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Intrathoracic Injection for the Study of Adult Zebrafish Heart

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Anna Jazwinska, PhD
Associate Professor
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TITLE:

Intrathoracic Injection for the Study of Adult Zebrafish Heart

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KEYWORDS:

intrathoracic injection, microinjection, adult zebrafish, heart regeneration, cardiac preconditioning, heart stimulation, epicardium

SUMMARY:

This method relies on the injection of 0.5−3 µL of solution into the thorax of adult zebrafish. The procedure efficiently delivers proteins and chemical compounds into the proximity of the zebrafish heart without damaging the organ. The approach is suitable for testing effects of exogenous factors on various tissues of the heart.

ABSTRACT:

The adult zebrafish heart provides a powerful model in cardiac regeneration research. Although the strength of this system is based on transgenic approaches, a rapid delivery of exogenous factors provides a complementary technique in functional studies. Here, we present a method that relies on administration of a few microliters of solution into the pericardial cavity without causing myocardial damage. Intrathoracic (IT) injections can efficiently deliver proteins and chemical compounds directly onto the heart surface. The injected substances diffuse through the epicardium into the underlying cardiac tissues. Compared to intraperitoneal (IP) injections, the main advantage of intrathoracic injections is the focal administration of the tested factors on the target organ. The delivery of molecules directly into the pericardium is a suitable strategy for studies of cardiac preconditioning and regeneration in adult zebrafish.

INTRODUCTION:

Among vertebrates, zebrafish possess a remarkable capacity to regenerate their hearts[1,2]. This ability has been reported in several injury models, namely ventricular apex resection, cryoinjury (CI) and genetic cardiomyocyte ablation[3-7]. After invasive injuries, the damaged wall of the ventricle becomes transiently healed by fibrotic tissue, which is progressively replaced by a new myocardium[8-11]. The early wound healing response involves epicardium activation and recruitment of immune cells[12-15]. Concomitantly, cardiomyocytes near the injured myocardium become activated, dedifferentiate, proliferate and progressively replace the wounded area

within 30−90 days[16-19]. Substantial progress in deciphering the molecular and cellular mechanisms of cardiac regeneration has been achieved thanks to the availability of genetic tools, such as cell-lineage tracing analysis, inducible gene overexpression, fluorescent tissue reporter lines, and CRISPR/Cas9 gene mutagenesis[20,21].

We have recently established a model of cardiac preconditioning in the adult zebrafish by thoracotomy[22,23]. Preconditioning increases expression of cardioprotective genes and elevates the re-entry into the cell cycle in the intact and regenerating hearts. These processes are associated with the recruitment of immune cells and matrix remodeling[22,24]. The mechanisms of preconditioning are poorly understood, and the establishment of new techniques is required to foster this area of research. In particular, optimized administration of secreted signaling proteins or other chemical compounds is essential to further investigate this topic.

Being aquatic animals, zebrafish can naturally absorb various substances dissolved in water through their gills and skin. This offers a possibility for non-invasive drug delivery through immersion of fish in solutions with diverse chemicals, such as pharmacological inhibitors, steroid hormones, tamoxifen, BrdU and antibiotics. Indeed, numerous studies from various laboratories, including ours[25-27], have taken advantage of this method, which is particularly valuable in the field of regenerative biology[6,28]. This approach is, however, not appropriate for delivery of peptides, DNA, RNA, morpholinos or molecules with a limited tissue permeability. In these cases, a more efficient delivery is achieved by microinjection into the body, for example, by inserting the capillary into the retro-orbital venous sinus, into the intraperitoneal or intrapericardial cavity[29-31]. Here, we describe a procedure of intrathoracic injection of a small amount of solution, as a suitable method to study heart regeneration and preconditioning in adult zebrafish.

PROTOCOL:

Animal care and all animal procedures described in the following protocol were approved by the cantonal veterinary office of Fribourg, Switzerland.

1. Tools and solutions for injections

1.1. Pull microinjection-adapted borosilicate glass capillaries using a needle puller according to **Figure 1A**. Store pulled capillaries in a 9 cm Petri dish with rails of modeling clay or adhesive tape.

1.2. Using common scissors, cut a piece of sponge (7 cm x 3 cm x 1 cm) and carve a fish-like silhouette in its middle.

1.3. Prepare small aliquots of injection solution with the tested proteins or other compounds. Adjust their concentration dependently on the assay by diluting the substance in 1x Hanks balanced salt solution (HBSS) complemented with 10% phenol red.

NOTE: Here, the concentration of the tested protein was 100 ng/Ml.

1.4. To prepare a stock solution of buffered tricaine anesthetics, dissolve 4 g of tricaine in 980 mL of distilled water. Adjust pH to 7.0−7.4 using 1 M Tris-HCl pH 9, and fill up with water to 1000 mL. Store the solution in dark at 4 °C.

1.5. To obtain the working concentration of anesthetics, add 1−2 mL of tricaine stock solution into 50 mL of fish water in a beaker.

NOTE: The working concentration of tricaine anesthetics should be prepared freshly before use.

2. Preparation of the injection station

2.1. Turn on the stereomicroscope with the light from the top and adjust magnification to 16x.

2.2. Soak the sponge with fish water, place it on a 9 cm Petri dish on the microscope stage and adjust focus.

2.3. Under the stereomicroscope, cut the end of a microcapillary at ~7 mm from the basis using an iridectomy scissors as shown in **Figure 1A**. The ideal tip diameter would be ~20 µm.

NOTE: Cutting the tip of the capillary in an oblique way is optimal for insertions into the tissue.

2.4. Insert the microcapillary into the needle holder of the microinjector apparatus.

2.5. Using microloader tips, load a control solution (i.e., 1x HBSS) to set up the pressure of injection, in order to obtain the appropriate flow ranging between 0.3 µL/s and 0.5 µL/s. Empty the needle.

2.6. Load the selected volume of the injection solution (i.e., ciliary neurotrophic factor [CNTF] diluted in 1x HBSS) into the tip of the capillary (**Figure 1B**). There should be no air bubble in the capillary.

NOTE: The maximal volume of injection solution depends on the fish size. For a standard length of 2.5−3 cm (distance from the snout to the caudal peduncle), the maximum injection volume that prevents exessive thoracic swelling and bleeding was determined to be 5 µL (**Figure 1F**). Larger volumes could be injected to bigger fish.

3. Preparation of the fish for intrathoracic injection

3.1. Catch an adult fish (*Danio rerio*) with a net and transfer it into the anesthetic solution.

3.2. After 1−2 min, when fish stops swimming and the movement of operculum is reduced, touch the fish with a plastic spoon to make sure it does not react to any contact.

3.3. Quickly and carefully transfer the fish with the spoon into the groove of the wet sponge, with ventral side up. The head of the fish should point away from the operator's dominant-hand.

4. Microinjection into the pericardium

4.1. Under the stereomicroscope, carefully observe the movement of the beating heart under the skin of the fish. Visually determine the injection point above the beating heart and in the middle of the triangle defined by the ventral cartilaginous plates (**Figure 1D**). Insert the tip of the capillary at 30−45° degree angle relative to the body axis (**Figure 1E**). Gently penetrate the skin with the tip of the microcapillary into the pericardium (**Figure 1C**). An optimal entry point is closer to the abdomen than to the head.

NOTE: Do not insert the capillary too deeply into the body and the heart, as this will cause injury to the organ. In case of heart puncture, the needle generally fills with blood. If this happens, remove the capillary and exclude the fish from the experiment.

4.2. Once the needle is inside the pericardium, complete injection by pressing the pedal of the microinjector device.

NOTE: Be careful not to inject air into the thoracic cavity.

4.3. After injection, gently withdraw the capillary from the thorax and immediately transfer the fish into a tank with system water for recovery.

4.4. Monitor the fish until total recovery from anesthesia.

4.5. Collect heart at the desired time point and prepare it for further analysis.

NOTE: In case the fish does not resume movement of the operculum within 30 s, reanimate the fish by squeezing water into the gills with a plastic pipette.

REPRESENTATIVE RESULTS:

After intrathoracic (IT) injections, the effects of exogenous solution can be analyzed. For this purpose, the fish should be euthanized and the hearts collected, fixed and histologically processed, according to previously published protocols[32,33].

To validate the method, we first performed two test experiments by injecting color and fluorescent dyes. First, we euthanized fish and post-mortem injected 3 µL of ink into the thorax. The hearts were collected after 5 min, washed in phosphate-buffered saline (PBS), fixed in 2% formalin, washed in PBS and photographed under the microscope. Second, we injected 3 µL of 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in vivo, and fixed the heart after 2 h. In both assays, whole-mount analysis revealed labeling of the entire heart including the ventricle, the atrium and the bulbus arteriosus (**Figure 2A,B**). These results reveal efficient spreading of the

injected solution on the heart surface.

A common protocol for delivery of exogenous substances into the adult fish is intraperitoneal (IP) injection. To compare the suitability of IT versus IP injections for heart studies, we injected a similar amount of DAPI using both methods and fixed the hearts after 5 min and 120 min (**Figure 3A**). The hearts were sectioned and stained with phalloidin Alexa Fluor (AF) 568 that labels F-actin in cardiac muscle. No DAPI-positive cells were observed in the hearts after IP injection at both time points (**Figure 3B**). By contrast, IT injection resulted in the presence of DAPI-labeled nuclei in the myocardium (**Figure 3B**). These results demonstrate that IT injection improved the delivery of the compound to the heart, as compared to the IP injection.

To test the suitability of this method for heart regeneration studies, we cryoinjured ventricles[8], and performed IT injections of 3 µL of 1 µg/mL DAPI and 1 µg/mL phalloidin AF649 at 3 and 7 days post-cryoinjury (dpci) (**Figure 4A**). At 1 h post-injection, the hearts were collected, fixed, sectioned and stained with phalloidin AF568 to visualize the intact myocardium. We found that both the myocardium and the injured tissue contained numerous DAPI positive cells, indicating an efficient penetration of this dye into the intact heart and fibrotic tissue (**Figure 4B**). Furthermore, injected phalloidin AF649 was also incorporated by cardiomyocytes of the peri-injury zone and some recruited fibroblasts of the wounded area. This experiment reveals that the drugs can cross the epicardium and penetrate into the underlying myocardium.

After testing the efficiency of IT injections using dyes, we analyzed the effects of injected proteins on the heart. We synthetized a cytokinine, called CNTF, which is upregulated after thoracotomy[24]. We investigated the effects of the exogenous CNTF cytokine on various processes, namely cardiomyocyte proliferation, extracellular matrix deposition, immune cell recruitment and cardioprotective gene expression. We found that all these biological aspects were activated by IT injection of CNTF, as compared to control immunoglobulins (**Figure 5**)[24]. These results demonstrate that the method of intrathoracic injection provides a suitable strategy for targeted delivery of proteins to study their effects on distinct heart tissues in a variety of assays.

FIGURE LEGENDS:

Figure 1: Intrathorascic (IT) injection in adult zebrafish. (**A**) Photograph of a pulled microinjection capillary with filament (6”, 1.0 mm in diameter) and values of the needle puller program used. (**B**) Photograph of a pulled microinjection capillary with filament (6”, 1.0 mm in diameter) filled up with 2.5 µL of solution containing 10% phenol red. The pulled tip of the needle is maximally 7 mm long. (**C**) Schematic representation of the IT injection procedure. (**D**) Photographs of the IT injection procedure. This figure has been modified from Bise et al.[24]. Numbers in panels C and D correspond to the same steps of the procedure: (1) fish is placed ventral side up on a humidified sponge. The puncture site (red dot in the triangle) is located in the center of the chest near the gills. (2) Penetration of the needle into the pericardium. Red dot indicates puncture site. (3) Injection is monitored by observing the spread of the red solution in the pericardial cavity. (**E**) Scheme of IT injection. Angle between the injection

capillary and the body axis should be between 30° and 45° to avoid heart puncture. (**F**) Photoraph of fish thorax at 1 hour after IT injection of indicated volumes. White arrows are pointing at the redish tissue, which might indicate internal bleeding.

Figure 2: IT injected solutions spread nearly uniformly on the heart surface. (**A**) Stereomicroscope images of whole hearts of fish subjected to post-mortem IT injection with 2.5 µL HBSS or 2.5 µL ink. Ink stained the surface of the ventricle (V), atrium (A) and bulbus arteriosus (Ba). Scale bar, 300 µm. (**B**) Bright-field and fluorescent stereomicroscope images of whole hearts of fish subjected to IT injection with HBSS and 3 µL of 1 µg/mL DAPI. DAPI fluorescence is detected on the heart parts soon after IT injection. Scale bar, 300 µm.

Figure 3: Comparison of two injection methods for the delivery of DAPI to the heart. (**A**) Scheme of the experimental design. Intraperitoneal (IP) and Intrathorascic (IT) injections were performed with the same amount of 1 µg/mL DAPI (3 µL). Hearts were collected at 5 and 120 minutes after injection. (**B,C**) Confocal microscopy images of heart sections stained with fluorescent phalloidin (red) that abundantly labels muscle fibers. Injected DAPI was visualized in the appropriate channel shown in green. (**B**) After IP injection, DAPI is not detected in the heart at any time point. (**C**) After IT injection, DAPI positive cells are present in the ventricle after both time points. Scale bar, 500 µm.

Figure 4: IT injection to study heart regeneration. (**A**) Scheme of the experimental design. At 3 and 7 days following cryoinjury, a mix of DAPI and phalloidin AF649 was IT-injected (3 µL of 1 µg/mL). Hearts were collected 1 hour after IT injection, fixed, sectioned and stained with phalloidin AF568 (red). (**B**) Confocal microscopy images of longitudinal heart sections at 3 and 7 dpci. Injected DAPI (green) and phalloidin AF649 (blue) label cells of the injured area (delimited by white dashed line) and the intact myocardium (red staining). White arrows are pointing at DAPI (green) distribution through intact compact and trabeculated myocardia and the epicardium. Scale bar, 500 µm.

Figure 5: Exogenous IT-injected CNTF stimulates several biological processes in the heart. (**A**) Scheme of the experimental design. First, 2.5 µL of a solution containing 250 ng of zebrafish CNTF or control immunoglobulins (hIgG) was injected into the pericardium of transgenic fish expressing nuclear DsRed2 in cardiomyocytes. Hearts were collected at 7 and 1 days post-injection (dpi) and analyzed by immunofluorescence and in situ hybridization, respectively. (**B-D**) Confocal microscopy images of ventricular sections of control and CNTF-injected hearts. (**B**) Immunostaining against a cell cycle marker, minichromosome maintenance complex component 5 (MCM5; green), reveals a higher number of proliferating cardiomyocytes in response to exogenous CNTF. Scale bar, 500 µm. (**C**) Immunostaining against collagen XII shows increased deposition of collagen XII in the myocardium after CNTF injection. In control heart, collagen XII is confined to the epicardium[34]. Scale bar, 500 µm. (**D**) Immunostaining against an immune cell marker, L-plastin, detects an enhanced recruitment of immune cells in the CNTF injected fish. Scale bar, 500 µm. (**E**) Bright-field microscope images of ventricular cross sections after in situ hybridization using an antisense mRNA probe against cystatin, a cardioprotective factor, displays transcriptional upregulation of this gene in the heart of CNTF-injected fish. Scale

bar, 500 µm. This figure has been modified from Bise et al.[24].

DISCUSSION:

Here, we describe a method for delivering exogenous compounds and proteins into the pericardial cavity in order to study their effects on the heart in adult zebrafish. The procedure is based on intrathoracic injection, which results in the delivery of a small volume of solution in the vicinity of the organ. This technique was developed and described for studying cardiac preconditioning and regeneration.

The critical step in this procedure is the penetration of the glass capillary into the thoracic cavity. This step depends on three parameters which are: the rigidity and sharpness of the capillary tip, the angle of penetration, and the puncture site. To optimize the penetration through the skin, the pulled part of the capillary should not to be too long, as such needles are too flexible and bend in contact with the skin. To avoid this, the rigidity can be adapted by reducing the tip size with the iridectomy scissor. Although the angle of penetration can vary between 30° and 45°, it can be adapted to the rigidity of the tip. Indeed, a thin tip will penetrate the skin better with a narrower angle.

In order to optimize the needle penetration, the injection site should be immediately above the beating heart. The risk of heart puncture is usually low ranging between 5% and 8%. Insertion of the needle posterior to the heart increases the risk of heart puncture, as seen by enhanced bleeding. In such cases, the animals should be removed from the experiments.

Another source of trouble during the IT injection occurs at the capillary level. Indeed the capillary can break when lateral forces are exerted on it. To avoid this, the needle must move along the axis of injection in a straight way. Occasionally, the capillary can be blocked by tissue residues that prevent the liquid from flowing. The needle can be unblocked by gently withdrawing the tip whilst injecting. If this does not improve the flow, we recommend completely withdrawing the needle from the thorax and replacing the needle.

Lesions can be caused by a too deeply inserted needle in the pericardium. In order to avoid lesions in the pericardial sac, the needle must not be inserted too much (1−2 mm) into the thorax. Some leaks were observed when the injection volume was bigger than 8 µL.

In zebrafish, the exact composition of the pericardial fluid is unknown. However, the volume of the pericardial cavity is estimated at ~10 µL[31]. Given that the volume of the adult zebrafish ventricle is approximately 1−2 mm^3, we assume that the pericardial cavity accordingly has a tiny volume, which has to be considered before injections. From our preliminary studies, we determined that the optimal range of the injected volume is between 0.5 and 3 µL for fish measuring 2.5−2.8 cm (distance from the snout to the caudal peduncle). This volume can be adapted depending on the size of the fish. Injection of up to 5 µL did not induce any lesion in the fish of this size. However, volumes from 8 µL were sufficient to cause bulging and internal bleeding as shown in **Figure 1F**. Based on this data, we estimate that an amount of solution bigger than 3 µL might cause physical and physiological stress on the organ. This limitation

infers the need to choose a higher concentration of molecules instead of increasing the amount of the injected solution.

Another important factor is the osmotic property of the injected solution, which should be in the physiological range. Indeed, to avoid a risk of osmotic stress, we recommend HBSS as injection medium.

In zebrafish, the common methods used to deliver drugs are through water treatment and intraperitoneal injection[30,35]. Although both of these techniques are suitable for many applications, IT injections provide experimental and economic advantages, by decreasing the risks of undesired systemic side effects and reducing the usage of costly molecules, respectively. This method can be suitable for delivery of tamoxifen to activate the Cre-ERT2 transgenic system used for cell lineage tracing analysis, and guide modified RNAs for functional studies in regeneration research.

The IT-injection method in zebrafish has been previously described[31,36]. In those reports, intrathoracic injections were performed with insulin needle, puncturing from the anterior side. In contrast, our protocol presents an alternative strategy with the pulled glass capillary inserted from the posterior direction. Specifically, our approach takes into account the anatomy of the fish pericardium to optimize the injection with a reduced risk of heart puncture. Furthermore, during the procedure, the fish is not held by metallic forceps, but by a moist and soft sponge, which is a more suitable method to avoid any external injury of the fish. Thus, the presented method might be better suited for studies of cardiac homeostasis, preconditioning and regeneration in adult zebrafish.

IT injections have already been established in mammalian model organisms. Indeed, this method has also been applied in experiments with pigs and clinical studies in humans[37,38]. In mice, transthoracic intramyocardial injections guided by ultrasounds have been used to challenge their heart[39]. Within this article we propose a detailed protocol to ease the use of IT injection for zebrafish. This will be particularly valuable to the field, in order to complement genetic approaches in cardiac homeostasis, preconditioning and regeneration research.

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DISCLOSURES:

The authors have nothing to disclose.

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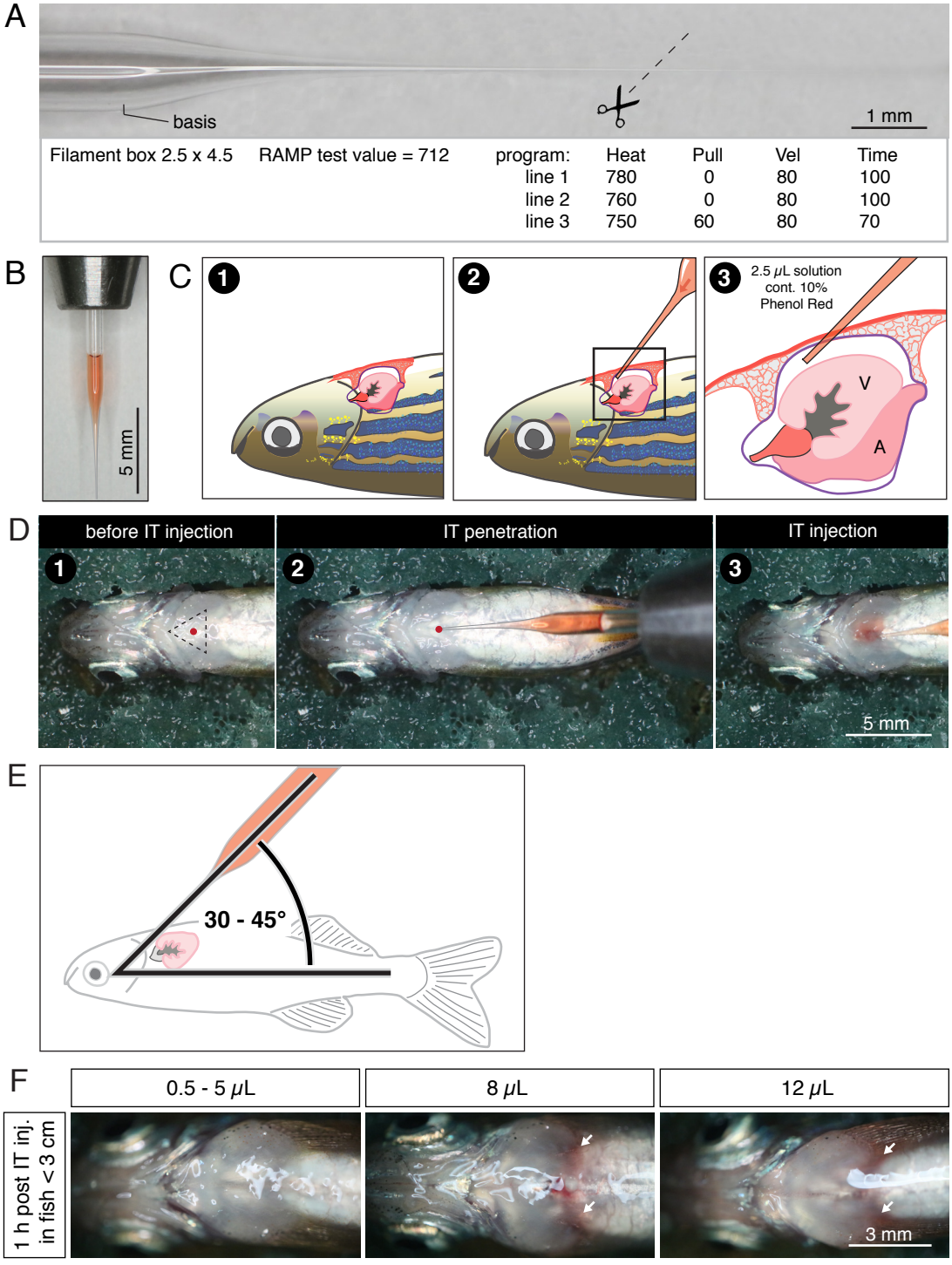
Figure 1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line 3 750 60 80 70
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Figure 2

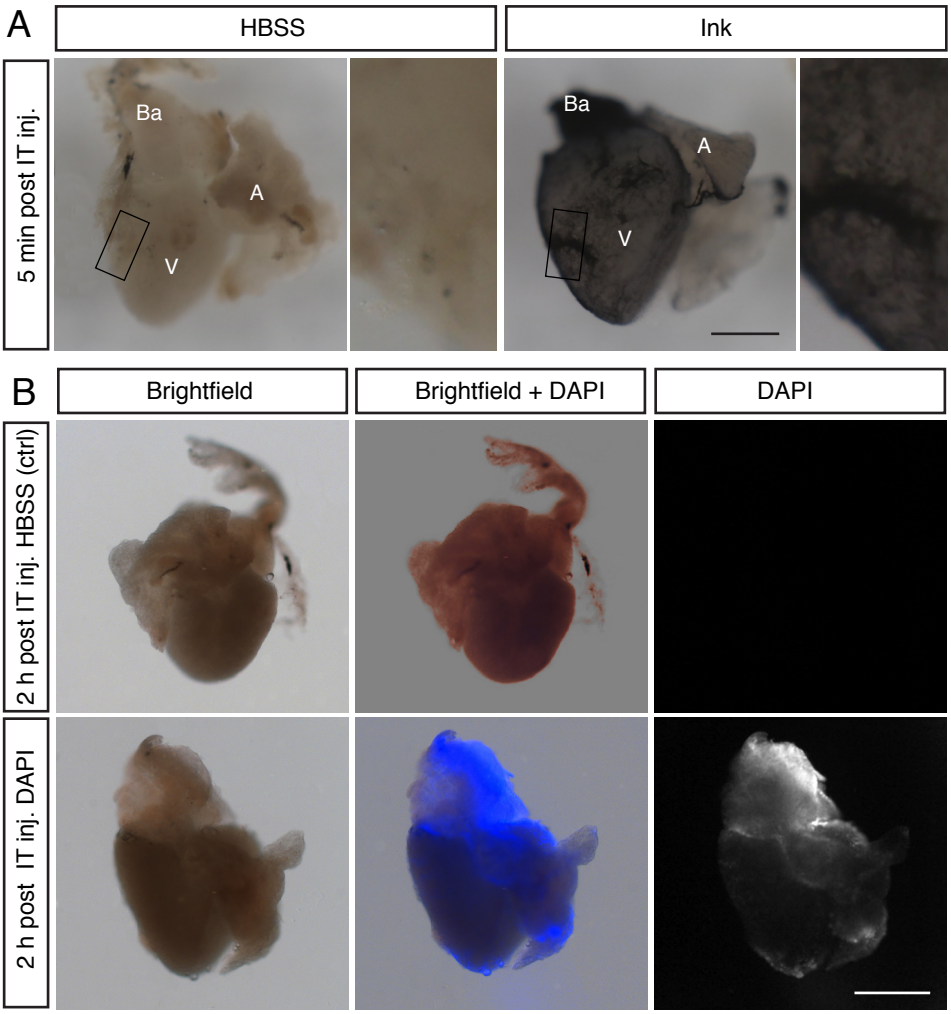

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Figure 3

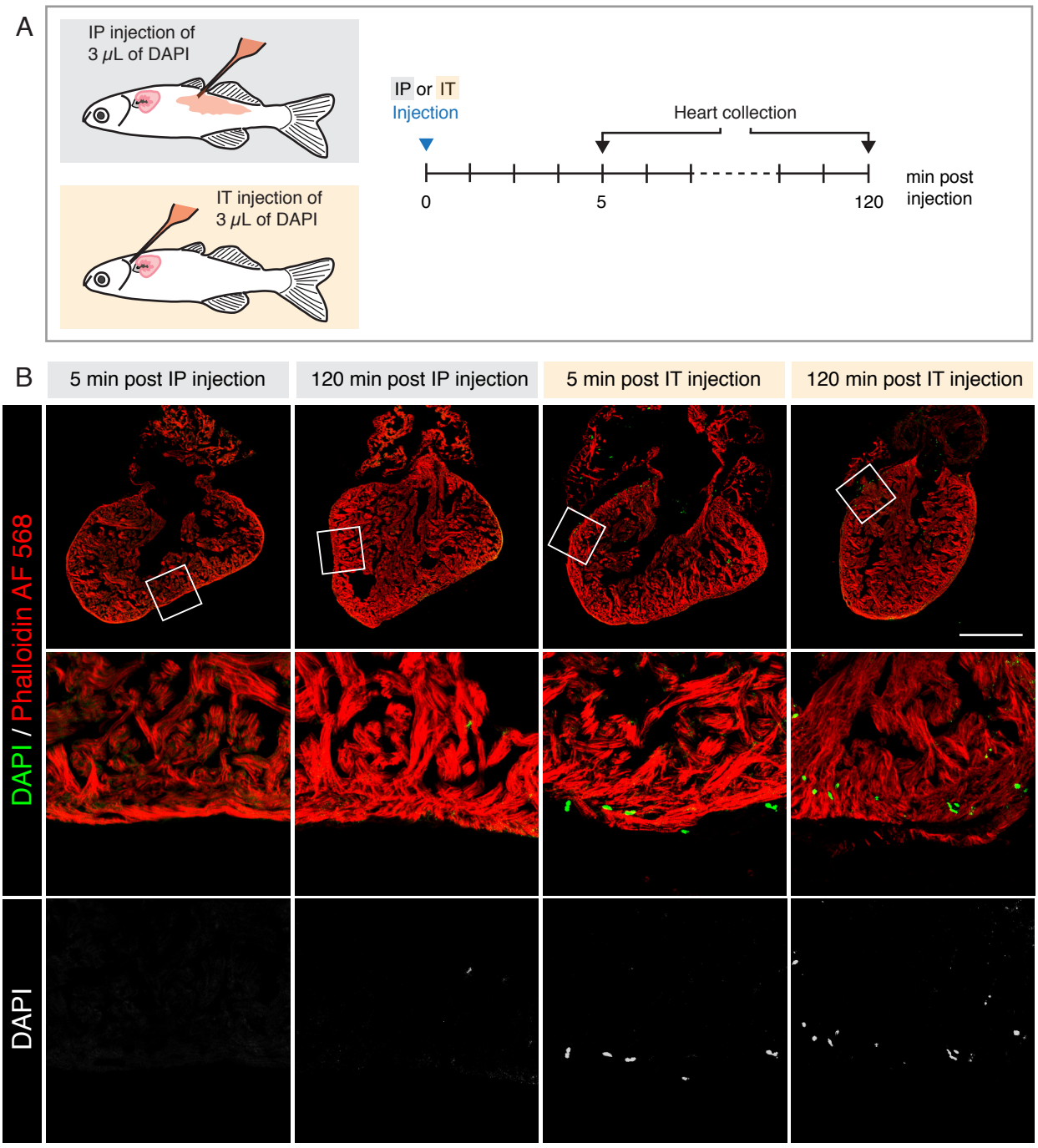

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Figure 4

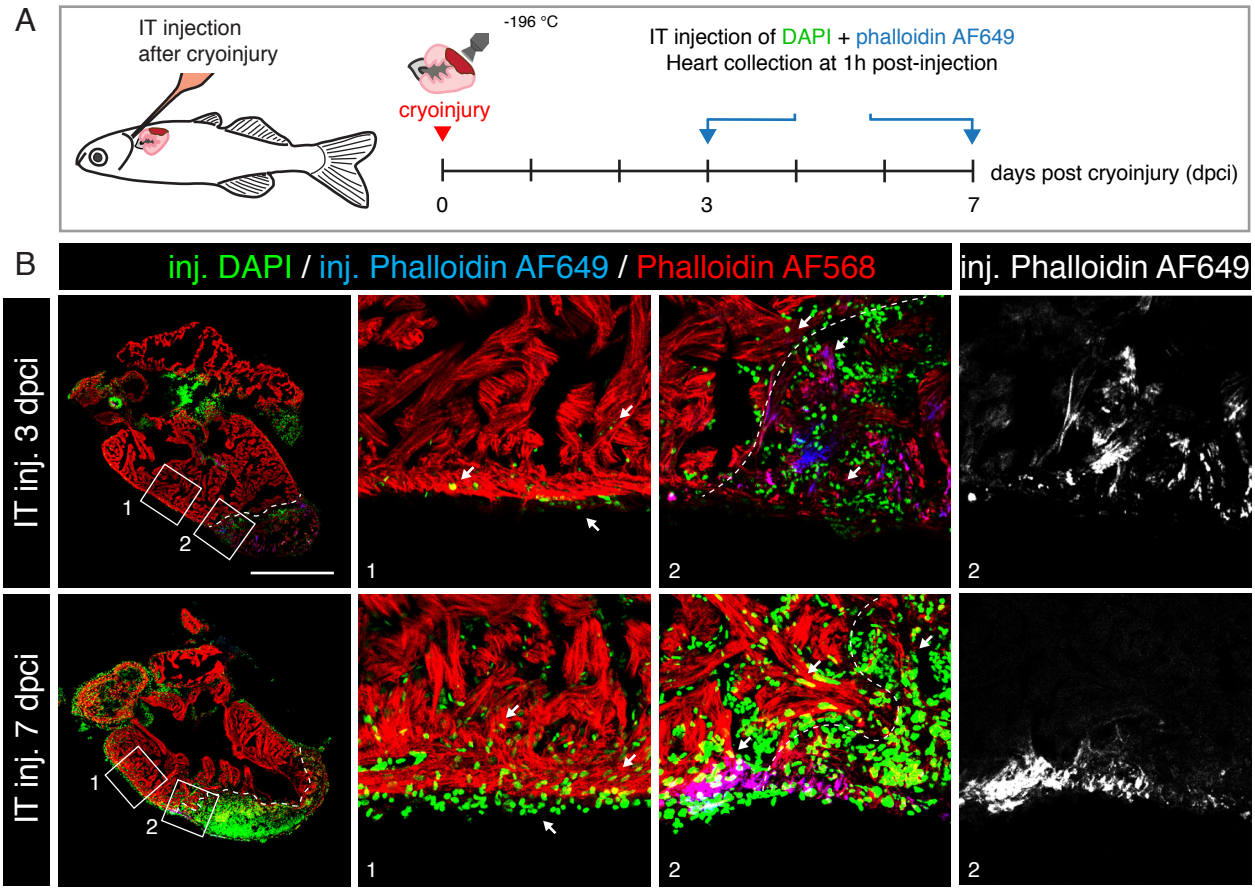
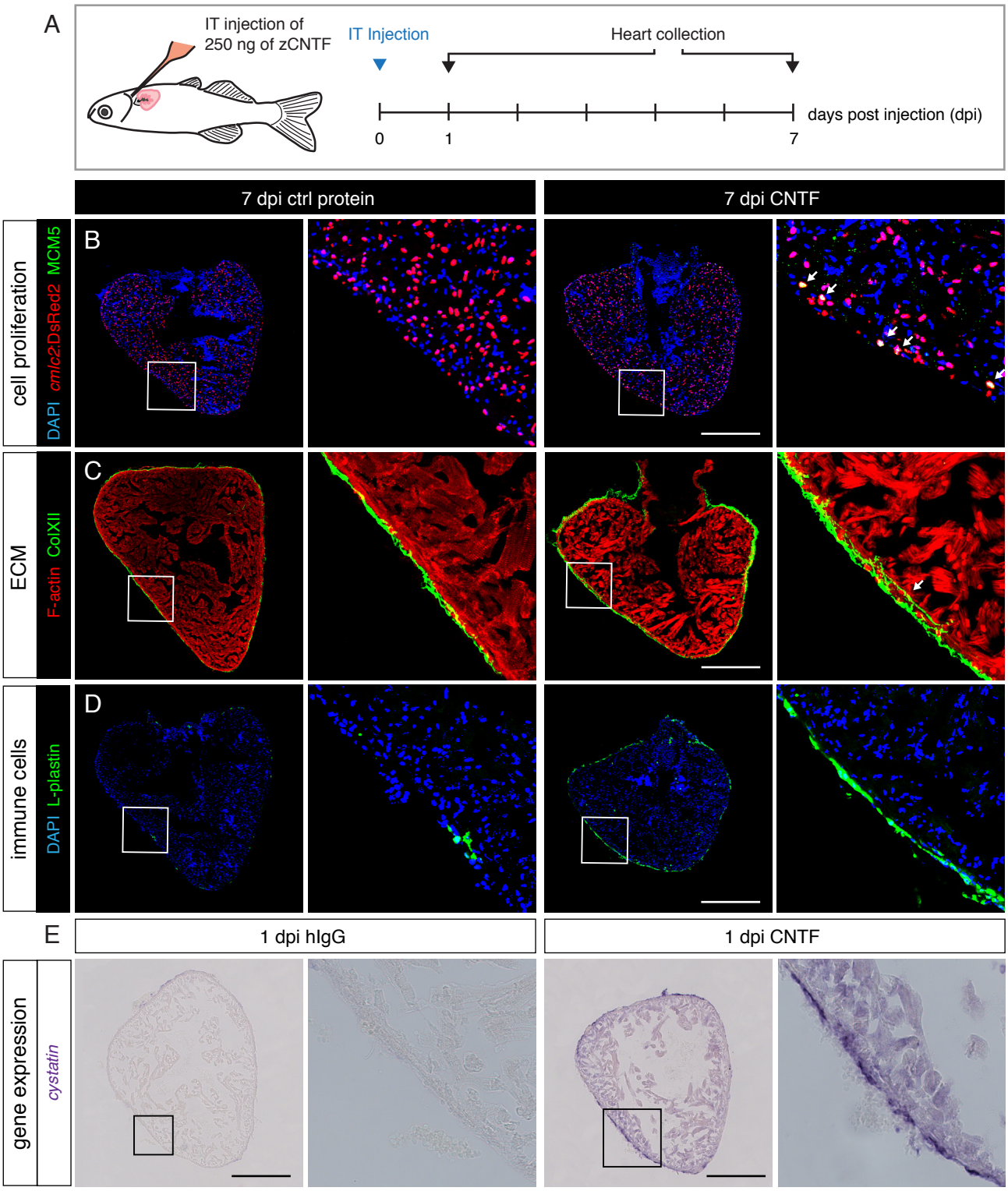


Figure 5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7 dpi ctrl protein
7 dpi CNTF
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DAPI cmlc2:DsRed2 MCM5
B
ECM
F-actin ColXII
C
immune cells
DAPI L-plastin
D
E
1 dpi hIgG
1 dpi CNTF
gene expression
cystatin

Table of materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Hanks Balanced Salt Solution	Gibco by Life technology	14065-056	
Iridectomy scissor	Roboz Surgical Instruments (	RS-5602	
Macroscope (binocular)	M400		with Apozoom
Micro-injector femtojet	Eppendorf	5247 0034 77	
Microloaders femtotips	Eppendorf	5242 956.003	
Micropipette glass needles type C	WPI	TW100F-6	thin-wall capillary
Micropipette puller model P-87	Flaming/Brown	20081016	filament box 2.5 x 4.5 mm
Sponge	any	any	dim. carved sponge 7cm x 3 cm x 1 cm
Tricaine (Anestethic)	Sigma	E10521	
Dyes and Antibodies	**Company**	**Catalog number**	**Concentration**
anti-Chicken Cy5	Jackson ImmunoResearch Laboratories		1 / 500
anti-Guinea pig Cy5	Jackson ImmunoResearch Laboratories		1 / 500
anti-Rabbit Cy5	Jackson ImmunoResearch Laboratories		1 / 500
Chicken l-plastin	gift from P. Martin, Bristol		1 / 1000
DAPI	Sigma	10236276001	1 / 2000 (1µg/ml); 1/100 IT injected
Guinea pig anti-ColXII	gift from Florence Ruggerio, Lyon		1 / 500
Phalloidin-Atto-565 (F-actin)	Sigma	94072	1 / 500
Phalloidin-Atto-647 (F-actin)	Sigma	95906	1 / 50 IT injected
Rabbit anti-MCM5	gift from Soojin Ryu, Heidelberg		1 / 500
Stamping Ink 4K	Pelikan	1 4k 351 197	1 / 1
ISH probe primers	**gene number**	**fw primer**	**Rev primer**
Cystatin	ENSDARG00000074425	GATTCACTGTCGGGTTTGGG	ATTGGGTCCATGGTGACCTC

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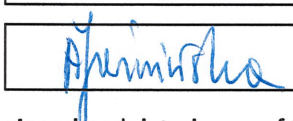
Signature:	
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Our response to Reviewers' comments:

We read reviews of our submission with great interest. We were gratified that both reviewers found substantial merit to our work that makes it potentially suitable for publication in JoVE. We have addressed the criticism of both reviewers, and we are submitting a revised manuscript for publication.

We thank both reviewers for their constructive comments, which improve our manuscript.

Reviewer #1:

Manuscript Summary:

In this manuscript entitled " Intrathoracic injection for the study of adult zebrafish heart," Bise and Jaźwińska present a method protocol to administrate a few microliters of solution into the pericardial cavity without causing myocardial damage, named as intrathoracic injection (IT). This method may provide a complementary, rapid delivery technique for functional analysis of exogenous chemicals, siRNA, RNA or proteins. The authors showed that the IT efficiently delivered proteins and chemical compounds onto the heart surface. As a result, it is a suitable strategy for studies of cardiac preconditioning and regeneration in adult zebrafish. The experimental procedure described in this paper is clearly and well written. However, there are a few questions need to be addressed by the authors.

Major Concerns:

1. In the discussion part, the authors conclude "With this report, the IT procedure of injection into the pericardium is established for zebrafish". However, as far as I know, several other studies have tried intrathoracic injections (Xiao et al., 2018; Diao et al., 2015). Moreover, it is more interesting to compare these two different intrathoracic injections rather than compare with intraperitoneal injection.

Our answer:

The studies mentioned by the reviewer (Xiao et al., 2018 ; Diao et al., 2015), use larger volumes for intrathoracic injections. In the revised manuscript, we precise that the maximal injection volume depends on the fish size. For standard lenghth of 2.5-3 cm (distance from the snout to the caudal peduncle), we determined that the maximum volume injected without causing exessive thoracic swelling and bleeding was 5 µL. However, larger volumes could be injected to bigger fish. This information is now provided in the Note for the point 2.6. We also included new data in Figure 1F.

2. One drawback of the approach is the potential for preferential targeting of the epicardium because these cells will be exposed to the highest protein and chemical compound concentration. What are not well addressed in this work is how the myocardium is efficiently targeted.

Our answer: We agree with the reviewer that the injected solution first contacts the epicardium. However, the epicardium does not form a tight barrier, it is a relatively thin tissue, permeable for many substances. To verify this statement, we performed an additional experiment in the revised manuscript. In the new Figure 4, we show that

certain chemical compounds, such as DAPI, Phalloidin, can pass through the epicardial layer and access the underlying myocardium within just one hour.

3. The authors concluded that delivery of more than 10 µl of solution impairs wellbeing of the animals. How did the authors measure the wellbeing of the animals? It would be better if they included their original data.

Our answer:
Indeed, we recommend the use of a volume ranging between 0.5 and 3 µL. The zebrafish heart is a very small structure, its ventricle has a diameter of approx. 1 mm, which corresponds to a volume of 1 µL. Injection of many times more solution than the size of the organ can be considered as a physiologically and physically stressful condition for the animal. In the revised manuscript, we now included an additional figure showing that the injection of larger volume causes aneurysm of the thorax and internal bleeding (a new Fig. 1F and a note for the point 2.6).

4. In protocol "4.1, what is the rate of causing heart puncture?

Our answer:
Like for any invasive procedure, the rate of causing heart puncture depends on the experience of the surgeon. In this manuscript, we provide a detail description of the procedure. If it is carefully followed and with some practice, we expect the rate of heart puncture is in the range of 5-8 %.

Minor Concerns:
1. The authors should define all abbreviations before use, like intrathoracic injection (IT).
2. In Figure 2, "HBS" should be "HBSS".

Our answer:
We corrected these comments.

Reviewer #2:
Manuscript Summary:
This manuscript described a detailed procedure for intrathoracic injection of proteins and chemical compounds for the purpose of studying adult zebrafish hearts. This can serve as a rapid functional assay to test different exogenous factors. The molecules will be delivered directly into the pericardium and will be used as a strategy to study cardiac preconditioning and regeneration of adult zebrafish hearts. This manuscript is very well written and the data are of high quality.

Major Concerns:
1. The sizes of adult fish hearts can vary significantly from fish to fish. Therefore, the pericardial cavity volume can be different as well. The authors should clarify how differences in heart size and pericardial cavity volume can affect the variation of the results. For instance, if a fixed amount of proteins or chemical compounds is injected, the effective concentration on the hearts might be different for individual fish.

Our answer:

It is important to use fish of the same size for control and experimental injections. In most of our experiments, we select fish that are 2.5-3 cm long (measured from the snout to the caudal peduncle). We added this specification in the manuscript (See a note for the point 2.6 and a new Figure 1F). In addition, we suggested that higher volumes can be injected to larger fish.

2. It seems that the injected proteins and chemical compounds might affect one side of the ventricle facing the pericardial cavity more than the other side attached to the atrium. How will this affect the results? The readouts such as cardiomyocyte proliferation or gene expression might show asymmetric distributions on the ventricle. For instance, in Figure 4 panel E, the cystatin expression (in situ) at 1dpi CNTF seems more upregulated on one side than the other side.

Our answer:
We cannot exclude an asymmetric response of the ventricle after IT injection. However, injection of ink and DAPI showed a rather uniform spreading of the reagent all around the heart. Thus, the effects of the injected substance are likely to be uniform along the heart circumference.

3. The authors discussed the importance of positioning the injection needle between 30 and 45 degrees to the body axis to avoid heart puncture. They should also discuss how to prevent needles from protruding through the anterior side of the pericardial sac (Fig. 1B-3, anterior to the left). If the needle pokes through the pericardial sac, the factors will leak out and might be not effective in affecting the heart ventricle.

Our answer:
Indeed the needle should not be inserted too deeply into the body. In the revised manuscript we modified the note at the point 4.1 to address this remark.

Reviewer #3:
This manuscript from Bise and Jazwinska describes a protocol to perform pericardial injections in adult zebrafish. The authors report a new system to deliver small amounts of drugs or peptides directly to the heart using a glass needle.
The article is very well written and clearly articulated. It describes a useful protocol, and I anticipate that it will be broadly used in zebrafish cardiac studies. The authors accompany the text with very illustrative cartoons. References are properly selected. Overall, I believe this is a very nice contribution to the field, although there are a few issues that need attention.

Specific comments:
1) To successfully use this protocol, readers will need to produce needles that are compatible with this application. These are probably quite different to the needles that most zebrafish laboratories use for embryo injection. Thus, the authors should provide a much more detailed protocol and instructions for producing the needles in Step 1.1. For example, they should explain the program that they used in their needle puller (temperature, time, ramps, etc.). Providing this description will ensure the reproducibility of this protocol in other laboratories.

Our response:

In the revised figure 1, we provide additional information about the program for the needle puller (Fig. 1A).

2) While I agree with the authors that this method is useful to study certain aspects of cardiac biology such as cardioprotection, I believe they have not properly demonstrated that this method is relevant for cardiac regeneration studies (as referenced in line 224-225). To induce injuries to the heart, the ventricle is exposed by opening the thorax. The body wall closes after a few days post injury, but this healed structure is significantly more fragile than the uninjured thorax. Is the regenerating pericardium leaky? Would it retain the injected volume? The authors should include in this manuscript some experiment to demonstrate that their method is also useful to study regeneration (for example, injecting some dye in the pericardial cavity at 3 or 7 dpi).

Our response:
We agree with the reviewer that our method has been developed and used mainly for preconditioning studies. However, this method is also useful for studying heart regeneration after cryoinjury, as reported in our recent publication (Bise et al., 2019). To demonstrate the application of the IT injection method after heart injury, we performed a new experiment, which is presented in a new Figure 4. Specifically, at 3 and 7 dpci, we injected a mixture of DAPI and Phalloidin Alexa Fluor 649, and analyzed the heart at 1 hour post-injection. Both molecules penetrated cryoinjured area. Furthermore, the initiation of diffusion of the drug into the myocardium becomes evident.

3) In the summary section, the authors claim that this method is suitable for "testing effects of exogenous factors on various tissues of the heart". Again, they have not properly demonstrated that this is the case. My understanding is that they try to do so in Figure 3, when they inject DAPI either intra-peritoneally or intra-thoracically. However, in vivo DAPI only labels damaged/dying cells. It would be more convincing if the authors use a vital dye (i.e., BODIPY ceramides or BODIPY 505) which would label all cells. This would be much more convincing than the present data.

Our response:
According to our experience and literature, DAPI can label living cells without a need of membrane permeabilization.
Here are the examples of previous articles reporting the suitability of DAPI for live-cell labeling:
https://doi.org/10.1016/S1046-2023(02)00289-X
https://doi.org/10.3727/096368908786576444
https://doi.org/10.3727/096368909788809811
https://doi.org/10.1111/jmi.12133

Furthermore, our new Figure 4 also demonstrates this feature of DAPI.

4) I would recommend expanding a bit the discussion to mention that this method could be potentially useful to deliver molecules such as tamoxifen (to induce CreERT2 lines used in regeneration studies), and even guide RNAs or modified RNAs to perform gain and loss of function studies.

We added this suggestion to the discussion.

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