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In vivo calcium imaging in mouse inferior olive

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TITLE:

In vivo calcium imaging in mouse inferior olive

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Neuroscience, inferior olive, in vivo, injection; calcium imaging, medulla, mouse

SUMMARY:

We present a protocol to expose the brainstem of adult mouse from the ventral side. By using a gradient-refractive index lens with a miniature microscope, calcium imaging can be used to examine the activity of inferior olive neural somata in vivo.

ABSTRACT:

Inferior olive (IO), a nucleus in the ventral medulla, is the only source of climbing fibers that form one of the two input pathways entering the cerebellum. IO has long been proposed to be crucial for motor control and its activity is currently considered to be at the center of many hypotheses of both motor and cognitive functions of the cerebellum. While its physiology and function have been relatively well studied on single-cell level *in vitro*, presently there are no reports on the organization of the IO network activity in living animals. This is largely due to the extremely challenging anatomical location of the IO, making it difficult to subject to conventional fluorescent imaging methods, where an optic path must be created through the entire brain located dorsally to the region of interest.

Here we describe an alternative method for obtaining state-of-the-art -level calcium imaging data from the IO network. The method takes advantage of the extreme ventral location of the IO and involves a surgical procedure for inserting a gradient-refractive index (GRIN) lens through the neck viscera to come into contact with the ventral surface of the calcium sensor GCaMP6s-expressing IO in anesthetized mice. A representative calcium imaging recording is shown to demonstrate the feasibility to record IO neuron activity after the surgery. While this is a non-survival surgery and the recordings must be conducted under anesthesia, it avoids damage to life-critical brainstem nuclei and allows conducting large variety of experiments investigating spatiotemporal activity patterns and input integration in the IO. This procedure with

modifications could be used for recordings in other, adjacent regions of the ventral brainstem.

INTRODUCTION:

The main goal of systems neuroscience is to understand how spatiotemporal activity patterns of neuronal networks contribute to generation of animal behavior. Thus, fluorescent imaging methodology utilizing calcium-sensitive probes has in the past decade become a main tool for examining neuronal network activity in living animals[1,2], as it allows visualization of such dynamics across spatial scales ranging from single cells to mesoscale circuitry. In recent years, the common approach where neural circuits in superficial brain structures (such as cerebral or cerebellar cortices) are imaged through a transparent cranial window[3] has been complemented with the use of gradient-refractive index (GRIN) lenses[4] allowing examination of network dynamics in deep brain structures. Currently-available GRIN lenses allow reaching into structures several millimeters deep, such as the mouse amygdala, hippocampus and basal ganglia[5]. However, many regions of interest such as various nuclei in the ventral medulla lie significantly deeper, placing them at the extreme of the GRIN lens reach.

Here, we describe how to overcome this difficulty by taking advantage of the relatively easy accessibility of medulla through the ventral aspect of the brain. Using adult mice where the inferior olive (IO), a nucleus in the ventral medulla, has been virally transfected with a calcium sensor GCaMP6s, we describe the surgical steps (modified from the method described originally in Khosrovani et al. 2007[6]) to place a GRIN lens on the ventral surface of the brain of an anesthetized mouse. Using a miniature microscope, we demonstrate the feasibility of recording neuronal activity in such extremely ventral brain regions. While the procedure is necessarily a non-survival surgery and no experimentation can be performed in awake animals, the method allows examination of intact network dynamics in the context of sensory or other afferent pathway stimulation, providing clear advantages over ex vivo-approaches such as using acute slice preparations.

PROTOCOL:

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Aseptic surgery techniques were applied to the stereotaxic virus vector injection.

1. Stereotaxic virus vector injection

NOTE: Virus carrying the genetic material for expressing GCaMP6s (AAV9.CAG.GCaMP6s.WPRE.SV40) is stereotaxically injected as previously described[7, 8] with following modifications.

1.1. Use quartz glass (external diameter: 1.14 mm, internal diameter: 0.53 mm) instead of borosilicate glass to fabricate a pipette with long and rigid taper for IO injection using a laser capillary puller with parameters adjusted as in the product manual. After pulling, cut off 1-2 mm from the tip of the pipette with a scalpel to acquire an 8-10 mm long taper with a 15-20 µm internal tip diameter (**Figure 1a**). Finalize the pipette by beveling its tip to a 30° needle shape

with a rotating micropipette beveler for easier brain penetration and less pipette bending (**Figure 1a**).

NOTE: The commonly used borosilicate glass pipette will be too flexible to correctly target deep areas when pulled to this length. A nanoliter injector was used with the glass pipette to deliver the virus in this study. Alternatively, a precision glass syringe or a pressure injector can also be used.

1.2. Make sure the mouse chest lies on the thermal pad so its neck is not stretched with enough body support, and be extremely careful with leveling the skull when fixing the mouse on the stereotaxic frame (**Figure 1b**).

NOTE: Bregma-lambda difference should be less than 0.05 mm in vertical dimension.

1.3. Use 6.2 mm caudal, ±0.5 mm lateral and 6.6 mm ventral relative to bregma as the the coordinates for targeting the principal IO nucleus (**Figure 1c**).

NOTE: Perfect leveling of brain will dramatically increase the success rate of injecting virus into IO. The coordinates must be confirmed by the experimenter as significant differences are expected between laboratories, mouse strains and individual researchers. For preparing video material for this publication, we used one male C57Bl/6J mouse.

1.4. Follow the relevant institutional guidelines for post-operative care and housing procedures for viral-transfected animals for 3-4 weeks.

[Place Figure 1 here]

2. Preparation of tools and consumables for ventral approach surgery

2.1. Prepare an intubation tube by cutting a 5 to 6 mm long and 0.8 mm wide-slit from the tip of a 20-gauge catheter so the intubated animal can breathe out (**Figure 2a**).

NOTE: The slit is needed for animal to breath out if a common isoflurane vaporizer with constant airflow is utilized. If a ventilator is used in addition to the vaporizer, the catheter can be left intact.

2.2. Prepare a blunt needle to support the trachea during the tracheotomy. Cut off the sharp tip of a 25-gauge needle with pliers. Smooth the fracture surface with sandpaper. Bend the blunt needle in the middle to about 15° with pliers.

NOTE: A curved needle lifts the trachea gently. This can decrease the deformation of the trachea.

2.3. Dilute 50 mg/mL ketamine with saline to 15%. The final concentration is 7.5 mg/mL.

2.4. Assemble the clean surgery tools and the consumables including lidocaine gel, saline, gelatin

sponges, absorbent swabs, petroleum jelly and cleaning tissue.

2.5. Switch on the isoflurane vaporizer. Set the animal heating pad to 38 °C.

2.6. Turn the nose cone of stereotaxic frame 180° horizontally, so the mouse can be fixed into the frame ventral side up. Adjust the nose cone height so that it is at the level of ear bars.

3. Administration of anesthesia and preparation of the mouse for the surgery

3.1. Weigh the animal with a weighing scale and calculate the amount of diluted ketamine for injection.

NOTE: The total amount of ketamine needed is 56.25 mg per kg body weight. Therefore, the volume of diluted ketamine is 7.5 mL per kg body weight. The drawbacks of using ketamine alone include poor muscle relaxation, tachycardia and enhanced muscle tone[9]. In this protocol, though, ketamine is only used to cover the 10-20 s period during which isoflurane administration is interrupted due to the tracheotomy. By keeping this step as brief as possible, the anesthetic effect of isoflurane is not significantly diminished and the drawbacks using ketamine are minimized.

3.2. Place the mouse into the anesthetic induction chamber prefilled with 5% isoflurane. When the animal is fully anesthetized shown by loss of righting reflex and deeper and slower breathing pattern, switch the isoflurane flow to the nose cone of stereotaxic frame and reduce the isoflurane concentration to 2.5%.

3.3. Fix the animal in the stereotaxic frame ventral side up (**Figure 2b**). Adjust the elevation and the pitch of the nose cone to make sure the animal can breathe easily. Keep the animal warm with the pre-warmed heating pad.

NOTE: Covering the lower part of the animal body with a piece of tissue paper or aluminum foil can help maintaining the animal temperature.

3.4. Remove the skin hair in throat and thigh areas with a shaver and hair removal cream (**Figure 2b**). Topically apply the lidocaine gel on the throat skin.

NOTE: The thigh clamp peripheral oxygen saturation (SpO_2) sensor, which will be used in procedure 6, works best on hairless skin.

3.5. Monitor the animal temperature with a rectal thermometer (**Figure 2b**).

3.6. Inject 1 mL of warm (37 °C) saline intraperitoneally to compensate for the fluid loss during surgery.

3.7. Assess the depth of anesthesia by strong pinch on hind limb toes. No detectable response

should be evoked.

[Place Figure 2 here]

4. Tracheotomy and intubation (20-25 min)

4.1. Exposing the trachea.

4.1.1. Make a vertical incision in throat skin along the midline. Separate the neck skin from the viscera under it by using the blunt dissection method and cut the skin off to reveal the salivary glands (**Figure 3a-b, 4a**).

4.1.2. Free salivary glands from the connective tissue and flip them laterally to expose the sternothyroid muscle covered trachea with forceps (**Figure 3b-c**).

NOTE: The petroleum jelly can be applied on the exposed tissue to keep them moisture. Avoid the trachea area to keep it clean for the following steps.

4.2. Tracheotomy

4.2.1 Inject the first dose of diluted ketamine (7.5 mg/mL) intraperitoneally, 5 mL per kg body weight (37.5 mg/kg).

NOTE: It takes 3-5 min for ketamine to onset, thus the first dose of ketamine should be injected before separating the trachea from surrounding muscles and blood vessels. Ketamine is administered in two injections because of the elevated risk of overdose effects when combined with isoflurane anesthesia.

4.2.2 Carefully split the sternothyroid muscle along the midline with the tip of a fine forceps to expose the trachea (**Figure 3c**). Detach the trachea from the blood vessels and the esophagus with forceps using the blunt dissection method.

4.2.3. Intraperitoneally inject the second dose of diluted ketamine (7.5 mg/mL), 2.5 mL per kg body weight (18.75 mg/kg).

4.2.4. Insert a blunt needle under the trachea crosswise to prop it up (**Figure 3d**). Hold this needle with fingers to support the trachea. Guide the suture thread around the third trachea ring caudal to the thyroid gland with a half-circle needle (**Figure 3d**). Make four instrument ties on this tracheal ring.

NOTE: The thread tied to the tracheal ring is used to secure the trachea to the animal chest. Do not cut the thread off from the half circle needle at this step. Tracheal rings are made of cartilage which is flexible but less strong than bones. Do not tie the suture thread too tight or the ring may break.

4.2.5. Pinch the chest skin with a pair of forceps. Pierce the chest skin with the same half-circle needle used in the last step and lead the thread through the skin, in preparation for securing the trachea to the chest in the next step (**Figure 3d**).

4.2.6. Gently lift the trachea by pulling the thread tied to the tracheal ring and cut the trachea rostral to the tied ring and caudal to the thyroid glands. Pull the caudal trachea towards the chest. Raise the opening of the trachea by adding a small piece of surgical sponge under it.

NOTE: Make sure more than 5 min have passed after the second injection of ketamine before cutting the trachea to ensure the anesthesia level.

4.2.7. Remove any remaining liquid inside the opening tip of the trachea with a thin strip of cleaning tissue. Switch the isoflurane flow from stereotaxic nose cone to the intubation tube. Insert the intubation tube into the trachea about 2 mm deep and make sure part of the slit in the tube remains outside of the trachea to allow breathing (**Figure 3e, 4b**).

NOTE: Carefully adjust the angle of the intubation tube and its insertion depth to make the mouse breath smoothly and to avoid damaging the trachea. Petroleum jelly can be applied on the outside of trachea to prevent it from getting dry, so the trachea does not rupture easily.

4.2.8. Fix the trachea to the chest skin by making 3-4 instrumental ties. Tie the trachea with the suture thread to secure the intubation tube (**Figure 3e, 4b**).

[Place Figure 3 here]
[Place Figure 4 here]

5. Exposing the brainstem (40-45 min)

5.1. Slit the sternothyroid muscle along the muscle fiber with the fine forceps. Cut the isolated part off with the spring scissors (**Figure 3e**).

5.2. Carefully free the left-over trachea and the larynx from muscles to minimize the damage on the blood vessels in muscles. Remove the left-over trachea and the larynx. Free the esophagus from the attached tissue with forceps and cut it off with spring scissors. (**Figure 5a**).

5.3. Remove the muscle covering the ventral arch and the anterior tubercle of atlas with fine forceps and spring scissors (**Figure 5b**).

NOTE: When removing the muscle, split part of it with the tip of the fine forceps. Cut the separated part off with a pair of spring scissors. Repeat this multiple times to expose the atlas ventral arch to minimize the risk of ripping blood vessels.

5.4. Cut the ventral arches of atlas with a rongeur (**Figure 4c, 5c**). Remove the anterior tubercle

of atlas. Remove the blood and fluid with surgical sponge to view the foramen magnum and the brain stem (**Figure 4c, 5d**).

5.5. Expand the foramen magnum by removing the occipital bone with a rongeur. (**Figure 5d-e**).

5.6. Remove the thin cartilage above the foramen magnum with fine forceps and spring scissors. Carefully peel the periosteal layer of the dura mater with fine forceps to have a clear view of the ventral brainstem (**Figure 5f**). Do not break the dura mater.

[Place Figure 5 here]

6. Calcium imaging

6.1. Clamp the SpO_2 sensor on the thigh of the mouse to monitor vital signs such as heart rate, oxygen saturation and breath rate (**Figure 2b**).

NOTE: The heart rate should be between 500 bpm and 600 bpm, the oxygen saturation should be higher than 90%, and the breath rate should be 50-70 breaths per minute[10, 11].

6.2. Mount the GRIN lens probe (9mm length. 1 mm diameter) on the implantation rod.

NOTE: Clean the GRIN lens with 70% ethanol-soaked cleaning tissue before imaging for good imaging quality.

6.3. Fix the implantation rod on the stereotaxic frame and mount the miniature microscope on the implantation rod.

NOTE: Preparation of the miniature microscope for imaging should be completed according to appropriate product user guidelines.

6.4. Add several drops of warm saline in the brainstem area for immersion of the GRIN lens.

6.5. Approach the brainstem with the GRIN lens (**Figure 4d, 5g**). Turn on the excitation blue LED (455 ± 8) in the miniature microscope. Locate the GCaMP6s-transfected IO neurons by monitoring the fluorescence image from the miniature microscope. Look for IO neurons in a rectangle-shaped region ~0.5-1.7 mm rostral to the remaining atlas and ~0.6-1.1 mm lateral to the midline in the superficial area of ventral brainstem (**Figure 5f**).

NOTE: When looking for an appropriate field of view for IO imaging, look for the location where the average diameter of somata matches that of IO neuron somata (about 15 μm[12]). The adjacent regions in medullary reticular formation consist of significantly larger cells[13, 14]. Be careful when moving GRIN lens vertically, as pressing it on brainstem too hard may kill animal.

7. Euthanizing animal following procedure

7.1. At the end of experiment, euthanize the animal with cervical dislocation or other method approved by local laboratory animal care regulation.

7.2. For further histology investigation, first anesthetize the animal with injectable drug, such as ketamine/xylazine combination (100 mg/kg and 10 mg/kg, respectively)[15] before heart perfusion with Ringer's solution followed by fixative solution to fix the brain.

8. Data processing

8.1. Pre-process the recorded calcium imaging video before analyzing data.

NOTE: The commercial data processing software accompanied with the miniature microscope was used for this step and the following protocol steps relate to that. Alternatively, free and open source software such as CaImAn[16], MINIPIPE[17], and MiniscoPy[18] can be utilized for both pre-processing and analysis.

8.1.1. Load the recorded calcium imaging video in the data processing software.

8.1.2. Click the **Preprocess** button. Define a crop area excluding regions without any fluorescent neurons and crop the video to decrease the file size for faster processing.

8.1.3. Click the **Spatial Filter** button. Set the low cut-off and the high cut-off for spatial filter to 0.005 pixel^{-1}, and 0.5 pixel^{-1} respectively. Apply the spatial filter on each frame of the video to increase the contrast and smooth the image.

NOTE: The spatial filter is a bandpass Gaussian filter. Low spatial frequency components originating in out-of-focus cells may confound motion correction in next step. High spatial frequency component may cause the video to appear less smooth.

8.1.4. Click the **Motion Correction** button. Apply the motion correction to the video by using the first frame as the reference frame to reduce the movement-related artefacts caused by blood flow in the brainstem.

NOTE: The motion correction uses an image registration method developed by Thevenaz et al[19].

8.1.5. Export the motion-corrected video as TIFF format.

8.2. Apply CNMF-E[20] on the motion-corrected video in MATLAB to identify single neurons, following the instructions for the CNMF-E MATLAB code in the online repository[21].

NOTE: CNMF-E is a constrained non-negative matrix factorization approach customized for one-

photon imaging. Demo scripts in the repository can be modified and used to process data.

REPRESENTATIVE RESULTS:

Here we present a representative recording obtained with the method as described. **Figure 6a** shows the location of brightly labeled IO cells visualized during the experiment. The dark diagonal stripes are blood vessels. Note the variable brightness of individual cells, resulting from variable transfection efficacy. In panel **Figure 6b** we show the mean-normalized fluorescence intensity (deltaF/F) traces obtained from the somata indicated with colors and numbers in panel a. Upward deflections represent transient increases in intracellular calcium. Note how different level of GCaMP6s expression (reflected in cell brightness in panel a) lead to variable signal-to-noise-ratios (SNR).

[Place Figure 6 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Stereotaxic virus vector injection. (**a**) The laser-pulled quartz glass pipette has a 10-12 mm long straight taper. After pulling, cut off 1-2 mm from the tip. The pipette is finalized by beveling the tip to a 30° needle shape. (**b**) The correct injection relies on the proper position of mouse body in the stereotaxic frame. Support the mouse chest to prevent stretching the neck. Level the mouse head by aligning the bregma and lambda horizontally. (**c**) The IO coordination relative to bregma is shown in dorsal view (left) of the mouse skull and coronal view (right top) of the brain. Injection reaches the lateral part of the principal (IOPr) and the dorsal (IOD) subnuclei of IO (right bottom).

Figure 2: Preparation of ventral approach surgery. (**a**) Prepare an intubation tube by cutting a 5-6 mm long and 0.8 mm wide slit in the tip of 20-gauge catheter. (**b**) Mount the animal ventral side up in a stereotaxic frame and adjust the nose cone angle to ensure the animal is breathing easily. Shave the skin around the throat and thigh areas. Attach the SpO_2 sensor to the thigh for monitoring mouse vital signs. Insert the rectal temperature probe for monitoring mouse body temperature.

Figure 3: Tracheotomy and intubation of mouse. (**a-c**) panels show the process of exposing trachea. (**a**) Remove the throat skin by cutting along the dashed lines. (**b**) Flip the salivary glands (SG) laterally to expose the trachea covered by the sternothyroid muscle (SM). (**c**) Slit open SM along the dashed line to expose the trachea. (**d-e**) panels show the tracheotomy. (**d**) Support the trachea with a blunt and curved needle. Tie the third trachea ring caudal to the thyroid gland for securing the trachea to the chest skin. (**e**) Apply isoflurane with an intubation tube with a slit in the tip. Secure the trachea to the chest skin with the suture thread. Secure the intubation tube to the trachea by tying them together. Scale bar in a=5 mm, applies to all panels.

Figure 4: Schematic diagram of ventral approach surgery from lateral view. (**a**) A schematic drawing with relevant anatomical parts indicated in their relative location when mouse is placed ventral side up. Abbreviations: muscle covering atlas (AM), longitudinal muscle (LM), salivary glands (SG), sternothyroid muscle (SM), thyroid gland (TG). (**b**) Schematic of arrangement of the

intubation tube in relation to the trachea when the tracheotomy is completed. The trachea is secured by the ties on the chest skin (T-trachea). The intubation tube (IT) is secured by the ties around the trachea end (T-tube). (**c**) Remove the atlas anterior tubercle (AAT) to clear the line of vision to the IO. (**d**) Schematic describing the positioning of the miniature microscope (MM) and the GRIN lens above the IO for imaging experiment.

Figure 5: Expose brainstem of mouse for calcium imaging. (**a-f**) panels show the process of exposing brainstem. (**a**) Remove the sternothyroid muscle (SM) labeled in **Figure 3e**. Cut off the larynx and the esophagus. (**b**) Remove the longitudinal muscle (LM) and the muscle covering atlas (AM). (**c**) Cut the atlas ventral arches (AVA) with a rongeur and remove the atlas anterior tubercle (AAT). (**d**) Cut off the occipital bone (OB) to expand the foramen magnum (FM). (**e**) Expanded FM. (**f**) The thin cartilage above the foramen magnum is removed. The periosteal layer of dura mater is peeled off. The square indicates the area containing superficial IO neurons. (**g**) Image the IO with GRIN lens. Scale bar in a=5 mm, applies to a-c. Scale bar in d=2 mm, applies to d-e. Scale bar in f=2 mm. Scale bar in g=2 mm.

Figure 6: Example recording of activity of IO neurons in anesthetized mouse. (**a**) Representative example frame from a recording after spatial filtering. Bright spots are IO neuronal somata, several of which have been indicated as regions of interests (ROIs, colored numbers). Dark stripes are blood vessels. (**b**) Example deltaF/F traces obtained from the ROIs indicated in panel a. Upwards deflections reflect increases in calcium signal. Scale bar in a=10 µm.

DISCUSSION:

As the surgical procedure involves operations performed in the throat region with numerous vitally critical structures (arteries, nerves), it is essential that it is conducted by a researcher with high-level surgical skills. In following, we highlight and comment on several key points of the procedure; however, it must be reminded that no amount of written advice can supplant the experience, skill, and intuition of the researcher.

The most critical step in the surgery is tracheotomy. It involves cutting the trachea, switching isoflurane from the nose cone to the intubation tube, securing the trachea to the chest skin and tying the trachea and the intubation tube together. All these operations must be completed in a smooth and fast manner to avoid accidents, such as inadequate anesthesia, inflow of fluid into trachea or intubation tube slip-off. One must keep the protocol clear in mind before cutting the trachea.

Hemorrhage is one of the major causes of animal death in this surgery. Since the neck area is dense with blood vessels, cut should only be executed when the line of sight is clear to avoid cutting unseen veins and arteries. Therefore, muscles and connective tissues obscuring vision must be removed, and blood from broken capillaries must be cleaned before advancing.

Animal can be kept alive for a long time (more than 8 hours) from the start of surgery. However, it is important to finish the surgical procedure quickly so there is more time to examine brainstem neurons when animal physiological condition is good. A skilled researcher can finish the whole

procedure in 70 min.

While the method provides a clean view of ventral brain surfaces, it is unfortunately impossible to do so without performing a tracheotomy as well as removing significant amount of tissue in the throat region. Therefore, the animal cannot be allowed to wake from anesthesia. Furthermore, even though it is possible to keep the animal alive for many hours with careful adjustment of anesthetic delivery, maintaining body temperature and hydration, it is inevitable that prolonged experimentation will eventually lead to weakening of the animal condition. It is left to the expertise of the researcher to consider the maximal duration of stable recordings.

Another potential limitation of the method as described here is that as the GRIN lens is not inserted into the brain parenchyma, only relatively superficial neurons (~150-200 µm) can be examined. While surgical implantation of GRIN lens is technically possible, acute surgery method does not allow sufficient time for neurons to recover from oxidative stress and presence of blood after implantation likely will degrade image quality beyond acceptable.

Despite the above concerns, we believe this is the first time a method for *in vivo* imaging of IO neurons is presented. It allows examination of spatiotemporal activity in the IO neurons in the context *in vivo* in the presence of intact afferent inputs from sensory systems as well as the signals from the cerebellar nuclei and the mesodiencephalic junction[22], a feat that has not been possible hitherto. With this method, the function of the IO can now be investigated in greater depth with combination of sensory and optogenetic stimulation. Notably, with the evolution of voltage imaging (such as our recent method for voltage imaging in the IO [23]), we hope the presented surgical method will inspire numerous researchers to take up the challenge of investigating how the IO contributes to the generation of cerebellar complex spikes.

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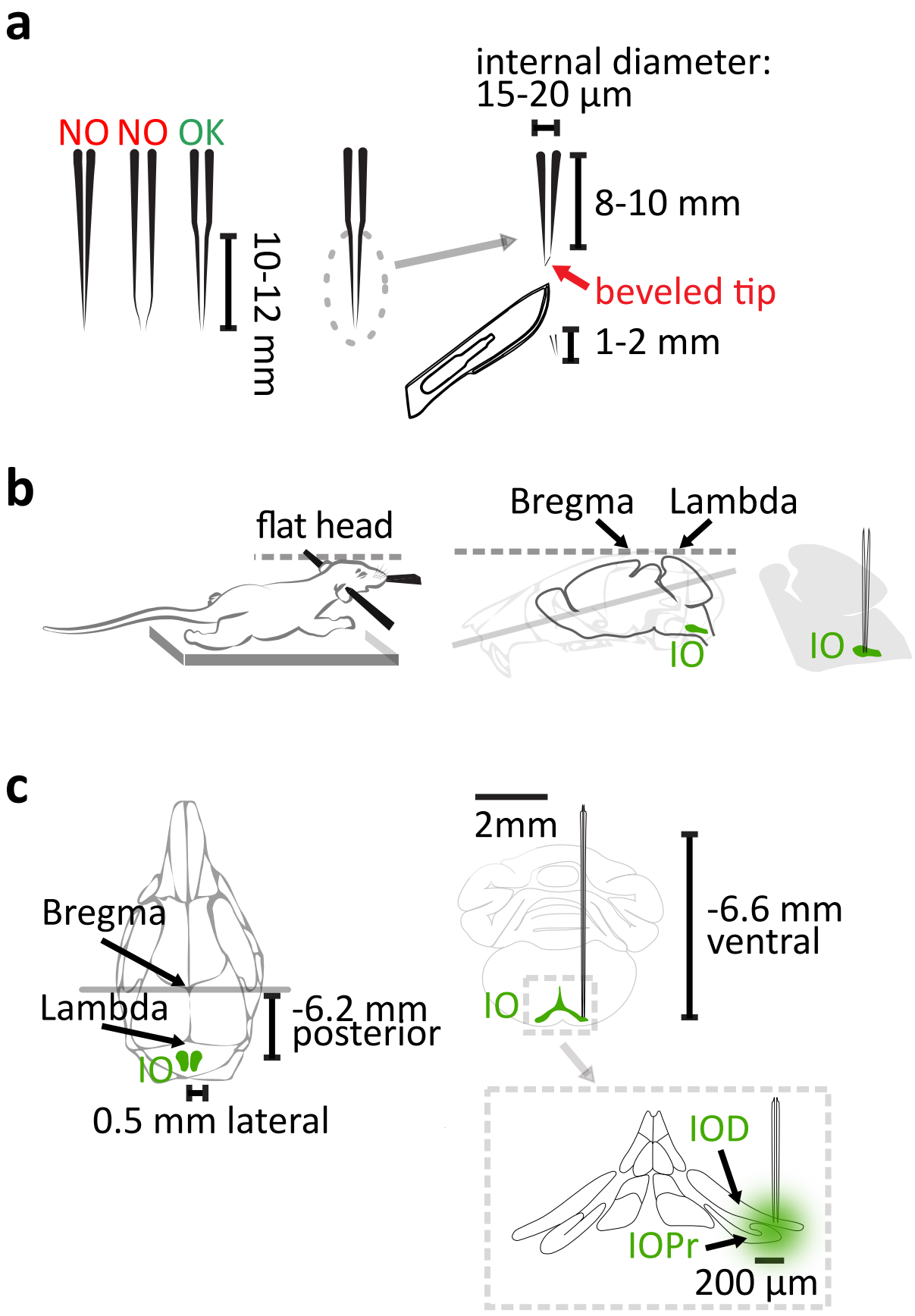
DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Russell, J.T. Imaging calcium signals in vivo: A powerful tool in physiology and pharmacology. *British Journal of Pharmacology*. doi: 10.1111/j.1476-5381.2010.00988.x (2011).
2. Zhang, T. *et al.* Kilohertz two-photon brain imaging in awake mice. *Nature Methods*. doi: 10.1038/s41592-019-0597-2 (2019).
3. Lin, X., Zhao, T., Xiong, W., Wen, S., Jin, X., Xu, X.M. Imaging neural activity in the primary somatosensory cortex using Thy1-GCaMP6s transgenic mice. *Journal of Visualized Experiments*. **2019** (143), 1–8, doi: 10.3791/56297 (2019).

4. Lee, H.S., Han, J.H. Successful In vivo Calcium Imaging with a Head-Mount Miniaturized Microscope in the Amygdala of Freely Behaving Mouse. *Journal of visualized experiments*. (162), 1–19, doi: 10.3791/61659 (2020).
5. Aharoni, D., Khakh, B.S., Silva, A.J., Golshani, P. All the light that we can see: a new era in miniaturized microscopy. *Nature Methods*. **16** (1), 11–13, doi: 10.1038/s41592-018-0266-x (2019).
6. Khosrovani, S., Van Der Giessen, R.S., De Zeeuw, C.I., De Jeu, M.T.G. In vivo mouse inferior olive neurons exhibit heterogeneous subthreshold oscillations and spiking patterns. *Proceedings of the National Academy of Sciences*. **104** (40), 15911–15916, doi: 10.1073/pnas.0702727104 (2007).
7. Osten, P., Cetin, A., Komai, S., Eliava, M., Seeburg, P.H. Stereotaxic gene delivery in the rodent brain. *Nature Protocols*. **1** (6), 3166–3173, doi: 10.1038/nprot.2006.450 (2007).
8. Berg, L., Gerdey, J., Masseck, O.A. Optogenetic Manipulation of Neuronal Activity to Modulate Behavior in Freely Moving Mice. *Journal of visualized experiments : JoVE*. doi: 10.3791/61023 (2020).
9. Green, C.J., Knight, J., Precious, S., Simpkin, S. Ketamine alone and combined with diazepam or xylazine in laboratory animals: A 10 year experience. *Laboratory Animals*. **15** (2), 163–170, doi: 10.1258/002367781780959107 (1981).
10. Tsukamoto, A., Serizawa, K., Sato, R., Yamazaki, J., Inomata, T. Vital signs monitoring during injectable andn inhalant anesthesia in mice. *Experimental Animals*. **64** (1), 57–64, doi: 10.1538/expanim.14-0050 (2015).
11. Ewald, A.J., Werb, Z., Egeblad, M. Monitoring of vital signs for long-term survival of mice under anesthesia. *Cold Spring Harbor Protocols*. **6** (2), 174–178, doi: 10.1101/pdb.prot5563 (2011).
12. Vrieler, N. *et al.* Variability and directionality of inferior olive neuron dendrites revealed by detailed 3D characterization of an extensive morphological library. *Brain Structure and Function*. **0** (0), 1677–1695, doi: 10.1007/s00429-019-01859-z (2019).
13. Esposito, M.S., Capelli, P., Arber, S. Brainstem nucleus MdV mediates skilled forelimb motor tasks. *Nature*. doi: 10.1038/nature13023 (2014).
14. Martin, E.M., Devidze, N., Shelley, D.N., Westberg, L., Fontaine, C., Pfaff, D.W. Molecular and neuroanatomical characterization of single neurons in the mouse medullary gigantocellular reticular nucleus. *Journal of Comparative Neurology*. doi: 10.1002/cne.22639 (2011).
15. Ketamine/Xylazine. *Cold Spring Harbor Protocols*. **2006** (1), pdb.rec702, doi: 10.1101/pdb.rec702 (2006).
16. Giovannucci, A. *et al.* CaImAn an open source tool for scalable calcium imaging data analysis. *eLife*. **8**, 1–45, doi: 10.7554/eLife.38173 (2019).
17. Lu, J. *et al.* MIN1PIPE: A Miniscope 1-Photon-Based Calcium Imaging Signal Extraction Pipeline. *Cell Reports*. **23** (12), 3673–3684, doi: 10.1016/j.celrep.2018.05.062 (2018).
18. Viejo, G., Kerjean, E. (Sciences S.U. GitHub - PeyracheLab/miniscoPy: A package to analyse calcium imaging data recorded with the Miniscope. at <https://github.com/PeyracheLab/miniscoPy>.
19. Thévenaz, P., Ruttimann, U.E., Unser, M. A pyramid approach to subpixel registration based on intensity. *IEEE Transactions on Image Processing*. doi: 10.1109/83.650848 (1998).
20. Zhou, P. *et al.* Efficient and accurate extraction of in vivo calcium signals from

microendoscopic video data. *eLife*. **7**, 1–37, doi: 10.7554/eLife.28728 (2018).
21. Zhou, P. GitHub - zhoupc/CNMF_E: Constrained Nonnegative Matrix Factorization for microEndoscopic data. at <https://github.com/zhoupc/CNMF_E>.
22. De Gruijl, J.R., Bosman, L.W.J., De Zeeuw, C.I., De Jeu, M.T.G. Inferior olive: All ins and outs. *Handbook of the Cerebellum and Cerebellar Disorders*. doi: 10.1007/978-94-007-1333-8_43 (2013).
23. Dorgans, K., Kuhn, B., Uusisaari, M.Y. Imaging Subthreshold Voltage Oscillation With Cellular Resolution in the Inferior Olive in vitro. *Frontiers in Cellular Neuroscience*. doi: 10.3389/fncel.2020.607843 (2020).



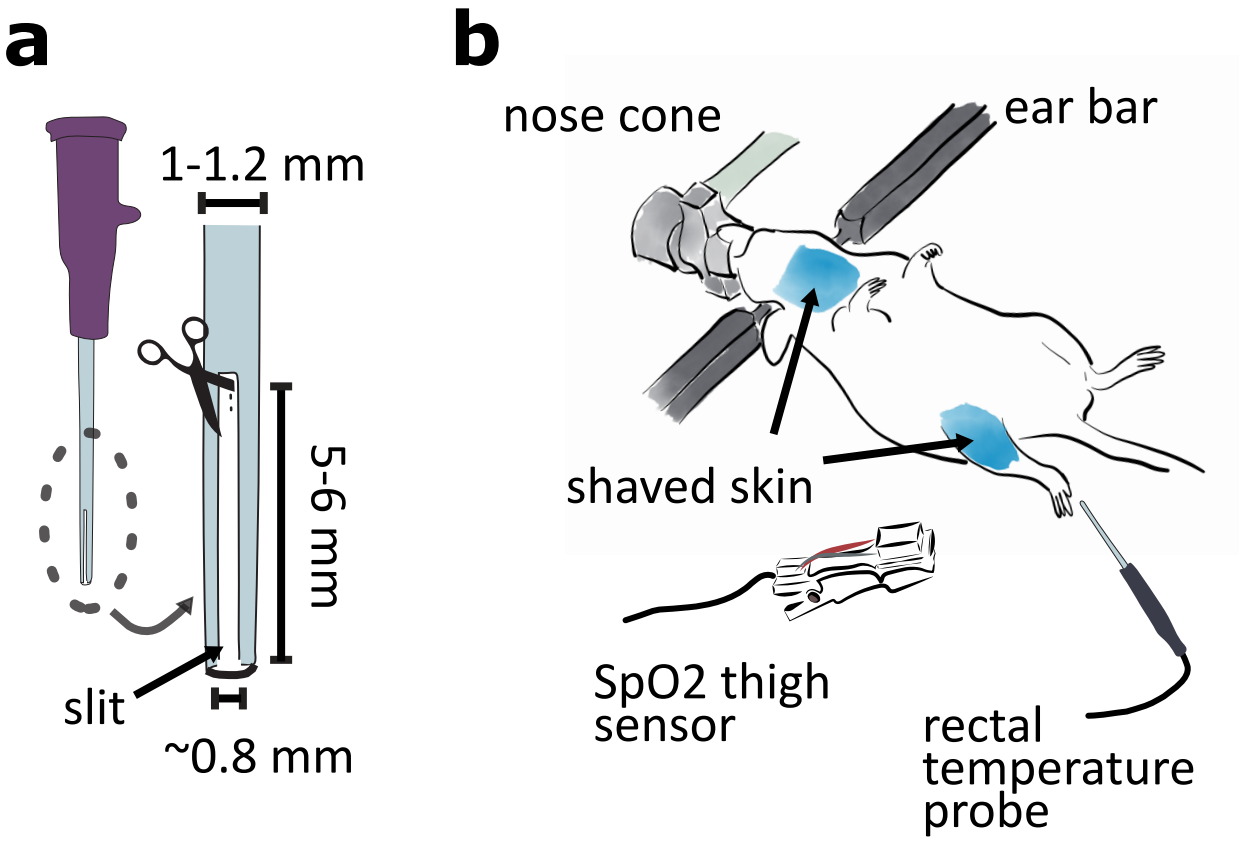
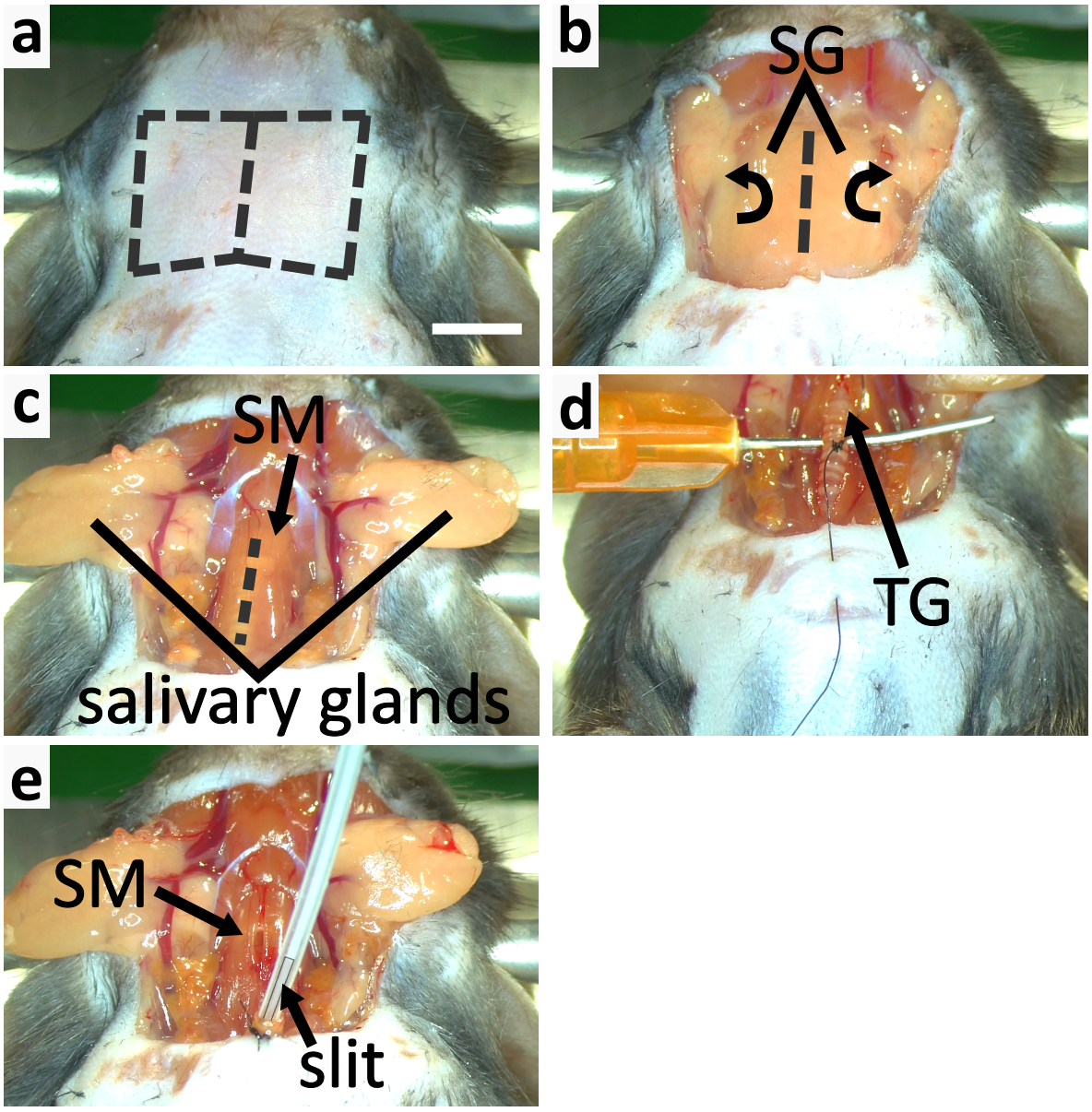
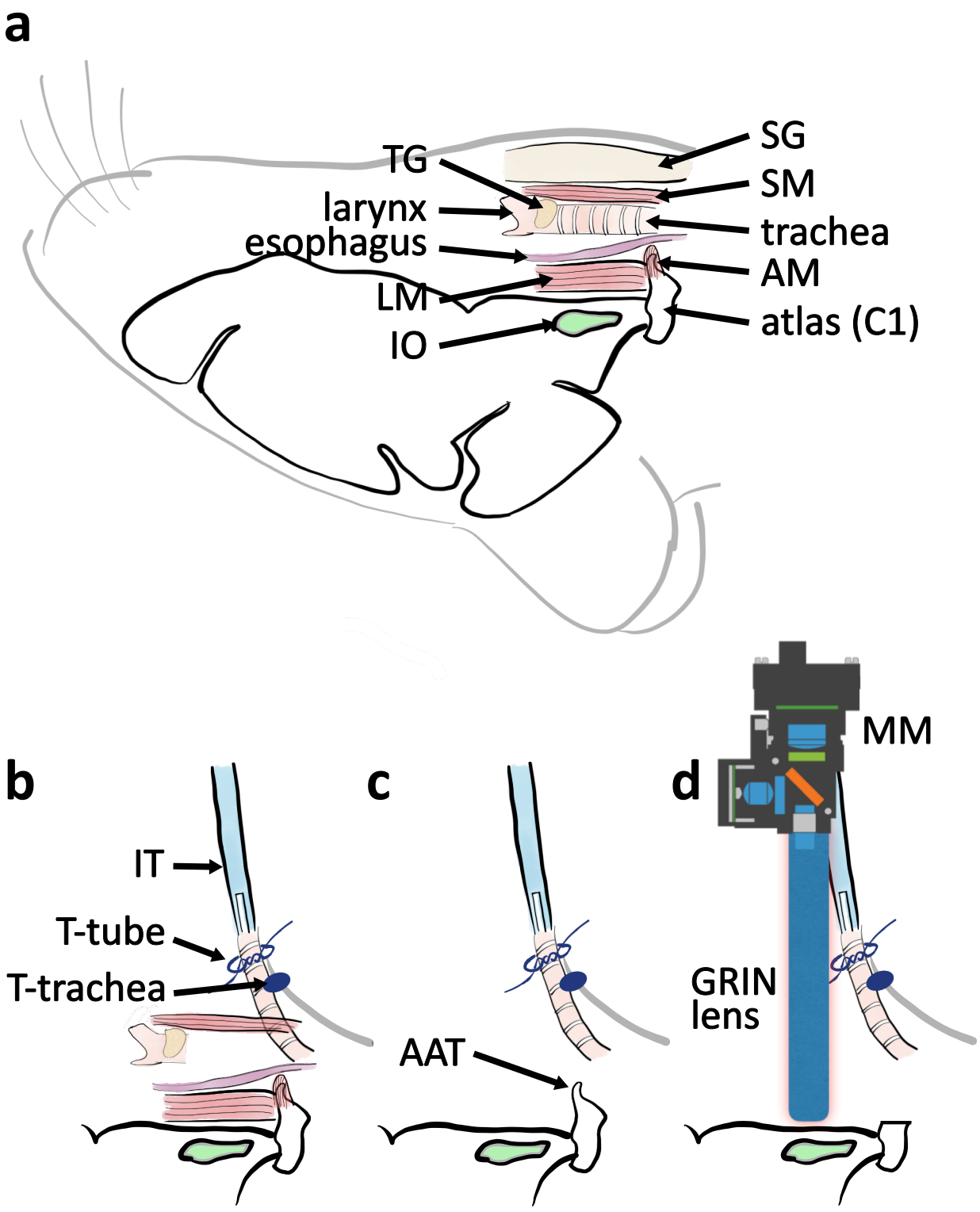
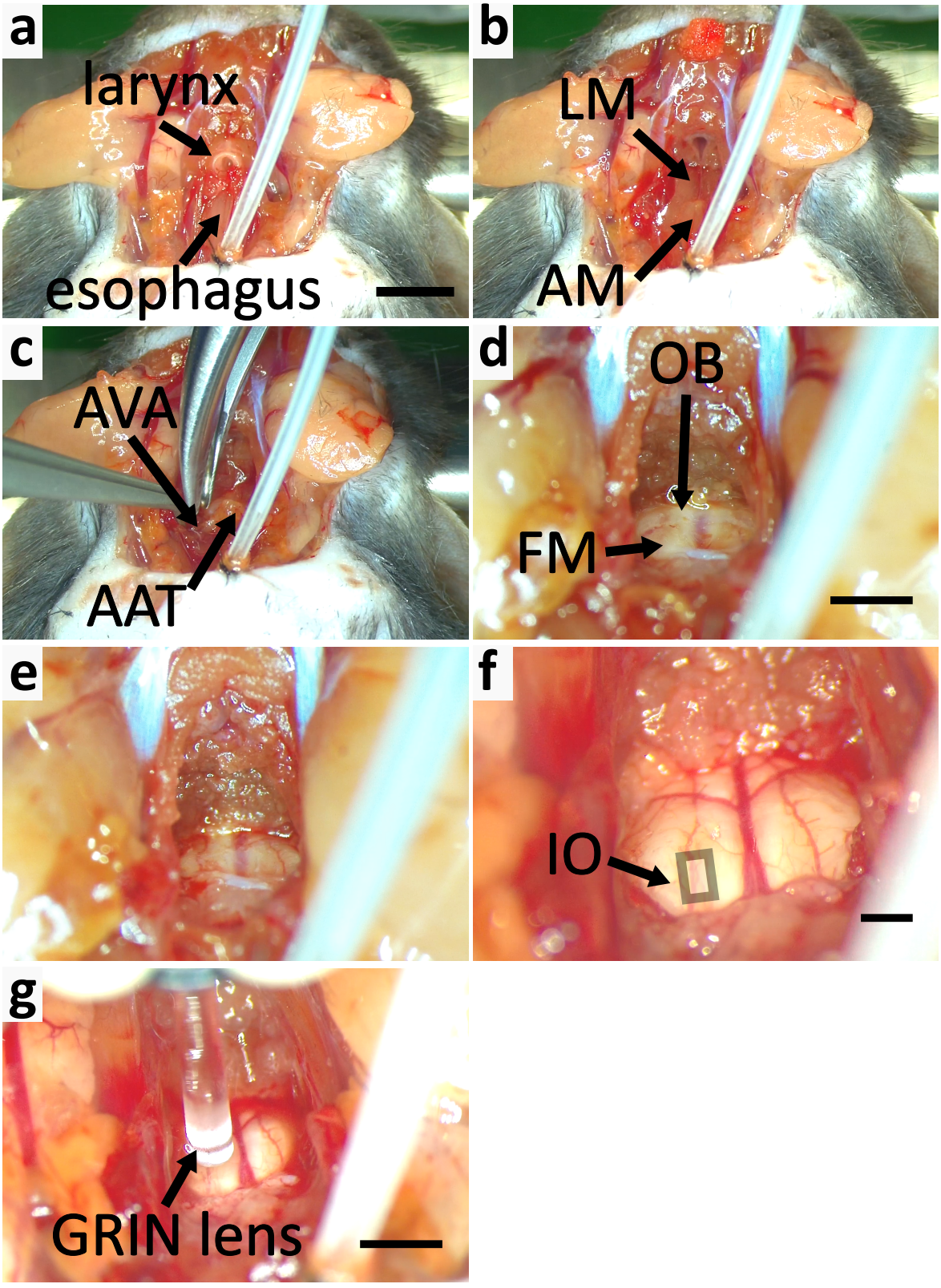
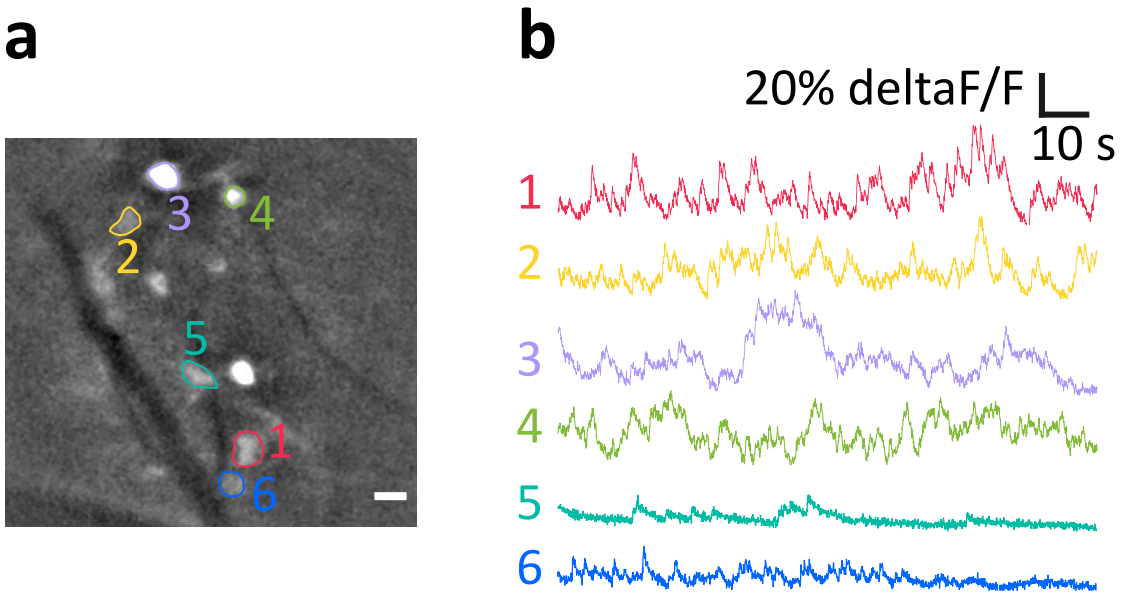


Figure 3









Name of Material/ Equipment	Company	Catalog Number
AAV.CAG.GCaMP6s.WPRE.SV40	Addgene, USA	100844-AAV9
Absorbable suture with 6 mm half circle needle	Natume, Japan	L6-60N2
Absorption triangles	FST, Germany	18105-03
Stereo microscopes	Leica, Germany	M50
Castroviejo curved tip needle holder with lock	FST, Germany	12061-01
cotton swabs	Sanyo, Japan	HUBY-340
Delicate suture tying forceps	FST, Germany	11063-07
Delicate Suture Tying Forceps	FST, Germany	11063-07
Dumont #5/45 forceps	FST, Germany	11251-35
Fine Iris scissors	FST, Germany	14060-09
Friedman-Pearson rongeur curved tip	FST, Germany	16221-14
Gelfoam absorbable gelatin sponge	Pfizer, USA	0315-08
Glass-Capillary Nanoinjection	Neurostar, Germany	n/a
Graefe Forceps with serrated tip	FST, Germany	11052-10
Implantation rod	Inscopix, USA	n/a
IsoFlo	Zoetis, UK	n/a
KETALAR FOR INTRAMUSCULAR INJECTION	Daiichi Sankyo, Japan	n/a
Kimwipes	Kimberly-Clark, USA	
Laser-Based Micropipette Puller	Sutter Instrument, USA	P-2000
Micropipette Beveler	Sutter Instrument, USA	BV-10
Motorized Stereotaxic based on Kopf, Model 900	Neurostar, Germany	n/a
mouseOxPlus with rectal temperature sensor and thigh clamp pulse oximeter	Starr Life Sciences, PA, USA	MouseOxPlus
nVoke2 integrarted Calcium imaging micro camera system	Inscopix, USA	1000-003026
Ohaus Compact Scales	Ohaus, USA	CS 200
Otsuka Normal Saline	Otsuka Pharmaceutical Factory, Japan	n/a
Physiological-biological temperature controller system	SuperTech Instruments, Hungary	TMP-5b
ProView Lens Probe 1.0 mm diameter, 9.0 mm length	Inscopix, USA	1050-002214
Q114-53-10NP glass capillaries	Sutter Instrument, USA	112017
Safety IV Catheter 20G	B. Braun, Germany	4251652-03
Sand paper	ESCO, Japan	EA366MC
Scalpel blade	Muromachi Kikai, Japan	10010-00

SomnoSuite low flow inhalation anesthesia system	Kent Scientific, USA	SOMNO
Surgic XT Plus drill	NSK	Y1002774
Syringe 1 ml	Terumo, Japan	SS-01T
Syringe needle 25G	Top, Japan	00819
Syringe needle 26G	Terumo, Japan	NN-2613S
Thrive 2100 Professional Trimmer	Thrive, Japan	n/a
Vannas-Tübingen spring scissors	FST, Germany	15004-08
Vaseline	Hayashi Pure Chemical, Japan	22000255
Veet sensitive skin	Veet, Canada	n/a
Xylocaine Jelly 2 % 30ml	Aspen Japan, Japan	871214

	Comments/Description
hook needle with thread	
Surgical sponges	
Surgery tool	
Surgery tool	
Surgery tool	
Surgery tool	
Surgery tool	
Surgery tool	
Hemostatic gelatin sponge	
For virus vector injection	
Surgery tool	
It is part of the nVoke2 system. It's designed to nVoke2 miniature microscpe and GRIN lens can be mounted on it	
Isoflurane	
Ketamine	
Cleaning tissue	
Stereotaxic frame	
Measures animal heart rate, arterial oxygen saturation (SpO2), breath rate, and temperature	
Miniature microscope	
Scale used to weight animal	
Thermal pad for mouse	
Gradient-refractive index (GRIN) lens	
Customized quartz glass capillaries	
20 gauage catheter used to prepare intubation tube	
Used to polish the tip of 25G needle to prepare curved and blunt needle	
Used to cut the tip of quartz glass pipette	

Provides precise control of isoflurane flow
For virus vector injection

Used to make blunt and bended needle

Shaver
Surgery tool

Hair removal cream

From the nRIM Unit

OIST

OKINAWA INSTITUTE
OF SCIENCE AND TECHNOLOGY
GRADUATE UNIVERSITY

1919-1 Tancha, Onna-son,
Okinawa, 904-0495 Japan
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http://www.oist.jp

Dear Editor,

We thank you, the reviewers and the vet for the generous comments and have edited the manuscript and the video to address the concerns.

In particular we have added more details to the protocol to ensure the replication of the method by both experts and researchers new to the field.

We hope that the manuscript and video are now suitable for publication in JoVE.

Marylka Yoe Uusisaari

Assistant Professor

On behalf of all authors.

Editorial and production comments:

Changes to be made by the Author(s) in the Text:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please use American English throughout.

We have proofread the manuscript to correct numerous spelling and grammar mistakes.

2. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: nVoke 2, Inscopix, CA, USA, B.Braun Introcan Safety IV Catheter, Kimwipe, Inscopix, CA, USA, Vaseline, etc.

All commercial language has been removed from the manuscript and replaced with common terminology.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

It has been added.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Non-imperative tense contents have been moved to "Note."

5. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed from the protocol.

6. The Protocol should contain only action items that direct the reader to do something.

Protocol has been modified accordingly.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? This can be done by including mechanical actions, button clicks in the software, command lines, (for the data analysis part), knob turns, etc.

We have added more details to many protocol steps where feasible.

8. Please include how you maintain sterility during the surgery process.

In this protocol, aseptic surgery techniques were only applied to the stereotaxic virus vector injection. This has been indicated at the beginning of the protocol. The rest procedures are non-survival surgery and aseptic surgical techniques are not necessary. Therefore, we only mention that the surgery tools should be clean at step 2.4.

9. *Please sort the materials table in alphabetical order.*

We have sorted the materials table in alphabetical order.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

Manuscript has been modified upon request. Manuscript figures have been added to the video.

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

Manuscript has been modified to be more homogenous with the narration.

3. Please equalize the volume throughout.

Audio volume has been equalized.

4. Please ensure that the subheadings in the protocol section are the same in the text and in the video.

Subheadings have been corrected to be the same in the manuscript and in the video.

5. Need graphics for the speakers with their names and institution. Unfortunately, we cannot do this in a video produced by authors.

We have added speakers' name with the institution logo.

6. Please spell Denionise as Deionized in the results section. Please use American English throughout.

We think what was referred to was “Denoised”. What we want to express is that representative result video is noise-removed.

7. Please include the title card at the end of the video as well.

It has been added.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe a novel ventral surgical approach that allows use of a GRIN lens to monitor neuronal activity in the Inferior Olive (IO) of the adult mouse in vivo. The extreme ventral location of the IO complicates a more traditional dorsal approach. The procedure is explained in detail and representative data are included from an IO recording. The manuscript is clearly written and I found it easy to follow the steps. All material, equipment is detailed in a list.

We thank the reviewer for the kind comments.

Major Concerns:
The manuscript does a nice job of explaining a technically challenging procedure. My main concern is that Figure 3, which is the photographic depiction of the steps in the procedure, may be too small to be clear. Perhaps this Figure could be split into 2 figures and each panel size increased?

We have split the Figure 3 into two figures (Figure 3 and 5) and increased the size of panels.

Minor Concerns:
1. Throughout the text there is a dearth of articles (e.g., "the") and prepositions (e.g., "a", "an", "in") and this makes it read a bit rough. This did not detract form the message getting through, however.

We have thoroughly proofread the text, hopefully it is acceptable now.

2. Spo2 sensor is undefined.

We have added the explanation for SpO2: peripheral oxygen saturation.

Reviewer #2:

Manuscript Summary:
New advances have been made in photonics, where calcium indicators and gradient-refractive index (GRIN) lenses, allow neuronal imaging deep within the brain. However, brain regions with a deeper anatomical arrangement are difficult to access, meaning that their functions remain unstudied. Guo and colleague's aim to develop a novel protocol for in vivo calcium imaging of the inferior olive (IO) of the medulla in mice brains. To test this aim, they developed a non-recovery surgery protocol, involving a tracheotomy to access the brainstem, before mounting a GRIN lens and miniature microscope above the IO for imaging of GCaMP6s-expressing neurons. This allowed investigation of neuronal activity by identifying GCaMP6s-expressing neurons and monitoring calcium transients of IO neurons. Authors

claim that this procedure provides a new method for in vivo calcium imaging of regions of the deep ventral brainstem. After reading this paper, I believe it is innovative by tackling the restrictive access to the ventral medulla by opting to approach it from a different angle. The paper is understandable, aided by a very informative video that takes you step-by-step through the procedure and coincides well with the written information. While a broader image of what this protocol may allow in the future is missing, along with some important aspects of the protocol (discussed below), it provides a well-established in vivo calcium imaging protocol for the ventral regions of the brain. Thus, I believe it will provide a novel approach that many labs may use in their experimental design for a more established recording of IO activity. On this note, I believe that the paper is acceptable for publication after subsequent corrections are completed.

We thank the reviewer for highlighting the relevance for ventral-region imaging studies, and indeed hope that it will be used for other regions that the IO as well.

Major Concerns:
Introduction/Abstract
* Line 35: In the abstract you mention that you combine calcium imaging with optogenetic stimulation. However, this is not described throughout the protocol or results. It would be best to remove this statement.

The mention of the optogenetic stimulation has been deleted from the abstract.

* Within the abstract, there is no mention of the results or conclusions of the paper. It may be worthwhile adding a couple of sentences describing that neurons were imaged, and calcium traces were identified to illustrate that this procedure has been tested and shown to be successful.

We have added a sentence in the abstract to indicate that calcium imaging was used to show the usefulness of the method.

Methods
* More information regarding the animals used in your protocol is required - breed, number used, sex, age - and any ethical comments should be provided in the text.

We have added information on mouse breed and sex in the note for 1.3. The ethic statement has been added at the beginning of protocol.

We have used a considerable number of animals while developing this protocol in the past years so that the reader does not have to. For the present manuscript we filmed the procedure on one animal.

*Also, information regarding how the animals were housed and animal care following viral injection surgery would be helpful.

We added a comment highlighting that sterile surgery techniques were only applied to the virus injection procedure and not the non-survival ventral surgery that is the focus of this protocol.

We follow our institutional guidelines for post-operative care and have now written in the manuscript that the reader should follow the guidelines of their institution.

* More information regarding the viral transfection is required. While information on the procedure is provided in another paper, it would be beneficial to have the procedure used in writing on this paper. For example, information regarding the anaesthetic used, how to attach the mice in the stereotaxic frame, how to identify the injection site and how to inject the virus. Additionally, information on the virus that is used in this experiment (virus used, promoters used, injection rate etc.) will be required for replication.

We have considered this request in depth. However, we respectfully would disagree. Viral injection procedures are on one hand standard laboratory methodology (search for "mouse brain viral injection" returns 197 articles in JoVE alone) and on the other hand, must be executed according to institutional safety regulations. Therefore, we do not believe it being useful for us to present it here. What we have provided are the specific details that distinguish IO-targeting injections from those to more easily-reachable targets, which accord with the JoVE guideline - "Well-established methods used within the protocol should be cited as necessary and any modification of the aforementioned procedures should be described".

Most importantly, this manuscript focuses on the surgery approach of IO from the ventral side which is a novel method that does not necessarily require viral injections (in case transgenic animals with IO-targeted GCamP6 are available, for example).

Thus, we would prefer not to include the description of the entire viral transfection part, as we believe it is not relevant for the main topic (ventral surgery). Also, we have no means of producing the additional video material on our own without JoVE video-editorial help within the requested time frame.

* In the step 2.4, clarify what "prepare isoflurane flow" means.

We have change it to switch on the isoflurane vaporizer.

* The timing of the injectable anaesthetic is unclear e.g., when to inject and at what stage of the surgery. It would be ideal if there was a diagram or flow-chart of this procedure to aid understanding of the correct methodology.

We have added a separated step, 4.2.3, for the second dose of ketamine to make the timing of injections more understandable.

* There is no information provided regarding the GRIN lens and the microscope in the main text, but only in the table, which does not seem to be referred. For replication, information on how this imaging system is set up and used is essential.

JoVE does not allow any commercial language in the manuscript, but only in the materials table. Thus, we cannot describe our nVoke system in any further detail. The specifics of how to set up a miniature

endoscope system vary greatly, so we have added a recommendation for the user to set up the system according to the product user manual.

* Greater explanation on how to identify the IO for recording would be useful.

We have added a comment on average soma diameter of IO neurons vs. adjacent medullary reticular formation neurons, with references, in the "Note" of 6.4.

Results
* Some readers may require further explanation of what 20% dF/F is and how it was calculated.

We have changed the dF/F to deltaF/F and added "the mean-normalized fluorescence intensity" in the sentence to explain it.

Conclusions
* As there is no conclusive statement, there is no validation that the aim of this study has been accomplished. The concluding remarks don't go into enough detail to justify why the protocol has been successful and what it will allow for future research. For example, you have not justified what this will now allow in terms of investigating the IO and inputs to the cerebellum. By doing so, this will add a final understanding to the paper and clarify its importance to the field.

We have added a paragraph at the end of the discussion to indicate the potential of this method.

* The majority of the discussion states the limitations of the protocol with no discussion of the protocol's success. This ends the paper with a lack of excitement of what you have been able to achieve. I suggest that while mentioning these limitations, you could provide a potential improvement for the future and to finish by mentioning the current success of the protocol.

We have added comments on possible future applications using various stimulations such as sensory and optogenetics, to help to investigate how the IO integrates information prior to transmitting it to the cerebellar cortex.

Minor Concerns:
Introduction/Abstract
* There is no emphasis on what functions this brain region may be involved in and why it is 'necessary' to undertake this surgical procedure for brain research. By explaining to the reader, the potential role of the IO, it will clarify the importance of this research.

We have added a sentence in the abstract to state that IO is a critical component of cerebellar functions related to both motor and cognitive behavior.

* The structure of the introduction does not flow from paragraph to paragraph. For example, there is an

explanation of how GRIN lenses have difficulty reaching deep layers of the brain, with the next sentence then describing how you are going to use GRIN lenses to image deep areas of the brain. Introducing more of a flow and justifying how the GRIN lens will be able to image deep areas in the brain will make it more understandable.

The first sentence of the second paragraph of introduction has been modified to "… we describe how to overcome this difficulty by taking advantage of the relatively easy accessibility of medulla through the ventral aspect of the brain" to have a better flow.

* The grammar in the abstract/summary needs some corrections. Line 22: "a protocol to expose brainstem of adult mouse from ventral side. By using gradient-refractive index lens" should be "a protocol to expose the brainstem of adult mice from the ventral side. By using a gradient-refractive index lens"

We have carefully checked our manuscript and hopefully corrected all grammar problems.

* Line 38: GCaMP6S should be GCaMP6s.

It is GCaMP6s now.

Methods
* It may be worthwhile to include some troubleshooting so common and fixable surgical errors can be corrected.

We have added more information to the protocol for the replication of the presented method. According to the JoVE guideline, we placed the troubleshooting in the discussion section.

* Figure legends in the paper could be made clearer e.g., start each legend with a title of what each panel shows and ensure that everything in the figure is described in the legend. Also, some of the figures have a lot of acronyms and while they can be seen throughout the legend, it would be more understandable if they were also listed at the end of each figure legend.

Legends have been modified to be clearer about the figure contents.

Missing abbreviations in each figure have been explained in the related figures. Please understand that we cannot add an additional list of abbreviations at the end of each figure legend. That would be too repetitive.

* In Figure 3, from D-F, it may be worthwhile including the tie to the chest at this point as seen in the video.

We have changed the "secure the trachea to the chest skin" to "secure the trachea to the chest skin with the suture thread" for easier understanding.

* The structure of protocol section 2 could be improved as it is slightly back and forth. For example, it

starts describing how to prepare the injectable drugs in section 2.3 but stops and re-starts in section 2.6. Therefore, I suggest having three different sub-sections within section 2: preparation of experimental tools, preparation of injectable anaesthetic, preparation of isoflurane anaesthetic.

We have moved the 2.6 to 3.1 and rename the 3 as "administration of anesthesia and preparation of the mouse for the surgery". The protocol is chronological, and the volume of diluted ketamine can only be calculated with the weight of the animal, therefore the step 3.1 must be separated from 2.3.

* Line 185: "the needle to lead thread go around" should be "the needle to lead thread around".

It has been changed accordingly.

Results

* There is a mention of differences in transfection efficacy, with stronger transfection having lower signal-to-noise ratio. However, a neuron that appears to have strong transfection efficacy has not been processed to illustrate its calcium trace. It could be worthwhile showing the results of this cell, to illustrate the strong calcium transients that can be recorded with your protocol.

The pointed-out neuron with strong fluorescence signal did not show any calcium transients. This most likely due to the over-expression of GCaMP6s. For this reason, a proper transfection time (3-4 weeks mentioned in the step 1.4) for virus is required to express adequate GCaMP6s in a good number of neurons. However, neurons with inadequate and/or overexpressed GCaMP6s are inevitable.

While a standard method of current neuroscientific laboratory, proper use of calcium imaging methodology demands that the researchers are deeply familiar with the genetic, molecular, metabolic, neurophysiological, optical and image processing aspects of the methodology. Discussion along these themes is beyond the scope of the present surgical method paper.

Conclusions

* The structure of the discussion section can be improved. It may be better to start by introducing the results of the paper and what was achieved, followed by a short discussion of the issues that may have occurred, finishing with a conclusive statement as to what this has achieved and what this means for the future.

According to the guideline of JoVE, this section should discuss:

- *Critical steps in the protocol*
- *Modifications and troubleshooting of the method*
- *Limitations of the method*
- *The significance of the method with respect to existing/alternative methods*
- *Future applications or directions of the method*

We are grateful that the reviewer believes in our method and suggests a less humble ending to the discussion. We hope our added sentences are acceptable.

Reviewer #3:

Manuscript Summary:
The manuscript of Guo et al. provides a detailed and well followable demonstration of a novel protocol for recording in vivo neuronal activity from the ventral brainstem. I believe the manuscript and the video are suitable for publication in the present form. Although, in some points I would suggest minor changes.

Major Concerns:
there are no major concerns

Minor Concerns:
I have a few suggestions for consideration.

51. From which company the GRIN lenses were purchased? Similarly, it is worth mentioning the source for stereotaxic frame, animal temperature controller and SpO2 sensor.

JoVE editors tell us they cannot publish manuscript with commercial language. Therefore, please find the details of materials in the separated table file.

70. What are the parameters of the virus carrying GCaMP6s gene? (which virus, serotype, plasmid, purchaser or any other source)

It is a AAV9 virus with CAG promoter. I have added the full name of this virus in section 1. Please find the catalog number in the materials table.

73. The Authors use quartz glass pipettes for injection. What are the advantages of pipettes compared to Hamilton syringes?

The quartz glass pipette functions as the "needle" to obtain extremely precise targeting. We used computer-controlled nanoliter injector to deliver precise volume of virus. We have clarified this in the "Note" of this sub section. We believe Hamilton syringe cold be used instead of the injector with this glass pipette by using an adaptor.

146. I would mention that Veet cream is a hair removal gel.

Agreed. We've changed Veet to hair removal cream.

In summary, the manuscript is acceptable in the present form but the Authors could consider my suggestions for a few minor changes.

Reviewer #4:

Manuscript Summary:
Authors Guo, Ozer, and Uusissaari have provided a very thorough and meticulous protocol for in vivo calcium imaging in the mouse inferior olive using a GRIN lens and miniature microscope. The paper is extremely well-written and would enable a reader to implement this technique for their own use. The authors do an excellent job outlining the myriad technical considerations relevant to the protocol. I especially appreciate the "notes" throughout the paper where important technical considerations are explained in more detail. This greatly adds to how helpful this protocol would be to others.

We are grateful to the reviewer's kind assessment of our work and detail, as we hope the method will be helpful for many researchers.

Major Concerns:
- None

Minor Concerns:
- In step 8.3, what is the justification for applying a bandpass filter set to 1Hz? Is this the typical frequency threshold used for lowpass filtering in calcium imaging data? If so, has this been used with data collected from brainstem neurons?

Lowpass filter with 1Hz as passband was used to smooth data for peak detection of noisy data. However, this kind of analysis is not the focus of this manuscript. We have deleted step 8.3 from the manuscript and only show the non-filtered, raw data traces in figure 5 to avoid confusion.

Vet's comments:

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes
1: Section 2.1, and section 4.2.6	1:03	(Improvements or clarification required) Text describes cutting a slit in the intubation tube so that the animal can breathe out. Can a reference to the source of this information be provided, as it's not a method I'm familiar with. If the flow rate is appropriate on the anesthesia machine, breathing should not be impaired with an intact tube. Leaving an opening allows for leakage of isoflurane, decreasing efficacy and potentially exposing personnel to waste gas.	Possibly, depending on response	Yes	If there is a specific reasoning for this technique, either based on past scientific data or a particular reference, this should be explained in the text, since this is most users are trained to use an unaltered catheter for intubation.
2: Section 2.3.1		(Improvements needed) Ketamine dilution is described as a percentage. Please provide the mg/ml of the dilution for clarity, since ketamine concentrations could potentially differ.	No	Yes	

3: Section 4.2.1		(Improvements needed) Ketamine doses are listed as ml/kg. Please list as mg/kg to ensure clarity for users who may dilute their ketamine differently.	No	Yes	

1. Isoflurane was administered directly to the animal trachea with a vaporizer dedicated to small animals, which has a constant airflow, in this protocol. Mouse is not able to breath out with the constant air pressure if the tube is intact. However, if a ventilator is used in addition to the vaporizer, the tube can be left intact. We have added this information to the note of 2.1.

2. The concentration of ketamine as mg/ml has been provided.

3. We have added the amount of ketamine as mg/kg in case reader want to dilute it differently.