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TITLE:

Using Baseplating and a Miniscope Preanchored with an Objective Lens for Calcium Transient Research in Mice

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SUMMARY:

The shrinkage of dental cement during curing displaces the baseplate. This protocol minimizes the problem by creating an initial foundation of the dental cement that leaves space to cement the baseplate. Weeks later, the baseplate can be cemented in position on this scaffold using little new cement, thereby reducing shrinkage.

ABSTRACT:

Neuroscientists use miniature microscopes (miniscopes) to observe neuronal activity in freely behaving animals. The University of California, Los Angeles (UCLA) Miniscope team provides open resources for researchers to build miniscopes themselves. The V3 UCLA Miniscope is one of the most popular open-source miniscopes currently in use. It permits imaging of the fluorescence transients emitted from genetically modified neurons through an objective lens implanted on the superficial cortex (a one-lens system), or in deep brain areas through a combination of a relay lens implanted in the deep brain and an objective lens that is preanchored in the miniscope to observe the relayed image (a two-lens system). Even under optimal conditions (when neurons express fluorescence indicators and the relay lens has been

properly implanted), a volume change of the dental cement between the baseplate and its attachment to the skull upon cement curing can cause misalignment with an altered distance between the objective and relay lenses, resulting in the poor image quality. A baseplate is a plate that helps mount the miniscope onto the skull and fixes the working distance between the objective and relay lenses. Thus, changes in the volume of the dental cement around the baseplate alter the distance between the lenses. The present protocol aims to minimize the misalignment problem caused by volume changes in the dental cement. The protocol reduces the misalignment by building an initial foundation of dental cement during relay lens implantation. The convalescence time after implantation is sufficient for the foundation of dental cement to cure the baseplate completely, so the baseplate can be cemented on this scaffold using as little new cement as possible. In the present article, we describe strategies for baseplating in mice to enable imaging of neuronal activity with an objective lens anchored in the miniscope.

INTRODUCTION:

Fluorescent activity reporters are ideal for imaging of the neuronal activity because they are sensitive and have large dynamic ranges[1-3]. Therefore, an increasing number of experiments are using fluorescence microscopy to directly observe neuronal activity[1-16]. The first miniaturized one-photon fluorescence microscope (miniscope) was designed in 2011 by Mark Schnitzer et al.[5]. This miniscope enables researchers to monitor the fluorescence dynamics of cerebellar cells in freely behaving animals[5] (i.e., without any physical restraint, head restraint, sedation, or anesthesia to the animals). Currently, the technique can be applied to monitor superficial brain areas such as the cortex[6,8,15,16]; subcortical areas such as the dorsal hippocampus[8,11,13,14] and striatum[6,17]; and deep brain areas such as the ventral hippocampus[14], amygdala[10,18], and hypothalamus[8,12].

In recent years, several open-source miniscopes have been developed[4-7,11,13,17,19]. The miniscope can be economically assembled by researchers if they follow the step-by-step guidelines provided by the University of California, Los Angeles (UCLA) Miniscope team[4,7,11,13]. Because optical monitoring of neural activity is restricted by the limitations of light transmission[7] to and from the neuronal population of interest, a miniscope was designed that requires an objective gradient refractive index (GRIN) lens (or objective lens) to be preanchored at the bottom of the miniscope to magnify the field of view that is relayed from a relay GRIN lens (or relay lens)[6-8,10,16,17]. This relay lens is implanted into the target brain region such that the fluorescence activity of the target brain region is relayed onto the surface of relay lens[6-8,10,16,17]. Approximately 1/4 of a full sinusoidal period of light travels through the objective GRIN lens (~ 0.25 pitch) (**Figure 1A1**), resulting in a magnified fluorescence image[6,7]. The objective lens is not always fixed at the bottom of the miniscope nor is the implantation

of the relay lens necessary[6,7,11,13,15]. Specifically, there are two configurations: one with a fixed objective lens in the miniscope and a relay lens implanted in the brain[8,10,12,14,16] (**Figure 1B1**) and another with just a removable objective lens[6,7,11,13,15] (**Figure 1B2**). In the design based on the fixed objective and implanted relay lens combination, the fluorescence signals from the brain are brought to the top surface of the relay lens (**Figure 1A1**)[7,8,10,12,14,16]. Subsequently, the objective lens can magnify and transmit the visual field from the top surface of the relay lens (**Figure 1A2**). On the other hand, the removable objective GRIN lens design is more flexible, which means preimplantation of a relay lens into the brain is not mandatory (**Figure 1B2**)[6,7,11,13,15]. When using a miniscope based on a removable objective lens design, researchers still need to implant a lens into the target brain region but they can either implant an objective lens[6,7,11,13,15] or a relay lens in the brain[6,7]. The choice of an objective or a relay lens for implantation determines the miniscope configuration that the researcher must use. For instance, the V3 UCLA Miniscope is based on a removable objective GRIN lens design. Researchers can choose either to directly implant an objective lens in the brain region of interest and mount the "empty" miniscope onto the objective lens[6,7,11,13,15] (a one-lens system; **Figure 1B2**) or to implant a relay lens in the brain and mount a miniscope that is preanchored with an objective lens[6,7] (a two-lens system; **Figure 1B1**). The miniscope then works as a fluorescence camera to capture livestream images of neuronal fluorescence produced by a genetically encoded calcium indicator[1-3]. After the miniscope is connected to a computer, these fluorescence images can be transferred to the computer and saved as video clips. Researchers can study neuronal activity by analyzing the relative changes in fluorescence with some analysis packages[20,21] or write their codes for future analysis.

The V3 UCLA Miniscope provides flexibility for users to determine whether to image neuronal activity with a one- or two-lens system[7]. The choice of the recording system is based on the depth and size of the target brain area. In brief, a one-lens system can only image an area that is superficial (less than approximately 2.5 mm deep) and relatively large (larger than approximately 1.8 x 1.8 mm^2) because the manufacturers only produce a certain size of objective lens. In contrast, a two-lens system can be applied to any target brain area. However, the dental cement for gluing the baseplate tends to cause misalignment with an altered distance between the objective and relay lenses, resulting in a poor image quality. If the two-lens system is being used, two working distances need to be precisely targeted to achieve the optimal imaging quality (**Figure 1A**). These two critical working distances are between the neurons and bottom surface of the relay lens, and between the top surface of the relay lens and the bottom surface of the objective lens (**Figure 1A1**). Any misalignment or misplacement of the lens outside of the working distance results in imaging failure (**Figure 1C2**). In contrast, the one-lens system requires only one precise working distance. However, objective lens size limits its application for monitoring of deep brain regions (the objective lens that fits the

miniscope is approximately 1.8 ~ 2.0 mm[6,11,13,15]). Therefore, implantation of an objective lens is limited for the observation of the surface and relatively large brain regions, such as the cortex[6,15] and dorsal cornu ammonis 1 (CA1) in mice[11,13] . In addition, a large area of the cortex must be aspirated to target the dorsal CA1[11,13]. Because of the limitation of the one-lens configuration that prevents imaging of deep brain regions, commercial miniscope systems offer only a combined objective lens/relay lens (two-lens) design. On the other hand, the V3 UCLA miniscope can be modified into either a one-lens or two-lens system because its objective lens is removable[6,11,13,15]. In other words, V3 UCLA miniscope users can take advantage of the removable lens by implanting it in the brain (creating a one-lens system), when performing experiments involving superficial brain observations (less than 2.5 mm in depth), or by preanchoring it in the miniscope and implanting a relay lens in the brain (creating a two-lens system), when performing experiments involving deep brain observations. The two-lens system can also be applied for superficially observing the brain, but the researcher must know the accurate working distances between the objective lens and relay lens. The main advantage of the one-lens system is that there is a decreased chance of missing the working distances than with a two-lens system, given that there are two working distances that need to be precisely targeted to achieve optimal imaging quality in the two-lens system (**Figure 1A**). Therefore, we recommend using a one-lens system for superficial brain observations. However, if the experiment requires imaging in the deep brain area, the researcher must learn to avoid misalignment of the two lenses.

The basic protocol for two-lens configuration of miniscopes for experiments includes lens implantation and baseplating[8,10,16,17]. Baseplating is the gluing of a baseplate onto an animal's head so that the miniscope can be eventually mounted on top of the animal and videotape the fluorescence signals of neurons (**Figure 1B**). This procedure involves using dental cement to glue the baseplate onto the skull (**Figure 1C**), but the shrinkage of dental cement can cause unacceptable changes in the distance between the implanted relay lens and the objective lens[8,17]. If the shifted distance between the two lenses is too large, cells cannot be brought into focus.

Detailed protocols for deep brain calcium imaging experiments using miniscopes have already been published[8,10,16,17]. The authors of these protocols have used the Inscopix system[8,10,16] or other customized designs[17] and have described the experimental procedures for viral selection, surgery, and baseplate attachment. However, their protocols cannot be precisely applied to other open-source systems, such as the V3 UCLA Miniscope system, NINscope[6], and Finchscope[19]. Misalignment of the two lenses can occur during the recording in a two-lens configuration with a UCLA Miniscope due to the type of dental cement that is used to cement the baseplate to the skull[8,17] (**Figure 1C**). The present protocol is needed because the distance

between the implanted relay lens and the objective lens is prone to shift due to the undesirable shrinkage of dental cement during the baseplating procedure. During baseplating, the optimal working distance between the implanted relay lens and the objective lens must be found by adjusting the distance between the miniscope and the top of the relay lens, and the baseplate should then be glued at this ideal location. After the correct distance between the objective lens and the implanted relay lens is set, longitudinal measurements can be obtained at cellular resolution (**Figure 1B**; *in vivo* recording). Since the optimal range of working distances of a relay lens is small (50 - 350 µm)[4,8], excessive cement shrinkage during curing can make it difficult to keep the objective lens and the implanted relay lens within the appropriate range. The overall goal of this report is to provide a protocol to reduce the shrinkage problems[8,17] that occur during the baseplating procedure and to increase the success rate of miniscope recordings of fluorescence signals in a two-lens configuration. Successful miniscope recording is defined as recording of a livestream of noticeable relative changes in the fluorescence of individual neurons in a freely behaving animal. Although different brands of dental cement have different shrinkage rates, researchers can select a brand that has been previously tested[6-8,10-16,22]. However, not every brand is easy to obtain in some countries/regions due to the import regulations for medical materials. Therefore, we have developed methods to test the shrinkage rates of available dental cements and, importantly provide an alternative protocol that minimizes the shrinkage problem. The advantage over the present baseplating protocol is an increase in the success rate of calcium imaging with tools and cement that can be easily obtained in laboratories. The UCLA miniscope is used as an example, but the protocol is also applicable to other miniscopes. In this report, we describe an optimized baseplating procedure and also recommend some strategies for fitting the UCLA miniscope two-lens system (**Figure 2A**). Both examples of successful implantation (n= 3 mice) and examples of failed implantation (n=2 mice) for the two-lens configuration with the UCLA miniscope are presented along with the discussions for the reasons of the successes and failures.

PROTOCOL

All procedures performed in this study were approved by the National Taiwan University Animal Care and Use Committee (Approval No.: NTU-109-EL-00029).

1. Assessment of the volume alteration of dental cement

NOTE: Changes in the volume of dental cement occur during the curing process. Test the volume changes of dental cement before implantation and baseplating. Researchers can test any brand of dental cement and use the brand with the lowest volume change to cement the

baseplate. An example is shown in **Figure 3**.

1.1. Weigh 0.5 g of each dental cement powder and mix it with its appropriate solution (1 mL).

NOTE: The recommended powder/liquid ratio in the dental cement instructions is 0.5 g of powder to 0.25 mL of the solution to obtain a solid form. This testing protocol dilutes the ratio to 0.5 g/mL so that the mixture can be aspirated in liquid form in order to measure the volume change in pipette tips.

1.2. Use 0.1–10 µL pipette tips to remove 2.5 µL of the dental cement mixture, and then seal the pipette tip with a light curing glue.

1.3. Place the tips on a rack, mark the top-most levels of the dental cement mixture, and measure the level of the dental cement mixture after 40 min (**Supplementary video S1**).

1.4. In addition to monitoring the level changes during the dental cement curing in the tips, measure the remaining dry dental cement density. To calculate the density, measure the weight of the dental cement, and measure the volume with Archimedes' principle.

1.4.1. In brief, place the remaining dry dental cement in a cup filled with water, and weigh the water that overflows.

1.5. Use the dental cement with the least shrinkage for all the protocols detailed below.

2. Anesthesia, surgical implantation, viral injection, and dummy baseplating

2.1. Anesthesia

NOTE: The present report aims to optimize the surgery and baseplating procedure for the UCLA miniscope; therefore, it was assumed that the optimal viral titer for infecting the target brain regions was known. The methods for finding the optimal viral titer can be found in steps 1 to 5 of Resendez et al.[8]. Once the viral dilution has been optimized, surgical implantation can be initiated.

2.1.1. Place the mouse in an induction container and provide oxygen (100% at an airflow rate of 0.2 L/min). Preoxygenate the mouse for 5 to 10 min.

2.1.2. Administer 5% isoflurane mixed with 100% oxygen (airflow rate: 0.2 L/min) until the mouse starts to lose its balance and is eventually anesthetized.

2.1.3. Remove the mouse from the induction chamber and inject atropine (0.04 mg/kg; subcutaneous injection), to prevent the accumulation of saliva and buprenorphine (0.03 mg/kg; subcutaneous injection) as an analgesic.

2.1.4. Shave the mouse's head.

2.1.5. Wear a mask, sterile gown, and gloves. Prepare a sterile space and sterile surgical instruments for surgery. Stabilize the mouse's head (maintain anesthesia with 3 to 2.5% isoflurane during this step) on the stereotaxic apparatus. After the analgesic and anesthetic procedures, ensure that the ear bars securely stabilize the head.

2.1.6. Ensure that the head is set in a straight position, sterilize the shaved head with betadine, and then spray the head with xylocaine (10%), a local analgesic.

2.1.7. Apply veterinary ointment on the eyes to prevent dryness.

2.1.8. Test the animal's deep pain reflex by pinching its hind paw. Once the animal shows no paw withdrawal reflex, proceed with surgical procedures.

2.1.9. Make a small incision along the mid-sagittal plane of the skull (starting from approximately 2 mm anterior to the bregma and ending approximately 6 mm posterior to the bregma). Then, clean the connective tissue on the skull, and anchor three stainless steel screws onto the left frontal and interparietal bones.

2.1.9.1. At this point, reduce the isoflurane to 1-2%. Monitor the breathing rate during the whole surgical procedure. If the breathing rate is too slow (approximately 1/s, depending on the animal), decrease the concentration of isoflurane.

2.1.10. Execute a craniotomy for the relay lens implantation under a surgical microscope or stereomicroscope by using a burr drill with a 0.7 mm tip diameter. Use a micro-drill to grab the burr drill bit and draw an outline of the intended circle area (the full thickness of the calvarium does not need to be penetrated).

NOTE: The relay lens used in the present protocol had a diameter of 1.0 mm, a length ~ 9.0 mm a pitch of 1, and a working distance range of ~100 µm - 300 µm; therefore, the craniotomy

had a diameter of 1.2 mm.

2.1.10.1. Gently deepen the outline until the dura is exposed.

2.1.10.2. Prepare 3 mL of sterile saline in a 3 mL syringe and cool it in an ice bucket. Frequently rinse the exposed area with 0.1 mL of saline from the syringe to cool the area and prevent heat damage and hemorrhage.

2.1.11. Lightly pick and peel away the dura with a 27G needle.

2.1.12. Put a marking on a 27 G blunt needle at 1 mm from the tip and use it to carefully aspirate the brain's cortex in order to create a window for lens implantation[8,11,13,17] (**Figure 2B1**). If needed, make a blunt needle by grinding down the tip of a 27 G injection needle with sandpaper. Then, connect the needle to a syringe, connect the syringe to a tube, and connect the tube to a source of suction to create a vacuum.

NOTE: The relay lens is blunt and will compress the brain tissue when it is placed into the deep brain area. Thus, aspiration of the cortex reduces tissue damage due to relay lens implantation. The cortex is approximately 1 mm thick in mice but is difficult to visualize under a stereomicroscope. The mark creates a landmark to allow the depth of the needle to be determined more precisely than with a stereomicroscope alone. In addition, one can determine whether the cortex region has been reached or passed depending on the resistance; the top portion of the cortex feels relatively soft, while the subcortical region feels dense. Therefore, once the texture begins to feel denser, stop the aspiration (**Figure 2B3**).

2.1.13. Prepare 3 mL of sterile saline in a 3 mL syringe and cool it in an ice bucket. Rinse the area with saline to stop the bleeding and decrease the possibility of brain edema.

2.2 Adeno-associated virus (AAV) injection

NOTE: AAV9-syn-jGCaMP7s-WPRE was used in the present experiment. jGCaMP7s is a genetically encoded calcium indicator that emits green fluorescence[3]. Because the present study used a wild-type mouse as a subject, a viral vector was needed to transfect the neurons with the green-fluorescent calcium indicator gene and enable the expression. Researchers using transgenic mice expressing the green-fluorescent calcium indicator as their subjects can skip protocol 2.2.

2.2.1. Mount the inner needle of a 20 G intravenous (i.v.) catheter onto the stereotaxic arm

and slowly puncture (~100 – 200 µm/min) the brain at the appropriate coordinates (the same coordinates used during relay lens implantation) (**Figure 2B3**).

2.2.2. Lower the microinjection needle at a speed of 100–200 µm/min into the ventral CA1 and infuse (~ 25 nL/min) 200 nL of the viral vector into the target region (-3.16 mm AP, 3.25 mm ML, and -3.50 mm DV from the bregma).

2.2.3. Wait for 10 min to allow the virus to diffuse and to minimize the back flow.

2.2.4. Withdraw (~100–200 µm/min) the microinjection needle.

2.3 Relay lens implantation

2.3.1. Sterilize the relay lens with 75% alcohol and then rinse with pyrogen-free saline. Soak the lens in cold pyrogen-free saline until implantation.

NOTE: A cold lens minimizes brain edema when placed into the brain.

2.3.2. Hold the relay lens using a micro bulldog clamp (**Figure 2B3**) whose teeth have been covered with heat-shrink tubing.

NOTE: The bulldog clamp can firmly hold the lens without damaging it. Refer to Resendez et al.[8] for the method to make the tubing-covered bulldog clamp.

2.3.3. Slowly place (~100 - 200 µm/min) the relay lens on top of the target region (-3.16 mm AP, 3.50 mm ML, and -3.50 mm DV from the bregma) and stabilize it with dental cement.

2.4 Dummy baseplating

NOTE: The purpose of "dummy baseplating" during the implantation surgery is to create a dental cement base in order to reduce the amount of dental cement that needs to be applied during the real baseplating procedure several weeks later. In this manner, the risk of a change in the volume of the dental cement is minimized.

2.4.1. Fasten the objective lens on the bottom of the miniscope and assemble the baseplate onto the miniscope (in this step, the assembly of the anchored objective lens, baseplate, and miniscope has not yet been placed over the mouse's skull).

2.4.2. Wrap 10 cm of paraffin film around the outside of the baseplate (**Figure 4A**).

2.4.3. Hold the miniscope with the stereotaxic arm probe using reusable adhesive clay.

2.4.4. Align the objective lens on top of the relay lens with as little space between the lenses as possible (**Figure 4B**).

2.4.5. Use dental cement to secure the positioning of the baseplate (such that the cement touches only the paraffin film).

2.4.6. When the dental cement has dried, remove the film.

2.4.7. Remove the baseplate and the miniscope. The dental cement base is hollow (**Figure 4B**).

2.4.8. To protect the relay lens from daily activities and from being scratched by the mouse, seal the relay lens with some molding silicone rubber until the relay lens is covered, and cover the silicone rubber with a thin layer of dental cement (**Figure 4B**).

2.4.9. Disengage the mouse from the stereotaxic instruments and place the mouse into a recovery chamber.

2.4.10. Inject carprofen (5 mg/kg; subcutaneous injection) for analgesia and ceftazidime (25 mg/kg; subcutaneous injection) to prevent any infection.

2.4.11. Watch the animal until it regains sufficient consciousness to maintain sternal recumbency.

2.4.12. House mice individually and provide ibuprofen in water (0.15 mg/mL) for one week to relieve postsurgical pain. Check the animal every day after surgery to make sure that it is eating, drinking, and defecating normally.

2.4.13. Perform baseplating after 3 or more weeks.

3. Baseplating

NOTE: Usually, the baseplating procedure (**Figure 5**) cannot be performed within 2-3 weeks of the initial procedure due to the recovery and viral incubation time. The induction procedures

for baseplating are similar to those described in steps 2.1.1 to 2.1.8. However, baseplating is not an invasive procedure, and the animal requires only light sedation. Therefore, 0.8-1.2% isoflurane is sufficient, as long as the animal cannot move on the stereotaxic frame. In addition, relatively light isoflurane anesthesia can also help facilitate the expression of the fluorescence transients of neurons during monitoring[8] (**Supplementary video S2**).

3.1. Carefully cut the thin layer of the dental cement roof with a bone rongeur and remove the silicone rubber. Clean the surface of the relay lens with 75% alcohol.

3.2. Fasten a set screw beside the baseplate to fix it to the bottom of the miniscope (**Figure 5A1**).

NOTE: The baseplate must be tightened securely under the bottom of the miniscope because the difference between a tightened and lightly fastened baseplate may cause an inconsistent distance.

3.3. Adjust the focus slide to be approximately 2.7 - 3 mm from the main housing (**Figure 5A2**).

NOTE: Although the length can be further adjusted during the baseplating procedure, fix it to approximately 2.7 mm, because simultaneously adjusting the miniscope length and the optimal length between the implanted relay lens and preanchored objective lens is very confusing.

3.4. Connect the miniscope to the data acquisition board and plug it into a USB 3.0 (or higher version) port on a computer.

3.5. Run the data acquisition software developed by the UCLA team.

3.6. Adjust the exposure to 255, the gain to 64x, and the excitation LED to 5%. These settings are recommended for initial baseplating; the specific settings will vary depending on the brain regions and the expression levels of the genetically encoded calcium indicator.

3.7. Click the **Connect** button to connect the miniscope to the data acquisition software and watch the livestream.

3.8. Align the miniscope objective lens to the relay lens by hand and begin searching for the fluorescence signals (**Figure 5B$_1$**).

NOTE: Initially, searching for the fluorescence signal manually facilitates adjustments. Because an AAV system was used to express the genetically encoded calcium indicator, both the promoter type and AAV serotype affected the efficiency of transfection[23,24]. Researchers should need to check the expression of the calcium indicator by finding individual neurons that show changes in fluorescence before proceeding with future experiments.

3.9. Drill the dental cement in any place where it blocks the miniscope (optional).

3.10. Watch the live stream of the data acquisition software and use the margin of the relay lens as a landmark (**Supplementary video S2**). The margin of the relay lens appears as a moon-like gray circle on the monitor (**Figure 5B1**; white arrows).

3.11. Once the relay lens is found, carefully adjust the various angles and distances of the miniscope until the fluorescence signals are found.

NOTE: The images of the neurons are whiter than the background. A precise neuronal shape indicates that the neurons lie perfectly on the focal plane of the relay lens. Round neurons suggest that they were near the focal plane. The neuronal shape is different across the brain, here ventral CA1 is shown as an example.

3.12. If no individual neuron displays changes in fluorescence as a function of time, reseal the base with silicone rubber. Fluorescence is not observed because the incubation period is also dependent on the property of the virus[23,24]. Perform steps 3.1-3.12 after an additional week. (This is optional).

3.13. Hold the miniscope at the optimal position and move the stereotaxic arm with a reusable adhesive clay towards the miniscope (**Figure 5B2**). Adhere the miniscope to the stereotaxic arm with reusable adhesive clay.

3.14. Slightly adjust the x, y, and z arms to search for the best view (**Supplementary video S2**). Next, adjust the angle between the surface of the objective lens and relay lens by turning the ear bar, tooth bar, or reusable adhesive clay on the z-axis. Viral expression and lens implantation is successful when at least one cell displays fluorescence transients (**Figure 6A**; **Supplementary video S2**) during baseplating (which also depends on the area of the brain, such as the CA1).

NOTE: If the monitor shows only white cells but no transients, there is another cause (see **Figure 6B,C**, **Supplementary videos S4**,S5, the Results section, and the troubleshooting

section).

3.16. Cement the baseplate (**Figure 5B2**) with the dental cement.

3.17. Monitor the region of interest during cementing to ensure that the optimal position does not change.

3.18. Use the smallest possible amount of cement while still firmly gluing the baseplate onto the dental cement base (**Figure 5B2**). Apply a second and third layer of cement around the baseplate but be careful not to affect the GRIN lens.

NOTE: Applying cement to the baseplate is easier in this step since the base of the baseplate was formed during the dummy plating procedure.

3.19. Carefully detach the miniscope from the baseplate when the cement is cured. Then, screw on a protective cap.

3.20. Move the animal to a recovery cage. Watch the animal until it regains sufficient consciousness to maintain sternal recumbency.

3.21. House mice individually. Make sure that it is eating, drinking, and defecating normally every day. Wait for 5 days for the mouse to fully recover and then check the calcium imaging.

NOTE: There is only a small amount of dental cement holding the baseplate. Therefore, if the baseplate has shifted considerably since the baseplating procedure, remove the dental cement with a drill perform the baseplating procedure again.

3.22. Euthanize (by intraperitoneal injection of a ketamine (87 mg/kg) /xylazine (13 mg/kg) mixture) and perfuse[25] the animal when all experiments are done.

REPRESENTATIVE RESULTS

Assessment of the dental cement volume alteration

Since the volume of dental cement changes during the curing process, it may significantly impact the imaging quality, given that the working distance of a GRIN lens is approximately 50 to 350 µm[4,8]. Therefore, two commercially available dental cements were tested in this case, Tempron and Tokuso, before the implantation and baseplating procedure (**Figure 5**). Videos were first evaluated to determine whether the cements had shrunk and then the volumes of

cements were compared when completely dried. The same concentration and volumes of the initial mixtures of dental cements were loaded into pipette tips and videotaped (**Supplementary video S1**). Video S1 demonstrated that both cements shrunk during the drying process. Tokuso performed better than Tempron (i.e, it had a larger volume than Tempron even after shrinking): **Figure 3B**; mean volume of Tempron: 0.93 ± 0.07 mL; mean volume of Tokuso: 1.18 ± 0.07 mL; t-test: $p = 0.048$, $t_{(6)} = 2.46$, suggesting that it shrunk less. The densities of the cements were also tested 4 times after complete drying. Tokuso had a lower mean density than Tempron (mean density of Tempron: 1.07 ± 0.12 g/mL; mean density of Tokuso: 0.93 ± 0.08 g/mL; t-test: $p = 0.38$, $t_{(6)} = -0.94$), implying that it had a lower shrinkage rate. Therefore, Tokuso was used for the following implantation and baseplating steps. The purpose in describing the experiment above is not to recommend any brands but rather to remind experimenters to find a dental cement that does not undergo a considerable volume change when curing.

Measurement of image shifting

We simulated the dummy baseplating and original baseplating procedures on a fixed object to investigate whether the dummy baseplating procedure results in a smaller shift in the baseplate location than the original procedure. A light-cure adhesive was used to fix a 23 G 2.5 cm syringe needle in the center of a baseplate with 1 cm sticking out of the baseplate (**Figure 3C**). Then, baseplating was simulated by holding the needles with the arm of a stereotaxic instrument. Instead of using laboratory animals, a sticker was attached onto the table of the stereotaxic instrument. The syringe needle pierced the sticker, thereby representing the original location. Then, the needle was elevated by 0.5 mm and the simulation surgeries were started. In a surgery mimicking the one-time baseplating procedure[8], dental cement was applied until it covered the baseplate, which was 1 cm above the sticker. Then, there was a 2 h wait period for the cement to cure before gently pushing the needle to pierce the sticker again. Since the light-cure adhesive did not completely stabilize the needle, the needle could be pushed deeper to create another hole, representing the location of the baseplate after the dental cement had dried. The distance between the initial hole and the second hole was measured to quantify the location shift of the needle. The results demonstrated that baseplate 1 cm above the skull resulted in a 1.76 ± 0.14 mm location shift, but the dummy baseplating resulted in shift of only 0.75 ± 0.17 mm (**Figure 3D**; mean shift distance of one-time baseplating versus dummy baseplating; t-test: $p < 0.01$, $t_{(8)} = 6.03$).

Additionally, we were curious about whether the height of the dental cement would influence the shift. Therefore, we further tested a shorter height (**Figure 3E**; 0.5 cm above) by using the one-time baseplating procedure. The baseplate that was anchored 0.5 cm above the skull shifted significantly less than the baseplate 1 cm above the skull (**Figure 5F**; mean shift

distance: 0.43 ± 0.11 vs 1.76 ± 0.14 mm; t-test: $p < 0.01$, $t_{(8)} = 9.73$). Data suggested that the height of the baseplate above the skull and the distance the baseplate shifted were positively correlated. Since the dummy baseplating procedure is followed by a second baseplating step that uses only a minimal amount of dental cement, this data also supports the hypothesis that dummy baseplating helps improve the precision of the baseplating procedure.

Imaging using a two-lens miniscope system

By following the surgical protocols for viral infusion, dummy baseplating, and baseplating (**Figure 2, Figure 4, Figure 5**), we observed fluorescence transients of individual neurons in three mice (n = 3/5) (**Figure 6A**, **Supplementary video S3**) during the baseplating procedure and after the baseplating procedure while the mice were freely moving, confirming that the working distance between the objective and relay lenses remained the same (**Figure 6A1** versus **Figure 6A3**, only a slight shift in position occurred) after the dental cement had completely dried. Here, we provide videos of the baseplating of one mouse (**Supplementary video S2**; mouse #5) and subsequent calcium imaging while mice were moving freely (**Supplementary video S3**; mouse #3, #4, #5; raw data). Please note that the data acquisition software for V3 does not contain functions for the background subtraction, and the contrast of the video can be improved by offline processing). For **Supplementary video S2**, a manual search was performed for the fluorescence signals and then the miniscope was attached to the stereotaxic arm for fine adjustments. Some fluorescence transients were observed during the probing (**Supplementary video S2**; **Figure 6A**). A transient is a smooth shift in brightness in gray or white spots, not a firing pattern resembling a flashing light; **Figures $6A_1$** to **$6A_2$** display images of transients. The video of freely behaving mice showed an acceptable spatial shift compared with the region of interest during the baseplating procedure (**Supplementary video S3**). The margins of the cells could not be significantly improved at this stage because we could not adjust the distance between the bottom of the relay lens and the active neurons. Notably, some issues may frequently occur during the baseplating procedure, including an absence of anything except a uniform background (**Figure 6B; Supplementary video S4**) or visibility of white cell margins without no fluorescence transients (**Figure 6C**; **Supplementary video S5**). Two examples of negative results are also provided (n = 2/5) (**Figure 6B,C**, **Supplementary videos S4, S5**). The potential reasons for the failures are examined in the Discussion section. The surgical procedures for these two mice were performed without using a 1 mm diameter needle to clear the pathway; thus, the relay lens was directly implanted. We propose that in mouse #2, the relay lens may have compressed the neurons and dragged some neurons down, resulting in damaged neurons. Therefore, fluorescence transients were not found.

FIGURE AND TABLE LEGENDS:

Figure 1: Mechanisms of the miniscope and the defects that usually cause imaging failure. (**A**) Cartoon diagram of the relay lens and objective lens displaying the mechanisms necessary for visualization of fluorescent cells in a two-lens system. The signaling waves of each lens emphasize the importance of and the mechanism involved in finding a working distance to visualize the fluorescent cells. (**A1**) The relay GRIN lens is placed above the target neurons within the working distance. Since the pitch of the relay lens is 0.5*N (where N is an integer), the lens enables visualization of target neurons by relaying light, bringing the original fluorescence signals to the top of the relay lens. Then, approximately 1/4 of a full sinusoidal period of light travels through the objective GRIN lens (~ 0.25 pitch) and results in a magnified image. (**A2**) The objective lens transmits the images to a complementary metal-oxide-semiconductor (CMOS) sensor located on the top (the green plate in the diagram) of the miniscope. (**B**) Cartoon diagram presenting the differences in the GRIN lens implantation procedures between a (**B1**) two-lens system and (**B2**) a one-lens system. In general, the major difference is that the one-lens system directly uses its objective lens to image the surface of the brain; as a result, the objective lens needs to be implanted on top of the target neurons. (**C**) In contrast, in the two-lens system, in comparison, an additional relay lens is implanted in the brain and the objective lens is preanchored in the miniscope. (**C1**) The shape of the baseplate for the V3 version of the UCLA Miniscope may cause an uneven shift after the dental cement completely dries. (**C2**) This shift causes misalignment or deviation from the working distance between the relay lens and the objective lens.

Figure 2: Modified protocols and detailed surgical protocols to improve the success rate of the two-lens miniscope system. (**A1**) Modified surgical protocol for the two-lens system. The locations of the viral injection, and relay GRIN lens implantation are shown. Dummy baseplating allows the working distance to accurately encompass the neurons of interest and reduces the positional shifting associated with the (**A2**) baseplating procedure. Hence, (**A3**) the probability of imaging fluorescent cells in freely behaving mice is increased. (**B**) Cartoon image of the steps for viral injection, and relay GRIN lens placement. (**B1**) First, mark the aspiration needle with a pen approximately 1 mm (for mouse experiments) from the tip to aid in assessment of the cortex aspiration depth. Then, aspirate the connective tissue and cortex (approximately 1 mm deep in mice) from the brain. (**B2**) Use the ventral hippocampus as the target area to implant a relay lens; notably, the tough corpus callosum is the landmark for stopping aspiration of the cortical region. The fibrous texture of the corpus callosum can be seen under the stereomicroscope during aspiration. (**B3**) Second, puncture the point of interest with an inner needle of a 20 G catheter (approximately 1 mm in diameter; the diameter is similar to that of the relay lens) to clear a pathway before viral infusion. Under the stereomicroscope, a dent (dashed circle in the picture) created by the needle should be visible. Lower the microinjection needle and relay lens at the dent (or only 250 μm away from the

center of the dent). Finally, place the relay GRIN lens (in the present study, we used a relay lens 1 mm in diameter).

Figure 3: Measurement of the volume change of the cured dental cement. (**A**) An example with cured dental cement of two brands. The black arrows indicate the original levels after we mixed the powder and solution. The white arrows indicate the levels after 10, 20, and 40 min of curing. The red arrows point to the bottoms of the tips, which were sealed with light-cure glue. (**B**) Mean volumes of the remaining dental cements after they completely dried. The bars and error bars are the means ± SEMs. * denotes significance ($P < 0.05$) as determined by Student's t-test. (**C**) Illustrations and a photograph explaining the method used to measure the location shift of the baseplate. A needle was glued through the baseplate and was used to simulate the original baseplating procedure (one-step baseplating) and the dummy baseplating procedure. The red and black arrows represent the locations of the needle before and after the dental cement was cured. (**D**) Mean distance changes of the tip of the needle (**E**) Illustrations of the method used to measure the relation between the baseplate above skull level and the location shift. (**F**) Mean distance changes of the tip of the needle. The bars and error bars are the means ± SEMs. ** denotes significance ($P < 0.01$) as determined by Student's t-test.

Figure 4: Detailed dummy baseplating procedure (executed immediately after relay lens implantation). (**A1**) Three-step cartoon of objective lens, baseplate, and paraffin film assembly on the miniscope. (**A2**) Photograph version of the assembly. (**B1**) Cartoon showing the dummy baseplating procedure. First, the assembled miniscope is aligned with the relay lens. The objective lens positioned is as close to the relay lens as possible. Second, dental cement is added to the parafilm surrounding the baseplate and applied onto the skull to make a female mold for future baseplating. Then, the baseplate is removed, and molding silicon rubber (pink) is added and topped off with dental cement to protect the female mold and relay lens. The animal is then allowed to recover from anesthesia. After at least 3 weeks, the baseplating procedure is performed. (**B2**) Photograph version of (**B1**).

Figure 5: Steps for the baseplating procedure. (**A1**) Attach the baseplate and (**A2**) manually adjust the focus slider to 2.7 to 3 mm. A setting of 2.7 to 3 mm (at the position shown in the picture) seems to be the best place to start before manually searching for the fluorescent cells. (**B1**) The first cartoon diagram illustrates the importance of the length between the objective lens and the relay lens for visualization of the fluorescent cells (fluorescent cells are usually observed at a distance between 0.5 and 2 mm). Before the fluorescent cell emerges, the researcher should see the margin of the relay lens (arrows in the top picture). It is helpful to first manually adjust the lens to the optimal viewing position because it is easier to make quick

adjustments by hand. (**B2**) Place reusable adhesive clay onto the stereotaxic arm probe, and then stabilize the miniscope by adhering it onto the arm with reusable adhesive clay. At this stage, the region of interest may have shifted, but this can be easily fixed by slightly adjusting the stereotaxic arm and the reusable adhesive clay. After checking for and finding the optimal region of interest, add a small amount of dental cement (represented in red). Glue the baseplate with as little dental cement as possible. Separate the miniscope and baseplate after the dental cement dries. If the baseplate is not stable enough, add additional dental cement. (**C**) This diagram illustrates the importance of every step for achieving success.

Figure 6: Examples of success and failure during the baseplating procedure. (**A**) An example of successful imaging during the baseplating procedure. The relay lens targeted the ventral hippocampus. Fluorescence transients were noticed when the animal was under anesthesia with isoflurane (see also **Supplementary video S2**). (**A2**) The contrast was increased for better visibility. The red dashed lines indicate blood vessels. (**A3**) Fluorescence transients five days after baseplating when the mouse (#5) was freely behaving in its homecage (see also **Supplementary video S3**; the images of mice #3 and #4 are also provided in the **Supplementary video S3**). Only a slight shift in position occurred. (**B1**) An example of a failure. During baseplating, only homogeneous white/gray areas were noticed that were not cell-like in shape (see also **Supplementary video S4**). (**B2**) The relay lens missed the target area and was located above a region with no infected cells. (**C1**) Another example of a failure. In this mouse, although many round cells were observed, no fluorescence transients were observed during the baseplating (after three weeks of incubation and while the mouse was anesthetized/sedated). Furthermore, no fluorescence transients were onserved when the baseplating procedure was performed again (in the fifth week of incubation) or even when the animal was freely behaving. The image was obtained during baseplating at the fifth week of incubation (see also **Supplementary video S5**). (**C2 and C3**) The relay lens missed the pyramidal layer of the ventral hippocampus. The blue dots are nuclei that were stained with DAPI.

Table 1: Troubleshooting chart.

Supplementary video S1: Dental cement volume test. The difference between the volume were measured at the beginning of the dental cement preparation and that the volume after the cement dried. Two brands of dental cement (e.g., Tempron and Tokuso Curefast) were tested. To test the volume of the dental cement after it dried, 0.5 g of each dental cement powder were weighed and mixed with its appropriate solution (1 mL). Then, 0.1–10 µL pipette tips were loaded with 2.5 µL of the dental cement mixture and sealed with light-cure glue. Then, the tips were placed on a rack, the surfaces of the dental cement mixtures were marked,

and videotaped for 40 minutes.

Supplementary video S2: Demonstrations of the baseplating procedure. The angle between the surface of the objective lens and relay lens was adjusted by turning the ear bar, tooth bar, or reusable adhesive clay on the z-axis. The margin of the relay lens appeared as a moon-like gray circle on the monitor. Successful viral expression and lens implantation should reveal at least one fluorescence dynamic (arrows) during baseplating.

Supplementary video S3: Demonstrations of fluorescence dynamics in freely behaving mice. Fluorescence transients from mice #3, #4, and #5.

Supplementary video S4: An example of a failure. A uniform background appeared during the baseplating procedure. Please note that the alteration in brightness was not because of the fluorescence transients of individual cells; it was caused by the search procedure.

Supplementary video S5: Demonstration of another failure example. A lack of fluorescence transients occurred during the baseplating procedure, but some round cell margins were still visible. Please note that the alteration in brightness was not caused by the fluorescence transients of individual cells; it was caused by the search procedure.

DISCUSSION

The present report describes a detailed experimental protocol for researchers using the two-lens UCLA Miniscope system. The tools designed in our protocol are relatively affordable for any laboratory that wishes to try *in vivo* calcium imaging. Some protocols, such as viral injection, lens implantation, dummy baseplating, and baseplating, could also be used for other versions of the miniscope system to improve the success rate of calcium imaging. Other than general problems with viral injection, and lens implantation that need to be fixed regardless of the miniscope version, the structure of the UCLA baseplate may cause a positional shift, which can lead to mismatched working distances between the objective and relay lenses (**Figure 1C**). A modified experimental protocol was developed to minimize the positional shift of the two lenses (**Figures 2, Figure 4, Figure 5**). Successful calcium imaging can be achieved through a combination of successful viral expression, lens implantation, and baseplating procedures (**Figure 5C**). Using a two-lens system to observe deep brain areas requires a high level of accuracy in the baseplating procedures because the relative positions of the implanted relay lens and the objective lens also need to be accurate. This report not only presents a protocol that solves baseplating issues but also discusses some tips for viral injection, and lens implantation. Below, we discuss the baseplating procedures first, followed by the viral injection, and lens implantation procedures, and we describe some examples of failures

(**Figure 6**).

Baseplating

The V3 version of the UCLA Miniscope is currently the most commonly used design and can be ordered online. The baseplate of this version consists of two edges without walls (**Figure 1C1**), but its shape can cause an uneven shift (**Figure 1C1, C2**) after the dental cement is completely dry. The dummy baseplating procedures in our protocol (**Figure 4**) minimizes the misalignment and baseplate shifting issues because it uses paraffin film to reduce the available space for the dental cement during real baseplating (**Figure 5B**). The remaining space is still sufficient for finding an optimal imaging position during the real baseplating procedure. Therefore, the researcher can glue the baseplate with as little dental cement as possible (**Figure 5B2**). In addition, dummy baseplating procedure takes relatively little time (approximately 15 to 20 min) since it does not require a perfect distance between the objective lens and relay lens to be achieved. However, dummy baseplating is just one of the key factors for successful calcium imaging; for example, during monitoring of fluorescence signals, neurons may appear blurry if they are slightly off the working plane (depending on the working distance of the relay lens; often approximately 100 ~ 300 μm). The ability to improve the working distance is limited, since the working distance of the neurons is predetermined by the relay lens implantation procedure. An experiment was performed without the dummy baseplating procedure and the region of interest was always (in four out of four mice) missed after one-time baseplating. Therefore, these solutions were devised to reduce the shrinkage problem. Some light-cure dental cements may avoid the problem of shrinkage during curing because they cure very quickly; these cements may help researchers adjust the distance during the baseplating procedure. Although the range of wavelengths for exciting the calcium indicators and curing the dental cement are different, photobleaching during the baseplating procedure needs to be prevented. In order to prevent photobleaching, the range of wavelengths generated by the light source needs to be fairly narrow to avoid cross-reactions between the calcium indicators and the light-cure dental cement. Therefore, the use of light-cure dental cement is not recommended for the baseplating procedure. In addition, some specific brands of dental cements are frequently used for gluing baseplates by other research teams[6-8,10-16,22]. The low-shrinkage properties of these cements may solve the problem, but we did not have the opportunity to test these brands due to difficulties in purchasing (importing) them. Medical-grade products may be subject to import regulations depending on the country/region. If researchers have difficulty accessing the cements mentioned in the literature[6-8,10-16,22], our protocol will solve the dental cement shrinkage problem.

In addition to the shrinkage problem, structural wear can occur in the miniscope itself and on

the baseplate (especially the magnets in the baseplate) over the course of many experiments. Any small gap caused by wear reduces the stability of the distance between the two lenses. Again, accuracy over the whole optical path is key to imaging of calcium transients in freely behaving mice using a miniscope.

Troubleshooting of viral injection/lens implantation

Before infusing the virus into the brain region, it is recommended to mount a new needle onto the stereotaxic arm and slowly puncture the brain at the appropriate coordinates. Because the new needle is very sharp, this puncturing serves as a preparation step to clear the path (or cut a path) for the actual microinjection needle and relay lens and minimizes tissue damage, which can be caused by compression from the microinjection needle or relay lens. The sharp needle cuts a path for the blunt relay lens; thus, the tip of the needle should be lowered to the same coordinates with the relay lens. The inner needle of a 20 G I.V. catheter was chosen because it had a diameter of 1 mm, which was similar to the diameter of our relay lens. A different needle gauge can be used depending on the diameter of the relay lens.

Although the present study did not focus on the properties and titers of viral vectors, these factors are still critical for successful calcium imaging (**Figure 5C**). It is necessary to pretest the expression quality of the viral vector. Steps 1 to 5 of Resendez et al.'s protocol provide detailed methods for the identification of an optimal viral titer for calcium imaging experiments[8]. If pretesting cannot be conducted for some reason, it is recommended that beginners try a relatively high titer first. One of the disadvantages of using a high viral titer iscytotoxicity[8]. Dead neurons give rise to autofluorescence and are visible as bright white spots under the miniscope[8]. However, these dead neurons are good landmarks for beginners to find and practice the baseplating procedure. On the other hand, although infusion of a low-titer virus has a lower risk of killing cells, the fluorescence may be masked by the background if the cells do not show any fluorescence transients during the baseplating procedure. It is difficult to pinpoint the reasons for failure because the target neurons may be missed due to relay lens implantation or due to the poor expression by the viral vector. Determining the cause is very confusing without histological confirmation. Therefore, using relatively high-titer viruses is recommend if the viral vectors cannot be pretested.

Resendez et al. recommend inserting the microinjection needle 250 µm lateral and ventral to the placement of the lens. Our observations demonstrated that infusion of the virus at the same depth as the relay lens can also lead to the expression of good calcium signals. We propose that due to the possibility of infection and spread, infusing the virus at the same depth as the coordinate of the relay lens is optimal for reducing tissue damage and increasing the accuracy of the distance between the infected neurons and the relay lens. Resendez et al

also recommend performing viral injection, and lens implantation during the same surgery given the narrow range of working distances between target cells and the relay lens[8]. However, some researchers follow a protocol in which the viral infusion step and lens implantation step are conducted two (or more) weeks apart[18,26]. Lengthening the time between steps reduces the individual operative time. But in this protocol these two steps were merged into one surgery, because in the one-surgery protocol, the surgeon can monitor the position between the microinjection needle and the relay lens when lowering them into the target area, which reduces the chance of failed targeting (**Figure 2B3**).

For two mice (mice #1 and #2), the use of a needle to create a path for the lens was also omitted, and in mouse #2, round cell-like gray spots were observed (**Figure 6C**). However, fluorescence dynamics (**Supplementary video S5**) were not observed. Upon examining the brain slice of this example (Figure 6C2, C3), we postulated that the relay lens dragged some cells down while it was being lowered. These dragged neurons were unhealthy, so no activity was noticed during the baseplating procedure or during the experiment.

We had one mouse with a completely homogenous gray signal and no visible cell-like images during the baseplating procedure (**Figure 6B1**). After sacrificing the mouse its brain slices were observed. It was noticed that the lens was on the medial side of the curved pyramidal layer; the target area (**Figure 6B2**) was completely missed. These unsuccessful attempts were critical experiences that led us to modify our surgery protocol and ultimately succeed with three mice (Supplementary video S3).

Microinjection needle

The corpus callosum is a tough fibrous tract that overlays dorsal hippocampus. Because of its stiffness, it resists penetration by a flat-tipped microinjection needle. Therefore, we recommend using a microinjection needle with a beveled tip. This type of needle not only reduces tissue damage but also reduces the possibility of failure to infuse the virus into the target area due to blockage by nearby fibers. We have summarized the abovementioned problems in a troubleshooting chart (**Table 1**).

Time frame

The time frame for the entire procedure is summarized in **Figure 2A**, with days as the units. In detail, the surgery part (viral injection, relay lens implantation, dummy baseplating) may take 3 h for beginners or 1.5 h for sufficiently trained researchers. Mice have a small body size and fast metabolism, and they are more susceptible to health problems caused by long periods under anesthesia than larger species[27]. Researchers should aim to keep the time spent in surgery under 3 h. Real baseplating can be performed after 3 to 6 weeks of calcium indicator

expression. This procedure may take 1 h. The subsequent longitudinal calcium activity observation can be continued for more than 1 month.

Conclusions

The UCLA Miniscope is an open-source *in vivo* calcium imaging device. Without a ready-made, preinstalled baseplate or lens module, individuals conducting experiments need to be able to accurately achieve the optimal distance between the objective lens and the relay lens during the baseplating procedure. The increased difficulty of using the two-lens UCLA Miniscope system lies in the volume change of the dental cement. We have optimized the original protocols and minimized the defects caused by the problems mentioned above, thus increasing the success rate of calcium imaging with the two-lens UCLA Miniscope system.

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DISCLOSURES

This is not an industry-supported study. The authors report no financial conflicts of interest.

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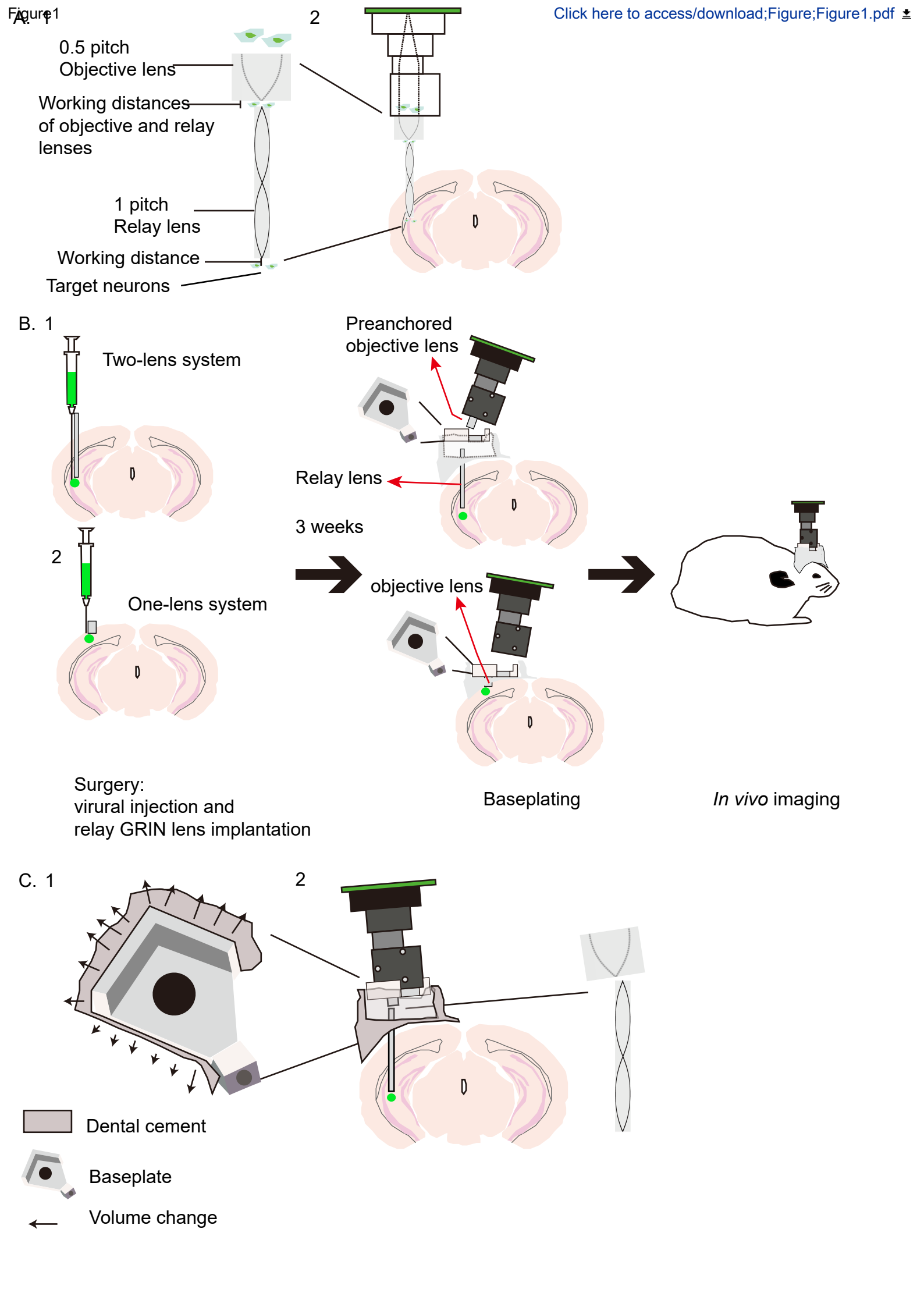
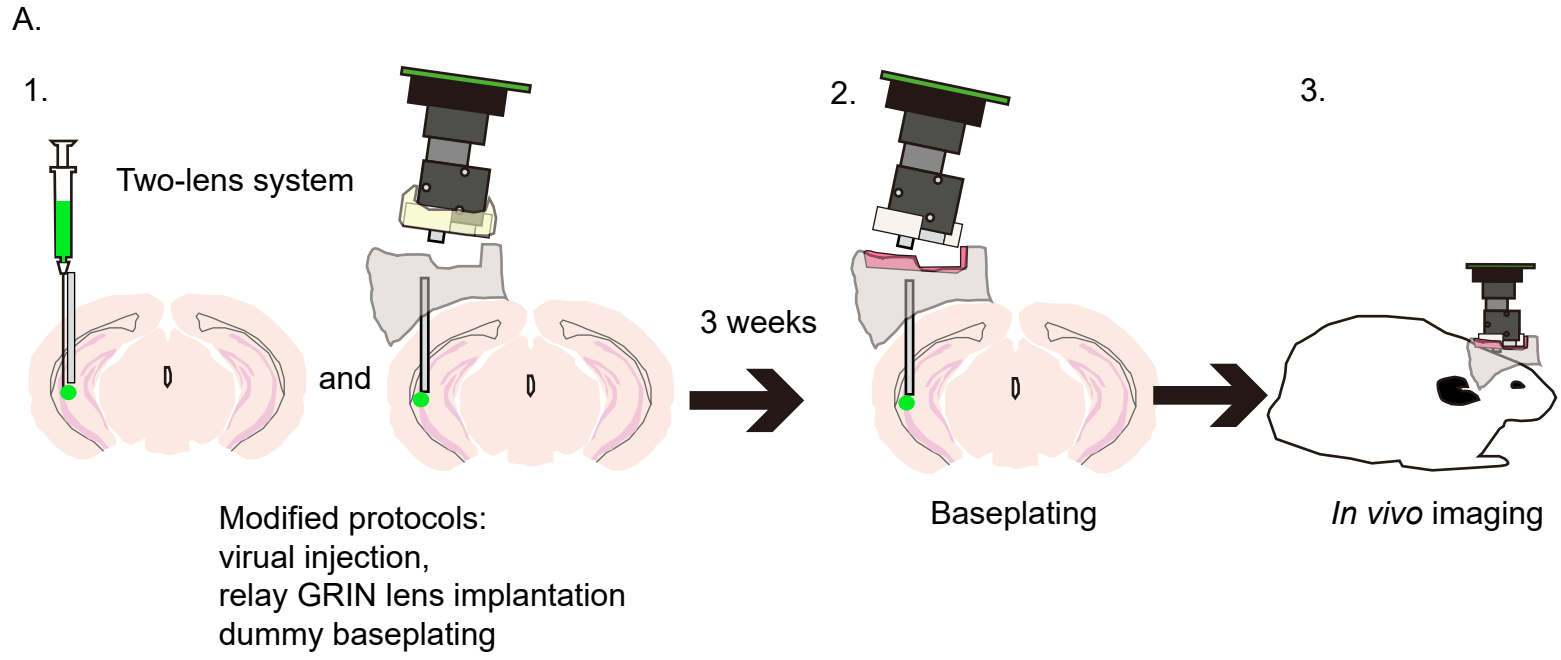
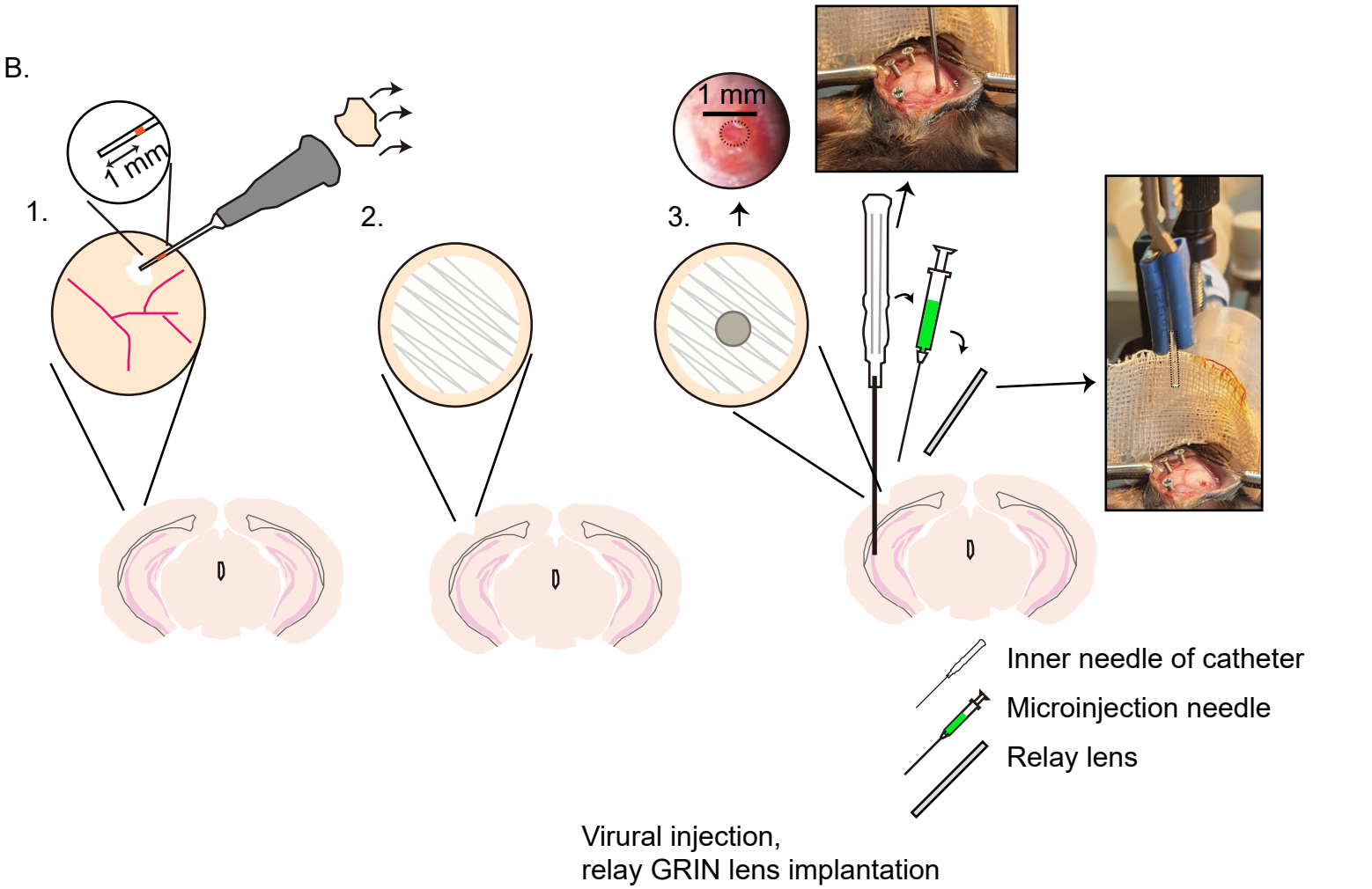
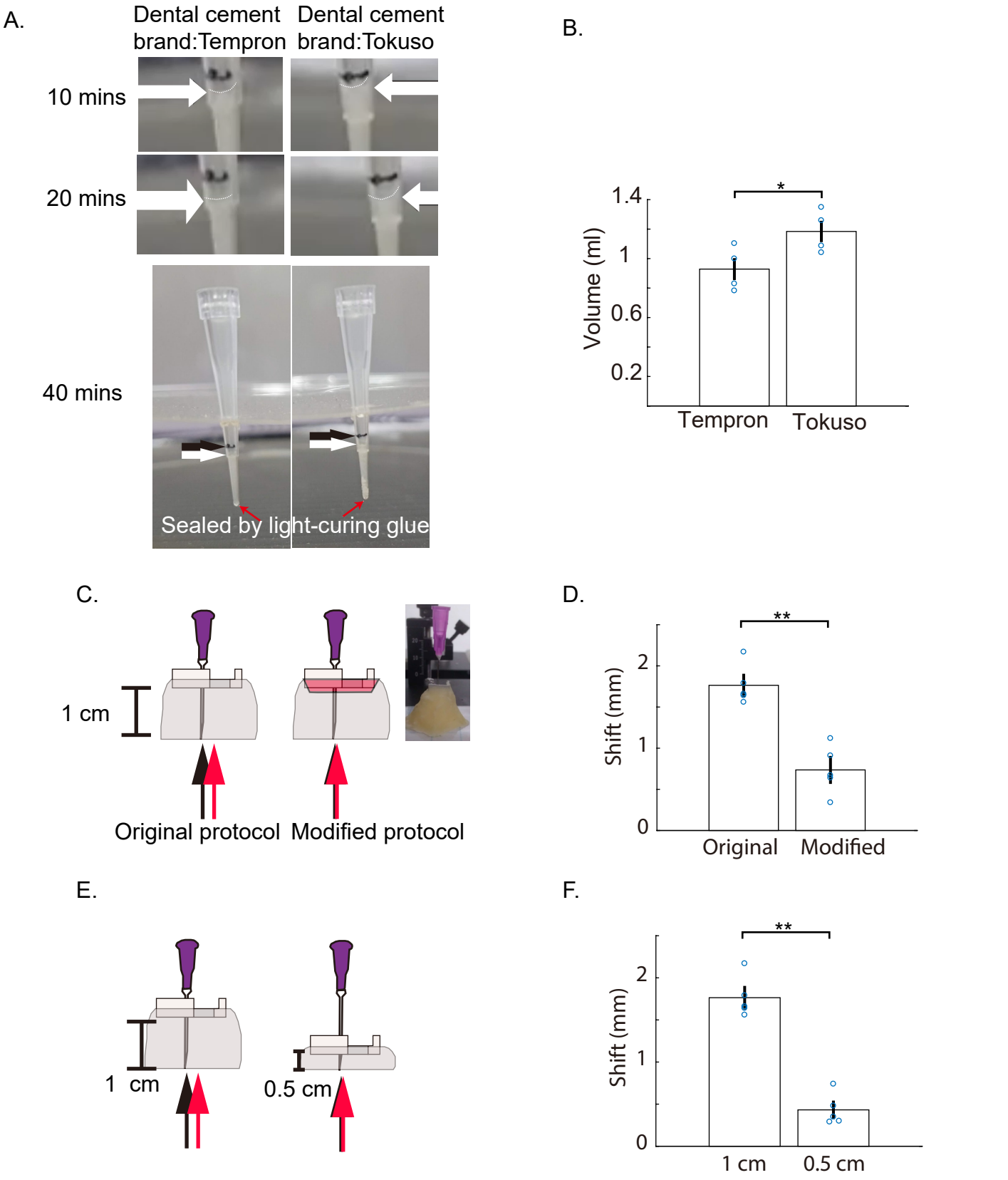


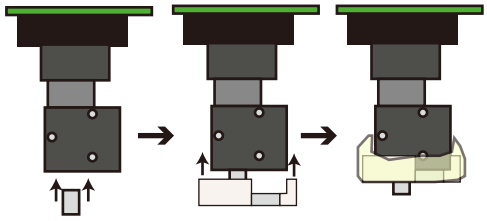




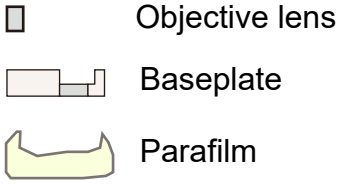


A.

1.



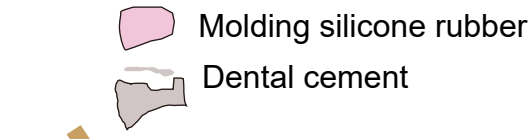
2.

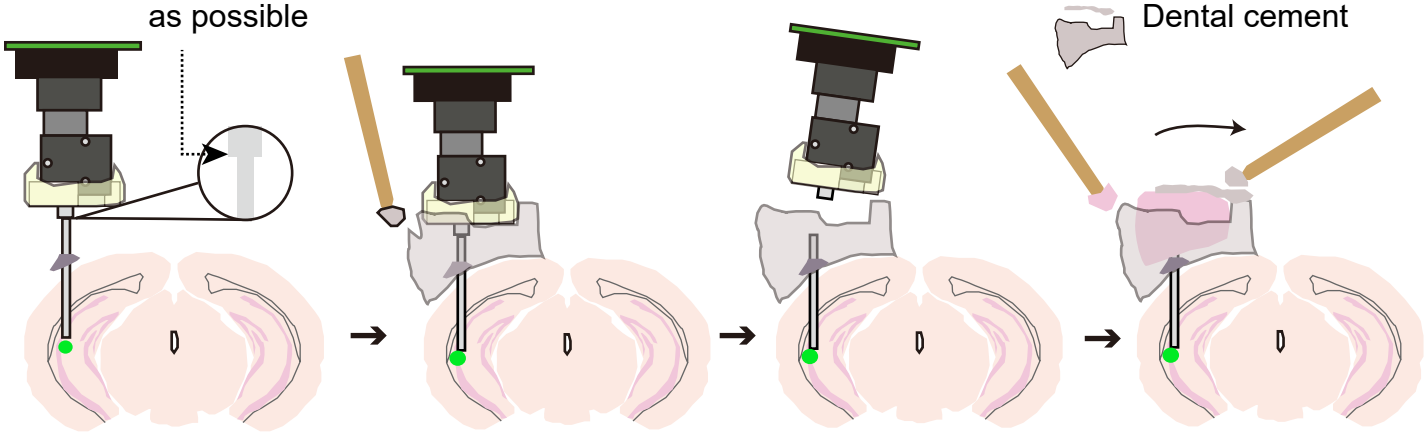


B.

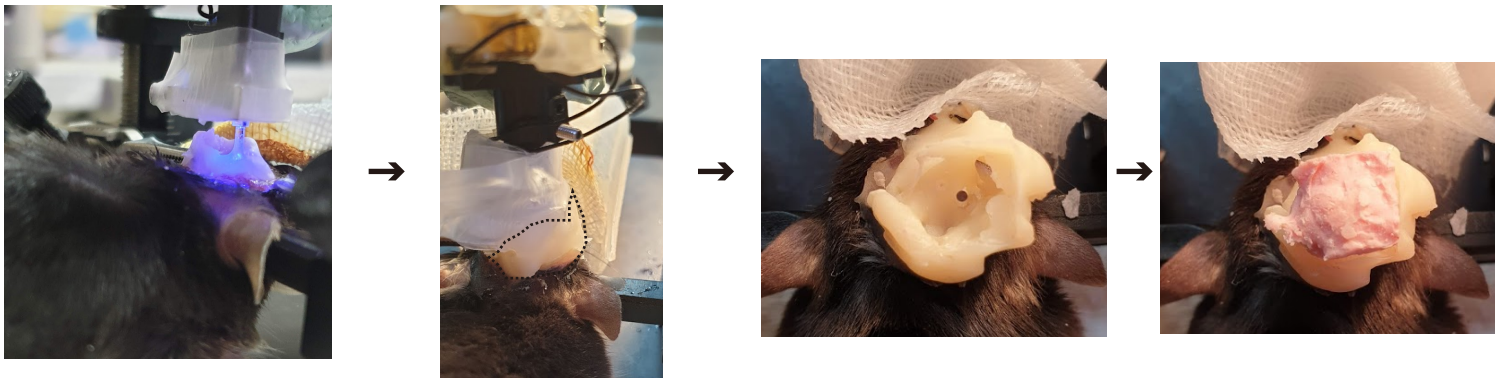
1.

As little space between as possible



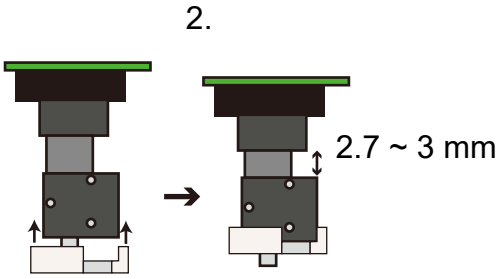


2.

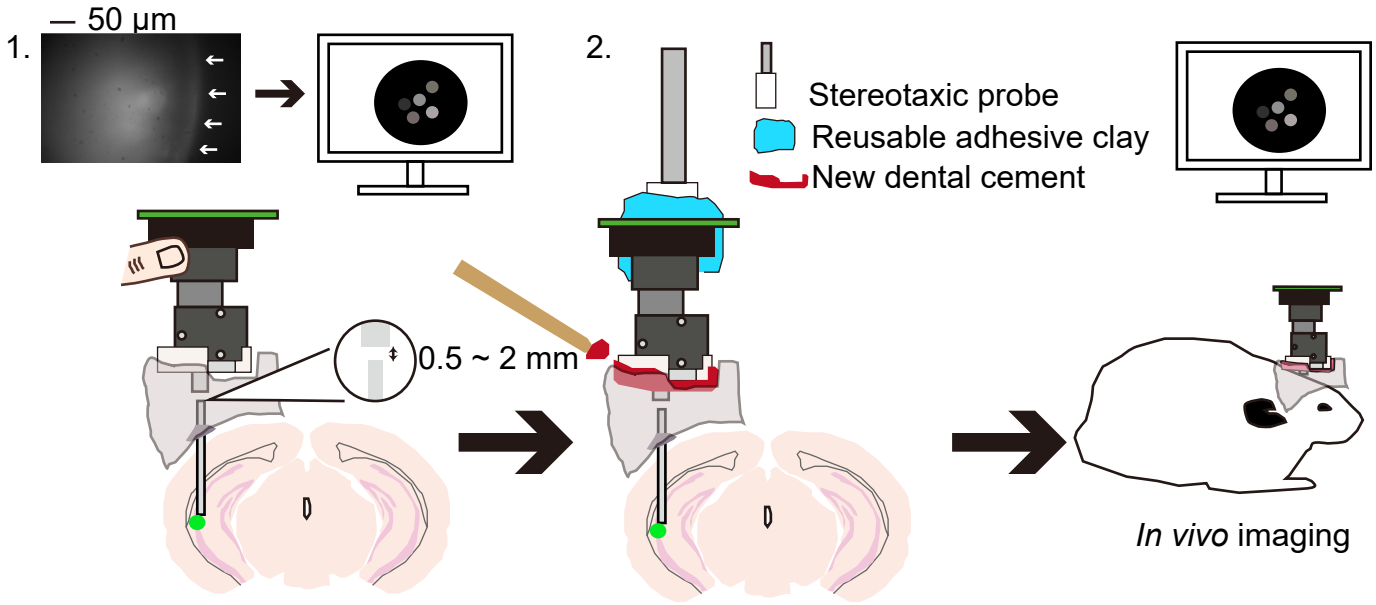


Dummy baseplating

A.

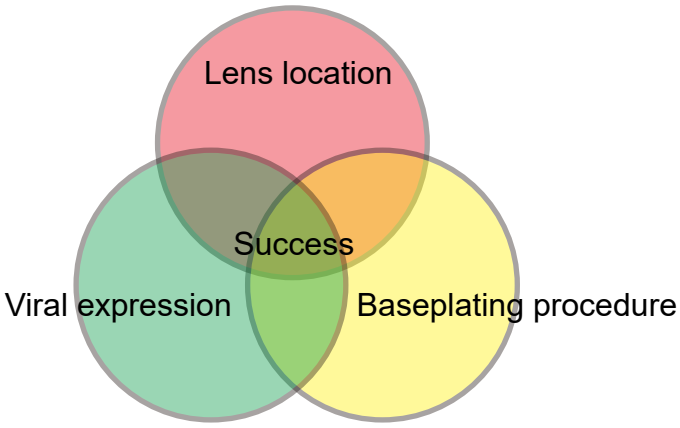


B.



Baseplating

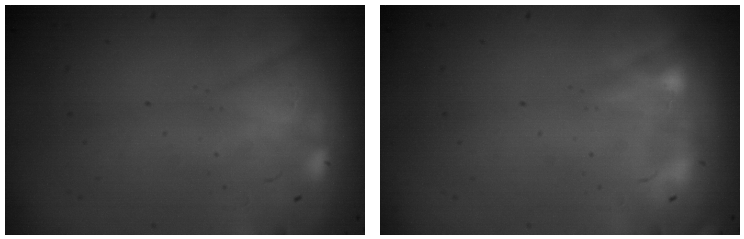
C.



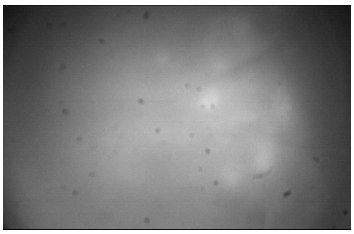
Mouse #5

A.

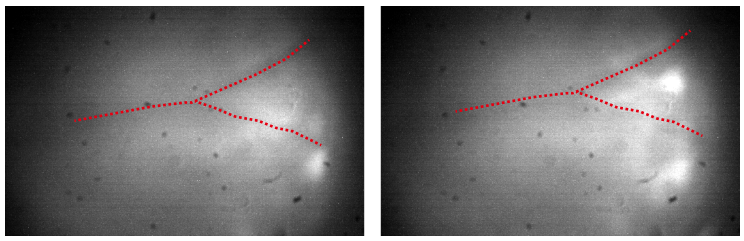
1.



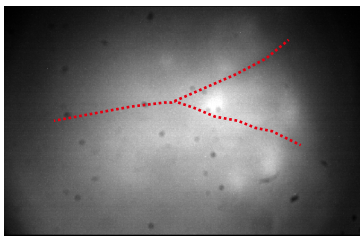
3.



2.



4.



Increase the contrast for better visibility

Fluorescence transients during baseplating procedure

Five days after the baseplating procedure while freely behaving

B.

Mouse #1

1.

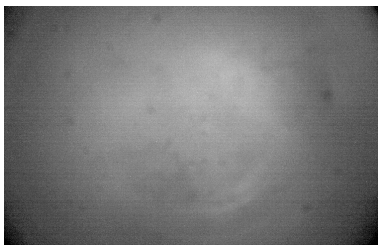
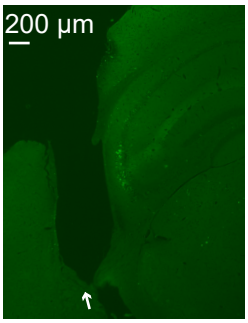


Image during baseplating

2.



Brain slice

C.

Mouse #2

1.

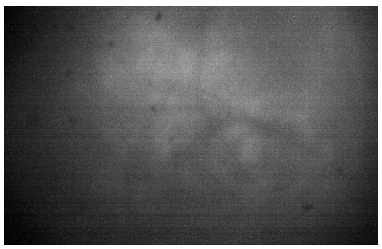
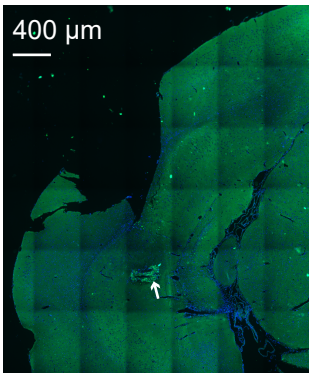
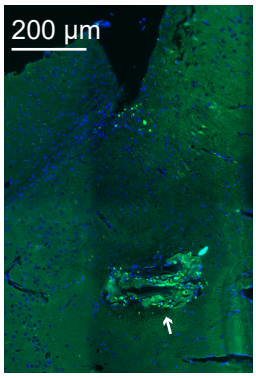


Image during baseplating

2.



3.



Brain slice

Figure 6

Supplementary video1

Click here to access/download

Video or Animated Figure

video1.wmv

supplementary video2

Click here to access/download

Video or Animated Figure

video2.wmv

supplementary video3

Click here to access/download

Video or Animated Figure

video3.wmv

supplementary video4

Click here to access/download

Video or Animated Figure

video4.wmv

supplementary video5

Click here to access/download

Video or Animated Figure

video5.wmv

Problem	Possible cause
Had good imaging when baseplating but completely out of focus the next day	Volume change upon curing the dental cement between the subject's skull and the baseplate
Has homogeneous white/gray areas without any cell-like shape during baseplating (also see supplementary video S4)	A. The relay lens missed the target area B. Bad expression of fluorescence indicator C. Forget to install an objective lens beneath the miniscope where the fluorescence indicator is actually expressed
Observe many cells but no fluorescence dynamics during baseplating	A. Unhealthy neurons or dead neurons B. Sparse fining type of neurons C. Deep anesthesia status

Solution
Proceed “dummy baseplating” after lens implantation procedure as suggested in the present report. Choose a dental cement that has less volume change.
A. Use dummy lenses for practicing surgeries. Check the coordinates of the dummy lens after the practicing implantation B. Find an optimal viral titer for calcium imaging experiments (referred to Resendez et al. 2016). If for some reason, pretesting cannot be conducted, we recommend that beginners try a higher titer first. (See Disscusion section for details) C. Screw on an objective GRIN lens (~ 0.25 pitch. ex: Edmund Optics; Stock #64-519) at the bottom of miniscope.
A. Be careful for every surgical procedure. Use a new syringing needle which is similar to the diameter of your relay lens to cut the infusion/implantation path first to release the pressure by lens implantation. Slowly infuse the virus and slowly implant the lens. B. Wait for a longer time to monitor the activities of neurons or pinch the tail a little bit to stimulate the mouse. C. Baseplating is not an invasive procedure, and the animal only needs sedation. Therefore, maintaining 1.2 ~ 0.8% isoflurane is sufficient, as long as the animal is unable to move on the stereotaxic frame.

Name of Material/ Equipment	Catalog Number	Company
0.7-mm drill bit	#19008-07	Fine Science Tools; USA
0.1–10 µl pipette tips	104-Q; QSP	Fisher Scientific; Singapore
20 G IV cathater	#SR-OX2032CA	Terumo Corporation; Tokyo, Japan
27 G needle	AGANI, AN*2713R	Terumo Corporation; Tokyo, Japan
AAV9-syn-jGCaMP7s-WPRE	#104487-AAV9; 1.5*10^13	Addgene viral prep; MA, USA
Atropine sulfate		Astart; Hsinchu, Taiwan
Baseplate	V3	http://miniscope.org
BLU TACK	#30840350	Bostik; Chelsea, Massachusetts, USA
Bone Rongeur Friedman	13 cm	Diener; Tuttlingen, Germany
Buprenorphine		INDIVIOR; UK
Carprofen	Rimadyl	Zoetis; Exton, PA
Ceftazidime		Taiwan Biotech; Taiwan
Data Acquisition PCB for UCL	purchased on https://www.labmaker.org/collections/neuroscience/prod	

Dental cement set	Tempron	GC Corp; Tokyo, Japan
Dental cement set	Tokuso Curefast	Tokuyama Dental Corp.; Tokyo, Japan
Dual Lab Standard with Mouse and Rat Adaptors	#51673	Stoelting Co; Illinois, USA
Duratear ointment		Alcon; Geneva, Switzerland
Ibuprofen		YungShin; Taiwan
Isoflurane		Panion & BF Biotech INC.; Taoyuan, Taiwan
Inscopix	nVista System	Inscopix; Palo Alto, CA
Ketamine		Pfizer; NY, NY
Normal saline		
Micro bulldog clamps	#12.102.04	Dimedo; Tuttlingen, Germany
Microliter Microsyringes, 2.0 µL, 25 gauge	#88400	Hamilton; Bonaduz, Switzerland
Molding silicone rubber	ZA22 Thixo	Zhermack; Badia Polesine, Italy
Objective Gradient index (GRIN) lens	#64519	Edmund Optics; NJ, USA
Parafilm	#PM996	Bemis; Neenah, USA

Portable Suction	#DF-750	Doctor's Friend Medical Instrument Co., Inc., Taichung, Taiwan
Relay GRIN lens	#1050-002177	Inscopix; Palo Alto, CA, USA
Stainless steel anchor screws	1.00 mm diameter, total length 3.00 mm	
Stereo microscope	#SL720	Sage Vison; New Taipei City, Taiwan
Stereotaxic apparatus	#51673	Stoelting; IL, USA
UV Cure Adhesive	#3321	Loctite; Düsseldorf, Germany
V3 UCLA Miniscope	purchased on https://www.labmaker.org/products/miniscope-complete-	
Xylazine	X1126	Sigma-Aldrich; St. Louis, MO
Xylocaine pump spray 10%		AstraZeneca; Södertälje, Sweden

Comments/Description
for surgery
for testing dental cement
for surgery
for surgery
for viral injection
for surgery/dummy baseplating/baseplating
for dummy baseplating/baseplating
Reusable adhesive clay; for surgery/dummy baseplating/baseplating
for baseplating
for surgery
analgesia
prevent infection
for baseplating

for testing dental cement

for testing dental cement/surgery/dummy baseplating/baseplating

for surgery/dummy baseplating/baseplating

for surgery/dummy baseplating/baseplating

analgesia

for surgery/dummy baseplating/baseplating

for comparison with V3 UCLA Miniscope

for euthanasia

for surgery

for lens implantation

for viral injection

for dummy baseplating

for dummy baseplating/baseplating

for dummy baseplating

for surgery

for dummy baseplating/baseplating

for surgery

for surgery/dummy baseplating/baseplating

for surgery/dummy baseplating/baseplating

for testing dental cement

for surgery/dummy baseplating/baseplating

for euthanasia

for surgery

From
Yi-Tse Hsiao, DVM, Ph.D.
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Taipei 106, Taiwan
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ythsiao@ntu.edu.tw

May 2021

Dear Editors,

We are grateful to the Editors and Reviewers for the positive comments and constructive feedback on our manuscript (Baseplating Procedure for Research on Calcium Transients in Mice using a Miniscope Preanchored with an Objective Lens (original title: Modified Surgery and Baseplating Procedures for the Two-Lens UCLA Miniscope System) JoVE62611R1). The Reviewers and Editors gave many informative suggestions.

We were able to address all comments and bring clarity. The following is our point-by-point responses to the Reviewer and Editorial comments (responses in red text). Attached files are clean version, marked version of revised manuscripts, revised Figures, and rebuttal letter.

We believe that this revised manuscript is appropriate for publication. Sincerely hope we can publish our work in Journal of Visualized Experiments! We like to share all the new ideals and modified baseplating procedures for anyone planning/(struggling) on executing in vivo calcium imaging experiments.

Best,

Dr. Yi-Tse Hsiao, DVM, Ph.D.
Department of Veterinary Medicine
National Taiwan University

The revised parts were marked with red. The **Line numbers** of the following responses come from the **tracked changes version** of manuscript. Clean version of manuscript is also available. Thank you very much for reviewing our article.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

2. Please address all the specific comments marked in the manuscript.

2.1 Please reword the title to reflect the protocol being presented.

We thank the editor for the suggestion. The Reviewer #5 think using "2-lens system" may let the reader think we are implanting 2 lenses in the brain. In combine with the Reviewer #5's suggestion, we modified the title to "Baseplating Procedure for Research on Calcium Transients in Mice using a Miniscope Preanchored with an Objective Lens "

2.2 SUMMARY: Pleases bring clarity with respect to the protocol being presented.

We rewrite whole summary that point out the problem and solutions within 50 words. **Line 26 to 30**. "*Shrinkage of dental cement during curing displaces the baseplate. The protocol minimizes this problem by creating an initial foundation of dental cement that leaves space for cementing of baseplate. Weeks later, the baseplate can be cemented in position on this scaffold using little new cement, reducing the problem of shrinkage.*"

2.3 SUMMARY: Original protocols of …. Please bring clarity.

We rewrite whole summary. The new summary brings out the problem and solution immediately in the text.

2.4 Abstract: The abstract only talks about the baseplating protocol but the title says surgery as well. Please align to clearly explain the goal of the protocol in 150-300 words.

We apologize for the confusion, the main goal of the protocol is improving the baseplating procedure; therefore, we remove "surgery" in the title and point out the goal of the protocol in the abstract and also the solution of the problem "*The goal of the present protocol is to minimize the misalignment problem caused by volume changes in dental cement. The protocol reduces the misalignment by building an initial foundation of dental cement at the time of relay lens implantation. The convalescence time after implantation sufficient for the foundation of dental cement to cure completely so that the baseplate can be cemented on this scaffold using as little new cement as possible.*" **Line 48 to 53**

2.5 Introduction:

Please ensure the Introduction include all of the following with citation:

a) **A clear statement of the overall goal of this method**

The revised introduction brings out the overall goal of this method in **Line 181 to 188**. ”*The overall goal of this report is to provide a protocol to reduce the shrinkage problems that occur during the baseplating procedure and to increase the success rate of miniscope recordings of fluorescence signals in a two-lens configuration. Successful miniscope recording is defined as recording of a livestream of noticeable relative changes in the fluorescence of individual neurons in a freely behaving animal.*”

b) **The rationale behind the development and/or use of this technique**

The rationale is using two lens system is prone to have shrinkage problem after baseplating, therefore we development a new baseplate protocol. The revised introduction brings out the rationale in **Line 160 to 165**. “*Misalignment of the two lenses can occur during recording in a two-lens configuration with a UCLA Miniscope due to the type of dental cement that is used to cement the baseplate to the skull (Figure 1C). The present protocol is needed because the distance between the implanted relay lens and the objective lens is prone to shift due to the undesirable shrinkage of dental cement during the baseplating procedure.*”

Line 186 to 191. “*Although different brands of dental cement have different shrinkage rates, researchers can select a brand that has been previously tested. However, not every brand is easy to obtain in some countries/regions due to the import regulations for medical materials. Therefore, we have developed methods to test the shrinkage rates of available dental cements and, importantly, we provide an alternative protocol that minimizes the shrinkage problem. “*

c) **The advantages over alternative techniques with applicable references to previous studies**

We apologize for not providing references from our lab because the relative experiments are still in process or is still in review process. We have saw many miniscope beginners come across baseplate problems; thus, we dedicate ourselves to solve the problems. Resendez et al and Zang et al’s protocol do not focus on shrinkage problem during baseplating too much and just mention “*lift the miniscope slightly after finding the best focal position*”. However, we think lift the miniscope is still not accurate. We also have long time trial and error test and finally development this technique. Therefore, we like to share the tips to the community before the actual research paper is out.

The revised introduction brings out the advantage in **Line: 191 to 193** “*The advantage over the present baseplating protocol is increase the success rate of*

calcium imaging with tools and cement that can be easily obtained in laboratories."

d) **A description of the context of the technique in the wider body of literature**
We added many new references and cited them in the relative descriptions of the introduction

e) **Information to help readers to determine whether the method is appropriate for their application**
We thank the Editor for the suggestion. We bring out the information to help reader in **Line 133 to 145**. "*In other words, the V3 UCLA Miniscope users can take advantage the of removable lens by implanting it in the brain (creating a one-lens system) when performing experiments involving superficial brain observations (less than 2.5 mm in depth) or by preanchoring it in the miniscope and implanting a relay lens in the brain (creating a two-lens system) when performing experiments involving deep brain observations. The two-lens system can also be applied to superficial brain observation, but the researcher must know the accurate working distances between the objective lens and relay lens. The main advantage of the one-lens system is that there is a decreased chance of missing the working distances than with a two-lens system, given that there are two working distances that need to be precisely targeted to achieve optimal imaging quality in the two-lens system (Figure 1A). Therefore, we recommend using a one-lens system for superficial brain observations. However, if the experiment requires imaging in the deep brain area, the researcher must learn to avoid misalignment of the two lenses.*"

2.6 Awake, non anesthetized?
Yes, non-anesthetized, sorry for the confusion. We modified the sentences to "*freely behaving animals (i.e., without any physical restraint, head restraint, sedation, or anesthesia to the animals).*" **Line 66 to 68**.

2.7 More clarity on this part is needed (objective gradient-refractive-index (GRIN) lens (or objective lens) to magnify and to combine with a relay GRIN lens (or relay lens) to observe the target brain region. Line 78 to 83) (Sorry we accidentally deleted your comment in the MS).
We modified the sentences to "*objective gradient refractive index (GRIN) lens (or objective lens) to be preanchored at the bottom of the miniscope to magnify the field of view that is relayed from a relay GRIN lens (or relay lens). This relay lens is implanted into the target brain region such that the fluorescence activity of the target brain region is relayed onto the surface of relay lens*" **Line 78 to 83**

We also added new arrows to indicate the location of the preanchored objective lens and implanted relay lens in Figure 1B.
We think the revised introduction and Figure 1 are clearer for readers.

2.8 So if I get this right – the miniscope is mounted on the brain region to view neuronal activity. How do you study the neuronal activity after mounting the miniscope? Is this attached to a software? Does this provide live recording? Please include details.
We apologize for not providing details. We added some sentences. "*For instance, the V3 UCLA Miniscope is based on a removable objective GRIN lens design. Researchers can choose either to directly implant an objective lens in the brain region of interest and mount the "empty" miniscope onto the objective lens (a one-lens system; Figure 1B2) or to implant a relay lens in the brain and mount a miniscope that is preanchored with an objective lens (a two-lens system; Figure 1B1). The miniscope then works as a fluorescence camera to capture livestream images of neuronal fluorescence produced by a genetically encoded calcium indicator1-3. After the miniscope is connected to a computer, these fluorescence images can be transferred to the computer and saved as video clips. Researchers can study neuronal activity by analyzing the relative changes in fluorescence with some analysis packages 20,21 or write their codes for future analysis.*" **Line 98 to 108**

2.9 What kind?
We modified the sentence to "*Misalignment of the two lenses can occur during recording in a two-lens configuration with a UCLA Miniscope due to the type of dental cement that is used to cement the baseplate to the skull*" **Line 160 to 165.**

2.10 Please somewhere first introduce that how miniscope work.
We add many descriptions to describe the how miniscope work in **Line 77 to 108 (about the mechanisms), Line 147 to 154 (about the basic protocols)**

2.11 How is this done?
We modified the sentence to "*the optimal working distance between the implanted relay lens and the objective lens must be found by adjusting the distance between the miniscope and the top of the relay lens,*" **Line 169 to 171**

2.12 Please bring clarity (Since the optimal range of distances is small (50 ~ 350 µm) it is difficult to stay within this range when using cement that shrinks excessively when it cures) (Line 179 to 174) (Sorry we accidentally deleted your comment in

the MS)

We modified the sentence to "*Since the optimal range of working distances of a relay lens is small (50 ~ 350 µm), excessive cement shrinkage during curing can make it difficult to keep the objective lens and the implanted relay lens within the appropriate range.*" **Line 173 to 176**

2.13 What kind of signals are being studied here. How do you study it? Please briefly introduce the protocol here.

We added some descriptions "*The miniscope then works as a fluorescence camera to capture livestream images of neuronal fluorescence produced by a genetically encoded calcium indicator. After the miniscope is connected to a computer, these fluorescence images can be transferred to the computer and saved as video clips. Researchers can study neuronal activity by analyzing the relative changes in fluorescence with some analysis packages or write their codes for future analysis.*" **Line 103 to 108**

And also "*Successful miniscope recording is defined as recording of a livestream of noticeable relative changes in the fluorescence of individual neurons in a freely behaving animal.*" **Line 184 to 186.**

2.14 How small?

We modified the sentence to "(*starting from approximately 2 mm anterior to the bregma and ending approximately 6 mm posterior to the bregma*)" **Line 270 to 272**

2.15 How is this done?

We added some descriptions "E*xecute a craniotomy for the relay lens implantation under a surgical microscope or stereomicroscope by using a burr drill with a 0.7 mm tip diameter. Use a micro-drill to grab the burr drill bit and draw an outline of the intended circle area (the full thickness of the calvarium does not need to be penetrated). (The relay lens used in the present protocol had a diameter of 1.0 mm, a length ~ 9.0 mmn a pitch of 1, and a working distance range of ~100 µm - 300 µm; therefore, the craniotomy had a diameter of 1.2 mm.) Gently deepen the outline until the dura is exposed. Prepare 3 ml of sterile saline in a 3 ml syringe and cool it in an ice bucket. Frequently rinse the exposed area with 0.1 ml of saline from the syringe to cool the area and thus to prevent heat damage and hemorrhage.*" **Line 278 to 286**

2.16 Do you mean removing some fluid from the cortex region or removing some of the cortex region to create a window for lens implantation? Citations if any to show that this is a standard procedure.

We remove the cortex region to create window for lens implantation. It is a standard procedure to implant 1 mm (or above) diameter GRIN lens. Without this procedure, the target neurons will be compressed and killed by the GRIN Lens. We added some citations here.
We modified the sentence to "*Put a marking on a 27 G blunt needle at 1 mm from the tip and use it to carefully aspirate the cortex of the brain in order to create a window for lens implantation8,11,13,17*" **Line 294 to 298**

2.17 How much saline?
We added some descriptions to **Line 310.** *"Prepare 3 ml of sterile saline in a 3 ml syringe and cool it in an ice bucket."*

2.18 What kind of AAV is used in your experiment? what kind of fluorescent reporters are present?
We added some descriptions to "*NOTE: AAV9-syn-jGCaMP7s-WPRE was used in the present experiment. jGCaMP7s is a genetically encoded calcium indicator that emits green fluorescence3. Because the present study used a wild-type mouse as a subject, a viral vector was needed to transfect the neurons with the gene of the green fluorescent calcium indicator and to enable expression of the indicator. Researchers who use transgenic mice expressing the green fluorescent calcium indicator as their subjects can skip protocol 2.2.*" **Line 313 to 319**

2.19 Does the miniscope remains attached to the animal's head?
No, it doesn't. This step is just assembling the equipment. The assembly will be placed over the mouse skull in protocol 2.4.4
Sorry for the confusion. We added some description to "*(in this step, the assembly of the anchored objective lens, baseplate, and miniscope has not yet been placed over the mouse's skull)*" **Line: 359 to 360**

2.20 Citation?
Citation added.

2.21 How is this done?
We added some descriptions in **Line 410** "*Fasten a set screw beside the baseplate to fix it to the bottom of the miniscope (Figure 5A1)*".

2.22 Reasons for checking calcium indicator? Link between AAV and calcium

indicator is needed somewhere.

The linkage between AAV and calcium indicator is added "B*ecause we used an AAV to express the genetically encoded calcium indicator, both the promoter type and AAV serotype affected the efficiency of transfection23,24. Researchers should need to check the expression of the calcium indicator by finding individual neurons that show changes in fluorescence before proceeding with future experiments.*" **Line 442 to 445.**

2.23 What kind of fluorescent signal is observed.

We modified the sentence in **Line 461** "*reseal the base with silicone rubber if no individual neuron that displays changes in fluorescence as a function of time. And redo protocol 3.1 to 3.12 after an additional week. (optional)*"

2.24 Not sure how this is obtained. Please bring out clarity.

We apologize for the confusion, we added some descriptions "*We measured the distance between the initial hole and the second hole to quantify the location shift of the needle.*" in **Line 545 to 546**

2.25 Please bring out clarity with respect to fluorescence, calcium imaging and AAV infusion.

We added some descriptions and also further explained the relationship between fluorescence, calcium indicator, AAV *"By following the surgical protocols for viral infusion, dummy baseplating, and baseplating (Figures 2, 4, 5), we observed fluorescence transients of individual neurons in three mice (n = 3/5) (Figure 6A, Supplementary video S3) during the baseplating procedure and after the baseplating procedure while the mice were freely behaving, confirming that the working distance between the objective and relay lenses remained the same (Figure 6A$_1$ vs 6A$_3$, only a slight shift in position occurred) after the dental cement had completely dried."* **Line 566 to 571.**

Other relationship among fluorescence, calcium imaging and AAV is in **Line 313 to 319; Line 442 to 445.**

3. The manuscript requires thorough proofreading.

We proofread the manuscript and rephrase/modified several sentences. The article currently is more fluent than previous version.

4. Please address all the reviewers' comments.

Please find our point-by-point responses to the Reviewer in the following section.

5. Once done please ensure that the highlighted section is no more than 3 pages including headings and spacings.
The highlighted sections are marked in yellow. We have counted the pages. If we remove the “Notes” in the highlighted steps and still include heading and spacing, it is approximately 2.8 pages.

Reviewers' comments:
Reviewer #4:
The authors have addressed the majority of the comments, I still have a comment about my original major revision point number 2

Although I understand the reasoning and the limitation due to not having a license to get medical grade cements, I still think that it is useful for other labs that can buy these cements to cite the existence of zero shrink cements on which people can still apply the same test done by the authors.
We are very appreciated for the suggestion; I think it is a good ideal letting readers understand that there are some brands of cement potentially can solve the shrinkage problem and letting them know that if their place can not obtain certain brand, they can still perform miniscope experiment by following our protocol. We added some description in introduction and discussion.

Line 186 to 193 *“Although different brands of dental cement have different shrinkage rates, researchers can select a brand that has been previously tested6-8,10-16,22. However, not every brand is easy to obtain in some countries/regions due to the import regulations for medical materials. Therefore, we have developed methods to test the shrinkage rates of available dental cements and, importantly, we provide an alternative protocol that minimizes the shrinkage problem. The advantage over the present baseplating protocol is increase the success rate of calcium imaging with tools and cement that can be easily obtained in laboratories.”*

Line 791 to 797 *“In addition, some specific brands of dental cements are frequently used for gluing baseplates by other research teams6-8,10-16,22. We believe the low-shrinkage properties of these cements may solve the problem, but we did not have the opportunity to test these brands due to difficulties in purchasing (importing) them. Medical-grade products may be subject to import regulations depending on the country/region. If researchers have difficulty accessing the cements mentioned in the literature6-8,10-16,22, our protocol will solve the dental cement shrinkage problem.”*

Reviewer #5:

Manuscript Summary:

In their manuscript, the authors introduce issues related to cement displacement during curing, which can prevent the use of 2 lenses (relay + objective) with miniscopes. The authors present a method to increase success in preserving baseplating field of view. The solution proposed by the author is well presented. I only have some reserved on the terminology ('2 lens system') and a few minor discussion points (see below)

We are grateful to the Reviewer for the positive comments and constructive feedback on our manuscript.

Major Concerns:

I find that the method presented is interesting for the miniscope community, and the authors touch on a difficulty that is well know when imaging with an objective lens. I must say that **the title is quite confusing**, and could suggest that the authors are **actually implanting 2 lenses**. I would suggest using the use of '**imaging with an objective lens**' expression when referring to this particular recording configuration where **one (relay) lens is implanted, and another GRIN lens is anchored in the miniscope**. This is actually the terminology used by the authors later in their schematics.

We thank the Reviewer for the constructive suggestion. We apologize for not realizing that the title will confuse the readers. In combine with the Editors suggestion, we modified the title to “Baseplating Procedure for Research on Calcium Transients in Mice using a Miniscope Preanchored with an Objective Lens “.

In the main text, we still use the term “one-lens system”, “two-lens system” but we have clearly define what is one-lens, two- lens meaning.

Abstract **Line 36 to 41** *“It permits imaging of fluorescence transients emitted from genetically modified neurons through an objective lens implanted on the superficial cortex (a one-lens system) or in deep brain areas through a combination of a relay lens implanted in the deep brain and an objective lens that is preanchored in the miniscope to observe the relayed image (a two-lens system).”*

Line 98 to 103 *“For instance, the V3 UCLA Miniscope is based on a removable objective GRIN lens design. Researchers can choose either to directly implant an objective lens in the brain region of interest and mount the “empty” miniscope onto the objective lens (a one-lens system; Figure 1B2) or to implant a relay lens in the*

brain and mount a miniscope that is preanchored with an objective lens (a two-lens system; Figure 1B1).”

Line 133 to 137 *“In other words, the V3 UCLA Miniscope users can take advantage the of removable lens by implanting it in the brain (creating a one-lens system) when performing experiments involving superficial brain observations (less than 2.5 mm in depth) or by preanchoring it in the miniscope and implanting a relay lens in the brain (creating a two-lens system) when performing experiments involving deep brain observations.”*

We also emphasize several time that the relay lens is implanted in the brain by using the words “implanted relay lens” in the introduction.

And we also modified the Figure 1 using arrows to indicate the location of preanchored objective lens, implanted relay lens (two-lens system), implanted objective lens (one-lens system).

We believe the readers will not be confused about where are the preanchored objective lens and implanted lens.

Minor Concerns:

Baseplating cement is not necessarily the only source of FOV shift: the authors should mention in their intro/discussion that the plastic used in miniscope construction can wear over time (especially when using delrin), as well as the baseplate magnets, which could explain displacements other than ones due to cement curing. This can matter especially when the FOV is zoomed in using long relay lenses.

That is correct, the miniscope protocol need extreme accuracy for all the optical pathway. We further bring the concern of the wearing of miniscope itself and baseplate in discussion.

Line 798 to 802 *“In addition to the shrinkage problem, structural wear can occur in the miniscope itself and on the baseplate (especially the magnets in the baseplate) over the course of many experiments. Any small gap caused by wear reduces the stability of the distance between the two lenses. Again, accuracy over the whole optical path is key to imaging of calcium transients in freely behaving mice using a miniscope.”*

The proposed surgery time are quite long. One should ideally aim to keep this type of surgery in the ~3 hours range, as prolonged or repeated exposure to isoflurane can

be problematic.

We totally agree, a well-trained operator can reduce anesthesia duration to about 1.5 hours. We added some descriptions to remind reader of aiming at finishing the anesthesia in 3 hours range.

Line 881 to 886 "*The time frame for the entire procedure is summarized in Figure 2A, with days as the units. In detail, the surgery part (viral injection, relay lens implantation, dummy baseplating) may take 3 hours for beginners or 1.5 hours for sufficiently trained researchers. Mice have a small body size and fast metabolism, and they are more susceptible to health problems caused by long periods under anesthesia than larger species27. Researchers should aim to keep the time spent in surgery under 3 hours.*"