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TITLE:

Labeling of Blood Vessels in the Teleost Brain and Pituitary using Cardiac Perfusion with a DiI-Fixative

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KEYWORDS:

DiI, cardiac perfusion, labeling, vasculature, blood vessels, fish, brain, pituitary

SUMMARY:

The article describes a quick protocol for labeling blood vessels in a teleost fish by cardiac perfusion of DiI diluted in fixative, using medaka (*Oryzias latipes*) as a model and focusing on brain and pituitary tissue.

ABSTRACT:

Blood vessels innervate all tissues in vertebrates, enabling their survival by providing the necessary nutrients, oxygen, and hormonal signals. It is one of the first organs to start functioning during development. Mechanisms of blood vessel formation have become a subject of high scientific and clinical interest. In adults however, it is difficult to visualize the vasculature in most living animals due to their localization deep within other tissues. Nevertheless, visualization of blood vessels remains important for several studies such as endocrinology and neurobiology. While several transgenic lines have been developed in zebrafish, with blood vessels directly visualized through expression of fluorescent proteins, no such tools exist for other teleost species. Using medaka (*Oryzias latipes*) as a model, the current protocol presents a quick and direct technique to label blood vessels in brain and pituitary by perfusing through the heart with fixative containing DiI. This protocol allows improvement of our understanding on how brain and pituitary cells interact with blood vasculature in whole tissue or thick tissue slices.

INTRODUCTION:

Blood vessels play an essential part of the vertebrate body as they provide the necessary nutrients, oxygen and hormonal signals to all organs. Also, since the discovery of their involvement in cancer development[1], they have received much attention in clinical research. Although a number of publications have investigated the mechanisms allowing blood vessel growth and morphogenesis, and a large number of genes important for their formation have

been identified[2], a lot remains to be understood regarding the interaction between cells or tissues and the circulating blood.

Visualization of blood vasculature in the brain and pituitary is important. Neurons in the brain require a high supply of oxygen and glucose[3], and the pituitary contains up to eight important hormone-producing cell types that use the blood flow to receive signal from the brain and send their respective hormones to different peripheral organs[4,5]. While in mammals, the portal system at the base of the hypothalamus named the median eminence, links the brain and the pituitary[6], such a clear blood bridge has not been described in teleost fish. Indeed, in teleosts, preoptico-hypothalamic neurons directly project their axons into the pars nervosa of the pituitary[7] and mostly innervate the different endocrine cell types directly[8,9]. However, some of these neurons have their nerve endings located in the extravascular space, in close vicinity to blood capillaries[10,11]. Therefore, the difference between teleost fish and mammals is not so clear, and the relationship between the blood vasculature and the brain and pituitary cells requires greater investigation in teleost fish.

Zebrafish has, in many aspects, an anatomically and functionally comparable vascular system to other vertebrate species[12]. It has become a powerful vertebrate model for cardiovascular research mostly thanks to the development of several transgenic lines where components of the vascular system are labeled with fluorescent reporter proteins[13]. However, exact circulatory system anatomy may vary between species, or even between two individuals belonging to the same species. Therefore, visualization of blood vessels may be of high interest also in other teleost species for which transgenesis tools do not exist.

Several techniques have been described to label blood vessels in both mammals and teleosts. These include in situ hybridization for vasculature-specific genes, alkaline phosphatase staining, microangiography, and dye injections (for a review see[14]). Fluorescent lipophilic cationic indocarbocyanine dye (DiI) was first used to study membrane lipids lateral mobility as it is retained in the lipid bilayers and can migrate through it[15-17]. Indeed, a molecule of DiI is composed of two hydrocarbon chains and chromophores. While the hydrocarbon chains integrate in the lipid bilayer cell membrane of the cells in contact with it, the chromophores remain on its surface[18]. Once in the membrane, DiI molecules diffuse laterally within the lipid bilayer which helps to stain membrane structures that are not in direct contact with the DiI solution. Injecting a DiI solution through cardiac perfusion, will therefore label all endothelial cells in contact with the compound allowing direct labelling of the blood vessels. Today DiI is also used for other staining purposes, such as single molecule imaging, fate mapping, and neuronal tracing. Interestingly, several fluorophores exist (with different wavelengths of emission) allowing the combination with other fluorescent labels, and the incorporation as well as the lateral diffusion of DiI can occur in both live and fixed tissues[19,20].

Formaldehyde, discovered by Ferdinand Blum in 1893, has been used widely to the present day as the preferred chemical for tissue fixation[21,22]. It shows broad specificity for most cellular targets and preserves the cellular structure[23,24]. It also preserves the fluorescent properties of most fluorophores, and thus can be used to fixate transgenic animals for which targeted cells

express fluorescent reporter proteins.

In this manuscript, a previous protocol developed to label blood vessels in small experimental mammalian models[25] has been adapted to the use in fish. The entire procedure takes only a couple of hours to perform. It demonstrates how to perfuse a fixative solution of formaldehyde containing DiI in the fish heart in order to directly label all blood vessels in the brain and the pituitary of the model fish medaka. Medaka is a small freshwater fish native to Asia, primarily found in Japan. It is a research model organism with a suite of molecular and genetic tools available[26]. Therefore, identification of blood vessels in this species as well as in others will allow to improve our understanding on how the brain and pituitary cells interact with blood vasculature in whole tissue or thick tissue slices.

PROTOCOL:

All animal handling was performed according to the recommendations for the care and welfare of research animals at the Norwegian University of Life Sciences, and under the supervision of authorized investigators.

1. Preparation of instruments and solutions

1.1. Prepare DiI stock solution dissolving 5 mg of DiI crystal in 1.5 mL plastic tube with 1 mL of 96 % EtOH. Vortex for 30 s and keep covered using aluminum foil.

NOTE: The DiI stock solution can be conserved in the dark at -20 °C for several months.

1.2. Prepare the fish holder (**Figure 1A**) by cutting a piece of polystyrene to 5 cm length, 3 cm width, and 2 cm thickness, and gluing it to a 9 cm-diameter plastic dish. Make a 3 cm boat-shaped hole with a scalpel blade in the polystyrene.

1.3. Prepare the perfusing system (**Figure 2**) by adding a 30–50 cm long plastic cannula at the extremity of the needle.

1.4. Prepare 40 mL of fresh 4% paraformaldehyde solution (PFA) by diluting 10 mL of 16% PFA with 30 mL of phosphate buffered saline solution (PBS) in a 50 mL plastic tube.

1.5. Prepare 10 mL of fixative/DiI solution by diluting 1 mL of DiI stock solution in 9 mL of freshly prepared 4% PFA in a 10 mL plastic tube. Keep in the dark until use.

NOTE: The DiI crystals that are not dissolved can be kept in the stock solution tube, and new ethanol can be added to prepare new DiI stock solution (see step 1.1).

1.6. Prepare 50 mL of tricaine (MS-222) stock.

1.6.1. Dissolve 200 mg of Tricaine powder in 48 mL of H_2O. Add 2 mL of 1 M Tris base (pH 9). Adjust to pH 7 with 1 M HCl and store at -20 °C.

1.7. Dilute 5 mL of Tricaine stock in 50 mL of clean water in a small glass.

1.8. Prepare several 5 cm glass pipettes (**Figure 2**) by stretching a glass capillary with a pipette puller following the manufacturer's instructions.

2. Dissection and perfusion

NOTE: PFA is a toxic volatile compound, therefore the dissection and perfusion should be performed in a hood or in a ventilated room, and the user must be wearing a gas mask.

2.1. Prepare the dissection tools including small scissors, and one sharp and one strong forceps before dissection.

2.2. Fill the syringe with the prepared solution of PFA/DiI by placing it in the 50 mL tube and drawing up. Then place the needle with the cannula at the extremity of the syringe (**Figure 2**).

2.3. Fix the syringe to the bench using several pieces of tape placed in different directions, adjust the position of the microscope and the seat to obtain a good position for dissection and for pressing the syringe piston (**Figure 1B**).

NOTE: In this protocol, the elbow is used to press down the syringe piston, but other possibilities options may be used, e.g., assistance from another person.

2.4. Euthanize the fish with an overdose of tricaine by placing the fish in the solution prepared in step 1.7. Wait 30 s and test the reflexes of the fish by grabbing its caudal fin with the forceps. The fish can be used when it has stopped responding to the stimuli.

2.5. Place the fish in the fish holder with its abdomen facing up and pin one needle into the extremity of the head and another one above the tail to keep the fish in place.

2.6. Open the anterior abdomen with the scissors by horizontally cutting the superficial layer of skin.

2.7. Using forceps, remove the skin above the heart until a clear access to the ventricle and the bulbus arteriosus is provided (**Figure 3**).

2.8. Add a glass pipette at the extremity of the capillary and break the end of the tip of the glass pipette.

NOTE: The hole of the broken tip should be big enough to let the liquid out, but still small enough to easily enter the tissue. The glass pipette can be reused if not broken or clogged.

2.9. Bring the glass pipette close to the ventricle and add pressure to the syringe piston with the elbow to force the liquid out.

2.10. Pin the heart ventricle with the glass pipette while adding pressure to the syringe.

2.11. Directly after, perforate the sinus venosus with forceps to enable blood to leave the circulation.

NOTE: The heart ventricle becomes more fragile because of the fixative. It also becomes less red as the blood is diluted with the fixative/DiI solution.

2.12. From the ventricle, adjust the angle of the glass pipette to find the entrance of the bulbus arteriosus. Bring the pipette opening inside the bulbus arteriosus as shown in **Figure 2**, and add more pressure to the syringe.

NOTE: The bulbus arteriosus is transparent and the tissue is elastic. When replacing blood with DiI/fixative, the glass pipette inside the heart should become more visible and by adding pressure, the size of the bulbus arteriosus should expand. This step is crucial to make sure that enough pressure has been used to replace all the blood with the fixative/DiI solution, and that all blood vessels have been reached. Often when successful, the PFA provokes muscle contractions and the fish will be moving.

2.13. Continue adding pressure on the syringe for 30–60 s still keeping the glass pipette inside the bulbus arteriosus.

2.14. Remove the glass pipette and the needles from the fish.

2.15. Dissect the brain and the pituitary, and incubate tissues in fresh 4% PFA in PBS for 2 h in the dark at room temperature.

NOTE: Dissection of brain and pituitary previously has been described in detail in two different ways by Fontaine et al.[27] and Ager-Wick et al.[28]. Because of the fixation, the tissue becomes quite hard and the fragile link between the brain and the pituitary may be broken. After dissection, post fixation processing can also be performed overnight at 4 °C.

2.16. Rinse the tissue twice in PBS for 10 min before preparing for imaging.

NOTE: The tissue can then be mounted between slide and cover slip with spacers in between and mounting medium for confocal imaging (see **Figure 4**), or sectioned with a vibratome as described in detail in Fontaine et al.[27] before mounting between slide and cover slip for imaging with a stereomicroscope (see **Figure 4**).

REPRESENTATIVE RESULTS:

This protocol demonstrates a step by step procedure to label blood vessels in the medaka brain and pituitary, and at the same time fix the tissue. After labelling by cardiac injection of a fixative solution containing DiI into the heart, blood vessels can be observed on slices using a fluorescent stereomicroscope (**Figure 4**) or on whole tissue using a confocal microscope (**Figure 5**). Either on the thick tissue slice or on the whole tissue, the architecture of the blood vasculature can be observed in three dimensions. Tissue slices can be labeled for specific targeted proteins using standard immunofluorescence protocols after the end of the fixation, and on transgenic lines where cells of interest express fluorescent reporter proteins (**Figure 6**). This allows investigations on interactions between blood vessels and other specific cell types. Here for example, the use of a transgenic line of medaka where green fluorescent protein (GFP)-producing cells revealed the location of pituitary Lh cells[29]. One can observe that these cells send extensions towards the blood vessels. Some blood vessels may not be labeled properly if perfusion is sub-optimal. This can be the case, for example, if the solution is injected in the heart ventricle instead of the bulbus arteriosus, or if using too low pressure and/or for too short a period on the syringe piston (**Figure 7**). Finally, by imaging the same tissue with the same imaging parameters, the intensity of the labeling was shown to decrease after four days, with the signal more spread out (**Figure 8**).

FIGURE LEGENDS:

Figure 1: Images of the holding plate for perfusion of the fish (A) and the injection setup (B).

Figure 2: Schema of the medaka fish heart and perfusion system shown with the idel location of the glass needle in the heart for successful perfusion. Arrow head show where to perforate the heart to allow the blood to leave circulation.

Figure 3: Image of the ventral side of the fish opened, with the heart exposed for perfusion.

Figure 4: Image of the blood vasculature in a slice of brain and pituitary tissue from medaka. An adult medaka was perfused with a fixative solution containing DiI. Brain and pituitary were dissected and fixed overnight. Tissues were mounted in 3% agarose and sectioned with a vibratome before imaging with a fluorescence microscope. All blood vessels labeled with DiI are seen through the whole section showing the vasculature in the brain and the pituitary. OT, optic tectum; Tel, telencephalon; Hyp, hypothalamus; Pit, pituitary; Cb, cerebellum; Hind, hindbrain.

Figure 5: 3D rendering of a confocal z-stack from the blood vasculature in medaka pituitary. An adult medaka was perfused with a fixative solution containing DiI. Brain-pituitary were dissected and fixed overnight. The pituitary was dissected from the brain and mounted between a slide and coverslip with spacers ib between, before imaging with a confocal microscope. Z-stack was recorded, and a 3D rendering was made using Fiji software and the 3D viewer plugin[30]. The entire pituitary blood vasculature could be observed in 3D. RPD, rostral pars distalis; PPD, Proximal pars distalis; PI, pars intermedia.

Figure 6: Z projection of confocal z-stack from a tissue slice of transgenic medaka where Lhβ promoter controls expression of green fluorescent reporter protein. An adult tg(*lhb*:hrGfpII)

medaka was perfused with a fixative solution containing DiI. Brain-pituitary were dissected and fixed overnight. Tissues were mounted in 3% agarose and sectioned with a vibratome. Some hormone producing cells, Lh cells in this case, sending extensions (arrow heads) towards the blood vessels can be observed, probably to sense signals from the blood and/or release their hormones into the circulation.

Figure 7: Image of blood vasculature in a slice of brain and pituitary tissue from poorly perfused medaka. An adult medaka was poorly perfused with a fixative solution containing DiI by injecting the solution in the ventricle only. Brain-pituitary were dissected and fixed overnight. Tissues were mounted in 3% agarose and sectioned with a vibratome before imaging with a confocal microscope. The blood vessels were poorly labeled with DiI and some of them were not labeled at all. OT, optic tectum; Tel, telencephalon; Hyp, hypothalamus; Pit, pituitary; Cb, cerebellum; Hind, hindbrain.

Figure 8: Time lapse imaging of labeled blood vasculature in medaka brain-pituitary tissue section. An adult medaka was perfused with a fixative solution containing DiI. Brain-pituitary were dissected and fixed overnight. Tissues were mounted in 3% agarose and sectioned with a vibratome before imaging with a stereomicroscope directly after mounting (**A**), and 4 days after mounting (**B**) with the same imaging parameters. Note that the labeling has decreased and spread out with time. OT, optic tectum; Tel, telencephalon; Hyp, hypothalamus; Pit, pituitary; Cb, cerebellum; Hind, hindbrain.

DISCUSSION:

Cardiac perfusion with DiI previously has been used to label blood vessels in several model species[25], including teleost fish[11,14,31].

As DiI is directly delivered to the endothelial cell membrane by perfusion in the vasculature, it is possible to increase the signal-to-noise ratio by increasing the DiI concentration in the fixative solution. In addition, the fluorophore provides intense staining when excited with minimal bleaching allowing relatively long-lasting emission[19,32]. Also, the labeling can remain for several days and be used in combination with other labeling techniques that require mild treatments, such as in immuofluorescence (IF)[33].

However, this technique has some limitations. After removal from the fixative solution, labeling and imaging of the tissues should be performed as quickly as possible because the intensity of the labeling will weaken, and the signal will diffuse with time (**Figure 8**). This process will be even faster when using detergents that increase porosity of the membrane. Also, because PFA is a toxic volatile compound, several precautions should be taken when performing this experiment. To minimize the hazardous aspects of of this protocol, one can perfuse a solution of DiI and PBS before dissecting the tissue of interest and fix it with the PFA solution right after perfusion. However, this can be performed only for small-sized tissues, as the penetration of PFA in larger tissue samples will be less efficient than during perfusion.

The success rate of this protocol is improved by training, so it is expected that researchers will need some time to get acquainted with the different steps of the technique. For instance, the perfusion of the solution in the bulbus arteriosus is a particularly critical step of the protocol and requires some training to achieve a good result. Also keeping the needle in the correct position for long enough during the perfusion is not trivial, and will require training by the manipulator.

Finally, this protocol is optimized for adult medaka but it can be used for other species with some adaptations. Different tissues of interest, or even different life stages of the animal within the same tissue will also require optimizations for the concentration of DiI and the time of perfusion as well as the material used (needle and syringe sizes for example).

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DISCLOSURES:

The authors have nothing to disclose

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Figure 1

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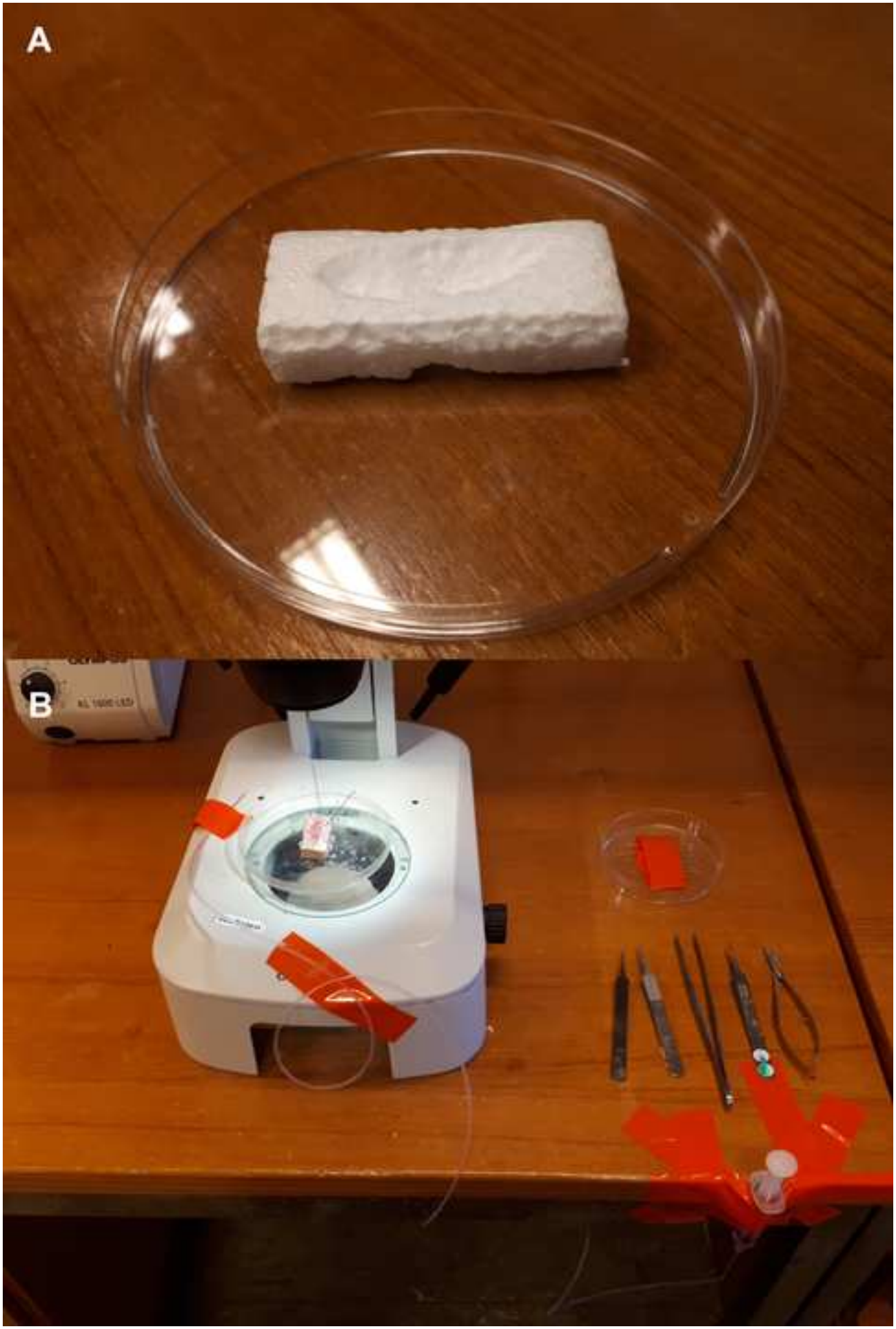

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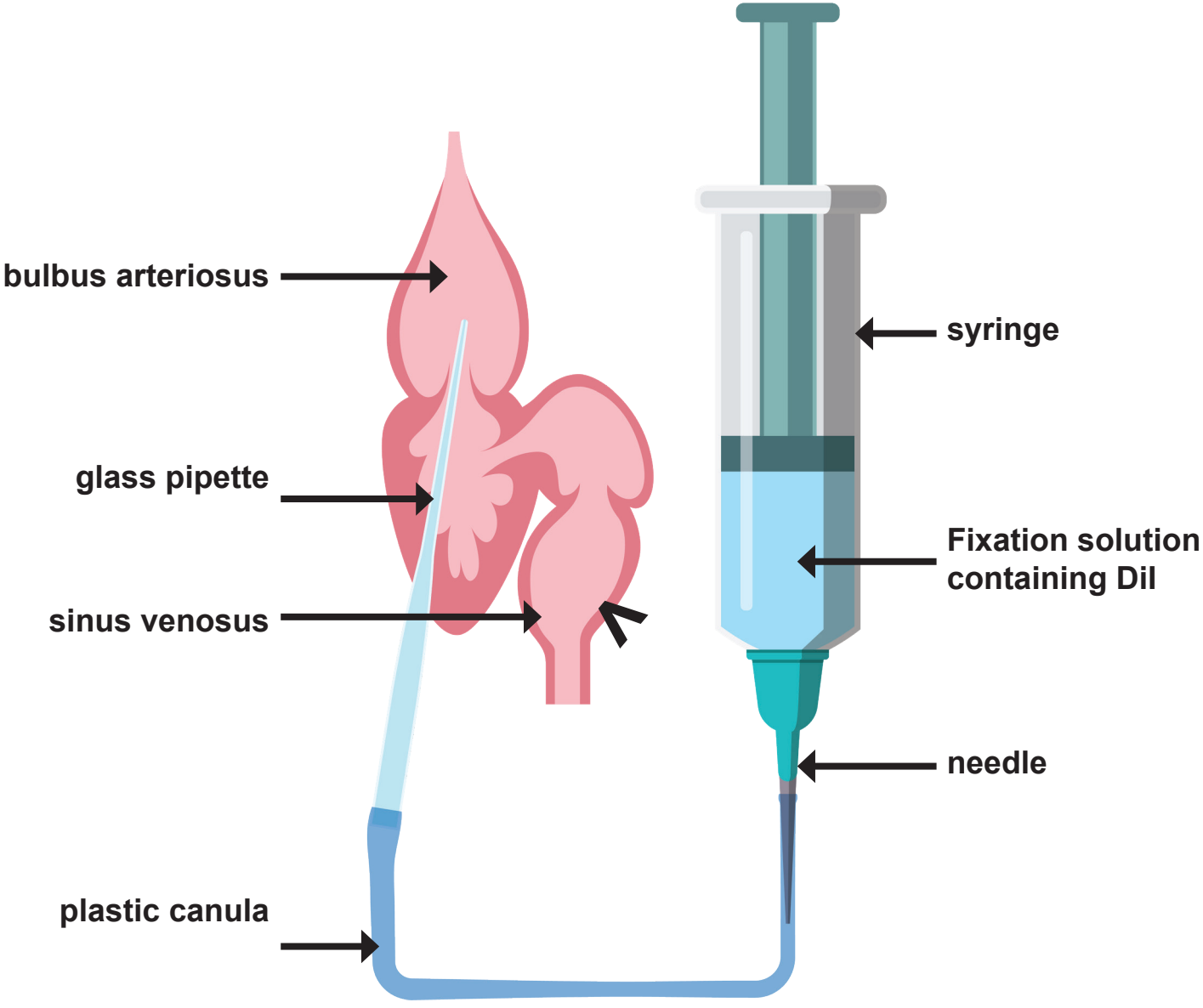

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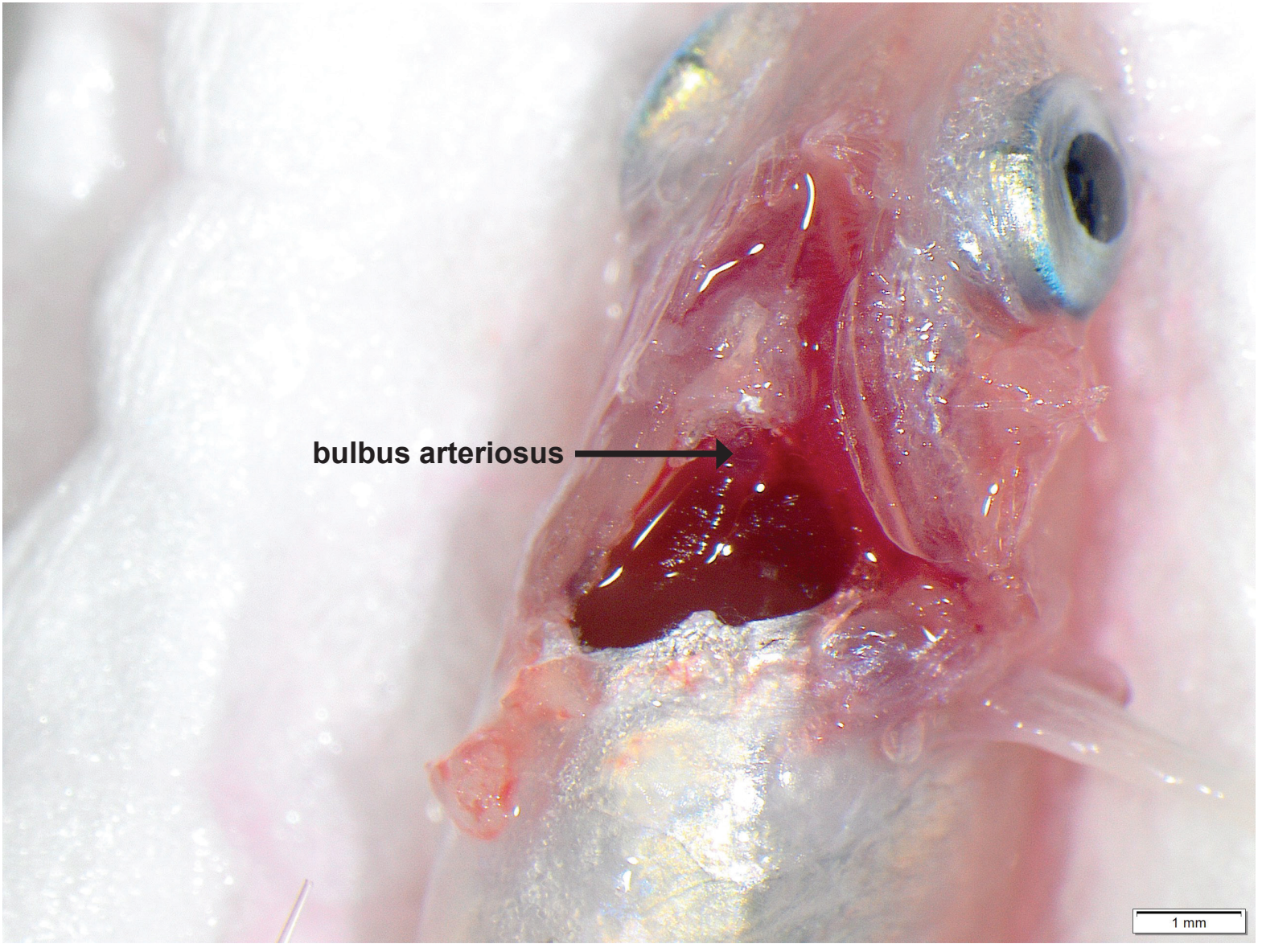

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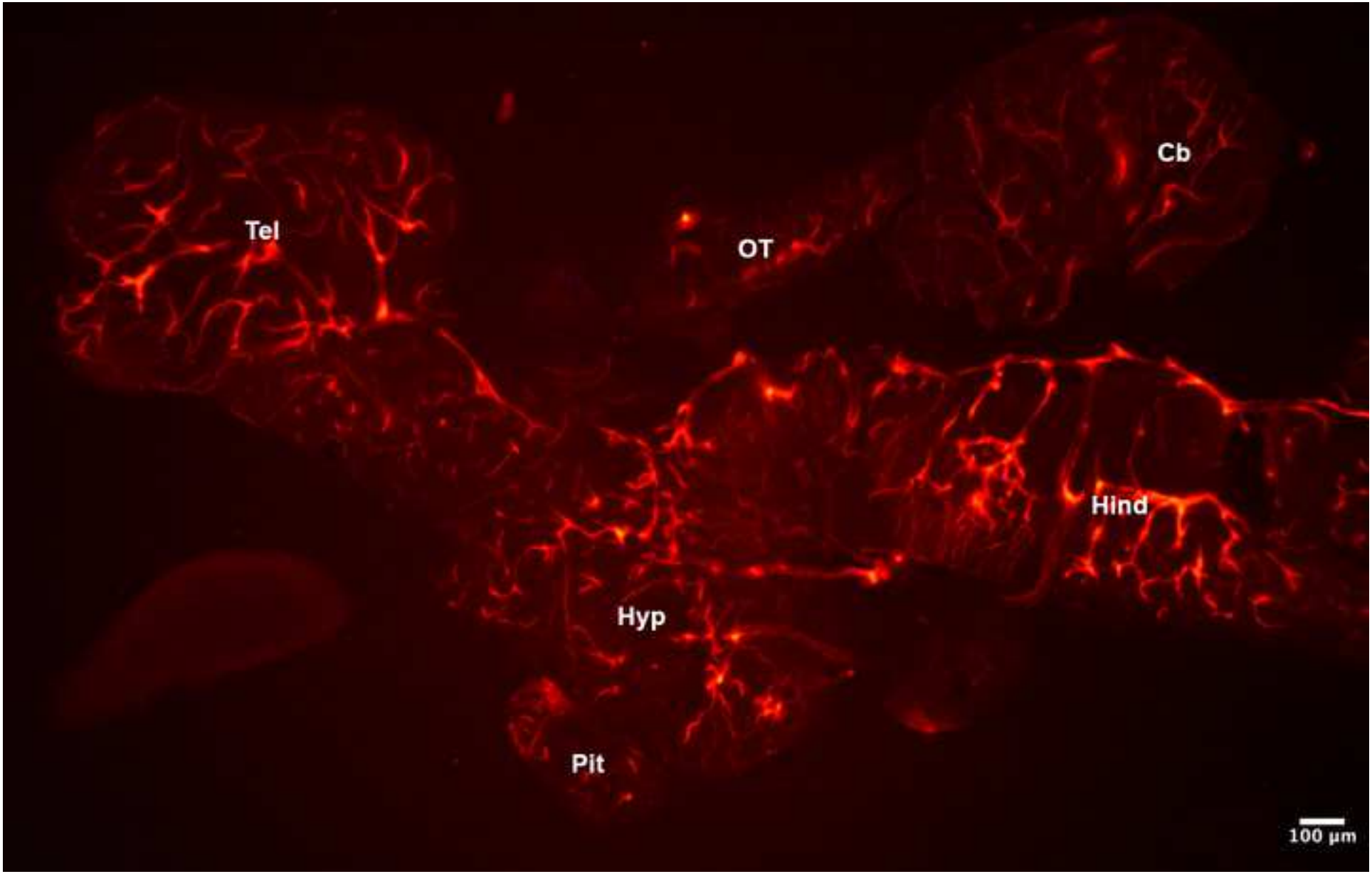

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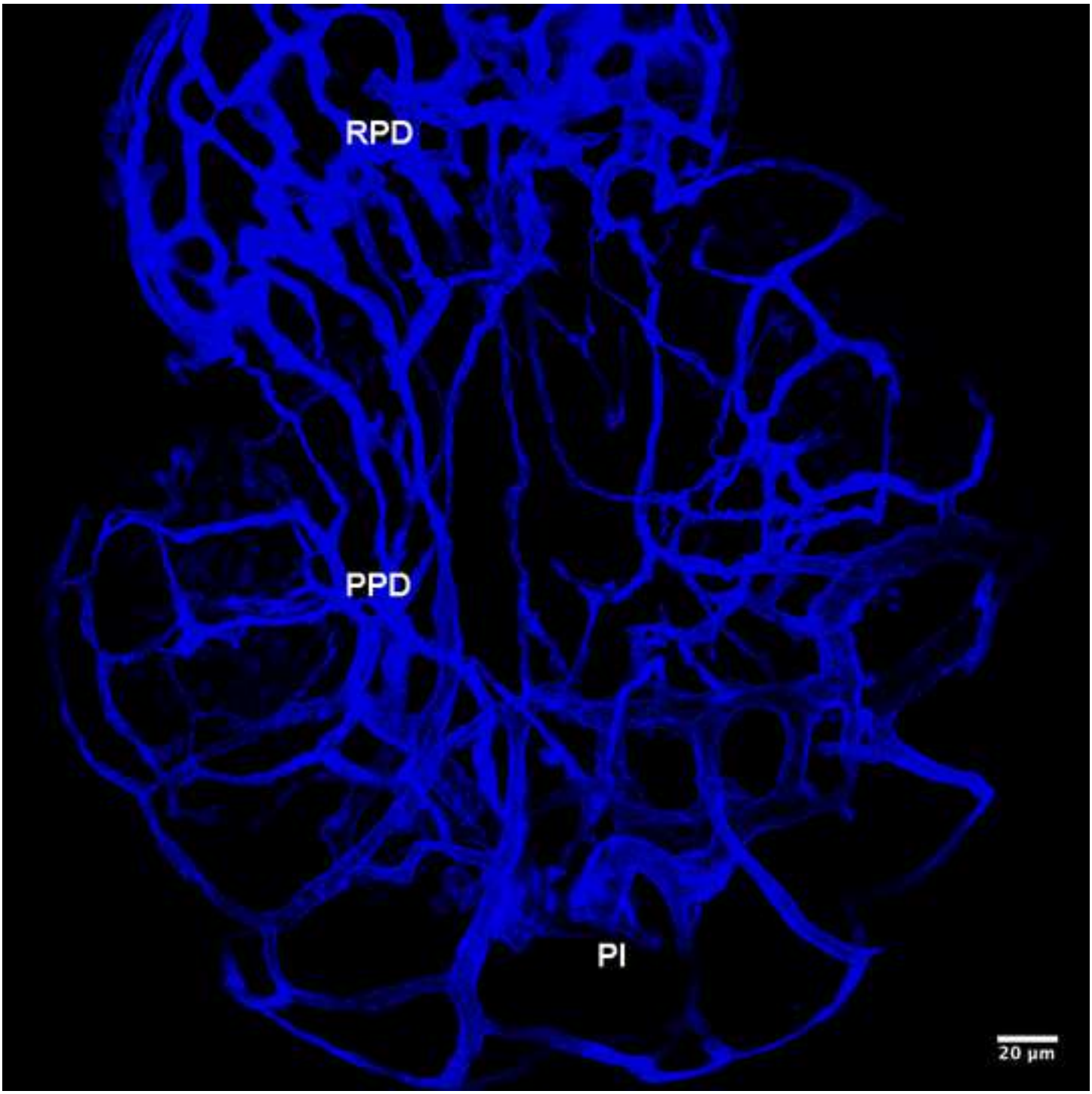

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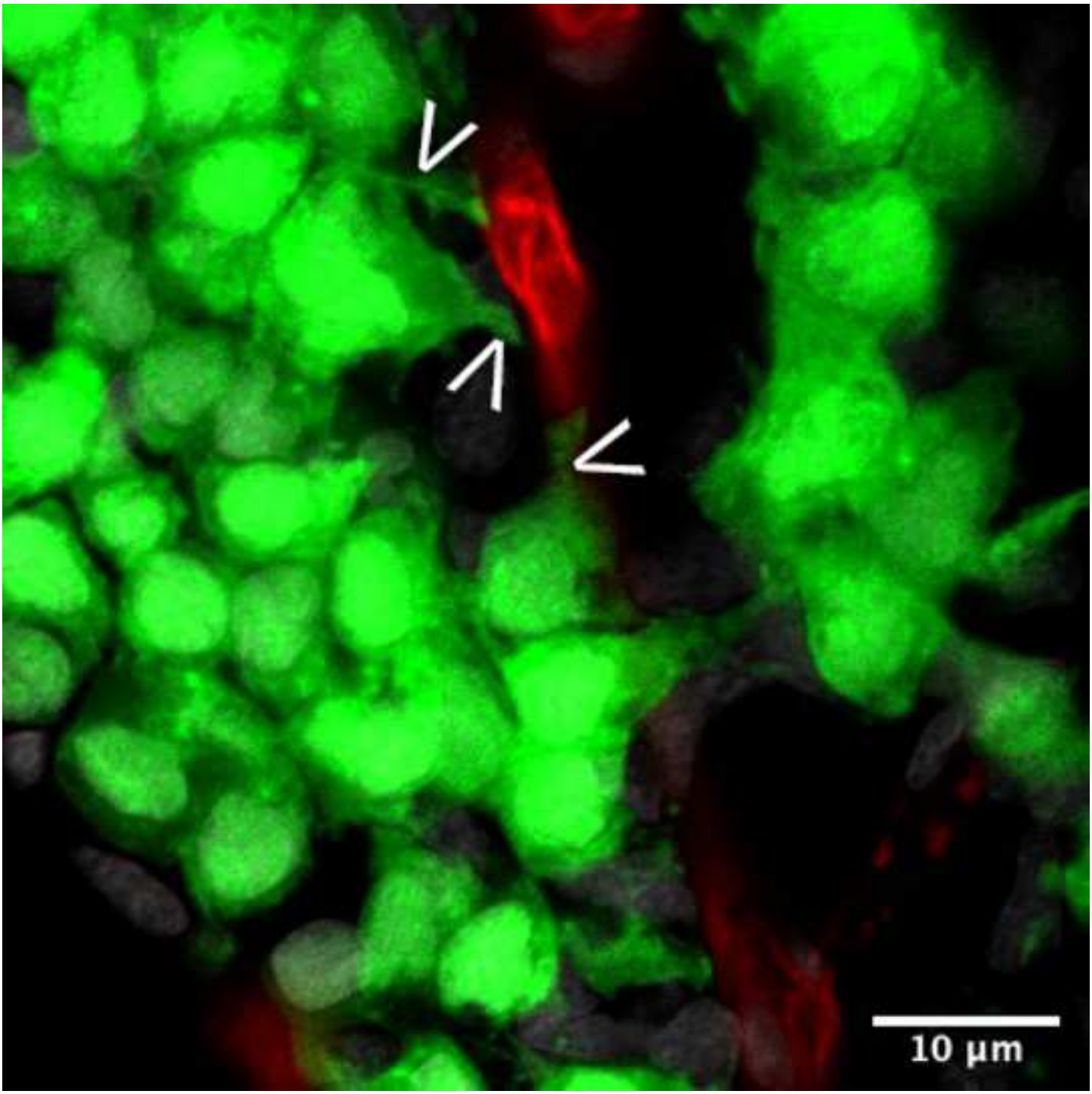

Figure 7

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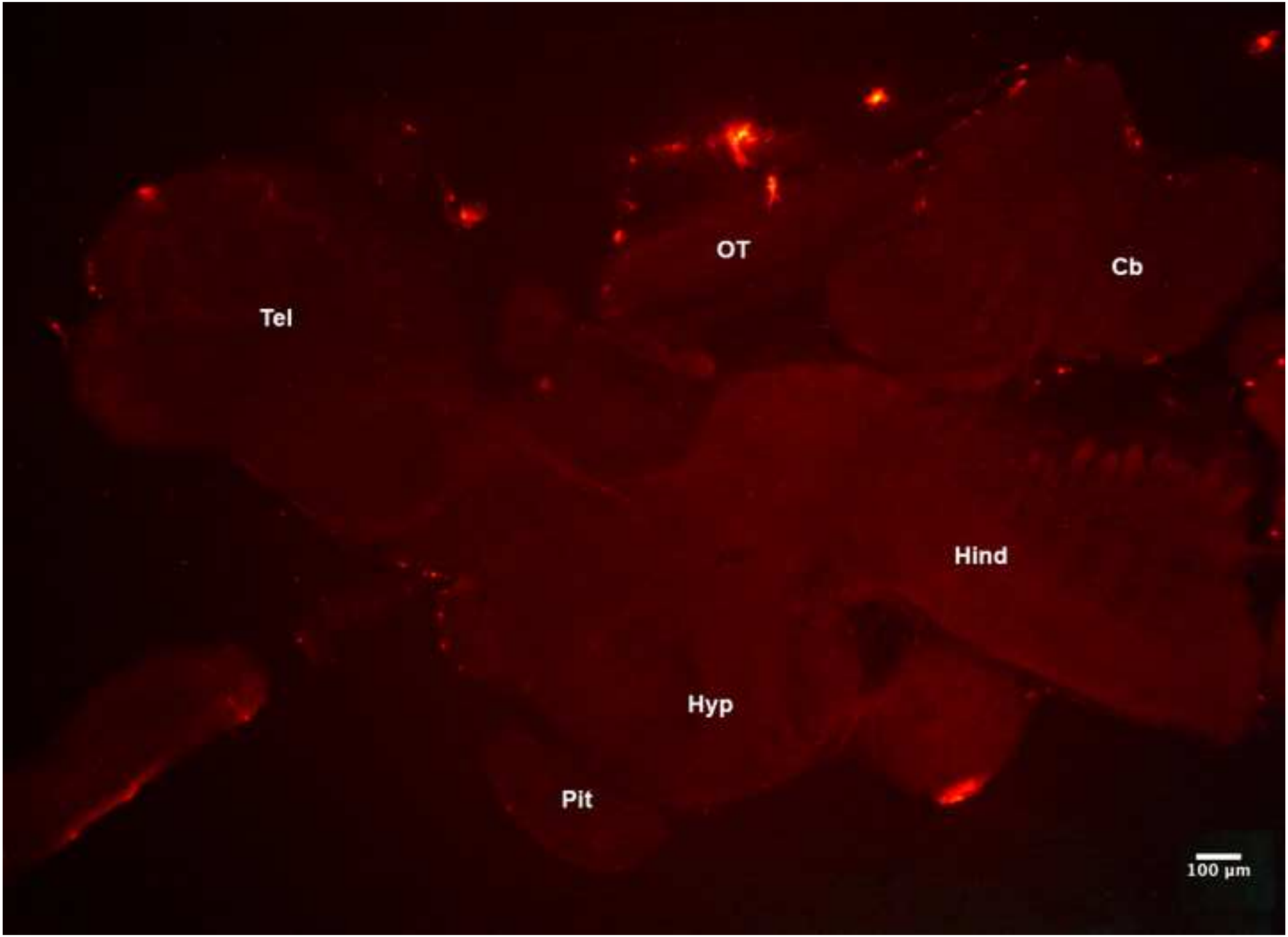

Figure 8

Click here to access/download;Figure;new figure 8.tif

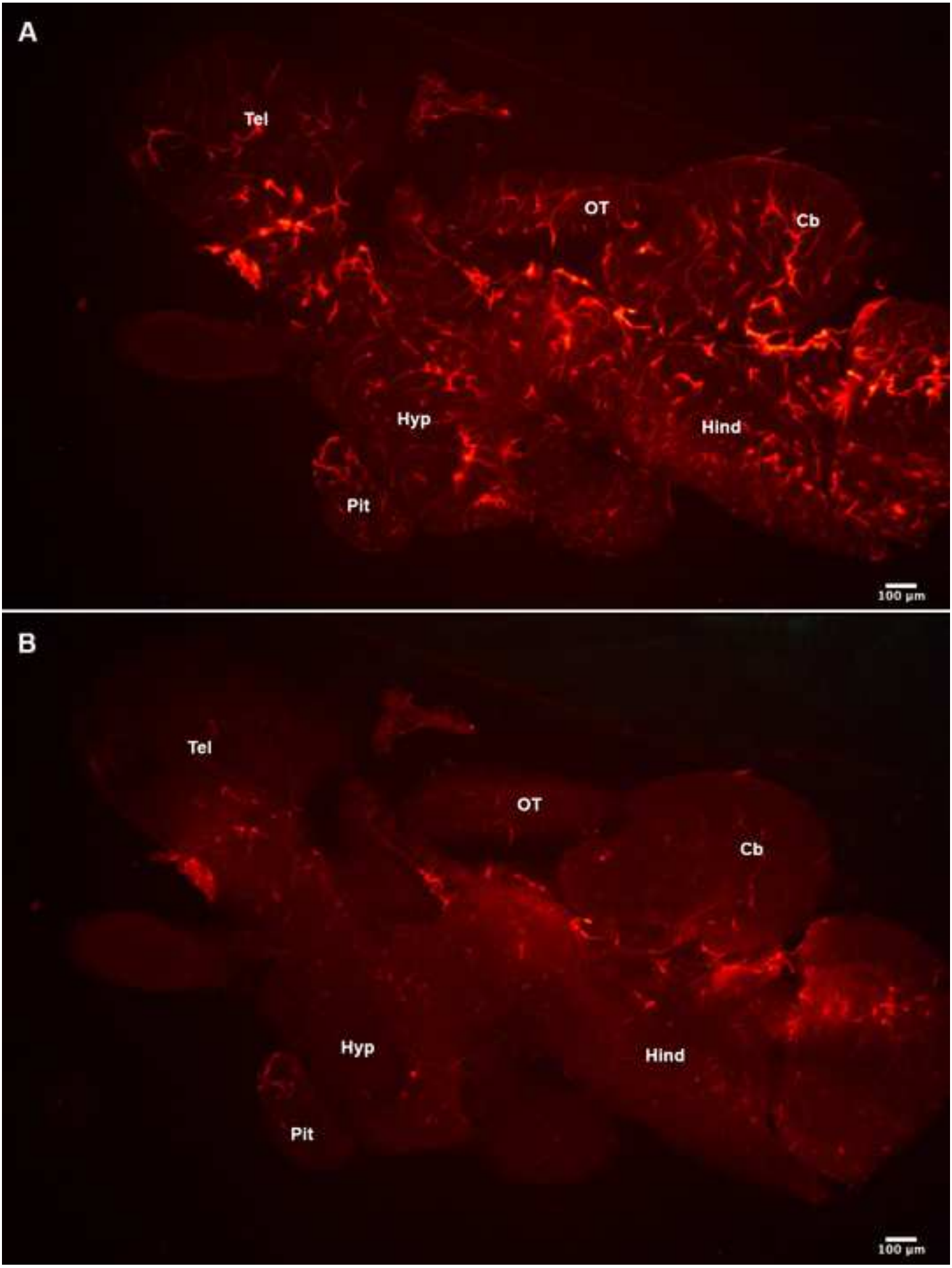

table material

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
16% paraformaldehyde	Electron Microscopy Sciences	RT 15711
5 mL Syringe PP/PE without needle	Sigma	Z116866-100EA
BD Precisionglide syringe needles	Sigma	Z118044-100EA
borosilicate glass 10cm OD1.2mm	sutter instrument	BF120-94-10
DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine	Invitrogen	D-282
LDPE tube O.D 1.7mm and I.D 1.1mm	Portex	800/110/340/100
Phosphate Buffer Saline (PBS) solution	Sigma	D8537-6X500ML
pipette puller	Narishige	PC-10
plastic petri dishes	VWR	391-0442
Super glue gel	loctite	c4356
tricaine (ms-222)	sigma	E10521-50G

Comments/Description
syringes
needles 18G (1.20*40)
glass pipette
canula

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Author(s): Romain FONTAINE and Finn-Arne WELTZIEN

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Title:	Professor
Signature:	
Date:	Jan 30 2019

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First of all, we would like to thank all reviewers and the editor for their comments which helped to improve the quality and clarity of our paper. We have carefully read all comments and adjusted the text accordingly. Please see our detailed answer to individual comments below.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We went through the whole manuscript and corrected the errors we found, we also improved the English language throughout.

2. Please revise lines 25-26, 27-29, 39-41, 72-74, 91-92, 279-281, 295-298 to avoid previously published text.
These lines have been revised to avoid already published text

3. Keywords: Please provide at least 6 keywords or phrases.
We added *fish*, *brain*, and *pituitary* as keywords to get more than 6.

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
We went through the whole manuscript and changed all abbreviations that were not according to SI

5. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
All pronouns have been removed from the protocol.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below.
We went through all steps and added more details where we found it necessary to help the reader to replicate the protocol.

7. 1.2: Please specify the size of the polystyrene and the dish as well the hole.
We added this information in the manuscript

8. 1.3: Please provide the size of the canula and the needle.
We added this information in the manuscript

9. 1.7: Do you mean beaker instead of Becker?
We corrected this language error

10. 1.8: Please add more details here such as the size and diameter at the end of the pipette.
We added the size of the pipette but at this step there is no opening, it is melted, and it will be broken in step 2.8. Also, there is no way to accurately control the diameter of the pipette tip as we break it. It should be small enough to enter in the tissue without pushing or destroying it. We made a note to explain this in the protocol

11. 2.2: How to fill the syringe, from the top?
We added this information in the manuscript

12. 2.3: How to fix the syringe to the bench?
We added this information in the manuscript

13. Please describe the procedures used to obtain images presented in Figures 4-8. For instance, move the method details in the figure legend to the protocol.
For presenting the results of this protocol we used two different advanced imaging techniques: fluorescent confocal microscopy (figure 5 and 6) and fluorescent stereomicroscopy (figure 4, 7 and 8). Both are complex techniques that would require a protocol of their own. We therefore focused this protocol on the labeling itself but added a note to provide information about the imaging possibilities at the end of the protocol.

14. Please consider highlighting more content for filming. For instance, preparation of instruments (fish holder and the perfusing system) is appropriate for filming.
We added 3 more steps for the filming (1.2 / 1.3 / 1.8)

15. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then “et al.”.
We corrected the bibliography by using the JoVE endnote style provided on your website.

16. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.
We added some missing reagents and materials in the table.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The proposal involves the dissemination of a very interesting method, using the perfusion technique, to study blood vessels in the brain and pituitary of fish. The justification presented is quite pertinent, because according to the authors, this method will allow the study of vessels in other species, besides zebrafish, where molecular markers are not available. The methodology is clear, and its efficiency has been proven by the authors in three different practical applications using several image analysis tools. In the opinion of this reviewer the proposal will bring many benefits to the scientific community. We also highlight an important aspect brought by the authors that the technique is involved with an important topic of discussion that is the presence or not (or in what intensity) of a portal system in fish.

Minor Concerns:
In my opinion, the step 2.11 is determining for the success of the infusion. This step does not appear in figures, therefore suggest authors (and this can be done in the video) to identify clearly where the perforation should be made.
This will be shown in the video but we agree that it would be interesting to have it also in the figure. Therefore, we added an arrow head to show where to make the perforation in figure 2.

After completing item 2.10, after how long does item 2.11 should start? I think this point will be more evident in the video as well.
2.11 should quickly follow 2.10 to avoid extra pressure in the blood vessels that may cause rupture. This could lead to leakage of DiI solution in the tissues. This will be highlighted in the video but we have now specified this time aspect in the protocol.

Reviewer #2:

Manuscript Summary:
The manuscript describes the protocol for labelling blood vessels in a teleost fish by cardiac perfusion of DiI diluted in PFA fixative. The protocol is well described and presents good results. The article is well ilustrated and the figures are in good quality. Also, this same technique could be applied in many other studies successfully.

Although well written, a general review of English language is required, since there are some poorly translated words in the text, as well as some missing punctuation.

Minor Concerns:
- From item 2.2 to item 2.13 the text is highlighted in yellow. I did not understand the reason! Is there something that should be added or changed in this section?
This is a requirement from JoVE, it highlights the part that will be recorded for the video.

- In the text it is used the term "bulbus arteriosus", however in the figure it is identified as "bulbus aortica". Please standardize the terms used in the manuscript.
Sorry for the mistake, we standardized the term used across the entire manuscript and the figures.

- References are not standardized, some are even incomplete. Please check the journal's standards and adjust them.
All the references have been corrected to correspond to the journal format (see also answer to comment #15 from the Editor above).

- Two tables are presented in the end of the manuscript. For the first one, it is necessary to include a title and it should be cited throughout the text. As for the second one, I did not understand the purpose of this table. My suggestion is to remove it.
Sorry, this is a result of the PDF builder system of the submission website. There is in fact only one table but because of its size, it has been cut and printed in two different pages in the PDF. We believe this will be fixed for the final printing by the journal.

Reviewer #3:

Manuscript Summary:
This study by Fontaine & Weltzien describes a systematic and detailed protocol for the visualization of the vessels

present in the encephalon and pituitary of the medaka fish model using a Dil-fixative.

Minor Concerns:
Due to the quality of the work presented, I only have minor concerns related to figure captions, which can be easily corrected.

Figure 4. To facilitate the understanding of this image by different readers, I suggest the insertion of markings in the image to delimit the regions comprised by the brain and the pituitary.
We agree with the reviewer that some anatomical nomenclature may help the reader, we therefore labelled the principal structures.

Figure 5. Please, if possible, indicate in this image the pituitary lobes (RPD, PPD and PI). This result can be so informative for many readers and future studies. We agree with the reviewer that this information may help the reader, we therefore included them in the figure and the legend.

Figure 6. The authors forgot to cite in the caption of the figure the arrowheads inserted in the image to indicate the cytoplasmic projections of Lh-expressing cells.
Sorry for this mistake, we added the information in the figure legend.

Figure 7 and 8. Please, indicate the pituitary location in these images.
Again, we agree with the reviewer that some anatomical nomenclature may help the reader, we therefore labelled the principal structures.