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High-Throughput Analysis of Mouse Embryos by Magnetic Resonance Imaging

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Abstract

Genetic studies in the mouse are crucial for uncovering new genes and signaling pathways associated with development. The identification of murine models with developmental malformations in high-throughput mutagenesis screens is made difficult because, after mid-embryogenesis, the embryo is opaque. Traditional phenotyping methods such as histological sectioning are labor intensive and destructive. We have developed and optimized a novel method for high-throughput multiembryo magnetic resonance imaging (MRI). Here we present our method for processing 32 mouse embryos for analysis by MRI. We describe the MR system, imaging software, and the reconstruction of two-dimensional (2D) and three-dimensional (3D) images. We also discuss the applications of this technique, highlight its advantages, and point out some disadvantages. Using this approach, we can identify developmental malformations in mutant embryos at high spatial resolution (voxel size $25.4 \times 25.4 \times 24.4 \mu\text{m}$). This technique can be easily used for mouse mutagenesis screens and thus provides an important tool for identifying new mouse models for human diseases.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose (SeaKem; Lonza)

Make a fresh 1% (w/v) solution in H_2O immediately before use. Keep warm while stirring on a hot plate. Cover flask with foil to prevent evaporation.

Gadolinium-diethylenetriamine pentaacetic anhydride (Gd-DTPA; Magnevist, Schering UK), 0.5 M stock solution

Hanks' balanced salt solution (HBSS) with 5 mM EDTA, prewarmed to 37°C before use (see Step 3)

Dilute HBSS (10 $\times$) (Sigma-Aldrich) to 1 $\times$ with H_2O and add 0.5 M EDTA (Sigma-Aldrich) to give a final concentration of 5 mM EDTA.

Mice, male and female

PBS(P) <R>

PFA (4%) with Gd-DTPA <R>

Equipment

Balance (for weighing embryos)

Computer (Apple Macintosh or PC) with dedicated graphic cards and at least 4 GB of random access memory

Dry ice or liquid nitrogen (see Step 7)

Forceps, straight (Dumont #5) (Interfocus/Fine Science Tools)

Heat pad (e.g., Morphy Richards)

Imaging setup

These studies require an experimental MR system with a vertical or horizontal magnet (magnetic field strength preferably >7 T), high-duty cycle, water-cooled gradient system (500 mT/m), dedicated radiofrequency (RF) coils optimized in geometry and loading properties for multiembryo samples (e.g., from Rapid Biomedical, Germany), and standard 2D and 3D spoiled gradient echo sequences. For example, we have used a Varian VNMRS MR system (Varian Inc.), which comprises a 9.4-T (400 MHz) horizontal magnet, shielded gradient systems with a maximal gradient strength of 600 mT/m or 1 T/m, and quadrature-driven birdcage-type coils with an inner diameter [ID] of 28 mm (coil length ~45 mm, outer diameter [OD] 59 mm, and 115 mm to fit into gradient systems). A 3D spoiled gradient echo sequence, 60°–90° excitation pulses with rectangular pulse shape, was used with a short repetition time (30 msec) to obtain strong T1 contrast. A long echo time (TE) of 10 msec enhanced tissue interfaces caused by susceptibility differences (Schneider et al. 2003). A matrix size of 608 × 608 × 1408 at field of view of 26 × 26 × 50 mm (3D volume sagittally orientated) yielded an experimental resolution of 43 × 43 × 36 mm. The total experimental time was ~12.3 h for 32 embryos (typically overnight runs), and each phase-encoding step was averaged four times to improve the signal-to-noise ratio.

Imaging software

We use custom-written software to reconstruct MR data and write stacks of TIFF images. 3D image analysis software is also required (e.g., Amira from Visage Imaging, Inc.).

Intuos3 pen tablet (Wacom)

Nuclear magnetic resonance (NMR) tubes, 28-mm OD (Fluorochem)

These tubes are fitted with an M4 nylon rod (RS Components) and a custom-made Teflon disk to allow stacking of embryos and facilitate easy removal after imaging (Fig. 1A).

Plates, six-well

Platform rocker (Grant-bio PMR-30)

Scissors, dissecting (Noyes and Vannas) (World Precision Instruments)

Teflon clamp ring (custom-built to fix the NMR tube centrally inside the RF coil)

Tubes, 1.5 and 2 mL (larger tubes may also be needed, depending on the age of the embryos; see Step 12)

METHOD

Embryo Preparation

1. Set up timed matings to produce embryos of the desired genotype. Check females every morning for the presence of a copulatory plug (taken as 0.5 d postcoitum [dpc]). Embryos are required at 13.5–18.5 dpc.

Embryos collected at 15.5 dpc are ideal as they allow high-throughput analysis; organogenesis is also complete by this stage. They are large enough to dissect and process on the open bench without the aid of a microscope, and four of these embryos can easily be placed in one layer within the 28-mm NMR tube. Embryos younger than 13.5 dpc are not suitable because of their size and contrast properties, and also, recovery from the agarose following MRI is difficult.
2. Kill pregnant dams by cervical dislocation.

Cervical dislocation is the most appropriate method for killing the pregnant dam and maintaining viable embryos. The administration of anesthetics may adversely affect or compromise heart rate, thereby reducing embryo viability and affecting the bleeding-out procedure.
3. Dissect the uterine horns from the dam and place them into warm HBSS with 5 mM EDTA. (EDTA functions as an anticoagulant.)
4. Dissect the embryos from the uterine horns, taking care not to disrupt the yolk sac. Place each embryo into a well of a six-well plate containing warm HBSS with EDTA. Ensure that the embryo is submersed.
5. For each embryo in turn, open the yolk sac to expose the umbilical vessels. Cut the umbilical vessels with Noyes scissors.

Take care to ensure good bleeding out. The umbilical cord should be cut cleanly with sharp scissors. Blood should flow out

immediately and with strong pulses at first. The remaining blood will pump out slowly and form a pool beneath the embryo.

See Troubleshooting.

6. Ensure that the embryos bleed out completely for a maximum of 20 min.

This procedure is best performed using a platform rocker in a warm room but can also be performed using a heat pad on the bench. Avoid bleeding out for >20 min as embryos can go into rigor mortis.

7. Collect the yolk sacs into 1.5-mL tubes for genotyping (by polymerase chain reaction [PCR] or Southern blotting) and freeze on dry ice or in liquid nitrogen.

8. Wash the embryos twice with cold PBS(P).

9. Photograph each embryo for a record of external phenotype.

10. Weigh the embryos.

Embryo weight is not only useful data to record as part of determining a mutant phenotype, but also provides information that can be used as a reference following volume measurements of internal organs. For example, organ volume can be expressed as a ratio with embryo mass.

11. Remove the left forelimb with Vannas scissors and freeze for genotyping or archiving. For large embryos (15.5 dpc), make slits on either side of the abdomen with Vannas scissors.

Removal of the left forelimb is performed to unequivocally mark the left side of the embryo. This can be clearly seen on the MRI images and ensures that the images have not been flipped and that visceral situs can be correctly identified. The sides of the embryo are slit to allow penetration of the fixative plus contrast agent into the embryo.

12. Transfer the embryos to tubes and immersion fix in 4% PFA with Gd-DTPA on ice.

An adequate volume of fixative is required to ensure good fixation. We find that embryos of 15.5 dpc and below will fit comfortably into a 2-mL tube that can be filled with fixative. In these tubes, large numbers of embryos can be conveniently stored in boxes. Larger embryos will need to be immersion fixed in a larger vessel with more fixative. Gd-DTPA is a paramagnetic material. It shortens the longitudinal relaxation time (T1) of water protons, thereby enhancing the contrast of the MRI scan.

13. Store at 4°C for at least 4 d.

A period of at least 4 d is required to allow full penetration of the fixative and Gd-DTPA through all tissues. Leaving the embryos to fix for longer (e.g., 7 d) may produce better contrast.

14. When the embryos are fixed, load them into a 28-mm NMR tube (Fig. 1B) as follows.

- i.** Add ~22 mL of 1% agarose containing 2 mM firm. Gd-DTPA to the 28-mm tube, filling to ~2 cm above the disk (i.e., ~4.5 cm from the bottom of the tube), and allow to set firm.
- ii.** Select four embryos for the first layer. Remove the left forelimb from each embryo. Then remove the tail from the first, the right hindlimb from the second, and the left hindlimb from the third embryo (see note below).
- iii.** Lay the embryos onto the agarose in the 28-mm tube.
- iv.** Pipette agarose containing Gd-DTPA onto the embryos until they are just covered. Ensure that no air bubbles form.
- v.** Repeat Steps 14.ii–14.iv until all layers are completed. The maximum distance from the bottom of the first layer to the top of the last layer should be 45 mm.
- vi.** Tubes can be stored upright for 5 d at 4°C until ready to image.
- vii.** Make a table showing embryo position and genotype.

Before embedding, embryos have other limbs and/or tails removed so that each embryo in a given layer (of four embryos) can be unequivocally identified. Position them so that alternate layers have embryos facing outward, then facing inward, so the stack is even and all the embryos are aligned vertically. Tubes containing embryos can be safely sent by courier (packed in ice) to a facility with a suitable MRI setup.

Magnetic Resonance Imaging

15 . Place the tube in the RF coil and secure using the Teflon clamp ring (Fig. 1C).

Align the NMR tube in the probe so that (1) when analyzing the images, the requirement for oblique slice is minimized, and (2) the transverse section most closely resembles the image in the Mouse Atlas. Although oblique slice is useful, it reduces resolution.

- 16 . Fix the RF coil/NMR tube arrangement inside the magnet and perform scout imaging (using a 2D multislice gradient echo sequence: sagittal orientation, field-of-view 60×30 mm, slice thickness 0.5 mm, 4 slices, gap 3 mm, 512×256 points, TE/TR [echo time/repetition time] = 10/30 msec, $\alpha = 90^\circ$) to position the embryo stack in the center of the RF coil.
- 17 . Manually tune and match the RF coil to adjust the impedance to 50Ω .
- 18 . Optimize the homogeneity of the static magnetic field using manual or automated shimming.

In the manual approach, the line width (full width at half maximum) of the water signal has to be minimized. Automated methods typically minimize the phase variation across the sample.
- 19 . Adjust the main frequency and the RF-pulse power manually or automatically.

The main frequency is determined by the frequency of the water signal. It is recommended to adjust the length required for a 180° or 360° pulse at a given power level. The required length τ_α for any other flip angle α at the same power level can then simply be derived from the equation: $\tau_\alpha = \alpha \times \alpha_{180^\circ}/180^\circ$.
- 20 . Load the 3D gradient echo method (sagittal orientation, field-of-view $50 \times 26 \times 26$ mm, no slice selection, $1408 \times 608 \times 608$ points, TE/TR = 10/30 msec, $\alpha = 90^\circ$, four averages).

The final raw data size is ~4 GB, and some MR systems may not be able to handle such a big file size. The scan needs to be split into partitions of an appropriate size. It is also recommended to turn off reconstruction during acquisition.
- 21 . Adjust read-out position and receiver gain, and start the scan.
- 22 . Transfer data upon completion to the reconstruction computer.

Data Reconstruction and Analysis

- 23 . Reconstruct the raw MR data into a stack of 2048 (for 32 embryos) 2D TIFF files (16-bit pixel resolution, 4 GB total size) using purpose-written c-software.
- 24 . Analyze the TIFF files using Amira software (Fig. 2).
 - i. Identify the start and finish of each layer of embryos by loading up a stack of TIFF files (e.g., 500) and scrolling up and down to find exactly in which TIFF files the first and last part of the embryo is visible. Repeat until all layers have been identified.
 - ii. Load up the correct TIFF files for analysis of the first layer of embryos.

- iii. Open an orthoslice tab to view embryos in sagittal section.
- iv. Open more orthoslice tabs and check the appropriate box to view embryos in transverse and coronal orientations.
- v. Adjust the contrast for each orthoslice.
- vi. Scroll through each embryo in each orientation to phenotype.
- vii. Display the scale bar and capture images as JPEG files using the camera button or print screen button.

See Troubleshooting.

25 . Create 3D reconstructions using the Image Segmentation Editor (Fig. 3).

- i. Crop a region of interest from a selected embryo. Make this region as small as possible to exclude unwanted material, but ensure that all relevant structures are included. Save the cropped TIFF stack as an AmiraMesh file.
- ii. Select “Label field” to open the Image Segmentation Editor.
- iii. Create a new field to label and select color.
- iv. Select the appropriate orientation (x - y , x - z , y - z) to model the structure.
- v. Use the Intuos3 pen tablet to draw around the desired structure and fill in. Repeat this process for each section until the structure is completed.

Alternatively, if the structure is well defined (e.g., dorsal aorta), the first section containing the structure of interest can be drawn around, the TIFF stack advanced (e.g., five to 20 slices), and the structure drawn around again, and the intervening sections automatically filled in using the interpolate function.

- vi. Fix the segmented sections by selecting the “+” button to prevent accidental loss of material.
- vii. Check the accuracy of the segmentation in each orientation and tidy up. Erase mistakes by drawing over the unwanted area and selecting the “-” button.
- viii. Many structures can be modeled in one segmentation window.
- ix. Create a 3D model and capture the image.
- x. Save as a network file.

Segmentation typically has to be performed manually or can at best be done semiautomatically because the contrast is dependent on the tissue of interest and the

location within the tube. Separation from the surrounding/unwanted tissue/media is probably the most critical aspect precluding a fully automated analysis.

- 26 . Tissue volumes for morphometric analysis can be measured using the Measure Tissue Statistics tool (Fig. 3). It is helpful to know the embryo mass for the final calculation. However, if this is not available, the whole embryo volume can be calculated.
- 27 . Write MR data and TIFF images as backup to a DVD.

TROUBLESHOOTING

Problem (Step 5): The embryos fail to bleed out.

Solution: Bleeding out is very important, as blood gives a strong MR contrast, making it difficult to visualize intracardiac anatomy. Consider the following:

1. If no bleeding out is seen, cut the umbilical cord further up (closer to the umbilical hernia), but take care not to damage the embryo itself.
2. Bleeding out that starts well but ceases can be caused by a clot forming at the end of the cut vessels. This can be pinched off with forceps.
3. Keep HBSS with EDTA at 37°C to maintain body temperature, thereby facilitating bleeding out.

Problem (Step 24): The MRI images are dark and lack contrast.

Solution: Check MR parameters such as RF-power calibration. If consistent, remove the embryos from the NMR tube and place them into PFA again. Make sure that Gd-DTPA has been added to the fixative. Re-embed and re-image 4–5 d later, ensuring that the agarose also contains 2 mM Gd-DTPA.

DISCUSSION

Background

Current major international efforts in mouse genetics are directed at creating ~20,000 mouse deletion mutants via the EUComm (European Conditional Mouse Mutagenesis), NorComm (North American Conditional Mouse Mutagenesis), and KOMP (Knockout Mouse Project) programs (Collins et al. 2007a,b). Analysis of the Mouse Genome Informatics Database shows that 1534 of 5166 gene mutations created to date have an embryonic or perinatal lethal phenotype. Approximately 70% of this lethality occurs after 14.5 d of gestation. Assuming this sample was ascertained without underlying bias, we would expect that about 6000 of the estimated 20,000 genes are required for normal embryonic development. Phenotyping these late gestational lethal mutations, and others created through approaches such as ENU (*N*-ethyl-*N*-nitrosourea) and transposon mutagenesis, is a major challenge. A particular difficulty with the mouse embryo is its

opacity in the organogenesis and late-gestational phases of development. Conventional methods of analysis, such as histology, are labor intensive, are time consuming, result in loss of 3D information, and lead to destruction of the embryo.

Ideally, the phenotyping tool of choice for high-throughput analysis of mouse embryos should be nondestructive (to allow subsequent analysis of the embryo by other means), be able to produce 3D information (which is essential for the interpretation of complex malformations), permit the analysis of relatively large numbers of embryos at one time, and permit embryos to be processed rapidly and without highly specialized operator skills. To enable us to examine embryos at high throughput for phenotype-driven mutagenesis screens, we have developed a method of imaging up to 32 fixed embryos simultaneously in a single unattended overnight run at high spatial resolution (Bamforth et al. 2004; Schneider et al. 2004). Following data reconstruction, a single trained individual can thoroughly examine each embryo in ~30 min. After high-throughput MRI screening, the embryos can be recovered for further analysis by, for example, high-resolution episcopic microscopy (Pieles et al. 2007) or classic histological sectioning. This may be necessary as small abnormalities with dimensions <20 μm , which is the limit of this technique, may be missed.

Advantages and Disadvantages of the Technique

The multiembryo MRI technique described here allows for high-throughput screening and identification of developmental abnormalities in the mouse embryo. At the spatial resolution achieved, it is possible to efficiently identify and quantitate relatively subtle cardiac and visceral malformations. However, smaller developmental defects that cannot be identified at this resolution ($25.4 \times 25.4 \times 24.4 \mu\text{m}$) may be missed, and alternative techniques such as standard histology should also be used. The multiembryo MRI technique has been optimized for late-gestation mouse embryos to identify those congenital defects that allow survival through most of gestation. Importantly, these defects resemble human congenital malformations and could provide mouse models for the study of these diseases. Also, more classical methods of phenotyping, such as histology, may result in incomplete analysis and loss of 3D information. Owing to the lack of contrast provided by mid-embryogenesis embryos, MRI is not appropriate for these embryos, and other phenotyping tools should be used, such as optical projection tomography or high-resolution episcopic microscopy (HREM). We have successfully used multiembryo MRI to identify complex developmental defects in mouse embryos lacking *Cited2* (Bamforth et al. 2004; MacDonald et al. 2008), *Ptdsr* (Schneider et al. 2004), *Cyclin E* (Geng et al. 2003), *Pinch1* (Liang et al. 2007), *Sox4* (Bogani et al. 2005), *Flna* (Hart et al. 2006), and *Pcsk5* (Szumska et al. 2008). For example, using this technique, we were able to identify and describe complex left–right patterning defects in *Cited2*-null embryos. By creating 3D models of the heart outflow tract and pharyngeal arch artery system, we were able to identify complex developmental defects such as double-outlet right ventricle, transposition of the great arteries, interrupted aortic arch, and vascular ring (Bamforth et al. 2004).

The limitations of this technique are the relatively low resolution when compared to standard histological sections. For example, fine detail such as the compact zone of the ventricular walls of the heart is difficult to analyze, and the coronary vessels cannot be identified.

However, as the multiembryo MRI technique is nondestructive, the selected embryos can be recovered following MRI and processed for histology to examine these structures.

A major advantage of MRI is the ability to send fixed embryos by courier from referring laboratories to the laboratory that performs the MRI analysis. This minimizes the expense of animal relocation, re-derivation, and breeding required to generate the embryos, and significantly reduces animal experimentation. The single-coil/multiple-embryo approach using a standard imaging sequence makes this technique readily available on any experimental MR system. In particular, it eliminates the need for any cost-intensive MR system upgrade or time-consuming technical developments.

MRI scanning of the multiembryo tube can be performed in unattended overnight runs, thereby utilizing the downtime of the MR system with minimal hands-on time (typical setting-up time is ~15 min). Data transfer and reconstruction can also be performed semiautomatically. The subsequent data analysis can easily be performed at a reasonable scale if multiple operators analyze the data in parallel, for example, when applied to mouse mutagenesis screens. Also, as the data are permanently stored on DVDs, they can be sent out to collaborators or other specialists for further and more detailed analysis.

RECIPES

PBS(P)

Reagent	Quantity (for 1 L)	Final concentration (10×)
NaH ₂ PO ₄	2.56 g	18.6 mM
Na ₂ HPO ₄	11.94 g	84.1 mM
NaCl	102.2 g	1.75 M

Adjust the pH to 7.4 using NaOH or HCl as necessary. This recipe produces a 10× stock solution; prepare 1× PBS(P) by diluting with H₂O. Both 1× and 10× PBS(P) can be kept indefinitely at room temperature

PFA (4%) with Gd-DTPA

0.5 M Gd-DTPA (gadolinium-diethylenetriamine pentaacetic anhydride; Magnevist, Schering UK) Paraformaldehyde (PFA; Sigma-Aldrich)

PBS(P) <R>

1. Make 1 L of a 4% (w/v) solution of PFA in PBS(P) and adjust the pH to 7.4. Store in 50 mL aliquots at −20°C. Thaw before use and keep on ice.
2. Add 40 µL of 0.5 M Gd-DTPA per 10 mL of 4% PFA to give a final concentration of 2 mM Gd-DTPA.

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FIGURE 1.

Multi-embryo setup. (A) A glass NMR tube with an OD of 28 mm is fitted with an M4 nylon rod and a custom-made Teflon disk to stack the embryos and facilitate removal after imaging. (B) Thirty-two PFA-fixed embryos are placed in eight layers of four embryos and loaded into the NMR tube with agarose containing the contrast agent. (C) The NMR tube with embryos is placed in the RF coil and secured using the clamp ring. Scale bars, 1 cm.

FIGURE 2.

Data analysis. The raw MR data are reconstructed into 2D TIFF files and analyzed using Amira software. (A) Section through the long axis of the NMR tube showing embryos in eight layers. (B) Sagittal section through layer 8 showing the four embryos in this layer. (C–E) Transverse, sagittal, and coronal sections through individual embryos in layers 5, 1, and 4, respectively. The voxel size is $25.4 \times 25.4 \times 24.4 \mu\text{m}$. The structures indicated are the spinal cord (sc), the right and left lungs (rl, ll), atria (ra, la) and ventricles (rv, lv), primary atrial and interventricular septa (pas, ivs), mitral valve (mv), midbrain roof (mbr), midbrain (mb), mesencephalic vesicle (mes), thalamus (tha), hypothalamus (hy), pons (po), cerebellum (c), medulla oblongata (mo), pituitary (pit), tongue (t), thymus (th), left superior vena cava (lsvc), left main bronchus (lmb), aorta (ao), liver (li), stomach (s), left adrenal (lad), left kidney (lk), pancreas (pa), intestines (i), umbilical hernia (uh), aqueduct of Sylvius (aq), fourth ventricle (fv), inner ear (ie), larynx (lar), right ventricular outflow tract (rvot), spleen (sp), and testes (te). Scale bars, 500 μm . Axes: (d) dorsal; (v) ventral; (r) right; (l) left; (a) anterior; and (p) posterior. (Reprinted, with permission, from Schneider et al. 2004.)

FIGURE 3.

Example of cardiac 3D reconstructions and lung volume measurements in *Vcc* embryos at 15.5 dpc. (A) MRI transverse section through a control embryo (at 15.5 dpc) showing a normal heart. The right and left atria and ventricles (RA, LA, RV, LV), mitral valve (MV), primary atrial septum (PAS), and interventricular septum (IVS) are indicated. (A') Corresponding section of a mutant littermate *Vcc/Vcc* embryo (Szumska et al. 2008) showing an ostium primum atrial septal defect (ASD) and common atrio-ventricular valve (CAVV). (B) 3D reconstruction of a control heart, left anterior oblique view. The aorta (Ao)

arises from the LV, and the main pulmonary artery (PA) from the RV. The aortic arch passes to the left of the trachea (Tr), over the left main bronchus. (B') Corresponding reconstruction of a mutant littermate *Vcc/Vcc* heart showing a ventricular septal defect (VSD), a common arterial trunk (CAT) arising from the RV, giving rise to both cranial and pulmonary arteries, and an aortic vascular ring (AoVR). The trachea is not separated from the oesophagus (Tr-O). (C,C') 3D reconstruction of lungs from wild-type (C) and littermate mutant *Vcc/Vcc* (C') embryos. The cranial (Cr), middle (Mi), caudal (Ca), and accessory (Ac) lobes of the right lung, and the left lung (LL) are indicated. (D) Lung volumes (mean $\pm$ SEM, corrected for embryo volume) are shown for control left and right lungs (Con-LL, Con-RL) and littermate mutant *Vcc/Vcc* left and right lungs (Mut-LL, Mut-RL). The mutant left lung is significantly smaller than the control (*t*-test, $p = 0.003$). Axes: (A) anterior; (P) posterior; (R) right; (L) left; (D) dorsal; (V) ventral. Scale bars, 0.5 mm. (Reprinted, with permission, from Szumska et al. 2008.)