

3D imaging in CUBIC-cleared mouse heart tissue: going deeper

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Abstract: The ability to acquire high resolution 3D images of the heart enables to study heart diseases more in detail. In this work, the CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) clearing protocol was optimized for thick mouse heart sections to enhance the penetration depth of the confocal microscope lasers into the tissue. In addition, the optimized CUBIC clearing of the heart enhances antibody penetration into the tissue by a factor of five. The present protocol enables deep 3D high-quality image acquisition in the heart allowing a much more accurate assessment of the cellular and structural changes that underlie heart diseases.

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1. Introduction

Heart diseases are often accompanied by structural changes in the vascular network and/or the myocardium that may result in impaired contractility or relaxation of the ventricles and eventually lead to heart failure [1, 2]. Despite advancements in the field, our understanding of the adult heart structure and its remodeling following disease is still limited by the inability to provide three-dimensional (3D) images of the myocardium at cellular resolution. There is a growing trend to study the 3D structure of organs and tissues, requiring researchers to work with volumes rather than thin sections [3]. Confocal microscopy is a well-established imaging technique that plays an important role in studying tissue with high magnification. However,

this technology is limited by the reduced light penetration depth (around ~100-200 μm) due to changes in the refractive indices in the biological tissue (opacity of the tissue) and the resulting light scattering effects [4, 5]. Furthermore, reduced antibody penetration represents an additional limitation when performing confocal microscopy in immunohistochemical studies using thick tissue sections.

In this work, we investigate a modified CUBIC tissue clearing protocol, optimized for its use in mouse heart, which minimizes light scattering and significantly increases light penetration depth as compared to conventional confocal microscopy. Additionally, our modified protocol allows a far deeper penetration of antibodies into the tissue (~250-550 μm) for performing immunohistochemical studies (IHC). This modified protocol provides a solution for overcoming technical limitations of confocal microscopy by enabling the generation of high quality 3D images of thick slices of cardiac tissue at cellular resolution.

2. Methods

2.1 Mice

Three adult C57BL/6J male mice (stock 0664, Jackson Labs) and three adult male $\text{LysMcre}^{+/-}, \text{mT/mG}$ mice were used in this study. The $\text{LysMcre}^{+/-}, \text{mT/mG}$ mice were generated from the crossing of a transgenic double-fluorescent Cre-reporter mT/mG mouse (B6.129(Cg)-Gt(ROSA)26Sort^{m4(ACTB-tdTomato,-EGFP)^{Luo}/J}, stock 7676, Jackson Labs) with a transgenic LysMcre mouse (B6.129P2-Lyz2^{tm1(cre)lfo}/J, stock 4781, Jackson Labs). All of them expressed membrane-targeted tandem dimer (Td) Tomato (a red fluorescent protein) in all cells except in those with a myeloid cell lineage that instead expressed membrane-targeted enhanced green fluorescent protein (EGFP) due to the Cre-mediated excision of floxed STOP codons within the transgene [6]. Mice were sacrificed with CO_2 .

All experimental procedures were conducted in conformity with European Union Directive 2010/63/EU and were approved by the Ethics Committee for Animal Experimentation of hospital (Comité de Ética en Experimentación Animal, CEEA; number ES280790000087).

2.2 Heart perfusion and tissue processing

To obtain the non-cleared (control) tissues, mice were transcidentally perfused with 20 ml of ice cold PBS followed by 50 ml of 4% paraformaldehyde (PFA). The heart was dissected and post-fixed in PFA 4% overnight. It was then embedded in a 2% agarose block and 750 μm thick coronal sections starting at the apex were cut with a vibratome. The sections were counterstained with 0.25 $\mu\text{g/ml}$ DAPI for 2.5 h, washed and stored in PBS at 4°C until imaged.

To obtain the cleared hearts, mice were perfused intracardially following the CUBIC-perfusion protocol [7, 8] with 30 ml of ice cold PBS, 150 ml of ice cold 4% PFA, 20 ml of PBS to wash the fixative solution and then with 30 ml of diluted CUBIC-Reagent 1 (R1) (1:1 in distilled water). R1 consists of Urea, 2-hydroxypropyl, Triton X-100 and distilled water. The heart was dissected, embedded in 2% agarose and cut into 750 μm thick transversal sections with a vibratome. The sections were further cleared by immersion in R1 for 24 h, washed in PBS, counterstained with 0.25 $\mu\text{g/ml}$ DAPI for 2.5 h, washed again in PBS and incubated in the clearing CUBIC-Reagent 2 (R2) for 24 h. These incubation times have been optimized for the clearing of heart slices and result in a 70% shortening of the duration of the entire protocol. R2 consists of Sucrose, Urea, Nitrotriethanol and distilled water. Sections were stored in R2 at 4°C until imaged.

2.3 Immunohistochemistry

For the control tissues, heart sections were incubated with 0.02 mg/ml of anti-CD31 (ab28364, Abcam) overnight, washed with 0.1% Tritón X-100 in PBS (PBS-T) and incubated

with 3,3 $\mu\text{g}/\text{ml}$ of anti-rabbit secondary antibody (Alexa 647, Molecular Probes) together with 0.25 $\mu\text{g}/\text{ml}$ of DAPI (DAPI, Molecular Probes) for six hours. These incubation times represent a 70% shortening of the original protocol. After the secondary antibody was washed with PBS-T, the sections were stored in PBS at 4°C until confocal image acquisition.

For the CUBIC cleared tissues, heart sections were immersed into R1 for 24 h. After this step, the sections were washed in PBS and incubated with 0.02 mg/ml of anti-CD31 overnight. The following day the sections were washed and incubated with 3,3 $\mu\text{g}/\text{ml}$ of secondary antibody and 0.25 $\mu\text{g}/\text{ml}$ of DAPI for 6 h. After washing, the sections were incubated in R2 overnight, and then stored in R2 at 4°C until confocal image acquisition.

2.4 Image acquisition

All images of cleared and non-cleared sections were acquired with a Leica TCS SPE Confocal Microscope using an ASC APO 10x/0.30 DRY and an ACS APO 20x/0.60/IMM objectives. Image contrast was linearly adjusted and a despeckle filter was applied. To obtain a Z-intensity profile, the images were thresholded to include only the intensity of the nuclei but not the background; the illumination power was the same for both cleared and non-cleared tissue. All processing was done with ImageJ software (ImageJ 1.50e, National Institute of Health, USA). The Topological 3D-viewer tool of LAS X, (Leica microsystems) was used for video processing.

3. Results and discussion

In this paper, we adapted the CUBIC clearing protocol to mouse heart slices by reducing the incubation times around 70% at several steps. To determine the potential of the modified CUBIC clearing protocol, we first used *LysMcre^{+/-},mT/mG* transgenic mice which present constitutive expression of EGFP in the plasma membrane of myeloid-derived (*LysMcre*-expressing) cells and expression of TdTomato protein in non-myeloid derived cells. Visual inspection confirmed the transparency of the heart sections treated with the modified CUBIC protocol, compared with the opaque tissue treated with the standard perfusion and fixation protocol [Fig. 1(A) and 1(D)]. In the non-cleared control heart sections, we achieved an imaging depth of 90 μm with the 10x magnification objective [Fig. 1(B), 1(G), 1(I) and [Visualization 1](#)] and 60 μm with a 20x objective [Fig. 1(C) and 1(H)]. In contrast, clearing with the optimized CUBIC protocol allowed an imaging depth of 550 μm with the 10x magnification objective [Figs. 1(E), 1(G), 2(D) and [Visualization 2](#)] and of more than 250 μm with the 20x objective [Figs. 1(F) and 1(H)]. With the 20x objective, its working distance and not the signal intensity was the limiting factor. Overall, clearing the heart sections with the modified CUBIC protocol enables 6-fold (for the 10x objective) and 4-fold (for the 20x objective) deeper light penetration compared to the non-cleared. This improvement was confirmed by the area under the curve (AUC), which showed a significant increase in signal intensity in the cleared tissue with a $p < 0,05$, Fig. 1(I). In our work this protocol has been optimized by using the shortest possible incubation time, in order to achieve the most straightforward procedure, but it would be interesting to measure quantitatively the increase of light penetration vs. incubation time. The increase of the signal intensity observed in the cleared tissue may be due to the composition of reagent 1, which includes Triton-X100 and polyalcohols which increase cell permeability enhancing antibody penetration.

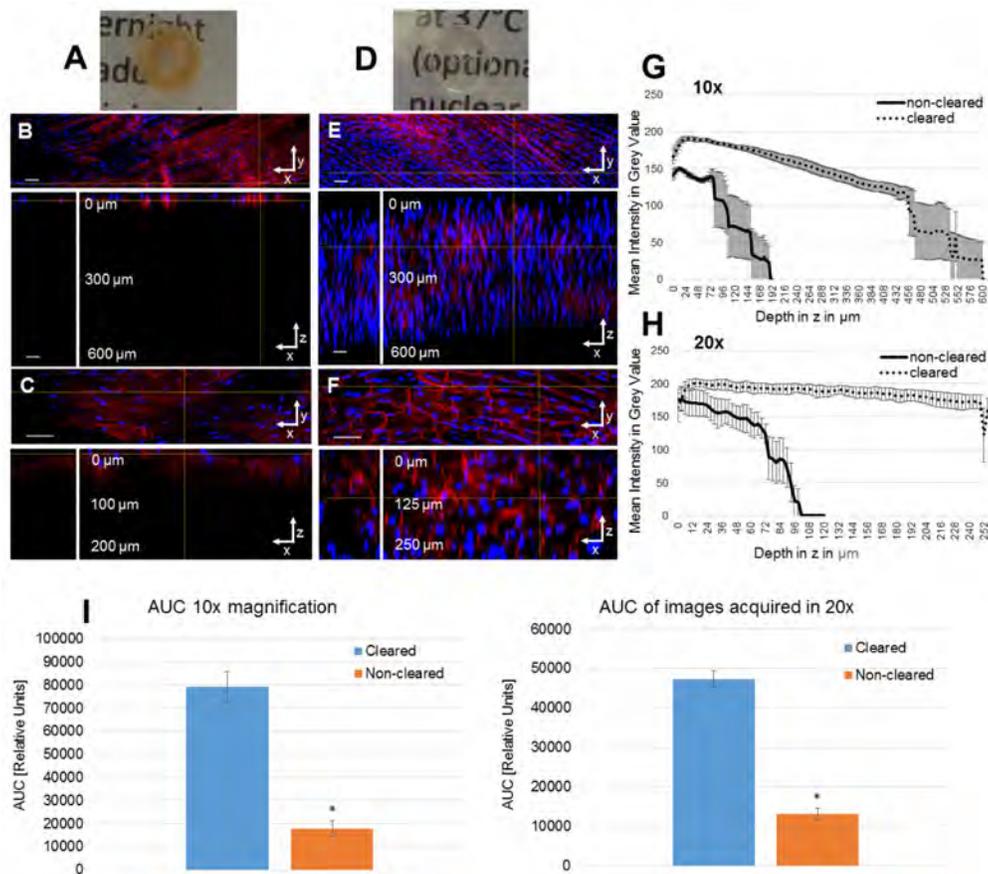


Fig. 1. The modified CUBIC protocol significantly enhances light penetration into cardiac tissue. **A**, Heart tissue section (750 μm -thick) from *LysMere^{+/-},mT/mG* transgenic mice were perfused with a standard protocol. **B**, **C**, Confocal images in the XY plane and their corresponding reconstructions in the XZ plane were obtained using a 10x (**B**) and a 20x (**C**) objective. Red, TdTomato; Blue, DAPI. The depth of the tissue in the XZ plane is indicated in microns. **D**, Heart tissue section (750 μm -thick) from *LysMere^{+/-},mT/mG* transgenic mice perfused following the modified CUBIC protocol. The background text allows to appreciate the transparency of the tissue. **E**, **F**, Confocal images in the XY and XZ planes were obtained using a 10x (**E**) or a 20x (**F**) objective. **G**, **H**, Z-profile mean intensity distribution \pm SD thresholded DAPI channel of the images acquired with 10x (**G**) and 20x magnification (**H**). Scale bars in all the images: 50 μm . **I**, The area under curve of the Z-profile (mean intensity distribution \pm SD) of thresholded DAPI channel of the images acquired of the cleared (blue) and non-cleared (orange) heart samples with 10x and 20x. $n = 4$ heart samples per condition. * $p < 0.05$, Mann-Whitney test.

We next investigated in wild-type C57BL/6J mice whether the modified CUBIC protocol improves the penetrance of the antibodies into the cardiac tissue, which represents a major hurdle in the study of 3D protein distribution in the heart. For this purpose, we used a primary antibody that recognizes the endothelial cell marker CD31 and its corresponding secondary antibody. Whereas the antibodies only entered about $\sim 50 \mu\text{m}$ into the non-cleared heart sections [Fig. 2(A) and Visualization 3], they entered up to $\sim 260 \mu\text{m}$ deep into the cleared heart sections [Fig. 2(B) and Visualization 4]. These results demonstrate that the clearing protocol improves antibody penetration into cardiac tissue, which represents a relevant additional advantage that will allow to obtain 3D image distribution of proteins and cells in the heart using conventional confocal microscopy.

The use of this tissue clearing protocol may also offer potential advantages with light sheet microscopy and optical coherence tomography (OCT). Clearing thick pieces of tissue, such as complete mouse organs or whole embryos, may enable 3D image acquisition with these techniques. Heart tissue of other widely used experimental animals (such as rats) could also be cleared and stained using this protocol provided while the section thickness is maintained.

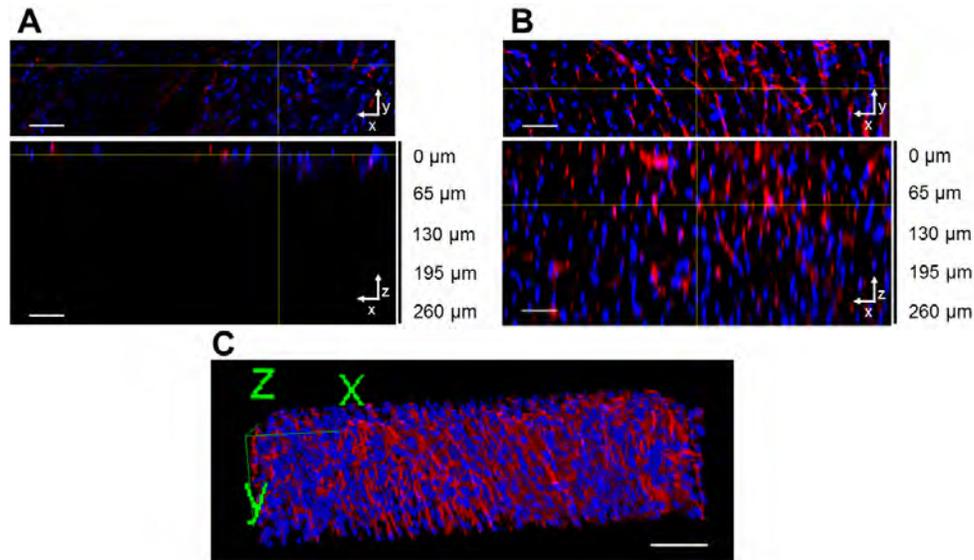


Fig. 2. The modified CUBIC protocol improves antibody penetrance into cardiac tissue sections A, B, Non-cleared sections (A) and sections cleared using the modified CUBIC protocol (B) were immunostained using an anti-CD31 antibody and confocal images of the XY plane and the corresponding XZ plane were obtained. C, 3D view of the vasculature of the CUBIC-cleared tissue. Red, anti-CD31; Blue, DAPI. Scale bars in all images: 50 μm .

In summary, we describe a modified CUBIC clearing protocol that makes it possible to obtain 3D images of thick cardiac tissue sections with high magnification and subcellular resolution using conventional confocal microscopy. Additionally, the modified clearing protocol could be used with light sheet microscopy and OCT imaging techniques to obtain 3D images of thick myocardial samples. The combination of the modified CUBIC clearing technique with immunohistochemical methods provides a new tool for researchers interested in gaining more profound and detailed information regarding protein and cell distribution within the heart.

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