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Award Number: DAMD17-00-1-0464

TITLE: Estimation of Tumor Angiogenesis with Constrast Enhanced Subharmonic Ultrasound Imaging

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REPORT DATE: July 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Estimation of Tumor Angiogenesis with Contrast Enhanced Subharmonic Ultrasound Imaging

5. FUNDING NUMBERS
DAMD17-00-1-0464

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

New contrast-specific imaging modalities such as harmonic imaging (HI) may improve the accuracy of breast ultrasound. Unfortunately, HI suffers form reduced blood-to-tissue contrast resulting form second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between denign and malignant lesions by combining injection of an ultrasound contrast agent with SHI.

To date, in vitro experiments comparing SHI to flow rates have been completed showing good correlation with contrast uptake slopes. Animal experiments with rabbits were abandoned due to problems associated with the perfusion measurements. A new protocol using dogs was approved by the TJU IACUC. Two new interations of SHI software have been developed for a new scanning platform (the Logiq 9) and initial testing has commenced (in a canine model). A probe (the 7L) was selected for human studies.

14. SUBJECT TERMS
Breast cancer, ultrasound imaging, ultrasound contrast agent

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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4. INTRODUCTION

The goal of any breast imaging modality is to improve the early detection of tumors and to improve the differentiation between benign and malignant lesions. While x-ray mammography is efficacious in diagnosing a high percentage of breast masses, it also produces a high rate of false positives [1]. The percentage of breast biopsies that are actually malignant vary between 10 % and 35 %. Thus, a technique that reliably differentiates between malignant and benign masses would improve the diagnosis of breast cancer and should, therefore, reduce the number of negative biopsies as well as the trauma of the patients. This proposal will attempt to establish such a technique through the novel and innovative use of subharmonic ultrasound contrast imaging.

Ultrasound imaging is currently an auxiliary modality in breast imaging. It is mainly used to differentiate between cystic and solid lesions [2]. Investigations into the possibility of breast cancer diagnosis based on Doppler ultrasound flow detection have produced mixed results, due to overlap between flow measurements in benign and malignant tumors [3-4]. One problem may be the lack of sensitivity in flow detection in small tumor vessels using ultrasound. This hypothesis is supported by reports in the pathology literature describing angiogenic vascular morphology as an independent predictor of metastatic disease [5].

Ultrasound contrast agents produce increases of 15 to 25 dB in the echo intensities of blood flow signals; especially when combined with new contrast-specific imaging modalities such as harmonic imaging [6-7]. However, harmonic imaging has been found to suffer from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. SHI should have much better lateral resolution due to the higher transmitting frequency and should allow tumor perfusion, a measure of angiogenesis, to be estimated via time-dependent subharmonic fractional blood volume estimates. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by using SHI.

Quantifiable parameters of tumor angiogenesis will be estimated from the subharmonic signal intensities. A pulse-echo system will be built to perform SHI and tested in vitro as well as in vivo (in animals). The ability of SHI to depict normal vascularity as well as tumor angiogenesis will also be assessed in rabbits. Currently, the NIH and DOD have funded a study at Thomas Jefferson University into the efficacy of ultrasound contrast in the diagnosis of breast disease. We propose to expand on that project by adding SHI in the third year of this proposal. Not only is the potential of SHI in itself innovative, but because of the NIH/DOD funded study it will be possible to compare a number of new and unique approaches to breast cancer diagnosis i.e., SHI, 2D power Doppler with and without contrast as well as harmonic imaging directly to x-ray mammography. Furthermore, this project is extremely cost-effective because the existing grants coves a majority of the personnel costs as well as all major equipment purchases. The amalgamation of the NIH/DOD project with the current proposal also allows for basic research

into the correlation between SHI flow signals and pathologically detected lesion vascularity. This will enable a deeper understanding of the relationship between tumor neovascularity and ultrasound flow measurements

Consequently, this project proposes the development of a novel contrast specific imaging mode called SHI and the derivation of quantitative tumor angiogenesis estimates from SHI data. The fundamental hypothesis is that the neovasculature of malignant lesions can be visualized and quantified with SHI, thus, improving the diagnosis of breast cancer.

5. BODY

The central hypothesis of this project is that the differentiation between benign and malignant breast lesions can be improved by detection and estimation of tumor neovascularity using contrast enhanced SHI. To investigate this hypothesis SHI will be investigated in vitro and then in vivo in rabbits with VX-2 tumors. Finally, approximately 50 women with breast lesions will be recruited in year three and imaged using contrast enhanced SHI. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

5.1 Methods

In Vitro experiments

A pulse-echo system was built to perform SHI (Fig. 1). The setup consists of a pair of confocally positioned broadband focused transducers (diameter: 2.54 cm). A pulse/function generator (8111A; Hewlett-Packard Company, Polo Alto, CA, USA) was used to generate 32 cycle busts with a PRF ranging between 20 and 100 Hz. An RF power amplifier (A150; ENI Technology Inc., Rochester, NY, USA) amplified this signal by 55 dB to generate pressure levels from 0.3 to 1.5 MPa. The transmitting transducer used was a 2.54 cm spherical focused, narrow bandwidth, 5 MHz transducer (13-0508S; Harisonic / Staveley Industries Plc, Croyden, UK). The backscattered signals were picked up using a wide band 2.54 cm spherical focused transducer with center frequency of 2.25 MHz (13-0208R; Harisonic / Staveley Industries Plc, Croyden, UK). This substantially improved the spatial resolution of the system, because scattered signals only come from the microbubbles in the small confocal region of the two transducers (1-4 mm^3 for 2 MHz transmission). The sampling frequency was 20 MHz. This setup was employed to investigate the subharmonic signal generation from a new contrast agent QFX (Nanfang Hospital, Guangzhou, China), which consists of perfluorocarbon-filled albumin microbubbles [8].

The current version of SHI software installed on a Logiq 700 scanner (GE Medical Systems, Milwaukee, WI) was initially tested in an in vitro flow phantom with an 8 mm vessel (ATS Laboratories, Bridgeport, CT) using a broad bandwidth curve-linear array (the 348c probe; bandwidth 2 - 4 MHz). The frequency of insonation was 4.4 MHz, as the resonance frequency

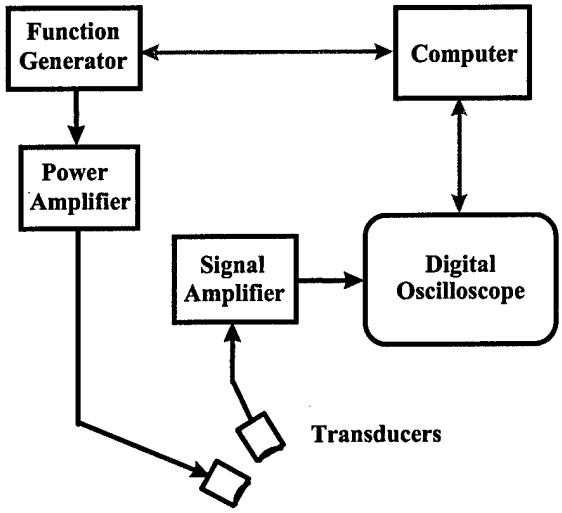

Figure 1. *The dual-transducer pulse-echo system built to perform SHI. A function generator produced a sequence of transmit pulses, which were amplified and then supplied to a single-element broadband focused transducer. Another broadband focused transducer (confocally positioned to the first) sensed signals scattered from the contrast bubbles. The received, amplified signals were digitized and processed using a digital oscilloscope.*

of Optison is around 2 MHz and this is the frequency range used in the ultrasound scanner employed for our preliminary SHI work [9-10]. This is in keeping with the concept of minimum threshold for subharmonic generation when the insonation frequency is twice the resonance frequency.

The contrast agent used for this part of the study was Optison® (manufactured by Mallinckrodt Inc, St. Louis, MO, USA and co-promoted by Amersham Health, Oslo, Norway). Optison is approved for use in echocardiography by the U.S. Food and Drug administration (for improved endocardial border delineation). It consists of a suspension of perfluoropropane-filled albumin microspheres with a concentration of 6.3×10^8 bubbles/ml and the bubbles have mean diameters in the range of 3 to 5 μm. In the flow phantom the contrast kinetics of the microbubbles within the flow (i.e., the uptake and washout of the contrast agent) was measured for different concentrations of Optison (1 % and 2 % by volume) and flow rates of 9.8 and 19.6 ml/min and the relative changes were compared.

In Vivo experiments

Initial animal studies were performed under supervision of a veterinarian and fully conformed to the National Institutes of Health guidelines for use of laboratory animals. All protocols were approved by the University's Animal Use and Care Committee. Four laboratory bred New Zealand white rabbits (mean weight 3.6 kg) and two mongrel dogs (mean weight 21 kg) were used in this project. The rabbits were sedated with 0.65 mg/kg of a mixture of Xylazine hydrochloride (Gemini, Rugby Laboratory, Rockville Centre, NY) and Ketamine hydrochloride (Ketaset, Aveco, Fort Dodge, IA) administered intramuscularly under the supervision of a

veterinary technician. The rabbits were maintained under anesthesia with 15 to 20 mg/kg/hr of 1 % Propofol (Diprivan®, Zeneca Pharmaceuticals, Wilmington, DE) as needed for the entire procedure. The dogs were premedicated with intramuscular administration of a mixture of 0.04 mg/kg atropine sulfate (Anthony Products, Arcadia, CA), and 0.75 mg/kg acepromazine (Promace; Aveco, Fort Dodge, IA). The dogs were placed on a warming blanket to maintain body temperature within normal range. A facemask with Isoflurane 4-5 % (Iso-thesia; Abbott Labs, N.Chicago, IL) was used for induction of anesthesia, which was maintained with 0.5 to 2 % of Isoflurane during the entire procedure.

Originally the intention was to use fluorescent labeled, polystyrene microspheres with a diameter of 15 μm (Triton Technology, San Diego, CA) to measure absolute perfusion (in ml/min/g). However, Triton Technologies has been taken over by anothew company and no longer provide this swervice. Instead a similar technology from BioPal (Worcester, MA) was adopted. Here the spheres are metalic and by exposing the tissues to gamma radiation (after harvesting and at BioPal's facilities) the spheres will temporarily become radioactive and the activity can be determined using spectroscopy. The ratio of the sphere number in tissue to that in a timed reference blood sample will provide an absolute measure of perfusion. This technology was employed in the rabbits.

Finally, the version of software for SHI installed on the Logiq 700 scanner (GE Medical Systems, Milwaukee WI) was found to be constrained by the hardware of the unit. Hence, an upgrade was required to run SHI on a more flexible unit (in terms of hardware and software). It was decided to replace the existing Logiq 700 with a completely new Logiq 9 scanner (GE Medical Systems, Milwaukee WI) to support this project. To date two new iterations of SHI software have been developed (for the m7c and 7L probes) and initial testing has commenced (in the canine model).

5.2 Results and Discussion

Figure 2 shows a comparison of the subharmonic backscatter from the contrast agent QFX at insonation powers of 0.4 and 0.6 MPa, respectively. Subharmonic signals from the contrast medium could be observed to increase 22 dB around 1.5 MHz with the increase in pressure. The amplitude of the subharmonic backscatter was about 14 dB lower than the fundamental signal at the 0.6 MPa pressure level (compared to 36 dB below at the 0.4 MPa level). These results were reported at an international conference [8].

In the flow phantom the contrast kinetics of the microbubbles within the flow was measured for different concentrations of 1% and 2% (by volume) and flow rates of 9.8 and 19.6 ml/min (Figure 3). The subharmonic intensity increased approximately linearly with time as the bubbles flow in and then decay exponentially as bubbles flow out. The slopes of the linear uptake curves were estimated to be 0.0074, 0.016 and 0.015 s^{-1}, respectively, and the decay rates of the wash-out curves were 0.09, 0.09 and 0.17 s^{-1}, respectively, in Figure 3(a-c). The slope is approximately doubled when either the concentration or the flow rate is doubled. The decay rate depends on flow rate but not on the concentration, as predicted by Schwarz et al [11]. Hence, the decay rate cannot be used as a predictor of local tumor perfusion.

In vivo experiments were initially carried out in rabbits. However, all 4 animals expired during the placement of the catheters needed for the absolute perfusion measurements (i.e., the gold standard against which SHI was to be compared). We established that the vessels in the rabbits are too small to allow for the required catheter placement. Hence, it was decided to switch the animal model to dogs and a new protocol to that effect was approved by the TJU IACUC (Appendix II). Due to the budgetary restrains of the grant, only 4 animals will be evaluated for perfusion measurements.

During the imaging phase of the rabbit experiments we found that the SHI software version installed on the Logiq 700 scanner lacked flexibility. Hence, an upgrade was required to run SHI on a more programmable scanner. It was decided to replace the existing Logiq 700 with a completely new Logiq 9 scanner (GE Medical Systems, Milwaukee WI) to support this project. This unit was purchased by the Department for clinical use, but it will be made available for this research project as well. This development and its successful resolution became the major focus of the second half of Year 3 somewhat limiting the progress made on the project in the third year.

Two new iterations of SHI software have been developed for the Logiq 9 scanner to permit SHI on the m7c and 7L probes (a curve-linear and a linear transducer, respectively). Initial testing has commenced in the canine model as demonstrated in Figure 4. In vivo SHI with Optison shows both flow in the larger hepatic vessels and perfusion in the kidney. Notice the excellent suppression of tissue echoes. The transmission frequency was 4.4 MHz and the receive frequency was 2.2 MHz. Comparison of the m7c and 7L probes showed the latter to be markedly more sensitive and we, therefore, selected the 7L transducer for the upcoming human studies.

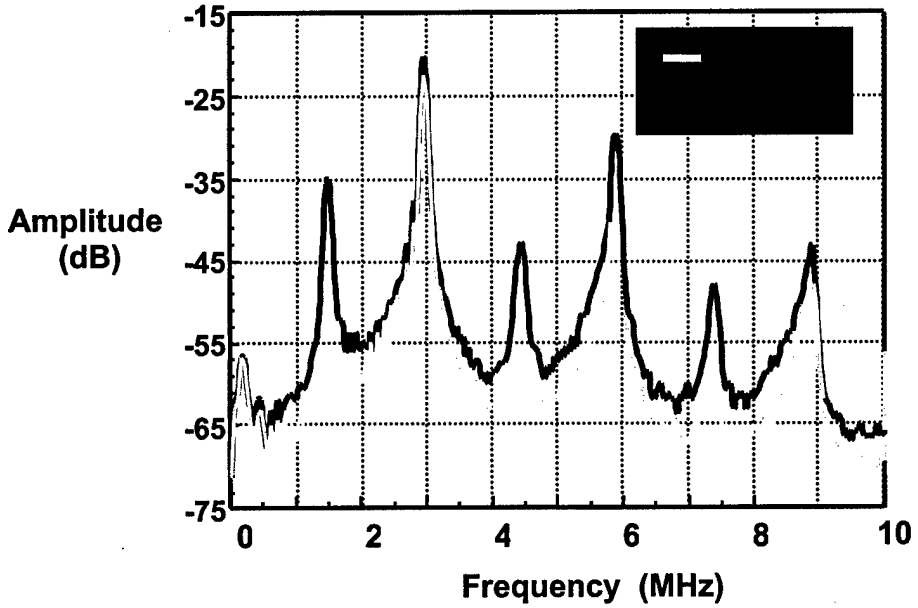

Figure 2. *Signal intensity vs frequency for QFX (concentration 1 μl/ml) obtained at insonation pressures of 0.4 and 0.6 MPa using a 3 MHz pulse of 32 cycles with a 5 Hz PRF.*

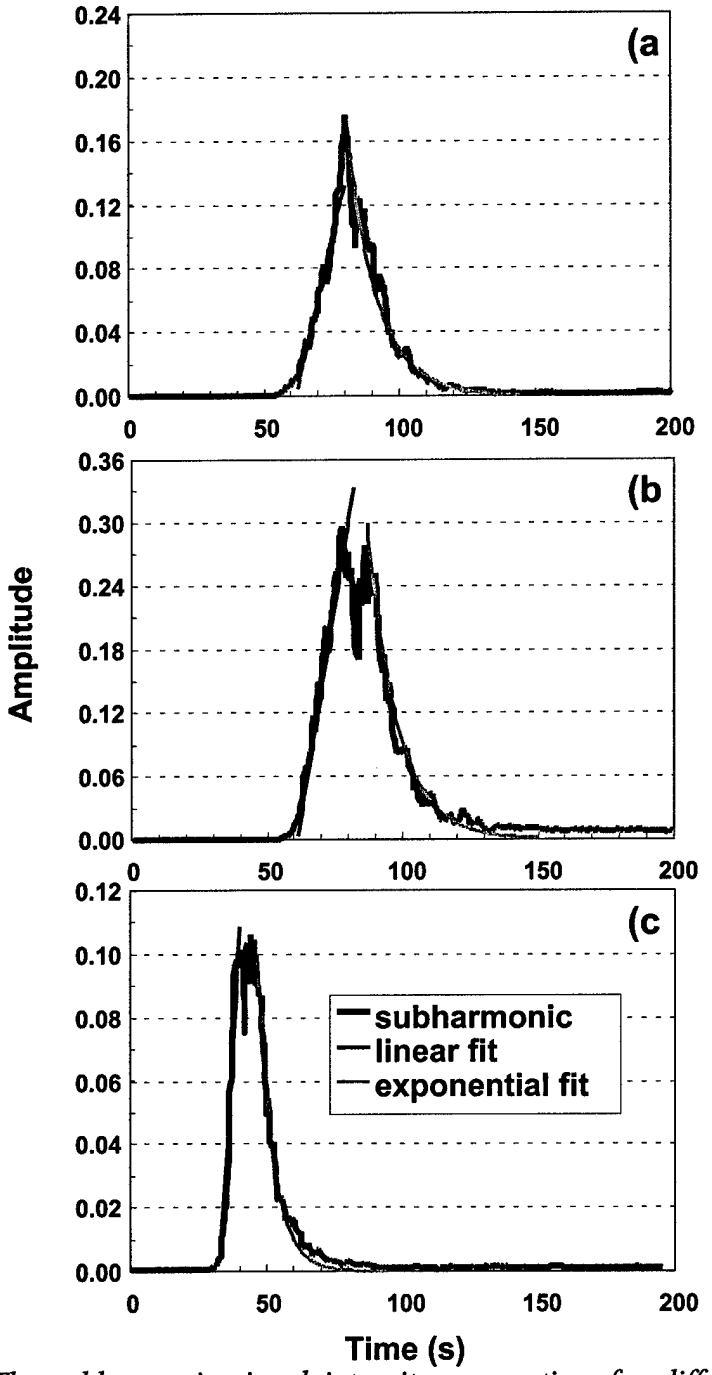

Figure 3. *The subharmonic signal intensity versus time for different concentrations and flow rates: (a) 1% and 9.8 ml/min, (b) 2% and 9.8ml/min, and (c) 1% and 19.6 ml/min.*

In order to obtain IRB approval for the human component of this project it is necessary to establish the acoustic power levels of the SHI software on the selected probe. This effort will be done at GE Medical Systems in Milwaukee and has been scheduled for the week of August 11th, 2003. We expect to obtain IRB approval sometime in early to mid September at which time the human studies will be initiated. These efforts represent the commencement of task 3a.

6. KEY RESEARCH ACCOMPLISHMENTS

- SHI experiments were conducted with QFX in vitro.
- SHI experiments were conducted with Optison and the Logiq 700 in the flow phantom.
- The contrast uptake slope doubles with concentration and flow rate. The decay rate depends on flow rate but not on the concentration.
- A new scanner platform was acquired for SHI.
- A new animal model for perfusion studies (canines) was selected.
- SHI was performed in dogs and the probe to be used for human studies (the 7L) was selected.

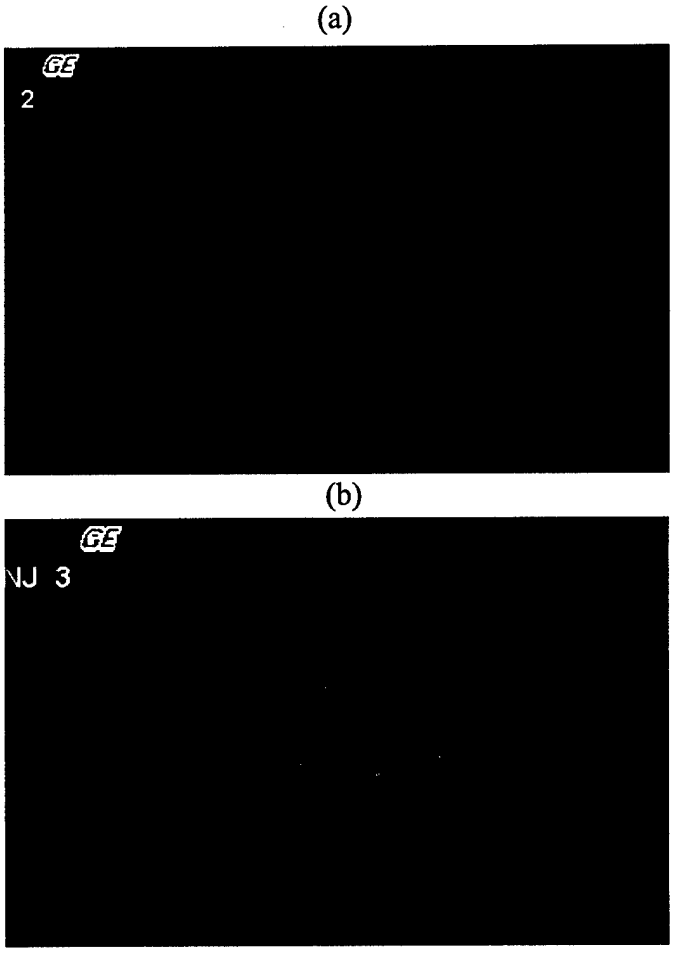

Figure 4. *In vivo SHI post injection of 0.1 ml/kg of Optison showing (a) larger vessels in the liver and (b) perfusion in the kidney obtained with a Logiq 9 scanner in a canine model. The transmission frequency was 4.4 MHz and the receive frequency was 2.2 MHz.*

7. REPORTABLE OUTCOMES

F Forsberg, G Bhagavatheeshwaran, WT Shi, PM Shankar. Contrast enhanced subharmonic ultrasound imaging. *Proc Era of Hope, DoD Breast Cancer Research Meet,* pp. P29-3, 2002.

F Forsberg, WT Shi. In vivo subharmonic imaging and pressure estimation. *Proc Leading Edge in Diag. Ultrasound*, B. B. Goldberg (ed.), Thomas Jefferson Univ., Philadelphia, pp. I45 - I48, 2003.

WT Shi, Y Liu, Z Lu, F Forsberg, D Zha, JB Liu, BB Goldberg. Nonlinear imaging with a new contrast agent. *Ultrasound Med Biol*, vol. 29, pp. S97, 2003.

G Bhagavatheeshwaran, WT Shi, F Forsberg, PM Shankar. Subharmonic generation from contrast agents in simulated neovessels. Submitted to *Ultrasound Med Biol*, May, 2003.

September 25 – 28, 2002	Era of Hope, Dept. of Defense Breast Cancer Research Meeting, Orlando, FL, USA. • Contrast enhanced subharmonic ultrasound imaging (poster).
May 13 - 16, 2003	The Leading Edge in Diagnostic Ultrasound, Philadelphia, PA, USA. • In vivo subharmonic imaging and pressure estimation.
June 1 - 4, 2003	The 47th Annual Convention of the American Institute of Ultrasound in Medicine, and the 10th Congress of the World Federation for Ultrasound in Medicine and Biology, Montreal, Canada. • Nonlinear imaging with a new contrast agent

8. CONCLUSIONS

In vitro the subharmonic backscatter from the contrast agent QFX was investigated (Fig. 2). In the flow phantom the contrast kinetics of the microbubbles was measured (Figure 3). The subharmonic intensity increased approximately linearly with time as the bubbles flow in and then decay exponentially as bubbles flow out. The slope is approximately doubled when either the concentration or the flow rate is doubled. The decay rate cannot be used as a predictor of local tumor perfusion.

Animal experiments with rabbits were abandoned due to the problems associated with the catheter placement required for perfusion measurements. A new protocol using dogs was approved by the TJU IACUC (Appendix II). During the imaging phase of the rabbit experiments we found that the SHI software version installed on the Logiq 700 scanner lacked flexibility. A completely new Logiq 9 scanner (GE Medical Systems, Milwaukee WI) was purchased by the

Department for this research project as well. This development and its successful resolution became the major focus of the second half of Year 3 somewhat limiting the progress made on the project in the third year.

Two new iterations of SHI software have been developed for the Logiq 9 scanner and initial testing has commenced (Figure 4). Moreover, we selected the 7L transducer for the upcoming human studies. The acoustic power testing of the SHI software on the 7L probe has been scheduled for the week of August 11th, 2003 at GE's testing facility.

In summary, tasks 1a, 1b and 1c have been completed while tasks 2a and 3a are ongoing, but due to the delay caused by the original animal model as well as the upgrade to a new hardware platform the project is approximately 12 months behind schedule. A one year no cost extension was consequently requested (and granted).

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Appendix I

The Statement of Work from the original proposal:

Objectives 1 - 2
Task 1: Software development and *in vitro* experiments (months 1 - 24)

a. Develop software for SHI and for FBV estimates to be produced from SHI data (months 1 - 24).
b. Design and implement pulse echo SHI setup (months 1 - 6).
c. Perform *in vitro* flow phantom experiments comparing SHI and FBV estimates to absolute perfusion and flow rates (months 6 - 12).

Objectives 2 - 3
Task 2: Animal experiments and data collection (months 13 - 24)

a. Perform *in vivo* experiments in 12 normal rabbits comparing FBV estimates to absolute flow rates and perfusion obtained with colored microspheres (months 13 - 20).
b. Perform *in vivo* experiments in 6 rabbits with renal VX-2 tumors implanted comparing FBV estimates to absolute tumor perfusion obtained with colored microspheres (months 20 - 24).
c. Evaluate the performance of SHI in the detection of rabbit VX-2 tumors compared to conventional ultrasound imaging, with and without contrast administration, as well as to harmonic imaging (months 13 - 24).

Objectives 4 - 5
Task 3: Human data collection and analysis (months 25 - 36)

a. Recruit 50 - 75 patients, which is about two-thirds of the anticipated number of patients being enrolled in the existing NIH/DOD supported contrast study (months 25 - 36).
b. Perform SHI contrast studies as part of the already funded NIH/DOD project. This involves an extra injection of contrast (within the permitted total dose) and will add no more than 20 minutes to the total duration of the contrast study (months 25 - 36).
c. Research coordinator to collect clinical information, pathology results, etc. (months 25 - 36).
d. Incorporate SHI findings into the existing database developed for the NIH/DOD supported study (months 25 - 36).
e. Perform ROC analysis in collaboration with the statistician (months 30 - 36).
f. Perform remaining statistical analysis in collaboration with the statistician (months 30 - 36).

Appendix II

The IACUC approval for the use of canines in this project:

Thomas Jefferson University | Jefferson Medical College

Institutional Animal Care and Use Committee

July 28, 2003

Founded 1824

Jefferson Medical College

Jefferson College of Graduate Studies

Jefferson College of Health Professions

Jefferson University Physicians

Flemming Forsberg , Ph.D.
763J
Main Building
132 South 10th Street
Philadelphia, PA 19107

Dear Dr. Forsberg,

The Institutional Animal Care and Use Committee (IACUC) at Thomas Jefferson University on 07/22/2003 **approved** your new protocol

264CC Estimation of Tumor Angiogenesis with Contrast Enhanced Subharmonic Ultrasound Imaging

You have been approved to use animals of the species **Dog**. This approval extends to 07/22/2004 unless revoked or suspended earlier by the IACUC.

Continuation of this study beyond the above stated expiration date depends on a review of your protocol by IACUC. This review process has been implemented to comply with the revised GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS (1996), The Health Research Extension Act (1985), and the PHS Policy on Humane Care and Use of Laboratory Animals (1986), and The Animal Welfare Act and subsequent amendments.

Any proposed change in your protocol during this approval period must be submitted to the IACUC before the change is implemented.

All research involving laboratory animals must be approved by the Institutional Animal Care and Use Committee before initiating the study.

This letter approving the RO-5 animal protocol is not to be used as substitute for the IACUC grant application approval letter. The animal activities described in the grant application must be matched and correlated with the RO-5 protocol before a grant approval letter will be issued to the Principal Investigator for transmittal to the sponsor.

Sincerely,

Linda Casey
Coordinator of the Office of Animal Resources

/lc