### STUDIES INTO MOLECULAR MECHANISMS OF SKELETAL MUSCLE CONTRACTION: APPLICATIONS TO TRANSGENIC MICE WITH INHERITED CARDIOMYOPATHIES

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#### LIST OF ABBREVIATIONS

**DTT** Dithiothreitol

EDC – 1 ethyl 3 (3'dimethylaminopropyl) carbodiimide

EDTA – Ethylene diamine tetra acetic acid

EGTA - Ethylene glycol bis( $\beta$ -aminoethyl)ether

ELC - Essential light chain

FHC - Familial hypertrophic cardiomyopathy

RLC - Regulatory light chain

TIRF - Total internal reflection fluorescence

FCS - Fluorescence Correlation Spectroscopy

TMRIA – tetramethylrhodamine 5 iodoacetamide dihydroiodide (5-TMRIA) (single

isomer)

SeTau – SeTau 647 mono maleimide

APD - Avalanche PhotoDiode

ACF - AutoCorrelation Function

DV - Detection Volume

FWHM - Full width at half maximum

PF - Polarization of fluorescence

S1 Myosin subfragment-1

- LMM Light Meromyosin
- SMD Single molecule detection
- STED Stimulated emission detection
- SRX Super relaxed state of muscle
- HS half sarcomere
- MLCK Myosin light chain kinase
- ECV Elliptical Confocal Volume
- FLC Fluorescence Lifetime Correlation
- TFP TriFluoroperazine

#### **CHAPTER 1**

#### **INTRODUCTION**

History of Muscle Research: One of the most widely researched areas of biology is muscular contraction. Studies on muscle research dates back to antiquity. Erasistratus, a famous anatomist and physiologist of III century B.C (Greek era) attributed the motion inherent in all living beings (spiritus animalis) to muscle. Galen, a roman physician of II century A.D delineated further that heart muscle pumps blood to circulate into arteries and veins. Leonardo da Vinci a renaissance man drew many anatomical images of musculoskeletal system as the basis for force generation. Hermann von Helmholtz, a German physician of 18 century explained the mechanism of energy conservation in his work on muscle metabolism. It was in 1864 that Kühne isolated the viscous protein from muscle and named it myosin, he also accounted myosin for the rigor mortis state of a dead body. Modern era begins with Muralt and Edsall (1935) who demonstrated the strong birefringent nature of myosin in solution because of its uniform shape and size. Engelhardt and Ljubimova (1939) purified fractions of myosin which had adenosinetriphosphatase activity. In 1942 at the University of Szeged, Bruno Straub along with his mentor Andrew Szent Györgi extracted a protein with low viscosity at low salt concentrations from muscle. They called this protein Actin and described that it is the interaction of two proteins actin and myosin responsible for muscular contraction <sup>4</sup>. Sliding filament theory proposed by Andrew F. Huxley and H.E. Huxley <sup>5</sup> in 1954 is the widely accepted model for the mechanism of muscle contraction <sup>6</sup>. The theory basically suggests that rotation of myosin cross-bridges pull actin thin filaments to generate force <sup>7</sup>.

<u>Myosin:</u> Myosin is a classical ATPase enzyme where it captures the energy by splitting ATP to generate directional force with a conformational change, its crystal structure was first determined by Rayment et al <sup>8</sup>. Of the several domains of myosin, cross-bridge or S1 is the main functional domain (Fig 1A).



Figure 1: A. Subdomains of S1 subunit of myosin <sup>1</sup>. The catalytic and the force generating domain of myosin is the cross-bridge or S1. The sub-domains of S1 include an Upper 50K domain (red), lower 50K domain or the actin binding domain, N terminus 25K domain (green), C terminus 20K domain, calmodulin like domains essential light chain (yellow) and regulatory light chain (magenta). B. Schematic of myosin molecule and the various fragments generated by enzymatic digestion (LMM – Light meromyosin, HMM – Heavy meromyosin) <sup>3</sup>.

Genetic cloning and expression of different subdomains of actin and myosin filaments lead to the identification of the critical role performed by each sub-domain. Myosin is made of a heavy chain and two light chains (120kDa). Cloned HMM (Heavy Mero Myosin) domain retains ATPase activity and can achieve directional movement in in vitro motility assays <sup>1,9</sup>. Fig. 1 A represents the tadpole shaped functional swinging cross-bridge of myosin. The boundary between the upper 50K domain and the N terminus 25K domain possess the site for nucleotide binding. It is about 5nm apart to the actin binding domain. X-ray crystallographic analysis provided evidence that cleft between the upper 50K domain and the lower 50K domain opens when actomyosin complex is formed and closes when myosin detaches from actin <sup>10</sup>.

<u>Kinetic Cycle of Muscular Contraction:</u> A breakthrough work by Ed Taylor and Richard Lymn led to the Lymn-Taylor model (1971) to explain the kinetic cycle of muscle contraction coupled to ATP hydrolysis <sup>2</sup>. The kinetic cycle of muscular contraction has four basic steps (Fig. 2) consisting of (1) ATP binding and detachment of myosin head from actin filaments (2) ATP hydrolysis (3) Binding of myosin head to actin filament with ADP and Pi (Pre-Power stroke) (4). Phosphate release triggers a force productive conformational change in myosin head (Post Power stroke). The myosin cross-bridge kinetic cycle has been extensively studied through biochemical assays and the presence of distinct conformational states of myosin has been speculated through in vitro and X ray diffraction studies. However, power stroke states of myosin cross-bridges were not shown in muscle and my first part of thesis project focuses on this aspect.



Figure 1: Lymn-Taylor model to explain the force productive mechanical changes in myofilaments coupled to ATP hydrolysis<sup>2</sup>. The model provides understanding of the mechanochemical steps during muscle contraction. Binding of ATP to myosin head and detachment of the actomyosin state initiates the cycle. Energy derived by splitting of ATP is utilized by myosin to extend and bind to actin. Phosphate release triggers a force productive conformational change in myosin head with the simultaneous pulling of actin filaments inwards (sarcomere shortening). ADP release reinforces the actomyosin bond characteristic of rigor state and completes one cycle of contraction.

<u>Swinging lever arm model of muscle contraction</u>: With emerging time resolved high resolution images of myosin II it has been postulated that during the working stroke of contraction, the upper 50k and lower 50k domain undergoes ~45° angular shift to bring the two domains to the upright 90° conformation.



Figure 2: Conformation changes in myosin S1 domain during power stroke of contraction. The picture depicts that it is the lever arm of myosin that undergoes majority of conformation change during contraction. It amplifies the subtle change in the myosin head domain.

The small change in the upper domains is amplified by the converter domain which is bound in a socket, resulting in a 70 ° angular shift of the lever arm consisting of ELC and RLC (Fig. 3). Since the majority of the conformational change during power stroke is localized to the lever arm, the swinging cross bridge model of muscle contraction is modified to swinging lever arm model of contraction <sup>11,12</sup>. Therefore tremendous research effort has been attempted to correlate the kinetics and biomechanics of lever arm during contraction. Recently polarized total internal reflection fluorescence technique was exploited to measure the azimuthal angular conformation undergone by myosin V lever arm <sup>13</sup>. Many other studies were attempted using fluorescent and paramagnetic probes at different sites on myosin and actin to reveal the conformational changes of actomyosin during contraction <sup>14-21</sup>. Much of the earlier studies come from synthesized actin

and myosin subdomains, where the chemo-mechanical steps don't reflect the original properties of muscle. Myosin II, myosin V and other non-conventional myosins were evolved to perform dynamically in a crowded environment. Therefore it is essential to study the actomyosin interaction in live working muscle. In live working myofibrils millions of actin and myosin molecules interact asynchronously to generate force. In order to study the conformational changes of myosin molecule, one has to sample only few cross-bridges. This is because the average value obtained by sampling millions of molecules doesn't reflect the precise spatiotemporal conformations of myosin. Therefore my study is aimed at elucidating the orientation changes of myosin at single molecule regime through a sensitive time resolved fluorescence polarization based assay.

Fluorescence Polarization Assay: The technique I have used for studying cross-bridges dynamics is called fluorescence polarization and was discovered by Jean Perrin in 1926 and later elaborated by Gregorio Webber in 1950's. When plane polarized light is used to excite a fluorophore, the emitted light is both vertically and horizontally polarized. The ratiometric difference of the intensities of the parallel and perpendicular emitted light is given by the formula Fluorescence Polarization FP =  $I_{\parallel}$ -I $\perp$  /  $I_{\parallel}$ +I $\perp$ . The optical alignment for fluorescence polarization assay in my experiments is schematically illustrated in figure 4. The absorption of light by a fluorophore is maximum when the electric dipole moment of incident light and the fluorophore are parallel to each other. Therefore, when polarized light impinges on a fluorophore immobilized on myosin cross-bridge, the absorption and fluorescence emission reflects the conformation of cross-bridges.



Figure 3: Optical alignment for fluorescence polarization assay on myofibrils. Alba Fluorescence Correlation Spectrometer (FCS) was used to follow the sensitive orientational changes of actin and myosin molecules during the different stages of muscular contraction. Parallel polarized light is used to excite the fluorescent probes immobilized on myofibrils in confocal volume. The emission fluorescence signal is split into 50/50, where one half is received by detector 1 with parallel polarized light and other half is received by detector 2 with perpendicular polarized light (Indicated by arrows). Picture adapted from ISS Website.

This is the working principle behind my research and this fundamental technique will be used to study the molecular aspects of muscle contraction in rabbit psoas myofibrils and to elucidate the molecular basis of familial hypertrophic cardiomyopathy. It is to be noted that the value of FP is characteristic of the orientation of fluorophore.

Familial Hypertrophic Cardiomyopathy: The first scientific problem to which I applied this technique is to the serious disease of the cardiac muscle, Familial Hypertrophic Cardiomyopathy (FHC). FHC is characterized by the increase in the ventricle wall thickness and myocyte disarray <sup>22,23</sup>. Abnormal mutations in the myocardium lead to compromised function of the heart. This results in activation of compensatory mechanisms that puts heavy load on the heart for pumping sufficient blood, which ultimately culminates in increased muscle mass in the heart tissue and hypertrophy. FHC affects 1 in 500 individuals and is the leading cause of sudden cardiac death (SCD) of younger individuals. The disease originates as classical autosomal dominant mutation in sarcomeric proteins with variable penetrance and expression. Over 140 mutations in the sarcomeric proteins were identified, 60% of which are clinically associated with FHC <sup>24</sup>. Idiopathic restrictive cardiomyopathy in children routinely goes undiagnosed and is also caused by mutations in sarcomeric proteins in heart <sup>25</sup>. Much of the pathophysiology of the disease is attributed to obstruction of blood outflow from the lower left chamber due to the thickened hypertrophic wall. Classical symptoms include dyspnea, palpitations, arrhythmias, syncope and death due to myocardial infarction <sup>24</sup>. It is also known that left ventricular hypertrophy, asymmetrical and heavy hearts are closely associated with ischemic stroke <sup>26</sup>. The disease has poor prognosis arising from heterogeneity of the mutations in the sarcomeric proteins regulating muscle contraction  $^{27}$ .

Cardiac muscle is involuntary striated muscle made up of alternating thick myosin and thin actin filaments. Cross-bridges are formed during muscle contraction by the association of myosin head to actin filament. Synchronized action of actin and myosin filaments is required for propelling blood from atria into the ventricles and into the blood vessels to enter the circulatory system. Mutations in myocardial proteins are clinically associated with several cardiovascular diseases

including FHC and coronary artery disease (CAD) leading to myocardial infarction (MI) <sup>24,28</sup>. The proposed work here addresses single point mutations in the ventricular muscle with malignant outcomes. Much of the pathology germane to these mutations arises because of altered myocyte structure and function <sup>29</sup>. FHC mutations in different sarcomeric proteins are listed in Table 1 and schematically represented in Fig. 5.

Protein	Gene	# of mutations
$\beta$ cardiac myosin heavy chain	MYH7	116
Myosin Regulatory light Chain	MYL2	10
Myosin Essential Light Chain	MYL3	3
Troponin T	TNNT2	27
Troponin I	TNN13	24
Tropomyosin	TPM1	10
Actin	ACTC	5
Myosin binding protein C	MYBPC3	72
Titin	TTN	1

Table 1: Mutations in sarcomeric proteins known to induce FHC



Figure 4: Mutations in Sarcomeric proteins leading to FHC

Most of the point mutations have been localized to myosin heavy chain and myosin binding protein C. These mutations have been extensively researched because of their frequency in clinical outcomes. Mutations in contractile proteins basically induce three different types of cardiomyopathies- hypertrophic, restrictive and dilated cardiomyopathies. Hypertrophic cardiomyopathies are the most common among them, and characterized by ventricular wall thickness and inefficient blood pumping. Restrictive cardiomyopathies are characterized by diastolic dysfunction arising from inability of the heart to relax completely. Dilated cardiomyopathies are more rare and characterized by dilation of part of the ventricular muscle leading to systolic dysfunction. Some of the debilitating mutations known to induce sudden cardiac death are localized to TNT, RLC and ELC proteins. My thesis work involves studying the molecular mechanism in cardiomyopathies induced by single point mutations listed in table 2.

Mutated Protein in the Sarcomere	Single Point Mutations
Troponin-T (TNT)	Arginine replaced with Cysteine at 278 (R278C)
Troponin-T (TNT)	Isoleucine replaced with Asparagine at 79 (I79N)
Troponin-T (TNT)	Phenylalanine replaced with Isoleucine at 110 (F110I)
Regulatory Light Chain of Myosin	Alanine replaced with Threonine at 13 (A13T)
Regulatory Light Chain of Myosin	Arginine replaced with Glutamine at 58 (R58Q)
Essential Light Chain of Myosin	Alanine replaced with Glycine at 57 (A57G)
Troponin – I (TNI)	Arginine replaced with Tryptophan at 145 (R145W)
Troponin – I (TNI)	Arginine replaced with Cysteine at 21 (R21C)

Table 2: Various single point mutations leading to FHC that will be characterized as part of my thesis project

Cardiovascular diseases rank the highest in the mortality rate and accounts for 650,000 deaths each year. Current therapeutics widely used for inherited cardiomyopathies are indirect and have systemic effect. Drugs targeting neurohormonal receptors such as  $\beta$ -blockers and Ca<sup>2+</sup> ion antagonists have many side effects such as elevated oxygen consumption, hypotension which exacerbate the condition and increases the mortality rate <sup>30</sup>. While these drugs offer a temporary symptomatic relief, they offer little benefits in terms of ameliorating the disease itself or in terms of regression of hypertrophy. Therefore, a great deal of research is focused on early identification of the disease and understanding the molecular derangements responsible for progression to clinical hypertrophic condition. I hypothesized that the transgenic mutant myocardium will show altered contractile properties owing to the degree of cross-bridge disarray. Such disarray may form a basis for rapid and inexpensive diagnosis of the disease. Currently, the underlying pathophysiologic processes responsible for clinical hypertrophic heart condition in these mutants remain obscure. My study will give deeper insight into the understanding of the root cause for the pathophysiology of FHC and possibly lead to therapeutic interventions.

Literature Review: Orientation of a cross-bridge is widely used as a parameter of the state of muscle. The conventional measurements of orientation, such as made by the wide-field fluorescence microscopy <sup>16,19,31,32</sup> or by Electron Paramagnetic Resonance (EPR), report the average orientation of 10<sup>12</sup>-10<sup>9</sup> myosin cross-bridges. Under the conditions where all the cross-bridges are immobile and assume the same orientation, for example in normal skeletal muscle in rigor, it is possible to determine the average orientation from such global measurements <sup>17,33-36</sup>. But when one measures signal from 10<sup>12</sup> cross-bridges arranged randomly along axis of muscle fiber, the error associated with the orientation is only 10<sup>-4</sup>% of the average value. The observable

error is contributed mainly by the shot noise of the detector and carries no information about the error in cross-bridge order. But the error associated with orientation carries important information about muscle, for example the degree of order may be disturbed because of a disease. The situation is exacerbated in transgenic (Tg) muscle in which a given protein mutation may be expressed at the low level. Suppose it is expressed at 10% level. The result is that muscle contains a mixture of 90% wild-type (WT) and 10% of mutated (MUT) protein. Such a small fraction of MUT protein may not lead to a significant change in the global contractile properties of muscle because the instrumental error and variation between muscles may be more than 10%. On the other hand, if only one cross-bridge is studied, 1 out of 10 experiments is expected to show 100% difference with WT cross-bridge. If two cross-bridges are studied 50% difference with WT cross-bridge is expected etc.

The best way to avoid averaging is to observe a signal originating from a single molecule. Fluorescence is the only signal strong enough to be used in this way. Motion of single molecules of contractile proteins in vitro has been studied extensively. Thus using fluorescence of rhodamine incorporated into smooth myosin it was possible to determine its conformational states <sup>37</sup> and the use of total internal reflection fluorescence (TIRF) to measure orientation of the light chain <sup>38</sup>. Using quantum dots it has been possible to observe diffusive movement of processive kinesin-1 on microtubules <sup>39</sup>. Motion of single molecule of kinesin labeled with Cy3 was determined to occur in hand-over-hand fashion <sup>40</sup>, myosin VI labeled with GFP or Cy3 was seen stepping via a <sup>41</sup> hand-over-hand mechanism <sup>42</sup>. Motion of myosin V, because of its large tail domain, has been investigated extensively. Thus three-dimensional structural dynamics of myosin V was measured by single-molecule fluorescence polarization <sup>43</sup>, processive motion of the lever arm of and head movements of myosin V have been observed simultaneously <sup>44</sup>. Also stepping and structural dynamics of unconventional myosin X has been determined <sup>45</sup>.

To observe single molecules in vivo is much more complex, mainly because of high local protein concentration and complicating effects of light scattering and autofluorescence. Particularly successful methods for decreasing the effective concentration involved the temporal separation of fluorophores. Central to these methods is the idea that the center of a point spread function (PSF) of the microscope objective can be determined with higher precision than its full width at half maximum (FWHM), and thus as long as only one molecule at a time exists in the detection volume, its precise location may be determined from the center of the diffraction expanded PSF. The effective concentration of fluorophores within a living cell is reduced to the necessary level by randomly activating/deactivating them with light pulses. The practical realization of this idea resulted in different flavors of PALM and STORM microscopy, where fluorescent images of a cell are obtained when fluorophores are randomly activated. The images are recorded and every molecule is analyzed computationally to determine the center of its PSF. This results in beautiful images with resolution far below the diffraction limit of conventional microscopes but the time resolution of the method is low. A related method of overcoming the diffraction limit involves minimizing the observational volume. In this technique, like in conventional confocal microscopy, fluorescent probes are attached to the molecules of interest and are excited by a focused laser beam and observed through a confocal aperture. However, unlike PALM, fluorophores are not randomly activated, but most of their fluorescence is depleted by a second, red-shifted, doughnut-shaped laser beam which is made to illuminate the sample before spontaneous emission of fluorescence occurs. This second beam stimulates the transition of

excited molecules back to their ground state. Stimulated emission has the same red-shifted wavelength as the doughnut-shaped beam. A bandpass filter rejects the stimulated photons and allows collection of the shorter wavelength fluorescence photons, which originate from the center of the doughnut. The second beam effectively quenches a subset of fluorophores at the periphery of the PSF, thereby reducing the effective PSF to the diameter of the "doughnut hole". The PSF produced by the non-quenched fluorophores is ~10 times smaller than that in a conventional or confocal microscope <sup>46,47</sup>. Therefore, STED microscopes produce spectacular images of the insides of cells <sup>48,49</sup>, but like PALM, STORM and related methods are too slow to follow rapid conformational changes.

<u>Mesoscopic Approach</u>: To avoid these problems, it is necessary to decrease the number of observed cross-bridges to a mesoscopic value (i.e. the value affected by fluctuations around the average ). In such mesoscopic regime, the averaging of the signal is minimal and steady-state behavior, whether in contracting, relaxing or rigor muscle can be examined in great detail. If the number of observed cross-bridges is, say 9, and they are arranged completely randomly along muscle axis, the error would be 33%, much larger than shot noise of the photodetector. So any observed error originates with the cross-bridges, not with the detector. In other words, one is able to quantify the degree of order.

To achieve the required time resolution in experiments on skeletal or cardiac muscle, I have adopted a different approach. The number of observed molecules is equal to the detection volume (DV) multiplied by the concentration of the fluorophore. Our approach has been to limit the DV and to minimize the concentration of fluorophore. The effective way to minimize the volume has been to use TIRF microscopy to minimize the height of the DV and to use a confocal

aperture to minimize its lateral dimension <sup>50</sup>. Alternatively, around-the-objective total internal reflection fluorescence microscopy can be used <sup>52</sup>. The volume could be further minimized by observing Surface Plasmon Coupled Emission (SPCE) fluorescence <sup>53-56</sup>, by two photon fluorescence or by Reverse Kretchmann illumination<sup>20</sup>. Ultimately, we found that the most effective way to decrease the DV was to use single myofibril as a sample. In this case the height of DV is equal to the height of a myofibril, which is typically 0.5 µm. This has the advantage that one does not have to rely on wide-field detection such as TIRF, which gives higher background autofluorescence from the glass elements of the objective where the exciting beam is focused. Instead, confocal point scanning detection can be used. At the same time we minimize the concentration of fluorophore by adding to a myofibril a small concentration of a probe without impairing ability of muscle to contract. If the degree of labeling is small enough there is only a few molecules in DV. In my experiments either myosin or actin are labeled. The myosin Light Chain 1 (LC1) is labeled with a fluorescent dye and exchanged with the native LC1 of a myofibril. The exchange is deliberately inefficient so that only one in ~60,000 myosin molecules carries the fluorophore. A small volume within the labeled myofibril (~1 fL, single halfsarcomere), is observed by confocal microscopy. For a myofibril that is inefficiently labeled, this volume contains only 6-9 fluorescent cross-bridges. This point is made schematically in Figure 6: The exciting light beam (green hourglass) is focused to a diffraction limited volume positioned on an A-band of a myofibril. The entrance aperture of the objective was slightly overfilled resulting in a better image at the expense of narrowness of FWHM. The Elliptical Confocal Volume (ECV) of the microscope (outlined by the dashed line) was estimated by measuring FWHM of 200 nm fluorescent beads imaged in X-Y and X-Z planes (Figure 6B). The FWHM's of the resulting Gaussians were 400 nm and 700 nm, respectively, and defined the waist ( $\omega_0$ ) and

height ( $z_o$ ) of the elliptical ECV =  $(\pi/2)^{3/2} \omega_o^2 z_o^{58,59} = 0.6 \ \mu\text{m}^3$ . Since ECV =  $(1/2)^{3/2} \cdot \text{DV}$  this gives DV = 1.7  $\mu\text{m}^3$ . ECV agrees well with the theoretical calculated value: ECV =  $(\pi/2)^{3/2} \omega_o^2 z_o^2 = 0.5 \ \mu\text{m}^3$  where a waist  $\omega_o$  is 0.5  $\mu\text{m}$  [i.e. equal to the diameter of the confocal pinhole divided by the magnification of the objective (60x)] and  $z_o = 1 \ \mu\text{m}$  is equal to the thickness of a typical myofibril.



Figure 6: A. The principle of observing few molecules in muscle, a small fraction of myosin carries fluorescently labeled essential Light Chain 1 (LC1, red) characterized by a single transition dipole (red arrow). The dynamics of the fluorophore is determined by periodically measuring polarization of fluorescence and calculating its autocorrelation function. The dispersion,  $\Delta \alpha$ , of the lever arm orientations is calculated from parallel ( $\parallel$ ) and perpendicular ( $\perp$ ) components the emitted fluorescent light. B: The beam profiles along X-Z and X-Y axes

The DV is less than the volume of a typical half-sarcomere. The number of myosin molecules in DV is determined experimentally by Fluorescence Correlation Spectroscopy (FCS) from the number of fluctuations of the free SeTau dye. The number of molecules (N) contributing to the autocorrelation function of these fluctuations is equal to the inverse of the value of the autocorrelation function at delay time 0 [G<sub>(0)</sub>], N=1/G<sub>(0)</sub><sup>60,61</sup>. The ACF's were obtained for

solutions of fluorophore in the range 5-20 nM. To eliminate contributions from random events and after pulsing, cross-correlation function between ch1 and ch2 was computed.



Figure 7: Estimating the number of molecules in the detection volume: The numbers of molecules in the DV were obtained from the inverse of the autocorrelation function (shown in the inset) extrapolated to a lag time 0. Average diffusion coefficient of SeTau and its correlation time were 250  $\mu$ m<sup>2</sup>/s and 10.5 ms, respectively. Inset autocorrelation function of 10 nM SeTau solution giving 6 molecules. The laser power 0.1  $\mu$ W

The  $1/G_{(0)}$  is plotted vs. number of SeTau molecules in the DV in Fig. 7. Extrapolation reveals that the concentration contributed by one molecule of the dye corresponds to ~75 counts per channel, i.e. the total fluorescence ( $I_{total}=I_{\parallel}+2*I_{\perp}$ ) from one molecule of SeTau is 225 counts/s. In a typical experiment we have signal of 10,000 counts/sec, i.e it comes from about 50 molecules,

Different states of muscle contraction such as nucleotide binding, hydrolysis and induced force parameters have been extensively investigated through biochemical, physiological, x-ray

diffraction and spectroscopic techniques. However, little is known on the spatial and temporal conformational changes of individual myosin cross-bridges in live crowded working muscle. It is crucial to follow the mechanical perturbations in myosin molecule after ATP hydrolysis. Research from non-muscle myosin, x-ray crystallographic studies and other in vitro assays have demonstrated the formation of two conformational changes in myosin during power stroke<sup>1</sup>. The first part of my thesis was aimed at elucidating the distribution of myosin cross-bridges during power stroke of muscle contraction. The question which I set to answer is whether spatial distribution of cross-bridges is different in different states of muscle. The second part of my thesis is focused on how the cross-bridge orientation and motility is being affected by regulatory light chain phosphorylation. One of the chief regulating steps during skeletal muscle contraction is the binding of  $Ca^{2+}$  ions to troponin complex and the induced conformational change in tropomyosin to expose the sites on actin for myosin binding. There is mounting evidence suggesting that Regulatory Light Chain (RLC) phosphorylation is another important regulating step during skeletal muscle contraction. Myosin light chain kinase (MLCK) phosphorylates (RLC) in a  $Ca^{2+}$  ion/Calmodulin dependent process. Phosphorylation of RLC drives myosin cross-bridges in relaxed state to activated form by increasing myosin ATPase activity. Recently, a new state of myosin cross-bridges called the super relaxed state (SRX) was characterized based on its slow ATPase activity <sup>62</sup>. One of my projects is dedicated to study the regulation of muscle contraction by RLC phosphorylation. The third part of my thesis work revolves around understanding the molecular basis of Familial Hypertrophic Cardiomyopathies (FHC). FHC is a genetically inherited disease caused by mutations in sarcomeric proteins necessary for pumping blood in the heart. FHC is the leading cause of Sudden Cardiac Death (SCD) in young athletes and teenagers and almost 1 in 500 individuals with FHC have SCD. Some of the common

symptoms include arrhythmias, dyspnea, angina, palpitations and ventricular hypertrophy. Humans are heterozygous for these mutations. Normal proteins and mutated proteins are interspersed in the diseased heart. This is one of the challenges to diagnose the condition at an early stage because of no apparent clinical symptoms. However, these cardiomyopathies remain silent until adulthood and are often surfaced by SCD. My project involves studying the deranged contractile properties at molecular level in transgenic mice with FHC. To summarize my thesis work is divided into the following 3 specific projects -

Project 1: Evidence for Pre- and Post-Power stroke states of cross-bridges of contracting skeletal myofibrils

Project 2: Orientation and rotational motion of cross-bridges containing phosphorylated and dephosphorylated regulatory light chain

Project 3: Motion and conformation of cross-bridges in healthy and hypertrophic hearts

### **CHAPTER 2**

#### **METHODS**

The following flowchart provides the experimental design for my research -



<u>Rabbit Skeletal Muscle Experiments:</u> The first and second project work of my thesis involved studies in rabbit psoas muscle ex-vivo. Upon arrival of rabbits, they were subjected to quarantine and maintained under IACUC approved protocols. Only rabbit psoas muscle was used for my studies. Psoas muscle obtained was immediately processed in cold room ( $4^{0}$ C) to make bundles of muscle fibers and preserved in glycerinating solution (150 mM KCl, 10 mM MgCl2, 5 mM EGTA, 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM ATP, 0.1% β-mercaptoethanol and 50% glycerol).

<u>Transgenic Mice Model:</u> A transgenic mice model was developed to assess the impaired cardiac function associated with familial hypertrophic cardiomyopathy disease. In these transgenic mice, human gene carrying mutations for sarcomeric proteins (Troponin-T and others) were expressed and hence the name transgene. Several transgenic mice bearing FHC mutations (Table 2) were bred by our collaborators at the Miller school of medicine, university of Miami. The degree of human gene expression in the Tg mouse hearts were analyzed by genotyping. All animals were anesthetized and euthanized according to the approved protocol by the animal care and use committee (IACUC). After euthanasia, the hearts were immediately frozen and stored at -  $80^{0}$ C until needed. The Tg hearts used in this study were ~ 3 to 7 months old. The frozen hearts were thawed and then briefly rinsed (no more than 30 s) with ice-cold 0.9% NaCl. Muscle strips from left and right ventricles and papillary muscles were dissected at 4°C in a cold room in ice-cold pCa 8 solution (10-8 M [Ca<sup>2+</sup>], 1 mM [Mg<sup>2+</sup>], 7 mM EGTA, 2.5 mM [MgATP<sup>2+</sup>], 20 mM MOPS, pH 7.0, 15 mM creatine phosphate, ionic strength = 150 mM adjusted with potassium propionate) containing 30 mM BDM and 15% glycerol <sup>63</sup>. After dissection, muscle strips were transferred to pCa 8 solution mixed with 50% glycerol and incubated for 1 h on ice. Then the muscle strips were transferred to fresh pCa 8 solution mixed with 50% glycerol and containing 1% Triton X-100 for 24 h at 4°C. Muscle strips were then transferred to a fresh batch of pCa 8 solution mixed 1:1 with glycerol and kept at – 20 °C until used for the preparation of myofibrils. A total of 6 animals were sacrificed for each of the 6 groups (transgenic wild-type F110I, R278C, I79N and transgenic mutant F110I, R278C, I79N) to perform my experiments. Myofibrils were prepared from either right or left ventricular muscle. Polarized fluorescence intensity fluctuations data was collected for a total of 25 experiments per group.

<u>Chemicals and solutions:</u> Tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA) (single isomer) (Cat. No. T-6006), Alexa Fluor 488, hydrazine (cat# A10436) and non-targeted Quantum Dots (QD) (21 nm size, cat # Q21031MP) were from Molecular Probes (Eugene, OR) and were used as cell tracers. SeTau-647-mono-maleimide was from SETA BioMedicals (Urbana, IL). All other chemicals were from Sigma-Aldrich (St Louis, MO). The composition of the solutions were: EDTA-rigor solution: 50mM KCl, 5mM EDTA, 10mM TRIS-HCl pH=7.5; Mg-rigor solution: 50mM KCl, 2mM MgCl<sub>2</sub>, 10mM TRIS-HCl pH=7.5; Ca-rigor solution: 50mM KCl, 0.1mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub>, 10mM TRIS-HCl pH=7.5. Contracting solution: the same as Ca-rigor plus 5mM ATP; relaxing solution: the same as Ca-rigor except that 0.1mM Ca was replaced with 2mM EGTA. The contracting solution contained a regenerative system (Carigor, 100 μM ATP, 20mM creatine phosphate and 10 units/mL of 1mg/mL creatine kinase.

LC1 Expression: A pQE60 vector containing recombinant LC1 with a single cysteine residue (Cys 178) was donated by Dr. Susan Lowey (University of Vermont). The plasmid DNA was

transformed into E.coli M15 competent cells and recombinant clones were selected by ampicillin resistance. The LC1-cDNA insert of the clones was confirmed by DNA sequencing of both strands (Iowa State University of Science and Technology). LC1 protein was over expressed in E. Coli M15 cells grown in Luria broth containing 100µg/ml of ampicillin by induction with IPTG. His-tagged LC1 protein was affinity purified on Ni-NTA column following the manufacturer's protocol. The imidazole eluted fractions were run on SDS-PAGE followed by Western analysis with Anti-LC1 antibodies (Abcam, CA). Fractions containing LC1 were pooled together and dialyzed with buffer (50mM KCl and 10mM phosphate buffer (pH 7.0)). Dialyzed protein showed a single ~25-kDa band on SDS-PAGE after Coomassie staining (Fig. 8). Protein concentration was determined using the Bradford assay. In some experiments, commercial skeletal human LC1 was used (Prospec, Ness Ziona, Israel).



Figure 8: Characterization of recombinant LC1 protein by SDS-PAGE and Western Blot analysis. A: Western blot of 25-kDa LC1 protein with anti-LC1 after induction with (+) and and with no induction by IPTG in E.coli M15 cells (two separate batches). B: SDS-PAGE of 25-kDa LC1 protein purified by Ni-affinity column and stained with Coomassie blue (16% SDS-PAGE). C: Western blot of the same 25-kDa protein band as in panel B labeled with rabbit polyclonal LC1 antibody (AbCam, CA). Arrows indicate MW=25KD.

<u>LC1 labeling</u>: Purified LC1 was dialyzed against the 50mM KCL and 10mM phosphate buffer (pH 7.0) and fluorescently labeled by incubating with a 5 molar excess of dye overnight on ice. Unbound dye was eliminated by passing labeled LC1 through a Sephadex G50 column.

Degree of labeling of LC1: The concentrations of LC1 protein and bound 5-TMRIA or SeTau were determined to estimate the degree of labeling. Protein concentration was determined by the Bradford assay and 5-TMRIA ( $\varepsilon = 87,000 \text{ M}^{-1}\text{ cm}^{-1}$  at 535 nm) or SeTau ( $\varepsilon = 200,000 \text{ M}^{-1}\text{ cm}^{-1}$  at 647 nm) concentration was determined from the peak absorbance obtained on a Varian Eclipse spectrometer (Palo Alto, CA). The concentration of both the protein and dye was found to be the same, indicating that the dye and protein are bound in a 1:1 ratio.

Preparation of phosphorylated and dephosphorylated muscle: Fresh rabbit psoas skeletal muscle was harvested and bundles of muscle fibers were prepared. Muscle fibers were dephosphorylated by storing in glycerinating solution containing 150 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM EGTA, 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM ATP, 0.1% β-mercaptoethanol and 50% glycerol. Phosphorylated muscle was prepared by inhibiting the activity of phosphatase enzyme with the addition of 20 mM sodium fluoride and 20 mM phosphate to the muscle fibers in glycerinating solution  $^{62}$  and incubated for a week at  $40^{0}$ C while rocking.

<u>Preparation of myofibrils:</u> Rabbit psoas muscle bundles were incubated in EDTA-rigor solution for 30 minutes followed by further incubation for an additional 30 minutes with fresh EDTA rigor solution on ice. EDTA rigor was then aspirated and replaced with Mg-Rigor solution and homogenized using a Heidolph Silent Crusher S homogenizer for 20 s (with a break to cool after 10 s). Myofibrils excised from muscle are then washed with Mg-Rigor and Ca-Rigor solutions.

LC1 Exchange into Myofibrils: The low exchange ratio, necessary for mesoscopic experiments, was achieved with very low SeTau-LC1 concentration (5-10 nM). The exchange procedure was the same as in <sup>64</sup>, except that exchange solution contained 15mM KCl, 5 mM EDTA, 5 mM DTT, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM ATP, 1 mM TFP, and 10 mM imidazole, pH 7 and the exchange was carried out by incubating myofibrils at 30°C for 20 min (not usual 37°C for 1/2 hr. 1 mg/mL of freshly prepared myofibrils were used.

<u>Cross-Linking</u>: In the presence of  $Ca^{2+}$  and ATP, the myofibrils shorten, making it impossible to record polarized intensities during contraction. The myofibrils were prevented from shortening by cross-linking with a water-soluble cross-linker 1-ethyl-3-[3 (dimethylamino)propyl]carbodiimide (EDC) <sup>63,65</sup>. 1 mg/mL of myofibrils in Ca-rigor solution containing 20 mM EDC were incubated for 20 min at room temperature . The reaction was stopped by adding 20 mM DTT. The pH of the solution (7.4) remained unchanged throughout the 20 min reaction. The cross-linking had no effect on the probability distribution or ATPase. The absence of shortening was verified by imaging myofibrils with differential contrast microscopy, and with fluorescence microscopy after labeling the myofibrils with a 10 nM rhodamine-phalloidin <sup>66</sup>.

<u>Isoelectric focusing:</u> The extent of myosin phosphorylation was analyzed by isoelectric focusing (IEF). The protocol for separating myofibrillar lysate and staining procedure was similar to the

one employed by Cooke et al. <sup>62</sup>. Phosphorylated and dephosphorylated muscle fibers were homogenized in 8 M urea and total protein concentration was analyzed by the Bradford assay. The homogenates were diluted to obtain a final concentration of 2.5 mg/ml in sample buffer (9 M urea, 130 mM DTT, 20% glycerol, 250 µl of 40% carrier ampholines pH 4-6 (BioRad), 10% Triton X-100). Denaturing isoelectric focusing gel (10 M urea, 18% acrylamide/bis-acrylamide, 20% glycerol, 10% Triton-X 100, 1 ml of 40% ampholines (pH 4-6), 60 µl of 10% APS, and 30 µl of TEMED) was run overnight at constant 350V and fixed (7% TCA, 50% methanol) for 2 hours. The gel was then washed with double distilled water for 10 minutes, stained with Diamond Pro-Q phosphoprotein gel stain (Invitrogen) for 3 hours in the dark and destained (50 mM sodium acetate, 20% acetonitrile) by shaking at room temperature for 30 min. The gel was imaged at 530 nm using a Chemi Doc XRS+ imaging system (BioRad). The result (Fig. 9) shows that the band corresponding to phosphorylated RLC in lane 2 is 4.8 times stronger than dephosphorylated RLC shown in lane 1.



Figure 9: Isoelectric focusing gel image of dephosphorylated (lane 1) and phosphorylated (lane 2) myofibrillar lysate. Notice the strong RLC phosphorylated band in lane 2.

<u>Myosin ATPase activity</u>: was determined using a Sensolyte MG Phosphate assay kit (Anaspec). This involves using malachite green reagent that reacts with free phosphate after ATP hydrolysis to form bluish-green end product. The amount of free phosphate generated can be quantified by measuring absorbance at 620 nm and compared with phosphate standard provided in the kit. The ATPase activity of myofibrils (5 mg/ml) from phosphorylated and de-phosphorylated muscle containing 100  $\mu$ M ATP was assessed. The initial slope (during the first minute of incubation) of phosphate/time curve had a slope of 34.7  $\mu$ M Pi/min/ $\mu$ M head and 16.6  $\mu$ M Pi/min/ $\mu$ M head for phosphorylated and de-phosphorylated myofibrils, respectively.

Confocal Imaging and probability distribution measurements: Fluorescence lifetime images and time traces were acquired on a PicoQuant MT 200 confocal system (PicoQuant, Berlin, Germany) coupled to an Olympus IX 71 microscope. It is the time-resolved fluorescence microscope system capable of Fluorescence Lifetime Imaging with Single Molecule Detection (SMD) sensitivity. A 635nm pulsed laser diode was used for excitation. The data will be collected by Time-Correlated Single Photon Counting electronics in Time-Tagged Time-Resolved mode where each photon is recorded individually. Even though the exciting laser light was linearly polarized, a polarizer will be inserted before the entrance to the microscope to avoid slight depolarization by the single mode optical fiber. Only those myofibrils which are oriented parallel to the incident polarized light was used in this study. Olympus 60x-1.2 NA water immersion objective will be used to focus the samples. The fluorescent light was passed through a 30  $\mu$ m pinhole before being split by a 50-50 prism, and the separated light beams was detected by separate Avalanche Photodiodes (APDs) with respective  $\|$  and  $\bot$  oriented analyzers. The APD's were carefully calibrated before each polarization experiment to give identical intensities
when exposed to an isotropic solution of the 50 nM rhodamine 700 dye (for this control, a dye with long fluorescence lifetime has to be used. The laser polarization was at a magic angle with respect to long axis of a myofibril.

Data Analysis for conformational studies: Fluorescence from myosin cross-bridges collected by two independent detectors was analyzed. First, the data was binned together to smooth it and give it the time resolution of 10 ms. Let ||I|| be the polarized intensity obtained with the exciting and detected light polarized parallel to the myofibril axis, and ||I<sup>\_</sup> be the polarized intensity obtained with exciting and detected light polarized parallel and perpendicular to the myofibril axis, respectively. The polarization of fluorescence was given  $PF = P_{\parallel} = (\|I\| - \|I\perp)/(\|I\| + \|I\perp)$ . From the PF data, histograms were plotted to estimate the dispersion of orientations of myosin cross-bridges. The order is best represented by the probability distribution of orientations. Probability distributions are plots of polarization values versus the number of times that a given orientation occurs during a 20-s experiment. A narrow probability distribution indicates that the cross-bridges are relatively uniformly oriented. Conversely, a broad probability distribution indicates that cross-bridges are relatively disordered. These histograms were fit with normal (Gaussian) distribution equation -  $y=a \exp[-0.5(x-x_0/b)^2]$ . From the Gaussian curves Full Width at Half Maximum (FWHM) were contrasted and compared between rigor, relaxation (dephosphorylated & phosphorylated muscle) and contraction states.

<u>Data analysis for kinetics of contraction:</u> Autocorrelation function (ACF) will be calculated from polarization of fluorescence values to extract kinetic information. The autocorrelation function of PF's is the normalized time average of PF's multiplied by the value of PF's at delay time later. It is possible to relate the decay of the correlation function to cross-bridge kinetics <sup>67</sup>. For a simple

2-state model of cross-bridge cycle, where cross-bridges can assume only two orientations (rigor or detached from actin) the decay parameters are related in a simple way to the rate of cross-bridge attachment ( $k_1$ ) and dissociation ( $k_2$ ) from actin.

Statistical analysis: It was carried out using Origin v.8.5 (Northampton, MA) and SigmaPlot 11 (Systat Software, San Jose, CA). Non-linear curve fitting was performed using the Levenberg-Marquardt algorithm for chi-square minimization. Chi-square minimization optimizes a parameterized fitting function with respect to a particular set of data by iteratively adjusting the fitting function's parameter values in order to minimize residuals. Residuals are the point-wise deviations between the fitting function (i.e., the theoretical curve) and the experimental data. For the current data a Gaussian (normal) fitting function was used. We saw no difference in the results when the Voigt or Lorentzian model was used. Goodness of fit was assessed by the reduced  $\chi^2$ . The code for generating the "velocity plots" (available on request) was written with Labview 2010. Velocities were calculated by taking the difference in consecutive polarization data points and dividing it by the difference in the time stamps associated with each data point. The plot is the array of velocity data points versus the array of polarization points on an x-y plot. The front panel of the Labview program is a view graph, buttons for initializing the process, storing the data as an Excel file, and exporting a graph of the data as a .jpg file. The program was compiled using Labview's compiler into a standalone executable.

$$R_2(t) = \frac{(a_1k_2 + a_2k_1)^2}{(k_1 + k_2)^2} + k_1k_2\frac{(a_1 - a_2)^2}{(k_1 + k_2)^2}\exp(-(k_1 + k_2)t)$$

Equation 1: 2-state model of cross-bridge cycle.  $k_1$ , and  $k_2$  are forward and reverse rate constants of cross-bridge binding,  $a_1$  and  $a_2$  are fluorescence intensities associated with conformational change.

Schematic summarizing the mode of data collection and analysis to interpret myosin cross-bridge orientation and motility in my research: In the following figure plane polarized light (blue) from the laser is used to image a single isolated myofibril. Only fluorescence emitted by the myofibril (green) passes through the dichroic mirror. Total fluorescence is split into two with a 50-50 beam splitter. Then both the beams are condensed and passed through a 50µM pinhole to reject the out of plane fluorescent light. Now the miniscule fluorescence coming through the pinhole is passed through two polarizers, one oriented parallel and other perpendicular to the long axis of the myofibril. Finally the polarized emission is collected by two avalanche photodiodes with single molecule detection capability. Polarized fluorescence intensity fluctuations are now plotted as histograms to characterize the cross-bridge orientation. Autocorrelation function of polarized fluorescence intensity fluctuations is computed to determine the rate of cyclical motion of myosin cross-bridges.



Confocal Fluorescence Correlation Spectroscopy

#### **CHAPTER 3**

## Project 1: Evidence for Pre- and Post-Power stroke states of cross-bridges of contracting skeletal myofibrils

#### **INTRODUCTION**

In this project orientational fluctuations of a small number of myosin molecules (~3) in working skeletal muscle myofibrils were assessed. Myosin Light Chain 1 (LC1) was labeled with a fluorescent dye and exchanged with the native LC1 of skeletal muscle myofibrils cross-linked with EDC [1-ethyl-3-[3(dimethylamino) propyl] carbodiimide] to prevent shortening. A small volume within the A-band (~ $10^{-15}$ L) was observed by confocal microscopy and cyclic fluctuations in orientation of the myosin neck (containing LC1) were measured by recording the parallel and perpendicular components of fluorescent light emitted by the fluorescently-labeled myosin LC1. Histograms of orientational fluctuations from fluorescent molecules in rigor were represented by a single Gaussian distribution. In contrast, histograms from contracting muscles were best fit by at least two Gaussians. These results provide direct evidence that cross-bridges in working skeletal muscle assume two distinct conformations, presumably corresponding to the pre-and post-power stroke states.

### RESULTS

<u>Imaging:</u> A typical fluorescence lifetime image of a rigor myofibril is shown in Fig. 10. All Abands were fluorescently labeled. The red circle in A indicates a 2D projection of the confocal aperture on the image plane, and its diameter  $(1.2 \ \mu m)$  is equal to the diameter of the confocal aperture (50  $\mu m$ ) divided by the magnification of the objective (40x). Images are shown at various degrees of labeling. Native LC1 of myofibrils were exchanged with 4 nM (A), 2 nM (B), 1.2 nM (C) and 0.8 nM (D) of fluorescently labeled light chain (R-LC1). In the reported experiments, the myofibrils were exchanged with 3 nM R-LC1.



Figure 10: Lifetime images of a rigor myofibril. The color bar to the right of image A is the lifetime scale from 0 (blue) to 6 (red) nsec. The red circle is a projection of the confocal aperture

on the sample plane (diameter 1.2  $\mu$ m). Native LC1 molecules were exchanged in myofibrils with 4 nM (A), 2 nM (B), 1.2 nM (C) and 0.8 nM (D) of R-LC1. Sarcomere length = 3.1  $\mu$ m (A), 3.0  $\mu$ m (B), 3.0  $\mu$ m (C) and 2.9  $\mu$ m (D). Images were acquired on a PicoQuant Micro Time 200 confocal lifetime microscope. The sample was excited with a 470 nm pulse of light and the emission collected on an APD through a LP500 filter.

LC1 is immobilized by the myosin neck: I measured the decay of anisotropy of R-LC1 exchanged into myofibrils to test whether rhodamine was rigidly immobilized on the surface of LC1 and the orientation of the transition dipole of the fluorophore reflects the orientation of the neck portion of the myosin head. Anisotropy is defined as  $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$ . The decay is shown in Fig. 11. The decay of free TMRIA was best fit by a double exponential curve r(t) =Ro+ a  $\cdot \exp(-t/\theta_1) + b \cdot \exp(-t/\theta_2)$ , where Ro = 0.05 is the value of anisotropy at infinite time and  $\theta_1 = 0.3$  and  $\theta_2 = 608.7$  ns are the rotational correlation times (Fig. 11A). The anisotropy decay comprised mostly of the fast decay (93.1%), and thus the slow correlation time comprised only 6.9% of the decay, probably contributed by aggregates of rhodamine. The decay of R-LC1 bound to myofibrillar myosin was best fit by the same double exponential function, but this time Ro = 0.33,  $\theta_1 = 67.7$  and  $\theta_2 = 0.9$  ns (Fig. 11B). The slow correlation time comprised 82.1% and the fast correlation time 17.9% of the decay. The short and long correlation times were most likely due to the rotation of rhodamine moiety on LC1 and to rotation of bound LC1, respectively. The maximum value of anisotropy was 0.384, and this high value of initial anisotropy indicates that the absorption and emission dipoles of rhodamine are nearly parallel.



Figure 11: Anisotropy decay of 0.1  $\mu$ M free TMRIA (A) and 50  $\mu$ M myofibrils (B) after exchange with 2.5  $\mu$ M R-LC1 into 1 mg/mL myofibrils for 5 min at 30°C.

Intensity fluctuations during rigor and contraction of muscle myofibrils: A myofibril was placed on the stage of the confocal microscope with long axis oriented vertically on the microscope stage. The linear polarization of the illuminating laser light was also vertical, i.e., parallel to the long axis of myofibril. The fluorescence emanating from the DV was split 50-50 by a beam splitter and projected through 50 μm confocal apertures onto the photosensitive surface of the two APDs. The original data was collected every 10 µsec, but to keep the data files to a manageable size, 1000 points were binned together to give the time resolution of 10 msec.

Skewness and Kurtosis: Fig. 12 shows examples of six histograms of polarized fluorescence randomly selected from a pool of 20 histograms obtained from myofibrils in rigor. A histogram can be quantitatively characterized by the values of kurtosis and skewness. A positive skewness means that the tail of the curve is directed towards positive values of the histogram relative to a Gaussian distribution. This was the case for both rigor and contraction (see below). A positive kurtosis is expressed by long tails and higher peaks compared to the Gaussian curves. This was the case for rigor myofibrils. The negative kurtosis means that the tails are smaller than those of Gaussian curves. This was the case for contracting myofibrils (see below). Zero skewness and kurtosis means that the histograms are Gaussian. This was the case for the un-cross-linked myofibrils in rigor. Table 3 shows the values of skewness and kurtosis. The differences in both skewness for rigor histograms were influenced by cross-linking. The difference was statistically significant.

Myofibrils	Skewness	Kurtosis	N
Rigor	$-0.02 \pm 0.04$	$0.06 \pm 0.18$	18
EDC-Rigor	$1.12 \pm 0.34$	$0.84 \pm 1.63$	18*
Relaxation	$1.04 \pm 0.19$	$0.20 \pm 0.81$	18
EDC Relaxation	$1.48 \pm 0.54$	$3.00 \pm 3.05$	18
Contraction	$0.86 \pm 0.16$	$-0.59 \pm 0.27$	18*

Table 3: Comparison of kurtosis and skewness parameters from fluorescence polarization histograms of rigor, relaxing and contracting skeletal myofibrils. Errors are given as SD and N is the number of experiments. \* Two histograms with extreme maxima and minima were rejected.



Figure 12: Examples of histograms of polarized fluorescence from rigor (top panel) and relaxed (bottom panel) myofibrils. Horizontal scale: The negative value of parallel fluorescence polarization. Vertical scale: The number of events during 20 s. The position of peaks for rigor myofibrils were (top panel, clockwise from the top left): P = -0.07, P = 0.05, P = 0.08, P = 0.02, P = 0.00, P = 0.07. The blue line shows the fit to a single Gaussian distribution. For relaxed myofibrils (bottom panel) the parameters (area, center, width, height) for each peak of a two Gaussian fit are given in clockwise order, starting from the upper left: Upper Left: minor peak (red) 0.85, 0.18, 0.10, 6.82; major peak (green) 14.89, -0.03, 0.21, 57.01. Upper Middle: minor

peak (red) 0.64, 0.03, 0.00, 115.88; major peak (green) 17.16, -0.02, 0.22, 61.66. Upper Right: minor peak (red) 0.11, 0.11, 9.86E-4, 86.96; major peak (green) 12.63, -0.07, 0.19, 51.74. Lower Left: minor peak (red) 1.09, 0.05, 0.09, 10.09; major peak (green) 12.10, 0.09, 0.18, 53.36. Lower Middle: minor peak (red) 0.23, 0.05, 0.02, 9.54; major peak (green) 14.24, -0.07, 0.21, 53.62. Lower Right: minor peak (red) 0.47, 0.01, 0.05, 7.57; major peak (green) 14.30, -0.00, 0.23, 48.84. The contribution of the minor peak (red line) makes little difference relative to the second peak (green line) of a double Gaussian distribution fit. The blue line is the sum of red and green line fits.

<u>Rigor histograms:</u> The fit of representative histograms in rigor with a 3-parameter Gaussian curve y=a exp[-0.5(x-xo/b)2] (blue line) is shown in the top panel of Fig. 12. The goodness of fit, assessed by Standard Error in the PeakFit program, showed that none of the curves were significantly improved by adding a second Gaussian. The ratios of the areas under the major (green) and minor (red) peaks were (clockwise from upper left) 17.5, 18.1, 11.6, 59.5, 12.6, 55.0 with an average of 29.0, indicating that adding the second Gaussian makes on average only a 3.4% difference. The average position of a single peak was  $P = 0.05 \pm 0.08$  (in an oriented, immobile system the fact that the polarization = 0 does not mean that the dipoles are randomly arranged). Since the absorption and emission dipoles of rhodamine are nearly parallel (time zero anisotropy  $\approx$ 0.4, Fig. 11) and the myofibrillar axis is parallel to the direction of polarization of exciting light, this indicates that the transition dipoles of rhodamine in rigor are oriented mostly perpendicular to myofibrillar axis. <u>Relaxation histograms</u>: Histograms of relaxed myofibrils were also best fit by a single Gaussian. The bottom panel of Fig. 12 shows the representative histograms during relaxation. The ratios of the areas under the major (green) and minor (red) peaks were (clockwise from upper left ) 17.4,26.8, 114.5, 12.0, 61.7, 31.1 with an average of 43.9, indicating that adding the second Gaussian makes on average only a 2.2% difference.



Figure 13: Examples of histograms of polarized fluorescence from contracting myofibrils. Horizontal scale: The negative value of fluorescence polarization. Vertical scale: The number of events during 20 s. The positions of minor (red line) and major (green line) peaks from a double Gaussian distribution fit are (clockwise, from the top left): P = -0.3 and 0.11, P = -0.29 and -0.06, P = -0.2 and -0.06, P = -0.33 and -0.14, P = -0.32 and -0.11, P = -0.31 and .012. The ratios of the major peak (green line) to the corresponding minor peak (red line) in the double Gaussian fits are (clockwise, from the top left) corner): 2.7, 1.1, 0.5, 2.7, 1.6 and 3.1. The blue line is the sum of red and green line fits.

<u>Contraction histograms</u>: The histograms of polarized fluorescence fluctuations obtained from contracting myofibrils were significantly different from those observed in rigor and relaxation. Fig. 13 shows examples of six histograms of polarized fluorescence randomly selected from a

pool of 20 histograms of contracting myofibrils. The average skewness and kurtosis were 0.86±0.16 (SD) and -0.59±0.27 (SD), respectively. Both skewness and kurtosis were statistically significantly different during rigor and contraction at a level of 0.69% for skewness (t = -2.87, P = 0.0069, 34 Degrees of Freedom) and at a level of <0.07% for kurtosis (t = -3.68, P = 0.00078, 34 Degrees of Freedom), respectively. The Standard Error test of the PeakFit program showed that 14 of 20 histograms were best fit by two Gaussians. The average positions of the peaks were  $P = -0.14 \pm 0.04$  for peak #1 and  $P = -0.31 \pm 0.05$  for peak #2. The positions of the two peaks were significantly statistically different at P<0.001% (t=9.92). Most of the power was contained in peak #1, although on two occasions the peak #2 was larger than peak #1 (e.g. Fig. 13, right upper corner). The average ratio, i.e., the relative contribution of both Gaussians was 5.7 ± 7.7. The overall experimental curve (blue) is the sum of the red (peak #1) and green (peak #2) Gaussians.

Myofibrils	Peak #1	Peak #2	Ν
Rigor	$0.04\pm0.02$	-	18
EDC-Rigor	$0.05\pm0.08$	-	20
Relaxation	$-0.03\pm0.03$	-	18
EDC Relaxation	$-0.06\pm0.02$		18
Contraction	$-0.14 \pm 0.04$	$-0.31 \pm 0.05$	16*

Table 4: Average polarization values from the Gaussian distribution fits. Errors are given as SD and N is the number of experiments. \* 20 histograms were analyzed in total, four of which were fit best by a single Gaussian distribution with the peak position corresponding to that reported by peak #1.



Figure 14: Schematic illustration of the cross-bridge power stroke. In the pre-power stroke state, corresponding to peak #1 of the polarized fluorescence histograms from contracting myofibrils, the upper and lower 50K domains (UP50 and L50, notation as in <sup>68</sup>) are in partially closed conformation and LC1 is pointing sidewise (low polarization). In the post-power stroke state, corresponding to peak #2 of the polarized fluorescence histograms from contracting myofibrils, the upper and lower 50K domains are in a closed conformation and LC1 is pointing downwards (high polarization). The rigor state is short-lived and the myosin head adopts a similar conformation to that of the post power state <sup>68</sup>, except that LC1 is now pointing completely sideways (0 polarization). This schematic does not take into account such complicating factors as disorganization of actin filaments and inhomogeneities between myofibrils. For simplicity it is assumed that the transition dipole of rhodamine is parallel to the long axis of LC1.

#### DISCUSSION

The main finding of this project is that polarization histograms from fluorescently-labeled LC1 on the myosin head during muscle contraction consist of two separate peaks. Since the peaks correspond to the number of times a cross-bridge assumes a given orientation during 20 s contraction, it makes sense to suggest that during contraction, cross-bridges assume two distinct orientations. There was a large difference in polarization between the two peaks. They most likely indicate cross-bridge conformations in the pre-and post-power stroke positions. The first peak corresponds to the pre-power stroke position, when the cleft between upper and lower subdomains of 50KDa fragment is partially closed, the lever arm is in the "up" orientation and the myosin active site contains ADP and Pi<sup>68</sup>. The second peak corresponds to the post-power stroke position when the cleft between upper and lower subdomains of 50KDa fragment is totally closed, the lever arm is in the "partially down" orientation and the myosin active site contains only ADP <sup>68</sup>. The area corresponds to the average number of times a cross-bridge assumes a given orientation during the 20 s contraction, and the average ratio of the area under peak #1 divided by the area under peak #2 was 5.7. Therefore, the fact that the pre-power stroke state is more populated than post-power stroke state is consistent with the notion that the transition from pre-to post-power stroke is rate-limiting.

Earlier work used EPR spectroscopy to detect ATP-induced changes in the structure of spinlabeled myosin heads in whole muscle fibers in rigor upon transition to relaxation and contraction <sup>69</sup>. Different conformational states were resolved on the basis of nanosecond rotational motion within the protein. In rigor, only a single conformation was detected, in

agreement with the present work. In relaxation and isometric contraction, the EPR spectrum showed two conformations and a small fraction of heads in the oriented, actin-bound conformation. Their rigor results are consistent with those reported here. Relaxation and contraction results differ, most likely because these authors used whole muscle fibers labeled at Cys707, while we observed 3 cross-bridges labeled at LC1. Fluorescent probes were also used to study the breadth of the orientation distribution of fluorescent probes bound to the regulatory light chain of myosin, which were shown by Ling et al. <sup>70</sup> and later Hopkins et al. <sup>17</sup> to be essentially the same in different physiological states. These results are consistent with the present work which also did not show consistent changes in the width of distributions.

In our experiments, the post-power stroke conformation of the lever arm is different than the rigor conformation. This implies that each cross-bridge executes a rotation in two steps. Fig. 14 illustrates schematically the presumed sequence of events. A cross-bridge is initially in the pre-power stroke state, represented by peak #1, and subsequently transits to the post-power stroke state (weak ADP binding state), represented by peak #2. If the rigor state (strong interaction of the apo-head with actin) is a part of the cross-bridge cycle, there must exist a second transition from the post-power stroke state to the rigor state. The rigor conformation is not seen during contraction, because this state is short lived in the presence of excess ATP. This interpretation is consistent with the model originally proposed by Huxley & Simmons <sup>6</sup> which suggested that the myosin head had a small number of sites, each of which was capable of combining reversibly with a corresponding site on F-actin.

Translation of the polarization values to the absolute orientation of transition dipoles with respect to the myofibrillar axis has not been attempted here because it is critically dependent on the model of arrangement of cross-bridges. For example, if it is assumed that the cross-bridges are arranged helically along the long axis of muscle and the fluorescence contains  $\alpha$ % contribution from a random, immobilized component, then the difference between pre-and post-power stroke angles corresponds to an angular change from 90° to 5° depending on the value of  $\alpha$ <sup>71</sup>. However, by calculating the average area under the Gaussian curves, we can estimate that the relative populations of cross-bridges are ~70% in the pre- power stroke states and ~30% in the postpower stroke states.

An additional possibility is that the two different peaks indicate two populations of cross-bridges, each executing distinct rotation. This is unlikely because the presence of two populations has not been detected in direct EM measurements of cryofixed, isometrically active, insect flight muscle <sup>72</sup>. It is also possible that cross-bridges do not rotate at all during isometric contraction, and our results reflect their static arrangement. In the absence of filament sliding, cross-bridges may not be able to rotate while attached, and cross-linking may prevent their reaching neighboring actin target zones while detached <sup>72</sup>. This interpretation does not alter the conclusions of this project. The observed distributions would then correspond to static positions assumed after the first molecule of ATP was split when a cross-bridge was still allowed to rotate against unstretched elastic element.

We want to emphasize that no correlation analysis (such as in  $^{22}$ ) of the kinetic data was used here. This is a significant advantage of the present method, because correlation analysis requires assumptions about a model of cross-bridge-actin interaction.

We have considered three possible artifacts and conclude that all are unlikely:

a) <u>The second peak is due to rotation of rhodamine moiety alone.</u> Although the mobile fraction was 10% (Fig. 11B), this is unlikely because (i) rotational motion at the scale of nanoseconds does not affect polarization of fluorescence measured on a scale of milliseconds, and (ii) the nanosecond rotation of rhodamine is clearly visible in a solution of free dye (Fig. 11), whereas the second peak is not visible in rigor myofibrils (Fig. 12).

b) <u>Addition of ATP alone is sufficient to induce appearance of the peaks.</u> However, in control experiments we saw no effect of ATP on the anisotropy or fluorescent lifetime of rhodamine-LC1.

c) <u>The fluctuations are caused by myofibril movement, not cross-bridge rotations.</u> This is unlikely because the control experiments, in which we measured sarcomere length as a function of concentration of cross-linker, showed no shortening upon addition of ATP when myofibrils were cross-linked with 20 mM EDC.

#### **CHAPTER 4**

# <u>Project 2: Orientation and Rotational motion of Cross-Bridges Containing Phosphorylated</u> <u>and Dephosphorylated Regulatory Light Chain</u>

#### **INTRODUCTION**

Calcium binding to thin filaments is a major element controlling active force generation in striated muscles. Recent evidence suggests that processes other than Ca<sup>2+</sup> binding, such as phosphorylation of myosin Regulatory Light Chain (RLC) also controls contraction of vertebrate striated muscle <sup>73</sup>. Electron Paramagnetic Resonance (EPR) studies using nucleotide-analog spin label probes showed that dephosphorylated myosin heads are highly ordered in the relaxed fibers and have very low ATPase activity. This ordered structure of myosin cross-bridges disappears with the phosphorylation of RLC<sup>62</sup>. The slower ATPase activity in the dephosphorylated moiety has been defined as a new super-relaxed state (SRX)<sup>73</sup>. It can be observed in both skeletal<sup>73</sup> and cardiac muscle fibers <sup>74</sup>. Given the importance of the finding that suggests a novel pathway of regulation of skeletal muscle, we aim to examine the effects of phosphorylation on cross-bridge orientation and rotational motion. We find that: (1) relaxed cross-bridges, but not active ones, are statistically better ordered in muscle where the RLC is dephosphorylated compared to phosphorylated RLC; (2) relaxed phosphorylated and dephosphorylated cross-bridges rotate equally slowly; and (3) active phosphorylated cross-bridges rotate considerably faster than dephosphorylated ones during isometric contraction but the duty cycle remained the same, suggesting that both phosphorylated and de-phosphorylated muscles develop the same isometric tension at full  $Ca^{2+}$  saturation.

#### RESULTS

The degree of order of relaxed cross-bridges: The upper and lower panels of Fig. 15 show representative histograms of relaxed myofibrils which contained phosphorylated and dephosphorylated RLCs, respectively. The FWHM values for these and 19 other half-sarcomeres are summarized in Table 5. FWHM and the average polarizations of phosphorylated and dephosphorylated cross-bridges is to plot instantaneous angular velocity v of a cross-bridge (defined as change of polarization in 10 ms) versus polarization of fluorescence. The overall velocity in isometric contraction is, of course 0, but plotting the data in 2D (i.e. v vs. PF) allows presenting the information contained in all 20 experiments on a single plot. Each experiment on phosphorylated or de-phosphorylated muscle consists of 2000 measurements of PF, so Fig. 16 which is a plot of 20 experiments on phosphorylated and 20 experiments on de-phosphorylated cross-bridges of relaxed myofibril contains 80,000 points. It is impossible to show so many data points in 1D plots. It can be seen that the points from de-phosphorylated muscle (green) are more narrowly distributed than points from phosphorylated muscle (red), and that phosphorylation decreases mean polarization.

The difference between FWHMs for phosphorylated and de-phosphorylated half-sarcomeres was statistically significant. A difference of 0.0188 had t value of 4.646 with 37 degrees of freedom, 0.95 percent confidence interval for difference of means was 0.0106 to 0.0271. Since the difference in the mean values of the two groups is greater than would be expected by chance, there is a statistically significant difference between the input groups (P<0.001). The differences between the average values of polarization were also statistically significant. The two-tailed P value was less than 0.0001, t = 10.9169 with 37 degrees of freedom. 95% confidence interval of

this difference ranged from -0.07944 to -0.05456. By conventional criteria, the difference is considered to be extremely statistically significant. Differences in total fluorescence intensities were not significant.

RLC	FWHM	Total Intensity	Average Polarization
Phosphorylated	$0.117\pm0.007$	$85.946 \pm 9.949$	$0.315\pm0.024$
De-phosphorylated	$0.098\pm0.016$	$88.128 \pm 20.529$	$0.382 \pm 0.013$

Table 5: Difference between the means  $\pm$  standard deviations of the distributions (FWHM), total fluorescence intensities and average polarizations of labeled myosin RLCs in relaxed half-sarcomeres containing phosphorylated and de-phosphorylated RLCs. (Averages of 20 experiments)



Figure 5 Upper panels: representative histograms of relaxed half-sarcomeres containing phosphorylated RLCs. Lower panels: representative histograms of a half- sarcomeres containing de-phosphorylated RLCs. Frequency (vertical scale) is the number of times a given polarization of fluorescence (horizontal scale) occurs in a given experiment. The green arrows in the first histogram indicate FWHM.



Figure 6: Angular Velocity Plots: The change of PF/10 ms plotted versus the entire 80,000 values of PF obtained from relaxed de-phosphorylated (green) and phosphorylated (red) muscle. It can be seen that the points from de-phosphorylated muscle are more narrowly distributed than points from phosphorylated muscle, and that phosphorylation decreases mean polarization. Note that the horizontal scale shows -PF.

<u>Rotation of contracting cross-bridges:</u> Cross-bridges in contraction execute random motions, powered by hydrolysis of ATP. We sought to determine whether the cross-bridges in contraction execute random motions, powered by hydrolysis of ATP. The question we asked whether dephosphorylated cross-bridges move differently than phosphorylated ones. As before, we took advantage of the fact that the number of cross-bridges in every HS was mesoscopic and so we were able to measure the autocorrelation function of polarized fluorescence. The rate of decay of ACF characterizes rapidity of rotational motions of the lever arm. As before, we define constants  $k_1$  and  $k_2$  forward and reverse rate constants associated with conformational change. There was significant statistical difference between rates of cross-bridge attachment or detachment between two phosphorylated and de-phosphorylated (Figure 17). Table 6 summarizes the results.



Figure 7: Autocorrelation functions of the rotational motion of contracting cross-bridges containing phosphorylated (top panels) and de-phosphorylated (bottom, panels) RLC

RLC	$k_1(s^{-1})$	$k_2(s^{-1})$
Phosphorylated	$1.28\pm0.46$	$0.10\pm0.04$
De-phosphorylated	$0.64\pm0.31$	$0.05\pm0.01$

Table 6: Differences between rates of rotational motion of contracting cross-bridges containing phosphorylated and de-phosphorylated RLC (Errors are standard deviations, averages of 21 experiments).

#### DISCUSSION

As mentioned before, the reasons we limit the number of molecules is to emphasize the differences between FWHMs, to be able to study the kinetics of the rotational motion of crossbridges and to minimize possible inhomogenitieties existing between different sarcomeres. We found that relaxed cross-bridges of muscle in which RLCs were de-phosphorylated were organized more tightly than cross-bridges of muscle in which RLC's were phosphorylated (Table 5, Fig. 16). This work confirms the original SRX finding by suggesting that the SRX state corresponds to a highly ordered array of myosin heads in relaxed muscle in the absence of RLC phosphorylation, and that phosphorylation of RLC can switch to a disordered array of myosin heads <sup>73</sup>. It is possible that the increase in organization was due to the fact that the dephosphorylated cross-bridges were lying close to the core of the thick filament, as suggested in <sup>73</sup>. During contraction, there was a significant difference between the ON and OFF rates of rotation of phosphorylated and de-phosphorylated heads (Table 6, Fig. 16). However, the ratio between ON and OFF rates (and the duty cycle=7.2%) remained exactly the same, suggesting that isometric force was not changed by phosphorylation of RLC. This was indeed observed at saturating Ca<sup>2+</sup> concentrations as observed in skeletal <sup>75</sup> and cardiac <sup>76</sup> muscle. A second pathway that may influence the population of the SRX is the phosphorylation of myosin binding protein-C (MBP-C), the role of myosin-binding protein C was not addressed in this study. MBP-C binds to the thick filament and has been shown to affect both thick filament structure and myosin function. MBP-C binds to the S2 portion of myosin adjacent to the head region, and it is thought to stabilize the binding of the head back to the core of the thick filament. The cardiac MBP-C has three sites for phosphorylation and can be phosphorylated by a variety of kinases,

including protein kinase-A, protein kinase-C and calcium-calmodulin-dependent kinase. Phosphorylation at some of these sites causes myosin heads to be released from the core of the thick filament and become disordered, leading to multiple consequences for cardiac myosin function. It is possible that protein C acts as a signaling molecule, reporting changes in cross-bridges to actin. It binds to both actin and myosin heads through the N-terminal region (containing domains C0, C1 and C2)<sup>77</sup>. Indeed, recent data suggests that MyBP-C may alter interaction between cross-bridges and actin<sup>78</sup>.



Figure 8: Schematics of the arrangement of cross-bridges on the surface of thick filament (white cylinder). Phosphorylated and de-phosphorylated RLC are indicated by blue and yellow spheres, respectively. Red sphere is the LC1 containing rhodamine; its transition dipole is indicated by red arrows. Top panel: relaxation – de-phosphorylated myosins are well oriented – the transition dipoles point approximately in the same direction. De-phosphorylated myosins are in SRX state. In contrast, phosphorylated myosins are not well oriented. Both phosphorylated and de-

phosphorylated myosins s are largely immobilized by thick filament core. Bottom panel: contraction – Now cross-bridges leave the surface of thick filaments to be able to interact with thin filaments (not shown). Both types of myosins are equally disorganized and rotating slow during relaxation, but phosphorylated ones are more mobile during contraction. Blue and yellow arrows imply rotation. Rotation occurs both in polar and azimuthal plane. Thickness of yellow and blue arrowheads indicates speed of rotation. De-phosphorylated heads (yellow arrows) move slower than phosphorylated ones (blue arrows).

#### **CHAPTER 5**

#### Project 3: Motion and conformation of cross-bridges in healthy and hypertrophic hearts

#### **INTRODUCTION**

In this project, I analyzed how the orientation and motion of myosin cross-bridges is different in normal W.T hearts and diseased hypertrophic hearts in a transgenic mice model. Mutations in the ventricular TnT, Tn and Tm mutations often alter Ca<sup>2+</sup> sensitivity of contractility. In the heart, severity of familial hypertrophic cardiomyopathy is related to the degree to which  $Ca^{2+}$ sensitivity is increased by mutations <sup>79,80</sup>. Mutations in other subunits of Tn have been extensively investigated e.g. TnT mutations are often associated with malignant outcomes. In this project we carried out measurements using single molecule detection (SMD). The rationale for SMD measurements is that humans are heterozygous for FHC. It follows that hypertrophic myocardium contains a mixture of the wild-type and mutated proteins. If the degree of expression of mutated protein is small, only a small fraction of myosins interact with actin that carries mutated TnT. In such muscles, it is possible that averaging over billions of molecules inherent in global measurements such as tension, ATPase or Ca sensitivity, will not reveal differences between mutated and wild type muscle. In contrast, if only a few molecules of myosin are observed, there is no averaging of responses from a large number of molecules and there is a good chance that experiments will reveal hypertrophic behavior. We have therefore worked in mesoscopic regime where fluctuations from the average are significant. We compared the distribution of orientations (polarized fluorescence) of ~5 myosin molecules of the healthy and diseased hearts.

#### RESULTS

To examine few cross-bridges, rather than to observe a global parameter of muscle, measurements on myofibrils were done as illustrated in Fig. 19. Panels A and B show typical orthogonal fluorescence intensity images of a single myofibril from the right ventricle of a transgenic mouse where phenylalanine is modified to isoleucine (F110I) in rigor. The image is fainter (A) when the emission polarization is perpendicular to the direction of polarization of exciting light, than when it is parallel (B) indicating that the absorption/emission dipoles of the dye are largely perpendicular to the axis of a myofibril. C is the sum of A and B. D is fluorescence lifetime image. In contrast to skeletal muscle, where nebulin prevents phalloidin from labeling all-but the pointed ends of actin filaments<sup>81</sup>, in the nebulin-free heart muscle the entire I-bands are labeled. The dark areas do not contain myosin. The red circle in A is a 2D projection of the confocal aperture on the image plane. Its diameter is equal to the diameter of the confocal aperture (50  $\mu$ m) divided by the magnification of the objective (40×). The data is collected from the detection volume (DV) of which the red circle in A is a projection.



Figure 9: Images of rigor myofibril with F110I mutation in TnT. Native LC1's of cardiac myofibrils exchanged with 3nM of R-LC1. The red circle in A is the projection of the confocal aperture on the sample plane (diameter 1.2  $\mu$ m). The numbers at right of A–C indicate the B/W scale with 0 corresponding to black and 255 to white. Polarization of laser is vertical. A. Emission polarization horizontal; B. Emission polarization vertical; C. Both emissions together and D is the lifetime image with blue corresponding to 0 and red to 8 ns. Bar in A is 2 $\mu$ m, sarcomere length=1.7 $\mu$ m. The images are indistinguishable from WT myofibrils. Images acquired with the PicoQuant Micro Time 200 confocal lifetime microscope. Excitation with a 470 nm pulse of light, emission through LP500 filter.

<u>Dispersion of rigor orientations in WT and Troponin-T mutants</u>: The rigor distribution of mutated and WT myofibrils differs in width and in the position of the center. Good qualitative

illustrations of the differences in the width are "velocity" plots where the "angular velocity" is plotted against PF. The "angular velocity" is defined as the difference of PF at consecutive times divided by 10 ms (hence "angular", because PF is related to the angle of the transition dipole of the dye). Of course it is not actual velocity, which is 0 in rigor. It merely has the units of velocity and is introduced here to create 2D plots of PF. The plots contain the information contained in all 25 experiments done for each mutation. One experiment contains 2000 measurements of PF, i.e. a velocity plot contains 50,000 points. Fig. 20 shows the differences between WT and MUT of rigor myofibrils for each mutation. It is clear that the distribution of polarizations is narrower in WT myofibrils (green) than in MUT myofibrils (red). The meridional lines in Fig. 21 arise because polarization, defined as normalized number of (polarized photons minus number of polarized photons, can assume only discrete values. Similarly, the equatorial lines arise because velocity is defined as  $\Delta PF/\Delta t$ . For each mutation 25 experiments were done. A difference between WT and MUT myofibrils in rigor was quantified by constructing histograms — plots of the number of events of a given polarization value occurring during 20 s experiment. The histograms were fitted with a Gaussian  $y = a * \exp \left[-0.5(x-x_0/b)^2\right]$ . The key finding is that the histograms of MUT myofibrils in rigor were more disperse than histograms of WT myofibrils in rigor (Fig. 20).



Figure 20: Typical histograms of polarized fluorescence of rigor-WT (A) and rigor-MUT (B, C and D) myofibrils from ventricular muscle with TNT mutations. The solid line is the best fit to the Gaussian 3 parameter function  $y=a \cdot \exp[(-0.5(x-x_o)/b)^2]$ .

The differences between histograms of WT and mutated hearts are shown in representative Fig. 20. Each figure shows representative histograms using different mutations. The trend was consistent for each mutation: mutated-rigor MFs were always more disperse and had different center PFs than rigor-WT MFs. Since FHC mutations are expressed at ~50% level it should be possible to subdivide data into two sets, clustering around different polarizations. This was indeed the case: the data from mutated myofibrils could always be divided into two approximately equal populations, clustering around small and large polarizations. This

presumably corresponds to myofibrils carrying a mutation, and myofibrils which were classified as mutated but that carried no mutation. The values of polarizations clearly are clustered around small (green) and large (red) values of PF. The dispersion of rigor histograms was quantified by the value of Full Width at Half Maximum of a single Gaussian fit. Table 7 shows that PF clusters around two distinct values. The differences between FWHM of histograms of WT and the value of high PF of MUT rigor myofibrils were statistically highly significant. This suggests that high PF of MUT myofibrils represents mutated fraction, and low PF of MUT myofibrils represents non-mutated fraction. No clustering occurs in experiments on WT myofibrils (Fig. 21, left panel). Similar results were obtained for other mutations.



Figure 21: Polarized fluorescence plotted against angular velocity of 24,000 measurements of W.T myofibrils which had small values of polarization (green) and 26,000 measurements of mutated myofibrils which had large values of polarization (red).

Sample	FWHM
Average for WT	$0.25 \pm 0.05$
F110I-MUT-lowPF	$0.14 \pm 0.02$
F110I-MUT-highPF	$0.48 \pm 0.09$
R278-MUT-lowPF	$0.29\pm0.03$
R278C-MUT-highPF	$0.31\pm0.06$
I79N-MUT-lowPF	$0.22 \pm 0.03$
I79N-MUT-highPF	$0.39 \pm 0.06$

Table 7: Rigor polarizations of mutated myofibrils showing that the values cluster around low and high values

Kinetic studies in WT and Arginine 145 Tryptophan (R145W) Troponin-I mutant transgenic mice: I hypothesized that kinetics and the degree of order of cross-bridges will be disturbed by the cardiac myopathy. The power of the mesoscopic approach is well demonstrated in our investigation of the effect of cardiac myopathy causing mutations of cardiac troponin I (cTnI) on dynamics and probability distribution of a mesoscopic number of cross-bridges in the left ventricle of transgenic mouse. cTnI inhibits actomyosin interactions in the absence of  $Ca^{2+82}$ . The R145W mutation in human cTnI is associated with restrictive cardiomyopathy (RCM). It is the least common of cardiomyopathies, but is associated with the greatest morbidity and mortality<sup>83</sup>. RCM is characterized by near normal myocardial wall thickness and cavity size<sup>84</sup>, but has impaired physiological functions including restrictive ventricular filling and reduced diastolic volume. Even though the systolic function is normal, RCM presents increased end diastolic pressure. Several mutations (L144Q, R145W, A171T, K178E, D190G, and R192H) in the highly conserved region of human cTnI correlate with RCM<sup>85</sup>. We investigated one of those mutations (R145W). The physiological measurements in skinned fibers reconstituted with this mutant showed an increase in the  $Ca^{2+}$  sensitivity of force development, in the basal force levels

in the absence of  $Ca^{2+}$ , and a decrease in the ability to inhibit actomyosin ATPase activity in the actin-Tm activated myosin-ATPase assay in the presence of 1.0 mM EGTA <sup>84,86</sup>. In the present study, we asked what molecular changes in myosin are associated with these impaired physiological functions caused by R145W mutation.

In order to find out whether the kinetics of cross-bridges was affected by myopathic mutations, we compared ACFs of contracting WT and R145W MUT cardiac muscle using the same approach as in skeletal muscle. Representative autocorrelation functions of WT and MUT myofibrils are shown in Fig. 22. Taking  $a_1 = 0$  and  $a_2 = 1$ , where  $k_1$ , and  $k_2$  are forward and reverse rate constants of cross-bridge binding and  $a_1$  and  $a_2$  are fluorescence intensities associated with conformational change, the solution of eq. 1 yields  $k_1 = 4.07 \text{ s}^{-1}$  and  $k_2 = 0.48 \text{ s}^{-1}$ for WT muscles and  $k_1 = 0.76 \text{ s}^{-1}$  and  $k_2 = 0.06 \text{ s}^{-1}$  for MUT muscles. In this particular case, the rate of myosin dissociation from actin during contraction was 8 times slower for MUT than for WT muscles. The same was true for A57G mutation. The difference in k<sub>1</sub>s of all experiments had t = 2.621, P = 0.013 with 36 degrees of freedom. The difference in  $k_2$  s of all experiments had t = 2.721, P = 0.010 with 36 degrees of freedom. We conclude that the difference in the mean values of the two groups is greater than would be expected by chance, i.e. cross-bridges in MUT myofibrils bind and dissociate from actin statistically significant slower than cross-bridges in WT myofibrils. To summarize contraction experiments: MUT myofibrils were unbinding from thin filaments relatively slowly. This reinforces the notion that diastolic dysfunction is caused by inability of cross-bridges to dissociate from actin in a timely manner.



Figure 22: Kinetics of orientation change of lever arms of WT (a) and MUT (b) R145W myofibrils. The black lines are best fits for WT and MUT muscles. The fluctuations are caused by change of orientation of lever arms only. The other possible source of fluctuations, i.e., fluctuations in the number of fluorophores in the DV (Magde et al. 1974<sup>60</sup>; Elson and Magde 1974) is not significant because PF is only sensitive to rotations and myofibrils were not twisting.

#### DISCUSSION

This project demonstrates that the hypertrophic phenotype associated with FHC mutations in TnT is manifested by large difference in the center and width of distributions of orientation of WT vs. MUT cross-bridges. The increase of FWMHs in MUT myofibrils indicates the loss of order of rigor attachment of the cross-bridges. This effect was clearly visible in the velocity plots (Fig. 21). The effect was qualitatively demonstrated in Figs. 20 which showed that the centers and widths of histograms of mutated myofibrils were dramatically larger than that of WT controls. Table 7 quantified this result. It must be noted that the fact that cross-bridges form improper rigor bonds may not be a direct result of TnT mutations but may be caused by posttranslational modifications status in transgenic animals, MyBP-C phosphorylation etc.

Earlier experiments <sup>79,80</sup> indicated that maximal force in all TnT mutations was decreased. Our results indicate that mutated muscles suffered a decrease in the number of cross-bridges attached in rigor in a stereo-specific manner. Such attachment is thought to be necessary for force generation <sup>68</sup>, and therefore absence of stereo-specificity in rigor suggests that some cross-bridges are unable to contribute to force development. This, in turn, suggests that MUT hearts may be subject to inefficient energy utilization by producing less contractile force than the WT hearts for the same supply of ATP. This would ultimately lead to metabolite accumulation in the mutated myocardium and compromised heart performance, because sustained or progressive demands on the heart can result in a series of compensatory responses including cardiac hypertrophy and myocardial remodeling. In other words, energy expenditure through inefficient energy use would lead to increased turnover of ATP, particularly during stress. From the clinical
standpoint, these multilevel changes in cardiac contractility would lead to severe FHC phenotype.

The PF values clustered around two distinct values of polarization (Table 7, Fig. 21). This was not surprising because fluorescence is contributed by cross-bridges which interact with actin carrying both WT and MUT TnT. We note that this effect can only be seen in mesoscopic measurements. Conventional measurements, containing contributions from billions of crossbridges, offer no hope of detecting two populations.

The polarizations observed in mutated hearts in rigor can, in principle, be translated into range of angles that the transition dipole of rhodamine assumes with respect to the myofibrillar axis. However, such translation has not been attempted here because it is critically dependent on the model of arrangement of cross-bridges. For example, in the model of Tregear and Mendelson<sup>71</sup> which assumes that the cross-bridges are arranged helically along the long axis of muscle and that polarized fluorescence contains  $\alpha$ % contribution from random immobilized component, the angle can range from 90° to 5° depending on the value of  $\alpha$ . In earlier global studies, fluorescent probes were used to study the breadth of orientation distribution of probes bound to RLC of skeletal muscle myosin. Thus Ling et al. <sup>70</sup> and later Hopkins et al. <sup>17</sup> reported that the breadth of distribution of cross-bridges in skeletal muscle was essentially the same in different physiological states. Similarly to TnT mutations, 25 experiments on FHC hearts containing A13T mutation in the regulatory light chain revealed a significant increase in the range of polarizations (data not shown). The average skewness of mutated myofibrils in rigor increased by 20% and kurtosis increased by 132% over WT myofibrils in rigor. Thus it is likely that an

increase in rigor disorder is a general phenomenon i.e. the stereospecific rigor attachment of force generating myosin crossbridges is necessary for the normal working of the heart. Any alteration of this important energetic state of the myosin motor could be a triggering factor of cardiomyopathy.

The question remains as to how our findings of the functional deficit at the molecular level explain the diastolic dysfunction? The diastolic dysfunction (decreased ability to relax) may be related to the increased Ca<sup>2+</sup> affinity to TnC in these mutations (slower off rate of Ca<sup>2+</sup> from TnC needed for relaxation). Since rigor bridges are known to increase the Ca<sup>2+</sup> affinity of TnC <sup>87</sup>, it is possible that altered rigor bridge binding increases Ca<sup>2+</sup> affinity and therefore contributes to diastolic dysfunction. We can also speculate that these TnT HCM mutations may have two different components that affect contraction: one component affecting the troponin Ca<sup>2+</sup> affinity and the other component affecting the maximal force. The component that affects Ca<sup>2+</sup> affinity would be correlated with diastolic dysfunction since it delays the Ca<sup>2+</sup> transient <sup>79</sup>. The other component affecting the rigor bonds would be correlated with the decreased maximal force (which is a trend seen in all these three TnT HCM mice).

To summarize: the cardiac muscle data showed that the rate of dissociation of cross-bridges from thin filaments was decreased in MUT muscle as compared to WT, while the probability distributions during rigor indicated a substantial loss of order. This was true not only in TnI R145W mutation but also in ELC-A57G, RLC-R58Q <sup>67</sup>, and TnT <sup>88</sup> mutations. This suggests that the diastolic dysfunction (decreased ability to relax) may be related to the increased Ca<sup>2+</sup> affinity to TnC (slower off rate of Ca<sup>2+</sup> from TnC needed for relaxation). Since rigor bridges are known to increase the Ca<sup>2+</sup> affinity of TnC <sup>87</sup>, it is possible that altered rigor bridge binding increases Ca<sup>2+</sup> affinity and therefore contributes to diastolic dysfunction. Thus, it is likely that a decrease in the rate of cross-bridge dissociation from thin filaments and an increase in rigor disorder are general phenomena, i.e. the stereo-specific rigor attachment of force generating myosin cross-bridges is necessary for the normal working of the heart. Any alteration of this important energetic state of the myosin motor could be a triggering factor of cardiomyopathy.

## CONCLUSIONS

The projects I have dissected out as part of my thesis work have demonstrated that the novel technique of SMD is a viable alternative to more conventional methods. First, in the mesoscopic regime the averaging of the signal is minimal and the dynamic and steady-state behavior can be examined in great detail. Second, the method provides information from a few cross-bridges confined to one half sarcomere, avoiding muscle heterogeneity. Third, it is well known that certain mutations in sarcomeric proteins of cardiac muscle cause severe cardiomyopathy. The mesoscopic method offers a possibility of examining mutations which are expressed at low levels. Often, the mutations can be generated in transgenic mouse with only  $\sim 10$  % incorporation of the mutated protein into a sarcomere. Such a small fraction may not lead to a significant change in the conventional contractile properties of muscle. The situation is different when examining few cross-bridges. They belong either to wild-type (WT) or mutated (MUT) subpopulation. If the means of subpopulations differ by 1 standard deviation, there is a 34 % chance that, by doing 10 independent experiments, the existence of the MUT sub-population that is expressed at 10 % level will not be revealed. But this number goes rapidly to zero as the number of experiments increases. The chance that MUT sub-population will not be revealed in 100 experiments is virtually zero. Fourth, the method provides a possibility of studying the distribution of orientations of sarcomeric proteins. My approach to investigate molecular mechanism of muscular contraction can be further improvised to provide insight into the functionality and behavior of individual molecules and subdomains of individual molecules. Combination of experimental approaches such as single molecule fluorescence and optical traps can be exploited to measure the mechanochemical steps and force generation simultaneously <sup>89</sup>

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## References

- Geeves MA, Holmes KC. Structural mechanism of muscle contraction. Annu Rev Biochem. 1999;68:687-728.
- Lymn RW, Taylor EW. Mechanism of adenosine triphosphate hydrolysis by actomyosin. Biochemistry. 1971;10:4617-24.
- 3. Slayter HS, Lowey S. Substructure of the myosin molecule as visualized by electron microscopy. Proc Natl Acad Sci U S A. 1967;58:1611-8.
- 4. Szent-Gyorgyi AG. The early history of the biochemistry of muscle contraction. J Gen Physiol. 2004;123:631-41.
- 5. Hanson J, Huxley HE. Structural basis of the cross-striations in muscle. Nature. 1953;172:530-2.
- Huxley AF, Simmons RM. Proposed mechanism of force generation in striated muscle. Nature. 1971;233:533-8.
- Huxley AF. Cross-bridge action: present views, prospects, and unknowns. J Biomech. 2000;33:1189-95.
- 8. Rayment I. The structural basis of the myosin ATPase activity. J Biol Chem. 1996;271:15850-3.
- 9. Spudich JA, Kron SJ, Sheetz MP. Movement of myosin-coated beads on oriented filaments reconstituted from purified actin. Nature. 1985;315:584-6.
- 10. Conibear PB, Bagshaw CR, Fajer PG, Kovacs M, Malnasi-Csizmadia A. Myosin cleft movement and its coupling to actomyosin dissociation. Nat Struct Biol. 2003;10:831-5.

- Cooke R. The mechanism of muscle contraction. CRC Crit Rev Biochem. 1986;21:53-118.
- 12. Houdusse A, Sweeney HL. Myosin motors: missing structures and hidden springs. Curr Opin Struct Biol. 2001;11:182-94.
- 13. Lewis JH, Beausang JF, Sweeney HL, Goldman YE. The azimuthal path of myosin V and its dependence on lever-arm length. J Gen Physiol;139:101-20.
- 14. Corrie JE, Brandmeier BD, Ferguson RE, Trentham DR, Kendrick-Jones J, Hopkins SC, van der Heide UA, Goldman YE, Sabido-David C, Dale RE, Criddle S, Irving M. Dynamic measurement of myosin light-chain-domain tilt and twist in muscle contraction. Nature. 1999;400:425-30.
- 15. Cooke R, Crowder MS, Thomas DD. Orientation of spin labels attached to cross-bridges in contracting muscle fibres. Nature. 1982;300:776-8.
- 16. Dos Remedios CG, Millikan RG, Morales MF. Polarization of tryptophan fluorescence from single striated muscle fibers. A molecular probe of contractile state. J Gen Physiol. 1972;59:103-20.
- 17. Hopkins SC, Sabido-David C, van der Heide UA, Ferguson RE, Brandmeier BD, Dale RE, Kendrick-Jones J, Corrie JE, Trentham DR, Irving M, Goldman YE. Orientation changes of the myosin light chain domain during filament sliding in active and rigor muscle. J Mol Biol. 2002;318:1275-91.
- 18. Borejdo J, Muthu P, Talent J, Akopova I, Burghardt TP. Rotation of actin monomers during isometric contraction of skeletal muscle. J Biomed Opt. 2007;12:014013.

- 19. Borejdo J, Putnam S, Morales MF. Fluctuations in polarized fluorescence: evidence that muscle cross bridges rotate repetitively during contraction. Proc Natl Acad Sci U S A. 1979;76:6346-50.
- 20. Borejdo J, Shepard A, Akopova I, Grudzinski W, Malicka J. Rotation of the lever arm of Myosin in contracting skeletal muscle fiber measured by two-photon anisotropy. Biophys J. 2004;87:3912-21.
- 21. Borejdo J, Talent J, Akopova I, Burghardt TP. Rotations of a few cross-bridges in muscle by confocal total internal reflection microscopy. Biochim Biophys Acta. 2006;1763:137-40.
- 22. Muthu P, Mettikolla P, Calander N, Luchowski R, Gryczynski I, Gryczynski Z, Szczesna-Cordary D, Borejdo J. Single molecule kinetics in the familial hypertrophic cardiomyopathy D166V mutant mouse heart. J Mol Cell Cardiol;48:989-98.
- 23. Orenes-Pinero E, Hernandez-Romero D, Jover E, de la Morena G, Valdes M, Marin F. An Insight of Novel Pharmacological Therapies in Hypertrophic Cardiomyopathy. Med Chem.
- Taylor MR, Carniel E, Mestroni L. Familial hypertrophic cardiomyopathy: clinical features, molecular genetics and molecular genetic testing. Expert Rev Mol Diagn. 2004;4:99-113.
- 25. Kaski JP, Syrris P, Burch M, Tome-Esteban MT, Fenton M, Christiansen M, Andersen PS, Sebire N, Ashworth M, Deanfield JE, McKenna WJ, Elliott PM. Idiopathic restrictive cardiomyopathy in children is caused by mutations in cardiac sarcomere protein genes. Heart. 2008;94:1478-84.

- 26. Di Tullio MR, Zwas DR, Sacco RL, Sciacca RR, Homma S. Left ventricular mass and geometry and the risk of ischemic stroke. Stroke. 2003;34:2380-4.
- 27. Rodriguez JE, McCudden CR, Willis MS. Familial hypertrophic cardiomyopathy: basic concepts and future molecular diagnostics. Clin Biochem. 2009;42:755-65.
- 28. Vassalli G, Winkelmann BR. Molecular genetics of myocardial infarction: many genes, more questions than answers. Eur Heart J. 2004;25:451-3.
- 29. Oberst L, Zhao G, Park JT, Brugada R, Michael LH, Entman ML, Roberts R, Marian AJ. Dominant-negative effect of a mutant cardiac troponin T on cardiac structure and function in transgenic mice. J Clin Invest. 1998;102:1498-505.
- 30. Malik FI, Hartman JJ, Elias KA, Morgan BP, Rodriguez H, Brejc K, Anderson RL, Sueoka SH, Lee KH, Finer JT, Sakowicz R, Baliga R, Cox DR, Garard M, Godinez G, Kawas R, Kraynack E, Lenzi D, Lu PP, Muci A, Niu C, Qian X, Pierce DW, Pokrovskii M, Suehiro I, Sylvester S, Tochimoto T, Valdez C, Wang W, Katori T, Kass DA, Shen YT, Vatner SF, Morgans DJ. Cardiac myosin activation: a potential therapeutic approach for systolic heart failure. Science;331:1439-43.
- 31. Aronson JF, Morales MF. Polarization of tryptophan fluorescence in muscle.
  Biochemistry. 1969;8:4517-22.
- 32. Dos Remedios CG, Yount RG, Morales MF. Individual states in the cycle of muscle contraction. Proc Natl Acad Sci U S A. 1972;69:2542-6.
- *33.* Thomas DD, Cooke R. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. Biophys J. 1980;32:891-906.

- 34. Borejdo J, Assulin O, Ando T, Putnam S. Cross-bridge orientation in skeletal muscle measured by linear dichroism of an extrinsic chromophore. J Mol Biol. 1982;158:391-414.
- 35. Berger CL, Craik JS, Trentham DR, Corrie JE, Goldman YE. Fluorescence polarization of skeletal muscle fibers labeled with rhodamine isomers on the myosin heavy chain. Biophys J. 1996;71:3330-43.
- 36. Sabido-David C, Brandmeier B, Craik JS, Corrie JE, Trentham DR, Irving M. Steadystate fluorescence polarization studies of the orientation of myosin regulatory light chains in single skeletal muscle fibers using pure isomers of iodoacetamidotetramethylrhodamine. Biophys J. 1998;74:3083-92.
- Warshaw DM, Hayes E, Gaffney D, Lauzon AM, Wu J, Kennedy G, Trybus K, Lowey S,
  Berger C. Myosin conformational states determined by single fluorophore polarization.
  Proc Natl Acad Sci U S A. 1998;95:8034-9.
- 38. Quinlan ME, Forkey JN, Goldman YE. Orientation of the myosin light chain region by single molecule total internal reflection fluorescence polarization microscopy. Biophys J. 2005;89:1132-42.
- *Lu H, Ali MY, Bookwalter CS, Warshaw DM, Trybus KM. Diffusive movement of processive kinesin-1 on microtubules. Traffic. 2009;10:1429-38.*
- 40. Yildiz A, Tomishige M, Vale RD, Selvin PR. Kinesin walks hand-over-hand. Science. 2004;303:676-8.
- 41. Mattheyses AL, Axelrod D. Fluorescence emission patterns near glass and metal-coated surfaces investigated with back focal plane imaging. J Biomed Opt. 2005;10:054007.

- 42. Yildiz A, Park H, Safer D, Yang Z, Chen LQ, Selvin PR, Sweeney HL. Myosin VI steps via a hand-over-hand mechanism with its lever arm undergoing fluctuations when attached to actin. J Biol Chem. 2004;279:37223-6.
- 43. Forkey JN, Quinlan ME, Shaw MA, Corrie JE, Goldman YE. Three-dimensional structural dynamics of myosin V by single-molecule fluorescence polarization. Nature. 2003;422:399-404.
- 44. Lu H, Kennedy GG, Warshaw DM, Trybus KM. Simultaneous observation of tail and head movements of myosin V during processive motion. J Biol Chem;285:42068-74.
- 45. Sun Y, Sato O, Ruhnow F, Arsenault ME, Ikebe M, Goldman YE. Single-molecule stepping and structural dynamics of myosin X. Nat Struct Mol Biol;17:485-91.
- 46. Hell SW, Wichmann J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt Lett. 1994;19:780-2.
- 47. Klar TA, Jakobs S, Dyba M, Egner A, Hell SW. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. Proc Natl Acad Sci U S A. 2000;97:8206-10.
- 48. Urban NT, Willig KI, Hell SW, Nagerl UV. STED nanoscopy of actin dynamics in synapses deep inside living brain slices. Biophys J;101:1277-84.
- 49. Tian Q, Pahlavan S, Oleinikow K, Jung J, Ruppenthal S, Scholz A, Schumann C, Kraegeloh A, Oberhofer M, Lipp P, Kaestner L. Functional and morphological preservation of adult ventricular myocytes in culture by sub-micromolar cytochalasin D supplement. J Mol Cell Cardiol;52:113-24.

- Burghardt TP, Ajtai K, Borejdo J. In situ single-molecule imaging with attoliter detection using objective total internal reflection confocal microscopy. Biochemistry. 2006;45:4058-68.
- 51. Muthu P, Talent JM, Gryczynski I, Borejdo J. Cross-bridge duty cycle in isometric contraction of skeletal myofibrils. Biochemistry. 2008;47:5657-67.
- 52. Burghardt TP, Hipp AD, Ajtai K. Around-the-objective total internal reflection fluorescence microscopy. Appl Opt. 2009;48:6120-31.
- Borejdo J, Calander N, Gryczynski Z, Gryczynski I. Fluorescence correlation spectroscopy in surface plasmon coupled emission microscope. Opt Express. 2006;14:7878-88.
- 54. Borejdo J, Gryczynski Z, Calander N, Muthu P, Gryczynski I. Application of surface plasmon coupled emission to study of muscle. Biophys J. 2006;91:2626-35.
- Gryczynski Z, Borejdo J, Calander N, Matveeva EG, Gryczynski I. Minimization of detection volume by surface-plasmon-coupled emission. Anal Biochem. 2006;356:125-31.
- 56. Burghardt TP, Charlesworth JE, Halstead MF, Tarara JE, Ajtai K. In situ fluorescent protein imaging with metal film-enhanced total internal reflection microscopy. Biophys J. 2006;90:4662-71.
- 57. Mettikolla P, Calander N, Luchowski R, Gryczynski I, Gryczynski Z, Borejdo J. Kinetics of a single cross-bridge in familial hypertrophic cardiomyopathy heart muscle measured by reverse Kretschmann fluorescence. J Biomed Opt;15:017011.
- 58. Borejdo J, Rich R, Midde K. Mesoscopic analysis of motion and conformation of crossbridges. In: Biophysical Reviews: Springer Berlin / Heidelberg; 2012:1-13.

- 59. K. Midde RR, V. Hohreiter, S. Raut, R. Luchowski, C. Hinze, R. Fudala, I. Gryczynski, Z. Gryczynski, and J. Borejdo. Rotation of myosin lever arms during isometric contraction of skeletal myofibrils. In: Willems M, ed. Skeletal Muscle: Physiology, Classification and Disease. Chichester, UK: Nova Science Publishers Inc; 2012.
- 60. Magde D, Elson EL, Webb WW. Fluorescence correlation spectroscopy. II. An experimental realization. Biopolymers. 1974;13:29-61.
- 61. Elson EL. Quick tour of fluorescence correlation spectroscopy from its inception. J Biomed Opt. 2004;9:857-64.
- 62. Stewart MA, Franks-Skiba K, Chen S, Cooke R. Myosin ATP turnover rate is a mechanism involved in thermogenesis in resting skeletal muscle fibers. Proc Natl Acad Sci U S A;107:430-5.
- 63. Herrmann C, Lionne C, Travers F, Barman T. Correlation of ActoS1, myofibrillar, and muscle fiber ATPases. Biochemistry. 1994;33:4148-54.
- 64. Ushakov DS, Caorsi V, Ibanez-Garcia D, Manning HB, Konitsiotis AD, West TG, Dunsby C, French PM, Ferenczi MA. Response of rigor cross-bridges to stretch detected by fluorescence lifetime imaging microscopy of myosin essential light chain in skeletal muscle fibers. J Biol Chem;286:842-50.
- 65. Tsaturyan AK, Bershitsky SY, Burns R, Ferenczi MA. Structural changes in the actinmyosin cross-bridges associated with force generation induced by temperature jump in permeabilized frog muscle fibers. Biophys J. 1999;77:354-72.
- 66. Midde K, Luchowski R, Das HK, Fedorick J, Dumka V, Gryczynski I, Gryczynski Z, Borejdo J. Evidence for pre- and post-power stroke of cross-bridges of contracting skeletal myofibrils. Biophys J;100:1024-33.

- 67. Mettikolla P, Calander N, Luchowski R, Gryczynski I, Gryczynski Z, Zhao J, Szczesna-Cordary D, Borejdo J. Cross-bridge kinetics in myofibrils containing familial hypertrophic cardiomyopathy R58Q mutation in the regulatory light chain of myosin. J Theor Biol;284:71-81.
- 68. Coureux PD, Sweeney HL, Houdusse A. Three myosin V structures delineate essential features of chemo-mechanical transduction. Embo J. 2004;23:4527-37.
- 69. Ostap EM, Barnett VA, Thomas DD. Resolution of three structural states of spin-labeled myosin in contracting muscle. Biophys J. 1995;69:177-88.
- Ling N, Shrimpton C, Sleep J, Kendrick-Jones J, Irving M. Fluorescent probes of the orientation of myosin regulatory light chains in relaxed, rigor, and contracting muscle. Biophys J. 1996;70:1836-46.
- 71. Tregear RT, Mendelson RA. Polarization from a helix of fluorophores and its relation to that obtained from muscle. Biophys J. 1975;15:455-67.
- 72. Tregear RT, Reedy MC, Goldman YE, Taylor KA, Winkler H, Franzini-Armstrong C, Sasaki H, Lucaveche C, Reedy MK. Cross-bridge number, position, and angle in target zones of cryofixed isometrically active insect flight muscle. Biophys J. 2004;86:3009-19.
- 73. Cooke R. The role of the myosin ATPase activity in adaptive thermogenesis by skeletal muscle. Biophys Rev;3:33-45.
- 74. Hooijman P, Stewart MA, Cooke R. A new state of cardiac myosin with very slow ATP turnover: a potential cardioprotective mechanism in the heart. Biophys J;100:1969-76.
- 75. Szczesna D, Zhao J, Jones M, Zhi G, Stull J, Potter JD. Phosphorylation of the regulatory light chains of myosin affects Ca2+ sensitivity of skeletal muscle contraction. J Appl Physiol. 2002;92:1661-70.

- 76. Muthu P, Kazmierczak K, Jones M, Szczesna-Cordary D. The effect of myosin RLC phosphorylation in normal and cardiomyopathic mouse hearts. J Cell Mol Med;16:911 9.
- 77. James J, Robbins J. Signaling and myosin-binding protein C. J Biol Chem;286:9913-9.
- 78. Colson BA, Locher MR, Bekyarova T, Patel JR, Fitzsimons DP, Irving TC, Moss RL.
  Differential roles of regulatory light chain and myosin binding protein-C
  phosphorylations in the modulation of cardiac force development. J Physiol;588:981-93.
- 79. Miller T, Szczesna D, Housmans PR, Zhao J, de Freitas F, Gomes AV, Culbreath L, McCue J, Wang Y, Xu Y, Kerrick WG, Potter JD. Abnormal contractile function in transgenic mice expressing a familial hypertrophic cardiomyopathy-linked troponin T (I79N) mutation. J Biol Chem. 2001;276:3743-55.
- 80. Hernandez OM, Szczesna-Cordary D, Knollmann BC, Miller T, Bell M, Zhao J, Sirenko SG, Diaz Z, Guzman G, Xu Y, Wang Y, Kerrick WG, Potter JD. F110I and R278C troponin T mutations that cause familial hypertrophic cardiomyopathy affect muscle contraction in transgenic mice and reconstituted human cardiac fibers. J Biol Chem. 2005;280:37183-94.
- Szczesna D, Lehrer SS. The binding of fluorescent phallotoxins to actin in myofibrils. J Muscle Res Cell Motil. 1993;14:594-7.
- 82. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle.
  Physiol Rev. 2000;80:853-924.
- 83. Artz G, Wynne J. Restrictive Cardiomyopathy. Curr Treat Options Cardiovasc Med. 2000;2:431-438.

- 84. Wen Y, Xu Y, Wang Y, Pinto JR, Potter JD, Kerrick WG. Functional effects of a restrictive-cardiomyopathy-linked cardiac troponin I mutation (R145W) in transgenic mice. J Mol Biol. 2009;392:1158-67.
- 85. Mogensen J, Kubo T, Duque M, Uribe W, Shaw A, Murphy R, Gimeno JR, Elliott P, McKenna WJ. Idiopathic restrictive cardiomyopathy is part of the clinical expression of cardiac troponin I mutations. J Clin Invest. 2003;111:209-16.
- 86. Gomes AV, Potter JD. Molecular and cellular aspects of troponin cardiomyopathies. Ann NY Acad Sci. 2004;1015:214-24.
- 87. Guth K, Potter JD. Effect of rigor and cycling cross-bridges on the structure of troponin C and on the Ca2+ affinity of the Ca2+-specific regulatory sites in skinned rabbit psoas fibers. J Biol Chem. 1987;262:13627-35.
- 88. Midde K, Dumka V, Pinto JR, Muthu P, Marandos P, Gryczynski I, Gryczynski Z, Potter JD, Borejdo J. Myosin cross-bridges do not form precise rigor bonds in hypertrophic heart muscle carrying troponin T mutations. J Mol Cell Cardiol;51:409-18.
- 89. Elting MW, Spudich JA. Future challenges in single-molecule fluorescence and laser trap approaches to studies of molecular motors. Dev Cell;23:1084-91.