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Customizable, wireless and implantable neural probe design and fabrication via 3D printing

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Abstract

This Protocol Extension describes the low-cost production of rapidly customizable optical neural probes for *in vivo* optogenetics. We detail the use of a 3D printer to fabricate minimally invasive microscale inorganic LED-based neural probes which can control neural circuit activity in freely behaving animals, thus extending the scope of two previously published protocols describing the fabrication and implementation of optoelectronic devices for studying intact neural systems. The 3D-printing fabrication process does not require extensive training and eliminates the need for expensive materials, specialized cleanroom facilities, and time-consuming microfabrication

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Author contributions

K.E.P., J.L., J.R.K., J.-W.J. and J.G.M. conceived the project and designed the detailed experimental protocol. K.E.P., J.L., J.R.K., C.K., C.Y.K., R.Q. and K.-I.J. performed the experiments. K.E.P., J.L., J.R.K., J.-W.J. and J.G.M. wrote the paper. J.-W.J. and J.G.M. acquired funding and supervised the project. K.E.P., J.L. and J.R.K. contributed equally to this work. J.-W.J. and J.G.M. are co-senior authors.

Code availability

The code and STL files used in this study are provided in Supplementary Data 1–3, and 7. Gerber and CAD files for the PCBs are provided in Supplementary Data 4. Code for the BLE SoC and smartphone app are provided in Supplementary Data 5 and 6 and are also available at https://github.com/juulee2011/3DPOPs_control_app_Android.git.

Competing interests

Dr. Jae-Woong Jeong, and Dr. Jordan G. McCall share two US Patents (US 10,617,300 B2 and 11,160,489 B2) for injectable and implantable cellular-scale electronic devices, but do not earn income related to these patents.

techniques typical of conventional manufacturing processes. As a result, the design of the probes can be quickly optimized, based on experimental need, reducing the cost and turnaround for customization. For example, 3D-printed probes can be customized to target multiple brain regions or scaled up for use in large animal models. This protocol comprises three procedures: 1) probe fabrication, 2) wireless module preparation, and 3) implantation for *in vivo* assays. For experienced researchers, neural probe and wireless module fabrication requires approximately two days, while implantation should take 30–60 minutes per animal. Time required for behavioural assays will vary depending on the experimental design and should include at least 5 days of animal handling prior to the implantation of the probe, to familiarize each animal to their handler, thus reducing handling stress which may influence the result of the behavioural assays. The implementation of customized probes improves the flexibility in optogenetic experimental design and increases access to wireless probes for *in vivo* optogenetic research.

Keywords

3D printing; wireless; neural probes; optogenetics; neuroscience

INTRODUCTION

The development of optogenetic approaches to study neural circuits in awake, freely behaving animals will help elucidate their function and regulation[1–3]. Conventional optogenetic studies have contributed significantly to the identification of neural circuits responsible for some aspects of animal behaviour, however these techniques typically rely on light delivery systems that require implanted optical fibres which are connected to an external light source. Although simple and effective, the tethered nature of this approach restricts experimental flexibility, decreasing the opportunity for experimental designs that can manipulate neural circuits in freely moving animals. The use of individual optic fibre implants may also hinder more complex experimental designs aimed at probing multiple brain regions simultaneously. Such whole-brain experiments may require photostimulation from distinctly targeted light sources, possibly with multiple wavelengths. To achieve different targeting locations, depths, and directions of light delivery, multiple optical fibres would need to be implanted simultaneously into a small mouse brain, making the approach impractical.

Here, we provide the procedures required for the fabrication and implementation of customizable 3D-printed optogenetic probes (termed 3D-POPs) where the shape and length can be easily modified[4]. 3D-POPs are thin (with a typical cross-section of 60 μm) optoelectronic devices that are constructed using microscale inorganic light-emitting diodes (μ-ILEDs), electrodes for μ-ILED control, and a 3D-printed substrate for probe construction. The key component to enabling the slim profile are the design of 3D-printed 20 μm micro-grooves (Fig. 1a). These grooves are filled with conductive paste to allow for simple fabrication of the electrodes necessary to power the μ-ILEDs. Typical electrical pin connectors on the 3D-POP enable easy connection to a variety of external power sources. Here, we build on earlier work describing the fabrication of wireless optoelectronic devices for photostimulation and for measuring physical properties of brain tissue[5], as well as that

of devices for concurrent photostimulation and pharmacological manipulations in deep brain tissue[6]. While the devices described in our earlier protocols remain of interest to the field, their use is nevertheless constrained by the need for specialized equipment and their limited adaptability due to time-consuming and expensive manufacturing and design processes. In this Protocol Extension, we provide an easily customizable and cost-effective technique for the rapid fabrication of customized optical neural probes to address the limitations to accessibility of our prior microfabrication techniques. 3D-POPs can be manufactured without extensive training or expensive equipment, and therefore increase the independence and flexibility with which neural implants can be designed.

Development of the protocol

This protocol overcomes the limitations of previous approaches to neural probe manufacturing[5–9]. In particular, many labs do not have direct access to, or the resources to attain access to, the specialized facilities and equipment such as mask aligners and metal evaporators required to reproduce the neural probe fabrication processes, we sought to develop an approach that reduces the overall costs and time required to fabricate wireless neural probes. To do so, we identified a high-resolution, commercially available 3D resin printing scheme that enables the cost-effective preparation of devices. This 3D printing approach can be easily adapted by individual users who will be able to tailor a series of probe designs for each experiment, as needed. The 3D-printed probes are lightweight, flexible and compatible with nearly any powering scheme – here we present options for both Bluetooth Low Energy (BLE) and tethered powering.

Overview of the procedure

This protocol is divided into three main sections: 1) Fabrication of 3D-POPs, 2) Preparation and implementation of wireless functionality in 3D-POPs, and 3) Implantation and application of 3D-POPs for behavioural assays. The entirety of Procedure 2 can be skipped by users intending to use these 3D-POPs solely with a wired or tethered approach. The use of a 3D-printed fabrication strategy allows for fast and simple construction of the probes, which is key to the customizability and cost-effective nature of this protocol. An example of this customisability can be found in Box 1, which details how users can multiplex fabrication to fabricate 3D-POPs in array configurations to target multiple brain regions at once. Additionally, researchers that have prior experience with traditional stereotaxic surgeries and tethered models of optogenetic probes would require minimal training to achieve proficiency implanting and implementing these probes for *in vivo* experimentation.

Advantages

Unlike the sophisticated thin-film deposition and photolithography approaches typically used for neural probe fabrication[5,6], the wireless neural probes are 3D-printed using a commercially available high resolution resin printer. These printing approaches not only eliminate the need for expensive materials, special cleanroom machines, and time-consuming microfabrication procedures, but also provide on-the-fly design customisation. These probes can be made with minimal skills, equipment, and training, thus significantly reducing the overall cost, time, and effort required for the construction of neural probes for specific target applications. Single- or multi-shanked devices can be made with almost

no impact on design time and fabrication cost (Fig. 1b). The design principles outlined here could further be used in neuroscience research to customize the illumination sources for *in vitro* applications, such as adding photostimulation to slice electrophysiology or devices for long-term optical manipulation of cultured cells[10–12]. This approach is based on an initial modest investment to purchase the printing hardware, which results in the fabrication of neural probes suitable for most standard neuroscience experiments for less than $1/probe. Even without the upfront purchase of the 3D printer, the designs can be commercially printed for around $7/probe (at time of writing). The protocol could be adopted by the user community of circuit neuroscientists, and could also provide a blueprint for further development to users of other open-source resources such as miniscope.org[13], open-ephys.org[14], and other related communities.

Limitations

Although the 3D-printed neural probes offer production and cost advantages over conventional wireless devices and fibre-optic implants, the technology is not necessarily intended to substitute existing techniques which can already be successfully used *in vivo*. Despite the reduction in cost of 3D-printed manufacturing compared to other technologies, the upfront cost for the equipment may still limit access to the technology, which also requires training and expertise with electronics and 3D-printing software.

As with any device, appropriate care must be taken when producing, assembling, and handling the probes. An important consideration to make is that many μ-ILED-based devices[5–9,21,22,27–31,36], including the default 3D-POP design, illuminate the tissue laterally (that is, the light is emitted from the side of the probe), whereas the direction of illumination found in most fibre-optic implants[35] and fibre-coupled LED systems[32] is aligned to the probe's axis (that is, the light is emitted from the tip of the probe). The illumination orientation affects neural activity and may also affect animal behavior[37–39] and should be taken into account when designing an experiment and customizing the design of a probe.

The 3D-printed probes have lightweight and thin profiles. For targeting small mammals such as mice or rats, integration with existing commercially-available μ-ILEDs allows 3D-POPs to be printed with a minimum width of 300 μm and with a thickness of 60 μm. Scaling up to larger organisms like pigs or non-human primates is only limited by the size of the print field, which varies across available printers, as larger prints will more easily accommodate multiple μ-ILEDs to illuminate larger brain structures. Importantly however, there is a necessary trade-off between minimum size and manufacturing schemes that should be well understood before adopting this strategy. Smaller form-factor designs are achievable with cleanroom fabrication processes and advanced equipment[40].

Applications

The primary function of the 3D-printed neural probes is for optogenetic stimulation *in vivo*. While we specifically implemented these probes in mice, they can easily be scaled to fit other rodents or larger animal models due to the ease of design and fabrication. As these are optoelectronic devices, it is straightforward and inexpensive to power and control each probe with either wired or wireless (e.g. BLE) schemes. As with other devices, 3D-POPs are not specifically intended for chronic long-term applications, however this protocol details encapsulation options that extend the working life of the devices after implantation to more

than a year. Our group has validated the use of these devices in driving food consumption through stimulation of agouti-related protein expressing neurons in the arcuate nucleus of the hypothalamus in mice[4]. However, nearly any behavioural assay requiring optogenetic stimulation should be possible with these devices. Further, 3D-POPs can be adopted for use in any animal model and customized to desired length, wavelength, direction, and number of light sources. With additional modifications, this fabrication technique could be used to produce implantable bioelectronic devices with other modalities (e.g. optical sensing[33,54], electrical[5,7,56,57], microfluidic[6,8,31,58], etc.) also making them compatible for use in other organs[59,60].

Alternative methods

While many different schemes are available to enable wireless or multi-site devices for optogenetic manipulation, the most advanced systems rely on labour-intensive and high-end fabrication processes[5–9,15–31]. Other approaches use a fibre-coupled external LED which can substantially reduce costs[32], but these approaches are often bulky and can weigh more than 10% of the animal's body weight which has served as a guiding principle for much neural device development. Though devices more than 10% of body weight can be worn by rodents, head-mounted devices beyond this limit can limit normal activity in freely behaving animals or require extensive training prior to experimental use[41–43]. The 3D-printed devices described here strike a balance between weight optimization and function. The intracranial probes weigh 80 mg and the whole head-mounted wireless control module weighs around 1 g. The flexible powering schemes enable connections via lightweight tethering (negligible weight), radio frequency (RF) power transfer schemes (tens of milligrams)[21,24,26–29,33], or, as shown here, battery-powered BLE systems (~1 gram)[9,30,31,34].

Experimental design

This protocol describes the fabrication, implantation, and operation of low-cost 3-D printed optogenetic probes that are easily customizable to fit diverse experimental needs. Researchers can choose the design of the probes based on the requirements of individual experiments. Depending on the desired probe and experimental designs, certain steps are not strictly necessary. If a tethered model is desired, Procedure 2 can be skipped and replaced with the 3-pin male connector soldered to an gauge wired for the external power source/signal generator. While this protocol was implemented in mice, probe design can be modified to accommodate almost any other vertebrate animal species.

Probe design.—Depending on the number and locations of the targeted neural circuits in the brain, the probes can be designed to have different layouts, lengths, and dimensions. 3D-printed substrates are the basis for designing 3D-POPs. The substrate consists of probe shank(s) where the μ-ILEDs are attached and an interface pad for connection to a μ-ILED controller (i.e., wireless control module or signal generator). Each 3D-POP shank has microgroove patterns for guiding silver paste to build electrodes that provide electrical connection between μ-ILEDs and the interface pad. The shank has a tapered tip to facilitate implantation into brain tissue. The typical design has two electrodes to operate μ-ILEDs, but this could be adapted for independently-addressable μ-ILEDs on the same shank. As the number of electrodes increases, the number of independently-controllable μ-ILEDs also

increases. The interface pad with microgroove structures should be designed considering the number of shanks and μ-ILEDs, as well as the shape of a connector. Researchers can fabricate any custom designed 3D-POPs by following Procedure 1.

Wireless control circuit design.—Procedure 2 is only required for wireless operation. The wireless control circuit introduced here uses a BLE system-on-chip (BLE SoC), which is a microcontroller supporting BLE. Programmed BLE SoC supplies voltage pulses to the connected 3D-POPs to control μ-ILEDs. The circuit shown in Supplementary Data 4 and the code for the BLE SoC included in Supplementary Data 5 are developed to provide independent control of up to two μ-ILEDs. If increasing the number of μ-ILEDs is necessary, the output pins, pin connector, and the software of BLE SoC should be modified. Flexible and double-sided printed circuit boards (PCBs) are recommended for lighter and smaller circuits. It usually takes about a week to order these PCBs from a PCB vendor.

***In vivo* applications.—**Procedure 3 focuses on surgical implantation and application of 3D-POPs in behavioral experiments. Although each behavioral assay will require distinct experimental designs, the principles established in Procedure 3, Steps 16–23 provide general guidance for the application of these probes *in vivo*. Any implanted 3D-POP can be powered and controlled by either the wireless BLE modules (Procedure 2) or by tethered connections to a signal generator. Depending on the duration and complexity of the behavioral task, inclusion of an electrical commutator[61–64] may be necessary to provide freedom of movement in tethered experiments.

Subjects.—This protocol and these devices have been optimized for use in adult (25–35 g) C57BL/6J mice and mutant mice backcrossed to the C57BL/6J mouse strain. Here, we describe the application of the protocol to male and female mice between 2–6 months of age. It is important to consider the sex of the animals in the experimental design, and its role as a variable that may significantly impact the interpretation of the results[51,52]. Additionally, because the printed and electronic components described in this protocol can be damaged by cage-mates' chewing, care must be taken when group housing animals following implantation. We recommend covering the entire implant with cement to deter cage-mates from chewing on the device. If this proves unsuccessful, individually housing animals may be required, which may in turn impact animal behaviour and must be considered in designing behavioural experiments[44–50]. Prior to beginning this protocol, all the procedures described herein should be approved by the relevant Animal Care and Use Committee of the investigating institution and conform to internationally recognised standards. 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. This protocol will enable researchers to perform *in vivo* experiments. The details of these experiments will be set by individual research teams, but this technology is compatible with the reduction and refinement of human animal research set forth by the "3Rs" measures for the Replacement, Refinement and Reduction of animals in research (Directive 2010/63/EU). Likewise, we recommend users follow the ARRIVE guidelines when planning experiments and documenting research results.

Controls.—Appropriate control animals are critical for optogenetic experimental design. We recommend that the control group consists of age- and sex-matched animals expressing a fluorescent reporter using the same genetic targeting scheme as used to express the opsin in the experimental group. This approach should control for animal strain as well as any off-target effects of related surgeries and protein expression in the cell-type of interest. Importantly, these controls account for possible heat- and light-induced artifacts of photostimulation[53]. Animals expressing the opsin of interest without receiving photostimulation is another important control for potential changes to baseline excitability and neuronal function. This can be achieved using the same individuals to be photostimulated, when behavioural tests allow for repeated testing, or using a separate cohort when behavioural tests cannot be repeated. Additionally, as optogenetic photostimulation typically involves tethered animals during behavioural assays, it is likely worthwhile to consider that there may be behavioural differences in untethered animals compared to tethered groups that arise simply from the additional freedom that wireless devices provide. Other reasonable considerations include accounting for the time of day the assays take place, the handler effects, the number of cagemates, and the age and sex of the animals.

MATERIALS

Biological materials

- **Mice.** Animals used in this protocol were adult male C57BL/6J (The Jackson Laboratory, cat. no. 000664) and *agrp*-IRES-Cre (The Jackson Laboratory, cat. no. 012899) mice backcrossed to C57BL/6J mice which were bred to Ai32 (The Jackson Laboratory, cat. no. 024109) mice. ! CAUTION Experiments involving live animals must be approved by the Animal Care and Use Committee of the investigating institution and must adhere to relevant institutional and national regulations. All experiments in this protocol were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

REAGENTS

Preparation of wireless optogenetic probe system

- Isopropyl alcohol (Chemitop) ! CAUTION Isopropyl alcohol is flammable and causes eye/skin irritation. Keep away from heat and flame. Use adequate ventilation and wear protective eyeglasses and gloves.
- Photopolymer (B9 Creations, cat. no. B9R-4-YELLOW)
- Kapton tape (Bedell, cat. no. 33-00356-02)
- Silver paste (Toluene: Silver powder: Ethyl lactate = 3: 5: 2, resistivity < 0.3 mΩ•cm; JIN Chemical, cat. no. ELCOAT P-100). Any conductive paste can be used as far as it has similar or better properties (e.g., TED PELLA. (cat. no. 16031)).

- Solder paste (CHIPQUIK, cat. no. SMDLTLFP10T5)
- Polydimethylsiloxane (PDMS; Dow Corning, cat. no. Sylgard 184)
- Parylene C dimer (Dichlorodi-para-xylylene; Daisan Kasei, cat. no. diX C)
- 5 Minute Epoxy (Permatex, cat. no. 84101)
- Adhesive film (Adhesive Research, cat. no. ARclear 8932EE)

Implantation of 3D-printed optogenetic probes (3D-POPs) into targeted brain structure

- Isoflurane, USP (Isothesia, Butler Schein, cat. no. 029405) ! CAUTION Experiments involving live animals is a regulated process that poses risks to the experimenter and animal subject alike. Prior to beginning this protocol, the relevant animal welfare committee of the investigating institution should approve all procedures and conform to national guidelines regarding the use of animals in research. All experiments in this protocol were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines. ! CAUTION Isoflurane is an anesthetic gas that is an eye and skin irritant, as well as a central nervous system toxicant. Ensure proper ventilation and gas scavenging methods are in place to prevent potential inhalation of excess isoflurane.
- Betadine solution (Purdue Products, cat. no. 6761815017)
- Ethanol (Sigma-Aldrich, cat. no. 362808) ! CAUTION Ethanol is flammable. Do not use near sparks or open flames.
- Hydrogen peroxide, 3% USP (Select Medical Products, cat. no. 117)
- Lidocaine ointment USP, 5% (Fougera)
- Ophthalmic ointment (Pivetal, cat. no. 21295075)
- Kwik-Sil (World Precision Instruments, cat. no. KWIK-SIL)
- Adhesive Luting Cement (C&B-Metabond, Parkell Inc., cat. no. S380) ! CAUTION This item is flammable. Do not use heat/sparks/open flames/hot surfaces. Use only outdoors or in a well-ventilated area. Wear protective gloves/protective clothing/eye protection/face protection. Avoid breathing dust/fume/gas/mist/vapors/spray. Wash face, hands, and any exposed skin thoroughly after handling.
- 0.9% Sodium Chloride Inj., USP ((9 mg/ml NaCl), Hospira, cat. no. RL-0497(9/04))
- Enroflaxin (Baytril, Bayer, cat. no. R30901)
- Antibiotic ointment (Neosporin, Johnson & Johnson, cat. no. 174-73087Q)
- Nylon suture (McKesson, cat. no. S662GX)

EQUIPMENT

Fabrication of 3D-printed optogenetic probes

- 3D CAD tool (Autodesk, Inventor)
- 3D printing system consisting of slicing software (B9 Creations, B9Create 1.0), 3D printer (B9 Creations, B9 Core 530), build head (B9 Creations, cat. no. B9S-BTA-0010), vat (B9 Creations, cat. no. B9A-VAT-500), post-curing solution (B9 Creations, cat. no. B9A-LCB-010) ▲CRITICAL A 3D printer with the minimum X/Y-axis resolution of 100 μm and the Z-axis resolution of 30 μm is necessary to execute the fabrication process.
- Glass substrate (Marienfeld, cat. no. 1000412)
- Rubber blade (AES Industry, cat. no. AES 87606)
- Digital multimeter (Fluke, 87V Industrial Multimeter)
- Microscale inorganic light-emitting diode (μ-ILED; CREE, cat. no. C470TR2227). TR2227 μ-ILED is also available from Marktech Optoelectronics (marktechopto.com).
- Stereo microscope (Omano, cat. no. OM4713)
- Reflow oven (SMTmax, cat. no. AS-5060)
- Vacuum chamber: desiccator (Scilab, cat. no. 27-03391-01), vacuum pump (Rocker, cat. no. 31-00812-01)
- Convection oven (SH Scientific, cat. no. DO100FG230)
- Parylene coater (Specialty Coating Systems, cat. no. PDS 2010)
- Three-pin female connector (Harwin, cat.no. M50-3140345)

Preparation of wireless control module

▲CRITICAL This section is not required for wired control of the 3D-POPs.

- Flexible printed circuit board (PCB; PCBWAY, custom design)
- Bluetooth Low Energy system-on-chip (BLE SoC; RF Digital, cat. no. RFD77101)
- Voltage regulator (ON Semiconductor, cat. no. NCP4624DMU30TCG)
- Three-pin male connector (Harwin, cat. no. M50-3630342)
- Two-pin connector: male (Harwin, cat. no. M50-3630242), female (Harwin, cat. no. M50-3130245)
- Indicator LEDs: red (Vcc, cat. no. VAOL-S4RP4), green (Vcc, cat. no. VAOL-S4GT4)
- Capacitors: 0.1 μF (Samsung Electro-Mechanics, cat. no. CL03A104KQ3NNNC)

- Resistors: 6.04 kΩ (Panasonic, cat. no. ERJ-1GEF6041C)
- Lithium polymer battery (PowerStream, cat. no. GM300910-PCB)

Development of smartphone application

▲CRITICAL This section is not required for wired control of the 3D-POPs.

- Integrated Development Environment (IDE) for Android development (Google, Android Studio) ▲CRITICAL Only Android-based devices are able to execute the application created through this IDE. However, many generic Android and iOS BLE apps (e.g., Nordic Semiconductor ASA, nRF connect, etc.) can be used to control the wireless modules.

Implantation of 3D-POP into targeted brain structure

- Stereotaxic alignment system (KOPF Model 942) ! CAUTION Prior to beginning this protocol, the relevant animal welfare committee of the investigating institution should approve all procedures and conform to national guidelines regarding the use of animals in research.
- Digital Stereo Microscope (Leica S9i)
- Stereotaxic drill (KOPF Model 1474) and #66 drill bit (KOPF cat. no. 8669)
- Stereotaxic single cannula holder (2.5mm ferrule) (Thorlabs cat. no. XCF)
- Stereotaxic adapter for cannula holder (Extended Data Fig. 1; Supplementary Data 1)
- Knurled head screw #8–32 × 3/8 inch (Everbilt)
- Needles (Becton-Dickinson, cat. no. 305111)
- Forceps (Fine Science Tools, cat. no. 11009-13)
- Scalpel Handle (Fine Science Tools, cat. no. 10003-12)
- Scalpel Blade #10 (Fine Science Tools, cat. no. 10010-00)
- Microspatula (Chemglass, cat. no. CG-1982-12)
- Electric clippers (Wahl, cat. no. 8064-900)

REAGENT SETUP

C&B-Metabond Quick! Luting Cement

The cement can be prepared using a modification of the “Bulk Mix” technique as described in the user instructions. Place the ceramic mixing dish on ice to ensure longer working time. Place one scoop of L-powder (radiopaque white) into the shallow well of the chilled ceramic dish. Add four drops of the Quick Base liquid and 1 drop of Universal Catalyst. Mix with a small, rounded spatula. Powder/liquid ratio may be adjusted if thinner or thicker viscosity is needed (adjusted by using 1–2.5 scoops). A thinner viscosity may be preferred during initial application to ensure best adherence to the skull, while thicker viscosity may

be preferred during later stages of forming the headcap as the luting cement is easier to apply and manipulate. (Extended Data Fig. 2). ▲CRITICAL Adjusting the viscosity alters the working time of the cement.

PROCEDURE 1

Fabrication of 3D-printed optogenetic probes (3D-POPs) ● TIMING 3–5 h

1. Create 3D models for desired probes using a 3D computer-aided design (CAD) (Extended Data Fig. 3a and Supplementary Data 2).

   ▲ **CRITICAL STEP** Including tapered tips in probe design may reduce tissue damage during implantation.

   ▲ **CRITICAL STEP** Include microgroove patterns (20 μm in depth) in the probe substrate designs to create electrodes for micro inorganic light-emitting diodes (μ-ILED).

2. Prepare a CAD file for printing by arranging multiple 3D probe models and convert the file type (filename extension: cpj). Add as many probe models as possible based on the printer build head area for maximum printing throughput. The number of models has a very minimal effect on the printing time, so this approach can reduce wait times for new probes and minimize excess resin waste.

3. Load the converted CAD file (filename extension: cpj) to the 3D printer using a flash drive.

4. Clean the printer build head and the vat with isopropyl alcohol and pour photopolymer in the vat. Set the build head and vat in the 3D printer. ▲ **CRITICAL** Gloves should be worn from step 4 to 15 in this procedure.

5. Start printing to create the probe substrates using photopolymer via stereolithography (Fig. 2a).

6. Take out the build head and rinse the printed probe substrates thoroughly with isopropyl alcohol (Fig. 2b). This process removes excess photopolymer from the probe substrates.

7. Carefully release the printed substrates from the build head from the corners using a rubber blade (Fig. 2c).

   ? TROUBLESHOOTING

8. Immerse the probe substrates in water and post-cure the substrates under ultraviolet chamber (intensity: 40 mW/cm^2; time: 10 min) to improve robustness. By soaking in water, ultraviolet can treat all sides of the substrates.

   ■ **PAUSE POINT** Probe substrates can be stored in petri dishes at room temperature (~15–25 °C) for more than a year.

9. Mount the probe substrates on a Kapton tape-coated glass substrate with a tweezer, and spread silver paste over the microgroove patterns of the probe

substrate using a rubber blade to form the electrodes (Fig. 2d,e). Thermally cure the silver paste on 70 °C hotplate for 30 min.

▲ **CRITICAL STEP** Using an ohmmeter, measure the resistance of each electrode after curing the silver paste. All electrodes must be isolated, and each electrode must be electrically connected from the probe tip to the base.

? TROUBLESHOOTING

10. Apply solder paste on the electrodes and transfer-print a μ-ILED (Fig. 2f).

▲ **CRITICAL STEP** Accurate and precise positioning of the μ-ILED is crucial because it determines the location of photostimulation in the brain. This process should be done under the microscope to precisely control the printing site.

11. Bake the solder paste in the reflow oven (temperature: from 138 °C to 165 °C, time: 30 s; the same soldering condition can be used throughout the entire procedure; Fig. 2g) and complete the soldering of the μ-ILED on the silver electrodes (Fig. 2h).

12. Carefully detach the probe from the Kapton tape to prepare for the encapsulation of the probe shank. Polymer (PDMS and parylene C) encapsulation is essential to provide biocompatible and waterproofing interfaces on the probe.

? TROUBLESHOOTING

13. Prepare a supporter and a stencil mask (Extended Data Fig. 4, Supplementary Data 3) using the 3D printer. The 3D-printed stencil mask (40 μm thick) has an opening with the shape of the probe shank (Fig. 2i, inset). The supporter has a groove structure (60 μm-deep) to keep the probe and the stencil mask flat during screen-printing.

14. Place the probe in the groove of the 3D-printed supporter and cover it with the stencil mask. Coat a thin PDMS layer (elastomer/curing agent ratio = 10:1; thickness < 40 μm) around the probe shank using a screen-printing technique (Fig. 2i). Cure it for 1 hour in an oven with a temperature of 70 °C.

15. Deposit parylene C (6 μm thick) on the PDMS-coated probe shank using a parylene coater. To avoid the probe base electrodes being coated with parylene C, cover the probe base with a thin PDMS piece (<1 mm thick) (Fig. 2j). For short-term (1–2 months) implantation, PDMS encapsulation is sufficient, therefore this step is optional depending on experimental aims. However, Parylene C coating provides enhanced durability (up to 2–5 years)[4,54] thus should be considered if longer in vivo experiments are desired.

16. Solder a pin connector to the probe base (Fig. 2k) which can provide electrical connection with a device control system.

17. Print a probe holder using a 3D printer (Extended Data Fig. 3b, Supplementary Data 2).

18. Mount the 3D-POP on the printed holder and secure them with epoxy for probe protection and easy handling (Fig. 2l).

19. For fabrication of 3D-POPs with array configurations, follow the instructions in Box 1.

PROCEDURE 2

▲ **CRITICAL** This section is not required for wired control of the 3D-POPs.

Preparation of the BLE control circuit ● TIMING 1 d

1. Design a circuit for wireless control of the 3D-POP (Extended Data Fig. 5, Supplementary Data 4). If experimental conditions require a different light intensity than would be native to the μ-ILED, then a current limiting diode can be added between the 3D-POP and the BLE SoC.

2. Build a flexible printed circuit board (PCB) through a PCB vendor or custom microfabrication to minimize the weight of the device.

3. Attach the PCB on a glass slide using Kapton tape making sure the BLE SoC layer is facing upwards and apply solder paste on the electrodes (cover ~70% of each electrode; Fig. 3a). Remove any excess solder paste with a sharp needle.

4. Under a microscope (at 10× magnification), mount a BLE SoC on the PCB to ensure precise alignment between electrodes of the chip and the circuit.

5. Cure solder paste in the reflow oven (Fig. 3b).

6. Turn the PCB over and solder the rest of the circuit components (i.e., pin connectors and indicator LEDs; Fig. 3c).

Device integration for a wireless optogenetic probe system ● TIMING 10 min

7. Prepare a combined BLE control circuit (Steps 1–6– of Procedure 2) and a rechargeable LiPo battery to construct a plug-n-play wireless control module (Fig. 4a). Attach a female pin connector to the battery to enable easy assembly and disassembly for powering the module.

8. Stack the BLE control circuit on the battery and wrap them with Kapton tape to make a wireless control module (Fig. 4b).

   ▲ CRITICAL STEP The pins of the connectors must be exposed through Kapton tape.

9. Plug the female pin connector of the battery into the male connector of the circuit to supply power to the wireless control module (Fig. 4c). Check the powering status through the indicator LEDs of the circuit (Supplementary Data 5).

10. Assemble the male pin connector of the control module into the female pin connector of a 3D-POP for wireless operation (Fig. 4d).

? TROUBLESHOOTING

Operation of a custom-designed smartphone app for wireless control● TIMING 10 min

11. Download the custom-designed control application “Wireless optogenetics” onto a smartphone from Google Play (Fig. 5a). Use the attached source code (Supplementary Data 6) if you need to modify the application.

▲ CRITICAL STEP This application is built for easy and straightforward control of these BLE modules to operate 3D-POPs with up to two shanks, but many generic BLE apps (e.g., Nordic Semiconductor ASA, nRF connect, etc.) exist for Android and iOS which can also control these modules with the proper commands.

12. Open the application using the Bluetooth connectivity of the phone to scan the active wireless control modules in the vicinity (Fig. 5b). Connect to the target devices by selecting desired devices and clicking the ‘CONNECT’ button. Connecting multiple devices is possible. ▲ CRITICAL STEP The smartphone’s Bluetooth must be switched on before using the application.

13. Control the target devices by selecting the desired operation conditions in the graphical user interface. The left panel displays a list of the selected devices. Each button shows unique parameters for μ-ILED operation.

14. Initiate operating the device by selecting μ-ILED operating parameters and clicking the ‘SEND’ button (Fig. 5c). Note that this is also the command to use to start the photostimulation during an experiment (in Step 20 of Procedure 3).

15. Terminate the μ-ILED operating conditions by clicking the “Sleep” button, which sets the devices to sleep mode. Alternatively, manually disconnect the control module from the 3D-POP after the experiment.

PROCEDURE 3

Implantation of 3D-POPs into targeted brain structure ● TIMING 1 d

1. To prepare the stereotaxic frame and 3D-POP cannula holder for implantation, place the 3D-POP into a cannula holder adapter and mount the adaptor onto the stereotaxic frame prior to surgery. Adjust the orientation of the 3D-POP in the cannula holder adaptor and the stereotaxic frame setup to ensure that the angle and direction of the probe are suitable for proper illumination of the opsin expressing brain region of interest. For this procedure, the probe is oriented to face perpendicular to the anterior-posterior axis.

2. To prepare the animal for implantation, carefully follow Steps 24–38 of our previous Protocol[5] and Step 39 of our previous Protocol Extension[6]. ▲ CRITICAL STEP Ensure that all surgical instruments are sterilized prior to each animal surgery. ▲ CRITICAL STEP To ensure secure bonding to the skull, score the skull with scalpel blade in a cross-hatched manner to aid in cement adhesion.

(Fig. 6a). ! CAUTION Experiments involving live animals must be approved by the Animal Care and Use Committee of the investigating institution and must adhere to relevant institutional and national regulations. All experiments in this protocol were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

3. Mount the 3D-POP device in the 3D-printed cannula holder adapter (see EQUIPMENT SETUP) (Fig. 6b,c and Extended Data Fig. 1). ▲ CRITICAL STEP The device must be placed along the midline of the adapter to achieve proper spatial targeting. ▲ CRITICAL STEP The probe must be handled with care during this procedure to prevent the tip from breaking or bending. We recommend using broad, non-serrated, and non-conductive forceps for this step. Use the forceps to handle the probe by the 3D-printed holder (Figure 2l and do not directly touch the tip. Once mounted, be mindful to avoid contacting the adapter and mounted 3D-POP device with surrounding surfaces.

? TROUBLESHOOTING

4. Using the determined stereotaxic coordinates (Paxinos and Franklin Brain Atlas), move the drill above the implantation site. Drill a small hole that is wide enough to accommodate the probe. The craniotomy should be approximately 1mm x 0.5mm. Ensure the drill hole fully penetrates the skull, but not the dura (Fig. 6a). ▲ CRITICAL STEP It is important not to penetrate/destroy the dura with the drill. Drilling through the dura prior to careful resection in Step 5 can cause widespread damage, bleeding, and inflammation under the skull beyond the craniotomy site. ? TROUBLESHOOTING

5. With a sterile 26-gauge needle, carefully remove the dura in the centre of the craniotomy. Take care to avoid damaging the brain tissue or any nearby blood vessel. ▲ CRITICAL STEP Intact dura at the site of implantation can potentially cause tissue damage and could prevent proper probe implantation.

6. Remove drill and attach cannula holder with 3D-printed adapter and probe.

7. To ensure proper implantation, realign the cannula holder with the attached probe to bregma, record or reset the coordinates, then position the implant above the drill hole (Fig. 6d).

8. At an insertion speed of approximately 50μm/sec, slowly lower the probe into the brain to the desired dorsal-ventral coordinates (Fig. 6e).

9. Silicone adhesives such as Kwik-Sil can be used to seal the hole at the site of implantation to help reduce the possibility of the luting cement (Metabond) directly contacting brain tissue.

? TROUBLESHOOTING

10. Use the spatula to carefully apply a layer of Metabond (see Reagent Setup and Extended Data Fig. 2) directly to the site of implantation and surrounding skull to fully secure placement of the probe (Fig. 6f). Mix and apply additional Metabond if needed.

11. Check that the first layer of Metabond is cured by using the spatula or forceps to gently tap the cement layer. The Metabond is cured when it does not yield under the pressure of the spatula or forceps and is fully hardened (the cement typically cures within 5–10 minutes after application) Then, remove the cannula holder adapter from the 3D-POP device (Fig. 6g).

    ? TROUBLESHOOTING

12. Finish the encapsulating the probe by applying Metabond to the 3D-POP to ensure complete coverage of the implant. ▲ CRITICAL STEP Be sure to leave the pin connector exposed and accommodate space for the BLE-module connection ((Fig. 6h).

13. After the second layer of Metabond is cured (refer to step 10), use a sterile spatula or forceps to detach skin from the cement to ensure the longevity and the durability of the headstage. ? TROUBLESHOOTING

14. Apply a topical antibiotic ointment and lidocaine ointment to the entire incision area.

15. Remove the animal from the stereotaxic frame and place it in a clean cage positioned on top of a heating pad or table during recovery from surgery. Once the animal has recovered and displays normal, awake locomotor behaviours, it can be returned to its home cage. ▲ CRITICAL STEP Due to potential damage to the probe, care should be taken when group-housing animals. Complete encapsulation of the probe (except for the pin connector) should ensure durability for the lifetime of experimentation.

Preparation of the behavioural assay for optogenetic stimulation ● TIMING ~ 5 days

16. At least five days before experimentation, experimenters should handle animals to acclimate the animals to the handling procedures.

17. Animals should also be habituated to the chosen powering scheme (i.e., wired or wireless). The animals should be connected to the BLE module or tethered power supply and allowed to explore their home cage for the same duration of the behavioural experiment once per day during the five-day habituation period. ▲ CRITICAL STEP To eliminate locomotor confounds, the animals must be habituated to carrying the added weight of the BLE-module.

Performing the in vivo optogenetic behavioural assay ● TIMING variable

18. Allow the animals to habituate to the behavioural experiment room for 30 minutes. ? TROUBLESHOOTING

19. Gently remove the animal from the home cage and carefully scruff the animal. Ensure that you are holding the animal by the skin at the base of the skull and take care to not press against the probe with the hand holding the mouse[65–67]. Appropriately orient the BLE module pins and align into the probe connector. Confirm connection by gently pinching the module and probe together (Fig. 7a–c). Alternatively, connect the tethered power cable (Fig. 7d,e)

20. Place the animal in the behavioural apparatus (Fig. 7f).

    ? TROUBLESHOOTING

21. Execute the desired photostimulation patterns using either the “Wireless optogenetics” application or through an external signal generator (Fig. 5; Extended Data Fig. 6).

22. Following the behavioural test, gently scruff the animal, and remove the BLE module from the headstage, and place it back into the homing cage.

23. After the final behavioural testing session, animals can be euthanized, and tissue collected for multiple types of postmortem evaluation. ! CAUTION Euthanizing animals is a regulated procedure. The method of euthanization must be chosen based on experimental needs prior to requesting approval by the Animal Care and Use Committee of the investigating institution and must adhere to relevant institutional and national regulations. Euthanization of animals in this protocol were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

TIMING

Procedure 1

Steps 1–19, Fabrication of 3D-printed optogenetic probes (3D-POPs): 3–5 h

Procedure 2

Steps 1–6, Preparation of BLE control circuit: 1 d

Steps 7–10, Device integration for a wireless optogenetic probe system: 10 min

Steps 11–15, Operation of custom-designed smartphone app for wireless control: 10 min

Procedure 3

Steps 1–15, Implantation of 3D-POPs into targeted brain structure:1 d (30–60 min per animal)

Steps 16–17, Preparation and performance of the behavioural assay for optogenetic stimulation: ~5d

Steps 18–23, Performing the in vivo optogenetic behavioural assay: variable (hours to weeks)

Box 1

Fabrication of POPs with array configurations (m × n probes): 4–6 h

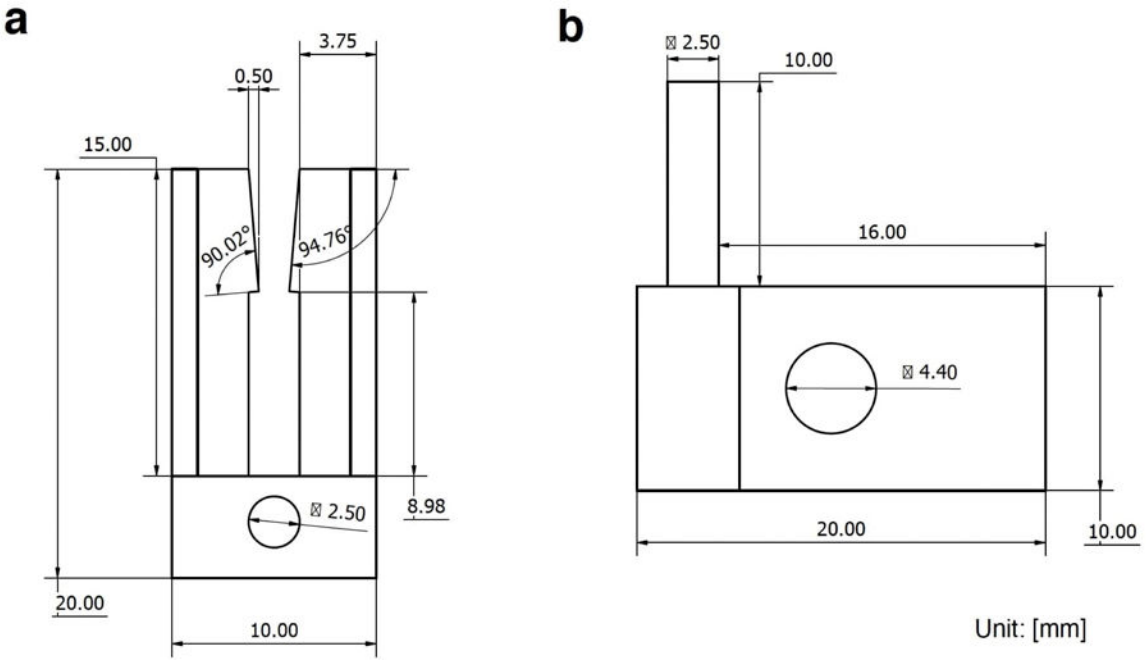
TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

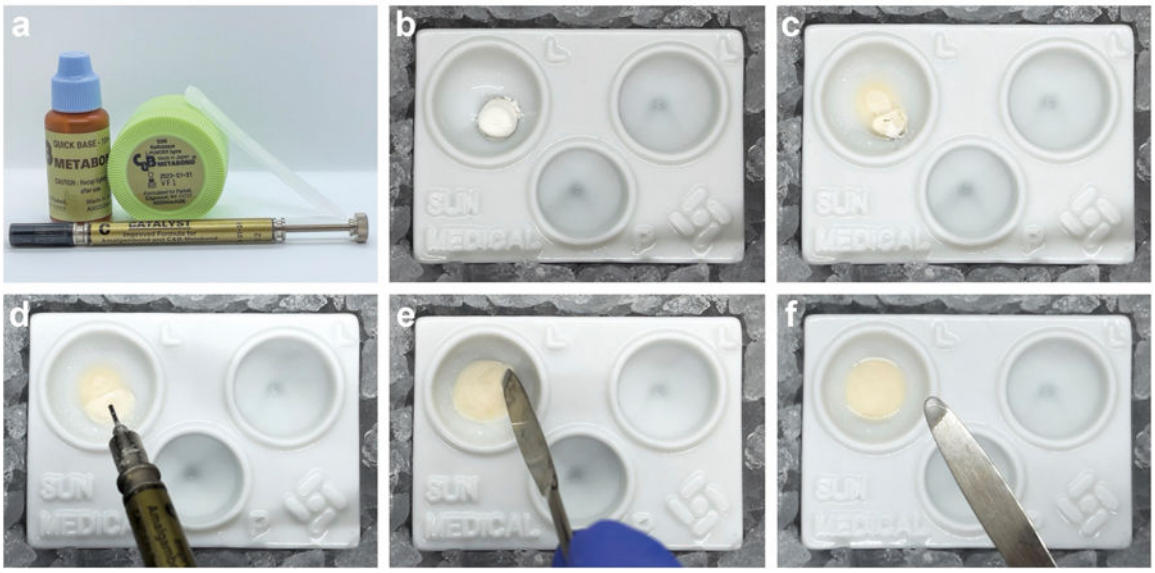
After successful completion of this protocol, users will be able to fabricate and implement 3D-printed neural probes with wireless photostimulation capabilities for *in vivo* animal behavioural assays. Importantly, researchers will be able to customize the devices for more complex experiments requiring particular specifications, with minimal time, cost, and training required compared to previous fabrication techniques which may have been prohibitive. Implanted devices can remain functional for several months; we have observed device functionality for up to 3 months *in vivo* and, following their removal from the brain, the probes remain functional for multiple years[4]. Though reuse of these devices is likely possible by following Box 2 of our previous protocol[5], it is likely not worth the time, effort, or costs to reuse across multiple animals. Instead, we recommend production of new 3D-POPs for each *in vivo* experiment. When possible, we recommend rigorous histological validation of implant site and the functional validation of the optogenetic construct activation prior to analysing behavioural outcomes. Researchers may need time to adjust to the practical and technical differences between 3D-POPs and other neural probes used in optogenetics. After this initial adjustment period, however, experimental success rates should easily be comparable to the levels expected with other neural probes such as fibre optic implants[35]. Cumulatively, the steps in this protocol will enable a broader community of neuroscientists to use optoelectronic devices for wirelessly manipulating neural circuit function in awake, freely behaving animals.

Extended Data



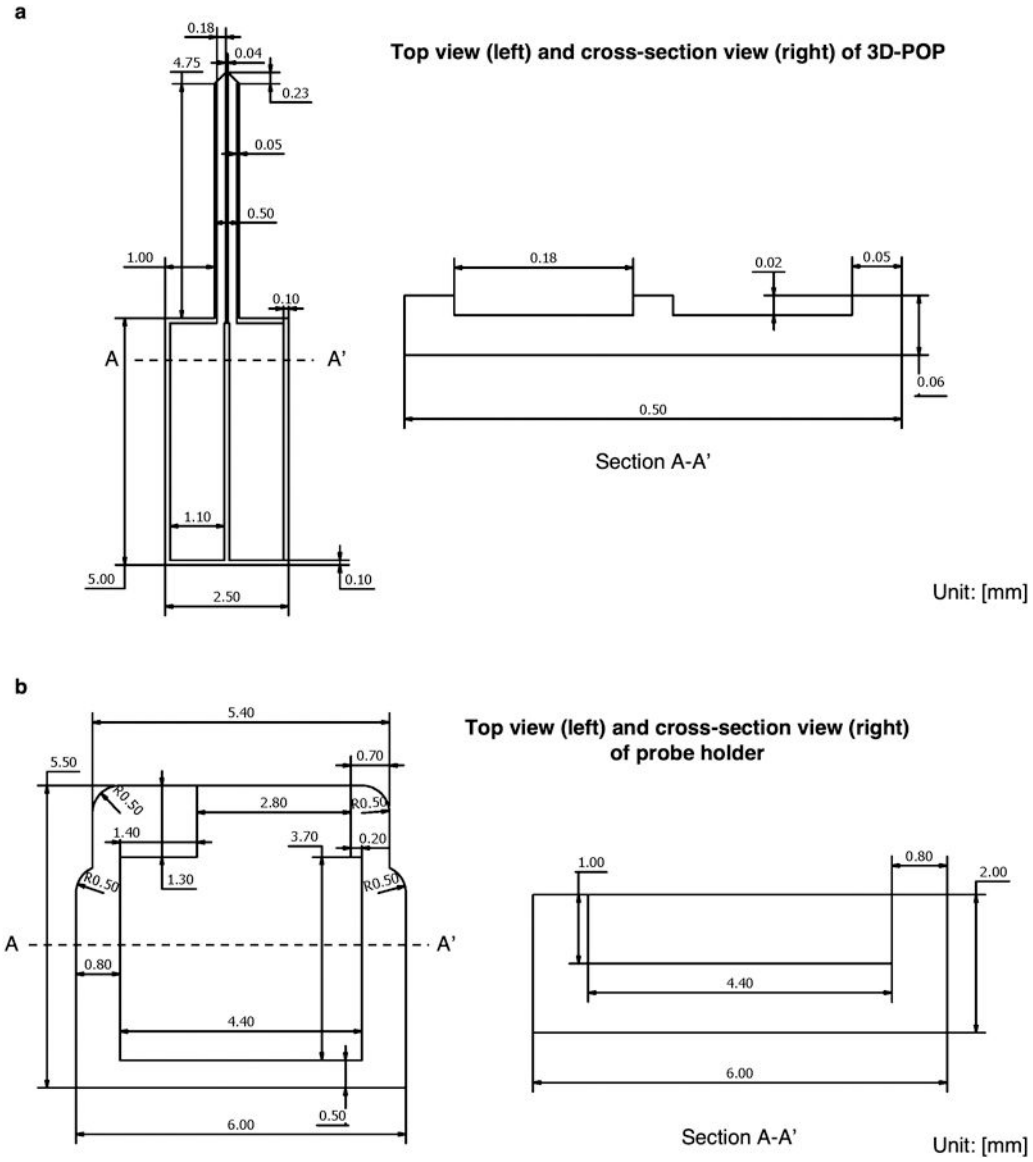
Extended Data Fig. 1. Technical drawing with dimensions for 3D-printed stereotaxic adapter for cannula holder

(a) Top and (b) side view of 3D-printed stereotaxic adapter.



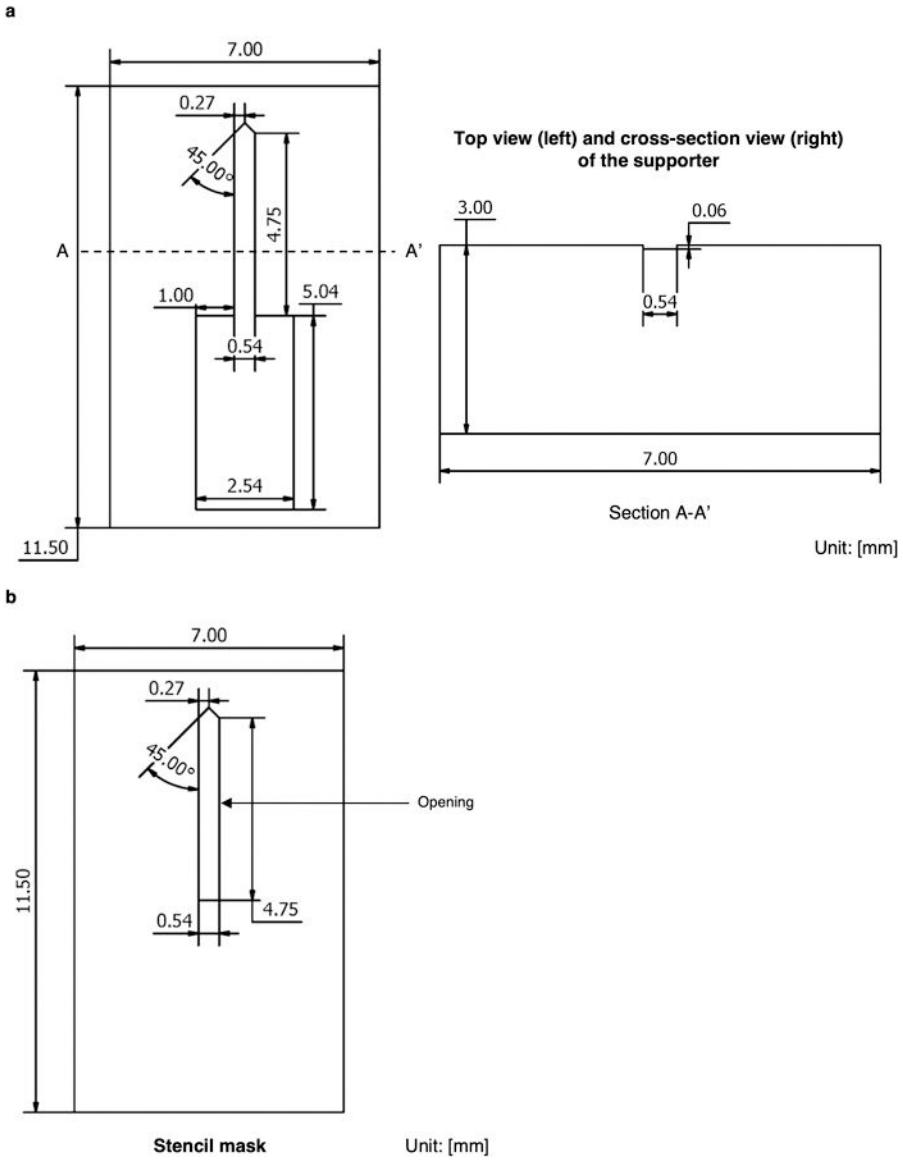
Extended Data Fig. 2. Bulk preparation of Metabond quick set cement kit

a, Selected components of the Metabond kit used to secure the implant in its position. b, Add one scoop of Metabond radiopaque L-powder to the chilled ceramic dish. c, Add 4 drops of Quick Base liquid. d, Add one drop of Catalyst liquid. e, Mix cement preparation with rounded spatula. f, Use spatula to apply it directly to the skull surface.



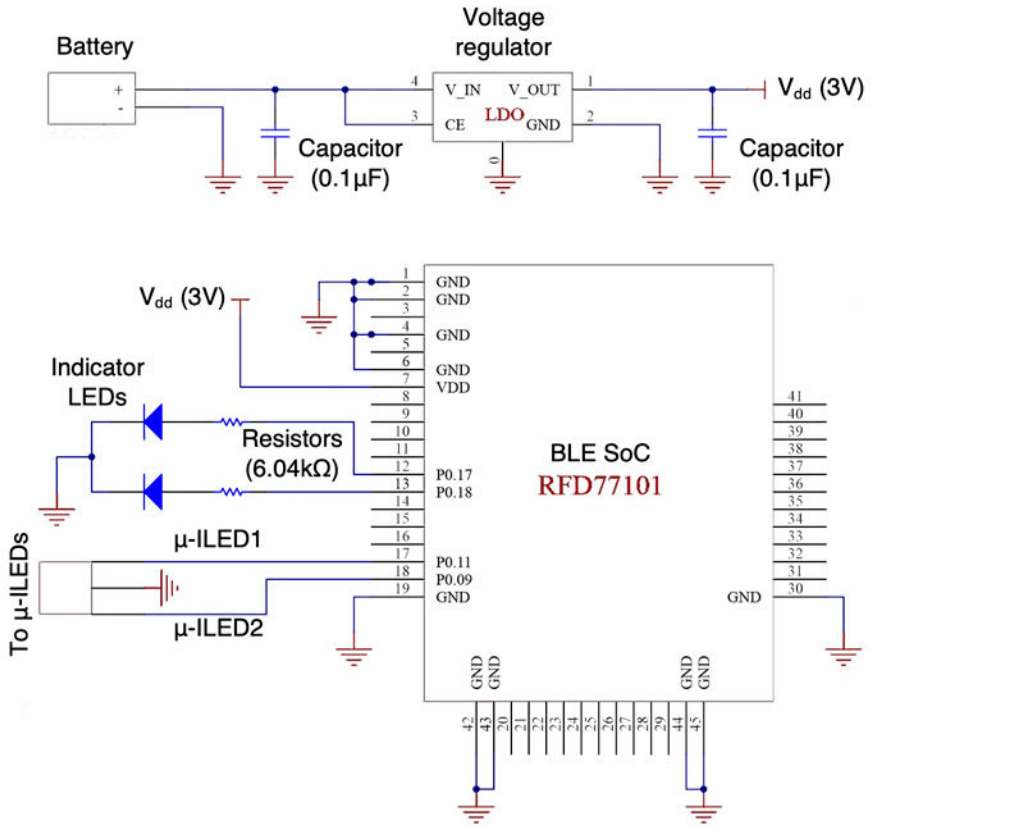
Extended Data Fig. 3. Technical drawing with dimensions for a 5mm-long unilateral 3D-POP and a probe holder

(a) Top view (left) and cross-section view (right) of 3D-POP. (b) Top view (left) and cross-section view (right) of probe holder.



Extended Data Fig. 4. Technical drawing with dimensions for a supporter and a stencil mask used for PDMS screen printing

(a) Top view (left) and cross-section view (right) of the supporter. (b) Top view of stencil mask.



V_IN (Voltage input): input of the voltage regulator, connected to the battery

V_OUT (Voltage output): output of the voltage regulator, connected to V_{dd}

V_{dd} (Supply voltage): power supply of BLE SoC

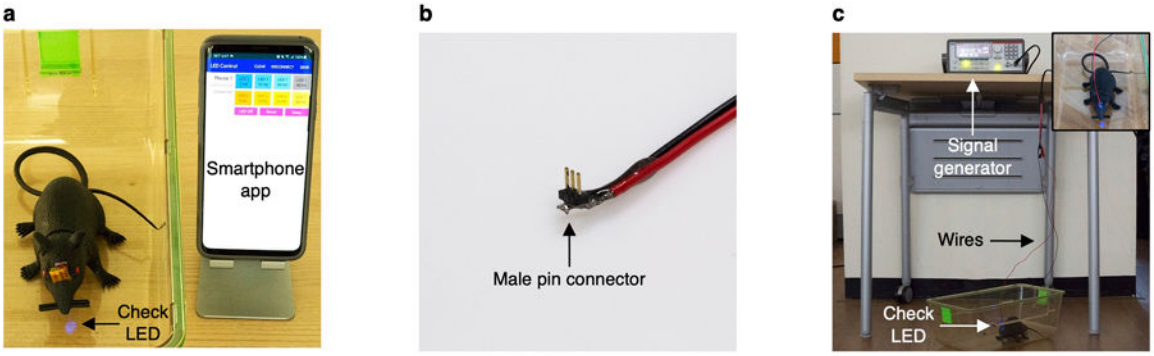
GND (Ground)

CE (Chip enable): turn on/off the voltage regulator

P0.09, P0.11, P0.17, P0.18: number of programmable output pins responsible for voltage pulse generation

Extended Data Fig. 5. Circuit diagram for the BLE control circuit

The circuit includes a Bluetooth Low Energy (BLE) system-on-chip (SoC), a voltage regulator, indicator LEDs (red, green), and connectors for the integration of a lithium polymer (LiPo) battery in a 3D-POP. The voltage regulator converts fluctuating input voltage from the LiPo battery into constant output voltage (3 V) and supplies it to the BLE SoC. The BLE SoC enables wireless control by smartphone and regulates the photostimulation condition of 3D-POPs. Indicator LEDs help to recognize the communication status of the wireless system.



Extended Data Fig. 6. Preparation of the behavioural assay for optogenetic stimulation

Preparation for wireless operation. Integrate a 3D-POP and the BLE wireless control module. Check LED operation using the smartphone app. (b) and (c) Preparation for a wired operation. (b) Solder wires to a male pin connector. (c) Connect a signal generator to a 3D-POP through the wire and apply electrical pulse signals for testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability

Experiments that support the findings of this protocol are described in our previous paper.[4] Further data are available from the corresponding authors upon request.

Related links

Key reference using this protocol

Lee, J. et al. *Adv. Funct. Mater.* **30**, 2004285 (2020): https://doi.org/10.1002/adfm.202004285

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Box 1 |

Fabrication of 3D-POPs with array configurations (m × n probes) ● TIMING 4–6 h

3D-printed neural probes with m × n probes, where m and n indicate arbitrary numbers, are needed to access and manipulate multiple neural circuits across various brain regions. Such 3D probes can be achieved by assembling multiple rows of probes (i.e., 1 × n probes). An illustrated example (Fig. 8) is given for the fabrication process of a device designed with 3 × 3 probes and the 3D models for the main construction parts are available in Supplementary Data 7. Detailed step-by-step procedures are described below.

Procedure

1. Design and print 1 × *n* probe substrates. Form the electrodes and mount μ-ILEDs as described in Procedure 1; Steps 9–15 (Fig. 8a).

   ? TROUBLESHOOTING

2. Prepare a base holder that can hold multiple 1 × n probes for the construction of a three-dimensional device. Fill the grooves on three sides of the holder with silver paste to make the electrodes (Fig. 8b). The cathode for the μ-ILED operation is on the backside of the holder. The anode is formed on the front, back, and side.

   ▲ **CRITICAL STEP** For the anode, cure the silver paste completely on one side and then create electrodes on the other side. Apply extra silver paste at the 90° corners to make sure to create electrical connection between the electrodes located on different sides.

   ? TROUBLESHOOTING

3. Print m plates to form m × n probes, which can be attached to each probe base, and prepare all the main components (i.e., probes, plates, a base holder - as many as needed) (Fig. 8c). The plates help to securely integrate the probes into the base holder.

4. Attach the plate at each probe base using adhesive film (Fig. 8d).

5. Plug plate-attached 1 × n probes into the base holder (Fig. 8e). Each time the probe is inserted, apply a small amount of silver paste between the electrodes of the probe to ensure proper electrical connection.

6. Solder a female pin connector on the backside of the holder and apply epoxy to firmly fix the connector, the base holder, and the probes (Fig. 8f).

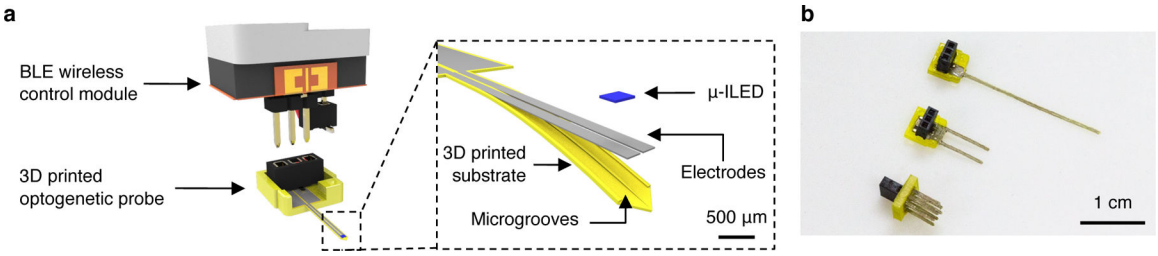

Fig. 1 |. Design of 3D-printed optogenetic probes (3D-POPs).

a, Schematic illustration of the overall system architecture for a wireless optogenetic probe with two main components: a Bluetooth Low Energy (BLE) wireless control module and a 3D-POP. The inset shows a magnified view of a 3D-POP. **b**, 3D-POPs with various designs (constructed with 1, 2 or 9 shanks).

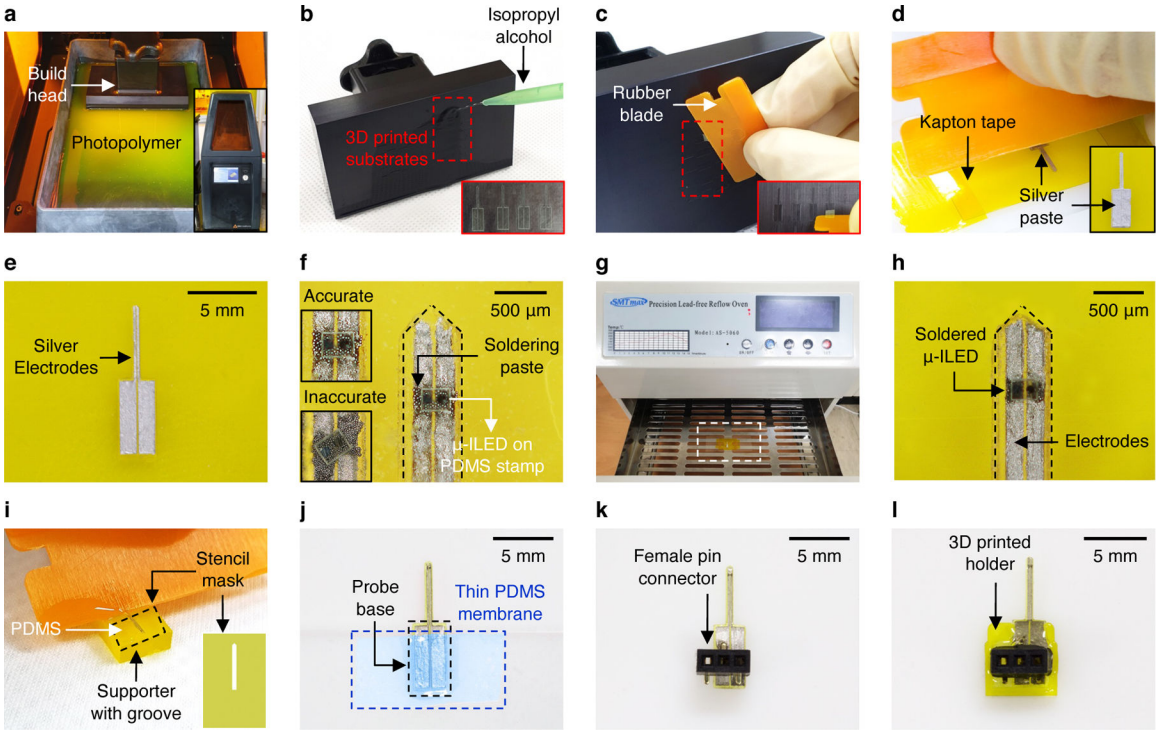

Fig. 2 |. Fabrication process for the 3D-POPs.

a, 3D-printing of the microelectrode probe substrates with a photopolymer via stereolithography using a computer-aided design (CAD) file loaded into the 3D printer (inset). **b**, The 3D-printed substrates are rinsed with isopropyl alcohol to wash out excess residue. The inset shows a magnified view of the printed probes on the printer build head after cleaning. **c**, The printed substrates are retrieved from the build head using a rubber blade. **d** and **e**, Create silver electrodes in the 3D-printed substrates by applying a thin layer of silver paste on the microgroove structure (inset) and scrape the excess using a rubber blade. **f**, Apply a small amount of solder paste onto the tip of the shank and place a micro inorganic light-emitting diode (µ-ILED) using the transfer-printing method. **g** and **h**, Solder the µ-ILED onto the ends of the electrode leads by baking the device in the reflow oven. **i**, Encapsulate the electrode shank with a thin layer of PDMS using screen printing. **j**, Protect the electrodes at the probe base with a thin layer of PDMS (blue) and coat it with Parylene C to ensure waterproofing. **k**, Attach a pin connector to the probe base. **l**, Mount the 3D-POP on a 3D-printed holder and secure it on its location using epoxy.

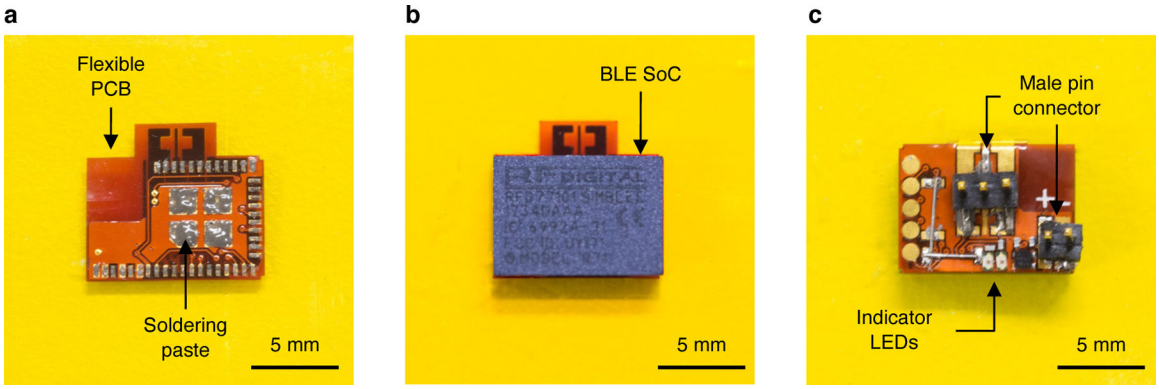

Fig. 3 |. Preparation of BLE control module.

a, Secure a flexible printed circuit board (PCB) on a glass substrate using Kapton tape and apply an appropriate amount of solder paste on the electrodes (cover ~70% for each electrode). **b**, Mount the BLE System-on-Chip (SoC) on the PCB and cure the solder paste by placing it inside a reflow oven. **c**, Turn the PCB over and attach the other circuit components (i.e., pin connectors and indicator LEDs).

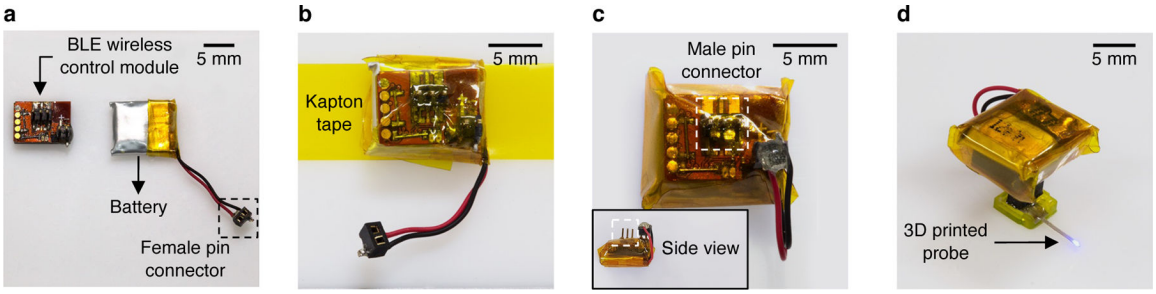

Fig. 4 |. Device integration with a BLE control module.

a, A BLE control module and a battery with a female pin connector form a plug-n-play powered wireless system. **b**, Place the BLE module on top of the battery and secure the device by wrapping it with Kapton tape. **c**, Connect the battery female pin connector to the BLE circuit's 2-pin male connector that extends through the Kapton tape for power supply. **d**, Plug the male 3-pin connector of the wireless control module into the female pin connector of a 3D-POP for wireless operation. Test the connectivity of the device.

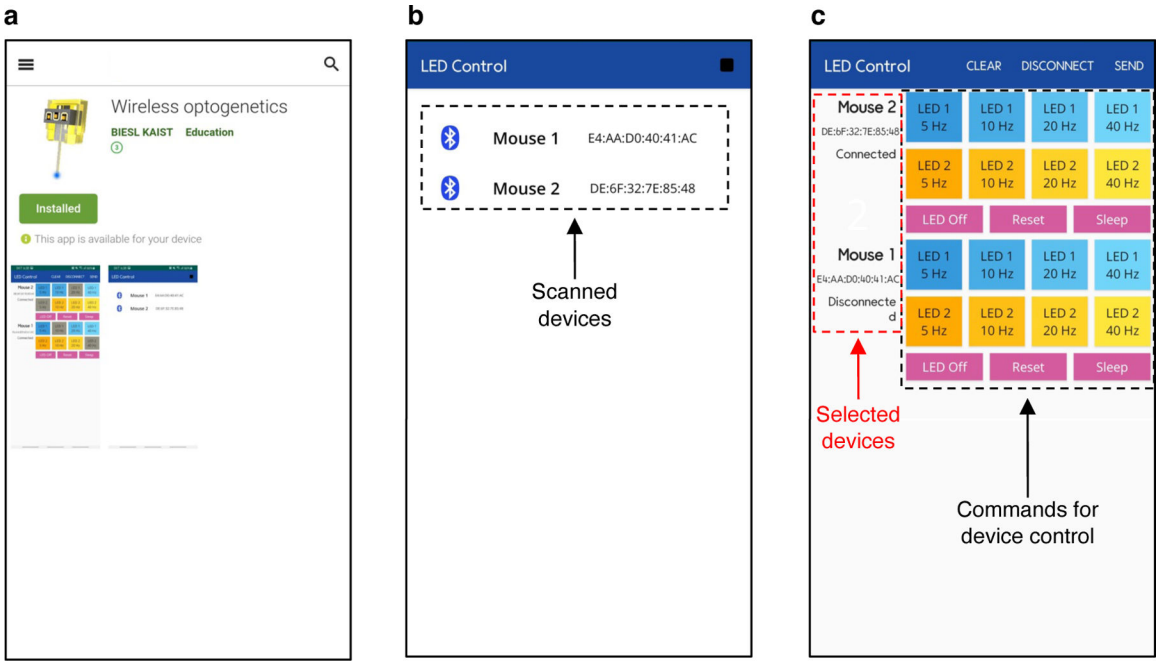

Fig. 5 |. Operation of the custom-designed smartphone application for wireless control.
a, Download the control application from Google Play. **b**, Start the application and scan the available devices near the vicinity to select the target devices to control. **c**, Select the LEDs intended for control and their operation parameters (i.e., pulse frequency) for target devices and click the ‘SEND’ button to start the wireless operation of 3D-POPs.

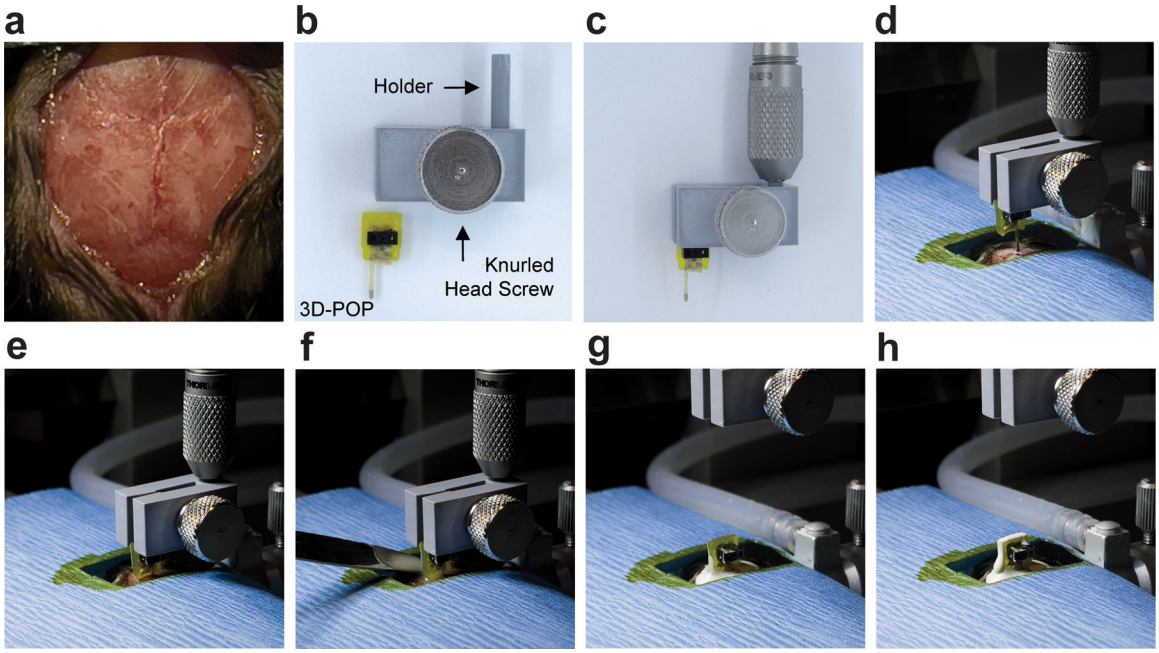

Fig. 6 |. Surgical procedure for the implantation of a 3D-POP into a mouse brain.
a, Score the mouse skull with a scalpel blade in cross-hatched pattern to ensure the adherence of cement to the skull surface. Perform the craniotomy. **b,** 3D-POP and 3D-printed device holder **c,** The 3D-POP is held in place via a screw and the holder is assembled with a stereotaxic attachment for implantation **d,** Align the 3D-POP to be inserted into the brain following the craniotomy. **e,** Lower the stereotaxic probe holder until the desired position for the μ-ILEDs is reached within the brain area of interest **f,** Directly apply Metabond to the skull surface, covering the entire exposed surface. **g,** After allowing Metabond to harden, release the 3D-POP from the holder by loosening the screw. **h,** Applying an additional layer of Metabond to the 3D-POP ensures complete coverage of the implant. All procedures were approved by the Animal Care and Use Committee of Washington University in St. Louis and conformed to NIH guidelines.

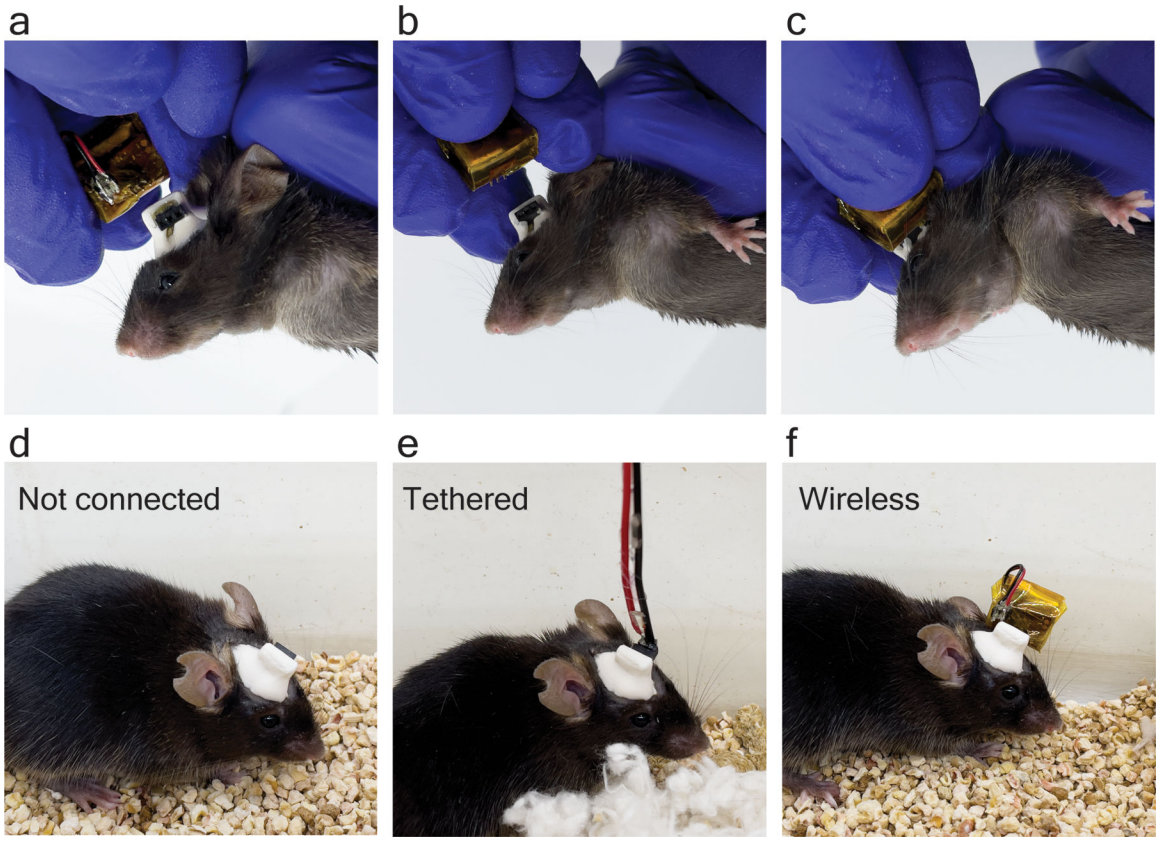

Fig. 7 |. *In vivo* behavioural study.

a, 3D-POP and BLE-module preparation for a mouse. **b,** Align the BLE-module allowing for pin and plug connectivity. **c,** Firmly squeeze the BLE-module and implant together to secure the connection. **d,** Mouse freely moving with no connection. **e,** Mouse with wired connection. **f,** Mouse with wireless BLE-module connection. All procedures were approved by the Animal Care and Use Committee of Washington University in St. Louis and conformed to NIH guidelines.

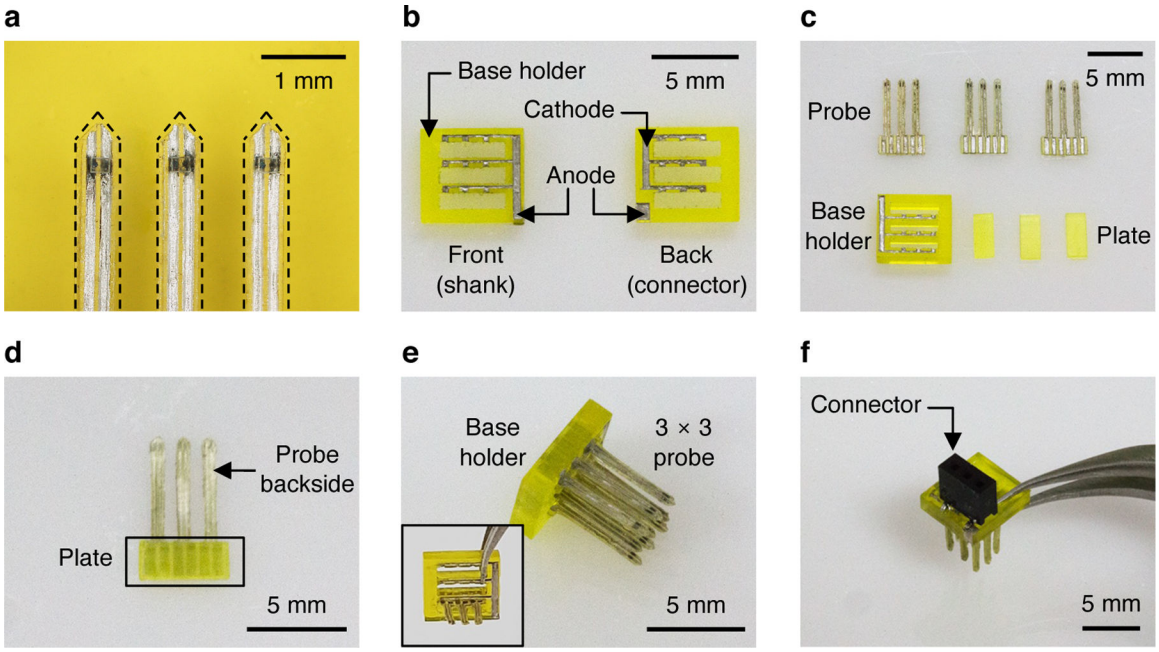

Fig. 8 |. Fabrication of 3D-POPs configured as an array (i.e., 3 × 3 probes).

a, Print substrates with 3 shanks in a row, i.e., 1 × 3 probe. Then, form electrodes using silver paste and mount μ-ILEDs. **b,** Print a base holder that can integrate the 1 × 3 probes and create electrodes on the groove patterns using silver paste. **c,** Prepare the main components (i.e., three 1 × 3 3D-POPs, three plates, and a base holder) to build the 3 × 3 probes before assembly. **d,** Stack each of 1 × 3 probes on the plate using adhesive film. **e,** Plug the plate-attached probes into the holder. The inset shows a photograph of the device after assembly of one 1 × 3 probe into the holder. **f,** Attach the 3-pin female electrical connector on the back side of the holder and apply epoxy to fix the connector and the probes.

Table 1 |

Troubleshooting table.

Step	Problem	Possible reason	Possible Solution
Procedure 1			
7	Printed substrates damaged during the release by a rubber blade	The rubber blade is dull or too thick to make clear-cut release of the printed substrate	Sharpen the edge of the rubber blade. A sharp blade can easily slip between the substrate and the build head
9, Box 1 (stepS 1, 2)	Unwanted connection between electrodes	Silver paste residues remain between the electrodes which form the electrical connection	Dry silver paste completely and clean the probe surface with a stainless steel blade until the residues are completely removed
11	Inoperative μ-ILEDs	Soldering between the μ-ILED and the electrodes is not secure	To prevent this problem, check if the amount of solder paste is adequate and two electrodes of a μ-ILED are precisely positioned on both electrodes of the 3D-POPs in Step 10. In case of trouble, remove the μ-ILED from the silver electrodes using a tweezer and re-solder it. If there was damage to the electrodes, fill in the silver paste again before proceeding.
12	Damage of the electrodes/ substrates while detaching the probe from Kapton tape	Kapton tape is too sticky	Reduce adhesion by scratching the surface of the Kapton tape several times with a stainless-steel blade
Procedure 2			
10	Failure in assembling the connectors	Excessive epoxy can fill the female connector, thereby blocking the connector holes	Apply minimal epoxy around the connector in Step 17 to prevent excess filling of epoxy into the connector holes
Procedure 3			
3, 4	Probe bends or shears upon insertion into the brain	If the dura is not completely removed or if the probe is not completely perpendicular to the site of insertion, the probe may flex during insertion.	Ensure the probe is perpendicular to the skull surface and the dura is completely removed before insertion
4	Probe shifts position during implantation	If the probe is not securely held in the adapter, the probe may move during implantation	Check before inserting probe that the probe is securely attached to the stereotaxic arm
	Widespread damage, bleeding, and inflammation around implantation site	Drill penetrates through the dura	Ensure magnification levels of the surgical scope during drilling are sufficient to visually assess whether the dura is intact after drilling. Excessive bleeding and visible brain tissue damage are signs that the drill has penetrated beyond the dura and has damaged the brain.
9	Metabond enters the drill hole, directly contacting brain tissue	The initial later of Metabond is a very thin viscosity or the researcher applies Metabond too close to drill hole	Apply Kwik-Sil to seal the hole at the implantation site prior to applying Metabond.
11, 18	Damage to connector or 3D-printed probe holder	Cagemates chew the connector	Ensure that the entire implant and connector are covered with Metabond leaving only plug openings or choose to house mice separately
13	Large portions of the skull are visible around the cement cap	The incision is too large for the cap size	Apply a suture to the posterior end of the incision to ensure proper healing
20	BLE module is disconnected prior to or during behavioural assay	Plug connection is too loose allowing the animal to remove with normal grooming or locomotion	Gently bend the prongs of the connector to create a tighter fit with the plug