Journal of Visualized Experiments

Optical Imaging of Isolated Murine Ventricular Myocytes

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60196R1
Full Title:	Optical Imaging of Isolated Murine Ventricular Myocytes
Keywords:	Myocyte, Enzymatic Isolation, Optical Action Potential, Calcium, Excitation-Contraction Coupling, Action Potential Duration
Corresponding Author:	Matthew KLOS, PhD UNIVERSITY HOSPITALS CLEVELAND MEDICAL CENTER Cleveland, OH UNITED STATES
Corresponding Author's Institution:	UNIVERSITY HOSPITALS CLEVELAND MEDICAL CENTER
Corresponding Author E-Mail:	MLK131@CASE.EDU
Order of Authors:	Matthew KLOS, PhD
	Shuxin Han
	Sherry Morgenstern
	Shreyas Suresh
	Ramiz Ahmad
	Isabella Pua
	Kayla Hicks
	Eric Devaney
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Cleveland, OH, USA

June 11th, 2019

JoVE Editorial Board
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editors:

Thank you for the review of our manuscript formerly entitled “Optical Imaging of Isolated Ventricular Myocytes Using the FluoVolt™ Membrane Potential Kit” and currently titled “Optical Imaging of Isolated Murine Ventricular Myocytes” which we submitted for exclusive consideration for publication as an article in the Journal of Visualized Experiments.

We found the reviews reasonable and insightful. To address the concerns raised by the reviewers, we significantly expanded the methodology and included three new figures. Because of this review, we feel the overall quality of our manuscript improved substantially.

In closing, thank you for your consideration of our work.

Sincerely,

Eric J. Devaney

Chief, Division of Pediatric Cardiac Surgery
Rainbow Babies & Children’s Hospital
Professor of Surgery
Case Western Reserve University
11100 Euclid Ave
Cleveland, OH 44106
Eric.Devaney@uhhospitals.org

TITLE:

Optical Imaging of Isolated Murine Ventricular Myocytes

AUTHORS AND AFFILIATIONS:

Matt Klos[1,*], Shuxin Han[2,*], Sherry Morgenstern[1], Shreyas Suresh[1], Ramiz Ahmad[1], Isabella Pua[1], Kayla Hicks[1], Eric Devaney[1]

[1]Pediatric Cardiac and Thoracic Surgery, University Hospitals Cleveland Medical Center, Cleveland, OH, USA

[2]Central Nodal (Anhui) Bioscience and Technology Research Center, Hefei, Anhui Province, China

*These authors contributed equally.

Email addresses of co-authors:

Matt Klos	(mlk131@case.edu)
Sherry Morgenstern	(sxm998@case.edu)
Shreyas Suresh	(sxs1654@case.edu)
Ramiz Ahmad	(rsa57@case.edu)
Isabella Pua	(iap17@case.edu)
Kayla Hicks	(kbh34@case.edu)

Corresponding authors:

Shuxin Han	(1439658593@qq.com)
Eric Devaney	(EricDevaney.@uhhospitals.org)

KEYWORDS:

myocyte, enzymatic isolation, optical action potential, calcium, excitation-contraction coupling, action potential duration

SUMMARY:

We present the methodology for the isolation of murine myocytes and how to obtain voltage or calcium traces simultaneously with sarcomere shortening traces using fluorescence photometry with simultaneous digital cell geometry measurements.

ABSTRACT:

The ability to isolate adult cardiac myocytes has permitted researchers to study a variety of cardiac pathologies at the single cell level. While advances in calcium sensitive dyes have permitted the robust optical recording of single cell calcium dynamics, recording of robust transmembrane optical voltage signals has remained difficult. Arguably, this is because of the low single to noise ratio, phototoxicity, and photobleaching of traditional potentiometric dyes. Therefore, single cell voltage measurements have long been confined to the patch clamp technique which while the gold standard, is technically demanding and low throughput. However, with the development of novel potentiometric dyes, large, fast optical responses to changes in voltage can be obtained with little to no phototoxicity and photobleaching. This

protocol describes in detail how to isolate adult murine myocytes which can be used for cellular shortening, calcium, and optical voltage measurements. Specifically, the protocol describes how to use a ratiometric calcium dye, a single-excitation calcium dye, and a single excitation voltage dye. This approach can be used to assess the cardiotoxicity and arrhythmogenicity of various chemical agents. While phototoxicity is still an issue at the single cell level, methodology is discussed on how to reduce it.

INTRODUCTION:

In order to study the heart during healthy and pathological states, it is often useful to examine the phenotype at the single cell level. While scientific advances have permitted the robust measurement of single cell calcium dynamics, single cell optical voltage measurements have remained scarce[1]. Arguably, this is because of the low signal to noise ratio (SNR), phototoxicity, and photobleaching of traditional potentiometric dyes[2,3]. Nonetheless, isolated myocyte optical action potentials have been obtained[2-4]. Further, with advances in the chemistry and the physics of voltage sensitive dyes, the SNR has improved[5]. Newer membrane potential probes (**Table of Materials**) respond to changes in membrane potential in sub-milliseconds and have a fluorogenic response range of approximately 25% per 100 mV. Further, the excitation/emission of the membrane potential kit (e.g., FluoVolt; **Table of Materials**) used in this protocol works with standard fluorescein isothiocyanate (FITC) or green fluorescent protein (GFP) settings[6].

The FITC and GFP excitation/emission spectra overlap with the fluo-4 calcium bound spectra[7]. Simultaneous acquisition of fluorescence photometry with digital cell geometry measurements traditionally has been used for the simultaneous acquisition of calcium and cellular shortening measurements[8]. This protocol describes in detail how to isolate murine myocytes and how to record calcium or voltage signals using standard FITC settings. Additionally, it describes how a simple switch in excitation/emission filters on the imaging workstation can be used to obtain calcium and shortening measurements using the ratio metric calcium dye fura-2. Compared to fluo-4, fura-2 has a higher affinity for calcium and is relatively resistant to photobleaching[9]. Consequently, using a single workstation this protocol allows for a thorough examination of singly myocyte excitation-contraction coupling.

PROTOCOL:

All methods and procedures described in this protocol have been approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

1. Preparation of solutions, instruments, and coverslips

NOTE: 1x solutions can be used for up to a month.

1.1. Make 10x Krebs-Henseleit buffer HEPES buffer without calcium (KHB-HB) by adding 68.96 g of NaCl, 3.57 g of KCl, 59.58 g of HEPES, 2.18 g of K_2HPO_4, 3.08 g of $MgSO_4$ and 19.82 g of glucose to 800 mL of double distilled water in a 1000 mL flask. After contents are fully dissolved, bring up to volume in a 1000 mL volumetric flask.

NOTE: Traditional Krebs Henseleit solution uses sodium bicarbonate as a buffer and the solution in this protocol uses Krebs Henseleit solution with HEPES buffer. Solution is stable for 6 months if sterile filtered.

1.2. Make 10x Tyrode's solution by adding 86.51 g of NaCl, 0.552 g of NaH_2PO_4, 2.03 g of $MgCl_2$, 9.91 g of glucose, 4.03 g of KCl, 2.65 g of $CaCl_2$, and 35.76 g of HEPES to 800 mL of double distilled water in a 1000 mL flask. After contents are fully dissolved, bring up to volume in a 1000 mL volumetric flask.

NOTE: Solution is stable for 6 months if sterile filtered.

1.3. Make 1x KHB-HB by measuring out 100 mL of the 10x stock and adding to 875 mL of double distilled water in a 1000 mL flask. Place flask in 37 °C water bath. Once the solution has reached 37 °C, use NaOH to increase the pH to 7.39. After adjusting the pH, bring solution to volume in a 1000 mL volumetric flask. Sterile filter the solution using a vacuum filtration system.

1.4. Make 1x Tyrode's solution by measuring out 100 mL of the 10x stock and adding to 875 mL of double distilled water in a 1000 mL flask. Place flask in 37 °C water bath. Once the solution has reached 37 °C, use NaOH to increase the pH to 7.39. After adjusting the pH, bring solution to volume in a 1000 mL volumetric flask. Sterile filter using a vacuum filtration system.

1.5. Make 1x modified Tyrode's solution by measuring out 100 mL of the 10x stock and adding to 875 mL of double distilled water in a 1000 mL flask. Dissolve 3.07 g of L-glutathione reduced in flask. Place flask in 37 °C water bath. Once the solution has reached 37 °C, use NaOH to increase the pH to 7.39. After adjusting the pH, bring solution to volume in a 1000 mL volumetric flask. Sterile filter the solution using a vacuum filtration system.

1.6. Make 100 mM blebbistatin stock solution by adding 855 µL of dimethyl sulfoxide (DMSO) to 25 mg of powder. Aliquot out in 20 µL increments and store in a -80 °C freezer for up to six months.

1.7. Make stopping buffer by adding 2 g of bovine serum albumin (BSA) and 1 vial of aliquoted blebbistatin stock to 100 mL of 1x KHB-HB and sterile filter the solution using a vacuum filtration system.

1.8. Make plating buffer by adding 5 mL of fetal bovine serum and 1 vial of the aliquoted blebbistatin stock to 95 mL of M199 HEPES. Sterile filter the solution using a vacuum filtration system.

1.9 Make myocyte culture buffer by adding one 1 vial of the aliquoted blebbistatin stock and 4 mls penicillin-streptomycin to 396 mL of M199 (25mM HEPES). Sterile filter the solution using a vacuum filtration system.

1.10. Autoclave 2 pairs of Dumont tweezers, 2 pairs of Iris curved scissors, 2 hemostats, one pair of plastic surgery forceps, 6 black braided silk 4-0 sutures arranged to be used as a surgical double-throw knot, and four 100 mL beakers.

1.11. Sterilize 22 x 22 glass coverslips. First, place a single coverslip in each well of a six well plate. Afterwards, with the lid removed, turn on the UV lamp of the biosafety cabinet and expose the coverslips to UV light for 1 h.

1.12. Make working laminin stock solution by first thawing the bottle on ice. Add contents of one bottle to enough cold sterile phosphate-buffered saline (PBS) to reach a final concentration of 0.04mg/mL. Aliquot out 1.3 µL into autoclaved 1.5 mL centrifuge tubes. Store at -80 °C.

NOTE: Each tube has enough laminin for a single six well plate. Avoid multiple freeze thaw cycles.

1.13. Coat sterilized coverslip by first thawing the working laminin solution on ice. Using a P1000 pipette, aspirate 200 µL of laminin. Gently drag the pipette tip along one edge of the coverslip to allow capillary action to pull out a minuscule amount of laminin to facilitate coverslip attachment to the six well plate.

1.14. Then, expel the remaining laminin in the center of the coverslip. In a circular motion, spread the laminin droplet across the coverslip. Place in a 37 °C incubator at least 1 h and up to 24 h before the isolation.

2. Preparation of the Langendorff apparatus

NOTE: The individual components of the Langendorff apparatus used in this protocol are listed in **Table of Materials**.

2.1. Turn on the circulating water bath. Set temperature so that perfusate has a temperature of 37 °C.

NOTE: With the solution reservoirs set to a height of 60 cm, the circulating waterbirth needs to be set to 41 °C to have the perfusate be 37 °C. Unlike previously reported protocols, the height of the reservoir does not need to be changed.

2.2. Rinse the Langendorff apparatus with 70% ethanol followed by two rinses with autoclaved double distilled water. After rinsing, fill reservoir with KHB-HB and oxygenate with 100% oxygen.

2.3. Prime the system by allowing oxygenated KHB-HB to first flow into a 100 mL beaker. Once 50 mL of solution has flowed into the beaker, switch the 3-way stop-cock position to stop flow from the KHB-HB reservoir. Pour 50 mL of oxygenated KHB-HB from the beaker into the collagenase reservoir.

2.4. Let the KHB-HB drain from the digestion reservoir until 5 mL remains in collagenase reservoir. While priming collagenase reservoir, switch the 3-way stop cock repeatedly between reservoirs to allow the lines to degas. After the system is primed, remember to use the degassing trap located on top of the heating coil to allow any remaining air to exit the system.

2.5. Make the collagenase solution. For rats combine 100 mg of type II collagenase, 100 mL of oxygenated KHB-HB, and 2 vials of the blebbistain stock. For mice, combine 100 mg of type II collagenase, 40 mL of oxygenated KHB-HB, and 2 vials of the blebbistain stock. Once mixed, the solution should be stable for 1 h.

NOTE: Myocyte viability can vary between type II collagenase lots. Take advantage of a collagenase sampling program to test a lot before bulk ordering.

3. Myocyte Isolation

3.1. Inject the animal with 1000 units of heparin. Wait 5 min.

NOTE: Mice and rats of any age can be used. However, in general the older or more diseased the animal, the lower the myocyte yield.

3.2. Sacrifice the animal by first anesthetizing it with isoflurane using the open-drop method (1 cc of Isoflurane per 500 cc volume) before euthanizing the animal with a pentobarbital mixture (150 mg/kg intraperitoneal).

3.3. Rapidly excise the heart by first grabbing the fur above the xiphoid process. With the iris scissors, make a small incision immediately below the xiphoid process and pull the fur upwards toward the head exposing the skin.

3.4. Grab the xiphoid process and cut the diaphragm exposing the thoracic cavity. Make a trap door incision, pull the sternum back using a hemostat, and use the curved forceps to excise the heart above the ascending aorta and place in cold KHB-HB.

3.5. Cannulate the heart using a stereo microscope and number 5 forceps. Make sure the heart is submerged and the cannula was primed before heart excision to prevent emboli. Confirm proper positioning of the cannula by visualizing the tip of the cannula approximately 1 mm above aortic insertion into the ventricle.

NOTE: The faster the cannulation time, the better the myocyte yield.

3.6. Start the flow of KHB-HB by rotating the stopcock on the Langendorff. Connect the cannula to the Langendorff. Perfuse the heart for 5 min.

NOTE: Since perfusion is supplied by a gravity-based system, flow through the heart will be a function of coronary artery compliance.

3.7. Switch perfusion from the KHB-HB reservoir to the digestion buffer reservoir. Once the digestion buffer reaches the heart, set a timer (5 min for mouse or 15 min for rat). Make sure to collect the perfusate in a sterile 100 mL beaker. Refill the digestion buffer reservoir as needed with the perfusate until the digestion time has expired.

3.8. After digestion, separate the chambers of the heart with forceps and the iris scissors in a sterile 100 mL beaker. Place each chamber into a separate well of a six well plate. Pour 5 mL of collagenase solution into each well.

3.9. Immediately start mincing the heart tissue using scissors. Tissue chunks should be approximately 1 mm^3. Using sterile transfer pipettes, gently triturate the minced heart tissue. The solution should turn cloudy.

3.10. Once the tissue chunks become white and feathery, examine the cells using an inverted microscope. If the number of viable cells is greater than 80%, proceed to strain the cells into a 50 mL conical tube using a 100 µm cell strainer. Use a different tube and strainer for each chamber of the heart.

3.10.1. If the number of viable cells is less than 80%, check the time it took to cannulate. If the cannulation time is over 5 min, try another heart. If not, assay new collagenase lots through the collagenase sampling program.

3.11. Pellet the cells by centrifuging at 215 x *g* for 2 min. The pellet should be compact and not loose. If the pellet is loose, the preparation contains many dead cells. In a tissue culture hood, resuspend the pellet in 10 mL of stopping buffer.

3.12. Pellet the cells by centrifuging at 215 x *g* for 2 min. The pellet should be compact and not loose. If the pellet is loose, the preparation contains many dead cells.

3.13. Resuspend the cells in 5 mL of plating buffer. Perform a cell count. Adjust the milliliters of plating buffer to reach a final myocyte concentration of 2 x 10^4 cells per mL.

3.14. Remove the laminin-coated coverslips from the incubator. Aspirate the laminin droplet.

3.15. Plate 200 µL of myocyte suspension on each coverslip. Place in a 37 °C incubator (21% O_2, 5% CO_2) for 2 h to allow attachment. After 2 h, aspirate the unattached cells, add 2 mL of culture media, and culture for up to 4 days.

4. Fura-2 dye loading

4.1. Make a 2 mM fura-2 acetoxymethyl ester (fura-2 AM) stock solution by adding 25 µL of DMSO to 50 µg of fura-2 AM powder (1 vial). Aliquot out into 6 µL aliquots. Take 1 aliquot of fura-2 AM and add to 6 mL of plating medium. Vortex to mix.

4.2. Remove 1 six well plate of myocytes from the incubator. Aspirate media. Add 1 mL of fura-2 media mixture to each well. Cover plate with foil, leave the plate at room temperature, and wait 15 min.

4.3. Aspirate fura-2 media mixture and add 1 mL of Tyrode's solution to each well. Cover with foil. Wait 20 min at room temperature to allow for dye washout before imaging.

5. Fluo-4 dye loading

5.1. Make a 1.82 mM fluo-4 acetoxymethyl ester (fluo-4 AM) stock solution by adding 25 µL of DMSO to 50 µg of fluo-4 AM powder (1 vial). Aliquot out into 8.333 µL aliquots. Take 1 aliquot of fluo-4 AM stock and add to 6 mL of plating medium. Vortex to mix.

5.2. Remove 1 six well plate of myocytes from the incubator. Aspirate media. Add 1 mL of fluo-4 AM media mixture to each well. Cover plate with foil, leave the plate at room temperature, and wait 15 min.

5.3. Aspirate fluo-4 AM media mixture and add 1 mL of Tyrode's solution to each well. Cover with foil. Wait 20 min at room temperature to allow for dye washout before imaging.

6. Membrane potential dye loading

6.1. Remove component A and component B from the membrane potential kit. In a 15 mL conical tube, combine 50 µL of component B and 5 µL of component A. Vortex to mix. Add 10 mL of plating media to the 15 mL conical tube containing the voltage dye mixture. Vortex to mix.

6.2. Remove 1 six well plate of myocytes from the incubator. Aspirate the media. Add 800 µL of the membrane potential dye mixture to each well. Cover plate with foil, leave the plate at room temperature, and wait 15 min.

6.3. Aspirate dye media mixture and add 1 mL of modified-Tyrode's solution to each well. Cover with foil.

7. Photometry and charge coupled device recordings

7.1. Turn on the equipment in the following order: microscope, arc lamp, hyperswitch, fluorescence interface system, Myocam power supply, field stimulator, and computer.

7.2. Make sure the excitation/emission filter sets are appropriate for the imaging dye.

NOTE: Fura-2 is excited at 340 nm and 380 nm of light. It emits at 510 nm of light. Fluo-4 and the voltage membrane dye are excited at 485 nm of light and emit at 520 nm of light.

7.3. Prime the system by turning on the vacuum, fully opening the hose clamp, and gently plunging each 60 mL syringe being used in the manifold. For calcium recordings use Tyrode's solution. For voltage recordings use modified Tyrode's solution.

7.4. Turn heater on and set flow by adjusting the roller clamp on the perfusion tubing. Make recordings at 36 ± 1 °C.

7.5. Open the acquisition software. Make sure the parameters are set for the correct imaging dye.

7.6. In the dark, remove the foil from the six well plate and place a coverslip in the pacing chamber. Make sure the stimulator is off during this step. Focus on the myocytes using the 10x objective.

7.7. Once in focus, start pacing by field stimulating at 1 Hz, 0.2 V. Gradually increase the voltage until 1:1 pacing is obtained. Then increase the voltage until 1.5x the threshold is reached.

NOTE: Because excitation-contraction coupling is temperature dependent, make sure the cells have been perfused for 15 min before recording. This allows for myocytes to recover from the shock of going from room temperature back to 37 °C as well as loosely attached cells to float away.

7.8. Switch from the 10x objective to the 40x objective. Focus in on a cell that is following a 1:1 pacing. Adjust the plastic shades so only one cell is in the field of view.

7.9. Using the software, place the area of interest box on well-defined sarcomeres. Start the acquisition software to initiate the excitation light. Using the neutral density filters, adjust the intensity setting accordingly to obtain an adequate SNR.

REPRESENTATIVE RESULTS:

Figure 1A shows the Langendorff apparatus. The oxygenator is in the KHB-HB reservoir. The collagenase solution is in the middle 60 mL syringe reservoir. The degassing line is connected to the empty 60 mL syringe reservoir. After a successful isolation, most of the cells should be rod shaped and striated. Under a 40x objective, most myocytes should have clear striations visible. **Figure 1B,C** shows examples of healthy rat myocytes. Once isolated, cells can be cultured up to 4 days while maintaining their morphology and electrical properties.

To measure excitation-contraction coupling, the cells are then placed in a heated pacing chamber. Because myocytes are sensitive to changes in temperature, it is important to allow the coverslip to equilibrate for 15 min in the chamber before recording. For fluorescence recordings, the excitation wavelength is generated by a 75 W xenon-arc bulb. Xenon-arc bulbs produce a light spectrum that mimics natural sunlight. The intensity of the light and the wavelength are controlled by neutral density/emission filers. The excitation light then passes through the objective to the myocyte. The emission wavelength is then collected by a photomultiplier tube.

Using the system described here, both the excitation and emission filters need to be changed manually.

Shortening on the other hand is obtained by a charge coupled device sensor. Measuring in real time up to 1000 times per second, the acquisition software performs an average of the lines within an area of interest to create a well resolved striation pattern. A fast Fourier transform (FFT) is then calculated. The peak within the power spectrum represents the average sarcomere spacing. Changes in the sarcomere spacing during pacing are then plotted and subsequently quantified.

Figure 2 shows calcium and shortening traces recorded from a C57/B6 mouse myocyte loaded with the calcium dye fura-2. The pacing protocol is a modification of pacing protocols described previously[10,11]. Healthy mouse myocytes should be able to be paced at their resting heart rate 10 Hz. **Figure 3** is quantification of ensembled averaged data obtained from a C57/B6 mice and their transgenic (TG) littermates who had a point mutation introduced into a potassium channel. Notice there is no difference between the groups except for the relaxation time at 10 Hz pacing.

Unlike fura-2 which is a dual excitation dye, the voltage dye and fluo-4 are single wavelength excitation dyes whose excitation/emission work with standard FITC excitation and emission spectrum (494/506 nm). Therefore, recordings of calcium and sarcomere shortening or voltage and sarcomere shortening can be obtained using this filter set.

Figure 4A shows a voltage tracing recorded from a C57/B6 mouse myocyte paced at 10 Hz. Compared to calcium signals, single cell voltage tracings are smaller in amplitude and need post-processing to obtain a useable signal. **Figure 4B** shows an ensembled averaged action potential (AP) made from the APs in **Figure 4A**. **Figure 4C,D** shows an ensembled average AP after a low pass Butterworth or a Savitzky-Golay digital filter was applied. Care must be taken when filtering the signal as not to distort the real data. Notice the subtle differences in the shape of the APs in **Figure 4B-D**.

Figure 5 shows traces recorded from rat myocytes paced at 1 Hz. In addition to the voltage signal being lower than the calcium signal, the contraction kinetics are different as well. This is because calcium dyes buffer calcium while voltage dyes do not.

As with the calcium transient (**Figure 3**), myocytes demonstrated pacing dependent changes in their optical action potential duration (APD) as well (**Figure 6**). While the fura-2 traces were ensembled averaged before being quantified, the voltage traces were filtered with a Savitzky-Golay polynomial smoothing filter (width 5, order 2) before being ensembled averaged and quantified.

As quantified in **Figure 6** and **Figure 7**, in addition to demonstrating pacing induced changes in APD, they also demonstrated drug induced prolongation of the AP. At 4 Hz pacing, concentration dependent blockade of the transient outward current (I_{to}) with 4-aminopyridine resulted in prolongation of the APD.

Finally, care must be taken to avoid cytotoxicity. **Figure 8** is the last 11 seconds of a 20 s recording. Indicated by the red arrows in **Figure 8**, prolonged exposure of myocytes to blue light leads to triggered activity.

FIGURE LEGENDS:

Figure 1: Constant pressure Langendorff apparatus. (**A**) The Langendorff Apparatus with each component labeled in white lettering. (**B**) Isolated Sprague-Dawley rat myocytes viewed through a 10x objective. (**C**) Isolated rat myocytes viewed through a 40x objective.

Figure 2: Representative calcium and sarcomere shortening traces recorded from C57/B6 myoyctes using fura-2. Calcium and sarcomere shortening traces recorded at 1, 2, 4, 10, 0.5 and 0.75 Hz.

Figure 3: Quantification of sarcomere shortening, peak calcium, relaxation time, and reuptake time recorded from a C57/B6 wild type (WT) and transgenic (TG) mice. (**A**) Sarcomere shortening. (**B**) Peak calcium. (**C**) Relaxation time defined as 90% return to baseline of the shortening trace. (**D**) Reuptake time defined as 90% return to baseline of the calcium trace.

Figure 4: Optical action potential recorded from a C57/B6 mouse myocyte paced at 10 Hz. (**A**) 1 second unfiltered trace. (**B**) Ensembled averaged optical action potential. (**C**) Ensembled averaged optical action potential after a lowpass Butterworth filter was applied. (**D**) Ensembled averaged optical action potential after a Savitzky-Golay polynomial smoothing filter was applied.

Figure 5: Representative calcium, voltage, and sarcomere shortening traces recorded from Sprague-Dawley rat myocytes paced at 1 Hz. (**A**) Calcium and sarcomere shortening traces recorded at 1 Hz pacing using fluo-4. (**B**) Voltage and sarcomere shortening traces recorded at 1 Hz pacing using the voltage dye.

Figure 6: Optical action potentials recorded from Sprague-Dawley rat myocytes paced at 1, 2, and 4 Hz pacing. (**A**) Filtered trace recorded at 1 Hz pacing. (**B**) Filtered trace recorded at 2 Hz pacing. (**C**) Filtered trace recorded at 4 Hz pacing. (**D**) Action potential duration 10, measured as 10% return to baseline. (**E**) Action potential duration 50, measured as 50% return to baseline. (**F**) Action potential duration 90, measured as 90% return to baseline.

Figure 7: The Effects of 4-aminopyridine on Sprague-Dawley rat optical action potentials recorded at 4 Hz pacing. (**A**) Ensembled averaged trace recorded at 4 Hz pacing with no 4-Aminopyridine in the solution. (**B**) Ensembled averaged trace recorded at 4 Hz pacing with 1 µM 4-Aminopyridine in the solution. (**C**) Ensembled averaged trace recorded at 4 Hz pacing with 10 µM 4-Aminopyridine in the solution. (**D**) Action potential duration 10, measured as 10% return to baseline. (**E**) Action potential duration 50, measured as 50% return to baseline. (**F**) Action potential duration 90, measured as 90% return to baseline.

Figure 8: Voltage dye induced phototoxicity in Sprague-Dawley rat myocytes after 20 seconds of continuous light exposure. Red arrows indicate cytotoxic events.

DISCUSSION:

Being able to isolate cardiac myocytes is a powerful method that can be used to understand cardiac physiology, pathology, and toxicology. In the above protocol, we described a method that utilizes a constant gravity pressure Langendorff apparatus to obtain single cardiac myocytes. Afterwards, using the fluorescence photometry system, we describe how to simultaneously acquire either calcium and shortening or voltage and shortening traces.

Because of the different kinetics between calcium dyes, care must be taken on which dye to select. For this protocol, both the fura-2 and fluo-4 used were engineered with AM esters necessitating a wash step to allow for intracellular esterases time to cleave the AM group and trap the dye in the cell. While both fura-2 and fluo-4 are considered high affinity calcium dyes, the Kd for fura-2 is 145 nM compared to the 345 nM for fluo-4[9]. Further, fura-2 is ratiometric. Because of this, it can be used to quantify intracellular calcium levels[9,12]. Fluo-4 on the other hand is a single wave calcium probe. The advantage of using fluo-4 is it produces a brighter fluorescence signal. Regardless of which calcium dye is used, compared to the calcium dye, membrane voltage probes have a lower SNR.

As shown in **Figure 4** and **Figure 5**, voltage traces compared to calcium traces are smaller in amplitude. Using the software's digital trace filtering, it is possible to increase the SNR and quantify the data (**Figure 4** and **Figure 7**). Once quantified, both calcium transients and optical APDs demonstrate restitution, shortening their duration at faster pacing frequencies (**Figure 2**, **Figure 3**, **Figure 6**, and **Figure 7**). Shorter APDs during faster pacing cycles are necessary to allow enough time for ventricular filling during diastole. Alterations in this phenomenon is thought to be indicative of an increase in the risk of arrythmias[13-16]. While alterations in APD can be caused by disease, they can also be caused by chemicals. As shown in **Figure 7**, when the predominant murine repolarizing potassium current, I_{to}, is blocked, the optical APD becomes longer.

Still, as reported previously with voltage sensitive dyes, light intensity and duration can alter the APD[2,5,17]. This is believed to be the result of the generation of reactive oxidative species (ROS)[5]. Previously, it has been shown that the addition of antioxidants to the recording solution can prevent voltage sensitive dye cytotoxicity[5]. As a result, we added the antioxidant L-glutathione (10 mM), to Tyrode's solution. Shown in **Figure 8** is the last 11 seconds of a 20 s recording obtained at 1 Hz pacing. As indicated by the red arrows, alterations in the APD did not occur until 15 s into the recording; therefore, while the modified Tyrode's solution did not prevent phototoxicity it delayed it significantly. Using modified Tyrode's solution, using a low light intensity setting and keeping the duration of the recording to under 5 s, it is possible to avoid any dye induced alterations in APD. This is important because without taking care to avoid phototoxicity, the data could be misinterpreted as causing early or delayed after depolarizations. In addition to limiting the exposure to blue light, there are additional precautions that can be taken to prevent misinterpretation of the data.

The first is to only record from cells that follow one to one pacing and have a resting sarcomere length greater than or equal to 1.75 µm. The 1.75 µm cutoff is taken from the observation by Gordon et al.[18] that tension rapidly declines once the sarcomere length is below this amount. Nonetheless, certain pathologies may result in significant alterations in resting sarcomere length. To be sure that the phenotype is real and not an artifact of the isolation, the following trouble shooting approaches should be taken.

If myocytes are consistently not following 1:1 pacing, have sarcomere lengths below 1.75 µm, heavy membrane blebbing, or do not survive the isolation, the first thing to check is the time it took to cannulate the heart. The longer the cannulation time, the lower the yield will be. If a long cannulation time is required, viability can be improved by placing the heart in a cardioplegic solution[19]. Nonetheless, because the collagenase is an enzyme, the activity and specificity of a specific lot change over time. If the overall yields progressively become worse despite good cannulation times, new lots should be assayed. While our protocol was optimized for 5 s recordings, if longer voltage traces are needed, additional neutral density filters will need to be purchased. The system described in the protocol comes with neutral density filters that reduce the transmitted light by 37%, 50%, 75%, 90%, and 95%.

In summary, we described a methodology that allowed for the isolation of adult murine ventricular myocytes that were used for calcium, voltage, and sarcomere shortening measurements.

ACKNOWLEDGMENTS:

We thank Dana Morgenstern for careful proofreading of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Hagen, B. M., Boyman, L., Kao, J. P., Lederer, W. J. A comparative assessment of fluo Ca2+ indicators in rat ventricular myocytes. *Cell Calcium.* **52** (2), 170-181 (2012).

2. Schaffer, P., Ahammer, H., Muller, W., Koidl, B., Windisch, H. Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes. *Pflügers Archiv: European Journal of Physiology.* **426** (6), 548-551 (1994).

3. Hardy, M. E., Lawrence, C. L., Standen, N. B., Rodrigo, G. C. Can optical recordings of membrane potential be used to screen for drug-induced action potential prolongation in single cardiac myocytes? *Journal of Pharmacological and Toxicological Methods.* **54** (2), 173-182 (2006).

4. Tian, Q. et al. Optical action potential screening on adult ventricular myocytes as an alternative QT-screen. *Cellular Physiology and Biochemistry*. **27** (3-4), 281-290 (2011).

5. Warren, M. et al. High-precision recording of the action potential in isolated cardiomyocytes using the near-infrared fluorescent dye di-4-ANBDQBS. *The American Journal of Physiology-Heart and Circulatory Physiology.* **299** (4), H1271-1281 (2010).

6. ThermoFisher Scientific. *FluoVolt™ Membrane Potential Kit*, <https://www.thermofisher.com/document-connect/document-

connect.html?url=https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0009668_FluoVolt_Membrane_Potential_Kit_UG.pdf&title=VXNlciBHdWlkZTogRmx1b1ZvbHQgTWVtYnJhbmUgUG90ZW50aWFsIEtpdA==> (2018).
7. ThermoFisher Scientific. *Fluorescence SpectraViewer*, <https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html> (2019).
8. IonOptix. *Calcium and Contractility in Isolated Myocytes*, <http://www.ionoptix.com/product/myocyte-calcium-and-contractility-recording-system/> (2019).
9. Paredes, R. M., Etzler, J. C., Watts, L. T., Zheng, W., Lechleiter, J. D. Chemical calcium indicators. *Methods.* **46** (3), 143-151 (2008).
10. Davis, J. et al. Diastolic dysfunction and thin filament dysregulation resulting from excitation-contraction uncoupling in a mouse model of restrictive cardiomyopathy. *The Journal of Molecular and Cellular Cardiology.* **53** (3), 446-457 (2012).
11. Ren, J., Davidoff, A. J. Diabetes rapidly induces contractile dysfunctions in isolated ventricular myocytes. *The American Journal of Physiology.* **272** (1 Pt 2), H148-158 (1997).
12. Roe, M. W., Lemasters, J. J., Herman, B. Assessment of fura-2 for measurements of cytosolic free calcium. *Cell Calcium.* **11** (2-3), 63-73 (1990).
13. Goldhaber, J. I. et al. Action potential duration restitution and alternans in rabbit ventricular myocytes: the key role of intracellular calcium cycling. *Circulation Research.* **96** (4), 459-466 (2005).
14. Weiss, J. N. et al. The dynamics of cardiac fibrillation. *Circulation.* 112 (8), 1232-1240 (2005).
15. Baher, A. et al. Short-term cardiac memory and mother rotor fibrillation. *The American Journal of Physiology-Heart and Circulatory Physiology.* **292** (1), H180-189 (2007).
16. Kleber, A. G., Rudy, Y. Basic mechanisms of cardiac impulse propagation and associated arrhythmias. *Physiological Reviews.* **84** (2), 431-488 (2004).
17. McPheeters, M. T., Wang, Y. T., Werdich, A. A., Jenkins, M. W., Laurita, K. R. An infrared optical pacing system for screening cardiac electrophysiology in human cardiomyocytes. *PLoS ONE.* **12** (8), e0183761 (2017).
18. Gordon, A. M., Huxley, A. F., Julian, F. J. The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *The Journal of Physiology.* **184** (1), 170-192 (1966).
19. Li, Y. et al. Three Preservation Solutions for Cold Storage of Heart Allografts: A Systematic Review and Meta-Analysis. *The Journal of Artificial Organs.* **40** (5), 489-496 (2016).

Figure 1

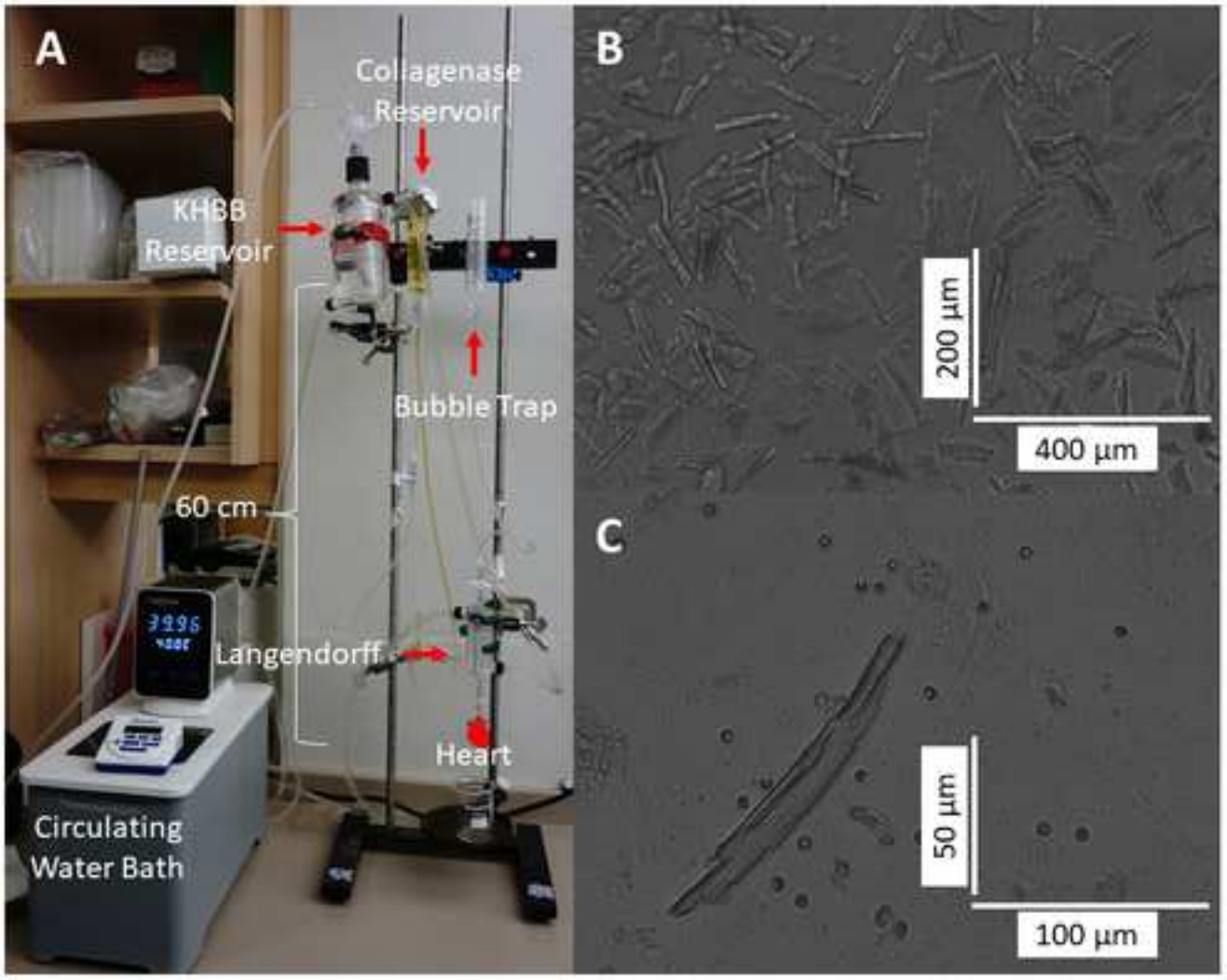


Figure 2

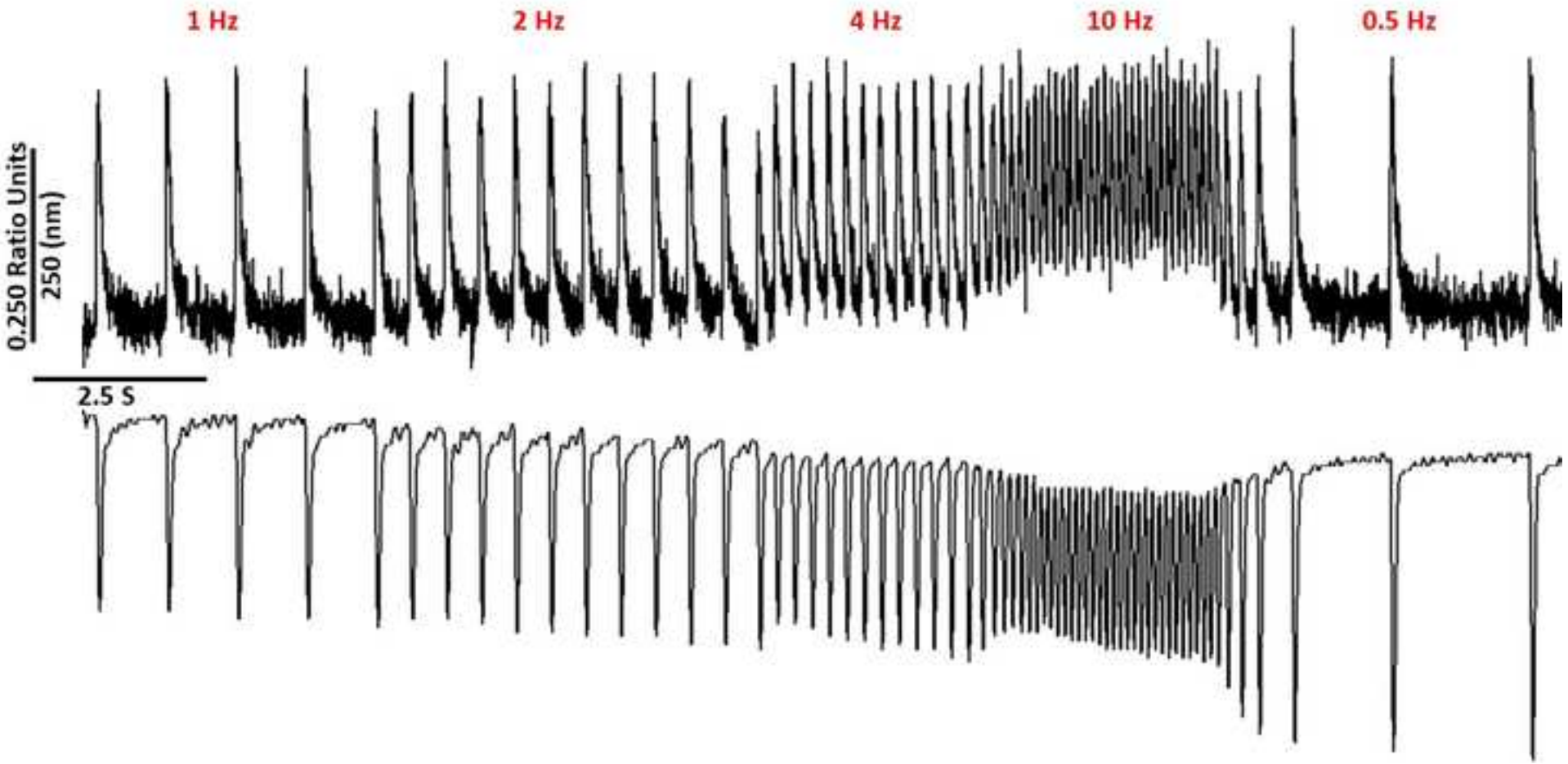


Figure 3

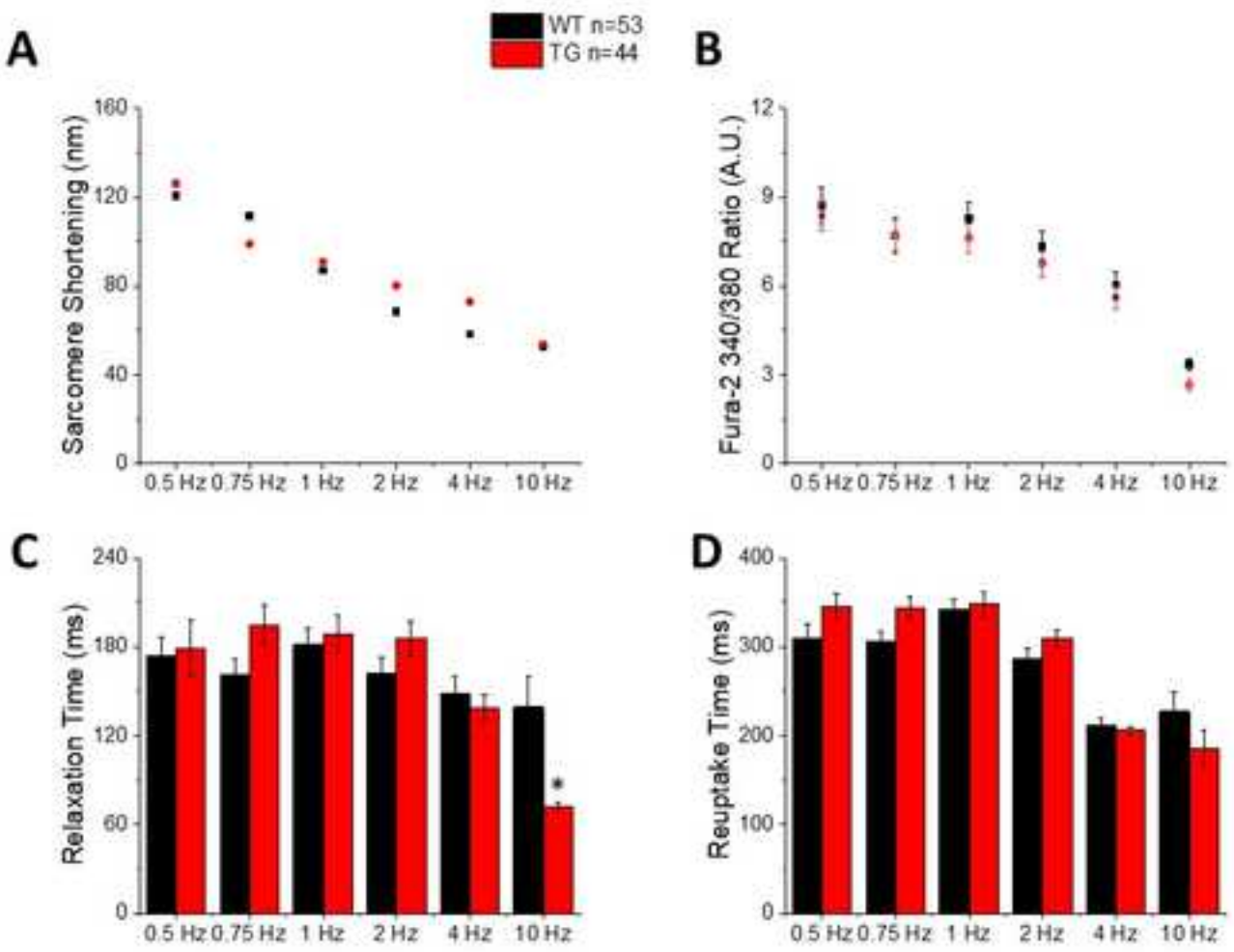


Figure 4

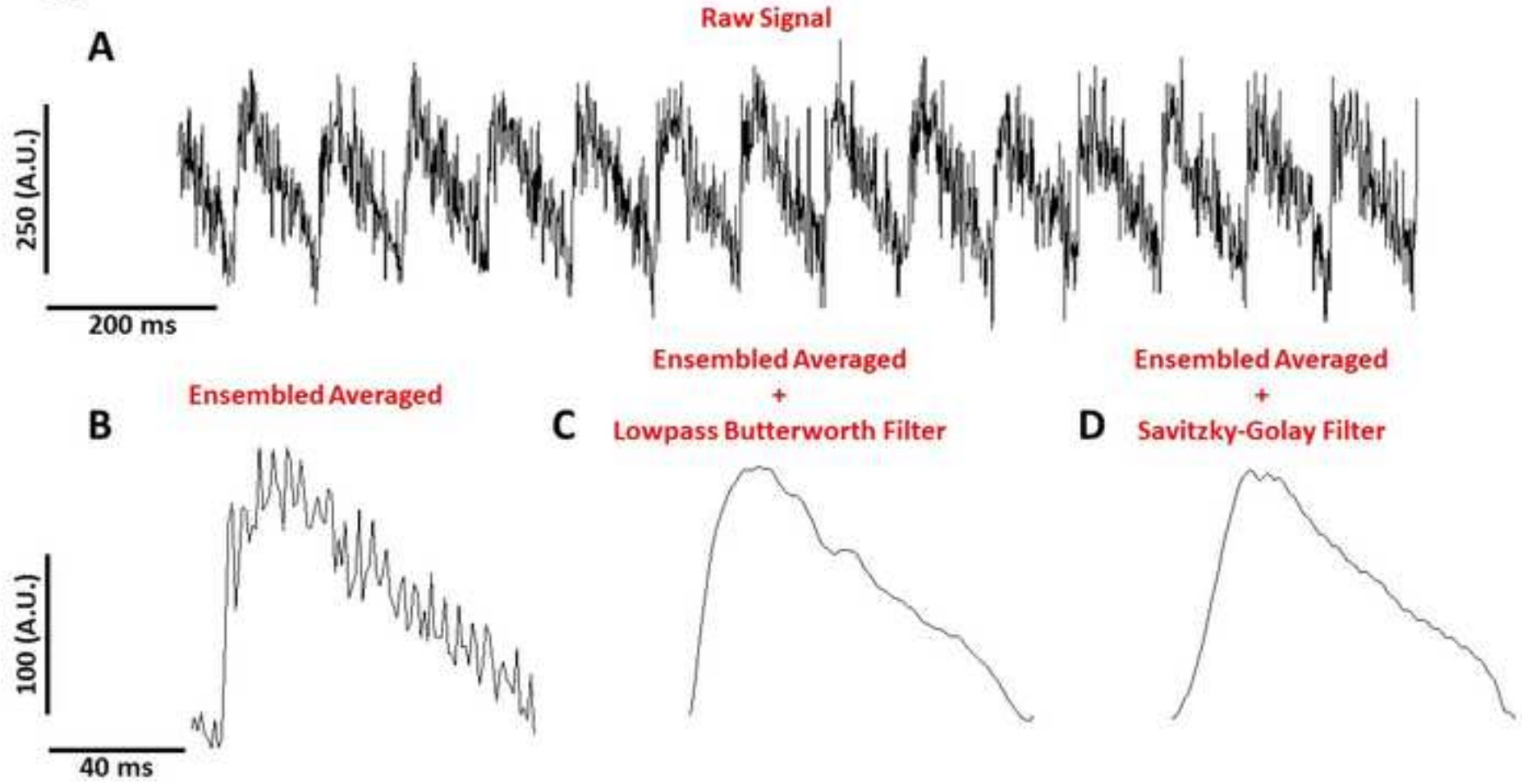
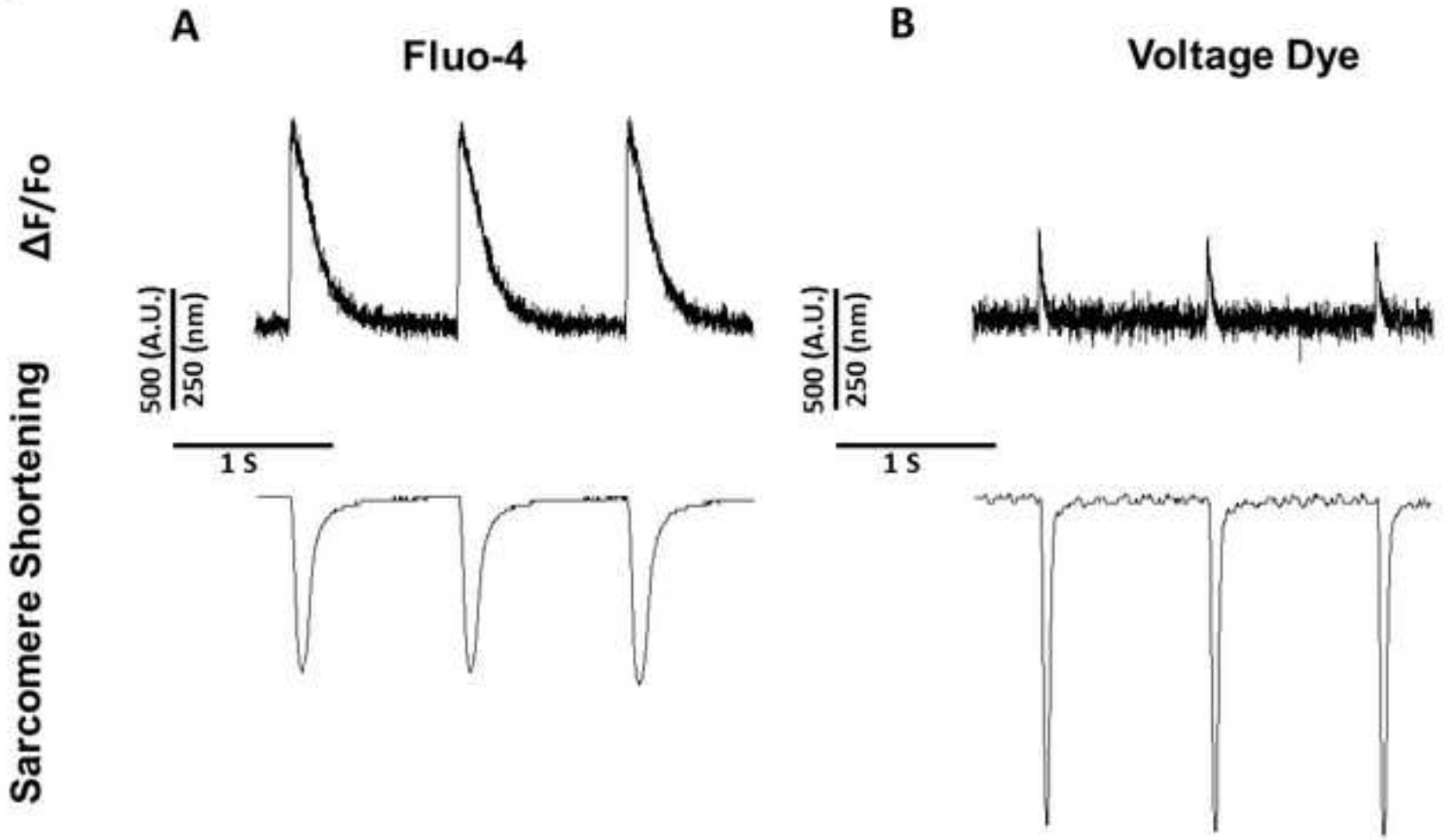
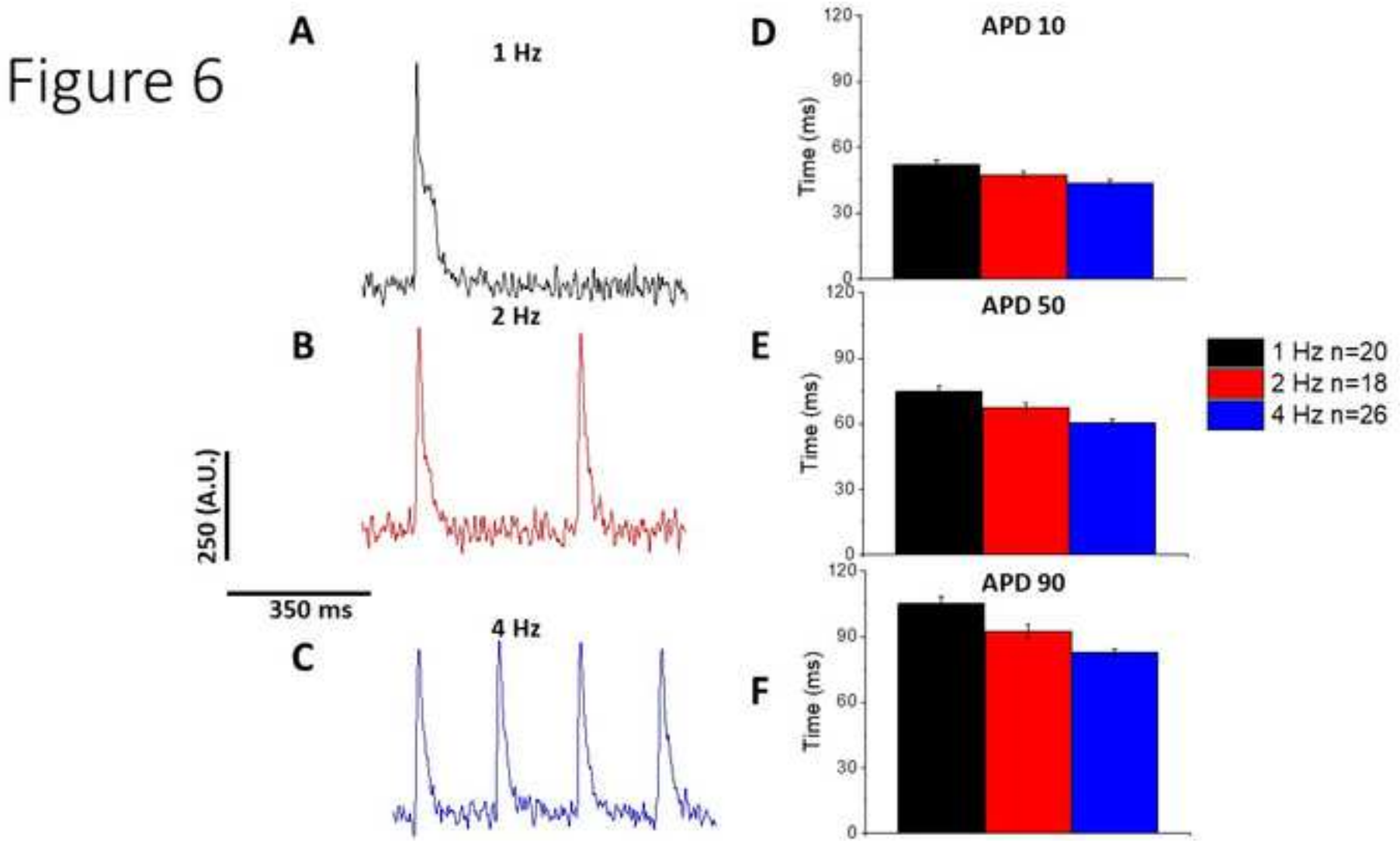


Figure 5





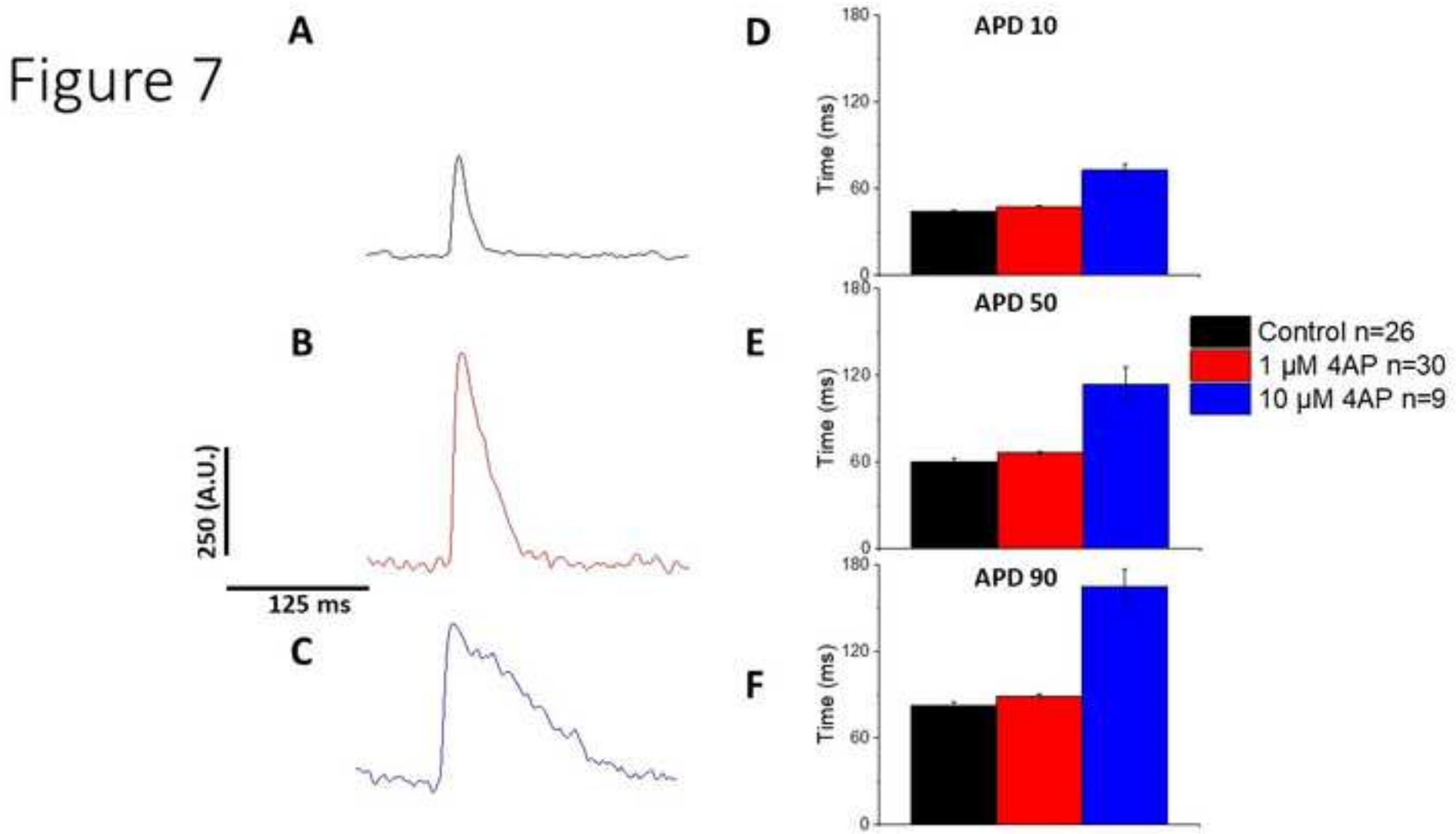
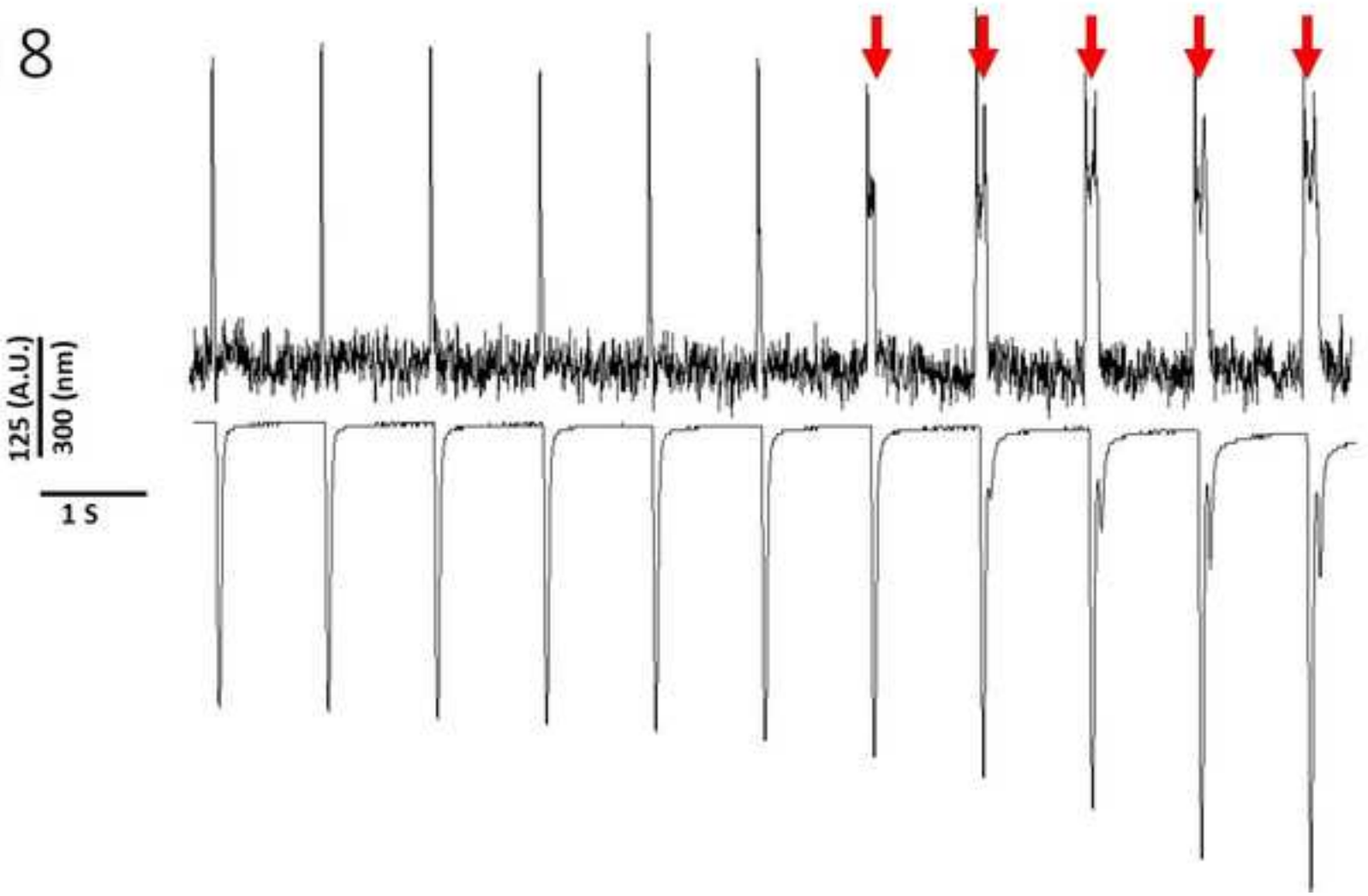


Figure 8



Name of Material/ Equipment	Company	Catalog Number
0.25 Liter Water Jacketed Reservoir	Radnoti, LLC	120142-025
1 liter volumetric flask	Fisher Scientific	10-205F
100 ml beaker	Fisher Scientific	FB-100-100
100 ml graduated cylinder	Fisher Scientific	08 562 5C
1000 ml flask	Fisher Scientific	FB-500-1000
2-Bar Lab Stand with Stabilizer Bar and 24” Stainless Steel Rods	Radnoti, LLC	159951-2
4-Aminopyridine	Sigma-Aldrich	275875
40X Oly UApo/340 Non-Immersion Objective (NA 0.9, WD 0.2mm)	IonOptix	MSCP1-40 (b)
60-mL syringe, BD Luer-Lok tip	BD	309650
Aortic Metal Cannulae	Harvard Apparatus	73-0112
Bovine Serum Albumin	Fisher Scientific	BP9703-100
C-6 Standard Heating Circulator	Chemyx	A30006
CaCl2	Fisher Scientific	BP510500
Cell framing adapter	IonOptix	CFA300
CellPr0 Vacuum Filtration System, 1 liter, 0.22µm,Cs/12	Labratory Product Sales, Inc	V100022
CellPro Vacuum Filtration System,250mL, 0.22µm,Cs/12	Labratory Product Sales, Inc	V25022
CellPro Vacuum Filtration System,500mL, 0.22µm,12/CS	Labratory Product Sales, Inc	V50022
CMC (mTCII) Temp Control w/ inline flow heater	IonOptix	TEMPC2
Cole-Parmer Large-bore 3-way, male-lock, stopcocks	Cole-Parmer	EW-30600-23
Cole-Parmer Luer fittings, Large-bore stopcocks, male lock, 4-way	Cole-Parmer	EW-30600-12
Cole-Parmer Stopcocks with Luer Connections; 1-way; male slip	Cole-Parmer	EW-30600-01
Collagenase Type II	Worthington	LS004177
Corning Sterile Cell Strainers	Fisher Scientific	07-201-432
Dell Optiplex 790 mini-tower, 4G RAM, 250G HD, Windows 7 Pro	IonOptix	CPUD7M
DMSO	Fisher Scientific	50980367
Dumont Tweezers Style 5	Amazon	B00F70ZDEQ
FHD Rapid Change Stimulation Chamber	IonOptix	FHDRCC1
Fluo-3/4 Optics Package	IonOptix	IonOP-Fluo
Fluorescence system interface – (w PCI-I/O card)	IonOptix	FSI700
Gibco Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	15-140-122
Glucose	Fisher Scientific	D16-1
Hemostat, Curved 5-1/2"	Amazon	B00GGAAPD0

HEPES	Fisher Scientific	BP310500
HyperSwitch dual excitation light source	IonOptix	HSW400
Inverted Motic Fluorescence Microscope	IonOptix	MSCP1-40 (a)
IonWizard Core + Analysis	IonOptix	IONWIZ
Iris Scissors, curved	Amazon	B018KRRMY6
K2HPO4	Fisher Scientific	P288-100
KCl	Fisher Scientific	BP3661
L-Glutathione reduced	Sigma-Aldrich	G4251
LOOK Silk Spool, Black Braided, 4-0, 100yds	SouthernAnesthesiaSurgical Inc.	SP116-EA
M199 Media	Fisher Scientific	12 340 030
MgCl2	Fisher Scientific	MP021914215
MgSO4	Fisher Scientific	BP2131
MyoCam-S Digital CCD video system	IonOptix	MCS100
MyoPacer Field Stimulator	IonOptix	MYP100
NaCl	Fisher Scientific	BP358212
NaH2PO4	Fisher Scientific	56-754-9250GM
Oxygenator Bubbler with Fluid Inlet for 0.25 Liter	Radnoti, LLC	140143-025
Photomultiplier sub-system	IonOptix	PMT400
PMT Acquisition add-on	IonOptix	PMTACQ
Radnoti Heating Coil 5 mL with Degasing Trap	Radnoti, LLC	158830
Ring Clamp 60 – 80mm Dia. for 250ml Reservoir	Radnoti, LLC	120141-025
Ring Clamp for Bubble Trap Compliance Chamber	Radnoti, LLC	120149RC
Saint-Gobain ACF000010 5/32 in.9/32 in.	Fisher Scientific	14-171-214
Saint-Gobain ACF000013 3/16 in.3/8 in.	Fisher Scientific	14-171-217
Saint-Gobain ACF000016 1/4 in.5/16 in.	Fisher Scientific	14-171-219
Saint-Gobain ACF000025 5/16 in.5/8 in.	Fisher Scientific	14-171-226
Saint-Gobain ACF00003 1/16 in.3/16 in.	Fisher Scientific	14-171-209
Saint-Gobain ACF00005 1/16 in.3/32 in.	Fisher Scientific	14-171-210
Saint-Gobain ACF00009 5/32 in.7/32 in.	Fisher Scientific	14-171-213
Sarcomere Length Recording add-on	IonOptix	SARACQ
T/C Adson Tissue Platic Surgery Forceps 4.75"	Amazon	B00JDWRBGC
VETUS Anti-Static Curved Tip Tweezers	Amazon	B07QMZC94J
Vistek 3200 Motic Vibration Isolation Platform	IonOptix	ISO100

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Optical Imaging of Isolated Ventricular Myocytes Using the FluoVolt™ Membrane Potential Kit
Author(s):	Matthew Klos, Shuxin Han, Sherry Morgenstern, Ramiz Ahmad, Isabella Pua, Kayla Hicks, Shreyas Suresh, and Eric Devaney

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

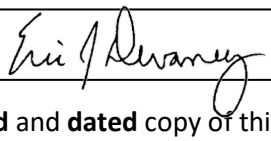
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Eric J. Devaney
Department:	Pediatric Cardiac and Thoracic Surgery
Institution:	University Hospitals Cleveland Medical Center
Title:	Chief, Pediatric Cardiothoracic Surgery, Professor of Surgery
Signature:	
Date:	04/30/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Reviewers' comments:

We would like to offer our earnest thanks to the reviewers for taking the time and effort to carefully critique our work. Because of their insights, we feel the quality of our manuscript has improved significantly. Our responses to the reviewers are bolded and italicized below. Significant changes within the manuscript are in red font color.

Reviewer #1:
The authors describe a protocol to isolate murine cardiac myocytes and perform single cell voltage or calcium imaging along with sarcomere shortening measurements. Using this technique, they confirm previously described APD restitution properties and response to 4-aminopyridine treatment. Interestingly, they also report reduction in sarcomere shortening due to reduced availability of calcium ions during calcium dye treatment and cytotoxicity due to prolonged FluoVolt dye excitation. The protocol very clearly details this procedure however there are some concerns that need to be addressed to improve the manuscript.

Major Concerns:
1. The abstract describes this manuscript as a protocol for murine cardiac cell isolation and imaging but the manuscript switches between rat and mouse protocols and rat hearts are used in figures while mouse heart protocols are described in corresponding manuscript text. For example, a rat heart is included in Figure 1A, so are the myocytes in the other Figure 1 panels also from rats? Clearly define scope of the manuscript and delineate rat and mouse sections.

We apologize for the confusion. We have used this protocol to isolate mouse, rat, and rabbit myocytes. The only thing that differs between species is the digestion time and concentration of the collagenase in the digestion buffer. In retrospect, while we started the study using mice, all the originally submitted data was recorded from rat. Therefore, to include mouse data, we added three new figures (Figure 2, 3, and 4). The manuscript has been changed accordingly.

2. Is the dye incubation and the following washout out phase done at room temperature or is the preparation returned to the incubator for this stage? If it is at room temperature, comment on the effects of temperature change on electrophysiology. Parameters like AP take about 15-20 minutes to recover from temperature shock of going between 37 C and room temperature. Also, include how long you allow the cells to recover at 36 C before you image them.

Excellent point. To address this concern, the following was added to the manuscript on page 6 line 287 "Because excitation-contraction coupling is temperature dependent, make sure the cells have been perfused for 15 minutes before recording. This allows for myocytes to recover from the shock of going from room temperature back to 37° C as well as loosely attached cells to float away."

3. The AP traces in Figure 3 is very noisy and this can affect the APD90 measurements. In Figure 4 the traces look much cleaner. Explain how this was accomplished and how was APD90 reliably measured in the former instance?

Another excellent point. The AP's in the former Figure 3 were filtered but not ensembled averaged. The ones in Figure 4 are ensembled averaged traces that were filtered with a

Savitzky-Golay Filter. For the voltage data presented in this manuscript, action potentials were filtered with a Savitzky-Golay Filter and ensembled averaged before being quantified. The fura-2 and sarcomere shortening data was ensembled averaged, but not filtered.

Regarding filtering, the Ionwizard has three digital filters that can be used to analyze data. A low-pass Butterworth filter, a zero-phase low-pass Butterworth filter, and a Savitzky-Golay polynomial smoothing filter. To illustrate the filtering process, a new figure was added (Figure 4). Because the digital filter settings can distort the real signal, hence alter the APD measurements, the following sentences were specifically added on page 7 at line 335: "Compared to calcium signals, single cell voltage tracings are smaller in amplitude and need post-processing to obtain a useable signal. Figure 4B shows an ensembled averaged AP made from the APs in Figure 4A. Figures 4C and 4D show an ensembled average AP after a low pass Butterworth or a Savitzky-Golay digital filter was applied. Care must be taken when filtering the signal as not to distort the real data. Notice the subtle differences in the shape of the APs in Figure 4B, 4C, and 4D."

Minor Concerns:

1. Image post-processing is mentioned in lines 266-267 but no further details on what processing methods are applied are given. Please describe. ***We apologize for our carelessness. The manuscript has been changed accordingly.***
2. Label parts of the system in Figure 1A, indicate where the rat heart is in this photo. ***Good catch. The parts of the system are now labeled, and the legend has been changed to appropriately describe the figure.***
3. It looks like Figure 2A and 2B are at different time scales but only 1 time scale bar is included. Please correct. ***We double-checked the scales. While it appears to be two different time scales, it is an optical illusion. Nonetheless, to avoid confusion we put a scale on each figure.***
4. Please describe how sarcomere shortening measurements were made. ***The following sentences were added to the manuscript on page 7,*** **line 317*****: "Shortening on the other hand is obtained by a CCD sensor. Measuring in real time up to 1000 times per second, the acquisition software performs an average of the lines within an area of interest to create a well resolved striation pattern. A fast Fourier transform (FFT) is then calculated. The peak within the power spectrum represents the average sarcomere spacing. Changes in the sarcomere spacing during pacing are then plotted and subsequently quantified."***
5. Traditional Krebs Henseleit solution uses sodium bicarbonate as a buffer, please make a note that the solution you use is a modified Krebs Henseleit solution with HEPES buffer. ***The following sentence has been added to page 1 line 85:*** **"Note traditional Krebs Henseleit solution uses sodium bicarbonate as a buffer and the solution in this protocol uses Krebs Henseleit solution with HEPES buffer. Additionally, KHB-B has been changed to KHB-HB"**
6. Include concentration of HEPES in culture media on line 195. ***25 mM has been listed.***
7. Mention duration of dye washout phase in line 221-222. ***The manuscript has been changed accordingly.***
8. Technically, you're not starting to pace at 0V, correct the statement in line 241-242. ***Another good catch. The manuscript has been changed accordingly.***

9. In line 196 you state that the cells can be cultured for 1 week and in line 259 you state 4 days. Please clarify and keep consistent***. It has been clarified.***

Reviewer #2:

Manuscript Summary:

The authors describe a method to isolate viable ventricular cardiomyocytes from mouse hearts using a standard Langendorff-perfusion based method; moreover, the authors comprehensively describe a simple and repeatable method to employ the largely available Ion-Optics fluorescence system (with the Fluo-3/4 filter option) to simultaneously record either sarcomere shortening and intracellular calcium (using Fluo-4) or sarcomere shortening plus membrane voltage (using FluoVolt)

Major Concerns:

1) the perfusion system is described as a constant-pressure Langendorff apparatus. Is the constant pressure provided by gravity? If so, what is the height at which the solution reservoir is positioned during heart perfusion?

We apologize for the confusion. The height of the solution reservoir is 60 cm. Figure 1 has been changed accordingly to indicate this.

In some mouse CM isolation protocols, the perfusion pressure is increased in the second half of collagenase perfusion, but the authors do not mention it here. At page 2 line 119 the authors describe a circulating water bath and suggest to set a flow rate of 12-14 mL/min. What is it used for? To refill the high reservoir? The authors should clarify these points as they make the procedure unclear.

Excellent observation. We apologize for the confusion. The text has been changed to the following on page 3 line 144: "With the solution reservoirs being set at a height of 60 cm, the circulating waterbirth needs to be set to 41° C to have the perfusate be 37° C. Unlike previously reported protocols, the height of the reservoir does not need to be changed."

2)Page 3 line 166. After perfusion, the heart chambers are placed into separate dishes and collegenase solution is added to each dish. How long should the chambers be kept in this digesting solution before mincing them with scissors? Or should they be minced immediately? If so, the authors should suggest the time allowed for mincing, straining and centrifuging the cells before the resuspension in stopping buffer, as persistence of isolated cells in enzymatic solution might damage cells.

We apologize for the omission. The text has been changed to the following on page 4 at line 198: "After digestion, separate the chambers of the heart. Place each chamber into a separate well of a six well plate. Pour 5 mL of collagenase solution into each well. Immediately start mincing the heart tissue using scissors. Tissue chunks should be approximately 1 cubic mm. Using sterile transfer pipettes, gently triturate the minced heart tissue. The solution should turn cloudy."

3)In your trial experiments, you paced the cells up to 4Hz. In my experience, good mouse

cardiomyocytes can easily be stimulated up to 7-8 Hz without damaging them. Have you tried stimulating them at 7-8Hz?

Specifically, 7-8 Hz, no. The pacing protocol is a variation of two previously published pacing protocols (please see newly included references 10 and 11). Nonetheless, your point is well taken. A sinus rate of 4 Hz would be a lethal bradycardia in a mouse. Therefore, in the past we have tried 10 Hz, which corresponds to a healthy resting heart rate in mice. In the revised manuscript, we have included 3 new figures to illustrate this.

4)Although the risk of fluovolt photodamage is known, in my experience it only occurs when a very powerful illumination is used. Have you tried adjusting the illumination intensity in order to reduce fluovolt photo damage?

Yes, we have. The IonOptix illuminating source is a Xenon Short Arc Lamp. To control the intensity, the IonOptix has 5 neutral density filters preinstalled. They are ND 0.2, ND 0.3, ND 0.6, ND1/0, and ND 1.3. They correspondingly reduce the transmitted light by 37, 50, 75, 90, and 95%. Using these we can get recordings of upwards of 15 seconds without phototoxicity. We have not tried other neutral density filters. In order to address this concern, the following sentences were added to page 10 line 457 of the manuscript: "While our protocol was optimized for 5 sec recordings, if longer voltage traces are needed, additional neutral density filters will need to be purchased. The system described in the protocol comes with neutral density filters that reduce the transmitted light by 37, 50, 75, 90, and 95%."

Minor Concerns:

1)Is fluo-4 or Fluovolt loading performed at room temperature? ***The loading of all dyes was performed at room temperature. The manuscript has been changed accordingly to reflect this.***

2)Figure 1: please add the Y scale for sarcomere length. ***A y-scale has been added.***

3)Figure 5: please describe the situation in the caption: how long was the cell paced and illuminated before developing damage? ***The manuscript has been changed accordingly.***