

Submission ID #: 71188

Scriptwriter Name: Kathyayani Sridharan

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**Title: Assessing Murine Aortic Intrinsic Stiffness Using Pin Myography:
A Translational Framework for Interrogating the Influence of the
Circulating Milieu**

Authors and Affiliations:

**Branden L. Nguyen¹, Ravinandan Venkatasubramanian¹, Mackenzie N.
Kehmeier¹, Ryan Calabrese¹, Sharmila Bc¹, Jenna Bowman¹, Vienna E. Brunt²,
Zachary S. Clayton¹**

¹Division of Geriatric Medicine, Department of Medicine, School of Medicine, University of Colorado Anschutz

²Division of Renal Diseases and Hypertension, Department of Medicine, School of Medicine, University of Colorado Anschutz

Corresponding Authors:

Zachary S. Clayton

Zachary.clayton@cuanschutz.edu

Email Addresses for All Authors:

Branden L. Nguyen

branden.nguyen@cuanschutz.edu

Ravinandan Venkatasubramanian

ravinandan.venkatasubramanian@cuanschutz.edu

Mackenzie N. Kehmeier

mackenzie.kehmeier@cuanschutz.edu

Ryan Calabrese

ryan.calabrese@cuanschutz.edu

Sharmila Bc

sharmila.bc@cuanschutz.edu

Jenna Bowman

jenna.bowman@cuanschutz.edu

Vienna E. Brunt

Vienna.brunt@cuanschutz.edu

Zachary S. Clayton

Zachary.clayton@cuanschutz.edu

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **Yes**

SCOPE shots: 2.1.2; 2.2.1; 2.2.2; 2.3.1; 2.3.2; 2.3.4; 3.4.4; 3.5.1;3.5.2;3.5.3; 3.8.2; 3.10.1

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 13

Number of Shots: 35

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Zachary Clayton:** Our research focuses on using pin myography for the aortic elastic modulus assessment as a measure of intrinsic aortic stiffness.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Zachary Clayton:** This protocol applies to both mice and rats, enabling investigation into how intrinsic properties contribute to overall *in vivo* aortic stiffness.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Branden Nguyen:** This protocol allows researchers to measure both the contributions of collagen and elastin to aortic intrinsic stiffness.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Zachary Clayton:** Accurate intrinsic aortic stiffness determination critically requires precise, consistent measurements of ring length, wall thickness, and diameter.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Branden Nguyen:** Future studies can use this platform to determine how proteins, RNA, or extracellular vesicles directly affect aortic elastic modulus.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What problem in your field of research does your JoVE article address?

- 1.6. **Zachary S. Clayton, PhD, Assistant Professor, Division of Geriatric Medicine, University of Colorado Anschutz:** (authors will present their testimonial statements live)
 - 1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What is the key takeaway you hope viewers remember after watching your video article?

- 1.7. **Zachary S. Clayton, PhD, Assistant Professor, Division of Geriatric Medicine, University of Colorado Anschutz:** (authors will present their testimonial statements live)
 - 1.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Anschutz Medical Campus

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

NOTE: All Screen capture and Scope shots are filmed by the videographer

2. Mouse Aorta Dissection

Demonstrator: Branden Nguyen

- 2.1. To begin, place the mouse in a dorsal recumbent position after anesthetizing with 2.5 percent inhaled isoflurane [1]. Following blood collection and secondary euthanasia, carefully remove the aorta from the arch to the abdominal segment [2].
 - 2.1.1. WIDE: Talent positioning the anesthetized mouse in dorsal recumbency.
Videographer: Please obtain CU shot as well for this shot.
 - 2.1.2. Show careful dissection and removal of the aorta from the aortic arch to the abdominal aorta.
- 2.2. Place the aorta in cold physiological saline solution, or CPSS (C-P-S-S) [1]. Use Vannas Spring Scissors with 2.5-millimeter blades to remove all connective and perivascular adipose tissue [2].
 - 2.2.1. Show placing the isolated aorta in CPSS.
 - 2.2.2. SCOPE: Show using the Vannas Spring Scissors to remove connective and perivascular adipose tissue from the aorta.
- 2.3. Carefully cut three 1-millimeter-wide aortic segments in the thoracic region just below the aortic arch [1]. Place two 1-millimeter aortic segments in a 1.5-milliliter tube containing CPSS [2] and store the tube at minus 80 degrees Celsius until stress-strain pin myography experiments [3]. Store the third segment in a cryomold filled with optimal cutting temperature compound for subsequent histological measurements [4].
 - 2.3.1. SCOPE: Show cutting three 1 mm-wide aortic segments from the thoracic region below the aortic arch.
 - 2.3.2. Show placing two aortic segments into a 1.5-milliliter tube filled with CPSS.
 - 2.3.3. Talent placing the tube into storage at minus 80 degrees Celsius.
 - 2.3.4. Show storing the third aortic segment separately for later histological measurements.

3. Stress-strain Pin Myography Experiments

Demonstrator: Zachary Clayton

- 3.1. Preheat the chambers containing approximately 6 milliliters of PBS (*P-B-S*), to 37 degrees Celsius [1] and thaw the 1-millimeter frozen aortic segments [2].
 - 3.1.1. Talent preheating the chambers containing phosphate-buffered saline to 37 degrees Celsius.
 - 3.1.2. Talent thawing the frozen 1-millimeter aortic segments.
- 3.2. Next, place the calibration bridge on the myograph [1] so that the T-balance bar does not touch the pin connected to the force transducer of the pin myography chamber [2].
 - 3.2.1. Talent placing the calibration bridge on the myograph.
 - 3.2.2. Close-up of the T-balance bar positioned without touching the pin connected to the force transducer.
- 3.3. Ensure the force transducer pin is not subject to any force [1]. When the relative force is stable, place a 2-gram weight on the T-bridge so that the T-balance bar applies approximately 2 grams of force on the force transducer pin [2].
 - 3.3.1. Talent checking that the force transducer pin is free of applied force.
 - 3.3.2. Talent placing a 2-gram weight on the T-bridge.
- 3.4. Confirm that the force readout is within 9.71 to 9.91 millinewtons to complete the calibration [1]. Determine the baseline micrometer reading [2] and record the initial value [3]. Use a dissection scope to determine the baseline micrometer distance at which the two parallel pins are nearly touching [4].
 - 3.4.1. SCREEN: Show the force readout within 9.71 to 9.91 millinewtons.
 - 3.4.2. Talent determining the baseline micrometer reading.
 - 3.4.3. Talent recording the initial micrometer value.
 - 3.4.4. SCOPE: Show the two parallel pins nearly touching while the baseline micrometer distance is determined.
- 3.5. Place the 1-millimeter aortic segment over the two pins [1]. Increase the pin width during mounting to create tension and prevent the aorta segment from being dislodged [2]. Use microcalipers to record the aortic segment width [3]. Next, connect the chamber unit to the pin myography system interface [4].
 - 3.5.1. SCOPE: Show the aortic segment being placed over the two pins.
 - 3.5.2. SCOPE: Show the pin width being increased to secure the aorta and prevent its dislodging.
 - 3.5.3. Show the measurement and record the width of the aortic segment using microcalipers.
 - 3.5.4. Talent connecting the chamber unit to the pin myography system interface.
- 3.6. After connecting the chamber and mounting it on the pin myography system, return

the micro positioner to the initial baseline position [1]. Increase the distance between the two pins by 1 millimeter from the initial baseline to stretch the aorta [2]. Next, return the pins to the initial micro-positioner reading to relax the aorta, then repeat the stretch-relax sequence 3 times [3].

3.6.1. Talent adjusting the micro positioner back to the initial position.

3.6.2. Talent increasing the distance between the two pins by 1 millimeter to stretch the aorta.

3.6.3. Talent returning the pins to the initial micro positioner reading.

3.7. On the pin myography system interface, zero the force reading for each chamber in use [1]. To commence the stress-strain experiment, adjust the micro-positioner to increase the force reading to approximately 1 milliNewton [2].

3.7.1. SCREEN: Show the pin myography system interface as the force reading is zeroed for each chamber in use.

3.7.2. Talent adjusting the micro positioner until the force reading reaches approximately 1 millinewton.

3.8. After approximately 1 millinewton force equilibration for 3 to 5 seconds, record the new micro-positioner reading at approximately 1 millinewton force to obtain the first force reading [1]. Perform 5 clicks or hashes on the instrument to increase the micro positioner by 50 micrometers, allowing the aorta segment to stretch for 3 minutes [2]. Then, record the force [3].

3.8.1. Talent recording the new micro positioner reading after equilibration.

3.8.2. SCOPE: Show increasing the micro positioner by 50 micrometers.

3.8.3. Talent recording the force after 3 minutes.

3.9. Continue increasing the micro-positioner by 50 micrometers every 3 minutes [1] and record the force until the yield point is reached [2].

3.9.1. Talent increases the micro positioner by 50 micrometers.

3.9.2. Talent continuing the sequence until the yield point is reached.

3.10. Confirm the yield point by stretching the aorta an additional 50 micrometers after the initial yield point [1] to ensure that the force value remains decreased or is slightly above the yield point [2]. Continue the three-minute stretch increments until the yield point is confirmed, then conclude the experiment and begin data analysis [3].

3.10.1. SCOPE: Show increasing the stretch by an additional 50 micrometers after the initial yield point.

3.10.2. SCREEN: Show the force value remaining decreased or slightly above the yield point.

3.10.3. Representative shot of talent performing the analysis.

FINAL SCRIPT: APPROVED FOR FILMING



Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

Results

4. Results

- 4.1. Representative stress-strain curves show that older aortas require higher stress to reach maximum expansion than younger tissue [1].

4.1.1. LAB MEDIA: Figure 8A. *Video editor: Please highlight the increase in height of the gray line in the stress-strain curves.*

- 4.2. While the elastic modulus in the low-force elastin region is higher in younger tissue [1], the high-force collagen region exhibits a significantly higher elastic modulus in older tissue [2].

4.2.1. LAB MEDIA: Figure 8B. *Video editor: Show the increased height of black bar relative to the grey bar in the elastin region graph.*

4.2.2. LAB MEDIA: Figure 8C. *Video editor: Show the increased height of the grey bar relative to the black bar in the collagen region graph.*

1. Dorsal
Pronunciation link: <https://www.merriam-webster.com/dictionary/dorsal>
IPA: /'dɔːrsəl/
Phonetic Spelling: DOR·suhl
2. Recumbent
Pronunciation link: <https://www.merriam-webster.com/dictionary/recumbent>
IPA: /rɪ'kʌmbənt/
Phonetic Spelling: rih·KUM·buhnt
3. Isoflurane
Pronunciation link: <https://www.merriam-webster.com/medical/isoflurane>
IPA: /ˈaɪsoʊˈfloʊrɪn/
Phonetic Spelling: eye·soh·FLOOR·ayn
4. Euthanasia
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanasia>
IPA: /ˌjuːθəˈneɪzə/
Phonetic Spelling: yoo·thuh·NAY·zhuh
5. Aorta
Pronunciation link: <https://www.merriam-webster.com/dictionary/aorta>
IPA: /eɪˈɔːrtə/
Phonetic Spelling: ay·OR·tuh
6. Aortic
Pronunciation link: <https://www.merriam-webster.com/dictionary/aortic>

- IPA: /er'ɔ:rtɪk/
Phonetic Spelling: ay·OR·tik
7. Physiological
Pronunciation link: <https://www.merriam-webster.com/dictionary/physiological>
IPA: /ˌfɪziə'lɑ:dʒɪkəl/
Phonetic Spelling: fiz·ee·uh·LAH·jih·kuhl
8. Vannas
Pronunciation link: No confirmed link found
IPA: /'vænəs/
Phonetic Spelling: VAN·uhs
9. Perivascular
Pronunciation link: <https://www.merriam-webster.com/medical/perivascular>
IPA: /ˌpɛrə'væskjələr/
Phonetic Spelling: pair·uh·VAS·kyuh·ler
10. Adipose
Pronunciation link: <https://www.merriam-webster.com/dictionary/adipose>
IPA: /'ædəˌpɔs/
Phonetic Spelling: AD·uh·pohss
11. Thoracic
Pronunciation link: <https://www.merriam-webster.com/dictionary/thoracic>
IPA: /θə'ræsɪk/
Phonetic Spelling: tuh·RASS·ik
12. Cryomold
Pronunciation link: No confirmed link found
IPA: /'kraɪoʊˌmoʊld/
Phonetic Spelling: CRY·oh·mohld
13. Histological
Pronunciation link: <https://www.merriam-webster.com/dictionary/histological>
IPA: /ˌhɪstə'lɑ:dʒɪkəl/
Phonetic Spelling: hiss·tuh·LAH·jih·kuhl
14. Myography
Pronunciation link: <https://www.merriam-webster.com/medical/myography>
IPA: /maɪ'ɑ:grəfi/
Phonetic Spelling: my·AH·gruh·fee
15. Phosphate-buffered
Pronunciation link: No confirmed link found
IPA: /'fɑ:sfɛrt 'bʌfərd/
Phonetic Spelling: FOSS·fayt BUF·erd
16. Myograph
Pronunciation link: <https://www.merriam-webster.com/medical/myograph>
IPA: /'maɪəˌgræf/
Phonetic Spelling: MY·uh·graf
17. Transducer
Pronunciation link: <https://www.merriam-webster.com/dictionary/transducer>

- IPA: /trænzˈduːsər/, /trænsˈduːsər/
Phonetic Spelling: tranz·DOO·ser / trans·DOO·ser
18. Millinewton
Pronunciation link: No confirmed link found
IPA: /ˈmɪlɪˌnuːtən/
Phonetic Spelling: MIL·lee·NOO·tuhn
19. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkrɑːmɪtər/
Phonetic Spelling: my·KRAH·muh·ter
20. Microcalipers
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊˈkælɪpərz/
Phonetic Spelling: my·kroh·KAL·ih·perz
21. Equilibration
Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibration>
IPA: /ɪˌkwɪləˈbreɪʃən/
Phonetic Spelling: ih·kwil·uh·BRAY·shuhn
22. Elastic
Pronunciation link: <https://www.merriam-webster.com/dictionary/elastic>
IPA: /ɪˈlæstɪk/
Phonetic Spelling: ih·LASS·tik
23. Modulus
Pronunciation link: <https://www.merriam-webster.com/dictionary/modulus>
IPA: /ˈmɑːdʒələs/
Phonetic Spelling: MAH·juh·luhs
24. Elastin
Pronunciation link: <https://www.merriam-webster.com/dictionary/elastin>
IPA: /ɪˈlæstɪn/
Phonetic Spelling: ih·LASS·tin
25. Collagen
Pronunciation link: <https://www.merriam-webster.com/dictionary/collagen>
IPA: /ˈkɑːlədʒən/
Phonetic Spelling: KAH·luh·juhn