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Title

Microendoscopy reveals positive correlation in multiscale length changes and variable sarcomere lengths across different regions of human muscle.

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Running Head

Sarcomere and fascicle length changes in passive human muscle

Keywords

muscle fascicle, fibre, second harmonic generation, biomechanics

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New and Noteworthy

- Sarcomere and fascicle lengths were measured *in vivo* from human muscle to examine the relationship between the different scales of organisation.
- Changes in fascicle length were moderately related to sarcomere length changes, however sarcomere length and number per fibre varied from proximal to distal regions of the muscle.
- Differences in average sarcomere operating lengths across the muscle suggests potentially different stresses or strains experienced within different regions of muscle.

Abstract

Sarcomere length is a key physiological parameter that affects muscle force output; however, our understanding of the scaling of human muscle from sarcomere to whole muscle is based primarily on cadaveric data. The aims of this study were to explore the *in vivo* relationship between passive fascicle length and passive sarcomere length at different muscle-tendon unit lengths and determine whether sarcomere and fascicle length relationships are the same in different regions of muscle. A microendoscopy needle probe capable of *in vivo* sarcomere imaging was inserted into a proximal location of the human tibialis anterior muscle at three different ankle positions (5° dorsiflexion [DF], 5° plantar flexion [PF], 15° PF) and one distal location at a constant ankle position (5° PF distal). Ultrasound imaging of tibialis anterior fascicles, centred on the location of the needle probe, was performed for each condition to estimate fascicle length. Sarcomere length and fascicle length increased with increasing muscle-tendon unit length, although the correlation between sarcomere length change and muscle fascicle length change was only moderate ($r^2 = 0.45$). Passive sarcomere length was longer at the distal imaging site than the proximal site ($P = 0.01$). When sarcomere number was estimated from sarcomere length and fascicle length, there were fewer sarcomeres in the fibres of distal location than the proximal location ($P = 0.01$). These data demonstrate that fascicle length changes are representative of sarcomere length changes, although significant variability in sarcomere length exists within a muscle, and sarcomere number per fibre is region dependent.

Introduction

The length of sarcomeres that are arranged in-series within a striated muscle fibre is one of the most important determinants of muscle force. Sarcomere length influences overlap of actin and myosin, which affects contractile force (20), calcium sensitivity and activation dynamics (17, 41) and muscle energetics (2). Consequently, to understand the mechanics of in-vivo muscle contraction it is important to understand how sarcomere length varies with muscle length changes (34). The relationship between muscle length and sarcomere length is dependent upon the number of sarcomeres in series within the muscle's fibres, and this number has a strong influence on sarcomere strains and strain rates during movement.

Sarcomere length and operating range vary considerably both within and across species (8). However, there is general consensus that the average operating length range of sarcomeres favours force production for the tasks required for that particular muscle (36, 48) and that sarcomere arrangement is likely to be an important adaptation of muscle to chronic changes in mechanical loading. For instance, stretching muscle passively or actively increases the number of sarcomeres in series within a muscle fibre (58), whereas muscle denervation in a shortened position can cause a reduction in sarcomere number for a given muscle (59). Such adaptations are variable and likely dependent on the specific mechanical stimulus experienced (9, 10) and on muscle architecture [e.g. pennation angle (22)].

There are several methods to assess sarcomere length in different muscle preparations. Muscle fixation followed by fibre dissection and direct measurement using light microscopy has been used to characterise the diversity of sarcomere lengths within different muscles (15, 16). Laser diffraction is another method that can be used in intact muscle (33), muscle biopsies (50) and fully dissected muscles (18). Laser diffraction has provided invaluable information about human sarcomere arrangement and adaptation (31, 32); however this method is relatively invasive and is typically done under surgical conditions. Microendoscopy using second harmonic generation (SHG) imaging is a promising new method to assess *in vivo* sarcomere lengths in both human and animal muscle (35). Recent investigations using novel needle probes have provided new information about the both the relationship of sarcomere length to joint position in passive muscles (11-13) and the time course of muscle twitches (49). These investigations have demonstrated variability in sarcomere lengths within and across muscles (13), which is in general agreement with similar measures made using table top SHG imaging on intact muscle (42) or direct imaging across frozen sections of muscle (44).

Although measures of passive sarcomere length within specific muscles are useful, to gain insight into the number of sarcomeres per fibre, estimates of muscle fibre lengths are also required (34). Ultrasound imaging has become a popular tool for measuring fascicle length, which is often used as a proxy for fibre length assuming the fibre length is the same as fascicle length (14). Classic studies using the ultrasound technique in human lower limb muscles have shown that during isometric contractions, muscle fascicles can shorten up to 35% of the initial length (27, 38, 43). This has implications when considering the relationship between passive and active sarcomere length measurements. It is common to use measures of human fascicle length as a proxy for sarcomere number because of ease of measurement. For example, by determining the optimum fascicle length during contraction and assuming an optimum sarcomere length of 2.64 (55), one can estimate the total number of sarcomeres within the imaged muscle fibres (37). The limitation of this approach is that it relies on local measures of fascicle length changes, ignores potential sarcomere and fibre length heterogeneity within the muscle (34, 54, 57), and makes assumptions about optimal sarcomere length.

Here we provide the first simultaneous *in vivo* measurement of both fascicle length and sarcomere length in passive human muscle, so that estimates of sarcomere numbers per fibre can be determined. The first aim was to use microendoscopy to explore the relationship between passive fascicle length and passive

sarcomere length within the same region of the human tibialis anterior muscle for different muscle-tendon unit lengths. We hypothesised that sarcomere number calculations should be consistent across muscle-tendon unit lengths, as sarcomere numbers should not change. We also hypothesised that as muscle-tendon unit length was passively changed, fascicle length changes would be correlated with sarcomere length changes, as is typically assumed (60). The second aim was to determine whether sarcomere and fascicle length relationships are homogenous across different regions of the human tibialis anterior muscle, at a single muscle-tendon unit length. We hypothesised that passive sarcomere length would vary across different regions, based on results from numerous studies in animal muscles (42, 44, 54, 57), and as such, that the sarcomere number estimated per fibre would vary depending on muscle fibre location.

Methods

Protocol

Eight healthy participants [6 male and 2 female; age = 31 ± 4 years; height = 1.78 ± 0.1 cm; mass = 73.4 ± 14.1 kg (mean ± standard deviation)] who were free from lower limb injury or neuromuscular disorders provided written consent to participate in this study. The Stanford University Institutional Review Board and The University of Queensland Human Research Ethics Committee approved the experimental protocol.

Participants sat in a chair with their knee flexed at a constant angle of approximately 15° from full extension and their foot strapped to a rigid foot-plate such that the ankle was in an anatomically neutral position (Figure 1). The angle of the foot plate could be adjusted to place the ankle in three different positions: 15° plantar flexion (15°PF), 5° plantar flexion (5°PF), and 5° dorsiflexion (5°DF), as measured by the angle made by the line between the fibula head and lateral malleolus and the line made by the base of the foot along the foot plate. The range of ankle angles (5°DF - 15°PF) was selected to correspond to the plateau and ascending limb of the length-tension relationship based on torque vs. angle data during maximum voluntary contractions, and to avoid passive tension in the muscle (40).

Sarcomere and fascicle length measures were first made in a proximal region of the tibialis anterior muscle using a microendscope needle probe (see Sarcomere Imaging and Analysis section for details) and B-mode ultrasound imaging (see Ultrasound Imaging and Analysis section). The needle probe was inserted so that the imaging site was approximately 1.5 cm deep in the superficial compartment (before the probe was drawn out) and 3 cm distal to the proximal end of the central aponeurosis, identified by ultrasound imaging (Figure 1). To image multiple muscle fibres within the muscle region, the needle/microscope was slowly drawn out of the muscle by up to 1cm (without removing it from the muscle) and reinserted to the initial depth when imaging was complete. Measurements were performed at each of the three different ankle angles in a randomly selected order. Between each ankle angle, the microscope attached to the needle probe was removed but the needle remained within the muscle to ensure that the same region of muscle fascicles was imaged across different ankle positions. Note to accommodate the length change of the fascicles, the needle probe rotated by approximately 15°, however the microscope could still be attached to the probe and held by the operator. Prior to moving between ankle positions, ultrasound images of the fascicles were collected such that the embedded needle sat directly next to the middle of the transducer (see Ultrasound Imaging and Analysis section for details and Figure 2).

Sarcomere and fascicle length measures were then made at a more distal location (approximately 4 cm from the distal end of the superficial compartment of the tibialis anterior muscle) with the ankle at 5 degrees PF by re-inserting the microendoscope needle probe (Figure 1). The time between removal of the needle probe from the proximal region to the insertion in the distal region was approximately 10 minutes, during which time the needle was placed in a disinfecting solution. The distance between insertion points was approximately 4-8 cm, depending on the length of superficial compartment of the TA muscle.

Sarcomere Imaging and Analysis

Sarcomeres were imaged using a microendoscope system that accessed the muscle via a needle probe (2 cm) with a side-mounted lens (49). A commercially available system (Zebrascope, Zebra Med Tech, CA) that uses second harmonic generation (SHG) imaging to visualise the repeating patterns of thick filaments (myosin) was used. A 1030 nm, femtosecond excitation pulse was directed out the side of a transmitting needle via a small lens centred 4 mm from tip of the needle. Unlike previous designs, which excite and receive the reflected signal in a single lens (35, 49), in the present study the emitted SHG signal was collected through a receiving lens in a separate needle that lay parallel to transmitting needle at a distance of 1 mm (Figure 2, inset). This has additional advantages in that the received signal strength is stronger and less susceptible to interference due to blood or fluid around the needles. The imaging distance was adjustable between 0 and 150 µm from the surface of the emitting needle.

The needle probe is attached to a housing that aligns the laser to a handheld microscope, which subsequently interfaces with the laser. The needles were inserted into the muscle using a spring-loaded device that rapidly inserts the needles. Prior to insertion, B-mode ultrasound imaging (LogicScan, Telemed, Lithuania) was performed using a flat shaped ultrasound transducer (6 cm transducer width, mean frequency 6 MHz) to determine the line of action of the muscle fascicles (Figure 2). The correct plane of the fascicles was assumed to be the plane where muscle fascicles were clearly visible and continuous throughout the image of the superficial compartment and where a clear central aponeurosis was visible and approximately perpendicular to the imaging plane (6). The ultrasound image was also used to define the proximal and distal insertion sites based on the criteria described above. The probe was then inserted so that the transmitting and emitting needles were inserted approximately in the middle of the image and along the plane of the image so that the line from one lens to the other was approximately perpendicular to the fascicle plane. As such, the fibres of interest should have been uncompromised between the two needles. The microscope was then attached to the needle probe to begin imaging.

A sequence of images was collected as the microscope and needle were slowly moved in and out of the muscle as has previously been reported (11, 12). Image depth was approximately 5-15 mm into the muscle, limited by the length of the needle and the thickness of skin and subcutaneous fat. Images were collected at 1 Hz with the operator being able to see the images in real-time. A second operator adjusted the image depth and power of the signal to obtain as clear images as possible as the images were recorded to file for subsequent analysis. Sequences of images ranging from 20 seconds to 2 minutes were collected while reasonable images were detected visually by the operators.

Image sequences were then analysed using a modified process that was previously reported (11, 12). First, a fast Fourier transform and a Gaussian filter were applied to the image. White noise was then subtracted from the Fourier image and the strongest frequency spectrum between that predicted for sarcomere lengths between 1.5 and 5 µm was calculated across the image. Feasible images were selected based on the intensity of the image and signals that fell within the set sarcomere length range and these images were used for further analysis. To ensure that single fibres were analysed separately, feasible images were then examined by an operator who placed regions of interest along the length of any separate visible fibres within the image (between 1 and 3 fibres can be distinguished at once). The same Fourier transform calculation of sarcomere lengths was then performed on each region of interest (Figure 2) to get the sample sarcomere length, which represents the average sarcomere length across the region of interest (~100 µm in length). Individual fibres were only selected once within a sample, to the best of the ability of the image analyser (GL). Between 6-90 separate muscle fibre images (mean 28.4 ± 19.2) were collected and used in the analysis for every ankle position (or location within the muscle), for each participant. The number of suitable images was assessed offline, post collection, depending on quality of image sequences.

Ultrasound Imaging and Analysis

B-mode ultrasound images were acquired when the microscope was removed from the needle, but while the needle was still embedded in the muscle for each insertion site and at each joint angle. The same ultrasound system used to determine the insertion point for the microscope needle probe (see above) was used to determine muscle fascicle length in the same region of muscle as the needle. Ultrasound images were acquired with the ultrasound transducer as close to the needle insertion point as possible by aligning the flat ultrasound transducer next to the needle connector (Figure 2) and in an orientation to obtain clear, continuous images of fascicles and aponeurosis in the superficial compartment (see image examples in Figure 1) and ensuring that the fascicles were at the maximum length. Although it was not possible to image the same fascicles that were imaged between the needles on the probe, fascicle images within approximately 5 mm from the imaging site and at the same proximo-distal location were imaged. Fascicle length is likely to be homogenous within this close range. Muscle fascicle length was determined as the straight-line distance from superficial aponeurosis to the central aponeurosis, along the line of action of the fascicles, within the middle of the image (14, 47). All distances were converted from the pixel scale to millimetre scale using the known depth and width calibration factors of the image.

Statistical Analysis

Sarcomere length data from each condition (proximal 5°DF, 5°PF, 15°PF and distal 5°PF) were averaged across each individual and a one-way repeated measures ANOVA was used to assess the effect of ankle joint angles on the sarcomere number, sarcomere length, and fascicle lengths at the proximal imaging location. A paired Student's t-test was used to assess the effect of proximal vs. distal imaging location (in the 5°PF ankle position only) on sarcomere number, sarcomere length and fascicle length. Multiple linear regression was used to establish potential relationships between fascicle length changes and sarcomere length changes across all measurements at the proximal location, using each participant as a categorical predictor (sarcomere length * participant) to account for multiple measurements made across participants in the data used in the regression (4). Fascicle and sarcomere length changes were expressed relative to the mean value across all measurements for each individual, to account for individual variation. To understand the variability of measurements, the coefficient of variation was determined for each individual and at each measurement site. All statistical tests were conducted in Matlab using SPM1D.org software (version 0.4) with the alpha level set at $P<0.05$.

Results

Individual participant sarcomere data were averaged across measurements made at each joint angle or location. This was based on the following average number of sarcomere measurements per participant at each of the joint angles or imaging locations: 5°DF: 21 ± 12 measurements per participant; 5°PF: 36 ± 19 measurements per participant; 15°PF: 21 ± 13 measurements per participant; 5°PF (distal location): 35 ± 27 measurements per participant.

A box and whisker plot (mean, 25^{th} and 75^{th} percentile) and individual average data points for sarcomere length, fascicle length and sarcomere number at different ankle flexion angles is shown in Figure 3. At the proximal site of imaging, sarcomere length increased significantly ($P = 0.016$) with ankle angle change from the dorsiflexed position (5°DF) to the plantar flexed position (15°PF). There was also a significant increase in length of the fascicles with the same change in ankle position ($P<0.001$). There was no significant difference in sarcomere number when estimated from the sarcomere and fascicle lengths at each of the three ankle joint angles in the proximal imaging position ($P = 0.502$).

A box and whisker plot and individual average data points for sarcomere length, fascicle length and sarcomere number in different regions of the muscle is shown in Figure 4. Sarcomere length was greater in

the proximal than distal imaging locations in 5°PF ankle position (P = 0.011). There was a tendency for shorter fascicles in the distal region, but this difference was not significant (P = 0.084). When sarcomere number was estimated from these two measures, there was a significantly lower sarcomere number in the distal location than the proximal location (P = 0.013).

Relationships between sarcomere length change and fascicle length change, for the proximal imaging location, are shown in Figure 5. Length changes were calcualted relative to the average lengths for each individual across all joint angles measured. There was a significant positive correlation (P < 0.006) between the length change of the sarcomeres and that of the fascicles. The variance in fascicle length change predicted 45% of the variance in the sarcomere length change when adjusted for individuals to account for multiple measures for each individual.

The variance in measurements across individuals and measurement sites is shown in Figure 6. There was a large variation of sarcomere lengths within individuals, with an average co-efficient of variation (CV) of 7.81% (± 2.48%). The variance was similar across all conditions (P = 0.449) (Figure 6B).

Discussion

In agreement with our first hypothesis, we found that estimates of sarcomere number are consistent across different muscle-tendon unit lengths when average measures are made from the same region of muscle using microendoscopy combined with ultrasound imaging. Measured sarcomere length and fascicle length both increased significantly with muscle-tendon unit length, and there was a moderate positive correlation between sarcomere length change and fascicle length change when using the microendoscopy technique to determine mean sarcomere length from a relatively large sample of images from the muscle. This relationship only explained 45% of the overall variance, which is best explained by variability in measurement within participants and potential errors in measurement of length for both microendoscopy (sarcomere) and ultrasound (fascicle) measurements. In support of our second hypothesis, sarcomere number per fibre was greater in the proximal region of the muscle than the distal region, despite similar muscle fascicle lengths. This result suggests heterogeneity of sarcomere number and length between regions of individual human muscles and has implications for how fascicle level mechanics can be interpreted in terms of the stresses and strains that muscle fibres might experience during contractions or movement.

Sarcomere number for a given fibre cannot change with changes in muscle length due to joint rotation. The finding of constant sarcomere number for average measures made from a single region of the muscle adds confidence in the use of the microendoscopy to quantify sarcomere lengths/numbers *in vivo*, without necessarily validating the measurements. Using ultrasound imaging to assess fascicle lengths is known to be susceptible to errors due to transducer alignment, although this is generally unbiased (7). While we used procedures to try ensure optimum alignment (6), errors in fascicle length certainly confound the relationship between fascicle length changes and sarcomere length changes. However, there are also additional considerations when applying the microendoscopy technique to measure sarcomere length that should also be considered. We first consider whether the sarcomere lengths measured agree with expected lengths.

The resting sarcomere lengths for all locations and ankle positions were considerably higher than the predicted optimum (2.64 µm) (55), even at 15° PF, which is the known optimal angle for maximum dorsiflexion force production (37). The likely explanation for this discrepancy is the large length changes that occur during contraction as the muscle stretches the in-series elastic tissues. Maganaris and Paul (39) measured TA muscle belly shortening during isometric contraction to be on the order of 18% (12 mm). Attributing this 18% shortening entirely to the sarcomeres would estimate that the active sarcomere length in the 15° plantar flexed position to be 2.56 µm, which is in the region of optimum sarcomere lengths that has been estimated for human muscle (32, 55). In another study, maximum isometric contractions were

performed at different ankle positions to estimate the optimal fascicle length (37). Assuming that optimal fascicle length corresponded to when most sarcomeres were at optimal lengths (2.64 µm), Maganaris (37) estimated the number of sarcomeres in the TA muscle fibres at 21 500 for a scanning location similar to the more proximal measurements made here. This estimate of sarcomere number is remarkably close to our estimates (mean 21 712 sarcomeres in proximal region) based on direct measures of passive sarcomere length and fascicle length. Our estimate is also extremely close to the direct measures of sarcomere number per fibre from the TA of human cadavers (21 751 sarcomeres per fibre) (56). Although the average sarcomere length measurements are in accordance with expectations, the interpretation further demonstrates the requirement to consider the influence of series compliance, as previously demonstrated (29, 33), when inferring optimal muscle fibre lengths or muscle-tendon unit lengths from passive measurements of sarcomere lengths.

There was considerable between- and within-subject variability in sarcomere length measurements within a single region of muscle at the same ankle position (Figure 6). This likely contributed to the moderate relationship between sarcomere length change and fascicle length change across all individuals. The between participant variability is to be expected, and is likely due to differences in how the muscle is used during everyday life. For instance, participants who regularly undertake exercise that involves eccentric contraction (e.g. downhill walking), might have shorter sarcomeres across at comparable ankle positions (10). However, we also found large within participant variability. The coefficients of variation averaged 7.81% across all measurement sites within individuals. A major proportion of this variability is likely explained by natural variation within the muscle and is within the range of reported variance measured from both dissected animal muscle (44) and *in situ* muscle (42). For example, a recent study using SHG imaging to examine sarcomere length within an in situ mouse muscle (42) found considerable variability both within and across different muscle regions, with a coefficient of variation of approximately 5% across all sites of the same muscles (and up to 8% at shortest lengths).

Several methodological details might also contribute to the variability of our measurements. To sample sarcomeres from multiple fibres in each region, we withdrew the needle probe through the muscle from deep to superficial regions. Due to the pennation angle of the fibres, we will have sampled from different regions of individual fibres, primarily from the mid- to distal-regions of the fibres. There is evidence from both isolated fibres (25, 26) and whole muscle (42, 52) suggesting that sarcomere lengths may differ between sites along a muscle and hence this could contribute to some of the variability we measured. The lack of systematic control of where we imaged in each fibre means that we have randomly sampled and makes it difficult to reconcile the source of variability in our measures. Sampling at different locations in the same fibre/s was beyond the scope of this study, but would certainly provide greater insight into the source of the variability and particularly the potential for sarcomere length change heterogeneity across and between individual fibres in different locations of the muscle. There is some early evidence that fibre strains in human muscle may be highly heterogeneous during both passive length changes (45) and light contractions (28). This could help explain the only moderate relationship between muscle length changes and measured sarcomere length changes and questions the assumption that fibre length changes directly reflect sarcomere length changes.

Some of the variance in our study is also likely attributable to the measurement technique. For instance, the imaged fibre section may be slightly distorted by up to 9% due to the needle or image plane (12), however this correction (which represents the maximum possible distortion) has recently been considered to be not required (51), likely because most of the fibres that are imaged are farther from the needle where distortion is minimal. Finally, it is also possible that some fibres may be damaged by the needles or some fibres might not be completely passive during imaging (i.e. low levels of underlying activation).

Overall, it is clear that with a sufficient number of measures, reasonable estimates of mean sarcomere length can be made, which result in consistent sarcomere number estimates at different muscle lengths. This highlights one limitation of the imaging methods – a relatively large sample size is needed to ensure a representative mean value is obtained and this is limited to relatively small areas of the muscle that is sampled. Another promising sarcomere imaging technique recently proposed, termed resonant reflection spectroscopy, samples much greater regions of muscle with good temporal resolution and minimal invasiveness. Although that technique may yield lower variability in individual measurements (61), there are presently no reports that have used this technique in human muscle.

In agreement with some previous literature (42, 44), we found different sarcomere lengths in different regions of the muscle despite no change in muscle-tendon unit length. Our study is unique in that we were also able to determine the fascicle length corresponding to the imaging region, and the fascicle length was similar for both the proximal and distal regions imaged. Combined with the longer sarcomere lengths, this resulted in significantly smaller sarcomere numbers in the muscle fibres in distal region of the human tibialis anterior muscle. This difference in sarcomere length could result in up to a 20% difference in force potential upon initial activation, based on a standard length-tension relationship of sarcomeres, scaled for human muscle (Figure 7). We speculate that the difference in the sarcomere number may relate to the strains experienced during active contractions or movement profiles. There is evidence that muscles fibres experience variable strains within different regions of muscle during passive length changes (52) and dynamic contractions (1). Simulation studies suggest that this is driven by differences in how muscles must distort during contraction (23) and other factors like myofascial force transmission (24, 62). Such differences in strain amplitudes could provide stimulus for having heterogeneity in sarcomere lengths across the muscle and may influence force generating capacity under different conditions, and this heterogeneity has been suggested to improve the force generating capacity of muscle through the range(23).

In human muscle, ultrasound imaging studies have reported conflicting reports regarding whether fascicles experience uniform strains during active contractions. The gastrocnemius has been shown to undergo relatively homogenous strain throughout (21, 30), however other human muscles such as the biceps femoris (3) and biceps brachii (46) muscles have shown some regional differences in fascicle length and shortening during contraction. It is possible that the sarcomere number may be regulated to ensure that sarcomeres operate at more uniform or optimum lengths during contraction, based on the shortening or strain experienced in the relevant portion of the muscle. Our hypothesis from the current data would be that if all fibres shorten a similar amount during contraction, fibres in the distal portion of tibialis anterior would undergo greater relative shortening during fixed-end muscle contraction than fibres in the proximal region of this muscle. Under this paradigm, sarcomeres in the distal part of the TA would reach similar lengths to sarcomeres in the proximal region once the muscle is in a contracted state, despite starting from a longer initial sarcomere length. This requires further experimentation and/or simulations to confirm.

The results of this study have important implications for understanding muscle mechanics and adaptation. First, it is clear from the present data that there is a moderate linear relationship between sarcomere length changes and fascicle length changes when stable estimates of measures are obtained from averaging multiple samples. Therefore, it is reasonable to assume that changes in fascicle length reflect changes in sarcomere length across the muscle. However, there was variability in individual measurements from the same muscle and hence a sufficient number of samples needs to measured from an individual muscle region to accurately determine average sarcomere lengths. Second, in lower limb muscles which have substantial in series compliance, such as the tibialis anterior, the passive sarcomere length may be substantially longer than the optimal length. It is presently technically difficult to sample sarcomere lengths from active muscle, however the significant shortening that is known to occur during isometric contraction should be accounted for when

trying to predict optimum lengths. Third, sarcomere numbers varied between different muscle regions, even when muscle fascicles were of similar length. This has implications for interpreting passive fascicle length differences in both cross-sectional and prospective studies. For instance, various concentric or eccentric strength training protocols (e.g. 5, 53) have been shown to induce changes in passive fascicle length at specific joint configurations. However, it is difficult to determine whether this would directly relate to changes in sarcomere number or overall lengthening of sarcomeres. It is also difficult to determine whether adaptations might be consistent across different regions across the muscle. For instance, there is some recent evidence that focal adhesion kinase (FAK), a mechanotransduction protein, is activated after eccentric and concentric exercise in a region-dependent manner in human muscle (VL), with the largest effects in the distal site of the muscle (19), which could regulate region-specific adaptations. However, the only way to assess whether sarcomere level adaptations occurs in different regions of the muscle would be through direct sarcomere length measurement, as has been achieved here. The method used here would therefore be generally useful for investigation of adaptation in structure and function in response to training, disuse or pathology.

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Author Contributions

All authors were involved in the conception or design of the research and drafting the work or revising it critically for important intellectual content. G.L., D.F., X.C., and S.D. were involved in acquisition, analysis and interpretation of data for the work.

Disclosures

S.D. has co-founded and has a financial interest in the company that developed the microendoscopy technology (Zebra Medical Technologies Inc.). All other authors have no other competing interests.

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Figure Legends

Figure 1: Imaging sites on the tibialis anterior muscle for both ultrasound and microendoscopy. Note that the microendoscopy needle was inserted in the mid-region of the ultrasound image, but the ultrasound transducer was placed adjacent to the needle, such that the fascicle image was made ~0.5 cm lateral to the sarcomere measures.

Figure 2: Experimental equipment and imaging setup. (Left) The needles (inset image) were inserted in the muscle (red square) and ultrasound imaging was conducted parallel to this site. (Left inset) Image showing needle probe used in study, which includes two needles (emitter and collector). (Right) Images collected using the SHG imaging technique. White box indicates the region where the sarcomere length was calculated using Fourier analysis. Ripples at left and right of image indicate borders of muscle fibre with adjacent fibres.

Figure 3: Sarcomere length and fascicle length increased significantly as the ankle was moved from a dorsi-flexed to a plantar flexed position, whereas calculated sarcomere number remained constant. Change in sarcomere length (A), fascicle length (B) and calculated sarcomere number (C – fascicle length divided by sarcomere length) are shown for each ankle position: 5° dorsi-flexion (5°DF '+' symbols), 5° plantar flexion 5°PF – 'o' symbols) and 15° plantar flexion (15°PF – 'x' symbols) when measured in the proximal location only. Average measurements for each individual (N = 8) are shown using points. Box indicates spread between the 25th and 75th percentile of the variance and whiskers indicate extreme data points, neglecting any outliers (red symbols).

Figure 4: Sarcomere lengths were significantly longer in the distal location compared to proximal, while fascicle length remained unchanged in the 5°PF position, resulting in reduced sarcomere numbers per fibre in the distal region. Sarcomere length (A), fascicle length (B) and calculated sarcomere number (C – fascicle length divided by sarcomere length) as a function of muscle imaging location: Proximal (5°PF 'o' symbols), Distal (5°PF '◊' symbols). Averages measurements for each individual (N = 8) are shown using points. Box indicates spread between the 25th and 75th percentile of the variance and whiskers indicate extreme data points, neglecting any outliers ('+' symbols outside whiskers).

Figure 5: A significant, but modest, correlation between fascicle length change and sarcomere length change across all individuals. Relationship between fascicle length changes (relative to average across all ankle joint positions) and sarcomere length changes (relative to average across all ankle joint positions) in the proximal insertion site. Data points are averages for each individual with different symbols representing points measured at different joint ankle angles (5°DF '+' symbols, 5°PF 'o' symbols, 15°PF 'x' symbols).

Figure 6: Variablity in sarcomere length measurments across different joint positions and locations. A) Individual data points for each individual participant (indicated by individual column of data points, each with a different colour, N = 8) at each joint position for proximal measurement and the distal measurement site. B) Coefficient of variation across all measurements of sarcomere length at each joint position for proximal measurement and the distal measurement site (Data are group mean ± s.d.).

Figure 7: Theoretical relationship between sarcomere length and force generating potential and mean ± standard deviation of sarcomere lengths for each measurement angle in the proximal imaging location (5°DF '+', 5°PF Proximal 'o', 15°PF 'x') and for the distal imaging location (5°DF Distal '◊'). The theoretical curve is based off the curve for vertebrate muscle reported by Burkholder & Lieber (8) and scaled based on an estimated optimal sarcomere length of 2.64 (55).