Title

Protocol to electroporate DNA plasmids into Ciona robusta embryos at the 1-cell stage

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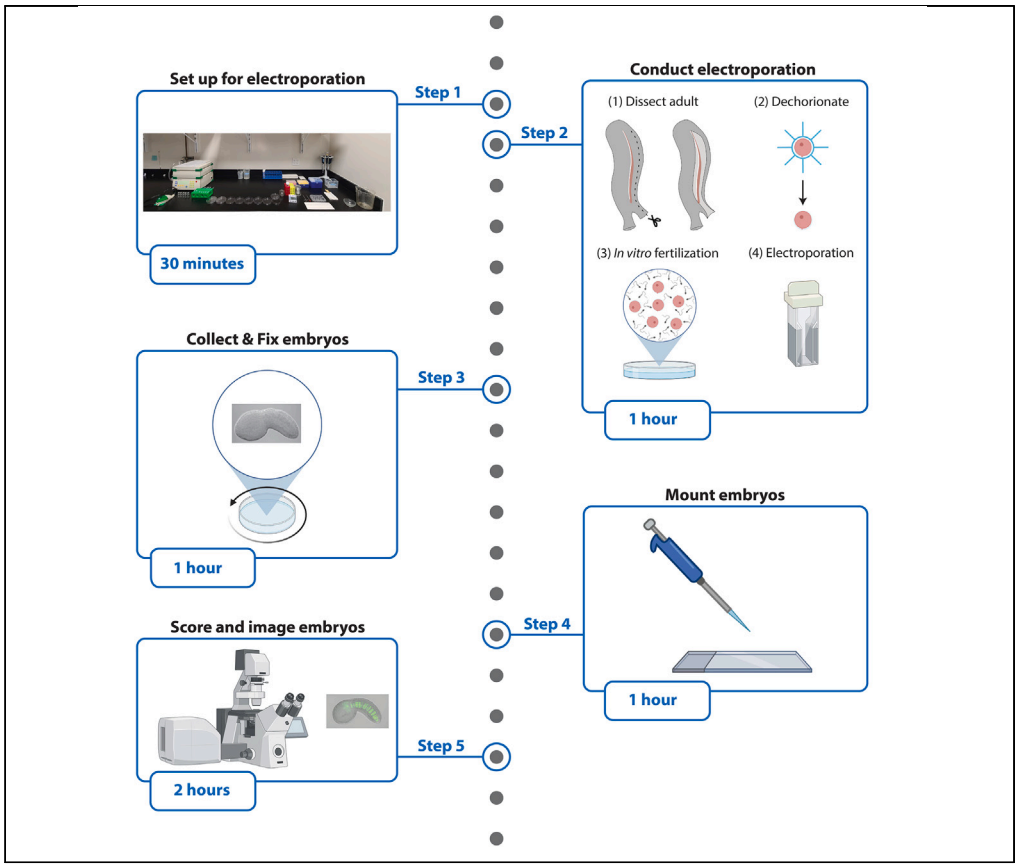

Electroporation is a technique to introduce DNA constructs into cells using electric current. Here, we present a protocol to electroporate DNA plasmids into *Ciona robusta* embryos at the 1-cell stage. We describe steps for setting up and conducting electroporation. We then detail procedures for collecting, fixing, and mounting embryos and counting expression. This protocol can be used to study the expression of enhancers via reporter assays, manipulating cells using genes or modified genes such as dominant negatives, and genome editing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for electroporating *Ciona robusta* embryos with DNA constructs of interest

Instructions for collecting, fixing, and mounting *Ciona robusta* embryos

Guidance on scoring and imaging electroporated *Ciona robusta* embryos

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Protocol to electroporate DNA plasmids into *Ciona robusta* embryos at the 1-cell stage

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SUMMARY

Electroporation is a technique to introduce DNA constructs into cells using electric current. Here, we present a protocol to electroporate DNA plasmids into *Ciona robusta* embryos at the 1-cell stage. We describe steps for setting up and conducting electroporation. We then detail procedures for collecting, fixing, and mounting embryos and counting expression. This protocol can be used to study the expression of enhancers via reporter assays, manipulating cells using genes or modified genes such as dominant negatives, and genome editing.

For complete details on the use and execution of this protocol, please refer to Song, et al.[1]

BEFORE YOU BEGIN

The first paper reporting electroporation of DNA constructs in *Ciona* was published more than 25 years ago.[2,3] Since then, updated protocols have been published on electroporation of *Ciona* embryos,[4,5] including a protocol that extends the method to four other Ascidian species.[6] How to culture *Ciona* has been described elsewhere.[7]

The protocol presented here represents our detailed protocol for *Ciona* electroporation. In this method, we complete the dechorionation step prior to fertilization. This approach ensures that fertilized eggs are electroporated at the one-cell stage and thus that there is homogenous uptake of DNA constructs. The electroporation protocol begins with preparing the gelatin plates used in the experiment, this step can be done in advance. We describe the experimental setup and provide a step-by-step protocol for performing the electroporation which includes dissecting eggs and sperm from the *Ciona* adults, dechorionation of the eggs, *in vitro* fertilization, electroporation of fertilized eggs and placing electroporated fertilized eggs onto gelatin coated plates to allow them to develop to the desired stage. The electroporation protocol takes about 1 h to complete. Plates with fertilized eggs are then left for the required amount of time to reach the chosen developmental stage. We describe our approach to fix, mount on slides and image electroporated embryos. The ability to electroporate constructs into *Ciona* makes this research organism an excellent system to study questions relating to development, evolution and gene regulation.[8,9]

Institutional permissions

Ciona are a marine invertebrate chordate and typically do not need permissions for experiments, but you should check permissions at your institution.

Prepare gelatin plates for *Ciona* embryos

Timing: 2 days

The electroporation experiment relies on putting eggs and sperm into 10 cm petri dishes, once eggs are dechorionated, fertilized eggs and embryos can stick to these plates and lyse; therefore we coat the 10 cm petri dishes in gelatin (gelatin plates) so that the eggs and embryos are not damaged during the experiment. Gelatin plates can be made in batches, we typically make 3 months of plates each time.

1. Prepare 5× gelatin coating (to be prepared fresh when making plates).
   a. Add 40 mL Ultrapure water to 0.25 g gelatin to a 50 mL falcon tube.
   b. Microwave for 20 s.
      i. Keep tube on a tube rack when microwaving.
      ii. Only lightly place cap on tube when microwaving (do not tighten cap).
      iii. Swirl to dissolve.
   c. Add 925 μL of 10% formaldehyde.
      i. Work in fume hood.
      ii. Pour small amount into new tube cap to take formaldehyde without putting pipette in formaldehyde bottle.
      iii. Discard leftover formaldehyde into waste container in fume hood.
   d. Fill the falcon tube to 50 mL with Ultrapure water.
   e. Invert gently to mix and avoid bubbles.
2. Prepare 1× gelatin coating (to be prepared fresh when making plates).
   a. Add 10 mL of 5× gelatin to 40 mL of Ultrapure water in a 50 mL falcon tube.
   b. Invert gently to mix and avoid bubbles.
3. Prepare gelatin plates (they are stable for at least 3 months at 20°C).
   a. Lay paper towels on bench – this is to avoid getting detergent from the bench on the plates, as detergent will lyse *Ciona* embryos.
   b. Pour 25 mL of 1× gelatin coating onto first plate, ensuring that it covers the entire surface of the plate.
      i. Stack the lids on a paper towel to prevent contact with detergent.
      ii. Make sure 1× gelatin touches the entire bottom surface of each plate.
   c. After a few seconds, pour the solution from this first plate into the next plate.
      i. After pouring gelatin into next plate, place empty coated plates upside down slightly leaning on top of each other to ensure maximum drying.
   d. Dry plates for 12–24 h.
   e. The next day, rinse plates with distilled water to wash off formaldehyde, use *Ciona*-specific beaker that has never been exposed to detergent to carry this water as the beaker must be detergent-free.
   f. Draw one black line down the side of plates to remind you that plates have been gelatin coated and washed with water.
   g. Dry plates for 12–24 h and store at 20°C in a detergent free container. We use them within a year.

⚠ CRITICAL: Avoid any contamination with detergent, as detergents lyse *Ciona* embryos.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Pronase	Sigma-Aldrich	10165921001
Sodium thioglycolate	Sigma-Aldrich	T0632-100G

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
10× PBS	Life Technologies	70013032
D-mannitol	Sigma-Aldrich	M4125-1KG
TAPS sodium salt	Sigma-Aldrich	T0647-500G
Glycine	Sigma-Aldrich	G7126-500G
NaOH	Sigma-Aldrich	S5881-500G
MOPS	Fisher Scientific	BP308500
NaCl	VWR	97061-270
Triton X-100	VWR	95043-474
$MgSO_4$	Sigma-Aldrich	793612-500G
EGTA	VWR	97062-288
10% formaldehyde	VWR	87001-890
NH_4Cl	VWR	97062-050
Experimental models: Organisms/strains		
Ciona robusta	M-Rep	
Recombinant DNA		
Plasmid: Lama1 bpFog > GFP	Song et al.[1]	N/A
Plasmid: Lama1 -E3 bpFog > GFP	Song et al.[1]	N/A
Plasmid: Lama1 –Z bpFog > GFP	Song et al.[1]	N/A
Plasmid: Lama1 RE3 bpFog > GFP	Song et al.[1]	N/A
Software and algorithms		
Olympus Fluoview and cellSens software	Olympus	
Other		
2.0 mL Snap Seal microcentrifuge tubes	USA Scientific	1480-2700
Cell strainer	Fisher Scientific	07-201-431
Sea water	Reliant Aquarium	
Culture tubes	Fisher Scientific	14-959-11B
Centrifuge 5424	Eppendorf	05-400-005
10 mL tips	Genesee Scientific	23-401
20 mL tips	Genesee Scientific	23-404
200 mL tips	Genesee Scientific	23-412
1,000 mL tips	Genesee Scientific	23-430
500 mL vacuum filtration system	Genesee Scientific	25-227
1 L vacuum filtration system	Genesee Scientific	25-229
Macherey-Nagel Midiprep Kit	Fisher Scientific	740410.100
0.2 mL PCR tubes	Genesee Scientific	27-125
Zymo clean and concentrator kit	Genesee Scientific	11-302
Zymoclean gel DNA recovery kit	Genesee Scientific	11-301
Zymo classic miniprep kit	Genesee Scientific	11-308A
500 mL plastic centrifuge bottles	Celltreat	229468
10 cm Petri dishes	Genesee Scientific	32-107
Ultrapure water	Fisher Scientific	10977023
15 mL Falcon tubes	Genesee Scientific	28-103
50 mL Falcon tubes	Genesee Scientific	28-108
4 mm electroporation cuvettes	Genesee Scientific	40-102
Gene Pulser Xcell total system	Bio-Rad Laboratories	1652660
Glass Pasteur pipettes	Fisher Scientific	1367820B
2 mL rubber bulbs	Fisher Scientific	15000506
ProLong Gold antifade mounting media	Life Technologies	P36930
Glass slides	Fisher Scientific	125442
Coverslips	Fisher Scientific	1254414
Dumont #5 tweezers	FST	11251-20
Metal scissors	VWR	82027-582
6-well plate for collecting eggs	Genesee Scientific	25-105
2 glass jars	Uline	S-15847P-W

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Orbital rocker	Fisher Scientific	10-320-100
Olympus FV3000	Olympus	

MATERIALS AND EQUIPMENT

MEM		
Reagent	Final concentration	Amount
MOPS pH 7.4 (1 M)	0.16 M	70 mL
NaCl (5 M)	0.79 M	70 mL
10% Triton X-100	0.079% Triton X-100	3.5 mL
$MgSO_4$ (1 M)	3.2 mM	1.4 mL
EGTA pH 8.2 (0.5 M)	1.6 mM	1.4 mL
Ultrapure water	N/A	294 mL
Total	**N/A**	**440.3 mL**
After mixing, filter into 500 mL bottle. Store at 4°C. Use within a year.		

PBTT		
Reagent	Final concentration	Amount
10× PBS	1×	100 mL
NH_4Cl (1 M)	50 mM	50 mL
10% Triton X-100	0.3%	30 mL
Ultrapure water	N/A	820 mL
Total	**N/A**	**1000 mL**
After mixing, filter into 1000 mL bottle. Store at 4°C. Use within a year.		

PBS + 0.01% Triton		
Reagent	Final concentration	Amount
10× PBS	1×	100 mL
10% Triton X-100	0.01%	1 mL
Ultrapure water	N/A	899 mL
Total	**N/A**	**1000 mL**
After mixing, filter into 1000 mL bottle. Store at 4°C. Use within a year.		

- 0.96 M D-Mannitol: Dissolve 52.4 g D-mannitol in 300 mL of MilliQ water. Use stir bar and heat to dissolve. Add 3 mL of 0.5 M TAPS and filter.

Aliquot in 15 mL tubes. Store at −20°C. To use, thaw aliquot in 55°C and keep at 20°C–22°C. Use within a year.

- 2.5% Pronase: Dissolve 1 g pronase in 40 mL of filtered seawater + TAPS. Put everything in 50 mL falcon tube and put on rocker for 5 min to dissolve.

Aliquot 800 µL to 50 1.7 mL tubes. Store at −80°C. Use within a year.

- 0.5 M TAPS (pH 8.2): Dissolve 66.32 g of TAPS sodium salt in 500 mL Ultrapure water. pH solution to 8.2 with HCl.

Filter into 500 mL bottle. Store at 4°C. Use within a year.

- 133 mM glycine: Dissolve 0.500 g glycine in 50 mL seawater + TAPS. Turn 50 mL tube upside down to dissolve. Use within a year.

Store at 20°C.

- 1 M NH_4Cl: Dissolve 10.7 g NH_4Cl in 200 mL MilliQ water. Dissolve with stir bar.

Store at 22°C. Use within a year.

- 10 M NaOH: Dissolve 20 g NaOH in 50 mL Ultrapure water. Only add 10 pellets at a time and swirl to dissolve.

Store at 20°C. Use within a year.

⚠ CRITICAL: Dissolving NaOH is an extremely exothermic reaction, so use glass beaker and be careful

- 1 M MOPS (pH 7.4): Dissolve 41.8 g MOPS to 200 mL of MilliQ water and pH solution to 7.4 with NaOH

Filter into 500 mL bottle. Store at 4°C, wrapped in aluminum foil. Use within a year.

- 5 M NaCl: Add 58.44 g NaCl to 200 mL of MilliQ water.

Dissolve with heat and a stir bar. Store at 22°C. Use within a year.

- 10% Triton X-100: add 5 mL Triton X-100 to 45 mL Ultrapure water.

Put on rocker for 2 h until dissolved, wrapped in aluminum foil. Store at 4°C, wrapped in aluminum foil. Use within a year.

- 1.4 M $MgSO_4$: Dissolve 8.52 g $MgSO_4$ in 50 mL Ultrapure water. Place in 50 mL tube and rock to dissolve.

Put on rocker for 2 h until dissolved, wrapped in aluminum foil. Store at 4°C, wrapped in aluminum foil. Use within a year.

⚠ CRITICAL: Dissolving $MgSO_4$ is an extremely exothermic reaction, also powder solidifies in water, so difficult to dissolve (should take 30 min on rocker)

- 0.5 M EGTA (pH 8.2): Dissolve 38.035 g EGTA in 200 mL of MilliQ water. Use heat and a stir bar to dissolve.

EGTA will dissolve starting at pH 6.2. pH solution to 8.2 with NaOH. Store at 22°C. Use within a year.

- Assembling egg collecting basket: Hold a razor blade with forceps and heat over a Bunsen burner flame then cut through the tube at the 25 mL mark with the heated razor blade. Cut the plastic tab on the cell strainer that juts out with a razor blade. Heat the tube (on the end that is used to screw onto cap) briefly to melt a little bit of the plastic. Push the melted plastic onto the cell strainer, ensuring that the tube is centered of the strainer. Use parafilm to wrap around the section where the tube and strainer joins (do not cover the mesh at all). Once made, this falcon tube basket should last many years.

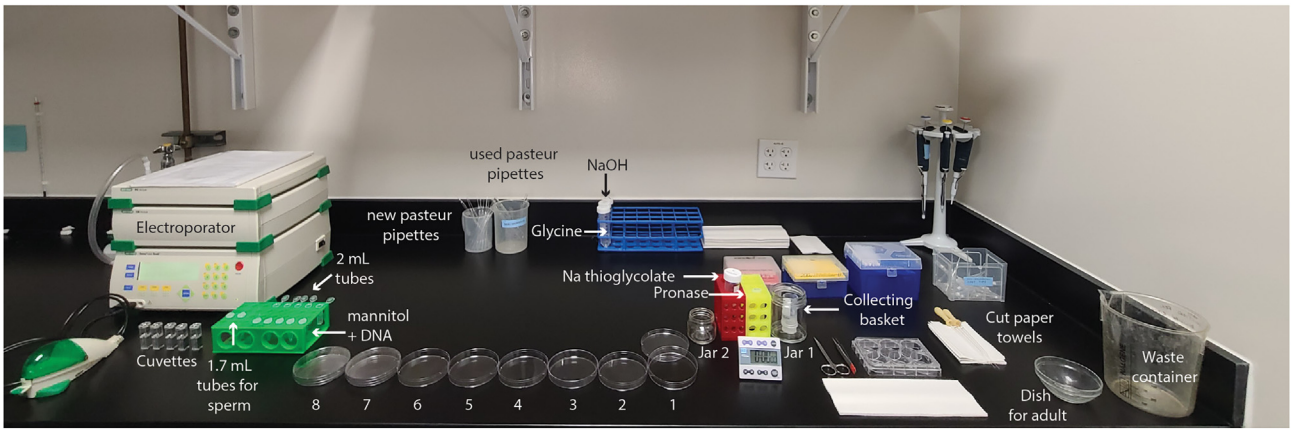

Figure 1. Setup for electroporation procedure

The gelatin-covered dishes are numbered from Dish 1 to Dish 8. This image shows how we set up the bench for the electroporation procedure (Step 2) with all the materials. We typically work right to left as the experiment progresses through the steps.

⚠ CRITICAL: Be careful when using Bunsen burner and blade.

Alternatives: Seawater made from Instant Ocean at a salinity of 35 can be used in place of Sea water at a salinity of 35.

Note: Items to be stored at 22°C are items left in the normal lab space, while items stored at 20°C are items stored in the *Ciona* room.

STEP-BY-STEP METHOD DETAILS

Electroporation of *Ciona* embryos at the 1-cell stage

Step 1: Set up for electroporation

Timing: 30 min

Setup for electroporation of fertilized 1-cell stage *Ciona* embryos with DNA constructs of interest. This part of the experiment takes about 30 min to set up and an hour for the electroporation protocol. We recommend making seawater + TAPS in advance of the experiment, the seawater can be stored for several weeks.

1. Set up for electroporation. Figure 1 shows the experimental set up.
   a. Prepare filtered seawater + TAPS.

   Note: We typically prepare 2–4 L at once and use for an entire week. This can be stored for longer.

      i. Take seawater out of bucket and check that salinity is 35 – we use fresh seawater, but artificial seawater can also be used.

   Note: If salinity is too high, add tap water.

      ii. Put 1 L seawater in 1 L vacuum filtration system (0.22 μM).
      iii. Add 10 mL 0.5 TAPS to seawater and filter.
   b. Prepare DNA.

i. Add 70 μg DNA (midiprepped with Macherey-Nagel kit to be endotoxin-free) in 100 μL total with Ultrapure water.

Note: The concentration of DNA needed will vary depending on the experimental design, e.g., Cas9, dominant negatives or reporters. Typically, we use 70 μg DNA cytoplasmic reporter constructs, and 10 μg DNA for histone reporter constructs.

ii. Add 400 μL 0.96 M Mannitol.
iii. Mix well by pipetting.

c. Bring items into *Ciona* room.
   i. 0.4 g sodium thioglycolate aliquot (from freezer).

Note: Sodium thioglycolate from supplier should be aliquoted into 50 mL tubes since sodium thioglycolate oxidizes over time.

   ii. 800 μL pronase aliquot (from −80 degree freezer).
   iii. 0.5 M TAPS (from fridge).

d. Prepare workspace for electroporation.
   i. Arrange 8 gelatin-coated petri dishes, 6-well plate for collecting eggs, 2 jars for collecting eggs and dechorionation, egg collecting bucket, paper towels, cut paper towels, scissors, tweezers and waste bucket on your work bench.
   ii. Rinse all petri dishes, 6 well plate, and jars out with seawater + TAPS once.
   iii. Refill dishes 2–8 to 20 mL, 6-well plate and Jar 1 with seawater + TAPS (Figure 1).
   iv. Add 1 mL 50 mM glycine to dishes 2 and 3 (Figure 1).

e. Preparing gelatin plates for incubation of electroporated embryos.

Note: Typically, we place the electroporated fertilized eggs electroporated with a plasmid into one or two plates to incubate until the correct developmental time. We use gelatin coated plates and use a permanent marker to print the name of the DNA construct on the top and bottom of the gelatin-coated petri dishes.

   i. After labeling, dishes are rinsed with seawater + TAPS, this solution is then poured into the waste container.
   ii. Then, add around 20 mL of seawater + TAPS into the rinsed gelatin coated plates.
   iii. Place these dishes on a surface nearby, but out of the way of your electroporation space.

Note: In addition to the constructs of interest in your experiments, we include a no electroporation control and a water electroporation control.

Step 2: Electroporate fertilized 1-cell stage Ciona *embryos with DNA constructs of interest*

Timing: 1 h

2. Conduct electroporation.
   a. Pick 3 *Ciona* that look healthy and have a lot of eggs (e.g., Figure 2A). Take one out at a time from the tank and transfer to your bench using the glass bowl.

Note: It is best to take one *Ciona* at a time as sperm can leak out of the animals if they are all compressed together in a jar. We use 3 adult individuals to minimize self-fertilization as *Ciona* prefer non-self-fertilization, and to ensure we have sufficient embryos for experiments.

   b. Remove eggs from each adult *Ciona*.

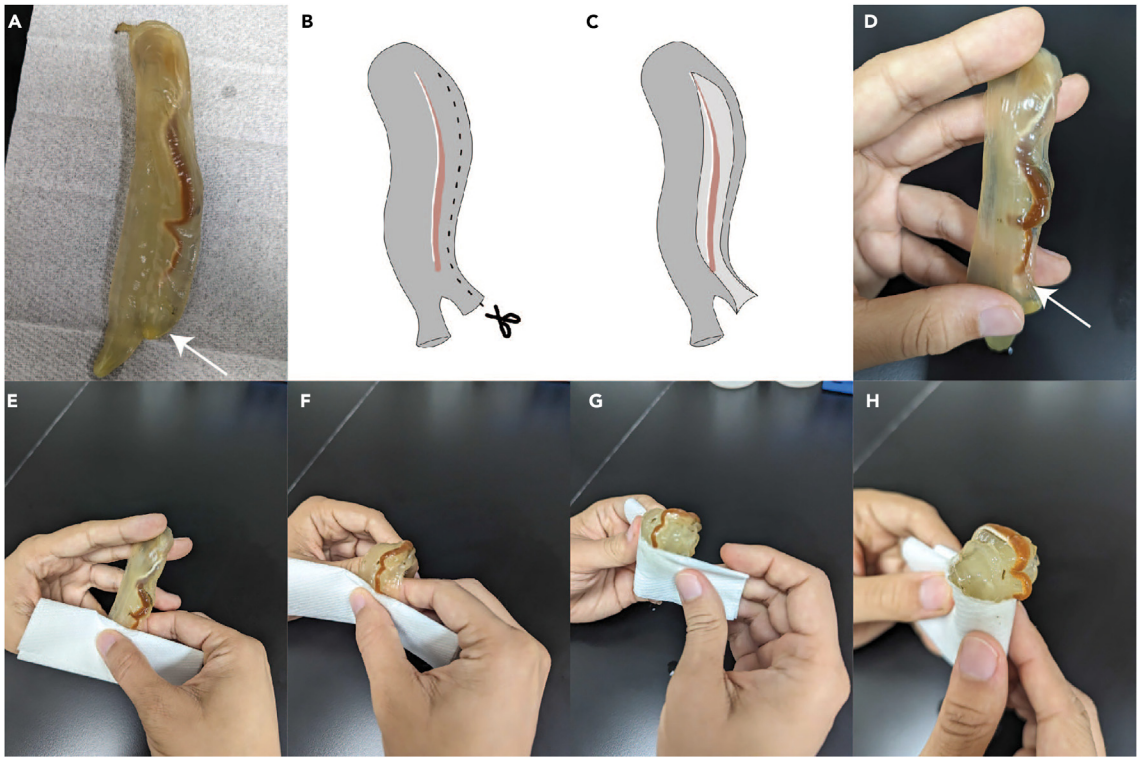

Figure 2. Steps during the dissection of an adult *Ciona*

(A) A gravid *Ciona* individual with atrial siphon (shorter siphon) marked by arrow.
(B) Schematic of how to cut *Ciona* to expose the egg duct.
(C) Schematic of the result after the cut.
(D) Resulting individual with the end of the sperm duct marked.
(E) Cover the end of the sperm duct with a cut paper towel.
(F) Fold individual *Ciona* so that egg duct is on top.
(G) Wrap cut paper towel around Ciona.
(H) The egg duct is now ready to be rinsed.

i. Hold adult so that the body is taut as shown, insert scissors into atrial siphon (shorter siphon) and keep scissors base in siphon while cutting. Go up the siphon until ~2/3 of the duct is exposed.
ii. Cut next to the egg duct (Figure 2B), so that membranes on top of egg duct are removed (Figures 2C and 2D).
iii. Fold *Ciona* in half and wrap *Ciona* in a cut paper towel (we use 1/2 of a piece of paper towel) covering the end of the sperm duct.

Note: This is to make extracting the eggs easier and to avoid sperm contamination (Figures 2E–2H).

iv. Rinse *Ciona* in seawater + TAPS using a glass pipette to remove intestinal debris from *Ciona* egg duct.
v. Place egg duct into the seawater in the 6-well plate.
vi. Ensuring that the egg duct is submerged in water, use tweezers to pierce a hole in egg duct, be careful to not pierce the sperm duct.
vii. Use the back of tweezers to gently push down the egg duct towards the puncture hole and make sure that the pierce egg duct is under water in the 6-well plate before pushing the eggs out so that the eggs directly go from duct to sea water.

Note: The 6-well plate is not coated in gelatin, as the eggs at this stage have chorions.

viii. Repeat this for each adult *Ciona*, placing the eggs from each adult into a different well within the 6-well plate.
ix. Once you have sufficient eggs for your experiment, check the quality of the eggs under an upright stereo microscope.

Note: Good quality eggs should be round, with a chorion, and the eggs should be dark yellow/red. Poor quality eggs will be non-spherical. If there are some wells with poor quality eggs, collect more eggs in another well of the 6-well plate.

x. Once you have confirmed you have good quality sperm-free eggs from at least 3 animals, transfer the eggs gently into the collecting basket sitting within the jar containing seawater + TAPS.

Note: Keep the glass pipette upright during transfer to ensure that eggs do not settle on the side of the glass pipette.

⚠ CRITICAL: If you see cloudiness or white thick solution in the well then you have sperm contamination, and these eggs cannot be used. Place a small corner piece of paper towel in this well to indicate that these eggs cannot be used. Make sure no sperm escapes into 6 well dishes.

c. Make Dechorionation solution.
   i. Add 40 mL of filtered seawater + TAPS to a 50 mL Falcon tube containing 0.4 g sodium thioglycolate.
   ii. Mix by inverting.
   iii. Add 168 μL 10 M NaOH.
   iv. Mix by inverting.
   v. Add 800 μL pronase aliquot.
   vi. Mix by inverting.

⚠ CRITICAL: It is critical to add these chemicals and enzymes in this order and to mix between adding as the pronase could be deactivated by the NaOH if added together or without mixing. 10 M NaOH is highly corrosive, so be careful when handling this solution.

d. Add half of Dechorionation solution to the second empty jar and half to the first empty gelatin coated plate. Empty gelatin-coated dish should sit on top of a lid so that it is at a slight angle with the solution collecting at the bottom of the plate.
e. Transfer collecting basket containing eggs to the second jar with Dechorionation solution and start timer immediately.

Note: Make sure that the eggs are not out of water at any point.

Note: Make sure the water is lower than the top of the sieve part when transferring, to avoid diluting the Dechorionation buffer.

f. Transfer eggs from the jar to dish 1 containing Dechorionation solution using a glass pipette.

Note: When transferring eggs, avoid producing bubbles and keep pipette upright (perpendicular to the bench).

Note: Do not touch the mesh of the basket with the pipette as this could rip the mesh and result in loss of your eggs.

g. Once all eggs are transferred, gently create circular current with glass pipet by gently sucking up liquid and pushing in back at an angle on the side of the plate.

⚠ CRITICAL: Make sure when pipetting, that you do not do this too hard and that the eggs do not immediately hit the side of the dish or the bottom of the dish.

h. Alternate between swirling eggs around the plate gently and letting the eggs sit in the solution.

Note: The pipetting provides mechanical force to help remove the chorion while the solution provides chemical dissociation to remove the chorion from the eggs.

i. At around 5 min, swirl the eggs to the center of the dish by placing your thumb on one side of the dish and your ring finger on the other edge of the dish and gently push the dish into the bench while moving your fingers in a clockwise manner to move the dish in a clockwise manner.

Note: Pushing the plate into the bench makes the swirling gentler and avoids the water spilling. Once eggs are at the center of the dish, look at them to determine if they are dechorionated.

j. When most are brick red, begin transferring the eggs to first dish with glycine (Dish 2) using a glass pipette to quench dechorionation reaction. Ensure all eggs are transferred by 6 min.

Note: Eggs will look brick red when dechorionated, and yellow debris (the chorions) will float above the brick red eggs. Keep the glass pipette upright (perpendicular to the plate) during the transfer to ensure that eggs do not settle on the glass pipette and lyse.

⚠ CRITICAL: Immerse glass pipet in solution perpendicular to bench when transferring the eggs from one plate to another, instead of dropping eggs onto the surface of the solution. When placing the eggs in the new dish, pipette the eggs into the dish and move the pipette as you plate the eggs, to ensure they are not all in one place within the dish. Do not scratch bottom of dish with glass pipet as this will remove the gelatin coating, eggs that settle on the scratch will lyse. Troubleshooting 1, Troubleshooting 2.

k. Swirl eggs together and gently pipet off the debris/chorionated eggs with glass pipet.

Note: The debris and chorionated eggs will float, while the dechorionated eggs will sit on the bottom of the plate and will be brick red.

   i. Only take up material that is above the eggs or around (usually yellowish color or white).
   ii. Do not disturb the eggs (brick-red color).

l. Transfer the dechorionated eggs to second dish with glycine (Dish 3). Troubleshooting 3.

m. Transfer through 3 plain filtered seawater + TAPS dishes of washing (Dishes 4–6).

Note: This is to ensure that there is no trace of the dechorionation buffer or glycine on the eggs. Once you have completed 3 washes, leave the eggs in the last dish. Remember to pipette these into the last dish under the surface of the water and distribute them evenly across the dish.

n. Collect sperm.
   i. To collect sperm, pinch sperm duct with tweezers, keeping the tweezers perpendicular to the sperm duct.
   ii. Push sperm out using a clean glass pipet and collect sperm with pipet tip.
   iii. Place sperm into an Eppendorf tube and repeat for all of the adults.
   iv. Once you have collected sperm from all the adult *Ciona*, mix sperm using glass pipet, avoiding bubbles.
   v. Add 10 μL of sperm to a second Eppendorf tube containing 1 mL of filtered seawater + TAPS, and invert to mix to ensure that the sperm is uniformly diluted within the seawater.
o. Add 168 μL sperm to eggs and start timer (counting up).

⚠ CRITICAL: Make sure that you distribute the sperm uniformly across the entire plate to ensure that all eggs are fertilized.

Note: At 2 min, check under the microscope to make sure the eggs are spinning, which indicates that fertilization of the eggs occurred. Egg spinning should occur between 2–3 min.

p. At the 4-min mark, start to transfer fertilized eggs from dish 6 to dish 7.
q. Transfer fertilized eggs into dish 7 with filtered seawater + TAPS dishes, distribute the eggs uniformly and gently into the dish, then swirl to center, remove any debris floating above the eggs.
r. Transfer fertilized eggs to dish 8, by uniformly distributing them across the dish, and then swirl to the center with goal to finish at 8 min post fertilization. Remove any debris floating above the eggs.
s. Transfer equal amounts of eggs to 2 mL microcentrifuge tubes (this brand is chosen because the *Ciona* eggs don't stick to the sides of the tube).
t. After eggs have settled to the bottom, remove liquid such that the meniscus should be between the 100 μL line and the 200 μL line.
u. Conduct the electroporation.
   i. Mix the DNA/ 0.98 M D-Mannitol solution by pipetting up and down (do water electroporation last).
   ii. Add the DNA/mannitol solution carefully to the eggs then mix the solution by pipetting up and down several times very gently.
   iii. Transfer all the liquid to the cuvette, move quickly to the next step (do not let the eggs settle).
   iv. Electroporate immediately with Voltage: 50 V, Capacitance: 1000 μF, Resistance: infinity, Cuvette length: 4 mm. Record the time constant on the dish and on electroporation spreadsheet.

Note: Electroporations should be completed by 18–20 min to avoid electroporation into dividing eggs and thus mosaic reporter signal. If embryos are electroporated later than the one-cell stage, mosaicism will occur. Troubleshooting 4.

v. Immediately after electroporation, transfer electroporated eggs into gelatin-coated labeled plate to develop until required stage.
   i. Add some liquid from plate to the cuvette, mix gently to dilute the mannitol, then take up all the liquid and evenly spread them onto plate.
   ii. Use the same glass pipet used in step u. per each DNA construct.
   iii. Complete steps quickly because want cells in 1-cell stage at the time of plating.
w. Cover plates with foil and let sit for 8 h, or until time point interested in, at 20°C.
x. Clean up following electroporation.

i. Rinse 2 mL microcentrifuge tubes and cuvettes by filling them completely with tap water and then discarding water.

Note: We reuse the 2 mL microcentrifuge tubes and cuvettes, this is because the eggs lyse on plastic of the new tubes and cuvettes. We have found rinsing these tubes removes the DNA from the electroporation and thus allows them to be reused and minimizes lysis of the eggs in the experiment. We reuse the cuvettes and tube for about 4 months. If a cuvette gives a high time constant, we discard the cuvette.

ii. Rinse 6 times or more.
iii. Tap dry on a paper towel.
iv. Keep them upside down on new paper towel.

y. Wash remaining metal items used with tap water and dry to avoid rust.
z. Wash other items with deionized water.

Pause point: Pause here to wait for embryos to develop to the right stage.

Step 3: Collect and fix Ciona embryos with DNA constructs of interest at developmental time of interest

Timing: 1 h

3. Collect embryos for downstream processing, in this case fixing embryos for visualizing reporter expression.

Note: At this point, embryos could also be collected for RNA isolation and sequencing,[10,11] fluorescence activated cell sorting,[12–14] isolation of nuclei for ATAC-seq,[15] transferred to Petri dishes that are 150 mm in diameter to grow up to the juvenile stage[16] or other downstream applications. Embryos could also be left in the dish to grow to the larva stage.[17]

a. Prepare MEM-FA solution for fix, fresh each time.
   i. 6.3 mL MEM.
   ii. 3.7 mL 10% formaldehyde.
   iii. Invert to mix.

⚠ CRITICAL: Perform steps with formaldehyde in fume hood.

b. Check if embryos have developed properly by looking at their morphology under an upright stereomicroscope. Troubleshooting 3, Troubleshooting 5.
c. Prepare a new 1.7 mL microcentrifuge tube to place your electroporated embryos and label each tube with the construct name corresponding to the name of the gelatin plate.
d. Harvest one batch of embryos.
   i. Swirl embryos from the plate to the center.
   ii. Using a clean glass pipette, take up the embryos, keeping the pipette perpendicular to the dish.
   iii. Transfer the embryos into the 1.7 mL microcentrifuge tube.

Note: The tube has a volume of 1.7 mL, so be careful to take up less volume than this. You do not have to take all eggs up at once.

e. Allow embryos to settle.

Note: Flicking/tapping the tube gently can help embryos settle and dislodge embryos that are stuck on the side of tube.

f. Remove as much sea water as possible from top to bottom using a glass pipet (down to 100 μL).

Note: If applicable, you can then add more embryos from the relevant dish to the tube again if you are not able to get all the embryos in step d.

g. Once you have transferred all embryos from your different construct electroporations into labeled 1.7 mL microcentrifuge tubes, take the tubes in a rack to the fume hood.
h. Place rack and tube in fume hood on paper towel to avoid detergent contamination.
i. Remove seawater from top to bottom using a glass pipet (down to ~100 μL).
j. Add ~1.5 mL of MEM-FA to each tube using glass pipet.
k. Allow embryos to settle in the dark (5 min).
l. Remove the solution down to ~100 μL.
m. Add MEM-FA to ~1.5 mL, close tubes and rock covered in foil for 10 min.
n. Place embryos back in rack and let them settle under foil (5 min).
o. Remove the MEM-FA leaving ~100 μL of solutions and embryos.
p. Add PBTT to the tube up to ~1.5 mL.
q. Let embryos settle in the dark for 5 min, and then remove the PBTT (this will contain traces of formaldehyde and thus needs to be discarded of in the formaldehyde waste container).
r. Add ~1.5 mL PBTT, wrap in foil and rock in cold room at 4°C for 12–24 h.

Pause point: Pause here to wait for the embryos to rock in cold room at 4°C 12–24 h.

Step 4: Mount fixed developed Ciona *embryos on slides*

Timing: 1 h

4. Mount embryos.
   a. Next morning, remove tubes from rocker and place in rack to allow embryos to settle.
   b. Remove solution leaving ~100 μL.
   c. Add ~1.5 mL of PBS + 0.01% Triton.
   d. Let embryos settle under foil for 5 min.
   e. Remove solution leaving ~100 μL, and then add ~1.5 mL of PBS + 0.01% Triton.
   f. Rock tubes covered in a piece of foil for 5 min.
   g. Let embryos settle in a tube rack under foil for 5 min.
   h. Prepare and label slides with construct, time constant, time since fertilization, temperature of development, date of fertilization.

Note: Use a pencil to label, rather than pen, as the dye in the pen can affect the fluorescence if it comes into contact with the mounting media.

   i. Remove the PBS + 0.01% Triton, leaving >100 μL of PBS + 0.01% Triton and embryos.
   j. Add 70 μL PBS + 0.01% Triton to the tube and mix by pipetting up and down gently.
   k. Pipet the solution with embryo onto a slide placed on dark surface so that the embryos can be seen easily.
      i. Mix by pipetting up and down before transfer.
      ii. Pipet the embryos in a long line along the slide, and then a second long line on the slide to ensure even distribution of the embryos across the slide.
      iii. Avoid putting embryos near the edge of the slides, and only cover an area the same size as the cover slip.

l. If there are remaining embryos in the tube, rinse remaining embryos in tube with 70 μL of PBS + 0.01% Triton and pipet them to the slide.
m. Use 10 μL unfiltered pipet tip to spread the solution and embryos across the slide.
    i. Use unfiltered tips.
    ii. Spread on the side of the slide and around embryos.
    iii. Do not touch embryos directly with tip, because might squish them.
    iv. Spread the solution instead of the embryos.
n. Remove the liquid with a vacuum, which has a glass pipette and a 10 μL unfiltered pipet tip on it.

Note: This will leave the embryos on the slide so that we can add mounting media and cover slip.

    i. Only remove solution with vacuum while tilting away from vacuum.
    ii. Tilt the slide such that all the solution falls to the end near the label, vacuum liquid off the other end of the slide (to avoid taking up embryos).
    iii. Tilt the slide around to spread the embryos, then repeat the step above and vacuum off liquid.
    iv. Ensure embryos are evenly spread on the slide (not clustered) but within the area of a cover slip.
    v. Change the 10 μL pipette tip for each slide.
o. Pipet 70 μL of mounting media onto the slide.
    i. Use cut 200 μL unfiltered tips, because mounting media is very viscous.
    ii. Avoid introducing air bubbles.
    iii. First pipet to the area with most embryos, so that it is possible to spread them out further.
p. Tilt slide to spread mounting media over all the embryos and cover slip area.
    i. Ensure mounting media covers all the embryos.
    ii. If there is a gap, use pipet tip to spread.
    iii. If there is a bubble, use vacuum to remove.
    iv. Ensure mounting media covers the area of the cover slip.
q. Slowly place coverslip onto slide with some tweezers onto the area with mounting media.
    i. Check to make sure that there are NOT 2 cover slides stuck together. This can be seen as a rainbow effect in the glass rather than being clear.
    ii. Note that cover slip is smaller than slide.
    iii. Place one side of cover slip at the end with more mounting media between your thumb and index finger, rest the other end of the cover slip with tweezer, slowly lower and then remove tweezers while holding the other side of the cover slip.
    iv. Be careful not to touch coverslip other than on its sides.
    v. Avoid tilting slide after coverslip has been placed because coverslip might slip off.
r. Allow slides to set for 24 h before viewing.
    i. Keep at 22°C and in a dark drawer for 24 h.
    ii. After 24 h, keep in fridge.

Pause point: Pause here to wait for mounting media to harden.

Step 5: Score and image developed Ciona *embryos with DNA constructs of interest*

Timing: 2 h

5. Score and image electroporated embryos.
    a. Have investigator not involved in the scoring process blind slides.

Note: This involves placing tape of the labeled part of the slide, randomly rearranging the order of the slides and then labeling the slides A, B, C, D, etc.

Table 1. Scoring data for Lama1 construct and versions with mutations

Construct	Repeat 1, percentage of embryos with notochord expression	Repeat 2, percentage of embryos with notochord expression	Repeat 3, percentage of embryos with notochord expression	Avg. percentage of embryos with notochord expression across replicates	*p*-value
Lama1	38	34	30	34	
Lama1 -E3	0	0	0	0	4.5 E−15
Lama1 -Z	0	0	0	0	4.5 E−15
Lama1 RE3	0	0	0	0	4.5 E−15

b. For scoring, use an epifluorescence microscope with a 20× objective lens.
   i. Get samples in focus using the bright-field setting, then switch to a fluorescence setting (depending on your reporter) to score enhancer activity in different cells within the embryo.

Note: The majority of *Ciona* embryos develop properly following electroporation. The fraction of well-developed *Ciona* embryos that receive plasmid is close to 100%, since electroporation of some constructs cause fluorescence in close to 100% of embryos.[8,18] The fraction of embryos that show fluorescence depends on the enhancer reporter construct, with weak enhancers not driving expression in embryos that receive less plasmid.

   ii. Use a scoring sheet to tally up tissues where reporter expression is detected in each embryo and score 50 embryos per slide. Our scoring sheet is included for your use (Figure S1).

Note: You may also want to take note of relative reporter expression levels in each embryo. *Ciona* tissues commonly scored include anterior sensory vesicle, posterior sensory vesicle, nerve cord, endoderm, mesenchyme, notochord, tail muscle, epidermis, and heart progenitor

   iii. Import scoring data into spreadsheet and use chi-squared tests to determine if your expression pattern and/or reporter signal intensity is statistically different from a wild-type sequence control (e.g., Table 1).

c. While slide is still blinded, take z-stack of embryo representative of the counting data (e.g., Figures 3A–3D).

EXPECTED OUTCOMES

Following this protocol, embryos should be electroporated with the desired construct. If electroporation is of reporter constructs, we anticipate that embryos should be well developed and have expression of the reporter in the expected location. Expected outcomes are shown in Figure 3.

QUANTIFICATION AND STATISTICAL ANALYSIS

We typically score 50 embryos for each replicate. In the example, we noted if fluorescence from the reporter construct was present in the notochord cells. Chi-squared tests were done comparing the wild-type Lama1 enhancer construct driving cytoplasmic GFP to Lama1 enhancer constructs with modifications to binding sites driving cytoplasmic GFP. Chi-squared can also be used if levels of expression were scored (e.g., weak, medium, strong, no fluorescence).

LIMITATIONS

The quality of eggs from *Ciona robusta* declines during the winter months and when it rains near where they are harvested. It will be more challenging to do *Ciona robusta* electroporations and have the embryos develop normally during these times.

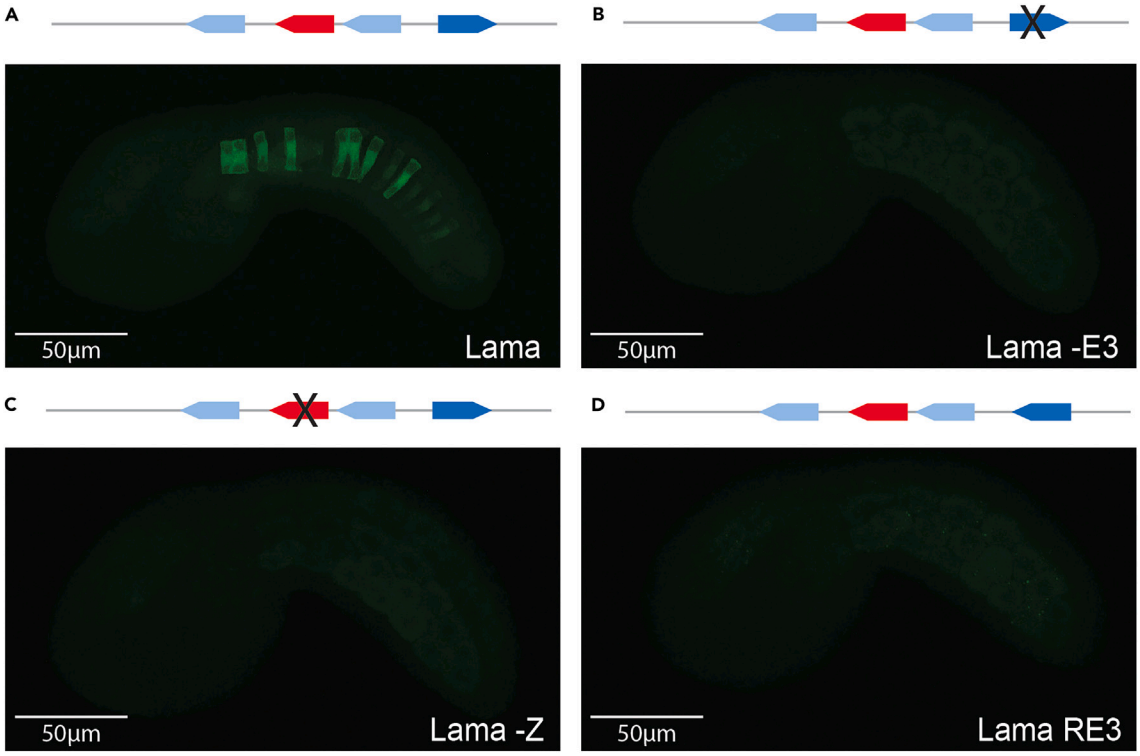

Figure 3. Zic and ETS grammar encode a notochord *laminin alpha* enhancer

(A) Embryo electroporated with the Lama enhancer (ZEE13); GFP expression can be seen in the notochord.

(B) Embryo electroporated with Lama -E3, where the transcription factor binding site ETS3 was mutated to be non-functional; no GFP expression detected.

(C) Embryo electroporated with Lama -Z, where the transcription factor binding site for Zic was mutated to be non-functional; no GFP expression detected.

(D) Embryo electroporated with Lama RE3, where the sequence of transcription factor binding site ETS3 was reversed; no GFP expression detected. Comparable results were seen when ETS1 was reversed. For each enhancer, three biological replicates were performed with 50 embryos per replicate (see Table 1). Each image in this figure is representative of the expression observed from three biological replicates. Scale bars, 50 μm.

Figure reproduced with permission from our previous paper.[1]

TROUBLESHOOTING

Problem 1

The dechorionation reaction does not occur properly (related to Step 2j).

Potential solution

- Use a fresh aliquot of Sodium thioglycolate.
- Make sure there is not glycine contamination in any of the dishes used before dechorionation.

Problem 2

The dechorionation reaction goes for too long, causing the embryos to not develop properly (related to Step 2j).

Potential solution

- Only have the dechorionation reaction proceed for 6 min, never longer than 8 min.
- Do not use too much mechanical force when pipetting the embryos around the plate during dechorionation.

Problem 3

Eggs or embryos are lysing (related to Step 2l, 3b).

Potential solution

- This could be due to detergent contamination within your solutions or consumables. Conduct electroporation in a room free of detergent.
- Do not use detergent to wash anything you will use for electroporation.
- If you have to take items for the clean detergent free area into an area in which detergent is used, take items on trays and place paper towels or pads down on the area before placing your items on surfaces to avoid detergent contamination.

Problem 4

The time constants shown upon *Ciona* electroporation are variable (related to Step 2u).

Potential solution

- Use new 4 mm electroporation cuvettes.
- Make sure you have the correct volume of solution in the 2 mL microcentrifuge tube, with the meniscus between the 100–200 μL line. Make sure you look at this at eye level.

Problem 5

The fraction of embryos that undergo proper development is not high (related to Step 3b).

Potential solution

- In the Pacific Ocean off of Southern California, *Ciona* gametes are of the highest quality from March through October. Runoff from rain in the area also lowers the quality of gametes.
- Lysis of embryos, taking too long to dechorionate the eggs, having the eggs out of water in the transfer basket, dropping eggs onto the surface of the water when transferring between plates, not keeping the glass pipettes upright to reduce settling and lysis of embryos on the side of the glass, using too much force when pipetting eggs during dechorionation all can lead to poor development.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Emma Farley (efarley@health.ucsd.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Emma Farley (efarley@health.ucsd.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103107.

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AUTHOR CONTRIBUTIONS

G.A.J., B.P.S., K.T., F.L., and E.K.F. designed the experiments. K.T. and B.P.S. conducted the experiments. E.K.F. designed the protocol. F.L. wrote the detailed protocol with images from G.A.J. and A.T.B. G.A.J., K.T., A.T.B., and E.K.F. prepared the manuscript. All authors were involved in editing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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