Electroporation-mediated gene transfer in free-swimming embryonic *Xenopus laevis*

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Abstract ***Xenopus laevis*** **are a rich resource for vertebrate embryology and cell biology. Transplantation and transgenesis have provided much information about the developmental mechanisms of embryogenesis and molecule function, however existing methods have faced limitations regarding either the precise localization of gene expression or flexibility in the timing of gene transfer. Here we have found that electroporation of tailbud (stage 29/30) embryos is a rapid and efficient method of combining cell-specific expression with variation in temporal delivery. At the low voltages required for electroporation, embryos resumed normal swimming behavior and development. We conclude that electroporation has wide experimental application to** ***Xenopus*** **developmental and cell biology.**

Key words: Electroporation; Gene transfer; Lacz; Transgenic; *Xenopus laevis*

1. Introduction

The tailbud stage of *Xenopus* (Nieuwkoop and Faber stage 29/30 [1]) is a useful stage for studying molecular contributions to cell migration, body patterning and organogenesis, yet relative few methods exist for precise spatial and temporal control of gene expression. Electroporation is a method of delivering brief electric field impulses to cells or tissue to allow DNA, enzymes, antibodies and other macromolecules to enter cells [2]. In other experimental systems, electroporation has offered advantages over viral methods of gene delivery by its immediate effects, lack of immunogenicity and unrestricted DNA size [3]. Also, electroporation appears equivalent or superior to other non-viral gene delivery methods (lipofection, gene gun, direct DNA injection), in terms of transfer efficiency [3]. Electroporation has most widely been applied to embryonic stem cells [4], cell lines [5] or primary cultures [6], however more recently it has also been successfully applied to intact chick embryos (chick) [7,8]. In the present study, we were interested in examining electroporation as a method of gene delivery and macromolecule uptake in swimming stage 29/30 of *Xenopus*. Expression plasmids using ubiquitous or cell-specific promoters were tested.

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2. Materials and methods

2.1. Animals

Xenopus embryos were generated by in vitro fertilization as previously described by Liu et al. [9]. Embryos were allowed to develop to Nieuwkoop and Faber stages 25–29/30, were briefly treated with 0.15% tricaine, then electroporated as described below.

2.2. Electroporation

For electroporation, embryos were placed in a 3 μl 1×MMR [10] ± plasmid DNA or propidium iodide (PI) (50 μg/ml; Sigma). PI is a useful marker of macromolecule delivery because it is impermeable to the membranes of viable cells, yet fluorescently labels cell nuclei with successful electroporation [11]. Plasmid DNA consisted of either pEF/myc/cyto/GFP (Invitrogen) or pGFA2Lac-1 (gift of M. Brenner, University of Alabama) at 0.5–1 μg/ml. pEF/myc/cyto/GFP is a 6.1 kb plasmid which drives green fluorescent protein (GFP) under the control of the elongation factor 1-α promoter. pGFA2Lac-1 is an 8.5 kb plasmid that drives the lacz gene under the control of the human glial fibrillary acidic protein promoter [12]. An ECM 803 electroporator (BTX, San Diego, CA, USA) was used for all experiments. Embryos were placed in a 3 μl drop of DNA between the tips of Genetrode (BTX) electrodes spaced 3–4 mm apart. Effective electroporations were performed at 10–20 V, 50 ms pulse length, 1 s pulse interval, 4–8 shocks. Following electroporation, embryos were photographed using an automated fluorescent photomicrographic system from Olympus (BX50, Lake Success, NY, USA).

PI and GFP embryos were analyzed live. For axial sections, embryos were euthanized in tricaine, fixed in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% paraformaldehyde, pH 7.4) for 1 h, then sectioned with a sharp microscissors.

2.3. Adeno-associated viral (AAV) injections

AAV DNA vector injections were performed as described by [13]. The nβgalITR expression vector was generously provided by Sylvia Evans (Scripps Institute). nβgalITR consists of a plasmid containing AAV inverted terminal repeats and the cytomegalovirus enhancer, 5′ untranslated region from the β-globin gene, and nuclear-localized lacz (β-galactosidase). Prior to injection, the plasmid was linearized with *Not*I, purified by alcohol precipitation and then injected into two cell embryos using a Nanoject injector (Drummond).

For lacz development, embryos were fixed in MEMFA for 1 h, washed in 0.1 M phosphate buffer (PB) for 5 min, then transferred embryos into βgal solutions consisting of 32.93 mg potassium ferricyanide, 42.24 mg potassium ferrocyanide and 0.1 ml 15% Xgal (15 mg in 100 μl DMF) in 10 ml 0.1 M PB [13].

3. Results

3.1. Electroporation-mediated delivery comparable to AAV-mediated transgenesis

Fig. 1 demonstrates the efficiency of electroporation-mediated delivery compared to conventional transgenic methods. Fig. 1A–C shows the head regions of stage 29/30 embryos expressing nuclear Lacz (A), vector control (B) or electroporated PI (C). Fig. 1D–F shows representative back regions of

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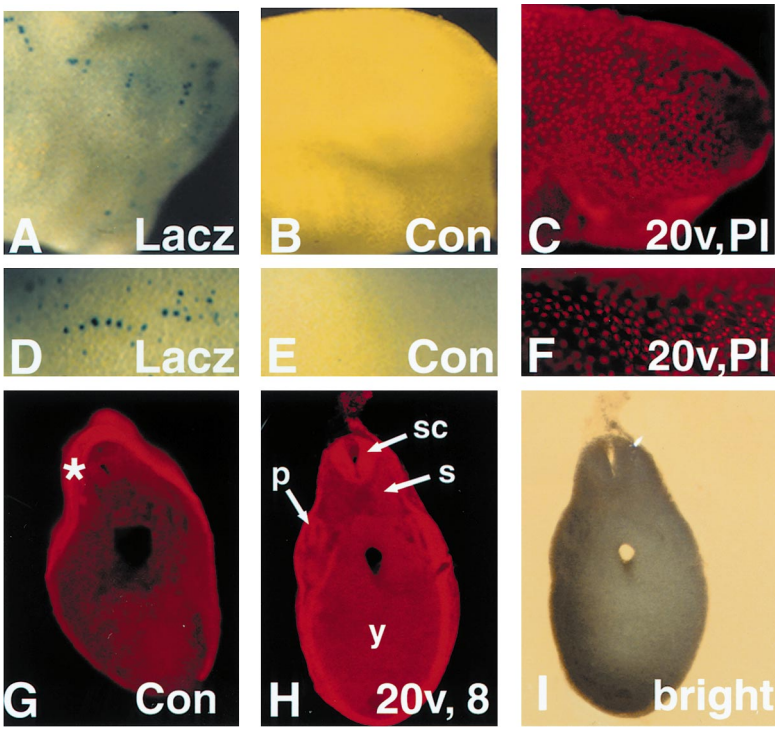


Fig. 1. Efficiency of electroporation-mediated PI delivery. A–C shows the head regions of stage 29/30 embryos expressing Lacz (AAV transgenesis), vector control (B), or electroporated PI (C, 20 V, 8 pulses). Representative back regions of these embryos are shown in D–F. G and H show efficient delivery by electroporation throughout the embryo in the axial plane. G and H are control (G) and electroporated (H, 20 V, 8 pulses) embryos that had been incubated briefly while live in PI, euthanized, fixed and sectioned. The asterisk in G shows non-specific PI staining in fixed epidermis. p, pronephric anlage, s, somite, sc, spinal cord, y, yolk mass. A brightfield view is shown in I.

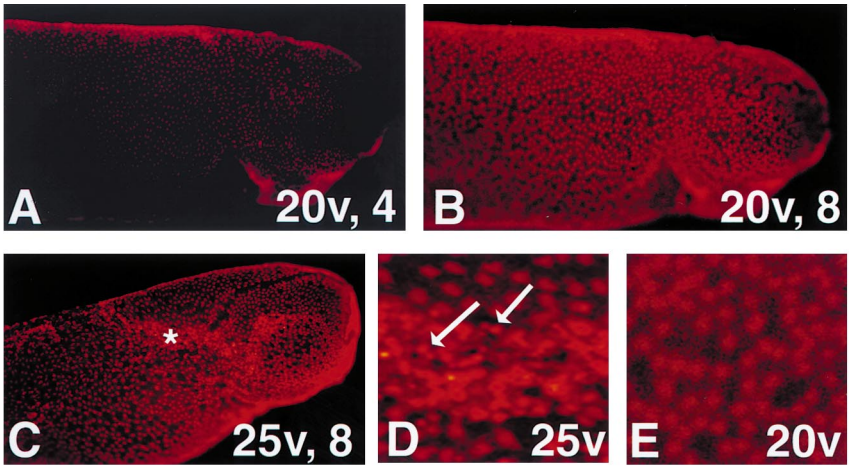
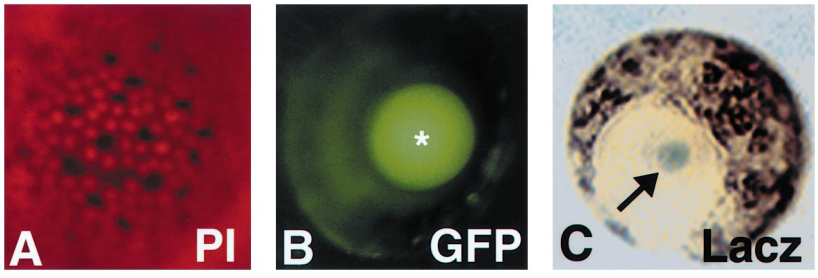


Fig. 2. Effect of shock number on electroporation-mediated delivery. A and B show the correlation of pulse number with the quantity of PI delivered to the nucleus. A and B are photographs of a single embryo after 4 (A) and 8 (B) pulses. All other electroporation parameters were held constant (20 V, 50 ms). At higher voltages (25 V) some tissue injury could be detected in embryos by irregularities in PI staining or kinking of tails. C shows a line of condensed PI-stained nuclei (asterisk) thought to reflect early apoptotic changes. D is a high magnification view of the asterisked region shown in C (arrows). E is a similar high magnification view of an embryo electroporated at lower voltage (20 V).



conventional transgenic (D), control (E) or electroporated (F) embryos. At 250 V/cm, 50 ms pulse length, 1 s pulse interval, pulse number 8, electroporation caused a robust and uniform uptake of PI throughout the embryo, with more definite and homogeneous labeling of each nuclei than seen with AAV plasmid injections (A). Under the optimal electroporation conditions, 100% of stage 29/30 embryos showed prominent PI nuclear staining, and few deaths occurred (3%, $n = 60$). In some cases, the brief exposure to tricaine may have contributed to deaths. No untoward effects were observed from electroporation at this setting, embryos awakened within seconds to minutes of electroporation/tricaine anesthesia, quickly resuming normal swimming and escape behaviors. Fig. 1G–I showed electroporation was able to deliver in a fairly uniform fashion throughout the embryo. The asterisk in Fig. 1G shows non-specific PI labeling (MEMFA-fixed) of PI-treated (but non-electroporated) controls. Control embryos were incubated in PI, but not electroporated. Fig. 1H shows that electroporation mediated fairly uniform delivery of PI throughout the embryo, including the spinal cord (sc), somites (s), pronephric anlage (p) and endodermal yolk mass (y). Fig. 1I is a brightfield view of the electroporated embryo shown in Fig. 1H.

In all cases, electroporation at optimized levels (250 V/cm, 50 ms, 8 pulses) did not appear to affect normal development. Stage 25 (hatched) embryos were earliest and stage 35/36 the oldest whole tadpoles to be electroporated in this study. By stages 40–45, electroporations appeared incomplete, most likely because of the larger size and increased thickness of the embryos.

3.2. PI uptake is proportional to shock number; higher voltages cause damage

Fig. 2A,B show the effect of increasing pulse number on PI uptake. At 20 V, 4 pulses (Fig. 2A), nuclear PI staining could be seen throughout the embryo, but the overall intensity of fluorescent staining was low, and some regional variations in fluorescence intensity could be seen. Fig. 2B shows the same embryo in Fig. 2A with 4 additional pulses. Increasing pulse number greatly increased PI uptake, consistent with a manipulation of shock numbers to vary macromolecule or gene delivery.

At 25 V (600 V/cm), we began observing some tissue damage from the electroporations. Some embryos developed kinked tails, poor swimming and alterations in PI staining within 5 h. Fig. 2C,D are low and high magnification views of an embryo with an electroporation-mediated damage (4 h post-electroporation). Fig. 2C shows a jagged line of (asterisk) condensed nuclei occurring in the vicinity of where the electrodes were positioned. At higher magnification (Fig. 2D), condensed nuclei were seen (arrows) consistent with early apoptotic changes [14]. Fig. 2E shows a similar high magnification view of an embryo electroporated at 20 V (250 V/cm) for comparison.

3.3. Cell-specific gene expression retained with electroporation

Fig. 3A–C demonstrates the variation in gene expression offered by different promoters in the developing eye. Fig. 3A shows the uniformity of electroporation-mediated PI uptake in the stage 29/30 embryo. Fig. 3B,C shows the results of electroporated DNA plasmids using different promoters (elongation factor 1-α, glial fibrillary acidic protein) analyzed at stage 42 (3 days following electroporation). Under the elongation factor 1-α promoter, GFP was expressed at high levels in the cornea (asterisk) as well as in the surrounding neural retina (Fig. 3B). In contrast, Fig. 3C shows a brightfield photograph of restricted lacz gene expression over the optic nerve (arrow). In *Xenopus* optic nerve, as with most vertebrate species, glial fibrillary acidic protein is expressed at high levels [15,16]. Because no neuronal cell bodies comprise the optic nerve, intense glial-specific expression can be observed overlying the optic nerve in whole mounts.

4. Discussion

In this paper, we conclude that electroporation is an efficient low morbidity technique for gene and macromolecule transfer at the tailbud stage in *Xenopus*. With 8 pulse repetitions at 250 V/cm, 50 ms pulse interval, high levels of PI uptake or gene delivery were achieved. Following electroporation, embryos quickly resumed their normal swimming behaviors and were indistinguishable from non-electroporated controls. Follow-up 2 weeks after electroporation showed no gross developmental alterations. Cell-specific expression was retained in electroporation, so that precise temporal control of foreign gene expression could be combined with cell-specific expression using selective promoters. In developing *Xenopus*, the combination of tight spatial and temporal control may be a powerful strategy for determining gene function during development. *Xenopus* tailbud embryos may develop from the 2-cell embryos in less than 2 days [17]. In the *Xenopus* retina, only 8 h separates the cessation of DNA synthesis in the central retina (stage 28) and arrival of the optic nerve in the diencephalon (stage 32) [18,19]. Given the rapid pace of development in this animal model, precise minute-to-hour control of gene delivery can facilitate the dissection of biological events. At present, conventional transgenic methods allow some spatiotemporal control of gene expression [13,19], however precise cellular restriction usually offers little leeway in the timing of gene delivery, and inducible systems offer little leeway in the restricting sites of expression [20]. Tetracycline-regulated gene shutoff may provide one alternative to conventional expression systems [21], however toxicity and the incompleteness of gene shut-off complicate this approach. In summary, we believe that electroporation will be a powerful addition to gene delivery techniques in developing *Xenopus*. Because of the ease of use, the immediacy of gene transfer, high and selective expression levels and low morbidity, it has broad applications in developmental, physiological and pharmacological studies involving this animal system.

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Fig. 3. Electroporation permits cell-specific expression in the *Xenopus* eye. A–C show the effect of electroporated PI (A), and electroporated GFP (B) and lacz (C) plasmids in wholemount stage 42 embryos. GFP was expressed under the ubiquitous promoter elongation factor 1-α, and lacz was expressed under the glial fibrillary acidic protein promoter (see Section 2). The asterisk in B indicates prominent green fluorescence in the cornea. In C, the arrow indicates the presence of lacz staining overlying the optic nerve.

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References

[1] Nieuwkoop, P.D. and Faber, J. (1956) in: Normal Table of *Xenopus laevis*, Daudin, Amsterdam.
[2] Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) EMBO J. 7, 841–845.
[3] Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997) Biochem. Biophys. Res. Commun. 230, 376–380.
[4] Doetschman, T., Maeda, N. and Smithies, O. (1988) Proc. Natl. Acad. Sci. USA 85, 8583–8587.
[5] Baum, C., Forster, P., Hegewisch-Becker, S. and Harbers, K. (1994) BioTechniques 17, 1058–1062.
[6] Kotnis, R.A., Thompson, M.M., Eady, S.L., Budd, J.S., Bell, P.R. and James, R.F. (1995) Eur. J. Vasc. Endovasc. Surg. 9, 71–79.
[7] Muramatsu, T., Nakamura, A. and Park, H.M. (1998) Int. J. Mol. Med. 1, 55–62.
[8] Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K. and Yasuda, K. (1999) Dev. Growth Differ. 41, 335–344.
[9] Liu, Z.Z., Zhu, L.Q. and Eide, F.F. (1997) J. Neurosci. 17, 8749–8755.
[10] Seidman, S. and Soreq, H. (1997) Transgenic *Xenopus*, Humana Press, Totwa, NJ.
[11] Harrison, R.L., Byrne, B.J. and Tung, L. (1998) FEBS Lett. 435, 1–5.
[12] Brenner, M., Kisseberth, W.C., Su, Y., Besnard, F. and Messing, A. (1994) J. Neurosci. 14, 1030–1037.
[13] Fu, Y., Kan, D. and Evans, S. (1999) Methods Mol. Biol. 127, 167–173.
[14] Fukuzuka, K., Rosenberg, J.J., Gaines, G.C., Edwards, C.K., Clare-Salzler, M., MacKay, S.L., Moldawer, L.L., Copeland, E.M. and Mozingo, D.W. (1999) Ann. Surg. 229, 851–858.
[15] Dahl, D., Crosby, C.J., Sethi, J.S. and Bignami, A. (1985) J. Comp. Neurol. 1239, 75–88.
[16] Taylor, J.S. (1993) J. Neurocytol. 22, 1007–1016.
[17] Hausen, P. and Riebesell, M. (1991) in: The Early Development of *Xenopus laevis*: an Atlas of the Histology, Springer, New York.
[18] Grant, P., Rubin, E. and Cima, C. (1980) J. Comp. Neurol. 189, 593–613.
[19] Amaya, E. and Kroll, K.L. (1999) Methods Mol. Biol. 97, 393–414.
[20] Wheeler, G.N., Hamilton, F.S. and Hoppler, S. (2000) Curr. Biol. 10, 849–852.
[21] Ridgway, P., Quivy, J.-P. and Almouzni, G. (2000) Exp. Cell. Res. 256, 392–399.