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](file:///R:\Research\Technical%203DUS%20Article\Revision\).

***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.  
  
o The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
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.  
o The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
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.*

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MoCap, HP259 Z440, Intel Xeon E5-1630v3, 16.0 GB RAM, NVIDIA Quadro K620, <http://www.fixforlifeembalming.com/>, NDI Optotrak , etc.  
1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.  
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.  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

***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 10th 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 10th 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