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TITLE

Optical clearing in cardiac imaging: A comparative study

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ABSTRACT

The optical clearing of the cardiac tissue has always been a challenging goal to obtain successful 3D reconstructions of entire hearts. Typically, the developed protocols are targeted at the clearing of the brain; cardiac tissue requires proper arrangements to the original protocols, which are usually tough and time-consuming to figure out. Here, we present the application of three different clearing methodologies on mouse hearts: uDISCO, CLARITY, and SHIELD. For each approach, we describe the required optimizations that we have developed to improve the outcome; in particular, we focus on comparing the features of the tissue after the application of each methodology, especially in terms of tissue preservation, transparency, and staining. We found that the uDISCO protocol induces strong fiber delamination of the cardiac tissue, thus reducing the reliability of structural analyses. The CLARITY protocol confers a high level of transparency to the heart and allows deep penetration of the fluorescent dyes; however, it requires long times for the clearing and the tissue loses its robustness. The SHIELD methodology, indeed, is very promising for tissue maintenance since it preserves its consistency and provides ideal transparency, but further approaches are

needed to obtain homogeneous staining of the whole heart. Since the CLARITY procedure, despite the disadvantages in terms of tissue preservation and timings, is to date the most suitable approach to image labeled samples in depth, we optimized and performed the methodology also on human cardiac tissue from control hearts and hearts with hyper trophic cardiomyopathy

Keywords: Fluorescence microscopy Tissue clearing Cardiovascular research Light-sheet microscopy

INTRODUCTION

Three-dimensionality is one of the most relevant characteristics of biological structures. Cellular self-organization and interconnection with the extracellular matrix are extremely complex and significantly impact the physiological function of the tissue. In the heart, the systematic contraction of the cardiac muscle, allowed by the action potential propagation within the organ, is highly affected by the cellular organization. Thus, structural alterations of the cardiac tissue can pave the way to electrical dysfunctions and arrhythmias (Cohn et al., 2000; Finocchiaro et al., 2021). Therefore, cardiac structural studies have been a subject of great interest over the years and ever more approaches have been developed to successfully image the heart at high resolution. The hematoxylin-eosin staining protocol has been widely used for at least a century and it is still the gold standard to study the morphometric characteristics of biological samples, due to its easiness and adaptability to all kinds of tissues (Fischer et al., 2008; Yi et al., 2018). Nevertheless, the major limitation of this method is that it only provides 2D images, requiring tissue sectioning and thus reducing the reliability of the analyses.

Recent advance in several optical imaging techniques, based on the use of fluorescence microscopes, allows the 3D reconstruction of large volumes of tissues (Lichtman and Conchello, 2017; Kel and Dodt, 2012). However, the capability of these techniques to image the tissue in depth is limited by the phenomenon of light scattering, related to the refractive index (RI) mismatch of the biomolecules (Ueda et al., 2020; Richardson and Lichtman, 2015). To overcome this limitation, a wide amount of optical clearing methodologies has been largely used to homogenize the RI mismatch within fixed organs (Costantini et al., 2019). In the beginning, the RI homogenization was achieved using organic based solvents (Dodt et al., 2007; Renier et al., 2014; Ertürk et al., 2012; Pan et al., 2016). These methodologies are based on a first step of tissue de-hydration, followed by the incubation in organic compounds characterized by a RI as high as the biological tissues one (Jacques, 2013). However, the use of these agents can lead to the quenching of endogenous fluorescent proteins, which can occur in a relatively short time (Li et al., 2018). In 2013, the CLARITY methodology (Chung et al., 2013) opened the way to different approaches based on tissue transformation. CLARITY is based on the selective and uniform removal of lipids from the tissue, after the covalent linking of proteins and nucleic acids to an acrylamide-based matrix, thus maintaining their physiological locations to achieve an accurate structural reconstruction.

This approach is ultimately able to confer complete transparency and preserve endogenous fluorescence. Several similar approaches have then been conceived: among these, the SHIELD protocol (Park et al., 2019) is one of the most recent, based on the use of a flexible epoxide to form multiple intra-molecular bonds, enhancing the protein's tertiary structure. Initially, all tissue transformation methodologies have been settled and applied in neuroscience, to successfully image and study the brain (Silvestri et al., 2016). However, compared to cerebral tissue, cardiac tissue displays several differences, e.g., the high concentration of myoglobin that increases significantly tissue coloration and thus optical absorption in the range of visible wavelengths (Di Bona et al., 2020). Therefore, the application of the original protocols does not perform as efficiently on cardiac tissue as on the brain, and has been mainly used for the 3D investigation of small portions of the heart (Pianca et al., 2019; Olianti et al., 2020). Furthermore, the optimization of such approaches for the heart is typically time-consuming and expensive.

Here, we employ the two most efficient tissue transformation protocols to achieve full transparency of cardiac tissues: CLARITY and SHIELD methodologies, comparing them to uDISCO (Erturk et al., 2016), one of the most effective organic solvent-based techniques. We compare the tissue features of the cleared and stained hearts and we describe in detail the required optimizations that we have developed to increase the performances on cardiac tissues.

MATERIALS AND METHODS

Animal models

Adult male C57-BL6J mice were used for the experiments. All animal procedures performed conform to the guide-lines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes; experimental protocol is approved by the Italian Ministry of Health on July 6, 2015; Authorization No. 944/2018-PR. All the animals were provided by ENVIGO, Italy.

Human cardiac samples

From 2019 to 2020, we enrolled two cardiac patients consecutively referred to surgical myectomy for relief of symptoms related to obstruction of the left ventricular outflow tract; one patient was affected by hypertrophic cardiomyopathy with obstruction, and one was affected by aortic stenosis with bulging septum. The sample from aortic stenosis patient was used here as control. Protocols for tissue collection and use were approved by the ethical committee of Careggi University-Hospital (2006/0024713; renewed May 2009). During cardiac surgery, a portion of the interventricular septum is removed. Septal specimens were immediately collected from the surgeon and rapidly washed with cardioplegic solution and were processed within 30 min from excision. Endocardial trabeculae were dissected, fixed in 4% of Paraformaldehyde (PFA) in PBS and used to evaluate the tissue clearing CLARITY protocol as described below.

Heart isolation and perfusion

Animals were deeply anesthetized with 2 mL of 3% Isoflurane and the heart was rapidly isolated. The heart has been cannulated through the proximal aorta and perfused with 30 mL of Phosphate Buffered Saline (PBS) (pH 7.6) with a constant pressure of 10 mL/ minute to remove blood from the vessels. Subsequently, the heart has been perfused with 24mL of a fixative solution containing 4% of PFA in PBS (pH 7.6). Heart was incubated in 30 mL of PFA 4% in PBS overnight (O/N) and the following day it was washed in PBS 3 times for 1 h. 2.4. CLARITY protocol Fifteen hearts were subjected to a passive tissue transformation protocol based on CLARITY methodology, modified to be used on cardiac tissue. Hearts were subjected to CLARITY protocol: they were incubated in 30 mL of Hydrogel Solution (4% Acryilamide, 0,05% Bis-Acryilamide, 0,25% Initiatior AV-044 in 0,01 M of PBS) for 3 days at 4 °C in gentle shaking. After 3 days, the samples were degassed using a drier (KNF Neuberger, N86KT.18) and oxygen has been replaced by nitrogen. To favor gel polymerization, the samples have been kept at 37 °C for 3 h and then incubated in 30 mL of clearing solution (Boric Acid 200 mM, 4% Sodium Dodecyl-Sulfate; pH 8,6) at 37 °C in shaking up to complete clearing of the tissue (Fig. 1).

SHIELD protocol

Twelve hearts were subjected to the SHIELD protocol, slightly modified for its application on the cardiac tissue. Hearts were incubated in 20 mL of SHIELD-OFF solution (25% of ddH2O, 25% of LifeCanvas Technologies SHIELD Buffer solution, 50% of LifeCanvas Technologies SHIELD Epoxy solution) at 4 °C in shaking for 5 days. The following day they were incubated in 20 mL of LifeCanvas Technologies SHIELD-ON solution at 37 °C in shaking for 24 h. The hearts were then incubated in SHIELD clearing solution (Sodium Dodecyl Sulfate 300 mM, Boric acid 10 mM, Sodium sulfite 100 mM; pH 9) at 37 °C in shaking up to the achievement of good transparency (Fig. 1).

uDISCO protocol

Four hearts were incubated in 20 mL of increasing concentrations (30%, 50%, 70%, 80%, 90%, 96% and 100%) of tert-butanol in ddH2O at 37 °C in shaking for 12 h each. The samples were then incubated at room temperature (RT) in BABB (benzyl alcohol þ benzyl benzoate 1:2, respectively) and diphenyl ether (DPE) at the ratio BABB-D 15:1, for 3e6 h (Fig. 1).

Tissue labeling

CLARITY- and SHIELD-treated samples were washed in warmed up PBS for 24 h at room temperature (RT) in shaking, and then in warmed-up PBS þ 0.1% of Triton-X (PBS-T 0.1x) for 24 h at RT in shaking.

→Membrane staining of whole murine hearts: CLARITY-cleared hearts were incubated in 1:100 WGA Alexa Fluor 633 (Thermo Fischer, W21404) in PBS-T 0.1x at RT in shaking for 5 days. The samples were then washed in PBS-T 0.1x for 24 h at RT in shaking; they were then fixed in PFA 4% in PBS for 15 min and washed in PBS for 5 min three times. SHIELD-cleared hearts were incubated in 1:100 WGA Alexa Fluor 633 in PBS-T 1x (Triton-X 1%) at RT in shaking for 7 days. The samples were then washed in PBS-T 1x for 24 h at RT in shaking; they were then fixed in PFA 4% in PBS for 15 min and washed in PBS for 5 min three times.

→Labeling of CLARITY-cleared human cardiac tissues CLARITY-cleared human tissues were incubated 1:100 WGA Alexa Fluor 594 (Thermo Fischer W11262) in PBS-T 0.1x at RT in shaking for 3 days. The samples were then washed in PBS-T 0.1x for 24 h at RT in shaking and incubated in 1:10.000 SytoxGreen in PBST 0.1x for 3 h. They were then fixed in PFA 4% in PBS for 15 min and washed in PBS for 5 min three times.

Refractive index matching

CLARITY-cleared hearts were incubated in increasing concentrations of 2-2' Thiodiethanol (Costantini et al., 2015) (TDE) in PBS: 20% TDE/PBS for 4 h at RT in shaking, 47% TDE/PBS for 4 h at RT in shaking, and 68% TDE/PBS O/N in shaking. SHIELD-cleared hearts were incubated in 20 mL of Easy Index (LifeCanvas Technologies) at RT for 3 days. Cautions have been taken to avoid hearts floating on the solution.

Image acquisition

CLARITY-cleared human tissues were acquired using a custom-made two-photon fluorescence microscope (TPFM) as described in Olianti et al. (2020). Briefly, we used an excitation wavelength of 780 nm and a refractive index tunable 25 objective lens (LD LCI Plan-Apochromat 25 x/0.8 Imm Corr DIC M27; Carl Zeiss, Oberkochen, Germany). Fluorescence and second-harmonic signals were collected using three independent GaAsP photomultiplier modules (H7422; Hamamatsu Photonics, Bridgewater Township, NJ, USA). Band-pass emission filters centered at 530 ± 55 nm and 618 ± 50 nm were used, respectively, for SytoxGreen and Alexa Fluor 594 detection, and a filter centered at 390 ± 18 was used for second-harmonic generation. Stacks of 450 -450 µm with a depth of 300e400 mm and a Z-step of 2 mm were acquired. Whole cleared hearts were acquired using a custom-made fluorescence light-sheet microscope, able to rapidly acquire images of a mesoscopic FoV with micron-scale resolution (MesoSPIM) as described in Giardini et al. (Giardini, 2021). Briefly, the microscope illuminates the cleared sample with an axially-scanned light sheet (Voigt et al., 2020) at 638 nm. For the detection, we used a sCMOS camera (Orca Flash V3.0, Hamamatsu) operating at 500 ms of exposure time and a frame rate of 1.92 Hz. A 2x magnification objective (Thorlabs, TL2X-SAP) was used to acquire the entire FoV in a single scan. A long-pass filter (Thorlabs, FELH0650) placed after the objective is used to select the fluorescence signal. Tomographic reconstructions were obtained by moving the sample at constant velocity of 6 mm/s providing a Z-step of the order of 3 mm.

RESULTS

Tissue features after clearing protocols

We evaluated the tissue features of the hearts after the performance of each clearing protocol. The level of tissue preservation and transparency of the cleared hearts is shown in Fig. 2A. Fig. 2B shows the different timings required to have an entire heart transparent. With uDISCO we can make a whole heart transparent in 5 days; CLARITY requires about 5 months for the clearing of an entire heart, whereas SHIELD requires about 3 months. With the uDISCO protocol, the heart becomes transparent after a few hours in BABB-D, the same solution in which images are then acquired (RI ¼ 1.52). With the CLARITY methodology, the heart has to be maintained in the clearing solution until it has achieved a high degree of transparency, which will be increased when incubated in TDE 68% (RI ¼ 1.46). SHIELD-cleared hearts have to be removed from the clearing solution before the achievement of the complete transparency, when the clearing step does not show any further improvement in tissue de-lipidation. The ultimate transparency will be obtained by incubating the samples in EasyIndex solution (RI ¼ 1.46). uDISCO and CLARITY protocols produce a variation in the volume of the heart: uDISCO, due to the first step of tissue dehydration, induces a reduction of the volume of the heart; CLARITY transforms the heart into a hydrogel-hybridized form and thus induces an expansion of the tissue; however, the following incubation of CLARITY-cleared hearts in TDE leads to a shrinkage of the tissue (which occurs over time) to a size even smaller than the original one. The SHIELD procedure is not based either on tissue dehydration nor hydrogel-hybridization and, for this reason, it does not affect the original size of the organ. he highest level of transparency is achieved in CLARITYand SHIELD-treated hearts; indeed, uDISCO confers a medium-high level of transparency. Moreover, the uDISCO methodology leads to fibre delamination in the cardiac tissue (Fig. 3), altering the tissue structure; in CLARITY-cleared hearts, the tissue structure is well preserved, but the hearts lose their robustness; on the contrary, SHIELD-cleared hearts keep their consistency without any tissue alteration. uDISCO-cleared hearts suffer significant fibre delamination (Fig. 3), probably due to the dehydration step. On the contrary, the tissue structure in CLARITYand SHIELD-cleared hearts is completely maintained and optimally preserved. However, it is noteworthy that CLARITY-cleared hearts become significantly gelatinous, and for this reason the manipulation of the specimen can easily introduce damages to the integrity of the organs. We also evaluated the preservation of the tissue over time. With the uDISCO protocol, the heart can be maintained in BABB-D solution for several weeks without altering the organ preservation. CLARITY-treated hearts can be maintained in clearing solution for months and do not lose their features but, once incubated in TDE, the imaging has to be performed as soon as they start to shrink. SHIELD-cleared hearts suffer slight deterioration when kept in clearing solution too much time; nevertheless, when removed after a proper time, they can be maintained in EasyIndex for several months without any structural change or damage. The most challenging procedure to be performed by the operator is the CLARITY one, due to critical steps (e.g., the de-gassing procedure) that affect the quality of the outcome; contrarily, uDISCO and SHIELD resulted to be relatively easy to perform. SHIELD is considerably expensive (about V1100 to clear 10 hearts), while the costs to perform CLARITY are lower (about V650 to clear 10 hearts). Finally, uDISCO is the cheapest approach (about V200 to clear 10 hearts). Table 1 summarizes the main features of the tissue after the three treatments.

Tissue labelling

We assumed that, due to the observed fibre delamination occurring in uDISCO-cleared hearts, tissue labelling would be misleading and not informative. Contrarily, we performed the staining of the cellular membrane in CLARITYand SHIELD-cleared hearts using a fluorescent Wheat-Germ Agglutinin (WGA Alexa Fluor 633), a small lectin with an extremely low molecular weight (see Fig. 4). Regarding the demonstrated compatibility of CLARITYcleared specimen with homogeneous labelling of the tissue, we performed the CLARITY methodology on human cardiac tissues from control hearts and hearts with hypertrophic cardiomyopathy (HCM). To properly study the tissue remodeling occurring in HCM patients, we labelled the cellular membranes with WGA-Alexa Fluor 594 and the nuclei with SytoxGreen. We imaged the tissues with a custom-made Two-Photon Fluorescent Microscope (TPFM) and we detected the collagen amount using the Second

Harmonic Generation (SHG) (Mostaço-Guidolin et al., 2017). Fig. 5 shows representative frames of both control and HCM human hearts, where the structural remodeling that occurs in the pathology is clearly noticeable by the increase of fibrosis and collagen amount with respect to the control heart.

DISCUSSION

Muscular tissues, like the heart, are more challenging to clear compared to the brain. We noticed that some optical clearing protocols proved to be highly efficient in neurosciences are not so effective when performed on the hearts. Hence, each clearing methodology requires proper optimization to be applied to heart samples. These enhancements are usually expensive and time-consuming; for this reason, we deemed useful to compare the most promising techniques and analyse the features of the tissue after each protocol. We think that our research could offer a useful overview of the potentials and limitations of the three screened methodologies, which can be considered in the design of the experiments according to the desired outcome. We performed one of the most successful organic solvent-based technique (uDISCO) and two tissue transformation approaches (CLARITY and the most recent SHIELD). The uDISCO methodology is based on the homogenisation of therefractive index within the tissue by simply incubating the sample in an organic-solvent based solution, after a step of tissue dehydration. It is certainly the fastest and cheapest protocol to clear the heart, but it does not provide a complete clearness of the muscular tissue and, moreover, it induces significant fibre delamination of the heart, thus limiting its reliability for structural analyses of the organ. With the CLARITY and SHIELD approaches, the tissue proteins and nucleic acids are crosslinked to a structural matrix (consisting of an acrylamide-based gel in the CLARITY protocol and a polyepossidic resin in the SHIELD one) and the refractive index homogenisation is achieved by, firstly, the removal of the lipid bilayers from cellular membranes and, subsequently, by the incubation of the samples in a refractive index matching solution (TDE 68% in PBS for CLARITY-cleared hearts and EasyIndex for SHIELD-cleared ones). These approaches are slower, more challenging, and expensive compared to uDISCO, but they provide a higher level of transparency of the heart and the tissue is optimally preserved.

In particular, SHIELD-cleared hearts are the most well preserved, since there is no change in their original size and they maintain their robustness over time; on the contrary, CLARITY-cleared hearts, due to the tissue hybridization of the Hydrogel, suffer a massive expansion during the clearing process, shrinking again when incubated in TDE 68%, even to a lower size than the original. Furthermore, the CLARITY-cleared hearts become very gelatinous and, so, they have to be handled very carefully and it is easy to introduce damages to the tissue while performing the following procedures. We can conclude that, in terms of tissue preservation and integrity, the SHIELD procedure is the most successful one when applied on entire hearts, and it also offers a good compromise in terms of clearing timings; moreover, it is very easy to perform, even though it is the most expensive approach among the screened ones. However, due to the compactness maintained by the tissue, it is very challenging to obtain homogeneous staining of the entire organ, with even the small-sized fluorescent dyes (e.g. WGA) remaining confined to the surface of the tissue.

We think that further improvements are required when performing the SHIELD procedure to successfully label the whole heart. In particular, other potentially useful approaches to be screened are (i) the use of different detergents for cellular permeabilization, which could help the diffusion of the dye in depth(Zhao, 2020), and (ii) the use of stochastic electrotransport (Kim et al., 2015), which has already been tested to increase the antibody penetration within millimeter-sized brain tissues (Park et al., 2019). In addition, to increase the staining performances we could exploit the ionic strength of different buffers of incubation of the sample with the dye, which could significantly support the homogeneous penetration of the fluorescent probes (Susaki et al., 2020). Due to the actual staining issue in SHIELD-cleared hearts, we can state that currently, the CLARITY protocol is the most complete and finalising approach to obtain 3D reconstructions of fluorescently labelled organs. Indeed, although the tissue requires long timings to achieve the complete

transparency, appears to be very fragile and loses its hardiness, the hydration-driven expansion of the tissue is effective in the staining step, allowing the fluorescent dyes to penetrate within the whole thickness of the organ, thus enabling to image and study the structural organization of the tissue. Due to that, we tested the approach on human cardiac tissue, which is more challenging to clear and stain compared to murine hearts.

After the CLARITY procedure, we stained the samples from control hearts and hearts with HCM with SytoxGreen for nuclear detection and WGA-Alexa Fluor 594 for cellular membrane staining. We obtained excellent results in terms of transparency, preservation, and compatibility with the staining. This result allowed us to visualize in detail the morphological features of the heart in HCM and to compare them to control hearts. Thus, this result confirmed the applicability of the methodology to a wide range of cardiac tissues. We are confident that, with few optimizations, it will be possible to successfully stain whole SHIELD-cleared samples, thus achieving even better improvements in terms of tissue preservation, manageability, and timings.

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TABLE 1

	uDISCO	CLARITY	SHIELD
Volume variation	Reduction	Expansion	None
Transparency	Medium - high	High	High
Tissue preservation	Low	Medium	High
Integrity over time	High	Medium	High
Clearing timing	5 days	5 months	2-3 months
Convenience	High	Medium - Low	High
Cost	Low	Medium	High

Fig. 1. To be drawn

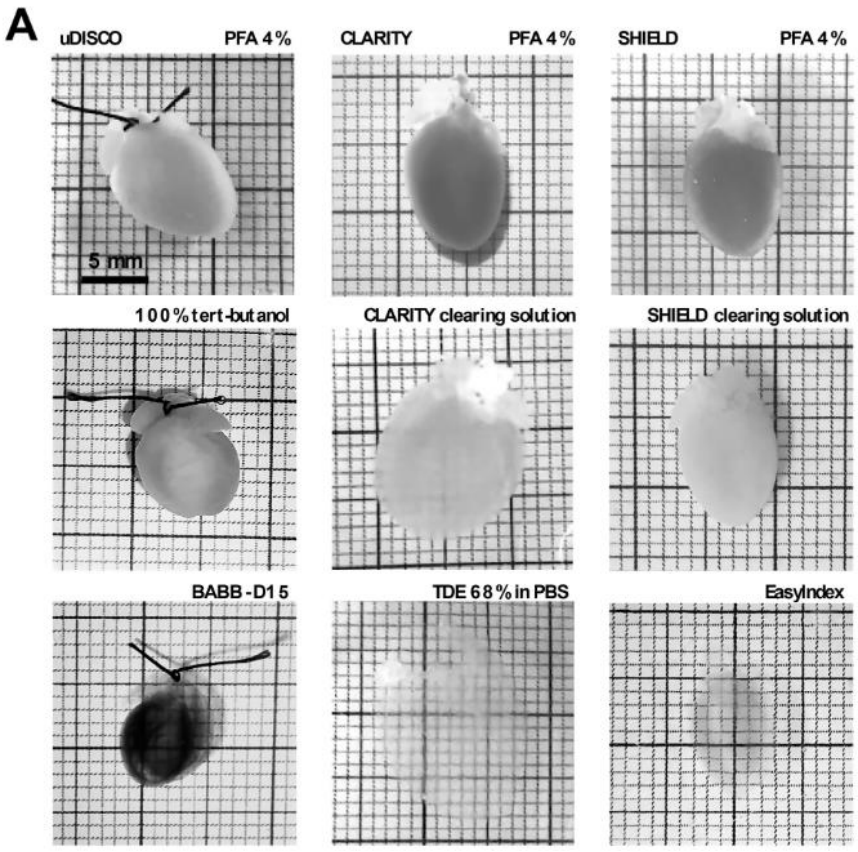

Fig. 2. Results of the three clearing protocols performed on isolated hearts. A) Levels of transparency obtained with the application of the three different protocols. uDISCO-cleared hearts do not achieve complete transparency, while CLARITY- and SHIELD-c

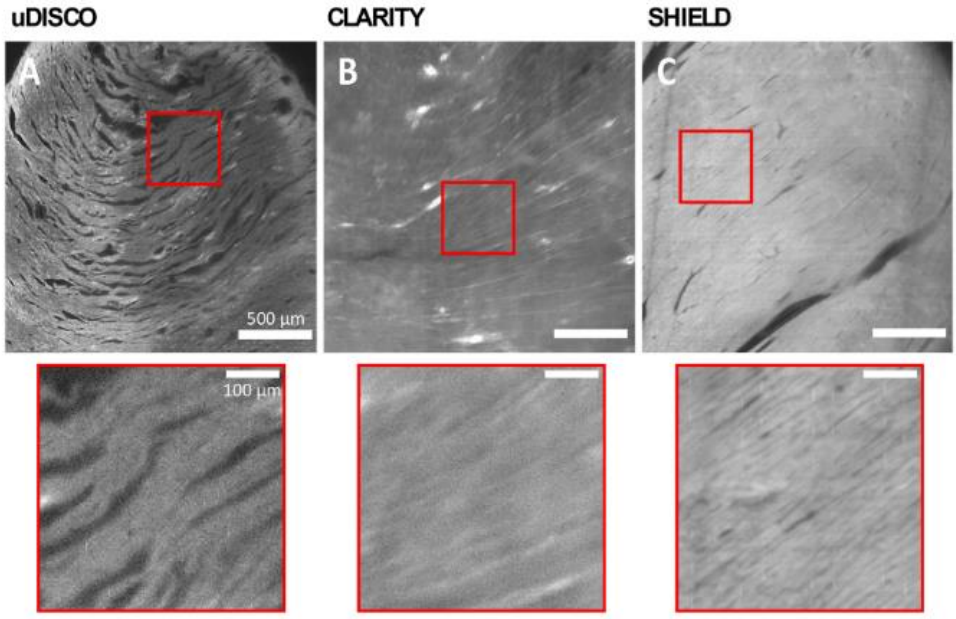

Figure 3. Tissue preservation and integrity of the hearts after the clearing protocols. A) uDISCO-cleared hearts encounter significant fibre delamination which affects the tissue

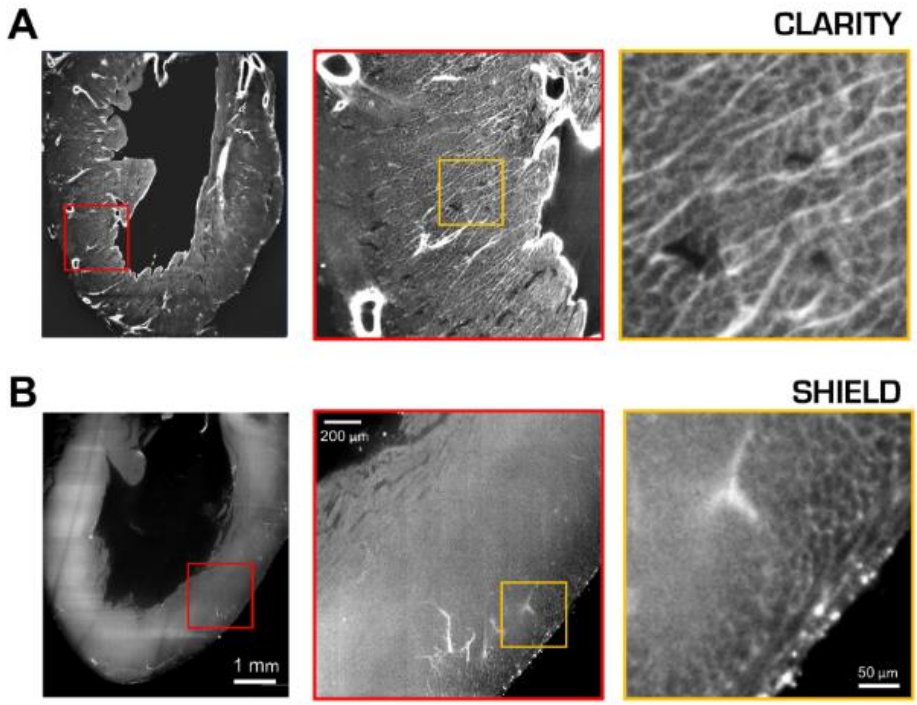

Figure 4 Shows a frame of both CLARITY (A)- and SHIELD (B)-cleared hearts, labelled with WGA e Alexa Fluor 633 and acquired with a custom-made LSFM.

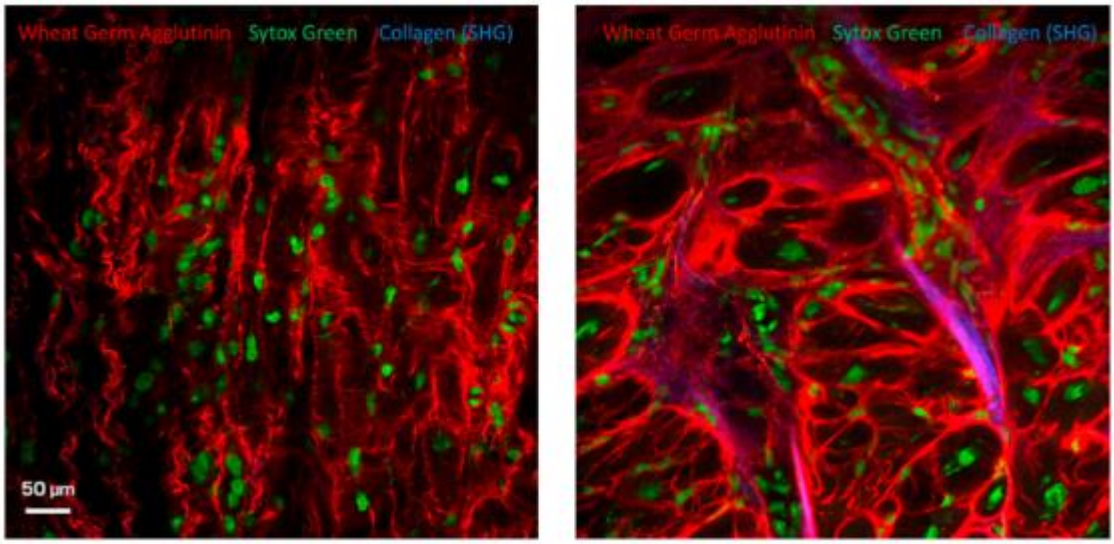

Figure 5 CLARITY-cleared human tissues of control heart (left) and heart with hypertrophic cardiomyopathy (right).