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TITLE:

Design and Microinjection of Morpholino Antisense Oligonucleotides and mRNA into Zebrafish Embryos to Elucidate Specific Gene Function in Heart Development

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KEYWORDS:

zebrafish, morpholino antisense oligonucleotide, microinjection, *hand2*, gene knockdown, loss of function, gene overexpression, mRNA, heart, cardiac development.

SUMMARY:

The present protocol describes designing, preparing, and microinjecting a translational-blocking morpholino against a representative cardiac gene; Heart And Neural Crest Derivatives Expressed 2 (*hand2)* into the yolk of newly fertilized zebrafish embryos to knock down gene function. It also shows a transient rescue of these "morphants" by co-injection of mRNA encoding this gene product.

ABSTRACT:

The morpholino oligomer-based knockdown system has been used to identify the function of various gene products through loss or reduced expression. Morpholinos (MOs) have the advantage in biological stability over DNA oligos because they are not susceptible to enzymatic degradation. For optimal effectiveness, MOs are injected into 1–4 cell stage embryos. The temporal efficacy of knockdown is variable, but MOs are believed to lose their effects due to dilution eventually. Morpholino dilution and injection amount should be closely controlled to

minimize the occurrence of off-target effects while maintaining on-target efficacy. Additional complementary tools, such as CRISPR/Cas9 should be performed against the target gene of interest to generate mutant lines and to confirm the morphant phenotype with these lines. This article will demonstrate how to design, prepare, and microinject a translation-blocking morpholino against hand2 into the yolk of 1-4 cell stage zebrafish embryos to knockdown *hand2* function and rescue these "morphants" by co-injection of mRNA encoding the corresponding cDNA. Subsequently, the efficacy of the morpholino microinjections is assessed by first verifying the presence of morpholino in the yolk (co-injected with phenol red) and then by phenotypic analysis. Moreover, cardiac functional analysis to test for knockdown efficacy will be discussed. Finally, assessing the effect of morpholino-induced blockage of gene translation via western blotting will be explained.

INTRODUCTION:

The utilization of zebrafish as a model for the study of cardiovascular development and disease offers a variety of advantages, including high conservation of gene function, optical transparency, rapid cardiovascular development, and cheaper cost when compared to traditional *in vivo* models[1]. Morpholino oligonucleotides (MOs) are the most commonly used antisense gene knockdown tools for the zebrafish model. MOs are frequently used to determine a phenotype or to probe gene function. Dr. James Summerton initially developed the morpholino delivery system for the *in vivo* inhibition of mRNA translation as an attempt to develop therapeutics for human developmental defects[2,3]. MOs have been used for *in vitro* and *in vivo* model organisms to knockdown genes and investigate the consequence of this knockdown on phenotype. This is done by observing alterations in the development of specific organs, for example, the heart. Knockdown of heart-specific genes in WT zebrafish embryos led to the failure of a proper heartbeat, attesting to the indispensable function of these genes for heart development[4,5]. These phenotypes were rescued by co-injection of mRNAs for the specific genes. A study involving cardiac troponin T (Tnnt2) showed that the expression of full-length *tnnt2* mRNA could rescue sarcomeric phenotypes caused by morpholino knockdown[6]. Another study revealed that the integrity of A-bands and Z-discs could be restored by overexpression of the regulatory myosin light chain ortholog (*cmlc2*) mRNA in *cmcl2* morphants[7].

MOs are commonly used to knock down gene expression by targeting pre-mRNA splicing or by blocking translation. Splice blocking MOs bind and inhibit pre-mRNA by inhibiting the splicesome. Translational blocking occurs when the MO binds to the 5'-untranslated region of complementary mRNA to hinder the ribosome assembly. MOs are the most widely used gene-specific method to knockdown gene expression for *in vivo* models; they are also the most efficient mRNA blocking agents used in cell cultures. The morpholino itself typically consists of a short-chain (around 25) of morpholino subunit bases. Each MO subunit includes a nucleic acid base, a morpholine ring, and a non-ionic phosphorodiamidate. The different mechanisms of action for the two types of MOs necessitate different tests to verify the efficacy of the knockdown. For translation blocking MOs, a western blot analysis is the most reliable test of efficacy, as the protein of interest should not be produced due to blockage of the ATG translation start site. MOs do not directly degrade their target mRNA; instead, they bind to specific regions and inhibit the expression until naturally degraded. However, the splice blocking MOs modify the pre-mRNA by

inducing splice modification, which can be assayed by reverse-transcriptase polymerase chain reaction (RT-PCR) and gel electrophoresis.

Three crucial parts of the MOs screening process must be standardized: (i) The MO dose curve must be tuned for phenotypic recognition. The dose curve also shows the lethal dose 50 (LD50: the dose at which 50% of injected embryos die) for each MO tested to improve the ability to optimize phenotypic 'signal' versus off-target 'noise'[3]. (ii) The phenotyping nomenclature that was adapted should be well documented; a precise and easily understandable phenotypic description is critical to provide extensive explanations based on existing literature and investigator experience to facilitate information sharing among those who did not directly examine the embryos. (iii) Having well-defined language makes it easy to collect data centrally from Morpholino Database[8].

In MO knockdown studies for cardiac genes, animals' heart activity and blood flow dynamics must be monitored in order to determine the impact of MO knockdown experiments on cardiovascular system function. Such analyses require real-time visualizing of the cardiovascular system at high resolution. Zebrafish skin is transparent for the first week of development, enabling visualization of the heart and blood circulation via microscopy. For assessment of heart function, the most calculated physiological parameters are heart rate and cardiac output as well as fractional shortening, fractional area change, and ejection fraction. Blood flow velocities can be measured by tracking moving RBCs, and these measurements are used to determine shear stress levels, a crucial mechanobiological factor on endothelial cells. Such an assessment requires recording time-lapse movies for beating heart and flowing blood via an inverted or a stereomicroscope equipped with a high-speed camera.

This paper shows how to design, prepare, and microinject a translational-blocking morpholino against a gene of interest into the yolk of freshly fertilized zebrafish embryos to knock down gene function. It will also show rescuing these "morphants" by co-injection of mRNA encoding this gene. We will then analyze the efficacy of the morpholino microinjections through phenotypic characterizations as well as cardiac structural and functional analyses. This approach will be demonstrated on a widely studied cardiac gene, *hand2.*

PROTOCOL:

All experiments were carried out in accordance with the accepted standards of humane animal care under the regulation of the IACUC at QU; animals were held in the zebrafish facility under Qatar University Biomedical Research Center (QU-BRC). All animals used in these experimental studies were under 3 days post-fertilization (dpf).

NOTE: For each experimental group, it is advisable to use at least 30 embryos for statistical rigor. The experimental groups are as follows:

Control group: This group includes embryos cultured in egg water without any injections. Results here will form the control baseline.

Negative Control group: This group includes embryos cultured in egg water injected with

scrambled MOs.

Injected group: This group includes embryos injected with *hand2* MO alone and *hand2* MO with hand2 mRNA to rescue the phenotype. Results here will confirm that observed phenotypes appeared due to injected MOs. Comparison of experimental groups will enable assessment of the influence of inhibiting and rescue of *hand2* on heart function precisely.

1. Morpholino designs for *hand2*.

NOTE: MO sequences can be adapted from the literature[9–11].Alternatively, these oligos can be designed online by Gene-tools. Gene-tools offers a free and fast online design service, which can be accessed through their website[12]. A custom MO can readily be designed by providing information about the genes of interest, such as sequence information or accession numbers. The following specific steps summarize how to design MOs against *hand2* in zebrafish:

1.1. First, search for details of the gene of interest from the NCBI database. For *hand2*, in zebrafish[13].

1.2. Get GenBank mRNA transcript ID from NCBI[14].

1.3. Obtain the mRNA sequence from NCBI[15] .

NOTE: Morpholino (MO)-modified antisense oligonucleotides for the zebrafish *hand2* gene are available and were previously published. *hand2* MO sequence[9–11]: 5'-CCTCCAACTAAACTCATGGCGACAG-3', *MO* sequences used in the study are listed in the **Table of Materials**.

1.4. Get a negative control from either a mismatched or scrambled MO (similar sequences with random base pair changes) from gene tools[16] to be injected. This would help attest to the specificity of the phenotype (s) observed in specific MO injections and minimize the risk that the observed effects are from an artifact of the injection procedure.

2. Preparation of morpholino injection

2.1. Stock preparation

2.1.1. Dissolve MO stocks with ddH_2O; Diethyl pyrocarbonate (DEPC) can damage MOs and be toxic to embryos. Hence, use DEPC-free ddH_2O. Upon ordering, MOs are delivered in a vial at a concentration of 300 nM.

NOTE: While Gene Tools recommend 1 mM stock solutions (~8 ng/nL), this can be too low, especially if the morpholino requires a high dose or is mixed with other MOs. Therefore, it is ideal for making various concentrations of MO stock solutions (2 or 3 mM).

2.1.2. Prepare a 0.2 mM working solution by sub-diluting the working solution (i.e., for 2 mM stock, 1:10 dilution). Prepare 10 µL of working solution by adding Danieaux's solution (7 µL), stock morpholino (1 µL), and of phenol red (2 µL from 10x stock). Phenol red gives the solution a dark red color to help trace the injected material in the embryo.

2.1.3. Keep MOs at room temperature (RT) in airtight containers to prevent evaporation. Do not keep MOs on ice on the benchtop, as the solution may precipitate. RT handling is most appropriate.

NOTE: Gene Tools recommends either of two storage methods for MOs: The working solution can be kept at -20 °C (for many months to years) or RT in a sealed tube for long-term storage. If kept at -20 °C, heat the oligonucleotide solution at 65 °C for 10 min and vortex to completely dissolve the morpholino before use (no need to warm it up if kept at RT).

2.2. *In vitro* transcription of mRNA

NOTE: The *in vitro* transcription was used to generate mRNA of Hand2 from Plasmid HAND2 (NM_021973) Human Tagged ORF Clone to be microinjected into zebrafish embryos for rescuing the phenotype due to knocking down zebrafish *hand2* using morpholinos, plasmid details used in the study are listed in the **Table of Materials**.

2.2.1. Linearize the plasmid DNA by an appropriate restriction enzyme and purify using a DNA purification kit (**Table of Materials**). Use the purified linearized DNA as a template for *in vitro* transcription to generate mRNA for injection as per the manufacturer's instructions (**Table of Materials**).

2.2.2. Finally, inject 250 pg of human mRNA for the gene of interest to perform the rescue of phenotype to test whether the lack of a phenotype is indeed from the loss of zebrafish gene by MO.

2.3. Preparing to inject

2.3.1. Pull a needle using a micropipette puller (**Table of Materials**), following steps 2.3.2–2.3.6.

2.3.2. Use 1 mm capillary tubes with filaments and a micropipette Puller. Turn the machine on and open the lid

2.3.3. Turn the mode selection knob to **NO.2** Heater. Using **NO.2** heater, adjust the knob to bring the heat to 68 °C

2.3.4. Turn the mode selection knob back to **Step 1** position. Use four weights (2 Type Light / 2 Type Heavy)

2.3.5. Place the capillary on the holder and close the lid. Press the **Start** button to get the pulled needle.

NOTE: This will generate capillary needles with a long taper

2.3.6. Cut the tip of the needles briefly, using forceps. Sharpen the tip of the pulled needle for easy penetration into zebrafish embryos using a capillary beveler for about 30 s, resulting in tips with diameters of about 15–25 µm.

2.3.7. Calibrate the needle to estimate the amount of injection following steps 2.3.8–2.3.13.

2.3.8. Briefly mix mRNA/morpholino solution before loading it into the needle to dissolve the particles that could clog the needle fully.

2.3.9. Add ~3–4 µL of MO solution to the back part of the injection needle using a micron-tipped pipet tip. The injection needles are equipped with a small trench to facilitate capillary backfilling.

2.3.10. Confirm that there are no bubbles in the injection needle. Mount the needle to the microinjector.

2.3.11. Check that the tip of the needle is not clogged and that MO solution can flow out of the needle by pressing the injection pedal.

2.3.12. Calibrate the volume of injection by measuring the droplet size. Put the tip of the needle in mineral oil on a micrometer slide. Press the injection pedal and adjust the injection (Eject) pressure (psi) and/or injection time (ms) until the ejected droplet diameter is 0.1 mm, corresponding to 0.5 nL.

2.3.13. Ensure that the injection volume is around 0.5–1 nL. Adjust the volume by changing the injection (Eject) pressure (psi) or time (ms).

2.3.14. For picoliter injector setup protocol, follow steps 2.3.15–2.3.16

2.3.15. Use picoliter injector for zebrafish injections. Turn on the injector's feed pump.

2.3.16. Prepare the injector according to the requirements shown below:

2.3.16.1. **P-balance**: Ensure that P-balance is around 0. Ensure that it is slightly negative to prevent yolk flow back into the needle, diluting the MO solution in the needle. Conversely, if the backpressure is too high, the MO will constantly flow out of the needle even without the exertion of pressure on the pedal, resulting in variability and inconsistencies in the injected dose and the observed phenotypes.

2.3.16.2. **P-inject**: 20-25 psi is ideal; change this to adjust injection volume. This can range from 10–30 psi but start with ~20 psi to check whether it delivers the desired volume.

2.3.16.3. **Injection time**: Ensure to reduce the injection time to 300 ms. Prior to adjusting the injection volume, make adjustments to the time of injection.

2.3.17. For morpholino injection, follow steps 2.3.18–2.3.19.

2.3.18. Prepare the injection chamber as follows: Make 2% agarose from 0.6 g of agarose and 30 mL of E3 medium. Pour the agarose into a Petri dish and place an injection mold on the agarose using a TU-1 injection mold. Once cooled in a freezer, furrows will form for placing and stabilizing embryos (**Figure 1A**).

NOTE: The 60x E3M stock solution recipe consists of 5.0 mM NaCl, 0.17 mM KCl, 0.16 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2 \cdot 2H_2O$ in 1 L ddH_2O with a final pH of 7.6.

2.3.19. Transfer the collected embryos to the furrows using a transfer pipette, remove all excess E3 medium around embryos to prevent the floating of embryos (**Figure 1B**).

3. Injection of MO and mRNA solution into the yolk

3.1. Insert the needle through the chorion, inject near the boundary of cell/yolk to the embryo side and press the injection pedal (**Figure 1C**).

NOTE: The injected MO solution/phenol red will not diffuse immediately within the yolk. A red spot will be observed in the yolk that gradually dissipates. The injected material should not exceed more than 10% of the embryo size. MOs can be microinjected into the yolk of newly–laid zebrafish eggs at up to the 8-cell stage because the MOs can easily be taken up in the cytoplasmic stream of the yolk sac by the developing cells[17,18]. In plasmid or capped mRNA injections, the injection should be performed into a single blastomere of a one-cell stage embryo.

3.2. Transfer the embryos into a Petri dish with E3 medium and incubate them at 28 °C until 3 dpf, with daily replenishment of E3 medium. At that stage, perform the phenotypic assessment using a microscope.

NOTE: MO solutions must be carefully diluted for each investigation to establish the lowest concentration needed to induce a specific phenotype. Embryonic lethality for each MOs must also be determined. At higher concentrations (above 4–10 ng, depending on the MO), MOs tend to elicit non-specific effects, such as brain or general cell death.

3.3. Co-inject MO with 250 pg of mRNA of human *hand2* in the same way to confirm the rescue of phenotype induced by loss of zebrafish *hand2* gene by MO.

4. Western blot to verify the success of morpholino knockdown

4.1. Collect the embryos at specific time points (48 hpf, 72 hpf) and dechorionated if needed using the Pronase enzyme.

4.2. Deyolk the embryos to remove Vitellogenin using a previously known methodology[19].

NOTE: Vitellogenin is a phospholipo-glycoprotein that serves as a source of nutrients for the growing embryo. High-resolution 2D gel electrophoresis and vastly improved western blotting are made possible by deyolking the embryos and removing Vitellogenin.

4.3. Perform western blotting as described previously[20].

5. Cardiac structure and function assessment:

5.1. Live imaging of zebrafish: Imaging the ventricle

NOTE: Several heart function/hemodynamics parameters can be calculated by visualizing the beating heart of zebrafish embryo[21,22]. These parameters include cardiac output (CO), ejection fraction (EF), stroke volume (SV), fractional shortening (FS), and fractional area change (FAC) (please see previous papers involving detailed protocols for cardiac function assessment for zebrafish embryos[22,23]). The following steps briefly explain how to calculate those parameters for zebrafish embryos at ~3 dpf, a stage when the skin of these organisms is transparent, enabling visualization with bright-field microscopy.

5.1.1. Put a drop of 3% methylcellulose solution (RT) in the concave well imaging slide.

5.1.2. Position the Zebrafish embryo in the well using a suitable plastic dropper (**Figure 2A**,**B**)

NOTE: Overfilling the well may cause fish displacement out of the well.

5.1.3. Gently mix the 3% methylcellulose drop with the E3 medium to stabilize the hatched embryo.

NOTE: To prepare 3% methylcellulose solution, dissolve 3 g of methylcellulose powder in 100 mL of PBS, or other mounting media, in a flask. Place a stirring magnet in the mixture's flask and place the flask on a magnetic stirring plate. Set the speed to "low" and keep it at 4 °C for ~1 day to dissolve all the clumps. Once the methylcellulose is completely dissolved, aliquot into small tubes and store at -20 °C.

5.1.4. Position the fish on its left, with its right side facing up and anterior point to the left to facilitate unambiguous imaging for the ventricle (**Figure 2C**).

5.1.5. Under the microscope, zoom in on the embryo heart with 100x magnification and start recording for ~5 s. Ensure that the ventricle borders are inside the imaging window (**Figure 2D**).

5.1.6. Record time-lapse movies using a high-speed camera and stereomicroscope at about 100 frames per second (fps) of the whole embryo (**Figure 2C**), beating ventricle (**Figure 2D**), and moving red blood cells (RBC) in major vessels, such as the dorsal aorta or the caudal vein (**Figure 2E**), for cardiac function analysis.

5.1.7. Save the movie in either AVI movie format or TIFF (or JPEG) image sequences format.

5.2. Calculate the heart rate

5.2.1. Calculate the time needed to record two consecutive frames. For 100 fps the time interval is equal to 0.01 s.

5.2.2. Choose a known point from any recorded cardiac cycle (i.e., end-diastole or end-systole). Calculate the number of frames needed to repeat the cycle.

5.2.3. Multiply the time interval from step 5.2.1 with the number of frames from step 5.2.2. The result is the time duration (in seconds) for one heartbeat.

5.2.4. To calculate beats per minute, divide 60 by the number obtained in the previous step. For normal 3 dpf embryos, this should be around 150 bpm (2.5 Hz).

NOTE: Many software applications can calculate heart rate automatically from beating heart recordings such as ViewPoint[24] and DanioVision[25].

5.2.5. Analyze the cardiac structure using a record time-lapse movie at about 100 fps to check the whole heart to detect the presence of cardiac edema or any other structural defect.

NOTE: **Figure 3A** shows a normal 3 dpf embryo heart, and **Figure 3B** shows a 3 dpf heart with cardiac edema and heart looping defect (elongated ventricle).

5.3. Calculate the fractional area change (FAC):

NOTE: Fractional area change (FAC) is a parameter used to compare ventricular end-diastole and end-systole areas to assess contractility of the ventricle.

5.3.1. Use a record time-lapse movie at about 100 fps to determine frames that represent one cardiac cycle. Extract the frames that show the end of both end-systole and end-diastole.

5.3.2. Calculate both end-diastole area (EDA) and end-systole area (ESA) using ImageJ or a similar image analysis software.

5.3.3. Use the following formula to calculate FAC[22,26,27]:

$$\text{FAC} = 100 \times \frac{\text{EDA}-\text{ESA}}{\text{EDA}}$$

5.4. Calculate the fractional shortening (FS):

NOTE: Fractional shortening (FS) is another parameter used to evaluate the contractility of the ventricle; to determine FS, the end-diastolic and end-systolic diameters of the ventricles must be measured[21].

5.4.1. Repeat step 5.3.1

5.4.2. Use ImageJ or any other equivalent image analysis program to measure the diameters of the ventricular walls at the end-diastole D_d and the end-systole D_s points. In most cases, short-axis diameters are employed to determine the fractional smoothness (FS) (**Figure 4**).

5.4.3. Use the following formula to calculate FS[22,27]:

$$\text{FS} = \frac{(\text{D}_\text{d}-\text{D}_\text{S})}{\text{D}_\text{d}}$$

5.5. Calculation of stroke volume (SV):

NOTE: For each heartbeat, the SV is the amount of blood pumped from the ventricle, which is easily computed from the end-diastole and end-systole volumes of the ventricles[28].

5.5.1. Repeat step5.3.1 and 5.3.2

5.5.2. Measure the End-diastole (D_L) and End-systole (D_S) diameters as shown in **Figure 4.**

NOTE: Assuming that the ventricles of zebrafish hearts have a shape of a prolate spheroidal[21,22], ventricle volume is calculated using the following formula.

$$\text{Volume} = \frac{1}{6} \times \pi \times D_L \times {D_S}^2$$

SV can be calculated following the below formula[27]. The normal SV range is 0.15–0.3 nL for 2–6 dpf embryos[29]. Here, EDV is end-diastole volume, and ESV is end-systole volume.

$$\text{SV} = (\text{EDV} - \text{ESV})$$

5.6. Calculate the ejection fraction (EF):

NOTE: EF is defined as the fraction of blood ejected from the ventricle with each heartbeat[28].

5.6.1. From the above formulas, extract EDV and ESV for the fish.

5.6.2. Calculate EF as follow[22,27]:

$$\text{EF (\%)} = \frac{(EDV-ESV)}{EDV} \times 100 = \frac{SV}{EDV} \times 100$$

5.7. Calculate the cardiac output (CO):

NOTE: CO is the volume of blood being pumped by the heart[28]. CO has a value of 10–55 nL/min for 2– 6 dpf embryos[29].

5.7.1. Calculate SV and HR as mentioned in the previous subsections 5.5 and 5.2.

5.7.2. Use the following formula to calculate CO[22,27]:

CO (nL/min) = SV (nL/beat) x HR (beats/min).

5.8. Measure the RBCs velocities:

NOTE: Cells speed determination is vital for evaluating flow rate, vessel diameter, and calculating the shear stress exerted on the vessel's endothelial cells (i.e., Shear stress, on the other hand, is the frictional force imposed on the endothelial cells by moving blood). For 2–5 dpf embryos, the average red blood cells (RBC) velocity is about 300–750 µm/s[29]. To measure the RBCs velocities:

5.8.1. Under the microscope, zoom in on the animal tail at a magnification of 100x. RBCs movement should be visible at this magnification (**Figure 5A**,**B**).

5.8.2. Start recording for about 8 s. Ensure that dorsal aorta (DA) and posterior cardinal vein (PCV), the two most important axial arteries, are 100–120 fps inside the imaging window. Track individual cells from sequential frames (**Figure 5A**,**B**).

5.8.3. Extract several frames using ImageJ or other similar image analysis software.

5.8.4. Calculate the difference in distance that an individual RBC moves (Δx).

5.8.5. Determine the difference in time between the consecutive frames (Δt).

5.8.6. Use the following formula for RBC velocity[22,27]:

$$\text{RBC velocity (µm/s)} = \frac{\Delta x\ (\mu m)}{\Delta t\ (s)}$$

NOTE: Maximum and average velocity can be extracted from the repetition of consecutive frames for individual cells[21]. RBCs velocity also represents blood flow velocity. Alternatively, a variety of commercially available software applications can be used to measure RBCs' velocity automatically. ViewPoint[24], as well as DanioVision[25], have such applications (**Figure 5C**). Furthermore, there are few available plugins with ImageJ, such as TrackMate[30] and MTrackJ[31], compatible with movies recorded above 100 fps.

5.9. Calculation of shear stress from measured cell velocities

Measured the RBC velocities in blood vessels also represent blood flow velocities (**Figure 5C**). From these measurements, calculate the shear stress $\boldsymbol{\tau}$ in a vessel of interest as follows with Poiseuille Flow assumption[22,27]:

$$\tau = \frac{4\mu V_{mean}}{D}$$

Where V is the average blood velocity (μm/s), μ is the blood viscosity (dynes/cm^2), and D is the vessel diameter (μm). For 3 dpf embryos, shear stress in DA[22,26,27] is about 4 dynes/cm^2.

REPRESENTATIVE RESULTS:

The graph in **Figure 6** illustrates the average percent of embryos surviving at 24, 48, and 72 hpf for both HAND2- specific MO and control scrambled MO-injected embryos. The 1 mM (8 ng/μL) and 0.8 mM (6.4 ng/μL) MO-injected embryos showed a significant reduction in survival percentage compared to control scrambled MO-injected embryos. This was observed across each measured time point where lethality or malformation was observed. The results indicated that a high concentration of HAND2MO had a significantly lower survival percentage due to off-target effects and toxicity of MO. While 0.4 mM (3.2 ng/nL) HAND2 MO-injected embryos did not show a significant reduction in survival percentage; however, some similar phenotypes in the experimental group were noted, such as delayed in heart development, elongated tub-like structure heart, and pericardial edema in comparison to control scrambled MO-injected embryos (**Figure 6**).

HAND2 knockdown embryos exhibited distinct and specific phenotypes. Phenotypes of the morphants were examined between 24 and 72 hpf under a Light Microscope. HAND2 loss of function gave rise to embryos with a delay in heart development and an elongated heart, shaped like a tube with pericardial edema (**Figure 7**). As a result, embryos with this defect in heart formation behaved differently in terms of cardiac performance in comparison to scrambled MO (**Figure 8**).

In our investigation, we have characterized the changes in the zebrafish heart shape, cardiac output, and blood flow in control and HAND2 knockdown animals. Blood flow velocity was measured by tracking down moving RBCs, which were used to determine shear stress levels, an important mechanobiological factor on endothelial cells.

Cardiac output and blood flow analyses showed that HAND2 knockdown triggered a delay in heart development in comparison to wild-type embryos. This alteration resulted in a decrease in dorsal aorta blood velocity, a drop in heart pulse, and a reduction in cardiac output (**Figure 8A**). In agreement with the negative impact of HAND2 knockdown on cardiac function, shear stress was also reduced significantly (**Figure 8A**) in comparison to the control scrambled MO-injected embryos. Most of the heart function failure was rescued when HAND2 MO was co-injected with human HAND2 mRNA, which resulted in an increase in dorsal aorta blood velocity, increase in heart pulse, and restoration in cardiac output (**Figure 8A**). These results are in agreement with cardiac output and blood flow analysis results for PCV (**Figure 8B**). Collectively, the data

suggested that HAND2 might play a critical role in cardiomyogenesis during zebrafish development.

FIGURE AND TABLE LEGENDS:

Figure 1: Steps for injection of MO solution into the yolk. (**A**) Injection chamber. (**B**) Using a transfer pipette, transfer the collected embryos to the furrows. (**C**) Insert the needle through the chorion, inject near the boundary of cell/yolk to the embryo

Figure 2: Mounting and imaging Zebrafish embryos. (**A**) The concave well imaging slide with zebrafish embryo under the microscope. (**B**) Magnified view of the well. Fill the wells ¾ of volume with E3 medium. (**C**) Positioning of the embryo should be on its left side. (**D**) Ventricle can be seen clearly in this configuration (zoomed image on left, ventricle borders are highlighted). Anterior is to the left. (**E**) Moving red blood cells (RBC) in major vessels, such as the dorsal aorta or the caudal vein. Anterior is to the left.

Figure 3: Analysis of the whole heart to detect the presence of cardiac edema and/or any structural defects. (**A**) A normal 3 dpf embryo heart. (**B**) Cardiac edema and looping defect in 3 dpf zebrafish embryo. Anterior is to the left.

Figure 4: Measurement of ventricle size. (**A**) Ventricular cavity and myocardial wall are highlighted. Long axis and short axis diameters are seen for (**B**) end-diastole and (**C**) end-systole.

Figure 5: Automatic detection of vessels using ZebraLab. (**A**,**B**) The two major blood vessels in zebrafish: the dorsal aorta (DA) and the posterior cardinal vein (PCV). (**C**) Viewpoint program used to quantify blood flow and vessel diameter in DA.

Figure 6: *Hand2* MO titration. Embryonic survival for different *Hand2* MO concentrations was converted to a percentage, averaged, and plotted. The percent survival of MO-injected embryos was calculated at 24, 48, and 72 hpf. All data points represent mean ± SEM (100 embryos were used in each group; experiment was performed in triplicate). The analysis was done by two-way-ANOVA with Dunnett test. *$*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Figure 7: *Hand2* morphants exhibit distinct and specific phenotypes. Hearts and tails of 72 hpf embryos were examined under a light microscope (100x magnification). (**A**,**B**) Control scrambled MO shows normal heart development without pericardial edema. (**C**,**D**) 0.4 mM HAND2 MO-injected embryos as positive control shows tube-like heart structure.

Figure 8: Assessment of cardiac function (**A**) Dorsal aorta blood flow analysis, (**B**) Posterior Cardinal Vein (PCV) blood flow analysis. 1-4 cell stage zebrafish embryos were injected as groups with scrambled MO, *Hand2* MO, *Hand2* MO + *Hand2* mRNA rescue, and scrambled MO + *Hand2* mRNA rescue. Un-injected embryos were used as control. All data are presented as mean ± SEM (6 embryos were used in each group; experiment was performed in triplicate). The analysis was done by one-way ANOVA with Sidak post hoc test. *$*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001*.

DISCUSSION:

Morpholino (MO) technology has been extensively used in zebrafish, xenopus, sea urchins, and more recently in cell culture model systems. With most methods, along with the benefits, there are also pitfalls that the experimenter should be aware of. One of the major pitfalls with MO technology includes the concern that phenotypic effects observed by the MO-mediated gene knockdown approach are not due to the loss of function associated with the primary gene product but that some other genes along with the primary gene or independent of the primary gene has been targeted, and thus this so-called "off-target" effects is the reason for the "observed" morphant phenotype. This point came to the forefront when poor correlation was observed between morphants and mutants for the same gene in zebrafish[32]. Thus, several recommendations and guidelines for morpholino use in zebrafish field[2,3] have been established, and it is recommended that experimenters follow these guidelines. In our view, when possible, a second independent complementary approach (CRISPR) confirming the morphant or mutant phenotype is desirable. A second limitation includes the ability to selectively knockdown a gene in space and time using MOs. Yes, photoactivatable MOs[33] are available, but genes with complex expression patterns and those that have distinct functions at different times in development (early vs. late) will be difficult to target using this method. Further, the use of high light intensities to uncage/activate the MOs may damage the embryonic tissue, which precludes its widespread use. A third limitation of MO is a general one for knockdown technology wherein only transient blocking of gene transcription is possible, and from a model system perspective, MOs will only work effectively in organisms like zebrafish, Xenopus, sea urchins where organogenesis occurs within a short time (2–3 days) period. This time frame limits the use of the technology in higher order organisms, and in adults. Some reports have emerged for in vivo MO use in mice[34] although, such approaches have not gained widespread adoption. Finally, non-coding RNAs that comprise the bulk of our genome is a challenge to target with MOs. MOs have successfully targeted short non-coding RNAs (miRNAs)[35] but long (>200 bp) non-coding RNAs without translational start site or definitive exon-intron splicing sequence information remains a challenge.

In this study, we investigate HAND2, a transcription factor that regulates cardiac development. Its expression domain determines the heart-forming region in the anterior lateral plate mesoderm (ALPM)[36-38]. HAND2 has an early impact on cardiac progenitor cells formation; consequently, HAND2 knockdown embryos were used as a positive control to assess defective heart development. To examine the involvement of HAND2 in zebrafish embryonic heart development, its translation was interrupted using antisense MO that was designed to target the 5' untranslated region (UTR). The lack of evidence describing the optimal concentration of MO required for HAND2 silencing in zebrafish necessitated the optimization of the MO dosage.

Zebrafish injected with scrambled MO had a similar survival percentage as compared to un-injected embryos. This finding rules out any embryo lethality attributed to damage from the morpholino injection solution. However, the low survival percentage before 12 hpf could be attributed to the injection of unfertilized embryos. Several concentrations were tested to ascertain the optimal dosage in which knockdown would be achieved while maintaining the

normal development of the fish. Fish should appear healthy and not developmentally stunted, which would indicate a delicate balance between phenotypic 'signal' versus off-target 'noise has been obtained[3]. For clarity, only the optimum amount used is shown in **Figure 6**.

After successfully optimizing the condition for MO injection, we further examined whether HAND2 knockdown affected heart function. Cardiac defects in HAND2 knockdown zebrafish embryos generally manifested as pericardial edema and faulty circulation at an early stage[39]. For functional analysis, we began with studying heart rate, as it is an important factor for the assessment of pathological heart function in zebrafish[27,40]. HAND2 MO-injected embryos exhibited a significant decrease in heart rate, confirming that HAND2 is important for heart function. Successful rescue of HAND2 expression was confirmed at both structural and functional levels by assessing the protein level as well as the embryo's phenotype, as shown in cardiac function analyses. The results in **Figure 8** show that HAND2 expression was significantly diminished and that expression was significantly rescued when the HAND2 MO was co-injected with human HAND2mRNA. This result indicated that HAND2 knockdown is the cause of the induced phenotype.

Our investigations confirmed that in comparison to wild-type, HAND2 knockdown embryos exhibited a delay in heart development accompanied by a reduction in the number of red blood cells and an overall decrease in blood flow and cardiac output.

The MO-based knockdown system has been used to identify the roles played by various gene products through a lack of functional testing. It has the advantage in biological stability over DNA oligos since MOs are not susceptible to enzymatic degradation. For optimal effectiveness, they are injected into 1–4 cell stage embryos. The temporal efficacy of knockdown is variable, and the oligo is believed to eventually lose its effect due to dilution. This protocol details the biological analysis and structural/functional assessment steps to determine if a specific phenotypic abnormality is produced when a cardiac-specific gene is targeted via MOs in zebrafish.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors declare no financial interests or other conflicts of interest.

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Figure 1

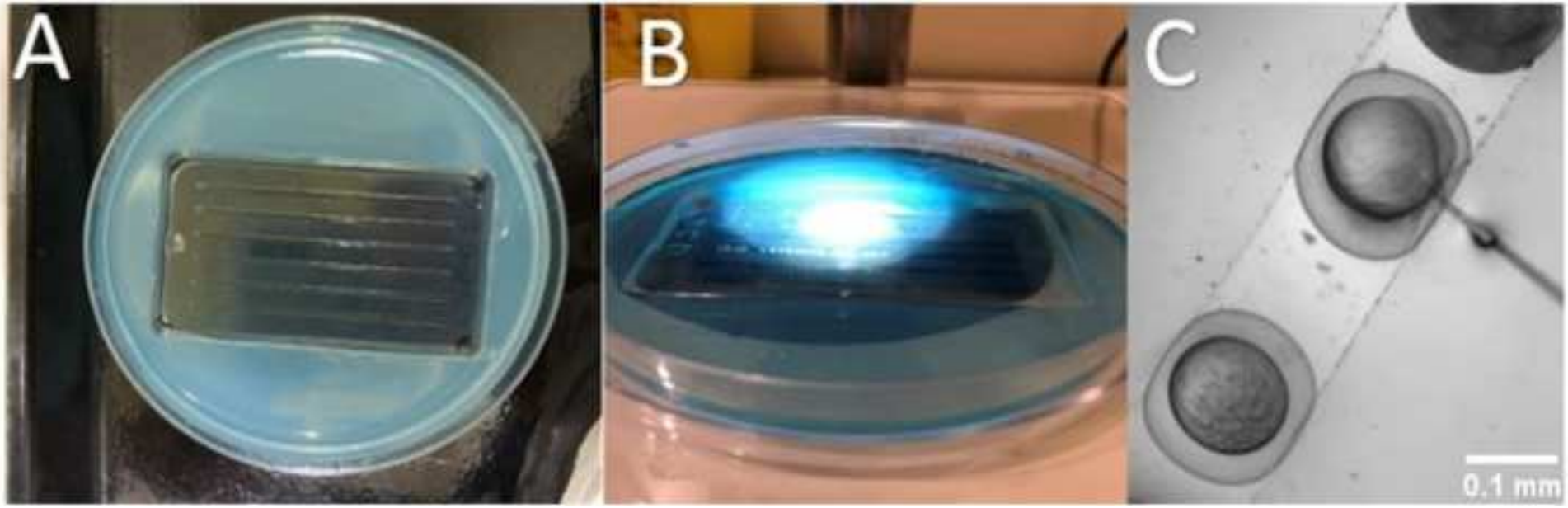

Figure 2

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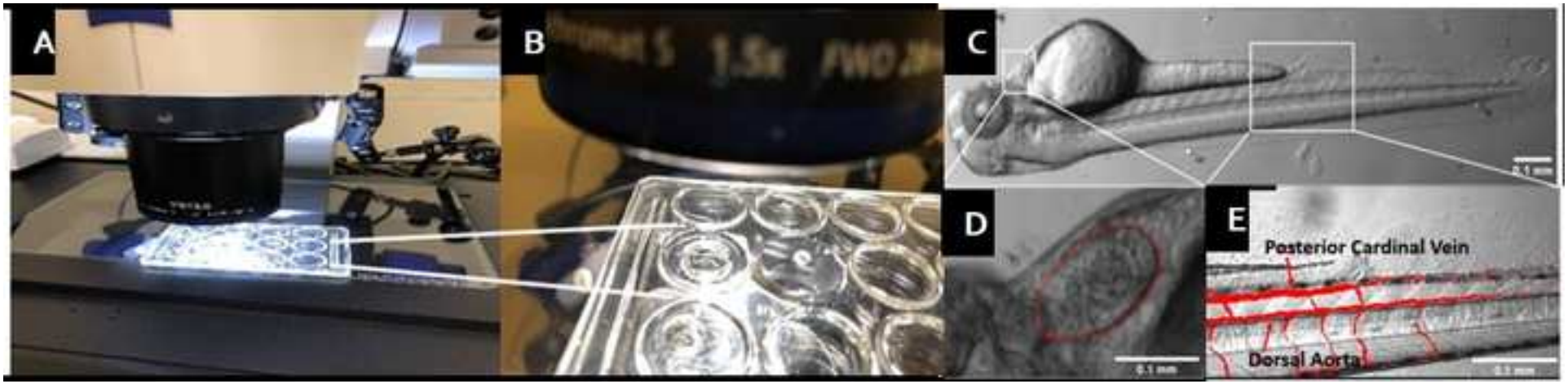

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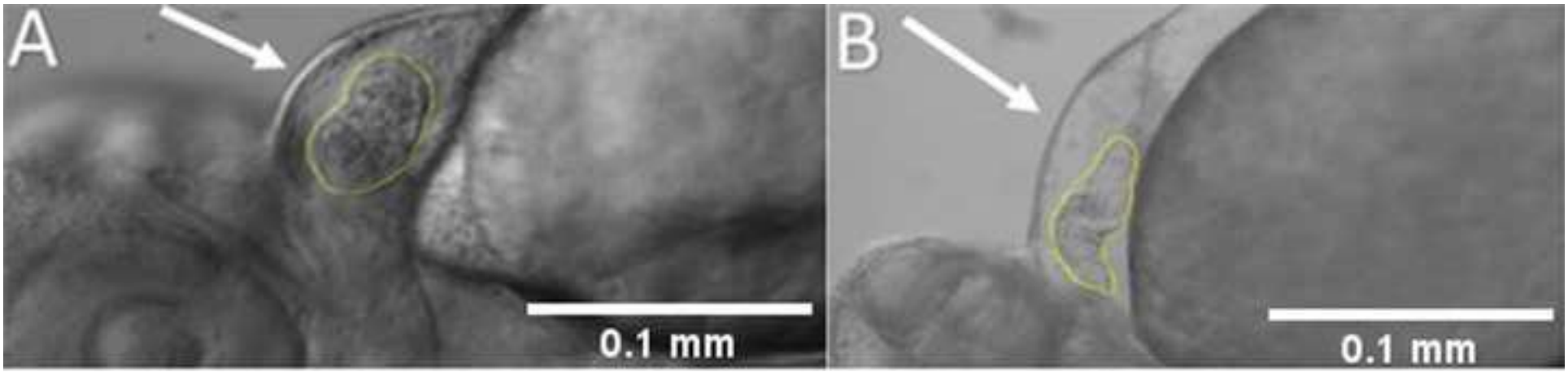

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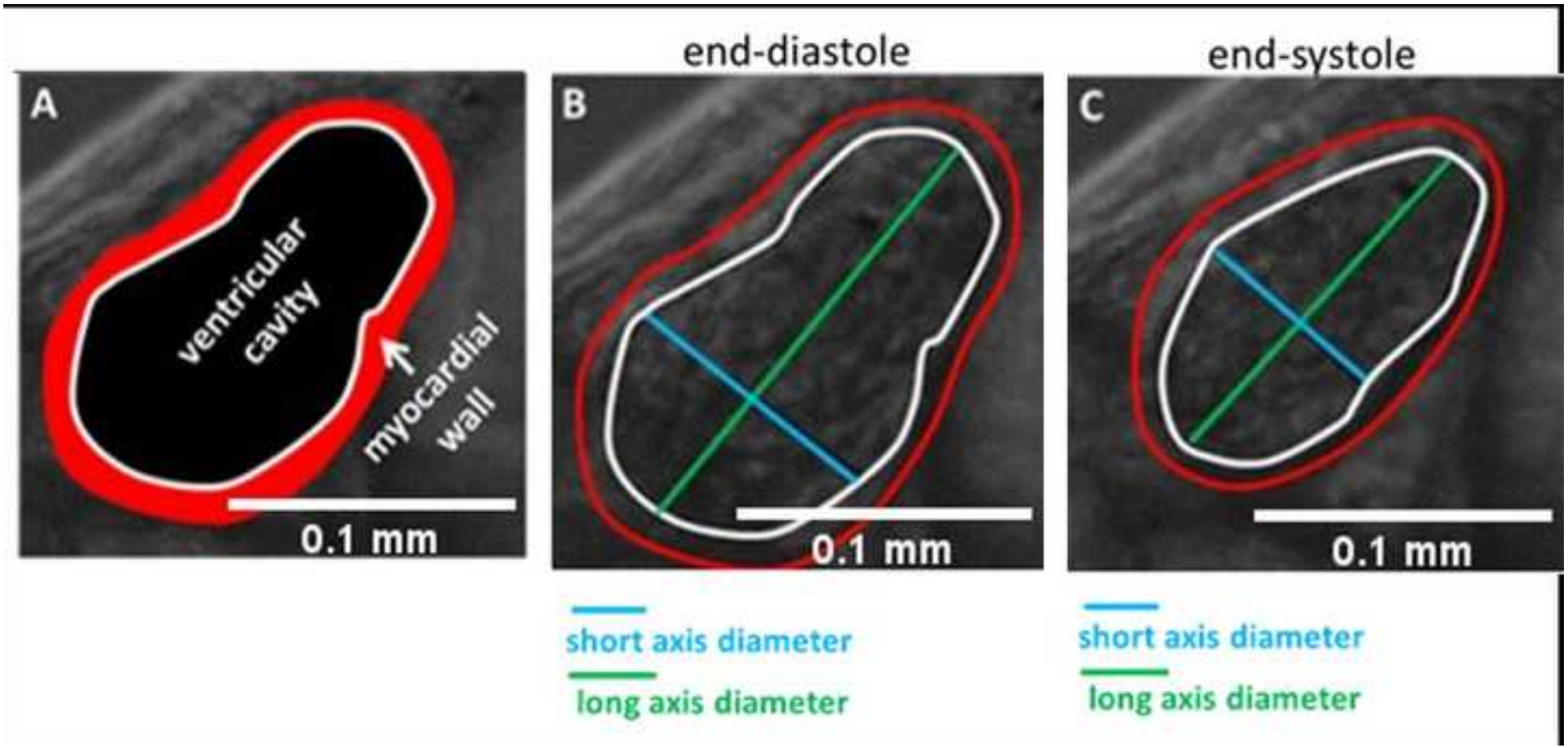

Figure 5

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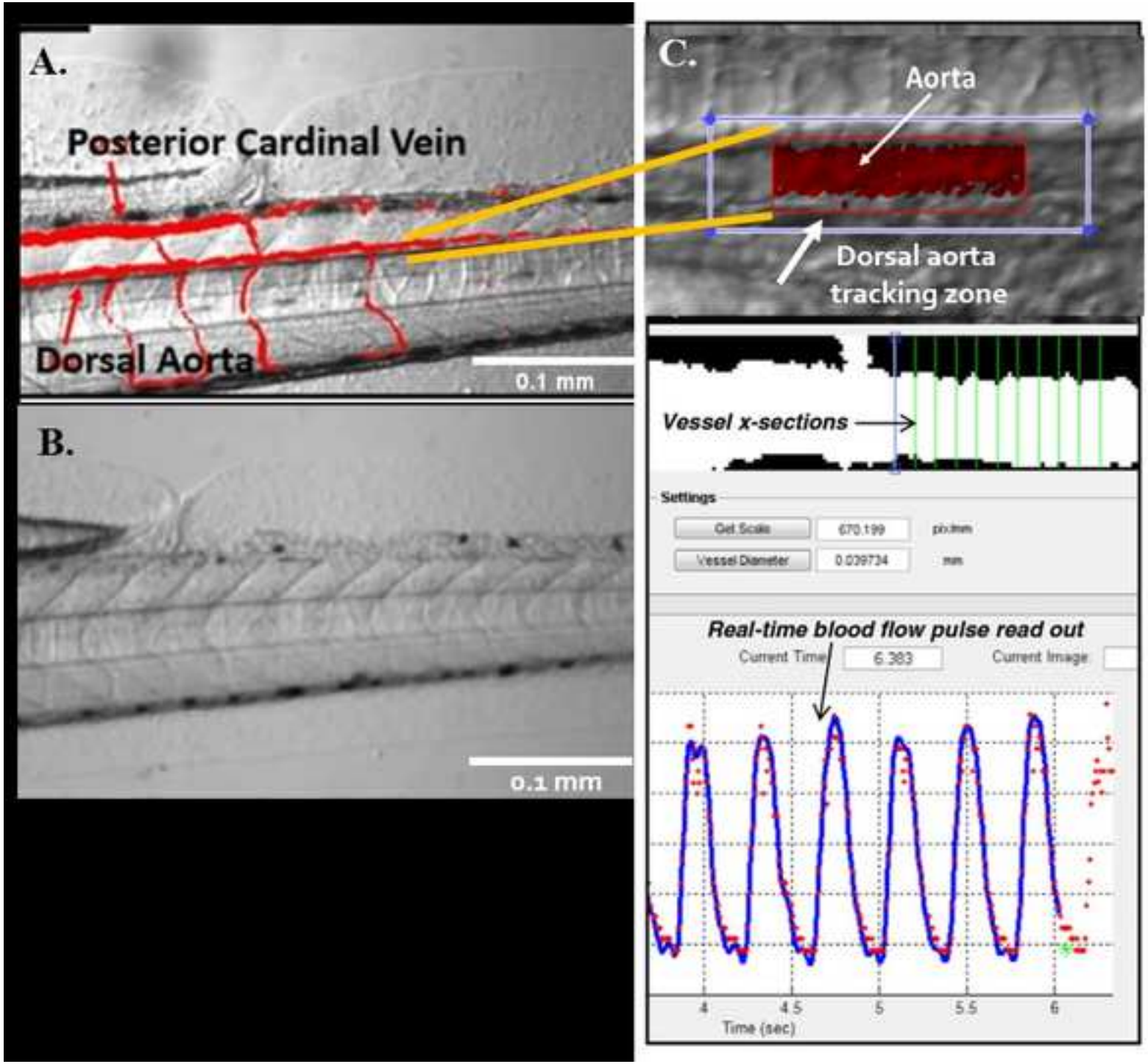

Figure 6

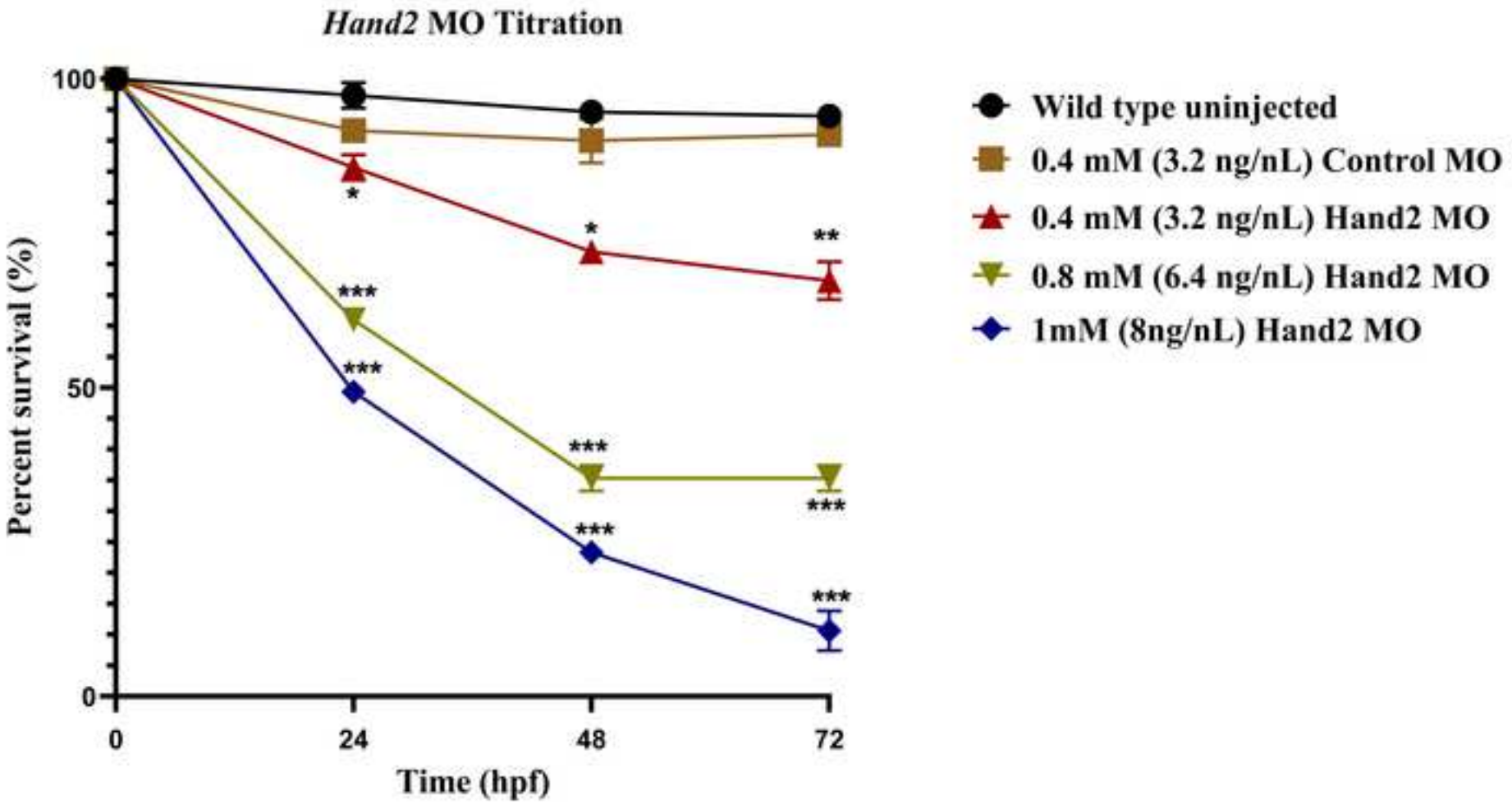

Figure 7

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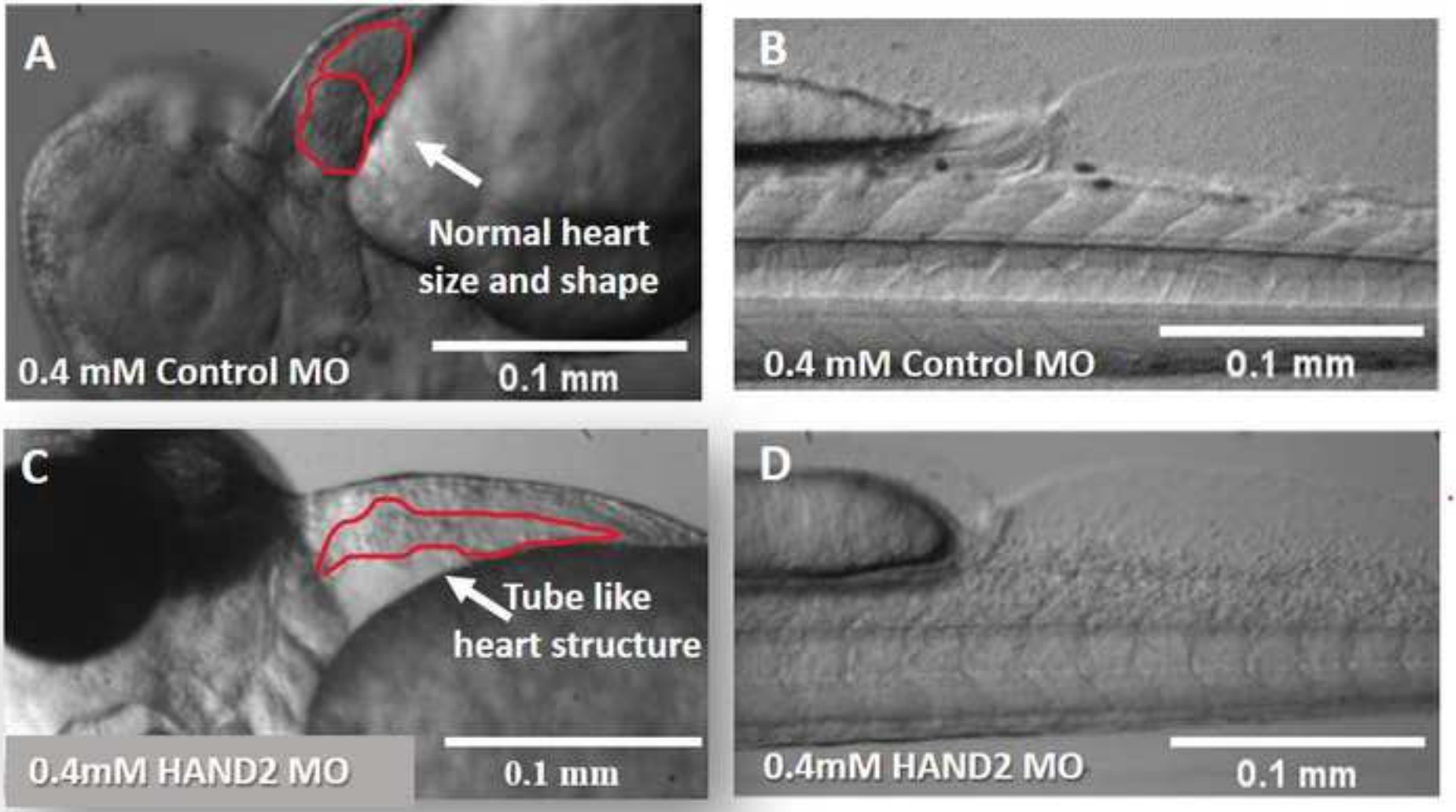

Figure 8

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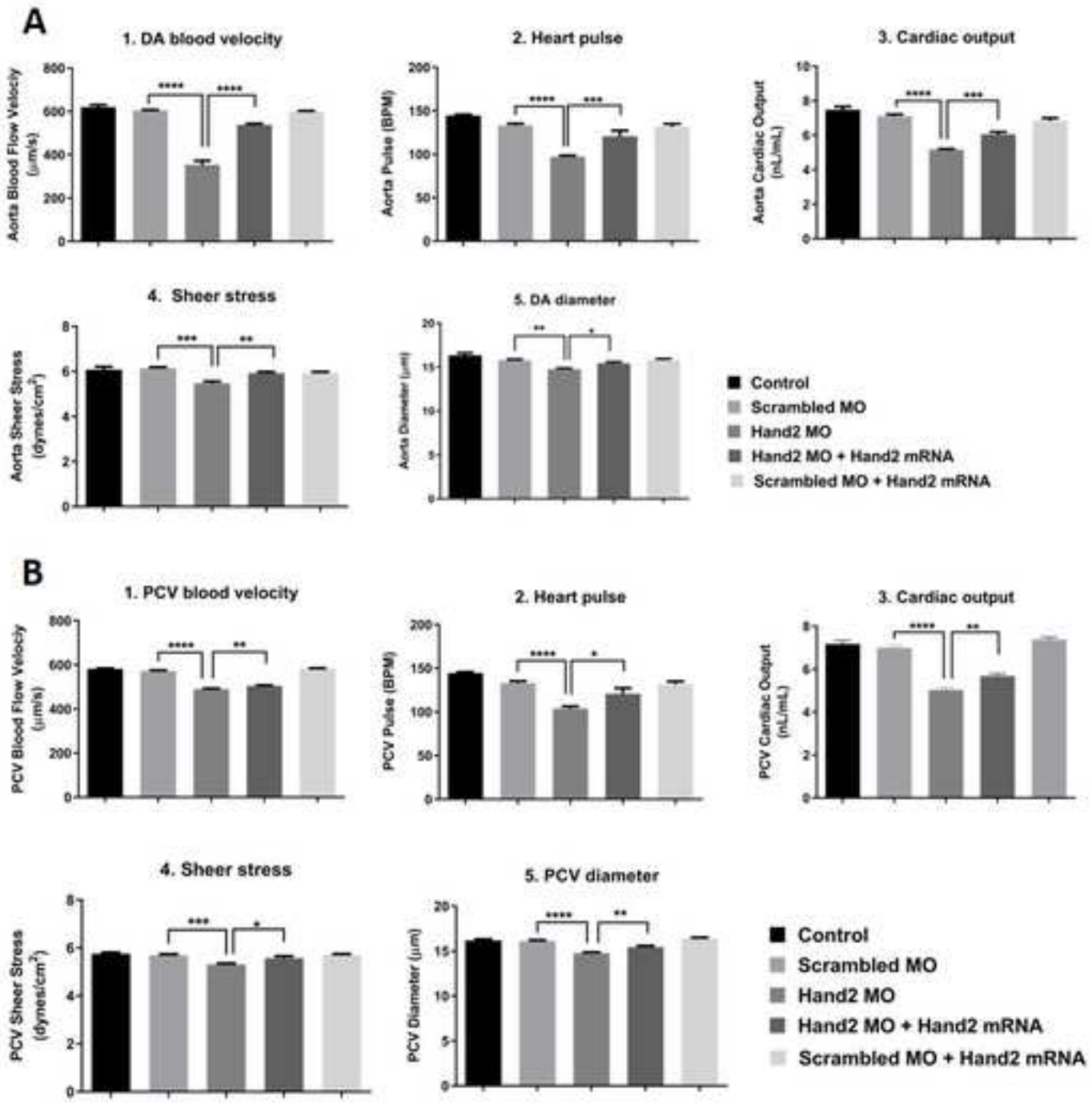

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Table of Materials

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: *We thank the editor for valuable critiques. We went thorogh the text and corrected all grammatical and spelling errors. We have made the changes in "Track-changes" in the revised manuscript.*

2. Please reword the following lines to avoid previously published work: 84-87, 88-90, 92-94, 192-195, 217-218, 220-221, 270-273, 357-362, 366-369, 396-397, 404-405.

Response: *The lines were reworded as requested*

3. Please provide an email for each author.

Response:

Zz1513224@qu.edu.qa
fatiha@qu.edu.qa
hyalcin@qu.edu.qa
seisabeygi@mcw.edu
rramchan@mcw.edu

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: *Text was revised and pronouns were removed*

5. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "The present protocol describes. …". Here the word limit is exceeding.

Response*: The summary was rephrased within the required word limit*

6. Please remove citations from the Abstract and include them in the Introduction section.

Response: *Citations were removed from the Abstract and included in the Introduction section*

7. Please format in-text journal references to appear as numbered superscripts after the appropriate statement(s).

Response: *Reference citations were fixed as requested*

8. Please ensure that all the abbreviations are defined at the first instance.

Response: *We revised the entire text for abbreviations and fixed.*

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Gene Tools, Orogene, Thermo Fischer, World Precision Instruments, Narishige, Slutter Instruments, Harvard Apparatus

Response: *All trademark symbols (™), registered symbols (®), and company names were removed and added to the table of materials*

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. All action steps should be numbered.

Response: *Numbering was corrected accordingly*

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response: *Protocol steps were revised accordingly*

12. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a “NOTE.”

Response: *Steps were revised with imperative tense*

13. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: *We revised the steps to explain in more detail and in brief statements as requested*

14. Please add more details to your protocol steps:
Line 113: Please add citations for the literature.
Line 409: Please include citations for the equations.

Response: *Citations were added accordingly*

15. Please remove the Figures/Tables embedded in the manuscript.

Response: *Embedde figures and tables were removed from the text as requested*

16. Please submit each Figure individually in a high-resolution format to your Editorial manager account. The file of Figure 3 is missing.

Response: *High resolution images were submitted seperately*

17. Figure 1C/Figure 2C,D,E/Figure 3A,B/Figure 4: Please include a scale bar.

Response: *Scale bar was added as requested*

18. Please provide the Tables in xlsx format and upload separately in your Editorial Manager account.

Response: *Tables were submitted separately as requested*

19. Please include a Representative Results section after the Protocol section. This section should contain at least one paragraph of text to explain the Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the Figures/Tables. Please include all the observations and conclusions you can derive from the Figures. The Results section should focus on the effectiveness of your technique backed up with data.

Response: *Results for a representative cardiac gene, hand2 was added to Results section*

20. Please include all the Figure/Table Legends together at the end of the Representative Results in the manuscript text. Each Figure/Table Legend should relevant include a title and a short description of the data presented in the Figure and symbols. The Discussion of the Figures should be placed in the Representative Results.

Response: *Figure/Table Legends were included together at the end of the Representative Results in the manuscript text*

21. Please include a Discussion section after the Figure/Table legend section. As we are a methods journal, please ensure that the Discussion cover the following in detail in 3-6 paragraphs with citations:
a. Critical steps within the protocol
b. Any modifications and troubleshooting of the technique
c. Any limitations of the technique
d. significance with respect to existing methods
e. Any future applications of the technique

Response: Discussion section was added covering the required details.

22. Please provide a list of all the gene sequences used, plasmids, and promoters used in the study in a separate Supplementary File. Please reference the file in the manuscript text and include a legend in the Figure/Table legend section.

Response: *All the Morpholinos sequences, plasmids used in the study were listed in a separate Supplementary File*

23. Please ensure that the Table of Materials contains all the details of all the essential supplies, reagents, and equipment in a single sheet. The present Table has six sheets. Please combine all these. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: *All the essential supplies, reagents, and equipment were listed alphabetically in a single sheet with all the details about the name, company, and catalog number.*

24. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references.

Response: *Bibliography was fixed as requested*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this paper Zakaria and colleagues describe, in a very detailed protocol, how to design, order, dilute and microinject morpholinos in fertilized zebrafish embryos to knock down a specific gene of interest. The authors also describe how to perform rescue experiments by the co-injection of exogenous mRNA of the targeted gene, in morphants embryos. Finally, the efficacy of the morpholino microinjections was showed by the authors through the analysis of cardiac structure and function of hand2 morphants. The paper is clear and well written, and each procedure is described. in detail, step by step. I have only few suggestions that in my opinion might improve this manuscript that are listed hereinafter

Minor Concerns:

1- The authors should mention in the text also the **limitations of the morpholino technique and stress a bit more that the morpholino injection can induce off-target effects associated with unspecific phenotypes such as cardiac oedema or microcephaly**.

Response: a paragraph about morpholinos limitations andoff-target effects was added to discussion section, as below

Morpholino (MO) technology has been extensively used in zebrafish, xenopus, sea urchins and more recently in cell culture model systems. With most methods, along with the benefits, there are also pitfalls that the experimenter should be aware of. One of the major pitfalls with MO technology include the concern that phenotypic effects observed by MO-mediated gene knockdown approach is not due to the loss of function associated with the primary gene product but that some other genes along with the primary gene or independent of the primary gene has been targeted, and thus this so called “off-target” effects is the reason for the “observed” morphant phenotype. This point came to the forefront when poor correlation was observed between morphants and mutants for the same gene in zebrafish (Kok et al., 2015). Thus, several recommendations and guidelines for morpholino use in zebrafish field (Bill et al., 2009;Stainier et al., 2017) have been established, and is recommended that experimenters’ follow these guidelines. In our view, when possible, a second independent complementary approach (CRISPR) confirming the morphant or mutant phenotype is desireable. A second limitation include the ability to selectively knockdown a gene at space and time using MOs. Yes, photoactivatable MOs (Sumanas, 2017) are available but gene targets with complex expression patterns and those that have distinct functions at different times in development (early vs. late) will be difficult to address using this method. Further, the use of high light intensities to uncage/activate the MOs that may damage the embryonic tissue has also precluded its widespread use. A third limitation of MO is a general one for knockdown technology wherein only transient blocking of gene transcription is possible, and from a model system perspective, MOs will only work effectively in organisms like zebrafish, Xenopus, sea urchins where organogenesis occurs within a short time (2-3 days) period. This time frame limits the use of the technology in higher order organisms, and in adults. Some reports have emerged for in vivo MO use in mice (Kyritsi et al., 2017) although, such approaches have not gained widespread adoption. Finally, non-coding RNAs that comprise the bulk of our genome is a challenge to a target with MOs. MOs have successfully targeted short non-coding RNAs (miRNAs) (Flynt et al., 2017) but long (>200 bp) non-coding RNAs without translational start site or definitive exon-intron splicing sequence information remains a challenge.

2- I also would suggest the authors to cite and comment in the ms the seminal paper of Stainier et al., 2017 (10.1371/journal.pgen.1007000) that represent a very interesting Guide for the use of morpholino in zebrafish.

Response: *The mentioned paper was added to the paper and discussed accordingly*

3- In line 81 of the ms the authors affirm that "It is important to standardize three critical elements of the MOs screening process: " I would ask the author to better explain the point ii)
Response*: We have expanded this part to make it more explanatory.*

ii) The phenotyping nomenclature used; a precise and easily understandable phenotypic description is critical to provide extensive explanations based on existing literature and investigator experience to facilitate information sharing among those who did not directly examine the embryos.
(iii) Having well-defined language made it easier to collect data centrally from Morpholino Database.(Bedell et al., 2011)

4- In line 145 I would suggest the author to remove the last part of the sentence "or to keep them frozen at -2°C." Actually, Gene Tools suggests to store morpholinos at room temperature in a sealed vial or freeze-dried (lyophilized).
https://www.gene-tools.com/sites/default/files/Storage_RTorLyophilized_v3.pdf ;
https://www.gene-tools.com/content/storage-morpholinos-refrigerate-or-room-temperature

Response: *We removed that sentence as requested*

5- Line 224: The authors should modify the sentence "MOs can be microinjected into the yolk of newly-laid zebrafish eggs, at up to the 8-cell stage" with "MOs can be microinjected into the yolk of newly-laid zebrafish eggs, at up to the 4-cell stage"

Response: *Requested modification was adapted*

6- Lines 307-309: I would suggest the authors to control point n. 7. There is a discrepancy between the images of figure 4 and their description in the text.

Response: *Text was corrected as requested*

Reviewer #2:
Manuscript Summary:
"Design and Microinjection of Morpholino and mRNA into Zebrafish Embryos to Elucidate Specific Gene Function in Heart Development" by Zakaria et al. provides a description of a protocol to induce gene knockdown using morpholinos and to rescue phenotypes using mRNA. Morpholino design, preparation, injection, and assessment are all covered in detail in this manuscript.

Major Concerns:

1- The most significant criticism is that the manuscript barely **glances over well-documented off-target effects of Morpholinos**. This must be elaborated upon.

Response: a paragraph about morpholinos limitations andoff-target effects was added to discussion section, as below
Morpholino (MO) technology has been extensively used in zebrafish, xenopus, sea urchins and more recently in cell culture model systems. With most methods, along with the benefits, there are also pitfalls that the experimenter should be aware of. One of the major pitfalls with MO technology include the concern that phenotypic effects observed by MO-mediated gene knockdown approach is not due to the loss of function associated with the primary gene product but that some other genes along with the primary gene or independent of the primary gene has been targeted, and thus this so called “off-target” effects is the reason for the “observed” morphant phenotype. This point came to the forefront when poor correlation was observed between morphants and mutants for the same gene in zebrafish (Kok et al., 2015). Thus, several recommendations and guidelines for morpholino use in zebrafish field (Bill et al., 2009;Stainier et al., 2017) have been established, and is recommended that experimenters’ follow these

guidelines. In our view, when possible, a second independent complementary approach (CRISPR) confirming the morphant or mutant phenotype is desireable. A second limitation include the ability to selectively knockdown a gene at space and time using MOs. Yes, photoactivatable MOs (Sumanas, 2017) are available but gene targets with complex expression patterns and those that have distinct functions at different times in development (early vs. late) will be difficult to address using this method. Further, the use of high light intensities to uncage/activate the MOs that may damage the embryonic tissue has also precluded its widespread use. A third limitation of MO is a general one for knockdown technology wherein only transient blocking of gene transcription is possible, and from a model system perspective, MOs will only work effectively in organisms like zebrafish, Xenopus, sea urchins where organogenesis occurs within a short time (2-3 days) period. This time frame limits the use of the technology in higher order organisms, and in adults. Some reports have emerged for in vivo MO use in mice (Kyritsi et al., 2017) although, such approaches have not gained widespread adoption. Finally, non-coding RNAs that comprise the bulk of our genome is a challenge to a target with MOs. MOs have successfully targeted short non-coding RNAs (miRNAs) (Flynt et al., 2017) but long (>200 bp) non-coding RNAs without translational start site or definitive exon-intron splicing sequence information remains a challenge.

2- Furthermore, the manuscript fails to mention recently published guidelines for Morpholino use in zebrafish (PMID: 29049395).

Response: *We included the mentioned guidelines in the manuscript*

3- In the introduction, the authors stress the importance of using a dose-response curve to optimize MO injections, but a dose-response curve is not presented among the figures. The dose-response curve should contain not only LD50, but also penetrance of on-target phenotypes (including cardiac defects) as well as known off-target phenotypes such as neural death (if observed). It may be worth noting that for pleiotropic genes such as hand2, different phenotypes may display different dose sensitivity as shown for cardiac and pectoral fin phenotypes of tbx5a (PMID: 32958829).

Response: *Hand2* a dose-response curve was *added in representative results section*

4- Similarly, rescue mRNA needs to be titrated and titration data has to be provided as an example.

Response: *Hand2 results were added in representative results section*

Minor Concerns:

1- In sections 4.7 and 4.8, the authors provide a description and calculation for using shear stress to measure cell velocity. While this description is detailed, a figure or a video demonstrating the method and quantifying the results would be beneficial.

Response: Figure 5 was added to show how to to measure cell velocity, to be use it for calculating shear stress, moreover, in the filming process of this article we showed a video for measuring cell velocity. Video 1 were added.

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