Closed-loop optogenetic intervention in mice

Caren Armstrong[1,2], Esther Krook-Magnuson[1,2], Mikko Oijala[1] & Ivan Soltesz[1]

[1]Department of Anatomy & Neurobiology, University of California, Irvine, School of Medicine, Irvine, California, USA. [2]These authors contributed equally to this work. Correspondence should be addressed to C.A. (cmarmstr@uci.edu).

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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 intervention 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 by using optogenetic intervention. This system performs with a very high sensitivity and specificity, and the strategy is not only relevant to epilepsy but also can also be used to react to diverse brain states in real time, with optogenetic or other interventions. The protocol presented here is highly modular and requires variable amounts of time to perform. We describe the basic construction of a complete system, and we include our downloadable custom closed-loop detection software, which can be used for this purpose.

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 on the basis of their projection patterns or to modulate specific intracellular cascades[5,7,22]. New optogenetic tools are continuously being developed that 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 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 for detecting seizures from intracranial electroencephalography (EEG) in real time; it responds by triggering light output, activating opsins in both a cell type–specific and a temporally specific manner[2].

We provide here a modular protocol detailing the 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 in cost. The detection software described in this protocol and the accompanying user guide (**Supplementary Manual**) are designed to provide selectivity and sensitivity for a range of signals. Specifically, this software was designed to detect, in real time, temporal lobe seizures, which can show variability with respect to key signal features[30] and are accompanied by intervening interictal spikes. Given these considerations, simpler detection[11,31] methods, such as amplitude crossing, would be insufficient. Therefore, the software builds on previous off-line detection algorithms[32] and provides a number of signal-detection conditions for the user to choose from on the basis of spike, frequency and power properties, with parameters that can be tuned to accomplish the desired sensitivity and specificity for each mouse. These additional features further increase applicability, making the software relevant to the detection of a range of events, even in conditions in which 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 mouse must be very stable to allow for prolonged recording and intervention. For optogenetic interventions, appropriate control mice (for example, opsin-negative littermate controls) must be 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 for generating a closed-loop system, as used in Krook-Magnuson *et al.*[2]. Specifically, as further outlined below, we detail how to construct implantable optical fibers for delivering light, how to combine these implantable fibers with electrodes to create optrodes, how to implant these components into a stable headstage and how to perform an on-line closed-loop intervention. We provide a modified version (**Supplementary Data**) of the stand-alone custom software that we developed for optogenetic intervention during seizures in chronically epileptic mice[2]. This version of the software has increased functionality and improved user interfaces compared with previous versions. Our software is highly flexible and customizable and requires no programming experience.

Preparation of fibers. Although 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 (<$2,000), we estimate that making 500 implantable fibers in-house in batches of 100 takes about

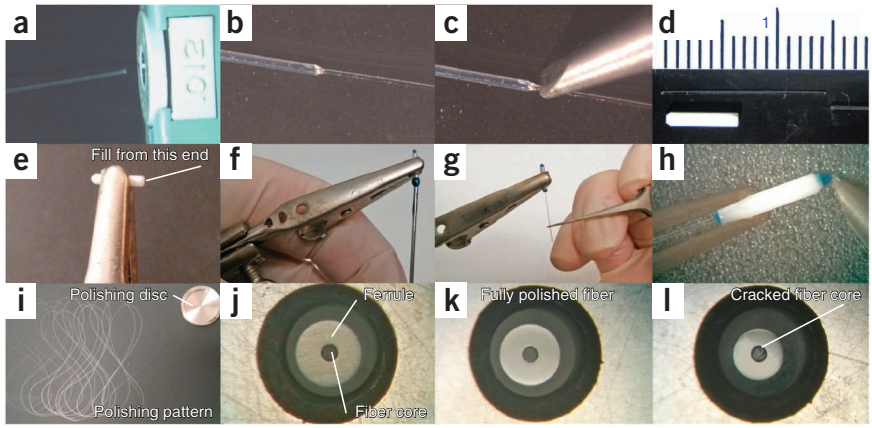

Figure 1 | Generating implantable optical fibers. (**a**) Insert the unstripped fiber into the appropriately sized fiber-stripping tool. (**b**–**d**) The stripped fiber (**b**) should then be cleaved off using (**c**) the diamond scribe and cut into short (10–15 mm) segments, which will be placed into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

100 person hours and costs just under $2,500 in reagents. At current prices, 500 implantable fibers can be purchased commercially for between $9,800 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; scroll down to the 'Ferrule Connectors' section)[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. Although 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 the potential for photoelectric effects. We used a simple premade twisted bipolar electrode from PlasticsOne to achieve a single differential intracranial EEG signal from each mouse[37]. The steps outlined below are illustrated in **Figure 2**.

The advantages of this protocol include the use of relatively inexpensive, readily available materials as well as an interface that does not require specialized combined headstage/amplifier/software systems. In addition, the placement of the different components of the headstage can be adjusted easily, providing flexibility with respect to the brain regions targeted. Thus, this protocol provides a simple but effective and flexible way of combining the recording of 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. Although it is 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 quite stable when created and implanted as described. Indeed, we have

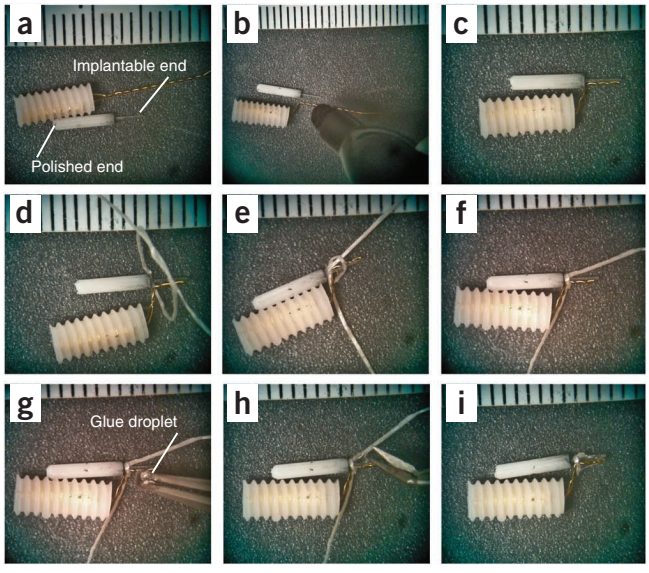

Figure 2 | Assembling optrodes for implantation. (**a**) To attach an implantable optical fiber (the product of the steps shown in **Fig. 1**, shown here with polished and implantable ends labeled) that has been cleaved to the desired length to a bipolar PlasticsOne electrode, bend the electrode to be almost parallel with the base of the pedestal and again at 90° to run alongside the optical fiber. (**b**,**c**) Measure and cut the electrode (**b**) such that it will be just shorter than the optical fiber (**c**). (**d**–**f**) Use a fine thread or suture material to tie the fiber and the electrode together. (**g**) Use a drop of glue on a syringe tip or fine forceps to secure the fiber to the electrode, taking care not to get any glue on the bare wire ends of the electrodes. (**h**,**i**) Trim the ends of the thread or suture material (**h**), and then allow the optrode to dry (**i**) before further bending the electrode into the final desired orientation for implanting.

had success in performing continuous tethered video-EEG recordings with optical intervention for several months.

On-demand event detection and light triggering. This portion of the protocol and the accompanying user guide (**Supplementary Manual**) explain the recording and analysis software, which allows real-time event detection and light delivery. The software allows the experimenter to use 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.*[2]).

Data analysis. The approach to data analysis will 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.*[2].

Limitations and 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 mice, 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 that can be used in isolation or in conjunction with one another, providing great flexibility. Therefore, not only is it capable of detecting a variety of seizure types, but also it can be adapted with minimal adjustment to respond to other electrical signals that have a characteristic appearance, including non-epilepsy-related patterns (sleep, brain rhythms and so on), electromyographic recordings and perhaps even 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 a Bayonet Neill-Concelman (BNC) connector, the software is not limited to use with the headstage system described in this protocol, and it 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[36,42–45]. In addition, this protocol makes use of separate optical and electrical commutators. This can be 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** for a list of ordering information and approximate costs for these materials.

REAGENTS

- Epileptic mice expressing opsins[2], 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 governmental regulations.
- Implantable optical fibers terminated in 1.25-mm diameter ferrules. These are available premade (ThorLabs or Doric Lenses), or see below for instructions to make your own
- Bipolar depth electrodes with pedestal (PlasticsOne, cat. no. MS303/3-A/SP)
- Cyanoacrylate gel glue (we prefer Loctite, cat. no. 130380)
- Thin thread or suture material (Patterson Veterinary Supply, cat. no. 07-8102709)
- Small screws (McMaster-Carr, cat. no. 91773A052)
- Dental cement (Teets Cold Curing, Pearson Dental, cat. no. C73-0076, C73-0060) **! CAUTION** Dental cement is flammable and may cause skin reaction or eye irritation. Use it in a well-ventilated area and avoid contact with skin and eyes.
- Weigh dishes for mixing dental cement (Fisher Scientific, cat. no. 02-202-100)
- Small (1-cm diameter, 1-mm height) plastic rings. We use 5-ml pipette tips cut into 1-mm segments (USA Scientific, cat. no. 1050-0700)
- Permanent marker or pencil
- Ceramic zirconia split sleeves, 1.25-mm inner diameter (Precision Fiber Products, cat. no. SM-CS125S)
- Kainic acid (Tocris, cat. no. 0222)

Additional reagents necessary to make your own implantable optical fibers

- Optical fiber: we used fibers with numerical aperture (NA) 0.37, low OH and a 200-μm diameter (ThorLabs, cat. no. FT200EMT)
- Epoxy and syringes for injecting epoxy (ThorLabs, cat. nos. F112 and MS403-10)
- Razor blades (American Safety Razor single edge, 66-0089-disp)
- Ethanol (70%, vol/vol) and distilled water in squeeze bottles **! CAUTION** Ethanol is flammable. Avoid eye and skin contact.
- Polishing sheets of decreasing grain size (5 μm, 3 μm, 1 μm, 0.3 μm; ThorLabs, cat. nos. LFG5P, LFG3P, LFG1P, LFG03P)
- Ferrules: we used 1.25-mm ceramic ferrules, inner diameter 225 μm (Kientec Systems, cat. no. FZI-LC-225)
- Lint-free wipes (Fisher Scientific, cat. no. 066-666-A)
- Small Petri dishes (Fisher Scientific, cat. no. 08-757-100B)

EQUIPMENT

- Forceps with fine points (Fine Science Tools, cat. no. 11253-25; WPI, cat. no. 500342)
- Size no. 0 screwdriver (McMaster-Carr, cat. no. 7026A18)
- Stereotaxic surgical setup, including a stereotaxic arm suitable for accommodating the ferrule holder stereotaxic adapter, reliable anesthesia-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, cat. no. SCH_1.25)
- Laser or light-emitting diode (LED) light source with transistor-transistor logic (TTL) trigger (various sources exist; we used Shanghai Laser & Optics Century, see **Supplementary Table 1**)
- Optical commutators (Doric Lenses, cat. no. FRJ_FC-FC, or ThorLabs)
- Fiber optic patch cords with appropriate connectors for connecting the light source to the commutator (e.g., ferrule connector/physical contact (FC/PC) on both ends), as well as for connecting the commutator (FC/PC)

to a bare 1.25-mm ferrule (to connect to the implanted optical fiber). When ordering cables, determine the necessary lengths before placing the order (Doric Lenses or ThorLabs) ▲ CRITICAL The cables that connect the commutator to the mouse should have as little slack as possible while still allowing the mouse to reach all corners of the cage comfortably.
- Electrical commutator and cables (PlasticsOne, cat. no. SL2C/SB, and cables). When ordering cables, determine the necessary lengths before placing the order ▲ CRITICAL The cables that connect the commutator to the mouse should have as little slack as possible while still allowing the mouse 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 a 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
- Digitizer (National Instruments, NI) ▲ CRITICAL The custom software supplied here is designed to pair with NI digitizers. We have tested both the eight-channel NI-USB-6221-BNC and the 16-channel NI-USB-6229-BNC digitizers. Other M-series NI digitizers may also work, but they have not yet been tested.
- A dedicated computer running Windows with at least 4 GB memory. We have tested the recorder program on two different Dell Optiplex (990 and 7010) computers running Windows 7 Professional (either 32 or 64 bit is acceptable) with i5 processors and either 4 or 16 GB memory. It is helpful to have a second, large-capacity (>500 GB) hard drive dedicated to data storage. We have tested the analyzer on computers running either Windows 7 or Windows XP
- MATLAB runtime environment 2011b (included in **Supplementary Data**)
- Low-light compatible USB webcams. We recommend using Logitech C270 HD webcams; others can be used (minimum frame rate of 5 frames per second), but proceed 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 night-time lighting in the specific room that is being used, as well as on the precision necessary for the behavior being monitored
- Normal white lighting and red lighting (for night-time recording) on day/night timers (Intermatic, cat. no. TN311)
- Clear cages with tall sides and open tops or with lids with built-in commutators. Also ensure that food and water are made available to the mouse without the risk of entangling the cords on water bottles

Additional highly recommended equipment
- Dissecting microscope (particularly if you plan on making your own optical fibers)
- Uninterrupted power supply (OfficeMax, cat. no. 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, cat. nos. PM100A, SV120VC). These are useful for checking both the power of the lasers and the quality of the implantable optical fibers
- External hard drives (OfficeMax, cat. no. 23192026), large capacity. For eight channels of intracranial EEG sampling at 500 Hz and four videos (which record at 5 frames per second), plan on using 1 GB of storage per hour. Thus, about 6 weeks of data can fit on a 1-TB 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, cat. no. S90W)
- Clamp or mounted alligator clip (A-M Systems, cat. no. 726200)
- Glass polishing plate (ThorLabs, cat. no. CTG913)
- Polishing disc for bare ferrules (ThorLabs, cat. no. D50-L)
- Fiber stripper appropriate for the fiber you choose (ThorLabs, cat. no. T12S21)
- Light power meter and sensor (ThorLabs, cat. nos. PM100A, SV120VC). These are also listed above as optional equipment, but they are especially useful if you are making your own implantable optical fibers

REAGENT SETUP

Mice For epilepsy induction, we use 50–100 nl of 20 mM kainic acid injected unilaterally into the hippocampus (at 2.0 mm posterior, 1.25 mm left and 1.6 mm ventral to bregma)[2]. The dose is adjusted by strain to minimize acute mortality and to reliably generate mice with late spontaneous seizures. We use mice that are at least postnatal day 46 in age, 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 Laboratory, stock no. 008069) or CamKII cells (Jackson Laboratory, stock no. 005359)) and crossed these to either floxed-halorhodopsin (Jackson Laboratory, stock no. stock 014539) or floxed–channelrhodopsin 2 (Jackson Laboratory, stock no. stock 012569) lines[19,46–48]. Of course, viral vectors containing Cre-dependent or Cre-independent constructs can alternatively be used to introduce opsins in mice. These techniques are described elsewhere[17,36,49].

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

PROCEDURE

(Optional) Creating your own implantable optical fibers ● TIMING ~17 h for 100 implantable fibers

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

! CAUTION Wear gloves and appropriate personal protective 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** The experiment can be paused indefinitely and materials can be left at room temperature (20–25 °C).

3| Use the diamond scribe to cut the stripped fiber into 10–15-mm-long pieces (**Fig. 1c**,**d**). To use the diamond scribe, hold it perpendicularly to the fiber and gently score the outside of the fiber. Thereafter, 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 in which we store the cut fiber pieces.

■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

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. Next, 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 (**Fig. 1e**,**f**).

7| Use forceps to gently slide one piece of fiber into the back (flat, concave) end of the ferrule (**Fig. 1g**).
▲ **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 (i.e., work fast).

10| Allow the epoxy to cure for at least 24 h 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** The experiment can be paused indefinitely and materials can be left at room temperature.

11| Score the end of the fiber that has emerged from the smaller, convex end of the ferrule as close to the bead of epoxy at the end as possible (**Fig. 1h**). 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 substantial and it will be difficult to polish the fiber enough to reveal intact fiber.

12| Use a razor blade to scrape off 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** Appropriately handle and dispose of sharps, including razor blades.
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

13| Use Kimwipes and a squeeze bottle with ethanol to thoroughly clean the glass polishing plate of all debris. While it is still slightly wet, lay the 5-μm 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 disk with the cleaved, smaller end emerging from the bottom of the disk. If you are using the polishing disk for bare ferrules, clamp the ferrule once it touches the plastic base provided.

15| Gently set the polishing disk on the 5-μm-grain polishing sheet. Begin making figure-eight patterns with the disk, taking care 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-eight patterns with increasing pressure until the fiber is flat with the end of the ferrule (**Fig. 1i**). If you are using the Lucent connector (LC) polishing disk (rather than the polishing disk 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 (**Fig. 1j**). 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** The experiment can be paused indefinitely and materials can be left at room temperature.

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 (the 3-µm 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 until the fiber appears free of scratches. Repeat for the entire batch of ferrules before proceeding to the next step.
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

19| Clean the glass plate with ethanol and a Kimwipe and place the finer, 1-µm 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 then put the polishing disk down such that the end of the ferrule is in the water. Moving slowly across the polishing sheet, polish the ferrule in 15 figure-eight patterns. After this step, the fiber should appear, under the dissecting microscope, to be quite shiny and free of scratches or dark spots.
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

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 using the 1-µm grain sheet (Step 19) it can be omitted. Clean the glass plate with ethanol and a Kimwipe and place the finest-grain (0.3-µm) 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 disk down such that the end of the ferrule is in the water. Moving across the polishing sheet, polish the ferrule in three figure-eight patterns. After this step, the polishing is complete (**Fig. 1k**).
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

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 you are working with lasers, always use protective eyewear appropriate for the wavelength and power of laser light.
? **TROUBLESHOOTING**

22| Turn on the laser and measure the power at the tip of the optical fiber.
? **TROUBLESHOOTING**

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 (**Fig. 1l**). Discard any fibers that do not produce sufficient light transmission.
! **CAUTION** Wear appropriate eye protection when working with lasers. Even when you are wearing appropriate safety goggles, do not look directly into the path of the laser.
? **TROUBLESHOOTING**
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

(Optional) Making an optrode from the implantable fiber and bipolar electrode ● TIMING 10 min per optrode
24| Measure the desired length of the optical fiber. Add ~1 mm to the desired depth to allow space for attaching the electrical fiber (**Fig. 2a**).

25| If necessary, cleave the end of the fiber to the desired length using the diamond scribe, taking care not to damage the fiber. For a description of how to cleave fibers, see Step 3.

26| Plan out the desired location of all optical and electrical fibers using a brain atlas[50]. Keep the size guidelines given in **Box 1** in mind.

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 starting[50]. Fiber targets must be 3.0 mm from one another to allow for the two 1.25-mm ferrules (assuming they are vertically oriented and assuming a patch cable with a protective metal flange; without the flange, this minimum distance is reduced).
- If the fibers are to be plugged in one at a time instead, the centers of the ferrules must be at least 1.5 mm apart, as the outer diameter of the split sleeve is 1.5 mm.
- Screws to stabilize the implant have a head diameter of 2.6 mm and a shaft diameter of 1.4 mm.
- The PlasticsOne bipolar electrode pedestal is 3.3 mm in diameter, and when plugged in, the receptacle has a diameter of 5.7 mm. 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 <4 mm.

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 then bend the bipolar electrode at an angle of 90° away from the pedestal at this distance (**Fig. 2a**,**b**).

29| Cut the bipolar electrode cleanly to be just shorter than the optical fiber protruding from the end of the ferrule (**Fig. 2c**).
▲ **CRITICAL STEP** There is a much greater chance to observe photoelectric effects if the electrical contacts sit under the path of the light.

30| Use a length of very thin, flexible thread or suture to tie the electrode to the optical fiber, and position it in an ideal arrangement (**Fig. 2d**–**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 cyanoacrylate 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, thus allowing it to climb partway down the fiber and electrode to adhere them to each other (**Fig. 2g**).
▲ **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 before implanting it (**Fig. 2h**,**i**).
■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

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 40 min–2 h per mouse**

34| Anesthetize the mouse, shave its head and place it securely in the stereotax (detailed surgical technique protocols can be found elsewhere[38–41]).
! **CAUTION** Use standard intraoperative and postoperative 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 with institutional and governmental 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 (**Fig. 3a**) 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 (**Fig. 3b**).
▲ **CRITICAL STEP** If any tissue remains between the skull and the implant when implantation is finished, the tissue may become necrotic and incite inflammation. This may cause the implant to become loose.

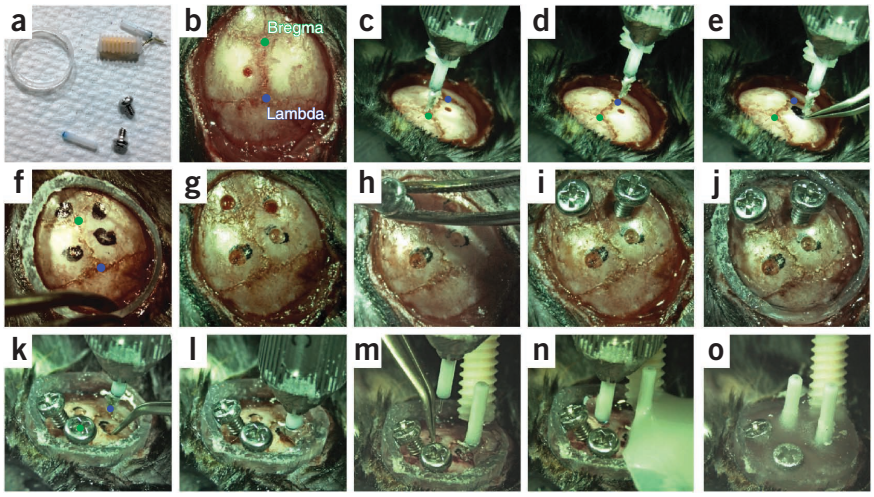

Figure 3 | Implanting the optical fiber and electrode headstage. (**a**) The implanted headstage consists of the fully assembled optrode, any additional implantable optical fibers, screws and a small plastic ring, which will hold the dental cement. (**b**) Expose the skull, remove all fascia in the area in which you will place the implant and note the locations of bregma and lambda. Note that this mouse 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. (**c**,**d**) 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. (**e**) Next, measure and mark the desired anterior-posterior and medial-lateral position for the optrode and any additional optical fibers. It may also be helpful to mark the locations for the screws for planning purposes. (**f**) Once the locations of the headstage components have been determined, fit the plastic ring to the shape of the skull and ensure that all components will fit. (**g**) Drill the holes in the skull, ensuring that those for the screws are just smaller than the thread size. (**h**) Use firm pressure to screw the screws into the holes, 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. (**i**) 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. (**j**) 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. 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. (**k**) Use pointed forceps or small scissors to break through the dura to allow the optrode to pass into the brain. (**l**) Place gel glue at the base of the ferrule, avoiding getting glue on the optical fiber itself and lower the optrode into the brain. (**m**) 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. (**n**) Mix dental cement until it is slightly thickened, but still pourable, and pour it into the implant, taking care not to get any cement on the upper sides of the optical fibers or electrode pedestal. (**o**) Allow the cement to harden and release the final implantable optical fiber from the stereotaxic adapter to complete the procedure. All procedures in live animals were performed with the approval of the University of California (UC) Irvine Animal Care and Use Committee.

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 and 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; the experimenter should flush the tissue with excess saline after using the hydrogen peroxide, as it can excessively dry the tissue or cause weakening of the skull.

38| Identify and measure the coordinates of bregma (**Fig. 3c**) and lambda (**Fig. 3d**) 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 remeasure 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 electrodes[50]. At each location, use a permanent marker or pencil to mark the location for drilling. It can also be useful to use fine forceps to scratch a target into the mark on the skull that was drawn by permanent marker to more precisely center the desired craniotomy location (**Fig. 3e**).

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 (**Fig. 3f**).

41| Use a hand drill or dental drill to carefully make holes in the skull for the optical fibers, electrodes and screws (**Fig. 3g**).
▲ 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 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 predrilled holes (**Fig. 3h**,**i**). Once the screws are secure in the bone, you can leave the screws in if bregma is not obscured, or you can remove the screws and place them later 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 cyanoacrylate gel glue on the bottom and outside of the ring.
▲ **CRITICAL STEP** Ensure that the ring sits on dry, well-cleaned skull at its most anterior and posterior points of contact.

44| Use another forceps to hold the skin back, and then 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 (**Fig. 3j**). Alternatively, if desired, this ring can be placed after some or all fibers are in place, but you should exercise caution when doing so to avoid displacing the fibers or altering the head position.

45| Readjust the mouse 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 mouse's head should not move after this step; thus, ensure that it is securely within 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 you are 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 forceps or scissors to gently nick and spread open the dura (**Fig. 3k**).

48| Place a layer of cyanoacrylate 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 (**Fig. 3l**). If you 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 (**Fig. 3m**). 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, as it should not be cemented into the implant. When the last ferrule has been placed, the stereotaxic arm can be left in place until the implantation procedure is complete.

51| If you have removed the screws in 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 (**Fig. 3n**).
▲ **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 mouse from being plugged in properly later.

53| Allow the cement to harden, apply postoperative antibiotics and analgesics (we use flunixin 2.5 mg kg^{-1} subcutaneously, a topical bacitracin and tetracaine powder at the incision site and enrofloxacin 5 mg kg^{-1} intramuscularly) and follow standard surgical recovery protocols (**Fig. 3o**).

54| House the mice 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 mice to allow the implant to stabilize and heal properly.
■ **PAUSE POINT** The experiment can be paused indefinitely and mice can be housed in their standard cages until you are ready to record.

Hardware and software setup ● TIMING variable

55| On the recording computer, install drivers for NI digitizers (http://joule.ni.com/nidu/cds/view/p/id/3622/lang/en, or go from the NI homepage to *Support* → *Downloads* → *Drivers* 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 ensure that installations work 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/en-us/support/hd-webcam-c270?crid=405&softwareid=10505&bit=64&osid=14&section=downloads).

57| On the recording computer, and on any computer running the analysis program, install MATLAB runtime environment 2011b (**Supplementary Data**, MCRInstaller (2011b).exe). This will be required to run the complied custom software. You only need to run this once on each computer before running the custom software.

58| Place the custom recorder executable file (**Supplementary Data**, Soltesz_Recorder_v1.exe) into a dedicated folder into which all files will be recorded. The analyzer executable (**Supplementary Data**, Soltesz_Analyzer_v1.exe) can be placed in a different folder or 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, thus creating a low-noise environment (**Fig. 4**).

61| Set up lighting using day/night timers. We use a bank of red lights that are constantly on and a timer that 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 Krook-Magnuson *et al.*[2], 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 these require different electrical cables.

63| Connect the appropriate laser and electrographic input to each channel as follows:

Channel 1: analog input BNC port AI0	Digital and timing I/O PFI 0/P1.0
Channel 2: analog input BNC port AI1	Digital and timing I/O PFI 1/P1.1
Channel 3: analog input BNC port AI2	Digital and timing I/O PFI 2/P1.2
Channel 4: analog input BNC port AI3	Digital and timing I/O PFI 3/P1.3
Channel 5: analog input BNC port AI4	Digital and timing I/O PFI 4/P1.4
Channel 6: analog input BNC port AI5	Digital and timing I/O PFI 5/P1.5
Channel 7: analog input BNC port AI6	Digital and timing I/O PFI 6/P1.6
Channel 8: analog input BNC port AI7	Digital and timing I/O PFI 7/P1.7

■ **PAUSE POINT** The experiment can be paused indefinitely and materials can be left at room temperature.

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 anesthetic such as isoflurane to briefly anesthetize the mouse. It may also be helpful to use a split

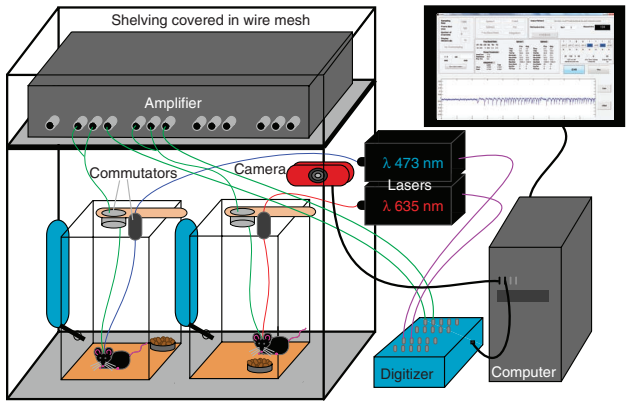

Figure 4 | Equipment setup. An example of the hardware setup for real-time optogenetic intervention is shown. Implanted mice (only two of the possible eight cages are shown here for simplicity) are tethered for both electrical recording (green cables) and laser stimulation (blue and red cables). Electrical 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 mouse 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 is triggered by the software (**Fig. 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 four 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.

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 or while placing the mouse into the cage, as it can be dislodged if you use excess force.
? TROUBLESHOOTING

65| Before starting the custom software, use the Logitech webcam software to ensure that the camera will record the mouse's entire cage.
▲ **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**) 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 eight) and video inputs (up to four) 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 Steps 71–88 below.

69| Record several hours of baseline intracranial EEG.

70| Note that as the mouse 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, to minimize false positives or false negatives, you can make the detector more or less strict, respectively. Steps 78–88 are an example of the tuning procedure. The **Supplementary Manual** explains the user interfaces and 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.
? TROUBLESHOOTING

72| Start the analyzer program (**Supplementary Data**). 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.

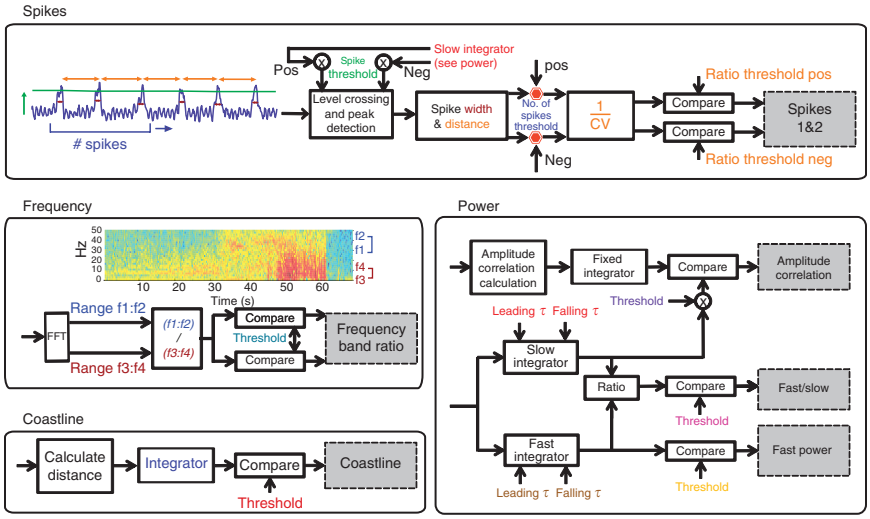

Figure 5 | Summary of detection algorithms. A number of features of the signal can be used to trigger detection, summarized here and detailed in the accompanying **Supplementary Manual**, as well as in Krook-Magnuson *et al.*[2]. In this figure, all words in color are user-defined parameters. Portions of this figure have been modified from Krook-Magnuson *et al.*[2]. Spikes: spike detection uses a user-defined amplitude threshold (green) on the basis of a user-adjustable slow integrator (red, also used in calculating power properties). In addition, 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 (no. 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 blue, and f3 to f4, in maroon). The signal is filtered using a fast 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 value of 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 the **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.

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.

75| Narrow the display window to target the area of interest (that is, the portion of time that contains the seizure or event of interest). 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 on the basis of 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) and the time window over which each parameter is integrated to determine a trigger are 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. Proper tuning reduces false positives and false negatives (**Fig. 6a**,**b**).

78| If you are detecting events on the basis of positive-going spikes (using Condition 1), first examine the threshold for spikes in the top panel of the Spikes1 plot, to determine whether all of the intended spikes are crossing the threshold. As illustrated in **Figure 6b**, if the threshold is set too high, events will be missed.

79| Next, consider the widths and interspike 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 on the basis of the current criteria; those that cross the threshold but lack a circle have failed to meet either the interspike interval or the width requirements. Adjust these

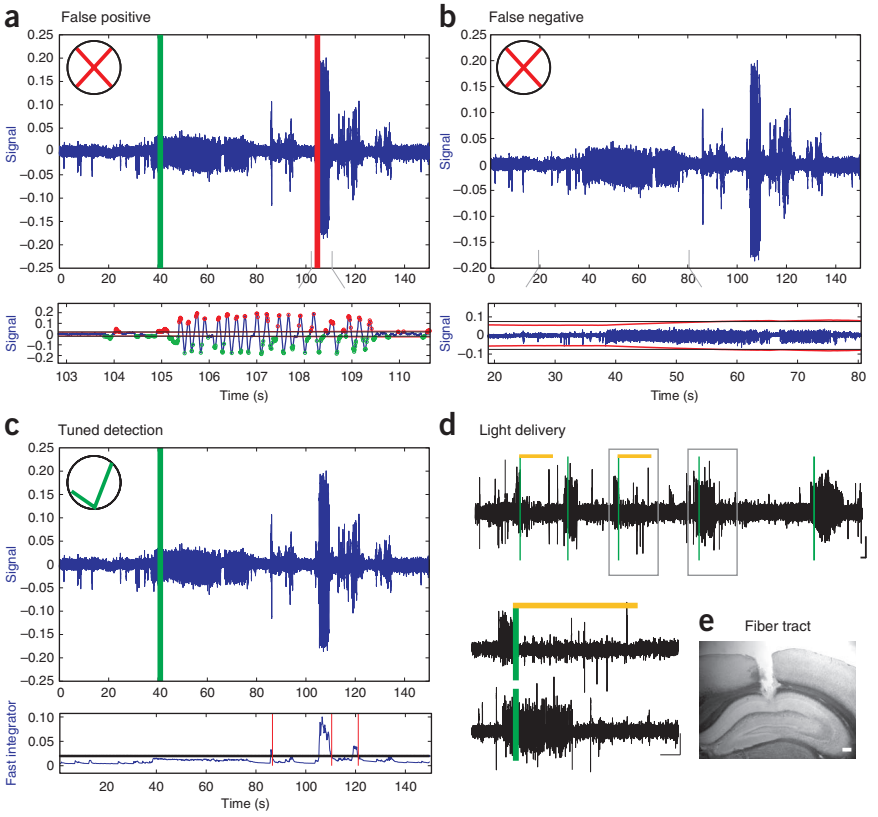

Figure 6 | On-line seizure detection and fiber tract location. (**a**–**c**) The detection software should be tuned for each mouse to avoid false positives (red vertical line) while detecting true events (green vertical line). Even in cases of rhythmic artifact (due to, for example, scratching), good 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, horizontal lines indicate thresholds). Note that, as detailed in the **Supplementary Manual**, 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 the system is 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, online seizure detection (vertical green lines indicate event detection) can trigger light delivery (indicated by horizontal orange lines) to a percentage 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 µm in diameter) implanted to deliver light to the hippocampus. Bright-field image of unstained tissue. Scale bars, (**d**) 100 µV, 5 s; (**e**) 200 µm. Panel **d** was modified with permission from ref. 2.

parameters ('Min Dist,' 'Max Dist,' 'Min Width,' 'Max Width' and the associated 'Threshold Low') until the spikes you wish to count are marked by circles. Tightening these parameters can be done to exclude artifacts (**Fig. 6a**).

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 interspike 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. Once it is calculated, the ratio value is displayed 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 satisfies the spiking requirements ('Trigger True') before the condition will trigger.

83| Continue to adjust these values (and periodically click on 'Analyze' to check the results) until the events you wish 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 large, rapid increase in the signal. These events can be excluded by the use of the fast integrator (condition 8), as illustrated in **Figure 6c**. 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 the 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 that 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 the 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 the values for all conditions are established and entered, view and analyze the entire length of the file (by default this length is 1 h). 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 had 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 and light triggering ● TIMING variable

89| Open the recorder program (**Supplementary Data**) if it is 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, re-start the recording.
? TROUBLESHOOTING

90| Enter all parameter and condition values, as determined in Steps 71–88 for the channel(s) of interest.

91| Enter 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, although 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 6d**.

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 the laser are appropriately attached to deliver the correct wavelength of light to the correct mouse and that the TTL output for the channel triggering the laser is connected to the laser. Ensure that the laser, in turn, is connected to the correct mouse and finally that the key for the laser is turned to 'ON'.

94| Press the 'Start' button to begin collecting data, event detection and light triggering. After a period of data collection, review the files and video to ensure that triggering is taking place as desired and that the light is turning on appropriately.
! CAUTION Turn all lasers to the off position before power-cycling the computer or digitizer.
▲ 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, ensure 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 The experiment can be paused indefinitely and materials can be left at room temperature. Between recording and analysis, data can be stored indefinitely, although prompt analysis is recommended if 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 event 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** To avoid introducing bias, only click 'Show LEDS' after analysis is complete.
▲ **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 1 h) 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.
! **CAUTION** Always turn off all lasers before power cycling the digitizer or computer.
? TROUBLESHOOTING

101| Analyze a large data set. We typically use 100 events.
? TROUBLESHOOTING

102| Perform appropriate statistical analysis. Note that there should be no difference in time to trigger between the light and no-light conditions. If a substantial difference is seen, an unintended bias may have been introduced, and the data must be re-examined.

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) (**Fig. 6e**) and power from the ends of the fibers.
? TROUBLESHOOTING

? TROUBLESHOOTING

Light emission through the implantable optical fibers is <30% (Steps 21–23)
Possible reasons: the fiber was nicked or broken inside the ferrule chamber, the ferule is defective (i.e., the hole for the fiber was off-center), or the epoxy was not fully polished away.

Possible solution: 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 and 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 re-polish the ferrule, taking care to polish away any visible cracks or defects (see **Fig. 1l** and compare with **Fig. 1k**).

The implant falls off (Steps 64–94)
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 mouse has become entangled in the cords, causing a torque on the implant.

Possible solutions: 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, it 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 before implantation. It may be helpful to make a larger initial incision and to clean a larger area of the skull before implanting the ring. Damage to the skull could also indicate that the mouse was entangled in the cables or on items in the cage. Use the video monitoring record to determine whether the mouse met resistance from the tethers before losing the implant, and resolve any sources of tension on the cables that might have arisen.

The mouse becomes lethargic or sick (Steps 64–94)

Possible reason: some mice do not adapt to being tethered as well as others. Most mice will seem unsure for the first few hours of being tethered, but they will 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 solutions: keep a record of the animals' weights as well as taking note of their food and water consumption. If a mouse 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 a mouse has difficulty adjusting, introduce it to the environment for a few hours before placing the tether. The mouse can also be habituated to the tether several hours per day over several days before extended recording periods.

The signal is missing or is composed primarily of movement artifact (Step 71)

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 solutions: 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, and ensure that they do not become covered by any insulating substances such as glue or cement. Ensure that implants are secured (see Troubleshooting for Steps 64–94). If the implant is suspect, remove the mouse 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, thus securing the implant better.

The implantable optical fiber slides out of the implant when the patch cable is removed (Steps 89–94)

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

Possible solution: 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 mice to avoid problems with the headstage.

After starting the custom software, the recorder will not run (Step 94)

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

Possible solutions: close all instances of the custom software and attempt to run the program as an administrator by right-clicking the executable and selecting 'Run as Administrator'. If that does not work, power cycle both the computer and digitizer, and then run the custom software as an administrator.

The laser is always on, or does not turn on when the program indicates that it should (Step 94)

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

Possible solutions: ensure that the mouse 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.

The desired light pulses are not being produced (Steps 94,100)

Possible reasons: 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 slowness.

Possible solutions: see the **Supplementary Manual** for more information about these parameters. The cycle time must be at least 1 s 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 (~2 ms) 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.

The signal degrades over time (Steps 94–101)
Possible reasons: 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 solutions: 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.

Light effects are visible in the recording, even in mice not expressing the opsin (Steps 96–100)
Possible reasons: the ends of the electrode are positioned in the path of the light, inducing a photoelectric current in the wires; or the light power is too high, causing tissue heating or perceptual cues to the mouse.

Possible solutions: When making optrodes, ensure that the electrode is shorter than the optical fiber. If you are 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 mice, and always compare effects with non–opsin-expressing control mice.

Not all video channels are being recorded for every file (Step 100)
Possible reason: the custom software may be unable to properly access the cameras.

Possible solution: restart the computer.

The optical fibers are not targeting the desired brain area (Step 103)
Possible reasons: the mouse was not in a head-flat position during implantation, the coordinates are not correct in the age/strain/sex of the mouse being used, the ferrule was not secured in the stereotaxic adapter (so was accidentally moved in the *z* direction before or during implanting) or the ferrule was not released fully before raising the stereotaxic arm (causing it to pull back out slightly).

Possible solutions: ensure that the mouse is secured in a 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 before 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.

● **TIMING**
(Optional) Steps 1–23, creating your own implantable optical fibers: 10 min (Made in batches of about 100, each fiber will take 10–15 min to make, spread over 2–3 d.)
(Optional) Steps 24–33, making an optrode from the implantable fiber and a bipolar electrode: 10 min per optrode
Steps 34–54, surgical implantation of fibers and electrodes: ~1 h (Depending on your proficiency, method of induction and the number of implanted fibers, this might take anywhere from 40 min to 2 h per mouse.)
Steps 55–70, hardware and software setup: variable (Once the hardware and cages are placed in a convenient location, connecting the hardware and installing the necessary drivers will take 1–2 h.)
Steps 71–88, initial assessment and tuning of signal: 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 15 min to a few hours tuning the detector to optimally pick up that mouse's seizures.)
Steps 89–94, on-demand seizure detection and light triggering: 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 mouse. Behavioral seizures were typically much less frequent, requiring weeks to months in order to achieve similar statistical power.)
Steps 95–103, data analysis: variable (Analysis of 100 events can be time consuming, and it will depend on the speed of the blinded observer, the parameters that will be measured, the number of files and so on. Generally, a data set of 100 events can be completed in a few hours.)

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 should be clean and the optrode should be positioned properly, providing an intracranial EEG signal with minimal movement or photoelectric artifacts. Provided that the mouse 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 (because of, e.g., scratching), detection criteria can be tuned to reduce both false positives and false negatives (**Fig. 6**). Once long-term stable recordings are achieved and the program is 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 mice expressing the inhibitory opsin halorhodopsin in excitatory cells rapidly truncates seizures (**Fig. 6d**).

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS M.O. designed the custom MATLAB software, E.K.-M. and C.A. contributed equally and performed the experiments and C.A., E.K.-M., M.O. and I.S. designed the experiments and prepared the manuscript.

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