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Super Harmonic Ultrasound Localization Microscopy

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***Abstract*—Microbubble contrast agents and high frame rate ultrasonography are capable of imaging microvascular structures beyond the diffraction-limited resolution of a typical ultrasound system using a form of localization microscopy. By recovering the locations of bubbles within a long series of sparsely populated contrast-enhanced images, the resolution of the final image is improved by an order of magnitude. The success or failure of this method is determined by the capability of the imaging platform to separate contrast signal from tissue background. Super harmonic imaging is a form of contrast-enhanced ultrasound which produces excellent contrast-to-tissue ratios by exclusively receiving in the frequency band dominated by the harmonic oscillations of microbubbles. In this work, we demonstrate the feasibility of super harmonic ultrasound imaging for localization microscopy *in vitro* and *in vivo*.**

Keywords— Super harmonic imaging; ultrasound localization microscopy; microbubble; contrast agent; microvasculature; angiogenesis

I. Introduction

Abnormal vascular morphology and aberrant angiogenesis are biomarkers for various diseases such as cancer, diabetes, and inflammatory conditions [1], [2]. These features can be noninvasively interrogated using ultrasonography and microbubble contrast agents. For example, irregular angiogenesis imaged by a method known as acoustic angiography has been utilized to quantitatively compare malignant and healthy animal models [3], [4], [5]. This technique is able to resolve structures on the order of 150 microns in diameter.

A new approach to microvascular imaging, ultrasound localization microscopy (ULM), has received warranted attention due to its ability to resolve blood vessels down to a diameter of a few microns at centimeters in depth *in vivo* [6], [7]. Desailly *et al.* estimate that for clinical applications such as breast imaging, ULM will achieve resolutions on the order of 2 microns when using a 7 MHz linear array transducer [8]. In rodent models, the potential diagnostic utility of super-resolution microvascular imaging has been demonstrated by comparing groups based on metrics of vessel morphology and blood flow [9], [10].

The accuracy of the localization of a microbubble in an ultrasound image is determined in part by the contrast-to-tissue ratio (CTR) of the received echoes [8]. As CTR increases, the standard deviation of the spatial localization error decreases. Super harmonic imaging (SHI) is a method of imaging contrast that excludes echoes below the third harmonic of the fundamental frequency of the transmission [11]. This technique results in a large increase in CTR compared to techniques such as pulse inversion or amplitude modulation. Bouakaz and colleagues reported a 40 dB increase in contrast-to-tissue ratio when comparing SHI (receiving third to fifth harmonics) to second harmonic imaging [11].

Whereas previous dual-frequency transducers have utilized a focused beam, recent work has led to the development of a

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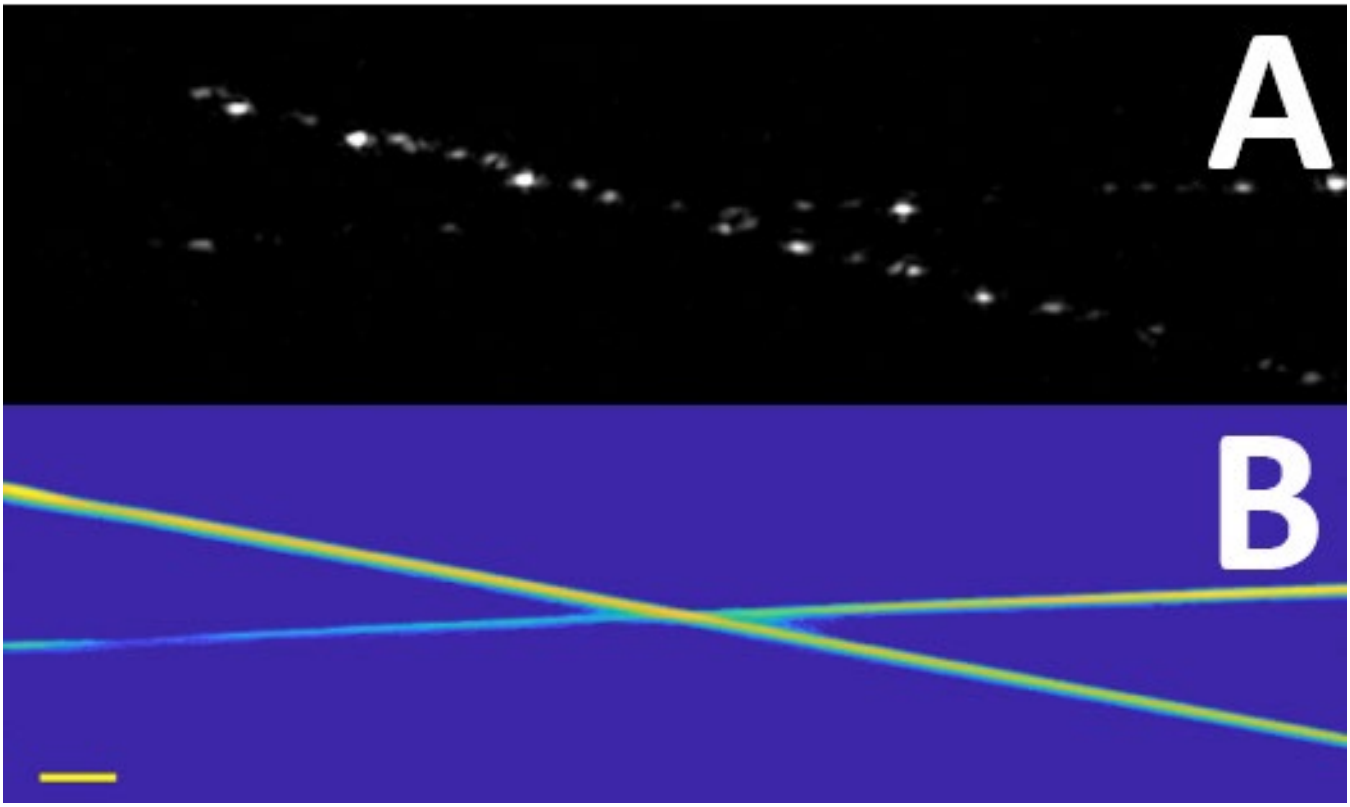

Fig. 1. An example of super harmonic imaging applied to ultrasound localization microscopy. (A) A single super harmonic image of two crossed microtubes in a water bath. The bright points are individual microbubble contrast agents. (B) The super-resolution image generated from 25,000 super harmonic images. Scale bar = 500 microns.

device for plane wave SHI that is capable of very high frame rates (>1 kHz) [12]. In this work, we use this device and demonstrate the feasibility of super harmonic imaging for ultrasound localization microscopy *in vitro* and *in vivo.*

II. Materials and Methods

A. Contrast Agent Preparation

Microbubbles were prepared in-house following the protocol outlined in [13]. In short, a 1 mM lipid solution composed of 10 mole % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (P2K) and 90 mole % 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was formulated in phosphate buffered saline (PBS) containing 15 % (v/v) propylene glycol and 5 % (v/v) glycerol. Aseptic lipid solution was distributed into 3 mL vials and the air headspace was vacuumed. Decafluorobutane (C_4F_{10}) was added to each vial and the microbubble emulsion was created by mechanical agitation (VialMix, Lantheus Medical Imaging, N. Billerica, MA). Concentration and size distribution of the microbubble contrast agent were measured using an Accusizer 780 AD (Entegris, Billerica, MA); typical concentration was 3×10^{10} microbubbles per mL with an average diameter of 1.0 microns.

B. Imaging System

A dual-frequency (DF) array described in [12] was used for this study. Two large 1.7 MHz elements were attached to a high-frequency (HF) 20 MHz linear array transducer (MS-250, FujiFilm VisualSonics, Toronto, Canada). The HF array is connected to a Vantage 256 ultrasound scanner (HF configuration, Verasonics, Kirkland, WA, USA) and can image normally or act as a receiver in dual-frequency mode. A single-cycle, cosine-windowed sine wave [14] with a frequency of 1.7 MHz was generated using an arbitrary waveform generator (AWG 2021, Tektronix, Beaverton, OR, USA) and amplified by 50 dB (240 L RF amplifier, ENI, Rochester, NY, USA) in order to drive the low-frequency (LF) elements. The pressure output of the LF elements was calibrated using a hydrophone (HNA-0400, Onda, Sunnyvale, CA, USA), and all imaging was performed at a mechanical index of 0.24.

C. In Vitro *Imaging*

A phantom was constructed with two microtubes (inner diameter = 66 microns) arranged in a cross. The center of the 'X' was placed in a water bath at approximately 20 mm in depth using HF B-mode as a reference. A solution of microbubbles (1×10^7 #/mL) were infused at a volume flow rate of 10 µL/min (Harvard Apparatus, Holliston, MA). 25,000 SHI frames were collected at a PRF of 500 Hz. Data were beamformed offline.

D. In Vivo *Imaging*

Kidney imaging was performed on a healthy female rodent (Fischer 344 rats, Charles River Laboratories, Durham, NC). All imaging procedures were conducted according to a protocol accepted by the Animal Care and Use committee at the University of North Carolina at Chapel Hill. A solution of contrast was prepared at a concentration of 1×10^9 #/mL and

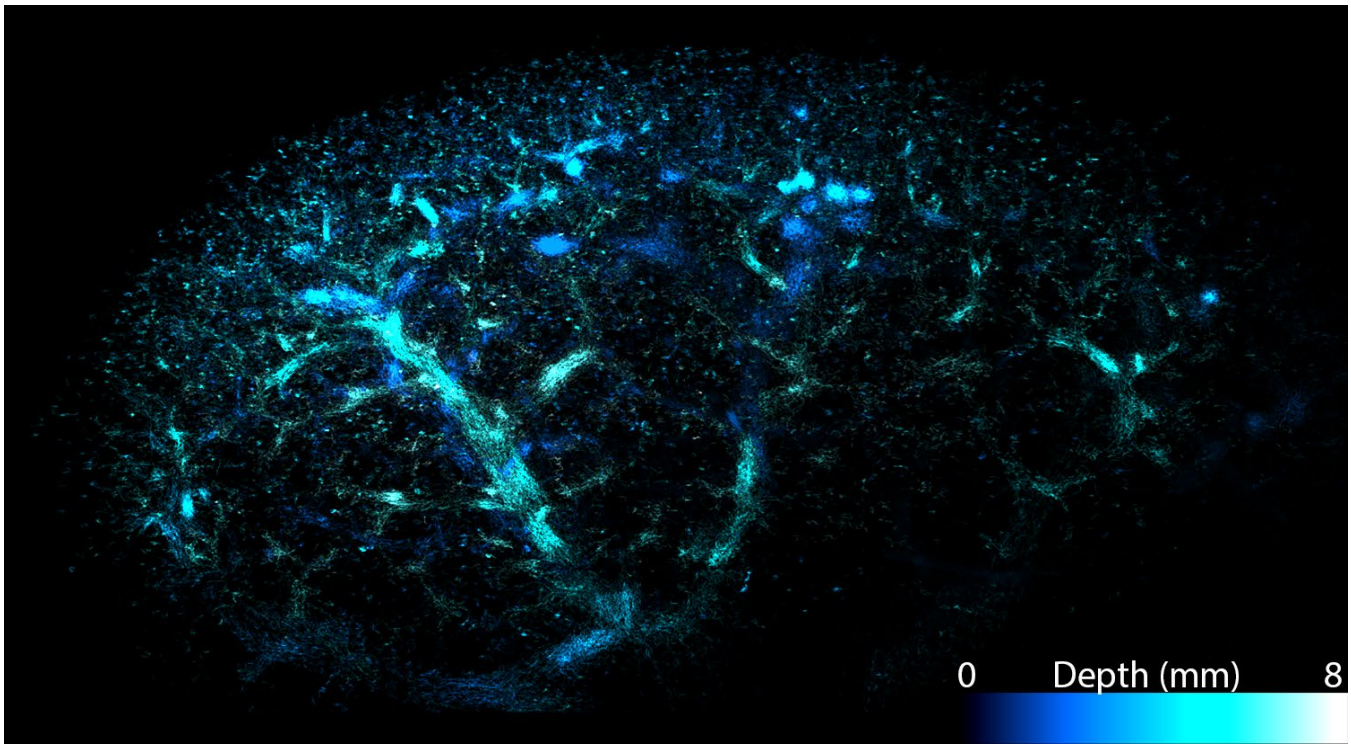

Fig. 1. A maximum intensity projection of 17 ultrasound localization microscopy images of a rodent kidney, each spaced by 500 microns. Each elevational slice is colored according to the depth chart in the lower right corner of the figure (indigo closest to the observer, cyan/white farthest from the observer). Created using Temporal Color Code plugin for ImageJ.

injected at a volume flow rate of 25 µL/min via tail vein for the duration of imaging. DF imaging was performed at a PRF of 500 Hz, and HF B-mode frames were interleaved after every 100 DF acquisitions. Using a linear motion stage (Velmex, Bloomfield, NY, USA), the array was translated in elevation by 500-micron steps (17 positions total). At each step, 25,000 DF images were acquired. Data were beamformed offline.

E. Super-resolution Processing

Radiofrequency data were processed offline using dynamic receive beamforming on a 10-micron grid. SHI images were processed with a threshold to remove features due to electrical noise. Afterwards, centroiding was used to estimate the locations of bubbles in each frame. Between adjacent frames, a nearest neighbors method was used to estimate bubble tracks. For the *in vivo* datasets, speckle tracking according to [15] was performed on the interleaved B-mode frames to estimate tissue motion and correct localization positions. Some frames were thrown away due to very large motion.

III. Results

A. In Vitro *Imaging*

An example super harmonic image of the crossed microtubes is shown in Fig. 1A. This image highlights the exceptional signal to noise ratio of the imaging modality, as well as the high resolution of the HF probe. The ULM image derived from 25,000 SHI frames is shown in Fig. 1B. Profiles from the tubes had an average full-width half-maximum (FWHM) of 44 microns.

B. In Vivo *Imaging*

A volumetric ULM image was created by mechanically scanning the transducer in the elevational dimension with a step size of 500 microns for a total scan length of 8 mm. 25,000 SHI frames were collected and processed at each position. Speckle tracking of interleaved B-mode frames was used to correct for nonrigid deformations when possible. The results of this 3D scan are shown in Fig. 2. These results demonstrate that ULM with SHI is possible *in vivo* in the presence of respiratory and cardiac motion.

IV. Discussion and Conclusion

In this work we have demonstrated the viability of super harmonic imaging for ultrasound localization microscopy. *In vitro* the technique resolves a sub-resolution tube in a water bath. The full-width at half-maximum of the average profile of either tube was measured to be 44 microns compared to the true diameter of 66 microns. It is possible that another method of measuring diameter would be more appropriate than FWHM for this scenario.

In vivo, this approach to ultrasound localization microscopy was utilized to image a rat kidney in 3D. While successful in reconstructing in-plane vessels, the 500-micron step size in elevation was simply too large to fully sample the vessels oriented in the elevational dimension. This is a limitation of super-resolution imaging in 3D with a 1D linear array transducer, but research into volumetric imaging at high

acquisition rates is very promising for improving ULM image quality.

CONFLICT OF INTEREST

P.A.D. declares that he is an inventor on a patent describing dual-frequency imaging and is a co-founder of SonoVol, Inc., which has licensed this patent.

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