Rapid 3D Phenotyping of Cardiovascular Development in Mouse Embryos by Micro-CT With Iodine Staining

Karl Degenhardt, MD, PhD; Alexander C. Wright, PhD; Debra Horng, BSc; Arun Padmanabhan, BA; Jonathan A. Epstein, MD

Background—Microcomputed tomography (micro-CT) has been used extensively in research to generate high-resolution 3D images of calcified tissues in small animals nondestructively. It has been especially useful for the characterization of skeletal mutations but limited in its utility for the analysis of soft tissue such as the cardiovascular system. Visualization of the cardiovascular system has been largely restricted to structures that can be filled with radiopaque intravascular contrast agents in adult animals. Recent ex vivo studies using osmium tetroxide, iodinated contrast agents, inorganic iodine, and phosphotungstic acid have demonstrated the ability to stain soft tissues differentially, allowing for high intertissue contrast in micro-CT images. In the present study, we demonstrate the application of this technology for visualization of cardiovascular structures in developing mouse embryos using Lugol solution (aqueous potassium iodide plus iodine).

Methods and Results—We show the optimization of this method to obtain ex vivo micro-CT images of embryonic and neonatal mice with excellent soft-tissue contrast. We demonstrate the utility of this method to visualize key structures during cardiovascular development at various stages of embryogenesis. Our method benefits from the ease of sample preparation, low toxicity, and low cost. Furthermore, we show how multiple cardiac defects can be demonstrated by micro-CT in a single specimen with a known genetic lesion. Indeed, a previously undescribed cardiac venous abnormality is revealed in a PlexinD1 mutant mouse.

Conclusions—Micro-CT of iodine-stained tissue is a valuable technique for the characterization of cardiovascular development and defects in mouse models of congenital heart disease. (Circ Cardiovasc Imaging. 2010;3:314-322.)

Key Words: micro-CT ▪ iodine ▪ mouse ▪ development ▪ PlexinD1 ▪ congenital heart disease

The ability to genetically manipulate the mouse has resulted in a powerful model system for the investigation of many disease processes. In particular, genetic studies in the mouse have enhanced our understanding of embryonic development, and by extension, of congenital defects. In humans, cardiac defects are the most common serious anomalies among live births with an estimated frequency of 0.6%.1 Cardiac development, and by extension, of congenital defects. In humans, cardiac defects are the most common serious anomalies among live births with an estimated frequency of 0.6%.1 Numerous mouse models of congenital heart disease have been generated and characterized, adding greater insight into the molecular and cellular origins of these defects.2 In addition, current research in the area of targeted gene deletions holds great promise to further elucidate mechanisms of cardiac development.

Although structurally similar to the human, the significantly reduced size of the murine cardiovascular system presents a number of technical challenges when attempting to stage anatomic features such as vascular structures. Identification and characterization of the phenotype of cardiovascular defects in mice traditionally has relied on histological analysis of sectioned specimens. Histology, however, is limited to fixed 2D views from which critical information may be lost, and it is prone to artifacts associated with sample fixation and preparation. These limitations make the visualization of features such as ventricular septal defects or subtle vascular abnormalities challenging. Three-dimensional reconstruction of photomicrographs is possible but is highly labor-intensive3,4 and prone to many of the same artifacts. Episcopic fluorescence image capture, on the other hand, is a promising new histological technique that can generate a 3D data set from sectioned material.5,6 Nevertheless, nondestructive imaging modalities, such as high-frequency ultrasound,7,8 micro-MRI,9-11 and microcomputed tomography (micro-

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CT),12,13 are available for assessing cardiovascular structural abnormalities in mice. Micro-CT has been used widely for morphological phenotyping of calcified tissues, primarily bone in small animals ex vivo, providing true 3D visualization of microstructure at isotropic resolutions as high as 5 to 10 μm.14–16 The great advantages of micro-CT are the nondestructive nature of the imaging, as compared with histological techniques, and the relative speed of data acquisition, as compared with micro-MRI techniques.17,18 However, micro-CT has been limited to the imaging of calcified or otherwise radiopaque tissues, as soft tissues provide little relative contrast in the images. Although it has been possible to visualize microvasculature with micro-CT by postmortem perfusion of a radiopaque fluid,19–21 contrast between different soft tissues is generally available because of the similarity of their x-ray attenuation. Recently, however, the possibility of obtaining high relative tissue contrast with micro-CT was demonstrated by first soaking the tissue in a solution containing an element of high atomic number, for example, osmium, in an aqueous solution of osmium tetroxide.12,22–24 In addition, commercially available iodine-based contrast agents used in clinical CT imaging have been shown to accomplish similar results,25 allowing for the quantification of tissue properties such as fixed charge density.26 Furthermore, inorganic iodine and phosphotungstic acid recently have been shown to provide adequate staining for the visualization of the developing chick embryo by micro-CT.13

We have independently developed a similar process for treating embryonic tissue samples before micro-CT scanning. In the present study, we demonstrate the ability to generate contrast for micro-CT imaging of soft tissues using a radiopaque contrast agent: aqueous potassium triiodide (K+ + I3−), also known as Lugol solution, and we show optimization of this technique for maximum resolution of soft tissues in embryonic and newborn mice with minimal artifact. We provide a detailed description of our protocol and an atlas of mouse development as visualized by micro-CT. Furthermore, we demonstrate that this technique is suitable for analysis of cardiovascular structures at various embryonic stages during mouse development. Finally, using PlexinD1 mutant mice as an example, we show that multiple cardiovascular defects can be illustrated in a single specimen, including a previously unappreciated phenotype. Such a capability will be of great utility for the nondestructive visualization and characterization of mouse mutants that serve as models of congenital heart disease.

**Methods**

**Iodine Staining Protocols**

Mouse embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), after which they were treated with Lugol solution as a micro-CT contrast agent. Samples were soaked in various preparations of Lugol solution for 24 to 72 hours. Lugol solution, or Lugol iodine,27 was prepared from 10 g KI plus 5 g I2 in 100 mL H2O. The potassium iodide in water dissociates, and on adding elemental iodine the formation of the triiodide ion is favorable, enhancing the aqueous solubility of iodine. The traditional (100%) Lugol solution thus has an iodine concentration of 986 mmol/L and has an osmolarity of 1204 mOsm/L, including all ionic species. However, normal saline (0.9% NaCl), which is physiologically isotonic, has an osmolarity of 308 mOsm/L. Thus, a 25% Lugol solution, made by diluting 100% Lugol with deionized H2O, will be approximately isotonic to biological tissues. Because

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**Figure 1.** Aqueous iodine stains soft tissue differentially in a concentration-dependent manner. Four neonatal mice were treated with a mixture of PBS and 100% Lugol solution in different ratios before scanning by micro-CT. Representative sagittal multiplanar reformatted images are shown. Windowing for each image was separately optimized to maximize anatomic visibility. A, Only calcified bony structures, such as vertebrae (arrows), are visible in a specimen treated in PBS alone. B, The 9:1 mixture of PBS and Lugol solution brings out many soft tissue structures; however, most tissues stain similarly, and overall signal-to-noise ratio is poor. Moreover, the central part of the specimen remains unstained. C and D, The 1:1 mixture of PBS and Lugol solution (C) and 100% Lugol solution (D) both impart differential attenuation to soft tissues, with brown fat (BF), liver, and blood staining most intensely. However, there is some tissue distortion. Note the space between the diaphragm and heart (asterisk).
Hypertonic solutions have the tendency to cause tissue shrinkage due to the extraction of water from the tissue, we tested different treatments of the specimens using various mixtures of Lugol solution and different durations of exposure of the specimens. The different treatments were qualitatively and quantitatively evaluated for degree of tissue shrinkage, soft tissue image contrast and signal-to-noise ratio, and uniformity of iodine penetration.

To quantify the apparent concentration of iodine in the tissues of each embryo, we first performed micro-CT scans on a set of calibration phantoms consisting of plastic tubes, each filled with a different serial dilution made with deionized water from the same preparation of 100% Lugol solution. Each tube was embedded in paraffin wax in a manner identical to that used for preparing embryos for micro-CT scanning. The mean reconstructed image intensity in Hounsfield units (HU) within a region of interest drawn on each image was plotted versus its corresponding iodine concentration. A linear fit to the data points yielded the so-called “K factor” as the slope of the fitted line. The equation of the fitted line thus provides a means to transform image intensity (HU) into apparent iodine concentration (mM) (Supplemental Figure 1). This provides only the apparent iodine concentration because no correction was made for the spectral filtering of the x-ray beam, for example, beam hardening, caused by the sample.

Another means to evaluate the contrast agent is by measuring its effective diffusion coefficient $D$. To do this, a 1D diffusion equation can be applied approximately to the sample preparation technique, where contrast agent concentration at position $x$ in the tissue and at time $t$ is given by

$$C(x,t) = C_0 \text{erfc} \left( \frac{x}{\sqrt{4Dt}} \right)$$

where $\text{erfc}$ is the complementary error function. Here, the assumption is made that a semi-infinite half-space of contrast agent solution...
at concentration $C_0$ is adjacent to a homogeneous semi-infinite half-space of tissue, in which case only diffusion occurring along the $x$-axis, which is orthogonal to the interface, is relevant. By measuring the concentration of stain at a point $x$ in the tissue at various times $t$, we can fit this equation to the data points to obtain an estimate of the effective diffusion coefficient $D$. Equation 1 was fitted to data points obtained from each mouse neonate soaked in a different preparation of Lugol solution: isotonic 25% and 12.5% solutions and a saturated 25% solution. Lugol solution (100%) was diluted 1:4 in deionized water, giving it a calculated osmolarity of 308 mOsm/L (isotonic, 25%). This solution was in turn diluted 1:1 in isotonic PBS (isotonic, 12.5%). Because elemental iodine ($I_2$) is relatively insoluble in water unless complexed with $I^-/HIO_2$, addition of $I_2$ crystals theoretically should not alter the osmolarity. We therefore also tested 25% Lugol solution saturated with $I_2$ (saturated, 25%). Specimens stained with each of these 3 solutions were imaged on 3 consecutive days after 24, 48, and 72 hours of treatment. A small, circular region of interest was drawn in the brain stem on a central sagittal slice of the micro-CT data acquired from each mouse at each time point to ensure a repeatable location in the brain stem. The region of interest had a diameter of 150 μm and its center (x-position) and was about 600 μm from the skin surface. The brain stem was chosen for its uniform intensity and relatively superficial location to approximate the assumptions behind Equation 1.

**Micro-CT Scanning**

The micro-CT scanner used in this study is an eXplore Locus SP specimen scanner (GE Healthcare, London, Ontario, Canada), and has been described previously. Resolution as high as $8 \times 8 \times 8$ μm$^3$ can be achieved in reconstructed images of objects approximately 1 cm in diameter or less, whereas objects up to 3 cm in diameter can be accommodated with some loss of resolution. Raw projection data were acquired using the following parameters: 80 kVp, 80 μA, 400 views, 0.5° increment, short scan (Parker) method, 1.7 s exposure time, 2x2 pixel binning, 16 μm isotropic resolution, 8 frame averages, and 2-hour scan time. Because signal-to-noise ratio and achievable resolution are inversely related, the signal-to-noise ratio was increased by averaging together 8 data acquisitions. The projection data were corrected for distortion and detector anomalies and then reconstructed by Feldkamp cone-beam filtered back-projection, resulting in a 3D image volume having isotropic resolution. Typical reconstructed voxel size was $16 \times 16 \times 16$ μm$^3$, although some images were reconstructed at $32 \times 32 \times 32$ μm$^3$. Intensity values in the reconstructed images conformed to the Hounsfield scale, with air set to $-1000$ HU and water set to 0 HU.

We embedded each embryo in paraffin wax in a small plastic test tube (inner/outer diameter, 10/12 mm) that fitted securely within the acrylic plastic sample tube supplied by the manufacturer of the micro-CT scanner. Initial scanning experiments were performed without wax by wrapping the iodine-stained specimen in thin plastic wrap before inserting it into the scanner sample tube. The plastic wrap prevented iodine from contaminating the walls of the scanner sample tube; however, with this configuration there was the possibility of slight sample movement during the 2-hour scan, leading to image blurring. Therefore, instead of plastic wrap we used paraffin embedding of the sample in a smaller-diameter tube that could be removed from the scanner sample tube. The latter method worked better because it effectively immobilized the sample during the scan and avoided iodine contamination of the scanner sample tube.

**Figure 3.** Visualization of mouse development by micro-CT. Three viewing modes are used to generate micro-CT images of mouse embryos at stages E10.5, E11.5, E13.5, E15.5, and E17.5. Also shown is a neonate at postnatal day 0 (skin removed). A through F, Volume rendering (VR) windowed to show external features allows for accurate staging. G through L, Maximum intensity projection (MIP) images show that blood is most intensely stained, allowing delineation of many vascular structures. M through R, Representative sagittal sections generated by multiplanar reformatted (MPR) show differential staining of soft tissues in all stages except E10.5. By E11.5, even structures such as somites (arrows) can be delineated. Scale bars, 200 μm (E10.5), 800 μm (E11.5 through E13.5), and 1 mm (E15.5 through postnatal day 0).
Results

Iodine Staining of Soft Tissues Produces Differential X-Ray Attenuation on Micro-CT

To assess the ability of iodine to stain and penetrate tissue, fixed neonatal mice first were skinned and placed in mixtures of PBS and 100% Lugol solution at various ratios for approximately 72 hours before imaging by micro-CT. Using pure PBS, only bony structures were clearly visualized (Figure 1A). However, with addition of a relatively small amount of Lugol solution, differential staining of soft tissues occurred as evidenced by variable attenuation (Figure 1B). Increased proportions of Lugol solution showed better differential staining, with liver and brown fat staining more intensely (Figure 1C and 1D). Higher ratios of Lugol also resulted in more uniform penetration; however, a greater degree of tissue shrinkage was noted (compare Figure 1B and 1C). These solutions contained PBS, which is isotonic, plus Lugol solution, which is hypertonic. The mixtures were therefore hypertonic, which we postulate may have contributed to tissue shrinkage and distortion.

In an effort to optimize the contrast observed by micro-CT, we next stained fixed neonatal mice in solutions that were physiologically isotonic but of different iodine concentrations for various periods of time (24, 48, and 72 hours) before micro-CT imaging. With 12.5% Lugol, uniform penetration of the specimen was not achieved, even after 72 hours of staining (Figure 2G through 2I). In contrast, saturated 25% Lugol showed uniform penetration by 48 hours but also began to show tissue distortion (shrinkage) by that time (Figure 2A through 2C). Staining with 25% Lugol for 48 hours resulted in complete and uniform tissue penetration with minimal shrinkage (Figure 2E). Thus, for subsequent experiments, we stained with 25% Lugol solution for 48 hours before scanning. It should be noted that despite the use of isotonic solutions, some degree of tissue distortion always occurred under conditions that gave adequate differential staining.

All 3 isotonic solutions showed evidence of progressive diffusion as staining of the central portion of the specimen increased over time, with the time course varying by concentration (Figure 2). When the apparent iodine concentration in tissue (see Supplemental Figure 1) was plotted versus time, the 1D diffusion equation (Equation 1) could provide a reasonably good approximation of the data (Figure 2J). The best fit of this equation gave an effective diffusion coefficient $D$ for each curve (Supplemental Table 1). The value of $D$ ranged from $3.95 \times 10^{-6} \text{mm}^2/\text{s}$ to $5.99 \times 10^{-6} \text{mm}^2/\text{s}$, approximately 250 times smaller than that for pure water. This is consistent with the observed delay in staining of the central portion of the embryo (Figure 2). The process of contrast optimization thus used both qualitative and quantitative analyses of the various staining protocols tested here.

Iodine Staining Enables Visualization of Embryonic Mouse Development by Micro-CT

Micro-CT of iodine-stained mouse embryos at multiple developmental time points was performed using the staining protocol as optimized. Representative volume rendered, maximum intensity projection, and multiplanar reformatted sagittal views are shown (Figure 3 and Supplemental Figure 2). Differential staining of embryonic tissues was seen in specimens as young as embryonic day 11.5 after conception (E11.5). Embryos at E10.5 stained relatively weakly and displayed mostly uniform image intensity. Interestingly, blood stained most intensely at E11.5 (see Figure 3G through 3L, maximum intensity projection images) and throughout development. Less intensely staining structures, such as somites, also could be distinguished easily from surrounding tissue (Figure 3N). In most specimens analyzed, the cardiac ventricles were partially or completely emptied of blood, leaving the chambers dark. Thus, trabeculation could be appreciated in many images. Because of space constraints, only a selection of sagittal views is shown. Complete image stacks can be viewed in Supplemental Movies 1 to 6.
Developing cardiovascular structures could be clearly appreciated by micro-CT. For example, at E11.5, both the truncus arteriosus and the ventricles are undergoing septation. The conotruncal region can be appreciated at early stages of septation (Figure 4A); the presumptive right and left ventricles have a partially formed septum; however, the bulboventricular foramen remains (Figure 4A). By E13.5, the aorta and pulmonary artery are divided, whereas the ventricular septum is not yet complete (Figure 4B and 4C). Quantitative analysis of aortic vessel diameters measured from either histological cross-sections or micro-CT data sets of E17.5 embryos yielded comparable results (Supplemental Figure 3).

Micro-CT of Iodine-Stained Blood Allows 3D Angiography

Because iodine stains blood intensely, micro-CT angiograms often could be generated without the need of injected contrast agents. Micro-CT data from a postnatal day 0 pup were used to generate a volume rendered image of vascular structures that illustrates the similarities between human and mouse arterial structures and differences in venous structures (Figure 5). Mice have a left-sided aortic arch, with the same branching pattern as that seen in humans. Unlike humans, mice have bilateral superior vena cavae and a left-sidedazygos vein. In addition, mice have a single pulmonary vein rather than 4 separate veins entering the left atrium.

Multiple Cardiac Defects Can Be Viewed by Micro-CT

Mice that are mutant for PlexinD1 have been shown to have multiple cardiovascular defects, including truncus arteriosus, aberrant origins of the coronary arteries, and abnormal branching of the arch arteries.32 We stained and imaged a PlexinD1 null mutant embryo at E17.5 and compared it with a wild-type embryo to see how each of these defects could be visualized. Multiplanar reformatted was used to generate images at planes that best illustrate each of the defects. Corresponding views were made of the wild-type control (Figure 6A through 6E). Truncus arteriosus was demonstrable along with a ventricular septal defect in an off-axis sagittal view (Figure 6F). A left arch with a retroesophageal right subclavian is best visualized in a cross-sectional view (Figure 6G). In other views, a coronary artery was shown with its origin at the sino-tubular junction rather than in the sinus of Valsalva (Figure 6H). Volume rendered images were used to show the truncus arteriosus and arch branching (Figure 6I and 6J, and Supplemental Movies 7 to 8). Hence, both intracardiac and extracardiac defects can be effectively demonstrated by micro-CT.

In addition to visualizing previously reported cardiovascular defects, we were able to identify a novel abnormal cardiac venous connection in a PlexinD1 mutant. Using multiplanar reformatted, an aberrant structure that appeared along the surface of the right ventricular outflow tract could be traced to the left superior vena cava (Figure 7A and Supplemental Movie 9). The structure ran along the left side of the truncus arteriosus. A similar cross-sectional view in the wild-type control revealed no such structure (Figure 7B). This result suggests that PlexinD1 is necessary not only for arterial patterning but for venous patterning as well.

Discussion

In the present study we have described the ability to image soft tissues by micro-CT using aqueous potassium triiodide (K⁺I⁻⁻), also known as Lugol solution. Through the use of this stain, we have obtained excellent image contrast with micro-CT, allowing clear visualization of the heart and major vascular structures of neonatal and embryonic mice. Without iodine staining, the soft tissues of the mouse are isointense by micro-CT; however, many structures are dramatically revealed after treatment with Lugol solution. Our initial experiments showed that differential staining of soft tissues was greater at higher iodine concentrations. However, these conditions also led to greater tissue distortion, evident by the increased spaces in the thoracic and abdominal cavities. This effect was reduced with the use of isotonic solutions and by
limiting staining to 48 hours, although it appears that some degree of shrinkage was unavoidable under conditions that maximized differential staining. Nevertheless, the tissue distortion did not significantly interfere with qualitative analysis of cardiovascular structures. We note that Metscher evaluated the use of 10% Lugol solution for micro-CT imaging of chick embryos, whereas we have arrived independently at a similar stain recipe (25% Lugol solution) and optimized this protocol for mice. The structural similarity between the murine and human heart, in combination with the ability to manipulate the mouse genome, has made the mouse a widely used tool in cardiovascular research. We conducted the present study with this in mind. The optimized micro-CT imaging protocol used here thus offers a fast, simple, and inexpensive method to characterize phenotypes in mouse models of cardiovascular development.

Shortening of the time needed for staining is limited by the diffusion properties of iodine. We were able to estimate the effective diffusion coefficient $D$ by fitting a 1D diffusion model to data points obtained from image intensities in the brain stems of mouse embryos subjected to different concentrations of iodine solution for different durations. The results indicated a relatively slow diffusion process, probably due to the existence of numerous barriers to iodine diffusion such as cell membranes and internal organ interfaces. The effective diffusion coefficients we estimated were consistent with the observed delay of staining in central portions of the specimens. The 1D model used here (Equation 1) potentially could be improved by using a 3D model having cylindrical geometry or with additional dedicated experiments beyond the scope of the present study.

The strength of any contrast agent lies in its ability to impart differential strength of signal. The images obtained in this study show that liver and brown fat stain strongly, but blood stains the most intensely. There are many gradations of...
lighter staining tissues as well. The brain and spinal cord stain moderately, with some distinction also evident between gray and white matter. Some muscles stain strongly, including the tongue and the myocardium. The inner walls of blood vessels are clearly visible when the lumen is not filled with blood, and tracing the pattern of vessel branching can be easily accomplished. Likewise, when lumens are filled with blood, angiographic images can be generated. Depending on the desired anatomic image, specimen preparation may be modified to either allow blood to drain or not. For analysis of vascular anatomy, contrast also can be generated by postmortem perfusion of a radiopaque liquid (eg, Microfil) consisting of a suspension of an element of high atomic number such as lead, bismuth, or barium that solidifies before scanning. This has enabled micro-CT to become an excellent imaging tool for the characterization of, for example, vascular patterning defects in a mouse model of human Notch signaling deficit.20 However, such studies are limited to adult animals because the immature vasculature of young animals does not tolerate the intravascular pressure needed to inject contrast. This is a particular problem in the study of cardiac development because many cardiac defects result in embryonic or perinatal death, hence the strength of the micro-CT imaging protocol described in the present study for imaging neonatal and embryonic mice.

PlexinD1 mutant mice represent an example of a well-characterized genetic model of congenital heart disease that exhibits perinatal lethality.25 Plexins are cell surface receptors for semaphorin ligands that mediate guidance cues. The expression of PlexinD1 on endothelial cells has been shown to be critical for normal cardiovascular development.32,34 Cardiovascular defects, including persistent truncus arteriosus, arch artery paucity defects, and aberrant coronary arteries, have been described previously.25 In this report, we have shown the ability to thoroughly evaluate these cardiovascular defects, taking advantage of the ability to visualize the 3D data set from a single specimen from multiple angles. This approach is relatively simple and rapid and proved sensitive enough to reveal a previously unappreciated abnormality of venous drainage in PlexinD1 null mice. The association of coronary artery abnormalities with persistent truncus in patients has been long appreciated.35 However, venous abnormalities in these same patients are less well described and may deserve further investigation.

We were able to generate images of embryonic murine cardiovascular tissues with high intersurface contrast, similar to that of MRI yet in a fraction of the time. The primary advantages of micro-CT over MRI and other modalities are that micro-CT can generate images at extremely high resolution (≤16 μm isotropic) of optically opaque samples in relatively short scan times (~2 hours). The results presented demonstrate the strength of micro-CT for visualizing soft tissues of the mouse at different embryonic stages. We have shown how micro-CT can be particularly applicable to the investigation of cardiac development and the analysis of mouse models of congenital heart disease. The technique is simple, fast, widely available, and relatively inexpensive.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Congenital heart disease is the most common serious birth defect in humans. Great strides have been made in our understanding of the molecular origins of cardiac defects through the use of the mouse as a model system. This is primarily because of our ability to create targeted mutations in mice. Microcomputed tomography, when used in conjunction with the staining methods described in the present study, offers a means to better analyze mutant mice to detect subtle cardiovascular abnormalities. This imaging methodology represents a powerful tool for better understanding the implications of genetic lesions that result in heart defects.
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Supplemental Methods

Animals

Analysis of normal development at different time points (E10.5 to P0) was carried out on embryos harvested from timed pregnancies in CD-1 mice (Jackson Laboratory). For each embryonic stage of development, we collected micro-CT data sets for three individual specimens while seven specimens were necessary to evaluate the various staining conditions we outlined in our manuscript for the single postnatal time point we investigated. Staging was confirmed morphologically at the time of dissection and on 3D volume renderings. *PlexinD1* mutant mice have been described previously (1) and a single plexinD1-null embryo and its wild type littermate control were utilized for the corresponding studies.

Image Analysis

The 3D micro-CT image volumes generated at the scanner are 16-bit data files of approximately 300 MB, and these were downsampled to 8-bit data (~150 MB) in order to make them easier to work with in the visualization software. We employed several image analysis tools for qualitative inspection of the image data and for quantitative measurements of image intensity and structural dimensions. These tools include the open source software packages MicroView (GE Healthcare), ImageJ (NIH), and OsiriX (www.osirix-viewer.com). Three visualization methods were used in this study: multi-planar reformatting (MPR), volume rendering (VR), and maximum intensity projection (MIP). Real-time interactive 3D visualization using these methods was enabled by running a 64-bit version of OsiriX on a Mac Pro workstation (Apple Computer, Inc.)
having two 3 GHz Intel Xeon Dual-Core processors, 12 GB RAM, and a high-end graphics card (512 MB VRAM).

The 3D isotropic nature of the micro-CT image data (voxel size \(16\times16\times16 \, \mu m^3\)) permits MPR at arbitrary oblique planes without significant loss of resolution, although a minor amount of smoothing occurs due to necessary interpolation between voxels. MPR thus enables visualization of heart and vascular structures at ideal orientations, permitting qualitative inspection of the internal anatomy and also quantitative thickness measurements of structures of interest. In addition, VR and MIP techniques provide complementary qualitative information. While both are based on ray tracing algorithms, VR makes use of depth cues, shading, and texturing to display a 3D representation of the data, whereas MIP reveals the brightest internal structures. By changing the image display settings, such as the window width and level, different aspects of the data can be emphasized. This is especially true for VR, and the VR images shown in this paper were generated in OsiriX using the following settings: muscle/bones color look up table, logarithmic inverse opacity table, shading coefficients: ambient = 0.15, diffuse = 0.90, specular = 0.30, specular power = 15.0, and various window width/level values.
References

**Supplemental Table 1.** Values of parameters $C_0$ and $D$, obtained by curve fitting

Equation 1 to the time course of iodine diffusion as measured at position $x$ in the brain stem of each mouse in Figure 2. Nominal [I] refers to the presumed iodine concentration in each bath of Lugol’s solution.

<table>
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<th>Lugol’s solution</th>
<th>nominal [I] (mM)</th>
<th>$x$ (µm)</th>
<th>$C_0$ (mM)</th>
<th>$D$ (mm$^2$/s)</th>
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<td>717.1</td>
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</tbody>
</table>
**Supplemental Figure Legends**

**Supplemental Figure 1.** Calibration curve of micro-CT image intensity from serial dilutions of Lugol’s solution shows a highly linear relationship between iodine concentration and X-ray attenuation. Serial dilutions of 100% Lugol’s solution were made using de-ionized water and were scanned as phantoms. Each point corresponds to the mean intensity of a ROI drawn on the reconstructed images of each phantom. Standard deviations of the means were only slightly larger than the size of the plotted symbols. The fitted solid line demonstrates the highly linear behavior ($R^2 = 0.9999$), as expected for X-ray attenuation by iodine. The line has a slope (K factor) of 5.37 HU/mM and an offset of 10.5 HU. This offset is well within the standard deviation of intensity values around the value of zero, which is the intensity of pure water on the Hounsfield scale. Unstained, non-fatty tissues have intensity values very close to that of water. Therefore, to convert images (HU) into maps of apparent iodine concentration (mM), we can apply the following formula to each pixel in the image: \( \text{mM} = (\text{HU} - 10.5)/5.37 \).

**Supplemental Figure 2.** Variations in display settings demonstrate the effectiveness of iodine staining for visualizing multiple anatomic features from a single micro-CT data set. (A) False color VR image of a wild type E17.5 embryo, windowed to show the entire embryo, gives excellent superficial detail. (B, C) Alterations in window settings highlight vasculature and internal organs. (D) MIP image highlights vascular patterning.

**Supplemental Figure 3.** Quantification of the vessel diameters by micro-CT is comparable to measurements taken from histological sections. Cross-sections through the
thoracic aorta result in an oval shape in embryos at E17.5. The vessel diameters were measured along the long- and short-axis of this oval just posterior to the carina as shown in panels (A) and (B) (micro-CT MPR and H&E stained section, respectively). There is no significant difference in the mean diameters as measured by either technique (C) (n=3 specimens for each measurement) (Es, esophagus; Br, bronchus).
Supplemental Movie Legends

Supplemental Movie 1. Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse embryo (stage E10.5), demonstrating very little inter-tissue contrast yet still providing good delineation of cardiovascular structures. The micro-CT data were reconstructed at an isotropic resolution of 16 μm, and are displayed with pixel interpolation performed in OsiriX software.

Supplemental Movie 2. Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse embryo (stage E11.5), demonstrating good inter-tissue contrast, although not as much as that of later stage embryos. The micro-CT data were reconstructed at an isotropic resolution of 16 μm, and are displayed with pixel interpolation performed in OsiriX software.

Supplemental Movie 3. Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse embryo (stage E13.5), demonstrating high inter-tissue contrast. The
micro-CT data were reconstructed at an isotropic resolution of 16 µm, and are displayed with pixel interpolation performed in OsiriX software.

**Supplemental Movie 4.** Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse embryo (stage E15.5), demonstrating high inter-tissue contrast. The micro-CT data were reconstructed at an isotropic resolution of 16 µm, and are displayed with pixel interpolation performed in OsiriX software.

**Supplemental Movie 5.** Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse embryo (stage E17.5), demonstrating high inter-tissue contrast. The micro-CT data were reconstructed at an isotropic resolution of 16 µm, and are displayed with pixel interpolation performed in OsiriX software.

**Supplemental Movie 6.** Iodine staining enables visualization of cardiovascular development and multiple anatomic features in embryonic and neonatal mice by micro-CT. The movie shows sagittal slices through a micro-CT data set of an iodine-stained wild type mouse neonate (stage P0), demonstrating high inter-tissue contrast. The micro-CT data were reconstructed at an isotropic resolution of 32 µm, and are displayed with pixel interpolation performed in OsiriX software.
**Supplemental Movie 7**

Three-dimensional volume rendered images of ventricles and great vessels in a wild type E17.5 mouse embryo reveals normal conotruncal anatomy and arch patterning.

**Supplemental Movie 8**

Three-dimensional volume rendered images of ventricles and great vessels in an E17.5 *PlexinD1* mutant mouse embryo demonstrating a truncal artery and aberrant right subclavian artery arising from the descending aorta.

**Supplemental Movie 9**

Serial axial micro-CT projections through an E17.5 *PlexinD1* mutant mouse embryo reveals an aberrant blood-filled vessel arising from the surface of right ventricular outflow tract and coursing along the left side of the truncal artery as it travels to towards the left superior vena cava.