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# Multiscale light-sheet for rapid imaging of cardiopulmonary system

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The ability to image tissue morphogenesis in real-time and in 3-dimensions (3-D) remains an optical challenge. The advent of light-sheet fluorescence microscopy (LSFM) has advanced developmental biology and tissue regeneration research. In this review, we introduce a LSFM system in which the illumination lens reshapes a thin light-sheet to rapidly scan across a sample of interest while the detection lens orthogonally collects the imaging data. This multiscale strategy provides deep-tissue penetration, high-spatiotemporal resolution, and minimal photobleaching and phototoxicity, allowing in vivo visualization of a variety of tissues and processes, ranging from developing hearts in live zebrafish embryos to ex vivo interrogation of the microarchitecture of optically cleared neonatal hearts. Here, we highlight multiple applications of LSFM and discuss several studies that have allowed better characterization of developmental and pathological processes in multiple models and tissues. These findings demonstrate the capacity of multiscale light-sheet imaging to uncover cardiovascular developmental and regenerative phenomena.

## Introduction

Real-time 3-dimensional (3-D) imaging of tissue development and regeneration remains an optical challenge. Conventional optical microscopes are limited by low tissue penetration and small working distance, which are prohibitive to long-term live imaging that requires rapid data acquisition to minimize photobleaching and phototoxicity to the specimens (1–3). In addition, samples must be mechanically sectioned, thereby distorting intrinsic tissue integrity and subsequently resulting in undersampling after 3-D reconstruction (4). While PET (5, 6), μCT (7, 8), MRI (9, 10), and bioluminescence imaging (11, 12) are capable of capturing 3-D images from live samples, the spatial resolution of these techniques is inadequate to capture organ morphogenesis in small-animal models (13–17). For these reasons, the advent of light-sheet fluorescence microscopy (LSFM) (18–22) has revolutionized multiscale imaging, allowing visualization of samples ranging from live zebrafish embryos (~ $0.4 \times 0.5 \times 0.6$ mm³) to adult mouse hearts (~8 × 8 × 10 mm³) with high-spatiotemporal resolution and minimal photobleaching and phototoxicity.

Unlike confocal and wide-field microscopy, LSFM has the capacity to localize 4-D (3-D spatial + 1-D time or spectra) cellular phenomena with multiple fluorescence channels (23–28). The theoretical principle of light-sheet imaging was first reported in 1903 (29); however, the experimental application of LSFM was not possible until the introduction of fast-rate charge-coupled devices/complementary metal–oxide–semiconductor (CCD/CMOS) camera for high-speed data acquisition in 2004 (23). Initially, LSFM was developed to image small-model organisms, such as *Caenorhabditis elegans* (30, 31), zebrafish embryos (32, 33), and *Drosophila* (34, 35). Subsequently, LSFM imaging of the entire 3-D mouse hippocampus (36–39) and cochlea (40–43) has been made possible with advancements in optical clearance techniques (Figure 1A).

The unique operation of LSFM resides in the orthogonal optical pathway. The illumination and detec-

tion pathways are linearly aligned in the inverted or upright microscope, whereas the illumination pathway is perpendicular to the detection pathway in the LSFM system. The sample is illuminated at the focal plane of a thin light-sheet of the detection lens (Figure 1, B and C). The emitted fluorescence is perpendicularly collected by the detecting objective lens connected to a fast-rate CCD/CMOS camera. The sample is placed at the intersection of the illumination and the detection axes. In addition to imaging transparent zebrafish embryos, LSFM offers the ability to visualize opaque specimens, including mouse organ systems, following the optical clearing techniques to render these specimens translucent with matching refractive indices (44, 45).

A continuous-wave laser is typically used as the illumination source for LSFM. The detection module is composed of a set of filters and a scientific CMOS for rapid multichannel acquisition. This module is perpendicularly installed to the illumination plane (Figure 1, D and E). The lateral resolution (*d*) of LSFM is determined by the numerical aperture (NA) of the objective lens and the wavelength of excitation light (*λ*), defined as *d* is proportional to *λ/NA*. For data acquisition, each image is acquired within tens of milliseconds of exposure time. Some of the raw data are further processed to remove stationary noise (46–48). In addition to the static light-sheet generated by a cylindrical lens (49–51), the digitally scanned light-sheet microscopy introduced rapid scanning with a Gaussian laser beam (32), further implementing with the two-photon excitation for deep and fast live imaging of *Drosophila melanogaster* embryos (34).

Recently, improvements in both hardware and software components have enhanced the LSFM-acquired images. First, additional structural illumination or pivoting the light-sheet allows rejection of out-of-focus background and shadows in dense tissues (52, 53). Second, computational processing methods have allowed for fusion of multiview images of the same sample (54, 55). Finally, four-lens systems have been developed to minimize rotation and registration efforts (35, 56, 57). Detailed advantages and applications among different light-sheet techniques are listed in Table 1. Distinct from the conventional fluorescence microscopy, LSFM has the capacity to achieve (a) deep penetration into light-scattering tissues; (b) selective optical sectioning of the tissue; (c) minimal photobleaching and phototoxicity; and (d) rapid and multiview acquisition.

In previous work, our group demonstrated the capacity of light-sheet imaging to uncover both mechanical and structural cardiac phenotypes at the cellular level without stitching image columns or pivoting the illumination beams (18–22, 28, 58, 59). In this review, we summarize the use of LSFM to (a) track a recently discovered subpopulation of neural crest–derived cells as they incorporate into the developing heart tube via specific labeling of cell lineages using the fluorescent reporters in zebrafish embryos, (b) capture the dynamics of atrioventricular (AV) valve leaflets throughout the cardiac cycle, (c) integrate dual-sided illumination into the LSFM system to allow for large-scale imaging of 3-D vascular calcification in an adult mouse model, (d) reveal the 3-D developmental lung mesenchyme in a young mouse model, and (e) image the ocular architecture and its retinal vasculature. Overall, the multiscale LSFM system has been shown to be useful in unraveling cardiovascular development and regeneration in models ranging from zebrafish embryos to adult mouse cardiovascular tissues that otherwise have been considered optically challenging for existing imaging modalities.

## Time-lapse imaging of neural crest cell incorporation into the developing heart tube

Genetic lineage-tracing studies have previously shown that cardiac neural crest cells integrate into the developing heart tube and differentiate into cardiomyocytes (60–63). The transgenic *NC:NfsB-mCherry* zebrafish line (*Tg[-5sox10:GAL4,UAS:Cre]^{la2326Tg}*; *Tg[UAS-E1b:NTR-mCherry]^{c264Tg}*; *Tg[myl7:NLS-EGFP]^{chb2Tg}*) was used for time-lapse imaging to understand the temporal dynamics of how this subpopulation of neural crest–derived cells integrates into the heart tube (Figure 2A). The transgenic *Tg(NC:NfsB-mCherry)* embryos express nuclear eGFP in cardiomyocytes (green) and mCherry in cells derived from the neural crest lineage (red) (Figure 2B). A series of time-lapse images taken from 26 hours after fertilization (hpf) to 30 hpf revealed the spatial and temporal migration of this subpopulation to the heart tube (Figures 2, C–G). Neural crest–derived cells were observed contacting the dorsal surface of the heart tube at 26–27 hpf (Figure 2, C and D). By 29 hpf, these neural crest–derived cells integrated into the heart tube and expressed nuclear eGFP (yellow cells in Figure 2, F and G). Thus, LSFM revealed the 4-D distribution of this subpopulation of neural crest–derived cells, providing an imaging platform for further investigation into the lineage-specific differentiation of these cells into cardiomyocytes (yellow in the right panel) and the future septal and valve mesenchyme.
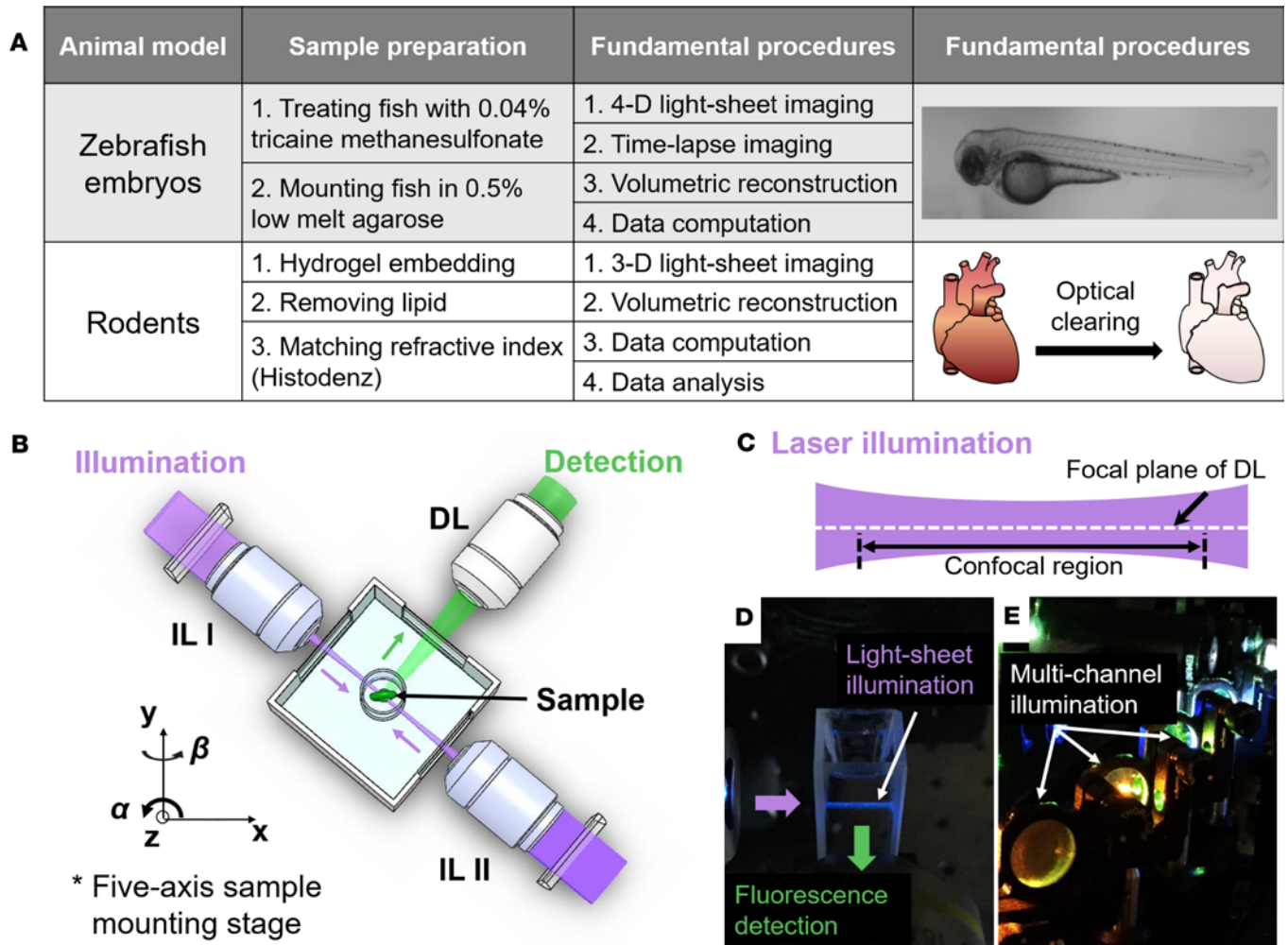
**Figure 1. Fundamental concept of the light-sheet imaging strategy.** (**A**) Crucial procedures of multiscale imaging are indicated from embryonic zebrafish and rodent models. (**B**) The sample holder is oriented by a five-axis mounting stage for scanning the biological specimen. The laser light-sheet is excited from the illumination lenses (IL I and IL II) in a 2-D plane, which is orthogonal to the detection lens (DL). (**C** and **D**) A schematic and a photo illustrate the conversion of laser light to a sheet that can transversely illuminate a thin layer of the sample. (**E**) This photo depicts an array of laser beams aligned for multichannel fluorescent detection. (Reproduced with permission from ref. 68.)

## Dual-channel imaging for 3-D valve leaflet dynamics

Hemodynamic forces are known to govern AV valve formation (64); however, rigorous in vivo visualization of AV leaflets remains an imaging challenge (65, 66). Using the transgenic *Tg(fli1:GFP;cmlc2:mCherry)* zebrafish line at 5 days after fertilization (dpf), we applied dual-channel imaging to capture the excursion of valve leaflets (green) in relation to the endocardium (green) and myocardium (red) (Figure 2H). At a rate of 100 frames per second (fps), dual-channel LSFM colocalized AV valve leaflet closure (Figure 2I) and opening (Figure 2J) with ventricular myocardial contraction and relaxation, respectively. These results demonstrate that LSFM imaging allows for time-dependent structure and function determination, which will be useful for studies of mechanosignal transduction of valvulogenesis (67).

In addition to the aforementioned applications of LSFM to examine developmental processes in zebrafish embryos, our group has used LSFM imaging to study vascular injury and regeneration in transgenic *Tg(fli1:GFP; gata1:DsRed)* and *Tg(fli1:GFP; cmlc2:mCherry)* zebrafish lines, which allowed us to track blood cells in the vasculature in response to tail amputation at 3 dpf (68). In the *Tg(fli1:GFP;gata1:DsRed)* line, GFP expression is driven by *fli1* promoter in the vasculature throughout embryogenesis and the *gata1* promoter drives the expression of DsRed in blood cells. These animals allowed us to simultaneously track the vascular loop connection between the dorsal aorta and the dorsal longitudinal anastomotic vessel at 3 dpf and blood cell trafficking to the injured site. This method may allow studies of nonlinear shear rates in

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**Table 1. Overview of different light-sheet techniques**

| Technique | Unique features | Advantages | Applications | References |
|---|---|---|---|---|
| Light-sheet techniques | | | | |
| OPFOS/HROPFOS | Single-sided light-sheet illumination | Optical sectioning | Fluorescent samples | (40–43) |
| TLSM | Sample immersed in the aquatic medium | Improved signal-to-background ratio | Aquatic microbes | (134) |
| SPIM/mSPIM/DSLM | Pivoting light-sheet; digitally scanned virtual light-sheet | Low photobleaching and deep penetration | Live imaging of developmental embryos | (18, 20, 23, 25, 32, 52) |
| UM/TSLIM | Dual-sided light-sheet illumination | Large murine or rodent models | Optically cleared samples | (21, 37–39, 50) |
| OCPI/soSPIM | Light-sheet illumination coupled to detection lens | Fast acquisition; super resolution | From cell aggregates to embryos | (135, 136) |
| HILO/OPM/SCAPE | Oblique light-sheet illumination and detection | Single lens for both illumination and detection | From single-cell to behaving organisms | (137–139) |
| MuVi-SPIM/SiMView/diSPIM/IsoView | Multiview reconstruction | Improved axial resolution and superior sample coverage | From live embryos to opaque specimen | (26, 30, 35, 54–57, 140–142) |
| LLSM/meSPIM/ALSM | Nondiffracting beam and structural illumination | Uniform light-sheet thickness with an invariant profile | From single-cell to live embryos | (31, 116, 143–145) |

Only the most representative citations are given. OPFOS, orthogonal-plane fluorescence optical sectioning; HROPFOS, high resolution OPFOS; TLSM, thin light-sheet microscopy; SPIM, selective-plane illumination microscopy; mSPIM, multidirectional SPIM; UM, ultramicroscopy; TSLIM, thin-sheet laser illuminating microscopy; OCPI, objective-coupled planar illumination; soSPIM, single objective SPIM; HILO, highly inclined and laminated optical sheet; OPM, oblique plane microscopy; SCAPE, swept confocally aligned planar excitation; SiMView, simultaneous multiview; IsoView, isotropic multiview; MuVi-SPIM, multiview SPIM; diSPIM, dual-view inverted SPIM; LLSM, lattice light-sheet microscopy; meSPIM, microenvironmental SPIM; ALSM, Airy-beam light-sheet microscopy.

a low-flow Reynolds number system (Re = 100–1,000). Genetic manipulation and use of ADAM10, which inhibits proteolytic cleavage of the Notch extracellular domain, also enabled us to elucidate a mechanism by which Notch-mediated vascular regeneration connects the loop between dorsal longitudinal anastomotic vessel and dorsal aorta. Thus, LSFM provides an entry point for the discovery of novel microcirculation phenomenon with clinical significance for injury and repair.

In summary, light-sheet imaging allows for analysis of the entire 3-D dynamic process of neural crest cell migration to the heart tube and differentiation to cardiomyocytes. Conventional optical microscopes are limited by the insufficient frame rate to 2-D planar images. In studies of cardiomyopathy mechanisms, LSFM has been used to associate neural crest cell–derived cardiomyocytes with trabeculation and cardiac contractility (69–75), with minimal photobleaching and phototoxicity during time-lapse imaging. In addition, this method has been used to quantify the velocity of circulating blood cells (76) in a transgenic zebrafish model of tail injury, allowing for correlation of microcirculatory shear rates with vascular regeneration and providing clinical relevance to the use of selective-plane illumination (77). Due to the cyclical rhythm of myocardial contraction and relaxation, LSFM has marked potential to deliver 4-D reconstruction of dynamic AV valve leaflet movements and, after applying spatial registration between images captured at different time points (20), can provide further insights into the mechanosignal transduction of valvulogenesis.

## Analysis of doxorubicin chemotherapy–induced cardiac injury and repair

The regenerating myocardium electrically couples with uninjured myocardium (78) and represents an evolutionarily conserved model of cardiomyopathy (79) in adult zebrafish. However, the small size of the two-chambered zebrafish heart limits precise morphologic assessment of regenerating heart tissue. Utilization of chemical clearing to achieve tissue transparency and laser light transmission, along with light-sheet imaging coupled with automated image segmentation based on histogram analysis, led to rapid and robust 3-D cardiac reconstruction, thereby unraveling the architecture of doxorubicin chemotherapy–induced cardiac injury and regeneration in adult zebrafish (Figure 2K) (58). Precise 3-D reconstruction further enabled

A 24 hpf — Eye, Heart, Yolk, Caudal vein

B 36 hpf — Head, Eye, Heart tube, Pharyngeal arches; Rostral, Ventral, Dorsal, Caudal; *myl7:nuc-eGFP*, *NC:mCherry*

C 26 hpf

D 27 hpf

E 28 hpf

F 29 hpf

G 30 hpf

H Myocardium, Endocardium, V, AV valve

I Myocardium, Endocardium, V, A, AV valve closure

J V, A, AV valve open

K AV Valve, VB Valve, Ventricular Cavity
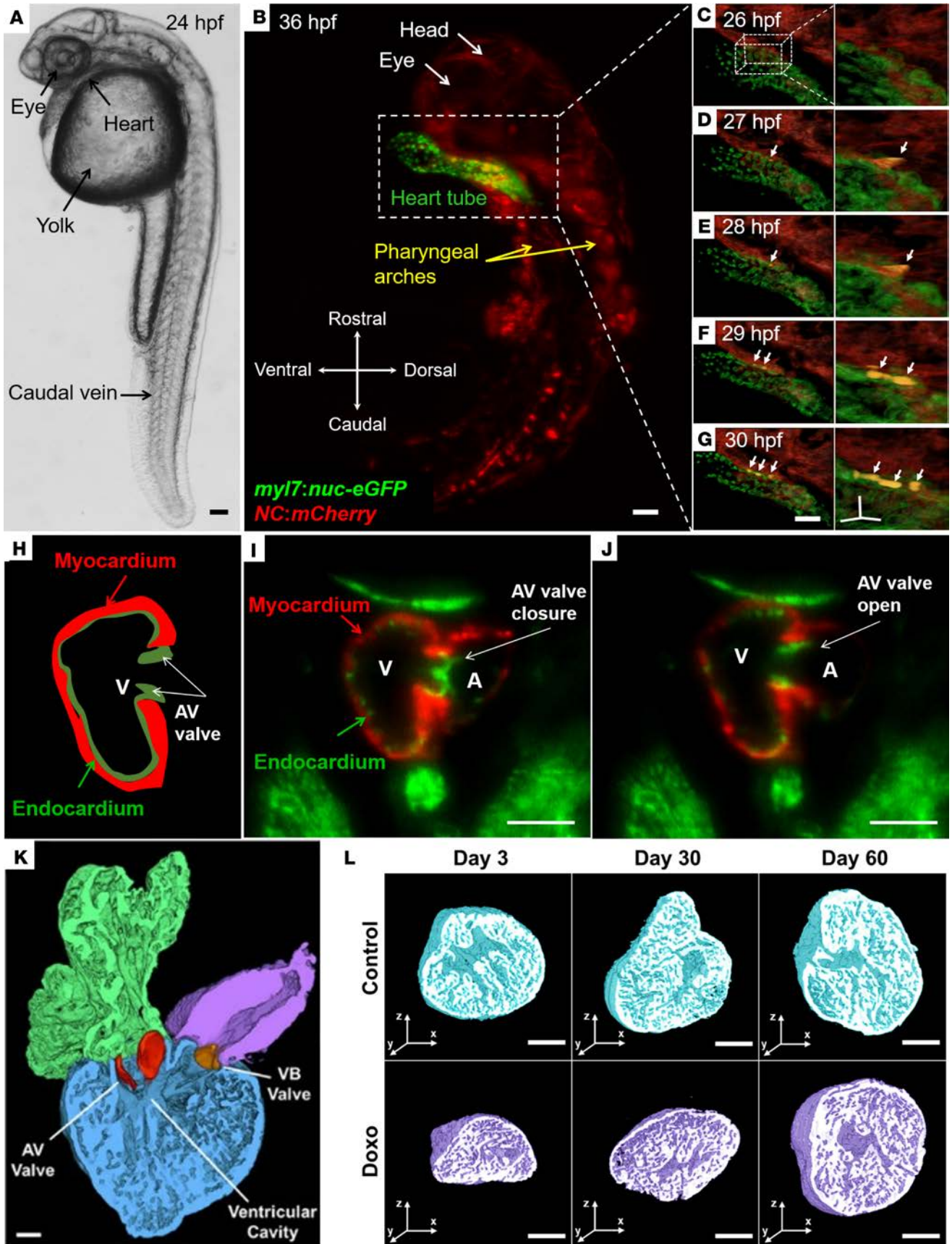
L Control / Doxo — Day 3, Day 30, Day 60

**Figure 2. In vivo visualization of the developing hearts from the live zebrafish embryos and high-resolution imaging of chemotherapy-induced cardiac injury and regeneration.** (**A–G**) Light-sheet imaging of neural crest incorporation into the developing zebrafish heart tube (transgenic zebrafish line, *Tg(-5sox10:GAL4,UAS:Cre)ᴵᵃ²³²⁶ᵀᵍ; Tg(UAS-E1b:NTR-mCherry)ᶜ²⁶⁴ᵀᵍ; Tg(myl7:NLS-EGFP)ᶜʰᵇ²ᵀᵍ*). (**A**) Bright-field microscopic image of a zebrafish embryo 24 hpf. (**B**) The transgenic *Tg(NC:NfsB-mCherry)* zebrafish embryos express nuclear eGFP in the cardiomyocytes (green) and mCherry (red) in cells derived from the neural crest lineage at 36 hpf. (**C–G**) Colocalization of eGFP and mCherry from 26–30 hpf indicates the presence of cardiomyocytes of neural crest origin (yellow, arrows). (**H–J**) Dual-channel LSFM to capture the dynamic movement of atrioventricular (AV) valve leaflets from the transgenic *Tg(fli1:GFP; cmlc2:mCherry)* zebrafish embryos. An illustration of the AV valve leaflets in relation to the myocardium (*cmlc:mCherry*) and the endocardium (*fli1:GFP*) (**H**). AV valve leaflet closure (**I**) and opening (**J**) were captured at 100 fps. Myocardium (red) and endocardium (green) were concurrently acquired by the dual-channel imaging system. (**K** and **L**) Cardiac architecture following doxorubicin treatment and 3-D rendering of the adult zebrafish heart. A cross-section through the atrium, ventricle, and bulbus arteriosus demonstrates the two leaflets of the AV valve (red) and of the ventriculo-bulbar (VB) valve (orange) (**K**). Throughout the duration of the study, control hearts exhibited a preserved architecture in comparison with doxorubicin-treated groups at days 3, 30, and 60 (**L**). (Reproduced with permission from ref. 58). Scale bars: 50 μm (**A–G**); 10 μm (insets of **C–G** on the right side); 200 μm (**I–L**).

quantitation of cardiac volumes at days 3, 30, and 60 after chemotherapy treatment (Figure 2L). Compared with control fish, doxorubicin-treatment fish had an acute decrease in myocardial and endocardial volumes at day 3, demonstrating global cardiac injury. Ventricular remodeling was notable at day 30, and by day 60, the injured heart had completely regenerated and normal architecture was restored. The results demonstrate the suitability of LSFM combined with automated segmentation as a high-throughput method to monitor 3-D cardiac ultrastructural changes in adult zebrafish, with translational implications for drug discovery and modifiers of chemotherapy-induced cardiomyopathy.

## Three-dimensional tracking of cardiac regeneration in neonatal and adult mouse hearts

The 3-D distribution of cardiac progenitor cells during both cardiac morphogenesis and regeneration remains poorly understood (80, 81). Two reporter systems were utilized along with LSFM imaging to identify cardiac progenitor lineages. A rainbow multicolor reporter system (82) was used to retrospectively identify the source of new cardiomyocytes in a mouse heart at P1 (Figure 3A) (83). In the *αMHC*ᶜʳᵉ *R26*ⱽᵀ²/ᴳᴷ mouse model, Cre-mediated recombination of paired loxP sites resulted in expression of all four fluorescent proteins (cerulean, GFP, mOrange, and mCherry). Another fetal mouse model, *Mesp1*ᶜʳᵉ/⁺ mice crossed with the *Rosa26*ᵗᵈᵀ/⁺ reporter mice, allowed evaluation of the contribution of tdT⁺ cells (yellow; hot) in an intact fetal mouse heart (Figure 3B). Embryos from timed mating of *Mesp1*ᶜʳᵉ/⁺ *Rosa26*ᵗᵈᵀ/⁺ transgenic mice were isolated at E16.5, when the four-chambered heart had formed. Three-dimensional LSFM imaging showed the majority of the tdT-labeled cardiac cells arose from a *Mesp1*-expressing cell origin and allowed tracing of their 3-D distribution, proliferation, and tissue formation. Thus, light-sheet imaging allows for the elucidation of the organ-specific differentiation of stem cells in cardiac development and regeneration.

In addition, the relative ratio of ventricular volume to wall thickness provides insight into the regenerative capacity of cardiomyocyte proliferation at neonatal stages. A WT postnatal heart at P7 was imaged entirely by autofluorescence (Figure 3C) (21, 84). The 3-D reconstruction of the heart highlighted the ventricular cavity in yellow and rendered the myocardium by blue by a user-defined intensity threshold. Three-dimensional, in toto imaging enables measurement of the thicknesses of the right ventricular wall (~300 μm), the septum (~650 μm), the left ventricular wall (~950 μm), and even ventricular volume in the intact mouse heart. Further, localization of ion channel distribution of in an adult heart is challenging to discern using conventional methods. With the use of LSFM, we revealed the spatial distribution of renal outer medullary potassium (ROMK) channels (green) after gene modulation and/or therapy in the intact heart (Figure 3D) (21, 85). An adult mouse heart of 7.5 months of age was imaged after tail vein injection of an adeno-associated virus vector 9 (AAV9) that employed a cardiac-specific troponin T promoter (cTnT) to drive cardiomyocyte gene expression of a customized construct in which the ROMK channel was fused at its C-terminus to one GFP molecule (AAV9-ROMK-GFP). Finally, this adult mouse heart was imaged in its entirety in 3-D to assess the cardiac-specific expression of an exogenous kidney potassium ion-channel that otherwise is not normally expressed in the heart.

## 3-D calcific vasculopathy in the *ApoE*⁻/⁻ mouse model of atherosclerosis

Calcific vasculopathy is associated with increased mortality and morbidity, especially in patients with renal disease (86, 87). In addition to localizing the distribution of aortic calcium mineral, LSFM revealed the formation of calcification with high spatial resolution (8 μm × 8 μm × 5 μm) in a 15-month-old *ApoE*⁻/⁻ female mouse that
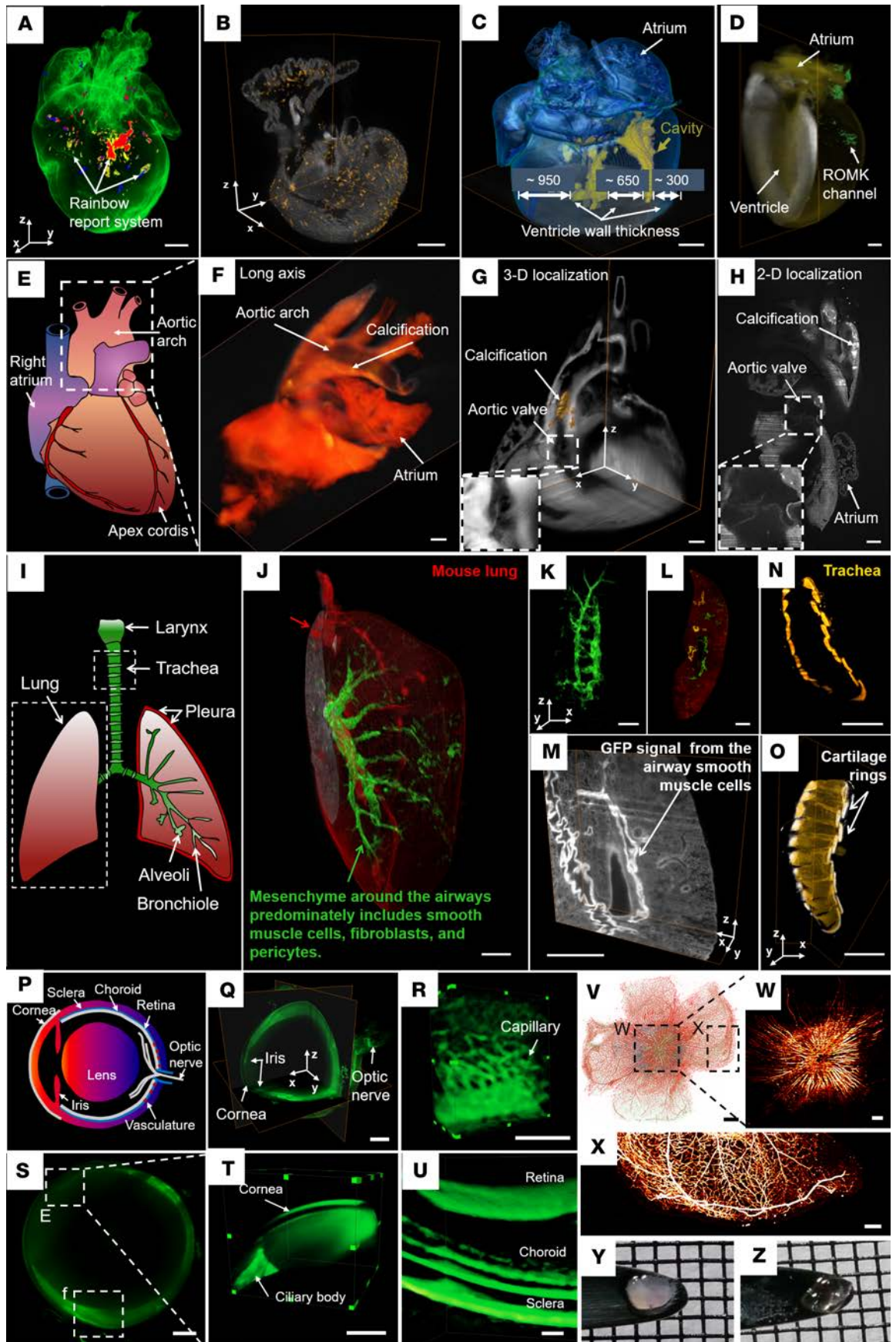
**Figure 3. Three-dimensional interrogation of cardiovascular development and regeneration in neonatal and adult mouse models.** (**A** and **B**) Cardiac progenitor lineage tracking in neonatal mouse hearts. Cre expression leads to cerulean, GFP, mOrange, and mCherry expression (**A**). Spatial distribution of tdT⁺ cells (hot) in a neonatal heart from a *Mesp1^{Cre/+}* mouse crossed with the *Rosa26^{tdT/+}* reporter line (**B**). (**C**) The reconstructed heart reveals the small ventricular cavity (yellow) in a thick wall at P7. (**D**) Detection of GFP-tagged renal outer medullary potassium (ROMK) channel (green) in a 7.5-month-old adult mouse heart. (**E–H**) Calcific vasculopathy in a mouse atherosclerosis model. Illustration of cardiac anatomy delineating the areas imaged, including the heart base and aortic arch (**E**). Three-dimensional reconstruction shows calcium mineral (yellow) in a 15-month-old female apolipoprotein-deficient mouse (**F**). A 3-D orthogonal slice (**G**) and 2-D raw data (**H**) show distribution of aortic calcium mineral (brown and white, respectively). (**I–O**) Three-dimensional localization of developing mesenchyme from an 8-week-old reporter mouse lung. Cre expression (mGFP, green) was detected in lung mesenchymal cells, including airway, vascular smooth muscle cells, a variety of fibroblasts, vascular endothelial cells, and pericytes. Cre-negative cells expressed mTomato (red) (**I–K**). Two-dimensional (**L**) and 3-D (**M**) raw data of airway smooth muscle cells with high GFP. Tracheal architecture is visualized by the C-shaped rings (yellow) of hyaline cartilage (**N** and **O**). (**P–Z**) Label-free imaging of an intact albino mouse eye and fluorescence imaging of an *rd10* eye using light-sheet microscopy. Illustration of a mouse eye includes the lens, cornea, sclera, choroid, retina, iris, and optic nerve (**P**). Light-sheet microscopy captured the 3-D orthogonal slice of the entire eyeball without changing the objective lens (**Q**). Three-dimensional vascular network in the posterior ocular system (**R**). Two-dimensional (**S**) and 3-D (**T**) structure of the cornea and ciliary body. Three-dimensional reconstruction of multilayer images reveals the retina, choroid, and sclera (**U**). The *rd10* mouse is a model of autosomal recessive retinitis (**V–X**). Bipolar and ganglion cells express GFP (blue), while amacrine cells and vasculature are labeled with Alexa 594 (red) (**V**). Maximum intensity projection (MIP) images are presented in single-channel neurons expressing GFP (**W**) and single channel of vasculature labeled with Alexa 594 (**X**). Retina prior to (**Y**) and after (**Z**) simplified CLARITY are presented. Scale bars: 500 μm (**A–D**, **Q**, **S–V**); 1 mm (**F–O**); 200 μm (**R**, **W** and **X**). (Reproduced with permission from refs. 21, 83, 88.)

had been injected with a fluorescent bisphosphonate probe (5-FAM-ZOL; Biovinc) that binds calcium (Figure 3E) (88) and renders areas where this mineral is present fluorescent after injection. Three-dimensional reconstruction shows that calcification (yellow) is evident in the aortic arch (Figure 3F). Both the 3-D orthogonal slices (Figure 3G) and 2-D raw data (Figure 3H) unraveled the spatial location of aortic calcium mineral (brown in Figure 3G and white in Figure 3H), and the aortic valve cusps were visible in the insets (Figure 3, G and H). Thus, LSFM provides large-scale scanning of an adult mouse model of vascular calcification with translational implications for optimally controlling calcium and phosphate homeostasis in patients with chronic kidney disease (89) and for evaluating the efficacy of potential therapeutic agents.

## Visualization of developing pulmonary mesenchyme for lung morphogenesis

In addition to the cardiovascular system, we have demonstrated that LSFM can be applied to investigate the developing pulmonary mesenchyme as a source of specification for lung development and function (Figure 3I) (90). An 8-week-old reporter mouse was generated by crossing the lung mesenchyme–specific *Tbx4* lung enhancer–driven Tet-On inducible Cre transgenic mouse (91) with a loxP-mTomato-STOP-loxP-mGFP (mTmG) fluorescent protein reporter mouse line (92). In this mouse, Cre-positive cells are green as a result of Cre-mediated floxed mTomato deletion, while Cre-negative cells are red as a result of mTomato expression (Figure 3J). The lung and trachea were harvested, perfused with 4% paraformaldehyde (PFA), and subjected to the simplified CLARITY protocol for tissue clearing (21, 85) after the mice were euthanized. Using light-sheet imaging, we demonstrated that doxycycline induction in the triple transgenic (*Tbx4-rtTA* TetO-Cre mT-mG) mouse line from E6.5–E18.5 resulted in mGFP expression only in lung mesenchymal cells, including airway and vascular smooth muscle cells, as well as a variety of fibroblasts, vascular endothelial cells, and pericytes. Using specific filtering thresholds, we were able to highlight an elevated GFP signal in airway smooth muscle cells; presumably, this intensity was due to the compacted nature of these cells as compared with low-intensity GFP from the scattered fibroblasts and pericytes (Figure 3K). The 2-D merged data (Figure 3L) and 3-D orthogonal slices (Figure 3M) also allowed for tracing the developing mesenchyme with high spatial resolution at 2 μm × 2 μm × 1 μm. Furthermore, light-sheet imaging revealed the cross-section of C-shaped rings (yellow) of hyaline cartilage (Figure 3N) from a 3-D trachea (Figure 3O). While previous studies of lung development have focused on the lung epithelium, light-sheet imaging enables investigators to further investigate the lung mesenchyme as a critical source of inductive cues for a host of complex cell lineages during lung development and function.

## Label-free imaging of an intact mouse ocular system

Ocular disorders are often early signs of cardiovascular disease (93, 94). While the existing imaging modalities — including fundus photography (95), confocal scanning laser ophthalmoscopy (96), and optical coherence tomography (97) — provide valuable 3-D ocular imaging, LSFM offers an advantage by enabling imaging of the entire globe with a single scan. This capacity for one-time scanning is achieved without the need to switch objective lenses when changing focus from anterior to posterior ocular structures due to the presence of the crystalline lens of the eye. An intact albino mouse was chosen for imaging because the

**Table 2. Overview of light-sheet techniques in comparison with other imaging modalities**

**Other imaging modalities**

| | | | | |
|---|---|---|---|---|
| MRI/CT/US/PET/SPECT | Versatile imaging modality with high image contrast | Anatomical, physiological, and molecular imaging | Clinical and preclinical practice | (146–151) |
| PAT | Optical illumination and ultrasonic detection | Absorption contrast and deep penetration | Vasculature, hemodynamics, oxygen metabolism. | (152, 153) |
| Confocal | Spatial pinhole for optical sectioning | Elimination of out-of-focus fluorescence | Fluorescence imaging of cells and tissues | (154, 155) |
| MPM | Nonlinear optical imaging | Deep penetration and 3-D imaging | From single-cell to behaving organisms | (156, 157) |
| STED/PALM/STORM | Far-field subdiffraction limit imaging | Single molecular localization of live ultrastructure | Molecular dynamics | (158, 159) |

MRI, magnetic resonance image; CT, X-ray computed tomography; SPECT, single photon emission computed tomography; US, ultrasound; PAT, photoacoustic tomography; MPM, multi-photon microscopy; STED, stimulated emission depletion; PALM, photoactivated localization microscopy; STORM, stochastic optical reconstruction microscopy.

relative lack of melanin pigment rendered the ocular architecture intact and translucent, allowing for visualization of the cornea, conjunctiva, iris, sclera, choroid, retina, optic nerve, blood vessels, and lens without fluorescence staining (Figure 3, P–U). Endogenous autofluorescence served as the imaging contrast. LSFM has also been used to image an isolated retina from a mouse model of retinal degeneration (*rd10*) (Figure 3, V–Z). In this model, the bipolar and ganglion cells in the retina expressed GFP (blue), while the amacrine cells and vasculature were labeled with Alexa 594 (red) (Figure 3V). The LSFM captured the neurons (Figure 3W) and vasculature (Figure 3X) in the *rd10* model with a spatial resolution of 2 μm × 2 μm × 3 μm. In addition, we compared retinas with and without optical clearing (Figure 3, Y and Z). Light-sheet imaging was applied to image the zebrafish eye during development (98) and to quantify rodent retina development (99), further demonstrating the capability of LSFM for large-scale imaging of intact ocular systems.

Thus, in rodent models, LSFM enables the detailed analysis of developmental and vascular biology. In terms of cell lineage determinations, LSFM provides a powerful method to concurrently trace the 3-D distribution of cardiomyocytes that differentiate from numerous progenitors in an intact heart by taking advantage of the multichannel and large-scale capacities of the light-sheet imaging system. LSFM-generated images of organ-specific stem cell differentiation allow a more detailed understanding of cardiac development and regeneration. Furthermore, in comparison with CT or conventional optical microscopes (100–102), LSFM provides specific fluorescence from mineralized tissue and superb spatial resolution for large-scale scanning of vascular calcification in the *ApoE*$^{-/-}$ mouse model of atherosclerosis, shedding light on translational optimization for regulating calcium and phosphate homeostasis in patients with chronic kidney disease (89). In addition, the demonstration of the role of developing pulmonary mesenchyme in mouse lung morphogenesis and the visualization of the entire mouse ocular system from the anterior to the posterior structures provide advances in optical imaging with great value to multiscale vascular biology and developmental cardiology.

## The future of LSFM

Live imaging has transformed biomedical sciences by enabling visualization and analysis of dynamic cellular processes as they occur in their native context (103, 104). The advent of LSFM has led to widespread exploration of in vivo biological processes beyond the coverslip (3, 105). Currently, 4-D live imaging has made it possible to visualize biophysical and biochemical interactions in the freely moving embryos or rodents (106–109). The digital micromirror device–based light-field technique further enhances temporal resolution of LSFM for real-time volumetric imaging (110).

Among these optical methods, adaptive optics transforms conventional LSFM to compensate for optical aberrations and scattering by controlling the wavefronts (111–113). Furthermore, integration of self-reconstructing beams with LSFM generates a long and uniform light-sheet (114, 115), resulting in single cell imaging with two-photon Bessel beams (116). Further development of an optical lattice enables ultrathin light-sheet imaging from cellular to embryonic specimens (31). Meanwhile, another advance of LSFM is the ability to control light-sheet thickness by two electrically tunable lenses, adapting the position of light-sheet and light exposure independently throughout organisms within milliseconds (117).

Parallel advances in pixel superresolution (118–120), deep learning (121–124), virtual reality (125–128), optogenetics (129), and laser ablation (130) further provide complementary opportunities to elucidate cardiovascular architecture and function. A subvoxel LSFM is technically implemented for high-resolution, high-throughput volumetric imaging of cardiovascular development in a large field of view (22). This iterative resolution recovery method is transformative to improve inadequate focusing capability. For postimage processing, the development of a novel convolutional or recurrent neural network for automatic segmentation would likely bypass manual segmentation of large data sets. The study of interactive virtual reality demonstrates an efficient and robust framework for creating a user-directed microenvironment that can be used to uncover developmental cardiac mechanics and physiology with high spatiotemporal resolution.

## Conclusion

In this review, we have summarized studies that have shown the capacity of LSFM for multiscale imaging to elucidate cardiopulmonary development, regeneration, and disease. Furthermore, we have shown that LSFM can provide detailed analysis of cardiovascular phenomena, ranging from embryonic heart development to calcific atherosclerotic disease in adult mice, and can be applied to samples, ranging from tissue clearing ex vivo to 4-D imaging in vivo. This framework builds on the high axial and temporal resolution for long-term, 3-D and 4-D visualization of in vivo cellular events, tissue morphogenesis, and organogenesis, with minimal photobleaching or phototoxicity. This strategy brings advanced imaging to studies of tissue injury, regeneration, and pathology, with multiscale applications to fundamental studies of cardiovascular development and translational work in cardiac anomalies and disease.

As compared with other optical imaging modalities, the unique feature of LSFM is the capacity to rapidly image the entire cardiovascular specimen within 30 seconds for zebrafish and 60 seconds for intact mouse hearts without the requiring image-column stitching. This methodology bypasses the need to move the tissue volume or light-sheet along the propagation of the illumination, thereby allowing for multiscale imaging for a wide range of specimens, from embryos to adults. This methodology further expands the field of view from hundreds of micrometers to tens of millimeters to cover the entire adult mouse heart with the spatiotemporal resolution needed to localize the progenitor cell fates. In addition to reducing the complexity of pre- and postprocessing of cardiac images, the optimized imaging strategy simplifies image acquisition and enhances the imaging system to provide dual-channel in vivo imaging at over 100 fps. The comparative advantages and applications among different imaging modalities are listed in Table 2.

The limitations of LSFM for studying cardiovascular development and regeneration are the false-positive signals that result from the presence of pigment or residual hemoglobin in the ventricle. Numerous wavelengths of excitation and fluorophores with narrow emission spectra are required for distinguishing overlapping spectra artifacts from fluorescently labeled tissues. Other limitations that degrade image quality include photon absorption, scattering, and out-of-focus light, prone to the presence of stripes or shadow artifacts. Thus, optimization of optical clearing techniques (131–133) is critical to minimize the reduction or loss in fluorescence following prolonged optical clearing required for rodent models. Overall, we believe that LSFM will be transformative, as this multifunctional framework has potential to combine with new advances in optical imaging to provide great value for fundamental and translational research.

## Author contributions

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