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Craniotomy Procedure for Visualizing Neuronal Activities in Hippocampus of Behaving Mice

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TITLE:

Craniotomy Procedure for Visualizing Neuronal Activities in Hippocampus of Behaving Mice

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KEYWORDS:

Neuroscience, cranial window, in vivo imaging, hippocampus, neuronal activities, calcium sensor.

SUMMARY:

This article demonstrates the preparation of a custom-made imaging window supplemented with infusion cannula and its implantation onto the CA1 region of the hippocampus in mice.

ABSTRACT:

Imaging neuronal activities at single-cell resolution in awake behaving animals is a very powerful approach for the investigation of neural circuit function in systems neuroscience. However, high absorbance and scattering of light in mammalian tissue limit intravital imaging mostly to superficial brain regions, leaving deep-brain areas, such as the hippocampus, out of reach for optical microscopy. In this video, we show the preparation and implantation of the custom-made imaging window to enable chronic *in vivo* imaging of the dorsal hippocampal CA1 region in head-fixed behaving mice. The custom-made window is supplemented with an infusion cannula that allows targeted delivery of viral vectors and drugs to the imaging area. By combining this preparation with wide-field imaging, we performed a long-term recording of neuronal activity using a fluorescent calcium indicator from large subsets of neurons in behaving mice over several weeks. We also demonstrated the applicability of this preparation for voltage imaging with single-spike resolution. High-performance genetically encoded indicators of neuronal activity and scientific CMOS cameras allowed the recurrent visualization of subcellular morphological details

of single neurons at high temporal resolution. We also discuss the advantages and potential limitations of the described method and its compatibility with other imaging techniques.

INTRODUCTION:

The hippocampus is a key brain region responsible for learning and memory[1] as well as for spatial navigation[2]. Hippocampal atrophy is associated with neurological and psychiatric disorders involving memory loss and cognitive decline[3-5]. In mice, the hippocampus is a very well-established model to study spatial, contextual, and associative learning and memory formation on the cellular and network levels[4,5]. The mechanistic studies of learning and memory require longitudinal interrogation of neuronal structure and function in behaving mice. Fluorescence imaging in combination with genetically encoded probes[6] provides unprecedented capabilities to record membrane voltage dynamics[7,8], calcium transients[9], and structural changes[10] over large subsets of neurons intravitally. However, optical access to the hippocampus in mice is obstructed by the cortex, which can reach over 1 mm in thickness. Here, we described a procedure for assembling a custom-made imaging device and its chronic implantation into the mouse head for long-term optical access to the CA1 subregion of the dorsal hippocampus in behaving mice. Infusion cannula integrated into the imaging implant allows administration of viruses or drugs directly onto the neurons in the field of view. The described preparation in combination with wide-field microscopy enables recurrent imaging of the large subsets of neurons in behaving mice over long periods of time. We utilized this preparation to express calcium and voltage genetically encoded indicators in hippocampal CA1 region via targeted injection of recombinant adeno-associated virus (rAAV) for neuronal activity recordings at single-cell resolution. We also performed longitudinal calcium imaging of the corresponding neuronal subsets at high spatiotemporal resolution in behaving animals. In addition, this preparation is compatible with multiphoton microscopy and microendoscopy, thus further expanding the toolbox of imaging techniques to study neuronal networks at cellular and subcellular levels in behaving mice. We described critical steps and troubleshooting of the protocol. We also discussed the possible pitfalls and limitations of the method.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Westlake University.

1. Implant assembling

NOTE: Assembling of the imaging implant is technically simple and requires only commercially available items (**Figure 1**, see also **Table of Materials**). The head plates can be manufactured at the local machine shop using stainless-steel or titanium plates. We suggest preparing a stock of fully assembled implants before starting the surgeries. After *in vivo* experiments are done, the implants can be recovered and reused multiple times. In some cases, it may only require reattaching the infusion cannula by soldering or replacing the cover glass.

1.1. Prepare all six key hardware components for assembling and installation imaging implants (**Figure 1**).

[Place Figure 1 here]

1.2. Turn on the welding machine and heat it up to the required temperature.

NOTE: The temperature depends on the welding tin used.

1.3. Polish the side surface of the imaging cannula using fine sandpaper to remove the oxidation layer and thus facilitate stronger soldering.

1.4. Adjust the position of the imaging cannula and injection cannula (guide cannula with inserted dummy cannula) using helping hands (**Figure 2A,B**).

1.5. Using a syringe with a needle, apply a small amount of appropriate type of flux onto the connection spot between imaging and injecting cannulas for 5 seconds, and then remove the droplet.

NOTE: For this preparation, we used a commercially available flux that is specified by the manufacturer to be for soldering stainless steel parts as imaging and infusion cannulas are made of stainless steel. In the case of other materials used to manufacture cannulas, end-user should select flux that can weld the selected material.

1.6. Melt soldering tin and apply it to the connection spot treated with flux (**Figure 2**).

NOTE: Avoid excess soldering tin, as it will require unnecessary larger craniotomy during the surgery.

[Place Figure 2 here]

1.7. Wait for the soldering tin to cool down. Usually, it takes several seconds.

1.8. Confirm that the injection cannula is not blocked by inserting the dummy cannula from both directions.

1.9. Apply UV-curing optical adhesive on the bottom side of the imaging cannula using a toothpick or 26 G syringe needle.

1.10. Use a fine tweezer to carefully place a cover glass of the corresponding size to the imaging cannula.

NOTE: The positioning of the glass must be done precisely onto the imaging cannula without moving the glass too much once it has touched the optical adhesive. Otherwise, the glass gets

dirty, thus reducing the quality of imaging.

1.11. Cure the adhesive for a least an hour by 350-400 nm UV illumination from a standard handheld UV lamp.

NOTE: The used adhesive must be optically transparent. Otherwise, it will decrease the quality of the imaging window.

CAUTION: Avoid skin and eye exposure by wearing UV-protecting glasses, gloves, and a lab coat.

1.12. Wash the cannula in 70% ethanol, air dry, and store in a sterile container until surgery.

NOTE: It is very important to keep the cover glass as clean and intact as possible. The used optical adhesive is chemically stable in 70% ethanol.

2. Window implantation

2.1. Preparation steps before the surgery

2.1.1. Sterilize all the surgical instruments in an autoclave.

2.1.2. Prepare 1x PBS and 70% ethanol in two separate Petri dishes.

2.1.3. Optionally: Disinfect the surgery area using UV light for at least 20 minutes before starting the surgical procedure.

NOTE: Operating under the most sterile conditions possible will result in successful and long-lasting (as long as 6 months) glass-covered cranial windows. Contamination may result in reduced window transparency or severe inflammation in most cases.

2.2. Surgical procedure

2.2.1. Sterilize the surgical area with 70% ethanol right before the surgery.

2.2.2. Weigh the animal and administer a pre-surgical dose of analgesic subcutaneously according to the IACUC approved animal protocol.

2.2.3. Anesthetize a mouse with isoflurane (4% for induction, 1.5-2% for maintaining, 0.3-0.5 L/min flow rate of air). Use a tail-pinch and toe-pinch technique to ensure the animal is fully sedated. Observe vital signs of the animal, such as respiration, SpO_2, and heart rate for the duration of the procedure.

2.2.4. Use a trimmer or depilatory cream to remove the fur from the back of the neck up to the eyes.

2.2.5. Place mouse in a stereotaxic frame over a surgery heating pad (maintaining 37 °C). Secure the head with ear bars. Slightly push the head in all directions to make sure the head is secured firmly.

2.2.6. Apply eye ointment to prevent the animal's eyes from drying out during the surgery.

2.2.7. Sterilize the surgical site with betadine followed by 70% ethanol three times before making an incision.

2.2.8. Remove the skin over the top of the skull, starting with a horizontal cut all along the base of the head, followed by two cuts in the rostral direction, almost reaching the eyelids, then two oblique cuts that converge at the midline.

2.2.9. With two sterile cotton swabs, retract the connecting tissue, as well as the musculature of the back of the neck, to the edges of the skull.

NOTE: Try to avoid damaging blood vessels (especially ones hidden in the muscle) during manipulation.

2.2.10. Apply a drop of lidocaine solution (~0.1 mL) to the surface of the periosteum for 2 minutes to avoid excessive pain. Optionally to reduce the brain from swelling after removing the skull, 0.1 mL of 1% dexamethasone can be injected subcutaneously.

2.2.11. Gently scrape the entire exposed area of the skull with a scalpel to create a dry and rough surface that allows glue and the dental cement to adhere better and thus resulting in chronic implantation.

2.2.12. Place the tip of the needle mounted on the stereotaxic station onto the bregma, set all three coordinates (AP: Anterior-Posterior; ML: Medial-Lateral; DV: Dorsal-Ventral) as 0.

2.2.13. Place tip of the needle onto the lambda and see if the AP coordinate is 0 to confirm that the head position is vertical, as well as if the ML coordinate is 0 to confirm the head is positioned horizontally. If not, adjust corresponding knobs on the stereotaxic station, until the AP and ML coordinates are both within 0.1 mm.

2.2.14. Move the tip of the needle to find the corresponding points for craniotomy and mark their positions on the skull using a fine marker. In the case of implantation for hippocampus, there are 4 points with the following coordinates (AP: -0.68, ML: -2.0) (AP: -3.68, ML: -2.0) (AP: -2.18, ML: -0.5) and (AP: -2.18, ML: -3.5)[11], as well as (AP: -4.0, ML: -2.0) for the most caudal point of the injection cannula.

NOTE: The marker used in this step must be sterilized using UV illumination for at least an hour before the surgery. The coordinates shown here are for 6-8-week old C57BL/6J mice. The

coordinates may differ due to different ages or strains of mice.

2.2.15. Draw a circle based on four marked points, as well as the outline of the injection cannula area on the caudal side of the circle (**Figure 3**).

[Place Figure 3 here]

2.2.16. Use a pneumatic drill at the speed of 10,000 rpm to gently draw" along the outline marked on the skull.

2.2.17. Drill the skull until a very thin layer of bone is left, which usually starts to wiggle under gentle touch in the center.

2.2.18. Apply a drop of sterile 1x PBS to the center of the craniotomy, lift the bone flap from the skull with very thin tip forceps or two 26 G needles approaching from opposite sides.

NOTE: The PBS will help remove the piece of skull and prevent possible bleeding of the dura[12].

2.2.19. Apply PBS, followed by gentle aspiration through a 26G blunt needle several times to clean the surface of the dura.

2.2.20. Gently remove the dura, either by aspiration or by ophthalmic scissors. Apply gentle suction (~-60kPa) to ablate the cortex, as well as the corpus callosum above the hippocampus.

NOTE: The cortex is often more yellow than the corpus callosum, and the corpus callosum is usually whiter than the hippocampus. The corpus callosum is usually easy to distinguish by neuronal fibers going in the vertical and horizontal directions when observed from the top (**Figure 3**).

[Place Figure 3 here]

2.2.20.1. Bleeding at this point will affect the visibility of the brain tissue in the craniotomy. Apply 1x PBS, followed by gentle suction, while aspirating the cortex to get rid of the blood.

NOTE: Continuous bleeding is inevitable during this step, and to some extent, continuous bleeding is a sign of normal blood pressure. Unlike cortex imaging window implantation, the presence of blood under the optical window is acceptable since it will be cleared several days after the surgery. Insertion of the imaging cannula to the created cavity as soon as possible after ablating the cortex is optimal.

2.2.20.2. If the craniotomy is larger by <0.5 mm than the imaging cannula, rescue the installation of the cannula to some extent by using extra Kwik Sil sealing before fixing the implant with SuperBond.

2.2.20.3. If the craniotomy is smaller by <0.5 mm than imaging cannula, rescue the surgical procedure to some extent by trimming the edge of the craniotomy using a fine tweezer or a pair of ophthalmic scissors since the remaining bone at the edge of craniotomy is thinner than the skull itself as a result of drilling.

NOTE: Craniotomies which are exceeding the ranges above 0.5 mm cannot be rescued. The corresponding actions in those cases should follow the termination procedure according to the animal protocol.

2.2.21. Gently insert the implant into the craniotomy.

2.2.22. Firmly press on top of the implant with the L-shaped needle to position the optical window of the implant as close as possible to the exposed surface of the hippocampus. Repeatedly apply PBS on the skull around the implant followed by suction to remove blood as much as possible during implant insertion. Then apply a thin layer of Kwik Sil between the implant and skull to prevent dental cement from penetrating under the skull (**Figure 4**).

2.2.22.1. Ensure the placement of the optical window of the implant is right against of hippocampus to avoid blood or other liquid accumulation underneath.

NOTE: The critical point is to make sure that the cover glass of the imaging cannula is placed right against the hippocampus, which may require gentle pressure on top of the cannula during the installation and sealing process. Whether the upper side of the imaging cannula is parallel to the skull is not critical for the final optical access as long as the optical window is placed against the hippocampus.

2.2.22.2. According to the average thickness of the cortex above the CA1 area, keep the upper surface of the imaging cannula above the skull surface by ~0.5 mm to facilitate attachment of the cannula to the skull (**Figure 4**).

2.2.23. Once the Kwik Sil is cured, which usually takes no more than ~1 min, apply Super-Bond C&B evenly on the surface of the skull, the surface of Kwik-Sil, and the upper surface of the implant.

2.2.24. Once the Super-Bond C&B is cured, apply denture base resin above the Super-Bond C&B, as well as the skin around the incision made at the beginning of the surgery.

NOTE: Alternative types of cement are available from multiple vendors. Follow the corresponding manufacturer's instructions.

2.2.25. After the denture base resin is cured, place the head plate on the resin around the implant and make it concentric with the imaging cannula. Apply more denture base resin around and above the head plate to fix its position. Let it cure for several minutes.

2.2.25.1. Avoid building up a thick layer of cement around the cannula to ensure better access to the imaging window with the objective lens (**Figure 4**). [Place Figure 4 here]

2.2.25.2. Dilute the denture base resin to decrease its viscosity, thus allowing it to fill the caveats which are hard to reach with an applicator.

2.2.26. Gently place an insulated rubber tape above the window to protect the window from possible contamination from animal bedding.

2.2.27. When the surgery is finished, inject anti-inflammatory drug subcutaneously to prevent an inflammatory response.

2.2.28. Place the animal in a warm cage until it recovers from anesthesia.

2.2.29. Check the health status of the mouse for 72 h post-surgery by observing general behavior. Anti-inflammatory drug and analgesic are injected subcutaneously for two-three days post-surgery every 24 hours to release pain and reduce the inflammatory response.

NOTE: Alternative monitoring procedures, drugs, and dosages are possible for postoperative care, refer to the IUCAC approved animal protocol for the exact procedure.

2.2.30. Check the window 5-7 days post-surgery to observe vasculature under the window. In the case of a clear window, the animal is ready for virus injection.

3. Virus Injection

NOTE: Virus injection is usually done in 5-7 days after the surgery. Before virus injection, it has to be confirmed that the imaging window is clear, and it is possible to observe brain vasculature (**Figure 5**). In some cases, it may take up to 14-16 days to clear the window, which is also acceptable if no brain inflammation is detected.

[Place figure 5 here]

3.1. Add Fast-green dye stock solution to virus solution, diluted to the desired titer, in the ratio of 1:9 in a PCR tube.

NOTE: Fast green dye is added to facilitate visualization of virus solution during the injection.

3.2. Connect the polyethylene tubing with the syringe, then backfill the tubing with mineral oil using a syringe pump.

3.3. Connect the internal cannula to the other end of the tubing, infuse and withdraw the mineral oil a few times to make sure that the internal cannula is not clogged.

3.4. Anesthetize the animal with isoflurane (4% for induction, 1.5-2% for maintaining, 0.3-0.5 L/min flow rate of air), fix the head in a stereotaxic frame over a heating pad (maintaining 37 °C), apply eye ointment.

3.5. Withdraw 600 nL of the virus solution, remove the dummy cannula and insert the internal cannula connected to the injection syringe into the guide cannula, infuse the virus at the speed of 50 nL/min for 10 minutes in total.

NOTE: Check if the dye is visible through the window using a stereomicroscope to confirm successful virus injection (**Figure 5**).

3.6. After injection, keep the internal cannula connected for 10 minutes to allow the virus to spread under the window.

3.7. Gently remove the internal cannula from the guide cannula and recap it with a dummy cannula.

3.8. Place the animal in a warm cage until it recovers from the anesthesia.

NOTE: Typically, mice are ready for imaging in 10-20 days after viral injection. Expression level and time depend on the virus serotype and promoter used to drive gene expression.

4. Imaging of awake mice under wide-field microscope.

NOTE: The prepared head plate provides extraordinary stability of imaging implant and thus allows for longitudinal imaging in awake and behaving mice with minimal motion artifacts.

4.1. Induce the mouse with 4% isoflurane for a few minutes, fix its head plate to the head fork, and then fix the head fork to the treadmill.

NOTE: The head fork and the treadmill are customized for the headplate used in this study, please see Supporting Materials for the corresponding cad files. Induction of mouse before head fixation is optional as it is possible to habituate animal for this procedure.

4.2. Move the treadmill under the microscope stage and position the optical window under objective lens.

4.3. Use a low magnification objective lens to find the best field of view (FOV) for functional imaging, then switch to a higher NA objective lens to record the neuronal activities at single-cell resolution.

NOTE: If the injection cannula is still an obstacle for the objective length to achieve its working distance, use a wire clipper to cut off the injection cannula from the head plate.

REPRESENTATIVE RESULTS:

In vivo imaging of neuronal activity using a genetically encoded calcium indicator. On average, *in vivo* imaging starts 3-4 weeks after implantation if a sufficient level of transgene expression is achieved. By this time, cerebral edema and hemorrhage are usually completely resolved, and brain vasculature can be easily observed through the optical window. Here we utilized the described preparation to perform the repeated recordings of neuronal activity in the dorsal hippocampal CA1 region in behaving mice under fluorescence wide-field microscope. To record neuronal activity, we used a bright genetically encoded calcium indicator, named NCaMP7[13], which exhibits similar calcium sensitivity and temporal resolution to that of GCaMP6s[14]. To express the NCaMP7 indicator in the hippocampus, we injected the rAAV/DJ-CAG-NCaMP7 virus using an infusion cannula and initiated longitude imaging at 14 days post-injection. To record neuronal activity, we used a 10x NA 0.3 air objective lens and Hamamatsu OrcaFusion sCMOS camera that allowed imaging at ~1.5x1.5 mm FOV at up to 100 Hz frequency. Green fluorescence was excited by a commercially available 470 nm LED using a standard GFP filter set. The average depth of imaging achieved in green channel is about 50-120 µm, which allows recording of neuronal activity mainly in stratum oriens and stratum pyramidale. Imaging depth in near-infrared channels can be up to 200 µm reaching the deeper layers of hippocampus[8]. The average recording time per FOV was 6-12 min, although much longer imaging sessions are possible as NCaMP7 is characterized by extremely high photostability and no detectable phototoxicity was observed (**Figure 6**).

[Place Figure 6 here]

To obtain fluorescence traces, the regions of interest (ROIs) corresponding to neuronal somas were segmented manually and analyzed by ImageJ software. Prior to the image analysis motion correction, common post-recording procedures in awake animals were not required as the acquired datasets did not exhibit motion artifacts due to the high stability of imaging implant. A representative single-trial optical recording of neuronal activities from the hippocampus in an awake behaving mouse is presented in **Figure 6**. 15 ROIs corresponding to neuronal somas were selected manually from the same FOV shown in **Figure 6B**, and the single-trial fluorescence traces within each ROI are shown in **Figure 6C**. **Figure 6D** shows two representative parts of fluorescence traces from two different ROIs. We performed 4 consecutive imaging sessions for the same FOV with 3-day intervals. It was possible to identify and image the same neurons in certain FOVs for at least two weeks (the longer imaging sessions were not performed for this study, however, the same preparation has been used for up to 6 month-long imaging study in mice previously[7]; **Figure 7**). In this study, we used AAV/DJ-CAG vector, which was driving a strong expression of the gene of interest even 21 days after virus delivery (**Supplementary Figure 1**). The continuous expression complicated long-term identification of the same neurons due to increased fluorescence background and appearance of new neurons expressing the calcium indicator. Therefore, selection of the AAV serotype and promoter to drive target gene expression should be one of the important considerations during experimental design in particular if longitudinal imaging of the same subset of neurons is required. The imaging quality allowed to resolve proximal dendrites as well as visualize blood vessels.

[Place Figure 7 here]

In vivo imaging of neuronal activity using a genetically encoded voltage sensor. In this study, we also used a novel genetically encoded voltage sensor, named SomArchon[7], which allows for voltage imaging with single-cell single-spike resolution in behaving animals[7,8]. To express SomArchon, we injected rAAV/DJ-CAG-SomArchon virus using an infusion cannula and performed voltage imaging in a head-fixed behaving mouse several days post-injection. To record neuronal activity, we used a 40x NA 0.8 objective lens and Hamamatsu OrcaFusion sCMOS camera that allowed us to image 150x40 µm FOV at up to 830 Hz acquisition rate. The GFP protein, which a part of SomArchon construct to facilitate visualization of expression in the visible range of the spectrum, can be easily imaged in the green channel (LED excitation at 470/20 nm, emission 525/50 nm) to locate cells of interest for voltage imaging. Optical voltage recordings were performed in the near-infrared channel (laser excitation 637 nm at 3.4 W/mm^2, emission 665 nm long pass) with 4 x 4 binning at 830 Hz acquisition rate. We recorded the spontaneous activity of a hippocampal neuron in an awake mouse with average SNR of 7 per action potential (**Figure 8**).

Histology

After functional imaging study is done, post-mortem analysis is used to confirm the correct placement of the implant, the area of virus expression, and localization of a protein of interest in imaged neurons. For histological verification of virus expression and placement of the implant, coronal sections of the PFA fixed brain were examined under a fluorescence wide-field microscope (**Figure 9A, C**). A confocal microscope was used to acquire high-resolution images of individual neurons expressing the calcium indicator, as well as the voltage indicator (**Figure 9B, D**). DAPI staining was used to visualize the overall morphology of the brain slice. In addition, the brain slices can be assessed using immunohistochemistry to verify astrogliosis or gliosis caused by window implantation and viral expression. Our previous studies demonstrated that the procedure did not induce noticeable gliosis[7].

[Place Figure 9 here]

FIGURE AND TABLE LEGENDS:

Figure 1: **Six key hardware components for assembling and installation of the imaging implant.** (**A**) Dummy cannula. (**B**) Guide cannula. (**C**) Imaging cannula. (**D**) Glass cover glass. (**E**) Headplate. (**F**) Internal cannula. Scale bar: 5 mm.

Figure 2: **Schematic of assembling injection cannula, consisting of guide cannula with inserted dummy cannula, with the imaging cannula.** (**A**) The angle between the injection cannula and the imaging cannula should be proximately 45 degrees. (**B**) The tip of the injection cannula should be right on the edge of the imaging cannula. (**C**) An appropriate size of the welding tin used to solder imaging and injection cannulas (red line indicates the outline of the tin droplet). (**D**) Inappropriate size of the welding tin that should be avoided during implant preparation (red line indicates the outline of the tin droplet).

Figure 3: **Stereotaxic coordinates of hippocampus location and brain ablation process.** (**A**) Four coordinates for the edges of the craniotomy area. (**B**) Complete craniotomy area. (**C-E**) Representative images acquired during the surgery (left) and their schematic diagram (right) indicating the different colors and directions of neural fibers of (**A**) Cortex (**B**) Corpus Callosum, and (**C**) Hippocampus visible during cortex ablation. Scale bar: 1 mm.

Figure 4: **Schematic diagram of window implantation in (A) coronal and (B) sagittal view.** (**a**) Headplate; (**b**) Denture base resin; (**c**) Superbond; (**d**) Kwik-Sil; (**e**) Imaging cannula; (**f**) Injection cannula; (**g**) Soldering tin. (**C**): Mouse with the installed implant after the surgery.

Figure 5: **Representative image of optical window (A) before and (B) after virus injection supplemented with FastGreen dye.** Arrow indicates the same vasculature structure. Scale bar: 1 mm.

Figure 6: **Recording of the neuronal activity in the hippocampal neurons using a green fluorescence genetically encoded calcium indicator.** (**A**) A selected FOV imaged under a wide-field fluorescence microscope in green channel. (**B**) The 15 ROIs corresponding to the single neurons shown in A and selected using the standard deviation projection of the whole recording. (**C**) Representative fluorescence single-trial traces of the 15 selected neurons in B. (**D**) A representative zoom-in view of 2 calcium traces shown in corresponding color boxes shown in C. Scale bar, 100 µm.

Figure 7: Image sequence of four different FOVs from the hippocampal area tracked over 12 days. The animal was implanted with the window on Day 0 and was injected with the rAAV/DJ-CAG-NCaMP7 virus on Day 7. Arrowheads indicate the neuron tracked within the FOV. Scale bars: 80 µm.

Figure 8: **Recording of the neuronal activity in the hippocampal neurons using a near-infrared fluorescence genetically encoded voltage indicator SomArchon.** (**A**) A selected FOV imaged under wide-field fluorescence microscope in the near-infrared channel. (**B**) Single-trial fluorescence trace of the neuron in A. (**C**) A representative zoom-in view of the voltage trace in the corresponding box shown in B. Scale bar: 25 µm.

Figure 9: Histological verification of the optical window position and virus expression. (**A**) A representative fluorescent image of the coronal section brain slice showing the placement of the optical window from an NCaMP-expressing mouse. Scale bar: 1 mm. (**B**) A representative confocal image of neurons expressing the NCaMP7 indicators. Scale bar: 25 µm. (**C**) A representative fluorescence image of the coronal section brain slice showing the placement of the optical window from a SomArchon-expressing mouse. Scale bar: 1 mm. (**D**) A representative confocal image of neurons expressing the SomArchon indicators. Scale bar: 25 µm.

Supplementary Figure 1: Quantitive analysis of relative fluorescence intensity along with expression time.

DISCUSSION:

Here we describe a method for long-term imaging of the hippocampus CA1 region in behaving mice. The method is based on the chronic implantation of a custom-made imaging window, which also enables the targeted administration of viruses or drugs directly to the neurons of interest. The present protocol consists of four major parts: i) assembling of imaging implant; ii) installation of imaging implant; iii) virus injection via imaging implant; iv) functional imaging in behaving mice. Below we describe and discuss critical steps in the protocol, troubleshooting, modifications, and limitations of the method. We also discuss the significance of the method and its potential alternative applications.

There are several critical steps in the described protocol that are rather important for successful surgery: (i) preparation of a high-quality imaging implant; (ii) sterile surgical conditions; (iii) aspiration of the cortex; (iv) precise placing of the imaging implant; (v) viral injection. As Step 1.6 indicates, excess soldering tin would require a larger craniotomy and thus increases the risk of inflammation. It is also very important to use an appropriate amount of the adhesive optical glue when fixing the cover glass to the imaging cannula, as indicated in Step 1.11, as an insufficient amount may result in leakage of cerebrospinal fluid into the imaging cannula and making it opaque. On the other hand, excess optical adhesive can result in the decreased transparency of the glass window. Possible contamination of the imaging implant can cause an active proliferation of connective tissue under the optical window and/or severe inflammation, which will lead to early termination of the experiment. Therefore, imaging implant assembling and preparation before the surgery is almost as important as the surgical procedure itself.

During the surgery, the part of the cortex under craniotomy is ablated by gentle aspiration, which results in inevitable bleeding. Blood at the surgical site significantly reduces the visibility of the brain tissue that must be removed. This complicates the precise assessment of the required depth of tissue ablation. Careful flushing of the surgical site with PBS every time before applying suction to remove the next portion of tissue provides better control of depth. The brain tissue should always be removed in small portions step-by-step confirming the depth of ablated tissue before proceeding with more suction. Finer control of suction can also be achieved with a thinner blunt needle. We suggest using a 26 G needle, however, smaller than 26 G diameter is more prone to clogging. Furthermore, it usually takes lots of practice to determine the precise depth of aspiration required for each animal, as the color of the cortex, corpus callosum, and hippocampus may vary from mouse to mouse (**Figure 3**).

Insertion and securing of the imaging implant should be done very precisely to ensure the closest possible position of the imaging window to the dorsal surface of the hippocampus. The size of the prepared craniotomy should closely match the implant and allow its insertion without significant resistance. At the same time, there should be no visible gap between the skull and the implant to ensure proper sealing and avoid brain tissue exposure. Gentle and stable pressure should be applied to the top of the implant during its sealing to the skull. It is almost unavoidable to have blood under the imaging window during the implant installation. If the surgical procedure is done properly, the window should clear out in 3-7 days, and brain vasculature becomes clearly

visible. It is also important to ensure that virus is properly injected under the window. In the case of failed expression, virus can be reinjected multiple times.

The major complication we encountered in some cases is reduced visibility of the imaging window. There are several possible reasons for poor imaging quality: i) ongoing inflammation; ii) outgrowth of connective tissue on the glass; iii) big gap between the window and hippocampus. Inflammation is usually caused by contamination during surgery or by not properly sterilized imaging implant. We suggest autoclaving the surgical instruments before and after each surgery, disinfect the surgery area right before the procedure, and wear clean personal protective equipment during surgery. Imaging implants should be cleaned after assembling, sterilized, and stored in sterile conditions. The outgrowth of connective tissue on the glass of imaging implant can be due to mechanical contamination on the surface of the glass or excessive trauma of the brain tissue during cortex ablation. After assembling the implant, it is important to confirm the glass surface is clean and smooth. Also, all pieces of damaged brain tissue must be carefully removed before inserting imaging implant into the craniotomy. In certain cases, the gap between the glass window and hippocampus results in the accumulation of cerebrospinal fluid, reducing the quality of imaging. Therefore, during implant installation, it is crucial to insert it all the way in ensuring good contact between hippocampus and glass window. Sometimes, it is difficult to identify the exact reason for opaque imaging window. We suggest performing post-mortem analysis to reveal conditions under the optical window and correspondingly adjust subsequent surgeries.

The method has several fundamental and technical limitations that should be taken into account before and during *in vivo* imaging. One of the major limitations is cortex ablation. Part of the visual and sensory cortex is removed during the surgery. While it is hard to precisely evaluate the impact of cortex ablation, as removed brain tissue does not directly project onto hippocampus, several studies demonstrated no noticeable impairment of hippocampal-dependent learning or other relevant hippocampus functions[15,16]. The optical limitations should also be considered, especially when high NA objective lenses are used. For example, in this study, we used a 1.75 mm long cannula with a 1.9 mm inner diameter. The geometry of this cannula will not preserve the full NA of air objective with NA more than ~0.5 or water objective with NA more than ~0.6 as it will clip some of light. Another limitation, common for all brain imaging implants, is that part of the brain is getting exposed, thus promoting heat loss[17,18]. However, physiological brain temperature can be easily restored during imaging by perfusion of a warm buffer.

The described method can be easily modified or adjusted for other applications. For example, the preparation can be adapted for imaging of striatum[7]. As the striatum lays slightly deeper than cortex, the longer imaging cannula should be used for assembling imaging implant. We suggest using 2.0 mm imaging cannula. The coordinates of the craniotomy should be adjusted correspondingly (AP: +0.8 mm, ML: −1.8 mm). In addition, virus injection via infusion cannula allows achieving the expression of a transgene in a thin layer of neurons when using AAV serotype with restricted spread[19,20]. It is particularly beneficial for one-photon imaging due to reduced out-of-focus fluorescence from deeper layers and, as a result, improved single-cell resolution imaging. Furthermore, injection cannula can also be used during functional imaging for the administration

of drugs or other chemicals directly onto the neurons in FOV (**Figure 5B**). Overall infusion cannula adds useful functionalities to the imaging implant, improving imaging quality due to the targeted viral expression and enabling pharmacological stimulation of neurons in FOV. The used head plate provides extraordinary stability of imaging implant minimizing motion artifacts even in actively moving animals on a treadmill. The head plate is small and light, causing minimal discomfort to animals, and remains stable for several months after installation. The imaging implant is also compatible with multiphoton imaging[15,16,21] and can be combined with micro-endoscopes[22,23]. A similar imaging implant was also used for multiphoton imaging of the deeper hippocampus structures, including stratum radiatum, stratum lacunose, and dentate gyrus[16,24-27]. However, targeting the deeper hippocampus structures with AAVs via infusion cannula may require further optimization of the AAV serotype and volume[19].

We believe that the described protocol will facilitate studies that aim to investigate neuronal activity with high spatiotemporal resolution in the hippocampus of behaving mice using simple and affordable one-photon imaging setups.

ACKNOWLEDGMENTS:

We would like to thank all the members of the Molecular BioEngineering Group at Westlake University for all the help and useful discussion. We also thank Jinze Li and Jie-Min Jia from Westlake University for the help with filming the surgical procedure.

This work was supported by start-up funding from the Foundation of Westlake University, 2020 BBRF Young Investigator Grant, and National Natural Science Foundation of China grant 32050410298 all to K.D.P.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

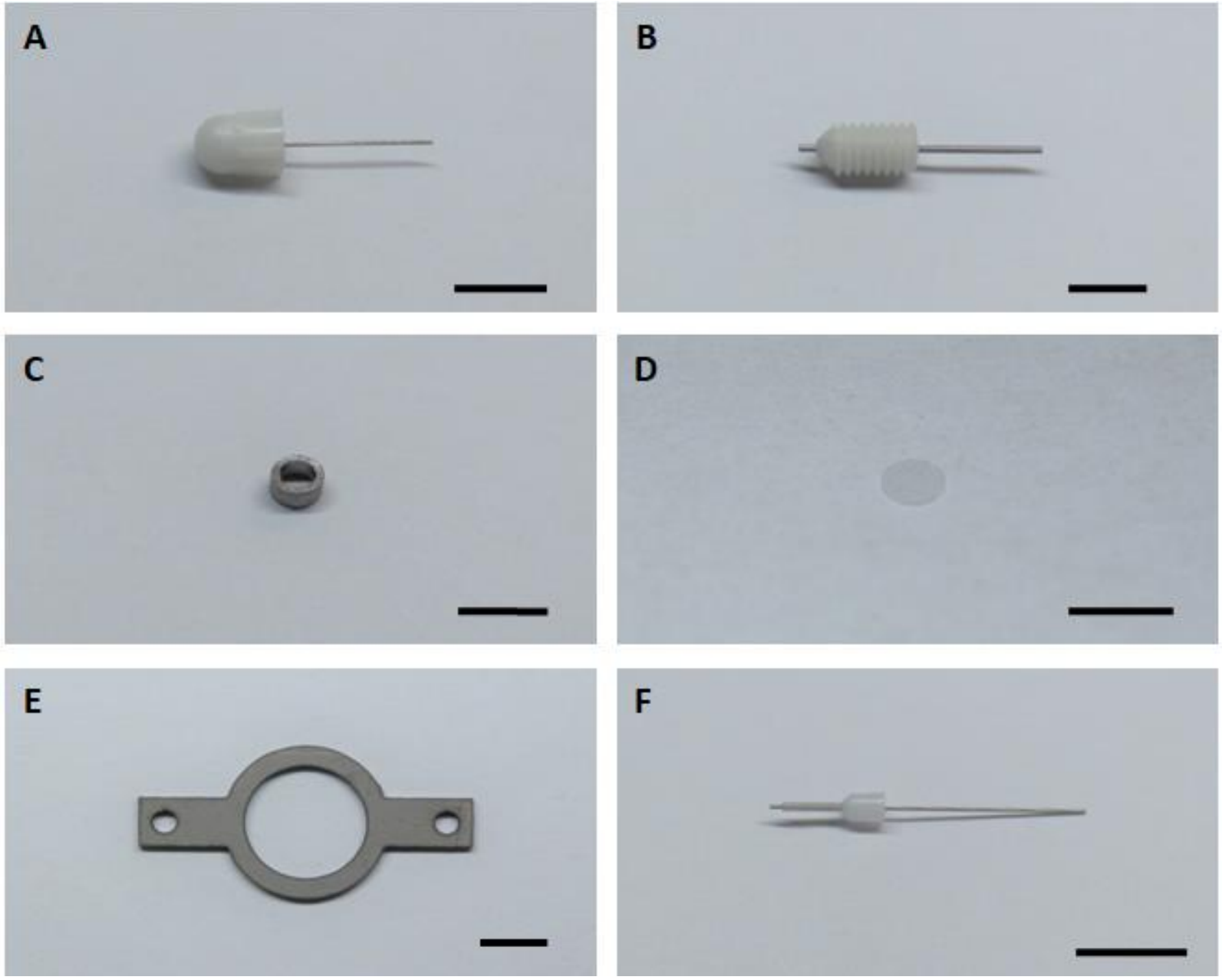

Figure 2

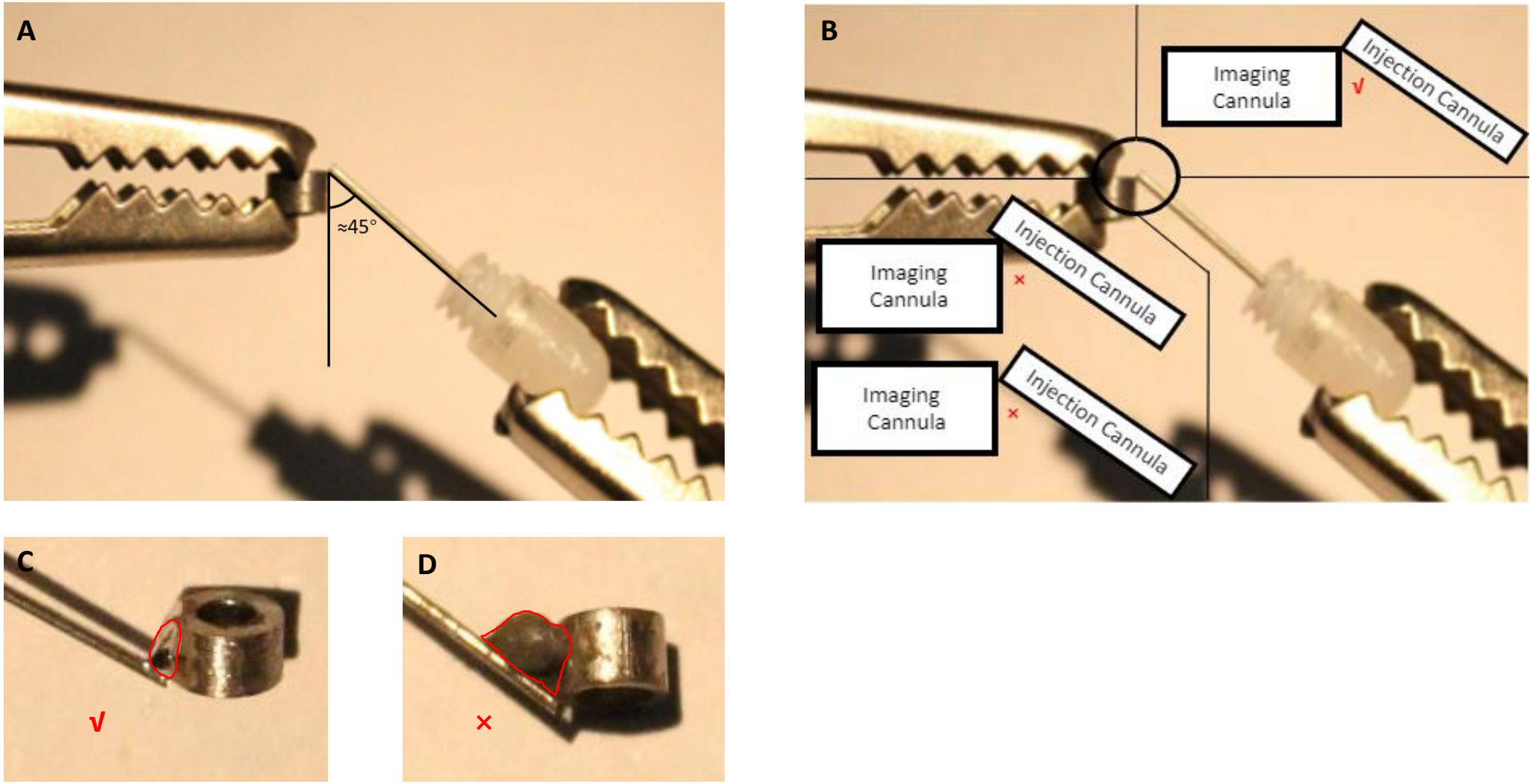

Figure 3

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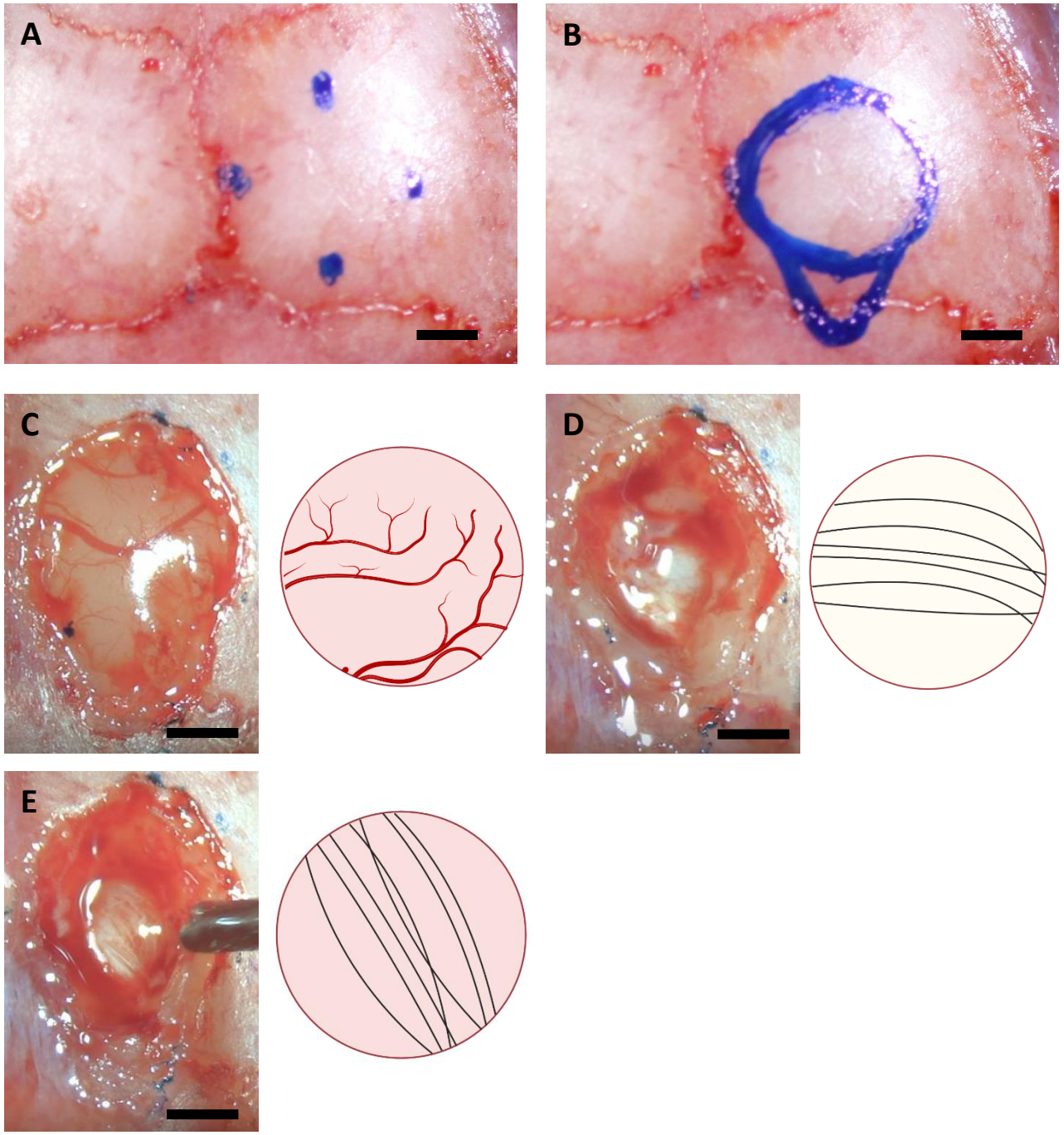

Figure 4

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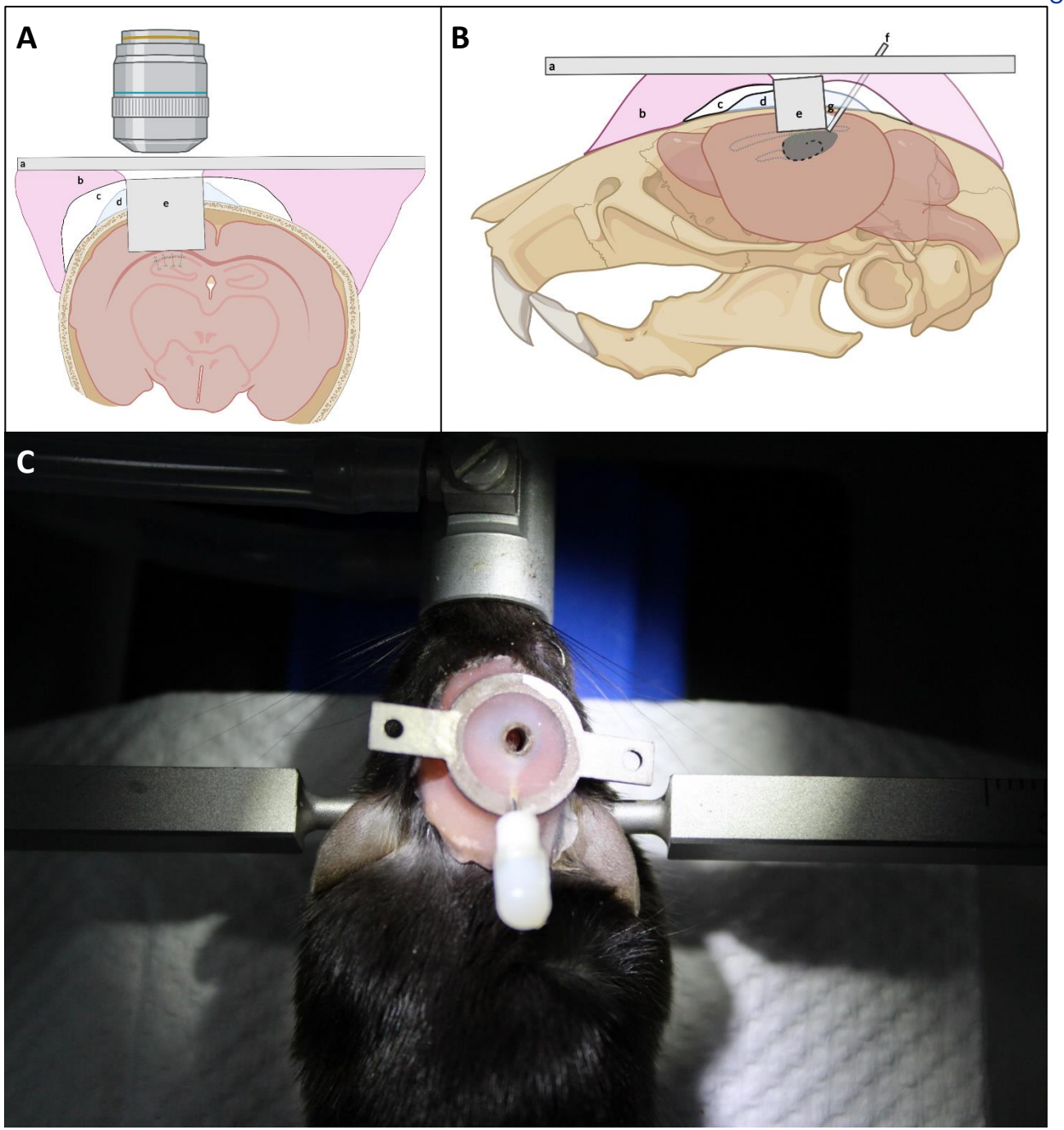

Figure 5

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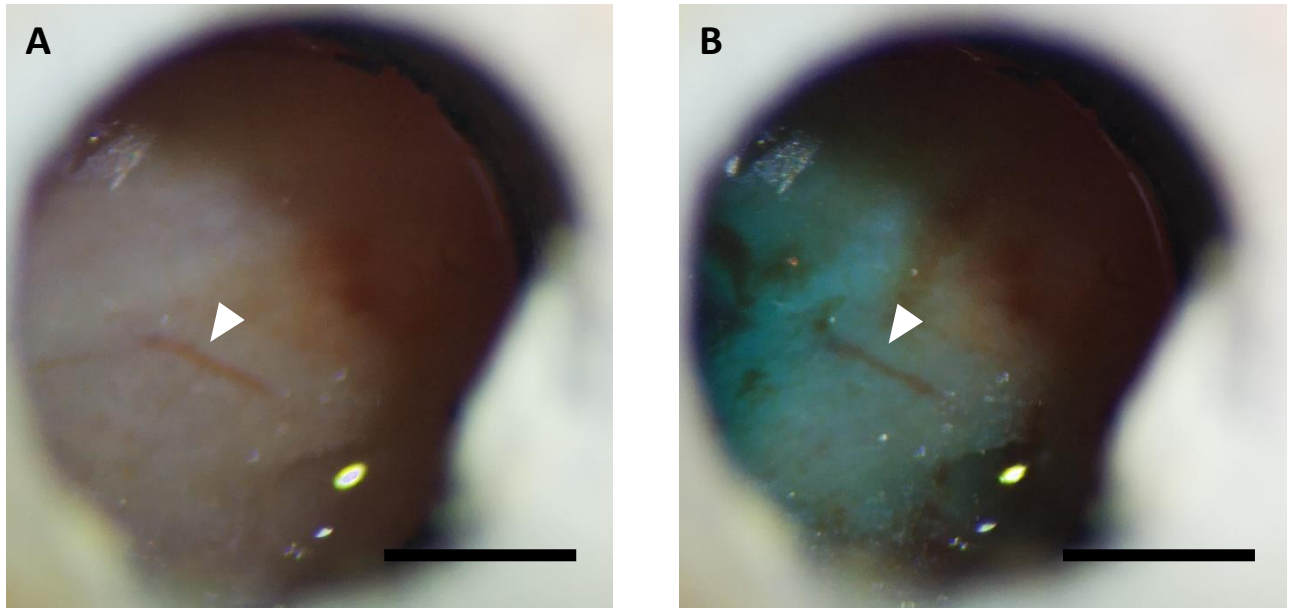
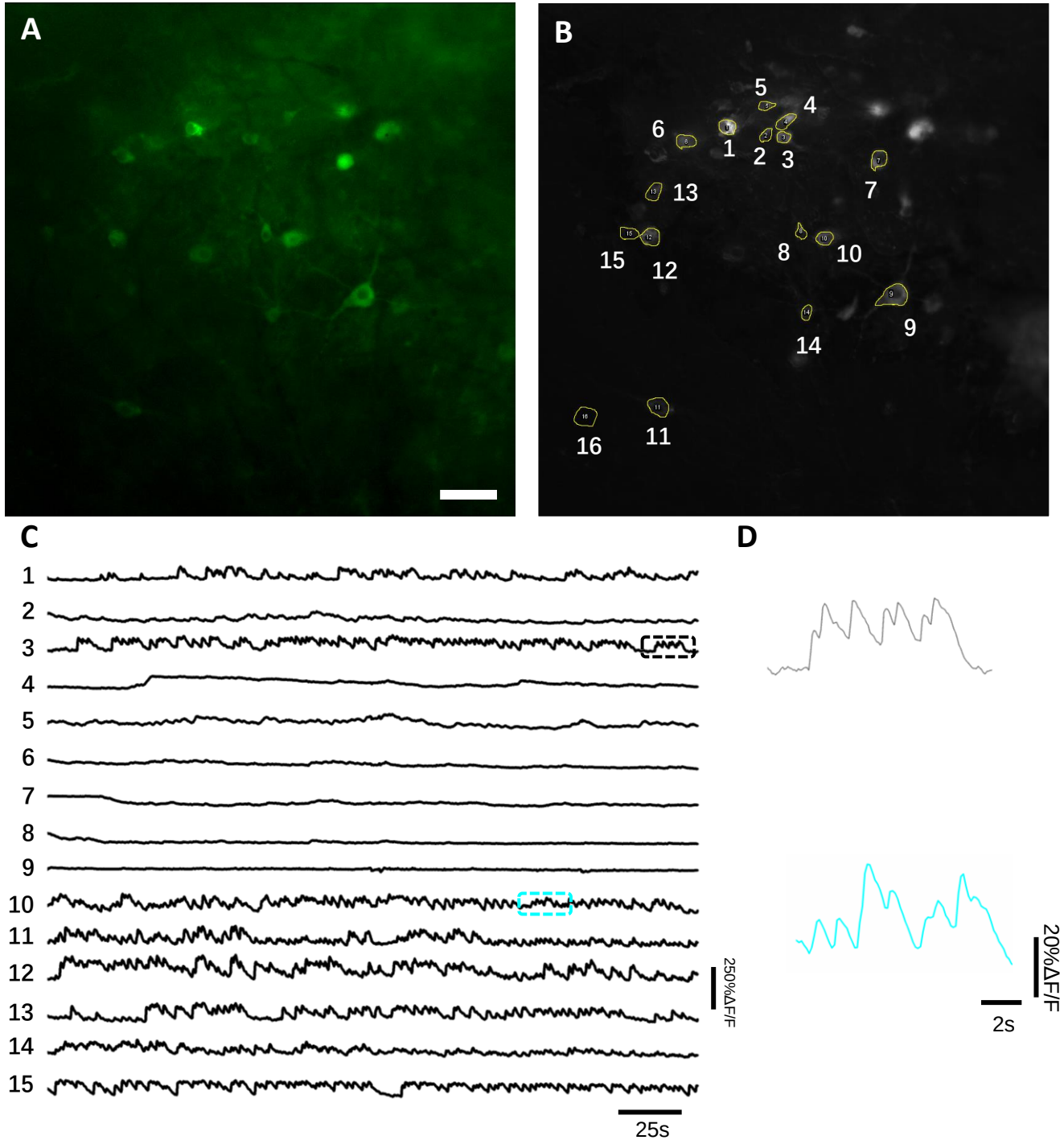


Figure 6



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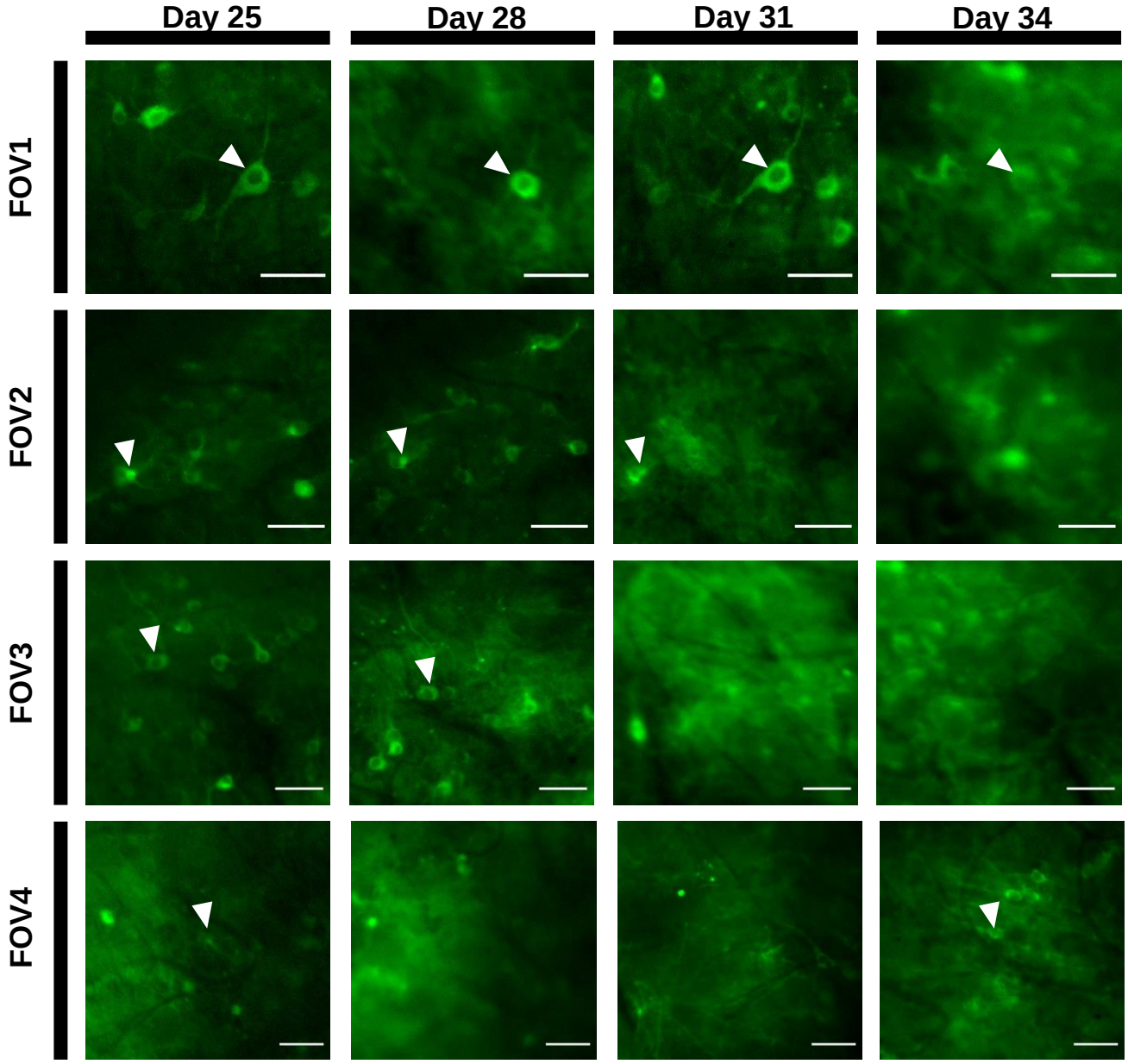

Figure 8

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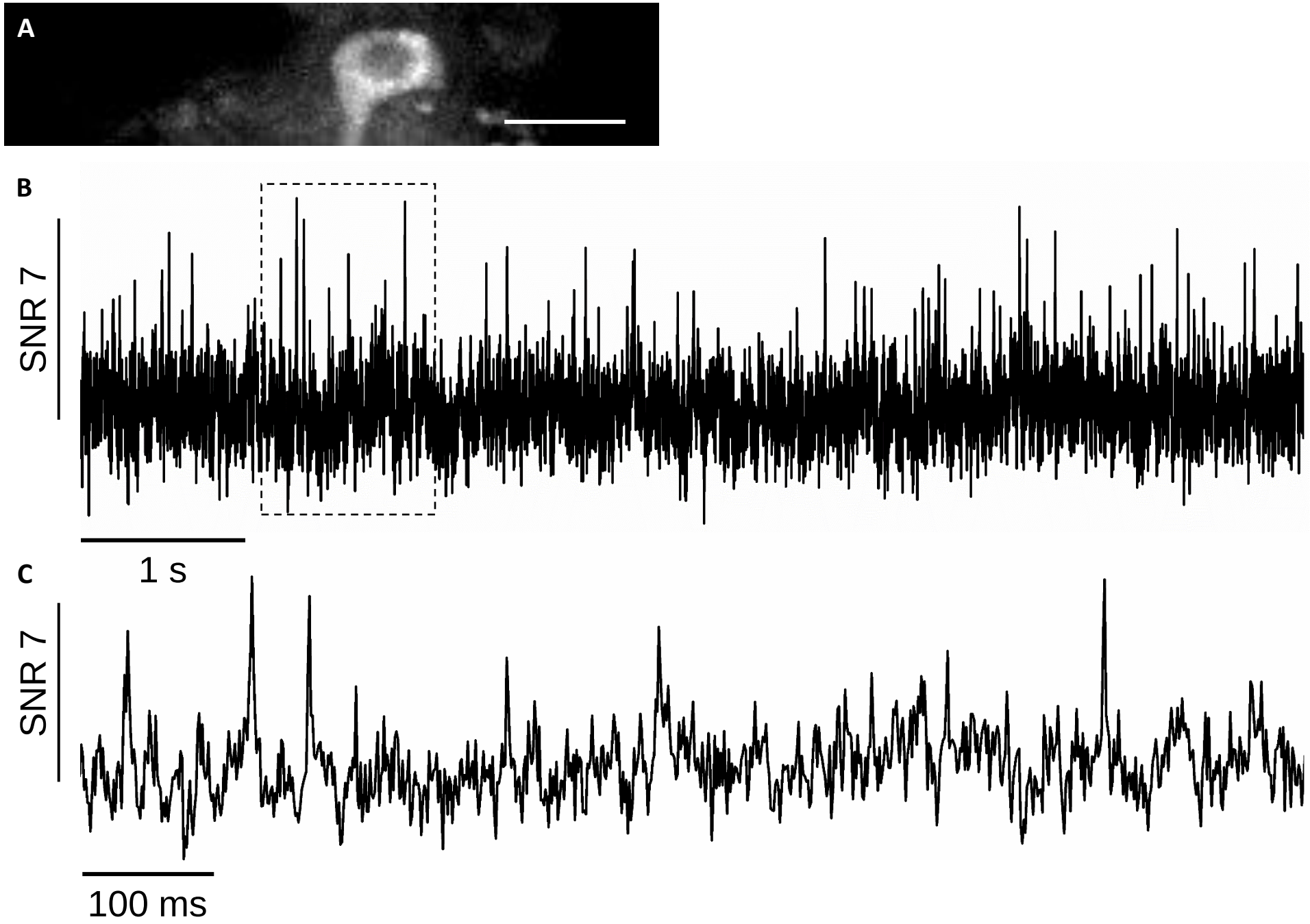

Figure 9

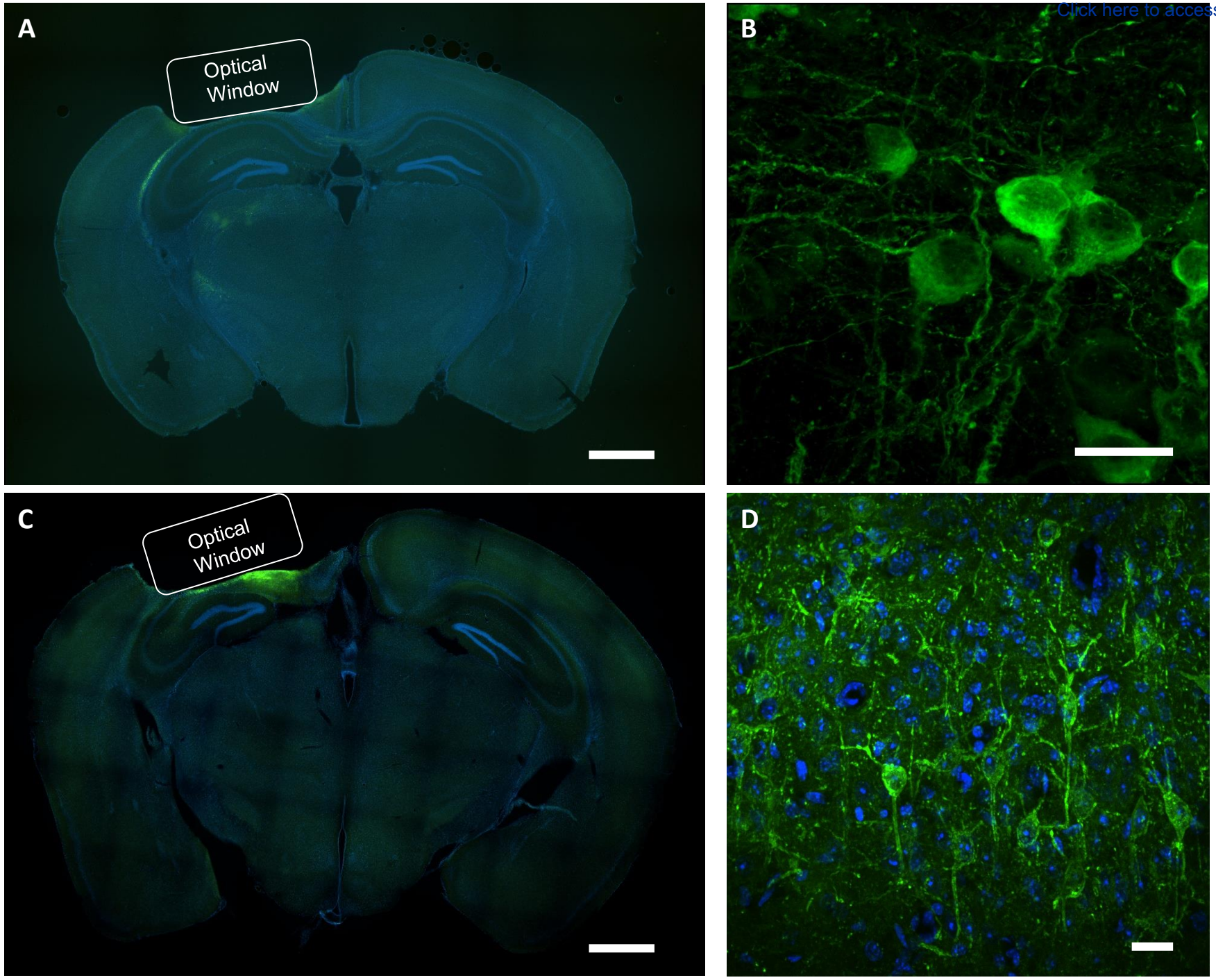

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Table of Materials

Material List.xlsx

We thank the editorial team and Reviewers for the valuable comments and suggestions that helped us greatly improve the manuscript. We were able to address all concerns and questions in full. In addition, we performed new experiments to demonstrate that the described procedure can be used for voltage imaging with single-cell resolution in head-fixed behaving mice. Below we provided detailed responses to every comment.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have run proofread to correct mistakes and typos as well as further improve English.

2. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
We have refilmed some of the scenes of the visual protocol as well as rearranged the written manuscript to ensure consistency between the written manuscript and the video. Please refer to the revised version of the manuscript.

Vet Review comments are attached as well.

The recorded surgical procedures shown in this visual protocol have been performed according to the IACUC approved animal protocol and involved administration of a pain killer drug before the surgery as well as during 48-72 h post-surgery as required. The vital signs were observed throughout the entire procedure. Anti-inflammatory drug was injected right after the surgery and during post-surgery recovery. We have added corresponding steps to the written manuscript. Please see Page 4 Step 2.2.2 and Page 6 Step 2.2.28.

Changes to be made by the Author(s) regarding the video:
1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have increased the consistency between text and video by adding newly filmed parts to the video file as well as improving narration. We also revised the written manuscript to make it consistent with the video. All figures that are shown in the video are now inserted into the written manuscript.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

We have changed the narration of the video file to correspond to the written manuscript. In particular, the narration of the revised video for all crucial steps is a word-by-word reading of the written manuscript.

3. Please include a more substantial results section in the video.

In the revised version of the video file, we significantly extend the presentation of the result section to cover performed fluorescence recording in more detail. We also have added a completely new part of the result section into the video presenting in vivo voltage imaging in an awake mouse. In particular, we have presented real-time optical traces along with the video of neurons in vivo. Please see corresponding changes at time 10:53.

4. JoVE Video Article Format Standards:
• Video must start with a main title card containing the title of the protocol, authors, and their corresponding affiliations.

We have revised the video file to reflect all required information.

• Please add on-screen text to identify all on screen speakers.

We have revised the video accordingly.

• Please add title section cards before each section: Introduction, Protocol, Results, Conclusion

The title section cards have been added to the video at the respective time point.

• Please add a Conclusion to wrap up the video.
The video has been revised to include the narration for the Conclusion section.

5. Video & Audio Editing:
• 01:11 This still image of the canula needs more screen time to be seen by the viewers. Consider extending the duration it is visible on screen by a couple seconds.

We have extended the duration of the assembled implant is shown on the screen. Please see corresponding changes at time 1:32.

• 02:03 The first sounds of this sentence are cut off. It sounds like the end of "sterilize". Edit this section to include the missing syllables.

The audio of the corresponding part has been edited to include the missing syllables. Please see corresponding changes at time 02:12.

• 05:35 There is a jump cut here along with an abrupt audio edit that is a possible error. It happens at the words: "...or repeatedly apply PBS..." This should be edited to include the full sentence and also consider adding a dissolve to the video portions to ease the pacing.
The audio of the corresponding part has been edited to include the full sentences. Please see corresponding changes at time 07:29.

6. Animal Use:
• 02:08 Please remove the shot of the anesthetization chamber. Descriptions of anesthesia are okay to include (so the narration is fine), depictions are not okay to include (the visuals are not okay).

The video has been edited to remove the shot of the anesthetization chamber. Please see corresponding changes at time 2:05.

• 07:55 Please remove this shot (visual) of the anesthetization chamber.
The video has been edited to remove the shot of the anesthetization chamber. Please see corresponding changes at time 10:23.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/5xnPncJOEtUsoDpcVudC?oref=e

Reviewers' comments:
Reviewer #1:
Remarks to the authors:
In this JOVE manuscript entitled "Craniotomy Procedure for Visualizing Neuronal Activities in Hippocampus of Behaving Mice" the authors show the preparation of an imaging window with infusion cannula and its implantation onto the hippocampus in mice. This preparation allows for the delivery of viral vectors and drugs. Further, the approach can be used for chronic in vivo imaging of CA1 in behaving animals.
In general, this is a helpful protocol for the field. Not substantial steps are missing and the steps listed in the procedure will lead to the described outcome.
There are just some comments and minor suggestions:
We thank Reviewer for the positive feedback and valuable suggestions that have been fully addressed.
Introduction:
1) "The descried preparation in combination with…": Spelling: "described"
The spelling of the word was corrected. Please see P2, Introduction.

Protocol:

Implant assembling
2) Imaging cannula information is missing in the Material list. It will be helpful to give information about the size of the imaging cannula (diameter, height) and of the three infusion cannulas (diameter, length) in the Material list or in this first section.

The Material list has been edited to include the dimensions of the imaging cannula and three parts of the infusion cannula.

3) 1.5: Which is the "appropriate type of flux" for in vivo experiments? This should be added.

For this preparation, we used a commercially available flux that is specified by the manufacturer to be for soldering stainless steel parts as imaging and infusion cannulas are made of stainless steel. In the case of other materials used to manufacture cannulas, end-user should select flux that is appropriate for the selected material. Please see P 3, step 1.5.

4) 1.10: It should be stressed explicitly that the positioning of the glass has to be done precisely onto the cannula, without moving the glass too much once it has touched the optical adhesive. Otherwise the glass gets dirty and this makes it hard to image.
This is a very good suggestion. We have revised the main text of the manuscript to stressed out this point. We have also refilmed the corresponding step in the visual protocol. Please see P3 Step 1.10, video time 01:45.

Window implantation
5) 2.2: Does the mouse need subcutaneous pain killer?

The recorded surgical procedures shown in this visual protocol have been performed according to the IACUC approved animal protocol and involved administration of a pain killer drug before the surgery as well as during 48-72 h post-surgery as required. The vital signs were observed throughout the entire procedure. Anti-inflammatory drug was injected right after the surgery and during post-surgery recovery. We have added corresponding steps to the written manuscript. Please see P4 Step 2.2.2 and P6 Step 2.2.28.

6) 2.2.12: AP coordinated must change if you move from Bregma to Lambda. ML should instead stay 0.

Thank you for pointing out this mistake in the text. We have revised the text accordingly to fix this typo. Please see P4 Step 2.2.12.

7) 2.2.14: Give some indications about the size of the injection cannula area, or suggest the coordinates (most caudal point for example).

We agree with the reviewer, this is important information that can help with following the procedure more precisely. Therefore, we have revised the manuscript to include the caudal point for the injection cannula. Please see P4 Step 2.2.13 of the revised manuscript.

8) 2.2.17: "Apply a drop of sterile 1X PBS applied to the center of the craniotomy...": Wording: remove "applied".

The sentence was corrected accordingly. Please see P5 Step 2.2.17.

9) 2.2.19: Clarify what "appropriate" means. "Gentle" may be the better wording, as mentioned in the discussion.

We have followed the reviewer's suggestions and changed "appropriate" to "gentle" and indicated the exact negative pressure used in this step. Please see P5 step 2.2.19.

10) 2.2.19 NOTE 2: Clarify if, in case of continuous bleeding, it could be stopped with a sponge or something that can be gently inserted into the hole.

Continuous bleeding is inevitable during this kind of surgery, and to some extent, continuous bleeding is a sign of the normal blood pressure. Unlike cortex imaging window implantation, the presence of blood under the optical window is acceptable since it will be cleared in several days after the surgery. Insertion of the imaging cannula to the created cavity as soon as possible after ablating the brain tissue is optimal. The corresponding note has been added to the main text of the revised manuscript. Please see P5 Step 2.2.19.

11) 2.2.20: Clarify the procedure in case the craniotomy would be too large for the cannula or in case the cannula does not fit through the craniotomy. Could these mistakes be rescued?

In the case of too large (less than 0.5 mm larger) craniotomy, the installation of the cannula can be rescued to some extent by using extra Kwik Sil sealing.

In the case of too small (less than 0.5 mm smaller) craniotomy, the surgical procedure can be rescued to some extent by trimming the edge of the craniotomy using a fine tweezer or a pair of ophthalmic scissors since it is thinner than the skull itself as a result of drilling.

Craniotomies which are exceeding the ranges above 0.5 mm could not be rescued, the corresponding actions in those cases should follow the termination procedure in the animal protocol. We have added these notes to the revised version of the manuscript. Please see P5 Step 2.2.20.

12) 2.2.21: It would be helpful to specify if the imaging cannula has to be pushed into the hole until it is at the same level of the skull or if the upper part has to be kept outside. Also, if it has to be parallel to the skull and if these aspects could affect the optical access.

The critical point is to make sure that the cover glass of the microscope cannula is placed right against the hippocampus, which may require gentle pressure on top of the cannula during the installation and sealing

process. Whether the upper side of the imaging cannula is parallel to the skull is not critical for the final optical access as long as the optical window is placed against the hippocampus.

According to the average thickness of the cortex above the CA1 area, it is suggested that the upper surface of the imaging cannula is kept above the skull surface by ~0.5mm to facilitate attachment of the cannula to the skull. The corresponding notes were added to the main text. Please see P5 Step 2.2.21.

Imaging of awake mice under wide-field microscope
13) 4.3: "Use 4X objective length to find…": Wording: "lens".
The spelling of the word was corrected. Please see P7 Step4.3.

Representative results:
14) "We performed 4 consecutive imaging sessions for the same FOV with 3-day intervals". Would it be possible to image more frequently?

The frequency of imaging can be increased. However, it can potentially reduce the longitudinal imaging due to the faster photobleaching of fluorescence and in the case of imaging behaving animals (for example, during locomotion) it can result in loosening up implant stability. The imaging frequency can be increased but the following cons should be noticed: a) increased imaging frequency can lead to faster photo-bleaching. B) increased imaging frequency can lead to loosening up the implant and thus decrease the potency of longitudinal imaging.

15) Figure 6: The long-term images are not very convincing. Some cells are not always visible. Is this an implant problem? Calcium indicator problem? How could these problems influence a longer imaging time? That should be "discussed".

We agree with the reviewer that in this study for some FOVs it was more difficult to trace the same neurons from one imaging session to another. One of the major factors complicating identification of the same neurons throughout longitudinal imaging was continuous expression of the fluorescent indicator that resulted in increased background fluorescence blurring the signal from target cells. To express the green fluorescent calcium indicator, we used AAV/DJ-CAG vector, which was driving a strong expression of the gene of interest even 21 days after virus delivery (please see Supplementary Figure 1 of the revised manuscript). Quantification of the green fluorescence from the same FOVs revealed more than 50% increase in fluorescence background and appearance of new neurons expressing the calcium indicator. Therefore, selection of the AAV serotype and promoter to drive target gene expression should be one of the important considerations during experimental design in particular if longitudinal imaging of the same subset of neurons is required. We have discussed this issue in the main text of the written manuscript to make readers aware of this experimental consideration. Please see P8 Paragraph 1 and P12 Paragraph 2.

Histology:
16) "...wide-field microscope (Figure 7A) Confocal microscope...": Wording: add full stop after

parenthesis.
The sentence was corrected. Please see P9 Histology section.

Discussion:
17) "(iv) precise placing imaging implant": English: precise placing of the imaging implant.

The wording was corrected accordingly. Please see P10 Discussion Paragraph 2.

18) "Furthermore, it usually takes lots of practice to determine the precise depth of aspiration required for each animal, as the color of the cortex, corpus callosum, and hippocampus may vary from mouse to mouse ": It will be useful to have example pictures of cortex, CC and final hippocampus.

We agree with the Reviewer's comment. To facilitate identification of the proper depth of cortex ablation we filmed new parts of the visual protocol to demonstrate these steps in more detail and pointed out important features that have to be watched out during brain tissue ablation. In addition, we have inserted the representative pictures of cortex, corpus callosum, and hippocampus to Figure 3 of the revised manuscript. Please see 05:33 and 06:59 of the video file and Page 5 Step 2.2.19 of the written manuscript.

19) Authors could suggest the deep structures you could reach with multiphoton imaging and micro-endoscopes. Moreover, it is not very clear if deeper structures (potentially reachable with these other microscopes) could be injected and reached with the infusion cannulas.

We thank the reviewer for this valuable comment. Indeed, it is important information that can help researcher to choose the right experimental approach for the desired brain region. The described imaging implant is mainly used for imaging of dorsal CA1 region of hippocampus, including alveus, stratum oriens, stratum pyramidale, stratum radiatum, as was also reported previously by Dombeck et al., 2010, Mizrahi et al., 2004, Busche et al., 2012, Attardo et al., 2018, Castello-Waldow et al., 2020. Using three-photon imaging it is possible to reach even deeper structures such as stratum lacunosum and dentate gyrus (Ulivi at al., 2019). While the infusion cannula is designed to deliver viruses right under the imaging window, i.e., dorsal CA1, the expression area will also depend on the AAV serotype and injection volume, which will define viral particle diffusion in the brain. For example, it was shown that AAV spread in the brain depends on serotype (Watakabe et al., 2015). We have added a corresponding discussion to the written manuscript. Please see Page 12, second paragraph.

20) Authors could add information about the tolerance of the mice to this surgery. How frequently can the surgery affect the health of the mouse and the impossibility to perform the imaging experiment?

We have not observed morbidity due to the craniotomy and cannula implantation in the course of this study. However, we encountered a few cases when the imaging was not possible :

A) the implant falls off from the animal and craniotomy is exposed (3 mice out of 38).

B) inflammatory liquid appears in the microscope cannula (2 mice out of 38).

In the above cases, it is suggested to perform termination procedures according to the approved animal protocol.

VIDEO:
1) Authors could add subtitles to the video. This could be helpful if the experimenter works with other people in the room.

We appreciate your comment, we will discuss it with the journal production team to get help with subtitles.

2) 00.33: prolong this framing to have enough time to read and understand all the information.

We have extended the duration of this frame on the screen. Please see corresponding changes at time 00:48 of the revised video file.

3) 00.20: add the list of material.

We have added the material list to the video file, please see time 00:35. We also have updated the material list in the written manuscript to include all important items description and lot numbers, please refer to the updated Material list file.

4) 00.38: it would be helpful to have a zoom of this moment to see the siringe, the flux drop and the soldering tin.

We have refilmed this scene to show the syringe, the flux drop, and the soldering tin at higher magnification. Please see time 00:56.

5) 00.44: if possible avoid out-of-focus during video.

We have refilmed this part of the video to avoid out-of-focus frames in the visual protocol. Please see time 01:23.

6) 01.11: prolong this framing and show how to test that the cannula is not blocked in both directions.

We have refilmed this part of the video to demonstrate testing of infusion cannula after implant assembling. Please see time 01:31.

7) 01.14: show the screen with the cannula in full screen modality in the video and not tilted.

We have adjusted the corresponding part of the video to show the cannula in the full-screen modality. Please see time 01:33.

8) 01.27: probably not too good to put the glass so displaced at the beginning and slide it after onto the optical adhesive. This would make it dirty. It is probably better to be more precise from the beginning and center it onto the imaging cannula.

We agree with the reviewer. To demonstrate the proper procedure, we have refilmed this part of the video to show the precise placing of the glass onto the imaging cannula. Please see time 01:45.

9) 02.55: the two oblique cuts are not very clearly shown with this framing.

We agree with the reviewer that this frame of the video is not shown very clearly. However, due to the limited access of the camera to the stereotaxic station, we could not get a better angle to refilm with the frame. We also believe that this is a very standard procedure for many craniotomy surgeries and viewers still can get the right guidelines with our video.

10) 04.10: write the coordinates for CA1 in this picture and give indications to draw the injection cannula area (maybe putting the coordinates of the most caudal point).

We have edited the video file to add coordinates of all five points required for drawing craniotomy area on the skull. Please see time 04:06.

11) 05.05: it would be helpful to have a magnification of this part, zooming on the exposed brain. Also, clarify how to distinguish the cortex from the CC and the CC from the hippocampus below to properly terminate the aspiration. Pictures of three different moments of the aspiration could be used with arrows pointing to the different tissues.

We thank the reviewer for the valuable comment. We took a video of the cortex ablation step at higher resolution and supplemented it with schematic representation to further improve the demonstration of this procedure. Please see time 05:33 and 06:59. We also added corresponding images to Figure 3 of the revised written manuscript.

12) 05.11: is it possible to gently clean the surface and stop the bleeding with a sponge?

Continuous bleeding is inevitable during this step, and to some extent, continuous bleeding is a sign for the normal blood pressure. Unlike cortex imaging window implantation, the presence of

blood under optical window is acceptable since it will be cleared in several days after the surgery. Insertion of the imaging cannula to the created cavity as soon as possible after ablating the brain tissue is optimal. We added the corresponding note to the written manuscript. Please see P5 Step 2.2.19.

13) 05.36: from the video it looks like the window will remain pretty much outside the skull. Giving an indication about this will help to understand how much the experimenter has to push the window inside the hole. Additionally: must the window be parallel to the skull surface? Will errors in this passage affect the optical accessibility under the microscope?

To provide more details on the implant insertion step we revised the video file, written manuscript, and add extra images to Figure 4. The critical point of this step is to make sure that the cover glass of the imaging cannula is placed right against the hippocampus, which may require gentle pressure on top of the cannula during the installation and sealing process. Whether the upper side of the imaging cannula is parallel to the skull is not critical for the final optical access as long as the optical window is placed right against the hippocampus. According to the average thickness of the cortex above the CA1 area, it is suggested that the upper surface of the imaging cannula is kept above the skull surface by ~0.5 mm to facilitate attachment of the cannula to the skull (Figure 4). Accordingly, please find the revised visualization of this step in the video file at time 07:10. We also added corresponding notes to the manuscript at Page 5 Step 2.2.21, and inserted a schematic representation of the mouse skull with the implant in Figure 4.

14) 08.15: virus injection: it would be nice to have a picture of the green dye under the window during virus injection.

We added images of FastGreen dye spread under the imaging window both to the video file at time 10:15 and introduced new Figure 5 showing imaging widow before and after injection of a virus supplemented with FastGreen dye.

15) 08.55: show an example picture of 4X best field of view.

We have edited the video file to show a representative image of hippocampus expressing green calcium sensor under 10x objective lens. Please see time 10:56.

16) 09.00: in the manuscript there is an indication to switch the lens for the imaging. Write in the video which objective lens has been used for this recording.

We have revised the video file to indicate the magnification of the objective lenses used for imaging. Please see time 10:56.

Reviewer #2:
Manuscript Summary:

In the manuscript and video, Wang et al describe the preparation and implantation of the custom-made imaging window to enable chronic in vivo imaging of the CA1 region in head fixed behaving mice. By combining this implantation with wide-field imaging, a long-term recording of neuronal activity in behaving mice was performed over several weeks. As a method paper, the manuscript and the video clearly demonstrated the method itself. Although the method itself is not entirely new, it will be useful for researchers interested in chronic imaging in hippocampus of behaving mice.

Minor Concerns:
I hope the authors could address my following concerns before publication.

(1) It is challenging to remove the cortex and the corpus callosum above the hippocampus, however, it is lacking information of how the operations were done in the video (~ 05:10 in the video). I would suggest the authors to provide more details about that.

We agree with the review that this part may need more details on the procedure. To improve visualization of the procedure, we took a video of the cortex ablation step at higher resolution and supplemented it with schematic representation to further improve the demonstration of this procedure. Please see time 04:50. In addition, we have inserted representative images of cortex, corpus callosum, and hippocampus taken during surgery into the written manuscript. Please see Page 5 2.2.19 and revised Figure 3.

(2) It is unclear about the strength of suction using 26G needle to ablate the cortex and the corpus callosum above the hippocampus. It will be better that the authors could provide a description for that.

To address this comment, we have measured the pressure created by the pump that was used for cortex ablation. The applied pressure was about -60 kPa. We have added the corresponding number to the main text of the revised manuscript. Please see Page 5 Step 2.2.19.

(3) In Figure 5, it is confusing about the ROIs and the corresponding neuronal signals, the authors should label the ROIs in Panel A and the neuronal activities in Panel B.
We thank Reviewer for the valuable suggestion. We have revised Figure 5 to number the ROIs and the corresponding optical traces in Panel B and C, respectively. Please see revised Figure 5.

(4) In Figure 6, it is unclear about the imaging data quality. Hence it would be very helpful if the authors could also explain how to evaluate the imaging data quality.

We agree with the reviewer that some images have some out-of-focus fluorescence reducing image quality. One of the major factors contributing to the reduced imaging quality was continuous expression of the fluorescent indicator, which resulted in more out-of-focus fluorescence. To express the green fluorescent calcium indicator we used AAV/DJ-CAG vector, which was driving strong expression of gene of interest even 21 days after virus delivery (please see Supplementary Figure 1 of the revised manuscript). Quantification of the green fluorescence

from the same FOVs revealed more than 50% increase in fluorescence background and appearance of new neurons expressing the calcium indicator. Therefore, selection of the AAV serotype and promoter to drive target gene expression should be one of the important considerations during experimental design in particular if longitudinal imaging of the same subset of neurons is required. We have discussed this issue in the main text of the written manuscript to make readers aware of this experimental consideration. Please see Page 12 Paragraph 2 and Supplementary Figure 1.

Reviewer #3:

Manuscript Summary:

The manuscript by Piatkevich et al. describes a craniotomy procedure for visualizing neuronal activities in the hippocampus of awake mice. The authors provide a detailed description of steps necessary for fabrication of the implant, successful implantation surgery, imaging from the hippocampus and post-hoc validation of implant location and RCaMP

Major Concerns:

- The 45 degree angle between the imaging and injection cannula requires a huge craniotomy (2-3 mm cannula diameter as I can estimate from Fig. 1C + additional 2-3 mm required for 450 angle attachment), and may not generate a lot of interest in the field. Have the authors tried to vary this angle to make it sharper and less invasive for the upper lying.

Indeed, we found that the infusion cannula would usually need around 0.5 mm bigger craniotomy. However, if this angle is too small, the imaging process would be very like to be affected due to the limitation from the working distance of the objective length as the infusion cannula will collide with objective lens before the focal plane of the objective reaches the field of interest. Typically, 45-degree angle of the infusion cannula would allow most of the objective lenses to be used for imaging, at the same time limiting the craniotomy area as much as possible.

- Virus injection is usually done in the same time with implantation surgery. It actually saves time for experiment as post-implantation recovery occurs in the same time with virus expression. The authors state in the Discussion that the method they use helps somehow to achieve a lower efficiency transfection to visualize single cells. I'm not sure how it works and what is the actual benefit of having virus to be injected after implantation surgery.

The described imaging widow is used for wide-field single-photon imaging at single-cell resolution. Since during wide-field excitation the fluorophore above and below the focal plane are excited with high efficiency creating significant out-of-focus fluorescence. Out-of-focus fluorescence reduces imaging quality of cells in the focal plane. One way to minimize out-of-focus fluorescence is to achieve the expression of fluorescent indicator in a thin layer of brain tissue. Injection of virus right under the imaging window helps to restrict expression of transgene to a thinner layer. Indeed, in case of cortical window implantation, it is convenient to inject virus in the same time with implantation surgery. However, this implantation procedure requires

cortex ablation that results bleeding. Viral injection during bleeding may wash away virus as well as lead to excess blood loss as injection usually takes 15-20 min. Therefore, to achieve optimal imaging quality under wide-field microscope, viral injection under imaging window after animal recovery is optimal.

The injection cannula allows for pharmacological manipulations with neuronal activity during chronic imaging. This is the major advantage of the described device and procedure. It would be nice to have a demonstration that it may work, because as of current it is unclear how far within imaging window the injected drugs can spread. Can you demonstrate it with Fast green for example and suggest the technical parameters for such local application? There is still a major doubt that it would be possible to apply drugs for distances exceeding 0.5 mm from the edge of the imaging cannula where the injection cannula is located, suggesting that imaging at the edge will be required if one wants to manipulate using drugs.

To address this comment, we have performed the experiment suggested by the Reviewer. We have imaged optical window before and after injection of virus supplemented with FastGreen. We observed that FastGreen quickly spread throughout the window going more than 1 mm away from the injection cannula. We have inserted representative images showing the diffusion of the FastGreen dye after injection to help visualizing the drug spreading situations. Please see Figure 4 of the revised manuscript.

Minor Concerns:
- I could not find the description of the imaging cannula in the Materials Table. Is it commercially available or custom-made?

The Material list has been edited to include the dimensions of the imaging cannula and three parts of the infusion cannula including lot numbers. Please refer to the updated Material list.

- Step 1.5: what do you consider as the "appropriate type of flux" in this procedure?

For this preparation, we used a commercially available flux that is specified by the manufacturer to be for soldering stainless steel parts as imaging and infusion cannulas are made of stainless steel. In the case of other materials used to manufacture cannulas, end-user should select flux that is appropriate for the selected material. Please see Page 3 Step 1.5.

- Step 1.12: can washing cannula in 70%-ethanol remove in part the adhesive and damage the whole implant?

The optical adhesive we chose to attach the cover glass with the imaging cannula could not be removed by 70% ethanol, so theoretically this washing procedure would not affect the quality of the implants. Moreover, we could even store prepared implants in 70% without affecting their

quality. We have added a corresponding note to the written manuscript. Please see Page 3 Step 1.12.

- Step 4.1: is it really necessary to anesthetize the animal to fix it on the treadmill? Training the animal to the fixation procedure would be desirable to avoid multiple anesthesia sessions during day-to-day chronic experiments.

We agree with the Reviewer that it is possible to habituate mouse for head fixation process without induction and it is even beneficial for day-to-day chronic experiments. We edited the written text to add a corresponding note. Please see Page 7 Step 4.1.

- Results: I doubt it would be possible to image up to 6-months, as overexpression of RCaMP may have undesirable side effects and extensive brain damage may even result in trauma-related seizures. Also, as shown in Fig. 6, by day 12, the quality of images starts to deteriorate and cells imaged previously are no longer detected. Consider limiting the imaging duration to maximum 30 days, which is currently a standard in the field.

The estimation of the chronic imaging duration is based on our previous publication describing population voltage imaging using SomArchon indicator (Piatkevich et al., 2019). In the main text of the manuscript, when we suggest the duration of the chronic imaging we refer to this paper "the same preparation has been used for up to 6 month-long imaging study in mice previously (Piatkevich et al., 2019)". However, we agree with the Reviewer that the quality of images starts to deteriorate in the later imaging sessions. One of the major factors contributing to the reduced imaging quality was continuous expression of the fluorescent indicator, which resulted in more out-of-focus fluorescence. To express green fluorescent calcium indicator we used AAV/DJ-CAG vector, which was driving strong expression of the gene of interest even 21 days after virus delivery (please see Supplementary Figure 1 of the revised manuscript). Quantification of the green fluorescence from the same FOVs revealed more than 50% increase in fluorescence background and appearance of new neurons expressing the calcium indicator. Therefore, selection of the AAV serotype and promoter to drive target gene expression should be one of the important considerations during experimental design in particular if longitudinal imaging of the same subset of neurons is required. We have discussed this issue in the main text of the written manuscript to make readers aware of this experimental consideration. Please see P8 first papragraph.

Supplemental Figure 1

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