Journal of Visualized Experiments

FUNCTIONAL EVALUATION OF OLFACTORY PATHWAYS IN LIVING XENOPUS TADPOLES

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58028R1
Full Title:	FUNCTIONAL EVALUATION OF OLFACTORY PATHWAYS IN LIVING XENOPUS TADPOLES
Keywords:	Xenopus; tadpole; synapse; presynaptic terminal; olfaction; olfactory receptor neuron; olfactory glomeruli
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Laboratory of Neurobiology, Faculty of Medicine, University of Barcelona and Bellvitge Biomedical Research Institute (IDIBELL), Pavello de Govern-Lab. 4112, 08907 L'Hospitalet de Llobregat, Spain

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11 July 2018

Dear Alisha,

Please find enclosed the reviewed version of our manuscript entitled *Functional evaluation of olfactory pathways in living Xenopus tadpoles (#JoVE58028).*

We would like to thank the reviewers for carefully reading our manuscript and for their suggestions. We have addressed all comments. The paper has been significantly improved and now contains 7 figures and two supplementary movies. We hope this current version is now suitable for publication in JoVE.

Yours,

Artur Llobet, PhD

TITLE:

Functional Evaluation of Olfactory Pathways in Living *Xenopus* Tadpoles

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KEYWORDS:

Tadpole, *Xenopus laevis*, *Xenopus tropicalis*, calcium, synapse, olfactory receptor neuron, presynaptic terminal

SUMMARY:

Xenopus tadpoles offer a unique platform to investigate the function of the nervous system *in vivo*. We describe methodologies to evaluate the processing of olfactory information in living *Xenopus larvae* in normal rearing conditions or after injury.

ABSTRACT:

Xenopus tadpoles offer a unique platform to investigate the function of the nervous system. They provide multiple experimental advantages, such as accessibility to numerous imaging approaches, electrophysiological techniques and behavioral assays. The *Xenopus* tadpole olfactory system is particularly well suited to investigate the function of synapses established during normal development or reformed after injury. Here, we describe methodologies to evaluate the processing of olfactory information in living *Xenopus* larvae. We outline a combination of *in vivo* measurements of presynaptic calcium responses in glomeruli of the olfactory bulb with olfactory-guided behavior assays. Methods can be combined with the transection of olfactory nerves to study the rewiring of synaptic connectivity. Experiments are presented using both wild-type and genetically modified animals expressing GFP reporters in central nervous system cells. Application of the approaches described to genetically modified tadpoles can be useful for unraveling the molecular bases that define vertebrate behavior.

INTRODUCTION:

Xenopus tadpoles constitute an excellent animal model to study the normal function of the nervous system. Transparency, a fully sequenced genome[1,2], and accessibility to surgical,

electrophysiological and imaging techniques are unique properties of *Xenopus* larvae that allow investigating neuronal functions *in vivo*[3]. Some of the multiple experimental possibilities of this animal model are illustrated by the thorough studies performed on tadpole sensory and motor systems[4-6]. A particularly well-suited neuronal circuit to study many aspects of information processing at the level of synapses is the *Xenopus* tadpole olfactory system[7]. Firstly, its synaptic connectivity is well defined: olfactory receptor neurons (ORNs) project to the olfactory bulb and establish synaptic contacts with dendrites of mitral/tufted cells within glomeruli to generate odor maps. Secondly, its ORNs are continuously generated by neurogenesis throughout life to maintain the functionality of olfactory pathways[8]. And thirdly, because the olfactory system shows a great regenerative capability, *Xenopus* tadpoles are able to entirely reform their olfactory bulb after ablation[9] .

In this paper, we describe approaches that combine imaging of olfactory glomeruli in living tadpoles with behavioral experiments to study the functionality of olfactory pathways. The methods detailed here were used to study the functional recovery of glomerular connectivity in the olfactory bulb after olfactory nerve transection[10]. Data obtained in *Xenopus* tadpoles are representative of vertebrates since olfactory processing is evolutionary conserved.

The methods described are exemplified using *X. tropicalis* but they can easily be implemented in X. *laevis*. Despite the larger size of adult *X. laevis*, both species are remarkably similar during tadpole stages. The main differences reside at the genomic level. *X. laevis* displays poor genetic tractability, mostly determined by its allotetraploid genome and long generation time (approximately 1 year). In contrast, X. *tropicalis* is more amenable to genetic modifications due to its shorter generation time (5-8 months) and diploid genome. The representative experiments are illustrated for wild-type animals and three different transgenic lines: Hb9:GFP (*X. tropicalis*), NBT:GFP (*X. tropicalis*) and tubb2:GFP (*X. laevis*).

The methodologies outlined in the current work should be considered alongside the genetic progresses in the *Xenopus* field. The simplicity and easy implementation of the techniques presented makes them particularly useful for evaluating already described mutants[11], as well as *Xenopus* lines generated by CRISPR-Cas9 technology[12]. We also describe a surgical procedure used to transect olfactory nerves that can be implemented in any laboratory having access to *Xenopus* tadpoles. The approaches used for evaluating presynaptic calcium responses and olfactory-guided behavior require specific equipment, albeit available at a moderate cost. Methodologies are presented in a simple form to promote their use in research groups and could set the bases of more complex assays either by implementing improvements or by the association to other techniques, *i.e.,* histological or genetic approaches.

PROTOCOL:

All procedures were approved by the animal research ethics committee at University of Barcelona.

Note: *X. tropicalis* and *X. laevis* tadpoles are reared according to standard methods[13,14]. Tadpole water is prepared by adding commercial salts (see **Table of Materials**) to water obtained by

reverse osmosis. Conductivity is adjusted to ~700 µS and ~1400 µS for *X. tropicalis* and *X. laevis* tadpoles, respectively. Larvae can be obtained either by natural mating or *in vitro* fertilization[14]. Embryos are dejellied with 2% L-cysteine prepared in 0.1x Marc's Modified Ringers (MMR), which contains (in mM): 100 NaCl, 2 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 5 HEPES, 0.1 EDTA, pH 7.8. Larvae are transferred after 2-3 days (stage 25) to 2 L tanks with tadpole water. When tadpoles reach stage 40 of the Nieuwkoop–Faber (NF) criteria[15], they are placed in 5 L tanks and maintained at a density of 10 animals/L. Temperature is kept constant at 23-25 °C and 18-20 °C for *X. tropicalis* and *X.laevis* tadpoles, respectively. Animals found at stages 48-52 of the NF criteria are used for experiments.

1. Transection of Olfactory Nerves

1.1. Prepare an anesthetizing solution of 0.02% MS-222 in 50 mL of tadpole water at room temperature.

1.2. Prepare a small tank (1-2 L) with tadpole water to allow recovery of animals after surgery.

1.3. Cut rectangular pieces of cellulose qualitative filter paper (4 cm x 3 cm, see **Table of Materials**).

1.4. Wet 2 pieces of cellulose qualitative filter paper in 0.02% MS-222 solution and place them under the dissecting scope.

1.5. Pick a tadpole from the tank and immerse it into the anesthetizing solution. The animal stops swimming within 2-4 minutes and does not react to mechanical stimuli applied at the tail level using tweezers.

1.6. Place the anesthetized tadpole on the rectangular pieces of filter paper. Position the animal with its dorsal side facing upward, so brain structures can be visualized.

1.6.1. Using vannas scissors (see **Table of Materials**) cut one or both olfactory nerves (depending on the type of assay to be carried out). Transect a single nerve for experiments that require an internal control of nerve injury.

1.6.2. For behavioral experiments, transect both nerves in order to suppress all odorant information arriving to the olfactory bulb. The efficiency of sectioning of olfactory nerves can be easily observed under the dissecting scope; however, pigmentation or animal position can be limiting factors.

Note: (Optional) The best way to certify the validity of the procedure is using transgenic tadpoles that express fluorescent reporters on their nervous system (see representative results). To this aim, it is necessary to use a dissection scope equipped with fluorescence (**Figure 1**). If only wild-type animals are available, tracing with CM-dil can be employed. Follow protocol 2 (see below) to inject a 0.5 mg/mL solution of CM-dil prepared in 0.3 M sucrose in the nasal capsule. See [16] for

details on preparation and storage of CM-dil. Leakage of dye out of the principal cavity must be minimize. To this aim, it is necessary to modify the pressure of injection and the opening of micropipettes. Fluorescence at the level of the glomerular layer of the olfactory bulb becomes obvious 24 h after application of CM-dil. The present work uses labeling with CM-dil just to certify the transection procedure; however, this method can also be used to obtain morphological information of olfactory glomeruli using conventional histological procedures.

1.7. Transfer animals to the recovery tank. Tadpoles should recover normal swimming within ~10 min. Perform a careful inspection for the presence of hemorrhages, which are expected in ~ 1% of animals subjected to surgery.

1.8. Euthanize injured animals in a 0.2% MS-222 solution.

2. Labeling of Olfactory Receptor Neurons with Fluorescent Calcium Indicators

2.1. Prepare a solution containing 12% Calcium Green-1-dextran (see **Table of Materials**), 0.1% Triton X-100, and 1 mM NaCl[17]. Store the solution at -20 °C or at -80 °C if it is not to be used within a month.

2.2. Prepare glass pipettes with tip openings ~1-2 µm (similar diameter to microelectrodes used for patch-clamp experiments) for microinjection using a micropipette puller (see **Table of Materials).**

2.3. Calibrate the volume of microinjections. Using distilled water, adjust pressure and injection time in order to obtain injection volumes of 0.15-0.3 µL.

Note: A simple procedure consists in counting the number of pulses required to empty a pipette filled with 1 µL of water. Typical parameters are a pressure of 30 psi and 50 ms injection time.

2.4. Place a pipette in the microinjector and load it with ~ 2 µL of calcium green-1 dextran solution.

2.5. Prepare a tadpole following steps 1.1 to 1.6.

2.6. Move the tip of the pipette into the principal cavity of the nasal capsule.

Note: See **Figure 2A** describing the location of olfactory pathways in a Xenopus tadpole.

2.7. Using settings obtained in 2.3, deliver a couple of puffs. Restrict dye presence to the nasal capsule.

2.8. Let the tadpole rest for 2-3 min. Using a Pasteur pipette, pour drops of 0.02% MS-222 solution on the more caudal parts of the animal to avoiding drying.

2.9. Transfer the animal to the recovery tank.

Note: It should recover normal swimming within ~10 min. Manipulation of animals might cause injuries.

2.10. Euthanize tadpoles that do not recover normal swimming behavior 15 min after injection using a 0.2% MS-222 solution.

2.10. Observe fluorescence at the level of the glomerular layer of the olfactory bulb on the day after injection.

3. Preparation of Tadpoles for Live Imaging of Presynaptic Responses

3.1. 24-48 h before conducting the experiment, coat 4-6 Petri dishes of 35 mm diameter with silicone elastomer (*e.g.*, Sylgard). Once the elastomer has polymerized, fabricate a rectangular well to fit the tadpole.

Note: Typical dimensions for *X. tropicalis* tadpoles found at NF stages 48-52 are 10 mm x 4 mm.

3.2. Prepare 100 mL of a 160 µM amino acid solution acting as an odorant stimulus for tadpoles. The solution can contain a mixture of several amino acids: methionine, leucine, histidine, arginine and lysine. Dilute amino acids in *Xenopus* Ringer's solution, composed of (in mM): 100 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, 240 mOsm/kg, pH 7.8. Ensure that pH is maintained at 7.8.

3.3. Fill an elevated reservoir with 20 mL of the amino acid solution. Connect the reservoir with polyethylene tubing to a 28 G capillary tube (see **Table of Materials**) placed above the nasal capsule.

Note: The capillary tube is mounted on a micromanipulator (see **Table of Materials**). Air bubbles must be absent from the perfusion system.

3.4. Achieve temporal precision in applying the amino acid solution using the transistor-transistor logic (TTL) control of solenoid pinch valves (see **Table of Materials**). A stimulator is used to generate TTL pulses (see **Table of Materials**). Check the temporal precision to deliver the odorant solution by changing the duration of TTL pulses, *i.e.,* 0.1 to 1 s.

3.5. Fill another elevated reservoir with 100 mL of *Xenopus* Ringer's solution.

3.6. Anesthetize a tadpole and place it under the dissecting scope (steps 1.1 to 1.6).

3.7. Prepare a tadpole for imaging. If albino tadpoles are available proceed to step 3.9, otherwise remove the skin above the olfactory bulb because it contains melanocytes that impair imaging (step 3.8).

Note: There are two ways to perform the experiment depending on the pigmentation of the animal. It is preferable to use albino animals. Albino strains are available for *X. laevis* and albino *X. tropicalis* lines have recently been generated by CRISPR-Cas9 [12] or TALENs[18].

3.8. Using vannas scissors, make a lateral incision on the tadpole skin on the edge of the central nervous system. Make the cut should be made at the level of the olfactory bulb and never reaching the position of tectum, which can be easily identified by the location of the optic nerve.

3.9. Pinch the cut skin using tweezers and pull it over the nervous system. Verify successful removal by the absence of melanocytes above the olfactory bulb. Keep the animal moist by pouring drops of 0.02% MS-222 solution using a Pasteur pipette.

3.10. Place the tadpole into the well of the coated dish (see **Table of Materials**). Put a glass coverslip coated with high vacuum grease above the animal. Position the coverslip to cover the top of the tectum to the end of the tail.

3.11. Ensure that the olfactory bulb and placodes remain exposed to the extracellular medium. Keep the tadpole immobile during imaging. Fill the Petri dish with *Xenopus* Ringer's solution containing 100 µM tubocurarine (see **Table of Materials**) to prevent muscle contractions.

Note: Tubocurarine is stored in aliquots at -80 °C no longer than 6 months.

3.12. Place the dish holding the tadpole under an upright microscope. Connect the reservoir containing *Xenopus* Ringer's solution with the dish using polyethylene tubing (see **Table of Materials**) for continuous perfusion of *Xenopus* Ringer's solution to keep the animal alive for > 1 h.

Note: Mini magnetic clamps (see **Table of Materials**) are very useful to stably connect tubing to the dish. Perfusion and suction tubes must be located in ~180° angle.

3.13. Start perfusing *Xenopus* Ringer's solution. Maintain the level of the solution in the dish constant throughout the experiment. Continuously evaluate tadpole viability by observing blood circulation through the vessels.

4. Live Imaging of Presynaptic Ca^{2+} Changes in Olfactory Glomeruli

Note: The imaging procedure is described for wide-field microscopy but could be easily adapted to a confocal microscope by adjusting the acquisition settings. Imaging should be carried out in an upright microscope mounted on an anti-vibration table.

4.1. Visualize the tadpole with a low magnification objective, for example 5X.

4.2. Move the micromanipulator axes to place the capillary delivering the odorant solution on the top of one nasal capsule forming a 90° angle with the olfactory nerve. The flow of odorant solution above the olfactory bulb should be avoided because it might cause turbulences that distort imaging.

4.3. Find the olfactory bulb located ipsilaterally to the nasal capsule (subject to stimulation) using a high magnification, long working distance, water immersion objective: 60X, 0.9 N.A.

4.4. Check the fluorescence emission by eye. Glomerular structures should be obvious (**Figure 2B**).

4.5. Perform live acquisition with a camera suitable for calcium imaging. Define a box containing the entire olfactory bulb, typically of 256 x 256 or 512 x 512 pixels. Set the acquisition frame rate acquisition to 20–40 Hz. Adjust the gain, so that the values of basal fluorescence are ~ 20% of saturation. Acquire a 5 s video.

4.6. Visualize the movie. Check image focus, the absence of movement artifacts and regions containing saturated pixels. Typical fluorescence values of glomerular regions should be of 5000 – 20000 a.u. if using a 16-bit camera. Proceed to the next step if imaging conditions are optimal. Repeat step 4.6 if needed to improve the image quality or adjust gain settings.

4.7. Start a time-lapse acquisition to record responses evoked by olfactory stimuli.

Note: Precise application of the odorant solution is controlled by TTL stimuli. A typical experiment contains a baseline period of 4 s, followed by stimulation times ranging from 0.1 to 0.5 s and a recovery period of 6-10 s.

4.8. Perform repetitive stimulations of odorants for time intervals >2 min. Set the flow rate to 1-1.5 $mL \cdot min^{-1}$. Since the global perfusion is on during all experiments, locally applied amino acids are washed out.

Note: The volume of solution in the dish is ~3 mL.

4.9. Image analysis

4.9.1. Detection of responses

4.9.1.1. Export movies to ImageJ.

Note: The goal is detecting the presence of glomerular regions responding to stimuli.

4.9.1.2. Transform the raw sequence of fluorescence images to a $\Delta F/F_0$ movie. Measure relative changes in basal fluorescence according to the following relationship: $(F-F_0)/F_0$, where F_0 indicates baseline fluorescence levels.

4.9.1.3. Draw regions of interest (ROI) around areas showing putative fluorescence increases during stimulation and record their position in the ROI manager (**Figure 2E**). Draw an ROI to detect background fluorescence levels in an area devoid of glomerular structures.

4.9.2. Quantification of responses.

4.9.2.1. Place the defined ROIs in the raw sequence of fluorescence images. Obtain the mean gray value of selected ROIs for each frame. Transfer the sequence of values obtained to an analysis program (*e.g.,* Igor Pro).

4.9.2.2. Subtract background fluorescence, and then calculate $\Delta F/F_0$ changes for each ROI (Figure. 2F). Plot the increases in $\Delta F/F_0$ for each one of the ROIs selected. Calculate the standard deviation of basal $\Delta F/F_0$ (before stimulation).

Note: A positive response is considered if increases in $\Delta F/F_0$ obtained during stimulation are larger than 2 standard deviations of basal values.

5. Olfactory-Guided Behavior Assay

Note: A schematic diagram of the setup for performing the assay is shown in **Figure 3**.

5.1. Make small holes to fit 1.57 mm O.D. x 1.14 mm I.D. tubing in the upper part of each well of a 6-well dish. Insert the tubing and seal using an epoxy adhesive (see **Table of Materials**).

Note: The modified dish can be re-used many times after thorough wash with distilled water.

5.2. Prepare 50 mL of an amino acid solution containing methionine, leucine, histidine, arginine and lysine (see step 3.2 for details). The concentrations can range from 160 µM to 1 mM. Place 20 mL of the solution in an elevated reservoir.

5.3. Do not feed tadpoles for at least 12 h before the assay. Take 6 tadpoles from their housing tank and place them in 2 L of clean tadpole water to minimize the exposure to odorants.

5.4. Place the modified 6 wells dish on a white LED-transilluminator (**Figure 3**).

5.5. Couple the perfusion inlets to the reservoir containing the amino acid solution using a manifold (see **Table of Materials**). Check the perfusion system and eliminate air bubbles. Fill the 6 wells simultaneously. Adjust the height of the reservoir to allow the delivery of ≥5 mL of odorant solution within ~ 30 s.

Note: Wash each well 4 times with double distilled water after exposure to the odorant solution.

5.6. Fill each well with 10 mL of tadpole water. Place 1 tadpole/well. Leave to rest for > 3 min.

5.7. Set up image acquisition. Use a conventional CCD camera that can acquire images at ≥5 Hz. Connect the camera to a computer. Here, use a Zeiss MRC5 camera controlled by Zen software but other equivalent configurations can be used. If it is necessary to increase frame rate, apply pixel binning. Images should show the whole 6-well dish.

5.8. Start image acquisition such that movies contain basal (30 s), stimulus (25-35 s) and recovery (30-60 s) periods.

5.9. Return animals from the 6 wells dish to tanks after imaging.

5.10. Analyze movies in ImageJ using plugins such as wrMTrck[19,20] that provide multiple parameters associated to motility.

5.11. To prepare images for analysis, first select a well by drawing a rectangular ROI of 35 x 35 mm (**Figure 4A**). Obtain a background image by calculating the maximum projection of the whole sequence. It should display an image of the well without the tadpole.

5.12. Subtract the maximum projection from the raw movie. Perform thresholding on the generated 32-bit movie and apply the wrMTrck plugin. Adjust WrMTrck parameters to reliably track animal movements. Transfer the obtained X,Y coordinates into an analysis program.

5.13. Using X-Y coordinates, calculate the Euclidean distance to the odorant source (perfusion inlet in the well), by applying the following equation:

$$D(os, tad). = \sqrt{((Xos - Xtad)^2 + (Yos - Ytad)^2)}$$

where os indicates the position of the odor source and tad indicates the tadpole position at a given time. See **Figure 4A** for details.

REPRESENTATIVE RESULTS:

In this paper, we present a combination of two complementary approaches to perform *in vivo* study of the functionality of the *Xenopus* tadpole olfactory system: i) a method for imaging presynaptic Ca^{2+} changes in the glomeruli of living tadpoles using a fluorescent calcium indicator, and ii) an odor guided behavioral assay that can be used to investigate the response to specific waterborne odorants. Since these approaches have been employed to evaluate the recovery of olfactory processing after injury[10], a simple method for transecting olfactory nerves is also described.

Transection of olfactory pathways in *Xenopus* tadpoles

There are two ways to certify the validity of the procedure. Both rely on the visualization of olfactory nerves using fluorescent reporters. One method is based on transgenic tadpoles that express fluorescent proteins on their nervous system. Two recommended lines that express GFP under a neuronal β-tubulin promoter are *X. laevis* tubb2b-GFP and *X. tropicalis* NBT-GFP (**Figure**

1, see **Table of Materials**). If only wild-type animals are available, CM-dil can be used (see step 1.8). **Figure 1** shows images of tubb2-GFP *X. laevis* tadpoles. Images are from four different animals where a single olfactory nerve was transected. The cut should be obvious under the dissection scope. The advantage of using transgenic lines is that reformation of the olfactory nerve can be followed over a period of time. When doing sequential observations, it is recommended to minimize exposure to fluorescent light to prevent photodamage. Sectioning of a single olfactory nerve is useful when an internal control is required, as for example, to compare normally developed *vs* rewired glomerular units. Sectioning of both olfactory nerves should be applied when the aim is completely suppressing the transmission of information.

Live imaging of presynaptic responses to olfactory stimuli

The correct labeling of ORNs with calcium green-1 dextran can be observed at the level of the olfactory bulb (**Figure 2A**) using widefield microscopy. Glomeruli are obvious (**Figure 2B**) and should appear distributed in different layers by moving the focus plane. The morphology of glomerular structures can be better resolved if a confocal microscope is used instead (**Figure 2C**). The number of labeled glomeruli depends on dye uptake at the level of the olfactory epithelium. Therefore, this procedure does not allow the visualization of all glomerular units. Animals showing a more intense fluorescence staining of the glomerular region should be selected before performing imaging experiments, since they contain more ORNs labeled. This maneuver is highly recommended in order to increase experimental throughput and should be performed under a dissecting scope equipped with a fluorescence lamp. Reject animals that do not show labeled glomerular units or that show fluorescence restricted to particular areas of the glomerular layer. Presynaptic Ca^{2+} responses can be observed as soon as 1 day after dye loading. For carrying out imaging experiments it is desirable to use objectives of high numerical aperture, typically ≥0.9.

Increases of presynaptic calcium levels can be evoked exposing dendritic knobs of ORNs to amino acids. It is important to position the capillary delivering the odorant solution above the nasal capsule. Care should be taken to avoid contact because it could clog the tip of the capillary and/or cause mechanical stimulation of ORNs. Transient increases in presynaptic calcium levels can be observed for stimuli ≥0.1 s (**Figure 2D**) and are indicative of a correct olfactory transduction. It is also important to visualize basal calcium levels with low camera gain. Presynaptic terminals of ORNs display high fluorescent increases and it is essential to avoid signal saturation. High temporal resolution can be achieved with widefield microscopy. For example, using an electron-multiplied CCD camera, it is possible to achieve frame rates of 50 Hz or higher. Use of confocal microscopy reduces temporal resolution but allows a better definition of glomerular structures.

The high affinity of calcium green for binding calcium (190 nM) is particularly useful to detect small responses. Transient intracellular calcium increases detected in the glomerular layer are variable. Some glomerular regions show changes in $\Delta F/F_0$ ≥0.2, while neighboring processes might not even respond (**Figure 2D**). The following factors contribute to the variability of the response of glomerular units: i) the overall number of labeled glomeruli, ii) the intracellular concentration of calcium green, and iii) the selectivity of ORNs to detect amino acids. Since a too low number of labeled glomeruli might preclude observing responses, it is absolutely necessary to carry out these experiments with animals containing as many labeled ORNs as possible.

Olfactory-guided behavior

Data analysis

Olfactory-guided behavior is studied using a custom-built system. **Figure 3** shows a schematic drawing of equipment used to carry out the assay. The test is based in the ability of tadpoles to detect the presence of amino acids, which act as odorants. A solution combining five different amino acids (methionine, leucine, histidine, arginine and lysine) is used for stimulation. The solution is locally delivered during 30 s to a 35 mm well containing a free-swimming tadpole. The immediate response of tadpoles to the incoming solution is an increase in motility. Random movements occurring during the initial ~5-10 s of solution application are followed by a direct swim towards the source of odorants. Tadpoles remain for several seconds in the vicinity of the nozzle during delivery of amino acids and gradually recover motility in random directions (see **Supplementary Videos 1, 2**).

The experimental conditions described allow the normal swim of *X. tropicalis* tadpoles stages 48-52; however, it must be taken into account that the motility of larger animals might be restricted in 35 mm wells. Tadpole movements are recorded with a CCD camera. Attraction for the odorant solution can be detected as a reduction of the Euclidean distance separating perfusion inlet from tadpole position (**Figure 4**). Tracking of tadpole head positions within an area of 35 mm x 35 mm (or the equivalent size in pixels) allows obtaining a quantitative analysis of olfactory-guided behavior (**Figure 4A**). Individual plots of tadpole movements are constructed using X-Y coordinates obtained by image analysis (**Figure 4B**). The extracted motility plots must faithfully reproduce video images.

There are two possible methods of interpreting olfactory-guided behavior experiments. The first approach is inspired on a previous study using zebrafishes[21]. Measurement of the time spent in the vicinity of the nozzle delivering odorants evidences the presence of a positive tropism. A region of interest of 8.75 mm radius (corresponding to ¼ of the well diameter) centered on the solution inlet it is used to classify the proximity of the animals to the odorant source (**Figures 4A, 4C**). Binning the time spent by tadpoles in the vicinity of the nozzle during defined periods, *i.e.,* 15 s intervals, allows identifying the ability to detect amino acid solutions (**Figure 4D**). The overall behavior of a population of tadpoles can be obtained by plotting the distribution of individual data (**Figure 5A**). A positive tropism can be detected when the solution of methionine, leucine, histidine, arginine and lysine is prepared either at 1 mM or 160 μM (**Figures 5A** and **5B**). Animals do not respond to water application (**Figure 5C**), thus discarding the participation of mechanosensitive mechanisms. Differences among time intervals defined in each experimental group can be established using nonparametric repeated measures ANOVA with Dunn's multiple comparisons test. The disadvantage of binning data in time intervals of 15 s is a reduced temporal resolution.

A way to increase temporal information of the behavioral response is by making average plots of Euclidean distances from the odor source. Although tadpole movements show an intrinsic variability, the average motility of a population of animals (typically ≥40) shows the olfactory-guided behavior. To carry out this analysis it is necessary to group animal positions before the

onset of stimulation. Since tadpoles are found in different locations when the odorant solution enters into the well it is required to set the Euclidean distance to 0 just before stimulation (**Figure 6A**, see also the inclusion criteria in **Figure 7**). Negative and positive values therefore indicate an attraction or a repulsion from the odor source, respectively. An attraction for odors is well described by a linear fit with regression coefficients ≥0.9. If water is delivered, the net changes of Euclidean distance are distributed around 0 and it is not possible to fit a line during odorant stimulation, thus indicating the absence of an olfactory-guided behavior (**Figure 6B**). Comparison of average plots of Euclidean distances for amino acid solutions prepared at 1 mM and 160 µM suggest different delays in the olfactory-guided response (compare **Figures 6A and C**). The time interval required to initiate the movement towards the source of odorants is shorter when amino acids are applied at a higher concentration. A lack of olfactory-guided behavior is observed in tadpoles with both olfactory nerves transected (**Figure 6D**).

A limitation of the described olfactory-guided behavioral assay is the establishment of complex fluid plumes. This can be seen if the amino acid solution is substituted by a dye, such as Fast Green, when setting up the system. The use of colored solutions verifies the formation of plumes and shows that waterborne odorants reach any region of the well within 5 s. Turbulences caused by the delivery of the solution are likely detected by the lateral line of tadpoles and probably contribute to the variability observed in animal motility but do not interfere with olfactory guided behavior. Control experiments carried out by using water instead of amino acid solutions reveal that tadpoles are capable to discriminate olfactory from mechanical stimuli. The estimation of time spent in a region of interest (**Figure 5**) and the average plot of Euclidean distances (**Figure 6**) are two complementary methods to describe the olfactory-guided response of tadpoles.

Inclusion criteria

Inclusion criteria must also be taken into account for data analysis. Some tadpoles show a resonant movement, which is illustrated by the fitting of the plot of Euclidean distances to a sinusoidal function (**Figure 7A**). Tadpoles displaying this behavior must be discarded from all analysis.

The exclusion of animals that at the onset of the application of odorant solutions are at a maximum (>30 mm **Figure 7B**) or a minimum Euclidean distance (< 5mm, **Figure 7C**) from the nozzle allows decreasing the variability of the average plots. The example illustrated in **Figure 7B** shows a positive tropism for the amino acid solution. The tadpole is located at a maximum distance of the solution inlet at the onset of stimulation. Therefore, this relative position can only reveal an attraction for the odor source. **Figure 7C** shows the opposite situation. Here, a tadpole is located in the vicinity of the nozzle delivering the amino acid solution. Quantification of the time spent near the odor source shows a response (method used in **Figure 5**); however, it cannot show a net movement towards the inlet.

In summary, the proposed assay of olfactory-guided behavior defines a binary test. This method can be used to detect the ability of an experimental group of tadpoles to respond to odorants. Further improvements are required if the aim is establishing differences among complex olfactory-guided responses, as for example, determining preferences for given odors.

FIGURE AND TABLE LEGENDS:

Figure 1. Transection of olfactory nerves. Representative images of tubb2-GFP *X. laevis* tadpoles obtained after transection of a single olfactory nerve (arrows). Nerves of tubb2-GFP tadpoles display strong fluorescence. Nerve transection is obvious immediately after surgery (D0). Regrowth of the olfactory nerve is evident 4 days after cut (D4). Eight days after surgery (D8) there is little difference between control and reformed nerves. Tadpoles were anesthetized in 0.02% MS-222 to collect images. Olfactory bulb (O.B.), olfactory nerve (O.N.), nasal capsule (N.C.), tectum (Tec), optic nerve (Op.N.). Arrows indicate the location of the transected nerve.

Figure 2. Labeling of olfactory receptor neurons with calcium green dextran and visualization of presynaptic calcium influx upon stimulation with amino acids. **A)** Transmitted light image of a tadpole showing the location of the olfactory epithelium, olfactory nerves and the glomerular layer of the olfactory bulb. **B)** Image of an olfactory bulb visualized by widefield (wf) microscopy. Neurons were labeled by application of calcium green-1 dextran at the nasal capsule. The fluorescence observed corresponds to presynaptic terminals from olfactory receptor neurons forming glomeruli. O.N: olfactory nerve; O.B: olfactory bulb. **C)** Confocal section located dorsally from the entry of the olfactory nerve to the bulb. The presynaptic component of olfactory glomeruli was labeled using calcium green-1 dextran, as in B). **D)** Presynaptic terminals transiently increase their calcium levels upon exposure of the olfactory epithelium to a solution containing five different amino acids. Relative changes in calcium fluorescence obtained before, during and after 1 s stimulation. **E)** Distribution of 10 different regions of interest (ROIs) used to quantify $\Delta F/F_0$ changes. ROI11 is outside of the glomerular layer and is used to define background levels of fluorescence. **F)** Individual $\Delta F/F_0$ responses for ROIs defined in E).

Figure 3. Olfactory-guided behavior assay. A) Schematic diagram showing the equipment used in the test. **B)** Example of motility tracks recorded over 90 s. Each circle represents a well containing a single animal.

Figure 4. Tracking of olfactory-guided behavior. **A)** Example showing a well used for the behavioral assay. Blue dotted lines indicate the location of X,Y coordinates (in mm) used to track tadpole movements (see also **Supplementary Video 1**). The green ellipse represents the position of the solution inlet. The dotted black line indicates the proximal area to the tube delivering the amino acid solution (see text for details). **B)** Motility of the tadpole shown in A) during the behavioral assay. Color-coded traces indicate the position of the animal before (gray) and after olfactory stimulation (violet). Movements during application of the odorant solution are illustrated in a temporal color gradient (30 s, red to blue). **C)** Using X,Y tadpole positions it is possible to calculate changes in the Euclidean distance from the tadpole head to the perfusion inlet. Distances shorter than 8.75 mm correspond to the proximal area of the nozzle. **D)** Plot of the time spent by tadpoles in the region defined by the dotted line in A). Each dot indicates a 15 s period. The animal is attracted by the odorant solution.

Figure 5. Tadpoles are attracted by amino acids. **A)** Time spent by tadpoles in the vicinity of the odor source. Each bin comprises a 15 s period. Box plots represents the median (black horizontal line), 25 to 75% quartiles (boxes), and ranges (whiskers) of data. Delivery of a 1 mM amino acid solution attracted tadpoles to the odor source. **B)** Tadpoles were attracted by the odorant solution when the concentration of amino acids was reduced to 160 μM. **C)** Delivery of water did not modify animal behavior. Repeated measures ANOVA with Dunn's multiple comparisons test, $p<0.05$.

Figure 6. Temporal response of tadpoles to odorants. A) Plot of the average Euclidean distance to the odor source as a function of time. The Euclidean distance was set to 0 before stimulation in each individual trace. Negative and positive values indicate a decrease and an increase in distance to the odor source, respectively. Attraction to the odor source can be described by a linear fit (r^2=0.98). **B)** Delivery of water does not modify the distance to the odor source. **C)** Tadpoles respond to the application of a 160 μM solution of amino acids as revealed by a linear fit (r^2=0.96). **D)** Tadpoles with both olfactory nerves transected do not respond to amino acids.

Figure 7. Inclusion criteria for the olfactory-guided behavior assay. **A)** Some tadpoles show a resonant movement. This behavior is revealed by successful fit of a sinusoidal function to the plot of the Euclidean distance to the odor source. Tadpoles displaying this activity should be excluded from the test. **B, C)** A way to reduce variability in the average temporal response to odorants (**Figure 6**) is by excluding animals located at a maximum (B) or a minimum (C) Euclidean distance at the onset of stimulation. Red dotted lines indicate the threshold value (30 mm and 5 mm). The Euclidean distance before the onset of stimulation (arrow, left plots) is set to "0" to report attractive or repulsive behaviors as negative or positive values (right plots), respectively.

Supplementary Video 1. Olfactory-guided behavior triggered by delivery of an amino acid solution. The movie shows a tadpole freely swimming on a 35 mm well. The blue ellipse indicates the position of the nozzle delivering the odorant solution. The onset and end of stimulation are indicated by green and red dots, respectively. **Figure 4** shows the analysis of the behavior observed.

Supplementary Video 2. Tadpole motility during delivery of water. The movie shows a tadpole freely swimming on a 35 mm well. The blue ellipse indicates the position of the nozzle releasing MQ water. The onset and end of water delivery are indicated by green and red dots, respectively.

DISCUSSION:

This paper describes techniques that are useful to investigate the functionality of olfactory pathways in living *Xenopus* tadpoles. The current protocol is particularly useful for those laboratories that work, or have access to *Xenopus*; however, it is also interesting for those researchers studying the cellular and molecular bases of neuronal regeneration and repair. Results obtained in *Xenopus* can be combined with data gathered in other vertebrate models to identify conserved mechanisms. The methods described will benefit from the development of genetically modified *Xenopus*[18,22,23] and are applicable to experimental models of nervous system diseases in tadpoles[24,25].

In order to obtain reproducible *in vivo* data, it is key to correctly rear *Xenopus* tadpoles. In particular, *X. tropicalis* is very sensitive to poor housing conditions. For example, they do not tolerate temperatures below 20 °C and should be kept in tanks or water systems in the range of 24 to 28 °C. It is also important to not increase animal density above established limits, regularly feed tadpoles and keep an optimal water quality[13]. Management of animal colonies following standardized conditions is absolutely required for gaining reproducibility of *in vivo* experiments.

The described method of calcium imaging is useful to detect a correct olfactory transduction of ORNs *in vivo*. Loading of ORNs with calcium green-1 dextran is achieved by transient permeabilization of the plasma membrane using a low concentration of Triton X-100, as previously reported[17]. The main advantage of this loading method is simplicity, because it only requires a microinjector. An important drawback is that Triton X-100 causes the transient elimination of olfactory cilia and microvilli. The olfactory epithelium of zebrafish regenerates within 48 h after treatment[17]. Regeneration in *Xenopus* tadpoles might be even faster since responses to odorants can be observed 1 day after dye loading (**Figure 2D**). However, a detailed morphological analysis is required to accurately estimate the regeneration time of the olfactory epithelium after Triton X-100 treatment.

Labeling ORNs with calcium green-1 dextran is only effective in a population of neurons, allowing the visualization of presynaptic terminals with a high signal-to-noise ratio (**Figures 2B** and **2C**). The almost complete absence of background is advantageous if compared to loading of the whole olfactory bulb with AM ester forms of calcium dyes. The number of fluorescent ORNs differs from animal to animal. It is thus necessary to use a broad olfactory stimulus of several amino acids. We have obtained successful results using a solution of methionine, leucine, histidine, arginine and lysine. Other combinations of different amino acids could also be effective. An alternative method to load ORNs with calcium indicators is electroporation[26], which is widely used to express genetically encoded fluorescent reporters in tadpole neurons[27]. Electroporation can be done using commercial or custom-made equipment and allows visualization of neuronal structures with an excellent signal-to-noise ratio[28]. Similarly to the described approach, cell populations labeled are heterogeneous and differ from animal to animal. Transgenesis is desirable if the aim is investigating a defined population of neurons[22]. For example, driving the expression of genetically encoded calcium indicators such as GCaMPs in a restricted group of ORNs, could be very useful to investigate the response of a defined set of presynaptic terminals to odorants.

The described method using calcium-green 1 dextran reports presynaptic terminal function *in vivo*. The observation of intracellular calcium increases is indicative of a correct olfactory transduction and release of glutamate at the level of glomeruli. Quantitative analysis of changes in fluorescence is, however, limited. Stimulation of presynaptic terminals increases intracellular calcium levels to the micromolar range and saturation of a high affinity calcium indicator such as calcium green must be taken into account. Results illustrated in **Figure 2** are obtained using widefield microscopy. This is the simplest approach and can be implemented in most laboratories. Improvement by using two-photon microscopy or genetically encoded fluorescent reporters could allow obtaining more quantitative estimates of presynaptic function.

For live imaging of calcium responses, it is critical that there is appropriate positioning of the capillary delivering the odorant solution. It should be located above the nasal capsule and always avoid direct contact with tissue. Both the correct delivery of the odorant solution and the flow of the perfusion should be checked before placing the tadpole under the microscope. All tubing used for the perfusion must be inspected for air bubbles. Flow changes of the amino acid solution have to immediately respond to successive opening and closing of the solenoid valve. Delays are indicative of the presence of air. It is also desirable to check for correct volume increases or decreases of solution delivered after changing the opening time, *i.e.,* from 0.1 s to 1 s or vice versa. Use a low light intensity while setting experimental parameters (step 4.6) in order to minimize photobleaching.

Although the importance of olfaction in the biology of tadpoles is well-established[29], there is a lack of tests directly assessing olfactory-guided behavior in *Xenopus* larvae. The method described in this paper is a simple test that allows the detection of a response to an odor stimulus in a large population of animals. The recent description of the odorant sensitivity of *Rana catesbeiana* tadpoles for the presence of chemicals in water illustrates the complex mechanisms coupling olfaction to motor behavior[30]. The assay described in this paper takes into account the intrinsic variability of olfactory-guided behavior in tadpoles. The use of a 6-well dish instead of single wells increases experimental throughput. Factors contributing to variability such basal motility, relative position to the perfusion inlet and plumes generated by the odorant solution are overcome by averaging many tadpoles. Approximately 40 independent measurements are required to describe the control attractive response for amino acids.

We propose two types of analysis for the olfaction test. The first approach quantifies the time spent near the odor source over a defined period. It is particularly well suited for statistical analysis. The second approach is based on the average plot of Euclidean distances from the odor source and is useful to describe the temporal response to odorants. Both types of analysis are complementary and come generated by the same data. Interpretation is binary and allows distinguishing animals that sense odorants from those that do not[10].

How could the methods described be useful to the *Xenopus* community? Although the methods are essentially illustrated for wild-type animals, it should be taken into account that genetic possibilities are continuously expanding in the *Xenopus* field. The combined study of *in vivo* ORN responses and the presence of olfactory-guided behavior can also be very useful to investigate the correct processing of olfactory information in *Xenopus* mutants created either by forward or reverse genetic screens[11]. The information provided by calcium imaging and the behavioral assay can be combined. For example, a mutation selectively affecting granule cells of the olfactory bulb would not modify the presynaptic response of ORNs but would probably impair olfactory-guided behavior.

Methods associating cellular and behavioral responses *in vivo* are particularly relevant for the genetic dissection of neuronal circuits. The interpretation of results can be aided by previous morphological works, which have provided an anatomical map of the glomerular layer in tadpoles[31]. Information obtained from olfactory bulb slices of *Xenopus* tadpoles[32] is also very

valuable. Calcium imaging of mitral/tufted cells in olfactory bulb slices has revealed fundamental characteristics of olfactory processing in *Xenopus* tadpoles, as for example the sensitivity of ORNs to different amino acids[33] or the relevance of response latencies in coding of olfactory information[7]. However, brain slices show a limited capacity to reproduce the complex integrative mechanisms associating different neuronal circuits due to the sectioning of numerous neuronal projections. Also, a characterization of the properties of individual glomerular units is yet elusive with the exception of the γ-glomerulus[34]. The question of whether single glomerular units determine specific behaviors in tadpoles will only be answered by combining genetic tools, *in vivo* imaging approaches and behavioral assays.

ACKNOWLEDGMENTS:

We thank the National *Xenopus* Resource RRID:SCR_013731 (Woods Hole, MA). This work was supported by grants from El Ministerio de Economía y Competitividad (MINECO; SAF2015-63568-R) cofunded by the European Regional Development Fund (ERDF), Fundació La Marató de TV3 (111530), and by competitive research awards from the M. G. F. Fuortes Memorial Fellowship, the Stephen W. Kuffler Fellowship Fund, the Laura and Arthur Colwin Endowed Summer Research Fellowship Fund, the Fischbach Fellowship, and the Great Generation Fund of the Marine Biological Laboratory, where a portion of this work was conducted under the auspices of these awards.

DISCLOSURES:

The authors have nothing to disclose.

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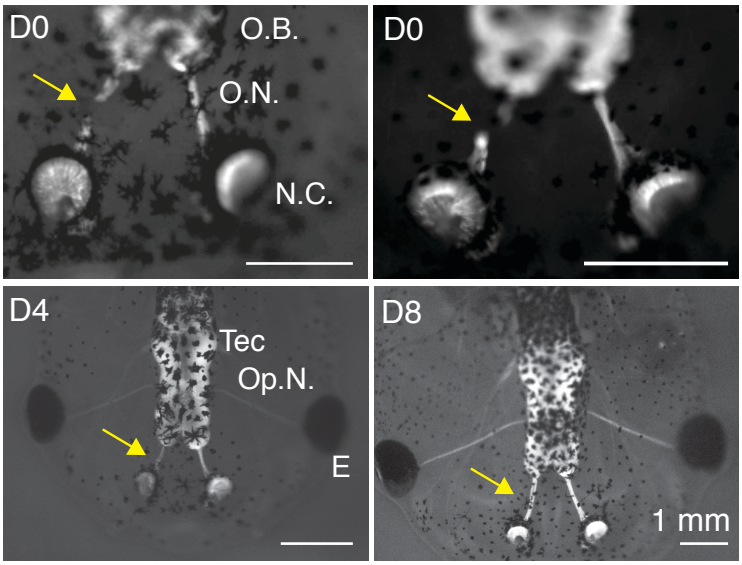
Figure 1



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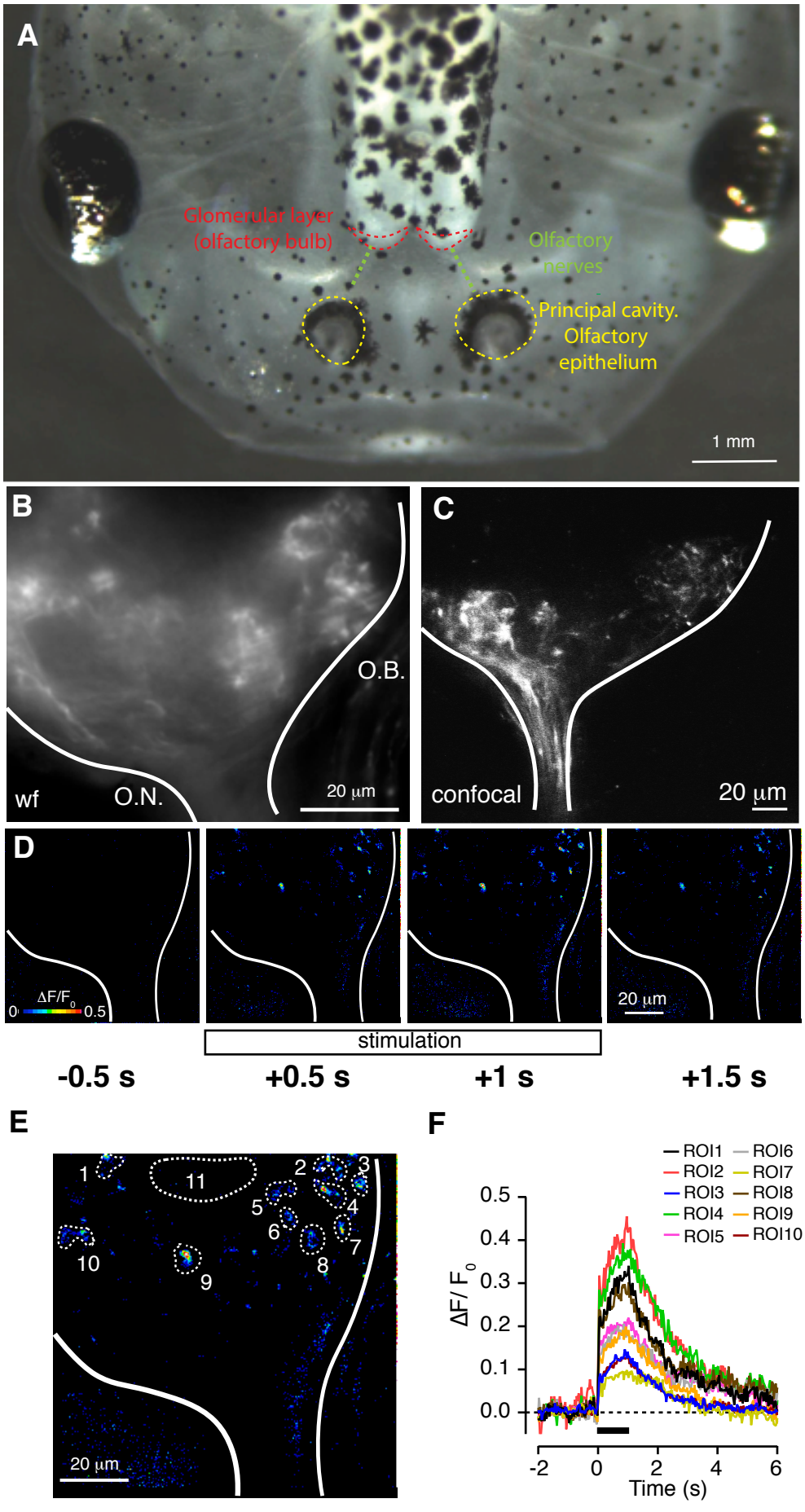

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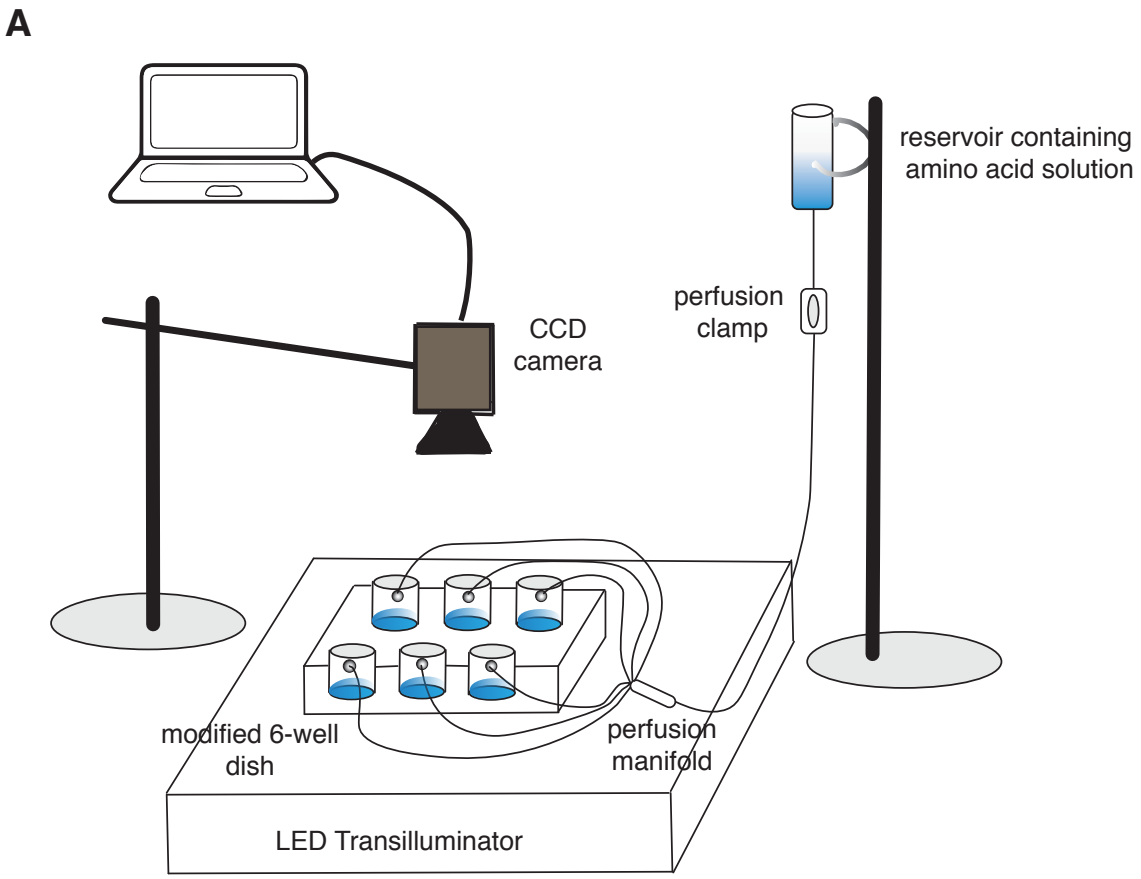

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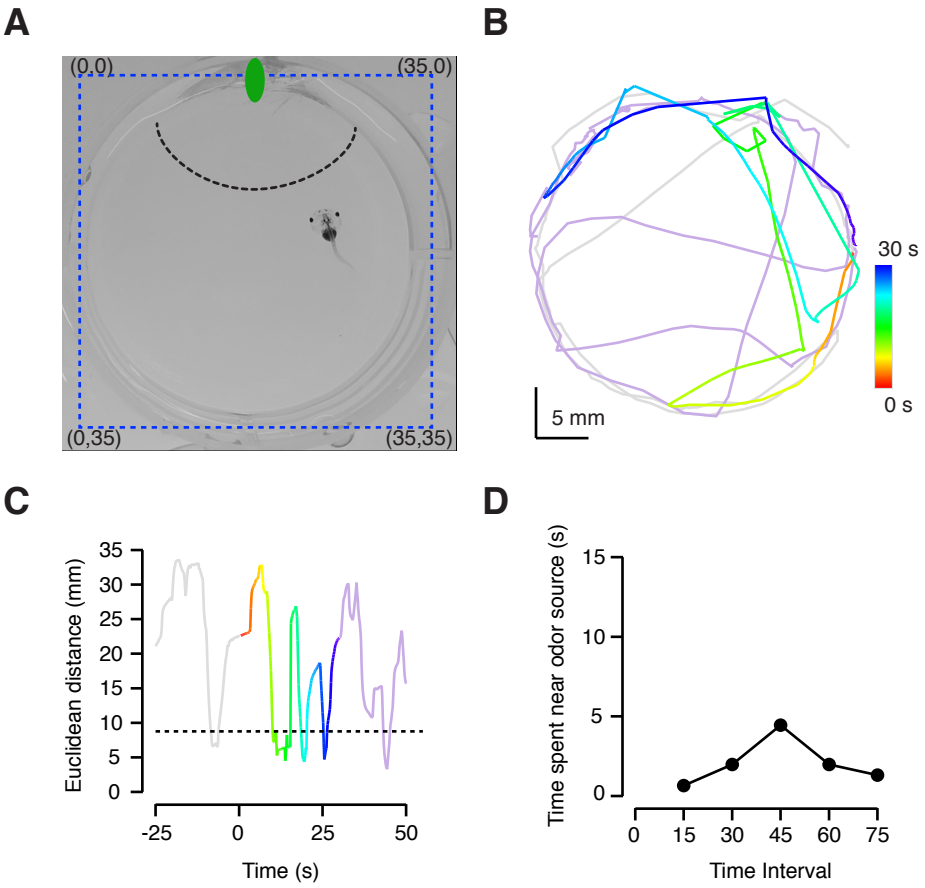

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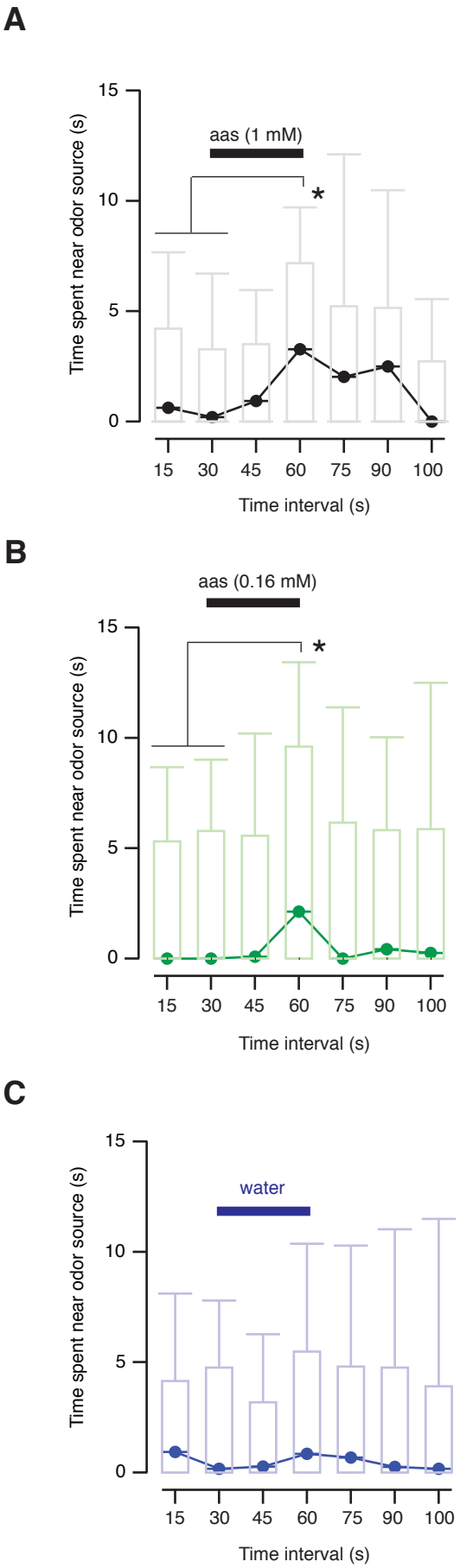

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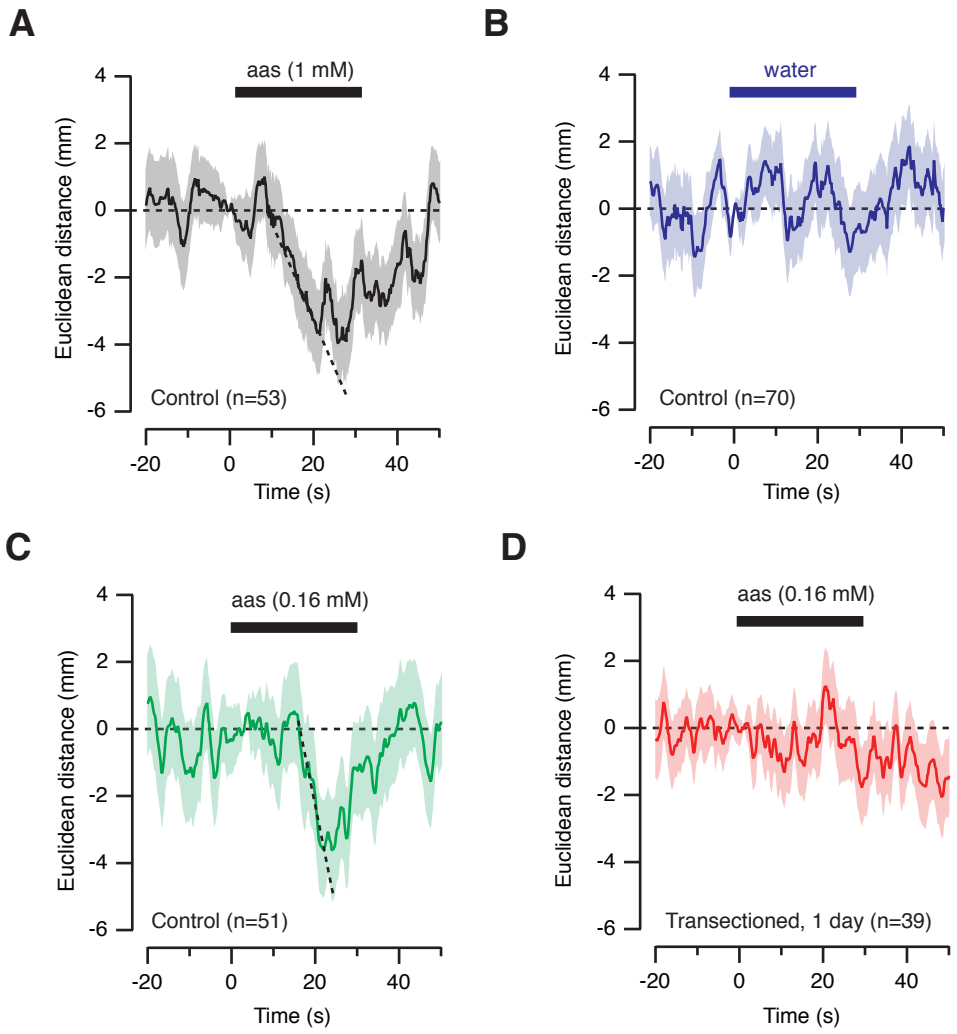
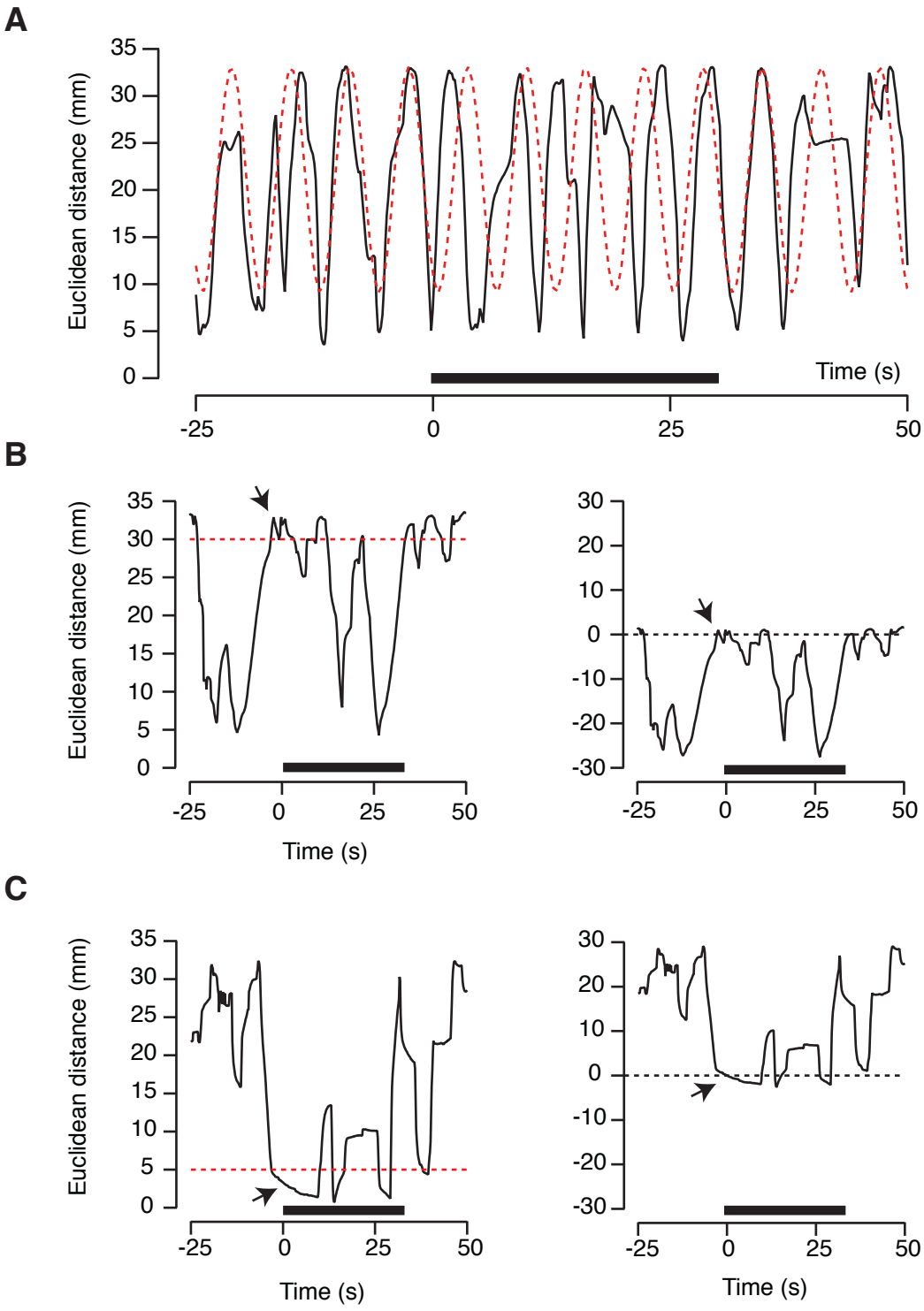


Figure 7



Movie 1

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Movie 2

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Table of materials

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Name of Material/ Equipment
Salts for aquariums (Instant Ocean Salt)
Tricaine (Ethyl 3-aminobenzoate methanesulfonate)
Tweezers #5 (tip 0.025 x 0.005 mm)
Vannas Scissors (tip 0.015 x 0.015)
Whatman qualitative filter paper
X. laevis tubb2-GFP
X.tropicalis NBT-GFP
CellTracker CM-DiI
Calcium Green dextran, Potassium Salt, 10,000 MW, Anioni
Borosilicate capillaries for microinjection
Puller
Microinjector
Sylgard-184
Microfil micropipettes
Upright microscope
Master-8 stimulator
CCD Camera
Solenoid valves
Dow Corning High Vacuum Grease
Tubocurarine hydrochloride
CCD Camera
camera lens

Epoxy resin
Manifold
Micromanipulator for local delivery of solutions
Mini magnetic clamps
Polyethylene tubing

Company

Tecniplast

Sigma-Aldrich

World Precision Instruments

World Precision Instruments

Fisher Scientific

National Xenopus Resource (NXR), RRID:SCR_013731

European Xenopus Resource Center (EXRC) RRID:SCR_007164

ThermoFisher Scientific

ThermoFisher Scientific

Sutter Instrument

Sutter Instrument

Parker Instruments

Sigma-Aldrich

World Precision Instruments

Zeiss

A.M.P.I.

Hamamatsu

Warner Instruments

VWR Scientific

Sigma-Aldrich
Zeiss
Thorlabs

RS Components
Warner Instruments
Narishige
Warner Instruments
Warner Instruments

Catalog Number

XPSIO25R

E10521

501985

501778

WH3030917

NXR_0.0035

C-7001

C-3713

B100-75-10

P-97

Picospritzer III

761028-5EA

MF28G-5

AxioImager-A1

Image EM

VC-6 Six Channel system

636082B

T2379
MRC-5 Camera
MVL8ML3

MP-6 perfusion manifold
MN-153
MAG-7, MAG-6
64-0755

Comments/Description

O.D.=1.0 mm., I.D.=0.75 mm.

Controlled by Zen software
There are multiple possibilities that should be adapted to the camera model used

O.D.=1.57 mm., I.D.=1.14 mm.

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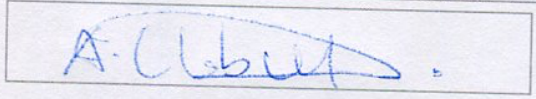
Signature:	 Date: 19 February 2018

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Reviewer #1:

We thank the reviewer for the careful evaluation of our manuscript. We appreciate all comments, which have been very helpful to substantially improve the present work.

Manuscript Summary: The authors outline their methods for using Xenopus tadpoles to measure neural and behavior responses to olfactory stimuli using both control animals and animals where the olfactory nerves are transected. The nerve transection procedure is detailed. To assess the neural responses, the authors provide methods for preparing the tadpoles, loading the olfactory receptor neurons with calcium indicator and using microscopy to measure the responses of these cells at the level of their inputs into the olfactory glomeruli in the olfactory bulb. The authors also describe their methods for delivering olfactory stimuli (amino acid solutions) to wells containing individual tadpoles and reference their methods for analyzing the tadpoles' movements in response to the stimuli.

Major Concerns:

Summary: I am concerned that there are too many details that are omitted from this paper and I fear that an investigator unfamiliar with the procedures or working with the Xenopus tadpoles would not be able to replicate these methods. Adding the details that I have listed in the "minor concerns" would greatly increase the reproducibility of the methods described.

We agree with this reviewer and have explained the methods in greater detail. Due to the particular format of JoVE, we were basically thinking on a video and not in a “real” paper when we submitted the manuscript. We have substantially modified the original manuscript.

The authors should describe their image analysis with much greater detail and add a new section to the PROTOCOL that includes a step-by-step procedure for selecting and analyzing the data.

Points 4.10 and 4.11 now provide all details for image analysis. New figures 2E and 2F also address this point.

I feel that there are major flaws with the behavior analysis section of the paper because appropriate controls have not been conducted (or recommended in the procedure). I have detailed my concerns below.

We are sorry for the lack of clarity explaining the behavioral assay. New figures 3,4,5,6 and 7 provide a thorough explanation of the method. We now show the response of tadpoles to two different odorant concentrations. We also include a detailed analysis of the response of tadpoles to water, as well as inclusion criteria for improving analysis. Two new representative movies are presented: movie 1 (delivery of amino acids) and movie 2 (delivery of water).

The following figures are copied from the author's previous paper [Terni B, Pacciolla P, Masanas H, Gorostiza P ,Llobet A. Tight temporal coupling between synaptic rewiring of olfactory glomeruli and the emergence of odor-guided behavior in Xenopus tadpoles.J Comp Neurol. 2017;525:3769-3783. https://doiorg.proxy.wm.edu/10.1002/cne.24303]. JOVE SUBMISSION vs. TERNI ET AL, 2017

Figure 1 "D4" vs Figure 3d, "D4:

Figure 2A vs. Figure 8a

Figure 2C vs. Figure 8C

Figure 2D vs. Figure 8E

Figure 4C (lower panel, recolored and rescaled) vs. Figure 2C

Please note that the authors of record are not identical between the Jove submission and the Terni et al. 2017 paper. Please confirm that the 2 authors excluded from the JOVE submission did not contribute to the data in the JOVE submission.

According to JoVE guidelines it is possible to reproduce figures from previously published papers. In the present version we keep all the examples shown in Figure 1. New figures 2B and 2D also correspond to the example shown in Terni et al., JCN 2017. Figure 4 comes from an experiment specifically carried out to reply this reviewer. New Fig. 4 is associated to movie 1.

Pau Gorostiza and Helena Masanas were co-authors of the paper published in JCN 2017 but did not contribute to the methods described in the present workr. The current version now includes Margalida Perelló, since she has done most of the new experiments related to the behavioral assay.

L229. Clarification/additional detail. The authors must provide more detail with regard to how to perform the image analysis. How do the authors measure the "F" versus the "ΔF"? What is the baseline response? How long of an interval is required to measure it? Do the authors make a region of interest around the glomeruli and measure the fluorescence only in that area? Is the region of interest made around multiple glomeruli? There is insufficient detail provided here so that others would be able to replicated this procedure.

This point is now addressed in 4.10 and 4.11. Figure 2B shows an image of the raw fluorescence visualized by widefield microscopy. Figure 2D shows images of $\Delta F/F_0$ changes before, during and after stimulation. Figure 2E illustrates the procedure for ROI selection. Figure 2F displays the $\Delta F/F_0$ changes obtained for selected ROIs.

Figure 1. Clarification/modification necessary.
*To make this paper more useful to those researchers relying on this paper who are not familiar with Xenopus, please add a diagram and label the anatomical structures listed in the manuscript, such as: olfactory placode, olfactory nerve, olfactory bulb optic tectum, and other anatomical landmarks.
Figure 1 now shows the location of anatomical structures. Figure 2A shows the position of olfactory pathways in a transmitted light image of a tadpole.

*Please specify how the images of the tadpoles were obtained Are these in vivo images? Does the image sequence show regeneration of the olfactory nerve in the same animal over time? If that is not the case, then provide an explanation to how the animals were selected and how they were prepared for the image acquisition.
Figure 1 shows pictures of living tadpoles. Images show 4 different examples obtained at the indicated times after transection. The goal of this figure is to illustrate a method used for validating the correct transection of an olfactory nerve (section 1, D0 images). Images at D4 and D8 are representative images of nerve reformation. Nerves can be tracked in a single animal over a period of time. See for example Fig. 7b in Terni et al, JCN 2017, however, this is not the purpose of the current work. Images were acquired under a dissection scope equipped with fluorescence. Tadpoles were anesthetized with 0.02% MS-222 to take images. These aspects are now included in figure 1 legend and section 1.9.

Figure 2. Clarification/modification necessary.
*This Figure would be improved by the addition of representative images of baseline/unstimulated fluorescence and the fluorescence evoked by the amino acid stimulus. I suggest expanding Figure 2C into 3 panels that reveal the baseline, stimulated and the ΔF/F % (as shown in Fig. 2C). Indicate in this figure (by drawing the regions of interest) what regions were selected in order to generate Figure 2D.
We have followed these suggestions. See now Figures 2D and E.

*What data is shown in Figure 2D? Are those representative traces from multiple glomeruli? Does the figure represent the response from the same glomerulus to repeats of the same stimulus? If so, which area in the figure was used to generate the data? In the text, the authors state that some glomeruli do not respond at all (as expected). Where is their data in this figure?
Sorry for the lack of clarity. Figure 2F now shows the $\Delta F/F_0$ changes obtained for ROIs indicated in 2E. We first inspected the $\Delta F/F_0$ movie generated in Image J to draw ROIs. The goal was identifying those glomerular regions that were presumably responding to stimulation. Defined ROIs were then placed on the raw fluorescence images and F values were collected. Data were exported to Igor Pro to calculate $\Delta F/F_0$. As it can be seen in Fig. 2F, selected ROIs respond synchronously to stimulation but peak changes in $\Delta F/F_0$ show different values. The heterogeneous sensibility of olfactory receptor neurons to the amino acids applied could contribute to the variability of responses, however, the involvement of other factors such as out-of-focus fluorescence, or differences in the concentration of calcium-1 green dextran in the visualized presynaptic processes could also participate.
The goal of Figure 2 is illustrating sections 2 and 3. We do not attempt to address the complexity of glomerular responses to odorants. Our goal is illustrating how to visualize and analyze the response of presynaptic terminals of olfactory receptor neurons upon olfactory stimulation in vivo.

*Figure 2E requires a scale bar and an outline of the olfactory bulb (as in figures A-C) for context.
OK. Thanks. Now shown in new Fig. 2E.

The example of the "spontaneous" calcium response is not convincing without examples from the time lapse that show the change in fluorescence. Again, without additional explanation it is impossible to know what is plotted in Figure 2F. Does it represent the change in fluorescence from a region around the varicosity labeled with the green arrow?
Sorry for the lack of clarity. Detection of spontaneous calcium transients is not critical for describing the current method. We have increased the details explaining calcium imaging and expanded all sections describing behavior. Since spontaneous calcium transients are occasionally observed we have removed them from the text.

Figure 4.*The authors state (L 371) that Figure 4B represents the "baseline subtracted" responses. Please clarify what is the "baseline."
Baseline makes reference to setting euclidean distance to 0 just before the onset of odorant stimulation. We now include section 6.3.1 that explains data analysis of olfactory-guided behavior. See lines 420-435. Figures 7B and C show the subtraction procedure in two different examples.

*It is impossible to interpret these data without quantification of the control behavior elicited when the input flow is turned on and just water is delivered. The animals could simply be moving in response to the flow of the solution into the well or the substantial increase in the volume of the well. The better way to perform this test would be to measure each animal's orientation response to a water-only input versus the animal's response to the amino acid solution. Paired tests/statistical measures for each individual's response should be conducted.

We have followed reviewer suggestions. Section 6.3. describes in depth how to obtain data and two different methods of analysis. Figure 4, which is associated to movie 1, shows an example of olfactory-guided behavior in a *X. tropicalis* tadpole. Figure 5 illustrates the analysis of the attractive response to odorants using the same amino acid solution at two different concentrations. The absence of effect when water is applied is also displayed. The method of Figure 5 is based on the use of 15 s bins. It is particularly well suited for statistics but compromises temporal resolution. Figure 6 shows an analysis of the temporal response to odorants. Data are shown for stimulation with the solution of amino acids prepared at a final concentration of 160 μM or 1 mM. The presence of a response to odorants can be detected by successful application of a linear fit (r^2>0.9). The behavioral response is not observed when water is delivered or both olfactory nerves are transected. Figure 7 provides further details on data analysis and describes inclusion criteria. For example, tadpoles displaying a resonant motility should be discarded from analysis.

*While the transection data (Figure 4C) supports the authors interpretation, it is not the only control needed because it is lacking a control for the surgical procedure itself. Are the animals with nerve transections not responding to the odorant or not moving because they have had a major surgery the day before the behavior test and have yet to recover from the injury? Compared to the individual control animals (Fig 4B), there appear to be many traces of individual animals in Figure 4C that appear to NOT be moving at all (long horizontal traces). The authors should quantify total spontaneous movement of control vs nerve-transection animals and show that there are no differences between the groups.

Transected animals recover well from surgery. Olfactory nerves are reformed in >80% of the tadpoles four days after transection. It is true that 1 day after transection tadpoles seem to show a reduced basal motility. They could be recovering from surgery or display a reduced exploratory behavior due to the absence of olfaction. We think that these issues are out of the goal of the current manuscript. The main objective of the manuscript is describing a method and it is well illustrated by new Figures 3-7 and section 6.3.

*The authors should use a sham-surgery control for these experiments - where the skin and tissue next to the olfactory nerve is transected.

We have no evidences that surgery might affect behavior. Animals recover very well. A completely normal behavior is observed 4 days after injury (Terni et al, JCN 2017).

6. Movie/Video. There is no explanation of the video. I presume the authors have indicated the inlet tubing blue so that it is more visible. What is the significance of the green and red indicators? (presumably flow On and flow off). I assume "control" signifies an animal that has not had an olfactory nerve transection and not a test where only water is delivered.

Sorry about this. We now show a new movie 1. It is associated to Figure 4. Movie legend is now included. We also show movie 2, which displays the lack of an olfactory-guided response to water.

Minor Concerns:

1. L79. Please clarify - does the author mean that the tadpoles maintain body temperature and avoid heat sources by moving? Not clear why this is relevant as an advantage to the tadpole. Not necessary for the methods presented in this paper.

We attempted to say that work with tadpoles allows performing true physiological experiments at room temperature. This part has been removed. Not relevant.

2. L97-98. Clarification. I'm confused about the animal husbandry. I think that the authors raise the animals using the methods described in reference 15 (Jafkins et al) - if so it would be useful to clarify if the embryos are dejellied and the need to use the MMR media until the embryos are ~2 days old (if that is the case)

Yes, we dejelly embryos. We now provide more details in lines 96-107.

3. L124. Clarification/additional detail. Are the animals restrained during the nerve transection? Can the authors please add detail about how they complete this nerve cut.

Yes, we use cellulose paper. This particular aspect will be visualized in the movie of the experiment. Steps 1.3 and 1.4.

4. L135. Clarification/additional detail. Do you make a high concentration stock solution of the CM-diI? Do you make aliquots and freeze them? Is there a limit to their storage?
5. L148. Clarification/additional detail. Is there a lifetime for the aliquots? How long can they be stored at -80? Do you aliquot the working solution or a concentrated stock solution?
Yes, we do stock solutions of CM-diI. A thorough description of diI labeling is out of the scope of the current manuscript. We quote the reference of Xu et al., Dev. Biology 2008 for further details on the use of diI.

6. L159. Clarification/additional detail. Is there an optimal angle for injecting the olfactory placode? Perhaps the author could elaborate their methods here? Could the authors describe common pitfalls or problems to avoid when injecting the animals
We believe that this question can be clearly shown in the video of the experiment. The main problem to avoid is the leakage of the indicator out of the placode (step 2.7).

7. L169. Clarification/additional detail. Could the authors describe what they consider sufficient labeling? When you screen the animals, what are you looking for?
We are looking for similar images to the examples shown in Fig. 2B and 2C. More detailed explanation is provided in lines 348-361.

8. L176. Clarification/additional detail. Would the authors please explain why one would want to use a mixture of amino acids versus a single amino acid solution?
We are not labeling all glomeruli. Therefore, we must be sure that we can stimulate visualized glomerular structures. A mixture of 5 amino acids acts as a broad olfactory stimulus. Discussed in lines 564-579.

9. L193. Clarification/additional detail. What is the MS-222 solution used here? Do the authors bathe the exposed brain in a MS-222/water solution? If so, does this not damage the exposed nervous system? Or MS-222 in ringer?
The solution of calcium green-1 dextran is applied to the olfactory epithelium. It contains a low concentration of Triton X-100 to allow the penetration of the indicator into cells. For successful loading it is necessary to maintain the presence of the solution in the nasal capsule for approximately 3 min. Tadpoles are kept moist by pouring drops of water containing anesthetic on the more caudal parts of the body. The solution with calcium indicator should not leak out of the nasal capsule. The presence of MS-222 is used to avoid movements. We will show the details of this part of the procedure in the movie of the article.

10. L195. Clarification/additional detail. I am having difficulty visualizing how the animals are restrained. A diagram of the petri dish/tadpole/coverslip/perfusion inlets for amino acids and for the ringer solution is necessary.
We understand and will follow this suggestion. We think that we can take advantage of the video to show this part accurately.

11. L198. Clarification/additional detail. Do the authors make stock solutions and aliquots of the tubocurarine solution? Are they frozen? Do they have an expiration (or, rather, how long have the authors been able to use them?)
We store aliquots of tubocurarine at -80 C. We use them within 6 months of their preparation. Now indicated in line 215-216.

12. L199-200. Clarification/additional detail. How do the authors maintain the constant perfusion? Is it gravity fed/ recirculating pump? What is the flow rate or approximate time to completely flush the 35mm dish? Is there an optimal rate? Where are the input/outs to the dish? Where are they in relation to the position of the tadpole in the dish? Does it matter with regard to washing out the amino acid solutions that are delivered?
These questions are now addressed in sections 3.11 and 3.12.

13. L218. Clarification/additional detail. How many labeled-glomeruli are optimal? Can the authors describe potential problems with the labeling and what would make them reject a tadpole from an experiment?
We cannot say a precise number of labeled glomeruli to carry out the experiment. Since the calcium indicator is only loaded in some olfactory receptor neurons and each glomerulus responds to a given stimulus, the goal is having as many labeled glomeruli as possible. Glomerular structures should be obvious in widefiled microscopy (Fig. 2B) and confocal microscopy sections (Fig. 2C). Those tadpoles that do not show any labeling, or fluorescence is concentrated in restricted areas of the glomerular layer, are rejected from the experiment. We always work with animals showing the most intense and widespread labeling of the glomerular layer, because they allow obtaining responses reliably. Lines 348-361.

14. L226-228. Clarification/additional detail. Is the 2 minute interval suggested because this is the time needed to flush the petri dish chamber? Is there an optimal rate of the global perfusion?

Flow rate is 1-1.5 mL·min^{-1}. The volume of solution in the sylgard dish is ~ 3 mL. The volume of odorant solution applied to the epithelium is ~ 50 µL. Under these experimental conditions a time window of 2 min accounts for the removal of amino acids from the olfactory epithelium.

15. L246. Clarification/additional detail. Please describe the LED transilluminator? Is there a commercial source for it?
Our LED transilluminator was built by inserting an LED strip in a box.

16. L290/291. Clarification/additional detail. Could you add detail please and describe what you consider to be "good" glomerular units? Is it the distinct edges of the glomeruli? The roundness? The separation between glomeruli? Is this affected only by the calcium green dextran labeling? The health of the animals? The position within the olfactory bulb? Is the labeling of "good" glomeruli achievable with practice? Is it achieved only by chance?
Sorry for the lack of clarity. We have modified section 6.2 to address these points. Sections 4.5 to 4.11 now include more details on the procedure. Images 2E-F are also related to these issues. The discussion now addresses the variability related to the procedure used to label olfactory receptor neurons. We compare our method to other established methodologies (lines 564-579).

17. L260. Clarification/additional detail. Would the authors please describe the type of data that is generated by wrMtrack? How do they manage it - what results do they extract from the Image J results in order to do this analysis? These distances are in 2 dimensions, yet the tadpole is capable of moving in the z-space (as well as the X and Y). The authors should consider what is missed because of the loss of this 3D data.
Sections 5.11 and 5.12 now address these questions. The reviewer is right that we are plotting 3D data in two dimensions. A pure movement in the Z-axis will not be detected and the tadpole will appear immobile. However, we are recording an attractive response that is essentially displayed by a translation in the X-Y plane. As shown in movies 1 and 2 tadpole movements are not restricted. Also, movements exclusively occurring in the Z-axis are quite rare for *X. tropicalis* tadpoles NF-stages 48-52.

18. L335-339. Clarification/additional detail & Recommendation. See the major concerns above. I strongly recommend that these are necessary control experiments and that they should be added to the methods. The procedure for eliminating mechanical stimulation should be elaborated, detail added and a new section added to the appropriate methods sections above. For example, I would add a section that details how an investigator could change the flow rate or position of the amino acid inlet tubes so that mechanical stimulation artifact could be detected, and if detected how it could be minimized.
The effect of water delivery on tadpole motility is now shown in Figs. 5C and 6B. See also movie 2.

19. Figure 4.
*To make the explanation clearer for Figure 4A, the authors might consider modifying the sentence at line 369: "Movements during the 35 second application of the amino acid odorant solution are illustrated in a temporal color gradient (yellow to black)." And add, "the amino acid solution (aas) perfusion inlet is labeled with the arrow.
*Adding an arrow along the time axis to the Euclidean distance graph to indicate when the amino acid solution was delivered would also help clarify the experiment.
Thanks. Figure 4 has been modified.

Finally, we would like to thank this reviewer for their thorough review. All the points raised have been useful to improve the original version of this manuscript.

Reviewer #2:

We thank the reviewer for their time and for their careful evaluation of our manuscript.

Manuscript Summary: The authors provide a protocol for experimental procedures to investigate functional aspects of the olfactory system of Xenopus tadpoles. This includes labeling of presynaptic terminals with calcium indicator dye and subsequent functional fluorescence imaging. As second approach to test olfactory perception, the authors observed tadpole swimming reactions after odor exposure. Furthermore, axotomy of the olfactory nerve was applied to disrupt olfactory function.

Major Concerns:
In general, the protocol lacks important protocol details and falls short on correct background information about the olfactory system of the used animal model. Unfortunately, the information about the olfactory system is not sufficient. Many facts stated by the authors are not precise enough and sometimes not even correct. Selected example: Olf. sensory neurons are not stimulated on their cell bodies. Olf. receptors are expressed in cilia on the dendritic knob of these neurons. It is critical that the authors try to match their observations with the known facts about the system. Amino acid sensitivity for example was reported to not be present in the whole olf. bulb as suggested by them. The whole text of the manuscript needs substantial adaptation to include only correct and complete information.

We are sorry if some information provided was not correct. We have corrected the sentence stating that stimulation was on cell bodies. We have reviewed other sentences related to amino acid sensitivity.

For a protocol, it is essential to cover the advantages/disadvantages of using the techniques described by the authors in comparison to already published and widely used methods. This information is missing and needs to be added. E.g., electroporation is a valuable tool for cellular labeling on level of individual cells and large groups of cells (there are numerous publications and protocols supporting this). In general, I am not convinced that the presented methods are robust enough in their present form.

We agree with the reviewer that it is necessary to compare the protocol described with other methods. We now discuss the method of labeling olfactory receptor neurons in the context of other techniques such as electroporation or the generation of transgenic *Xenopus* lines expressing genetically encoded calcium indicators (lines 565-580). We have included more details on calcium imaging (Fig. 2) and the olfactory-guided assay (Figs 3-7). We believe that the methods are robust and can be replicated in any laboratory. Our goal is presenting two complementary assays to evaluate olfactory pathways in living tadpoles. To our knowledge, this is the first time that these types of approaches are described.

Injection of calcium indicator:In stage 48-52 no actual olfactory placode is present anymore. Do the authors mean the olfactory organ/nasal epithelium? Where exactly is the dye solution injected? Concise information about where in the olfactory organ the injection was localized is essential. Main olfactory epithelium or vomeronasal epithelium? Into the nasal cavity without piercing the sensory epithelium or directly into the sensory epithelium? Lateral? Medial? Which cellular layer?
We have substituted the term placode by nasal capsule and olfactory epithelium. Thanks for this comment. The dye was microinjected in the principal cavity without piercing the sensory epithelium.

It is surprising that one injection should be sufficient to label the whole population of olfactory receptor neurons.
We did not say this. Sorry, if it was not clear enough. It would be great to label the whole population of olfactory receptor neurons with a single dye injection but we just label a population. The number of ORNs varies from animal to animal. It is preferable to choose tadpoles with as many neurons labeled as possible. This point is better explained in lines 348-361.

How many of the total number of olf. sensory neurons can be reliably labeled using this method?
We have not quantified the proportion of ORNs labeled.

Is the labeling efficacy uniform between the different regions of the olf. bulb?
A uniform labeling of the glomerular layer was achieved. We did not observe a preference to label lateral or medial regions of the olfactory bulb. A representative population of glomeruli can be visualized with the method used. As we mention in lines 351-359, it is necessary to select animals showing the most uniform labeling of the glomerular layer before carrying out experiments.

Why is TritonX-100 included in the injection solution? TritonX-100 leads to ablation of olf. cilia in zebrafish and has been shown to impair olf. sensory neurons to detect odorant stimulations (Friedrich and Korsching, 1997; here externally applied into the olf. organ). How is the dye taken up by the neurons? How can the authors make sure that injection of TritonX-100 into the nasal cavity or directly into the sensory epithelium does not damage cells or has negative side effects in general? I assume that this approach is not frequently used anymore because of better alternative methods. The authors absolutely need to provide more details on this procedure.
A low concentration of Triton X-100 is used to induce a transient permeation of the cell membrane to allow the entry of calcium green-1 dextran into cells. The application of Triton X-100 might damage cilia of the olfactory epithelium as in zebrafish, however, the observation of the glomerular response to odorant application shows that the possible damage is reverted 1 day after loading with the indicator. Please see steps 2.5 to 2.10 and lines 554-563 of the discussion. It is not clear to us what does the reviewer mean by "better alternative methods". We think that establishing stable transgenic lines expressing genetically encoded fluorescent reporters is definitely better than calcium green injections, albeit way more complex. This view is now described in lines 577-579.

Functional imaging: Why have the authors chosen Calcium Green as functional dye? Alternative available dyes, e.g. Oregon Green Bapta-1, have strongly improved properties and are much better suited.
Calcium green is a great indicator. The use of calcium green dextran avoids compartimentalization and works nicely in our preparation. High affinity calcium indicators, such as calcium green or of oregon green bapta-1 are very good showing if presynaptic terminals respond to stimuli. They have a similar Kd and a comparable brightness. These comments are now included in the text, lines 373-380, 582-587.

The authors do not mention the advantages of their approach to an ex vivo whole mount preparation of the olf. system.
This is a good point. It is now indicated in the discussion, lines 628-640.

The brain of fluorophore-labeled tadpoles is exposed by cutting a window into the "skin" under anesthesia with MS222. I think this preparation needs more details. During the imaging procedure, tadpoles are immobilized by a muscle-relaxant, but are NOT anesthetized with MS222 anymore. The authors report survival times of 1 hour or greater. I am not sure if this preparation even falls into the category "in vivo" or "living animal". I cannot approve this procedure as an ethical approach. There are for sure more gentle experimental procedures to achieve the same experimental goals. It is essential that the authors make sure that this procedure complies with national and European guidelines for animal experimentation using vertebrates.
An ethical committee has previously approved all procedures described (Generalitat de Catalunya, project #9275). We are not allowed and we do not carry out animal experiments that do not comply with European regulations, as in any other country member of the EU. It is necessary to remove melanocytes for in vivo imaging. We now include a new paragraph explaining that albino animals can be used to skip this step (section 3.6). Albino *X. laevis* are widely used. The no-privacy line of *X. tropicalis* has an albino phenotype but reproduces the Hermansky-Pudlak Syndrome (Nakayama et al., 2017). Recently generated lines using TALENs or CRISPR-Cas9 have become available and will be very useful for imaging. Although animals are viable in a time window of 1 h, the recording time is usually 30 minutes or less.

Behavioral assay:Have other groups already used behavioral assays with Xenopus or other amphibian larvae? This should be included in the manuscript.
Yes. We mention two studies: one in zebrafish (line 406) and another one in *Rana catesbeiana* (line 604).

It is unclear what additional information this experiment provides to supplement the calcium imaging.
The goal of the current paper is to evaluate the functionality of olfactory pathways in *Xenopus* tadpoles in vivo. Calcium imaging is useful to visualize the function of ORNs but cannot report on the functionality of the entire circuit. On the other hand, the olfactory-guided assay evaluates a response to odorants without reporting cellular information. We therefore think that both methods provide complementary information. The presence of presynaptic calcium responses indicates a proper olfactory transduction but cannot tell if information is correctly processed downstream. For example, a mutant selectively impairing the function of granule cells could have an impact on olfactory-guided behavior but would not affect olfactory transduction. See lines 621-626 of the discussion.

The wells are very small and the tadpole is very restricted to move. Also the water level is pretty low. As tadpoles move at an inclined angle, I wonder if a natural olf. behavior is even possible here.
Well dimensions do not restrict swimming of *X. tropicalis* tadpoles found at NF stages 48-52. (see movies 1 and 2).

Supported by the presented figures, the authors conclude that their behavioral assay is highly variable. Overall this already questions if the chosen setup is optimally suited for the experimental question asked. I see multiple factors that could be responsible for the high variance. The large volume of abruptly added stimulation solution via gravity

feed is 20/6 ml in comparison to 10 ml solution in the tadpole well. This leads inevitably to pronounced turbulences in the well. Amphibian larvae with their lateral line organ are very sensitive to disturbances in the water column. This in conclusion leads to a massive behavioral response to the mechanical stimulation going along with the stimulation. I think that adding much smaller volumes of olf. stimulation at a gentle speed would lead to less unspecific reactions of the tadpoles. This would decouple mechanical from olfactory stimulation and improve the information content of the tadpole reactions.
In Fig.4A the animal already started its trajectory towards the odor source before the stimulus was added (green trace). After addition of the odor, one could argue that it is actually swimming away from the application site. The presented odorant stimulation lacks a proper control with only tadpole water as suggested also by the authors (P8, L335). This should be easy to include in Fig.4 as additional panel (comparable to B and C). In addition, the reader would benefit from a representative movie file of a behavioral control experiment with no olfactory stimulus included in the gravity feed. This allows the direct comparison of a stimulation and a proper control.

The analysis of olfactory-guided behavior faces variability. This is because we are not looking at a reflex carried out by a well-defined group of neurons and few synapses. Tadpoles sense odorants and elaborate a *behavioral* response. Under the current experimental conditions we see an attraction for the source of odorants, which is reminiscent of the behavior observed in zebrafishes (Koide et al, 2009). Olfactory-guided behavior is initiated by chemotransduction in ORNs and finishes with the activation of certain pools of motor neurons. Since information is processed at multiple levels of the nervous system, we cannot expect an identical response of all animals.
Having said this, the reviewer is right that the lateral line could be involved in the responses observed. We apologize for not showing all data obtained with the delivery of water. We now include this information in Figures 5C and 6B. We also include new movies 1 and 2, which show the response to amino acids and water, respectively.

I also think that the time that the tadpoles have to get used to the experimental tank before the actual experiment is critical to decrease variability: 3 mins after transfer seems too short.
We found that 3 min is a valid time window for the described experimental conditions. The animal was previously placed in a tank with clean water during >10 min to minimize exposure to odorants.

The evaluation is not clear in how background activity was removed.
It is now better explained in lines 421-429. See also Figs. 7B and C.

Minor Concerns:Abstract:P1, L45: The authors show no processing of information.
We consider that odorants are information; therefore, the methods described evaluate the processing of olfactory information in vivo.

P1, L46: The methods presented are not used in combination. It is not clear how they should be purposefully combined.P1, L52:
We think that methods presented can be used alone or in combination. For example, they can be combined to detect when tadpoles recover the ability to process olfactory information after nerve transection (Terni et al., JCN, 2017).

Detectable presynaptic function is already proving an odorant stimulus detection. I think further comprehensive analysis in conjunction with a binary behavioral test is very limited.
We do not agree. As we said before, interpretation of odorant stimuli is complex. A correct chemotransduction does not necessarily imply the proper processing of odorant information. For example, the methods proposed can be very useful to detect if mutant tadpoles generated by forward or reverse genetics correctly process olfactory information. Calcium imaging of ORNs only detects if chemotransduction is correct. Putative downstream defects can be identified by the analysis of olfactory-guided behavior (lines 618-626).

Introduction:

P2, L63: "they" missing. Reference missing
Thanks. Corrected.

.P2, L73: How can ORN regeneration be a fundamental function of the OB?
Rephrased. Functionality of olfactory pathways.

P2, L77: I do not like the comparison of tadpole to rodents. It is unnecessary and does not even point out relevant advantages. A long, accessible olf. nerve suited for axotomy could be a well suited one. Why is olf. stimulation with air mixture a problem?

We respect the opinion of this reviewer but many experiments investigating the olfactory system are carried out in rats or mice. We think that it is easier to prepare a solution rather than making mixtures in air, however, we agree that this is a subjective point of view and have removed it.

Protocol:P3, L118: No definition for anesthetizing solution given.
Now specifically stated that the MS-222 solution is the anesthetizing solution (line 112).

Connect to MS222.P3, L122: What does dorsal position mean?
Dorsal side facing upward (line 123).

Clarify.P3, L128: The successful axotomy is critical for the correct interpretation of subsequent experiments. I think it is crucial to only use animals that were correctly and completely axotomized. "Usually" implies that this was not the case.
All animals used were correctly axotomized. We have substituted usually by easily. We used the term "usually" to indicate that very often was possible to observe transection by eye, immediately after surgery. Point 1.8 is linked to 1.9, stating methods to certify the validity of the procedure. We always inspected nerve fluorescence after surgery in NBT-GFP or tubb2-GFP tadpoles (Figs. 1A and B) when we were setting up the procedure. We are completely sure of the efficiency of the surgery applied. Nerve transection is optimally carried out in lines showing fluorescent nerves but can be perfectly applied to WT animals. This is why we suggest the use of DiI.

P4, L157: What kind of microinjector? Check for naming of the used materials.P4,
Parker Instruments, picospritzer III (table of materials)

L159: Replace placode with a more meaningful and more concise location. See above.
OK. Principal cavity of the nasal capsule.

Results:P6, L275: Why did the authors choose 4 different animals for figure 1? Would be nicer to see the change in an individual animal.
The aim is displaying illustrative images of the procedure and the reformation of the olfactory nerve. A sequence of images from the same tadpole is shown in Terni et al., JCN 2017 Fig 7b.

P7, L295: ORNs are not activated by stimulating their cell bodies with amino acids.
Sorry about this confusion. Corrected (dendritic knobs).

P7, L300: What is low/high? Too vague. Better give clear suggestions
We now provide quantitative parameters. See section 4.7.

.P7, L301: It is essential to avoid signal saturation.
OK. Corrected.

P7, L304-309: Information given here is completely decoupled from the properties of the olf. system. Of course, different glomerular modules have a variation in the activation pattern depending on the stimulus. This is different from the labeling efficacy that needs to be addressed separately. I doubt that all or most glomerular units can be activated just by multiple amino acids. The authors need to check if their results are in line with previous reports on the olf. system of Xenopus. I assume that the labeling with calcium green is not complete.
We have now addressed this issue throughout the paper. We do not label all glomerular units with the indicated procedure. If we choose animals with very few glomeruli labeled we will not get a response. However, animals showing a strong labeling of the glomerular layer reliably respond to stimulation with a mixture of 5 amino acids. See section 6.2 and 565-579 of the discussion.

P7, L317: How is this done and how is the head defined? Include proper definition.
Eyes are easily identified in images. The head corresponds to pixels located in the middle of both eyes.

P8, L324: How are net changes determined and calculated? Please provide more details.
Yes. Now indicated in lines 420-428. See also Figs. 7B and C.

Figure legends:P8, L349: It is not clear that fig 2 depicts the olf. bulb.
We now include new Fig. 2A and indicate the position of the olfactory nerve and olfactory bulb in 2B.

P8, L354: see above. The amino acids are not a source of natural odorants.
OK. Corrected.

Discussion:P9, L395: Provide reference.
The goal of this sentence is to highlight the importance of rearing tadpoles in standardized conditions. Poor housing conditions affect animal welfare and will likely impair many functions of the nervous system. This sentence has been rephrased in more general terms.

P10, L412: Provide reference.
OK. Terni et al., JCN 2017. Now line 617.

P10, L421: Why is a red fluorescent reporter useful?
For example, genetic labeling of ORNs with mCherry could be combined with imaging of calcium green responses. The ratio between green and red fluorescence could be used to obtain a more quantitative analysis of calcium signals. Red fluorescence could also be informative of the morphology of axonal processes and be useful to evaluate the location of calcium green signals.

Fig 2:The olf. bulb is big and has different regions. On which level of the olf. bulb were the confocal images taken. Provide localization details about the specific regions, specifically dorsal/ventral parts of the bulb. It would help to overlay the regions of interest that the activity time courses were extracted from.
Fig. 2C shows a confocal section taken dorsally from the entrance of the olfactory. Now indicated in the figure legend.

Fig 4:Panel B and C include wrong y-axis labels.
Y-axes are correctly labeled. Now the procedure for obtaining plots of euclidean distances is better explained throughout the manuscript.

Reviewer #3:

We thank the reviewer for their time and for their careful evaluation of our manuscript.

This paper outlines a set of methods for studying olfactory-mediated neuronal and animal behavior using video imaging and microscopy in Xenopus tadpoles. The general utility of the method provided seems rather limited to tadpoles, but could potentially of interest for studying more general phenomena like nerve regeneration or aversive odorant conditioning. There are two serious concerns I have about this paper. First is the poor quality of the English writing, which was too severe for this reviewer to correct completely, though some suggestions are given. Copy editing by a native speaker of English should absolutely be performed BEFORE resubmitting.
We are sorry for the quality of the English writing of our previous version. We have carefully reviewed the manuscript. The current version contains three more figures and movie 1 has been substituted.

A more serious second concern is that the experimental data presented in figure 4 and in the video appear to misrepresent the experimental procedure. It is apparent in the video that what is presented as olfactory-guided behavior is much more likely to simply be suction of the animal toward a nozzle by water pressure. Tadpoles NEVER swim backwards, yet there are clear examples in the video of a tadpole apparently caught in a backflow of medium that appear to be erroneously presented as guidance behavior. While I am not certain that intentional misrepresentation has occurred here, I would definitely need to see convincing evidence that the flow of medium is not impacting the movement of the animal.
The odorant solution was kept in an elevated reservoir. Therefore, there was always a positive pressure of solution flowing into the well. The observation of the reviewer might be related to the turbulences caused by fluid plumes. We acknowledge the point raised by the reviewer and now include new movie 1, which is analyzed in Fig. 4. Movie 2 now shows the (lack of) response to delivery of water.

Line 41, the word "gather" does not make sense here.
Thanks. Corrected.

Line 49, Rephrase as "Experiments are presented using both wild-type and transgenic animals expressing fluorescent reporters in central nervous system cells."
Corrected

Line 51 remove word "performing"
Corrected

Line 53 change "relevant to unravel" to "powerful for unraveling"
Corrected

Line 63 insert "THEY" between "because" and "are".
Corrected

Line 63 "Regenerate" is a better word than "reform"
Corrected

Actually the quality of the English throughout the paper is unacceptable and should be fully edited by a native speaker.
We rushed to deliver the manuscript on the deadline agreed with the editor. We are sorry for the poor quality of the English writing. Several members of the lab have now read the paper. One of them is a native English speaker. We hope that the quality of English writing is not an issue in the current version of the manuscript.

Line 86 refers to secretion of molecules by glia (ref 14), but this reference does not contain details about Xenopus glia or transgenics and is thus inappropriate for this sentence.
Thanks. Corrected.

Line 93 "among" should be "of"
We have changed the sentence.

Line 101 what is "in vitro fertilization of adult animals"?

Sorry. In vitro fertilization.

Line 104 Nieukoop and Faber reference should appear on line 102.
Corrected.
Line 122 "in dorsal position" should be "dorsal side facing upward"
Corrected.

Line 125 IT is necessary.
Corrected.

Line 145 2% MS-222 is too highly concentrated. Most standard euthanasia solutions use 0.2%
Thanks for highlighting this issue. 2% MS-222 is the highest concentration used for euthanasia. We usually employ 0.2%. Now corrected.

Line 160, is it one pulse or 150-300nL volume that you inject?
Yes. "Deliver a single puff".

Line 211 how do you prevent animals from moving? If they are still MS222 anesthetized, won't this alter olfactory responses? MS222 is thought to be a Na channel blocker, presumably necessary for transmission of signal from the olfactory pits to the olfactory bulb.
Animals are anesthetized with MS-222 for removing the skin above the olfactory bulb. After surgery is completed, tadpoles are transferred to the imaging chamber. Tadpoles can be kept alive for >1h under continuous perfusion with *Xenopus* Ringer. This Ringer contains tubocurarine to prevent muscle contractions. The response of olfactory receptor neurons is not affected by tubocurarine.

Line 353 How are regions of interest determined? Please provide more detail and if possible a drawing on the image.
Yes. Now indicated in Figs. 2B-F.

Line 418 raises the possibility of using transgenic animals, but they are not really a critical part of this paper as written.
We obviously did not convey our message. The described in vivo assays could be particularly relevant in the context of genetically modified animals. For example, Fig.1 shows the importance of transgenic tadpoles to visualize the transection and reformation of olfactory nerves. But this is just an example. We do all imaging with calcium green-1 dextran. The same imaging approach could become very powerful substituting calcium-green by GCaMPs (line 577-580). The behavioral assay could be useful to evaluate the processing of olfactory information in mutant tadpoles (lines 85-87 and 618-626).

No legend is provided for the video. This makes it difficult to understand exactly what is shown, but I presume it is a demonstration of attraction of a tadpole toward an odorant source?
We are deeply sorry for this mistake. It is unacceptable. The legend was not included. The reviewer is right in their interpretation.

It is apparent at 0:25, 0:33 and 0:40 that the tadpole, or debris in the well are being actively sucked toward the odorant source by negative pressure. Tadpoles cannot swim backwards as appears to occur at 0:25. At the other times you can see small particles rapidly moving toward the odorant "inlet". This raises the very serious concern that the "attraction" of the tadpole by the odorant is in fact an "artifact" of this very powerful suction rather than an olfactory-driven behavior. I presume the authors have made a mistake by including this data in the paper, but it raises questions about the data presented in Figure 4. I would like to see this video presented together with supporting evidence that there is not a net negative pressure pulling the animal toward the odorant source.
There is no suction by the nozzle because there is a constant delivery of the solution. The olfactory-guided response is not a reflex. Tadpoles sense odorants and elaborate a *behavioral* response. Under the current experimental conditions we see an attractive response, which is reminiscent of the behavior observed in zebrafishes (Koide et al, 2009).
Olfactory-guided behavior is initiated by chemotransduction in ORNs and finishes with the activation of certain pools of motor neurons. Since information is processed at multiple levels of the nervous system, we cannot expect an identical response of all animals. The contribution of mechanosensation is now illustrated by the (lack of) response to water (Figs. 5C and 6D, movie 2). The section of representative results of olfactory-guided behavior has also been expanded.