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In utero ultrasound backscatter microscopy of early stage mouse embryos

Daniel H. Turnbull*

Skirball Institute of Biomolecular Medicine and Departments of Radiology and Pathology, New York University School of Medicine, 540 First Avenue, New York, NY 10016 USA

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Abstract

A high resolution ultrasound imaging technique, ultrasound backscatter microscopy (UBM), has previously been shown to be useful for in utero imaging of mouse embryos, and for direct manipulation of mouse embryos through UBM-guided injections. UBM images from mouse embryos staged between 8.5 and 10.5 days of gestation are presented to demonstrate the range of anatomical structures which can be studied with this approach. Ultrasound contrast agents have been injected into the forebrain ventricle of 10.5 day embryos to characterize the resulting three-dimensional distribution of the injected agents. These studies provide important background data relevant to future use of this technique for in utero analysis of early brain and heart development, and for in utero manipulation of mouse embryos through UBM-guided injections.

Keywords: Ultrasound imaging; Ultrasound backscatter microscopy; Mouse embryos; Brain and heart development

1. Introduction

The mouse is the primary model of mammalian development. With the recent introduction of transgenic and gene targeting methods, the mouse has also been used to develop increasing numbers of human disease models. Alteration or misexpression of genes involved in normal development and many diseases often lead to phenotypic changes which are first evident at early embryonic stages. Previously, we demonstrated that high frequency (40–50 MHz), high resolution (50–100 μm) ultrasound imaging, referred to as ultrasound backscatter microscopy (UBM), is a useful method for visualizing live mouse embryos in utero [1]. More recently we have used UBM for noninvasive analysis of early mouse cardiac development [2], and have developed a UBM-guided Doppler system for quantitative measurement of blood flow in early mouse embryos [3]. UBM has also been developed as a tool for in utero guided injections into mouse embryos, allowing transplantation of cells between different regions of the developing brain [4] and gene misexpression studies through the use of transfected cells [5] and retroviruses.

Prior to the introduction of UBM techniques, the study of embryogenesis in the mouse was limited to histological analysis [6], and the use of high resolution imaging of fixed specimens using techniques such as scanning electron microscopy (SEM) [6] and magnetic resonance microscopy (MRM) [7]. While MRM images of live midgestation mouse embryos have been demonstrated recently [8], at this time UBM is the only easily accessible, real-time imaging method for noninvasive analysis of mouse embryos, and the only method available for image-guided manipulation of mouse embryos, in utero. In this study, in utero UBM images of early stage mouse embryos, from 8.5 to 10.5 days of gestation (E8.5–E10.5) are presented. The images of E8.5 mouse embryos, approximately equivalent to three week human embryos [6], provide the first demonstration of UBM to image structures at this early stage of development. The embryos shown in this paper (E8.5–10.5) are representative of very early stages of brain and heart patterning and cell differentiation. These imaging studies therefore provide important background data for future investigations of neural and cardiac development using UBM imaging and UBM-guided injections into early stage mouse embryos.

2. Materials and methods

2.1. Animals

All animals used in these studies were maintained under protocols approved by the Institutional Animal Care and Use Committee of the New York University Medical

* Tel.: + 1 212 263 7262; Fax: + 1 212 263 8951; e-mail: turnbull@saturn.med.nyu.edu

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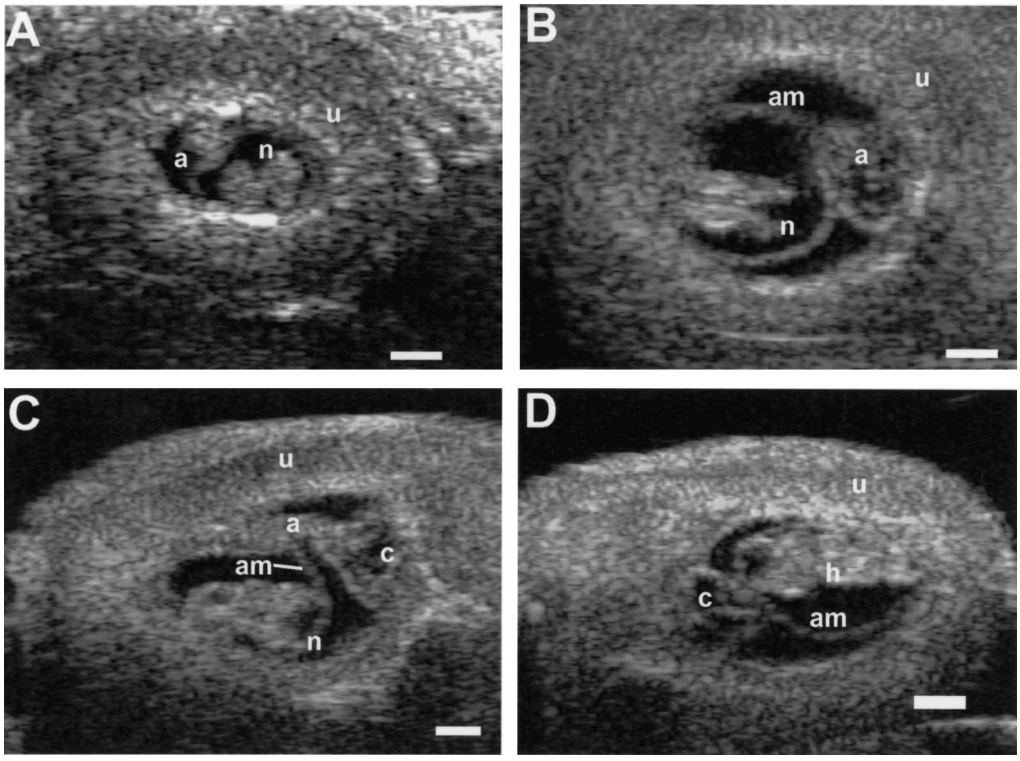

Fig. 1. UBM images of E8.5 mouse embryos. (A): Transabdominal image of E8.5 embryo, demonstrating the neural folds (n) and allantois (a) inside the uterine wall (u). (B,C,D): After laparotomy and exposure of the uterus (u), the neural folds (n), allantois (a), chorion (c) and amnion (am) are clearly visualized. Cardiac activity was detected on real-time UBM in the primitive heart (h). Scale bars (A,B,C,D) = 500 μm.

Center. Timed pregnant Swiss-Webster mice (Taconic, Germantown NY) were anesthetized with sodium pentobarbital (0.5 mg/10 g body weight, injected intraperitoneally) mixed with magnesium sulfate ($MgSO_4 \cdot 7H_2O$, 1 mg/10 g body weight) as a mild muscle relaxant. Noninvasive, transabdominal imaging of the embryos was performed as described previously [1,2]. Briefly, the abdomen and lower back region of the anesthetized mouse were wet shaved and a small water bath fitted over the skin to provide fluid coupling between the transducer and tissue. In utero imaging was accomplished by mechanically scanning the UBM transducer over the shaved skin of the pregnant mouse. UBM imaging of the surgically exposed uterus and injection into mouse embryos was performed as described previously [4,5]. In this case, a small incision was made in the skin and peritoneal wall, and a piece of the uterus positioned under the ultrasound transducer in a bath of sterile phosphate buffered saline (PBS) attached over the mouse's abdomen. In these studies embryos were staged in days of gestation, where day 0.5 (E0.5) was defined as noon of the day a vaginal plug was detected after overnight mating.

2.2. UBM imaging and UBM-guided injections

The UBM system used in these studies [3] was very similar to that described previously for high resolution ultrasound imaging [9]. Focused UBM transducers were mechanically scanned over the tissue to produce 8 mm × 8 mm, 512 × 512 × 8 bit images at frame rates of 4–8 images per second. The transducers were operated at frequencies between 40 and 50 MHz, with measured axial resolution between 25 and 30 μm, and lateral resolution between 60 and 90 μm [3,9]. Penetration of ultrasound at these high frequencies was between 6 and 10 mm, which was adequate for transabdominal imaging of most embryos [2]. Three-dimensional (3-D) UBM data were acquired as a series of 2-D images, incrementing the position of UBM image plane in steps of 50–100 μm using a precision motion stage (Model 105021P-20M, Parker-Daedal, Harrison City PA) and stepper motor (Model 5023-094, Applied Motion Products, Watsonville CA). 3-D data sets consisting of 50–80 UBM images were acquired in less than 30 s. UBM-guided injections were performed with pulled, sharpened glass microcapillary needles, mounted on a three-axis micromanipulator stage (Model MO-155, Narishige, Tokyo), and using an oil filled microsyringe pump (Stoelting, Wood Dale IL) to control the injection, as described previously [4,5].

2.3. Contrast injections and 3-D visualization

Suspensions of cells were prepared and injected into the E9.5 neural tube cavity as described previously [5]. One microliter of an experimental ultrasound contrast agent (LU 95101-1, Schering AG Berlin) was injected into the

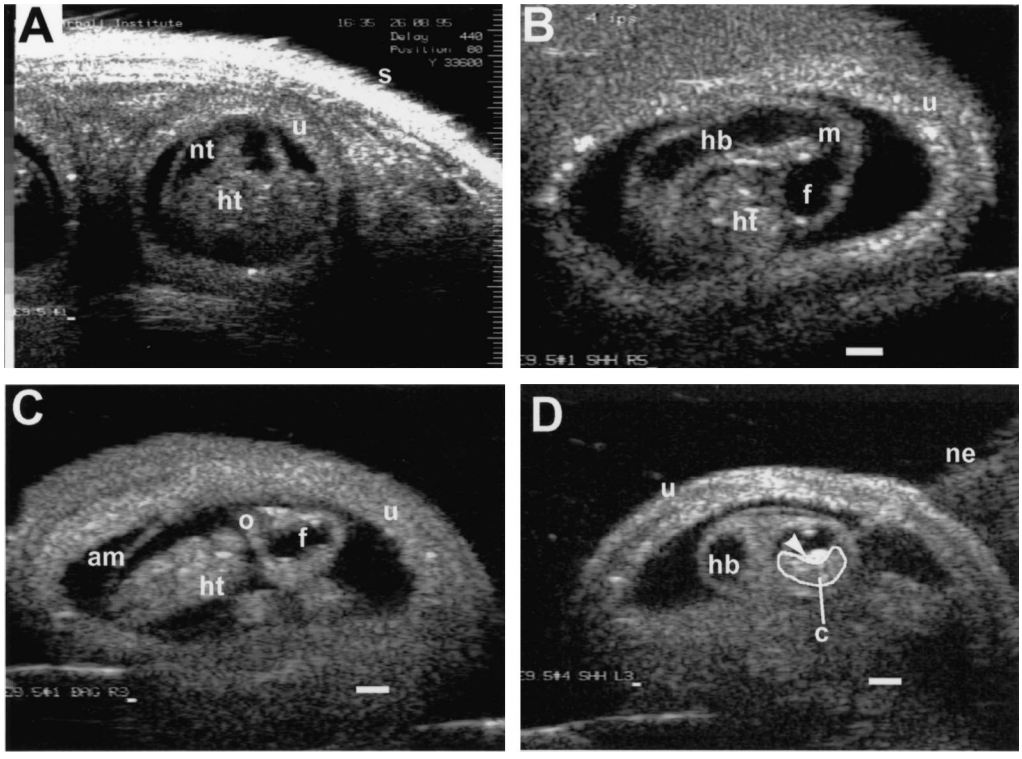

Fig. 2. UBM images of E9.5 mouse embryos. (A): Transabdominal sagittal image of E9.5 embryo, demonstrating the heart (ht) and neural tube cavity (nt). Smallest scale increments (right) = 100 μm. (B): Similar sagittal view of an E9.5 embryo after surgical exposure of the uterus. (C): Coronal view of an E9.5 embryo. (D): E9.5 embryonic brain with the tip (arrowhead) of an injection needle (ne) in the forebrain ventricle. Injected cell suspension (outlined in white) can be visualized filling the ventricle. Labels: am, amnion; c, cell suspension; f, forebrain; hb, hindbrain; ht, heart; m, midbrain; o, optic vesicle; s, skin; u, uterus. Scale bars (B,C,D) = 500 μm.

forebrain ventricle of E10.5 embryos to study the resulting spatial distribution of agents in the neural tube cavity. The contrast agent consisted of encapsulated microbubbles, with a distribution of diameters ranging between 0.1 and 5 μm. A volumetric image rendering and analysis program (Voxelview 2.5, Vital Images, Fairfield IA) running on an off-line workstation (Indigo2, Silicon Graphics, Mountain View CA) was used to segment, reconstruct and display surface renderings of the contrast agent distribution from 3-D UBM data. Segmentation of the contrast agent distribution was obtained using a seed fill algorithm which detected all contiguous voxels greater than a user-defined threshold value.

3. Results

3.1. In utero UBM imaging

Live mouse embryos were imaged in utero, over a range of post-implantation stages from 8.5 to 10.5 days post coitus (E8.5–E10.5). These are critical stages of brain patterning through early neurogenesis, and are also the earliest stages of heart development. Morphologically, the embryo goes through a complicated turning process between E8.5 and E9.5 [6], during which the dorsal region of the embryo is transformed from being the concave (inside) surface of the ''U-shaped'' embryo at E8.5, to being the convex (outside) surface at E9.5. During this same period, the open cephalic neural folds (E8.5) grow out and turn over, closing to form a fluid-filled neural tube (E9.5) which later develops into the CNS of the adult mouse. The primitive heart is transformed from a straight tube (E8–E8.5) through a looping process to form a single atrium and ventricle by E9.5. The initial stages of atrial and ventricular septation around E10.5 give rise to recognizable precursors of the final four cardiac chambers.

Figs. 1–3 demonstrate the main features of early brain and heart development which are apparent in UBM images of E8.5–E10.5 mouse embryos. Each of these figures shows one image acquired transabdominally (i.e., completely noninvasively), and three additional in utero images acquired after surgical exposure of part of the uterus (see Materials and methods, Section 2). This approach was taken for several reasons. As one of the major applications foreseen for UBM imaging is to directly manipulate mouse embryos through guided injections [4,5], the images presented in this study provide important background data to aid future users of this technology in identifying anatomical structures for UBM-guided injections. Surgical exposure of the uterus also made it easier to obtain standard orientations in the UBM image planes, and the resulting image resolution was somewhat improved after removing

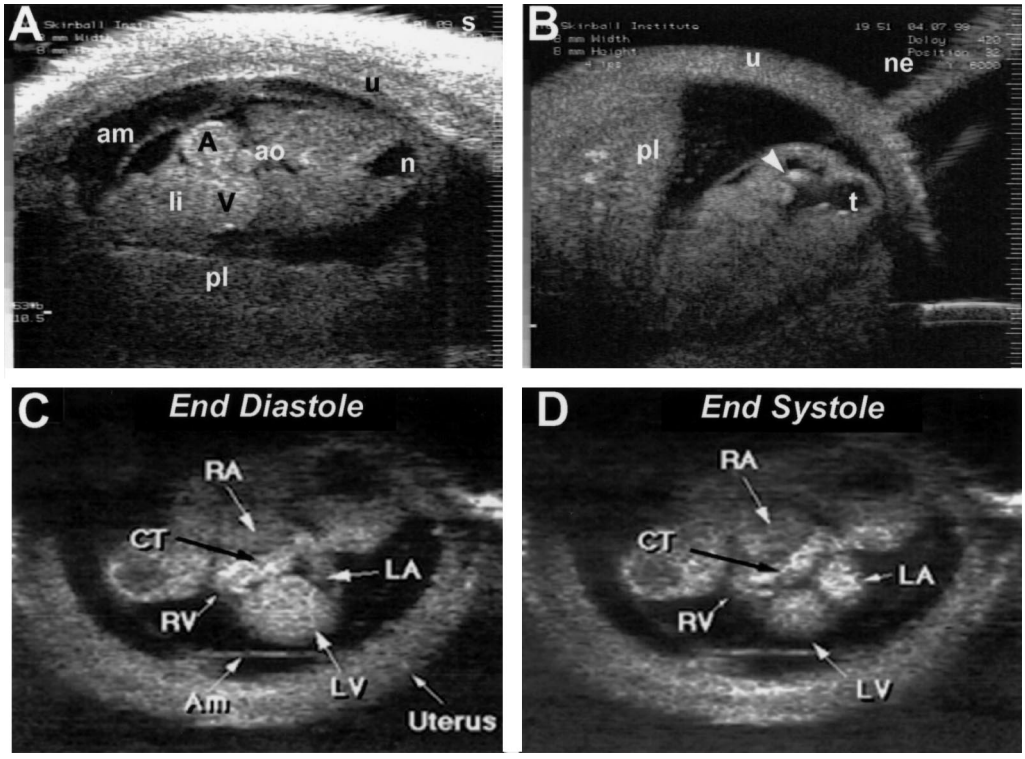

Fig. 3. UBM images of E10.5 mouse embryos. (A): Transabdominal image of E10.5 embryo demonstrating the visualization of neural tube (n), liver (li) and cardiac structures: A, atrium; ao, aorta; V, ventricle. Other labels: am, amnion; pl, placenta; u, uterus. (B): E10.5 embryonic brain with tip (arrowhead) of injection needle (ne) inserted through the uterus (u) and into the telencephalic ventricle (t). Smallest scale markers (right; A, B) = 100 μm. (C,D): Detailed cardiac anatomy visualized by UBM of surgically exposed uterus, with image frames captured at end diastole (C) and systole (D). (C,D) have been oriented to allow direct comparison with SEMs of similar stage mouse embryo heart ([6]: Plate 65n). Labels: Am, amnion; CT, conotruncus; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

the intervening skin and muscle layers. Nevertheless, it is obvious from the images presented that all of the structures which were visualized in the exposed uterus could also be identified using noninvasive transabdominal UBM embryo imaging [1–3]. This is important for performing serial imaging studies of individual embryos and for noninvasive detection and in utero analysis of mutant embryo phenotypes.

3.2. UBM images of E8.5–E9.5 mouse embryos

At E8.5, the most prominent features evident on UBM images were the open neural folds, which made it straightforward to differentiate between embryonic and extra-embryonic tissues (Figs. 1A,B). In more advanced E8.5 embryos, the initial formation of the chorio-allantoic placenta could be visualized (Fig. 1C) and real-time UBM images made it possible to detect cardiac activity in the beating primitive heart tube (Fig. 1D). By E9.5, the neural tube and beating heart were prominent on real-time UBM images (Figs. 2A,B,C). The major subdivision of the neural tube into forebrain, midbrain and hindbrain regions, and even smaller ventricular subdivisions such as the optic and otic vesicles, were identified. Cells and other agents could be injected into the closed neural tube cavity using UBM-guidance [5]. In addition, cell suspensions injected into the neural tube cavity produced a high-intensity signal which was easily identified and monitored on UBM images (Fig. 2D).

3.3. UBM images of E10.5 mouse embryos

At E10.5, the shape and subdivisions of the neural tube cavity were easily identified on UBM images [1], (Fig. 3B, Figs. 4A,C,E), making injections into specific regions, such as the telencephalic ventricles (Fig. 3B, Fig. 4A), straightforward. The significant thinning of the uterine wall between E8.5 (Fig. 1) and E10.5 (Fig. 3) made UBM-guided injections easier with increasing embryonic stage. The embryonic heart was sufficiently well developed at E10.5 to make out the developing atria and ventricles (Figs. 3A,C,D). Previously we showed that noninvasive, dynamic measurements of cardiac chamber dimensions could be performed with UBM imaging starting at E10.5 [2]. The liver primordium, which undergoes its first expansion at E10.5, could be identified lying posterior to the heart (Fig. 3A). The high UBM signal from blood and the resulting moving speckle pattern on real-time images [2,3] made it possible to identify major blood vessels such as the aorta (Fig. 3A), conotruncus or outflow tract (Figs. 3C,D) and

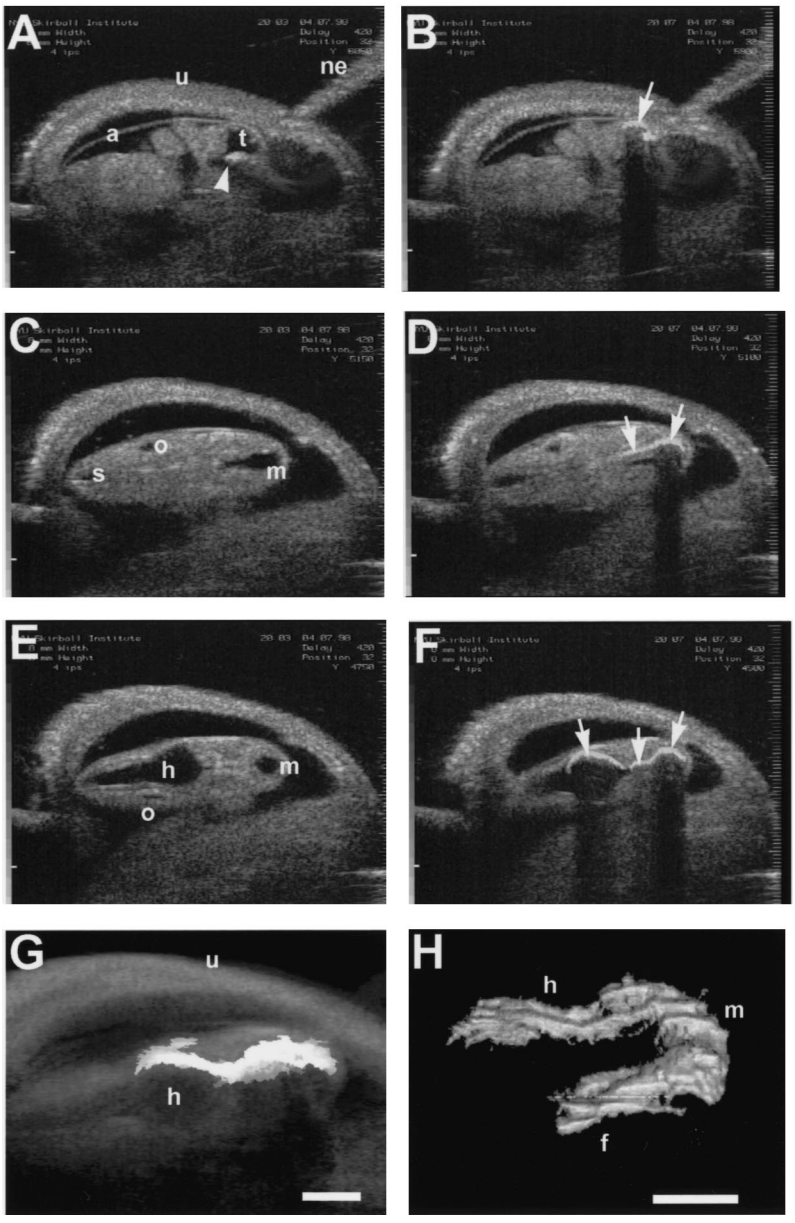

Fig. 4. Contrast injection into E10.5 forebrain ventricle. Coronal images of an E10.5 mouse embryo before (A,C,E) and after (B,D,F) injection of an experimental contrast agent. (A) The tip (arrowhead) of the injection needle (ne) is shown inside the telencephalic ventricle (t). (B,D,F) After injection, the contrast agent is detected in the forebrain ventricle (arrow), shadowing the underlying image (B). Contrast agent (arrows) is also detected 800 μm (D) and 1200 μm (F) posterior to the injection site, in the midbrain and hindbrain ventricles. Smallest scale markers (right; A–F) = 100 μm. (G): Volumetric rendering of the distribution of contrast agent in the neural tube cavity with the surrounding tissue set to 50% opacity. (H): Surface rendering of the contrast agent with the surrounding tissue removed. Scale bars (G,H) = 500 μm. Labels: a, amnion; f, forebrain; h, hindbrain; m, midbrain; ne, injection needle; o, otic vesicle; s, spinal cord; t, telencephalon; u, uterus.

umbilical vessels [2,3]. This should be most important for future measurements of blood flow parameters in mouse embryos using UBM-guided Doppler [3].

3.4. Neural tube contrast injections

Cell suspensions injected into the neural tube cavity were visualized filling the brain ventricles (Fig. 2D), even in regions far removed from the initial injection site [5]. In order to characterize the spatial distribution of agents injected into the anterior neural tube, an experimental ultrasound contrast agent was injected into the telencephalic ventricles of eight E10.5 embryos. In each case, the injected volume was 1 μl, which is similar to the volume of cell suspensions injected in previous studies [4,5]. The contrast agent produced a very high UBM signal after injection (Figs. 4 B,D,F). Indeed the backscatter signal from the contrast agent was so high that the UBM image underlying the agent was completely shadowed, with the result that the image data only showed the top surface of the contrast agent

distribution. Nevertheless, it was apparent in all eight embryos that the injected volume (1 μl) quickly dispersed from the injection site and distributed itself throughout the neural tube cavity from the telencephalon well back into the hindbrain cavity (Figs. 4G,H). This is consistent with previous findings that cell suspensions injected into the E9.5 forebrain ventricle, resulted in cell aggregates attached randomly throughout the anterio-posterior extent of the neural tube [5]. Note that the irregularities in the surface rendering of the contrast agent (Fig. 4H) are predominantly because of motion, caused by the breathing of the pregnant mouse, during 3-D data acquisition.

4. Discussion

This study demonstrates the feasibility of noninvasive analysis of early embryonic brain and heart development in mouse embryos over a range of postimplantation stages from 8.5 to 10.5 days of gestation (E8.5–E10.5). In particular, these are the first UBM images of E8.5 mouse embryos. This is an important stage of brain development, when many genes involved in patterning of the CNS are first expressed. UBM-guided injections at E8.5 should be feasible. For example, it would be interesting to inject retroviruses into the amniotic fluid at E8.5 to infect neuroepithelial cells of the open head folds in order to misexpress specific genes or trace cell lineages in the early developing CNS. The representative UBM images presented here, covering three days of normal early embryonic development, should be useful to those employing this technology to compare abnormal and normal developmental events through the use of defined mutant mouse strains, to investigate dynamic developmental events such as structure/function relationships in the cardiovascular system, to perform serial imaging studies of individual embryos in utero, and to screen pregnant mice for mutant or transgenic embryos. This last application is important in view of recent interest shown in searching for new developmental genes in the mammalian genome using mutagenesis screens in mice [10]. The images presented here also provide background data for investigations using UBM-guided injections into early stage mouse embryos [5]. In particular, these results demonstrate that injections of small volumes into the early neural tube cavity can be expected to result in a wide initial distribution of the injected agents throughout the developing CNS. The use of ultrasound contrast agents with UBM-guided embryo injections provides a simple and instantaneous method to assay the effectiveness and precision of targeted injections into parenchymal tissues. For survival studies in embryos, the ultrasound contrast agents must first be tested for possible toxic effects, which has not been done at this time.

Three-dimensional (3-D) UBM image acquisition and visualization has been employed to characterize the distribution of contrast agent in the neural tube cavity. Previously, a similar approach was used to segment the neural tube cavities in E10.5 mouse embryos and to quantify the mid-hindbrain deletion in embryos homozygous for a null mutation in the gene *Wnt-1* [1]. The routine application of 3-D imaging methods to UBM has the potential to greatly improve the technology, providing more accurate confirmation of the target site in UBM-guided injections and to enable the use of reformatted image planes and segmentation of specific anatomical structures to facilitate the detection and analysis of mutant mouse phenotypes at early embryonic stages. At this time, the most significant impediment to progress in this area are image artifacts caused by respiratory motion. A possible solution to this problem would be the implementation of triggered 3-D UBM image acquisition, where each image frame in a 3-D data set is captured at the same phase in the respiratory cycle.

5. Summary

In conclusion, UBM is the only method available currently for in utero imaging of early stage mouse embryos, and for manipulation of mouse embryos through UBM-guided injections. The combination of this high resolution ultrasound technology with advances in mouse genetics provides a new and powerful method to study mammalian developmental processes in the mouse. This paper demonstrates representative in utero UBM images of mouse embryos staged between 8.5 and 10.5 days of gestation, which should provide important background data for the future use of this approach to analyze early embryonic brain and heart development, and to perform targeted injections into mouse embryos using UBM image guidance.

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Daniel H. Turnbull was born in Niagara Falls, Ontario, Canada in 1955. He received the B.Sc. degree in Mathematics in 1978 from Brock University, St. Catharines, Ontario, Canada and the M.S. degree in Applied Mathematics in 1981 from the California Institute of Technology, Pasadena, California. He also received the M.A.Sc. degree in Mechanical Engineering in 1983 and the Ph.D. degree in Medical Biophysics in 1992, both from the University of Toronto, Toronto, Ontario, Canada.

From 1983 to 1986, he was a Systems Engineer at Imatron Inc., a medical imaging company in South San Francisco, California. He worked as a Medical Physicist at the Toronto-Sunnybrook Regional Cancer Center from 1991 to 1994 and was made an Assistant Professor of Medical Biophysics, University of Toronto in 1994. Since September 1994, he has been an Assistant Professor of Radiology and Pathology at the Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY. His broad research interests involve the application of high resolution ultrasound and magnetic resonance imaging methods for in vivo investigations of embryonic development and human disease models in transgenic mice.