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Targeting the Zebrafish Optic Tectum Using *In Vivo* Electroporation

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INTRODUCTION

In vivo electroporation is a method for delivery of plasmids and other oligonucleotide reagents that offers precise temporal control. In zebrafish, *in vivo* electroporation has been shown to be particularly well-suited to delivering GFP expression vectors to the developing central nervous system. This protocol describes a modification of *in vivo* electroporation that can be used to specifically target the developing optic tectum of zebrafish embryos beginning at 24 hpf. The electroporation electrodes required for this approach can be easily constructed from relatively inexpensive materials. Microinjection of plasmid DNA to the midbrain ventricle followed by precise positioning of the electroporation electrodes allows for the targeting of developing neurons of only one hemisphere of the optic tectum. Using this protocol, the optic tectum can be effectively targeted in a high percentage of expressing embryos (79% in our hands). This method can also be used to simultaneously deliver both expression vectors and loss-of-function reagents, which should provide precise temporal control of the knockdown of gene function.

RELATED INFORMATION

This protocol was adapted from Kera et al. (2010).

MATERIALS

Reagents

agarose, low-melting-point (Sigma, A9414)

egg water [0.24 g/l sea salts] (Nusslein-Volhard and Dahm, 2002)

electroporation buffer [RECIPE: 180 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.2] (Cerdeira et al., 2006)

electroporation buffer plus 0.017% tricaine (Western Chemical, #10209)

DNA plasmid of interest (here a Gal4/UAS EGFP expression system)

plasmid midi- or maxi-prep kit (e.g. Qiagen, #12643)

sterile water + 0.03% phenol red (Sigma, P3532)

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Equipment

capillary glass with filament; ID = 0.78 mm; OD = 1.0 mm (Sutter Instruments, #BF100-78-10)

dissecting microscope

electrical tape

epoxy, fast setting (e.g. Devcon, #S-208)

fine forceps

Grass SD-9 Stimulator (Grass Technology, Model SD-9)

Grass platinum subdermal electrodes, straight needle (Grass Technology, #E2)

microcentrifuge

micro-loader pipette tips (Eppendorf, #X22703R)

nail polish, clear acrylic

pipette puller (e.g. Sutter, Model P-30)

plastic rod or other non-conducting hand-held probe (~5 mm in diameter)

plastic petri dishes, 60 mm (Falcon, 35-1007)

pressure injection apparatus (Applied Scientific Instruments, Model MPPI-2)

METHOD

Constructing Electroporation Electrodes

NOTE: This step can be skipped by purchasing prefabricated electroporation electrodes, which are available from several commercial sources. However, we've found that these electrodes are relatively inexpensive and easy to construct, and perform well using the below electroporation protocol.

1. Using narrow (1-2 mm) strips of electrical tape, initially secure two Grass subdermal platinum electrodes to the end of a plastic rod or other non-conducting hand-held probe (~5 mm in diameter) (Fig. 1A). It helps if the end of the probe is tapered such that the electrodes approach the optimal separation of 1 mm (here we use a small plastic knitting needle). Ensure that the ends of the platinum electrodes are even (Fig. 1A). Next, permanently secure the electrodes to the probe using fast-setting epoxy. To ensure that the electrodes are firmly secured to the probe, epoxy should be applied in multiple steps, inverting the electrode between each application (Fig. 1B).

NOTE: Platinum wire can be used in place of the Grass subdermal electrodes, but we found that the Grass electrodes were more rigid than platinum wire, allowing them to more consistently maintain the appropriate 1 mm spacing during repeated use. In addition, the Grass electrodes come attached to wire leads, which avoids having to solder wire leads to the platinum part of the electrode.

2. Secure the wire leads to the plastic probe using electrical tape at multiple positions along the length of the probe. The wires should be sufficiently attached such that the electrodes are not disturbed during hand-held use (Fig. 1D).

NOTE: When securing the wire leads be sure that you can still identify which lead goes to which electrode because it will be important to know which electrode is connected to the positive terminal of the voltage source.

3. Once the electrodes have been fully secured with glue and tape, manually bend the platinum electrodes such that the ends are separated by 1 mm (Fig. 1C).

4. Coat the platinum electrodes with clear acrylic nail polish all the way down to about 1 mm from the end. If using the Grass electrodes, the electrodes should be coated down to the point where the electrode starts to taper. Having only about 1 mm of the electrodes exposed allows you to direct the electric field to the appropriate region of the embryo.

NOTE: When coating the electrodes, apply several very light coats of polish to avoid dripping toward the ends that should remain uncoated. Also, have the ends of the electrodes pointing upward to prevent drips from running toward the ends.

DNA Plasmid Preparation

5. Isolate and purify the GFP-expression plasmid using a midi- or maxi-prep kit (e.g. Qiagen, etc.). Do the final isolation step using sterile water instead of TE buffer so that the plasmid can be concentrated.

6. Concentrate plasmid for injections by drying down 20 micrograms of plasmid using a speed-vac. Resuspend the plasmid at 0.5 µg/µl in sterile water + 0.03% phenol red.

Note: Here we use a Gal4-VP16 activator/effector system to maximize GFP expression (kind gift of Dr. Reinhard Koster). This requires the inclusion of two plasmids in the injection solution: (1) a plasmid including the coding sequence for a fusion protein of the Gal4 DNA binding domain and the VP16 transcriptional activation domain, under control of the ubiquitous EF-1α promoter; (2) a plasmid including the coding sequence EGFP under the control of 14 tandem UAS (Gal4 binding) sequences and the fish basal promoter E1b (Koster and Fraser, 2001). Other GFP expression vectors using the CMV promoter can also be used (e.g. pCS2+), although they yield lower levels of expression.

7. Thirty minutes before injections, spin down the concentrated plasmid solution for 30 minutes at max-speed in a microcentrifuge. This pellets any particulates that can clog injection pipettes.

Mounting Embryos for Injection and Electroporation

8. Make a 0.5% solution of low-melting-point agarose in electroporation buffer plus 0.017% tricaine. Dissolve agarose by microwaving. Place agarose in a 32°C incubator and allow temperature to equilibrate.

9. Using fine forceps, free 24 hpf zebrafish embryos from their chorionic membranes. Transfer embryos to a dish containing electroporation buffer plus 0.017% tricaine. Allow at least 2 minutes for the anesthetic take effect.

10. On a 60 mm plastic petri dish, place a large drop (~ 500 µl) of the agarose solution to the center of dish. Transfer several (6-10) embryos from the tricaine solution in as little buffer as possible and add them to the agarose drop.

11. Using fine forceps, position the embryos such that they are aligned in a row and are all facing the same direction (Fig. 2A). Embryos should be lying on their side, tilted slightly such that an injection pipette can access the developing brain on the dorsal side of the embryo (Fig. 2C). To speed up the solidification of the agarose, the alignment can be performed with the embryo dish sitting on top of a larger Petri dish with 2-3 mm of ice. Once the agarose has solidified (1-2 minutes on ice), flood the dish with electroporation buffer plus 0.017% tricaine. Be sure that the agarose is completely covered with buffer.

NOTE: The row of embryos should be aligned such that the micromanipulator-mounted injection pipette can access the embryos from one side (Fig. 2C), and the hand-held electroporation electrode can access the embryos from the other side (Fig. 2B). Ensure that each embryo is orientated such that access of the pipette or electroporation electrode does not disrupt other embryos in the row (Fig. 2A).

DNA Injections

12. Microinjection pipettes should be fabricated using either a horizontal or vertical pipette puller. Heat and pull-strength settings will vary depending on the type of puller, the type of heating element, and the type of glass capillary tubes. Using a Sutter P-30 vertical puller, we found that a heat setting of 980 and a pull-force of 960 yielded long and tapered sharp pipettes (capillary glass with filament; ID = 0.78 mm; OD = 1.0 mm). These pipettes are sealed and must be broken back before injection.

13. Add 1-2 μ l of DNA solution (see steps 6 and 7 above) to the injection pipettes using a microloader pipette tip. Mount the loaded pipette into the pressure injection system pipette holder. Break-back the loaded pipette with fine forceps until pressure pulses lead to puffed release of the red DNA solution. Alternatively, tips can be broken back by quickly penetrating a submerged, folded kimwipe.

14. Inject DNA solution into the midbrain ventricle of the first embryo in the row. Inject with multiple injection pulses until the ventricle slightly distends and is completely full of the red DNA solution.

NOTE: Proceed to the electroporation step as soon as possible after injection because the DNA begins to immediately diffuse and dilute to other parts of the embryo. Electroporate after injection of each embryo. We can typically electroporate within 10 seconds of the injection.

Electroporation

15. Position the hand-held electroporation electrodes (described above, steps 1-4) such that the positive electrode is lateral and slightly dorsal to the hemisphere of the optic tectum to be targeted. Depending on how the embryos is oriented, this typically requires that the positive electrode is tucked underneath the embryo (Fig. 2D-E). This should position the negative electrode lateral and ventral to the opposite eye of the embryo. This orientation of the electric field will specifically target only one hemisphere (right or left) of the developing optic tectum. Thus, when imaged 96 hours after electroporation, expression of GFP is restricted to one side of the brain (Fig. 3B-D).

NOTE: The electroporation electrodes should be 1 mm apart at their tips. With a 1 mm separation, positioning the electrodes as described above should be feasible without touching the embryo. To avoid damage to the embryos always ensure that the electrodes are not physically contacting the embryos during electroporation pulses.

16. Manually initiate 7 electroporation pulses using the single mode switch on the SD-9 stimulator, separating each pulse by about 1 second. A typical voltage protocol for embryos at 24 hpf would be 70 volts, 5 ms duration, with the SD-9 stimulator set to biphasic pulses.

NOTE: Which voltages are appropriate will vary depending on the age of the embryos and the voltage source used. Younger embryos require lower voltages to avoid embryo damage, while older embryos require higher voltages to initiate electroporation (see Kera et al., 2010). When using the SD-9 stimulator, there is a big difference in the voltage dependence whether you use the monophasic or biphasic setting. We've found using the biphasic setting on the SD-9 stimulator that 70-75 volts works well with 24 hpf embryos, while 90-95 volts is required for 96 hpf embryos. Given the variability with setup and embryo age, we recommend measuring the voltage dependence for expression and viability when first setting up *in vivo electroporation in the lab*.

17. Allow the embryos to recover for at least 10 minutes after electroporation before freeing them from the agarose. Using fine forceps, free the embryos by gently tracing the outline of the embryos, while avoiding actual contact with the embryos. Once freed, return the embryos to egg water and keep then at 28°C until they are to be imaged for GFP expression. If using PTU to block pigment formation, wait 3-4 hours after electroporation before returning the embryos to egg water plus PTU.

DISCUSSION

Although not as widely used as in other model organisms, *in vivo* electroporation in zebrafish has been shown have excellent spatial resolution (Bhatt et al., 2004; Teh et al., 2005; Hendricks and Jesuthasan, 2007; Tawk et al., 2009). Here we describe a modified version of a previously described *in vivo* electroporation approach (Kera et al., 2010), which can reliably target neurons in the developing optic tectum. Targeting is efficient, yielding tectal expression of GFP in 79% of the embryos electroporated (Fig. 3A), and expression is restricted to one hemisphere of the developing brain (Fig. 3B-D). Although here we characterize the electroporation of embryos at 24 hpf, *in vivo* electroporation has been shown to have excellent temporal resolution (Teh et al., 2005), and embryos can be efficiently targeted from at least 24 to 96 hpf (Kera et al., 2010). Furthermore, in addition to DNA expression plasmids, electroporation can be used to incorporate either morpholino- or RNAi-based loss-of-function reagents (Thummel et al., 2006; Cerda et al., 2006; Kera et al., 2010). Thus, by simultaneously delivering both an expression plasmid and a loss-of-function reagent, *in vivo* electroporation could provide a very powerful time-resolved genetic approach for analysis of zebrafish development.

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Figure 1. Construction of *in vivo* electroporation electrodes

Electrode construction is greatly simplified using Grass E2 subdermal platinum electrodes as the starting material. This avoids tricky soldering and other manipulations required if using platinum wire. The two Grass platinum electrodes are initially secured to a suitable hand-held plastic probe using small strips of electrical tape (**Fig. 1A**). In this case we are using a small plastic knitting needle for the probe. When fastening the electrodes to the probe, ensure that the end of the platinum electrodes are even (**Fig. 1A**). Next, permanently secure the electrodes using fast-setting epoxy (**Fig. 1B**). Once the electrodes are firmly attached to the probe, bend the platinum electrodes such that the ends are separated by 1 mm (**Fig. 1C**). Finally, secure the wire leads to the hand-held probe using electrical tape (**Fig. 1D**).

Figure 2. Injection and electroporation of zebrafish embryos

For injections and electroporations, the embryos are suspended in low-melting-point agarose and then positioned in a row using fine forceps (Fig. 2A). The row of embryos should be aligned such that the micromanipulator-mounted injection pipette can access the embryos from one side (Fig. 2C), and the hand-held electroporation electrode can access the embryos from the other side (Fig. 2B). For electroporations, position the hand-held electroporation electrodes such that the positive electrode is lateral and slightly dorsal to the hemisphere of the optic tectum to be targeted (Fig. 2D). This requires that the positive electrode is tucked underneath the embryo (Fig. 2E). Note that the electroporation electrodes do not physically contact the embryo.

Figure 3. Using *in vivo* electroporation to target GFP expression to the developing zebrafish optic tectum

Zebrafish embryos were electroporated at 24 hpf using the method described in this protocol. The percentage of embryos expressing GFP within neurons of the optic tectum was determined by fluorescent microscopy (Olympus BX60). Twenty four hours after electroporation, 79% (S.D. 2.7%) of the embryos showed GFP expressed in the optic tectum (Fig. 3A), and 100% of these embryos were expressing GFP in the optic tectum 96 hours after electroporation (Fig. 3A). The orientation of the electric field used for this protocol leads to GFP expression only in one hemisphere of the developing optic tectum (Fig. 3B-D, 96 hours after electroporation). A majority of the cells expressing GFP had the typical morphology of tectal neurons (Fig. 3D). Note, however, that expression is not strictly limited to the optic tectum – significant expression is seen in the developing hindbrain (Fig. 3B-D).