Mesoscopic optical imaging of whole mouse heart

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10 **TITLE:**
11 Mesoscopic Optical Imaging of Whole Mouse Heart

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

43

44 **ABSTRACT:**
45 Both genetic and non-genetic cardiac diseases can cause severe remodeling processes in the heart.
46 Structural remodeling, such as collagen deposition (fibrosis) and cellular misalignment, can affect
47 electrical conduction, introduce electromechanical dysfunctions and, eventually lead to arrhythmia.
48 Current predictive models of these functional alterations are based on non-integrated and low-
49 resolution structural information. Placing this framework on a different order of magnitude is

50 challenging due to the inefficacy of standard imaging methods in performing high-resolution imaging
51 in massive tissue. In this work, a new methodological framework is described that allows imaging of
52 whole mouse hearts with micrometric resolution. The achievement of this goal has required an
53 impressive technological effort where advances in tissue transformation and imaging methods have
54 been combined. First, we describe an optimized CLARITY protocol capable of transforming an intact
55 heart into a nanoporous, hydrogel-hybridized, lipid-free form that allows high transparency and
56 deep staining is described. Then, a fluorescence light-sheet microscope able to rapidly acquire
57 images of a mesoscopic field of view (mm-scale) with the micron-scale resolution is described.
58 Inspired by the mesoSPIM project, the conceived microscope allows the reconstruction of the whole
59 mouse heart with micrometric resolution in a single tomographic scan. We believe that this
60 methodological framework will allow clarifying the involvement of the cytoarchitecture disarray in
61 the electrical dysfunctions and pave the way for a comprehensive model that considers both the
62 functional and structural data, thus enabling a unified investigation of the structural causes that lead
63 to the electrical and mechanical alterations after the tissue remodeling.
64
65 **INTRODUCTION:**
66 Structural remodeling associated with cardiac diseases can affect electrical conduction and
67 introduce electromechanical dysfunctions of the organ[1,2]. Current approaches used to predict
68 functional alterations commonly employ MRI and DT-MRI to obtain an overall reconstruction of
69 fibrosis deposition, vascular tree, and fiber distribution of the heart, and they are used to model
70 preferential action potential propagation (APP) paths across the organ[3,4]. These strategies can
71 provide a beautiful overview of the heart organization. However, their spatial resolution is
72 insufficient to investigate the impact of structural remodeling on cardiac function at the cellular
73 level.
74
75 Placing this framework at a different order of magnitude, where single cells can play individual roles
76 on action potential propagation, is challenging. The main limitation is the inefficiency of standard
77 imaging methods to perform high-resolution imaging (micrometric resolution) in massive
78 (centimeter-sized) tissues. In fact, imaging biological tissues in 3D at high resolution is very
79 complicated due to tissue opaqueness. The most common approach to perform 3D reconstructions
80 in entire organs is to prepare thin sections. However, precise sectioning, assembling, and imaging
81 require significant effort and time. An alternative approach that does not demand cutting the sample
82 is to generate a transparent tissue. During the last years, several methodologies for clarifying tissues
83 have been proposed[5–8]. The challenge to produce massive, transparent, and fluorescently-labeled
84 tissues has been recently achieved by developing true tissue transformation approaches (CLARITY[9],
85 SHIELD[10]). In particular, the CLARITY method is based on the transformation of an intact tissue into
86 a nanoporous, hydrogel-hybridized, lipid-free form that enables to confer high transparency by the
87 selective removal of membrane lipid bilayers. Notably, this method has been found successful also
88 in cardiac preparation[11–14]. However, since the heart is too fragile to be suitable for an active clearing,
89 it must be cleared using the passive approach, which requires a long time to confer complete
90 transparency.
91
92 In combination with advanced imaging techniques like light-sheet microscopy, CLARITY has the
93 potential to image 3D massive heart tissues at micrometric resolution. In light-sheet microscopy, the
94 illumination of the sample is performed with a thin sheet of light confined in the focal plane of the
95 detection objective. The fluorescence emission is collected along an axis perpendicular to the
96 illumination plane[15]. The detection architecture is similar to widefield microscopy, making the
97 acquisition much faster than laser scanning microscopes. Moving the sample through the light sheet
98 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.

245

246 5.5. Gently fix the tailored plug above the internal cuvette with two screws.

247

248 5.6. Mount the sample to the microscope stage using the magnets.

249

250 5.7. Translate the vertical sample stage manually to immerse the internal cuvette into the external
251 one.

252

253 5.8. Turn on the excitation light source (wavelength of 638 nm), setting a low power (in the order of
254 3 mW).

255

256 5.9. Move the sample using the motorized translator to illuminate an inner plane of the tissue.

257

258 5.10. Turn on the imaging software (HCImageLive) and set the camera **Trigger** on **External Edge**
259 **Trigger (light-sheet)** mode to drive the acquisition trigger of the camera by the custom software
260 controlling the entire setup.

261

262 5.11. Enable **Autosave** in the **Scan Settings** panel and set the output folder where the images need
263 to be saved.

264

265 5.12. Manually adjust the sample position in the XY plane with the linear translators to move the
266 sample to the center of the FoV of the camera sensor.

267

268 5.13. Move the sample along the Z-axis using the linear motorized translator to identify heart
269 borders for tomographic reconstruction.

270

271 5.14. Increase the laser power to ~20 mW, ready for the imaging session.

272

273 5.15. Start the tomographic acquisition, click the **Start** button in the **Capture** panel of the imaging
274 software, and at the same time move the sample along the Z-axis at the constant velocity of 6 μm/s
275 using the motorized translator.

276

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

282

283 [insert **Figure 1** here]

284

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

288

289 [insert **Figure 2** here]

290

291 Once the heart was cleared, cellular membranes were stained with an Alexa Fluor 633-conjugated
292 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

342   generated by the synchronization between the camera rolling shutter operating at 1.92 Hz and the
343   light beam position driven by the tunable lens. The FWHM of the light intensity profile is evaluated
344   along the FoV and results are shown in **D**.

345
346   **Figure 5: Point Spread Function in the Z-axis**. The Point Spread Function (PSF) of the optical system
347   is estimated by imaging fluorescent sub-micron-scale nanospheres (excited with a light sheet with a
348   wavelength of 638 nm) with a pixel size of 3.25 µm × 3.25 µm × 2.0 µm. PSF intensity profile along
349   the optical axis (Z) is represented as black dots. PSF profile is fitted with a Gaussian function with µ
350   = 18.6 µm and σ = 2.7 µm. The FWHM of the PSF estimated by the fit is 6.4 µm.

351
352
353   **Figure 6: Mouse heart tissue reconstruction.** The clarified heart was stained with WGA conjugated
354   to Alexa Fluor 633 and excited by a laser source with a wavelength of 638 nm. (**A**) Coronal and (**B**)
355   transverse representative sections. (**C–D**) Tissue transformation produces high tissue transparency,
356   allowing to resolve small structures in the wall depth. The optical system shows an axial resolution
357   sufficient to resolve micrometric structures (panel. **D**). (**E**) 3D low-resolution heart rendering.

358
359   **DISCUSSION**:
360   In this work, a successful approach to clear, stain, and image a whole mouse heart at high resolution
361   was introduced. First, a tissue transformation protocol (CLARITY) was optimized and performed,
362   slightly modified for its application on the cardiac tissue. Indeed, to obtain an efficient
363   reconstruction in 3D of a whole heart, it is essential to prevent the phenomenon of light scattering.
364   The CLARITY methodology allows us to obtain a highly transparent intact heart, but it requires long
365   incubation times when performed passively (about 5 months). With respect to the brain, the cardiac
366   tissue is not suitable for an active clearing, which takes advantage of an electric field. Even at low
367   voltages, the electric field leads to damages and tissue breakages. Here, a passive clearing approach
368   was optimized to obtain a completely cleared heart in about 3 months. After isolating and
369   cannulating the heart through the proximal aorta, the CLARITY methodology was performed as
370   described in section 3 of the protocol. To speed up the procedure, a homemade passive clearing
371   setup was arranged (**Figure 1**), which ended up decreasing the timing of tissue clearing by about
372   40%. The setup is composed of a container for the clearing solution, a water bath, a peristaltic pump,
373   several chambers containing different sample holders, capsule filters for each chamber, and a tubing
374   system for the recirculation of the solution. The pump extracts and circulates the solution from the
375   container in succession through each of the chambers, where the samples are held for clearing.
376   Before entering the chambers, the solution flows through a capsule filter to trap the lipids flushed
377   away from tissues during the clearing. The optimal temperature for the clearing solution, between
378   37–45 °C, is maintained within the chambers during the recirculation by keeping the solution
379   container in a water bath at 50 °C. It is advised to change the clearing solution in the container once
380   a week during the procedure. All components used are listed in detail in the **Table of Materials**. The
381   optimized solution presented here allows us to obtain a whole passively cleared mouse heart in a
382   significantly shorter time with respect to the standard passive clearing technique, thus reducing the
383   required experimental time without damaging the organ. The staining approach was also optimized
384   for homogeneous labeling of the cellular membranes and endothelium, using a fluorescent lectin
385   (WGA – Alexa Fluor 633).

386
387   The heart cytoarchitecture has been reconstructed by developing a dedicated mesoSPIM that axially
388   sweeps the light-sheet across the sample (https://mesospim.org). The custom-made fluorescence
389   light-sheet microscope (**Figure 3**) was able to rapidly acquire images of a mesoscopic FoV (of the
390   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.

DISCLOSURES:
Nothing to disclosure.

REFERENCES:
1.   Cohn, J. N., Ferrari, R., Sharpe, N. Cardiac remodeling-concepts and clinical implications: A consensus paper from an International Forum on Cardiac Remodeling. *Journal of the American College of Cardiology*. **35**, 569–582 (2000).
2.   Finocchiaro, G. et al. Arrhythmogenic potential of myocardial disarray in hypertrophic cardiomyopathy: genetic basis, functional consequences and relation to sudden cardiac death. *EP Europace.* **2**, 1–11 (2021).
3.   Bishop, M. J. et al. Development of an anatomically detailed MRI-derived rabbit ventricular model and assessment of its impact on simulations of electrophysiological function. *American Journal of Physiology – Heart and Circulatory Physiology*. **298** (2), H699–H718 (2010).
4.   Bishop, M. J., Boyle, P. M., Plank, G., Welsh, D. G., Vigmond, E. J. Modelling the role of the coronary vasculature during external field stimulation. *IEEE Transaction on Biomedical Engineering*. **57**, 2335–2345 (2010).
5.   Tainaka, K. et al. Whole-body imaging with single-cell resolution by tissue decolorization. *Cell*. **159,** 911–924 (2014).
6.   Ueda, H. R. et al. Whole-brain profiling of cells and circuits in mammals by tissue clearing and light-sheet microscopy. *Neuron* **106,** 369–387 (2020).
7.   Richardson, D. S., Lichtman, J. W. Clarifying tissue clearing. *Cell*. **162**, 246–257 (2015).
8.   Silvestri, L., Costantini, I., Sacconi, L., Pavone, F. S. Clearing of fixed tissue: a review from a microscopist's perspective. *Journal of Biomedical Optics*. **21**, 081205 (2016).
9.   Chung, K. et al. Structural and molecular interrogation of intact biological systems. *Nature*. **497**, 332–337 (2013).
10.   Park, Y. G. et al. Protection of tissue physicochemical properties using polyfunctional crosslinkers. *Nature Biotechnology.* **37**, 73 (2019).
11.   Ding, Y. et al. Light-sheet fluorescence microscopy for the study of the murine heart. *Journal of Visualized Experiments: JoVE.* **139**, 57769 (2018).
12.   Olianti, C. et al. 3D imaging and morphometry of the heart capillary system in spontaneously hypertensive rats and normotensive controls. *Scientific Reports*. **10**, 1–9 (2020).
13.   Pianca, N. et al. Cardiac sympathetic innervation network shapes the myocardium by locally controlling cardiomyocyte size through the cellular proteolytic machinery. *The Journal of Physiology*. **597**, 3639–3656 (2019).
14.   Di Bona, A., Vita, V., Costantini, I., Zaglia, T. Towards a clearer view of sympathetic innervation of cardiac and skeletal muscles. *Progress in Biophysics and Molecular Biology* **154**, 80–93 (2020).

488   15.   Voigt, F. F. et al. The mesoSPIM initiative – open-source light-sheet microscopes for imaging
489   cleared tissue. *Nature Methods*. **16** (11), 1105–1108 (2019).
490   16.   Costantini, I. et al. A versatile clearing agent for multi-modal brain imaging. *Scientific*
491   *Reports*. **5**, 9808 (2015).
492   17.   Judd, J., Lovas, J., Huang, G. N. Isolation, culture and transduction of adult mouse
493   cardiomyocytes. *Journal of Visualized Experiments: JoVE.* **114**, 54012 (2016).
494   18.   Yi, F. et al. Microvessel prediction in H&E stained pathology images using fully convolutional
495   neural networks. *BMC Bioinformatics*. **19** (1), 64 (2018).
496   19.   Susaki, E. A. et al. Versatile whole-organ/body staining and imaging based on electrolyte-gel
497   properties of biological tissues. *Nature Communications*. **11** (1), 1982 (2020).
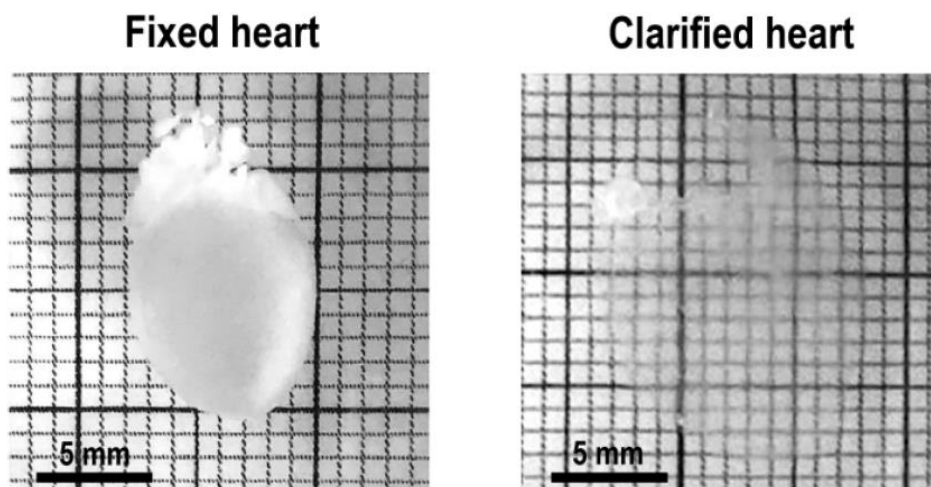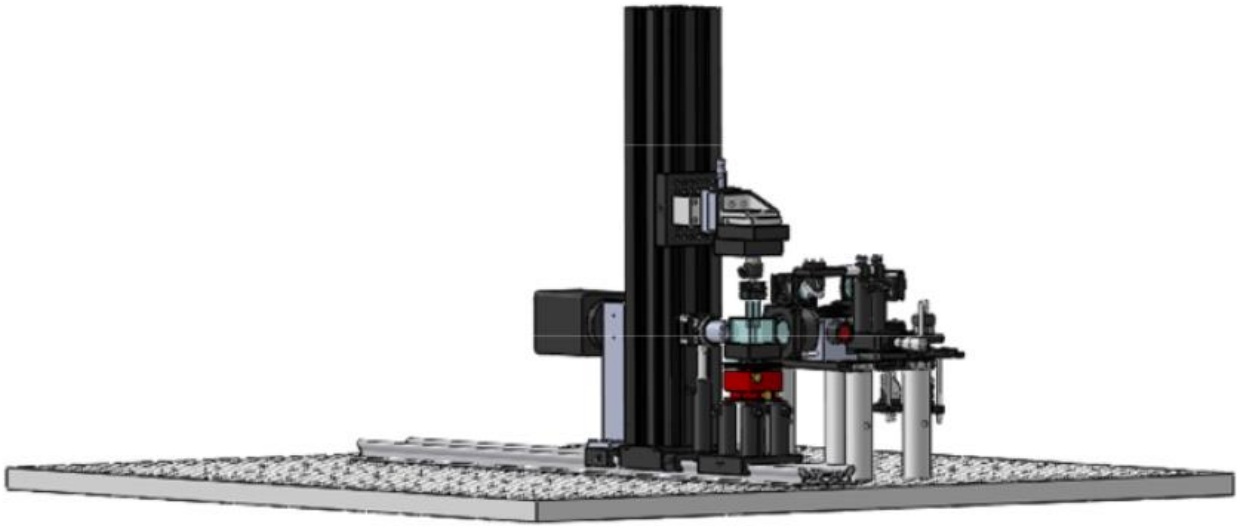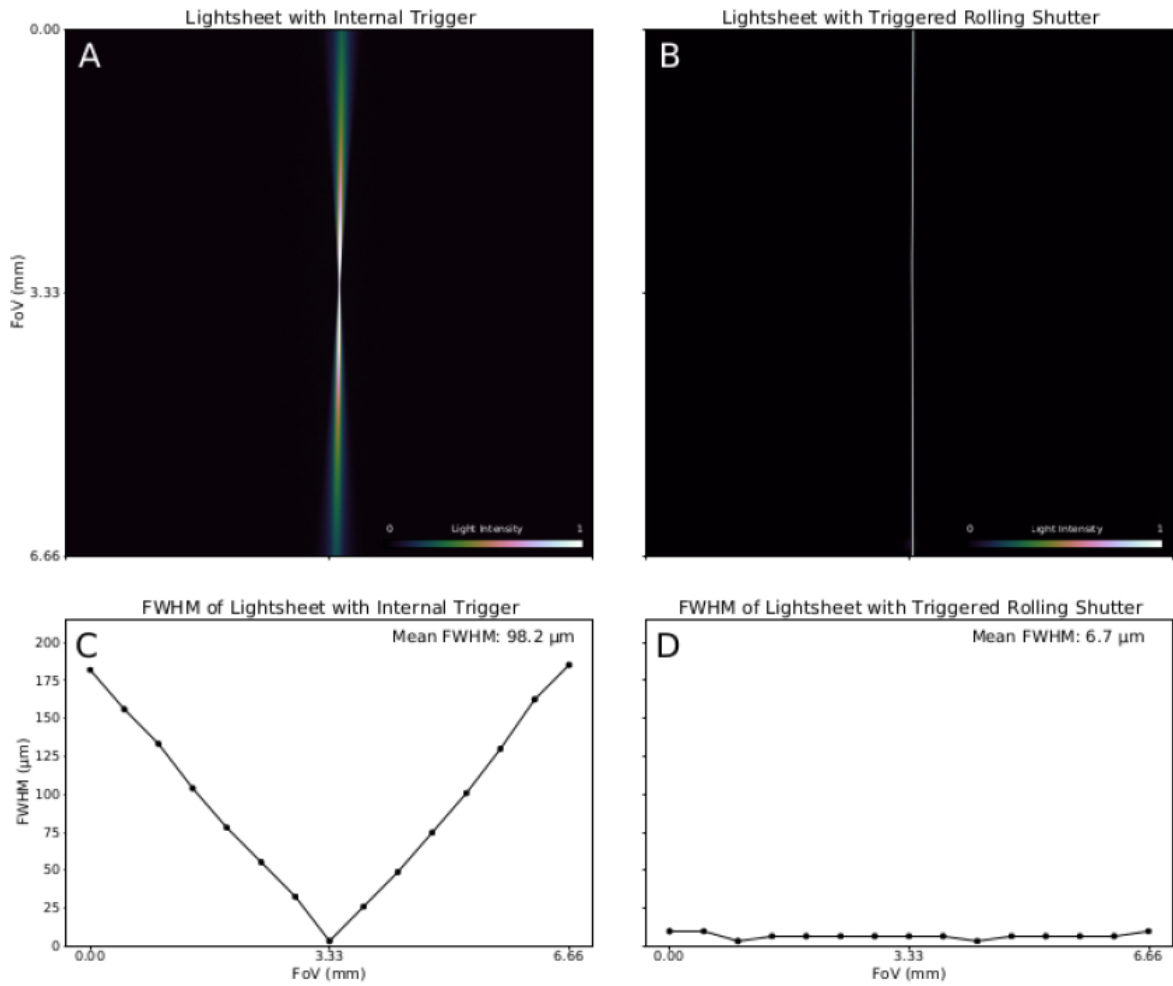
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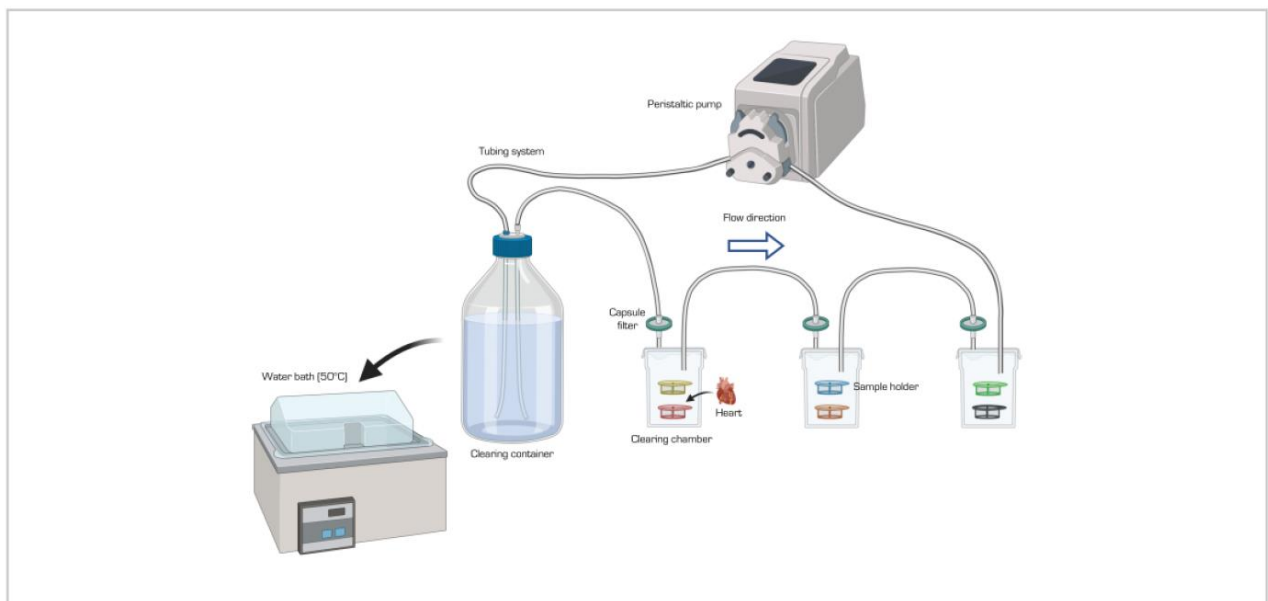
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6