Chapter 15

Intracranial Injection of an Optogenetics Viral Vector Followed by Optical Cannula Implantation for Neural Stimulation in Rat Brain Cortex

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Abstract

Optogenetics is rapidly gaining acceptance as a preferred method to study specific neuronal cell types using light. Optogenetic neuromodulation requires the introduction of a cell-specific viral vector encoding for a light activating ion channel or ion pump and the utilization of a system to deliver light stimulation to brain. Here, we describe a two-part methodology starting with a procedure to inject an optogenetic AAV virus into rat cortex followed by a second procedure to surgically implant an optical cannula for light delivery to the deeper cortical layers.

Key words Optogenetics, Neuromodulation, Optical cannula, Rat brain, Intracranial injection, Neural stimulation

1 Introduction

Development of brain stimulation techniques in which penetrating electrodes deliver current pulses to stimulate neural circuitries in deep brain objects introduced a new era in treating neurological diseases. Previously, the main approach in treating mental disorders has been the chemical imbalance paradigm in which we hypothesize that a mental disorder is the result of an imbalance in the concentration of chemicals, such as neurotransmitters, within the central or peripheral nervous system. Such diseases can be treated by controlling the concentration of the appropriate chemicals through pharmacologic manipulation. In contrast, interventional psychiatry is based on the idea that brain disease can be treated by directly modulating neuronal activity. One example of this new approach is the implantation of electrodes for deep brain stimulation (DBS) used to treat Parkinson's disease. Although DBS can be quite successful, electrode-based stimulation has at least three inherent deficiencies. One is the lack of practical strategies to

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target specific cell-types of interest. Typically, different cell-types are intermixed within most brain regions with each cell-type being involved in different microcircuits or data processing pathways. Injection of electrical current generally stimulates all cells in the vicinity of the electrode with little or no specificity thus potentially causing unintended side effects. Another limitation is that electrical stimulation typically causes an increase in neuronal firing while suppressive effects are minimal or difficult to control precisely. Moreover, due to these limitations the development of high density, arrays that can provide complex yet focally specific patterned stimulation/recording is problematic. Electrode arrays that are made for neuroprosthetic applications usually contain no more than few hundred electrodes at best and even for arrays with limited number of electrodes the data acquisition process is quite cumbersome.

Optogenetics is a new neuro-stimulation modality with the potential to remarkably improve upon previous technologies [1–3]. With optogenetics, specific cell-types of interest are genetically targeted to express light-sensitive ion channels or ion pumps [4, 5]. Once these proteins are produced within a cell, its activity can be increased or suppressed by exposing the cell to light of appropriate wavelengths. Cell-specific genetic targeting is achieved by controlling the gene delivery mechanism through choice of appropriate promoters and/or viral receptors.

Only the targeted cells are transfected efficiently and respond to light pulses. During the last few years, the list of light-sensitive proteins that are used for optogenetic stimulation has significantly expanded. Currently, there are proteins that function as cation channels to stimulate excitable cells or as anion pumps to hyperpolarize cells thus causing neural inhibition once exposed to light. By co-expressing different optogenetic proteins, it is possible to reversibly excite or inhibit cellular activity by simply changing the wavelength of the stimulation light [5]. Optogenetic stimulation also benefits from the inherent parallelism of optics. Using state-of-the-art technology, it is possible to generate complex patterns of stimulation with high spatial and temporal resolution making it feasible to manipulate the dynamics of extended cortical networks. As a result, optogenetics provides new opportunities for neuroscience research and has revolutionized the development of optoelectronic brain interface technologies [6].

Optogenetic tools are proteins that function as light-sensitive ion channels or ion pumps. These proteins, which are mostly categorized as microbial rhodopsin molecules, were originally isolated from microorganisms but are being used to manipulate the activity of mammalian cells such as neurons or muscle cells [5]. The most widely used member of this family is the channelrhodopsin (ChR) molecule which was isolated from the fresh water algae

Chlamydomonas reinhardtii and functions as a cation channel that opens when the protein is exposed to blue light with maximum spectral sensitivity around 445 nm [3]. When the pore of this channel opens, it allows the passage of positive ions, including Na^+ and Ca^{2+}, across the membrane along the direction of the diffusion force induced by electrochemical gradients. The influx of these cations depolarizes the cell and increases cellular activity. Another member of this family is halorhodopsin (HR) protein which is isolated from *Archaebacterial* and functions as an anion pump. When exposed to yellow light, wavelengths around 590 nm, it pumps negative ions, mostly Cl^-, into the cell which causes hyperpolarization and reduces activity [4, 5].

Light sensitivity in almost all rhodopsin molecules is achieved by isomerization of a covalently bound retinal cofactor. The electron–photon interaction in this molecule causes a conformational change from all-*trans* to 13-*cis* which reconfigures the structure of the protein so that ions transport across the membrane (*see* Fig. 1). Since the required exogenous cofactor all-trans-retinal (ATR) already exists in mammalian cells, both halorhodopsin and channel rhodopsin can function in these species without the addition of ATR; a positive aspect that facilitates the design of optogenetic experiments and simplifies the process for potential translation of optogenetics to humans in future clinical use.

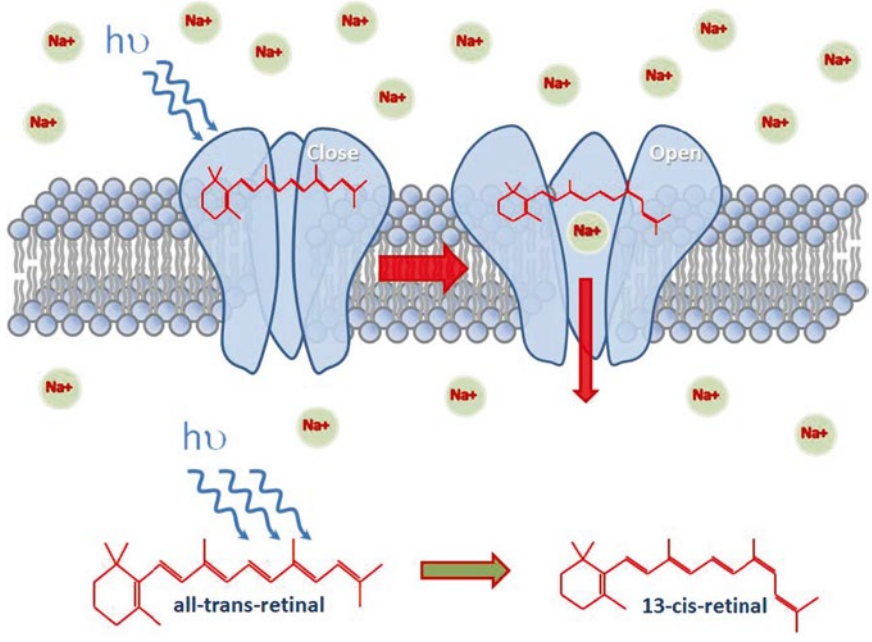

Fig. 1 Light sensitivity mechanism of ChR2: When the protein is exposed to blue light, the electron–photon interaction in this molecule causes a conformational change from all-*trans* to 13-*cis* which reconfigures the structure of the protein and opens the pore of the channel

Different strategies have been used to develop a wide range of opsins for optogenetic stimulation. In one approach, known as bioprospecting, researchers investigated a variety of different species to discover other opsin molecules with different optical or kinetic properties [7]. For example, the protein VChR1 was isolated from *Volvox carteri* and was first discovered by searching the genome database from the US Department of Energy Joint Genome Institute [3]. The spectral response of this protein is approximately 50 nm red-shifted compared with ChR2 allowing it to stimulate cells deeper within the tissue. However, VChR1 photo currents are usually less than half of the photo current passing through the stimulated ChR2 channels. Another approach to optimizing optogenetic proteins is genetic engineering. For instance, initial tests with inhibitory microbial rhodopsins, such as NpHR, were derived from prokaryotes and were not sufficiently compatible with mammalian cells. Higher level expression of these ion pumps was required to achieve augmented inhibitory function but caused accumulation of the protein to toxic levels within the cell [8]. To address this problem, signaling peptides from endogenous ion channels were added to each end of the microbial rhodopsin, which optimized the efficiency of the membrane targeting of the NpHR molecule and improved the amplitude of the induced photocurrent.

Over the last decade, significant effort was focused on developing new optogenetic tools or optimizing protein kinetics, sensitivity, or spectral response. For instance, light-sensitive cation channels with distinct spectral sensitivities are required to target multiple cell populations intermingled within the same brain area. To reach deeper inside the brain and to minimize photodamage or phototoxicity, the spectral response of the proteins must be shifted toward red wavelengths. To make optogenetic stimulation more light efficient the kinetics of optogenetic proteins can be slowed down so that once the channel opens, it remains open for a longer period. This allows more cations to pass through each channel and reduces the number of open channels required to generate a requisite level of depolarization. This, in turn, reduces the number of effective electron-photon interactions required to generate an action potential. However, this increase in light efficiency then comes at the price of a considerable decrease in temporal resolution. If the generation of high frequency action potential bursts is required, a less efficient protein with fast kinetics may be the better choice. Ultimately, optogenetic protein structures can be optimized for a variety of different applications. It was also shown that green light exposure facilitates closing the channel and, as a result, these opsins can operate as bi-stable actuators with blue light triggering the ON state and green light triggering the OFF-state (closed channel) [1, 3]. Moreover, some point mutations can alter the selectivity of the ion channel increasing the conductance of cations such as Ca^{2+} for example. These proteins can then be used to control the intracellular concentration of specific ions.

Detailed information regarding the structure of ChR2 has been revealed recently through a sequence of protein crystallography experiments [9]. This information will help protein engineers to tailor their approaches to precisely design new variants of ChR2 with diverse spectral response, optical sensitivity, biological compatibility, ion selectivity, or kinetic properties [7].

An essential first step in optogenetic stimulation is the delivery of a new gene into a target cell [3, 5]. For future human therapeutic applications this is a potentially controversial issue that could limit adoption [10]. Based on the specific application, different techniques are adapted to deliver the gene to the cell populations of interest. In cultured cells, genes can be delivered by nonviral delivery methods such as calcium phosphate transfection or electroporation. However, viral gene delivery is still the most popular method for in-vivo application and for delivery of genetic constructs smaller than a few kb [11]. Viral gene delivery is often easy, robust, has high infectivity compared to other methods and, so far, has shown no significant iatrogenic effect. By controlling the process of viral injection, the spatial distribution of the expressed protein can be limited almost to one or a few brain sites. Cell-type-specific targeting can be obtained by using appropriate promoters and engineering of the genetic constructs. In recent years, lenti- and adeno-associated viral (AAV) vectors have been exploited to target both neurons and astroglia in rodents and nonhuman primates. The AAV vectors have become more popular for optogenetic gene delivery since they are less immunogenic and offer better transduction efficiency for targeting larger tissue volumes. For applications where more uniform distribution of opsins is required, as for manipulation of large cortical networks, transgenic animals are usually a better choice. Using transgenic animals also simplifies the experimental procedures and reduces the cost and effort needed for each experiment. Different opsin expressing optogenetic animals are produced among which the most famous example is the transgenic mice generated under Thy1 promoter [11]. This promoter is used to target projection neurons in layer V of neocortex.

In this methods manuscript, we will describe a two-part methodology starting with a procedure to inject an optogenetic AAV virus into rat cortex followed by a second procedure to surgically implant an optical cannula for light delivery to the deeper cortical layers.

2 Materials

2.1 Viral Vector

1. Vector: AAV [Serotype: 2 ChR2: AAV-hSyn-hChR2(H134R)-EYFP] was purchased from the University of North Carolina Vector Core (UNC Vector Core, Chapel Hill, North Carolina, USA) (http://www.med.unc.edu/genetherapy/vectorcore). We thaw the 100 μl frozen vector sample upon arrival at our facility and pipette the material into smaller 10 μl aliquots. The small aliquots are then stored in a −80 °C freezer for later use.

2.2 Stereotaxic and Injecting Equipment

1. Stereotaxic: Lab Standard Rat Stereotaxic Instrument with non-rupture ear-bars. We utilize this instrument to make precise distance measurements on the rat skull for injection site location. This device is equipped with a mask to deliver gas anesthesia to the subject rat.
2. Anesthesia Setup: Our laboratory is equipped with a gas anesthesia delivery system that includes a gas-mixing flow-meter, isoflurane Vaporizer, and hospital grade vacuum system to remove excess anesthetic. We use a heated water circulation blanket with a rectal temperature feedback system to maintain constant temperature in the subject rat during surgical procedures.
3. Injection System: We use Quintessential Stereotaxic Injector (QSI) to achieve accurate control of the volume and flow rate of injections.
4. Syringes: Two glass-made Nanofil syringes are needed, one for viral injections into experimental animals and another for vehicle injections into sham animals to prevent cross contamination.
5. Needles: Nanofil 35 gauge blunt needle or 33 gauge beveled needle is required for these experiments.

2.3 Surgical Tools and Micro Drills

1. Surgical Tools: The following tools are needed: scalpel handle, scalpel blades, two ultra wide curved hemostats, Halsey needle holder, small bone curette 0.5 mm, Dumont forceps, two micro spatulas, and a hot bead sterilizer.
2. Electrocautery: Veterinary Electrosurgical Unit.
3. Micro Drills: 0.3 mm carbide twist drill, and Preclinical Drill.
4. Suture: Standard sterile chromic gut suture for wound closure.
5. Drugs: Cefazolin (1 g per vial), Carprofen (50 mg/mL), Lidocaine Hydrochloride (2 % Jelly).
6. General Surgical Supplies: iodine scrub, rubbing alcohol, sterile gauze, sterile needles, sterile plastic syringes, and sterile cotton swabs.

2.4 Skull Fixation Supplies

1. Dental Cement: Orthojet BCA liquid.
2. Glue: Loctite 454.
3. Skull Screws: Nylon Mounting Screws $080 \times 3/32$ Diameter ($0–80 \times 3–32$ N).

2.5 Optical Cannula

1. Optical Cannula: Fiber Optic Cannula, Ø1.25 mm Ceramic Ferrule, Ø200 μm Core, 0.39 NA, length = 2 mm.

3 Methods

All procedures approved by Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) and adhere to the "The Guide for the Care and Use of Laboratory Animals." This protocol consists of two surgical procedures separated by 2–3 weeks.

3.1 Intracranial Injection

1. Safety precautions: we perform our surgeries in a Biosafety Level II facility. We use surgical caps, masks, eye protection, and gloves while working with the virus, and load the syringe with the virus inside of a safety cabinet. We use sterile surgical technique for all survival procedures involving animals.
2. Anesthetize the rat in an induction chamber with 4 % isoflurane. Secure the rat in a stereotaxic apparatus with a nose cone attachment and reduce isoflurane to 1.5–3 % as needed for maintenance of the anesthetic plane (Fig. 2).
3. Prepare the rat by shaving the fur off of the scalp and washing with iodine followed by rubbing alcohol. Repeat the washing process twice more.
4. Give preoperative injections of cefazolin (30 mg/kg) antibiotic subcutaneously and carprofen (5 mg/kg) analgesic intramuscularly, and apply lidocaine jelly subcutaneously in the scalp at the injection site. Use a piece of gauze to massage in the lidocaine.
5. Make a small (approximately 2 cm) midline incision in the rat scalp to expose the dorsal surface of the calvaria; be sure to

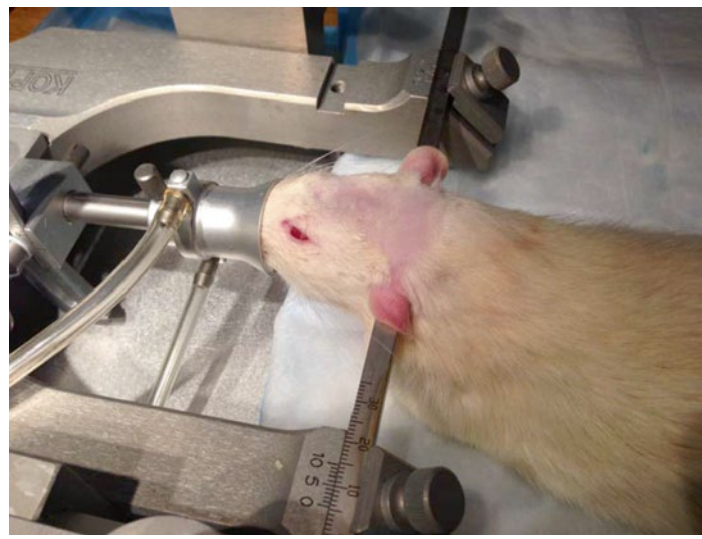

Fig. 2 Anesthetized rat secured in stereotaxic holder with shaved scalp

include bregma and lambda in this area to ensure the accuracy of other coordinate measurements.

6. Bluntly dissect the tissue to expose the bone. Clean the skull with hydrogen peroxide to highlight the cranial sutures. Use electrocautery as needed to achieve hemostasis and eliminate any bleeding from scalp tissue vessels.
7. Using a marker attached to a stereotactic arm, find the dorsoventral coordinates of bregma and lambda. Adjust the bite bar position until these readings are equal. This ensures the accuracy of further stereotactic depth measurements. Mark bregma and record its coordinates (Fig. 3a, b).
8. Calculate the coordinates of the region of interest and the location of the injection sites. Mark these using the stereotactic system with the marker, and afterward remove the marker and stereotactic arm (Fig. 3c, d) (*see* **Note 1**).
9. Drill a small hole through the calvaria at each injection site using a 0.3 mm micro drill bit by hand using a light touch (Fig. 4a)

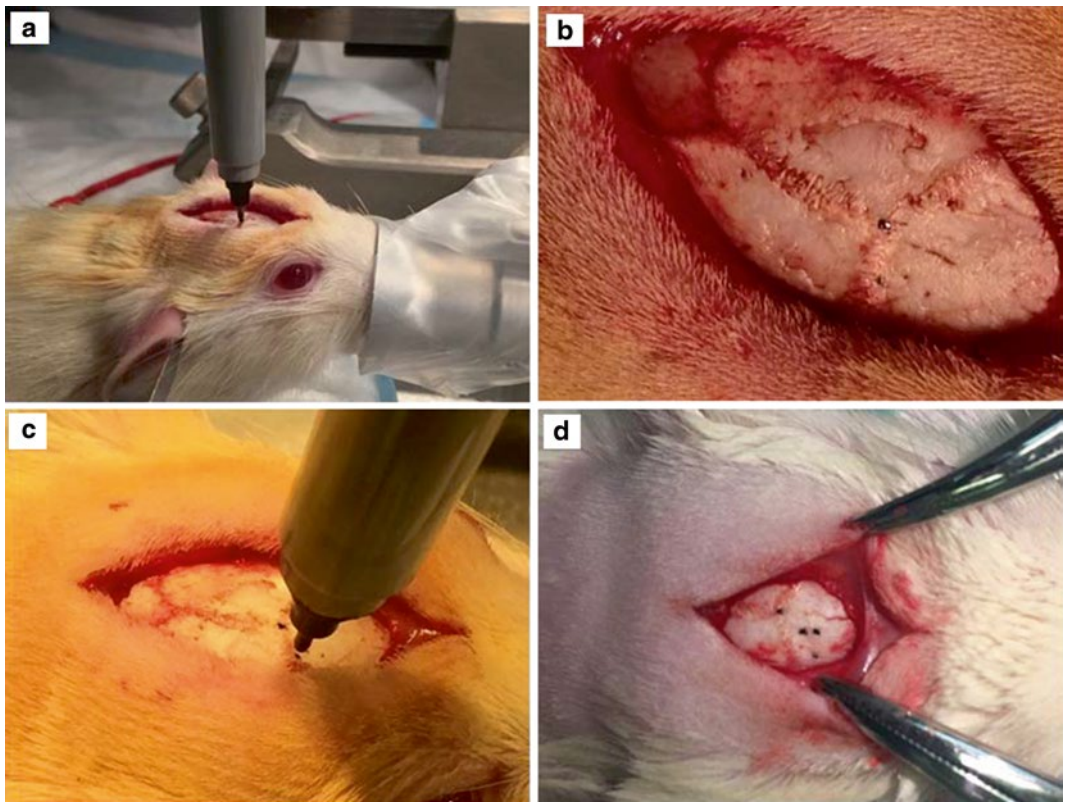

Fig. 3 (**a**) Use a surgical marker to highlight bregma. (**b**) Cleaned skull surface indicating bregma. (**c**) Use a surgical marker to indicate injection sites. (**d**) Skull surface prior to virus injection featuring marks at bregma and three injection sites

(*see* **Note 2**). Nick the dura with the tip of the drill bit if you are using a blunt needle for virus injection to ensure that the needle will penetrate into the brain tissue.

10. Fill the needle with the desired amount of virus, drawing up 0.5–1 μl extra virus. Affix to the stereotactic arm of the injection system. Set the pump to inject at 0.01 μl/min until you acquire your chosen volume. Advance the pump manually (on the touch screen) until a small drop of virus can be seen at the tip of the needle.
11. Advance the needle on the stereotaxic holder until it is at the surface of the skull over the first injection site (Fig. 4b). Record the dorsoventral coordinate. Calculate the coordinate of your desired injection depth. Slowly advance the needle to this depth (*see* **Note 3**). Wait 5 min for the brain to equilibrate to the presence of the needle (*see* **Note 4**).
12. Begin the injection of the virus. When the pump has finished injecting, wait 5 more minutes to allow the brain to equilibrate to the added volume of the injection before slowly withdrawing the needle. At this point you may adjust the setup or touch the rat if needed (*see* **Note 3**).
13. Repeat **steps 9** and **10** for each injection site.

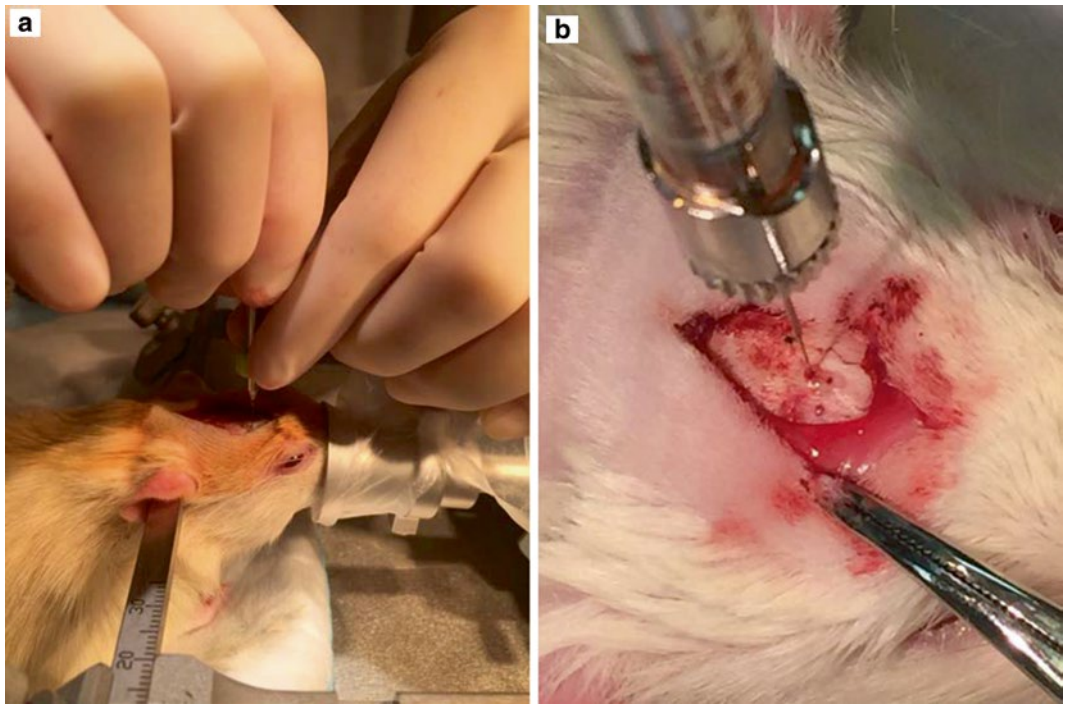

Fig. 4 (**a**) Drill a small hole through the skull by hand for viral injection using a micro drill bit. (**b**) Viral injection process

14. When the injections are completed and the needle withdrawn, suture the skin closed. It is not necessary to cover the holes in the skull before closing the skin.
15. Provide standard post-surgical care including secondary doses of cefazolin (30 mg/kg) and carprofen (5 mg/kg) subcutaneously.

3.2 Optical Cannula Implantation

1. Two to three weeks after the first protocol prepare the rat as for the injection procedure, **steps 1–3**.
2. Make a midline incision about 3.5 cm long in the rat scalp. Expose the calvaria, bluntly dissecting away the temporalis muscle to expose the sides of the skull as well.
3. Clean the skull with hydrogen peroxide and dry, using electrocautery to stop any persistent bleeding.
4. Using a marker attached to a stereotaxic arm, mark bregma and record its coordinates (Fig. 3a, b). Realign the dorsoventral coordinates of bregma and lambda as in the injection procedure, **step 5**.
5. Calculate and mark the location for the cannula placement (*see* **Note 5**).
6. Drill a small hole for the cannula by hand using the 0.3 mm micro drill bit (Fig. 5a). Puncture the dura with the tip of the drill bit or with a sterile hypodermic needle to ensure adequate penetration of the optical fiber.
7. Affix the cannula to a stereotactic arm and position above the implantation site (Fig. 5b) (*see* **Note 6**).
8. Lower the cannula into the implantation site, leaving approximately a 0.5 mm gap between the base of the cannula and the surface of the skull (Fig. 5c). Apply a dot of Loctite glue to this area and then lower the cannula until it touches the skull. Allow the glue to dry (Fig. 5d) (*see* **Note 7**).
9. Expose the side of the calvaria. Clean with peroxide and dry; cauterize if necessary to attain hemostasis (Fig. 6a).
10. Drill a hole in the side of the calvaria by hand with the smaller drill bit (Fig. 6b).
11. Enlarge the outer edge of the hole by hand with the larger drill bit. Use the small bone curette to smooth the edges of the hole (Fig. 6b).
12. Trim the nylon screw to 0.5–1 mm in length.
13. Carefully position the nylon screw and screw it fully into the hole. Dry around the screw with a piece of gauze or a cotton swab.
14. Apply a dot of Loctite glue to the skull and the screw. Allow glue to dry (*see* **Note 7**).

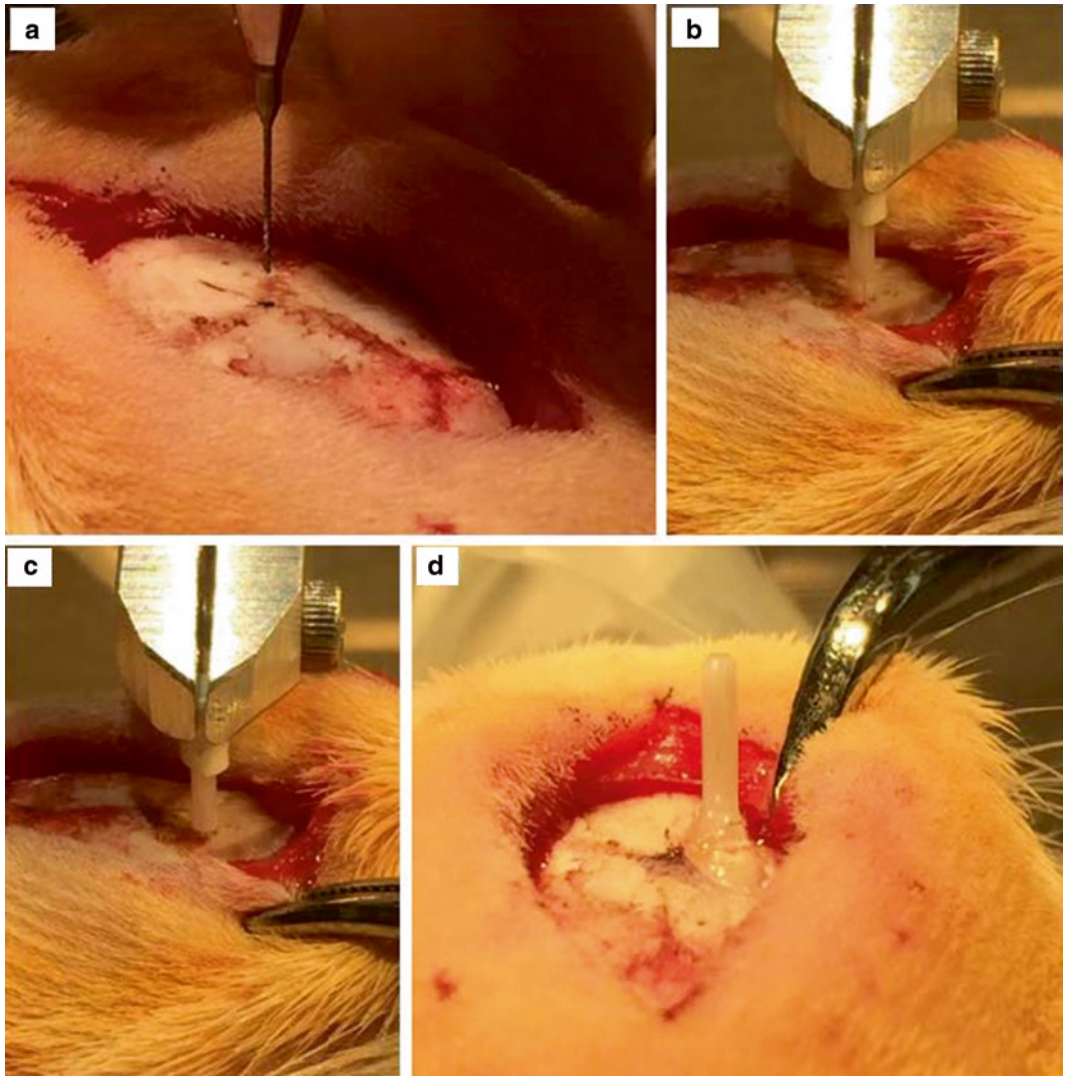

Fig. 5 (**a**) Drill hole for cannula by hand. (**b**) Align the fiber optic cannula with the hole. (**c**) Advance the implant to 0.5–1 mm above surface of skull. (**d**) Completed cannula dotted with superglue and fully advanced

15. Mix dental cement to a thick consistency and apply around screw using the micro spatulas, covering entirely. Allow to harden. Mix and apply more cement as needed to ensure an even coating (*see* **Note 8**). Also place a trail of cement over the top of the calvaria to the cannula (Fig. 6c).
16. Repeat **steps 9–15** on the other side of the skull.
17. Mix and apply more dental cement to the top of the skull, making a small mound around the cannula. Leave enough space to fit the connector and the fiber optic cable to the laser (*see* **Note 9**). Allow dental cement to harden (Fig. 6d).
18. Suture the skin closed, leaving space for the cannula to poke through (*see* **Note 10**).

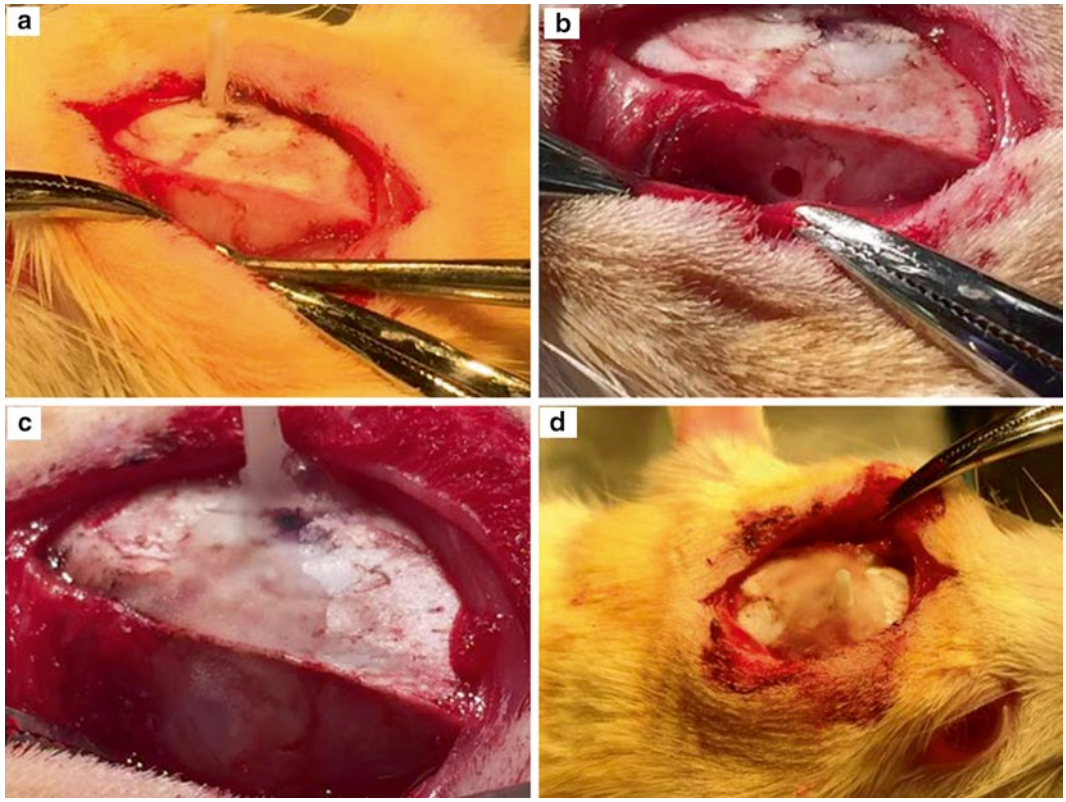

Fig. 6 (**a**) Expose the calvaria. (**b**) Hole in calvaria ready for screw insertion. (**c**) Spread dental cement over screw in calvaria and implant site. (**d**) Final cemented cannula ready for closure

4 Notes

1. All published rat atlases contain brain coordinates from bregma and other skull markers that are specific for a particular strain of rat within a specified size, age, and weight range (for example *see*: [12]). Please refer to the atlas for listed conversion factors when utilizing any other rat strains or ages/weights outside the specified strain used to construct the atlas.
2. Drilling the hole by hand decreases the risk of damaging the brain at the injection site.
3. You can vary the injection process in many ways. Such as injecting small amounts of virus across different cortical layers. The best approach to this method is to start the injections at the desired bottom cortical layer first and then continuing with additional micro injections as you advance the needle towards the brain surface.
4. It is important not to jar the operating surface or the stereotactic setup for the entire time that the needle is in the brain. We recommend not touching the setup or the operating surface

through the entire injection process, except in case of emergency.

5. It may be of benefit not to place the cannula directly over one of the injection sites. This is the case for many imaging applications where the cannula may interfere with imaging the injection sites and the cannula is only needed for light delivery.
6. The optical implant manufacturer includes a small sheath to protect the cannula in the shipping process. We affix this sheath to the stereotactic arm and fit the cannula into the sheath. This holds the cannula firmly enough to be stable for the implantation, but loosely enough that we can easily remove the arm once the cannula is secured in place.
7. A drop of the liquid component of Ortho-Jet BCA dental cement applied directly to the Loctite glue will act as an accelerant, causing it to harden immediately. This greatly reduces the surgical time.
8. Thicker dental cement dries much faster than when mixed to a thinner consistency, so mix it in small batches to reduce waste.
9. In our experience, the connector can be placed mostly onto the fiber optic cable, needing only a millimeter space free on the cannula.
10. If the cannula is more than about 2 mm lateral, you may need to make a small lateral incision to avoid stress on the cannula from the skin tension.
11. Several formulations are developed to model the distribution and penetration depth of light within the brain tissue [13, 14]. These models are used to find a reasonable estimation for the amount of light required to efficiently stimulate target cells within the region of interest. One simple yet practical formulation for optogenetic applications is developed based on the Kubelka-Munk model in which the brain tissue is considered as a highly scattering homogeneous turbid medium in which the absorption is negligible. Based on this model, the relative intensity of 473 nm laser at depth z (mm) along the optical axis of the fiber versus the intensity at the surface of an optical fiber of diameter $\varnothing$ (mm) and numerical aperture NA is given by [13]:

$$\frac{I(z)}{I(z=0)} \cong \frac{\rho^2}{(11z+1)(z+\rho)^2}$$

$$\rho = \frac{\varnothing}{2}\sqrt{\left(\frac{1.36}{\mathrm{NA}}\right)^2 - 1}$$

The $I(z)/I(z=0)$ ratio for a fiber with $\varnothing = 0.2$ mm and NA = 0.22 is shown in Fig. 7. Based on this curve, if we inject 10 mW of optical power into the tissue through this fiber, in

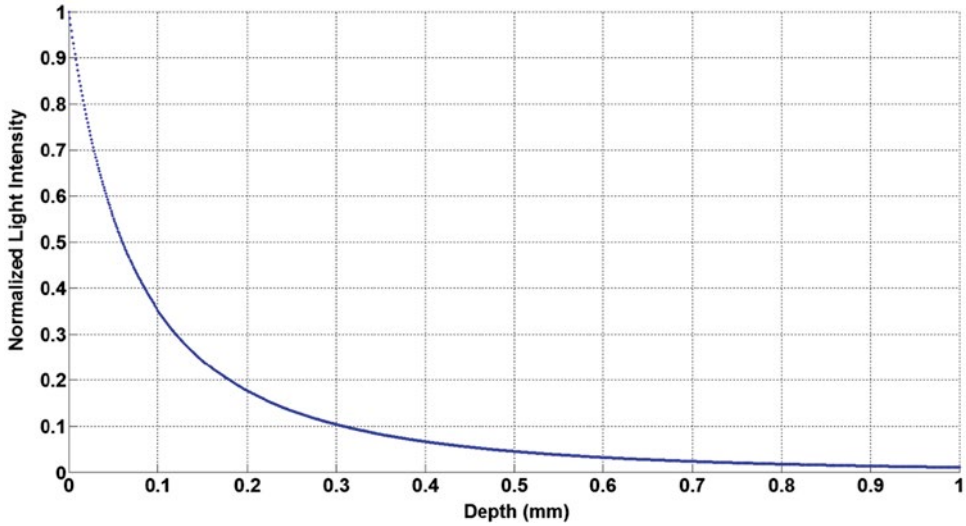

Fig. 7 Normalized intensity of light at different depths along the optical axis of an optical fiber of 0.2 mm diameter and 0.22 numerical aperture

about 0.3 mm distance from the fiber tip, the intensity of light drops to about 1 mW, which is almost the threshold level for the ChR2 protein to be efficiently activated. In case we use the ChR2(H134R) variation of the protein which is about two times more sensitive to light, the penetration depth for effective optogenetic stimulation increases to 0.5 mm or slightly more.

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