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

Adapted from:
Abstract
The development goal of 3D ultrasound imaging was to engineer a modality to perform 3D morphological ultrasound analysis of human muscles. 3D ultrasound images are constructed from calibrated freehand 2D B-mode ultrasound images, which are positioned into a voxel array. Ultrasound 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 m. vastus lateralis and m. gastrocnemius medialis. 3D ultrasound imaging facilitates standardization using 3D anatomical references. This approach provides a fast and cost-effective approach for quantifying 3D morphology in skeletal muscles. In health care and sports, information on the morphometry of muscles is very valuable in diagnostics and/or follow-up evaluations after treatment or training.
3D ultrasound imaging is a valid and reproducible technique to assess muscle morphology non-invasively

Reference: Weide & vd Zwaard et al., J Vis Exp, 2017
**Introduction**

In health care and sports, information on the morphology of muscles is very valuable in diagnostics and/or follow-up evaluations after treatment or training. Ultrasound (US) imaging is a tool commonly used for visualization of soft tissue structures in muscle diseases, critical illness, cardiovascular diseases, neurological disorders and to assess effects of physical training. US imaging enables quantification of muscle size, fascicle length and angle of pennation. These morphological variables are important determinants of muscle force and the muscle's length range of force exertion.

Currently, US 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). However, MRI is expensive and not always available. Also, visualization of muscle fibres requires special MRI sequences, such as diffusion tensor imaging (DTI). 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. 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. However, previous approaches 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 sequential ultrasound images.
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*Note:* A 5-cm linear-array probe (12.5 Mhz) is used to generate B-mode images (25 Hz). Before each measurement, the 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 **Motion Capture (MoCap) system** to the measurement computer.

1.3. Rigidly connect a MoCap cluster marker to the ultrasound probe in order 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 sound waves create a distinct artefact in the US image at system initiation (Figure 3.1A, Arrow).

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

2. Calibrate

*Note:* Perform a spatial calibration to calculate a transformation matrix ($^{*}T_{from}$) from the US images with respect to the probe coordinate system. This calibration process has been described previously. 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 ($P_{xyz}$ Figure 3.1B, 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 ($G_{l}$).

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

2.5. Submerge the head of the US probe ($P_{r}$) 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 ($I_{m}$).

2.6. Stop data acquisition.

2.7. 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 (described in step 3.4.1).
2.8. Identify relevant US images in which the crosswire is clearly visible and track the position of the crosswire in these US images (Im<sub>sys</sub>), correct position for water temperature.

2.9. Determine position of the crosswire with respect to the moving Pr by a series of transformations from Ph to Pr (Equation 3.1) at time instances (i = 1:n) corresponding to crosswire identification in step 2.8.

\[
\begin{bmatrix}
i(1) Pr_x \\
i(1) Pr_y \\
i(1) Pr_z
\end{bmatrix} \cdots \begin{bmatrix}
i(n) Pr_x \\
i(n) Pr_y \\
i(n) Pr_z
\end{bmatrix} = Pr_i T_{Gl} \times Gl_i T_{Ph} \times \begin{bmatrix}
Ph_x \\
Ph_y \\
Ph_z
\end{bmatrix}
\] (3.1)

2.10. Calculate the Im to Pr transformation matrix (Pr<sub>T</sub>Im) by solving Equation 3.2 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).

\[
Pr_i T_{Im} = \begin{bmatrix}
i(1) Pr_x \\
i(1) Pr_y \\
i(1) Pr_z
\end{bmatrix} \cdots \begin{bmatrix}
i(n) Pr_x \\
i(n) Pr_y \\
i(n) Pr_z
\end{bmatrix} / \begin{bmatrix}
i(1) Im_x \\
i(1) Im_y \\
i(1) Im_z
\end{bmatrix} \cdots \begin{bmatrix}
i(n) Im_x \\
i(n) Im_y \\
i(n) Im_z
\end{bmatrix}
\] (3.2)

Figure 3.1. Schematic of 3DUS algorithm. A) 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 artefact (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. With a complete series of known transformations, images from the Im can be transformed into any voxel array coordinate system (Va).

3. Experimental protocol
The experimental protocol describes two commonly performed protocols involving 3DUS imaging, i.e. morphometry of the m. gastrocnemius medialis (GM) and the m. vastus lateralis (VL) (Figure 3.2A).
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.

3.1.1.4. Connect the custom-made torque wrench with attached goniometer to the footplate. Find the footplate angle corresponding to an externally applied torque, e.g. 0 Nm (Figure 3.2A).

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 3.2A, 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 major and the latter with the epicondyle lateralis femoralis) to 95° by changing 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.

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

This is done for guidance of the 3D ultrasound examination and for post-experimental quantification of the subjects upper-, lower leg and foot posture. Identify and record 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: most prominent dorsal aspects of the medial and later femur epicondyles, the centers of the malleoli of 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 on the calcaneus.

3.2.2. For the VL measurement

3.2.2.1. Identify the following landmarks by palpation and mark using a surgical skin marker: the medial and lateral malleoli (as above), most proximal insertion of the patella tendon of the tuberositas tibiae, medial and lateral epicondyles (as above), 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 frame grabber 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 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 artefact in the US image at the instance of
MoCap initiation (Fig. 3.1A, 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 thigh. Perform a sweep (as described in section 3.3.1-3.3.5) in proximo-distal direction along the medial border of the GM, ensuring visibility within the anatomical cross-sectional images of the medial border of GM, and Achilles tendon all the way down to its insertion on the os calcaneus.

3.3.7.1. 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 3.2B). 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 3.2B). 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 affects 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.

3.4. 3D ultrasound voxel array reconstruction

Reconstruct a single 3DUS voxel array (3DUS 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 using a custom-script. In order to reconstruct a 3DUS voxel array, take the following post-experimental steps.
3.4.1. Synchronize MoCap data and US images by identifying the first US frame containing the piezo crystal created artefact and cropping the US image sequence accordingly with VirtualDub software. 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 (last skin contact) and press the end button. Press the F7 button to export the cropped image sequence.

3.4.2. 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.

3.4.3. 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 3.3).

\[
\begin{bmatrix}
V \alpha_{x(1)} & \ldots & V \alpha_{x(n)} \\
V \alpha_{y(1)} & \ldots & V \alpha_{y(n)} \\
V \alpha_{z(1)} & \ldots & V \alpha_{z(n)} \\
\end{bmatrix} = V \alpha_T \times P \times P \times I \times
\begin{bmatrix}
I_m x(1) & \ldots & I_m x(n) \\
I_m y(1) & \ldots & I_m y(n) \\
I_m z(1) & \ldots & I_m z(n) \\
\end{bmatrix}
\]

(3.3)

**Note:** 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 (Im), 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).
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Note: \( P_r^{-1} T_{gl} \) indicates the inverse of the previously described transformation matrix (i.e. a \( P_r \) to \( G_l \) transformation matrix).

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

#### 3.4.4.1. Create a bin-filled binary voxel array, in which all filled voxels are labelled. 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 binary images. By performing these steps one after another, outside boundaries remain while gaps inside are removed.

#### 3.4.5. Fill the identified gaps using an “inpaint procedure” using surround grey-valued voxels.

Note: This inpaint technique we chose can be used to: “fill gaps with a smooth interpolant based upon minimizing the sum of squares of the second derivative at each labelled voxel measured by finite differences on the grid”.

#### 3.4.6. Equalize voxel dimensions of the \( V_a \) 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 \( V_a \) coordinate system to merge multiple sweeps.

#### 3.5.2. Create a new \( V_a \) coordinate system, sized to accommodate all individual reconstructed sweeps.

#### 3.5.3. Place the individual \( V_a \)'s step by step into the larger \( V_a \), if a voxel is already assigned by another \( V_a \), 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 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.
4.2. 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 that are evenly distributed along the muscle belly length (Figure 3.3A).

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 left mouse button and moving the cursor following the muscles 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 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 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 ‘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 labelled volume size using a custom-script.

4.3. Find the orientation of the mid-longitudinal fascicle plane of the muscle belly, containing full length of fascicles (Figure 3.3)\textsuperscript{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 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 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 3.3B).

*Note:* Previously, it proved necessary to take into account the, sometimes curved, orientation of the distal aponeurosis\(^{38}\), which is illustrated by an anatomical cross-sectional image taken halfway between origin and distal end of the muscle belly (Figure 3.3B).

![Figure 3.3. Schematic of 3DUS analysis](image)

**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 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 the muscle belly and rotated until it matches the direction of underlying fascicles. The intersections of this line with the proximal and distal aponeuroses (connected by thick solid yellow line) represents 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.

**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 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 GM and VL were removed. A mid-longitudinal section was cut, taking the orientation of the distal aponeurosis into account. Using a caliper, fascicle length was measured, halfway between 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
calculated from the difference\textsuperscript{40}. Fascicle length and volume were measured three times and mean and standard deviation values were calculated. Criterion validity between 3DUS method and dissection measurements was tested using a Pearson’s correlation for average fascicle length and muscle volume. Intra-rater reliability of 3DUS method derived fascicle length and volume measurements was quantified using a two-way mixed model intra-class correlation coefficient (ICC\textsubscript{3,3})\textsuperscript{41}, and the coefficient of variation (CV) was calculated after logarithmic transformation of the data. 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 3DUS method derived measurements of fascicle length and volume was high (ICC\textsubscript{3,3} 0.983, CV 7.3% and ICC\textsubscript{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 the GM (Table 3.1).

### Table 3.1. Cadaver validation data.

<table>
<thead>
<tr>
<th>Cadaver</th>
<th>Muscle</th>
<th>Dissection Volume (mL)</th>
<th>Dissection Fascicle length (cm)</th>
<th>3DUS Volume (mL)</th>
<th>3DUS Fascicle length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>GM</td>
<td>122</td>
<td>4.1</td>
<td>121 ± 10</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>VL</td>
<td>274</td>
<td>7.5</td>
<td>285 ± 13</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>C2</td>
<td>GM</td>
<td>131</td>
<td>4.4</td>
<td>133 ± 5</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>VL</td>
<td>380</td>
<td>8.7</td>
<td>366 ± 4</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>C3</td>
<td>GM</td>
<td>143</td>
<td>3.0</td>
<td>138 ± 4</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>VL</td>
<td>539</td>
<td>7.6</td>
<td>500 ± 16</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>C4</td>
<td>GM</td>
<td>106</td>
<td>4.0</td>
<td>106 ± 7</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>VL</td>
<td>292</td>
<td>10.3</td>
<td>290 ± 18</td>
<td>10.1 ± 1.4</td>
</tr>
</tbody>
</table>

Notes: C# is Cadaver number, GM is m. gastrocnemius medialis, VL is m. vastus lateralis. Dissection are results from cadaver dissection, 3DUS are the average results from three determinations of volume and fascicle length with 3DUS image analysis of cadavers (mean±SD).

### Discussion

A valid and reliable 3DUS technique is presented that allows fast analysis of morphometric variables of skeletal muscles. Different 3DUS approaches for soft tissues imaging have been available for approximately a decade\textsuperscript{42,43}, however, 3DUS approaches are still not commonly used. Magnetic resonance imaging (MRI) is considered to be a ‘gold standard’ for estimation of in vivo muscle volumes\textsuperscript{44–20}. MRI validity has been tested and confirmed in studies comparing either phantoms or cadaveric organs of known volume to MRI-based volume estimates\textsuperscript{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 MR scanners. Typical MR images generate insufficient contrast to perform measurements of variables of muscle geometry (fascicle length and angles). However, 3D muscle geometry can be assessed also using MRI, by using additional techniques, e.g. diffusion tensor imaging (DTI) technique\textsuperscript{21}. Similar to MRI, US imaging provides adequate distinction at interfaces between different types of tissues, providing a valid modality for soft tissues (i.e. visible within US images) volume assessment\textsuperscript{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, to study larger muscles as well. This new 3DUS method provides a potential tool for clinical assessment of muscle morphology. This method can also be used 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 hours was necessary for reconstructing only one sweep yielding 750 US images (30 seconds at 25 Hz)\(^1\text{5,25,30}\). With the present 3DUS method the same sweep takes only 50 seconds reconstruction time (improving ‘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 by 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\(^{-1}\), with a target voxel size of 0.2×0.2×0.2 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 \((p\|T_{\text{sw}})\).

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 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 seconds.

**Limitations and critical steps**

Several aspects of the 3DUS imaging should be taken into account:

1. **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 artefact-free distinction of interfaces 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.

2. **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 dimensions of 0.2×0.2×0.2 mm\(^3\) using a MoCap system with an accuracy of 0.1 mm, providing
ample accuracy to reconstruct the 3DUS voxel array.

3. **Sample frequency.** The lowest temporal resolution of either the US images or MoCap data stream determines the highest 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 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 artefacts.

4. **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.

5. **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 and aponeurosis length) between subjects and monitoring within subjects in longitudinal studies. Note that the morphology assessed in rest may alter during muscle activation. For example, for the VL experiment, knee extensor morphology during maximal contraction may demonstrate a high pennation angle and shorter fascicles in 60 degrees knee flexion in comparison with morphology at rest\(^5\). In certain conditions (e.g. spasticity), EMG may be used to verify resting muscle activity levels during examination.

6. **Probe pressure and tissue deformation.** 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 (i.e. non skin-contact) 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 at the center of the probe, which is typically not the region of overlap with other sweeps.

7. **Imaging and anatomical knowledge.** Another important consideration in using any imaging modality is that knowledge of both anatomy and the imaging modality is essential to obtain meaningful interpretation. Anatomical variation between subjects and image artefacts 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. However in atrophied muscle (i.e. elderly, in case of pathology, or cadaver), clear identification is even more complicated because of a smaller size, decreased image contrast and therefore less distinct tissue interfaces (Figure 3.4). 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. Also detailed anatomical information on curvature of the distal aponeurosis was essential for an adequate selection of the mid-longitudinal plane in all subjects.

**Figure 3.4. Variation and quality of reconstructed anatomical cross-sectional 3DUS images of the quadriceps muscle halfway the thigh.** A) Example of a male human cadaver shows an image of an atrophied state at death (age at death: 81 y). Identification of the boundaries of individual heads of quadriceps muscle is difficult. B) Example of a sedentary male (aged 30 y). C) Example of a male athlete rower (aged 30 y). The white squares for scale represent 1 cm x 1 cm.

**Future applications**

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. Using 3DUS for monitoring patients who are at risk of losing muscle mass is important and potentially allow adjustment of the treatment. Another potential application of 3DUS lies in monitoring morphological adaptation of muscle in response to (training) intervention 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 meaningful morphological parameters of m. vastus lateralis and m. gastrocnemius medialis proved to be valid and reliable.
References


37. MITK. http://mitk.org/wiki/MITK.