

# *In vivo* Optical Spectroscopy of Acoustically Induced Blood Stasis

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**Abstract:** Ultrasound-induced blood stasis has been observed for more than thirty years. Most of the literature has been focused on the health risks associated with this phenomenon and methods employed to prevent stasis from occurring during ultrasound imaging. To date, experimental observations have been either *in vitro* or invasive. The current work demonstrates ultrasound-induced blood stasis in murine tumor and nontumor tissue, observed through noninvasive measurements of optical spectroscopy, and discusses possible diagnostic uses for this previously undesirable effect of ultrasound.\*

## I. INTRODUCTION

Stationary sound waves have long been known to create banding effects when solids are suspended in liquids; sand in air (in a cylinder), bubbles in water, etc. In 1971, Dyson et. al. reported that stationary ultrasound waves can create bands of red blood cells *in vitro*, using chick embryos removed from the egg shell but kept alive in saline solution [1]. Later ter Haar and Wyard showed that the banding was due to the standing pressure wave created by the ultrasound [2]. Nyborg later demonstrated that even a traveling pressure wave, with small amounts of reflection at the tissue boundaries can cause banding of blood cells in the plasma medium [3]. Many have continued to study the diagnostic limits and dangers of ultrasound and ultrasound-induced stasis [4-7], but to the best of our knowledge, no one has investigated the diagnostic potential.

A limiting factor in studying this ultrasound-induced phenomenon has been the difficulty of measuring the blood flow alterations. Previous works have required the blood vessels to be dissected from the abdomen of mice [5] or the removal of chick embryos from their shells [1] so as to be seen with microscopes and stereoscopes. The phenomenon has only been observed invasively and only in a few vessels immediately on the tissue surface or in vessels separated from the surrounding tissue. Methods have been suggested to avoid prolonged blood stasis during diagnostic imaging [4] and ultrasound intensity limits have been established to avoid tissue damage and to allow the blood flow to rebound. The current experiments have been conducted within the FDA therapeutic ultrasound limits (SPTA = 0.720 mW/cm<sup>2</sup>) and blood stasis and banding have been observed to be reversible under these conditions.

It has been shown that oxy and deoxyhemoglobin have signature absorption and scattering effects visible in steady-

state broadband diffuse reflectance optical spectroscopy [8]. Furthermore, oxyhemoglobin saturation can be determined using spectroscopic measurements of light reflected from tissue and analyzed with the diffusion approximation or the higher order P<sub>3</sub> approximation [9-10]. Spectral analysis performed with a P<sub>3</sub> approximation fit has been shown to be sensitive to the dynamic changes of hemoglobin oxygen saturation due to changes in oxygen content of air being inhaled by mice [11]. The current experiments combine the above techniques, focused standing wave ultrasound-induced blood stasis and optical spectroscopy to develop a noninvasive imaging tool with potential use in tissue diagnostics.

Cells require a constant supply of oxygen for metabolic processes. Normally, as the cells consume oxygen, hemoglobin molecules in the blood continually replenish the oxygen supply as the blood flows through the vessels. When standing wave ultrasound is used to slow or stop the blood flow, the oxyhemoglobin saturation decreases as the available oxygen is depleted. When the blood flow is stopped or slowed for short periods of time, the oxyhemoglobin saturation can be observed to decrease, using optical spectroscopy measurements, and return to pre-ultrasound levels shortly after the ultrasound radiation is stopped.

The processes involved in this phenomenon are neither simple nor straightforward and many physiological questions remain unanswered concerning the ultrasound-induced effects. Although the ultrasound intensities employed have been shown to create very little heating of the tissue and have not been shown to damage tissue, the effects of ultrasound on vessel diameter have yet to be addressed, i.e. does standing wave ultrasound constrict or expand the vessels? High intensity traveling ultrasound waves have been shown by Dalecki to exert pressure on the walls of frog heart cavities [12]. The pressures required to cause banding in moving blood are much lower than the intensities needed to deform the tissue of the heart. The current experiments were designed to remain below tissue heating, tissue damage and tissue pressure thresholds and below the current FDA limits of diagnostic ultrasound intensities.

## II. METHODS AND MATERIALS

Ultrasound was generated by a ≈1 MHz piezoelectric ceramic crystal (Channel Industries) mounted behind a concave aluminum lens with a focal length of 7 cm. At 1

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MHz the -6 dB focal zone diameter was 2 mm and the focal zone length was 30 mm. The ultrasound signal was created by a function generator (Agilent 33250A) and amplified by an RF amplifier (Amplifier Research 25A250A), monitored and recorded by an oscilloscope (Tektronix TDS 2022). The ultrasonic field was measured and characterized using a hydrophone (Onda Co. HNR 500). The intensity of the ultrasound was maintained at Spatial Peak Temporal Average Intensity (SPTA)  $\approx 0.7 \text{ mW/cm}^2$ , averaged over the burst cycle.

Initial tests of the ultrasound setup included a repeat of Dyson's seminal experiment, but with lower acoustic intensities (SPTA =  $0.7 \text{ mW/cm}^2$ ) and lower frequencies ( $f \approx 1 \text{ MHz}$ ). The ultrasound was observed visually to stop blood flow, causing bands to form for short periods of time. In order to perform a non-invasive test regarding the efficacy of the ultrasound in the mouse leg, a laser Doppler system (Transonic BLF21) was used to verify blood flow alterations or stasis due to ultrasound. This technique relies on the interference between an LED

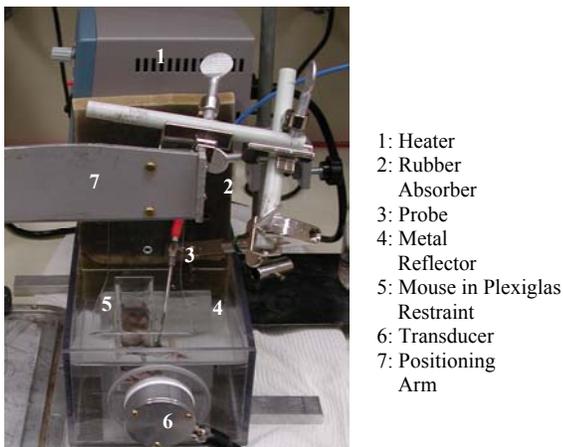


Fig. 1. A picture of the apparatus with the mouse leg located in the focal region of the ultrasound.

signal beam and the reflected beam, revealing the velocity profiles of the reflecting objects visualized as sidebands to the original beam. From the laser Doppler measurements, it is clear that in a small target volume the average velocity of the blood is at least slowing. When this same volume element is inspected with white light, there are measurable changes in the oxyhemoglobin saturation.

All experiments were conducted in a Plexiglas water tank. Distilled water was autoclaved for 45 minutes to remove ions and micro-bubbles in order to prevent cavitation and scattering of the acoustic field. A 2.5 cm thick piece of aluminum was used for the acoustic reflector and a 2.5 cm thick rubber block was placed behind the aluminum to absorb any scattered acoustic energy. During experiments the water was heated to  $37^\circ\text{C}$  using a circulating water heater and the rubber block was positioned to shield the data collection area from most of the water currents since moving water can interfere with spectroscopy measurements (Fig. 1).

Diffuse reflectance spectra were collected with a single 600 micron fiber, numerical aperture (N.A.) = 0.22, residing

at the center of a seven fiber probe (Ocean Optics, R600-7-VIS/NIR). The center collection fiber was connected to a 2048 pixel room temperature spectrometer (Ocean Optics, USB 2000-VIS/NIR) fitted with a grating for spectrum analysis between 200 nm and 1100 nm. The outer six fibers were connected to a broadband halogen light source (Ocean Optics, HL 2000). The source detector separation was 1 mm, resulting in an inspection volume of  $\approx 9 \text{ mm}^3$ , mostly within 1 mm of the tissue surface. The optical signal was weighted by the intensity curve of the light source. The intensity curvature was measured with a diffuse reflectance standard.

Six-eight week old C3H mice were inoculated intramuscularly to the right thigh with  $10^6$  MCA-35 mammary carcinoma cells, with the left hind leg used as control for the diagnostic portion of this experiment. To avoid scattering of the acoustic field, hair was removed from the hind legs using a depilatory agent (Nair®) one day prior to the experiment. The mice were sedated using a Ketamine (60 mg/kg) Xylazine (4 mg/kg) mixture injected intraperitoneally and placed in a Plexiglas restraint which positioned the leg to be examined away from the body. The probe was then fixed on the skin of the mouse leg using a positioning arm, ensuring contact but without skin compression. During data collection, the probe was held stationary, maintaining a constant pressure on the mouse skin. Optical spectroscopy measurements of hemoglobin *in vivo* are greatly dependent upon surface pressure since any changes will alter the blood volume alterations and consequently, hemoglobin volumes. Once a baseline spectrum was achieved ( $\approx 4$  minutes), the mouse/ probe were moved such that the focus of the ultrasound was  $\approx 2 \text{ mm}$  directly under the location of the optical probe. The direction of propagation of the ultrasound and light were kept orthogonal so that the metallic probe did not enter the focus of the ultrasound and obstruct or scatter the standing acoustic wave. Also, this increased the probability of intersecting the acoustic focal region with the volume of optical inspection.

During each experiment, ultrasound was administered in 5 second bursts, with 55 second relaxation periods between bursts and a total of six bursts per leg per experimental collection. For each mouse, both legs, one with a tumor (diameter  $\approx 10 \text{ mm}$ ) and one without, were subjected to ultrasound and optical spectroscopy to compare the effects in tumor versus nontumor tissue. The order of inspection of the legs was altered to diminish the possibility that the results were influenced by the depth of the anesthesia, which can directly affect the blood velocity.

Ultrasound pulse information for each experiment was monitored and collected with the oscilloscope and stored for later signal correlation studies. Optical spectra were collected with the supplied Ocean Optics software at 500 ms intervals in order to decrease the Signal to Noise Ratio (SNR) and to reduce the appearance of unwanted higher frequency signals and noise.

### III. ANALYSIS

The raw optical spectra were corrected for the curvature of the light source intensity. The intensity curve of the light

source was obtained using a diffuse reflectance standard. The optical signal then was cropped to avoid the spectral regions of low light ( $< 400$  nm) and regions near the end of the spectrometer's sensitivity ( $> 1000$  nm). Ultimately, the spectra were cropped to regions between 475 nm and 650 nm where significant changes in optical absorption are present due to oxy/deoxyhemoglobin shifts and few other absorbers affect this region of the spectrum. Several isolated wavelengths were initially considered (515 nm, 528 nm, 540 nm, 560 nm, 579 nm and 578 nm), but eventually the ratio of intensities ( $I$ ) at two wavelengths, 560 nm and 540 nm, was chosen. The ratio of  $I_{560}/I_{540}$  has been shown to be significantly affected by the presence (or absence) of the ultrasound. Since the intensities at 560 nm and 540 nm are dependent upon the oxy/deoxyhemoglobin saturations, it can be demonstrated that the ratio  $I_{560}/I_{540}$  correlates to oxyhemoglobin concentrations.

By visual comparison of  $I_{560}/I_{540}$  with the ultrasound signal, one can generally establish visual temporal correlation between these two signals in the non-tumor scans and the absence of correlation in the tumor scans (Fig. 2a and 2b). The drops in the  $I_{560}/I_{540}$  ratio signal have been consistently observed in nontumor scans and are mostly absent in tumor scans. It has been demonstrated since that the mathematical correlation between the ratio signal and the ultrasound signal in general is significantly higher in nontumor cases, than in tumor cases (Fig. 3).

#### IV. DISCUSSION

As predicted from prior studies, the presence of the standing wave ultrasound caused changes in the blood flow (Fig. 4), and, consequently, changes in the hemoglobin concentrations. These changes in the hemoglobin concentration affected optical absorption and the experimental spectroscopic measurements demonstrated this phenomenon.

These changes in oxy/deoxyhemoglobin concentration are believed to be caused by metabolic consumption of oxygen. The rate of metabolic consumption varies from mouse to mouse and from tumor to nontumor tissue types. It is generally believed that tumor cells have higher metabolic rates and therefore consume more oxygen in relation to nontumor tissue. This alone would lead one to expect that oxyhemoglobin concentrations would drop faster and more emphatically in tumor tissue than in nontumor tissue. In general, such drops were not observed in our experiment, although, in some instances tumor tissue did exhibit oxyhemoglobin drops when the optical probe was located in close proximity to visible (large) surface vessels. In these cases, higher initial oxyhemoglobin concentrations were also observed and the ultrasound-induced contrast was visible in the measured ratio signal. When the probe was carefully moved to a location distant (1-2 mm) from the visible vessel, the initial oxyhemoglobin concentration dropped and the contrast was much less pronounced. The ultrasound-induced contrast was much less dependent upon location in the nontumor leg.

Initial tumor and nontumor tissue oxyhemoglobin concentrations varied from mouse to mouse, probably due to

the depth of the anesthesia. Some mice with higher initial oxyhemoglobin concentrations demonstrated ultrasound-induced contrast in the tumor tissue. This contrast however was generally less substantial than the contrast observed in the nontumor tissue of the same mouse. Comparison of the ultrasound-induced contrast of each leg to the other leg of the same mouse was a technique used to reduce the mouse to mouse variance of oxyhemoglobin saturation. As noted in Ref. 11, there are significant differences in the diffuse reflectance spectra gathered from different mice and from various locations of a single tumor in each mouse.

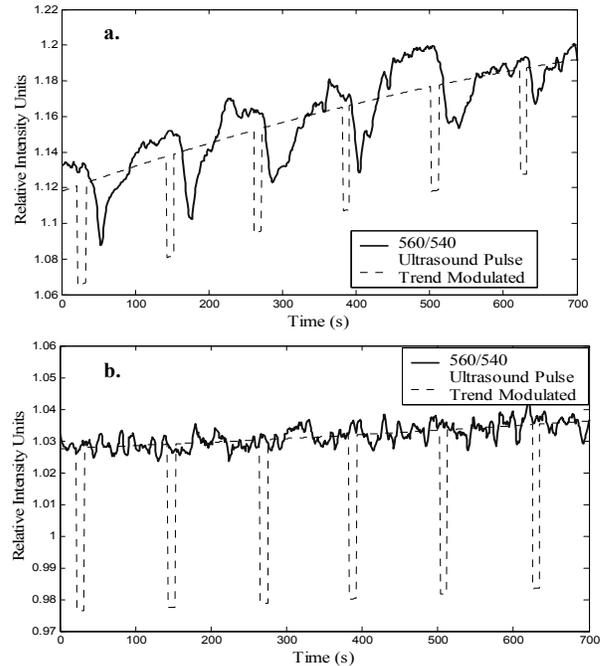


Fig. 2. The ratio  $I_{560}/I_{540}$  signal with the trend modulated ultrasound pulse signal superimposed for a typical (a) nontumor tissue and (b) tumor tissue. Notice the much larger ultrasound induced contrast in the nontumor signal.

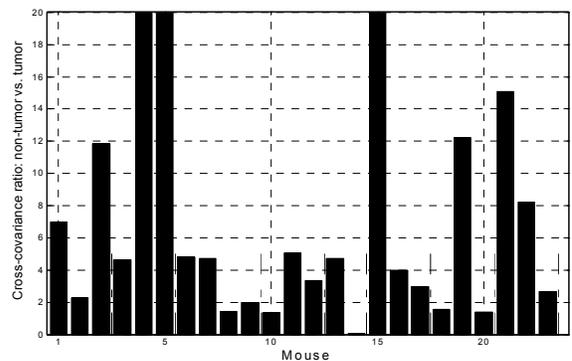


Fig. 3. The  $I_{560}/I_{540}$  signal in nontumor tissue is better correlated to the ultrasound than the same signal for tumor tissue, measured for the same mouse to compensate for differences in mice population. 79 % of the cases have a correlation ratio higher than two.

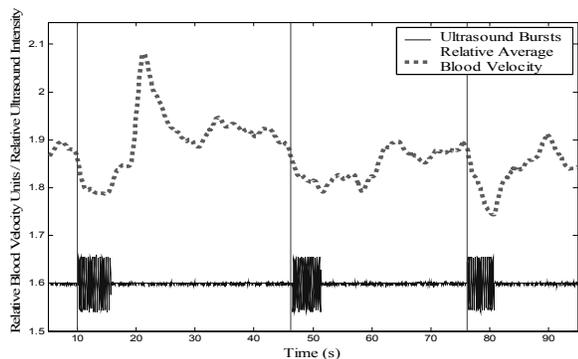


Fig 4. The top curve represents the Laser Doppler relative average volume velocity with the bottom curve representing the ultrasound bursts

For each mouse, the difference in ultrasound-induced oxyhemoglobin concentration changes between tumor and nontumor tissue could result from differences in blood vessel counts and vessel orientation within the tissue, as well as differences in the metabolic rates of each tissue. The MCa-35 tumor is a highly vascularized, well perfused metastatic model and develops an extremely chaotic mesh of blood vessels. It is generally well oxygenated when compared to other tumor species models [13]. When these tumors were analyzed with laser Doppler, the ultrasound appeared to have little effect on the blood flow, possibly due to the multidirectional and interwoven mesh of blood vessels which lack the orderly flow of skeletal vessels. In addition, the tumors generally had lower initial oxyhemoglobin saturations than the nontumor tissue of the other leg. Alternative tumor models with lower oxygenation, fewer vessels, and decreased blood flow would be expected to show less spectral contrast due to ultrasound.

In general, the ultrasound-induced contrast was much more pronounced when initial hemoglobin concentrations were elevated. This elevation could be due to inflammation of the skin, abrasion, higher vessel counts, large vessels in close proximity to the probe, less pressure exerted by the probe on the skin, etc. Although, experimental procedures attempted to decrease the effects of inflammation, abrasion, and probe force on the skin, it was unclear which factor predominated in producing the varying spectral responses between mice. Additional studies are needed to further investigate these factors.

Decreases in the observed  $I_{560}/I_{540}$  ratios were predictable, generally corresponding to the ultrasound bursts, but not always easily observable (Fig. 5). In some experiments it was hard to distinguish between responses of tumor and nontumor tissue. If the probe was positioned in the proximity of a major blood vessel in the tumor, the observed signal behaved in a manner similar to the nontumor cases (i.e., substantial decreases in the signal were correlated to the ultrasound bursts). Also, the relatively small size of the tumors made experiments sensitive to the probe positioning with respect to the surface vessels and the position of the ultrasound; operator skill was a critical factor in experiment success.

In summary, the current study demonstrated that there are substantial and predictable ultrasound-induced changes in the  $I_{560}/I_{540}$  ratio signal obtained through *in vivo*

spectroscopic measurements of diffuse light reflected from tumor and nontumor mouse tissue. The ratio signal is better correlated to the ultrasound signal for nontumor tissue than tumor tissue and appears highly promising for noninvasive tissue diagnostics.

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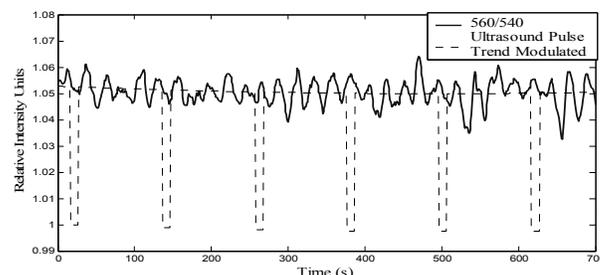


Fig. 5. The  $I_{560}/I_{540}$  ratio signal for non tumor tissue with the trend modulated ultrasound pulse superimposed. The ultrasound-induced drops are difficult to distinguish.

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