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Closed-Loop Optogenetic Intervention in Mice

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Abstract

Optogenetic interventions offer novel ways of probing, in a temporally specific manner, the roles of specific cell types in neuronal network functions of awake, behaving animals. Despite the unique potential for temporally specific optogenetic interventions in disease states, a major hurdle in its broad application to unpredictable brain states in a laboratory setting is constructing a real-time responsive system. We recently created a closed-loop system for stopping spontaneous seizures in chronically epileptic mice using optogenetic intervention. This system performs with very high sensitivity and specificity, and the strategy is relevant not only to epilepsy, but can also be used to react in real time, with optogenetic or other interventions, to diverse brain states. The protocol presented here is highly modular and requires variable time to perform. We describe the basic construction of a complete system, and include our downloadable custom closed-loop detection software which can be employed for this purpose.

Keywords

epilepsy; automated seizure detection; intracranial EEG; optrode

Introduction

Optogenetic techniques are extremely useful, cell type-specific tools for probing the functions of specific elements of neuronal networks in slices as well as in live animals^{1–21}. Light-sensitive opsins can be used not only to excite or inhibit cell types of interest in different ways, but also to affect cells based on their projection patterns, or to modulate specific intracellular cascades^{5, 7, 22}. New optogenetic tools are continuously being developed which will further enhance our ability to affect specific cells in specific ways using light. As optogenetic tools become a standard method of interrogating the functions *in vivo* of select neuronal cell types during specific brain states, the ability to introduce light with temporal precision in a responsive manner to state changes becomes increasingly important. Despite the now widespread use of optogenetics *in vivo*, a major barrier to

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harnessing the unique opportunity afforded by optogenetics for spatiotemporally precise interventions to unpredictable events, which are especially relevant to neurological disorders^{2, 11}, is the availability of a simple to use yet highly customizable system that can perform closed-loop responsive intervention over long time periods. To this end, we recently developed a custom closed-loop system to detect seizures from intracranial EEG in real time and to respond by triggering light output, activating opsins in both a cell type and temporally specific manner².

We provide here a modular protocol detailing preparation of implantable optical fibers, a simple combination of optical fiber and electrode, implantation of optrodes, and finally our closed-loop event detection and light delivery software. A number of optrode designs exist^{23–29}; our implants are straightforward to construct, compatible with long-term recordings and real-time event detection, relatively simple to use, and low-cost. The detection software described in this protocol and accompanying User Guide (Supplementary Manual) is designed to provide selectivity and sensitivity for a range of signals. Specifically, it was designed to detect, in real time, temporal lobe seizures which can display variability regarding key signal features³⁰ and are accompanied by intervening interictal spikes. Given these considerations, simpler detection^{11, 31}, such as amplitude crossing, would be insufficient. Therefore, the software builds on previous off-line detection algorithms³² and provides a number of signal detection conditions for the user to choose from based on spike, frequency, and power properties, with parameters which can be tuned to accomplish the desired sensitivity and specificity for each animal. These additional features further increase applicability, making the software relevant to the detection of a range of events, even in conditions where artifacts are present in the recorded signal.

Experimental Design

Implementing our custom closed-loop system requires several distinct elements. Foremost is a set of hardware and software capable of rapidly analyzing and responding in real time to signals of interest^{2, 11, 31}. To facilitate this, the signal of interest must be clear enough and persist long enough to allow for detection and intervention. In addition, the headstage implanted onto the animal must be very stable to allow for prolonged recording and intervention. For optogenetic interventions, appropriate control animals, for example, opsin-negative littermate controls, must included to control for light effects not related to the activation of opsins (e.g., visual sensory stimulation, tissue heating, or photoelectric effects^{33, 34}). In this protocol, we describe a very simple, inexpensive, and highly flexible method of generating a closed-loop system, as used in Krook-Magnuson et al., 2013². Specifically, as further outlined below, we detail how to: construct implantable optical fibers for delivering light; combine these implantable fibers with electrodes to create optrodes; implant these components into a stable headstage; and perform online closed-loop intervention. We provide in the supplementary material a modified version of the stand-alone custom software which we developed for optogenetic intervention during seizures in chronically epileptic animals². This version of the software has increased functionality and improved user interfaces. Our software is highly flexible and customizable, requires no programming experience, and is available here for download.

Preparation of fibers—While it is temptingly time-efficient to purchase premade implantable optical fibers (available from ThorLabs or Doric Lenses), it is fairly easy and considerably cheaper to make your own. As an example, once initial equipment costs are taken into account (<\$2000), we estimate that making 500 implantable fibers in-house in batches of 100 takes about 100 person hours and costs just under \$2500 in reagents. With current prices, 500 implantable fibers can be purchased commercially for between \$9800 and \$20,000.

Please note that you can find similar directions on making implantable optical fibers to those provided here from a number of sources (http://www.openoptogenetics.org/index.php?title=Fiberoptic_Guides_and_Connectors#Ferrule_Connectors_.28fiberoptic_cannula.29)^{35, 36}, as well as detailed instructions for parts of the procedure from ThorLabs' website (<http://www.thorlabs.us/thorcat/1100/FN96A-Manual.pdf>; <http://www.syntheticneurobiology.org/protocols/protocoldetail/35/9>). Below is a brief outline of our protocol for making implantable optical fibers. Key steps are illustrated in Figure 1.

Preparation of optrodes—While it is also possible, and in some cases desirable to implant the electrode and implantable optical fiber separately, if the fiber and electrode are targeting the same location, it can be helpful to make an assembly of the two before implanting, ensuring that the electrical wires are positioned in a way that minimizes potential for photoelectric effects. We used a simple premade twisted bipolar electrode from PlasticsOne to achieve a single differential intracranial EEG signal from each animal³⁷. The steps outlined below are illustrated in Figure 2.

Advantages of this protocol include the use of relatively inexpensive, readily available, materials and an interface which does not require specialized combined headstage/amplifier/software systems. Additionally, the placement of the different components of the headstage can easily be adjusted, providing flexibility regarding brain regions targeted, etc. Thus, this protocol provides a simple but effective and flexible way of combining recording electrical activity and optogenetic intervention.

Implanting the optical fibers and optrodes—For intervention during unpredictable events such as seizures, long-term stable headstages for performing recordings and interventions are essential. While similar to established surgical implantation techniques^{38–41}, we describe here our own protocol for a relatively low profile headstage which is well tolerated by the mice and, when performed as described, quite stable. Indeed, we have had success in performing continuous tethered video-EEG recordings with optical intervention for several months.

On-demand event detection & light triggering—This portion of the protocol, and the accompanying User Guide (Supplementary Manual), explains the recording and analysis software, which allows real-time event detection and light delivery. The software allows the experimenter to utilize a number of features for detecting events of interest including spike, frequency, and power properties. Parameters determining detection can be independently selected and tuned to the specific signal of interest, providing not only flexibility (that is, being able to selectively target diverse types of signals), but also sensitivity and specificity

(that is, when tuned properly, the system yields low false negative and false positive rates as detailed in the supplementary material of Krook-Magnuson et al., 2013).

Data analysis—The approach to data analysis will of course largely depend on the experimental details and scientific questions to be addressed. We include a brief description of a general analysis process similar to that used in Krook-Magnuson et al., 2013.

Limitations & applications—This protocol will be most useful to neuroscientists seeking responsive optogenetic or other intervention technologies. The system described here was originally developed for detecting and responding in real time to spontaneous seizures in chronically epileptic animals, but portions of the protocol are highly modular (and can be used independently of other sections). In addition, the seizure detection program consists of independent algorithms which can be used in isolation or in conjunction with one another, providing great flexibility. Therefore, it is not only capable of detecting a variety of seizure types, but can also be adapted with minimal adjustment to respond to other electrical signals which have a characteristic appearance, including non-epilepsy related patterns (sleep, brain rhythms, etc.), electromyographic, and even perhaps electrocardiographic recordings.

The implanted headstages described in this protocol for intracranial EEG recording and light delivery are designed to be maximally flexible, relatively inexpensive and simple to implement. They therefore use (and the analysis of the incoming signals are based on) a single intracranial EEG channel for each mouse. The size of the prefabricated electrical pedestals used in the implanted headstages described here also limits the number of implanted items that can fit on one mouse. However, because the program is able to use any input to BNC, utilizing the software is not limited to use with the headstage system described in this protocol, and requires only that the output of the amplified signal have no delay and be convertible to a BNC input to the digitizer. Thus, researchers may find it useful to interface portions of this protocol with their own existing methods of acquiring recordings^{36, 42–46}. Additionally, this protocol makes use of separate optical and electrical commutators. This is somewhat cumbersome, as the cables can become twisted, requiring monitoring and occasional untwisting; combined optical and electrical commutators are commercially available which may address this problem, requiring only different cables and recording equipment.

Materials

CRITICAL Also see Supplementary Table 1: Equipment and Reagents for a list of ordering information and approximate costs for these materials.

Reagents

- Epileptic mice expressing opsins², see Reagent Setup.

CAUTION: All procedures involving animals must be approved by your institutional animal care and use committee and must comply with institutional and national regulations.

- Implantable optical fibers terminated in 1.25mm diameter ferrules. These are available pre-made (ThorLabs or Doric Lenses), or see below for instructions to make your own.
- Bipolar depth electrodes with pedestal (PlasticsOne MS303/3-A/SP)
- Gel super glue (we prefer Loctite brand 130380)
- Thin thread or suture material (Patterson Veterinary supply 07-8102709)
- Small screws (McMaster-Carr 91773A052)
- Dental cement (Teets Cold Curing, Pearson Dental, C73-0076, C73-0060)
CAUTION: Flammable, may cause skin reaction or eye irritation—use in a well ventilated area and avoid contact with skin and eyes.
- Weigh dishes for mixing dental cement (Fisher Scientific 02-202-100)
- Small (1cm diameter, 1mm height) plastic rings. We use 5mL pipette tips cut into 1mm segments (USA Scientific 1050-0700).
- Permanent marker or pencil
- Ceramic zirconia split sleeves, 1.25mm ID (Precision Fiber Products SM-CS125S)

Additional reagents necessary to make your own implantable optical fibers

- Optical fiber: We used 0.37NA, Low OH, 200µm diameter fiber (ThorLabs FT200EMT)
- Epoxy and syringes for injecting epoxy (available from ThorLabs F112, MS403-10)
- Razor blades (American Safety Razor single edge, 66-0089-disp)
- Ethanol (70%) & distilled water in squeeze bottles
CAUTION: Ethanol is flammable, avoid eye and skin contact.
- Polishing sheets of decreasing grain (5µm, 3µm, 1µm, 0.3µm; ThorLabs, LFG5P, LFG3P, LFG1P, LFG03P)
- Ferrules: We used 1.25mm ceramic ferrules, 225µm ID (Kientec Systems, Inc. FZI-LC-225)
- Lint-free wipes (Fisher Scientific, 066-666-A)
- Small petri dishes (Fisher Scientific, 08-757-100B)

Equipment

- Forceps with fine points (Fine Science Tools, 11253–25; WPI, 500342)
- Size #0 screwdriver (McMaster-Carr, 7026A18)
- Stereotaxic surgical setup, including a stereotaxic arm suitable to accommodate the ferrule holder stereotaxic adapter, reliable anaesthesia induction methods, analgesia

and antibiotics, a drill, and sterilized surgical instruments (see Supplementary Table 1 for suggestions). CAUTION All procedures involving animals must be approved by your institutional animal care and use committee.

- Ferrule holder stereotaxic adapter (Doric Lenses, SCH_1.25)
- Laser or LED light source with TTL trigger (various sources exist, we used Shanghai Laser & Optics Century, see Supplementary Table 1)
- Optical commutators (Doric Lenses FRJ_FC-FC, or ThorLabs)
- Fiber optic patch cords with appropriate connectors for the light source to the commutator (e.g., FC/PC on both ends), as well as for the commutator (FC/PC) to a bare 1.25mm ferrule (to connect to the implanted optical fiber). When ordering cables, determine the necessary lengths prior to making the order (Doric Lenses or ThorLabs). CRITICAL: The cables that go from the commutator to the mouse should have as little slack as possible, while still allowing the animal to reach all corners of the cage comfortably.

- Electrical commutator & cables (PlasticsOne SL2C/SB, and cables). When ordering cables, determine the necessary lengths prior to making the order.

CRITICAL: The cables that go from the commutator to the mouse should have as little slack as possible, while still allowing the animal to reach all corners of the cage comfortably.

- Analog amplifier compatible with the electrical cables and digitizer used. In order to be compatible with the cables and digitizers listed here, the amplifier should accept banana plug input (banana to BNC converters are readily available), and the output of the amplifier should be (or be convertible to) BNC. We used a Brownlee 410 patch clamp amplifier.
- National Instruments Digitizer.

CRITICAL: The custom software supplied here is designed to pair with NI Digitizers. We have tested both the 8 channel NI-USB-6221-BNC and the 16 channel NI-USB-6229-BNC digitizers. Other M series NI digitizers may also work, but have not yet been tested.

- Dedicated computer running Windows with at least 4GB memory. We have tested the recorder program on two different Dell Optiplex (990 and 7010) computers running Windows7 professional (either 32 or 64 bit is acceptable) with i5 processors and either 4 or 16GB memory, respectively. It is helpful to have a second, large capacity (>500GB) hard drive dedicated to data storage. We have tested the analyzer on computers running either Windows7 or WindowsXP.
- Low light compatible USB web cams. We recommend using Logitech C270 HD webcams—others can be used (minimum frame rate of 5 frames per second), but with caution as some webcams may cause problems with video/EEG synchronization. The minimum light sensitivity needed will depend on the ambient

light levels provided by the red nighttime lighting in the specific room that is being used as well as the precision necessary for the behavior being monitored.

- Normal white lighting and red lighting (for nighttime recording) on day/night timers (Intermatic, TN311)
- Clear cages with tall sides and open tops, or with lids with built-in commutators. Note also that you will want food and water available to the animal without risk of entangling the cords on water bottles, etc.

Additional highly recommended equipment:

- Dissecting microscope (particularly if you plan on making your own optical fibers)
- Uninterrupted Power Supply (OfficeMax, 21880582; without this, an interruption in power can cause the computer and/or software to stop running, which may cause the lasers to turn on, compromising your experiments).
- Light power meter and sensor (ThorLabs, PM100A, SV120VC). This is useful to check both the power of the lasers and the quality of the implantable optical fibers (see below).
- External Hard Drives (OfficeMax, 23192026), large capacity. For 8 channels of intracranial EEG sampling at 500Hz and 4 videos (which record at 5 frames per second), plan on using 1GB of storage/hour. Thus, about 6 weeks of data can fit on a 1TB external drive. This is largely dependent on the size and number of the video files.

Additional equipment necessary for making your own implantable optical fibers

- Diamond scribe (ThorLabs, S90W)
- Clamp or mounted alligator clip (A-M Systems, 726200)
- Glass polishing plate (ThorLabs, CTG913)
- Polishing disc for bare ferrules (ThorLabs, D50-L)
- Fiber stripper appropriate for the fiber you choose (ThorLabs, T12S21)
- Light power meter and sensor (ThorLabs, PM100A, SV120VC). This is also listed above as optional equipment, but is especially useful if you are making your own implantable optical fibers.

Reagent setup

Mice

For epilepsy induction, we use 50–100nL of 20mM kainic acid (Tocris 0222) injected unilaterally into the hippocampus (at 2.0mm posterior, 1.25mm left, and 1.6mm ventral to bregma)². The dose is adjusted by strain to minimize acute mortality, and to reliably generate animals with late spontaneous seizures. We use animals that are at least postnatal day 46, which allows reliable targeting of the hippocampus and induction of epilepsy.

For opsin expression, we used mouse lines expressing Cre in specific cell populations (either PV cells, Jackson stock 008069, or CamKII cells, Jackson stock 005359), and crossed these to either floxed halorhodopsin (Jackson labs, stock 014539) or floxed channelrhodopsin2 (Jackson labs, stock 012569) lines^{19, 47–49}. Of course, viral vectors containing Cre dependent or independent constructs can alternatively be used to introduce opsins in animals. These techniques are described elsewhere^{17, 36, 50}.

Control animals are important to include in every experiment, since they allow differentiation between opsin-mediated effects and effects due to tissue heating, photoelectric effects, or visual perception of the light stimulus by the animal^{33, 34}. In our experiments, in addition to no-light internal controls, we used epileptic opsin-negative littermate control animals to ensure an opsin-mediated light effect.

Procedure

(Optional) Creating your own implantable optical fibers: Timing: ~17 hours for 100 implantable fibers

1. Cut and strip a length of fiber (Figure 1 a–b). The fiber stripping tool can only handle about 10cm of fiber at a time, so we typically cut a length of 30cm or so and then work down the piece of fiber, stripping short segments. Do this 6–7 times.

CAUTION: Wear gloves and appropriate personal protect equipment when working with the fiber, as microscopic shards of glass can get in your eyes or under your skin.

2. Wipe the stripped fibers with a wet Kimwipe to clean them

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

3. Use the diamond scribe to cut the stripped fiber into 10–15mm long pieces (Figure 1 c–d). To use the diamond scribe, hold it perpendicularly to the fiber and gently score the outside of the fiber. Then, firmly pull the two ends of the fiber apart to break it cleanly. The fiber pieces will have a lot of static cling. We pick them up with forceps and then firmly tap the forceps over a petri dish where we store the cut fiber pieces.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

4. Once you have more than 100 pieces (we usually do batches of 100 at a time to save time and epoxy costs), prepare the mounted alligator clip or a clamp to hold the ferrule while you fill it.
5. Mix the epoxy and pour it into the back of the filling syringe with the cap on. Then, flip the syringe over, remove the tip, allow the epoxy to run down to the stopper, and remove excess air before replacing the tip.

6. Clamp one ferrule in place and fill the larger (flat, concave) end with epoxy (Figure 1 e–f).
7. Use forceps to gently slide one piece of fiber into the back (flat, concave) end of the ferrule (Figure 1 g).

CRITICAL STEP! Take care not to bend or scratch the fiber with the forceps, and if the fiber breaks inside the ferrule, use another piece of fiber to push it through and replace it with an intact fiber.

8. Once the fiber has emerged from the smaller, convex end of the ferrule, remove the ferrule from the clamp and place it on a smooth disposable surface (we use disposable petri dishes), taking care to get as little epoxy on the outside of the ferrule as possible. Note that the fiber remaining at the other end of the ferrule (the flat, concave end) will be the part that is inserted into the brain; ensure that this is sufficiently long to reach the brain area of interest.
9. Repeat for as many ferrules as you can before the epoxy gets too hard (work fast).
10. Allow the epoxy to cure for at least 24h, or until it is hardened. The ferrules will be easiest to work with once the epoxy is fully cured; dry excess epoxy will flake off smooth surfaces when scraped.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

11. Score the end of the fiber that has emerged from the smaller, convex end of the ferrule as close to bead of epoxy at the end as possible (Figure 1 h). It is helpful to perform this step using a dissecting microscope.

CRITICAL STEP! Take care not to shatter the fiber close to the ferrule—if it breaks inside the ferrule the light losses will be significant, and polishing the fiber enough to get to intact fiber will be difficult.

12. Use a razor blade to scrape any excess epoxy from the outside wall of the ferrule—any epoxy remaining can become lodged in the polishing disk and will make it difficult to insert the ferrules.

CAUTION: Handle and dispose of sharps, including razor blades, appropriately.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

13. Use a squeeze bottle with ethanol and Kimwipes to thoroughly clean the glass polishing plate of all debris. While still slightly wet, lay the 5µm grain (this is the coarsest grain) polishing sheet, matte surface up, onto the glass plate. Also clean the matte surface with ethanol and a Kimwipe.
14. Insert the ferrule into the polishing disc with the cleaved, smaller end emerging from the bottom of the disc. If using the polishing disc for bare ferrules, clamp the ferrule in once it touches the plastic base provided.

15. Gently set the polishing disc on the 5 μ m grain polishing sheet. Begin making figure eights with the disc, being careful not to press down too hard and break the fiber.

CRITICAL STEP! If the fiber breaks at this step, it is likely to shatter well into the ferrule shaft and the fiber will be unusable.
16. Continue to polish in figure eights with increasing pressure until the fiber is flat with the end of the ferrule (Figure 1 i). If using the LC connector polishing disc (rather than the polishing disc for bare ferrules), use a fingernail to gently place a constant downward pressure on the ferrule.

CRITICAL STEP! Take care not to break or put pressure on the fiber to be implanted into the brain, which will be sticking up during the polishing steps.
17. It is very helpful at this stage to use a dissecting microscope to inspect the polished end of the ferrule (Figure 1 j). Once the epoxy is nearly polished away and the fiber appears free of cracks or deep scratches, it is ready for the next step. To save time, repeat for the entire batch of ferrules before proceeding to the next step.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).
18. Continue the polishing process similarly using polishing sheets of decreasing grain. Clean the glass plate with ethanol and a Kimwipe and place the next most coarse grain, 3 μ m polishing sheet on the glass plate, matte side up. Clean the matte surface with ethanol and a Kimwipe. Polish the fiber again in figure eight patterns until the epoxy is completely removed from the ferrule and the fiber appears free of scratches. Repeat for the entire batch of ferrules before proceeding to the next step.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).
19. Clean the glass plate with ethanol and a Kimwipe and place the finer, 1 μ m polishing sheet on the glass plate, matte side up. Clean the matte surface with ethanol and a Kimwipe. Place several drops of water on the polishing sheet, and put the polishing disc down such that the end of the ferrule is in the water. Moving slowly across the polishing sheet, polish the ferrule in 15 figure eights. After this step, the fiber should appear, under the dissecting microscope, to be quite shiny and free of scratches or dark spots.

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).
20. If desired perform a last polishing step with the finest grain polishing sheet. This allows for a very smooth surface, but if sufficient light transmission for the desired application is already achieved after the 1 μ m grain sheet (step 19), it can be omitted. Clean the glass plate with ethanol and a Kimwipe and place the finest grain, 0.3 μ m polishing sheet on the plate, matte side up. Clean the matte

surface with ethanol and a Kimwipe. Place several drops of water on the polishing sheet, and put the polishing disc down such that the end of the ferrule is in the water. Moving across the polishing sheet, polish the ferrule in 3 figure eights. After this step, the polishing is complete (Figure 1 k).

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

21. To test the power coming from each of the implantable optical fibers, prepare the power meter to respond to the correct wavelength of light and use the patch cables and a split sleeve to attach one of the newly made implantable optical fibers to the laser.

CAUTION: When working with lasers, always use protective eyewear appropriate for the wavelength and power of laser light.

22. Turn on the laser and measure the power at the tip of the optical fiber.
23. Depending on the strength of the laser and the patch cords used, the actual power at the tip of a usable implantable fiber will vary. Good fibers will transmit at least 80% of the light from the end of the patch cord to the tip of the implantable fiber. Implantable fibers with lower light transmission may be acceptable for the desired application provided the light source is strong enough. However, fibers should be discarded if the fiber has been damaged such that light escapes from within the ferrule (the ferrule stick will glow in this case, if you are using ceramic zirconia ferrules) or from the side of the implantable fiber. In the latter case, the protruding fiber can be recleaved above the damaged portion to salvage the implantable fiber. Fibers that transmit <30% of the light at the end of the patch cord may be broken, scratched, cracked, or still coated in epoxy (Figure 1 l). Discard any fibers that do not produce sufficient light transmission.

TROUBLESHOOTING

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).

(Optional) Making an optrode from the implantable fiber and bipolar electrode:

Timing: 10 minutes per optrode

24. Measure the desired length of the optical fiber. Add ~1mm to the desired depth to allow space for attaching the electrical fiber (Figure 2 a).
25. If necessary, cleave the end of the fiber to the desired length using the diamond scribe, being careful not to damage the fiber. For a description of how to cleave fibers, see step 3 above.
26. Plan out the desired location of all optical and electrical fibers using a brain atlas⁵¹. Keep the size guidelines given in Box 1 in mind.

27. Bend the bipolar electrode to be almost parallel with the base of the pedestal. As mentioned above, it will conserve space to angle the pedestal slightly away from the ferrule.
28. Measure the desired distance along the bipolar electrode from the center of the pedestal to the location of the optical and electrical implant site, and bend the bipolar electrode at a 90° angle away from the pedestal at this distance (Figure 2 a–b).
29. Cut the bipolar electrode cleanly to be just shorter than the optical fiber protruding from the end of the ferrule (Figure 2 c).
CRITICAL STEP! If the electrical contacts sit under the path of the light, the chance for observing photoelectric effects is much higher.
30. Use a length of very thin, flexible thread or suture to tie the electrode to the optical fiber, and position in an ideal arrangement (Figure 2 d–f). It can be helpful to use a bit of tape stuck to the table to hold things in place during this process.
31. Place a drop of super glue on a napkin or other disposable surface. Use a needle or forceps to pick up a drop of glue, and place it over the suture or thread material, allowing it to climb part way down the fiber and electrode to adhere them to each other (Figure 2 g).
CRITICAL STEP! Only glue the top part of the assembly. Do not let the glue touch the bare ends of the wire. This will block the electrical signals and will result in a poor intracranial EEG signal.
32. Trim the suture or thread material and allow the assembly to dry for several hours prior to implanting (Figure 2 h–i).
Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C).
33. Once the glue has dried, gently adjust the angle of the pedestal in relation to the ferrule.

Surgical implantation of fibers and electrodes: Timing: 40min–2h per animal, depending on the skill of the surgeon and the complexity of the desired implant.

34. Anaesthetize the animal, shave its head, and place it securely in the stereotax (detailed surgical technique protocols can be found elsewhere^{38–41}).
CAUTION: Use standard intra-operative and post-operative analgesia techniques approved by your institutional animal care and use committee. All procedures involving animals must be approved by your institutional animal care and use committee and must comply to institutional and national regulations.
35. Use forceps to lift the scalp, and scissors to remove a circle of skin centered between bregma and lambda. Alternatively, use a scalpel to cut down the

midline, and pull skin to the sides to reveal the skull. Figure 3 outlines the key steps to implant the materials (Figure 3 a) into a stable headstage.

36. Use a scalpel to clear away the fascia under the scalp and to expose the skull from several millimeters in front of bregma to several millimeters behind lambda. Continue to clear fascia from the skull until none remains adherent to the bone. Avoid removing the neck muscle attachments to the skull unless you need to place optical fibers very posteriorly. However, be sure to remove any tissue that will be within the implant area (Figure 3 b).

CRITICAL STEP! If any tissue remains between the skull and the implant, when the implant is finished, the tissue may become necrotic and incite inflammation. This may cause the implant to become loose.

37. If desired, place a drop of hydrogen peroxide on the cleaned skull for several seconds, and then wipe it away to highlight both the skull sutures as well as any remaining tissue to be scraped off of the skull (the hydrogen peroxide will cause the remaining tissue to become white).

CAUTION: Avoid putting the hydrogen peroxide on the free edges of the scalp and flush the tissue with excess saline as it can excessively dry the tissue or cause weakening of the skull.

38. Identify and measure the coordinates of bregma (Figure 3 c) and lambda (Figure 3 d) using the implant placed in the stereotaxic adapter for holding ferrules.

CRITICAL STEP! For accurate targeting, the head must be placed in a head flat position. Use the z coordinates of the stereotaxic arm to ensure that bregma and lambda are located at the same z coordinates. Adjust the position of the mouth bar and re-measure the coordinates of bregma and lambda as necessary to achieve a head flat position.

39. Use the stereotaxic arm to measure the desired location(s) for optical fibers and electrodes⁵¹. At each location, use a permanent marker or pencil to mark the location for drilling. It can also be useful to scratch a target into the permanent marker to more precisely center the desired craniotomy location (Figure 3 e).
40. Prepare the small plastic ring for fixation to the skull. Fixing this ring to the skull at the margins of the implant area will provide a well in which to pour the dental cement to prevent it from spilling. It can be helpful to bend the ring into a teardrop shape to better fit the shape of the skull and allow more anterior-posterior space for placement of implants (Figure 3 f).
41. Use a hand drill or dental drill to carefully make holes in the skull for the optical fibers, electrodes, and screws (Figure 3 g).

CRITICAL STEP! Do not allow the drill to touch the surface of the brain. When the skull is thinned enough, it should be possible to use a sharp pair of forceps or fine scissors to make a very tiny hole in the skull, and to remove the remaining thinned bone. This will ensure that unnecessary damage to the cortex does not occur.

CRITICAL STEP! The holes for the screws should be just smaller than the diameter of the screw threads.

42. Fit the screws into the pre-drilled holes (Figure 3 h–i). Once the screws are secure in the bone, you can leave the screws in if bregma is not obscured, or you can remove them for later placement if bregma is not accessible with the screws in place.

CRITICAL STEP! Use slight pressure to encourage the threads of the screws to bite into the bone. Secure screws are key to having a stable implant.

43. Hold the plastic ring with forceps and place a generous amount of gel super glue on the bottom and outside of the ring.

CRITICAL STEP! Be sure that the ring will sit on dry, well-cleaned skull at its most anterior and posterior points of contact.

44. Use another pair of forceps to hold the skin back, and place the ring firmly on the skull. Use the forceps to place the edges of the scalp into the glue on the outside of the ring. Gently press down to ensure that the ring is well fixed to the skull at its anterior and posterior borders (Figure 3 j). Alternatively, if desired, this ring can be placed after some or all fibers are in place, but exercise caution when doing so to avoid displacing fibers or altering head position.

45. Readjust the animal in the head bars if necessary, ensuring that the head is still flat. Measure bregma with the implant in the stereotaxic adapter.

CRITICAL STEP! The animal's head should not move after this step, so ensure the animal is securely in the head bars, and be careful not to make any jarring movements.

46. Position the stereotaxic arm at the desired medial-lateral and anterior-posterior coordinates, and gradually lower it toward the craniotomy. If placing the combined optical and electrical probe, ensure that the fiber and the electrode will both be able to reach their desired location as you lower the fiber assembly into the brain.

47. Identify the site at which the fiber will penetrate the dura and use a closed sharp forcep or scissors to gently nick and the spread open the dura (Figure 3 k).

48. Place a layer of gel glue onto the base of the ferrule, avoiding the fiber itself. Also place a drop of glue on the base of the electrode pedestal.

CRITICAL STEP! Make sure not to get any glue on the bare electrical wire tips.

49. Lower the fiber and electrode to the desired z position in the brain. Ensure that the glue is touching both the ferrule and the skull (Figure 3 l). If you will place another ferrule stereotaxically, wait several minutes for the glue to dry before releasing the ferrule from the stereotaxic holder.

CRITICAL STEP! Do not move the ferrule as you release it.

50. Repeat steps 46–49 until all ferrules have been placed (Figure 3 m). If you are placing ferrules very close together, you can use a split sleeve to extend the reach of the stereotaxic arm to avoid knocking over the previously placed ferrules. However, ensure that you place the sleeve only on the tip of the ferrule, since it should not be cemented into the implant. When the last ferrule has been placed, the stereotaxic arm can be left in place until after the implant is finished.
51. If you removed the screws in the step 42 to allow bregma to be measured, gently screw the screws back in, being very careful not to move the skull or knock over any ferrules. Because they were fitted earlier, they should screw in without force.
52. Mix the dental cement and solvent in a weigh dish until it is slightly thickened (the consistency of maple syrup) and carefully pour the cement into the plastic ring, avoiding the ferrules and electrode pedestal, until it covers the screws and the bases of the ferrules and pedestal (Figure 3 n).

CRITICAL STEP! If any cement remains more than about halfway up the sides of the ferrules or the electrode pedestal, gently scrape it off, as it will prevent the animal from being plugged in properly later.

53. Allow the cement to harden, apply post-operative antibiotics and analgesics (we use Flunixin 2.5mg/kg subcutaneously, a topical bacitracin and tetracaine powder at the incision site, and Enrofloxacin 5mg/kg intramuscularly) and employ standard surgical recovery protocols (Figure 3 o).
54. House the animals singly from this point on, as other mice will chew on the implants, which can damage the implanted optical fibers. Wait several days between implanting and attaching the tethers to the animals to allow the implant to stabilize and heal properly.

Pause Point: experiment can be paused indefinitely and animals can be housed in their standard cages until ready for recording.

Hardware & software setup: Timing: Variable, 1–2 hours once shelving and equipment have been arranged in a satisfactory location.

55. On the recording computer, install drivers for NI digitizers (<http://joule.ni.com/nidu/cds/view/p/id/3622/lang/en>, or go to National Instruments home→ support→ Drivers and Updates and select NI-DAQmx 9.6.1, or enter your digitizer's number and select a more recent version). This is a large file, so leave ample time for this installation.

CRITICAL STEP! Note that the MATLAB software was designed for computers running Windows. Always right click the executable and select 'run as administrator' (in Windows 7) to avoid installations not working properly. Before running the software and collecting data, install the necessary software.

56. On the recording computer, install drivers as needed for any cameras. Logitech webcam software can be downloaded here (<http://www.logitech.com/enus/>

support/hd-webcamc270?crd=405&softwareid=10505&bit=64&osid=14§ion=downloads).

57. On the recording computer, and any computer running the analysis program, install MATLAB runtime environment 2011b (MCRInstaller (2011b).exe). This will be required to run the compiled custom software. You only need to run this once on each computer prior to running the custom software.
58. Place the custom recorder executable file (Supplementary Data 1b, Soltesz_Recorder_v1.exe) into a dedicated folder into which all files will be recorded. The analyzer executable (Supplementary Data 1c, Soltesz_Analyzer_v1.exe) can be placed in a different folder, and even on a different computer.
59. Disable all automatic updates and other timed actions by the computer. Also disable automatic hibernation or shutdown of hard drives. Remember to manually update Windows and antivirus software periodically to properly maintain your computer.
60. Once the appropriate software has been installed, set up the hardware. Arrange the mouse cages, electrical commutators, amplifier, and video cameras in an area of low electrical noise. We used wire mesh to enclose a set of shelves, creating a low noise environment (Figure 4).
61. Set up lighting using day/night timers. We use a bank of red lights that are constantly on, and a timer which turns on a lamp with white light during the day.
62. Connect the appropriate cables between the commutators, amplifier, digitizer, computer, and lasers or LEDs, as diagrammed in Figure1 of Krook-Magnuson et al., 2013², placing the electrical and optical commutators close to each other over the center of the cage. Note that combined optical/electrical commutators are now available commercially (for example, from Doric Lenses), but require different electrical cables.
63. Connect the appropriate laser and electrographic input to each channel as follows:
 - a. Channel 1: Analog input BNC port AI0, Digital & Timing I/O PFI 0/P1.0
 - b. Channel 2: Analog input BNC port AI1, Digital & Timing I/O PFI 1/P1.1
 - c. Channel 3: Analog input BNC port AI2, Digital & Timing I/O PFI 2/P1.2
 - d. Channel 4: Analog input BNC port AI3, Digital & Timing I/O PFI 3/P1.3
 - e. Channel 5: Analog input BNC port AI4, Digital & Timing I/O PFI 4/P1.4

- f. Channel 6: Analog input BNC port AI5, Digital & Timing I/O PFI 5/P1.5
- g. Channel 7: Analog input BNC port AI6, Digital & Timing I/O PFI 6/P1.6
- h. Channel 8: Analog input BNC port AI7, Digital & Timing I/O PFI 7/P1.7 Pause Point: experiment can be paused indefinitely and materials left at room temperature (**20–25°C).

64. Attach the electrical patch cable to the headstage of the mouse and the electrical commutator. It may be helpful to use a short acting inhaled anaesthetic such as isofluorane to briefly anaesthetize the animal. It may also be helpful to use a split sleeve to connect the optical patch cable to the implanted optical fiber(s) at this time. However, the lasers should not be turned on until the tuning process has been completed (see below).

CRITICAL STEP! Be careful not to pull on the headstage during this process, nor while placing the animal into the cage, as it can be dislodged with excess force.

65. Prior to starting the custom software, use the Logitech webcam software to ensure that the camera will record the entire cage of the animal.

CRITICAL STEP! Completely close the Logitech software before attempting to start the custom MATLAB software.

66. Right click on the Soltesz_Recorder_v1.exe file (Supplementary Data 1b) and choose 'Run as Administrator'. On subsequent occasions, it should be sufficient to double click the executable file.
67. Select the number of intracranial EEG channels (up to 8) and video inputs (up to 4) and the desired folder name for the files. The file date and time are automatically added to the end of the files.
68. Initial recording should be done without adjusting the detection parameters. Select Start Multi File Recording to record baseline signals.
- CRITICAL STEP! Ensure that lasers are in the off position until you have tuned the detector. See Initial assessment and tuning of intracranial EEG signal below.
69. Record several hours of baseline intracranial EEG.
70. Note that as the animal moves around the cage, the electrical and optical cables will need to occasionally be untwisted.

Initial assessment and tuning of intracranial EEG signal: Timing: Variable

71. Once initial data has been collected, assess the quality of the signal. If seizures (or other events of interest) have been recorded, you are ready to tune the detector to the specific attributes of the events you wish to detect. A summary of different detection parameters is shown in Figure 5. This process will vary

depending on the signal and your experimental goals. For example, you can make the detector more or less strict, to minimize false positives or false negatives, respectively. Steps 78–88 are an example tuning procedure. Note that a detailed “User Guide” to both the Recorder and Analyzer programs is included with the executables in the supplementary material as a Supplementary Manual. This “User Guide” explains the user interfaces, as well as the various available parameters for detection. The optimal parameters for detecting an event will vary depending on the nature of the signal. For our studies in the intrahippocampal kainate model of epilepsy in mice, we found spike features, along with power-based exclusion criteria useful in a majority of cases. However, some signals were best identified using other combinations of parameters, so the optimal parameters should be determined empirically.

72. Start the analyzer program (Supplementary Data 1c). You may need to right click on `Soltesz_Analyzer_v1.exe` and ‘Run as Administrator’. Note also that the first time the program is opened, and in general on computers with lower available memory, this can take a few moments.
73. Open a previously recorded file. To do this, click “Open Input File” and select a file.
74. Select the channel of interest. To do this, click the Ch1 button until the appropriate channel number appears on the button.

See TROUBLESHOOTING

75. Narrow the display window to target the area of interest (that is, the portion of time that contains the seizure). Note that the User Guide (Supplementary Manual) contains detailed instructions on how to interact with the software, including how to change the duration of the signal in the display window.
76. Decide on initial parameters to investigate. For example, are there positive going spikes occurring at regular intervals? Open the plot control window, and select the parameters you wish to see displayed when you analyze the signal. Note that in addition to the frequency band ratio plot, you can also select “Spectrum” to see the relative energies of various frequency bands during the event.
77. Adjust the parameters for the conditions you have chosen and click analyze. The selected plots will appear based on the chosen parameters. Use the data in the plot windows for the different parameters to observe how the selected thresholds and settings affect the triggering for that parameter. Note that both the frequency at which the occurrence of triggers is calculated (frame size) as well as the time window over which each parameter is integrated to determine a trigger is user specified. Detailed information can be found in the User Guide (Supplementary Manual), but an example of tuning a signal is given in steps 78–88 below. Proper tuning reduces false positives and false negatives (Figure 6 a–b).
78. If you are detecting events based on positive going spikes (using Condition 1), first examine the threshold for spikes in the top panel of the Spikes1 plot. Are all

of the intended spikes crossing the threshold? As illustrated in Figure 6 b, if the threshold is set too high, events will be missed.

79. Next, consider the widths and inter-spike intervals of the spikes you wish to be counted. Examine the spikes in the top panel of the Spikes1 plot. Those marked with circles are counted as valid based on the current criteria; those that cross the threshold but lack a circle have failed to meet either the interspike interval or width requirements. Adjust these parameters (Min Dist, Max Dist, Min Width, Max Width, and associated Threshold Low) until the spikes you wish to be counted are marked by circles. Tightening these parameters can be done to exclude artifacts (Figure 6 a).
80. Decide on the minimum number of valid spikes required within the user-defined *time*. Note that this time window can be adjusted separately for each selected parameter to determine the size of the sliding window to be analyzed. The number of spikes in the selected *time* can be estimated by simple visual inspection of the events.
81. Next, adjust the required inter-spike interval regularity at which the spiking must occur (ratio). Note that, as further discussed in the User Guide (Supplementary Manual), the ratio value will not be calculated until the minimum number of valid spikes has been met. The ratio value is displayed, once it is being calculated, in the middle (for positive going spikes) and bottom (for negative going spikes) panels of the Spikes1 plot.
82. Finally, set the number of frames that the spiking requirements must be satisfied (Trigger True) before the condition will trigger.
83. Continue to adjust these values, and periodically click on analyze to check the results, until the spikes and finally, the entire events you are wishing to detect are detected with optimal timing.
84. Consider using additional parameters, as necessary. Enter all conditions to be used together in the first AND window. If desired, enter another set of conditions, to be used as an OR for triggering, in the second window. Select “Combined” through “Plot Control” and click on “Analyze” to see which events meet these combined requirements. For example, if you have tuned your signal to detect positive going spikes, but you would also like to detect other events with negative going spikes, first tune both upward (condition 1) and downward (condition 2) going spikes in the Spikes1 plot. Place a **1** in the first AND window and a **2** in the second AND window, and the program will trigger for either positive or negative going spikes. Alternatively, you may wish to detect only events with BOTH positive and negative going spikes in the same event. In this case, enter both **1** and **2**, separated by a space, into the first AND window.
85. To improve selectivity of event detection, you may also wish to invoke exclusion criteria. For example, movement artifact will often cause a rapid, large, increase in the signal. These events can be excluded by use of the Fast Integrator (Condition 8), as illustrated in Figure 6 c. Examine the fast integrator

signal during a true event. To do this, zoom in to the event in the display window, select “Amplitude Correlation, Fast/Slow Ratio” in “Plot Control”, and click on analyze. Note the maximum value in the bottom panel reached during a true event. Set the threshold for Fast Integrator just above this value. Note that the axis scales automatically to the signal and the set threshold. You can zoom in, however, as necessary. Include condition 8 in the AND window along with previously selected criteria, select “Combined” from “Plot control” and click on “analyze” to ensure that the desired event is still detected. Next, view movement artifact or any other signal which you wish to exclude. Analyze this portion of the signal to confirm that condition 8 exceeds the threshold in its plot window and that the event is now excluded in the combined plot window.

86. You may wish to adjust the Fast Integrator time constants as well (click “Integrators” to access this window) to control the speed at which the fast integrator responds to changes in the signal power. Repeat this process with a number of desired and undesired events until you are satisfied with the level of both sensitivity and specificity.
87. Once values for all conditions are established and entered, view and analyze the entire length of the file (by default this length is one hour). Analyzing the entire hour throughout the tuning process is not recommended as it will take a long time to analyze this length of data. However, it can be useful to do this when scanning for desired and undesired events as a final check in the tuning process, or for offline analysis. When the analyze button is pushed, the list of events to the right of the signal display is updated for the triggers using the current parameters and conditions set in the analyzer and each of these events can then be viewed by clicking on them. To reset to the triggers that occurred during the actual recording (detected on-line), right click on “Entire File”. Note that you will need to right click “Entire File” also to access events listed in this window for other channels.
88. Once parameters are optimized in the analyzer program, set these conditions and parameters for this channel in the recorder program to begin on-line detection.

On-demand seizure detection & light triggering: Timing: Variable

89. Open the recorder program (Supplementary Data 1b), if not already open. If the program is already running (that is, actively collecting data), stop the program before making changes. Once the changes are made, restart the recording.
90. Enter in all parameter and condition values, as determined in Initial assessment and tuning of Intracranial EEG signal for the channel(s) of interest.
91. Enter in the desired probability of the laser being triggered per detected event for the channel(s) of interest. For example, if you want half the events to trigger the light, enter 50%. Note, however, that which triggers will cause the light to turn on is determined by a random number generator. Therefore, while the probability of any event activating light will be 0.5, the actual percentage of

events receiving light, particularly for smaller numbers of events, may not be exactly 50%. To have the light turn on for every detected event, enter 100%. An example of seizure events with 50% light delivery is shown in Figure 6 d.

92. Enter the on/off light pulse parameters desired, the total duration of pulsed light, and the cycle duration. This latter value establishes a minimum time before the next trigger can occur. See the User Guide (Supplementary Manual) for more details on these values.
93. Ensure that the patch cords and laser are appropriately attached to deliver the correct wavelength of light to the correct mouse, the TTL output for the channel triggering the laser is connected to the laser connected to the correct mouse, and finally that the key for the laser is turned to the ON position.
94. Press the start button to begin collecting data, event detection, and light triggering. After a period of data has been collected, review the files and video to ensure that triggering is occurring as desired and that the light is turning on appropriately.

CRITICAL STEP! Watch for a moment after pressing start to ensure that settings are correct, that the laser is off at baseline, and if possible, that triggering activates the light with the correct duty cycle.

CRITICAL STEP! The appearance of intracranial EEG signals often changes with time. Monitor your signal and retune the detector as necessary.

TROUBLESHOOTING

Pause Point: experiment can be paused indefinitely and materials left at room temperature (20–25°C). Between recording and analysis, data can be stored indefinitely, though prompt analysis is recommended in case more data collection is needed.

Data Analysis: TIMING variable

95. Open the analysis program. Open the file to be analyzed. Click through to the channel of interest.
96. Click on an event detected in the event list.
97. Ensure that this is a true event (and not a false positive, due to e.g., movement artifact).

CRITICAL STEP! Use the signal only before the time of the trigger to decide which events to include as being appropriate triggers. Otherwise, it is possible for even a blinded observer to introduce bias into the analysis, particularly when strong light effects are present.

98. For each included event, measure the time from the start to the trigger, the time after detection, and the time from the end of the event until the start of the next event. Note that when you click on an event in the list to the right of the display

window, the event will center in the display window, with a vertical bar indicating time of detection.

99. Once analysis is complete, click on “Show LEDS” to see which events were light and which were no-light conditions. An asterisk will appear next to events receiving light. **CRITICAL STEP!** Only click “Show LEDS” after analysis is complete, to avoid introducing bias.

CRITICAL STEP! If implants are not constructed properly, light can produce a light artifact in the signal which may preclude analysis of the effect of light on the true intracranial EEG signal^{33, 34}. Ensure that you have a clean signal when light is present before analyzing or giving data to a blinded observer.

100. To ensure that all cables were connected properly and light was properly delivered, view the video for a few events marked as receiving light. Note that the alignment of the video and intracranial EEG signal can be off by a few seconds. The video and recorded signal are synchronized by keeping track of the total number of video frames recorded for each file (typically one hour) and assuming that the frame rate is constant throughout the whole file. However, there can be some drift in the video frame rate during the recording, resulting in some desynchronization in some parts of the file on the order of seconds. Note that light is delivered at the time of the trigger, even if the video appears to show it turning on slightly before or after the trigger.

See TROUBLESHOOTING

101. Analyze a large data set. We typically use 100 events.
102. Perform appropriate statistical analysis. Note that there should be no difference in time to trigger between light and no-light conditions. If a significant difference is seen, an unintended bias may have been introduced, and the data must be reexamined.

See TROUBLESHOOTING

103. Once all data has been collected, euthanize the mouse according to your animal care and use committee approved protocol, and confirm the location of the optical fiber(s) (Figure 6 e) and power from the ends of the fibers.

See TROUBLESHOOTING

Timing

(Optional) Creating your own implantable optical fibers (steps 1–23): 10 minutes. Made in batches of about 100, each fiber will take 10–15min to make, spread over 2–3 days.

(Optional) Making an optrode from the implantable fiber and a bipolar electrode (steps 24–33): 10 minutes per optrode

Surgical implantation of fibers and electrodes (steps 34–54): on average, 1 hour. Depending on your proficiency, method of induction, and number of implanted fibers this might take anywhere from 40min to 2h per mouse.

Hardware & software setup (steps 55–70): Variable. Once the hardware and cages are placed in a convenient location, connecting the hardware and installing the necessary drivers will take 1–2 hours.

Initial assessment & tuning of signal (steps 71–88): Variable. Depending on the seizure frequency, the initial baseline recording will take a variable amount of time. Once the first seizure has been identified, expect to spend 15min to a few hours tuning the detector to optimally pick up that animal's seizures.

On-demand seizure detection and light triggering (steps 89–94): Variable. Again, this will depend on the seizure frequency. For the intrahippocampal kainate model of epilepsy, the frequency of electrographic seizures can be quite high—several per hour. We recorded at least 100 events from each mouse for statistical analysis, which can require days of data per animal. Behavioral seizures were typically much less frequent, requiring weeks to months to achieve similar statistical power.

Data Analysis (steps 95–103): Variable. Analysis of 100 events can be time consuming, and will depend on the speed of the blinded observer, the parameters that will be measured, the number of files, etc. Generally, a data set of 100 events can be completed in a few hours.

Troubleshooting

Step: 21–23

Problem: light emission through the implantable optical fibers is less than 30%

Possible reasons: The fiber was nicked or broken inside the ferrule chamber, the ferrule is defective (i.e. the hole for the fiber was off-center), or the epoxy was not fully polished away.

Possible resolution: While the fiber is attached to the light source through the patch cable, examine the light path through the implantable optical fiber—if light emerges from the ferrule stick, discard the ferrule. If light emerges from the side of the implantable optical fiber, recleave the fiber above this level and retest the output. Examine the end of the ferrule to determine whether the fiber is centered & therefore properly aligned with the light from the patch cord. If it is not, discard that ferrule. If no escaping light is visible and the fiber appears centered in the ferrule, attempt to repolish the ferrule, taking care to polish away any visible cracks or defects (see Figure 1 l and compare to panel k).

CAUTION: Wear appropriate eye protection when working with lasers. Even when wearing appropriate safety goggles, do not look directly into the path of the laser.

Step: 103

Problem: the optical fibers are not targeting the desired brain area

Possible reasons: The animal was not in a head flat position during implantation, the coordinates are not correct in the age/strain/sex of mouse being used, the ferrule was not secured in the stereotaxic adapter (so was accidentally moved in the z direction prior to or during implanting), or the ferrule was not released fully before raising the stereotaxic arm (causing it to pull back out slightly).

Possible resolution: Ensure that the animal is secured in head flat position in the stereotax throughout the procedure (check repeatedly before implanting). Ensure that the ferrule is solidly in the stereotaxic adapter, and that the glue and/or cement are sufficiently dried prior to releasing the ferrule such that it does not move upon release. If the targeting is consistently incorrect, it may be an issue of strain, age, or sex differences, and in this case, adjust the stereotaxic coordinates in the direction of the desired structure.

Step: 64–94

Problem: the implant falls off

Possible reasons: The screws are not securely fastened to the skull, there is skin between the plastic ring and the skull, tissue was trapped between the skull and the cement and has become necrotic or inflamed, the commutator is not working properly, or the animal has become entangled in the cords, causing a torque on the implant.

Possible resolutions: If the skull is intact, the most likely problem is that the screws were not securely fastened. Use smaller craniotomy holes for the screws to ensure a strong hold. If there is damage to the skull near the site of the screw, the problem is more likely that the cement was not properly adherent to the skull, indicating that the plastic ring was not firmly attached to clean, dry skull, or that the tissue on the skull was not completely cleared away prior to implantation. It may be helpful to make a larger initial incision, and to clean a larger area of the skull prior to implanting the ring. Damage to the skull could also indicate that the animal was entangled in the cables or on items in the cage. Use the video monitoring record to determine whether the animal met resistance from the tethers prior to losing the implant, and resolve any sources of tension on the cables that might have arisen.

Step: 89–94

Problem: the implantable optical fiber slides out of the implant when the patch cable is removed

Possible reasons: The ferrule was not adherent enough to the skull or the cement.

Possible resolution: Ensure that the base of the ferrule has been glued to the skull, and that the cement is covering the glue and the bottom half of the ferrule. If a ferrule seems loose, some glue at the junction of the ferrule with the cement may help. In general, be gentle when plugging and unplugging the animals to avoid problems with the headstage.

Step: 71

Problem: the signal is missing or is composed primarily of movement artifact

Possible reasons: Patch cables are incorrectly connected, the bare wire ends of the electrodes became insulated by glue or cement, or the implant is loose.

Possible resolutions: Ensure that the patch cables are plugged into the correct outlets of the commutator and that all patch cables are connected to the appropriate channels of the amplifier and digitizer. Be mindful of the bare wire ends of the electrode throughout the implant procedure, ensuring that they do not become covered by any insulating substances such as glue or cement. Ensure that implants are secured (see previous troubleshooting tips for “implant falls off”). If the implant is suspect, remove the animal from the recording chamber for days to weeks to allow more healing to occur, as bone may heal around the screws with time, and the skin around the implant may also heal in place, securing the implant better.

Step: 96–100

Problem: light effects are visible in the recording, even in animals not expressing the opsin

Possible reasons: The ends of the electrode are positioned in the path of the light, inducing a photoelectric current in the wires, the light power is too high, causing tissue heating, or perceptual cues to the animal.

Possible resolutions: When making optrodes, ensure that the electrode is shorter than the optical fiber. If separately implanting electrodes and optical fibers, ensure that they are sufficiently spaced to prevent light shining directly onto the bare wire portion of the electrode. Use a minimum amount of light necessary to achieve a relevant effect in the opsin expressing animals, and always compare effects to non-opsin expressing control animals.

Step: 94

Problem: after starting the custom software, the recorder will not run

Possible reason: Occasionally, especially the first time the software is run from a new computer or new location, it may need to be run as administrator to work properly.

Possible resolutions: Close all instances of the custom software and attempt to run the program as administrator by right clicking the executable and selecting ‘run as administrator’. If that does not work, power cycle both the computer and digitizer, then run the custom software as administrator.

CAUTION: turn all lasers to the off position before power cycling the computer or digitizer.

Step: 94–101

Problem: The signal degrades over time

Possible reason: Scar tissue builds up around the electrode, the implant becomes loose, tension on the cables causes the electrode to become dislodged or broken, or electrical noise becomes introduced into the signal.

Possible resolutions: These problems may be unavoidable to some degree; however, high quality, stable implants and attention to reducing tension on the tethering cables may minimize such problems.

Step: 64–94

Problem: The animal becomes lethargic or sick

Possible reason: Some animals do not adapt to being tethered as well as others. Most animals will seem unsure for the first few hours of being tethered, but gradually begin to explore and resume normal grooming and feeding behaviors. Others, however, will not resume normal behaviors and will eat and drink very little while tethered.

Possible resolutions: Keep a record of the animals' weights as well as taking note of their food and water consumption. If the animal loses more than 15% of its original body weight, remove it and allow it to recover for at least a week before attempting to tether it again. If an animal has difficulty adjusting, introduce the animal to the environment for a few hours before placing the tether. The animal can also be habituated to the tether several hours per day over several days prior to extended recording periods.

Step: 94

Problem: The laser is always on, or does not turn on when the program indicates that it should.

Possible reason: Settings or connections are incorrect, or the computer is not communicating properly with the digitizer.

Possible resolutions: Ensure that the animal is connected to the correct laser, that the TTL output to the laser is connected securely to the correct output on the digitizer, the laser is powered on, the laser key is turned to the on position, and that the laser is in the correct TTL setting. Note also that when the digitizer is not controlled by the program (for example, when the computer is turned off, restarts, or loses power), the digitizer is not able to properly control the lasers. In this circumstance, many TTL signals 'float high' and the lasers may then stay on. Ensure that you have an uninterrupted power supply and always turn lasers off before turning off the digitizer or computer.

Step: 100

Problem: Not all video channels are being recorded for every file

Possible reason: The custom software may be unable to properly access the cameras.

Possible resolutions: Restart the computer.

CAUTION: Always turn off all lasers before power cycling the digitizer or computer.

Step: 94, 100

Problem: The desired light pulses are not being produced

Possible reason: The program has specific requirements for light pulse trains. Also, particularly for very short duration pulses, the software requires processor memory for timing, and there is a potential for ongoing system activities to cause slowing.

Possible resolutions: See the User Guide in the Supplementary Manual for more information about these parameters. The cycle time must be at least 1s longer than the duration of the light pulses, and the duration of light pulses must be at least 1.5 full cycles to achieve a full-length light pulse. If these requirements are not met, only a brief (~2ms) pulse will occur. Minimize the number of processes that are occurring on the computer at a given time (including all automatic updates). Use a function generator between the digitizer and light source if very precise short duration pulses are desired.

Anticipated Results

The above procedures allow stable long-term intracranial EEG recordings with on-demand optogenetic intervention. For implanted optical fibers, light transmitted should be close to 80% of that measured at the end of the patch cord. The implant should be mechanically very stable, the ends of the electrode clean, and the optrode positioned properly, providing an intracranial EEG signal with minimal movement or photoelectric artifacts. Provided that the animal adapts well to being tethered, a stable intracranial EEG signal can be recorded for weeks to months. With appropriate tuning, the detector provides sensitive and specific closed-loop intervention. Even in signals with rhythmic movement artifacts, due to e.g. scratching, detection criteria can be tuned to reduce both false positives and false negatives (Figure 6). Once long-term stable recordings are achieved, and the program appropriately tuned, light can be delivered to a subset of detected events, in a random fashion. In the intrahippocampal mouse model of temporal lobe epilepsy, light delivery to the hippocampus of epileptic animals expressing the inhibitory opsin halorhodopsin in excitatory cells rapidly truncates seizures (Figure 6 d).

Supplementary Material

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Box 1: Size guidelines for planning location of optical and electrical fibers

- > If you plan to simultaneously plug multiple fibers in, use a brain atlas to plan your implant before starting⁵¹. Fiber targets must be 3.0mm from one another to allow for the two 1.25mm ferrules (assuming they are vertically oriented, and assuming a patch cable with a protective metal flange; without the flange, this minimum distance is somewhat reduced).
- > If instead, the fibers are to be plugged in one at a time, the centers of the ferrules must be at least 1.5mm apart, as the OD of the split sleeve is 1.5mm.
- > Screws to stabilize the implant have a head diameter of 2.6mm and a shaft diameter of 1.4mm.
- > The PlasticsOne bipolar electrode pedestal is 3.3mm in diameter, and when plugged in, the receptacle has a diameter of 5.7mm. Thus, it is helpful to mount the pedestal at an angle away from the other components of the headstage to conserve space.
- > As a general estimate, expect the distance from bregma to lambda in adult mice to be just less than 4mm.

Figure 1. Generating implantable optical fibers

Insert the unstripped fiber into the appropriately sized fiber stripping tool (a). The stripped fiber (b) should then be cleaved off using the diamond scribe (c) and cut into short (10–15mm) segments which will be placed into the ferrule (shown together in d, small tick marks on the ruler are 1mm apart). Place the ferrule into an alligator clip or clamp (e) and note that there is a smaller convex end which will be polished, and a larger, concave end into which the epoxy and fiber will be loaded, and from which the implantable end of the fiber will emerge. Use a syringe to fill the larger, concave end of the ferrule until a bead of epoxy emerges from the other end (f). Next, gently pick up one segment of stripped fiber and insert it into the same end of the ferrule (g), being careful not to nick or shatter the fiber, and leaving as much fiber behind as you may want to implant (note that fibers can be further cleaved later). After allowing the epoxy to cure at least 24 hours, cleave the fiber emerging from the end to be polished as close to the epoxy bead as possible (h). Prepare the polishing surface with the 5µm polishing paper, and place the fiber into the polishing disk. Use very gentle, gradual pressure to polish the cleaved end in figure 8s, being careful not to break the fiber and working across the paper until the fiber and ferrule are flat with the polishing disk (i). Examine the end of the fiber in the polishing disk under a dissecting scope (j). Note that after the first polish, while no dark areas or cracks are visible in the fiber, fine scratches still appear on the ferrule and fiber core. A fully polished fiber (k) should appear quite shiny and free of scratches, particularly in the fiber core. If any cracks are visible after the last polishing step (l), the fiber should be re-polished, starting again from the 5µm polishing sheet, making sure to polish beyond all cracks before continuing.

Figure 2. Assembling optrodes for implantation

To attach an implantable optical fiber (the product of the steps shown in Figure 1, shown here in panel **a**, with polished and implantable ends labeled), which has been cleaved to the desired length, to a bipolar PlasticsOne electrode (also shown in panel **a**), bend the electrode to be almost parallel with the base of the pedestal, and again at 90 degrees to run alongside the optical fiber. Measure and cut the electrode (**b**) such that it will be just shorter than the optical fiber (**c**). Use a fine thread or suture material to tie the fiber and the electrode together (**d–f**). Use a drop of glue on a syringe tip or fine forceps to secure the fiber to the electrode (**g**), taking care not to get any glue on the bare wire ends of the electrodes. Trim the ends of the thread or suture material (**h**), and allow the optrode to dry (**i**) before further bending the electrode into the final desired orientation for implanting.

Figure 3. Implanting the optical fiber and electrode headstage

The implanted headstage consists of the fully assembled optrode, any additional implantable optical fibers, screws, and a small plastic ring which will hold the dental cement (a). Expose the skull, remove all fascia in the area in which you will place the implant, and note the locations of bregma and lambda (b). Note that this animal was previously injected in the left hippocampus with kainate to induce epilepsy, so there is a prior craniotomy present in the skull to the left of the sagittal suture. Measure bregma (green circles, c) and lambda (blue circles, d), and adjust the mouth bar as necessary to ensure that these points are at the same z position. Next, measure and mark the desired anterior-posterior and medial-lateral position for the optrode (e) as well as any additional optical fibers. It may also be helpful to mark the locations for the screws for planning purposes. Once the locations of the headstage components have been determined, fit the plastic ring to the shape of the skull (f) and ensure that all components will fit. Drill the holes in the skull, ensuring that those for the screws are just smaller than the thread size (g). Use firm pressure to screw the screws into the holes (h), keeping in mind that secure screws generated by the teeth of the screw biting into the edges of the craniotomy are key to a successful implant. Note that the screws are positioned here in such a way as to distribute any force from the tethers across more than one skull bone and suture (i). Use glue on the base of the plastic ring to fix the ring to the skull, and pull the edges of the incision around the ring into the glue (j). Next, insert the first optical fiber into the stereotaxic adapter, measure the location of bregma and navigate to the correct anterior-posterior and medial-lateral position. Use pointed forceps or small scissors to break through the dura to allow the optrode to pass into the brain (k). Place gel glue at the base of the ferrule, avoiding getting glue on the optical fiber itself, and lower the optrode into the brain (l). Carefully release the optical fiber from the stereotaxic adapter and place any other optical fibers, being careful not to knock over previously placed implanted fibers (m). Mix dental cement until it is slightly thickened, but still pourable, and pour it into the implant, being careful not to get any cement on the upper sides of the optical fibers or electrode pedestal (n). Allow the cement to harden and release the final implantable optical fiber from the stereotaxic adapter (o) to complete the procedure. All procedures in live animals were performed with the approval of the UC Irvine Animal Care and Use Committee.

Figure 4. Equipment setup

An example of the hardware setup for real-time optogenetic intervention is shown. Implanted mice (only 2 of the possible 8 cages are shown here for simplicity) are tethered for both electrical recording (green cables) and laser stimulation (blue and red cables). Electrical and optical commutators are positioned above the cages to allow the animal to freely move about the cage. The cables have enough slack that the animal can freely enter all areas of the cage. The electrical signal is then passed on to the amplifier, and the output of the amplifier is routed to the appropriate channel of the digitizer. The digitizer is connected by USB cable (black) to the computer running the custom recording software. Once an event is detected and light triggered by the software (see Figure 5), the digitizer output (purple cables) send a TTL signal to the laser, and the laser will then transmit the signal back to the mouse (both a blue and a red laser are shown here as examples). A camera connected to the computer by USB should be positioned in such a way as to capture the desired cages. Up to 4 cameras can be used at one time. It may be helpful to cover the shelving in a wire mesh to reduce electrical noise from the environment.

Figure 5. Summary of Detection Algorithms

A number of features of the signal can be used to trigger detection, summarized here and detailed in the accompanying User Guide (Supplementary Manual), as well as in the supplementary Material of Krook-Magnuson et al., 2013². In this figure, all words in color are user-defined parameters. Portions of this figure have been modified from Krook-Magnuson et al., 2013². **Spikes:** Spike detection uses a user-defined amplitude threshold (green) based on a user-adjustable slow integrator (red, also used in calculating Power properties). Additionally, minimum and maximum spike width (maroon arrows) at a specific relative height, and spike distance (orange) are user defined. Once the number of valid spikes in a user specified sliding time window crosses a user defined threshold (# of spikes threshold, blue), the program will begin to calculate the regularity of the spikes --the inverse of the coefficient of variation (CV, orange). When the user-defined ratio threshold based on this calculation is reached, the Spikes condition will trigger. This is done separately for positive (pos) and negative (neg) spike directions. **Frequency:** An example power spectrum of the frequencies present during a seizure is shown. The user can examine this spectrum to determine the specific ranges of frequencies that change most during the signal of interest (shown here as f1 to f2, in maroon, and f3 to f4, in blue). The signal is filtered using a Fourier transform (FFT) and the ratio of the power of the signal in the two user specified bands of frequencies is calculated. When the value of this calculation is either above or below a user-specified threshold (turquoise), the Frequency Band Ratio condition will trigger. **Power:** The power of the signal is examined in one of three ways. The properties of the slow (red) and fast (brown) integrators (i.e., how fast the signal changes in response to a change in the signal power) are user-specified by changing the leading and falling edge time constants (τ). Top: The user-defined threshold (purple) scaling of the slow integrator is compared with the amplitude correlation to determine triggering (see User Guide (Supplementary Manual) for more details, including equations) of the Amplitude Correlation condition. Middle: By comparing the values of the slow and fast integrators, the speed at which the signal changes can be used to exclude sudden movement artifacts using the Fast/Slow exclusion threshold (pink). Bottom: When the Fast integrator is below a user specified threshold (gold), triggering can occur. **Coastline:** The path length between each sample of the signal is determined and integrated in a user-specified manner over time. When the value exceeds a user-specified threshold, the Coastline condition is triggered.

Figure 6. On-line seizure detection and fiber tract location

a–c. The detection software should be tuned for each animal, to avoid false positives (red vertical line) while detecting true events (green vertical line). Even in cases of rhythmic artifact, due to for example scratching, good event detection can be achieved. **a.** If detection criteria are too broad, false positives can result. In this example, the movement artifact (expanded in the bottom trace), has wide spikes. As a result of poor, broad, tuning, these spikes are included (as indicated by the dots riding each spike). **b.** Poor tuning can also result in false negatives. In the example shown, the threshold for spike detection is set too high to detect the true event (bottom panel, horizontal lines indicate thresholds). Note, as detailed in the accompanying User Manual, that the threshold value entered by the user is not the same as the simple amplitude of the signal, and the user should inspect the plot to ensure the threshold set is appropriate to detect the events of interest. **c.** Once appropriately tuned, false negatives and false positives are avoided. Exclusion criteria, such as the Fast Integrator (bottom panel), can additionally be used to improve specificity. When the Fast Integrator value exceeds the user set threshold (horizontal black bar), detection is suppressed. Thin vertical red lines indicate where the Fast Integrator values return to below the threshold. **d.** Once tuned, on-line seizure detection (vertical green lines indicate event detection) can trigger light delivery (indicated by horizontal orange lines) to a percent of events, in a random fashion. In mice expressing the inhibitory opsin halorhodopsin in excitatory cells, light delivery to the hippocampus rapidly stops seizures. **e.** Post hoc

analysis confirms the location of an optical fiber (200 μ m in diameter) implanted to deliver light to the hippocampus. Bright field image of unstained tissue. Scale bars **d**: 100 μ V, 5s. **e**: 200 μ m. Panel **d** modified from Krook-Magnuson et al., 2013².