Video Article

The Olfactory System as a Model to Study Axonal Growth Patterns and Morphology *In Vivo*

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Abstract

The olfactory system has the unusual capacity to generate new neurons throughout the lifetime of an organism. Olfactory stem cells in the basal portion of the olfactory epithelium continuously give rise to new sensory neurons that extend their axons into the olfactory bulb, where they face the challenge to integrate into existing circuitry. Because of this particular feature, the olfactory system represents a unique opportunity to monitor axonal wiring and guidance, and to investigate synapse formation. Here we describe a procedure for *in vivo* labeling of sensory neurons and subsequent visualization of axons in the olfactory system of larvae of the amphibian *Xenopus laevis*. To stain sensory neurons in the olfactory organ we adopt the electroporation technique. *In vivo* electroporation is an established technique for delivering fluorophore-coupled dextrans or other macromolecules into living cells. Stained sensory neurons and their axonal processes can then be monitored in the living animal either using confocal laser-scanning or multiphoton microscopy. By reducing the number of labeled cells to few or single cells per animal, single axons can be tracked into the olfactory bulb and their morphological changes can be monitored over weeks by conducting series of *in vivo* time lapse imaging experiments. While the described protocol exemplifies the labeling and monitoring of olfactory sensory neurons, it can also be adopted to other cell types within the olfactory and other systems.

Video Link

The video component of this article can be found at https://www.jove.com/video/52143/

Introduction

The lifelong turnover of sensory neurons distinguishes the olfactory system from many other sensory and neuronal systems[1,2]. Newly formed sensory neurons are continuously generated in the basal portion of the olfactory epithelium[3] and extend their axons into the olfactory bulb, the first relay station of the olfactory system[4]. However, the cellular and molecular mechanisms controlling the formation and maintenance of the olfactory map are far from being fully understood[4,5].

Here, we describe a protocol for labeling sensory neurons of the olfactory organ of larval *X. laevis* by *in vivo* electroporation of fluorophore-coupled dextrans. The presented protocol allows visualization of axonal morphology and connectivity, track axonal development over time and study mechanisms regulating axonal wiring and guidance.

Electroporation is a well established method to introduce charged macromolecules, like dextran-coupled dyes and DNA, into cells[6,7]. The cell membrane is permeabilized by application of short voltage pulses and the molecules are electrophoretically delivered into the cytosol[8]. Spatially restricted electroporation using a micropipette permits selective labeling of cells including neurons and has been applied in various neuronal systems including the visual system of *X. laevis*[9,10].

We show how the electroporated animals can be used to study axonal growth patterns and morphology in living animals using confocal laser-scanning or multiphoton microscopy. The described procedure allows identifying the coarse topology of axonal projections of sensory neurons of the main and accessory olfactory system[11,12]. Using *in vivo* time lapse imaging, it is also suitable to supervise the glomerular connections of single mature sensory neurons, and to monitor the evolution of the axonal projection patterns of immature sensory neurons[12]. The described protocol can be applied to investigate the structure and formation of olfactory circuits in the intact animal and can be adapted to other cell types within the olfactory and other neuronal systems.

Protocol

NOTE: Animal handling and experiments were performed as approved by the Göttingen University Committee for Ethics in Animal Experimentation.

1. Preparation of Instruments and Pipette Fabrication

1. Ensure that the electroporation setup consists of a stereomicroscope with large working distance and is equipped with fluorescent illumination and filter sets for the used dye.
2. For electroporation use either a dedicated single cell electroporator or a generic square pulse generator attached to an oscilloscope. Connect the electroporator outputs to a pipette holder and a bath electrode. Connect the positive terminal of the pulse generator to the micropipette holder and the negative terminal to the bath electrode. Ensure both the pipette holder and the bath electrode contain silver wires coated with a thin layer of silver chloride.
3. Mount the pipette holder on a micromanipulator to allow exact positioning.
4. Fabricate electroporation micropipettes from borosilicate glass capillaries with internal filament.
5. Use a horizontal micropipette puller and apply a modified protocol for the fabrication of pipettes for patch clamp experiments as described by Bestman *et al.*[13].
6. Adapt parameters to produce a longer shank and a smaller tip opening resulting in a higher pipette resistance of around 15-20 MOhm for single cell electroporation. The pipette resistance should be lower, *e.g.*, 3-4 MOhm, for labeling of groups of cells.
7. Measure the pipette resistance either directly with a dedicated single-cell electroporator or calculate it with Ohm's Law after measuring current flow with an oscilloscope after application of a defined voltage pulse.

2. Preparation of Electroporation Solution

1. Dissolve fluorophore-coupled dextran in frog ringer (98 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, 5 mM Na-pyruvate, 10 mM HEPES, adjusted to pH 7.8, osmolarity was 230 mOsmol/l) at a concentration of 3 mM. The cells may not be so brightly labeled if lower dye concentrations are used. Prepare a large volume stock solution and divide it into small aliquots. Freeze them for storage (stable for months).
   NOTE: Dextrans are available in different sizes and a variety of emission/excitation spectra (*e.g.*, Alexa 488-dextran 10kD, Alexa 546-dextran 10kD, Alexa 568-dextran 10kD, Alexa 594-dextran 10kD, TMR-dextran 3kD).
2. Backfill the micropipette with an elongated pipette tip with a small volume of dextran solution (1-5 µl). Carefully flick the micropipette with the finger to remove residual air bubbles from the pipette tip.
3. Mount the micropipette in the pipette holder. Make sure that the silver wire inside the pipette is in contact with the dye solution.

3. Selection of Larval *X. laevis*

1. Use albino larvae of *X. laevis* for the experiments. Wild type animals possess pigment-filled melanophores that show autofluorescence emission during confocal/multiphoton imaging and are therefore not suitable for the described experiments.
2. Stage premetamorphotic tadpoles after Nieuwkoop and Faber[14]. Select tadpoles of stages 45-53 for the experiments.

4. Electroporation of Fluorophore-coupled Dextrans

1. Place a small piece of tissue in a petri dish and cover it with a small volume of water containing 0.02% tricaine (Ethyl 3-aminobenzoate methanesulfonate, adjusted to pH 7).
2. Anesthetize the tadpoles in water containing 0.02% tricaine. After some minutes the animals cease moving. Confirm proper anesthesia by touching tadpoles. They should be non-responsive.
3. Carefully transfer the tadpole from the anesthetic to the tissue covered petri dish.
4. Make sure the bath electrode closes the electroporation circuit. Ensure that the electrode is in contact with the wet tissue; a direct contact to the tadpole is not necessary.
5. Position the micropipette tip close to the olfactory organ using the micromanipulator.
6. Penetrate the skin covering the olfactory organ with the pipette tip and cautiously advance the tip into the centrally located sensory neuron layer of the main olfactory epithelium or vomeronasal epithelium.
7. Trigger positive square voltage pulses to transfer dye into sensory neurons. Apply a single voltage pulse (*e.g.*, 25 V, pulse length 25 msec) or trains of multiple pulses (*e.g.*, 50 V, pulse length 300 µsec, 400 msec train duration at 200 Hz).
   NOTE: Determine optimal voltage pulse parameters for the desired extend of labeling. Reduce voltage pulse amplitude, duration and number of repetitions to lower the number of labeled cells. Apply higher voltage pulse amplitude, duration and number of pulses for a more widespread labeling.
8. Visualize successful dye extrusion and electroporation by triggered pulses using fluorescent illumination of the stereomicroscope. The dye spreads quickly into cell body and dendrite after successful electroporation.
9. Repeat steps 4.5-4.9 for the second olfactory organ of the tadpole.
10. Transfer the tadpole into a beaker filled with fresh water for recovery. After ca. 5 min the tadpoles wake from anesthesia and start normal swimming movements.
11. After 24 hr the electroporated dye spreads in the sensory neurons and eventually reaches the olfactory bulb via axonal transport.

5. Mounting Animals for *In Vivo* Visualization of Cells and Axonal Processes

1. Anesthetize the tadpoles in water containing 0.02% tricaine.
2. Carefully transfer tadpoles into an imaging chamber, *e.g.*, a small silicon rubber covered petri dish with a tadpole-sized recess.
3. Cut a small rectangle into a stripe of Parafilm. Cover the tadpole with the Parafilm, leaving the anterior telencephalon exposed through the cut-out window. Fix the Parafilm with needles on the dish without injuring the tadpole.
4. Make sure the tadpole is submerged in sufficient water containing 0.02% tricaine.

5. Mount the imaging chamber on the stage of an upright multiphoton microscope or confocal microscope.
6. Acquire a three dimensional stack of images of the olfactory bulb. Ensure that the imaging procedure is as short as possible and does not exceed 10-15 min.
7. Return the tadpole into normal water in a separate tank and prevent exposure to light. After 5 min, the tadpole wakes from anesthesia.
8. Repeat steps 5.1-5.7 after specified time intervals, *e.g.*, every day.

6. Mounting Animals for *Ex Vivo* Visualization of Cells and Axonal Processes

1. Alternatively to section 5 of the protocol, use an excised brain preparation to visualize the labeled sensory neurons.
2. Anesthetize and kill the tadpole by transection of the brain at the transition to the spinal cord. Excise a block of tissue containing the olfactory organs, the olfactory nerves and the anterior telencephalon.
3. Transfer the tissue block to frog ringer solution and remove connective tissue with fine scissors to expose the ventral side of the brain.
4. Transfer the tissue block to an imaging chamber and fix it with a platinum frame stringed with nylon threads.
5. Mount the imaging chamber on the stage of a confocal/multiphoton microscope.
6. Acquire a three dimensional stack of images of the olfactory bulb.

7. Image Processing and Data Evaluation

1. Optimize and apply non-local means of filtering to improve image quality and visualization of neuronal structures as described by Coupé, P. *et al.* [15].
   NOTE: Data from imaging experiments in deeper tissue layers of live specimens are often noisy.
2. Create maximum intensity projections of the image stacks for an overview.
3. For time-lapse imaging experiments screen data sets for single unambiguously identifiable axons of sensory neurons.
4. Reconstruct cellular morphology via software-assisted tracing of neuronal processes as described by Peng *et al.*[16].

Representative Results

The described protocol can be successfully applied for electroporation and *in vivo* visualization of axonal processes of sensory neurons of the olfactory system of anesthetized *X. laevis*, using confocal laser-scanning or multiphoton microscopy (**Figure 1**).

Electroporation allows fluorophore-coupled dextrans to enter and to rapidly spread inside cells of the olfactory organ. It is helpful to apply fluorescent illumination to verify successful labeling after triggering of voltage pulses. Depending on the electroporation parameters, *e.g.*, pipette resistance, groups of cells (**Figure 2A, B**) or single cells (**Figure 2C**) of the olfactory organ are labeled. Axons of labeled sensory neurons can be visualized in the olfactory nerve (**Figure 2D**) and axonal processes can be observed also in the olfactory bulb, usually 24 hr after successful electroporation (**Figure 3**). Electroporation of groups of sensory neurons allows visualizing the coarse wiring patterns in the olfactory bulb (**Figure 3A**). Single cell electroporation can be applied to investigate individual axonal projection patterns, axonal bifurcations and connectivity to glomerular structures in the olfactory bulb (**Figure 3C-E**).

Transparent albino tadpoles of *X. laevis* permit *in vivo* confocal or multiphoton imaging of labeled sensory neurons in the intact brain of anesthetized tadpole. The development of axonal growth patterns can be tracked over several days/weeks by repeated *in vivo* visualization of the same labeled sensory neuron (**Figure 4**). Depending on the maturity of the sensory neuron at the time point of electroporation the axon pattern in the olfactory bulb can vary considerably. Mature neurons already possess elaborate axonal branches that feature tufted areas connected to glomeruli. The axonal growth pattern of mature neurons is rather stable, but elongation of axon branches and refinements of glomerular tufts are observable (**Figure 4A-E**). Sensory neurons can be observed *in vivo* for extended time periods, *e.g.*, longer than three weeks (**Figure 4F-I**). On the other hand, axons of immature neurons are still in the process of initial growth, do not possess tufted arborizations and have not yet connected to their final glomerular targets. The experimental protocol allows tracking the development of these neurons during maturation, *e.g.*, axon elongation, bifurcations and establishment of tufted arborizations (**Figure 4J-L**). For more examples also see Hassenklöver and Manzini[12].

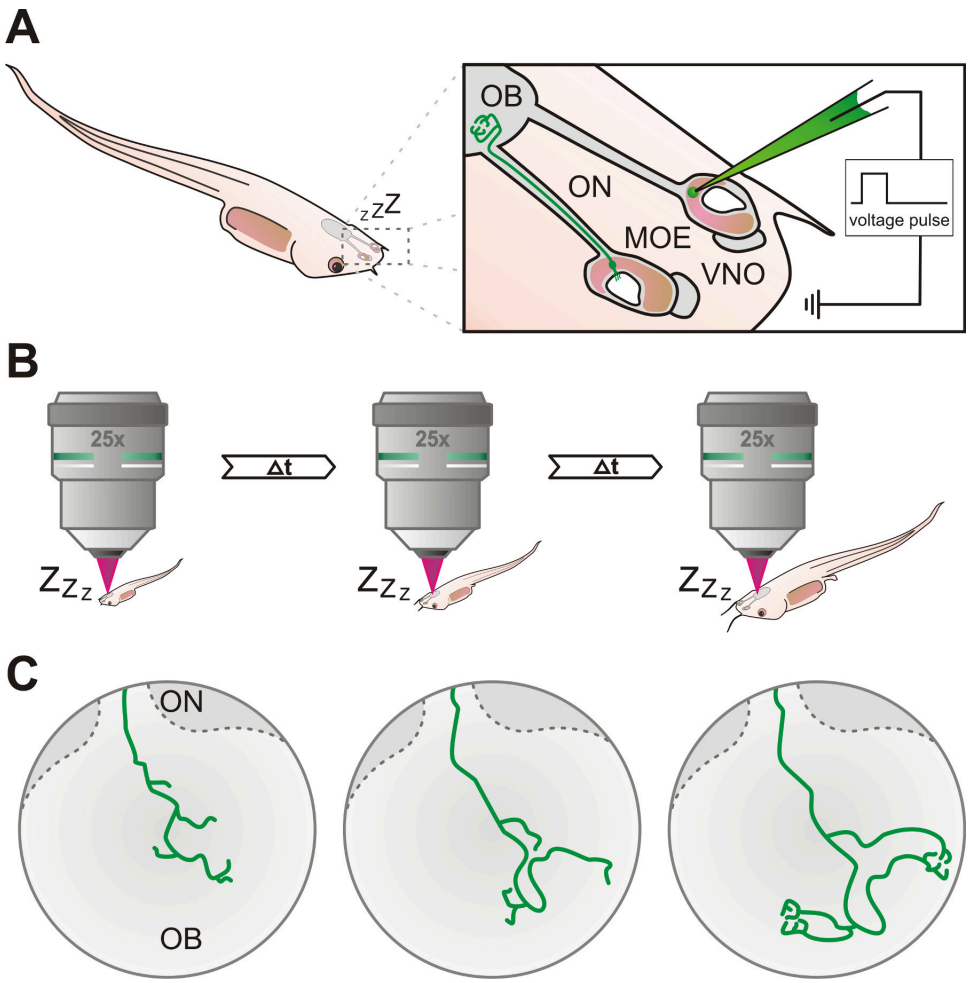

Figure 1. Schematic overview of the experimental protocol. (A) Sensory neurons in the main olfactory epithelium (MOE) or vomeronasal organ (VNO) of the olfactory organ of anesthetized *X. laevis* larvae are labeled via electroporation using a glass pipette filled with fluorescent dextran solution. The axons of labeled neurons can be followed through the olfactory nerve (ON) and eventually reach the olfactory bulb (OB). **(B)** The tadpole is anesthetized and the labeled neurons are repeatedly investigated using a confocal or multiphoton microscope in specific time intervals. **(C)** The incremental development of axonal growth patterns of labeled cells can be followed over time spans of days to weeks.

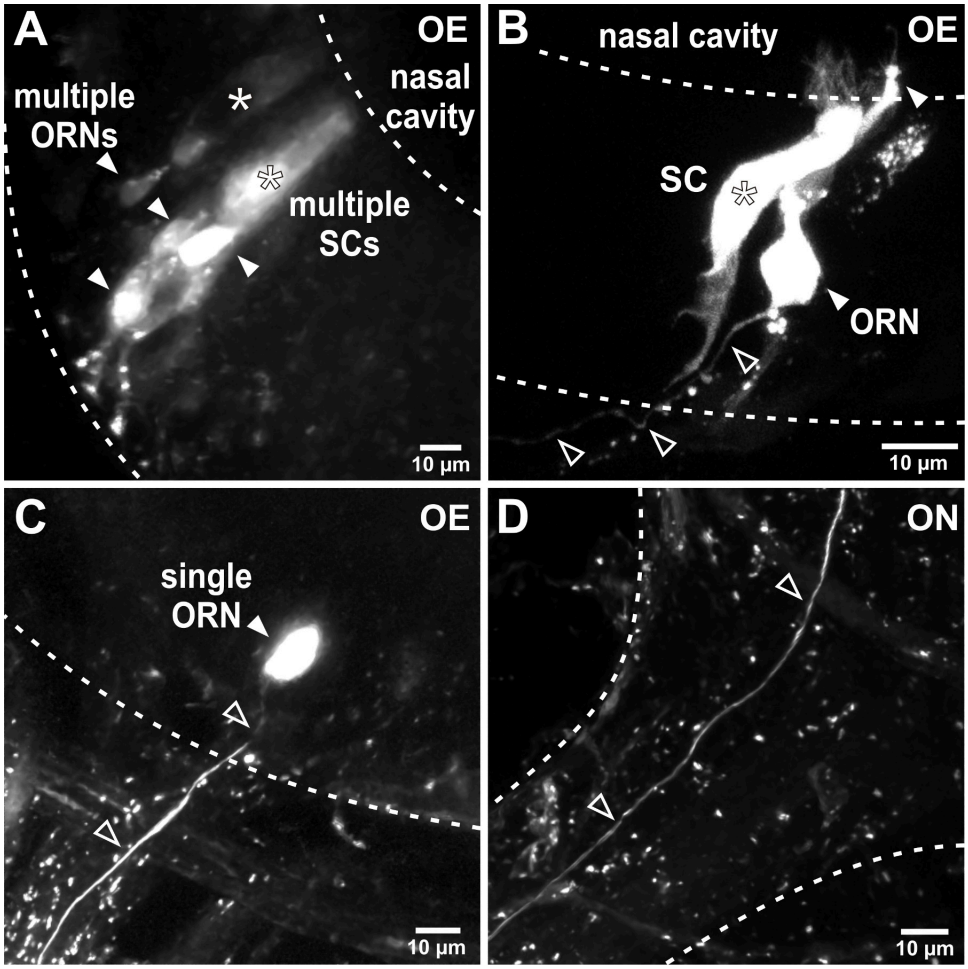

Figure 2. Electroporation of sensory neurons in the olfactory organ. (A) Electroporation with low resistance pipettes leads to labeling of multiple cells in the olfactory organ. In this representative example multiple olfactory receptor neurons (ORNs, filled arrowheads) and two columnar-shaped supporting cells (SCs, asterisks) were stained. Dashed lines demarcate the borders of the olfactory epithelium (OE). **(B)** Refining the electroporation parameters, *e.g.*, increasing the pipette resistance, restricts the number of labeled cells. In this example, a single sensory neuron (filled arrowheads) and one adjacent supporting cell (asterisk) were stained after electroporation. Note the single axon leaving the olfactory epithelium (open arrowheads). **(C)** Successful single cell electroporation leads to exclusive labeling of an individual sensory neuron (filled arrowhead) and its connected axon (open arrowheads) in the olfactory epithelium. **(D)** The single labeled axon (open arrowheads) can be followed through the olfactory nerve (ON) into the olfactory bulb.

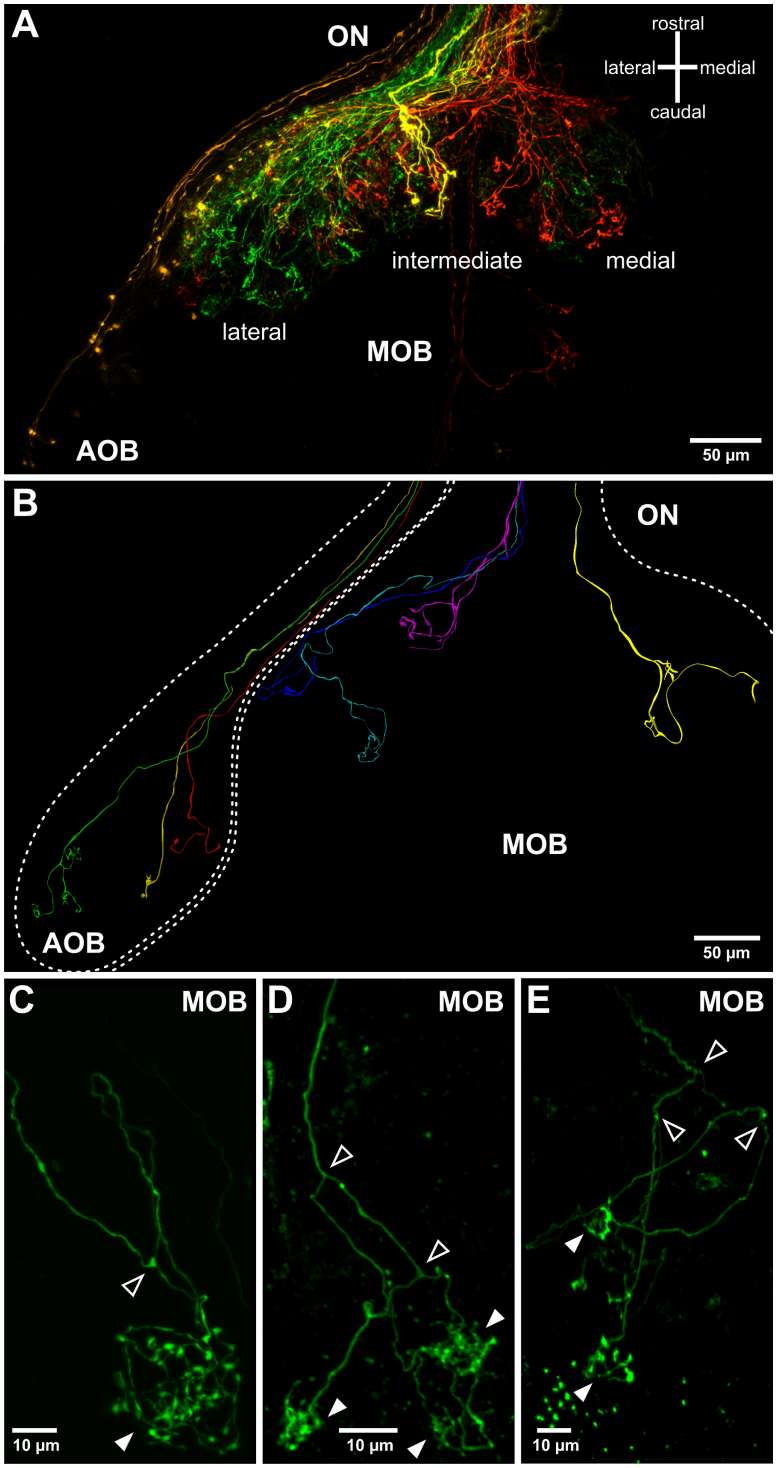

Figure 3. Visualizing olfactory axon projections in the excised olfactory bulb. (A) The coarse topology of axonal projections of sensory neurons in the olfactory bulb can be visualized using lower resistance electroporation pipettes. One olfactory bulb hemisphere and its associated olfactory nerve (ON) are depicted. Four dextrans coupled to different fluorophores were electroporated at four distant locations of the olfactory organ: lateral (green), intermediate (yellow), medial MOE (red) and VNO (orange). This allows visualizing the accessory olfactory bulb (AOB) and the three main projection fields of the main olfactory bulb (MOB). **(B)** Different axonal growth patterns of single olfactory sensory neurons superimposed on the structure of the olfactory bulb. Depicted are combined three dimensional reconstructions of multiple single cell stainings derived from different larval specimens. **(C-E)** Examples of single olfactory axons projecting into the olfactory bulb and forming tufted arborizations/synapses in spherical glomeruli (filled arrowheads). Note that in *X. laevis* olfactory axons bifurcate regularly (open arrowheads) before connecting to one, two or multiple glomeruli (filled arrowheads, also see Hassenklöver and Manzini[12]).

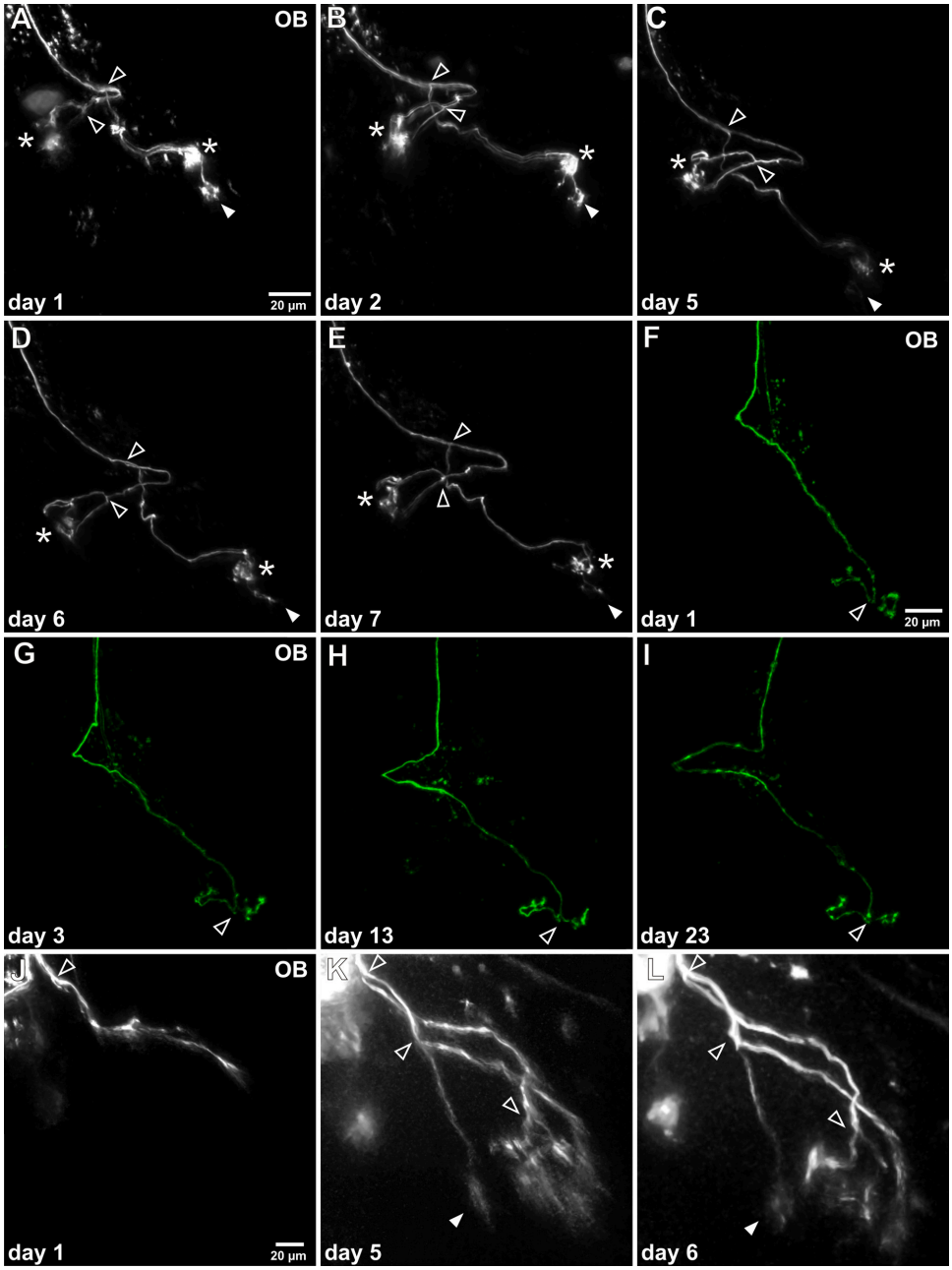

Figure 4. ***In vivo*** **time-lapse imaging of single olfactory neuron axons. (A-E)** After successful single cell electroporation the labeled axon can be repeatedly visualized in the olfactory bulb (OB). This example shows an individual axon that was investigated for one week. The overall morphology does not change considerably and two major branching points can be identified (open arrowheads). Note that over the time course, one glomerular tuft undergoes continuous reduction (filled arrowhead), whereas the other two glomerular tufts remain stable (asterisks). **(F-I)** This representative example shows the viability of long-term observations as this specific axon was investigated for more than three weeks. No apparent change of its growth pattern can be detected. **(J-L)** An example of an immature sensory axon in the process of growth is depicted. It has not yet connected to glomeruli and characteristic glomerular tufts are missing. After 5 days the axonal branches are elongated, the axon bifurcated multiple times and fine arborizations are established.

Discussion

The experimental procedure described here allows labeling sensory neurons of the olfactory organ of larval *X. laevis* by electroporation of fluorophore-coupled dextrans and subsequent visualization of sensory axon growth in the living animal. By varying the parameters of the *in vivo* electroporation it is possible to control the number of labeled sensory neurons. It is thereby possible to label large groups of neurons of a sensory epithelium, very few or even single cells.

To ensure the desired extend of neuronal labeling it is important to be particularly cautious about the micropipette characteristics and the electroporation pulses. Higher pipette resistances and reduction of voltage pulse amplitude, duration and number of repetitions can reduce the amount of labeled cells, whereas decreasing pipette resistances and higher voltage pulse amplitude, duration and number of pulses can lead

to more widespread labeling. The use of fluorescent dextrans for electroporation provides immediate visual feedback if the applied settings are appropriate. Be careful that using parameters for amplitude, duration and number of pulses that exceed the values provided in the protocol can potentially lead to cell damage or even cell death[17]. Clogged or broken tips of micropipettes can also hinder successful electroporation.

In vivo electroporation in the olfactory organ of *X. laevis* is limited to larval stages since the skin of postmetamorphotic frogs is tougher and cannot be easily penetrated with a micropipette. The *in vivo* visualization of neuronal processes can be hindered by scattering of excitation/ emission light in deeper brain areas or by blood vessels. This problem becomes especially apparent in higher larval stages due to a larger brain and can lead to noisy signals making the clear identification of fine axonal processes more difficult.

The presented protocol permits to visualize sensory neurons in the intact olfactory system without dissecting the animal, damaging the cells during labeling, preparing tissue slices or fixing the tissue as necessary for alternative methods, like labeling in whole-cell patch-clamp experiments[18]. When combining the labeling of few or single sensory neurons with *in vivo* time lapse imaging, it is possible to visualize the glomerular connections of single mature sensory neurons over long time intervals. This way it is also possible to monitor the development of the axonal projection patterns of immature sensory neurons over several weeks. This latter option is particularly interesting as it allows monitoring the growth patterns of single axons in the living animal. This opens the possibility to investigate cellular and molecular mechanisms that control axon guidance and pathfinding. Several factors, including odorant receptor expression, various axon guidance molecules and odorant-induced/ spontaneous activity of sensory neurons have been shown to regulate the target finding of sensory neuron axons[4,5].

The application of the protocol is not restricted to olfactory sensory neurons, but can also be applied to study other cell types, *e.g.*, stem/ progenitor cells of the neurogenic zones of the developing brain or mitral cells of the olfactory bulb. In addition the demonstrated technique can also be used in combination with calcium sensitive dextrans or injected membrane-permeable calcium dyes to get functional information about the labeled neuron and/or the connected circuitry[7,19]. The availability of a wide range of fluorophores coupled to dextrans permits labeling of multiple individual cells or populations with different colors. Also plasmid DNA solution, for example encoding for fluorescent proteins, is suitable for electroporation and can further enhance the versatility and usefulness of the technique[6]. The protocol can further be enhanced to allow the combined electroporation of dextrans and DNA or charged morpholinos to manipulate gene expression[13,17].

The described method certainly represents a new tool to investigate the complex and still not fully understood processes that regulate axonal guidance in the vertebrate olfactory system.

Disclosures

The authors have nothing to disclose.

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