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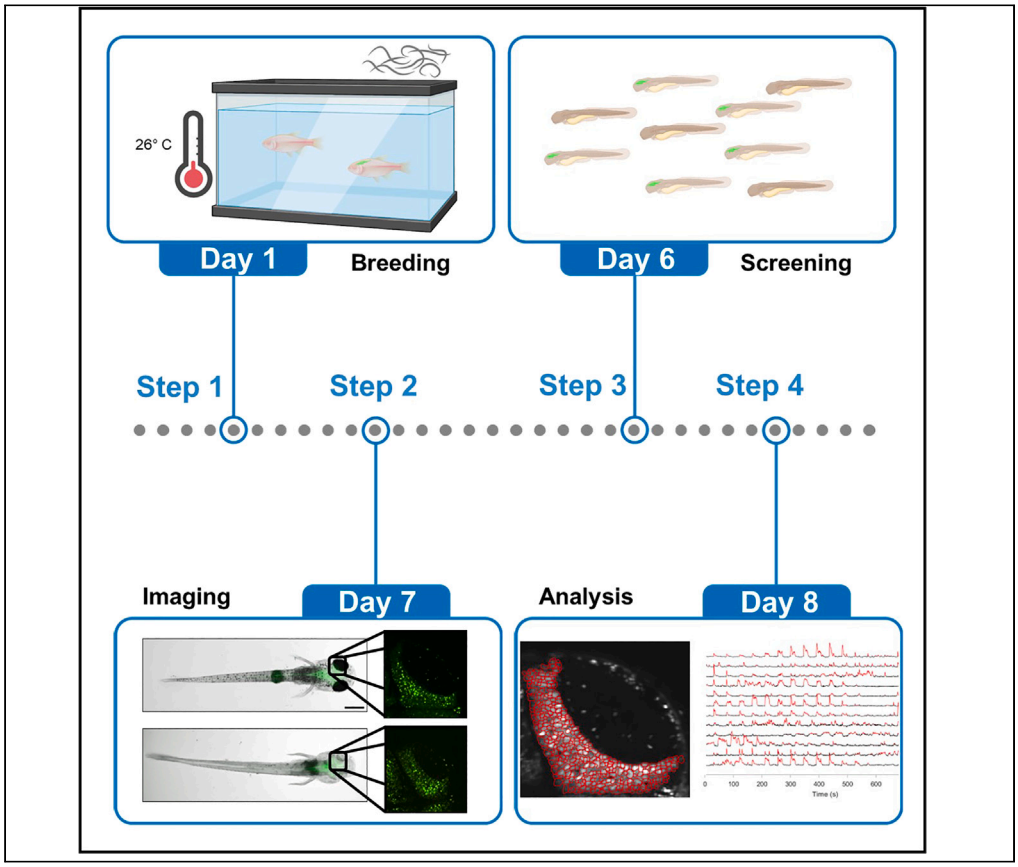
A protocol for whole-brain Ca^{2+} imaging in *Astyanax mexicanus*, a model of comparative evolution



In this protocol, we describe a comparative approach to study the evolution of brain function in the Mexican tetra, *Astyanax mexicanus*. We developed surface fish and two independent populations of cavefish with pan-neuronal expression of the Ca^{2+} sensor GCaMP6s. We describe a methodology to prepare samples and image activity across the optic tectum and olfactory bulb.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Evan Lloyd, Martin Privat, German Sumbre, Erik R. Duboué, Alex C. Keene

emlloyd@tamu.edu (E.L.)
keenea@tamu.edu (A.C.K.)

Highlights

Transgenic GCaMP lines allow for comparative brain imaging in cavefish

Current protocols allow for imaging brain activity in response to light and odorants

Analysis of imaging data enables prediction of circuit connectivity

This system can be applied to measure brain activity under diverse conditions

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A protocol for whole-brain Ca^{2+} imaging in *Astyanax mexicanus*, a model of comparative evolution

Evan Lloyd,[1,4,*] Martin Privat,[2] German Sumbre,[2] Erik R. Duboué,[3] and Alex C. Keene[1,5,*]

[1]Department of Biology, Texas A&M University, College Station, TX 77840, USA

[2]Institut de Biologie de l'ENS (IBENS), Département de Biologie, École Normale Supérieure, CNRS, INSERM, Université PSL, 75005 Paris, France

[3]Harriet Wilkes Honors College, Florida Atlantic University, Jupiter, FL 33458, USA

[4]Technical contact

[5]Lead contact

*Correspondence: emlloyd@tamu.edu (E.L.), keenea@tamu.edu (A.C.K.)
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SUMMARY

In this protocol, we describe a comparative approach to study the evolution of brain function in the Mexican tetra, *Astyanax mexicanus*. We developed surface fish and two independent populations of cavefish with pan-neuronal expression of the Ca^{2+} sensor GCaMP6s. We describe a methodology to prepare samples and image activity across the optic tectum and olfactory bulb.

BEFORE YOU BEGIN

Small fish models are widely used for studying the evolution of brain function and behavior.[1,2] Evolved trait differences that derive from inhabiting different ecosystems have been used to investigate the genetic basis of behavior in many fish species including African cichlids, the three-spine stickleback, and the killifish.[3–5] The Mexican tetra *Astyanax mexicanus* is unique because it exists as surface fish and at least 30 cave populations of the same species.[6–8] While these fish have been studied for over 80 years, the evolved differences in brain function have been largely inaccessible. Recently, we developed transgenic lines expressing the Ca^{2+} sensor GCaMP6s that allow for comparative brain imaging between populations of fish.[9] The basic approaches to image acquisition and functional imaging are largely derived from studies in zebrafish that have led in the development of brain-wide imaging approaches.[10–12] In this review we highlight husbandry required for maintenance of breeding in *A. mexicanus*, modifications specific to *A. mexicanus* imaging and comparative analysis used to identify evolved differences between the populations. While we focus on sensory systems, this approach can be broadly applied to imaging in diverse brain regions and conditions. Overall, this protocol is flexible and can be applied across diverse biological contexts.

Institutional permissions

All methods for handling *A. mexicanus* described in this protocol have been approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC). Before following this protocol, obtain approval from your relevant institutions to ensure compliance with federal, state, and local government regulations and animal welfare organization guidelines.

Husbandry and genetics

Timing: 1 week

This section described the husbandry required to breed and grow transgenic *A. mexicanus*.

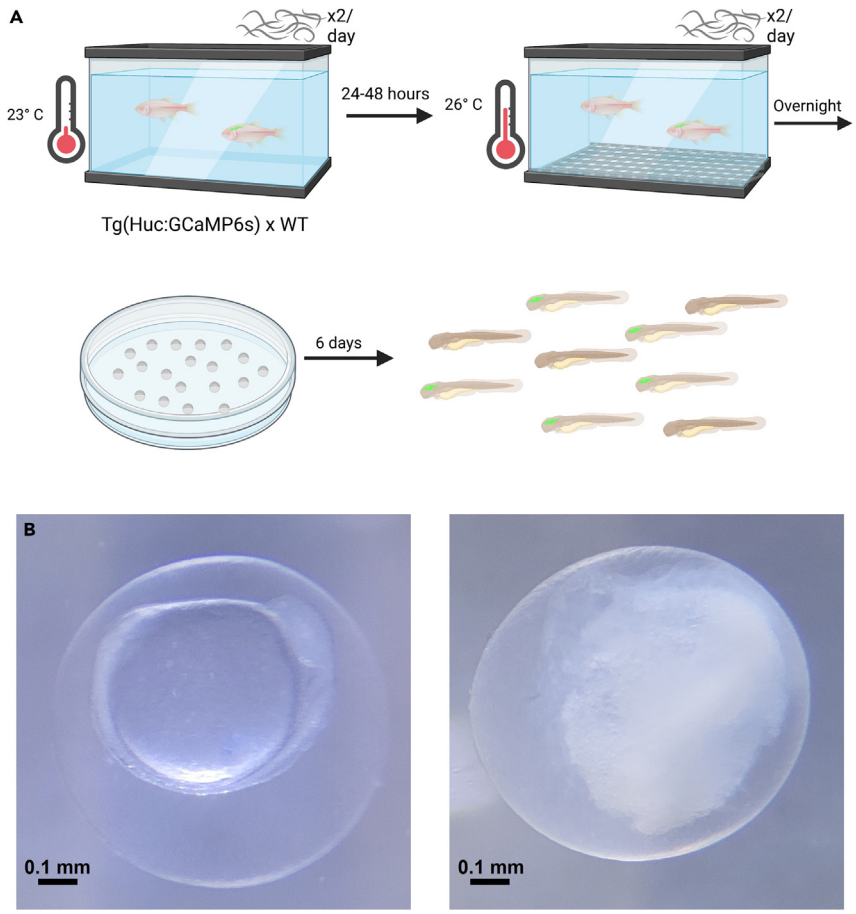

Figure 1. Husbandry and genetics

(A) Timeline of breeding and screening for transgenic animals.

(B) Examples of healthy live embryos (left), and unviable embryos (right).

1. Set up a cross to start the experiments.

Note: In zebrafish keeping homozygous lines can lead to in-breeding related problems. To avoid this, transgenic fish from each population are maintained as heterozygotes. In general efforts are made to maintain heterozygosity in pure-breeding stocks. For experiments they are outcrossed to a non-transgenic fish of the same population, resulting in ~50% transgenic fish in the progeny (Figure 1A).

a. Separate male and female fish 1–2 weeks prior to the experiment. Fish can be bred as individual pairs or in groups.
b. Feed males and females a high calorie diet consisting of at least two daily feedings of frozen bloodworms supplemented by standard fish food.

Note: To maintain water quality, avoid feeding more than the fish can consume within 10 min.

c. Approximately 48 h prior to the desired breeding time, place fish together in a tank, along with a heater to raise the temperature from 23°C to 26°C.

Note: Alternative protocols are often used, including ramping up the temperature 1°C each day, over a three-day period.[13]

d. Prior to onset of the dark period, place a mesh net (approximately 3 cm mesh size) in the bottom of a 10-gallon tank. This will prevent fish from cannibalizing eggs.

Note: If spawning does not occur after the first night, breeding procedures may be continued for up to 2 additional days. After this period, spawning becomes less likely as the fish acclimate to the higher temperature. If this occurs, fish should be separated and allowed to re-acclimate to normal temperature (23°C) before attempting breeding.

2. Collect and clean eggs following fertilization. Typically, fish will drop eggs during the night. If the time of drop is not observed, they can be staged using a developmental atlas.[14]
   a. Collect eggs using a fine mesh aquarium net, and transfer to a volume of fresh water.
   b. Sort live eggs within 8 h of fertilization and discard dead or unfertilized eggs.

Note: that healthy cavefish eggs appear to be more yellow in color. Dead/unfertilized eggs of both populations are opaque, while live eggs are transparent and readily distinguishable (Figure 1B).

⚠ CRITICAL: *A. mexicanus* embryos are very sensitive to water quality conditions in the first 24 h after fertilization. Always remove dead embryos as soon as possible, and keep embryos in as large a water volume as is practical. At least 5 mL per embryo is recommended.

   c. At 24 h post-fertilization (hpf), GCaMP6s fluorescence is readily identifiable in all transgenic embryos. If the breeding pair is heterozygous for the transgene sort and retain all GCaMp6s expressing embryos under a fluorescent dissection microscope.
   d. Place fertilized eggs in fresh system water. Replace water daily removing any dead embryos through six-days post fertilization (dpf).

Note: Both adult and larval fish should be kept on a daily light:dark cycle and at a constant temperature. *A. mexicanus* of both surface and cave varieties were maintained at 23°C and on a 14:10 h light:dark cycle. Adult fish may be fed standard flake food (e.g., Tetramin Pro) when not being prepared for breeding. Feed only as much food as can be consumed within 10 min.

Reagent preparation

Timing: 15–30 min

This section describes how to prepare reagents for olfactory testing of *A. mexicanus*.

3. Mivacurium chloride (paralytic):
   a. To prepare 2 mL of mivacurium chloride solution at 0.5 mg/mL, dissolve 1 g of mivacurium chloride powder into 2 mL of aquarium water.
   b. Aliquot into 100 µL single-use portions, and freeze at −20°C for up to 1 year.
4. Low-Melting Point (LMP) Agarose (embedding medium):
   a. To prepare 50 mL of 2% LMP solution, add 1 g of LMP Agarose powder to 50 mL of aquarium water in a 50 mL conical tube, and place in a beaker filled with water.
   b. Heat in 15 s increments in the microwave, until the powder has completely dissolved. Do not allow the agarose solution to boil.
   c. Prepared LMP agarose solution can be aliquoted and stored at room temperature or maintained at 45°C in a water or dry bath indefinitely.
5. L-Serine Solution: (olfactory stimulus):
   a. To prepare 500 mL of L-Serine solution at a 1 mM concentration, dissolve 52.5 mg of L-Serine powder (MW 105.09 g/mol) in 500 mL of aquarium water.

Note: Amino acid solutions should be made on the day of the experiment to minimize potential for degradation of the solution.

6. Aquarium (system) water:
   a. To prepare 1 L of aquarium water, start with 1 L of deionized water, adjust the pH to between 6.8–7.2 using sodium bicarbonate.
   b. Adjust the conductivity to approximately 500 μS using aquarium salt.
7. 0.2 mM Phenylthiourea (PTU) solution.
   a. To prepare 100 mL of 0.2 mM PTU solution, dissolve 3 mg of PTU powder (MW 152.22) in 100 mL of Aquarium water.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Mivacurium chloride	Sigma-Aldrich	CAS: 106861-44-3
Phenylthiourea	Sigma-Aldrich	P7629
L-Serine powder	Thermo Fisher	A11179.14
Low-melting point agarose	Thermo Fisher	CAT#17856
Aquarium salt	Instant Ocean	SS15-10
Experimental models: Organisms/strains		
Surface *A. mexicanus*, 6 dpf	Lloyd et al.[9]	Tg(*Huc:H2B-GCaMP6s*)
Molino *A. mexicanus*, 6 dpf	Lloyd et al.[9]	Tg(*Huc:H2B-GCaMP6s*)
Pachón *A. mexicanus*, 6 dpf	Lloyd et al.[9]	Tg(*Huc:H2B-GCaMP6s*)
Software and algorithms		
MATLAB 2020a	MathWorks	https://mathworks.com/
Romano et al. MATLAB toolbox	GitHub	https://github.com/zebrain-lab/Toolbox-Romano-et-al
FIJI 2.13.1	ImageJ	https://imagej.net/software/fiji/
"moco" Plug-In for FIJI	GitHub	https://github.com/NTCColumbia/moco
Python 3.9	Python	https://www.python.org/
CaImAn	GitHub	https://github.com/flatironinstitute/CaImAn
Other		
Nunc glass bottom dish	Thermo Fisher	CAT#150680
Forceps	Fine Science Tools	Dumont #5
Arduino Uno	Arduino	https://www.arduino.cc/
LED light strip	LEDSupply	R6030AA-NWT-03
Perfusion chamber	Warner Instruments	JG-23
Perfusion system	Automate Scientific	SKU: 17-21-20
Confocal microscope Nikon A1R	Nikon	AX R

STEP-BY-STEP METHOD DETAILS

Microscope and sample preparation

Timing: 15 min

This section describes the steps necessary to prepare the equipment and samples prior to live imaging of *A. mexicanus*.

1. Bring LMP agarose to ~45°C in a dry bath/heating block.
2. Prepare the microscope
   a. Power on the laser
   b. Activate the laser lines.

c. Start the confocal image acquisition software
d. Configure appropriate laser settings for the imaging conditions.

Note: For imaging tectal light response in larva expressing *GCaMP6s*, the following equipment and settings were used: Nikon AXR confocal microscope, 25× water immersion objective, 1.5× digital zoom, Galvano scanner, resolution 512 × 512 pixels, for an effective frame rate of 2 Hz.

3. Prepare larvae for immobilization in agarose by sedation or paralysis.

Note: Sedation in cold water is considered less aversive than paralysis, and is usually sufficient to maintain immobility for short periods. For longer experiments (>15 min), or when total immobility is required, paralysis is preferred, as cavefish are especially prone to attempt movement even when immobilized in agarose. In both cases, experiments should be performed immediately following immobilization, to minimize stress on the larvae. Additionally, while sedation on ice is reversible, paralysis in mivacurium chloride is a terminal treatment and requires euthanasia following experiment completion. Although mivacurium is considered a reversible neuromuscular junction blocker, at the concentrations used, larvae are not expected to recover from paralysis. In our hands, larvae remained healthy for up to 1 h after paralysis; longer imaging times were not attempted.

a. Preparation for imaging paralyzed larvae.
   i. Defrost mivacurium to room temperature prior to isolating experimental animals.
   ii. Transfer a single larva to a petri dish or similar container.

Note: Remove as much water as possible to limit the dilution of the paralytic to less than 10%. Then add ~100 μL of 0.5 mg/mL mivacurium chloride.

   iii. Maintain larva in mivacurium chloride for 1 min.
   Remove larva from mivacurium chloride and place them in a Petri dish containing system water. A.
   Allow 2–3 min after removal from paralytic for larvae to cease movement.
   iv. Immediately transfer larva to a mounting plate with a disposable 3 mL pipette and begin mounting procedures.
b. Sedation of un-paralyzed larvae:
   i. Fill an insulated bucket with ice and position a 250 mL bowl in the center, submerging the sides of the bowl in ice.
   ii. Add chilled system water (maintained at 4°C) to the bowl, and wait ~5 min for additional chilling.
   iii. Transfer a single larva to water bowl immediately prior to the initiation of mounting. Sedation should take effect within 1–2 min.
   iv. Transfer the larva to the imaging dish and begin mounting procedures after larva has ceased movement for at least 30 s. Prolonged chilling can lead to death; larvae should be sedated immediately prior to mounting and removed as soon as sedation is achieved.
4. Initiation of mounting procedure.
   a. Transfer sample to the imaging dish using a disposable 3 mL pipette.
   b. Remove as much liquid as possible using a P200 Pipette.
   c. Using the P200 pipette, immediately embed larva in a small drop (~50–200 μL) 2% LMP agarose that is warmed to 45°C.

Note: The volume of agarose added should be adjusted to the mounting and imaging conditions. Larger volumes enable easier manipulation of the larva into position, but care must be taken to ensure the larva brain is near the surface of the agarose, especially when using objectives with a short working distance.

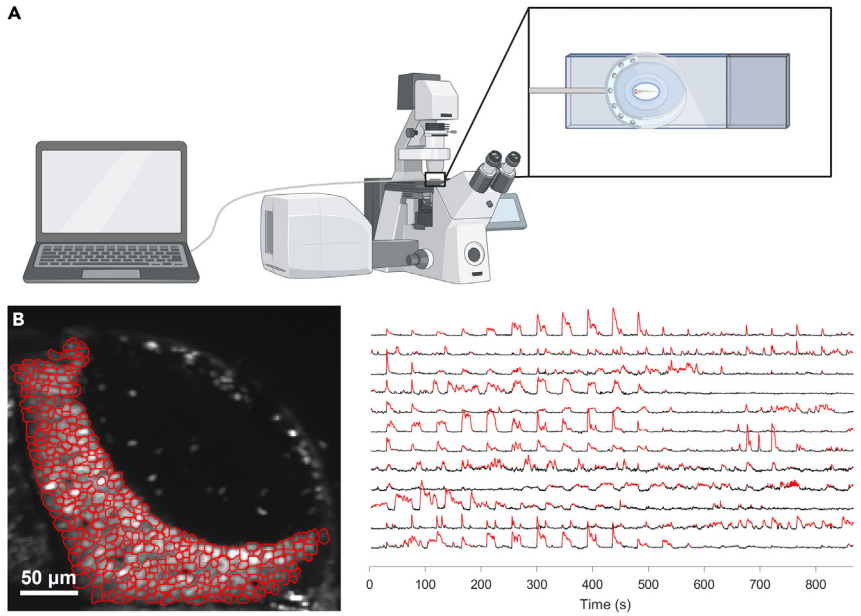

Figure 2. Functional imaging of light response in the tectum

(A) The visual imaging system. Inset: a larva mounted in agarose, with white light LED system arranged in the field-of-view of the larva.

(B) Results of post-processing of visual imaging; a surface fish optic tectum, with individual neurons programmatically segmented (left); representative fluorescence traces of neurons during visual stimulation, with significant Ca^{2+} transients highlighted in red (right).

5. Mount larvae dorsal side up for upright microscopes and dorsal side down against the cover slip for an inverted scope (Figure 2A).
   a. Gently orient the sample using two pairs of forceps. Maintain this position with the forceps until the agarose begins to solidify. This step should take 10–30 s.
   b. Alternatively, use a single pair of forceps, or a gentle instrument such as a thread from a toothbrush, to nudge the larvae into position until the agarose starts to solidify, then remove forceps tip.
   c. Once fully solidified (appearing slightly opaque after approximately 30 s), add a small amount (<1 mL) of system water on top of sample, affix LEDs to imaging chamber using a temporary adhesive (e.g., tape) and place under microscope.

⚠ CRITICAL: The mounting instrument should be removed after the larva is stable, but before the agarose has fully solidified. Removing the instrument too late will lead to a gap in the agarose, which can contribute to unwanted movement of the larva.

6. Place sample on microscope.
   a. For *in vivo* GCaMP imaging, be sure to prepare the confocal microscope (e.g., Nikon AX R) prior to mounting to minimize the time between immobilization and data acquisition.

Note: The protocol described can be used for confocal or two-photon microscope.

7. Navigate to your region of interest (e.g., the optic tectum) and collect time-lapse, and, optionally, volumetric images.

Note: The scanning speed, resolution, zoom and laser power should be optimized for the brain region under investigation and microscope used for acquisition. Most confocal microscopes are equipped with Galvano scanners, which are typically capable of imaging at a

rate of 2–4 Hz. Resonant scanners are capable of imaging at frame rates of up to 30 Hz or higher, although averaging may need to be used to achieve single-cell resolution. In general, for measuring Ca^{2+} using GCaMP6s imaging at speeds of 2–4 Hz is sufficient.

Imaging response to visual stimuli

Timing: 10–60 min

This section describes the process of imaging neural activity in *A. mexicanus* in response to visual stimulation.

8. Begin imaging, recording at a rate of ≥2 Hz.
9. Image for 60 s to establish baseline fluorescence of neurons.

Note: The excitation laser will cause some inherent visual stimulation. Laser power should be kept as low as possible while maintaining sufficient signal-to-noise ratio to visually resolve individual neurons. In our hands, a period of 30 s was sufficient in all cases for neuronal fluorescence to return to a stable baseline.

10. To identify functional cell types, immediately apply a whole-field light stimulus for a period of 30 s.

Note: Although structured stimuli may be presented with the use of a projector, a single LED light strip, placed directly in the field of view of the larva, will be sufficient to identify an array of functional neuronal responses (Figure 2B).

11. Repeat the stimulus up to ten times to establish response probability of neurons, with each stimulus separated by at least 30 s of image acquisition with no stimulation (Figure 2B).

⚠ CRITICAL: To ensure accurate identification of cell responses, the stimulus must be precisely timed, and the time points and duration of the stimulus logged accurately. This may be achieved either through manual logging, or through the use of hardware synchronization.[15]

Imaging response to olfactory stimuli

Timing: 10–60 min

This section describes the process of imaging neural activity in *A. mexicanus* in response to olfactory stimulation.

12. For olfactory imaging, mounting should be performed as described above, but the larva should be mounted in a perfusion chamber (e.g., Science Products, OAC-1), which allows for the inflow and outflow of liquid in a controlled manner (Figure 3A).

Note: The larvae may be fully embedded in agarose without the need to clear the head area, because agarose is a gel matrix which permits water flow along with any dissolved molecules.

13. Begin perfusion of system water over the larva, and then begin imaging, at a rate of ≥2 Hz (Figure 3B).

Note: Flow rate of the water will vary based on multiple factors including inner diameter and orientation of the perfusion hose, height of the system relative to the perfusion chamber, etc.

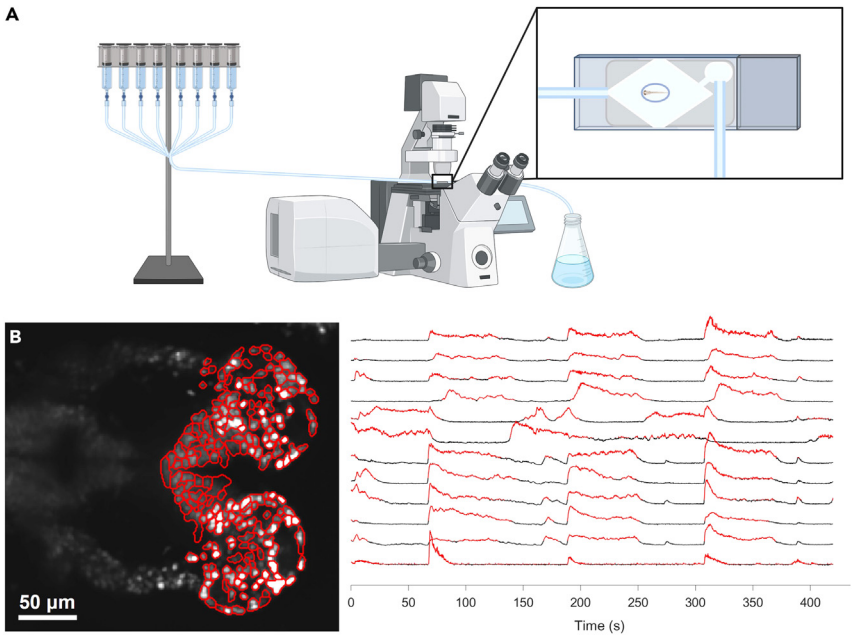

Figure 3. Functional imaging of olfactory response in the olfactory bulb

(A) The olfactory imaging system. Inset: a close-up view of the larvae, mounted in agarose, with odor inflow on the left, and outflow chamber on the right.

(B) Results of post-processing of olfactory imaging; a cavefish olfactory bulb with individual neurons programmatically segmented (left); representative fluorescence traces of neurons during olfactory stimulation, with significant Ca^{2+} transients highlighted in red (right).

These conditions should be standardized across all imaging sessions. The flow rate may affect the duration of stimulus required to elicit a response, as well as effective washout periods. The timing of these experiments was designed with a flow rate of approximately 10 mL per minute.

14. Image for 60 s to establish baseline fluorescence of neurons.
15. Perfuse the L-Serine over the larvae for 60 s.

Note: A short delay in response is to be expected, the timing of which will be dependent on the flow rate and chamber size, both of which should be standardized to allow comparison across trials.

16. Repeat the stimulus three or more times to establish probability of response (Figure 3B). Alternatively, apply a range of concentrations (e.g., 10 μM, 100 μM, 1 mM), or a series of different odorants, interspersed with a 60 s washout period of system water.

Post-experiment procedures

Timing: 10–45 min

This section describes how to handle larvae following imaging procedures.

17. Paralyzed larvae should be euthanized immediately following your experiment, according to your institution's approved guidelines (e.g., immersion in ice-cold water, followed by secondary euthanasia).

Note: Paralyzed larvae are stable for up to one hour post-paralysis, but are not expected to recover from paralysis at the concentrations used.

18. Sedated larvae may be carefully removed from the agarose using forceps and transferred to system water for recovery.

Note: Time under sedation post-chilling may vary; in general, larvae can be expected to begin normal swimming behavior approximately 30–45 min post-sedation.

Image analysis

Timing: 30–60 min

This section describes how to analyze functional imaging data.

19. Motion Correction: Prior to extraction of fluorescence time courses of individual neurons, unwanted subject motion in the X and Y planes may be eliminated through the use of ImageJ/FIJI (https://imagej.net/software/fiji/) and the "moco" plugin (https://github.com/NTCColumbia/moco).
20. Several freely available programs exist for post-processing and analysis of fluorescence time courses such as those acquired with genetically encoded calcium indicators (GECIs). Post-processing analyses may include number of cells, spike rate, peak ΔF/F, correlation of activity, etc.
    a. The MATLAB toolbox published by Romano and colleagues (https://github.com/zebrain-lab/Toolbox-Romano-et-al) provides a pipeline and a step-by-step explanation for automated motion correction, segmentation of individual neurons, extraction of fluorescence time courses, spike estimation, and other post-processing analyses.[16]
    b. CaImAn (https://github.com/flatironinstitute/CaImAn) represents an alternative method that provides many of the same functions, within the python environment.[17]

EXPECTED OUTCOMES

The generation of transgenic surface and cavefish allows for whole-brain imaging and a comparison of neural activity between independently evolved populations of *A. mexicanus*. The protocol described here, and previous work, is focused on imaging from the visual and olfactory centers of the brain. These analyses can define differences in response to stimuli, as well as baseline differences in neural activity and connectivity between *A. mexicanus* populations. For instance, in Lloyd et al., these protocols were used to identify subtle responses to visual stimuli in cave populations of *A. mexicanus*, as well as alterations in inter-neuronal activity correlation.[9] These same protocols can be readily adapted to image neural responses to other sensory processes, and other brain regions.

LIMITATIONS

Although the sample preparation and imaging steps of this protocol may be easily adapted to live imaging for most larval-stage fish species expressing activity reporters, the procedure is limited to organisms which fit easily on the stage of a confocal microscope, and so studies of later developmental stages or larger organisms will not be possible with the protocol as described. Additionally, as *A. mexicanus* larvae develop past 2–3 weeks of age, it is expected that gill perfusion will become necessary to maintain the organism during imaging, as in zebrafish.[18]

The analysis procedures described are only applicable to time-lapse images of single, two-dimensional planes of neuronal activity, which limits the conclusions for analysis of larger, more 3-dimensional brain regions. Volumetric images can be acquired with minimal alterations to the described procedures but will require additional post-acquisition analysis steps not outlined here.

TROUBLESHOOTING

Problem 1

Melanin in surface fish prevents imaging of desired neuronal populations (related to step 7).

Potential solution 1

Like zebrafish, surface morphs of *A. mexicanus* are mostly transparent at 6dpf, but do produce some melanophores over the cranium. In general, *A. mexicanus* exhibits much less melanization at 6dpf relative to zebrafish, and clearing of melanin is not necessary for most applications.

- If clearing of melanin is deemed necessary (e.g., when imaging at later developmental stages, which exhibit greater melanization), treatment with 0.2 mM phenylthiourea (PTU) beginning from 12–24 hpf until prior to imaging will be sufficient to inhibit formation of melanophores.
- Care should always be taken when performing experiments with PTU, as it has documented effects on diverse aspects of teleost growth, and, in our hands, can interfere with normal visual function.[19]
- Any experiments conducted in PTU-treated surface fish should use PTU-treated cavefish for comparison. A caveat is that PTU may have different effects in each population of *A. mexicanus* and this should be avoided whenever possible.

Problem 2

Acquired images are blurry, and lack single-cell resolution (related to step 7).

Potential solution 1

Due to the larger brain size of the Mexican tetra relative to zebrafish, imaging at deeper tissue depths can be difficult on a scanning-confocal microscope. Use of a two-photon microscope will improve the penetration of the excitation light and increase the clarity of the image.

Potential solution 2

In the case of very deep brain regions (e.g., the ventral hypothalamus), mounting the larva in an inverted position (ventral side up on an upright microscope, dorsal side up on an inverted microscope) may provide better clarity.

Problem 3

Responses are not consistent across subjects, or stimuli that previously exhibited responses no longer do (related to steps 7 and 13).

Potential solution 1

When performing comparative experiments, it is important to control for differences in brain size and shape across populations. This is most easily done by using shared neuro-anatomical features to calibrate imaging location.

- For example, when imaging in the superficial tectum, calculate the depth of the tissue by identifying the top and bottom of the structure during imaging, and always image at the same depth proportional to each subject. Because the sizes of certain brain regions, including the tectum, vary between surface and cave populations, measurements should always be made proportional to individual subjects measurements to ensure consistency (e.g., 1/3rd of the total depth of the structure to image the superficial layer).

Potential solution 2

In the case of olfactory imaging, chemical stimuli may degrade once in solution. Always prepare solutions the day of the experiment to minimize variation in stimulus concentration caused by degradation.

Problem 4

The subject drifts during imaging, so that neurons imaged in the beginning of the experiment are no longer visible by the end (related to steps 7 and 13).

Potential solution 1

Due to dilation of the agarose matrix, the sample may drift within the field of view during imaging. Although small movements in the X and Y dimensions are easily corrected (see analysis section), excessive drift in the Z dimension will complicate data analysis and need to be corrected at the imaging step.

- To correct this, first ensure that the agarose is the appropriate temperature; LMP agarose should always be used, and should be maintained just above gelling temperature to ensure quick solidification.
- Once the agarose has fully solidified, add water immediately to minimize dehydration of the agarose, and allow a few minutes for the agarose to fully rehydrate. Most issues with drift will be corrected by following these guidelines.

Problem 5

Maintaining larval health throughout the procedure (related to steps 7 and 13).

Potential solution 1

Excessive stress on the larva can cause it to expire during imaging. Always minimize the stress placed on the larva during and after mounting.

- Perform all sedation/paralysis and subsequent mounting procedures as quickly as possible, and as near to the imaging chamber as possible. Never immerse the larva in the sedative or paralytic longer than necessary to achieve sedation/paralysis.
- Ensure the larva has been reared in clean system water, and is properly fed (if applicable for its developmental stage) in the day(s) leading up to imaging.

RESOURCE AVAILABILITY

Lead contact

Requests for *A. mexicanus* fish should be directed to, and will be fulfilled by, the lead contact Alex Keene (keenea@tamu.edu).

Materials availability

This study generated unique transgenic *A. mexicanus*. These will be made available upon request.

Data and code availability

This study did not generate any new datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

E.L. developed the protocol, designed, and carried out the experiments. A.C.K., E.R.D., G.S., M.P., and E.L. conceived the experiments and experimental design and wrote the manuscript. A.C.K. and E.R.D. secured funding for this work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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