Measuring myosin cross-bridge attachment time in activated muscle fibers using stochastic versus sinusoidal length perturbation analysis

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Running title:
White-noise length perturbation analysis in muscle fibers

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Abstract

The average time myosin cross-bridges remain bound to actin ($t_{on}$) can be measured by sinusoidal length perturbations (sinusoidal analysis) of striated muscle fibers using recently developed analytical methods. This approach allows measurements of $t_{on}$ in preparations possessing a physiologically relevant myofilament lattice. In this study we developed an approach to measure $t_{on}$ in 5-10% of the time required for sinusoidal analysis by using stochastic length perturbations (white-noise analysis). To compare these methods we measured the influence of [MgATP] on $t_{on}$ in demembranated myocardial strips from mice, sampling muscle behavior from 0.125-200 Hz with a 20 second burst of white-noise versus a 300 second series of sinusoids. Both methods detected a similar >300% increase in $t_{on}$ as [MgATP] decreased from 5 to 0.25 mM, differing by only 3-14% at any [MgATP]. Additional experiments with Drosophila indirect flight muscle fibers demonstrated that faster cross-bridge cycling kinetics permit further reducing the perturbation time required to measure $t_{on}$. This reduced sampling time allowed strain-dependent measurements of $t_{on}$ in flight muscle fibers by combining 10 s bursts of white-noise during periods of linear shortening and lengthening. Analyses revealed longer $t_{on}$ values during shortening and shorter $t_{on}$ values during lengthening. This asymmetry may provide a mechanism that contributes to oscillatory energy transfer between the flight muscles and thoracic cuticle to power flight. This study demonstrates that white-noise analysis can detect underlying molecular processes associated with dynamic muscle contraction comparable to sinusoidal analysis, but in a fraction of the time.

Keywords: muscle mechanics, cross-bridge kinetics, cardiac and insect flight muscle, biological systems identification, motor proteins
**Introduction**

Viscoelastic behavior in activated muscle tissue arises from cyclical binding of the motor protein myosin with actin to create a force producing cross-bridge. Traditional system analysis techniques for analyzing cross-bridge kinetics examine the relationship between muscle stress ($\sigma$, force divided by cross-sectional area) and muscle strain ($\varepsilon$, the change in muscle length divided by the original length). These techniques employ either a step change in muscle length [4, 5, 11, 18], a series of sinusoidal strains [19, 20, 26, 27, 44, 46], or a variety of stochastic strains [3, 14, 42], each of which embodies benefits and limitations for investigating linear versus non-linear muscle responses. The likelihood of observing non-linearities in the force response increases with length changes greater than 0.5-1% muscle length (ML) [26], thus small amplitude length changes maintain system linearity and enhance the capacity of mathematical models for describing cross-bridge kinetics the underlie the measured stress-strain relationship [1, 5, 8, 20, 23, 35, 46].

Sinusoidal analysis of activated muscle fibers is based on a series of small amplitude sinusoidal length perturbations applied at several discrete frequencies, thus characterizing the complex modulus as a complex sum: 

$$Y(\omega) = E_e(\omega) + iE_v(\omega)$$

(see Eq. 1), where $E_e =$ elastic modulus, $E_v =$ viscous modulus, and $\omega =$ the angular frequency defined as $2\pi \times$ frequency (Fig. 1). Frequency-dependent characteristics of the complex modulus reflect the enzymatic cross-bridge behavior measured during force generating portions of the cross-bridge cycle. In particular, the frequency parameter of the historically defined C-process, $c$ in Eq. 1 [20], provides an estimate of mean myosin cross-bridge attachment duration as $t_{on} = (2\pi c)^{-1}$ [35], making sinusoidal analysis a
powerful technique for examining cross-bridge kinetics underlying force production in muscle preparations retaining myofilament lattice structure.

Although white-noise analysis in physiological systems encompasses a history of linear and non-linear analysis [28], we focused on linear aspects of muscle mechanics for comparison with the intrinsically linear systems analysis method of sinusoidal analysis. Prior studies have assessed the viscoelastic dynamics of intact and skinned muscle fibers through random changes in muscle length [14, 15, 17, 33, 42, 43], but none have linked measured system responses to varied kinetics at specific portions of the cross-bridge cycle as described here for shifts in $t_{on}$ with [MgATP] and load.

A potent advantage of white-noise analysis [35] over sinusoidal analysis is the ability to simultaneously sample a desired frequency range of the muscle response in a fraction of the time required to complete the multiple frequency sweeps of sinusoidal analysis (Fig. 2). This feature elevates the measured information content to facilitate extracting $t_{on}$ from the ‘noisy’ system response often associated with these measurements. The benefit of simultaneously sampling a specified frequency range enables layering the white-noise analysis upon linear shortening and lengthening transients to probe the effect of strain, or load, on actomyosin cross-bridge cycling kinetics. The following study not only confirms that similar values for $t_{on}$ can be obtained from sinusoidal and white-noise analysis, but clearly demonstrates that white-noise analysis is a robust and useful method for probing strain-dependent molecular processes underlying muscle contraction.
Materials & Methods

Solutions

Solutions were prepared according to a computer program that solves the ionic equilibria [13]. Unless listed otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO.) and concentrations are expressed in mmol/L (mM). For experiments using mouse myocardium, relaxing solution (pCa 8.0, where pCa = -log10[Ca2+] ) consisted of 20 N,Nbis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 35 creatine phosphate (CP), 300 U/mL creatine phosphokinase (CPK), 1 DTT, 5 EGTA, 1 Mg2+, 5 MgATP, at an ionic strength of 200 mEq adjusted with sodium methane sulfate, and pH 7.0. Activating solution was similar to relaxing solution, but at pCa 4.0. Skinning solution for the mouse myocardium experiments was similar to the relaxing solution described above, except for having an ionic strength of 190 mEq, 30 2,3-butanedione monoxime (BDM), 50% (wt/vol) glycerol, and 1% (wt/vol) Triton X-100. Mouse storage solution was similar to skinning solution with 10 μg/ml leupeptin and no Triton.

Skinning and storage solutions for insect flight muscle experiments were described previously [29]. Relaxing solution consisted of pCa 8, 20 BES, 20 CP, 450 U/mL CPK, 1 DTT, 5 EGTA, 1 Mg2+, 12 MgATP, 2 P, ionic strength of 200 mEq adjusted with sodium methane sulfate, and pH 7.0. Activating solution was similar to relaxing solution, but at pCa 4.0.

Mouse cardiac strips

Mouse cardiac muscle is well described by Eq. 1 [34, 35, 36], which is used to estimate \( t_{on} \) from sinusoidal and white-noise analysis measurements. The response of mouse cardiac muscle to
varying MgATP is well characterized by Eq. 1, in contrast with other striated muscles such as slow twitch skeletal muscle [31]. For these reasons, we chose mouse cardiac muscle as the most appropriate tissue for applying and assessing the white-noise technique to calculate the response of $t_{on}$ to varying [MgATP].

All procedures were reviewed and approved by the Institutional Animal Care and Use Committees of University of Vermont College of Medicine and complied with the Guide for the Use and Care of Laboratory Animals published by the National Institutes of Health (NIH). Hearts were removed from mice after cervical dislocation and placed into Krebs solution containing 30 mM BDM bubbled with 95% O$_2$-5% CO$_2$ at room temperature. Papillary muscle strips were prepared as previously described [35], and demembranated (skinned) strips were stored at −20° C for no more than 4 days.

Myocardial strips were dissected to 120-180 μm diameter. At the time of study aluminum T-clips were attached to the ends of a strip ~300 μm apart. The strip was mounted between a piezoelectric motor (model P-841.40, Physik Instrumente, Auburn, MA) driven by a custom built amplifier and a strain gauge (model AE801, SensorNor, Horten, Norway), lowered into a 30 μL droplet of relaxing solution at pCa 8 maintained at 27° C. Strips were adjusted to 2.2 μm sarcomere length as determined by digital fast Fourier transform (FFT) analysis (IonOptix, Milton, MA). Strips (n=2, from a single papillary muscle) were calcium activated to pCa 4.8 and MgATP concentration was varied from 5 to 0.25 mM. At each MgATP condition sinusoidal length perturbations of amplitude 0.125% muscle length (ML) were applied at 0.125-200 Hz as
previously described [38, 39]. Gaussian white-noise length perturbation was also applied as described below. Data were sampled at 5 kHz.

Insect flight muscle fibers

Insect flight muscle is stiffer and possesses faster myosin kinetics than cardiac muscle. These attributes enhance the signal-to-noise ratio for measures of elastic and viscous moduli using white-noise analysis, contributing to a more precise calculation of $t_{on}$ in insect flight muscle than in cardiac muscle (Table 1) and thus enabled better detection of small changes in the frequency characteristics of the muscle response. For these reasons and our familiarity with fruit fly flight muscle mechanics [7, 29, 30, 45], we used this preparation to detect $t_{on}$ during muscle shortening and lengthening.

Newly eclosed female *Drosophila melanogaster* (Oregon-R strain) were placed in 35-ml plastic vials containing standard cornmeal fly food and maintained at 25°C, 70% humidity, and a 12/12 h light/dark cycle. At 2- to 3-days old, flies were anesthetized with CO$_2$ and single dorsolongitudinal muscle fibers were isolated from half-thoraces. Fibers were then split lengthwise to ~100 μm diameter to reduce the cross-sectional area. The fibers were demembranated in skinning solution for 1 h at 4°C, clipped with aluminum T-clips at both ends ~300 μm apart. Where fibers were not used immediately, they were transferred to storage solution at -20°C and used within 2 days of dissection. Fibers were mounted between a piezoelectric motor (model P-841.10, Physik Instrumente) driven by an amplifier (model E662, Physik Instrumente) and a strain gauge (model AE801, SensorNor). As previously described [7, 29, 45], fibers (n=9, from six flies) were then lowered into 30 μL of relaxing solution maintained
at 15° C, then stretched 5% from just taut and activated to pCa 4.5, from which followed
additional stretches in 2% increments until oscillatory work production reached a stable
maximum. Additional mechanical analyses were then performed using white-noise length
perturbations, where data were sampled at 8 kHz.

**Mechanical analysis**

A digital FFT was applied to the strain and stress signals, and the complex modulus \( \text{Y}(\omega) \)
where \( \omega \) is angular frequency) is calculated from the quotient of these FFTs (stress/strain, as
depicted in Fig. 1). Elastic and viscous moduli are defined as the in-phase (real part) and out-of-
phase (imaginary part) portions of complex modulus, respectively. Fitting Eq. 1 to these data
provides an estimate of mean myosin attachment time \( (t_{\text{on}}) \), where \( t_{\text{on}}=(2\pi)^{-1} \) [35].

\[
Y(\omega) = A(\omega)^k - B \left( \frac{i\omega}{2\pi b + i\omega} \right) + C \left( \frac{i\omega}{2\pi c + i\omega} \right) \quad \text{Eq 1.}
\]

Mechanical measurements of \( Y(\omega) \) arise from for passive structure elements of the muscle cell
and force-producing cross-bridges. The A-process reflects the passive elements, where length
perturbations produce force responses of constant relative phase, independent of frequency, as
indicated by straight line in plots of elastic versus viscous moduli [35]. In our interpretation, the
parameter \( A \) represents the combined mechanical stiffness of the filaments and the number of
strongly bound cross-bridges [34], and \( k \) describes the degree of the viscoelastic response as
elastic \( (k \to 0) \) versus viscous \( (k \to 1) \). The power-law frequency response described in the A-
process provides a good description of almost all viscoelastic materials including living cells,
relaxed muscle [9], and the passive elements parallel to and in series with sarcomeres in activated muscle.

The B- and C-processes reflect enzymatic cross-bridge behavior in activated muscle and are sensitive to concentrations of MgATP, MgADP and P_i [21, 22] as well as to temperature with a Q_{10} ~ 2-3 [35]. It should be noted that using 2πc to estimate the mean t_{on} requires that a single exponential function reasonably describe the probability distribution of t_{on} [35]. A single exponential distribution is reported from single myosin molecule experiments in the laser trap under low (<0.1 mM) MgATP conditions [2, 10]. To demonstrate the flexibility of the single exponential assumption under high MgATP conditions, Palmer et al. [35] successfully used computational simulations to validate the technique using a double exponential distribution, which could represent a relatively long ADP state convolved with a short P_i state. These studies [2, 10, 35] and current measurements herein suggest a valid use of 2πc to calculate mean t_{on} in demembranated muscle fibers under low and high MgATP conditions. Our current methods do not, however, provide a description of time detached, t_{off}.

Sinusoidal analysis provides the complex modulus at several discrete frequencies, each calculated separately. In contrast, one can combine multiple sinusoids covering a specified frequency range to create a particular type of stochastic signal termed band-limited Gaussian noise (Fig. 2). In principle, this noise analysis should describe identical system behavior as sinusoidal analysis when applied to muscle fibers (Fig. 2 A). In practice, band-limited Gaussian noise is created a priori by summing a series of individual sinusoidal length perturbations [28]. The ‘band-limited’ adjective refers to the frequency content represented by the signal, which
includes sinusoids with frequencies corresponding to integer values of the fundamental frequency ($\omega_0=2\pi/T$) up to a prescribed cutoff frequency ($f_c$). As an example, for the fruit fly measurements the stimulus period ($T$) was 10 s and $f_c$ was 1000 Hz, thereby comprising sinusoids with frequencies from 0.1 to 1000 Hz at every 0.1 Hz. The ‘Gaussian’ adjective refers to the amplitude distribution taking on a Gaussian profile (Fig. 2 B) as a consequence of the central limit theorem, created by each individual sinusoid having a constant amplitude ($A$) with a random phase ($\phi_n$, drawn from a uniform distribution between $-\pi$ to $\pi$). The ‘white-noise’ adjective describes a flat power spectrum for the stochastic stimulus across the frequency range $\omega_0$ to $f_c$ (Fig. 2 C), indicating that the stimulus contains equal power across the specified bandwidth.

For our white-noise analysis, the standard deviation of the strain amplitude distribution was 0.1 % and 0.075 % ML, with $T$ of 20 and 10 s for the myocardial strip and insect flight muscle fiber measurements, respectively. Insect flight muscle experiments combined this white-noise stimulus with linear lengthening and shortening transients (of strain rate 0.02 % ML/s) to probe strain dependence of $t_{on}$. Similar to sinusoidal analysis, elastic and viscous moduli were calculated from the quotient of the FFTs for stress and strain (example magnitudes for these FFTs are depicted in Fig. 2 C). The mean slope across $T$ was subtracted from the stress and strain profiles prior to Fourier analysis. For each strain stimulus ($T$ seconds long) we wrapped the final and initial 2.5 seconds of the stimulus onto the front and back ends, respectively, creating a stimulus ($T+5$ seconds long) to mitigate discontinuities at the edges of the intermediate $T$ seconds of data analyzed to calculate the complex modulus. This approach also decreased mechanical heterogeneities in the measured stress response due to the abrupt length transitions at
the start and end of the white-noise stimulus. To increase visual clarity, the elastic and viscous
moduli plotted in Figs. 3 and 5 were down sampled every 10 data points, although the complex
moduli were calculated using complete data sets. Curve-fitting of these data (using complete
data sets) allowed us to estimate $t_{on}$ using white-noise analysis techniques, similar to the
sinusoidal analysis techniques described above.

Statistical analysis

All values are mean ± SEM. Statistical analyses were performed using SPSS (v.14.0; SPSS,
Chicago, IL) and Matlab (v 7.9.0, The Mathworks, Natick, MA.). Non-linear least squares
fitting of Eq 1 to recorded complex moduli was performed using a Levenberg-Marquardt routine
(IDL 7.0, ITT Visual Information Solutions, Boulder, CO). Most data were examined using a
one-way ANOVA followed by a multiple comparison of the means to access significance. To
explore whether there were population specific trends for model parameters from Eq. 1 or
whether $t_{on}$ varied with [MgATP] or muscle shortening and lengthening transients, data were
initially examined using a repeated-measures ANOVA followed with a one-way ANOVA and
multiple comparisons of the means to evaluate significance. For all analysis, data were
significant for p<0.05.
Results

Comparing sinusoidal and white-noise analyses for detecting $t_{on}$

We compared sinusoidal and white-noise analyses using mouse myocardial strips under maximal Ca$^{2+}$-activation (pCa 4.8) and varied [MgATP] conditions (Fig. 3). These experiments demonstrated that sinusoidal and white-noise analyses measure similar shifts in mechanical behavior as cross-bridge kinetics varied with [MgATP]. Leftward or rightward shifts in the plots of moduli versus frequency represent slower and faster kinetics, respectively. Thus, the leftward shift as [MgATP] decreased from 5 to 0.25 mM (Fig. 3) indicates slower cross-bridge cycling rates, as shown by previous measurements using sinusoidal analysis [21, 47].

While both methods described similar biomechanical phenomena, it is important to note that white-noise analysis took only 20 s while the sinusoidal analysis took ~300 s to complete. However, the discrete period (T) for white-noise analysis presents a trade off in measurement duration versus the ability to resolve strain and stress amplitudes at the smallest frequencies, due to fewer cycles as $\omega$ approaches $2\pi/T$. Thus, there appears more variability between adjacent moduli values at the lowest frequencies (particularly for the semi-log plots in Fig. 3), although standard error in moduli values was similar for both analysis methods.

Fitting Eq. 1 to the moduli data also illustrated similarities between the two techniques, where average fits for the noise data (lines in Fig. 3) closely resembled sinusoidal analysis. These fits show similar responses among all model parameters for both analysis methods (Fig. 4), and that
[MgATP] significantly affected the trend observed for each parameter value, except for $B$ (Fig. 4 B) which was not sensitive to [MgATP]. Importantly, this comparison further demonstrates both analysis methods are sampling and describing similar biomechanical phenomena, although systematic differences in the average parameter values returned by each method are evident.

Model parameters $A$, $k$, and $c$ (Fig 4 A, D, and F) were most sensitive to [MgATP]. As [MgATP] decreased, the increased $A$ values and decreased $k$ values demonstrate an increasingly elastic response due to the augmented population of strongly bound cross-bridges. The nearly 4-fold change in $A$ and $c$ suggests a 4-fold increase in cross-bridge duty-cycle as [MgATP] decreases, given the minimal change in the cross-bridge recruitment rate indicated by relatively small shifts in the B-process parameters with [MgATP] (Fig. 4 B and E). These results show that [MgATP] only modestly effects work producing portions of the cross-bridge cycle, while decreasing [MgATP] significantly increases myosin attachment time, $t_{on}$ (Fig. 5A).

Decreasing [MgATP] produced a non-linear increase in $t_{on}$, with only a 4-13% divergence in estimated $t_{on}$ values between both methods (correlation coefficient=0.99, Fig. 5A). As MgATP decreased from 5 and 0.25 mM, $t_{on}$ ranged from 3.3±0.1 to 12.9±0.8 ms for sinusoidal analysis and from 3.8±0.1 to 12.4±0.2 ms for white-noise analysis, suggesting less MgATP prolongs the rigor state of the cross-bridge cycle (Fig. 5B) [12]. This small divergence across a broad range of values further supports the premise that these two analysis techniques describe similar physiological processes.
Strain dependence of $t_{on}$ detected by white-noise analysis

White-noise analysis was applied to maximally activated (pCa 4.5) indirect flight muscle (IFM) fibers from fruit flies (Fig. 6). The super-fast actomyosin kinetics underlying IFM contraction helped to reduce white-noise analysis to 10 s (Fig. 6 B) versus ~200 seconds for sinusoidal analysis. We probed strain-dependent behavior by applying white-noise stimuli during periods of muscle shortening and lengthening (Fig. 6 A and C), capitalizing on white-noise analysis simultaneously sampling the entire system response. Mean strain-rates (0.02 % ML/s) were kept small during shortening and lengthening transients to maintain system linearity.

Viscous and elastic moduli values from the no-slope (mean strain = 0) measurements consistently fell between moduli values measured during shortening and lengthening. Although variability in the average moduli values made it difficult to resolve differences at individual frequencies, Nyquist plots depicting average fits to Eq. 1 (lines inset in Fig. 6E, with data and fits shown in Supplemental Figure 1) demonstrate strain-dependent shifts in the moduli. The effects of shortening and lengthening transients on mechanics and cross-bridge cycling kinetics were evaluated statistically by fitting Eq. 1 to the data (Fig. 7) and comparing parameter values. Analysis of the estimated model parameters using a repeated measures ANOVA only showed significant (P<0.05) strain sensitive effects for $A$, $k$, and $c$ (Fig. 7 A, B, D). The population response among all fibers for the entire set of $c$ values (Fig. 7 E) showed a strain-dependent shift in $c$ for most fibers, averaging a ~3 Hz decrease or increase with shortening and lengthening, respectively. Even though the resolution of $c$ is 1.5% (Table 1) the repeated measures analysis...
White-noise length perturbation analysis in muscle fibers

highlights the strength of the white-noise analysis for resolving very small kinetic changes within a single fiber.

The fits of Eq. 1 to the data demonstrated strain-dependent shifts in $t_{on}$ (Table 2), showing $t_{on}$ increased with shortening and decreased with lengthening. The minimum viscous moduli during shortening, no-slope, and lengthening conditions (Table 3) are directly proportional to the maximum oscillatory work produced within a single oscillatory cycle (work=$-E_v \pi A^2$ [29]). The lower viscous modulus at ~135 Hz during lengthening (Fig 6) may reflect reduced energy loss as a consequence of shorter $t_{on}$ (i.e., less frictional resistance to lengthening). Likewise, the higher viscous modulus at ~135 Hz during shortening (Fig 6) may reflect increased energy loss as a consequence of the longer $t_{on}$. 
Discussion

This study describes similarities and differences between sinusoidal and white-noise analysis techniques for measuring mechanical characteristics of mouse myocardial strips. Both methods yield similar values for cross-bridge attachment time ($t_{on}$) in Ca$^{2+}$-activated muscle fibers exposed to various MgATP concentrations. Sinusoidal analysis individually samples the muscle response at one applied frequency, requiring a sweep across multiple frequencies to reconstruct a composite muscle response over a desired frequency range. White-noise analysis, on the other hand, has a distinct advantage in sampling the muscle response simultaneously over a desired frequency range. Thus, the data acquisition time required for estimating $t_{on}$ can be significantly reduced using white-noise analysis (10-20 s versus 200-300 s for comparable sinusoidal analysis measurements), which also minimizes possible errors due to altered muscle behavior over the duration of an experiment. Because muscle fiber integrity and function often deteriorates with activation duration throughout an experiment, the reduced time required for white-noise analysis represents an important benefit to experimental protocols.

The fixed duration of a white-noise stimulus, on the other hand, represents a significant limitation in some applications because the relationship between strain and stress becomes most difficult to resolve for the lowest frequency data. White-noise stimuli contain more periodic cycles for the highest frequency data (near $f_c$) and one cycle for the lowest frequency data ($f_0$; Fig. 2). Thus, the signal-to-noise ratio for resolving elastic and viscous moduli increases with the number of cycles sampled, illustrated by diminished scatter in moduli values from $f_0$ to $f_c$ (Fig. 3). Although more obvious for mouse myocardium measurements due to the log-scaling of
abscissa (Fig 3), this phenomenon can be seen in the insect flight muscle measurements as well (Fig. 6). Consistently, variability in the estimate of $t_{on}$ decreases as cross-bridge cycling rates increase, shown by the lower variability in $t_{on}$ for insect flight muscle (Table 1) and the relatively higher variability in $t_{on}$ as its value increases with reduced [MgATP] in mouse myocardium (Fig. 5A). Thus, white-noise analysis and sinusoidal analysis impart different systematic errors that arise from inherent differences in frequency content and differences in signal-to-noise ratio at any given frequency. These different systematic errors underlie the small differences in absolute values for the estimated model parameters from Eq. 1 shown in Fig. 4. Nevertheless, these analytical methods clearly describe and track the same MgATP-dependent biochemical phenomena in activated muscle fibers (Fig. 3-5). One can further optimize white-noise analysis by varying the measurement period to appropriately sample kinetics associated with faster and slower muscles (Fig. 3 and 6).

Applying white-noise during periods of shortening and lengthening in Ca$^{2+}$ activated fruit fly flight muscle allowed us to take advantage of its high-strain sensitivities and super-fast cross-bridge kinetics [6, 32, 40, 41, 45] to detect strain-dependent shifts in $t_{on}$. We found that $t_{on}$ increased during shortening and decreased during lengthening (Table 2), which may be the fundamental mechanism leading to positive work production throughout an oscillatory cycle. As posited by Palmer et al [37], our reported strain dependence in $t_{on}$ would increase duty ratio during shortening and decrease duty ratio during lengthening (given no changes in myosin off time), leading to the force response lagging the length perturbation to generate positive work. This asymmetric molecular behavior may underlie viscoelastic energy transfer between the muscles and thoracic cuticle throughout a wing beat. In contrast with more traditional force-
velocity protocols that link macroscopic measurements with coarse changes in cross-bridge
cycling rate [16], white-noise analysis estimates kinetic changes of the cross-bridge cycle when
myosin is bound to actin with high resolution (e.g., changes in $t_{on}$ of the order of 1%). Strain
rates during lengthening and shortening translate into $\sim 0.3$ nm half-sarcomere$^{-1}$ s$^{-1}$ (assuming
sarcomere length 3.2 $\mu$m), well within the $\sim 7$ nm power-stroke for flight muscle [24]. Such
lengthening and shortening transients would strain an attached myosin head an average of $\sim 0.2$
$\text{pm}$, given a mean $t_{on}$ of roughly 0.6 ms (Table 2). Although small, this strain appears sufficient
to measurably bias myosin cross-bridge kinetics in Drosophila (Fig 7).

Cross-bridge behavior in Drosophila indirect flight muscle is highly sensitive to variations in
$[\text{MgATP}]$, with exceptionally fast release of MgADP [45]. Under our experimental conditions
(12 mM [MgATP] and 2 mM [Pi]) Pi release becomes the rate limiting step of the cross-bridge
cycle [45], likely due to cross-bridges rebinding Pi to reverse the power stroke. Single molecule
studies of $t_{on}$ for myosin II also show that a very short-lived population of events develops for
$[\text{Pi}]=40$ mM, which is not present in the absence of Pi [2]. Therefore, the elevated Pi sensitivity
in insect flight muscle could shorten $t_{on}$ via Pi rebinding and reduce the likelihood of myosin
completing a full MgATP-dependent cycle. However, strain dependencies in $t_{on}$ (Table 2 and
Fig. 7.) may also reflect kinetic differences in Pi release and rebinding that vary with load.

Linear system analysis methods enable us to estimate $t_{on}$ in a muscle strip or fiber, where cross-
bridge kinetics are influenced by the organized structure of the myofilament lattice. Over the
past few years we have validated the use of sinusoidal length perturbation analysis to estimate
mean $t_{on}$ by computational modeling and empirical assessments of $t_{on}$ due to the known effects of
varying myosin heavy chain isoform and temperature [35], and now [MgATP]. These tests have provided a consistent linear systems interpretation of the C-process of Eq. 1 expected from enzymatic cross-bridge cycling behavior [12, 25]. The present study shows that white-noise analysis can likewise robustly measure $t_{on}$ in muscle strips and illustrates the benefits and limitations of the white-noise versus sinusoidal analysis to probe mechanical and kinetic behavior in muscle.
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Disclosures

The authors declare no conflicts of interest.
Bibliography


White-noise length perturbation analysis in muscle fibers


White-noise length perturbation analysis in muscle fibers


**Figure Legends**

**Figure 1:** (A) A common engineering approach for mechanical system identification applies a sine wave stimulus (system input) to probe characteristics of a viscoelastic system, which acts as a filter on the stimulus to produce the measured response (system output). Applying similar analysis to muscle fibers (B), the length stimulus is normalized to muscle length (strain) and the measured force response from the muscle is normalized via fiber cross-sectional area (stress). (C) These normalizations facilitate calculating the viscous and elastic moduli, by separating the total stress response into an in-phase component that aligns temporally with the measured strain and an out-of-phase component shifted $\pi/2$ radians compared to the measured strain. The ratios of this stress to strain amplitude (arrows in panel C) represent the elastic and viscous moduli. As frequency of the length stimulus changes the viscoelastic muscle response changes, characterizing enzymatic behavior of actin-myosin cross-bridges in activated muscle fibers.

**Figure 2:** (A) System analysis similar to the description in Fig. 1 can be applied to muscle fibers using a white-noise stimulus. (B) The strain signal represents band-limited Gaussian white noise, constructed by summing a series of sine waves of the same amplitude but of random phase, and covering a frequency range up to a prescribed cutoff frequency ($f_c$). Theoretically this creates a strain signal with a Gaussian amplitude distribution and a flat power spectrum. See Methods for further detail. (C) Representative Fourier transforms of the measured strain stimulus and stress response illustrate the measured behavior from a demembranated fruit fly dorsolongitudinal muscle fiber.
Figure 3: Direct comparisons of sinusoidal and white-noise analyses illustrate consistent shifts among measured system behaviors as [MgATP] varied for demembranated myocardial strips from mice. Elastic and viscous moduli are plotted against frequency for measurements at 27°C (panels A and B). Lines represent the average curve-fits to Eq. 1 for the white-noise analysis data at each condition. Just as each sinusoidal data point is an average among strips (with error bars representing SEM), each white-noise point represents the average among strips with error bars omitted (where SEM at any frequency is comparable to that depicted by the sinusoidal analysis data).

Figure 4: The effect of [MgATP] on estimates of model parameters (panels A-F) from fits of Eq. 1 to the elastic and viscous moduli from mouse cardiac muscle (example data shown in Fig. 3). Data are plotted as mean±SEM, with error bars extending downward for sinusoidal analysis and upward for white-noise analysis.

Figure 5: (A) Estimates of actin-myosin attachment time ($t_{on}$) from sinusoidal and white-noise analysis are shown (mean±SEM) as a function of [MgATP] for the mouse myocardial measurements from Fig. 3, representing the expected increases in $t_{on}$ with decreasing [MgATP]. (B) Schematics of cross-bridge attachment events illustrating a longer rigor state as [MgATP] decreases from 5 to 0.25 mM (labeled High MgATP and Low MgATP, respectively), showing unitary force production ($F_u$) versus time throughout a single cross-bridge cycle.

Figure 6: Condensed temporal requirements of white-noise analysis facilitated combining normal stimuli (B) with linear shortening (A) and lengthening (C) transients to probe the effect of...
load on cross-bridge behavior using fruit fly dorsolongitudinal muscle fibers. Dashed lines show the mean length stimulus during the 10 s duration used to calculate the elastic ($D$) and viscous ($E$) moduli, plotted against frequency. Each point represents the average among fibers at a single frequency. Solid lines inset in panel E show average curve-fits to Eq. 1, depicting Nyquist behavior for each condition. Temperature, 15°C.

**Figure 7:** The effect of shortening (Short.), no-slope (No) and lengthening (Len.) on estimates of model parameters (panels A-D) from fits of Eq. 1 to the elastic and viscous moduli from insect flight muscle. The effect of strain on $c$ for the entire population of fibers (E) indicates a significant trend, better illustrated by the change in $c$ from the no-slope value (F). Except for panel E, data are plotted as mean±SEM.
Table 1: Coefficients of variation (%) among curve fits to Eq. 1. from repeated measurements (n=5) within a single fiber.

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</tr>
<tr>
<td>k</td>
<td>2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>1.3</td>
<td>4.9</td>
</tr>
<tr>
<td>b</td>
<td>3.8</td>
<td>2.4</td>
</tr>
<tr>
<td>C</td>
<td>5.2</td>
<td>15.5</td>
</tr>
<tr>
<td>c</td>
<td>3.5</td>
<td>5.6</td>
</tr>
<tr>
<td>$t_{on}$</td>
<td>3.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>
Table 2: Myosin cross-bridge attachment time ($t_{on}$) varies with strain in flight muscle fibers from fruit flies.

<table>
<thead>
<tr>
<th></th>
<th>Shortening</th>
<th>No Slope</th>
<th>Lengthening</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{on}$ (ms) †</td>
<td>0.62± 0.01</td>
<td>0.61± 0.01</td>
<td>0.60± 0.01</td>
</tr>
<tr>
<td>Change in $t_{on}$ (%) †</td>
<td>1.1± 0.8‡</td>
<td>0</td>
<td>-0.9± 0.4‡</td>
</tr>
</tbody>
</table>

† Repeated measures ANOVA indicate significant strain dependency represented by the three conditions (p<0.05).
‡ Normalized measurements within each fiber to the ‘No Slope’ value indicate significant differences for the relative change in $t_{on}$ due to lengthening or shortening (p<0.05).
Table 3: Minimum viscous modulus varied with strain in flight muscle fibers from fruit flies.

<table>
<thead>
<tr>
<th></th>
<th>Shortening</th>
<th>No Slope</th>
<th>Lengthening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnitude (kPa)</td>
<td>94.0±8.6†</td>
<td>119.6±11.3</td>
<td>133.5±13.0‡</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>138±3</td>
<td>134±3</td>
<td>133±3</td>
</tr>
</tbody>
</table>

† Repeated measures ANOVA indicate significant strain dependency represented by the three conditions (p<0.05).

‡ Different from the No Slope value (p<0.05).
Viscoelastic system

A System input

Total response

Elastic component

Viscous component

Time

Stress

Strain

(viscous stimulus)

(viscous response)

Ee = $S_e E_e$

Ev = $S_v E_v$

Elastic and viscous moduli:

Strain (length stimulus)

Stress (force response)

~200 μm

System input System output

Figure 1:
White-noise length perturbation analysis in muscle fibers

Figure 2:

\[
\text{Strain}(t) = \sum_{n=1}^{N} A \sin(n \omega_0 t + \phi_n)
\]
Figure 3:
Figure 4
Figure 5:

A

Mouse

- Sinusoid
- Noise

$\text{t}_{\text{on}}$ (ms)

ATP (mM)

B

High ATP

\[ \text{F}_u \]  

Time

Cross-bridge state

- ADP-P$_i$
- ADP
- Rigor

Low ATP

\[ \text{F}_u \]  

Time

Figure 5:
Figure 6:
Figure 7