

Fluoromicrometry: A Method for Measuring Muscle Length Dynamics with Biplanar Videofluoroscopy



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ABSTRACT

Accurate measurements of muscle length changes are essential for understanding the biomechanics of musculoskeletal systems, and can provide insights into muscular work, force, and power. Muscle length has typically been measured *in vivo* using sonomicrometry, a method that measures distances by sending and receiving sound pulses between piezoelectric crystals. Here, we evaluate an alternative method, fluoromicrometry, which measures muscle length changes over time by tracking the three-dimensional positions of implanted, radio-opaque markers via biplanar videofluoroscopy. To determine the accuracy and precision of fluoromicrometry, we simultaneously measured length changes of an isolated muscle, the frog sartorius, in an *in vitro* setup using both fluoromicrometry and a servomotor. For fluoromicrometry to perfectly match the results of the servomotor, the relationship between the two measurements should be linear, with a slope of 1. Measurements of muscle shortening from fluoromicrometry and the motor were compared across 11 isotonic contractions. The precision of fluoromicrometry was ± 0.09 mm, measured as the root mean square error of the regression of fluoromicrometry versus servomotor muscle lengths. Fluoromicrometry was also accurate: the mean slope of the fluoromicrometry–servomotor regressions did not differ significantly from the ideal line once off-axis motion was removed. Thus, fluoromicrometry provides a useful alternative for measuring muscle length, especially in studies of live animals, as it permits long-term marker implantation, wireless data collection, and increased spatial sampling. Fluoromicrometry can also be used with X-Ray Reconstruction of Moving Morphology to simultaneously measure muscle shortening and skeletal kinematics, providing a potent new tool for biomechanics research. *J. Exp. Zool.* 00:1–10, 2016. © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

Muscles are the motors of most animal movements, and many of their functions are directly linked to changes in length. In addition to shortening to produce skeletal motion, the magnitude and rate of length changes influence the force, work, and power produced by the muscle (Hill, '38; Rome et al., '93; Lutz and Rome, '94). Therefore, knowledge of muscle length changes is necessary for understanding the biomechanics of animal movements. However, muscle length change often cannot be directly inferred from external motions of the organism. Series elastic structures such as tendons can decouple muscle length from joint movement (Roberts and Azizi, 2011), and complex motions within the muscle such as dynamic gearing or length change heterogeneity can cause further departure (Ahn et al., 2003; Azizi et al., 2008).

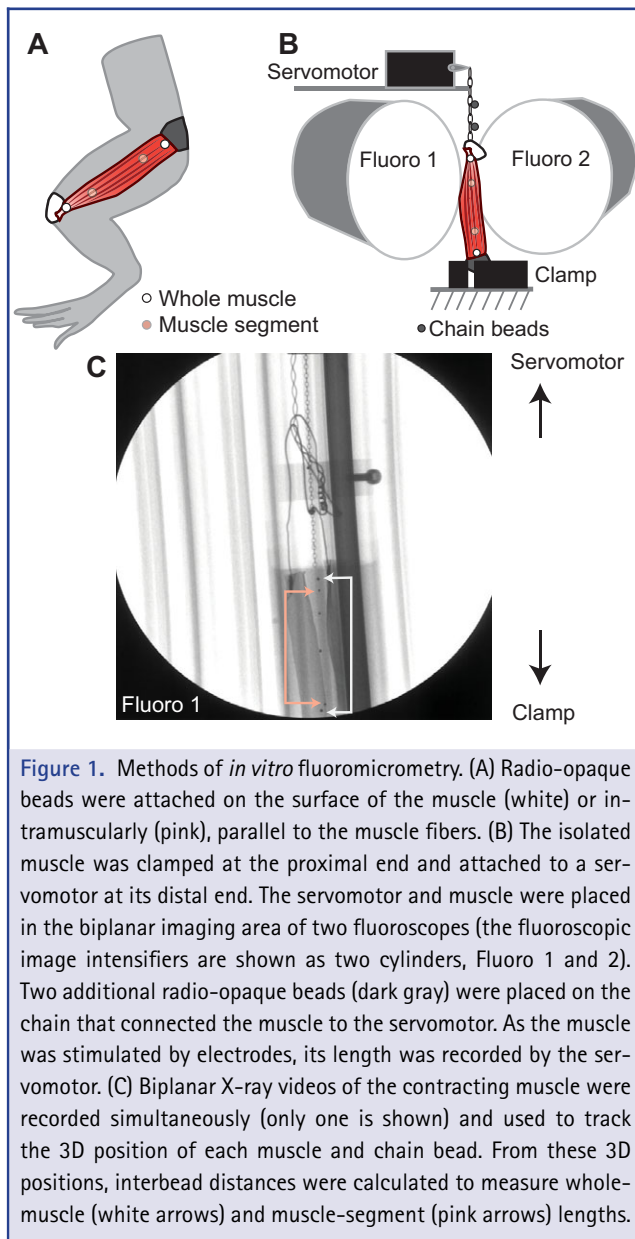
Consequently, direct measurement of muscle length dynamics *in vivo* is essential for understanding muscle function. The current standard method is sonomicrometry, in which two or more piezoelectric crystals are implanted into a muscle along the line of action of the fascicles (Rushmer et al., '56). An electrical pulse sent to one crystal causes it to vibrate, and this vibration is converted back into an electrical signal when it reaches another crystal. The time between the pulse being sent and received is measured, allowing distance to be calculated based on the speed of sound in an aqueous medium. The precision of this distance measurement is ultimately limited by the wavelength of the sound pulse (Newman et al., '84) and the frequency at which the time of the pulse's transit is measured (Goodman and Castellana, '82), both of which can vary between sonomicrometry setups to allow measurements as precise as ± 0.03 – 0.012 mm under optimal conditions. However, the precision and accuracy in experimental conditions is likely lower, as additional error can be introduced by the arrangement of the sonomicrometry crystals and ultrasound echoes and obstructions (Horiuchi et al., 2012).

Sonomicrometry has been applied to a wide range of muscles and movements and significantly advanced our understanding of the length changes muscles undergo *in vivo*. However, sonomicrometry presents some technical challenges. Animals must be tethered to equipment via wires to the sonomicrometer crystals, and the presence of wires also sets a practical limitation to the duration of implants. Signal strength and quality can vary depending on crystal orientation and the presence of external sources of electromagnetic interference. The number of crystals from which signals can be recorded is also limited. Additionally, sonomicrometry only records the distances between pairs of crystals; it does not provide the absolute three-dimensional (3D) position of each crystal through time. This limitation makes

it challenging to confirm the position and orientation of crystals throughout a muscle contraction, or to measure changes in muscle fiber length or orientation relative to the muscle's line of action or skeletal kinematics.

In this paper, we evaluate an alternative method for measuring muscle length changes, which we term "fluoromicrometry." In fluoromicrometry, bioinert, spherical metal beads (0.5–1 mm diameter) are surgically implanted into muscles, and the 3D position of each bead is recorded using biplanar X-ray videography as the animal moves. Techniques for the implantation, recording, and tracking of radio-opaque bone markers have already been developed and validated (Brainerd et al., 2010) for measuring skeletal motion using X-ray Reconstruction of Moving Morphology (XROMM). In fluoromicrometry, beads are implanted into muscles, rather than bones, and thus the change in distance between beads provides a measure of the change in muscle length. Uniplanar and biplanar X-ray video has been used previously to measure 2D muscle length and soft tissue movement (Leshin et al., '72; Shoukas et al., '81; Knight et al., '90; Westbury, '91; Monti, 2003), generally in isolated muscles or anesthetized animals that are not freely moving. However, the increasing availability of biplanar fluoroscopy systems and the analysis tools developed for XROMM now allows 3D tracking of the position and length of muscles in a range of animals and natural behaviors (Astley and Roberts, 2012; Camp and Brainerd, 2014; Camp et al., 2015) and soft tissue (Azizi and Roberts, 2009) through fluoromicrometry. In contrast to sonomicrometry, this technique allows the use of dozens of beads simultaneously, wireless data collection, simple surgical techniques, and long-term persistence of implanted markers. Because fluoromicrometry data provide both the interbead distances and the 3D positions of each implanted bead, the position and relative motion of the beads can be measured throughout a behavior. Additionally, fluoromicrometry can be combined with XROMM to provide simultaneous recording of muscle length changes and skeletal kinematics (Astley and Roberts, 2012; Camp and Brainerd, 2014; Konow et al., 2015).

Here, we quantify the accuracy and precision of fluoromicrometry as a method for measuring muscle length and discuss the advantages and limitations of this technique. While fluoromicrometry is well suited for measuring *in vivo* muscle fiber lengths in live animals, the use of an *in vitro* setup was necessary for this validation study to provide an independent measure of muscle length. We compared fluoromicrometry measurements in an isolated muscle to the whole muscle lengths measured directly by a servomotor during a series of isotonic muscle contractions to evaluate the precision and accuracy of the approach.



METHODS

We measured the length change of an isolated frog sartorius muscle with fluoromicrometry and compared it to simultaneously recorded length measurements from an analog servomotor. A live muscle was implanted with radio-opaque beads, isolated, and then stimulated to contract while biplanar X-ray videos were recorded (Fig. 1). From these videos, we used fluoromicrometry to measure length change of the muscle by tracking the change in distance between the beads. The precision of fluoromicrometry depends on the precision of this tracking (which we quantified and has been established previously for the XROMM

technique (Brainerd et al., 2010)) and the 3D reconstruction of the beads' movement. The accuracy of fluoromicrometry depends on how well the motions of the intramuscular beads track the motions of the muscle, so that all changes in muscle length are reflected by equal changes in interbead distance. Under ideal conditions of perfect accuracy and precision, the length changes recorded with fluoromicrometry (L_{fluoro}) and those recorded by the servomotor (L_{motor}) should have a linear relationship with a slope of 1 and intercept of 0, with all points falling perfectly on this line.

Animals

We used muscles from two adult bullfrogs (*Rana catesbaenia*) obtained from a licensed commercial supplier. Frogs were housed communally in tubs with access to both water and land areas, and maintained at a temperature of $18 \pm 2^\circ\text{C}$ and a standard (12 L: 12 D) light cycle. Vitamin-enriched crickets were provided bi-weekly. All housing and procedures were approved by the Brown University IACUC.

Bead Implantation

We obtained muscle length data from the sartorius muscle, chosen for its nearly parallel-fibered architecture and surgical accessibility. Prior to bead implantation, frogs were euthanized via double pithing, after which the frog was placed in a bath of oxygenated amphibian Ringer's solution maintained at 22°C . The sartorius muscle was exposed in the thigh, and 0.8 mm spherical tantalum beads implanted proximally and distally to span as much of the muscle length as possible and allow a direct comparison to the whole-muscle length measured by the servomotor. However, because of the extreme thinness of the muscle at its origin and insertion, beads at the most distal and proximal ends of the muscle had to be affixed to the external surface of the muscle with cyanoacrylate glue. These "whole muscle" beads spanned at least 90% of the measured resting length of the muscle (Fig. 1A). Additional "muscle segment" beads, spanning only a segment (~30–70%) of the muscle length, were also implanted in the muscle belly using an 18-gauge hypodermic needle and a 0.25 mm diameter steel rod plunger. Following implantation, the muscle was removed and mounted in a muscle chamber (Aurora Scientific Inc., Ontario, Canada). A custom-made clamp was used to fix a small piece of bone at the proximal muscle attachment, and the distal bony attachment was secured with silk suture to a metal chain attached to the servomotor (Fig. 1B). An additional two beads were rigidly affixed to the chain using cyanoacrylate glue in order to track the position of the chain.

In Vitro Experimental Data Collection

During measurements, the muscle was submerged in a chamber containing an oxygenated Ringer's bath maintained at 22°C . The proximal muscle clamp was rigidly affixed to the bottom of the

chamber, and the chain was attached to a servomotor muscle lever system (Model 310 B-LR; Aurora Scientific, London, Ontario, Canada) to record the force and length changes of the muscle. Analog force and length data were recorded at 10 kHz from the servomotor using a 16-bit A/D converter (PCI-MIO-16; National Instruments) controlled by Igor Pro software (Wavemetrics Inc., Lake Oswego, OR, USA). The muscle was stimulated to contract via two bilateral platinum plate electrodes connected to a Grass S48 stimulator (Grass Instruments, Warwick, RI, USA). Before initializing the experimental contractions, the optimal stimulus voltage was determined. The muscle was stimulated at increasing single-pulse (twitch) voltages until no increase in force was detected; the resultant voltage was increased by 1 V and used for all subsequent contractions as the supramaximal stimulus voltage. All subsequent tetanic stimuli had 0.2 msec pulse lengths and were delivered at a rate of at least 100 pulses per second for a duration of at least 200 msec. To characterize the relationship between fluoromicrometry beads and muscle shortening, we performed a series of at least five isotonic contractions at decreasing force thresholds to allow fascicles to actively shorten.

Video Fluoroscopy

High-speed, biplanar X-ray videos were collected synchronously with muscle force and length measurements to record the motion of the muscle and chain beads during all contractions. Two C-arm fluoroscopes (OEC Model 9400) operating at 90 kVp and 20 mA, with $2\times$ magnification, generated X-ray images. These fluoroscopes were equipped with high-speed cameras (Photron Fastcam 1024; Photron Inc., San Diego, CA, USA) that recorded the X-ray images at $250 \text{ frames}\cdot\text{sec}^{-1}$, as described in Brainerd et al. (2010). To keep the muscle in view and limit electromagnetic interference from the motor, the servomotor and chamber containing the muscle were attached to a vertical aluminum support wall about 0.5 m in height. The setup was positioned in the field of view of the C-arm fluoroscopes, oriented approximately 90° to each other (Fig. 1B), and the aluminum support wall was placed as close as possible to the two perpendicular image intensifiers. Prior to the placement of the muscle testing equipment, standard undistortion grid images were taken. After grid images were captured, the muscle testing apparatus was placed in position. In order to account for any distortion of the X-ray images introduced by interference from the servomotor, a standard calibration object was placed into the muscle testing setup such that the muscle would be within the calibration cube's volume. X-ray images of the calibration object were then recorded while the servomotor was on.

Fluoromicrometry

Using the X-ray videos, we tracked the 3D position and motion of all beads, and then calculated muscle length change with fluoromicrometry. The videos were undistorted, calibrated,

and the beads tracked using the XrayProject program (www.xromm.org) running in MATLAB (R2009b; MathWorks, Natick, MA, USA), as described in Brainerd et al. (2010). For each contraction, all beads were tracked for the entire duration of the contraction and subsequent relaxation, as well as at least 30 frames precontraction and postrelaxation. For each bead, XYZ coordinates were generated, and muscle length was calculated from the unfiltered coordinates as the vector distance between the proximal-most and distal-most beads (Fig. 1B).

Analysis of Precision and Accuracy

The precision of fluoromicrometry depends on the precision with which beads are tracked. In XROMM studies, this tracking precision is calculated as the standard deviation of the distance between two beads placed in a single bone, and is typically 0.1 mm or less (Brainerd et al., 2010). Because each bone is assumed to be a rigid body, this interbead distance should be constant and any deviation reflects errors in the correction of distortion introduced by the fluoroscopes, calibration, and/or bead tracking. We calculated tracking precision in a similar way for fluoromicrometry, using the mean standard deviation of the distance between two beads attached to the chain connecting the muscle to the servomotor. Because this chain is effectively rigid when under tension from the muscle and motor, the distance between these chain beads should be constant and any deviation reflects errors in the bead tracking.

To determine how precisely and accurately fluoromicrometry measured muscle length, we compared the length changes recorded by fluoromicrometry to those recorded by the servomotor (L_{motor}). All analyses were performed in Igor (WaveMetrics Inc.), using a custom procedure and raw (unfiltered) data from both the motor and fluoromicrometry. Length data from the motor were resampled to 250 Hz to match the frequency of the fluoromicrometry data, and in both sets data length was measured relative to its initial value prior to contraction. Because the beads did not span the whole length of the muscle, we performed a segment-length correction on muscle length measured with fluoromicrometry. Length changes from the two data sets were made directly comparable by dividing the length from fluoromicrometry by the ratio of initial interbead distance to the resting length of the muscle, as measured with digital calipers. These length-corrected fluoromicrometry measurements (L_{fluoro}) were compared to the length measurements of the motor by plotting L_{fluoro} against L_{motor} for the entire isotonic portion of each contraction (0.11–0.30 sec, depending on contraction speed; Fig. 2A).

For each contraction, a linear least squares regression was performed to determine the slope of L_{fluoro} versus L_{motor} and to measure the root mean square error (RMSE). The RMSE was the metric for the precision of fluoromicrometry, reflecting the raw data scatter around the regression line fit to the $L_{\text{fluoro}}-L_{\text{motor}}$ data. The accuracy of fluoromicrometry is represented by the

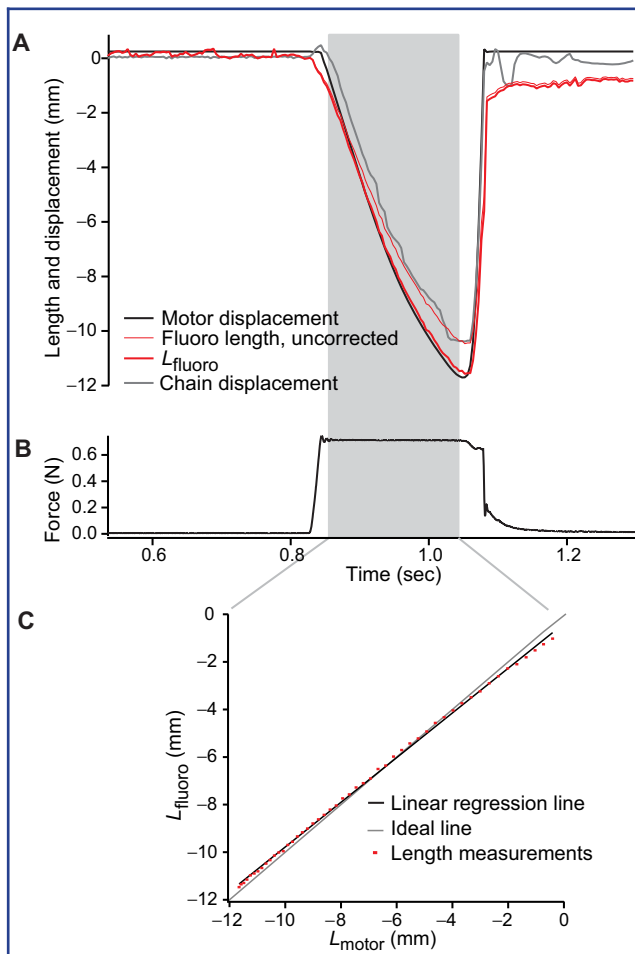


Figure 2. Muscle length change and force from a sample isotonic contraction. (A) Muscle length change was measured both by the servomotor (L_{motor} , black line) and by fluoromicrometry (red lines). The raw fluoromicrometry length (thin red line) was corrected (L_{fluoro} , thick red line) to account for the beads not spanning the entire length of the muscle. The displacement of a bead on the motor chain (gray line) was also measured. (B) The servomotor recorded muscle force during each contraction, and only the isotonic portion (gray-shaded region) was analyzed. (C) Muscle length change measured by L_{fluoro} was plotted as a function of that measured L_{motor} (red dots), and fit with a linear regression (black line). The RMSE of the points relative to the regression line was calculated to measure precision, and the slope of the regression line to measure accuracy. An ideal line (gray) represents perfect precision (RMSE = 0) and accuracy (slope = 1).

difference in slope between the $L_{\text{fluoro}}-L_{\text{motor}}$ line and the ideal line with a slope of 1. Two-tailed t -tests were used to determine if the slope of $L_{\text{fluoro}}-L_{\text{motor}}$ differed significantly from 1, which would indicate bias in the fluoromicrometry measurements.

Analysis of Single-Axis Muscle Length

Bead XYZ coordinates were also used to calculate muscle shortening exclusively along the line of action of the muscle. Only length changes along this single, proximodistal axis were recorded by the servomotor, and our *a priori* expectation is that bead motion also occurred solely along this axis. However, if the pairs of beads were not implanted in perfect alignment with the muscle's line of action—or if the muscle moves at all laterally during the contraction—this off-axis motion will then be included in the 3D vector distance between beads and result in a mismatch between fluoromicrometry and the servomotor. Because fluoromicrometry data include not only inter-bead distances, but also the 3D position of each intramuscular bead at every frame, such off-axis motion can be quantified and excluded when there is a known axis of muscle shortening.

Therefore, we recalculated the XYZ coordinates such that x -axis translations represented motion solely along the muscle's line of action. This recalculation was performed in the animation program Autodesk Maya, using custom scripts from the XROMM Maya Tools package (available at www.xromm.org). An anatomical coordinate system (ACS) was created and placed with its origin at the most bead, its x -axis pointing to a bead attached to the chain, and the y - and z -axes perpendicular to the x -axis and to each other. This resulted in the x -axis of the ACS aligned proximodistally, such that x -axis translations represent motion solely along the muscle's line of action. Any translations along the y - or z -axes represented lateral, off-axis motion relative to this line of action. The ACS was aligned at a frame prior to the onset of shortening for each contraction, and the XYZ coordinates of each intramuscular bead were then recalculated relative to the ACS at every frame.

We calculated muscle length change along this single axis as the change in x -axis distance between the whole-muscle beads. This single-axis muscle length was then length corrected using the ratio of initial interbead distance to the resting length of the muscle to make it directly comparable to L_{motor} . The accuracy and precision of these length-corrected, single-axis fluoromicrometry measurements were then measured using a linear least squares regression to determine the slope of these data versus L_{motor} and to measure the RMSE, as was done for L_{fluoro} .

Chain Bead Accuracy and Precision

Similar to the fluoromicrometry measurements of muscle length, the XYZ coordinates of a chain bead were used to measure the displacement of the chain during each contraction. This displacement could then be directly compared to the length changes measured by the servomotor (L_{motor}) to determine if there was any systematic bias (inaccuracy) in our X-ray-based measurements. The chain connecting the muscle to the motor did not change length, but rather we measured the displacement of the chain bead at each frame as the vector distance between its current position and its initial position. This L_{chain} was compared to

L_{motor} using a linear regression, as described previously for fluorimetry, although for L_{chain} no segment length correction was needed. The chain bead analysis was also repeated using single-axis translations detailed above to determine if off-axis motions of the chain affected the accuracy measurements.

Analysis of Muscle Segment Lengths

We also calculated length from the muscle segment beads (Fig. 1) and compared the resulting length changes to those of the servomotor. These beads spanned a shorter percentage (72% or 53%) of the resting muscle length, and in one muscle an intramuscular bead fell out of the muscle during the first contraction, reducing the span to 35% for the remaining contractions. Therefore, the segment-length correction ratio was based on the initial distance between these muscle segment beads for each contraction and used to calculate the corrected muscle length (L_{segment}). The accuracy of these muscle segment beads was measured by calculating the slope of the $L_{\text{segment}}-L_{\text{motor}}$ line, as described previously for L_{fluoro} .

RESULTS

We compared muscle length changes measured by fluorimetry to those measured by a servomotor in a total of 11 muscle contractions from two muscles. These contractions ranged in maximum excursion from 2.5 to 14.8 mm of muscle shortening (corresponding to about 5–30% of resting muscle length). During each isotonic contraction, muscle force rapidly increased as a result of stimulation and then plateaued, during which time the muscle shortened (Fig. 2). We did not observe obvious shifts in the position of any beads during the experiments, although one implanted bead fell out of the muscle during the initial contraction in one muscle. We calculated the accuracy and precision of fluorimetry individually for each contraction, and report the mean results (from $N = 11$ contractions), along with the standard error (SE).

Bead Tracking Precision

The tracking precision of fluorimetry, measured as the mean standard deviation of the distance between two beads attached to the chain, was 0.09 mm. As expected, this is similar to the precision reported previously for marker-based XROMM studies (Brainerd et al., 2010; Dawson et al., 2011; Miranda et al., 2011; Gidmark et al., 2012).

Precision of Fluorimetry

Fluorimetry had submillimeter precision, calculated as the RMSE of the $L_{\text{fluoro}}-L_{\text{motor}}$ data relative to its regression line. The average precision across all contractions was 0.09 mm (± 0.003 SE), with precision for individual contractions ranging from 0.03 to 0.16 mm. Measured precision when using single-axis measurements of muscle length change was also within this range (mean of 0.11 ± 0.005 mm).

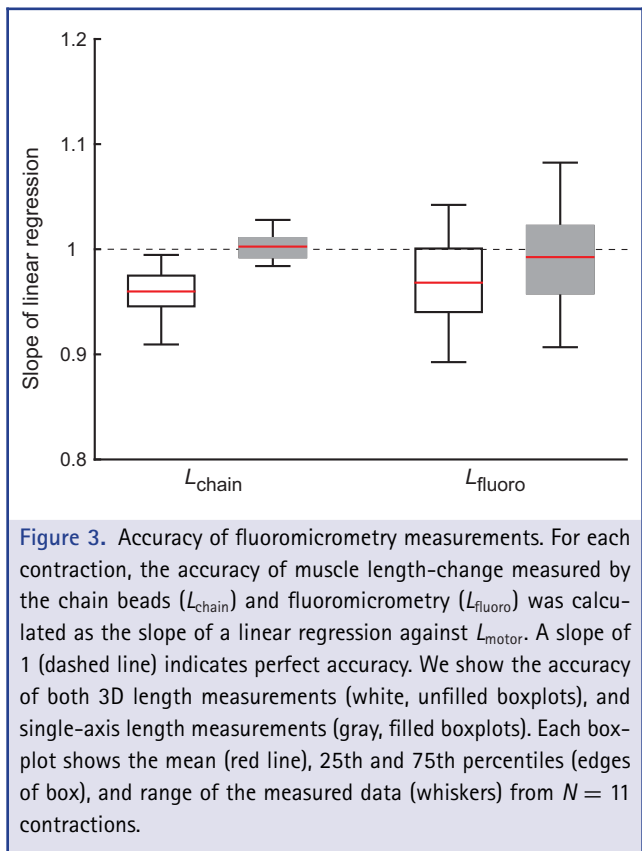


Figure 3. Accuracy of fluorimetry measurements. For each contraction, the accuracy of muscle length-change measured by the chain beads (L_{chain}) and fluorimetry (L_{fluoro}) was calculated as the slope of a linear regression against L_{motor} . A slope of 1 (dashed line) indicates perfect accuracy. We show the accuracy of both 3D length measurements (white, unfilled boxplots), and single-axis length measurements (gray, filled boxplots). Each boxplot shows the mean (red line), 25th and 75th percentiles (edges of box), and range of the measured data (whiskers) from $N = 11$ contractions.

Accuracy of Fluorimetry

Fluorimetry provided an accurate measure of muscle length (Fig. 3), with the slope of the $L_{\text{fluoro}}-L_{\text{motor}}$ line averaging $0.97 (\pm 0.036 \text{ SE})$ and the slopes of individual contractions ranging from 0.89 to 1.04. However, the slope was significantly different from the ideal value of 1 (two-tailed t -test, $t = -2.28$, $P = 0.046$), indicating a small but significant bias toward overestimating muscle length change from the raw 3D bead positions. There was no significant bias in the corrected single-axis muscle lengths, which had a mean slope of $1.02 \pm 0.02 \text{ SE}$ and did not differ significantly from 1 (two-tailed t -test, $t = -0.5779$, $P = 0.5761$).

The accuracy of the chain bead length change (i.e., displacement from its initial position) relative to the length measurements of the motor was similar to fluorimetry and also showed a bias unless off-axis motion was removed (Fig. 3). The $L_{\text{chain}}-L_{\text{motor}}$ regression line had an average slope of $0.96 (\pm 0.002 \text{ SE})$, which differed significantly from 1 (two-tailed t -test, $t = -5.616$, $P = 0.0002$). However, when only the single-axis motion of the chain bead was analyzed, the average slope was $1.0 (\pm 0.0013 \text{ SE})$ mm, and did not differ significantly from 1 (two-tailed t -test, $t = -0.1639$, $P = 0.8731$). Because the edge—rather than the center—of each bead was affixed to the taut chain,

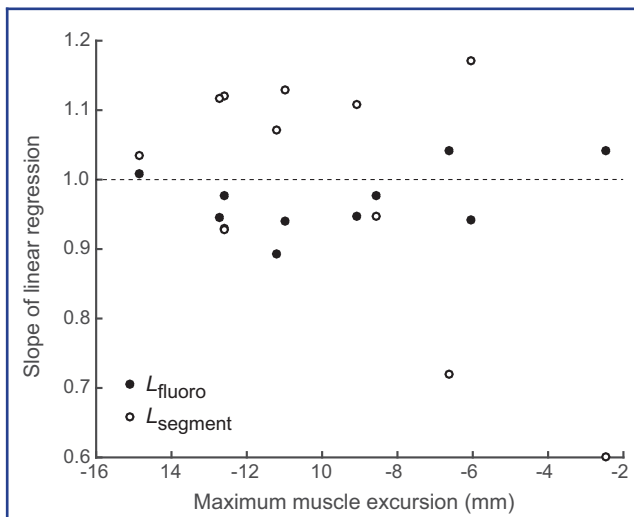


Figure 4. Comparison of whole-muscle (L_{fluoro}) and muscle-segment (L_{segment}) fluoromicrometry. The accuracy of each method of fluoromicrometry was calculated as the slope of a linear regression against L_{motor} , with a slope of 1 (dashed line) indicating perfect accuracy. For each muscle contraction, the accuracy of L_{fluoro} (black circles) and L_{segment} (white circles) is plotted as a function of the maximum muscle excursion recorded by the motor during that contraction. All of the plotted slopes are from three-dimensional length data and have not been corrected to single-axis motion.

rotational movements of the chain beads occurred around the central axis. In this way, it was possible for the chain beads to have off-axis motion, even though the motion of the chain itself was, by definition, in line with the muscle's shortening.

Fluoromicrometry using the muscle segment length beads, which spanned only ~30-70% of the muscle's resting length, generally produced similar mean accuracy as using the whole-muscle beads (mean slope of 1.0 ± 0.02). The segment beads both overestimated and underestimated whole-muscle length changes (Fig. 4), and at the shortest muscle excursions (<8 mm), muscle segment beads had considerably lower accuracy with slopes as low as 0.6. To determine if these muscle segment beads drifted over time, we compared the position of each bead and the length of the muscle (L_{motor}) before and after each contraction. Across all beads and all contractions ($N = 35$), the average bead drift was 0.45 ± 0.069 mm (about half of a bead's diameter), accompanied by minimal changes in the whole muscle's resting length measured by L_{motor} (mean of 0.0072 ± 0.0062 mm) and the passive tension (0.011 ± 0.0026 N) measured by the servomotor.

DISCUSSION

We show that fluoromicrometry provides accurate and precise measurements of muscle length changes when compared to

servomotor recordings during *in vitro* muscle contractions. Measured precision for fluoromicrometry in this study was 0.09 mm. These results confirm previous studies that have used fluoromicrometry to measure muscle shortening in live animals (Astley and Roberts, 2012; Camp and Brainerd, 2014; Camp et al., 2015). Indeed, it is in measuring such *in vivo* muscle length dynamics that fluoromicrometry is likely to prove most useful.

Precision of Fluoromicrometry

Fluoromicrometry measured muscle length with a precision of 0.09 mm, as measured by the RMSE of the $L_{\text{fluoro}} - L_{\text{motor}}$. This matches the precision of bead tracking alone, which is similar to published precision values ± 0.10 mm for tracking markers in X-ray videos using the XROMM program. The tracking precision includes errors due to the undistortion, 3D calibration and tracking of the X-ray videos, and therefore sets the limit for the most precise measurements possible.

Accuracy of Fluoromicrometry

Fluoromicrometry provided an accurate measure of whole-muscle length changes, as shown by the good agreement with motor-based length measurements, that is, slopes of $L_{\text{fluoro}} - L_{\text{motor}}$ near 1 (Fig. 3). Across all contractions, these slopes were slightly but significantly <1, suggesting a bias in the fluoromicrometry measurements; however, this likely reflects the difference between the 3D distances measured by fluoromicrometry and the linear distances measured by the motor. When fluoromicrometry muscle lengths were measured along the same single axis used by the motor, the resulting slopes did not differ from 1. Similarly, when the displacement of the chain bead was measured along a single axis, the slopes of $L_{\text{chain}} - L_{\text{motor}}$ were not significantly different from 1 (Fig. 3). This suggests that the bias measured in L_{fluoro} reflects inclusion of off-axis motion, rather than an inherent inaccuracy in fluoromicrometry.

For fluoromicrometry or sonomicrometry to measure muscle length changes accurately, the beads or crystals must not slide relative to the muscle fascicles during contraction. For fluoromicrometry, the implanted muscle-segment beads seem most vulnerable to shifting within the muscle, especially as they were implanted in an isolated muscle with no "healing-in" (i.e., without time to be surrounded by a fibrous capsule) that may occur when beads are implanted in the muscles of live animals (Leshin et al., '72). However, we did not find any evidence of such shifts over the course of a contraction. When we compared the positions of these beads before and after a contraction, the beads generally moved less than a bead diameter (mean of 0.45 ± 0.069 mm). The implanted segment beads did show more variability in accuracy than the glued-on whole muscle beads (Fig. 4), but still had a mean slope was 1.0 when regressed against L_{motor} . While this cannot rule out dynamic motions of the beads relative to the surrounding muscle, there were no obvious shifts in bead positions as a result of activation and relaxation.

This analysis of $L_{\text{fluoro}}-L_{\text{motor}}$ slopes does not exclude the possibility that beads were sliding over short timescales, such as the initial rise or final decline of muscle force. If a bead was loose in a “pocket” within the muscle, one could imagine that the rise in force as the muscle is activated could push the bead to one end of the pocket. The bead would maintain this position while the muscle is fully active, resulting in the accurate length measurements we recorded over the isometric portion of the contraction. However, this sliding behavior would result in rapid shifts in the XYZ coordinates of the beads during force rise and force decay. We did not observe any such shifts in our raw data (Fig. 2), suggesting that the beads did not slide, or did so only over very small distances.

To minimize the possibility of beads sliding within the muscle, we recommend implanting beads *in vivo* and allowing them time to potentially heal into position. Previous studies have shown that such implanted intramuscular beads maintain their positions across behaviors (Astley and Roberts, 2012) and over days (Camp and Brainerd, 2014) or even weeks (ALC, unpublished data). We also recommend using the smallest beads possible, as these seem least likely to slide within the muscle. The inertia of large, dense beads could cause them to be left behind as the muscle begins to contract, thereby underestimating the actual length change. Small beads are also likely to cause minimal disruption of natural muscle contraction behavior and shorter healing times. Beads with a 0.8 mm diameter have been successfully used with muscles ~50 mm long (this study) or larger (Camp and Brainerd, 2014), while 0.5 mm diameter beads are likely more appropriate for smaller muscles (Konow et al., 2015). Regardless of bead size and implantation technique, it is important for fluoromicrometry studies to test for evidence of bead shifting by measuring bead positions when the animal is in a standardized position (e.g., rest) and comparing these positions before and after each contraction or behavior.

Muscle-Segment versus Whole-Muscle Fluoromicrometry

Fluoromicrometry using implanted muscle-segment beads had similar mean accuracy but more variability, relative to the whole-muscle beads that were glued to the surface of the muscle (Fig. 4). This variability likely reflects strain heterogeneity in the isolated muscles tested *in vitro*. The muscle-segment beads spanned only 35, 53, or 72% of the muscle's resting length (Fig. 1), while the servomotor measured the length of the entire muscle. If the distal and/or proximal muscle segments beyond the muscle-segment beads shortened more or less than the rest of the sartorius (i.e., strain was heterogeneous across the muscle), muscle-segment beads would over- or underestimate the total change in muscle length. Previous experiments with sonomicrometry have showed substantial strain heterogeneity across leg muscles in toads (Ahn et al., 2003) and guinea fowl (Carr et al., 2011). Additionally, the conditions of the *in vitro* setup used here are likely to produce strain heterogeneity that may

not be representative of *in vivo* patterns. In particular, the field stimulation used here may result in uneven or unsynchronized stimulation of the entire muscle in a way that results in strain heterogeneity. While not the focus of this study, these results highlight the potential of fluoromicrometry for exploring if such strain heterogeneity also occurs in *in vivo* muscle contractions.

Given the possibility of strain heterogeneity, we believe that the particularly low slopes recorded at low excursions for the muscle segment beads in Figure 4 result from differences in the percent of the muscle spanned by each set of beads, rather than in implantation techniques: intramuscular (muscle-segment) and glued to the external surface of the muscle (whole-muscle beads). In other words, both sets of beads would have returned the same results had they been placed at the same position along the muscle. As stated above, this and previous studies have found no evidence that intramuscular beads are less accurate or prone to movement within the muscle. In the *in vivo* studies, where fluoromicrometry is anticipated to be most useful, we in fact recommend implanting beads intramuscularly, as was done for the muscle-segment beads. Beads attached to the external surface of the muscle were only used in this study because of constraints of the *in vitro* setup: the isolated muscle was extremely thin at its origin and insertion, and direct comparison to the servomotor required beads as close to the origin and insertion as possible to avoid any confounding strain heterogeneity.

These issues demonstrate the difficulties of using an *in vitro* setup and servomotor to measure the accuracy and precision of fluoromicrometry. Because any series elasticity will decouple motor length from fluoromicrometry length, we were limited to the use of isotonic contractions of a muscle with minimal series elastic components. The *in vitro* setup also imposed conditions that may have fostered strain heterogeneity across the muscle, and necessitated fluoromicrometry beads being attached externally to the extreme ends of the muscle. While this setup was necessary to determine accuracy and precision, it imposed limitations and alteration to the methods used for fluoromicrometry under *in vivo* conditions in previous studies.

Advantages of Fluoromicrometry

Fluoromicrometry provides several advantages for studying *in vivo* muscle length, compared to the current standard in the field, sonomicrometry. Sonomicrometry, which uses principles of sound conductance to measure the distance between two piezoelectric crystals, has been in use since the 1950s (Rushmer et al., '56). The precision of sonomicrometry can theoretically be as good as 0.03 mm, depending on the wavelength of sound and frequency of time measurements being used (Goodman and Castellana, '82; Newman et al., '84). However, additional error can be introduced during the experiment due to ultrasound echoes and obstructions, and the orientation of the crystals relative to each other will affect the accuracy and precision of length recordings (Horiuchi et al., 2012), thus special

care must be taken during placement of crystals in surgery. Sonomicrometry only measures the length between pairs of crystals; it does not record the absolute 3D position or motion of the crystals themselves. While the 3D positions of crystals can be calculated relative to one another (using a set of four, noncoplanar crystals), these relative positions cannot easily be related to the absolute position of the whole muscle, limb, or animal. The most obvious drawback to sonomicrometry is the necessity of the crystal's wire leads emanating from the animal, which can result in the animal altering its behavior, or damaging or removing the crystals. In-dwelling transducers connected to wire leads cannot remain in an animal for an extended period of time without careful monitoring and some limitations of natural behavior. Also, sonomicrometry is limited in the number of input channels available for recording, and typically no more than eight crystals can be used simultaneously.

Fluoromicrometry records *in vivo* length changes wirelessly, allowing for a wider range of behaviors and species to be studied. For example, fluoromicrometry has been used to record muscle length in free-flying bats (Konow et al., 2015), whose small size and unsteady flight behavior pose challenges for using wired transducers. Additionally, the biologically inert tantalum beads may remain in place indefinitely (Camp and Brainerd, 2014; Camp et al., 2015), allowing true longitudinal studies of age-associated changes in tissue mechanics, such as in heart and muscle fibers. There is even the potential for incorporating fluoromicrometry into mark-recapture field studies to assess more natural aging conditions.

Fluoromicrometry can also provide richer datasets for measuring 3D muscle length and shape changes in an anatomical context. Because the number of fluoromicrometry markers is limited only by the ability to consistently track and identify them in the field of view, it is possible to simultaneously record motion of multiple fascicles, dimensions, or segments of a muscle. Fluoromicrometry records not only the length between beads, but also the 3D position and motion of each bead. This allows length changes to be calculated along specific axes (such as the single-axis length analysis used in this study), which is not possible with sonomicrometry. This single-axis correction may be quite useful for muscles with complex architectures, where the 3D orientation and shortening of different fibers is important to the overall muscle function. Finally, because both fluoromicrometry and XROMM employ biplanar videoradiography, they can easily be performed simultaneously (Astley and Roberts, 2012; Camp and Brainerd, 2014; Camp et al., 2015), allowing synchronous high-speed tracking of muscle length changes and skeletal movement. This allows muscle shortening to be measured and visualized from an anatomically informed perspective.

Limitations of Fluoromicrometry

Fluoromicrometry will clearly not be appropriate for all studies and there are limitations to this method of measuring

muscle length. First, fluoromicrometry requires specialized equipment (biplanar X-ray video system), whose cost and space requirements may be prohibitive. The system used in this study—refurbished C-arm fluoroscopes—cost <\$140,000 (USD, not including high-speed video cameras) at the time of construction (Brainerd et al., 2010). However, similar biplanar X-ray systems are being used increasingly for zoological research at institutions across the United States, and in Australia, Canada, China, France, Germany, and the United Kingdom, and therefore may be available through local collaborations.

Second, the X-ray imaging volume and tracking precision will impose limitations on the size of animal and muscle that can be measured with fluoromicrometry. The imaging volume is limited to the intersections of two conical projections from the X-ray source to the image intensifier, and therefore strongly limited by the image intensifier size. The X-ray video system used in this study can only image a volume approximately 6,000 cm³ (i.e., about the size of a soccer ball) (Brainerd et al., 2010). While some newer systems can image slightly larger volumes, motions that require larger spaces may be only transiently viewable, though in some cases this can be remedied by use of treadmills, wind tunnels, or flumes and careful positioning of the fluoroscopes and animal. Conversely, the precision of fluoromicrometry and marker tracking (~0.1 mm) will limit how small of a muscle can be successfully measured with fluoromicrometry.

Lastly, there is not currently a method for viewing fluoromicrometry length measurements in real time as they are being recorded. While recent advances in X-ray video processing and marker tracking have substantially reduced the analysis time, it still takes longer than the biological behaviors likely to be studied. Therefore, when real-time feedback on muscle length is needed during the experiment (such as in many *in vitro* preparations), sonomicrometry is likely to be a more useful technique.

CONCLUSIONS

The precision and accuracy reported here demonstrate the utility of fluoromicrometry for measuring muscle lengths across a wide range of experiments and behaviors. While these values are a useful guide, the precision of fluoromicrometry is likely to vary between experimental setups and will depend on imaging parameters such as magnification level, camera resolution and focus, bead size, calibration, and image quality. We therefore recommend that users measure and report bead tracking precision for every study, using a frozen specimen with muscle beads implanted. The specimen should be moved in the X-ray beams to reproduce approximately the frequency and amplitude of bead motions in world space in the actual studies (Menegaz et al., 2015; Brainerd et al., 2016). Since the specimen is frozen, all interbead distances should be constant, and the standard deviation of these distances will be the measure of precision for that study.

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Authors' contribution

All authors contributed to the designing of the study. H. C. A., A. L. C., and A. M. H. performed data collection and analysis, wrote the manuscript, and created figures. All authors discussed the results and edited the manuscript and have read the manuscript and agreed to be listed as authors.

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