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40-MHz Annular Array Imaging of Mouse Embryos

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Abstract

Ultrasound biomicroscopy (UBM) has emerged as an important *in vivo* imaging approach for analyzing normal and genetically-engineered mouse embryos. Current UBM systems use fixed-focus transducers, which are limited in depth-of-focus. Depending on the gestational age of the embryo, regions of interest in the image can extend well beyond the depth-of-focus for a fixed-focus transducer. This shortcoming makes it particularly problematic to analyze 3D datasets and to generate accurate volumetric renderings of the mouse embryonic anatomy. To address this problem, we have developed a 5-element, 40-MHz annular array transducer and a computer controlled system to acquire and reconstruct fixed- and array-focused images of mouse embryos. Both qualitative and quantitative comparisons showed significant improvement with array-focusing including an increase of 3–9 dB in signal-to-noise ratio and an increase of at least 2.5-mm in depth-of-focus. Volumetric rendered images of brain ventricles demonstrated the clear superiority of array-focusing for 3D analysis of mouse embryonic anatomy.

Keywords

High frequency arrays; Ultrasound biomicroscopy; 3D reconstruction; micro-imaging; mouse imaging

INTRODUCTION

With the acceptance of the mouse as a genetic model organism for studies of human development and disease, the need for effective *in vivo* micro-imaging approaches tailored to the mouse has become obvious. Ultrasound biomicroscopy (UBM), a high frequency pulse-echo method, has emerged as an important imaging modality for *in utero* analysis of both normal and genetically-engineered mouse embryos (reviewed in Turnbull and Foster 2002). Indeed, UBM provides a unique real-time micro-imaging method for studying mouse cardiovascular development (reviewed in Phoon and Turnbull 2003), and for direct manipulation of mouse embryos via UBM-guided injection of cells, viruses and other agents (Olsson et al. 1997; Gaiano et al. 1999; Wichterle et al. 2001).

In the decade since UBM was first introduced for imaging mouse embryos (Turnbull et al. 1995), UBM technology has progressed significantly in terms of higher image frame-rates (currently up to ~100 frames/s), multiple imaging frequencies (over the 30–60 MHz range) and newer digital image processing methods (e.g., Foster et al. 2002; Goertz et al. 2003). Nevertheless, current UBM systems continue to be based on single-element focused

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polyvinylidene difluoride (PVDF) transducers, much the same as those described in the original UBM systems (Sherar and Foster 1989). For imaging mouse embryos, the geometrical extent of many regions of interest, such as brain ventricles or vascular structures, can be close to an order of magnitude greater than the depth-of-focus (DOF) of a fixed-focus transducer. This makes volumetric analysis of developing mouse embryos, including effective segmentation of three-dimensional (3D) anatomy from UBM images, difficult or impossible in many cases.

An obvious approach to increase DOF in UBM images is to employ multi-element array transducers. Linear arrays are most common for conventional ultrasound imaging, because of the advantages of electronic focusing and steering, eliminating the need for mechanical scanning of the transducer. However, the technical challenges of fabricating linear array transducers with large numbers of elements, and element-element spacing on the order of a wavelength or less has impeded progress for high frequency UBM (Ritter et al. 2002). An alternative approach is to employ a high frequency annular array transducer, with a relatively small number of annular elements to focus the beam in the axial direction, resulting in UBM images with significantly increased DOF although mechanical scanning is still required to form 2D images (Brown et al. 2004; Brown and Lockwood 2005).

Previously, we described the development of a 5-element, 40-MHz PVDF annular array transducer for UBM imaging (Ketterling et al. 2005). The operational capability of this transducer was recently verified using an offline synthetic array-focusing method (Ketterling et al. 2006). Wire phantom measurements demonstrated that the two-way echo amplitude was enhanced over approximately a 10-mm range about the passive focus. Furthermore DOF (−6 dB) was increased from approximately 1-mm (fixed-focus) to 5-mm (array-focus), and a lateral resolution of 80 μm was maintained over a 6 mm depth range about the passive focus (Ketterling et al. 2006). It is expected that heterogeneous and attenuating biological media will degrade annular array performance compared to wire phantom experiments. The aim of the current study was to determine the potential of this 40-MHz annular array transducer for imaging mouse embryos, *in utero*. To this end, we have analyzed fixed- and array-focused volumetric UBM images acquired from the same mouse embryos, directly comparing image quality, 3D renderings and volumes of cerebral ventricles, and quantitative estimates of signal-to-noise-ratio (SNR) and DOF from embryo images. Our results show an increase of approximately 2.5-mm in DOF after array-focusing, and demonstrate a clear superiority of the annular array transducer for both 2D and 3D UBM imaging.

MATERIALS AND METHODS

Array Fabrication

Details of the array fabrication and characterization have been reported previously (Ketterling et al. 2005). Briefly, an equal area, five-element annular array pattern was etched into a copper clad polyimide membrane using standard printed circuit board techniques. A 9 μm PVDF piezoelectric film, with a chrome-gold electrode on one side, was bonded to the polyimide film and pressed fit into a Teflon tube with a ball bearing. The tube was back filled with epoxy, and after curing the resulting plug was incorporated into a UHF connector.

The finished transducer had a total aperture of 6-mm, geometrically focused at 12-mm and with a center frequency close to 40 MHz. A small PCB board was used to link the signal traces from the array elements to the coaxial cables used to connect to the front-end electronics. Surface mount inductors were soldered to the board in order to facilitate impedance matching on the individual annuli. Finally the PCB board was housed in a metallic box to provide electrical shielding (Fig. 1).

RF Data Acquisition

Detailed information of the RF data acquisition and processing has been reported previously (Ketterling et al. 2006). The image acquisition instrumentation (Fig. 1) was software controlled from a PC using Labview (v7.1, National Instruments, Austin TX). The array transducer was linearly scanned using an automated 3-axis motion controller (PCI-7534, National Instruments). For a given image line, each array element was sequentially pulsed and a complete set of RF data from all 5 elements, 25 transmit / receive pairs, was acquired in five passes using a bi-directional crosspoint switch (CXL/8X8, Cytec, Penfield NY). An expander (DEX-3, Matec, Northborough MA) and limiter (1N50B, Anritsu, Richardson TX) protection circuit was incorporated between the pulser (AVB2-TB-C, Avtech, Ogdensburg NY) and the crosspoint switch. The RF data from four of the array elements were digitized using a digital storage oscilloscope (DSO) (6050A Lecroy, Chestnut Ridge, NY), and the RF data from the fifth element were digitized using a PCI digitizer (DP110 Acquiris, Monroe, NY). Each RF data line was amplified before digitization (45 dB AU-1313, Miteq, Hauppauge NY).

Synthetic Focusing Algorithm

The stored RF data were processed using an offline synthetic focusing algorithm to simulate array- focusing, as described previously (Ketterling et al. 2006). Each RF line was divided into 41 focal zones, each zone being 0.17- mm wide, and appropriate time delays were applied for each of the 25 transmit / receive pairs. After applying the time delays, RF lines were summed to simulate array-focusing. Images simulating a fixed- focus transducer were formed by directly summing the RF data (without delays) from the 25 transmit / receive pairs. The fixed- and array-focus RF data were demodulated in Labview software using a Hilbert transform and mean- filtered to produce bitmapped image files for qualitative and quantitative analysis.

Acquisition software was also developed to provide a "fast" imaging mode that output a fixed-focus image every 2 seconds by pulsing the center element and receiving the RF signals from all five elements. This low resolution imaging mode enabled iterative adjustment of the field-of-view and the imaging plane before full data acquisition. Data for a complete 2D UBM image consisted of 381 lines with line-to- line separation of 25- μm and an acquisition time of 40 seconds. Full 3D datasets consisted of stacks of 2D images with 50- μm separation between image planes.

Animals

All mice used in these studies were maintained under protocols approved by the Institutional Animal Care and Use Committee of the New York School of Medicine. Mouse embryos were imaged at embryonic day (E) 11.5 and E13.5, where E0.5 was defined as noon of the day a vaginal plug was found after overnight mating. In these studies to determine the feasibility of annular array imaging, the anesthetized pregnant mice were euthenized humanely by cervical dislocation immediately before image acquisition to eliminate breathing motion during the long data acquisition times. A midline laparotomy was performed to expose a selected uterine conceptus containing one embryo. The mouse was then positioned for imaging in a custom built plexiglass mouse holder. The embryo, intact within the uterus, was exposed through an opening in a rubber membrane into a petri dish full saline solution at room temperature (Fig. 1b; Olsson et al. 1997). With the array transducer in the saline solution, the low-resolution imaging mode was used to position the image planes close to one of the standard slice orientations. Subsequently, the full 3D RF datasets from each embryo were acquired, as described above. Although *in vivo* imaging was obviously not our goal in these first annular array UBM studies, we observed clear evidence of embryonic heart beating and blood flow on the low-resolution images, showing that the embryos were still alive during image acquisition.

UBM image analysis

Quantitative analysis was performed to estimate signal- to-noise ratio (SNR) and depth-of-focus (DOF) in fixed- and array- focused UBM images of mouse embryos. Both SNR and DOF were computed by importing both fixed- and array-focused images into ImageJ (v1.34p, Public domain software, NIH) image processing software. To compute SNR a square region-of-interest (ROI) was placed in the embryo (signal) and water (noise) and the mean intensity of each ROI measured. In this way, signal and noise values were computed for each image and SNR was calculated as the ratio of signal : noise. To compute DOF a vertical rectangular ROI was defined covering the entire embryo, and an averaged profile was calculated. This profile data was then smoothed using a 20-point averaging window in Origin (v7, OriginLab, Northampton, MA). The dynamic range for each of the B-scan images was measured from the RF data, before processing, by computing the ratio of the peak signal value to the root mean square of the background noise. For presentation, the dynamic range for all the B-scan image displays was 50 dB.

Volumetric segmentation, rendering and analysis were performed using Amira (v3.1, Mercury Computer Systems, San Diego, Ca). In order to objectively segment out the cerebral ventricles with minimal operator input, a semi-automatic segmentation tool was used. Seeds were placed in the ventricles throughout the 3D stack from which an initial contour was computed. Under software control, the contour was expanded until the edges were detected, based on the initial image and edge sensitivity parameters. These parameters were kept constant for each dataset of fixed- and array- focused stacks. For visual comparisons of segmented volumes, the fixed- and array-focused reconstructions were color-coded and overlaid with the transparency of the array-focused volume set to 50%.

RESULTS

Annular array focusing improves the quality of mouse embryo images

Mouse embryos were imaged with the annular array transducer at two different developmental stages (Fig. 2): E11.5 (n=4) and E13.5 (n=5). At both stages, the fixed- focus images showed a characteristic enhanced intensity band close to the passive focus (Figs. 2a, 2c), similar to UBM images made with traditional single element transducers. After processing the image data using the synthetic focus algorithm, the array-focused images showed an obvious increase in image SNR and DOF (Figs. 2b, 2d). For E11.5 embryos, the improvement was revealed most clearly in the improved visualization of the edges of the brain ventricles and the amniotic membrane, which was sharply resolved surrounding the embryo (Fig. 2b). With the passive focus positioned superficially in E13.5 embryos, the underlying ventricles were not resolved in the fixed- focus images (Fig. 2c). After synthetic focusing, the obvious increase in DOF resulted in clear visualization of the brain ventricles, even those furthest from the transducer (Fig. 2d). As in the E11.5 embryo, synthetic focusing in the E13.5 embryo resolved the amniotic membrane and the small gap separating the membrane, at depths up to 3- mm below the passive focus (Fig. 2d). Dynamic range was measured from 2D UBM images acquired at both E11.5 (n=11) and E13.5 (n=15), showing an improvement with array-focusing at both stages: Mean $\pm$ standard deviation at E11.5, 34 ± 4 dB (fixed- focus) vs. 37 ± 3 dB (array- focus); and at E13.5, 40 ± 4 dB (fixed-focus) vs. 45 ± 4 dB (array focus). All UBM images were displayed with a full dynamic range of 50 dB.

Array focusing significantly increases SNR and DOF

To perform quantitative analysis of DOF and SNR, a series of image datasets were acquired from an E13.5 embryo, moving the passive focus of the transducer over a 6- mm depth range in 1- mm steps, starting with the passive focus situated just above the uterus wall (Fig. 3). At each depth, images were generated using the fixed- and array- focusing algorithms.

Qualitatively, these images demonstrated a clear improvement in resolution of brain ventricles, and increased image uniformity, SNR and DOF with array- focusing (Fig. 3a). Quantitative measurements were made of SNR as a function of passive focal depth. At each focal depth, an average SNR value was calculated from four ROIs covering the entire depth range of the embryo in a uniform part of the brain. One of the ROIs used in this calculation is shown in Figure 3a. Measurements of SNR over the range of passive focal depths within the embryo (positions 1–5, Fig. 3a) demonstrated an increase of 3–9 dB with annular array- focusing (Fig. 3b), with the largest difference in SNR occurring when the passive focus was positioned in a superficial region of the embryo. This likely indicates that focusing with our annular array is more effective in the far-field than in the near- field of the transducer, which can also be appreciated from the series of B-scan images (Fig. 3a). DOF was assessed in images acquired with the passive focus 2- mm below the surface of the uterus, in the superficial region of the embryo (Fig. 3a). Profile data across a relatively uniform region of brain tissue demonstrated an increase in the –6 dB full width from approximately 2- mm (fixed- focus) to 4.5-mm (array- focus), an increase of approximately 2.5- mm (Fig. 3c).

Array focusing improves volumetric segmentation and analysis

To assess the potential of array- focusing for volumetric UBM analysis, 3D stacks of 2D UBM images were acquired for E11.5 (n=1) and E13.5 (n=1) embryos. For each stage embryo, both fixed- and array-focused stacks were generated and loaded into the Amira software package and the brain ventricles segmented. At the earlier stage (E11.5), major features of the embryo were identified including the amniotic membrane, uterus, and brain ventricles in both fixed and array- focused UBM images (Fig. 4). Since the embryonic brain was positioned close to the passive focus, the brain ventricles were readily segmented with either fixed- (Fig. 5a) or array- focusing (Fig. 5b). Registration of the two volume renderings showed only small geometrical differences between the two reconstructed ventricles (Figs. 5c) with several obvious errors in the fixed- focus reconstruction and a 6% volume increase using array- focusing compared to fixed-focusing.

For the later stage embryo (E13.5), when the passive focus was placed centrally in the body of the embryo, features such as the uterus, internal cavities and the limb buds were resolved in both fixed and array-focused images (Fig. 6a–b). More anterior in the 3D stack, when the passive focus lay in a superficial region of the embryonic brain, only the array-focused images were able to resolve the deeper brain ventricles (Fig. 6c–d). Consequently, a comparison of volumetric renderings of E13.5 embryos demonstrated much larger errors in the fixed- focus reconstructions (Fig. 7). In this case, accurate reconstruction of the entire ventricular system was only possible using array- focusing, and there was a 74% volume increase using array- focusing compared to fixed- focusing (Fig. 7a–c). The high, uniform contrast between the embryo proper, amniotic fluid and uterus also enabled threshold segmentation of the embryo surface revealing a number of features such as the ear, eye and limb (Fig. 7d,e).

DISCUSSION AND CONCLUSIONS

We have demonstrated that a 40 MHz, 5-element PVDF annular array transducer significantly improves image quality for mouse embryo UBM imaging. The increase in DOF, SNR and the array's ability to maintain two-way lateral resolution over the field of view facilitates effective and accurate segmentation, and 3D visualization and analysis of structures in the developing embryo. We have quantitatively verified an increase in DOF of approximately 2.5- mm, consistent with results obtained previously on wire phantom measurements (Ketterling et al. 2006).

A major advantage of this array transducer lies in the simplicity with which it can be fabricated. The fabrication procedure is robust and flexible enough to allow testing of multiple variations

in the array pattern. In combination with the approaches described in this paper, array fabrication and testing can be performed in a timely fashion to evaluate performance for imaging biological tissue. One of the main advantages of UBM compared to other imaging modalities is the ability to image in real time. In our current implementation of annular array imaging, data were acquired relatively slowly via a digital storage oscilloscope, and images were reconstructed off-line. In the future, implementation of a faster PC-based digitizer will improve 2D acquisition times by an order of magnitude, to under 5 seconds. We are also implementing the synthetic focusing algorithm in hardware, on a custom PC-board to speed up image reconstruction. For full functionality, in the future, the annular array system will require dedicated beamforming hardware to provide real time image frame rates (Brown and Lockwood 2005).

In the future, the ability to perform *in vivo* real time annular array embryo imaging will significantly improve current techniques and will likely lead to new applications. With the demonstrated ability of the array to segment and visualize 3D structures of interest in the developing embryo, longitudinal studies will enable efficient and accurate volumetric analysis of normal and abnormal development including mutant phenotypes with brain defects (Turnbull et al. 1995). Array imaging in combination with volumetric analysis will also significantly improve UBM-guided injections by providing accurate 3D localization of the injection needle (Olsson et al. 1997; Gaiano et al. 1999; Wichterle et al. 2001). Finally, embryonic cardiovascular imaging will benefit from real time array imaging (Ji et al. 2003; Phoon et al. 2004). In this area, array-focusing should improve the resolution of the heart and extended vascular system, which ultimately requires 3D analysis to understand the complex, developing cardiovascular anatomy.

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Fig. 1.

(a) Schematic diagram of the RF data acquisition system. The crosspoint switch enabled each array element to be excited and the RF signals collected from all five elements. The RF data was amplified (filled triangles), digitized with a DSO and PC based digitizer, and stored in the PC for later processing. A protection circuit was incorporated between the pulser and the crosspoint switch, consisting of an expander (E) and limiter (L). (b) Immediately after euthanasia, a pregnant mouse was positioned, in a mouse holder (H) with an exteriorized uterine conceptus containing one embryo (black arrow) exposed in a saline water bath. The annular array transducer (A) and the shielded matching network (B) were linearly scanned to produce a UBM image.

Fig. 2. Fixed and array focused UBM images of E11.5 (a, b) and E13.5 (c, d) mouse embryos, where the level of the passive focus (12-mm) is indicated by the black arrowheads. After array focusing, the amniotic membrane (white arrowheads) was resolved in both E11.5 (b) and E13.5 (d) embryos. Labels: brain ventricles, V; uterus, U; placenta, P. Scale bar = 1 mm (d).

Fig. 3. SNR and DOF were assessed qualitatively and quantitatively from UBM images of an E13.5 mouse embryonic brain. (a) Images were acquired over a 6- mm range of focal depths (black arrowheads) in 1- mm steps. (b) SNR was analyzed as a function of focal depth, comparing image intensities of ROIs in the embryo (S) and water (N) (top left panel). Mean SNR values are plotted, and error bars represent the standard deviations of 4 SNR measurements from signal regions covering the entire depth of the embryo. (c) DOF was analyzed over a vertical strip through the embryo (right panel at 2-mm focal depth), to generate profiles of average intensity from the fixed and array focused images. Labels: uterus, U; embryo top, ET; embryo bottom, EB. Scale bar = 1 mm (top left panel, a).

Fig. 4. Two representative transverse sections from a 3D stack of 120 planes through an E11.5 embryo, comparing fixed (a, c) and array (b, d) focusing. Volumetric data covered 7- mm x 7- mm in each image and 6- mm in the stack direction (passive focus indicated by black arrowheads). Labels: amniotic membrane, A; uterus, U; ventricle, V.

Fig. 5.

Brain ventricles were segmented from the 3D stacks shown in Fig. 4. The surface-rendered images show top views reconstructed from the fixed-focus stack (yellow, a) and array- focused stack (magenta, b), and a registration of the segmented ventricles from the two reconstructions (c). Corresponding side views of the segmented ventricles are also shown (d, e, f). The black arrows indicate obvious errors in the fixed-focus reconstructions (a, d). The forebrain (F), midbrain (M) and hindbrain (H) were clearly identified in both cases.

Fig. 6.

Two transverse sections from a 3D stack of 100 planes for an E13.5 embryo. The selected sections show abdominal (a, b) and head (c, d) regions of the embryo. The passive focus was placed 1.5-mm above the middle of the image in the upper portion of the embryo (black arrow). The developing limb buds (LB) and uterus (U) could be identified in both the fixed- and array-focus images (a, b). The brain ventricles, positioned several mm below the passive focus, were only visualized with array focusing (d). Labels: lateral ventricle, lv; third ventricle, 3v; fourth ventricle, 4v.

Fig. 7.

Volumetric analysis of the 3D stacks shown in Fig. 6. The brain ventricles were segmented from fixed- focused (yellow, a) and array-focused (magenta, b) focused image stacks. Registration of the two volumetric reconstructions revealed gross errors in the fixed- focus images (c). Surface renderings of the embryo proper from array- focused data enabled visualization of limbs and facial features such as the eye and ear (d). Registration of the embryo and ventricle reconstructions showed the orientation of the ventricles with respect to the embryonic head (e).