Fabrication and application of flexible, multimodal light-emitting devices for wireless optogenetics

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The rise of optogenetics provides unique opportunities to advance materials and biomedical engineering, as well as fundamental understanding in neuroscience. This protocol describes the fabrication of optoelectronic devices for studying intact neural systems. Unlike optogenetic approaches that rely on rigid fiber optics tethered to external light sources, these novel devices carry wirelessly powered microscale, inorganic light-emitting diodes (μ-ILEDs) and multimodal sensors inside the brain. We describe the technical procedures for construction of these devices, their corresponding radiofrequency power scavengers and their implementation *in vivo* for experimental application. In total, the timeline of the procedure, including device fabrication, implantation and preparation to begin *in vivo* experimentation, can be completed in ~3–8 weeks. Implementation of these devices allows for chronic (tested for up to 6 months) wireless optogenetic manipulation of neural circuitry in animals navigating complex natural or home-cage environments, interacting socially, and experiencing other freely moving behaviors.

INTRODUCTION

Optogenetics is a relatively new field of neuroscience that gives researchers the ability to control cellular signaling and neural activity in a cell type–selective manner. *In vivo* applications of optogenetics have rapidly aided in the understanding of neural circuit function in behavioral models[1–10]. Despite the success of these studies, tethered fiber-optic approaches have restricted opportunities for the study of more complex, ethologically relevant behavioral paradigms such as enclosed home-cage behavior, spontaneous pain, wheel running and freely moving social interactions. Here we present a protocol for the fabrication of flexible devices that carry wirelessly powered microscale μ-ILEDs and multimodal sensors to study neural circuitry in awake, freely moving animals. The devices described in this protocol are robust, self-contained, multifunctional and they are capable of wireless operation with conventional electronics and power supplies. These fully electronic systems eliminate the need for high-powered light sources, fiber coupling fixtures and optomechanical hardware for *in vivo* optogenetic experiments. The following protocol is based on technology and approaches developed jointly in our two laboratories[11–16].

Development of the protocol and comparison with traditional light sources

This protocol is the result of advances in material science that have led to the development of flexible electronics, biodissolvable adhesives, microscale sensors and high-efficiency μ-ILEDs[12–14,17,18]. Although other groups have successfully implemented wireless schemes and LEDs for optogenetics[19–22], the protocol described here provides a completely customizable approach for combining various materials engineering approaches to design and implement devices that can be optimized for an individual laboratory's experimental needs.

The current standard in neuroscience for light delivery into the depth of the brain is to use chronically implanted fiber optics[23]; this approach offers substantial advantages over acute delivery of fibers via metal cannulae[1,11]. These chronic implants, however, have their own limitations. Principally, light from fiber-optic implants can only escape from the tip of the implant to illuminate ventral brain structures. Although adaptations to this ventral light delivery are possible[10,24,25] (available commercially at http://www.doriclenses.com), the range of customizability can be limited and often restricts the user to only delivering light without the capability of observing physiology. Furthermore, μ-ILEDs create opportunities to restrict or expand spatial targeting by selecting from a range of sizes (625–10,000 μm^2), by altering the number and arrangement of μ-ILEDs, and by using reflective materials to direct light. This protocol provides a basis from which any combination of μ-ILEDs and sensors can direct light within the brain and measure physiological function without the restriction of enforced light trajectory.

Various strategies have been used for delivering multiple wavelengths of light into the same animal[24–26]. These approaches require establishing an extensive network of optics and tethered optical equipment external to the behaving animal. Depending on the laboratory behavioral space, such setups can restrict experimental possibilities, and they can require advanced experience

with optics to maintain optimal conditions. This protocol may require access to external facilities for some laboratories, but the end result is a device that can be operated with basic laboratory equipment that is already likely to be present in most neuroscience laboratories. Furthermore, the rescue and recycling of these devices for reuse is relatively easy, meaning that these devices can be used for several rounds of behavioral experimentation with different sets of animals. For light-evoked activation of channelrhodopsin-2 [ChR2 (H134)], optically sensitive seven-transmembrane domain receptors (i.e., OPTO-α_1, OPTO-β_2), and other blue light–sensitive optogenetic constructs, GaN μ-ILEDs are appropriate[2,11,27]. A device that uses these 450-nm-emitting μ-ILEDs is the focus of this protocol. However, it is important to note that μ-ILEDs emitting at other relevant wavelengths are also possible to fabricate for use in other contexts[11,28]. The combination of μ-ILEDs of different wavelengths provides the user with access to activation spectra of multiple optogenetic constructs with a single implanted, electronic device. Furthermore, the electronic nature of these devices ensures that they can be operated wirelessly. Wireless optogenetic manipulation of neural circuitry has been achieved by other means[20,21], but these approaches can restrict behaviors accessible to study because the animal is required to remain in a fixed environment. By using radiofrequency (RF) power scavenging, the devices and approaches contained in this protocol free the user of constraints on behavioral assays, thereby allowing for experimental testing in any space.

Applications of the protocol

Although the focus of this protocol is on the creation of devices engineered to deliver μ-ILEDs into the brain for optogenetic applications, this same protocol can be used to fabricate devices to measure electrophysiological, thermal and other properties of intact brain tissue (**Box 1**). The flexible nature of these devices provides the potential to extend their application into other intact tissues such as the peripheral nervous system and the circulatory system/cardiac tissue of larger organisms.

Experimental design

Subjects. This protocol and these devices have been optimized for use in adult (25–35 g) male C57BL/6J mice and mutant mice backcrossed to the C57BL/6J mouse strain. However, as the optogenetic toolbox expands to other mammals[29–34], these devices will probably have broader utility in other animal models. Specifically, larger organisms such as rats and nonhuman primates will tolerate the ~700-mg wireless antenna with greater ease than mice. Unlike animals with polished fiber-optic implants, animals with chronically implanted μ-ILEDs can be housed with other animals, as the metal pin connectors cannot be damaged by cage mates. Before beginning this protocol, all the procedures described herein should be approved by the Animal Care and Use Committee of the investigating institution and conform to US National Institutes of Health (or other relevant governmental guidelines) guidelines regarding animal research. For this protocol, all procedures were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

Controls. As noted elsewhere[2,11,23], the proper controls for *in vivo* optogenetics are mice that express genetically encoded fluorescent reporters in the absence of an opsin. These control mice will account for any disturbance from viral injection, device implantation, heating confounds from light delivery and fluorophore fluorescence within the brain. Another important confound to consider when conducting light-evoked single-unit electrophysiological experiments is the potential for generating neural activity downstream of retinal stimulation. These devices prevent the external light escape that is common with fiber-optic implants, but activity from the visual system has the potential to activate the retina and retinal ganglion cells from within the brain, depending on the brain region[35]. Recordings performed with external light stimuli can control for these effects. In addition, properly counter-balanced within-subject experimental designs are possible with these devices by withholding power to the devices during the behavioral testing period.

Limitations. Although these devices offer numerous advantages over traditional fiber-optic implants, the technology is not intended to replace protocols that work well to manipulate neural circuitry *in vivo*[1,23]. The greatest limitation associated with this procedure is the accessibility to the facilities and the technical skill required to fabricate these devices and their scavenging antennas. That being said, subsequent recovery and refabrication of the devices is straightforward and accessible to any laboratory. Once the protocol is followed, there are a few considerations when designing behavioral experiments. First, depending on the panel antenna used, the signal can be polarized. In current designs, both the panel antenna and the antenna on the scavenger are directional with linear polarization. A maximum efficiency of power transmission and reception is achieved when the polarizations of both antennas are aligned. The power transmitted by the panel antenna can typically be adjusted to accommodate mismatches that can occur in most practical situations. Such issues can be avoided entirely by the use of a transmission antenna with circular polarization or multiple-panel antennas. Care should also be taken to ensure that the animal's surroundings do not interfere with the RF signal by powering a free-standing device in the behavioral context[11]. Wireless control of these devices is compatible with a wide range of behavioral assays. In our hands, the devices have performed well in tests of anxiety-like behavior (open field test, elevated zero maze), reward-related behavior (conditioned place preference, operant behaviors and self-stimulation), social behaviors (social defeat stress and social aversion), pain behaviors (Hargreaves test) and home-cage behaviors[11]. In our experience, commonly used materials for behavioral apparatuses (wood, polyvinyl chloride, poly(methyl methacrylate) and the metal from cage lids) do not interfere with the RF signal, but they can reduce the overall power if they are positioned between the panel antenna and the headstage scavenger (see TROUBLESHOOTING).

The current protocol only provides wireless access to power the μ-ILEDs and not to receive information from the sensors. This backward data-stream restriction is largely based on the weight that an animal can endure on the headstage. For a mouse, electrophysiological sensors, temperature sensors and photosensors currently require a wired connection. For larger mammals, the devices can be integrated with existing telemetry setups (commercially available at http://www.plexon.com), provided that they do not interfere with the RF signal[36,37].

Box 1 | Combining multifunctional sensors and optoelectronics ● TIMING 1–4 d

One hallmark of these devices is their ability to both deliver light into the brain and to record information from the brain. Depending on the experiment, users may wish to outfit the devices with a variety of multiple and optional modalities. Each different modality can be combined with any other modality; devices can have between one and four different modalities incorporated. However, if the electrophysiological sensor is incorporated it should be the outermost layer to have direct exposure to the tissue.

1. The following steps provide information on adding functional layers (temperature sensors, option A, see also ; μ-IPDs, option B; and μ-electrodes for electrophysiological sensors, option C) to the devices. If these optional modalities are desired, **Box 1** should be completed before Step 16 of the PROCEDURE.

(A) Fabrication of temperature sensors

(i) Generate PR patterns on a needle-shaped piece of PET (same substrates used in Step 12) and deposit platinum (Pt, 10 nm) by sputtering. Remove PR by acetone and generate Cr/Au (15/300 nm) metal lines that connect to the Pt resistor.

(ii) Calibrate the temperature sensor. Dip the temperature sensor into DI water and measure reference resistance using a digital multimeter. With precisely controlled temperature of the DI water, measure the changes in resistance. As the resistance change (ΔR) of the Pt resistor depends linearly on the temperature change (ΔT), the formula for estimated temperature is $\Delta T \sim k \times \Delta R$, where k is constant.

(B) Fabrication of μ-IPDs

▲ CRITICAL (2nd layer shown in **Fig. 3**) Additional details of this method are shown in a previous publication[50].

(i) Deposit SiO_2 by PE-CVD on a silicon-on-insulator (SOI) wafer, with a top silicon layer thickness of ~1 μm.

(ii) Generate PR patterns to pattern the SiO_2 with HF.

(iii) Remove the PR and clean the wafer surface by RCA cleaning. First perform SC (standard cleaning)-1 with 1:1:5 solution of ammonium hydroxide (NH_4OH):hydrogen peroxide (H_2O_2):DI water at 80 °C for 15 min. Second, remove the SiO_2 layer using 1:50 solution of HF:DI water. Finally, perform SC-2 with a 1:1:6 solution of hydrochloric acid (HCl):H_2O_2:DI water at 80 °C for 15 min (RCA cleaning: http://inside.mines.edu/fs_home/cwolden/chen435/clean.htm).

(iv) Carry out solid-state doping of the silicon to form a p-type region.

(v) Repeat steps i–iv for n-type doping.

(vi) Generate PR hole patterns on the entire wafer surface and etch the silicon layer, to define the lateral dimensions of the photodetectors.

(vii) Etch the buried oxide (BOx) layer using HF.

(viii) Retrieve an ~1-μm-thick Si membrane photodiode using a PDMS slab and release it onto the needle-shaped PET substrate (described in Step12).

(ix) Form metal (Cr/Au, 15/300 nm) lines for interconnection.

(B) Fabrication of μ-electrode for electrophysiological sensor

(i) Generate PR (AZ 2030) lift-off patterns (1st layer shown in **Fig. 3**).

(ii) Deposit Pt (30 nm), a needle-shaped piece of PET (described in Step 12), by sputtering. Generate a pattern of SU-8 2 (2 μm thick) with a 20 × 20 μm^2 square opening.

▲ CRITICAL STEP An additional oxygen de-scum to the remove residual PR layer is recommended. The impedance of the Pt μ-electrode should be ~1.0 MΩ at 1 kHz.

Finally, independent control of individual μ-ILEDs is also possible with these devices, but this protocol has not been optimized for these conditions in a wireless control mode. If independent control is a desired feature, it is recommended that smaller connections featuring a number of channels be used over the pin connectors that we present here (many options are commercially available at http://www.omnetics.com).

Upon completion of the protocol presented here, users can expect to have devices with four μ-ILEDs that can be wirelessly powered with suitable light output from 2 m away. In our experience, more μ-ILEDs can be added to a single device with relative ease (the maximum we have tested is 25 (5 × 5) μ-ILEDs from a single power scavenger). Furthermore, the RF power-scavenging approach is appropriately suited for powering multiple μ-ILED devices (presumably in multiple animals) by using a different power-scavenging headstage antenna for each device. The upper limit on the number of simultaneously powered devices is primarily dependent on the spatial constraints of the experimental space and apparatus to achieve equal powering to all devices.

Notes on the materials and equipment used in this protocol. Accurate injection of these devices into brain tissue requires a stereotaxic adaptor for standard cannula holders. The basic principle of this adaptor is to allow the μ-needle to be mounted in line with the existing stereotaxic system. The dimensions of such an adaptor will vary by stereotaxic alignment system make and model, but most university machine shops should be able to fashion such an adaptor. The adaptor presented in this protocol was specifically designed for use with the KOPF single cannula holder (model 1966). It is also possible that other commercially available electrode holders may be capable of accurately targeting the devices (e.g., KOPF model 1768). More detailed information on the machining and dimensions of the adaptor for the KOPF model 1966 can be found in **Supplementary Figure 1**.

To deliver pulse trains of light, the devices can be controlled using a traditional function generator to drive amplitude modulation of the wireless powering equipment. Amplitude modulation is an internal function available on most RF generators. This function allows an internally or externally supplied modulating

signal to control the amplitude of the output RF signal. The internal modulating signal is usually a sinusoidal waveform with a much lower frequency compared with that of the RF output. For the positive region of the sinusoidal signal, the RF generator can output an RF signal whose amplitude is modulated by the low-frequency sinusoidal signal, and it can change from 0 to the maximum set power and then back to 0. For the negative region of the modulating signal, the output RF signal maintains at 0. An external transistor-transistor logic (TTL)-modulating signal is preferable in terms of modulating the amplitude of the output RF signal. Here, the high state of the TTL causes the RF generator to output a constant set power, whereas in the low state of the TTL the RF generator outputs 0 power. Thus, the TTL-modulating method is more suitable for generating constant light intensity during the high state of the TTL. The pulse width and frequency of photostimulation should be determined and based on physiologically relevant conditions[7,10,38–40].

Postmortem rescue of the devices for reuse is a delicate but straightforward process. The dental cement presented here (Lang Dental) allows for such rescue. Other cements and bonding agents can inhibit the process and destroy internal components. It may be necessary to use a stronger bonding agent in some scenarios (see TROUBLESHOOTING). In such cases, the reusability might be compromised.

MATERIALS

REAGENTS

Preparation of releasable polymer template

- UV-curable epoxy (SU-8 2 and 100 photoresist (PR); Microchem)
  ! CAUTION Partially cured or uncured epoxy needle can induce excess chemical contamination in the brain.
- SU-8 developer (Microchem)
- Isopropyl alcohol **! CAUTION** Isopropyl alcohol is flammable.
- Acetone **! CAUTION** Acetone is flammable.
- Silk adhesive (details are shown in other papers[13,17,18]; see Reagent Setup)
- Benzocyclobutene (BCB, DS-4022 35; Dow Corning)
- BCB developer (advanced developer, DS2100, Dow Corning)
- Water-soluble tape (3M)
- PRs (AZ 1518, AZ 2070; Capital Scientific)
- AZ 300 MIF developer (Capital Scientific)
- Hydrofluoric acid (HF) **! CAUTION** HF is extremely corrosive. Wear gloves and use eye protection when using HF.
- Chrome (Cr) etchant, gold (Au) etchant, palladium (Pd) etchant (Transene)
- Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning)
- Polyethylene terephthalate (PET; polyester film, 2.5 and 6 μm thick, Mylar film, Chemplex)

Preparation of μ-ILEDs

- Gallium nitride (GaN) LED epitaxial materials grown on sapphire substrates (Cermet)

Injection of virus and μ-ILEDs into targeted brain structure

- Isoflurane, USP (Isothesia, Butler Schein, cat. no. 029405) **! CAUTION** Before beginning this protocol, the Animal Care and Use Committee of the investigating institution should approve all procedures and conform to US National Institutes of Health (or other relevant governmental guidelines) guidelines regarding animal research **! CAUTION** Ensure that proper ventilation and gas scavenging methods are in place to prevent potential inhalation of excess isolflurane.
- Betadine solution (Purdue Products, cat. no. 67618015017)
- Ethanol (Sigma-Aldrich, cat. no. 362808) **! CAUTION** Ethanol is flammable.
- Hydrogen peroxide, 3% (wt/vol) USP (Select Medical Products, cat. no. 117)
- Lidocaine ointment USP, 5% (wt/vol) (Fougera)
- Ophthalmic ointment (Altalube Ophthalmic Product)
- Adhesive luting cement (C&B-Metabond, Parkell, cat. no. S380) **! CAUTION** Methyl methacrylate monomer, stabilized is a flammable liquid. It may also cause skin irritation; avoid contact with skin, eyes and clothing. Use it with adequate ventilation.
- Light cured bonding adhesive (VLC one-etep prime and bond adhesive, cat. no. 305-006-030) **! CAUTION** Methyl methacrylate monomer, stabilized is a flammable liquid. It may also cause skin irritation; avoid contact with skin, eyes and clothing. Use it with adequate ventilation.
- Dental cement (Jet denture repair, Lang Dental, cat. no. 1223, see Reagent Setup) **! CAUTION** Methyl methacrylate monomer, stabilized is a flammable liquid. It may also cause skin irritation; avoid contact with skin, eyes and clothing. Use it with adequate ventilation.
- Sodium chloride injection, 0.9% (wt/vol), USP, 9 mg ml^{-1} NaCl (Hospira, cat. no. RL-0497(9/04))
- Enrofloxacin (Baytril, Bayer, cat. no. R30901)
- Antibiotic ointment (Neosporin; Johnson & Johnson, cat. no. 174-73087Q)
- Viruses of interest (adeno-associated virus and lentiviruses are available from the Washington University of St. Louis Hope Center Viral Core, https://hopecenter.wustl.edu/?page_id=99 and/or the University of North Carolina Viral Vector Core, http://genetherapy.unc.edu/services.htm). Herpes simplex viruses are available from the Massachusetts Institute of Technology (MIT) Viral Core, http://mcgovern.mit.edu/technology/viral-core-facility)
  ! CAUTION Follow the appropriate safety precautions pertaining to the particular virus in use. It may be necessary to obtain a higher Biosafety Level certification before use.
- Artificial cerebral spinal fluid (ACSF; Tocris, cat. no. 3525 or custom, see Reagent Setup)[1,41]
- Animal(s) to be injected with the μ-ILED device. The procedure describes how to use a μ-ILED device with a mouse, as previously described[11]
  ! CAUTION Before beginning this protocol, the Animal Care and Use Committee of the investigating institution should approve all procedures and conform to US National Institutes of Health guidelines (or other relevant governmental guidelines) regarding animal research.

Behavioral procedures using RF power scavenging

- Thermal grease (Wakefield Solutions, cat. no. 120-2)

EQUIPMENT

Preparation for μ-ILEDs and multifunctional sensors

- Sputter (AJA international, ATC 200) metal deposition for Au (gold), Ni (nickel), Pt (platinum), etc.
- Rapid thermal annealing (RTA) **! CAUTION** High temperature, semi-transparent L-shaped current spreading layer on p-side on GaN LED should be used in thin metal layers (15/15 nm Ni/Au) followed by annealing at 500 °C.
- Mask aligner (Karl Suss, MJB)
- Inductively coupled plasma etcher (ICP; PlasmaTherm SLR-700, etching for GaN)
- Reactive ion etcher (RIE, PlasmaTherm 790, etching for SiNx)
- Plasma enhancement-chemical vapor deposition (PE-CVD; STS-mixed frequency nitride deposition system)
- Wafer bonder (Electronic Visions, EV501)
- Laser lift off (LLO; Krypton Fluoride (KrF), 0.9 J cm^{-2}, 248 nm wavelength (IPG Photonics (http://www.ipgphotonics.com/microprocessing.htm)) or Yttrium aluminum garnet (YAG):Nd laser (0.3 J cm^{-2}, 266 nm single pulse with 5 ns exposure, Sandia National Lab) **! CAUTION** Avoid eye contact with the laser; always wear eye protection.
- Digital multimeter (Fluke 115 or other commercially available multimeter)
- Printed circuit board (PCB) for headstage connection (General Circuits)
- Male pins for headstage PCB, 2.54-mm spacing male pins (Sunlight Inc., cat. no. P2540-H254-S180)

Measurement of μ-ILED

- Ocean Optics (Ocean Optics, HR4000; for measuring wavelength spectrum, light output power)
- Probe station (Agilent 4155; current-voltage (I-V) characteristics)
- Pulse generator (Global specialties, 4001)

- Oscilloscope (Agilent, DSOX2004A, 70 MHz)
- IR camera (QFI InfraScope II)

Injection of virus and μ-ILEDs into targeted brain structure

- Stereotaxic alignment system (KOPF model 1900) ! CAUTION Before beginning this protocol, the Animal Care and Use Committee of the investigating institution should approve all procedures and conform to US National Institutes of Health guidelines (or other relevant governmental guidelines) regarding animal research.
- Stereotaxic alignment indicator (KOPF model 1905)
- Centering microscope, 40× (KOPF 1915)
- Stereotaxic drill (KOPF model 1911) and no. 66 drill bit (KOPF, cat. no. 8669)
- Stereotaxic single cannula holder (KOPF model 1966)
- Stereotaxic adaptor for cannula holder (see **Supplementary Fig. 1**, made in your institutional machine shop or other)
- Anchoring screws (CMA Microdialysis, cat. no. 7431021)
- Needles (Becton-Dickinson, cat. no. 305111)
- Microinjection syringes (Hamilton, cat. no. 88011)
- Infusion pump and controller (UltraMicroPump III, World Precision Intstruments, cat. nos. UMP3 and UNC4)
- Forceps (Miltex, cat. no. 6-100)
- Surgical scissors (Miltex, cat. no. 18-1430)
- Hemostats (Miltex, cat. no. MH7-26)
- Microspatula (Chemglass, cat. no. CG-1982-12)
- Electric clippers (Wahl, cat. no. 8064-900)

Behavioral procedures using RF power scavenging

- RF generator (Agilent, N5181 MXF)
- Function generator with standard TTL (AMPI Master-9 or other)
- RF amplifier (Empower RF systems, 1100/BBM2E4AJP)
- RF antenna (ARC wireless solutions, ARC-PA0913B01, ARC 902–928 MHz, 12.5 dBi, flat panel)
- DC power supply (Mastech, cat. no. HY5005E-2)
- Relevant behavioral assay apparatus (university machine shop, MED Associates, Harvard Apparatus or other)
- Wireless headstage antenna (see PROCEDURE)
- PCB for wireless headstage antenna (General Circuits)
- Ceramic antenna (W3012; Pulse Electronics)
- Schottky diode (Digi-Key, cat. no. MMDL301T1G)
- Power meter (Bird Electronic, Thruline 43)
- Two SMA-male to N-male precision cables (Pasternack, cat. no. PE304-120)
- Fan-cooled heat sink (Fischer Elektronik, cat. no. LA 17/200 24V)

Behavioral procedures using a wired connection

- Animal(s) with injected μ-ILED device ! CAUTION Before beginning this protocol, the Animal Care and Use Committee of the investigating institution should approve all procedures and conform to US National Institutes of Health guidelines (or other relevant governmental guidelines) regarding animal research.
- Relevant behavioral assay apparatus (university machine shop, MED Associates, Harvard Apparatus or other)
- Function generator for standard TTL (AMPI Master-9 or other)
- BNC cables (Cables to Go, various lengths and cat. nos.)
- Electrical rotary joint (Moog, cat. no. SRA-73683 (ref. 42))
- BNC-to-banana plug adaptor (Fluke cat. no. BP881 or other)
- Wire, 30 gauge (Artistic Wire) and female connector (single modality devices: TE Connectivity, cat. no. AMP 3-640441-2; multimodal devices or independent μ-ILED control: TE Connectivity, cat. no. AMP 87631-4)

REAGENT SETUP

Silk adhesive Boil cocoons of the *Bombyx mori* silkworm for 30 min in a solution of 0.02 M Na_2CO_3 to remove the sericin protein. Rinse the extracted fibroin with dH_2O and dry it in ambient air for 12 h. After drying, dissolve the fibroin in a 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20 wt% aqueous solution, and subsequently dialyze the solution against dH_2O by using a dialysis cassette at room temperature (20–25 °C) for 48 h until the solution reaches a concentration of 8 wt%. Silk adhesive can be stored at room temperature for up to 6 months.

Dental cement The dental cement can be prepared as described elsewhere[23] by combining the Jet denture repair powder with the Jet liquid (methyl methacrylate). A starting point is 350 l of methyl methacrylate monomer added to 225 mg of powder. In some cases, the viscosity must be adjusted (by increasing or decreasing the amount of methyl methacrylate in the mixture) to properly secure the flexible device in place and/or mount the PCB in the headcap. Dental cement can be stored at room temperature for up to a year. ▲ CRITICAL Adjusting the viscosity alters the working time of the cement.

Virus Each type of virus has different handling instructions, but all must be stored on ice until immediately before injection. In some cases, dilution of stock titers requires obtaining more of the reagent in which the viral preparation was initially concentrated.

ACSF If not purchased, the solution can be prepared according to recipes described elsewhere[1,41]. It can be stored as a stock solution at 4 °C for up to 1 month and at room temperature as a working solution for 1 d.

EQUIPMENT SETUP

Preparation of amplifier with proper heat sink The RF amplifier requires an additional cooling system during operation. Thermal grease applied on top of the heat sink facilitates thermal contact with the amplifier, which is affixed to the sink using screws. The DC power supply operates both the amplifier and the fan attached to the heat sink by supplying power with voltages of 24 V and 10 V, respectively.

PROCEDURE

Preparation of μ-ILEDs ● TIMING 7 d

1| Clean the wafer surface of GaN LED stacks (450-nm emission wavelength; p-type GaN/multi-quantum well (MQW)/spacer/n-type GaN/undoped GaN) grown on sapphire substrate with 5 wt% diluted HCl for 5 min.

▲ CRITICAL STEP Proper cleaning enables reduced turn-on voltages and improved efficiencies, both of which minimize the production of heat by the operating μ-ILEDs.

2| Deposit metals (Ni/Au, 15/15 nm) immediately after cleaning.

▲ CRITICAL STEP The deposition should be carried out at degree vacuum levels of ~10^{-6} torr or less.

3| Form L-shape patterned metals (Ni/Au) onto the p-type GaN semiconductor layer by photolithography and wet etching with Cr and Ni etchants; follow by removal of the PR with acetone (**Fig. 1a**).

4| Anneal the wafer for 10 min at 500 °C in 20% oxygen and 80% argon atmosphere to generate *p*-ohmic contact.

▲ CRITICAL STEP The annealed metal layers should be semitransparent after this step.

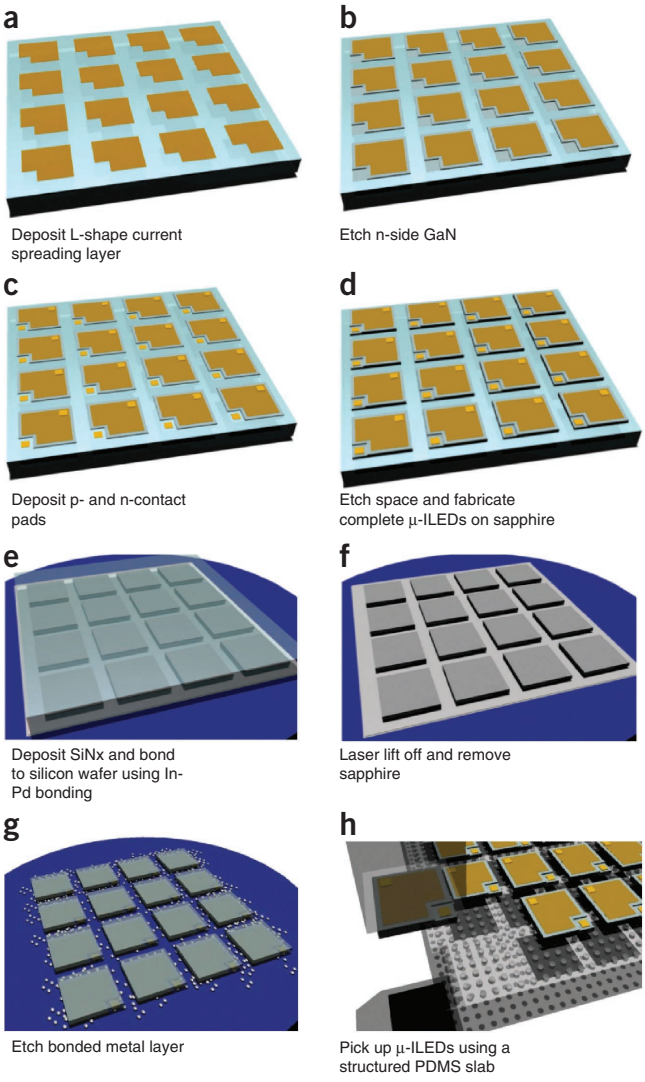

Figure 1 | Fabrication procedure for injectable μ-ILEDs. (**a**) An L-shaped current spreading layer (patterning of Ni/Au (15/15 nm) layers followed by 500 °C annealing) is formed on GaN blue lighting LED stacks grown on sapphire. (**b**) To expose n-type GaN semiconductors, etch rectangular shapes of p-type one, multi-quantum well (MQW) and spacer layers by reactive ion etching. (**c**) Generation of n- and p-contact pads (Cr/Au (15/300 nm) 25 × 25 μm^2 squares) on n-and p-type GaN sides. (**d**) Etch a 20-μm-width trench to define lateral dimensions of 100 × 100 m^2 or smaller. (**e**) Bonding μ-ILEDs on sapphire with a silicon wafer using an In-Pd metallic alloy bond. (**f**) Expose the laser on sapphire and mechanically remove the sapphire from μ-ILEDs. (**g**) Wet-etch the bonded metallic layer located on the wafer. (**h**) Transfer of all μ-ILEDs onto structured PDMS slabs and selectively pick up single μ-ILEDs by using a polydimethyl siloxane (PDMS) stamp. Panels **d**, **f**, **g** and **h** are reproduced from ref. 12 with permission from Wiley.

5| Generate 40 × 40 μm^2 square hole patterns in a negative-tone PR (AZ 2070 PR) and etch (chlorine gas–based RIE etching) the p-GaN layer, MQW, spacer to open n-side GaN (**Fig. 1b**).

6| Generate 25 × 25 μm^2 square hole patterns in PR for n- and p-type metal contact pads and deposit Cr/Au (10/300 nm) by using an electron beam evaporator. Then, remove PR (**Fig. 1c**).

7| Deposit a low-stress layer (200 nm) of SiNx on the substrate, by using PE-CVD. Next, pattern a negative-tone PR (AZ 2070) to serve as a mask for etching the SiNx and the GaN to define the lateral dimensions of the array of μ-ILEDs. Devices with dimensions of 100 × 100 μm^2 or less are formed with 20-μm spacings (**Fig. 1d**).

8| Deposit a layer of SiNx onto the μ-ILEDs to protect the devices and metal pads from further processes. Bond the LED wafer to a silicon substrate with an indium (In)-palladium (Pd) metallic alloy. This bonding uses Cr/Pd (15/150 nm) on the LED substrate and Cr/Pd/In (15/150/900 nm) on the silicon. The bonding occurs on contact with a pressure of 400 bar and a temperature of 220 °C for 2 h (**Fig. 1e**).

9| Use laser illumination through the sapphire to release the μ-ILEDs (**Fig. 1f**). Details of this LLO technique appear elsewhere[12,43].

10| Etch the unalloyed metal by immersion in 5 wt% HCl for 30 min (**Fig. 1g**).

11| Retrieve all μ-ILEDs onto the substrate of PDMS with patterns of surface relief, in the geometry of cylindrical pillars with 3 μm diameter and 1.4 μm height and 5 μm spacing. Remove residual metals, including In-Pd alloy and Cr, by Pd and Cr etchants (**Fig. 1h**).

12| Prepare a PDMS stamp with a single relief feature consisting of a post with 100 × 100 μm^2 lateral dimensions and 100 μm height. By using a mask aligner for photolithography, retrieve a single μ-ILED with the stamp and transfer it to a needle-shaped structure of PET adhered to a thin layer of PDMS on a transparent temporary substrate (**Fig. 2**).

13| Remove the passivation layer, SiNx (deposited in Step 8), with RIE and spin-coat a photosensitive BCB (6-μm-thick) onto the μ-ILED.

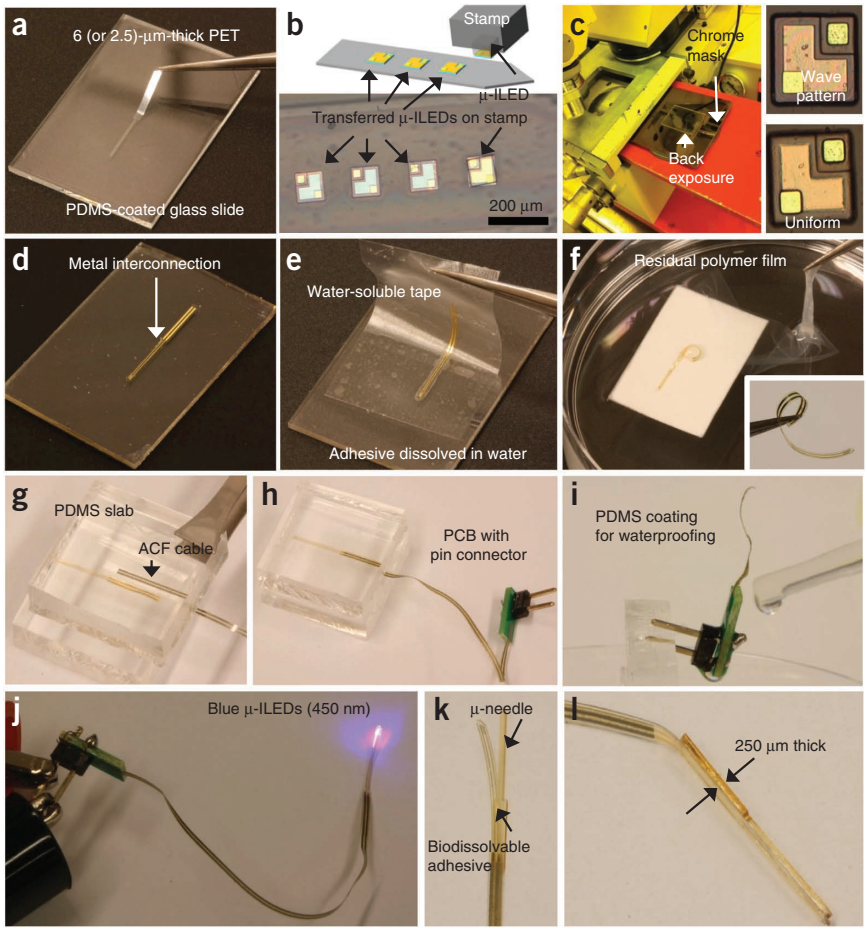

Figure 2 | Procedure for fabrication of injectable, multifunctional electronics. (**a**) Thin (~2.5-μm thick) needle-shaped PET is attached to a temporary PDMS-coated glass substrate. (**b**) Schematic and photograph demonstrating the transfer printing of four μ-ILEDs onto the tip of the PET using a PDMS stamp. (**c**) Passivation approach with photocurable BCB polymer. The back of the BCB-coated substrate is exposed to UV light. The wave pattern in the upper inset shows nonuniform coating of BCB. The lower inset shows successful uniform coating. (**d**) The metal interconnection (Cr/Au) is generated by sputtering, photolithography and metal etching to electrically connect the four μ-ILEDs. (**e**) The connected device is picked up with water-soluble tape. (**f**) The substrate is separated from the tape after the adhesive is dissolved in the water. The inset shows the μ-ILEDs on free-standing thin, flexible, needle-shaped PET. (**g**) The device is electrically connected to the ACF cable. The PDMS slabs on the top and bottom are compressed using high temperature (~150 °C) to bond the ACF cable. (**h**) The other side of the ACF cable is connected to the PCB with pin connector for wireless or wired powering schemes. (**i**) The ACF cable and PCB are coated with PDMS for waterproofing. (**j**) Blue (450 nm) μ-ILEDs are powered. The μ-ILEDs and electrical connection should be checked before injection. (**k**) The device is assembled with injection μ-needle using biodissolvable silk adhesive. (**l**) Image of a completed device ready for injection into brain tissue.

14| Pass UV light through the back of the substrate and develop the BCB with BCB developer (**Fig. 2c**) to obtain holes for n- and p-side contacts (**Fig. 2c**, lower inset). After further curing with BCB, anneal the samples at 250 °C on a hot plate for 3 h.

▲ **CRITICAL STEP** Make sure that BCB covers the μ-ILED surface when curing. Nonuniform coatings of BCB generate interference fringes that are readily visible (**Fig. 2c**, upper inset).

▲ **CRITICAL STEP** The annealing should be carried out in Ar atmosphere to avoid high-temperature damage to the BCB and polymeric layers.

? TROUBLESHOOTING

15| Deposit Cr/Au (15/300 nm) by sputtering, and form metal interconnects using positive-tone PR (AZ 15198), followed by metal etching (**Fig. 2d**).

Deterministic device assembly by transfer printing and formation of electrical interconnects ● TIMING 1 d

16| Remove μ-ILEDs fabricated on each temporary substrate, as shown in **Figure 2a**, with water-soluble tape (**Fig. 2e**). Remove the water-soluble tapes in deionized (DI) water (**Fig. 2f**) and place the devices on slabs of PDMS (**Fig. 2g**). If appropriate, follow instructions in **Box 1** to assemble a multifunctional device (**Fig. 3**).

▲ **CRITICAL STEP** Steps 16–20 describe the device assembly for μ-ILEDs alone. If you are assembling a multifunctional device, follow the instructions in **Box 1** before proceeding.

17| Connect each of the devices with anisotropic conductive film (ACF) cable to a PCB board (**Fig. 2h**).

▲ **CRITICAL STEP** Apply pressure and high temperature (~150 °C) during ACF cable bonding.

? TROUBLESHOOTING

18| Assemble all devices onto an injection μ-needle formed in SU-8 epoxy. The entire region of the injection device should be electrically insulated for *in vivo* use. Cover the flexible device with SU-8 polymer and the other parts with PDMS

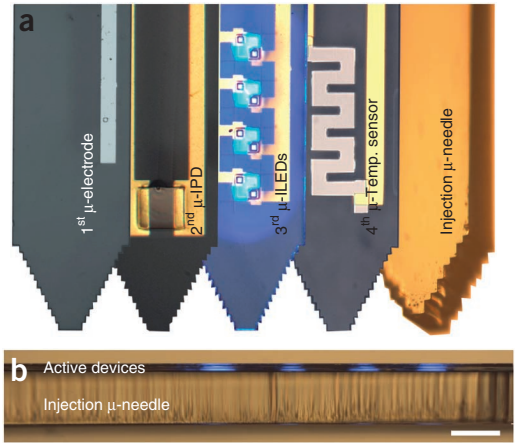

Figure 3 | Multifunctional sensors and optoelectronics. (**a**) Representative scheme for multifunctional, injectable electronics formed on an injectable needle. The devices include an electrophysiological sensor (μ-electrode; 1st layer), a silicon photodiode (μ-IPD; 2nd layer), four microscale μ-ILEDs (3rd layer), and a temperature sensor (μ-temperature (μ-temp.) sensor; 4th layer) based on a platinum resistor are formed on an injectable μ-needle fabricated from epoxy polymer. (**b**) Side view of such a device reveals the ultrathin nature of the active components of the device. Scale bar, 200 μm.

for waterproofing (**Fig. 2i**). Temporarily fix the device to the PDMS slab. Drop PDMS solution (Sylgard 180, Dow Corning, weight ratio = 10:1 (silicone elastomer precursor: curing agent), see http://www.dowcorning.com/applications/search/default.aspx?r=131en) onto the ACF cable and PCB, except for the pin connection area. After baking the PDMS in the oven at 70 °C for 2 h, the entire area except the pin adaptor is electrically passivated by PDMS.

19| To check functionality, power the device by connecting it to a power supply capable of delivering more than 1 mW (**Fig. 2j**). Use silk adhesive to bond the devices to the injection μ-needle (**Fig. 2k**,**l**).

Fabrication of wireless power harvester and preparation of wireless operation ● **TIMING 1 d**

20| Build either the robust, PCB-based (option A) or thin, flexible (option B) wireless power harvester.

▲ **CRITICAL STEP** Both options perform equally, but each has its advantages. The PCB-based option is more easily constructed and more durable, but it weighs more than the flexible version. We recommend using the PCB-based harvester for experiments that involve social interaction, whereas the flexible version is ideal for experiments involving only a single animal.

(A) Construction of the PCB-based wireless power harvester

(i) Build the wireless power harvester from two stacked PCB circuits (**Fig. 4**; general circuits). Each PCB includes top and bottom layers that are mostly covered by solid copper ground planes.

(ii) Mount the electrical components on the PCB boards through soldering. The first PCB circuit (labeled as 5 in **Fig. 4b**) contains a ceramic antenna operated at 915 MHz with a clearance space of 10.8 × 8.25 mm to the surrounding ground plane.

(iii) Connect a capacitor (8.2 pF in capacitance) between the feed line of the antenna and the ground plane to match the impedance of the antenna with the following circuit. A secondary PCB circuit (labeled as 6 in **Fig. 4b**) contains a voltage multiplier circuit constructed with six pairs of capacitors (47 nF in capacitance) and Schottky diodes (MMDL301T1G) in cascaded connection[11].

(iv) Mount the connector that is used for the μ-LEDs to the secondary PCB circuit. Join the PCB circuits and the connector with flexible copper wires (**Fig. 4c**), and then bond them together with resin epoxy (**Fig. 4d**). **Figure 4i** shows a schematic illustration of the power harvester. The circuit parts, which are labeled with numbers 5–7, correspond to the same numbers shown in **Figure 4b**,**e**.

(B) Construction of the thin, flexible wireless power harvester

(i) Fabricate the flexible PCB circuit on a 75-μm-thick Kapton film (**Fig. 4f**). Coat the film with a 6-μm-thick layer of copper by using electron beam evaporation.

(ii) Pattern the copper to form pads for the electrical components and interconnection. Spin-cast and pattern a 1-μm-thick polyimide film to expose the electrical contact pads, while passivating the interconnection (**Fig. 4f**).

(iii) Attach a ground plane made of copper tape (**Fig. 4g**) to the bottom of the Kapton film and connect it with the copper pattern on the top of the Kapton film through channels filled with solder. A schematic illustration of the flexible PCB circuit appears in **Figure 4j**.

21| Set up the wireless power transmission system. The system consists of an RF signal generator (N5181A, Agilent Technologies), an RF amplifier (1100/BBM2E4AJP, Empower RF Systems), a DC power supply (U8031A, Agilent Technologies), an antenna (ARC-PA0913B01, ARC Wireless) and a power meter (Thruline 43, Bird Electronic) (**Fig. 4a**). Connect the amplifier to the RF signal generator and an antenna by using coaxial cables (SMA-male to N-male precision cable with 160-series co-ax, Pasternack). The RF signal generator provides a 915-MHz RF signal with a power of −15 to −20 dBm. The signal is amplitude-modulated through the internal function of the generator to create a pulsed signal with a frequency of 10 Hz and

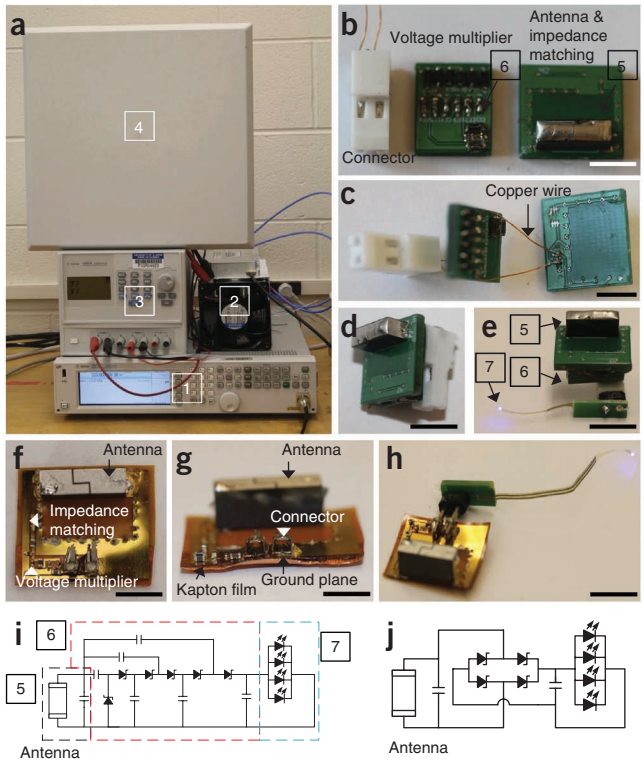

Figure 4 | Wireless operation and equipment. (**a**) An experimental setup for wireless power transmission. The setup contains an RF signal generator (1), an RF power amplifier (2), a DC power supply (3) and a panel antenna (4). Components for the wireless power harvester for μ-ILED powering with stacked PCB circuits (5: the circuit contains a ceramic antenna, and a capacitor is connected between the feed line of the antenna and the ground plane to match the impedance of the antenna with the next circuit. (**b**,**c**) A second circuit contains a voltage multiplier constructed with six pairs of capacitors and Schottky diodes in a cascaded connection(6)), before (**b**) and after (**c**) connecting with a copper wire. (**d**,**e**) A completed wireless power harvester alone (**d**) and with (**e**) connection to the μ-ILED device (7) for wireless operation. (**f**,**g**) Top (**f**) and side views (**g**) of a flexible wireless power harvester on Kapton film with similar components as the wireless harvester on PCB circuits. (**h**) A completed flexible wireless power harvester with connection to the μ-ILED device for wireless operation. Scale bars, 5 mm. (**i**) A schematic of the PCB-based power harvester. The numbered circuit components correspond to the same numbers shown in panels **b** and **e**. (**j**) A schematic of the wireless power harvester.

a carrier frequency of 915 MHz. Alternatively, an external TTL function generator can be connected to the RF signal generator to modulate the pulse width and frequency. This signal is directed to the RF amplifier, which amplifies the signal to 30–33 dBm.

22| Connect the power supply to the RF amplifier and the exhaust fan of the heat sink of the amplifier (see Equipment Setup) to provide DC voltages of 24 V and 10 V to the RF, respectively. Measure the intensity of the output power of the amplifier, and then connect the amplifier to the antenna to RF power to power the μ-LEDs at a 1~2 m distance (**Fig. 4e**,**h**).

Injection of the virus and μ-ILEDs into targeted brain structure ● TIMING 1 d, plus expression time

23| Mount the μ-ILED device in the custom-built cannula holder adaptor or another electrode or device holder (see Equipment Setup) (**Fig. 5a** and **Supplementary Fig. 1**). To do so, grasp the exposed region of the μ-needle (**Fig. 5b**).
▲ **CRITICAL STEP** The device must be placed along the midline of the adaptor to achieve proper spatial targeting.

24| Anesthetize the mouse in an isoflurane induction chamber using 4% (wt/vol) isoflurane and an oxygen flow rate of 1.5 liters per min.
! CAUTION Before beginning this protocol, all procedures should be approved by appropriate Animal Care and Use Committees, and they should conform to institutional and governmental regulations.
▲ **CRITICAL STEP** Ensure that the animal is sufficiently anesthetized before transferring it to the stereotaxic frame. The animal should have no response to a toe pinch, and its breathing rate should reduce to ~1 Hz.
? TROUBLESHOOTING

25| Transfer the animal to the stereotaxic frame, ensuring proper airflow of isoflurane to the nose cone (~2.5% (wt/vol) isoflurane; 1.5 liters per minute O_2).
▲ **CRITICAL STEP** Isoflurane levels should be monitored throughout the surgery to maintain sufficient anesthesia (no toe-pinch response) and breathing (1 Hz). For longer-duration surgery, isoflurane levels may be decreased to as low as 1% (wt/vol). Sterile, aseptic conditions should be used at all times to avoid infection.
▲ **CRITICAL STEP** Proper placement in the stereotaxic frame is required for accurate injection of virus and devices.
? TROUBLESHOOTING

26| Apply ophthalmic ointment to protect the animal's eyes during surgery.

27| Shave a 2 × 1 cm area on the animal's scalp (**Fig. 5c**).

28| Inject 0.1 ml of enrofloxacin into the hind limb (i.m.) and 0.3 ml of saline solution (0.9% (wt/vol), s.c.) to prevent infection and dehydration, respectively.

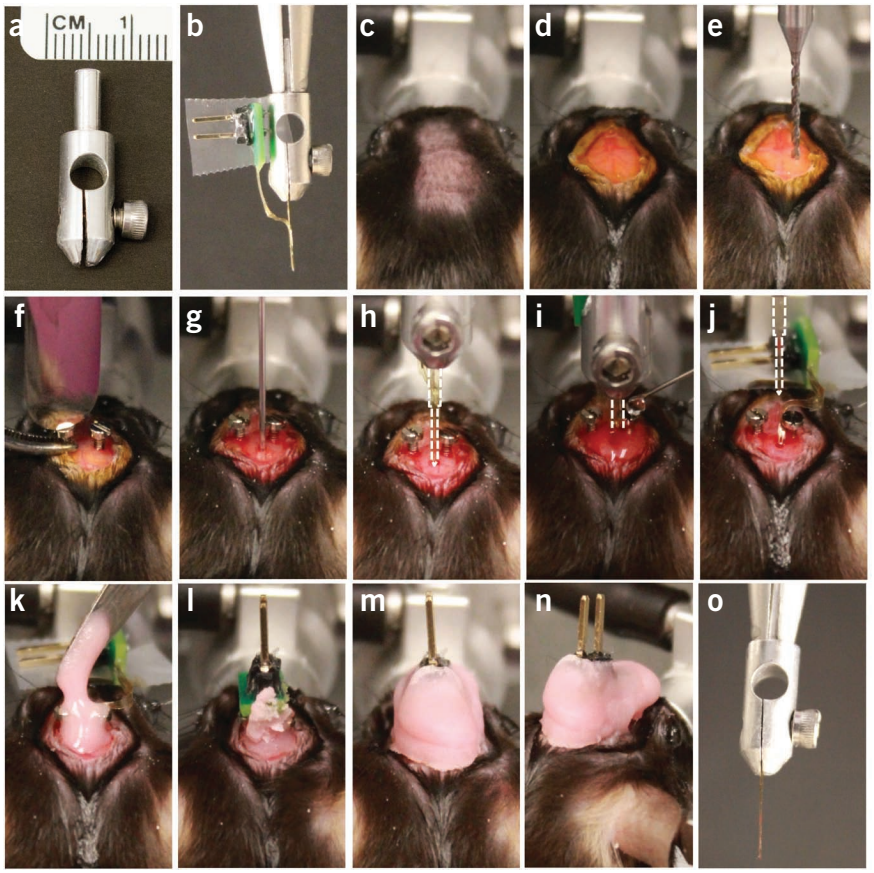

Figure 5 | Surgical procedure for injection of virus and μ-ILED devices into mouse brain. (**a**) Custom-built adapter for accurate stereotaxic placement of device (see Equipment Setup). (**b**) Mounted μ-ILED device, ready for injection into the animal. The exposed μ-needle is grasped with the adapter and a small piece of tape is used to secure the PCB during surgery. (**c**) A properly mounted mouse, with head shaved and eyes lubricated, is ready for surgery. (**d**) Betadine and ethanol are used to prevent infection and the scalp is open to expose the skull. (**e**) After leveling the skull, the drill is used to create pilot holes for the bone screws. (**f**) Forceps and a spatula or jewelry screwdriver are used to drive the screws into the skull. (**g**) The syringe needle is lowered to the desired coordinates to deliver the virus containing the optogenetic construct. (**h**) A μ-ILED device prepared to be driven into the brain using the same craniotomy as the viral injection. Dashed lines outline the shape of the device for clarity. (**i**) The μ-ILED device is lowered into the tissue and ACSF is applied to the skull surface to dissolve any external silk adhesive. (**j**) After a 15-min waiting period, the μ-needle is carefully retracted from the skull. (**k**) Dental cement is applied directly to the craniotomy site to secure the μ-ILED device in its targeted position. (**l**) The PCB connector is secured above the bone screws using a second layer of dental cement. (**m**,**n**) The PCB connector is completely encapsulated in dental cement, taking care to ensure that no bonds are made directly to the soft tissue. (**o**) The adapter is shown after surgery, containing only the μ-needle. All procedures were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

29| By using a cotton swab, apply 70% (vol/vol) ethanol and Betadine to the shaved area.

30| Carefully grasp the tissue with the forceps and use the scissors to make an incision the length of the shaved area of the animal's scalp (**Fig. 5d**).
▲ **CRITICAL STEP** Ensure that all surgical instruments are sterilized before each animal surgery.

31| After the skin separates, use the forceps and scissors to clean and remove any remaining periosteum on the skull surface.

32| Identify the skull sutures, lambda and bregma. Carefully level the skull according to the manufacturer's recommended steps for your stereotaxic frame. For the KOPF model 1900, use the stereotaxic alignment indicator. It is crucial that both lambda and bregma be level, and you must achieve a level plane laterally across the skull.
? TROUBLESHOOTING

33| Once the skull is level, move the drill to the coordinates you have selected for anchor screw placement. Carefully drill a hole with a diameter just wide enough for the screws to catch and not so deep as to penetrate the skull (**Fig. 5e**).
▲ **CRITICAL STEP** Proper anchoring is crucial to ensure that the headcap remains affixed to the skull for the duration of the behavioral experiments. Generally, anchor screws should be placed on either side of the midline within 2 mm of the site of implantation.
? TROUBLESHOOTING

34| Use the forceps and microspatula to properly anchor the screws into the skull (**Fig. 5f**).

35| Move the drill above the injection site. Drill a hole that penetrates the skull, but not the dura.
▲ **CRITICAL STEP** Drilling through the dura can cause widespread damage, bleeding and inflammation under the skull.

36| Align the infusion pump and lower the injection needle to the dorsal-ventral stereotaxic coordinates of the targeted structure of interest (**Fig. 5g**).
▲ **CRITICAL STEP** A beveled needle can penetrate the dura safely. If a blunt needle is used, the dura should be pierced using a sharp, sterile needle.
? TROUBLESHOOTING

37| By using the microcontroller, infuse the virus at a maximum rate of 100 nl min^{-1}. The volume of the virus will vary depending on the brain structure and serotype of virus used[1,2,4,44–47].
▲ **CRITICAL STEP** All viruses should be kept on ice before use, but special care should be taken with lentiviruses and herpes simplex viruses to prolonged exposure to temperatures above 4 °C.
? TROUBLESHOOTING

38| Once the infusion is complete, allow the injection needle to remain in place for 1 min for every 100 nl of virus infused. Slowly remove the needle from the injection site.

39| Position the cannula holder above the drill hole (**Fig. 5h**). Take care to orient the μ-ILED and other functionalities in the direction suitable for the experiment in order to properly illuminate the opsin-expressing brain region of interest.
▲ **CRITICAL STEP** For injection into rodents, rinse the device with ethanol to sterilize it before injection. For other mammals and primates, it might be necessary to use room-temperature ethylene oxide gas sterilization (http://www.anpro.com/sterilizers/anprolene/indexanprolene.html).
? TROUBLESHOOTING

40| Slowly lower the device into the brain to the desired dorsal-ventral coordinates.

41| By using a syringe needle, slowly apply ACSF to the skull to dissolve the silk-based adhesive. The brain tissue will dissolve the adhesive inside the skull. Wait for at least 10 min to allow for complete dissolution of the adhesive (**Fig. 5i**).
▲ **CRITICAL STEP** The adhesive must be completely dissolved before μ-needle removal. If not, the final placement of the flexible substrates will be affected.

42| Slowly remove the μ-needle. Monitor the position of the flexible substrates to ensure that zero movement occurs. If the adhesive is completely dissolved, the μ-needle will remove with ease and without movement (**Fig. 5j**).

43| Prepare the dental cement as described in Reagent Setup.

44| Use the microspatula to carefully apply a layer of dental cement directly to the point of injection to fully secure the placement of the device (**Fig. 5k**).
▲ **CRITICAL STEP** Allow this layer of cement to completely cure before building the structure of the remaining headcap.

45| Once the initial layer of cement is fully cured, position the PCB-based connector in the desired orientation. Apply a small amount of dental cement to secure it to the base layer (**Fig. 5l**).
▲ **CRITICAL STEP** The orientation of the connector will determine the orientation of the RF headstage antenna.

46| Finish the headstage by completely encapsulating the device-related hardware in dental cement. Be sure to leave the pins of the connector exposed to allow for connections to RF or wired functional generator (**Fig. 5m**,**n**).

47| Use a sterile spatula to detach any scalp skin from the cement. If the skin and the cement are connected, the headstage will be less stable over time.

48| Liberally apply (~1 g) the antibiotic ointment and lidocaine ointment to the entire incision area.

49| Remove the animal from the stereotaxic frame and place it in a clean home cage positioned on top of a heating pad for recovery. The animal should recover rapidly (<15 min), and it can be returned to its home cage once it displays normal,

Box 2 | Recycling the optoelectronic devices for reuse ● TIMING 2 d

1. After euthanizing the animal, use forceps to forcibly remove the headcap from the surface of the skull. Thoroughly remove any visible biological tissue or other build-up from the sides and bottom of the headcap. Take care not to damage the flexible aspect of the device. Once it has been removed and cleaned, the headcap is ready for dissolution (**Fig. 6a**).
2. Place the headcap into a glass beaker and add methyl methacrylate until the headcap is entirely submerged (**Fig. 6a**). Cover the beaker with tin foil and place in a properly ventilated fume hood overnight.
! CAUTION Methyl methacrylate monomer is a flammable liquid. It may also cause skin irritation; avoid contact with skin, eyes and clothing. Use it with adequate ventilation.
3. After overnight incubation, the dental cement should be completely dissolved. The device and bone screws should be clearly visible in the beaker (**Fig. 6c**). While you are wearing gloves and using forceps, remove these items from the beaker. The screws can be cleaned, sterilized and used for another surgery. The device will normally become inactive after dissolution of the headcap due to concurrent dissolution of the adhesive connecting the ACF cable to both the device and the PCB (**Fig. 6d**,**e**). Before proceeding to Step 4, use a multimeter to check that all μ-ILEDs still function properly (**Fig. 6f**). If any of the μ-ILEDs have electrical failure, discard the device and begin the fabrication process anew.
? TROUBLESHOOTING
4. Discard the original PCB and reassemble the device as described in Step 17 (**Fig. 2g**,**h**).
5. Apply PDMS to the entire device construction to provide passivation and waterproofing for operation *in vivo* as in Step 19 (**Fig. 2i**).
6. Attach the μ-needle using the silk adhesive and test the device to ensure proper electrical connection as done previously in Steps 18 and 20 of the PROCEDURE (**Figs. 2j–l** and **6g**,**h**). The device is now ready for injection into a fresh animal starting at Step 24 of the PROCEDURE.

awake locomotor behaviors. Because the only exposed portions of the devices are the metal connector pins, the animals can be group-housed for the duration of their experimental lifetime. However, if one chooses, the pins can be capped by using a dummy connector.

50| The μ-needle should be clear of any components of the device. Remove the adaptor from the cannula holder and sterilize it for reuse (**Fig. 5o**) as described in **Box 2** and **Figure 6**.

Preparation for behavioral testing ● TIMING ~5 d

51| House mice until the time at which proteins would be expected to be expressed. The choice of viral expression system will determine the expression time, and thus the wait time, before behavioral experimentation. This duration will vary from 1 to 6 weeks. For adeno-associated viral expression, typical wait times are 2–3 weeks for expression at cell bodies[1,2,9,11,44].

52| At least 5 d before experimentation, handle the mice to acclimate them to manipulation. In particular, connectors should be fitted to the headstage, and any areas of the body that will be injected should be gently touched in order to

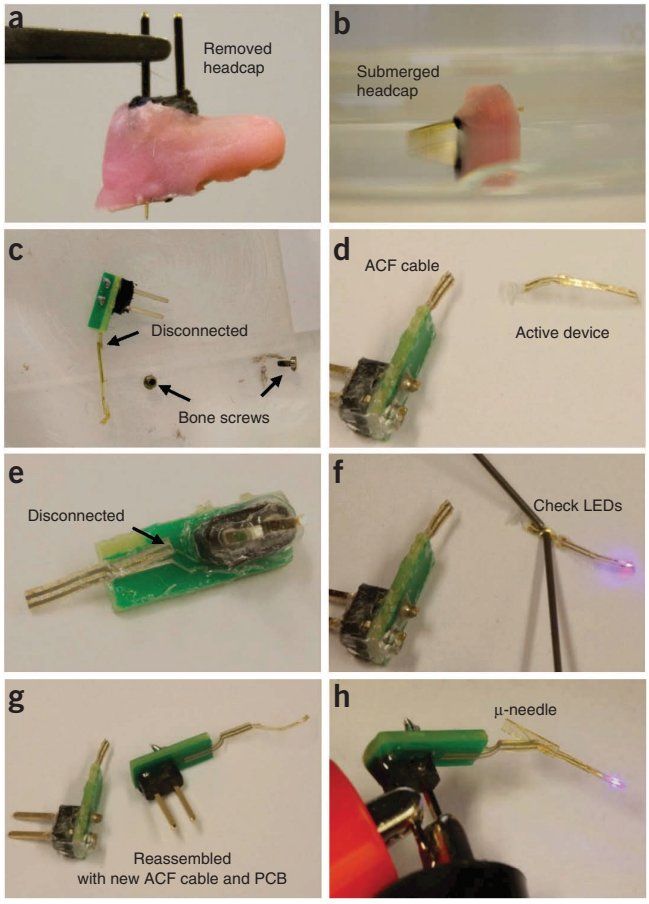

Figure 6 | μ-ILED device recycling and re-fabrication for subsequent use. (**a**) The same headcap from **Figure 5**, after being removed from the animal postmortem and cleaned of biological material. (**b**) The headcap should then be fully submerged in methyl methacrylate. (**c**) After overnight incubation in the stabilized methyl methacrylate monomer, the PCB, connector, μ-ILED device and bone screws will be freely available in the solution. (**d**,**e**) Both connections with the ACF cable will also dissolve, rendering the device inactive. (**f**) The device should be checked for reuse. If it is nonfunctional, the device should be discarded and a new device should be fabricated. (**g**) The working device is reassembled with new ACF cable and PCB. (**h**) The electrical connection through the new ACF cable should be checked after the device is attached to the injection μ-needle again.

habituate the animal to the manipulations that they will experience during behavioral testing (e.g., the nape of the neck for s.c., the abdomen for i.p., etc.).

53| Also habituate the animals to the method of powering the devices. If a wired approach is used, animals should be connected to the wires and allowed to explore a home-cage environment for at least 20 min once a day for 3 d. If a wireless approach is used, the RF headstage antenna should be connected in the home cage for the same duration.

▲ **CRITICAL STEP** To eliminate locomotor confounds, the animals must be habituated to carrying the added weight of the antennas.

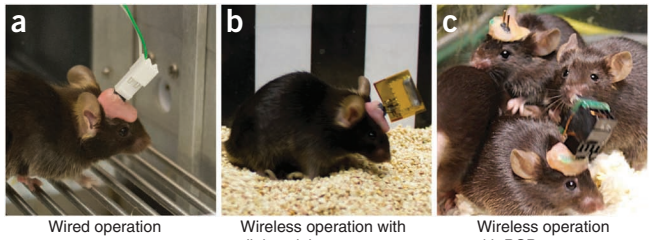

Figure 7 | Expected results after viral and device injection. Once a device is injected, the standard connection allows for temporary coupling multiple means of powering in a variety of behavioral assays. (**a**) A mouse connected for wired powering in a standard operant behavioral chamber. (**b**) The same mouse prepared for wireless powering with the lightweight, flexible power scavenger in a conditioned place preference environment. (**c**) Two mice with implanted devices among cage mates. The mouse in the foreground has a PCB-style RF scavenger for powering in a home-cage environment. All procedures were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

54| Perform wired (option A) or wireless (option B) behavioral tests.

▲ **CRITICAL STEP** The timing of the photostimulation will depend on the parameters and goals of the study, but the pulse-generation options are well suited for a wide variety of approaches. Any traditional function generator can either power the devices or provide TTL input into the RF signal generator to modulate the pulse width and frequency of the light pulses. If the photostimulation needs to be contingent on the animal's behavior, this can be achieved by using live video tracking connected to a TTL output (Noldus Ethovision 9.0 with trial and hardware control and I/O box or other) or triggering a TTL signal from IR beam breaks (Med Associates or other).

(A) Wired optogenetic behavioral control ● TIMING variable

(i) Once habituated to the connecting cables, power the devices by using a traditional function generator. The pulse width and frequency of photostimulation should be determined and based on physiologically relevant conditions[7,10,38–40]. The timing of the photostimulation will greatly depend on the parameters and goals of the study.

(ii) Connect the cable to the function generator by using the BNC-to–banana plug adaptor.

(iii) Route the cabling. For most wired behaviors, the best approach is to route the cabling through an electrical rotary joint as described previously[42].

(iv) Carefully scruff the mouse and connect the free end of the cable to the headstage of the animal. Place the animal in the behavioral apparatus (**Fig. 7a**) and perform the desired behavioral test.

(v) After the behavioral test, scruff the animal and remove the cable from the headstage.

(B) Wireless optogenetic behavioral control ● TIMING variable

(i) Connect the function generator to the RF signal generator, the RF signal generator to the RF power amplifier and the RF power amplifier to the panel antenna. Be sure that the power supply is connected to the heat sink (see Equipment Setup) to avoid damaging the amplifier. Connect the power supply to the RF amplifier. It is reasonable to assume that wireless operation of the devices would be compatible with any behavioral assay in which the behavioral apparatus itself does not interfere with the RF signal, but care should be taken to ensure proper powering of the devices in every behavioral apparatus used.

▲ **CRITICAL STEP** To avoid unnecessary powering of the devices, do not engage the power supply until the beginning of the behavioral session.

? TROUBLESHOOTING.

(ii) Carefully scruff the animal(s) and attach the RF scavenging antenna(s). Place the animal(s) in the behavioral apparatus and perform the behavioral test (**Fig. 7b**,**c**).

(iii) After the behavioral test, carefully scruff the animals and remove the scavenging antenna.

(iv) After the final behavioral session, if desired, kill the animals and use their tissue for any manner of post-mortem evaluation[11].

? TROUBLESHOOTING.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
14	Failure of the electrical device	Nonuniform coating of BCB passivation on μ-ILED	Spin-coat the BCB again and re-do the process
17, **Box 2** (step 3)	Failure of the ACF cable bonding	Insufficient temperature and/or pressure when bonding	Be certain to apply enough pressure (~1 or 2 MPa) and temperature (150 °C)
24	Device is damaged before or during mounting for surgery	The electrical components are grasped by the cannula holder adaptor	Only grasp the upper, exposed portion of the μ-needle. No portion of the electrical components should be grasped with the cannula holder adaptor. Be careful to apply appropriate pressure so as to avoid crushing the μ-needle
25, 32	Inaccurate tissue targeting	Improperly aligned skull	Follow the instructions provided for your laboratory's stereotaxic equipment for ear bar placement and skull leveling. Species-specific ear bars may be required. If the suture lines for identifying bregma/lambda on the skull are difficult to visualize, a surgical microscope and/or treating the skull with hydrogen peroxide can be a helpful addition. It may be necessary to wait longer after penetration before removal of the μ-needle
33, 36	Headcap falls off in the home cage or during behavior	Insufficient anchoring or cement application	Dental cement is ideal for reusablility, but other adhesives such as VLC one-step adhesive and C&B Metabond offer stronger bonds to the screws and skull surface. Although two screws are normally sufficient, larger PCB interfaces may require more anchor screws
37, 54B(iv)	Little or no expression of the viral construct	Improper handling and injection of the virus	Thaw virus as close in time to the surgery as possible. Keep the virus on ice until the time of injection. Depending on the promoter that drives expression, some systems will not provide sufficient opsin expression. This should be empirically determined before any attempted behavioral experiments
39	Dissolution of silk adhesive during sterilization	Too much exposure to ethanol	Most aqueous solutions will dissolve the silk adhesive. Do not soak the devices in ethanol, as this will promote dissolution of the adhesive. In our experience, rinsing the device with ethanol for ~15 s is sufficient to prevent widespread inflammation in mouse brain tissue[11]. Care should be taken to use appropriate sterilization techniques in higher mammals to avoid immune responses, such as ethylene oxide gas sterilization
54B(i)	Insufficient RF power is reaching the headstage scavenger	The behavioral apparatus is between the panel antenna and the scavenger (in an enclosure)	Connect a freestanding device to the scavenging headstage and place it in the behavioral enclosure. Using the gain on the RF signal generator adjust the RF power until sufficient power is available to power the μ-ILEDs to the desired light output. If problems powering the device persist, consider reducing the distance between the panel antenna and the scavenger
Box 2	Headcap does not fully dissolve	Insoluble dental cement was used, insufficient solvent was used, or there was limited duration of exposure to the solvent	Some dental cements will not dissolve in methyl methacrylate. Consider using the exact recommended cements where possible. If using Jet denture repair, add more solvent or wait longer for the cap to dissolve

● TIMING

Steps 1–22, preparation of μ-ILEDs, deterministic device assembly and fabrication of wireless power harvester: 9 d
Steps 23–50, viral and device injection: 1 d for procedure, 1–6 weeks for expression
Steps 51–54, behavioral experimentation: 5 d for preparation, variable depending on the experiment
Box 1, combining multifunctional sensors and optoelectronics: 1–4 d depending on modalities
Box 2, rescuing the optoelectronics for re-use: 2 d

ANTICIPATED RESULTS

After successful fabrication and implementation of μ-ILEDs, wireless manipulation of intact mammalian neural circuitry is possible. Although our group has demonstrated the feasibility of using these devices to investigate reward-related and anxiety-like behaviors[11], nearly any behavioral assay should be accessible with these devices. In particular, behaviors that require complete freedom of movement such as social interactions, wheel running and home-cage behaviors are now possible (**Fig. 7c**). Users can expect injected devices to function for many months, as we have tested devices up to 6 months after injection and observed that the devices retain their operational functionality[11]. Furthermore, we show here that these devices can be reused in other animals to avoid the lengthy process of remaking new devices for each experiment. Depending on the nature of the experiment, a single panel antenna can power numerous headstage devices. If the stimulation parameters are the same across animals, this approach can greatly increase the experimental throughput for a variety of behavioral assays. Although not presented in detail here, a reasonable extension of this protocol would be to incorporate other existing microscale sensors such as those for pH, blood oxygen, glucose levels or neurochemical detection onto μ-ILED devices[48,49]. In sum, the protocol described here should provide researchers with the ability to use optogenetics to wirelessly control and study intact mammalian neural circuitry in a minimally invasive manner.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Machining of the cannula holder adapter. This adapter is specifically designed for use with the KOPF Model 1966 Cannula Holder. The adapter is fashioned from aluminum with an 8 mm stalk (3 mm in diameter) that can be held by the Model 1966. The main body of the adapter is 14 mm in length with a 7 mm diameter. There are two orthogonal bore holes through the body. The first is a 5 mm hole from which the center slit is created through to the tip of the adapter. The second is a 2 mm screw-hole so that a screw can be tightened to reduce the size of the center slit to hold the μ-needle. It is important that the center point of the adapter be in-line with the center point of the cannula holder itself to ensure accurate device injection. Note that this adapter is merely a suggestion, but we acknowledge there can be many other solutions to the problem of accurate injection of the devices. Most stereotaxic instrument manufacturers offer custom-built holders and it is likely that many standard electrode holders can be modified to suit the needs of the individual laboratory (e.g. KOPF Model 1768).