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3D Subharmonic Ultrasound Imaging In Vitro and In Vivo

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Abstract

Rationale and Objectives—While contrast-enhanced ultrasound imaging techniques such as harmonic imaging (HI) have evolved to reduce tissue signals using the nonlinear properties of the contrast agent, levels of background suppression have been mixed. Subharmonic imaging (SHI) offers near-complete tissue suppression by centering the receive bandwidth at half the transmitting frequency. In this work we demonstrate the feasibility of 3D SHI and compare it to 3D HI.

Materials and Methods—3D HI and SHI were implemented on a Logiq 9 ultrasound scanner (GE Healthcare, Milwaukee, Wisconsin) with a 4D10L probe. Four-cycle SHI was implemented to transmit at 5.8 MHz and receive at 2.9 MHz, while 2-cycle HI was implemented to transmit at 5 MHz and receive at 10 MHz. The ultrasound contrast agent Definity (Lantheus Medical Imaging, North Billerica, MA) was imaged within a flow phantom and the lower pole of two canine kidneys in both HI and SHI modes. Contrast to tissue ratios (CTR) and rendered images were compared offline.

Results—SHI resulted in significant improvement in CTR levels relative to HI both *in vitro* (12.11 ± 0.52 vs. 2.67 ± 0.77 , $p < 0.001$) and *in vivo* (5.74 ± 1.92 vs. 2.40 ± 0.48 , $p = 0.04$). Rendered 3D SHI images provided better tissue suppression and a greater overall view of vessels in a flow phantom and canine renal vasculature.

Conclusions—The successful implementation of SHI in 3D allows imaging of vascular networks over a heterogeneous sample volume and should improve future diagnostic accuracy. Additionally, 3D SHI provides improved CTR values relative to 3D HI.

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Keywords

Subharmonic imaging; 3D Imaging; ultrasound contrast agents; contrast-enhanced ultrasound

Introduction

Despite its advantages in cost, portability, and real-time nature, ultrasound suffers from low blood-to-tissue contrast relative to other imaging modalities. Segmentation of vascular formations from the surrounding tissue is clinically useful in a variety of radiological applications. For example, malignant lesions often show a more tortuous, sporadic vascular network relative to benign lesions [1]. This more chaotic growth pattern can often be quantified by number of bifurcations [2], microvascular density [2], or fractal dimensionality [3] as a means for differentiation of diseased from healthy tissue. Additionally, temporal parameters within the vasculature such as perfusion or filling time of intravenous contrast can also be used for identification and classification of diseased tissue [4-6].

While feasibility of experimental, nano-scale agents has been demonstrated [7-8], clinically approved ultrasound contrast agents are small gas bubbles stabilized by an outer shell (generally composed of protein or phospholipids), with diameters ranging from 1 to 8 μ m that constrain their distribution within the body to the blood supply [4]. The difference in compressibility and acoustical impedance of the gas core within these microbubbles relative to the surrounding fluid (roughly 20,000X and 250X, respectively for most gases) result in up to 30 decibels (dB) of vascular enhancement [9]. Numerous reports on the usage of contrast-enhanced ultrasound for lesion characterization have been reported, although results have been mixed [10-12], in part due to an inability to distinguish signals from the contrast agent, perfused within the micro-vasculature, and signals from the surrounding tissue.

When insonated at sufficient acoustic pressures and frequencies, these agents act as nonlinear scatterers, emitting signal at the fundamental transmission frequency (f_0) consistent with a linear scatterer, but also at higher harmonics of the original transmitted frequency ($n \cdot f_0$) and the subharmonic ($f_0/2$) [4]. Frequency filtering of received radio frequency (RF) data can be used for improved contrast detection modes. Harmonic imaging (HI) modes are now available on most commercial ultrasound scanners, and these modes greatly improve their ability to detect ultrasound contrast agents. However, HI still suffers from reduced blood to tissue contrast due to second harmonic generation and accumulation within the surrounding tissue [13]. The ability to generate signals at the subharmonic frequency is exclusive to ultrasound contrast agents, and provides near complete tissue suppression [14]. The feasibility of subharmonic imaging (SHI) has been shown by both our group [15-18] and others [19-22]. Previously, we showed that cumulative maximum intensity projections from SHI provided significantly higher diagnostic accuracy relative to mammography in a small pilot study of 16 lesions in 14 women (90 vs. 76%, $p = 0.03$) [18, 23]. Additionally, the rates of contrast inflow from this study were analyzed and shown to vary significantly ($p = 0.0014$) between benign and malignant lesions [24].

Despite these advances, previous implementations of SHI were limited to a single 2D plane. This results in discontinuity within the vascular depiction, and often a limited representation of a heterogeneous volume. Thus, the goal of this work is to show the feasibility of 3D SHI on a commercial scanner and to compare its performance to 3D HI.

Materials and Methods

Modification of a Commercial Scanner

Ultrasound imaging was performed using a modified Logiq 9 ultrasound scanner (GE Healthcare, Milwaukee, Wisconsin) with a 4D10L probe (50 × 58 mm footprint, 3.5 – 11.0 MHz bandwidth, 37.4 mm field of view). Three-dimensional SHI was implemented transmitting 4 cycle pulses at 5.8 MHz and receiving at 2.9 MHz by equalization filtering. This mode is not currently available commercially. Three-dimensional HI (commercially available) was performed on the same probe (2 cycle transmit pulses at 5 MHz and receiving at 10 MHz). A longer pulse length was required for SHI to avoid signal transmission within the SHI bandwidth. Pulse inversion was implemented for both imaging modes to further suppress linear signal components from the received signal. Harmonic and subharmonic filtering without pulse inversion was not investigated because the goal of this work was to compare the best SHI possible with the current HI clinical standard (in which pulse inversion is employed). The acoustic output of the system at the maximum transmit settings applied in this study, was measured using a 0.5 mm needle hydrophone (Precision Acoustics, Dorset, UK) and found to have peak mechanical indexes of 0.33 for SHI and 0.36 for HI at the focus, which is well below the FDA's mechanical index limit of 1.9 [25].

Validation of Subharmonic Generation in vitro

The modified system was first used to acquire raw radio frequency (RF) data from solutions of Definity (Lantheus Medical Imaging, North Billerica, MA) to ensure generation and detection of subharmonic behavior was occurring. Circulating Definity was imaged at a concentration of 0.4 ml/l (of isotonic diluent) at 20 ° C within a flow phantom (Model 524; ATS Laboratories, Bridgeport, CT) with a 6 mm diameter vessel embedded at a depth of 2 cm. *In vitro* concentration was selected by incrementally increasing the dosage until complete vessel filling was observed in 2D HI and SHI. Contrast agent was circulated using a roller pump (S10K II; Sarns Inc., Ann Arbor, MI) at a rate of approximately 250 ml/min and the solution maintained homogenous using a magnetic stirrer. The acoustic output of the scanner was changed from 0 to 100% and the frequency response of the backscatter signal computed in Matlab (The MathWorks, Natick, MA) from the saved RF data. Spectra were normalized to the peak fundamental for comparison.

In vitro HI and SHI

Definity was imaged within the same flow phantom system described above. Scanning was performed by an experienced radiologist. Standard grayscale 2D B-mode data was first collected to orient the imaging plane. Data was then acquired for five seconds in both 3D HI and SHI modes. An approximate volume of 2.5 cm × 2.5 cm × 2.5 cm was acquired with a volume angle of 19 (resulting in 45-50 slices of data per volume). This resulted in a volume frame rate of 1.8 to 2.2 Hz depending on depth of the selected region of interest (ROI). Acquisitions were performed in triplicate and both the volumetric slice data and rendered image filters were stored for later analysis.

In vivo HI and SHI

In vivo imaging was performed in two open abdomen canines (22.1 and 22.7 kg) as part of an ongoing perfusion estimation study. The study was approved by Thomas Jefferson University's Institutional Animal Care and Use Committee. Canine sedation was achieved by intravenous injection of propofol (Abbott Laboratories, Chicago, IL; dose of 7 ml/kg) and anesthesia maintained with 2-4% isoflurane (Iso-thesia; Abbott Laboratories, Chicago, IL) throughout the study. Over the course of the study, the canine's body temperature was

maintained by a warming blanket and vital signs were monitored by a qualified veterinarian technician.

All scanning was performed by a radiologist with 8 years of ultrasound imaging experience. The ultrasound probe was covered by a transducer cover (Civco Medical Instruments, Kalona, IA) and placed on the lower pole of the kidney. Image orientation was first determined using standard B-mode ultrasound. Acoustic output was set to 12 % (reduced relative to the optimal *in vitro* settings due to shallower depths) with a gain of 20 dB, to minimize tissue signal without overly suppressing contrast. These selections were based on qualitative observations from earlier trial injections using both HI and SHI. After selection of the ROI, either 3D HI or SHI was performed. Canines then received a weight based, bolus injection of Definity followed by a 5 ml saline flush through an 18-gauge catheter placed in a forelimb vein. Contrast injections of 5 μ l/kg for HI and 20 μ l/kg for SHI were used based on previous experiences with each imaging mode [18, 26]. These differences in optimal *in vivo* concentrations are attributed to the ability of harmonic imaging to more easily detect agent within the microvasculature (considerably smaller than the 6 mm *in vitro* flow phantom) due to higher resolution at increased frequency. All injections were repeated in triplicate with at least 15 minutes between injections. Data was recorded over the entire contrast wash in and wash out phase and stored for later analysis.

Image Analysis

The vessel to tissue signal ratio was determined using the formula for contrast to tissue ratio (CTR) and calculated from volumetric slice data from both SHI and HI in triplicate. This parameter is defined as:

$$CTR = \frac{2(\gamma_V - \gamma_T)^2}{\sigma_V^2 + \sigma_T^2}, \quad (1)$$

where γ_V and γ_T represent the mean backscatter signal strength in the vessel and tissue respectively; and σ_V^2 and σ_T^2 represent the variance in the respective ROIs [27]. *In vitro* CTRs were calculated from the ROIs as shown below in Fig 1. The vessel ROI was selected as a square region of 45-55 pixels (approximately 20 mm²) within the vessel lumen. Four equidistant, equal sized tissue ROIs along the same horizontal and vertical axis as the vessel ROI were then selected and averaged together. This process was performed for each plane within the volume (45-50 slices/volume), for each volume over the 5 second period (9-11 volumes depending of volume acquisition rate). Final *in vitro* CTR was calculated as the average of 3 repetitions of 5 second acquisitions.

In vivo ROIs were selected from the enhanced vasculature and surrounding parenchyma to calculate CTR values. A square ROI was selected from the enhanced vasculature along the periphery of the kidney. Two, equal sized square tissue ROIs were then selected from the unenhanced renal pelvis and outside the kidney boundary. Whenever possible, all three ROIs were located along a straight line to reduce selection bias, although some degree of selection bias is unavoidable due to motion and changing orientations prohibiting an automated selection technique. An example of ROI selection is shown in Figure 2. The process was repeated for three planes (midline and the fifth frames from midline in each direction) and averaged for 8 volumes (4 volumes at roughly 1/2 second intervals leading to peak enhancement, and 4 at roughly 1/2 second intervals immediately post peak enhancement). This process was then repeated for 3 injections in 2 canines.

Statistical significance was determined using a Student's t-test with $\alpha = 0.05$. All statistics were analyzed using Prism version 5.0 (Graphpad Software, San Diego California, USA).

CTR values from tissue slice data were computed in Matlab. Image rendering for display purposes was performed using 4D View software (version 7, GE Healthcare, Munich, Germany).

Results

Unfiltered RF data from Definity within a flow phantom acquired using the modified unit is shown in Figure 3, normalized to the peak fundamental. At 10% acoustic output (Fig 3a) the transmitting pulse is clearly shown at approximately 5.8 MHz with no subharmonic generation. At 20% acoustic output (Fig 3b), growth of the subharmonic peak (approximately 2.9 MHz) occurs, and reaches saturation at 32% power (Fig 3c, with limited growth of the subharmonic as shown in Fig 3d; 60% power). This examination of the RF data demonstrates the modified scanner is capable of both eliciting and detecting a subharmonic response from the ultrasound contrast agent.

Figure 4 shows raw unprocessed slice data obtained sequentially in both HI and SHI modes. For both *in vitro* and *in vivo* imaging cases, HI resulted in slightly less contrast signal intensity, but much stronger tissue signals, resulting in a less favorable contrast-to-tissue ratio. Figures 4a and 4b show 2D slices extracted from 3D HI and SHI images of Definity within the flow phantom. While the overall image resolution of each 2D slice is modest, SHI shows better overall differentiation of the flow vessel from the surrounding tissue mimicking material. Figures 4c and 4d show similar *in vivo* images taken from the center plane of a canine kidney approximately 9.5 seconds post contrast injection. Again, while the individual 2D slices of data are quite pixilated due to the need to maintain reasonably high volume acquisition rates, SHI provides superior differentiation of the larger feeding renal artery from the surrounding parenchyma.

Averaged CTR values over the spatial and temporal sample volumes are shown in Table 1. Subharmonic imaging resulted in statistically significant improvements in CTR relative to HI in both cases, presumably due to increased levels of tissue suppression.

Rendered images of Definity within the flow phantom from both 3D imaging modes are shown in Figure 5. Both rendered images show a clear volumetric representation of the contrast agent filled vessel. However, 3D SHI (Fig 5b) results in better suppression of the surrounding tissue-mimicking material compared to 3D HI (Fig 5a), and thus better segmentation of the reconstructed vessel.

Figure 6 shows representative sequences of 3D HI (left column) and SHI (right column) while imaging the lower pole of a canine kidney. Baseline images (prior to contrast arrival at $t = 0.5$ seconds) illustrated SHI's superior ability to suppress tissue signals relative to HI. During initial contrast arrival ($t = 9.5$ seconds post contrast injection) the renal artery becomes enhanced. Immediately afterwards, full perfusion filling of the renal artery takes place ($t = 10.5$ seconds post injection) as well as portions of the peripheral microvasculature. At this stage differentiation of the vasculature containing contrast becomes easier in SHI mode due to better suppression of the central parenchyma. The lowest two panels ($t = 14.0$ seconds) show the lower pole with each imaging mode after complete contrast filling. While demarcation of the smaller vessels becomes more difficult in both cases due to saturation of the contrast agent, isolation of the larger vessels is still easier in SHI mode due to suppression of avascular regions.

Discussion

Three dimensional HI and SHI have successfully been employed on a modified commercial ultrasound scanner. This implementation allows 3D imaging in HI and SHI modes and

storage of both image intensity data at each acquired plane and the overall rendered volume. At equivalent acoustic output levels, HI resulted in similar received signal levels from the contrast agent to SHI. However, SHI's superior levels of tissue suppression resulted in significantly higher CTR levels both *in vitro* when viewed in a flow phantom and *in vivo* when imaged within the renal vasculature of two canines (Table 1). These improvements are demonstrated in the rendered canine images (Fig 6), in which SHI provides better isolation of the renal vasculature from the surrounding parenchyma throughout the course of contrast perfusion.

An additional benefit of SHI relative to HI is evident in the increased penetration observed in the *in vitro* data in Fig 4. While the signal intensity is homogeneous throughout the vessel when imaged in SHI, HI shows a dramatic decrease in signal intensity with depth due to increased scattering and attenuation at higher frequencies. While HI resulted in similar *in vitro* and *in vivo* CTR levels, there was over a two-fold reduction in CTR when SHI was applied to *in vivo* relative to the tissue-mimicking phantom. This drop in performance is attributed to the flow phantom representing an 'ideal' situation of a vessel surrounded by homogenous tissue material, while living tissues will inherently contain acoustic scatterers and interface mismatches which will confound pulse-inversion and frequency filtering approaches to some degree. More physiological akin phantoms have been described [28], but were not investigated for this pilot study.

Isolation of ultrasound contrast agent signals from surrounding tissue can be clinically useful for a variety of applications. Previous studies have shown that monitoring the perfusion of ultrasound contrast agents with HI is useful for differentiating diseased from healthy tissues [29-31]. However, improved suppression of tissue signals through SHI presents a better representation of the vascular network for identifying malignant lesions and may further improve results. Previous results from our group have shown that 2D SHI of breast lesions sufficiently isolates vascular networks to improve characterization of breast lesions, with the area under the receiver operating characteristics curve (A_z) improving from 0.68 for contrast enhanced power Doppler to 0.78 with SHI [18]. Additionally, the use of cumulative maximum intensity projections further improved SHI's A_z to 0.90, which was significantly better than mammography ($p = 0.03$, mammography $A_z = 0.76$) [23]. Quantitative analysis of SHI images can also be used for lesion characterization. The perfusion of contrast in SHI images was shown to vary significantly between carcinoma and benign lesions (0.1 ± 0.03 vs. 0.05 ± 0.02 intensity units/ second, $p = 0.0014$) [24]. Skeletonization algorithms which reduce the SHI image to a vascular tree can also be used to quantify bifurcations and vessel length, although no significant difference was seen between malignant and benign lesions ($p > 0.4$) [32]. While these results are promising, they inherently relied on imaging a single plane within a heterogeneous volume.

The benefits of 3D ultrasound relative to 2D in cancer imaging have been well documented [33-35]. Ohishi *et al.* showed that 3D power Doppler of hepatic lesions can be used for improved and more complete visualization of lesion vascularity [33]. Kurjak *et al.* showed that analysis of vascularity in ovarian lesions using 3D power Doppler increased the sensitivity and specificity of reads from 66.7 and 93.8% respectively in 2D Doppler to 77.8 and 97.5% [34]. Contrast-enhanced 3D HI has been shown to provide the visualization of a vascular network over an entire sample volume [36]. Recently, Hoyt *et al.* showed the importance of performing 3D measurements when using contrast-enhanced ultrasound for monitoring tumor blood flow [37]. Using HI in a human breast cancer model in mice, the group showed that errors varied from 6.4 to 40.3% depending on both the blood flow marker and offset of the image plane [37]. These findings illustrate the need to measure tumor vascularity over the entire volume and the potential improvements 3D SHI offers over previous 2D results.

Implementation of SHI in 3D may further improve previously demonstrated uses. Changes in subharmonic signal have been used for the estimation of hydrostatic pressures in a variety of clinical applications and 3D SHI may be expanded to provide 3D pressure maps for diagnostic use [38–41]. Subharmonic imaging has also been applied to molecular imaging with UCA coated with target-specific binding peptides in the hopes of better segmenting attached agent from either unattached or surrounding tissue [Ref 42–43]. These approaches could be readily applied in 3D for a better 3D target representation (often a heterogeneous tumor).

While imaging advances from this implementation are promising, future improvements may further increase SHI's clinical usefulness. As seen in Figure 3, the extracted slice data appears quite pixilated and has resolution limited by the trade-off with 3D acquisition parameters. This results from the inherently reduced resolution of SHI (decreases in ultrasound frequency results in decreased resolution), low pulse lengths and duty cycles, and the need to limit data acquisition to maintain sufficiently high volume rates. While the acquired data is still sufficient for rendering purposes (see Figs 5, 6), further improvements may result from increased processing power and higher transmitting frequencies. Additionally, the current implementation of 3D SHI requires orientation using 2D B-mode or HI (due to a lack of sonographical landmarks in SHI) before switching to SHI. This process increases scan time and uncertainty due to the possibility of motion occurring, while switching between modes prior to contrast arrival. While a lack of sonographical landmarks is a fundamental drawback in SHI, dual grayscale/SHI imaging has been previously implemented on the same ultrasound scanner in 2D mode and may be incorporated in the future to reduce this uncertainty [44]. A final drawback in SHI is the need for a contrast dosage of 4X higher than that of HI. However, the applied *in vivo* dose of Definity (20 μ l/kg) is still feasible in humans and should translate to clinical applications relatively easily [18].

In conclusion, 3D SHI has been implemented on a commercial ultrasound scanner. This setup benefits from SHI's improved CTRs relative to HI and 3D imaging's ability to quantify vascularity over an entire volume. Future work will explore the system's effectiveness in breast imaging.

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Figure 1. Schematic diagram showing *in vitro* ROI selections (1 = vessel ROI, 2-5 = tissue ROIs averaged together). Measurements were performed for each plane for each acquired volume and averaged.

Example of ROI Selection for CTR Calculations

Figure 2.

Example of ROI selection from an in vivo slice dataset with color bar corresponding to signal intensity. One square vessel area (V) was selected from the peripheral region of the kidney, while two equal sized tissue regions (T) were selected from outside the kidney boundary and within the cortex. Whenever possible all three regions were selected along a straight line (shown in red on figure) to reduce selection bias.

Figure 3.

Normalized frequency spectrum responses from Definity within a flow phantom generated and acquired by the modified unit. As acoustic power increases the subharmonic peak (frequency and amplitude shown by the dotted line) shows clear stages of baseline levels at the noise floor (a), occurrence (b), and full saturation (c-d).

Figure 4.

Raw intensity data acquired from a flow phantom in HI (a) and SHI (b), and from the midline of a canine kidney roughly 9.5 seconds post injection in HI (c) and SHI (d). White arrows in 4d show the renal vessels. The color bar represents harmonic or subharmonic signal intensity in arbitrary units.

Figure 5. Rendered images of Definity within a flow phantom in 3D HI mode (a), and in 3D SHI mode (b).

Figure 6. Rendered images of 3D HI (left column) and SHI (right column) from the lower pole of a canine kidney pre-contrast injection ($t = 0.5$ sec), during initial contrast arrival at the renal artery ($t = 9.5$ sec) during perfusion of the peripheral microvasculature ($t = 10.5$ sec), and at peak enhancement ($t = 14.0$ sec).

Table 1*In vitro* and *in vivo* Contrast to Tissue Ratios

	3D Harmonic Imaging	3D Subharmonic Imaging	Significance
In vitro CTR	2.67 ± 0.77	12.11 ± 1.52	p = 0.0007
In vivo CTR	2.40 ± 0.48	5.74 ± 1.92	p = 0.04