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Title: Successful In Vivo Calcium Imaging with a Head-Mount Miniaturized Microscope in the Amygdala of Freely Behaving Mouse

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   Videographer: Screen captures provided, do not film

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **50**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Han-Sol Lee**: Many challenges can arise when using miniaturized microscopy for in vivo calcium imaging of the amygdala. This protocol provides time saving guidelines for achieving a successful in vivo calcium imaging **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Han-Sol Lee**: In vivo calcium imaging allows the continuous, simultaneous imaging of genetically defined cell populations over a period of up to several weeks, a process that was previously not possible **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Animal Ethics Committee at Korea Advanced Institute of Science and Technology.

Protocol

2. Adeno-Associated Virus (AAV) Injection

2.1. For AAV (A-A-V) injection, with the anesthetized mouse secured in a stereotaxic frame **[1-TXT]**, align the height of the bregma and lambda with the pin **[2-TXT]** and identify the specific location on the exposed and disinfected skull for the craniotomy **[3]**.

2.1.1. WIDE: Talent fixing pin with probe holder *Videographer: More Talent than mouse in shot* **TEXT: Anesthesia: pentobarbital 83 mg/kg i.p.**
2.1.2. Height being aligned **TEXT: See text for full mouse preparation details**
2.1.3. Location being identified

2.2. Use a drill equipped with a 0.6-millimeter bit to carefully make a 1.6-millimeter craniotomy with an 18,000 revolutions per minute drill speed **[1]** and wash the skull with sterile PBS **[2-TXT]**.

2.2.1. Skull being drilled
2.2.2. Cotton tipped swabs being dipped in PBS and skull being washed, with PBS container visible in frame **TEXT: Remove any remaining bone debris as necessary**

2.3. Subtract the dorsal-ventral difference between the bregma and the exposed brain surface from 4.2 millimeters, the dorsal-ventral coordinate of the lens implantation based on a bregma to calculate the Value A distance **[1]**, and load 3 microliters of mineral oil and 0.7 microliters of a greater than 1 x 10^{13} viral genomes/milliliter of the AAV solution into a glass pipette **[2]**.

2.3.1. Shot of coordinates *Videographer: Important step; Video Editor: please emphasize distance between bregma and exposed brain surface*

2.3.2. Oil being added to pipette, with oil and AAV solution containers visible in frame *Videographer: Important step*

2.4. Remove the pin from the stereotaxic probe holder to allow the pipette to be fixed to the frame **[1]** and position the pipette over the prepared injection site **[2]**.

2.4.1. Pipette being fixed to frame
2.4.2. Pipette being positioned over injection site

2.5. When the bleeding has completely stopped, insert the glass pipette tip 4.3 millimeters

into the brain tissue from the dorsal-ventral bregma coordinate **[1]** and use an injection pump to deliver the entire volume of virus **[2]** before slowly removing the pipette **[3]**.

2.5.1. Tip being inserted
2.5.2. Virus being delivered
2.5.3. Pipette being removed

3. Gradient Refractive Index (GRIN) Lens Implantation

3.1. When all of the virus has been injected, drill the skull two more times as demonstrated to allow the implantation of two skull screws **[1]** and wash the skull fractions with PBS **[2]**.

3.1.1. WIDE: Talent drilling skull *Videographer: More Talent than mouse in shot*
3.1.2. Skull being washed

3.2. Next, implant the 70% ethanol-sterilized screws as deeply as possible **[1]** and install a motorized surgery arm on the stereotaxic frame **[2]**.

3.2.1. Screw being implanted
3.2.2. Talent installing arm *Videographer: More Talent than mouse in shot*

3.3. Connect the required hardware to a laptop computer on which the controlling software is installed **[1]** and use a cannula holder to stably secure a 26-gauge needle onto the stereotaxic frame **[2]**.

3.3.1. Talent connecting hardware to computer
3.3.2. Needle being secured to holder on frame

3.4. After calculating the coordinates for the needle insertion site, use the stereotaxic manipulator to position the needle tip at the exposed surface of the brain **[1-TXT]** and hydrate the brain tissue with 2-3 drops of PBS **[2]**.

3.4.1. Needle being positioned *Videographer: Important step* **TEXT: AP: -1.6 mm; ML: -3.55 mm; DV -3.7 mm**
3.4.2. Drops being applied *Videographer: Important step*

3.5. Enter the appropriate dorsal-ventral and lateral amygdala coordinates into the software and click **Go To [1]** to lower the needle **[2]**.

3.5.1. SCREEN: 3.5.1: 00:03-00:11
3.5.2. Needle being lowered

3.6. When the needle has completely stopped, enter 45 millimeters to the start position box and click **Go To** to elevate the needle **[1]**.

3.6.1. SCREEN: 3.6.1: 00:03-00:11

3.7. When the needle stops moving, remove the PBS from the brain surface **[1]** and uninstall the needle and cannula holder from the stereotaxic frame **[2]**.

3.7.1. PBS being removed
3.7.2. Needle and/or cannula holder being uninstalled

3.8. Attach the lens holder to the stereotaxic rod **[1]** and fix the GRIN (grin) lens to the lens holder **[2]**.

3.8.1. Talent attaching holder
3.8.2. Talent placing lens onto holder

3.9. Sterilize the lens with 70% ethanol **[1]** and attach the stereotaxic rod to the stereotaxic frame **[2]**.

3.9.1. Lens being washed/wiped
3.9.2. Talent attaching rod to frame

3.10. Position the tip of the GRIN lens at the designated site on the brain surface **[1]** and subtract the absolute value of "Value A" from the dorsal-ventral coordinate of the brain surface to determine the dorsal-ventral coordinate for lens implantation **[2]**.

3.10.1. Tip being positioned *Videographer: Important step*
3.10.2. Shot of skull/coordinates, with surgery sheet visible in frame *Videographer: Important step; Video Editor: please emphasize distance from coordinate to implantation site*

3.11. Hydrate the brain tissue with an additional 2-3 drops of PBS **[1]** and use the motorized arm to set the lowering depth to 1000 micrometers and the raising height to 300 micrometers **[2]**.

3.11.1. PBS being added to brain *Videographer: Important step*
3.11.2. SCREEN: 3.11.2: 00:01-00:06

3.12. When the GRIN lens reaches the target site, remove the PBS **[1]** and carefully apply resin dental cement around the GRIN lens, screws, and screw sidewalls **[2]**.

3.12.1. Shot of lens in position, then PBS being removed
3.12.2. Resin being applied

3.13. When the dental cement has hardened, remove the lens holder from the lens **[1]** and apply acrylic cement to the skull surface **[2]**.

3.13.1. Lens holder being removed
3.13.2. Cement being applied

3.14. Next, replace the lens holder with a head plate taped to the tip of the rod in a horizontal orientation **[1-TXT]**.

3.14.1. Head plate being attached **TEXT: Tilted plate causes tilted view**

3.15. Slowly lower the rod until the head plate is positioned at the top of the lens cuff **[1]** before lowering the head plate 1000 micrometers until the lens is located at the right edge of the internal ring of the headplate **[2]**.

3.15.1. Rod being lowered
3.15.2. Head plate being lowered, with internal ring of head plate visible in frame

3.16. Use acrylic cement to attach the head plate to the cement layers **[1]** and use paraffin film to protect the implanted GRIN lens surface from dust **[2]**.

3.16.1. Cement being applied
3.16.2. Film being applied

3.17. Then apply removable epoxy bond onto the paraffin film to secure the film to the lens surface **[1]** and return the animal to its cage with monitoring until full recumbency **[2-TXT]**.

3.17.1. Bond being applied
3.17.2. Talent placing mouse into cage *Videographer: More Talent than mouse in shot* **TEXT: See text for full post-operative care details**

4. GRIN Lens Implantation Validation

4.1. To evaluate the quality of the GRIN lens implantation, place a carbon cage under the anesthetized mouse on the head bar of a mobile home cage system **[1-TXT]** and turn on the airflow so that the cage moves freely without friction **[2]**.

4.1.1. WIDE: Talent placing cage under mouse *Videographer: More Talent than mouse* in shot **TEXT: Anesthesia: 1.5% isoflurane**

4.1.2. Talent turning on air flow

4.2. Use a microscope gripper and the stereotaxic manipulator to vertically align a miniaturized microscope to the frame **[1-TXT]** before lowering the microscope until the surface of the implanted GRIN lens appears in the image field of view within the software **[2]**.

4.2.1. Microscope being aligned vertically **TEXT: See text for full microscope setup details**

4.2.2. Microscope being lowered

4.3. Align the center of the implanted GRIN lens with the center of the field of view and capture the image of the implanted GRIN lens surface **[1]**.

4.3.1. SCREEN: 4.3.1 *Video Editor: can speed up*

4.4. Then slowly raise the objective lens of the microscope while observing the image for the appearance of GCaMP (G-camp)-expressing cells **[1-TXT]**.

4.4.1. SCREEN: 4.4.1 *Video Editor: please speed up* **TEXT: Adjust imaging conditions (Gain, LED power, exposure time) as necessary**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.3., 3.4., 3.10., 3.11.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.11. To ensure lowering and raising lens in constant speed, we use motorized surgery arm controlled by computer software.

Results

5. Results: Representative GRIN Lens Implantation Validation and GCaMP Signal Evaluation

5.1. In animals with a successful lens implantation **[1]**, both GCaMP-expressing cells **[2]** and blood vessels can be clearly observed within a single focal plane range **[3]**.

5.1.1. LAB MEDIA: Figures 2A and 2B
5.1.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize bright signal in Figure 2A*
5.1.3. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize dark vessels in Figure 2A*

5.2. In contrast, in animals with off-target implantation, a clear image of GCaMP-expressing cells is not observed within the focal plane range **[1]**, although the blood vessels can be observed with some difficulty **[2]**.

5.2.1. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize Figure 2C*
5.2.2. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize vessel in Figure 2C*

5.3. In the case of an out-of-view lens, neither blood vessels nor GCaMP-expressing cells can be visualized **[1]** and a bright edge can be observed on the implanted GRIN lens **[2]**.

5.3.1. LAB MEDIA: Figures 2E and 2F *Video Editor: please emphasize Figure 2E*
5.3.2. LAB MEDIA: Figures 2E and 2F *Video Editor: please emphasize bright edge in Figure 2F*

5.4. In animals with a successful GRIN lens implantation **[1]**, approximately 50-150 likely spontaneously active cells typically display a significant fluorescence change within the field of view in the lateral amygdala even without tone **[2]**.

5.4.1. LAB MEDIA: Figure 3A
5.4.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize outlined cells*

5.5. Upon tone presentation, only a few cells display a tone-specific change of GCaMP signal **[1]** as determined by delta F image analysis **[2]**.

5.5.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize colored shapes*
5.5.2. LAB MEDIA: Figures 3C and 3D

5.6. When the same analysis is conducted on mice injected with GFP-expressing AAV as a control **[1]**, GFP-expressing cells are detected within the focal plane range **[2]** and no cells display a significant fluorescence change with or without tone **[3]**.

5.6.1. LAB MEDIA: Figure 3E
5.6.2. LAB MEDIA: Figure 3E *Video Editor: please emphasize red square*
5.6.3. LAB MEDIA: Figures 3E and 3F *Video Editor: please emphasize Figure 3F*

5.7. Histological verification of GCaMP expression and GRIN lens targeting **[1]** reveals no sign of tissue damage due to brain tissue inflammation around the GRIN lens **[2]**.

5.7.1. LAB MEDIA: Figure 4
5.7.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4C*

Conclusion

6. Conclusion Interview Statements

6.1. **Han-Sol Lee**: If the speed of the needle or the lens is not consistent, it can cause motion artifacts and a poor image quality. Using a motorized surgery arm can help **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.5., 3.11.)

6.2. **Han-Sol Lee**: If the mobile home cage system is not ready during the baseplate attachment step, a low concentration of isoflurane gas can be used to anesthetize the mouse during the baseplate attachment **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera