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PROTOCOL

Embryo microinjection of the lecithotrophic sea urchin *Heliocidaris erythrogramma*

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ABSTRACT

Microinjection is a common embryological technique used for many types of experiments, including lineage tracing, manipulating gene expression, or genome editing. Injectable reagents include mRNA overexpression, mis-expression, or dominant-negative experiments to examine a gene of interest, a morpholino antisense oligo to prevent translation of an mRNA or spliceoform of interest and CRISPR-Cas9 reagents. Thus, the technique is broadly useful for basic embryological studies, constructing gene regulatory networks, and directly testing hypotheses about cis-regulatory and coding sequence changes underlying the evolution of development. However, the methods for microinjection in typical planktotrophic marine invertebrates may not work well in the highly modified eggs and embryos of lecithotrophic species. This protocol is optimized for the lecithotrophic sea urchin *Heliocidaris erythrogramma*.

Keywords: direct development, *Heliocidaris erythrogramma*, lecithotroph, sea urchin, microinjection

BACKGROUND

There several methods to manipulate the gene product contents of a zygote. Reagents such as mRNAs, RNAi, splice- or translation-blocking reagents, CRISPR reagents, or proteins are often introduced into experimental embryos by microinjection or electroporation. Microinjection has the advantage that individual cells of cleavage-stage embryos can be injected to allow linage tracing or selective manipulation of a sub-population of the embryo's cells. Reliable techniques for microinjection of typical marine embryos are well established (*e.g.*, [1,2]).

The evolution of life history is a long-standing and growing area of interest for evolutionary developmental biologists. In particular, losses or truncations of a feeding, metamorphic larval life stage present opportunities to ask direct questions at the intersection of evolution and development. This trait, called direct development or lecithotrophy, has evolved independently many times and species pairs or groups with differing degrees of direct development are increasingly popular model systems for evolutionary developmental biology. Examples include intraspecific variation and plasticity [3,4] and speciation events that fixed differences in life history [5-7].

An ongoing increase in interest and tools is rapidly expanding the selection of tractable laboratory model species. To advance beyond descriptive studies to experimentally probe how developmental mechanisms and gene regulatory networks change in life-history evolution, methods for working on secondarily lecithotrophic relatives of established experimental systems are needed. Standard methods for injecting planktotrophic marine embryos do not work on such embryos. The degree of optimization necessary to work on sister species is much greater than that for a new but typical species of the same order.

Previous work with the lecithotrophic sea urchin *Heliocidaris erythrogramma* (*H. erythrogramma*) has used microinjection of tracer dyes to partially fate map the embryo [8-10] and of *in vitro* transcribed mRNA to examine the phenotypic effects of overexpression of a few key developmental genes [11]. However, detailed, optimized methods sufficient to replicate published results for such experiments are largely contained within unpublished lab resources, so we wished to share this technique broadly with researchers interested in developing methods for other lecithotrophic species and with those who may wish to replicate our results in the future.

Experimental manipulation of live zygotes' cytoplasmic or nuclear contents is the key to progressing beyond simply descriptive and comparative studies. For example, examining allele-specific expression in inter- and intra-specific hybrid crosses is one way to tease apart cis- from trans-regulatory effects [12-15]. However, only direct manipulation can demonstrate that any single change is relevant to the developmental phenotype. To build developmental gene regulatory networks and test

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hypotheses about their evolution requires manipulation of the individual component genes' expression [16-17]. With the advent of gene editing in sea urchins [18,19], CRISPR-Cas9 experiments targeting individual fixed changes to cis-regulatory elements between lecithotrophic and planktotrophic congenerics will allow direct probes of cis-regulatory evolutionary consequences.

MATERIALS

Reagents

- ✓ Filtered natural sea water (FSW)
- ✓ Agarose (Sigma, cat. #: A9539)
- ✓ Gelatin (Sigma, cat. #: G1890)
- ✓ Potassium chloride (Sigma, cat. #: P9541)
- ✓ Ficoll 400 (Sigma cat. #: F-9378)
- ✓ Molecular grade glycerol (Sigma cat. #: G5516)
- ✓ Fixable tetramethylrhodamine-dextran (TMR) 10000 MW (Sigma cat. #: 73766)
- ✓ Penicillin-streptomycin 100× stock solution: 10000 units penicillin and 10 mg streptomycin/ml (Sigma cat. #: P4333)
- ✓ RNase-free water (not DEPC-treated)
- ✓ Experimental reagent(s) of choice, *e.g.*, IVT-mRNA, morpholino oligos, CRISPR guides and Cas9
- ✓ 0.1% gelatin, 0.05% paraformaldehyde (to coat tools)

Recipes

- ✓ 2× microinjection solution (RNase-free/molecular-grade reagents), for 20 µl:
  - 3.5 µl water
  - 6.5 µl lysine-fixable fixable tetramethylrhodamine (TMR) dextran stock, 10000 MW (150 mg/ml) (ThermoFisher D1868)
  - 2.0 µl 4M KCl (Sigma cat. #: 60128)
  - 8.0 µl glycerol (Sigma cat. #: G5516)
- ✓ Pasteurized filtered sea water (PFSW): Coarsely filter natural sea water through a gravity-fed glass filter. Pasteurize at 65°C 30 min at temperature (heated stir plate or water bath) and returned to room temperature. Alternatively, vacuum filter with 0.22 µm filter.
- ✓ Spawning injection solution: 0.5 M KCl (Sigma cat. #: P9333)
- ✓ 1× Penicillin-Streptomycin in PFSW
- ✓ 2% w/v Ficoll 400 in PFSW

Equipment

- ✓ Embryo-dedicated labware:
- ✓ 1 L glass bottles (VWR cat. #: 10754-820)
- ✓ 500 ml beakers (VWR cat. #: 10754-956)
- ✓ 250 ml flask (VWR cat. #: 10536-914)
- ✓ Stir bars (VWR cat. #: 58948-138)
- ✓ Lidded plastic boxes
- ✓ 10 ml syringe with needle
- ✓ Heated stir plate
- ✓ Microwave oven
- ✓ Needle puller (Sutter P97 Flaming/Brown Micropipette Puller) equipped with standard 2.5 × 2.5 mm box filament
- ✓ Microinjector (General Valve Picospritzer II)
- ✓ Nitrogen tank and regulator
- ✓ Miniature ruler (Ted Pella 13623) or micrometer
- ✓ Dissecting microscope (Olympus SZH) with white light
- ✓ Epifluorescence dissecting microscope with correct light source and fluorescence filter set for the selected dye
- ✓ P10 pipettor and tips (RNase-free if injecting mRNA)
- ✓ Humid chambers (dedicated airtight lidded plastic boxes with moistened absorbent material such as a sponge)

Consumables

- ✓ Coarse filters for FSW (Millipore AP25 prefilters AP2504700)
- ✓ 60 × 15 mm petri dish lids (Falcon 351007)
- ✓ 4 well IVF dishes (Multidish Polystyrene 4 Well Nunclon Round w/Lid Sterile 66 × 66 MD4 for IVF 15 mm Well) (Thermo 176740)
- ✓ 6 well plates (*e.g.*, Falcon 351146), coated with gelatin or Corning® Costar® Ultra-Low Attachment 6-well plates
- ✓ Plastic transfer pipettes
- ✓ 5" glass Pasteur pipettes
- ✓ Pasteur pipette bulbs
- ✓ Latex tubing
- ✓ Needle stock: thin wall borosilicate glass capillaries with inner filament, OD 1.0 mm, ID 0.75 mm (World Precision Instruments TW100F-6)
- ✓ 150 micron nitex filter basket

Equipment setup

- ✓ Inject under a dissecting microscope with a black background.

PROCEDURE

Prepare tools and reagents

1. Pull needles using the following parameters: heat = 760 (100% ramp value), pull = 80, delay = 120. The needle's taper should begin approximately 9 mm from the tip. The tip of a representative needle is shown in **Figure 1**.
2. Gelatin-coat 6-well plates to use as culture dishes (or use IVF dishes). Fire-polish and gelatin-coat glass Pasteur pipettes to transfer embryos.
3. Filter, pasteurize, and cool to room temperature natural sea water.
4. Prepare injection dishes.

4.1. Make 2% agarose in 50% FSW and melt gently. Into the lids of 60 mm petri dishes, pour just enough agarose to cover the surface of each overturned lid. Allow agarose to solidify.

4.2. Fill injection dishes with PFSW for at least 1 h before use. Store dishes filled with PFSW and protected from dust, at room temperature. Use within 24 h.

HINTS: Prepare pasteurized, filtered natural seawater (PFSW) at least 24 h before planned injection. Prepare injection dishes no more than 24 h and no less than 1 h in advance (2–8 h optimal).

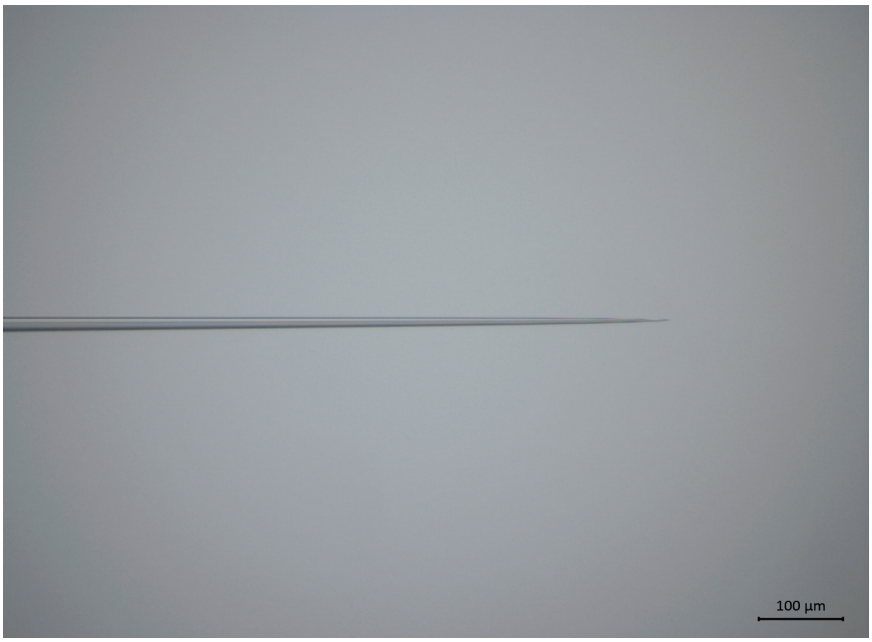

Figure 1. Example of a pulled needle's tip. The preferred needle size is relatively fine compared to the size of *H. erythrogramma* eggs.

Prepare gametes

5. Spawn female and male urchins by injecting ~0.5–1 ml 0.5 M KCl into an adult urchin using a syringe fitted with a 24-gauge hypodermic needle.

6. Collect sperm dry into a microfuge tube and store at 4°C for up to 1 week. Sperm are activated in the presence of seawater and the sperm will be compromised if any gets into the tube.

7. Collect eggs. Allow female to spawn at the bottom of a beaker of coarsely filtered seawater (**Fig. 1**). With a transfer pipette, move the eggs from the top of the beaker to a clean 500 ml beaker of FSW.

8. Wash and partially de-jelly eggs by washing 3× with ~500 ml FSW. Draw off FSW with a bulb syringe through a basket made with 150 µm mesh attached to either a plastic beaker with the bottom removed or a small section of PVC pipe. Allow eggs to rise to surface after last wash.

9. Transfer washed eggs from surface into a small volume (~50–100 ml) PFSW in a 500 ml container. Dilute sperm by adding 500 µl FSW to 5 µl sperm. Fertilize washed eggs with diluted sperm.

10. After 5–10 min, check under magnification for fertilization envelopes to confirm fertilization. The perivitelline space tends to be relatively small in this species, so look for a shiny surface. If most do not have visible fertilization envelopes at 5 min, add more sperm to synchronize the time of fertilization.

11. After confirming fertilization, wash out excess sperm by filling dish with PFSW. Allow zygotes to rise to the top, and transfer into a clean dish of PFSW.

HINTS: Good quality *H. erythrogramma* eggs are deep orange and can be seen as individual with the naked eye (**Fig. 2**). They are released as strings of beads connected with jelly and float to the surface. Low quality eggs may look pale and clumped, and rise to the surface more slowly than healthy eggs.

It aids in screening to fertilize with a higher concentration of sperm than would be used to establish a culture and then rapidly wash it out. This better synchronizes the time of fertilization, which will vary considerably using

the standard sperm concentration for *H. erythrogramma* in the lab. The trade-off is an elevated risk of polyspermy. An additional fertilization control using a lower concentration of sperm can establish whether a high rate of developmental arrests is resulting from excessive sperm.

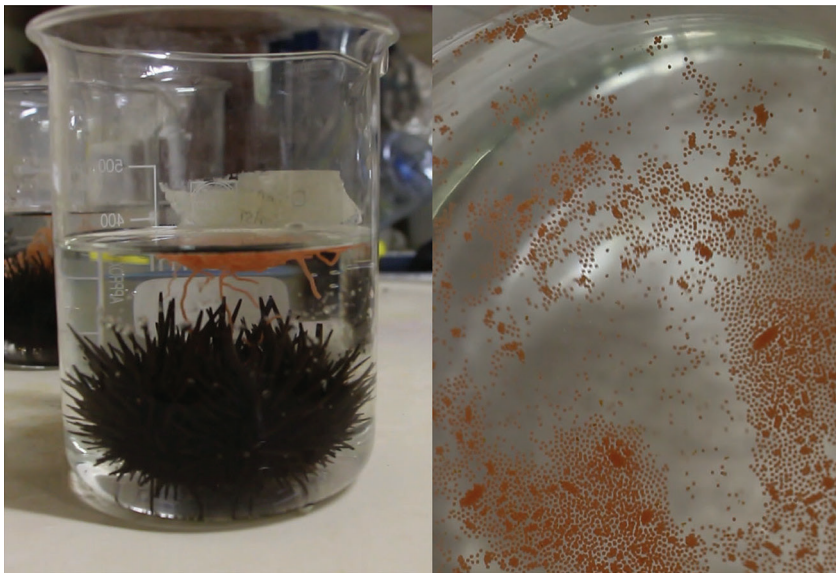

Figure 2. Healthy *H. erythrogramma* eggs. They are positively buoyant and bright orange in color. Left: Spawning female. Right: Washed eggs floating on the surface of FSW.

Set up injection dishes

12. Pick best-looking fertilized zygotes into a clean dish filled with pasteurized FSW for storage until injected.

13. Transfer 20–50 embryos into an injection dish containing 2% w/v Ficoll 400 in PFSW. Keep approximately constant the number of embryos per plate for the experiment and simply inject more plates to produce more experimental embryos.

14. Draw off the solution and replace with fresh 2% Ficoll-PFSW. Swirl the dish to bring zygotes to float near the dish's center (**Fig. 3A**).

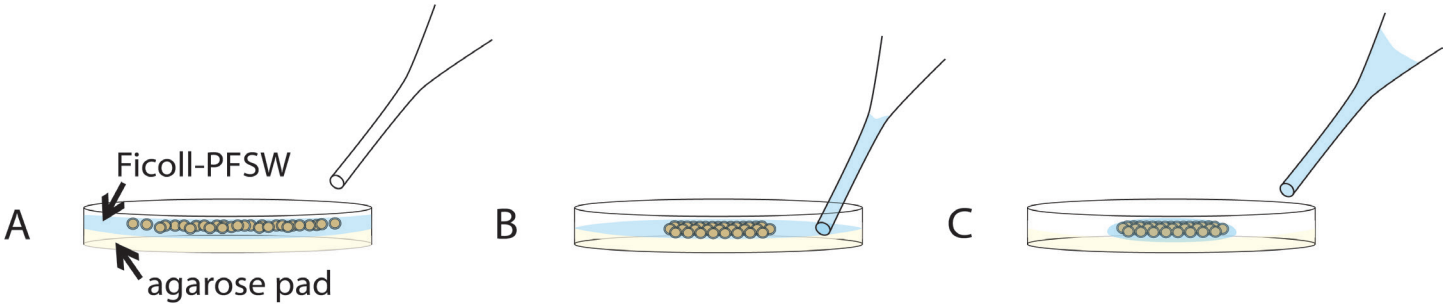

Figure 3. Set up of injection dish immediately before injection. A. Allow zygotes to equilibrate in Ficoll-PFSW in the injection dish. **B.** Draw off Ficoll-PFSW using a fire-polished Pasteur pipette. **C.** Continue to remove Ficoll-PFSW until the embryos are immobilized against the agarose pad, leaving just enough solution to cover them.

15. Draw off Ficoll-PFSW until zygotes make firm contact with agarose pad (**Fig. 3B** and **3C**). Wait 5–10 s, and then gently add Ficoll-PFSW dropwise to each cluster of zygotes until the meniscus is beyond their cell membranes. If they float away, the volume is too large; draw it off and try again.

HINTS: Injections 25–70 min post-fertilization have the best rates of survival. Alternatively, you may try injecting and then transferring into FSW and fertilizing after 20 min recovery time as in [11]. Needles may be loaded 5 min to 1 h before injections.

The eggs are positively buoyant and also fragile; this method allows them to stick to the plate without agents such as protamine sulfate during injection but working in a small volume risks osmotic stress as the water evaporates. Fewer embryos per plate are best so that the water volume may be restored quickly. Prepare one plate at a time for injection and hold a second plate at step 14 (in Ficoll solution). After each plate is completed, bring a new plate to step 14 and advance the previous plate to inject.

The timing and volumes used to set up the embryos in the injection dishes must not stray from the protocol. If the zygotes spend too little time in Ficoll-PFSW before injection, survival rates decline. However, if the embryos are incubated in Ficoll-PFSW before placing on injection dish and drawing off the liquid, they do not stick to the surface as well and injection efficiency is decreased. The small volume necessary to keep the embryos from lifting from the surface of the injecting dish evaporates rapidly, subjecting the embryos to osmotic stress. Thus, it is important to set up 2 dishes to be injected plus a control before the first injection.

Inject

16. Load needles with 1–2 µl reagent of choice in 1× injection mix. Use pipettor to place solution on the back end of the needle, where the microcapillary will draw it to the sharpened end.
17. Turn on microinjector and gas supply. Set the Picospritzer air pressure to 30 PSI. Use the constant pressure setting until the needle is broken.
18. Load a needle into the microinjection rig. Under dissecting microscope, break needle on the ridges on the edge of the injection plate. Alternatively, forceps or a hypodermic needle may be used to break open the needle.
19. Switch to the pulse setting on the Picospritzer. Check that the microinjector settings and needle opening create the desired bolus size at ~100 ms pulse duration. The ideal bolus is no greater than 1/10 the embryo volume. Adjust the pressure duration setting accordingly.
20. Approach at a 30°–45° angle so that the needle enters near a relatively large volume of Ficoll-PFSW. Insert the needle 1/5–1/3 diameter of the egg. The dye will rapidly spread through the embryo if it is properly injected (**Fig. 4**; **Video S1**).
21. Immediately after injecting the whole plate, flood with Ficoll-PFSW and place in a humid chamber.

HINTS: Inject experimental and control reagents for the same comparison in succession, or alternate experimental and control plates, rather than injecting all experimental plates together and all control plates together to prevent confounds between time of injection and survival or phenotype.

The bolus volume can be checked using the method in [20]. Briefly, use equation $v = 4/3\ (\pi r3)$, where v is the volume in nL and r is the radius of the sphere in µm. There are several options for making the measurement but a miniature ruler (or any stage micrometer) is a straightforward option. A test bolus can be held on the tip of the needle or placed into a dish of oil to approximate its radius.

Pick and culture injected embryos; complete assays

22. Sort experimental embryos between 4-cell stage and early blastula (**Fig. 4D**). Transfer injected embryos from injection dish to culture dish with gelatin-coated pipette. Under a fluorescent dissecting microscope, transfer evenly injected, similarly bright embryos (**Fig. 5**) and transfer into coated culture dishes (IVF plates or gelatin-coated multi-well plates) containing 1× Penicillin-Streptomycin in PFSW (Pen-Step PFSW).
23. Culture embryos in a humid chamber at 17°C–22°C in Pen-Step PFSW. Change water and remove dead embryos twice daily. Culture density for first 24 h should not exceed 10 embryos/ml.
24. Fix appropriately for the intended assay at a predetermined time point.

HINTS: Many injected embryos arrest at the 2-cell stage so it is best to wait until they are 4-cell or beyond to sort. Embryo care and culture after injection are crucial for success.

ANTICIPATED RESULTS

Microinjection techniques can be applied to many kinds of questions. The expected results depend primarily upon the reagents injected. Morphological phenotypes should be screened under both a dissecting microscope and with cleared whole-mount samples under a compound microscope. Expected molecular phenotypes can be assayed at the appropriate stage by *in situ* hybridization, immunohistochemistry, qPCR, and similar techniques.

Positive results can be confirmed using multiple lines of evidence.

First, the phenotype should be robust across individual crosses. Complete at least three independent biological replicates for each experiment. Be sure to use the appropriate controls, meaning negative control should be as close as possible to the experimental reagent; a dye-only control is not as good as a control morpholino [21] (or a control mRNA in the case of mRNA injection) but better than an uninjected control. Numerically score the experiments and conduct appropriate statistical tests to identify significant differences from controls. Consider ahead of time how to score expected phenotypes, such as presence/absence or levels of a target cell type or downstream transcript.

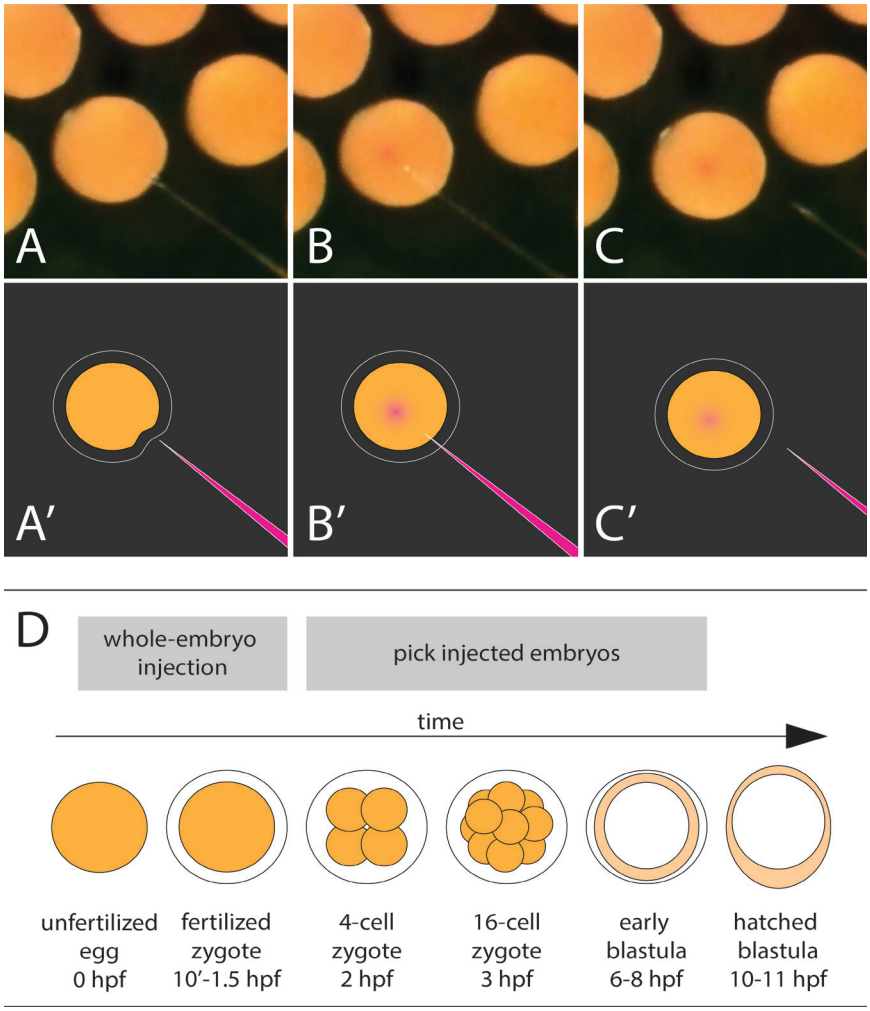

Figure 4. Microinjection of *H. erythrogramma* embryos. Top row: photomicrograph of an injection. Middle row: cartoon illustration of focal embryo in top row. Bottom row: Schematic of early of *H. erythrogramma* development. **A** and **A'**. The needle dents the fertilization envelope and the embryo itself. **B** and **B'**. Injection solution in pulsed into the embryo. **C** and **C'**. The needle is withdrawn and the injected solution disperses evenly within the embryo. **D.** Approximate timing of *H. erythrogramma* development from fertilization to hatching at 22°C. Grey boxes indicate timing windows for injection and sorting. See also **Video S1**.

Inject a known positive control reagent to ensure that the injection technique is working. For example, Axin2 translation-blocking morpholino (sequence: CTAGACTCATGTCTGCACATTGTAG) is a good positive control with an early molecular phenotype and a dramatic phenotype at post-gastrula stages.

Check the phenotype at multiple time points and with multiple assays, if possible. An early phenotype may differ from a later phenotype, which might represent different gene regulatory network roles for the same gene or simple recovery; a molecular phenotype observed with *in situ* hybridization may not have a morphological consequence. Experimental phenotypes generally should have a dose-response relationship; inject a lower dose to demonstrate such a relationship. However, this rule does not apply to all types of manipulations. For example, a CRISPR experiment may show varying penetrance or mosaic development at lower doses but no dose-response to the genome editing *per se*.

Use alternative reagents to phenocopy the result. For example, a small-molecule inhibitor targeting the same signaling pathway and a morpholino knockdown of one of its key components should have similar phenotypes. An alternative morpholino, such as a splice-blocking and a translation-blocking morpholino, for the same target can help to confirm one another, as can knocking down two different members of the same pathway.

Reagents may have expected opposing phenotypes. For example, a knockdown or dominant-negative and overexpression may have opposing phenotypes, although they may not depending on the underlying biology. Members of the same pathway could be used for this type of

experiment (*e.g.* reagents targeting GSK3 and β-catenin should have opposing phenotypes).

Demonstrating the capacity to rescue is the gold standard in most embryological experiments. A rescue experiment could be an overexpression construct engineered to differ from the morpholino-targeted sequence, co-injected with the morpholino.

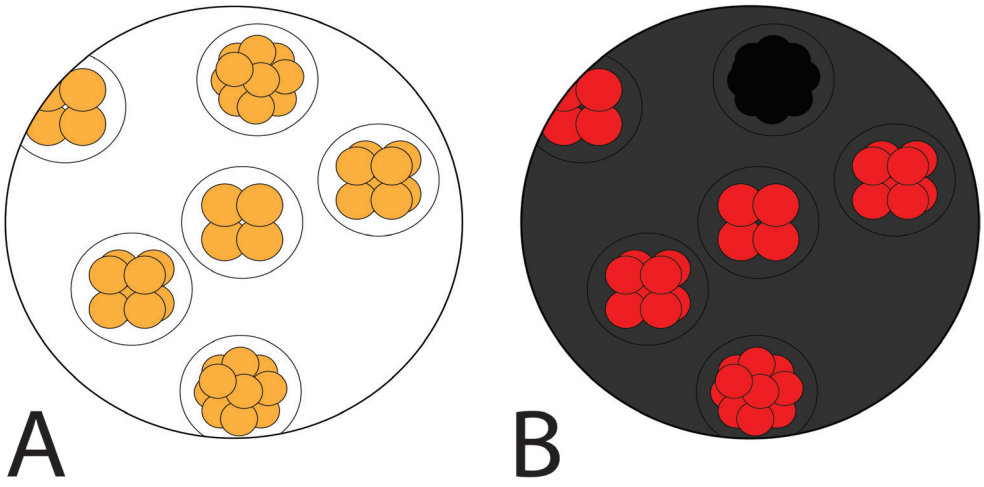

Figure 5. Screen injected embryos. Use a fluorescent dissecting microscope. Pick only evenly injected embryos cleaving normally at 4-cell stage or beyond and transfer into a fresh, low-attachment plate to culture. **A.** Diagrammatic view of injected embryos under white light. **B.** Diagrammatic view of injected embryos under fluorescent light; dark embryos are not injected.

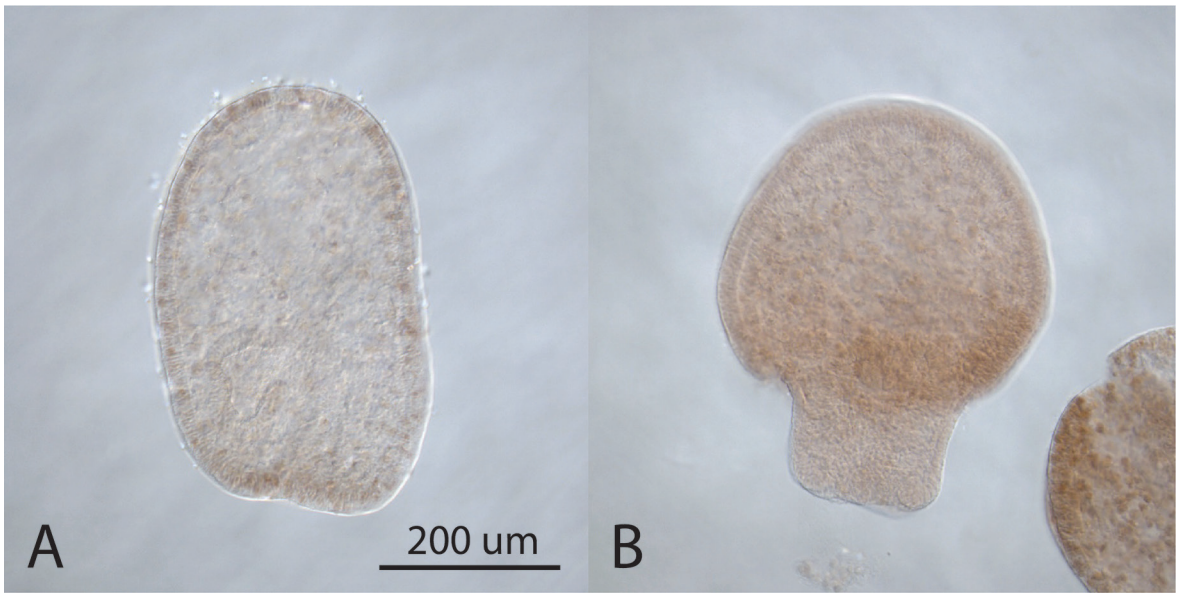

Figure 6. Representative results. A. Control embryo, injected with 1× injection mix and fixed at late gastrula stage. **B.** Experimental embryo, injected with 50 nM Axin2 translation-blocking morpholino and fixed at late gastrula stage.

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 1**. Suspected false negative results may reflect the following possibilities:

The reagent was degraded. Follow manufacturer instructions for reconstituting or dissolving fresh reagents and for troubleshooting precipitated or degraded morpholinos. Use standard RNase-free materials and techniques for RNA. Check integrity of mRNA by electrophoresis. Incorporate a positive control with an easily screened phenotype, such as pigmentation [18].

Insufficient quantities of the reagent were injected. Double-check stock concentration by spectrophotometer. Titrate the dose by injecting a lethal concentration of the reagent and decreasing concentrations from there.

A reagent such as a morpholino is mismatched to its target [21].

Check the target region of the transcript of interest in the sub-population of interest by PCR and Sanger sequencing to be sure the morpholino sequence is correct. Check the transcript for alternative start sites.

Test alternative reagents such as small molecule inhibitors targeting the same pathway, or a dominant-negative mRNA to phenocopy a morpholino or vice versa.

Table 1. Troubleshooting.

Step	Problem	Cause	Suggestions
10	eggs fail to fertilize	sperm concentration too low	add more sperm
10	eggs fail to fertilize	sperm not viable	collect fresh sperm; always store sperm dry
10	eggs fail to fertilize	eggs not viable	spawn another female
15	uninjected zygotes spontaneously rupture	too much water removed	set up a new injection dish removing less water; work quickly after removing water
20	injected zygotes rupture	needle too large	break fresh needle closer to tip
20	injected zygotes leak	Ficoll % too low	follow steps 13–14 exactly
20	injected zygotes leak	needle too large	break fresh needle closer to tip
20	visible spots of dye remain at zygote surface	needle too large	break fresh needle closer to tip
20	visible spots of dye remain at zygote surface	needle not inserted deep enough	insert the needle 1/5–1/3 diameter of the egg
20	needle clogs	occasional needle clogs are unavoidable	use "clear" function on microinjector; gently wipe needle on agarose pad; re-break needle
20	needle clogs	needle bore too large	break fresh needle closer to tip
20	needle clogs	needle not removed from zygote immediately after injection	pull out needle immediately after injecting the bolus
22	abnormal cleavage planes	polyspermy	fertilize again using less sperm in step 9 and/or washing more in step 11
23	injected embryos die at much higher rate than uninjected control	too much material injected	reduce bolus size
23	injected embryos die at much higher rate than uninjected control	infection	change water more often
23	experimental embryos die at much higher rate than injection control	reagent is lethal at current dose	titrate reagent dose

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Supplementary information

Video S1. View through dissecting scope during injection, showing broken needle with appropriate bolus size relative to the embryo and rapid dye dispersal upon injection of the bolus.

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/292.