Bulk Electroporation of Retinal Ganglion Cells in Live *Xenopus* Tadpoles

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Protocol

Bulk Electroporation of Retinal Ganglion Cells in Live *Xenopus* Tadpoles

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Individual neurons in the developing nervous system of *Xenopus laevis* can be visualized by the targeted delivery of a fluorophore. The fluorophore can be delivered as a fluorescent dye or DNA that encodes a fluorescent protein. Local iontophoresis is a method that works well for transfer of fluorescent dye to retinal ganglion cells (RGCs) in the eye, but it does not give a high yield for delivery of DNA. This is largely because the degree of pigmentation of the eyes, even in albino strains, makes it difficult to visualize RGC somata during pipette positioning. Bulk retinal electroporation is a better approach for delivery of plasmid DNA to RGC. The method described here works best in tadpoles older than stage 42.

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

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.

Plasmid DNA (0.1–5.0 µg/µL)

An endotoxin-free plasmid purification kit is recommended for plasmid preparation. DNA solutions can be kept indefinitely at −20°C. A small amount of Fast Green dye can be added (<0.2 µL of a 0.1% solution in water) to a working volume (~3 µL) of plasmid DNA solution.

Steinberg's rearing medium <R>

Xenopus laevis tadpoles (at appropriate stage of development)

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

Equipment

Coarse micromanipulators (e.g., Narishige M-152)

This procedure requires two micromanipulators: one to position the injection micropipette and one to position the electroporation electrodes. The electrodes are mounted ~1 mm apart at the end of a rod held by the second micromanipulator. Each plate should be fixed in place and soldered to copper wires that are connected to the two leads of the electrical stimulator.

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

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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 from Grass Technologies)

To produce exponentially ramped pulses, connect a 3-μF capacitor in parallel.

Fine brush

Glass micropipettes

These can have fairly crude tips several micrometers in diameter because they will be used exclusively to inject plasmid solution into the target structure. However, if the tips are too long, they will bend and break on the skin of the tadpole. Use borosilicate standard wall pipettes with filament (1.0 mm OD, 0.75 mm ID), pulled to a fine tip using any micropipette puller. Break back by gently touching the tip to a moist laboratory tissue.

Illumination source

Laboratory tissues (e.g., Kimwipes)

Platinum plate electroporation electrodes (~1 × 2-mm)

These can be fabricated in the laboratory by carefully shaping old filaments from the Sutter P-97 puller or they can be purchased commercially.

Pressure injection system (e.g., Picospritzer III)

Although precise microinjection systems can be purchased, a 10-mL syringe equipped with a three-way stopcock that can be covered with your thumb to permit instantaneous release of pressure and connected to polyethylene tubing also works adequately.

Transfer pipettes (plastic)

METHOD

1. Connect the circuit as a direct loop across the plate electrodes, with the capacitor in parallel, as follows: Positive lead of stimulator → one lead to capacitor → copper wire to one plate electrode → tadpole → second plate electrode → other lead of capacitor → negative lead of stimulator.

   Although the two plate electrodes can be positioned independently, we usually mount them on a single rod to ensure consistent distance between the electrodes. DNA is negatively charged and will transfect cells on the side nearest the positive electrode (Fig. 1B).

2. Back-fill a glass micropipette with the DNA solution. Mount it onto the pipette holder held by the micromanipulator. Break back the tip until solution can first be seen with the dissection microscope coming from the tip when pressure is applied.
3. Anesthetize a tadpole by immersing it in 0.02% MS-222 for ~1 min.
4. Use a plastic transfer pipette and fine brush to transfer the tadpole to a moist tissue to immobilize it while the pipette is inserted into its eye.

   Folds in the tissue should be used to provide support and to orient the animal optimally.

5. 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 (Fig. 1A).
6. 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.
7. Pressure-inject just enough of the DNA solution into the vitreous humor (or brain ventricle for brain electroporation; see Discussion) to see the Fast Green dye accumulate in the target area (Fig. 1A).

   If the lens distends slightly as a result of the increased intravitreal pressure, the positioning is good, but if solution accumulates around the eye, it is likely that the pipette tip is too deep or shallow.

8. Withdraw the pipette. Lower the plate electrodes so that they barely touch opposite sides of the tadpole's eye (Fig. 1B).

   The part of the retina closest to the positive electrode will be electroporated. The efficiency of transfection falls dramatically if one of the two electrodes is dirty or not securely touching the eye.

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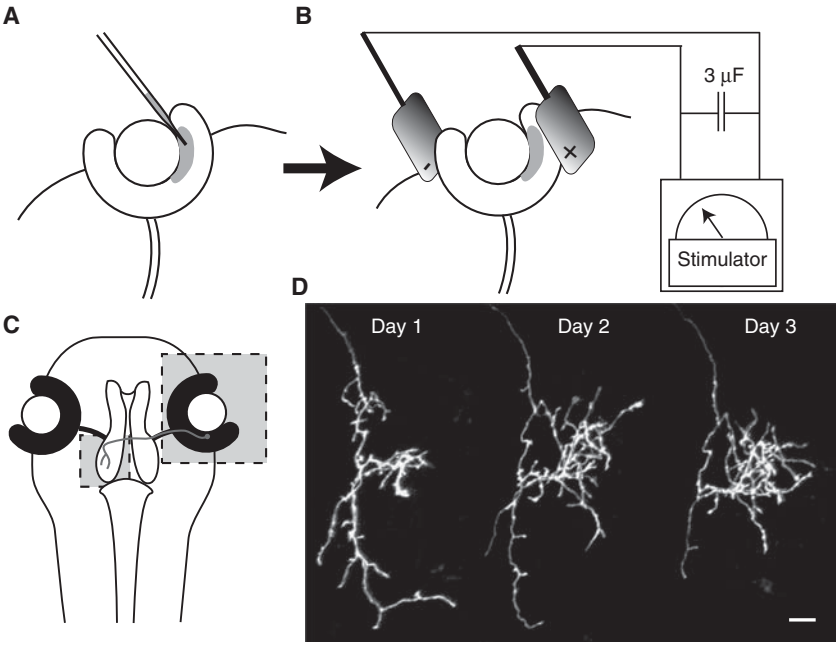

FIGURE 1. Retinal electroporation of plasmid DNA. (*A*) Plasmid solution, with Fast Green to provide contrast, is pressure-injected into the vitreous humor by insertion of a micropipette at the margin of the lens and sclera. (*B*) Platinum plate electrodes are placed on the surface of the tadpole just across the eye. Negatively charged DNA will move toward the positive electrode. A capacitor, placed in parallel, creates the exponential decay pulse shape for optimal transfection efficiency. (*C*) Schematic diagram of the tadpole head with the eye and tectum indicated in squares. (*D*) Within 24–48 h, labeled axons are visible in the contralateral optic tectum. This example of an RGC axon expressing EGFP in a stage-48 tadpole can be observed to refine its arbor in the optic tectum over 3 d. Scale bar, 10 μm.

9. Apply approximately five pulses of 1.6-msec duration at 30–60 V across the electrodes.

   The capacitor in parallel will convert this pulse from a square wave into a lower-intensity pulse with an exponential decay. This waveform is thought to be ideal for first creating micropores in the plasma membranes of cells and then driving DNA into the electroporated cells. Small bubbles should appear at the electrode, indicating that current is flowing. A large number (>10) of small bubbles is a better indication of successful electroporation than fewer larger bubbles.

10. Because only cells on the positive side are electroporated, one might wish to increase the number of cells that are labeled. In this case, reverse the polarity of the stimulator and repeat the electroporation.

11. Retract the electrodes. Transfer the tadpole, using a transfer pipette or brush, to a bowl containing fresh rearing solution.

12. Clean the electrodes gently by wiping them with a few moist tissue fibers held by fine forceps.

DISCUSSION

Green fluorescent protein (GFP)-expressing RGC axons should first be visible in the optic tectum between 24 and 48 h after electroporation (Fig. 1D). The number of labeled axons is highly variable and appears to decrease with the age of the tadpole at the time of electroporation. Electroporation of retinal cells in tadpoles at stage 45–46 generally gives rise to one to five well-labeled axons, although this number is influenced by the volume and concentration of the DNA solution. Brightly labeled axons can be followed for periods up to several weeks in vivo.

For live imaging experiments, it is particularly useful to be able to unambiguously distinguish an individual cell from the group, but bulk electroporation with plate electrodes, as opposed to single-cell electroporation with a micropipette, tends to transfect large clusters of cells. We have developed two

techniques to permit discrete labeling of individual neurons in bulk-electroporated tissue. The first of these involves cotransfection of photoactivatable-GFP (PA-GFP) together with mCherry red fluorescent protein (Patterson and Lippincott-Schwartz 2002). PA-GFP is a variant of GFP that increases its fluorescence by two orders of magnitude on exposure to intense ultraviolet light. Photoactivation can also be induced on a two-photon microscope by illumination with laser light at 780–800 nm. Thus, when coexpressed as a free cytoplasmic molecule together with mCherry, it is possible to select individual neurons on the red emission channel for photoactivation and subsequent time-lapse imaging. Remarkably, the diffusion of the activated fluorophore is fast enough to backfill an entire RGC axon terminal arbor merely by focusing activating light onto the growth cone of one of the branches of the cell (Fig. 2A,B). This method has proved highly useful whenever any part of the cell (e.g., the soma or an axonal or dendritic process) can be optically isolated from other cells in the field for photoactivation. Following photoactivation, imaging of the GFP can be performed exactly as one would image EGFP, but taking care not to expose the sample to any ultraviolet light.

The second method takes advantage of the fact that efficiency of gene transfer by electroporation, both with respect to number of cells transfected and copy number of plasmids transferred, is proportional to the concentration of DNA in the plasmid solution used. This poses a serious challenge for achieving both sparse and bright expression of GFP when bulk electroporating. This problem is solved by coelectroporating extremely low concentrations (0.1–0.5 ng/μL) of plasmid expressing Cre recombinase together with higher concentrations (>1 μg/μL) of an LNL-GFP plasmid that has a "floxed" stop codon upstream of EGFP. Thus, LNL-GFP will be present in a large number of cells, but only in those cells that also received at least one copy of the Cre-expressing plasmid is the EGFP transcribed. Because one molecule of Cre recombinase can "activate" an unlimited number of copies of the LNL-GFP plasmid, amplification occurs, resulting in only a small number of fluorescent cells, most of which exhibit bright fluorescence (Fig. 2C).

It is worth noting that bulk electroporation is also useful for transfecting large groups of cells in the brain of the tadpole. For example, to electroporate groups of cells in the optic tectum, the DNA solution can be injected into the brain ventricle and the current passed across the midbrain by placing the electrodes directly on the overlying skin. By changing the size, contact area, and positioning of the plate electrodes, this method can be used to electroporate cells along any part of the rostrocaudal extent of the brain or spinal cord. To target individual neurons with fluorescent dye, see **Dye Labeling Retinal Ganglion Cell Axons in Live *Xenopus* Tadpoles** (Ruthazer et al. 2013).

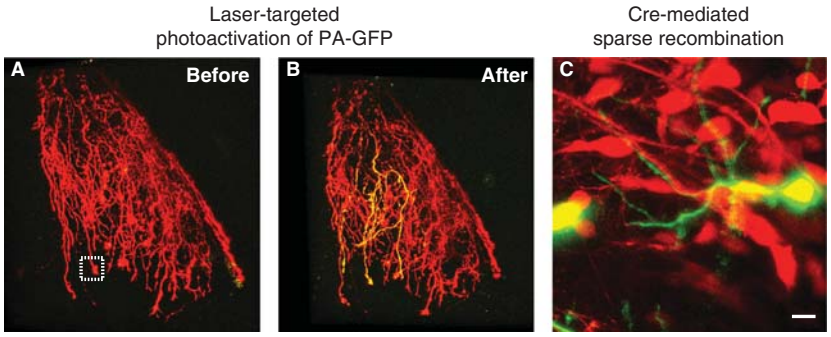

FIGURE 2. Alternative methods for sparse labeling of neurons by electroporation. (*A*) A large number of RGC axons were coelectroporated in the eye with plasmids encoding mCherry and PA-GFP. At 990 nm excitation, the mCherry but not the unactivated PA-GFP can be imaged. To activate the PA-GFP, the microscope was set to scan for 1 sec at 800 nm at high zoom over a small isolated region into which only one axon extended (dashed box). (*B*) Less than 5 min following photoactivation of the axon tip, the entire single axon can now be visualized at 990 nm on the GFP channel. (*C*) Another method to achieve sparse labeling of cells is to cotransfect LNL-GFP (with a floxed stop codon before the GFP sequence) together with very low concentrations of Cre recombinase. In this example, 0.2 ng of Cre, 1 μg of LNL-GFP, and 1 μg of mCherry per 1 μL of water were bulk-coelectroporated into the tadpole brain, resulting in sparse, bright GFP labeling of individual neurons. Scale bars, 7.5 μm (*A,B*), 10 μm (*C*).

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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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