Electroporation of DNA, RNA, and morpholinos into zebrafish embryos

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Abstract

The combination of accessible embryology and forward genetic techniques has made zebrafish a powerful model system for the study of vertebrate development. One limitation of genetic analysis is that the study of gene function is usually limited to the first developmental event affected by a gene. In vivo electroporation has recently matured as a method for studying gene function at different developmental time points and in specific regions of the organism. The focal application of current allows macromolecules to be efficiently introduced into a targeted region at any time in the life cycle. Here we describe a rapid protocol by which DNA, RNA and morpholinos can all be precisely electroporated into zebrafish in a temporally and spatially controlled manner. This versatile technique allows gene function to be determined by both gain and loss of function analyses in specific regions at specific times. This is the first report that describes the electroporation of three different molecules into embryonic and larval zebrafish cells.

1. Introduction

Gene transfer techniques have been used extensively in cultured cells, tissues and whole organisms for assaying eukaryotic gene function. Traditionally, the introduction of genes into cells, or into genomes, has relied on transfection, viral infection or microinjection. Ideally, one should be able to study the effects of a gain or loss of gene function effect in the intact, live organism to monitor the effects of genetic modifications in a physiological context. Transgenesis and gene targeting (knock outs) have been invaluable in the assignment of gene function but they present problems when the gene of interest has multiple roles during development. While tissue specific knock outs or inducible expression systems are elegant ways to circumvent this problem, they are expensive, laborious, and limited to certain organisms.

Electroporation has served as an effective method for introducing DNA into bacteria, yeast, and mammalian cells [1]. This technique uses electric pulses to make small holes in the cell membrane through which DNA molecules can enter the cell. The application has been extended in past years to embryos of several species, most notably the chick, facilitating analyses at the molecular level, which traditionally have been difficult to tackle in many model systems [2,3]. Electroporation into mouse embryos is also possible, though embryos must be cultured after the procedure, a situation that can be extended for a limited time [4]. More recently, *Xenopus* tadpoles have been electroporated with minimal cell death and excellent survival [5]. Electroporation of embryos has been made feasible by altering voltage and current parameters to minimize damage. Most effective are square wave pulses of low voltage and longer durations

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compared to conditions used in cultured cells [6–8]. Electroporation of DNA and RNA into embryos of different stages has thus introduced the possibility of modifying gene activity late in development in organisms that are not genetically tractable. Even when mutations are available, it is often important to analyze the effects of eliminating gene function in a specific tissue while the rest of the animal develops unperturbed. Electroporation provides a means for creating localized loss of function using overexpression of dominant-negative proteins, siRNA [2] or antisense oligonucleotides such as morpholinos [9].

Electroporation of DNA into the zebrafish embryonic neural tube [10] and adult fin [11] have been reported, and this technique was recently used to manipulate Fgf signaling in the zebrafish midbrain/hindbrain region [12]. However, the electroporation of DNA constructs into other tissues, and of mRNA and antisense morpholinos (MOs) into zebrafish, have not been reported. Here we describe a greatly simplified yet precise method for introducing these molecules into neural, retinal, and somitic tissue in the zebrafish embryo. These techniques will extend the use of electroporation and facilitate the analysis of gene function in any tissue and at any time in development.

2. Method for electroporation

2.1. Embryo preparation and mounting

Zebrafish (*Danio rerio*) of the Tübingen or TL strains were maintained on a 14–10-h light–dark cycle and bred in our laboratory according to standard conditions [13]. Embryos obtained from mass matings were kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$, and 0.1% methylene blue), staged in hours postfertilization (hpf) [14], dechorionated, and anesthetized using MS222 (Sigma). 20–24 hour embryos were dechorinated by incubating in pronase (125 µg/ml dissolved in E3 medium) for 2 min followed by three washes in E3 medium. 16–20 hpf embryos were dechorionated by hand, as pronase treatment tended to make them more fragile during mounting. Dechorionated embryos were placed in Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl$_2$, 5 mM Hepes pH 7.2).

To mount embryos for electroporation, a 1% LMT agarose solution was prepared in electroporation Ringers (ER), a slightly modified Ringers solution that facilitates current flow (180 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, pH 7.2). The agarose was made in a 15 ml conical tube and maintained at 50 °C in a water bath until needed. Embryos were pipetted into the tube, allowed to sink, then quickly pipetted onto a 2% agarose (in ER) covered 100 mm petri dish with one embryo per drop of agarose. For speed, embryos were not oriented, with up to 60 embryos being placed in a single dish. Orientation was not necessary when two micromanipulators were used to position the electrodes. The plate was then filled with ER and positioned under a stereomicroscope equipped with two micromanipulators, one controlling each electroporation electrode (Fig. 1).

2.2. Preparation for microinjection

Circular GFP encoding plasmid DNA (Gibco, Cat. # 10642.015) and in vitro synthetized mRNA were diluted to the desired concentration in injection buffer (0.5% Fast Green, 200 mM Tris–Cl pH 7.0, 20 mM EDTA). Morpholinos (Gene Tools, Inc.) were diluted in PBS or water without fast green, as fast green was shown to inhibit MO uptake [9]. DNA (0.7–2 µg/µl) was prepared using a Qiagen plasmid Maxi kit, while mRNA (0.5–1.0 µg/µl) was synthesized using the Message Machine kit (Ambion). Morpholinos were synthesized with a lissamine side chain to allow visualization within the electroporated embryo. 0.2–0.5 mM of the *ptc1* MO (CATAGTCCAAACGGGAGGCAGAAGA) [15] was injected in these experiments. Embryos were pressure injected using a MPPI Pressure injector (ASI, Eugene, OR, USA), with the injection needle being positioned by hand. Injection needles were made using 1 mm capillaries with filament (Cat. # TW100F-4, WPI, Sarasota, FL, USA) and a Sutter P-97 electrode puller and backfilled by pipetting a small drop into the blunt end of the capillary. Electrode tips were broken to the desired diameter using fine forceps. 10–30 nl of solution was microinjected in the somites, eyes, or brain ventricles.

2.3. Microinjection and electroporation

Two different electroporation devices were used to generate electric pulses for these experiments: (1) the ElectroSquare Porator ECM830 (BTX, San Diego, CA, USA) and (2) the TSS20 Ovodyne Electroporator (Intracel, Roysten Herts, UK) without the accessory current amplifier. Both electroporators were effective, however we found that the Intracel unit allowed for a wider range of electroporation conditions. Tungsten electrodes (0.010", Cat. # 573500, A-M Systems Inc., Carlsberg, WA, USA) were used for all experiments. To avoid current leakage it was crucial to insulate the electrodes completely using parafilm, leaving a few millimeters of metal exposed at the tip to contact the target tissue. Electrodes were positioned using micromanipulators such that the resulting current would pass through the desired tissue. For DNA and mRNA, the positive electrode was placed just outside the embryo in the desired location, while the negative electrode was positioned so that it could quickly be inserted into the tissue near the injection site. For lissamine-coupled MOs, the reverse polarity was used. Embryos were then injected, the negative electrode was inserted into the tissue, and current was quickly applied using a foot switch.

Optimizing the electroporation parameters is imperative as the application of high-voltage pulses can kill many cells. Parameters of voltage, pulse length, pulse intervals, and the number of pulses for DNA, mRNA, and MO electroporation are specified in Table 1.
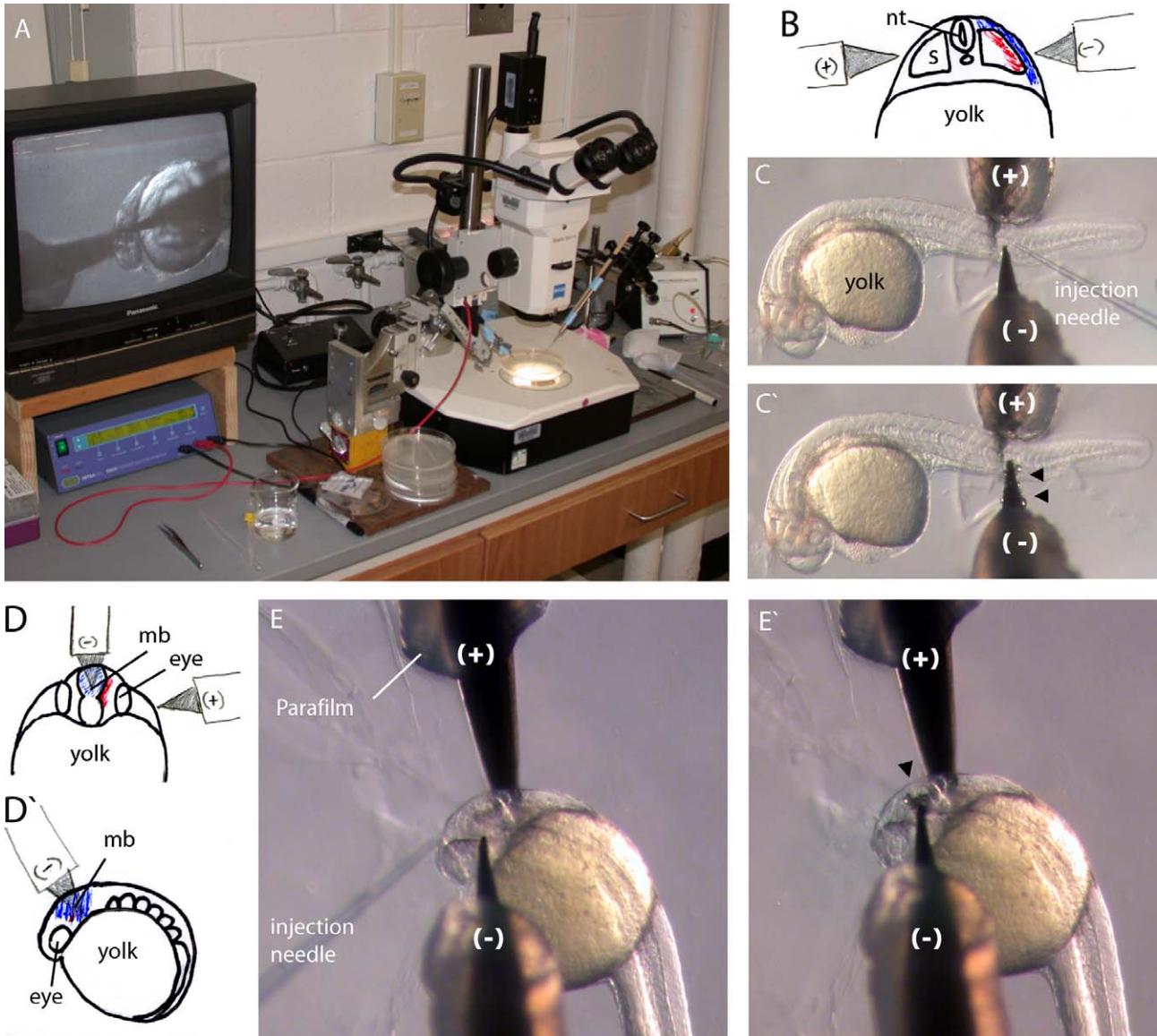
Fig. 1. Electroporation setup and procedure. (A) A Zeiss SV11 stereomicroscope was outfitted with two micromanipulators that held the electroporation electrodes, while the injection needle was hand held. Foot switches controlled both the electroporator (under monitor) and the pressure injector (far right). (B) Diagrammatic cross section showing position of electrodes for somite electroporation. Light shading shows position of injected solution, dark shading shows location of electroporated cells. (C) Higher magnification views of an embryo being electroporated in the somites with the electrodes positioned and the injection needle in place. (C′) The same embryo after electroporation. Note the appearance of bubbles on the uninsulated portion of the negative electrode (arrowheads), an indication that current has been applied. (D and D′) Diagrams showing position of electrodes for midbrain electroporation in cross section (D) and side view (D′). Same shading as in (B). (E) Embryo being electroporated in the midbrain with electrodes and injection needle in place. (E′) Same embryo just after current has been applied. The negative electrode has been inserted into the third ventricle after injection and prior to the application of current. Arrowhead points to bubbles in the midbrain. Note: electrode polarity shown needs to be reversed for MO electroporation. mb, midbrain; nt, neural tube; s, somite.

These parameters serve as a starting point and should be optimized for each electroporator and electrode set-up, as well as for each tissue and developmental stage being analyzed. After electroporation, the embryos were gently removed from the agarose with the help of forceps and placed in petri dishes containing zebrafish Ringer's solution with penicillin–streptomycin (50–100 U/ml) and incubated at 28 °C. GFP and lissamine fluorescence were visualized in live embryos using both dissecting and compound microscopes (Fig. 2).

3. Concluding remarks

The ability to express or inactivate a gene in a directed fashion is a powerful means of analyzing its role in development. Electroporation is a simple procedure for spatially and temporally regulating gene function that can be performed more easily and quickly than any other transfection method. The electroporation protocol we describe here has several advantages over those previously described for zebrafish. First, by replacing both electrodes with

Table 1
Electroporation conditions and efficiencies for DNA, mRNA, and MOs

Molecule (location)	Stage	Optimal voltage protocol	Survival rate (%)	Electroporation rate for survivors (%)	*n*
DNA-pGL (midbrain)	13–48 hpf	5–8 V; 1 ms; 2×[b]	87	77	246
		13–17 V; 1 ms; 3×[a]			
DNA-pGL (somites)	13–16 hpf	16 V; 1 ms; 2×[b]	86	41	51
DNA-pGL (eye)	24 hpf	12–16 V; 1 ms; 3×[a]	80	40	25
DNA-pGL (midbrain)	3–5 dpf	13–19 V; 1 ms; 3×[a]	63	73	63
mRNA-GFP (eye)	3 dpf	6–12 V; 1 ms; 3×[a]	89	53	76
mRNA-GFP (midbrain)	5 dpf	12 V; 1 ms; 3×[a]	82	45	63
mRNA-GFP (trunk)	5 dpf	8–11 V; 1 ms; 2×[a]	100	45	20
MO-lissamine (midbrain)	15 hpf	5 V; 1 ms; 2×[b]	76	83	223
		2–4 V; 1 ms; 3×[a]			

dpf, days postfertilization; hpf, hours postfertilization; pGL, green lantern plasmid encoding GFP under the control of a CMV promoter.

[a] Parameters for Intracel Ovodyne Electroporator.

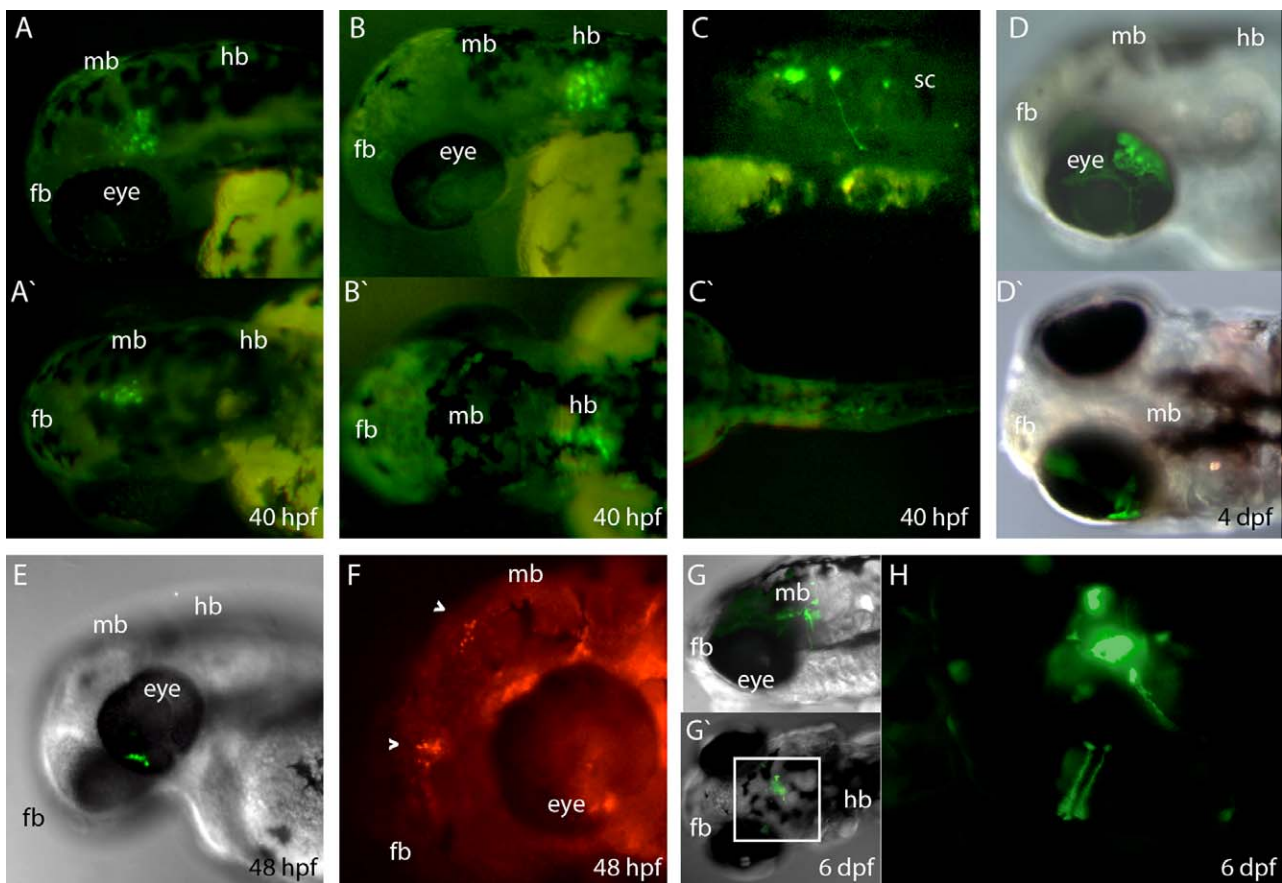[b] Parameters for the BTX Electro-Square Porator.

Fig. 2. Expression of GFP and localization of MOs following electroporation. (A and A′) Lateral and dorsal views (respectively) of a 40 hpf embryo that was electroporated in the midbrain at 24 hpf with the GFP encoding plasmid pGl. (B and B′) Lateral and dorsal views of a 40 hpf embryo that was electroporated in hindbrain at 24 hpf with pGl. (C and C′) Lateral and dorsal views of a 40 hpf embryo electroporated in the trunk at 16 hpf with pGl. (D and D′) Lateral and dorsal views of a 4 dpf larva that was electroporated in the eye at 3 dpf with GFP-encoding mRNA. (E) Lateral view of a 48 hpf embryo electroporated in the eye at 24 hpf with pGl. (F) Lateral view of a 48 hpf embryo electroporated at 14 hpf with the lissamine conjugated *ptc1* morpholino. Open arrowheads indicate electroporated cells in the dorsal forebrain and midbrain. (G and G′) Dorsal and lateral views of a 6 dpf larva that was electroporated in the midbrain at 5 dpf with pGl. (H) Higher magnification view of labeled midbrain neurons seen in (G). fb, forebrain; hb, hindbrain; mb, midbrain; sc, spinal cord.

sharpened tungsten needles we were able to narrow and precisely position the electric field, allowing for very precise targeting of macromolecules into the brain. Second, this technique allowed for highly efficient introduction of macromolecules while maintaining high survival rates. Third, by rapidly mounting embryos, we greatly increased the speed of the protocol, allowing many more experiments to be performed in a given time. Finally, we show that DNA, mRNA, and morpholinos can all be precisely targeted into zebrafish tissue.

'Conditional mutagenesis' techniques such as Cre–Lox recombination in the mouse [16] and the Gal4–UAS system in the fly [17] and zebrafish [18] allow tissue specific and time-restricted analysis, but the process can be complicated and is dependent on having promoters that will drive expression in the correct tissue and/or at the desired time. Further, the analysis of two or more genes is especially difficult using these methods. Using electroporation it is now relatively easy to perform both gain-of-function and loss-of-function experiments in any tissue. A key advantage of electroporation is the ease with which two or more expression constructs can be simultaneously introduced into cells. Morpholinos, dominant negative constructs, and/or constitutive activator constructs for different genes can be combined to simultaneously manipulate multiple genes in the same cells. Since in vivo electroporation can alter gene expression in a small area, this method will be helpful for the generation of region specific transient knockouts and will allow analyses in embryonic areas for which specific transcriptional control sequences have not been identified.

Our studies illustrate that GFP-construct electroporated cells can be easily visualized in the zebrafish, both in the live embryo (Fig. 2), as well as after fixation and using the anti-GFP antibody (data not shown). We were able to detect strong fluorescence from the GFP transgene product both 12 h and 24 h after electroporation.

By co-electroporating constructs encoding GFP or other cellular markers it will thus be possible to track the developmental fates of cells with altered gene expression as well as to determine the effects on surrounding cells. Importantly, these studies can be done at any time in embryonic or larval development, or in adult tissue, greatly expanding the ability to assess gene function. This approach is especially useful for studying postembryonic gene activity, as early essential gene function can be bypassed. This will greatly facilitate the study of processes such as regeneration, tumorigenesis, or response to injury in differentiated tissue.

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