TITLE PAGE

Title of the article: Corrosion casting of the cardiovascular structure in adult zebrafish for analysis by scanning electron microscopy and x-ray microtomography

Running title: Zebrafish corrosion casting

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With 5 figures.

Summary/Abstract

Zebrafish have come to the forefront as a flexible, relevant animal model to study human disease, including cardiovascular disorders. Zebrafish are optically transparent during early developmental stages, enabling unparalleled imaging modalities to examine cardiovascular structure and function *in vivo* and *ex vivo*. At later stages however, the options for systematic cardiovascular phenotyping are more limited. To visualize the complete vascular tree of adult zebrafish, we have optimized a vascular corrosion casting method. We present several improvements to the technique leading to increased reproducibility and accuracy. We designed a customized support system and use a combination of the commercially available Mercox II methyl methacrylate with the Batson's catalyst for optimal vascular corrosion casting of zebrafish. We also highlight different imaging approaches, with a focus on scanning electron microscopy (SEM) and x-ray microtomography (micro-CT) to obtain highly detailed, faithful three-dimensional reconstructed images of the zebrafish cardiovascular structure. This procedure can be of great value to a wide range of research lines related to cardiovascular biology in small specimens.

Keywords: Zebrafish, cardiovascular physiology, corrosion casting, scanning electron microscopy, X-ray microtomography

Introduction

The use of animal models has strongly advanced the knowledge of the structure and function of the cardiovascular system, allowing for the development of new strategies to address disorders affecting this organ system. Zebrafish (*Danio rerio*) have emerged as a flexible model, complementary to mammalian animal models, not only for fundamental biological research but also as an efficient tool for the discovery of new (pharmacological) treatments for human disease, particularly for heritable conditions (Asnani & Peterson, 2014, Wang, Sips, Khin, Rotival, Sun et al., 2016, Zhao, Zhang, Sips & Macrae, 2019). A large body of work exists on the physiological and biochemical phenotyping of zebrafish embryos and larvae, which due to zebrafish size and fecundity can be assayed at scale. For cardiovascular research, the combination of genetic tractability and optical transparency allow for efficient visualization of cardiac and blood vessel structures during early developmental stages *in vivo* and *ex vivo* using genetically encoded reporters and specialized microscopy techniques (Lawson & Weinstein, 2002, Shin, Pomerantsev, Mably & Macrae, 2010). Recent technological advances (i.e. CRISPR/Cas9 to generate new genetically manipulated lines, light sheet fluorescence microscopy for fast high-resolution three-dimensional imaging) have provided a strong boost to this field. However, the options for studying the cardiovascular phenotype in adult zebrafish remain limited.

Studies in adult zebrafish can provide important information related to progressive conditions, and can be used for the validation of results obtained in large-scale embryonic or larval studies before proceeding to larger animal models. Although an overview of the three-dimensional structure of the vasculature *in situ* can be achieved efficiently in the embryonic or larval stage using transgenic zebrafish expressing a fluorescent marker in endothelial cells (Lawson & Weinstein, 2002), this technique cannot be used in later stages due to the loss of transparency. In adult zebrafish, *in vivo* echocardiography can be used to obtain cardiac function measurements (Wang, Huttner, Santiago, Kesteven, Yu et al., 2017, Huttner, Wang, Santiago, Horvat, Johnson et al., 2018), while several structural features can be visualized using standard histochemical techniques (Hu, Yost & Clark, 2001). However, the study of the complete three-dimensional microvascular network is more complicated. For this purpose, vascular corrosion casting represents an interesting alternative technique.

Vascular corrosion casting has long been an established method to visualize the vascular tree in vertebrates (Rogers, Sherman & Spieler, 2014, Cornillie, Casteleyn, Von Horst & Henry, 2019). The corrosion casting technique consists of injecting a liquid resin into the circulatory system and allowing it to harden. This provides a negative replica of the hollow space filled with the resin, which can be visualized after chemical maceration (corrosion) of the soft tissue. The combination of

microvascular corrosion casting with scanning electron microscopy and micro-CT enables the three-dimensional analysis of the vasculature at high resolution (Murakami, 1971, De Spiegelaere, Cornillie, Erkens, Van Loo, Casteleyn et al., 2010, Debbaut, Segers, Cornillie, Casteleyn, Dierick et al., 2014, Junaid, Bradley, Lewis, Aplin & Johnstone, 2017).

The first documented applications of the casting techniques date back to Leonardo da Vinci (1505-7) who injected hot bee wax into the ventricles of the brain. Since then, a variety of substances have been explored to visualize the vascular tree. The initial substances to make the casts came with considerable drawbacks, as some substance required high temperatures, or other substances showed considerably shrinkage upon hardening (Cornillie, Casteleyn, Von Horst & Henry, 2019). Most of these drawbacks were overcome with the introduction of resins that harden due to polymerization. Currently a wide array of commercially available vascular corrosion casting resins exists with different characteristics such as viscosity, elasticity, speed of polymerization temperature and curing time, shrinkage, etcetera (Rogers, Sherman & Spieler, 2014). The choice of the casting media largely depends on the size of the specimens (costs and processing time), the intended use (elasticity) and the desired level of detail (viscosity). One of the most fluid casting media available is the Mercox II resin, which closely resembles the viscosity of blood and therefore is the resin of choice to visualize small blood vessels, including fine capillaries (Rogers, Sherman & Spieler, 2014, Cornillie, Casteleyn, Von Horst & Henry, 2019).

Despite its wide use in mammal model systems, such as mice, rats and rabbits, only a few reports are available of vascular corrosion casting in zebrafish. Kamei et al. (Kamei, Isogai, Pan & Weinstein, 2010) described a technique to perform vascular corrosion casting in zebrafish which was used in a subsequent study to map the development of the vasculature of the brain and the spinal cord (Kimura, Isogai & Hitomi, 2015). We here describe an adapted, alternative method for vascular corrosion casting of adult zebrafish.

Materials & Methods

Zebrafish (Danio Rerio)

Wild-type zebrafish with an AB genetic background aged 11 – 12 months, obtained from the European Zebrafish Resource Center (EZRC, Karlsruhe, Germany), were used for the casting experiment. Zebrafish experiments were approved by the local ethical committee and conducted in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals and in compliance with the Directive 2010/63/EU. All applicable international,

national and institutional guidelines for the care and use of animals and the conduction of animal experiments were followed.

Preparation of specimens

Prior to casting, the zebrafish were euthanized by immersion in a lethal dose of tricaine (4 g/L). After confirmation of death, the animal was placed on a 3D printed support designed to keep the zebrafish immobilized during dissection and injection of the polymer.

Microdissection scissors and forceps were used to make a long ventral incision from the anal fin to the base of the operculum. The heart was exposed by pulling apart the skin at the incision site using forceps. The skin was then fixed on a polyethylene hard foam base using needles punctured through the skin near the pectoral fins (Fig. 1).

Chemicals

The methyl methacrylate-based Mercox II resin and the benzoyl peroxide catalyst were obtained from Ladd Research (Williston, VT, USA). Batson's no. 17 catalyst was from Polysciences Inc. (Warrington, PA, USA).

Cardiac injection

Borosilicate glass capillaries (O.D. 1.5 mm, I.D. 1.10 mm, Sutter Instrument, Novato, CA, USA) were filled with the polymerization solution using a setup consisting of Tygon® ND 100-80 medical tubing which connects an 18G needle fitted on a 1 mL syringe containing the solution with the capillary.

To inject the polymer solution, a micromanipulator (MM33, Märzhäuser Wetzlar, Wetzlar, Germany) connected to the microinjection system (FemtoJet 4i, Eppendorf, Hamburg, Germany) was used. The needle was placed into the grip head, positioned in the direction of the zebrafish head in a 45° angle, and slowly inserted into the ventricle using a Leica M80 stereomicroscope (Leica Microsystems, Wetzlar, Germany) for visualization (Fig. 1). Minor movement of the needle was sometimes necessary to place the needle into the cavity of the ventricle in order to facilitate flow of the polymerization solution. An incision was made at the distal end of the trunk just anterior of the tail fin, to allow drainage of blood from the vasculature.

Injection was performed at a constant pressure using the 'Continuous Flow' function of the FemtoJet microinjector. Starting at a pressure of 5 hPa, the pressure was slowly increased over several minutes until a pressure of 25 hPa is reached. A higher pressure may result in deformation and damage of the vascular walls. Injection time varies from 15 to 20 minutes, depending on the hardening of the polymer. Injection volume depends on factors such as the preparation of the polymer solution, the position of the needle in the ventricle and possible leakage upon injection. This may result in failure of injecting the full content of the needle which, if fully loaded, contains about 45 µL of the polymer solution. Since the blood volume in an adult zebrafish volume is estimated at approximately 20 µL, the injection of half the volume of the needle should suffice.

After injection, the apparatus was not manipulated for 30 minutes to allow the initial hardening of the polymer. Afterwards, the specimen was placed in system water (obtained from the aquatics facility, to avoid tissue swelling due to osmosis) at least overnight to ensure full polymerization.

Corrosion and washing

The final step of the procedure is the dissolution of the tissues surrounding the cast by incubation of the specimen in 20% potassium hydroxide (KOH) solution at room temperature, overnight.

After the chemical maceration of the surrounding tissue was completed, the casts were thoroughly washed with deionized water to avoid the formation of mineral crystals. The casts were air dried after a short rinse in 70% methanol to minimize the risk of local deformations caused by evaporation of water. Methanol was chosen over ethanol as a volatile solvent due to the slightly increased chemical resistance of the poly(methyl methacrylate) to this alcohol. The casts must be completely dried before attempting manipulation, as they tend to break more easily when wet.

Microscopy

Brightfield images were captured using a Leica M165FC microscope with a DFC450 C color camera (Leica Microsystems, Wetzlar, Germany). Fluorescent imaging was performed using a Zeiss Axio Zoom.V16 microscope with an Axiocam 305 color camera (Carl Zeiss Microscopy, Jena, Germany). Z-stack recordings were reconstructed to a single in-focus image using the complex wavelet-based Extended Depth of Field plugin in ImageJ (FIJI distribution, version 1.52n, NIH) (Forster, Van De Ville, Berent, Sage & Unser, 2004, Schindelin, Arganda-Carreras, Frise, Kaynig, Longair et al., 2012). For scanning electron microscopy (SEM), the sample was mounted on an aluminium base and sputtered

with platinum using the JEOL JFC 1300 Auto Fine Coater (Jeol Ltd., Tokyo, Japan). The sample was examined with a JEOL JSM 5600 LV scanning electron microscope (Jeol ltd.). Multiple images were stitched together using the ImageJ plugin developed by (Preibisch, Saalfeld & Tomancak, 2009).

Micro-CT imaging

The sample was scanned at the Ghent University Centre for X-ray Tomography (UGCT) with the Medusa scanner. The Medusa scanner is the re-designed version of the first UGCT sub-micron CT system, which is described in (Masschaele, Cnudde, Dierick, Jacobs, Van Hoorebeke et al., 2007). The sample was scanned in traditional cone beam mode, using a Photonic Science VHR detector with a pixel size of 9^2 µm², used in binning 4 mode. Octopus Reconstruction software version 8.9.4.2 was used to obtain a stack of reconstructed TIFF images at isotropic voxel size of 5.5^3 µm³. The casted cardiovascular structure was segmented from this stack in Mimics 20.0 (Materialise, Leuven, Belgium) using a conventional thresholding approach based on the difference in grey values. As expected, the grey values where rather binary, except from the graphite mould on which the sample was mounted for imaging. The remaining graphite could be detected and separated from the cast structure during the segmentation. The automatic thresholding approach already resulted in an accurate segmentation which was then checked for irregularities. Minor flaws were (semi-)manually corrected using the interpolation tool of Mimics. The resulting 3D volume was lightly smoothed (20 iterations, smoothing factor 0.2) and shrinkage resulting from the smoothing operation was compensated.

Results

Optimization of the casting procedure

Initial experiments were performed using the commercially available Mercox II kit which is based on a methyl methacrylate monomer solution mixed with a benzoyl peroxide catalyst in order to initiate polymerization to the solid poly(methyl methacrylate) plastic. However, due to the small volumes required for casting the zebrafish vasculature, difficulties were encountered to mix the appropriate amount of the semi-solid Mercox II catalyst paste with the monomer solution. Therefore, we chose to switch to the liquid, low viscosity catalyst formulation which is included with the Batson's no. 17 anatomical corrosion kit. This catalyst mixture contains the same active ingredient, benzoyl peroxide, to initiate polymerization of the methyl methacrylate solution, albeit in a different concentration (~6-10% in Batson's catalyst vs. 40% for the Mercox II catalyst according to the manufacturers'

specifications). We therefore empirically optimized the ratio of Batson's catalyst to Mercox II methyl methacrylate solution to reach an ideal curing time of about 10-15 min, which was suitable for our experimental approach. In our hands, the optimal concentration of the Batson's catalyst was 80-100 µL per 1 mL Mercox II monomer resin, depending on the batch.

Another point that required optimization was the immobilization of the zebrafish specimen for injection with the polymerization mixture. Since the heart is located superficially on the ventral side of the zebrafish, ideally the animal needs to be fixed with the ventral side facing up in a rigid holding device to minimize movements during the microinjection procedure. To enable a reproducible immobilization of adult zebrafish, we made use of a custom 3D-printed design, consisting of a narrow trench for positioning of the animal and a set of clamps operated by micro screws to secure the head and tail in place. Below the dorsal side of the zebrafish, a replaceable polyethylene hard foam insert is included which functions as a support for pinning the needles puncturing the skin (Fig. 2).

Microscopic imaging of cardiovascular corrosion casts from adult zebrafish

Using our optimized technique, we successfully performed corrosion casting experiments on adult zebrafish. We successfully obtained casts from major blood vessels down to smaller capillaries, as evidenced by the visualization of the gill vasculature. We performed both brightfield and fluorescent imaging, taking advantage of the autofluorescence of the polymerized cast (Fig. 3). Considering the size of the adult zebrafish cardiovascular system, with major arteries having a diameter in the range of 50-150 µm while capillaries are in the low µm range, more advanced imaging methods are necessary to extract all information from the obtained casts. Therefore, we also performed imaging using a SEM setup to capture the full detail of the (micro)vasculature, as evidenced from our visualizations of the heart and the gill vasculature (Fig. 4). This technique allows for imaging in the sub-µm range, and thanks to its large depth of focus can provide some information about the spatial arrangement of different structures.

Micro-CT imaging of zebrafish corrosion casts

High-resolution X-ray tomography (micro-CT) is a well-suited technique to obtain the maximum three-dimensional information from complex casts. The latest generation of micro-CT scanners can achieve resolutions in the low µm range, depending on the size and conformation of the sample. For the micro-CT scan of the heart specimen (Fig. 5), an isotropic voxel size of 5.5^3 µm³ was chosen by using a source to object distance of 34.03 mm and a source to detector distance of 222.27 mm in

order to capture the entire sample. This imaging modality allowed us to visualize the striking morphological differences between the lumen of atrium and ventricle, the former having a smooth surface while the latter showed many indentations due to the strongly trabeculated nature of the ventricular endocardium, which can clearly be appreciated in a full three-dimensional rendering of the imaging data (Supplementary Movie 1).

Discussion

In this paper, we have presented an optimized technique to generate vascular corrosion casts from adult zebrafish specimens, as well as several options for imaging the polymerized structures. The major challenge lies in the small size of zebrafish specimens, with blood vessel diameters down to the low µm range. This aspect significantly complicates the implementation of the existing routine corrosion casting protocols used for larger vertebrate animal models. The technical optimizations presented in this manuscript will likely prove to be very valuable to researchers embarking on vascular corrosion casting experiments.

A key point for every corrosion casting experiment is the choice of the correct materials. Considering the small size of zebrafish blood vessels, a resin is required with a very low viscosity and a minimal amount of shrinkage during polymerization. We have adapted the use of the Mercox II resin, which answers to both criteria (Haenssgen, Makanya & Djonov, 2014, Cornillie, Casteleyn, Von Horst & Henry, 2019) for reproducible preparation of smaller volumes using a different, liquid catalyst. Once the ideal catalyst amount is determined, this quantity can be exactly measured out using a micropipette, which is not feasible using the semi-solid Mercox II catalyst paste. In our hands, this has significantly improved reproducibility of the resin handling and curing time. It needs to be stressed that the resin components must be mixed thoroughly in order to avoid areas of weak polymerization which can lead to cast breakage or incomplete penetration. It is also prudent to empirically check the polymerization time using different ratios of Mercox II solution : catalyst every time a new batch of one of the components is used.

Although a number of publications recommend prior flushing of the vasculature with a saline solution containing anticoagulant (Kamei, Isogai, Pan & Weinstein, 2010), with or without additional perfusion with a fixative, we have opted to directly inject the resin mixture without any pretreatment. This simplifies the procedure significantly, especially considering the small size of the injection needles which would otherwise need to be switched in between the different injection solutions. Using this approach, we have obtained high-quality results, in line with the findings of other researchers who found no difference in the quality of vascular casts with or without prior

lavage (Martin-Orti, Stefanov, Gaspar, Martin & Martin-Alguacil, 1999). Nevertheless, it is possible that Mercox II resin extravasation, which we have observed on occasion in our casts, can be prevented by prefixation of the vascular lumen (Gassner, Lametschwandtner, Weiger & Bauer, 1994), so this option can be considered to reduce this type of artefacts if necessary.

Several other points can be considered to achieve optimal results. We found that extending the amount of time to allow the polymerization to finalize after injection, by leaving the zebrafish specimen in water at room temperature up to 48h, increases the integrity of the final cast. After chemical maceration in the KOH solution, great care has to be taken when handling the casts to avoid breakage. For SEM imaging, we recommend transferring the cast to the aluminium support immediately after the 70% methanol rinse and allowing it to air-dry *in situ* in order to preserve the structure. An alternative method to preserve the fine structure of the capillaries could be the use of freeze drying as used in zebrafish by (Kamei, Isogai, Pan & Weinstein, 2010).

The laterally flattened body of the zebrafish makes it difficult to position and fix the zebrafish dorsally. In the protocol proposed by (Kamei, Isogai, Pan & Weinstein, 2010) a paraffin bed is used with a depressed notch to orient the zebrafish. In our initial experiments we made use of expanded polystyrene (EPS) foam in which a small wedge was cut to position the zebrafish. This was a workable but cumbersome approach, considering the difficulty to cut a wedge of the right size. In addition, any leakage of Mercox resin on the EPS foam resulted in the partial melting of the support. Our 3D-printed custom device, which was designed to be chemically resistant to the resin, enabled us to standardize the immobilization procedure for implementation within the integrated setup using a micromanipulator. Obtaining a whole-body zebrafish vascular cast remains challenging. However, using our optimized procedure we can obtain large vascular structures in most animals tested. Considering the steep learning curve for this procedure, researchers can expect to achieve more accurate and reproducible results through continuous improvement of their technique.

Depending on the goal of the study, several imaging options can be considered, which have been highlighted in this manuscript. SEM and micro-CT imaging are considered state-of-the-art techniques to achieve the maximum resolution and spatial information, respectively. By definition, the SEM method is limited to observing superficial structures which were coated with an electron-dense material. More complex internal structures can only be visualized using SEM after physical dissection of the region of interest. CT imaging on the other hand yields a full three-dimensional rendering of the structure, enabling virtual cross-sections through the sample to investigate internal structures. These data are suitable for detailed structure analysis and measurements. Due to technical reasons the resolution of a CT scan depends on the sample size, enabling submicron resolution for the

imaging of small dissected fragments of interest using nano-CT systems (Wagner, Van Loo, Hossler, Czymmek, Pauwels et al., 2011).

In conclusion, the method outlined in this paper can be applied to faithfully evaluate the cardiac and (micro)vascular structure of adult zebrafish, which cannot be captured with the same amount of morphological information using other available techniques. This approach has potential applications in different areas of research, including angiogenesis, comparative cardiovascular biology, tumour vascular biology, and a range of disease models. Zebrafish are particularly well suited for the study of genetic disease, such as heritable syndromic and non-syndromic disorders with progressive cardiovascular complications, which will strongly benefit from vascular corrosion casting data.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1: The setup for injection of the polymerization mixture into the zebrafish heart. A) Overview showing the microinjection setup, dissection microscope, and zebrafish specimen mounted on the 3D printed support. B) Surgical preparation of the zebrafish, showing the ventral incision to expose the heart. C) Detail showing the injection needle puncturing the heart for injection of the polymerization mixture. a: atrium, f: FemtoJet 4i, h: 3D-printed holding device, i: injection needle, m: micromanipulator, v: ventricle.

Figure 2: The 3D-printed and assembled support for immobilization of the zebrafish specimen during injection of the resin. A) Euthanized zebrafish specimen prepared for injection. B) Schematic overview of the design of the support device. c: control knobs, pe: PE hard foam, r: rubber anti-slip lining, s1: slider 1, s2: slider 2, zf: zebrafish specimen. Red arrows in panel B indicate the direction of movement of the sliders.

Figure 3: Imaging of zebrafish vascular corrosion casts using conventional microscopy techniques. A) Brightfield and B) fluorescent (FITC filter) image of venous trunk vasculature. cc: common cardinal vein, pcv: posterior caudal vein, isv: intersegmental vein, hpv: hepatic portal vein. C) Brightfield and D) fluorescent (DAPI filter) image of gill vasculature cast.

Figure 4: SEM images of zebrafish vascular corrosion casts. A) Structure of the luminal surface of the atrium (a), ventricle (v), and sinus venosus (sv) of the zebrafish heart. Asterisk: artefactual cavity caused by the injection site of the Mercox resin. Representation is stitched from multiple recordings. B) Zebrafish gill vasculature. Lamellar blood vessels (asterisks) can be seen sprouting from the filament arteries. C) Higher magnification view of white rectangle area from panel B, showing the microvascular sinusoid structure of the gill lamellae.

Figure 5: Reconstructed rendering of a micro-CT scan of the zebrafish atrium (a) and ventricle (v). The sinus venosus (sv) can be seen draining into the atrium. The branchial vein (b) runs laterally next to the ventricle. The ventricle is color-coded in red, while the atrium and the connected venous vessels are highlighted in blue. Asterisk marks the Mercox injection site.

Supplementary Movie 1: Three-dimensional representation of the reconstructed micro-CT images of an adult zebrafish heart. Color coding as in Figure 5.

Figure 1

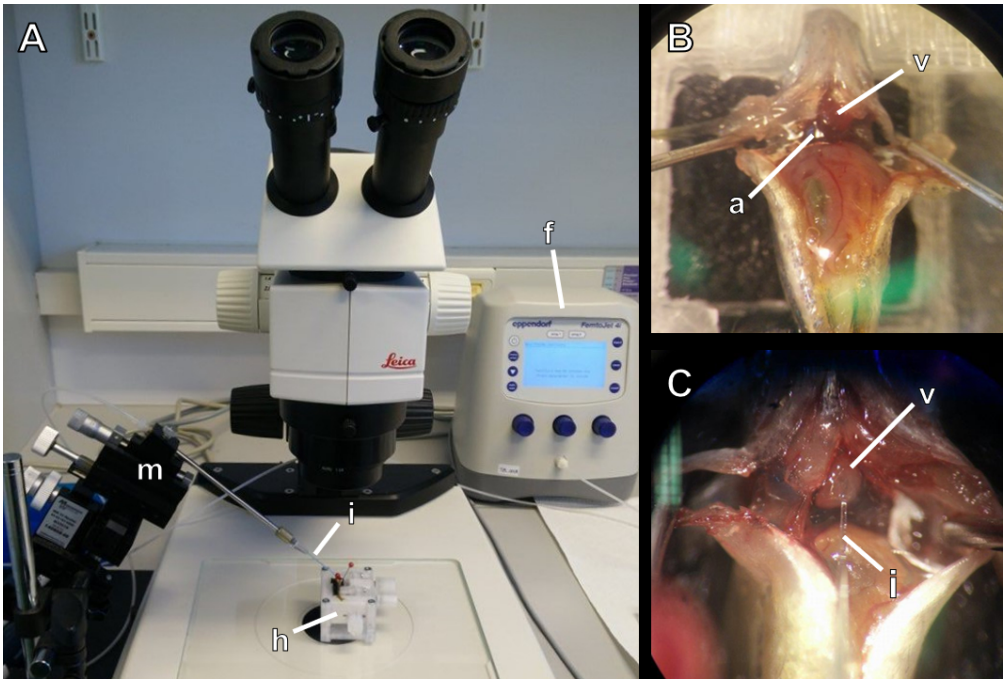

Figure 2

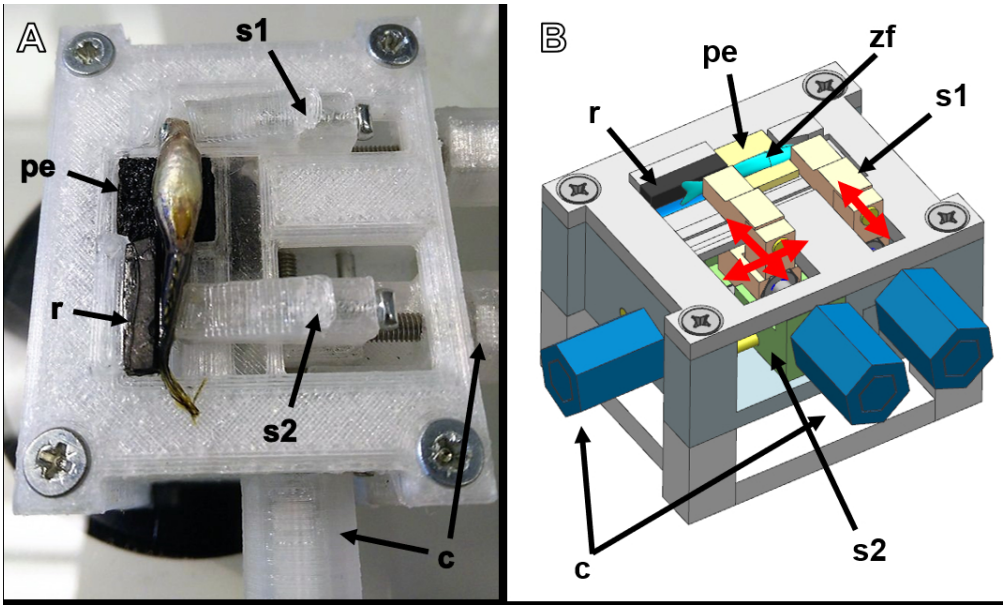

Figure 3

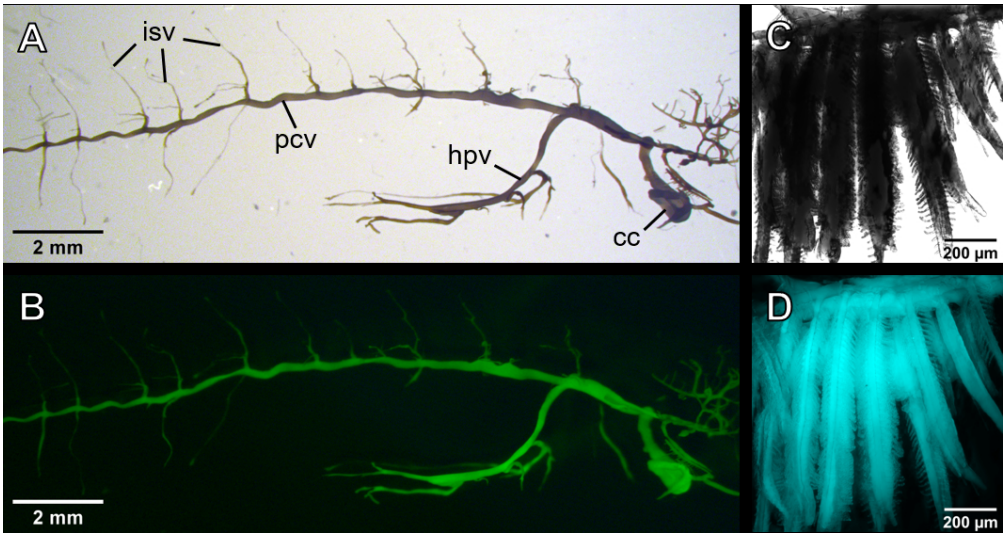

Figure 4

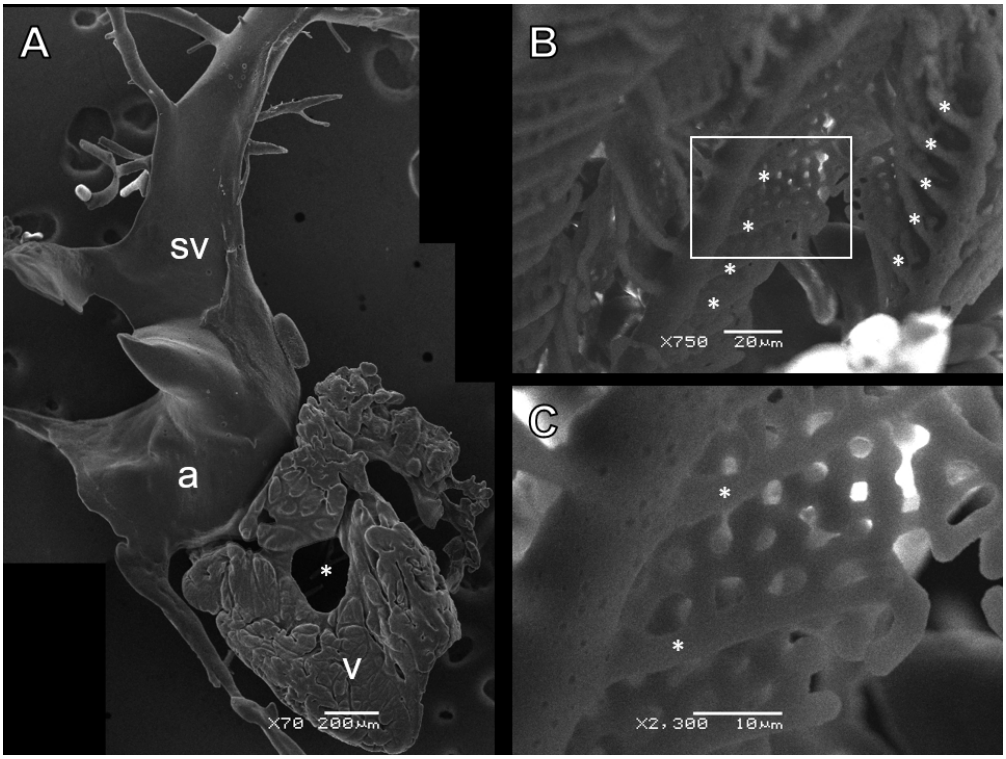

Figure 5