strong negative correlation between vulnerable state duration and processivity (Figure 5-1F). Recent gold-nanoparticle tracking results from Isojima et al. showed that human kinesin-1 with cys-lite modifications in 12 mM PIPES buffer containing saturating ATP had an increased 1HB duration when the NL was shortened to 13 amino acids, and slightly decreased duration when the NL was increased to 20 amino acids (six glycine insert) (30). However, kinesins with a 13 amino acid NL have a reduced run length (36) and Cys-lite mutants with various 20 amino acid NLs have a surprisingly increased run length (51). Hence, their results follow the same negative correlation between vulnerable state duration and processivity. Additionally, our 17 amino acid NL constructs did not show the unbinding events, sideways steps, or futile hydrolysis (Figures 5-1 and 5-11) that 20 amino acid NL cys-lite did (30, 41), indicating that not all elongated NL constructs should be lumped together for comparison.
One possible explanation for why elongating the NL increases the 1HB duration is that the longer NL increases the conformational search volume of the tethered head. However, an argument against this model is that *Drosophila* kinesin-1 constructs with NLs from 15-20 residues all have roughly the same RL (27, 35). A second possibility is that elongating the NL alters the interaction of the tethered head with the bound head while in the 1HB state. Cross and colleagues used biochemical and structural data to argue that in the ATP waiting state, the tethered head is nestled next to the bound head, and chemical events in the bound head release the tethered head to step to the next binding site (52). If extending the NL disrupted the ability of the bound head to free the tethered head, this would increase the measured 1HB duration and time spent in the vulnerable state. A third possibility is that a NL of 14 amino acids provides the ideal geometry for the tethered head to find its next binding site, perhaps because the docked NL is rigid, and any lengthening or shortening of this domain causes mis-registration between the tethered head and the next binding site, thereby reducing the attachment rate.

### 5.3.2 Electrostatic interactions stabilize the kinesin-microtubule complex in the vulnerable state

We found that adding or subtracting the three amino acids DAL to the NL affected the tethered head attachment rate but not the bound head detachment rate. Thus, kinesins were paired by NL length when observing attachment (Figure 5-1) and paired by motor domain when observing detachment (Figure 5-3). The bound-head detachment rate was evaluated by proxy using the dwell time in saturating ADP, an approach justified by the close agreement of the measured $k_{Detach}^{ADP}$ values (Figure 5-3C) with the $k_{Detach}$ values calculated from measured RLs and $\Delta \tau_{1HB}$ (Table 5-1). The effect most likely comes from electrostatic interactions between positively-charged residues in kinesin and negatively charged residues in the microtubule, particularly in the tubulin C-terminal tail (50). Consistent with this, cleaving off the C-terminal tail with subtilisin has been shown to
decrease kinesin processivity (50, 53, 54). It is notable that $k_{Detch}$ can also be affected by adding positive residues outside the motor domain. Adding lysines to the neck-coil has been shown to increase processivity (50). Adding lysines when elongating the kinesin-1 NL has been shown to decrease the run length in BRB80 but not in BRB12 (35, 41), a result best-understood by offsetting changes in $k_{Attach}$ (longer NL) and $k_{Detach}$ (ionic strength dependence). We verified this by showing that K117 with a lysine (KAL inserted) had a stronger ionic strength dependence of run length and dwell time in ADP than K117 without a lysine (DAL inserted; Figure 5-12).

### 5.3.3 Slowing ADP release reduces processivity by reversing tethered head attachment

When mantATP is used to power kinesin-2 stepping, the slow mantADP release rate increases the probability that the newly-bound front head fails to complete its step by releasing its ADP, and instead reverts to the 1HB vulnerable state. This effect only decreases run length about 2-fold (Figure 5-4C), which is surprisingly mild and suggests a first-passage model: attachment of the tethered head is the irreversible ratcheting step, and rapid ADP release subsequently locks-in the 1HB to 2HB transition. Our results and model predictions show that kinesin-1 and -2 both rectify steps with nearly 100% efficiency in cold ATP, meaning that the vulnerable state is only entered once per cycle the majority of the time. In Figure 5-4, the efficiency of capturing the forward step was compromised chemically by using a high-affinity nucleotide analog. It can also be compromised mechanically by increasing intramolecular tension: Isojima et al. showed that kinesin-1 with a 13 residue NL occasionally returned to the 1HB state after tapping down on the microtubule-binding site, indicating an increased $k_{Detach}^{FH}$ (30). Multiple isoforms of kinesin have been shown to undergo one-dimensional diffusion when forced into the ADP state (26, 55–58), and some low-processivity kinesins such as MCAK utilize diffusion in the ADP or ADP-Pi state in the cell to perform their role in mitosis (58). It follows that these kinesins have a greatly slowed ADP
off-rate (59), such that the efficiency of locking-in steps is reduced and kinetic flux is forced into the 1HB ADP-Pi and ADP states.

5.3.4 Assisting loads decrease run length by enhancing detachment

This work also provides insights into how external loads alter kinesin processivity. Optical trapping experiments on wild-type kinesin-1 have shown that hindering loads reduce motor velocity and run length, while assisting loads have no effect on velocity but strongly reduce run length (27, 33, 60). Our results show that either $k_{\text{Attach}}$ or $k_{\text{Detach}}$ can affect run length, but of these two only $k_{\text{Attach}}$ affects velocity. Thus, the decrease in run length under hindering load may result from changes to $k_{\text{Attach}}$ and $k_{\text{Detach}}$, but the decrease in run length under assisting load must result solely from changes to $k_{\text{Detach}}$. This makes intuitive sense, as it not clear how an assisting load would decrease $k_{\text{Attach}}$, and assisting loads have been shown to rescue the irregular stepping of mutants (41). It also means that assisting loads must speed $k_{\text{Detach}}$ to a greater degree than do hindering loads. External loads may also increase the front head or rear head detachment rates from the 2HB state (Figure 5-4A state 3→2 and 3→5 transitions, respectively), and thus increase the flux into vulnerable states in a fashion similar to mantATP (Figure 5-4).

Here we show that processivity, an important biophysical determinant of intracellular function, is set by a kinetic race between attachment of the tethered head and detachment of the bound head when kinesin is in a vulnerable one-head-bound state. This simple and general model provides a link between kinesin structure and function that should apply across the kinesin superfamily in both healthy and disease states.

5.4 Materials and methods
5.4.1 Constructs and protein preparation

All kinesin proteins used were expressed in BL21(DE3) bacteria (New England Biosciences) with a C-terminal 6x His tag and purified by affinity chromatography followed by buffer exchange as reported previously (28, 43). The kinesin-1 construct used was *Drosophila* KHC truncated at amino acid 559, and the elongated neck linker K17 construct was made by adding the three amino acids DAL at position 345 just preceding α7, consistent with previous works (34–36). The kinesin-2 construct used was human KIF3A motor and neck linker homodimerized using the kinesin-1 coiled-coil (345-559) (35), and the K214 construct was made by deleting the DAL sequence at the end of the NL and replacing the kinked proline at position 355 with an alanine (35). The GFP constructs included a C-terminal eGFP directly preceding the His tag (35, 36, 61). The N-terminal avi-tag constructs were described previously (28). For the kinesin-1 and -2 constructs the linkers GG and GGAGG respectively were added directly downstream of the avi-tag. Biotin was added after induction, typically 0.5 to 2 hours before cell lysis in order to empirically control the degree of biotinylation of the homodimers. Biotinylation was quantified by comparing the biotin concentration as measured by the HABA assay (Sigma) to total protein concentration as measured by absorbance at 280 nm, and all motors prepared had <0.2 moles of bound biotin per mole of dimer motor. Kinesins used for biochemical assays were truncated a position 406 rather than 560 to enable higher yields (28, 43). Insertions and deletions were made either using Q5 or Gibson Assembly (New England Biosciences).

5.4.2 Single-molecule experiments

Gold nanoparticles were imaged using a custom-built total internal reflection dark field microscope, employing a Coherent Sapphire-LP 532 nm laser (5-10 mW at sample). Images were recorded using a Basler Ace acA640-750um CMOS camera (1,000 frames per second, 945 μs exposure) accessed by custom LabVIEW software. Microtubules were adhered to cleaned cover
slips as previously (28). All assays, unless otherwise noted, were carried out in imaging solution: 0.5 mg/mL casein, 10 µM taxol, 20 mM glucose, 20 µg/mL glucose oxidase, 8 µg/mL catalase, 0.2 mg/mL BSA, 1:200 β-mercaptoethanol, and 2 mM MgATP in BRB80 (80 mM K-PIPES, 1 mM EGTA, 1 mM MgCl$_2$, pH 6.8). Biotinylated motors were incubated with a stoichiometric excess of streptavidin-coated 30 nm gold nanoparticles (BBI Solutions) on ice for 30 min, then spun down at 20,000 g for 4 min and resuspended at working levels (100-300 pM gold) in imaging solution. Point spread functions were fit using FIESTA (39) to obtain (X,Y,t) data.

GFP was imaged by total internal reflection fluorescence microscopy using a Nikon TE2000 inverted microscope as previously (28, 35, 36). Frame rates were set to 3 and 20 frames per second for ATP and ADP experiments, respectively. GFP motors were used at 10-300 pM. ATP movies were analyzed using FIESTA (39), and velocity and run length were determined from the returned distance over time trace. Population run lengths were determined by fitting the cumulative density function (after removing runs <5 pixels, 71 nm/pixel) with an exponential with an X-offset. Mant run lengths were measured in imaging solution with 0.1 mM mantATP. ADP durations were measured manually from kymographs drawn at positions coincident with Cy5 labelled microtubules in ImageJ (http://imagej.nih.gov/ij/) (43). All experiments were performed at 22-23°C.

5.4.3 Stopped-flow spectrofluorimetry and steady-state biochemistry

ATP half-site experiments were performed on an Applied Photophysics SX20 spectrofluorometer (43). Data acquisition and fitting were performed in Pro-Data SX software (Applied Photophysics). One syringe was filled with 2-6 µM microtubules, 10 µM taxol, 500 nM mantADP, and 200-600 nM motor in BRB80 buffer (1:10 molar ratio motor dimer to microtubule). For kinesin-2, 500 nM mantGDP was used instead of mantADP due to the higher affinity of the motor for mantADP (43). The second syringe was filled with 4 mM ATP in BRB80. Excitation
was set to 356 nm for the mantADP nucleotide and 450 nm emission was collected using a HQ480SP emission filter (Chroma). An integration time of 1 ms was set for the photomultiplier tube. Each experiment contained 1,200 uL split into 60 uL shots, and the ensemble average fluorescence transient generated upon mixing was fit with a double exponential in the range of 1-500 ms. The faster rate constant was reported. The experiment was repeated at least 9 times for each motor, and data sets were taken on at least two separate days for each motor. Solution exchange of cold versus mantADP to determine relative affinity was done as previously (43). ATPase experiments were carried out as previously described (43) with 5-10 nM motor. ATP turnover rates were determined from the Michaelis-Menton fit to the microtubule-dependence of the observed reaction rate, corrected for initial kinesin concentration as determined by mant-ADP exchange (25). All experiments were performed at 22-23°C.

5.5 Supplementary information

5.5.1 Hidden Markov Model for fitting short and long states in high-resolution (X,Y,t) data

Nanometric (X,Y) position versus time data returned from point spread function fitting to gold nanoparticle movies were fit with a Hidden Markov Model (HMM) in order to determine points of state transition between the long state on the microtubule binding sites (16.4 nm intervals) and the short state in between the microtubule binding sites. First, the X,Y data were rotated to minimize standard deviation in the X direction, thus aligning Y with the microtubule axis. Next, the experimental noise in Y, σ, was determined using the pair wise differences method described previously (28, 62). Then, the tDetector model-free step-finding algorithm (62) was applied to the Y data, and the Y data as a whole were translated to best align the output staircase with an ideal staircase with 8.2 nm spacing. Next, a transition probability matrix was structured using the state space drawn in Figure 5-5:
\[
A = \begin{bmatrix}
1 - g & g/3 & g/3 & g/3 & 0 & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
0 & 1 - g & 0 & g/2 & g/2 & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
0 & 0 & 1 - g & g/2 & g/2 & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
0 & 0 & 0 & 1 - g & g & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
0 & 0 & 0 & 0 & 1 - g & g/3 & g/3 & g/3 & 0 & \cdots & 0 \\
0 & 0 & 0 & 0 & 0 & 1 - g & 0 & g/2 & g/2 & 0 & \cdots & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 1 - g & g/2 & g/2 & 0 & \cdots & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 - g & g & 0 & \cdots & 0 \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \ddots & \ddots
\end{bmatrix}
\]

Where \( g \) is defined as the number of potential 8.2 nm steps in the trace divided by the number of data points in the trace. Row and column indices matched the state numbering in Figure 5-5.

Next, an emission matrix containing parameters for a 2D uncorrelated symmetric Gaussian distribution was structured:

\[
B = \begin{bmatrix}
0 & 0 & \sigma \\
0 & -3\sigma & \sigma \\
0 & 3\sigma & \sigma \\
8.2 & 0 & \sigma \\
16.4 & 0 & \sigma \\
16.4 & -3\sigma & \sigma \\
16.4 & 3\sigma & \sigma \\
24.6 & 0 & \sigma \\
\vdots & \vdots & \vdots
\end{bmatrix}
\]

Where the first column represents mean Y position values of Gaussian emitters placed according to the diagram in Figure 5-5, the second column represents mean X position values, and the third column represents standard deviation values. Row indices match the numbering in Figure 5-5. 3\( \sigma \) was used as the minimum detectable positional change in X. The transition matrix \( A \), emission matrix \( B \), and experimental data were then used as input to the Viterbi algorithm (63), which returned the mostly likely sequence of hidden states. Indices of state transition were then used to call boundaries between successive long and short states. Traces had to be split into \( \sim 100-200 \) nm subtraces to be amenable to the HMM fitting algorithm, as a perfect 8.2 nm register in the experimental data was gradually lost over time due to system drift. The model was implemented in MATLAB software (MathWorks).
5.5.2 Calculation of rate constants for predicting run lengths under first passage model

In order to calculate all the necessary rate constants for modeling run lengths according to the model set up in Figure 5-4A, the following system of equations was solved for each kinesin-1 and kinesin-2 using the cold ATP 1HB duration and GFP run length data:

\[
\begin{align*}
\tau^{14}_{1HB} &= \tau_{Hydrolysis} + \tau^{14}_{Attach} \\
\tau^{17}_{1HB} &= \tau_{Hydrolysis} + \tau^{17}_{Attach} \\
RL^{14} &= (8.2) \frac{\tau_{Detach} + \tau^{14}_{Attach}}{\tau^{14}_{Attach}} \\
RL^{17} &= (8.2) \frac{\tau_{Detach} + \tau^{17}_{Attach}}{\tau^{17}_{Attach}} \\
\tau_{Detach} &= \frac{\tau_{FH}}{\tau_{Detach}} = \frac{\tau_{RH}}{\tau_{Detach}}
\end{align*}
\]

State times \( \tau \) are the inverse of the state exit rate \( k \), and 8.2 is the distance per transition in nm.

These equations assume that extending the neck linker does not affect \( k_{Hydrolysis} \) or \( k_{Detach} \), the latter of which was shown experimentally in Figure 5-3C. Solutions to these equations are shown in Table 5-1. The following transition probability matrix was then set up:

\[
P = \begin{bmatrix}
1 & 0 & 0 & 0 & 0 & 0 \\
0 & P_{21} & 0 & P_{23} & 0 & 0 \\
0 & 0 & P_{32} & 0 & P_{34} & 0 \\
0 & 0 & 0 & P_{35} & 0 & 0 \\
0 & 0 & 0 & 0 & P_{56} & 0 \\
0 & 1 & 0 & 0 & 0 & 0
\end{bmatrix}
\]

Where row and column indices correspond to the state numbers in Figure 5-4A. Transition probabilities were calculated from the rate constants, for example \( P_{34} = \frac{k_{Off}^{ADP}}{(k_{Detach}^{RH} + k_{Off}^{ADP} + k_{Detach}^{FH})} \). The absorbing state (row and column one) was then removed to create a matrix of transient states \( P^T \), and the expected number of time periods the motor spends in each transient state per run was calculated as \( S = (I - P^T)^{-1} \), where \( I \) is the identity matrix. \( S_{15} \), the expected number of time periods the motor spent in the ATP waiting state given that it started in the 1HB post-hydrolysis state, was taken as the processivity, and converted to a run length by multiplying by 8.2 nm. Simulations and all data analysis was done in MATLAB (MathWorks).
5.5.3 Supplemental figures

Figure 5-5 State space diagram for 2D Hidden Markov Model used to fit long and short states in high-resolution tracking data. The nanoparticle-labeled motor domain takes full 16.4 nm steps, with ~8.2 nm substeps that may or may not have a positional component perpendicular to the microtubule. Long states (where the labelled head is bound to the microtubule) are shown as black circles, and short states (labeled head is off the microtubule) comprise the three red circles in between each long state. Arrows denote potential transitions. σ denotes the standard deviation of the particular trace in plateau regions between stepping events; ±3σ is set as the minimum detectable lateral displacement.

Figure 5-6 Empirical cumulative density functions of long and short states for all motors. (A) Distributions of N=336 long states (black) and N=300 short states (blue) for K114. Data come from 44 molecules. (B) Distributions of N=336 long states (black) and N=300 short states (red) for K117. Data come from 37 molecules. (C) Distributions of N=370 long states (black) and N=326 short states (cyan) for K214. Data come from 47 molecules. (D) Distributions of N=349 long states (black) and 310 short states (magenta) for K217. Data comes from 73 molecules.
Figure 5-7 Velocity and run length distributions for four motor constructs at various ionic strengths. Black lines in run length distributions show exponential plus X-offset fits. **Leftmost column** shows $K_{14}$, with fit run lengths in $\mu$m 2.00±0.21 (N=399) 1.85±0.24 (N=322) 1.21±0.16 (N=350), and 0.86±0.12 (N=365); velocity mean values in $\mu$m-s$^{-1}$ 0.57±0.06, 0.63±0.06, 0.67±0.07, and 0.70±0.07 for BRB12, BRB20, BRB40, and BRB80, respectively. **Second column** from left shows $K_{17}$, with fit run lengths in $\mu$m 0.91±0.09 (N=364), 0.72±0.09 (N=350), 0.59±0.12 (N=138), and 0.40±0.04 (N=353); velocity mean values in $\mu$m-s$^{-1}$ 0.42±0.04, 0.51±0.05, 0.49±0.05, and 0.52±0.05. **Third column** from left shows $K_{24}$, with fit run lengths in $\mu$m 3.66±0.40 (N=303), 2.56±0.33 (N=308), 2.07±0.30 (N=360), and 0.91±0.11 (N=416); velocity mean values in $\mu$m-s$^{-1}$ 0.29±0.03, 0.33±0.03, 0.38±0.04, and 0.49±0.05. **Rightmost column** shows $K_{27}$, with fit run lengths in $\mu$m 2.21±0.32 (N=321), 2.10±0.31 (N=334), 1.30±0.23 (N=209), and 0.55±0.07 (N=424); velocity mean values in $\mu$m-s$^{-1}$ 0.28±0.03, 0.31±0.03, 0.31±0.03, and 0.40±0.04. Run length errors were obtained by bootstrapping (50), and velocity errors we obtained from the standard error of the mean (SEM) plus a 10% added error for 1°C temperature fluctuations.
Figure 5-8 Example ATP half-site transients from stopped-flow spectrofluorometry. Traces show the drop in fluorescence signal associated with release of mantNDP from the motor domain into solution. Kinesin-2 was observed to have a larger fluorescence enhancement for mant nucleotides than kinesin-1. Kinesin-1 traces were carried out with mantADP, while Kinesin-2 traces were carried with mantGDP due to the motor’s high mantADP affinity.

Figure 5-9 Raw data for microtubule dwell times for four motors in 2 mM ADP, as a function of ionic strength. **Leftmost column** shows $K_{14}$ with exponential fits in seconds: 1.59±0.20 (N=311), 1.37±0.18 (N=305), 1.05±0.13 (N=303), and 0.51±0.05 (N=326) for BRB12, BRB20, BRB40, and BRB80, respectively. **Second column** from left shows $K_{17}$ with exponential fits in seconds: 1.34±0.17 (N=310), 1.32±0.15 (N=309), 0.91±0.11 (N=312), and 0.51±0.06 (N=309). **Third column** from left shows $K_{24}$ with exponential fits in seconds: 2.30±0.31 (N=389), 1.84±0.20 (N=303), 1.52±0.24 (N=306), and 0.46±0.05 (N=303). **Rightmost column** shows $K_{27}$ with exponential fits in seconds: 2.52±0.30 (N=314), 2.06±0.26 (N=305), 1.60±0.18 (N=319), and 0.43±0.04 (N=311). All errors were determined by bootstrapping(50). Example kymographs (20 frames per second) shown below. All scale bars are 1 second.
Figure 5-10 Kinesin-1 solution ADP/mantADP exchange and Kinesin-1 and -2 motor performance in mantATP. (A) At left, kinesin-1 motors incubated in cold ADP were flushed against excess mantADP. Data were fit to a rising exponential with rate constant of 0.033 s$^{-1}$, corresponding to the (rate-limiting) off-rate for cold ADP. At right, motors incubated in mantADP were flushed against excess cold ADP. Data were fit by a falling exponential with a rate constant of 0.022 s$^{-1}$, corresponding to the off-rate for mantADP. The similarity in these off-rates demonstrates that kinesin-1 has a similar affinity for mantADP and cold ADP. (B) GFP motor run lengths and velocity distributions in mantATP. Leftmost column (blue) shows K1$_{14}$ velocity 0.42±0.04 μm·s$^{-1}$ and run length 0.90±0.11 μm (N=425). Second column (red) shows K1$_{17}$ velocity 0.37±0.04 μm·s$^{-1}$ and run length 0.52±0.05 μm (N=326). Third column (cyan) shows K2$_{14}$ velocity 0.21±0.02 μm·s$^{-1}$ and run length 0.52±0.07 μm (N=325). Rightmost column (magenta) shows K2$_{17}$ velocity 0.25±0.03 μm·s$^{-1}$ and run length 0.36±0.06 μm (N=268). Run length fits (black lines) were to an exponential distribution with X-offset, and errors were obtained by bootstrapping (50). Velocity errors were obtained from the SEM plus a 10% added error due to 1°C temperature fluctuations.
Figure 5-11 Elongating the neck linker does not disrupt chemomechanical coupling. (A) Solution ATPase rates normalized to the active kinesin concentration as determined by mantADP exchange. All data points show mean plus or minus SEM for N=5 measurements. The $k_{\text{cat}}/K_M$ values for $K_{14}$, $K_{17}$, $K_{214}$, and $K_{217}$ in s$^{-1}$ and μM are 67.1±10.6/0.62±0.32, 53.1±5.8/0.98±0.32, 53.2±4.3/0.75±0.19, and 50.2±4.4/0.89±0.24, respectively. (B) Fit $k_{\text{cat}}$ values from solution ATPase normalized to motor stepping rates. Stepping rates were determined by the inverse of the sum 1HB and 2HB durations reported in Figure 5-1E. A value of 1.0 indicates that one ATP turnover event occurs per step. Thus, no motors showed evidence of futile hydrolysis cycles.
Adding lysines to the neck linker changes the ionic strength dependence of run length through changes to $k_{\text{Detach}}$. (A) Raw data for kinesin-1 with an extended NL including a lysine ($K_{17}\text{KAL}$) at various ionic strengths. Leftmost column shows velocity distributions in 2 mM ATP, with mean values in $\mu\text{m}\cdot\text{s}^{-1}$ 0.69±0.07 (N=328), 0.72±0.07 (N=330), 0.70±0.07 (N=392), and 0.70±0.07 for BRB12, BRB20, BRB40, and BRB80, respectively. Center column shows run length distributions in 2 mM ATP, with fit values in $\mu\text{m}$ 2.65±0.28, 1.82±0.20, 1.02±0.12, and 0.64±0.06. Rightmost column shows dwell time distributions in 2 mM ADP, with fit values in $\text{s}$ 2.20±0.24, 1.39±0.15, 1.02±0.10, and 0.56±0.07. (B) Compiled raw data for wildtype kinesin ($K_{14}$) versus kinesin with DAL inserted into the NL ($K_{17}\text{DAL}$) and kinesin with KAL inserted into the NL ($K_{17}\text{KAL}$). At high ionic strength, all three motors have an equal ADP dwell time and thus an equal $k_{\text{Detach}}$, but different run length values and thus different $k_{\text{Attach}}$ values. At low ionic strength, $K_{17}\text{KAL}$ had a much larger increase in run length and ADP dwell time than $K_{17}\text{DAL}$ and $K_{14}$. This is emphasized in the normalized data (C): $K_{17}\text{KAL}$ had a 4-fold increase in both values, whereas $K_{17}\text{DAL}$ and $K_{14}$ had only a 2.5-fold increase. We propose that this difference in scaling is due to the positive charge on the lysine residue. This difference in scaling means that at low ionic strength, $K_{17}\text{KAL}$ has a similar run length to $K_{14}$—its $k_{\text{Attach}}$ value is still lower, but its strongly depressed $k_{\text{Detach}}$ value compensates.
References


Table 5-1 Calculated rate constants. The tabulated values were gathered from experiments (Figures 5-1 and 5-3) and used for the processivity modeling in Figure 5-4.
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Chapter 6

Resolving conflicts in high-resolution kinesin tracking

6.1 Introduction

The data shown in Chapter 4 of this thesis was published in December 2015 (1). Shortly after, in January 2016, another paper was published from the lab of Dr. Michio Tomishige that also focused on the high spatiotemporal resolution tracking of kinesin-1 molecules (2). The Tomishige Lab initially presented some of this data at the Biophysical Society Annual Meeting in 2013, approximately half a year prior to the start of this dissertation. Dr. Tomishige’s contribution to the kinesin field has been very large (3–7), particularly with respect to single-molecule experiments.

The aim of this chapter is to explore the major differences between work published by the Hancock Lab and the Tomishige Lab, and to suggest resolutions. Differences between the experimental constructs and setups are first enumerated, and differences in results are then stated. New, unpublished results from the Hancock Lab that replicate the Tomishige results are next shared. Based on a global view of all the data from both labs, a more refined version of the ATP-waiting state is suggested. This new version of the ATP waiting state, which we term “unbound-undisplaced”, is sufficient for resolving all apparent conflicts between the Hancock and Tomishige papers, and provides a solution while maintaining the integrity of both works.

6.2 Similarities and differences between the Hancock and Tomishige papers

The paper from the Hancock Lab (Chapter 4 of this dissertation), and the paper from the Tomishige lab (2) both investigated the stepping patterns single kinesin-1 dimers by tagging one head of one motor domain with a gold nanoparticle. However, different kinesin mutants, different gold sizes, different buffers, and different microscopes were used. At first glance, there are some apparent conflicts between the results of the two papers.
6.2.1 Major experimental differences

Both the Hancock and Tomishige papers reported on in vitro reconstitution experiments where kinesin molecules with one head labeled with gold were tracked using point spread function fitting while walking on top of immobilized microtubules. However, there are several critical differences between the how the experiments were set up and run.

First, different kinesin-1 mutants were used. The Tomishige paper used a “Cys-Lite” mutant of truncated human Kinesin-1 (KIF5B), in which six separate solvent-exposed cysteine residues were mutated to serine or alanine (C7S/C65A/C168A/C174S/C294A/C330S/C421A). A seventh point mutation was then made to introduce a new cysteine at a desired location for labeling (S55 and E215 were both separately used). Cys-Lite kinesin-1 was instrumental for early kinesin stepping experiments(3, 8), but more recent work has definitively shown that the large number of mutations introduce marked changes to the force-velocity and mechanochemistry of the motor(9).

The Hancock paper used truncated kinesin 1 heavy chain from Drosophila melanogaster with an N-terminal biotinylated Avitag (K560-AviN). K560-AviN was shown to have no difference in velocity or run length relative to wildtype kinesin-1(1). As explored in detail in section 6.4 these different constructs have the gold bound on different faces of the kinesin motor domain.

Second, different microscope techniques were used. The Tomishige paper used TIRDFM, while the Hancock paper used iSCAT. However, as highlighted in Chapter 5 of this dissertation, later K560-AviN measurements made by the Hancock Lab in TIRDFM replicated the iSCAT results(10). Hence, the different microscope designs are not the root of any disparities in the results.

Third, different buffers were used. The Tomishige paper used low ionic strength BRB12, while the Hancock paper used physiological-like BRB80. As explored in detail in Chapter 5 of this dissertation, ionic strength has a large effect on kinesin mechanochemistry by strengthening motor-microtubule interactions.
Fourth, different size nanoparticles were used. The Tomishige paper used streptavidin coated 40-nm diameter gold nanoparticles, while the Hancock paper used streptavidin coated 30-nm diameter gold nanoparticles. Both of these nanoparticle sizes are much larger than the kinesin heads, which are closer to about 5 nm in diameter.

Fifth, although biotin-streptavidin linkages were used in both papers, different sized linkers were used. The Tomishige paper used a biotin-maleimide polyethylene glycol linker, which has a small contour length (2.91 nm). The Hancock paper used the 14 amino acid Avitag, which has an approximately 5.1 nm contour length.

Sixth, different image types and frame rates were used. The Tomishige paper used 8-bit AVI files taken at 18,000 frames per second (downsampled to 353 frames per second for analysis), whereas the Hancock paper used 12-bit TDMS files taken at 1,000 frames per second.

6.2.2 Major differences in results

There were two major differences between the kinesin stepping data in the Tomishige paper and the Hancock paper (Figure 6-1). First, the nature of the ATP waiting state was different. In the Tomishige paper, the authors observed clear “bound” and “unbound” regimes during each 16.4 nm step (Figure 6-1A). As they reduced the ATP concentration, they saw that the unbound duration became progressively longer. Hence, they concluded that the ATP waiting state is unbound, and thus part of a one-head-bound (1HB) state (Figure 6-1B). In the Hancock paper, we observed clear “on-lattice” and “off-lattice” substeps (Figure 6-1C). As we reduced the ATP concentration, we saw that the on-lattice substep got progressively longer. Hence, we concluded that the ATP waiting state is undisplaced, and thus part of a two-heads-bound (2HB) state (Figure 6-1D). The apparent discrepancy between the two papers is thus whether the ATP waiting state is 1HB or 2HB. It is worth noting, however, that in both papers assignment to the 1HB or 2HB came from author interpretations of the stepping patterns.
A second difference between the two papers was whether or not kinesin-1 swings to the right while stepping. The Tomishige paper clearly and repeatedly saw a rightward swing (Figure 6-1A), while the Hancock paper did not (Figure 6-1C).

**Figure 6-1 Highlighted differences from separate kinesin gold tracking experiments.** (A) An example trace of Cys-Lite kinesin-1 walking at low (10 μM) ATP from Isojima et al.(2). Clear bound (red) and unbound (blue) segments are visible. Kinesin moves to right while in the unbound state. (B) Measured 1HB and 2HB durations as a function of ATP from Isojima et al.(2). The 1HB duration is said to increase at low ATP since the unbound observation elongates. (C) Example traces of K560-AviN walking at low ATP from Mickolajczyk et al.(1). Clear on- (black) and off-lattice (red) substeps are visible (lattice spacing marked as horizontal lines). Kinesin does not move off of the lattice during the ATP waiting state, and does not swing right while stepping. (D) The measured 1HB and 2HB durations as a function of ATP from Mickolajczyk et al.(1). The 2HB duration is said to increase at low ATP since the on-lattice observation elongates.

### 6.3 Three models for the ATP waiting state

In looking at both our work and the Tomishige work, it is clear that different criteria were used for determining whether the motor was in the 1HB or 2HB state (Figure 6-1). The Tomishige
paper focused on whether the kinesin head was “bound” or “unbound” based on whether or not the gold-trace was displaced to the right and having a high standard deviation. They did not, however, use the information of whether or not the kinesin head was currently on or off the microtubule lattice position, since they did not see consistent substeps in the direction of stepping. In our work, we focused on whether the kinesin head was on or off the previous microtubule lattice position. However, we did not consider whether the kinesin was bound or unbound, since we did not see any apparent change in the standard deviation. We note here that sampling at only 1,000 frames per second together with using a longer tether for the gold likely diminished the standard deviation changes due to better mixing within the exposure time.

Combining the data from our paper and Tomishige’s paper, we now consider three different options for the true nature of the ATP waiting state. One option is “bound-undisplaced”, which means that the rear head is truly bound to the microtubule. A second option is “unbound-undisplaced”, which means that the rear head is not tightly bound to the microtubule, but its mean position is still at the microtubule lattice site. A third option is “unbound-undisplaced”, which means that the read head is both unbound from the microtubule and forward displaced along the microtubule axis.

6.4 Changing the gold position changes the apparent ATP waiting state

In order to resolve the differences between our work and the Tomishige work, we made their plasmid constructs, recombinant expressed the resulting protein, and carried out experiments on them using our microscope setup. All assays were done in TIRDFM, in BRB80 buffer, with 30-nm gold nanoparticles, and at 1,000 frames per second. Interestingly, under these conditions, we able to replicate the measurements from the Tomishige paper. When using the S55 (rearward label) tagging position, as was done in their paper, we saw that the motor swung to the right with high fluctuations during the ATP waiting state (Figure 6-2). Hence, we were able to confirm that the
ATP waiting state is unbound. This observation was even more clearly seen when we tagged the Cys-Lite construct at the E215 (frontward label) position (Figure 6-2). However, we also repeated our measurement with K560-AviN, and again saw that the ATP waiting state led to no displacement in either X or Y (Figure 6-2). Hence, we also were able to confirm that the ATP waiting state is undisplaced. We conclude that the best model for the ATP waiting state is unbound-undisplaced.

Figure 6-2 Tracking results at 10 μM ATP for different kinesin-gold tagging strategies. Leftmost in red, data is shown for Cys-Lite with a cystein introduced at S55. As shown from the crystal structure, this tag position put the gold nanoparticle on the rear-center of the kinesin head. The stepping trace shows clear movements to the right accompanied with high fluctuations and small displacements along the microtubule axis (Y) during the ATP waiting state. Center in blue, data is shown for Cys-Lite with a cystein inroduced at E215. As shown from the crystal structure, this tag position put the gold nanoparticle on the front-center of the kinesin head. The stepping trace shows clear movements to the right accompanied with high fluctuations and large backward displacements along the microtubule axis (Y) during the ATP waiting state. Rightmost in black, data is shown for K560-AviN. As shown from the crystal structure, this tag position put the gold nanoparticle on the right side of the kinesin head. The stepping trace shows no clear movements in X or Y during the ATP waiting state, nor are increases in fluctuations evident. All X position tick mark lines are 25 nm. Dark colored traces are downsampled from 1,000 to 333 frames per second. Gold nanoparticles not to scale. Crystal structure from Shang and Sindelar(11).

The unbound-undisplaced ATP waiting state begins to make more sense when we consider the relative position of gold and the kinesin head (Figure 2-6). For the S55 construct, the gold tag is on the middle-rear of the kinesin head. Since the biotin maleimide linker is very small, and since
the gold is about 6 times larger than the kinesin, it is safe to assert that when the kinesin head is bound, as in a 2HB state, then for the S55 construct the gold will be systematically biased to positions behind the kinesin head along the reference axis. However, when the kinesin head becomes unbound and gains rotational freedom, this systematical positional bias goes away and the gold accurately reports the position of the head. Said another way, when the kinesin head goes from bound to unbound, the gold rotates and causes an apparent forward displacement. This is indeed what we see in our traces (Figure 6-2 red). Since the systematic biasing is all along the microtubule axis (Y), we interpret the rightward X displacements to be real.

For the E215 construct, the gold nanoparticle is in the front of the kinesin head. Hence, when the head is tightly bound to the microtubule, the gold position is systematically biased to positions in front of the kinesin head (towards the microtubule plus end). When the kinesin head becomes unbound and gains rotational freedom, this bias goes away and the gold accurately reports the position of the head, leading to an apparent backwards displacement of the gold. This is exactly what we see in our traces (Figure 6-2 blue), with the dramatic backward movements clearly standing out. Since the systematic biasing is all along the microtubule axis (Y), we interpret the rightward X displacements to be real.

For the K560-AviN construct, the gold nanoparticle is on the right side of the kinesin head. Hence, when the kinesin head is tightly bound to the microtubule, the gold position is systematically biased to positions to the right of the kinesin head. When the kinesin head becomes unbound and gains rotational freedom, this bias goes away and the gold accurately reports the position of the head, leading to an apparent leftwards displacement of the gold. In our data (Figure 6-2 black), we do not see displacements in the X or Y position during the ATP waiting state. We interpret this to mean that the true rightward positional displacement of the head and the apparent leftward correction of the gold cancel out. Importantly, since the correction of the systematic gold positional bias is all in the direction perpendicular to the microtubule axis (X), the K560-AviN construct is actually the
best for determining whether there is any Y-displacement when the motor does into the ATP waiting state, and we see that there is not.

6.5 Conclusions and future directions

Here we show that the apparent disputes between the Tomishige and Hancock papers can easily be resolved simply by paying attention to the gold-label position and being careful about how 1HB and 2HB states are determined. We conclude that the ATP waiting state is unbound-undisplaced, and that kinesin does indeed swing to the right while stepping. The original conclusion that ATP binding drives the 2HB to 1HB transition remains true. The gold nanoparticles used are significantly larger than the kinesin heads. Hence, when the heads are bound, the gold position is biased approximately half a gold diameter to whatever side the label is on. When the heads are unbound, they have rotational freedom and thus the position of the gold accurately reports the position of the head. The “half-a-gold” apparent movement associated with going into the ATP waiting state needs to accounted for, as it dominates the observable stepping pattern. If the gold is placed on the front or back of the kinesin head, then the “half-a-gold” movement will occur in the direction of the microtubule axis (Y). In this case, positional changes in the Y direction cannot be interpreted as true movements of the head itself, but changes in the X direction can. If the gold is placed on the side of the kinesin head, then the “half-a-gold” movement will occur in the X direction. Thus, in this case, positional changes in the X direction cannot be trusted to be real movements of the head, but positional changes in the Y position can. Only by looking at the stepping patterns of two tagging strategies (front and side) can we reveal the entire X,Y picture.

This study highlights the important of being extremely careful in how data is interpreted, especially when relatively large gold nanoparticles are used. It is tempting to say that “seeing is believing”, and that the position of the gold always accurately represents the position of the kinesin head, but this is not so. Changes in the degree of rotation freedom induce and release systematic
positional biases of the gold position, and these biases dominate the apparent substep positional changes. It is worth noting, however, that the kinetic arguments are immune to this effect—even is the gold position is not representative of the head position, the information that the head changed its mechanical state is still true. To that end, it is worth noting that if 1HB and 2HB are swapped, the kinetic measurements from the Tomishige and Hancock papers are in excellent agreement (Figure 6-1B and D).

The arguments made in this section are mostly based in inductive reasoning, with the assumption that the small tethers will lead to systematic biases of the gold position. This line of reasoning takes us quite far and is capable of explaining all of the experimental data quite well. However, it is not enough for a scientific publication. Future work thus includes approaching this line of reasoning with a quantitative model. Brownian dynamics simulations can easily be used to model the relative positions of the gold nanoparticles and kinesin heads. Tethered diffusion on work-like-chain polymer tethers with controlled contour and persistence lengths can reveal how the robustly the systematic positional biases occur. Switching between state of zero (2HB) and high rotational (1HB) freedom of the read head can reveal how big of a positional change the release of positional biases can cause. Such a model, together with the data shown in this chapter, can make a strong case for the unbound-undisplaced ATP waiting state and rightward swing of the kinesin head, and can resolve the apparent differences between the Tomishige and Hancock papers in a peaceable way.

6.6 Materials and methods

6.6.1 Constructs and protein preparation

Drosophila KHC truncated at amino acid 559 with an N-terminal avi-tag was biotinylated and purified as reported previously(1). To prepare human constructs, a plasmid containing cys-lite
KIF5B (C7S/C65A/C168A/C174S/C294A/C330S/C421A) was obtained from Addgene (#24430). Cysteins were placed at position S55 or E215 using site-directed mutagenesis performed on-demand by Genscript. All constructs had a C-terminal 6x His tag. The plasmids were transformed into BL21(DE3) bacteria (New England Biosciences) for expression. Proteins were purified by affinity chromatography and desalted into phosphate buffer saline (pH 7.0) with 10 μM ATP. Proteins were then mixed with maleimide-PEG2-biotin (Thermo Fisher 21901BID) with a 2-10 fold molar excess of protein (to ensure low biotinylation), and incubated on ice for 30 minutes prior to quenching with ~71.5 mM β-mercaptoethanol and desalting into 25A200 plus 10 μM ATP. The biotinylation percentage was determined by dividing the biotin concentration (measured using the Piece Fluorescence Biotin Quantitation Kit, Thermo Fisher 46610) by the total protein concentration (measured at A₂₈₀). The biotinylation percentage was 11.2% for S55C, 7.0% for E215C, and 20.0% for K560-AviN.

6.6.2 Single-molecule experiments

Kinesins tagged with gold nanoparticles were tracked using a custom-built total internal reflection dark field microscope, as previously(10). Images were recorded using a Basler Ace acA640-750um CMOS camera (1,000 frames per second, 945 μs exposure). Flow cell preparation was done as previously(10). All experiments were done in imaging solution: 0.5 mg/mL casein, 10 μM taxol, 20 mM glucose, 20 μg/mL glucose oxidase, 8 μg/mL catalase, 0.2 mg/mL BSA, 1:200 β-mercaptoethanol, and 2 mM MgATP in BRB80 (80 mM K-PIPES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8). Lowly-biotinylated kinesin motors (8-20% biotinylation) were incubated with a 3-10 fold molar excess of 30-nm diameter streptavidin coated gold nanoparticles (BBI Solutions; ratio of biotin:gold) on ice for 20-30 minutes. The gold nanoparticles were then spun down at 20,000 g for 4 minutes (to remove all non-biotinylated motors) and resuspended in imaging solution at ~300 pM gold. Motor position over time was determined using FIESTA software(12).
6.7 References

Chapter 7

Application of kinesin mechanochemistry to solving cell biology problems

7.1 Introduction

The generalized mechanochemical model built in Chapters 4 and 5 provides a useful tool for linking kinesin structure to function (1, 2). Small changes in structure, such as elongation of the neck linker (NL) and addition of charged residues at the motor-microtubule binding site can tune the rate of mechanochemical transitions, and thereby change biophysical properties of the motor such as velocity (2–4), processivity (2, 5–7), side-stepping ability (8, 9), and force-sensitivity (10–12). Changes in biophysical abilities, in turn, specify a particular motor for a particular job in the cell. The goal of this chapter is to take a step back and to make and test predictions about how differences in mechanochemistry can specialize kinesin-1 versus kinesin-2 for carrying out particular cellular roles. In this broader context the lack of functional overlap between these two motors—why knockdowns of kinesin-1 are fatal (13) even though both motors are ubiquitously-expressed and can attach to the same cargoes (14, 15)—gains a mechanochemical basis.

A simplified version of the most critical mechanochemical transition in the kinesin stepping cycle is shown in Figure 7-1. Kinesin starts in the vulnerable one-head-bound (1HB) state, where a kinetic race occurs between attachment of the tethered (blue) head and detachment of the bound (green) head (2). Motors that are structurally like kinesin-1 have a short NL and thus proceed from the 1HB state to the two-heads bound (2HB) state quickly and efficiently. The residence time in the 1HB state is therefore minimized. Motors that are structurally like kinesin-2 have a longer NL and thus proceed from the 1HB state to the 2HB state more slowly. These motors therefore have an increased residence time in the vulnerable 1HB state. Kinesin-2 also has more charged residues at its motor-microtubule binding interface, and therefore has stronger electrostatic interactions to make and maintain attachment to the microtubule than kinesin-1.
In the context of the generalized mechanochemical model, it is possible to make and test predictions about how kinesin-1 and -2 should be different in terms of biophysical abilities. Kinesin-1 transitions from 1HB to 2HB faster than kinesin-2, and therefore should have higher velocity and processivity. Work for this dissertation(2, 9, 16), bolstered by older work in the literature(3, 6, 9), has shown that this is true. Kinesin-2 spends more time in the vulnerable state and therefore should be more susceptible to falling off the microtubule under applied loads. This prediction is also supported by reports in the literature(11, 12).

One new, untested prediction is that kinesin-2 should sidestep more than kinesin-1. Sidestepping refers to the case when a kinesin switches from one protofilament of the microtubule to another. Kinesin-1 has been shown to rarely sidestep and to mostly travel along a single protofilament(17, 18). Sidestepping may be useful in cases of microtubule crowding. Multiple microtubule associated proteins (MAPs) occupy the same microtubules as kinesins in cells and can serve as roadblocks that inhibit transport. Previous work has shown that kinesin-2 is less sensitive to microtubule crowding by MAPs than kinesin-1(8), setting up the possibility that this motor may sidestep more often. In observing the generalized mechanochemical model (Figure 7-1), it is clear that sidesteps must come from the 1HB state rather than the 2HB. Hence, motors that spend more time in the 1HB state should have a higher probability of going “off-pathway” and taking a sidestep rather than a forward step. To test this prediction, single-molecule experiments were used here to detect and count the number of sidesteps each motor takes as it travels. This data is reproduced from a second author paper, where the first author is Dr. Gregory Hoeprich(9).

A second, untested prediction is that kinesin-2 should be a more efficient team player in multimotor transport than kinesin-1. Most transport in cells is carried out by teams of motors that need to coordinate their activities(15, 19–21). The transport environment in the cell is very crowded, and motors teams need to coordinate and adapt in order to circumnavigate geometric blockages such as other microtubules and actin filament networks(14, 22). In observing the
mechanochemistry of kinesin-1, it is clear that this motor should not work particularly well in teams. Since it spends a minimal amount of time in the vulnerable 1HB state, it has a high velocity and little sensitivity to load. Hence it will always try to pull and often get stuck. Kinesin-2, on the other hand, spends an increased amount of time in the vulnerable 1HB state and thus travels more slowly and can be dissociated from the microtubule more easily. Kinesin-2 can hence detach and be free to reattach whenever a blockage is encountered (as long as another motor is tethering the ensemble to the microtubule). We thus have the prediction that, in multimotor transport, kinesin-1 should rarely detach/reattach but often get pulled to a pause, whereas kinesin-2 should readily detach/reattach but rarely pause. To test this prediction, single-molecule experiments using DNA origami to create controlled pairs of motors were performed. Using high-resolution tracking, individual detach/reattach events and pausing events were resolved. This data is reproduced from a second author paper, where this first author is Qingzhou Feng(16).

![Figure 7-1 Mechnochemical transition rates govern the biophysical abilities of kinesin motors.](image)

Motors transfer between a one-head-bound (1HB) state and a two-heads-bound (2HB) state during each step. Kinesin-1, and generally motors with short neck linkers (NL), have a fast 1HB-2HB transition rate. Kinesin-2, and generally motors with longer NLs, have a slow 1HB-2HB transition rate. Detachment from the 1HB state is mediated by electrostatic interactions. Kinesin-1 has fewer charged residues and hence this electrostatic effect is less pronounced. Kinesin-2 is the opposite. Overall, the relative tuning of these transition rates determines the biophysical abilities of the motors by changing the manner and responsiveness of the stepping pattern. Small structural changes can lead to large functional changes by tuning mechanochemical transition rates and thereby modifying biophysical abilities.
7.2 Results

7.2.1 Kinesin-2 navigates crowded microtubules more effectively than kinesin-1

To test the hypothesis that kinesin-2 sidesteps more often than kinesin-1, we first repeated a run length control experiment in the presence of roadblocks. Individual GFP-kinesin dimers were observed using total internal reflection fluorescence microscopy (TIRFM) as they walked along immobilized microtubules loaded with MAPs. One MAP used was Tau, a neuronal protein associated with Alzheimer’s disease(8, 18, 23, 24). A “low Tau” condition had 1 Tau molecule per every 5 tubulin dimers (prior to mixing), and a “high Tau” condition had 1 Tau per every 3 tubulin dimers. We note that, depending on the binding affinity, only a fraction of the Tau will actually be bound to the microtubule lattice. A second MAP used was rigor kinesin (RK), which is not a physiologically-relevant MAP but it useful as a tool since it does not cluster, does not diffuse, and binds irreversibly with high affinity. A “low RK” condition had 1 RK molecule per every 12.5 tubulin dimers, and a “high RK” condition has 1 RK molecule per every 7.5 tubulin dimers (prior to mixing). The measured run lengths (Figure 7-2) show that both kinesin-1 and -2 have their run length shortened by either MAP. However, the kinesin-2 run length only becomes significantly shorter at high MAP concentrations, whereas the kinesin-1 run length becomes attenuated even at low MAP concentrations. Hence, kinesin-2 is less sensitive to MAPs than kinesin-1, potentially due to sidestepping. We note that a proper graded response of kinesin-1 run length attenuation is seen for RK, but not for Tau. We interpret this to mean that Tau begins to aggregate and form patches on the microtubule at higher concentrations, a phenomenon that has been observed previously(25). Kinesin-2 is unable to circumnavigate patches as effectively as it does individual Tau molecules, and hence its run length decreases at high Tau but not low Tau. In contrast to Tau, RK binds to the microtubule with proportionally increasing occupancy as the concentration is increased, leading to a graded run length attenuation response.
Kinesin-2 switches protofilaments more often than kinesin-1

The data in Figure 7-2 suggests that kinesin-2 may be sidestepping more than kinesin-1. In order to test this claim directly, we performed single-molecule experiments with higher resolution in order to directly detect sidesteps. Kinesin motors were prepared with a C-terminal biotinylated Avitag and conjugated to streptavidin-coated quantum dots. This approach enabled imaging at 20 frames per second with subpixel spatial precision. Although the sidesteps themselves only lead to an approximate 6-nm displacement in the lateral direction (based on the microtubule lattice), the length of the kinesin coiled-coil and the size of the Qdot magnify this displacement to about 25-nm (Figure 7-3A). Displacements of this magnitude are possible to detect with the spatial resolution of the TIRFM setup. Example traces for kinesin-1 and -2 and shown in Figures 7-3C and 7-3D, respectively. Traces were rotated to match the microtubule axis, and model-free step-finding(28) was performed on the lateral position versus time traces. Example detected sidestep positions and magnitudes are shown in Figures 7-3C and 7-3D. The average number of detected sidesteps per
micron traveled in a processive run was calculated, and repeated for an entire population of processive runs (Figure 7-3E). The population data showed clearly that kinesin-2 sidesteps significantly more often than kinesin-1, with an approximately fourfold increase.

**Figure 7-3 Kinesin-2 sidesteps fourfold more often than kinesin-1.** (A) Single-molecule experimental design. Kinesin motors with a quantum dot conjugated to the tail walk along microtubules decorated with tau roadblocks. (B) Sidesteps will appear as apparent 25 nm lateral movements due to the length of the coiled-coil and the size of the quantum dots. (C) Example trace of kinesin-1 walking along a microtubule. The lateral position over time shows the detected sidesteps. (D) Example trace of kinesin-2 walking along a microtubule. The lateral position over time shows multiple detected sidesteps. (E) The measured sidestepping frequency of each motor. Kinesin-2 sidesteps significantly more than kinesin-1 (Mann-Whitney U test). Data shown as mean±standard error of the mean (SEM) for N=30 and 31 traces for kinesin-1 and -2, respectively. Experiments done by Greg Hoeprich, motors prepared and data analysis tool written by Keith Mickolajczyk. Figure adapted from (9).

### 7.2.3 Kinesin-2 detaches and reattaches more often than kinesin-1 during multimotor transport

The second prediction based on their differing mechanochemistry was that pairs of kinesin-2 should detach/reattach more often and pause less often than pairs of kinesin-1. To test this prediction experimentally, we developed a DNA origami scaffold system that, through the use of unique single-stranded DNA staples, enables exactly two motors of known type to be connected in a controlled manner(16). Briefly, unique single-stranded DNA oligomers were attached to GFP-tagged kinesin-1 or kinesin-2 molecules using a GFP binding protein (GBP). The oligomers were then hybridized to the DNA origami backbone via base pair binding of complementary sequences.
Since there are only two open, single-stranded sites on the DNA origami for the oligomers to bind, it is known and controlled than only two motors attach to the origami. In this fashion, we made pairs of kinesin-1 motors (Kin1-Kin1) and pairs of kinesin-2 motors (Kin2-Kin2) amenable for observation by single-molecule microscopy. One motor in each pair had a biotinylated N-terminal Avitag, such that a 30-nm gold nanoparticle could be attached and the motor pair could be tracked using high-resolution total internal reflection dark field microscopy (Figure 7-4).

Example high-resolution traces of Kin1-Kin1 and Kin2-Kin2 pairs are shown in Figure 7-4A. In these traces, the Y position corresponds to the microtubule axis, and time is encoded in color. As highlighted in Figure 7-4B, only one head of one kinesin in the pair has a gold nanoparticle on it. Hence, if both kinesins in the pair are engaged with the microtubule, then regular 16-nm steps should be observed (albeit with velocity modified by the second kinesin). If the unlabeled kinesin detaches or reattaches, no translocation of the gold nanoparticle should occur. However, if the labeled kinesin detaches or reattaches, then a large translocation of the gold should occur. Since the two kinesins in the pair can walk on separate protofilaments of the same microtubule, or indeed on separate microtubules altogether, it is possible that these large translocations can have an X (perpendicular to microtubule axis) component. In observing the example traces in Figure 7-4A, multiple large translocations are apparent. We scored these detachment/reattachment events as any instantaneous positional jump greater than 40 nm in the Y direction or 15 nm in the X direction, as these magnitudes of jumps are never seen in single kinesin traces(2). These events are marked as “r” in Figure 7-4A, and it is immediately apparent that they appear more often for Kin2-Kin2 pairs than for Kin1-Kin1 pairs. Indeed, for a large population of traces, Kin2-Kin2 pairs were seen to detach/reattach 5.3-fold more often on a per micron basis than Kin1-Kin1 pairs (Figure 7-4C). The faster apparent reattachment rate of kinesin-2 is consistent with complementary single-molecule and biochemical studies(16), and goes back to the idea that the kinesin-2 binding interface has enhanced electrostatic interactions with microtubules (Figure 7-1).
We also scored pausing events within the traces, which we defined as areas where no positional change occurred for at least ten full step durations. Dwell times of this duration never occur in single kinesin experiments (1, 2). Pausing events are marked as “p” in Figure 7-4A. The population measurements revealed than Kin1-Kin1 pairs paused 3.1-fold more often on a per micron basis than Kin2-Kin2 pairs.

Figure 7-4 High-resolution single-molecule tracking reveals that kinesin-2 reattaches more often and pauses less often than kinesin-1. (A) Example 1,000 frames per second traces of Kin1-Kin1 (blue-red) and Kin2-Kin2 (blue-yellow) pairs. A single motor domain of one motor was tagged with a 30-nm gold nanoparticle (shown in diagram in B), and the particle position imaged by dark-field total internal reflection microscopy (see Materials and Methods). Time information is encoded in color. Of note are abrupt positional changes that intersperse normal stepping, indicating reattachment events, and areas of high versus low variance, indicating whether labeled motors are engaged with the microtubule. Scored rebinding events (r) and pauses (p) are highlighted on each trace. (C) Kin2-Kin2 pairs reattach more often than Kin1-Kin1 pairs. Reattachments were scored as jumps \(>40 \text{ nm in the Y position (parallel to the microtubule)}\) or \(>15 \text{ nm in the X position. Kin1-Kin1 pairs reattached 1.54 \pm 0.19 times, whereas Kin2-Kin2 pairs reattached 8.16 \pm 0.58 times per micron traveled (mean \pm \text{SEM}; N=29 and N=33 traces, respectively, with plot showing one point per trace and mean values as red bars). A 2-sample t-test indicated that the difference in reattachment frequency was significant (p <0.00001). (D) Kin1-Kin1 pairs pause more often than Kin2-Kin2 pairs. Pauses were scored
as instances of no positional change lasting longer than 10 step durations (137 ms for kinesin-1 and 410 ms for kinesin-2). Kin1-Kin1 pairs paused 0.86±0.21 times per micron traveled (mean±SEM, N=29 traces), whereas Kin2-Kin2 paused 0.28±0.09 times per micron traveled (mean±SEM, N=33 traces). A Mann-Whitney U-test indicated that the difference in pausing frequency was significant (p < 0.01). DNA origami prepared by Qingzhou Feng, and experiments and data analysis done by Keith Mickolajczyk. Figure reproduced from (16).

7.3 Discussion

The data shown in this chapter substantiates two predictions that can be made from observing the differences in mechanochemistry between kinesin-1 and kinesin-2. Kinesin-2 is seen to sidestep more often than kinesin-1, and also to detach/reattach more frequently and pause less frequently than kinesin-1. These results details how the tuning of mechanochemical transitions rates between kinesin isoforms can lead to functional specialization and enable a specific kinesin to perform a specific job in the cell (Figure 7-5).
7.3.1 Kinesin-2 trades off run length for the ability to sidestep

Kinesin-2 has an elongated NL, and therefore is less efficient in making the 1HB to 2HB transition (Figure 7-1) than kinesin-1(2). At first glance, this structural change in the NL region appears to be disadvantageous, as it effectively reduces how far kinesin-2 can transport cargos(2, 3, 5, 6, 8, 9), and makes it more sensitive to applied load(11, 12). However, as highlighted in Figures 7-2 and 7-3, this structural change is actually an important design evolution, as it enables the motor to sidestep around MAPs. Hence, in physiological scenarios where microtubules are highly crowded by MAPs, the choice of a long NL makes kinesin-2 an effective transporter (Figure 7-2). Since run length and sidestepping ability step from the same mechanochemical transition (Figure 7-1), it is necessary to trade off one for the other.

7.3.2 Kinesin-2 trades off resistance to load for the ability to work effectively in teams

A second apparently adverse effect of elongating the NL is force sensitivity. Kinesin-2 spends more time than kinesin-1 in the vulnerable state, and thus its probability of detaching from the microtubule as a function of applied force is much higher(11). This seems like a fatal flaw for bidirectional transport, where dynein motors consistently provide antagonistic forces(20, 21, 29). However, kinesin-2 has a more charged microtubule binding interface (Figure 7-1) and a fast reattachment rate (Figure 7-4), meaning that it is capable of rapidly coming on and off the microtubule. In the context of multimotor transport (Figure 7-5), kinesin-2 is therefore capable of dynamic tethering. It detaches and reattaches at a new position whenever the cargo becomes stuck, helping the cargo to navigate around obstacles. This ability makes kinesin-2 more capable of cooperative motor transport through crowded environments than kinesin-1, which commonly pauses whenever its motion is impeded (Figure 7-4D).
7.3.3 **Kinesin-1 and -2 motors are tuned for different cellular roles**

In describing the mechanochemical basis of functional diversity in the kinesin superfamily, it is important to determine how small structural changes between motors from different families make them capable of performing some jobs, but incapable of performing others. The distinct cellular roles of kinesin-1 and -2 are highlighted in Figure 7-5. Kinesin-1, with its fast 1HB to 2HB transition rate, walks faster, farther, and with less sensitivity to applied loads than kinesin-2(2, 11). It hence can play a role in active pulling, moving the cargo while resisting the antagonizing loads being produced by dynein motors(16, 29). However, the downside of a fast 1HB to 2HB transition rate is that it leads to a reduced ability to sidestep (Figure 7-3) and a reduced ability to detach from and reattach to the microtubule when the cargo is stuck at an obstacle (Figure 7-4). Kinesin-2, with its slowed 1HB to 2HB transition rate, can perform the sidestepping and detachments/reattachments that kinesin-1 cannot, but is unable to resist antagonistic forces the way that kinesin-1 can. Overall, without both motors, effective cargo transport is not possible— with kinesin-1 alone the cargos would commonly get stuck, and with kinesin-2 alone the bidirectional tug of war would be lost to dynein. This explains why kinesin-1 knockouts are fatal(13), even though kinesin-2 is ubiquitously expressed and capable of getting onto cargoes similarly to kinesin-1(14, 15).

7.4 **Materials and methods**

7.4.1 **Protein preparation for roadblock and sidestep experiments**

All kinesin constructs contained *Drosophila* kinesin-1 neck and stalk domains (residues 346-559) fused with either a C-terminal eGFP/hexahistidine tag expressed and purified as previously published in (5) or a C-terminal Biotinylated Avi tag. Biotinylation was performed intracellularly in BL21(DE3) bacteria (New England Biolabs, Ipswich, MA) as previously published(1). Kinesin-1 constructs contained the N-terminal motor domain and neck-linker from
Drosophila KIF5 (residues 1-345), while kinesin-2 constructs contained the motor domain and neck-linker from mouse KIF3A (residues 1-359). The rigor kinesin obstacle was modified from monomeric Rattus norvegicus KIF5C motor domain (residues 1-354), which was a generous gift from Dr. Kathy Trybus. A T93N point mutation and a FLAG-Tag positioned at the C-terminal end were introduced by QuikChange II XL site-directed mutagenesis (Stratagene, La Jolla, CA). Rigor kinesin was expressed in BL21-CodonPlus(DE3)-RP E. coli cells (Stratagene, La Jolla, CA) using the isopropyl 1-thio-β-D-galactopyranoside-inducible pET vector system (Novagen, Madison, WI) and purified with the FLAG® monoclonal antibody (Sigma-Aldrich, St Louis, MO). The 3RS Tau isoform, which was a generous gift from Dr. Stephen King, was expressed and purified as previously described(25). Tau and rigor kinesin concentrations were determined using the Bicinchoninic Acid Protein (BCA) Assay (Pierce, Rockford, IL) using desalted, lyophilized, 3RS-Tau or BSA, respectively, for standards. Samples were dialyzed against BRB80 (80 mM PIPES, pH 6.9 at room temperature, 1 mM EGTA, and 1 mM MgCl₂). Tubulin was isolated from bovine brain (obtained from Vermont Livestock & Slaughter, Ferrisburgh, VT), using high molarity PIPES buffer (1M PIPES, pH 6.9 at room temperature, 10 mM MgCl₂, and 20 mM EGTA). Tubulin concentration was determined using a spectrophotometer (extinction coefficient at A₂₈₀ nm = 115,000 M⁻¹ * cm⁻¹).

7.4.2 Single-molecule roadblock and sidestepping experiments

Flow chambers were prepared by adhering ARTUS shims (Englewood, NJ) with Norland Optical Adhesive (Cranbury, NJ) to siliconized glass cover slips. Samples were prepared by incubating the flow chamber with monoclonal anti-β III (neuronal) antibodies (Sigma-Aldrich, St Louis, MO) at 33 µg/mL in MAB for 5 minutes or NEM myosin (used during protofilament switching experiments), which was a generous gift from Dr. Kathy Trybus. The chambers were washed and blocked with 0.5 mg/mL of bovine serum albumin (BSA) in MAB for an additional 2
minutes before the addition of 1 µM of the desired microtubule preparation. After an 8-minute incubation, the chambers were washed with MAB followed by 50 pM of the desired kinesin construct (with 1 mM or 0.1 mM ATP), which was added just prior to image acquisition for all experimental conditions examined. It should be noted that due to differences in buffer conditions our observed kinesin run lengths in MAB were different than previously measured values in BRB80 with the same constructs (5).

For experiments completed in the presence of rigor kinesin, tubulin polymerization was performed as described above, without rhodamine-labeled tubulin. After the microtubule incubation and wash in the flow cell (as described above), a 5-minute incubation with Alexa 532 labeled monomeric rigor kinesin-1 (at either 1:12.5 rigor kinesin to tubulin or 1:7.5 rigor kinesin to tubulin dimers) was then added followed by another MAB wash. Finally, 50 pM of the desired kinesin construct in MAB, supplemented with desired concentration of ATP, was added just prior to image acquisition.

For Qdot labeled kinesin motors, C-terminal biotinylated kinesin constructs were incubated with a fivefold higher concentration of streptavidin coated 655 Qdots for 30 minutes on ice in MAB and supplemented with desired concentration of ATP.

Total internal reflection fluorescence microscopy (TIRFM) was performed at room temperature using a Nikon Eclipse Ti-U inverted microscope equipped with a 100X plan apochromatic objective lens (1.49 NA), an auxiliary 1.5X magnification and a custom "Micro Optic Fiber Launch TIRFM" (patent pending). Images were obtained using an XR/Turbo-Z intensified 10-bit camera running Piper Control software v2.3.39 (Stanford Photonics, Palo Alto, CA). Kinesin-eGFP and Qdot 655 labeled kinesin constructs were excited with a 473 nm argon laser and imaged through emission 525/50 and 655/70 band-pass filters, respectively. The pixel resolution was 93.0 nm, kinesin-eGFP movies were acquired at 5 Hz and Qdot 655 labeled kinesin movies were acquired at 20 Hz. All experimental conditions tested were performed at least three times.
7.4.3 Data analysis for roadblock and sidestep experiments

Run length motility data was collected with eGFP kinesin constructs and measured using the MTrackJ plug-in for ImageJ software, version 1.46r (National Institutes of Health, Bethesda, MD) and track lengths were measured using the segmented line tool in ImageJ. Characteristic run length measurements, significance testing and power calculations were calculated as previously described(26). For detecting the frequency and lateral displacement of protofilament switches, movies of Qdot-labeled kinesin constructs were analyzed in MATLAB (MATLAB 2012b, MathWorks) using FIESTA(30) to track particle positions and Tdetector1(28) to identify statistically significant lateral displacements.

7.4.4 Protein purification for multimotor experiments

Kinesin-1 assemblies consisted of Drosophila KHC truncated at 559 and fused to a C-terminus eGFP and His6 tag(5). Kinesin-2 consisted of the head and 17 amino acid neck-linker domain of M. musculus KIF3A fused to the coiled-coil of Drosophila KHC followed by eGFP and His6 tag, as previously described(5). Motors were bacterially expressed, purified by Ni column, and stored at -80 °C, following previously published protocols(5). For high-resolution tracking experiments, N-term biotinylated kinesin-1 and kinesin-2 motors were generated and attached to streptavidin-coated 30 nm gold nanoparticles (BBI Solutions) as previously described(1). Tubulin was purified from bovine brain as described(5). SNAP-tagged, His6-tagged GFP nano-body (GBP) (a gift from the Grischuck lab, University of Pennsylvania) was bacterially expressed and purified following protocols developed for motors(5).
7.4.5 Generating oligo-functionalized GBP

Benzylguanine (BG) functionalized oligonucleotides were generated by reacting Benzylguanine-GLA N-hydroxysuccinimide (New England BioLabs) with C6-amine modified oligonucleotides in a 50 mM HEPES pH 8.5 buffer for 30 mins, followed by purification through a Sephadex G-25 Superfine desalting column (GE Healthcare). BG oligos were then mixed with SNAP-tagged GBP for 1 h at 4 °C, followed by purification through the Ni column to remove un-reacted BG-oligos. GBP1 and GBP2 concentrations were quantified by mixing with varying known concentrations of complementary strands and running on SDS-PAGE gels to determine the concentration needed to completely shift the band to the higher molecular weight.

7.4.6 High-resolution tracking of multimotor pairs

High-resolution tracking of gold nanoparticles was carried out using a custom-built total internal reflection dark field microscope, as described previously(2, 31). All imaging conditions matches those described previously(2).

7.5 References


Chapter 8

Conclusions and future directions

8.1 New microscopy techniques enable single-molecule mechanochemistry

In Chapters 2 and 3 of this dissertation, detailed instructions were provided for how to custom build a total internal reflection dark field microscopy (TIRDFM) and an iSCAT microscope. Both of these microscope designs are cutting edge (1–5), and enable very high spatiotemporal measurements of single molecules to be made. They are, however, not commercially available and need to be built on an optical table. The instructions provided are merely suggestions for a working design, and there is a large amount of space for improvements depending on experimental needs. Both TIRDFM and iSCAT can be run in the 10-100 kHz regime (4, 6, 7), but in this dissertation were only run at 1 kHz. Very high frame rate measurements tend to simply resolve the gold-tether dynamics, but in some cases this is very useful information.

The breakthrough that TIRDFM and iSCAT provided in this dissertation is the ability to resolve substep transitions in the kinesin-1 mechanochemical cycle. It has long been known that kinesin must process through a number of nucleotide dependent states in order to take a step, but those substep intermediates are highly elusive and difficult to measure (8–14). In resolving a substep mechanical transition, we obtained a vital tool for ordering and quantifying mechanochemical transitions. In the approach used in Chapter 4, the chemical conditions were controlled and altered and the effects on the mechanical transition rates was measured. In the approach used in Chapter 5, the motor domain type or neck linker length was altered and the effects on the mechanical transition rates were measured. In this way, single-molecule mechanochemistry could be performed on various kinesin motors. High-resolution tracking now represents a go to approach for elucidating the mechanochemistry of new motors of interest.
8.2 Solving the mechanochemical cycle of kinesin-1

The high-resolution tracking results under multiple nucleotide conditions in Chapter 4 combine to tell the story of how kinesin-1 uses ATP to take a step. In this minimal mechanochemical cycle, kinesin begins in an ATP waiting state with the rear head unbound but undisplaced from the previous binding site. ATP to the front head binding drives the two-heads-bound (2HB) to one-head-bound transition (1HB) of the rear head. ATP hydrolysis in the bound head then occurs, followed by attachment of the tethered head. The newly bound front head releases its ADP, and the rear head then releases its phosphate to return to the ATP waiting state. The mechanochemical cycle of kinesin-1 is important because it informs both how the chemical energy from ATP hydrolysis is transduced into mechanical work, and how the biophysical abilities of a motor are derived. The work in Chapter 6 resolving conflicts in the field underscores this importance.

8.3 Mechanochemical basis of processivity

The cross-family functional analysis utilizing high-resolution tracking in Chapter 5 revealed an important and general model for kinesin processivity. In this model, a critical mechanochemical transition occurs during each kinesin step where a kinetic race occurs between attachment of the tethered head and detachment of the bound head. The tethered head attachment rate was found to be mostly determined by the neck linker length, while the bound head detachment rate was found to be mostly determined by the strength of the electrostatic interaction between the motor domain and the microtubule. Hence, by making small structural changes to the motor domain or neck linker domain, the attachment or detachment kinetics can be modified and processivity can be controlled. This study provided novel insights into how very small structural differences between motors can lead to large functional differences.
8.4 Mechanochemical basis of functional diversity

Knowing the differences in mechanochemical tuning between kinesins -1 and -2 enabled us in Chapter 7 to make and test predictions about how these two motors should be capable of carrying out unique jobs in the cell. The most critical transition is the 1HB to 2HB attachment rate. Slowing this transition by elongating the neck linker leads to lower velocity, lower processivity, and increased sensitivity to applied force. However, it was also seen to increase the ability to sidestep around roadblocks and detach/reattach to circumnavigate obstacles in multimotor scenarios. In this way, kinesin-1 with its short neck linker plays the role of active pulling in multimotor transport, while kinesin-2 plays the role of dynamic tethering. Both kinesin-1 and kinesin-2 are needed for effective transport, and their functional specialization ultimately has a mechanochemical basis.

8.5 Outlook for other kinesin isoforms

The experimental impetus moving forward is to solve the mechanochemical cycle and measure the mechanochemical transition rates for other kinesin isoforms. The generalized mechanochemical cycle solves in Chapter 4 has been shown to be true for all motors tested in the lab thus far. In work not shown in this dissertation, kinesin-5 was found to share this mechanochemical cycle, and to spend the vast majority of its cycle in the 2HB state(15). This result was highly consistent with the strong resistance to applied load that is characteristic for kinesin-5(16). Hence, kinesins-1, -2, and -5 all share the same cycle, albeit with very different transition rates. Note that kinesin-5 is a mitotic kinesin, whereas kinesin-1 and -2 are transport kinesins. It is generally expected that mitotic kinesins should spend very large amounts of time in the 2HB, as this will bolster their ability to resist loads and perform jobs such as crosslinking microtubules to separate poles(17, 18).
A kinesin of particular interest is kinesin-3. Kinesin-3 motors make up the largest family of kinesins in mammals (19–21), with KIF1A being the prototypical isoform. Initial work showed that kinesin-3 may act as a processive monomer (22), but subsequent work highlighted its ability to dimerize on vesicles (23). The most recent work, led by the lab of Dr. Kristen Verhey at the University of Michigan, has cemented that KIF1A can work as a homodimer (24–26). Excitingly, multiple kinesin-3 isoforms have been shown to display very high velocities as well as “superprocessivity”, loosely defined as a run length that is so long that runs usually end because the motor reaches the end of the microtubule (24–26). In contrast to expectation, kinesin-3 has an elongated neck linker (27), which would predict that it has a slowed 1HB to 2HB transition rate. Consistent with a long 1HB duration, kinesin-3 has been shown to be more sensitive to applied loads than any other N-terminal kinesin tested (16). Using the processivity model presented in Chapter 5, we therefore predict that if kinesin-3 is not deriving its processivity from a fast 1HB to 2HB transition rate, then it must be doing so using a slowed detachment rate from the vulnerable 1HB state (1). Consistent with this idea, kinesin-3 has a large, highly charged insert termed the K-loop (23, 26). Because the detachment from the 1HB state was shown in this dissertation to be mediated by electrostatic interactions between positive charges in the motor and negative charges in the microtubule, such an insertion would be perfect for stabilizing the vulnerable state. Work from Dr. Ron Vale’s Lab showed clearly that Unc104 had superprocessivity with its K-loop, and that replacing the K-Loop with the analogous uncharged loop from kinesin-1 made it lose its superprocessivity. Work from the Verhey Lab showed that removing that swapping the kinesin-3 K-loop into kinesin-1 doubled its run length (26). The Verhey Lab initially reported mixed results on the effect of removing the K-loop from various kinesin-3 isoforms (26), but more recent unpublished work presented at the Biophysical Society 2017 Annual Meeting, supported the role of the K-loop in conveying superprocessivity. Also consistent with the idea of superprocessivity
stemming from a slow 1HB detachment rate, mutation of separate charged residues in the kinesin-3 microtubule binding domain all led to decreases in run length (25).

Overall, we have clear predictions of how superprocessivity should work for kinesin-3. We expect that due to its long neck linker, kinesin-3 has a 1HB-2HB transition rate that is closer to kinesin-2 than it is to kinesin-1. It is, however, an extremely fast motor with a velocity nearly twice that of kinesin-1 (23, 24). Each step is therefore only about 6 ms on average. We therefore expect that kinesin-3 spends a minimal amount of time in the 2HB state. Consistent with this prediction, kinesin-3 is extremely sensitive to applied loads (16). We expect that kinesin-3 utilizes its K-loop to minimize its detachment rate from the vulnerable 1HB state. We note and strongly emphasize here that most kinesin-3 studies to date have been done in low ionic strength BRB12 buffers (23, 24). In Chapter 5 of this thesis, we showed clearly that in low ionic strength buffers, run lengths were the most strongly increased so long as there is sufficient charge in the motor-microtubule binding interface. High-resolution single-molecule tracking provides the most direct way to test the predicted mechanochemical basis of kinesin-3 superprocessivity.

There are many other kinesins of interest to study, with mitotic kinesins in particular having high clinical relevancy. The homodimer mitotic motor Kinesin-6 (KIF23) is an extremely interesting target, with the *C. elegans* ortholog Zen4 being a common model motor (28). Zen4, together with homodimeric Cyk4 makes the heterotetrameric centralspindlin complex (29). Centralspindlin is absolutely essential for cytokinesis, and tethers the spindle to the cytoskeleton to allow for initial furrow ingression (29, 30). Recent work, not reported as part of this thesis, showed that Zen4 exhibits a backwards-docked neck linker in the apo state, and that this backwards docking depends on an “arginine gate” that helps mediate intramolecule tension (28). Of intense interest, we found that introducing the arginine gate to kinesin-1 via a single point mutation (A276R), increased the kinesin-1 run length substantially (28). Even more interesting, introducing the arginine gate via A276R to a kinein-1 mutant with its neck linker shortened to 13 amino acids, which normally has
no processivity (27), rescued the processivity (28). We note that the backward docking of the neck linker, enabled by the arginine gate, occurs in the ATP waiting state, where the rear head is unbound and undisplaced from its previous binding site. But how does the apparent stabilizing of this ATP waiting state enhance processivity? Much more work on the arginine gate, and on kinesin-6 in general, is needed to find out.

8.6 For every problem, an engineering solution

This dissertation emphasizes the importance of engineering for advances in biology. The optics and electronics knowledge needed to put together the TIRDFM and iSCAT microscopes falls outside the bounds of biology training. Many physicists, physical chemists, and electrical engineers have the training necessary to build these types of instruments, but they lack the biological question. The strength of the bioengineer is the ability to be question driven—to have a deep understanding of the underlying biology and to be able to perform hypothesis-driven biological research—as well as the ability to follow through on whatever type of experiment is needed to answer the question. The iSCAT microscope was built only because it is the best possible way to measure kinesin mechanochemistry. It is tempting to fall to one side of the spectrum or another—to become immersed in the biology and forgo advancement of highly technical engineering skills, or to become fully immersed in technology and instrumentation and to build devices without prior thought to what exact question the device will help answer. A good bioengineer must wear two hats. Biology is full of unknowns and unsolved problems, and every problem has an engineering solution.

8.7 References

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