Video Article

Functional Evaluation of Olfactory Pathways in Living *Xenopus* Tadpoles

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/58028/

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[4,5,6]. 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 #1,400 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). 1x MMR 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. Prepare an anesthetizing solution of 0.02% MS-222 in 50 mL of tadpole water at room temperature.
2. Prepare a small tank (1–2 L) with tadpole water to allow recovery of animals after surgery.
3. Cut rectangular pieces of cellulose qualitative filter paper (4 cm x 3 cm, see **Table of Materials**).
4. Wet 2 pieces of cellulose qualitative filter paper in 0.02% MS-222 solution and place them under the dissecting scope.
5. Pick a tadpole from the tank and immerse it into the anesthetizing solution. The animal stops swimming within 2–4 min and does not react to mechanical stimuli applied at the tail level using tweezers.
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. 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.
   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-diI can be employed. Follow protocol 2 (see below) to inject a 0.5 mg/mL solution of CM-diI prepared in 0.3 M sucrose in the nasal capsule. See [16] for details on preparation and storage of CM-diI. 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-diI. The present work uses labeling with CM-diI just to certify the transection procedure; however, this method can also be used to obtain morphological information of olfactory glomeruli using conventional histological procedures.

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

2. Labeling of Olfactory Receptor Neurons with Fluorescent Calcium Indicators

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. 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**).
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.
4. Place a pipette in the microinjector and load it with ~2 μL of calcium green-1 dextran solution.
5. Prepare a tadpole following steps 1.1 to 1.6.
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.
7. Using settings obtained in 2.3, deliver a couple of puffs. Restrict dye presence to the nasal capsule.
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.
9. Transfer the animal to the recovery tank.
   NOTE: It should recover normal swimming within ~10 min. Manipulation of animals might cause injuries.
10. Euthanize tadpoles that do not recover normal swimming behavior 15 min after injection using a 0.2% MS-222 solution.
11. 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

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.
2. Prepare 100 mL of a 160 µM to 1 mM 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. 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.
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.
5. Fill another elevated reservoir with 100 mL of *Xenopus* Ringer's solution.
6. Anesthetize a tadpole and place it under the dissecting scope (steps 1.1 to 1.6).
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].
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.
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.
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.
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 solutioncontaining 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.
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.
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.

1. Visualize the tadpole with a low magnification objective, for example 5x.
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.
3. Find the olfactory bulb located ipsilaterally to the nasal capsule (subject to stimulation) using a high magnification, long working distance, water immersion objective: 60Xx, 0.9 N.A.
4. Check the fluorescence emission by eye. Glomerular structures should be obvious (**Figure 2B**).
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.
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 5,000–20,000 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.
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.
8. Perform repetitive stimulations of odorants for time intervals >2 min. Set the flow rate to 1–1.5 mL·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.
9. **Image analysis**
   1. Detection of responses
      1. Export movies to ImageJ.
         NOTE: The goal is detecting the presence of glomerular regions responding to stimuli.
      2. Transform the raw sequence of fluorescence images to a ΔF/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.

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.

2. Quantification of responses.
   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).
   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**.

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.
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.
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.
4. Place the modified 6 wells dish on a white LED-transilluminator (**Figure 3**).
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.
6. Fill each well with 10 mL of tadpole water. Place 1 tadpole/well. Leave to rest for >3 min.
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.
8. Start image acquisition such that movies contain basal (30 s), stimulus (25–35 s) and recovery (30–60 s) periods.
9. Return animals from the 6 wells dish to tanks after imaging.
10. Analyze movies in ImageJ using plugins such as wrMTrck[19,20] that provide multiple parameters associated to motility.
11. To prepare images for analysis, first select a well by drawing a rectangular ROI of 35 mm 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.
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.
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-diI 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 (**Figure 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 (**Figure 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 **Figure 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.
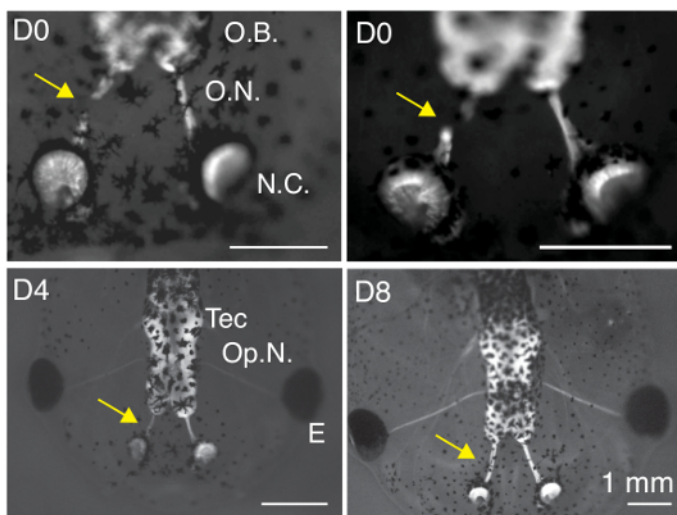

Figure 1: Transection of olfactory nerves. Representative images of tubb2-GFP *X. laevis* tadpoles obtained after transection of a single olfactory nerve (arrows). Nerves of tubb2-GFP tadpoles display strong fluorescence. Nerve transection is obvious immediately after surgery (D0). Regrowth of the olfactory 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. Please click here to view a larger version of this figure.
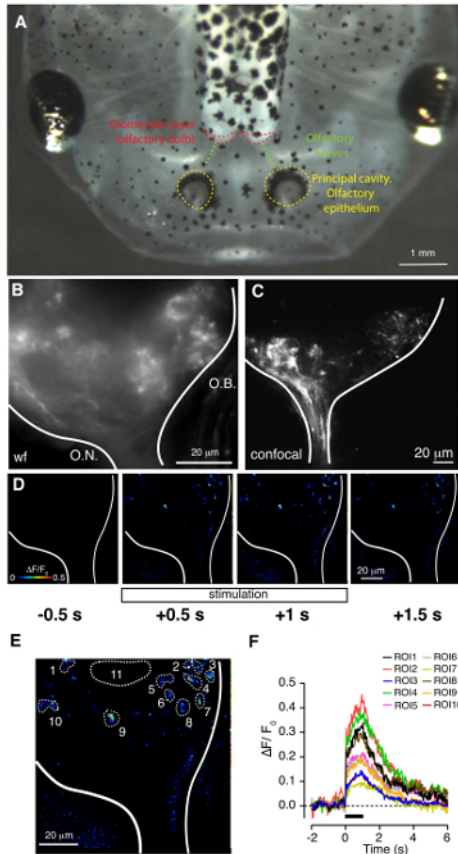
Figure 2: Labeling of olfactory receptor neurons with calcium green dextran and visualization of presynaptic calcium influx upon stimulation with amino acids. (**A**) Transmitted light image of a tadpole showing the location of the olfactory epithelium, olfactory nerves and the glomerular layer of the 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). Please click here to view a larger version of this figure.
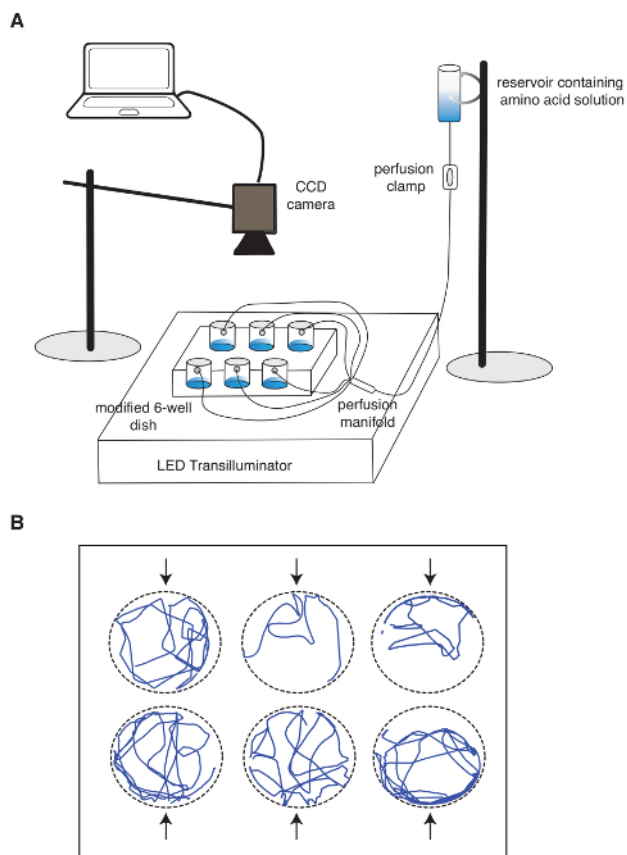
Figure 3: Olfactory-guided behavior assay. (**A**) Schematic diagram showing the equipment used in the test. (**B**) Example of motility tracks recorded over 90 s. Each circle represents a well containing a single animal. Please click here to view a larger version of this figure.
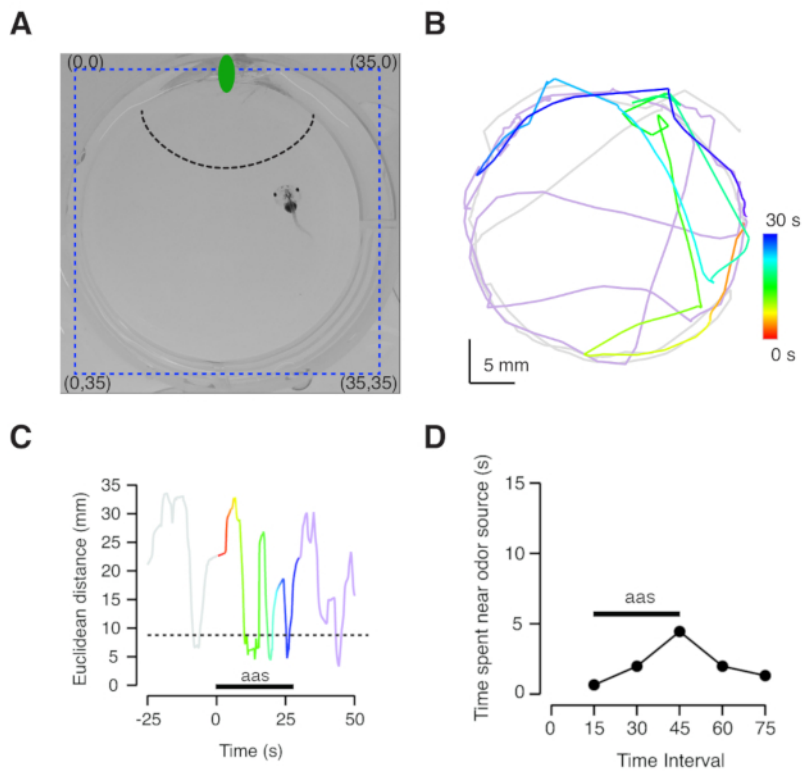
Figure 4: Tracking of olfactory-guided behavior. (**A**) Example showing a well used for the behavioral assay. Blue dotted lines indicate the location of X,Y coordinates (in mm) used to track tadpole movements (see also **Supplementary Video 1**). The green ellipse represents the position of the solution 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. Please click here to view a larger version of this figure.
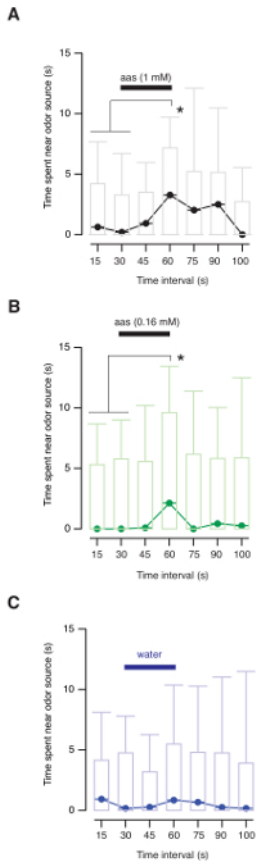
Figure 5: Tadpoles are attracted by amino acids. (**A**) Time spent by tadpoles in the vicinity of the odor source. Each bin comprises a 15 s period. Box plots represents the median (black horizontal line), 25 to 75% quartiles (boxes), and ranges (whiskers) of data. Delivery of a 1 mM amino acid solution 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. Please click here to view a larger version of this figure.
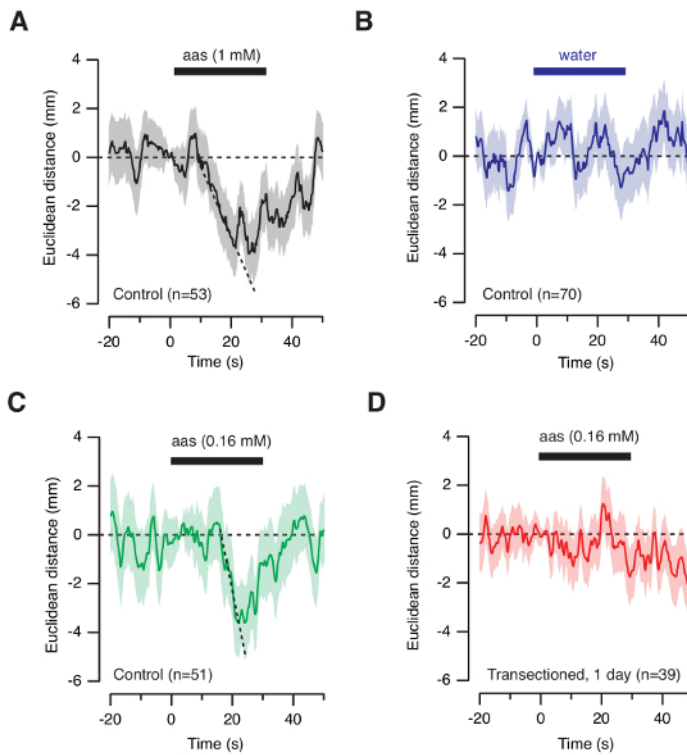
Figure 6: Temporal response of tadpoles to odorants. (**A**) Plot of the average Euclidean distance to the odor source as a function of time. The Euclidean distance was set to 0 before stimulation in each individual trace. Negative and positive values indicate a decrease and an increase in distance to 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. Please click here to view a larger version of this figure.
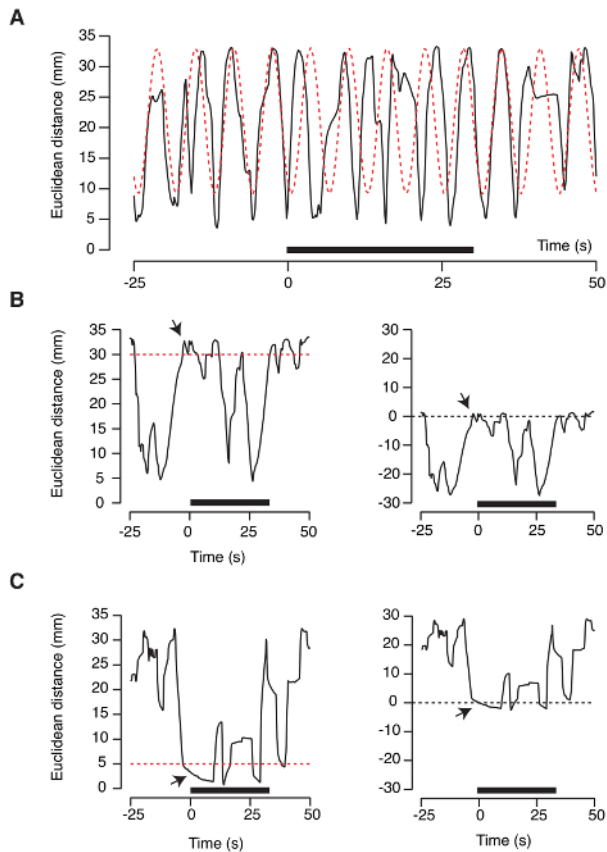
Figure 7: Inclusion criteria for the olfactory-guided behavior assay. (**A**) Some tadpoles show a resonant movement. This behavior is revealed by successful fit of a sinusoidal function to the plot of the Euclidean distance to the odor source. Tadpoles displaying this activity should be excluded from 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. Please click here to view a larger version of this figure.
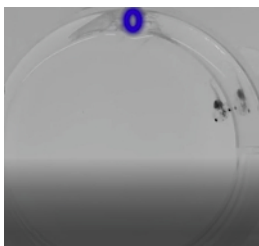
Supplementary Video 1: Olfactory-guided behavior triggered by delivery of an amino acid solution. The movie shows a tadpole freely swimming on a 35 mm well. The blue ellipse indicates the position of the nozzle delivering the odorant solution. The onset and end of stimulation are indicated by green and red dots, respectively. **Figure 4** shows the analysis of the behavior observed. Please click here to view this video. (Right-click to download.)
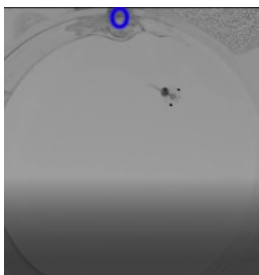
Supplementary Video 2: Tadpole motility during delivery of water. The movie shows a tadpole freely swimming on a 35 mm well. The blue ellipse indicates the position of the nozzle releasing MQ water. The onset and end of water delivery are indicated by green and red dots, respectively. Please click here to view this video. (Right-click to download.)

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 (**Figure 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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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