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Corresponding Author:	Chung-Hao Lee University of Oklahoma Norman, OK UNITED STATES
Corresponding Author's Institution:	University of Oklahoma
Corresponding Author E-Mail:	ch.lee@ou.edu
Order of Authors:	Colton Ross
	Devin Laurence
	Yi Wu
	Chung-Hao Lee
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SCHOOL OF AEROSPACE & MECHANICAL ENGINEERING

November 26, 2018

Dr. Nandita Singh
Senior Science Editor

Dr. Alisha DSouza
Senior Review Editor

Journal of Visualized Experiments
1 Alewife Center Suite 200
Cambridge, MA 02140, USA

RE: Cover Letter for the Revised Manuscript (JoVE59170) Resubmitted to the Journal of Visualized Experiments (JoVE)

Dear Dr. Singh and Dr. DSouza,

Enclosed please find our revised manuscript for resubmission to the Journal of Visualized Experiments (JoVE), titled "*Biaxial Mechanical Characterizations of Atrioventricular Heart Valves*," authored by Colton Ross, Devin Laurence, Dr. Yi Wu, and myself.

We are greatly grateful for the constructive and thoughtful comments received from the review, and we have taken these comments into consideration in the revision and improvement of our manuscript. Changes in the manuscript, along with our responses to both the editorial comments and the reviewers' comments, are provided in the "Rebuttal Document" as part of our resubmission.

In addition, all authors were fully involved in the study and preparation of the manuscript and that the material within has not been and will not be submitted for publication elsewhere. The novelty and research highlight are briefly summarized below:

- A biaxial mechanical testing procedure has been, for the first time, documented for mechanical characterization of atrioventricular heart valve leaflets;
- Such a novel, unified tissue mechanical characterization experiment consists of (i) a force-controlled biaxial testing protocol, (ii) a displacement-controlled biaxial testing protocol, and (iii) a biaxial stress-relaxation testing protocol.

The experimental protocol presented in this revised manuscript is applicable not only to heart valve leaflet tissues but also to other soft biological tissues, or polymers/rubber-type materials. The provided scheme would provide for full characterization of any such materials compatible with a biaxial testing device.

Telephone: 405-325-4842 FAX: 405-325-1088 E-mail: ch.lee@ou.edu

We believe this paper will be of great interest to the audience of the JoVE. We hope that you will see the work in a similar light.

Thanks for your time and consideration.

Sincerely,

Chung-Hao Lee, Ph.D.
Assistant Professor
School of Aerospace and Mechanical Engineering
Faculty Member
Institute for Biomedical Engineering, Science and Technology
The University of Oklahoma

TITLE:

Biaxial Mechanical Characterizations of Atrioventricular Heart Valves

AUTHORS AND AFFILIATIONS:

Colton Ross[1,*], Devin Laurence[1,*], Yi Wu[1], Chung-Hao Lee[1,2]

[1]Biomechanics and Biomaterials Design Laboratory (BBDL), School of Aerospace and Mechanical Engineering, The University of Oklahoma, Norman, OK, USA

[2]Institute for Biomedical Engineering, Science and Technology (IBEST), The University of Oklahoma, Norman, OK, USA

*These authors contributed equally.

E-mail addresses of co-authors:

Colton Ross (cjross@ou.eu)

Devin Lawrence (dwlaur@ou.edu)

Yi Wu (yiwu@ou.edu)

Corresponding author:

Chung-Hao Lee (ch.lee@ou.edu)

Assistant Professor, School of Aerospace and Mechanical Engineering (AME)

Affiliated Faculty Member, Institute for Biomedical Engineering, Science and Technology (IBEST)

The University of Oklahoma

865 Asp Ave., Felgar Hall Room 219C

Norman, OK 73019, USA

Office: (405) 325-4842

KEYWORDS:

Biaxial mechanical testing; mitral valve; tricuspid valve; tissue biomechanics; stress & strain calculations; digital image correlation; histological analysis; stress relaxation

SUMMARY:

This protocol involves characterizations of atrioventricular valve leaflets with force-controlled, displacement-controlled, and stress-relaxation biaxial mechanical testing procedures. Results acquired with this protocol can be used for constitutive model development to simulate the mechanical behavior of functioning valves under a finite element simulation framework.

ABSTRACT:

Extensive biaxial mechanical testing of the atrioventricular heart valve leaflets can be utilized to derive at optimal parameters used in constitutive models which provide a mathematical representation of the mechanical function of those structures. This presented biaxial mechanical testing protocol involves (i) tissue acquisition, (ii) the preparation of tissue specimens, (iii) biaxial mechanical testing, and (iv) postprocessing of the acquired data. First, tissue acquisition requires obtaining porcine or ovine hearts from a local food administration-approved abattoir for later

dissection to retrieve the valve leaflets. Second, tissue preparation requires using tissue specimen cutters on the leaflet tissue to extract a clear zone for testing. Third, biaxial mechanical testing of the leaflet specimen requires the use of a commercial biaxial mechanical tester, which consists of force-controlled, displacement-controlled, and stress-relaxation testing protocols to characterize the leaflet tissue's mechanical properties. Finally, postprocessing requires the use of data image correlation techniques and force and displacement readings to summarize the tissue's mechanical behaviors in response to external loading. In general, results from biaxial testing demonstrate that the leaflet tissues yield a nonlinear, anisotropic mechanical response. The presented biaxial testing procedure is advantageous to other methods since the method presented here allows for a more comprehensive characterization of the valve leaflet tissue under one unified testing scheme, as opposed to separate testing protocols on different tissue specimens. The proposed testing method has its limitations in that shear stress is potentially present in the tissue sample. However, any potential shear is presumed negligible.

INTRODUCTION:

Proper heart function relies on appropriate mechanical behaviors of the heart valve leaflets. In situations where heart valve leaflet mechanics are compromised, heart valve disease occurs, which may lead to other heart-related issues. Understanding heart valve disease requires a thorough understanding of the leaflets' proper mechanical behaviors for use in computational models and therapeutic development, and as such, a testing scheme must be developed to accurately retrieve the healthy leaflets' mechanical properties. In previous literature, this mechanical characterization has been conducted using biaxial mechanical testing procedures.

Biaxial mechanical testing procedures for soft tissues vary throughout the literature, with different testing frameworks utilized to retrieve different characteristics[1–19]. Testing methods have been extended for investigations of the mechanical characteristics of heart valve leaflets. In general, biaxial mechanical testing involves loading the heart valve tissue with simultaneous forces in the two principal directions, but how this testing is performed varies based on the biomechanical properties to be observed. Some of these testing protocols include (i) strain-rate, (ii) creep, (iii) stress-relaxation, and (iv) force-controlled testing.

First, strain-rate testing has been utilized to determine the time-dependent behaviors of the tissue leaflets[18,20]. In this testing protocol, leaflets are loaded to a maximum membrane tension at different half-cycle times (i.e., 1, 0.5, 0.1, and 0.05 s) to determine if there is a significant difference in peak stretch or hysteresis between loading times. However, these tests have demonstrated a negligible difference in the observed stretch with varying strain-rates. Second, in creep testing, the tissue is loaded to the peak membrane tension and held at peak membrane tension. This testing allows a demonstration of how the tissue's displacement creeps to maintain the peak membrane tension. However, it has been shown that the creep is insignificant for heart valve leaflets under physiologically functioning[3,20]. Third, in stress-relaxation testing, the tissue is loaded to the peak membrane tension and the associated displacement is held constant for an extended period of time[3,21,22]. In this type of testing, the tissue stress has a notable reduction from the peak membrane tension. Lastly, in force-controlled testing, tissues are cyclically loaded at various ratios of the peak membrane tension in each direction[17,23]. These tests reveal the

material's anisotropy and nonlinear stress-strain response, and by loading the tissue under various ratios, potential physiologic deformations may be better understood. These recent investigations made it apparent that stress-relaxation and force-controlled protocols prove most beneficial to perform a mechanical characterization of heart valve leaflets. Despite these advances in heart valve biomechanical characterization, the testing has not been performed under one **unified** testing scheme, and there are limited methods to investigate the coupling between directions.

The purpose of this method is to facilitate a full material characterization of the heart valve leaflets by a unified biaxial mechanical testing scheme. A unified testing scheme is considered as one where each leaflet is tested under all testing protocols in one session. This is advantageous, as tissue properties are inherently variable between leaflets, so a full characterization for each leaflet proves more accurate as a descriptor than performing each protocol independently on various leaflets. The testing scheme consists of three main components, namely (i) a force-controlled biaxial testing protocol, (ii) a displacement-controlled biaxial testing protocol, and (iii) a biaxial stress-relaxation testing protocol. All testing schemes utilize a loading rate of 4.42 N/min, and 10 loading-unloading cycles to ensure stress-strain curve replicability by the 10th cycle (as found in previous work).[23] All protocols are also constructed based on the membrane tension assumption, which requires that the thickness be less than 10% of the effective specimen lengths.

The force-controlled protocol used in this presented method consists of 10 loading and unloading cycles with peak membrane tensions of 100 N/m and 75 N/m for the mitral valve (MV) and tricuspid valve (TV), respectively[15,17]. Five loading ratios are considered in this force-controlled testing protocol, namely 1:1, 0.75:1, 1:0.75, 0.5:1, and 1:0.5. These five loading ratios prove useful in describing the stresses and strains correspondent to all potential physiologic deformations of the leaflet in vivo.

The displacement-controlled protocol presented in this method consists of two deformation scenarios, namely (i) constrained uniaxial stretching and (ii) pure shear. In the constrained uniaxial stretching, one direction of the tissue is displaced to the peak membrane tension while fixing the other direction. In the pure shear setup, the tissue is stretched in one direction and judiciously shortened in the other direction, so the area of the tissue remains constant under deformation. Each of these displacement-controlled testing procedures is performed for each of the two tissue directions (circumferential and radial directions).

The stress-relaxation protocol used in the presented method is achieved by loading the tissue to the peak membrane tension in both directions and holding the tissue at the correspondent displacements for 15 min to monitor the tissue's stress relaxation behavior. The detailed experimental procedures are discussed next.

PROTOCOL:

All methods described were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Oklahoma. All animal tissues were acquired from a United States

Department of Agriculture (USDA)-approved slaughterhouse (Country Home Meat Co., Edmond, OK).

1. Tissue acquisition and cleaning

1.1) Retrieve the animal hearts on the same day as the animal is slaughtered and store the hearts in an ice chest to ensure the tissue freshness. Transport the hearts to the laboratory space.

1.2) Upon arrival at the lab, submerge the heart in a bucket of phosphate-buffered saline (PBS) solution to rinse off any excess blood. Retrieve forceps, a placemat, a surgical blade, a bucket of PBS solution, bleach, and a plastic bag. Prepare the placemat by laying it on the dissection counter, allowing for an easier cleanup of blood-related mess. After the heart has been sufficiently rinsed, place the heart on the placemat (**Figure 1a**).

1.3) Using the forceps, locate the parting line between the atria and the ventricle on each side of the heart. Using a razor blade, carefully make an incision along this parting line and reveal the heart valves and the ventricles (**Figure 1b**). Make the incision along the entire outer circumference of the heart, such that the atria and all heart material superior to the ventricles may be removed.

1.4) With the forceps, carefully pull out any observed blood clots in the ventricles (**Figure 1c**). If an attempt is made to remove a blood clot but it does not move, ensure the chordae tendineae or leaflets have not been grabbed. Place blood clots in the biohazard bag for waste disposal.

1.5) When all blood clots have been removed from the ventricles, rinse the heart one final time in a bucket with PBS solution. Place the clean heart in the plastic bag and store it in a freezer.

1.6) Using a solution of 10% bleach and 90% water, mix the blood with the bleach solution and stir continuously for approximately 10 min. Look for a successful bleach treatment, indicated by the solution transitioning from red to yellow. Dispose of the bleach-treated blood through drainage.

CAUTION: Bleach is a toxic substance and can be harmful if ingested.

NOTE: The protocol can be paused here.

2. Heart dissection and examination of anatomy

2.1) Retrieve the previously cleaned heart and allow it to thaw in a warm water bath. The required materials for dissection include forceps, surgical blades, placemats, PBS, and small storage containers. After the heart is completely thawed, put it on a placemat to absorb any remaining blood.

2.2) Hold the heart for a top-down (superior) view to optimally observe the valve structures. Beginning with the MV on the left side of the heart, use forceps to carefully manipulate the leaflets and identify a commissure, or parting line, between the leaflets.

2.3) Make an incision along the commissure and carefully cut through the ventricular wall, making sure not to damage the leaflets. It may be necessary to cut the chordal attachments during this process to fully open the ventricle. Once the full incision is made, open the ventricle (**Figure 2a**).

2.4) Identify the MV anterior and posterior leaflets and use a surgical blade to sever the chordal attachments to the papillary muscles. Using forceps, stretch the leaflets taut and make cuts to separate the leaflets from the annulus. Place the excised leaflets in an appropriately labeled container filled with PBS solution and store it in a refrigerator at approximately 4 °C.

2.5) Hold the heart for a top-down view and identify the TV on the right side of the heart. Locate the commissures and make an incision through one of the commissures and the ventricular wall (**Figure 2b**).

2.6) Identify the TV septal, posterior, and anterior leaflets, and perform the leaflet extraction as done in step 2.4. Place all obtained leaflets in a labeled container filled with PBS solution and store the container in a refrigerator at approximately 4 °C.

NOTE: The protocol can be paused here. However, tissue biomechanical testing and the subsequent histology analyses should occur within 2 days of the heart dissection.

3. Tissue dissection

3.1) Retrieve a leaflet from the fridge, the tissue cutter for the specified sectioning size, a surgical pen, forceps, razor blades, and a cutting mat.

3.2) Using forceps, remove the specimen from the PBS solution and lay it flat on the cutting mat with the radial direction (R) aligned to the Y-direction, and the circumferential direction (C) aligned to the X-direction (**Figure 3a**). Identify the leaflet's **central region** as the testing section.

3.3) Align the tissue cutter so that the desired tissue testing region is within the boundaries of the razor blades. Make one cut horizontally and another vertically to form a square region of the desired dimensions (**Figure 3b**). Using the surgical pen, label the tissue's radial direction (**Figure 3b**).

3.4) Using the razor blade, trim any chordal attachments by stretching the chordae from the leaflet with the forceps and making a careful cut without causing any damage to the leaflet.

NOTE: The protocol can be paused here. If the protocol is paused, store the sectioned tissue in a labeled container filled with PBS solution, and store the container in a refrigerator at

approximately 4 °C (as explained in step 2.6). However, tissue testing should occur within 2 days of the dissection.

4. Thickness measurement and biaxial tester setup

4.1) Retrieve the sectioned tissue specimen, digital calipers, and a small metal spatula. Using the digital calipers, measure and record the thickness of the metal spatula.

4.2) Using forceps, lay the tissue specimen flat on the metal spatula. Using the digital calipers, measure the thickness of the spatula-tissue pair (**Figure 3c**) at three different leaflet locations. Subtract the spatula's thickness from each measurement and record the average thickness.

4.3) Prepare a PBS bath at 37 °C, which corresponds to the tissue's physiologic conditions.

5. Tissue mounting and fiducial marker placement

5.1) Retrieve forceps, the tissue specimen, mounting hardware, a fine-tipped tool, glass beads (with diameters of 300–500 µm), and super glue.

5.2) Mount the tissue to the biaxial testing system (**Figure 3d,e**). While mounting, ensure that the tissue's circumferential and radial directions are aligned with the machine's X- and Y-directions.

5.3) For the fiducial marker placement, place glass beads into one small open-faced container and a small pool of super glue in another container. Using the fine-tipped tool, coat the tip with a small amount of super glue and stick an individual bead to the tip of the tool.

5.4) Carefully use the tool to transfer the bead to one corner of the middle third of the tissue's testing region (**Figure 3f**). Repeat this step until a square array of four beads is formed (**Figure 3g**).

NOTE: It is crucial that excess glue is avoided, and that the fiducial markers do not stick together as later data image correlation (DIC) techniques will produce useless tracking results. It is important that the square array must be within the middle third of the tissue's testing region.

6. Preconditioning step and duration timing

6.1) To compute the appropriate membrane tension, obtain the tissue's effective testing edge length and use the following equation.

$$T = diag[T_C, T_R] = diag[f_C, f_R]/L \quad (1)$$

NOTE: 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.

6.2) Create a preconditioning protocol so that the tissue will undergo 10 loading/unloading cycles at the forces associated with peak membrane tension at a loading rate of 4.42 N/min, including a preload of 2.5% of the maximum force (**Figure 4**).

6.2.1) Create a new arbitrary testing directory to temporarily store the preconditioning data, because it is not necessary for future calculations. Establish a loading rate of 4.42 N/min for subsequent testing.

6.2.2) Create a new set of testing parameters and set the name of the protocol as **Preconditioning0** (**Figure 4a**). For the X- and Y-axes, set the **control mode** to be **force** and the **control function** to be **step**. Set the **load magnitude** as the force associated with targeted peak membrane tension (cf. step 6.1) (**Figure 4b**). Set the **preload magnitude** as 2.5% of the maximum force for the first repetition only (**Figure 4c**). Set the **stretch duration** and **recovery duration** both to be 25 s. Set the **number of repetitions** to be 10 (**Figure 4e**).

6.3) When the preconditioning step finishes, make a note of the tissue's deformation in the X- and Y-directions. Prepare a protocol to move the specimen to the maximum force, beginning from the recorded size.

6.3.1) Retrieve a stopwatch for timing purposes. Begin the maximum force loading protocol and start the stopwatch simultaneously when the machine begins actuation (**Figure 5a**). Stop the stopwatch when the actuation stops. Stopping will be obvious through auditory cues.

6.3.2) Record the post-preconditioning peak tissue deformation alongside the time from the stopwatch representing the tissue's optimal stretch time (**Figure 5b**).

7. Biaxial mechanical testing

7.1) Prepare a force-controlled protocol at a loading rate of 4.42 N/min.

7.1.1) Open a new testing directory and name the test. Set the data to save to a known location for use in later stress and strain calculations. Move the specimen back to the original mounting configuration.

7.1.2) Create a protocol set titled **FirstImage**. Set the **X-axis** and **Y-axis control mode** to **force** and the **control function** to **step**. Set the **load magnitude** to **0 mN**. Set the **stretch duration** and **recovery duration** each to **1 second**. Set the **number of repetitions** to **1**. Set the **data output frequency** and **image output frequency** each to **1 Hz**.

7.1.3) Construct a new testing set, named **PreconditioningA**. Establish the testing parameters such that the tissue will undergo 10 repetitions of cyclic loading/unloading to the targeted force for the desired membrane tension exactly as was prepared in step 6.2. Note that now, the stretch time and recover time should be the time recorded in step 6.3.2. No images are captured in the **A** testing set, but data is captured at 15 Hz.

7.1.4) Construct another testing set, named **PreconditioningB**. All testing parameters should be identical to those as mentioned in the previous step, with the exception that **image output frequency** is set to **15 Hz**, and no preload is applied.

7.1.5) After the preconditioning protocol, create testing protocols so that the tissue is loaded to the 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, and 1:0.5 (**Figure 6**). Retrieve data from the last two cycles of each loading ratio for subsequent data processing and analyses described in section 10. Refer to **Table 1** for a detailed description of the protocols to be established.

7.2) Prepare a displacement-controlled testing protocol at a loading rate of 4.42 N/min as follows. (i) **Biaxial stretching** in the X-direction and Y-direction to the displacements associated with the peak circumferential and radial stretches, respectively (**Figure 7a**). (ii) **Pure shear** along the X-direction—stretching in the X-direction associated with the peak circumferential stretch and shortening in the Y-direction, while keeping the dashed area constant under deformation (**Figure 7b**). (iii) **Constrained uniaxial stretching** along the X-direction (**Figure 7c**). (iv) **Pure shear** along the Y-direction (**Figure 7d**). (v) **Constrained uniaxial stretching** along the Y-direction (**Figure 7e**).

7.2.1) Between each of these steps, construct a rest "cycle" of 1 min that holds the tissue at the original mounted configuration. Retrieve data from the last two cycles of each loading ratio for data processing and analyses (section 9). Refer to **Table 2** for a detailed description of the protocols to be established.

7.3) Prepare a stress-relaxation protocol so that the tissue is loaded in each direction, at a loading rate of 4.42 N/min, to the displacements associated with the peak membrane tensions (step 7.2) and held at that displacement for 15 min (**Figure 8** and **Figure 9**). After 15 min, the protocol should be set to recover the tissue to its original mounting configuration.

NOTE: In the case of tissue tearing, abort the test immediately to prevent any potential damage caused to the biaxial testing system.

8. Tissue fixation for histology analysis

8.1) 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 at approximately 4 °C. Fix the tissue for 24–48 h, depending on the tissue's thickness.

CAUTION: Formalin is a known carcinogen and, if breathed in, an excess may cause lungs to become fixed. All work with formalin should be performed in a fume hood with adequate ventilation.

8.2) After the tissue has been fixed in formalin for 24–48 h, transfer the tissue to an 80% ethanol solution for later histology. The tissue should be stored in solution in a refrigerated environment at 4 °C.

NOTE: The protocol can be paused here. Once the tissues are fixed, the specimens can be analyzed at any time. If the protocol is paused, store the tissue in a labeled container filled with 80% ethanol, and store the container in a refrigerator at approximately 4 °C (as explained in step 8.2).

8.3) Prepare the tissue for commercial histology analysis as per the vendor's instructions. If a certain leaflet constituent, such as collagen, elastin, glycosaminoglycans, etc., is of the study's interest, ensure that the appropriate histology stain is employed.

NOTE: Histology slides may be visualized using a microscope to observe desired constituents (**Figure 10**).

8.4) Using the image processing program ImageJ, perform color deconvolution methods to determine the percentage of each stained constituent in the tissue. For more details on these procedures, please refer to Ruifrok and Johnston[24].

9. Biaxial testing data postprocessing procedures

9.1) Perform DIC-based tracking on the four fiducial markers from the images taken during the biaxial mechanical testing (**Figure 11**) to determine the time-dependent marker positions.

$$\boldsymbol{x}_I = \boldsymbol{X}_I + \boldsymbol{d}_I, \text{ for } I = 1, 2, 3, 4 \tag{2}$$

NOTE: Here, $\mathbf{X}_I$ and $\mathbf{x}_I$ are the undeformed and deformed positions of the markers, respectively, and $\mathbf{d}_I$ is the displacement vector of each marker.

9.1.1) If it is desired to perform the analysis with respect to the mounting configuration, let $\mathbf{X}_I$ be the marker positions in the undeformed state at the beginning of the biaxial test. If it is desired to perform the analysis with respect to the post-preconditioning deformation, let $\mathbf{X}_I$ be the marker positions at the end of the preconditioning protocol.

NOTE: The subsequent steps will be conducted in the same manner, regardless of the reference configuration chosen.

9.2) Compute the deformation gradient (**F**) of the fiducial markers using a four-node bilinear finite element[2,23,25].

$$\boldsymbol{F} = \boldsymbol{F}(\boldsymbol{X}, t) = \frac{\partial \boldsymbol{x}}{\partial \boldsymbol{X}} = \begin{bmatrix} \sum_{I=1}^{4} B_{XI} u_I(t) & \sum_{I=1}^{4} B_{YI} u_I(t) \\ \sum_{I=1}^{4} B_{XI} v_I(t) & \sum_{I=1}^{4} B_{YI} v_I(t) \end{bmatrix} \tag{3}$$

NOTE: Here, B_{xI}'s and B_{yI}'s are the finite element shape function derivatives in the X- and Y-directions for node I, respectively, and $u_I(t)$ and $v_I(t)$ are the time-dependent X- and Y-

displacements, respectively, as previously determined from step 9.1. Note that the X- and Y-coordinates are aligned to the tissue's circumferential and radial directions.

9.3) Compute the right Cauchy–Green deformation tensor (**C**) and the Green strain tensor (**E**).

$$\boldsymbol{C} = \boldsymbol{F}^T\boldsymbol{F}, \text{ and } \mathbf{E} = \frac{1}{2}(\boldsymbol{C} - \boldsymbol{I}) \quad (4)$$

NOTE: Here, **I** is the second-order identity tensor. Determine the circumferential and radial stretches by taking the square roots of the principle values of **C**.

9.4) Determine the first Piola-Kirchhoff (1st-PK) stress tensor (**P**).

$$\boldsymbol{P} = \frac{1}{t}\begin{bmatrix} T_C & 0 \\ 0 & T_R \end{bmatrix} \quad (5)$$

NOTE: Here, t is the specimen's thickness, and T_C and T_R are the applied membrane tensions in the circumferential and radial directions, respectively.

9.5) Also, compute other stress tensors, such as the Cauchy stress tensor (**σ**) and the second Piola-Kirchoff (2nd-PK) stress tensor (**S**).

$$\boldsymbol{\sigma} = J^{-1}\boldsymbol{P}\boldsymbol{F}^T \text{ and } \mathbf{S} = \mathbf{F}^{-1}\boldsymbol{P} \quad (6)$$

NOTE: Here, J is the Jacobian of the deformation gradient tensor **F**.

REPRESENTATIVE RESULTS:

Stress-stretch data from the force-controlled biaxial mechanical testing reveals a nonlinear curve with some resemblance to an exponential curve (**Figure 12**). Regarding the response in each principal direction, the material behavior is transversely isotropic, with the radial stretch greater than the circumferential deformation. In some cases, the anisotropy's directions may flip, with the circumferential direction exhibiting greater compliance than the radial direction. This flipped response is observed in the TV more often than in the MV.

From displacement-controlled testing, stress-stretch data follows a nonlinear response for the principal direction undergoing tension (pure-shear, constrained uniaxial tension [**Figure 13**]). When the tissue shortens in the other principal direction, a "negative (compressive) stress" is observed. In the constrained uniaxial tension protocol, there also exhibits an increasing stress-stretch response in the constrained direction, demonstrating the coupling of applied stretching in the other principal direction.

From stress-relaxation testing, normalized membrane tension-time data follows a nonlinear decaying curve (**Figure 14a,b**). Both the MV and TV leaflet tissues exhibit a greater stress reduction in the radial direction compared to that in the circumferential direction.

Representative histologic results of the mitral valve anterior leaflet (MVAL) and tricuspid valve anterior leaflet (TVAL) tissues using Masson's trichrome are presented in **Figure 10**. The Masson's trichrome stain demonstrates typical constituents found in atrioventricular heart valves, such as collagen fibers (blue) and valvular interstitial cells (red cytoplasm and black nuclei). Other stains can be used to visualize constituents such as elastin (Verhoeff-van Gieson stain) and glycosaminoglycans (Alcian blue stain).

FIGURE LEGENDS:

Figure 1: Experimental photos of porcine hearts retrieved from a local slaughterhouse. (**a**) A whole heart is rinsed of blood with PBS solution. (**b**) A cut is made between the atria and ventricle to reveal both the mitral and tricuspid valves. (**c**) Blood clots are then removed from the heart before storage.

Figure 2: Experimental photos of the opened porcine heart revealing the five atrioventricular heart valve leaflets and other components of the valve apparatus. (**a**) The mitral valve with the dissection of the left heart along the commissure between the two leaflets, showing the anterior leaflet (MVAL) and posterior leaflet (MVPL), and (**b**) the tricuspid valve with a similar dissection on the right side of the heart, revealing the anterior leaflet (TVAL), posterior leaflet (TVPL), and septal leaflet (TVSL).

Figure 3: Experimental photos of the excised leaflet being prepared for biaxial mechanical testing. Heart valve leaflet testing requires (**a**) the bulk leaflet to be sectioned into (**b**) a 10 mm x 10 mm 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.

Figure 4: Example protocol parameters for the preconditioning testing of a mitral valve anterior leaflet of a 7.5 mm x 7.5 mm testing region. The preconditioning protocol is created by setting (**a**) the protocol name, (**b**) the testing control mode and force in the X-axis, (**c**) the preload conditions, (**d**) the Y-axis parameters to be the same as in the X-axis, and (**e**) the cycle parameters.

Figure 5: Example protocol parameters for the timing step for a mitral valve anterior leaflet of a 7.5 mm x 7.5 mm testing region. The timing step requires (**a**) moving the tissue from the post-preconditioning deformation to the peak membrane tension (and corresponding peak deformation) while simultaneously starting a stopwatch to record the stretch time. When the target force has been reached, (**b**) the post-preconditioning deformation is recorded.

Figure 6: Schematic of the force-controlled biaxial testing procedure for testing mitral and tricuspid valve leaflets. The testing protocol consists of an equibiaxial loading preconditioning step to exercise the tissue to its in vivo state, followed by various loading ratios of the peak membrane tension in each tissue direction (Tx:Ty): 1:1, 0.75:1, 1:0.75, 0.5:1, and 1:0.5. Each subsection of the force-controlled testing protocol is performed for 10 loading/unloading cycles.

Figure 7: Schematic of the displacement-controlled biaxial testing procedure for testing mitral and tricuspid valve leaflets. The testing protocol consists of (**a**) biaxial displacements associated with the peak membrane tensions, (**b**) pure shear in the X-direction, (**c**) constrained uniaxial displacement in the X-direction, (**d**) pure shear in the Y-direction, and (**e**) constrained uniaxial displacement in the Y-direction. Each subsection of the displacement-controlled testing protocol is performed for 10 loading/unloading cycles.

Figure 8: Example stress-relaxation testing parameters for a mitral valve anterior leaflet with an effective testing region of 7.5 mm x 7.5 mm. Testing set parameters for stress-relaxation testing for a mitral valve anterior leaflet where targeted displacement is the peak tissue deformation specific to this tissue.

Figure 9: Schematic of the 15 min stress-relaxation testing procedure for testing mitral and tricuspid valve leaflets. The testing protocol involves holding biaxial displacements associated with the peak membrane tensions for 15 min, after which the tissue is returned to the mounting configuration.

Figure 10: Example histological data from the atrioventricular heart valves' anterior leaflets. Representative histology images of (**a**) the mitral valve anterior leaflet and (**b**) the tricuspid valve posterior leaflet. Both are stained with a Masson's trichrome stain: collagen in blue, cytoplasm and keratin in red, and nuclei in black. The scale bar = 200 μm.

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 tissue mounting configuration. (**b**) The configuration after the preconditioning step. (**c**) The deformed configuration associated with the tissue specimen under mechanical loading.

Figure 12: Representative data from the force-controlled protocols for the mitral valve anterior leaflet (MVAL). Representative data demonstrates the material anisotropy and nonlinear strain response of the tissues under biaxial loading at varying loading ratios of peak membrane tension in each tissue direction (Tx:Ty): (**a**) 1:1, (**b**) 0.75:1, (**c**) 1:0.75, (**d**) 0.5:1, and (**e**) 1:0.5.

Figure 13: Representative data from the displacement-controlled protocols for the mitral valve anterior leaflet (MVAL). Representative data demonstrates the material anisotropy and nonlinear strain response of the tissues during (**a**) biaxial displacements associated with the peak membrane tensions, (**b**) pure shear in the X-direction, (**c**) constrained uniaxial displacement in the X-direction, (**d**) pure shear in the Y-direction, and (**e**) constrained uniaxial displacement in the Y-direction.

Figure 14: Representative data from the stress-relaxation protocols for the mitral and tricuspid valve anterior leaflets. Representative data for (**a**) the MVAL and (**b**) the TVAL, illustrating the exponential stress reduction over time.

Table 1: Full testing parameters for all protocols of the force-controlled testing scheme. Forces (in millinewtons) are written as *F* to represent the force associated with the targeted peak membrane tension. Stretch time is written as *t* to represent the stretch time (in seconds) specific to the tissue being tested.

Table 2: Full testing parameters for all protocols of the displacement-controlled testing scheme. Displacements (in percentages) are written as d_x and d_y to represent the peak post-preconditioning percentage elongation in the X- and Y-directions, respectively. Stretch time is written as *t* to represent the stretch time (in seconds) specific to the tissue being tested. Abbreviations: PS = pure shear; CU = constrained uniaxial.

DISCUSSION:

Critical steps for this biaxial mechanical testing include (i) the proper orientation of the leaflet, the proper biaxial tester setup for negligible shear, and (ii) a careful application of the fiducial markers. The orientation of the leaflet is crucial to the obtained mechanical characterization of the leaflet tissue as the material is anisotropic in nature. Thus, the radial and circumferential directions need to be known for properly aligning the tissue specimens with the testing X- and Y-directions. It is also essential that the biaxial tester is properly calibrated so that the specimen is mounted to the system with negligible shear stress introduced. If a non-negligible amount of shear is observed, the results can be greatly skewed in subsequent tissue strain and stress calculations. Special attention is required to the application of the four fiducial markers to ensure that none of the markers stick to the others to avoid inaccurate calculations of tissue strains. With regard to the tissue strain calculations, interested readers are referred to the procedures as detailed in previous studies[2,23,25].

Some modifications that could be made to the current protocols include adding strain-rate and creep testing to the testing framework. These tests allow for insight into different viscoelastic properties of the aortic heart valve (AHV) leaflet, but it has been shown in previous literature that the strain-rate and creep are insignificant for heart valve leaflet tissues under physiologically functioning conditions.

Limitations of this method include the potential for shear introduction in cases of improper planar alignment of the specimen and stuck fiducial markers that invalidate data, as aforementioned. Other limitations of this method include the use of tines for specimen mounting, as the specimen is only controlled by five points on each edge, rather than a full clamping to control specimen edges. The use of tines over clamping methods causes issues with uniaxial testing protocols such that tines may allow small deformations despite the displacement of the tine-end attached to the biaxial testing system being constant. However, this deformation from individual tine movement can be presumed negligible.

This method is significant in its advantages compared to other methods because all testing protocols (force-controlled, displacement-controlled, and stress-relaxation) are performed in one unified tissue specimen. Alternatives to the presented methodology may only perform one testing protocol for each tissue, rather than three combined testing protocols. This entails that

those alternatives may not be as accurate in their description of tissue behaviors, as tissue properties can significantly vary between tissues from different animal subjects.

This method can be extended by application to other materials besides the atrioventricular heart valve leaflets. For example, these methods may be useful in characterizing other soft tissues, or polymers/rubber-type materials. The provided scheme would provide for the full characterization of any such materials compatible with a biaxial testing device, provided there is an adequate setup, such as an appropriate load-cell capacity and specimen size.

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DISCLOSURES:

The authors have nothing to disclose.

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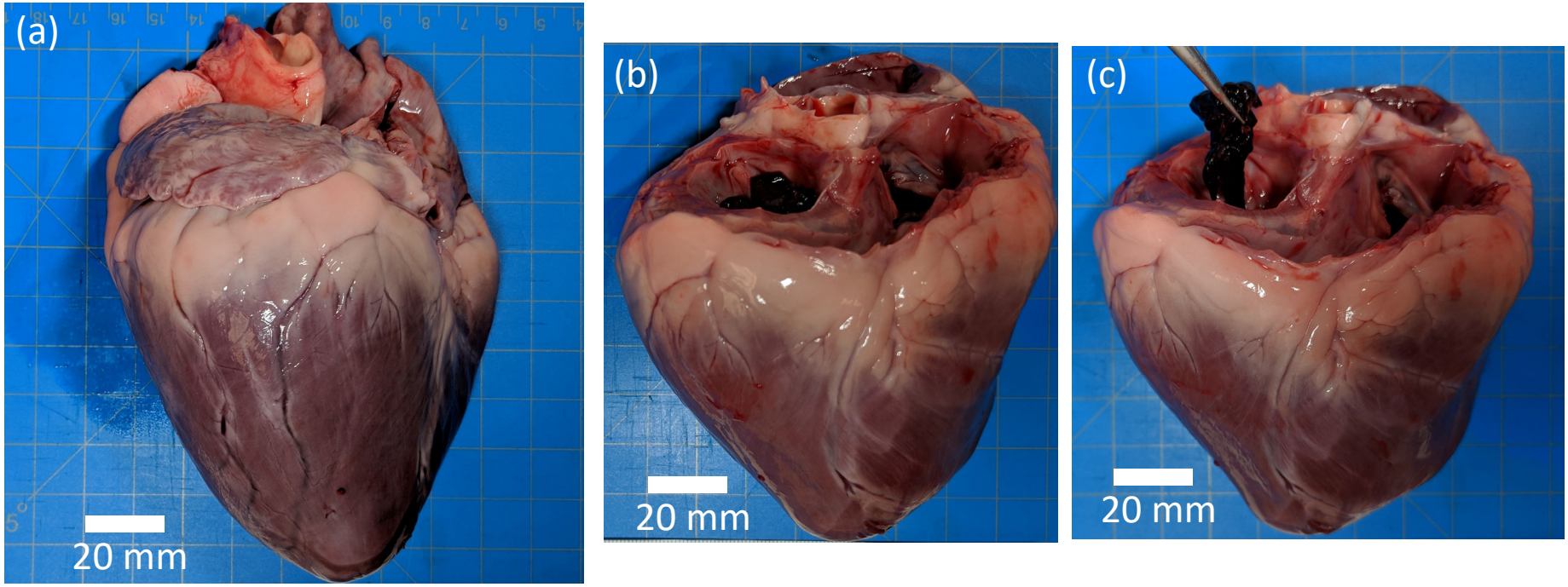
Figure 1



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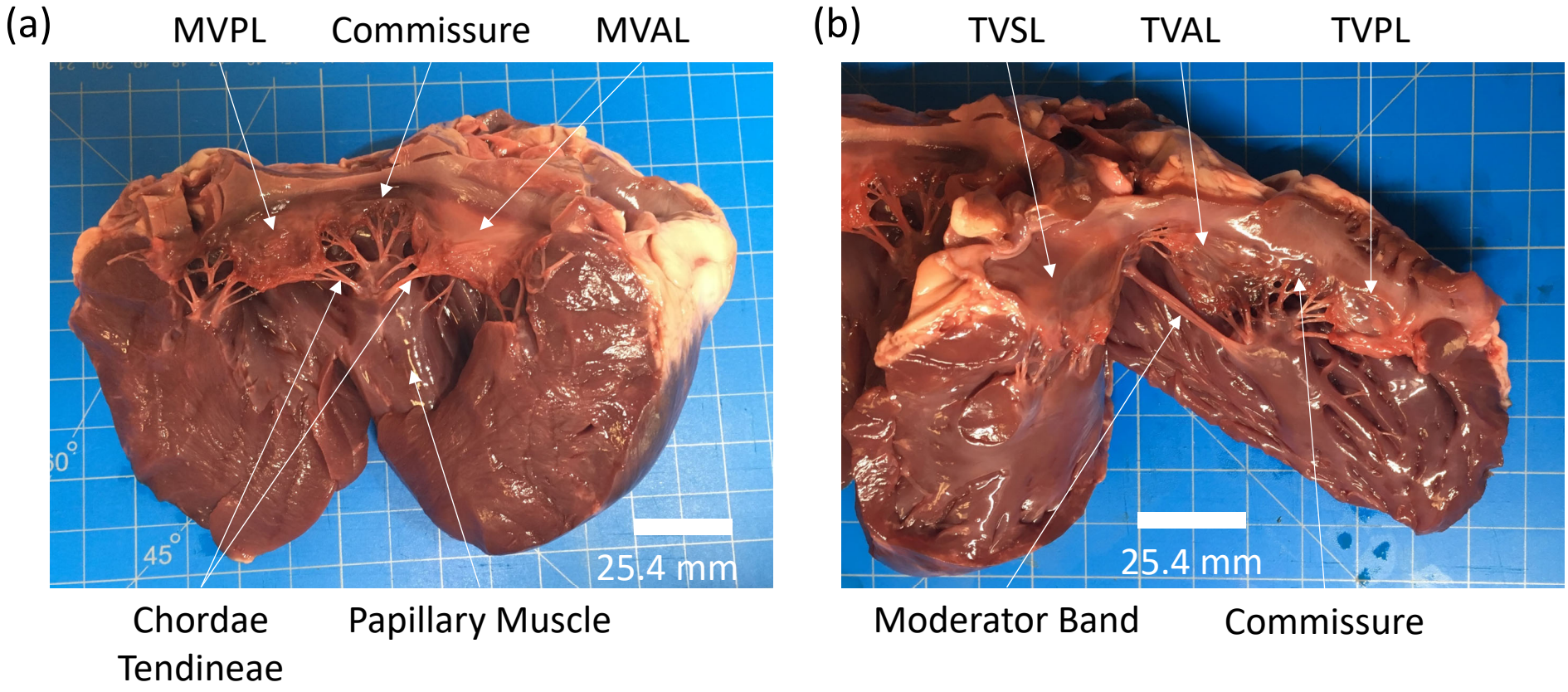

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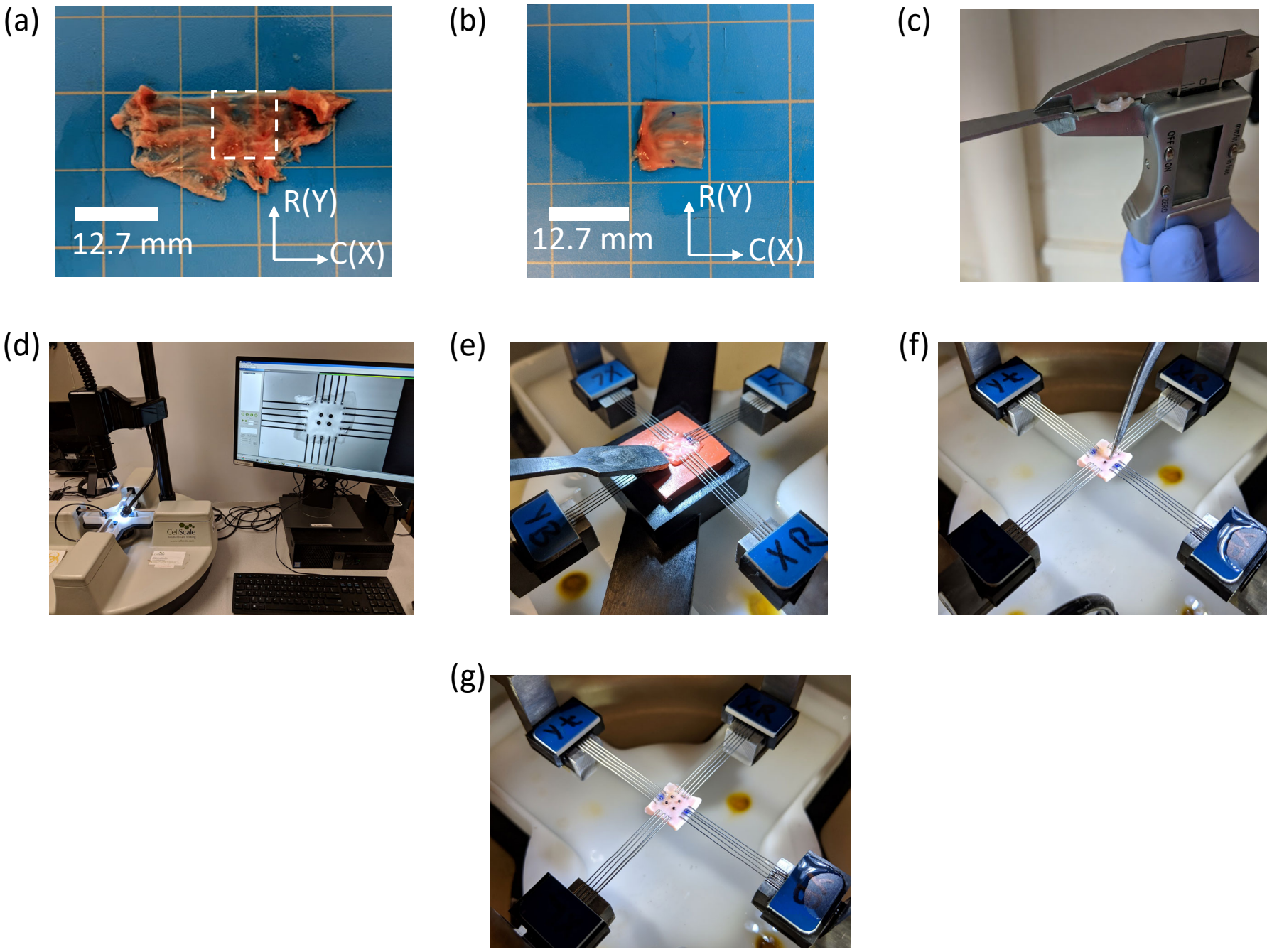

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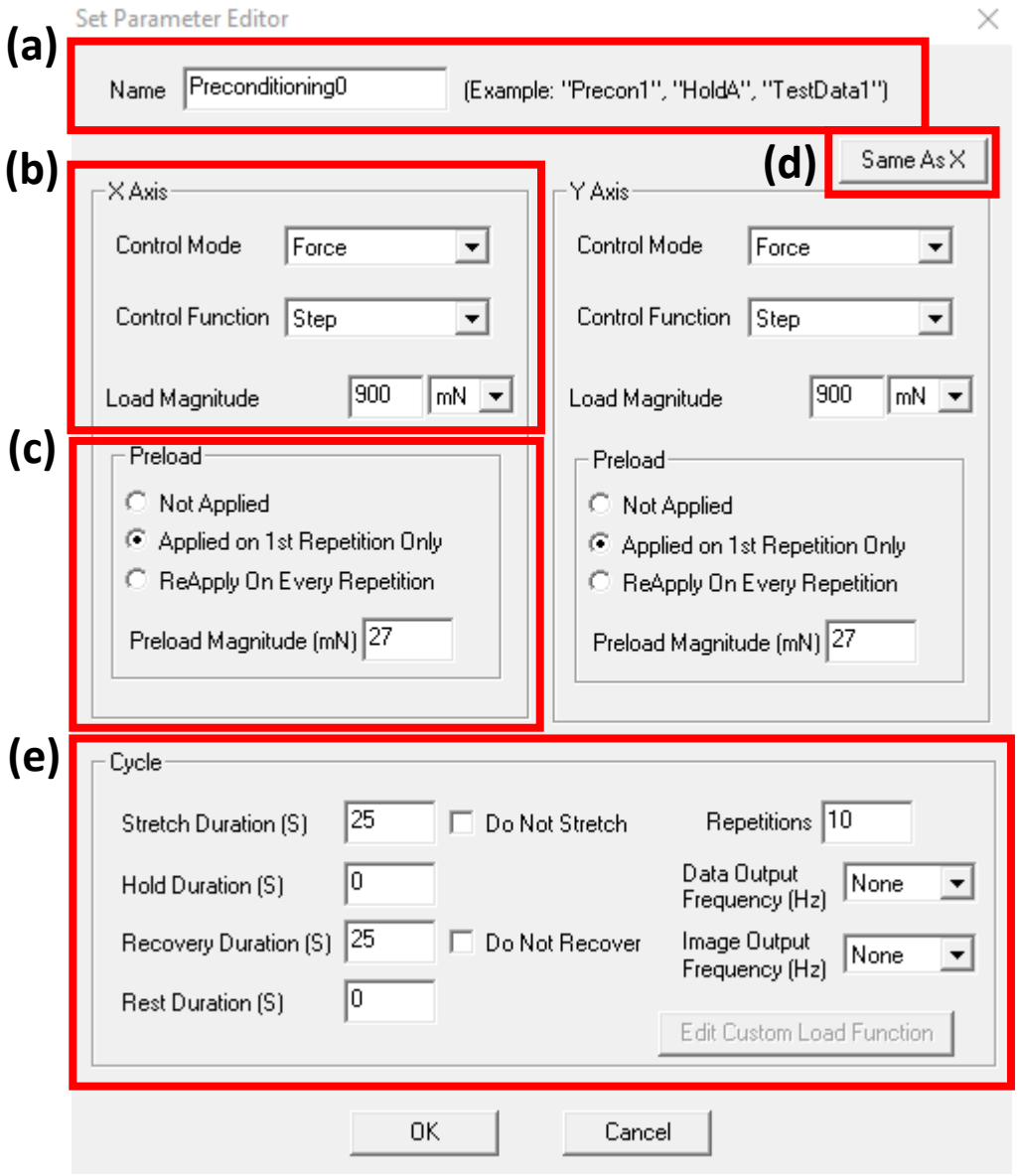

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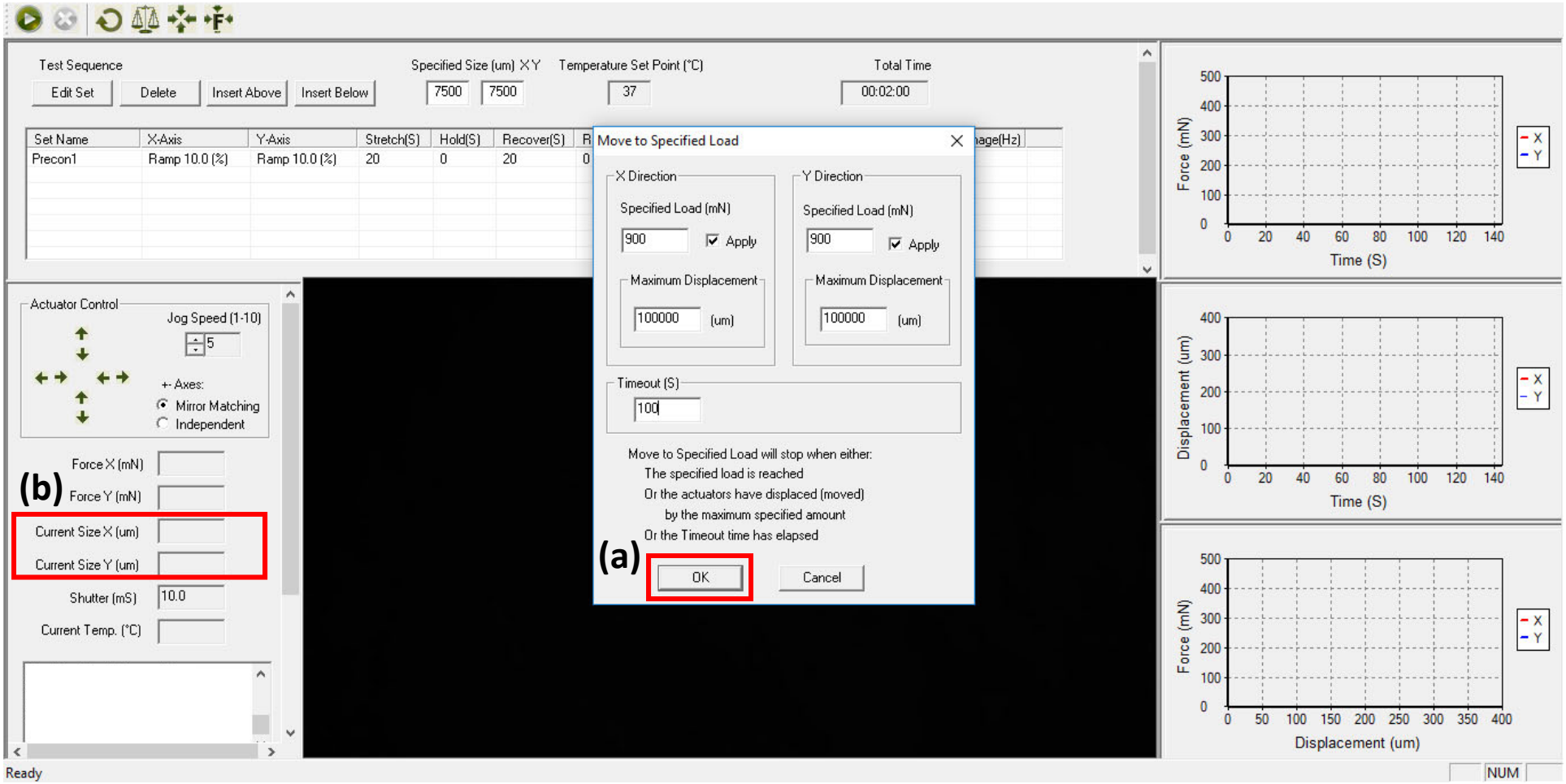

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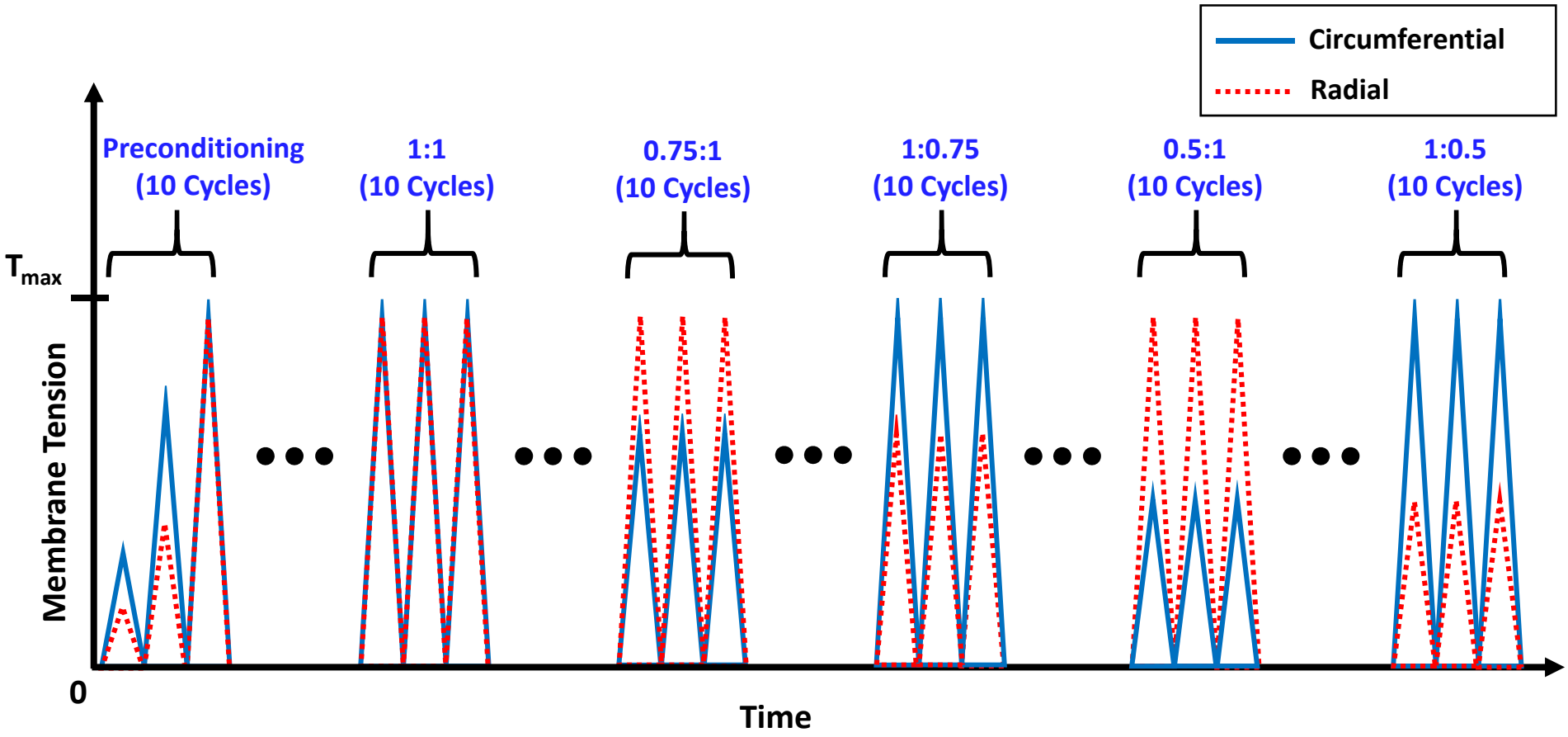

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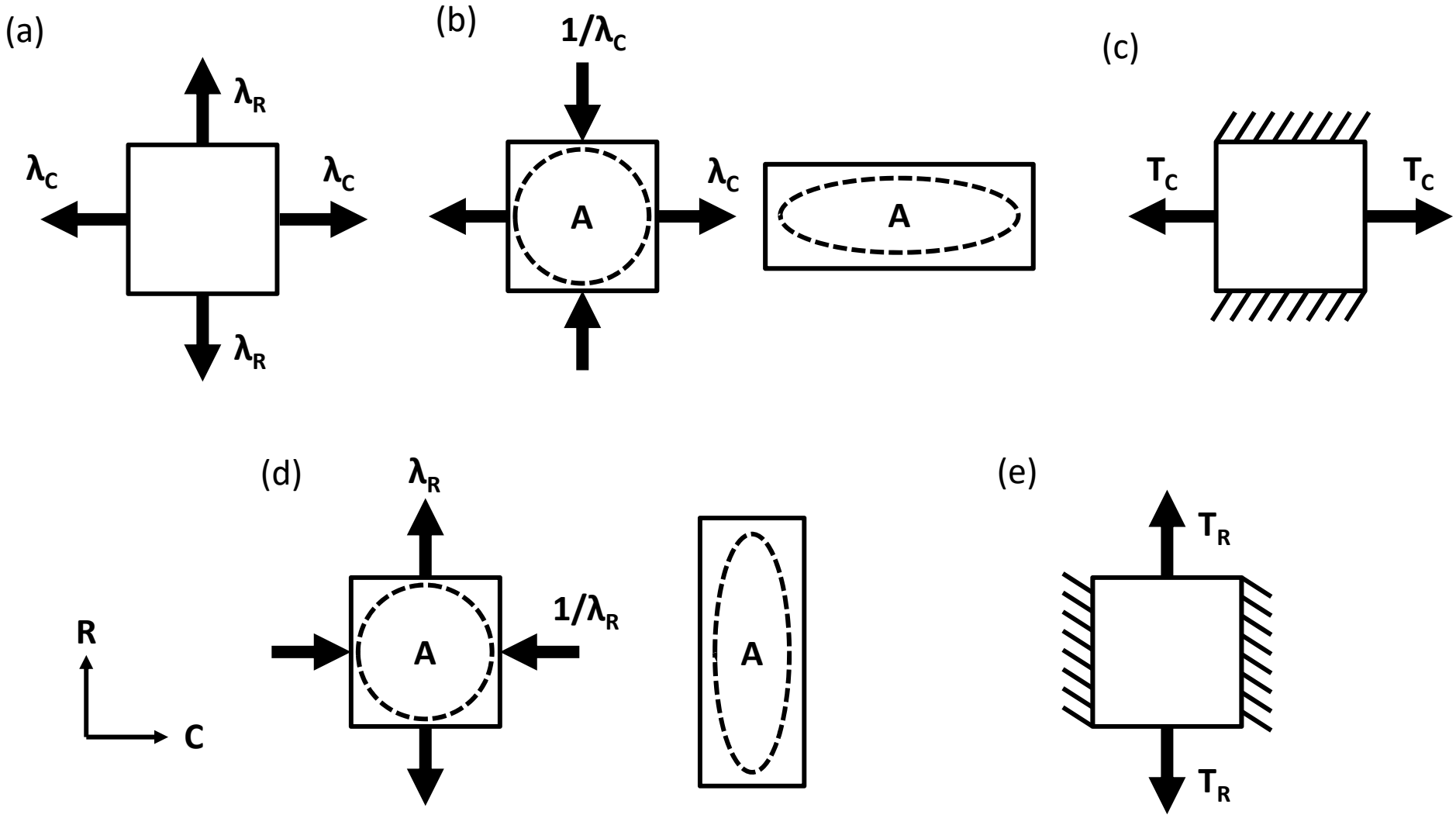

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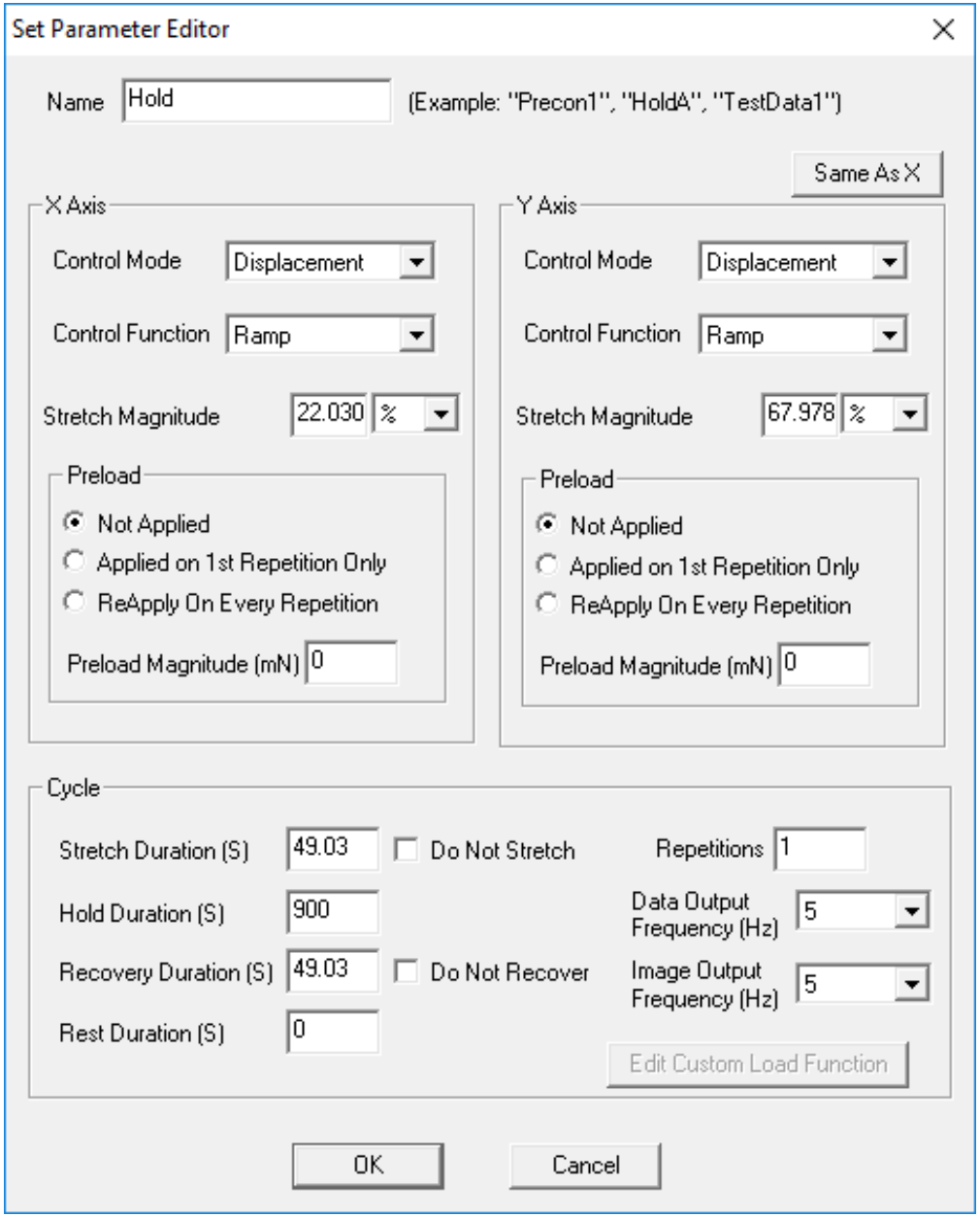

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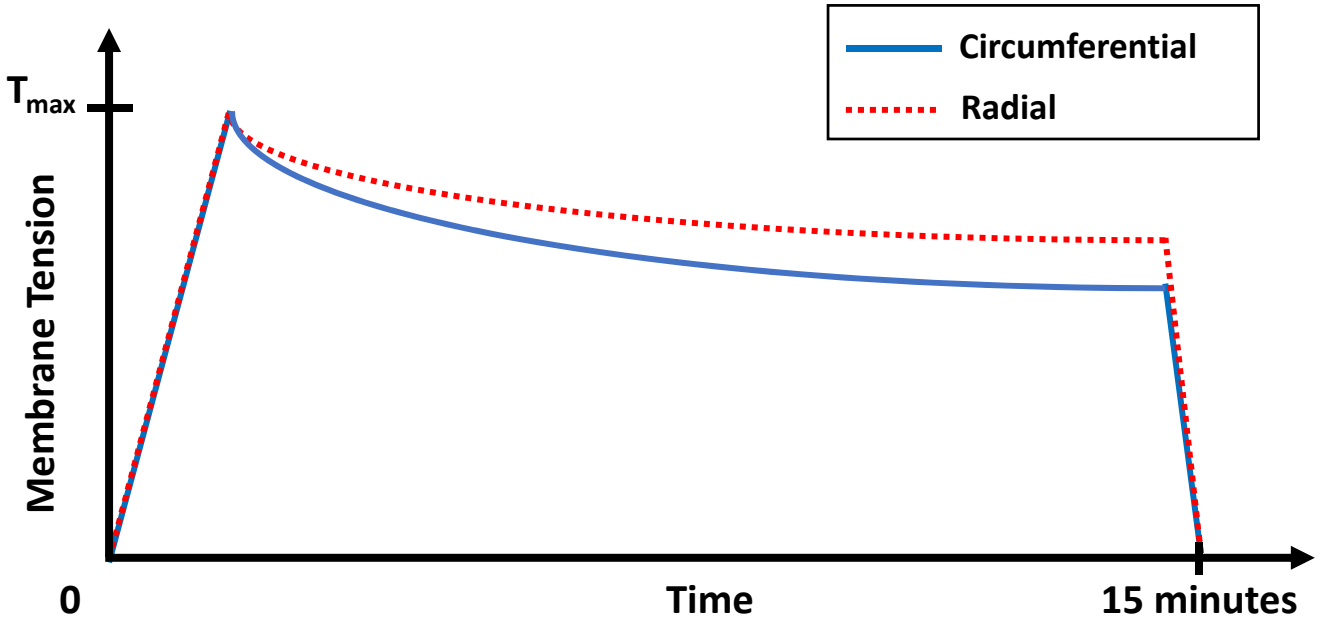

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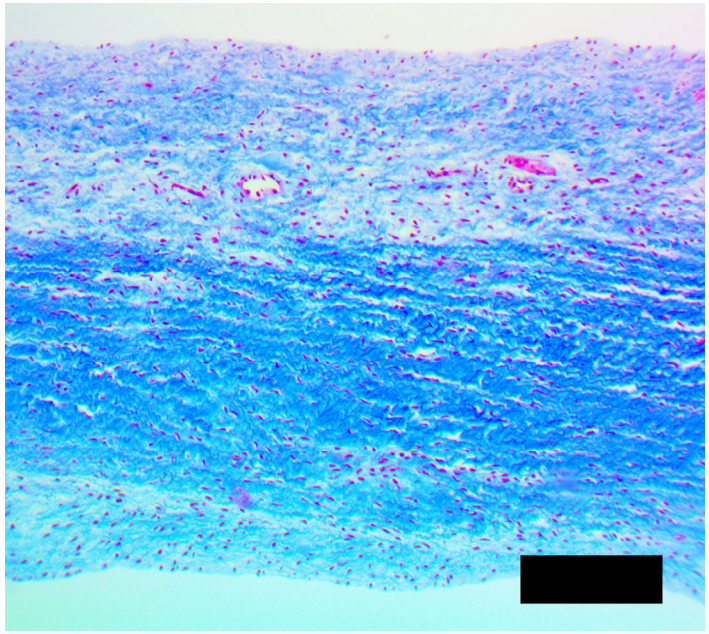

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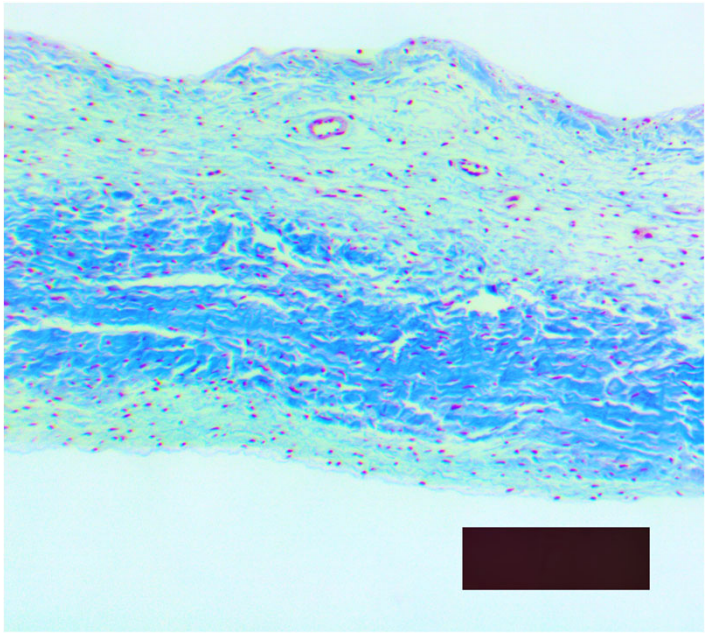

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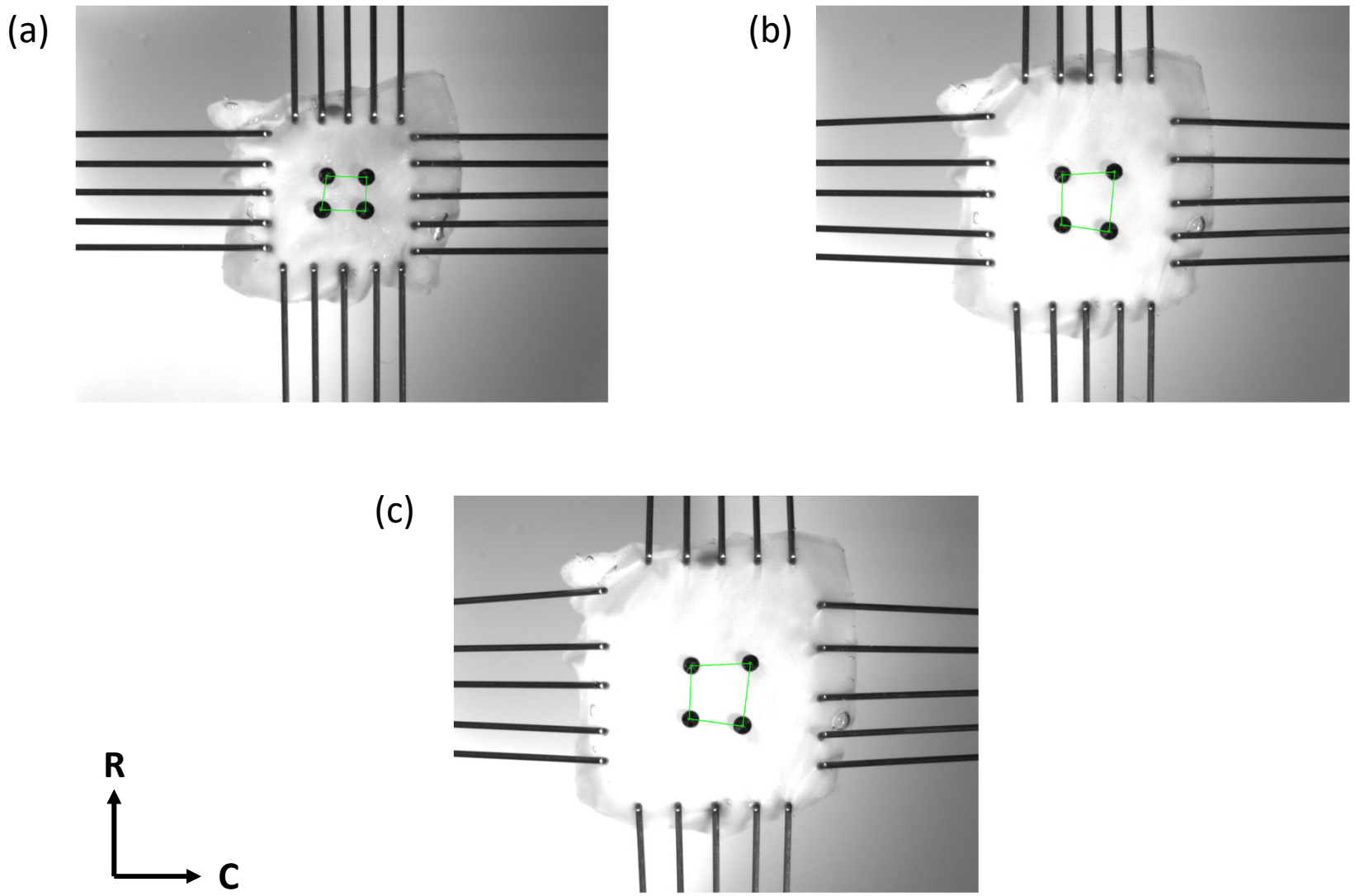

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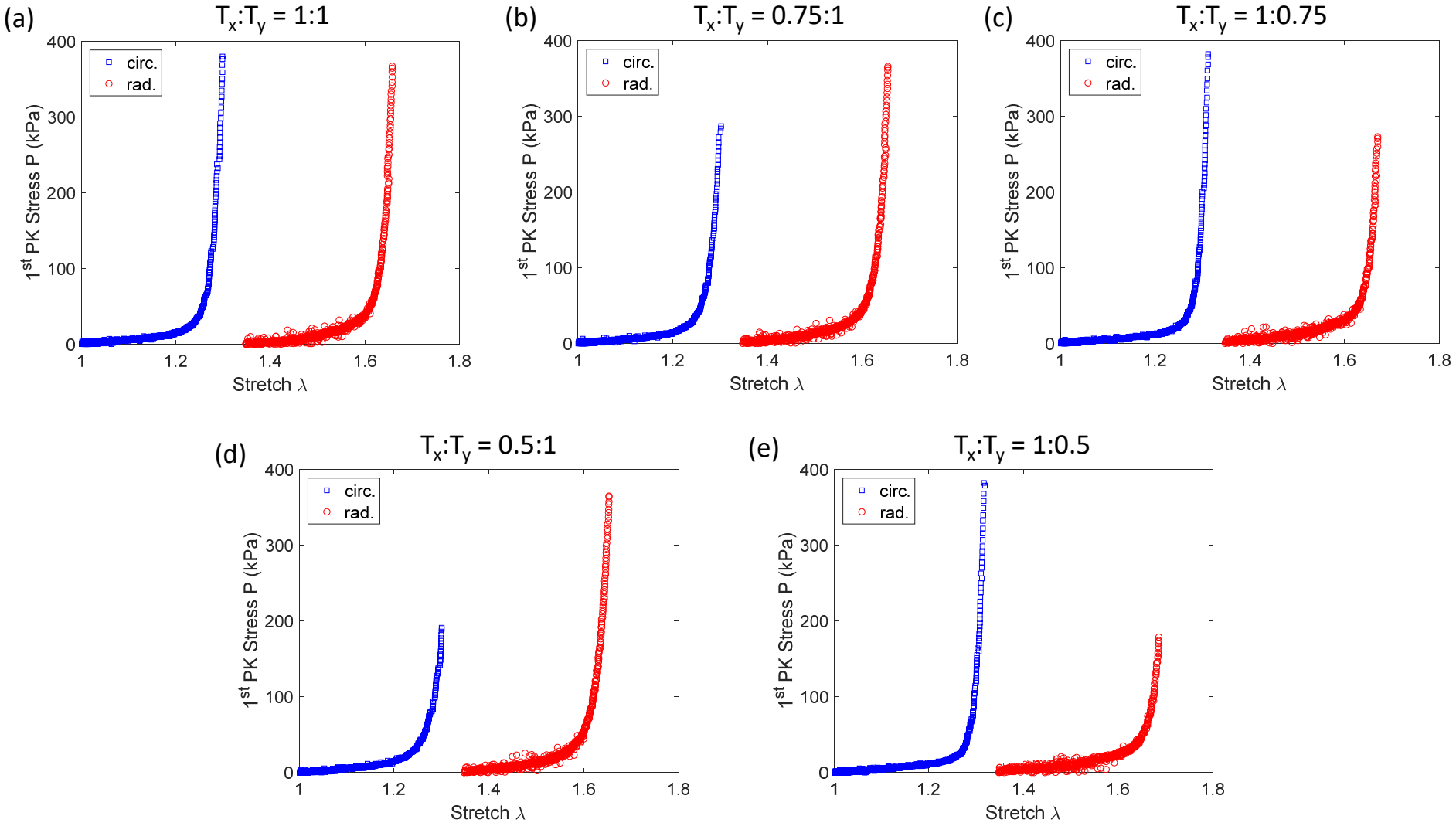

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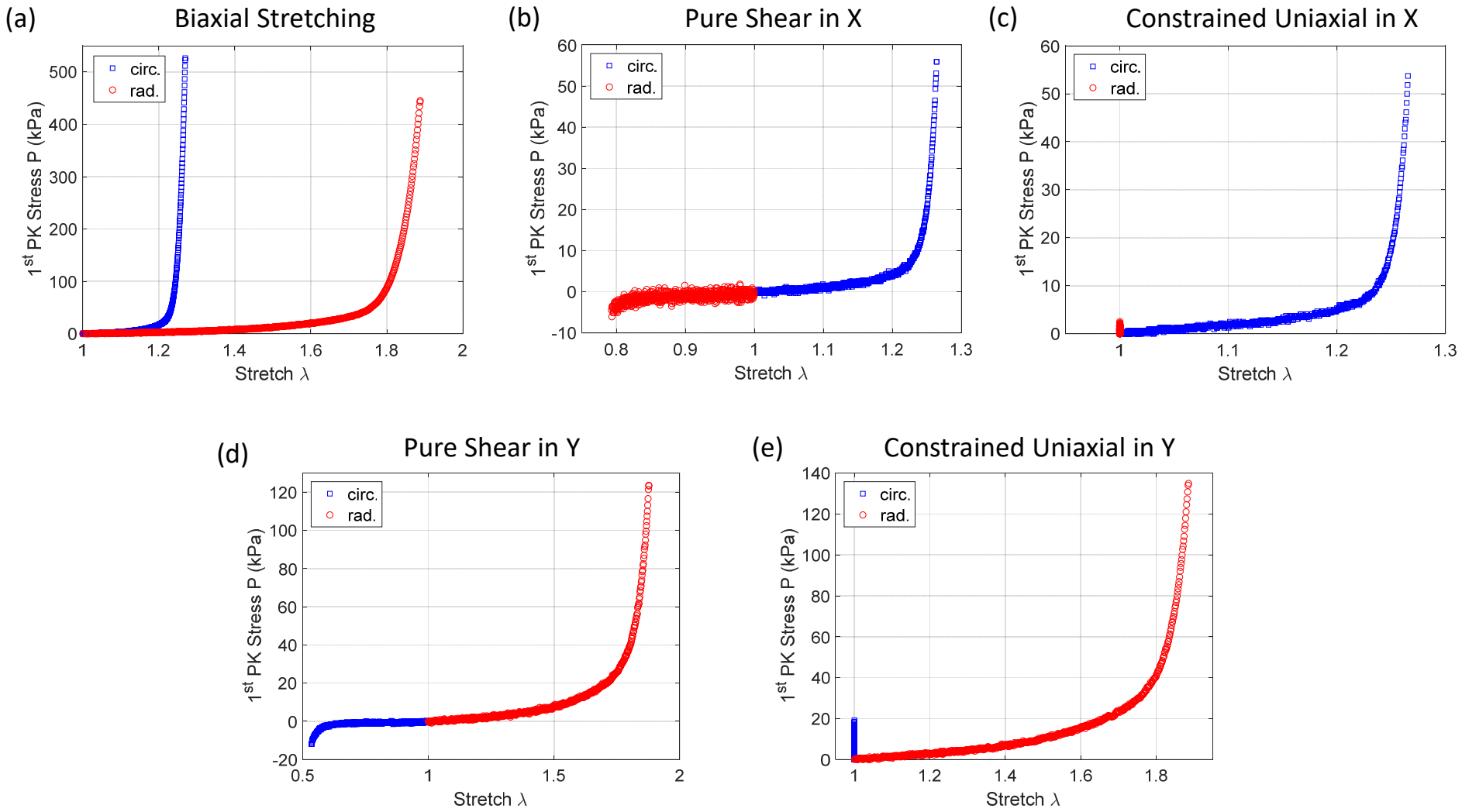

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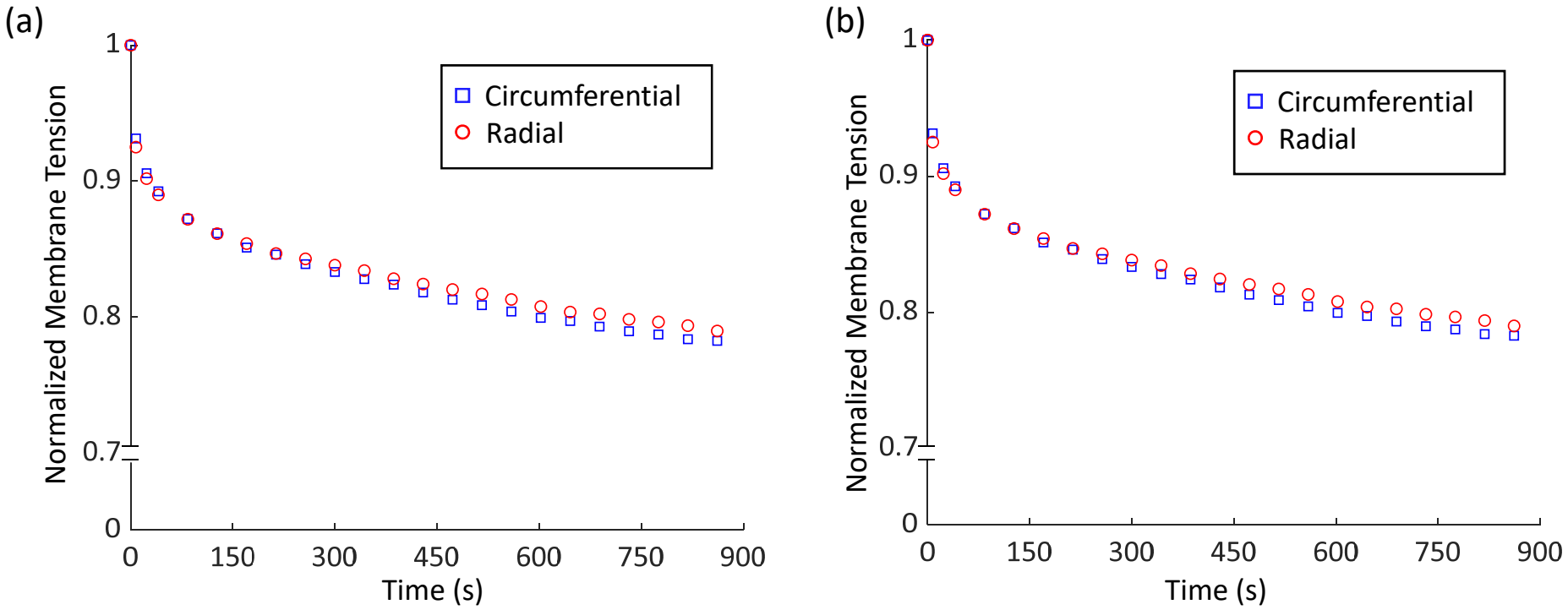

Table 1

Set Name	X-Axis	Y-Axis	Stretch (S)	Hold (S)	Recover (S)	Rest (S)
FirstImage	Step 0.0 (mN)	Step 0.0 (mN)	1	0	1	0
PreconditioningA	Step *F* (mN)	Step *F* (mN)	*t*	0	*t*	0
PreconditioningB	Step *F* (mN)	Step *F* (mN)	*t*	0	*t*	0
1:1A	Step *F* (mN)	Step *F* (mN)	*t*	0	*t*	0
1:1B	Step *F* (mN)	Step *F* (mN)	*t*	0	*t*	0
0.75:1A	Step (0.75**F*) (mN)	Step *F* (mN)	*t*	0	*t*	0
0.75:1B	Step (0.75**F*) (mN)	Step *F* (mN)	*t*	0	*t*	0
1:0.75A	Step *F* (mN)	Step (0.75**F*) (mN)	*t*	0	*t*	0
1:0.75B	Step *F* (mN)	Step (0.75**F*) (mN)	*t*	0	*t*	0
0.5:1A	Step (0.5**F*) (mN)	Step *F* (mN)	*t*	0	*t*	0
0.5:1B	Step (0.5**F*) (mN)	Step *F* (mN)	*t*	0	*t*	0
1:0.5A	Step *F* (mN)	Step (0.5**F*) (mN)	*t*	0	*t*	0
1:0.5B	Step *F* (mN)	Step (0.5**F*) (mN)	*t*	0	*t*	0

XPreload (mN)	YPreload (mN)	Reps	Data (Hz)	Image (Hz)
0.0 (First)	0.0 (First)	1	1	1
0.025*F (First)	0.025*F (First)	8	15	0
None	None	2	15	15
None	None	10	15	0
None	None	2	15	15
None	None	10	15	0
None	None	2	15	15
None	None	10	15	0
None	None	2	15	15
None	None	10	15	0
None	None	2	15	15
None	None	10	15	0
None	None	2	15	15

Table 2

Set Name	X-Axis	Y-Axis	Stretch (S)	Hold (S)	Recover (S)	Rest (S)
FirstImage	Step 0.0 (mN)	Step 0.0 (mN)	1	0	1	0
1:1A	Ramp d_x (%)	Ramp d_y (%)	*t*	0	*t*	0
1:1B	Ramp d_x (%)	Ramp d_y (%)	*t*	0	*t*	0
Rest	Ramp 0.0 (%)	Ramp 0.0 (%)	0	0	0	60
PSXA	Ramp d_x (%)	Ramp $1/d_y$ (%)	*t*	0	*t*	0
PSXB	Ramp d_x (%)	Ramp $1/d_y$ (%)	*t*	0	*t*	0
Rest	Ramp 0.0 (%)	Ramp 0.0 (%)	0	0	0	60
PSYA	Ramp $1/d_x$ (%)	Ramp d_y (%)	*t*	0	*t*	0
PSYB	Ramp $1/d_x$ (%)	Ramp d_y (%)	*t*	0	*t*	0
Rest	Ramp 0.0 (%)	Ramp 0.0 (%)	0	0	0	60
CUXA	Ramp d_x (%)	Ramp 0.0 (%)	*t*	0	*t*	0
CUXB	Ramp d_x (%)	Ramp 0.0 (%)	*t*	0	*t*	0
Rest	Ramp 0.0 (%)	Ramp 0.0 (%)	0	0	0	60
CUYA	Ramp 0.0 (%)	Ramp d_y (%)	*t*	0	*t*	0
CUYB	Ramp 0.0 (%)	Ramp d_y (%)	*t*	0	*t*	0

XPreload (mN)	YPreload (mN)	Reps	Data (Hz)	Image (Hz)
0.0 (First)	0.0 (First)	1	1	1
0.025*F (First)	0.025*F (First)	10	15	0
None	None	2	15	15
None	None	1	15	0
None	None	10	15	0
None	None	2	15	15
None	None	1	15	0
None	None	10	15	0
None	None	2	15	15
None	None	1	15	0
None	None	10	15	0
None	None	2	15	15
None	None	1	15	0
None	None	10	15	0
None	None	2	15	15

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials Biaxial Testing Revised.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
10% Formalin Solution, Neutral Bufffered	Sigma-Aldrich	HT501128-4L
40X-2500X LED Lab Trinocular Compound Microscope	AmScope	SKU: T120C
BioTester - Biaxial Tester	CellScale Biomaterials Testing	
ImageJ	National Institute of Health, Bethesda, MD	Version 1.8.0_112
LabJoy	CellScale Biomaterials Testing	Version 10.66
MATLAB	MathWorks	Version 2018b
Phosphate-Buffered Saline	n/a	
Single Edge Industrial Razor Blades (Surgical Carbon Steel)	VWR International	H3515541105024

Comments/Description

1.5N Load Cell Capacity

Recipe for 1L 1X PBS Solution: 8.0g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4

Razord blades for tissue retrieval and preparation procedures

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CORRESPONDING AUTHOR:

Name:	Chung-Hao Lee
Department:	School of Aerospace and Mechanical Engineering
Institution:	The University of Oklahoma
Article Title:	Biaxial Mechanical Characterizations of Atrioventricular Heart Valves
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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.

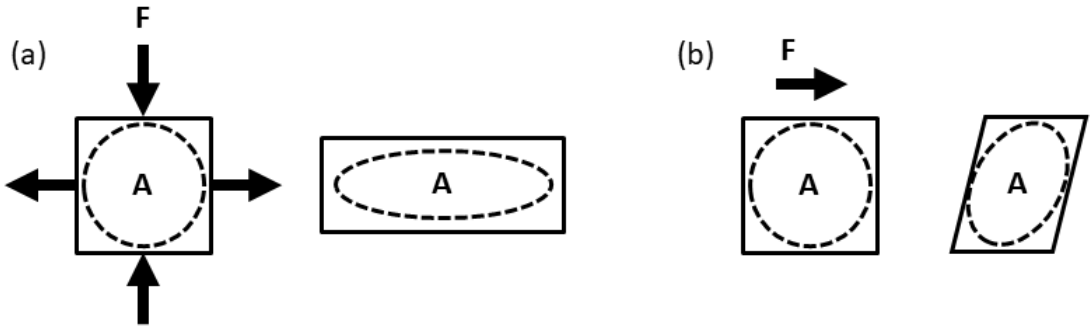

Figure R1: (a) Pure shear involves elongation of one direction of a 2D element while the other direction is shortened (irrotational shear). (b) Simple shear involves the rotation of a sample about a fixed point in the direction of applied displacement.

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.

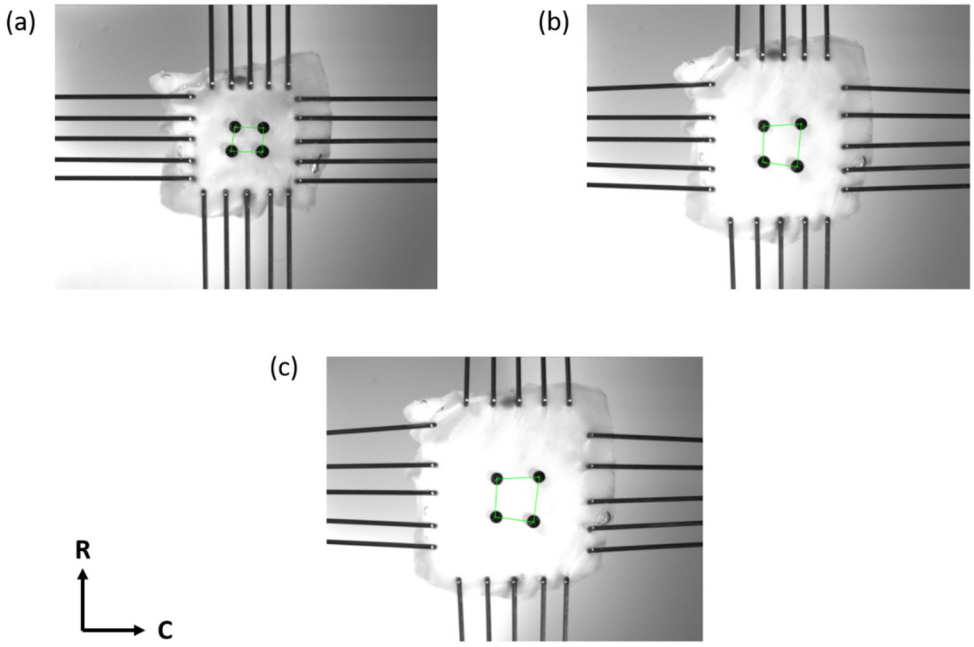

Figure 11: Representative images illustrating the tracking of the coordinates of four fiducial markers during biaxial mechanical testing using a data image correlation (DIC) technique: (a) the mounting configuration, (b) the reference configuration after the preconditioning step, and (c) the deformed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

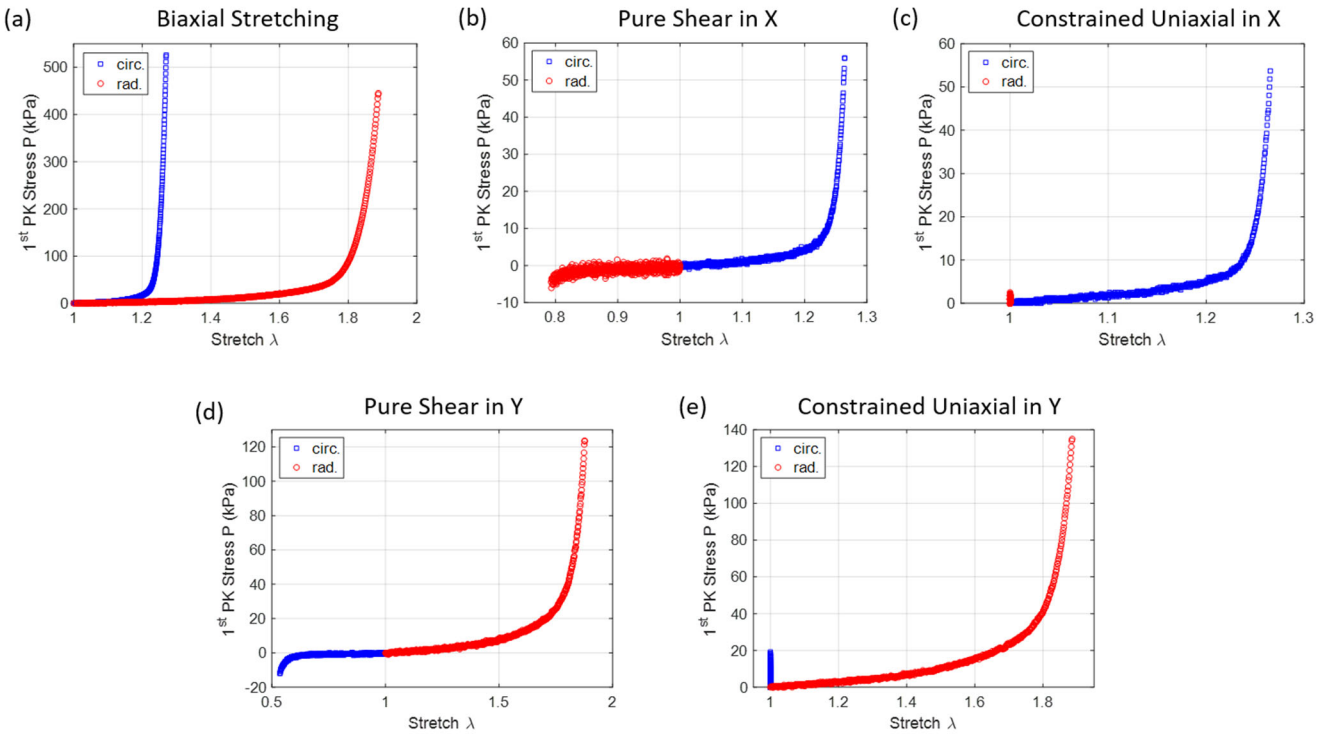

Figure 13: (a-e) Representative data from displacement-controlled protocols for the mitral valve anterior leaflet are provided.

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.

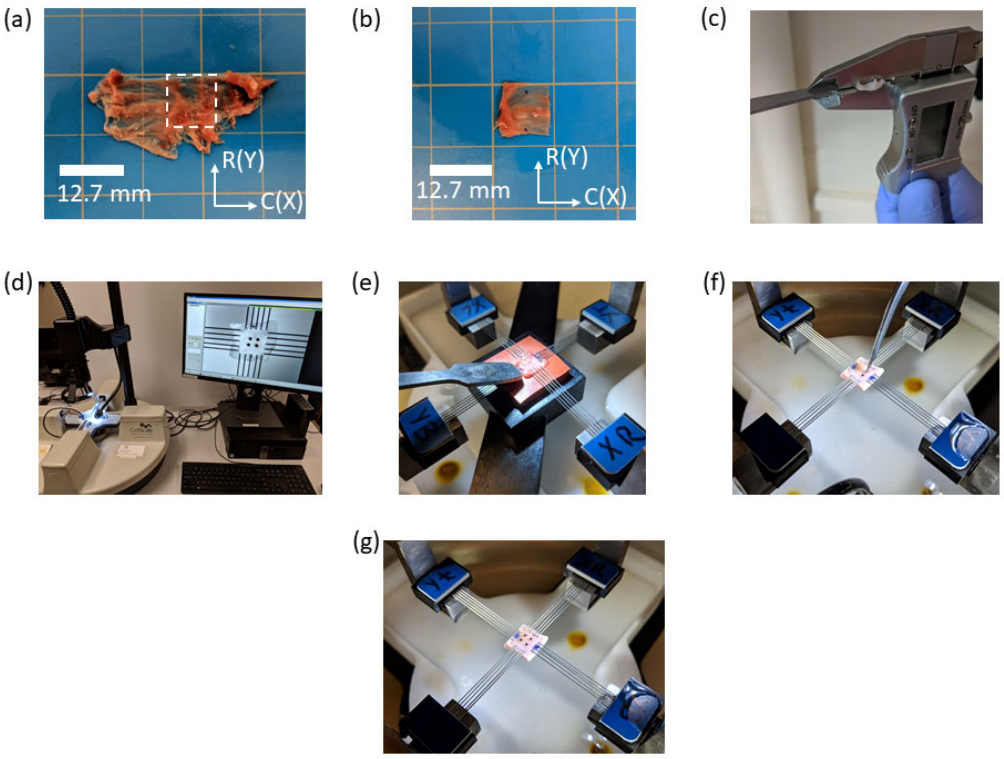

Figure 3: Heart valve leaflet testing requires the (a) bulk leaflet be sectioned into a (b) 10mm x 10mm testing region (radial direction noted by surgical pen markers). (c) The leaflet thickness is measured. Specimens are mounted to (d) the biaxial testing system by (e) piercing the tissue with metal 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).
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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 (4^{o}C) environment.

2. *5.1)* Prepare a PBS bath at 37^{o}C 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.

3. 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.

4. 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.

5. To compute the appropriate membrane tension, obtain the tissue's effective testing edge length and use the equation: $T = diag[T_C, T_R] = diag[\, f_C, \, f_R]/L$. 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.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. 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.

12. 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.

13. 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 ($4^{o}C$).

14. 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 (4°C) 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.

15. 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:

$$\mathbf{x}_I = \mathbf{X}_I + \mathbf{d}_I, \text{ and } I = 1,2,3,4 \quad (1)$$

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).

16. 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.

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 with changes highlighted in blue. Below is a summary of our modifications made in the revised manuscript, for addressing the Editorial comments and for improving the clarity and readability.

- Highlighted portions of the manuscript for filming have been adjusted to better illustrate our procedure in a concise manner.
- Figures and Tables have been added which better demonstrate how to establish the biaxial mechanical testing parameters.
- Greater clarity has been made on software-specific steps in the protocol, while also avoiding commercial language.

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

1. **6. Preconditioning Step and Duration Timing**

   It appears that each of these steps is a collection of numerous actions. It will be better to exclude this from filming, and allow readers to refer to the supplementary file instead. If you'd like viewers to see the supplementary file, please edit it to standardize the text color, and remove the rebuttal comments from it.

   This portion of the protocol has been greatly revised to include information that was previously presented in the supplementary file. Because of this revision we have suggested that this portion of the protocol be filmed, as it is important to the overall procedure.

2. **7. Biaxial Mechanical Testing**

   In order to film a step, it must be described in detail in the manuscript. This ensure that the portion to film is within our length limits (2.75 pages of highlighting). We have flagged steps that are lacking details for filming. Pleas expand the description of these items within this document.

   We apologize for not including greater detail of the procedure within the manuscript. We have expanded the descriptions of each step to better address how they are performed. This has also included the addition of two tables and 3 figures which better demonstrate the procedure.

3. 7.1.2) Retrieve data from the last two cycles of each loading ratio for the subsequent data processing and analyses described in Step 10.

   Please update.

We apologize for our oversight in the numbering of our steps after subsequent revisions of the manuscript. This has been updated to state the correct step, **Step 9**.

4. 7.4) Sequentially run the three testing protocols constructed in Steps 8.1-8.3. After each testing protocol, move the tissue back to the original specimen size.

   Update?

   We apologize for our oversight in the numbering of our steps after subsequent revisions of the manuscript. This has been updated to state the correct step, **Steps 7.1-7.3**.