Video Article

Optical Imaging of Isolated Murine Ventricular Myocytes

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/60196/

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[2,3,4]. 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. 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 1,000 mL flask. After contents are fully dissolved, bring up to volume in a 1,000 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.
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.
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 1,000 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.
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 1,000 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 1,000 mL volumetric flask. Sterile filter using a vacuum filtration system.
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 1,000 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 1,000 mL volumetric flask. Sterile filter the solution using a vacuum filtration system.
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.
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.
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.
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 (25 mM HEPES). Sterile filter the solution using a vacuum filtration system.
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.
11. Sterilize 22 x 22 mm^2 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.
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 0.04 mg/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.
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.
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**.

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. 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.
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.
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.
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

1. Inject the animal with 1,000 U 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.
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. 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.
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.
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.
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.
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.
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.
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.
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.
    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.
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.
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.
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.
14. Remove the laminin-coated coverslips from the incubator. Aspirate the laminin droplet.
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

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.
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.
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

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.
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.
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

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.
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.
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

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

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.
4. Turn heater on and set flow by adjusting the roller clamp on the perfusion tubing. Make recordings at 36 ± 1 °C.
5. Open the acquisition software. Make sure the parameters are set for the correct imaging dye.
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. 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.
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.
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 1,000 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 s of a 20 s recording. Indicated by the red arrows in **Figure 8**, prolonged exposure of myocytes to blue light leads to triggered activity.

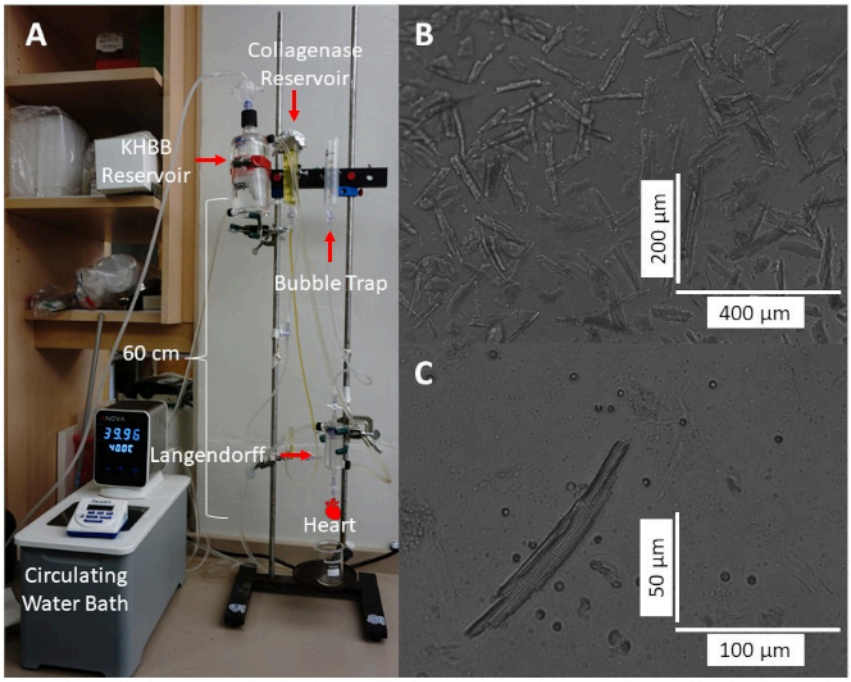

Figure 1: Constant pressure Langendorff apparatus. (**A**) The Langendorff Apparatus with each component labeled in white lettering. (**B**) Isolated Sprague-Dawley rat myocytes viewed through a 10x objective. (**C**) Isolated rat myocytes viewed through a 40x objective. Please click here to view a larger version of this figure.

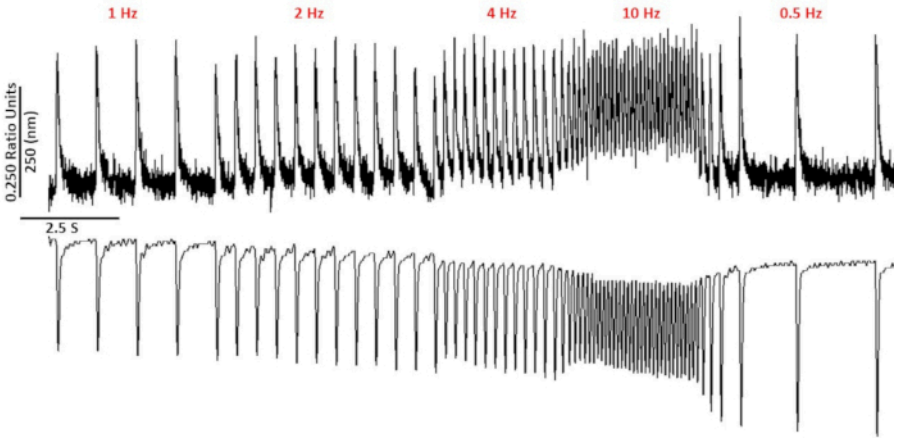

Figure 2: Representative calcium and sarcomere shortening traces recorded from C57/B6 myoyctes using fura-2. Calcium and sarcomere shortening traces recorded at 1, 2, 4, 10, 0.5 and 0.75 Hz. Please click here to view a larger version of this figure.

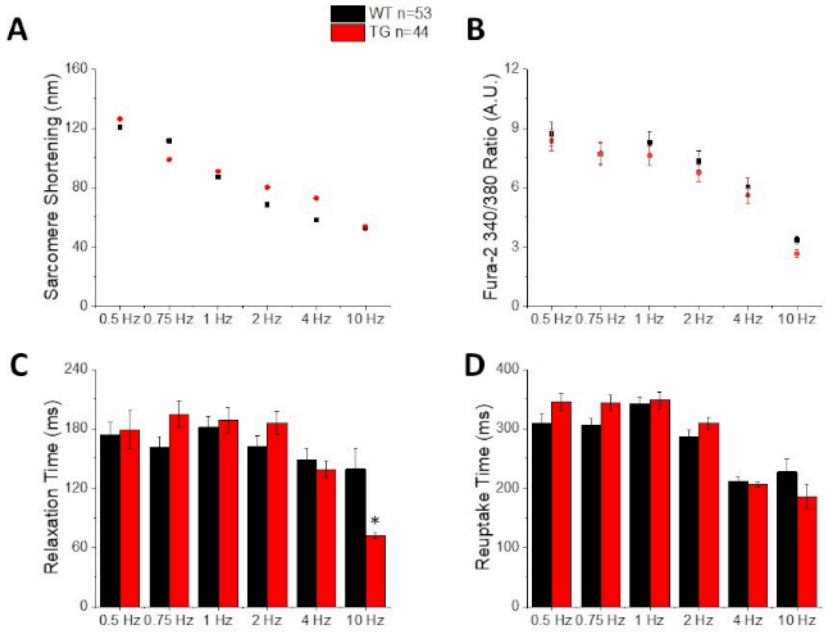

Figure 3: Quantification of sarcomere shortening, peak calcium, relaxation time, and reuptake time recorded from a C57/B6 wild type (WT) and transgenic (TG) mice. (**A**) Sarcomere shortening. (**B**) Peak calcium. (**C**) Relaxation time defined as 90% return to baseline of the shortening trace. (**D**)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

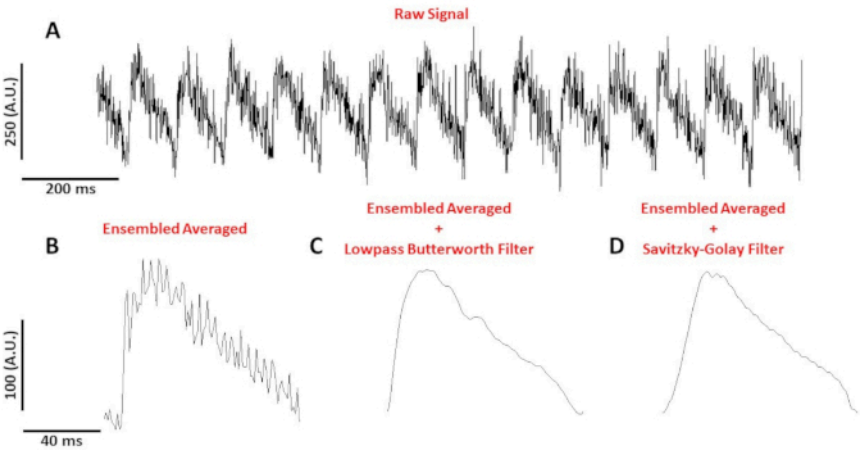

Figure 4: Optical action potential recorded from a C57/B6 mouse myocyte paced at 10 Hz. (**A**) 1 second unfiltered trace. (**B**) Ensembled averaged optical action potential. (**C**) Ensembled averaged optical action potential after a lowpass Butterworth filter was applied. (**D**) Ensembled averaged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

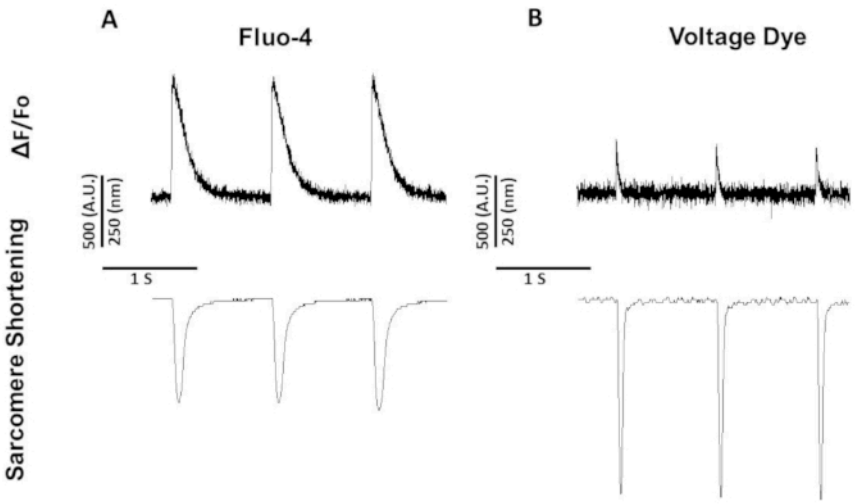

Figure 5: Representative calcium, voltage, and sarcomere shortening traces recorded from Sprague-Dawley rat myocytes paced at 1 Hz. (**A**) Calcium and sarcomere shortening traces recorded at 1 Hz pacing using fluo-4. (**B**) Voltage and sarcomere shortening traces recorded at 1 Hz pacing using the voltage dye. Please click here to view a larger version of this figure.

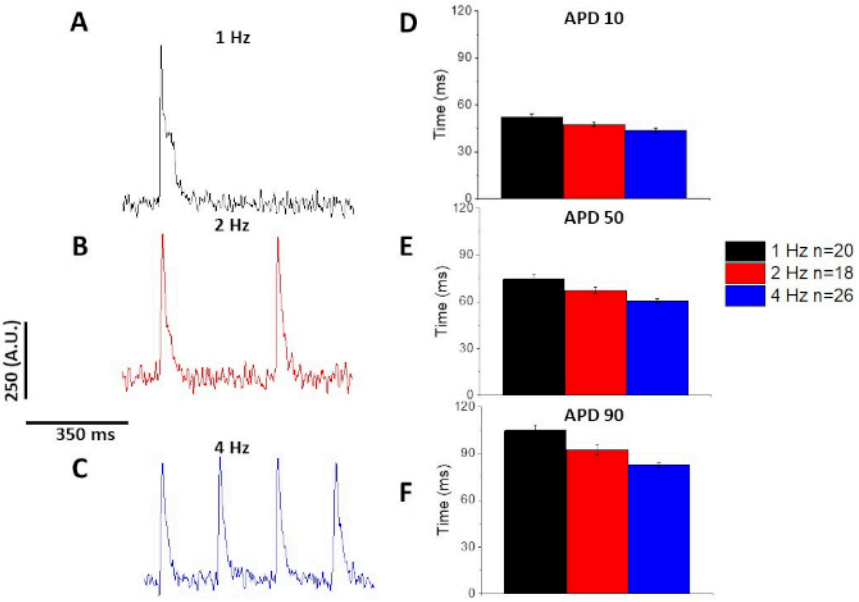

Figure 6: Optical action potentials recorded from Sprague-Dawley rat myocytes paced at 1, 2, and 4 Hz pacing. (**A**) Filtered trace recorded at 1 Hz pacing. (**B**) Filtered trace recorded at 2 Hz pacing. (**C**) Filtered trace recorded at 4 Hz pacing. (**D**) Action potential duration 10, measured 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. Please click here to view a larger version of this figure.

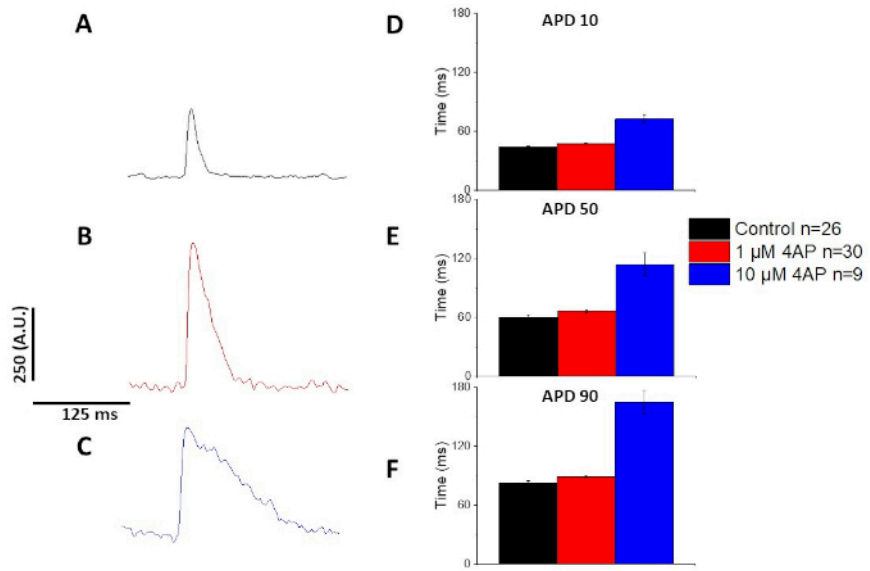

Figure 7: The Effects of 4-aminopyridine on Sprague-Dawley rat optical action potentials recorded at 4 Hz pacing. (**A**) Ensembled averaged trace recorded at 4 Hz pacing with no 4-Aminopyridine in the solution. (**B**) Ensembled averaged trace recorded at 4 Hz pacing with 1 µM 4-Aminopyridine in the 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. Please click here to view a larger version of this figure.

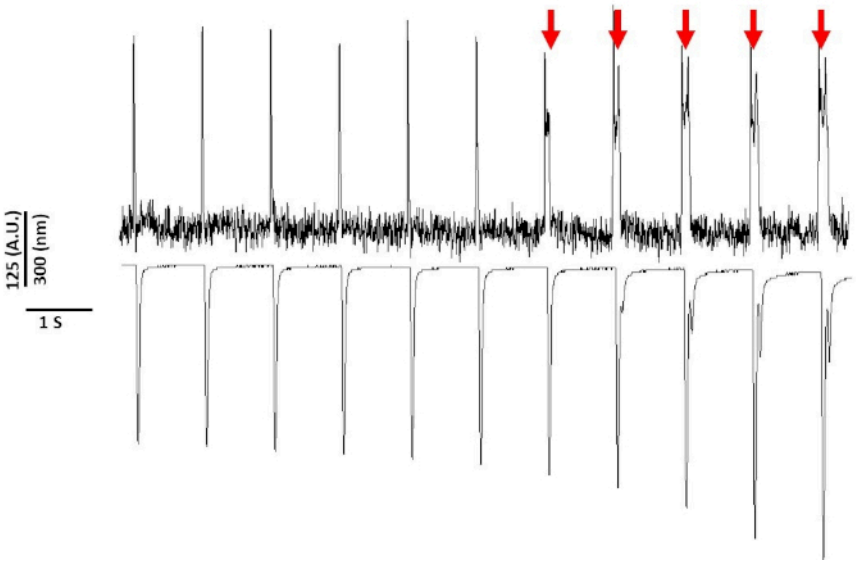

Figure 8: Voltage dye induced phototoxicity in Sprague-Dawley rat myocytes after 20 s of continuous light exposure. Red arrows indicate cytotoxic events. Please click here to view a larger version of this figure.

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[13,14,15,16]. 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 s 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.

Disclosures

The authors have nothing to disclose.

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