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Cover Letter

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April 24, 2018

To whom it may concern:

Enclosed please find our manuscript, entitled "***Ex vivo* imaging of cell-specific calcium signaling at the tripartite synapse of the mouse diaphragm**", by Heredia *et al*., to be considered for publication in *JoVE.* This manuscript describes the methods to analyze the activation of the three distinct cell types at the tripartite neuromuscular junction using cell-specific targeting of genetically-encoded calcium indicators. We provide a protocol for generating the mice, dissecting the tissue, imaging the calcium responses, confirming these responses with electrophysiology, and processing the data with imaging software. We employ an image splitter to show dual-wavelength imaging could be used to image two distinct cell type responses simultaneously.

As I have to file for tenure this year, I would be most grateful if, dependent on a positive outcome of peer review, this article could be in press by July 31st, the day before the tenure submission deadline.

Sincerely,

Thomas Gould

Thomas Gould

TITLE:

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

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KEYWORDS:

Calcium imaging, genetically encoded calcium indicators, GCaMPs, muscle, Schwann cell, motor neuron, neuromuscular junction, transgenic, diaphragm.

SHORT ABSTRACT:

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

LONG 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.

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.

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 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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Figure 1

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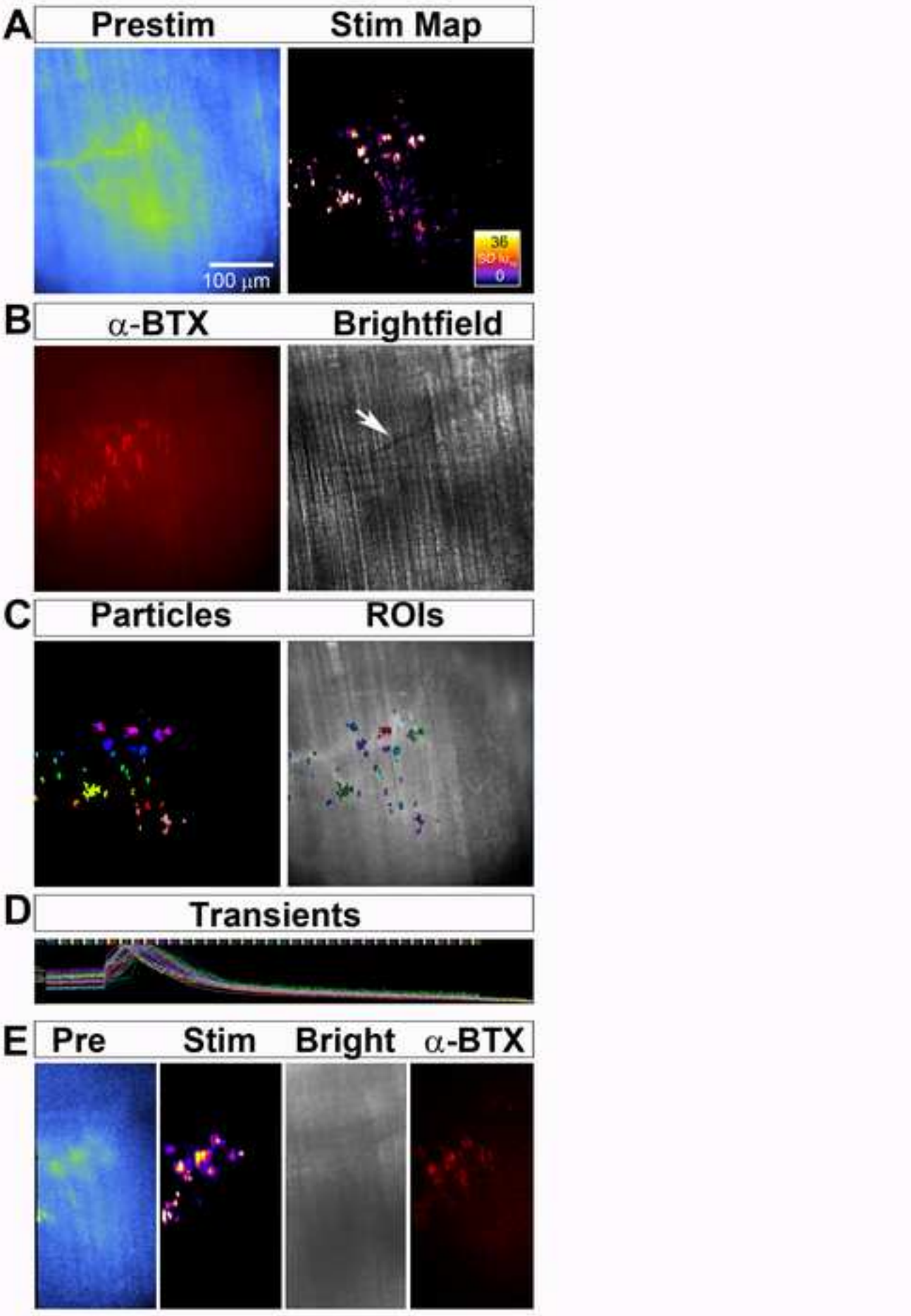

Figure 2

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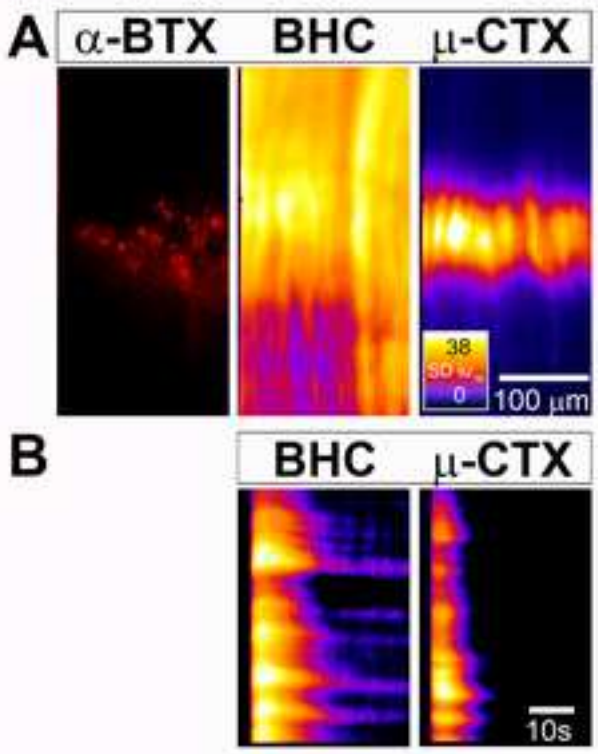

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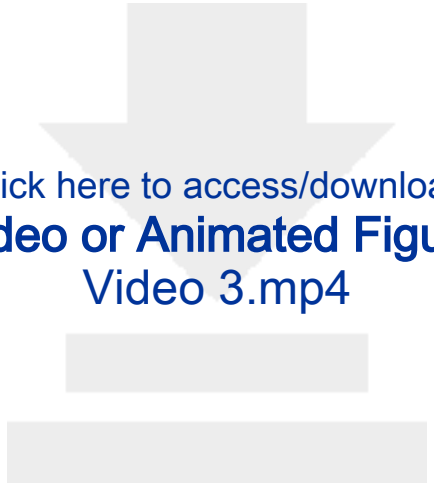
Video 2.mov

Video 3

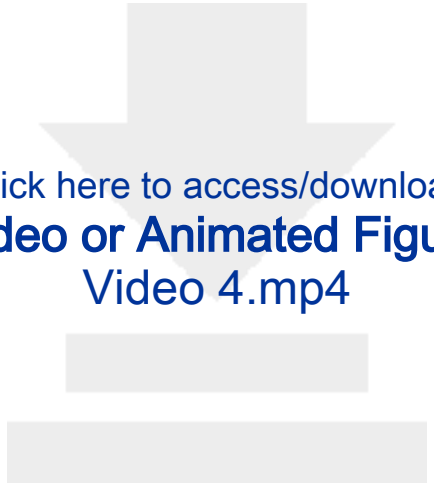


Click here to access/download

Video or Animated Figure

Video 3.mp4

Video 4



Click here to access/download

Video or Animated Figure

Video 4.mp4

Table of Materials

Click here to download Table of Materials JoVE_Materials.xlsx

Name of Material/ Equipment	Company
Myf5-Cre mice	Jax
Wnt1-Cre mice	Jax
Sox10-Cre mice	Jax
Conditional GCaMP3 mice	Jax
Conditional GCaMP6f mice	Jax
BHC (3-(N-butylethanimidoyl)-4-hydroxy-2H-chromen-2-one)	Hit2Lead
CF594-α-BTX	Biotium
μ-conotoxin GIIIb	Peptides Int'l
Silicone Dielectric Gel; aka Sylgard	Ellswoth Adhesives
Minutien pins (0.1mm diameter)	Fine Science Tools
Eclipse FN1 upright microscope	Nikon
Basic Fixed Microscope Platform with Manual XY Microscope Translator	Autom8
Manual micromanipulator	Narishige
Microelectrode amplifier	Molecular Devices
Microelectrode low-noise data acquisition system	Molecular Devices
Microelectrode data analysis system	Molecular Devices
Square wave stimulator	Grass
Stimulus Isolation Unit	Grass
Borosilicate filaments, 1.0 mm outer diameter, 0.5mm internal diameter	Sutter
Borosilicate filaments, 1.5 mm outer diameter, 1.17mm internal diameter	Sutter
Micropipette Puller	Sutter
1200x1200 pixel, back-illuminated cMOS camera	Photometrics
Light Source	Lumencor
Infinity-corrected fluorescent water immersion objectives, W.D. 2mm	Nikon
Fiber Optic Illuminator with Halogen lamp	Sumita
W-View Gemini Image Splitter	Hamamatsu
Single-band Bandpass Filters (512/25-25 and 630/92-25)	SemRock
560 nm Single-Edge Dichroic Beamsplitter	Sem Rock
Imaging data acquisition system	Nikon
Wavelength control module	Nikon
Emission splitter hardware module	Nikon
Imaging data analysis system	NA

Catalog Number

#007893
#003829
#025807
#029043
#024105
#5102862
#00007
#CONO20-01000
Sil Dielec Gel .9KG
26002-10
MBA74100
MXMScr
M-152
Axoclamp 900A
Digidata 1550
PCLAMP 10 Standard
S48
PSIU6
FG-GBF100-50-15
BF150-117-15
P-97
Prime 95b
Spectra X
CFI60
LS-DWL-N
A12801-01
FF01-512/25-25; FF01-630/92-25
FF560-FDi01-25x36
NIS Elements - MQS31000
MQS41220
MQS41410
Volumetry 8D[5], Fiji

Comments/Description
Drives muscle cell expression as early as E13[6]
Drives expression into all Schwann cells at E13 but not P20[9]
Drives Schwann cell expression at older ages
Expresses GCaMP3 in cell-specific fashion
Expresses GCaMP6f in cell-specific fashion
Blocks skeletal muscle myosin but not neurotransmission[6]
Labels acetylcholine receptor clusters at NMJ
Blocks Nav1.4 voltage-dependent sodium channel[8]
Allows for the immobilization of the diaphragm by minutien pins
Immobilizes diaphragm onto silicone dielectric gel
Allows staging and observation of specimen
Allows movement of specimen
Holds recording and stimulating electrodes
Allows sharp electrode intracellular electrophysiological recording
Allows electrophysiological data acquisition
Performs electrophysiological data analysis
Stimulates nerve to excite muscle
Reduces stimulation artifacts
Impales and records nerve-evoked muscle potentials
Lengthened and used for suction electrode
Pulls and prepares recording electrodes
Sensitive camera that allows high-resolution, high-speed imaging
Provides illumination from LEDs for fluorescence obsevation
Provide long working distances for visualization of specimen
Provides illumination for brightfield observation
Projects 1 pair of dual wavelength images separated by a dichroic to single camera
Permits dual band imaging
Dichroic mirror which separates beams of light to allow dual-wavelength imaging
Allows imaging data acquisition
Module for imaging data acqusiition
Module for imaging data acqusiition
Allows analysis of fluorescence intensity and other imaging data

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Author(s): **Dante J. Heredia, Grant W. Hennig, Thomas W. Gould**

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CORRESPONDING AUTHOR:

Name:	**Thomas Gould**
Department:	**Physiology and Cell Biology**
Institution:	**University of Nevada School of Medicine**
Article Title:	**Ex vivo imaging of cell-specific calcium signaling at the tripartite synapse of the mouse diaphragm**
Signature:	Thomas Gould
Date:	**4/25/2018**

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Dear Dr. Gould,

Your manuscript, JoVE58347 Ex vivo imaging of cell-specific calcium signaling at the tripartite synapse of the mouse diaphragm, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jun 01, 2018**.

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Best,

Nam Nguyen, Ph.D.
Manager of Review
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Editorial comments:
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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please provide a scale bar for all microscope images. In Figure 1, does the scale bar apply to each panel? √ Yes, and we mentioned it. (Line 210)
3. Please provide an email address for each author. √ (lines 11,12)
4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). √
5. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method √See 2 of final 4 sentences starting with “In order to address these issues…” and “Here, utilizing…” (Lines 60, 66)
b) The rationale behind the development and/or use of this technique √The rationale precedes the

sentence starting with “In order to address these issues…” (lines 48-60)
c) The advantages over alternative techniques with applicable references to previous studies √ New sentence, third from last, starting with “Conventional”
d) A description of the context of the technique in the wider body of literature √ We added this to the second-to-last sentence: “expressing genetically encoded calcium indicators designed to measure cell specific calcium signaling using genetic techniques” (lines 66-67)
e) Information to help readers to determine whether the method is appropriate for their application √ We added a sentence at the end (line 70-72) to provide this info.
6. 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. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. √ We added one such note on line 176
7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Where specifically? Cannot find
8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Where specifically?
9. I. Equipment/Tools should be moved to the Materials Table. √
10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. √
11. II.A.1: How is the dissection done? Please provide explicit details if this is to be filmed or a citation if it will not be filmed. √ (lines 94-95)
12. B1: What is a significant length? Please quantitate. √ (line 97)
13. Please specify all surgical tools used and all experimental parameters. How large are the incisions? √ (line 95)
14. Please specify all volumes and concentrations used throughout. √
15. What is the perfusion rate? √ (line 103)
16. Please highlight 2.75 pages or less 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. √
17. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
18. Please do not highlight the data analysis (D). √
19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol √ Lines 254-264
b) Any modifications and troubleshooting of the technique √ Lines 265-269

c) Any limitations of the technique √ Lines 270-277
d) The significance with respect to existing methods √ Lines 278-282
e) Any future applications of the technique √ Lines 282-303
20. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. √
21. Please do not abbreviate journal titles. √

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes the use of the calcium indicators GCaMP3 and GCaMP6f to monitor intracellular calcium concentration in the three components of the neuromuscular junction - the nerve, the muscle, and the perisynaptic Schwann cells - in the ex vivo phrenic nerve-diaphragm preparation from the mouse. The authors demonstrate three experiments: (1) Schwann cell Ca2+ responses following stimulation of the phrenic nerve; (2) muscle cell Ca2+ responses following stimulation of the phrenic nerve in the absence and presence of the Nav1.4 antagonist μ-conotoxin; and (3) nerve cell Ca2+ responses following application of high KCl. They also present dual wavelength images of GCaMP3-mediated Ca2+ responses and CF594-α-bungarotoxin fluorescence to demonstrate the potential use of this technique to simultaneously image two indicators, e.g. for intracellular Ca2+ and voltage. The authors discuss the potential utility of this method for investigating multiple questions that require the simultaneous monitoring of populations of cell types within a tissue or organism.

Major Concerns: I have two major concerns with the manuscript as it stands. First, the distribution of NMJs based on the staining with CF594-α-bungarotoxin does not co-localize perfectly with the Ca2+ responses in the muscle. There may be a reasonable explanation for this, but one is not obvious to me. With the absence of an action potential in the muscle, the depolarization and resulting Ca2+ release should spread passively in both directions away from the end-plates. This is not what is shown in Figure 2 and thus requires explanation. √ We re-did another couple experiments, this time with the Gemini splitter, and provided the images and movies from one. The calcium response in the presence of mu-conotoxin (and therefore mediated by external calcium ingress through AChRs) is restricted to the CF594-α-bungarotoxin-labeled endplate zone, at least at these early postnatal stages.
My second concern is with the data shown in Figure 3. The cause of the particular pattern of fluorescence is not obvious. Why does high K increase Ca2+ in the axons? Is the Ca2+ entering through voltage-gated channels in the nerve terminal? If so, there should be a spread of fluorescence away from the terminals. If another mechanism is predicted, this should be stated. Why was KCl used, rather than electrical stimulation of the nerve as in the other cases? Could you see any signal in the nerve terminals?

If such could be demonstrated, the manuscript would be significantly strengthened, as this would be the most relevant compartment in the nerve with respect to the neuromuscular junction. . √ For the first few mice we did at E14.25, we had trouble getting suction electrode to drive contraction, a problem we routinely encounter at this stage (dissections of the nerve are tricky). Thus, we added potassium. We did observe dynamic responses at the endplate zone of the diaphragm as well in response to potassium. However, when we looked at older stages, when it is much easier to draw the nerve into the suction electrode, we were unable to detect Ca^{2+} responses at endplates. We couldn't get them with potassium either. At older ages, non-muscle cells besides motor neurons also exhibited Ca^{2+} responses in the muscle in response to potassium (fibroblasts?), complicating the imaging. Finally, we've had difficulty generating Islet1-Cre mice recently. Collectively, these issues led to our decision to remove this data on the basis that it is too preliminary. We have accordingly removed the appropriate text in the introduction, results and discussion. We are still pursuing this line of inquiry however, as it would be a very useful tool to parse out sequence of effects of stim/drugs, particularly with dual recording of red and green sensors in neurons and Schwann cells, should the tools become availlable. We feel that the removal of this data is not sufficient to undermine the significance or credibility of the manuscript, since all the data with Schwann cell and muscle cell Cre-drivers is valid and (we hope) useful.

Minor Concerns: There are some mistakes in the Protocol.
Lines 104-5. BHC does not block action potentials. √
Lines 107-8. μ-conotoxin blocks action potentials, not endplate potentials. √
Line 121. Change "as" to "at" √
Line 146. Change mΩ to MΩ √
Lines 229-231. Not a complete sentence. √

Reviewer #2:

Manuscript Summary:
The authors describe a protocol for imaging of calcium signals using the genetically encoded calcium indicator GCaMP in the neuromuscular junction of mouse diaphragm by targeting expression of GCaMP separately to the motor nerve, muscle, or Schwann cell using available Cre lines. They also discuss how this protocol could be extended or modified to include multi-color imaging of multiple components simultaneously or modification of NMJ function by drugs.

Major Concerns:
None

Minor Concerns:
line 130 - "...maintain a significant length of phrenic nerve." Be more explicit/descriptive. √
line 140 - define BHC √, on line 104
lines 140-144 - The DMSO dilution described for adding BHC results in 1/2000 dilution of DMSO. The authors state that 1/1000 dilution of DMSO or higher concentration leads to failure of the experiment. This does not seem like a large window between successful experiment and failure. Maybe

elaborate/discuss more. √ We have found recently that we can use higher DMSO concentrations without causing non-specific, non-transient fluorescence in GCaMP3-+ cells if we pre-dilute drugs like BHC into an intermediate volume of Krebs-Ringers before adding to dish. So we added a sentence explaining that and deleted the original sentence stating that failure occurs with higher DMSO concentrations.

line 160 - "add potassium chloride (KCl) to diaphragm preparations" Specify concentration of KCl √ We removed Islet1-GCaMP3 experiments; see below.

lines 112 and 178 - the software Volumetry 6c is recommended, then Volumetry 8d is recommended. Clarify if specific versions are needed or if any version will work √ We changed the version to 8d on line 112.

lines 176-202 - formatting of protocol step numbers is different than earlier in the document √