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Selective imaging of adherent targeted ultrasound contrast agents

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Abstract

The goal of ultrasonic molecular imaging is the detection of targeted contrast agents bound to receptors on endothelial cells. We propose imaging methods that can distinguish adherent microbubbles from tissue and from freely circulating microbubbles, each of which would otherwise obscure signal from molecularly targeted adherent agents. The methods are based on a harmonic signal model of the returned echoes over a train of pulses. The first method utilizes an ‘image–push–image’ pulse sequence where adhesion of contrast agents is rapidly promoted by acoustic radiation force and the presence of adherent agents is detected by the signal change due to targeted microbubble adhesion. The second method rejects tissue echoes using a spectral high-pass filter. Free agent signal is suppressed by a pulse-to-pulse low-pass filter in both methods. An overlay of the adherent and/or flowing contrast agents on B-mode images can be readily created for anatomical reference. Contrast-to-tissue ratios from adherent microbubbles exceeding 30 dB and 20 dB were achieved for the two methods proposed, respectively. The performance of these algorithms is compared, emphasizing the significance and potential applications in ultrasonic molecular imaging.

1. Introduction

Molecular imaging has the potential to detect molecular changes before phenotypic changes become apparent, and therefore holds promise for highly sensitive detection of disease. Additionally, the capability to image physiologic processes based on molecular signatures could promote rational drug discovery and development (Jaffer and Weissleder 2005, Lanza and Wickline 2003). Key components of molecular imaging with ultrasound include the development of targeted contrast agents specific for biochemical markers and the development of methods to detect the agents at the target site (Lindner 2004b, Dayton and Ferrara 2002). Targeted microbubbles, liposomes and nanoparticles have been developed through the conjugation of a peptide, antibody or other ligand to the contrast agent shell. Since ultrasound contrast agents are blood pool agents, biomarkers in ultrasonic molecular imaging are generally located on endothelial or blood cells available to targeted agents. Detection of inflammation (Christiansen *et al* 2002), heart transplant rejection (Weller *et al* 2003), thrombus (Unger *et al* 1998, Wright *et al* 1998) and angiogenesis (Leong-Poi *et al* 2003, Weller *et al* 2005) have been reported using ultrasonic molecular imaging.

Oscillating bubbles can be eight orders of magnitude more efficient than purely passive scatterers in active sound emission (Hilgenfeldt *et al* 1998), and encapsulation by a lipid shell further increases their stability and provides opportunities for incorporation of targeting ligands (Klibanov 2005, Lindner 2004a). Single microbubbles can be detected in the bloodstream with various ultrasound signal-processing techniques (Klibanov *et al* 2004), and these agents provide spatial resolution on the order of hundreds of microns.

Although initial studies have been promising, some major challenges remain in ultrasonic molecular imaging. The first issue is the poor targeting efficiency. The density of adherent microbubbles retained in the vasculature at a target site has been shown to be extremely low with intra-vital microscopy, on the order of 10 microbubbles per mm³ (Schumann *et al* 2002, Leong-Poi *et al* 2003). However, the targeting efficiency can be greatly increased with the application of ultrasound radiation force (Zhao *et al* 2004, Rychak *et al* 2005).

Imaging methods to sensitively detect and distinguish adherent targeted contrast agents from freely circulating agents and tissue are unavailable (Christiansen and Lindner 2005). Methods that perform well for imaging free microbubbles may be applied to adherent microbubbles. However, the high background signal from free microbubbles and tissue obscures echoes from adherent microbubbles and thus the potential sensitivity of molecular imaging is lost. Due to the lack of selectivity and sensitivity, current strategies for imaging adherent agents require waiting for clearance of freely flowing microbubbles and fragmentation of adherent agents (Christiansen and Lindner 2005). This multi-step process is generally inefficient due to the multiple pulse sequences and waiting time required.

In this paper, we present two selective and non-destructive detection strategies for targeted adherent agents from a harmonic signal model of tissue, free agents and adherent agents, and compare the performance in terms of tissue rejection and free agent signal suppression. These new methods will allow rapid and selective detection of adherent agents in ultrasonic molecular imaging.

2. Theory

2.1. Model of received signals from single scatterers

Assuming it is stationary, the echo from a single scatterer (either a bubble or tissue scatterer) located at z at time t is

$$f(t) = s(t - 2z/c) \sum_n a_n \cos[n\omega_0(t - 2z/c)] \quad (1)$$

where $s(t)$ is the signal envelope in baseband (assuming a real envelope), a_n is the amplitude of n th harmonic, ω_0 is the fundamental frequency and c is the speed of sound. At time $u = t + T$, where T is the pulse-repetition interval, the same scatterer moves to a new location $z' = z + vT \cos \alpha$ and the echo becomes

$$\begin{aligned} f(u) &= s(u - 2z'/c) \sum_m a_m \cos[m\omega_0(u - 2z'/c)] \\ &= s(u - 2z/c - 2vT \cos \alpha/c) \sum_m a_m \cos[m\omega_0(u - 2z/c - 2vT \cos \alpha/c)] \end{aligned} \quad (2)$$

where v is the velocity of the scatterer and α is the beam-vessel angle. The time delay caused by scatterer movement is $\Delta t = 2\Delta z/c = 2vT \cos \alpha/c$. For a relatively long pulse, this time delay is small relative to the pulse duration, so the change of the envelope can be ignored for a short time T .

As an example let the phase change caused by scatterer movement equal 2π at the fundamental frequency ($m = 1$),

$$\Delta\phi = \omega_0 \Delta t = \omega_0 2vT \cos \alpha/c = 2\pi, \quad (3)$$

then

$$vT = \lambda_0 / (2 \cos \alpha). \quad (4)$$

For a fundamental frequency of 4 MHz and a 60° angle, the product of velocity and time equals one wavelength or ~0.4 mm. If the velocity is 10 mm s⁻¹, then $T \approx 0.04$ s, about four pulse periods at a pulse repetition frequency (PRF) of 100 Hz. Higher order harmonics will change multiples of 2π within the same period. Therefore, averaging the received echoes over multiple pulses in a time period longer than T can suppress the flowing agent signal.

The correlation

$$\begin{aligned} R_f(T) &= E[f(t)f(t+T)] \\ &= E[f(t)f(u)] \\ &= E\left[s(t-2z/c) \sum_n a_n \cos[n\omega_0(t-2z/c)] \right. \\ &\quad \left. \cdot s(u-2z'/c) \sum_m a_m \cos[m\omega_0(u-2z'/c)]\right]. \end{aligned} \quad (5)$$

Ignoring the envelope,

$$\begin{aligned} R_f(T) &\approx E\left[\sum_n a_n \cos[n\omega_0(t-2z/c)] \cdot \sum_m a_m \cos[m\omega_0(u-2z'/c)]\right] \\ &= E\left[\sum_n a_n \cos[n\omega_0(t-2z/c)] \cdot \sum_m a_m \cos[m\omega_0(t+T-2(z+vT\cos\alpha)/c)]\right] \\ &= E\left[\sum_n a_n \cos[n\omega_0(t-2z/c)] \cdot \sum_m a_m \cos[m\omega_0(t-2z/c-2vT\cos\alpha/c)]\right] \\ &\approx 1/2 \sum_n a_n^2 \cos[n\omega_0 2vT\cos\alpha/c]. \end{aligned} \quad (6)$$

Note that $\omega_0 T = 2k\pi$, where k is an integer, in practical ultrasound systems and odd and even orders of harmonics cannot be zero at the same time. For a simplified case with no harmonics, the correlation equals zero when $\omega_0 2vT\cos\alpha/c = \pi/2$, therefore $vT = \lambda_0/(8\cos\alpha)$. If the angle is 60°, then the product vT equals a quarter of a wavelength when the correlation is zero. Similarly, for echoes with harmonics with a beam–vessel angle of 60°, the correlation equals zero when the product of vT equals a quarter wavelength at the effective centre frequency.

2.2. Model for received signal from tissue, free and adherent agents

The returned signal from a group of scatterers (including bubbles and tissue scatterers) with harmonics can be written as a summation of contributions from all scatterers delayed according to their spatial locations and weighted by lateral beam weighting (Ferrara and Algazi 1994):

$$\begin{aligned} r(t) &= \sum_i \sum_k b[r_i(t)] s[t-2z_i/c - kT(1+2v(\tilde{r}_i)\cos\alpha/c)] \\ &\quad \cdot \sum_n a_{in} \cos[n\omega_0(t-2z_i/c - 2kTv(\tilde{r}_i)\cos\alpha/c)] \end{aligned} \quad (7)$$

where i and k are the scatterer index and pulse index, respectively, $b[r_i(t)]$ describes the lateral beam weighting, z_i and r_i describe the position of the i th scatterer in a cylindrical coordinates system of a transducer, $\tilde{z}_i$ and $\tilde{r}_i$ are the position of the scatterer in a cylindrical coordinates system of a vessel, $v(\tilde{r}_i)$ is the velocity of the i th scatterer (assuming laminar flow) and a_{in} is the amplitude of n th harmonic of the i th scatterer.

The autocorrelation is

$$\begin{aligned}
R_r(t, u) = E[r(t)r(u)] = E \left\{ \sum_i \sum_k b[r_i(t)] s[t - 2z_i/c - kT(1 + 2v(\tilde{r}) \cos \alpha/c)] \right. \\
\cdot \sum_n a_{in} \cos[n\omega_0(t - 2z_i/c - 2kT \cdot v(\tilde{r}) \cos \alpha/c)] \\
\cdot \sum_j \sum_l b[r_j(u)] s[u - 2z_j/c - lT(1 + 2v(\tilde{r}) \cos \alpha/c)] \\
\cdot \left. \sum_m a_{jm} \cos[m\omega_0(u - 2z_j/c - 2lT \cdot v(\tilde{r}) \cos \alpha/c)] \right\}.
\end{aligned} \tag{8}$$

In the case of tissue scatterers or monodisperse microbubbles, scattering amplitudes can be assumed to be the same for all scatterers, i.e. all a_{in} simplify to a_n .

At relatively low driving pressure (100–200 kPa), tissue propagation can be approximated as a linear filter, therefore only a_1 would be significant and $a_n \approx 0$, $n \geq 2$, for tissue at the low pressures employed here. On the other hand, even at relatively low pressures, both free and adherent agents can produce higher order harmonics, therefore, $a_n \neq 0$, $n \geq 1$ for microbubbles.

2.3. Numerical evaluation of signal model and signal correlation

The Field II program (Jensen and Svendsen 1992) is used to simulate the received echoes from microbubbles flowing at a mean velocity of 10 mm s^{-1} in a $200 \mu\text{m}$ vessel with a beam–vessel angle of 60° . In order to model the active acoustic response from microbubbles, a simulated bubble echo (Morgan *et al* 2000) is used as the excitation for a transducer whose impulse response is a delta function. Parameters include a microbubble diameter of $1.2 \mu\text{m}$, and either a 4 MHz ten-cycle or 2 MHz five-cycle Blackman-windowed pulse at 210 kPa (the same as in the experimental studies). The bubble size distribution is assumed to be monodisperse. Echoes from the moving microbubbles in this model are the summation of the simulated single bubble echoes delayed according to spatial locations, with effects of pulse envelope and beam weighting included, as in (7). One hundred echoes are received at a PRF of 1 kHz and the correlations are calculated, as in (8). This process is repeated 20 times and the correlations are averaged and compared to the correlation of experimental echoes at the same conditions.

The correlation of echoes from adherent agents is calculated using experimental echoes since adherent microbubbles are assumed to be stationary and bubble fragmentation can cause signal decorrelation.

2.4. Detection of adherent agents based on signal difference before and after the radiation force pulse: the ‘image–push–image’ method

To facilitate the description of the following parts, the signal in (7) is rewritten in a two-dimensional form as

$$\begin{aligned}
r(t, k) = \sum_i b[r_i(t)] s[t - 2z_i/c - kTv(\tilde{r}) \cos \alpha/c] \\
\times \sum_n a_{in} \cos[n\omega_0(t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c)]
\end{aligned} \tag{9}$$

where t and k are the indices in fast time and slow time, respectively. Here ‘fast time’ refers to propagation time, where distance and time are simply related by the speed of sound; ‘slow time’ refers to firing index, i.e., the time between individual excitations of the ultrasound transducer. Depending on the imaging system and choice of transducers, n can be 3, 4 or even higher.

Adherent agents are assumed to be absent from the imaging region before the radiation force pulse and the returned signal is

$$\begin{aligned}
 r_1(t, k) &= \sum_i b[r_f(t)]s[t - 2z_i/c]a_{i1,t} \cos[\omega_0(t - 2z_i/c)] \\
 &+ \sum_i b[r_f(t)]s[t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c] \\
 &\times \sum_n a_{in,f} \cos[n\omega_0(t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c)] \\
 &= r_{t,1}(t, k) + r_{f,1}(t, k)
 \end{aligned} \tag{10}$$

where ‘ t ’, ‘ f ’ and ‘ b ’ in the subscripts denote tissue, free and bound agents, respectively. As mentioned previously, averaging over multiple pulses would suppress the flowing agent signal and thus estimate the tissue component:

$$r_1(t) = \frac{1}{N} \sum_k r_1(t, k) = \frac{1}{N} \sum_k [r_{t,1}(t, k) + r_{f,1}(t, k)] \approx \hat{r}_t(t). \tag{11}$$

After the radiation force pulse, the returned signal is

$$\begin{aligned}
 r_2(t, k) &= \sum_i b[r_f(t)]s[t - 2z_i/c]a_{i1,t} \cos[\omega_0(t - 2z_i/c)] \\
 &+ \sum_i b[r_f(t)]s[t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c] \\
 &\times \sum_n a_{in,f} \cos[n\omega_0(t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c)] \\
 &+ \sum_i b[r_f(t)]s[t - 2z_i/c] \sum_n a_{in,b} \cos[n\omega_0(t - 2z_i/c)] \\
 &= r_{t,2}(t, k) + r_{f,2}(t, k) + r_{b,2}(t, k).
 \end{aligned} \tag{12}$$

After averaging (12) becomes

$$r_2(t) = \frac{1}{N} \sum_k r_2(t, k) = \frac{1}{N} \sum_k [r_{t,2}(t, k) + r_{b,2}(t, k) + r_{f,2}(t, k)] \approx \hat{r}_t(t) + \hat{r}_b(t). \tag{13}$$

The difference between the averages after and before the radiation force pulse gives an estimate of the bound agent signal:

$$r_2(t) - r_1(t) \approx \hat{r}_b(t). \tag{14}$$

The averaging process in (11) and (13) can be generalized as a linear filter

$$r(t) = \sum_k e(k)r(t, k) \tag{15}$$

where $e(k)$ are the coefficients of the filter and $e(k) = 1/N$ for (11) and (13). Therefore, a slow-time low-pass filter can be applied instead of averaging in implementation.

In summary, a strategy to detect bound agents includes multiple steps. First, the received echoes during the first imaging period are low-pass filtered in slow time to reduce the component from freely floating agents. A long low intensity pulse is then used to produce radiation force which deflects flowing agents to the tube wall resulting in rapidly increased binding. Slow-time filtering is repeated in the second imaging period, and finally the filtered echoes from before and after the radiation force pulse are subtracted (figure 1). Importantly, this sequence of

operations can be implemented without waiting for the clearance of freely circulating agents. This method will be referred as ‘image–push–image’ and can be implemented on a system with limited bandwidth.

2.5. Incorporation of spectral filtering: the ‘harmonic’ method

The previous method for detecting adherent agents requires the incorporation of radiation force and assumes bound agents are absent before the sequence. A second method is now described in which a fast-time high-pass filter is applied for tissue echo rejection. Although radiation force will contribute to this second method by enhancing targeted contrast agent adhesion rate and efficiency, it is not required.

Following transmission of a pulse with a peak negative pressure in the hundreds of kilopascals, high order harmonics from tissue are relatively weak and high-pass filtering of the received signal in fast time can reject tissue echoes (Kruse and Ferrara 2005, Bouakaz *et al* 2003).

The received signal with tissue, free and bound agents all present,

$$\begin{aligned}
 r(t, k) &= \sum_i b[r_i(t)]s[t - 2z_i/c]a_{i1,t} \cos[\omega_0(t - 2z_i/c)] \\
 &+ \sum_i b[r_i(t)]s[t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c] \\
 &\times \sum_n a_{in,f} \cos[n\omega_0(t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c)] \\
 &+ \sum_i b[r_i(t)]s[t - 2z_i/c] \sum_n a_{in,b} \cos[n\omega_0(t - 2z_i/c)] \\
 &= r_f(t, k) + r_b(t, k) + r_b(t, k).
 \end{aligned} \tag{16}$$

After a fast-time high-pass filter,

$$\begin{aligned}
 r_{hp}(t, k) &= \sum_i b[r_i(t)]s[t - 2z_i/c] \sum_{n=2} a_{in,b} \cos[n\omega_0(t - 2z_i/c)] \\
 &+ \sum_i b[r_i(t)]s[t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c] \\
 &\sum_{n=2} a_{in,f} \cos[n\omega_0(t - 2z_i/c - 2kTv(\tilde{r}) \cos \alpha/c)] \\
 &= r_{b,hp}(t, k) + r_{f,hp}(t, k).
 \end{aligned} \tag{17}$$

Averaging over multiple pulses to suppress free agent signal:

$$r_{hp}(t) = \frac{1}{N} \sum_k r_{hp}(t, k) = \frac{1}{N} \sum_k [r_{b,hp}(t, k) + r_{f,hp}(t, k)] \approx \hat{r}_{b,hp}(t). \tag{18}$$

As shown in (15), this averaging process can also be generalized as a slow-time low-pass filter. The relative number of freely flowing versus adherent agents can be estimated from the signal power change after the slow-time low-pass filter. This method will be referred as the ‘harmonic’ method.

By combining the filters in fast time and slow time, four different images can be formed using this imaging strategy as labelled A, B, C and O in figure 2: A, a contrast only image with both free and adherent agents in harmonics band; B, anatomical reference image with tissue and adherent agents in the fundamental band; C, freely flowing agents only in harmonic band and O, adherent agents only in harmonic band.

3. Experimental methods

3.1. Experiment setup

Experiments were conducted on a custom-built ultrasound prototype system with a three-axis motion stage described previously by Kruse and Ferrara (2002). The system was synchronized by the 62.5 MHz clock of the A/D board. A ten-cycle Blackman-windowed pulse at 4 MHz or a five-cycle Blackman-windowed pulse at 2 MHz was generated on an arbitrary wave generator (AWG 2021, Tektronix, Irvine, CA), and amplified by a radio frequency (RF) amplifier (ENI 3200L, ENI, Rochester, NY) to provide transducer excitation. A 5 million cycle, 4 MHz, low amplitude (~50 kPa) pulse was used as a 'push' pulse to displace the flowing agents to the vessel wall via the mechanism of radiation force. A custom-built 2.25 MHz outer annular element (V3966, OD 0.75 inch and ID 0.47 inch, Panametrics-NDT, Waltham, MA) was used for transmission and a 15 MHz inner element (IM1502HR, 0.25 inch, Valpey Fisher Corp., Hopkinton, MA) was used for reception, each with a 1 inch focal length in a coaxial and confocal setup. The received signal was amplified 40 dB by a Panametrics receiver (5900PR, Panametrics-NDT, Waltham, MA), and digitized by an A/D board (PDA12, Signatec Inc., Newport Beach, CA) at the PC at a sampling rate of 125 Ms s⁻¹.

The pressure output from the 2.25 MHz transducer was calibrated with a needle hydrophone (HNZ-0400, Onda Inc., Sunnyvale, CA) for both the imaging pulses and radiation force pulse. The imaging system was characterized with a 1.5 mm diameter steel ball positioned at the mutual focus with the imaging pulses. The one-way -6 dB bandwidth was ~120% (0.9–3.6 MHz) and 90% (~10–23 MHz) for the outer and inner elements, respectively. The 2.25 MHz annular element transducer produces side lobes in the lateral direction, approximately -12 dB at 1.2 mm and 0.7 mm at 2 MHz and 4 MHz, respectively.

A pair of 200 μ m cellulose tubes was held tightly against a piece of excised bovine muscle tissue. One of the tubes was coated with an avidin receptor while the other served as a control. Biotin-targeted contrast agents were pumped through the two tubes using a dual syringe pump (PhD 2000, Harvard Apparatus, Holliston, MA) at a concentration of ~5000 microbubbles per microlitre. The preparation of the avidin tube and biotin-targeted contrast agents were previously described by Zhao *et al* 2004. The tubes were held vertically to eliminate the retention of large microbubbles at the top of the tube due to floatation. The tube pair was positioned at approximately the mutual focus of the two transducers. Tissue, tubes and transducers were all immersed in a water bath for acoustic coupling.

All data were processed off-line in MATLAB® (The MathWorks, Inc., Natick, MA) as described in figures 1 and 2. All performance measurements are reported as contrast-to-tissue ratio, which is ultimately limited by the noise floor as perfect tissue rejection leaves only noise. The noise-free dynamic range of the system used in this study was approximately 45 dB for all of the data presented, but this figure is expected to be at least 100 dB on commercial scanners.

3.2. Image-push-image

Targeted contrast agents were pumped through both the control and targeted tubes at the same flow rate of 25 mm s⁻¹. Twenty B-mode images containing tissue and the tubes were initially acquired. This imaging was immediately followed by a sequence of 20 radiation force pulses at a pulse repetition frequency (PRF) of 0.2 Hz to promote rapid microbubble adhesion in the target tube. At a PRF of 0.2 Hz, one radiation force pulse was fired every 5 s, although each pulse itself lasted 1.25 s. There was a 3.75 s rest time between every two pulses. The radiation force pulses were delivered while the transducer was scanning. Following the radiation force sequence, a second set of 20 additional B-mode images was acquired. Optical observation had

previously demonstrated that the radiation force pulses cause bubble adhesion in the target but not control tube (Zhao *et al* 2004).

After imaging, high amplitude break pulses were applied to fragment the microbubbles in the sample volume and the tube was rinsed with water. A set of ten B-mode images of tissue only was then acquired, providing a tissue only background for contrast signal quantification. Images were captured at a PNP of 210 kPa at centre frequencies of 2 MHz and 4 MHz. The image field of view was 4 mm for a transmitted centre frequency of 4 MHz and 8 mm for a centre frequency of 2 MHz.

An image of adherent microbubbles was formed following the steps in figure 1 as follows: the images before and after push pulses were averaged and the difference was calculated by image subtraction. During this process, the contrast-to-tissue ratio (CTR) and tissue suppression due to background subtraction were calculated. Freely flowing signal suppression due to averaging was also be quantified with the help of the available tissue only image.

Digital low-pass filters were applied in the received signal to simulate the limited bandwidth of an imaging system. The cut-off frequency of these filters was carefully chosen to keep signals of fundamental only or fundamental and higher harmonics.

3.3. Harmonic method

A 'swept' imaging mode described by Kruse *et al* was used in this second study (Kruse *et al* 1998). A swept mode image is a B-mode image which is oversampled in lateral direction. This unique property implies that the RF lines are highly correlated, and thus slow-time filters can be applied continuously. In this study, the lateral distance between consecutive lines in the lateral direction was 2 μm .

Again, targeted contrast agents were pumped through both the control and targeted tubes at the same flow rate of 25 mm s^{-1} . Ten radiation force pulses were applied to promote adhesion instead of 20, since the lateral scanning distance was reduced to 2 mm. Images were captured in swept-scan mode with 4 MHz transmission with 15 MHz reception. These data included echoes from tissue as well as both freely flowing microbubbles and adherent microbubbles in the same sample volume.

A second set of data was acquired similar to the previous set except that the two tubes were rinsed with water for about 3 min after the radiation force pulse sequence so that non-adherent microbubbles were washed out. This data set contained signals from only tissue and adherent microbubbles, and was used as a reference to verify detection of adherent microbubbles from the first experiment.

All data were processed off-line as described in figure 2. The fast-time filters in figure 2 were implemented as fourth-order Chebyshev type II digital filters. The stop band edge frequency is 4.5 MHz for the high-pass filter and 8 MHz for the low-pass filter, respectively, and the stop band ripple is -25 dB.

To facilitate the comparison between the two methods, the slow-time low-pass filter was implemented as a moving average over 20 pulses, i.e., the same as in image-push-image method. The slow-time high-pass filter was a finite impulse response (FIR) filter of order 20.

3.4. Effect of flow rate, centre frequency and pressure on harmonic signal power

In order to study the effect of flow rate and pressure, the first set of experiments in section 3.3 was repeated for a pressure of 210 kPa at three flow rates of 10, 5, 2.5 mm s^{-1} . The transmission centre frequency was 4 MHz. For each pressure level and flow rate, experiments were repeated

ten times. The signal power in the frequency band from second harmonic up to 20 MHz was quantified.

Experiments were then repeated with matched pulse pairs at 2 MHz and 4 MHz at a pressure of 210 and 290 kPa and a flow rate of 10 mm s^{-1} . The frequency response of the receiving transducer was measured experimentally and used to correct the received signal power before the comparison.

3.5. M-mode data for calculation of signal correlation

Experiments were repeated in M-mode with tissue removed. Echoes from freely flowing and adherent agents were captured with imaging pulses both at 4 MHz and 2 MHz. The flow rate was 10 mm s^{-1} , the PRF was 100 Hz and the beam-flow angle was $\sim 60^\circ$. The first 100 echoes after pulsing started were used for the calculation of the echo correlation. The correlation was then averaged over ten independent data sets.

4. Results

4.1. Signal correlation

For transmission at both 2 and 4 MHz, the correlation of simulated echoes from flowing microbubbles decreases nearly to zero when the product of vT is about 0.05 mm, which indicates that the effective or equivalent centre frequency of the bubble echoes is about 8 MHz (figure 3). The correlation of the experimental echoes from free microbubbles reaches zero after one pulse (vT equals 0.1 mm), which implies that free microbubble echoes from both 2 MHz and 4 MHz experiments are uncorrelated from pulse to pulse.

In contrast, echoes from adherent microbubbles remain highly correlated even after 30 pulses at 100 Hz PRF, or 0.3 s, at three pressures and two transmission frequencies (figure 4). The correlation remains at ~ 0.8 after 30 pulses at 290 kPa with a transmission centre frequency of 4 MHz and ~ 0.6 at 210 kPa with a transmission centre frequency of 2 MHz. For the 210 kPa case, the correlation was ~ 0.9 and ~ 0.7 after 20 pulses with a transmission centre frequency of 4 MHz and 2 MHz, respectively. The faster decorrelation with a transmission at 2 MHz in contrast to 4 MHz is likely due to microbubble destruction, which is known to be greater at lower centre frequency (Chomas *et al* 2001a, 2001b). In each of these cases, the adherent echoes remain highly correlated over 20 pulses, indicating that averaging over 20 pulses can suppress the flowing agent signal while preserving the signal from adherent agents.

4.2. Image–push–image

Following the image–push–image procedures (figure 1), an image of a control tube, a targeted tube and tissue was obtained for transmission and reception at 4 MHz (figure 3(a)). This image displays a high contrast between the targeted tube and tissue background (CTR = 37 dB). The CTRs at different steps in figure 1 are provided in table 1. Two parameters are of particular interest, i.e., suppression of free agent signal due to averaging (from I to A or B to C) and suppression of the tissue signal due to signal subtraction (from C to O), both of which are crucial to the good performance of the proposed image–push–image strategy.

The change in the signal intensity resulting from slow-time averaging in the control tube is ~ 10 dB before radiation force pulses (I–A), and is 13 dB afterwards (B–C), corresponding with the rejection of the signal from free agents. In the targeted tube, slow-time averaging reduces the signal by ~ 4 dB before the radiation force pulse (I–A), which may indicate the existence of some adherent agents; and ~ 0.1 dB afterwards (B–C), indicating that the adherent agent signal is dominant in the targeted tube after radiation force enhanced adhesion (table 1). The suppression of tissue echoes by signal subtraction (O–C) results in an increase of CTR of ~ 33

dB for the control tube and ~29 dB for the targeted tube. When the reception bandwidth is extended to include the second and third harmonic (row 3 compared with row 1 within each group in table 1), the suppression of free agent signal (I–A and B–C) increases less than 1 dB while tissue suppression (O–C) decreases less than 2 dB in all cases.

Side lobes are clearly visible for the targeted tube in the image (figure 5(a)). Side lobes are –14 dB relative to the main lobe, which is in agreement with the –12 dB measured with a 1.5 mm diameter steel ball during initial calibration.

An image of a wider region was obtained with a matched pulse at 2 MHz (figure 5(b)). Since the pulse length was the same, the axial extent of the targeted tube in the image is similar compared to that obtained with transmission at 4 MHz. However, because the wavelength is doubled at 2 MHz, the lateral extent of the targeted tube is also doubled, which indicates a lower lateral resolution at 2 MHz. CTRs at different steps in figure 1 were calculated for different reception bandwidths (table 2). As the bandwidth for reception increases, the CTR at the output (O) increases for the control tube while decreasing slightly for targeted tube (tables 1 and 2).

4.3. Harmonic method

The combination of filters in fast time and slow time (figure 2) was used to separate echoes from free microbubbles, adherent microbubbles and tissue. Data are illustrated for 4 MHz transmission with 15 MHz reception (figure 6). After fast-time high-pass filtering, the image shows signals from freely flowing agents in both tubes, although only the targeted tube had contrast agents bound to the vessel wall (figure 6(a)). A subsequent slow-time high-pass filter removed the signal from adherent agents (figure 6(c)) but did not change the CTRs for control and targeted tube substantially (the CTR increases from 13.6 dB to 14.0 dB for the control tube and decreases from 13.4 dB to 13.0 dB for the targeted tube). Reception at 15 MHz provides the resolution to separate the two tubes despite the fact that they were less than a millimetre apart (figure 6(a)).

The targeted tube is clearly highlighted after slow-time low-pass filtering (figure 6(d)) due to the strength of the coherent signal from adherent bubbles (CTR = 21 dB). The signal power decreased by ~12.5 dB in the control tube and ~2.4 dB in the targeted tube after the slow-time low-pass filter, indicating that the targeted tube contained adherent agents.

Detection of adherent agents was confirmed by imaging after rinsing the two tubes with water (figure 7). No flowing agents can be observed after fast-time high-pass filtering (figures 7(a) and (c)) which confirms the removal of flowing agents by the rinse. The remaining signal after further slow-time low-pass filtering (figure 7(d)) confirms the detection of adherent agents. The signal power in the targeted tube changed by only 0.3 dB after the slow-time low-pass filter, again indicating the absence of flowing agents in the targeted tube. The CTRs for control and targeted tubes in figures 6 and 7 are summarized in table 3.

4.4. Effect of flow rate, centre frequency and pressure

For both free and adherent microbubbles, harmonic signal power increased with increasing pressure (figure 8(a)). At the pressure levels studied, the harmonic signal power was higher when transmitting at 2 MHz than 4 MHz for both free and adherent microbubbles (figure 8(a)). However, while harmonic signal power increased with increasing flow rate for free agents, the correlation of flow rate with signal power was absent for adherent microbubbles (figure 8(b)). The higher signal power of free agents compared to adherent agents in figure 8(a) was likely due to the concentration of free contrast agents, which was about four times greater in this experiment than other experiments described in this paper.

5. Discussion

5.1. Tissue rejection and flowing agent signal suppression: comparison of the two methods

Tissue rejection and freely flowing agent suppression are the two key components of a selective imaging method for adherent agents. Examination and comparison of different techniques to achieve these goals provides insight as to when and how these new methods should be applied (table 4). A mathematical formalism was developed and used to propose two methods for the differentiation of free and adherent agents. This formalism will be employed to optimize these methods in future studies. One interesting result is the indication that the correlation of the harmonics generated by freely floating agents changes with the harmonic number, i.e., higher harmonics can be used to differentiate slower flowing agents with a given pulse train.

Tissue rejection in the image–push–image method was provided by signal subtraction, therefore the performance will be affected by tissue motion. In the harmonic method, the separation of echoes from tissue and contrast agents was based on their spectral difference. This process is in fast time and thus immune to tissue motion. One drawback of this technique is that the fast-time high-pass filter suppresses both the tissue echoes and microbubble echoes at the fundamental frequency. From our observations, the power at the fundamental frequency for adherent microbubbles is greater than that from freely flowing microbubbles, and this spectral difference is lost during the fast-time filtering process (Zhao *et al* 2006). Other tissue rejection methods such as phase inversion can also be employed.

Flowing agent signal suppression in both methods was realized by averaging over multiple frames, i.e. a slow-time low-pass filter, and the performance depends on the number of frames available, the decorrelation between frames, as well as tissue motion. In the presence of tissue motion, correlation-based or other similar algorithms can be used for tissue motion tracking and correction. Additionally, better performance can be expected by optimizing the filter parameters.

The most substantial differences (highest contrast) between adherent and flowing microbubbles are expected in a relatively large vessel (e.g. 200 μm diameter as in this study) because a large number of adherent agents can concentrate on the vessel wall to provide strong echoes. For a capillary, the number of adherent microbubbles is limited within a single vessel but the total number of adherent agents available within a sample volume may still be large because of the high density of capillaries. Due to higher reception centre frequency and wider bandwidth, the harmonic method can provide better resolution for imaging small vessels.

Although when using our single-element system, the application of radiation force over our entire sample volume required the use of 20 pulses (~ 1 min) due to the small sample volume and the mechanical scanning of the single-element transducer, we anticipate this time would be substantially reduced by using an array transducer, where radiation force could be applied to a large sample volume simultaneously (~ 1 s).

The addition of targeting enhancement using radiation force makes the first proposed detection method substantially more efficient than previous techniques. The application of a radiation force pulse promotes rapid adhesion of targeted agents, achieving far greater densities of adherent bubbles in seconds than would otherwise be achieved within the relatively brief circulation time of the contrast agents. The signal from adherent contrast agents is strongest following the radiation force pulse, when the largest number of adherent agents is available. During the long waiting period in previous methods, some adherent contrast agents detach from the target site due to shear stress, become smaller due to diffusion or disappear due to their limited life time. By employing radiation force to rapidly promote contrast agent adhesion, real-time selective and non-destructive imaging of adherent targeted agents becomes possible.

5.2. Effect of pressure and flow rate

The increase of harmonic signal power with pressure (figure 8(a)) indicates that increasing transmission power can increase the returned harmonic signal power, thus providing better CTR and sensitivity. However, adherent microbubbles may be fragmented quickly at a high pressure and no longer available for imaging.

The harmonic signal power for flowing microbubbles increases with increasing flow rate (figure 8(b)). It is likely that some bubbles are fragmented even at low pressures and the sample volume is not refreshed at lower flow rates. Additionally, in our system a higher flow rate increases the chance of receiving stronger echoes from larger more echogenic bubbles which otherwise tend to get trapped along the top of the injection tube. In contrast, this direct relationship between harmonic signal power and flow rate is missing for adherent agents. The received signal power is believed to be determined by the quantity and size of the adherent microbubbles, and is thus independent of flow rate. However, the volume of bubbles displaced from the flow stream due to radiation force and remaining adherent to the tube wall can be affected by flow rate (Zhao *et al* 2004).

5.3. Effect of centre frequency

The received signal power was higher at a transmission centre frequency of 2 MHz than at 4 MHz in our experiments (normalized by the receiving transducer frequency response).

Tissue generates less nonlinear response at lower centre frequencies, which benefits the separation of tissue and contrast agents by receiving harmonics. An additional benefit is that due to frequency-dependent attenuation lower frequency insonation will provide more signal penetration. The main drawback of lower frequency insonation is that the pressure threshold for microbubble destruction decreases as centre frequency decreases (Chomas *et al* 2001b).

One limitation of our experimental system is the noise-free dynamic range, which particularly limits our measurement of the contrast-to-tissue ratio in the harmonic method. For current commercial systems, the noise-free dynamic range is much larger (over 100 dB generally), therefore, we expect that the CTR will be higher on a commercial system.

6. Conclusion

Two new selective and non-destructive imaging methods have been proposed for detecting adherent targeted agents. Both methods can distinguish the signal of adherent agents from that of tissue and freely flowing agents, therefore the methods can be implemented within hundreds of milliseconds, without waiting for the clearance of freely circulating agents. Contrast-to-tissue ratios from adherent microbubbles exceeding 30 dB and 20 dB were achieved for the image-push-image and harmonic methods, respectively. Incorporation of radiation force pulses promotes rapid adhesion of targeted contrast agents, reduces waiting periods for accumulation of targeted agents and increases the number of agents available for imaging. The detection techniques described in this paper represent the first examples of real-time methods to sensitively detect adherent targeted contrast agents based on their unique echo response, thus overcoming one of the main challenges with targeted imaging (Lindner 2004b). Implementation of signal-processing strategies such as those described here could play an important role in both clinical and research settings.

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Figure 1.

Flow chart for the image–push–image method detailing the process for detection of targeted contrast agents. I (input), original data contain echoes from free agents plus tissue; A , free agent signal is suppressed by a slow-time low-pass filter (s-LP) and an estimate of tissue signal is formed as in (11); B , following the radiation force pulse, the acquired data set includes tissue, free and bound agents as in (12); C , following the radiation force pulse and a slow-time low-pass filter, the data set represents an estimate of echoes from tissue plus bound agents as in (13); O (output), subtracting A and C yields an estimate of the bound agents as in (14).

Figure 2.

Flow chart for the harmonic method detailing signal-processing steps. In the input (I), echoes from adherent targeted agents overlap those of tissue and circulating agents. Combination of filtering in fast time and slow time can provide additional information about: A, free and adherent agents in harmonics band; B, anatomical reference in the fundamental band and C, freely flowing agents only in harmonic band. Output data contain echoes from adherent agents only as in (18).

Figure 3.

Echo correlation from simulations and experiments for flowing agents in a 200 μm vessel with a 60° beam-flow angle. The transmission centre frequency was either 2 MHz or 4 MHz and PNP was 210 kPa.

Figure 4. Echo correlation from experiments for adherent agents. The transmission centre frequency was either 2 MHz or 4 MHz, and PNP was 130, 210 or 290 kPa.

Figure 5.

Images of a pair of tubes containing targeted contrast agents surrounded by tissue using the image–push–image method with PNP of 210 kPa and a transmission centre frequency of 4 MHz (a) and of 2 MHz (b). The CTR is 37 dB and 34 dB for the targeted tube, and 10 dB and 16 dB for the control tube in (a) and (b), respectively.

Figure 6.

Images of a pair of tubes containing targeted contrast agents surrounded by tissue using the harmonic method with PNP of 210 kPa and a transmission centre frequency of 4 MHz. Images are shown for: (a) all contrast agents; (b) tissue background; (c) flowing agents only and (d) adherent only, as labelled A, B, C and O in figure 2, respectively.

Figure 7.
Same as figure 6, except tubes have been rinsed to remove free agents.

Figure 8.

Received harmonic signal power for free and adherent agents. (a) data for 2 MHz and 4 MHz, at PNP of 210 and 290 kPa and 10 mm s^{-1} . (b) data for 4 MHz, 210 kPa, and flow rates of 2.5, 5 and 10 mm s^{-1} .

Contrast-to-tissue ratios in dB for variables labelled as in figure 1, image data presented in figure 5(b) and image-push-image processing method. The transmission centre frequency was 4 MHz and PNP was 210 kPa. Change of CTR from I to A or B to C indicates suppression of free agent signal and from C to O indicates suppression of tissue signal.

Table 1

	I (input)	A	I-A	B	C	B-C	O (output)	O-C
Control tube	Fund	-18.8	9.6	-10.8	-23.7	12.9	9.6	33.3
	Fund + 2nd	-18.4	10.0	-9.8	-22.9	13.1	9.6	32.5
Targeted tube	Fund + 2nd + 3rd	-17.6	10.3	-8.8	-21.8	13.0	10.2	32.0
	Fund	-10.9	4.7	6.9	6.8	0.1	36.7	29.8
	Fund + 2nd	-10.7	4.9	6.9	6.8	0.1	35.8	29.0
	Fund + 2nd + 3rd	-10.4	5.2	6.9	6.8	0.1	34.8	28.0

Contrast-to-tissue ratios in dB for variables labelled as in figure 1, image data presented in figure 5(b) and image-push-image processing method. The transmission centre frequency was 2 MHz and PNP was 210 kPa. Change of CTR from I to A or B to C indicates suppression of free agent signal and from C to O indicates suppression of tissue signal.

Table 2

		I (input)	A	I-A	B	C	B-C	O (output)	O-C
Control tube	Fund	1.0	-6.0	7.0	-7.7	-12.0	4.3	15.8	27.8
	Fund + 2nd	2.9	-5.8	8.7	-4.0	-10.4	6.4	15.9	26.3
	Fund + 2nd + 3rd	4.6	-5.2	9.8	-1.5	-9.7	8.2	16.6	26.3
Targeted tube	With all harmonics	7.5	-3.6	11.1	1.9	-8.3	10.2	18.2	26.5
	Fund	1.1	-4.0	5.1	8.7	8.5	0.2	33.7	25.2
	Fund + 2nd	3.0	-2.7	5.7	8.8	8.4	0.4	32.8	24.4
	Fund + 2nd + 3rd	4.4	-2.2	6.6	9.2	8.5	0.7	32.2	23.7
	With all harmonics	6.6	-1.6	8.2	9.7	8.5	1.2	30.8	22.3

Table 3

Contrast-to-tissue ratios in dB for variables labelled A, C and O in figure 2, image data presented in figures 6 and 7 and harmonic processing method. Transmission centre frequency of 4 MHz and PNP of 210 kPa. CTR at output demonstrates performance with targeted versus control agents.

		A	C	O (output)
Figure 6	Control	13.6	14.0	9.0
	Targeted	13.4	13.0	21.2
Figure 7	Control	0.0	0.2	-1.8
	Targeted	11.8	1.8	19.8

Table 4

Comparison of the two proposed methods.

	Image–push–image	Harmonic method
Tissue rejection	Signal subtraction produced 25–30 dB tissue rejection in this study.	Harmonic (fast-time) filter produced 18 dB of tissue rejection in this study.
Free agents suppression	Slow-time low-pass filter produced ~10 dB suppression.	Slow-time low-pass filter produced ~10 dB suppression.
Final CTR	~30 dB.	~20 dB.
Bandwidth requirement	Fundamental frequencies with/without harmonics.	Fundamental frequencies with higher order harmonics.
Pressure dependence	Low pressure operation possible.	Enough pressure to generate harmonics.
Limitations	Requires radiation force pulse. May require tissue motion correction.	Depth of penetration of harmonics may limit applications. May require tissue motion correction.
Assumption	No adherent agents before radiation force pulse.	None.
Resolution	Determined by the operating centre frequency.	Higher, due to higher reception frequency and wider bandwidth.