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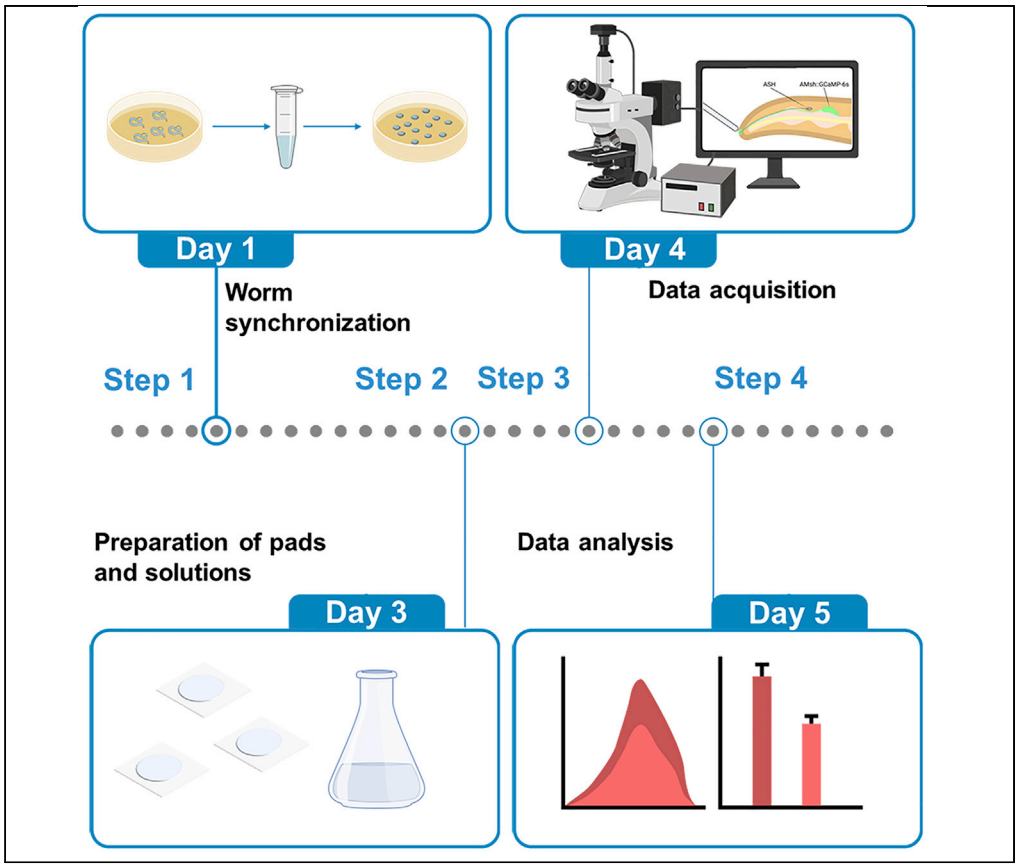
Protocol

A protocol for imaging calcium and chloride in *C. elegans* glia upon touch stimulation



Glia are important for the function of touch receptors. Here, we present a protocol in *Caenorhabditis elegans* for calcium and chloride imaging in worm glia upon nose touch stimulation. We describe aspects of the procedure that are essential for data reproducibility, including worm immobilization, poking angle, and applied force. We then detail data processing and analysis of calcium and chloride transients in glia. This protocol can be used for other types of mechanical stimulations or for stimulation using odorants.

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Highlights

Fluorescence imaging in *C. elegans* upon nose touch stimulation

Gluing worms for *in vivo* recordings

Processing and analysis of calcium and chloride transients in glia

Useful for other types of mechanical stimulations or for stimulation using odorants

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Protocol

A protocol for imaging calcium and chloride in *C. elegans* glia upon touch stimulation

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SUMMARY

Glia are important for the function of touch receptors. Here, we present a protocol in *Caenorhabditis elegans* for calcium and chloride imaging in worm glia upon nose touch stimulation. We describe aspects of the procedure that are essential for data reproducibility, including worm immobilization, poking angle, and applied force. We then detail data processing and analysis of calcium and chloride transients in glia. This protocol can be used for other types of mechanical stimulations or for stimulation using odorants.

For complete details on the use and execution of this protocol, please refer to Fernandez-Abascal et al., 2022.

BEFORE YOU BEGIN

The protocol described here was used to record changes in intracellular calcium and chloride in the Amphid sheath (AMsh) glia upon nose touch stimulation. However, it can also be used to record calcium changes, and potentially also chloride changes, in ASH polymodal nociceptors upon nose touch stimulation (Fernandez-Abascal et al., 2022). The nematode *Caenorhabditis elegans* is maintained at 20°C on Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* (strain OP50) as food source (Brenner, 1974).

Preparation of NGM agar plates

Timing: 3 days

1. For 1 L (100 plates) of NGM (see table in materials and equipment), dissolve 2.5 g of bacto peptone, 3 g of NaCl, 17 g of agar, and 0.2 g of streptomycin in 975 mL of double distilled water (ddH_2O). You can dissolve the mix using a magnetic stir bar and a stirrer.

Note: Use an Erlenmeyer flask with a volume capacity double of your final volume.

2. Cover the flask with aluminum foil, secured with autoclaving tape, and autoclave for 30 min using the liquid cycle.
3. Air-cool the flask to 55°C while stirring. Keep the foil on the flask to prevent contamination.

Note: A hotplate magnetic stirrer set at 55°C can be used to ensure the maintenance of the right temperature.

Alternatives: The flask can be water cooled with gentle stirring for 10–15 mins to achieve the desired temperature.

Alternatives: Stir for 30 min and monitor the temperature with an infrared thermometer.

4. Add 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of (5 mg/mL) cholesterol (in 95% ethanol), 1.25 mL of (10 mg/mL) nystatin (in 70% ethanol), and 25 mL of 0.5 M KP buffer (pH 6.0, see table in materials and equipment) and mix well.

⚠ CRITICAL: Working in aseptic conditions (near a Bunsen burner flame or under the hood) is important to ensure sterility of the media. All stock solutions are sterilized by autoclaving except for cholesterol and nystatin, which are dissolved in ethanol.

5. Pour the NGM medium into petri dishes using a sterile plastic pipette (10 mL in 60 mm dishes, 4 mL in 35 mm dishes).

Note: 100 mm petri dishes can be used to grow larger number of worms. Pour 25 mL of NGM medium in these dishes.

Note: Purchase non-coated Petri dishes. Coating that facilitates cell attachment is not needed for this application.

Note: To avoid bubbles in the poured plates, aspirate 2–3 extra mL of NGM medium so when pouring the desired amount, there will always be a remaining 2–3 mL in the tip of the pipette.

6. Leave the NGM plates at room temperature (~22°C) for at least 24 h to allow enough drying and evaporation of excess moisture.

Note: Do not leave the plates too long at room temperature, otherwise the plates will over-dry.

7. Stack NGM plates and place them upside-down in an air-tight container to prevent steam condensation on the lid and store at 4°C. Plates stored at this temperature last about 1 month.

Food source preparation

Timing: 3 days

8. Pick one isolated streptomycin resistant colony of OP50 *E. coli* from a Luria Broth (LB) agar plate (see table in materials and equipment) and inoculate it into 25 mL of LB liquid medium (see table in materials and equipment). Incubate at 37°C for 12–14 h in a shaker incubator. Verify growth of the bacteria by visual inspection: the LB medium should look cloudy.
9. Working under sterile conditions, add ~100 µL of the liquid cultured bacteria in the center of a 60 mm NGM plate and spread to create a larger lawn.

Note: Do not spread the bacteria throughout the entire surface of the plate. Keep the bacterial lawn in the center of the plate to facilitate the picking of the worms.

Alternatives: Pour 10 mL of liquid cultured OP50 *E. coli* in a sterile 60 mm petri dish and dip a sterilized metal spreader in it, then spread the bacteria in the NGM plates. Sterilize the spreader every 6 plates by dipping it in ethanol and carefully flaming it. Wait 10 s after the flame is gone to dip again into the liquid bacteria.

10. Let the bacterial lawn grow and dry for 12–16 h at room temperature (~22°C).

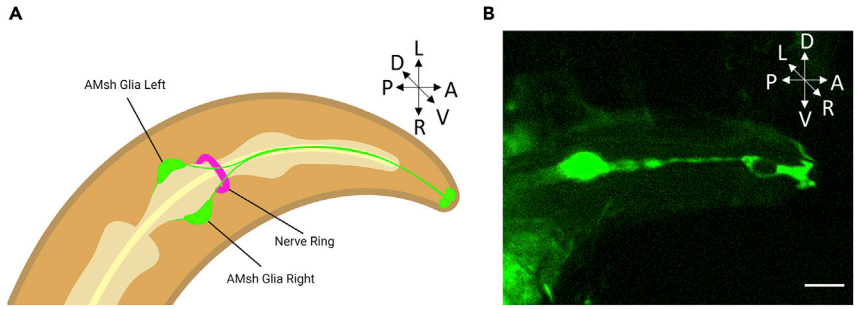

Figure 1. The Amphid sheath glial cells of *C. elegans*

(A) Drawing depicting the left and right AMsh glial cells (green) on each side of the pharyngeal bulb. In purple a schematic of the nerve ring. Image represented from a dorsal point of view.

(B) A transgenic worm expressing GCaMP-6s in AMsh glia under the control of the glial promoter *T02B11.3*. Image represented from the worm's right side. Scale bar is 20 μm. Coordinates are represented in both panels for better orientation (A: Anterior; P: Posterior; D: Dorsal; V: ventral; R: Right; L: Left).

11. Store the plates at 4°C for up to 1 month.

Note: The seeded bacteria can also be grown at 37°C for 6–8 h, if needed.

Worm synchronization

Timing: 3 days

A pair of AMsh glial cells is in the head of the worm, on the dorsolateral sides of the terminal bulb of the pharynx and posterior to the nerve ring (Figure 1A). Each cell projects a process towards the tip of the nose where they enwrap the dendrites of the sensory neurons (Ward et al., 1975). To image calcium in the AMsh glia, we used the strains BLC402, expressing GCaMP-6s under the control of the glial promoter *T02B11.3*, (Figure 1B) and BLC498, expressing the chloride sensor Superclomeleon under the control of the same promoter (Fernandez-Abascal et al., 2022). The strain BLC402 also expresses the co-injection marker *Punc-122::GFP*. *unc-122* is expressed in the seam cells which are located away from the head of the worm (Loria et al., 2004). Thus *Punc-122::GFP* fluorescent signal does not interfere with GCaMP-6s imaging in the AMsh glia.

Alternatives: Other types of genetically encoded calcium indicators, including FRET-based ones, may be used (Perez Koldenkova and Nagai, 2013). Depending on the sensor, the microscope optical filters and the recording conditions may have to be adjusted.

12. Rinse a plate full of healthy gravid adults with 2 mL of M9 (see table in materials and equipment) and collect the liquid containing the worms into a 15 mL tube.
13. Spin the tube at 4,000–5,000 rpm for 5 min.
14. Aspirate and discard the supernatant.

Note: You can use a disposable Pasteur pipet to discard most of the supernatant. When few hundred microliters are left, use a micropipette to discard the remaining liquid. This also applies to eggs in steps 19 and 22.

⚠ CRITICAL: Pelleted worms detach easily from the bottom of the tube when aspirating the supernatant. So, it is important to do this carefully. You can monitor this step under a stereo microscope for better results.

15. Resuspend the pellet in 400 μL of hypochlorite solution (see table in materials and equipment).

16. Monitor 10 μL under the microscope to observe worms' breakage and egg release.

Note: Stop the reaction when 85%–90% of the worms have released their eggs (5–10 min from the inoculation of the bleach solution) (see next step).

Note: Carefully flick the tube every few minutes to ensure that the worms are evenly exposed to the bleaching solution.

⚠ CRITICAL: To ensure effectiveness of the bleaching solution use fresh NaOH solution and fresh bleach. The NaOH solution should be kept for less than 1 month. Use fresh commercially available standard bleach with no added fragrances. Purchase small bottles so that a new bottle can be opened and kept for only a week.

17. Stop the reaction by adding 14 mL of M9 solution.
18. Spin at 4,000–5,000 rpm for 5 min.
19. Discard supernatant. See note of step 14.
20. Add another 14 mL of M9 solution to the pellet and resuspend the eggs by pipetting.
21. Spin at 4,000–5,000 rpm for 5 min.
22. Discard supernatant. See note of step 14.
23. Resuspend in 100 μL.
24. Take 10 μL and count the number of eggs under the microscope to calculate the approximate concentration of eggs.
25. Seed as many microliters as needed to have 200 animals per plate.

⚠ CRITICAL: More than 200 eggs/plate can be seeded if needed. However, do not seed more than 400 eggs/plate to avoid crowding and consequent starvation.

Alternatives: The worm synchronization step can be replaced by picking L4 fluorescent worms from a non-synchronized plate the day before the data acquisition. The following day, the researcher will have only fluorescent Day 1 adults in the same plate, which will facilitate worm picking during image acquisition.

Imaging chamber

Timing: 1 day

An imaging chamber to hold the cover glass coated with the agarose pad is needed for this protocol (Figure 2 and Methods video S1). This chamber keeps the pad and the worm submerged in the imaging buffer (see table in materials and equipment) during the recording. The chamber is 25 × 75 mm with a smaller indented chamber on the bottom measuring 61 × 22 mm, to which a coverslip glass is glued using silicone to serve as the bottom wall of the chamber (Figures 2A and 2C). On the top side, the chamber is smaller (19 × 19 mm) and has two small indentations that allow the use of forceps to remove the glass coated with the agarose pad (Figures 2B and 2D). A 3D model of the chamber can be downloaded (Data S1). The chamber is reusable and can be printed in multiple copies by any 3D printer at a very low cost. This study used a chamber printed with Polylactic acid (PLA plastic) which is commonly used in 3D printing.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli	CGC	Strain OP50

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Peptone	BD Biosciences	Cat#211677
Sodium Chloride	Sigma-Aldrich	Cat#S7653
Agar	bioPLUS	Cat#30620001
Streptomycin	Sigma-Aldrich	Cat#S9137
Calcium dichloride	Sigma-Aldrich	Cat#223506
Magnesium sulfate	Sigma-Aldrich	Cat#230391
Cholesterol	Sigma-Aldrich	Cat#C8503
Nystatin	Sigma-Aldrich	Cat#N3503
Monopotassium phosphate	Fisher Scientific	Cat#BP362
Dipotassium phosphate	Fisher Scientific	Cat#P290
Tryptone	BD Biosciences	Cat#211705
Yeast extract	BD Biosciences	Cat#212750
Disodium phosphate	Sigma-Aldrich	Cat#S7907
Sodium hydroxide	Sigma-Aldrich	Cat#S8045
Sodium hypochlorite	CLOROX	Cat#41H893
Gluture	Fisher Scientific	Cat#NC0632797
Agarose	Denville Scientific	Cat#GR140-500
Potassium chloride	Sigma-Aldrich	Cat#P9541
D-Glucose	Sigma-Aldrich	Cat#G5767
HEPES	Sigma-Aldrich	Cat#H3375
Experimental models: Organisms/strains		
C. elegans: BLC401: blcEx446[Psra-6::GCaMP-6s; Punc-122::GFP]. Day 1 adults, hermaphrodites.	(Fernandez-Abascal et al., 2022)	N/A
C. elegans: BLC498: blcEx481[pT02B11.3::Superclomeleon]. Day 1 adults, hermaphrodites.	(Fernandez-Abascal et al., 2022)	N/A
Software and algorithms		
Fiji (ImageJ)	NIH (Schindelin et al., 2012)	RRID:SCR_002285 http://fiji.sc
Micro-manager	Vale Lab, UCSF	https://micro-manager.org/ RRID:SCR_000415
PIMikroMove	Physik Instrumente	https://www.physikinstrumente.com/en/products/motion-control-software/
Clampfit	Molecular Devices	RRID:SCR_011323 http://www.moleculardevices.com/products/software/pclamp.html
GraphPad Prism	GraphPad software	RRID:SCR_002798 http://www.graphpad.com/
Biorender	BioRender	RRID:SCR_018361 http://biorender.com
Microsoft Excel (Office 365)	Microsoft Corporation	RRID:SCR_016137 https://office.microsoft.com/excel
Other		
Olympus Inverted Microscope	Olympus	Model IX70
Microscope camera	PCO	Model SensiCam
Fluorescence illumination system	Sutter Instrument Co.	Model Lambda DG-4
YFP Excitation Filter	Semrock	Part# FF01-500/24-25
CFP Excitation Filter	Semrock	Part# FF02-438/24
CFP Emission Filter	Semrock	Part# FF01-483/32
YFP Emission Filter	Semrock	Part# FF01-542/27
Borosilicate capillary glass	Warner Instruments	Cat#G150F-6
Fire polisher – Microforge	Narishige	Cat#MF-830
Micropipette puller	Sutter Instrument Co.	Cat#P-1000
Mercury Servo Controller	Physik Instrumente	Cat#C-863
Zeiss dissecting stereomicroscope	Zeiss	Model Stemi 305

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
60 mm Petri Dish	NEST Scientific	Cat#754001
100 mm Petri Dish	NEST Scientific	Cat#753001
150 mm Petri Dish	Falcon	Cat#1058
18 × 18 mm Cover Glass	Baxter	Cat#M6045-1
22 × 22 mm Cover Glass No.1	Thermo Scientific	Cat#3306
Supplemental information	Mendeley	Mendeley data: https://doi.org/10.17632/tpvptsck7r.1

MATERIALS AND EQUIPMENT

NGM Agar plates		
Reagent	Final concentration	Amount
NaCl	51.33 M	3 g
Agar	50.55 M	17 g
Peptone	10.23 M	2.5 g
Streptomycin	343.9 mM	0.2 g
ddH_2O		up to 975 mL
Autoclave. Stir until the temperature cools to 55°C. Then add the following ingredients:		

Reagent	Final concentration	Amount
$CaCl_2$ (1 M)	1 mM	1 mL
$MgSO_4$ (1 M)	1 mM	1 mL
Cholesterol (5 mg/mL in 95% ethanol)	12.9 μM	1 mL
KP Buffer	12.5 mM	25 mL
Nystatin (10 mg/mL in 70% ethanol)	13.5 mM	1.25 mL
Pour 10 mL into 60 mm dishes and leave it at room temperature for 24 h. Storage at 4°C for up to 2 months.		

KP Buffer		
Reagent	Final concentration	Amount
KH_2PO_4	441 mM	30 g
K_2HPO_4	57.4 mM	5 g
ddH_2O		up to 0.5 L
Adjust to pH 6.0 and autoclave. Store at 4°C for up to 2 months.		

LB Agar plates		
Reagent	Final concentration	Amount
Tryptone	140.7 mM	5 g
Yeast extract	15.7 mM	2.5 g
NaCl	171.1 mM	5 g
Agar	44.6 mM	7.5 g
ddH_2O		up to 0.5 L
Autoclave. Cool to 55°C and pour it in 90 mm petri dishes. Store at 4°C for up to 1 month.		

Liquid LB medium		
Reagent	Final concentration	Amount
Tryptone	140.7 mM	5 g
Yeast extract	15.7 mM	2.5 g
NaCl	171.1 mM	5 g
ddH_2O		up to 0.5 L
Autoclave and store at 4°C for up to 1 month.		

M9 buffer		
Reagent	Final concentration	Amount
KH_2PO_4	22.1 mM	3 g
Na_2HPO_4	42.3 mM	6 g
NaCl	85.6 mM	5 g
ddH_2O		up to 1 L
Autoclave and cool to 55°C. Add 1 mL of sterilized (1 M) $MgSO_4$. Store at 4°C for up to 2 months.		

Hypochlorite solution		
Reagent	Final concentration	Amount
NaOCl (commercial bleach)	20.4 mM	200 µL
NaOH (10 M)	8.2 mM	80 µL
ddH_2O		700 µL
Make fresh every use. The NaOH stock solution (10 M) should not be more than 3 months old.		

Equipment

Imaging buffer		
Reagent	Final concentration	Amount
NaCl	145 mM	29 mL (1 M)
KCl	5 mM	1 mL (1 M)
$CaCl_2$	1 mM	200 µL (1 M)
$MgCl_2$	5 mM	1 mL (1 M)
D-Glucose	20 mM	721 mg
HEPES	10 mM	477 mg
ddH_2O		Up to 200 mL
Adjust to pH 7.2 and filter the solution with a bottle top filter (0.22 µm). Store at 4°C for up to two weeks.		

- An Olympus IX70 microscope with a X10 objective and a PCO SensiCam camera was used in this study. Additional equipment was added to the microscope:
  - A Lambda DG-4 illumination system equipped with the excitation filters listed in the key resources table.
  - To acquire specific emission wavelengths, the microscope was equipped with a Lambda 10-2 optical filter changer. The emission filters listed in the key resources table were installed.
  - To control the probe for nose touch stimulation, the C-863 Mercury Servo Controller was installed on a platform adjacent to the objective.
- A dissecting Zeiss Stemi 305 stereomicroscope with standard 10× eyepieces and objectives which range from 0.8× to 4× is also used to glue the worms on the agarose pads.

STEP-BY-STEP METHOD DETAILS

Preparation of the probes for mechanical stimulation

Timing: 30 min

This step describes how to prepare probes for nose touch stimulation.

1. Place a 15 cm length borosilicate capillary glass in the Sutter P-1000 micropipette puller to fabricate two needles using the following settings:

Heat	Pull	Vel	Time	Pressure	Ramp
874	5	50	200	300	892

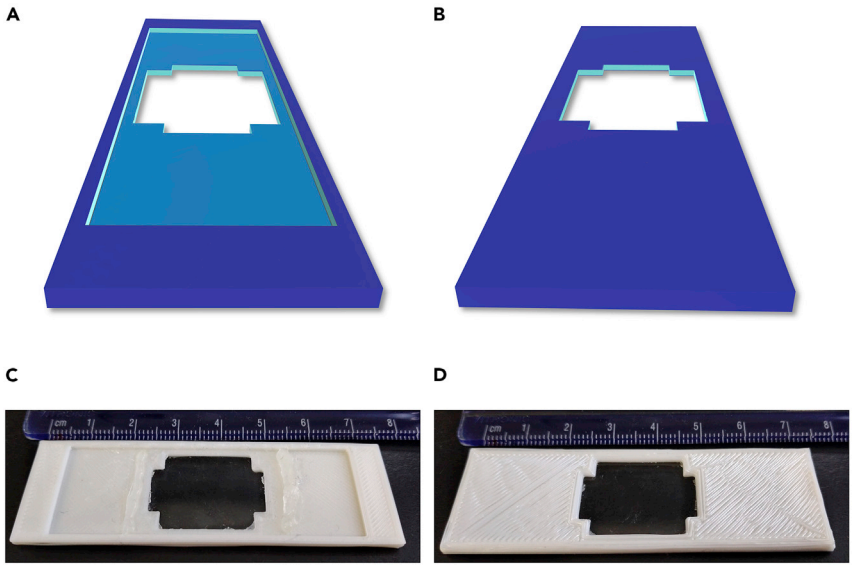

Figure 2. The imaging recording chamber

(A and B) Drawings depicting the recording chamber from the bottom (A) and from the top (B). A glass coverslip is glued onto the bottom of the chamber using silicon. The light blue in A represents the indented chamber of the size of 61 × 22 mm that houses the glass coverslip.

(C and D) Pictures of the 3D printed imaging chamber next to a ruler to show the size. C and D are the bottom and top view respectively. In C the glued coverslip is visible.

Note: The exact settings on the Sutter P-1000 are determined by the heating filament. Follow the manufacturer's instructions to test the filament and to establish the exact settings. The "heat" parameter is the one that most likely will require the most adjustment.

Note: Other types of pullers can be used. The goal is to fabricate needles with a long tip, like the ones used for injections in *C. elegans* or *Xenopus* oocytes.

Note: A borosilicate glass (OD=1.50 mm and ID=0.86 mm) with standard wall with filament was used in this study. Other borosilicate glasses can be used but they will need to be tested under the same experimental conditions first.

Note: Pull 8–10 needles as they are easy to break during the manipulation steps.

2. Using the fire polisher, heat the tip of the needles to melt it into the shape of a bulb of the size of 10–20 μm (Figure 3).

Note: This shape of the probe works well for nose touch stimulation. Other shapes may be used for touch stimulation to other parts of the *C. elegans*' body (Suzuki et al., 2003; O'Hagan et al., 2005; Kindt et al., 2007; Kang et al., 2010; Geffeney et al., 2011; Han et al., 2013).

Preparation of the agarose pads

Timing: 30 min

This step describes the process of making 2% agarose pads on which worms are glued.

3. Add 1 g agarose to 50 mL of imaging buffer (2% w/v) and heat the mix in a microwave until fully dissolved.

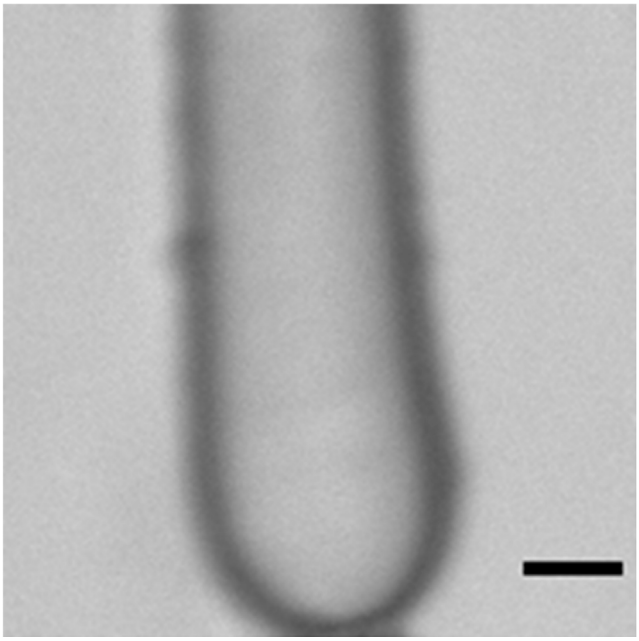

Figure 3. Photograph of the tip of a glass needle used as probe to mechanically stimulate the nose of the worm
Scale bar is 10 μm.

Note: You can leave the heated agarose at room temperature for 2–3 min while you prepare the coverslips to allow some cooling. Cooler agarose will make slightly thicker pads that will stay moist for longer.

Note: The flask in which the agarose was heated can be sealed with parafilm and stored at 4°C for up to a month. When reheating the agarose, use reduced microwave power to avoid water evaporation and consequently agar concentration. Limit to 2–3 reuses of the same agarose.

4. Place ~25 18 × 18 mm cover glass on a flat surface and with a disposable Pasteur pipette add one drop (~100 μL) of 2% agarose on the center of one slide. Immediately after, while the agarose is still in liquid form, take a 22 × 22 mm cover glass and gently lay it down on top of the agarose drop. The weight of the top cover glass will be enough to create a thin pad. Repeat with the next cover glass until finishing the 25 slides (Figure 4).

   Note: Using two different sizes of cover slides will facilitate the separation of the two glasses in the next step.

5. With the help of a razorblade, carefully separate the bigger cover glass from the smaller one, making sure the pad remains on the small glass.

   Note: By the time you are done with step 4, the glass cover slides are ready to be separated.

   Note: Sometimes the pad remains on the larger cover glass. If that happens, drag the smaller one on top of the pad in small circles until it is separated from the larger glass.

6. Store the pads in a 150 mm petri dish and use them immediately.

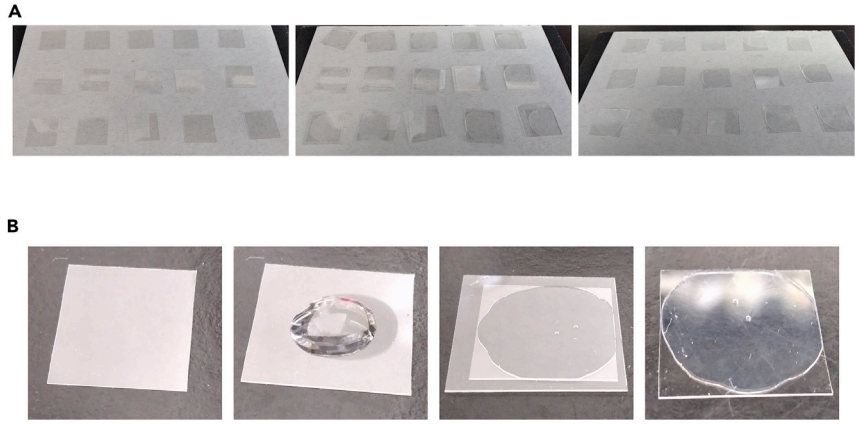

Figure 4. Preparation of the agarose pads

(A) 18 × 18 mm glass coverslips are laid out on a clean piece of printer paper (left), a drop of 2% agarose is then placed in the center of each coverslip and 22 × 22 mm coverslips are placed on the agarose drops (middle), finally, the top larger coverslips are removed (right).

(B) Closeup of the procedure described in (A) for one coverslip.

Note: A wet wipe tissue can be placed in the middle of the dish (not in contact with the pads) to keep the pads moist. Store the pads in the dish containing the wet wipe tissue at 4°C for up to a week.

⚠ CRITICAL: The pads can easily dehydrate depending on the storage conditions. Always check for dryness before using. Make them fresh every 2–3 days for better performance.

Gluing worms

Timing: 2 min

This step describes the process of gluing worms onto agarose pads so they can be immobilized for imaging. See also Methods video S2.

7. With an eyelash, pick a fluorescent young adult and place it on the agarose pad.

Note: To facilitate worm picking, the day before the recordings you can pick several fluorescent L4 onto a fresh plate. Alternatively, you can pick fluorescent Day 1 adults into a new plate before the recordings.

⚠ CRITICAL: Pick and transfer the worms very gently to avoid stimulation of the AMsh glia.

8. Carefully dip the tip of a probe like the ones used for nose touch stimulation into the glue (Gluture) and place a drop of glue on the pad, close to the tail of the worm.
9. With the probe still covered in glue, touch the tail of the worm so it is glued onto the agarose pad, and it stops moving forward.
10. Dip the probe in the drop of glue and start gluing one of the sides of the worm until the glue has reached the tip of the nose but it has not covered it (Figure 5).

Note: Under our conditions, the posture of the worm when it is glued is not relevant to the outcome of the recordings.

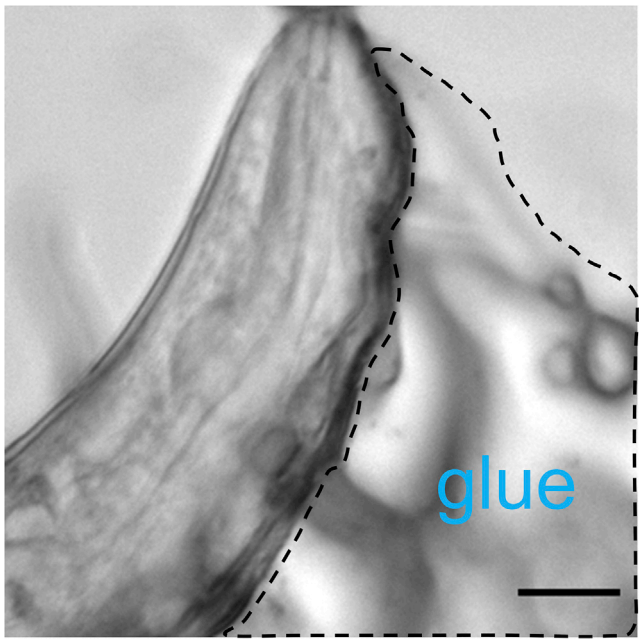

Figure 5. Glued worm head
Close up of the head of a worm glued onto an agarose pad. The dotted line indicates the borders of the glue. Note that the glue does not cover the tip of the nose. Scale bar is 20 μm.

⚠ CRITICAL: Gluing worms is the most important step in this protocol as the tip of the nose must remain exposed (not glued).

⚠ CRITICAL: Placing small amounts of liquid glue close to the posterior part of the worm will allow the glue to travel to the anterior part of the body by capillarity. This ensures better gluing without too much mechanical stimulation of the body of the animal. If the drop of glue is too big, it may move too fast to the nose and cover it.

Note: Gluing worms requires a lot of practice but, once the skills are acquired, gluing a worm should take only 20–30 s.

11. Place the cover glass containing the pad with the glued worm in the calcium imaging chamber.
12. Fill the chamber with imaging solution.

⚠ CRITICAL: Sometimes air bubbles get stuck onto the nose of the worm because air gets trapped under the glue. It is easy to remove the bubbles using the probe once the worm is placed under the microscope. This requires a careful manipulation of the probe (it is recommended to use the probe manipulator) to avoid stimulating the nose of the worm, otherwise it will affect subsequent experiments.

Image acquisition

Timing: 2 min

This step describes the process of image acquisition at baseline and during nose touch stimulation.

13. Open micromanager or another software that controls the microscope.
14. Open PIMikroMove or another software that controls the movement of the probe.

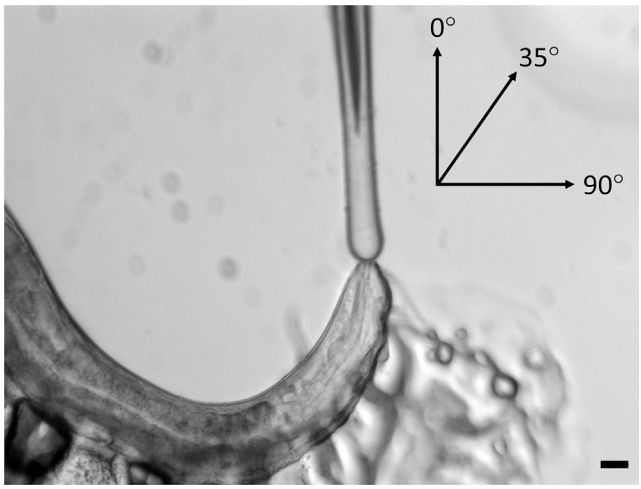

Figure 6. Position of the probe for nose touch stimulation

The probe is placed close to the nose of a glued worm, it is retracted 150 μm for 30 s, and then pushed forward 200 μm. Finally, the probe is retracted 200 μm. AMsh glia are best stimulated by a probe placed straight on the nose of the worm like shown in this picture. Scale bar is 20 μm.

15. Using bright field illumination and a low magnification objective, find the worm. Position it so the tip of the nose faces the probe at the desired angle. Focus on the tip of the nose.

Note: The angle of the probe with respect to the nose of the worm is very important to achieve the correct stimulation and may vary depending on the cell of interest. In our experience, ASH neurons respond to a wide range of angles of stimulations, from 0° to up to a 90° angle (perpendicular to the nose). On the contrary, AMsh glia respond to stimulations from 0° to up to a 35° angle (Figure 6).

16. Move the probe close to the tip of the nose and adjust the angle of action to achieve the best results (Figure 6).

⚠ CRITICAL: Setting the Z axis for the probe position is essential to get a good activation of the cell of interest. If the position of Z axis is too high, then the probe will end up brushing the top of the worm body without directly stimulating the nose. If the Z axis position is too low, then the probe will brush the agarose pad, thus decreasing the force applied to the nose. Using the two black parallel lines of the mouth of the worms as a point of reference is helpful.

17. Adjust the focus on the computer view and position the probe as close as possible to the nose of the worm without touching it.
18. Choose your emission and excitation filters. For GCaMP-6s in AMsh glia, we used the YFP excitation filter and no emission filters since there is not overlap with any other fluorophore. For SuperClomeleon, we used the CFP excitation filter and the CFP and YFP emission filters to record CFP and YFP fluorescence separately.
19. Switch to fluorescence and focus again on your cell of interest. Adjust the exposure time that better suits your experiment.

Note: For AMsh glial cells expressing GCaMP-6s we used 50 ms exposure time. For chloride imaging using animals expressing SuperClomeleon in AMsh, we used 20 ms. In our experience though, smaller cells such as ASH neurons require longer exposure time (100 ms). These

settings may vary depending on the power of your imaging set up, the expression level of your fluorophore, and the size of the cell of interest.

20. Start the recording and the mechanical stimulation protocol (see code below for PIMikroMove software). The protocol that was used in this study consisted in retracting the probe 150 μm for 30 s and then pushing it forward 200 μm. Finally, the probe is retracted 200 μm, ready for subsequent touch stimulations.

```
MVR A 0.15
WAC ONT? A = 1
DEL 30000
MVR A -0.200
WAC ONT? A = 1
DEL 0
MVR A 0.200
```

Note: The length of the recording may vary depending on the type of experiment. For longer recordings, it is recommended that the frequency of acquisition is reduced to limit photobleaching, unless short transient changes in intracellular calcium or chloride are expected. For AMsh glia we acquired at a frequency of 1 and 2 Hz (1 and 2 frames per second) for SuperClomeleon and GCaMP-6s recordings respectively.

Note: The time the probe spends on the nose will be important depending on the type of cells that the researcher is stimulating, as they have different sensitivities. For ASH and AMsh it is enough with just the touch and 0 s spent during the indentation. Other cells may need to re-adjust this time as they will need a constant stimulation for them to get activated.

⚠ CRITICAL: Since the movement of the probe and the image recording are controlled by two separate software, it is important to synchronize the initiation of the two programs. Set the software buttons always at the same distance. This will facilitate the start of both programs virtually at the same time and will also ensure that touch is delivered always on the same frame number from the start. However, this must be double checked during data analysis.

⚠ CRITICAL: The protocol used for touch stimulation will determine the force used to stimulate the nose of the worm. To calculate the force, use the following Equation 1:

$$F = m \cdot a$$

Where m is the mass of the probe. In this study, the probe weighted 145 g. The weight included the glass probe and probe holder. The acceleration (a) is calculated using Equation 2:

$$V_f = V_0 + a \cdot t$$

In this study the velocity of the probe was 300 μm/s, the time that the probe took to travel the 150 μm distance was 0.5 s and the velocity of the probe at time 0 (V_0) was 0 μm/s. Thus:

$$a = \frac{V_f - V_0}{t} = \frac{300\ \mu m/s}{0.5\ s} = 600 \mu m \Big/ s^2$$

If we apply the calculated acceleration to Equation 1, then:

$$F = 145\ g \cdot 600 \mu m/s^2 = 87\ \mu N$$

Using the code for nose touch stimulation shown above and modifying the mass of the probe, speed of the probe, and distance, one can adjust the force applied.

21. Once the recording is finished, save it, and start the following recording if you want to record the response to a second touch stimulation (steps 13–17). Alternatively, you can discard the current pad, clean the chamber with some imaging buffer, and start with a new worm (step 7).

Note: The repetition of the recordings in the same animal may vary depending on the type of stimulation, the cell of interest and the imaging equipment. For ASH and AMsh, recordings of 3–4 consecutive stimulations can be achieved if the worm is well glued and healthy. However, due to the length of the recordings, consecutive stimulations should require the implementation of additional steps to avoid starvation or structural damages.

EXPECTED OUTCOMES

The imaging of AMsh glia shows that touch stimulation induces a slow and transient increase in intracellular calcium that travels from the tip of the nose to the cell body (30 s from nose touch stimulation to peak in the cell body) (Methods video S3) (Fernandez-Abascal et al., 2022). A second touch stimulation, delivered 2 min after the first touch stimulation, elicits little to no change in intracellular calcium. In chloride imaging experiments, a steady increase in chloride concentration in the cell body of the AMsh glia is observed upon first touch stimulation. This change in intracellular chloride does not recover to the pre-stimulation level during the recording. During the second touch stimulation, in wild type worms there is a slow decrease in intracellular chloride that also does not recover to pre-stimulus levels (Fernandez-Abascal et al., 2022). While the changes in intracellular calcium can be readily observed during the recording as they are proportional to the GCaMP-6s fluorescence, changes in intracellular chloride are not easily seen in real time. This is because they are proportional to the YFP/CFP ratio. Whereas intracellular calcium changes upon touch stimulation are normally transient, less is known about how intracellular chloride changes upon touch stimulation or stimulation by other types of sensory cues.

QUANTIFICATION AND STATISTICAL ANALYSIS

The analysis of the imaging files follows a 3-steps process:

1. Raw data acquisition from the file.
   a. Calcium imaging (Data S2).
      i. Open the file using Fiji (ImageJ) software.
      ii. Open the ROI manager in Analyze/Tools/ROI manager.
      iii. Select the region of interest (ROI) in the video: the cell body of the AMsh glia (Figure 7).

Note: It is important to be familiar with the shape of your cell of interest, and to select the ROI as close as possible to the borders of the cell to obtain accurate results. Avoid selecting the fluorescent area surrounding the cell (Figure 7).

Note: Sometimes it is helpful to adjust the brightness and contrast of the video to facilitate the visualization of the cell. It is important to reset it to default values once the ROI is selected.

      iv. Add the ROI to the ROI manager.

v. Correct the bleaching of the fluorophore by running the bleach correction tool at Image J/Adjust/Bleach correction. We use the exponential fit as correction method (Figures 8A and 8B).

Note: Sometimes the video is not corrected because there is minimum to no fluorescence decay.

vi. With the corrected resulting image, go to ROI manager and run the multi measure tool under the "more>>" tab.

Note: If a background subtraction is going to be applied during the data processing, a ROI close to the cell body must be selected in the raw video. Data from this ROI can be then extracted as described above (Figure 8C).

vii. The results page that pops up can be copied onto an excel file (Data S2). Among other default parameters, it will contain the mean fluorescence of the ROI for each frame of the video.

viii. To quickly visualize your raw data, select the mean fluorescence column and plot a dispersion graph (Figures 8A and 8B).

b. Chloride imaging (Data S3).
   i. Open the file with Fiji (ImageJ) software.
   ii. Split channels at Image/Color/Split channels.
   iii. Perform steps ii-viii as described above (Figures 9A, 9B, and 9D–9F).

⚠ CRITICAL: For YFP/CFP fluorophores, it is important to perform a background subtraction. Changes in fluorescence will be more readily visible after data processing.

Note: CFP fluorescence does not photobleach in our recordings; however, YFP fluorescence bleaches over time and should be corrected as explained in point 1.a.v of calcium imaging (Figures 9D and 9E).

2. Data processing.
   a. Determine within the original video, at what frame did the probe touch the nose of the worm.
   b. The given frame will allow to establish the frames within the recording that will be used as baseline (10 s before the touch).

Note: The number of frames to be used for the 10 s baseline will depend on the recording frequency. For calcium imaging, in this study we imaged at a frequency of 2 Hz. Thus the 10 s baseline will consist of 20 frames. For chloride imaging, in this study 1 frame per channel every 1 s was recorded. Thus, the baseline will consist of 10 frames.

⚠ CRITICAL: The calculation of the 10 s before touch is important because it is going to set the baseline of your analysis. Any mistake in this step will cause incorrect data analysis, data distortion, and misinterpretation.

   c. Calcium imaging (Data S2) (Figures 8D–8G).
      i. In excel, calculate the average of the 10 s before touch. This will constitute your F_0.
      ii. Per each frame, apply the following formula, where F is the mean fluorescence of each frame:

$$\Delta F \Big/ F_0 = \left(\frac{(F - F_0)}{F_0}\right) \cdot 100$$

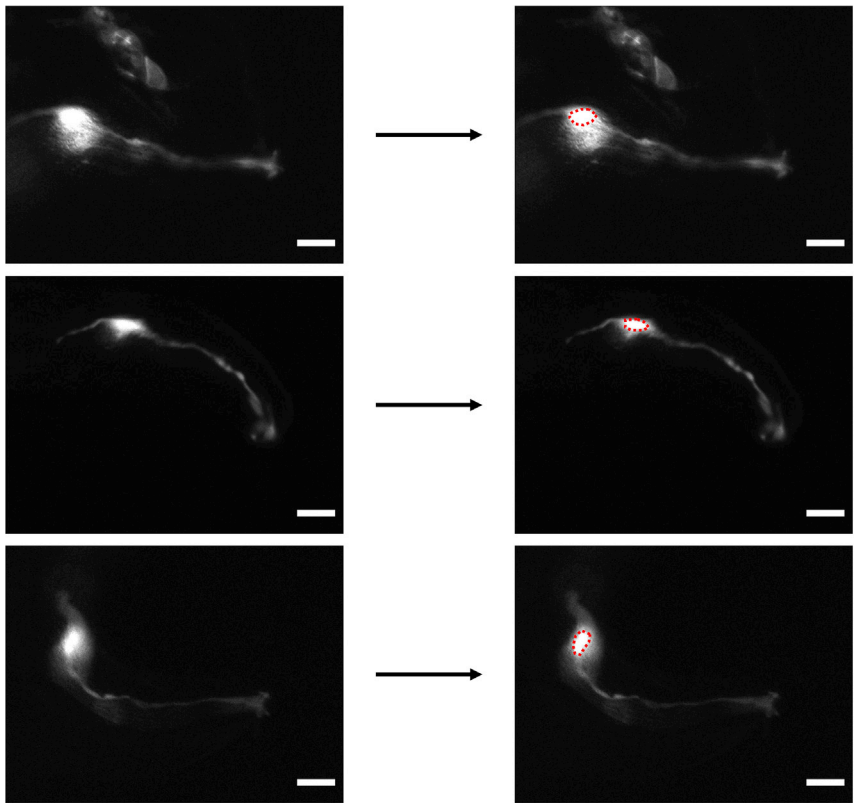

Figure 7. ROI selection

Example of 3 raw images showing the AMsh expressing GCaMP-6s and the ROI selection (red dashed lines) of the cell body. Scale bar is 20 μm.

Note: If a background subtraction is applied to the data analysis, calculate the average pixel intensity of the background ROI from the 10 s before touch until the end of the recording. Then subtract that value from each frame and continue with step ii.

iii. The resulting values of each frame will be used to plot the final graph and will represent the relative fluorescence of each point to the baseline.

d. Chloride imaging (Data S3) (Figure 9).
   i. In excel, calculate the CFP and YFP fluorescence after background subtraction (Figures 9C and 9G).
   ii. Calculate the YFP/CFP ratio (R) from data generated in the previous step (Figure 9H).
   iii. Calculate the average baseline ratio (R_0) of the 10 s before touch.
   iv. Plot the R/R_0 ratio (Figure 9I).

Note: The raw YFP/CFP ratio can also be used to plot changes in intracellular chloride (Figure 9J).

3. Statistical analysis.
   a. Calcium Imaging (Data S2).
      i. For each recording, a maximum value of $\Delta F/F_0$ corresponding to the peak of the curve can be obtained (Figures 8F and 8H, left panels).
      ii. To calculate the decay time (τ), copy the $\Delta F/F_0$ data of each recording and paste it in clampfit software.
      iii. Create a graph for each recording.

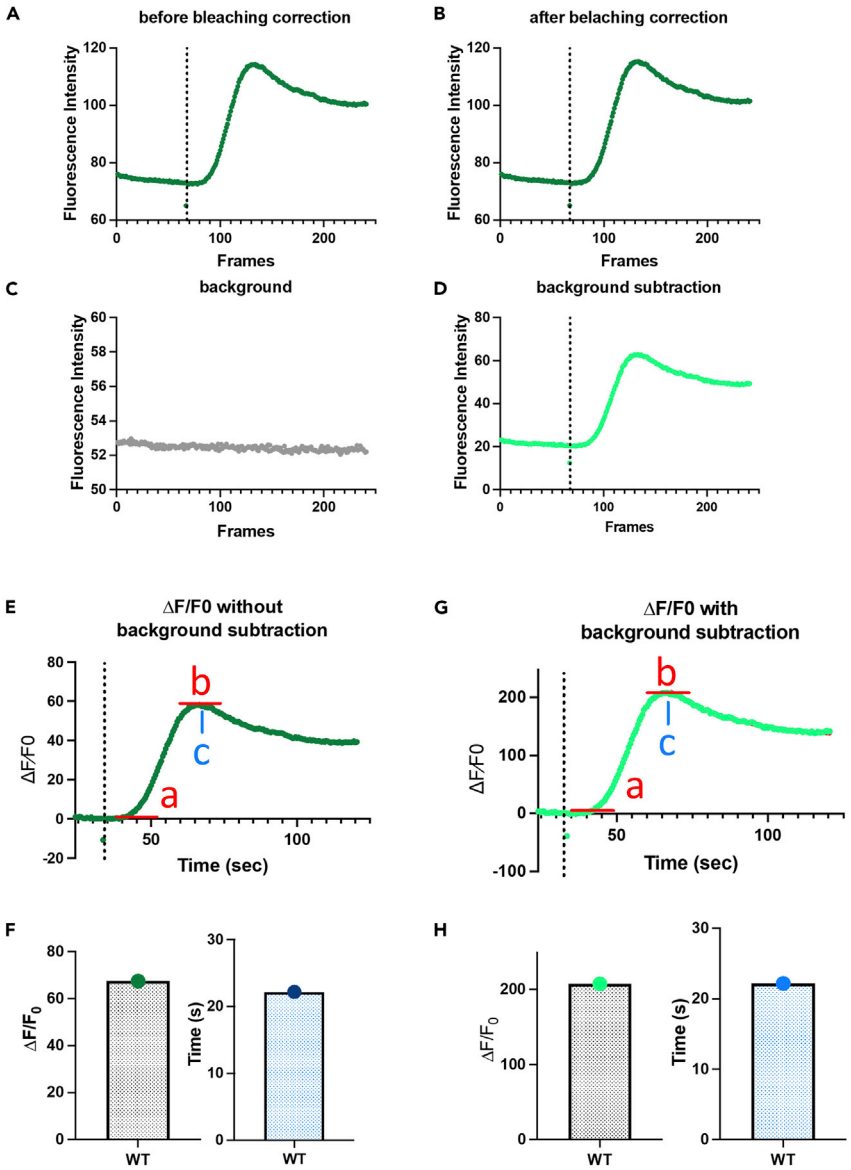

Figure 8. Calcium imaging analysis

(A) Change in GCaMP-6s fluorescence upon touch stimulation (dotted line) across the acquired frames.

(B) Same as in A but after bleaching correction using imageJ.

(C) Background fluorescence of a ROI close to the AMsh cell body.

(D) GCaMP-6s fluorescence after background subtraction.

(E and F) $\Delta F/F_0$ calculated without background subtraction. F_0 was obtained by averaging the 10 s before touch stimulation. The number of frames was converted into time according to the frequency of frames' capture used. The two horizontal red lines (a and b) represent the levels used to calculate the peak of $\Delta F/F_0$ (shown in F, left panel). The vertical blue line (c) indicates the point from which the data were fitted with an exponential decay (tau value shown in F, right panel).

(G and H) same as in E and F for $\Delta F/F_0$ calculated with background subtraction.

iv. Select the region of the graph from the maximum $\Delta F/F_0$ value to the end of the recording.

v. Fit the curve using the exponential standard equation.

vi. In the newly generated tab, τ will be displayed.

vii. Plot the resulting values and perform statistical analysis based on your hypothesis and conditions (Figures 8F and 8H, right panels).

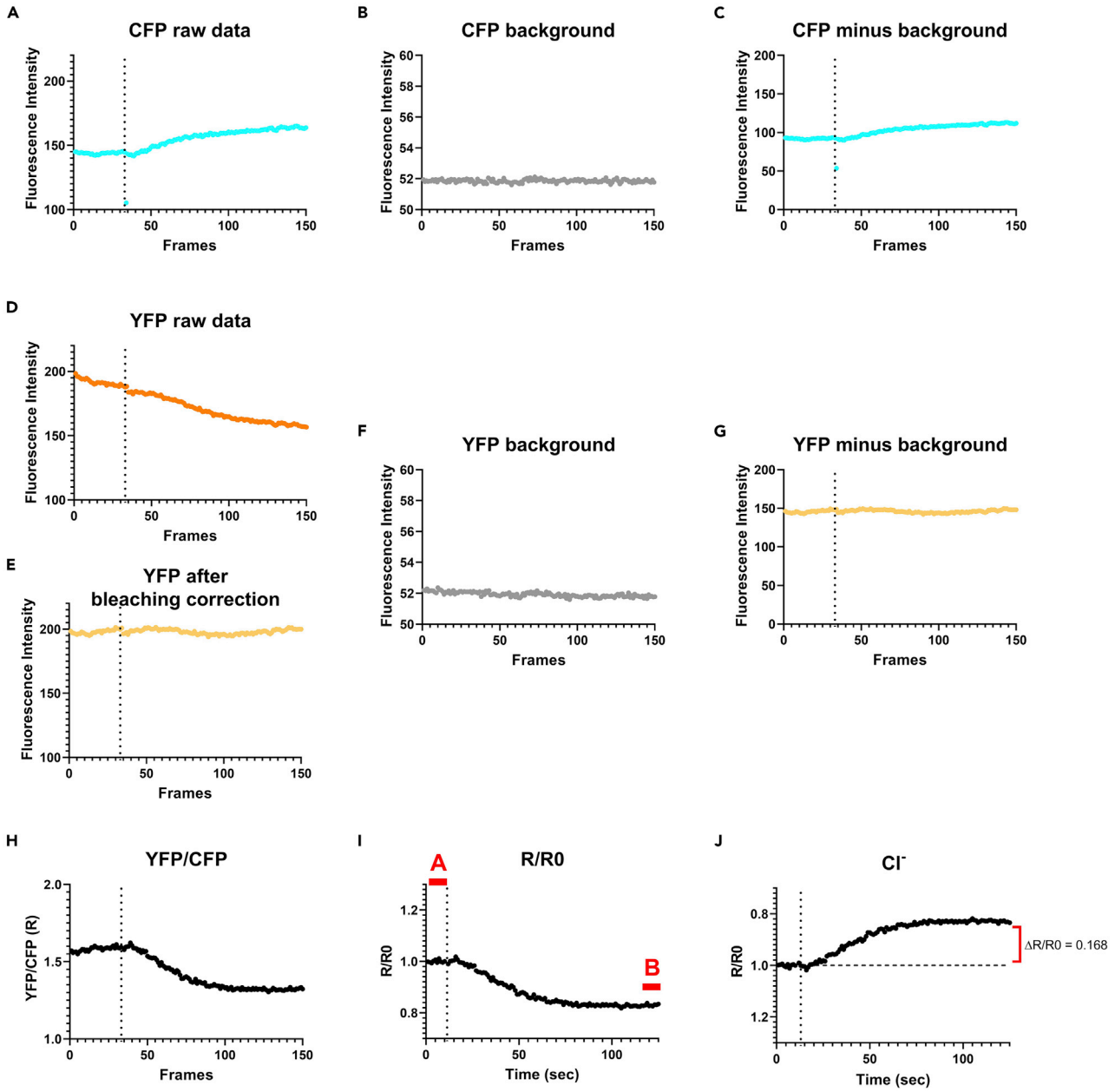

Figure 9. Chloride imaging analysis

(A) Change in the fluorescence intensity of CFP in SuperClomeleon upon touch stimulation (dotted line) across the acquired frames.

(B) Background CFP fluorescence of a ROI close to the AMsh cell body.

(C) CFP fluorescence after background subtraction.

(D) Change in the fluorescence intensity of YFP in SuperClomeleon upon touch stimulation (dotted line) across the acquired frames.

(E) Change in YFP fluorescence after bleaching correction.

(F) YFP background.

(G) YFP fluorescence after background subtraction.

(H) Ratio of YFP/CFP (R) across the acquired frames.

(I) R/R_0 over time. The number of frames was converted into time according to the frequency of frames' capture used. A and B (in red) indicate the range of frames used to calculate the $\Delta R/R_0$.

(J) Because R/R_0 decreases as intracellular Cl^- rises, the Y axis of R/R_0 was inverted to show the change in intracellular Cl^- which in this example was 0.186, as shown on the right of the panel. The dashed line corresponds to the basal Cl^- level.

Note: To calculate decay time (τ), the exponential decay fitting function of Prism or similar software can also be used.

⚠ CRITICAL: The τ value obtained corresponds to number of points (1/frame) in the graph. Adjust your value to match the time scale. In our conditions, 1 s of recording would correspond to 2 points. Thus, to obtain the real value, we must divide it by 2 (Figures 8F and 8H, right panels).

b. Chloride Imaging (Data S3).
   i. Calculate the average YFP/CFR or R/R_0 ratio of the 10 s before touch (section A) and the last 10 s of the recording (section B) (Figure 9I).
   ii. Calculate the difference B-A.
   iii. The obtained values can be used as a measure of change in chloride concentration over time and can be plotted in a graph or used for statistical analysis (Figure 9J).

LIMITATIONS

This protocol describes the *in vivo* study of the AMsh glia activity upon nose touch stimulation using a manual approach to position the stimulating probe in the Z axis. It is hard to determine for every touch how much the probe is rubbing the agarose pad, thus decreasing the force of stimulation. This limiting factor creates the possibility that upon running the protocol, the cell does not get activated. This limitation is even more of a concern in chloride imaging recordings where changes in fluorescence are hard to see during data acquisition. The experience of the researcher will be critical to achieve consistent results.

TROUBLESHOOTING

Problem 1

The fluorescence intensity of the AMsh glia is too low, too high, or shows vacuolization of the cell (step 19).

Potential solution

AMsh glia are touch sensitive. Sometimes the cell can become activated during worm transfer or gluing. This leads to high and long-lasting GCaMP-6s fluorescence, as the calcium is buffered very slowly in these cells. To avoid this, handle the worms gently and try to avoid touching their head area.

Very low fluorescence or the presence of vacuoles in the cell suggests that the animal is not healthy. In this case we recommend discarding the animal and selecting another one for recordings.

Problem 2

Too many bubbles in the imaging solution (critical note after step 12).

Potential solution

Bubbles can get trapped under the glue or under the glass coverslip coated with the agarose pad. Gently push these bubbles out using the gluing probe viewing the recording chamber under the stereotaxic microscope. Sometimes, bubbles can be also effectively pushed out using the touch probe under the recording microscope. If the entire solution contains a lot of bubbles, then normally this suggests that it is old and needs to be replaced.

Problem 3

The cell body or the nose of the worm moves before or after mechanical stimulation (steps 16–21 and related to the gluing process: steps 7–12).

Potential solution

Gluing is a critical step in this protocol and requires practice. The better the worm is glued onto the agarose pad, the less it will move during the recording. Modest movement can be corrected though. The ROI can be tracked across the video either using automated plugins in Fiji (ImageJ) such as TurboReg or manually. After touch stimulation, the cell body is sometimes slightly displaced. Readjust the ROI to its new position and continue measuring the average intensity for each frame.

Problem 4

The cell is not responding to touch (step 20).

Potential solution

The reasons why one cell does not respond to touch are many, and the whole protocol should be reviewed in order to find the cause of the problem.

It is common to not set up the speed of the probe to the right value, as by default, the software always initializes with half of the maximum speed.

The position of the probe and the angle of impact are also important, and better performance can be obtained if the stimulation of the worms is done so the probe faces towards the glue.

Despite the probe seem to move and to hit properly the worm, it may be brushing the pad just enough to not apply the desired force and not be noticed by human view. Reposition the probe and try again.

Check the buffer and the pads to make sure the worms are healthy.

The cell was stimulated during the gluing process, and it may still be recovering.

Rarely, some worms just do not respond and it is better to move to a new one. If the problem persists after 2–3 worms, then something in the protocol is wrong.

Problem 5

The pads detach from the slide during the recording (step 20 and related to steps 3–6).

Potential solution

Make sure to have fresh pads prepared for your experiments. However, it is recommended to prepare them a few hours or even a day before the recording so the pad dries just enough to be well attached to the slide. Under our conditions, covering the whole area of the slides with the pad usually favors the detaching (for example in round slides).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Laura Bianchi (l.bianchi@med.miami.edu).

Materials availability

DNA constructs and *C. elegans* strains generated in this study are available upon request.

Data and code availability

Data reported in this paper are provided in Data S2 and S3.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Supplemental information can be obtained from https://doi.org/10.17632/tpvptsck7r.2

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101282.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.F.A. and L.B.; Methodology, J.F.A. and L.B.; Software, J.F.A.; Validation, J.F.A. and L.B.; Formal Analysis, J.F.A.; Investigation, J.F.A.; Resources, L.B.; Data Curation, J.F.A.; Writing – Original Draft, J.F.A. and L.B.; Writing – Review & Editing, J.F.A. and L.B.; Visualization, J.F.A. and L.B.; Supervision, L.B.; Project Administration, L.B.; Funding Acquisition, L.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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