TITLE:

Ex Vivo Imaging of Cell-specific Calcium Signaling at the Tripartite Synapse of the Mouse Diaphragm

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SUMMARY:

Here, we present a protocol to image calcium signaling in populations of individual cell types at the murine neuromuscular junction.

ABSTRACT:

The electrical activity of cells in tissues can be monitored by electrophysiological techniques, but these are usually limited to the analysis of individual cells. Since an increase of intracellular calcium (Ca^{2+}) in the cytosol often occurs because of the electrical activity, or in response to a myriad of other stimuli, this process can be monitored by imaging techniques, but would typically depend on the restricted delivery of the fluorescent calcium indicator dye to individual cells within the tissue. In contrast, genetically encoded calcium indicators (GECIs) can be expressed by an individual cell type and fluoresce in response to an increase of intracellular Ca^{2+}, thus permitting the imaging of Ca^{2+} signaling in entire populations of individual cell types. Here, we apply the use of the GECIs GCaMP3/6 to the mouse neuromuscular junction, a tripartite synapse between motor neurons, skeletal muscle, and terminal/perisynaptic Schwann cells. We demonstrate the utility of this technique in classic *ex vivo* tissue preparations. Using an optical splitter, we perform dual-wavelength imaging of dynamic Ca^{2+} signals and a static label of the neuromuscular junction (NMJ) in an approach that could be easily adapted to monitor two cell-specific GECI or genetically encoded voltage indicators (GEVI) simultaneously. Finally, we discuss the routines used to capture spatial maps of fluorescence intensity. Together, these optical, transgenic, and analytic techniques can be employed to study the biological activity of distinct

cell subpopulations at the NMJ in a wide variety of contexts.

INTRODUCTION:

The NMJ, like all synapses, is composed of three elements: a presynaptic terminal derived from a neuron, a postsynaptic neuron/effector cell, and a perisynaptic glial cell[1,2]. While the basic aspects of synaptic transmission were first demonstrated at this synapse[3], many aspects of this process remain unknown, in part owing to the expression of the same molecules by the distinct cellular elements of this synapse. For example, receptors for both the purine adenine nucleotide ATP and acetylcholine (ACh), which are co-released by motor neurons at the vertebrate NMJ, are expressed by muscle, Schwann cells, and motor neurons, thus complicating the interpretation of any functional effect exerted by these substances (e.g., transmitter release or response, muscle force generation)[4]. Moreover, although the tripartite components of the NMJ are simple compared to, for example, neurons in the central nervous system which often exhibit multiple synaptic inputs, whether motor neurons, muscle cells, or Schwann cells vary in response to stimuli based on their intrinsic heterogeneity (e.g., embryonic derivation, fiber subtype, morphology) is unclear. In order to address each of these issues, it would be advantageous to simultaneously track the response of many cells within one synaptic element, as well as track, at the same time, such a response in either of the other separate elements. Conventional strategies using chemical dyes to measure calcium signaling cannot achieve these two goals, because bath-applied dye is taken up by multiple cell types after application to tissue, and intracellularly loaded dye can only be used to visualize individual or small cohorts of cells. Here, utilizing transgenic mice expressing GECIs designed to measure cell-specific calcium signaling using genetic techniques, together with specific imaging and software tools[5], we demonstrate the first of these two overall goals and discuss how the addition of new transgenic tools would help achieve the second. This technique will be useful for anyone interested in tracking calcium dynamics or other cellular signaling events observable through gene-encoded optical sensors in multiple cell populations at the same time.

PROTOCOL:

Animal husbandry and experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the IACUC at the University of Nevada.

1. Preparation of the diaphragms and phrenic nerves from transgenic mice

1.1. Purchase transgenic mice and oligonucleotide primers to genotype these mice.

NOTE: The primers are listed on the “Information” page for each of these mice.

1.1.1. Breed a 3- to 6-month-old mouse expressing one copy of the appropriate transgenic/knock-in Cre-driver allele and zero copies of the conditional *GCaMP3/6* allele with a second mouse of the same age expressing one or two copies of the conditional *GCaMP3/6* allele and zero copies of the Cre-driver allele.

1.1.2. Genotype the pups and mark the ones that have both Cre and conditional GCaMP3/6 alleles—these will henceforth be called double-transgenic mice (e.g., *Myf5-Cre*, *conditional GCaMP3*[6].

NOTE: This way, all data will derive from mice expressing one copy of both Cre and conditional GCaMP3/6 alleles. This is particularly important when adding in other mutant mice (e.g., knockouts) to these crosses.

1.2. When the double-transgenic mice are of the appropriate age (e.g., postnatal day 0 or 5 [P0 or P5] or adult), euthanize the mice by decapitating them with scissors (for mice younger than P10) or by placing them in an isoflurane inhalation chamber—when they are no longer responsive to pinching the tail with a pair of forceps, they are ready for sacrifice.

1.3. Sacrifice the animal by decapitation with a pair of scissors.

1.4. Transversely section across the entire animal just below the liver and just above the heart and lungs with iridectomy scissors.

1.5. Dissect away the liver, the heart, and the lungs, being careful to maintain a length of the phrenic nerve that is sufficiently long to be drawn into a suction electrode (i.e., 1 - 2 cm).

NOTE: The left phrenic nerve can be identified as a white piece of tissue that enters the medial portion of the left diaphragm. It must not be cut when removing the lungs. The right phrenic nerve runs within a piece of fascia that also contains the superior vena cava and is thinner and whiter than the vena cava. Together, they both penetrate the right medial diaphragm.

1.6. Further remove the ribcage and the vertebral column, except for the thin ridge around the diaphragm.

1.7. Place the diaphragm and the phrenic nerve sample in a microfuge tube with Krebs-Ringer solution with 1 µg/mL 594-αBTX for 10 min in the dark.

NOTE: This concentration of 594-αBTX labels ACh receptors (AChRs) without blocking their function (personal observation).

2. Stimulation and recording of the muscle action potentials

2.1. Using minutien pins, immobilize the diaphragm by pinning it onto a 6-cm dish coated with silicone dielectric gel and filled with ~8 mL of oxygenated Krebs-Ringer solution and place it onto the microscope stage. Perfuse the diaphragm with more Krebs-Ringer solution (8 mL/min) for 30 min.

NOTE: This rinses the unbound 594-αBTX, as well as equilibrates the tissue after dissection.

2.2. Make a suction electrode according to the established methods[7].

2.2.1. At 4x magnification, using a micromanipulator, move the suction electrode over the left phrenic nerve and apply suction by pulling out the barrel of a 5-mL syringe connected to the tubing that is attached to the suction electrode.

NOTE: When successfully drawn into the suction electrode, the phrenic nerve is taut. Turn on the stimulator and stimulate the phrenic nerve by flipping the **manual** switch 1x.

2.2.2. Ensure that the diaphragm contracts in response to the 1-Hz stimulation by visually examining it with brightfield illumination. If not, adjust the voltage by turning the voltage knob incrementally to achieve a supramaximal pulse, which can be verified by a visual examination of muscle contraction. If still not visible, blow out the nerve with the syringe and attempt to draw it in again by applying suction.

2.3. Turn off the perfusion and add the muscle-specific myosin inhibitor BHC[6] or the voltage-gated sodium channel antagonist μ-conotoxin[8] to a final concentration of 100 μM.

2.3.1. To make 100 μM BHC, pipette 4 μL of 200 mM stock in DMSO and predilute it in 1 mL of Krebs-Ringer solution.

2.3.2. Remove 1 mL of Krebs-Ringer solution from the dish.

2.3.3. Add the prediluted BHC, to the dish.

NOTE: This predilution helps prevent the induction by undiluted DMSO of a non-transient fluorescent response in GCaMP3-expressing cells.

2.3.4. Wait 30 min and then, turn on the perfusion of fresh Krebs-Ringer solution for another 20 - 30 min.

2.4. Prepare the recording electrode.

2.4.1. Wearing gloves, place a borosilicate filamented glass with an outer diameter (OD) of 1 mm and an inner diameter (ID) of 0.4 mm into a micropipette puller and tighten the dials to clamp it into position. Close the puller door.

2.4.2. Using a P-97 puller, program the following setting: heat at 900, pull at 120, velocity at 75, time at 250, pressure at 500, and no additional loops.

NOTE: Resistance (R) is measured using software controls of the amplifier: the data acquisition software confirms resistance by solving the formula V = IR. The software controller passes a known current (I) (typically 1 nA) through the electrode and measures the change in voltage

(V), thus enabling us to solve for R.

2.4.3. For embryonic diaphragms, ensure that the resistance is near 60 MΩ, and for older diaphragms, 10 - 20 MΩ. Load the recording electrode with 3 M KCl.

2.5. At 10X magnification, lower the electrode into muscle, using a second micromanipulator on the opposite side of the stage as a stimulating electrode.

2.6. Using electrophysiological data acquisition software, wait until the resting membrane potential changes from 0 to -65 mV or below.

2.7. Stimulate at 1 Hz and verify the presence of a muscle action potential by checking for a large potential that exhibits a modest overshoot (potential that rises above 0 mV when it starts at -65 mV or below). Do not confuse stimulation artifact with an action potential.

NOTE: Potentials are significantly longer in duration (~5 ms) than stimulation artifacts.

3. Imaging of the fluorescence of the sample

3.1. At 20x magnification, locate the endplate band at the center of the muscle by looking for 594-αBTX–labeled NMJs under green/yellow light excitation (550 nm). Switch to the blue light excitation (470 nm) to image Ca^{2+} responses in muscle, motor neuron, or Schwann cells.

3.2. If desired, set up the image splitter with bandpass filters and a dichroic single-edge filter for the dual-wavelength imaging.

3.3. In order to calculate the maximal fluorescence (Fmax) exhibited by GCaMP3/6-expressing tissue, add 12 µL of 3 M potassium chloride (KCl) to the diaphragm preparations[6].

3.3.1. Perform experiments with the brightness bar on the lookup table bar set to 110% of the level at which the GCaMP3/6-expressing tissue exhibits saturation at 20X magnification, without binning in response to KCl.

NOTE: In general, neuromuscular GCaMP3/6-expressing mice produce a robust signal that can be easily detected by the Prime 95B camera, without binning, at a variety of magnifications (10X - 100X). For instances where the signal is fainter (e.g., at 4X), the camera sensor can be binned up to 2x, which will accordingly reduce the image resolution by half.

3.4. Record at 20 frames per second to not miss any fast events.

3.5. Stimulate with 1 - 45 s of 20 - 40 Hz of nerve stimulation by delivering a train of impulses using the suction electrode or add pharmacological agonists by bath application or by perfusion and collect dynamic fluorescent Ca^{2+} responses in one cell subtype together with the static 594-αBTX NMJ signal.

NOTE: If tissue-specific red or far-red GECI or GEVI mice become available for use at the NMJ, they can be used to collect two dynamic signals reflecting two distinct cellular elements at the NMJ.

3.6. When the imaging or electrophysiological experiments are finished because the desired results have been achieved, perfuse water through the perfusion lines and suck water 2x - 3x through the suction electrode to ensure that salts do not build up.

4. Export and analysis of the data by a standard deviation map of fluorescence intensity (SD_{iu16})

4.1. Record image sequences recorded as 16-bit TIFF stacks and load them into the desired imaging data analysis system for analysis.

4.2. In the software's 8d file menu, select **Image stack of interest** and click to load.

4.2.1. Once the video loads, scan through the time to identify a section that has no cellular fluorescent activity.

NOTE: This region will be used to create a background sample.

4.2.2. Hold **Shift** and click to draw a region of interest (ROI) box in the area identified as the background sample area.

4.2.3. After creating the box, press the **space bar** to generate a plot of background activity change.

4.2.4. Right-click the trace and select the **assorted** option to present the option to **Dump ROI as text** to make the trace as an xy coordinate text file.

4.3. Moving back to the video of interest, scan again to identify the time region where the activity of interest is occurring.

4.3.1. Using the middle mouse button, select this time region in the yellow time box.

4.3.2. Right-click on the video and select **Stack OPS** and then **Stat map** option 5.

NOTE: This will generate a standard deviation map (SD map) in the left window.

4.3.3. Click on the SD map and then press the **]** key 19x to apply the appropriate color heat map.

4.3.4. Right-click the SD map and select **STM load and save**, which will present the option **Save stm as tiff** to save the SD map.

4.3.5. Then, press the **[** key 19x to return to a grayscale color map.

4.3.6. Press **C** and then **D** to bring up density mapping tools. Using the left mouse button and the center mouse button, adjust the threshold to include all fluorescent activity shown in the SD map.

4.3.7. Press **C** to close the density tools while maintaining the threshold settings.

4.3.8. Right-click the SD map and select **STM particles** and then **Find PTCLS**.

NOTE: This will identify individual cells expressing fluorescent activity.

4.3.9. Right-click the SD map once more and select **Create Particle ROIs**.

NOTE: This will superimpose the selected cells on the original video of interest.

4.3.10. While holding **Shift**, right-click on any one of the now identified particle ROIs on the original video.

4.3.11. Select **ROI Marker** and **Measure Int in ROI**.

NOTE: This will generate individual fluorescent activity plots for each identified ROI in the video of interest. These can be saved by right-clicking any one of these and selecting **Assorted**, followed by **Dump ROI as text**.

4.4. For detailed logic underlying these operations, please see the source code file[9].

REPRESENTATIVE RESULTS:

Several examples of fluorescence intensity changes, mediated by increases of intracellular Ca^{2+} within defined cell types of the NMJ, show the utility of this approach. These results are presented as spatial fluorescence intensity maps, which provide the location of responding cells, as well as the intensity of their responses, thus allowing for the evaluation of how many cells respond and how much each cell responds to a particular stimulus. For example, as shown in **Figure 1**, we took videos of the Ca^{2+} responses in a population of terminal/perisynaptic Schwann cells (TPSCs) at the NMJs of the diaphragm of a P7 *Wnt1-Cre*; *conditional GCaMP3*-expressing mouse in response to stimulation of the phrenic nerve and identified the subpopulations of the responding cells by spatial fluorescence intensity maps. These maps of fluorescence intensity are presented as heat maps and color-coded according to a Fire color lookup table (Fire CLUT). We recorded these videos with and without splitting the image to simultaneously view the clusters of α-BTX-labeled AChRs in the middle of the diaphragm (**Videos 1** and **2**), an approach that could easily be adapted to capture dynamic GECI or GEVI responses from two distinct cell types, provided that each of them exhibits non-overlapping excitation and emission spectra.

In **Figure 2**, we performed the same nerve stimulation experiment on the diaphragm of a P4 *Myf5-Cre; conditional GCaMP3*-expressing mouse and imaged the Ca^{2+} responses in muscle cells.

Interestingly, when we used either the myosin blocker BHC or the skeletal muscle-specific voltage-gated sodium channel ($Na_v1.4$) blocker μ-conotoxin (**Figure 2A** and **Video 3** or **Figure 2B** and **Video 4**, respectively), we visualized Ca^{2+} transients that travel the full length of the muscle fiber, representing the action potential and mediated by the release of Ca^{2+} from the sarcoplasmic reticulum, or merely the length of the endplate band, representing the endplate potential and mediated by extracellular Ca^{2+} influx through the AChR. In addition to identifying subpopulations of responding cells with spatial fluorescence intensity maps (SD maps), as in **Figure 1**, we also measured the change in fluorescence over time in a population of these muscle cells with spatiotemporal (ST) maps. Each of these experiments represents a different cell type, a different age, a different treatment (nerve stimulation *vs.* nerve stimulation in the presence of different drugs) and different types of analysis (spatial *vs.* spatiotemporal fluorescence intensity maps). These figures also illustrate one of the most useful features of transgenic GCaMP-expressing mice, namely the ability to repeatedly stimulate and image the same sample and, therefore, test the effect of different treatment conditions.

FIGURE AND TABLE LEGENDS:

Figure 1: Measurement of activity-induced Schwann cell Ca^{2+} responses in the diaphragm and phrenic nerve of P7 *Wnt1-Cre*; *conditional GCaMP3* mice. (**A**) (Left) An average fluorescence intensity image, showing background levels of fluorescence in Schwann cells along the phrenic nerve branches and at the neuromuscular junction (NMJ), was captured before nerve stimulation (Prestim). The values of this background fluorescence were subtracted from fluorescence values obtained after nerve stimulation. (Right) A spatial map of the standard deviation of 16-bit fluorescence intensity units ($SDiu_{16}$) of Ca^{2+} responses generated after 30 s of 40 Hz of phrenic nerve stimulation (Stim Map or SD Map) shows a robust response in the terminal/perisynaptic Schwann cells (TPSCs) at the NMJ. The fire CLUT heatmap is in $SDiu_{16}$ and the scale bar is in microns. All images in panels **B** - **E** are the same magnification as those in panel **A**. (**B**) (Left) The same diaphragm was labeled with 594-conjugated α-bungarotoxin (α-BTX), which binds to and labels acetylcholine receptors (AChRs), and excited with green/yellow light to identify the NMJ. (Right) This panel shows a brightfield image of the same diaphragm, showing the tip of an intracellular recording electrode (arrow), which can be guided to an NMJ, based on α-BTX labeling. (**C**) The Ca^{2+} transient features (e.g., intensity, onset after stimulation, duration) of individual cells or groups of cells can be evaluated by demarcating individual regions in the spatial intensity map as particles (left), representing them as color-coded regions of interest (ROIs), and (**D**) plotting their intensities over time. (**E**) This panel shows dual-wavelength images of GCaMP3-mediated fluorescent Ca^{2+} responses and 594-α-labeled NMJs in the same diaphragm using the Gemini image splitter after nerve stimulation.

Figure 2: Measurement of activity-induced muscle cell Ca^{2+} responses in the diaphragm of P4 *Myf5-Cre*; *conditional GCaMP3* mice. (**A**) (Left) Nicotinic AChR clusters of the centrally located endplate band of the diaphragm are labeled with 594-α-BTX. (Middle) A spatial map of Ca^{2+} transient intensities (SD map), generated after 30 s of 40 Hz of phrenic nerve stimulation in the presence of the myosin inhibitor BHC, shows a response throughout the entire region of all diaphragm muscle cells. (Right) In contrast, an SD map generated from the same diaphragm after

the same stimulation, but in the presence of the $Na_v1.4$ antagonist μ-conotoxin (μ-CTX), exhibits a spatially restricted response in the medial region of all diaphragm muscle cells that corresponds to the AChR cluster-enriched endplate band. The fire CLUT heatmap is in $SDiu_{16}$ and the scale bar is in microns. (**B**) This panel shows spatiotemporal maps of Ca^{2+} transient intensities over time (ST maps) in a population of muscle cells (*y*-axis) followed over time (*x*-axis). The scale bar is in seconds.

Video 1: Movie without image splitting of activity-induced Schwann cell Ca^{2+} responses at P7, as described in detail in the Figure 1 legend.

Video 2: Movie with image splitting of activity-induced Schwann cell Ca^{2+} responses and 594-α-BTX-labeled AChRs at P7, as described in detail in the Figure 1 legend.

Video 3: Movie of activity-induced muscle cell Ca^{2+} responses in the presence of the myosin blocker BHC at P4, as described in detail in the Figure 2A legend.

Video 4: Movie of activity-induced muscle cell Ca^{2+} responses in the presence of the $Na_v1.4$ antagonist μ-conotoxin at P4, as described in detail in the Figure 2B legend.

DISCUSSION:

Here we provide some examples of measuring Ca^{2+} responses in specific cells in intact neuromuscular tissue using GECI-expressing mice. In order to successfully perform these experiments, it is imperative not to injure the phrenic nerve during the dissection. To image Ca^{2+} responses in Schwann cells at either low or high power (i.e., 20X or 60X), it is necessary to use either BHC or μ-conotoxin to block movement. For low-power imaging of Ca^{2+} responses in muscle cells, it is possible to measure them in the absence of these drugs, thus permitting the simultaneous acquisition of muscle Ca^{2+} transient intensities and muscle length changes during high-frequency nerve stimulation[6]. When performing multiple experiments on the same sample, it is necessary to separate each one by at least 15 min, during which time the sample can be perfused. These steps allow for the repeated imaging of stimulation-induced Ca^{2+} responses from the same field of view in the same sample for at least 3 - 5 hours. It is also critical to predilute drugs dissolved in DMSO as described for BHC, as DMSO applied directly onto GCaMP-expressing tissue induces irreversible, stimulus-independent fluorescence responses.

We found that for reasons that are unclear, *Wnt1-Cre*; *conditional GCaMP3/6* mice fail to exhibit nerve stimulation or agonist-induced Ca^{2+} responses in Schwann cells after P15 - P20. However, *Sox10-Cre*; *conditional GCaMP3/6* mice continue to exhibit these responses at least as late as P56, the oldest age that we have examined. In contrast, *Myf5-conditional GCaMP3/6* mice exhibit responses as old as one year, the oldest age examined.

While GECI-expressing mice provide unique opportunities for imaging Ca^{2+} responses in whole populations of cells of a specific subtype, there are some limitations, such as the inability to perform ratiometric imaging and, thus, extract quantitative Ca^{2+} measurements. There are also limitations to the amount of depth of tissue from which these responses can be imaged using

widefield fluorescence microscopy (i.e., as opposed to using confocal or multiphoton microscopy). Therefore, while the thinness of the diaphragm is amenable for the application of the techniques presented here, capturing cell-specific Ca^{2+} responses in cell types of the NMJ in other muscles that are thicker may require sub-dissection or other kinds of fluorescence microscopy.

These genetic and optical tools represent a significant advancement over previous Ca^{2+} imaging techniques, by which only multiple cell types or a few individual cells within one cell type could be imaged. An additional advantage is that Ca^{2+} responses can be repeatably imaged for long periods of time from the same cells using GECI mice, whereas this is not easily possible using traditional chemical Ca^{2+}-binding fluorescent dyes. Finally, using an image splitter, we perform dual-wavelength imaging of a dynamic signal within one cell type (Schwann cells) and a fixed label within a second (muscle cells) and, thus, show how multiple cell-specific calcium or voltage responses can be evaluated (e.g., a Schwann cell Cre-driving mouse crossed to a conditional Cre-dependent GCaMP mouse as reported here, crossed to a transgenic Cre-independent mouse expressing a muscle cell-specific GECI or GEVI with non-overlapping fluorescence excitation/emission spectra[10], would allow simultaneous tracking of dynamic Ca^{2+} and/or voltage changes in both Schwann and muscle cells). Such tools could help evaluate whether the response of one cell type to a specific stimulus, such as the purine ATP or its breakdown product adenosine, is direct or indirectly mediated by a direct effect on another cell type at the NMJ.

The main goal of these studies was to evaluate the spatiotemporal Ca^{2+} response pattern of cell subtypes to nerve stimulation, but the techniques employed to achieve this can be deployed toward other goals. For instance, they can be used to analyze Ca^{2+} responses in the presence of certain antagonists or in certain mutant backgrounds, such as in specific animal models of motor neuron disease, muscular dystrophy, or Charcot-Marie Tooth disease, to analyze the Ca^{2+} response to specific agonists to evaluate receptor expression, to assess the heterogeneity of Ca^{2+} response features within a cell subtype to a stimulus, or to compare Ca^{2+} responses in a cell subtype to other functional responses within that type (electrophysiologically recorded muscle endplate or action potentials, optically imaged muscle shortening, force-transducer-recorded muscle tension, etc.) or to other parameters (e.g., *post hoc* evaluation of nerve/muscle Schwann cell morphology or molecular expression *via* immunohistochemistry). Together, these studies show how cell-specific GECI or GEVI mice can be used to illuminate a wide spectrum of physiological processes at a synapse composed of genetically identifiable, cell-specific inputs.

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DISCLOSURES:

The authors have nothing to disclose.

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