Published in final edited form as:

Birth Defects Res. 2017 June 01; 109(10): 778–790. doi:10.1002/bdr2.1048.

Phenotyping Cardiac and Structural Birth Defects in Fetal and Newborn Mice

Xiaoqin Liu, Andrew J. Kim, William Reynolds, Yijun Wu, and Cecilia W. Lo*

Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15201

Abstract

Mouse models are invaluable for investigating the developmental etiology and molecular pathogenesis of structural birth defects. While this has been deployed for studying a wide spectrum of birth defects, mice are particularly valuable for modeling congenital heart disease, given they have the same four chamber cardiac anatomy as in humans. We have developed the use of noninvasive fetal ultrasound together with micro-computed tomography (micro-CT) imaging for high throughput phenotyping of mice for congenital heart defects (CHD) and other developmental anomalies. We showed the efficacy of fetal ultrasound and micro-CT imaging for diagnosis of a wide spectrum of CHD. With fetal ultrasound, longitudinal scans can be conducted to track the developmental profile of disease pathogenesis, providing both structural information with two-dimensional (2D) imaging, as well as functional data from hemodynamic assessments with color flow and spectral Doppler imaging. In contrast, with micro-CT imaging, virtual necropsies can be conducted rapidly postmortem for diagnosis of not only CHD, but also other structural birth defects. To validate the CHD diagnosis, we further showed the use of a novel histological technique with episcopic confocal microscopy (ECM) to obtain rapid 3D reconstructions for accurate diagnosis of even the most complex anatomical defect. The latter histological imaging technique when combined with the use of ultrasound and micro-CT imaging provides a powerful combination of imaging modalities that will be invaluable in meeting the accelerating demands for high throughput mouse phenotyping of genetically engineered mice in the coming age of functional genomics.

Keywords

fetal ultrasound imaging; micro-CT; congenital heart disease; structural birth defects; mouse model; forward genetic screen

Introduction

Mouse models have become invaluable resources for studying the cellular and molecular etiology for a variety of human diseases. This development has been greatly facilitated by the production of knockout (KO) mouse models using standard homologous recombination

techniques with embryonic stem cell gene targeting. Thus, with the NIH Knockout Mouse Project (KOMP), KO ES cells for thousands of genes have been systematically created, many of which have been converted into mice and put through a phenotyping pipeline (Austin et al., 2004; Collins et al., 2007). In addition, with the recent development of the highly efficient CRISPR/Cas9 gene targeting techniques, it is now possible to generate KO mice by direct injections of mouse oocytes (Wang et al., 2013; Yang et al., 2014), bypassing the need for ES cell gene targeting. Furthermore, CRISPR technology can be used to generate knockin mutations, allowing direct assessment of specific mutations recovered from patients in mouse models. These reverse genetic approaches have created a plethora of mouse models.

Adding to the boom in mouse model production is the renewed interest in the use of forward genetics with phenotype driven screens to recover mutations that may contribute to a variety of human diseases. This flurry of activity in high throughput screening in mice has been inspired with the recent demonstration that mouse whole exome sequencing is a highly effective strategy to recover mutations from the genome of the mutant mice, alleviating the previous bottleneck in mutation recovery efforts. Thus in the past, mutation recovery would require mapping of the mutation followed by candidate gene sequencing, an expensive and laborious process that often took years. Now high throughput sequencing has changed the landscape for pursuit of forward genetic screens. As a result, the challenge now is the identification of suitable high throughput and sensitive phenotyping methods for screening mice in large scale forward genetic screens.

In this brief review, we will describe several imaging modalities that have proved invaluable in our own large scale mouse forward genetic screen with ENU chemical mutagenesis (Li et al., 2015). This includes the use of noninvasive ultrasound imaging (Liu et al., 2014) for live assessment of fetal cardiovascular function and structural anomalies, as well as micro-CT imaging (Kim et al., 2013) for rapid postmortem virtual necropsies. We have carefully documented the detection sensitivity and specificity of these imaging modalities, using histopathology as the gold standard for the defect diagnosis. The latter entails the use of a novel imaging technique, episcopic confocal microscopy (ECM), which allows rapid 3D reconstruction of serial histological imaging stacks that are well suited for detailed anatomical assessments that are especially critical for the diagnosis of complex congenital heart defect (CHD) phenotypes. Together these three imaging modalities are well suited for high throughput phenotyping. Our focus has been the use of these imaging modalities for cardiovascular phenotyping, but also to provide some examples of their utility for detection and analysis of other structural birth defect phenotypes.

Ultrasound Phenotyping

Ultrasound imaging, being noninvasive, can be deployed as a rapid high throughput method for interrogating fetal development. With ultrasound imaging, longitudinal assessments can be conducted on the same fetuses over a period of days to examine the developmental etiology and progression of disease phenotypes. Ultrasound imaging has been well developed in the clinical setting for assessment of the health and development of human fetuses, allowing the noninvasive *in utero* detection of a wide range of birth defects.

Moreover, ultrasound imaging with fetal echocardiography is an exquisite tool for the sensitive detection of defects in the cardiovascular system, yielding both structural and functional information on the developing fetuses. This has made it possible to detect and diagnose prenatally, CHDs. Currently, two major types of ultrasound systems are commercially available, one platform comprising equipment developed for imaging in the clinical setting, and the other is an ultra-high frequency system developed for research studies with ultra-high resolution suitable for small animal imaging, including ultrasound phenotyping the developing cardiovascular system in fetal mice.

Both ultrasound systems provide three imaging modalities that include 2D imaging, color flow and spectral Doppler imaging, and M-mode imaging. The main difference between the clinical and research ultrasound systems is the nearly ten times higher 2D imaging resolution of the research ultrasound system. This is invaluable for detection of structural heart defects in the small embryonic hearts of fetal mice. The color flow, spectral Doppler, and M-mode imaging, however, perform similarly in both the clinical and research ultrasound systems. The color flow and spectral Doppler imaging provide hemodynamic assessment of flow velocities, as well as the detection of regurgitant flows that together can suggest the presence of structural heart defects. With M-mode imaging, measurement can be obtained of chamber wall thickness and lumen dimensions in systole and diastole, which together can be used to quantitatively assess cardiac contractility, and detect hypertrophy/hyperplasia of the ventricular myocardium.

The phenotyping pipeline we have developed in our recent large scale mutagenesis screen for CHD has entailed a two stage ultrasound screening protocol. First, we conduct ultrasound scans with the clinical ultrasound system, and if abnormal findings are observed, we then pursued additional interrogation with the ultra-high frequency ultrasound system. Typically, the first tier ultrasound scan of a litter of 5–8 mouse fetuses will require ~10–15 minutes, and a similar amount of time or somewhat longer for further diagnosis of the CHD using the ultra-high frequency system.

Clinical Ultrasound

Among the many clinical ultrasound systems available, the Acuson Sequoia has been used most extensively for imaging fetal mice (Spurney et al., 2006; Tobita et al., 2010). This is usually conducted using a 15 MHz high frequency vascular transducer that can provide axial resolution of 300um axial and 500 um lateral resolution. With this increased resolution, it is still challenging to image the very small fetal mouse hearts that are less than 2–3 um in diameter. However, when combined with color flow and spectral Doppler imaging, the relative position of inflow and outflow can be ascertained, and together with the hemodynamic assessments for shunts, outflow velocity increase, or regurgitant flow, it is possible to identify fetuses with structural cardiovascular anomalies. This assessment can be conducted in fetuses from E14.5 to term.

While the detection of CHD is possible with the Acuson, its detection sensitivity is limited as is the specificity, which largely precludes the diagnosis of specific structural heart defects with 2D imaging on clinical ultrasound systems (Table 1). For example, the detection of shunts may indicate valvular abnormalities, but it is not possible to diagnose the specific

CHD (Fig 1. A,I,N,S) (Shen et al., 2005; Yu et al., 2004). Rather, the assessment of cardiovascular anatomy is largely indirect, inferred from the color flow imaging and spectral Doppler assessments. The definitive diagnosis of specific cardiac defects requires follow up analysis of the fetuses using other imaging modalities.

Overall, clinical ultrasound imaging performed with a 15-MHz transducer is well suited for high throughput phenotypic screening of fetal mice to monitor fetal growth, measure heart rates and flow velocities, assess cardiac contractility, and detect valvular regurgitation, pericardial effusion, and fetal hydrops. While the detection of structural heart defects may be indicated, the diagnosis of specific structural heart defects requires follow up analysis with necropsy or virtual necropsy with micro-CT (see below) and histopathology examination.

Ultra-high Frequency Ultrasound

The ultra-high frequency ultrasound system now commonly used for mouse phenotyping is a research grade system by Visualsonics, referred to as the ultrasound biomicroscope (UBM). UBM can provide 30um axial $\times$ 68um lateral resolution and ultrasound transducers are available with different ultrasound frequencies (30–50 MHz), suitable for *in utero* imaging of mouse embryos requiring different penetration depths. As with clinical ultrasound systems, in addition to 2D imaging, it is equipped with color flow and spectral Doppler, and M-mode imaging.

Given the much higher 2D imaging resolution, the UBM is particularly advantageous for imaging small fetal mouse hearts. With the much higher 2D imaging resolution of the Visualsonics Vevo2100, mouse fetuses can be interrogated from much earlier developmental stages, allowing detailed 2D imaging of the cardiovascular system prior to cardiac and outflow tract separation at E12.5. Together with the availability of color flow spectral Doppler imaging for hemodynamic assessments, it is now possible to directly visualize and diagnose specific structural cardiac anomalies, even in mouse embryos at E11.5 or younger (Moran et al., 2013; Tobita et al., 2010).

Using the Vevo2100 UBM, a wide spectrum of CHD can be diagnosed (Table 1), including outflow tract malalignment defects such as double outlet right ventricle (DORV) (Figure 1T–Y), transposition of great arteries (TGA), and persistent truncus arteriosus (PTA)/pulmonary atresia (PA) (Figure 1O–R), a variety of cardiac septation defects, including ventricular septum defect (VSD), atrioventricular septa defects (AVSD) (Figure 1T–Y), left or right heart obstructive lesions comprising pulmonary stenosis, tricuspid stenosis or atresia, aortic stenosis or atresia, mitral valve stenosis or atresia, coarctation, hypoplastic right heart syndrome (Figure 1B–F), and hypoplastic left heart syndrome (Figure 1G–M). Also detected by UBM are coronary artery fistulas and cardiac situs anomalies (dextrocardia, heterotaxy, Figure 2A–C). However, Vevo2100 imaging cannot distinguish between PTA *versus* PA, DORV *versus* overriding aorta, or secundum atrial septal defect *versus* foramen ovale. It is also difficult to assess by UBM aortic arch anomalies.

The efficacy of Vevo2100 in CHD diagnosis was evaluated in our previous large-scale ENU mutations screen (Liu et al., 2014), in which histopathology carried out using episcopic confocal microscopy (ECM) served as the gold standard for structural heart defect diagnosis

(Table 1). This analysis showed consistently high specificity of Vevo2100 in diagnosing a wide variety of CHDs (Table 1). However, sensitivities varied from 30% to 100%, with the lowest associated with aortic anomalies, valvular defects, and coronary fistulas (Table 1). Overall, the study showed excellent diagnostic capability of Vevo2100 UBM for a wide spectrum of CHDs. For septal defects, low sensitivity mainly reflects the failure to detect very small VSDs. For OFT defects, transposition of the great arteries and DORVs are prone to be missed. Accuracy in diagnosis of these defects will improve, as advances are made in further increasing the ultrasound imaging resolution of the UBM.

Ultrasound Phenotyping of Noncardiac Structural Birth Defects

Using the Vevo2100 UBM, various other structures and organs can be visualized besides the heart, including the brain, lung, liver, stomach, kidney, and bladder. Also observable are the craniofacial structures, limbs and digits, vertebral column, and rib cage. Hence using the UBM, it is possible to identify a variety of noncardiac defects, such as cleft palate, kidney cysts, body wall defect encompassing omphalocele or gastroschisis (Figure 2D), polydactyly (Figure 2E), or syndactyly. It has also proved to be a useful tool for *in vivo* determination of left–right patterning defects, such as dextrocardia or dextrogastria, where the heart/stomach are misplaced to the right, rather than the normal left sided position in the body (Figure 2A–C). Ultrasound imaging also can visualize the spinal column and brain, and thus can detect neural tube closure defects, such as exencephaly (Figure 2H), or various brain anomalies such as microcephaly (Figure 2F, G).

Micro-Computed Tomography

Micro-CT is a powerful imaging modality that utilizes x-ray attenuation to acquire high-resolution 3D images, traditionally from mineralized tissue such as bone or teeth. Like ultrasound imaging, micro-CT is non-invasive and allows small animals including fetal mice to be analyzed in a non-destructive and noninvasive manner, thereby preserving integrity of the specimen for further downstream analysis such as histology. One of the previous limitations with micro-CT was the inability to image soft tissue. This is due to the inherent low density of soft tissue, and hence there is insufficient x-ray attenuation, giving poor image contrast (Metscher, 2009a; Metscher, 2009b). However, with recent development and optimization in the use of iodine-based contrast agents for tissue staining, it is now possible to use micro-CT for post-mortem soft tissue imaging, even imaging of intact newborn mouse pups. Thus formalin-fixed mouse embryos/pups stained with iodine by simple immersion in 0.025 N potassium tri-iodide (Lugol) solution can provide excellent contrast, enabling micro-CT scans to be used for performing virtual necropsies (Degenhardt et al., 2010). This can be particularly helpful in the screening and phenotyping of structural birth defects (SBD), including CHD (Savolainen et al., 2009). Indeed recent technological improvements in micro-CT systems can provide high spatial resolution at near cellular level, although imaging at such high resolution provides very small field-of-view and typically requires much longer scan times (Cheng et al., 2011; Schambach et al., 2010).

Experience gained from using micro-CT imaging for phenotyping mice and newborn pups in a large-scale mouse *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen has shown the

efficacy of micro-CT for high throughput postmortem detection of a wide range of CHD and other structural birth defects. Micro-CT scans were typically conducted at 15–45 μm resolution with iodine stained fetal mice or newborn pups. A typical scan takes approximately 1 hour, during which four to eight fixed embryos (embryonic day 12–16) or 12 fixed newborn mouse pups can be scanned at a resolution of 15 μm or 45 μm , respectively. The imaging data collected in this manner can provide remarkably accurate visualization of fetal and newborn mouse anatomy (Kim et al., 2013; Li et al., 2015). Notably, most of the major CHD observed clinically can be detected by micro-CT with high accuracy, including outflow tract defects, as well as atrioventricular and ventricular septal defects, ventricular hypertrophy or hypoplasia, aortic arch anomalies, and even coronary fistulas (Figure 2, Table 2). High specificity (>99%) and high sensitivity (>90%) was observed for all, but VSD (specificity of 91%; sensitivity of 84.9%), as small VSDs were often difficult to distinguish. Interestingly, aortic arch anomalies which were difficult to discern by ultrasound imaging (detection sensitivity of 31%; Table 2), showed high detection sensitivity (94.4%) and specificity (100%) (Table 3).

Since with micro-CT imaging every major organ can be visualized, visceral organ situs abnormalities also can be detected (Figure 2, Table 2). Some examples of other SBDs that can be detected are shown in Figure 3, including diaphragmatic hernias, lung cysts, and lung lobation abnormalities, to renal abnormalities including cystic/dysplastic kidneys and hydronephrosis. Also readily detected are enlarged or mega bladder, as well as gastrointestinal abnormalities, including megacolon, and gut malrotation. Also easily observed are craniofacial and head defects, including eye defects, micrognathia, cleft palate, holoprosencephaly, hydrocephaly, Chiari malformation, microcephaly, and other head/brain defects. With the ability to simultaneously image 8–12 fetuses/pups per scan, mutant mice can be readily identified from an entire litter with just a single scan, greatly accelerating the diagnosis of CHD and other structural birth defects.

Despite significant advancements in the use of micro-CT to study birth defects, some technical challenges remain. In particular, tissue shrinkage using iodine-based contrast agents remains an issue, particularly in well-hydrated tissues (e.g., brain and lungs), and further refinements to the staining protocol are needed to minimize morphological distortion (Wong et al., 2012). Altering the concentration of contrast agents, together with shortening the duration of contrast agent exposure, may help mitigate this issue. This will need to be experimentally tested to determine the optimal conditions for tissue staining, which may vary depending on whether a whole animal or isolated organ or tissue is being processed. Other strategies include the use of alternative contrast agents based on other high-density atoms (e.g., osmium, uranium, zinc, or tungsten) or combinatorial approaches with hydrogels (Hiraiwa et al., 2013; Liu et al., 2013; Wong et al., 2013). With the expanding use of micro-CT as a diagnostic and screening tool, it is likely that the development of new protocols will help further improve the reproducibility and reliability of micro-CT imaging for anatomical assessments (Solomon et al., 2016).

3D Histopathology Using Episcopic Confocal Microscopy

Histopathology is the ultimate gold standard for validating the diagnosis of CHD or other structural birth defects made using ultrasound or micro-CT imaging (Savolainen et al., 2009). This is carried out using a novel histological technique referred to as episcopic confocal microscopy (ECM). With ECM imaging, the paraffin embedded specimen is sectioned using a sliding microtome with a fixed photoposition, and the block face is serially imaged after each cut using a laser scanning confocal microscope mounted over the microtome (Figure 6). In this manner, the 2D serial image stack collected of the specimen is in registration, allowing rapid 3D reconstructions to be generated, and it is also possible to digitally reslice the serial 2D image stack in any imaging plane. In this manner, it is possible to make a complete assessment of any structural cardiac anomaly, regardless of the embedding plane of the specimen. This ECM histopathology has all the conveniences of micro-CT or micro-MRI data, but with the much higher resolution achieved by histology.

This ECM technique provides a highly efficient method for CHD diagnosis, as well as the diagnosis of other birth defects. Given its exquisite resolution, it is possible to assess for brain abnormalities, including the detection of cerebellar hypoplasia, dysplasia of the hippocampus, holoprosencephaly (Figure 7), as well as defects involving the corpus callosum, olfactory bulb, and other structures of the brain. Given the ease for digital resectioning of the images, and in making 3D volumes, it is possible to assess for cleft palate and other craniofacial anomalies, as well as other defects involving the pharynx and the airway. It is because of this ability to examine the ECM data in any imaging plane, that complex anatomical defects can be deconvoluted, not only for complex CHD as shown above (Figures 1,2), but also for other complex structural birth defects, such as tracheoesophageal fistulas or anomalies, such as cloacal septation defect, resulting in abnormal connection between the intestine and bladder (Figure 7A,B). Thus, ECM imaging, when combined with functional assessments afforded by ultrasound imaging and structural assessments by micro-CT imaging, can provide an imaging tool box that can facilitate deep mouse phenotyping. This will be invaluable for analyzing the flood of mutant mouse models expected to emerge in the near future, with the wide implementation of CRISPR gene targeting.

Summary

The combined use of ultrasound and micro-CT imaging will be invaluable for meeting the accelerating demands for high throughput mouse phenotyping of genetically engineered mice in the coming age of functional genomics. Our studies showed ultra-high frequency can be used to diagnose complex CHD in fetal mice. With ultrasound, insights gained from the 2D imaging and color/spectral Doppler analyses can help delineate alterations in structure and function for the diagnosis of CHD. Ultrasound imaging also can detect other structural birth defects, such as limb and craniofacial/brain abnormalities. In contrast, micro-CT imaging allows for rapid postmortem virtual necropsies and identification of anatomical alterations for the diagnosis of CHD and other birth defects. The CHD diagnosis made by these imaging modalities are complemented by the use of ECM imaging for high resolution 3D histological reconstructions that serves as the gold standard for CHD and structural birth

defect diagnosis. Together these imaging modalities provide a powerful tool kit for high throughput assessment of CHD and other structural birth defects in fetal/neonatal mice.

Acknowledgments

Supported by NIH grants U01-HL098180, R01-HL132024, R01-GM104412, S10-OD010340 to C.W.L.

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Figure 1. Ultrasound diagnosis of a variety of congenital heart defects

(A–F). Hypoplastic right heart syndrome. Acuson Doppler imaging detected an E17.5 fetus with increased velocity in the inflow tract (A, asterisks). Vevo2100 revealed a hypoplastic right ventricle (B and C) with hypoplastic pulmonary artery (PA). Necropsy (D) confirmed hypoplastic PA, with ECM imaging (E,F) showing a hypoplastic right ventricle, hypoplastic tricuspid valve (TV) compared with the mitral valve (MV; arrowheads in E), and hypoplastic PA (arrowhead in F).

(G–M). Hypoplastic left heart syndrome. Vevo2100 imaging of E16.5 fetus in sagittal view (G) showed reversed aortic blood flow during diastole (H) and confirmed by spectral Doppler (I). Short-axis view (J) showed hypoplastic left ventricle. Necropsy of stillborn pup (K) revealed hypoplastic aorta (Ao) and small LV. ECM histology (L,M) revealed small mitral valve orifice, small LV, and hypoplastic aorta, confirming HLHS. DA, ductus artery; DAo, descending aorta

(N–R). Persistent truncus arteriosus. Acuson spectral Doppler showed an E17.5 fetus with outflow tract (OFT) regurgitation (arrowhead in N; OFT shown by asterisks; forward flow). Vevo2100 revealed a single OFT overriding the ventricular septum with a ventricular septal defect (VSD; arrowhead in O). This was associated with OFT regurgitation, indicated by red forward and blue reverse blood flow (O and O'), further confirmed with necropsy (P) and

ECM analysis (Q,R). Also observed is ventricular noncompaction (Q,R) and muscular VSDs (arrowheads in R).

(S–Y). Double outlet right ventricle with hypoplastic pulmonary artery and atrioventricular septal defect. Acuson imaging detected an E16.5 fetus with outflow tract regurgitation (OFT R in S). Vevo2100 imaging (T,T') revealed a RV–LV shunt, indicating a ventricular septal defect (VSD; arrowheads in T). Vevo2100 imaging in the transverse view (U,U') showed inflow tract forward (U, arrow) and regurgitant (U', arrow) flows, whereas the sagittal view (V) showed the aorta (Ao) mostly connects to the RV with an adjacent very hypoplastic pulmonary artery (arrowhead, PA) and a VSD. Necropsy (W) and ECM imaging (X,Y) confirmed a hypoplastic PA (V,Y) with large VSD (Y, arrowhead). A common atrioventricular valve (CV in X) was observed in the transverse view.

Scale bar, 1 mm. A, anterior; Cd, caudal; Cr, cranial; L, left; P, posterior; R, right; CT, common trunk, CVF, common atrioventricular valve forward flow; CVR, common atrioventricular valve reverse flow. From Liu et al., 2014.

Figure 2. Diagnosis of a variety of noncardiac defects

(A–C), **Ultrasound diagnosis of heterotaxy.** Vevo2100 imaging showed heterotaxy with heart apex on left (A, arrowhead) and stomach to right (S in B), which was confirmed by necropsy (C). Panel (D) showed a fetus with body wall closure defect with an omphalocele (arrowhead). Panel (E) shows ultrasound detection of fetus with polydactyly (asterisks). In panel (G), ultrasound imaging showed a fetus with microcephaly with no lower jaw (arrowhead) and beak-like face as compared to normal fetus (F). Fetal ultrasound imaging also can detect hydrops and exencephaly with brain tissue protruding from the cranium (H). Scale bar, 1 mm. A, anterior; L, left; P, posterior, R, right. Cd, caudal; Cr, cranial. S, the stomach.

Figure 3. Micro-CT detection of outflow tract defects

Micro-CT in the coronal viewing plane revealed outflow tract mal-alignment defects, including double outlet right ventricle (DORV; F), transposition of the great arteries (TGA; G), and persistent truncus arteriosus (PTA; H). Necropsy and EFIC images of each heart is shown in (A–D) and (E–L). Scale bars=1 mm (A–H), 0.5 mm (I–L). Ao indicates aorta; LA, left atrium, LV, left ventricle; PA, pulmonary artery; RA, right atrium, and RV, right ventricle. Reprinted from Kim *et al.* Microcomputed tomography provides high accuracy congenital heart disease diagnosis in neonatal and fetal mice. *Circulation: Cardiovascular Imaging*. 2013;6:551–559.

Figure 4. Micro-CT detection of visceral organ situs defects

Necropsy (A–C) and corresponding micro-CT images (D–I) of newborn mice with normal placement of visceral organs (A, D, and G), situs inversus totalis with complete mirror reversal of organ situs (B, E, and H), and heterotaxy with left-right randomized organ situs (C, F, and I). The mutant pup exhibiting situs inversus possesses a right-sided heart (dextrocardia), left-sided inferior vena cava, inversion of lung and liver lobes, and a right-sided stomach (A, D, and G), whereas the mutant pup exhibiting heterotaxy showed dextrocardia, duplicated inferior vena cavae, left-pulmonary isomerism, normal liver lobation, and a left-sided stomach (C, F, and I). Arrows denote the direction to which the heart apex is pointing; asterisks indicate mirrored organ positioning. Scale bars=2.5 mm. Stm = stomach. Reprinted from Kim *et al.* Microcomputed tomography provides high accuracy congenital heart disease diagnosis in neonatal and fetal mice. *Circulation: Cardiovascular Imaging*. 2013;6:551–559.

Figure 5. Micro-CT detection of other structural birth defects

Diaphragmatic herniation can be detected as illustrated by necropsy (A) and micro-CT (B) in the coronal viewing plane. Note the location of the diaphragm. A liver lobe and a portion of the small intestine are visible above the diaphragm and displace lung lobes. Right pulmonary isomerism, characterized by a mirror duplication of each lobe of the right lung (asterisks) is shown by necropsy (C) and micro-CT (D). Likewise, left pulmonary isomerism is illustrated by necropsy (E) and micro-CT (F) and is shown by the presence of a large, duplicated left lobe (asterisks). Hydronephrosis, as indicated by distended renal pelvis (asterisks), affects the right kidney and can be seen by necropsy (G, H), histology via H&E staining (I), and micro-CT (J). Note that the ureter (#) continuous to the renal pelvis is also distended (I, J). Cystic kidneys can also be detected, as illustrated by necropsy (K, L), histology (M, N), and micro-CT (O). Arrows highlight notably large cysts throughout both kidneys.

Scale Bars = 1mm (A–H, J–L, O); 500µm (I, M, N). Diaph. = diaphragm; Ht = heart; L = lung lobe; LvL = liver lobe; Panc. = pancreas; Sm. Int. = small intestine; Stm = stomach.

Figure 6. Episcopic confocal microscopy set up for serial 2D and 3D histopathology

The episcopic confocal microscopy technique utilizes a Leica SM2500 heavy-duty sliding microtome and Lecia True Confocal Scanner (TCS) Large Scale Imaging (LSI) microscopy system equipped with 488, 532, 635 nm 10 mW lasers. This system is mounted on a TMC Active Vibration Isolation Table (pneumatic).

Figure 7. Diagnosis of structural birth defects by episcopic confocal microscopy
 (A,B) ECM imaging of a wildtype (A) vs. mutant (B) newborn mouse revealed abnormal cloacal septation defect in the mutant leading to abnormal communication between the intestine and bladder. From Cui et al., 2014. (C–F) ECM imaging revealed brain abnormalities in mutant (D,F,H) vs. control (C,E,G) newborn mice. Shown are the findings of severe alobar holoprosencephaly (D), cerebellar dysplasia (F), and hippocampal abnormalities (H).

Table 1

Efficiency of Cardiovascular Phenotyping with Acuson vs. Vevo2100

Ultrasound Diagnosis	Acuson*	Vevo2100*	p-value [†]
Abnormal Echocardiography Findings n=(1457)			
Cardiac Structural Defects (n=352; 24%)			
Septal Defects	17 (4.8%)	241 (68.5%)	<0.01
VSD	15 (4.3%)	176 (50%)	<0.01
AVSD	2 (0.6%)	65 (18.5%)	<0.01
Outflow Tract Defects	12 (3.4%)	127 (36.1%)	<0.01
DORV/Overriding Aorta	9 (2.6%)	75 (21.3%)	<0.01
-With VSD	9 (2.6%)	57 (16.2%)	<0.01
-With AVSD	0 (0%)	18 (5.1%)	<0.01
-With pulmonary stenosis	0 (0%)	17 (4.8%)	<0.01
PTA/PA	3 (0.9%)	38 (10.8%)	<0.01
-With VSD	3 (0.9%)	25 (7.1%)	<0.01
-With AVSD	0 (0%)	13 (3.7%)	<0.01
TGA	0 (0%)	14 (4.0%)	<0.01
-With VSD	0 (0%)	13 (3.7%)	<0.01
-With AVSD	0 (0%)	1 (0.3%)	1.000
-With pulmonary stenosis	0 (0%)	1 (0.3%)	1.000
Left Heart Obstruction Lesions	7 (2.0%)	23 (6.5%)	<0.01
Mitral Atresia/Stenosis	0 (0%)	8 (2.3%)	<0.05
Aortic Atresia/Stenosis/Coarctation	7 (2.0%)	15 (4.3%)	0.087
Hypoplastic Left Heart Syndrome	0 (0%)	4 (1.1%)	0.133
Right Heart Obstruction Lesions	10 (2.8%)	50 (12.2%)	<0.01
Tricuspid Atresia/Hypoplasia	0 (0%)	15 (4.3%)	<0.01
Pulmonary Stenosis	10 (2.8%)	35 (9.9%)	<0.01
Hypoplastic Right Heart Syndrome	0 (0%)	11 (3.1%)	<0.01
Coronary Artery Fistula	4 (1.1%)	8 (2.3%)	0.247
Cardiac Situs Anomalies	0 (0%)	40 (11.4%)	<0.01
Dextrocardia	0 (0%)	34 (9.7%)	<0.01
Mesocardia	0 (0%)	6 (1.7%)	<0.05
Non-Specific Abnormal Findings (n=1105)			
Total Arrhythmia	262	262	1.000
Without Structural Defects	103	103	1.000
Total Hydrops/Pericardial Effusion	598	635	0.165
Without Structural Defects	324	364	0.08
Total Hemodynamic Perturbations[§]	1030	1100	0.190
Mild Without Structural Defects	670	678	0.766

* Values in parentheses represent percentage of affected fetuses amongst 352 fetuses scanned by Acuson and UBM and identified with CHD.

[†] p values based Chi-square analysis.

[§]Hemodynamic perturbations including inflow and OFT increased velocity and regurgitation.

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Table 2

Accuracy of Vevo2100 Ultrasound Diagnosis of Congenital Heart Disease*

CHD diagnosis	True positive diagnosis	True negative diagnosis	False positive diagnosis	False negative diagnosis	Sensitivity	Specificity	Accuracy
Septal Defects [‡]	156	292	7	69	69.3%	97.7%	85.5%
Outflow Tract Defects [‡]	105	376	18	25	80.8%	95.4%	91.8%
HLHS [§]	4	520	0	0	100%	100%	100%
HRHS	7	513	2	2	77.8%	99.6%	99.2%
MS/MA [#]	6	514	2	2	75%	99.6%	99.2%
AS/AA/COA ^{**}	9	489	6	20	31%	98.8%	95.0%
Tricuspid Hypoplasia/Atresia	6	510	4	4	60%	99.2%	98.5%
Pulmonary Stenosis	9	499	1	15	37.5%	99.8%	96.9%
Coronary Artery Fistula	5	508	2	9	35.7%	99.6%	97.9%
Cardiac Situs Defects	18	501	0	5	78.3%	100%	99%
Total Diagnoses	325	4722	42	151			

* Total number of fetuses/pups analyzed =524, including 277 identified by Vevo ultrasound as with CHD+161 littermates of the affected fetuses but Vevo ultrasound identified as without CHD + 86 fetuses from litters Vevo ultrasound identified as without CHD (see Figure S2).

[‡] VSD, AVSD.

[‡] TGA, DORV and PTA/PA,

[§] HLHS= hypoplastic left heart syndrome,

^{||} HRHS=hypoplastic right heart syndrome.

[#] MS/MA= mitral valve stenosis /mitral valve atresia,

^{**} AS/AA/COA= aortic stenosis/aortic atresia/coarctation.

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Table 3**EFIC Confirmation of Micro-CT Diagnosis of CHD in Fetal and Neonatal Mice**

Cardiac Diagnosis	CHD Diagnosis Confirmed [‡]	Missed CHD Diagnosis [§]	Sensitivity	Specificity [#]	Accuracy ^{**}
Neonates					
VSD	62/78	11/178	84.9% (62/73)	91.3% (167/183)	89.5%
Outflow Tract Defects [*]	22/24	5/232	81.5% (22/27)	99.1% (227/229)	97.3%
Aortic Arch Defects [‡]	17/17	1/239	94.4% (17/18)	100% (238/238)	99.6%
AVSD	9/10	1/246	90.0% (9/10)	99.6% (245/246)	99.2%
Coronary Artery Fistula	5/6	5/268	50.0% (5/10)	99.6% (263/264)	97.8%
Tricuspid Hypoplasia/Atresia	3/3	0/253	100% (3/3)	100% (253/253)	100%
Dextrocardia	4/4	0/270	100% (4/4)	100% (270/270)	100%
Fetuses					
VSD	10/10	1/8	90.9% (10/11)	100% (7/7)	94.4%
Outflow Tract Defects [*]	9/9	0/9	100% (9/9)	100% (9/9)	100%
Aortic Arch Defects [‡]	4/4	0/14	100% (4/4)	100% (14/14)	100%
AVSD	5/5	1/13	83.3% (5/6)	100% (12/12)	94.4%
Coronary Artery Fistula	---	---	---	---	---
Tricuspid Hypoplasia/Atresia	1/1	0/17	100% (1/1)	100% (17/17)	100%
Dextrocardia	---	---	---	---	---
Neonates + Fetuses					
VSD	72/88	12/186	85.7% (72/84)	91.6% (174/190)	89.8%
Outflow Tract Defects [*]	31/33	5/241	86.1% (31/36)	99.2% (236/238)	97.4%
Aortic Arch Defects [‡]	21/21	1/253	95.5% (21/22)	100% (252/252)	99.6%
AVSD	14/15	2/259	87.5% (14/16)	99.6% (257/258)	98.9%
Coronary Artery Fistula	5/6	5/268	50.0% (5/10)	99.6% (263/264)	97.8%
Tricuspid Hypoplasia/Atresia	4/4	0/270	100% (4/4)	100% (270/270)	100%
Dextrocardia	4/4	0/270	100% (4/4)	100% (270/270)	100%

VSD = ventricular septal defect; AVSD = atrioventricular septal defect.

^{*} Transposition of the great arteries, double outlet right ventricle and persistent truncus arteriosus.[‡] Right aortic arch and coarctation/interrupted aortic arch.

$$\text{CHD Diagnoses Confirmed} = (\text{No. of confirmed micro-CT, "CHD", diagnoses}) / (\text{total no. of micro-CT, "CHD", diagnoses}).$$

$$\text{\# Missed CHD Diagnosis} = (\text{no. of confirmed false-negative "No-CHD", Micro-CT diagnoses}) / (\text{total no. of Micro-CT "No-CHD", diagnoses}).$$

$$\text{\# Sensitivity} = (\text{no. of confirmed micro-CT, "CHD" diagnoses}) / (\text{no. of confirmed CHD} + \text{no. of confirmed false-negative "No-CHD" diagnoses}).$$

$$\text{\# Specificity} = (\text{no. of confirmed "No-CHD" diagnoses}) / (\text{no. of confirmed "No-CHD" diagnoses} + \text{no. of false-positive "CHD" diagnoses}).$$

$$\text{\# Accuracy} = (\text{no. of confirmed "CHD"} + \text{no. of confirmed "No-CHD" diagnoses}) / (\text{total no. of micro-CT scanned animals}).$$
 From Kim et al., 2013.