

AD _____

Award Number: W81XWH-04-1-0239

TITLE: Early Detection of Breast Cancer by Fluorescence Molecular Tomography

PRINCIPAL INVESTIGATOR: Vasilis Ntziachristos, Ph.D.

CONTRACTING ORGANIZATION: The General Hospital Corporation
Massachusetts General Hospital
Boston, MA 02114

REPORT DATE: July 2006

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-07-2006		2. REPORT TYPE Annual		3. DATES COVERED 1 Jul 2005 – 30 Jun 2006	
4. TITLE AND SUBTITLE Early Detection of Breast Cancer by Flourescence Molecular Tomogaphy				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0239	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Vasilis Ntziachristos, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The General Hospital Corporation Massachusetts General Hospital Boston, MA 02114				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. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT Early detection of breast tumors continues to be a significant diagnostic challenge and thus remains the focus of attention of a number of medical research groups. Molecular targeting approaches have tremendous potential for early-detection because they rely on elucidation of abnormal gene-expression, rather than on discovery of retarded anatomical changes inflicted by growing tumors upon their microenvironment. Originally, we proposed an investigation of whether highly-sensitive fluorescence molecular tomography (FMT) could be used to detect breast cancer at its earliest and smallest stages via the detection of injected, protease-activatable molecular probes. We enter year 3 with the goals of the previous 2 years fully achieved. We have now not only constructed and fully optimized the proposed FMT imaging system using phantoms, but have also completed extensive in-vivo imaging studies ahead of schedule. Similarly, we have made significant progress toward the goals for aim 3 and year 3, in regard to the translation of our findings to a clinical setting, by researching the construction of appropriate breast-like phantoms.					
15. SUBJECT TERMS molecular imaging, fluorescence, tomography, early detection, specificity, breast cancer					
16. SECURITY CLASSIFICATION OF:			UU	18. NUMBER OF PAGES 25	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	10
Key Research Accomplishments.....	20
Reportable Outcomes.....	21
Conclusions.....	22
References.....	23
Appendices.....	

Introduction

Breast cancer is the second most common female malignancy in the United States. The ability to detect malignancies in their earliest stages is of tremendous importance. Early detection has been shown to increase likelihood of survival [1, 2] and to reduce the risk of disease recurrence [3 – 6]. However, early detection continues to be a significant diagnostic challenge and thus remains the focus of attention of a number of medical research groups.

Well-established detection techniques, most notably mammography, remain limited in their sensitivity to small-volume disease. Though mammography has been of great importance for detection of breast malignancies, many tumors have evaded detection by this method largely due to its limited contrast. The technique is particularly hampered by background interference such as increased tissue density or scarring from prior surgery. Functional imaging, which includes Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET), has been used as a compliment to anatomical imaging and has shown significant potential to image functional characteristics of tumors [7]. However, the high cost and complex operational logistics of these modalities may limit their practicality for use in large-scale screening studies.

An additional parameter that plays a vital role in breast cancer detection is the ability to detect specific disease signatures based on the molecular profile of the disease. Molecular imaging, i.e., imaging of cellular and sub-cellular processes that are associated with molecular onset and evolution of disease, can lead to increased sensitivity and specificity and can improve the overall performance of a diagnostic study. Improved specificity, based on the detection of molecular profiles, can lead to the overall reduction in unnecessary biopsies, which are associated with increased healthcare costs and patient distress, and thus can lead to personalized medicine. It is foreseen that such methods will help optimize therapeutic strategies and will enable the effects of treatment to be more closely followed in time scales of hours or days instead of the longer timeframe of weeks or months that is the current standard for on anatomical imaging methods. Some of our recent review articles [8, 9] provide more detailed discussions of molecular imaging in general, and one of our recent studies [10] demonstrates the ability of optical imaging to monitor disease behavior.

Optical methods have emerged as economic, safe, and effective alternatives for functional imaging. Diffuse Optical Tomography (DOT) has been developed for imaging of breast cancers because of its ability to non-invasively quantify oxy- and deoxy-hemoglobin concentrations, which can be used to characterized angiogenesis and hypoxia [11-15]. In addition, DOT has been used in combination with MRI to detect tumors based on the extrinsically-administered contrast agent Indocyanine Green (ICG) that marks angiogenesis and permeability. However, these markers, though they enable DOT to effectively image well-developed tumors, are not ideal for detection of the earliest stages of disease. Consequently, while DOT may certainly be a useful tool for evaluating larger tumors, a more effective optical modality is needed for early detection.

Fluorescence Molecular Tomography (FMT) is a relatively new optical tomographic imaging approach that capitalizes on the strengths of molecular imaging. It bases detection on specific molecular signatures, using highly targeted fluorescent probes, rather than on anatomical or functional changes within tumor microenvironments. This approach yields a shift in the radiological paradigm that has traditionally focused on anatomy and physiology [16]. Unlike DOT, FMT can three-dimensionally resolve protein expression, protease activity, receptor regulation, and similar molecular markers that play vital roles in carcinogenesis. It has been demonstrated in-vivo that NIR photons can penetrate more than 12 centimeters into the breast, and that fluorochromes accumulating in small tumors are detectable through the human breast [17]. We have previously shown that FMT is capable of resolving phantoms placed less than 1 mm apart [18], and the sensitivity of this technology has been predicted to be adequate for detection of human breast lesions smaller than 5 mm [19]. More recently it has been shown that the technique can visualize apoptosis as a response to treatment [10]. Furthermore, because non-ionizing radiation is used, the technology allows for repeated imaging for screening or monitoring of disease. FMT thus holds great promise for early detection of breast malignancies.

At the time of our grant proposal, several elegant targeted fluorescent probes had already been developed [20-22] for use with optical methods such as FMT. Among the most notable of these were the so-called “smart” probes (also termed “activatable probes”), which are optically silent until they interact with a specific enzyme, such as a protease, which then activates the probe, converting it to a brightly fluorescent state [20]. These probes are powerful not only because of their specificity for cancer-associated enzymatic activity, but also because they significantly minimize background levels and exhibit several-fold amplification of fluorescence upon activation, enabling dramatic improvements in signal-to-noise contrast. Because activatable probes fluoresce only in the presence of specific tumor phenotypes, they can easily be localized and quantified against a virtually “dark” background, allowing for highly sensitive and specific detection.

Our proposal described using a smart probe that is activated by major cathepsins, which are members of the cysteine family of proteases and play a central role in tumorigenesis and matrix invasion [23-26]. For example, it has been demonstrated that high levels of cathepsin B expression correlates positively with aggressive behavior and progression of human tumors and negatively with patient survival [23]. We therefore chose to evaluate the performance of this probe for imaging breast cancer development and progression.

Until the study was supported by this grant, in-vivo imaging of such probes were limited to basic feasibility studies, generally involving imaging of xenographic implants in nude mice. Generally, it was unknown how early in disease progression such probes could be used to reliably detect malignancies, as the in-vivo sensitivity limits of FMT have not been rigorously explored. The goals of our proposal were primarily (1) to develop and optimize optical technology for highly sensitive detection; and (2) to evaluate the early-detection limits of FMT by conducting a blind screening study of transgenic mice that develop spontaneous tumors of the mammary fat pad,

closely mimicking the natural development and progression of human breast cancer. We proposed developing imaging tools designed to systematically reduce background noise levels and to improve fluorochrome localization and quantification. The study was designed to investigate screening efficacy and to predict sensitivity limits of human breast cancer.

The hypothesis of this proposal is that highly sensitive fluorescence imaging (tomosynthesis) of molecular contrast will enable earlier detection of breast tumors based on signals associated with tumor growth and matrix invasion. We predicted that this technology could achieve high detection specificity.

Our study had three major goals:

Aim 1. To develop appropriate photon illumination and detection strategies and reconstruction methods for highly sensitive fluorescence detection and quantification.

Aim 2. To utilize optimal photon technology from Aim 1 to study in-vivo detection of spontaneous disease in transgenic mice and to follow local disease progression and distant metastases.

Aim 3. To translate the animal study findings into clinically-relevant detection schemes.

The first of these aims was designed to find an optimal illumination technology and processing method in order to obtain the highest detection sensitivity of fluorochromes in tissues. We proposed investigating limited angle projections versus multi-angle illumination, planar illumination, and transillumination schemes.

The second aim focused on the clinical relevance of FMT. We proposed a screening study involving transgenic mice that spontaneously develop breast tumors. Our interest was and continues to be primarily (1) to characterize detection capacity as a function of disease progression and tumor growth; and (2) to quantify fluorescence concentration in-vivo and determine whether it correlates with tumor expression profiles. This screening study would enable us to establish early-detection limits.

The third aim was to translate the copious data acquired from exploring the first two aims into an evaluation of the clinical feasibility of FMT for early-detection of breast cancer.

In the grant proposal we presented a timeline for accomplishing our primary aims for years 1 – 3 of the study (Table 1).

Table.1 Overview of aims and time line

	Year 01	Year 02	Year 03
<u>Aim 1: Method Development & Optimization</u>			
• Construction of 4-mode imaging chamber	X		
• Developing imaging algorithms	X		
• Optimizing imaging parameters with phantoms	X		
• Optimize imaging parameters with orthotopic mouse model(s)		X	
<u>Aim 2: In vivo Screening study</u>			
• Establish appropriate transgenic mouse model(s)	X		
• Breed and screen mice for early detection of tumors		X	X
• Study local and distant disease progression.		X	X
<u>Aim 3: Clinical translation</u>			
• Construction of breast-like phantoms		X	X
• Experiments with phantoms			X
• Simulations			X

To date, we have not only met, but also exceeded the goals for year 2, and we have also made significant progress in reaching the goals for year 3. The following sections provide a detailed evaluation of our progress.

Quick Glimpse At The Achievements of Last Year

We successfully completed construction of a 4-mode, integrated illumination/detection small animal imaging system. A schematic of which is presented in Figure 1. This system is based on a previously-reported scanner [18] with key advancements to accommodate all four illumination schemes that were described in the grant proposal, namely *reflectance imaging*, *transillumination*, *tomosynthesis*, and *tomography*:

Reflectance Imaging The illumination branch (Figure 1, f, g) illuminates the animal through the chamber window, and reflected intrinsic or fluorescence photons are recorded on the CCD using band-pass filters. Figure 2 presents intrinsic and fluorescence reflectance images of a mouse with two subdermal tumors within the mammary fat pads,

Transillumination. In this mode, each of the 46 source fibers directed toward the back of the imaging chamber (Figure 1, e) is sequentially switched on and the transmitted pattern is filtered with appropriate intrinsic or fluorescence band pass filters, and photons are recorded on the CCD camera. The

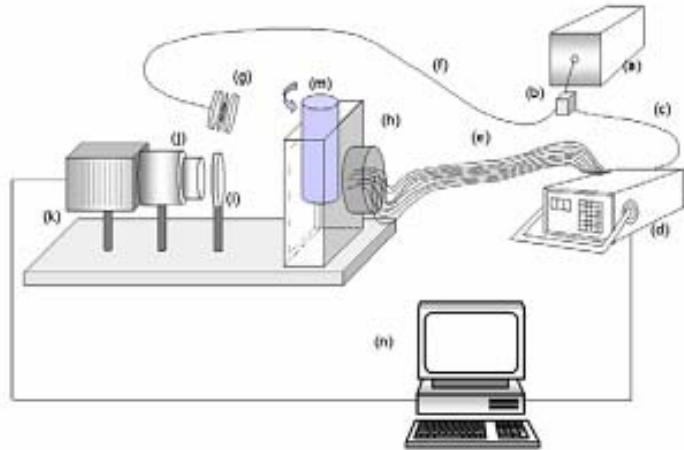


Figure 1: FMT imaging system.

resulting images are added together to create a composite image, which is geometrically similar to an X-ray mammogram. The fluorescence composite image may also be divided by the intrinsic composite image to generate a normalized image, which significantly reduces sensitivity to background heterogeneity and thus provides a more accurate image. In the second year we have further upgraded this system in a fiber-free implementation by using motor stages to translate a single optical fiber emitting light focused on the animal surface, thus implementing significantly higher illumination versatility.

Tomosynthesis. In year 1 we made significant progress with implementing limited angle projection FMT, the equivalent of FMT tomosynthesis. For this approach data is gathered in the same manner as in the transillumination approach; however, unlike transillumination, tomosynthesis involves applying algorithms to the data that solve a volume integral equation relating spatially-distributed fluorochrome concentrations (in physical units) to the transillumination measurements. This offers more accurate quantification compared to transillumination.

Tomography. To achieve superior imaging performance it is important to illuminate using a large number of projections and detect signal around the animal, similarly to other tomographic techniques such as X-ray CT, PET or SPECT. For this reason we have implemented the rotational device (Figure 1, m) which rotates the object of interest in front of the illumination path and CCD camera to implement a number of projections. In this mode data can be collected in 360° projections and offer true three-dimensional tomographic imaging.

Software In the first year we also gained considerable ground in way of tomographic algorithms. In particular, we developed imaging algorithms for transillumination as well as advanced background fluorescence subtraction techniques (reported in reportable outcomes). We also evaluated algorithms for robust imaging within highly heterogeneous backgrounds, suitable for in-vivo imaging. Lastly, we adapted algorithms already designed for tomosynthesis to complete-projection imaging. Through implementation of several phantom studies, we then evaluated the developed algorithms in order to assess the overall performance of the method. Bearing our future goals in mind, we specifically constructed highly heterogeneous phantoms (of solid and liquid diffusive media), intended to best simulate the varying optical properties that are likely within breast tissue.



Figure 2: Intrinsic (left) and fluorescence (right) reflectance images of a mouse with two HT1080 subcutaneous tumors (red arrows in right image). The liver is indicated with a white arrow. The mouse was injected with 2 nmol of a cathepsin-activatable probe 24 hours prior to imaging.

Imaging studies Significant progress was achieved with in-vivo imaging. A total of 16 mice resulted through our controlled breedings and were subsequently subjected to imaging of tumor growth. In-vivo imaging further enabled the optimization of imaging (reconstruction) parameters and experimental refinement necessary for full completion of the large-scale screening study within the allotted time, as originally stated in the grant proposal. An example of tomographic imaging performed in year 1 is shown in Fig 3.

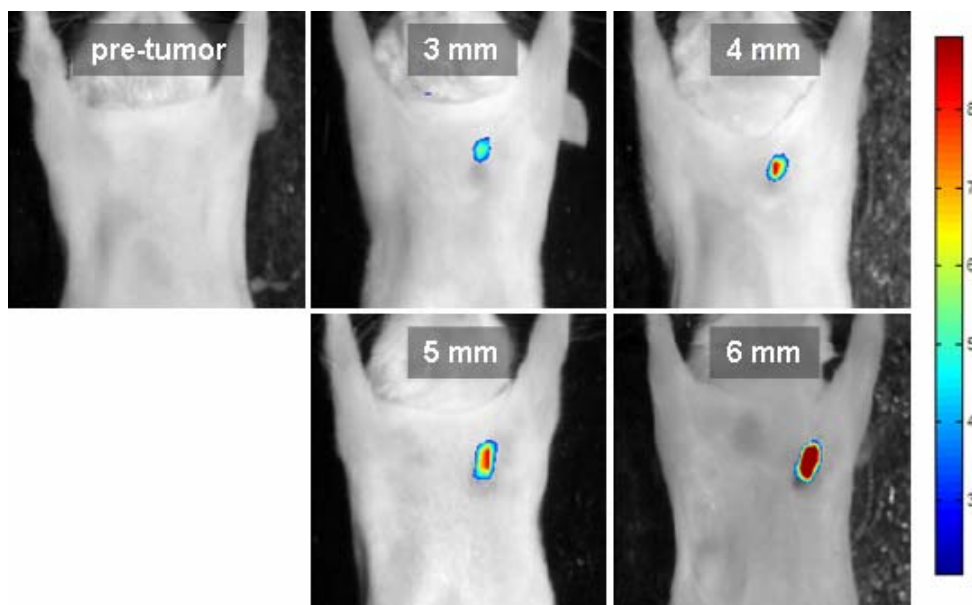


Figure 3: A mouse imaged prior to visible manifestation of tumors and subsequently imaged on a weekly basis to follow tumor growth from 3 mm to 6 mm in diameter. The mouse was injected via tail-vein with 2 nmol of a cathepsin-activatable NIR probe 24-hours prior to each imaging session.

Body

We have completed all of the goals set for the first 2 years of our investigation, which encompasses all of Aims 1 and 2 and a small portion of Aim 3. While Aim 1 was fully achieved in year 1, the following is a detailed description of accomplishments and reported outcomes achieved over the past year:

Aim 2. *To utilize optimal photon technology from Aim 1 to study in-vivo detection of spontaneous disease in transgenic mice and to follow local disease progression and distant metastases.*

While imaging of disease progression was established in year 1, in this year we have further solidified our ability to in-vivo image cancer with improved accuracy and imaged a total of 25 animals as described in the following:

A2.1: Transgenic mouse models

We have established our own MMTVneu transgenic mouse colony (c-neu oncogene; strain name FVB/N-TgN(MMTVneu)202Mul, Jackson Laboratory, Bar Harbor, Maine) In this model, the mouse mammary virus tumor (MMTV) promoter directs expression of the c-neu oncogene to the mammary fat pads. The model was originally reported to develop mammary tumors in 50% of females by 89 days, but ultimately there is 90% penetrance by 5-6 months. The major reason of establishing a colony we are eliminating all availability issues that may arise when requesting such a popular transgenic strain from an outside supplier, expediting the progress of our intended studies.

Expansion of Animal Sources

We are also currently receiving retired breeder females that we requested from the existing colony at Jackson Laboratory. Many of these females are already of age for spontaneous tumor development. These animals will provide for immediate data collection and avoidance of any unnecessary downtime while awaiting tumor development among females of our own colony. Lastly, upon securing a second source of the MMTVneu strain from another “in house” investigator, we have received and genotyped (Figure 4) 10 additional females. Now nearly 4 months old, we anticipate imaging any developing tumors even sooner than originally expected.

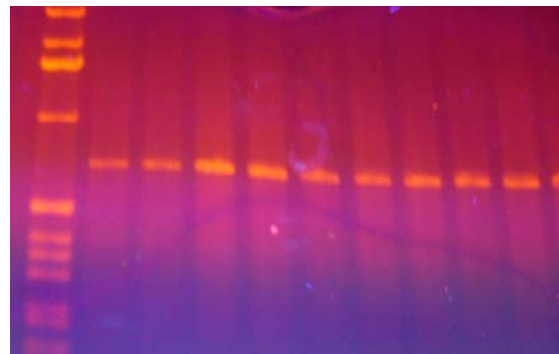


Figure 4: Agarose gel displays results of genotyping of MMTVneu transgenic females. PCR was performed using primers designed specifically for the rat Her-2/neu oncogene. The appropriate 600-700 bp neu transgene is present in all animals received.

Diversification into Human Cell Lines

Intent upon generalizing our findings on breast cancer as it occurs in women, we have expanded and diversified the animal models utilized in order to investigate a small number of xenograft models of human cancer cell lines. We are convinced that the potential for variation in way of prognostic biomarkers, receptor expression and general cellular profiles introduced through the use of different human cancer cell lines will prove invaluable in best depicting the lesions that typify human breast cancer. Thus, we have begun orthotopic injections of human cell lines: MDA-MB-231 and MDA-MB-435s, representing breast adenocarcinoma and mammary ductal carcinoma respectively. In this way we have broadened our base for *in vivo* imaging (as outlined in Aim 2) allowing for more extensive evaluation of local as well as distant disease progression. For instance, the highly invasive and estrogen independent MDA-MB-231 line should contrast well with the notably poorer metastatic potential of MDA-MB-435s type carcinoma. We have performed orthotopic injection of 5 athymic (nu/nu) mice to date and in year 3 we will investigate imaging performance in this case as well. The inclusion of such xenograft models might permit us to evaluate not only the detection capacity but importantly the ability to evaluate the effectiveness of targeted therapies *in-vivo*. For instance, given the high COX-2 expression of the MDA-MB-231 cell line, treatment with an appropriate inhibitor could provide the opportunity for subsequent observation of matrix metalloproteinase (MMP) or serine proteinase expression levels. Such investigation strives to meet the need for clinical propagation of this technology for identifying better clinical endpoints in monitoring the efficacy of drugs intended to block enzymatic function and serine proteases *in vivo* within the intact tumor environment.

A2.2: Multi-spectral imaging

An important feature that was enabled in this year 2 of the development was the ability to perform dual wavelength imaging. While technologically straightforward, this development has significant biomedical application, since it allows the ability to simultaneously resolve and differentiate probe bio-distribution and activation. The ability to simultaneously monitor different functional and molecular characteristics *in vivo* can offer complementary information and significantly improve the accuracy of the findings. This can be achieved by elucidating coupled pathways at the cellular and sub-cellular level. This is a significant development over the first year, and it is not customarily applied in *in-vivo* studies, as described in more detail in the following:

Technical developments

The overall design is based on the system shown on Figure 1. This design achieves high-spatial sampling of photons that propagate through the animal in trans-illumination mode and uses now a motorized single fiber using translational stages to trace a user programmable source pattern and offers high flexibility in the field of view implemented. Dual wavelength capability was achieved by the addition of an additional laser and set of filters. The selection of the wavelengths was done based on the commercial availability of efficient fluorescent dyes. We selected illumination at 671nm and 748nm to illuminate popular fluorochromes that are

commercially available and are typically used in the synthesis of fluorescent probes, for example the Cy5.5 dye (Amersham, Piscataway, NJ) or AlexaFluor 680 dye (Invitrogen, Carlsbad, CA) for the 671 wavelength and AlexaFluor 750 dye (Invitrogen, Carlsbad, CA) 748nm. Illumination in both cases was performed with CW laser diodes (B&W Tek, Newark, DE) which are time-multiplexed using a 2x1 channel optical switch Fig.1(ix) (DiCon FiberOptics, Berkeley, CA). The output of this switch is connected to the motorized fiber and its output is focused on the animal surface. Photon detection is based on a 512x512 element cooled CCD camera fig:SetupFigure 1(iii) (Roper Scientific, Trenton, NJ) coupled to a AF Nikkor 35mm, f/2.8D lens (Nikon, Melville, NY) which is focused on the chamber's detection window. The CCD detector offers quantum efficiency of 60% or better for the spectral region of 370nm to 830nm and therefore it is well suited for multi-spectral application in the NIR.

Filter selection and system characterization

3-cavity band-pass (Andover Optics, Salem, NH) and long-pass filters (Omega Optics, Brattleboro, VT) were used to effectively block excitation light and minimize cross-talk between fluorescent dyes while maximizing transmission at the selected wavelengths. Filters were also used for the excitation wavelengths. Laser diode signals were pre-conditioned with a 671DF10 bandpass filter (Omega Optics, Brattleboro, VT). Pre-filtering the laser to remove unwanted wavelengths and harmonics improved cross-talk by a 4 fold decrease. Excitation measurements were performed through bandpass filters (Andover Optics, Salem, NH). Emission data was collected through a combination of a long pass and band pass filter (Longpass filter: 695nm, bandpass 710nm +/-20nm for the Cy5.5 measurements and longpass filter: 770nm and bandpass at 800nm +/- 20nm for AF750 based measurements)

We performed extensive characterization of the separation ability of this system. A related result based on experimental phantom measurement is shown in Fig. 5 . The figure shows two tubes placed in the system imaging chamber and photographed before the addition of intralipid with optical properties simulating those of the human breast tissue. The left tube contained 250nM of the Cy5.5 dye (emission max: 690nm) and 500nM of the AF750 dye (emission max: 770nm). The right tube contained 500nM of Cy5.5. and 250nM of AF 750. The fluorescence of the tubes, when imaged with the dual wavelength FMT system clearly shows the 1:2 and 2:1 ratios when the tubes are imaged at the Cy5.5 (top row) and AF750 channels (bottom row) as shown in the middle column marked "FRI". Finally, FMT images

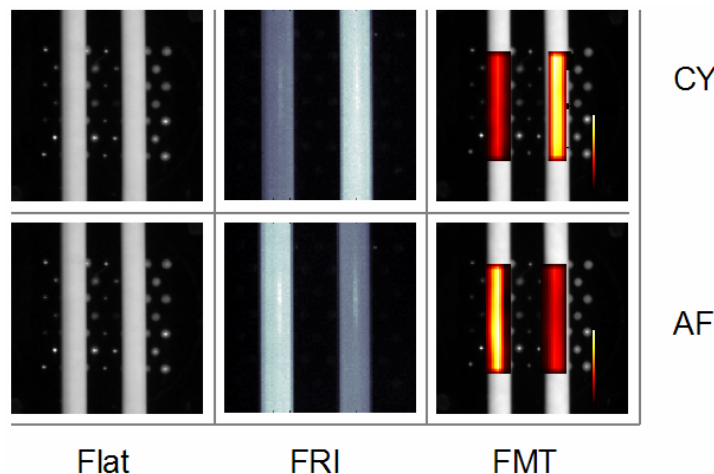


Figure 5: Separation of fluorochromes at different wavelengths using the dual wavelength system developed in year 2. (see text for details)

performed after the tubes were immersed in a highly diffusive fluid also demonstrated the same separation. In all cases the cross-talk between the two channels was found to be less than 1% using the filter combination described above.

Imaging studies

The system developed was utilized herein to differentially resolve fluorescence probe bio-distribution and protease activity. While 16 animals have been already imaged using the single wavelength system, another 9 animals were imaged using dual wavelength. This is achieved through the simultaneous injection of 2nmol of an NIR cathepsin-activatable probe (ProSense, Visen Medical, Woburn, MA) and an NIR vascularization agent (AngioSense, Visen Medical, Woburn, MA) via tail vein 24 hours prior to imaging of transgenic mice bearing developing tumors. The Angiosense is essentially the same molecule as the Prosense, albeit with less fluorochromes loaded to avoid quenching. Therefore the probe is always “on” and allows for visualization of the biodistribution of ProSense which generally activates only in the presence of proteases (cathepsins). Figure 6 demonstrates FMT of a her2/neu mouse bearing a 7mm tumor. The cathepsin-activatable NIR probe shows strong signal selectivity within the region of the tumor, suggesting elevated cathepsin activity at this location (top row). At the same time, the images in the bottom row resulted from FMT of the NIR vascularization agent and clearly indicate increased probe distribution not only within the tumor but throughout the surrounding area, consistent with increased tumor vascularity. Thus inclusion of the fluorescent *in vivo* blood pool imaging agent not only provides reliable, non-invasive imaging of vascularity but also enables quantification of delivery of both probes by serving as an internal control.

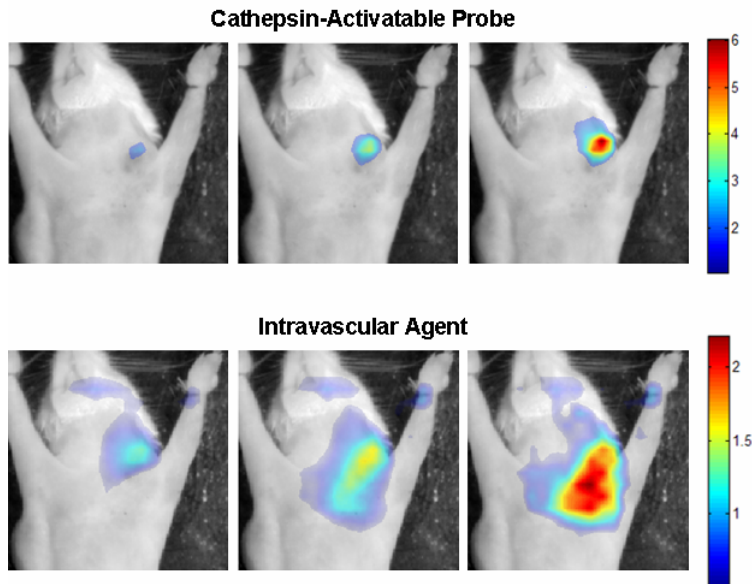


Figure 6: FMT of mouse with 7 mm tumor. **Top row:** FMT of cathepsin-activatable NIR probe shows strong signal selectively within the region of the tumor, indicative of increased cathepsin activity within the tumor and its microenvironment. **Bottom row:** FMT of NIR vascularization agent, showing increased probe distribution not only throughout the tumor but also the surrounding regions, which is consistent with increased tumor vascularity. Note that the measurement of activated cathepsin probe is contained within the measurement of probe bio-distribution, as expected.

The dual wavelength approach can be particularly useful in instances where limited fluorescence activity is recorded from regions of interest. For example, if low activity is recorded

in the ProSense channel, the dual wavelength approach can readily identify if this is due to lack of probe delivery and target accessibility or otherwise if it is due to lack of enzymatic activity.

Figure 7 shows a similar imaging session to the one shown in Fig.6 from a different mouse with a tumor grown in the lower left mammary region measuring ~4mm in size. Similarly this MMTVneu female mouse was injected with the NIR vascular contrast agent (AngioSense 750) and the cathepsin activatable (ProSense 680) smart probe 24 hours prior to imaging. In this example we were again able to explore the biodistribution of the probe through simultaneous detection of the vascular agent. The AngioSense signal may be again noted as extending far beyond the region of the tumor itself suggesting that an increase in local vasculature has occurred. We have performed dual wavelength studies in 9 animals so far (figure 8). These results demonstrate an average signal/background ratio of 2.8 across this specific group. Dual wavelength imaging sessions will continue in year 3 to study earlier disease stages as well as improve the statistics, as outlined in the grant proposal. Statistics will be based on the animals imaged in dual wavelength as this strategy allows for more accurate observations since it can differentiate between delivery and protease presence. So far we have seen highly consistent results.

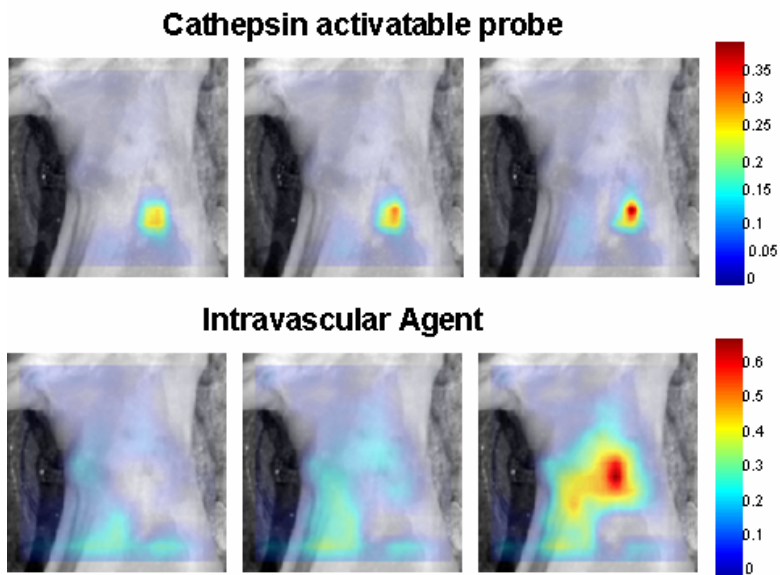


Figure 7: FMT of mouse with 4 mm tumor. Similarly to Fig.6 **Top row:** FMT of cathepsin-activatable NIR probe shows strong signal selectively within the region of the tumor, indicative of increased cathepsin activity within the tumor and its microenvironment. **Bottom row:** FMT of NIR vascularization agent, showing increased probe distribution not only throughout the tumor but also the surrounding regions.

By establishing feasibility to consistently image in dual wavelength mode, we have more recently shifted our attention to address each tumor in each mouse individually and confirm in-vivo imaging studies with the underlying fluorescence activity and histology. For instance, as apparent in the reflectance images shown below (figures 9 and 10), fluorochrome concentration clearly varies across the respective tumor samples all excised from the same MMTVneu female at approximately 6 months of age. The images were collected 24 hours after 2nmol probe administration, both AngioSense 680 (vascular imaging agent) and ProSense 750 (cathepsin-activated smart probe) via tail vein injection. Proceeding clock-wise from the red arrow in each figure are tumor samples taken from the following general anatomical locations: right medial

mammary, left medial mammary, left lateral mammary (axillary), lower right mammary (hip), right lateral mammary (axillary), and at center is normal muscle tissue serving as a contrasting negative control tissue sample. These tumors, excised from a retired breeder with multiple tumors demonstrate a clear heterogeneity of tumor uptake even in the same animal. Through implementation of our dual imaging approach we are now able to address whether variation in fluorophore concentration is merely a function of blood/probe distribution or a reflection of tumor size. To achieve the images of Figure 9, 10 we implemented a separate fluorescence imaging system under other funding. In year 3 we plan to combine in-vivo imaging findings, such as the ones shown in Figures 6,7 with individual assessment of ex-vivo tumor signals, such as the ones shown in Figs 9,10 before submitting for histopathology, in order to further confirm the in-vivo findings on a tumor to tumor basis.

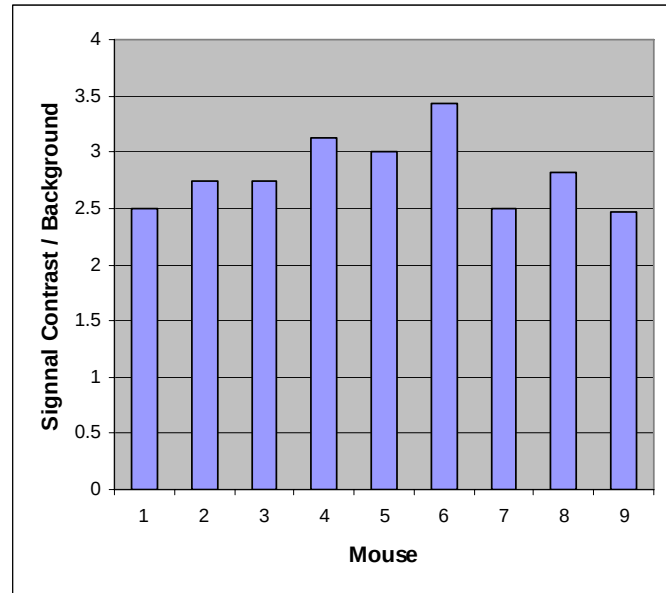
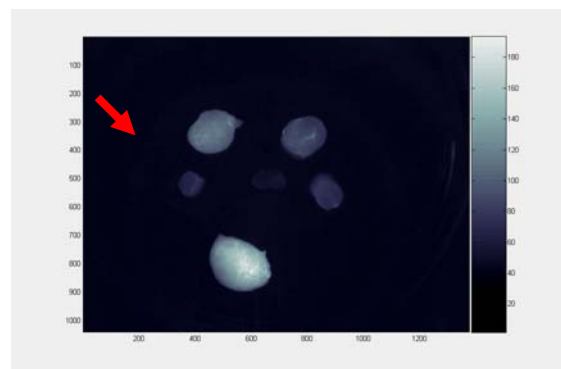


Figure 8: Nine Mice imaged using dual wavelength parameters. The highest intensity of signal reconstructed in the ProSense 750nm channel normalized to background levels has been presented for each animal.

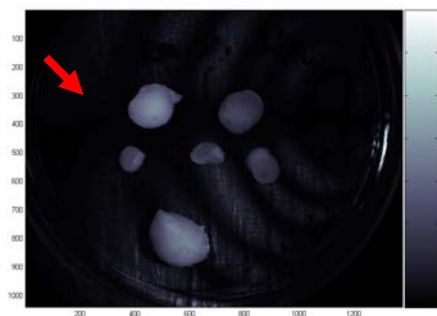


**Excitation Wavelength
(750 nm)**

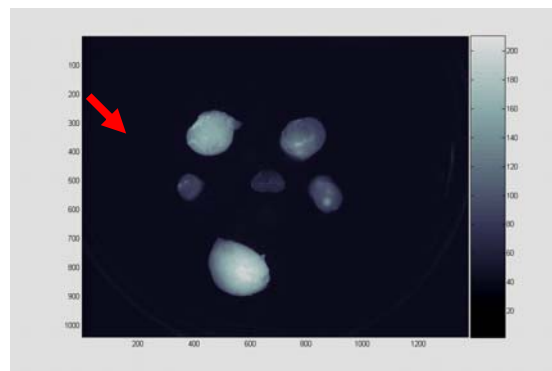


**Fluorescence Emission
Wavelength (750 nm)**

Figure 9



**Excitation Wavelength
(680 nm)**



**Fluorescence Emission
Wavelength (680nm)**

Figure 10

A2.3 Imaging parameter and algorithmic optimization

Recent work pertaining to our fluorescence molecular tomography (FMT) system has concentrated on establishing a uniform basis from which to compute reconstructions. This allows for greater consistency between data sets, which in turn enables easier and more accurate analysis of the resulting images. We have previously shown that by taking the ratio of emission over excitation signals, information about the noise in those signals is obscured, and intensity is no longer an appropriate indicator of signal to noise ratio (SNR). Because of this, when using all data points to compute an image, highly noisy data points may be heavily weighted, resulting in a poor result. The previous solution to this dilemma was to remove data pairs from the inverse solution based on minimum signal thresholds for each of the fluorescence and excitation signals. Data pairs were then removed from the reconstruction algorithm when one or both of the received signals failed to meet the minimum requirements. While effective, this method was highly variable, and required a considerable amount of user input in order to operate effectively. This increased the variability of the reconstructions, not only from one mouse to another, but even with regards to a single mouse imaged at different times. As such, direct comparison of such reconstructions was more challenging.

To address this issue, the approach we have taken is to look at the Born ratio from a statistical perspective. We began by constructing and experimentally validating noise models for each of the received signals. These were then used to construct a probability density for the overall ratio. This allowed us to then form a maximum likelihood (ML) problem, and obtain a solution which optimally weights each of the individual data points. While certain minimal signal requirements are still in place, these levels are very low, and fixed from one data set to another. The weighting of each remaining data pair is automatically determined, and requires no user input. Starting from the complete statistical problem statement, we then constructed

different inversion approaches that require no user input and are more reliable in handling various amounts of signal and noise in the data. The first solution, referred to as the Full Fixed Point solution, formally obtains the complete maximum likelihood ML solution using a fixed point iteration. The Reduced Fixed Point method eliminates several terms from the full ML problem, enabling us to use more efficient methods of solution. Finally, the Weighted Least Squares method eliminates the need for the fixed point iteration, further reducing the computation required. The validity of each of these statistically weighted methods was checked using existing data sets, and compared against the reconstructions obtained using the existing threshold method. The details of the method have now been submitted for publication as reported in “Reportable outcomes”.

Across a wide range of data sets, including phantoms in intralipid, euthanized mice with implanted tubes, and in-vivo imaging sessions, the three inverse methods we have developed show a remarkable consistency, and correlate well with previously obtained reconstructions. The results shown in Fig. 11 are from a set of in-vivo data from the Her2/neu mammary tumor mouse-model. As can clearly be seen, the results are extremely similar between each of the four reconstruction methods. The advantage that these new methods have is in their automated nature. Rather than requiring multiple inversions in order to determine the best threshold levels, our statistical methods need only be run once. Importantly, it requires no user input that makes it a very robust approach for quantification studies in multiple animal and imaging sessions, such as the ones proposed herein; yet the resulting answer is of equal or better quality than those obtained using threshold methods, and is more consistent across multiple data sets.

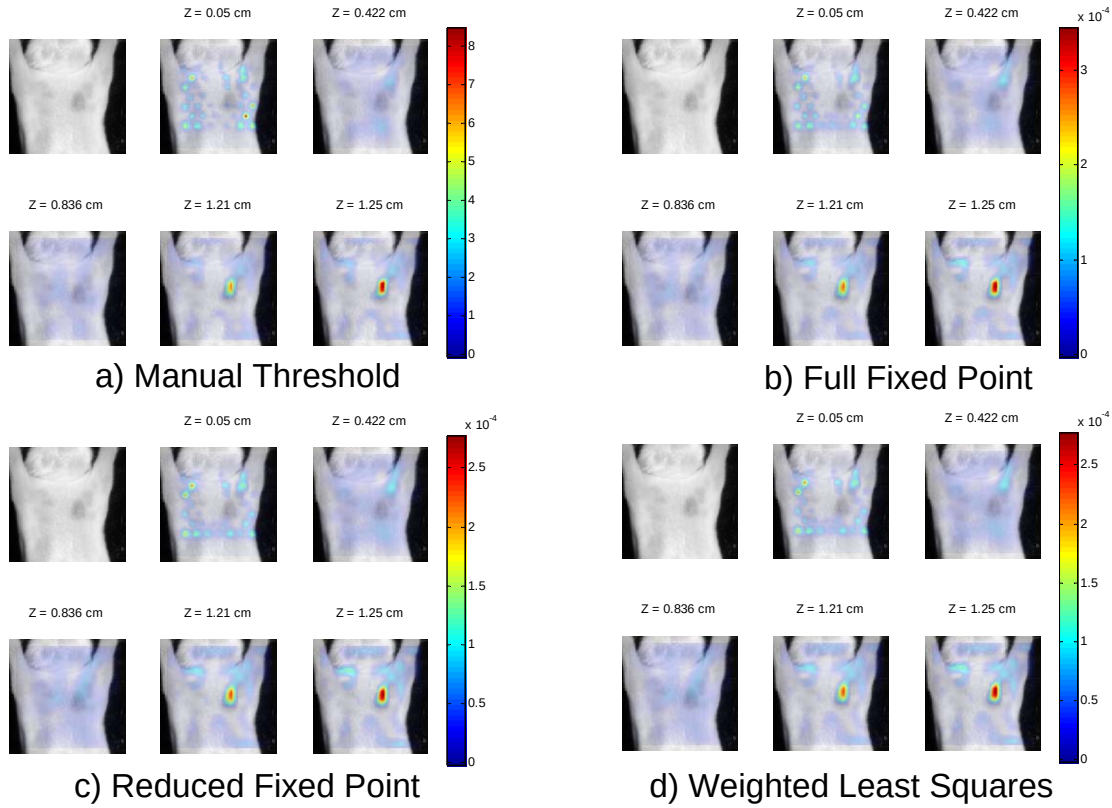


Figure 11: Processing of the same data set (shown in Fig.3) using a) a user-input threshold and b-c) statistically based methods for optimizing inversion. (see text for details).

Aim 3: To translate animal study findings into clinically-relevant detection schemes.

Consistent with the time plan of the proposal in the second year we have initiated the construction of phantoms that simulate the human breast, both geometrically and in terms of optical properties and we will be performing feasibility studies to identify clinical utility.

A.3.1 Phantoms for Breast Imaging

To develop breast models in order to address the goals of Aim 3 we have researched two manufacturing methods for developing diffusing phantoms that can be fluorescing. Specifically, silicone rubber and polyester casting resin are used as base materials, and silicone pigments and TiO_2 / India Ink are added to vary the optical properties (scattering and absorption), respectively. Fluorophores are then added to the silicone and resin, if the phantom is to be made fluorescent,

and solidifying agents are added to cure the samples. Currently, hydrophobic (IR-780 Iodide) and hydrophilic (Cy5.5) fluorophores have been tested successfully in the silicone and resin standards, respectively. Photograph of one phantom constructed can be seen in Fig.12

Quantification of the phantom's optical properties has been done using time-resolved methods. In short, a wavelength tunable femto-second laser (MaiTai, Spectra-Physics, Mountain View, California) with a pulsewidth of approximately 100 fs was coupled to a scanning galvanometer. The laser light could be scanned in a non-contact manner across a sample as desired. The transmitted light was detected with a Cooke SensiCamQE CCD camera coupled with a gated image intensifier (LaVision Picostar HR12, LaVision GmbH, Goettingen, Germany). A high-rate imager (Kentech Instruments Ltd., Oxfordshire, England) and a picosecond delay unit (Kentech) allowed acquisition of images with gate widths of 200 ps with a temporal step size of 25 ps.

Using computer software, the images can be analyzed to yield multiple photon-profile curves as a function of time and radial position from the source. By fitting these curves with the time-dependent diffusion equation, the optical coefficients can be obtained with high accuracy. Thus, by varying the concentration of one particle in the sample while holding the concentration of the other particle constant, a relationship between the particle concentration and the respective optical coefficient can be determined. These relationships were then used for determining the necessary amount of each particle to add. An example is shown below.



Figure 12. Silicon breast phantom constructed

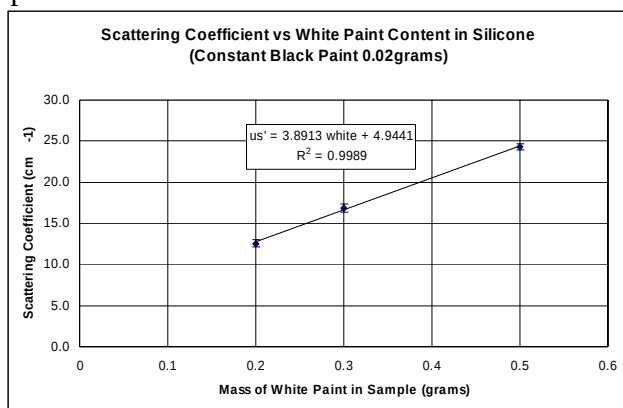


Figure 13: Relationship between the mass of white paint added to a silicone sample and the scattering coefficient. Typical human breast values for the reduced scattering coefficient are ~10 – 15cm⁻¹.

Using such phantoms, manufactured at appropriate sizes, shapes and varying optical properties, it would be possible in year 3 to propagate findings from the animal models to understanding the feasibility in detecting fluorescence contrast in human breast cancer detection, from a technological (sensitivity) stand point.

Evaluation of status of achieving year-2 goals

We have completed our year two goals, and easily anticipate completion of imaging studies for the remaining MMTVneu females by the end of this year, especially in regard to imaging distant disease, utilizing significantly improved imaging methods and strategies as was developed in this second year. We have so far imaged 16 Her2/neu animals with the single wavelength system and 9 animals with the dual wavelength system. We have also already made significant progress toward achieving the goals outlined for year 3. Multi-spectral imaging and improved FMT algorithms further allow for more accurate imaging. In year 3 we will continue to breed mice to achieve the necessary number of subjects and perform the necessary statistical analysis to overall evaluate the performance of the technique as a function of tumor size. We fully anticipate achieving our target animals proposed ahead of schedule. Additionally, we have already begun the construction of phantoms to correlate our finding to the potential of clinical applicability.

Key Research Accomplishments

- Establishment of own MMTVneu transgenic mouse colony and expansion to additional providers of appropriate animal models in interim.
- Diversification to orthotopic implants of human mammary cell lines
- Development of multi-spectral imaging analyses for improving the accuracy of the observation and providing internal controls.
- Development of automatic – threshold statistical methods for inversion to eliminate user input and thus standardize quantification.
- Imaging of 16 animals in single wavelength and 9 animals in dual wavelength studies.
- Construction breast-like phantoms

Reportable Outcomes Yr 1 & 2

Journal Publications & Proceedings:

Ntziachristos V, Turner G, Dunham J, Windsor S, Soubret A, Ripoll J & Shih HA, “Planar fluorescence imaging using normalized data” J. Biomed. Optics 10(6): 064007 (2005).

Soubret A, Ripoll J, Ntziachristos V, “Accuracy of fluorescent tomography in presence of heterogeneities: Study of the normalized Born ratio”, IEEE Med. Imag. **24**(10): 1369-1376 (2005).

Soubret A, Windsor SD, Turner GM, Ripoll J, Ntziachristos V, “Accuracy of fluorescence tomography in the presence of background fluorescence,” in press, Phys. Med. Biol 2006.

Hyde D, Miller E, Brooks DH, Ntziachristos V, “A Statistical Approach to Inverting the Born Ratio”, submitted IEEE Trans. Med. Imag.

Dunham J, Soubret A, & Ntziachristos V, “Dual wavelength fluorescence molecular tomography of breast cancer in Her2/neu mice” in preparation

Shih H, Ntziachristos V, “In vivo Characterization of Her-2/neu Carcinogenesis in Mice Using Fluorescence Molecular Tomography”. Optical Society of America Topical Meeting on Biomedical Optics, Ft. Lauderdale, MI (2006)

Windsor SD, Shih HA, and Ntziachristos, V, “In-vivo fluorescence molecular tomography of mammary adenocarcinomas in transgenic mice bearing an activated c-neu oncogene,” *U.S. Army Era of Hope Conference*, Philadelphia, PA, June 2005.

Windsor SD, Shih HA, Weissleder R, Ntziachristos V, “Simultaneous imaging of protease expression and biodistribution in Her-2/neu mice using dual-wavelength fluorescence molecular tomography,” *Society of Molecular Imaging Conference*, Cologne, Germany, Sept. 2005.

Presentations:

Windsor SD, “In-vivo fluorescence molecular tomography of mammary adenocarcinomas in transgenic mice bearing an activated c-neu oncogene,” U.S. Army Era of Hope Conference, Philadelphia, PA, June 09, 2005.

Conclusions

In year 2 we achieved all proposed goals and met all milestones. In addition we began our transition toward more clinically relevant detection schemes through initial construction and experimentation with breast-like phantoms, originally planned for year 3. We were able to identify tumors as early as 2-3mm and we were able to follow their growth as a function of time, which was demonstrated already in Year 1. In year 3, we will continue studies towards understanding the targeting ability and early detection based on protease probes and examine the potential for clinical translation. We will further summarize all studies, in particular the dual wavelength studies, with appropriate statistical measures and prepare appropriate publications on the biomedical findings of this study.

References

- [1] Humphrey LL, Helfand M, Chan BKS, and e. al., "Breast cancer screening: a summary of evidence for the U.S. preventive services task force," *Annals of Internal Medicine*, vol. 137, pp. 347-60, 2002.
- [2] Fletcher SW and E. JC., "Clinical practice. Mammographic screening for breast cancer," *N Engl J Med*, vol. 348, pp. 1672-80, 2003.
- [3] Arriagada R., Le M. G., Contesso G., Guinebretiere J. M., Rochard F., and S. M., "Predictive factors for local recurrence in 2006 patients with surgically resected small breast cancer," *Ann. Onc.*, vol. 13, pp. 1404 - 1413, 2002.
- [4] Arriagada R, Le MG, Rochard F, and C. G., "Conservative treatment versus mastectomy in early breast cancer: patterns of failure with 15 years of follow-up data. Institut Gustave-Roussy Breast Cancer Group," *J Clin Oncol*, vol. 14, pp. 1558-1564, 1996.
- [5] Mirza NQ, Vlastos G, Meric F, Buchholz TA, Esnaola N, Singletary SE, Kuerer HM, Newman LA, Ames FC, Ross MI, Feig BW, Pollock RE, McNeese M, Strom E, and H. KK., "Predictors of locoregional recurrence among patients with early-stage breast cancer treated with breast-conserving therapy.," *Ann Surg Oncol.*, vol. 9, pp. 256-65, 2002.
- [6] Wallgren A, Bonetti M, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, Holmberg SB, Lindtner J, Thurlimann B, Fey M, Werner ID, Forbes JF, Price K, Coates AS, and C. J, "Risk factors for locoregional recurrence among breast cancer patients: results from International Breast Cancer Study Group Trials I through VII.," *J Clin Oncol.*, vol. 21, pp. 1205-1213, 2003.
- [7] Weissleder R and Mahmood U, "Special review: Molecular imaging," *Radiology*, vol. 219, pp. 316-333, 2001.
- [8] Weissleder R and Ntziachristos V, "Shedding light onto live molecular targets," *Nat Med*, vol. 9, pp. 123-128, 2003.
- [9] Ntziachristos V, Ripoll J, Wang LV, and Weissleder R, "Looking and listening to light: the evolution of whole-body photonic imaging", *Nat Biotech*, vol. 23, pp. 331-320, 2005.
- [10] Ntziachristos V, Schellenberger EA, Ripoll J, Yessayan D, Graves E, Bogdanov A, Josephson L, and Weissleder R, "Visualization of antitumor treatment by means of fluorescence molecular tomography with an annexin V-Cy5.5 conjugate," *PNAS*, vol. 101, pp. 12294-12299, 2004.

- [11] Ntziachristos V and Chance B, "Probing physiology and molecular function using optical imaging: applications to breast cancer," *Breast Cancer Res*, vol. 3, pp. 41-46, 2001.
- [12] Hawrysz DJ and Sevick-Muraca EM, "Developments toward diagnostic breast cancer imaging using near-infrared optical measurements and fluorescent contrast agents," *Neoplasia*, vol. 2, pp. 388-417, 2000.
- [13] Colak SB, van der Mark MB, Hooft GW, Hoogenraad JH, van der Linden ES, and Kuijpers FA, "Clinical optical tomography and NIR spectroscopy for breast cancer detection," *IEEE Journal of Selected Topics in Quantum Electronics*, vol. 5, pp. 1143-1158, 1999.
- [14] Pogue BW, Poplack SP, McBride TO, Wells WA, Osterman KS, Osterberg UL, and Paulsen KD, "Quantitative Hemoglobin Tomography with Diffuse Near-Infrared Spectroscopy: Pilot Results in the Breast," *Radiology*, vol. 218, pp. 261-266., 2001.
- [15] Jakubowski DB, Cerussi AE, Bevilacqua F, Shah N, Hsiang D, Butler J, and Tromberg BJ, "Monitoring neoadjuvant chemotherapy in breast cancer using quantitative diffuse optical spectroscopy: a case study" *J Biomed Opt*, vol. 9, pp. 230-238, 2004.
- [16] Weissleder R, "Molecular imaging: exploring the next frontier," *Radiology*, vol. 212, pp. 609-14, 1999.
- [17] Ntziachristos V, Yodh AG, Schnall M, and Chance B, "Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement," *Proc Nat. Acad Sci U.S.A.*, vol. 97, pp. 2767-72, 2000.
- [18] Graves EE, Ripoll J, Weissleder R, Ntziachristos V, "A submillimeter resolution fluorescence molecular imaging system for small animal imaging," *Med Phys*, vol. 30, pp. 901-911, 2003.
- [19] Ntziachristos V, Ripoll J, and Weissleder R, "Would near-infrared fluorescence signals propagate through large human organs for clinical studies?," *Optics Letters*, vol. 27, pp. 333-335, 2002.
- [20] Weissleder R, Tung CH, Mahmood U, and Bogdanov A, "In vivo imaging of tumors with protease-activated near-infrared fluorescent probes," *Nature Biotech*, vol. 17, pp. 375-8, 1999.
- [21] Tung C, Mahmood U, Bredow S, and Weissleder R, "In vivo imaging of proteolytic enzyme activity using a novel molecular reporter," *Cancer Research*, vol. 60, pp. 4953-8, 2000.
- [22] Tung C, Bredow S, Mahmood U, and Weissleder R, "Preparation of a cathepsin D sensitive near-infrared fluorescence probe for imaging," *Bioconjug Chem*, vol. 10, pp. 892-6, 1999.
- [23] Koblinski JE, Ahram M, and Sloane BF, "Unraveling the role of proteases in cancer," *Clin Chim Acta*, vol. 291, pp. 113-35., 2000.

- [24] Yan S, Sameni M, and Sloane BF, "Cathepsin B and human tumor progression," *Biol Chem*, vol. 379, pp. 113-23, 1998.
- [25] Sameni M, Elliott E, Ziegler G, and e. al., "Cathepsin B and D are localized at the surface of human breast cancer cells," *Pathol Oncol Res*, vol. 1, pp. 43-53, 1995.
- [26] Levicar N, Kos J, Blejec A, and e. al., "Comparison of potential biological markers cathepsin B, cathepsin L, stefin A and stefin B with urokinase and plasminogen activator inhibitor-1 and clinicopathological data of breast carcinoma patients.," *Cancer Detect Prev*, vol. 26, pp. 42-9, 2002.
- [27] Ntziachristos V, Weissleder R., "CCD-based scanner for tomography of fluorescent near-infrared probes in turbid media," *Medical Physics*, vol. 29, pp. 803-809, 2002.