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3D ultrasound imaging: fast and cost effective morphometry of musculoskeletal tissue

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TITLE:
3D Ultrasound Imaging: Fast and Cost-Effective Morphometry of Musculoskeletal Tissue

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SHORT ABSTRACT:
3D ultrasound imaging (3DUS) allows fast and cost-effective morphometry of musculoskeletal tissues. We present a protocol to measure muscle volume and fascicle length using 3DUS.

LONG ABSTRACT:
The developmental goal of 3D ultrasound imaging (3DUS) is to engineer a modality to perform 3D morphological ultrasound analysis of human muscles. 3DUS images are constructed from calibrated freehand 2D B-mode ultrasound images, which are positioned into a voxel array. Ultrasound (US) imaging allows quantification of muscle size, fascicle length, and angle of pennation. These morphological variables are important determinants of muscle force and length range of force exertion. The presented protocol describes an approach to determine volume and fascicle length of *musculus vastus lateralis* and *musculus gastrocnemius medialis*. 3DUS facilitates standardization using 3D anatomical references. This approach provides a fast and cost-effective approach for quantifying 3D morphology in skeletal muscles. In healthcare and sports, information on the morphometry of muscles is very valuable in diagnostics and/or follow-up evaluations after treatment or training.

INTRODUCTION:

In healthcare and sports, information on the morphology of muscles is very valuable in diagnostics and/or follow-up evaluations after treatment or training[1]. Ultrasound (US) imaging is a tool commonly used for visualization of soft tissue structures in muscle diseases[2], critical illnesses[3,4], cardiovascular diseases[5], neurological disorders[6–8], and effects of physical training[6,9,10]. US imaging enables quantification of muscle size, fascicle length, and angle of pennation. These morphological variables are important determinants of muscle force and length range of force exertion[11–15].

Currently, US imaging measurements are mostly performed in 2D images, with the examiner choosing a presumably, suitable orientation and location of the ultrasound probe. Such 2D methods restrict morphological measurements to one image plane, while the parameter of interest may not be present within this plane. Morphological analysis requires a 3D approach, providing out-of-plane measurements using 3D reference points. Such a 3D morphological representation of soft tissues is known to be provided by Magnetic Resonance Imaging (MRI)[16–20]. However, MRI is expensive and not always available. Also, visualization of muscle fibers requires special MRI sequences, such as diffusion tensor imaging (DTI)[21]. A cost-effective alternative to MRI is 3D ultrasound (3DUS) imaging. The 3DUS approach provides several advantages over MRI techniques, *e.g.*, it imposes less space limitations for positioning the subject during an examination. 3DUS imaging is a technique sequentially capturing 2D (B-mode US) images and positioning them into a volume element (voxel) array[22–24]. The process of 3DUS image reconstruction consist of five steps: (1) Capturing a series of freehand 2D US images; (2) Tracking the position of the US probe, using a Motion Capture (MoCap) system; (3) Synchronizing the MoCap position and US images; (4) Calculating the location and orientation of the ultrasound images within the voxel array using a calibrated system of reference; and (5) Placing these images into this voxel array.

The 3DUS approach has been successfully applied for assessment of morphology of skeletal muscle[15,25–29]. However, previous approaches[7,15,25,30] have proved cumbersome, time consuming and technically limited, as only small segments of large muscles could be reconstructed.

To improve the 3DUS approach, a new 3DUS protocol has been developed that allows reconstruction of complete muscles within a short period of time. This protocol article describes the use of 3DUS imaging for morphometry of the *m. vastus lateralis* (VL) and *m. gastrocnemius medialis* (GM).

PROTOCOL:

All procedures involving human subjects have been approved by the medical ethics committee of the VU medical center, Amsterdam, the Netherlands.

1. Instrumentation

1.1. Connect the **ultrasound device** to the **measurement computer**. If needed, use frame-grab

hardware and/or software to store the sequential ultrasound images.

Note: A 5-cm linear-array probe (12.5 MHz) is used to generate B-mode images (25 Hz). Before each measurement, imaging depth, acoustic frequency, and power are optimized to visualize interfaces of extra- and intramuscular connective tissues. During the measurement, these settings are not changed.

1.2. Connect the **MoCap system** to the measurement computer.

1.3. Rigidly connect a MoCap cluster marker to the ultrasound probe to track the position and orientation of the US probe.

1.4. Connect the **synchronization device** (piezo crystal) to the trigger input of the MoCap system.

Note: Activation of the synchronization device momentarily activates the piezo crystal, sending sound waves towards the transducer. The received soundwaves create a distinct artifact in the US image at the system initiation (**Figure 1A**, Arrow).

1.5. Fill the **custom-made calibration frame** (phantom) with water.

2. Calibrate

Note: Perform a spatial calibration to calculate a transformation matrix $\left({}^{to}\boldsymbol{T}_{from}\right)$ from the US images with respect to the probe coordinate system. This calibration process has been described previously[22]. Please see below for a brief description.

2.1. Place the phantom filled with water, holding a crosswire (*i.e.*, two submerged crossing wires) at a known position within the phantom coordinate system ($\boldsymbol{Ph}_{xyz}$ **Figure 1B,** arrow), on a stable surface.

2.2. Measure the water temperature with a thermometer.

2.3. Use the MoCap pointer tool to record the position and orientation of the phantom in the global coordinate system (***Gl***).

2.4. Start the US image sampling and activate MoCap data acquisition (described in step 3.3.3).

2.5. Submerge the head of the US probe (***Pr***) in the water. Translate and rotate the US probe for 40 s (sampling at 25 Hz) in all directions, maintaining visibility of the crosswire in the US images (***Im***).

2.6. Stop data acquisition.

2.7. Synchronize the MoCap data and US images by identifying the first US frame containing the

piezo crystal-created artifact and crop the US image sequence accordingly (described in step 3.4.1.1).

2.8. Identify the relevant US images in which the crosswire is clearly visible and track the position of the crosswire in these US images ($_{i}\boldsymbol{Im}_{xyz}$),and correct the position for water temperature.

2.9. Determine the position of the crosswire with respect to the moving ***Pr*** by a series of transformations from ***Ph*** to ***Pr*** (**Equation 1**) at time instances (i = 1:n) corresponding to the crosswire identification in step 2.8.

$$\begin{bmatrix} {}_{i(1)}\boldsymbol{Pr}_x & \cdots & {}_{i(n)}\boldsymbol{Pr}_x \\ {}_{i(1)}\boldsymbol{Pr}_y & \cdots & {}_{i(n)}\boldsymbol{Pr}_y \\ {}_{i(1)}\boldsymbol{Pr}_z & \cdots & {}_{i(n)}\boldsymbol{Pr}_z \end{bmatrix} = {}^{Pr}_{i}\boldsymbol{T}_{Gl} \times {}^{Gl}\boldsymbol{T}_{Ph} \times \begin{bmatrix} \boldsymbol{Ph}_x \\ \boldsymbol{Ph}_y \\ \boldsymbol{Ph}_z \end{bmatrix} \qquad (\textbf{Equation 1})$$

2.10. Calculate the ***Im*** to ***Pr*** transformation matrix (${}^{Pr}\boldsymbol{T}_{Im}$) by solving **Equation 2**, involving all identifications of the crosswire in ***Im*** (measured in step 2.8) at the time matched (i = 1:n) coordinates of the crosswire in ***Pr*** (calculated in step 2.9).

$${}^{Pr}\boldsymbol{T}_{Im} = \begin{bmatrix} {}_{i(1)}\boldsymbol{Pr}_x & \cdots & {}_{i(n)}\boldsymbol{Pr}_x \\ {}_{i(1)}\boldsymbol{Pr}_y & \cdots & {}_{i(n)}\boldsymbol{Pr}_y \\ {}_{i(1)}\boldsymbol{Pr}_z & \cdots & {}_{i(n)}\boldsymbol{Pr}_z \end{bmatrix} / \begin{bmatrix} {}_{i(1)}\boldsymbol{Im}_x & \cdots & {}_{i(n)}\boldsymbol{Im}_x \\ {}_{i(1)}\boldsymbol{Im}_y & \cdots & {}_{i(n)}\boldsymbol{Im}_y \\ {}_{i(1)}\boldsymbol{Im}_z & \cdots & {}_{i(n)}\boldsymbol{Im}_z \end{bmatrix} \qquad (\textbf{Equation 2})$$

[Place figure 1 here]

3. Experimental Protocol

Note: The experimental protocol describes two commonly performed protocols involving 3DUS imaging, *i.e.*, morphometry of GM and VL (**Figure 2A**).

3.1. Subject positioning

3.1.1. **For the GM experiment:**

3.1.1.1. Ask the subject to lie prone on an examination table with both feet overhanging the edge of the table.

3.1.1.2. Align the lower leg horizontally, by placing a support under the tibia. Fix the thigh to the examination table with padded lashing straps to prevent knee extension during the experimental protocol.

3.1.1.3. Fit the foot of the leg to be scanned into the custom-made footplate[31].

3.1.1.4. Connect the custom-made torque wrench with an attached goniometer to the

footplate[31]. Find the footplate angle corresponding to an externally applied torque, *e.g.*, 0 Nm (**Figure 2A**).

3.1.1.5. Fix the footplate in the orientation corresponding to the 0 Nm net dorsiflexion moment, by using an extendable rod which is connected to the table (**Figure 2A**, arrow).

3.1.2. **For the VL experiment:**

3.1.2.1. Ask the subjects to lie supine on an examination table.

3.1.2.2. Set the knee flexion angle (*i.e.* operationalized as the angle between the lines connecting the centers of the *malleolus lateralis* with *epicondylus lateralis* and the latter with the *trochanter major*) to 60°, by positioning the lower legs on a support.

3.1.2.3. Place a triangular shaped beam underneath the buttocks to prevent hip movement.

3.1.2.4. Fix the lower leg to the support with two padded lashing straps to prevent leg movement during the experimental protocol.

3.1.2.5. Set the hip angle (*i.e.*, operationalized as the angle between lines connecting *os coracoides* with the *trochanter maj*or, and the latter with the epicondyle *lateralis femoralis*) to 95 °, by changing the angle of the back support of the examination table.

Note: This described pose has been chosen, as it mirrors joint angles during optimal isometric knee extension measurements[32,33].

3.2. **Localization of bony landmarks and region of interest (ROI)**

Note: This is done for guidance of the 3D ultrasound examination and for post-experimental quantification of the subject's upper leg, lower leg, and foot posture. Identify and record the positions of the anatomical bony landmarks in the global coordinate system using the MoCap pointer tool.

3.2.1. **For the GM experiment:**

3.2.1.1. Identify the following landmarks by palpation and mark them using a surgical skin marker: the most prominent dorsal aspects of the medial and lateral femur epicondyles, and the centers of the malleoli of the tibia and fibula.

3.2.1.2. Using the US device, identify and mark using a surgical skin marker the most superficial point of the medial and lateral femur condyles (on the dorsal side of the leg) and the most proximal location of the insertion of the GM on the calcaneus.

3.2.2. **For the VL measurement:**

3.2.2.1. Identify the following landmarks by palpation and mark them using a surgical skin marker: the medial and lateral malleoli (as above); the most proximal insertion of the patella tendon of the *tuberositas tibiae*; the medial and lateral epicondyles (as above); the apex of the patella and the most medial, proximal, and lateral insertion boundaries on the patella; and *os coracoides* on the shoulder.

3.2.2.2. Identify with the US device and mark the most superficial aspect of the *trochanter major* and most proximal insertion of the VL on the *trochanter major*.

3.2.3. For all muscles, use the MoCap pointer tool to record the marked landmarks (described in sections 3.2.1 and 3.2.2) in the global coordinate system. Move the MoCap pointer tool to the identified anatomical landmarks, and use the MoCap software to record the position by pressing the "record" button.

3.2.4. Use ultrasound to identify the medial and lateral muscle border; mark the medial and lateral borders on the skin using a surgical skin marker.

3.3. **3D ultrasound examination**

3.3.1. Instruct the subject not to move during the 3D ultrasound examination.

3.3.2. Apply ample ultrasound gel on the ROI to ensure proper contact between the skin and US probe.

Note: Such application of gel allows limiting probe pressure and thus tissue deformation necessary to get a clear US image.

3.3.3. Open the frame grabber software (*e.g.*, WinDV[34]) on the measurement computer and start the US image acquisition by clicking on the "record" button.

3.3.3.1. Subsequently, initiate and activate the MoCap data acquisition by pressing the "start" button on the synchronization device; this automatically activates the synchronization device (*i.e.*, piezo crystal) located close to the US probe, creating a distinct artifact in the US image at the instance of MoCap initiation (**Figure 1A**, Arrow).

3.3.4. While exerting minimal probe pressure yet ensuring image quality, move the probe at a constant speed over the ROI; this is referred to as a "sweep". Make sure that clear anatomical cross-sectional US images of the target muscle are recorded.

3.3.5. Visually check for movement of the subject during the examination; if the subject moves, abort the sweep and repeat from step 3.3.1.

3.3.6. **Sweep protocol for the GM experiment**

3.3.6.1. Place the US probe proximally to the femur condyles on the medial aspect of the thigh. Perform a sweep (as described in sections 3.3.1–3.3.5) in the proximodistal direction along the medial border of the GM, ensuring visibility within the anatomical cross-sectional images of the medial border of GM and the Achilles tendon, all the way down to its insertion on the calcaneus.

3.3.6.2. Add additional sweeps (as described in section 3.3.3–3.3.5) until the entire ROI is scanned and the medial border of the muscle is imaged completely (**Figure 2B**). Use the trace in the gel of the previous sweep to guide the next sweep, slightly overlapping (0.5 cm) the previous swept area.

3.3.7. **Sweep protocol for the VL experiment**

3.3.7.1. Place the US probe on the lateral aspect of the tibia plateau. Start a sweep in distal-proximal direction over the lateral border of the VL, ensuring visibility of the lateral border of VL, all the way up to the origin on the *trochanter major*.

3.3.7.2. Add additional sweeps (as described in section 3.3.3-3.3.5) until the entire ROI is scanned and the medial border of the VL is imaged completely (**Figure 2B**). Use the trace in the gel of the previous sweep to guide the next sweep, slightly overlapping (0.5 cm) the previous swept area.

Note: During the sweep protocol, movement of the subject should be prevented, as movements negatively affect positioning of the 2D US images in the voxel array. The number of sweeps are determined by the width of the probe and the width of the target muscle. Typically, with a probe width of 4 cm and a muscle width of 12 or 18 cm, 5 or 7 sweeps, respectively are needed to cover the ROI including the borders.

[Place figure 2 here]

3.4. **3D ultrasound voxel array reconstruction**

3.4.1. Reconstruct a single 3DUS voxel array (3DUS image) from a single sweep over the skin of a specific anatomical ROI (*e.g.*, muscle, tendon) by bin-filling and inpainting the 3DUS voxel array using a custom-script. In order to reconstruct a 3DUS voxel array, take the following post-experimental steps.

3.4.1.1. Synchronize the MoCap data and US images by identifying the first US frame containing the piezo crystal-created artifact and cropping the US image sequence accordingly with VirtualDub software[35]. First, place the frame selection slider at the identified starting frame and press the “home” button on the keyboard. Next, move the slider to the end of the measurement (the last skin contact) and press the “end” button. Press the “F7” button to export the cropped image sequence.

3.4.1.2. Define a voxel array (***Va***) coordinate system which can be filled with US images, using a

custom-script. Ensure that the ***Va*** is oriented in accordance with the scanning direction and sized to fit all the US images of a single sweep.

Note: Initially, the ***Va*** consists of rectangular-shaped voxels, with the longer axes in the direction of the sweep; this shape improves filling efficiency.

3.4.1.3. Assign the voxels in the ***Va*** with pixel grey-values from the US images, using a custom script. This process is described as forward mapping or bin-filling (**Equation 3**; **Figure 1C**)[23,24].

$$\begin{bmatrix} {}_{i}\boldsymbol{Va}_{x(1)} & \cdots & {}_{i}\boldsymbol{Va}_{x(n)} \\ {}_{i}\boldsymbol{Va}_{y(1)} & \cdots & {}_{i}\boldsymbol{Va}_{y(n)} \\ {}_{i}\boldsymbol{Va}_{z(1)} & \cdots & {}_{i}\boldsymbol{Va}_{z(n)} \end{bmatrix} = {}^{\boldsymbol{Va}}\boldsymbol{T}_{\boldsymbol{Gl}} \times {}^{\boldsymbol{Pr}}_{i}\boldsymbol{T}^{-1}_{\boldsymbol{Gl}} \times {}^{\boldsymbol{Pr}}\boldsymbol{T}_{\boldsymbol{Im}} \times \begin{bmatrix} {}_{i}\boldsymbol{Im}_{x(1)} & \cdots & {}_{i}\boldsymbol{Im}_{x(n)} \\ {}_{i}\boldsymbol{Im}_{y(1)} & \cdots & {}_{i}\boldsymbol{Im}_{y(n)} \\ {}_{i}\boldsymbol{Im}_{z(1)} & \cdots & {}_{i}\boldsymbol{Im}_{z(n)} \end{bmatrix} \quad \textbf{(Equation 3)}$$

Note: This shows forward mapping of 2D US images in the ***Va*** according to the orientation and position of the images in the *Va* coordinate system. In short, the positions of all pixels of one image ($\boldsymbol{Im}_{xyz(1:n)}$) at time instance (i), are simultaneously mapped forward into the voxel array. The bin-filling procedure only fills the addressed voxels, leaving the non-addressed voxels empty (*i.e.*, black).

Note: ${}^{\boldsymbol{Pr}}_{i}\boldsymbol{T}^{-1}_{\boldsymbol{Gl}}$ indicates the inverse of the previously described transformation matrix (*i.e.*, a ***Pr*** to ***Gl*** transformation matrix).

3.4.1.4. Using a custom script identify gaps inside the voxel array (*i.e.*, black voxels). Take the following steps by using binary image processing:

3.4.1.4.1. Create a bin-filled binary voxel array in which all filled voxels are labeled. Use binary image dilation and erosion, with the same size structuring-element, to label all relevant voxels (*i.e.*, grey-valued voxels) inside the scanned region. Detect gaps by subtracting the bin-filled binary voxel array (with gaps) from the relevant voxels (no gaps).

Note: Subsequent dilation and erosion operations are image-processing steps to complete the binary images. By performing these steps one after another, the outside boundaries remain while the gaps inside are removed.

3.4.1.5. Fill the identified gaps using an "inpaint procedure" and surround grey-valued voxels[36].

Note: This inpaint technique can be used to: "fill gaps with a smooth interpolant based upon minimizing the sum of squares of the second derivative at each labeled voxel measured by finite differences on the grid"[36].

3.4.1.6. Equalize the voxel dimensions of the ***Va*** by 'bicubic' interpolation and save the voxel array as a stacked .tiff image (3DUS image).

3.5. **Multiple sweeps reconstruction**

3.5.1. Reconstruct all individual sweeps (described in section 3.4) covering one larger ROI according to the same ***Va*** coordinate system to merge multiple sweeps.

3.5.2. Create a new ***Va*** coordinate system, sized to accommodate all individual reconstructed sweeps.

3.5.3. Place the individual ***Va***'s step-by-step into the larger ***Va***. If a voxel is already assigned by another ***Va***, this voxel will only be overwritten if the new voxel has a grey value ≥ 10 on an 8-bit scale, otherwise the new voxel grey value is discarded.

4. Measurement of Variables of Muscle Morphology

4.1. Use the Medical Interaction Toolkit[37] (MITK) to load the 3DUS image and retrieve the coordinates of the origin, insertion, and distal end of the muscle belly.

4.1.1. After loading the 3D image, set the slicing to 'Coupled crosshair rotation'. Align the axes with muscle or bony structures to precisely retrieve the coordinates.

Note: MITK is preferred over other 3D imaging analysis software for the assessment of anatomical points, because it allows fast and interactive voxel array slicing in any direction ("Coupled crosshair rotation"), facilitating the identification procedure.

4.2. In order to measure muscle volume, use MITK to identify the muscle belly boundaries between the origin and distal end of the muscle belly. Use the built-in MITK segmentation to manually segment the multiple anatomical cross-sections evenly, distributed along the muscle belly length (**Figure 3A**).

4.2.1. Open the 'segmentation tool' and create a 'new segmentation'. Start segmenting the muscle boundaries identified in a cross-section halfway along the muscle belly. Press 'A' on the keyboard to add a manual segmentation and draw by pressing the left mouse button and moving the cursor following the muscle boundaries. Press 'S' to remove parts of segmentation.

4.2.2. Press the key corresponding to the last selected mode (*i.e.*, 'A' or 'S') to move the crosshair to other cross-sections along the muscle belly. Repeat step 4.2.1 to segment the new selected cross-section. Repeat this step for at least 6 times, before continuing to the next step.

4.2.3. Set 'Interpolation' to 'enable', review the proposed segmentations of the muscle boundaries (yellow lines) in all cross-sections along the muscle belly length.

4.2.4. Add additional segmentations in the cross-sections in which the proposed interpolated segmentation (yellow line) does not match the muscle boundary in the image. Repeat step 4.2.2.

4.2.5. Press the 'Confirm for all slices' button and select the plane in which the segmentations

were made.

4.2.6. Save the binary volume as a nearly raw raster data (NRRD) file and calculate the labeled volume size using a custom-script.

4.3. Find the orientation of the mid-longitudinal fascicle plane of the muscle belly, containing the full length of fascicles (**Figure 3A**)[38].

Note: The mid-longitudinal plane is defined by three points. The origin and distal end of the muscle belly are the first two points. The third point is found in an anatomical cross-sectional image halfway between the origin and distal end of the muscle belly. Within this anatomical cross-sectional image, the midpoint between the first two points projected onto the tangent of the distal aponeurosis yields a third point that together with the origin and distal-end of the muscle belly defines the mid-longitudinal plane.

4.4. From the mid-longitudinal plane, measure the fascicle length at a pre-defined standardized position between the origin and distal end of the muscle belly (*e.g.*, 50%). Segment the muscle boundaries. Place a line halfway and rotate this line until it matches the direction of the underlying fascicles. The intersection of this line with the muscle boundaries represents the estimate of the fascicle length (**Figure 3B**).

Note: Previously, it proved necessary to take into account the, sometimes curved, orientation of the distal aponeurosis[38], as seen in an anatomical cross-sectional image (**Figure 3B**), taken halfway between origin and distal end of the muscle belly.

[Place figure 3 here]

REPRESENTATIVE RESULTS:

The described 3DUS technique was used to collect morphological data of the GM and VL in four male human cadavers, age at death 76.8 ± 7.9 years (mean ± SD). The cadavers were obtained via the donation program of the Department of Anatomy and Neuroscience of the Vrije Universiteit Medical Center (VUmc), Amsterdam, the Netherlands. The bodies were preserved using an embalming method aimed at maintaining the morphological features of the tissue[39].

Prior to dissection, a 3DUS image was made of the GM and VL according to the methodology described. During dissection, skin, subcutaneous tissue, and fasciae overlaying the GM and VL were removed. A mid-longitudinal section was cut, taking the orientation of the distal aponeurosis into account. Using a caliper, the fascicle length was measured, halfway between the origin and the distal end of the muscle belly. Subsequently, after tenotomy, the muscle belly was dissected and submerged in a calibrated water column. Using ImageJ, the volumes were measured on photographs of the water column with and without the muscle belly, and muscle volume was calculate from the difference[40]. Fascicle length and volume were measured three times and the mean and standard deviation values were calculated. Criterion validity between the 3DUS method and dissection measurements was tested using a Pearson's correlation for

average fascicle length and muscle volume. Intra-rater reliability of the 3DUS method derived fascicle length and volume measurements was quantified using a two-way mixed model intra-class correlation coefficient ($ICC_{3,3}$)[41], and after logarithmic transformation of the data, the coefficient of variation (CV) was calculated. The validity of fascicle length, as well as muscle volume, was confirmed with significant and high correlations ($r = 0.998$, $p < 0.01$ and $r = 0.985$, $p < 0.01$, respectively). Intra-rater reliability of the 3DUS method derived measurements of fascicle length and volume was high ($ICC_{3,3}$ 0.983, CV 7.3%, and $ICC_{3,3}$ 0.998, CV 5.4%, respectively). It is concluded that the 3DUS approach presented is a valid and reliable tool for volume and fascicle length assessment of human VL and GM (**Table 1**).

[Place table 1 here]

TABLE & FIGURE LEGENDS:

Figure 1: Schematic of the 3DUS algorithm. (**A**) Motion Capture (MoCap) system is used to track a cluster of markers rigidly connected to the ultrasound probe, within the global coordinate system (***Gl***). Synchronization of MoCap and ultrasound data is accomplished making use of an artifact (arrow) introduced by Optotrak triggered piezo crystal. (**B**) The position and orientation of the ultrasound image coordinate system (***Im***) is calculated relative to the probe coordinate system (***Pr***) by identifying a known point within the ***Pr*** and ***Im***. For this purpose, a custom-designed phantom is used filled with water, holding a crosswire (*i.e.*, two submerged crossing wires) at a known position within the phantom coordinate system (***Ph***). (**C**) With a series of transformations, this known point is calculated in the ***Pr***. (**D**) With a complete series of known transformations, images from the ***Im*** can be transformed into any voxel array coordinate system (***Va***).

Figure 2: Schematic of the experimental setup and sweeps of the ultrasound probe over the target muscles (*m. gastrocnemius medialis* (GM) setup and *m. vastus lateralis* (VL) setup). (**A**) Specific joint configurations of the subject for the two experimental conditions. The objects displayed in green are adjustable to set the position and orientation of the limbs. Arrow indicates an extendable rod that is used to fix the footplate angle. (**B**) Path of multiple sweeps of the ultrasound probe over the regions of interest. The blue arrows represent single sweeps over the region of interests. Left: sweeps over the GM; Right: sweeps over the VL.

Figure 3: Schematic of the 3DUS analysis. (**A**) Identification and segmentation of the target muscle boundaries in an anatomical cross-sectional image halfway along the muscle belly. The solid green line represents the orientation of the mid-longitudinal plane (*i.e.*, oriented perpendicular to the orientation of the distal aponeurosis (blue dotted line). (**B**) Measurement of the fascicle length is performed within the mid-longitudinal fascicle plane. The red transparent region is segmented by identification of muscle boundaries. A dotted yellow line is placed halfway on the muscle belly and rotated until it matches the direction of the underlying fascicles. The intersections of this line with the proximal and distal aponeuroses (connected by thick solid yellow line) represent the estimate of fascicle length. The solid green line represents the position and orientation of the anatomical cross-sectional plane. Top: GM (*m. gastrocnemius medialis*)

and bottom: VL (*m. vastus lateralis*) muscle. The white squares for scale represent 1 cm x 1 cm.

Figure 4: Variation and quality of reconstructed anatomical cross-sectional 3DUS images of the quadriceps muscle halfway along the thigh. (**A**) Example of a male human cadaver shows an image of an atrophied state at death (death age: 81 years). Identification of the boundaries of individual heads of the quadriceps muscle is difficult. (**B**) Example of a sedentary male (aged 30 years). (**C**) Example of a male athlete rower (aged 30 years). The white squares for scale represent 1 cm x 1cm.

Table 1: Cadaver Validation Data. C# is Cadaver number, GM is *m. gastrocnemius medialis*, VL is *m. vastus lateralis*. "Dissection"shows results from the cadaver dissection, and "3DUS" shows the results from the 3DUS image analysis of the cadavers.

DISCUSSION:

A valid and reliable 3DUS technique is presented that allows for the fast analysis of morphometric variables of skeletal muscles. Different 3DUS approaches for soft tissue imaging have been available for approximately a decade[42,43], however the 3DUS approaches are still not used commonly. MRI is a 'gold standard' for estimation of *in vivo* muscle volumes (*e.g.*,[16–20]). MRI validity has been tested and confirmed in studies comparing either phantoms or cadaveric organs of known volume to MRI-based volume estimates[44,45]. However, MRI availability for research is limited and scans are time consuming and costly. In addition, experimental subject postures are limited by the bore seize of the MRI scanners. Typical MR images generate insufficient contrast to perform measurements of variables of muscle geometry (fascicle lengths and angles). However, 3D muscle geometry can be assessed also using MRI by using additional techniques, *e.g.*, DTI technique[21]. Similar to MRI, US imaging provides adequate distinction at interfaces between different types of tissues (*i.e.*, visible within US images), providing a valid modality for soft tissues volume assessment[1,30,44,46–49]. In contrast to MRI, 3DUS images have sufficient contrast to perform analysis on both volume and muscle geometry from the same measurement.

In addition, the technique presented allows combining images of multiple sweeps into one array, for the study of larger muscles. This new 3DUS method provides a potential tool for clinical assessment of muscle morphology. This method can be used also for imaging soft-tissue structures other than muscle (*e.g.*, tendons, internal organs, arteries).

Modifications to Improve Offline Processing Time:

Modifications of the 3DUS approach were mainly aimed at improving processing time and measuring larger muscles. The offline processing time of a 3DUS image depends on voxel array settings, sampling frequency, size of ROI, duration and speed of the sweep, number of sweeps, and the used workstation. Previously, a reconstruction time of ≈ 2 h was necessary for reconstructing only one sweep yielding 750 US images (30 s at 25 Hz)[15,25,30]. With the present 3DUS method, the same sweep takes only 50 s reconstruction time (improving the 'offline' processing time by ≈ 99%). This improvement can be explained by the enhanced filling algorithm that utilizes large vector operations to fill the voxels frame-by-frame, instead of pixel per pixel

and increased random access memory (RAM) of workstations to construct larger voxel arrays. With the new 3DUS approach, a typical reconstruction representing a sweep length of 30 cm at a speed of 1 cm/s, with a target voxel size of 0.2 x 0.2 x 0.2 mm^3 and a sampling frequency of 25 Hz, takes the following time to reconstruct:

a. Approximately 10 s to identify the synchronization pulse and select relevant US images.
b. Approximately 120 s to determine the calibration transformation matrix ($^{Pr}\boldsymbol{T}_{\boldsymbol{Im}}$).
c. Approximately 10 s for the bin-filling stage.
d. Approximately 30 s for executing the gap-filling steps.

In total, taking 170 s. Note, step b only needs to be performed once, assuming a rigid connection of the MoCap markers to the probe, leaving 50 s for the reconstruction of a single sweep. Combining two single sweep reconstructed voxel arrays takes approximately 10 s.

Limitations and Critical Steps:

There are several 3DUS imaging aspects that should be taken into account:

i. **US image quality:** Higher spatial resolution of 2D US images provide more pixels to be placed within the voxel array. This would allow the voxel dimensions to decrease, leading to higher voxel density. Several currently available ultrasound machines use spatial compounding to reduce the noisy granular texture, allowing for better artifact-free distinction of the interfaces of tissues. Another option to reduce speckle is edge enhancement. However, it should be noted that this approach is not desirable, since it deforms the image in an attempt to create distinct interfaces, thereby distorting the true anatomical position of the interfaces.

ii. **MoCap accuracy:** Pixels can only be accurately placed into a voxel, if the position sensor accurately quantifies the coordinates of the probe. With an increase in image resolution, MoCap accuracy becomes more important. The presented 3DUS setup works best with a voxel dimension of 0.2 x 0.2 x 0.2 mm^3, using a MoCap system with an accuracy of 0.1 mm, providing ample accuracy to reconstruct the 3DUS voxel array.

iii. **Sample frequency:** The lowest temporal resolution of either the US images or MoCap data stream determines the sample frequency. This affects the sweep time or the voxel array settings. For instance, doubling the sample frequency from 25 to 50 Hz allows a sweep to be performed in half the time. Alternatively, not changing the sweep speed, provides more images to fill the voxel array, leaving fewer gaps to be filled and thereby potentially increasing the voxel array resolution. However, increasing the voxel array resolution, without increasing the sampling frequency, requires a slower scan, which will increase the potential of movement artifacts.

iv. **Image reconstruction time:** Fast reconstructions require a powerful workstation with sufficient available RAM. In addition, reconstruction time varies largely based on the voxel array volume and complexity of the gap-filling process.

v. **Experimental protocol:** Standardization of the experimental protocol, as exemplified in the present study for the VL and GM, is essential for comparison of morphological measurements (*e.g.*, fascicle length, fascicle angle, muscle belly length, tendon length, aponeurosis length) between subjects and monitoring within subjects in longitudinal studies. However, note that the morphology assessed at rest may alter during muscle activation. For example, for the VL experiment, the knee extensor morphology during maximal contraction may demonstrate a high pennation angle and shorter fascicles in 60 ° knee flexion, in comparison with morphology at rest[50]. In certain conditions (*e.g*., spasticity), electromyography (EMG) may be used to verify resting muscle activity levels during examination.

vi. **Probe pressure and tissue deformation:** If ample ultrasound gel is applied on the ROI, the amount of pressure to remain for full contact between probe and the skin is limited. As guidance, we advise that scanning a ROI should feel like hovering over the skin, and pressure should only be applied to keep in contact with the gel and thereby the skin. However, slight tissue deformation may be inevitable, even with a generous amount of ultrasound gel. Probe size and a curved ROI affect the required amount of pressure or gel used. Larger probe size and a more curved ROI require more pressure and/or more gel, than smaller probes with a similar curved ROI. Another possible solution is to discard the reverberation (*i.e.*, non-skin-contact) region of the US images. In addition, tissue deformation is most likely to occur in the first tissue layers, such as skin and subcutaneous adipose tissue layers. Note that subjects with little to no subcutaneous adipose tissue are therefore more prone to adverse effects of pressure. In addition, the tissue deformation occurs most likely at the center of the probe, which is typically not the region of overlap with other sweeps.

vii. **Imaging and anatomical knowledge:** Another important consideration in using any imaging modality is that the knowledge of both the anatomy and the imaging modality is essential to obtain meaningful interpretation. Anatomical variation between subjects and image artifacts needs to be recognized and taken into account in the identification process of anatomical structures. Even with healthy and/or well developed muscles, clear identification can be difficult because it requires anatomical knowledge to differentiate between different components of one muscle or between muscle groups[51]. However, in atrophied muscle (*i.e.*, elderly, in the case of pathology, or a cadaver), the clear identification is even more complicated because of a smaller size and decreased image contrast, and therefore less distinct tissue interfaces (**Figure 4**). We believe that without prior anatomical knowledge, we would have been limited in making correct judgements in designing this 3DUS approach and in performing the 3DUS measurements. For example, for GM experiments, different footplate angles do not necessarily cause expected changes in muscle tendon complex lengths, due to deformation within the foot[7]. Also detailed anatomical information on curvature of the distal aponeurosis was essential for an adequate selection of the mid-longitudinal plane in all subjects[38].

[Place figure 4 here]

Future Applications:

The 3DUS approach provides an imaging tool that can be used for various purposes and settings in sports and clinics. In clinical interventions effectiveness is related to the physical fitness level[52]. Using 3DUS for monitoring patients who are at risk of losing muscle mass is important (*e.g.*,[53–55]) and potentially allows for adjustment of the treatment. Another potential application of 3DUS lies in monitoring the morphological adaptation of muscle in response to intervention (training) and/or injury.

This protocol described a cost- and time-effective method of measuring soft tissue structure of the human body based on freehand 3DUS sweeps. Moreover, assessment of the meaningful morphological parameters of *m. vastus lateralis* and *m. gastrocnemius medialis* proved to be valid and reliable.

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DISCLOSURE:
The authors have nothing to disclose.

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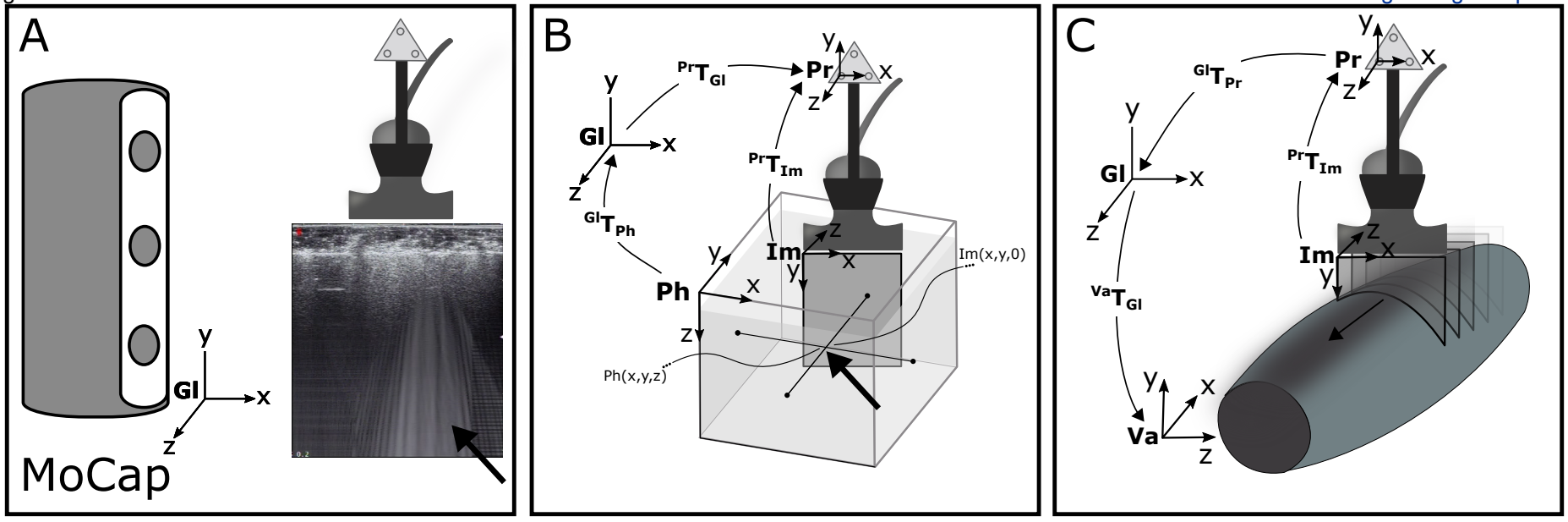

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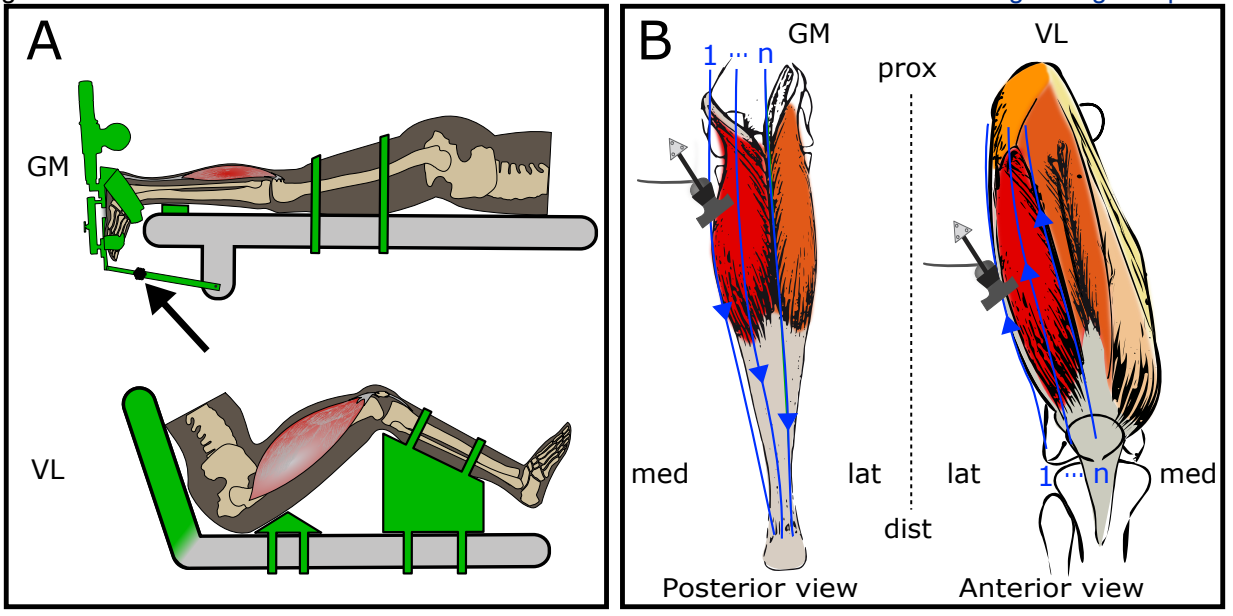

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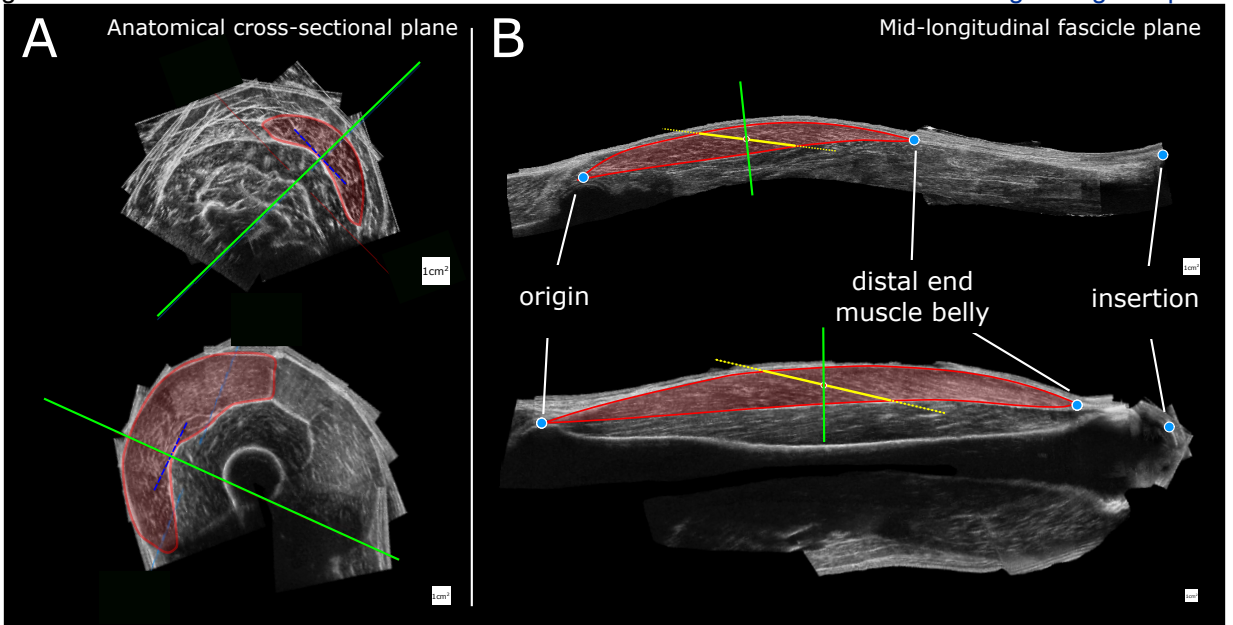

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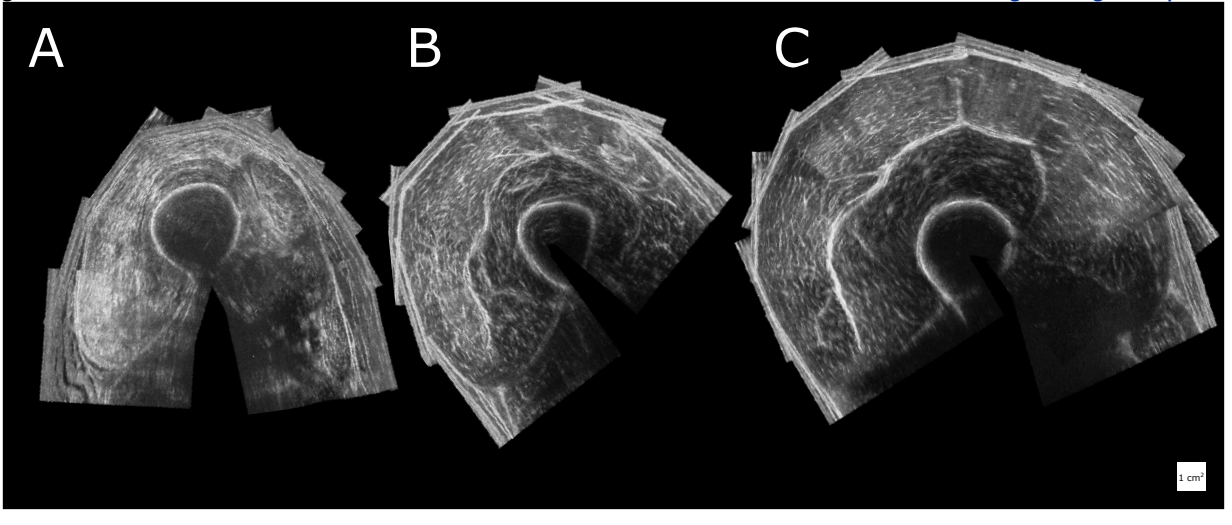

Table 1

Cadaver	Muscle	Volume(ml)		Fascicle length(cm)	
		Dissection	3DUS (mean±SD)	Dissection	3DUS (mean±SD)
C1	GM	122	121±10	4.1	4.2±0.4
	VL	274	285±13	7.5	7.5±0.3
C2	GM	131	133±5	4.4	4.5±0.3
	VL	380	366±4	8.7	7.9±0.1
C3	GM	143	138±4	3	3.4±0.2
	VL	539	500±16	7.6	8.3±0.9
C4	GM	106	106±7	4	3.6±0.2
	VL	292	290±18	10.3	10.1±1.4

Table of Materials/Equipment

Click here to download Table of Materials/Equipment JoVE_Materials_3DUS.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ultrasound device (Technos MPX)	Esaote, Italy	NA	
Linear array probe (12.5 Mhz, 5 cm)	Esaote, Italy	NA	
Workstation (HP Z440)	HP, USA	http://www8.hp.com/us/en/workstations/z440.html	
Framegrabber (Canopus, ADVC 300)	Canopus, Japan	ADVC 300	
Motion Capture System (Certus)	NDI, Canada	http://www.ndigital.com/msci/products/optotrak-certus/	
Synchronisation device	VU, NL	Contact corresponding author	
Calibration frame	VU, NL	Contact corresponding author	
Thermometer	Greisinger, Germarny	GTH 175/PT	
Examination table	NA	NA	Any examination table
Inclinometer	Lafayette instrument, USA	ACU001	
Adjustable Footplate	VU, NL	Contact corresponding author	
Torque wrench	VU, NL	Contact corresponding author	
Extendable rod	VU, NL	Contact corresponding author	
Goniometer (Gollehon)	Lafayette instrument, USA	1135	
Triangular shaped beam	NA	NA	Made out a piece of stiff foam
Lashing straps	NA	NA	Any lashing strap
Surgical skin marker	NA	NA	Any surgical skin marker

Ultrasound transmission gel	Servoson	NA	A sticky gel type is recommended

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Article Title: 3D ULTRASOUND IMAGING: FAST AND COST EFFECTIVE MORPHOMETRY OF MUSCULOSKELETAL TISSUE

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Dear editor,

We would like to thank the editor and reviewers for investing time in reading the paper and formulating their comments, which we felt were useful for improving the manuscript. We have done our utmost to accommodate the reviewers' concerns regarding our manuscript*: "3D ultrasound imaging: fast and cost effective morphometry of musculoskeletal tissue".* Our motivation for the adjustments is given in the responses to the comments below.

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response:
The manuscript has been thoroughly proofread multiple times. We have done our utmost best to free the manuscript of spelling or grammatical errors.

• **Formatting:** Please use 12 pt font and single-spaced text throughout the manuscript. Please adjust the line spacing to "Single" with 0 pts before and after. Please adjust the page margins to 1 inch on each side, and delete headers and footers. Please review the latest instructions for authors for complete formatting requirements by following the link http://www.jove.com/files/Instructions_for_Authors.docx.

Response:
We have now changed the manuscript according to the latest formatting instructions.

• **Abstracts:**
1) Please revise the Long Abstract to focus more on the protocol being presented rather than the results obtained and provides a detailed overview of the technique and a brief summary of its advantages, limitations and applications. The Long Abstract must clearly state the goal of the protocol.. The Long Abstract must clearly state the goal of the protocol. For example, "This protocol describes…"
2) Please remove the superscript citations from the long abstract, and reorder the references accordingly.

Response:
We have revised the Long Abstract. We now describe the goal of the protocol instead of the results. We have also removed the superscript citations.

• **Introduction:** Please ensure that your Introduction includes the following: 1) A clear statement of the overall goal of this method 2) The rationale behind the development and/or use of this technique 3) The advantages over alternative techniques with applicable references to previous studies 4) Description of the context of the technique in the wider body of literature 5) Information that can help readers to determine if the method is appropriate for their application.

Response:

In line with the changes made in the long abstract, the introduction has been revised to be more focused on the protocol.

•**Protocol Language:** The JoVE protocol should be almost entirely composed of short steps (2-3 sentences each) written in the imperative tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please re-write your ENTIRE protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
1) For example, Section 1 of your protocol is a listing of instruments without any stepwise description of actions, this needs to be rewritten appropriately.

Response:
We have changed the protocol according to the instructions.

2) Please review the following example JoVE publications for ideas on protocol language style (please disregard the text formatting):
a) https://www.jove.com/video/55733/subsurface-defect-localization-structured-heating-using-laser
b) https://www.jove.com/video/55810/switchable-acoustic-optical-resolution-photoacoustic-microscopy-for

3) Lines 309- 328 should be part of the results section.

Response:
These lines have been transferred to the results section.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action. For all software-based steps, please mention what buttons are clicked on in the software, or which menu items need to be selected.

Response:
We have changed the protocol into an imperative language style and added more details I to the protocol steps.

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Response:

changed as suggested.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

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o Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
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o Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
o Notes cannot be filmed and should be excluded from highlighting.
o Please bear in mind that software steps without a graphical user interface and calculations cannot be filmed.

Response:
the protocol text is now within the 10 page limit, we have highlighted 2.5 pages text as parts that should be visualized.

• **Results:** Please expand the results section to describe how these results show the technique, suggestions about how to analyze the outcome etc. Please specify the format of quantitative results presented, for example, was mean ± standard deviation used? Please mention the statistical tests performed.

Response:
In order to improve the representative results, we have added more details on how to analyze the 3DUS images.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please rewrite the discussion so that it covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. Also, The JoVE format does not include a "Conclusion" section. Please move the text in this section to the Discussion.

• **Figures 3,4 :**: Please provide scale bars, and define them in the figure legend.

Response:
We have added scale bars and defined then in the legend.

• **Tables:**: Please upload each table as an individual Excel file.

Response:
We have now uploaded the tables as individual Excel files.

• **References:** Please move the in-text http weblinks into the reference list, and use superscripted citations (e.g. Lines 314, 323).

Response:
We have all the http weblinks into the reference list.

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2) Please remove the word "optotrax" from Fig 1A.

• Please define all abbreviations at first use.

• Please use standard abbreviations and symbols for SI Units such as μL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

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Response:
We have taken the editorial comments into account changed the manuscript accommodating the above mentioned instructions and suggestions. (See the tracked

changes for all the changes made)

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
Thank you for letting me review this paper. It describes a 3D Ultrasound methods for deriving muscle volumes and architectural parameters which is exemplified using the medial gastrocnemius and vastus medial muscles. First off, I congrat the authors for their very nice work which builds upon a series of past high quality investigations. The authors state that they made considerable advancements in processing time of their analysis technique which will probably attract a lot of attention from researchers and clinicians in future. I encourage the author's to put the code open source, too.

Response*:*
Thank you for your appreciation of our technique and the constructive comments.

General comments:
-Is there any need to control for muscle activity during the assessments? How do the authors usually handle this?

Response:
Yes, in order to obtain reproducible estimates of muscle morphology, muscle activity should be standardized and should not vary during the scanning procedure. Previous studies have shown that in a supported condition normalized EMG activity is typically well below 10%.
We have now mentioned how we handle standardization in the discussion section (Line: 455-463).
Line 462: " In certain conditions (e.g. spasticity), EMG may be used to verify resting muscle activity levels during examination".

-Comment on the us probe (e.g. length) used and needed. Do you have any recommendation?
-How do you control for the pressure applied during the sweeps? Any special suggestions?
-Do you recommend to use any specific geltype material, e.g. sonokit, to accommodate for the rounded surface shapes of the calf or thigh? Would that decrease the amount of sweeps that are usually required? Would this also have the potential to reduce the amount of sweeps that needs to be taken and necessitate less time to stay still?

Response:
We have used a 5 cm linear probe, which is described in the methods section. Indeed with a larger probe it is possible to scan a larger tissue volume within one sweep and depending on the muscle volume this may reduce the number of sweeps and the risk of movement of the subject.
However, a drawback of a larger probe is that more central pressure is needed on a rounded surface to make full skin contact, which potentially causes tissue deformation. Using a large amount of gel may decrease the amount of pressure needed to make skin

contact. We prefer using a sticky type of gel to create a layer that sufficiently accommodates the flat head of the probe.

We now have mentioned this in the method section and in addition the probe size and pressure issue are discussed in the "consideration section" of the discussion (Line: 464-477).

Line 464-477: "If ample ultrasound gel is applied on the ROI, the amount of pressure to remain full contact between probe and the skin is limited. As guidance, we would advise that scanning a ROI should feel like hovering over the skin, pressure should only be applied to keep in contact with the gel and thereby the skin. However, slight tissue deformation may be inevitable, even with a generous amount of ultrasound gel. Probe size and a curved ROI affect the required amount of pressure or gel used. Larger probe size and a more curved ROI, requires more pressure and/or more gel, than smaller probes with a similar curved ROI. Another possible solution would be to discard the reverberation region of the US images. In addition, tissue deformation is most likely to occur in first tissue layers, such as skin and subcutaneous adipose tissue layers. Note that subjects with little to no subcutaneous adipose tissue are therefore more prone to adverse effects of pressure. In addition, tissue deformation occurs most likely in the center of the probe, which is typically not the region of overlap with other sweeps. An alternative solution could be to attach a sound permeable flexible layer to the probe to absorb pressure and thereby further limit tissue deformation."

-What is the procedure in case of motion occurring during sweeps?

Response:

In case of limb motion during the US imaging procedure, the procedure is repeated. Typically we take 2 sets of sweeps and reconstruct two 3DUS voxel arrays. If the first 3DUS voxel array contains unintended motion artefacts we use the second 3DUS voxel array.

We now have addressed this issue in the 3D ultrasound examination section (3.3):
Line 224:"Visually check for movement of the subject during the examination, if the subject moves abort the sweep and repeat from step 3.2.6."

-Would you recommend a higher sampling frequency in patient populations that are prone to movements during tests?

Response:

Yes, we prefer using high sampling frequencies, as this will reduce the imaging time and chance to have adverse (leg) movements during the imaging procedure. Currently, ultrasound sampling frequency is the limiting factor in our setup. By increasing ultrasound sampling frequency to 50 Hz, examination time could be reduced by more than 50%. This would drastically reduce the likelihood of adverse movements to occur during the examination.

This consideration is now mentioned in the discussion (line 444-451):
": The lowest temporal resolution of either the US images or MoCap data stream determines the sample frequency. This affects the sweep time or the voxel array settings. For instance; doubling the sample frequency from 25 to 50 Hz; allows a sweep to be performed in half the time. Alternatively, not changing the sweep speed, provides more images to fill the voxel array, leaving fewer gaps to be filled thereby potentially increase the voxel array resolution. However, increasing the voxel array resolution, without increasing the sampling frequency, requires a slower scan, which will increase the potential of movement artefacts."

-If I understood correctly, there was no need to put any marker cluster on the subjects?
-Would you recommend to use markers on the subjects to capture their movement during tracking?

Response:
Typically, in our experiments ankle, knee and hip postures are fixed and therefore potential movements during the assessment are limited. Rigidly connected cluster markers may hinder the 3DUS scanning procedure and may be subjected to errors related to tissue deformation during limb movement. If these difficulties could be overcome, it could be a helpful addition to online monitor adverse movements. In this protocol article we will not recommend nor discourage using markers because using markers adds both new possibilities and also limitations beyond the scoop of this work.

-How well does your technique work for tendinous tissue, e.g. the patellar or Achilles-tendon?

Response:
Tendon length and thickness can be measured with 3DUS, however we did not describe it in this protocol.

-How many persons do you need for running the experiment? Sounds like one is running the mocap software, while another one is handling the probe?

Response*:*
The protocol contains many steps, and can be run by a single examiner. However, during the experiment there are quite some processes that need to be monitored. With two persons, one can monitor the MoCap system while the other can handle the probe. Since this is not a constraining factor for the technique, we have not mentioned this in the protocol.

Abstract:
Maybe put voxel into plane language

Response:
Voxel is derived from the words volume (vox) and element (el).
We have made the following changes to the manuscript:

Line 59: "…images and positioning them into a volume element (voxel) array."

Provide SEM values in units of measurement, next to ICC
Later in the paper also the smallest detectable difference could be mentioned

Response: *In addition to the ICC, the CV provides a measure of the standard error. The SEM depends on the number of observations and as these are low in the current study (proof of principle) we argue that the CV provides a better indication of the measurement error.*

Intro:
l. 70 Put voxel, array in plane language at least at first use during the paper

Response:
We have changed voxel at the first use (line 59) into volume element array. Thereafter, we consistently use voxel array, as we consider this to be the most plane language.

We have added the abbreviation after volume element array (voxel array).

l. 75 get into more detail about dimensions

Response:
Dimension should be morphology. We have changed the text accordingly.

l. 77 specify large anatomical structures

Response:
Large anatomical structures refers to muscles.
We have changed anatomical structures into muscles.

l. 86 I guess the ultrasound videos could also be stored directly on the computer that runs the motion capture system, is that correct?

Response:
Yes, this is correct.
We changed the manuscript instrumentation section to explain how the instruments are connected and what is stored where.

l. 87 Despite the cluster on the probe there are no further clusters on the subjects, is that correct?

Response:
Yes, this is correct. Visual inspection is used to detect subject movement during the sweeps.

l. 96 Maybe use plain language for phantom

Response:

Phantom refers to a custom-made calibration frame..
We have changed the first use of phantom into calibration frame to describe what the phantom is.

l. 105 Can you describe the artefact in detail? The related picture in the figure is not so clear.

Response:
The artefact resembles a strong reflection (echo). However, the perceived sound did not originate from the probe, instead it was produced by a single piezo crystal sending sound waves towards the transducer, yielding an instant echo response in the image. We changed the manuscript text in the instrumentation section describing the details of the synchronization device and the artefact. We have added a description of the artefact. The text now reads as follows: Line 105: "Activate MoCap data acquisition, this automatically initiates the synchronisation device (i.e. momentarily activated piezo crystal, sending a soundwave towards the transducer) which creates a distinct artefact in the US image at the instance of MoCap system initiation. (Fig. 1A, Arrow)"

l. 111 How many pictures do you usually need for a proper calibration

Response:
On average, ≈35 clear identifications (i.e. pictures/images) of the cross-wire are used per calibration. In order to get enough identifications, the probe is translated and rotated around the crosswire for 40 seconds. Sampling at 25 Hz yields 1000 images. From this 1000 images each 10^{th} image is viewed ((i.e. 100 images in total), from these 100 images ≈35 are selected in which the crosswire is clearly visible. Position and orientation of the probe between these 35 images need to be as dissimilar as possible, for this reason we use every 10^{th} image.
We made the following changes to the manuscript:
*Line 109: "Submerge the head of the US probe (**Pr**) in the water and translate and rotate the US probe for 40 s (sampling at 25 Hz) in all directions, maintaining visibility of the crosswire in the US images (**Im**)"*

l. 120 step 2.1.13 is missing (at least the header…)

Response:
This should be step 2.10, we changed the manuscript accordingly.

Experimental protocol
Maybe provide a real picture in addition to the schematic drawings of Fig 2 A and B. Also a picture of the custom-made foot plate would be fine but that is probably very well shown in the video?

Response:
Thank you for the suggestion. Schematic drawings are preferred to emphasize key elements of our setup. In the video the true setup will be visible, including the custom-made foot plate.

l. 176 the marking of the roi on the skin is not clear, do you draw a circumference?

Response:
We do not draw a circumference. Instead, only the medial and later boundaries are marked. The sweep starts ≈5 cm before and ends ≈5 cm after the ROI. During the imaging, it has to be checked whether the medial and lateral boundaries of the muscle are completely visible in the 2D US image.
We have changed the following sentence in the manuscript to make it more clear:
Line 202: "Use ultrasound to identify the medial and lateral muscle border, mark the medial and lateral border using a surgical pen."

l. 178 What do you do if leg movements occur? How much movement is really disturbing? Can you actually fully prevent such motion, e.g. in pediatric patients?

Response:
If leg movement is detected, the scanning procedure will be repeated. We typically take more than one sequence of sweeps.
We have made the following changes in the manuscript:
Line 224:"Visually check for movement of the subject during the examination, if the subject moves abort the sweep and repeat from step 3.2.6."

I was wondering if it would be reasonable to track the motion of the limbs, too.

Response:
It is reasonable and important to use a method to detect unwanted conditions (i.e. muscle activation or misplaced images). If motion tracking of the limbs is possible without hindering the scanning procedure, this would be a good addition.
We have made adjustments to the "consideration section" in the discussion to address this issue.

l. 187 What was your strategy to control for the speed of probe motion. 1 cm/s is rather slow, do you recommend verbal counting or is it just very slow.

Response:
For novice examiners we advise to get a feeling for the speed and the consistency of speed by using a clock.

Based on your experience, how many sweeps do you usually perform, e.g. depending on the muscle or size of the patients (e.g. in an adult or child gastrocnemius or vastus)

Response:
The number of sweeps is determined by the width of the probe and the width of the ROI. Typically, with a probe width of 4 cm and a 12 or 18 cm ROI width, 5 or 7 sweeps are needed, respectively, to cover the ROI including the borders.
We have added the response to this question as a note to the manuscript protocol section.

l. 196 How do you online check that the whole region of interest has been covered?

Response:
For this purpose we use the traces made in the gel.
We changed the text in the manuscript to clarify how we did this.
Line 238 and 247: "Use the trace in the gel of the previous sweep to guide the next sweep, slightly overlapping (0.5 cm) the previous swept area."

l. 227 Can you describe the forward mapping and bin filling in plain language?

Response:
Pixel-grey values of the ultrasound images are assigned to the nearest voxels in the voxel array.
The manuscript has been adapted to provide more detail on this manner (line 267).
Line 267: "Voxels in the Va are assigned with pixel grey-values from the ultrasound images"

l. 239 Can you describe dilation and erosion in plain language?

Response:
Subsequent dilation and erosion operations are image processing steps used on binary images to complete lattices. By performing these steps one after another, outside boundaries remain while gaps inside are removed.
We have changed the manuscript by adding the above response as a note to the manuscript.

l. 276 What does the MITK stand for? Maybe I missed it.

Response:
MITK stands for Medical Interaction Toolkit. ***We have now spelled this out in full in the manuscript.***

l. 283 The boundaries of the muscle in the 'added' cross-sections are manually drawn, is that correct? Can you describe the process in further detail? How many cross-sections do you usually analyze to gain an estimate of the muscle volume? How well does the automated segmentation work?

Response:
All segmentations are performed manually. Based on the manual segmentations, MITK interpolates the segmentations of the anatomical cross-sections. Based on the complexity of muscle boundaries, additional manual segmentations may need to be added to match the complete shape of the muscle. Muscle morphology and the complexity of the muscle boundaries determine the necessary number of manual segmentations.

We have added more detail in section 4 (measurement of variables of muscle morphology) of the manuscript.

l. 337 Why is the transformation needed? Can you provide SEM in units of measurement?

Response:
A logarithmic transformation was used to provide a more accurate coefficient of variation (CV) over the small range of subjects. In addition to the ICC, the CV provides a measure of the standard error. The SEM depends on the number of observations and as these are low in the current study (proof of principle) we argue that the CV provides a better indication of the measurement error.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This is a well written piece on the technical approach to using freehand 3D ultrasound to measure muscle volumes, which is potentially a useful imaging technique in various clinical populations where MRI imaging may be not suitable or too expensive for some purposes. In general the technique is described well, however I think a few aspects could be improved or more detail added as per the below two main points.

Response:
Thank you for your time reviewing this paper, and thank you for your constructive comments.

Major Concerns:
N/A

Minor Concerns:
The pressure applied to the muscle would seem to have a major impact on the volume reconstruction when multiple sweeps are made. Deformation of one sweep would not be accounted for in the next, where deformation would mean that the same region of muscle would be in a different region of voxels in subsequent sweeps. Can the authors provide guidance on how much pressure should be applied in order to get reasonable reconstructions?

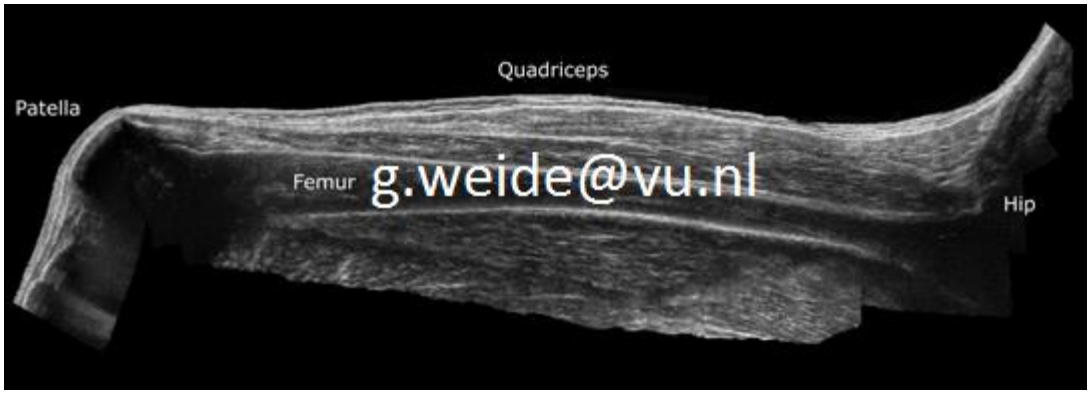
Response:
If ample ultrasound gel is applied on the ROI, the amount of pressure to remain full contact between probe and the skin is limited. As guidance, we would advise that

scanning a ROI should feel like hovering over the skin, pressure should only be applied to keep in contact with the gel and thereby the skin. However, slight tissue deformation may be inevitable, even with a generous amount of ultrasound gel. Probe size and a curved ROI affect the required amount of pressure or gel used. Larger probe size and a more curved ROI, requires more pressure and/or more gel, than smaller probes with a similar curved ROI. Another possible solution would be to discard the reverberation region of the US images.
In addition, tissue deformation is most likely to occur in first tissue layers, such as skin and subcutaneous adipose tissue layers. Note that subjects with little to no subcutaneous adipose tissue are therefore more prone to adverse effects of pressure. In addition, tissue deformation occurs most likely in the center of the probe, which is typically not the region of overlap with other sweeps.

We have added a paragraph "Probe pressure and tissue deformation" to the consideration section in the discussion containing the above response (Line: 465-477)

I also felt that the technique biases the data from later muscle sweeps, or even later images in the same sweep, if the voxels that have been collected in one image and are then re-populated from another image scan where the image sits across the same voxel region. Is this actually how this is dealt with and what limitations does this pose when using multiple scans?

Response: *In 3DUS, a reflecting object can be imaged from multiple directions. When a certain region from an US image from one direction contains for example shadowing artefact and this same region is imaged from another direction containing no shadow region, then the shadow region will be overwritten, providing a 3DUS image with regions that would have 'normally' been shadow regions (e.g. underneath bone), are now visible. This technique does not necessarily bias the 3DUS image, but more likely enriches it (see example image in which shadow regions under the femur are filled).*



Additional Comments to Authors:

N/A

Line 93: What type of transducer is used and what is its frequency, please add a note to describe it briefly and add it to the table of materials? Please include ALL parameter settings used (e.g imaging depth, sample freq, power etc)

Response:

We have added 3 lines describing the type of transducer and the settings.

The following text has been added to the manuscript:

"A 5-cm linear-array probe (12.5 Mhz) is used to generate B-mode images (25 Hz). Before each measurement imaging depth, acoustic frequency and power are optimized to visualize interfaces at extra-and intramuscular connective tissues. During the measurement, these settings are not changed."

Line 175: Please provide an example

Response:

We have made the following adjustments to the manuscript:

Line 173-178:

"Fit the foot of the leg to be scanned into the custom-made footplate.[31]

Connect the custom-made torque wrench with attached goniometer to the footplate[31] *Find the footplate angle corresponding to externally applied torque, e.g. 0Nm (Fig. 2A).*

Fix the footplate in the orientation corresponding to the 0Nm net dorsiflexion moment, by using an extendable rod which is connected to the table (Fig. 2A, arrow)."

.

Line 230: These need to be updated, please check. Also, there are no steps below that describe how the landmarks are recorded. Please add these details here and include button clicks.

Response:

We have made the following adjustments:

Line 228-231: "For all muscles, use the MoCap pointer tool to record the marked landmarks (described in sections (3.2.1 and 3.3.2) in the global coordinate system. Move the MoCap pointer tool to the identified anatomical landmarks, and use the MoCap software to record the position by pressing on the "record" button."

Line 294: 2x Please mention explicit button clicks and software selections

Response:

We have changed the manuscript to explicitly mention button clicks and software selection.

"Open framegrab software (e.g. WinDV) on the measurement computer and start US image acquisition by clicking on the record button. Subsequently, initiate the MoCap data acquisition and activate MoCap data acquisition by pressing a button on the synchronization device, this automatically activates the synchronization device (i.e. piezo crystal) located close to the US probe creating a distinct artefact in the US image at the instance of MoCap initiation (Fig. 1A, Arrow)."

Line 252: We assumed that this happens automatically without user input, if you need to interact with the system please mention explicit software actions.

Response: We have removed this sentences and adjusted editorial changes.
Line

Line 252-257: "*While exerting minimal probe pressure yet ensuring image quality, move the probe at a constant speed over the ROI; this is referred to as a "sweep". Make sure that clear anatomical cross-sectional B-mode US images of the target muscle are recorded.*

Visually check for movement of the subject during the examination, if the subject moves abort the sweep and repeat from step 3.2.6."

Line 262: Please fix.

Response: We have fixed the section reference.

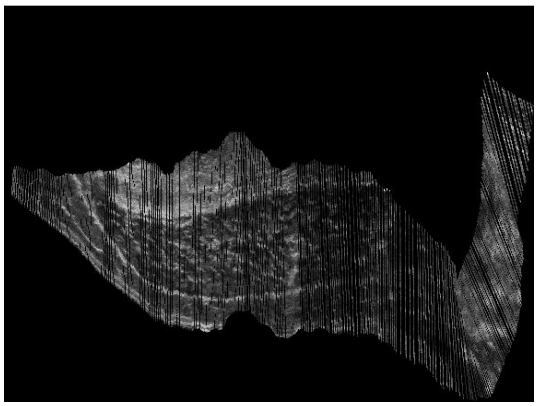
Line 266: Please mention explicit button clicks and software selections.

Response: We have made the following adjustment to explain how additional sweeps are made.
Line 266-269: "Add additional sweeps (as described in section 3.3.3-3.3.5) until the entire ROI is scanned and the medial border of the muscle is imaged completely (Fig. 2B). Use the trace in the gel of the previous sweep to guide the next sweep, slightly overlapping (0.5 cm) the previous swept area."

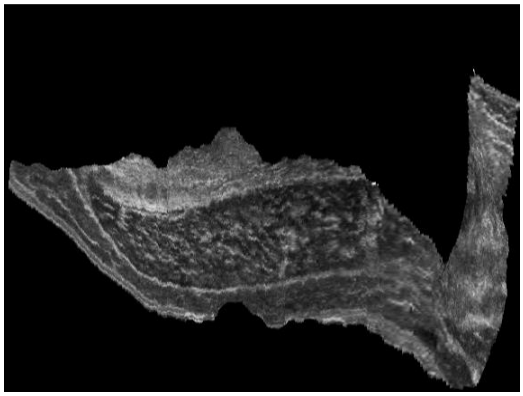
Line 292: As there are no details of the reconstruction steps it is unclear what you wish to show here. Are any of the steps below performed in GUI-based software?

Response: We could shortly visualize the 2 main reconstruction steps.

Using custom-made software assign the voxels in the ***Va*** with pixel grey-values from the ultrasound images (Bin-filling):



Fill the gaps using an “inpaint procedure”:



We have made the following adjustments to the manuscript mentioning these 2 main reconstruction steps:

Line 292 -294: “Reconstruct a single 3DUS voxel array (3D image) from a single sweep over the skin of a specific anatomical region of interest (ROI) (*e.g.* muscle, tendon) by bin-filling and inpainting the 3DUS voxel array.”

Line 297: How? Is this all done using custom scripts?

Response: We have added detail on how we perform this step

Line 297-301: *“Synchronize MoCap data and US images by identifying the first US frame containing the piezo crystal created artefact and crop the US image sequence accordingly using VirtualDub software. When using VirtualDub, place the slider at the identified frame and press the home button on the keyboard. Next, move the slider to the end of the measurement and press the end button. Press the F7 button to export the cropped image sequence.”*

Line 303: How? Is this all done using custom scripts?

Response: We now mentione that we did this by using a custom-script.

Line 303-305*: "Using a custom script define a voxel array (**Va**) coordinate system which can be filled with US images. Ensure that the **Va** is oriented in accordance with the scanning direction and sized to fit all the pixels of a single sweep."*

Line 310: How? Is this all done using custom scripts?

Response: We now mention that we did this by using a custom-script.

*Line 310-312: Using a custom script assign the voxels in the **Va** with pixel grey-values from the ultrasound images. This process is described as forward mapping or bin-filling (eq. 3) (Fig 1C)[23,24].*

Line 323: How? Is this all done using custom scripts?

Response: We had not mentioned that we do this with a custom script and have now mentioned that more detail is given in the next step.

"Using a custom script identify gaps inside the voxel array (i.e. black voxels). Take the following steps by using binary image processing:"

Line 344: Again, as in 3.4, it is unclear what we would show here. We can likely state once that the images are reconstructed to produce the 3D volumes, so 3.4.1 can stay highlighted but 3.5 and 3.5.1 should be unhighlighted.

Response: the importance of this section is to mention that not just a single sweep is reconstructed, instead multiple single sweeps are reconstructed and merged (step 3.5.3 is now highlighted)). We can graphically show how the single sweeps are merged, to form MRI like images. (similar to figure 4)

Line 346-354: *"Reconstruct all individual sweeps (described in section 3.4) covering one larger ROI according to the same **Va** coordinate system (section 3.4).*

*Create a new **Va** coordinate system, sized to accommodate all individual reconstructed sweeps.*

*Place the individual **Va**'s step by step into the larger **Va**, if a voxel is already assigned by another **Va**, this voxel will only be overwritten if the new voxel has a grey value ≥ 10 on an 8-bit scale, otherwise the new voxel grey value is discarded."*

Line 358: Please add step to mention whether the 2D images or 3D volumes are loaded, and mention how.

Response: We have modified the text. Now we mention that the 3DUS images are

loaded in the MITK software.
Line 358-362: *"Use Medical Interaction Toolkit[35] (MITK) to load the 3DUS voxel array and retrieve the coordinates of the origin, insertion and distal end of the muscle belly.*

Set the slicing to 'Coupled crosshair rotation'. Align the axes with muscle or bony structures to precisely retrieve the coordinates."

Line: 373: How? What is done here?

Response: We now explain how and why the segmentation is performed:

Line 373-379: *" In order to measure muscle volume use MITK to identify muscle belly boundaries between origin and distal end of the muscle belly. Use the build-in MITK segmentation to manually segment multiple anatomical cross-sections evenly distributed along the muscle belly length (Fig. 3A).*

Open the 'segmentation tool' and create a 'new segmentation'. Start segmenting the muscle boundaries on an anatomical cross-section halfway along the muscle belly. Press 'A' on the keyboard to add a manual segmentation and draw by pressing left mouse button and moving the cursor over the muscles boundaries, Press 'S' to remove parts of segmentation."

Line 391: "If this is to be filmed, explicit details are needed. If you leave this as is it will only be stated in the video."

Response: This step cannot be visualized, stating it in the video will be sufficient.

Line 394: Unclear what is done here, this should be unhighlighted.

Response: The how to determine the orientation of the mid-longitudinal plane was described in the next Note. We have now included this note into the protocol, to give direction to the reader. These steps can be visualized, as shown in figure 3.

Line 394-400: "*Find the orientation of the mid-longitudinal fascicle plane of the muscle belly, containing full length of fascicles (Fig 3)[36].*

The mid-longitudinal plane is defined by three points. The origin and distal end of the muscle belly are the first two points. The third point is found in an anatomical cross-sectional image halfway between the origin and distal end of the muscle belly. Within this anatomical cross-sectional image, the midpoint between the first two points projected onto the tangent of the distal aponeurosis yields a third point that together with the origin and distal-end of the muscle belly defines the mid-longitudinal plane."

Line 402: How? This also need not be highlighted

Response: We have made the following adjustments to the manuscript.

Line 402-406: “From the mid-longitudinal plane, measure fascicle length at a pre-defined standardized position between origin and distal end of the muscle belly (*e.g.* 50%). Segment the muscle boundaries. Place a line halfway the muscle and rotate this line until it matches the direction of the underlying fascicle. The intersection of this line with the muscle boundaries represents the estimate of the fascicle length (Fig 3B).”