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Corresponding Author:	Dmitry V. Samigullin, Ph.D. Kazan Institute of Biochemistry and Biophysics Kazan, Tatarstan RUSSIAN FEDERATION
Corresponding Author's Institution:	Kazan Institute of Biochemistry and Biophysics
Corresponding Author E-Mail:	samid75@mail.ru
Order of Authors:	Nikita Zhilyakov
	Arsenii Arkhipov
	Eduard Khaziev
	Marat Mukhamedyarov
	Dmitry V. Samigullin, Ph.D.
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TITLE:

Registration of Calcium Transients in Mouse Neuromuscular Junction with High Temporal Resolution using Confocal Microscopy

AUTHORS AND AFFILIATIONS:

Nikita V. Zhilyakov[1], Arsenii Y. Arkhipov[1], Eduard F. Khaziev[1,2], Marat A. Mukhamedyarov[3], Dmitry V. Samigullin[1,2] *

[1]Laboratory of Biophysics of Synaptic Processes, Kazan Institute of Biochemistry and Biophysics, FRC Kazan Scientific Center of RAS, 420111, Kazan, Russia

[2]Department of Radiophotonics and Microwave Technologies, Kazan National Research Technical University named after A.N. Tupolev, 420111, Kazan, Russia

[3]Department of Normal Physiology, Kazan State Medical University, 420012, Kazan, Russia

***Corresponding Author:**

Dmitry V. Samigullin (samid75@mail.ru)

Email addresses of co-authors:

Nikita V. Zhilyakov (kiosak71@gmail.com)
Arsenii Y. Arkhipov (senjaarh@rambler.ru)
Eduard F. Khaziev (eduard.khaziev@gmail.com)
Marat A. Mukhamedyarov (maratm80@list.ru)
Dmitry V. Samigullin (samid75@mail.ru)

SUMMARY:

The protocol describes the method of loading a fluorescent calcium dye through the cut nerve into mouse motor nerve terminals. In addition, a unique method for recording fast calcium transients in the peripheral nerve endings using confocal microscopy is presented.

ABSTRACT:

Estimation of the presynaptic calcium level is a key task in studying synaptic transmission since calcium entry into the presynaptic cell triggers a cascade of events leading to neurotransmitter release. Moreover, changes in presynaptic calcium levels mediate the activity of many intracellular proteins and play an important role in synaptic plasticity. Studying calcium signaling is also important for finding ways to treat neurodegenerative diseases. The neuromuscular junction is a suitable model for studying synaptic plasticity, as it has only one type of neurotransmitter. This article describes the method for loading a calcium-sensitive dye through the cut nerve bundle into the mice's motor nerve endings. This method allows the estimation of all parameters related to intracellular calcium changes, such as basal calcium level and calcium transient. Since the influx of calcium from the cell exterior into the nerve terminals and its binding/unbinding to the calcium-sensitive dye occur within the range of a few milliseconds, a speedy imaging system is required to record these events. Indeed, high-speed cameras are commonly used for the registration of fast calcium changes, but they have low image resolution parameters. The protocol presented here for recording calcium transient

allows extremely good spatial-temporal resolution provided by confocal microscopy.

INTRODUCTION:

The problem of measuring fast calcium waves in excitable cells is one of the most important and challenging aspects of studying signal transmission in the central and peripheral nervous systems. Calcium ions play an important role in triggering neurotransmitter release, synaptic plasticity, and modulation of the activity of various intracellular proteins[1–5]. Studying calcium signaling is also important for finding ways to treat neurodegenerative diseases[6]. To measure changes in the calcium levels, fluorescent calcium-sensitive dyes are commonly used, and changes in their fluorescence level are analyzed[7–9].

Loading of calcium dyes into cells can be achieved in different ways. Predominantly, cell-permeant dyes are used[10,11]. However, in such a case, it is not only difficult to control the concentration of a dye inside the cell, but it is also hard to select target cells for loading. This method is not applicable for studying peripheral nerve endings since the dye enters postsynaptic cells. Instead, cell impermeant dyes are more suitable for such preparations. In this case, the dyes are delivered to the cells by microinjection or through a patch pipette[12–14]. There is also a method of loading through a nerve stump. The latter method is most suitable for neuromuscular junction preparations[15–20]. It allows performing staining for only cells of interest. Although this method does not provide an accurate evaluation of the concentration of the dye in the target cell, the concentration can be estimated approximately by comparing the level of fluorescence of the cells at rest in solutions with a known concentration of calcium[21]. In this study, a modification of this method applied to synapses of mammals is presented.

Calcium entry during the depolarizing phase of the action potential is a fast process, especially in the neuromuscular junction; therefore, for its registration, appropriate equipment is required[1]. A recent study using a voltage-sensitive fluorescent dye demonstrated that the duration of the action potential in the peripheral synapse of a mouse is approximately 300 µs[22]. Calcium transient, evaluated using calcium-sensitive dyes in the peripheral synapses of the frog, has a longer duration: the rise time is about 2–6 ms and the decay time is about 30–90 ms, depending on the calcium dye used[23,24]. To measure fast processes with the help of fluorescent dyes, CCD or CMOS cameras are generally used, with fast and sensitive CCD matrices. However, these cameras have the disadvantage of low resolution, limited by the size of the sensitive elements of the matrix[25–28]. The fastest cameras with sufficient sensitivity to record both action potentials and calcium transients in response to low frequency stimulation of cells have a scanning frequency of 2,000 Hz, and a matrix with a dimension of 80 x 80[29]. To obtain signals with a higher spatial resolution, confocal microscopy is used, especially if it is necessary to assess some volumetric changes in the signal[30–32]. But it should be kept in mind that confocal microscopy has a high scanning speed in line scan mode, but there are still significant limitations on the speed of recordings of fast processes when building a spatial image[33]. There are confocal microscopes based on rotating Nipkow disks (slit-scanning microscopy) and Multipoint-Array Scanners, which have a higher scanning speed. At the same time, they are inferior to the classical confocal microscopes in confocal image filtering (pinholes crosstalk for microscopes with a Nipkow disk)[32,34,35]. Confocal imaging with resonance scanning can also

provide a high spatio-temporal resolution required for high temporal measurements[36]. However, take into account that the registration of weak fluorescent responses at a high scanning speed when using resonance scanners requires highly sensitive detectors such as hybrid detectors[36].

This article presents a method for increasing the temporal resolution of signals recorded with the Laser Scanning Confocal Microscopy (LSCM) while maintaining the spatial resolution[37]. The current method is a further development of the methods described earlier and transferred to the LSCM platform[38–40]. This approach does not require changes in the microscope hardware and is based on the application of an algorithm for recording periodically evoked fluorescent signals with a time shift relative to the moment of stimulation.

PROTOCOL:

Experiments were performed on isolated nerve-muscle preparations of *levator auris longus* (m. LAL) from the Mice BALB/C (20–23 g, 2–3 months old)[41]. The experimental procedures were performed in accordance with the guidelines for the use of laboratory animals of the Kazan Federal University and the Kazan Medical University, in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol met the requirements of the European Communities Council Directive 86/609/EEC and was approved by the Ethical Committee of the Kazan Medical University.

1. Preparation of the Ringer's and Filing solutions

1.1. Prepare the Ringer's solution for mammalian muscle by mixing the following ingredients: NaCl (137 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), NaH_2PO_4 (1 mM), $NaHCO_3$ (11.9 mM), and glucose (11 mM). Bubble through the solution with 95% O_2 and 5% CO_2 and adjust its pH to 7.2–7.4 by adding HCl/NaOH if necessary.

1.2. Prepare the dye loading solution.

1.2.1. Prepare HEPES (10 mM) solution with pH in the 7.2–7.4 range. 500 µg of commercial dye comes in a 500 µL vial. Dissolve the dye in 14 µL of the HEPES solution to obtain a dye concentration of 30 mM. Shake well and centrifuge until totally dissolved.

1.2.2. Dilute the solution of Ca^{2+} indicator with HEPES solution down to 1 mM concentration. Keep it in a freezer (-20 °C) and avoid exposure to light.

2. Dye loading procedure

NOTE: The dye loading procedure is performed according to the protocol for loading through the nerve stump, adapted from the protocols previously published[19,42–46].

2.1. Dissect LAL muscle according to the dissection procedure for this preparation as described in the previously published protocols[47,48].

2.1.1. Fix the tissue slightly stretched (no more than 30% from initial length) in the elastomer-coated Petri dish with fine stainless-steel pins and add Ringer's solution until the muscle is fully covered.

NOTE: The Petri dish was pre-filled with elastomer according to the manufacturer's instructions (see **Table of Materials**).

2.2. Prepare the Filling Pipette

2.2.1. Using a micropipette puller (see **Table of Materials**), prepare a micropipette with a fine tip which is as sharp as possible for the intracellular recordings. Use capillaries without internal filaments (1.5 mm in outer diameter and 0.86 or 1.10 mm in inner diameter).

2.2.2. Break off the micropipette tip after scoring the taper with an abrasive, leaving the tip open to about 100 µm in diameter. Fire-polish the tip down to limit when the internal diameter shrinks from >80 µm to 12–13 µm. Attach a silicone tube to one side of the Filling Pipette and a syringe (without a needle) to the the other side.

2.3. Under a stereomicroscope, find the place where the nerve trunk turns into separate nerve branches. Place the Filling Pipette with the mounted tube and the syringe on the Petri dish using wax. Move the pipette tip until it stands above the nerve.

2.4. With fine scissors, cut the nerve close to the muscle fiber, leaving a small piece of the nerve stump about 1 mm long. Gently aspirate the nerve stump together with some Ringer's solution, without pinching it, into the tip of the Filling Pipette. Remove the silicone tube from the Filling Pipette.

2.5. Draw some amount of the dye loading solution (~0.3 µL) using a syringe with a long filament. This volume corresponds to approximately 3 cm of the filament.

NOTE: Initially, it is necessary to make a filament from a pipette tip with a volume of 10 µL by pulling on the fire using an alcohol lamp or a gas burner.

2.6. Gently insert the filament tip with loading solution into the Filling Pipette. Release the mixture directly onto the nerve stump. Incubate the preparation at room temperature in dark for 30 min.

2.7. After that, rinse the preparation with fresh Ringer's solution and incubate at 25 °C for up to 2 h in a glass beaker with 50 mL (or more) Ringer's solution (preparation must be covered with the solution). During this time, the dye will reach the synapses.

3. Video capture with confocal microscopy

NOTE: Registration of calcium transients is performed with a laser scanning confocal microscope (LSCM) (see **Table of Materials**). To register fast calcium transients, an original protocol that permitted recordings of signals with a sufficient spatial and temporal resolution was used. The method has been described thoroughly in the publication by Arkhipov et al[37]. The microscope was equipped with a 20x water immersion objective (1.00 NA). The 488 nm laser line was attenuated to 10% intensity and emission fluorescence was collected from 503 to 558 nm.

3.1. Mount the preparation into the silicon elastomer-coated experimental chamber and fix it, slightly stretched, with a set of steel micro-needles. Rinse the preparation extensively with Ringer's solution.

NOTE: A simple custom-made perfusion experimental chamber made of organic glass with the bottom of the chamber covered with an elastomer (prepared in accordance with the manufacturer's instructions; see **Table of Materials**) was used. The chamber has a solution supply tube. The solution is pumped out *via* a syringe needle, mounted on a magnetic holder (see **Table of Materials**). As an experimental chamber, a Petri dish could be used (like the one used for incubation of the preparation) but with attached supply and suction tubes.

3.2. Install suction electrode which will be used to stimulate the nerve.

NOTE: Construction of the electrode is similar to what was published in the 2015 paper by Kazakov et al[49]. Place and fix the electrode by waxing beside the bath. Move the tip close to the nerve stump and aspirate it into the electrode.

3.3. Mount the preparation chamber onto the microscope stage and place the inlet and outlet fittings into the chamber.

3.4. To perfuse the preparation, use a simple gravity-flow-driven system. Turn on the perfusion suction pump to remove the excess solution.

3.5. Plug the stimulating suction electrode into an electric stimulator and ensure that muscular contractions occur after stimuli. See section 3.9–3.12 for stimulation conditions and recording.

3.6. Fill up the perfusion system with the Ringer's solution with d-tubocurarine (10 μM).

NOTE: This solution helps to prevent muscular contractions. D-tubocurarine or alpha-bungarotoxin-specific blockers of nicotinic acetylcholine receptors on the postsynaptic membrane would completely or partially block muscle contractions[50]. Also, for preventing muscular contractions, specific blockers of postsynaptic sodium channels such as μ-conotoxin GIIIB could be used[51].

3.7. Switch on the perfusion suction pump and start perfusion of the preparation with the Ringer's solution containing d-tubocurarine.

3.8. Set imaging parameters in the LSCM software as follows.

3.8.1. In the LSCM software (LAS AF; see **Table of Materials**), choose **Electrophysiology**.

NOTE: In this mode, when an image is captured at the time point, a synchronizing pulse is sent to the stimulator with the help of the trigger box. This elicits action potential generation in the preparation (**Figure 1**; stimulator unit).

3.8.2. Select **Acquisition Mode**. For triggering the stimulator using the microscope sync pulse, in the **Job** menu settings, select the **Trigger** settings. Set the **Trigger Out On Frame** field to the **out1** channel.

3.8.3. Use the following settings: **Scanning Mode**: XYT, **Frequency of Scanning**: 1400 Hz, **Zoom Factor**: 6.1, **Pinhole**: fully open. Ensure that sequential trans-passing Bidirectional X mode is on.

3.8.4. Set minimum time to form a frame at 52 ms and frames to be collected in a raw video at 20 frames.

NOTE: These settings permit image capturing with a resolution of 128 x 128 pixels while taking a single frame every 52 ms.

3.8.5. Set excitation wavelength of the argon laser at 488 nm with 8% of output power.

[place Figure 1 here]

3.8.6. Press the **Live Mode** button to switch to Live mode, which helps to get a preview of nerve terminals loaded with the dye.

[place Figure 2 here]

3.9. Stimulation unit

NOTE: In this work, the stimulator described in the article by Land et al.[52] was used. This device allows for setting temporal parameters of stimulation *via* the MatLab software.

3.9.1. Create a new file, paste the code from the above-mentioned article to the MatLab code window, and save the file. Click on **Run**, so a window with stimulation parameters appears. Set the delay time and duration of the stimulus.

NOTE: The delay determines the temporal resolution of the reconstituted fluorescent signal. The electric pulse of 0.2 ms duration is delayed, and then sent to the isolation unit. The latter forms the amplitude and polarity of the stimulating pulse and electrically isolates the biological object from the recording equipment.

3.9.2. To stimulate the nerve, select supramaximal amplitude of the stimulating impulse (25%–50% greater than the maximum stimulation intensity necessary to activate all the nerve fibers).

NOTE: The presented method is based on a special algorithm for recordings of single fast fluorescent signals using LSCM with the minimized sweep. At each step of the developed algorithm, the recorded fluorescent signal is shifted from the previous one by a time interval that is shorter than the microscope sweep. The value of time shifts determines the temporal resolution of the required signal. The number of steps (shifts) in the algorithm depends on the required temporal resolution and original temporal resolution. With this method of registration, the stimulation of the preparation is carried out with a frequency of 0.25 Hz.

3.10. In the Live mode, search for the ROI and obtain the best focus. Run the data acquisition software.

3.11. Shift the delay on the stimulator by 2 ms less relative to the previous value and run the data acquisition software.

3.12. Repeat step 3.11 26 times to acquire 26 sequences, with each sequence shifted by 2 ms from the previous one.

4. Video processing

NOTE: A series of video images acquired by the confocal microscope is exported in the TIFF format with the free software LAS X (see **Table of Materials**). This series was divided into frames and exported to a folder. For generating the image sequence with higher time resolution, the ImageJ software, which has an open initial code for the analysis and processing of the data, was used. The algorithm of signals processing is represented schematically in **Figure 3**.

[place Figure 3 here]

4.1. Run LAS X software. Open the project which was created during performing experiment. Click on **Export**, and then on **Save As** to save frames in .tiff format in the destination folder.

4.2. Run the ImageJ software. Click on **File** > **Import** > **Image Sequence**.

4.3. In the **Open Image Sequence** window, choose the destination folder and open the first frame.

4.4. In the **Sequence Options** window, in the **Starting Image** field, set the frame number to 1 for the first frame. In the **Increment** field, set the value equal to the number of frames in the initial signal recording (20 for the present case) and click on **OK**.

4.5. To save the generated file of stitched first frames in a separate folder, click on **File** > **Save** > **Folder**.

4.6. Repeat steps 4.3–4.5 for the next 19 frames. In the **Sequence Options** window, set the corresponding frame number in the **Starting Image** field.

4.7. To generate the full high-time-resolution video, stitch all the frames together. To do this, click on **File** > **Import** > **Image Sequence** and select 1 in the **Starting Image** and **Increment** fields. The result will be the final video with increased temporal resolution. Save the file in .tiff or any other suitable format.

5. Video analysis

NOTE: In ImageJ, select ROI and background. Subtract background from ROI. Data is represented as the ratio, $(\Delta F / F_0 - 1) * 100\%$, where F_0 is the intensity of fluorescence at rest and ΔF is the intensity of fluorescence during stimulation.

5.1. Click on **Image** > **Stacks** > **Tools** > **Stack Sorter**. Then, click on **Analysis** > **Tools** > **ROI Manager**.

5.2. Drag and drop the .tiff file saved in step 4.7 into the ImageJ window. Expand the image for a better view. To improve image visualization, click on **Image** > **Adjust** > **Brightness/Contrast** > **Auto**. This step will not affect the data.

5.3. Set the background close to the nerve terminal by drawing ROI. Add it to the ROI manager. Calculate the background by clicking on **More** > **Multi Measure**. Copy mean values, paste to the Spreadsheet program, and calculate the average.

5.4. Subtract the calculated average value from the stacks by clicking on **Process** > **Main** > **Subtract**. Enter the value.

5.5. Draw ROI around a nerve terminal *via* a polygon line. Add it to the ROI manager.

5.6. Measure the intensity of the nerve terminal: Click on **More** > **Multi Measure**. Copy mean values and paste them to the Spreadsheet program.

5.7. Calculate the average offset of signals.

NOTE: Use the corresponding points depending on the delay time before stimulation. This step establishes the F0 value that will be used in subsequent calculations.

5.8. Divide the signal values by the average offset value.

NOTE: After this step, the signal does not contain the contribution of the background and raw

fluorescence to the amplitude values for the selected ROI.

5.9. Subtract 1 from values obtained in step 5.8, and then multiply by 100%.

5.10. Plot a graph of Ca^{2+}- transient and calculate the amplitude.

REPRESENTATIVE RESULTS:

After loading the preparation with dye according to the presented technique, most of the synapses located close to the nerve stump had a sufficient level of fluorescence (see **Figure 2**). After loading preparation with the dye and applying the described method of registration and image processing, calcium transients with the desired spatial and temporal resolution were obtained (see **Figure 4**). The calcium transient has been recovered by the proposed method (see **Figure 3**).

Amplitude and time parameters of the recovered signals were also analyzed. Average data are presented in **Table 1**.

[place Figure 4 around here]

[place Table 1 here]

Calcium transient analysis makes it possible to assess the amplitude-dynamic characteristics of changes in the presynaptic calcium level in the nerve ending during the action potential[11]. The change in the amplitude of the calcium transient correlates well with the change in the quantal content[53]. Calcium transient amplitude analysis is commonly used to study the effect of physiologically active compounds associated with modulation of presynaptic calcium levels on synaptic transmission[54,55]. The time course of the calcium transient reflects the kinetics of calcium binding with the dye and its dissociation[23,56]. It is obvious when using dyes with different affinity for calcium[23,56]. Although the temporal parameters of the calcium transient reflect the kinetics of the calcium sensitive dye, and do not represent the kinetics of free calcium in the nerve terminal, mathematical modeling methods based on experimental data can restore the behavior of free calcium in the cell and calculate the concentration of calcium buffers[23].

FIGURE AND TABLE LEGENDS:

Figure 1: The schematic of the experimental setup. 1. Laser Scanning Confocal Microscope (LSCM). 2. Synchronization module of LSCM (trigger box). 3. Stimulator. 4. Isolation unit. 5. The biological sample. 6. Suction electrode for electrical stimulation of nerve. 7. Perfusion systems (7a: perfusate reservoir, 7b: dropper, 7c: flow regulator, 7d: vacuum flask). Arrows point to the direction of propagation of synchronizing pulse.

Figure 2: Mouse nerve and terminals loaded with the Ca^{2+} indicator.

Figure 3: Scheme for compiling a high-resolution video file (2 ms on frame) from original

video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta, and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time of 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n - 1) * t, where t is time shift (2 ms), and n is the number of shift iterations. k denotes the number of frames in the original video files (lines 2–4) and depends on the duration of the recorded signal. In this case, to register a signal with a duration of 1 s, it is necessary to select k = 20 (52 ms * 20 = 1040 ms). t_0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52 ms) must be divided by the required temporal resolution (2 ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained.

Figure 4: Representative trace of calcium signal from one experiment. Some important parameters of signal, such as mean amplitude (MA), rise time (RT), and decay time (DecT) and its projections on axes are indicated. MA is calculated by averaging points at the peak, colored in green. RT is the time taken for the amplitude to rise from 20% to 80%, which is calculated as the difference between projections on the x-axis colored in blue. DecT is the time over which the amplitude decreases by e times, which is calculated as the difference between projections on the x-axis colored in red.

Table 1: The averaged parameters of the Ca^{2+} transient. Data are presented as mean ± s.e.m., and n is the number of measurements in the distinct nerve-muscle junctions. Peak ΔF/F is the mean amplitude of ΔF/F.

DISCUSSION:

The method for loading Ca^{2+}-sensitive dye into mouse nerve endings through the nerve stump and for registering a fast calcium transient using a confocal microscope is presented in this article. As a result of the implementation of this loading method, most of the synapses located close to the nerve stump had a sufficient level of fluorescence to enable registration of the entry of calcium into the nerve endings in response to low-frequency stimulation of the motor nerve.

Unlike the previously presented protocols for loading calcium dyes through the stump, this protocol is designed for use on mammalian synapses. Previous protocols used in cold-blooded animal preparations required an overnight incubation[43]. In this protocol, the required incubation with the drug is only 2 h. Depending on the length of the nerve segment that remained after the cutting, the speed of dye loading, and the number of loaded terminals may vary. It was possible to reduce the incubation time even further in dye solution for shorter nerve stumps. A similar method is described for the loading of fly synapses[18,19]. Also, the incubation of the muscle was carried out with a slight increase in temperature above the room

temperature, which improves the diffusion of dye along the nerve. It thus helped to reduce the incubation time and, therefore, current dye loading method can be used for loading synapses of mammals that do not tolerate prolonged incubations. It is important to choose the appropriate preparations for the study. The LAL muscle is well suited for this method, as it is possible to cut a nerve stump close enough to the synaptic terminals. Other thin muscles can also be suitable for such application[57]. One of the most important steps in this protocol is that the nerve stump has to be placed into the dye-containing solution in the first few minutes after cutting the nerve. Since the length of the nerve is short, a specific design of the suction electrode must be used for stimulation. In this research, both glass and plastic electrodes were used, with the diameter comparable with that of the nerve segment.

Acquiring calcium transients should be carried out using special equipment. Recordings of long-lasting changes in calcium level in response to frequency stimulation of the motor nerve require regular cameras or a confocal microscope. While registration of calcium transients is in response to rare stimulation of the nerve, sensitive cameras are needed when the signal from the dye has low intensity and high speed[11]. Highly sensitive CCD cameras or matrices consisting of photodiodes are mainly used to register these fast processes. However, cameras with high sensitivity and speed tend to have low resolution. If high-resolution registration is required, confocal microscopy methods are more suitable. In confocal microscopes, photomultipliers are used to register fluorescence, and more recently, hybrid detectors came to use. They have a very high sensitivity compared to CCD cameras and are well suited for detecting weak fluorescence. But the main disadvantage of LSCM is low scanning speed when building a spatial image. In this study, to record the calcium transient, the original registration method was used *via* LSCM, which is described thoroughly in the article by Arkhipov et al. related to the synapses of the frog[37]. Using this method, it was possible to estimate the proximal-distal gradient of calcium transient in the elongated frog synapses[37]. This method of registration can be useful for assessing subcellular calcium dynamics in excitable cells, for example, in dendrites and spines in brain slice preparations. In the present study, it was applied to mammalian synapses. It allowed obtaining confocal video images with 2 ms sampling of signals to analyze the parameters of the calcium transient.

The described method deals with fluorescent signals, triggered by an external stimulus and has a fixed delay before the stimulus has come. Varying the delay on the stimulator makes it possible to change the point of signal start and register the shifted signal by LSCM. Then, the original signal with a high temporal resolution is restored, using inverse convolution according to the algorithm described previously. One of the limitations of the current method is that the original signals must have little variability in parameters and have good reproducibility. For applying the method, one needs to perform several scans to get enough data for convolution. In the considered case, 26 trials with 20 frames each, i.e., 520 frames in total were recorded. The duration of imaging and the number of trials depends on the required time resolution and signal duration. So, focus position stability of the preparation during imaging is required. The accuracy of the signal recovery by the proposed method is mostly determined by the size of the ROI. The smaller the ROI size, the less time it takes to scan and the fewer errors occur during signal recovery with the required temporal resolution[37].

This study presented a method for loading fluorescent dyes into peripheral synapses of mammals and a method for recording fast fluorescent calcium signals *via* a confocal fluorescence microscope. Using the described method, it was possible to register a signal with good spatial and temporal resolution. Registration of calcium transients is a powerful tool in studying cellular processes, such as regulation of neurotransmitter release and synaptic plasticity[54,55].

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DISCLOSURES:

The authors have nothing to disclose.

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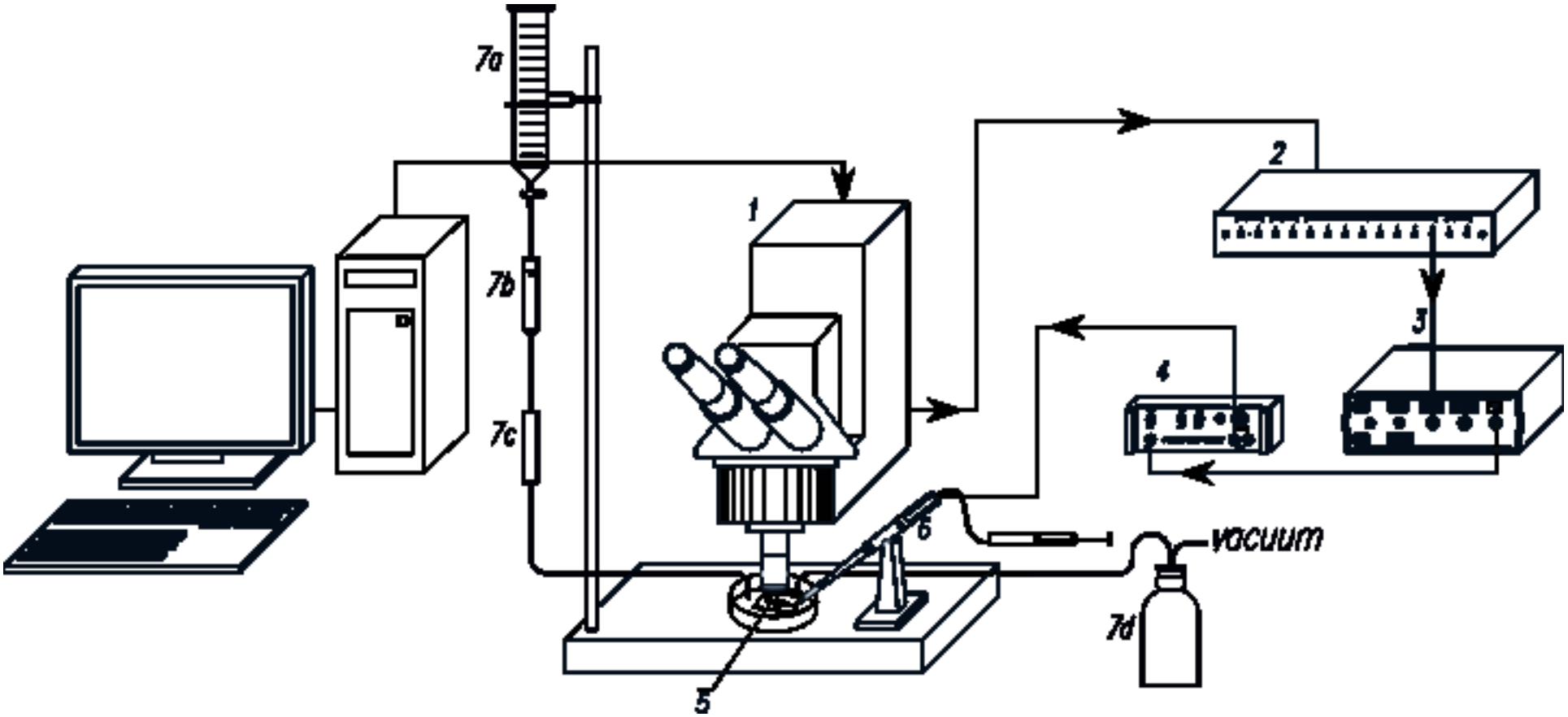
Figure

Click here to access/download;Figure;Fig_1.tif



Figure

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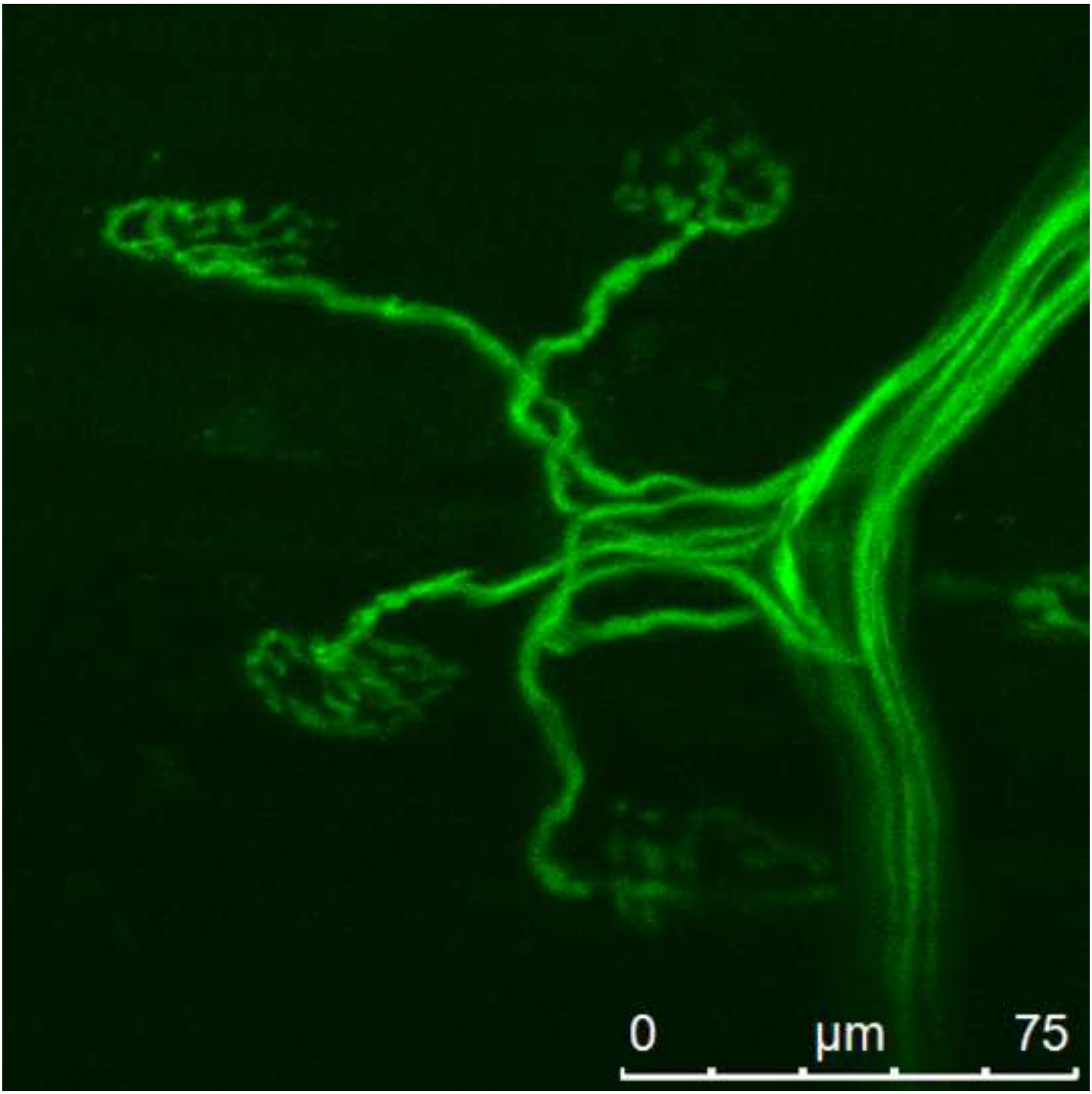

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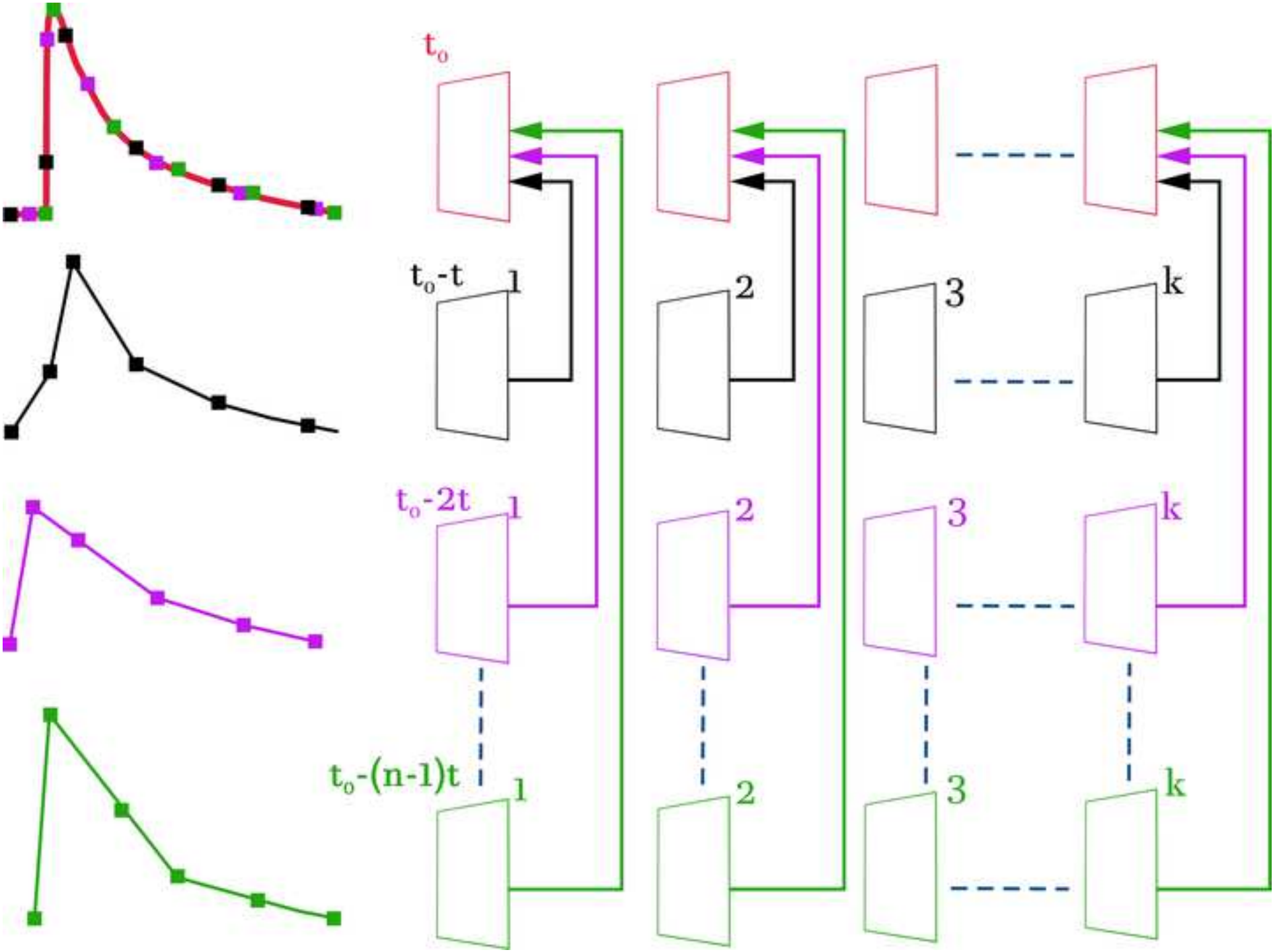

Figure

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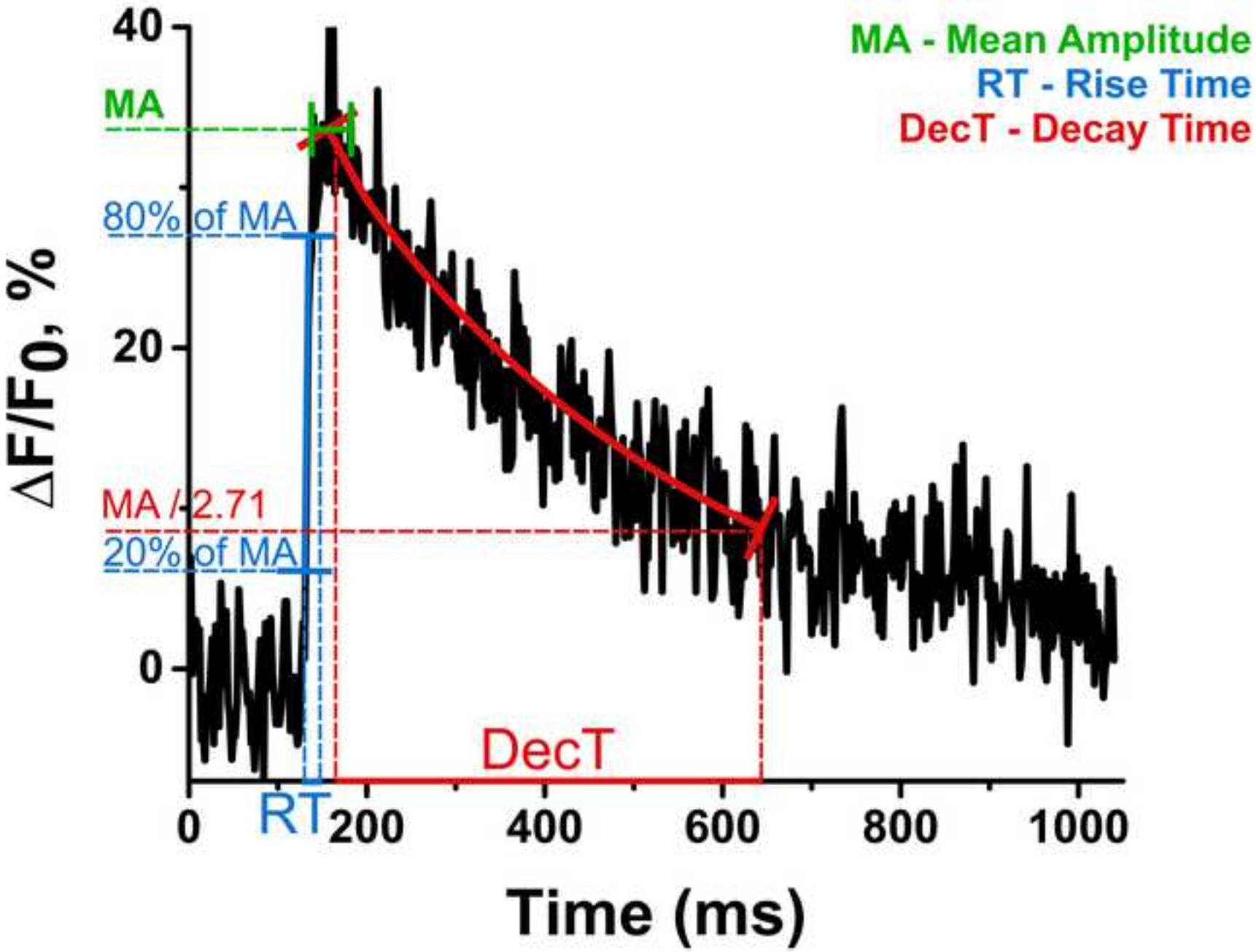

Table

Peak ΔF/F (%)	Rise time 20%-80% (ms)	τ (ms)
27.0±4.6 (n=5)	6.8±0.48 (n=5)	456±53(n=5)

Dear Dr. Deepika Mittal!

Thank you for letter and valuable Referee´s comments. We revised our manuscript according to these comments. We are resubmitting our revised manuscript JoVE63308 "Registration of calcium transients in mouse neuromuscular junction with high temporal resolution using confocal microscopy". In accordance with your recommendations and comments of reviewers, we have rewritten abstract, introduction, and added the text to result section and discussion to the manuscript. We would like to thank the reviewers and you personally for a thoughtful revision of our manuscript and many good suggestions on how to improve its scientific value and readability. I hope that we did an honest effort to reply to all questions and concerns raised by the reviewer to the best of our ability. We also hope that with the revisions accomplished our manuscript now will meet all high standards formulated by your journal for the manuscripts acceptable for publication. During resubmission we loaded two versions of manuscript: one - with highlighted changes in the text (red color – deleted text, green color - added text) and the second file is the copy with accepted corrections. Answers to the issues raised by Editor and Reviewers are also provided. Thank you in advance for your kind consideration.

Since, in order to answer all the questions of the reviewers and make all the nessrsery corection to the text and carry out additional editing of the text, we needed the help of a colleague. We add one more co-author to the co-authors list

On behalf of all co-authors

Sincerely,

Dmitry Samigullin

Editorial comments:

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer

We have corrected language and grammatical errors in revised manuscript.
We have edited the manuscript by a native English speaker.

2. Please provide an Abstract between 150-300 words to more clearly state the goal of the protocol.

Answer

We reworked the abstract according to your comments. All corrections in the original text are highlighted. Hopefully, the purpose of the protocol is now clearer.

3. Please revise the following lines to avoid previously published work: Line 94-101, 122-123, 128-129, 137, 155-156, 164, 177, 208-214, 216-219, 221, 267-290. See attached file.

Answer

We have changed the text in the indicated lines according to your comments.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Leica Microsystems, Heidelberg, Germany, etc.

Answer

We have excluded all commercial language in the text and transferred them to the Table of Materials.

5. The Protocol should contain only action items that direct the reader to do something. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Answer

We tried to follow your recommendations and marked the additional text as Notes. Reviewers asked for more information on some of the steps.

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer

We have changed the text according to your recommendations

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible.

Answer

We have changed ALL text in the protocol section, so it is written now in the imperative Tense

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Readers of all levels of experience and expertise should be able to follow your protocol.

Step 1.2.4: What was the initial concentration of the dye? What solution was used for diluting the dye?

Answer
We have changed the text according to your recommendations:

1.2.2. Dissolve the dye in 14 µL of the HEPES solution. Thus, the concentration of the dye will be 30 mM.

1.2.4. Dilute the solution of Ca^{2+} indicator with HEPES solution down to 1 mM concentration. Keep at freezer(-20^0C) and avoid exposure of light.

Step 2.1.1.: How was the tissue stretched? How was the elastomer-coating done? The volume of Ringer’s solution was added.

Answer
We have changed the text according to your recommendations:

2.1.1. Fix the tissue slightly stretched (no more than 30% from initial length) in the elastomer-coated Petri dish with fine stainless still pins and add Ringer’s solution until the muscle is fully covered.

Note: The petri dish was pre-filled with elastomer according to the manufacturer's instructions supplied. (see the Table of Materials).

Step 2.12.: Volume of dye loading solution used. What does 3 cm indicate?

Answer
We have changed the text according to your recommendations:

2.9. Draw some amount of the dye loading solution (~0.3 µl) using a syringe with a long filament. This volume corresponds to approximately 3 cm of liquid filling the filament.

Note: Initially it is necessary to make a filament from a pipette tip with a volume of 10 µl by pulling on the fire using an alcohol lamp or a gas burner.

Step 3.1: Was a special experimental chamber used? If yes, please describe the chamber setup briefly here? How was the sample fixed? Please describe.

Answer
We have changed the text according to your recommendations:

3.1. Mount the preparation into the silicon elastomer-coated experimental chamber and fix it, slightly stretched, with a set of steel micro-needles.

Note: A simple self-made perfusion experimental chamber made of organic glass, the bottom of the chamber is covered with an elastomer (prepared in accordance to the manufacturer's instructions

supplied; see Table of Materials) was used. The chamber has a solution supply tube. The solution is pumped out via a syringe needle, mounted on a magnetic holder (see Table of Materials). As an experimental chamber, a Petri dish could be used, the same as for incubation of the preparation, but with attached the supply and suction tubes.

Step 3.4-3.5: How was the chamber setup? How was the sample placed along with the electrode? Please describe in brief.

Answer

We have changed the text according to your recommendations see previous Answer.

How exactly the sample will be located in the camera and the electrode will be installed will be presented in detail in the video.

Step 3.6.: what was the strength of the electrical stimulation given to observe contractions? How was this determined?

Answer

We have changed the text according to your recommendations and add information in section Stimulation unit :

3.6. Plug in stimulating electrode to an electric stimulator and make sure occurring muscular contractions after stimuli. See section 3.10-3.15 for stimulation conditions and recording.

See lines 264-266

To stimulate the nerve, the supramaximal amplitude of the stimulating impulse is selected (25% –50% greater than the maximum stimulation intensity necessary to activate all the nerve fibers).

.

Step 3.11: How was the stimulation software setup? Please describe in brief.

Answer

We have changed the text according to your recommendations:

3.10 Stimulation unit

In this work the stimulator described in the article by Land et al. [51] was used. This device allows set temporal parameters of stimulation via the MatLab software. Create file, copy and paste the code from mentioned article to MatLab code window, then save. Click Run, a window with stimulation parameters will appear. Set delay time and duration of the stimulus. The delay determines temporal resolution of the reconstituted fluorescent signal. Electric pulse of 0.2 ms duration is delayed, then sent to the isolation unit. The latter forms the amplitude and polarity of the stimulating pulse and electrically isolates the biological object from the recording equipment. To stimulate the nerve, the supramaximal

amplitude of the stimulating impulse is selected (25% –50% greater than the maximum stimulation intensity necessary to activate all the nerve fibers).

Presented method based on special algorithm for recordings of single fast fluorescent signals using LSCM with the minimized sweep. At each step of developed algorithm, recorded fluorescent signal is shifted from the previous one by time interval which is shorter than the microscope sweep. The value of time shifts determines temporal resolution of required signal. Number of steps (shifts) in algorithm is depends on required temporal resolution and origin one. With this method of registration, the stimulation of the preparation is carried out with a frequency of 0.25 Hz.

9. Please include a single line space between all the steps. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Answer

We have changed the text according to your recommendations

10. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures, except for figures showing the experimental set-up. Data from both successful and sub-optimal experiments can be included.

Answer

We have changed the text according to your recommendations:

See line 360-384

REPRESENTATIVE RESULTS

After loading the preparation with dye according to the presented technique, most of the synapses located close to the nerve stump had a sufficient level of fluorescence (see Figure 2).

After loading preparation with the dye and applying the described method of registration and image processing, calcium transients with the desired spatial and temporal resolution were obtained (see Figure 4). The calcium transient has been recovered by the proposed method (see Figure 3).

Amplitude and time parameters of the recovered signals were also analyzed. Average data presented on table 1.

[place figure 4 around here]

[place Table 1 here]

Calcium transient analysis makes it possible to assess the amplitude-dynamic characteristics of changes in the presynaptic calcium level in the nerve ending during the action potential.[11]. The change in the amplitude of the calcium transient correlates well with the change in the quantal content[52]. Calcium transient amplitude analysis is commonly used to study the effect of physiologically active compounds associated with modulation of presynaptic calcium levels on synaptic transmission[53, 54]. The time course of the calcium transient reflects the kinetics of calcium binding with the dye and its dissociation[23, 55]. It is obvious when using dyes with different affinity for calcium[23, 55]. Although the temporal parameters of the calcium transient reflect the kinetics of the calcium sensitive dye, and do not represent the kinetics of free calcium in the nerve terminal, it could be suggested and the concentration of calcium buffers calculated via mathematical modeling based on experimental data[23].

11. Please include a short description of the data presented in the Figure and relevant symbols of each figure and/or table. Please include what the x-axis and y-axis represent.

Answer

We have add information in description figure legend:

See line 386-418:

FIGURE AND TABLE LEGENDS:

Figure 1: The schematic of the experimental setup. 1: Laser Scanning Confocal Microscope (LSCM). 2: Synchronization module of LSCM (trigger box). 3: Stimulator. 4: Isolation unit. 5: The biological sample. 6: Suction electrode for electrical stimulation of nerve. 7: Perfusion systems a-perfusate reservoir, b-dropper, c-flow regulator, d-vacuum flask. Arrows point to the direction of propagation of synchronizing pulse.

Figure 2: Mouse nerve and terminals loaded with the Ca2+ indicator.

Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained.

Figure 4: Representative trace of calcium signal from one experiment. Some important parameters of signal, such as mean amplitude (MA), rise time (RT) and decay time (DecT), and its projections on axes are indicated. MA – calculated by averaging points at peak, colored in green. RT – the time while amplitude rise up from 20 to 80% and calculated as difference between projections on x axis, colored in blue. DecT– the time when amplitude decrease at e times and calculated as difference between projections on x axis colored in red

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Zhilyakov et al. describes a method for imaging fast calcium transients in the presynaptic nerve terminal of the mouse neuromuscular junction. While similar approaches have been used by some in other preparations, and calcium imaging has been performed previously at the mouse neuromuscular junction, this report is still valuable as it will provide a detailed procedure and video to aid investigators who are interested in using this approach at motor nerve terminals. There are a few issues that the authors should address.

Major Concerns:

None

Minor Concerns:

1. On line 48-49 when the authors are comparing cell permeant dyes with cell impermeant dyes, they state that when using cell permeant dyes it is difficult to control the concentration of the dye inside the cell. In fact, this is also an issue when using cell impermeant dyes in the manner described in this manuscript. Loading dyes through the cut end of the nerve does not allow the experimenter to know the concentration of dye within the nerve terminal. This should be made clear.

Answer

Thank you for suggestion, you're right. We discussed this in the introduction.

.

See line 63 -68:

"There is also a method of loading through a nerve stump. The latter method is most suitable for neuromuscular junction preparations[15–20]. It allows performing staining only cells of interest. Although this method does not provide an accurate evaluation of the concentration of the dye in the target cell, it can be estimated approximately by comparing the level of fluorescence of the cells at rest in solutions with a known concentration of calcium[21]. In this study, we present a modification of this method applied to synapses of mammals."

2. On line 57, the authors comment that calcium entry during action potentials in the mammalian neuromuscular junction is a very fast process. The authors could cite a recent report that demonstrates that presynaptic action potentials have an exceptionally fast time

course at the mammalian neuromuscular junction (Ojala et al., J Biol Chem. 2021 Jan-Jun; 296: 100302).

Answer

Thank you very much for the information, we have added this to the text and a cite this work.

See line 69-75

"Calcium entry during the depolarizing phase of the action potential is a fast process, especially in the neuromuscular junction; therefore, for its registration, appropriate equipment is required[1]. Recent study using a voltage-sensitive fluorescent dye, demonstrated that the duration of the action potential in the peripheral synapse of a mouse is 300 µs approximately[22]. Calcium transient, evaluated using calcium-sensitive dyes in the peripheral synapses of the frog, has a longer duration: the rise time is about 2-6 ms and the decay time is about 30-90 ms, depending on the calcium dye used[23, 24]"

3. On line 104-105 and 333, the authors cite prior publications that employ calcium dye loading through the cut end of the nerve. They should consider adding two others: Luo et al., J Neurosci. 31:11268-81; Luo et al., J Neurophysiol. 2015 113:2480-9.

Answer

Thank you very much for the information, we have added cites in text:

See line 130-131

The dye loading procedure is performed according to the loading protocol through the nerve stump, adapted from the protocols previously published[19, 42–46].

4. Figure 3 is not described completely enough in the figure legend to easily understand the figure. A more detailed legend should be written.C

Answer

Thanks for the suggestion, we have added details to the description of the figure.

See line 396-411:

"Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends

on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained."

5. The manuscript implies that the described method will allow the investigator to accurately measure the fast time course of the presynaptic calcium transient. However, the data shown in Figure 4 and the measured parameters of that signal in Table 1 reflect the kinetics of the calcium sensitive dye, and do not represent the kinetics of free calcium in the nerve terminal. The choice of dye is critical here and the authors should acknowledge this fact. This is nicely demonstrated by Sabatini and Regehr, Biophys J. 1998, 74:1549-63.

Answer

Thanks for the comment; we added a discussion of this to the results.

See line 374-384:

"Calcium transient analysis makes it possible to assess the amplitude-dynamic characteristics of changes in the presynaptic calcium level in the nerve ending during the action potential.[11]. The change in the amplitude of the calcium transient correlates well with the change in the quantal content[52]. Calcium transient amplitude analysis is commonly used to study the effect of physiologically active compounds associated with modulation of presynaptic calcium levels on synaptic transmission[53, 54]. The time course of the calcium transient reflects the kinetics of calcium binding with the dye and its dissociation[23, 55]. It is obvious when using dyes with different affinity for calcium[23, 55]. Although the temporal parameters of the calcium transient reflect the kinetics of the calcium sensitive dye, and do not represent the kinetics of free calcium in the nerve terminal, it could be suggested and the concentration of calcium buffers calculated via mathematical modeling based on experimental data[23]."

"

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors describe a protocol for live-imaging of synaptic calcium transients at the neuromuscular junction using the levator auris longus muscle from BALB/C mice. This method and approach will be valuable in the field for enabling real-time capture of these calcium transients involved in neuronal signaling events, in the mammalian setting.

Overall the protocol seems to be straightforward, however more detail is needed in several places for execution using solely this article. Other previously published works are referenced, but the manipulations included from those papers are not described at all here, and the authors move forward onto later steps that would be problematic for a naïve user. As an individual familiar with similar protocols and usage of the software programs included

herein, it also seems that several steps should be grouped together as step/sub-step processes rather than individual new steps, as indicated in the comments section.

It is the impression of this reviewer therefore, that with sufficient editing and attention to fine detail, that this manuscript will be suitable for publication with revision and will provide a novel and extremely useful tool for other scientists looking to perform similar studies.

Thank you for evaluating our work

Major Concerns:
Suggest a proofreading by a native English speaker. There are frequent inaccuracies in word agreements, usage of articles, and general phrasing.

Answer

We have edited the manuscript by a native English speaker.

The authors do not present evidence of efficacy of their stimulation and recording, or a troubleshooting section. The sole figure which speaks to a positive response (Figure 4) is from a single synaptic region, and does not show the value of total magnitude of signal, or the length of the full time period that is represented. The authors fail to mention what a reasonable deltaF/F value is for a successful stimulation. Importantly, they also do not discuss the sensitivity of the assay- can this differentiate synapses that are less effective at firing, or is it an all-or-nothing response? They also do not discuss how technical and biological replicates can and should be combined, and therefore it is unclear how larger studies exploring synaptic maturation, degeneration, or therapeutic intervention could be designed or interpreted.

Answer
Thanks for the comment

The main purpose of this article is to provide a protocol for loading a calcium dye and registering a calcium transient using a confocal microscope. Figure 2 shows the result of loading peripheral terminals using this technique. Usually all loaded terminals respond in response to stimulation with a calcium transient. This can be assessed with the eyes when previewing the specimen through the eyepieces. Figure 3 shows the result of registration and restoration of a calcium transient from one synaptic terminal. Table 1 provides statistics for several experiments. We have added material to the results section, which will expand the understanding of how to interpret the results obtained and further use the presented methodology. The average amplitude of the registered signals is 26 + _ (table), which makes it possible to evaluate the main characteristics of the transient.

See line 360-384:

“After loading the preparation with dye according to the presented technique, most of the synapses located close to the nerve stump had a sufficient level of fluorescence (see Figure 2).

After loading preparation with the dye and applying the described method of registration and image processing, calcium transients with the desired spatial and temporal resolution were obtained (see Figure 4). The calcium transient has been recovered by the proposed method (see Figure 3).

Amplitude and time parameters of the recovered signals were also analyzed. Average data presented on table 1.

[place figure 4 around here]

[place Table 1 here]

Calcium transient analysis makes it possible to assess the amplitude-dynamic characteristics of changes in the presynaptic calcium level in the nerve ending during the action potential.[11]. The change in the amplitude of the calcium transient correlates well with the change in the quantal content[52]. Calcium transient amplitude analysis is commonly used to study the effect of physiologically active compounds associated with modulation of presynaptic calcium levels on synaptic transmission[53, 54]. The time course of the calcium transient reflects the kinetics of calcium binding with the dye and its dissociation[23, 55]. It is obvious when using dyes with different affinity for calcium[23, 55]. Although the temporal parameters of the calcium transient reflect the kinetics of the calcium sensitive dye, and do not represent the kinetics of free calcium in the nerve terminal, it could be suggested and the concentration of calcium buffers calculated via mathematical modeling based on experimental data[23]."

The authors state in their discussion (line 327-329) "most of the synapses located close to the nerve stump had a sufficient level of fluorescence to enable registration of the entry of calcium into the nerve endings in response to low-frequency stimulation of the motor nerve". There is no data within this manuscript or referenced which speaks to this claim. A percentage of labelled synapses or a readout of effective stimulation tests per region tested would be necessary.

A similar loading method is used for various preparations and is described in the literature, cite to which we give. For example, Suzuki, S. et al. 2000 described:

«When loading of the Ca2+ indicator was successful, all the terminals near the main nerve bundle innervating the muscle fluoresced strongly enough for [Ca2+]i to be measured, although the terminals far from the main bundle fluoresced only weakly, and were not used to measure [Ca2+]i.»

The number of stained terminals may vary depending on the loading conditions (length of the nerve stump, the amount of dye trapped in the nerve, the number of synapses close to the main trunk, etc.) We noted this in the discussion.

See line 434-437:

"Depending on the length of the nerve segment remained after the cutting, the speed of dye loading and the number of loaded terminals may vary. It was possible to even further reduce the incubation time in dye solution for shorter nerve stumps. A similar method is described for the loading of fly synapses[18, 19]"

It is difficult to estimate the percentage of loaded synapses. Because only loaded synapses can be visualized. Unloaded ones need to be dyed separately and counted for each prep. This is the subject of a separate study.

Minor Concerns:

Usage of the abbreviation LSCM on line 69 is presented without first informing the audience of what this stands for.

Answer

Thank you. We have added a transcript of the abbreviation to the text

See line 94:

"This article presents a method for increasing the temporal resolution of signals recorded with the Laser Scanning Confocal Microscopy (LSCM) while maintaining the spatial resolution[37]."

Preparing the dye loading solution: The value of 14uL in step 1.2.2 seems like a very small volume to dissolve 500ug of dye in, and would lead to an incredibly high concentration of dye if it all can dissolve in this amount. Please double check this value.
Similarly in step 1.2.4, dyes are typically used in the uM range rather than the mM range so this value seems high. How much total of this dye liquid is typically made at a time, the authors later state 3cm in a syringe per muscle preparation.

Answer

There was no dilution error. We have added a concentration value of 30mM. This is basic concentration. We used this concentration when loading the nerve endings of the frog (Samigullin, DV, et. All Loading a Calcium Dye into Frog Nerve Endings Through the Nerve Stump: Calcium Transient Registration in the Frog Neuromuscular Junction. Journal of Visualized Experiments. 2017 (125) , e55122, doi: 10.3791 / 55122 (2017).). A similar concentration is used by other researchers when loading the dye in a similar way. For example:

"Briefly, the nerve was immersed in a drop of 30 mM dye dissolved in distilled water for 6-8 h at room temperature." (Luo, F., Dittrich, M., Cho, S., Stiles, JR, Meriney, SD Transmitter release is evoked with low probability predominately by calcium flux through single channel openings at the frog neuromuscular junction.Journal of Neurophysiology. 113 (7), 2480–2489, doi: 10.1152 / jn.00879.2014 (2015).)

Then we diluted the dye to 1 mM and used this concentration as a working concentration when applying the dye to the stump.

Yes, dyes are usually used in μM concentrations. But in our case, after the dye loading solution enters the loading pipette and the nerve, it is diluted with a ringer, etc. and already

in the peripheral nerves, the concentration will be about 40-150 µM (Suzuki, S. et al. Ca2 + dynamics at the frog motor nerve terminal. Pflugers Archiv: European journal of physiology. 440 (3), 351–65, doi: 10.1007 / s004240000278 (2000).). We have added this information to the introduction.

See line :65-67:

“Although this method does not provide an accurate evaluation of the concentration of the dye in the target cell, it can be estimated approximately by comparing the level of fluorescence of the cells at rest in solutions with a known concentration of calcium[21].”

Step 2.1: References a previous protocol for the dissection of the LAL muscle. Following this dissection, when the tissue is fixed in step 2.1.1, does it need to be entirely submerged in Ringers solution? Similarly, in step 2.21 how much Ringers solution should be added (or should the sample be submerged) to prevent it from drying out over the 2hr incubation period? Should the entire setup be covered to prevent evaporation?

Answer

Thanks for the comment. We made a text correction and added information

See line 136-140:

“2.1.1. Fix the tissue slightly stretched (no more than 30% from initial length) in the elastomer-coated Petri dish with fine stainless still pins and add Ringer’s solution until the muscle is fully covered.

Note: The petri dish was pre-filled with elastomer according to the manufacturer's instructions supplied. (see the Table of Materials).”

See line 181-184:

“2.1.1. Fix the tissue slightly stretched (no more than 30% from initial length) in the elastomer-coated Petri dish with fine stainless still pins and add Ringer’s solution until the muscle is fully covered.

Note: The petri dish was pre-filled with elastomer according to the manufacturer's instructions supplied. (see the Table of Materials).”

Steps 2.2-2.5 seem like they should be a single step with sub-steps, for the preparation of the pulled "filling pipette".

Answer

Thanks for the comment. We made a text correction and added information

See line 142-151.

It is recommended that the authors include a representative figure or definitely film steps 2.6- 2.13. There is no representative figure for this portion of the protocol at this time, and without visual aids it would be quite challenging for a novice experimenter to complete these steps correctly.

Answer

Thanks for the comment, of course, these stages will be given special attention when preparing the video.

The phrasing "to stimulate the nerve" is confusing in step 3.3, as this is not the step when stimulation is applied. According to the protocol the stimulation unit is not set until step 3.11 and not applied until step 3.14. Similarly, the phrase "observe muscular contractions under the microscope" in step 3.6 should be followed with the phrase "See section 3.11-3.16 for stimulation conditions and recording", as no stimulation is yet applied at this step.

Answer

Thanks for the comment. We made a text correction and added information

See line 205 :

“3.3. Install suction electrode which will be used to stimulate the nerve.”

See line 216-217:

“ 3.6. Plug in stimulating electrode to an electric stimulator and make sure occurring muscular contractions after stimuli. See section 3.10-3.15 for stimulation conditions and recording.”

Sections 3.9- 3.10 seem like they should be a single step with sub-steps, for the "setup of imaging parameters".

Answer

Thank you for your comment. We made corrections and took into account your comments.

See line 229-253

Step 3.7: There is no mention as to why b-turbocurarine is included in the Ringers solution. Also, there is no representation of the perfusion system included in the Figure 1 description of the overall experimental setup.

”

Answer

Thank you for your comment. We add perfusion system in the Figure 1 . We made corrections and took into account your comments.

See line 219-224

“3.7 Fill up the perfusion system with the Ringer’s solution with d-tubocurarine (10 μM).

Note: This solution helps to prevent muscular contractions. D-tubocurarine or alpha-bungarotoxin - specific blockers of nicotinic acetylcholine receptors on postsynaptic membrane, would completely or partially block muscle contractions[49]. Also for preventing muscular contractions specific blockers of postsynaptic sodium channels - μ-conotoxin GIIIB could be used[50].”

3.10: There are no details on how to initiate or use live mode given in this step. The associated Figure (Figure 2) is very pixelated and does not have good resolution. As this protocol is promoting a high-quality resolution using these parameters, a better representative image should be included with this step.

Answer

We've added information about enabling live mode.

See line 251-252

“3.9.2. Live mode helps to get a preview of nerve terminals loaded with the dye.

Press the "Live mode" button to switch to Live mode”

Figure 2 is presented for a representative purpose in order to show what the peripheral nerve endings look like loaded with calcium dye according to the proposed method. It is made with a resolution of 512 by 512 pixels, which is sufficient for most applications. This photograph was taken with a confocal microscope. Using confocal filtration, and confocal scanning of the specimen. This ensures the fixation of the fluorescent signal from a small volume of the preparation during scanning at each separate moment of scanning time. And this provides a higher resolution compared to shooting with fluorescent cameras and lighting the entire sample.

As for the protocol, the concept of high-quality resolution is primarily related to the temporal parameters (high temporal resolution) in the application of confocal microscopy and the comparison of these parameters with high-speed cameras. On high-speed cameras that provide high-speed shooting, the resolution is 80 * 80 pixels, which is much lower than what confocal microscopy can offer.

Sections 4.1- 4.11 should be an independent "video processing" section than steps 4.12-4.24 which is "video analysis". This is particularly evident since there is a paragraph titled

"next step", lines 257-259 which state that the steps following are a separate process.

Answer

Thank you for your comment. we have corrected the text according to your recommendations

Step 4.7 The number of frames (20) indicated in this step, does not match with the number of 2ms shifts indicated in step 3.16 (26).

Answer

The number of frames during video recording depends on the duration of the signal (calcium transient). In fact, this is the number of frames k multiplied by the time required to form one frame. Since one frame (frame) is formed in 52 ms, it is necessary to choose k = 20 so that the duration of the video recording is 52 ms * 20 = 1040 ms, or about one second. The number of steps, in turn, depends on what kind of temporal resolution we want to get. Since we need a temporal resolution of 2 ms to adequately estimate the rise time, we need to take the number of repeated records n = 26 or n-1 = 25 steps, if we do not take into account the first record. the number of recording iterations is calculated by the formula n = T / t = 52ms / 2ms = 26, where T is the time required for the confocal microscope to form one frame, t is the required time resolution.

We have added this information to the description for Figure 3.

See line 396-411:

“Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained.”

Line259: The directive: "Click Image >adjust> brightness/contrast" needs more detail and explanation. What are these values adjusted to, and is it applied uniformly to all images?

Answer

Thank you for your comment. we have corrected the text according to your recommendations

See line 355-366

"5.5. To improve image visualization click Image > adjust > brightness/contrast > auto. This step will not affect data".

Step 4.20 should denote at the end that this step has now performed actions of background subtraction and recording of adjusted raw fluorescence values for the selected ROI.

Answer

Thank you for your comment. we have corrected the text according to your recommendations

See line 355-357

5.12. Divide signal values by average offset value. After this step, the signal does not contain the contribution of background and raw fluorescence to the amplitude values for the selected ROI.

Step 4.21 should indicate that this step is the establishment of the F0 value that will be used in subsequent calculations.

Answer

Thank you for your comment. we have corrected the text according to your recommendations

See line 351-353

"5.11. Average offset of signals

Note: Use the corresponding points depending on delay time before stimulation. This step is the establishment of the F0 value that will be used in subsequent calculations. "

Figure 4: Details of how this experiment were performed are required in the Figure legend. The image itself needs additional information, such as the overall magnitude of the deltaF/F reached, and the overall duration of the recording.

Answer

We have added information to the Figure and in the Figure legend

See Figures, 1,3,4

See line 389-419:

“**Figure 1: The schematic of the experimental setup.** 1: Laser Scanning Confocal Microscope (LSCM). 2: Synchronization module of LSCM (trigger box). 3: Stimulator. 4: Isolation unit. 5: The biological sample. 6: Suction electrode for electrical stimulation of nerve. 7: Perfusion systems a-perfusate reservoir, b-dropper, c-flow regulator, d-vacuum flask. Arrows point to the direction of propagation of synchronizing pulse.

Figure 2: Mouse nerve and terminals loaded with the Ca^{2+} indicator.

Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained.

Figure 4: Representative trace of calcium signal from one experiment. Some important parameters of signal, such as mean amplitude (MA), rise time (RT) and decay time (DecT), and its projections on axes are indicated. MA – calculated by averaging points at peak, colored in green. RT – the time while amplitude rise up from 20 to 80% and calculated as difference between projections on x axis, colored in blue. DecT– the time when amplitude decrease at e times and calculated as difference between projections on x axis colored in red

”

4.24 More details are needed in this bullet point and in Table 1. Is the value presented the peak deltaF/F value? The authors state in the representative results that they "analyzed amplitude and calculated time parameters of recovered signals", however there is no description in the manuscript text or in the figure legend as to how this was done.
It is rather unclear as to how the authors arrive at their final "peak" fluorescence value. The

authors just have a listing of end output results for Table 1, rather than a visual illustration of how the calculations were performed.

Answer

We have added information to the Figure4 and in the Figure4 legend

See Figure 4 and line 414-419:

“Figure 4: Representative trace of calcium signal from one experiment. Some important parameters of signal, such as mean amplitude (MA), rise time (RT) and decay time (DecT), and its projections on axes are indicated. MA – calculated by averaging points at peak, colored in green. RT – the time while amplitude rise up from 20 to 80% and calculated as difference between projections on x axis, colored in blue. DecT– the time when amplitude decrease at e times and calculated as difference between projections on x axis colored in red”

In line 334-335 of the discussion, the authors say that "it was possible to even further reduce the incubation time for shorter nerve stumps". Could the authors please include a specific example or further details on this?

Answer

Thank you for your comment. we have corrected the text according to your recommendations

See line

In lines 344-346 the authors stress the importance of the suction electrode. It would be advantageous to point this out as a note in the protocol section as a particular point to pay close attention to.

Answer

Thank you for your comment. we have corrected the text according to your recommendations

See line 435-438

“Depending on the length of the nerve segment remained after the cutting, the speed of dye loading and the number of loaded terminals may vary. It was possible to even further reduce the incubation time in dye solution for shorter nerve stumps.”

Reviewer #3:

Manuscript Summary:

In this manuscript, N. Zhilyakov et al. report on two different protocols for characterizing calcium transients in mouse neuromuscular junctions. The first one is about the labeling of the neurons to be characterized. Specifically, they describe how to perform the loading of a calcium-sensitive dye through the nerve stump into the nerve endings of a mouse. The second protocol is about the imaging of the calcium transients using confocal microscopy.

Major Concerns:
In my opinion, the information in the manuscript is insufficient to reproduce the experiments described. Further information is needed, as described below.

1) A list of all the materials needed should be specified before describing the protocol.

Answer

According to the rules of the journal, the list with materials is presented in a special table Table of Materials. It is downloaded separately.

2) The steps for the dye-loading procedure are not clear. For instance:
- Line 92: The dye is not specified.

Answer

According to the rules of the magazine, the names of firms and commercial names of materials cannot be used in the text.

«JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials».

The specify of the dye is presented in Table of Materials

Oregon Green 488 BAPTA-1 pentapotassium salt, Molecular Probes, USA, O6806500 500 µg

- Line 116: "Break off the tip of the electrode after score of the taper with an abrasive." Which electrode are the authors referring to?

Answer

This refers to a micropipette, the manufacture of which was described in paragraph 2.2.

We changed the text to make it clearer

See line 148-149

“2.2.2 Break off the tip of the micropipette after scoreing of the taper with an abrasive. Leave the tip open to about 100 microns in diameter. “

- Line 137: "Remove the suction tubing from the blunt end of the Filling Pipette." Which suction tubing?

Answer

Meaning the tube that was put on for suction of the nerve into the micropipette
See step 2.3 (line 154-155). "Put on silicone tube on the back of the Filling Pipette from the one side, and on syringe without needle from the other".

All these steps will be presented in detail in the video.

- Line 139: "Draw some amount of the dye loading solution (~3 cm) using a syringe with a long filament." What type of filament?

- A schematic representation of the different steps is needed.

Answer
We've added some text for a more complete explanation, and the video will show you how to make filament.

See line 171-174:

"2.9. Draw some amount of the dye loading solution (~0.3 µl) using a syringe with a long filament. This volume corresponds to approximately 3 cm of liquid filling the filament.

Note: Initially it is necessary to make a filament from a pipette tip with a volume of 10 µl by pulling on the fire using an alcohol lamp or a gas burner".

3) More information is needed for the video capture with the confocal microscopy section.
- The confocal microscope used is equipped with a system for electrophysiology. No details of such a system are provided.

Answer

Registration of the video-images was accomplished in the regime "Electrophysiology" set-up on the LSCM. In this regime, at the time point when image is captured, a synchronizing pulse is sent to the trigger box. This in turn triggers stimulation pulse, eliciting AP generation in the preparation.
We have added clarification to the text

See line 233-236:

"In Leica Microsystems LAS AF choose **Electrophysiology**;

Note: In this mode, at the time point when image is captured, a synchronizing pulse is sent to stimulator with help the trigger box. This eliciting action potential generation in the preparation (see Figure 1 and Stimulation unite)."

- Line 166: "Use suction electrode to stimulate the nerve." Which suction electrode?

Further in the text there is an explanation and a reference to the article describing the construction of the suction electrode

See line 205-208

“3.3. Install suction electrode which will be used to stimulate the nerve.

Note: Construction of the electrode is similar to what was published in the paper by Kazakov et. al., 2015. Place and fix by wax the electrode beside the bath. Move the tip close to the nerve stump and aspirate it into the electrode. “

- Line 182: "Switch on the pump and start perfusion of the preparation with the Ringer's solution with d-tubocurarine." Which concentration of d-tubocurarine is needed?

Answer

The previous sentence indicates the concentration of d-tubocurarine

See line 219:

“3.7 Fill up the perfusion system with the Ringer’s solution with d-tubocurarine (10 μM).

- Line 208: "In our work, we used the programmable stimulator described in in the paper by Land et al.". Details of the stimulator should be provided in the current protocol.

Answer

We've added a description of the stimulation unite

See line 255-272

“3.10 Stimulation unit

In this work the stimulator described in the article by Land et al. [51] was used. This device allows set temporal parameters of stimulation via the MatLab software. Create file, copy and paste the code from mentioned article to MatLab code window, then save. Click Run, a window with stimulation parameters will appear. Set delay time and duration of the stimulus. The delay determines temporal resolution of the reconstituted fluorescent signal. Electric pulse of 0.2 ms duration is delayed, then sent to the isolation unit. The latter forms the amplitude and polarity of the stimulating pulse and electrically isolates the biological object from the recording equipment. To stimulate the nerve, the supramaximal amplitude of the stimulating impulse is selected (25% –50% greater than the maximum stimulation intensity necessary to activate all the nerve fibers).

Presented method based on special algorithm for recordings of single fast fluorescent signals using LSCM with the minimized sweep. At each step of developed algorithm, recorded fluorescent signal is

shifted from the previous one by time interval which is shorter than the microscope sweep. The value of time shifts determines temporal resolution of required signal. Number of steps (shifts) in algorithm is depends on required temporal resolution and origin one. With this method of registration, the stimulation of the preparation is carried out with a frequency of 0.25 Hz."

4) As it is written, the protocol for data analysis is difficult to follow.
- Line 250: Why is 20 frames mentioned, when the actual number of acquisitions is 26? The description of the steps for processing images with ImageJ does not yield the results described in Figure 3.

Answer

We have add description to the to Fig. 3 and we hope that the description of the protocol has become clearer

See line 397-412

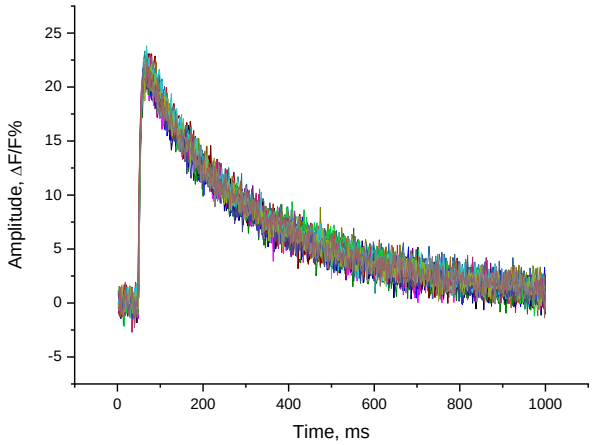
"**Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame).** The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames (52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained."

5) In the Discussion section, the authors should explain how to implement the inverse convolution of images.
The authors also state that "One of the limitations of current method is that the original signals must have little variability in parameters and have a good reproducibility". The reproducibility of the signal should be properly characterized by recording calcium transients obtained after identical stimulation conditions.

Answer

Application of our method assumes that underlying events triggered by external stimulation are reputable and reproducible in magnitude and kinetics, so that one can use time increments in subsequent stimulation cycles to reconstitute high-resolution signal. So, this technique cannot be used to describe differences between individual events sampled. The events sampled must be uniform in magnitude and kinetics. Our group has devoted many

years to the study of calcium transient. And our early observations and data from other authors suggest that calcium transient meet this condition. The figure below shows, for example, 18 realizations of signals recorded by a speed camera.



6) The authors state that Line 59: "However, their main disadvantage is low resolution, limited by the size of the sensitive elements of the matrix". The problem of using cameras for characterizing calcium transients is not due to their reduced number of pixels or low spatial resolution. It is their low temporal resolution. This is explained in: M. Duocastella et al., Acousto-optic systems for advanced microscopy, Journal of Physics: Photonics, 3, 012004 (2021).

Answer

Cameras capable of registering fast and low intensity processes exist we have added information and a link to the introduction. But the problem is that calcium transients in response to rare stimulation are fast and low in intensity. So cameras capable of registering such low-intensity processes should be not only high-speed, but also have sufficient sensitivity. So those cameras that fit have a low-resolution monochrome sensor.

For example, the camera, which is at the disposal of the team of authors http://www.redshirtimaging.com/redshirt_neuro/hardware_neuroccdsm.htm,

allows to register calcium transients with a time resolution of 2 ms and has a matrix of 80 to 80 pixels.

7) Similarly, the problem of confocal microscopy is not the low scanning speed, as the authors mention in Line 63. Note that the authors use a microscope capable of scanning at 1400 Hz! The problem is the sequential nature of confocal microscopes and the need for point-by-point scanning. More details on this topic can be found in: J. Mertz, Optical sectioning microscopy with planar or structured illumination, Nature Methods, 8, 811-819 (2011)

Answer

Thank you very much for your comment and helpful information. We have corrected this phrase and added a cite

See line 82-84

“But it should be kept in mind the fact that confocal microscopy has a high scanning speed in line scan mode, but there are still significant limitations on the speed of recordings of fast processes when building a spatial image[33]”

For information

The frequency 1400 Hz characterizes scanning along one X axis. The transition to the next scan line in the XY scanning mode in the microscope takes a long time and apparently depends on the speed of the mirrors. We carried out the corresponding measurements during the preparation of the publication (A. Y. Arkhipov, E. F. Khaziev, A. I. Skorinkin, E. A. Bukharaeva, and D. V. Samigullin, Microscopy and Microanalysis (2020).) And they are included in the supplementary materials:

«For the window of 512 by 64 pixels and the frequency of scanning equal to 1400 Hz, the LSCM required 44 ms to generate a single frame (these parameters coincide with the scanning parameters in the biological experiments described in paper). Within this time span, 22.75 ms are required for scanning process itself and then, the rest 21.5 ms the laser is shut down. The latter corresponds to the time required to return the mirror of the Y axis displacement from the ending to the starting point. At the bottom of Fig. E the signals are presented that describe generation of lines by the laser during scanning. The laser requires 234 us to generate the line during displacement along the X axis. After that the laser is shut down for 122 us. This interval corresponds to the time required for transition to a new line along the Y axis. This means, that generation of a frame requires 22.78 ms ((=122 us + 234us)*64); the rest of the time (21.5 ms) is spent for return of the laser beam to the starting point of scanning. The parameters measured correspond to the parameters of scanning used in our experiments described in this paper. They can vary depending on the setting of scanning parameters of the microscope, such as scanning frequency, the size of scanning area, the regime of scanning, zooming etc.»

Reviewer #4:

Manuscript Summary:

This manuscript presents an approach for loading fluorescent dyes into peripheral synapses of mammals and a method for recording fast fluorescent calcium signals via confocal microscopy. The manuscript is well-written and makes an important contribution. However, some deficiencies are noted (described below). Authors are encouraged to addressed all the comments to improve the presentation.

Major Concerns:

Introduction:

1. This section will benefit from including more specific technical details. For example, "fast process" does not say much. Please specifically mention biological timescales of interest and how current imaging modalities match up to thee timescales. Same comment holds for spatial resolution.

Answer

We have add information in introduction

see Lines 69-92:

"Calcium entry during the depolarizing phase of the action potential is a fast process, especially in the neuromuscular junction; therefore, for its registration, appropriate equipment is required[1]. Recent study using a voltage-sensitive fluorescent dye, demonstrated that the duration of the action potential in the peripheral synapse of a mouse is 300 µs approximately[22]. Calcium transient, evaluated using calcium-sensitive dyes in the peripheral synapses of the frog, has a longer duration: the rise time is about 2-6 ms and the decay time is about 30-90 ms, depending on the calcium dye used[23, 24]. To measure fast processes with the help of fluorescent dyes, CCD or CMOS cameras are generally used, with the fast and sensitive CCD matrices. However, these cameras have a disadvantage which is low resolution, limited by the size of the sensitive elements of the matrix[25–28]. The fastest cameras with sufficient sensitivity to record both action potentials and calcium transients in response to rare stimulation of cells have a scanning frequency of 2000 Hz, and a matrix with a dimension of 80 * 80[29]. To obtain signals with a higher spatial resolution, confocal microscopy is used, especially if it is necessary to assess some volumetric changes in the signal [30–32]. But it should be kept in mind the fact that confocal microscopy has a high scanning speed in line scan mode, but there are still significant limitations on the speed of recordings of fast processes when building a spatial image[33]. There are confocal microscopes based on rotating Nipkow disks (slit-scanning microscopy) and Multipoint-Array Scanners, which have a higher scanning speed. At the same time, they are inferior to the classical confocal microscopes in confocal image filtering (pinholes crosstalk for microscopes with a Nipkow disk)[32, 34, 35]. Confocal imaging with resonance scanning can also provide the high spatial–temporal resolution required for high temporal measurements[36]. However, take into account that the registration of weak fluorescent responses at a high scanning speed when using resonance scanners, requires highly sensitive detectors, such as hybrid detectors.[36]."

2. Please include background on the biological importance of this work and its clinical implications. The rationale for measuring calcium is not fully described.

Answer

The first paragraph of the introduction focuses on the importance of studying calcium signaling in biology. We have added additional information

See Lines 125-130:

"The problem of measuring fast calcium waves in excitable cells is one of the most important and challenging in the field of studying signal transmission in the central and peripheral nervous systems. Calcium ions play an important role in the triggering neurotransmitter release, synaptic plasticity and

modulation of the activity of various intracellular proteins[1–5]. Studying calcium signaling is also important for finding ways to treat neurodegenerative diseases[6]. To measure changes in the calcium levels, fluorescent calcium-sensitive dyes are commonly used, and changes in their fluorescence level are analyzed[7–9]”

3. I agree that 3D volumetric imaging without compromising temporal resolution is challenging. However, this point needs to be elaborated with suitable examples and a comparison of other imaging techniques. Since this is a methods paper, a short table identifying specific spatio-temporal challenges issues/associated with imaging calcium oscillations and the limitations of current imaging approaches for the same would be appropriate.

Answer

The main aim of this research is devoted to a topic: loading a calcium dye and a description of the method for registering calcium transinet using LCSM methods with high temporal resolution. We have added information in the introduction provides on comparing methods and comparing the main disadvantages and advantages. A more detailed comparison of the methods requires a separate study, and many review articles are devoted to this, which we refer to in this work. Including with tables of comparison of methods. For example, Bullen A. et. all (Bullen A, Patel SS, Saggau P. High-speed, random-access fluorescence microscopy: I. High-resolution optical recording with voltage-sensitive dyes and ion indicators. Biophys J. 1997; 73 (1): 477-491 . doi: 10.1016 / S0006-3495 (97) 78086-X)

4. In addition to multipoint array scanner, confocal imaging with resonance scanning can also provide the necessary spatio-temporal resolution required for such measurements. This can be mentioned in the introduction. One recent reference: https://doi.org/10.1002/bit.27465

Answer

Thanks for the information, we've added a text and cite in introduction

See line 88-92:

“Confocal imaging with resonance scanning can also provide the high spatial–temporal resolution required for high temporal measurements[36]. However, take into account that the registration of weak fluorescent responses at a high scanning speed when using resonance scanners, requires highly sensitive detectors, such as hybrid detectors.[36]. “

5. Please clearly but briefly state at the end of the introduction how your approach overcomes the spatio-temporal limitations discussed with current imaging modalities.

Answer

See line 93-98

"This article presents a method for increasing the temporal resolution of signals recorded with the Laser Scanning Confocal Microscopy (LSCM) while maintaining the spatial resolution[37]. The current method is a further development of the methods described early and transferred to the LSCM platform[38–40]. This approach does not require changes in the microscope hardware and is based on the application of an algorithm for recording periodically evoked fluorescent signals with a time shift relative to the moment of stimulation."

Methods:

1. Although appropriate references are given to previous work, this paper needs to stand on its own. So, where appropriate, please provide all the details in the protocols for someone to replicate the data based on this paper alone.

Answer

Since the article actually describes two protocols: dye loading and registration with signal processing, in order not to overload the material, we omitted the part devoted to dissection, based on the fact that this journal has a separate publication with a video on how to dissect this particular preparation - LAL (Wright, M., Kim, A., Son, Y.-J. Subcutaneous Administration of Muscarinic Antagonists and Triple-Immunostaining of the Levator Auris Longus Muscle in Mice. Journal of Visualized Experiments. (55), e3124, doi: 10.3791 / 3124 (2011); Burke, SRA, Reed, EJ, Romer, SH, Voss, AA Levator Auris Longus Preparation for Examination of Mammalian Neuromuscular Transmission Under Voltage Clamp Conditions.Journal of Visualized Experiments. 2018 (135), e57482, doi : 10.3791 / 57482 (2018)). The rest of the necessary steps are presented in the protocol and will be presented in the video.

2. What is the dye being used? It's not mentioned in main text but only in the table of materials.

Answer

According to the rules of the magazine, the names of firms and commercial names of materials cannot be used in the text.

«JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials».

The specify of the dye is presented in Table of Materials

Oregon Green 488 BAPTA-1 pentapotassium salt, Molecular Probes, USA, O6806500 500 µg

3. Please provide a real-time photograph or a detailed technical schematic of the imaging set-up, imaging chamber and perfusion set-up described in steps 3.1 to 3.9 and mark all the parts to match with the description in the text. Replace Figure 1 with this. The current Figure 1 does not add much value to the manuscript.

Answer

We have added information to the scheme. The diagram allows you to accurately separate the main components of the setup. Also the diagram shows the connection and direction of the sync pulse. It doesn't work in the photo. The real components of the installation will be presented in the video.

4. Section 3.11: please provide more details of the approach used to improve temporal resolution. For example, how long is the imaging conducted and how often are images captured?

Answer

Our registration method is described in detail in the article “Arkhipov, A.Y., Khaziev, E.F., Skorinkin, A.I., Bukharaeva, E.A., Samigullin, D. V. Enhancement of the Temporal Resolution of Fluorescent Signals Acquired by the Confocal Microscope. Microscopy and Microanalysis. 26 (2), 204-210, doi: 10.1017 / S1431927620000148 (2020) ". Which we are referring to in the post test. In this publication (according to the subject of the journal), we focus on the signal registration protocol using this technique. The method is illustrated in Figure 3. We have written a more detailed description in the caption to this figure. And we also correct 3.10.

According to the updated description to Fig. 3, it takes about 1 min to restore the signal with the required time resolution of 2 ms. The time is made up of the time required to generate 26 videos with a low temporal resolution (52 ms per frame), each of which has a duration of 1040 ms and consists of 20 frames, and the time required to change the time delay between recordings (it depends on the characteristics of the stimulator). in our case, the frequency of stimulation of the drug depends on the frequency of the formation of the video file and is 0.25 Hz or one stimulus in 4 s

See line 397-412

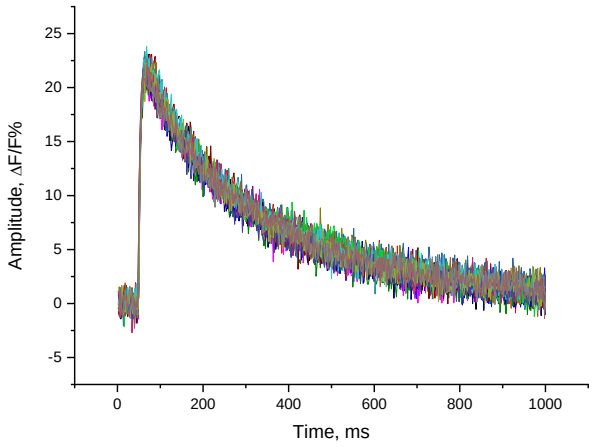
“Figure 3: Scheme for compiling a high resolution video file (2 ms on frame) from original video files with a low temporal resolution (52 ms on frame). The original video files and the corresponding signals are colored in black, magenta and green. The compiled video file and the resulting signal are colored red. The scheme on the right, line by line, shows the video images obtained with a confocal microscope. On the left, the corresponding signals of fluorescence change from the selected ROI. The topmost line is formed frame by frame from the received frames according to the scheme. The result is a video image consisting of the entire array of frames so that there is a delay time is 2 ms between frames instead of 52 ms. Each line corresponds to an offset of the stimulation signal by (n-1)*t, where t - time shift (2 ms), n - number of shift iterations. k- the number of frames in the original video files (2nd-4th lines) depends on the duration of the recorded signal. In this case, in order to register a signal with a duration of 1 second, it is necessary to select k = 20 (52 ms * 20 = 1040 ms), t0 is the required delay before stimulation. To calculate the number of shift iterations n, the initial temporal resolution between frames

(52ms) must be divided by the required one (2ms). In this case, n = 26, which corresponds to 26 registered sweeps. As a result of the performed manipulations, a video image consisting of n * k = 520 frames is obtained.”

Results:
1. Fig.4: Please report multiple calcium transients over a reasonable time period to show evidence of temporally varying calcium oscillations. Please quantify and report inter-spike distance.

Answer

Application of our method assumes that underlying events triggered by external stimulation are reputable and reproducible in magnitude and kinetics, so that one can use time increments in subsequent stimulation cycles to reconstitute high-resolution signal. So, this technique cannot be used to describe differences between individual events sampled. The events sampled must be uniform in magnitude and kinetics. Our group has devoted many years to the study of calcium transient. And our early observations and data from other authors suggest that calcium transient meet this condition. The figure below shows, for example, 18 realizations of signals recorded by a speed camera .



We have added information about the frequency of stimulation to the manuscript.

See line 271-271

“With this method of registration, the stimulation of the preparation is carried out with a frequency of 0.25 Hz.”

Discussion:
1. High spatial and temporal resolution is mentioned a few times. However, the terms "high" and "low" are subjective. Please discuss the biological implications of your results and also

where this spatio-temporal resolution may be considered sufficient and in what (biological/clinical) scenarios the current resolution might not be sufficient.

Answer

Thanks for the comment. We have added this information to the discussion.

See line 461-466

"In this study, to record the calcium transient, the original registration method was used via LSCM, described thoroughly in the article by Arkhipov et al., applied to the synapses of the frog [37]. Using this method, it was possible to estimate the proximal-distal gradient of calcium transient in the elongated frog synapses[37]. This method of registration can be useful for assessing subcellular calcium dynamics in excitable cells, for example, in dendrites and spines in brain slice preparations."

Minor

Concerns:
Methods:
1. Please avoid the word plastic in the protocols, mention the exact name of the plastic (e.g., polystyrene, polypropylene)

Answer

Thanks, we fixed it

2. The table of materials has typographical errors that need to be corrected.

Answer

Thanks, we try fixed all the mistakes