Video Article

Imaging Cleared Embryonic and Postnatal Hearts at Single-cell Resolution

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Abstract

Single clonal tracing and analysis at the whole-heart level can determine cardiac progenitor cell behavior and differentiation during cardiac development, and allow for the study of the cellular and molecular basis of normal and abnormal cardiac morphogenesis. Recent emerging technologies of retrospective single clonal analyses make the study of cardiac morphogenesis at single cell resolution feasible. However, tissue opacity and light scattering of the heart as imaging depth is increased hinder whole-heart imaging at single cell resolution. To overcome these obstacles, a whole-embryo clearing system that can render the heart highly transparent for both illumination and detection must be developed. Fortunately, in the last several years, many methodologies for whole-organism clearing systems such as CLARITY, Sca*l*e, SeeDB, ClearT, 3DISCO, CUBIC, DBE, BABB and PACT have been reported. This lab is interested in the cellular and molecular mechanisms of cardiac morphogenesis. Recently, we established single cell lineage tracing via the *ROSA26-Cre*^{ERT2}; *ROSA26-Confetti* system to sparsely label cells during cardiac development. We adapted several whole embryo-clearing methodologies including Sca*l*e and CUBIC (clear, unobstructed brain imaging cocktails and computational analysis) to clear the embryo in combination with whole mount staining to image single clones inside the heart. The heart was successfully imaged at single cell resolution. We found that Sca*l*e can clear the embryonic heart, but cannot effectively clear the postnatal heart, while CUBIC can clear the postnatal heart, but damages the embryonic heart by dissolving the tissue. The methods described here will permit the study of gene function at a single clone resolution during cardiac morphogenesis, which, in turn, can reveal the cellular and molecular basis of congenital heart defects.

Video Link

The video component of this article can be found at https://www.jove.com/video/54303/

Introduction

Cardiac morphogenesis is a sequential event that requires the spatiotemporal organization of four different types of cardiac progenitor cells into distinct sectors of the heart, and also requires multiple genetic regulatory networks to orchestrate this process to form the functional heart[1,2]. Cardiac specification, differentiation, patterning, and chamber maturation are regulated by cardiogenic transcription factors[3]. Genetic mutation or posttranscriptional aberration of these factors in cardiac progenitor cells could result in either embryonic lethality or congenital heart defects (CHD)[4]. The study of cardiac morphogenesis requires an understanding of inherent structural details in three dimensions (3D) and single labeled cardiac progenitor cell lineage tracing during cardiac development will promote the understanding of cardiac morphogenesis. A number of high-resolution section based tomography methods have been developed in the past few decades to image organ structure[5,6]; however, these methods require expensive, specialized instruments, extensive labor, and lack detailed structural organization at single cell resolution in the final volumetric reconstructed image[7,8].

3D volumetric imaging at the single cell level provides a means to study progenitor cell differentiation and cellular behavior *in vivo*[7]. However, tissue light scattering remains the primary obstacle to imaging cells and structures in 3D deep inside the intact heart. Lipids are a major source of light scattering, and the removal of lipids and/or adjustment of the refractive index difference between lipids and their surrounding areas are potential approaches for increasing tissue transparency[8]. In the past several years, a number of tissue clearing methods were developed, which reduce tissue opacity and light scattering, like BABB (benzyl alcohol and benzyl benzoate mixture) and DBE (tetrahydrofuran and dibenzylether); but in these methods, fluorescence quenching remains an issue[8-10]. The solvent based hydrophilic methods, such as SeeDB (fructose/thioglycerol) and 3DISCO (dichloromethane/dibenzylether), preserve fluorescent signals, but do not render the whole organ transparent[7,8,11]. In comparison, the CLARITY tissue-clearing method renders the organ transparent, but it requires a specialized electrophoresis device to remove lipids[8,12], as does PACT (passive clarity technique), which also requires hydrogel embedding[7,13]. For detailed information regarding all available tissue-clearing methods, refer to **Table 1** in Richardson and Lichtman, *et al.*[7].

In 2011, Hama *et al.* serendipitously discovered a hydrophilic mixture 'Sca*l*e' (urea, glycerol and Triton X-100 mixture) that renders the mouse brain and embryo transparent while completely preserving fluorescent signals from labeled clones[14]. This allows for the imaging of the intact brain at a depth of several millimeters and large-scale reconstruction of neuronal populations and projections at a subcellular resolution. Susaki

et al. further improved Sc*a*l*e* by adding aminoalcohols and developed the 'CUBIC' (clear, unobstructed brain imaging cocktails and computational analysis) tissue clearing method, which increased phospholipid solubilization, reduced clearing time, and allowed for multicolor fluorescent imaging[8]. In the present study, taking advantage of the Sc*a*l*e* and CUBIC tissue clearing techniques and high resolution 3D optical sectioning, individual clones inside the heart during cardiogenesis were traced using *Rosa26Cre*^{ERT2} [15], *R26R-Confetti*[16], αMHC-Cre[17], *cTnT-Cre*[18], *Nfatc1-Cre*[19], and *Rosa26-mTmG* (mTmG)[20] mouse lines. The combination of the whole mount staining (WMS) method developed previously[21,22] with tissue clearing methods further allowed for the staining of other proteins in labeled clones and for the study of their behavior in a 3D volumetric context. The combination of tissue clearing and WMS allows for a better understanding of the roles of different genes and proteins during cardiac development, and the etiology of congenital heart defects. This protocol can be applied to study other progenitor cell differentiation, cellular behavior, and organ morphogenesis events during development.

Protocol

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Albany Medical College and performed according to the NIH Guide for the Care and Use of Laboratory Animals.

1. Solution Preparations

NOTE: The *Rosa26Cre*^{ERT2} [15], *R26R-Confetti*[16], αMHC-Cre[17],*and Rosa26-mTmG* (mTmG)[20] mouse lines were purchased commercially. *cTnT-Cre*[18] was a gift from Dr. Jiao at University of Alabama. *Nfatc1-Cre* was a gift from Dr. Bin Zhou at Albert Einstein College of Medicine[19]. Mice were euthanized by carbon dioxide inhalation in a closed chamber for at least 60 seconds followed by physical means of death verification by bilateral thoracotomy, decapitation or cervical dislocation.

1. Prepare Tamoxifen. Dissolve 10 mg of Tamoxifen in 10 ml of sunflower seed oil. Once it is completely dissolved, aliquot and store at -20 °C.
2. Prepare phosphate buffered saline (PBS). Dissolve Na_2HPO_4 (1.41960 g), KH_2PO_4 (0.24496 g), NaCl (8.0669 g) and KCl (0.20129 g) in 1 L of distilled water and adjust the pH to 7.4.
3. Prepare PBST:0.1% Tween-20 solution in PBS by dissolving 100 µl of Tween-20 in 100 ml of PBS.
4. Prepare blocking buffer. Make 3% bovine serum albumin (BSA) blocking solution by dissolving 3 g of BSA in 100 ml of PBST containing 0.2% Tween-20.
5. Prepare CUBIC-1 (reagent 1). Mix 25 wt% urea, 25 wt% N, N, N', N'-tetrakis (2-hydroxypropyl) ethylenediamine and 15 wt% Triton X-100 in dH_2O.
6. Prepare CUBIC-2 (reagent 2). Mix 50 wt% sucrose, 25 wt% urea, 10 wt% 2, 2', 2"-nitrilotriethanol and 0.1% (v/v) Triton X-100 in dH_2O.
7. Prepare Sc*a*l*e* A2: 4 M urea, 10% w/v glycerol and 0.1% w/v Triton X-100 in dH_2O. Adjust the pH to 7.7.
8. Prepare Sc*a*l*e* B4: 8 M urea and 0.1% w/v Triton X-100 in dH_2O. Adjust the pH to 8.7.
9. Prepare Sc*a*l*e* 70: 4 M urea, 70% w/v glycerol and 0.1% w/v Triton X-100 in dH_2O.

2. Tamoxifen Induction, Embryo Isolation and Fixation

1. Tamoxifen Induction and Embryo Isolation
    1. Using a curved feeding tube approved protocol (ACUP 13-12007), gavage the female R26Cre^{ERT2} Confetti mice (plugged by R26Cre^{ERT2} male mice) with 10 µg/g of Tamoxifen at embryonic developmental day E7.75[33]. At embryonic day 9.5 (E9.5) or E10.5, euthanize the female mice with CO_2 and cervical dislocation.
    2. After verification of mouse death (see Section 1 Note), open the mouse abdominal cavity with scissors and dissect out the uterine horn, remove the uterine tube layers with the help of scissors and tweezers, and release the embryos from the uterine tube[23,24].
        1. Transfer the embryos to a petri dish containing PBS (pH 7.4). Under the dissecting microscope, remove the non-embryonic layers (chorion, yolk sac and amnion) with the help of tweezers.
    3. Using tweezers and scissors, collect the female mouse and embryo tail samples for genotyping as previously reported[22]. Using a plastic pipette, transfer each embryo to a well of a 48 well plate containing 1 ml of 1x PBS.
    4. Under a fluorescent microscope, identify the GFP/RFP/CFP positive embryos via an inverted microscope with a camera. Peel away the pericardium using tweezers and rotate the heart/embryo with the help of curved tweezers to determine the number of fluorescent clones on both sides of the heart with the GFP/RFP/CFP filters. No cuts are needed.
2. After identifying GFP/RFP/CFP positive embryos, quickly wash the embryos twice (2 min each) with 1x PBS in the 48 well-plate on a plate shaker at room temperature (RT). This will remove excess blood.
3. Fix the embryo/embryonic heart using 4% paraformaldehyde (PFA) at RT. The fixation time depends on the embryonic age and tissue size. For E9.5 fix the embryo for 2 hr at RT. The late embryonic stage embryos require longer fixation time, which can even be prolonged to 24 hr for postnatal heart.
   CAUTION: Paraformaldehyde is toxic, avoid contact with skin, eyes and mucous membrane.
4. After fixation, wash the embryo/heart in the 48 well-plate twice (10 min each) with 1x PBS on a plate shaker at RT. This removes the excess PFA from the embryo/heart. The embryos are then further processed for whole mount staining and tissue clearing.

3. Whole Mount Staining

NOTE: After PFA fixation the embryo/heart is subjected to whole mount staining as follows.

1. Permeabilize the embryo/heart in a 48 well-plate using 1 ml of 0.1% PBST for 2 hr on plate shaker at RT.
2. Following permeabilization, immerse the embryo/heart in 1 ml of blocking buffer, for 2 hr or overnight on a plate shaker at 4 ºC.

3. Immerse the embryo/heart to the endothelial cell-specific antibody PECAM (CD31), diluted (1:100) in 0.3 ml blocking buffer for 48 hr on plate shaker at 4 ºC.
4. Wash the embryo/heart using PBST 5 times, 30 min each at RT on plate shaker. This removes the excess non-specific bound primary antibody from the tissue.
5. Immerse the embryo/heart in Alexa Fluor 647 goat anti-rat secondary antibody, diluted (1:1,000) in 1 ml blocking buffer for 48 hr on plate shaker at 4 ºC.
6. Repeat step 3.4 to wash the secondary antibody.
7. Post-fix the embryo/heart with 4% PFA for 1 hr at RT on plate shaker and wash twice with PBS 5 min each.
8. Stain the embryo/heart with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (diluted in PBST at a concentration of 0.01 mg/mL) for 10 min on plate shaker at RT. Follow with two PBS washes of 5 min each.

4. Embryo/heart Clearing with Sca*l*e or CUBIC Method

NOTE: After whole mount staining, the embryo/heart is subjected to either the Sca*l*e or CUBIC tissue clearing method. The choice of method depends on the tissue size and embryonic age. For E11.5 or earlier age embryos, use Sca*l*e tissue clearing method, while for older embryos or postnatal hearts the CUBIC tissue clearing method is preferred.

1. Scale Tissue Clearing Method
    1. Incubate the embryo/heart with 1 ml of Sca*l*e A2 solution in a scintillation vial (wrapped in aluminum foil) with occasional gentle shaking (by hand) for 48 hr at 4 °C.
    2. After Sca*l*e A2 incubation, incubate the embryo/heart with 1 mL of Sca*l*e B4 solution at 4 °C in the same vial with occasional gentle shaking for another 48 hr.
    3. Incubate the embryo/heart for another 48 hr with 1 ml Sca*l*e 70 at 4 °C with occasional gentle shaking. Mount embryo using 90% glycerol (see section 5.1).

2. CUBIC Tissue Clearing Method
    1. For whole embryo/heart CUBIC clearing, immerse each embryo/heart in 1 ml of CUBIC-1 with occasional gentle shaking for 48 hr at 37 ºC[7]. After 48 hr, replace the solution with the same volume of fresh CUBIC reagent 1, and incubate the sample for an additional 48 hr at 37 °C with occasional shaking with hand.
    2. Wash the CUBIC-1 treated heart/embryo with 1x PBS several times at room temperature with gentle shaking with hand. This removes the excess CUBIC-1 solution.
    3. After washing out CUBIC-1 with PBS, immerse the embryos/hearts in CUBIC-2 solution (1 g per embryo/heart) for 48 hr at 37 ºC with occasional gentle shaking with hand. This follows embryo/heart mounting using glycerol (see section 5.2).

5. Sample Mounting and Imaging

NOTE: The cleared embryos or organs in Sca*l*e 70 or CUBIC-2 solution can be imaged directly with Scale 70 and CUBIC-2 solution as the imaging medium, respectively. However, considering the vulnerability of the embryonic samples to clearing reagents, if the samples are not be imaged immediately, but stored long-term, store the samples in 90% glycerol following the protocol below.

1. Directly immerse the Sca*l*e cleared sample in 1 ml of 90% glycerol and store at 4 °C until imaging.
2. Wash the CUBIC treated sample with 1x PBS twice, 5 min each and sequentially incubate the sample with 1 ml of 30%, 50%, 70% and 90% glycerol solution each for 30 min.
3. For imaging, transfer the sample to glass bottom Petri dish with a minimal amount of 90% glycerol and image clones with a confocal microscope equipped with two photon capabilities.
4. Image the heart or embryo in the glycerol with a 10X/0.3 NA, a 25X/0.8 NA immersion, or a 40X/1.2 NA water corrective objective lens. Image DAPI using 2-photon excitation from a coherent titanium:sapphire chameleon laser. The choice of microscope and objective lens will depend on the purpose of the experiments, *e.g.* for super-resolution, a light sheet fluorescent microscope could be utilized.

Representative Results

Imaging the cleared embryonic heart

Vertebrate heart formation is a spatiotemporally regulated morphogenic process and depends on the organization and differentiation of progenitor cells from four different sources[1]. Cells from the first heart field of the cardiac crescent will fold toward the ventral midline to form a linear heart tube. The cells from the second heart field, initially residing dorsomedially to the first heart field, subsequently translocate to the pharyngeal and splanchnic mesoderm, from where they migrate to the pre-existing scaffold of the linear heart tube. The cells of second heart field will contribute to the right ventricle, outflow tract (OFT) myocardium and to some endocardium[25-28]. Cardiac neural crest cells (CNCC), originating from postotic rhombomeres 6, 7 and 8, will migrate to the caudal pharynx and contribute significantly to the smooth muscle layer and endocardial cushions in the OFT. They also contribute to the formation of the aorticopulmonary septum[29,30]. The fourth population consists of epicardial cells derived from the pro-epicardial organ (PEO), and contributes to fibroblasts, smooth muscle cells and potentially other cardiac cell types[31]. The epicardium regulates coronary vascular development, cardiac growth and morphogenesis[21].

The most important concept about cardiac morphogenesis is that during heart development, abnormal cell migration/differentiation of the four progenitor cell populations will result in malformations or congenital heart defects[4,22,32], which is the number one cause of birth defects in the world. Determining the mechanisms of cardiac morphogenesis at the cellular and molecular level is an essential step toward improving diagnosis and potential treatments for CHDs. Therefore, it is critical to image the process of heart morphogenesis at the whole heart level with single cell resolution. To achieve that, we labeled the cardiomyocytes by crossing the Cre line under the control of cardiac specific troponin T (cTnT), which is specifically expressed in cardiomyocytes[18], with mTmG reporter line. The embryos at E8.5 were harvested and stained for PECAM to distinguish endocardial and endothelial cells from cardiomyocytes. The embryos were then cleared by Sca*l*e and the heart was imaged. The myocardium was labeled by GFP due to cTnT-driven Cre-mediated recombination of the mTmG reporter, and the endocardium was labeled with PECAM (**Figure 1A** and **Supplementary Movie 1**). The cardiomyocytes can be observed at a single cell resolution (**Figure 1A**). The heart structures of the imaged portion, such as the primitive ventricle and OFT can be clearly identified after 3D reconstruction using 3D reconstruction software (**Figure 1B** and **Supplementary Movie 2**).

Imaging single clones of the cleared embryonic heart

Cardiac morphogenesis depends on cardiac lineage differentiation and individual cell organization at the whole heart level[22], and thus, it is essential to image single cardiac progenitor cell differentiation to different cardiac cell types and to also image cell morphology at single cell resolution. To achieve that, *Rosa26Cre*^{ERT2} [15] was crossed to *R26R-Confetti*[16], and the pregnant female was gavaged with Tamoxifen at a low concentration. After 48 hr, the embryo was harvested at E9.5, and whole mount stained for PECAM, and then the whole heart was imaged. We found that there was only one cluster of cells, localized to the OFT, and this clone had 8 cells based on the reconstructed image and consecutive sections (**Figures 2A**, **2B** and **Supplementary Movie 3**), indicating that these cells are derived from a single progenitor cell. The cellular morphology and cellular behavior such as cellular proliferation and migration can be inferred from the ultimate positioning of the cells (**Figure 2A**). We also applied the CUBIC method to clear the embryonic heart at E9.5 and E10.5, and found that even after only 24 hr of incubation with reagent 1, the embryos were subtly dissolved and the tissue structure was adversely affected (Data not shown).

Imaging of cleared postnatal and late embryonic hearts

We applied both Sca*l*e and CUBIC to clear postnatal hearts. P2 hearts with the genotype of αMHC-Cre; mTmG (αMHC-Cre is specifically expressed in cardiomyocytes[17]) were cleared with Sca*l*e for 48 hr, but did not display any more transparency than the hearts treated with PBS only (Data not shown), indicating that Sca*l*e clearing is not effective for postnatal hearts. When P2 hearts were cleared with CUBIC reagent 1 for 48 hr, and reagent 2 for 96 hr, they were much more transparent than the hearts cleared with reagent 1 for 48 hr and reagent 2 for 48 hr. The imaging depth was significantly increased with 96 hr of reagent 2 incubation (**Supplementary Movies 4** and **5**), indicating that longer incubation with the CUBIC reagents promotes transparency and provides for an increase in imaging depth. The shape and size of each cardiomyocyte can be identified by the membrane localized GFP (**Figure 3A**), which can be used to identify hypertrophy.

We also cleared *Nfatc1-Cre;Confetti* (Confetti female plugged with Nfatc1Cre male) E17.5 hearts (Nfatc1-Cre is expressed in cardiac endocardial cells[19]) with CUBIC reagent 1 for 48 hr and reagent 2 for 48 hr. The hearts are transparent, and fluorescent proteins including GFP, YFP, CFP and RFP were expected to label the endocardial cells and their derived cells; however, only GFP and YFP could be imaged through the whole heart (**Figures 3B**, **3C** and **Supplementary Movies 6** and **7**), and the CFP and RFP could not be detected (Data not shown). The staining for PECAM (Alexia Fluor 647) also could not be detected (Data not shown), suggesting that the CUBIC reagent quenches the CFP, RFP and the antibody conjugated fluorophore in this protocol.

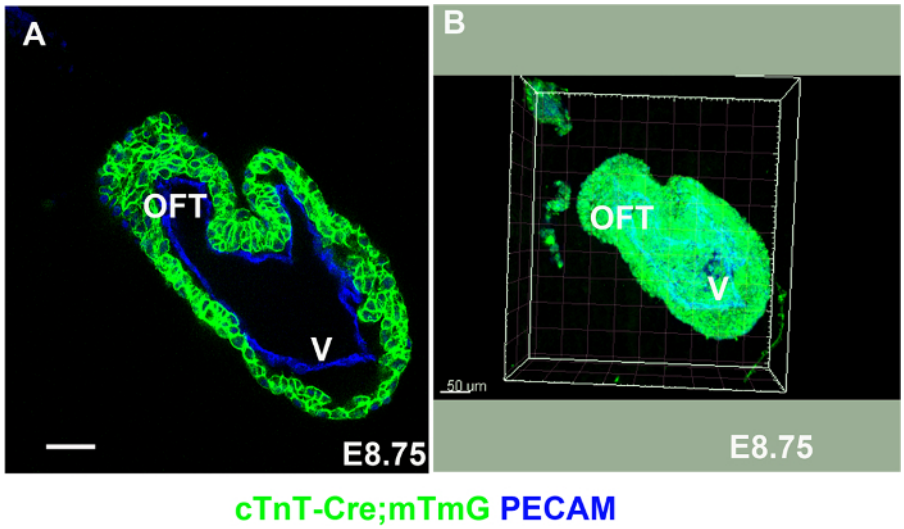


Figure 1: Imaging the Sac/e cleared embryonic heart. (**A**) One optical slice of an E8.75 heart (cTnT-Cre;mTmG) is shown. V: ventricle; OFT: outflow tract. (**B**) Shows the reconstructed heart structure of 93 consecutive optical slices with a total depth of 110.4 μm. Scale bar = 20 μm in A. Please click here to view a larger version of this figure.

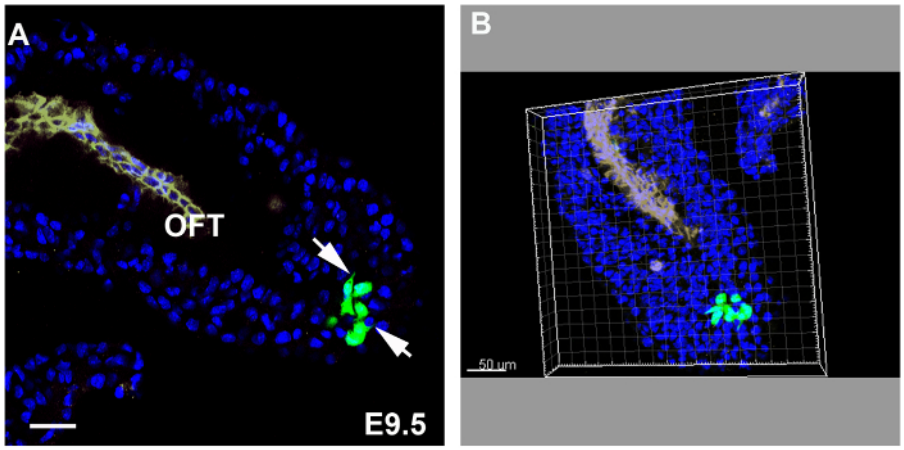


Figure 2: Imaging single clone of Sca/e cleared embryonic heart. (**A**) One optical section of a clone from an E9.5 heart with the genotype of ROSA26-Cre^{ERT2}; ROSA26-Confetti. The arrow points to a cellular protrusion of a cardiomyocyte. (**B**) Shows the reconstructed clone in the OFT region of the E9.5 heart with 10 optical slices and 36 µm total depth. Scale bar = 20 µm in A. Please click here to view a larger version of this figure.

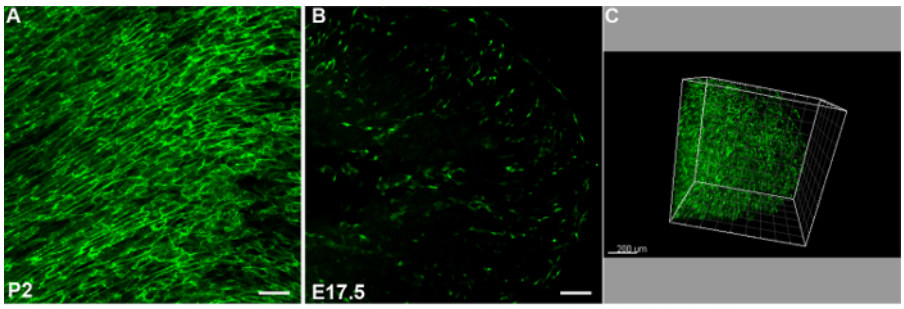
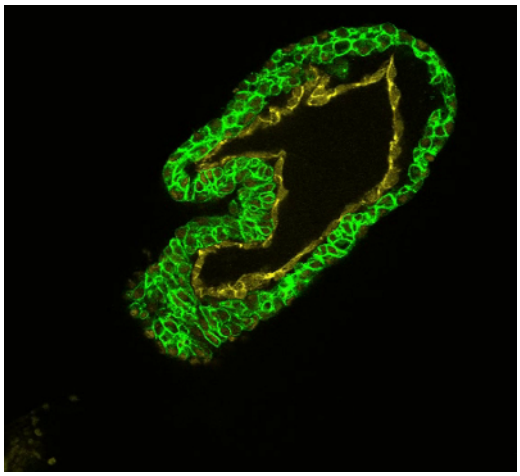
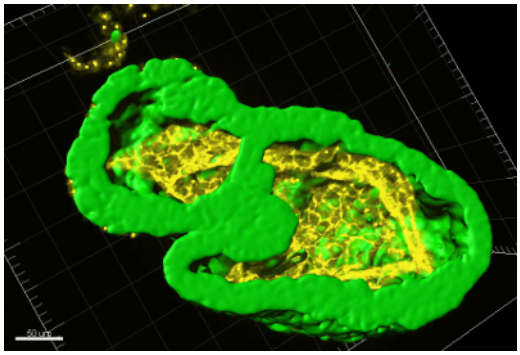


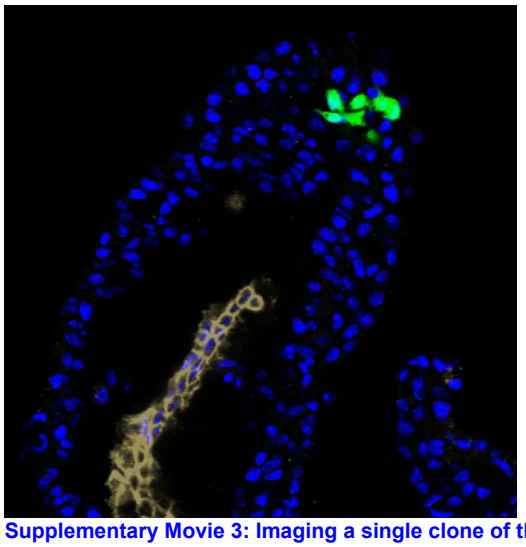
Figure 3: Imaging the CUBIC cleared postnatal and late embryonic hearts. (**A**) Shows one optical section of a P2 heart with the genotype of αMHC-Cre;mTmG; all of the optical sections are shown in Supplementary Movie 5. (**B**) Shows one section of an E17.5 heart with the genotype of Nfatc1-Cre;ROSA26-Confetti. (**C**) Shows the reconstructed heart structure from 106 optical slices with a total depth of 630 µm. The Scale bar = 20 µm in A and is 100 µm in B. Please click here to view a larger version of this figure.



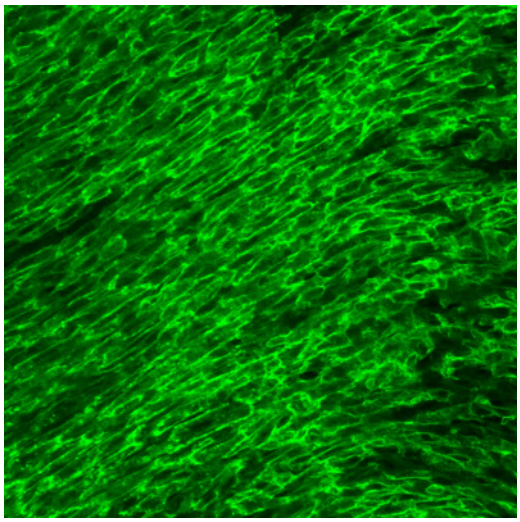
Supplementary Movie 1: Imaging the Sca*/e* cleared embryonic heart (Right click to download). Movie 1 shows optical sections of a Sca*/e* treated E8.75 heart (cTnT-Cre;mTmG) from heart surface to heart lumen. The depth is 110.4 µm and there are 93 slices in total.



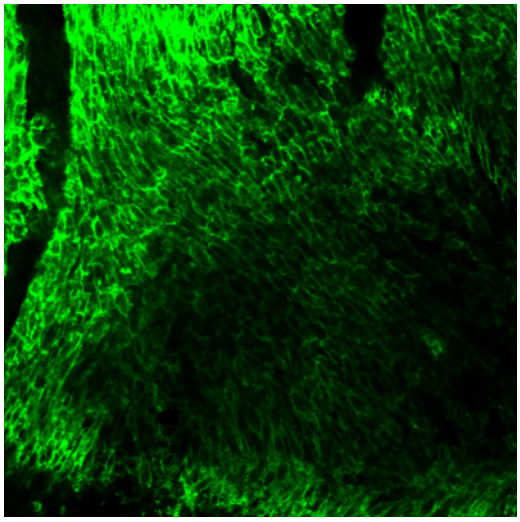
Supplementary Movie 2: Imaging the Sca*l*e cleared embryonic heart (Right click to download). Movie 2 shows the 3D surface picture of the reconstructed heart in Movie 1, reconstructed via the 3D reconstruction software. Yellow marks the PECAM expression and green labels all the cardiomyocytes.



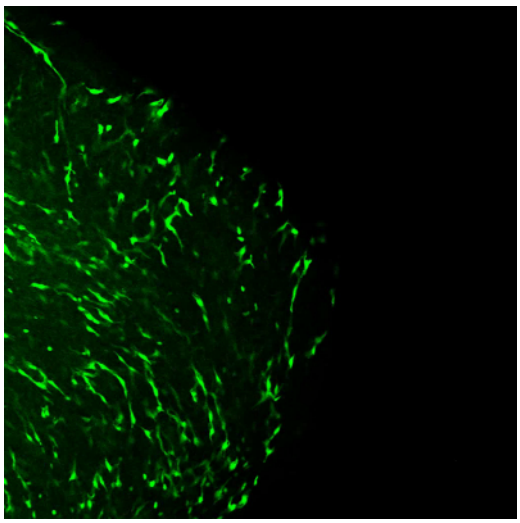
Supplementary Movie 3: Imaging a single clone of the Sca*l*e cleared embryonic heart (Right click to download). Movie 3 shows sections of a clone from an E9.5 heart with the genotype of ROSA26-Cre^{ERT2}.



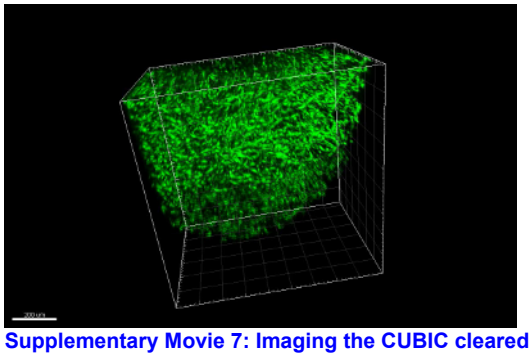
Supplementary Movie 4: Imaging the CUBIC cleared postnatal hearts (Right click to download). Movie 4 shows sections of a CUBIC treated P2 heart (αMHC-Cre;mTmG) from heart surface to heart lumen. This P2 heart was cleared with CUBIC reagent 1 for 48 hr, and CUBIC reagent 2 for 96 hr.



Supplementary Movie 5: Imaging the CUBIC cleared postnatal hearts (Right click to download). Movie 5 shows a heart that was cleared with reagent CUBIC 1 for 48 hr and reagent CUBIC 2 for 48 hr. The depth is 136 µm with 6 µm per section in movie 4 and the thickness is 76 µm with 6 µm per section in movie 5.



Supplementary Movie 6: Imaging the CUBIC cleared late embryonic heart (Right click to download). Movie 6 shows sections of a CUBIC treated E17.5 heart (Nfatc1-Cre;ROSA26-Confetti) from heart surface to heart lumen. The depth is 630 µm with 6 µm per section.



Supplementary Movie 7: Imaging the CUBIC cleared late embryonic heart (Right click to download). Movie 7 shows the 3D surface picture of the heart in Movie 6, reconstructed via the 3D reconstruction software.

Discussion

The embryo isolation is a very critical step. E9.5 embryos are very fragile and small in size, so extra care should be taken not to damage the embryo/heart structures during isolation. The non-embryonic extra layers enveloping the embryo/heart should be removed carefully especially when imaging the whole embryo. This allows antibody and clearing mixture penetration deep inside the embryonic tissues, and also helps in removing the background signal when imaging. Multiple antibodies including antibodies against PECAM, acetylated α-tubulin and integrin β1

were examined and all were successfully detected (data not shown)[33]. This demonstrates that the molecular integrity, cellular integrity and antigen compatibility were not disrupted during this clearing process.

Transfer the embryo/heart using a wide mouth transfer pipette (cut the tip). This avoids damage during handling. The GFP/YFP/RFP/CFP expression pattern and number of clones should be carefully recorded in the heart to determine the appropriate dosage to label one clone per heart or any other organs. Due to the low dosage of Tamoxifen (10 µg/g), sometimes the heart does not contain any clones or is difficult to detect any clones. Therefore, it is recommended to use the yolk sac to determine if the embryo has any GFP/YFP/RFP/CFP clones.

The embryo or heart fixation is a critical step, as over-fixation will result in a high background while under-fixation will result in poor staining. It is recommended to fix the E8.5-E10.5 whole embryo for 2 hr at RT on a plate shaker. While for E11.5-E15.5 embryos, the recommendation is to carefully isolate the heart and fix it with 4% PFA for 2 hr at RT. After ensuring to remove the layers surrounding the heart if processing the whole embryo, the E11.5- E13.5 embryos can also be fixed for 3-4 hr at RT but that requires increased tissue clearing time. The E16.5-P1 (postnatal day 1) hearts are fixed for 3-4 hr at RT on a plate shaker. When fixing the whole embryo, the fore and hind limbs should be removed as they hinder the heart imaging.

The samples should be treated with CUBIC and Sca*l*e in scintillation vials to minimize sample drying. We have observed better clearing of samples with the CUBIC tissue clearing method at 37 °C, while Sca*l*e clearing is optimally done at 4 °C. After imaging, the samples can be stored short-term in CUBIC-2 or Sca*l*e 70. The samples should always be covered with aluminum foil to prevent fluorescence bleaching.

Embryonic tissues and organs have less lipid and extracellular matrix deposition, which results in less clearing time. On the other hand, they are more vulnerable to the clearing reagents. The clearing reagents might affect the integrity of the tissue structure and antigen, resulting in the quenching of CFP, RFP and antibody conjugated fluorophore. Therefore, longer fixation times might prevent the quenching. The quenching of the fluorescent proteins could also be due to their sensitivity to chemicals and pH as previously reported[34]. Other chemical recipes, pH, fixatives, and incubation length should be explored to minimize the quenching of the fluorescent proteins.

The method of whole organ transparentizing or tissue clearing has revolutionized volumetric 3D imaging. The previous imaging method for cardiac morphogenesis, which involved the manual 2D or 3D reconstruction of serial sections, is time consuming and instrument demanding[5,6]. High resolution episcopic microscopy (HREM) in conjunction with 3D modeling can be applied to the study of the morphogenesis of the mouse heart and provides for excellent anatomic description of the trabecular architecture in the developing mouse embryo[35]. However, HREM does not allow the identification of the fluorescent protein labeled cells or antibody stained cells from the rest of cells[35]. Using the current protocol, with minimal effort and high cost effectiveness, we have successfully preserved the fluorescent signals in the embryonic heart using Sca*l*e or CUBIC tissue clearing methods (**Figures 1**-**3**). Combined with a low dose (10 µg/g) of Tamoxifen to induce Cre recombination events and generate a few clones inside the whole embryo and in the heart, this system can be used to trace and study single cardiac progenitor cell differentiation and cellular behaviors *in vivo* (**Figure 2A**)[33]. In addition, combined with Cre mediated gene deletion or overexpression and Cre mediated single cell labeling, this imaging system can be applied to study genetic loss of function or gain of function on progenitor cell differentiation and cellular behavior during cardiac morphogenesis, and thus provides a robust tool to study the etiology of congenital heart defects.

The two-tissue clearing systems Sca/e and CUBIC used in this study showed different clearing efficiencies, which further differentiates the systems based on embryonic age, tissue thickness and duration of tissue clearing time. For early stage embryos E9.5-E10.5, the Sca/e system not only rendered the tissue clear but also protected the embryo morphology and preserved the fluorescent signals, while the CUBIC treatment resulted in embryo dissolution. This might be the result of additional aminoalcohols and a higher concentration of Triton X-100 in the CUBIC solutions[8]. For older hearts, the Sca/e system failed to clear the tissue completely even with a longer incubation time. The CUBIC clearing solution rendered the older tissue clear (**Figure 3**). However, increased incubation time resulted in weaker signals and only protected endogenous GFP, while RFP, CFP and antibody conjugated signals were lost. For this reason, a more optimized recipe of the clearing reagents and better incubation time would be required to volumetrically image hearts or organs with a greater spectrum of fluorescent labels.

This study demonstrated that one can label, trace, and image single cardiac clones in combination with a whole mount staining of endocardial/endothelial cell marker PECAM of the developing heart. The whole mount staining of PECAM and DAPI in this system not only helps to identify the cell types and cell morphology of the labeled cells inside a heart, but also allows the study of the clonal pattern, migration and behavior during cardiac morphogenesis. In **Figure 2A** we observed a clone with 8 cells in OFT. Using the number of cell present in this clone with reference to the labeling time, the cell proliferation rate can be easily calculated. In another study using this same system we identified the oriented cell division pattern and migration pattern of single cardiomyocyte during trabeculation process[33]. The application of this protocol to study the effects of genetic loss of function or gain of function spatiotemporally on the labeled cardiac progenitor cell differentiation, clonal pattern and cellular behavior is feasible, and will help in studies of the etiology of congenital heart defects at genetic and molecular level in a 3D context. In addition, this protocol can be applied to study of morphogenesis in other organ systems.

Disclosures

The authors declare no competing financial interests.

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