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SINGLE-MOLECULE STUDIES OF UNCONVENTIONAL MOTOR PROTEIN MYOSIN VI

BY

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DISSERTATION

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ABSTRACT

Myosin VI is one of the myosin superfamily members that are actin-based molecular motors. It has received special attention due to its distinct features as compared to other myosins, such as its opposite directionality and a much larger step size than expected given the length of its “leg”.

This dissertation presents the author’s graduate work of several single-molecule studies on myosin VI. Special attention was paid to some of myosin VI’s tail domains that consist of proximal tail (PT), medial tail (MT), distal tail (DT) domains and cargo-binding domain (CBD).

The functional form of myosin VI in cells is still under debate. Although full length myosin VI proteins in cytosolic extracts of cells were monomers from earlier studies, there are several reasons why it is now believed that myosin VI could exist as a dimer. If this is true and dimerization occurs, the next logical question would be which parts of myosin VI are dimerization regions? One model claimed that the CBD is the sole dimerization region. A competing model claimed that there must be another region that could be involved in dimerization, based on their observation that a construct without the CBD could still dimerize.

Our single-molecule experiment with progressively truncated myosin VI constructs showed that the MT domain is a dimerization region, supporting the latter model. Additional single-molecule experiments and molecular dynamics (MD) simulation done with our collaborators suggest that electrostatic salt bridges formed between positive and negative amino acid residues are mainly responsible for the MT domain dimerization.
After resolving this, we are left with another important question which is how myosin VI can take such a large step. Recent crystal structure showed that one of the tail domains preceding the MT domain, called the PT domain, is a three-helix bundle. The most easily conceivable way might be an unfolding of the three-helix bundle upon dimerization, allowing the protein to stretch and reach a larger distance. The single-molecule stepping data with mutant full-length construct that lacks two helices out of three in the PT domain tell that it is indeed the case.

In this dissertation, more details of myosin VI PT/MT domain experiments will be explored along with background information on the single-molecule experiment methods used in these studies.
To my parents
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CHAPTER 1
INTRODUCTION

1.1. Why single-molecule?

The environment inside a cell is inherently very complex and complicated. Ensemble measurements for a protein of interest, which examine bulk properties of sample, can give us some rough information on it, but have unavoidable limitations. However, the advents of single-molecule studies which examine individual molecules, have shed new light on biology, and have been extremely powerful tools.

If we investigate molecules of a given species, what we see at a certain time reveals various conformations. Therefore, the synchronization of target molecules to see the same conformation at the time is of the utmost importance in the ensemble measurements. However, this sort of synchronization is not required in the single-molecule experiments. Single molecule experiments, as name suggests, are a way of obtaining different information from individual molecules without the worry of getting averaged information. Furthermore, single-molecule methods enable us to observe a short-lived transient intermediate state of a molecule that can hardly be observed in the ensemble measurements (Fig 1.1).

Owing to their power, single-molecule studies are gaining popularity. According to a PubMed (http://www.pubmed.gov) search done by Cornish and Ha, the growth of the number of publications with a “single-molecule” title is exponential [1].
There are numerous single-molecule techniques. A partial list includes FIONA (Fluorescence Imaging with One-Nanometer Accuracy) [2], FRET (Fluorescence/Föster Resonance Energy Transfer) [3], SHRImP (Single-molecule High-Resolution Imaging with Photobleaching) [4], SHREC (Single-molecule High-REsolution Colocalization) [5], optical tweezers (optical traping) [6], magnetic tweezers [7], AFM (Atomic Force Microscopy) [8], and so on. Here, some of them are based on light (fluorescence), the others are force-based techniques.

Among those single-molecule techniques, the author mainly used the FIONA technique in his myosin VI studies. We will exclusively discuss FIONA in the following section.

1.2. FIONA*

1.2.1. Diffraction-limited spot

The intensity profile of light from a small circular aperture was first theoretically derived by Sir George Biddell Airy (1801-1892), mathematician, astronomer, and Astronomer Royal of England [9]. When the radius of small circular aperture is \( a \), the intensity profile at an axial distance \( R \) from the center of the aperture is described as

\[
I(\theta) = I(0) \left[ \frac{2J_1(ka \cdot \sin(\theta))}{ka \cdot \sin(\theta)} \right]^2
\]

(Equation 1)

where \( I(0) \) is the intensity at the center of image plane and \( \theta \) is the angle from the center of the aperture.

* Part of section 1.2 is submitted to the FIONA chapter of Encyclopedia of Biophysics (Springer) as an invited review book chapter (HyeongJun Kim and Paul R. Selvin).
aperture [10] (See Fig. 1.2). $J_1$ is the first order Bessel function and $I(\theta)$ has the first minimum when $J_1(ka \cdot \sin(\theta))$ is zero, where $ka \cdot \sin(\theta) = kar/R = 3.83$ (See Fig. 1.3. $r$ is a radial distance in the image plane as shown in Fig. 1.2).

Using $k = \frac{\lambda}{2\pi}$, $D = 2a$, $f \approx R$, and $\frac{f}{D} = \frac{1}{2 \cdot NA}$ (where NA stands for numerical aperture) [10], the distance from the maximum intensity peak to the first minimum is calculated as $0.61 \cdot \frac{\lambda}{NA}$.

Depending on the definition, the width (measured from the intensity peak) of diffraction-limited spot from a small point-like diffraction-limited source is either $0.61 \cdot \frac{\lambda}{NA}$ or $\approx \frac{1}{2} \cdot \frac{\lambda}{NA}$ [2, 11].

This implies that the width of visual light from that source is roughly in the 150~250 (nm) range. Figure 1.4 is a single quantum dot 565 imaged onto the EMCCD camera. Note how big it looks (roughly 250 nm) despite the small physical size of the quantum dot (around 20 nm). For organic dyes, their physical size is even smaller (usually less than 5 nm), but diffraction blurs their images to a diffraction-limited size.

### 1.2.2. Theoretical background

The physical property of light, diffraction, limits our ability to locate small dyes or fluorophores with a precision better than $\sim 200$ nm, despite them being only tens of nanometers in size. Nevertheless, there are some techniques that can localize their positions much more accurately than $\approx \frac{1}{2} \cdot \frac{\lambda}{NA}$. The question of how to circumvent the diffraction limit of light and obtain nanometer accuracy in measuring position is a key feature of many single molecule studies. In
2002, Thompson et al. proposed a theoretical formula that determines a localization-accuracy as shown in (Eq. 2), when the signal is fitted by two-dimensional Gaussian function [12].

\[
\sigma_{\mu_i} = \sqrt{\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}} \]  
(Equation 2)

* \( \sigma_{\mu_i} \): Standard error of the mean \( (i : x \text{ or } y \text{ direction}) \)

s_i: standard deviation of the Gaussian function in the \( i \) direction

N: collected photon number,

a: detector pixel size

b: uncertainty (standard deviation) of the background noise

The first term is from a well-known statistical formula of the standard error of the mean, which is based on the Central Limit Theorem (CLT), and accounts for photon noise. The second term is pixilation noise due to finite detector pixel size. The numerator \( "a^2/12" \) came from the variance of the expected photon arrival site within the pixel of size \( a \), when a top-hat distribution is assumed. The third term accounts for background noise [2, 11, 12].

This formula suggests that, if we collect enough photons \( (N \text{ is large}) \), we can determine the center of the point-spread function (PSF) fitted with two-dimensional Gaussian very accurately.

For example, when \( s_i \) is 150 (nm) and \( N \) is 10,000, we can approximate the (Eq. 2) into \( s_i / \sqrt{N} \). It yields \( \sigma_{\mu_i} = 1.5 \) (nm), meaning that the position of a dye can be determined by \( \pm 1.5 \) (nm) accuracy. In fact, with the FIONA technique, current generation fluorophores make it possible to gather 20,000 or more photons during the detector exposure time, so achieving one nanometer accuracy with FIONA is feasible.
However, experimental localization precision is about 30% worse than its theoretical value from (Eq. 2) [2, 11, 12]. It is reasoned that several approximations and assumptions (for example, a top-hat distribution of photons arriving at a single pixel) underestimated the theoretical value [12]. In situations where precision is critical, maximum-likelihood fitting will do a better job [13].

1.2.3. Jablonski diagram

A starting point for modelling the physical process behind fluorescence is the Jablonski diagram, named after Polish professor Alexander Jablonski. In figure 1.5, $S_0$, $S_1$, and $S_2$ represents the singlet ground, first, and second electronic states, each of which consists of excited vibrational states (gray lines in the figure 1.5. Excited rotational states are not denoted in the figure). $T_1$ represents the first triplet electronic state.

When an external photon comes in, it excites the energy level of a fluophore from the singlet ground state to the singlet first (or even second) excited states in around a femtosecond ($10^{-15}$ second) [14]. In general, the fluorophore is excited to vibrational level with higher energy within the $S_1$ state by the absorbed photon, and transits into the lowest vibrational state within about a picosecond ($10^{-12}$ second) [14]. This process is called internal conversion, and it is a non-radiative process. The energy is, then, emitted as a photon with lower energy (compared with that of the absorbed photon) by transitioning electronic energy state into the $S_0$ ground state. The emitted light is called fluorescence, and its lifetime is around ten nanosecond ($10^{-8}$ second) [14]. However, the fluorophore sometimes (although relatively rarely) transits to the first triplet state
by intersystem crossing. Since transition from T<sub>1</sub> to S<sub>0</sub> is forbidden [14], the fluorophore stays for a long time (milliseconds to seconds) in the triplet state. This causes blinking.

As seen in (Eq. 2), getting more photons during camera exposure time will enable us to determine the center position of a fluorophore more accurately. Since the blinking results in smaller collected photon number N, a lot of effort had been directed towards minimizing blinking.

A fluorophore can also be irreversibly photobleached, meaning that it loses the ability to fluoresce, by light-induced chemical reactions.

### 1.2.4. How FIONA works

So far, we have learned from the (Eq. 2) that, to locate a fluorophore with the maximum accuracy, one needs to

1. extract as many photons as possible from the fluorescent dye,
2. send the photons to a detector with minimal loss, and
3. reduce the background noise.

The success of FIONA can be attributed to successfully achieving (1) through (3).

As pointed out in the previous section, fluorophores are intrinsically unstable due to blinking.
and photobleaching. The most prominent strategy to delay photobleaching is to remove oxygen molecules from a buffer solution. While oxygen molecules reduce T_1 triplet state population, they can cause photobleaching via interaction with the excited states of the fluorophore [14]. Oxygens can be removed by an oxygen scavenger system such as glucose oxidase and catalase system [15]. Alternatively, a PCA (protocatechuic acid) / PCD (protocatechuate-3,4-dioxygenase) system [16] or a reducing and oxidizing system (ROXS) [17] can be used to suppress the photobleaching of organic dyes. (If a quantum dot is used, there is essentially no photobleaching.)

In addition to oxygen scavenger system, reducing agents such as β-mercaptoethanol (BME) or Dithiothreitol (DTT) [11] or 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) [18] are used to suppress blinking. Interestingly, the ROXS system removes blinking, too [17].

The background noise is significantly reduced by adopting total internal reflection fluorescence microscopy (TIRFM). With this microscopy scheme, incoming laser light whose incident angle is larger than the critical angle impinges on the water (or medium, the index of refraction is n_{water} = 1.33) - glass (n_{glass} = 1.518) interface and reflected away from the interface. However, an evanescent field is generated in the aqueous medium. Since the intensity of excitation is decreasing exponentially with distance (in the z-direction) from the water-glass interface, only fluorophores in limited region, defined in (Eq. 3), are excited [14] (See Fig. 1.6). Other regions of the sample outside this range are essentially not excited, such that background levels can be significantly reduced compared to that of epi-fluorescence microscopy.
Penetration Depth \( d = \frac{\lambda_0}{4\pi \sqrt{n_{\text{glass}}^2 \sin^2 \theta - n_{\text{water}}^2}} \)

\[ = 118^\circ \quad \text{for} \quad \lambda_0=532 \text{ (nm)}, \theta=65^\circ \]  

(Equation 3)

The background can be further reduced by proper selection of dichroic mirror and emission / excitation filters. The dichroic mirror and emission filter are selected to keep reflected laser light and Raman-scattered light from entering the detector, while sending as much emission signal from excited fluorophores to the detector as possible. A notch filter is sometimes inserted in the emission beam path to cut off any reflected or scattered laser light. An excitation filter placed in front of dichroic mirror helps clean up unwanted laser bandwidth and block the off-axis light, allowing further reduction in background.

In addition to the proper dichroic mirror and filter sets, employing an objective with a high numerical aperture (NA) and a high-performance detector allows the maximum amount of photons to be registered for final FIONA data analysis. Due to modern cutting-edge technology, we now have the high NA objectives (generally 1.40-1.49, even 1.65 is available), and the high performance electron multiplying charge-coupled device (EMCCD) camera with almost 100% quantum efficiency.

Once data consisting of fluorophore images are gathered, the center of the fluorophore is found by a two-dimensional Gaussian fitting using custom (Matlab, IDL, SigmaPlot and so on) programming code. Although the PSF is described as Airy pattern, it has been shown that the error caused by a Gaussian fitting is negligible [12].
1.2.5. Applications of FIONA

One early application of FIONA was to precisely measure the step sizes of a variety of motor proteins. It has also been applied to *in vitro* [2], *in vivo* [19], and in living organism [20] studies. FIONA has achieved the time resolution of as fast as 2 milliseconds, which made it feasible for *in vivo* studies. Due to the nature of TIRF microscopy, FIONA is suitable for single-molecule studies on surface-tethered proteins/nucleic acids or membrane proteins in a cell.

1.3. Actin

Cells are referred to as building blocks of living organisms. They sometimes maintain their shapes against external forces, and other times they change their shapes to move into different positions. The cytoskeleton plays an important role in both cases and in various cellular processes [21]. There exist three kinds of cytoskeletal proteins in eukaryotic cells: actin filaments, intermediate filaments, and microtubules [22] (See Fig. 1.7). Here we will briefly review only actin filaments.

A building block of the actin filament is a 42 kDa globular protein called actin. Actin is a very abundant protein in the cell - occupying 10% of the total cell protein by weight [22], and the gene encoding it is highly conserved among different species. Although it is also called G-actin due to its globular shape, high-resolution crystal structure of actin revealed that this is not the case [23], as shown in Fig. 1.8. It has four sub-domains and is roughly divided in half by a cleft that functions as the nucleotide (ATP, ADP) binding site [23].
Upon an increase in the salt concentration (such as K\(^+\), Mg\(^{2+}\)), G-actins begin to be polymerized into actin filament, called F-actin. (The feature that F-actin polymerization is initiated by high ion concentration is used when we prepare for F-actin for myosin VI experiment. The detailed polymerization protocol can be found in the appendix of this dissertation.) The F-actin is the thinnest component of the cytoskeleton in eukaryotic cells, with a diameter of 8~9 nm [22], and its high-resolution structure was very recently solved [24]. F-actin shows polarity, because when G-actins are assembled, the same side points toward the same direction. Conventionally, the direction where the nucleotide cleft is facing is designated as the (-) end or the pointed end. The other direction is designated as the (+) end or the barbed end (See Fig. 1.9A). F-actin has a double right-handed helix structure, and it has a pseudo 36 nm repeat (See Fig. 1.9B).

Although much is not known, some cytoskeleton homologues in prokaryotic cells were discovered. For example, ParM is an actin homolog and it polymerizes into double helical filaments [25] in an ATP-dependent manner [26]. MreB is another example of actin homologue [27].

### 1.4. Background information on myosin VI

**1.4.1. Cytoskeleton-based motor proteins**

If we observe any eukaryotic cell under the microscope, we soon realize that there are various types of movement happening inside it. Movement can be caused by thermal diffusion. However, thermal diffusion alone is not sufficient to perform complicated tasks inside cells, especially in bigger eukaryotic cells. In reality, many movements inside cells are driven by cytoskeleton-
based molecular motor proteins.

There are three kinds of cytoskeletal motor proteins: myosins, kinesins, and dyneins (See Fig. 1.10). Myosins are actin-based molecular motors, while kinesins and dyneins use microtubule as their “track”. Although the motor proteins are categorized into three families, in fact, each motor protein consists of various sub-classes. For example, myosin has, at least, 35 sub-classes [28]. These motors use ATP (adenosine triphosphate) as their energy source, and converts chemical energy into mechanical energy. When ATP hydrolysis occurs, 20~25 $k_B T$ (or ~ 100 pN·nm) of energy is released [29, 30] (Fig. 1.11), and motor proteins use the energy with around 60% efficiency or higher (even ~95 % according to Dr. Ron Vale’s lecture at iBioSeminars whose webpage is found at “http://www.ibioseminars.org”).

Due to different kinds of tracks that myosin and kinesin use, they had been thought to have little connections. However, further research suggested that myosin and kinesin have a common ancestor that might be even related to the G-protein superfamily [31, 32].

1.4.2. Myosins

The molecular motor myosin uses actin filaments as “tracks”, and its role in muscle contraction is best studied and known [33]. In addition to their role in muscle contraction, myosins are responsible for numerous cellular events that will be explained in this section.

Myosin is further classified into different superfamily members such as myosins I, II, III, V, VI,
VII, X, XI, and so on by comparison between core motor domain sequences (Figure 1.12) [34]. According to Odronitz and Kollmar’s study, myosins are classified into 35 classes [28]. Due to both of historical reason and convenience, Cheney and Mooseker defined myosin II, that plays various roles in muscle, as a conventional myosin, and other myosins as unconventional myosins [35]. Indeed, unconventional myosins play various roles such as cell movement, organelle / particle / vesicle movements, endocytosis, exocytosis, melanosome transport, maintenance of stereocilia in inner hair cells, leukocyte differentiation and so on [36] (Fig. 1.13).

Myosins are characterized by a motor domain, a neck domain (lever arm), and (a) tail domain(s) (See Fig. 1.14). The motor domain is a conserved ~80 kDa catalytic domain [37], containing an actin-binding domain and nucleotide binding domain. It is followed by the neck domain with various number of IQ motifs that serve as calmodulin (CaM) or CaM-like light chain binding sites [36]. Sometimes a term “head” is used to simultaneously describe the motor domain and the neck domain. The tail domain is where there exist numerous variations as seen in Fig. 1.14. It could have coiled-coil (CC) regions to allow dimerization of the two myosin monomers, a cargo-binding domain, as its name suggests, to bind a cargo, or special motifs (MyTh4, PH domains, etc.) Also, this region could be the alternatively spliced to make the motor perform different tasks. For example, melanosomes are transported only by myosin V that keeps an exon-F in the tail domain after splicing [38].

The affinity of myosin for F-actin is well-studied. ATP (adenosine triphosphate) binding to myosin results in myosin’s dissociation from the actin-filament, while myosin is in strong actin-bound state when it has an ADP or no nucleotide in it [39]. Phosphate release is coupled to the
powerstroke representing the neck domain movement [39].

1.4.3. Myosin VI
Myosin VI is an actin-based molecular motor, originally identified in *Drosophila Melanogaster* [40], and plays various roles in cells such as maintenance of Golgi morphology and protein secretion [41], maintenance of stereocilia in the inner hair cells [42, 43], spermatogenesis in *Drosophila* [44], transport of vesicles [45], regulation of the morphogenesis of cadherin cell-cell contacts [46] and so on (See Fig. 1.15). (The roles of myosin VI are reviewed in several wonderful papers [47-50].

The structure of myosin VI roughly divides into three domains: motor domain, lever arm, and tail domain (See Fig. 1.16). The motor domain is a catalytic domain where Mg\(^{2+}\)-ATP binds and its hydrolysis occurs. It is also the region that actin-filament can bind to. Following the motor domain is an α-helical lever arm. The lever arm contains a myosin VI-specific region referred to as unique insert-2, and one CaM (Calmodulin)-binding site called IQ motif. It had been thought that a myosin VI monomer has only one CaM-binding site based on the number of IQ motifs, but Bahloul *et al.* demonstrated that the insert-2 is another CaM-binding site [51]. While 4Ca\(^{2+}\)-CaM binds to the insert-2, apo-CaM binds to the IQ-motif [52]. Compared to the motor domain and lever arm, the tail domain is the least-understood region. It contains unknown structure which permits lever arm extension and dimerization, and a globular tail as a cargo-binding domain. In the chapter 2 of this dissertation, sub-domains of the tail domain will be re-defined based on new findings.
Compared to other unconventional myosins, myosin VI has several unique features. First of all, it moves toward the pointed minus end of actin filament, in the opposite direction of other myosins [53]. In 2002, truncated myosin 9b was reported to move toward the pointed minus end of actin filament [54] like myosin VI, but in 2003, native myosin 9b was shown to be a (barbed) plus-end-directed molecular motor [55], and a recent study also concluded that myosin 9b moves toward the plus end of actin filament [56]. These studies left myosin VI as the only minus-end-directed myosin super family member up to now. A high-resolution crystal structure of rigor-like state (the state with myosin and actin complex in the absence of nucleotide) shows that the insert-2 repositions the lever arm by 120°, that reverses myosin VI’s directionality [52] (See Fig. 1.17).

Another interesting feature that myosin VI has is its large step size. For other previously characterized myosins, the step size was determined by their lever arm lengths. For example, myosin V has six CaMs on six IQ-motifs and takes around 37-nm center-of-mass step [2]. But, when the number of IQ-motifs is varied, what Sakamoto et al. observed was that the step size of myosin V was proportional to the number of IQ-motifs, that is, the length of the lever arm [57]. As mentioned above, myosin VI has only two CaM-binding sites, yet takes large steps (30-36 nm center-of-mass movement) that are comparable to those of myosin V [58-61]. A hint came from crystal structure study of myosin VI in pre-powerstroke state where ATP hydrolysis products (ADP and P_i) are trapped in the motor domain. The converter domain had been thought to be a rigid structure, but surprisingly, this crystal structure revealed that the converter itself adopts different conformation [62]. Owing to this conformational rearrangement of the converter domain itself in the prepowerstroke state [62] (See Fig. 1.18), myosin VI has a large
(12-18 nm) powerstroke size [62-65], that corresponds to the distance the lever arm travels through ATP products release. Yet, the large average step size is difficult to explain in the context of the short lever arm. What we know is that there should be a flexible domain after the lever arm for myosin VI to be processive and take a large step [65].

Based on predicted coiled-coil sequences, myosin VI had been assumed to be capable of dimerization. But, Lister et al.’s findings, that both expressed full-length and native myosin VI from cell extract are monomeric based on gel filtration, sucrose density gradients, and negative staining electron microscopy data [64], raised questions on whether myosin VI can exist as a dimer. However, Park et al. showed that the full-length and C-terminal truncated constructs (991 and 1050) can dimerize and walk processively when their concentrations are high mimicking the cargo-binding onto to the cargo-binding domain (equivalent to the globular tail domain in Fig. 1.16) [66]. The details of the two truncated constructs (991 and 1049) will be discussed in the chapter 2.
1.5. Figures for chapter 1

[Figure 1.1] (A) Biological objects have different conformations or are in different states. (B) With the ensemble measurement, what we might observe is the averaged information of A. (C) For a specific object, we can get detailed information from it in real time, in this case, conformational changes with single-molecule techniques. Also, we can observe the transient intermediate state (marked with a red star), that can hardly be seen in the ensemble measurement.
[Figure 1.2] Light passing through small circular aperture with radius a. Light green panel on the right represents image plane.

[Figure 1.3] Airy pattern graph. $I(\theta) / I_0$ is drawn as a function of $ka \cdot \sin(\theta)$. The minimum occurs at $ka \cdot \sin(\theta) = kar/R = 3.83$. Figure is adapted from “http://commons.wikimedia.org/wiki/File:Airy_Pattern.svg”.
[Figure 1.4] An image of quantum dot 565 (left) and its point-spread function graph. Note that the quantum dot looks much bigger than its actual size (around 20 nm) due to diffraction. Scale bar represents 400 (nm), and 1 pixel is around 107 (nm).

[Figure 1.5] Jablonski diagram. S and T denote the singlet and the triplet states, respectively. The subscript numbers 0, 1, and 2 represent the ground, the first excited, and the second excited states, respectively. IC (internal conversion) and ISC (intersystem crossing) are non-radiative processes.
[Figure 1.6] Schematic of TIR. Laser impinges on the water(buffer)-glass interface with angle $\theta$, which is over the critical angle. Evanescent field is generated and its intensity exponentially decays. Fluorescent dyes within penetration depth $d$ are essentially excited. The index of refractions of cover glass (gray), the index-matching immersion oil (brown), and objective lenses (not drawn in this figure) are all the same.
[Figure 1.7] In this cell image, microtubules are stained in green, actin filaments in red, and nuclei in blue. The image is due to Dr. Jan Schmoranzer in Freie Universität Berlin.

[Figure 1.8] Crystal structures of G-actin with ATP (A) or ADP (B) at the central cleft. The size is 5.5 nm in the longest side [22], and there are four subdomains. These crystal structure figures are adapted from [23].
[Figure 1.9] (A) F-actin has polarity showing that one end is distinct from the other end. This feature is important, since actin-based molecular motor myosins also have polarity. Numbers denote sub-domains of the previous figure. (B) F-actin exhibits double-helix structure and its pseudo-helical repeat spans 36 nm. Figure (A) is adapted from Schmid et al. [67], and figure (B) is adapted from Ökten et al. [59].
[Figure 1.10] Cytoskeletal molecular motors. They include kinesins, myosin, and dynein. Some motor proteins can exist as dimers. Figure is adapted from [68] with a permission.

[Figure 1.11] ATP hydrolysis. $\gamma$-phosphate of ATP is cleaved, and the resulting products are ADP (adenosine diphosphate) and inorganic phosphate $P_i$ and 20~25 $k_B T$ (or $\sim 100$ pN-nm) of energy. Structures of ATP and ADP were taken from “http://en.wikipedia.org/wiki/ATP_hydrolysis”, and modified.
A myosin phylogenetic tree reveals that myosin consists of multiple superfamily members, classified by comparison between core motor domain sequences. This figure was obtained from “http://www.mrc-lmb.cam.ac.uk/myosin/trees/trees.html” and copied here as supplied. Almost the same figure can be found from [34], published by the same research group.
[Figure 1.13] A schematic that depicts various tasks that unconventional myosins perform in a cell. Numbers refer to myosin class. Note that some myosins are involved in multiple roles. The list of functions that myosins perform is expanding even now. The figure was from [36] with permission.
Myosins consist of the motor domain (colored in light blue), the neck domain with (an) IQ-motif(s) (black vertical bar), and the tail domains (beyond the neck region). Note the structural diversity especially in the tail domain. “CC (coiled-coil)”, “MyTH4”, and so on depicts specific motifs of the tail domain. The figure is modified from [36] with permission.
[Figure 1.15] Schematics that show several examples on the roles of myosin VI. It transports a cargo during the endocytosis, maintains stereocilia, involves in spermatogenesis, and maintains the Golgi complex, etc. Figures are adapted from references [47] and [50] with permissions.

[Figure 1.16] A schematic diagram of myosin VI structure. It consists of motor domain, lever arm region, and tail domain. Two calmodulins (CaMs) bind to insert-2 and IQ-motif of the lever arm, respectively. The tail domain also divides into a few sub-domains for lever arm extension, dimerization, and cargo-binding (globular tail). As will be seen in later chapter, sub-domains of the tail domain will be re-defined again. Figure modified from [50].
Crystal structures for part of myosin VI (left) and myosin V (right). N-terminus (Nter.), upper 50 kDa (U50kDa), lower 50 kDa (L50kDa), converter (Conv.) are sub-regions of the motor domain. For myosin VI, insert-2 repositions the lever arm to the opposite direction of that of other myosins [52]. The first (proximal) part of the insert-2 contacts with the converter domain, and the second (distal) part is a calmodulin-binding site [52]. Figure modified from [52] with a permission.
[Figure 1.18] The conveter rearrangement in the prepowerstroke state of myosin VI and large powerstroke size. (A) Note the conformational differences of the conveter domain. (B) Due to conveter rearrangement, myosin VI has a large powerstroke (lever arm movement). Another noticeable difference of myosin VI powerstroke is that it has a large (~180°) powerstroke [62-64, 69-71], while that of myosin V is ~72° [72]. The figure is modified from [71] with a permission.
CHAPTER 2 ++
PROXIMAL AND MEDIAL TAIL DOMAINS OF MYOSIN VI

2.1. Background information

As mentioned in the previous chapter, the observation that a full-length myosin VI exists as a monomer in cell extract [64] was surprising because of the previous prediction that myosin VI can exist as a dimer due to predicted coiled-coil region in its tail domain [40]. The two conflicting points of view motivated Park et al. to postulate that monomer myosin VIIs might undergo dimerization upon cargo-binging [66]. Although no cargo-binding mediated dimerization mechanisms were reported from any of myosin superfamily members at that time, one example, Unc104 was already known from the kinesin superfamily to dimerize upon binding to cargo [73]. To test their prediction, Park et al. prepared a full-length myosin VI construct and several truncated constructs, and let the construct monomers approach each other in close proximity (mimicking cargo-binding) in the hope that monomers could be induced to dimerize [66]. They observed that, indeed, the full-length construct did dimerize and walk processively with a step size similar to that of an artificially zippered dimer [66]. They also found that not only the full-length construct, but also 991 and 1049 constructs (Fig. 2.1), that lack the C-terminal cargo-binding domain, can dimerize and walk with the same step size as the full-length construct [66]. Following additional experiments with other truncated constructs,

++ The results of this chapter were published in [74] as follows:
Myosin VI Dimerization Triggers an Unfolding of a Three-Helix Bundle in Order to Extend Its Reach.
Monalisa Mukherjea (*), Paola Llinas (*), HyeongJun Kim (*), Mirko Travaglia, Daniel Safer, Julie Ménétrey, Clara Franzini-Armstrong, Paul R. Selvin, Anne Houdusse, and H. Lee Sweeney
Molecular Cell (2009), 35, 305-315.
(*) Equally contributed
Single-molecule experiments are done by the Selvin lab, biochemistry experiments are done by the Sweeney lab, and crystal structure was solved by the Houdusse lab.
they proposed a working model that cargo-binding makes myosin VI monomers dimerize and some (if not all) dimerization regions lie below the cargo-binding domain [66].

The aim of this chapter is to present our experiments which answer several questions about myosin VI dimerization. We demonstrate the location of the myosin VI dimerization region and explore how myosin VI can take such large steps. In addition, a more plausible working model of myosin VI is proposed.

A schematic myosin VI structure can be found in Figure 1.16, but in our study, as shown in Fig. 2.1, we re-defined the domains of myosin VI following Spink et al.’s definitions [75]. The tail domain which mediates cargo-binding and dimerization is of key importance for our studies. It is divided into four regions: the proximal tail (PT), the medial tail (MT), the distal tail (DT), and the cargo-binding domains (CBD).

Spink et al. [75] demonstrated that the PT domain is largely \( \alpha \) helical and forms a highly compacted domain. They postulated from the Rosetta 2.2.0 structure prediction algorithm that the PT domain shape was most consistent with a three-helix bundle. They further suggested that the MT domain that lies between the PT domain and the DT is a stable single \( \alpha \)-helix (SAH) that forms the lever arm extension (LAE) necessary for the large step size of myosin VI [75]. Lastly, they claimed that the only region responsible for dimerization is the cargo-binding domain [75] (See Fig. 2.2).

However, Spink et al.’s model [75] is inconsistent with Park et al.’s observations [66] that the
991 and 1049 constructs which lack the cargo-binding domain have the ability to dimerize and processively move with a step size identical to that of the full-length dimer. One possible explanation for this discrepancy is that the data of Park et al. might be compatible with Spink et al.’s model in which a SAH domain (mostly the MT domain) provides the necessary LAE of myosin VI if there exists an additional component of weak dimerization below the cargo-binding domain but at the end of the constructs examined by Park et al. We will examine if this is indeed the case.

To narrow down the dimerization region(s) of myosin VI and discover the mechanism that allows it to achieve its 30-36 nm large step size (center-of-mass dimer movement), we made additional C-terminal truncations. By doing this, we could further delineate the location of the LAE and the minimal length structure that is required for dimerizing with a normal step size. Surprisingly, we observe that the truncated 940 construct extending only 28 amino acids beyond the PT domain is able to form a processive dimer, with step sizes identical to that of the full-length dimer. In addition, a crystal structure of monomer myosin VI that includes only the lever arm with its associated calmodulins (CaMs) and the proximal tail domain confirms that the PT domain (the ~80 amino acids C-terminal region immediately following the lever arm) is indeed the three-helix bundle. From our data, we hypothesize that, upon dimerization, the three-helix bundle must unfold and form the lever arm extension (LAE). Additional experiments with another truncated construct and fluorescence quenching provide us with evidences of our hypothesis.

Detailed experimental designs and results are discussed later in the appendix.
2.2. Results

To investigate the location of the dimerization regions of myosin VI and test discrepancies between Park et al.’s [66] and Spink et al.’s [75] models, we further truncated myosin VI at Arg940 (previously truncated at 991), and created a myosin VI 940 (MVI-940) construct. According to the model proposed by Spink et al., the 940 construct should not dimerize due to the lack of a cargo-binding domain, DT, and significant portion of the MT domain. In addition, according to the Spink et al.’s model shown in Figure 2.2, even if dimerization occurs by any mechanism in the 940 construct, its step sizes must decrease due to the loss of significant portion of the lever arm extension (LAE).

2.2.1. Result (1) - Truncated construct can dimerize and step processively

We discovered that that the truncated 940 construct (MVI-940) can dimerize and the dimer is sufficiently stable to function as a processive molecular motor. As stated in section A.1, the population of the MVI-940 monomers, which were sparsely labeled, was bound to an actin filament in the absence of ATP to induce dimerization. Following addition of ~20 μM ATP, 12% of the CY3-labeled molecules were observed to initiate processive movement on actin, indicating dimerization with an unlabeled monomer 940 construct (See Fig. 2.5 for stepping examples of processive dimers). Our observation of dimerization was not expected according to Spink et al.’s model [75]. Furthermore, measurements of step size from these actin-induced processive dimers yield a value of 54.3±19.4 nm (Fig. 2.3), that is indistinguishable from those of the full-length dimer, the zippered HMM 991 construct, or the dimerized construct truncated at 991 without an added Leucine zipper (See Table 2.1).
Additionally, the MVI-940 dimers have an average run length (the travel length of motor protein until it falls off of actin filament track) of 0.9 μm (Fig. 2.4), longer than any construct reported except the full-length dimer (Table 2.1).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Percentage of molecules that were processive</th>
<th>Average step size (nm±SD. CY3 on IQ-Calmodulin)</th>
<th>Average run length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVI-917</td>
<td>0a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>MVI-940</strong></td>
<td>12a</td>
<td><strong>54.3±19.4</strong></td>
<td><strong>0.9</strong></td>
</tr>
<tr>
<td>MVI-991</td>
<td>10a</td>
<td>54.2±17.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MVI-991-GCN4 (zippered dimer)</td>
<td>&gt;98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.2±19.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Full-length</td>
<td>15-30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.2±17.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

[Table 2.1] Step sizes and run lengths of various constructs. “a” and “b” are values reported in [76] and [61].

In addition to the single-molecule data, measurements<sup>*</sup> of actin-activated ATPase activity of the MVI-940 reveal that \( V_{\text{max}} \) (maximum rate of actin-activated ATPase activity) decreases from 7.5±1.0 (head<sup>-1</sup> second<sup>-1</sup>) to 3.1±0.7 (head<sup>-1</sup> second<sup>-1</sup>) and 2.9±0.8 (head<sup>-1</sup> second<sup>-1</sup>) after antibody binding and actin saturation, respectively. (See section A.1 for the details of these two methods.) These gatings (decreased ATPase activity per head) are signatures of a dimer.

Further evidence from rotary shadowing EM images<sup>*</sup> supports the single-molecule observation that the MVI-940 construct can dimerize. As shown in Fig. 2.5, some 940 construct are dimers. In fact, the percentage of the 940 dimers (10±3 %) is small as compared to that of the zippered

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<sup>*</sup> Actin-activated ATPase activity data and rotary shadowing EM images in the following page were obtained by the Sweeney lab.
HMM construct (98±1 %). There are two reasons why the dimer percentage of the MVI-940 is small. First of all, as time goes on between release from actin and spraying onto the EM grid, more dimers become monomers. Also, the glycerol needed for rotary shadowing destabilize the dimerization. Nonetheless, this number (10±3 %) is a smaller percentage of dimers than Park et al. [66] observed for the MVI-991 or MVI-1049 constructs (50-70 %), suggesting that additional interactions distal to amino acid 940 further stabilize dimerization.

From our observations that the MVI-940 construct can dimerize and walk processively with the same step size as that of the full-length construct, we are led to believe that Spink et al.’s model [75] does not likely represent the “real” myosin VI. Furthermore, the observations that the MVI-917 does not dimerize [66] (also see table 2.1) but the MVI-940 does suggest that some region between 917 and 940 is involved in dimerization. In other words, the medial tail (MT) domain is a dimerization region.

2.2.2. Result (2) - The three-helix bundle unfolds when myosin VI dimerizes

We have learned that the MT domain is a dimerization region, but still have limited information on other tail domains. Solving the puzzles of the myosin VI’s large step size started with solving a crystal structure of them. Attempts to crystallize myosin VI construct (residues 1-917) coexpressed with CaM were not successful#. Nonetheless, a crystal structure# of residues 770-913 with two bound CaMs was solved from the attempts at 2.7 Å resolution (See Fig. 2.6). The

(#) The crystal structure was solved by the Houdusse group.
770-913 consists of the full lever arm (FLA) containing two CaMs bound to insert 2 and an IQ motif followed by the 77 residues (835-913) of the heavy chain. The 77-residue heavy chain (proximal tail domain) is shown to be an anti-parallel three-helix bundle, as was postulated from the Rosetta 2.2.0 structure prediction algorithm [75], and its starting point P835 (proline at 835) results in a 30° kink relative to the lever arm. The three-helix bundle of the PT domain is around 4 nm long, and it acts as a lever arm extension (LAE) by 4 nm. Several residues at the proximal part of the bundle form a compact hydrophobic core, while the distal part is very loose with much less packing of hydrophobic side chains. Helix 1 (starting from the end of the lever arm) is shorter and has a long loop (K848-K864) connecting helices 1 and 2. Although the proximal part possesses some stability, the bundle may not be very stable due to its short side chains (even in the proximal part of the bundle) and, hence, the bundle is loosely packed (especially in the distal part.) (See section A.9).

Although some crystal structures of myosin VI exists, including that of 770-913, the mechanism behind the large step size of the dimerized MVI-940 construct (and that of the dimerized full-length construct) is still elusive. With the three-helix bundle folded and assuming 36 nm spacing between two monomer motor domains, there must be an additional LAE of 9 nm from each monomer as illustrated in Fig. 2.7. Even if the region between the end of the three-helix bundle and 940 was a stable α-helix (SAH), this would only provide another 4 nm from each side, and the 4 nm is not long enough for them to dimerize. A possible solution is that dimerization lets the three-helix bundle unfold and become three separate alpha-helices connected by hinges (Fig. 2.7). This hypothesis implies that the bundle would be stable in the monomeric form of the full-length construct, but not as dimer.
To prove our hypothesis that dimerization induces an unfolding of the three-helix bundle, several experiments with different approaches were designed and performed.

The first evidence came from single-molecule FIONA experiments for a truncated full-length construct. We deleted the second and third helices and their connecting loops (848-909) from the full-length construct (again, CY3 is sparsely labeled on the IQ-CaM), and designated this construct as “MVI-FL 848-909 del”. An underlying idea behind this construct design is that a significant step size decrease is expected if the three-helix bundle indeed unfolds upon dimerization, since approximately two-thirds of the unfolded helix length is shortened. More specifically, the center-of-mass step size is predicted to decrease from 30-36 nm to ~15 nm (See Fig. 2.8). However, if the three-helix bundle does not unfold, then this deletion would have minimal impact on the step size, since the length of remaining helix (835-847) is approximately similar to that of the folded bundle (4 nm in length) as shown in Fig. 2.9.

To test the “MVI-FL 848-909 del” construct, the actin-saturation method was again employed. After saturating F-actin with the construct in rigor, ATP was added and properly dimerized constructs walked processively along the actin filaments (See Fig. 2.10).

Then, measurements of step sizes of the ‘MVI-FL 848-909 del’ construct using the single-molecule techniques yielded values of 33.1±16.8 (nm) (N=63) and -15.4±8.7 (nm) (N=3) for forward and backward steps, respectively (See Fig. 2.11). The forward step size value is in good agreement with the predicted model in Fig. 2.8, demonstrating our hypothesis that the three-helix bundle unfolds upon dimerization. A large number of smaller steps possibly corresponding
to the detection of stepping by two actin monomers were found. Also, larger steps are sometimes detected, which may be possible if the weak dimerization in the MT domain occasionally dissociates. We think that the dissociation of the weak dimerization may underlie the extremely large steps sometimes seen for the wild-type molecules [66]. Another notable observation is that this construct has obviously shorter step sizes than those of the MVI-940 and the full-length constructs. This makes sense in the context of the model in Figure 2.8, since contraction of the PT requires one of the motor domains bind to the off-axis actin monomer ahead, that induces torsion to the myosin construct.

The second evidence that dimerization induces an unfolding of the three-helix bundle PT domain comes from fluorescence quenching experiments***. In these studies, a property of tetramethylrhodamine (TMR) dye was exploited. The TMR dye is such that when two of them are positioned very close with proper orientations, the fluorescence becomes quenched via exciton coupling [77, 78]. For this purpose, a specific labeling scheme was needed, so two “cys-lite” constructs were prepared, where native, reactive cysteines were mutated to other residues [79]. One is a monomeric construct (MVI-917-Cys-lite) that lacks dimerization region (MT domain), and the other is a dimeric construct (MVI-991-GCN4-Cys-lite) which was shown to exist almost exclusively in a dimeric form [66] due to a GCN4 Leucine zipper. For the both construct, two Cysteine residues were introduced within the three-helix bundle at positions T845 and A880. (Also as a control, the same constructs but with only one Cysteine residue at A880 were prepared and tested.) Judging from the crystal structure of the three-helix bundle, the

*** The fluorescence quenching experiments were performed by the Sweeney lab
distance between residues 845 and 880 in folded-form of the bundle is about 18 Å (See Fig. 2.12A). This distance and orientation of two TMR dyes are such that the fluorescence should be quenched in this folded form. However, if the bundle unfolds, the distance between them is predicted to increase possibly up to 59 Å (See Fig. 2.12B), which would allow unquenched fluorescence signals. The experimental results show that the MVI-917-Cys-lite (T845C, A880C), indeed, displays low fluorescence (24.5±4.6 fluorescence ratio), consistent with the two TMRs being in close proximity as in Fig. 2.12A, as compared to high fluorescence (266.2±28.1 fluorescence ratio) of the control construct MVI-917-Cys-lite (T845C). On the contrary, in the MVI-991-GCN4-Cys-lite (T845C, A880C), a high fluorescence signal (196.7±20.7 fluorescence ratio) was observed, meaning that the two rhodamines are too far apart (bundle is unfolded) to be quenched.

Another line of evidences is from single-molecule FIONA step size measurements for a construct with a Leucine zipper immediately following the three-helix bundle. In the publication of Spink et al. [75] in 2008, the addition of a GCN4 Leucine zipper immediately following the three-helix bundle PT domain (MVI-920-GCN4) resulted in a nonprocessive molecule with measured powerstroke sizes of ~23 nm in single-molecule optical trap assays. They claimed from this powerstroke size that the MT domain is the key region that allows myosin VI to take such a large step. However, it has been reported by Rock et al. that GCN4 alone does not maintain dimers when diluted to the low concentrations required for single-molecule experiments [65]. This suggests that MVI-991-GCN4 remains (almost 100%) dimerized even at pM concentration by some additional dimerization region below residue 991 in conjunction with GCN4 [65]. Thus, we think that Spink et al. [75] must have been examining the behavior of
monomers, not dimers. This issue was tested by a nearly identical construct (MVI-919-GCN4) that is truncated at Gln919 followed by GCN4 and GFP. We noticed that the construct lost its ability to participate in ATPase gatings regardless of its clustering on actin. The $V_{\text{max}}$ of 7.2/s/head is similar to that of the monomeric form of MVI-940. However, as long as the motor possesses a high duty ratio, the loss of gating could still maintain processive movement. This led us to perform the FIONA stepping assays. As expected (since GCN4 alone cannot maintain dimerization at single-molecule concentrations), without clustering on actin, no molecules exhibited processive movement. So, we saturated F-actin with the MVI-919-GCN4 construct in rigor (without ATP) to greatly increase the effective concentration, and then added ATP. The addition of ATP revealed processive molecules (See Fig. 2.13). These molecules displayed a shorter average step size 47.5±21.2 (nm) than MVI-940 (54.3±19.4 nm). (But, the step size is still large, and that cannot be explained without the three-helix bundle unfolding.) Interestingly, the step size distribution is still broad as other myosin VI constructs tested in this study, but the mean step size was reduced due to a large increase in the number of extremely small steps (~25-30 nm) (See Fig. 2.14), which might be possible for the construct with the three-helix bundle folded. We speculate that one possible underlying mechanism could be the dynamic folding and unfolding of the three-helix bundle, and this might explain large step size distribution. The local structural perturbation due to the insertion of GCN4 may have simply shifted the equilibrium more toward the folded conformation.

Finally, evidences from circular dichroism (CD) measurements (Fig. 2.15) also suggests the

[@] ATPase assays were performed by the Sweeney group

[%] Circular dichroism measurements were also performed by the Sweeney group
three-helix bundle unfolding upon dimerization. Brief descriptions are as follows:

The CD spectrum of the PT domain (834-917) shows a high portion of α-helix judging from deep trough at 222 nm, and its melting curve shows a steep transition (a cooperative melting) at 55°C. This is consistent with a three-helix bundle. The counterpart of the MT domain (906-991) also indicates a high α-helical content, but its melting curve is non-cooperative (gradual), consistent with a stable single α-helix (SAH). And those for PT+MT (834-991) are the sum of the PT and MT spectra, and cooperative transition shifted to around 67°C. Possibly this may imply that the three helix bundle of the PT domain is further stabilized by interactions between the PT and MT domains. The most revealing evidence from the CD experiments came with the examination of the construct of 834-991-zip, where GCN4 is appended at the end of the PT-MT construct to induce dimerization. For this construct, the steep transition that we ascribed to melting of the three-helix bundle was not found. Instead, gradual and noncooperative melting curve was observed, suggesting the unfolded three helix bundle even in the absence of the myosin head. However, the α-helical content is still maintained even after dimerization. The data also rule out the presence of a large segment of coiled coil. However, there might be a short segment of coiled-coil structure in the dimerized MT domain in addition to the coiled-coil in GCN4, since there appears to be a small degree of cooperative melting centered on ~77°C. Most majority portion of the MT domain consists of alternating four positive and four negative amino acid residues, and the domain has a small number of hydrophobic residues. We initially thought that the short segment of it with hydrophobic residues was responsible for dimerization. That is why only the short region of the MT domain contacts with the other MT domain counterpart in Fig. 2-12B. However, we later found that this was not the case. In chapter 3, we will investigate
the dimerization mechanism of the MT domain in greater detail, and present a different point-of-view.

In summary, single-molecule FIONA stepping measurements for two different myosin VI construct after actin-saturation, fluorescence quenching experiments, and circular dichroism measurements all directly and indirectly demonstrate our hypothesis that myosin VI dimerization induces an unfolding of the three-helix bundle.

2.3. Summary and discussion

2.3.1. A dimerization domain lies outside of the cargo-binding domain

In 2006, Park et al. [66] showed that a myosin VI construct truncated at Arg991 (MVI-991) can be induced to dimerize by holding monomers in close proximity. Contrary to this observation, what Spink et al. [75] concluded in 2008 is that the cargo-binding domain of the full-length construct is solely responsible for dimerization, and left Park et al.’s observation [66] as a question mark. To address this issue, we prepared the MVI-940 construct, which lacks additional ~50 residues on the C-terminal side compared to the MVI-991. From single-molecule stepping assays, rotary shadowing EM images, ATPase assays, we demonstrated that dimerization must occur between Leu913 (last residues of the three helix bundle of the PT domain) and Arg940. Obviously, the MVI-940 construct does not include the cargo-binding domain (CBD) and the distal tail (DT) domain (See Fig. 2-1), and its capability of dimerization rebuts the Spink et al’s model [75]. As previously noted [75, 80], a prominent feature of most of the myosin VI MT domain sequence (From Leu913 to Arg980) is its alternating pattern of
positively and negatively charged amino acids that are known to form a stable single α-helix (SAH) [81]. However, there is a short sequence between amino acids Leu913 to Lys936 that may contain a sufficient number of appropriately spaced hydrophobic and polar amino acids to form a short coiled coil. Furthermore, the experiment with the “MVI-FL 848-909 del” construct that lacks the last two helices of the three-helix bundle provides strong evidence that the sequence immediately following the three-helix bundle participates in dimerization. The dimerization mechanism of the MT domain will be further discussed in next chapter, and with this we can increase the level of detail that accompanies our model for this protein.

2.3.2. Role of the cargo-binding domain in dimerization

Spink et al. [75] reached the conclusion that the cargo-binding domain is the only dimerization region from the following observations. First, they observed dimerization of a construct that starts from the beginning of the PT domain to the end of the CBD in the μM concentration range. Second, the MT-DT construct that does not possess the CBD did not dimerize at the similar μM concentration range. Their second observation is consistent with our data, since dimerization required a much higher effective concentration. For example, in the case of the MVI-940 construct, we could induce dimerization only when monomers were brought into close proximity by actin clustering which tremendously increases the effective myosin concentration.

It has been known that there exist head-tail interactions that may inhibit dimerization in the absence of cargo [66]. In our model of cargo-mediated dimerization, it is the cargo itself that allows the two myosin VI monomers to be brought together (likely disrupting interactions
between the CBD and the rest of the myosin VI molecule), promoting internal dimerization. It is likely that interactions within either the CBD or the sequence just proximal to it initiate internal dimerization that propagates to the region immediately distal to the three-helix bundle, triggering its unfolding. Later, cargo-mediated dimerization was experimentally demonstrated from Yu et al’s work [82] with clathrin-coated vesicle adaptor protein Dab2 (Disabled-2) and Phichith’s work [83] with optineurin and Dab2.

2.3.3. Unfolding of a three-helix bundle as a mean of extending a lever-arm

The crystal structure revealed that the PT domain is a three-helix bundle (Fig. 2-6), and while its proximal part possesses some stability, its distal part is fairly loose. Single-molecule FIONA stepping assays, fluorescence quenching assay for monomeric and dimeric forms of myosins, and CD data all suggest that the bundle must unfold upon myosin VI dimerization. We are not aware of its exact mechanisms, but speculate that dimer formation could recruit residues either immediately following or perhaps even within the last helix, which would destabilize the three-helix bundle. Alternatively, when two bundles are brought in close proximity, steric hindrance between them could induce the bundle unfolding. The bundle is stable in a monomer, but induced to unfold in a dimer perhaps by either mechanism.

2.3.4. Working model

So far, what we have learned that

(1) The PT domain is a three-helix bundle in a monomer.
(2) The PT domain must unfold in a dimeric form of myosin VI.

(3) The MT domain is a dimerization region. Perhaps the region immediately following the PT domain forms a short segment of coiled-coil.

(4) There is a certain kind of unknown head-tail interaction in the full-length construct that inhibits dimerization. Cargo-binding may break this interaction.

Based on this information, our working model (see Fig. 2-16) is that cargo-binding to the CBD disrupts the interactions that used to inhibit dimerization. Then, internal dimerization is initiated and propagated to the proximal region. By an unknown mechanism, the three-helix bundle is disrupted and unfolds. The unfolded bundle plays a role of lever-arm extension so that myosin VI can take a large (30-36 nm) step.

### 2.3.5. Perspective

Although the roles of the proximal and medial tail domains were identified in this study, additional work is required to better understand those two domains. Two example questions which remain to be answered are as follows:

(1) How does the dimerization of the MT domain induce the unfolding of the three-helix bundle? Can we experimentally identify the underlying mechanisms?

(2) Three-helix bundle unfolding results in exposure of hydrophobic residues of the bundle to aqueous environment. How can myosin VI remedy this problem? Does an external protein (for example, calmodulin) protect the exposed hydrophobic residues?
2.4. Figures for chapter 2

[Figure 2.1] A newly defined schematic diagram of myosin VI structure. The motor domain and the lever arm region are the same as Figure 1-16. The main differences lie at the tail domain. Following Spink et al.’s terminologies [75], it is re-defined as four sub-domains: proximal tail (PT), medial tail (MT), distal tail (DT), and cargo-binding domains (CBD). Numbers represent protein sequence numbers. Note that the 991 construct is truncated at the DT domain and does not have the CBD. The 1049 construct lacks most of the CBD. The figure is from reference [74].
[Figure 2.2] A myosin VI dimer model proposed by Spink et al [75]. The proximal tail (PT) domain colored in blue is a compact structure, which they postulated to be a three-helix bundle. The medial tail (MT) domain is a region for the lever arm extension (LAE), and the cargo-binding domain (CBD) is solely responsible for dimerization. Two numbers 919 and 940 are truncation positions that we used in our study. This figure is from [74], and slightly modified. All color schemes are the same as those in Figure 2.1.
[Figure 2.3](Left) Two examples of Myosin VI 940 construct displacement. Blue dots are raw data, and red line represents fitted line by Student’s t-test. (Right) Step size histogram of the processive (dimerized) 940 construct. Forward step size is $54.3 \pm 19.4$ nm (N=87), and backward step size is $-24.6 \pm 3.4$ nm (N=2). Note that CY3 is labeled on one of the IQ-CaMs, meaning that step sizes of the center-of-mass movement are approximately the half of these values. Note a high population of large steps (roughly over 60 nm). Especially those large steps cannot be explained by Spink et al.’s model [75].
[Figure 2.4] Run length histogram of properly dimerized MVI-940 construct. It was fitted with exponential decay function, and its decay constant is 0.9 μm.
[Figure 2.5] Rotary shadowing EM images of myosin VI taken by the Sweeney group. (A) Dimerized 940 construct. (B) artificially dimerized HMM construct (991 + Leucine zipper). (C) Monomer 940 construct.
[Figure 2.6] (A) Myosin VI structure for 771-913 (Full-length lever arm with the insert-2 and IQ motif, followed by the three-helix bundle of the PT domain). The PT domain is a three-helix bundle and it extends lever arm by 4 nm. The structure was solved by the Houdusse group. (B) Some detailed interactions between the PT domain (three-helix bundle) and the apo-CaM, and between helices. (C) The three-helix bundle (PT domain, Lever arm extension (LAE)) is stabilized by apolar interactions. Note that there are more interactions in the proximal part than the distal part. (D) Sequence of the PT domain. Colored dots represent the residues found within the bundle. K848-K864 is a long loop but their electron densities are not observed.
With the three-helix bundle of the PT domain folded, it increases the length of lever arm only by 4 nm. Given that two motor domains of dimerized full-length myosin VI construct span 36 nm, the regions from the motor domain to the PT domain explain only 18 nm (9 nm x 2), and the regions between the end of the PT domain and 940 only explain another 8 nm (4 nm x 2). There must be something that can explain, at least, additional 10 nm. (B) One imaginable hypothesis is an unfolding of the three-helix bundle of the PT domain. Since the PT domain has a folded conformation in the crystal structure of monomeric form, the hypothesis implicates that the dimerization induces unfolding of the three-helix bundle.
[Figure 2.8] This figure describes a model of the (dimer) full-length construct without two helices of the three-helix bundle (MVI-FL 848-909 del). Expected distances between two motor domain is ~15 nm (three monomer actin).

[Figure 2.9] Approximate length comparison of wild-type and truncated proximal tail domain. (A) Wild-type proximal tail domain is ~4 nm in length. Region between helices 1 and 2 is not seen in crystal structure, so is arbitrarily drawn as a dotted curve. (B) Truncated PT in “MVI-FL 848-909 del” construct is approximately estimated to be ~2 nm in length. Note that the lengths of (A) and (B) are similar to each other (compared to 36 nm). The image (A) was rendered with VMD [84].
[Figure 2.10] Two examples of the “MVI-FL 848-909 del” construct stepping. Green circles and lines represent raw data and fitted steps by Student’s t-test, respectively. Numbers are step sizes in nanometer. The upper trajectory is the one with the longest run length. This construct has shorter run length compared to that of the MVI-940 and the full-length constructs.
[Figure 2.11] Step size histogram of the “MVI-FL 848-909 del” construct after applying actin-saturation method. Forward step size average is 33.1±16.8 (nm) (N=63), and backward step size average is -15.4±8.7 (nm) (N=3).
[Figure 2.12] Experimental design for the TMR dye quenching experiments done by the Sweeney lab. (A) When the three-helix bundle remains folded, the distance between T845C, and A880C is only 18 Å according to the crystal structure. TMR dye signal quenching is expected with this distance and orientation. (B) When the bundle is unfolded, predicted distance between the two TMR dyes are around 60 Å. With this distance, two dyes must fluoresce normally.
[Figure 2.13] Stepping example of the “MVI-919-GCN4” construct (in blue). CY3 is sparsely labeled on the IQ-CaM, and step sizes are found after t-test fitting. Numbers denote the step sizes. Stepping graphs for the “MVI-940 (Fig. 2-3)” and the “MVI-FL 848-909 del (Fig. 2-10)” are also shown as references.
[Figure 2.14] Step size histogram of the “MVI-919-GCN4” construct after applying actin-saturation method. Forward step size average is $47.5\pm21.2$ (nm) ($N=108$), and backward step size average is $-25.0\pm5.4$ (nm) ($N=3$). Like other constructs, CY3 is sparsely labeled onto the IQ-CaM.
[Figure 2.15] Circular dichroism of the various tail domains. (A) CD spectra, (B) melting curve
[Figure 2.16] Working model of dimerized myosin VI. Please refer to the text of section 2.3.4 for details.
CHAPTER 3++

DIMERIZATION MECHANISM OF THE MEDIAL TAIL DOMAIN OF MYOSIN VI

3.1. Background information

We have shown that the medial tail (MT) domain of myosin VI is a dimerization region [74] in chapter 2. The most prominent property of the MT domain (residue numbers 913-980) is that it exhibits a pattern of four positive residues followed by four negative residues, called the ER/K motif, that is repeated, except a few hydrophobic residues near the N-terminal side of the MT domain (See Fig. 3.1). The properties of ER/K motif are such that the MT domain forms a stiff, ~10 nm long, stable single α-helix (SAH) with a 15 nm persistence length [75, 81, 85]. In the previous chapter and our previous study [74], we hypothesized that the hydrophobic residues act as a hydrophobic core, forming a short segment of coiled-coil. In this chapter, we will investigate how far the ER/K motif contributes to the dimerization of the myosin VI MT domain. For this purpose, we employ molecular dynamics (MD) simulation and experimental single-molecule methods.

Our MD simulation results confirm that positive and negative residues across different monomers can interact to form a stable homodimer. In order for this to happen, the two identical monomers must assume, however, a spatial offset with respect to each other, such that positive

(++) The results of this chapter were published in [86] as follows:
Formation of Salt Bridges Mediates Internal Dimerization of Myosin VI Medial Tail Domain.
HyeongJin Kim (*), Jen Hsin (*), Yanxin Liu (*), Paul R. Selvin, and Klaus Schulten
Structure (2010), 18, 1443-1449. (*) Equally contributed
Single-molecule experiments are done by the Selvin lab, and molecular dynamics (MD) simulation was done by the Schulten group.
residues on one monomer interact with negative residues on the other. In agreement with the simulation, experimental data support the key roles of Coulomb attraction between positive and negative residues participating in myosin VI dimerization. It is observed, furthermore, that mutations of hydrophobic residues on the MT domain do not abolish myosin VI dimerization, suggesting that Coulomb attraction in the form of salt bridges is the major contributor to dimerization. This conclusion is also supported by calculations of dimerization energies.

3.2. Experimental methods used in this chapter

The details on the dimerization initiation method were fully discussed in section A.1. The single molecule FIONA experiments with the wild- and mutant-type of the MVI-940 construct were performed using the same methods in section A.3 except only a few differences.

(1) Concentrations of the construct added into the flow chamber to saturate the actin-filament are 100 nM for experiments with 52 mM and 149 mM ionic strengths, and 50 nM for experiments of wild- and mutant-type comparison.

(2) ATP concentrations were 20 μM for step size measurement, and 40 μM for the calculation of percentage of processive dimer movement.

(3) Imaging buffer contains M6+ buffer, ATP, and oxygen-scavenging system. All of concentrations of individual constituents are the same as those explained in section A.3, except KCl concentration. 25 mM KCl was used for 52 mM ionic
strength, and 122 mM KCl was used for 149 mM ionic strength.

Experimental data analysis was performed as mentioned in section A.4.

3.3. Results

3.3.1. Self-association of the MT domain was seen in the MD simulation

In 2006, Park et al. showed that the truncated 991 construct (residues 1-991) can be induced to dimerize despite the lack of the cargo-binding domain (CBD) [66]. Later, we showed that even the 940 construct (MVI-940. residues 1-940) also has a capability of dimerization [74]. Together with other data, we concluded that the MT domain is a dimerization region. To discern how the MT domain dimerizes, MD calculations were performed for two different constructs of the MT domain:

(1) A full MT domain segment consisting of residues 907-980 (MT-907-980)

(2) A truncated MT domain segment consisting of residues 907-940 (MT-907-940)

Since no crystal structure of the MT domain was available, we aimed at establishing viable models of the MT domain. For this purpose, a microsecond simulation was conducted with two free MT segments, initially separated by 30 Å (Fig.3.2A). This simulation was performed in a so-called coarse-grained representation (more specifically, residue-based coarse graining representation, RBCG) (See Fig. 3.2A), since it permits surveying of the microsecond time-scale

(*) The MD simulation was performed by the Schulten group. For smooth logical flows, the simulation results are also described in this dissertation.
relevant for self-assembly processes [87, 88] with reduced computational time compared to the all-atom simulation. During the RBCG simulation, the two MT domain segments were seen to associate after 500 ns starting from the N-terminal ends, and propagate toward C-terminal ends. To answer the question of what molecular interactions are involved during this dimerization process, all-atom (AA) resolution was recovered from the coarse-grained representation using the molecular dynamics flexible fitting (MDFF) method [89-91] (See Fig. 3.2B. See section A.10 for details of the recovery of all-atom resolution).

During this AA simulation, the still separated C-terminal ends were seen to also associate, indicative of the completion of dimerization. The dimerization is also recognized through a rise of buried molecular surface between the two associating segments (See Fig. 3.3).

The contact map depicting interactions between residues in one MT segment and those in the other MT segment is very revealing and provides detailed information on what interactions are involved in the dimerization. As shown in Fig. 3.4, the interactions involves a vertical offset between the two segments, with one segment positioned few residues above the other. What the vertical shift means is that it brings the charged residues in one segment in contact with oppositely charged residues of the other segment, permitting thereby formation of salt bridges. Indeed, five salt bridges are seen to form in the modeled MT domain dimer, shown in the inset of Fig. 3.4.

The dimerization simulation for the MT-907-940 was also performed through the same MD protocol as the MT-907-980, since we previously observed that the MVI-940 can dimerize upon
actin-saturation (mimicking the cargo-binding). Spontaneous association of the two MT-907-940 segments was observed again in a microsecond coarse-grained MD simulation, and they remained stable as a dimer during the subsequent AA simulation. The results of the MT-907-940 dimerization are summarized in section A.11.

3.3.2. Buffer with higher ionic strength impedes dimerization of myosin VI

The above simulations suggest that the electrostatic interactions between the positive and negative residues in each MT domain play an essential role in domain-domain aggregation. Consequently, increasing the ionic strength of the myosin VI buffer (M6 buffer) is expected to reduce the propensity for dimerization.

To cross-check the simulation results, we designed an experiment based on this prediction. Again, the actin saturation method was employed to induce dimerization. First, actin filaments were decorated with high concentration (100 nM) of the truncated myosin VI 940 (MVI-940) construct (a gift from the Sweeney lab) in the absence of ATP. This tends to bring the monomeric myosins in close proximity. Then, we added ATP, and this allowed a certain percentage of the properly dimerized myosins to begin processive movements along the actin filament. The whole processes were performed with two different buffer conditions, one with 52 mM ionic strength, the other with 149 mM ionic strength. (Please refer to the sections 3.2 and A.3 for experimental methods.)

As expected, the buffer with higher ionic strength impeded the dimerization and processivity of
the MVI-940 construct. While 14.0% ± 3.8% (average ± standard deviation) of the construct was processive with the 52 mM ionic strength buffer, only 3.8% ± 2.9% of the myosins were processive with the 149 mM one (Fig. 3.5).

3.3.3. Mutations of hydrophobic residues in the medial tail domain do not abolish dimerization

From the simulation and experiments above, we are led to believe that the bonding of positive and negative residues on different monomers is a key for the dimerization of myosin VI tail domain. However, a question remains whether the hydrophobic residues of the MT domains also contribute to dimerization. To address this question, we reasoned that disrupting hydrophobic residues in the MT should inhibit dimerization if hydrophobic interactions were the predominant dimerization mechanism. Specifically, five glycines were substituted for hydrophobic residues in the MVI-940 construct, namely, L909, L913, L926, I929, and M933.

Again the actin saturation method (with 50 nM of myosin concentration) was employed. Surprisingly, the mutant 940 construct (a gift from the Sweeney lab) showed processive movements after ATP addition, indicating properly dimerized constructs. We then determined the step sizes of the mutated construct by tracking the position of a CY3 dye labeled on the IQ-CaM using the single-molecule FIONA technique. The mutant construct showed a typical stair-like displacement (Fig. 3.6), and the average step size (± standard deviation) was 53.3 ± 19.1 nm for forward steps (Fig. 3.7). This value is nearly indistinguishable from that of the wild-type (WT) 940 construct of 54.3 ± 19.4 nm (For experimental method, please refer to sections 3.2
and A.3).

After observing no significant difference between the step sizes of wild-type and mutant constructs, we proceeded to determine if there is a difference in the efficiency of dimerization. When experiments were performed in the same conditions, the percentages (± standard deviation) of processive myosin constructs for the mutant and the WT 940 constructs were 5.1±3.5 % and 6.6±4.3 %, respectively, showing again no significant difference between them. (See Fig. 3.8).

3.3.4. The dimerization of the 940 Mutant construct was also confirmed in MD*

The experiments in the previous section demonstrate that dimerization is still possible between the mutated 940 construct where five hydrophobic residues were mutated into five glycines. To further support our experimental observations, all-atom simulations* were performed for the both of the wild-type (WT) and mutant 940 construct MT segments (907-940). In the both all-atom simulations, the dimers remained associated, as shown in fig. 3.9A for the WT, and fig. 3.9B for the mutant type. Those simulation results are consistent with those we have seen from the experiments. To quantitatively address the dimerization propensity of both WT and mutant constructs, free energy calculations were performed on both dimers (the truncated MT segments; residue number 907-940) to provide an estimate of the dimer dissociation energy (See Fig. 3.10. Red and black traces represent the wild- and mutated types, respectively). The dimerization free

(*) The MD simulation was performed by the Schulten group. For smooth logical flows, the simulation results are also described in this dissertation.
energy was estimated from the plot of the potential of mean force (PMF) as a function of the separation of helices. The energies were calculated to be $15.8 \pm 0.4 \text{ kcal/mol}$ $(1.67 \pm 0.04 \text{ kcal/mol per helical turn})$ for the WT construct, and $12.6 \pm 0.2 \text{ kcal/mol}$ $(1.33 \pm 0.02 \text{ kcal/mol per helical turn})$ for the mutant construct. The calculation results suggest that the WT construct has a slightly higher (a few kcal/mol) dimerization strength, and this is consistent with the experiment where we found no significant difference between the ability to dimerize between the WT and mutant myosin VI 940 constructs. The figure 3.10 indicates the mutant dimer prefers closer helix-helix packing judging from the location of the local energy minimum. This is possibly due to the smaller side-chain size of the glycine.

3.4. Summary and discussion

The stable single $\alpha$-helix (SAH), which is the most prominent motif of the myosin VI MT domain, was first experimentally identified for myosin X among myosin superfamily members [80], and recently, several more charged single $\alpha$-helices (CSAH), particularly the ER/K motifs, have been identified [81, 92], with the MT domain of myosin VI being a prominent example. Although the properties of the ER/K motif have been investigated by various groups [81, 85, 92, 93], no detailed study on the interaction between two ER/K motifs in myosin VI had been reported so far. In our previous myosin VI publication ([74] and also see the previous chapter), we simply speculated that a few hydrophobic residues in the MT domain form a short coiled-coil structure. Indeed, the PAIRCOIL algorithm [94] predicts a strong coiled-coil tendency in the MT domain based on its sequence [65] (Fig. 3.11). However, it was known that PAIRCOIL could misrepresent CSAHs [92]. The MD simulation performed in the present study suggest that
electrostatic interactions through several interhelical salt bridges, made possible by a small vertical offset (~10 Å) between the MT helices, are a key contributor to the potential dimerization of MT segments. (However, the formation of salt bridges is possibly nonspecific; we do not claim that dimerized conformation observed in the simulation is the only possible one.) It is then understandable why our prior circular dichroism (CD) data of an artificially dimerized PT-MT construct (834-991-GCN4) did not find a significant amount of coiled-coil, but rather demonstrated a high level of α-helical structure (without forming a large segment of coiled coil along the entire MT domain). We do note, however, that formation of a short coiled coil from the few MT domain hydrophobic residues, not giving a discernable change in the CD spectra, is still possible.

The experimental observation of lower dimerization propensity with higher ionic strength in the buffer further implicates electrostatic interaction mediated by salt bridges to play a role in the MT domain dimerization. If formation of salt bridges were not the dimerization mechanism, we would not have been able to observe such a significant dimerization dependence on ionic strength of buffer. In a buffer with higher ionic strength, interstitial ions weaken interhelical interactions between two MT domains (and perhaps also intrahelical interactions that are responsible for the formation of SAH).

Additional evidence for a key role of salt bridges in the dimerizaton of the MT domains further supports our findings. If hydrophobic interactions were the only contributor to dimerization, then substitution of five hydrophobic residues in the MVI-940 construct (residue number 1-940) into five (small size) glycines should significantly lower the dimerization propensity of the
construct. However, we found that this is not the case. The single-molecule experiments showed that the mutant 940 construct actually does not show a significant decrease in terms of probability of dimerization compared to that of the WT 940 construct (5.1 % versus 6.6 %). It should be noted, though, that we do not rule out the participation of hydrophobic interactions in the dimerization of the MT helices, but it is unclear how much the hydrophobic residues actually contribute to the dimerization, given the close values (5.1 % versus 6.6 %). Certainly, the experiments with the mutant construct reveal that hydrophobic interactions (if any) are not the dominant dimerization mechanism and strongly suggest that there must be something else controlling dimerization. The MD simulation also appears to support these experimental observations. Calculations on the dimerization free energy of the WT and mutant dimers confirm that both constructs require significant amount of energy to disassociate.

Combining all experimental and simulation data, we are led to believe that the key role in the MT domain dimerization is played by the formation of interhelical salt bridges.

As mentioned in the previous chapter, two groups showed that cargo binding mediates dimerization of myosin VI [82, 83], implying that cargo binding plays a role in bringing two monomers close enough for self-association. According to our previous model (see the previous chapter and [74]), cargo binding initiated dimerization at the distal end of the tail domains brings about the dimerization of the more proximal parts via a short coiled-coil formation in the hydrophobic amino acid region of the MT domain; then the three-helix bundle of the PT domain unfolds and provides a large portion of the step size [74, 83]. Our present study modifies this model by suggesting that internal dimerization of the MT domain is held together via the
formation of salt bridges alone or in combination with a short coiled-coil (Fig. 3.12).

3.5. Figures for chapter 3

[Figure 3.1] Schematic diagram of myosin VI construct. This diagram is essentially the same as Fig. 2.1, but amino acid sequences of the MT domain are displayed on the right. Blue, red, and green colors denote positively and negatively charged residues and hydrophobic residues, respectively. Note that the most prominent feature of the MT domain is the alternating charged amino acid pattern (repeating four positive and four negative residues). At the proximal side (N-terminal side) of the MT domain contains small number of hydrophobic residues. In the previous chapter, we speculated that these properly spaced hydrophobic residues form a short segment of coiled-coil.
[Figure 3.2] MD simulation of the MT domain (residue number 907-980) dimerization. (A) RBCG simulation starts with two MT segments that are 30 Å apart. (B) At 950 ns, all-atom representation is recovered by the molecular dynamics flexible fitting (MDFF) method.

[Figure 3.3] Buried molecular surface area of the full-MT domain (residue number 907-980) in time is drawn in black. The red trace represents the buried molecular surface area of the hydrophobic residues.
[Figure 3.4] The contour map that depicts interactions between residues of the MT helices 1 and 2. Blue color in the map represents strong interactions. The main inter-helical interactions are seen to lie below the diagonal, indicating a vertical offset between the helices. Five insets shows salt bridge formations between positively-charged residues (in blue) and negatively-charged residues (in red).
[Figure 3.5] Percentage of properly dimerized myosins at different ionic strength. The percentages of processive myosins were measured with 52 and 149 mM buffer. The percentages were calculated from six separate measurements. A total of 764 and 1037 myosins were counted for buffers with 52 and 149 mM ionic strength, respectively. Error bars represent the standard deviations. The details of the buffer can be found at sections 3.2 and A.3.
[Figure 3.6] One example stepping graph of the mutant myosin VI 940 construct where five glycines were substituted for hydrophobic residues. Raw data were fitted by the Student’s t-test algorithm.

[Figure 3.7] Measured step size histogram for the mutant 940 construct. The average forward step size is $53.3 \pm 19.1$ nm ($N=143$) and the average backward step size is $-26.3$ nm ($N=1$).
[Figure 3.8] Comparison of the percentage of properly dimerized myosin VI for WT and mutant-type constructs. A fraction of 5.1±3.5 % and 6.6±4.3% (average±SD) of myosin VI was seen to be processive for mutant and WT constructs, respectively. Percentages were calculated from nine separate measurements; a total of 1007 and 995 myosins was counted for mutant and WT, respectively. Ionic strength of the buffer was 52 mM for the both cases.
All-atom molecular dynamics simulation support the experimental observation that both of the wild- and mutant-type 940 constructs are able to self-associate and form dimers. (A) WT-type truncated medial tail (MT) segments (907-980), (B) Mutant-type truncated MT segments (907-980).

Potential of mean force (PMF) as a function of separation between two MT truncated segments (907-940). Red trace for the wild-type construct and black trace for the mutant construct.
[Figure 3.11] PAIRCOIL [94] scores of a part of the myosin VI tail domain. A strong tendency of forming the coiled coil in the medial tail domain (highlighted in yellow) is predicted. The PAIRCOIL score was calculated from “http://groups.csail.mit.edu/cb/paircoil/cgi-bin/paircoil.cgi”
[Figure 3.12] Myosin VI working model. The color scheme is adopted from Figure 2.18 (Purple: CBD, Orange: DT domain, Green: MT domain, Blue: PT domain). This model is essentially almost the same as that in Figure 2.16. However, a difference (based on the experimental and simulation data) is that salt bridge formation of the MT domain is a key dimerization mechanism of the MT domain, resulting in ~10 Å spatial offset. A big grey sphere represents a cargo. The cargo brings two myosin VI monomers into close proximity, and it initiates dimerization.
APPENDIX

A.1. How to induce dimerization of the MVI-940 construct

To induce dimerization, we need a way to bring two monomers in close proximity. For this purpose, we employed either the antibody method or the actin-saturation method, as was developed by Park et al. [66] (See Fig. A.1). The antibody method takes advantage of the fact that the myosin construct has a FLAG tag (encoding GDYKDDDDD) [95, 96] at the end of the C-terminus for purification purposes. Incubation of the myosin construct with anti-FLAG antibodies allows two myosin VI monomers to stay very close with respect to each other. They can be used as they are, attached to an antibody, or can be used after removal of the antibodies by access to the FLAG peptide. For the actin saturation method, an actin filament is saturated with a high concentration of myosins in the absence of ATP. Adding ATP releases undimerized constructs, and successfully dimerized constructs are expected to walk processively.

A.2. Fluorescent labeling and studies of the MVI-940 construct

To measure the step size of the construct, a fluorescent dye needs to be attached to it. To track the stepping behavior of myosin VI with nanometer precision, it is important to maximize the number of photons collected during imaging with an EMCCD and collect images over long time periods during which many steps occur. The choice of a fluorescent label is governed by the need for bright, long-lasting fluorescence. There is substantial variation in photostability among organic dyes that are commonly used in fluorescent imaging. We count the total number of photons obtained from a fluorescent dye before it photobleaches to gauge (See Fig. A.2). Our
lab found out that CY3, Alexa 647, ATTO 647N, CF 633 are fairly photostable with optimized buffer condition. In this study, we selected CY3 as the dye for myosin VI 940 construct labeling due to its photostability and ability to react with our construct.

Having selected a dye, the next consideration is the attachment of the dye to the protein. CY3-maleimide reacts with Cysteine (Cys), but we were not able to label the specific position of myosin VI, since it has multiple Cysteines on it. Instead, a different strategy was employed using the fact that calmodulin (CaM) does not have any Cysteines on it. Instead of Threonine at 146 of CaM, single point mutation with Cysteine (T146C) was introduced. The mutated CaM reacts with CY3, and then myosin construct is mixed with the CY3-labeled CaMs. Increasing the calcium concentration of the buffer results in the detachment of wild-type CaMs from the IQ-domain of myosin VI. At a 100 µM concentration of calcium, insert-2 CaMs are reported to remain bounded to the insert-2 [51]. Once the native CaM has detached and been removed from the myosin VI in solution, the excess calcium ions are quenched by adding EGTA. This allows the CY3-labeled CaMs to bind to the IQ-motif. The labeling efficiency of this protocol is sufficiently low that a dimerized construct (if they can dimerize) is very unlikely to contain two CY3-CaMs.

A.3. Experimental method details

1. Flow chamber is prepared. Detailed explanations are found at later section A.5.

2. Following reagents and buffer are prepared in advance.
(1) M6 buffer
- 20 mM HEPES (pH 7.0-7.2) (Molecular weight: 238.3)
- 2 mM MgCl$_2$ (Molecular weight: 203.3)
- 25 mM KCl (Molecular weight: 74.56)
- 1 mM EGTA (Molecular weight: 468.28)
- Storage temperature: 4 °C

(2) M6+ buffer
- Add calmodulin (stock concentration: 0.55 mg/ml in M6 buffer) just before doing myosin VI experiment.
- Final calmodulin concentration in M6+ buffer is ~ 0.09 mg/ml.

(3) 10 mg/ml biotinylated BSA (Bovine Serum Albumin)
- 10 mg powder of albumin, biotinamidocaproyl labeled bovine (Sigma-Aldrich catalog number: A6043) is dissolved into 1 ml of M6 buffer
- Storage temperature: 4 °C

(4) 5 mg/ml neutravidin
- 10 mg powder of neutravidin (Thermo Scientific, Pierce catalog number: 31000) is dissolved into 2 ml of ddH$_2$O or buffer
- Storage temperature: 4 °C

(5) 10 mg/ml BSA (Bovine Serum Albumin)
- 10 mg powder of albumin from bovine serum (Sigma-Aldrich catalog number: A9085) is dissolved into 1 ml of M6 buffer
- Storage temperature: 4 °C

(6) 20 mg/ml casein
- Storage temperature: -20 °C (thaw it just before using it.)

(7) 4X buffer

- To make 50 mL of 4x F-buffer, mix up 4.47 g KCl, 0.41 g MgCl₂, and 1.91 g HEPES with ddH₂O and make its pH 7.0~7.2

- This buffer will be used in actin filament polymerization. Final buffer concentration after actin polymerization step will be 300 mM KCl, 10 mM MgCl₂, and 40 mM HEPES.

(8) Biotinylated F-actin

- G-actin (stock concentration: 181.9 μM) and biotinylated G-actin (stock concentration: 51.2 μM) are generous gifts from Sweeney lab.

- Phalloidin stock concentration is 1300 μM.

<!> When handling with phalloidin, special care is required.

- Final target concentrations are 2.00 μM, 0.33 μM, and 10.00 μM for G-actin and biotinylated G-actin and phalloidin, respectively.

- To make 100 μL of F-actin,

  1) In separate eppendorf tube, put 1.10 μL of G-actin.
  2) Add 0.64 μL of biotinylated G-actin.
  3) Wait for 1~2 minutes.
  4) Add 25.0 μL of F-buffer.
  5) Add 72.49 μL of ddH₂O.
  6) After 10~12 minutes, add 0.77 μL of phalloidin.
  7) Leave it at 4°C overnight.

3. Experimental procedures
(1) Add 1.6 mg/ml biotinylated BSA to flow chamber. When the biotinylated BSA was diluted from stock concentration, M6 buffer was used.

(2) After 5~10 minutes, wash excess biotinylated BSA with M6 buffer.

(3) Add 0.8 mg/ml neutravidin to flow chamber. When the neutravidin was diluted from stock concentration, M6 buffer was used.

(4) After ~5 minutes, wash excess neutravidin with M6 buffer.

(5) Add 20~25 times diluted F-actin to flow chamber. The F-actin has one wild-type G-actin and six biotinylated G-actin. When the F-actin was diluted from stock concentration, M6 buffer was used.

(6) After ~5 minutes, wash excess F-actin with M6 buffer.

(7) Add 5 mg/ml casein to block the surface of cover glass and prevent non-specific bindings.

(8) After 5~6 minutes, add desired (high) concentration of myosin VI construct to flow chamber. When the construct was diluted from stock concentration, M6+ buffer was used, and it also contains additional surface blocking proteins (0.8 mg/ml BSA, 0.4 mg/ml casein).

(9) Allow 5~10 minutes before step (10). Meantime, turn on laser (CrystaLaer), EMCCD (Andor Technology, Ixon+) camera, and so on. Adjust knobs in the focusing lens unit just nearby the microscope, and let the incoming laser light is focused on the back focal plane of an objective of the microscope. Also let the angle of incoming laser light be such that the total internal reflection (TIR) is achieved. For details on how to achieve the TIR, please refer to section A.6.

(10) Add imaging buffer including 20 μM ATP to flow chamber. This will initiate processive movements of the properly dimerized myosin construct. In addition to ATP, the imaging
buffer contained 2.5mM PCA (protocatechuic acid) / 50 nM PCD (protocatechuate-3,4-dioxygenase) as an oxygen scavenging system [16], 20% trolox (we saturated trolox in the buffer at physiological pH, then filtered, and 20 μl was used for a total of 100 μl imaging buffer) [18], and surface blocking proteins (0.8 mg/ml BSA, 0.4 mg/ml casein).

(Note) Instead of BSA-biotin and neutravidin linkage (See (1)~(4)) to immobilize F-actin, there are two other ways: One with using α-actinin [97], the other with NEM (N-ethylmaleimide)-modified skeletal muscle myosin [98]. Both methods are especially useful when myosin construct itself (or CaM itself) has a biotin on it.

A.4. Data analysis methods

The tracks of fluorescently labeled myosin VI are recorded in the TIFF file format and analyzed with custom image analysis software. The center coordinates of the diffraction-limited CY3 signal were calculated using the FIONA (Fluorescence Imaging with One-Nanometer Accuracy) technique [2] with code written in IDL (ITT visual information solution). Myosin steps were identified by a t-test also written in IDL. The IDL code for the FIONA can be found in the section A.8, and a brief explanation on the t-test can be found in the later section A.7. For the calculation of the percentage of properly dimerized myosins, myosins showing movements longer than 3 pixels (around 0.3 μm) after adding ATP were considered to be properly dimerized processive myosins. The percentage was calculated from the number of moving spots, which satisfied this threshold, divided by the number of total spots in the field-of-view (around 30x30 pixels) of interest.
A.5. Flow chamber preparation (Related to section A.3)

1. Put a glass slide on clean kim wipe, and mark two spots where holes will be made (Fig. A.3).

2. Bring the glass slide to electrical drill. Properly position the glass slide so that hole will be made at the right spot where you intended with the mark. Put some water on the glass slide before drilling (Fig. A.4), and make sure that right drill bit is used. If the diameter of the drill bit is big, then it will cause a difficulty in adding solution to the interior of the chamber. We use the one (catalog number: 1-0500-100, size: 3/4 mm) from Kingsley North Inc. (www.kingsleynorth.com)

3. Turn on the power of the drill and make two holes by contacting the drill bit. Try to push the both sides of drill handle, and pay special attention to safety concerns (Fig. A.5).

4. Wash the surface briefly with ddH₂O by rubbing it by a hand (with a glove on it).

5. Put the glass slides in the beaker. Also put cover glasses on rack and then, put them in different beaker. The material of the rack should be chemical-resistant, for example, teflon. We use Invitrogen C-14784 for the rack. Pour acetone into beakers, then sonicate them for 20 minutes (Fig. A.6). Then dispose of acetone and fill the beakers with 1 M methanol, instead. Sonicate them for another 20 minutes.

6. Dispose of the methanol, and wash cover glasses and glass slides with ddH₂O.
7. Put a clean lens cleaning paper on the desk, and put glass slide on it. Attach double-sided tape, and put cover glass over the tape. Rub the surface of the cover glass smoothly. This will allow tight taping and removal of air between the tape and cover glass, or between the tape and glass slide.

8. Remove excess tape, and epoxy the side area of cover glass.

9. Final flow chamber will look like Fig. A.7.

A.6. How to achieve TIR (Related to section A.3)

1. First adjust the height of objective and image a fluorescent bead sample. When you see a clear image, stop changing the objective height. For this step, the incident angle of the laser light is not critical.

2. Remove the bead sample from the microscope and send the laser beam to the ceiling (See Fig. A.8). This can be done by adjusting the knobs of the TIR lens (also called a focusing lens).

3. Check the laser beam shape on the ceiling. If incoming laser light was focused onto the back focal plane of the objective, you will see small compact circle shape collimated beam on the ceiling. Otherwise, the size of the beam will be larger as seen at the bottom right in the Fig. A.8. In the case that the incoming laser light is not focused the back focal plane of the objective, it can be fixed by rotating knob (B) (micrometer (B)) of the focusing lens (TIR lens) unit (Fig.
A.9. Rotating the knob (B) will change the position of the lens toward or away from microscope. (Fig. A.9)

4. Using knobs (A) or (C) in Fig. A.9, change the outgoing beam angle (See Fig. A.10). Put bead sample back on the objective, and image it. Increase the outgoing angle until it is totally internally reflected as far as possible. This will be at a point just before the illumination region disappears completely.

A.7. Student’s t-test (Related to section A.4)

To locate myosin steps in our data, we used the Student’s t-test instead of using an “eye”. The t-test is a very well-known statistical tool in comparing two samples, and is slightly modified by Sheyum Shed (former Selvin lab member) to be used specifically in finding steps of molecular motors as briefly explained below.

1. From raw data, pick a point “j”. Make two groups before and after the point j. The number of data points in the group is designated as “g”, where g=2, 3, …, n. (See Fig. A.11.)

2. Calculate weighted averages and standard deviations of these two groups.

\[
X_{jk} = \frac{\sum_{i}^{i+g-1} w_i x_i}{\sum_{i}^{i+g-1} w_i}
\]

\[
\sigma^2_{jk} = \frac{g \sum_{i}^{i+g-1} w_i (x_i - \bar{x}_i)^2}{(g - 1) \sum_{i}^{i+g-1} w_i}
\]
where \( k = 1, 2 \) (j1 and j2 represent groups before and after the point “j”), and \( w \) is weight.

3. Now calculate the t-value.

\[
t_j = \frac{x_{j1} - x_{j2}}{\sqrt{(\sigma_{ji}^2 + \sigma_{j2}^2) / g}}
\]

where the denominator is the standard error of the mean.

4. Calculate \( P_j \) (probability that difference between mean values of group j1 and j2 is statistically significant). \( P_j \) is calculated from incomplete \( \beta \) function. Compare it with threshold value to see whether or not step exists.

\[
P_j = I\beta = \frac{\eta(\eta + t^2)}{\eta^2} \int_0^\eta x^{\eta/2-1} (1 - x)^{-\frac{1}{2}} \, dx
\]

where \( \eta = 2(g-1) \)

5. All of t-test calculations are done by IDL software programming (ITT visual information solutions) coded for by Sheyum Shed (former Selvin lab member). However, the IDL code does not know what are optimal group number and threshold value. Thus, in reality, we designate the minimum and maximum numbers of groups, and the minimum and maximum values of threshold in the code. The code uses various combinations of them, and calculates reduced chi-square values against raw data, then determines the optimized values and fitting.

In addition to the t-test method, there are a few other step determining methods such as the hidden Markov method (HMM) [99, 100], and Kerssemakers et al.’s step-finding algorithm
A.8. IDL code for FIONA (Related to section A.4)

In this section, IDL codes necessary in doing FIONA fitting with motor protein data are displayed.

1. In the full EMCCD field-of-view, there are multiple CY3 signals. Below code is to chop the image into smaller one so that only one CY3 signal is seen.

   (1) IDL code

   ```idl
   PRO spotslicerE, x, y, s, startFrame, endFrame, inFile, outFile
   subFrameVec = [(x-s/2), (y-s/2), s, s]
   for imageIndex=startFrame, endFrame do begin
       frame = read_tiff(inFile, IMAGE_INDEX=imageIndex-1, SUB_RECT=subFrameVec)
       write_tiff, outFile, frame, /APPEND, /LONG
   endfor
   end
   ```

   (2) Example

   ```idl
   spotslicere, 100, 200, 12, 1, 100, 'c:\data\myosin.tif', 'c:\image\chopped.tif'
   ```

   => It will chop ‘c:\data\myosin.tif” image into 12x12 pixel image around (x, y)=(100, 200)

   2. The next code will open the chopped image and do two-dimensional Gaussian FIONA fitting from the first frame to the last. Then the least-square-fitted line will be determined from all of
data point coordinates. (The line corresponds to actin-filament track.) Projected coordinates of the raw data onto the line will be recorded and saved as output file.

(1) IDL code

```
PRO stepperE, pix_size, d, filename

; open the file
openr, 1, filename+'.TIF'

; s is an object that contains information about the file
dummy = query_tiff(filename+'.TIF', s)

; This variable is left over from when the script was able to fit multiple spots. I set it to "1" rather than replace each instance of "spotnum" by hand.
spotnum = 1

; initialize the array that the frame to be fit is read into
frame = intarr(s.dimensions(0,0), s.dimensions(1,0))

small_array = intarr(15,15)

; initialize the array that stores result of the 2D Gaussian fit to small_array
fit = fltarr(s.num_images,spotnum,7)

; step through the data frame by frame and fit the spot to a two-dimensional gaussian using GAUSS2DFIT
for i=0, s.num_images-1 do begin
    frame = read_tiff(filename+'.TIF', image_index=i)
    print, i
    for j=0, spotnum-1 do begin
        small_array = frame(0:d-1, 0:d-1)
        dummy = GAUSS2DFIT(small_array, result, /tilt)
        fit(i,j,*) = result
```
; get rid of bad fit points by performing a simple sanity check
  for  \( z=0,5 \) do begin
    if \( \text{fit}(i,j,z) \lt 0 \) or \( \text{fit}(i,j,z) \gt 66000 \) THEN BEGIN

; arrays are always initialized with zeros.
; exploit this fact to replace all elements of a bad frame with zeros in one line of code
  \( \text{fit}(i,j,*) = \intarr(7) \)
  endif
endfor
endfor
endfor

; use this linear fit \((x,y)\) pairs of data to rotate bases so that the myosin steps all occur along one line,
; even if the myosin was not walking in the x- or y-direction.
; line is a two-component vector, \([A, B]\) where \( y = A + Bx \). the line is, essentially, the actin
line = linfit(fit(*,0,4)*pix_size, fit(*,0,5)*pix_size)
steps = fltarrr(s.num_images)
steps(0) = cos(atan(line(1)))*(fit(1,0,4) - fit(0,0,4)) + sin(atan(line(1)))*(fit(1,0,5) - fit(0,0,5))
for i=1, s.num_images-2 do begin
  steps(i) = (cos(atan(line(1)))*(fit(i+1,0,4) - fit(i,0,4)) + sin(atan(line(1)))*(fit(i+1,0,5) - fit(i,0,5)))*pix_size + steps(i-1)
endfor
steps(s.num_images-1) = steps(s.num_images-2)

; plot, fit(*,0,4), fit(*,0,5)

; close the file for reading
close,1

; open a new file for writing the fits. it will be a text file giving it the "csv" extension will cause
; Windows to associate it with a spreadsheet, which can make opening the file a little more convenient.
openw,1,filename+.csv

; now write everything to the file
for i=0,spotnum-1 do begin
  for j=0,s.num_images-1 do begin
printf 1, fit(j,i,1), fit(j,i,2), fit(j,i,3), fit(j,i,4)*pix_size, fit(j,i,5)*pix_size, steps(j)
endfor
endfor

; now close it
close, 1
end

(2) Example
steppere, 106.7, 12, ‘c:\image\chopped’

=> It will open the 12x12 pixel chopped image “chopped.tif” in ‘c:\image\’ folder. In final output file, the numbers are in nanometer by multiplying values in pixel by 106.7.

A.9. Comparison of three-helix bundles (Related to section 2.2.2)
The proximal part of the three-helix bundle of myosin VI has some stability by hydrophobic interactions (but, their side chains are short) and two hydrogen bonds. Its distal part is less stable due to the shortness of helix 1, giving rise to a loose packing of the hydrophobic core. However, other three-helix bundle structures (B and C of the Fig. A.12) show that the hydrophobic patch lies tightly along their whole length.

A.10. Recovery of all-atom resolution from the coarse-grained representation (Related to section 3.3.1)
Once the two MT monomers are seen to self-associate from the residue-based coarse-grained (RBCG) representation [102] where each amino acid is described by two beads (except glycine)
(Fig. A.13A), the beads are converted to atoms by placing the center of mass position of a group of atoms to that of a corresponding bead (Fig. A.13B). However, as seen in the inset of the Fig. A.13B, bonds are distorted in the AA representation. Thus, annealing cycle is needed to regain the proper bond length (Fig. A.13C). Although secondary structures of the MT domain are not fully recovered even after the annealing cycle, an artificial density map is generated (Fig. A.13D). Now, two all-atom MT domain segments that have the correct secondary structures are placed in the density map by rigid-body docking (Fig. A.13E), and then the segments are steered into the density map using the molecular dynamics flexible fitting (MDFF) method [89-91] (Fig. A.13F). The resulting all-atom structure (Fig. A.13G) will have the correct secondary structure characteristics with the overall conformation that matches with Fig. A.13A.

A.11. Self-association of the MT-907-940 (Related to section 3.3.1)

As is mentioned in the chapter 2, we have experimentally shown that the MVI-940 monomers, which lack the CBD, DT, and 40-residues of the MT, is induced to dimerize upon saturating the actin-filament [74]. Furthermore, the dimerization is strong enough that the dimeric MVI-940 construct can walk processively with average 0.9 μm run length when examined by the FIONA technique [74].

This experimental observation that the MVI-940 can dimerize was also confirmed by the MD simulation for the MT-917-940 domains. The spontaneous association for the truncated MT construct (MT-917-940) was seen in a 1-μs RBCG simulation, and 50-ns AA representation. Furthermore, the contact map for the dimer construct (Fig. A.14) shows that salt bridges are
formed between oppositely-charged amino acid residues, as was seen by strong interactions below diagonal of the contact map.

A.12. Figures for appendix

[Figure A.1] Two methods for inducing dimerization of monomer myosin VI constructs. (A) The antibody method takes advantage of the constructs having a short FLAG tag at the C-terminus. Incubation of myosins with anti-FLAG antibody allows two monomers to locate very close. (B) The actin saturation methods also allow monomers to stay very close by adding high concentration of myosin VI constructs onto the actin filament. Color schemes of myosin 940 construct are the same as those in Figure 2.1 except the motor domain. Note that amino acid residue 940 lies at (the middle of) the medial tail domain (in green). The F-actin figure in (B) is reproduced from [59], with permission.
[Figure A.2] An example of a histogram for the total number of photons collected by an EMCCD camera before an organic fluorescent dye photobleaches. For this specific example, it is for Atto532-labeled DNA oligos. The number is dependent on the photostability of the dye, emission filter set used in the microscope, buffer condition.

[Figure A.3] Using a Sharpie, make markers
[Figure A.4] Before drilling, put some water on the glass slide.

[Figure A.5] Apply equal forces to both drill handles.

[Figure A.6] Sonicator setup
[**Figure A.7**] Schematics of flow chamber (Top view, and side views)

[Figure A.8] (Left) Microscope setup. Laser light is intentionally sent to the ceiling. *(Top right)* Laser light image on the ceiling when incoming laser light is focused onto the back focal plane of the objective. *(Bottom right)* Laser light image on the ceiling when incoming laser light is not focused onto the back focal plane of the objective
[Figure A.9] There are three knobs (micrometers) in the focusing lens (TIR lens) unit. Knob (B) will move it toward or away from the microscope so that it enables us to focus the incoming laser light onto the back focal plane of the objective. Knobs (A) and (C) will change the angle of the beam out of the objective.

[Figure A.10] Turning the knob (A) or (C) in the previous figure will allow us to steer outgoing laser beam up and down, left and right. Use this ability to achieve the TIR.
[Figure A.11] Does the point “j” in this figure correspond to the first point of another step? To answer this question using the Student’s t-test, we group raw data before and after the point “j”, where the point “j” itself is included in the latter group. In this example, both groups circled in blue and green contain 5 raw data points, respectively, meaning that “g” is 5.
[Figure A.12] Hydrophobic core interactions of three helix-bundles. (A) Myosin VI proximal tail (PT) domain (by Houdusse group), (B) N-terminal domain of α-C protein (pdb 1YWM), (C) C-terminal sub domain of HSP70 (pdb 2P32).
[Figure A.13] Schematic diagrams depicting a recovery of all-atom (AA) resolution from a residue-based coarse graining (RBCG) representation. All of the simulations here are performed by the Schulten group. Detailed explanations can be found at the section A.10.
[Figure A.14] A contact map for the dimerized MT-917-940 construct. The significant inter-helical interactions are seen to lie consistently below the diagonal, indicating a spatial vertical offset between the helices. Salt bridge formations are identified as shown in the insets. Blue and red colors in the insets represent side chains of positively- and negatively-charged amino acids. The map is generated from the MD simulation done by the Schulten group.
REFERENCES


AUTHOR’S BIOGRAPHY

HyeongJun Kim was born in a harbor city, Masan, in Korea in 1979. He entered GyeongNam science high school for his interest in science. In 1998, he entered Yonsei University in Seoul, Korea, majored in physics, and graduated with Magna Cum Laude. While a student in Yonsei University, he also studied at the University of California at Santa Barbara as an exchange student. After graduation, he worked at EPIPLUS, a company in GyeongGi-Do, Korea, as a part of the national obligatory duties. He joined a physics PhD program of the University of Illinois at Urbana-Champaign in the summer of 2005, and joined the Selvin lab in 2006.

During his graduate study, he published papers as follows:


