We are grateful to the review editor for their careful reading and thoughtful comments on the previous version of our submitted manuscript to the Journal of Visualized Experiments (**JoVE59170**). We have thoroughly examined each comment and carefully addressed them in our revised manuscript. Below is a summary of our modifications made in the revised manuscript, for addressing the Editorial comments and for improving the clarity and readability.

* Some steps of the protocol have been revised for greater clarity, specifically regarding:
  + How the heart valve tissues are to be stored when the protocol is paused
  + The refrigeration temperatures for heart valve leaflet storage
* Supplemental information has been provided which details commercial software and hardware specific steps for internal review by the Journal of Visualized Experiments.
* Figure titles have been provided for all figures, and captions adjusted to be more fully detailing the contents of the figures.

**Responses to Editorial Comments Added to Our Resubmitted Manuscript (as shown in Green)**

1. Note: The protocol can be paused here. However, tissue testing should occur within two days of dissection.

*How are the leaflets stored?*

We thank the editor for providing additional feedback. The step has been rewritten to detail the storage methods for pausing the protocol, which includes storing the tissue in PBS solution in a refrigerated (4⁰C) environment.

1. *5.1)* Prepare a PBS bath at 37⁰C following the biaxial testing system’s instruction manual before any subsequent testing. This temperature is correspondent to physiologic conditions.

*Can this section simply be merged with the next one? A Single step under a protocol section is a bit awkward.*

We agree that a section with only one step is awkward. The manuscript has been revised such that Step 5.1 is merged with the previous section. Numbering of subsequent sections has been updated to reflect this change.

1. Retrieve forceps, tissue specimen, mounting hardware, fine-tipped tool, glass beads, and liquid super glue. Mount the tissue specimen to the biaxial testing device by following the device’s instructions (Fig. 3d-e). While mounting, ensure that the tissue’s circumferential and radial directions are mounted with respect to the machine’s X and Y directions, as the material is anisotropic in nature.

*Mention diameter*

The manuscript has been updated to include these details that the glass beads should be of diameters of 300-500 µm.

1. Retrieve forceps, tissue specimen, mounting hardware, fine-tipped tool, glass beads, and liquid super glue. Mount the tissue specimen to the biaxial testing device by following the device’s instructions (Fig. 3d-e). While mounting, ensure that the tissue’s circumferential and radial directions are mounted with respect to the machine’s X and Y directions, as the material is anisotropic in nature.

*In order to film this, please mention how it is mounted in brief.*

We have made a “Supplementary Information” to provide the detailed procedures. Please see **Pages 1-3** of the **Supplementary Information** document.

1. To compute the appropriate membrane tension, obtain the tissue’s effective testing edge length and use the equation:  . Here, T is the membrane tension in a unit of force/length, f is the force, and L is the specimen’s effective testing length.

*Calculations cannot be filmed, until there is a visual output (e.g. screencapture) to show. Please unhighlight.*

This step has been unhighlighted in the revised manuscript.

1. Create a preconditioning protocol such that the tissue will undergo 10 loading/unloading cycles at the appropriate force coinciding with desired peak membrane tension at a loading rate of 4.42 N/min, including a preload of 2.5% of the maximum force, and a stretch and recovery time of 25 seconds.

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally please provide a screenshot for this step as a supplementary file (for internal use)*

We have included details of all software actions in the **Supplementary Information** document. Please see **Pages 4-7** for more details.

1. When the preconditioning step finishes, make a note of the current size in the X- and Y-directions. Prepare a protocol to move the specimen to the maximum force beginning from the recorded size. This will be used to determine the peak total tissue stretch and the time required to reach the peak membrane tension.

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally please provide a screenshot for this step as a supplementary file (for internal use)*

We have included details of all software actions in the **Supplementary Information** document. Please see **Pages 4-7** for more details.

1. Retrieve a stopwatch for timing purposes. Simultaneously start the move to load protocol and the stopwatch to record the time to peak membrane tension. Upon completion, stop the stopwatch and record the measured time as well as the final X- and Y-dimensions.

*Mention button clicks for scripting/filming purposes.*

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally, please provide a screenshot for this step as a supplementary file (for internal use).*

We have included details of all software actions in the **Supplementary Information** document. Please see **Page 8** for more details.

1. Prepare a force-controlled protocol such that the tissue will be subjected to the preconditioning protocol, as described in Step 7.1, before subsequent force-controlled testing.

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally please provide a screenshot for this step as a supplementary file (for internal use)*

We have included details of all software actions in the **Supplementary Information** document. Please see **Pages 9-13** for more details.

*Is this performed a second time. It is unclear why it is repeated here.*

The first preconditioning step described in new Steps 6.1-6.4 was to bring the tissue to its *in vivo* physiologically functioning condition. The loading/unloading duration timing and the deformed size determination were done afterwards, which provided information for the subsequent biaxial mechanical testing in new Step 7. Since the tissue needs to be restored to the mounting configuration before the force-controlled biaxial testing (owing to Labjoy software interface), the same preconditioning step was performed again to ensure adequate retrieval of tissue’s *in vivo* biomechanical properties.

1. After the preconditioning protocol, create testing protocols such that the tissue is loaded to peak membrane tension in the following circumferential-to-radial loading ratios at a loading rate of 4.42 N/min: 1:1, 0.75:1, 1:0.75, 0.5:1, 1:0.5 (Fig. 4). Make sure in this protocol that data and an image are recorded of the mounting configuration before any mechanical testing is performed.

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally, please provide a screenshot for this step as a supplementary file (for internal use)*

We have included details of all software actions in the **Supplementary Information** document. Please see **Pages 9-13** for more details.

1. 7.2.1) Conduct biaxial stretching in the X-direction and Y-direction to the displacements associated with corresponding peak circumferential and radial stretches, respectively (Fig. 5a).

*For 8.2.1 to 8.2.5, if you which to film the software work, please provide more software detail. Can the stretching on the specimen be filmed here?*

We have included details of all software actions in the **Supplementary Information** document. Please see **Pages 14-15** for more details. Additionally, we would suggest that the tissue stretching be filmed for all testing protocols, as it should provide better visualization of the testing performed.

*I edited 8.2.1 to match our style. Please double check that it is correct, and also edit 8.2.2 to 8.2.5 to match 8.2.1.*

We apologize for not complying to standard manuscript style. We have checked the revised section and have ensured it is correct.

1. Prepare a stress-relaxation protocol such that the tissue is loaded in each direction, at a loading rate of 4.42 N/min, to the displacement associated with the peak membrane tension (Step 7.2) and held at that displacement for 15 minutes (Fig. 6). Ensure stretch time is at least 5 seconds greater than that found in Step 7.3. After 15 minutes, the tissue is recovered to its original mounting configuration.

*Unclear what is done and what we would show here. Please describe all software actions performed including button clicks and menu selections. Additionally please provide a screenshot for this step as a supplementary file (for internal use)*

We have included details of all software actions in the **Supplementary Information** document. Please see **Page 16** for more details.

*How is this done?*

The manuscript has been revised to clarify that this should be a function of the protocol that is to be developed. The manuscript has been revised to clarify this protocol development.

1. Unmount the tissue from the biaxial testing system. Place the tissue into a container filled with 10% formalin, and then place the container in a refrigerated environment. Let the tissue be fixed for 24-48 hours, depending on the tissue’s thickness.

*Temperature?*

We have revised the manuscript to describe refrigeration temperature (4⁰C).

1. Note: The protocol can be paused here. Once tissues are fixed, specimens can be analyzed at any time.

*How are they stored?*

This note has been revised for greater understanding, describing that the tissue should be stored in a refrigerator (4⁰C) following transfer to ethanol. Also, this note has been moved to be after Step 8.2 rather than Step 8.1, as the order made better sense than what is previously presented.

1. Perform data image correlation (DIC) based tracking on the four fiducial markers from the images taken during the biaxial mechanical testing (Fig. 8) to determine the time-dependent marker positions:



where XI and xI are the undeformed and deformed positions of the markers, respectively, and dI is the displacement vector of each marker.

*Unclear.*

We apologize for the lack of clarity in our notation (1~4). We have written this equation more explicitly for better clarity (1,2,3,4).

1. FIGURE LEGENDS

*Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.*

We have expanded each figure caption to include a title and more detail such that the figures would be self-explanatory and more easily understood.