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

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Summary

The rise of optogenetics provides unique opportunities to advance materials and biomedical engineering as well as fundamental understanding in neuroscience. This protocol describes the

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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 utilize flexible substrates to 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 approximately 3–8 weeks. Implementation of these devices allows for chronic (tested up to six months), wireless optogenetic manipulation of neural circuitry in animals experiencing behaviors such as social interaction, home cage, and other complex natural environments.

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 homecage 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, inorganic light-emitting diodes (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 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^{11–15}.

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^{12–14,16,17}. While other groups have successfully implemented wireless schemes and LEDs for optogenetics^{18–21}, 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 optics²², which offer 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. While adaptations to this ventral light delivery are possible^{10,23,24} (available commercially at 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), altering the number and arrangement of μ -ILEDs, and 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 employed for delivering multiple wavelengths of light into the same animal^{23–25}. 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 requires advanced experience with optics to maintain optimal conditions. This protocol may require access to external facilities for some labs, but the end result is a device that can be operated with basic laboratory equipment already likely to be present in most neuroscience laboratories. Furthermore, the rescue and recycling of these devices for re-use is relatively easy, meaning that these devices can be used for several rounds of behavioral experimentation with different sets of animals (See Box 2 for details). 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^{2,11,26}. A device utilizing these 450 nm-emitting μ -ILEDs will be 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^{11,27}. 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^{19,20}, but these approaches can restrict behaviors accessible to study because the animal is required to remain in a fixed environment. By utilizing 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 method

While 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 for the study of electrophysiological, temperature, and other properties of tissue (See Box 1 for details). 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^{28–33} these devices will likely have broader utility in other animal models. Specifically, larger organisms such as rats and non-human 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. Prior to 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 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^{2,11,22}, 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 region³⁴. Recordings performed with an external light stimuli can control for these effects. Additionally, properly counter-balanced within-subject experimental designs are possible with these devices by withholding power to the devices during the behavioral testing period.

Limitations—While 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*^{1,22}. 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 re-fabrication of the devices is straightforward and accessible to any laboratory. Once the protocol is followed there are few considerations when designing behavioral experiments. First, depending on the panel antenna employed, 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 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 context¹¹. In our experience, commonly used materials for behavioral apparatus (wood, polyvinyl chloride (PVC), Poly(methyl methacrylate) (PMMA; Plexiglass/Perspex) and the metal from cage lids) do not interfere with the RF signal, but can reduce the overall power if they are positioned between the panel antenna and the headstage scavenger (see ? TROUBLESHOOTING).

Second, the current protocol only provides wireless access to power the μ -ILEDs and not to receive information from the sensors. This backwards data stream restriction is largely one based on the weight that an animal can endure on the headstage. For a mouse,

electrophysiological, temperature, and photo sensors currently require a wired connection. For larger mammals, the devices can be integrated with existing telemetry setups, provided they do not interfere with the RF signal^{35,36} (commercially available at www.plexon.com). Finally, independent control of individual μ -ILEDs is also possible with these devices, but the described 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 are employed over the pin connectors that we present here (many options commercially available at www.omnetics.com).

Upon completion of the protocol presented here, users can expect to have devices with 4 μ -ILEDs that can be wirelessly powered with suitable light output from two meters 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 radiofrequency power scavenging approach is appropriately suited for powering multiple μ -ILED devices (presumably in multiple animals) 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 adapter for standard cannula holders. The basic principle of this adapter is that the μ -needle can be mounted in-line with the existing stereotaxic system. The dimensions of such an adapter will vary by stereotaxic alignment system make and model, but most university machine shops should be able to fashion such an adapter. The adapter 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 adapter for the KOPF Model 1966 can be found in Supplementary Fig. 2.

Post-mortem 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 re-usability might be compromised.

MATERIALS

REAGENTS

Preparation of releasable polymer template—

- UV curable epoxy (SU-8 2 & 100 photoresist, Microchem) ! CAUTION Partially cured or uncured epoxy needle can induce excess chemical contamination in the brain.
- SU-8 developer (Microchem)

- Isopropyl alcohol (IPA) !CAUTION Isopropyl alcohol is flammable.
- Acetone !CAUTION Acetone is flammable.
- Silk adhesive (provided by Tufts university, Details are shown in other papers^{13,16,17}).
- BCB (Benzocyclobutene, DS-4022 35, Dow corning)
- BCB developer (Advanced Developer, DS2100, Dow corning)
- Water soluble tape (3M)
- Photoresists (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 Inc.)
- Polydimethylsiloxane (PDMS, Sylgard184, Dow corning)
- Polyethylene terephthalate (PET, polyester film, 2.5 and 6 µm thick, Mylar® film, Chemplex® Inc.)

Preparation of microscale LED—

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

Injection of virus and µ-ILEDs into targeted brain structure—

- Isoflurane, USP (Isothesia, Butler Schein, cat. no. 029405)! CAUTION Prior to 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 regarding animal research. ! CAUTION Ensure proper ventilation and gas scavenging methods are in place to prevent potential inhalation of excess isoflurane.
- Betadine solution (Purdue Products, cat. no. 67618015017)
- Ethanol (Sigma-Aldrich, cat. no. 362808) ! CAUTION Ethanol is flammable.
- Hydrogen peroxide, 3% USP (Select Medical Products, cat. no. 117)
- Lidocaine ointment USP, 5% (Fougera)
- Ophthalmic ointment (Altalube Ophthalmic Product)
- Adhesive Luting Cement (C&B-Metabond, Parkell Inc., cat. no. S380) ! CAUTION
- Light cured bonding adhesive (VLC One Step Prime & Bond Adhesive, cat. no. 305-006-030) ! CAUTION

- 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 with adequate ventilation.
- 0.9% Sodium Chloride Inj., USP ((9 mg/ml 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 WUSTL 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 MIT Viral Core, <http://mcgovern.mit.edu/technology/viral-vector>) ! CAUTION Follow the appropriate safety precautions pertaining to the particular virus in use. It may be necessary to obtain a higher Biosafety Level certification prior to use.
- Artificial cerebral spinal fluid (ACSF; Tocris, cat. no. 3525 or custom, see REAGENT SETUP^{1,37})

Behavioral procedures using RF power scavenging—

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

EQUIPMENT

Preparation for μ -LEDs 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 500°C annealing.
- Mask aligner (Karl Suss, MJB)
- Inductively coupled plasma (ICP) etcher (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/cm², 248 nm wavelength, IPG Photonics (<http://www.ipgphotonics.com/microprocessing.htm>)) or Yttrium aluminum garnet (YAG):Nd laser (0.3 J/cm², 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 Co.)
- Male pins for headstage PCB (2.54 mm spacing male pins (Sunlight Inc, 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 Model1900) ! CAUTION Prior to 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 regarding animal research.
- Stereotaxic alignment indicator (KOPF Model 1905)
- 40X Centering Microscope (KOPF 1915)
- Stereotaxic drill (KOPF Model 1911) and #66 drill bit (KOPF cat. no. 8669)
- Stereotaxic single cannula holder (KOPF Model 1966)
- Stereotaxic adapter for cannula holder (See Supplementary Fig. 2, WUSTL Machine Shop or other)
- Anchoring screws (CMA Microdialysis, cat. no. 7431021)
- Needles (Becton-Dickinson, cat. no. 305111)
- Micro-injection syringes (Hamilton, cat. no. 88011)
- Infusion pump and controller (UltraMicroPump III, World Precision Instruments, 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—

- Animal(s) with injected μ -ILED device ! CAUTION Prior to 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 regarding animal research.
- RF generator (Agilent, N5181 MXF)
- Function generator with standard transistor-transistor logic (TTL) (AMPI Master-9 or other)
- RF amplifier (Empower RF systems Inc. 1100/BBM2E4AJP)
- RF antenna (ARC wireless solutions, ARC-PA0913B01, ARC 902-928 MHz 12.5dBi, Flat Panel)
- DC power supply (Mastech, cat. no. HY5005E-2)
- Relevant behavioral assay apparatus (university machine shops, MED Associates, Harvard Apparatus or other)
- Wireless headstage antenna (see PROCEDURE)
- Printed circuit board (PCB) for wireless headstage antenna (General Circuits Co.)
- Ceramic antenna (W3012, Pulse Electronics)
- Schottky diode (Digi-Key, cat. no. MMDL301T1G)
- Power meter (Bird Electronic Corp., 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 Prior to 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 regarding animal research.
- Relevant behavioral assay apparatus (university machine shops, MED Associates, Harvard Apparatus or other)
- Function generator for standard transistor-transistor logic (TTL) (AMPI Master-9 or other)
- BNC cables (Cables to Go, various lengths and catalog numbers)
- Electrical rotary joint (Moog, cat. no. SRA-73683³⁸)
- BNC-to-banana plug adapter (Fluke BP881 or other)

- Wire (30 gauge wire, 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

Dental cement—The dental cement can be prepared as described elsewhere²². 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. A starting point is 350 μ l of methyl methacrylate monomer added to 225 mg of powder. **▲CRITICAL STEP** 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 prior to the injection. In some cases, dilution of stock titers requires obtaining more of the reagent in which the viral prep was initially concentrated.

ACSF—If not purchased, the solution can be prepared following the recipes described elsewhere^{1,37}.

EQUIPMENT SETUP

Preparation of amplifier with proper heat sink—The RF amplifier (1100/BBM2E4AJP, Empower RF Systems Inc.) requires an additional cooling system during operation. Thermal grease applied on top of the heat sink (LA 17/200 24V, Fischer Elektronik Inc.) 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 Additional information on production of μ -ILEDs appears in Supplementary Fig.1 and a previous manuscript¹².

1 | Purchase GaN LED stacks (450 nm emission wavelength; p-type GaN/multi-quantum well (MQW)/spacer/n-type GaN/undoped GaN) grown on sapphire substrate and clean the wafer surface with 5 wt % diluted HCl for 5 min. **▲CRITICAL STEP** Proper cleaning enables reduced turn-on voltages and improved efficiencies, both of which minimize 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 degree vacuum levels of $\sim 10^{-6}$ 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 followed by removal of the photoresist (PR) with acetone (Supplementary 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 semi-transparent after the procedure 4.
- 5 |** Generate 40 × 40 μm² 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 (Supplementary Fig. 1b).
- 6 |** Generate 25 × 25 μm² square hole patterns in PR for n- and p-type metal contact pads and deposit Cr/Au (10/300 nm) using an electron beam evaporator. Then, remove PR (Supplementary Fig. 1c).
- 7 |** Deposit a low stress layer (200 nm) of SiN_x on the substrate, using plasma enhanced-chemical vapor deposition (PE-CVD). Next, pattern a negative tone PR (AZ 2070) to serve as a mask for etching the SiN_x and the GaN to define the lateral dimensions of array of μ-ILEDs. Devices with dimensions of 100 × 100 μm² or less are formed with 20 μm spacings (Supplementary Fig. 1d).
- 8 |** Deposit a layer of SiN_x onto the μ-ILEDs to protect the devices and metal pads from further processes. Bond the LED wafer to a silicon substrate using 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 temperature of 220 °C for 2 hr (Supplementary Fig. 1e).
- 9 |** Use laser illumination through the sapphire to release the μ-ILEDs (Supplementary Fig. 1f). Details of this laser lift-off (LLO) technique appear elsewhere^{12,39}.
- 10 |** Etch unalloyed metal by immersion in 5 wt% HCl for 30 min (Supplementary Fig. 1g).
- 11 |** Retrieve all μ-ILEDs onto 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. Residual metals, including In-Pd alloy and Cr are then removed by Pd and Cr etchants (Supplementary Fig. 1h).
- 12 |** Prepare a PDMS stamp with a single relief feature consisting of a post with 100 × 100 μm² lateral dimensions and 100 μm height. 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. 1a and 1b).
- 13 |** Remove passivation layer, SiN_x (deposited in procedure 8) with RIE and spin coat a photosensitive benzocyclobutene (6 μm thick, BCB) onto the μ-ILED.
- 14 |** Pass ultraviolet (UV) light through the backside of the substrate and develop the BCB with BCB developer (Fig. 1c) to obtain holes for n- and p-side contacts (Fig. 1c, lower inset) !CAUTION Make sure that BCB covers the μ-ILED surface. Non-uniform coatings of BCB generate interference fringes that are readily visible. (Fig. 1c, upper inset) After further curing BCB, anneal the samples at 250 °C on a hot plate for 3 hr. **▲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. 1d and Supplementary Fig. 1i).

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 Fig 1a with water-soluble tape (Fig. 1e). Remove water-soluble tapes in DI water (Fig. 1f) and place the devices on slabs of PDMS (Fig. 1g). ▲ CRITICAL STEP Steps 16-20 describe device assembly for μ -ILEDs alone. Optional assembly of multifunctional devices can 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. 1h). ▲ CRITICAL STEP Apply pressure and high temperature ($\sim 150^\circ\text{C}$) during ACF cable bonding. ?TROUBLESHOOTING

18 | Assemble all devices onto injection μ -needle formed in SU-8 epoxy The entire region of injection device should be electrical insulated for *in vivo* use. Cover the flexible device with SU-8 polymer and the other parts with PDMS for waterproofing (Fig. 1i). 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 details at <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 hrs, the entire area except pin adapter is electrically passivated by PDMS.

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

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

20 | The wireless power harvester is constructed from two stacked PCB circuits (Fig. 3b; General Circuits Co., Ltd.). Each PCB includes top and bottom layers that are mostly covered by solid copper ground planes. Electrical components are mounted on the PCB boards through soldering. The first PCB circuit (labeled as 5 in Fig. 3b) contains a ceramic antenna (W3012, Pulse Electronics) operated at 915 MHz with a clearance space of 10.8×8.25 mm to the surrounding ground plane. A capacitor (8.2 pF in capacitance) is connected 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. 3b) contains a voltage multiplier circuit constructed with 6 pairs of capacitors (47 nF in capacitance) and Schottky diodes (MMDL301T1G) in cascaded connection¹¹. A connector that is used for the μ -LEDs is mounted to the secondary PCB circuit. The PCB circuits and the connector are joined with flexible copper wires (Fig. 3c), and bonded together with resin

epoxy (Fig. 3d). Fig. 3i shows a schematic illustration of the power harvester. The circuit parts, which are labeled with number 5 to 7, corresponding to the same number shown in Fig. 3b and 3e.

21 |The flexible PCB circuit is fabricated on a Kapton film with thickness of 75 μm (Fig. 3f). The film is coated with a layer of copper with 6 μm thickness by electron beam evaporation. The copper is then patterned to form pads for electrical components and interconnection. A polyimide film with 1 μm thickness is spin cast and patterned to expose the electrical contact pads, while passivating the interconnection (Fig. 3f). A ground plane made of copper tape (Fig. 3g) is attached on the bottom of the Kapton film and connected with copper pattern on the top of the Kapton film through channels filled with solder. A schematic illustration of the flexible PCB circuit appears in Fig. 3j

22 |The experimental setup for wireless power transmission consists of an RF signal generator (N5181A, Agilent Technologies, Inc.), an RF amplifier (1100/BBM2E4AJP, Empower RF Systems Inc.), a DC power supply (U8031A, Agilent Technologies, Inc.), an antenna (ARCPA0913B01, ARC Wireless), and a power meter (ThruLine 43, bird electronic corp.) (Fig. 3a). 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 frequency of 10 Hz, and 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 to 33 dBm. The power supply provides DC voltages of 24 V and 10 V to the RF amplifier and an exhaust fan of a heat sink of the amplifier, respectively. The amplifier is connected to an RF signal generator and an antenna using coaxial cables (SMA Male to N Male Precision Cable Using 160 Series Coax, Pasternack Inc.). The output power of the amplifier is first input into the power meter to measure the intensity. The power meter is then switched to the antenna for transmitting RF power to power the μ -LEDs at a 1~2 meter distance (Fig. 3e and 3h).

Injection of virus and μ -ILEDs into targeted brain structure ● TIMING 1 d

23 |Mount the μ -ILED device in the custom-built cannula holder adapter or other electrol device holder (see EQUIPMENT SETUP) (Fig. 4a and Supplementary Fig. 2). To do so, grasp the exposed region of the μ -needle (Fig. 4b). ▲ CRITICAL STEP The device must be placed along the midline of the adapter to achieve proper spatial targeting. ?

TROUBLESHOOTING

24 |Anesthetize the mouse in an isoflurane induction chamber using 4% isoflurane and a flow rate of 1.5 L/min O_2 . ▲ CRITICAL STEP Prior to beginning this protocol, all procedures should be approved by the Animal Care and Use Committee of the investigating institution and conform to US National Institutes of Health guidelines regarding animal research. ▲ CRITICAL STEP Ensure that the animal is sufficiently anesthetized prior to transferring to the stereotaxic frame. The animal should have no response to a toe pinch and its breathing rate should reduce to ~ 1 Hz.

- 25** | Transfer the animal to the stereotaxic frame, ensuring proper airflow of isoflurane to the nosecone (~2.5% isoflurane; 1.5 L/min O₂). ▲ **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%. 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 the ophthalmic ointment to protect the eyes during surgery.
- 27** | Shave a 2 × 1 cm area on the animal's scalp (Fig. 4c).
- 28** | Inject 0.1 mL of Enrofloxacin into the hindlimb (i.m.) and 0.3 mL saline solution (0.9% (w/v), s.c.) to prevent infection and dehydration, respectively.
- 29** | Using a cotton swab, apply 70% ethanol (vol/vol) and betadine to the shaved area.
- 30** | Carefully grasp the tissue with the forceps and, using the scissors, make an incision the length of the treated area (Fig. 4d). ▲ **CRITICAL STEP** Ensure that all surgical instruments are sterilized prior to 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 recommend steps for your stereotaxic frame. For the KOPF Model 1900, use the stereotaxic alignment indicator. It is critical that both lambda and bregma be level, as well as achieving 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. 4e). ▲ **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. 4f)
- 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. 4g). ▲ **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.

37 I Using the microcontroller, infuse the virus at a maximum rate of 100 nl/minute. Volume of virus will vary depending on the brain structure and serotype of virus used^{1,2,4,40–43}. ▲ **CRITICAL STEP** All viruses should be kept on ice prior, but special care should be taken with lentiviruses and herpes simplex viruses to prolonged exposure to temperatures above 4°C. ? TROUBLESHOOTING

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

39 I Position the cannula holder above the drill hole (Fig. 4h). Take care to orient the μ -ILED/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 with ethanol to sterilize the device prior to injection. For other mammals and primates it might be necessary to use room temperature Ethylene Oxide (EtO) gas sterilization (<http://www.anpro.com/sterilizers/anprolene/indexanprolene.html>) ? TROUBLESHOOTING

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

41 I 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 at least ten minutes to allow for complete dissolution of the adhesive (Fig. 4i). ▲ **CRITICAL STEP** The adhesive must be completely dissolved prior to μ -needle removal. If not, the final placement of the flexible substrates will be affected.

42 I 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. 4j).

43 I Prepare the dental cement according the REAGENT SETUP.

44 I Using the microspatula, carefully apply a layer of dental cement directly to point of injection to fully secure the placement of the device (Fig. 4k). ▲ **CRITICAL STEP** Allow this layer of cement to completely cure prior to building the structure of remaining headcap.

45 I 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. 4l). ▲ **CRITICAL STEP** The orientation of the connector will determine the orientation of the RF headstage antenna.

46 I 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. 4m and 4n).? TROUBLESHOOTING

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

48 | Liberaally apply (~1g) 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 homecage position on top of a heating pad for recovery. The animal should recover rapidly (<15 minutes) and can be returned to its homecage once it displays normal, awake locomotor behaviors. Because the only exposed portion 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 using a dummy connector.

50 | The μ -needle should be clear of any components of the device and can be removed from the cannula holder adapter and sterilized for re-use (Fig. 4o) as described in Box 2.

Preparation for behavioral testing ● TIMING ~5 days

51 | The choice of viral expression system will determine expression time, and hence wait time prior to behavioral experimentation. This duration will vary from one to six weeks. For adeno-associated viral expression typical wait times are 2-3 weeks for expression at cell bodies^{1,2,9,11,40}.

52 | At least five days before experimentation, experimenters should handle animals to acclimate the animals 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 habituate the animal to the manipulations they will experience during behavioral testing (e.g. the nape of the neck for subcutaneous, the abdomen for intraperitoneal, etc.).

53 | Animals should also be habituated 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 homecage environment for at least 20 minutes once a day for three days. If a wireless approach is used, the RF headstage antenna should be connected in the homecage for the same duration. ▲ CRITICAL STEP To eliminate locomotor confounds, the animals must be habituated to carrying the added weight of the antennas.

Wired optogenetic behavioral control ● TIMING variable

54 | Once habituated to the connecting cables, the devices can be powered using a traditional function generator. The pulse width and frequency of photostimulation should be determined and based on physiologically relevant conditions^{7,10,44-46}. The timing of the photostimulation will greatly depend on the parameters and goals of the study.

55 | Connect the cable to the function generator using the BNC-to-banana plug adapter.

56 | For most wired behaviors, the best approach is to route the cabling through an electrical rotary joint as described previously³⁸.

57 | Carefully scruff the animal and connect the free end of the cable to the headstage of the animal. Place the animal in the behavioral apparatus (Fig. 5a).

58 | Following the behavioral test, scruff the animal, and remove the cable from the headstage.

Wireless optogenetic behavioral control ● TIMING variable

59 | 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 homecage behaviors¹¹. 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^{44–46}, but care should be taken to ensure proper powering of the devices in every behavioral apparatus used ?

TROUBLESHOOTING. Once habituated to the RF scavenging headstage antennas, 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 to 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 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 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, while 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^{7,10,44–46}. ▲ **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 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 infrared beam breaks (Med Associates or other).

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

61 | Carefully scruff the animal(s) and attach the RF scavenging antenna(s). Place the animal(s) in the behavioral apparatus (Fig. 5b and 5c).

62 | Following the behavioral test, carefully scruff the animal and remove the scavenging antenna.

63 | After the final behavioral session, animals can be sacrificed and tissue can be used for any manner of post-mortem evaluation¹¹. ? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Steps 1–22, Preparation of μ -ILEDs, deterministic device assembly, and fabrication of wireless harvester: 9 d

Steps 22–50, Viral and device injection: 1 d for procedure, 1–6 weeks for expression

Steps 51–63, Behavioral experimentation: 5 d for preparation, variable by experiment

ANTICIPATED RESULTS

Following successful fabrication and implementation of μ -ILEDs, wireless manipulation of intact mammalian neural circuitry is possible. While our group has demonstrated the feasibility of using these devices to investigate reward-related and anxiety-like behaviors¹¹, 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 homecage behaviors are now possible (Fig. 5c). Users can expect injected devices to maintain function for many months, as we have tested devices up to six months following injection and observe that the devices retain their operational functionality¹¹. Furthermore, we show here that these devices can be reused in subsequent animal groups can be tested with the same device 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. Though not presented in detail here, a reasonable extension of this protocol would be to incorporate other existing microscale sensors such as pH, blood oxygen, glucose levels, or neurochemical detection onto μ -ILED devices^{48,49}. In sum, the protocol described here should provide researchers the ability to use optogenetics to wirelessly control and study intact mammalian neural circuitry in a minimally invasive manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1**Combining multifunctional sensors and optoelectronics**

One hallmark of these devices is their ability to both deliver light into the brain and record information from the brain. Depending on the experiment, users may wish to outfit the devices with a variety of multiple and optional modalities. The following steps provide information on adding functional layers to the devices. If these optional modalities are desired, Box 1 should be completed prior to Step 16 of the main PROCEDURE.

Fabrication of temperature sensors (4th layer shown in Fig. 2)

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

Temperature sensor calibration ● TIMING 1 hr

- 3** | Dip the temperature sensor into deionized (DI) water and measure reference resistance using digital multimeter. With precisely controlled temperature of the DI water, measure the changes in resistance. Since the resistance change (ΔR) of the Pt resistor depends linearly on the temperature change (ΔT), the formula for estimated temperature is $\Delta T \sim k \cdot \Delta R$ where k is constant.

Fabrication of μ -IPDs (2nd layer shown in Fig. 2), Additional details of this method are shown in a previous publication⁴⁷.

- 4** | Deposit SiO₂ by PECVD on a silicon-on-insulator (SOI) wafer, with a top silicon layer thickness of approximately 1 μ m.
- 5** | Generate PR patterns to pattern the SiO₂ using hydrofluoric acid (HF).
- 6** | Remove PR and clean the wafer surface by RCA cleaning which involves a first step (called SC (standard cleaning)-1) carried out with 1:1:5 solution of ammonium hydroxide (NH₄OH):Hydrogen peroxide(H₂O₂):DI water at 80 °C for 15 min. The second step involves removal of the SiO₂ layer using 1:50 solution of HF:DI water. The final step (SC-2) is performed with 1:1:6 solution of hydrochloric acid (HCl):H₂O₂:DI water at 80 °C for 15 min (RCA Cleaning: http://inside.mines.edu/fs_home/cwolden/chen435/clean.htm).
- 7** | Carry out solid state doping of the silicon to form a p-type region.
- 8** | Repeat above Steps 4 to 6 from for n-type doping.
- 9** | Generate PR hole patterns on the entire wafer surface and etch the silicon layer, to define the lateral dimensions of the photodetectors.
- 10** | Etch the buried oxide (BOx) layer using HF

11 | Retrieve a ~1 μm thick thin Si membrane photodiode using a PDMS slab and release it onto the needle-shaped PET substrate (described in PROCEDURE, Step 12)

12 | Form metal (Cr/Au, 15/300 nm) lines for interconnect.

Fabrication of μ -electrode for electrophysiological sensor (1st layer shown in Fig. 2)

13 | Generate PR (AZ 2030) lift off patterns

14 | Deposit Pt (30 nm) a needle-shaped piece of PET (described in PROCEDURE Step 12) by sputtering. Generate a pattern of SU-8 2 (2 μm thick) with $20 \times 20 \mu\text{m}^2$ square opening.

▲ **CRITICAL STEP** Additional oxygen descum to remove residual PR layer is recommended. The impedance of the Pt μ -electrode should be ~1.0 M Ω at 1 kHz.

Box 2**Recycling the optoelectronic devices for re-use**

- 1 |** After sacrificing 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 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. 6b). 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 with adequate ventilation.
- 3 |** Following overnight incubation, the dental cement should be completely dissolved. The device and bone screws should be clearly visible in the beaker (Fig. 6c). Wearing gloves and using forceps, remove these items from the beaker. The screws can be cleaned, sterilized, and used for another surgery. ? TROUBLESHOOTING 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 and 6e). Prior to 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.
- 4 |** Discard the original PCB, and reassemble the device as described in Step 17 (Figs. 1g and 1h).
- 5 |** Apply PDMS to the entire device construction to provide passivation and waterproofing for operation *in vivo* as in Step 19 (Fig. 1i).
- 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. 1j-l, 6g, and 6h). The device is now ready for injection into a fresh animal starting at Step 24 of the PROCEDURE.

Figure 1.

Procedure for fabrication of injectable, multifunctional electronics. (a) Thin (~ 2.5 μ m thick) needle-shaped polyethylene terephthalate (PET) is attached on temporary Polydimethylsiloxane (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 benzocyclobutene (BCB) polymer. The backside of BCB coated substrate is exposed to ultraviolet light. The wave pattern in the upper inset shows ununiformed coating of BCB. The lower inset shows successful uniformed 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 freestanding thin, flexible, needle-shaped PET.

(g) The device is electrically connected to the ACF cable. The PDMS slabs on top and bottom are compressed using high temperature ($\sim 150^\circ\text{C}$) 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 is coated with PDMS for waterproofing. (j) Blue (450 nm) μ -ILEDs are powered. The μ -ILEDs and electrical connection should be checked prior to injection (k) The device is assembled with injection μ -needle using biodegradable silk adhesive. (l) Image of a completed device ready for injection into brain tissue.

Figure 2.

Multifunctional sensors and optoelectronics. (a) Representative scheme for multifunctional, injectable electronics formed on injectable needle. The devices include electrophysiological sensor (μ -electrode; 1st layer), silicon photodiode (μ -IPD; 2nd layer), four microscale inorganic light-emitting diodes (μ -ILEDs; 3rd layer), and temperature sensor (μ -temp. sensor; 4th layer) based on platinum resistor are formed on 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 applies to both panels and = 200 μ m.

Figure 3.

Wireless operation and equipment. (a) An experimental setup for wireless power transmission. The setup contains a RF signal generator (1), a 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: 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. 6: a second circuit contains a voltage multiplier constructed with 6 pairs of capacitors and Schottky diodes in a cascaded connection). before (b) and after (c) connecting with copper wire. A completed wireless power harvester alone (d) and with (e) connection to μ -ILED device (7) for wireless operation. 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 μ -ILED device for wireless operation. All scale bars are 5 mm. (i) A schematic of the PCB-based power harvester. The numbered circuit components correspond to the same number shown in Fig. 3b and 3e. (j) A schematic of the wireless power harvester.

Figure 4.

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 is 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 is 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 minute 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 following surgery, containing only the μ -needle.

Figure 5.

Expected results following 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 using the lightweight, flexible power scavenger in a conditioned place preference environment, (c) Two mice with implanted devices amongst cage mates. The mouse in the foreground has a PCB-style RF scavenger for powering in a homecage environment.

Figure 6.

μ -ILED device recycling and refabrication for subsequent use. (a) The same headcap from Fig. 4, removed from the animal post-mortem and cleaned of biological material. (b) The headcap should then be fully submerged in methyl methacrylate. (c) Following 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 non-functional. (f) The device should be checked for reusing. If non-functional, 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 with injection μ -needle again.

Table 1**7TROUBLESHOOTING**

Step	Problem	Possible reason	Possible solution(s)
14	Electrical device failure	Un-uniform coating of BCB passivation on μ -ILED	Spincoat BCB again and redo the process.
17, Box 2	ACF cable bonding failure	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 prior to or during mounting for surgery	The electrical components are grasped by the cannula holder adapter	Only grasp the upper, exposed portion of the μ -needle. No portion of the electrical components should be grasped with the cannula holder adapter. 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 prior to removal of the μ -needle.
33, 36	Headcap falls off in homecage/during behavior	Insufficient anchoring or cement application	Dental cement is ideal for reusability, but other adhesives such as VLC One Step Adhesive and C&B Metabond offer stronger bonds to the screws and skull surface. While two screws are normally sufficient, larger the PCB interfaces may require more anchor screws.
37, 63	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. Maintain 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 prior to any attempted behavioral experiments.
40	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 seconds is sufficient to prevent widespread inflammation in mouse brain tissue ¹¹ . Care should be taken to use appropriate sterilization techniques in higher mammals

Step	Problem	Possible reason	Possible solution(s)
			to avoid immune responses, such as EtO gas sterilization.
60	Insufficient RF power is reaching the headstage scavenger	The behavioral apparatus is between the panel antenna and 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, not enough solvent, or extend duration of exposure to 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 a longer durations for cap to dissolve.