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**.  
  
To submit a revision, go to the [JoVE submission site](https://na01.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.editorialmanager.com%2Fjove&data=01%7C01%7Ctgould%40med.unr.edu%7C0ec22a7f4e524b5dc2d008d5bcc2837f%7C523b4bfc0ebd4c03b2b96f6a17fd31d8%7C1&sdata=l7rbXWl%2FfH3ckUBs3lGk4z8gh2PjkNMLMUtFLuwXxH4%3D&reserved=0) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Nam Nguyen, Ph.D.  
Manager of Review  
[JoVE](https://na01.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.jove.com%2F&data=01%7C01%7Ctgould%40med.unr.edu%7C0ec22a7f4e524b5dc2d008d5bcc2837f%7C523b4bfc0ebd4c03b2b96f6a17fd31d8%7C1&sdata=GawB9V1fiBAzF4pbVmDJtrIclZy74AdBJZfk%2F84A5nI%3D&reserved=0)  
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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 Ca2+ responses at endplates. We couldn’t get them with potassium either. At older ages, non-muscle cells besides motor neurons also exhibited Ca2+ 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 √