Dye Labeling Retinal Ganglion Cell Axons in Live *Xenopus* Tadpoles

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Protocol

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Individual neurons in the developing nervous system can be visualized by the targeted delivery of a fluorophore. In this article, we describe a method for introducing a fluorescent dye via iontophoresis into retinal ganglion cell (RGC) axons in albino *Xenopus laevis* tadpoles. Iontophoresis is the enhanced permeation of molecules across biological membranes under the influence of an electrical field. Lipophilic dyes such as DiI are well suited to this method—being insoluble in the aqueous environment of the eye, they precipitate instantaneously, and only cells in contact with the dye crystal are labeled as the dye diffuses through the plasma membrane. A dissection stereomicroscope is used to allow a wide range of approach angles for the micropipette. The goal is to introduce a small bolus of dye into the neural retina where the ganglion cell somata are located and the axons course, with the expectation that it will be taken up by a small enough number of axons to allow individual cells to be distinguished. Because RGC axons will typically be imaged in the tectum far from the injection site, a relatively large injection can be made, increasing the probability of labeling axons without obscuring their visualization at the target. This approach is particularly useful under conditions in which it might be too difficult to perform juxtacellular electroporation because of limited visibility or access.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

DiI (0.01%–0.05% [w/v] in absolute ethanol)

Other color lipophilic dyes such as DiD or DiO are also available.

Fluorescent dextran (5% [w/v] in 0.1 M phosphate buffer or water)

Alexa Fluor-conjugated dextrans are more expensive but provide better photostability and less phototoxicity than traditional xanthene dyes such as FITC and TRITC. It is economical to use the former for experiments and the latter for practice.

MS-222 (0.02% [w/v] in 10% Steinberg's rearing medium) (Sigma-Aldrich)

The solution can be stored for 1 mo at 4°C.

Steinberg's rearing medium <R>

Adapted from *Imaging in Developmental Biology* (ed. Sharpe and Wong). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Xenopus laevis tadpoles (at appropriate stage of development)

Imaging is facilitated in tadpoles from an albino strain, which are transparent.

Equipment

Coarse micromanipulator (e.g., Narishige M-152)
Dissection stereomicroscope
Electrical stimulator capable of producing square wave pulses at a range of amplitudes (0–100 V) and frequencies (0–200 Hz) (e.g., SD9 [Grass Technologies] or Axoporator 800A [Molecular Devices])
Fine brush
Glass micropipettes

These should resemble patch pipettes with 0.5–1.0-µm tips to give ~10 MΩ resistance when filled with standard recording internal solution. If the tips are too long, they will bend and break on the skin of the tadpole. Borosilicate standard wall pipettes with filament (1.5 mm OD, 0.86 mm ID), pulled on a Sutter P-97 puller using a box filament, are recommended. Tip shape is critical to avoid labeling multiple cells and to prevent clogging. A good tip can be reused in multiple tadpoles in one session, but should not be saved for later use.

Laboratory tissues (e.g., Kimwipes)
Silver wire leads (0.25-mm diameter, ~4-cm length, soldered onto a standard copper wire)
Transfer pipettes (plastic)

METHOD

1. Prepare the labeling solution containing an appropriate fluorescent dye.

   The solution can be stored for at least 1 mo at –20°C, although fluorescent dyes are generally best made fresh from powder. Care should be taken to minimize exposure of fluorescent dyes to light at all times.

2. Backfill a micropipette with the labeling solution. Mount the pipette on a micromanipulator set up on the dissection microscope.

3. Connect the circuit as follows: Positive lead of stimulator → silver wire → labeling solution in micropipette → [gap] → tadpole → moist tissue → silver ground wire → negative lead of stimulator.

   To monitor for blockage of the pipette tip, a 10-kΩ resistor can be inserted in series anywhere along the circuit, and an oscilloscope can be used in parallel to measure the potential drop across the resistor. This method is also applicable to the delivery of plasmid DNA, but yields are much lower than for lipophilic dyes. For an alternative method of DNA delivery, see ***Bulk Electroporation of Retinal Ganglion Cells in Live Xenopus Tadpoles*** *(Ruthazer et al. 2013). For electroporation with DNA, the polarity of the stimulator must be reversed: DNA is negatively charged and will move toward the positive electrode.*

4. Anesthetize a tadpole by immersing it in 0.02% MS-222 for ~1 min.

5. Transfer the tadpole onto the stage of the microscope with a large-bore dropper pipette (e.g., a plastic transfer pipette with the tip cut back to ~5-mm diameter). Carefully orient the tadpole dorsal side up on a moist tissue by gently sliding it with a fine brush.

6. Orient the tadpole so that the dye-filled pipette, when advanced, will enter the eye, perpendicular to its surface at the border between the lens and retina.

7. Advance the pipette, under visual guidance, to the part of the neural retina directly behind the lens, entering the eye at the margin of the lens.

   Be careful to avoid the large blood vessel surrounding the lens of the eye. If this is damaged, there is a high probability that the tadpole will not survive because of blood loss. A common error is to eject dye too deep in the eye.

   See Troubleshooting.

8. Apply pulses from the stimulator. The dye should visibly eject to form a focal deposit in the eye.

   Settings for amplitude and pulse duration must be set empirically for each tissue and pipette shape. For fluorescent dextrans, but usually not for lipophilic dyes, many more pulses can be delivered over the course

of several minutes to increase the probability of successful labeling. However, this greatly increases the risk of labeling multiple axons that cannot be optically differentiated when imaging.

See Troubleshooting.

9. Withdraw the pipette and transfer the tadpole into fresh Steinberg's rearing medium.
10. Perform image analysis.

TROUBLESHOOTING

Problem (Step 7): If the pipette tip is not sharp enough or the angle of approach is bad, the eye will rotate in its socket, preventing the tip from entering the eye.

Solution: Carefully reorient the tadpole and micromanipulator so that the pipette hits the juncture between the lens and retina perfectly perpendicularly. The eye might compress slightly, but as long as the tip is not too dull it will enter.

Problem (Step 8): The dye does not come out.

Solution: Try reversing the polarity briefly to unblock the tip. In addition, the tip can sometimes be withdrawn and broken back very slightly against a moist Kimwipe, although this will eventually reduce the effectiveness of the micropipette for delivering tracer, especially for lipophilic dyes such as DiI that crystallize on contact with external medium.

RELATED INFORMATION

The local iontophoresis method presented here is appropriate for the transfer of fluorescent dye, but for high efficiency transfer of DNA, use the protocol described in **Bulk Electroporation of Retinal Ganglion Cells in Live *Xenopus* Tadpoles** (Ruthazer et al. 2013).

RECIPE

Steinberg's Rearing Medium

Reagent	Final concentration
NaCl	58.2 mM
KCl	0.67 mM
$Ca(NO_3)_2$	0.34 mM
$MgSO_4$	0.78 mM
HEPES	50 mM

Use at 10% strength and adjust the pH to 7.4.

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REFERENCES

Ruthazer ES, Schohl A, Schwartz N, Tavakoli A, Tremblay M, Cline HT. 2013. Bulk electroporation of retinal ganglion cells in live *Xenopus* tadpoles. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot076471.