

Microinjection of mRNA or morpholinos for reverse genetic analysis in the starlet sea anemone, *Nematostella vectensis*

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We describe a protocol for microinjection of embryos for an emerging model system, the cnidarian sea anemone, *Nematostella vectensis*. In addition, we provide protocols for carrying out overexpression and knockdown of gene function through microinjection of *in vitro*-translated mRNAs or gene-specific oligonucleotide morpholinos (MOs), respectively. Our approach is simple, and it takes advantage of the natural adherence properties of the early embryo to position them in a single layer on a polystyrene dish. Embryos are visualized on a dissecting microscope equipped with epifluorescence and injected with microinjection needles using a picospritzer forced-air injection system. A micromanipulator is used to guide the needle to impale individual embryos. Injection takes ~1.5 h, and an experienced researcher can inject ~2,000 embryos in a single session. With the availability of the published *Nematostella* genome, the entire protocol, including cloning and transcription of mRNAs, can be carried out in ~1 week.

INTRODUCTION

The starlet sea anemone, *N. vectensis*, is a morphologically simple basally branching animal that has a surprisingly complex genome¹. For example, the molecular complexity that is thought to have evolved in the vertebrate lineage, such as an entire complement of *wnt* genes² and the basic genomic architecture¹, appears to predate the cnidarian (e.g., corals, hydra, anemones and jellyfish) and bilaterian (e.g., insects and vertebrates) split^{1,2}. Its phylogenetic position and molecular complexity make *Nematostella* an attractive system for investigating the evolution of mechanisms that regulate animal development. *Nematostella* is also highly regenerative, capable of regenerating all adult structures after bisection^{3,4}. These properties make *Nematostella* an important animal model for investigating the relationship between the mechanisms that regulate development and regeneration of distinct cell types. In contrast, other models for investigating regeneration (planarians and *Hydra*) do not reproduce sexually or generate few embryos, making them less desirable for such comparisons. Vertebrate models are well suited for investigating development, but they often show limited regenerative capacity as adults and are expensive to maintain in culture. Taken together, these considerations provide compelling reasons to further develop the inexpensive invertebrate *Nematostella* for functional genetic investigation.

Microinjection of molecules into zygotes is one of the simplest methods for introducing RNA, DNA, protein, MOs and vital dyes into living embryos. The large egg size (200–300 μm; ref. 5) and accessibility to large numbers of eggs (100–1,000 per female)⁶ of *Nematostella* make microinjection in anemone embryos a fairly straightforward and powerful approach. There are a number of techniques for microinjection that rely on magnification and stabilization of the egg or zygote, a method to manipulate an injection needle in order to bring the embryo and needle into alignment, and the ability to visualize the injection of fluid into the egg. Standard dissecting scopes provide sufficient magnification for injecting the majority of the embryo types typically used in the laboratory including *Nematostella* (Fig. 1). Methods for stabilizing the embryo range

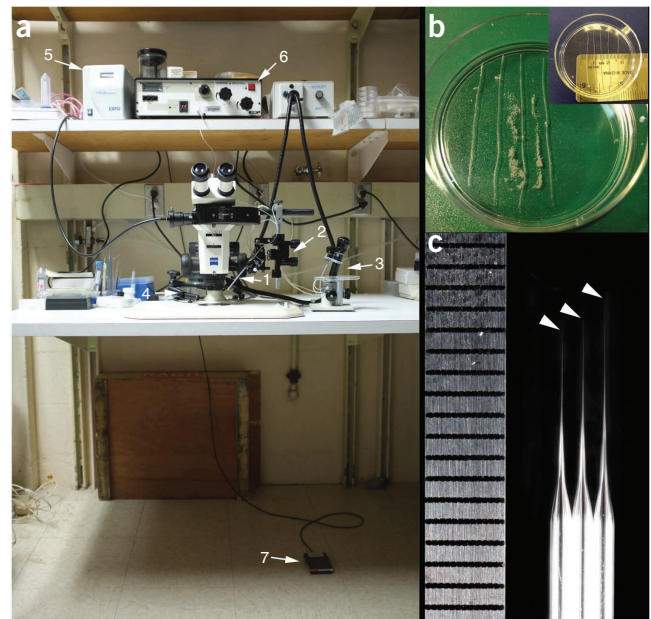
from using a suction capillary tube⁷, constructing wells for holding the embryos (http://zfin.org/zf_info/zfbook/chapt5/5.1.html) or physical adherence to a substrate⁸. *Nematostella* embryos can be stabilized using a suction capillary. However, *Nematostella* embryos transiently adhere to polystyrene Petri dishes, making this a simpler alternative approach to stabilizing embryos for injection (Fig. 1b). Visualization of the injectant entering an embryo is often achieved by co-injection with a tracer dye (e.g., a fluorescent dextran, Fast Green or phenol red). The size and opacity created by the high yolk content of *Nematostella* embryos make assessing the volume of fluid injected by using Fast Green and phenol red difficult. Thus, co-injection with a fluorescent tracer dye using an epifluorescence dissecting scope is the preferred method.

Misexpression of genes can be accomplished through microinjection of *in vitro*-synthesized mRNA coding for a gene of interest^{9–11}. This approach has been used in a variety of organisms ranging from invertebrates such as *Drosophila*¹² to vertebrates such as *Xenopus*¹³. The injection of mRNA into the fertilized embryo results in ubiquitous expression of the injected mRNA (Fig. 2), enabling researchers to assess the ability of the injected gene to affect a particular biological process. Recent papers have demonstrated the use of mRNA injection in *Nematostella* embryos to investigate the result of overexpressing a neurogenic gene⁹, to investigate the result of overexpressing truncated or dominant functioning forms of a particular gene^{10,14} and to rescue the phenotypes induced by knocking down of gene function using MO technology^{9,15}. These studies highlight the importance of mRNA injection-directed gene expression in that it improves the interpretation of MO gene knockdown experiments, and it enables researchers to misexpress a particular wild-type gene, as well as dominant-active, dominant-negative or individual domains of that gene.

MO-induced gene knockdown is an established technique for inhibiting the function of a specific gene by reducing the amount of functional protein *in vivo*¹⁶. MOs are synthetic nucleotide sequences designed to be complementary to the sequence of either a splice



Figure 1 | Microinjection. (a) Image of the injection apparatus. Shown is a typical setup for a right-handed injector. Injections are carried out using a standard stereo-dissecting microscope outfitted with a forced-air micropipette injection needle mount (1). A coarse (2) and fine (3) micromanipulator allow for mechanical control of the needle during the injection process. A single oblique-angled light source (4) allows the researcher to manipulate the dish and use their hand to both manipulate the injection dish and modulate white light. Fluorescence is excited by an external light source (5). A picospritzer (6) is used to control the flow of air to the needle, and an injection foot pedal (7) allows the injector to pulse air for injection while leaving hands free to hold the injection dish and the micromanipulator. (b) Example of injection dish with two rows of embryos positioned and ready for injection. Inset in **b** shows a 1-cm ruler with scale of spacing for scratches on the bottom of an injection dish. (c) Three injection needles with tips indicated by white arrowheads. Ruler scale is 1 mm per line. All three needles are sufficient for injection. The one on the left is stout and cannot be broken back as much as the other two if there is clogging. The one on the right has a very fine tip and can be flimsy, making injections for beginners more difficult. The middle needle combines the properties of both stoutness and a fine flexible tip.



site (Fig. 3) or a translation start site (Fig. 3a,e) in nascent mRNA molecules¹⁷. The binding of the MO to the target mRNA results in reduction of the protein encoded by the targeted gene. The use of MOs is distinct from other reverse genetic approaches (e.g., RNAi) because no additional cellular mechanisms are required to affect gene knockdown. In addition, when coupled with mRNA injection, it is possible to introduce gene products not targeted by the MO (Fig. 3c) and thus to identify phenotypes specifically induced by the knockdown of a particular gene versus off-target phenotypes. Our laboratory described the first successful use of MO technology in *Nematostella*, wherein a splice-blocking MO was shown to inhibit proper splicing of an endogenous transcript¹⁸, and the first morphant phenotype using a translation-blocking MO was described the following year¹⁹. MO-knockdown approaches have been a valuable technique deployed consistently since that time^{9,14,15,19–23}.

In *Nematostella*, microinjection has been used to introduce a wide range of biological molecules and vital dyes^{9,10,18}. Here we provide the protocol we use for microinjection of mRNA or MO molecules into the *Nematostella* embryo^{9,10,14,18,24}. Of note, this method has recently been used to introduce reporter gene DNA into *Nematostella*, in some cases resulting in stable transgenic animals²⁵. Regardless of the molecule being injected, the approach is essentially the same. Briefly, embryos are dejellied and maintained in sterile artificial seawater (ASW). Embryos are then adhered to a plastic surface and injected using a forced-air injection system and micromanipulator. Finally, embryos are allowed to recover and develop to the desired stage.

Experimental design

Microinjection. To microinject *Nematostella* eggs, zygotes or embryos, adult animals must be prepared for spawning in the evening before the day of injection^{26,27}. Ideally, male and female anemones are in separate bowls so that the researcher can control the timing of fertilization. However, male and female gametes show reduced fertility by 2 h after spawning occurs²⁷. After animals have spawned and fertilization has taken place, the eggs are dejellied using a 4% (wt/vol) cysteine solution²⁷ and washed in sterile 1/3× ASW. Embryos are then transiently adhered to polystyrene dishes in sterile 1/3× ASW and injected with the desired solution. If minimally disturbed, embryos will remain adherent to polystyrene dishes

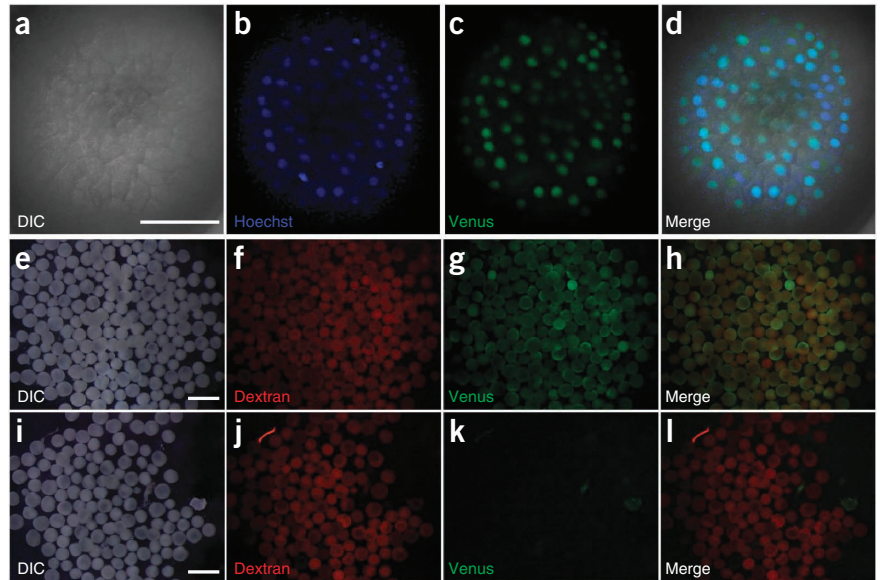
for ~3–4 h. First cleavage of *Nematostella* embryos occurs 1–2 h after fertilization, depending on the temperature^{5,10,27}, thereby providing sufficient time to carry out the injection protocol before the loss of adherence. Maintaining a cool (~17 °C) injection room slightly extends the time to first cleavage, which allows additional time for injection. If fluorescent dyes are used as co-injectants, as we recommend, injection rigs should be set up in a room in which the lights can be dimmed to improve visualization of tracer dye, which increases the ability to control the volume of material injected. With ~2 h between fertilization and first cleavage, an efficient researcher can dejelly and wash embryos in 30 min, leaving 1.5 h for injection. In 1.5 h, between 700 and 2,000 embryos can be injected, depending on the experience of the researcher.

A key for rapid and successful microinjection is the preparation of suitable injection needles. There are several commercial options for instruments to fashion glass micropipettes. Each instrument has parameters that can be varied to influence the tip size, taper length and so on. In addition, there are different kinds of glass that can be used (e.g., borosilicate or aluminosilicate), which influence the characteristics of the injection pipette. Some researchers prefer shorter, stiffer pipettes (Fig. 1c, left needle), whereas others prefer longer, more flexible tips that can be broken back in case of tip clogging (Fig. 1c, right needle). Figure 1c provides a range of suitable needles for the injection of *Nematostella*; in Equipment Setup, we provide details on setting variables on the Sutter pipette puller.

mRNA microinjection. To inject mRNA into any animal, including *Nematostella*, one must first generate and isolate the mRNA molecule of interest. The coding region of a gene of interest is identified and subcloned into a vector designed for *in vitro* mRNA transcription, typically such that a fluorescent fusion protein is generated or such that the protein encoded by the mRNA can be visualized with a commercially available antibody (anti-FLAG, HA, GST and so on). When choosing possible tags and their positions, it is important to consider the basic organization of conserved domains in the protein encoded by the mRNA. In general, it is best to subclone the cDNA so that possible tags are as far as possible from functional domains. For instance, if a conserved domain exists toward the C terminus of

PROTOCOL

Figure 2 | Expression of Venus protein in *Nematostella* injected with *NvashA:venus* mRNA. (a–d) Z-projection (8 μm) of cleavage stage embryo 3 h after injection with *NvashA:venus* mRNA. Venus expression can be clearly observed in c,d. Nuclear localization of Venus is observed by using Hoechst as a counterstain to label DNA (b–d). Nuclear localization of Venus is observed by using Hoechst as a counterstain to label DNA (b–d). Nuclear localization is expected because the *venus* coding sequence is fused in-frame with the *NvashA* transcription factor coding sequence, which contains a nuclear localization signal. (e–l) Low magnification views of unsorted embryos grown at 17 °C for 24 h after injection. Embryos were co-injected with dextran and *NvashA:venus* mRNA (e–h) or dextran alone (i–l). Nearly all embryos co-injected with the dextran and mRNA show strong fluorescence from the Venus protein (g,h), whereas control animals do not show green fluorescence (k,l). DIC, differential interference contrast. Scale bars (a–d), 100 μm ; (e–l), 500 μm .



your protein, then generating an N-terminal tag is less likely to cause unexpected effects on protein function. It is important to remember that although fusion proteins can usually be used without issue, there is the possibility that a fusion protein will behave differently than its wild-type untagged counterpart. After designing and subcloning, mRNA is generated by *in vitro* transcription and purified before injection into zygotes. There are a number of kits and methods available for purifying mRNA. However, we find that using the protocol described below leads to reliable and stable expression *in vivo*, and thus we prefer it to other methods.

For each functional experiment, an appropriate effective concentration of mRNA must be determined for each gene (Box 1). In our hands, most effective mRNA concentrations fall within the range of 150–700 $\text{ng } \mu\text{l}^{-1}$. To determine the appropriate concentration, it is useful to monitor injected embryos to determine whether they are viable and for expression of the protein of interest. mRNA concentrations that are too high are toxic to the embryo. The toxic effect can induce death even before any cleavages are observed. This is indicated by a breakdown in cell membrane and the release of cytoplasmic contents. Embryos may also go through a number of cleavages before failing. In this case, cells fail to adhere to one

another and late-cleavage stage embryos disassociate. One issue is that the overexpression of mRNA may cause a lethal phenotype but not because of toxicity. One control for determining whether the overexpression of mRNA induces nonspecific toxicity is to co-inject the mRNA and an MO designed to block translation of that mRNA. This experiment should rescue lethal phenotypes induced by the overexpression of the protein encoded by the mRNA in question. Concentrations of mRNA that are too low are more difficult to determine; however, the inability to detect the fluorescent fusion protein, which is properly cloned in-frame, is a good indicator that mRNA levels are too low. Protein detection can be observed either directly with fluorescence imaging when using a fluorescently tagged protein (Fig. 2) or by immunofluorescence if an antibody exists against your protein of interest or if you have cloned your mRNA in-frame with a tag against which a commercial antibody exists (e.g., FLAG, HA, GST and so on). Animals injected with the optimal concentration of mRNA are sorted on the basis of the presence of the protein product encoded by the injected mRNA (usually as judged by expression of the fused fluorescent tag).

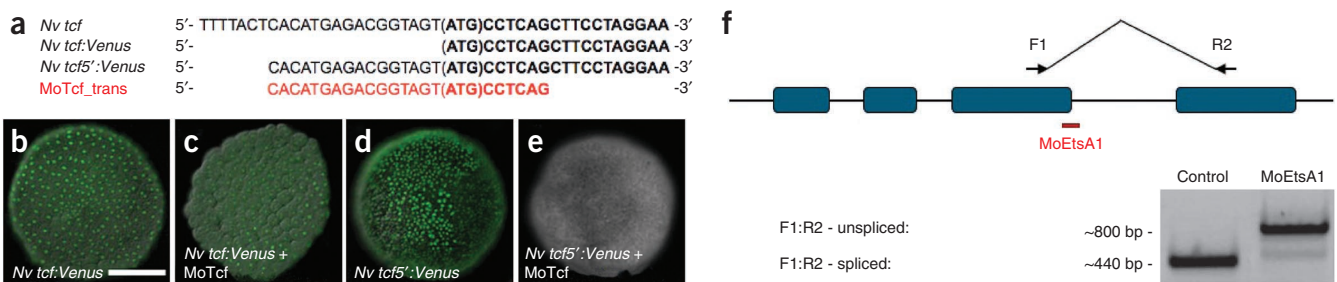


Figure 3 | Assaying MO efficiency. (a) Sequence information for *Nvtcf* (wild-type), *Nvtcf:Venus* (only ORF), *Nvtcf5':Venus* (containing part of the 5' UTR) and the target sequence for MoTcf_trans. (b–e) Overexpression of *Nvtcf:Venus* (b) or *Nvtcf5':Venus* (d) alone or in presence of MoTcf_trans (c,e) showing that MoTcf has no effect on *Nvtcf:Venus* translation, whereas MoTcf inhibits translation of *Nvtcf5':Venus*. The green dots correspond to the nuclear localization of the injected product. (f) Schematic of the genomic organization of *NvetsA1*. The approximate positions of the exons are indicated by blue boxes. F1 and R2 indicate the positions of the primers used to assay the efficiency of the splice-blocking MO. In uninjected controls, only the amplicons corresponding to spliced versions of the transcript (440 bp) are detected, whereas in MoEtsA1-injected embryos the large majority of the PCR product corresponds to an unspliced version of the transcript (800 bp). The low level of the 440-bp fragment compared with the 800-bp fragment in injected embryos demonstrates the efficiency of the used MO. Scale bar in b–d, 100 μm .

Box 1 | Optimizing mRNA or MO concentration for microinjection ● TIMING ~ 1 d

1. Follow Steps 2–19 of the main PROCEDURE to inject 100–300 embryos with each concentration of mRNA or MO to be tested. For most genes an mRNA concentration range of 150, 250 and 500 ng μl^{-1} , and an MO concentration range of 300–900 μM will be suitable for determining an effective concentration at which to carry out subsequent experiments.
2. Incubate embryos after injection and score them at ~4 and 24 h after injection for toxicity and effectiveness of injected reagent as described below.

? TROUBLESHOOTING

Scoring for toxicity

Toxicity is usually associated with high concentrations of mRNA or MO, and it is usually evident by death and/or failed cleavage of the embryo. Death induces a breakdown in cell membrane and the release of cytoplasmic contents. Embryos may go through a number of cleavages before death, but cells eventually fail to adhere to one another and late-cleavage stage embryos will dissociate.

Scoring for effectiveness of injectant

Scoring for effectiveness of injected mRNA is most easily achieved by cloning the gene of interest in frame with a detectable fluorescent tag (e.g., GFP, RFP or Venus; **Fig. 2**). Detection of the fluorescent tag indicates that mRNA levels are sufficiently high to result in detectable translated protein. Alternatively, if an antibody exists against the protein encoded by the mRNA or a commercially available tag included in the mRNA coding sequence (e.g., FLAG, HA, GST), immunofluorescence or western blotting could be used to show an increase in protein levels after injection of the mRNA. There are multiple ways to determine whether MO injections are effective at achieving gene knockdown. The ideal method for determining effectiveness of both translation- and splice-blocking MOs is to perform a western blot analysis using an antibody that recognizes the endogenous target protein. This approach allows the quantification of the reduction of protein levels between experimental and control groups. However, if no antibody exists, translation-blocking MO can be tested by co-injection of an mRNA encoding for the target gene that is fused to a fluorescent tag and is recognized by the MO. Loss or reduced fluorescence compared with injection of the mRNA alone indicates effective MO knockdown (**Fig. 3e**). Alternatively, you can perform an *in vitro* translation assay to show that the presence of MO inhibits the translation of the mRNA recognized by the MO^{15,19}. It should be noted that these two approaches do not identify how effective a MO is at inhibiting the translation of endogenous transcripts, but they provide some indication that the MO is blocking translation. Splice-blocking MO effectiveness can be assayed by performing RT-PCR on cDNA generated from RNA extracted from MO-injected and control animals. This allows for a quantification of the relative abundance of properly to improperly spliced transcripts in both the control and MO-injected animals (**Fig. 3f**).

Once proper concentration has been determined, perform future experiments using the optimized concentration of mRNA and allow embryos to proceed to the desired developmental stage for phenotypic analysis. Injection controls include the following: (i) inject fluorescent dextran alone (eliminates possible phenotypes induced by the act of injection); (ii) inject an mRNA that encodes a protein that should not affect the biological process of interest, such as fluorescent protein alone (this eliminates the possibility that the fluorescent tag or translation load owing to foreign mRNA is the source of the phenotype); and (iii) conduct a rescue experiment by co-injecting a translation-blocking MO that specifically recognizes the mRNA to ensure that the phenotypes resulting from injection are specifically due to the overexpression of the gene of interest.

MO microinjection. MO-based gene knockdown is carried out using essentially the same protocol as is used for mRNA gene misexpression. Splice- or translation-blocking MOs are designed against a gene of interest with the help of the GeneTools MO design team (<https://oligodesign.gene-tools.com/request/>). MOs can be fluorescently labeled after production or co-injected with a vital fluorescent dye (which is a less expensive alternative to MO after modification). As with mRNA injections, the appropriate concentration of MO must be determined before conducting the experiment (**Box 1**). A general approach is to use a series of MO concentrations ranging from 100 to 1,000 μM . Determine possible toxicity induced by the injection of each concentration of MO as described above for mRNA. The efficacy of the MO must also be determined so that the extent of gene knockdown can be assessed. If you are using a splice-blocking MO, collect RNA from injected

animals and controls. Thereafter, generate cDNA and determine the ratio of spliced versus mis-spliced target transcript present in the cDNA by RT-PCR (**Fig. 3f**; refs. 14, 15, 19). To demonstrate the effectiveness of a translation-blocking MO, perform western blotting using an antibody against the protein encoded by the target transcript²³, co-inject an mRNA recognized by the MO fused to a fluorescent protein (**Fig. 3e**) or perform an *in vitro* translation assay^{15,19}. However, only quantification of endogenous protein levels encoded by the targeted mRNA can determine the true efficacy of the MO. The goal is to define the lowest concentration of MO that provides the most complete knockdown of the gene of interest.

Once the proper concentration of MOs is determined, perform future experiments with the optimized concentration and allow animals to age to the desired developmental stage for phenotypic analysis. MO knockdown may not completely eliminate gene function, and splice variants inadvertently generated with a splice MO¹⁶ may have phenotypes that are inconsistent with reduced gene function; thus, when possible, it is desirable to design two MOs against the same gene to ensure that similar phenotypes are obtained when each MO is injected individually. Control experiments are critical to properly interpret phenotypes induced by MO gene knockdown²⁸. Controls to inject are the following: a control MO (often a similar MO to the experimental MO that contains five mismatches) and a rescue experiment in which an mRNA is co-injected that codes for a functional protein but is not recognized by the MO (to ensure that the phenotypes are specific to the knockdown of the anticipated target and not due to off-target effects of MO injection) (**Fig. 3a–e**).

MATERIALS

REAGENTS

For mRNA injection

- Transcription vector containing cDNA of interest (we obtained pSPE3—Rvenus and pCS2-gfp vectors from P. Lemaire and T. Lepage, respectively (see Acknowledgments). Additional varieties of the pCS2 vectors can be ordered from Addgene, and the pSPE3 Gateway cloning vectors can be ordered from Invitrogen)

For morpholino injection

- MO(s) (Gene Tools, must be custom designed to target sequence)
- Agarose (ISC BioExpress, cat. no. E-3119-500)
- ASW (Instant Ocean, cat. no. SS15-10)
- Dextran, Alexa Fluor 555 dye (20 mg ml⁻¹ in nuclease-free water; Invitrogen, cat. no. D34679)
- Dextran, Alexa Fluor 488 dye (20 mg ml⁻¹ in nuclease-free water; Invitrogen, cat. no. D22910)
- EDTA (Sigma-Aldrich, cat. no. E5134-500G)
- Ethanol (Pharmco-Aaper, cat. no. 111000200)
- Glacial acetic acid (Fisher Scientific, cat. no. BP2401SI-212)
- Glycogen (20 mg ml⁻¹; Roche Applied Science, cat. no. 10901393001)
- Isopropanol (Fisher Scientific, cat. no. A4164) **! CAUTION** Isopropanol is hazardous if it is ingested, inhaled or if it comes into contact with the skin or eyes.
- L-Cysteine (Sigma-Aldrich, cat. no. C7353-100G)
- MegaClear RNA purification columns (MegaClear kit; Ambion, cat. no. AM1908)
- mMessage RNA *in vitro* translation kit (SP6, T7 or T3; e.g., mMessage machine T3; Ambion, cat. no. AM1348)
- Nuclease-free water (Ambion, cat. no. AM9937) **▲ CRITICAL** Use non-DEPC-treated water because DEPC interferes with MO efficiency.
- NucleoBond PC100 (Clontech, cat. no. 740573)
- Phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.0) saturated with 10 mM Tris (Sigma-Aldrich, cat. no. P2069-400ML) **! CAUTION** This reagent is hazardous if it is ingested, inhaled or if it comes into contact with the skin or eyes.
- Phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) saturated with 10 mM Tris (Sigma-Aldrich, cat. no. P2069-400ML) **! CAUTION** This reagent is hazardous if it is ingested, inhaled or if it comes into contact with the skin or eyes.
- Qiagen Plasmid Plus midi kit (Qiagen, cat. no. 12943)
- TAE electrophoresis buffer
- Tris base (Trizma base; Sigma-Aldrich, cat. no. T6066)
- NaOH

EQUIPMENT

- Nalgene vacuum filter (0.2 μm; Thermo Scientific, cat. no. 154-0020)
- Glass needles with filament (1 mm; thin-wall glass capillaries with filament; World Precision Instruments, cat. no. TW100F-4)
- Wall Superthane ester-based tubing, 130 PSI at 70 °F (1/8-inch tubing; US Plastic, cat. no. 56402)
- 1/8-inch inner diameter (i.d.) × 1/4-inch outer diameter (o.d.) × 1/16 inches
- Falcon tubes (15 ml; ISC BioExpress, cat. no. C-3394-2)
- Plastic Petri dishes (60 × 15 mm; Becton Dickinson, cat. no. 351007)
- Dissecting microscope
- Electrode storage jar (World Precision Instruments, cat. no. E210)
- Glass micropipette puller (Sutter Instruments, cat. no. BF100-78-10)
- Glass Pasteur pipettes (Corning, cat. no. 7095B-5X)
- Fluorescent lamp and appropriate filters
- Microcentrifuge
- Micromanipulator apparatus (Narishige, 3D mechanical course manipulator, cat. no. M-152; three-axis joystick oil hydraulic micromanipulator, cat. no. MO-202U)
- Model PLI-OHN output hose (1-mm tubing; Harvard Apparatus, cat. no. 650010)
- Orbital shaker
- Picospritzer (Harvard Apparatus, cat. no. PLI-90) with external compressor
- Stainless steel pipette holder, 130 mm for 1–1.5-mm o.d. glass pipettes (needle holder; Harvard Apparatus, cat. no. 650013)
- Thermocycler

REAGENT SETUP

ASW, 1/3-strength (1/3× ASW) Combine 12 g of Instant Ocean with 900 ml of dH₂O. Mix thoroughly and adjust the volume to 1 liter with dH₂O. The resulting seawater should have a salinity of ~12 parts per thousand (p.p.t.). Store ASW indefinitely at 17–22 °C.

Cysteine dejelling solution (4% wt/vol cysteine) Dissolve 0.4 g of cysteine in 10 ml of 1/3× ASW and adjust the pH to 7.5–7.6 with 5 M NaOH. Freshly prepare the solution before every use.

Alexa Fluor dye stock solution, 100× Dissolve Alexa Fluor dye in nuclease-free water to a concentration of 20 mg ml⁻¹. Test the dye solution for RNase activity by incubating it with single-stranded RNA at 37 °C for 1 h. Assay for degradation on an agarose gel. Divide the solution into aliquots and store them at –20 °C for up to 2 years.

Agarose gel Prepare a 1% (wt/vol) agarose solution in TAE. Freshly prepare the gel before every use.

Sterile-filtered 1/3× ASW Filter 1/3× ASW through a 0.2-μm Nalgene filtration system according to the manufacturer's instructions.

Phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) Combine the entire contents of the equilibration buffer by following the manufacturer's instructions. Mix the buffer gently and allow the phases to separate for 2–4 h before use. Store the reagent for up to 1 year at 4 °C.

TAE stock solution, 50× (2 M Tris, 125 mM EDTA and 1 M glacial acetic acid, pH 8.5) Combine 242 g of Trizma base, 37.2 g of EDTA, 57.1 ml of glacial acetic acid and 700 ml of dH₂O. Mix and adjust the pH of the solution to 8.5 with 10 N NaOH and bring the volume to 1 liter with dH₂O. Dilute the solution to 1× as needed and store it indefinitely at room temperature (20–25 °C).

EQUIPMENT SETUP

Glass needles for injection The exact protocol will vary according to personal preference and the effect of local environment on the glass pipettes during pulling of needles for injection. The following is a starting point for input values based on using the glass pipette puller model indicated above: Heat = 545; P = 200; Pull = 90; Vel = 80 and Del = 80. See the manufacturer's manual and the 'Micropipette Cookbook' (http://www.sutter.com/contact/faqs/pipette_cookbook.pdf) for more details. In general, needles should be pulled as finely as possible to enable optimal microinjection. **Figure 1c** provides examples of three different needles that are all suitable for injecting *Nematostella* embryos. After pulling, store glass needles upright with the tips pointed down in an electrode storage jar.

Microinjection rig setup To perform microinjections under a dissecting scope, it is optimal to have the injection needle appear in the middle of the visual field of the microscope and to bring the cells to be injected to the tip of the needle. The oil-filled hydraulic joystick micromanipulator has a limited range that the researcher uses for the final impalement of the cell. The hydraulic manipulator is also attached to a coarse manipulator that is used to position the needle in the center of the field at the onset of each injection session. Take care not to damage the hydraulic oil-filled tubing that links the micromanipulator needle holder support to the joystick.

The glass injection pipette is held in a stainless steel holder that is mounted to the hydraulic manipulator (**Fig. 1a** and **Supplementary Fig. 1**). The stainless steel pipette holder is attached to the picospritzer by 1-mm tubing to the 'p-out' adaptor on the front of the instrument (provided by the manufacturer). The picospritzer is a device that uses gas (normally air) pressure to force fluid through the micropipette. The Harvard apparatus picospritzer requires an external air compressor that enters the instrument through the 'p-in' valve on the back of the instrument with non-expandable thick-walled 1/8-inch tubing (provided by the manufacturer).

The injection micropipette is placed in the open end of the holder, through the internal o-ring, and the end piece is tightened gently by hand. The stainless steel holder is then mounted on the hydraulic manipulator. The volume of material injected is controlled largely by the amount of pressure released and the duration of release on the picospritzer. Typical pressure values are 5–25 psi over times of 10–30 ms. Actuation of each injection event is generally performed using a foot pedal, allowing one hand free to position the dish of embryos at the tip of the injection pipette, and the other hand to operate the hydraulic joystick manipulator.



PROCEDURE

Preparation of mRNA or MOs for microinjection

1| A number of different molecules can be injected into *Nematostella* embryos. Here we provide details for preparing mRNA (option A) or MOs (option B) for microinjection.

(A) Preparation of mRNA for microinjection ● TIMING ~7–10 d

▲ **CRITICAL** The cDNA of interest must be subcloned into a vector suitable for *in vitro* transcription before beginning this protocol. The vector choice and subcloning strategy can be carried out by conventional recombinant DNA techniques that are not described here. We use the pCS2:GFP plasmid¹¹ and the Gateway pSPE3 system²⁹ for *in vitro* transcription vectors.

- (i) By using an appropriate restriction enzyme, linearize 5–10 µg of transcription vector containing coding sequence for the gene of interest.
- (ii) Carry out electrophoresis on 100–200 ng of DNA from restriction enzyme digestion on a 1% (wt/vol) agarose gel to verify complete digestion.
- (iii) Bring the volume of the remaining restriction-digested sample to 200 µl with nuclease-free water.
- (iv) Add 200 µl of phenol:chloroform:isoamyl alcohol to the restriction-digested sample.
- (v) Shake the sample vigorously for 15 s.
- (vi) Incubate the sample for 5 min at room temperature.
- (vii) Centrifuge the sample at 12,000g for 15 min at 4 °C.
- (viii) Transfer the aqueous phase to an RNase-free microcentrifuge tube.
- (ix) Add 200 µl of isopropanol and 0.5 µl of 20 mg ml⁻¹ glycogen to the tube. Mix the contents well.
- (x) Incubate the sample at room temperature for 10 min.
- (xi) Centrifuge the sample at 12,000g for 15 min at 4 °C.
- (xii) Wash the pellet with 1 ml of RNase-free 75% (vol/vol) ethanol. Vortex the pellet briefly.
 - **PAUSE POINT** The DNA precipitate can be stored for 1 week at 4 °C or for at least 1 year at –20 to –80 °C.
- (xiii) Centrifuge the DNA at 7,500g for 5 min at room temperature.
- (xiv) Remove the ethanol and either air-dry the pellet (~10 min) or use a SpeedVac to dry the pellet.
- (xv) Resuspend the pellet in 15 µl of nuclease-free water.
- (xvi) Measure the OD at 260 nm (OD₂₆₀) with a spectrophotometer for determining DNA concentration.
 - **PAUSE POINT** The precipitated DNA can be stored for 1 week at 4 °C or for at least 1 year at –20 to –80 °C.
- (xvii) Use up to 1 µg of the linearized DNA template (transcription kit can accommodate up to 6 µl volume of linear DNA) and set up the transcription reaction as outlined in the manufacturer’s instructions (e.g., Ambion). The final volume of the reaction mix is 20 µl.
- (xviii) Incubate the reaction mix at 37 °C for 2 h.
 - ▲ **CRITICAL STEP** Use a thermocycler to produce more consistent yields than 37 °C heat blocks or incubators.
- (xix) Add 1 µl of RNase-free DNase. Mix gently.
- (xx) Incubate the reaction mix at 37 °C for 15 min.
- (xxi) (Optional) Carry out electrophoresis of 0.5 µl of the reaction mix on a 1% (wt/vol) agarose gel to verify transcription, or proceed directly to mRNA purification (Step 1A(xxii)).
- (xxii) Bring the final volume of the transcription reaction to 100 µl with MegaClear elution buffer.
- (xxiii) Add 350 µl of MegaClear binding buffer concentrate and mix well by pipetting.
- (xxiv) Add 250 µl of RNase-free 100% ethanol and mix well by pipetting.
- (xxv) Add the mixture from Step 1A(xxii–xxiv) to the MegaClear column.
- (xxvi) Spin the column at 6,000g in a microcentrifuge for 1 min at room temperature.
- (xxvii) Discard the flow-through and wash the column with 500 µl of MegaClear wash solution.
- (xxviii) Spin the column at 6,000g in a microcentrifuge for 1 min at room temperature.
- (xxix) Repeat Step 1A(xxvii,xxviii).
- (xxx) Spin the column at 6,000g in a microcentrifuge for 1 min to remove residual wash buffer.
- (xxxi) Place the column in a new collection tube, and add 50 µl of MegaClear elution buffer (prewarmed to 90 °C) to the column.
- (xxxii) Spin the column at 6,000g in a microcentrifuge for 1 min at room temperature and leave the eluted mRNA in the tube.
- (xxxiii) Add another 50 µl of MegaClear elution buffer (prewarmed to 90 °C) to the column.
- (xxxiv) Spin the column at 6,000g in a microcentrifuge for 1 min at room temperature and retain the eluted mRNA.
- (xxxv) Add 100 µl of phenol:chloroform:isoamyl alcohol to the eluted mRNA from Step 1A(xxxiv).
- (xxxvi) Shake the mixture vigorously for 15 s.
 - ▲ **CRITICAL STEP** Do not vortex.



PROTOCOL

- (xxxvii) Incubate the tube at room temperature for 5 min.
- (xxxviii) Spin the sample at 12,000*g* in a microcentrifuge for 15 min at 4 °C.
- (xxxix) Remove the aqueous phase to a new RNase-free microcentrifuge tube.
 - (xl) Add 10 µl of 5 M ammonium acetate (provided in the MegaClear kit) and 275 µl of RNase-free 100% ethanol.
 - (xli) Incubate the sample at –20 °C for 1 h.
 - **PAUSE POINT** Incubation can be left at –20 °C overnight.
 - (xlii) Pellet the RNA by spinning it in a microcentrifuge at 12,000*g* for 15 min at 4 °C.
 - (xliii) Remove the supernatant. Wash the RNA pellet with 1 ml of ice-cold RNase-free 75% (vol/vol) ethanol. Vortex the RNA pellet briefly.
 - (xliv) Spin the column in a microcentrifuge at 12,000*g* for 5 min at 4 °C.
 - (xlv) Repeat Step 1A(xliii, xliv).
 - (xlvi) Remove the ethanol and allow the pellet to air-dry for ~10 min at room temperature.
 - ▲ **CRITICAL STEP** Do not dry the pellet completely or it will be difficult to resuspend.
 - (xlvii) Resuspend the pellet in 30 µl of nuclease-free water. Typical total yield is 30–40 µg of mRNA.
 - (xlviii) Perform electrophoresis of mRNA (1–5 µl) on a 1% (wt/vol) agarose gel in order to ensure that it is not degraded.
 - **PAUSE POINT** mRNA can be stored for up to a year at –80 °C.
 - (xlix) Prepare 15 µl of injection mix (3 µl of 5× Alexa Fluor, mRNA stock to desired concentration and nuclease-free water to a 15-µl total volume). The optimal concentration of mRNA to use can be determined as described in **Box 1**. Include the following injection controls: fluorescent dextran alone; mRNA coding an mRNA that should not affect the biological process of interest; and a co-injected translation-blocking MO that recognizes the mRNA of interest (Experimental design).

(B) Preparation of MO for microinjection ● **TIMING 1 h**

- (i) Resuspend MO in nuclease-free water to the desired concentration. We recommend preparing a 3 mM stock solution.
 - ▲ **CRITICAL STEP** Do not use DEPC-treated water because DEPC can interfere with MO activity. However, use nuclease-free water so that future co-injection mixes with mRNA can be made without concern for RNA degradation.
- (ii) Divide stock solutions of MOs into aliquots and store them at –20 °C.
 - ▲ **CRITICAL STEP** If stock MO solutions are stored frozen, they can precipitate out of solution. Therefore, before use, heat the solution at 65 °C for 5 min before injection.
- (iii) Prepare 15 µl of injection mix (3 µl of 5× Alexa Fluor, MO stock to the desired concentration and nuclease-free water to a 15-µl total volume). The optimal concentration of MO to use can be determined as described in **Box 1**. Include the following injection controls: fluorescent dextran alone; a control MO; and a co-injected MO and mRNA that represents the target gene of interest but that is not recognized by the MO (Experimental design).

Embryo preparation and microinjection ● **TIMING ~1–4 h**

- 2| Set up the adult anemones for spawning on the evening before the day of injection²⁶.
- 3| Fertilize the egg masses with sperm-containing water from male-only or mixed-sex bowls^{26,27,30}. Incubate the bowls at room temperature for 15 min.
- 4| Dejelly fertilized embryos at room temperature until the jelly is dissolved and embryos are released (usually 10–20 min in 10 ml 4% (wt/vol) cysteine in a 15-ml Falcon tube on a rocker)²⁷.
 - ▲ **CRITICAL STEP** Do not rock embryos too hard, as this will cause them to deform into an elongated shape. Medium setting on most orbital shakers works well.
- 5| Wash the embryos three times with 10 ml of sterile-filtered 1/3× ASW.
- 6| Transfer the embryos to a glass dish and maintain them in 1/3× ASW.
 - ▲ **CRITICAL STEP** Embryos must be maintained in glass. They will adhere to most plastics and will be difficult to transfer.
- 7| Pull the glass micropipette needles. Programs vary according to machines, preference pullers and so on (Equipment Setup).
- 8| Microcentrifuge the injection cocktail from Step 1A(xlix) or Step 1B(v) at 13,000*g* at 4 °C for 2 min to pellet debris that could clog the micropipette needle.

9| Load each needle with 0.5 μ l of injection cocktail.

▲ CRITICAL STEP Do not overload the needle with liquid. The cocktail will travel to the tip of the needle via capillary action in 1–3 min.

10| Scratch the bottom of a plastic Petri dish (60 \times 15 mm) with forceps to form a series of parallel scratches spaced \sim 0.5 cm apart (**Fig. 1b**).

▲ CRITICAL STEP Use the recommended Petri dishes to ensure suitable adherence of the embryos to the dish during injection.

11| Add enough filtered 1/3 \times ASW to cover the bottom of the scratched Petri dish but not too much to interfere with clear visibility during the injection protocol.

▲ CRITICAL STEP Add water immediately before adding embryos. Embryo adherence decreases if water rests in the dish before the addition of embryos.

12| By using a Pasteur pipette, transfer the desired number of embryos in single lines between and parallel to the scratches (**Fig. 1b**).

? TROUBLESHOOTING

13| Insert a pulled needle into the picospritzer needle holder.

14| Adjust the needle so that it is centered in the field of view through the oculars at lowest magnification.

▲ CRITICAL STEP Keep the needle at a steep ($\geq 45^\circ$) angle. This angle is ideal for the injecting process. Lower angles can result in the embryos being pushed around rather than being pierced by the needle.

15| Slowly lower the needle and locate the tip as it breaks the surface of the water.

16| Increase the magnification and lower the needle to the bottom of the dish.

17| Break the tip of the needle by using the micromanipulator to tap the needle against the bottom of the dish or against the side of the scratches. Set the balance pressure on the picospritzer such that the dye is barely flowing out of the tip of the needle.

? TROUBLESHOOTING

18| By using fluorescent dye as a tracer, adjust the pressure and time on the picospritzer so that a single pulse will fill \sim 3–5% of the embryo volume estimated by eye using the small sphere present immediately after a pulse is injected and before the tracer dye begins to diffuse. This is equivalent to \sim 10 pL. Estimating by eye is easier than measuring the volume, as adjustments often need to be made during injection to cope with issues such as decreased flow when the needle clogs slightly or increased flow when the tip breaks slightly during injection.

? TROUBLESHOOTING

19| By using the micromanipulator, insert the needle just inside the top surface of each embryo, and inject each embryo with a single pulse.

20| Inject a second dish of embryos with appropriate control(s).

21| Transfer the embryos to the desired incubation temperature, although it may be best to leave the embryos at cooler temperatures (17 $^\circ$ C) for 2–4 h before moving them to a warmer final temperature (typically 22 $^\circ$ C). Determine the success of the injection by determining whether injected animals are cleaving and are not dying, which can be indicated by loss of cell adhesion and the apparent disintegration of embryos.

22| Score for the phenotype of interest using an appropriate method.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

PROTOCOL

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Box 1, step 2	Embryos die even at low concentrations of mRNA	Residual phenol from RNA preparation is killing the embryos	Reprecipitate mRNA and perform extra washes with 70% (vol/vol) ethanol. Check RNA before resuspending in water to ensure there is no phenol odor
		Overexpression has an early lethal phenotype	Co-inject a translation-blocking MO that inhibits translation of your <i>in vitro</i> -transcribed mRNA
	Embryos die even at low concentrations of MO	MO has an early lethal phenotype	Co-inject an mRNA that codes for the MO target gene but is not targeted by the MO
	Protein expression is not detected	If you are using a fluorescent tag, cloning may have resulted in a reading frame shift	Sequence the clone to ensure that no reading frame shift has occurred, resulting in no fluorescent protein being produced
		If you are not using a fluorescent protein tag, your protein may have a reading frame shift or stop codon introduced by PCR and cloning	Sequence the clone used to generate mRNA to ensure there are no errors
	MO does not block translation or splicing as predicted	RNA is being degraded	Check all pipettes, tubes and dextrans for RNase activity. In addition, use a fresh package of glass needles
		MO concentrations are too low	Use higher concentration of MO, if animals can survive injection of a higher dose
Though predicted to work, MO does not bind the target sequence <i>in vivo</i>		Try additional MOs targeting the same gene. If you are using a translation-blocking MO it is possible to perform an <i>in vitro</i> translation assay or to co-inject the MO with mRNA fused to a fluorescence reporter to determine the effectiveness of the MO <i>in vitro</i>	
12	Embryos do not adhere to the dish	Filtered water is old	Filter fresh 1/3× ASW
		A different brand of Petri dish was used	Use the recommended brand of Petri dish or be sure that your dish is polystyrene
		Water is too warm	Anemones adhere better in cold water. Chill 1/3× ASW to 17 °C before adding it to the Petri dish
17	Needle tip will not break open	Needle is too thin and flexible	Adjust parameters of the needle puller to generate shorter, stubbier needles Use fine-tipped tweezers or a diamond pen to break the needle. Adjust the needle so the tip flexes and lies flat on the bottom of the dish. Break as close to the end as possible
		Needle is too stubby and stout	Adjust the parameters of needle puller to generate longer, thinner needles
18	Dye is flowing too slowly and is difficult to inject	Needle is clogged	Press 'Clear' on the picospritzer If the needle is thin enough, use fine-tipped tweezers to break the needle back further
		Dye is flowing too quickly out of the needle	Reduce the balance pressure on the picospritzer
		Needle opening is too large	Replace needle or adjust settings on needle puller

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
22	No phenotype is observed when mRNA is injected	The mRNA has a missense mutation in a codon encoding a critical residue	Sequence the clone to ensure there are no errors
	No phenotype is observed when MO is injected	The relative amount of knockdown is insufficient to produce a phenotype	Design additional MOs against the same target gene and co-inject multiple MOs to increase gene knockdown

● TIMING

Step 1A(i–xvi), preparing template for transcription reaction: ~7 d to subclone
 Step 1A(xvii–xxi), *in vitro* transcription reaction: ~2 h
 Step 1A(xxii–xlvi), mRNA purification: ~3 h
 Step 1A(xlix), preparing microinjection cocktails: ~1 h
 Step 1B(i,ii), preparation of MO: ~30 min
 Step 1B(iii), preparation of injection mix: ~30 min
 Steps 2–6, embryo preparation: ~1 h
 Steps 6–13, microinjection apparatus setup: ~5–15 min
 Steps 14–22, microinjection: ~1–2 h
Box 1, optimizing mRNA or MO concentration for microinjection: ~1 d

ANTICIPATED RESULTS

Microinjection

On average, 90% of embryos survive the injection procedure and appear normal at 24 h after injection (Fig. 4a). However, this is variable and can range from 85 to 95% (Fig. 4a). More importantly, the percentage of embryos from uninjected controls that survive the first 24 h is similar to that observed for animals injected with MO, mRNA or dextran (Fig. 4a), suggesting that injection alone does not markedly disrupt animal development. After the first 24 h, survival stabilizes and animals develop normally. On average, 90% of the uninjected controls present at 24 h form normal four-tentacle juvenile polyps by day 6. We observe similar numbers of development for dextran-, mRNA- or MO-injected animals (Fig. 4b), suggesting that major disruptions to development are not induced by the injection of dye alone, of control MO or of mRNA encoding GFP. Taken together, these data show that *Nematostella* development is robust and not substantially affected after the injection of control molecules.

mRNA injection

Typically, more than 95% of surviving injected embryos show protein expression after mRNA injection (Fig. 2g). Fluorescence of the expressed protein from *venus* mRNA is routinely detected ~2 h after injection and subcellular localization of the protein of interest can also be detected early (Fig. 2a–d). Fluorescence after mRNA injection can be observed at least 1 week after the injection (Supplementary Fig. 2). The overall stability of the mRNA and the translated protein are variable depending on the mechanisms acting to regulate each gene during development. For example, *NvashA:venus* (Fig. 2) expression can only be detected in about 50% of the animals at 48 h after

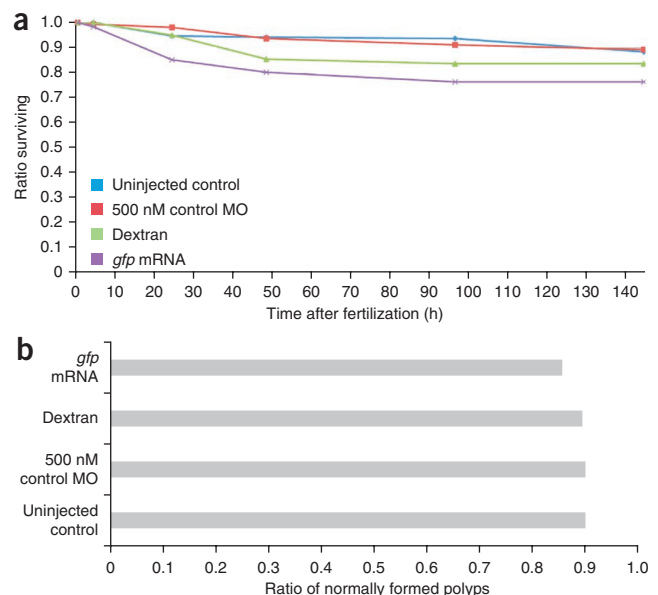


Figure 4 | Development after injection. (a) Survival curve of uninjected control (blue; *n* = 190), 500 nM control MO injected (red; *n* = 159), 200 ng μl^{-1} dextran (green; *n* = 221) and 300 ng μl^{-1} *gfp* mRNA (purple; *n* = 183). Animals were developed at 22 °C. (b) Ratio of surviving animals that had developed to the four-tentacle juvenile polyp stage by 144 h after fertilization. Control *n* = 168, control MO *n* = 142, dextran *n* = 185 and *gfp* mRNA *n* = 140.



fertilization in animals grown at 25 °C as observed on a dissecting scope, whereas injection of an unfused *gfp* coding sequence could still be detected 6 d after injection (**Supplementary Fig. 1**).

MO injection

As with mRNA injection, ~90% of the animals survive the injection protocol after MO injection. The benefit of MOs is that they are stable and do not degrade after injection. Reports have described morphant phenotypes in animals over 10 d old¹⁹. Quantification of the MO efficacy is crucial in some cases, as phenotypes are typically hypomorphic rather than the amorphic phenotypes associated with genetic null alleles. For example, with a splice-blocking MO, it is typical to be able to detect the presence of wild-type mRNA by RT-PCR (**Fig. 3f**; refs. 14,18,19).

Note: Supplementary information is available in the online version of the paper.

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