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Dr. Vineeta Bajaj,
Review Editor
Journal of Visualized Experiments
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Dear Dr. Bajaj:

Thank you for serving as Editor for our manuscript entitled "**Single-Cell Electroporation across Different Organotypic Slice Culture Ages of Mouse Hippocampal Excitatory and Class-Specific Inhibitory Neurons**" (manuscript #: JoVE61662). We greatly appreciate the Editor's and Reviewers' comments and have revised the manuscript in response to these reviews. Our point-by-point responses to the critiques are presented below. We hope the revised version is now acceptable for publication in the Journal of Visualized Experiments.

Thank you again for your kind consideration.

Sincerely yours,

Kenny Futai

We appreciate the positive and constructive comments from the Editor and three reviewers. We have addressed these critiques point-by-point in the revised version of our manuscript. As presented below, comments are copied in *italic* and our responses are written in blue. Revisions in the manuscript are highlighted in yellow.

Editor:

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.

We have thoroughly proofread the manuscript to check for spelling and grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have used the recommended formatting.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

The Short Abstract/Summary has been rephrased as suggested.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

The long Abstract is now within the word limit specified.

5. Please make subsections in the protocol. Each heading will be numbered as 1, 2, 3 and action steps as substeps 1.1., 1.1.1 etc

Subsections have been added to the Protocol as requested.

6. 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, alphabets, or dashes.

We thank the Editor for bringing this to our attention. The numbering of the Protocol has been corrected.

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. 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.

The Protocol has been updated to be consistent with the use of imperative tense.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

The Protocol is now written with discrete steps.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Addressed.

10. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Animal protocol approval following the animal care guidelines at our institution has been added at the beginning of the Protocol.

11. Please ensure you answer the "how" question, i.e., how is the step performed? For this please include all mechanical actions, button clicks in the software, knob turns in the instruments, command lines etc.

We have included additional details in the Protocol to address how each step was performed.

12. 2: How do you prepare the DNA construct? Which construct is used by you for this experiment?

Details about the DNA construct are now included in **Section 2** of the Protocol.

13. 1: What kind of slice culture was used in your experiment?

More emphasis has been placed on stating the use of mouse organotypic slice cultures.

14. 3: How do you perform the preincubation, temp, time, Carbon dioxide levels, etc?

These details are now included in **Section 1.2** of the Protocol.

15. 4,5,6,7: How is this done? Some of the actions are missing. Please ensure that all actions are described in a stepwise manner.

We thank the Editor for this comment. We have updated the Protocol to fully describe all actions in a stepwise manner.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

We have changed the font color to aqua for the section of the Protocol to be used for the video.

17. Please ensure the result are described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

We have expanded the **Results** and **Discussion** to better describe the experimental findings and interpretations.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We would like to clarify that all of the figures we submitted were created for this manuscript. We did not reuse any figures from a previous publication.

19. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

The **Discussion** has been expanded to better cover these points. We thank the Editor for helping us to improve the impact of this section.

20. Please do not abbreviate the journal-title.

We found that there are no such abbreviations in the manuscript.

21. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please sort the table in alphabetical order.

Addressed.

Reviewer #1:

Manuscript Summary:

Keener et al. describe the technique of a single-cell electroporation (SCN) in hippocampal organotypic slices targeting 3 types of neuronal cells at 3 time points of in vitro incubation. This is potentially an important method for the neuroscience community and as such will be interesting to JoVE viewers. I have only minor comments that should bring more clarity and additional information to the protocol.

Minor Concerns:

1. When describing the alternative methods, the authors omitted microinjection, which is not only a classical method for targeting dissociated single cells but is also used for targeting single neural cells (mainly neural progenitors) in organotypic brain slices. This should be mentioned in the Introduction and compared to SCE in Discussion. Generally, Discussion should contain a better comparison of SCE to alternative methods, not only previous versions of SCE. This should help disseminate this method further.

We thank the reviewer for bringing this to our attention. We have included text about microinjections and a more detailed discussion of SCE alternatives.

2. Throughout the manuscript (particularly in the Discussion) authors should better address the potential problems with SCE (like off-target labeling, cellular toxicity and death etc). The authors should make use of "Notes" in the Protocol section to deliver additional important information, including suggestions on how to minimize the potential problems (for example an ideal range of plasmid concentrations etc).

We have amended the current manuscript to include more information addressing these points. Notes have also been added in the Protocol to include additional important details. We have used only one plasmid concentration (0.1 µg/µl) and are not able to recommend an ideal range.

3. Protocol section could be better organized. It would be nice to list steps in a chronological sequence of events since this would help other scientists in applying the protocol. The authors should describe which

steps need to be performed one or more days before the experiment (starting the slice culture, preparation of glass pipettes etc) and clearly separate those from the preparations done on the day of the experiment.
We agree that the Protocol would be much improved by being organized this way, and we thank the reviewer for pointing it out so that we can make that improvement. The Protocol is now described as a chronological sequence of events.

4. Step 1. The authors should better describe the organotypic slice culture. What is the final thickness of the slice? What is the composition of the Slice culture medium? It also appears that the list of surgical tools used for this step is incomplete in the Methods section.
Additional details about the organotypic slice culture and surgical tools used are now included in **Section 1** and the **Material/Equipment** file, respectively. Unfortunately, we do not have a suitable method to measure the exact final thickness of the slices. However, we have stated that hippocampi are sliced at a thickness of 400 µm using a tissue chopper.

5. Step 6.3 Biological reasoning for adding TTX should be provided (either in a note, or elsewhere in the manuscript).
Addressed.

6. Step 6.7 A reference or a better explanation of side effects would be helpful here (related to my comment 2).
A reference explaining side effects in greater detail has been added in **Section 5.6.1**.

7. Step 7.5 What is the time interval between cycles?
The time interval between cycles (essentially, as little time as is physically possible) has now been mentioned.

8. Step 7.6 How long should the peak resistance be held before sending the pulse?
The duration of time to hold the peak resistance before sending the pulse has been added.

9. Figures: Are those fixed samples imaged by a confocal microscope? Where the slices re-sectioned? The low-mag images are too small. The authors should make a proper-size panel with low-mag images along with a nuclear counter-staining so that readers can understand the anatomy of the targeted region.

Addressed. We included a new **Section 7** that states the procedure for fixation and imaging. The slice conditions for fluorescence imaging have been added. Low magnification images of whole hippocampus with nuclear counterstaining is now presented in **Figure 2A.**

10. Do the authors determine the projection neurons only by morphology? They should perform a counter-staining with relevant neuronal markers as control, since at the moment the results lack any control experiments.

In the CA1 region, interneuron morphology can be clearly distinguished from pyramidal cells, and we use recently validated fluorescent mouse lines[1]. Therefore, we believe counterstaining is not necessary to label the fluorescently labeled interneurons. Hippocampal CA1 pyramidal neurons were identified based on the anatomy presented in **Figure 2A**.

Reviewer #2:

Manuscript Summary:

In this manuscript, David Keener et al established a single cell electroporation technique for mouse hippocampal neurons in vitro. Using this method can successfully transfect the target gene into hippocampal CA1 pyramidal neurons and endogenous neurons. The transfected brain slices can survive for 21 days in vitro, which provides a good experimental technology for the study of gene function of neurons in vitro.

Major Concerns:

1.In this method, the components of organotypic slice culture medium are not provided. In the process of slice culture, the culture medium is very important. I suggest the author list the culture medium in detail.

The components of the culture medium are now described in **Section 1** of the Protocol.

2.In this method, the author uses a large glass electrode, but does not provide a figure and specific operation process. I think the most important thing for a method is to define the specific operation process.

We have included additional details about the large glass electrodes in **Section 3** and **Figure 1.**

3. In Figure 1, it is recommended to provide a full view of the culture tissue slice.

Addressed. We thank this reviewer for this recommendation.

Minor Concerns:

It is better to provide method operation video.

If our paper is accepted, we plan to work with JoVE to create a method operation video. We agree with this comment!

Reviewer #3:

Manuscript Summary:

The paper is titled ´Single-Cell Electroporation across Different Organotypic Slice Culture Ages of Mouse Hippocampal Excitatory and Class-Specific Inhibitory Neurons` in which the authors discuss electroporation of single cells in organotypic slice culture. The authors state that the electroporation of excitatory and inhibitory neurons in organotypic slice cultures can be done by existing electrophysiology equipment and techniques with relative ease, low cost and efficiency.

Minor Concerns:

The use of speakers described in the step 6.6 and sub-heading 6: Prepare the rig for electroporation, is not clear. The use of speakers has to be elaborated as it is done in step 7.4 sub-heading 7: Electroporate the cells of interest

We thank the reviewer for the opportunity to clarify the purpose of the speakers. These details have now been elaborated in the Protocol in a note under **Section 4.2** as well as in the text of **Section 6.4.1**.

References in the rebuttal letter

1 **Uchigashima, M. *et al.* A Specific Neuroligin3-αNeurexin1 Code Regulates GABAergic Synaptic Function in Mouse Hippocampus. *Submitted.* doi:https://doi.org/10.1101/2020.05.27.119024, (2020).**