Improved Preparation of Chick Embryonic Samples for Magnetic Resonance Microscopy

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Previous work demonstrated the power of three-dimensional (3D) magnetic resonance microscopy (MRM) to follow complicated morphologic development in the embryonic cardiovascular system. In this study we describe a new dual-contrast method for specimen preparation that combines perfusion fixation and immersion in fixative with macro- and small molecular gadolinium agents to provide enhanced definition of both the heart wall and chamber. MRM was performed at 9.4 T with image resolutions of 25, 31, and 50 μm isotropic voxels for three stages of chick embryos (day 4, day 5.5, and day 9), and compared to histological sections of the same embryos. The results show considerable improvement of image quality over previous efforts, with better signal-to-noise ratio (SNR) and contrast between the cardiac chamber and myocardial wall. Excellent correlation was shown between the MRM images and histological sections. Thus, 3D high-resolution MRM in combination with the dual-contrast technique is useful for acquiring quantitative 3D morphologic data regarding heart development. Magn Reson Med 49:1192–1195, 2003. © 2003 Wiley-Liss, Inc.
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The developmental processes that transform the primitive tubular heart, with its peristaltic-like contractions, into the mature four-chambered heart of higher vertebrates have long attracted attention (1,2). Various imaging techniques, including video light microscopy, ultrasound, confocal electron microscopy, and scanning electron microscopy, have been applied to study embryonic heart development. However, many of these imaging methods have limitations that frequently force investigators to analyze cardiac phenotypes using postmortem histopathology. A major limitation of histological sections is that morphologic abnormalities cannot clearly be identified, because it is difficult to understand a three-dimensional (3D) structure with curves and loops, such as the heart, by examining two-dimensional (2D) sections.

The first 3D MR images of the chick heart were acquired in our laboratory in 1986 (3). Since that time, a number of additional studies have used MRM for the morphological analysis of embryonic hearts (4–8). In these studies, 3D images of the embryonic heart were acquired at very high magnetic fields (9.4T and 17.6T), with an intracardiovascular perfusion of a mixture of gadolinium agent and gelatin. These investigations demonstrated the ability of MRM to document cardiovascular morphogenesis and dysmorphogenesis (7,8). However, these investigators provided imaging of the heart chamber and vessel lumens only as an internal cast, and internal and external cardiac structures, such as the ventricular wall, myocardial trabeculations, cardiac jelly, and endocardial cushions could not be observed (8).

We describe a new dual-contrast technique that combines perfusion with immersion fixation, along with macro- and small molecular gadolinium agents, to improve cardiac structural studies of chick embryos. The improvements include more complete perfusion of the vasculature to inhibit collapse of the vascular lumens, and effective immersion fixation to enhance image contrast between the myocardial wall and the heart lumen. These innovations will enable us to perform volumetric morphologic analysis of chick hearts at different stages, and lead to a better understanding of cardiac development.

MATERIAL AND METHODS

Perfusion Solution Preparation

Gadolinium-diethylenetriaminepentaacetic acid-bovine serum albumin (Gd-DTPA-BSA) was freshly synthesized for each experiment. The T_1 relaxivity of Gd-DTPA-BSA in 3% gelatin (v/v) solution, as determined from T_1 measurements, was 4.4 s^{-1} mM^{-1} at 9.4T. We used a 3.5 mM Gd-DTPA-BSA solution in the perfusion, which resulted in a T_1 of 80 ms. The solution was colored with Evans blue so that we could observe the perfusion.

Embryo Collection

Fertilized Hubert Ross chicken eggs (Gold Kist Hatchery, Siler City, NC) were incubated at 37°C and 97% humidity, and harvested at Hamburger-Hamilton (HH) (9) stages 22–23 (day 4), 28 (day 5.5), and 35 (day 9). The embryos were placed ventral side up on a 2% agarose support for perfusion. The peripheral circulatory system of the embryo was opened by cutting a few peripheral vessels, which allowed the embryonic blood to be washed out of the cardiovascular system during the perfusion. The warm (37°C) perfusion solution was loaded into a fine borosilicate glass capillary pipette and injected manually via the proximal vitelline vein. Pressure was controlled such that no expansion of the vascular compartment was observed

FIG. 1. Early cardiovascular system of a day-4 chick embryo. **a:** MIP image of the whole embryo (3D MRM). The looped heart tube and the connected vascular system, such as aortic arch arteries (aaa), dorsal aorta (dao), presumptive ventricle (v), sinus venosus (sv), and inflow (i) and outflow (o) limbs of the heart tube, are clearly visible. **b:** An optical image of a day-4 chick embryo shows the vitelline vessels (vv), which served as the injection site for the perfusion. **c:** Zoomed MIP of the heart in the transverse plane reveals detailed cardiac structures, such as the right and left atria (ra and la), atrial septum (as), atrioventricular endocardial cushions (ec), aortic sac (aos), and trabeculated myocardium of the presumptive ventricle. Bar: (**a** and **b**) 1 mm.
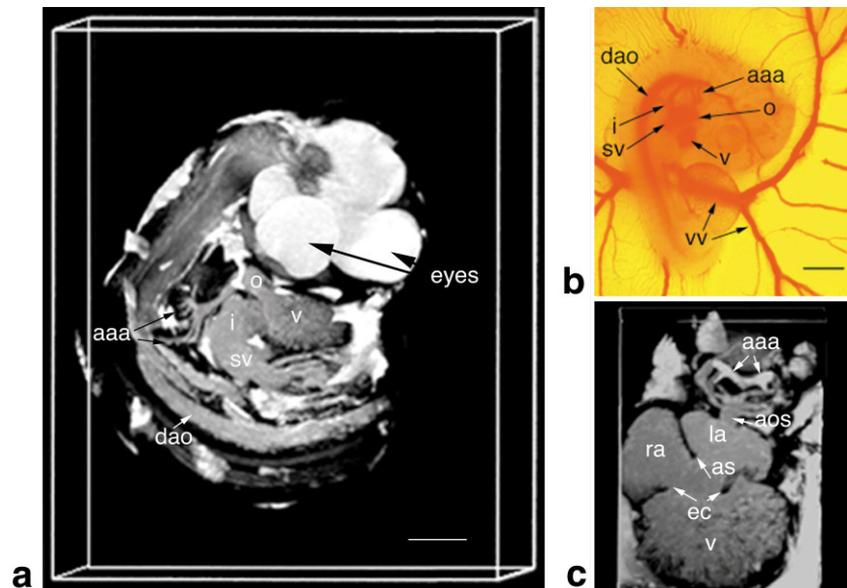
under the dissecting microscope. The heart continued to beat during the injection, which allowed more complete distribution of the solution throughout the vascular compartment. The heart was stopped on the cardiac cycle in diastole by dropping a 1.8% buffered potassium chloride solution directly onto the heart. The perfused embryo was placed on dry ice for 10 min to solidify the gelatin, followed by immersion-fixation with 25 mM of gadoteridol (ProHance; Bracco Diagnostics Inc., Princeton, NJ) and formalin (Bouin's fixative; LabChem Inc., Pittsburgh, PA) at 4°C for 30 min. This reduced the T_1 relaxation time of the embryo to about 120 ms, as determined from T_1 measurements with additional embryos. The smaller embryos (day 4 and day 5.5) were isolated and mounted in a flat acrylic slide containing a cylindrical chamber (10 mm diameter, 5 mm deep). The chamber was then was filled with proton-free perfluoro-polyether Fomblin (Ausimont, Thorofare, NJ) and covered with a screw cover-slip. The larger perfused day-9 embryo was placed in a 10-mm-diameter plastic tube filled with Fomblin and covered with a plastic cap. Total preparation time was typically ~1 hr.

Magnetic Resonance Microscopy (MRM)

MR images were acquired using an 89-mm vertical-bore 9.4 T magnet interfaced to a GE Signa 5X console modified for MRM (GE Medical Systems, Waukesha, WI). To optimize the signal-to-noise ratio (SNR) for the specimens of different sizes, a custom-designed Helmholz pair RF coil (10 mm diameter and 6 mm at separation) was used for day-4 and day-5.5 embryos, and a solenoid coil (10 mm diameter, 16 mm long) was used for the day-9 embryo. 3D T_1-weighted spin warp encoding images were acquired through the sagittal long axis of the heart. The imaging parameters were as follows: repetition time (TR) = 100 ms, echo time (TE) = 5 ms, bandwidth = ±31.25 KHz, matrix size = 256 × 256 × 256, and field of view (FOV) = 6.4 × 6.4 × 6.4 mm, 8 × 8 × 8 mm, and 12.8 × 12.8 × 12.8 mm

for day-4, day-5.5, and day-9 embryos, respectively. The total imaging time was 29 hr 10 min (number of acquisitions (NEX) = 16). The reconstructed image data were volume-rendered on a Silicon Graphics Workstation using VoxelView ULTRA 2.5, Slicer Dicer (PIXOTEC; LLC, Renton, WA) software, and Image-J (http://rsb.info.nih.gov/ij/).

Histology

After the embryos were scanned by MRM, they were processed for histology. The embryos were sectioned transversely at 7 μm and stained with hematoxylin-eosin. The correspondence between images acquired by MRM and histology was determined by visual assessment of the best match of anatomical landmarks.

RESULTS

The dual-contrast technique permits a reduction of the T_1 relaxation time of the embryos from 2000 to < 120 ms, yielding an effective T_1 contrast between the myocardium and the Gd-DTPA-BSA gelatin solution in the heart chamber. The contrast (heart chamber signal: myocardium signal) was 26%, defined as (chamber intensity-myocardial intensity)/myocardial intensity.

The 3D data obtained from the whole-mount embryos at three stages of development (days 4, 5.5, and 9) showed a high contrast-to-noise ratio (CNR) and a high isotropic spatial resolution (25, 31, and 50 μm³, respectively), which substantially improved the image postprocessing. Maximum intensity projection (MIP) was employed to observe the heart chamber and vascular system (Fig. 1a). All structures of the heart (about 2 × 2 × 2 mm) and the connected vascular system shown in the whole-mount image of a day-4 embryo (Fig. 1b) are depicted in the MIP. The distal portion of the outflow limb of the early heart tube (truncus) is connected to the aortic sac, which splits into the paired aortic arch arteries. The arch arteries then
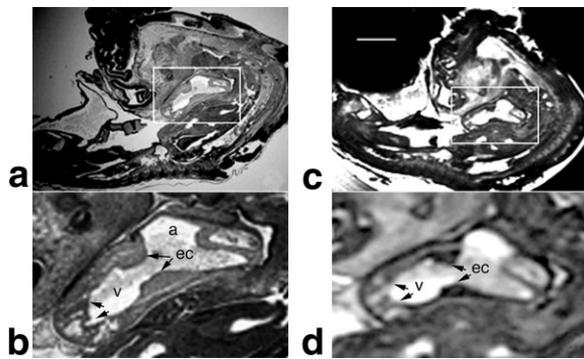
FIG. 2. Comparison of histological sections with MRM of the same embryo. **a:** Histological section of a day-4 chick embryo. **b:** The heart in a close-up view. **c** and **d:** Corresponding MRM of the same embryo. Note the excellent correspondence between the MRM image and the histological section of the heart. The single heart chamber, presumptive atrium (a), atrioventricular endocardial cushions (ec; arrows in **b** and **d**), and the trabeculated myocardium (arrows in b and d) of the presumptive ventricle (v) can easily be identified in both the histological section and the MRM image. Bar: (**a** and **c**) 1 mm.

of the technique is incomplete perfusion of the chambers and vascular system due to thrombus formation and the viscosity of the perfusion solution. Although thrombus formation could be minimized by washing the cardiovascular system with saline solutions prior to perfusion of the Gd-DTPA-BSA and gelatin solution (10), we chose to use a continuous in vivo perfusion technique, which enabled us to observe the blood being flushed out directly and the completeness of the perfusion. This continuous in vivo perfusion also avoids the introduction of air bubbles into the vascular compartment by multiple insertions. For an optimal perfusion to be obtained, a suitably viscous solution is critical. Hogers et al. (7) noted that a balance was necessary between the optimum perfusion of the smallest embryonic vessels (low viscosity) and the maximum gadolinium fixation inside the vessels (more viscous agent). The gelatin concentration used in the Hogers study was 5%, which is half that used in other studies (7). In our study, we used a 3% gelatin concentration with 3.5 mM Gd-DTPA-BSA. The relatively low concentration of gelatin improved the completeness of the perfusion and provided
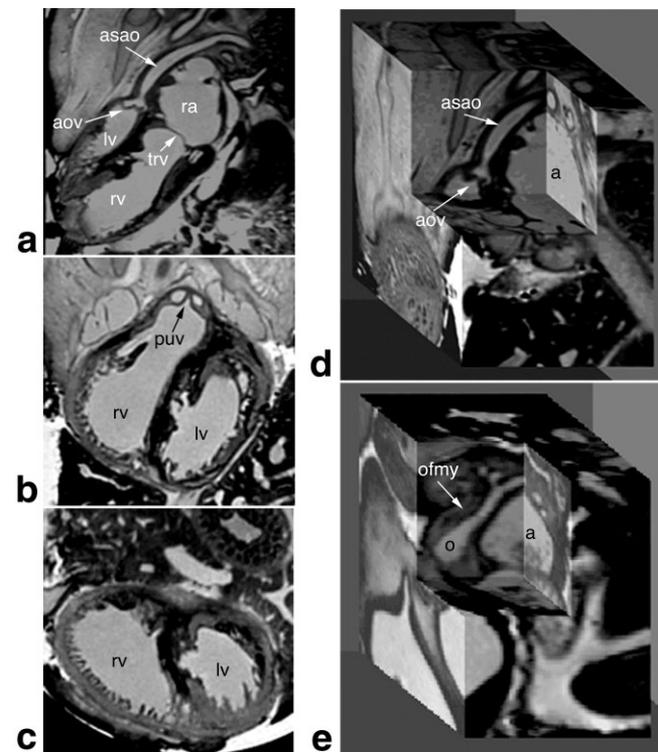
connect to the paired dorsal aortae. The right and left atria, atrial septum, atrioventricular endocardial cushions, and trabeculated myocardium of the presumptive ventricle are also shown (Fig. 1c).

Figure 2 shows an excellent correspondence between the MRM images and histological sections of the same heart for a day-4 chick embryo. The single atrial chamber, atrioventricular endocardial cushions, and trabeculated myocardium of the presumptive ventricle can easily be identified in both the histological sections and the MRM images.

MRM images can be selected in arbitrary planes, since the data are isotropic. Figure 3a–c shows the detailed cardiac anatomy of a day-9 chick heart (about 4 × 4 × 4 mm) in the sagittal, coronal, and transverse planes. Cardiac structures, such as left and right ventricles, right atrium, aortic and pulmonary valves, and ascending aorta are depicted. Note the difference in shape and trabeculation pattern between the right and left ventricles. Figure 3d and e, a cut-away of 3D MRM reconstruction, shows the outflow limb of the embryonic heart at days 9 and 4, respectively. Note that the aortic valve is clearly visible in the day-9 embryo, wherein the heart is fully formed, and is absent in the day-4 embryo, wherein the valve cushions protect the outflow limb from regurgitation.

DISCUSSION

Our laboratory previously developed a successful 3D MRM technique for imaging perfusion-fixed hearts of rodent, chicken, and human embryos (3–6). More recently, embryonic imaging at 17.6T was reported to improve image quality in smaller vessels (7). As a result of the embryonic cardiovascular system being perfused, the primary heart chamber or tube, and even smaller blood vessel diameters can be observed. However, a potential limitation

FIG. 3. MRM image of a fully formed day-9 chick heart in the (**a**) sagittal, (**b**) coronal, and (**c**) transverse planes. MRM provides anatomic information, ranging from sectional images along arbitrary anatomical planes to volumetric reconstruction of organs. asao = ascending aorta, aov = aortic valve, ra = right atrium, lv = left ventricle, trv = tricuspid valve, and rv = right ventricle. **d** and **e:** Cut-away 3D reconstructions of the embryonic hearts at (**d**) day 9 and (**e**) day 4. Note that the aortic semilunar valve is clearly visible in the day-9 embryo, wherein the heart is fully formed, and is absent in the day 4 embryo, wherein the valvular cushions of the outflow tract are present. asao = ascending aorta, aov = aortic valve, a = atrium, o = outflow, ofmy = outflow myocardium.

a stable intravascular cast when the gelatin was solidified with dry ice. However, the perfusion technique is labor-intensive and depends on the skill of the operator for microsurgical isolation of the vascular structures, cannulation, and then injection of the solution.

The visibility of a structure is dependent on both the spatial resolution and the CNR. Given a structure with N voxels, the tissue visibility (V) can be defined as V = CNR $\sqrt{n}$ (11). To reduce the effective T_1 relaxation time, we used the dual-contrast technique, which reduced the T_1 of the solution in the chamber to 80 ms and in the myocardium from 2000 ms to ~120 ms. For a given minimal TR (100 ms), the gain in tissue contrast between the chamber and the myocardium was 26%. The results show considerable improvement of image quality for the smaller vessels and myocardium compared to previous studies (3–6). For a structure as complicated as the heart, isotropic resolution is essential. The improvement in resolution is best discussed in terms of the voxel volume. The work shown here at 25 μm isotropic volume (1.56×10^{-5} mm³) had a ~2× higher resolution than that obtained in previous studies by Hogers et al. (7) in the chick heart. The resolution was more than 2500× higher than in our first images of the chick embryo (3). Most importantly, the dual-contrast technique can define structures that have not heretofore been clear.

High-resolution MRI combined with specific and sensitive MR probes is one of the most promising techniques currently available for molecular imaging. This technique has been used to visualize gene expression (12,13) and to image the transgenic mouse in studies linking genomics to disease development (14). Research into the mechanisms that control heart development has shown exciting progress in elucidating asymmetric gene expression in the primitive heart tube, which provides important clues and markers for normal and abnormal looping (15). The techniques developed in this study enable the 3D visualization of the endocardium and myocardium of the heart tube, and have potential applications for studies of, e.g., the correct spatial location and timing of genes involved in developmental processes.

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