Live animal calcium imaging of the geniculate ganglion

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Method Article

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Abstract

Calcium imaging has served as a standard technique to study neuronal function for decades. However, recording calcium activity from the living animal has not flourished until recent use of genetically encoded calcium indicators \(GCaMPs)[1,2]. GCaMPs and mouse genetics have enabled researchers to study calcium activity in various neuron types, including sensory neurons. The geniculate ganglion is one of the sensory ganglia in the peripheral gustatory system. Neurons in the geniculate ganglion innervate taste buds on the anterior tongue and the palate via the chorda tympani and greater superficial petrosal nerves, respectively. Electrophysiological recordings have been made from single fibers in the chorda tympani and from neurons in the geniculate ganglion[3-5]. However, data from those methods is limited. Here we describe a novel method of recording calcium activity from large assemblies of cells in the geniculate ganglion in transgenic mice that express GCaMP3 in sensory neurons \(GCaMP3 mice) \(ref 6). Calcium imaging cannot collect information as detailed as with the electrophysiological recordings, but calcium imaging enables one to record a number of neurons simultaneously. The procedures are: 1) to expose the ganglion in GCaMP3 mice, 2) to view the ganglion using scanning laser confocal microscopy, 3) to record GCaMP3 fluorescence while applying taste solutions to the oral cavity. The surgery to expose geniculate ganglia in rats has been described previously[3]. We adapted and optimized the surgery to mice[7].

Reagents

Animals \(see Reagent Setup) NaCl \(Sigma, cat. no. S9625) KCl \(Sigma, cat. no. P3911) $CaCl_2$ \(Sigma, cat. no. C5080) $MgCl_2$ \(Sigma, cat. no. M2670) Sucrose \(Sigma, cat. no. S5016) Monosodium glutamate \(Sigma, cat. no. 49621) Cycloheximide solution \(Sigma, cat. no. C4859) Quinine HCl \(Sigma, cat. no. Q1125) Citric acid \(FisherBiotech, cat. no. BP339-500) Ketamine solution \(KetaVed, NDC 50989-996-06) Xylazine solution \(AnaSed, NADA# 139-236) Sterile 0.9% NaCl solution \(APP Pharmaceuticals, NDC 63323-186-02) Mineral oil \(Sigma, cat. no. M5904) Kwik-Stop, styptic powder with benzocaine \(ARC laboratories, cat. no. 86001) **Reagent setup:** Animals: The protocol described below is optimized for 22-32 gram mice[6]. For best results, use male mice at 3-5 months old and female mice at 5-8 months old. Artificial Saliva: Dissolve 0.4383g NaCl, 0.8201g KCl, 0.2205g $CaCl_2$, and 0.0609g $MgCl_2$ in dH_2O; add dH_2O to final volume of 500ml. Adjust pH to 5.8 ± 0.58. Taste solutions: Make taste solutions of sucrose, NaCl, MSG, cycloheximide, QHCl and citric acid in the artificial saliva.

Equipment

Kent Scientific Gaymar T/Pump \(Stryker T/Pump) Kent Scientific Warming Pad \(Model. TPZ-0510EA) Physitemp Thermometer \(Model. BAT-7001H) Physitemp Rectal Probe \(Model. RET-4) Narishige Head Holder \(Model. SG-4N) Customized stage to fit Narishige head holder and Olympus confocal microscope 3cm Goldstein Retractor \(World Precision Instruments, cat. no. 501968) Customized retraction hook \(see Equipment Setup) High Temperature Cautery \(World Precision Instruments, cat. no. 500389)

Disposable High Temp Loop Tip \(World Precision Instruments, cat. no. 500394) 1 pair #2 Inox forceps \(Fine Science Tools, cat. no. 11223-20), 1 pair #3c Inox forceps \(Fine Science Tools, cat. no. 11231-20), 1 pair #5 Inox forceps \(Fine Science Tools, cat. no. 11251-20), 1 pair #55 Inox forceps \(Fine Science Tools, cat. no. 11255-20) 1 pair #5SF Inox forceps \(Fine Science Tools, cat. no. 11252-00) 1 pair #5 Carbon steel forceps \(Fine Science Tools, cat. no. 11251-10) 1 pair Iris scissors \(World Precision Instruments, cat. no. 14218-G) 1 pair student vannas spring scissors \(Fine Science Tools, cat. no. 915009-09) Arkansas stone \(Fine Science Tools, cat. no. 29008-01) Instrument oil \(Fine Science Tools, cat. no. 29055-00) Kimwipes wiper \(Kimtech science) 18G IV Catheter \(Becton Dickinson, cat. no. 381444) Sterile1ml syringe \(Becton Dickinson, cat. no. 309659) Sterile 30G needle \(Becton Dickinson, cat. no. 305106) Polyethylene tubing PE 160 \(Becton Dickinson, cat. no. 427430) Polyethylene tubing PE 60 \(Becton Dickinson, cat. no. 427415) SILASTIC silicon tubing \(VWR, cat. no. 62999-224) Nylon mesh \(Component Supply, cat. no. U-CMN-255) Olympus dissection microscope \(Model. SZX9) Olympus confocal microscope \(Model. FV1000) Olympus 4x long working distance objective \(Model. LMPLFLN) Olympus 20x long working distance objective \(Model. LMPLFLN) AutoMate Scientific Automate perfusion system Master-Mite Heat Gun \(Model. 10008) **Equipment setup:** Esophageal tube+ pad: cut PE 60 tube into 3.5cm length, with one ending flat and the other ending angled, cut nylon mesh into 4cm*7cm rectangle with a tail in the middle of the short side, attach the flat end of tube to the tail with super glue. Trim the pad to fit the mouth of the mouse \(fig1). Tracheal cannula: Detach the plastic tubing from 18G I.V. catheter \(fig1). Suction pipette: use the heat gun to heat the PE tube until it becomes transparent, and then pull it to the right diameter to make the suction pipette. Two pipettes are needed: for pipette I, the final diameter should be ~ 100um; for pipette II, the final diameter is the same as pipette I, but it also needs to be curved to extend into the tympanic cavity and be positioned near the geniculate ganglion \(fig1). Retraction hook: curve the point of a dissecting pin and tie the end of it to a weight \(any weight could work, fig1). 

Procedure

Mouse anesthesia \(Timing: 40min) 1. Attach the Gaymar T/Pump with the warming pad and fill it with water. Turn on the pump to pre-warm the warming pad. 2. Weigh the mouse. Prepare the anesthetic: add 2.4 ul of ketamine per gram of body weight, 1ul of xylazine per gram of body weight, and sterile 0.9% NaCl solution to a final volume of 200 ul \(solution A). Add 2.4 ul of ketamine per gram of body weight and 0.9% NaCl solution to a final volume of 200 ul \(solution B). Inject Solution A for the initial anesthesia. After 1 hour, inject 50 ul of solution B every 30min or as needed \(use paw pinch reflex to test whether more anesthesia is required). _For example: for a mouse of 25 grams, mix 60ul ketamine and 25 ul xylazine, and add 115 ul 0.9% NaCl solution to make 200 ul of solution A. Mix 60 ul ketamine and 140 ul 0.9% NaCl solution to make 200 ul of solution B._ 3. Inject 100 ul of solution A \(i.p.) with a 1ml syringe and 30G needle. Start the timer right after the injection \(T=0min). The mouse should become anesthetized in 3 min. 4. Transfer the mouse onto the warming pad at T=3min. Mount the mouse to the nose-holder at T=10min in a supine position \(fig2). Insert the rectal probe to monitor the body temperature. Adjust Gaymar T/Pump to maintain the mouse body temperature at 37 degrees. Caution:

after anesthesia, rinse the tongue with artificial saliva every 5-10min during the surgery to prevent the tongue from dehydrating. 5. Pinch the mouse paw to check if the mouse is fully anesthetized at T=15min. Wait until the mouse loses the pinch reflex to begin surgery. It usually takes 15 to 20min to reach an adequate surgical plane of anesthesia. **Tracheotomy \(Timing: 5min)** 6. Apply mineral oil to the neck and upper chest area \(fig2). Make a 1 cm midline incision to expose the salivary glands. \(fig3)  7. Separate the two lobes of the salivary glands with blunt dissection. You should be able to see the trachea and the trachealis muscle. Blunt dissect the muscle to expose the trachea. 8. Make a horizontal cut in the trachea with spring scissors but do not cut it through completely. Insert the tracheal cannula about 0.5cm \(fig4); no sutures are needed if using catheter tubing. **Insert esophageal tube \(Timing: 10min)** 9. Locate the esophagus underneath the trachea and carefully separate it from the surrounding tissue. Place a suture thread underneath the esophagus. Make a horizontal cut in the esophagus caudal to the thread. Do not cut through completely the esophagus. 10. Insert the esophageal tube into the oral cavity between the tongue and the palate. Gently press the tube through the throat until the end of the tube exits the incision of the esophagus made in step 9. 11. Pull the tube out through the incision until the pad lies between the tongue and the palate. Tighten the suture around the incision to fix the tube in place. **Expose the tympanic bulla \(Timing: 20min)** 12. Retract the skin and the trachea to expose the digastric muscle on the left side using the 3cm Goldstein retractor as shown in fig5. Separate the tendon of the digastric muscle slightly and cauterize the tendon to detach the caudal part of the digastric muscle. Be careful not to disturb the blood vessels underneath the digastric muscle. After cauterization, the surgery should be as shown in fig 6. The hyoglossus muscle covers the tympanic bulla \(fig6)[9].  13. Blunt dissect the hyoglossus muscle to expose the tympanic bulla. Clean the muscles and connective tissue around this area as much as possible \(fig7). **\(? Troubleshooting1)** **Expose the geniculate ganglion \(Timing: 60min)** 14. Retract the jaw rostrally with the hook to enlarge the area above the tympanic bulla \(fig8). Using #2 Inox forceps, break the transparent portion of the bulla and the opaque part of the bulla. Be careful to not damage the tympanic membrane while breaking the bulla. The interior structure of the tympanic cavity is shown in fig 9.  15. Within the tympanic cavity, carefully remove the stapes of the ossicles with #5 Inox forceps. Cut the tendon connecting the tensor tympani muscle to the ossicles with #55 Inox forceps and remove the muscle. There usually is minor bleeding mixed with tissue fluid. The chorda tympani nerve travels around the malleus of the ossicles and along the tympanic membrane. You must be very careful not to damage these structures \(fig 10,11). 16. Attach suction pipette I to the vacuum to remove any the tissue fluid and blood that accumulates. Grasp the tensor tympani muscle by #55 Inox forceps and remove it. It usually takes several attempts to remove the muscle completely. The temporal bone will be exposed after removing the tensor tympani muscle.  17. Rotate the mouse and the stage to a horizontal position \(clockwise 90 degrees) to enable a better view of the tympanic cavity. Break and remove the cochlear promontory using forceps \(fig12, 13). There will be large quantity of tissue fluid and possibly some bleeding while removing the promontory, remove the fluid and blood by suction pipette I. The tissue fluid will appear for the rest of the surgery, it is important to remove the tissue fluid continuously.  18. \(CRUCIAL STEP) At this point, the facial nerve and the greater superficial petrosal \(GSP) nerve will be visible. Figure 13 shows the location of the geniculate ganglion \(GG). The dashed white lines indicate the facial nerve and GSP nerve. The GG lies at

the junction of the facial and GSP nerves, as indicated by dashed green lines. Note, there are several blood vessels around GG and the nerves, as indicated by dashed red lines. During the surgery, use the facial and GSP nerves as markers to localize the GG. Use #5 carbon steel forceps to break the temporal bone along the edges of GG, inside the dashed red lines in fig 13 to avoid blood vessels. Remove the bone by breaking into large fragments. Avoid generating too many small bone fragments. If there is bleeding around the GG, remove the blood with small pieces of kimwipe. **\(? Troubleshooting2)** 19. Use #5SF Inox forceps to remove the bone fragments directly over the GG \(fig14). Do not touch the ganglion with forceps. Remove the fluid with suction pipette I continuously. **Calcium imaging \(Timing: 30min)** 20. Tighten the ear bars and the nose clamp to minimize any movement of the geniculate ganglion. Install suction pipette II close to the geniculate ganglion to remove the fluid continuously while imaging \(fig15, 16). Fix the suction pipette by taping it to the head holder. 21. Transfer the mouse with the warming pad to the confocal microscope and fix the stage under the confocal microscope. Connect suction micropipette II to vacuum. 22. Geniculate ganglia in GCaMP3 mice have a faint green fluorescence. Locate the ganglion using epifluorescence with a DAPI filter and a 4x objective. Check the fluorescence of geniculate ganglion neurons using the 20x long working distance objective. 23. Use the 488nm laser at 30% power to scan the geniculate ganglion. Set the pinhole at 400 um and adjust hv around 600-700. You should be able to see the bright green fluorescence of individual neurons. **\(? Troubleshooting3)** 24. Load taste solutions into reservoirs of the perfusion system and connect the perfusion manifold to the esophageal tube. Place a suction tube close to the mouth of the mouse to withdraw taste solutions. 25. Record the fluorescence at a scanning speed ~ 300 ms/scan with Olympus' Fluoview software \(). Perfuse taste solutions into the oral cavity. Different numbers of neurons will respond based on the preparation and the taste stimuli. **\(? Troubleshooting4)**

Timing

Timing: Mouse anesthesia: 25min \(expert), 40min \(beginner) Tracheotomy: 2min \(expert), 5min \(beginner) Insert esophageal tube: 3min \(expert), 10min \(beginner) Expose the tympanic bulla: 10min \(expert), 20min \(beginner) Expose the geniculate ganglion: 35min \(expert), 60min \(beginner)

Troubleshooting

See figure in Figures section.

Anticipated Results

Anticipated result: Figure 17 is a representative image of how neurons appear under a confocal microscope. Regions of interest \(ROIs) have been superimposed on neurons. Shown to the right are representative traces of GCaMP3 fluorescence signals of the ROIs, illustrating responses to 10 s application of 10 mM citric acid in the oral cavity. The color of each trace corresponds to that of the ROI on the left. **Discussion** If the surgery is correctly done and many healthy neurons are visible but few neurons respond, the potential reasons could be: 1. Anesthesia: some mice might be too weak to

maintain nervous activity when anesthetized; this might be the strain or that individual mouse. Use 25-30 gram male mice. 2. Application: the receptive field of the neuron \(taste buds on the tongue and the palate) might not be stimulated effectively. 3 The chorda tympani nerve was damage during surgery, most likely at step 15.

References

1 Nakai J, Ohkura M, and Imoto K. A high signal-to-noise Ca2+ probe composed of a single green fluorescent protein. Nature Biotechnology 19, 137-141 \(2001). 2 Broussard GJ, Liang R and Tian L. Monitoring activity in neural circuits with genetically encoded indicators. Frontiers in molecular neuroscience 7, 97 \(2014). 3 Sollars SI and Hill DL. In vivo recordings from rat geniculate ganglia: taste response properties of individual greater superficial petrosal and chorda tympani neurones. The Journal of physiology 564, 877-893 \(2005). 4 Lundy RF Jr and Contreras RJ. Gustatory Neuron Types in Rat Geniculate Ganglion. Journal of neurophysiology 82, 2970-2988 \(1999). 5 Frank ME, Contreras RJ and. Hettinger TP. Nerve fibers sensitive to ionic taste stimuli in chorda tympani of the rat. Journal of neurophysiology 50, 941-960 \(1983). 6 Kim YS. et al. Central terminal sensitization of TRPV1 by descending serotonergic facilitation modulates chronic pain. Neuron 81, 873-887 \(2014). 7 Barretto, R. P. et al. The neural representation of taste quality at the periphery. Nature 517, 373-376 \(2015). 8 Hirata S, Nakamura T, Ifuku H & Ogawa H. Gustatory coding in the precentral extension of area 3 in Japanese macaque monkeys; comparison with area G. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 165, 435-446 \(2005). 9 Weiinen JA, et al. Main trajectories of nerves that traverse and surround the tympanic cavity in the rat. J Anat 197 \(2000).

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Figures

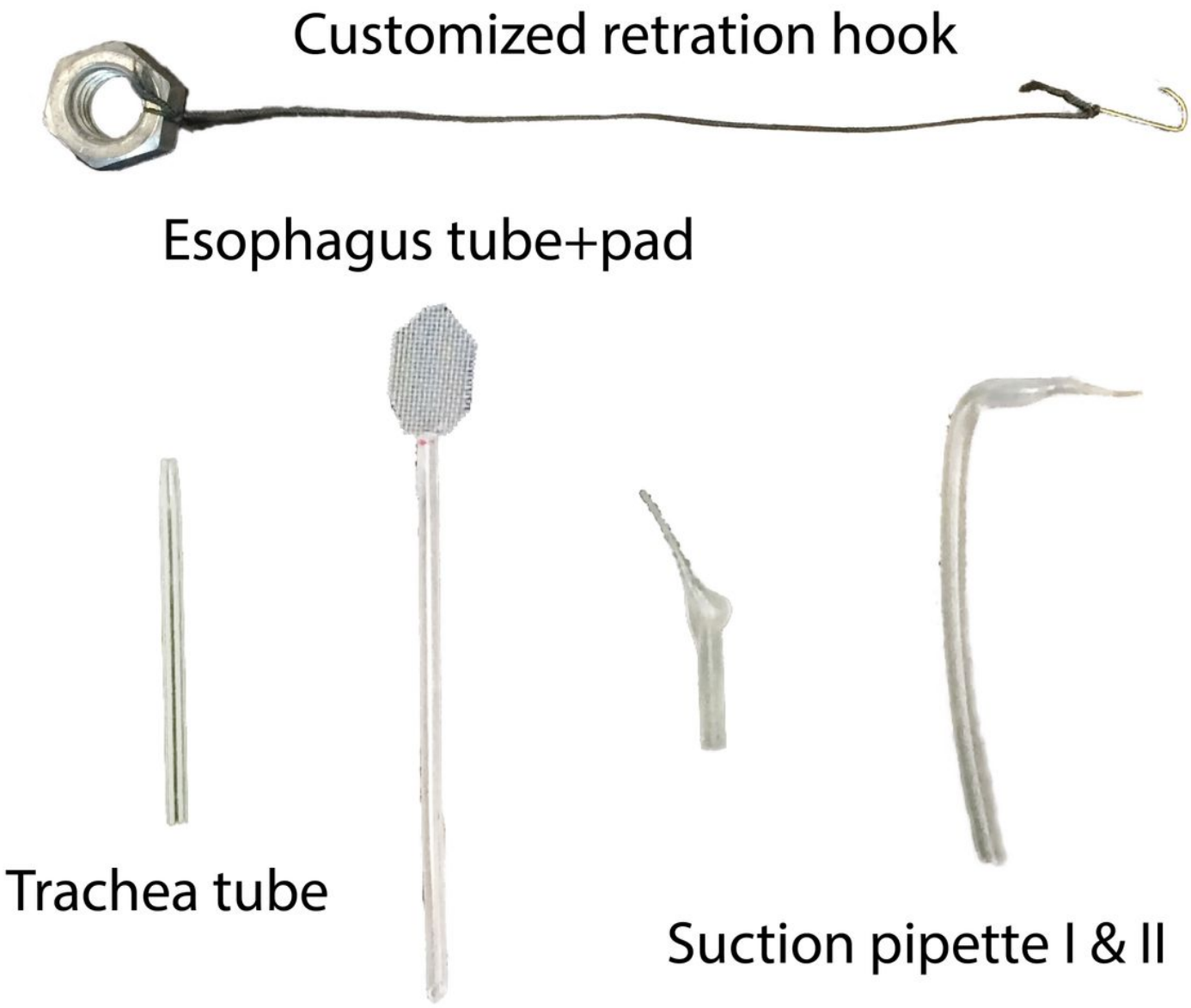

Figure 1

figure1 The equipment: customized retraction hook, trachea tube, esophageal tube+pad, suction pipette I & II.

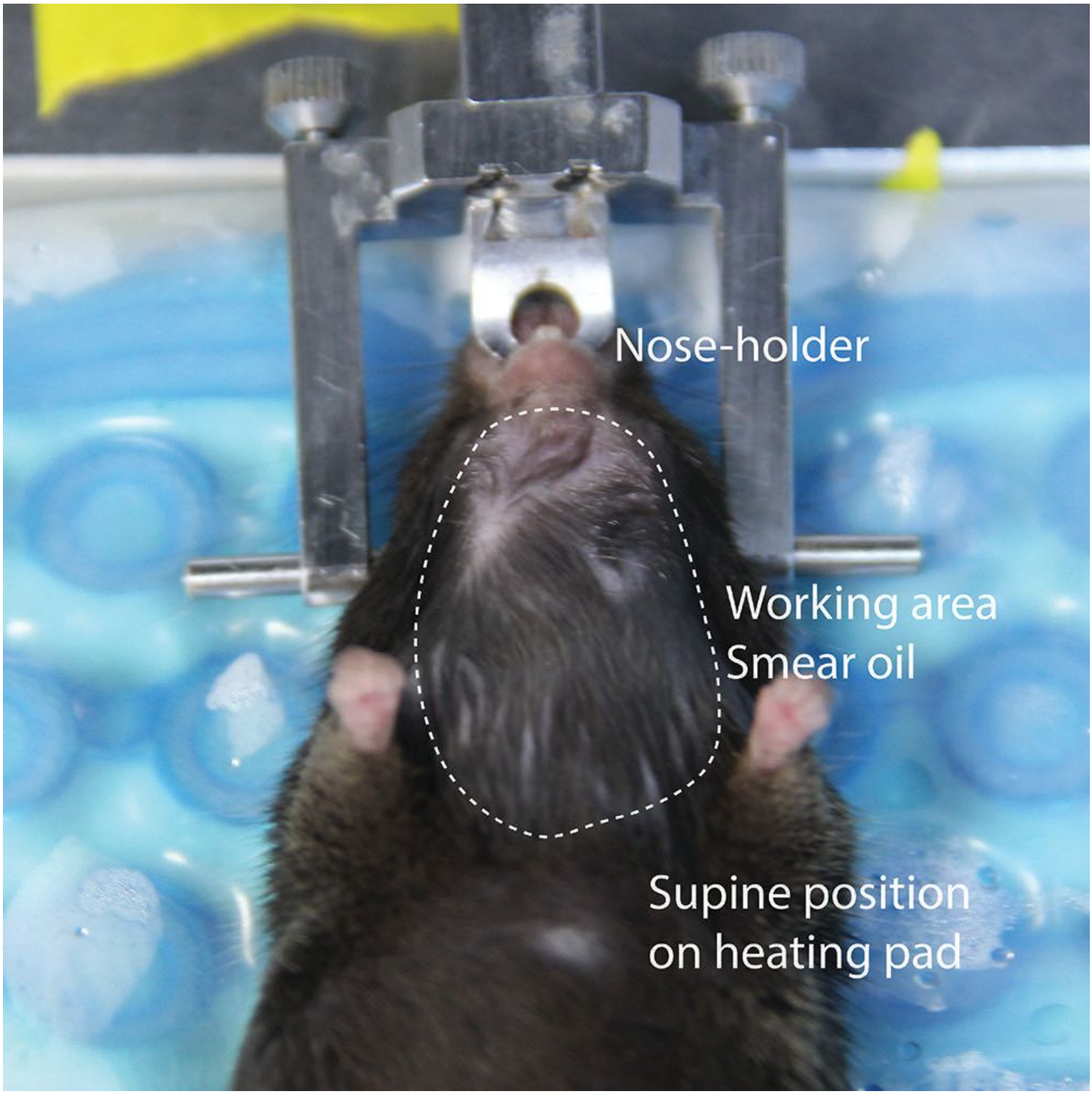

Figure 2

figure2 Mount the mouse on the noseholder and smear oil on the neck area.

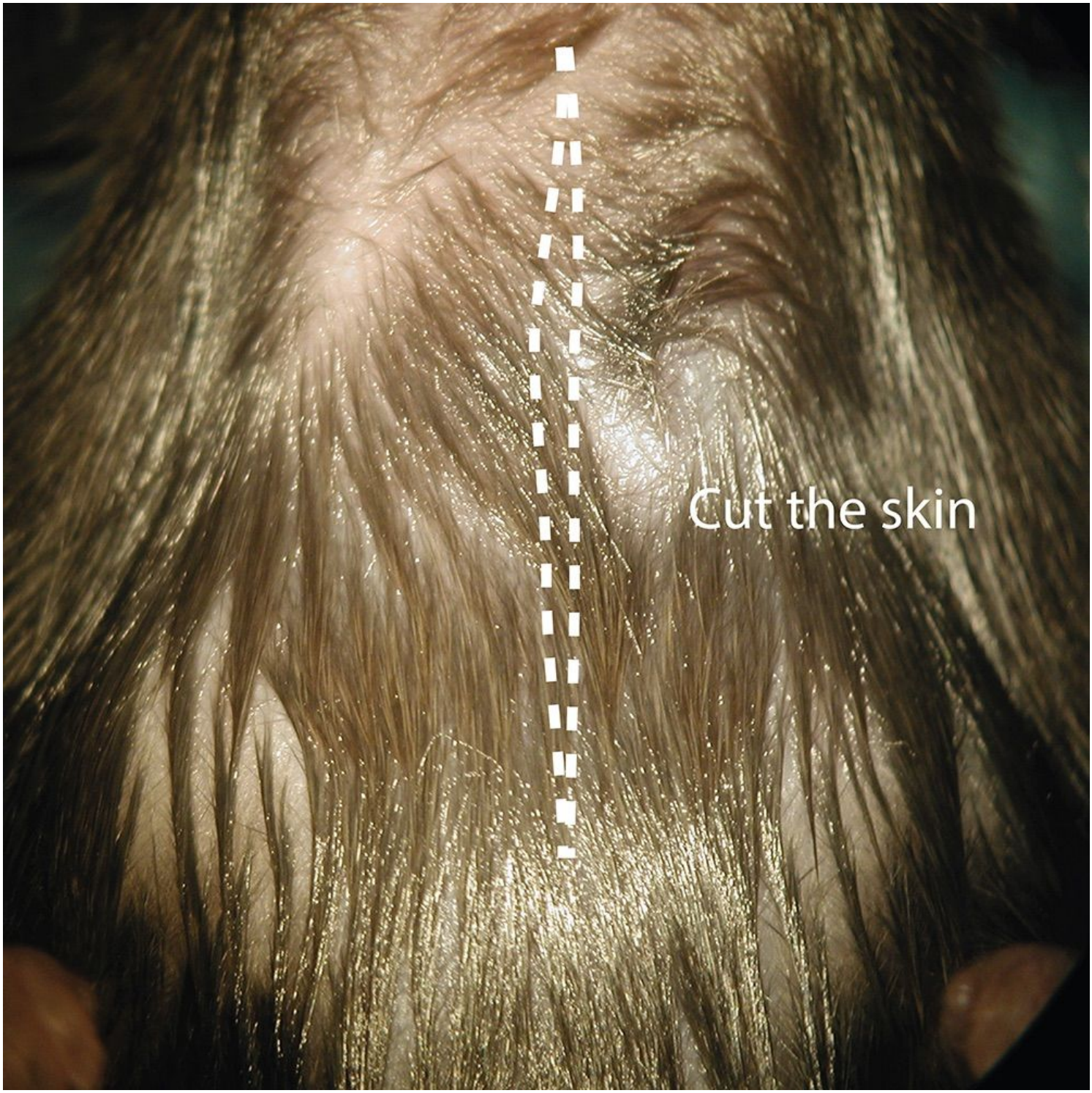

Figure 3

figure3 Cut the skin on the neck area.

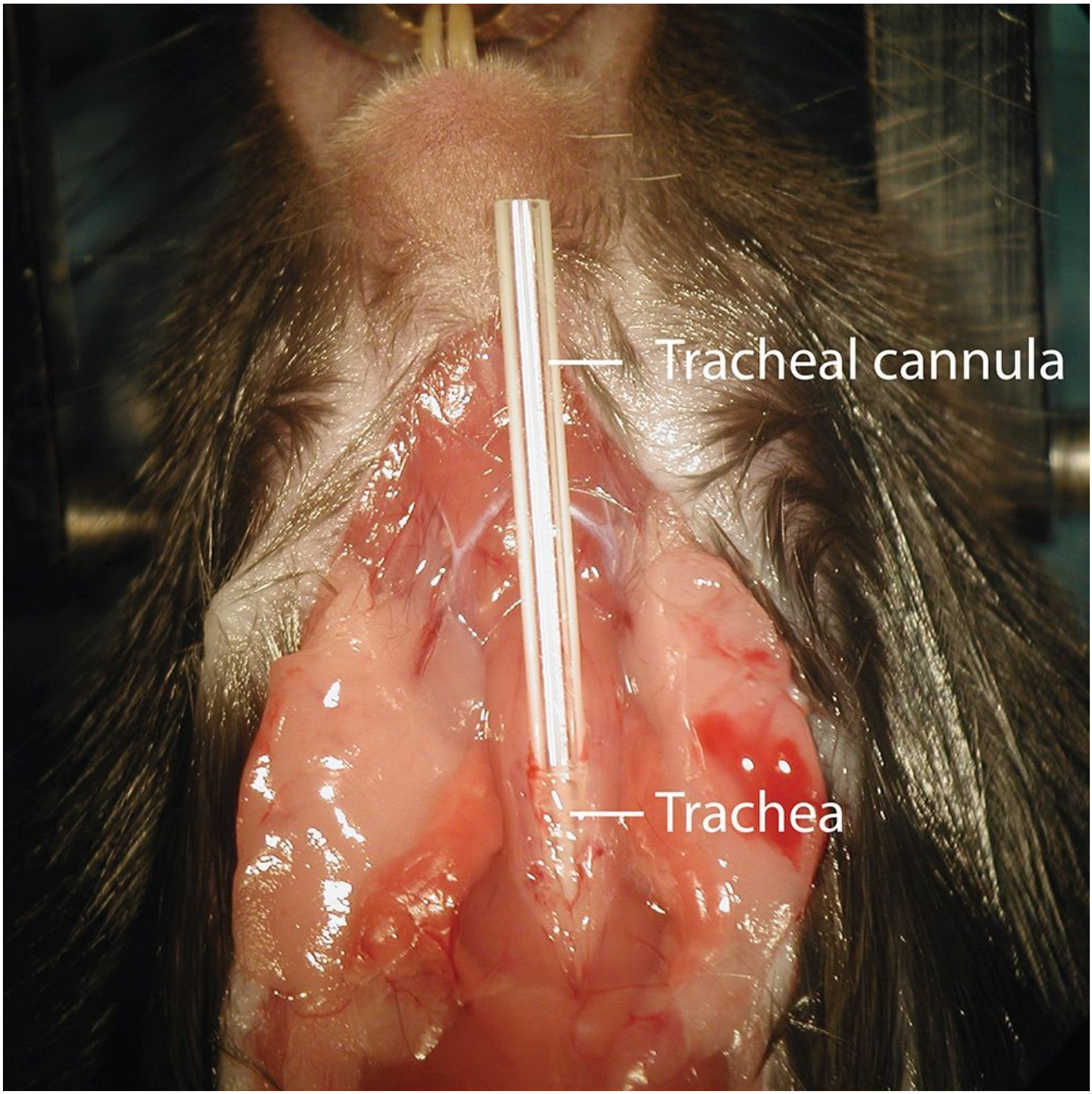

Figure 4

figure4 Insert the tracheal cannula.

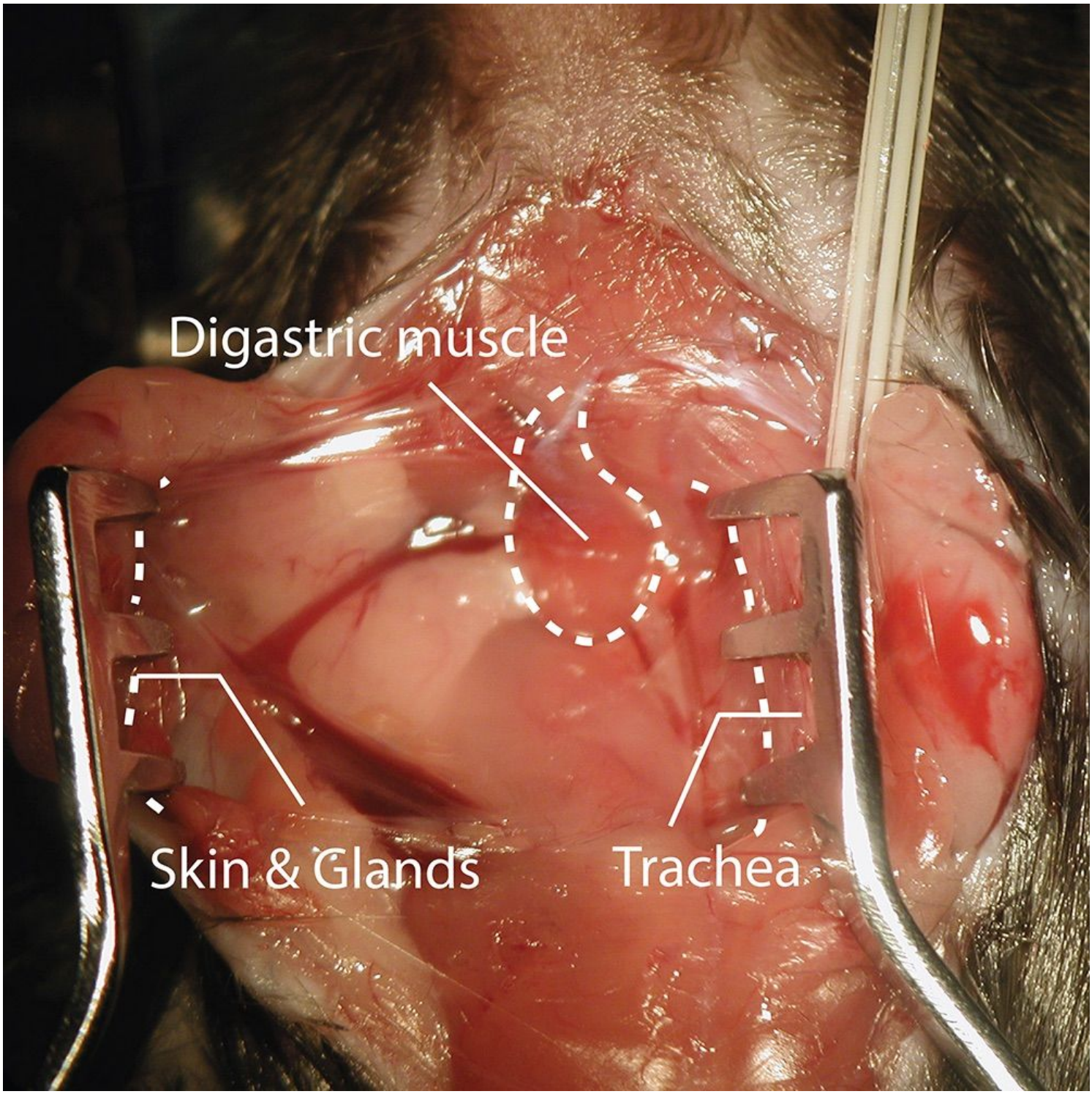

Figure 5

figure5 Retract the skin and salivary gland on left side.

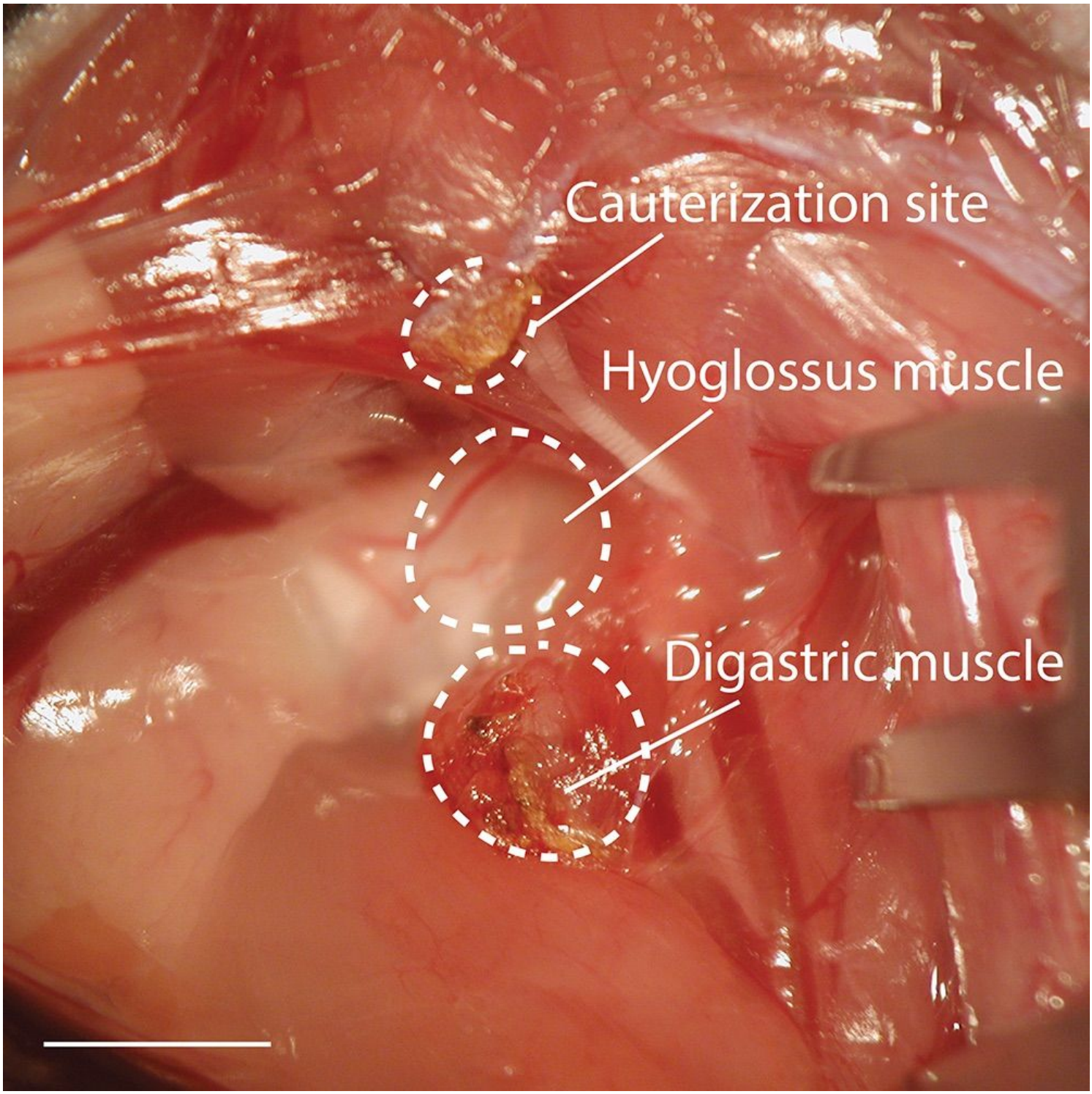

Figure 6

figure6 Cauterize the digastric muscle and remove the hyoglossus muscle. Scale bar: 5 mm.

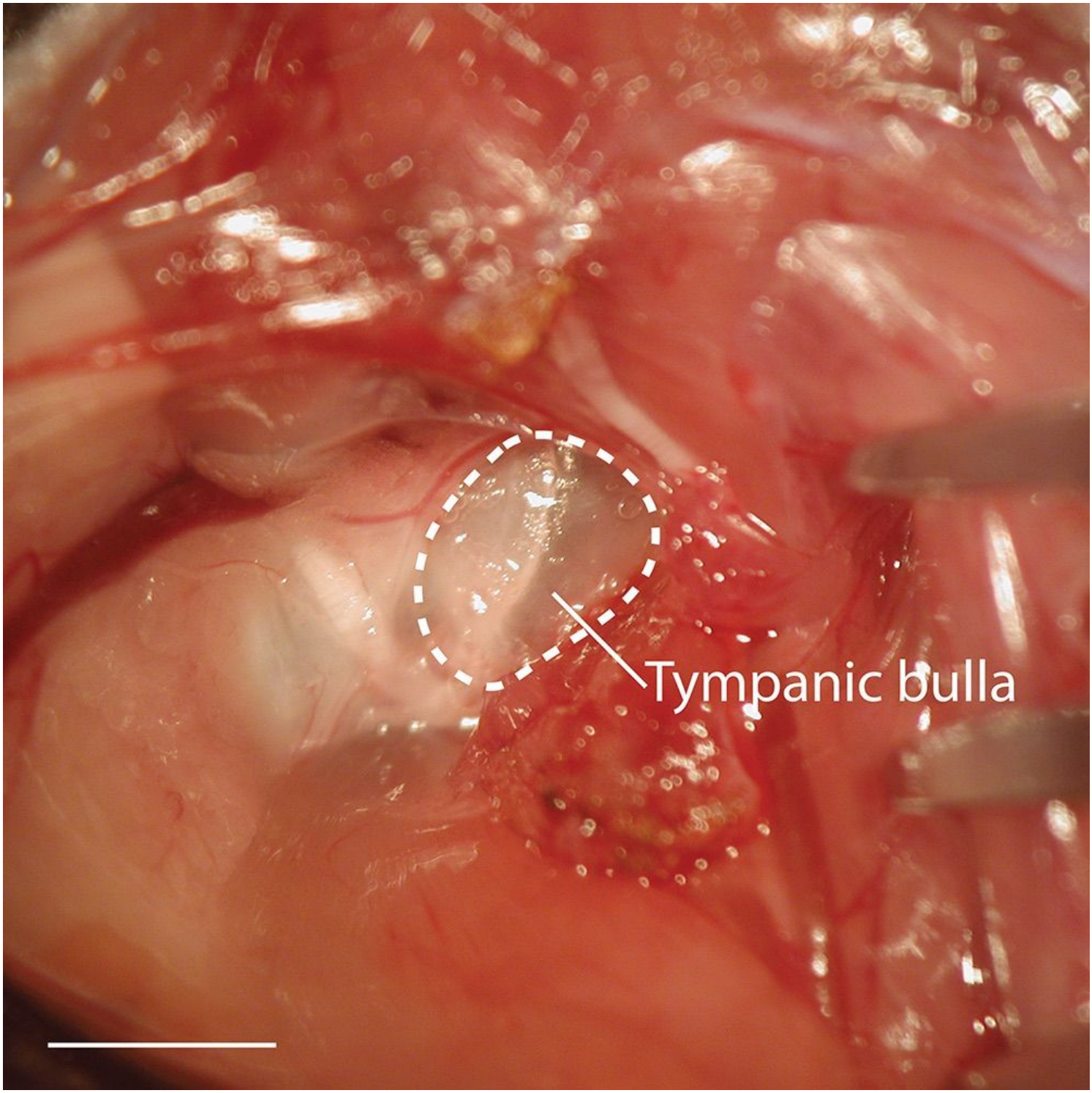

Figure 7

figure7 Expose the tympanic bulla. Scale bar: 5 mm.

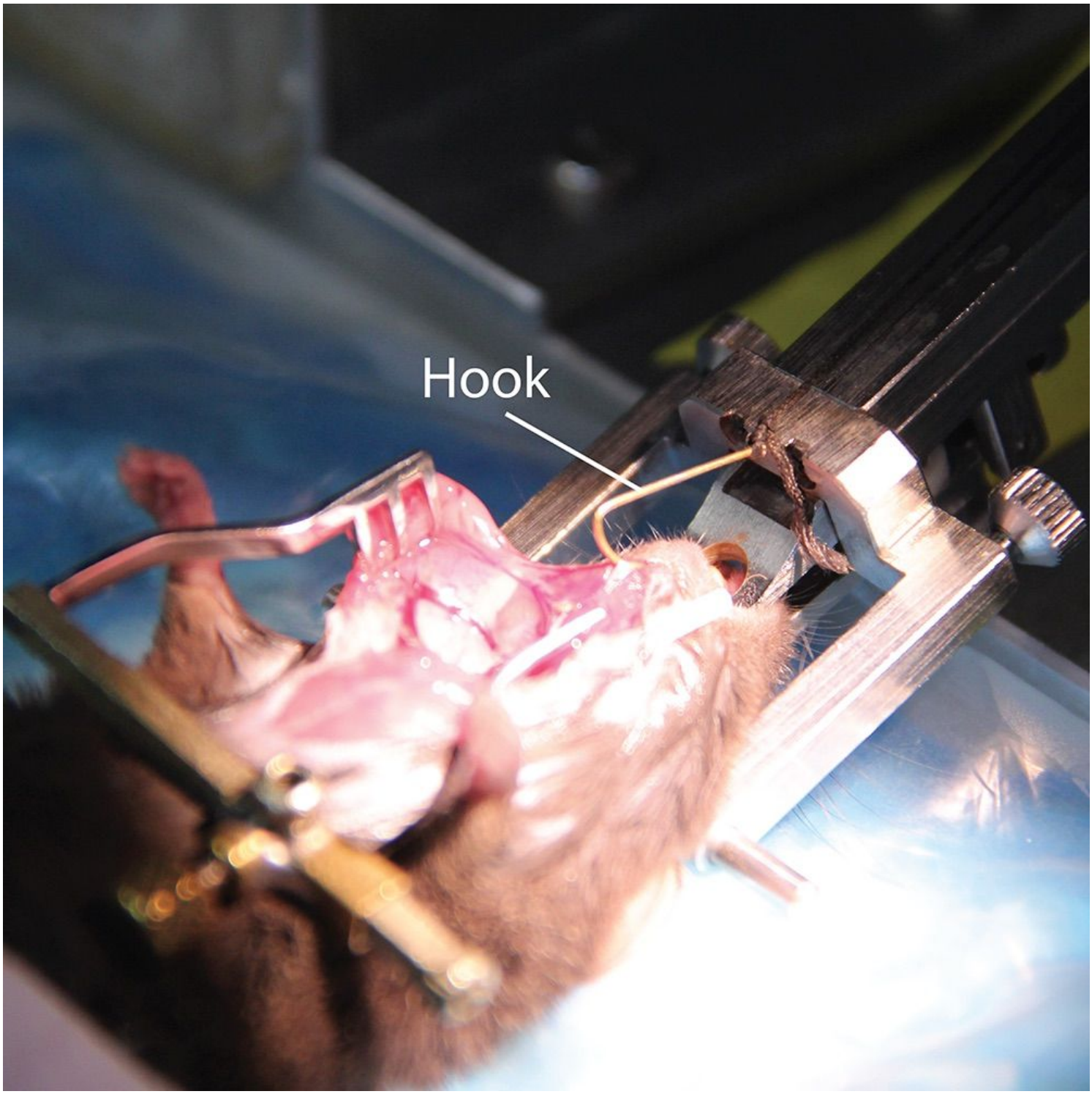

Figure 8

figure8 Use the customized hook to retract the skin and muscle rostrally.

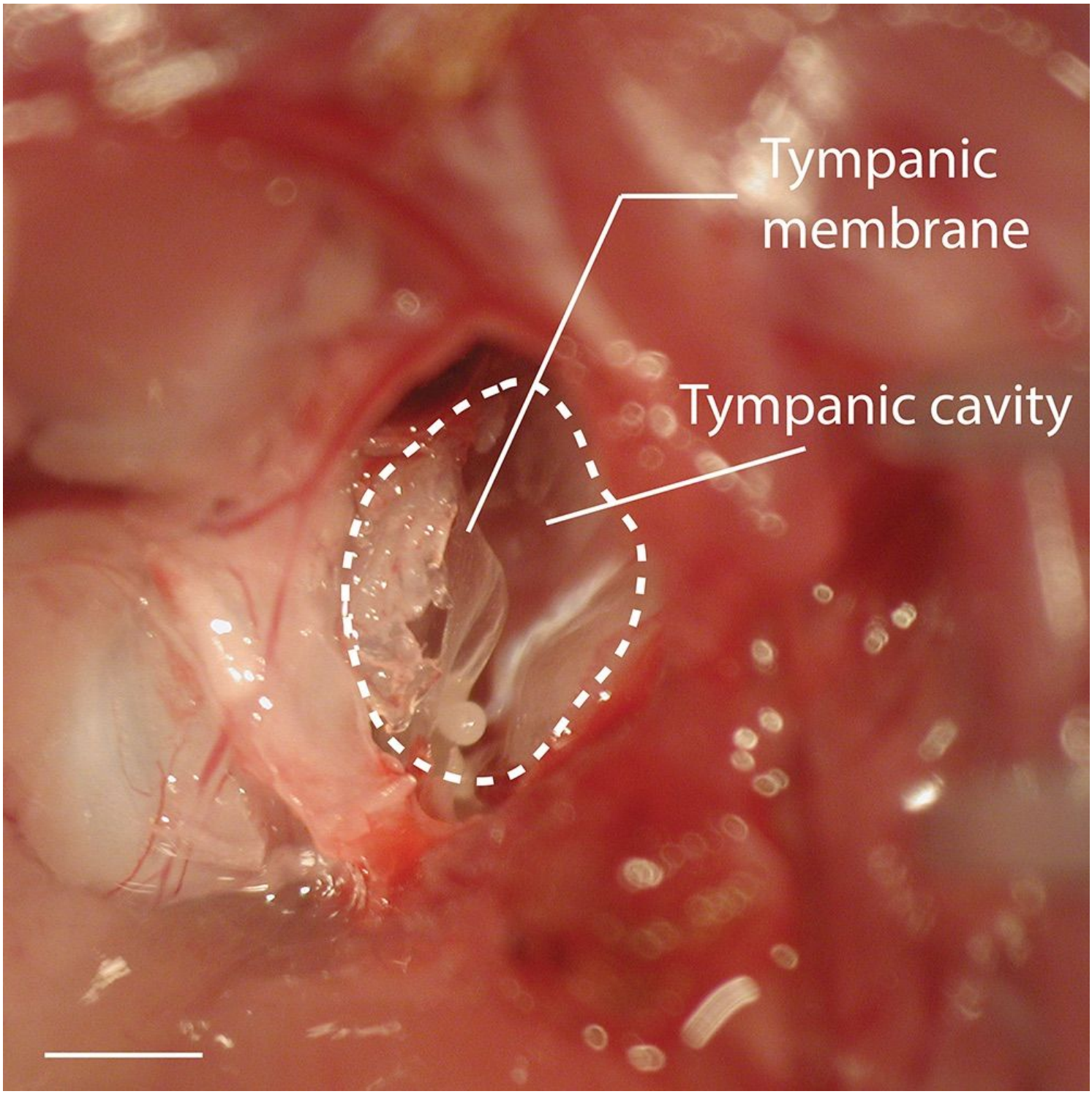

Figure 9

figure9 Penetrate the tympanic bulla and expose the tympanic cavity. Scale bar: 2.5 mm.

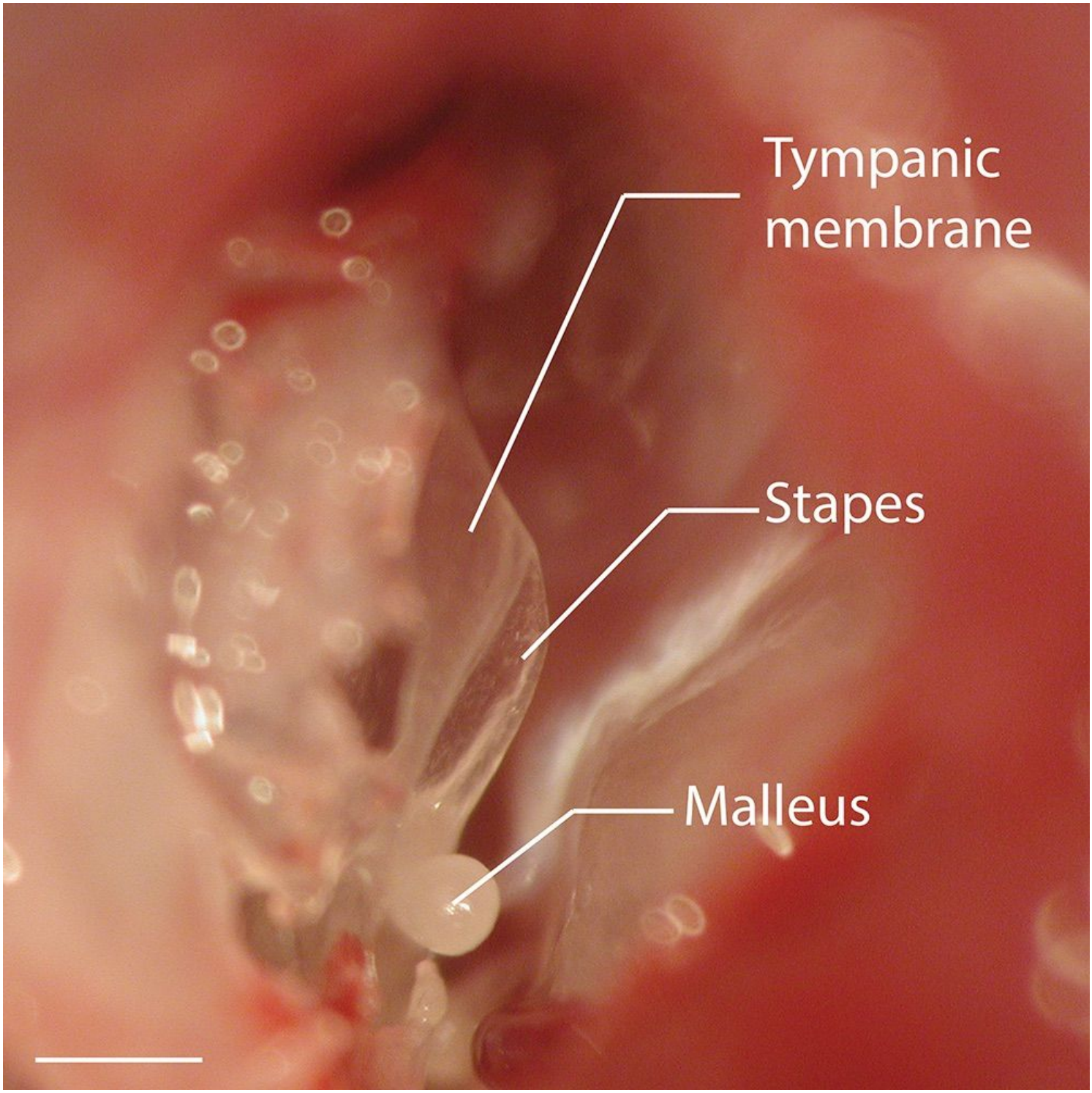

Figure 10

figure10 Inside the tympanic cavity, the staples of the ossicles connect with the tympanic membrane. Carefully remove the staples without breaking the tympanic membrane. Scale bar: 1 mm.

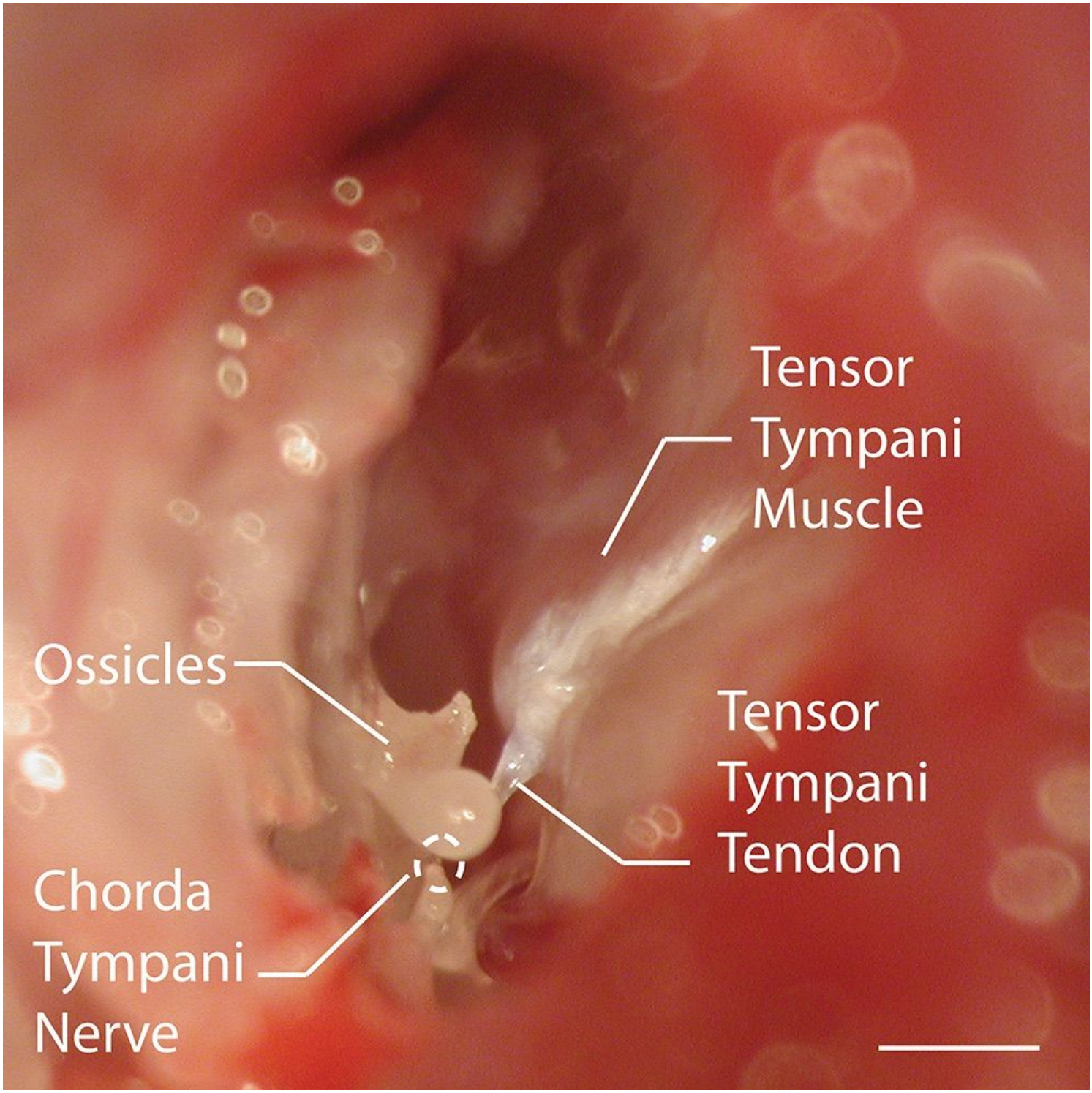

Figure 11

figure11 After removing the staples, remove the tendon of the tensor tympani muscle that connects with the ossicles. The chorda tympani nerve lies alongside the ossicles; be careful to avoid damaging the chorda tympani nerve. Scale bar: 1 mm.

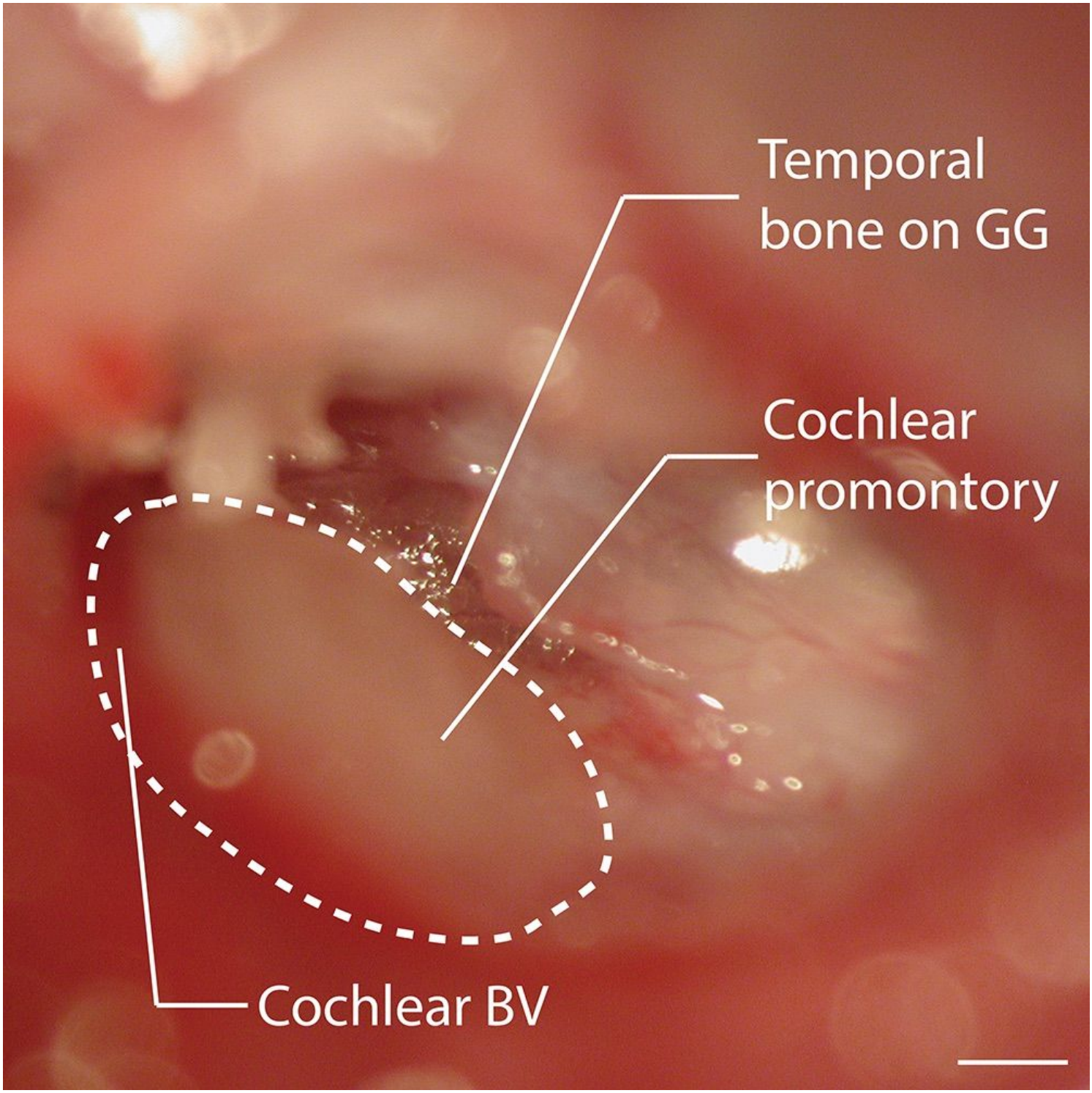

Figure 12

figure12 Break away the cochlear promontory and avoid the cochlear blood vessel (BV). Scale bar: 500 μm.

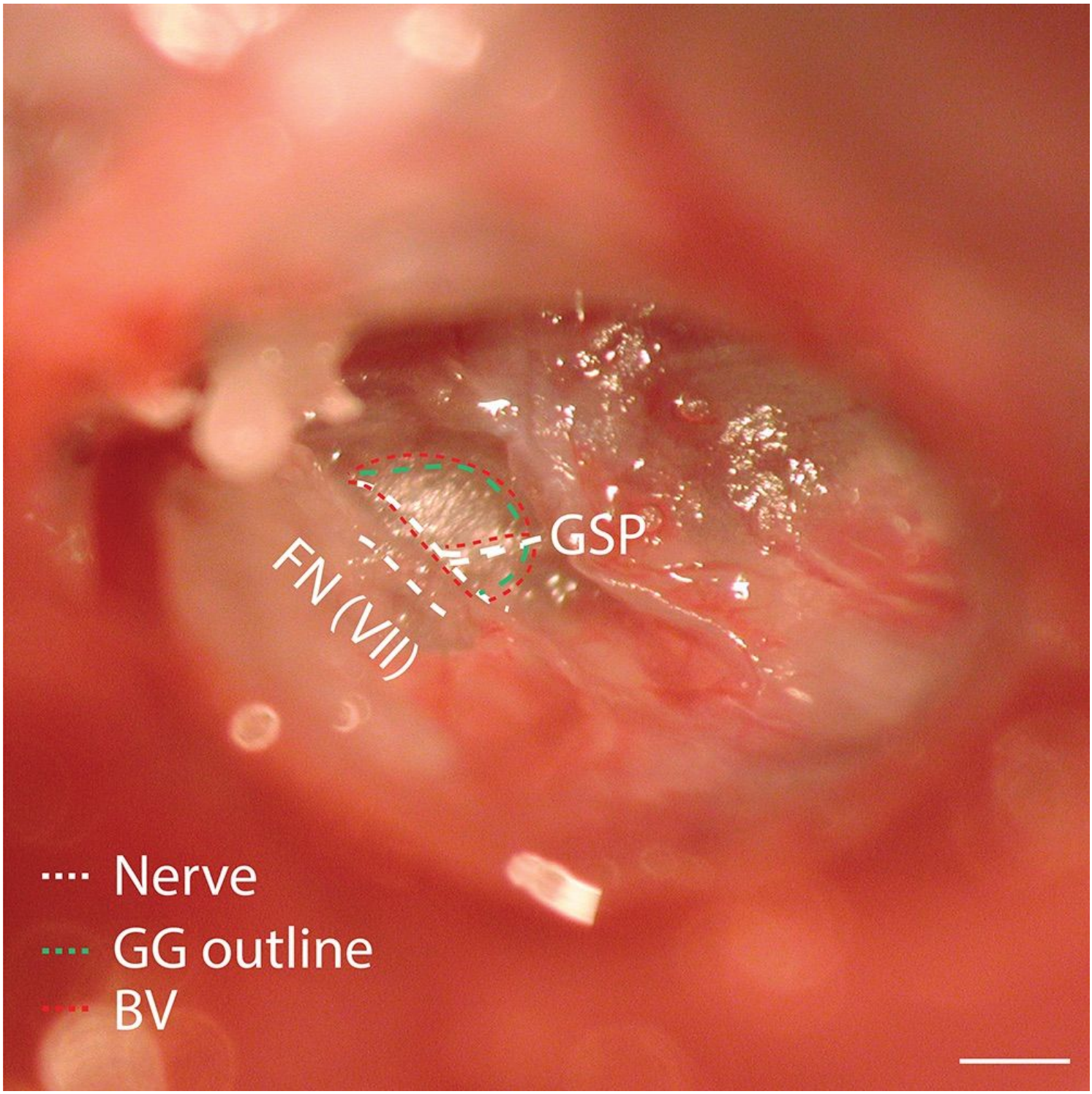

Figure 13

figure13 Exposing the geniculate ganglion After removing the cochlear promontory, the greater superficial petrosal nerve (GSP) and the facial nerve (FN) are exposed as indicated in the figure by dashed white lines. The dashed green line illustrates the approximate location of the geniculate ganglion (GG). The ganglion is surrounded by blood vessels that are indicated as dashed red lines. Scale bar: 500µm.

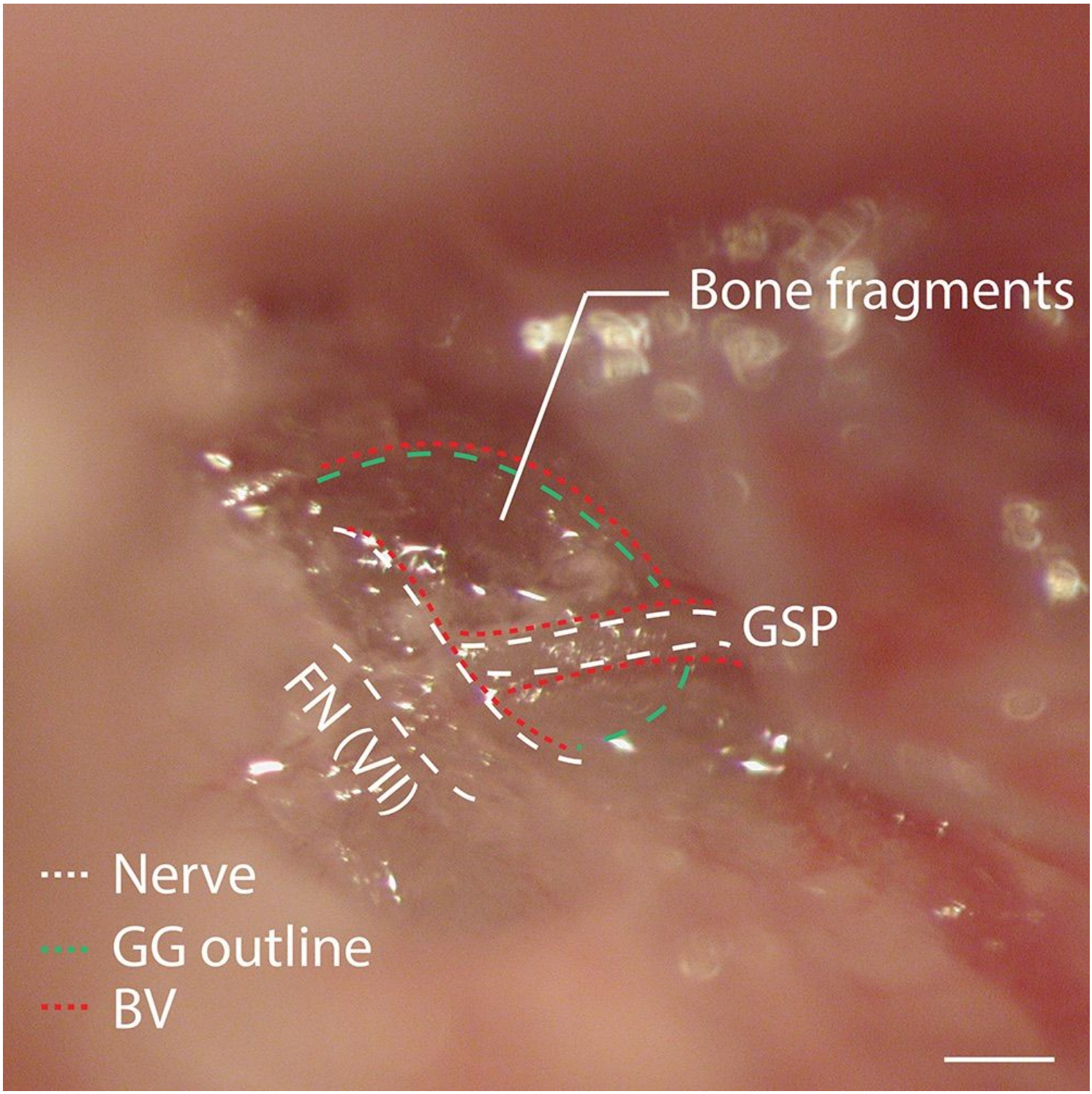

Figure 14

figure14 After breaking the temporal bone covering the geniculate ganglion, remove the bone fragments over the ganglion with 5SF forceps. Scale bar: 250μm.

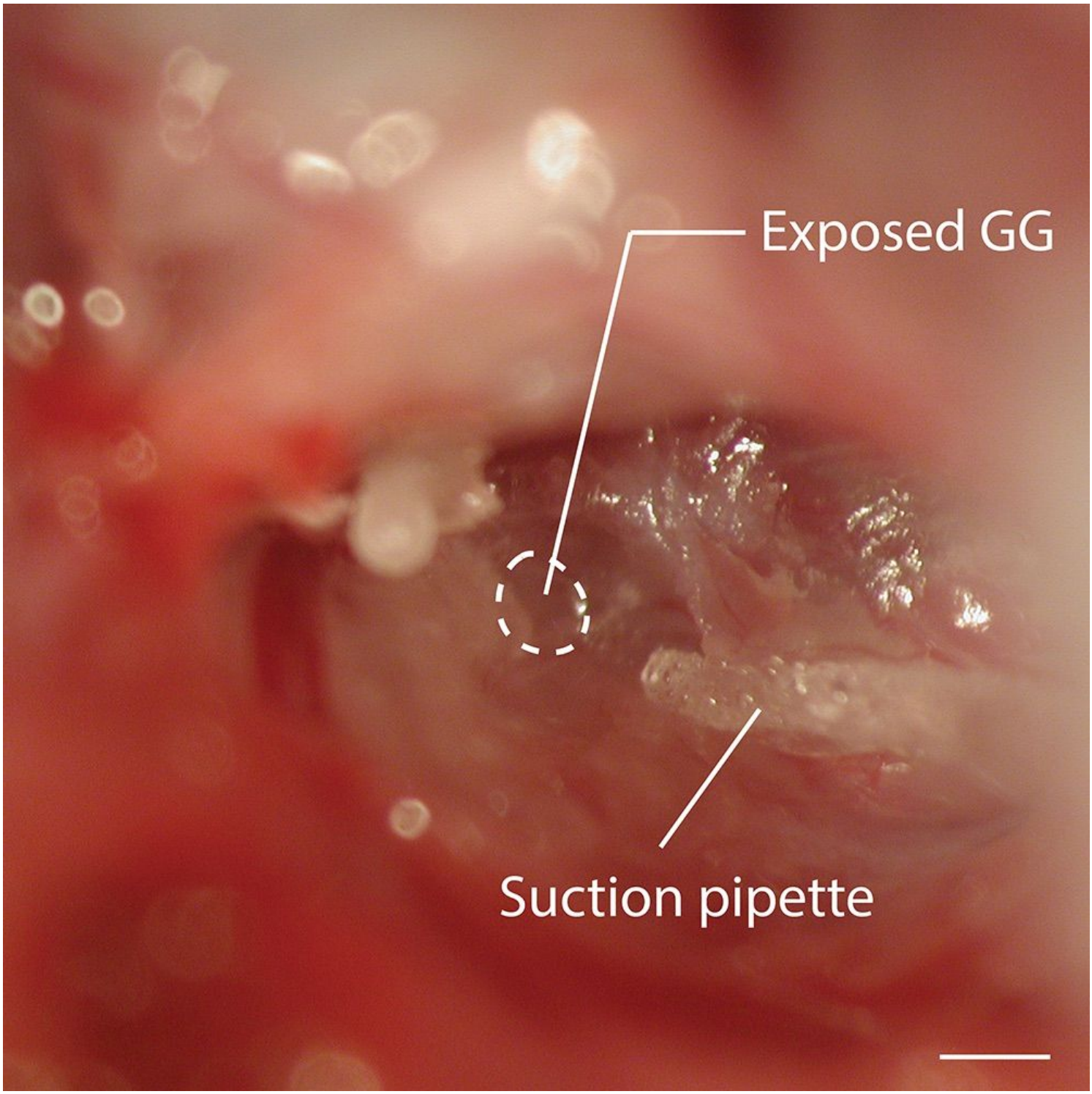

Figure 15

figure15 Postion a suction pipette close to the ganglion to draw off the fluid continuously after the surgery and during the recording. Scale bar: 500μm.

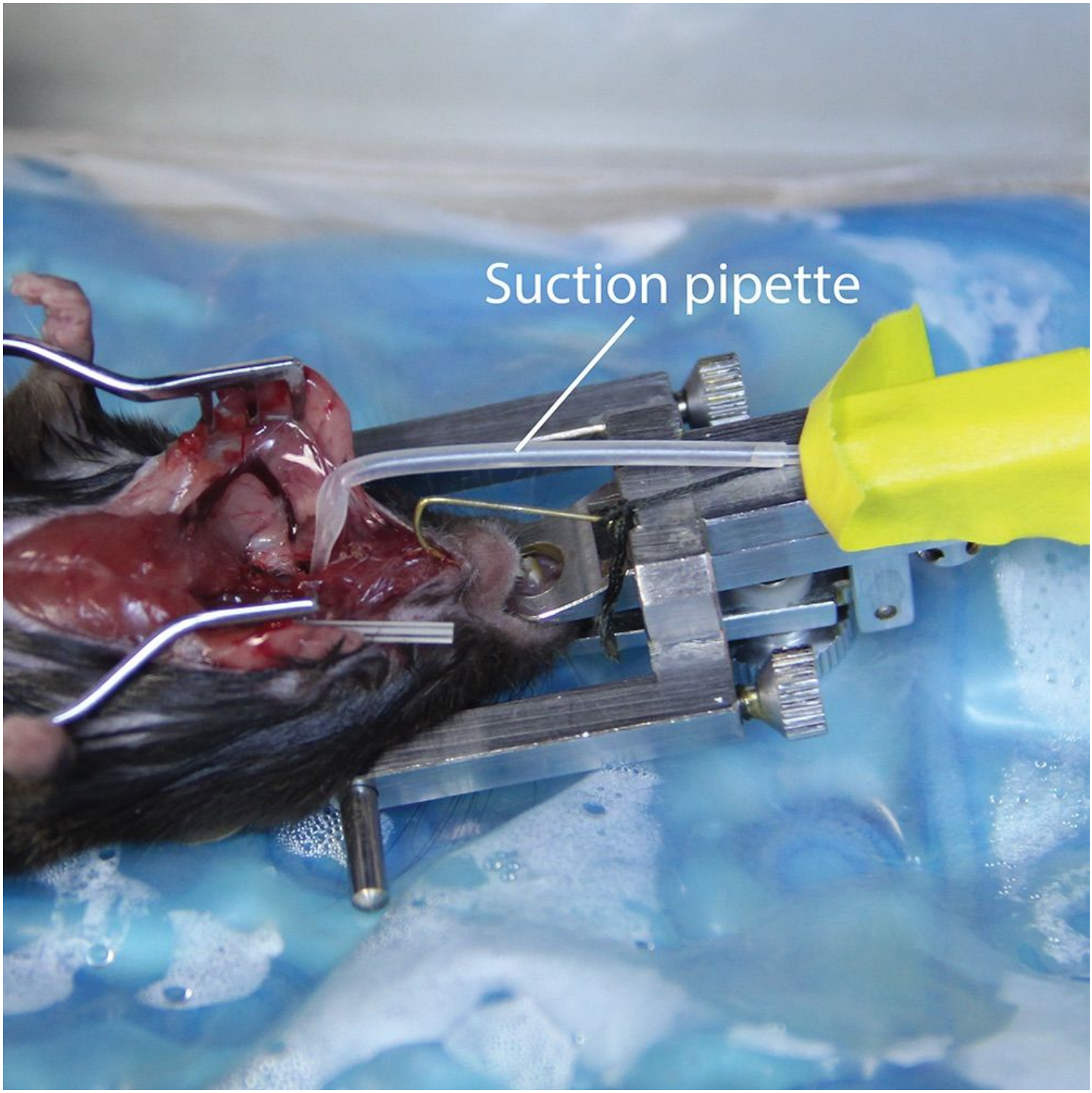

Figure 16

figure16 Fix the suction pipette on the mouse noseholder.

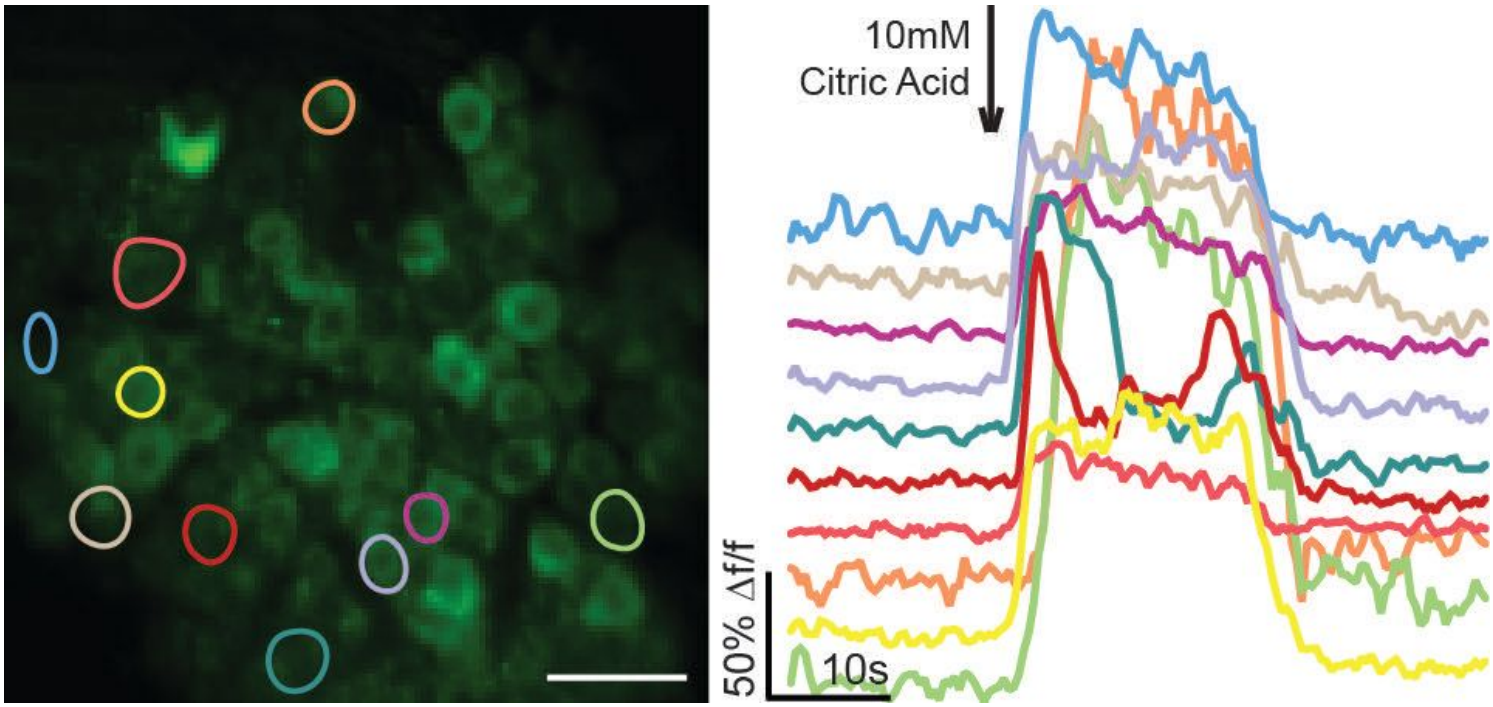

Figure 17

figure17 Imaging Left, view of neurons in the geniculate ganglion under the confocal microscope. Right, GCaMP3 fluorescence traces (ΔF/F0) in neurons responding to 10mM citric acid oral stimulation (as the arrow indicates). The color of ROIs in the left panel corresponds to the color of each trace in the right panel. Scale bar: 50μm.

No.	Problem	Probable cause	Solution
1	Unable to retract well;	Fail to place retractor in correct position;	The left side of the retractor should hold the gland and the skin while the right three hooks of the retractor should all hold the trachea;
2	1) Unable to see facial nerve and GSP nerve; 2) unable to use forceps to break temporal bones; 3) bleeding while breaking temporal bones;	1) The tensor tympani muscle is not completely removed, 2) forceps are not sharpened correctly, angle problem, 3) blood vessels underneath the temporal bone are broken;	1) Remove the tensor tympani muscle completely; 2) sharpen the forceps routinely, change the angle of the mouse to make it easier to break the temporal bone; 3) avoid breaking blood vessels around the GG;
3	1) Unable to see fluorescence in neurons or fluorescence is dim, 2) cannot see individual neuron or neuron is misshapen, 3) neuron is very bright but with strange shape	1) Suction micropipette is not working properly, surgery is not correctly done, 2) bleeds too much or the bone pieces are not correctly removed, 3) the pipette is too close to GG, the connective tissue around GG might be damaged	1) Check suction pipette position and reinstall if necessary, 2) avoid bleeding and remove bone pieces gently, 3) do not put the micropipette on top of the GG or too close to the GG, do not remove or damage the connective tissue that covers the GG.
4	1) Fluorescence declines or disappears during recording 2) image moving or shift of focal plane	1) Suction pipette II is not working properly, 2) mouse is not fixed to stage or stage is not fixed to confocal microscope correctly	1) Check the suction pipette position and reinstall it, 2) check the stage and the mouse, fix them correctly

Figure 18

Table 1 Troubleshooting