**Response to Reviewers’ Comments: JoVE59013**

**Title: Ultrasound Imaging of the Thoracic and Abdominal Aorta in Mice to Determine Aneurysm Dimensions**

We thank the Editor and Reviewers for their constructive comments and suggestions to improve this manuscript. The Editor’s and Reviewers’ comments are in **bold**followed by our responses point by point. Changes in the revised manuscript are distinguished in red color.

We hope that this version has successfully incorporated the Editor’s and Reviewers’ points, and the scientific quality has been improved substantially.

**Response to the Editor:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

Authors’ reply:

We have carefully checked spelling and grammar.

**2. Please do not highlight notes for filming.**

Authors’ reply:

This has been corrected.

**3. Please use h, min, s for time units.**

Authors’ reply:

All time units have been revised as requested.

**4. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.**

Authors’ reply:

We have revised Section 6 to provide the detailed steps of measuring aortic dimensions using the Vevo 2100 system. Example image of the computer screen is shown in Supplemental Figure 1.

**5. Please do not abbreviate journal titles for all references.**

Authors’ reply:

Journal titles in the references section have been revised as requested.

**6. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.**

Authors’ reply:

All figures have been uploaded individually as a PNG file.

**Response to the Editor:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

Authors’ reply:

Thank you for your comments. We have corrected all spelling and grammar issues.

**2. 2.1: Please specify the age, gender and strain of mouse. Please indicate the concentration of isoflurane.**

Authors’ reply:

Ten to twelve week-old C57BL/6J male mice (n = 5) were used for the experiments in this study. We have included this information in the figure legends.

We used 1.5 – 2.5 % vol/vol isoflurane with 1 L/min oxygen. We have revised (1.6) and (2.2) to include the concentrations of isoflurane and oxygen.

**3. 2.2: Please mention how to confirm that mouse is anesthetized.**

Authors’ reply:

Loss of withdrawal reflex was used as the index to confirm the depth of anesthesia. We have added this information to (2.3).

**4. 2.6: Providing an estimate for the delivery rate of anesthesia may be helpful.**

Authors’ reply:

We used 1.5 – 2.5 % vol/vol isoflurane with 1 L/min oxygen. We have revised (1.6) and (2.2) to describe the concentrations of isoflurane and oxygen.

**5. 3.4 and 3.5: Are these done by using the instrument software? Please specify.**

Authors’ reply:

Images were cropped using a standard tool in the Vevo 2100 ultrasound system during ultrasound imaging. We have revised (3.5) and (3.6).

**6. Lines 169-178; Please include these details in a separate step and write them in imperative tense if possible. Any text that cannot be written in the imperative tense should be added as a “Note”.**

Authors’ reply:

This paragraph has been moved to the “Note” of (3.4).

**7. Line 196: Please avoid the use of any personal pronouns.**

Authors’ reply:

Personal pronouns have been replaced.

**8. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.**

Authors’ reply:

The text has been modified in accord with these suggestions.

**9. Discussion: Please discuss critical steps within the protocol and any limitations of the technique.**

Authors’ reply:

Critical steps and limitation has been included in a paragraph in the discussion.

**10. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).**

Authors’ reply:

The corresponding reference numbers have been revised as requested.

**11. References: If there are six or more authors, list the first author and then “et al.”. Please do not abbreviate journal titles.**  
  
Authors’ reply:

The author lists have been corrected.

**Response to Reviewer #1:**

**Major Concerns:**

**1) Preparation of Mouse (section 2.1) - "Place the mouse in isoflurane/O2-filled induction chamber." It is actually recommended in codes of practice that the mouse is placed in the induction chamber before filling with isoflurane as this reduces stress experienced by the mouse and avoids any unwanted cardiovascular effects from anaesthetic shock that may affect the experiment. The induction chamber should ideally be pre-filled with oxygen and then gradually filled with anaesthetic once the mouse is in the chamber. This is particularly important when running longitudinal studies.**  
  
Authors’ reply:

We appreciate this important suggestion. Section (1.6) – (2.2) have been edited to include this suggestion. As suggested by the Reviewer #1, to minimize unwanted cardiovascular changes due to anesthesia, experimental mice should be placed in an O2-filled chamber and then anesthesia should be filled gradually in the chamber.

**2) Analysis of abdominal aortic images (section 6.2.2) - I would say it is essential to measure at a consistent phase of the cardiac cycle, even in the abdominal aorta, as not to do so would introduce an unwanted (and unnecessary) extra layer of variability in the analysis. A recent ultrasound study of mouse abdominal aorta actually quantified % distension from the change in aortic diameter over the cardiac cycle. In a naïve, WT mouse this can be as high as 20% on average. This point is actually made later on in the Discussion, therefore the ambiguity in 6.2.2 should be removed.**

Authors’ reply:

Thank you very much for your comments. For accurate measurements in both the thoracic and abdominal aortas, the cardiac cycle should be taken into consideration. Section (6.2.2) has been revised to remove the ambiguity.

**Minor Concerns:**  
  
**1) Preparation of Mouse (section 2) - there is no mention of use of an anaesthetic scavenging system in the text, although it is evident in Figure 1. Also, was temperature monitored during imaging (e.g. using rectal thermometer probe)?**

Authors’ reply:

Information on the scavenging system has been included in (1.4) and Figure 1. In this protocol, mouse was placed on the heated platform at 37.0 °C, but temperature was not monitored using a rectal thermometer probe.

**2) Imaging of thoracic aorta (section 3) - it would be a good idea to list typical ultrasound parameters used (e.g. frame rate, depth, gain, etc), possibly in a table, to assist an inexperienced ultrasound operator.**

Authors’ reply:

The usual ultrasound parameters are now included in the table (3.7).

**3) Imaging of the abdominal aorta (section 4.2) - suggest including pulsed wave Doppler in the imaging protocol to confirm pulsatile blood flow. Without this step, vena cava / portal vein may be mistaken for the abdominal aorta by an inexperienced operator, particularly in an aneurysm model where the artery is enlarged.**  
  
Authors’ reply:

As the Reviewer #1 suggested, pulse wave Doppler is useful method to measure the blood velocity. However, it is often difficult to detect the blood flow accurately in the abdominal aorta, because the axis of the abdominal aorta is not parallel to echo beam in this method. Thus, the abdominal aorta was distinguished from the vena cava and portal vein by color Doppler and pressing down on the probe. The vena cava and portal vein are compressible, while the aorta maintains its patency. We have added this step into (4.2).

**4) Analysis of thoracic aortic images (section 6.1.2) - which phase of the cardiac cycle do you typically use for analysis? This relates to Major Concern no. 2. The cardiac phase used should be stated clearly in the protocol. Also, more detail could be provided on how the measurements were performed in the abdominal aorta. Assuming image acquisition is reproducible, the analysis method is the key to reliability and reproducibility of the method as a whole. The authors should state whether the measurement was inner edge to inner edge of the vessel lumen (as stated for thoracic aorta measurements), outer to outer, etc.**  
  
Authors’ reply:

We routinely measure aortic diameters at the end of diastole, partially based on the ease of defining this phase in the ECG. We have added these comments into (6.1.2).

As with thoracic measurements, abdominal aortic diameter is measured from the inner edge to inner edge of the vessel lumen. This suggestion is now included in (6.2.2) and (6.2.4).

**Response to Reviewer #2:**  
  
**Major Concerns:**

**1. No mention is made regarding the infrarenal aorta. While many mouse models of AAAs are suprarenal, infrarenal models obviously do exist. Further, the vast majority of human abdominal aortic aneurysms occur in the infrarenal portion. The authors may want to consider including a section on imaging the infrarenal aorta and the best strategies for this region (i.e. changing the table orientation if abdominal gas creates an artifact).**

Authors’ reply:

This protocol can also be used to measure the diameter of the infra-renal aorta. We have revised Section 4 to reflect this ability.

**2. Line 355: As AAAs enlarge, they too can become tortuous. Keeping the same probe position for all images may result in artificially larger measurements as they could be taken off axis. Recommend mention of staying along the true transverse axis of the vessel.**

Authors’ reply:

The text of the note is (4.6) has been amended to include “adjustment of the probe angle is needed for accurate aortic imaging”.

**3. Line 357: The discussion fails to mention recent advancements in 3D and 4D vascular ultrasound. Recommend adding this to the discussion as many of the issues associated with M-mode and B-mode are alleviated with these volumetric approaches (PMIDs: 15364813, 10511652, 29234935, 29234935, 29966517).**

Authors’ reply:

The advantages of 3D and 4D imaging have been included in the discussion section.

**4. Line 371: The discussion on vessel "elasticity" is lacking. Ultrasound images can be used to measure vessel distention or strain. Calculations of vessel elasticity, stiffness, or pressure-strain elastic modulus require measurements and assumptions related to vessel loading (i.e. systolic, diastolic, and pulse pressures). Aneurysms certainly lead to stiffer vessels due to elastin breakdown and increased collagen deposition (PMID: 21071686, 26064906, 28186882), but ultrasound cannot measure vessel stiffness directly. Recommend rewording and expanding this section to clarify for the reader.**

Authors’ reply:

The discussion has been revised and expanded to explain aortic elasticity, strain, and stiffness.

**Minor Concerns:**

**1. No mention is made of a method to determine depth of anesthesia. I would suggest mention of the withdrawal reflex.**

Authors’ reply:

In accord with the reviewer’s suggestions, “monitoring the withdrawal reflex” has been added to (2.3).

**2. No mention is made of adjusting overall gain or time gain compensation. Most ultrasound systems now are user friendly and need very little tweaking, but these (especially overall gain) seem too important to leave out.**

Authors’ reply:

As the Reviewer #2 mentioned, ultrasound settings are important to image tissues accurately. A table (Table 1) has been included to describe the ultrasound setting including overall gain in (3.7).

**3. Throughout the paper the authors instruct the reader to do something in a specific direction. The authors use both the mouse's orientation and the reader's orientation. For example, lines 170-177 reference the mouse's left parasternal area, but in line 184 the reader is instructed that the "notch" should be on the left. Is this the mouse's left? Or the reader's left? To avoid confusion, I would recommend being clear and sticking with either the mouse's orientation or self-orientation, not both.**

Authors’ reply:

We apologize for these ambiguous instructions. The text has been modified (4.1) to the mouse’s orientation.

**4. Line 127: The authors instruct the reader to apply depilatory cream to the chest and abdomen. Most studies will only be looking at one area. As depilatory cream removes hair using chemicals, thoughtful restraint to be careful not to scar the mouse's skin and limit the area in which it is applied is recommended.**

Authors’ reply:

In accord with the reviewers suggestions “Use of depilatory creams should be kept to the minimum to avoid irritation” has been added to (2.7).

**5. Line 135: 400-500 bpm seems slow. Our mice typically have anesthetized heart rates above 500 unless they are hypothermic. Recommend adjusting this range.**

Authors’ reply:

We apologize for this mistake. Mice show typically around 500 bpm of HR during anesthesia. However, some mice with aneurysms have slightly lower heart rate than control mice during the procedure, even when on a heated platform and anesthetized using an appropriate dose of isoflurane. We have revised appropriate heart rate range to 450 – 550 bpm.

**6. Line 148: New users may not understand what the "notch" is, especially since it varies between ultrasound systems. A picture or brief explanation could demystify this.**

Authors’ reply:

We acknowledge that the “notch” was not an appropriate word for new users. The “reference marker” has been revised and added black arrows to indicate the marker in Figure 2A-D, and provided a brief explanation of the reference marker in the text (3.2).

**7. Line160: Cropping the ultrasound image does not improve spatial resolution. Lateral resolution changes only with focal depth and axial resolution is dependent on spatial pulse length, which can only be improved with higher frequency.**

Authors’ reply:

We apologize for this mistake. Images are cropped to increase the frame rate. This error has been corrected.

**8. Figure 2B/Line 171: In this figure the stage looks like it has been tilted back to level again. Previously the stage was tilted to the mouse's left. Is the stage still tilted?**

Authors’ reply:

For the right parasternal long axis view, the stage tilts to the mouse’s left. Conversely, in the left parasternal long axis view, the stage is flat or tilts to the mouse’s right slightly. This information has been added in (3.4).

**9. Line 185: Color Doppler signal may occasionally fail to appear in the vessel if the Doppler angle is at 90 degrees. Recommend briefly mentioning Doppler steering parallel to the vessel.**

Authors’ reply:

The reviewer’s suggestion has been incorporated in (4.2).

**10. Line 199: This needs clarification: do the authors recommend taking one cine loop from the SMA all the way down to the renal arteries? Or is one cine loop simply capturing several heart beats at one specific location? If so, what location is recommended?**

Authors’ reply:

We apologize for the insufficient explanation. One cine loop is captured at one specific location showing the maximum expansion in region of interest of the abdominal aorta. The imaging location is described in (4.7).

**11. Line 211: Ultrasound machines are expensive and very sensitive to chemicals. I would suggest the authors include a line recommending the reader check with the ultrasound vendor to determine what chemicals are acceptable for cleaning the ultrasound system.**

Authors’ reply:

Cleaning of the transducer has been included in (5.4) after consultation with FUJIFILM VisualSonics, Inc.. After every imaging session, the probe should be wiped gently with a soft cloth and isopropyl alcohol or glutaraldehyde wipes.

**12. Scant grammatical errors, such as line 245: In the context the authors are using it, mean is not a verb. Suggest replacement with the word average; Line 234: remove the word "and"; Line 173: insert the word "an" between the words "acquire" and "aortic".**

Authors’ reply:

Grammatical issues have been corrected.

**13. Line 333: Avoid stating there are "modes" of probe placement. For the average imaging researcher, the word modes refers to B-mode, M-mode, etc.**

Authors’ reply:

In accord with the reviewer’s suggestion, “modes” has been replaced with “approach”.

**14. Figure 4: One other option when estimating diameter from short axis views is to calculate an "effective diameter" based on the Area of the vessel and the assumption of a circular cross-section (A = Pi/4\*D^2). This is likely a better way than simply drawing a linear measurement at one orientation.**

Authors’ reply:

The aorta is cylindrical, but its cross section is not a perfect circle. In this protocol, the maximum diameter was measured. Thus, the assumption of a circular cross-section based on this measurement has a potential to cause over estimation of aortic diameter. In this protocol, actual area was obtained by drawing the inner edge of the aorta, instead of a calculated aortic area.

**Response to Reviewer #3:**  
  
**Major Concerns:**

**Although current manuscript concisely describes how to obtain the data, the reproducibility of the data has not been demonstrated in the current manuscript. Addition of several points would further strengthen the current manuscript.**

Authors’ reply:

Thank you for your constructive suggestion. This revision includes figures showing the reproducibility of this protocol. To examine the reproducibility, ultrasonography was performed on two different days using the same mice by two different investigators; an experienced cardiologist and a non-experienced student. Aortic diameter measurements between these two operators were in close agreement (Figure 5B, C).

**Minor Concerns:**

**1. The authors mentioned "confounding factors" and "confounders" in the manuscript without defining them. Please define these factors.**

Authors’ reply:

Probe position and cardiac cycle have an impact to cause measurement errors as confounders. These factor are now included in the abstract and discussion.

**2. It would be important to demonstrate the reproducibility of the data that were obtained by the provided method. Please provide the intra-observer and inter-observer variabilities of the data.**

Authors’ reply:

Figures have been added to demonstrate the intra- and inter-observer variabilities of this protocol in Figure 5B and C. There was no major variability of intra- and inter-observers. These data have been included in the representative results section.

**3. Please state anatomical landmark(s), if available, for the thoracic aorta to judge whether the obtained images are appropriate for the reproducible morphometry in a single animal and among different animals.**

Authors’ reply:

The aortic valve and innominate and pulmonary arteries were used as anatomical landmarks, as now stated in (3.4).

**4. Two approaches are recommended for the measurement of the thoracic aorta; right and left parasternal approaches. Providing the representative images for each approach would be helpful. Also, please summarize the advantages and disadvantages of each approach in a concise manner.**

Authors’ reply:

Section (3.4) has been revised and a table (Table 2) added to describe the advantages and disadvantages of right and left parasternal long axis views.

**5. Please state how "mid-systole" is defined.**

Authors’ reply:

The term ‘mid-systole” is defined as the cardiac phase when the aorta is maximally expanded. Section (6.1.2) has been revised to define the mid-systole.

**6. The term "dilated" should be consistently used for diseased aorta, not for the expansion of aorta due to the physiological beating.**

Authors’ reply:

To avoid confusion, “dilated” has replaced “expanded”.

**7. What are "Pre" and "Post" in Figure 4?**  
  
Authors’ reply:

We apologize for this confusing mistake. “Pre” and “Post” have been deleted from the Figure 4.

**Response to Reviewer #4:**  
  
**Major Concerns:**

**Despite the fact that the authors have decided to focus only on aortic diameter measurements in mice; it is important to note the majority of recent publications in the field include measurements of Pulse Wave Velocity (PWV) as a proxy for aortic wall stiffness in mice, which has significant values in animal studies, and can be compared with with elasticity index measured in human aneurysm patients. For this reason, I think this manuscript will be significantly improved and become more of interest to the wide audience if the author includes a section on methods for PWV measurements in mice. In addition, I would like to suggest that the authors use the phrase "Ultrasound Imaging" instead of "Ultrasonography", as the former seems to be more common in current literature.**  
  
Authors’ reply:

We appreciate your constructive comments. Measurements of PWV have been added in the discussion section. The manuscript has been edit to state “ultrasound imaging” instead of “ultrasonography”.

**Minor Concerns:**  
**\* Line 85; replace "cylindrical shape" with "cylindrical organ"**

Authors’ reply:

This has been corrected.

**\* Line 90; replace "high resolution" with "high-resolution"**

Authors’ reply:

This has been corrected.

**\* Line 97; replace "… for mice is performed" with "… for mice was performed"**

Authors’ reply:

This has been corrected.

**\* Line 101; replace "Eye ointment" with "Eye lubricant"**

Authors’ reply:

This has been corrected.

**\* Section 3.1; replace "to the left of mouse" with " to the left side of the mouse"**

Authors’ reply:

This has been corrected.

**\* Section 3.3; Would the use of color Doppler help with finding the best position at this stage?**

Authors’ reply:

The use of color Doppler has been included in section (3.3).

**\* Section 3.3; under the "Note" section explain what are the situations in which the entire ascending aorta cannot be captured in one scan? If the image is captured separately, what are the limitation with respect to data analysis?**  
Authors’ reply:

Aortic pathologies such as aortic dilation and tortuosity could cause this difficulty and measurement errors. Section (3.4) has been revised.

**\* Section 6.1.2; How do you select your images? how many images do you capture for each mouse? How many systole/diastole cycles would you include in your image analysis. Please create a section explaining your image selection for analysis.**  
  
Authors’ reply:

In this protocol, we used 401 frame rates and the mouse heart rate was around 500 beat per minute. Ultrasound images were captured for 300 frames. Therefore, 6 to 7 beats can be technically detected in one cine loop. Three images are selected for measurements from each cardiac cycle in the cine loop. The revised manuscript describes image selection (6.1.2, 6.1.5).

**\* Section 6.1.3; How do you determine the center of the lumen? How do you make sure that you are consistent between your samples while drawing the central line?**  
  
Authors’ reply:

Since the center line was only used for the guide of drawing measurement lines of aortic dimension, the center line was drawn based on visual inspection.

**\* Sections 6.1.5 and 6.2.5; are measurements for three cycles (hear beat) enough? There are publications in which they suggest using at least 5 cycles.**  
  
Authors’ reply:

There was no major deviation in our data of inner- and intra-observer variabilities for aortic measurements. Therefore, we consider three cardiac cycles are sufficient accurate measurements of aortic dimension.

**\* Line 276; Please clarify for the readers why you decide to capture the aortic images at mid-systole and not end-systole.**  
  
Authors’ reply:

In mice, it is technically difficult to define the end-systole. In addition, the aorta should be maximally expanded in the mid-systole, but not in the end-systole. Therefore, aortic images were captured at the mid-systole. The text has been revised to provide the rationale for measurement at the mid-systole.

**\* Clarify in your figure legends and text that the yellow line shows respiratory cycle vs. green for ECG.**

Authors’ reply:

Explanations of yellow and green lines have been added to the figure legend.