Video Article

Assessment of Calcium Sparks in Intact Skeletal Muscle Fibers

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URL: https://www.jove.com/video/50898
DOI: doi:10.3791/50898

Keywords: Physiology, Issue 84, flexor digitorm brevis (FDB), sarcoplasmic reticulum, SR Ca^{2+} release, calcium signaling, ryanodine receptor, confocal imaging, muscle physiology

Date Published: 2/24/2014

Citation: Park, K.H., Weisleder, N., Zhou, J., Gumpper, K., Zhou, X., Duann, P., Ma, J., Lin, P.H. Assessment of Calcium Sparks in Intact Skeletal Muscle Fibers. *J. Vis. Exp.* (84), e50898, doi:10.3791/50898 (2014).

Abstract

Maintaining homeostatic Ca^{2+} signaling is a fundamental physiological process in living cells. Ca^{2+} sparks are the elementary units of Ca^{2+} signaling in the striated muscle fibers that appear as highly localized Ca^{2+} release events mediated by ryanodine receptor (RyR) Ca^{2+} release channels on the sarcoplasmic reticulum (SR) membrane. Proper assessment of muscle Ca^{2+} sparks could provide information on the intracellular Ca^{2+} handling properties of healthy and diseased striated muscles. Although Ca^{2+} sparks events are commonly seen in resting cardiomyocytes, they are rarely observed in resting skeletal muscle fibers; thus there is a need for methods to generate and analyze sparks in skeletal muscle fibers.

Detailed here is an experimental protocol for measuring Ca^{2+} sparks in isolated flexor digitorm brevis (FDB) muscle fibers using fluorescent Ca^{2+} indictors and laser scanning confocal microscopy. In this approach, isolated FDB fibers are exposed to transient hypoosmotic stress followed by a return to isotonic physiological solution. Under these conditions, a robust Ca^{2+} sparks response is detected adjacent to the sarcolemmal membrane in young healthy FDB muscle fibers. Altered Ca^{2+} sparks response is detected in dystrophic or aged skeletal muscle fibers. This approach has recently demonstrated that membrane-delimited signaling involving cross-talk between inositol (1,4,5)-triphosphate receptor (IP_3R) and RyR contributes to Ca^{2+} spark activation in skeletal muscle. In summary, our studies using osmotic stress induced Ca^{2+} sparks showed that this intracellular response reflects a muscle signaling mechanism in physiology and aging/disease states, including mouse models of muscle dystrophy (*mdx* mice) or amyotrophic lateral sclerosis (ALS model).

Video Link

The video component of this article can be found at https://www.jove.com/video/50898/

Introduction

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is a versatile and important secondary messenger that regulates multiple cellular functions in excitable cells such as neurons, cardiac, skeletal and smooth muscles (for review see Stutzmann and Mattson[1]). Regulated Ca^{2+} mobilization and cross-talk between sarcoplasmic reticulum (SR) and T-tubule (TT) membranes are fundamental regulators of muscle physiology. Furthermore, changes in Ca^{2+} signaling had been shown to be an underlying mechanism of contractile dysfunction in various muscle diseases.

Ca^{2+} sparks are localized elementary Ca^{2+} release events originating from opening of the ryanodine receptor (RyR) channel on the sarcoplasmic reticulum (SR) membrane[2]. In cardiac muscle, sparks occur spontaneously through opening of the RyR2 channel by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism [3-5]. In skeletal muscle, RyR1 is strictly controlled by the voltage sensor at the TT membrane[6,7]. Thus Ca^{2+} sparks are suppressed and rarely detected in resting conditions in intact skeletal muscle fibers. Until recently, the sarcolemmal membrane needed to be disrupted by various chemical or mechanical "skinning" methods to uncouple the suppression of the voltage sensor on RyR1 and allowed for Ca^{2+} spark events to be detected in skeletal muscle[8,9]. One method previously described required permeabilization of the membrane of muscle fibers by saponin detergent[10].

In 2003, we discovered that either transient hypoosmotic stress or hyperosmotic stress could induce peripheral Ca^{2+} sparks adjacent to the sarcolemmal membrane in intact muscle fibers[11]. This method has since been modified to study the molecular mechanism and modulation of Ca^{2+} release and dynamics[12-16]. Here we outline the details of our experimental approach for induction, detection and analysis of Ca^{2+} sparks in intact skeletal muscle. We also present our custom-built spark analysis program that can be used to quantify the elemental properties of individual Ca^{2+} sparks in skeletal muscle, *e.g.* spark frequency and amplitude (Δ F/F0, which reflects the open probability of the RyR channels and the Ca^{2+} load inside the SR); time to peak (rise time) and duration (FDHM, full duration at half-maximal amplitude) of sparks, as well as the

spatial distribution of Ca^{2+} sparks. In addition, we present evidence that links altered Ca^{2+} sparks to the various pathophysiological states in skeletal muscle, such as muscular dystrophy and amyotrophic lateral sclerosis.

The advantage of this technique involves the ability to measure Ca^{2+} in relatively undamaged cells, instead of stripping the muscle fibers, allowing recording of Ca^{2+} sparks in conditions closer to physiologic. Additionally, our custom-designed program provides more accurate calculations of the properties of the spark in relation to muscle fibers.

Protocol

1. Setting up Osmotic-stress Perfusion System

Figure 1 is a schematic protocol of calcium sparks assessment in intact skeletal muscle fibers.

1. Set up a three-axis (xyz) micromanipulator capable of positioning the outlet tip of the perfusion system containing a minimum of two channels. This could be made with disposable Luer-syringe barrel with attached *three-way Luer-Lok* stopcock to switch on and/or off of the flow of perfusion solutions. These perfusion channels should be capable of delivering >1 ml/min of solution through a single perfusion tip with a >0.2 mm diameter.
2. Load two channels of the perfusion system, one with Isotonic Tyrode Solution and the other with Hypotonic Tyrode Solution.

2. Preparing Intact Single Flexor Digitorum Brevis (FDB) Muscle Fibers from Mouse

1. Remove the foot from a mouse euthanized following NIH and IACUC guidelines using a pair of heavy dissecting scissors by cutting through the leg above the ankle joint.
2. Pin the foot onto a dissection chamber filled with Minimal Ca^{2+} Tyrode Solution with the plantar surface facing up.
3. Dissect FDB by cutting the tendon and gently pulling up on the tendon to separate the muscle from the surrounding tissue.
4. Finish dissection by cutting the distal tendon where it branches into individual digits in the deep layers of the muscle.
5. Transfer the FDB muscle by picking up with forceps via its tendons to avoid additional damage to the muscle fibers and place into a tube with a thawed aliquot of Collagenase Digestion Solution prewarmed to 37 °C.
6. Incubate the tube containing FDB upright on an orbital shaker at 37 °C for 60-90 min with a speed of 160 rpm. This muscle bundles dissociation protocol needs to be experimentally examined for different strains of animals, especially for those disease/mutant fibers vulnerable to membrane damage.
7. Transfer the digested FDB into tube with 700 µl of Isotonic Tyrode Solution and gently triturate to release the fibers by drawing the muscle several times through a series of 200 ml-micropipette tips of gradually decreasing diameter via cutting with a clean razor blade to remove the tips of 200 ml micropipette. To avoid damaging fibers in particularly weak muscles from disease, make sure the tip is just large enough to allow the fiber bundle to pass through without sticking. Do not force the bundle through too small an opening.
8. Plate out the FDB fibers by gently tapping and resuspending FDB fibers in the tube and then drawing out 70 ml (1/10 of total fibers) with a cut 200 ml micropipette into the center of a 35 mm Delta TPG dish containing 1 ml of Isotonic Tyrode Solution to reduce diffusion across the dish.
9. Determine the number of intact single fibers in the dish using a dissection microscope. Add additional aliquots of FDB fibers to obtain 3-4 intact fibers on the dish.
10. Store additional isolated muscle fibers at 4 °C and use within 6 hr.

3. Fluo-4 AM Dye Loading and Ca^{2+} Imaging (Sparks Measurement)

1. Transfer 500 ml of Isotonic Tyrode Solution from dish with plated FDB fibers into a tube with 10 ml Fluo-4 AM stock (1 mM), mix and add back to the dish to a final Fluo-4 AM concentration of 10 mM. Alternatively, pluronic acid can be used to increase dye loading efficiency.
2. Load for 60 min at room temperature in the dark.
3. Wash the fibers by carefully drawing out 500 ml of Fluo-4 AM-containing Isotonic Tyrode Solution from the dish with an uncut 200 ml-plastic micropipette tip and replace with equal volume of fresh Isotonic Tyrode Solution.
4. Repeat this process 3 more times.
5. To visualize mitochondrial function, transfer 500 ml of Isotonic Tyrode Solution from dish with FDB fibers into a tube with 5 ml TMRE stock (1 mM), mix, and add back to the dish for a final concentration of 50 nM.
6. Incubate at room temperature for 10 min.
7. Wash the fibers by carefully drawing out 500 ml of TMRE-containing Isotonic Tyrode Solution from the dish with an uncut 200 ml plastic micropipette tip and replace with equal volume of fresh Isotonic Tyrode Solution.
8. Repeat this process 3 more times.
9. Transfer the dish to the confocal microscope and select an intact fiber with clear striations and a smooth sarcolemmal membrane for experimentation.
10. Position the tip of the perfusion system 400-500 µm away from the target muscle fiber and off center using micromanipulator controls.
11. Begin flow of the Isotonic Tyrode Solution to ensure the FDB fiber stays in place.
12. Record the fluorescence signal from Fluo-4 AM with a 40X oil immersion objective and excite Fluo-4 AM with an argon laser (excitation wavelength 488 nm) and record emission signals (wavelength 510-580 nm). The intensity of the fluorescent signal is proportional to the relative level of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).
13. Induce Ca^{2+} transients by changing the osmotic pressure of the extracellular solution in order to produce osmotic stress on fiber. Initially perfuse the fibers with Isotonic Tyrode solution (290 mOsM) (baseline collection for 60 sec) and then with Hypotonic Tyrode solution (170 mOsM) for 100 sec to induce swelling. Perfuse the FDB fibers back with Isotonic Tyrode solution. Generally, only one osmotic shock is applied to a single intact fiber in one delta TPG dish and the spark events last 10 min for data acquisition.

14. Record spatial localization of Ca^{2+} sparks (**Figure 2**) using a time-series of *xy* two-dimensional images with scan speed of 166 lps (line per second, and each line comprised of 512 pixels resulting in 3.08 sec/frame) for each condition of isotonic (1 min), hypotonic stress (100 sec) and post-hypotonic stress (5 min). Store signals for offline analysis to evaluate the distribution and frequency of Ca^{2+} sparks following completion of the experiment.
15. Find a new fiber on a new dish and follow the same osmotic shock protocol to induce Ca^{2+} transients. Use a confocal line scan (*xt*) mode (512 pixels in length, sample rate of 2 msec/line scan to record Ca^{2+} transients for one minute (*i.e.* 30,000 lines) with the confocal scan line placed directly underneath sarcolemmal membrane (**Figure 3**). Change the line scan to different focal planes to record three sequential series (at 1-min intervals) of linescans for analysis of the magnitude and kinetics of individual Ca^{2+} sparks.

4. Data Analysis

1. Collect a large number of *xt* line scan traces to evaluate the morphology and kinetics of individual Ca^{2+} sparks parameters, *i.e.* the amplitude (F/F_0; where F_0 is the resting Ca^{2+} fluorescence), rise time, time to the peak, duration (FDHM, full duration at half magnitude), and spatial width (FWHM, full width at half magnitude) of the recorded sparks. These parameters represent the basic gating properties of RYR Ca^{2+} channels such as the Ca^{2+} flux (amplitude), and gating kinetics of RYRs (duration).
2. Analyze digital image data with a custom-developed, semi-automatic algorithm which was created with image-processing language IDL (Interactive Data Language), as described previously[11,16]. Because the unique kinetics of Ca^{2+} release in the case of Ca^{2+} sparks induced in FDB fibers by osmotic stress, it is better to combine the manual and automated features of Ca^{2+} spark events identification and processing with these custom-build image analysis programs[11]. This algorithm is very useful when it's necessary to manually identify ROI (region of interest) in some muscle disease models. Once a ROI is defined, the algorithm could automatically derive the above mentioned spark parameters which could then be exported to Excel file. Alternatively, the public available image processing software, *e.g.* SparkMaster in ImageJ, can be used to define the kinetic properties of Ca^{2+} sparks and their spatial distribution in skeletal muscle[17].

Representative Results

Earlier studies showed that transient hypoosmotic stress induced peripheral Ca^{2+} sparks adjacent to the sarcolemmal membrane in intact muscle fibers[11]. **Figure 1** shows the images of intact single muscle fibers with smooth sarcolemmal membrane and characteristic clear striations. **Figure 2** shows typical Ca^{2+} sparks (as *xy* images) were induced by transient (100 sec) treatment with a hypoosmotic solution that swells the FDB muscle fibers from young, healthy mice. As the muscle fiber shrinks back to the original volume, a robust Ca^{2+} spark response will be directly under the sarcolemma of the muscle fiber. These *xy* images are used to calculate spark frequencies and plot the decay profile for this response. **Figure 3** shows typical line scans (*xt*) generated with this mode of spark analysis. The confocal scan line is located directly underneath of the sarcolemmal membrane (**Figure 3A**). The time series of spark events are captured and the specified ROIs (region of interest) could be identified with our custom-developed, semi-automatic "Spark Fit" program (**Figure 3B**). **Figure 3C** shows a typical Spark Fit analysis of amplitude and kinetics of given Ca^{2+} spark. The Ca^{2+} spark intensity is calculated as the amplitude change of Fluo-4 AM signals ($\Delta F/F_0$, where F is the peak Fluo-4 intensity and F_0 is the resting Fluo-4 intensity before spark arising). The duration of the spark is represented by FDHM (full duration of half magnitude) and the rise time as well as the time to peak can also be calculated. A typical 3D (three-dimensional) representation can be made from specified ROIs to show Ca^{2+} intensity change (**Figure 3D**). **Figure 4** demonstrates images of Ca^{2+} spark linescans from skeletal muscle fibers under different physiological and pathophysiological states. Line scan (*xt*) series of Ca^{2+} sparks derived from healthy, young (3 months) wild type muscle (**Figure 4A**), and aged (22 months) muscle (**Figure 4B**). Note the reduced signal that occurs in aged muscle fibers. The young *mdx* muscle fibers display robust Ca^{2+} sparks (**Figure 4C**). **Figure 5** shows Ca^{2+} sparks (*xy* images) from extensor digitorum longus (EDL) muscle fibers from wild type (**Figure 5A**) and amyotrophic lateral sclerosis (ALS) disease, a motor neuron disease with characteristic muscle atrophy and damaged mitochondria (**Figure 5B**). The Ca^{2+} intensities are show by Fluo-4 signals (green, left panels) and the mitochondria respiratory states are shown by TMRE signals (red, right panels). Note that the damaged mitochondria (losing TMRE signal) harbors robust Ca^{2+} sparks.

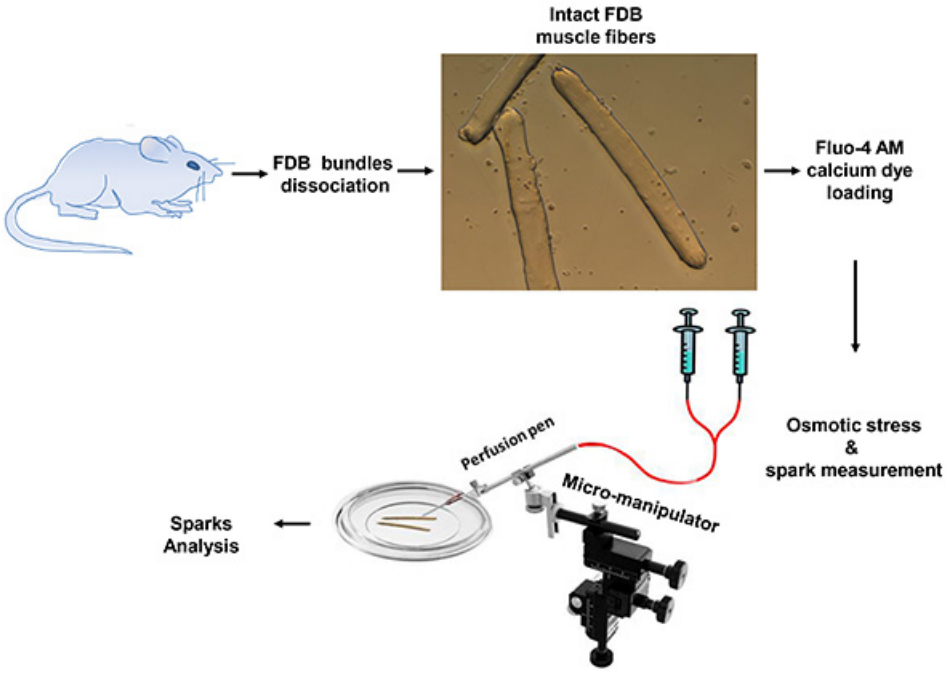

Figure 1. Schematic protocol of calcium sparks assessment in intact skeletal muscle fibers. Schematic protocol of calcium sparks assessment in intact skeletal muscle fibers. This shows step-wise procedure to obtain intact FDB muscle fibers for analysis and the set-up of perfusion system to induce osmotic stress on fibers. Click here to view larger image.

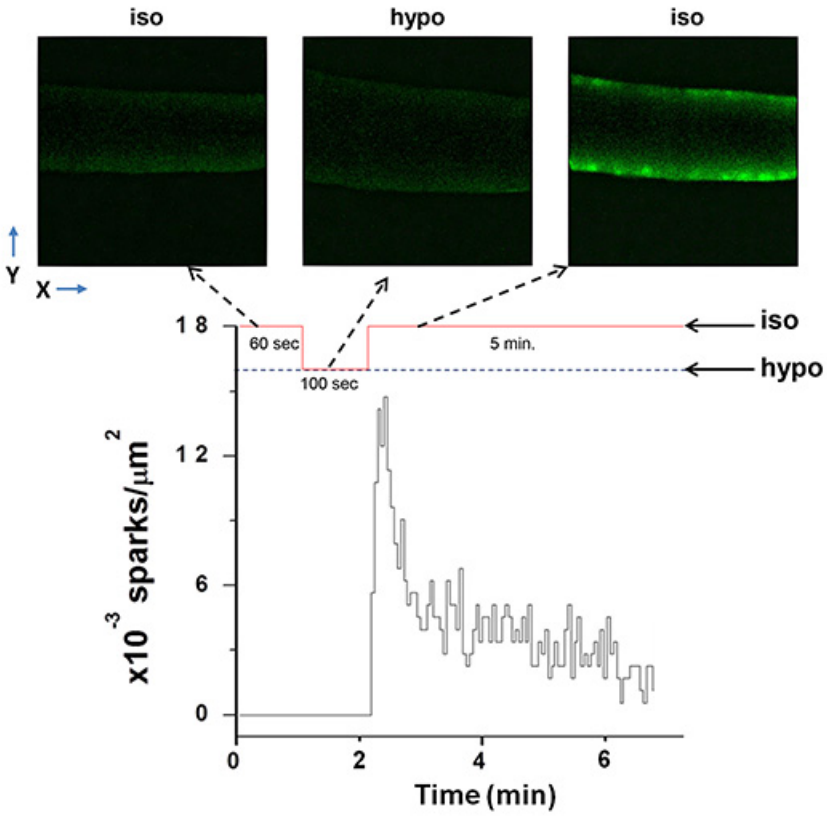

Figure 2. Representative Ca^{2+} sparks (XY images) episodes of skeletal FDB muscle fibers. The middle trace shows the timeline and the corresponding xy images (top panels) to the sequential osmotic pressure change in the protocols. Top left panel - fiber treated with Isotonic Tyrode solution; top middle panel - fiber treated with Hypotonic Tyrode solution; and top right panel - fiber returned to treatment with Isotonic Tyrode solution. A representative histogram of the spark episodes observed with xy imaging is shown on the bottom. Click here to view larger image.

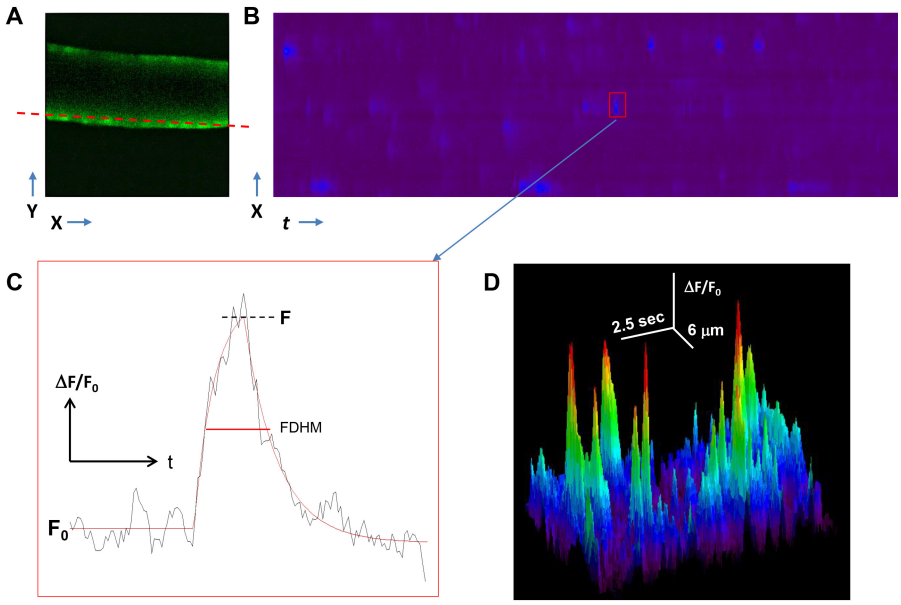

Figure 3. Representative Ca^{2+} sparks (line scans xt images) and Spark Fit analysis. (**A**) Confocal line scan (red dashed line) is placed right underneath the sarcolemmal membrane of muscle fiber; (**B**) Osmotic stress evoked discrete Ca^{2+} sparks are presented as line scan (xt) mode. (**C**) Spark analysis with Spark Fit algorithm to demonstrate the Ca^{2+} intensity ($\Delta F/F_0$) which is proportional to the SR Ca^{2+} release flux, and kinetics (duration, represented as FDHM). (**D**) Three-dimensional plot of a Fluo-4 line scan image showing the osmotic stress-evoked sparks in spatio-temporal mode and their intensities. Click here to view larger image.

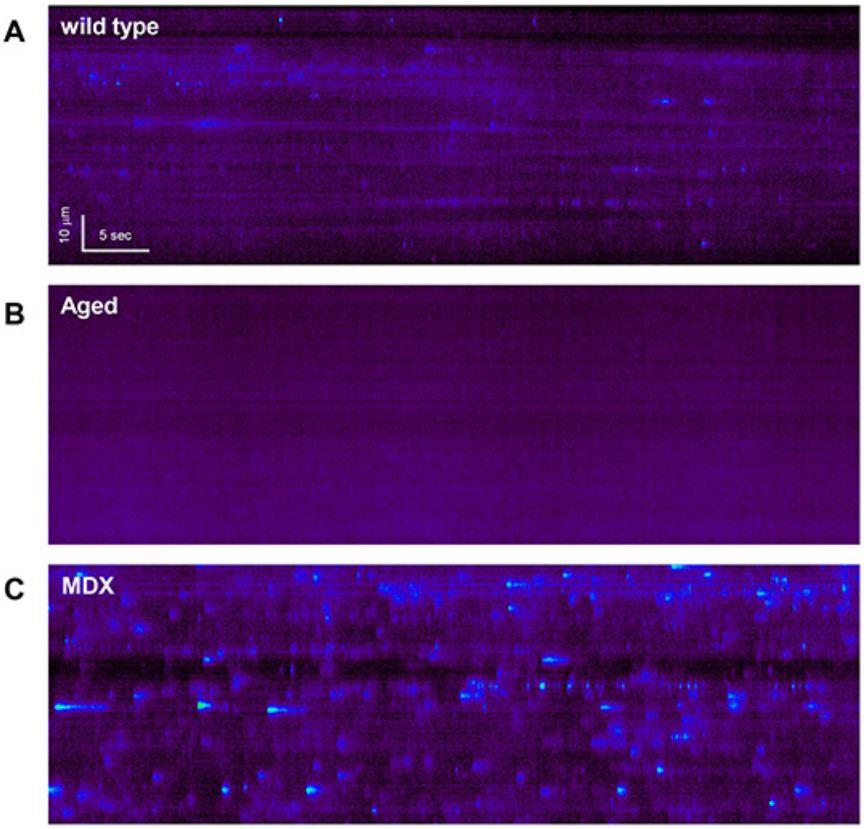

Figure 4. Representative Ca^{2+} sparks in muscle physiological and pathophysiological states in response to osmotic stress. (**A**) Line scans (xt images) of Ca^{2+} sparks derived from young (3 months) healthy FDB muscle fibers; (**B**) sparks from aged (22 months) FDB muscle fibers and (**C**) sparks from young (3 months) dystrophic mdx mouse FDB muscle. Click here to view larger image.

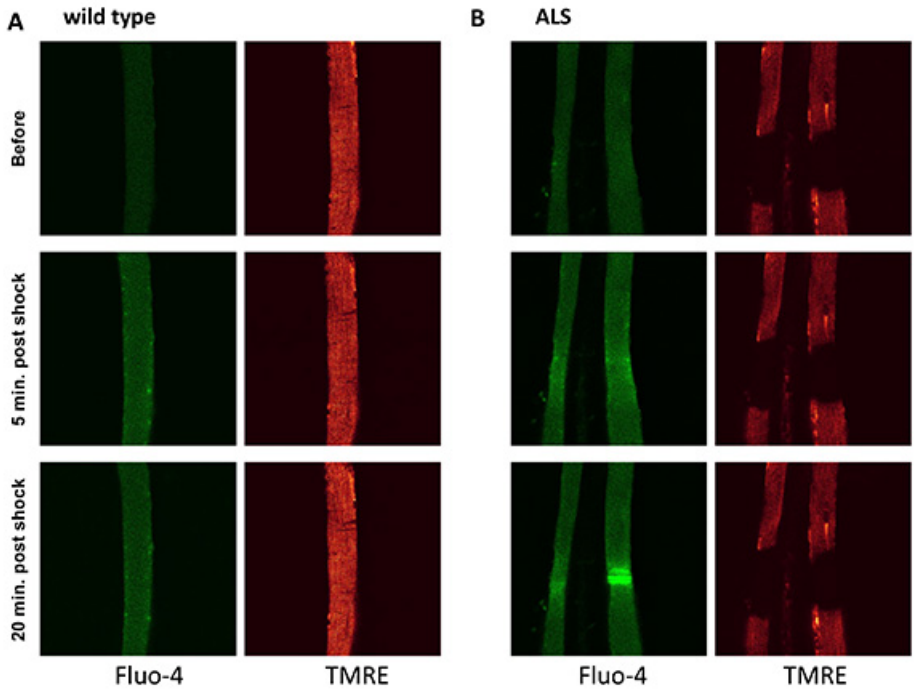

Figure 5. Representative Ca^{2+} sparks (XY images) from FDB muscles in response to osmotic stress. (**A**) Ca^{2+} sparks (XY images) from wild type FDB muscles and (**B**) Ca^{2+} sparks from ALS muscles. FDB muscle fibers simultaneously labeled with Fluo-4 AM (Ca^{2+}, green signals, left panels) and TMRE (mitochondrial, red signals on the right panels). Images show transients before and after osmotic pressure changes. Click here to view larger image.

Discussion

This method of assessing Ca^{2+} sparks in intact skeletal muscle is a useful tool for muscle physiology and disease research. We showed that the Ca^{2+} spark response was altered in different conditions, including muscular dystrophy[11], aging[18,19], as well as in amyotrophic lateral sclerosis[20]. Our recent study also revealed functional coupling between IP_3 receptor and RyR representing a critical component that contributes to Ca^{2+} sparks activation in skeletal muscle fibers[21]. Several other research groups also adapted this technique to explore the functional activities and the efficacy of gene rescue strategies of dystrophic muscles[12], and link Ca^{2+} spark signaling to altered redox stress in some disease states[13,22]. The fact that membrane deformation by osmotic stress to relieve the suppression of DHPR on RYR correlates nicely with the essential role of physical interaction (coupling) of DHPR-RYR in modulating Ca^{2+} sparks in differentiating skeletal muscle[23]; and thus these findings further implicate Ca^{2+} sparks as important physiological events. Further expansion of this technique to apply some physiological agents or inhibitors to modulate Ca^{2+} sparks will provide insightful information on Ca^{2+} signaling in the cells[24].

The most critical step of our assay is to isolate and identify intact muscle fibers under microscope before Ca^{2+} dye loading. One crucial step in this protocol is the collagenase digestion time and temperature as changing these conditions could potentially render cell membrane damage during fiber isolation. A fiber must be firmly attached to the bottom of the delta TPG dishes and display intact morphology with characteristic straight, rod-like appearance, size of 70-100 µm (length) and 8-18 µm (cross diameter), uniform striation pattern and a smooth sarcolemmal membrane to be useful for Ca^{2+} sparks measurement. An improvement upon previous limitations of data collection is taking measurements at several focal planes within each fiber to capture the most accurate readings possible of the Ca^{2+} sparks. Although we use a custom-derived "Spark Fit" algorithm to analyze spark frequency, dynamics and characteristics, similar programs are available to public domain for analyzing spark parameters characteristics such as "SparkMaster" in ImageJ[17]. Slight modifications to this method could allow better visualization of Ca^{2+} sparks in other tissue types that respond to osmotic-stress mediated triggering of Ca^{2+} release from the ryanodine receptor.

Visualization of local and global Ca^{2+} signals in adult skeletal muscle fibers is a very useful tool for muscle physiology and cardiovascular research. Expansion of this method for detection of Ca^{2+} sparks in skeletal muscle together with different animal models of human disease research and implementation of the powerful molecular biology approaches *(e.g.* gene overexpression or gene silencing) should produce vast information on the regulation of Ca^{2+} signaling in human health and diseases.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by RO1- AG028614 to JM, RO1- AR063084to NLW, and RO1-AR057404 to JZ.

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