Video Article

Electroporation of Craniofacial Mesenchyme

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URL: http://www.jove.com/video/3381
DOI: doi:10.3791/3381

Keywords: Developmental Biology, Issue 57, craniofacial, electroporation, *Xenopus laevis*, frog, cartilage, mesenchyme

Date Published: 11/28/2011

Citation: Tabler, J.M., Liu, K.J. Electroporation of Craniofacial Mesenchyme. *J. Vis. Exp.* (57), e3381, doi:10.3791/3381 (2011).

Abstract

Electroporation is an efficient method of delivering DNA and other charged macromolecules into tissues at precise time points and in precise locations. For example, electroporation has been used with great success to study neural and retinal development in *Xenopus*, chicken and mouse [1-10]. However, it is important to note that in all of these studies, investigators were not targeting soft tissues. Because we are interested in craniofacial development, we adapted a method to target facial mesenchyme.

When we searched the literature, we found, to our surprise, very few reports of successful gene transfer into cartilaginous tissue. The majority of these studies were gene therapy studies, such as siRNA or protein delivery into chondrogenic cell lines, or, animal models of arthritis [11-13]. In other systems, such as chicken or mouse, electroporation of facial mesenchyme has been challenging (personal communications, Dept of Craniofacial Development, KCL). We hypothesized that electroporation into procartilaginous and cartilaginous tissues in *Xenopus* might work better. In our studies, we show that gene transfer into the facial cartilages occurs efficiently at early stages (28), when the facial primordium is still comprised of soft tissue prior to cartilage differentiation.

Xenopus is a very accessible vertebrate system for analysis of craniofacial development. Craniofacial structures are more readily visible in *Xenopus* than in any other vertebrate model, primarily because *Xenopus* embryos are fertilized externally, allowing analyses of the earliest stages, and facilitating live imaging at single cell resolution, as well as reuse of the mothers [14]. Among vertebrate models developing externally, *Xenopus* is more useful for craniofacial analysis than zebrafish, as *Xenopus* larvae are larger and easier to dissect, and the developing facial region is more accessible to imaging than the equivalent region in fish. In addition, *Xenopus* is evolutionarily closer to humans than zebrafish (˜100 million years closer) [15]. Finally, at these stages, *Xenopus* tadpoles are transparent, and concurrent expression of fluorescent proteins or molecules will allow easy visualization of the developing cartilages. We anticipate that this approach will allow us to rapidly and efficiently test candidate molecules in an *in vivo* model system.

Video Link

The video component of this article can be found at http://www.jove.com/video/3381/

Protocol

Part 1A. Equipment

Microscope: upright stereo-dissecting scope with low power objective

- **Voltage/Pulse Generator:** BTX ECM 830 Square Wave Electroporation System
- **Pipette puller:** P-87 Micropipette Puller (Sutter Instrument Company, CA)
- **Manipulator:** coarse, or combined coarse and fine depending on preparation.
- **Micropipette holder:** Fine Science Tools
- **Electrode:** homemade
- **Electroporation chamber:** homemade

L-shaped electrodes:

1. Cut 8 cm of high-purity 0.4 mm tungsten wire (Goodfellow) and affix at midpoint a 1ml syringe using putty (we use Blu-Tack). Leave 4 cm tungsten wire exposed from the tip of the syringe and bend tip into L-shape, 1 cm from end (Fig. 1A).
2. Trim tip so that the end measures 0.5 mm in length. This tip is the electrode terminus.
3. Run excess tungsten wire parallel to syringe and use it to connect the electrode pulse generator.
4. Repeat process making a pair of electrodes.
5. Attach electrodes to square wave pulse generator via DC cables.

Electroporation chamber

1. Line bottom of 90 mm dish with #5 mm non-toxic plasticine.
2. Fill dish with electroporation media.

3. Using No. 5 watchmaker's forceps carve a T-shaped well (Fig. 2). The long well should measure #2 mm x 2 mm x 10 mm and the short #2 mm x 2 mm x 5 mm. The electroporation dish can be washed and reused.

Part 1B. Reagents

DNA or charged macromolecules

- **Micropipettes:** 1 mm wide 4" long borosilicate glass capillaries (WPI, TW100-F)
- **Culture media: Normal Amphibian Media (NAM)**
- >10 X stock: 1100 mM NaCl, 20 mM KCL, 10 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM EDTA.
  - Autoclave and store at 4 °C.
  - 1 X NAM: Dilute from 10x stock, buffer with 0.1 mM $NaHCo_3$ and 0.2 mM Na_3PO_4.
- ***Xenopus laevis* tadpoles, stage 28**

DNA preparation:

1. Prepare expression plasmids using standard protocols.
2. Resuspend DNA to a final concentration of 1 µg/µl in nuclease free H_20.

* We have had success with vectors containing a strong CMV promoter, such as pCS2+ [16]. For lineage analysis, we usually include DNA encoding green fluorescent protein (pCS2+GFP) at a final concentration of 0.1 µg/µl. [DNA concentrations between 0.1-3 µg/µl were also tested. We found that concentrations below 0.8 µg/µl inefficiently labelled cells, whereas DNA concentrations greater than 2 µg/µl did not improve electroporation efficiency.]

Morpholino oligonucleotide preparation:

(Note: MOs need to be fluoresceinated (3'-carboxyfluorescein modified) or otherwise charged.)

1. Resuspend morpholino oligonucleotides (MOs) (Genetools, www.genetools.com) at a concentration of 2 mM in nuclease free H_20.
2. Heat aliquot of stock solution at 65°C for 5 minutes.
3. Dilute to final concentration of 0.5 mM in nuclease free water.

* 0.1-1mM MO solutions were tested. 0.5 mM MO solutions were sufficient for electroporation of many mesenchymal cells.

Micropipettes

1. Prepare micropipettes from borosilicate glass capillaries (1 mm wide, 4" long, WPI no. TW100-F). Use needle puller to prepare micropipettes with an 8-12 mm long taper and fine tip.
2. Crush tip #2 mm from tip using forceps, creating a jagged break.

Media

1. Incubation media: Prepare fresh 3/4 Normal Amphibian Media (NAM) from 1x stock. Add 0.025 mg/ml gentamycin.
2. Electroporation media: as above, with 0.1% Benzocaine (Sigma, 06950).

2. Electroporation

Micropipette setup

1. Fill micropipette with #1 µl injection solution.
2. Secure micropipette in micromanipulator and attach to microinjector (Picospritzer II).
3. Angle micropipette at 50° from the tabletop.
4. Set injection pressure at 20 PSI.
5. Calibrate micropipette to inject 30 nl per pulse.

Tadpole preparation

1. Anesthetize stage 28 *Xenopus* larvae by incubating in electroporation media for 5 minutes.
2. Transfer anaesthetised tadpole into electroporation chamber filled with electroporation media. Position embryo within the long well so that the head rests in the T-junction with dorsal side down and ventral side exposed. The head should be slightly elevated compared to the tail.
3. Using forceps, gently secure tadpole in well with surrounding plasticine. (Note: If the tadpole is not secured, it may twitch and contact electrode during electroporation. In this case discard the tadpole as facial tissues will be severely damaged.)

Electroporation

1. Insert micropipette tip immediately posterior to the cement gland and into facial mesenchyme.
2. Inject 30 nl solution into mesenchyme.
3. Retract micropipette.
4. Quickly align electrode tips parallel to the head of the embryo (Fig. 3).
5. Apply 8 50 ms, 20 V square pulses.

6. Retract electrodes.
7. Using forceps carefully release tadpole from well and transfer to 3/4 NAM, 0.025 mg/ml gentamycin.
8. Tadpoles can be incubated in 3/4 NAM, 0.025 mg/ml overnight, or longer.
9. Screen embryos for efficient electroporation by fluorescence microscopy after 24 hours.

3. Representative Results:

The use of fluorescent molecules allows easy screening of electroporated embryos. Figure 4 shows a typical batch of MO electroporated tadpoles #12, 48 and 96 hours after electroporation, incubated at 14.5°C. Using fluorescence microscopy, MOs can be visualised immediately after electroporation and persist for several days after electroporation. In our experience, fluorescence is weakly evident at stage 46 (#5 days later). In the cartilages, fluorescence decreases dramatically after the onset of differentiation (#st 42); however, MO fluorescence persists more strongly in other cell types such as the pharyngeal endoderm. Fluorescence microscopy shows that oligonucleotides are incorporated into several craniofacial tissues including cartilage. Oligonucleotide fluorescence can often be visualised in tissue on either side of the head. This is likely due to rapid diffusion of the injection solution throughout the loose craniofacial mesenchyme prior to electroporation.

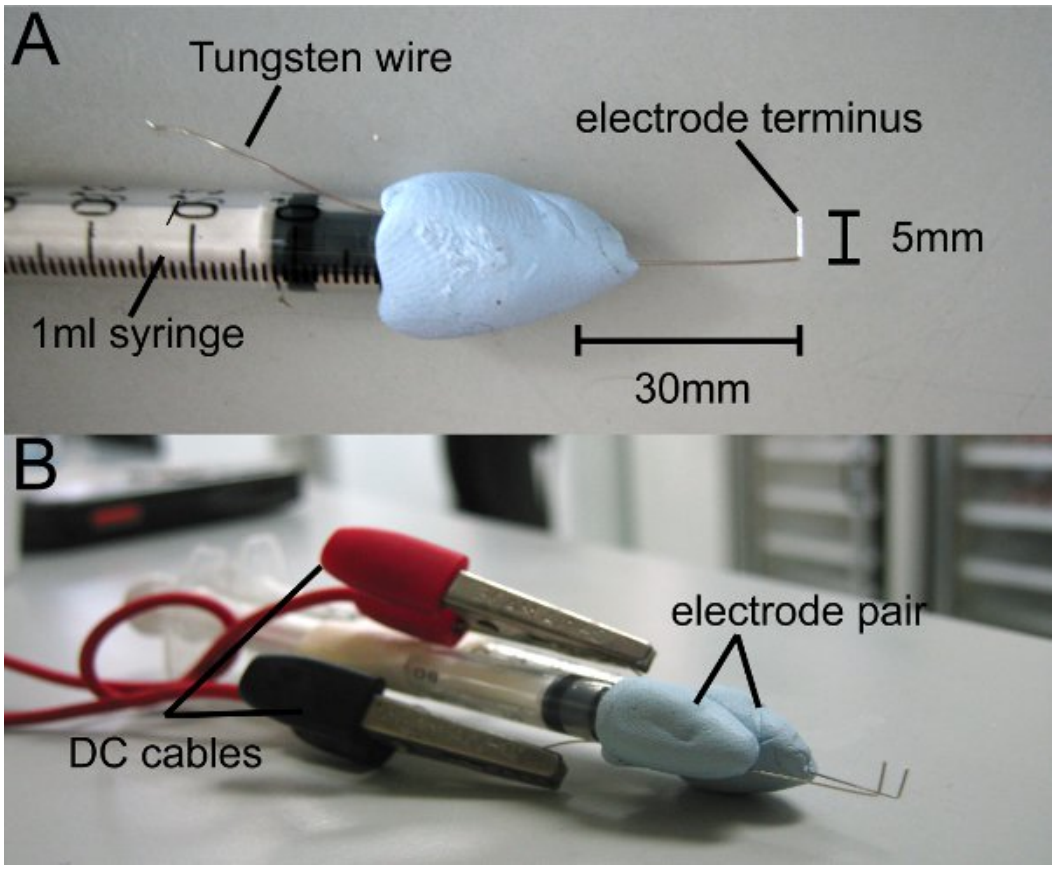

Figure 1 Homemade electrodes. L-shaped tungsten wire is attached to a 1 ml syringe using non-toxic clay or putty. (A) The electrode terminus measures 5 mm. (B) Attach a pair of electrodes, such that the termini run parallel. Electrodes are attached to pulse generator by DC cables.

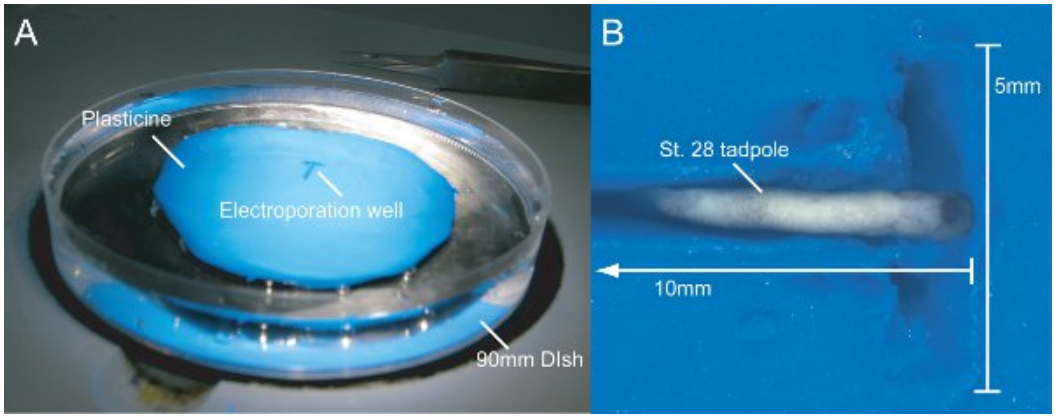

Figure 2 Electroporation chamber. (A) 90 mm dish lined with plasticine is filled with media and a T-shaped chamber carved with No 5 watchmaker's forceps. (B) The long side measures 2 mm X 2 mm X10 mm whilst the short measures 2 mm X 2 mm X 5 mm. The head of the embryo rests in the T-junction, ventral side up.

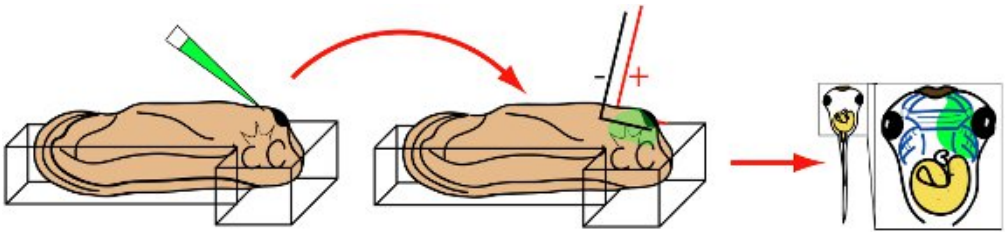

Figure 3 Schematic illustrating electroporation procedure. St. 28 tadpole is placed in electroporation chamber, ventral side up. Micropipette is inserted into facial mesenchyme underlying cement gland. Inject. Micropipette is removed and L-shaped electrodes are aligned parallel flanking the head. Apply eight 50 ms, 20 V square pulses. Retract electrodes. Grow tadpoles to desired stages. Visualise MOs or GFP expression using fluorescence microscopy.

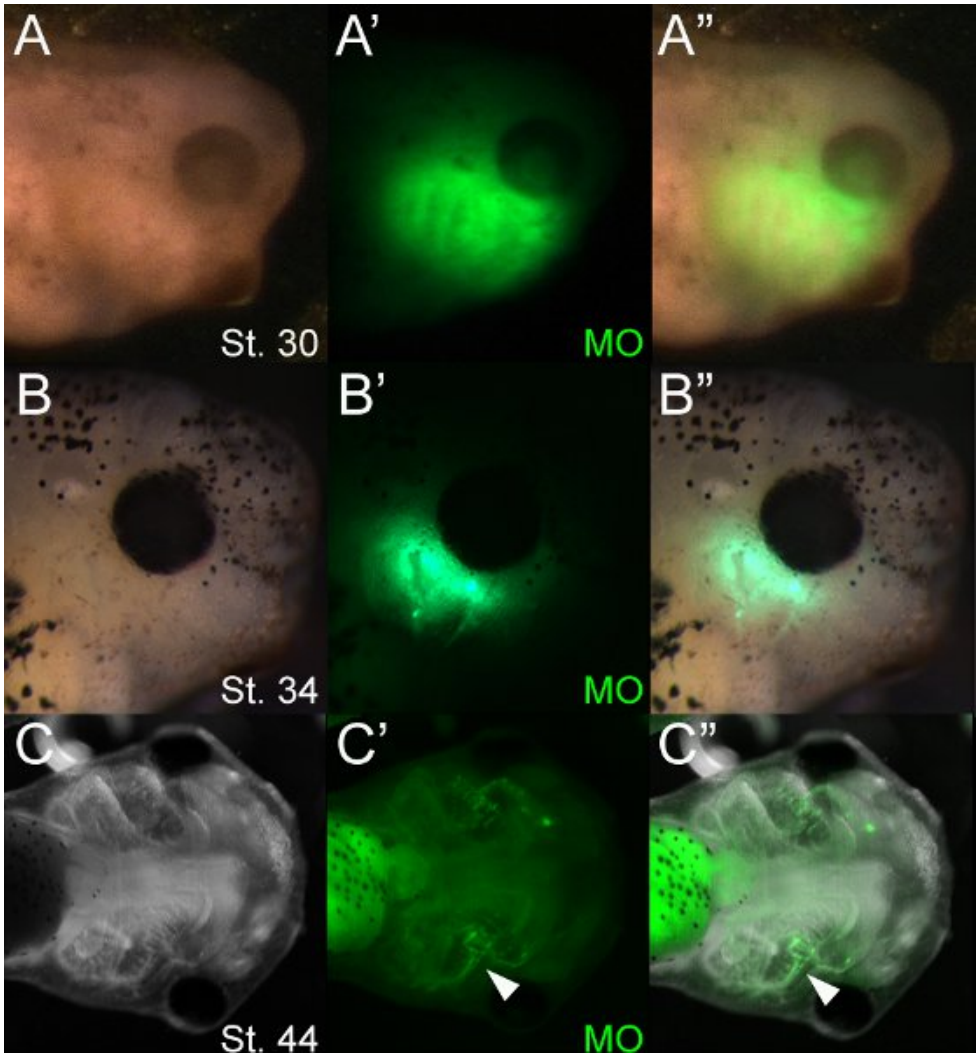

Figure 4 Representative tadpoles 12 (A), 48 (B), and 96 (C) hours post electroporation (stages 30, 34 and 44 respectively). (A"-B") Fluorescent MO can be visualised within craniofacial mesenchyme at stages 30 and 34. Fluorescence can be detected in cartilages at stage 44 (arrowhead, C'-C"). The gut is highly autofluorescent.

Discussion

In this video, we have demonstrated the feasibility of electroporation-mediated gene delivery into the facial mesenchyme of *Xenopus* tadpoles. Using this approach, we can bypass early developmental effects of manipulating gene function allowing us to target specific tissues at later time points. Our studies show that heterogenous populations of craniofacial mesenchymal cells can be affected, allowing us to examine lineage of electroporated cells as well as cell autonomous requirements for proteins of interest. Combined with live imaging, we can use this approach to study gene function, over time, during craniofacial development. This novel method highlights the tractability of *Xenopus* for the study of organogenesis. We anticipate that this method can be broadly adapted to study morphogenesis and differentiation of other tissues as well.

Disclosures

The authors have no conflict of interests.

Acknowledgements

We are grateful to Nancy Papalopulu and Boyan Bonev for assistance with *Xenopus* electroporation. We also thank Marc Dionne for critical reading, Jeremy Green and John Wallingford for helpful discussions and members of the Liu lab for their support. This work was funded by grants from the BBSRC (BB/E013872/1) and the Wellcome Trust (081880/Z/06/Z) to KJL.

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