We are grateful to the reviewers and 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 with changes highlighted in blue. Below is a summary of our modifications made in the revised manuscript, for addressing both the Editorial and Reviewers' comments and for improving the clarity and readability.

- The sections of the manuscript that have been selected for filming have been highlighted.
- Some steps of the procedure have been modified for greater clarification, especially regarding which tools are utilized in certain steps, and including references to other studies with additional information where appropriate.
- Figures have been revised:
    - Figure 3: Experimental photos have been updated to better reflect correct fiducial marker placement.
    - Figure 5(a): A schematic of the biaxial stretching for displacement-controlled testing has been provided. **(Now Figure 7 after two revisions in response to Editorial Comments.)**
    - Figure 9: For demonstration of results, representative data provided was selected from one of the five atrioventricular heart valve leaflets (mitral valve anterior leaflet), and protocols have been separated into individual graphs for greater clarity. **(Now Figure 12 after two revisions in response to Editorial Comments.)**
    - Figure 10: For demonstration of results, representative data provided was selected from one of the five atrioventricular heart valve leaflets (mitral valve anterior leaflet), and protocols have been separated into individual graphs for greater clarity. **(Now Figure 13 after two revisions in response to Editorial Comments.)**
- Representative Results section has been expanded to include discussion of the provided example histology results.

Please find the following our responses to the Editorial comments and each Reviewer's comment.

(1) Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

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

   We thank the editor for the advice. The manuscript has been thoroughly proofread to ensure that the spelling and grammar are correct.

2. *Please provide an email address for each author.*

   Author emails are provided as follows:
   Colton Ross: cjross@ou.edu;
   Devin Laurence: dwlaur@ou.edu;
   Dr. Yi Wu: yiwu@ou.edu;
   Dr. Chung-Hao Lee: ch.lee@ou.edu

   These email addresses have also been incorporated into the revised manuscript.

3. 1.3: What surgical instrument is used to make an incision and how large is the incision?

A razor blade is used to make the incision along the parting line of the atria and ventricles. The incision is made along the entire outer circumference of the heart, such that the atria and all heart material superior to the ventricles may be removed.

The manuscript (**Step 1.3**) has been revised for clarification regarding this step of the protocol.

4. Please specify the surgical instrument used in each step.

The manuscript has been revised to clarify which surgical tool is being used in each step of the protocol.

5. 1.6: Please describe how to bleach treat and provide composition of the bleach used.

A solution of 10% bleach and 90% water was used to bleach treat the blood. In this procedure the blood is mixed with the 10% bleach solution and mixed continuously for approximately 10 minutes. Successful bleach treatment is indicated by the solution transitioning from a red color to a yellow color. The bleach-treated blood is then disposed of through a drainage pipe.

The manuscript has been revised (**Step 1.6**) to better clarify this bleach treating procedure.

6. 9.2 and 9.3: Please provide specific details about the histology analysis and image deconvolution methods. If they are not going to be filmed, relevant references can be added.

We have decided the histological analysis and image deconvolution methods would not be filmed for this work. We believe that the primary focus of this work should be on the mechanical characterizations of the atrioventricular heart valve leaflets. The histological analysis is considered secondary to the mechanical characterizations. Although it does provide valuable information regarding the microstructure of the tissue, it is not essential to the biaxial mechanical testing procedure described in this manuscript. As such, relevant references have been added for those histology analysis sections of the manuscript for the reader's reference if they desire additional information.

7. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The manuscript has been revised to highlight 2.75 pages of material for filming (using green bracket **[**…**]**). The highlighted steps are those central to the overall testing scheme and allows for a cohesive story to be told in the video.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step *includes at least one action that is written in imperative tense.*

When highlighting portions of the manuscript to be filmed, we ensure that complete sentences, rather than portions of sentences, were highlighted, and that the highlighted step has some imperative tense associated with the action. Any changes made to the steps to abide to the imperative tense are highlighted in blue.

9. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

We thank the editor for this information. We have ensured that all necessary sub-items have also been highlighted.

10. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Each figure has been individually converted to a .tiff file and uploaded to the Editorial Manager account. The .tiff file type was chosen for its ability to preserve high resolution images.

11. Figure 7: Please ensure that the panels are of the same dimensions if possible.

Originally, Figure 7 (now Figure 10) was created such that the images were sized where the scale bars are of the same length, which resulted in one panel being of a varying dimension. Now, the panels have been resized such that they are of equal dimension.

12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

The Table of Equipment and Materials has been reordered to be in alphabetic order in accordance to the Name of Material/Equipment.

13. References: Please do not abbreviate journal titles.

We apologize for the incorrect citation style being used in the first submission of this manuscript. References have been updated in our revised manuscript.

(2) Reviewers' comments:

(2-a) Reviewer #1:

Manuscript Summary:

The goal of the manuscript is to provide a protocol of how to obtain biaxial mechanical properties of atrioventricular heart valves. Using porcine or ovine, the authors describe the process of dissecting the valves and assembling them to a planar biaxial testing device. The samples undergo force-controlled, displacement-controlled mechanical tests and a stress-relaxation test. Finally, the process to obtain strain measurements from fiduciary markers is described.

We thank the reviewer for the summary of our presented work.

Major Concerns:

The protocol described by the authors is not novel. It has been done extensively by many groups and in different tissues. Groups carrying out this protocol have been published in many manuscripts and thesis dissertations (i.e., one of the leaders in building these testing devices and carrying out the mechanical tests of valves and myocardium is Michael Sacks or Gerard Holzapfel).

We thank the reviewer for providing the constructive comment.

We would also like to recognize the biaxial mechanical protocols described in our manuscript are not some completely new development, which have been discussed previously in PhD dissertations as well as in journal publications.[1-12]

Nevertheless, there is benefit in the use of one unified testing scheme, which is *the first of its kind*, where the same tissue specimen could be systematically biaxially tested under force-controlled, displacement-controlled (including pure shear testing), and stress-relaxation protocols. Therefore, our protocol attempts to fill this gap by providing a testing scheme for one individual tissue under the various loading conditions, which has not been comprehensively documented in previous literature.

In addition, to our understanding, JoVE does not have a published manuscript detailing these procedures specifically for heart valves. Hence, this work still adds its important value to the field of heart valve biaxial mechanical testing, which is readily applicable for characterizing other biological tissues.

Furthermore, there are many gaps in terms of choices made by the authors:

1. *Working with muscles, the question of contractility and viability comes up. The authors include in their protocol the usage of PBS to transport and store heart and valves. But solutions like cardioplegic solution is large more acceptable if you are to decrease the metabolic demand of the tissue and be able to preserve it longer. There are other solutions or modified versions of PBS to exclude Ca and Mg.*

   We agree with the reviewer that cardioplegia solution should be used during transport for lowering metabolic demand of tissues. This solution is especially useful in cases where living tissue is a necessity, such as in the construction of a Langendorff system or Working Heart model. However, for the mechanical testing procedure provided, the heart valve tissues are not required to be in a living state, as the observed mechanical properties will be similar with the dead tissue. This is primarily because the heart valves were found to be functionally passive and do not exhibit contractility as observed in muscles or myocardium tissues. Additionally, the use of phosphate buffered saline solution in transportation and testing is common practice in biaxial mechanical testing of heart valve tissues, further supporting our methods.[3,13-19]

2. Along with temperature and solution types, to mimic closer the environment of these tissues it is important to include oxygen. Here the authors make no mention of it or do they include it in their protocol.

We gratefully thank the reviewer for their concern regarding our testing method. However, for the testing scheme provided, the heart valve tissues are not required to be in a living state, as stated previously. This is because the observed mechanical properties will be similar whether the specimen is a living or non-living tissue. Because of this, the tissue does not require an ***oxygenated***, phosphate-buffered saline solution (PBS). This is further supported by previous studies in which biaxial mechanical testing is performed with non-oxygenated PBS solution.[3,13-19]

3. For the biaxial test to be representative of the mechanical properties of a given tissue have to satisfy the membrane assumption, usually considered the thickness less than 10% the length and width. This is not mentioned.

We thank the reviewer for noticing our missing justification in the description of the membrane tension stress measure. Indeed, the membrane assumption is valid for all the valve leaflet tissues we have worked with: tissue thickness=0.3 mm for the TV and 0.4 mm for the MV; effective specimen size (tin-to-tin distance) = 7.5 mm.

We have also provided clarification regarding the use of this stress measure in the revised manuscript (**Introduction**).

4. Mechanically testing tissues with rigid arms impose a set of artificial boundary conditions and prevents the sample to rotate. Thus, it is a limitation in their testing approach and prevents from obtaining "pure shear".

We thank the reviewer for pointing out this issue.

The testing apparatus (CellScale Biotester with BioRake fixture) used in the presented methodology is limited to an irrotational testing scheme, due to the rigid arms, and, thus, *simple shear* is definitely not obtainable in the current setup. Future investigations may be warranted to induce rotational, simple shear on the tissue specimens, but this would require a suture-type tissue mounting mechanism allowing rotations.[20]

In contrast, what we considered in our testing procedure is the “pure shear” protocol, which was designed for investigating the combined shear effect by allowing elongation in one direction and shortening in the opposite direction, while maintaining a constant area. Through this extension and compression, the tissue experiences shear stress, which could be further incorporated in the development of valve-specific constitutive models.

Please refer to **Figure R1** below for an illustration of the differences between pure shear and simple shear.

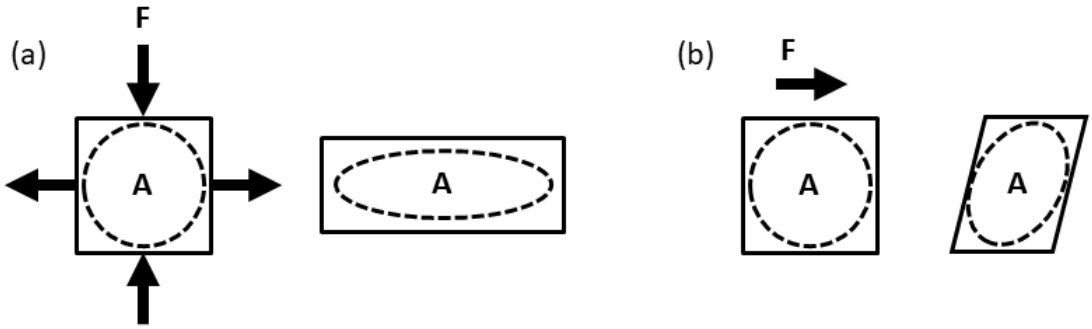

Figure R1: (a) Pure shear involves elongation of one direction of a 2D element while the other direction is shortened (irrotational shear). (b) Simple shear involves the rotation of a sample about a fixed point in the direction of applied displacement.

5. Preconditioning of a tissue varies hence saying 10 cycles is not accurate. It should be based on the cycles needed to reach reproducibility of the stress-strain curves (or force-displacement curves).

We gratefully thank the reviewer for the constructive feedback.

We totally agree with the reviewer that the number of loading/unloading cycles for the preconditioning step should be determined based upon whether the force-displacement curve between 2-3 consecutive cycles should reach a reproducible state. Based on our prior study on porcine mitral and tricuspid valves (2018 JMBBM paper), it usually took about 6-7 cycles to reach reproducibility of the force-displacement curves, by using a loading rate of 4.42 N/min. Thus, 10 cycles were selected to ensure the tissues stress-strain curves are uniform at the end of the preconditioning cycle.

The manuscript has been revised to clarify this in our biaxial mechanical testing protocols (**Introduction**).

6. How the authors described the calculation of strain based on the marker position is poor - need more details and *or references. Based on their description it would be hard for someone to reproduce.*

We thank the reviewer for providing the constructive feedback.

With regards to calculating the marker positions using a data image correlation (DIC) technique, the procedure will vary by software. In the case of the LabJoy software, as used in this protocol, the DIC method outputs an Excel document containing locations of the markers in terms of micrometers for each image. (c.f. https://cellscale.com/wp-content/uploads/2017/01/BioTester-User-Manual-v7.4.pdf)

Based on the captured/tracked history of the fiducial marker positions, we derived the marker locations at the reference configuration, $\mathbf{X}_I$, and the deformed marker locations, $\mathbf{x}_I$. Then, we followed the methods described in our manuscript. We have included citations to our previous work and the studies that originally incorporated these methods (using a single quadrilateral finite element) to the revised manuscript for clarification (**Discussion**).[3,21,22]

7. Not sure what Figure 7 adds.

Figure 7 (now Figure 10) was provided as an example of using histology (Masson's trichrome staining) to show the microstructural constituents in the mitral (**Fig. 7a**) and tricuspid (**Fig. 7b**) valve leaflets. The primary purpose was to provide an example data set for the reader to reference to supplement the histology procedures, similar to what was done for the biaxial mechanical testing.

We apologize for not providing enough information in the original manuscript about the purpose of this figure. Discussion regarding these representative results have been included in the corresponding section of the manuscript (**Representative Results**).

8. Markers are placed too close to the edges. It has been studied how to avoid artificial effects from grips and to better distribute stress across the tissues, the markers need to be place in the middle third of the sample.

We thank the reader for providing the valuable comments regarding the placement of fiducial markers on the tissue specimens.

We have acknowledged this comment by making a clarification in the revised manuscript. We also recognized our original **Figure 8** (now Figure 11) was not representative of this requirement and have selected another set of pictures to illustrate the marker positioning.

In addition, we have revised **Figure 3**, as the original experimental pictures provided did not have the appropriate marker placement depicted. We apologize for our initial oversight in the selection of these photos. Sometimes, newer lab undergraduate researchers, who are still familiarizing themselves with the procedures, may place their markers slightly outside of the required middle third area. For data analysis and preparation of data for publications, we ensure data used has proper marker placement, but missed it for preparation of this manuscript. In the revised **Figure 3**, we have provided experimental photos that better demonstrate the correct fiducial marker placement and what we consider for data analysis.

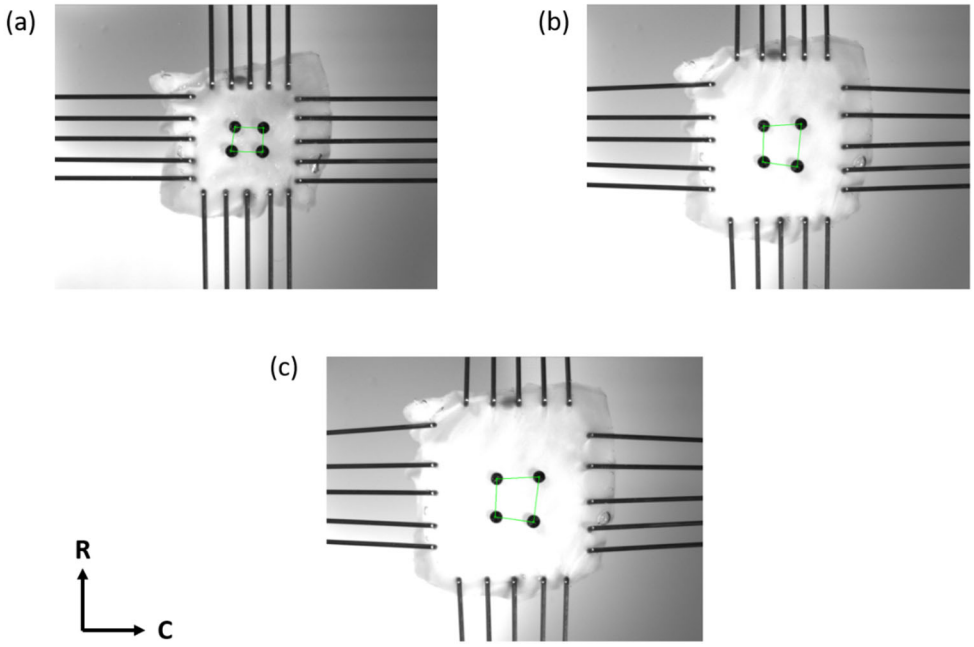

Figure 11: Representative images illustrating the tracking of the coordinates of four fiducial markers during biaxial mechanical testing using a data image correlation (DIC) technique: (a) the mounting configuration, (b) the reference configuration after the preconditioning step, and (c) the deformed configuration associated with tissue specimen under mechanical loading.

9. There is no mention of reference configurations - how to choose and process data based on the reference chosen *for analysis.*

We apologize for missing the information about the reference configuration.

Basically, three configurations/states are pertinent to our biaxial mechanical testing experiments: (i) the original configuration (Ω_0) when tissue was just mounted to the system; (ii) the post-preconditioning configuration (Ω_1) when the tissue specimen underwent 10 loading/unloading cycles for reaching its *in vivo* physiological state; (iii) the loaded configuration (Ω_t).

As for the displacement-controlled testing, the original configuration (Ω_0) was chosen as the reference configuration (c.f. **Figure 13**). On the other hand, for the force-controlled testing, we typically generate the stress-stretch plots with respect to both the original configuration (Ω_0) and the post-preconditioning configuration (Ω_1). The figures in our previous manuscript submission showing the representative force-controlled and displacement-controlled testing data (**Figure 12** and **Figure 13**) were not prepared in the most clear presentation, i.e., force-controlled testing data with respect to Ω_1, whereas displacement-controlled data with respect to Ω_0.

For better clarity and avoiding confusions, in our revised manuscript (c.f. **Step 9.1.1**), we have used the same configuration as the reference configuration (Ω_0) for plotting the stress-stretch data for both the force-controlled and displacement-controlled tests.

10. How do you account for the offset/shift of stretch in Figure 10.

We apologize for the confusion and misleading of our Figure 10 for showing the representative displacement-controlled testing data.

In fact, the stress-stretch data from all the displacement-controlled testing protocols started at the relaxed/stress-free state (1.0, 0.0), as the original configuration (Ω_0) was chosen for its stress-strain calculations. Please also see our response to Reviewer #1's Comment 9 for more details about the reference configuration.

For improving the clarity, Figure 10, as also shown on the next page, has been updated by separating the results from each testing protocol into each individual subplot.

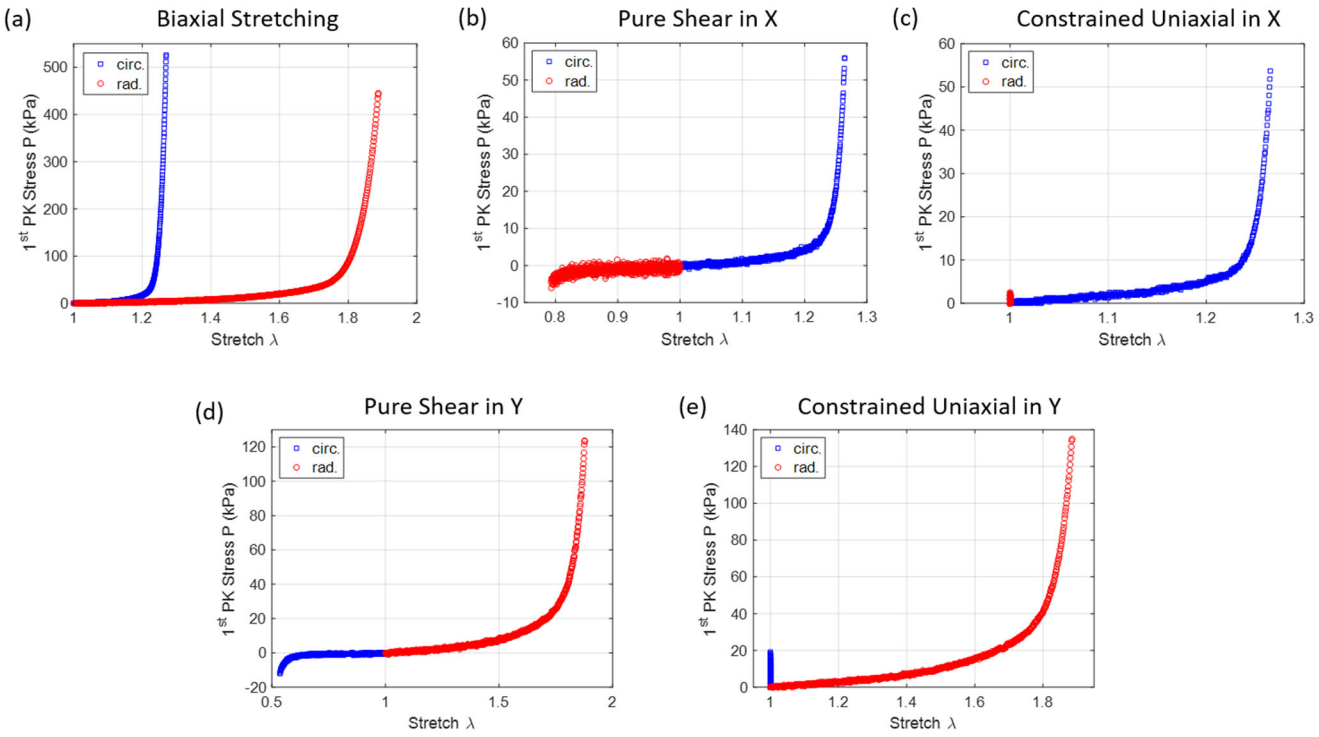

Figure 13: (a-e) Representative data from displacement-controlled protocols for the mitral valve anterior leaflet are provided.

Minor Concern:

1. To fix a tissue, after formalin the sample needs to go in ethanol - this is missing in their protocol.

We thank the reviewer for pointing out our missing information.

Standard histology procedures in this work, including dehydration, paraffin-embedding, sectioning, and staining, were usually performed within a few days after complete fixation in 10% formalin. If the fixed tissue specimens need to be stored for a longer term (e.g. > 48 hours), we would store them in 80% ethanol after complete formalin-fixation.

The manuscript has been updated to reflect this change in a new **Step 8.2**.

(2-b) Reviewer #2:

Manuscript Summary:

This paper presented biaxial mechanical testing protocol from tissue acquisition, preparation of tissue specimens, biaxial mechanical testing, to postprocessing of the acquired data. The paper briefly summarized the significance; the preparation and test protocol were clearly described, and the results were well illustrated.

We thank the reviewer for the summary of our presented biaxial mechanical testing procedures.

Major Concerns:

There is no major concern.

Minor Concerns:

1. Please specify (or approximately estimated) the loading rate of biaxial test.

We apologize for not including this information in the manuscript. In our biaxial mechanical testing, we used a loading rate of 4.42 N/min.

The manuscript has been revised (**Introduction, Step 6.1, Step 7.1, Step 7.2, and Step 7.3**) to clarify the loading rate used in the presented methodology.

2. Please specify the specific staining in histology.

We thank the reviewer for the constructive comment.

The specific stain used in **Figure 7** (now Figure 10) is Masson's Trichrome (collagen: blue, black: nuclei, red: cytoplasm & keratin). We intended to write our protocol as an open-ended one regarding the selected stain. The readers/researchers would allow to select specific stain(s) necessary to quantify the certain microstructural constituents of the tissues.

The caption for **Figure 7** (now Figure 10), in the revised manuscript, specifically details the stain used in those specific images.

3. It seems that there is missing information in the protocol of how the isotropy of the tissue is identified before the specimen is mounted.

We thank the reviewer for pointing out this oversight or the unclearness.

We included information about identifying the anisotropy of the tissue in **Step 3.4** by using surgical pen markers in the radial direction at the time of leaflet sectioning but did not clarify the purpose of this action. **Step 5.2** has been revised to include information about using the identified isotropy of the tissue in mounting.

In addition to this clarification in the procedures, **Fig. 3b** has been revised to visualize the use of markers for identifying the valve leaflet tissue's radial direction after sectioning.

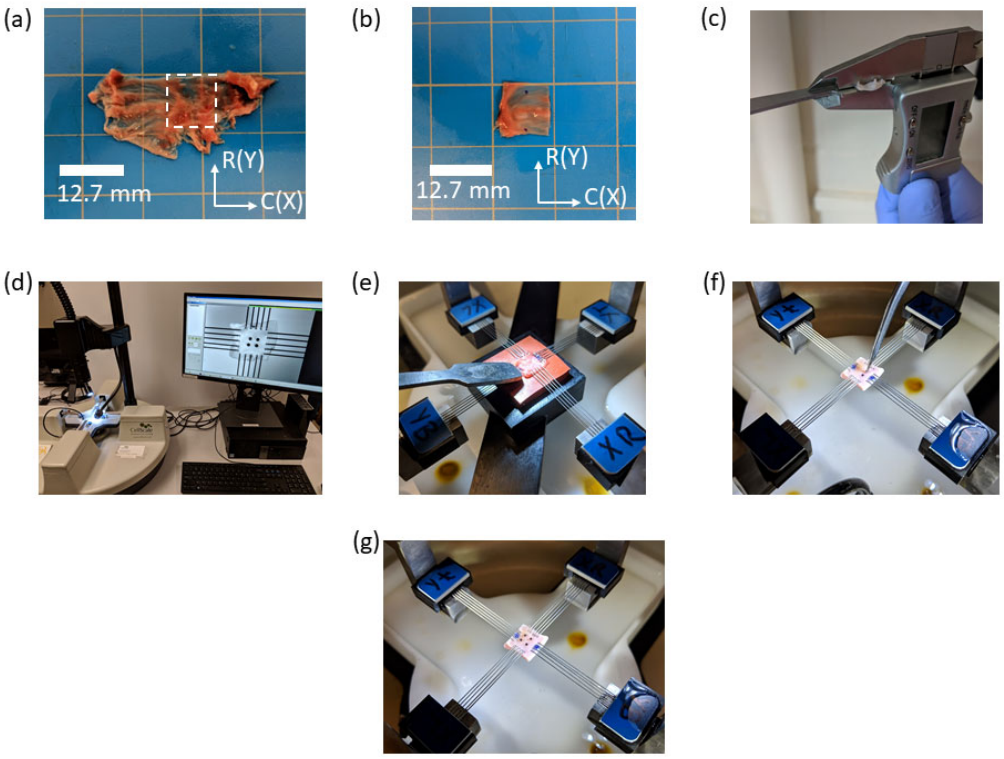

Figure 3: Heart valve leaflet testing requires the (a) bulk leaflet be sectioned into a (b) 10mm x 10mm testing region (radial direction noted by surgical pen markers). (c) The leaflet thickness is measured. Specimens are mounted to (d) the biaxial testing system by (e) piercing the tissue with metal tines. After mounting, (f) fiducial markers are glued onto the surface of the tissue before (g) submersion in PBS solution at 37°C.

Literature Cited in This Rebuttal Letter

1 Sacks, M. A method for planar biaxial mechanical testing that includes in-plane shear. *Journal of biomechanical engineering.* **121** (5), 551-555, (1999).
2 Sun, W., Sacks, M. S. & Scott, M. J. Effects of boundary conditions on the estimation of the planar biaxial mechanical properties of soft tissues. *Journal of biomechanical engineering.* **127** (4), 709-715, (2005).
3 Sacks, M. S. Biaxial mechanical evaluation of planar biological materials. *Journal of Elasticity.* **61** (1), 199, (2000).
4 May-Newman, K. & Yin, F. Biaxial mechanical behavior of excised porcine mitral valve leaflets. *American Journal of Physiology-Heart and Circulatory Physiology.* **269** (4), H1319-H1327, (1995).
5 Lanir, Y. & Fung, Y. Two-dimensional mechanical properties of rabbit skin—II. Experimental results. *Journal of Biomechanics.* **7** (2), 171-182, (1974).
6 Sacks, M. & Chuong, C. Biaxial mechanical properties of passive right ventricular free wall myocardium. *Journal of biomechanical engineering.* **115** (2), 202-205, (1993).
7 Waldman, S. D. & Lee, J. M. Effect of sample geometry on the apparent biaxial mechanical behaviour of planar connective tissues. *Biomaterials.* **26** (35), 7504-7513, (2005).
8 Sun, W., Sacks, M. S., Sellaro, T. L., Slaughter, W. S. & Scott, M. J. Biaxial mechanical response of bioprosthetic heart valve biomaterials to high in-plane shear. *Journal of biomechanical engineering.* **125** (3), 372-380, (2003).
9 Schulze-Bauer, C. A., Mörth, C. & Holzapfel, G. A. Passive biaxial mechanical response of aged human iliac arteries. *Journal of biomechanical engineering.* **125** (3), 395-406, (2003).
10 Sommer, G., Regitnig, P., Költringer, L. & Holzapfel, G. A. Biaxial mechanical properties of intact and layer-dissected human carotid arteries at physiological and supraphysiological loadings. *American Journal of Physiology-Heart and Circulatory Physiology.* **298** (3), H898-H912, (2009).
11 Tong, J., Cohnert, T., Regitnig, P. & Holzapfel, G. A. Effects of age on the elastic properties of the intraluminal thrombus and the thrombus-covered wall in abdominal aortic aneurysms: biaxial extension behaviour and material modelling. *European journal of vascular and endovascular surgery.* **42** (2), 207-219, (2011).
12 Sommer, G. & Holzapfel, G. A. 3D constitutive modeling of the biaxial mechanical response of intact and layer-dissected human carotid arteries. *Journal of the mechanical behavior of biomedical materials.* **5** (1), 116-128, (2012).
13 Billiar, K. L. & Sacks, M. S. Biaxial mechanical properties of the natural and glutaraldehyde treated aortic valve cusp-Part I: Experimental results. *Transactions-American Society of Mechanical Engineers Journal of Biomechanical Engineering.* **122** (1), 23-30, (2000).
14 Stella, J. A. & Sacks, M. S. On the biaxial mechanical properties of the layers of the aortic valve leaflet. *Journal of biomechanical engineering.* **129** (5), 757-766, (2007).
15 Grashow, J. S., Yoganathan, A. P. & Sacks, M. S. Biaixal stress–stretch behavior of the mitral valve anterior leaflet at physiologic strain rates. *Annals of biomedical engineering.* **34** (2), 315-325, (2006).
16 Tower, T. T., Neidert, M. R. & Tranquillo, R. T. Fiber Alignment Imaging During Mechanical Testing of Soft Tissues. *Annals of biomedical engineering.* **30** (10), 1221-1233, (2002).
17 Grashow, J. S., Sacks, M. S., Liao, J. & Yoganathan, A. P. Planar Biaxial Creep and Stress Relaxation of the Mitral Valve Anterior Leaflet. *Annals of biomedical engineering.* **34** (10), 1509-1518, (2006).
18 Prot, V., Skallerud, B., Sommer, G. & Holzapfel, G. A. On modelling and analysis of healthy and pathological human mitral valves: two case studies. *Journal of the mechanical behavior of biomedical materials.* **3** (2), 167-177, (2010).
19 Eckert, C. E. *et al.* On the biomechanical role of glycosaminoglycans in the aortic heart valve leaflet. *Acta Biomaterialia.* **9** (1), 4653-4660, (2013).
20 Potter, S. *et al.* A Novel Small-Specimen Planar Biaxial Testing System With Full In-Plane Deformation Control. *Journal of biomechanical engineering.* **140** (5), 051001, (2018).
21 Jett, S. *et al.* An investigation of the anisotropic mechanical properties and anatomical structure of porcine atrioventricular heart valves. *Journal of the mechanical behavior of biomedical materials.* **87** 155-171, (2018).
22 Billiar, K. & Sacks, M. A method to quantify the fiber kinematics of planar tissues under biaxial stretch. *Journal of Biomechanics.* **30** (7), 753-756, (1997).