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Using neuron spiking activity to trigger closed-loop stimuli in neurophysiological experiments.

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TITLE:

Using Neuron Spiking Activity to Trigger Closed-Loop Stimuli in Neurophysiological Experiments

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KEYWORDS:

Electrophysiology; neuronal population recordings; closed-loop stimulation, spike sorting, neuronal packets, rodents.

SUMMARY:

This protocol demonstrates how to use an electrophysiological system for closed-loop stimulation triggered by neuronal activity patterns. Sample Matlab code that can be easily modified for different stimulation devices is also provided.

ABSTRACT:

Closed-loop neurophysiological systems use patterns of neuronal activity to trigger stimuli, which in turn affect brain activity. Such closed-loop systems are already found in clinical applications, and are important tools for basic brain research. A particularly interesting recent development is the integration of closed-loop approaches with optogenetics, such that specific patterns of neuronal activity can trigger optical stimulation of selected neuronal groups. However, setting up an electrophysiological system for closed-loop experiments can be difficult. Here, a ready-to-apply Matlab code is provided for triggering stimuli based on the activity of single or multiple neurons. This sample code can be easily modified based on individual needs. For instance, it shows how to trigger sound stimuli and how to change it to trigger an external device connected to a PC serial port. The presented protocol is designed to work with a popular neuronal recording system for animal studies (Neuralynx). The implementation of closed-loop stimulation is demonstrated in an awake rat.

INTRODUCTION:

The goal of this protocol is to demonstrate how to implement closed-loop stimulation in neurophysiological experiments. The typical setup for closed-loop experiments in neuroscience involves triggering stimuli based on the online readout of neuronal activity. This, in turn, causes

modifications in the brain activity, thus closing the feedback loop[1,2]. Such closed-loop experiments provide multiple benefits over standard open-loop setups, especially when combined with optogenetics, which allows researchers to target a specific subset of neurons. For example, Siegle and Wilson used closed-loop manipulations to study the role of theta oscillations in information processing[3]. They demonstrated that stimulating hippocampal neurons on the falling phase of theta oscillations had different effects on behavior than applying the same stimulation on the rising phase. Closed-loop experiments are also becoming increasingly important in preclinical studies. For instance, multiple epilepsy studies have shown that neuronal stimulation triggered on seizure onset is an effective approach to reduce the severity of seizures[4-6]. Moreover, systems for automated seizure detection and the contingent delivery of therapy[7,8] showed significant benefits in epilepsy patients[9-12]. Another application area with rapid advancement of closed-loop methodologies is the control of neuroprosthetics with cortical brain–machine interfaces. This is because providing instantaneous feedback to users of prosthetic devices significantly improves accuracy and capability[13].

In recent years, several labs have developed custom systems for the simultaneous electrical recording of neuronal activity and delivery of stimuli in a closed-loop system[14-18]. Although many of those setups have impressive characteristics, it is not always easy to implement them in other labs. This is because the systems often demand experienced technicians to assemble the required electronics and other necessary hardware and software components.

Therefore, in order to facilitate the adoption of closed-loop experiments in neuroscience research, this paper provides a protocol and Matlab code to convert an open-loop electrophysiological recording setup[19-22] into a closed-loop system[2,6,23]. This protocol is designed to work with the Digital Lynx recording hardware, a popular laboratory system for neuronal population recordings. A typical experiment consists of the following: 1) Recording 5-20 minutes of spiking data; 2) Spike sorting to create neuronal templates; 3) Using these templates to perform online detection of neural activity patterns; and 4) Triggering stimulation or experimental events when user-specified patterns are detected.

PROTOCOL:

All procedures described here were performed under an Animal Research Protocol approved by the University of Lethbridge Animal Welfare Committee.

1. Surgery

NOTE: The surgery procedures used to implant probes for neurophysiological recordings have been presented in other publications[24-26]. The exact details of the surgery for closed-loop stimulation depend on the type of recording probes used and the brain areas targeted. In most cases, however, a typical surgery will consist of the following steps.

1.1. Bring to the surgery room a cage with a rat to be implanted with a silicone probe or electrode array to record neuronal activity.

1.2. Anesthetize the rodent with 2-2.5% isoflurane and fix the head in a stereotaxic frame. Ensure that the animal is unconscious during surgery by observing any motoric reaction to mild tactile stimuli[25].

1.3. Apply an eye ointment to minimize dryness during the surgery.

1.4. Shave the surgical area and disinfect the skin with 2% chlorhexidine solution and 70% isopropyl alcohol.

1.5. Inject lidocaine (5 mg/kg) under the scalp over the brain area where electrodes will be implanted.

1.6. Make an incision of the scalp over the area of future implant, and use a scalpel and cotton swab to clear the periosteum from the exposed skull[25].

1.7. Drill 4-8 holes in the skull for implantation of anchor screws (~0.5 mm) as structural support for the implant[25]. Attach the screws to the skull by inserting them in the holes and ensure that they are held firmly in place.

1.8. Drill the craniotomy at the specified coordinates, and inset the microdrive/probe implant.

NOTE: The described protocol for closed-loop stimulation will work for any brain region in which the electrodes are inserted.

1.9. Fix the microdrive/probe and any required electrical interface connector to the skull using dental acrylic. The amount of dental acrylic should be enough to firmly attach the implant, but it should not come in contact with the surrounding soft tissue[25].

1.10. After surgery, closely monitor the animal until it has regained sufficient consciousness to maintain sternal recumbency[25]. For the subsequent 3 days, administer subcutaneously an analgesic (e.g. Metacam, 1 mg/kg), and an antibiotic to prevent infection (e.g. enrofloxacin, 10 mg/kg).

NOTE: Animals are typically left to recover from surgery for one week prior to any testing or recording.

2. Software installation

NOTE: This was tested on Windows 10, 64 bit version.

2.1. Install data acquisition and processing software.

2.1.1. Install the data acquisition system Cheetah 6.4

(https://neuralynx.com/software/category/sw-acquisition-control), which includes libraries to interact with the Cheetah Acquisition System.

2.1.2. Install SpikeSort3D (https://neuralynx.com/software/spikesort-3d) or any other software that uses KlustaKwik[27] for spike sorting. The online detection software uses the cluster definitions from the KlustaKwik engine. This software may run on the same computer, or it may run on separate computers that are on the same network.

2.1.3. Install the NetComDevelopmentPackage (https://github.com/leomol/cheetah-interface/blob/master/NetComDevelopmentPackage_v3.1.0), which can be also downloaded from https://neuralynx.com/software/netcom-development-package.

2.2. Install Matlab (https://www.mathworks.com/downloads/; code was tested on Matlab version R2018a). Make sure that Matlab is enabled in the Windows firewall. Normally a pop-up will come up during the first connection.

2.2.1 Log in to a Matlab account. Choose the licence. Choose the version. Choose the operating system.

2.3. Download the following library for online event triggering from: https://github.com/leomol/cheetah-interface and extract files to the computer's 'Documents/Matlab' folder. A copy of the code is provided in the accompanying supplemental materials.

3. Initial data acquisition

3.1. Start data acquisition using Cheetah software.

3.2. Record a few minutes of spiking data to populate template waveforms.

3.3. Stop the data acquisition and perform spike sorting on the recorded data.

3.3.1. Open SpikeSort3D, click **File | Menu** | **Load Spike File**, and select a spike file from the folder with recorded data.

3.3.2. Click **Cluster** Menu and then **Autocluster using KlustaKwik**, leaving the default settings and click **Run**.

4. Closed-loop experiment

4.1. Resume data acquisition in Cheetah.

4.2. Open Matlab.

4.2.1. Open **ClosedLoop.m** and click on **Run**. Alternatively, in the Matlab command window, execute ClosedLoop(). Ensure that ClosedLoop.m is on the Matlab path. If the user wants to employ a custom function to call on every trigger, execute ClosedLoop('-callback', customFunction) instead, where customFunction is a handle to that function.

4.2.2. Load the spike information defined on the initial recording by clicking on **Load**, browsing to the recording folder, and selecting one of the spiking data files (.ntt, .nse).

4.2.3. Select one or multiple neurons that will trigger stimulation by clicking the checkbox under the plotted waveforms.

4.2.4. Define the minimum number of neurons that will trigger stimulation by typing an integer in the "*min matches"* text box; and define the time window in which spikes matching different waveforms are considered co-active by typing a number in the "window" text box.

4.2.5. Click **Send** to start. This will begin online triggering of events (tones as default) based on spiking activity of selected neurons.

REPRESENTATIVE RESULTS:

Fisher-Brown Norway rats born and raised on-site were habituated to handling for two weeks prior to the experiment. A recording drive was surgically implanted, similar to methods described previously[28-34]. The neuronal signals were recorded at 32 kHz with a digital acquisition system. Neuronal signals were first amplified with a unity gain wireless head-stage, then amplified with a gain of 1000, and band pass filtered between 600 and 6,000 Hz. Neuronal spikes exceeding a manually set amplitude threshold (typically 48-60 µV) were automatically saved, and then sorted into distinct clusters. Thus, each cluster presumably corresponds to spikes from a different neuron[27]. For this protocol demonstration, the rat was resting on a flowerpot, which was a familiar resting place during breaks in behavioral experiments (**Figure 1**).

A representative screenshot from the recording computer is shown in **Figure 2**. It shows the simultaneous running of the recording software (left) and the Matlab program, which displays spike waveforms acquired in real time. This Matlab script is included in the Supplemental Materials. When spikes from predefined triggering clusters are detected, the waveforms are displayed with a bold dashed line in the Matlab figure window (**Figure 2**), and it triggers a tone, providing a closed-loop system. This closed-loop experimental setup allows, for example, to study neuroplasticity, where one can test if pairing neuronal activity with an external stimuli (tone) can affect the receptive fields of those neurons.

FIGURE AND TABLE LEGENDS:

Figure 1. Photograph of a rat with the wireless head-stage (board with pre-amplifiers and a blue LED) attached to an implanted silicone probe. The probe is positioned under the dental acrylic (pink material) and is not visible.

Figure 2. **Screenshot of the recording and closed-loop software.** Leftward panels are windows that are part of the Cheetah recording system used for visualizing and controlling the data acquisition. The window on the right side of the screen shows a Matlab session running the described software. The middle window shows the waveform of an online detected spike matching a pre-defined template. Spikes belonging to that cluster were used to trigger sound in the presented video.

Figure 3. **Schematic pipeline for the data flow.**

Figure 4. **Test of stimulus latency.** (**A**) Histogram of delays between time of generating an artificial spike and time of triggered signal. (**B**) Schematic of a microprocessor board setup to generate artificial spike waveforms. The black and orange connections output an RC like waveform (ranging from 0 to 810 mV) and it is connected to the head-stage via a "signal-mouse" interface that reduces the voltage to 810 µV. Components plugged in the same column of the breadboard are connected (resistors: 110 Ohm; 220 Ohm; 1000 Ohm; capacitor: 10 µF). The Arduino was connected to a PC via USB/UART, which triggered Arduino spikes and received back signals from both the Arduino circuit and the acquisition software API. The Arduino was instructed to generate 1000 spikes.

DISCUSSION:

The protocol described here, shows how to use a standard neurophysiological recording system to perform closed-loop stimulation. This protocol allows neuroscientists with limited expertise in computer science to rapidly implement a variety of closed-loop experiments with little cost. Such experiments are often necessary to study causal interactions in the brain.

After preparing an animal and installing the software (Steps 1 & 2), the closed-loop experiment consists of two separate stages. First, initial data acquisition (Step 3) to collect data to define templates corresponding to activity of single neurons (i.e., spike sorting; Step 3.5). Secondly, the closed-loop stimulation, where newly recorded spikes will be automatically assigned to predefined clusters in real time and trigger stimulation if spikes form specified neurons are detected (Step 4). The presented Matlab scripts (see **Supplemental Materials**) demonstrate the triggering of different stimuli based on the activity of a single neuron, and on the activity of multiple selected neurons. The latter is a particularly important option, because neurons are believed to process information as an assembly (e.g., packets[35,36]). Triggering stimuli based on neuronal population patterns may thus be a key tool for answering a wide range of research questions. The data flow during closed-loop control is illustrated in **Figure 3**.

In this protocol demonstration, a 3 kHz tone stimuli was used. This pure tone can be replaced by an arbitrary sound waveform by changing the variable "tone0". Also, note that instead of a speaker, many other devices could be connected to the computer's audio output to trigger a stimulation. For example, the audio output was used to drive a vibration motor to deliver low frequency (20 Hz) tactile stimuli[22]. Alternatively, the Matlab code could be used to send a TTL signal to a device connected to a computer serial port. This can be accomplished by replacing the 'sound()' command with the following code: obj=serial('COM1'); fopen(obj); obj.RequestToSend

= 'on'. A sample implementation of this method is provided in the **Supplemental Materials** (see pulse.m). Similarly, Matlab could be used to send signals to external devices through a USB port. Thus, the code presented here allows users to send closed-loop triggers in a variety of ways to multiple devices.

Tests showed that the time delay between a neuronal spike and the trigger signal is about 13 ms (min 9 ms; max 15 ms). The distribution of time delays is illustrated in **Figure 4A**. For this latency tests, an Arduino was used to send an artificial spike to the acquisition system (via the head-stage). The delay was recorded as the time between the spike and the trigger signal from the acquisition PC running the closed-loop Matlab script. The schematic of the Arduino setup to generate spikes is shown in **Figure 4B**.

The approach presented here is implemented in software, and thus may not be able to deliver stimuli with temporal accuracy of systems with dedicated hardware. For example, TDT (Tucker-Davis Technologies) offers systems for spike triggered stimulation which can deliver stimuli within milliseconds. However, the advantage of the Matlab solution presented here is its low cost for users who own Cheetah recording hardware, its flexibility in defining the activity patterns to trigger stimuli, and its flexibility in defining neuronal templates. Moreover, single millisecond precision is not required in many experiments, so the implementation ease of this approach could offer a major advantage.

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DISCLOSURES:

Authors do not have any conflict of interests related to this work.

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Fig 1

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Fig 2

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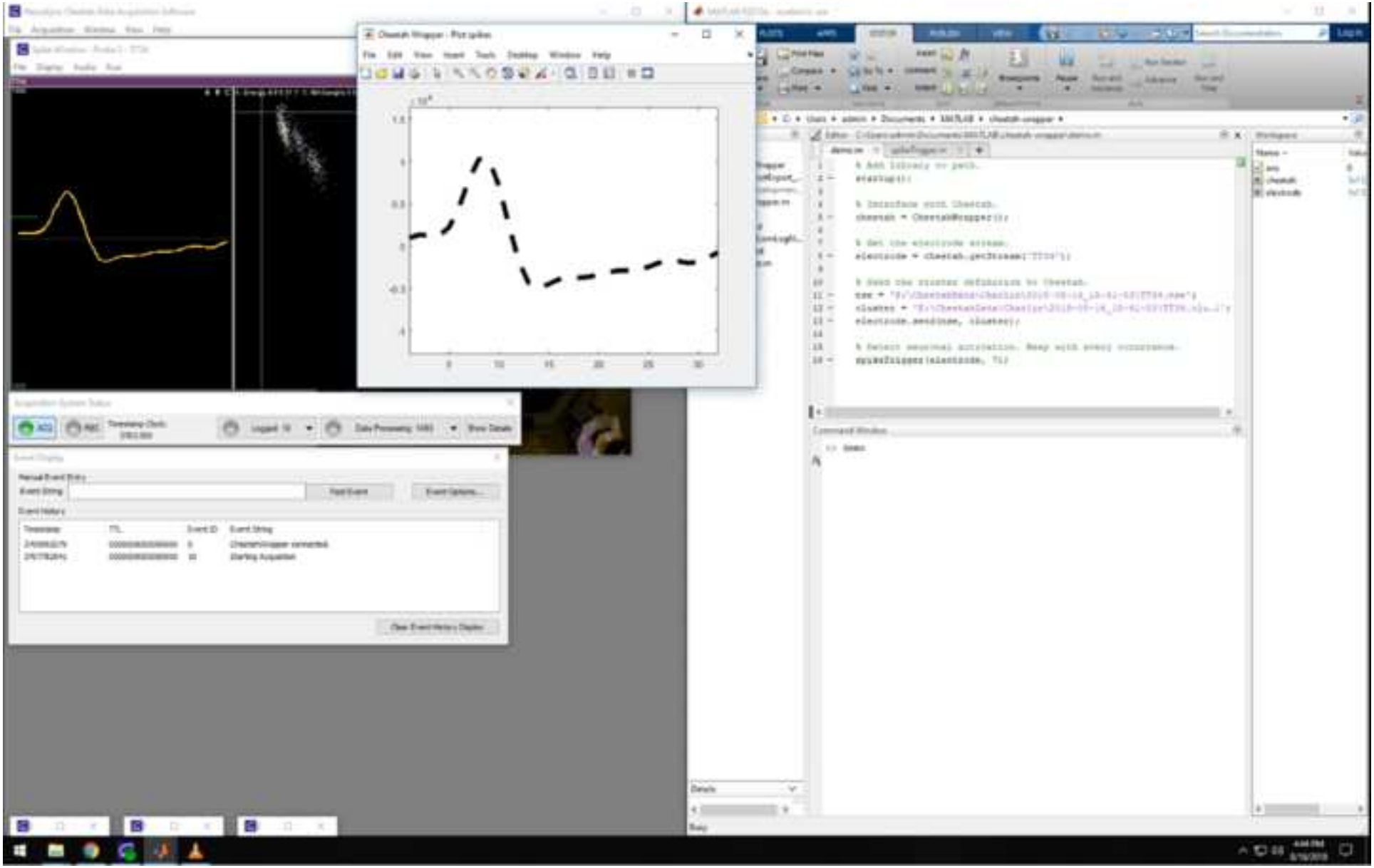

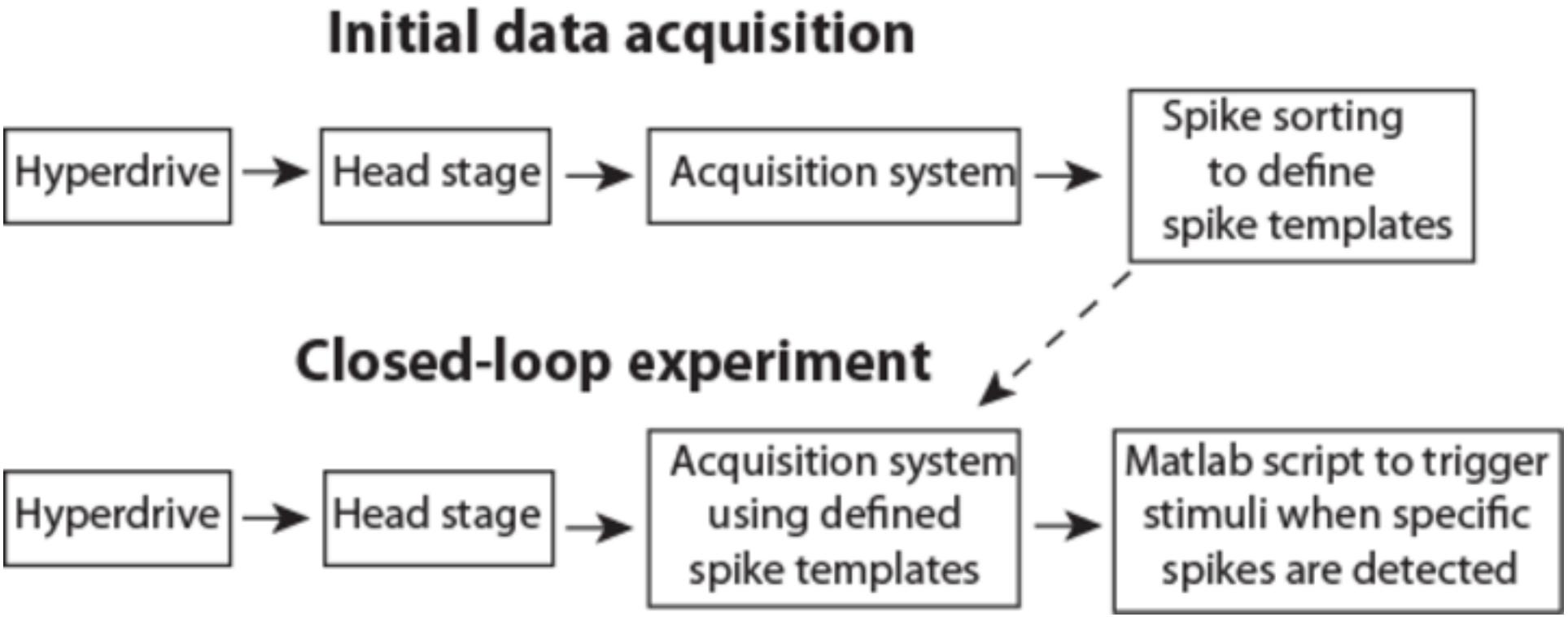

Fig 4

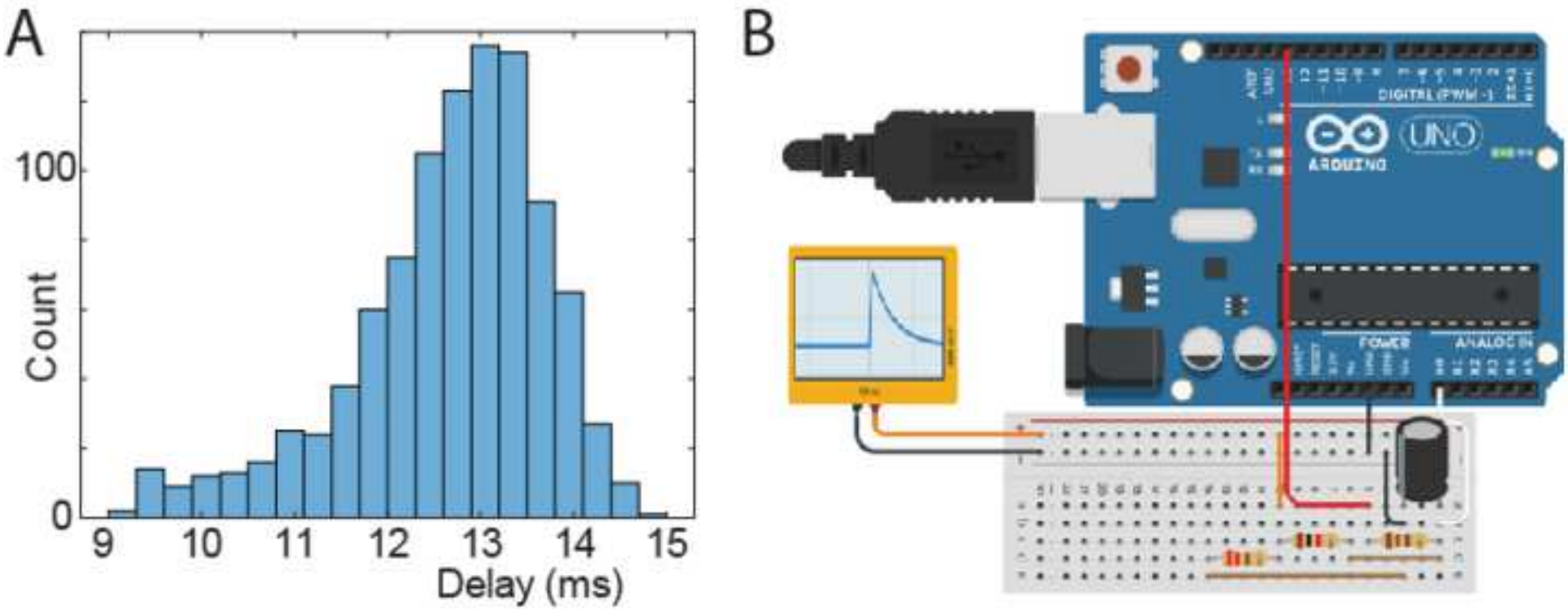

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Baytril	Bayer, Mississauga, CA	DIN 02169428	antibiotic; 50 mg/mL
Cheetah 6.4	NeuraLynx, Tucson, AZ	6.4.0.beta	Software interfaces for data acquisition
Digital Lynx 4SX	NeuraLynx, Tucson, AZ	4SX	recording equipment
Headstage transmitter	TBSI	B10-3163-GK	transmits the neural signal to the receive
Isoflurane	Fresenius Kabi, Toronto, CA	DIN 02237518	inhalation anesthetic
Jet Denture Powder & Liqud	Lang Dental, Wheeling, US	1230	dental acrylic
Lacri-Lube	Allergan, Markham, CA	DIN 00210889	eye ointment
Lido-2	Rafter 8, Calgary	DIN 00654639	local anesthetic; 20 mg/mL
Matlab	Mathworks	R2018b	software for signal processing and trigge
Metacam	Boehringer, Ingelheim, DE	DIN 02240463	analgesic; 5 mg/mL
Netcom	NeuraLynx	v1	Application Programming Interface (API)
Silicone probe	Cambridge Neurotech	ASSY-156-DBC2	implanted device
SpikeSort 3D	NeuraLynx, Tucson, AZ	SS3D	spike waveform-to-cell classification tool
Wireless Radio Receiver	TBSI	911-1062-00	transmits the neural signal to the Digital

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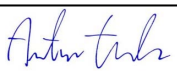
Signature:	
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Dear Dr. Luczak,

Your manuscript, JoVE59812R2 "Using neuron spiking activity to trigger closed-loop stimuli in neurophysiological experiments.," 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: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Jul 16, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

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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.

We did additional careful proofreading to eliminate any grammatical errors.

2. 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.

We inserted references in multiple steps to address this.

3. 1.6: What are the hole diameters? How many holes?

Now we clarify that typically, 4-8 screws with diameter ~0.5mm are used to anchor the implant.

4. 1.7: What are the coordinates?

We now clarify that the presented protocol for closed-loop stimulation will work properly, regardless if coordinates of implanted electrodes are set to motor cortex, sensory cortex or any other brain area.

5. 1.8: How much acrylic is applied?

Now we clarify that the amount of dental acrylic should be enough to sturdily attached implant but it should not extend beyond implant to contact soft tissue.

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We now eliminated personal pronouns.

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We would appreciate your advice on this topic. Our software is designed to work only on one type of recording system out of multiple types available on the market. Thus not mentioning explicitly in the main text for which system our protocol is designed may cause significant confusion. It is like reading instruction how to fix something, only to discover on the last page that it does not apply to my case. With other publishers, we never experienced restriction on specifying company names in the main text to disambiguate products. Therefore we kindly ask to allow us to mention right in the front, that our protocol is specifically designed only for Neuralynx system.

Reviewers' comments:
Reviewer #1:
The authors addressed all of my concerns and recommendations.
I am happy to support it being accepted.

Thank you.

Reviewer #3:

Manuscript Summary:
This article presents an easy to implement matlab software for stimulation upon detection of single or multiple neuronal activity. It is nice to present this as an method to be implemented in research. I think that both Introduction and Discussion do not discus many articles and the methods of closed-loop systems that they use. That could be interesting to enable comparison between different methods and recommendations when other methods are good to use and when this method outstands all the others.

Major Concerns:
- I think optogenetics is just one of the possible stimulation options. In human patients, there is a lot of interest in electrical stimulation. Is this also possible to apply using your software? If that is the case, you should mention one of the Neuropace studies as well, since they have applied electrical closed-loop stimulation to suppress epilepsy in a large amount of patients (Heck et al. 2014 Epilepsia, for example) in introduction. Another system for detection and delivering of therapy is Peters et al. J Clin Neurophysiol 2001.

Thank you for the suggestion. Those studies are now added in the Introduction.

- I am not familiar with Neuralynx. Is this system especially used in animal studies? This should be added in abstract line 35.

Yes, this system is mostly used in animal studies, especially in rodents. This is now specified in the Abstract.

- Page 3 line 129: I do not understand what kind of spike information is loaded. Is this an electrocorticography with epileptic spikes, or is this neuronal spiking data. I would like to see an example of the data than can be loaded.

This is neuronal spiking data, not epileptic spikes. Example of single neuronal spike is shown in Figure 2.

- Page 3 line 131. I do not understand what you mean with neuronal ensemble. Is this the kind of event you would like to detect to trigger stimulation? I would like to see an example of such a neuronal ensemble.

By neuronal ensemble we mean group of neurons. We now clarify that users select one or multiple neurons to trigger stimulation.

- Page 4 line 152, I only see a bold dashed line in figure 2, but not the displayed spike waveforms in real time, which you mention in line 152. Could you update the figure with such a plot as well?

Spike waveforms is plotted in the left top corner of Figure 2 but it was not clearly visible, as it was a dark blue trace on a black background. We have now changed the color of the spike waveform to yellow to better illustrate it.

- Page 4 line 147: what kind of clusters were observed when sorting the spikes? What were the properties of these clusters, and what were the criteria for a spike belonging to a cluster, and did all clusters trigger a stimulation?

This is a good question. Each cluster corresponds to spikes from a different neuron. For example, if a neuron is close to an electrode, then spikes from that neuron will have larger amplitude than spikes from neurons located farther away from the recording electrode. Thus, spike waveform features, like amplitude, will form separate clusters corresponding to different neurons. Differentiating spike feature clusters is often difficult, and there are multiple methods developed to facilitate this process. We now clarify this point in the text and we provide reference for more details on this topic.

- Discussion: I do not see that many references to other articles using a closed-loop system and what they're protocols are. What makes this protocol different/better than others?

We added 6 new references (including suggested above by the Reviewer) in the Introduction where we provide an overview of closed-loop literature. In the Discussion, we specify that the advantage of our method is that it only requires software installation, and it does not require any new hardware for users who already own a Cheetah recording system. Thus, for many labs studying animal electrophysiology, our protocol offers a low cost solution to implement closed-loop stimulation.

Minor Concerns:
- Page 2, line 102, Software installation: on separate computers that are. This is just one of the weird grammar examples (see also Abstract line 29-30, page 4 line 134). This should be checked!

Thank you for pointing it out. We corrected those sentences and we carefully checked grammar in the rest of manuscript.

Reviewer #4:

Manuscript Summary:
Paper describe the protocol for using closed-loop systems with optogenetics, specifically for triggering stimuli based on the activity of single neurons. Paper is well written and clear. Protocol and scripts are step-by step explained in a clear way. Authors well addressed all potential critical points. No further significant concerns has raised.

Major Concerns:
non major concerns are present

Thank you.

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