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● *Original Contribution*

SUBHARMONIC, NON-LINEAR FUNDAMENTAL AND ULTRAHARMONIC IMAGING OF MICROBUBBLE CONTRAST AT HIGH FREQUENCIES

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Abstract—There is increasing use of ultrasound contrast agent in high-frequency ultrasound imaging. However, conventional contrast detection methods perform poorly at high frequencies. We performed systematic *in vitro* comparisons of subharmonic, non-linear fundamental and ultraharmonic imaging for different depths and ultrasound contrast agent concentrations (Vevo 2100 system with MS250 probe and MicroMarker ultrasound contrast agent, VisualSonics, Toronto, ON, Canada). We investigated 4-, 6- and 10-cycle bursts at three power levels with the following pulse sequences: B-mode, amplitude modulation, pulse inversion and combined pulse inversion/amplitude modulation. The contrast-to-tissue (CTR) and contrast-to-artifact (CAR) ratios were calculated. At a depth of 8 mm, subharmonic pulse-inversion imaging performed the best (CTR = 26 dB, CAR = 18 dB) and at 16 mm, non-linear amplitude modulation imaging was the best contrast imaging method (CTR = 10 dB). Ultraharmonic imaging did not result in acceptable CTRs and CARs. The best candidates from the *in vitro* study were tested *in vivo* in chicken embryo and mouse models, and the results were in a good agreement with the *in vitro* findings. (E-mail: v.daeichin@erasmusmc.nl) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound contrast agent, Contrast imaging, High-frequency ultrasound, Chicken embryo, Contrast-to-tissue ratio, Non-linear propagation artifact, *In vitro*, *In vivo*.**

INTRODUCTION

The need for high-resolution ultrasound imaging has increased the diagnostic use of high-frequency ultrasound (HFU, >15 MHz) (Brown et al. 2007; Foster et al. 2009, 2011; Lukacs et al. 2006; Needles et al. 2010; Ritter et al. 2002). In addition to its numerous applications in small animal imaging (Foster et al. 2011), HFU is widely used in the clinical diagnosis of intravascular, dermatologic and ophthalmologic pathology (Shung et al. 2009). Within the field of HFU, the use of ultrasound contrast agents (UCAs) is also growing (Foster et al. 2011; Needles et al. 2010). Although UCAs have been used and studied extensively at frequencies below 15 MHz (Wilson and Burns 2010), the behavior of UCA microbubbles above 15 MHz is not fully understood (Foster et al. 2011).

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The stronger scattering behavior of UCAs is the key factor in linear contrast detection techniques (Ritter et al. 2002). However, these methods cannot provide sufficient contrast in images for many applications, such as detection of small capillaries in tissue perfusion or in the presence of tissue motion. Therefore, UCA-specific non-linear contrast imaging techniques are essential at high frequencies (>15 MHz). Conventional non-linear imaging techniques, at lower frequencies, focus mainly on detection of higher harmonics (Burns et al. 1994; Chang et al. 1995; de Jong et al. 2000; Deng and Lizzi 2002; Simpson et al. 1999). Similar techniques have been implemented at higher frequencies (Cachard et al. 1997; Lyshchik et al. 2007; Moran et al. 2002; Needles et al. 2010; Rychak et al. 2007; Willmann et al. 2008). However, the performance of these methods is degraded in HFU imaging because the driving frequency is much higher than the resonance frequency of the UCA. In addition, the propagation of the transmitted acoustic wave in tissue is more non-linear at higher frequencies (Blackstock 2000). Therefore, techniques exploiting higher harmonics are hampered because of the increase

in the amplitude of harmonics produced by tissue (Goertz et al. 2005). These limitations motivate the exploitation of the non-linear components of the UCA response at lower frequencies: the non-linear fundamental (NF), subharmonic (SH) and ultraharmonic (UH) frequencies.

Contrast imaging focusing on the NF component of the UCA works best if the excitation frequency is close to the resonance frequency of the microbubbles. To obtain a strong enough NF signal from the currently used UCAs at frequencies above 15 MHz, high transmit pressures are required, which may disrupt the microbubbles. Another challenge in NF imaging is the so-called non-linear propagation artifact or far-wall artifact (Renaud et al. 2012; Tang et al. 2010; ten Kate et al. 2012; Thapar et al. 2012; Yu et al. 2010) produced by non-linear propagation of the excitation wave through the UCA which is backscattered by the tissue behind it, resulting in a false response at the site of the linearly scattering tissue.

Use of the emission of energy by the UCA at half the excitation frequency f_0, the SH response (Eller and Flynn 1969), was proposed as a new imaging modality (Shankar et al 1998). No SH signal is generated during propagation in tissue, and no SH scattering is produced by tissue. The backscattered SH signal is attenuated less in tissue than the signals at fundamental and higher harmonic frequencies, providing a potentially powerful diagnostic tool for clinical examinations (Forsberg et al. 2000). Nevertheless, the excitation bursts required for SH imaging should also be considered. The SH signal is strongly dependent on the applied acoustic pressures, the ambient pressure variations (Eller and Flynn 1969; Tjotta and Tjotta 1980) and the envelope of the excitation signal (Cosgrove and Lassau 2009; Daeichin et al. 2012). Previously, we reported that the self-demodulation (S-D) signal can enhance the SH response of phospholipid-coated microbubbles by up to 20 dB at 10 MHz (Daeichin et al. 2012). The S-D signal is a low-frequency signal component produced by weakly non-linear propagation of an ultrasound wave. It is proportional to the second time derivative of the squared envelope of the transmitted signal (Averkiou et al. 1993; Berktay 1965; Daeichin et al. 2012; Vos et al. 2010). Rectangular envelopes were found to produce strong SH stimulation of the UCA.

The UH response (at $^3/_2 f_0$) constitutes another UCA-specific signal that is not generated by tissue (Maresca et al. 2013). UH contrast imaging can be performed with a low-bandwidth probe (40% bandwidth), whereas SH or higher harmonic imaging requires at least 70% bandwidth (Maresca et al. 2013). Also, the lateral resolution in UH imaging is higher than that in SH and NF imaging (Goertz et al. 2005). The drawbacks are the relatively weak level of the UH signal from the UCA and the higher attenuation of the UH signal because of frequency-dependent attenuation.

In the study described here, we systematically compared, in an *in vitro* setup, the value of three different frequency components: SH, NF and UH. We used excitation bursts with a rectangular envelope together with conventional non-linear contrast detection methods: pulse inversion (PI) (Simpson et al. 1999), amplitude modulation (AM) (Brock-Fisher et al. 1996) and a combination of PI and AM (PIAM) (Haider and Chiao 1999). The comparison is based on measurements of the ratio of the detected UCA signal to the residual tissue signal (termed the *contrast-to-tissue ratio* [CTR]) and the ratio of the detected UCA signal to the far-wall artifact signal (termed the *contrast-to-artifact ratio* [CAR]). Target depth, pulse duration, UCA concentration and transmit pressure were systematically varied to find the optimal non-linear imaging approaches for HFU applications in different conditions. Finally, those combinations with the highest CTRs and CARs *in vitro* were chosen for *in vivo* verification in mouse and chicken embryo models.

The results described in this article are the primary findings of our comprehensive *systematic in vitro* analysis (108 measurements and 648 analyses in total). The results and details of the *in vivo* experiments are presented in the online supplement (available online at http://www.umbjournal.org), referred to as Supplementary Material throughout this article. All CTR and CAR values referred to in this article are highlighted and numbered from ❶–❻ in Supplementary Tables 2–10 in the Supplementary Material.

METHODS

A high-frequency ultrasound scanner with linear array transducer (Vevo 2100 with MS250 probe, VisualSonics, Toronto, ON, Canada) was operated at three frequencies (15, 22 and 30 MHz). We insonified the UCA at transmit frequency f_0 and examined the frequency responses at around $^1/_2 f_0$ for SH imaging, f_0 for NF imaging and $^3/_2 f_0$ for UH imaging. The MS250 probe has a center frequency of 22.5 MHz and a –6-dB two-way frequency bandwidth of 70% (15–30 MHz) (Needles et al. 2010). To benefit from the transducer's sensitivity in both transmission and reception, the transmit frequencies were selected as follows: 15 MHz for UH imaging, resulting in a received UH component at 22.5 MHz; 22 MHz for NF imaging; and 30 MHz for SH imaging, with a received SH component at 15 MHz. Please note that in this study the terms SH and UH are defined with respect to the frequency of the transmitted ultrasound signal, not with respect to the resonance frequency of the UCA microbubbles.