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TITLE:

In Vivo Calcium Imaging of Mouse Geniculate Ganglion Neuron Responses to Taste Stimuli

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KEYWORDS:

calcium imaging, taste, geniculate ganglion

SUMMARY:

Here we present how to expose the geniculate ganglion of a live, anesthetized laboratory mouse and how to use calcium imaging to measure the responses of ensembles of these neurons to taste stimuli, allowing for multiple trials with different stimulants. This allows for in depth comparisons of which neurons respond to which tastants.

ABSTRACT:

Within the last ten years, advances in genetically encoded calcium indicators (GECIs) have promoted a revolution in in vivo functional imaging. Using calcium as a proxy for neuronal activity, these techniques provide a way to monitor the responses of individual cells within large neuronal ensembles to a variety of stimuli in real time. We, and others, have applied these techniques to image the responses of individual geniculate ganglion neurons to taste stimuli applied to the tongues of live anesthetized mice. The geniculate ganglion is comprised of the cell bodies of gustatory neurons innervating the anterior tongue and palate as well as some somatosensory neurons innervating the pinna of the ear. Imaging the taste-evoked responses of individual geniculate ganglion neurons with GCaMP has provided important information about the tuning profiles of these neurons in wild-type mice as well as a way to detect peripheral taste miswiring phenotypes in genetically manipulated mice. Here we demonstrate the surgical procedure to expose the geniculate ganglion, GCaMP fluorescence image acquisition, initial steps for data analysis, and troubleshooting. This technique can be used with transgenically encoded GCaMP, or with AAV-mediated GCaMP expression, and can be modified to image particular genetic subsets of interest (i.e., Cre-mediated GCaMP expression). Overall, in vivo calcium imaging of geniculate ganglion neurons is a powerful technique for monitoring the activity of peripheral gustatory neurons and provides complementary information to more traditional whole-nerve chorda tympani recordings or taste behavior assays.

INTRODUCTION:

A key component of the mammalian peripheral taste system is the geniculate ganglion. In addition to some somatosensory neurons that innervate the pinna of the ear, the geniculate is comprised of the cell bodies of gustatory neurons innervating the anterior tongue and palate. Similar to other peripheral sensory neurons, the geniculate ganglion neurons are pseudo-unipolar with a long axon projecting peripherally to the taste buds, and centrally to the brainstem nucleus of the solitary tract[1]. These neurons are activated primarily by the release of ATP by taste receptor cells responding to taste stimuli in the oral cavity[2,3]. ATP is an essential neurotransmitter for taste signaling, and P2rx receptors expressed by the gustatory ganglion neurons are necessary for their activation[4]. Given that taste receptor cells express specific taste receptors for a particular taste modality (sweet, bitter, salty, umami, or sour), it has been hypothesized that gustatory ganglion neuron responses to taste stimuli would also be narrowly tuned[5].

Whole nerve recordings have shown both the chorda tympani and the greater superior petrosal nerves conduct gustatory signals representing all five taste modalities to the geniculate ganglion[6,7]. However, this still left questions about the specificity of neuronal responses to a given tastant: if there are taste modality specific neurons, polymodal neurons, or a mixture of both. Single fiber recordings give more information about the activity of individual fibers and their chemical sensitivities[8-10], but this methodology is limited to collecting data from small numbers of fibers. Similarly, in vivo electrophysiological recordings of individual rat geniculate ganglion neurons give information about the responses of individual neurons[11-13], but still loses the activity of the population and yields relatively few neuron recordings per animal. In order to analyze the response patterns of neuronal ensembles without losing sight of the activity of individual neurons, new techniques needed to be employed.

Calcium imaging, especially using genetically encoded calcium indicators like GCaMP, has provided this technical breakthrough[14-18]. GCaMP uses calcium as a proxy for neuronal activity, increasing green fluorescence as calcium levels within the cell rise. New forms of GCaMP continue to be developed to improve the signal to noise ratio, adjust binding kinetics, and adapt for specialized experiments[19]. GCaMP provides single neuron resolution, unlike whole nerve recording, and can simultaneously measure responses of ensembles of neurons, unlike single fiber or single cell recording. Calcium imaging of the geniculate ganglia has already provided important information about the tuning profiles of these neurons in wild-type mice[16,20], and has identified peripheral taste miswiring phenotypes in genetically manipulated mice[18].

One major difficulty to applying in vivo calcium imaging techniques to the geniculate ganglion is that it is encapsulated within the bony tympanic bulla. In order to obtain optical access to the geniculate, delicate surgery is required to remove the layers of bones, while keeping the ganglion intact. For that purpose, we have created this guide to help other researchers access the geniculate ganglion and image the GCaMP mediated fluorescent responses of these neurons to taste stimuli in vivo.

PROTOCOL:

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas San Antonio.

1. Pre-operative setup

NOTE: Please note that initial setup of equipment is not addressed here, as it will vary based on pump system, microscope, camera, and imaging software used. For setup instructions please refer to instructional materials provided by equipment vendor. For equipment used by the authors, please see the **Table of Materials**.

1.1 Ensure liquid flows through all vehicle (water) and tastant lines. If line is blocked disconnect and flush with water. If the line is kinked, massage until liquid flows. Ensure that liquid starts and stops on cue.

1.2 Once all lines are confirmed unblocked, run vehicle for 10 s then close all valves.

1.3 Ensure imaging software is ready with all required variables (e.g., trial length, file names, frame rate, etc). Using µManager, an open-source image acquisition software package, input 200 ms into the field labeled **Exposure Time** for a frames per second of 5Hz, select x2 under **binning**, and press the button labeled **Live**. When the video starts, press the button on the left side labeled **ROI**. This will result in a 512x512 field of view.

2. Anesthetizing and immobilizing the animal

NOTE: The following protocol is a terminal procedure optimized for mice of either sex weighing 18-35 g. It is recommended for use with animals between 10 and 12 weeks of age. It may be used with transgenic animals expressing Genetically Encoded Calcium Indicators (GECIs) such as the Snap25-GCaMP6s, or animals stereotaxically injected with viral GECIs. Gloves, lab coat, and face mask should be worn for entirety of protocol.

2.1 Scruff animal and perform an intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (10 mg/kg). Assess the depth of anesthesia via toe pinch before continuing.

2.2 Shave the top of the head and neck.

2.3 Turn on the heating pad and place the animal prone on the pad.

2.4 Apply ointment to the animal's eyes to avoid drying of the eyes.

2.5 Make an incision (~1 cm) at the midline of the head to expose the animal's skull. Remove connective tissue using a sterile swab so that the bare bone is accessible. Use a cotton

tipped applicator to ensure the skull is dry.

2.6 Apply vet bond to the skull. Be sure to cover the exposed skull. Wait for the glue to dry.

2.7 In a Petri dish lid, mix and apply a layer of dental cement to skull. The back end of the cotton tipped applicator used in step 2.5 will work well for this process. Place headpost on top of the dental cement and apply a second layer of dental cement to sandwich the headpost in place on the skull.

2.8 Let it sit until the dental cement is dry and solid. Break the cotton tipped applicator in half and use the pointed ends to poke the dental cement to test. If the dental cement does not yield to being poked the animal may be turned to a supine position.

3. Tracheotomy

3.1 Make a midline incision ~ 2 cm in the skin of the throat from the sternum to the chin.

3.2 Retract the skin and sub-maxillary glands, being sure to fully expose the digastric muscles.

3.3 Find the seam in the paratracheal musculature, separate it with blunt dissection, and retract open.

3.4 Carefully cut an opening in the top of the trachea large enough to fit polyethylene tubing (I.D. 0.86 mm, O.D. 1.27 mm). Do not cut more than halfway through the diameter of the trachea. Insert tubing into the trachea towards the lungs.

3.5 Reposition retractors to release paratracheal musculature and retract the submaxillary glands.

3.6 Glue paratracheal musculature together over tubing with a minimal amount of veterinary glue (see **Figure 1A**).

4. Breaking open the tympanic bulla

4.1 Gently tease desired digastric muscle (left or right) up and pull apart the connective tissue. Cut at the anterior end of the muscle, avoiding blood vessels, and pull back posteriorly until clear of the tympanic bulla.

4.2 Tilt the head back slightly to lift the tympanic bulla. Locate the branch of the carotid artery anterior to the posterior insertion point of the digastric muscle. Feel just posterior to this blood vessel for the convex structure of the tympanic bulla.

4.3 Look for a seam in the musculature at this location (see **Figure 1B**). Using two sets of fine forceps, blunt dissect at the seam until the bone of the tympanic bulla is visible. Use retractors to keep a clear view of the tympanic bulla.

4.4 Find the seam running anterior to posterior on the bulla (see **Figure 1C**). Using a surgical probe, poke a hole in the bone at the center of this seam. Use a set of fine end scissors to cut a circular area in the bone, taking care not to cut blood vessels anterior, posterior, and deep beneath the bulla.

5. Exposing the geniculate

5.1 Within this hole is a convex bit of bone, this is the cochlea. Anterior to the cochlea is a muscle, the tensor tympani (see **Figure 1D**). Using the spring scissors, cut the tensor tympani and remove it.

5.2 Perform a toe pinch if animal responds give ketamine (30 mg/kg).

5.3 Prepare irrigation fluid and a suction line. Using the surgical probe, poke a hole in the cochlear promontory. Immediately irrigate the liquid that flows out and remove it with suction. This liquid will flow more or less continuously from this point and will need to be addressed periodically.

5.4 Enlarge the hole in the cochlea. Take care with the blood vessel encircling the cochlea to the posterior and lateral edge.

5.5 Tilt the mouse's head forward. Locate the hole in the temporal bone beneath what was the cochlea (see **Figure 1E**). Take note of the ridge anterior to this hole, this ridge sits directly over the seventh nerve.

5.6 Insert a surgical probe into the hole and carefully lift the temporal bone to expose the seventh nerve (see **Figure 1F**). Take stock of how much of the seventh nerve is visible and if the geniculate is not fully exposed, tilt the animal's head back and attempt to pull up bone from anterior to the nerve.

5.7 If the ganglion is still not fully visible, pull up more bone from beneath. Be very careful not to place the probe deep beneath the bone as doing this may damage the geniculate.

[Place **Figure 1** here]

6. Run tastant panel

6.1 Use suction to remove liquid from over the geniculate. Optionally place an absorbent point to help mitigate seepage and aid in microscope navigation.

6.2 Place the animal on absorbent pad under the microscope. Locate the geniculate ganglion: useful landmarks include the hole left in the bulla, the hole in the temporal bone, and the seventh nerve. Using the FITC/GFP filter on the epifluorescence scope, check for individual GCaMP-expressing geniculate ganglion neurons. A 10x objective (workind distance 10mm) will provide sufficient resolution to track the activity of individual cells, but a 20x objective (working distance 12 mm) can also be used.

6.3 Place dispensing needle for tastant line firmly in animal's mouth. Place a Petri dish below the animal's mouth to catch fluid.

6.4 Ensure that the camera is viewing the microscope's field of view. Synchronize start of the video recording with the start of tastant presentation.

6.5 During recording, watch live feed for responses, drift, and seepage.

6.6 If seepage occurs, suction the liquid until the view of the geniculate is clear and repeat. If drift occurs, check that all parts of the head post are firmly tightened. If no responses occur check that liquid is flowing and that the microscope and camera are focused on the proper location without anything obscuring the field of view.

6.7 Repeat until desired number of videos have been obtained. Gently ease retractors, then repeat steps 3-6 on the opposite side.

6.8 After the desired videos have been obtained for all desired ganglia, euthanize the animal via cervical dislocation.

REPRESENTATIVE RESULTS:

Following the protocol, a transgenic Snap25-GCaMP6s animal was sedated, geniculate ganglia were exposed, and tastant was applied to the tongue while video was recorded. The aim of the experiment was to define which tastants elicited responses from each cell. Tastants (30 mM AceK, 5 mM Quinine, 60 mM NaCl, 50 mM IMP + 1 mM MPG, 50 mM Citric Acid)[18] were dissolved in DI water and were applied to the tongue for 2 s separated by 13 s of DI water.

[Place **Figure 2** here]

As can be seen in **Figure 2**, taste stimuli applied to the tongue should result in a rapid, transient increase in GCaMP fluorescence, causing a noticeable change in brightness among responding neurons. The video can be analyzed with a variety of software packages to produce traces displaying the changes in fluorescence over baseline (dF/F) over time of individual regions of interest (such as individual neurons), thereby showing the responses of each cell to the tastant panel. In a successful surgery, in a Snap25-GCaMP6s transgenic line, it is typical to see responses in 20-40 neurons within a single ganglion/field of view. This may change depending on the transgenic line used or if AAV-GCaMP is used instead. Note that baseline fluorescence may be affected by a number of factors, including the expression level of the GCaMP, and

possible damage to the cells during the surgery. Changes in fluorescence intensity above a threshold level (typically df/f > 3-fold above the average noise)[20,21] is considered a positive response.

To determine the timing of stimulus delivery, the time it takes for liquid to flow through the lines should be measured in order to know when a fluid change actually contacts the tongue. To reduce this delay, use a moderate flow rate (5-10 ml/min) and a short length of tubing leading from the perfusion manifold to the oral cavity. Typically, with the stimuli described here, fluorescence starts almost immediately after tastant is applied to the tongue and will begin to fade almost immediately after the tastant is stopped and the oral cavity is washed with vehicle solution. When working with an unknown stimulus it can be helpful to observe the change in fluorescence of a region without responding neurons to compare overall changes in the image.

FIGURE AND TABLE LEGENDS:

Figure 1: Surgical exposure of the geniculate ganglion. (**A**) Image of the mouse neck cavity post tracheotomy. Arrow is pointing to the digastric muscle lying over the surgical area explored in the rest of the figure. (**B**) Image of region under the previously indicated digastric muscle. Arrow indicates the seam in musculature for blunt dissection. (**C**) Image of the Tympanic Bulla. Arrow indicates seam in the bone to break with a surgical probe. (**D**) Image of surgical area after opening the bulla. Lower left arrow indicates the cochlea, upper arrow points to the tensor tympani. Boxed line indicates area in (**E**) and (**F**). (**E**) Image of surgical area after cochlea has been broken and the contents removed. White arrow indicates where to place surgical probe referenced in protocol step 5.6. (**F**) An image of the exposed geniculate ganglion. Arrow indicates body of the seventh nerve, dashed triangle surrounds the geniculate ganglion. Panels A-B, Scale = 5 mm. Panels C-F, Scale = 1 mm.

Figure 2: Responses of geniculate ganglion neurons to tastants using in vivo GCaMP6s imaging. (**A**) Epifluorescent image of the geniculate ganglion of a Snap25-GCaMP6s transgenic mouse during baseline as water is perfused over the tongue. Dashed lines indicate the approximate boundaries of the geniculate ganglion. The seventh cranial nerve is labeled as such. (**B**) Snapshot of the same ganglion in (**A**) as a sweet tastant (AceK 30 mM) is applied to the tongue of the mouse. Notice several individual neurons increase in fluorescence intensity. Dashed line box indicates sweet responding cell used in (**C**) end. (**C**) Traces from five neurons indicating the amplitude of their GCaMP6s mediated fluorescence in response to a panel of tastants comprising sweet (30 mM acesulfame K), bitter (5 mM quinine); salty (60 mM NaCl); umami (50 mM monopotassium glutamate and 1mM inosine monophosphate); and sour (50 mM citric acid). Colored bars show the placement and duration (2 s) of the stimulus over the time course of the experiment. These representative data do not include a response to umami. Individual neurons commonly respond to both bitter and sour stimuli (bottom trace) [16,18,20]. Panels A-B, Scale = 5 mm. Panel C, horizontal scale bar indicates 6.5 seconds, vertical scale bar indicates threshold of 4% dF/F.

DISCUSSION:

This work describes a step-by-step protocol to surgically expose the geniculate ganglion and visually record the activity of its neurons with GCaMP6s. This procedure is very similar to that described previously[17], with a few notable exceptions. First, the use of a head post allows for easy adjustment of head positioning during surgery. Second, regarding stimulus delivery, the approach by Wu and Dvoryanchikov flows taste stimuli through esophageal tubing[17], whereas this protocol delivers liquid directly into the mouth with a dispensing needle. Either method can be used to successfully evoke geniculate ganglion neuron responses by stimulating the fungiform and palatal taste buds.

A note on maintaining a clear imaging field: after breaking the cochlea, there will be continuous seepage of fluid within the cavity, including directly over the geniculate ganglion. It is also possible that bleeding will obscure the geniculate ganglion. While a small amount of seepage may not be sufficient to prevent imaging, even small amounts of blood can entirely occlude the ganglia. These issues can be addressed in a couple of ways. First, if seepage is relatively minor, it can be removed using a suction line fitted with a blunt dispensing needle between trials. Alternatively, liquid can also be wicked away from the field by carefully placing absorbent points posterior or lateral to the seventh nerve. If the flow is particularly bad, it may be necessary to apply suction to the cavity during imaging. A carefully placed suction line can keep the ganglia clear while being applied to a lateral location, so as to avoid obscuring the ganglia during imaging.

The imaging itself can be accomplished using different styles of microscope setups, each with its attendant advantages and limitations. When using an epifluorescence scope[18,21], it is only possible to image the more superficial neurons. Another issue with epifluorescence imaging is that signals from deeper cells will come out as changes in background fluorescence (out of focus light) so be careful with the analysis not to pick up fluorescence changes from other cells in the ROI. For particularly thin structures, such as the geniculate ganglia, these issues may not be problematic. Use of a 2-photon[16] or confocal[20] can potentially allow for imaging cells in deeper layers.

It is important to highlight a few critical steps and ways to troubleshoot common issues. First, it must be noted that analysis will vary considerably depending on the software used. The open-source software, ImageJ provides tools sufficient for preliminary analysis. First, remove small motion artifacts using the Image Stabilizer plugin[22], Next use ImageJ to calculate the change in fluorescence divided by baseline fluorescence (dF/F). This can be accomplished using one of many open source macros for ImageJ[23], the referenced macro provides detailed installation notes. For other macros please refer to their documentation. After correcting for dF/F, utilize the forward and reverse buttons at the bottom of the image stack to observe cell responses to stimuli. Using the lasso tool from the tool bar, select fluorescing cells individually. After selecting a cell use **Image | Stacks | Plot-Z-Axis**. This will provide sufficient information to determine response profiles and analyze the time related events of each region of interest (ROI). More advanced analysis was long the domain of custom scripts in Matlab, R, etc. However, the popularity of calcium imaging has gradually led to the development of multiple open-source resources for analysis including CaImAn, EZCalcium, and more[24,25].

Calcium imaging is a powerful tool for examining the activity of neural ensembles with single neuron resolution. Because of the geniculate ganglion's small size, this protocol is especially powerful because the entire ganglion can be visualized within a single field. However, there are some limitations to this technique. In addition to the limitations common to all calcium imaging experiments, the surgical approach described here is invasive and must be carried out under anesthesia. This is a terminal procedure – the animal must be euthanized immediately after imaging. Therefore this surgical approach is appropriate for awake/behaving recordings.

Over the past few years, researchers have used this technique to study response profiles of the neurons of the geniculate ganglion[16,20,18]. Recent work has focused on potential genetic markers that could be used to manipulate subpopulations within the ganglia and has shown how transgenic Cre driver lines and Cre-dependent GCaMP can be used to identify the response profiles of these populations[26]. Other work may use GCaMP with photo-activated proteins such as pa-mCherry to first identify and then mark cells activated by tastants to then be used in immunohistochemistry or in situ hybridization[27]. It may also be possible to utilize calcium dependent photo-convertible proteins such as CaMPARI to the same effect while using experimental methods very similar to those described here[28]. Regardless of the specific questions and experiments, calcium imaging offers a powerful tool for exploring the response profiles of neurons engaged in any number of activities, and its usefulness in exploring the taste system is only beginning.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have no conflict of interest to report.

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Figure 1

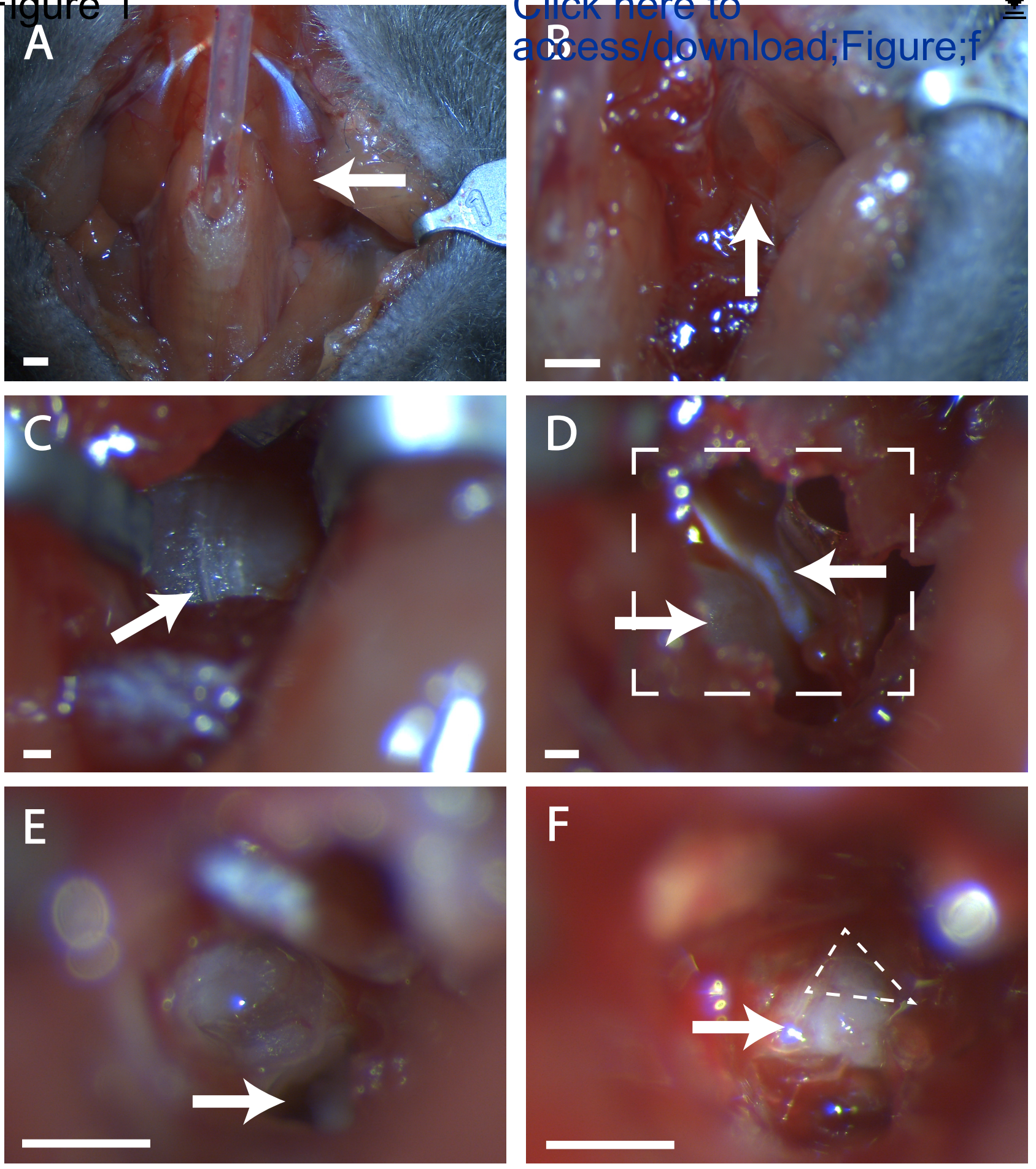

Figure 2

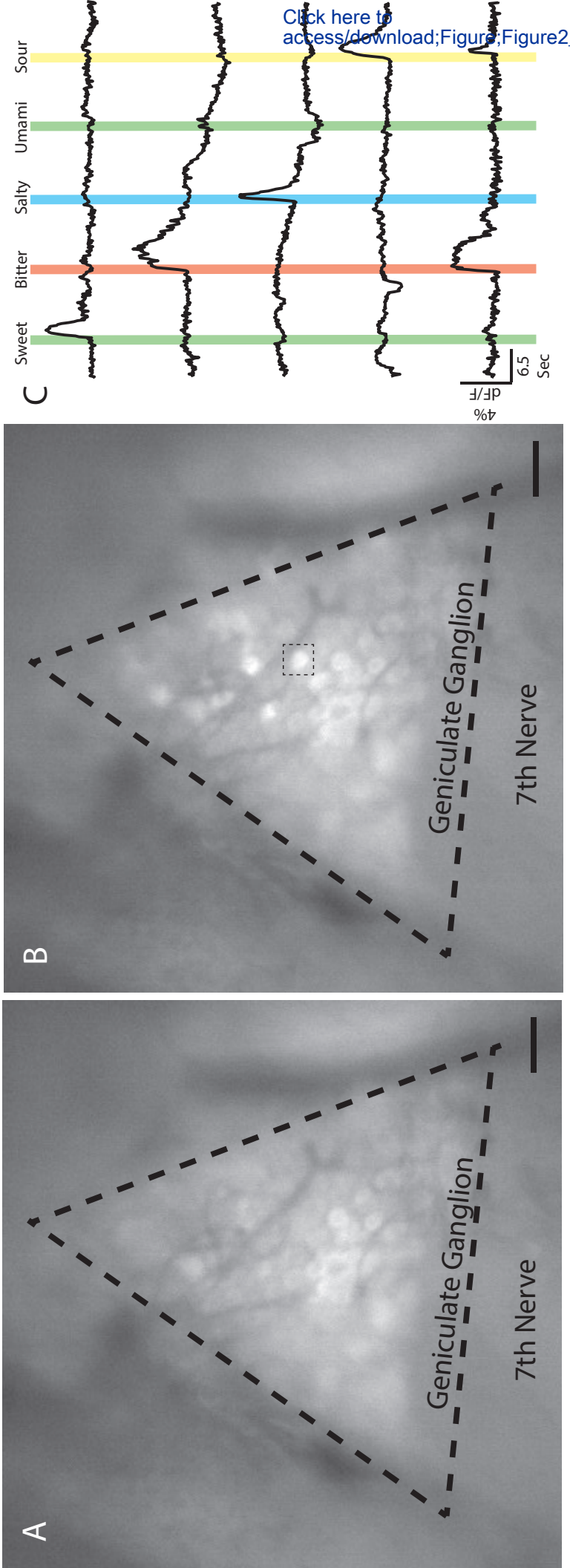

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 x #5 Inox Forceps	Fine Science Tools	NC9792102	
1ml Syringe with luer lock	Fisher Scientific	14-823-30	
2 x #3 Inox Forceps	Fine Science Tools	M3S 11200-10	
27 Gauge Blunt Dispensing Needle	Fisher Scientific	NC1372532	
3M Vetbond	Fisher Scientific	NC0398332	
4-40 Machine Screw Hex Nuts	Fastenere	3SNMS004C	
4-40 Socket Head Cap Screw	Fastenere	3SSCS04C004	
Absorbent Points	Fisher Scientific	50-930-668	
Acesulfame K	Fisher Scientific	A149025G	
Artificial Tears	Akorn	59399-162-35	
BD Allergist Trays with Permanently Attached Needle	Fisher Scientific	14-829-6D	
Blunt Retractors	FST	18200-09	
Breadboard	Thor Labs	MB8	
Citric Acid	Fisher Scientific	A95-3	
Cohan-Vannas Spring Scissors	Fine Science Tools	15000-02	
Contemporary Ortho-Jet Liquid	Lang	1504	
Contemporary Ortho-Jet Powder	Lang	1520	
Cotton Tipped Applicators	Fisher	19-062-616	
Custom Head Post Holder	eMachineShop		See attached file 202410.ems
Custom Metal Head Post	eMachineShop		See attached file 202406.ems
DC Temperature Controller	FHC	40-90-8D	
Digital Camera, sCMOS OrcaFlash4 Microscope Mounted	Hamamatsu	C13440	
Disection Scope	Leica	M80	
Hair Clippers	Kent Scientific	CL7300-Kit	
IMP	Fisher Scientific	AAJ6195906	

Ketamine	Ketaved	NDC 50989-996-06
LED Cold Light Source	Leica Mcrosystems	KL300LED
Luer Lock 1/16" Tubing Adapters	Fisher	01-000-116
Microscope	Olympus	BX51WI
Mini-series Optical Posts	Thorlabs	MS2R
MPG	Fisher Scientific	AAA1723230
MXC-2.5 Rotatable probe Clamp	Siskiyou	14030000E
NaCl	Fisher Scientific	50-947-346
petri dishes	Fisher Scientific	FB0875713A
Pressurized air	Airgas	AI Z300
Quinine	Fisher Scientific	AC163720050
Self Sticking Labeling Tape	Fisher Scientific	159015R
Silicone Pinch Valve Tubing 1/32" x 1/16" o.d. (per foot)	Automate Scientific	05-14
Sola SM Light Engine	Lumencor	
Snap25-2A-GCaMP6s-D	JAX	025111
Student Fine Scissors	Fine Science Tools	91460-11
Surgical Probe	Roboz Surgical Store	RS-6067
Surgical Probe Holder	Roboz Surgical Store	RS-6061
Thread	Gütermann	02776
BD Intramedic Tubing	Fisher Scientific	22-046941
Two Stage Gas Regulator	Airgas	Y12FM244B580-AG
Tygon vinyl tubing - 1/16"	Automate Scientific	05-11

Valvelink8.2 digital/manual controller	Automate Scientific	01-18
Valvelink8.2 Pinch Valve Perfusion System	Automate Scientific	17-pp-54
Xylazine	Anased	NADA# 139-236

Dear Dr. Bajaj,

Thank you and the reviewers for your helpful comments to strengthen the manuscript. We now submit a revised manuscript to address those comments and suggestions. We now include an additional figure to address concerns about descriptions of surgical landmarks, and provide a point-by-point response to the specific comments below. Original comments are in black, acknowledgement and rebuttal are in blue.

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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Done.

2. Please revise the following lines to avoid overlap with previously published work: 243-247
Section has been revised.

3. Please provide an email address for each author.
An email address has been added for both authors.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Use of "we" and "you" has been eliminated from the protocol.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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: BD Intramedic polyethylene tubing; vetbond glue;
Done, brand specific names have been changed to generic names such as "veterinary glue," "polyethylene tubing (specifications)", etc.

6. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
a) Please move information about using transgenic Snap25-GCaMP6s animal to the beginning of the protocol along with information about age, sex etc
Snap25 animals have been addressed as a potential transgenic line to use for this experiment at the beginning of the protocol. Suggested ages and sex have been included with the previously included suggested weight.

b) What happens to the animal after this procedure? Please specify the euthanasia method without highlighting it.
The fact that this is a terminal procedure has been highlighted at the beginning of the protocol. Preferred method of euthanasia has been briefly addressed at the last step of the protocol.

c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.
This step has been added to the protocol.

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.
N/A. See point 6B.

e) Discuss maintenance of sterile conditions during survival surgery.
N/A. See point 6B.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
N/A. See point 6B.

g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
N/A. See point 6B.

7. 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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Tense has been shifted to the imperative.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Major issues have been addressed with the addition of pictures for the particularly difficult portions of the protocol.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
The specifications have been adopted and the desired section has been highlighted.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
Scale bar is addressed in both figures for every panel.

11. As we are a methods journal, please add the following points to the Discussion:
a) Any limitations of the technique
Discussion now directly addresses limitations due to the invasive nature of this procedure.

b) The significance with respect to existing methods
This is now addressed within the discussion.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.
Endnote with the JoVE style was used. Aberrant references have been corrected independently.

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

Spatiotemporal activity of geniculate ganglion neurons offers a valuable information on how taste information is encoded and processed. Yet, microscopic access to the geniculate ganglion has been difficult due to the complex anatomy. This manuscript describes the detailed protocol for in vivo calcium imaging on geniculate ganglion neurons from surgical preparations to imaging. This reviewer believes this manuscript will provide a helpful guide for readers who wants to adopt this technique. I have only a few minor comments.

Minor Concerns:
1. I think the prior protocol worked on the same topic by An Wu & Gennady Dvoryanchikov (Protocol Exchange, 2015) needs to be cited. It would be also helpful if the authors discuss improvements over the prior protocol.
Done.

2. Although the video will be provided, I think the schematics or photographs on the anatomy can be highly helpful. For example, opening up of tympanic bulla and removal of temporal bone is not easy to follow without any prior knowledge on the anatomy.
Figures have been added for more complex parts of the protocol. Specifically: an initial image to orient readers to the surgical areas, where to blunt dissect musculature to expose the bulla, where to break into the bulla, a schematic of what the interior of the tympanic bulla should look like with critical features labelled (tensor tympani and cochlea), an image of post cochlea breaking showing the point used to pull up the temporal bone, and an image of the completed procedure showing an exposed geniculate.

Reviewer #2:
Fowler and Macpherson elegantly describe their techniques for in vivo imaging of mouse geniculate ganglion using GECIs. This manuscript provides a brief, yet informative introduction on the geniculate ganglion and the taste system. The authors describe other current methods for measuring peripheral nerve activity including whole nerve recording and single fiber recording and clearly explain the advantages of the herein described calcium imaging. Previous studies using this technique (utilizing 2-photon, confocal, or epifluorescence microscopy) are cited and clearly demonstrate how useful this technique is to the field of gustatory sciences. Lastly, the expression of GECIs is covered as the authors suggest the possibility of using Cre-mediated expression or viral delivery.
The protocol itself is logically prepared and easy-to-follow from equipment prep to data analysis. I have done similar mouse preps and had no trouble following the steps, even without pictures or video. I only have a few clarifications, listed below.
Overall, this is an excellent description of a delicate surgical prep. This technique is an invaluable addition to the quiver of methods to study gustatory peripheral nerve responses and has the potential to answer many yet outstanding questions in the field of gustatory science.

Questions/clarifications
1) Would the authors comment on potential feedback from the CNS? Typically in gustatory whole nerve recording, the nerve is transected distal to the ganglion body prior to applying stimulus.
This is a potentially interesting area to investigate. This method for calcium imaging keeps the entire ganglion and it's peripheral and central processes intact. Therefore, there is the potential for modulation by the CNS. We are not aware of any studies that have compared CT nerve responses to taste stimuli in transected vs intact preparations.

2) What does the future hold for this technology? The authors touch on this in their discussion of using photoconvertible fluorophores. But perhaps the authors could expand on this a bit more such as, could this be adapted for awake, behaving mice?
This is now addressed more extensively within the discussion, and the fact that this is best suited as a terminal procedure has been highlighted.

3) Line 72: change wording to imply that green fluorescence increases with increasing Ca
Changed to reflect that increased Ca concentration causes increased green fluorescence.

4) Line 138: what size/diameter PE tubing?
Tubing entry has been amended to declare the I.D. and O.D.

5) Line 192: would you provide more details on the objectives used? Specifically the minimum required working distance (or even an estimate, knowing there will be variation in preps and mice)
This information is now provided within the protocol.

Reviewer #3:
Manuscript Summary:
In the manuscript, titled "In vivo calcium imaging of mouse geniculate ganglion neuron responses to taste stimuli" Bryan Fowler et. al. have demonstrated imaging of individual geniculate ganglion in response to taste stimuli using GCaMP. The surgical procedure to expose the geniculate ganglion followed by fluorescence image acquisition by using a microscope and digital camera to record the taste-evoked response has been presented. The technique is presented very elaborately and the surgical procedure is easily understandable.

Major Concerns:
While the description for the experiment is in detail, it would be better to have some clear pictures of the surgical setup and a photo (or a cartoon) which shows the anatomy of the mouse geniculate ganglion which can help the reader understand overall procedures in addition to the video.

Figures have been added for more complex parts of the protocol. Specifically: an initial image to orient readers to the surgical areas, where to blunt dissect musculature to expose the bulla, where to break into the bulla, a schematic of what the interior of the tympanic bulla should look like with critical features labelled (tensor tympani and cochlea), an image of post cochlea breaking showing the point used to pull up the temporal bone, and an image of the completed procedure showing an exposed geniculate.

Minor Concerns:
1. Regarding figure 1, there is no explanation for the responses of umami and sour which responded differently than the others. Also, the figure contains many neurons and it would be better to indicate which cells responded to the specific five tastes.
The figure (now figure 2) has been updated to address the lack of significant umami response and the fact that sour and bitter co-responders are more common than other co-responders. The figure contains a picture of a snapshot pre stimulus and of the ganglion during a single tastant trial, AceK.

2. Line 230: "although bitter tastants such as quinine and cycloheximide can producemore prolonged responses" reviewer demands the representation or reference of this fact.
Claim has been removed as it references currently unpublished work.

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