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Mesoscopic optical imaging of whole mouse heart

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Corresponding Author:	Leonardo Sacconi ITALY
Corresponding Author's Institution:	
Corresponding Author E-Mail:	sacconi@lens.unifi.it
Order of Authors:	Francesco Giardini
	Erica Lazzeri
	Camilla Olianti
	Giada Beconi
	Irene Costantini
	Ludovico Silvestri
	Elisabetta Cerbai
	Francesco Pavone
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TITLE:

Mesoscopic Optical Imaging of Whole Mouse Heart

AUTHORS AND AFFILIATIONS:

Francesco Giardini[1,*], Erica Lazzeri[1,*], Camilla Olianti[1,*], Giada Beconi[1], Irene Costantini[1,2], Ludovico Silvestri[1,3,4], Elisabetta Cerbai[1,5], Francesco S. Pavone[1,3,4], Leonardo Sacconi[1,3,#]

[1]European Laboratory for Non-Linear Spectroscopy, Sesto Fiorentino, 50019, Italy.
[2]Department of Biology, University of Florence, Sesto Fiorentino, 50019, Italy.
[3]National Institute of Optics, National Research Council, Florence, 50125, Italy.
[4]Department of Physics and Astronomy, University of Florence, Sesto Fiorentino, 50019, Italy.
[5]Department of Neurosciences, Psychology, Drugs and Child Health, University of Florence, Italy.

* The authors contributed equally to the work.

Email addresses of co-authors:
Francesco Giardini (giardini@lens.unifi.it)
Erica Lazzeri (lazzerierica@gmail.com)
Camilla Olianti (olianti@lens.unifi.it)
Giada Beconi (giada.beconi@gmail.com)
Irene Costantini (costantini@lens.unifi.it)
Ludovico Silvestri (silvestri@lens.unifi.it)
Elisabetta Cerbai (cerbai@lens.unifi.it)
Francesco S. Pavone (pavone@lens.unifi.it)
Leonardo Sacconi (sacconi@lens.unifi.it)

[#]Corresponding author:
Leonardo Sacconi (sacconi@lens.unifi.it)

SUMMARY:

We report a method for mesoscopic reconstruction of the whole mouse heart by combining new advancements in tissue transformation and staining with the development of an axially scanned light-sheet microscope.

ABSTRACT:

Both genetic and non-genetic cardiac diseases can cause severe remodeling processes in the heart. Structural remodeling, such as collagen deposition (fibrosis) and cellular misalignment, can affect electrical conduction, introduce electromechanical dysfunctions and, eventually lead to arrhythmia. Current predictive models of these functional alterations are based on non-integrated and low-resolution structural information. Placing this framework on a different order of magnitude is challenging due to the inefficacy of standard imaging methods in performing high-resolution imaging in massive tissue. In this work, a new methodological framework is described that allows imaging of whole mouse hearts with micrometric resolution. The achievement of this goal has required an impressive technological effort where advances in tissue transformation and imaging methods have been combined. First, we describe an optimized CLARITY protocol capable of transforming an intact heart into a nanoporous, hydrogel-hybridized, lipid-free form that allows high transparency and deep staining is described. Then, a fluorescence light-sheet microscope able to rapidly acquire images of a mesoscopic field of view (mm-scale) with the micron-scale resolution is described. Inspired by the mesoSPIM project, the conceived microscope allows the reconstruction of the whole

mouse heart with micrometric resolution in a single tomographic scan. We believe that this methodological framework will allow clarifying the involvement of the cytoarchitecture disarray in the electrical dysfunctions and pave the way for a comprehensive model that considers both the functional and structural data, thus enabling a unified investigation of the structural causes that lead to the electrical and mechanical alterations after the tissue remodeling.

INTRODUCTION:

Structural remodeling associated with cardiac diseases can affect electrical conduction and introduce electromechanical dysfunctions of the organ[1,2]. Current approaches used to predict functional alterations commonly employ MRI and DT-MRI to obtain an overall reconstruction of fibrosis deposition, vascular tree, and fiber distribution of the heart, and they are used to model preferential action potential propagation (APP) paths across the organ[3,4]. These strategies can provide a beautiful overview of the heart organization. However, their spatial resolution is insufficient to investigate the impact of structural remodeling on cardiac function at the cellular level.

Placing this framework at a different order of magnitude, where single cells can play individual roles on action potential propagation, is challenging. The main limitation is the inefficiency of standard imaging methods to perform high-resolution imaging (micrometric resolution) in massive (centimeter-sized) tissues. In fact, imaging biological tissues in 3D at high resolution is very complicated due to tissue opaqueness. The most common approach to perform 3D reconstructions in entire organs is to prepare thin sections. However, precise sectioning, assembling, and imaging require significant effort and time. An alternative approach that does not demand cutting the sample is to generate a transparent tissue. During the last years, several methodologies for clarifying tissues have been proposed[5–8] . The challenge to produce massive, transparent, and fluorescently-labeled tissues has been recently achieved by developing true tissue transformation approaches (CLARITY[9] , SHIELD[10]). In particular, the CLARITY method is based on the transformation of an intact tissue into a nanoporous, hydrogel-hybridized, lipid-free form that enables to confer high transparency by the selective removal of membrane lipid bilayers. Notably, this method has been found successful also in cardiac preparation[11–14] . However, since the heart is too fragile to be suitable for an active clearing, it must be cleared using the passive approach, which requires a long time to confer complete transparency.

In combination with advanced imaging techniques like light-sheet microscopy, CLARITY has the potential to image 3D massive heart tissues at micrometric resolution. In light-sheet microscopy, the illumination of the sample is performed with a thin sheet of light confined in the focal plane of the detection objective. The fluorescence emission is collected along an axis perpendicular to the illumination plane[15]. The detection architecture is similar to widefield microscopy, making the acquisition much faster than laser scanning microscopes. Moving the sample through the light sheet permits obtaining a complete tomography of big specimens, up to centimeter-sized samples. However, due to the intrinsic properties of the Gaussian beam, it is possible to obtain a very thin (of the order of a few microns) light-sheet only for a limited spatial extension, thus drastically limiting the field of view (FoV). Recently, a novel excitation scheme has been introduced to overcome this limitation and applied for brain imaging, allowing 3d reconstructions with isotropic resolution[16].

In this paper, a passive clearing approach is presented, enabling a significant reduction of the clearing timing needed by the CLARITY protocol. The methodological framework described here allows reconstructing a whole mouse heart with micrometric resolution in a single tomographic scan with an acquisition time in the order of minutes.

PROTOCOL:

All animal handling and procedures were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and conformed to the principles and regulations of the Italian Ministry of Health. The experimental protocol was approved by the Italian Ministry of Health (protocol number 647/2015-PR). All the animals were provided by ENVIGO, Italy. For these experiments, 5 male C57BL/6J mice of 6 months of age were used.

1. Solution preparation

1.1. Prepare 4% Paraformaldehyde (PFA) in Phosphate-Buffered Saline (PBS) (pH 7.6) in a chemical hood. Store the 4% PFA aliquots at -20 °C for several months.

1.2. Prepare Hydrogel solution: Mix 4% Acrylamide, 0.05% Bis-acrylamide, 0.25% Initiatior AV-044 in 0.01 M PBS in a chemical hood. Keep the reagents and the solution on ice during the entire preparation. Store the hydrogel aliquots at -20 °C for several months.

1.3. Prepare Clearing solution: Mix 200 mM Boric acid, 4% Sodium Dodecyl-Sulfate (SDS) in deionized water; pH 8.6 in a chemical hood. Store the solution between 21–37 °C to avoid SDS precipitation.

1.4. Prepare fresh Tyrode solution on the day of the experiment: Add 10 mM Glucose, 10 mM HEPES, 113 mM NaCl, 1.2 mM $MgCl_2$, and 4.7 mM KCl; titrate to pH 7.4 using 1 M NaOH.

2. Heart isolation

2.1. Inject 0.1 mL of 500 I.U. Heparin subcutaneously 30 min before the heart isolation procedure.

2.2. Fill a 30-mL syringe and three 6-cm Petri dishes with fresh Tyrode solution. Make a small rift (3–4 mm in depth) on the border of one of the Petri dishes and place it under a stereoscopic microscope.

2.3. Fix a 1 mm-diameter cannula to the syringe and insert it in the rift of the Petri dish. Make sure there are no air bubbles in the syringe.

2.4. Fill a 20-mL syringe with 4% PFA and keep it in the chemical hood. Prepare an empty Petri dish under the hood.

2.5. Anesthetize the mouse with 3% Isoflurane/oxygen at a flow rate of 1.0 L/min and sacrifice it by cervical dislocation according to animal welfare rules in force.

2.6. After the sacrifice, remove the fur over the chest and open the chest to have full access to the heart.

2.7. Isolate the heart, immerse it in the Petri dish previously filled with 50 mL of Tyrode Solution. Use surgical scissors to cut the aorta immediately near the aortic arch to have the heart exposed.

2.8. Transfer the heart under a stereoscopic microscope and carefully perform the cannulation. Do not insert the cannula too deep into the aorta (no more than 2 mm) to avoid tissue damage.

2.9. Use a little clamp and a suture (size 5/0) to fix the heart to the cannula.

2.10. Perfuse the heart with 30 mL of the Tyrode solution with a constant pressure of 10 mL/min to remove blood from the vessels.

2.11. Detach the cannula from the syringe and place the heart in the Petri dish filled with Tyrode solution. Be careful not to have air bubbles in the cannula; otherwise, remove the air bubbles properly.

2.12. Attach the 20-mL syringe filled with cold 4% PFA to the cannula and perfuse the heart at the same constant pressure.

2.13. Incubate the heart in 10 mL of 4% PFA at 4 °C overnight (O/N). To avoid tissue degradation, perform steps 2.6–2.13 in the shortest time possible.

3. Heart clearing

3.1. The following day, wash the heart in 0.01 M PBS 3 times at 4 °C for 15 min.

NOTE: After this step, the heart can be stored in PBS + 0.01% sodium azide (NaN3) at 4 °C for several months.

3.2. Incubate the heart in 30 mL of Hydrogel solution in shaking (15 rpm) at 4 °C for 3 days.

3.3. Degas the sample at room temperature using a dryer, a vacuum pump, and a tube system that connects the dryer to both the pump and a nitrogen pipeline.

3.3.1. Place the sample in the dryer and open the vial, keeping the cap on it.

3.3.2. Close the dryer and remove the oxygen from the tube by opening the nitrogen pipeline.

3.3.3. Turn on the vacuum pump to remove the oxygen from the dryer for 10 min.

3.3.4. Turn off the pump and use the knob of the dryer to open the nitrogen pipeline. Once the pressure is equal to the atmospheric pressure, carefully open the dryer and quickly close the vial.

3.4. Keep the heart in the degassed Hydrogel solution at 37 °C for 3 h at rest.

3.5. When the Hydrogel is properly polymerized and appears entirely gelatinous, carefully remove the heart from it and place it in the sample holder.

3.6. Insert the sample holder with the heart in one of the clearing chambers and close it properly to avoid leaks of the clearing solution.

3.7. Switch on the water bath where the clearing solution container is placed and the peristaltic pump to start the recirculation of the clearing solution.

3.8. Change the clearing solution in the container once a week to speed up the clarification procedure.

4. Cellular membrane staining

4.1. Once the heart appears completely clarified, remove it from the sample holder and wash it in 50 mL of warmed-up PBS for 24 h. Wash again in 50 mL of PBS + 1% of Triton-X (PBS-T 1x) for 24 h.

4.2. Incubate the sample in 0.01 mg/mL Wheat Germ Agglutinin (WGA) – Alexa Fluor 633 in 3 mL of PBS-T 1x in shaking (50 rpm) at room temperature for 7 days.

4.3. After the 7-day incubation, wash the sample in 50 mL of PBS-T 1x at room temperature in shaking for 24 h.

4.4. Incubate the sample in 4% PFA for 15 min and then wash it 3 times in PBS for 5 min each.

NOTE: After this step, the heart can be stored in PBS + 0.01% NaN3 at 4 °C for several months.

4.5. Incubate the heart in increasing concentrations of 2,2'-Thiodiethanol (TDE) in 0.01 M PBS (20% and 47% TDE/PBS) for 8 h each, up to the final concentration of 68% TDE in 0.01 M PBS to provide the required refractive index (RI = 1.46). This is the RI matching medium (RI-medium) to acquire images[16] .

5. Heart mounting and acquisition

NOTE: All the components of the optical system are listed in detail in the **Table of Materials**.

5.1. Gently fill about 80% of the external cuvette (quartz, 45 mm × 45 mm × 42.5 mm) with the RI-medium.

NOTE: Here, it is possible to use different non-volatile solutions that guarantee a RI of 1.46.

5.2. Gently fill the internal cuvette (quartz, 45 mm × 12.5 mm × 12.5 mm) with the same RI-medium.

5.3. Immerse the sample inside the internal cuvette. The sample incubations described above allow the sample to remain stable inside the RI-medium without being held.

5.4. Gently move the sample to the bottom of the cuvette using thin tweezers and arrange the heart with its longitudinal axis parallel to the cuvette's main axis to minimize the excitation light path across the tissue during the scanning.

5.5. Gently fix the tailored plug above the internal cuvette with two screws.

5.6. Mount the sample to the microscope stage using the magnets.

5.7. Translate the vertical sample stage manually to immerse the internal cuvette into the external one.

5.8. Turn on the excitation light source (wavelength of 638 nm), setting a low power (in the order of 3 mW).

5.9. Move the sample using the motorized translator to illuminate an inner plane of the tissue.

5.10. Turn on the imaging software (HCImageLive) and set the camera **Trigger** on **External Edge Trigger (light-sheet)** mode to drive the acquisition trigger of the camera by the custom software controlling the entire setup.

5.11. Enable **Autosave** in the **Scan Settings** panel and set the output folder where the images need to be saved.

5.12. Manually adjust the sample position in the XY plane with the linear translators to move the sample to the center of the FoV of the camera sensor.

5.13. Move the sample along the Z-axis using the linear motorized translator to identify heart borders for tomographic reconstruction.

5.14. Increase the laser power to ~20 mW, ready for the imaging session.

5.15. Start the tomographic acquisition, click the **Start** button in the **Capture** panel of the imaging software, and at the same time move the sample along the Z-axis at the constant velocity of 6 µm/s using the motorized translator.

REPRESENTATIVE RESULTS:

The developed passive clearing setup allows to obtain a cleared adult mouse heart (with a dimension of the order 10 mm x 6 mm x 6 mm) in about 3 months. All the components of the setup are mounted, as shown in **Figure 1**. The negligible temperature gradient between each clearing chamber (of the order of 3°C) allows maintaining the temperature in a proper range across all chambers.

[insert **Figure 1** here]

Figure 2 shows the result of the clearing process of an entire heart. As already reported by Costantini et al.[16], the combination of the CLARITY methodology with TDE as RI-medium does not significantly change the sample's final volume nor leads to anisotropic deformation of the specimen.

[insert **Figure 2** here]

Once the heart was cleared, cellular membranes were stained with an Alexa Fluor 633-conjugated WGA to perform the cytoarchitecture reconstruction of the entire organ. The custom-made fluorescence light-sheet microscope (**Figure 3**) was able to ensure 3D micron-scale resolution across the entire FoV.

[insert **Figure 3** here]

Considering the numerical aperture (NA = 0.1) of the detection optics, the radial (XY) Point Spread Function (PSF) of the system can be estimated in the order of 4–5 µm. On the other hand, the excitation optics produce a light-sheet with a minimum waist of about 6 µm (Full width half maximum, FWHM) that diverges up to 175 µm at the edge of the FoV (**Figure 4A–C**). The

synchronization of the camera rolling shutter with the axial scan of the light-sheet waist ensures to excite the sample with the thinnest portion of the light-sheet, resulting in an average FWHM of about 6.7 µm along the entire FoV (**Figure 4B–D**).

[insert **Figure 4** here]

The Z-PSF of the microscope was also estimated by a tomographic reconstruction of the fluorescent nanosphere (**Figure 5**). An FWHM of 6.4 µm can be estimated by the fit, in good agreement with the previous assessment.

[insert **Figure 5** here]

Owing to the high transparency of the tissue, it was possible to illuminate the whole heart without significant distortion of the axially scanned light-sheet at an excitation wavelength of 638 nm. The fluorescence signal was collected by the sCMOS sensor operating at 500 ms of exposure time and a frame rate of 1.92 Hz. Based on previous quantification, the tomographic acquisition was performed using a Z-scan velocity of 6 µm/s, and assuming a frame rate of 1.92 Hz, one frame every 3.12 µm was acquired, oversampling the system Z-PSF by about two times. Two representative frames (on the coronal and transverse planes) of the left ventricle chamber are shown in **Figure 6**. This result confirms the potentiality of the system to resolve single cellular membranes in three dimensions with a sufficient Signal/Noise ratio in the entire organ (**Figure 6**).

[insert **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the passive clearing setup. The clearing solution (after being filtered) circulates in succession through the sample chambers with the help of the peristaltic pump. The maintenance of the solution container in a water bath set at 50 °C allows the solution temperature to be between 37–45 °C within the chambers. Image created with Biorender.com.

Figure 2: **Representative image of a heart before (on the left) and after (on the right) the CLARITY protocol.** The hearts become fully transparent and slightly oversized.

Figure 3: MesoSPIM. CAD renderings of the custom-made fluorescence light-sheet microscope.

Figure 4: Light-sheet generation and characterization. (**A**) An excitation light-sheet generated with a laser source of 638 nm is focused on the center of the Field of View (FoV) and acquired with a pixel size of 3.25 µm and an Exposure Time of 10 ms. Light intensity is normalized and reported with a colormap. The Full Width Half Maximum (FWHM) of the light intensity profile is evaluated in 15 different positions along the FoV. Results are shown in **C**. (**B**) Image of the excitation light-sheet generated by the synchronization between the camera rolling shutter operating at 1.92 Hz and the light beam position driven by the tunable lens. The FWHM of the light intensity profile is evaluated along the FoV and results are shown in **D**.

Figure 5: Point Spread Function in the Z-axis. The Point Spread Function (PSF) of the optical system is estimated by imaging fluorescent sub-micron-scale nanospheres (excited with a light sheet with a wavelength of 638 nm) with a pixel size of 3.25 µm × 3.25 µm × 2.0 µm. PSF intensity profile along the optical axis (Z) is represented as black dots. PSF profile is fitted with a Gaussian function with μ = 18.6 µm and σ = 2.7 µm. The FWHM of the PSF estimated by the fit is 6.4 µm.

Figure 6: Mouse heart tissue reconstruction. The clarified heart was stained with WGA conjugated to Alexa Fluor 633 and excited by a laser source with a wavelength of 638 nm. (**A**) Coronal and (**B**) transverse representative sections. (**C–D**) Tissue transformation produces high tissue transparency, allowing to resolve small structures in the wall depth. The optical system shows an axial resolution sufficient to resolve micrometric structures (panel. **D**). (**E**) 3D low-resolution heart rendering.

DISCUSSION:

In this work, a successful approach to clear, stain, and image a whole mouse heart at high resolution was introduced. First, a tissue transformation protocol (CLARITY) was optimized and performed, slightly modified for its application on the cardiac tissue. Indeed, to obtain an efficient reconstruction in 3D of a whole heart, it is essential to prevent the phenomenon of light scattering. The CLARITY methodology allows us to obtain a highly transparent intact heart, but it requires long incubation times when performed passively (about 5 months). With respect to the brain, the cardiac tissue is not suitable for an active clearing, which takes advantage of an electric field. Even at low voltages, the electric field leads to damages and tissue breakages. Here, a passive clearing approach was optimized to obtain a completely cleared heart in about 3 months. After isolating and cannulating the heart through the proximal aorta, the CLARITY methodology was performed as described in section 3 of the protocol. To speed up the procedure, a homemade passive clearing setup was arranged (**Figure 1**), which ended up decreasing the timing of tissue clearing by about 40%. The setup is composed of a container for the clearing solution, a water bath, a peristaltic pump, several chambers containing different sample holders, capsule filters for each chamber, and a tubing system for the recirculation of the solution. The pump extracts and circulates the solution from the container in succession through each of the chambers, where the samples are held for clearing. Before entering the chambers, the solution flows through a capsule filter to trap the lipids flushed away from tissues during the clearing. The optimal temperature for the clearing solution, between 37–45 °C, is maintained within the chambers during the recirculation by keeping the solution container in a water bath at 50 °C. It is advised to change the clearing solution in the container once a week during the procedure. All components used are listed in detail in the **Table of Materials**. The optimized solution presented here allows us to obtain a whole passively cleared mouse heart in a significantly shorter time with respect to the standard passive clearing technique, thus reducing the required experimental time without damaging the organ. The staining approach was also optimized for homogeneous labeling of the cellular membranes and endothelium, using a fluorescent lectin (WGA – Alexa Fluor 633).

The heart cytoarchitecture has been reconstructed by developing a dedicated mesoSPIM that axially sweeps the light-sheet across the sample (https://mesospim.org). The custom-made fluorescence light-sheet microscope (**Figure 3**) was able to rapidly acquire images of a mesoscopic FoV (of the order of millimeters) with micrometric resolution. In this way, single cardiomyocytes can be resolved and mapped into a 3D reconstruction of the entire organ. The microscope illuminates the cleared sample with a light-sheet, dynamically generated by scanning a laser beam at 638 nm using a galvanometric mirror. A sCMOS camera characterizes the detection arm in a 2x magnification scheme which enables it to acquire the entire FoV in a single scan. The fluorescence signal was selected by placing a long-pass filter after the objective. The camera was set to work in rolling shutter mode: at any time, the line of active camera pixels (i.e., exposed to the image) is synchronized with the in-plane shift of the focal band of the light-sheet, performed by an electrically tunable lens. This approach maximized the optical sectioning capability in the whole FoV by only acquiring images in the thinnest part of the focused light-sheet. This solution differs from conventional configurations,

where acquisition involves the entire range of focal depth of the light-sheet, preventing peak optical sectioning resolution in large part of the FoV. An integrated sample stage supports cuvettes, thereby optimizing positioning and enabling axial movement of the sample during the imaging process. In this way, tomographic reconstructions are possible by acquiring consecutive internal sections. The images obtained have a mesoscopic FoV and a micrometric resolution, while the acquisition time required for a whole mouse heart is ~ 15 min. The synchronization between the camera rolling shutter and the excitation light beam sweeping the FoV allows acquiring the entire image plane with a high spatial resolution (**Figure 4**). This allows direct reconstruction of the sample in a single tomographic acquisition, without the necessity of sample radial displacement and multi-adjacent-stacks-based imaging. Notably, the microscope allowed the reconstruction of the entire organ of about (10 mm x 6 mm x 6 mm) in a single imaging session, with a near-isotropic voxel size and a sufficient signal-to-background ratio to resolve single cells across the whole organ potentially.

It is noteworthy that the proposed protocol presents some critical steps that must be performed carefully to achieve good results. In particular, the cannulation of the heart through the proximal aorta can be quite difficult, but it is an essential step to wash and fix the organ properly. Judd et al.[17], showed how to perform this step effectively. Moreover, the degassing procedure needed by the CLARITY protocol is quite complex too, but it is essential for tissue preservation; if this step is not performed properly, the tissue could encounter damages and decay during the incubation in clearing solution.

Furthermore, although the presented experimental workflow is suitable for small fluorescent probes, the use of immunohistochemistry does not always provide good efficiency in the staining due to the higher molecular weight of the antibodies. Each immunostaining protocol requires proper optimization, and different approaches have been conceived to improve the antibody penetration, for example, tissue expansion[18] and/or variations in pH and ionic strength[19].

The mesoSPIM setup also presents two main limitations: i) the light-sheet preservation across the sample is strongly dependent on the tissue transparency, and ii) the dimension of the camera sensor limits the FoV. Guaranteeing a perfect refractive index matching inside the entire heart is very challenging, and small variations on the refractive index can produce light scattering, leading to degradation of the image quality. In this respect, a dual-side illumination scheme can be introduced. Two excitation arms can generate two distinct and aligned dynamic light sheets with maximally focused illumination by alternating the illumination from one side to the other of the specimen. Also, the FoV can be improved by using a new generation high-resolution back-illuminated sCMOS with very large sensors in combination with high numerical aperture telecentric lenses with low field distortion. This implementation would allow us to reconstruct bigger organs or expanded tissues maintaining the same optical section capability and thus producing micron-scale 3D images of centimeter-sized cleared samples.

Although the presented protocol still requires a long time for sample preparation and a high level of transparency to obtain a reliable cytoarchitecture reconstruction of the entire organ, the main significance of the approach resides in the improvements of the clearing protocol and the capability to perform mesoscopic reconstruction in a single scan at micrometric resolution. In the future, these advances can be combined with a multi-staining protocol to achieve whole-organ reconstruction integrating different biological structures.

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DISCLOSURES:
Nothing to disclosure.

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Figure

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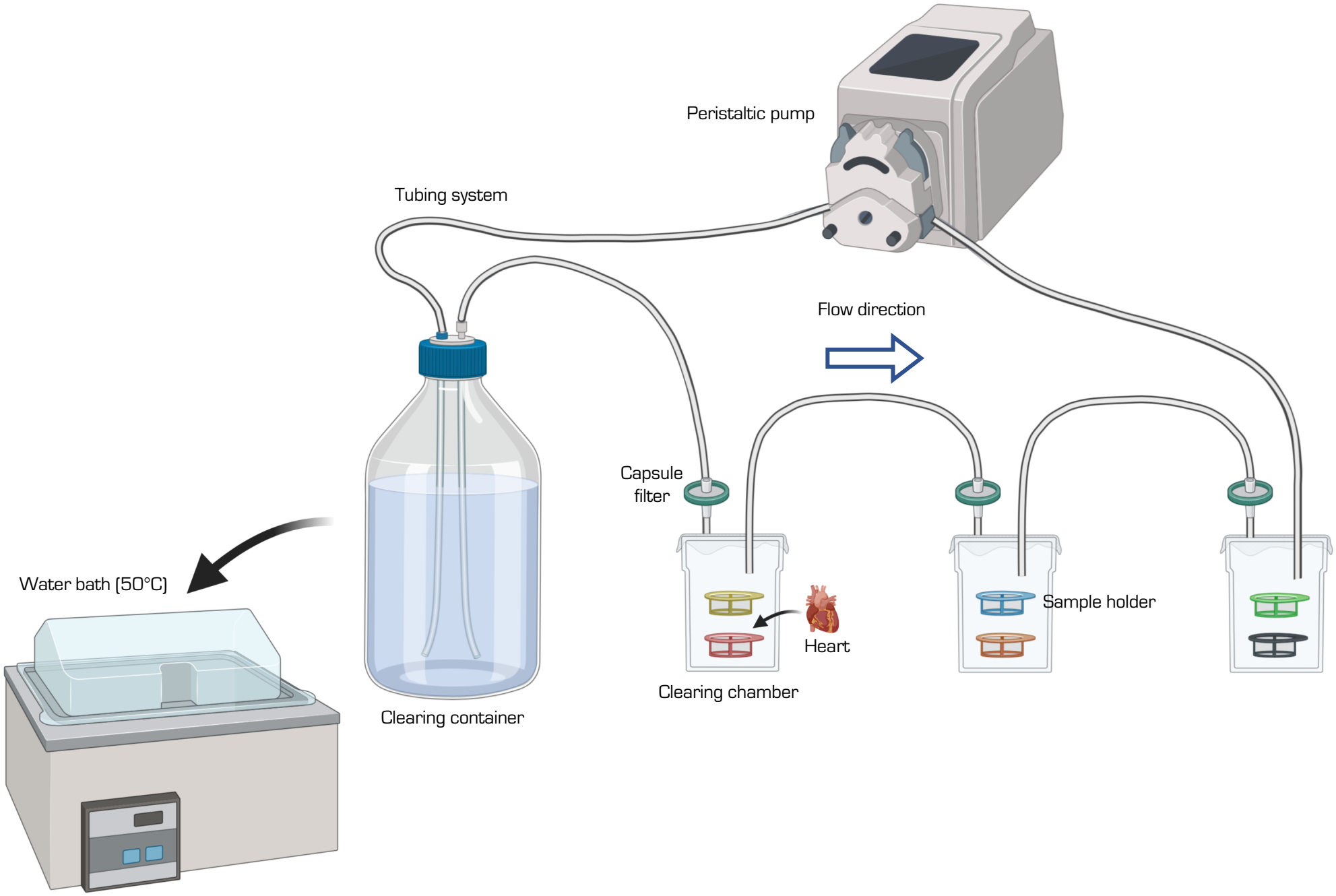

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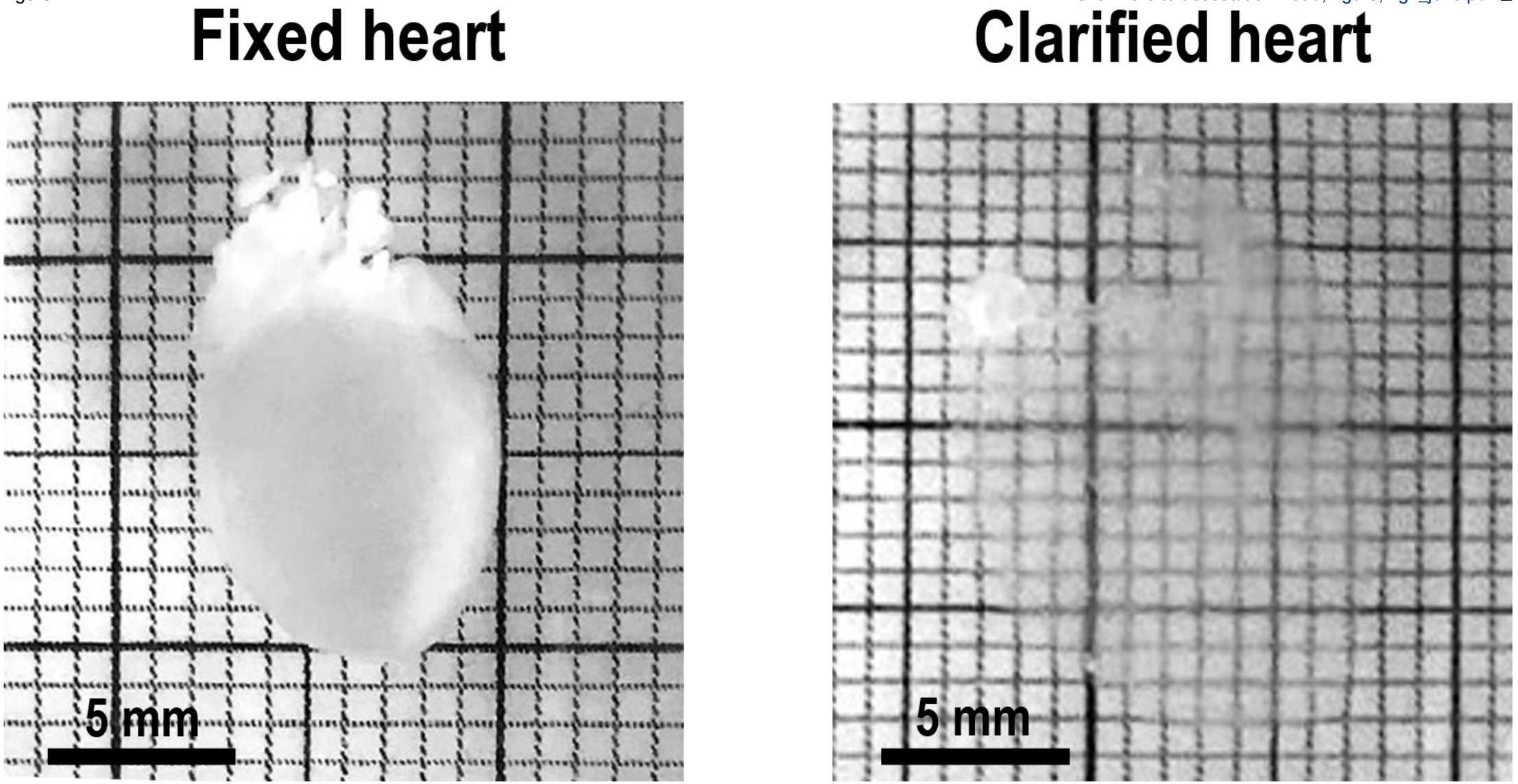

Figure

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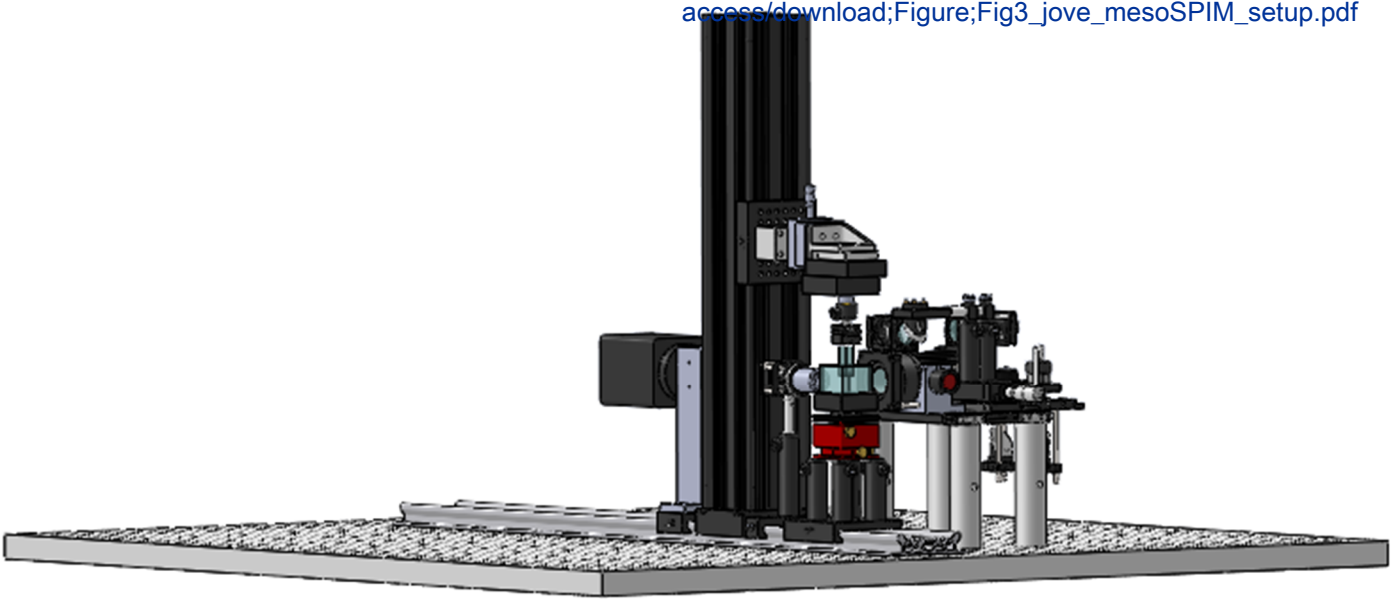

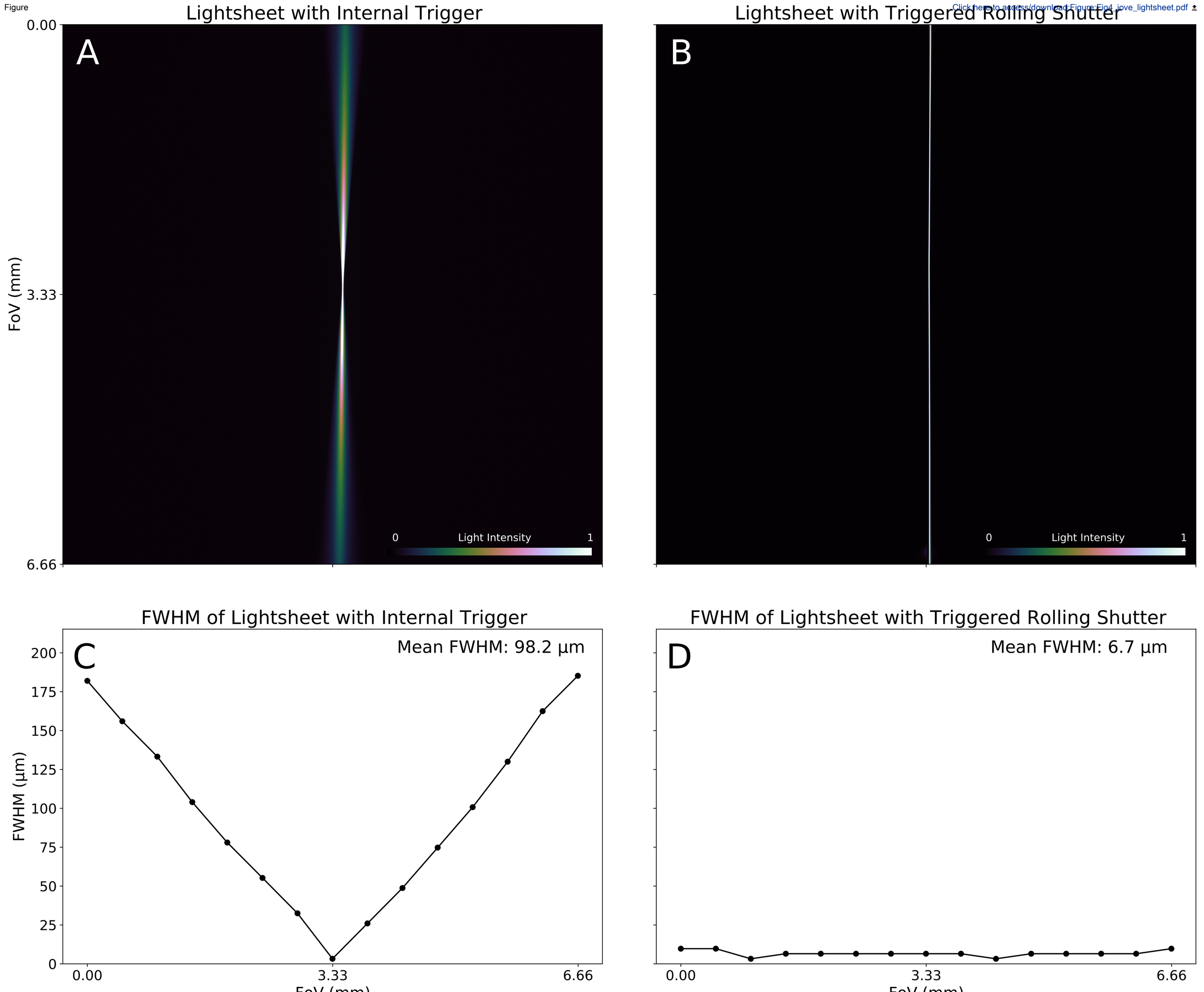

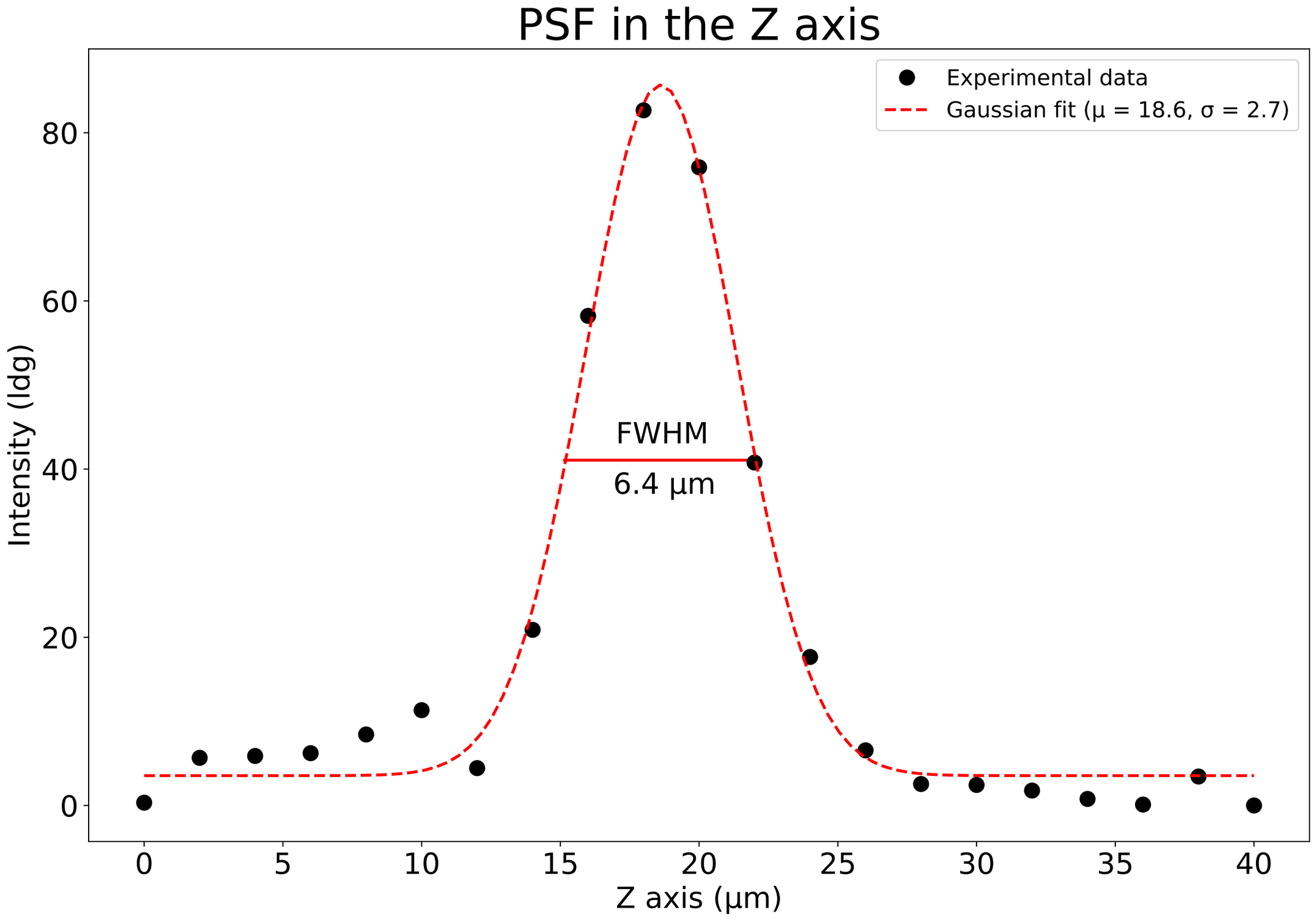

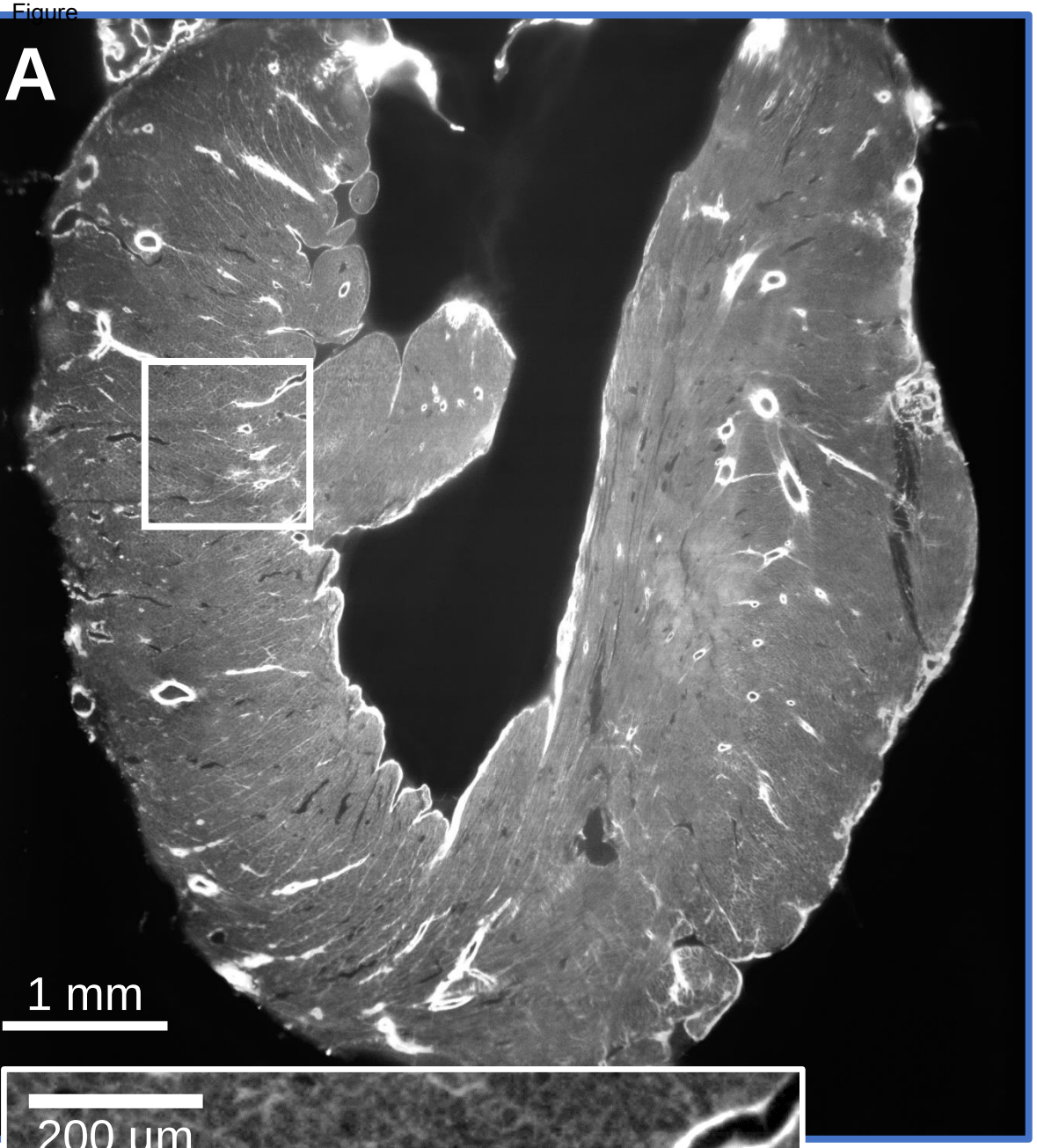

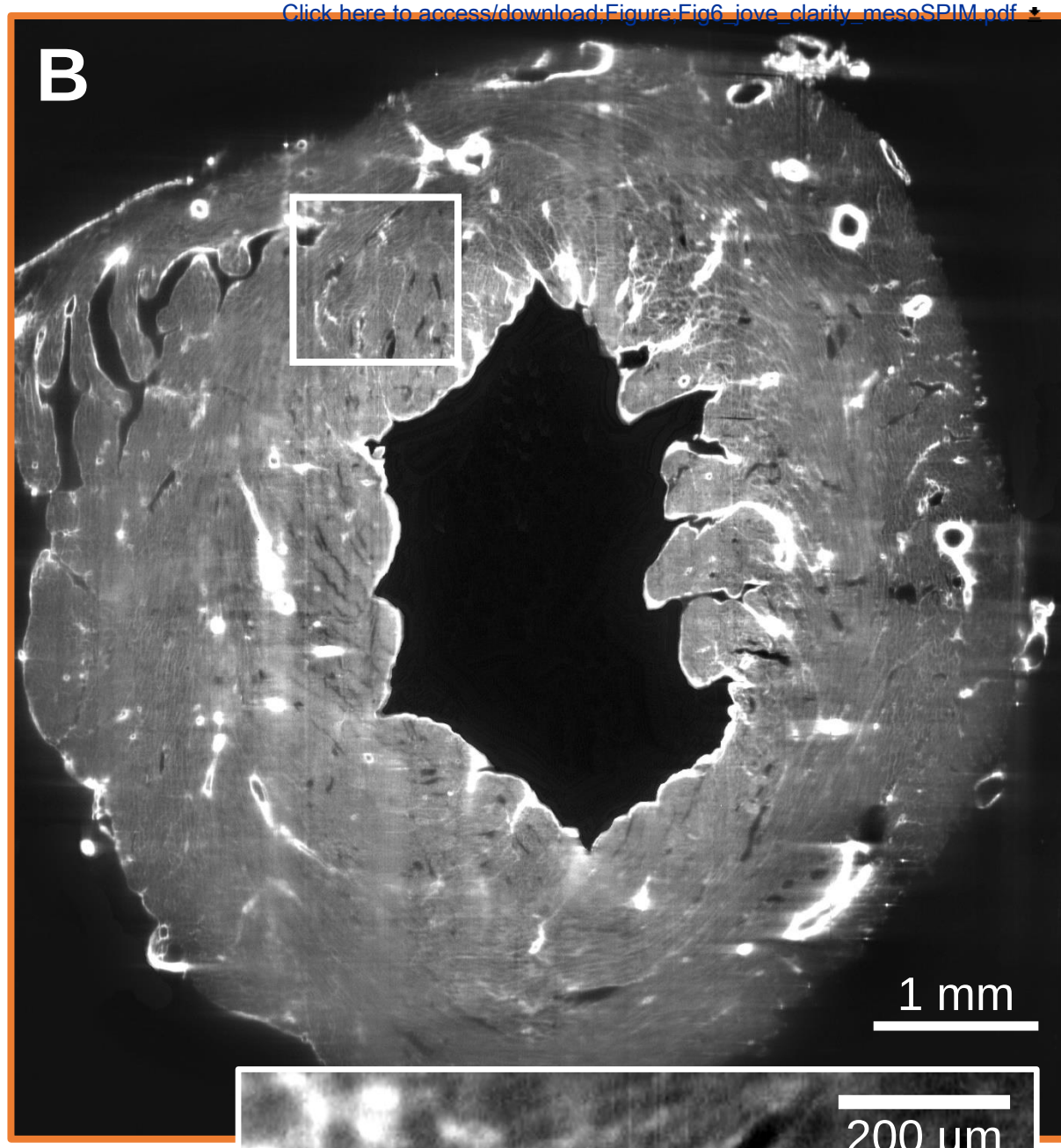

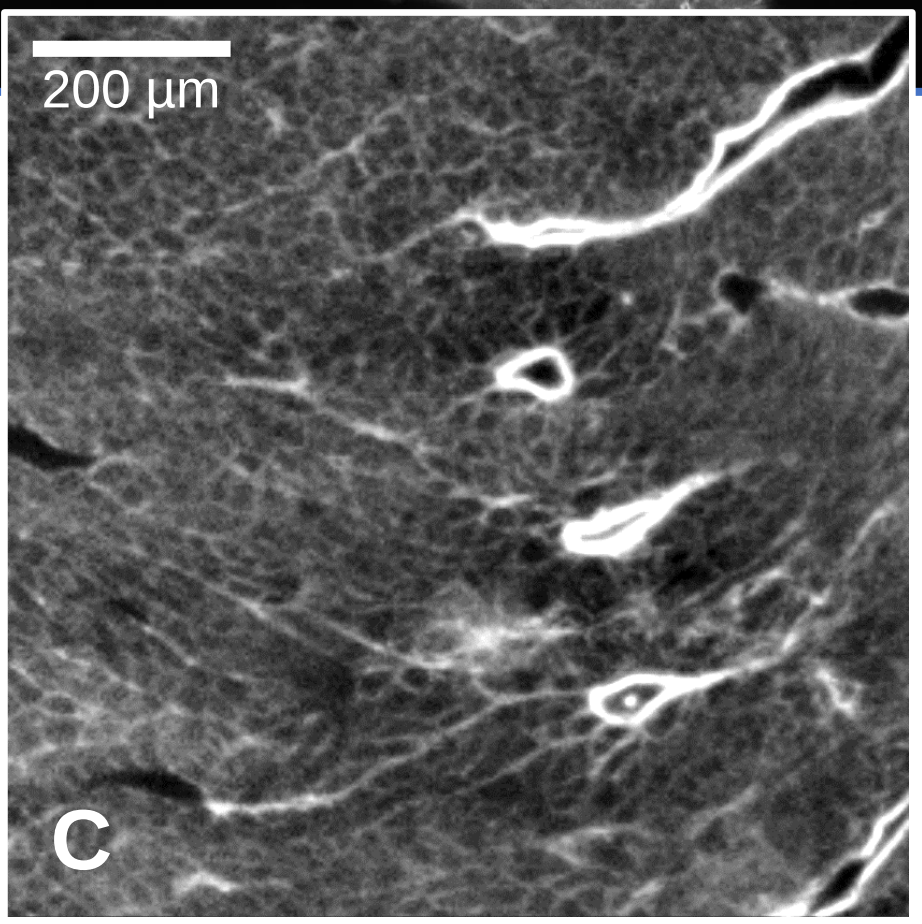

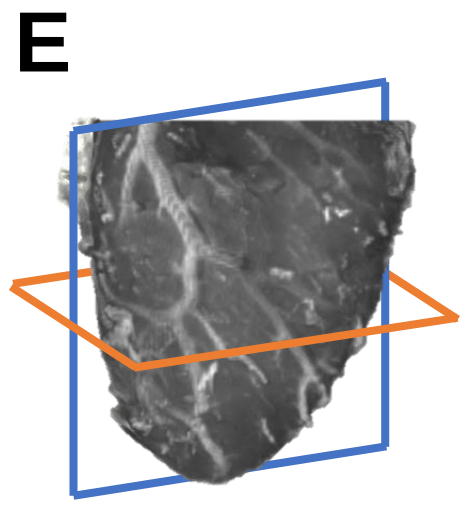

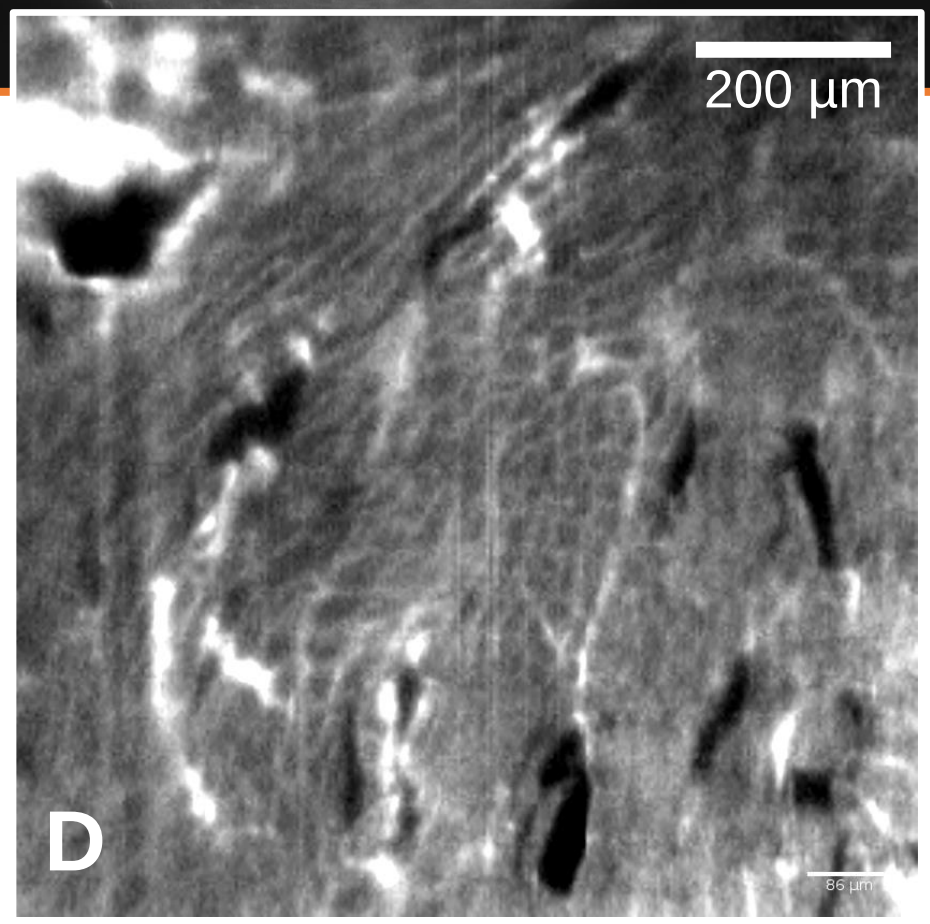

REPLY TO THE EDITORS

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We performed an accurate proofreading of the text.

2. Please revise the following lines to avoid previously published work: 40-43, 55-65, 67-70.
We revised the text and we checked the citations.

3. Please ensure that abbreviations are defined at first usage.
We performed an accurate proofreading of the text.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.
All commercial languages are removed from our manuscript and referenced in the Table of Materials.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The individual steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.
We adjusted the protocol section.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Accordingly, Step1 can be moved to the Discussion section.
We thank the editor for this suggestion. We moved Step 1 to the discussion accordingly.

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

8. Please add more details to your protocol steps.

Step 1: Please specify the age/gender/strain of the mouse used.
Step 2.1.3: The term Milli-Q water can be changed to a generic one, deionized water.
Step 2.2.5: Please use “cervical dislocation” instead of “capital dislocation”. After this, decapitation was performed? Please clarify. Also, specify how the method was performed.
Step 2.2.6: Please specify how much the chest should be opened. What equipment was used to cut the aorta?

Step 2.2.7: Please specify the depth for the cannula to be inserted.
Step 2.2.8: Please elaborate on the perfusion process.
Step 2.3.3: Please elaborate on the de-gassing process.
Step 2.3.4: Please specify how to check proper polymerization. Also mention for clarification, should the heart be kept at 4 °C?
Step 2.4.1: How to make sure that the clarification process is complete?
Step 2.4.2: What was the final concentration of the fluorophore used??
Step 2.4.6: What is meant by increasing concentrations?
Step 2.5.1: Is this a glass cuvette?
Step 7: What was the excitation light source used here?
Step 2.5.8: Which software was used? In the software, please ensure that all button clicks and user inputs are provided throughout. Also please ensure that the button clicks are bolded.

All the requested information has been added.

9. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Also, the highlighted part must be in line with the title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

10. Please rewrite the Results section to include all the observations and conclusions that can be derived from the Figures.
We expanded this section.

11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We expanded this section covering the suggested topics.

12. Figure 1: Please label all the components of the setup.
We modified the figure, we thank the editor for the suggestion.

13. Please combine the two Tables of Materials (1 and 2) in a single one.
We generated a new unique table.

14. Please spell out journal titles in all the references. Also include volume and issue numbers for all References.
We accurately checked the references.

REPLY TO REVIEWERS:

Reviewer #1:

***Manuscript Summary**:*
This paper presents a protocol that combines tissue clearing (a modified CLARITY) and optical imaging (light-sheet microscopy) for whole mouse heart imaging. To demonstrate the efficacy of the protocol, a reconstructed optical image of a sagittal plane from the mouse heart is presented. In my opinion, if the following issues can be addressed, this paper can be considered for publication.

Major Concerns:

1.The authors claim that they can achieve optical imaging at micron resolution (3.25 × 3.25 × 3.12 µm3) as well as sub-cellular resolution. But I cannot agree with it lying on the reason that presented result only show the individual cardiomyocytes but not organelles. To be more specific, to visualize sub-cellular cardiac structures, it require the imaging capacity of submicron voxel resolution.

We thank the reviewer for the comment. We have changed the resolution definition in "micrometric resolution".

2.To our best knowledge, some modified CLARITY protocols for mouse heart clearing have been reported before [1, 2]. The authors should list them in the introduction and clearify the difference and advantage of the presented protocol.

We added the two references and explained the advantages of our approach in the text.

3.In tissue clearing, non-linear expansion of the tissue is inevitable. It is an important indicator for evaluating transparent protocol. But the authors did not give a quantitative assessment, which should be added.

As reported in Costantini et al., 2015, Scientific Reports, we found that the tissue expansion in CLARITY-cleared tissues can be isotropically reverted by the incubation of the specimen in TDE as Ri matching medium. We thank the reviewer for the suggestion and we added an explanatory sentence in the results section.

4.On line 250, the authors mentioned that "The developed protocol allows to obtain a cleared heart in about 3 months". Does this mean that the clearing protocol can only be applied to heart samples within a specific size, i.e., 10 x 6 x 6 mm3? If so, it should be pointed out in the article.

This point has been mentioned on the revised manuscript.

5.In the Discussion, the authors mentioned that "the microscope allowed the reconstruction of the entire organ in a single imaging session, with a near-isotropic voxel size and a sufficient signal-to-background ratio to resolve single cells across the whole organ." But lack of representative results to prove it, which should be added.

With this sentence we mean that the optical system is *potentially* able to reach that result in terms of resolution and sensitivity, but of course a perfectly cleared and stained sample is necessary. We modified the sentence to highlight the points and, more importantly, we added a transverse plane

of the representative reconstruction of the cleared heart to show the resolution capacity in the three axes.

Minor Concerns:

1.On line 283, typo "Tehe" should be "The".

Typo has been fixed.

Reviewer #2:

Manuscript Summary:

Authors describe a new method to clarify, image and reconstruct whole mouse heart. They have developed a modified version of CLARITY to render the heart transparent. In addition, they have setup a lighsheet mesoscope based on the mesoSPIM to image rapidly with a cellular spatial resolution the whole heart of mice. I think that the paper is very well written, clear and provides enough technical information to allow other research groups to us ethis new approach. I just have few minor comments.

Major Concerns:

No Major concerns

Minor Concerns:

-There are some sentences in the manuscript where Authors used qualifying adjectives where they should provide some numbers (e.g. line 55: "massive" or line 73 "Big specimen", l74 "thin lighsheet" or line 75 "limits the FOV", l98 "decreasing the timing ")
The manuscript has been improved with more quantitative statements.

-Concerning the multiple chambers approach for clarification, could Authors precise whether there is a temperature gradient across the different chambers and if this may be an issue or not ?
-line 165: DO Authors really mean "100% oxygen" ?
-line 166: Do the Authors refer to "cervical discolation" instead of "capital dislocation"?

We made the proper corrections suggested.

-points 2.2.7 to point 2.2.10: could the Authors mention the maximum time it should take to do these steps ?

To avoid tissue degradation, steps 2.6-2.13 have to be performed in the shortest time possible. The manuscript has been revised highlighting this important aspect.

- point 2.4.6: Could Authors precise how excately should be processed the heart with increasing concentrations of TDE ? How many steps, for how long ? Also, could Authors mention whether

reaching a 68 % TDE in PBS is necessary to reach a certain RI and if this percentage can be adjusted to adjust the refractive index of the tissue?

We elaborated on this step. The percentage of TDE in PBS can be adjusted to tune the RI of the medium; however, since the delipidated tissue has a RI = 1.46, the 68% TDE in PBS is the suitable percentage, because it provides the same RI.

-point 2.5.1 Could Authors mention if it is an option to fill the external cuvette with Cargill oil that a lot of researchers in the field are using ?
We don't have any experience with the Cargill oil, but theoretically it is possible to use it if it ensures the right refractive index. We added a sentence about that possibility.

-point 2.5.3: COuld Authors precise how the heart in held in place inside the cuvette, during imaging ?
We elaborated this point in 5.3 and 5.4. We thank the reviewer for the suggestion.

-Imaging part: Could Authors shows a horizontal reslice of the dataset to illustrate that the axial resolution is enough and homogenous?
We added the requested images.

-line 283: typo error The instead of Tehe
-line 287 whole instead of wall

Typos have been fixed.

-line 295: I am confused here. Do the clarification process take 3 or 5 months (3 months indicated on line 251)

The CLARITY protocol performed using the standard passive clearing approach requires about 5 months to reach the complete transparency of a whole heart. The approach that we are proposing, thanks to the clearing solution recirculation, allows us to reduce the timings to about 3 months.

-line 300: Authors mention that they use a fluorescent lectin to label "cardiac cellular membranes". What are eaxctely these cells ? Cardiomyocites?
In additon, lectins are often used to label endothelial cells of blood vessels. Are authors labelling blood vessels or specific cells here ? Could Authors make this point more clear ?

Wheat Germ Agglutinin (WGA) is a lectin that binds glycoproteins; for this reason, it binds the cellular membranes of both cardiac cells and endothelium. This point has been clarified in the revised version of the manuscript.

-line 310 typo mistake remove "resulted"
References 4 and 15 are not correctly formatted.
Table of materials 2: typo error External

We corrected the mistakes.

Reviewer #3:

Manuscript Summary:
This is an interesting and well planned methodology paper.

No major concern

Specific comments:

1. What is the mesoscopic stands for in your paper, please explain, such as resolution, etc.
A quantitative statement of mesoscopic imaging has been provided in the revised manuscript.

2. Please provide the strain, age, and gender and number of the mice used
3. Authors should provide a statement of ethical approval for the study

Both points 2 and 3 have been elaborated and corrected in the text.

4. Why animal need to wait for 30 min after Heparin is injected. Based on this reviewer experience, 5 min is longer enough for preventing blood clots in the heart.

In our experience, we have observed that waiting for a longer time allows for the animals to return to a quiet and calm state after the injection.

5. It would be better to show some lightsheet images
We included a new figure (Fig. 4) to fill this gap. It includes a characterization of the lightsheet in the field of view in both camera configurations (internal trigger and synchronous rolling shutter trigger) and the estimation of the light-sheet thickness.