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Electroporation of short hairpin RNAs for rapid and efficient gene knockdown in the starlet sea anemone, *Nematostella vectensis*

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ABSTRACT

A mechanistic understanding of evolutionary developmental biology requires the development of novel techniques for the manipulation of gene function in phylogenetically diverse organismal systems. Recently, gene-specific knockdown by microinjection of short hairpin RNA (shRNA) was applied in the sea anemone *Nematostella vectensis*, demonstrating that the shRNA approach can be used for efficient and robust sequence-specific knockdown of a gene of interest. However, the time- and labor-intensive process of microinjection limits access to this technique and its application in large scale experiments. To address this issue, here we present an electroporation protocol for shRNA delivery into *Nematostella* eggs. This method leverages the speed and simplicity of electroporation, enabling users to manipulate gene expression in hundreds of eggs or embryos within minutes. We provide a detailed description of the experimental procedure, including reagents, electroporation conditions, preparation of *Nematostella* eggs, and follow-up care of experimental animals. Finally, we demonstrate the knockdown of several endogenous and exogenous genes with known phenotypes and discuss the potential applications of this method.

1. Introduction

The starlet sea anemone, *Nematostella vectensis*, is an established cnidarian model organism for in vivo studies of developmental processes (Layden et al., 2016). *Nematostella* has several unique advantages to study evolutionarily conserved pathways that regulate gene function in metazoans (Genikhovich and Technau, 2009c). First, *Nematostella* is a broadcast spawner, allowing massive numbers of gametes to be collected in a single spawn. Second, *Nematostella* features relatively rapid embryonic and larval development, and the embryos are easy to visualize and manipulate. Third, the *Nematostella* genome has been sequenced, with transcriptomic profiles available for a variety of developmental stages and tissues (Putnam et al., 2007). Last but not least, comparative genomic analysis indicates that despite a simple adult body plan, a large number of bilaterian genes and signaling pathways are conserved in *Nematostella*. (Genikhovich and Technau, 2009c). For example, *Nematostella* possesses the majority of known bilaterian signaling cascades including the Wnt (Guder et al., 2006), Notch (Fritz et al., 2013; Marlow et al., 2012), Bone Morphogenetic Protein (BMP) (Saina et al., 2009), Fibroblast Growth Factor (FGF) (Matus et al., 2007; Rentzsch et al., 2008), and Hedgehog pathways (Matus et al., 2008). Furthermore, a high percentage of introns and the overall intron-exon structure of human genes are also relatively conserved in *Nematostella* (Sullivan et al., 2006; Zimek and Weber, 2008). For these and additional reasons, *Nematostella* is an outstanding model organism for comparative studies of cellular, developmental and evolutionary biology.

Since the early 2000s, *Nematostella* researchers have developed several protocols including maintenance and spawning procedures (Genikhovich and Technau, 2009b), isolation of protein and nucleic acids (Stefanik et al., 2013), in situ hybridization (Genikhovich and Technau, 2009a), microinjection and morpholino-based gene knockdown (Layden et al., 2013; Wolenski et al., 2013), meganuclease-mediated transgenesis (Renfer and Technau, 2017) and CRISPR/Cas9-based gene editing (Ikmi et al., 2014).

The biological activity of miRNAs (Moran et al., 2014) and evidence for siRNA-mediated silencing of a target gene of interest in *Nematostella* was investigated previously (Moran et al., 2014; Pankow and Bamberger, 2007). Recently, gene-specific knockdown by microinjection of short hairpin RNA (shRNA) was reported in *Nematostella* (He et al., 2018). This method allows robust and cost-effective gene silencing, but the reliance on microinjection introduces experimental limitations, particularly the number of eggs that can be injected and later recovered. In addition, the precision control of

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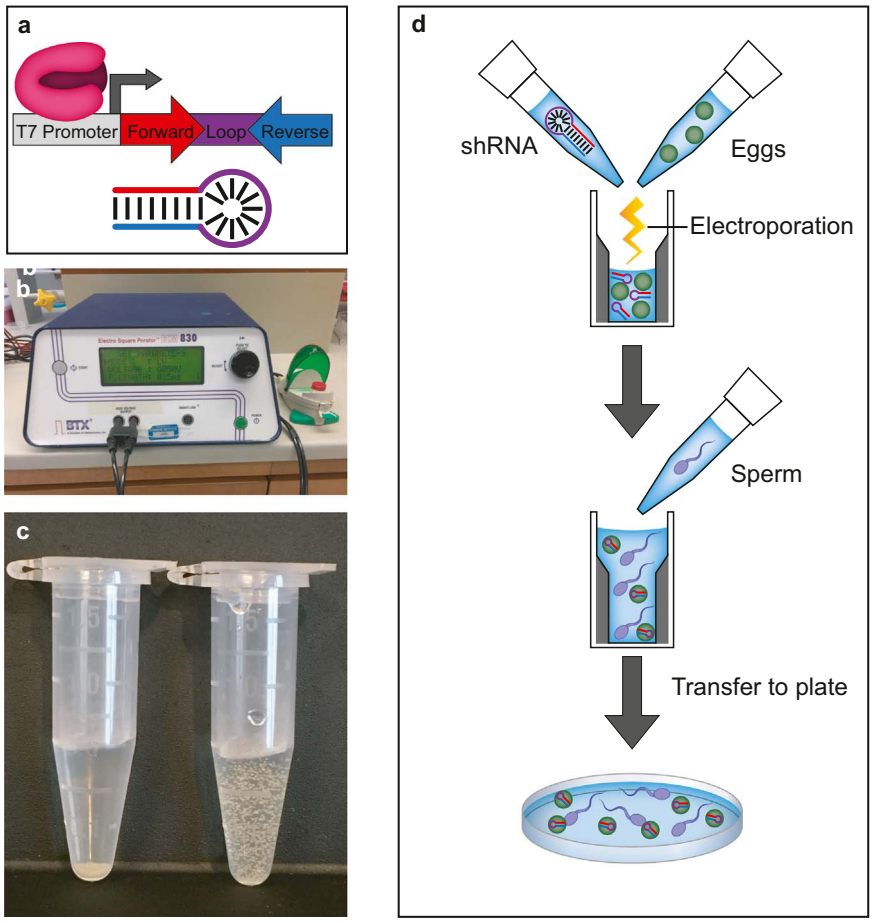

Fig. 1. Procedure for electroporation of shRNA into *Nematostella* eggs. **(a)** in vitro transcription using T7 RNA Polymerase and the template. **(b)** EMM 830 square wave electroporator (BTX instruments) with attached Gene Pulser Xcell ShockPod Cuvette Chamber (Bio-rad) and a 4 mm Cuvette (Mirus). LCD display indicates Electroporation settings (50 V, 25 ms, single pulse). **(c)** Suspension of eggs using 15% Ficoll PM 400 in 12 p.p.t salt water. Left: Eggs in 12 p.p.t salt water. Right: Eggs suspended in 12 p.p.t. salt water with 15% Ficoll PM400. **(d)** Illustration of the electroporation procedure.

injection volume is difficult. Thus, a more efficient method for shRNA delivery would be ideal for medium-and high-throughput applications. As an alternative to microinjection, electroporation has been utilized for biomacromolecule delivery into *Hydra* (Brennecke et al., 1998) and *Ciona (*Kari et al., 2016*;* Vierra and Irvine, 2012*).*

Here, we report an optimized method that combines the advantages of electroporation with the specificity of the shRNA-based gene knockdown approach in *Nematostella*. Unlike microinjection, this electroporation protocol can be performed by novice users, requires minimal effort, and can quickly deliver shRNA to a large number of eggs simultaneously. This method should be applicable in various aspects of *Nematostella* biology as well as in related cnidarian species where eggs are easily accessible.

2. Experimental design

2.1. Synthesis and preparation of shRNA

The shRNA-mediated knockdown method is based on in vitro transcription (IVT) of shRNA from a template DNA using T7 RNA Polymerase. An illustration of the IVT reaction is provided in (Fig. 1**a**). We used a 19 bp gene targeting motif size, which was found to be selective and cost-effective for gene knockdown. DNA templates for IVT were assembled by direct annealing of complimentary forward and reverse oligos. Primers were mixed 1:1 to a final concentration of 50 μM per primer and denatured at 98 °C for 5 min and cooled to 24 °C at the maximum ramp rate (5 °C/s). The assembled duplex was kept at room temperature for immediate use as the template for IVT. We typically allowed transcription reactions to continue for 5–7 h. DNase was then added to remove the template, and the reaction mix was purified using the Direct-zol™ RNA MiniPrep Plus. A single IVT reaction using this kit typically yielded 2–3 μg/μl purified shRNA in a 30μl elution volume as assessed by a spectrophotometer (we considered purity adequate at a 260/280 ratio above 1.80). In general, we have observed that a lower purity is not toxic to eggs when used for electroporation. Considering the typical yield, a researcher could potentially bypass the purification step and use the IVT product directly for electroporation if the aim is a large phenotypic screen. The shRNA templates used in this study were listed in Table 1.

2.2. Electroporation of shRNA

Electroporation uses pulses of electricity to temporarily form pores throughout the plasma membrane. During this temporal window, shRNA can be efficiently delivered into the egg. To exploit this, we used a standard laboratory electroporation setup to knock down genes of interest using shRNA. Electroporation efficiency depends on the parameters of voltage, pulse duration, and number of pulses. We therefore performed multivariate experiments to determine electroporation efficiency by evaluating embryo survival and subsequent knockdown penetrance for the conserved Wnt pathway component *Nvβ-Catenin* (Supplemental Table 1). Optimal parameters were determined to be 50 V delivered in a single 25 millisecond pulse in 4 mm cuvettes (Mirus Bio) using an Electroporator ECM830 (Genentronics Inc.; Fig. 1**b**). These conditions efficiently delivered shRNA into eggs without significant loss of viability (Table 2).

Table 1
Template sequences used for shRNA synthesis. The DNA Oligo Template-F sequence indicates the positions of the 5' T7 promoter (**black**), 19 nucleotide motif (underlined), a linker loop (blue), corresponding anti-sense motif (green) and two thymidine bases at 3'.

DNA Oligo Template	TAATACGACTCACTATA**GCAACACGCAGAGTCGTAA**TTCAAGAGA TTACGACTCTGCGTGTTGCTT
Control-F	TAATACGACTCACTATAGCAACACGCAGAGTCGTAATTCAAGAGAT TACGACTCTGCGTGTTGCTT
Control-R	AAGCAACACGCAGAGTCGTAATCTCTTGAATTACGACTCTGCGTG TTGCTATAGTGAGTCGTATTA
EGFP-F	TAATACGACTCACTATAGACGTAAACGGCCACAAGTTTCAAGAGAA CTTGTGGCCGTTTACGTCTT
EGFP-R	AAGACGTAAACGGCCACAAGTTCTCTTGAAACTTGTGGCCGTTTA CGTCTATAGTGAGTCGTATTA
NvBMP5/8-F	TAATACGACTCACTATAGGTAGACGCCCAAAGATCTTTCAAGAGAA GATCTTTGGGCGTCTACCTT
NvBMP5/8-R	AAGGTAGACGCCCAAAGATCTTCTCTTGAAAGATCTTTGGGCGTC TACCTATAGTGAGTCGTATTA
NvChordin-F	TAATACGACTCACTATAGGGTTACCGCCACAGAATTTTCAAGAGAA ATTCTGTGGCGGTAACCCTT
NvChordin-R	AAGGGTTACCGCCACAGAATTTCTCTTGAAAATTCTGTGGCGGTAA CCCTATAGTGAGTCGTATTA
Nvβcatenin-F	TAATACGACTCACTATAGTGGCACCAAACGTATCATTTCAAGAGAA TGATACGTTTGGTGCCACTT
Nvβcatenin-R	AAGTGGCACCAAACGTATCATTCTCTTGAAATGATACGTTTGGTGC CACTATAGTGAGTCGTATTA
NvAnthox1a-F	TAATACGACTCACTATAGGTCTGACGACGAATGTGATTCAAGAGAT CACATTCGTCGTCAGACCTT
NvAnthox1a-R	AAGGTCTGACGACGAATGTGATCTCTTGAATCACATTCGTCGTCA GACCTATAGTGAGTCGTATTA

Nematostella eggs have a higher density than sea water and tend to settle at the bottom of electroporation cuvettes, potentially resulting in non-uniform electroporation. To solve this problem, we suspended eggs in 15% Ficoll PM 400 in artificial sea water (12 p.p.t) for electroporation (Fig. 1**c**). shRNA was then added to the cuvette and mixed by gentle shaking for 10 s. Following these steps, eggs should be evenly suspended in the cuvette. These settings provided efficient shRNA delivery and gene knockdown with an acceptable amount of egg loss (15–20%). The overall workflow is illustrated in (Fig. 1**d**). Factors that might affect egg recovery include the number of unfertilized eggs per cuvette, the age of the eggs (hours post-spawn), and the care given to post-electroporation handling. In our experience, eggs from a fresh spawn are necessary for optimal survival. After electroporation, eggs tend to display abnormal morphologies and frequently exhibit a single protrusion on one side. These typically recover but are fragile and should be immediately transferred to a petri dish, fertilized, and incubated at low density without disruption. At higher densities eggs may fuse inside the cuvette and hence the number of eggs (ideally around 300–600 per cuvette) should be monitored. Electroporation with high numbers of eggs in the cuvette also resulted in reduction of

efficiency in addition to embryo fusion. The upper limit without significant loss of efficiency was approximately 1000 eggs per cuvette. At a maximum, we electroporated 1702 eggs with *Nvβ–catenin* shRNA and observed 89% phenotypic penetrance. This level is slightly lower than the 95–99% penetrance achieved with 300–600 eggs per cuvette. *Nvβ–catenin* shRNA produces a very strong loss-of-function phenotype that emerges during early embryonic development. Thus, penetrance levels might be significantly lower with other targets. Notably, embryo survival was comparable to that achieved with lower number of eggs.

Nematostella is an estuarian organism adapted to variable environmental conditions and the eggs can tolerate the dilution of seawater for short periods of time. We tested up to a 1:4 dilution of the egg suspension by adding different volumes of distilled water to a fixed shRNA concentration (up to a final salinity of 3 p.p.t.). Knockdown effects were similar in these conditions and the final salinity did not significantly impact the results within this range. We also found that electroporation can be performed with fertilized eggs, which do not fuse in the cuvette. Nevertheless, we recommend using unfertilized eggs for electroporation as this approach provides more flexibility in the timing of fertilization and enables the use of sperm from different male lines.

3. Validation and results

Following electroporation, the extent of target gene knockdown should depend on the shRNA dose. To test this, we electroporated either *EGFP* shRNA or a scrambled shRNA control into *Actin-EGFP* transgenic animals and analyzed EGFP protein levels by fluorescence microscopy (Fig. 2**a-i**). In this case, the optimal EGFP shRNA concentration was approximately 300 ng/μl, as higher concentrations did not produce a stronger knockdown effect (Fig. 2**f**). However, based on our experience with multiple experiments, the optimal shRNA concentration is gene-dependent. In general, *Nematostella* eggs can tolerate high concentrations of shRNA. Here, we electroporated up to 500 ng/μl shRNA and did not observe any obvious toxicity (Fig. 2**j-r**). In other experiments, concentrations as high as 900 ng/μl were used without any obvious effects on embryo survival (**e.g.** Fig. 6). To analyze the level of gene-specific knockdown at varying doses of shRNA, we next electroporated 100, 200 and 400 ng/μl *EGFP* shRNA into *Actin-EGFP* transgenic animals. Fluorescent EGFP signal was visibly reduced at all three concentrations (Fig. 3**a-d**). In each case some fully EGFP positive embryos were recovered, an effect that may be due to a variable position of eggs within the cuvette (Fig. 3**e**). Hence careful mixing of eggs and shRNA within the cuvette may improve the efficiency of gene knockdown. To quantify the results at a molecular level, we analyzed relative EGFP mRNA levels by RT-qPCR. In keeping with the image analysis, we observed a marked reduction in target mRNA levels at all three shRNA concentrations (100, 200, and 400 ng/μl; Fig. 3**f**).

In the experiments described in Figs. 2 and 3, we observed a slight variability of electroporation efficiency for shRNA doses below the threshold concentration where EGFP is completely knocked down. We further assessed variability in the experimental procedure by electroporating incremental concentrations of *EGFP* shRNA (0, 50, 100, 200, 300, 400 ng/μl) into *Actin-EGFP* transgenic eggs (Supplementary Fig. 1). We again found that knockdown efficiency varied at sub-threshold concentrations of shRNA (below 200 ng/μl). However, this variation was minimal in replicates performed above the threshold concentration (Fig. 3 and Supplementary Fig. 1). In general, phenotypic penetrance was not significantly different in experiments conducted on the same day using the same shRNA preparation. Conceivably, numerous parameters including the quality of shRNA, egg quality, and pipette handing errors could introduce variability between experiments. Since the threshold dose can vary slightly, we recommend that a dose-response curve followed by shRNA doses over threshold should be used to ensure reproducibility.

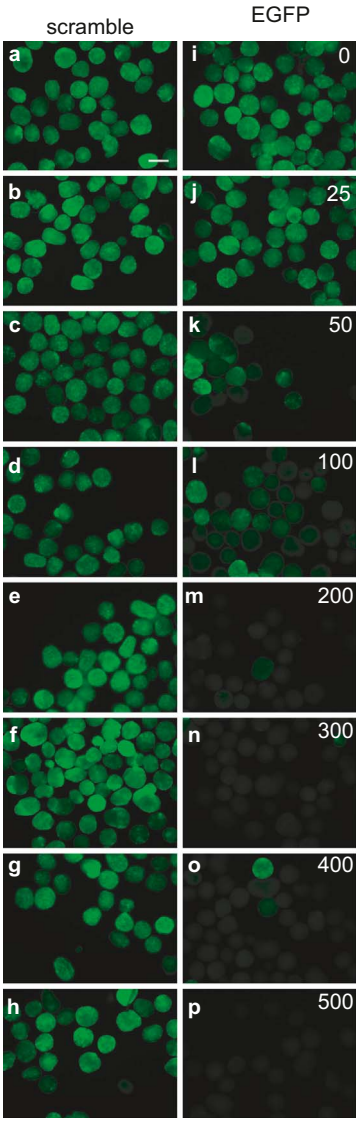

Fig. 2. Typical dose response results of varying concentrations of either EGFP shRNA or scramble control shRNA after electroporation into *Actin-EGFP* transgenic animals. Images were acquired 72 h after electroporation. **(a-h)** Incremental doses of control shRNA (ng/μl). (i-p) Corresponding incremental doses of EGFP shRNA. (Scale Bar = 200 μm).

In addition to knockdown of an exogenous GFP reporter gene, we also used electroporation to deliver shRNAs targeting four endogenous loci (*Nvβ–catenin*, *NvChordin*, *NvBMP5/8* and *NvAnthox1a*). Electroporation of shRNA targeting *Nvβ-catenin* blocked gastrulation and disrupted cell-cell adhesion by approximately 20 h post-fertilization (hpf) with 99% phenotypic penetrance (Fig. 4**b**). This finding is similar but more severe than the published morpholino phenotypes (Leclere and Rentzsch, 2014). In addition, electroporation of shRNAs against *NvBMP5/8* and *NvChordin* resulted in animals with highly elongated body columns and produced loss-of-function phenotypes at 52% and 78% penetrance, respectively (Fig. 5**d & f**). Confirming our approach, animals electroporated with NvBMP5/8 shRNA phenocopied a previously reported morpholino effect. Intriguingly, the similar *NvChordin* knockdown phenotype was not previously reported at this stage of development; further studies will be needed to understand its role in BMP signaling pathway at this stage of development.

Also in keeping with previous data, electroporation of shRNA targeting the *Nematostella* Hox gene *NvAnthox1a* phenocopied an expected tentacle fusion and bifurcation phenotype with 64% penetrance (Fig. 6**d**). In a previous study, microinjection of *NvAnthox1a*

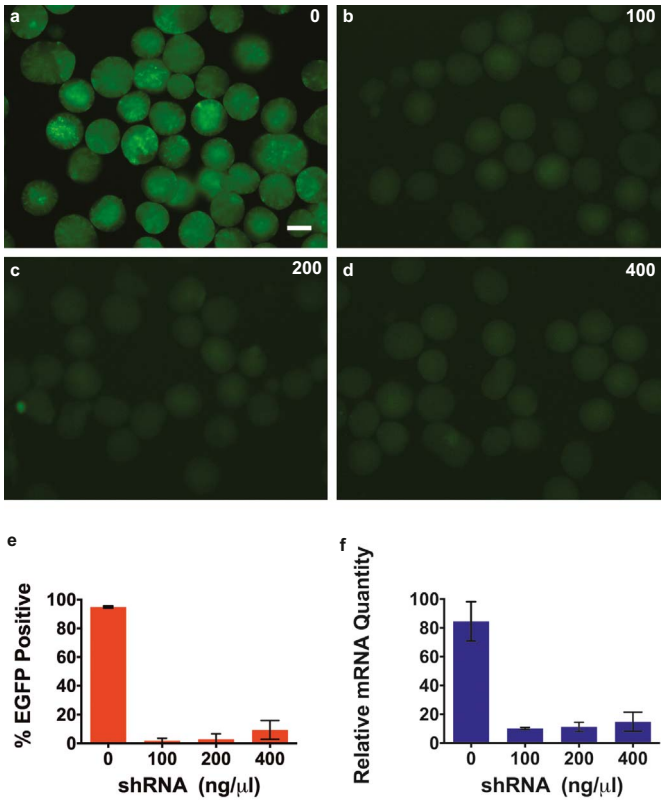

Fig. 3. Quantification of gene expression levels and percentage of embryos positive for each dose after electroporation into *Actin-EGFP* transgenic animals. **(a)** 0 ng/µl, **(b)**100 ng/µl **(c)** 200 ng/µl **(d)** 400 ng/µl indicate the doses of EGFP shRNA. Images were acquired 48 h after electroporation. **(e)** Percentage of EGFP positive embryos and **(f)** corresponding mRNA levels after electroporation. (Scale bar = 250 µm).

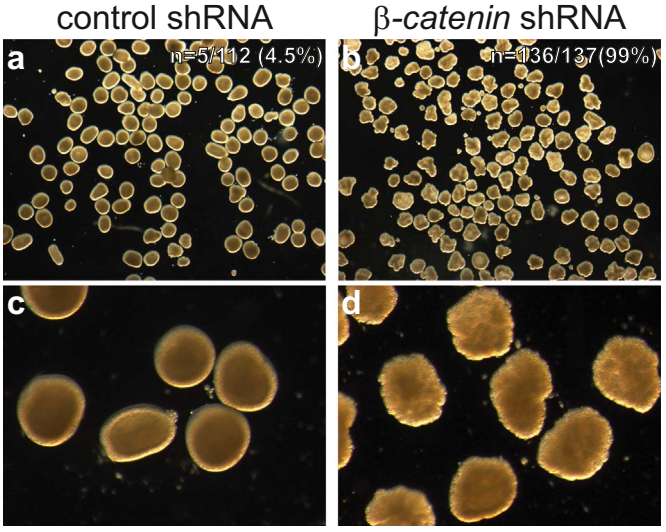

Fig. 4. Phenotypes of **(a)** control shRNA (300 ng/µl) and **(b)** shRNA targeting *β-catenin* (300 ng/µl) after electroporation. Close up images of **(c)** control shRNA **(d)** *β-catenin* shRNA animals. Images were acquired 72 h after electroporation.

shRNA produced this phenotype at 84.6% penetrance, higher than achieved by electroporation using the same shRNA sequence (He et al., 2018). The increased penetrance observed with microinjection could be explained by the selective enrichment for shRNA-injected embryos, which are typically labeled with tracer dyes. Since electroporation does not allow for enrichment of affected embryos, it is not possible to exclude animals that received sub-threshold levels of shRNA.

Combined, our results indicate that electroporation-mediated

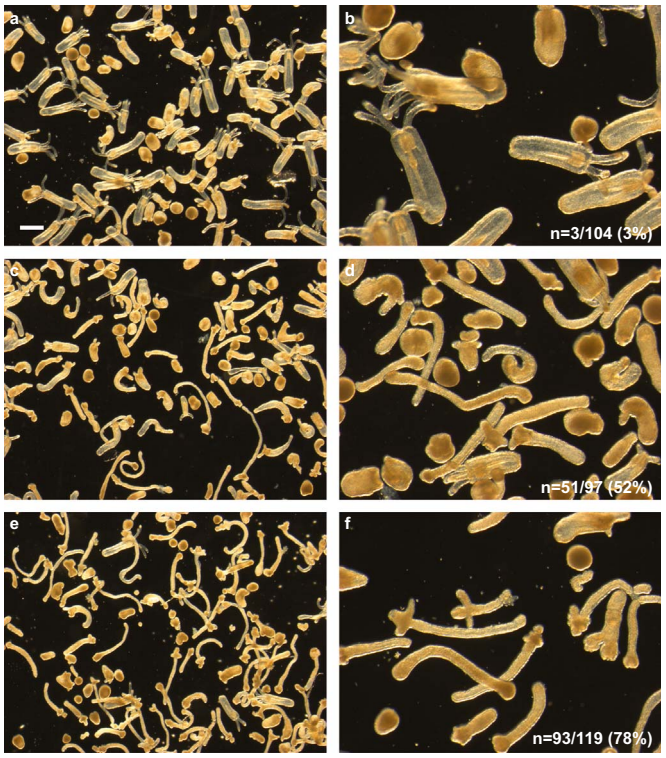

Fig. 5. Phenotypes of **(a)** Scrambled control shRNA (300 ng/µl) and **(b)** shRNA scramble control at high magnification. **(c)** shRNA BMP5/8 (300 ng/µl) and **(d)** shRNA BMP5/8 at high magnification, **(e)** shRNA chordin (300 ng/µl) and **(f)** shRNA chordin animals at high magnification. Numbers indicate counts and percentage of knockdown versus wildtype phenotypes in images (a), (c) and (e) respectively. Images were acquired 6 days after electroporation. (Scale Bar = 250 µm).

shRNA knockdown is a specific and potent tool for rapid analysis of gene function in *Nematostella*. While high doses of shRNA are tolerated (500–900 ng/µl), knockdown-dependent lethal phenotypes are frequently observed at much lower doses. To determine the phenotype for an unknown gene, we recommend a dose response, starting with a high concentration i.e. 500 ng/µl and a lower concentration, i.e. 200 ng/µl. Above, we tested up to 900 ng/µl shRNA without any observed toxicity (Fig. 6), suggesting that a broad range of shRNA concentrations can be used to knock down a target of interest. We further recommend that a scrambled shRNA as well as shRNAs that

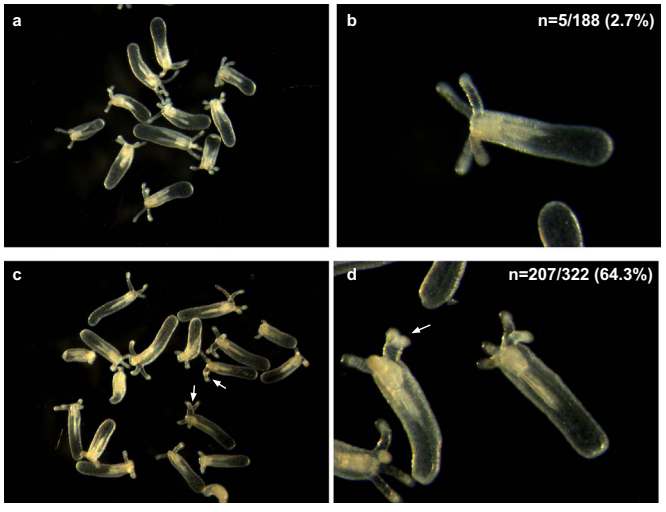

Fig. 6. Phenotypes of **(a)** Scramble shRNA (900 ng/µl) and **(b)** Scramble shRNA at high magnification. **(c)** *NvAnthox1* shRNA (900 ng/µl) and **(d)** *NvAnthox1a* shRNA at high magnification. Images were acquired 9 days after electroporation.

produce known phenotypes be used as negative and positive controls, respectively. As presented here, *Nvβ-catenin*, *NvBMP5/8* and *NvChordin* shRNAs all produce obvious phenotypes and can be used to validate both electroporation and knockdown efficiency. Additionally, non-electroporated controls should be included to evaluate general fertilization efficiency and viability for each experiment. Importantly, the distinct phenotypes of *NvChordin*, *NvBMP5/8*, *Nvβ-catenin* can be used as positive controls for phenotypic analysis at different time points. For example, knockdown of Nv*β-catenin* can be used as a positive control for early-expressed genes at 24hpf, whereas *NvBMP5/8*, *NvChordin* and *NvAnthox1a* can be used as a positive control for later phenotypes that manifest in the range of 5–7 days post-fertilization.

4. Conclusion

Electroporation is an efficient and scalable method to deliver shRNA into *Nematostella* eggs which should be applicable in diverse cnidarian species. This procedure is reproducible and should provide a similar knockdown efficiency as microinjection in a significantly reduced timeframe. We recommend 500 ng/μl and 200 ng/μl as starting shRNA concentrations. In our experience, shRNA should efficiently knock down a gene of interest at 500 ng/μl.

5. Protocol

5.1. Reagents

5.1.1. For shRNA synthesis and purification

- Forward and reverse primers for template annealing.
- AmpliScribe™ T7-*Flash*™ transcription kit (Lucigen, Inc).
- Direct-zol™ RNA MiniPrep Plus (Zymo Research, R2070).
- Tri-Reagent (Ambion, Inc).
- Nuclease-free water.
- Oligonucleotides (Integrated DNA Technologies).

5.2. Equipment

5.2.1. For shRNA synthesis and purification

- Thermal Cycler (Bio-rad DNA engine) or equivalent.
- Benchtop Centrifuge (Eppendorf, 5430R) or equivalent.
- Microcentrifuge tubes (Denville, Cat. C2170).

5.2.2. For egg preparation

- Nutator (Clay Adams).
- Conical bottom tubes (15 ml; Grenier Bio-One, Cat. 188261).
- Disposable Graduated Transfer Pipettes, (VWR, Cat. No. 414004–014).
- Crystallizing Dishes, (Kimax Kimble, Cat. No. 23000).
- L-Cysteine (Sigma, 168149).

5.2.3. For electroporation

- ECM 830 Electro Squire Porator (BTX™, Cat. No 450052).
- Electroporation Cuvettes (4 mm; Mirus, Cat. No. MIR50123).
- Gene Pulser Xcell ShockPod Cuvette Chamber (Bio-rad, Cat. No. 1652669).
- Laboratory wash bottle (500 ml; VWR 47750–710).
- Conical tubes (15 ml; Grenier Bio-One, cat. no. 188261).
- Microcentrifuge tubes (Denville, Cat. No. C2110).
- Plastic petri dishes (60 mm X 15 mm) (Falcon,351007).
- Disposable Graduated Transfer Pipets (VWR North America, Cat. No. 414004–014).
- Microcentrifuge transfer pipet (RPI; Cat. No. 147500).
- Single edge razor (VWR, Cat. No. 55411–050).

5.2.4. For image acquisition

5.2.4.1. Inverted fluorescence microscope. Images were acquired on a Zeiss Axiovert 200 M with Fluar 5 × 0.25NA equipped with Micro-Manager1. Image processing was done in Fiji. Individual embryos were segmented using custom thresholding, separated using watershed and mean intensity quantified. Processing macros and plugins can be found at https://github.com/jouyun/ (Edelstein et al., 2014). Brightfield microscope images were acquired in a Leica MZ16 Stereomicroscope. Embryos were counted and distinguished by using thresholding, segmentation using watershed and particle analysis in Fiji.

5.2.4.2. For RT-qPCR. We used the QuantStudio(TM) 7 Flex System (Thermo Fisher). Promega ImProm-II™ Reverse Transcriptase (cat. no. A3801) was used for RT reactions.

https://www.promega.com/products/pcr/rt-pcr/improm_ii-reverse-transcriptase/?catNum=A3801

qPCR was performed with NEB Luna® Universal qPCR Master Mix (M3003) at10μl reaction volume. The cycles were according to the supplier's manual. https://www.neb.com/products/m3003-luna-universal-qpcr-master-mix#Product%20Information

The following Primers were used for the qPCR reaction:

RT-qPCR-GFP-F	AGAACGGCATCAAGGTGAAC
RT-qPCR-GFP-R	TGCTCAGGTAGTGGTTGTCG
GAPDH-F	GGACCAAGTGCCAAGAACTG
GAPDH-R	GGAATGCCATACCCGTCAG
EF1α-qPCR-F	GGGTCTCGATTCTATCCTACCT
EF1α-qPCR-R	CACTGTTCCAATACCTCCAATC

5.3. Reagent preparation

5.3.1. Nematostella artificial saltwater (ASW), 12 ppt

Dissolve 1.2 g of artificial sea salt (Instant Ocean) into deionized water to a final volume of 1 L. ASW can be stored at room temperature for approximately one month.

5.3.2. Egg dejellying solution

Dissolve 1 g of L-Cysteine hydrochloride into 25 ml ASW. Add 5 drops of 5 M NaOH to adjust the pH and place on a rocker until L-Cysteine is dissolved. The solution must be freshly prepared and used immediately before de-jellying.

5.3.3. Egg suspension medium:15% (wt/vol) Ficoll PM400

In a 50 ml Falcon tube, dissolve 3 g Ficoll PM400 in 15 ml ASW. Vortex for 2 min and shake in a rotary shaker for 30 min. Adjust the volume to 20 ml by adding ASW. Vortex for 2 min, let the tube settle. Solution will initially look cloudy but will gradually clear within ten minutes.

Note: Ficoll dissolves in ASW slowly at room temperature. When dissolved, the solution should be clear and slightly viscous. If Ficoll doesn't dissolve well, incubate the Ficoll in ASW for 20 min at 50 °C and vortex every 5 min during incubation.

Table 2
Possible problems and solutions.

Step	Problem	Reason	Solution
16	Precipitate observed during IVT reaction assembly.	The IVT reagents were not warm enough.	Incubate the reaction mix before enzyme addition at 37 °C for 10 min, until the solution is clear, then add T7 RNA Polymerase.
22	Very low shRNA yield.	IVT mix precipitated after enzyme addition.	Inspect the IVT reaction mix for precipitation. If precipitation is observed, after enzyme addition, heat the IVT mix at 37 °C for 10 min. If there is precipitation, discard the tube and make a new reaction mix.
32	Rapid Resettlement of Eggs suspended in ASW 15% Ficoll	Excess water is left in the tube before ASW – 15% Ficoll addition.	Resuspend by gentle tapping before transfer to the cuvette
42	Very Low Fertilization Rate	Reduced sperm quality. Poor Contact of Sperm with Egg.	Add fresh sperm when possible. Keep sperm at 18 °C until use. Gently swirl the plate to mix sperm with electroporated eggs
44	Embryos and primary polyps are fused and look unhealthy.	Embryos clumped at the center of the plate.	Disperse eggs carefully and avoid crowding at the center of the petri dish after electroporation. Move slowly while carrying the plates to a different location.
47	Very low numbers of embryos with known phenotype.	shRNA is not mixed well. Low quality shRNA. shRNA concentration is low.	Check the quality of shRNA by running an aliquot of the IVT product in an agarose gel. Thoroughly mix shRNA and egg suspension by gentle shaking the cuvette for 30 s. If phenotype is present, electroporate a higher concentration of shRNA.
47	Unexpectedly high number of normal embryos in positive control dishes.	Pipetting error. Eggs stick to the non-conductive regions of the cuvette during embryo and shRNA transfer.	Wipe the cuvettes with Kim Wipes before transferring eggs to the cuvette.
		If Sperm source is a mixed-sex dish, it might be contaminated with eggs	If the sperm is originated from a mixed dish, inspect sperm dish for egg contamination before use.

5.4. Equipment setup

5.4.1. Electroporation

1. Connect the Gene Pulser Xcell ShockPod Cuvette Chamber (Bio-rad) cable.
2. Turn on the ECM830 electroporator. Wait for the device to boot and push the knob to select the parameters (i.e. volts, pulse duration, number of pulses and pulse interval). Adjust settings as follows: 50 V, 25 ms, 1 pulse. The device should be set ready for electroporation. Most comparable laboratory electroporators should be sufficient to achieve these conditions in a 4 mm gap cuvette.

5.4.2. Cleaning and re-use of electroporation cuvettes

- 4 mm cuvettes can be reused at least 10 times if washed and dried thoroughly after each electroporation. Clean the cuvettes with de-ionized water followed by a second wash with 100% ethanol. After the ethanol washes, dry at room temperature or 37 °C. Avoid using detergents to wash the cuvette. Cuvettes can be kept at room temperature indefinitely.

6. Detailed procedure

6.1. shRNA design TIMING (1–3 h)

1. Retrieve cDNA sequences of the target genes from Joint Genome Institute's *Nematostella* genome portal. (https://genome.jgi.doe.gov/pages/blast-query.jsf?db=Nemve1)
2. Open shWizard software. http://www.invivogen.com/sirnawizard/index.php
3. Set motif size to 19 nucleotides and paste cDNA sequence of interest.
4. Click search button, several candidate target sequences will be generated.
5. Select two or three motifs.
6. Click design button and retrieve the sense and anti-sense sequences. **See** Table 1**.**

   Note: Motifs must have reasonable complexity, a CG content between 40% and 55% and must have a relatively low CG content at the 3′ end. Total length of the template is 66 bases.
7. BLAST candidate sequences to JGI website for the *Nematostella* Genome database. Eliminate sequences with multiple hits.
8. In the program settings box, set the “expect” box to 1.02E-2 for short sequences.
9. Copy sequences with a single match in the *Nematostella* genome.
10. Design the IVT template by inserting the motifs according to the highlighted positions indicated in Table 1**, Row 1.**

Note: It is essential that the final DNA template is 66 base pairs long with the T7 promoter at the 5′ end, which is necessary for IVT. The final DNA template length must be 66 base pairs to avoid misfolding of the synthesized shRNA. Use mfold software from the University of Albany (http://unafold.rna.albany.edu/?q=mfold) to analyze the secondary structure of the shRNA to ensure that there is only one conformer. (Rouillard et al., 2003)

6.2. shRNA synthesis and production TIMING (8 h)

11. To construct the IVT template, first order forward and reverse primers as described in Step 10.

    Note: The final template should have 19 base sense and anti-sense targeting sequences as described in (Table 1**, Row 1).**
12. Dissolve the primers in nuclease free water to a final concentration of 50 μM.
13. In a PCR reaction tube, mix 5μl of forward and reverse primers to a final volume of 10 μl.

    Note: Mix with caution. Avoid mixing slightly higher or lower volumes of each primer as this changes the final duplex concentration for the IVT reaction.
14. In a thermal cycler, denature the primer mix at 98 °C for 5 min and immediately cool down to 24 °C (5 °C/s) to anneal the forward and reverse primers.
15. Use Ampliscribe™ T7-Flash™ Transcription Kit for the following steps with modifications of the protocol.
16. Prepare the IVT reaction mix at room temperature. For a 20 μl reaction:

Nuclease Free Water	4.5μl
10X T7 Reaction Buffer	2μl
DTT	2μl
ATP	2μl
CTP	2μl

UTP	2μl
GTP	2μl
RNase Inhibitor	0.5μl
T7 RNA Polymerase	2μl
Template DNA	1μl
Total	20μl

Note: Kit components may precipitate after preparation of the reaction mix. If this occurs, heat the components at 37 °C for 10 min.

17. Allow the IVT reaction to continue for 7 h at 37 °C. The reaction can be continued for 15 h or overnight if desired. However, no significant increase in yield will be achieved.
18. At this point, the reaction mixture can be temporarily stored at 4 °C as needed. Move to −20 °C for storage longer than 24 h.
19. To eliminate the IVT DNA template, add 1μl of RNase-free DNase to the reaction mix and incubate for 15 min at 37 °C.
20. At room temperature, dilute the reaction product 10 fold with DEPC-treated water. Add an equal volume of ethanol (95–100%) as described below.

T7 Reaction Mix	20μl
DEPC treated water	180μl
100% Ethanol	200μl
Total	400μl

21. Purify shRNA from the mixture using a Direct-zol™ RNA MiniPrep Plus spin column according to the manufacturer's instructions.
22. Following elution in nuclease-free water, measure shRNA concentration by Nanodrop or an equivalent spectrophotometer. The OD (260/280) ratio should be above 1.80.

    Note: The OD (260/280) reading can be above the measurable range. Dilute 1μl of eluted product with 99μl nuclease-free water to obtain accurate readings.
23. As an optional control, run 1 μl of the eluent in an agarose gel to confirm the spectrophotometer results. The IVT Reaction Mix can be stored up to 2 months at −20 °C.

6.3. Dejellying and preparation of eggs for electroporation TIMING (40 min)

24. Induce spawning of adult males and females in separate dishes according to the previously-described protocol (Genikhovich and Technau, 2009b).
25. Collect unfertilized egg sacs in 15 ml conical tubes and distribute 2 ml of eggs per tube. Add 12 ml L-Cysteine solution (to the 14 ml mark) using a 1 ml pipette.
26. Mix tubes on a nutator for 10 min.
27. Let eggs settle to the bottom for approximately 1 min. Remove water down to 2 milliliters and replace with fresh ASW.
28. Gently invert the tube to suspend eggs. Perform 3 washes with ASW.

    Note: Transfer pipettes can damage the eggs. To avoid this, cut off the end of the pipet to increase the diameter of the opening.
29. After the final wash, remove water and use a pipette to resuspend eggs in ASW. Transfer eggs into a 90 × 50 crystallizing dish containing ASW (12 p.p.t.). Keep at 18 °C until electroporation.

Note: Keeping the sperm at 18 °C should lengthen stability, but use within 2 h is recommended. Eggs can be kept for up to 4 h at 18 °C after dejellying. Sperm can be kept for 2 h at 23 °C.

6.4. Electroporation procedure TIMING (20 min)

30. Use a transfer pipette to move eggs from the crystalizing dish to a 15 ml conical tube. Allow eggs to settle and then transfer concentrated eggs to a fresh tube with a volume for the desired number of electroporations.

    Note: Each electroporation requires 100 μl of eggs suspended in 15% Ficoll ASW. Hence for 10 electroporations, transfer concentrated eggs to a 1.5 ml microcentrifuge tube. For larger-scale experiments, transfer to a 15 ml conical tube.
31. Remove ASW and replace with ASW containing 15% Ficoll PM 4000.
32. Suspend the eggs by pipetting the mixture with a Gelatin- or Ficoll-coated 1 ml transfer pipet.

    Note: Eggs can settle over time. (i.e. 5 min). If this happens gently tap the tube to re-suspend the eggs. Prepare and use coated plastic polypropylene pipettes for all transfers. Eggs stick to pipette tips if the tips are not coated with Ficoll or Gelatin.
33. Use a razor blade to cut a 200 μl pipette tip to increase the diameter.
34. Carefully transfer suspended eggs into a 4 mm cuvette using a 200 μl pipette.

    Note: Avoid touching the walls of the cuvette with the tip. Eggs that are stuck to the sides of the chamber or otherwise excluded from the main reaction volume will not be subjected to electroporation.
35. Add purified shRNA directly to the main reaction volume in the electroporation cuvette.
36. Gently mix shRNA and eggs by shaking side to side.

    Note: Shake 10–30 s to ensure complete mixing before electroporation.
37. Turn on ECM 830 Electroporator or equivalent device.
38. The device should be in low voltage mode (LV) as displayed on the LCD screen. Set the electroporation conditions to 50 V, 25 ms pulse duration, 1 pulse.
39. Insert the electroporation cuvette into the Bio-Rad Shock Pod cuvette receiver.
40. Press start button to electroporate. A short buzzing sound will be heard. The LCD screen will display the actual voltage, pulse duration and number of pulses. I.e. 46 V 25 ms, 1 pulse.

    Note: Each experiment should include controls with and without electroporation. These controls will provide information about the general fertilization rate for that day and embryo survival after electroporation along with the shRNA controls.
41. Remove the cuvette. On close examination, small bubbles should be observed on the walls of the cuvette.
42. Immediately use a transfer pipette to fill the cuvette with *Nematostella* sperm water from a dish of sex-sorted males.
43. Add enough sperm water to cover the bottom of a plastic petri dish (60 × 15 mm).
44. Transfer the entire volume of electroporated eggs into this petri dish using the same transfer pipette.

    Note: Make sure the developing embryos are well dispersed in the (60 ×15 mm) petri dish for incubation. Embryos can stick to each other and may form conjoined animals.
45. Incubate the plates at 18 °C or 23 °C overnight.
46. After 24 h remove debris and perform 2 or 3 exchanges of ASW. Using a transfer pipette, gently transfer the embryos to a new clean plate filled with ASW.
47. Monitor development relative to controls and perform more detailed phenotypic studies at the desired stage.

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Author contributions

M.G. conceptualized and supervised the project. A.K. developed the electroporation protocol. S.H. developed the method for shRNA-based gene knockdown. A.K. and S.H performed the experiments. A.K. S.H. C.C. and S.M. analyzed the data. M.G. and A.K. wrote the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2019.01.005.

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