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Single-Cell Electroporation across Different Organotypic Slice Culture of Mouse Hippocampal Excitatory and Class-Specific Inhibitory Neurons

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TITLE:

Single-Cell Electroporation across Different Organotypic Slice Culture of Mouse Hippocampal Excitatory and Class-Specific Inhibitory Neurons

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KEYWORDS:

single-cell electroporation, gene delivery, neuron, interneuron, electrophysiology, hippocampus, organotypic slice culture, mouse

SUMMARY:

Presented here is a protocol for single-cell electroporation that can deliver genes in both excitatory and inhibitory neurons across a range of in vitro hippocampal slice culture ages. Our approach provides precise and efficient expression of genes in individual cells, which can be used to examine cell-autonomous and intercellular functions.

ABSTRACT:

Electroporation has established itself as a critical method for transferring specific genes into cells to understand their function. Here, we describe a single-cell electroporation technique that maximizes the efficiency (~80%) of in vitro gene transfection in excitatory and class-specific inhibitory neurons in mouse organotypic hippocampal slice culture. Using large glass electrodes, tetrodotoxin-containing artificial cerebrospinal fluid and mild electrical pulses, we delivered a gene of interest into cultured hippocampal CA1 pyramidal neurons and inhibitory interneurons. Moreover, electroporation could be carried out in cultured hippocampal slices up to 21 days in vitro with no reduction in transfection efficiency, allowing for the study of varying slice culture developmental stages. With interest growing in examining the molecular functions of genes across a diverse range of cell types, our method demonstrates a reliable and straightforward

approach to in vitro gene transfection in mouse brain tissue that can be performed with existing electrophysiology equipment and techniques.

INTRODUCTION:

In molecular biology, one of the most important considerations to an investigator is how to deliver a gene of interest into a cell or population of cells to elucidate its function. The different methods of delivery can be categorized as either biological (e.g., a viral vector), chemical (e.g., calcium phosphate or lipid), or physical (e.g., electroporation, microinjection, or biolistics)[1,2]. Biological methods are highly efficient and can be cell type-specific but are limited by the development of specific genetic tools. Chemical approaches are very powerful in vitro, but transfections are generally random; further, these approaches are mostly reserved for primary cells only. Of the physical approaches, biolistics is the simplest and easiest from a technical point of view, but again produces random transfection results at a relatively low efficiency. For applications which require transfer into specific cells without the need for developing genetic tools, we look toward single-cell electroporation[3,4].

Whereas electroporation used to refer only to field electroporation, over the past twenty years, multiple in vitro and in vivo single-cell electroporation protocols have been developed to improve specificity and efficiency[5-7], demonstrating that electroporation can be used to transfer genes to individual cells and can, therefore, be extremely precise. However, the procedures are technically demanding, time-consuming, and relatively inefficient. Indeed, more recent papers have investigated the feasibility of mechanized electroporation rigs[8,9], which can help to eliminate several of these barriers for investigators interested in installing such robotics. But for those looking for simpler means, the problems with electroporation, namely cell death, transfection failure, and pipette clogging, remain a concern.

We recently developed an electroporation method that uses larger-tipped glass pipettes, milder electrical pulse parameters, and a unique pressure cycling step, which generated a much higher transfection efficiency in excitatory neurons than previous methods, and enabled us for the first time to transfect genes in inhibitory interneurons, including somatostatin-expressing inhibitory interneurons in the hippocampal CA1 region of mouse organotypic slice culture[10]. However, the reliability of this electroporation method in different inhibitory interneuron types and neuronal developmental stages has not been addressed. Here, we demonstrated that this electroporation technique is capable of transfecting genes into both excitatory neurons and different classes of interneurons. Importantly, transfection efficiency was high regardless of days in vitro (DIV) slice culture age tested. This established and user-friendly technique is highly recommended to any investigator interested in using single-cell electroporation for different cell types in the context of in vitro mouse brain tissue.

PROTOCOL:

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. Slice culture preparation, plasmid preparation, and electroporation are also detailed in our previously published methods and can be referred to for additional information[10].

1. Slice culture preparation

1.1 Prepare mouse organotypic hippocampal slice cultures as previously described[11], using postnatal 6- to 7-day old mice of either sex.

1.1.1 Prepare dissection media for organotypic slice culture consisting of (in mM): 238 sucrose, 2.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose in deionized water, then gas with 5% CO_2/95% O_2 to a pH of 7.4.

1.1.2 Prepare organotypic slice culture media consisting of: 78.8% (v/v) Minimum Essential Medium Eagle, 20% (v/v) horse serum, 17.9 mM $NaHCO_3$, 26.6 mM glucose, 2 M $CaCl_2$, 2 M $MgSO_4$, 30 mM HEPES, insulin (1 µg/mL), and 0.06 mM ascorbic acid, pH adjusted to 7.3. Adjust the osmolarity to 310-330 osmol using an osmometer.

1.1.3 Dissect hippocampi out from the whole brain by using two spatulas, and slice (400 µm) using a tissue chopper. Separate slices by using two forceps and transfer to 30 mm cell culture inserts in a 6 well plate filled with culture media (900 µL) underneath the inserts.

1.2 Store organotypic slice cultures in a tissue culture incubator (35 °C, 5% CO_2) and change the slice culture media every two days.

2. Plasmid preparation

2.1 Prepare the plasmid for the gene of interest.

2.1.1 Subclone enhanced green fluorescent protein (EGFP) gene into a pCAG vector.

2.1.2 Purify pCAG-EGFP plasmid with an endotoxin-free purification kit and dissolve in an internal solution that consists of diethyl pyrocarbonate-treated water containing 140 mM K-methanesulfonate, 0.2 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.3 with KOH (plasmid concentration: 0.1 µg/µL).

3. Glass pipette preparation

3.1 Pull borosilicate glass pipettes (4.5 – 8 MΩ) on a micropipette puller (**Figure 1A**).

NOTE: The glass pipettes used for whole-cell patch clamp recordings are ideal for electroporation.

3.2 Check the size of the pipette tip under a dissection microscope to approximate the electrical resistance.

3.2.1 Verify pipette resistance by attaching the pipette to the electroporation electrode and use the micromanipulator to maneuver the pipette tip into artificial cerebrospinal fluid (aCSF)

containing (in mM): 119 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 5 $MgCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4 and 11 glucose in deionized water, gassed with 5% CO_2/95% O_2 to a pH of 7.4. Confirm the actual resistance using the readout on the electroporator.

NOTE: The sharper the pipette tip, the larger the electrical resistance. The pipette resistance should be below 10 MΩ. Glass pipettes with high pipette resistance (**Figure 1B**) often clog at the tip during repeated electroporation.

3.3 Bake glass pipettes overnight at 200 °C to sterilize.

4. Electroporation rig setup

4.1 Install the electroporator to a standard whole-cell electrophysiology rig, equipped with an upright microscope mounted on a shifting table with a micromanipulator and peristaltic pump.

4.2 Install the headstage of the electroporator onto a micromanipulator and connect a pair of speakers to the electroporator. Connect the electroporator to a foot pedal which can be used to send a pulse when ready.

NOTE: The speakers emit a tone when turned on, which is an indicator of the electrical resistance at the electrode. This makes it possible to determine relative changes in resistance without pulling attention away from the procedure.

5. Electroporation preparation

5.1 Transfer slice culture inserts from 6 well plates to 3 cm Petri dishes loaded with 900 µL of culture media and store in a tabletop CO_2 incubator until ready to perform electroporation.

5.1.1 Preincubate fresh culture inserts with slice culture media (1 mL) for at least 30 min in a 3.5 cm Petri dish to culture slices after electroporation.

5.2 Clean and prepare the rig for electroporation.

5.2.1 Perfuse the lines with 10% bleach for 5 min to sterilize the tubing and chamber prior to beginning the experiment for the day.

5.2.2 Perfuse the lines with deionized autoclaved water for at least 30 min to rinse completely.

5.2.3 Perfuse the lines with filter-sterilized aCSF containing 0.001 mM tetrodotoxin (TTX).

NOTE: TTX minimizes cellular toxicity and death due to overexcitation of interneurons[10].

5.3 Set the electroporator's pulse parameters: amplitude of –5 V, square pulse, train of 500 ms, frequency of 50 Hz, and a pulse width of 500 µs.

5.4 Fill glass pipette with 5 µL of plasmid-containing internal solution.

5.4.1 Remove any trapped air bubbles from the pipette tip by flicking and gently tapping the tip multiple times.

5.4.2 Check the tip for damage by visualizing it under a dissection microscope or by repeating step 3.2.1 to check the pipette resistance.

NOTE: If the tip is damaged, the glass pipette must be discarded, and this step must be repeated with a new glass pipette previously prepared in step 3.

5.5 Securely attach the pipette tip to the electrode and turn the speakers on. Record the readout (the pipette's resistance) of the electroporator when the tip has contacted the aCSF medium.

5.6 Cut the culture insert membrane using a sharp blade and isolate one slice culture. Carefully transfer the slice culture to the electroporation chamber by using sharp angled forceps and fix its position with a slice anchor.

5.6.1 Do not keep the slice culture outside of the incubator for more than 30 min at a time to prevent side effects such as changes in neuronal health or function[12].

6. Electroporate cells of interest

6.1 Apply positive pressure to the pipette with mouth or by using a 1 mL syringe (0.2 - 0.5 µL pressure) attached to the tubing.

6.2 Use the micromanipulator's 3-dimensional knob controls to maneuver the pipette tip near the surface of the slice culture.

6.3 Choose a target cell and approach it, keeping the positive pressure applied until a dimple forms on the cell surface, visible on the microscope.

6.4 Perform pressure cycles.

6.4.1 Quickly apply mild negative pressure by mouth so that a loose seal forms between the pipette tip and the plasma membrane, indicated visually by the membrane going up into the pipette tip somewhat. Observe an increase (~2.5x the initial resistance) in pipette resistance by listening for an increase in tone coming from the speakers. Quickly re-apply positive pressure so that the dimple re-forms.

6.4.2 Immediately complete at least two more pressure cycles without pausing, then hold negative pressure for 1 s.

NOTE: Pausing between cycles, applying too much pressure, or holding the negative pressure for too long can cause significant cell damage and possibly cause the cell to die during electroporation.

6.5 Quickly pulse the electroporator once using the foot pedal when the tone from the speakers reaches a stable apex in pitch, indicating peak electrical resistance. Do not wait at the peak resistance for more than 1 s before sending the pulse.

NOTE: There is no off-target electroporation observed by our protocol[10]. Only the cells in contact with the glass pipette during pressure cycles were transfected. Positioning the pipette near other neurons does not result in gene transfection.

6.6 Gently retract the pipette approximately 100 µm from the cell without applying pressure.

6.7 Re-apply positive pressure, verifying that the resistance is similar to the recorded readout in step 5.5, then approach the next cell.

6.7.1 Remove potential clogs, indicated visually or by a significantly increased (>15% higher) pipette resistance after electroporation, by applying positive pressure.

NOTE: If there is no visible clog and the resistance is still significantly higher, discard the pipette and use a new one. On an average, a pipette can be used for up to 20 electroporation events if the user is careful[10].

6.8 After electroporation, transfer the slice culture onto a fresh culture insert, and incubate at 35°C in the incubator for up to 3 days.

7. Fixation, staining and imaging of organotypic hippocampal slice cultures

7.1 Fix electroporated organotypic slice cultures 2 – 3 days after transfection with 4% paraformaldehyde and 4% sucrose in 0.01 M phosphate buffered saline (1x PBS) for 1.5 h at room temperature.

7.2 Remove fixative and incubate slices in 30% sucrose in 0.1 M phosphate buffer (1x PB) for 2 h.

7.3 Place slices on a slide glass and freeze them by putting the slide glass on top of crushed dry ice. Thaw the slices at room temperature and transfer them to a 6 well plate filled with 1x PBS.

7.4 Stain the slices with mouse anti-GFP and rabbit anti-RFP antibodies in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 450 mM NaCl, and 32% 1x PB, pH 7.4) for 2 h at room temperature.

7.5 Wash slices with 1x PBS three times at room temperature for 10 min each wash.

7.6 Incubate slices with anti-mouse Alexa 488-conjugated secondary antibody and anti-rabbit Alexa 594-conjugated secondary antibody in GDB buffer for 1 h at room temperature. Incubate slices with DAPI (4 µg/mL) in 1x PBS for 10 min at room temperature.

7.7 Wash slices with 1x PBS three times at room temperature for 3 min each wash.

7.8 Mount slices on glass slides using mounting medium and perform fluorescence imaging.

REPRESENTATIVE RESULTS:

Our single-cell electroporation is capable of precisely delivering genes into visually identified excitatory and inhibitory neurons. We electroporated three different neuronal cell types at three different time points. Parvalbumin (Pv) or vesicular glutamate type 3 (VGT3) expressing neurons were visualized by crossing Pv^{cre} (JAX #008069) or $VGT3^{cre}$ (JAX #018147) lines with TdTomato (a variant of red fluorescent protein) reporter line (Jax #007905), respectively named Pv/TdTomato and VGT3/TdTomato lines. Organotypic slice cultures were prepared from C57BL/6J, Pv/TdTomato, and VGT3/TdTomato mice.

First, electroporation was performed in CA1 pyramidal neurons (Py) at either 7, 14, or 21 days in vitro (DIV). EGFP was transfected into 5-20 pyramidal neurons in the hippocampal CA1 area across these slice culture ages (**Figure 2B-D**). CA1 pyramidal neurons were identified using differential interference contrast (DIC). To demonstrate the anatomical distribution and morphological differences between pyramidal neurons and inhibitory interneurons in slice culture, CA1 pyramidal neurons were electroporated with EGFP in a DIV7 Pv/TdTomato mouse and nuclear counterstaining was performed to display the distinct location of EGFP-positive neurons in the CA1 pyramidal cell layer (**Figure 2A**).

Next, this protocol was also applied to TdTomato-positive Pv and VGT3 interneurons. EGFP electroporation was carried out in 1-10 fluorescently labeled interneurons. TdTomato-positive Pv (**Figure 3**) and VGT3 (**Figure 4**) neurons were successfully electroporated in the hippocampal CA1 area. Interestingly, transfection of the EGFP gene of interest in all of these inhibitory neuronal types was not significantly affected by DIV and did not differ with the transfection efficiency (~80%) observed in CA1 pyramidal neurons (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Two representative glass pipette images. (**A**) Display lower resistance (6.5 MΩ) pipettes, used in this protocol, and (**B**) higher resistance (10.4 MΩ) pipettes typical for electroporation protocols.

Figure 2. Organotypic hippocampal slice cultures were electroporated with EGFP (green) at three different time points. (**A**) Representative organotypic hippocampal slice culture in a DIV7 Pv/TdTomato mouse. CA1 pyramidal neurons were electroporated with EGFP (green, white arrowheads) and showed no overlap with TdTomato (TdT)-positive Pv interneurons (red, yellow

arrowheads). DAPI nuclear counterstaining (blue) was performed. DG: dentate gyrus. (**B-D**) CA1 pyramidal neurons were electroporated with EGFP at three different time points: (**B**) DIV7, (**C**) DIV14 and (**D**) DIV21. Organotypic slice cultures were fixed with 4% sucrose, 4% paraformaldehyde/ 1x PBS and imaged without further sectioning. Top left, low magnification images of the hippocampal CA1 area. Arrowheads represent individual CA1 pyramidal neurons targeted for electroporation. Transfected neurons with yellow arrowheads are zoomed in the bottom panels. White arrowheads signify additional electroporated neurons outside of the high magnification view. Top right, low magnification images of superimposed (Sup) fluorescent and Nomarski images. Scale bars: 500, 50, 100, 20 µm respectively.

Figure 3: Pv/TdTomato organotypic hippocampal slice cultures were electroporated with EGFP (green) at three different time points. (**A**) DIV7, (**B**) DIV14 and (**C**) DIV21. Overlap with Pv-labeled TdTomato (TdT)-positive cells (red) was observed in the hippocampal CA1 pyramidal cell layer and oriens. In the low magnification insets (top row), yellow arrowheads represent individual Pv interneurons targeted for electroporation. White arrowheads signify additional TdTomato-positive Pv interneurons electroporated outside of the high magnification view. Scale bars: 50, 20 µm.

Figure 4: VGT3/TdTomato organotypic hippocampal slice cultures were electroporated with EGFP (green) at three different time points. (**A**) DIV7, (**B**) DIV14 and (**C**) DIV21. Overlap with VGT3-labeled TdTomato (TdT)-positive cells (red) was observed in the hippocampal CA1 pyramidal cell layer and oriens. In the low magnification insets (top row), yellow arrowheads represent individual VGT3 interneurons targeted for electroporation. White arrowheads signify additional TdTomato-positive VGT3 interneurons electroporated outside of the high magnification view. Scale bars: 50, 20 µm.

Figure 5: Comparable levels of transfection efficiency in CA1 pyramidal neurons, Pv/TdTomato, and VGT3/TdTomato interneurons at three different *in vitro* slice culture ages. Summary bar graphs of three different slice culture ages: DIV7 (left), DIV14 (middle) and DIV21 (right). Each symbol represents the transfection efficiency obtained from one organotypic slice culture CA1 Py: DIV7 (12 slice cultures from 2 mice), DIV14 (8/2), DIV21 (8/2); Pv: DIV7 (5/2), DIV14 (6/2), DIV21 (6/2); VGT3: DIV7 (6/2), DIV14 (7/2), DIV21 (6/2). One-way ANOVA; n.s. (not significant). Data shown are mean ± SEM.

DISCUSSION:

We describe here an electroporation method that transfects both excitatory and inhibitory neurons with high efficiency and precision. Our optimized electroporation protocol has three innovative breakthroughs to achieve highly efficient gene transfection. Our first modification was to increase pipette size compared with previously published protocols[3,5,6]. This change enabled us to electroporate many neurons without pipette clogging. In addition, it is possible that the lower resistance pipettes allow for the use of milder electrical pulse parameters compared with previous methods while still achieving the desired result[3]. Next, repeated pressure cycling before electroporation markedly reduced cell death[10]. We often observed that with the application of a single pulse of negative pressure before electroporating, plasma membrane stuck to the inside

of the pipette tip, damaging the cell. The pressure cycles helped far less plasma membrane stick to the pipette, which improved cell survival and recovery during the procedure[10]. Finally, the addition of TTX into aCSF greatly improved the success of electroporation in inhibitory neurons[10]. We consider that electroporation can cause lethal cell overexcitation which can be prevented by TTX. Above all, these critical improvements offer remarkably high success rates in electroporation to both excitatory and inhibitory neurons (**Figure 5**). While the method shows no electrophysiological abnormalities[10] in the targeted cells, it has been reported that local pH changes during electroporation reduce cell viability and the optimization of electroporation parameters can be relatively difficult compared with other gene transfection methods[13,14]. Therefore, it is important to consider unforeseen side effects of electroporation and to re-optimize the electrical parameters as needed to achieve a high transfection efficiency for any specific application.

Microinjection technology has also been used as a powerful approach to deliver transgenes to cells[2,15,16]. However, this approach generally produces a lower yield of transfection and requires a high level of skill. In contrast, our method can be used with relative ease and at minimal cost to laboratories that routinely perform whole-cell electrophysiology studies.

Recently, we showed that multiple genes can be transfected into both excitatory and somatostatin expressing inhibitory neurons with no side effects on electrophysiological properties[10]. In this study, we demonstrate that this electroporation method is highly efficient in CA1 pyramidal neurons (**Figure 2**) and Pv (**Figure 3**) and VGT3 (**Figure 4**) inhibitory interneurons. Moreover, this electroporation technique allows us to transfect genes of interest with an ~80% success rate regardless of cell type or number of days in vitro (**Figure 5**). Although the technique has only been tested in vitro, organotypic hippocampal slice cultures at the same DIV time points we tested have been shown to follow age-matched in vivo synaptic morphology and activity, as seen in acutely prepared hippocampal slices[17]. Moreover, as we have only tested this method in hippocampal neurons, it is possible that there could be unanticipated challenges in applying this technique to neurons in other brain regions. The method invites exploration of genes during organotypic slice culture development in which synaptic transmission and density, among other properties, change over time.

This protocol is an improvement over previously established ones in that it is simultaneously low-cost, less technically challenging, and more efficient in generating single-neuron gene transfection[5-9]. It also appears to be an improvement over previous methods in terms of lower rates of cell damage or death, as we observed that ~80% of cells were successfully transfected and healthy after the procedure. This method, therefore, provides a new opportunity to examine the roles of genes in multiple neuronal cell types using fluorescent mouse models. Future studies using this method can focus on protein-protein interactions between cells to examine specific molecular or physiological functions, including trans-synaptic protein interactions.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

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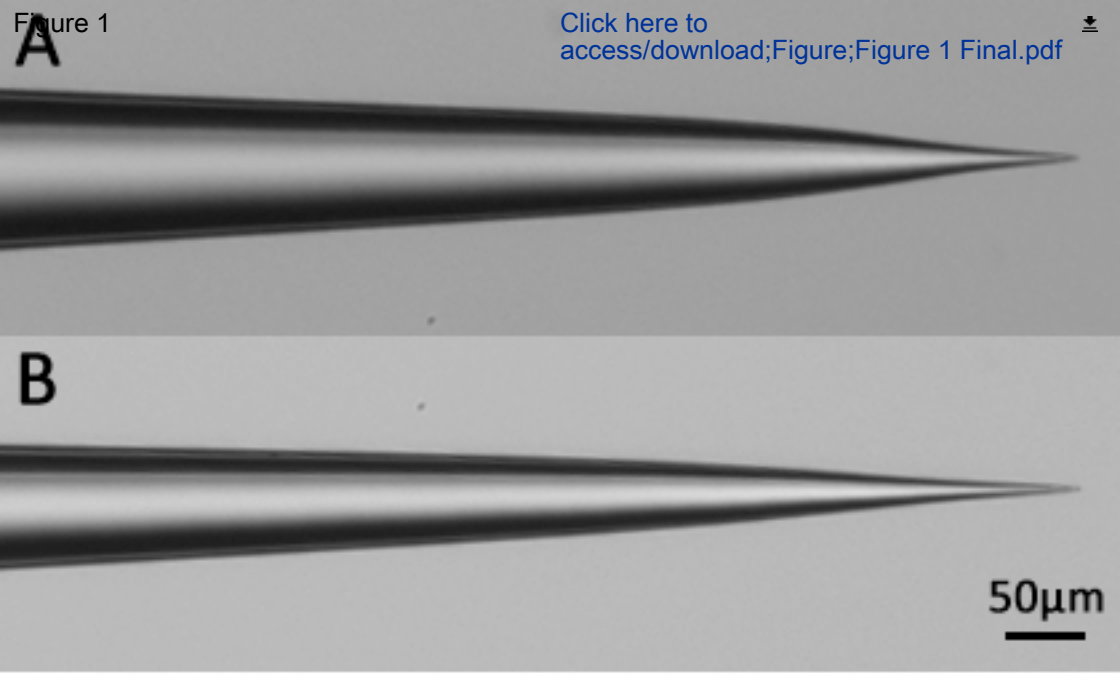

Figure 1

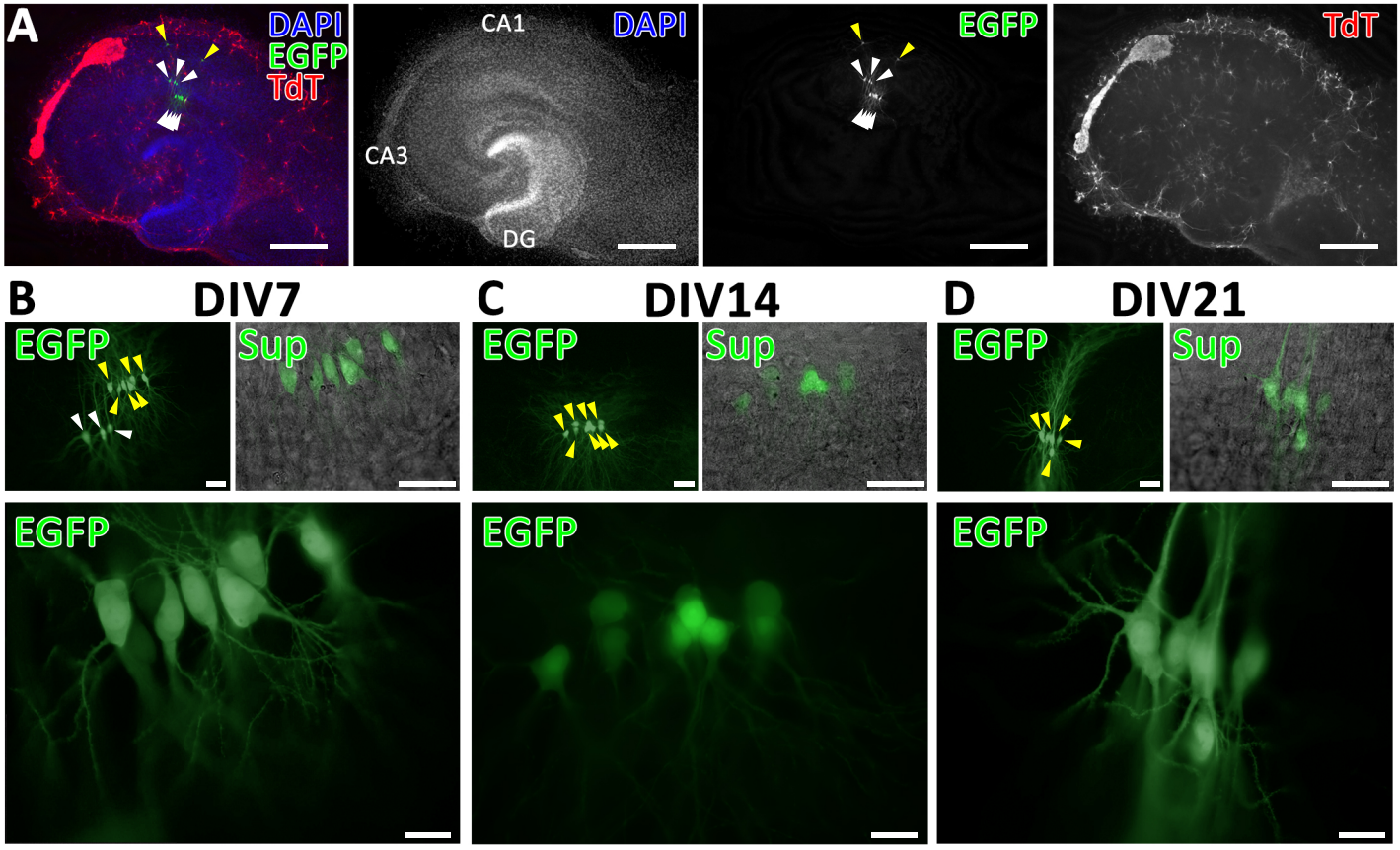

Figure 2

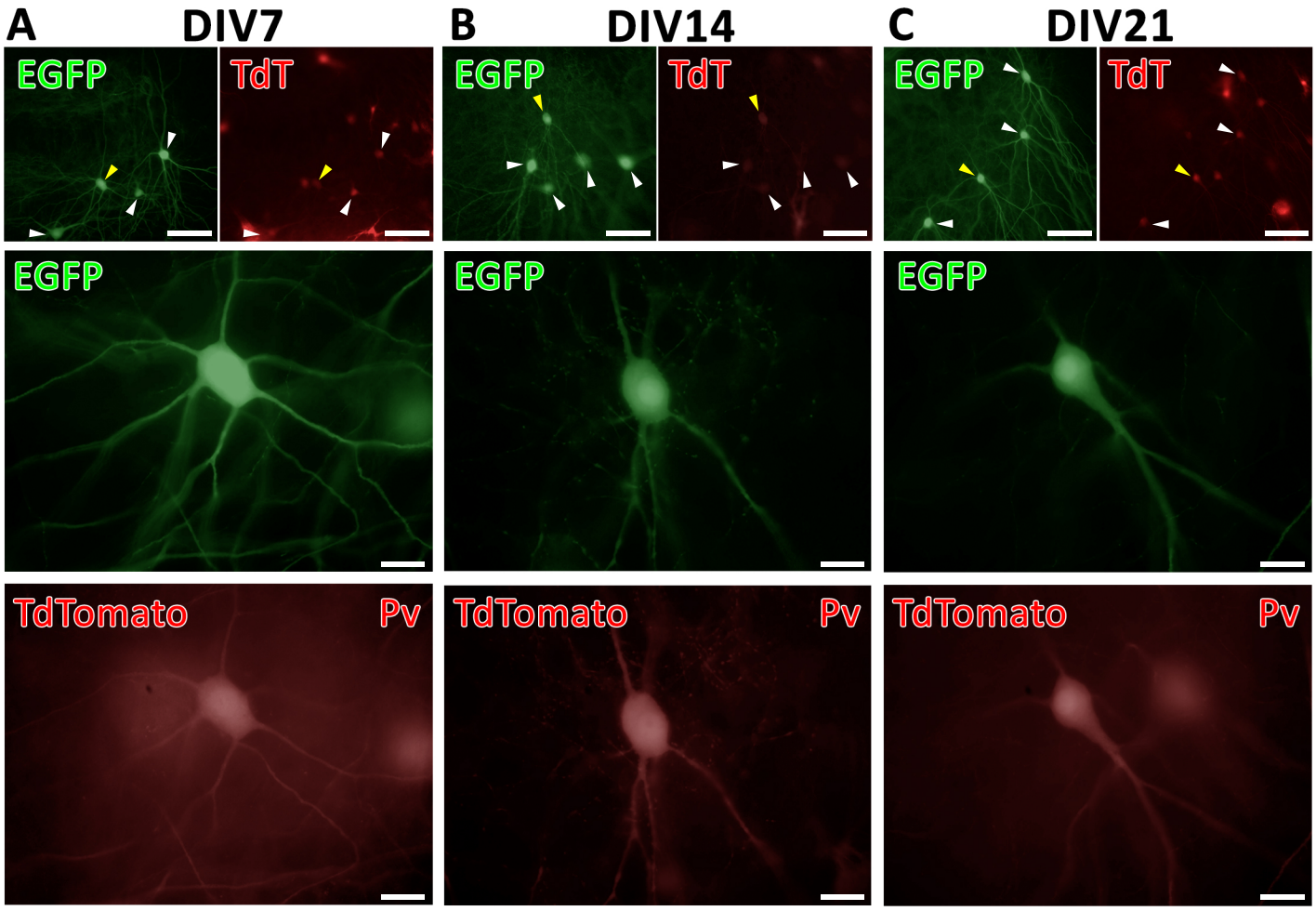

Figure 3

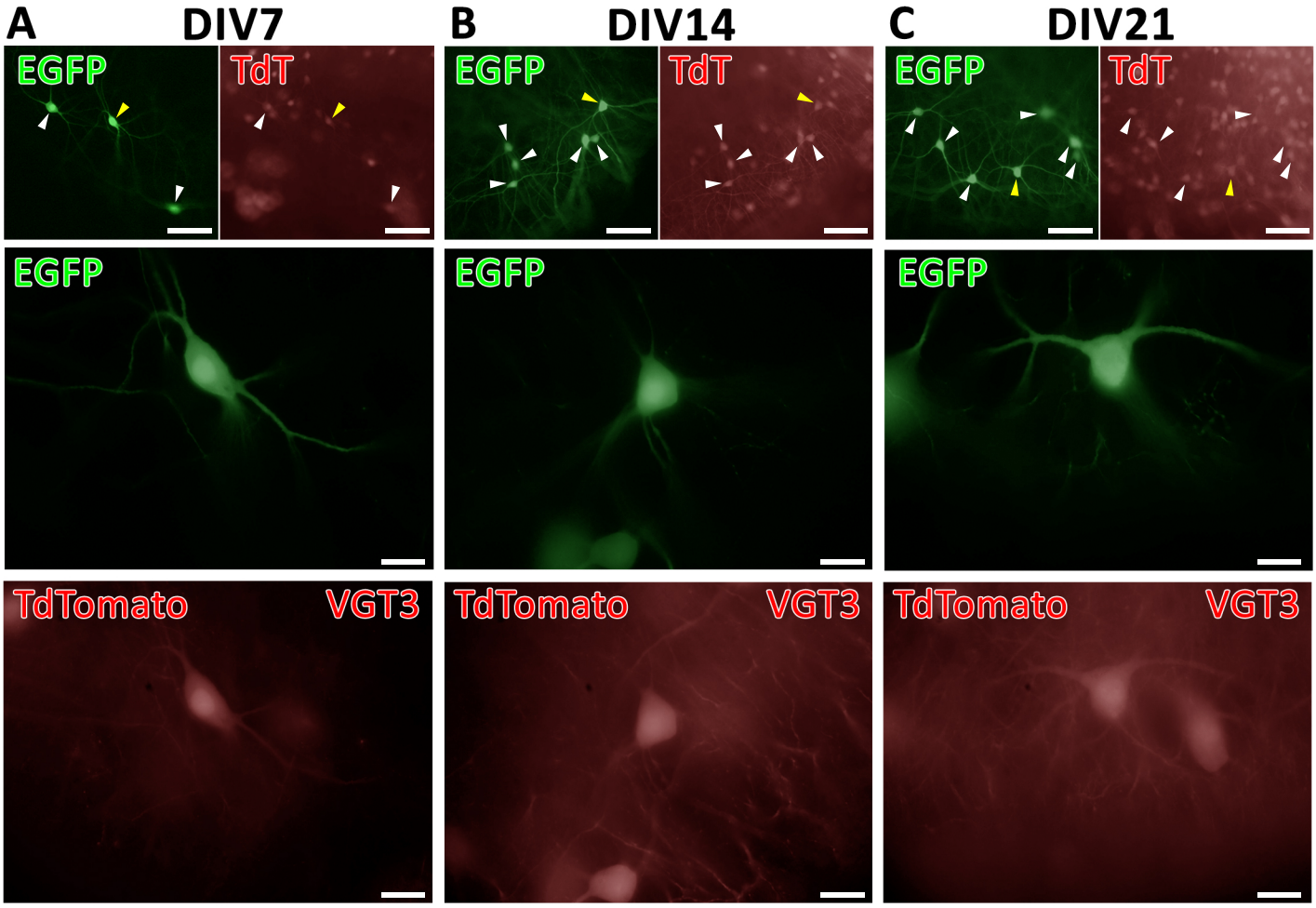

Figure 4

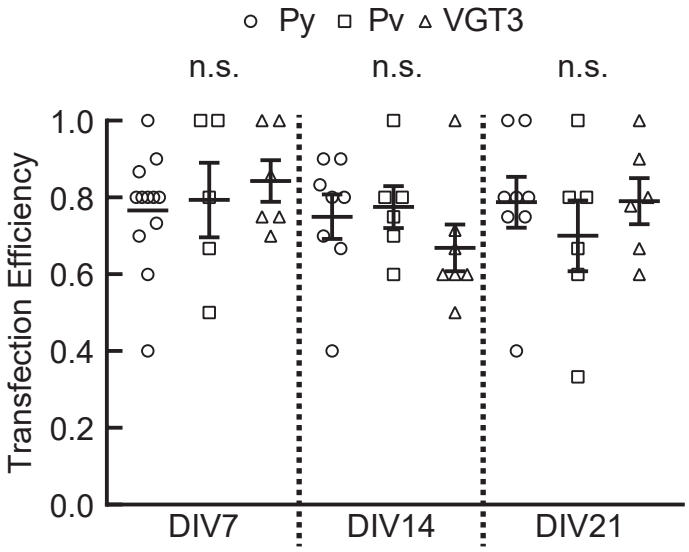

Figure 5

Name of Material/ Equipment	Company	Catalog Number
Plasmid preparation		
Plasmid Purification Kit	Qiagen	12362
Organotypic slice culture preparation		
6 Well Plates	GREINER BIO-ONE	657160
Dumont #5/45 Forceps	FST	#5/45
Flask Filter Unit	Millipore	SCHVU02RE
Incubator	Binder	BD C150-UL
McIlwain Tissue Chopper	TED PELLA, INC.	10180
Millicell Cell Culture Insert, 30 mm	Millipore	PIHP03050
Osmometer	Precision Systems	OSMETTE II
PTFE coated spatulas	Cole-Parmer	SK-06369-11
Scissors	FST	14958-09
Stereo Microscope	Olympus	SZ61
Sterile Vacuum Filtration System	Millipore	SCGPT01RE
Electrode preparation		
Capillary Glasses	Warner Instruments	640796
Micropipetter Puller	Sutter Instrument	P-1000
Oven	Binder	BD (E2)
Puller Filament	Sutter Instrument	FB330B
Single-cell electroporation and fluorescence imaging #1		
3.5 mm Falcon Petri Dishes	BD Falcon	353001
Airtable	TMC	63-7512E
CCD camera	Q Imaging	Retiga-2000DC
Electroporation System	Molecular Devices	Axoporator 800A
Fluorescence Illumination System	Prior	Lumen 200
Manipulator	Sutter Instrument	MPC-385

Metamorph software	Molecular Devices	
Peristaltic Pump	Rainin	Dynamax, RP-2
Shifting Table	Luigs & Neuman	240 XY
Speaker	Unknown	
Stereo Microscope	Olympus	SZ30
Table Top Incubator	Thermo Scientific	MIDI 40
Upright Microscope	Olympus	BX61WI

Fluorescence imaging #2

All-in-One Fluorescence Microscope	Keyence	BZ-X710

Comments/Description

Angled dissection forceps for organotypic slice culture preparation
Filtration and storage of culture media

Tissue chopper for organotypic slice culture preparation
Organotypic slice culture inserts

Filtration and storage of aCSF

Puller

Puller

Camera
Electroporator

Manipulator

Image acquisition
Perfusion pump

Speakers connected to the electroporator

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UMASS

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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).**