

Video Article

Embryo Microinjection and Electroporation in the Chordate *Ciona intestinalis*

Willi Kari¹, Fan Zeng¹, Lena Zitzelsberger¹, Johannes Will¹, Ute Rothbächer¹¹Department of Evolution and Developmental Biology, Zoological Institute, University InnsbruckCorrespondence to: Ute Rothbächer at Ute.Rothbaecher@uibk.ac.atURL: <http://www.jove.com/video/54313>DOI: [doi:10.3791/54313](https://doi.org/10.3791/54313)Keywords: Developmental Biology, Issue 116, Functional genomics, electroporation, microinjection, *Ciona*, ascidians, chordates, GATEWAY cloning, phylogenetic footprinting, gene regulatory network, subcellular localization

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Abstract

Simple model organisms are instrumental for *in vivo* studies of developmental and cellular differentiation processes. Currently, the evolutionary distance to man of conventional invertebrate model systems and the complexity of genomes in vertebrates are critical challenges to modeling human normal and pathological conditions. The chordate *Ciona intestinalis* is an invertebrate chordate that emerged from a common ancestor with the vertebrates and may represent features at the interface between invertebrates and vertebrates. A common body plan with much simpler cellular and genomic composition should unveil gene regulatory network (GRN) links and functional genomics readouts explaining phenomena in the vertebrate condition. The compact genome of *Ciona*, a fixed embryonic lineage with few divisions and large cells, combined with versatile community tools foster efficient gene functional analyses in this organism. Here, we present several crucial methods for this promising model organism, which belongs to the closest sister group to vertebrates. We present protocols for transient transgenesis by electroporation, along with microinjection-mediated gene knockdown, which together provide the means to study gene function and genomic regulatory elements. We extend our protocols to provide information on how community databases are utilized for *in silico* design of gene regulatory or gene functional experiments. An example study demonstrates how novel information can be gained on the interplay, and its quantification, of selected neural factors conserved between *Ciona* and man. Furthermore, we show examples of differential subcellular localization in embryonic cells, following DNA electroporation in *Ciona* zygotes. Finally, we discuss the potential of these protocols to be adapted for tissue specific gene interference with emerging gene editing methods. The *in vivo* approaches in *Ciona* overcome major shortcomings of classical model organisms in the quest of unraveling conserved mechanisms in the chordate developmental program, relevant to stem cell research, drug discovery, and subsequent clinical application.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54313/>

Introduction

Recently, genome sequence and transcribed gene repertoires have become accessible in a number of model organisms. Determining gene functional links, however, and how these connections are hardwired in the DNA to execute transcriptional activity throughout embryonic time and space, remain a major challenge, especially in vertebrates. The complexity of regulatory interactions and the complexity of genomes^{1,2}, notably in vertebrates, slow down efficient functional analyses, in particular at the DNA level *in vivo*. Indeed, no GRN is currently analyzed from fertilization to the final, terminally differentiated state of the developing organism.

The ascidian *C. intestinalis* represents a model organism where such complete GRN analyses may be possible. It has an unduplicated genome typical of invertebrates with little gene redundancy. Vertebrates, in contrast have undergone two rounds of genome duplications that have generated larger gene families and functional diversification but also redundancy between paralogs. The compact *cis*-regulatory sequences (the control units of GRNs) of *C. intestinalis*, and an invariable embryonic lineage with few cells are reminiscent of *C. elegans*. Furthermore, its unique phylogenetic position as an invertebrate sister group to vertebrates within chordates allows developmental observation, at cellular resolution, of the forming chordate body plan³⁻⁶.

The key issue that ensures the future success of functional genomics in model organisms is the generation of large numbers of embryos for quantitative *in vivo* perturbations. The development of the microinjection technique in *Ciona* eggs⁷, and the possibility to quickly obtain many transiently transgenic animals by *in vivo* electroporation in hundreds of *Ciona* zygotes^{8,9} has been a breakthrough in *Ciona* functional genomics. Here, we first present the more laborious microinjection technique for targeting individual genes that remains the method of choice for morpholino (MO)-mediated gene knockdown, notably of maternal transcripts. MO oligos typically target the initiator ATG or the splice sites of the RNA and interfere with protein translation. We then show how electroporation in fertilized eggs of *Ciona* allows for large numbers of embryos to be analyzed in a quantitative fashion, thus opening avenues of efficient analysis of enhancers *in vivo* and of tissue-specific gain- or loss-of function approaches, with the possibility for simultaneous manipulations and analyses. We further demonstrate how *Ciona* community tools are utilized for *in silico* prediction of regulatory regions and efficient cloning of full open reading frames (ORFs) in expression vectors. Finally, we demonstrate differential subcellular localizations of fluorescently tagged or labeled proteins upon overexpression by electroporation.

Protocol

1. Preparations for Microinjection and Electroporation in *Ciona* Eggs and Zygotes

1. Prepare 10 L artificial seawater with HEPES (ASWH) for culturing of embryos. Dissolve 420 mM NaCl, 9 mM KCl, 10 mM CaCl₂·2H₂O, 24.5 mM MgCl₂·6H₂O, 25.5 mM MgSO₄·7H₂O, and 2.15 mM NaHCO₃ in double distilled water (ddH₂O) by stirring overnight. Add 5 mM HEPES pH 8.0, and verify/adjust the pH to 8.0. Store the ASWH at 4 °C. Warm it up to 18 °C and filter it with filter paper before use.
2. Prepare 20x inactivated dechoriation solution: 20% sodium thioglycolate, 1% pronase in ASWH. Store 500 µl aliquots at -20 °C, and dilute to a final volume of 10 ml ASWH before use.
3. Prepare 15 to 20 culture dishes for the eggs/embryos. Pour melted 1% agarose in ASWH into 3.5, 5, 9 or 15 cm petri dishes, coating them with a 1-2 mm thin layer. Let the agarose solidify and cover the plates with ASWH. Stock them at 4 °C for 1-2 weeks maximum. Replace the ASWH before use.
4. Prepare special injection dishes.
 1. Pour a 5-7 mm thick layer of melted 1% agarose into a 5 cm Petri dish, and place a mold on the agarose (such as a zip lock closing glued to a plastic cover slip) to produce an indentation upon solidification of the agarose that can hold the eggs. Prepare 4 to 5 injection dishes and cover with ASWH. Store at 4 °C and replace with fresh ASWH before use. Use only fresh plates for injection (maximum one day old).
5. Prepare β-galactosidase (LacZ) staining buffer: 1 mM MgCl₂·6H₂O, 3 mM K₄[Fe(CN)₆]·3H₂O, 3 mM K₃[Fe(CN)₆] in phosphate-buffered saline (PBS) with Tween (PBT) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.05% Tween). Store in the dark at 4 °C. Prepare LacZ substrate 5-Bromo-4-chloro-3-indolyl β-D-Galactopyranoside (X-gal, 10 U/ml) in aliquots of 40 mg/ml in dimethylformamide (DMF) and store stocks at -20 °C until use.
6. Soak Pasteur pipettes in tap water overnight to prevent the embryos from sticking.
7. Prepare injection needles.
 1. Set up a micropipette puller at conditions such as heat 425, pull 75, velocity 75 and time 200 (from a filament ramp test of 415).
 2. Pull 4 to 5 filament-containing glass capillaries to produce 8 to 10 long, fine needles normally closed at the tip. Store the needles in a dust free needle box, attached to double adhesive tape on an elevated support, to avoid breaking the tips and which is convenient for needle filling.
NOTE: Test the obtained needles by injecting a few eggs with green vital dye (as described in section 6) and adjust the needle pulling conditions to obtain a tip shape that allows for fine-tuned and repetitive injection as described in 6.6.
8. Prepare injection solution. Prepare 4 µl of injection solution, e.g., composed of 1 µl of 2 mM MO, 2 µl of 10 µg/µl green vital dye and 1 µl ddH₂O. Add a final concentration of 0.05-0.5 µg/µl capped mRNA or 20 ng/µl of plasmid DNA according to the experimental question. Mix well. Keep on ice if the solution contains mRNA.

2. Collection of Gametes

1. Dissect *Ciona* adults in an 18 °C cooled embryo room. Use small scissors and start from the end that opposes the siphons on the side of the exhaling (shorter) siphon under which the oviduct and the sperm duct run.
2. Expose the oviduct and delicately make an incision in the oviduct using the tip of the scissors. Drop the outflowing eggs directly into a 6-well plate containing ASWH by gently pressing the oviduct with the closed scissors stripping off the eggs. Transfer the remaining eggs with a Pasteur pipette pre-rinsed with ASWH.
3. Deposit the eggs from different animals in different wells. Observe the egg quality under the dissecting scope.
NOTE: Well developed eggs are round and pink to slightly yellow in color. They are surrounded by a non-cellular vitelline coat, the chorion that forms a well-defined perivitelline space around the egg. The chorion is associated with test cells at its inside and star-shaped follicle cells at its outside. Eggs of low quality often lack the perivitelline space or the follicle cells. They appear less round, more granular or different in color. Immature oocytes are smaller and have a silver white color.
4. Cut the sperm duct with the scissors and collect concentrated sperm into a 1.5 ml tube using a separate Pasteur pipette. Pool the sperm from different animals (at least two) into the same tube. Store sperm at 4 °C for several days.

3. Fertilization

1. Collect sperm and eggs at 18 °C as described in step 2.
2. Activate the sperm.
 1. Prepare 1 ml ASWH in a 1.5 ml tube and mix with 50 µl of 1 M Tris pH 9.5. Then add 20 µl of concentrated sperm and gently mix by inverting the tube.
 2. Verify the sperm activation in a drop of sperm placed onto a cover of a small Petri dish or a glass slide and observe under the dissecting scope. The spermatozooids should be swimming frantically.
3. Add 100-200 µl of the activated sperm solution to each well of eggs (containing between 100 and 1,000 eggs), mix well by pipetting up and down, so the eggs are floating in the medium. Wait for 10 min without moving the embryos.

4. Dechoriation of Eggs and Embryos

1. Dilute a 500 μ l aliquot of 20x dechoriation solution to 10 ml in ASWH. Activate the dechoriation solution by adding drop-wise 200 μ l of 2.5 M NaOH to the 10 ml 1x dechoriation solution. A milky precipitate will form. Gently mix by inverting the tube.
2. Collect the eggs or zygotes (after the 10 min wait in step 3.3) into glass tubes using a Pasteur pipette. Pool the eggs from 2 to 3 animals in one tube (around 1,000-5,000 eggs/tube). Sediment with a hand centrifuge by spinning at high speed (1,200 x g) for about 20 sec to form an embryo pellet at the bottom of the tube. Slowly stop the centrifuge and remove the ASWH with a Pasteur pipette.
3. Add 4 ml of activated dechoriation solution to the pelleted eggs/zygotes in each tube. Suspend the eggs/zygotes by gently pipetting them up and down using a tap water-treated Pasteur pipette topped with a small rubber pear. The solution should turn yellowish after 1-3 min.
4. Follow the dechoriation by removing a small aliquot of the dechorinating egg/zygote suspension using the Pasteur pipette. Deposit a drop on a slide and observe under the dissecting scope. Keep pipetting eggs/zygotes up and down and check every 20-30 sec.
NOTE: First follicle cells detach, then the chorion turns yellow and opaque, and finally it detaches from the zygotes. Pink dechorinated eggs/zygotes will sink to the bottom of the glass tube. The dechoriation should not take more than max. 5 min.
5. Fill the tube with ASWH once 50% of eggs/zygotes are dechorinated.
NOTE: Very gently centrifuge (8 x g) for about 10-15 sec corresponding to a very low speed just enough to sediment the dechorinated eggs/embryos. Slowly stop the centrifuge as to not perturb the pellet of eggs/zygotes at the bottom of the tube.
6. Remove nearly all the liquid from the glass tube including the floating material with incompletely dechorinated eggs/zygotes and replace with ASWH. Slowly pipet up and down to wash and gently centrifuge again as in step 4.2. Alternatively, wait for zygotes to settle down by gravity. Wash once more until no chorion debris is left.
7. Transfer eggs/zygotes to freshly rinsed culture dishes using a Pasteur pipette. Keep the zygotes (not eggs) at low density (around 200 embryos per 10 cm dish) to avoid their sticking together. Culture the embryos to the desired stage at temperatures between 13 and 20 °C. Alternatively, electroporate the zygotes (Step 5) or inject the unfertilized eggs (Step 6).

5. Electroporation

1. Fertilize the eggs and dechorionate the zygotes at 18 °C as in sections 3 and 4. Provide between 50 and 400 eggs per electroporation sample.
2. Prepare 50 μ l plasmid DNA in a 1.5 ml tube (max. 100 μ g plasmid DNA in 50 μ l ddH₂O) and add 200 μ l 0.95 M mannitol. Mix well by vortexing.
3. Dispatch and gravity sediment the dechorinated zygotes in siliconized 1.5 ml tubes. Remove the ASWH down to the 100 μ l mark on the tube.
4. Proceed, consecutively, for each zygote tube as follows: add DNA/mannitol solution using a Pasteur pipette. Gently mix with the zygotes and instantly take up the DNA/mannitol/zygote suspension and transfer to a 4 mm electroporation cuvette.
5. Place the cuvette immediately into the electroporation holder and give a single pulse of 16 msec at 50 V.
6. Remove the zygotes from the cuvette using the same Pasteur pipette and spread them out on a culture dish containing fresh and filtered ASWH. Pipette the zygotes before they start cleaving around 1 hour post fertilization (hpf) at 18 °C.
7. Rinse the pipette between the samples in a beaker of ASWH.
8. Culture the zygotes at 15-20 °C at low enough density of around 200 embryos per 10 cm dish to avoid their sticking together.

6. Microinjection

1. Prepare Dechorinated Eggs for the Injection.
 1. Dechorionate the eggs separately, from 2-4 animals, as described in section 4. Keep the unfertilized and dechorinated eggs in separate small agarose coated culture dishes. Wash the eggs several times in the dishes to remove the debris.
 2. Test fertilize small batches of the dechorinated eggs (around 50) from each individual. Use a separate 5 cm dish for each batch and apply 2 μ l activated sperm (step 3.2). Mix well by swirling the dishes and spread out the eggs in the dish by moving the dishes sideways. Keep the sperm and eggs together for about 10 min without moving.
 3. Eliminate a maximum of sperm as follows: transfer the fertilized eggs to a fresh culture dish or rinse twice with fresh ASWH. Leave the cleaving embryos unperturbed till the 32-cell stage (at 18 °C for about 3 hours post fertilization). Choose eggs from the best developing batch (best percentage of fertilization and most regular cleavage pattern) for the microinjection.
2. Set up the Injection Needles.
 1. Backfill 4 injection needles in a vertical position to allow for gravity flow along the filament. Deposit 0.5 μ l injection solution (step 1.8) containing green vital dye on the back end of the needles that are positioned needle tip down. Wait for the liquid to fill the needle tip at the bottom.
 2. Reposition the needles horizontally and backfill them with mineral oil using a fine and long metal or plastic pipette tip. Slowly overlay the injection solution with mineral oil expelling all the air bubbles while filling the needle.
3. Setup the Micromanipulator in an 18 °C Cooled Room.
 1. Connect the plastic tubing of the needle holder to a 10 ml glass syringe filled with mineral oil. Backfill the tubing and the needle holder with oil and expel any air bubbles.
 2. Insert the needle in the needle holder and position the holder on the micromanipulator.
 3. Adjust the needle holder movement along a straight line at an angle of 45° relative to the surface.
4. Orient the dechorinated eggs along the agarose indentation of the injection dish so that the eggs can be injected one by one under the dissection microscope.
5. Break the needle tip, if necessary, by gently pushing the needle against the agarose or against a piece of glass cover slip placed on the agarose. Slightly pressure the syringe knob to verify that the needle tip is open (green needle contents will expel).

6. Inject the unfertilized eggs one by one by first introducing the needle into the egg and slightly aspirating to break the egg membrane followed by injection. Inject green injection solution into the middle of the egg to a maximum of 1/3 of the cell diameter. (This corresponds to approximately 30 μ l for an egg diameter of 140 μ m).
7. Transfer the injected unfertilized eggs to a fresh culture dish and incubate them at 15–18 °C until fertilization.
8. Fertilize the injected eggs with freshly activated sperm as in the test fertilizations (step 6.1.2). Eliminate a maximum of the sperm by transferring the zygotes to a fresh culture dish. Spread out the embryos and do not perturb them during cleavage stages (up to the 32-cell stage).
9. Culture the embryos to the desired stage at a temperature comprised between 13 and 20 °C.
10. Collect the embryos by swirling them into the middle of the plate and transferring them into siliconized 1.5 ml tubes. Fix and stain, or mount (Steps 7 and 8).
11. Compare the obtained phenotypes to the control injected embryos.
NOTE: Inject a second, non-overlapping MO that targets the same transcript to obtain identical phenotypes. Rescue the specific MO phenotype(s) with a modified mRNA encoding your protein of interest but that is not recognized by the MOs. Inject a control MO that gives no phenotype.

7. LacZ Staining

1. Collect the embryos at a desired stage in siliconized 1.5 ml tubes and fix them in 0.2% glutaraldehyde in 1 ml ASWH for 15 to 30 min maximum.
2. Wash 2x 10 min with 1 ml 1x PBT.
3. Rinse once in 500 μ l LacZ staining buffer. Replace with X-Gal substrate supplemented 1 ml staining buffer (use 10 μ l/ml of 40 mg/ml X-Gal stock). Transfer the embryos into a 12 well plate for better observation of the staining.
4. Incubate in the dark. Vary incubation from 0.5 hr to overnight at 4 °C, at RT or at 37 °C depending on the strength of the driver expressing LacZ.
5. Wash the embryos up to 4 times in 1 ml 1X PBT to remove staining buffer.
6. Post-fix for 10 min at room temperature with 2% paraformaldehyde (PFA) in 1 ml 1x PBT or 0.2% glutaraldehyde in 1 ml 1x PBT for interpretation and visualization of the stained embryos.

8. Mounting of Fluorescently Labelled Wmbryos

1. Fix the embryos of a desired developmental stage for 20 min in 2% PFA in 1 ml ASWH.
2. Wash the embryos 2x in 1 ml 1x PBT. Avoid any light exposure by keeping the embryos in dark conditions.
3. Transfer up to 50 embryos to one slide. Remove a maximum of PBT first with a pipette and carefully with a paper towel.
4. Add 20–25 μ l mounting medium.
5. Add a coverslip by positioning it in an angle (from one side first) and slowly lower it with a pointed object (needle, forceps, pipette tip) avoiding bubbles till the sample is covered. Fix the coverslip at its edges using dots of a transparent nail polish.
6. Seal the entire coverslip with a nail polish after drying. Store in the dark at -20 °C.

Representative Results

Microinjection for gene regulatory network studies

Embryos of the ascidian *Ciona intestinalis* are well suited for gene functional or gene regulatory studies at cell lineage level and with cellular resolution. mRNAs, MOs or labels can be microinjected into the unfertilized egg or into individual blastomeres following fertilization. MO mediated gene knockdown using the microinjection technique is described in step 6. MOs specifically target the mRNA of selected genes and prevent their translation into protein. MO mediated loss-of-function (LOF) of developmental genes changes the expression of a broad array of downstream genes, overall revealing a glimpse into the embryonic GRN¹⁰. Such analyses in *Ciona* provided the first whole embryo regulatory blueprint in a metazoan¹¹. **Figure 1** displays an example of how microinjection was utilized to study the upstream regulation of the expression of the conserved *Ci*-Myelin Transcription Factor (*Ci*-MYT) at the gastrula stage of *Ciona* embryos. Monitored by *in situ* hybridization (ISH), endogenous expression (**Figure 1a**, schematized in **Figure 1a'**) is observed in the 6-row neural plate precursors, of notably the brain (rows III/IV, in red) and the spinal cord (row I, in green). In addition, *Ci*-MYT upstream regulation was analyzed by MO injection targeting several factors that are expressed in or adjacent to neural precursors prior to *Ci*-MYT expression onset (**Figure 1b–g**). MO knockdown of early embryonic factors, such as the Forkhead box A-a (FoxA-a) transcription factor and the fibroblast growth factor 9/16/20 (FGF9/16/20) (**Figure 1b** or **1c**, respectively) eliminates overall *Ci*-MYT expression. Other transcription factors only partially affect *Ci*-MYT expression resulting in MO-mediated downregulation in a subset of brain precursors (blue arrow heads in **Figure 1d**, **f** and **g**). Conversely, *Ci*-MYT expression is ectopically activated upon downregulation of Nodal (**Figure 1e**, red arrow heads), suggesting that Nodal normally represses *Ci*-MYT expression in these lateral nerve cord precursors.

Electroporation for efficient gene functional and enhancer studies

Electroporation of plasmid DNA into *Ciona* zygotes is an efficient method for transient transgenesis and subsequent observation of phenotypic changes *in vivo*. Unlike microinjection of mRNA, electroporation allows for tissue specific overexpression, in which gene coding regions (ORFs, open reading frames) are expressed under control of tissue specific drivers. These normally constitute *cis*-regulatory regions of well-known genes (such as the brachyury enhancer for expression in notochord precursors⁸). Conversely, electroporation is instrumental for the efficient analysis of novel regulatory regions where reporter genes (LacZ or green fluorescent protein, GFP) reveal their activity *in vivo*. The workflow for the identification and subsequent electroporation mediated analysis of such a novel regulatory region (for *Ci*-MYT) is shown in **Figure 2**.

A vast repertoire of *Ciona* community data, readily accessible in ascidian specific genome browsers^{12,13}, like the Ascidian Network for In situ Expression and Embryological Data (ANISEED)²³, is inspected for the *in silico* identification of regulatory regions (**Figure 2A**). Subsequent polymerase chain reaction (PCR) based cloning of these regions into expression vectors allows for electroporation mediated testing *in vivo*. (**Figure 2B**). The genome browser screen-shot in **Figure 2A** shows the upstream region of the KH transcript model for *Ci-MYT* (first three exons, in orange, and two introns are visible). Different genome browser tracks annotate functional genome data predicted for this region, such as chromatin immunoprecipitation (ChIP) data¹⁴ (here shown for *ZicL*, *Ci-zinc finger of the cerebellum L*), sequence conservation between two related *Ciona* species^{15,24} or nucleosome occupancy^{16,17} (from top to bottom in **Figure 2A**). Generally, exonic protein coding regions are highly conserved (alignment track in **Figure 2A**). Additional high conservation peaks appear in non-coding regions upstream and in the first intron. Two of these are predicted to be nucleosome free according to a sequence based algorithm^{16,17} (red frames in **Figure 2A**) suggesting accessibility to transcription factors. Indeed, Chip-on-ChIP signals¹⁴ for *Ci-ZicL* transcription factor binding (green bars in **Figure 2A**) are enriched in these two conserved regions. Furthermore, using an interface²⁵ that searches for potential transcription factor binding sites, we identified ZIC sites located within the genome sequences marked by *Ci-ZicL* ChIP clusters (orange arrows and sequences with underlined ZIC-core, **Figure 2A**). The binding of *Ci-ZicL* transcription factor in conserved and predictably nucleosome free regulatory regions of *Ci-MYT* is consistent with the downregulation of *Ci-MYT* expression observed in MO-injection mediated knockdown experiments targeting *ZicL* (**Figure 1d**).

After determining potential *cis*-regulatory regions *in silico* for *Ci-MYT* expression, these are PCR amplified from *Ciona* genomic DNA and inserted by recombination cloning into LacZ reporter plasmids¹⁹. The constructs are then electroporated into fertilized *Ciona* eggs (**Figure 2B**) and analyzed for their activity by LacZ staining (**Figure 3**).

Figure 3 shows that by such an *in silico* approach it was possible to identify two separate *cis*-regulatory sequences that recapitulate *Ci-MYT* mRNA expression domains (compare **Figure 1a**). Generally, transiently transgenic embryos electroporated with plasmid DNA show mosaic expression in those cells only that have incorporated the DNA. Mosaic LacZ expression is thus driven by *pmyt-R3* and can be found in all the precursors that also express *Ci-MYT* mRNA i.e. neural plate precursors of the posterior row I and the anterior rows III/IV at gastrula and early neurula stages (**Figure 3a, b**, purple and red arrow heads, respectively). In contrast, *pmyt-R5* is active only in posterior (row I) neural plate precursors and starting at the later neurula stage (**Figure 3c**, red arrow heads). The two regions thus encode two separate spatial and temporal aspects of *Ci-myt* gene regulation. Interestingly, both regulatory regions also stain additional precursors not seen in the ISH, notably in the anterior and lateral neural plate and in muscle (**Figure 3a, b**, pink, white, and yellow arrow heads, respectively). Such wider expression may point to repressive elements that are not contained in the isolated regulatory fragments (such as for Nodal that represses lateral neural fate, see **Figure 1e**) or to low expression levels detected with accumulating LacZ enzymatic signal.

In addition to enhancer studies, electroporation in *Ciona* also facilitates the functional analyses of *Ciona* coding genes by their overexpression. A large collection of *Ciona* full length ORFs is available to the community, and was furthermore annotated for human orthologs, including disease-associated genes¹⁸. Individual genes or groups of genes from this recombination-cloning compatible full ORF cDNA library (**Figure 2B**) are easily transferred into destination vectors containing appropriate drivers to be expressed in embryos. Resulting expression clones can furthermore be co-electroporated with *cis*-regulatory reporter constructs to test their putative role in enhancer activation. **Figure 2B** illustrates the isolation of full ORF clones for the transcription factors *Ci-ZicL* and *Ci-GATAa* and their recombination into adapted destination vectors that may contain translational tags¹⁹ (e.g. green fluorescent Venus- or viral hemagglutinin (HA)-tag) and a tissue specific driver such as the Friend Of Gata regulatory region (*pfog* driver) for early pan-ectodermal expression¹⁵ used in the present study. The effect of *Ci-ZicL* overexpression in ectodermal and neuroectodermal tissues was analyzed by co-electroporation of *pmyt-R3*>LacZ and *pmyt-R5*>LacZ reporters (**Figure 3b', b'' and c', c''**, respectively). Our electroporation analyses suggest that *Ci-ZicL* may activate *Ci-MYT* expression through *pmyt-R3* and *pmyt-R5* enhancer regions as both reporter constructs showed ectopic LacZ expression in ectodermal regions (green arrow heads in **Figure 3b', b'' and c', c''**). As shown in **Figure 3d**, electroporation of plasmid DNA in hundreds of *Ciona* eggs allows for statistically relevant quantification of expression changes, illustrated for ectopic *pmyt-R3*>LacZ expression upon concentration dependent, pan-ectodermal *Ci-ZicL* expression.

Electroporation for *in vivo* subcellular localization

Figure 4 depicts the subcellular localization of tagged transcription factors when expressed upon electroporation in ectodermal blastomeres using adapted expression constructs (schematized in **Figure 2B**). By C-terminally tagging transcription factors (such as *Ci-GATAa*) with a Venus tag (**Figure 4b**) or a HA-tag (**Figure 4c**) and overexpressing them in ectodermal tissues (*pfog*) we could observe that *in vivo*, *Ci-GATAa* is mostly localized to nuclei, as expected for a transcription factor, whereas Venus expression is ubiquitous, including the cytoplasm. Similar experiments may be performed to study the dynamics of subcellular localization over time, of individual or combinations of transcription factors, possibly revealing their co-localization with different tags.

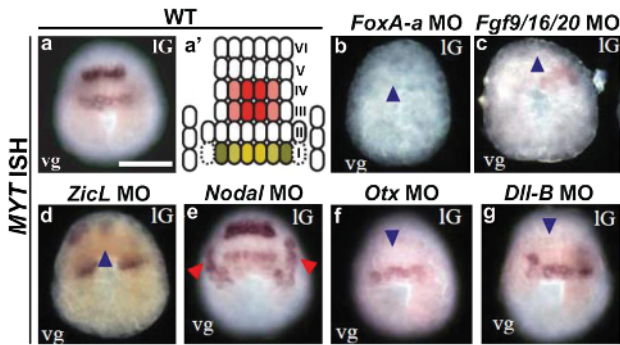


Figure 1: Assessing *Ci*-MYT regulation by microinjection in *Ciona* eggs. *Ci*-MYT mRNA expression in WT and Morpholino (MO) injected embryos. (a) WT expression pattern of *Ci*-MYT in the neural plate. (a') Schematic representation of stained precursors in the neural plate (NP). Row I/II: posterior neural fate; row III/IV: anterior neural fate; row V/VI: palp fate. (b-g) *Ci*-MYT expression upon microinjection of various MOs for knocking down indicated genes that participate in the early *Ciona* GRN (pictures adapted from Imai *et al.*, 2006)¹¹. *Ci*-MYT, *Ci*-myelin transcription factor; WT, wild type; blue arrowheads: loss of *Ci*-MYT signal; red arrowheads: ectopic *Ci*-MYT signal. Scale bar = 100 μ m refers to all images. [Please click here to view a larger version of this figure.](#)

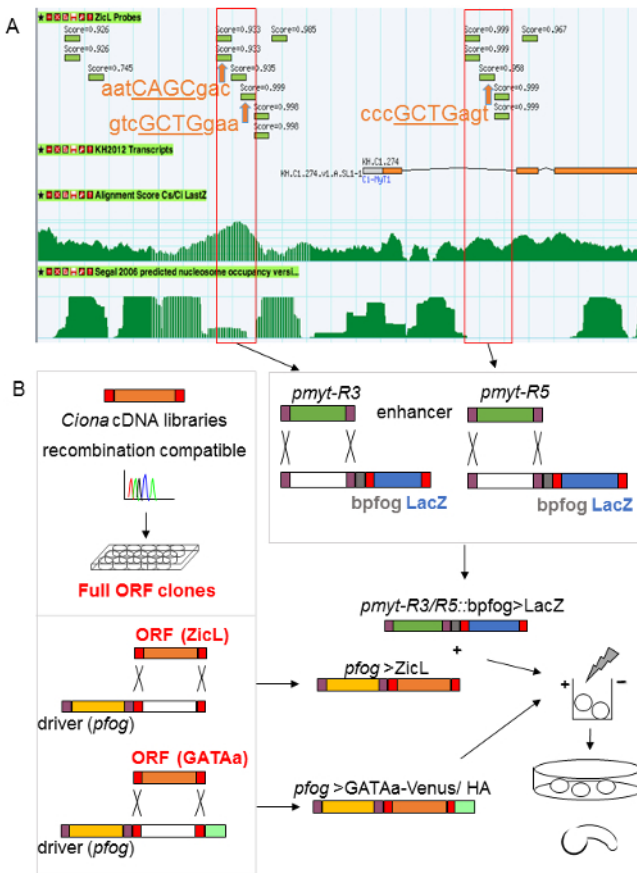


Figure 2: Workflow for *in silico* search of enhancer regions and recombination cloning for transient transgenesis by electroporation in *Ciona* embryos. (A) A snapshot from the ANISEED²³ ascidian genome browser identifies upstream regulatory sequences of *Ci-myf*. Red frames mark two potential *cis*-regulatory regions, *pmymt-R3* (scaffold_196:76883..77079) and *pmymt-R5* (scaffold_196:75856..76041) that were selected for analysis by electroporation. Names of the tracks: ZicL probes: CHIP-on-Chip data¹⁴; KH2012 Transcripts: *Ciona intestinalis* transcript models; Alignment Score Cs/Ci LastZ: sequence conservation between *Ciona savignyi* (Cs)/*Ciona intestinalis* (Ci)^{15,24}; Segal 2006 predicted nucleosome occupancy version 1: chick trained computational algorithm by Segal *et al.*, 2006¹⁶ applied to *Ci* as in Khoueiry *et al.*, 2010¹⁷. Green bars: *Ci*-ZicL ChIP peaks¹⁴; orange arrows: predicted ZIC sites; GCTG: core ZIC binding site. (B) Scheme for generating overexpression constructs from a *Ciona* full length ORF cDNA library, recombined into destination vectors containing tissue specific drivers (*pfog*) and protein tags subsequently co-electroporated with reporter constructs (such as *pmymt-R3*>LacZ or *pmymt-R5*>LacZ) for *in vivo* readout. *Ci*-MYT, *Ci*-myelin transcription factor; *Ci*-ZicL, *Ci*-Zinc finger of the cerebellum L transcription factor; *pfog*, regulatory region of *Ci*-Friend of GATA. [Please click here to view a larger version of this figure.](#)

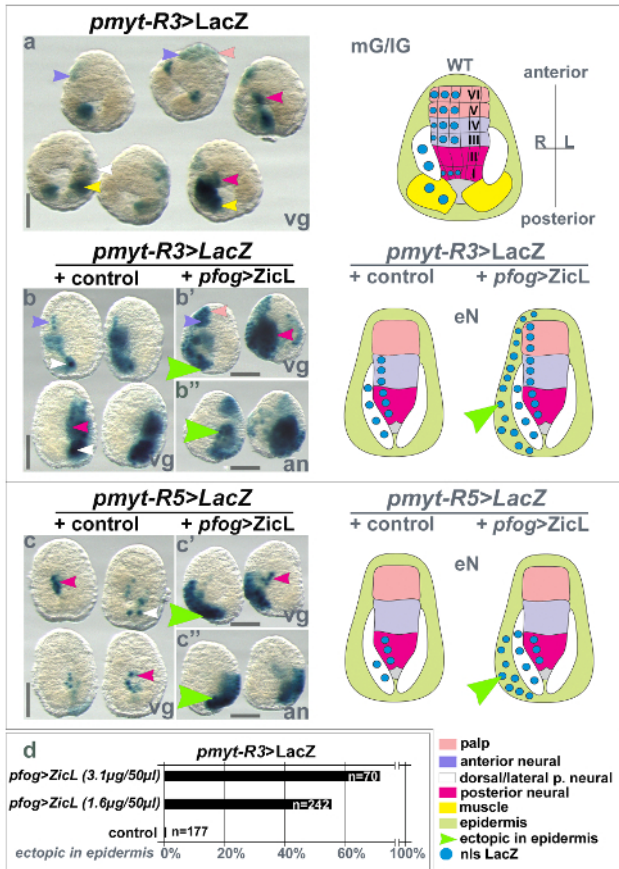


Figure 3: Electroporated *Ciona* embryos showing *Ci*-ZicL inducible spatio-temporal *cis*-regulation of *Ci*-MYT. LacZ stained embryos at mid-gastrula (mG)/late-gastrula (IG) or early neurula (eN) stage are shown that were co-electroporated with either *pmyt-R3>LacZ* or *pmyt-R5>LacZ* and a control construct (*pfog>mCherry*) or *pfog>ZicL*. The *pfog* driver¹⁹ mediates ectopic (pan-animal) ZicL expression in ectoderm and neuroectoderm and causes ectopic LacZ stain in these cells. (a) *pmyt-R3* LacZ activity in mG/IG stage embryos (small arrowheads, left panel) or in correspondingly colored schematic territories (blue circles, right panel); vegetal view (vg). Row I/II: posterior neural fate; Row III/IV: anterior neural fate; Row V/VI: palp fate. (b) *pmyt-R3* activity at eN stages in tissues as in a. (b') Ectopic *pmyt-R3* activity upon *Ci*-ZicL co-electroporation is indicated by green arrowheads. (b'') Same embryos as in b', oriented animal pole up (an). (c) *pmyt-R5* LacZ activity at eN stages (c') Ectopic *pmyt-R5* activity upon *Ci*-ZicL co-electroporation is indicated by green arrowheads. (c'') Same embryos as in c' but oriented animal pole up (an). (d) Quantification of representative experiments for *Ci*-ZicL over-activating *pmyt-R3* at low concentrations. One biological repeat is represented. *Ci*-MYT, *Ci*-myelin transcription factor; *Ci*-ZicL, *Ci*-Zinc finger of the cerebellum L transcription factor; *Ci*-FOG, *Ci*-Friend of GATA; WT, wild type; mG, mid-gastrula; IG, late-gastrula; eN, early neurula; an, animal view; vg, vegetal view; nls LacZ, nuclear localization signal for LacZ. Scale bar = 100 µm. [Please click here to view a larger version of this figure.](#)

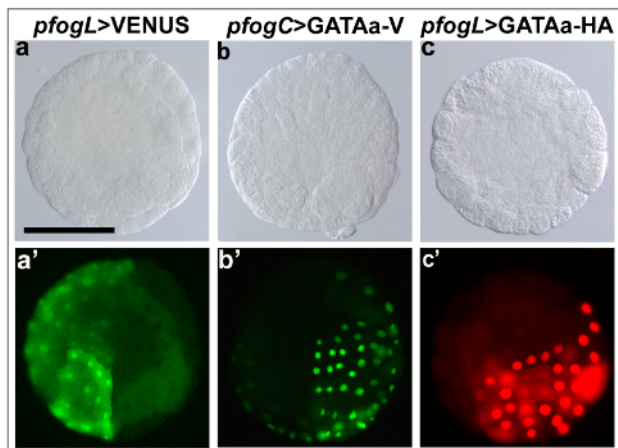


Figure 4: Differential subcellular localization of proteins upon electroporation. Localization of Venus-tagged or Hemagglutinin- (HA)-tagged proteins overexpressed in ectodermal cells using the *pfgog* driver¹⁹. (a-c) DIC images. (a', b') Corresponding fluorescence images. (c') Corresponding fluorescence image upon immune histological labelling with TRITC. Scale bar = 100 μ m refers to all images. DIC, Differential Interference Contrast. [Please click here to view a larger version of this figure.](#)

Discussion

There are two major techniques to generate transgenic *Ciona* embryos: microinjection of RNA or DNA and electroporation of DNA. These techniques for gene perturbation in *Ciona* embryos were first used in the 1990s^{7,8} and from then on successively improved, and now utilized in *Ciona* (and other ascidian genera)²⁰ worldwide.

Critical steps in the protocol

Successful transgenesis into *Ciona* gametes that are easily collected from adult animals depends on a number of critical factors. The quality of gametes depends on the spawning season (generally from May to December in the Northern Atlantic, changing with water temperature and oxygenation), on the sea water quality in the aquariums (at correct salinity, adults stay healthy for 2-4 weeks), and finally on correct handling of gametes upon extraction from the adults, as detailed below for the various steps in the protocol.

During preparation steps, incubating Pasteur pipettes with tap water will prevent embryos from sticking and sea water pre-incubation of agarose-coated petri dishes will remove toxic substances released from the agarose. Generally, dishes may be prepared up to 2 weeks before use, stored at 4 °C to avoid bacterial growth and rinsed freshly before use. One day old, small dishes seem best for injections.

Efficiency and length of dechoriation is a crucial step in the protocol as prolonged treatment of eggs/embryos will harm development. Careful preparation (no shaking) and correct storage (at 4 °C for up to one week) preserve the quality of the dechoriation solution. Proteases in the solution lose efficiency if either mixed too violently at preparation or over time, notably if kept at room temperature for longer hours/days. We have optimized this critical step by using fresh dechoriation solution conveniently prepared from frozen aliquots of 20x stock solution.

Correct handling of fragile dechorionated eggs/embryos is essential and shearing or stabbing of embryos while pipetting will be detrimental. When pipetting, embryos are maintained as much as possible in suspension (by gently 'blowing' liquid to swirl them up followed by smooth but quick pipetting), while holding the glass pipette in vertical rather than horizontal position. Such care applies to all pipetting steps for washing, resuspension and transferring eggs/embryos in and out of tubes, cuvettes or plates, before and after fixation. Upon fixation, special care should also be taken to separate pipetting devices for live versus fixed embryos to avoid any toxicity from remains of fixatives.

Microinjection is rather tricky in *Ciona* eggs/embryos due to their small size and the resilience of the vitelline membrane and critically depends on an optimized tip size of the injection needle, a perfect angle of injection (needle movement in one line, along the egg radius) and a finely tuned, manual breaking of the vitelline membrane (by suction prior to aspiration for injection). The latter is only possible if air bubbles are entirely eliminated from the injection tubing that contains mineral oil for smoothing the suction/injection pressure. In addition, the smallest pieces of dust or egg debris will clog the needle, and are easily avoided by clean working conditions and rinsing steps for eggs prior to the injection dish. Post injection, the transfer of eggs onto fresh dishes will avoid toxic effects from dead embryos not having survived the injection procedure.

Troubleshooting

Potential problems in the procedure may be addressed by the following solutions. Low fertilization rates may arise from insufficient sperm activation, low egg cleanliness or large fertilization volumes. Use freshly pooled and less diluted sperm. Verify the sperm movement upon activation. Wash the eggs with ASWH prior to fertilization as described in step 4.2. Reduce the water volume and reduce the amount of eggs per fertilization dish/well. Losing embryos during the preparation steps can be avoided by adding a drop of dechoriation solution to more efficiently centrifuge down the undehorionated eggs/embryos. Dechoriation time should be optimized as partially dechorionated eggs will not sediment and prolonged dechoriation is toxic, causing the embryos to explode.

Asynchronous development may arise from residual sperm released during dissection. Keep the egg batches separated from different animals to avoid uncontrolled cross-fertilization. Abnormal development can have various causes. At the beginning and at the end of the season, after

keeping the embryo from different individuals separated, the best batches may be selected. Avoid perturbing the embryos for the 10 min that follow fertilization to avoid interfering with their ooplasmic segregation. Ensure that the embryos didn't start dividing while being transferred as pipetting separates the sister blastomeres and results in partial embryos. Use less sperm for dechorionated eggs to avoid polyspermy. Use freshly prepared but rinsed agarose dishes. Ensure that the pipetting device is fixative free. Keep control embryos that were not dechorionated and not electroporated to detect at which step development was disrupted. Supplement the embryos with antibiotics if contamination and decomposition occurs.

If the injection device has 'clogged,' the injection needle opening may be verified by expelling some of its stained content. Clogging material may be wiped off by carefully dragging the needle tip through the agarose. Air bubbles in the injection tubing and/or needle should be removed. Change the needle for better control of the injection volume as the needle tip may break or clog along injection. Rinse the eggs and place them in a clean injection dish if debris has accumulated in the dish. Centrifuge the injection solution prior to filling fresh needles in order to sediment any small particles or crystals. Also helpful is practicing injection only with vital dye. Verify the injection technique by fertilizing injected and non-injected eggs. Finally, consulting with an experienced colleague can be helpful to improve injection technique.

To control for off-target effects a second non-overlapping MO and rescue with a modified mRNA that is not recognized by the MOs can faithfully rule out unspecific phenotypic effects in *Ciona*.

Microinjection: significance and limitations

Microinjection in *Ciona* was utilized to generate the first blueprint for a whole embryo gene regulatory network (GRN) in a chordate¹¹. However, despite such a remarkable achievement, microinjection in *Ciona* embryos has limitations, due to the relatively small size (0.14 mm in diameter) of *Ciona* eggs. Compared to those of other chordate species where foreign DNA/mRNA is introduced by microinjections, *Ciona* eggs are comparatively difficult to handle and the rate of injected *Ciona* embryos per experiment remains low (on average 30 to 50 embryos per experiment), impeding quantitative analyses. Nevertheless, for perturbing maternal factors in *Ciona*, microinjection of MOs is the method of choice also to study their involvement in zygotic onset of development from the 16 to 32 cell stages¹⁵. In addition, it is possible though much more difficult to microinject individual blastomeres up to the 16-32 cell stage. Finally, microinjection remains the most efficient technique for generating transgenic embryos (by mRNA or DNA injection) in ascidian species other than *Ciona*, for which fully optimized electroporation protocols do not exist.

Electroporation: significance and limitations

To overcome the low numbers and other constraints of microinjection, most of the *Ciona* community has used electroporation of plasmid DNA since it was developed and published as an optimized and standardized protocol by Zeller *et al.* in 2004⁹. Indeed, thousands of transgenic *Ciona* embryos can be generated within one hour with up to 500 embryos simultaneously electroporated in one cuvette using a single 50 V pulse of 16 msec. The approach of generating massive numbers of transgenic embryos is still unique amongst chordate model organisms. This had led *Ciona* to be quickly recognized as a powerful model system for performing *in vivo* analyses of potential *cis*-regulatory regions and cellular/subcellular localization, as exemplified here.

Although electroporation in *Ciona* zygotes has fundamentally revolutionized the chordate GRN research field, the technique nevertheless faces some limits. First, plasmids are transiently introduced into *Ciona* embryos and are most likely inherited as extrachromosomal arrays, making the stability of electroporated plasmid DNA speculative. Furthermore, it is very difficult, perhaps even impossible, to control the copies of electroporated plasmids that will be taken up per zygote, thus resulting in phenotypic fluctuations. A further problem that makes it difficult to interpret electroporation outcomes is a phenomenon that we call mosaicism. Electroporated plasmid DNA can be taken up at different positions of the one-cell stage embryo; which determines which and how many quarters of the zygote will receive plasmid to be inherited by the descendant blastomeres. Such variations clearly make the interpretation of quantitative effects more difficult. However, most problems that come with electroporation variability are solved by an appropriate amount of biological samples, with counting and statistics of LacZ (or GFP) positive cells, which is easily obtainable in a single batch.

Versatility and potential of electroporation for genetic engineering

Electroporation eventually allows for mid-throughput screening of potential *cis*-regulatory regions and quantitative analyses of gene function once cloned into reporter or expression plasmids. Due to the compact *Ciona* genome, identifying and cloning of potential regulatory regions is fairly straightforward³. A strategy for using the wealth of community data is demonstrated in **Figure 2A**. Cloning of reporter and gene coding plasmids is further facilitated by the generation of *Ciona* adapted, GATEWAY compatible vector suites¹⁹ and a compatible full length ORF library¹⁸. Both tools are available to the community and allow for efficient, restriction enzyme-free recombination of regulatory drivers and coding regions, as depicted in **Figure 2B**.

In recent years, electroporation was successfully adapted to achieve tissue-targeted gene manipulation with plasmids that express gene editing tools like the CRISPR/Cas9 components under the control of tissue specific drivers^{21,22}. Such approaches will quickly advance our understanding of the dynamics of GRNs and the potentially conserved signaling mechanisms, including transcription factor codes involved in tissue formation and the stepwise exit from pluripotency. Furthermore, tissue-specific loss- or gain-of-function approaches (by CRISPR/Cas9 or by overexpression) using electroporation will be instrumental to study *Ciona* orthologs of human disease-associated genes. Indeed, catalogues for *Ciona* orthologs of human disease genes and their full length ORF coding clones are readily available for analysis in *Ciona*¹⁸. Due to the genome wide duplications in vertebrates, most human genes possess functionally redundant paralogs that complicate analyses of gene function¹. *Ciona* being a simpler system with the typical chordate tissue arrangement in larvae should uncover fundamental roles of such important, often functionally conserved genes.

Disclosures

The authors declare that they have no competing financial interests.

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