Chapter 7

Measuring Cardiomyocyte Contractility and Calcium Handling In Vitro

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Abstract

In vitro measurements of cardiomyocyte contractility and Ca^{2+} handling have been used as a platform for determining physiological consequence of various genetic manipulations and identifying potential therapeutic targets for the treatment of heart failure. The Myocyte Calcium and Contractility System (IonOptix) offers a simultaneous trace of sarcomere movements and changes of intracellular Ca^{2+} levels in a single cardiomyocyte. Herein, we describe a modified protocol for the isolation of adult cardiomyocytes from murine hearts and provide a step-by-step description on how to analyze cardiomyocyte Ca^{2+} transient and contractility data collected using the IonOptix system. In our modified protocol, we recommend a novel cannulation technique which simplifies this difficult method and leads to improved viability of isolated cardiomyocytes. In addition, a comprehensive analysis of intracellular Ca^{2+} handling, SR Ca^{2+} load, myofilament Ca^{2+} sensitivity, and cardiomyocyte contractility is described in order to provide important insights into myocardial mechanics.

Key words Intracellular calcium, Calcium handling, Cardiomyocyte, Contractility, Heart disease

1 Introduction

Intracellular calcium (Ca^{2+}) cycling plays a critical role in regulating systolic and diastolic function of cardiomyocytes [1, 2]. Numerous ion channels, transporters, and other intracellular Ca^{2+} handling proteins are involved in maintaining proper Ca^{2+} homeostasis and defects in these essential molecules often result in a severe malfunction of cardiomyocytes [3–6]. Therefore, analyses of Ca^{2+} handling in healthy and disease conditions can greatly advance our understanding of cardiac disease. Due to the lack of suitable immortalized cardiac cell lines and significant limitations of neonatal cardiomyocytes, isolated adult cardiomyocytes provide a powerful platform for studying cardiac dysfunction and complement murine models of heart disease [7–11].

In the present protocol, we describe a fast and reproducible method for isolating adult murine cardiomyocytes and provide

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instructions on how to obtain a variety of measurements of cardiomyocyte contractility and Ca^{2+} handling using the Myocyte Calcium and Contractility System (IonOptix). Standard analysis of Ca^{2+} handling and cardiomyocyte contraction, evaluation of the sarcoplasmic reticulum (SR) Ca^{2+} reserve and the rate of diastolic Ca^{2+} removal, as well as the assessment of myofilament Ca^{2+} sensitivity are discussed in detail. Such measurements can be utilized in a variety of experimental settings to study the effects of small molecules, environmental stressors, infections, or genetic manipulations on cardiomyocyte function. Together, the use of these techniques may provide important mechanistic insights into Ca^{2+}-related signaling pathways and physiologically relevant characterization of cardiac disease.

2 Materials

2.1 Drugs

1. Heparin sodium: 1000 units/mL.
2. Ketamine–xylazine cocktail: ketamine and xylazine, at a ratio of 5:1, e.g., 65/13 mg/kg.

2.2 Equipment

1. Delicate suture tying forceps (2×): length: 9 cm, tip dimensions: 0.4 × 0.3 mm, curved.
2. Extra fine Graefe forceps (2×): length: 10 cm, tip dimensions: 0.5 × 0.5 mm, straight.
3. Spring scissors: length: 10 cm, tip diameter: 0.2 mm, cutting edge: 8 mm, straight.
4. Fine scissors: length: 10.5 cm, cutting Edge: 26 mm, straight.
5. Surgical suture: silk, 4-0.
6. Homemade perfusion cannula: *See* Fig. 1c.
7. Homemade cannula holder: *See* Fig. 1c.
8. Cell Strainer: Nylon, 100 μm pore size.

2.3 Apparatus

1. Dissecting microscope: Zoom range 1–8×.
2. Fluidic inline heater: internal dead volume is 35 μL/tubing.
3. Syringe pump: for caffeine injection.
4. Langendorff system: constant flow model (3 mL/min).
5. The Myocyte Calcium and Contractility System (IonOptix).

2.4 Solutions

1. Tyrode solution: 125 mM NaCl, 5 mM KCl, 2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM pyruvate, 11 mM glucose, 5 mM creatine, 5 mM L-carnitine, 5 mM taurine, 10 mM 2,3-butanedione-monoxime (BDM), 25 mM HEPES, pH 7.2.

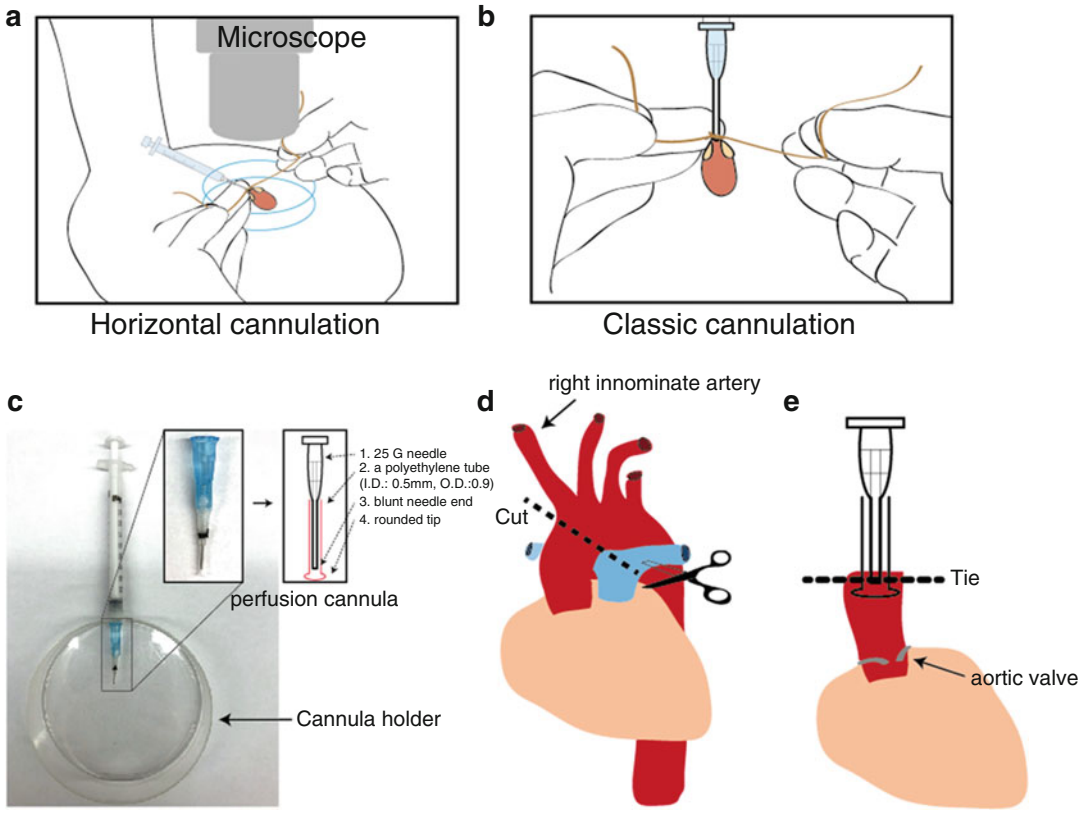

Fig. 1 General overview of the cannulation procedure. (**a**) Illustration of the horizontal cannulation setup described herein and (**b**) the commonly used (classic) cannulation setup. (**c**) A detailed schematic of the homemade horizontal cannulation apparatus. (**d**) Schematic diagram of a mouse heart illustrating the location of where the aortic arch should be cut off and (**e**) a proper ligation of the aorta to the Langendorff perfusion cannula

2. Enzyme solution: 250 units/mL Collagenase type B, 60 units/mL Hyaluronidase, Tyrode solution.
3. Ca^{2+} buffer: 1.2 mM $CaCl_2$, Tyrode solution.
4. Blocking solution: 5% bovine serum albumin (BSA), Tyrode solution.
5. Caffeine solution: 10 mM caffeine, Tyrode solution.
6. Coating solution: 5 μg/mL laminin, PBS.
7. Fura-2 AM (acetoxymethyl ester form): 1 μM Fura-2 AM, Ca^{2+} buffer.
8. PBS (regular phosphate-buffered saline): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
9. Culture medium: 0.1% bovine serum albumin (BSA), 1× insulin–transferrin–selenium (ITS), 10 mM BDM, 1× CD lipid (chemically defined lipid concentrate), 1× penicillin/streptomycin solution, M199 medium.

3 Methods

3.1 Isolation and Culture of Cardiomyocytes

1. Inject mouse with 150 units of heparin sodium into the peritoneal cavity.
2. Anesthetize mouse via intraperitoneal injection of ketamine–xylazine. After few minutes, confirm that the animal is fully anesthetized by the lack of toe pinch reflex.
3. Open the abdominal cavity under the xiphoid process with surgical scissors, lift the xiphoid process and open the chest. After cutting the inferior vena cava, inject Tyrode solution into the left ventricular apical region to remove blood from the chamber (*see* **Note 1**). Remove the pericardium, slightly lift the heart with curved forceps, identify the aortic arch, and excise the heart from the root of the aorta. Place the heart into a dish of ice-cold PBS.
4. Hold the aorta with two microdissecting forceps and mount it onto the Langendorff perfusion cannula (Fig. 1). Firmly ligate the aorta onto the cannula using surgical sutures (*see* **Notes 2** and **3**).
5. Transfer cannulated heart onto the Langendorff perfusion apparatus. Inspect the tubing and the cannulation needle to make sure there are no bubbles trapped in the perfusion system (*see* **Note 4**).
6. Perfuse the heart with Tyrode solution for 5 min at a rate of 3 mL/min. Switch the buffer to the enzyme solution and perfuse for 20 min (*see* **Note 5**).
7. Use forceps to grab the aorta and transfer the digested heart into a dish full of fresh blocking solution. Cut off the atria, the right ventricle and the atrioventricular junction area, leaving only the left ventricle (*see* **Note 6**).
8. Use fine scissors and forceps to cut the left ventricle into smaller pieces (~10 mm). Gently pipette the cell suspension up and down using a sterile 5 mL pipette to further dissociate the tissue.
9. Filter the cell suspension through a 100 μm nylon mesh filter into a 15 mL conical tube. Centrifuge at 50 × *g* for 60 s and discard the supernatant (*see* **Note 7**).
10. Resuspend the cell pellet in a desired volume of culture media (10 mL) and plate the cell suspension on laminin-coated coverslips in an appropriate sized dish. Incubate at 37 °C and 2% CO_2 for 60 min to allow the cells to equilibrate and adhere to coverslips.
11. Remove plating media from the dish and replace with culture media. Depending on the experimental conditions, cardiomyocytes can be cultured for up to 72 h (Fig. 2).

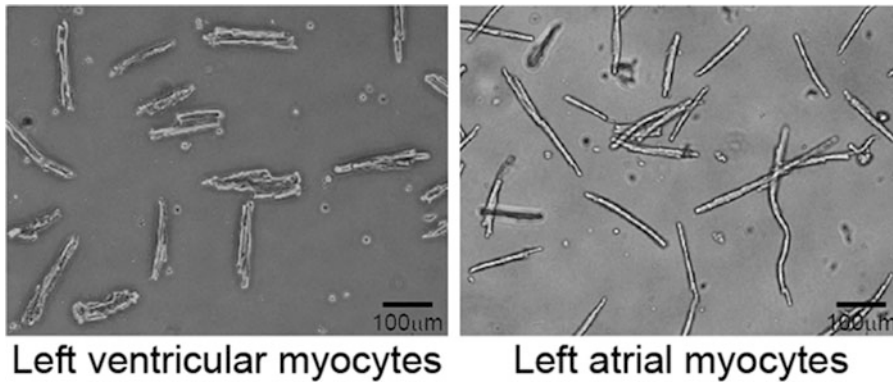

Fig. 2 Representative example of ventricular and atrial myocytes

3.2 Simultaneous Measurements of Intracellular Ca^{2+} Transients and Contractility

1. Power the IonOptix system ensuring that the arc lamp is initiated first.
2. Prime the buffer circulation system with Ca^{2+} buffer. Temperature should be held at 37 °C throughout the experiment using the fluidic inline heater.
3. Incubate cardiomyocytes with Fura-2 AM (1 μM) for 10 min (*see* **Note 8**).
4. Mount a glass coverslip with cardiomyocytes into the perfusion chamber and fasten.
5. Start perfusing the chamber (1.5 mL/min) with Ca^{2+} buffer and pace cardiomyocytes with 1 Hz field stimulation using MyoPacer field stimulator (10 V, 4 ms) for 5 min.
6. Use 40× objective lens to select a healthy cardiomyocyte (rod shape, sharp edge, clear cross striations, and no spontaneous contraction) for recording.
7. Center the selected cardiomyocyte in the field of view, line it up horizontally, and minimize the background area by adjusting the cell framing adapter. Adjust the focus of the microscope until sarcomere striations are clearly visible.
8. To measure cardiomyocyte shortening/relengthening, two recording tasks are available in the IonOptix system (*see* **Note 9**):
   (a) Sarcomere length recording task: Align the sarcomere zone control (purple rectangular box) on an area of the cardiomyocyte containing well-defined sarcomeres and adjust the focus to optimize the peak of the power spectrum (red peak).
   (b) Cell length recording task: Align the red and green selection lines on both edges which clearly show contrast between the cell edge and the background. Check whether the cursor indicates the edge during contraction.

9. Record the changes of sarcomere/cell length (15–20 stable contractions) under field stimulation. Since the IonOptix system provides simultaneous measurements of the change in the sarcomere/cell length of cardiomyocytes and Ca^{2+} recording of the Fura-2 AM fluorescence (excitation at 360/380 nm and emission at 510 nm), no additional set up is necessary to obtain Ca^{2+} transient data.

3.3 Analysis of Cardiomyocyte Contractility and Ca^{2+} Handling

Once the Ca^{2+} transients and contractility traces are acquired, data are analyzed with the IonWizard software according to the following instructions:

1. Create an ensemble average from 10 to 20 traces by selecting "Average Events" from the "Operations" pull-down menu (Fig. 3a).
2. Exclude any abnormal traces from the source data using inclusion indicators. Any bars drawn in red represent traces excluded from the current average (Fig. 3b).
3. Select the duration/end time to be analyzed in the ensemble average trace using transient mark editor.
4. Click the trace analysis button (M Tran) to analyze the ensemble average trace (Fig. 3c).
5. Contractility can be analyzed in a variety of ways:

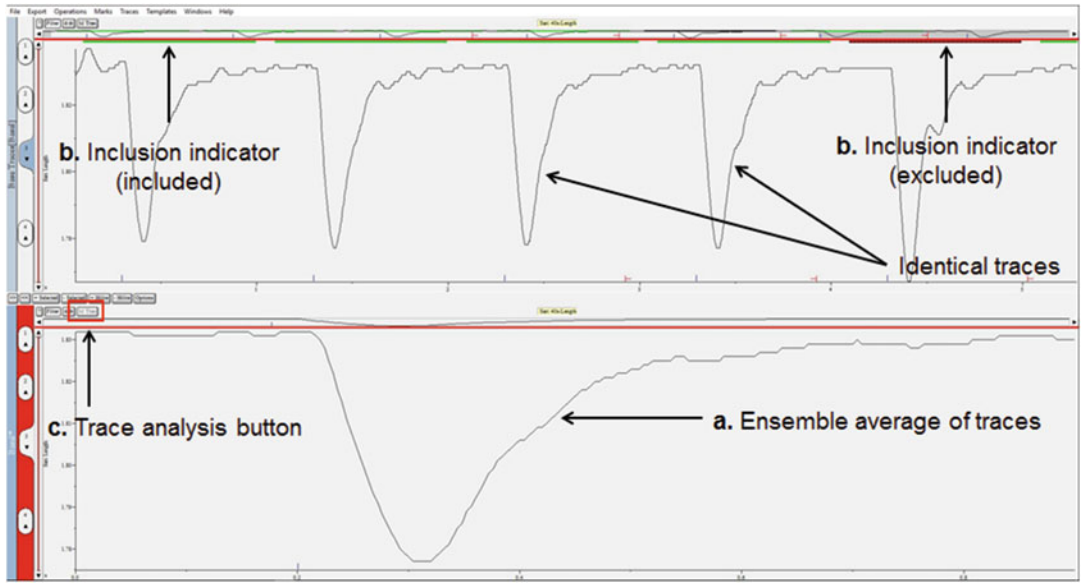

Fig. 3 Representative example of sarcomeric length measurement using the IonOptix system. (top panel) Sarcomere length tracing showing traces included and excluded (**b**) from the analysis. (bottom panel) Ensemble average of selected contractility traces (**a**) shown in the top panel. (**c**) Trace analysis button is indicated

For systolic function, one can assess the magnitude of contraction with fractional shortening or speed of contraction with time to peak shortening (bl% peak h, Fig. 4a), and maximal contraction velocity (dep v, Fig. 4b). Diastolic function can be analyzed by calculating time to 50% relaxation (t to bl 50%, Fig. 4c) and relaxation velocity (ret v, Fig. 4d).

6. For calcium transient analysis, baseline (bl, Fig. 4e), Ca^{2+} amplitude (peak h, Fig. 4f), tau (sin exp tau, Fig. 4g), and time to 50% baseline (t to bl 50%, Fig. 4h) parameters can be used to compare different data sets.

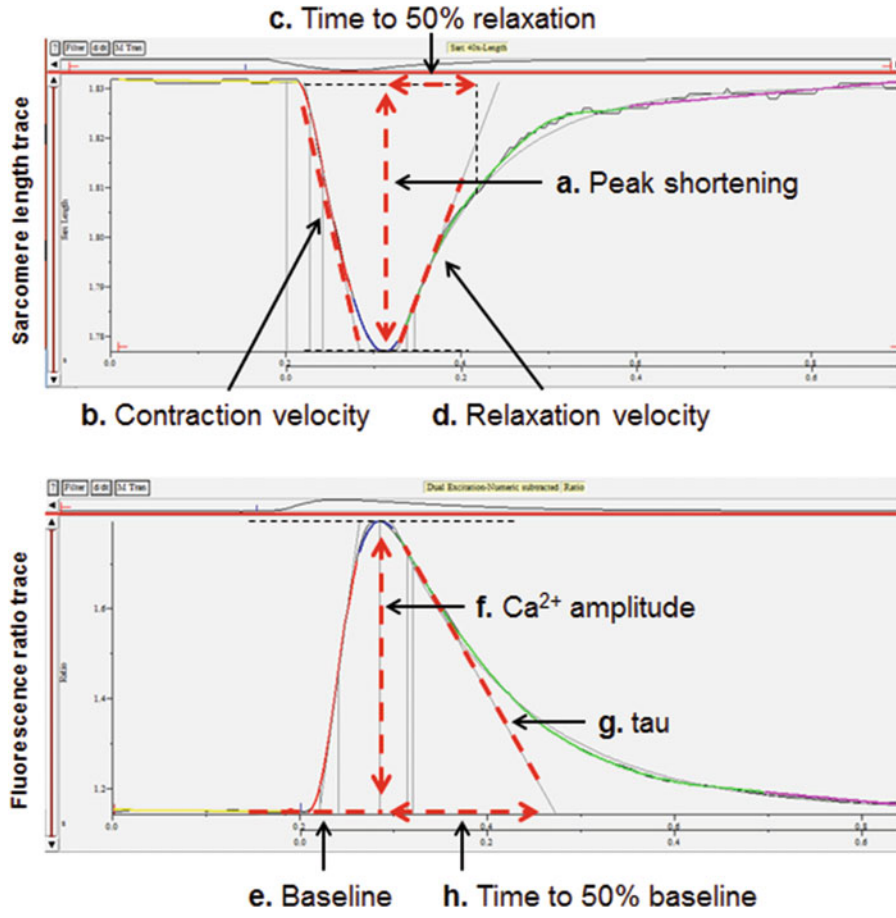

Fig. 4 (top panel) Ensemble average of contractility tracings after analysis. (**a**) Peak shortening, (**b**) contraction velocity, (**c**) time to 50% relaxation, and (**d**) relaxation velocity parameters are indicated. (bottom panel). Ensemble average of calcium transients after analysis. (**e**) Baseline, (**f**) Ca^{2+} amplitude, (**g**) tau, and (**h**) time to 50% baseline parameters are indicated

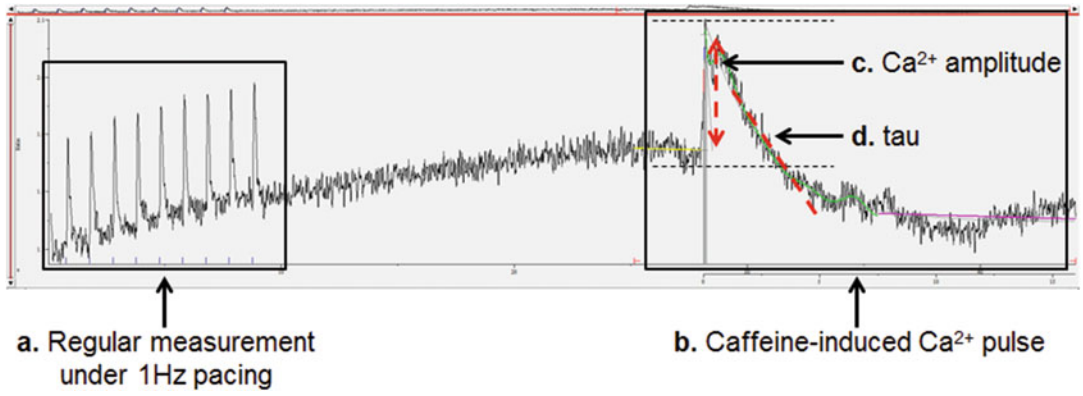

Fig. 5 Representative calcium transient trace from a SR Ca^{2+} reserve and diastolic Ca^{2+} removal measurement. (**a**) Initial phase of the experiment during which cardiomyocytes are paced at 1 Hz. (**b**) Ca^{2+} transient after the addition of caffeine into the system. (**c**) Ca^{2+} amplitude and (**d**) tau parameters are indicated

3.4 Assessment of SR Ca^{2+} Reserve and Diastolic Ca^{2+} Removal

Here, we describe how to record caffeine-induced Ca^{2+} pulses in isolated cardiomyocytes that can be used to extrapolate data which primarily reflect the contribution of SERCA2a and NCX to diastolic Ca^{2+} removal [12–14].

1. Prime a syringe pump with 60 μL of 80 mM caffeine solution (final caffeine concentration is 10 mM).
2. Start the recording and measure basal Ca^{2+} transient and contractility at 1 Hz pacing with Ca^{2+} buffer for 30 s (Fig. 5a).
3. Pause the recording and carefully adjust the position of the micropipette tip filled with caffeine solution nearby the target cardiomyocyte.
4. Resume the recording and inject caffeine solution rapidly.
5. To analyze caffeine-induced Ca^{2+} pulses (Fig. 5b), select only the pulse with a steep wave for analysis of the calcium removal function.
6. Measure the amplitude of the caffeine-induced Ca^{2+} pulse (peak h, Fig. 5c), indicating SR Ca^{2+} reserve.
7. Obtain the decay time constant of caffeine-induced Ca^{2+} pulse (sin exp tau, Fig. 5d), indicating NCX activity.

3.5 Assessment of Myofilament Ca^{2+} Sensitivity

Here, we describe a protocol used to assess the changes in myofilament Ca^{2+} sensitivity in isolated adult mouse cardiomyocytes.

1. Measure Ca^{2+} transients and contractility as described in Subheading 3.2.
2. Average the sarcomere length and Ca^{2+} transient data at steady-state (10–20 traces) and plot the phase-plane loop of the changes in sarcomere shortening vs. intracellular Ca^{2+} levels (Fig. 6).

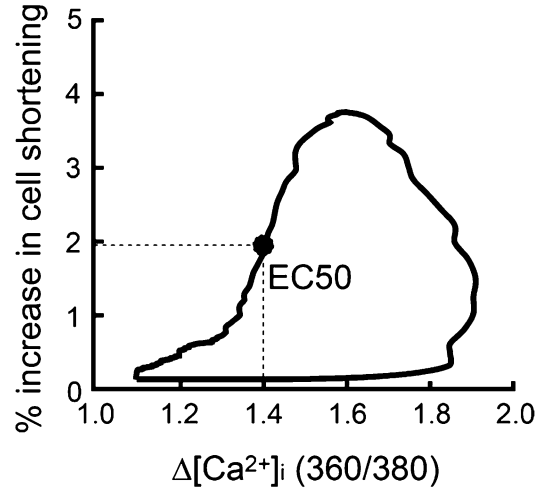

Fig. 6 Phase-plane plot of the delta changes in sarcomere shortening vs. intracellular Ca^{2+} level (Fura-2 ratio, 360/380) with the EC_{50} value (intracellular Ca^{2+} level at 50% sarcomere shortening) indicated

3. In each plot, define intracellular Ca^{2+} level at 50% sarcomere shortening (EC_{50}). Compare the loop plot and EC_{50} values for each condition to assess myofilament Ca^{2+} sensitivity between the tested groups (*see* **Note 10**).

4 Notes

1. Injection of Tyrode solution into the left ventricular apical region improves enzyme digestion of the heart by reducing the possibility of blood clotting inside the coronary artery.
2. In this protocol, horizontal cannulation is highly recommended as it presents several advantages over the classic (vertical needle insertion) cannulation method (Fig. 1a, b). First, the horizontal cannulation procedure can be performed under a dissecting microscope, which allows for easy visualization of the aorta leading to improved mounting onto the perfusion cannula. Second, cannulation time can be minimized down to 1 min increasing the yield of healthy cardiomyocytes. Moreover, the horizontal cannulation gives a chance to check the condition of cannulation and rectify it. The condition of cannulation can be tested by perfusing Tyrode solution through the cannula. If cannulation is successful, blood is removed from the coronary artery. If the blood vessel that was mounted on the needle is not the aorta, the cannulation procedure must be repeated. Finally, horizontal cannulation reduces strain on the heart during mounting, allowing for easier and more accurate tying of the surgical suture to the cannulation needle.

3. For cannulation of mouse hearts, blunt end needle (25 G) covered with a polyethylene tube (I.D.-0.50 mm, O.D.-0.90 mm) is used (Fig. 1c). The distal tip of the tube is rounded by high heat. Alternatively, a commercial animal-feeding needle (24G) can be used. The rounded polyethylene tube or the animal feeding needle will allow to secure the suture over the groove and prevent the heart from slipping off the cannula during perfusion.
4. Before cannulation make sure to trim the aorta as indicated in Fig. 1d (ideally below the aortic root distal to the right innominate artery). It is important to ensure that the end of the needle rests in the ascending aorta but that it does not extend through the aortic valve into the left ventricle (Fig. 1e).
5. The heart should appear scarlet over time indicative of proper enzyme digestion of the connective tissue. The perfusion should be stopped if the color of the heart turns pale as this means that cardiomyocytes have already started dying. At this point it is best to start the isolation again using another mouse heart.
6. It is important to note that the cell morphology and functional properties of cardiomyocytes differ significantly depending on the source. Therefore, before dissociating the heart tissue with forceps, it is possible to separate the heart into respective chambers in order to isolate chamber specific cardiomyocytes as desired (Fig. 2).
7. The supernatant contains nonmyocyte cells, including cardiac fibroblasts. To culture primary cardiac fibroblasts, centrifuge the supernatant at 500 $\times g$ for 10 min and discard the supernatant (cell debris). Resuspend the cell pellet in 5% serum medium and plate the suspension onto cell culture dishes (uncoated). Incubate at 37 °C in a cell culture incubator with 5% CO_2 for 2 h (preplating). At this time viable and healthy fibroblasts should have adhered to the dish. Check the cell confluence under the microscope (It should be around 50%.). Fibroblasts should resemble small spherical bodies.
8. Fura-2 is light-sensitive. Perform all loading procedures and experiments in the dark.
9. The IonOptix system offers two methods to measure contractility: one based on measuring the changes of the total cardiomyocyte length and second based on the changes of sarcomere length. Total cardiomyocyte length measurements trace the movement of the two distal cell edges. Some studies report increases in the full length of cardiomyocytes at diastole as an index of cardiac hypertrophy [15, 16]. However, the length of cardiomyocytes from different compartments of the heart (e.g., atrium vs. ventricle) varies significantly. Usually, the

variability is higher than the changes in cell length due to hypertrophy. Therefore, using cardiomyocyte length as an index of cardiac hypertrophy is not recommended. If necessary, cardiomyocyte isolation from only one part of the heart (e.g., septum only) should be performed to minimize diversity in cell length. Another method to measure contractility is based on tracing the movement of sarcomere length. The average sarcomere length of healthy cardiomyocytes at diastole is 1.8 μm. If the cardiomyocyte isolation is not successful and the viability of cells is low, the intracellular Ca^{2+} is increased in diastole leading to shortening of the sarcomere length (~1.5 μm). Therefore, routine monitoring of the sarcomere length should be helpful to maintain the consistency between Ca^{2+} transient and contractility measurements.

10. Higher EC_{50} value and a rightward shift of the loop trajectory are indicative of myofilament Ca^{2+} desensitization. This process is primarily caused by changes in the phosphorylation status of TnI and myobinding protein C which greatly affects myofilament's ability to bind Ca^{2+} [17–19].

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