Ultrasound backscatter microscope analysis of early mouse embryonic brain development

DANIEL H. TURNBULL*†‡, TIMOTHY S. BLOOMFIELD§, H. SCOTT BALDWIN§, F. STUART FOSTER§, AND ALEXANDRA L. JOYNER†

*Division of Medical Physics, Toronto–Sunnybrook Regional Cancer Centre, and ‡Reichmann Research Building, Sunnybrook Health Science Centre, Department of Medical Biophysics, University of Toronto, 2075 Bayview Avenue, Toronto, ON, Canada M4N 3M5; †Division of Cardiology, The Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Philadelphia, PA 19110; and §Division of Molecular and Medical Genetics, University of Toronto and Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, ON, Canada M5G 1X5

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ABSTRACT The history of developmental and genetic analysis in the mouse has made it the model of choice for studying mammalian embryogenesis. Presently lacking is a simple technique for efficiently analyzing early mouse mutant phenotypes in utero. We demonstrate application of a real-time imaging method called ultrasound backscatter microscopy for visualizing mouse early embryonic neural tubes and hearts. This method was used to study live embryos in utero between 9.5 and 11.5 days of embryogenesis, with a spatial resolution close to 50 μm. Ultrasound backscatter microscopy images of cultured embryos made it possible to visualize the heart chambers. This noninvasive imaging method was also used for analyzing a neural tube defect. The midhindbrain deletion associated with a null mutation of the Wnt-1 protooncogene was easily recognizable on ultrasound backscatter microscopy images of 10.5- and 11.5-day embryos. Computer-generated volumetric renderings of the neural tube cavities were made from three-dimensional image data. This allowed a much clearer definition of the Wnt-1 mutant phenotype. These imaging techniques should be of considerable use in studying mouse development in utero.

It has not been possible in mammals to image embryogenesis in utero with microscopic resolution, because the embryos are enclosed in the maternal uterus. With the introduction of transgenic techniques to produce targeted mutations in mice (1), genetic analysis of development in the mouse is becoming increasingly prevalent. With gene targeting techniques in embryonic stem cells now being relatively efficient, the number of new targeted mouse mutants is increasing dramatically. What has not kept pace with these developments is our ability to rapidly and effectively analyze the mutant phenotypes. Many of the mutations in developmental genes have their first effects during embryogenesis, a stage that is particularly difficult to analyze in vivo. An easily accessible in utero imaging method would be invaluable for studying the dynamic progression of mutant phenotypes of such developmentally important genes.

Ultrasound backscatter microscopy (UBM) is a method for subsurface imaging of biological tissues with close to optical resolution (2), employing the principles of pulse-echo or backscatter ultrasound imaging at much higher frequencies (40–100 MHz) than clinical ultrasound (3–10 MHz) (3). A UBM system developed for clinical skin imaging has been described in detail (4, 5). This scanner employs mechanically scanned, focused transducers, operating between 40 and 100 MHz, and produces two-dimensional (2D) images at close to real-time frame rates (up to 10 images per sec). Three-dimensional (3D) image data can be acquired as a series of 2D sections in ~1 min. At 50 MHz, the UBM system has a measured spatial resolution close to 50 μm and a penetration depth of 4.5 mm. These characteristics should make UBM ideal for high-resolution imaging of early mouse embryos, which are located quite superficially in utero.

In this report, we demonstrate that 50-MHz UBM can be used to image living mouse embryos in utero and has the potential to study normal and abnormal development of the nervous system. Preliminary results also indicate that UBM could be developed to image cardiac development and to assess cardiac function noninvasively in early mouse embryos. The first targeted mutants to show a major early embryonic phenotype in the nervous system were those in the protooncogene Wnt-1 (6, 7). We show that UBM is a sensitive method for distinguishing the midhindbrain deletion of 10.5- and 11.5-day Wnt-1 mutant embryos. Furthermore, the neural tube cavity was computer segmented from 3D UBM data and displayed in a volumetric rendering, allowing quantitative comparisons to be made between mutant and normal embryos. Taken together, these results demonstrate that this relatively inexpensive technique could have many applications in studying the biological roles of genes in mice.

MATERIALS AND METHODS

UBM. Full details of the UBM system used in this report have been given (4, 5). The images from the UBM system are 512 × 512, 8-bit pixels covering an area of 8 × 8 mm². The image frame rate was between 3 and 10 frames per sec, and 3D data were collected as a series of 2D images. The measured spatial resolution at the focus of the 50-MHz transducer is 30 μm in the axial (depth) direction and 60 μm in the lateral (horizontal) direction. This frequency was chosen to obtain adequate penetration (4–5 mm) in order to image the mouse embryos in utero. For excised, fixed embryos, a 60-MHz transducer was used with measured spatial resolution of 20 μm (axial) and 35 μm (lateral), but the depth of field with this transducer (1–1.5 mm) was too narrow for in utero imaging. Contrast in the UBM images results from the reflectivity or ultrasonic backscatter properties of the tissue through which the ultrasound propagates.

Embryo Imaging. Animals used in these studies were maintained according to protocols approved by local Animal Care and Use Committees. Wild-type mouse (CD1; Charles River Breeding Laboratories) embryos ranging in age from 9.5 to 11.5 days of embryogenesis were imaged with UBM. Day 0.5

Abbreviations: UBM, ultrasound backscatter microscopy; 2D, two dimensional; 3D, three dimensional; MR, magnetic resonance.
†Present address: Skirball Institute of Biomolecular Medicine, New York University Medical Center, 540 First Avenue, New York, NY 10016.
‡To whom reprint requests should be sent at the present address.
is defined as noon of the day a vaginal plug was found after overnight mating. In preliminary studies, embryos were dissected out of the uterus and fixed in buffered formalin. Each embryo was laid on its side in an ultrasonic coupling gel immersed in a small bath of phosphate-buffered saline for scanning with the UBM system. In utero images were made of embryos in both recently sacrificed and anesthetized pregnant mice [10% (vol/vol) sodium pentobarbital, 10 μl per g of body weight]. In either case, the abdomen and lower back regions of the pregnant mice were shaved to provide a clear window to the embryos, and a small water bath was fitted onto the skin to provide a fluid coupling medium for the transducer (see Fig. 2a). Cultured embryos (see below) were imaged with UBM by placing the embryos in ultrasonic coupling gel covered with Tyrode’s salt solution in a stage warming device (Biopotech, Butler, PA).

**Whole Embryo Culture Model.** Since the mouse embryo can be cultured in vitro for periods of up to 72 hr throughout the major periods of cardiac organogenesis, it provides an excellent model to study in situ cardiac development. Embryos were removed from the uterus and decidual mass on day 8 of gestation. Reichert’s membrane was removed and the embryos were then cultured in a roller bottle apparatus at 38°C in a mixture of 75% rat serum/25% Tyrode’s solution under a gas phase of 5% O2/5% CO2/90% N2 until being removed for UBM examination. Developmental milestones, total protein content, and total DNA content of embryos cultured by this technique are comparable to embryos developing in utero (6).

**3D Image Analysis.** An image manipulation program was used to reformat arbitrary image planes through a 3D set of image data. This program runs on a Macintosh Quadra computer, reconstructing oblique planes in close to real time (program provided by Shane Dunne and Aaron Fenster, Roberts Research Institute and University of Western Ontario, London, ON, Canada). Volumetric renderings of the neural tube cavities were produced with a 3D image workstation (Allegro; ISG Technologies, Mississauga, ON, Canada). In this case, the 3D image data were smoothed to reduce the speckle pattern inherent in the ultrasound images, and thresholds were set to enhance the neural tube region. The computer program was then used to detect the boundary of the neural tube cavity in each 2D UBM image and to reconstruct the surface of the neural tube, which can be viewed by rotating the image and adjusting several light sources.

**RESULTS AND DISCUSSION**

To test the feasibility of UBM for imaging embryos, 9.5- to 11.5-day embryos were dissected from the mother and fixed either intact in the uterus or after dissection from the uterus. The fixation process, which hardens the tissue, made it difficult to penetrate through the fixed uterus with UBM to image the embryos inside. However, the excised, fixed embryos could be imaged with a highly focused 60-MHz transducer (see Materials and Methods). Fig. 1 a–d shows two comparisons between the UBM images and matched histological sections of a 10.5-day embryo. These images are part of a complete set of 80 coronal images, each separated by 50 μm, extending 4 mm from the dorsal to the ventral side of the embryo. A 3D image manipulation program (see Materials and Methods) was used to reconstruct other image planes. Fig. 1e shows a midsagittal plane, which displays excellent contrast between the neural cavities and the surrounding tissue. The somites running along the back of the embryo are also evident on the UBM image as a series of faint lines.

Volumetric image data were collected from numerous mouse embryos and analyzed in several ways. The capability of reconstructing arbitrary planes from 3D UBM image data is important in imaging embryos, since the images that can be acquired in utero are not necessarily those of the most interest. The ability to

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**Fig. 1.** Comparison between UBM and histological sections of excised embryos. Displayed are two UBM (a and c) and histology (b and d) (stained with haematoxylin and eosin) coronal sections of a fixed 10.5-day mouse embryo. The UBM images and the histological sections have been matched in both size and position in the embryo. (c) A 3D image manipulation program was used to reconstruct the midsagittal plane from the set of coronal images, revealing the neural tube cavities outlining the morphology of the brain. F, forebrain; M, midbrain; H, hindbrain; T, telencephalic vesicle; MB, mandible; OV, otic vesicle; HT, heart; DA, dorsal aorta; HL, hindlimb; FL, forelimb; S, spinal cord. (Bar = 500 μm.)
To explore the use of UBM for imaging live embryos, pregnant mice were either sacrificed or anesthetized, and the embryos were imaged in utero (see Materials and Methods). A 50-MHz transducer was used for this application, since it provided more penetration and a greater depth of field (3–4 mm) than that used to image the fixed embryos (Fig. 2). UBM images of 9.5- to 11.5-day embryos showed clearly the developing neural tube cavity, which is visualized as a dark region in the image, void of echoes. Fig. 2b shows the in utero UBM image of an 11.5-day embryo, demonstrating that the neural cavities outlining the morphology of the developing brain are delineated almost as clearly as in the excised fixed embryos. The heart was also evident as an echogenic (bright) structure (Fig. 3a) and real-time images of live embryos highlighted the neural cavities and the beating heart. It should be noted that after imaging several mouse embryos each day from day 9.5 to 11.5, we let the pregnancies go to term and observed no deleterious effects on the live-born animals.

The high embryonic heart rate together with the respiratory and cardiac motion of the pregnant mother makes it difficult to clearly visualize the developing heart chambers with the existing UBM system, since there is significant motion during the time the image data are acquired. Using cultured 9.5- and 10.5-day embryos, the maternal motion was eliminated and the heart rate could be reduced to close to 60 beats per minute by lowering the temperature of the saline in which they were imaged (see Materials and Methods). At this lower heart rate, the lumens of the atria and ventricles were clearly visible using the same transducer and scanner settings that were used for in utero imaging. Fig. 3b shows an image of a 9.5-day cultured embryo, demonstrating the lumen of the ventricle and the left and right components of the common atrial chamber. Using the higher-resolution 60-MHz transducer, we were able to identify the lumen of the primitive heart tube in an 8.5-day embryo, which measured <100 μm in diameter (not shown). These results suggest that UBM could be developed to assess early heart function and to compare quantitatively normal and abnormal heart development, either by developing fast quantitative imaging techniques or by augmenting the UBM system to include Doppler ultrasound blood flow measurements. The most feasible imaging method would be an “M-mode” technique, in which the transducer is held stationary and the image displays the single depth-dependent ultrasound signal on the y axis as a function of time on the x axis. A frame rate of at least 30 images per sec could be achieved by this approach.

The high contrast in the neural tube cavity indicated that UBM could be used to compare normal and abnormal embryonic neural development in mice in utero. To address whether this should be possible, embryos homozygous for a targeted mutation in the Wnt-1 protooncogene (7) were imaged and compared to wild-type littermates. Homozygous Wnt-1 mutant embryos have been shown to develop a deletion of the mesencephalon and rostral hindbrain that is obvious starting at day 9.5 (7, 8). Wnt-1 heterozygous intercrosses were set up and the mothers were sacrificed at day 10.5 or 11.5 of embryogenesis. To facilitate localizing comparable sagittal sections of the embryos with UBM, freshly dissected uteri were imaged in a small bath of phosphate-buffered saline.
oriented to produce sagittal sections of each embryo. A complete 3D image set was collected for most of the embryos, using the same transducer and scanner settings that were used to produce the *in utero* images shown above. Immediately after the imaging procedure, each embryo was dissected out of the uterus and fixed in buffered formalin for confirmation of phenotype and subsequent histological analysis.

Three litters of mouse embryos were imaged, consisting of 7 homozygous mutants and 24 normal embryos. The midhindbrain deletion in each of the 7 mutant embryos was obvious from the UBM images and was confirmed by examining the dissected whole embryos and, in some cases, the histological sections. For two of the litters, the genotypes of most of the embryos were confirmed by Southern blot analysis of yolk sac tissue (7). Fig. 4 shows a comparison between a 10.5-day homozygous mutant *Wnt-1* embryo and a normal littermate, demonstrating the clear differentiation of mutant and normal phenotypes on UBM.

Computer-reconstructed volumetric renderings (see Materials and Methods) of the isolated neural tube cavities were performed to allow quantitative comparisons to be made between mutant and normal embryos. A deletion of likely all of the midbrain and the rostral hindbrain stood out in these reconstructions (Fig. 4 g and h). The rendered display of the isolated neural cavity provides an intuitive format, which can be rotated on the computer monitor making it easier to appreciate the 3D structure than a series of 2D images. It should be noted that these UBM data were collected from dissected uteruses, so the problems of image misregistration due to motion were eliminated. Obviously, robust algorithms for registering the sequential images must be developed in order to make volumetric image display a useful tool for *in

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**Fig. 4.** UBM analysis of *Wnt-1* homozygous mutant embryos. Comparison is shown between a 10.5-day *Wnt-1* mutant embryo (*a, c, e, and g*) and a normal littermate (*b, d, f, and h*), which shows the whole, dissected embryos (*a and b*), midsagittal UBM images (*c and d*), midsagittal histological sections (*e and f*), and volumetric renderings of the cavity of the anterior neural tube (*g and h*). F, forebrain; M, midbrain; H, hindbrain. (Bar = 500 μm.)
**CONCLUSIONS**

These studies demonstrate that UBM is a technique allowing *in utero* imaging of early embryonic development in live mice. Furthermore, 3D image data can be exploited both to reformat oblique image planes and to determine volumetric changes in developing tissues. The only other method we are aware of that might yield similar image data from developing mouse embryos is magnetic resonance (MR) microscopy. Using high-strength magnetic fields and gradients, MR microscopy can obtain volumetric image data with resolution between 10 and 50 μm. However, the imaging times are 30 min or more (often many hours), thus excluding real-time imaging of moving structures such as the beating heart or surgical procedures (see below). Smith *et al.* (10) have shown MR microscopy of excised and fixed 9.5- to 16.5-day mouse embryos, including visualization of the skeletal and vascular systems of mouse embryos and volumetric renderings of the segmented neural tube. Jacobs and Fraser (11) have demonstrated the potential of MR microscopy for studying cell lineages *in vivo* in developing frog embryos with a gadolinium-based contrast agent. It would be interesting to explore the possibility of using ultrasound contrast agents (12) to perform similar functional studies in mouse embryos by UBM.

We have presented *in utero* images of early mouse embryos by UBM and have shown the potential for identification and quantitation of genetic alterations affecting brain development in the mouse by a noninvasive imaging method. The application of UBM to the study of the development of early abnormalities in mouse mutants will allow for gross analysis of live embryos to pinpoint when important phenotypic changes occur. This could be particularly important in genetic screens for embryonic lethals. With the rapid expansion of the numbers of neurological mutants being generated by targeted mutagenesis (13), there is an urgent need for improved methods of analyzing phenotypic defects. UBM could be of considerable use in studying neurological mutants. Preliminary results suggest that with further technological development, UBM could be useful for studying heart development and assessing embryonic cardiac function *in utero*. Another promising application of UBM is for neural transplantation and injection studies. At present, day 12.5 of mouse embryogenesis is the earliest stage that is easily amenable for injecting cells or agents into the neural tube *in utero*. It should be possible to perform cell transplantation or marking studies at earlier stages (days 9-10.5) by using UBM guidance to introduce cells or retroviruses in specific regions of the neural tube.

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