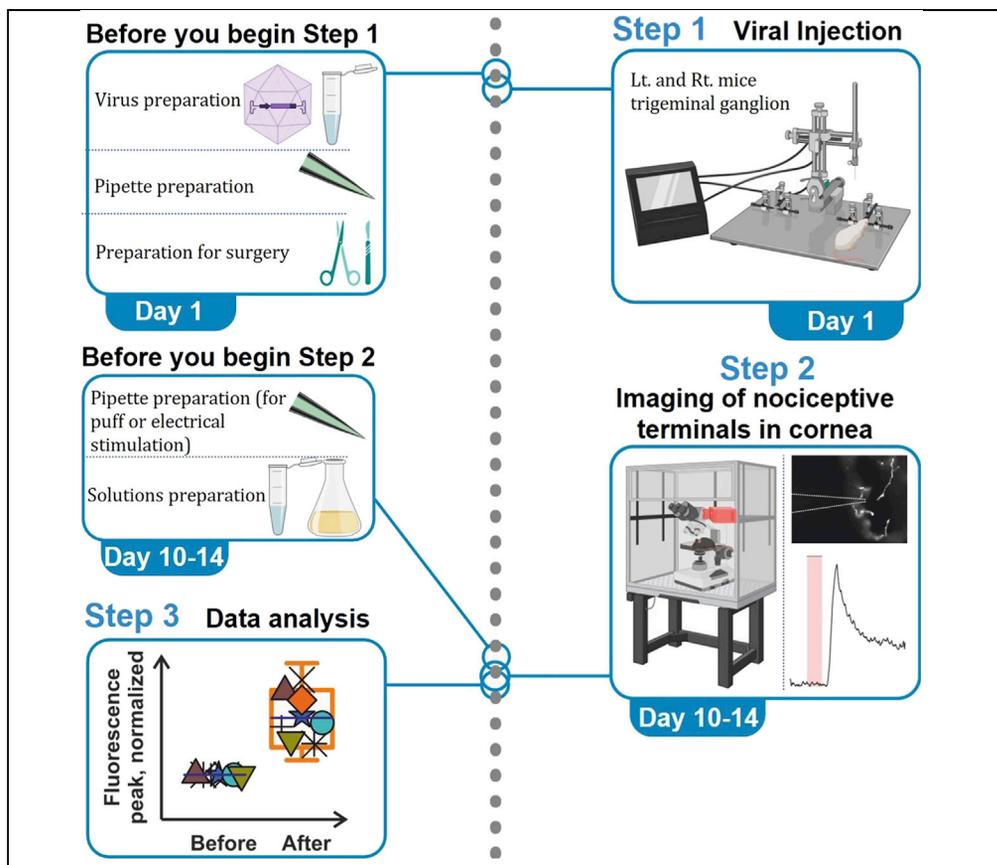


## Protocol

# *In vivo* optical recordings of ion dynamics in mouse corneal primary nociceptive terminals



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### Highlights

A protocol for measuring activity of mouse nociceptive terminal endings *in vivo*

Uses fast, high-sensitive optical recording from nociceptive terminals and fibers

Permits studying ion dynamics underlying encoding of noxious stimuli

This protocol aims to measure ion dynamics in nociceptive terminal endings in intact mice *in vivo*. We describe viral injection of GCaMP6s + RFP into trigeminal ganglia (TG) of mice, followed by calcium imaging of corneal nociceptive terminals that express GCaMP6s and RFP. This fast and high-resolution optical recording technique enables studying a nociceptive terminal's functional molecular network in physiological and pathological conditions. This platform can be applied to studying the physiology of terminals of other neurons.

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## Protocol

# *In vivo* optical recordings of ion dynamics in mouse corneal primary nociceptive terminals

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## SUMMARY

This protocol aims to measure ion dynamics in nociceptive terminal endings in intact mice *in vivo*. We describe viral injection of GCaMP6s + RFP into trigeminal ganglia (TG) of mice, followed by calcium imaging of corneal nociceptive terminals that express GCaMP6s and RFP. This fast and high-resolution optical recording technique enables studying a nociceptive terminal's functional molecular network in physiological and pathological conditions. This platform can be applied to studying the physiology of terminals of other neurons. For complete details on the use and execution of this protocol, please refer to Goldstein et al. (2019).

## BEFORE YOU BEGIN

The protocol below describes the specific steps for measuring evoked calcium dynamics in terminal endings and distal fibers of primary nociceptive terminals *in vivo*. The protocol consists of several steps: (1) viral injection of trigeminal ganglion neurons with viruses carrying a genetically encoded calcium indicator and tracer; (2) mice and solution preparations for the recording; (3) recording and (4) data analysis.

All procedures described here are carried out in accordance with the guidelines of the Animal Ethics Committee of the Hebrew University of Jerusalem and were approved by the Ethic committees of The Hebrew University.

## Preparations for viral injection

⌚ Timing: 30–45 min

1. Viruses AAV1.Syn.TurboRFP.WPRE.RBG (RFP) and AAV1.Syn.GCaMP6s.WPRE.SV40 (GCaMP6s) are ordered from Addgene (see [key resources table](#)), delivered on dry ice, and stored at  $-80^{\circ}\text{C}$ .
  - a. Thaw the viruses on ice.
  - b. Prepare the mixture of the two viruses, the GCaMP6s expressing virus and the RFP expressing virus (both of them with a titer of  $\geq 1 \times 10^{13}$  vg/mL):
    - i. Add 33  $\mu\text{L}$  of RFP stock into a 100  $\mu\text{L}$  of the GCaMP6s virus stock ampoule. This mixture and ratio were determined after conducting a series of calibration experiments to ensure co-expression of both RFP and GCaMP6s.



**Table 1. Protocol for pulling pipettes from capillaries for the viral injection**

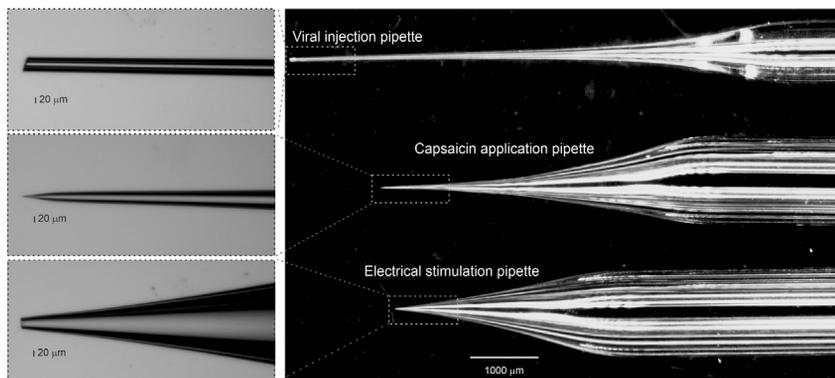
Steps	Heat	Pull (force)	velocity	Time	pressure
Cycle 1	330°C	120	140	25	500

Ramp-319, heat is always determined from the Ramp heat.

- ii. Aliquot the mixture to 9  $\mu\text{L}$  (approximately 4.5  $\mu\text{L}$  per animal) for each Eppendorf.
  - iii. Store at  $-80^{\circ}\text{C}$ .
2. Pull glass pipettes from capillaries (specified below in the [key resources table](#)) according to the pulling protocol for virus injections, detailed in [Table 1](#).
  - a. Use a scalpel to cut the pulled capillary's tips under a binocular, to a length of approximately 7 mm (internal diameter (ID) is approximately 15  $\mu\text{m}$ , [Figure 1](#), upper panel), so that the pipette is thin enough to penetrate the brain tissue all the way to the trigeminal ganglion (TG), where the viral mixture is injected.
3. Prepare a mixture of ketamine and medetomidine for intraperitoneal (IP) injection to anesthetize the mice.
  - a. Mix 1 mL ketamine (1 gr/10 mL) with 0.8 mL medetomidine (1 mg/mL) and add saline (NaCl 0.9%) to the final volume of 10 mL. This solution constitutes the anesthetizing mixture with a final concentration of ketamine at 10 mg/mL and medetomidine at 80  $\mu\text{g}/\text{mL}$ . Store the anesthetizing mixture at room temperature for up to a week.
  - b. For animals weighing between 20–25 gr, use 100  $\mu\text{L}$  of the anesthetizing mixture, consisting of 40–50 mg/kg ketamine and 0.36–0.4 mg/kg medetomidine.
4. Add isoflurane to a vaporizer to provide continuous anesthesia during the viral injection. For anesthesia induction, set the vaporizer to 2%. For anesthesia maintenance, set the vaporizer to 0.4%–0.6%. Store isoflurane at room temperature according to the expiration date.
5. Prepare meloxicam solution for analgesia. Using a 300  $\mu\text{L}$  syringe, take 20  $\mu\text{L}$  of meloxicam (5 mg/mL) and dilute with 280  $\mu\text{L}$  saline (NaCl 0.9%). For animals weighing between 20–25 gr, use 100  $\mu\text{L}$  of the solution (consisting of 1.32–1.65 mg/kg of meloxicam). Discard the remaining diluted meloxicam after use. Store undiluted meloxicam at room temperature according to the expiration date.
6. Prepare atipamezole solution for anesthesia reversal. Using a 300  $\mu\text{L}$  syringe, take 10  $\mu\text{L}$  of atipamezole (5 mg/mL) and dilute with 290  $\mu\text{L}$  saline (NaCl 0.9%). For animals weighing between 20–25 gr, use 150  $\mu\text{L}$  of the solution (consisting of 1–1.25 mg/kg of atipamezole). Discard the remaining diluted atipamezole after use. Store undiluted atipamezole at room temperature according to the expiration date.
7. Prepare instruments and accessories for performing the surgery and viral injections (all are specified in the [key resources table](#)), which include:
  - a. Hair clipper for shaving the fur on the mouse skull.
  - b. Hair removal cream.
  - c. Surgical tools.
  - d. A dental driller attached to the stereotactic adaptor.
  - e. A dental drill head of approximately 0.5 mm diameter.
  - f. A cannula holder.
  - g. A 10 mL syringe connected to a silicon tube. The syringe will be attached to the pulled pipette to produce negative (for filling the pipette with the viral solution) or positive pressure (for viral injection).
  - h. 1–10 mL pipette

### Preparations for *in vivo* optical recording of calcium signals

⌚ **Timing:** For the preparation of eye stabilizer and bath - 2 h. Note that once prepared, it could be used indefinitely. For the preparation of the SES - 40–45 min. Note that SES could

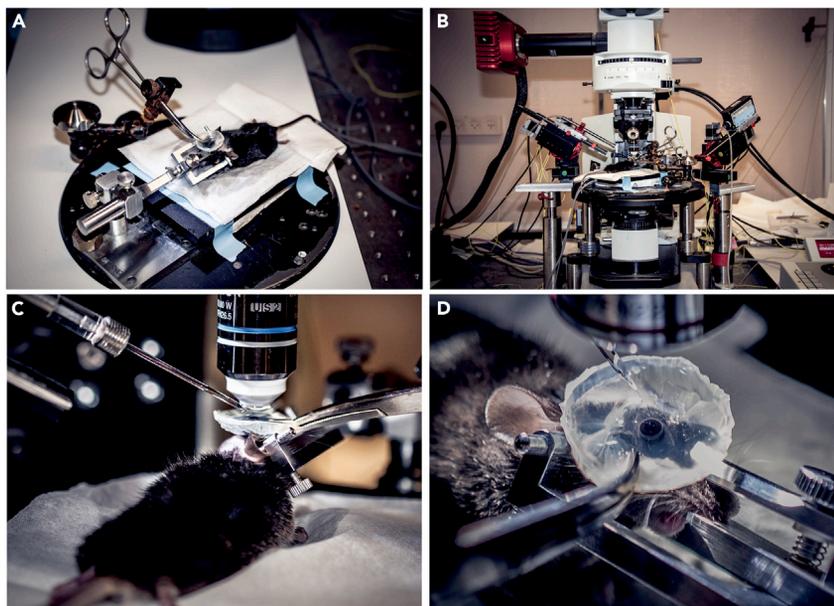


**Figure 1. Pipette shapes used for viral injection (upper panel), capsaicin application (middle panel), and generation of electrical stimulation (lower panel)**

*Insets: magnified images of pipette tips.*

be used for up to 6 months once prepared. Capsaicin and SR101 solutions and glass pipettes pulling should be prepared before each experiment - 30–40 min.

8. Prepare the eye stabilizer and bath (eyecup). The eye stabilizer and bath is used to stabilize the eye and immerse the eye in the solution during the experiments. The eye stabilizer and bath can be prepared customarily or manufactured by a 3D printer (Figures 2 and 3).
  - a. For custom preparation: The custom-made eye stabilizer and bath is prepared by cutting a silicon dental cement mixing cup (specified in the [key resources table](#)) to the dimensions



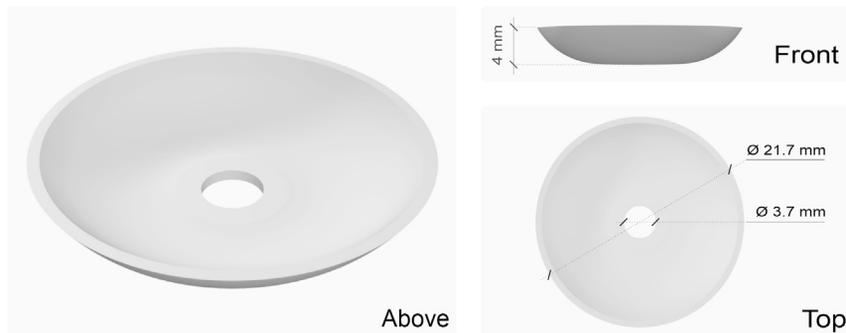
**Figure 2. Experimental setup**

(A) A mouse is placed on a heating pad, and the head is stabilized with a three-point head stabilizer. An eye stabilizer and bath, held by a hemostat fixed to a Noga arm which is connected to the plate, is placed over the eye and filled with SES.

(B) An overview of the experimental setup with a mouse prepared for data acquisition.

(C) Enlarged image depicting the position of a mouse, the puff pipette, the eye stabilizer and bath, and the lens under the microscope.

(D) Enlarged image showing placement of the eye stabilizer and bath together with the puff pipette. Note that the mouse head is tilted to place the imaged cornea in the horizontal plane.



**Figure 3. Scheme depicting the proportions and measurements of the eye stabilizer and bath**

shown in [Figure 3](#). A centered hole is made using a biopsy tissue puncher with a 3 mm diameter (specified in the [key resources table](#)), which is then enlarged to 3.7 mm to accommodate the eye firmly.

- b. For 3D printer manufacturing, TPU 95A filament (2.85 mm diameter, black and flexible, specified in the [key resources table](#)) is used, and the cup is molded according to the dimensions shown in [Figure 3](#). A file with a 3D printer design for the eye stabilizer and bath is available [here](#).
9. Prepare standard extracellular solution (SES) according to the protocol shown in the [materials and equipment](#) section. SES is used to fill the eye stabilizer and bath to create a bath encompassing the eye, hydration of the non-imaged eye, and to prepare the solution filling the puff pipette.
10. Prepare the solution for filling the puff pipette:
  - a. Dilute capsaicin from the 1 mM stock solution (see [materials and equipment](#) section) with SES to a final concentration of 500 nM. Capsaicin is aimed at activating TRPV1 channels in the terminals.
  - b. Add Sulfa-rhodamine 101 (SR101, from the 1.65 mM stock solution, see [materials and equipment](#) section) to the puff solution to a final concentration of 9  $\mu$ M. SR101 is used for tip pipette visualization using the wide-field epifluorescence microscope set to RFP acquisition parameters.
  - c. Keep the puff solution on ice during the experiment.
11. Pull glass pipettes for puff application of capsaicin or electrical stimulation from Sutter instrument capillaries using a Sutter instrument P-1000 pipette puller (see the [key resources table](#)) ([Figure 1](#), *middle and lower panels*, respectively) according to the protocols shown in [Tables 2 and 3](#), respectively.
12. The x, y position and the angle of the puff pipette holder mounted to the micromanipulator are calibrated to assure the access of the puff pipette tip to the cornea. To that end, a calibration experiment is performed in which naïve mice are anesthetized and placed under the microscope as described in steps 19–21 of the [calcium imaging of corneal nociceptive terminals, expressing GCaMP6s and RFP](#) stage, below. The correct angle and height of the puff pipette allowing uninterrupted access of the puff pipette tip to the cornea, are established and recorded. Slight adaptations of the holder position may be needed for each experiment.

**Table 2. Protocol for pulling puff pipettes from capillaries for the focal puff application of capsaicin**

Steps	Heat	Pull (force)	velocity	pressure
Cycle 1	355°C	19	30	400
Cycle 2	355°C	30	30	
Cycle 3	355°C	10	75	
Cycle 4	355°C	0	83	

Ramp-323, heat is always determined from the Ramp heat.

**Table 3. Protocol for pulling pipettes from capillaries for the electrical stimulation**

Steps	Heat	Pull (force)	velocity	Delay	pressure
Cycle 1	295°C	0	10	5	500
Cycle 2	295°C	0	10	5	
Cycle 3	295°C	0	20	5	

Ramp-313, heat is always determined from the Ramp heat.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
AAV1.Syn.TurboRFP.WPRE.RBG	James M. Wilson	Addgene AAV1; 105552-AAV1; RRID: Addgene_105552 <a href="http://addgene.org/105552">http://addgene.org/105552</a>
AAV1.Syn.GCaMP6s.WPRE.SV40	Chen et al., 2013	Addgene AAV1; 100843-AAV1; RRID: Addgene_100843 <a href="http://addgene.org/100843">http://addgene.org/100843</a>
<b>Chemicals, peptides, and recombinant proteins</b>		
Atipamezole 5 mg/mL (Antisedan)	Orion Pharma	N/A
Capsaicin	Sigma-Aldrich	Cat #M2028
D-glucose	Sigma-Aldrich	G7528
Hair removal cream	Veet	Cat #5000146980322
HEPES	Sigma-Aldrich	H3375
Isoflurane	Piramal Critical Care	N/A
Ketamine 1 gr/10 mL (Clorketam)	Vetoquinol	N/A
Medetomidine 1 mg/mL (Domitor)	Orion Pharma	N/A
Meloxicam 5 mg/mL (Rheumocam)	Chanelle Pharma	N/A
Povidone iodine	Dr. Fischer	Cat #1731
Sulforhodamine 101 (SR101)	Sigma-Aldrich	S7635
Synthomycine 5%	Rekah Pharmaceutical Industry	N/A
<b>Experimental models: Organisms/strains</b>		
C57BL/6JOLAHsd mice	Envigo-Israel	N/A
<b>Software and algorithms</b>		
CorelDraw	Corel Corporation	Version X6
Fiji	ImageJ, NIH	Version 1.4
MATLAB	MathWorks	Version R2016a
NeuroPlex	RedShirt Imaging	N/A
NIS Elements AR software	Nikon	Version 4.20.02
Office Suite	Microsoft	Version 2016
OriginPro	OriginLab Corporation	Version 2020
pCLAMP	Molecular Devices	Version 10.2
Turbo-SM	RedShirt Imaging	N/A
<b>Deposited data</b>		
The 3D printer design for the eye stabilizer and bath	Goldstein et al. (2019)	Mendeley data: <a href="https://data.mendeley.com/datasets/wzfn6czy9/1">https://data.mendeley.com/datasets/wzfn6czy9/1</a>
MATLAB script for analysis of changes in fluorescence	Goldstein et al. (2019)	Mendeley data: <a href="https://data.mendeley.com/datasets/66j6dymfxs/1">https://data.mendeley.com/datasets/66j6dymfxs/1</a>
<b>Other</b>		
Absorption triangles	Inami	MQA170310
A/D interface	Molecular Device	Digidata 1440A
Biopsy tissue puncher with a 3 mm diameter	Miltex	N/A
Cannula holder for stereotactic apparatus	Kopf Instruments	Model 1766-AP

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Capillaries (for capsaicin and electrical stimulation)	Sutter Instruments	Cat #BF150-86-10
Capillaries (for virus injections)	VWR	Cat #53432-706
Custom-made eye stabilizer and bath (eyecup)	Made in the lab using silicone dental cement mixing cup (below in the table, explained in step 8 of <a href="#">preparations for in vivo optical recording of calcium signals</a> ; see also <a href="#">Figure 3</a> for the specifications)	N/A
Dental drill head #006 (0.5 mm diameter)	Busch	020002
Differential amplifier with battery pack (9V)	Iso Flex, AMPI	N/A
Drill holder for stereotactic apparatus	Kopf Instruments	Model no. 1464
Filament for 3D manufacturing	Sinova	Ultimaker TPU 95A
Fluorescent camera, NeuroCCD-SMQ-II imaging system (RedShirt)	RedShirt Imaging	NCS01-t
Fluorescent camera	QImaging	Exi Aqua
Fluorescent microscope	Olympus	BX51WIF
Glass Bead Dry Sterilizer	CellPoint Scientific	Germinator 500
Hair clipper	Philips	NT9145/1318
Hemostat	Fine Science Tools	13002-10
Holding System Arm	Noga Engineering	NF60003
Isoflurane vaporizer	Datex Ohmeda	Isotec 5
LED gooseneck illuminator	Fisher Science Education	SO1461
LED illumination system (380 nm, 440 nm, 490 nm, 565 nm)	CoolLed	N/A
Micro-manipulator (SM7) with the adaptor for picospritzer pipette holder	Luigs & Neumann	UNIT Junior RE
Paraffin tape	Parafilm	PM-996
Pipette, 1–10 $\mu$ L	Gilson	Pipetman, F144055M
Pipette puller	Sutter Instruments	P-1000
Picospritzer	WPI	Pneumatic PicoPump, PV820
Silicone dental cement mixing cup	Keystone Industries	#5920490
Small micro spring scissors	Fine Science Tools	15024-10
Small scissors	Fine Science Tools	11251-30
Stereotactic apparatus	Kopf Instruments	Model 940, small animal
Surgical dental drill	Foredom	P-DP70
Surgical stitching kit	Ethicon	K890H
Surgical tweezers	Fine Science Tools	#5
Syringe (0.3 mL)	OMG	N/A
Syringe (10 mL)	BD	N/A

**MATERIALS AND EQUIPMENT**

**Standard extracellular solution (SES)**

Compounds	Final concentration (mM)	Amount (mass in grams for 1 liter of SES)
NaCl	145	8.47
KCl	5	0.373
CaCl <sub>2</sub>	2	0.294
MgCl <sub>2</sub>	1	0.203
HEPES	10	2.383
D-glucose	10	1.802
ddH <sub>2</sub> O	–	Up to 1L

pH titration to 7.4 using NaOH or HCl; Osmolarity 328 mOsm. Store at 4°C for 6 months.

- **Capsaicin solution:**

Stock solution: Dissolve 3.054 g of capsaicin powder in 10 mL of 100% ethanol to a final stock concentration of 1 mM. Store in  $-20^{\circ}\text{C}$  for up to 6 months.

The working solution is prepared by diluting the stock solution in SES to a final concentration of 500 nM.

△ **CRITICAL:** Capsaicin – a specific agonist of the noxious heat sensing channel, TRPV1 (Caterina et al., 1997), which, if swallowed or comes in contact with any parts of the body, will cause irritation and burning pain.

Wear protective gloves for skin protection eye/face protection, and use respiratory protection equipment. Work in a chemical hood.

**Alternatives:** Several other agonists such as resiniferatoxin (RTX) and bivalent tarantula toxin DkTx have been shown to activate the TRPV1 channel (Bohlen et al., 2010; Carnevale et al., 2016; Elokely et al., 2016). Moreover, agonists for other transducer channels which are expressed by nociceptive terminals such as AITC for TRPA1 (Jordt et al., 2004), menthol (Peier et al., 2002) or WS-12 (Bödding et al., 2007) for TRPM8, can also be used.

- **SR101 solution:**

Dissolve 10 mg of sulfa-rhodamine 101 (SR101, specified in the [key resources table](#)) in 10 mL SES to a final stock concentration of 1.65 mM. Store at  $4^{\circ}\text{C}$  for up to 6 months.

## STEP-BY-STEP METHOD DETAILS

### Viral injection of GCaMP6s + RFP into trigeminal ganglia (TG) of mice

⌚ **Timing:** the total procedure duration is approximately 40–60 min per animal. This time includes anesthesia induction, animal and surgical area preparation, cranium drilling, viral mixture injection into trigeminal ganglia, and scalp suturing.

This section describes in detail the technical steps required for mice surgery, in which the viral mixture is injected into trigeminal ganglia to express the genetic calcium indicator GCaMP6s and RFP tracer in the corneal nerve endings and terminal fibers. The injection coordinates are determined such that the viruses will infect the ophthalmic segment (V1) of the ganglion, which contains peripheral afferent nociceptive sensory neurons innervating the cornea (modified from (Whitehead et al., 2003)).

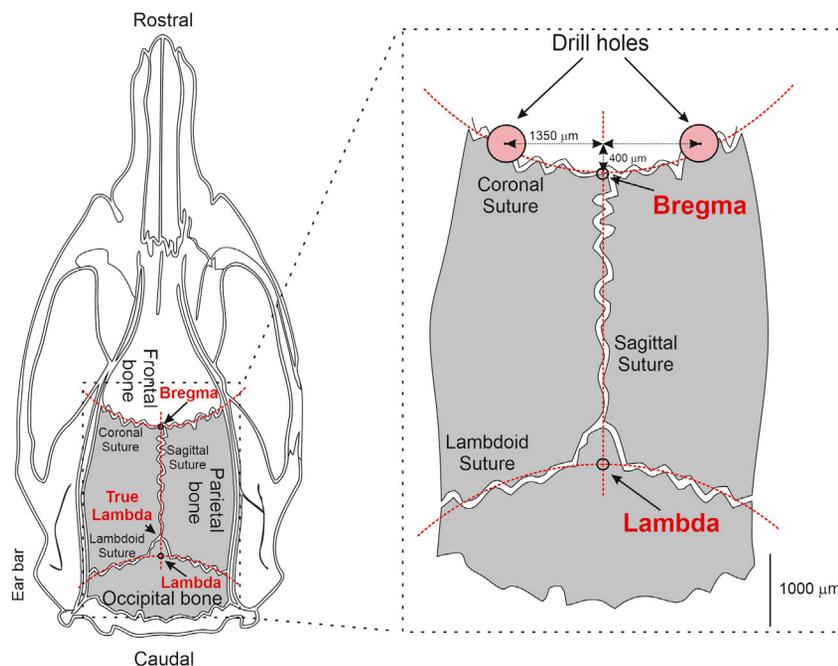
This stage allows for performing the main experiment, which is detection, characterization, and imaging corneal nociceptive terminals *in vivo*.

1. Sterilize the surgical tools using the glass bead dry sterilizer (specified in the [key resources table](#)).
2. Anesthetize a 4–6 weeks old adult male mouse by intraperitoneal (IP) injection of 100  $\mu\text{L}$  of anesthesia mixture (described above in [preparations for viral injection](#) section, step 3).
3. Shave the top of the mouse scalp.
4. Place the mouse in the stereotactic frame on a heating pad set at  $37^{\circ}\text{C}$ .
  - a. Attach the teeth to the mouthpiece.
  - b. Assemble the nose cone connected to the isoflurane vaporizer. Set vaporizer to 2% for anesthesia induction. Set the oxygen flow rate in the vaporizer to 350–400 mL/min.
  - c. Apply eye ointment (synthomycine, specified in the [key resources table](#)) to both eyes to avoid dryness which could cause eye irritation.

- d. Monitor pain reflexes by observing the responses to paw pinching.
- e. Once the anesthesia level is achieved and reflexes are diminished, decrease the isoflurane percentage to 0.4%–0.6% for anesthesia maintenance.
- f. Visually monitor the breathing rate during the entire procedure.

**Note:** The breathing rate is expected to be around 55–85 breaths per minute. If abrupt changes in the breathing rate occur, reevaluate the pain reflex, and readjust the anesthesia levels by slightly increasing the isoflurane percentage at the vaporizer.

5. Stabilize and fix the head in the stereotactic frame using non-rupture ear bars.
6. Inject meloxicam subcutaneously (SC) for pain relief at a concentration of 1.32–1.65 mg/kg (see [preparations for viral injection](#) section, step 5).
7. Use hair removal cream for removing the remaining fur after shaving. Clean and sterilize the scalp with alternating swabs of 70% ethanol and 10% povidone iodine (specified in the [key resources table](#)), and then dry the area.
8. Make a 1 cm midline incision to expose the Bregma and Lambda focal points.
  - a. Separate the scalp and the connecting tissues in the mediolateral axis, about 2.5–3 mm apart. To that end, use either surgical tweezers and scissors or a mini retractor.
  - b. Clean and dry the surface of the exposed bone by using triangle sponges and cotton swabs.
9. Determine Bregma and Lambda focal points by defining two points along an extrapolated straight line delineating the longitudinal sagittal suture, which intersects with an extrapolated continuous parabolic line delineating the anterior coronal suture (Bregma) and the posterior lambdoid suture (Lambda, [Figure 4](#)).
10. Attach the dental drill with its appropriate drill head (see the [key resources table](#)) to the stereotactic holder, and mount it to the stereotactic apparatus.
  - a. In order to align Bregma and Lambda in the horizontal plane (z-axis), bring the drill to the Bregma focal point under the binocular and zero its position in the z-axis.
  - b. Bring the drill to the Lambda focal point, and measure its position.



**Figure 4.** Scheme of the drill hole locations for the viral injections into the V1 area of the trigeminal ganglion

**Note:** no further changes are needed if the drill is still positioned at  $0 \pm 0.05$  mm on the z-axis. Otherwise, fine-tune the stereotactic apparatus (by adjusting the mouthpiece, which affects the position of the head on the z-axis) such that Bregma and Lambda focal points are at the same horizontal plane.

11. Determine the locations of cranial drilling (to enable injecting the virus into the V1 area of the two TGs). The locations of the drilling holes (approximately 0.5 mm diameter) are according to the following coordinates:  $+0.4 \text{ mm} \pm 0.02 \text{ mm}$  to Bregma on the anterior-posterior axis, and  $+/-1.35 \pm 0.02 \text{ mm}$  to Bregma on the mediolateral axis (Figure 4).
  12. According to the abovementioned coordinates (step 11), lower the dental drill slowly until the bone resistance diminishes, indicating the transition from bone to soft brain tissue. At this point, stop the drilling.
    - a. Wash the remaining bone and blood using saline (0.9% NaCl) and dry with swabs.
  13. For injection of the viral mixture, replace the drill with a pipette holder. Mount the glass pipette into the holder, and connect it with a silicon tube to a 10 mL syringe.
  14. Load 4.5  $\mu\text{L}$  of the viral mixture with a 1–10  $\mu\text{L}$  pipette (specified in the [key resources table](#)) onto a paraffin tape strip (Parafilm, specified in the [key resources table](#)), creating a droplet.
    - a. Bring the parafilm strip with the viral mixture droplet under the fixed pipette.
    - b. Load the viral mixture into the pipette using negative pressure applied by the syringe.
  15. Place the pipette filled with the viral mixture above the Bregma focal point. Confirm the Bregma to Lambda alignment as explained in step 10a-b, this time using the pipette instead of the dental drill.
    - a. Lower the pipette into the drill hole to a final depth of  $-6.39 \pm 0.04 \text{ mm}$ , on the z-axis.
    - b. Inject 1–2  $\mu\text{L}$  of the viral mixture per ganglion, at a rate of about 1  $\mu\text{L}$  per 10 s.
- △ CRITICAL:** Injection of air through the pipette could be lethal. To avoid air injection, visually monitor the viral mixture's fluid level. Perform the injection only when the fluid level in the pipette can be observed, and it is higher than the margins of the drilled hole.
- △ CRITICAL:** Following the injection of the virus, the injection pipette should not be removed and should remain in place for a 5-minute pause time to allow pressure equalization.
- c. After 5 min, lift out slowly the glass pipette, and place it at the zero-reference point (Bregma).
  - d. Examine the pipette tip integrity.
  - e. If pipette tip integrity is preserved, move the glass pipette to the second drill hole at the exact opposite coordinates in the x-axis for injecting the viral mixture into the second TG. If pipette tip integrity is not preserved, replace the pipette and repeat steps 13 and 14. Then move the glass pipette to the second drill hole at the exact opposite coordinates in the x-axis for injecting the viral mixture into the second TG.
  - f. Repeat steps 15a–15c at the new location.
  - g. Wash and sponge the drill holes with saline 0.9%, and then dry.
16. Suture the incision over the open dried and cleaned drill holes. Apply synthomycine over the sutured incision.
17. Inject the mouse with atipamezole 150  $\mu\text{L}$  IP (see [preparations for viral injection](#) section, step 6).
18. For animal recovery:
  - a. Place the mouse in a cage under warming light and soothing conditions, and constantly monitor until the animal appears to recover (normal gait, grooming behavior, and overall appearance). An animal is considered to be recovered from anesthesia when a mouse can maintain himself upright and move purposefully.
  - b. Put mouse into a separate cage for 2–3 days to recover from the surgical procedure and only then place it into a common cage with 2–4 littermates.
  - c. Mice are taken for imaging experiments 10–14 days post-viral injection.

△ **CRITICAL:** The 10–14-day experimental window was determined by performing a calibrating experiment on a separate group of mice to detect the optimal expression of the RFP tracer. These experiments were performed by monitoring RFP fluorescence in corneal nerve terminals using an epifluorescent microscope in anesthetized mice (as detailed in section [calcium imaging of corneal nociceptive terminals, expressing GCaMP6s and RFP](#)) every day following the viral injection. We found that the optimal expression of RFP in the corneal terminals and fibers depends on the viral titer and the injected amount. It is achieved at 10–14 days post-viral injection in the described conditions. This time window is also optimal for the recordings of GCaMP6s signals.

△ **CRITICAL:** Attempts to record from the terminals with prolonged post-injection periods allow clear RFP visualization, but in all cases, show low, if at all, evoked GCaMP6s fluorescence.

### Calcium imaging of corneal nociceptive terminals, expressing GCaMP6s and RFP

Ⓞ **Timing:** 3–6 h per animal (the timing differs and depends on the pharmacological compounds applied and each experiment's aim).

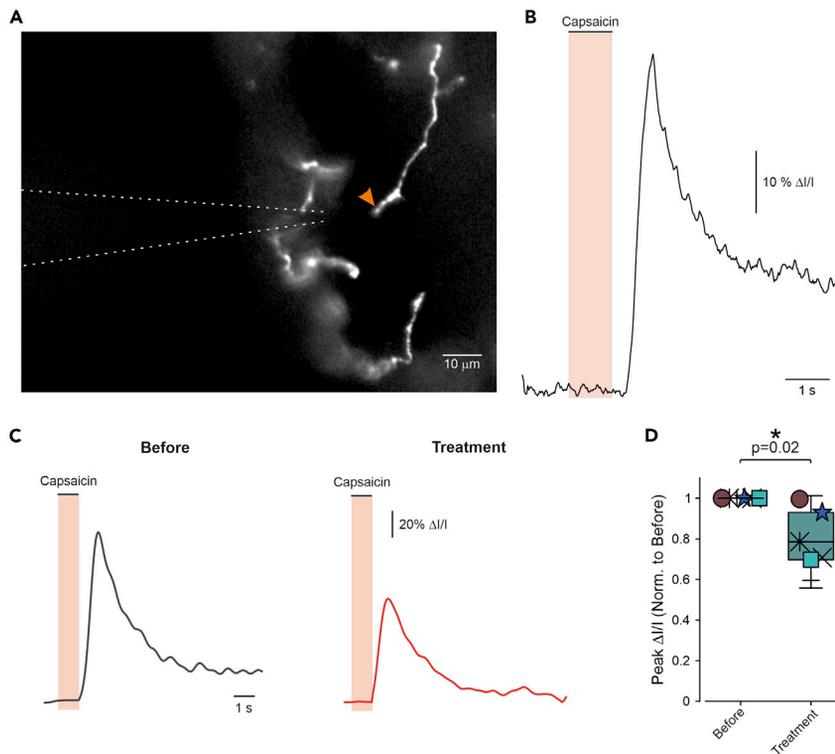
This section describes a detailed step-by-step process for wide-field epifluorescence and calcium imaging of single nociceptive terminals and distal fibers in the mouse cornea. Furthermore, this section describes the main principles governing the handling of the mouse, the optics, and the imaging apparatus, which enables data acquisition in precise spatial and temporal parameters.

19. For anesthesia, inject the mouse that has undergone viral injection, IP with 220–250  $\mu\text{L}$  of the anesthesia mixture (described in step 3 of section [preparations for viral injection](#)). Mice are considered anesthetized when there is no paw pinch reflex. It usually takes about 15 min.
20. Hydrate both eyes by applying approximately 10  $\mu\text{L}$  SES per eye to avoid eyes dryness. The drop should stay on the non-imaged eye during the experiment. If the SES drop slips or vaporizes from the non-imaged eye, reapply the drop.
21. Place the mouse on a heating pad set to 37°C, located on a recording plate ([Figure 2A](#)). Stabilize its head with a three-point head stabilizer (SGM-4, Narishige, Japan, specified in the [key resources table](#), [Figure 2A](#)).
  - a. Tilt the head such that the imaged eye is in the horizontal plane ([Figures 2C and 2D](#)), at an angle allowing placing the eye stabilizer and bath above it so that the bath can hold the solution (further checked and tuned in steps 21biii, 21bv).
  - b. The eye stabilizer and bath is held by a hemostat fixed to a Noga arm (specified in the [key resources table](#)), attached to the recording plate ([Figure 2A](#)).
    - i. Place the eye stabilizer over the mouse eye, and gently stretch the skin around the eyeball aside using two fingers to bulge out the eyeball.
    - ii. Carefully push the bulged eyeball through the hole of the eye stabilizer and bath (squeezed through, careful not to make any lateral movements which might lead to tissue damage, [Figures 2C and 2D](#)). The hole size of the eye stabilizer and bath ([Figure 3](#)) is as large as the eyeball or a bit smaller such that once the eyeball is squeezed through the hole, the solution does not leak out between the eye stabilizer and the eye ([Figure 2D](#)).
    - iii. Inspect to ensure that the eye stabilizer and bath is parallel to the horizontal plane. If this is not the case, lift the eye stabilizer gently and readjust it. It is also possible to adjust the angle of the mouse head if needed.
    - iv. Fill the eye stabilizer and bath with SES ([Figures 2A and 2D](#)).
    - v. Inspect again to ensure that there is no tilt of the chamber in relation to the horizontal plane. If the solution does not fill the eyecup in a homogenous manner around the eyeball, remove the solution and readjust, as explained above in 17biii.
  - c. Place the plate with the mouse on the microscope stage ([Figures 2B and C](#)).

22. Use an Exi Aqua (QImaging) monochromatic camera for wide-field epifluorescence image acquisition (bright field imaging and RFP fluorescence).
  - a. Center the cornea, and adjust the field of view via the eye-piece of the microscope using the 5X objective. At this stage, the eye is illuminated via an external LED light source attached to a gooseneck (LED gooseneck illuminator, see [key resources table](#)), and the microscope's filter turret contains an empty filter box.
  - b. Use a 40× water immersion high NA objective and RFP filter set (a 565 nm excitation LED and RFP filter set: Ex 560, Em 630, dichroic Lp 585; Chroma) to select a superficial terminal. Use a "live view" mode of the NIS elements imaging software (specified in the [key resources table](#)). The RFP imaging is performed with an exposure time of 300–500 ms, binning of 2 × 2, and a hardware gain of 10–20. The light source is at maximal power, with 3.1 mW/cm<sup>2</sup> flux at the focal plane of the objective. At this time point of the procedure, GCaMP6s basal fluorescence is non-detectable since intra-terminal calcium levels are low.
23. Use the prepared glass pipette (as detailed in [preparations for \*in vivo\* optical recording of calcium signals](#) section, step 11) and fill it with the solution containing SES, capsaicin (500 nM) and SR101 (9 μM), as described above in the [preparations for \*in vivo\* optical recording of calcium signals](#) section, steps 9–10.
  - a. Discard the trapped air bubbles at the tip of the puff pipette by gently flicking the pipette before placing it in the Picospritzer holder.
  - b. Place the filled puff pipette into the Picospritzer holder attached to the Picospritzer holder adapter on the micromanipulator (SM7, described in the [key resources table](#)), and control its movements via an external module.

**Note:** The holder and the manipulator positions are calibrated to assure that the puff pipette tip has access to the cornea (see [preparations for \*in vivo\* optical recording of calcium signals](#) section, step 12).

24. To bring the loaded pipette to the terminal field of view, lift the 40× objective several hundreds of μm above the epithelial plane without changing its x and y coordinates and replace it with a 5× objective.
  - a. Under the external light source, with the empty filter turret, bring the pipette above the bath solution to the objective focal plane and adjust the pipette tip to the center of the imaged field.
  - b. Place the 40× objective, and readjust the focal plane to bring the pipette tip into focus and to the center of the field of view.
  - c. Switch the optical pathway from the eye-piece to the QImaging camera, and set parameters to allow RFP fluorescence acquisition (detailed above, step 22b).
  - d. Perform a short test puff to ensure that the pipette is not blocked or leaking. This test is done by monitoring the change in SR101 fluorescence (using RFP fluorescence acquisition parameters) around the pipette tip after applying a puff.
25. To position the loaded pipette in close proximity to the terminal of interest, using a micromanipulator (described above and in the [key resources table](#)), gradually lower the objective (40×) and the pipette consecutively, starting with increments of 20–40 μm, narrowing the range when reaching closer to the terminal tip plane. Repeat this process until the pipette tip is placed 2–5 μm above the corneal surface (**CRITICAL:** without disturbing the cell layer) and approximately 10 μm in the x-y plane from the terminal end ([Figure 5A](#)).
26. Still using the parameters for detection of RFP fluorescence, change the optical pathway so that the fluorescent emission is directed towards the RedShirt camera (back-illuminated 80 × 80 pixels cooled and fast CCD camera). The RedShirt camera detection parameters are: 40–125 Hz acquisition rate, medium gain, and "live view" mode. Since the two cameras do not have the same optical pathway, and the field of view between them is very different in size, re-focus the microscope and bring the terminal to the center of the frame.
27. For performing the GCaMP6s imaging (calcium imaging) of the selected terminal:



**Figure 5. In vivo recording of capsaicin-induced calcium signals in corneal nociceptive terminals**

(A) Epifluorescent image of RFP fluorescence in the corneal terminals detected with QImaging camera and NIS elements acquisition program. The position of the puff pipette is outlined by the dotted lines. The orange arrow indicates the representative region of interest (ROI) from which the recordings of changes in GCaMP6s fluorescence are performed.

(B) A representative trace of optical recordings of the change in GCaMP6s fluorescence, from the terminal at the ROI shown in A, acquired by the RedShirt camera, and analyzed using Turbo-SM imaging analysis software and Matlab program (see section [quantification and statistical analysis](#)), following a 1s application of 500 nM of capsaicin.

(C and D) An exemplary experiment. (C) Representative traces of optical recordings of changes in GCaMP6s fluorescence following application of capsaicin (*Before*) and changes in GCaMP6s fluorescence following application of capsaicin to the same terminal and recorded from the same ROI, but during treatment with a drug (*Treatment*). (D)

Analysis of the data from 5 different terminals assessed from 5 eyes from 5 mice. The data are represented as boxplots and individual paired values of the terminal peak fluorescence intensities ( $\Delta I/I$ ). The boxplots depict mean; 25<sup>th</sup> and 75<sup>th</sup> percentile and SD. The values of peak fluorescence intensities are obtained by normalizing the fluorescence intensity values measured during the experiment ( $\Delta I$ ) to the values of the baseline obtained 10 s before applying capsaicin ( $I$ ). The effect of treatment on the peak fluorescence is measured from the same ROI at each terminal before and after treatment application. The values are then normalized to the values obtained before the treatment application. One sample t-test.

- a. Set GFP fluorescence parameters (a 490 nm excitation LED and GFP filter (Ex 480, Em 535, dichroic Lp 510; Chroma)). The light source is at maximal power and has 5.4 mW/cm<sup>2</sup> flux at the focal plane of the 40× objective.
- b. For detecting calcium-mediated changes in the GCaMP6s fluorescence, choose the regions of interest (ROIs) according to the examined compartment of nociceptive terminal and fiber:
  - i. For detecting calcium-mediated changes in the GCaMP6s fluorescence at the *terminals*, choose the regions of interest (ROIs) within 5–10 µm proximal to the terminal tip.
  - ii. For detecting calcium-mediated changes in the GCaMP6s fluorescence at the *fibers*, choose the regions of interest (ROIs) within 15–30 µm proximal to the terminal tip.
  - iii. For detecting calcium-mediated changes in the GCaMP6s fluorescence at the *plexuses*, choose the regions of interest (ROIs) within 60–100 µm proximal to the terminal tip.

△ **CRITICAL:** Regardless of the investigated areas (terminals, fibers, or plexuses), the application of capsaicin is always made above the terminal tip, as explained in step 25 above.

28. The ROI is visualized using the recording software (Turbo-SM), which is also used to trigger the puff and start recording the changes in GCaMP6s fluorescence.
29. Turbo-SM, via the RedShirt camera's analog-to-digital (A/D) converter, triggers a Digidata 1440 A/D interface (Molecular devices, specified in the [key resources table](#)), which in turn triggers the picospritzer (Pneumatic PicoPump, specified in [key resources table](#)) to initiate the puff. The experimental parameters are set using the pClamp software (Molecular Devices, specified in the [key resources table](#)).
30. The experimental protocol is as follows: 1,000 ms recording before puff application, 1,000 ms puff duration, and 5,500 ms recording after puff ends.
31. For focal puff application of capsaicin or vehicle, use a pipette with 4–6 MΩ resistance (when filled with SES, see the protocol detailed in [Table 2](#)) connected to a picospritzer (specified in the [key resources table](#)). The parameters of the puff are: 1 s puff, 2 pounds per square inch (psi). These parameters have been devised after calibrating the dispersion profile of the puffed solution ([Goldstein et al., 2017](#)).
32. Puff calibration: the dispersion profile of puffed substances was calibrated by measuring changes in sulfa-rhodamine 101 (SR101, 9 μM) fluorescent intensity profile, with distance, from the pipette tip according to

$$\frac{F_{x_1,y_1}}{F_{x_0,y_0}} \times C_{x_0,y_0} = C_{x_1,y_1} \quad (\text{Equation 1})$$

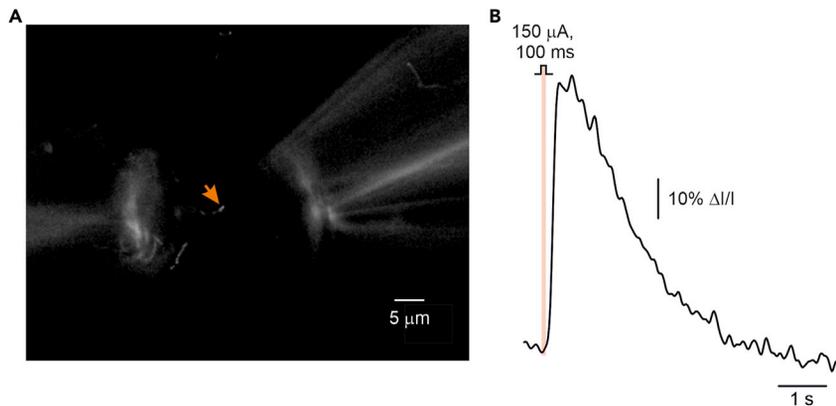
where F is fluorescence intensity at x, y coordinates and C is the concentration.

We calibrated the puff characteristics to achieve a substantial decrease in fluorescence at a distance of about 15 μm from the pipette tip on the horizontal plane (y-axis), thus preventing an effect on neighboring terminals ([Goldstein et al., 2017](#)). According to the calibration parameters, we place the pipette about 10 μm from the terminal tip in the following experiments. Thus, in these conditions, the concentration of capsaicin at the terminal is close to the half-maximal effective concentration (EC50) of TRPV1 channels to capsaicin (~150 nM ([Yang et al., 2015](#))). This is an estimated peak concentration during a 1 s puff, which dissipates in the medium when the puff ends. Additionally, considering that the minimal concentration of capsaicin to activate TRPV1 channels is ~30 nM ([Yang et al., 2015](#)), the prominent decrease in concentration along the y-axis renders the concentration mostly ineffective once it reaches other parts of the fiber or adjacent terminals.

33. Following terminal activation, calcium-induced signals at designated ROIs are recorded using the Turbo-SM imaging software.
34. Image data collected from the RedShirt camera are further processed and analyzed from selected ROIs (defined with a kernel size of two (2 × 2 pixels), using the NeuroPlex imaging analysis software) and other software and algorithms mentioned in the [key resources table](#) and section [quantification and statistical analysis](#).

△ **CRITICAL:** The puff pipette should not touch the corneal surface at any stage. If it does, replace the pipette and use a different terminal located far away (at least 100 μm) from the initial terminal (step 25 above).

△ **CRITICAL:** The puff pipette tends to move from its original and specific location after a puff. Therefore, when performing several puff applications to the same terminal, mark the pipette location (delineate the location of the tip on the image produced) before initiating the puff and return the pipette to its original location after each puff application.



**Figure 6. In vivo recording of electrical stimulation-evoked calcium signals in corneal nociceptive terminals**  
(A) Epifluorescent image of the RFP expressed by a corneal terminal and the position of the stimulating electrodes. The orange arrow indicates the representative region of interest (ROI) from which the changes of the GCaMP6s fluorescence are recorded using the RedShirt camera.  
(B) A representative trace of optical recordings of change in GCaMP6s fluorescence from the terminal at the ROI shown in A, following 100 ms application of a “rheobase” current (150  $\mu$ A).

**Optional: Electrical stimulation:** To examine the activity of the terminals’ voltage-gated channels, we have devised a way to stimulate a single terminal electrically. To that end, we apply field electrical stimulation via two pulled glass pipettes (tip diameter  $\sim 10 \mu\text{m}$ ,  $\sim 50\text{K}\Omega$ , [Figure 1](#), lower panel, see also the pipette pulling protocol, detailed in [Table 3](#)) filled with SES and an Ag/AgCl electrode, which is threaded into each pipette. The electrodes are connected to an Iso-flex battery pack and controller (AMPI, specified in the [key resources table](#)), commanded by the Digidata 1440 A/D interface (Molecular Devices, specified in the [key resources table](#)). The stimulation current is assessed by adding a known resistor to the circuit and measuring its potential with an oscilloscope. The stimulating pipettes are placed slightly above (2–5  $\mu\text{m}$ ) the corneal surface, without disturbing the cell layer, at a distance of approximately 5–10  $\mu\text{m}$  from the activated terminals, each stimulating pipette on opposing sides of a designated terminal ([Figure 6A](#)). For each terminal, a series of current stimuli are applied to determine a “rheobase” stimulus (the minimal stimulus that triggers an increase in the intra-terminal calcium). The “rheobase” stimulus is used throughout the experiment.

## EXPECTED OUTCOMES

Following the co-injection of the two viruses (a GCaMP6s expressing virus and an RFP expressing virus), it is expected to be able to see the RFP fluorescence, which delineates the structure of the primary nociceptive terminal ends and distal fibers of the mice cornea, including their bifurcations into deeper focal planes. Assuming we get a good fluorescent signal of the anatomical structures of interest (SNR of about 5–12:1, comparing the signal at the terminal compartments to the background noise), we choose ROIs and record capsaicin-induced or electrical stimulation-evoked calcium influx changes, which are inferred from the changes in the fluorescent signals of GCaMP6s ([Figures 5B](#) and [6B](#)). We expect to see an apparent deviation from the baseline fluorescence following puff applied capsaicin or electrical stimulation ([Figures 5B](#) and [6B](#)). Any deviation from the baseline fluorescence exceeding 2% of the mean baseline fluorescence is considered a response. Importantly, since only some of the corneal terminals express TRPV1 channels ([Alamri et al., 2015](#); [Belmonte et al., 2004](#); [González-González et al., 2017](#)), we expect to see a response in  $\sim 60\%$  of the examined terminals (see also [limitations](#)).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Image data collected from the RedShirt camera is further processed and analyzed using NeuroPlex 10.2.0, MATLAB software, and OriginPro 2020 (OriginLab Corporation). Using the NeuroPlex

program provided by RedShirt, the raw files of the ROI fluorescent intensities recorded are assessed. The data is further analyzed by MATLAB to obtain the changes in the fluorescence and  $I/I_0$  ( $F/F_0$ ) calculations. To that end, the fluorescent traces are normalized to baseline fluorescent intensities ( $I_0$ ), and the peak is calculated (Figures 5C and 5D, see also (Goldstein et al., 2019)). The MATLAB script we are using for the analysis is available here.

Due to possible variance in fluorescent intensities at different focal planes, compare only the values acquired at the same focal plane at the same ROI. Normalize peak fluorescent intensities to the first puff activation, and use paired statistical analysis to compare the fluorescent intensities at the same ROI before and after treatment. Since all the values are normalized to the values before the treatment, use a one-sample t-test (if the values are normally distributed) or Wilcoxon signed-rank test (for nonparametric values, Figures 5C and 5D).

### LIMITATIONS

The success rate for the expression of RFP is about 80%. Lack of success is related to reduced virus quality or could result from the incorrect injection site inferred by the mouse age/weight. RFP expression predicts the availability of GCaMP6s signals in 100% of the cases. The success rate for detecting a capsaicin-induced signal at the terminal is about 60% (Goldstein et al., 2019). It correlates with the percentage of TRPV1-expressing terminals in the cornea (Alamri et al., 2015; Belmonte et al., 2004; González-González et al., 2017).

Although the cornea is considered a transparent tissue, the attenuation of the fluorescent signals becomes more prominent with recordings from deeper layers such as sub-basal nerves and plexuses. We have determined that imaging in layers up to 50  $\mu\text{m}$  depth yields a good signal-to-noise ratio. The decrease in fluorescent intensity at deeper layers affects the recorded amplitude. Therefore, the comparison is not made between ROIs at different focal plains but rather for each ROI in different conditions (i.e., before and after drug applications), at one specific focal plane.

The terminals can degenerate throughout the experimental procedures due to dry eyes, protocols for induction of inflammation in the eyes, or even extended anesthesia time. In all these cases, the anatomical structure of the terminal is impaired, which affects calcium dynamics. It is suggested not to initiate/continue an experiment in these conditions. Due to the reasons mentioned above, it is recommended not to exceed 3 h for a single experiment in one eye.

Since the protocol is an *in-vivo* experiment under anesthesia, many aspects have to be constantly monitored and adjusted as they may affect the imaging stability. These factors include breathing rate and amplitude, which may be affected by the anesthesia level.

Our protocol measures calcium influx following TRPV1 activation. Several consecutive activations within a short period may result in impaired calcium signals due to TRPV1 desensitization. We devised a protocol to avoid this in our conditions (Goldstein et al., 2019). We suggest calibrating the minimal time between the applications, which does not decrease terminal response.

### TROUBLESHOOTING

#### Problem 1

[Step-by-step method details](#), section – viral injection, step 2.

[Step-by-step method details](#), section – calcium imaging, step 19.

The mice could show enhanced reflexes during surgery under anesthesia which may produce undesired movements during the surgery or the experiment. Importantly it indicates that animals feel pain, and therefore, it needs to be immediately resolved.

**Potential solution**

Breathing rate is visually assessed along the surgery to be around 55–85 breaths per minute to ensure a deep anesthesia level. The response to the pinch reflex is also monitored periodically. If the breathing rate changes or pinch reflex appears during the surgery, the level of isoflurane should be increased by small increments until the level of anesthesia is back to normal. If the change in breathing rate or response to pinch reflex appears during the experiment, 10–20  $\mu\text{L}$  of ketamine 10  $\mu\text{g}/\text{mL}$  should be added (by IP injection).

**Problem 2**

[Step-by-step method details](#), section – viral injection, step 5.

The mice are placed in a stereotactic frame and fixed by ear bars, after shaving but before exposing the skull. Therefore, it is difficult to identify if the skull is leveled, such that the two hemispheres are in the same plain.

**Potential solution**

Immediately after the incision (step 8), use the dental drill head attached to the stereotactic apparatus to determine height differences between the two hemispheres. The differences in the hemisphere position should not exceed 50  $\mu\text{m}$ . If the head is misaligned, loosen the ear bars and attach the head again until the difference is annulled.

**Problem 3**

[Step-by-step method details](#), section – viral injection, step 15a.

In some instances, due to the significant difference in the radius of the dental drill head compared to the pipette, the pipette’s accurate location falls on the rim or outside the drill hole.

**Potential solution**

Lift the pipette and broaden the drill hole using the dental drill. It can be done either by hand, applying very gentle pressure with the drill on the hole rims, or preferably (although more time-consuming) by replacing the pipette holder with the drill holder.

**Problem 4**

[Step-by-step method details](#), section – viral injection, step 15b.

The viral mixture may not flow out upon injection. One reason could be that the pipette has not penetrated through the trigeminal capsule. The other option could be that the pipette tip is blocked.

**Potential solution**

Option 1: Move the pipette up and down in fast increments of 100 microns (z-axis coordinates of  $-6.35$  to  $-6.45$  mm). This approach will most likely break the TG capsule, allowing the pipette penetration and subsequent injection.

Option 2: Lift the pipette. Slightly and minimally trim the pipette tip with micro scissors under the binocular. It is essential to recalibrate the z-axis as the pipette has been shortened.

**Problem 5**

[Step-by-step method details](#), section – calcium imaging, step 25.

While lowering the puff pipette towards the selected terminal, the pipette might touch the epithelial layer. The pipette interrupting the epithelial layer injures the epithelial cells and leads to the activation of the terminals of nociceptive neurons.

### Potential solution

Slowly remove and replace the pipette. Exclude all the terminals in 100  $\mu\text{m}$  radius from the experiment, and experiment on a different terminal located far away (at least 100  $\mu\text{m}$ ) from the initial terminal.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alexander M Binshtok, [alexander.binshtok@mail.huji.ac.il](mailto:alexander.binshtok@mail.huji.ac.il)

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

The 3D printer design for the eye stabilizer and bath has been deposited at the Mendeley data repository and is publically available here: Mendeley Data: <https://data.mendeley.com/datasets/wzfn6czyz9/1>

The MATLAB code for data analysis has been deposited at the Mendeley data repository and is publically available here: Mendeley Data: <https://data.mendeley.com/datasets/66j6dymfxs/1>

All the raw data or any additional information are available on request from the lead contact.

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### AUTHOR CONTRIBUTIONS

Conceptualization, R.N.G., D.G., and A.M.B.; methodology, R.N.G., D.G., L.A., S.L., and A.M.B.; investigation, R.N.G., D.G., L.A., S.L., and A.M.B.; writing – original draft, D.G., S.L., and A.M.B.; writing – review and editing, D.G., R.N.G., S.L., and A.M.B.; funding acquisition, A.M.B.; project administration, A.M.B.; supervision, A.M.B.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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