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TITLE: Noninvasive Label-Free Detection of Micrometastases in the Lymphatics With Ultrasound-Guided Photoacoustic Imaging

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14. ABSTRACT

The overall goal of this project is to design, build, and test a clinical ultrasound and photoacoustic imaging system that will detect functional changes associated with lymph node metastasis in breast cancer patients. Our efforts in the first year have focused on preclinical imaging studies of a metastatic mouse model of breast cancer and on clinical system development. While the animal studies are ongoing, we have demonstrated that bioluminescent/fluorescent breast cancer cells can reliably metastasize to the regional lymph nodes and optical imaging can be used to guide dissection. We have also successfully integrated a programmable ultrasound machine (Verasonics Vantage) and tunable pulsed laser (Opotek Phocus)that will form the basis of the clinical system. We have programmed a graphical user interface that enables real-time acquisition and display of co-registered ultrasound and photoacoustic images. Next steps include finalizing the design of an integrated ultrasound and photoacoustic handheld transducer and obtaining IRB approval.

15. SUBJECT TERMS
Lymph Node Metastasis, Ultrasound Imaging, Photoacoustic Imaging, Breast Cancer

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1. **INTRODUCTION:**

In this proposal, we aim to develop tools that can distinguish aggressive breast cancer from indolent cancers; overcome the problems of over diagnosis and overtreatment. Specifically, we will create a combined spectroscopic photoacoustic (sPA) and ultrasound (US) clinical imaging system to detect the widespread functional changes in lymph nodes that result from micrometastatic invasion. In addition, we will study the origin of these functional changes by performing imaging studies on a small animal model of breast cancer.

2. **KEYWORDS:**

Lymph Node Metastasis, Ultrasound Imaging, Photoacoustic Imaging, Breast Cancer

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

The overall goals are to develop a clinical imaging system and image processing methods to noninvasively detect lymph node metastases in breast cancer patients. This will be achieved through the following specific aims:

1. Study the mechanisms of SLN metastasis-induced hypoxia in a metastatic model of breast cancer.
2. Design and optimize a clinical US/sPA imaging system.
3. Perform US/sPA imaging on breast cancer patients

The original timeline from the Statement of Work as well as our current progress toward completion is outlined in the table below:

	Proposed Timeline	**Completion Date**
Specific Aim 1: Study the mechanisms of SLN metastasis-induced hypoxia in a metastatic model of breast cancer	**09/2014 – 09/2016**	**09/2016 (75% Completed)**
Major Task 1: Establish the mouse model	08/2014 – 02/2015	05/2015
Milestone 1: IACUC approval	08/20/2014	08/11/2014
Milestone 2: ACURO approval	01/01/2015	09/08/2014
Milestone 3: A stable set of luciferase transfected cell lines is created	02/2015	05/15/2015
Major Task 2: Perform *in vivo* longitudinal imaging studies	03/2015 – 03/2016	12/2015 (80% Completed)

Major Task 3: Analyze data and optimize image processing algorithms	07/2015 – 09/2016	07/2016 (25% Completed)
Milestone 4: Submit a journal manuscript on the ability of US/sPA imaging to detect the lymphatic spread of breast cancer tumors in mice	09/2016	09/2016 (0% Completed)
Specific Aim 2: Design and optimize a clinical US/sPA imaging system.	09/2014 – 03/2016	06/2016 (50% Completed)
Major Task 4: Design and construct integrated US/sPA imaging transducer	09/2014 – 05/2015	12/2015 (50% Completed)
Milestone 5: An integrated US/sPA transducer is assembled.	05/2015	12/2015 (50% Completed)
Major Task 5: Program the clinical system for real-time imaging	06/2015 – 11/2015	04/2016 (75% Completed)
Major Task 6: Test the clinical system on tissue mimicking phantoms	06/2015 – 03/2016	06/2016 (40% Completed)
Milestone 6: a fully-functional clinical system is constructed and optimized	03/2016	06/2016 (50% Completed)
Specific Aim 3: Perform US/sPA imaging on breast cancer patients.	03/2016 – 09/2017	06/2016 – 09/2017 (0% Completed)
Major Task 7: Obtain approval for human studies	03/2016 – 09/2016	06/2016 – 12/2016 (0% Completed)
Milestone 7: Approval to initiate human studies is obtained	09/2016	12/2016 (0% Completed)
Major Task 7: Perform human imaging studies	09/2016 – 09/2017	01/2017 – 09/2017 (0% Completed)
Milestone 8: Submit a journal manuscript on the ability of US/sPA imaging to detect the lymphatic spread of breast cancer tumors in human patients	09/2017	09/2017 (0% Completed)

- **What was accomplished under these goals?**

Activities in this reporting period have been focused on the first two specific aims: 1) imaging of a metastatic mouse model of breast cancer and 2) development of a clinical US/sPA imaging system. The third specific aim will be addressed in the final year of the project.

We have performed a series of experiments aimed at establishing the functional changes that occur with the onset of lymph node metastases in a mouse model of breast cancer. As a critical step in this process, we have demonstrated that we can effectively initiate and monitor a metastatic mouse model of breast cancer (**Fig. 1**). The bioluminescent MDA-MB-231 cell line enables noninvasive monitoring of tumor progression. In addition, by performing bioluminescence imaging during tissue excision, we have been able to accurately detect and extract metastatic tissue for histological evaluation. Our studies to date have demonstrated a high efficiency of metastasis, with each mouse averaging at least 2 metastatic lymph nodes. While image processing and statistical analysis efforts are still underway, these preliminary optical imaging results are encouraging for the success of these studies. We are currently preparing the tissue samples for histological analysis, where detection of the MDA-MB-231 cell will be further aided by their expression of green fluorescent protein.

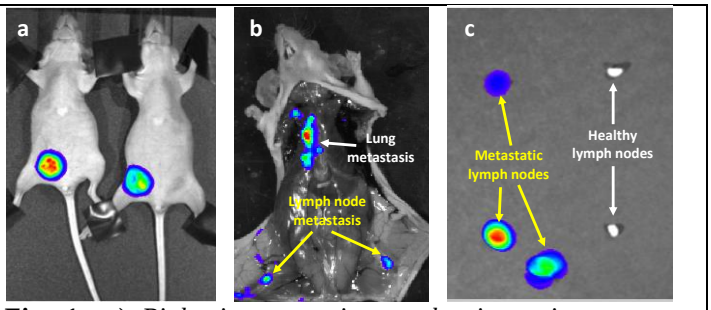

Fig. 1: *a) Bioluminescence image showing primary tumor progression; b) after removal of the primary tumor, residual metastases in the regional lymph nodes and lungs are visualized. This signal is used to guide excision of metastatic tissue; and c) bioluminescence imaging can be used to determine metastatic state of lymph nodes prior to histology.*

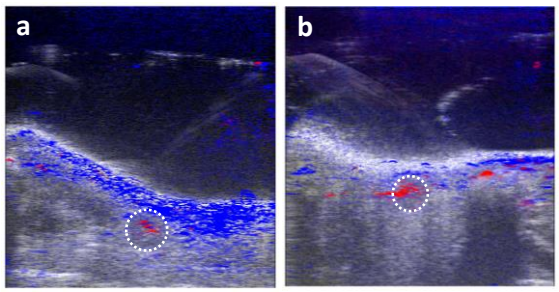

Fig. 2: *Representative combined US (grayscale) and sPA (red/blue) images of a) an axillary lymph node and b) an inguinal lymph node. Lymph nodes are outlined with a white dashed circle. Red corresponds to oxygenated hemoglobin and blue corresponds to deoxygenated hemoglobin.*

We have captured complete longitudinal imaging data on five mice. This includes capturing US and sPA images in regions which contain the primary tumor as well as the regional axillary, inguinal, and subilliac lymph nodes (all of which have the potential to develop metastases) once weekly. In addition, bioluminescence images were acquired once weekly. Imaging was performed for 4-6 weeks, until the mice showed distress which necessitated euthanasia. The initial evaluation of the images shows trends which agree with our previous studies in head and neck cancer. First, the lymph nodes are easily identifiable using high frequency US images (**Fig. 2**, white dashed regions). Second, photoacoustic signal from blood is clearly evident throughout the imaging volume, including within the lymph nodes. Finally, we noticed that the lymph nodes exhibited an elevated blood oxygen saturation when compared to the surrounding tissue (as seen by the red color in **Fig. 2**). Indeed, this is entirely consistent with what we observed in the cervical lymph nodes. Efforts are currently underway to analyze the blood oxygen saturation throughout all lymph nodes as the disease progresses to verify that the same trends (i.e., decreased oxygenation with increased metastatic invasion) exist in this breast cancer model. This evaluation, along with histological validation of all lymph nodes, will be completed within the next several months. We will also pursue additional immunohistochemical analysis of the tissue slices to elucidate the source of functional changes in metastatic nodes. Overall, based on our previous results and the high number of metastatic and healthy nodes which we observed in each animal, we are confident that we will have the statistical power needed to detect functional changes in the lymphatics with this set of experiments.

In addition to the small animal experiments, work has also progressed on the development of the clinical imaging system. We have successfully interfaced our laser (Opotek Phocus Mobile HE) with the programmable ultrasound machine (Verasonics Vantage). We have synchronized the signals to enable interleaved acquisition of US and PA images, and automated tuning of the laser wavelength and energy. We have also developed a graphical user interface which provides real-time (10 Hz) visualization of the US and PA images, and the ability to adjust a variety of parameters (**Fig. 3**). We will continue to fine-tune the interface by working with physicians and sonographers.

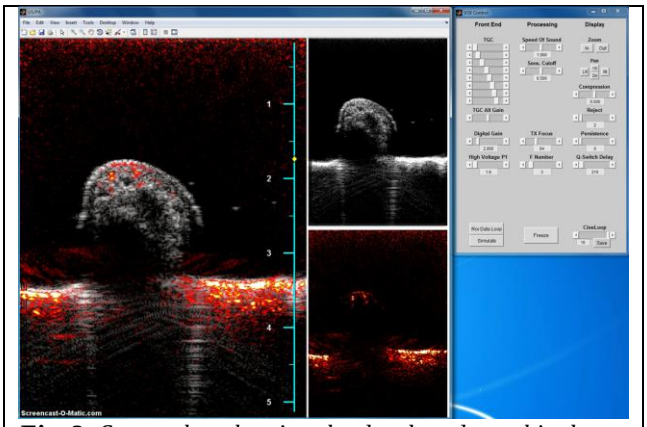

Fig. 3: *Screenshot showing the developed graphical user interface which enables real-time visualization of coregistered US (grayscale) and PA images (red/yellow). The control panel (right) allows for dynamic modification of focus, US transmit energy, laser energy, optical wavelength, and display parameters.*

The other key component of the clinical imaging system is the design of the handheld transducer which integrates US and optical delivery to the tissue. Our initial experiments with the US transducer proposed in the original application have indicated that it suffered from poor sensitivity. Therefore, we have recently moved to a lower-frequency transducer in order to boost imaging sensitivity, which is critical for deep-seated lymph nodes. Efforts are currently underway to acquire a three-dimensional scan of the new transducer (L11-4v, Verasonics Inc.) and build a housing which effectively couples fiber optic light delivery.

- **What opportunities for training and professional development has the project provided?**

Dr. Luke and Dr. Emelianov attended several conferences and presented their findings with large emphases on biomedical imaging and cancer applications. These include the Biomedical Engineering Society Annual Meeting, SPIE Photonics West, the World Molecular Imaging Congress, OSA Frontiers in Optics, and IEEE Ultrasonics Symposium. In addition, Dr. Emelianov and Dr. Luke participated in scientific review sessions for the National Cancer Institute and CDMRP BCRP. These opportunities were made available in part by the experience gained on this project.

- **How were the results disseminated to communities of interest?**

In addition to the several conference presentations listed below, Dr. Luke presented an overview of the research to an engineering class in Kealing Middle School in Austin, TX as part of a series of lectures covering a broad range of engineering career options.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we will conclude the small animal imaging studies and all associated analysis. It is our goal to submit a peer-reviewed manuscript summarizing the studies by the end of Year 2 of funding. Furthermore, we will complete the development and programming of the clinical sPA/US imaging system and associated transducer. We will begin to fully characterize and

optimize the system using tissue-mimicking phantoms. Finally, we plan to obtain IRB approval and have initiated federal approval for human studies by the end of Year 2. This will put us on track for final system optimization and human studies in Year 3.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

Our clinical US/sPA imaging system is one of the first that enables real-time acquisition and display of the images. This is a critical technological advance that needs to be perfected in order for this imaging technology to make an impact in the clinic. Furthermore, our next steps to include simultaneous acquisition of Doppler US and real-time spectroscopic visualization will present unique advancements in the field. We hope that other research groups will build on the capabilities of our imaging system to extend PA imaging to other clinical applications.

- **What was the impact on other disciplines?**

While similar mouse models have been published in the literature, our refinement and characterization of the metastatic breast cancer model will help other researchers in diverse fields initiate the model for their studies. We will disseminate this information to the research community by including specific details about the protocol, imaging parameters, and sites of metastatic involvement in a peer-reviewed journal article.

- **What was the impact on technology transfer?**

Our research is leading to a patentable design for the clinical sPA/US system; we intend to pursue intellectual property protection in the next year. Furthermore, we have begun collaborating with a new imaging company – Rockport Imaging – which is focused on clinical photoacoustic imaging for anesthesiology applications. While we have not yet begun collaborating on this BCRP-funded project, we hope that this relationship will provide a path for future commercialization.

- **What was the impact on society beyond science and technology?**

While this project is still in its early stages, the biggest potential impact is in the area of clinical diagnosis and treatment of breast cancer. If we are able to provide physicians with an accurate evaluation of the extent of metastatic invasion in the lymphatics, then more appropriate treatment plans can be developed. The end result could be improved cancer survival rates and decreased use of invasive surgical procedures.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

We have made two minor changes to our original proposed plan. First, rather than transfecting our own cell lines, we have opted to purchase a MDA-MB-231 cell line which has already been stably transfected to express luciferase and green fluorescent protein. We chose this route after two batches of cells arrived from ATCC contaminated and we had one unsuccessful attempt at transfection. Therefore this new approach helped to avoid further delays and the combination of

luciferase and green fluorescent protein will enable tracking of metastases *in vivo* and in excised tissue.

Second, we have changed the selection in our US transducer for the clinical system. Instead of using the proposed LA09 linear array transducer from Alpinion (10-MHz center frequency), we have instead opted for the L11-4v transducer from Verasonics (7-MHz center frequency). The former suffered from poor sensitivity, which is critical for this application. By shifting to a lower frequency transducer, we have sacrificed a small amount of spatial resolution to gain additional sensitivity.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

One source of delay has occurred in the first year of this project: Dr. Luke transitioned to a tenure-track position at Dartmouth College and Dr. Emelianov joined the faculty at Georgia Institute of Technology. Because there is no one remaining at the University of Texas at Austin to complete the project, we have requested that the award be transferred to Dartmouth College. Dr. Luke has already ordered (with start-up funds) all necessary equipment to replicate the clinical system at The University of Texas at Austin, and all items should arrive within 2-3 months. We are currently proceeding with finalizing the transducer design and completing the animal studies in the down-time. Another key change that comes with this move is the location of the clinical studies. The long distance between Dartmouth College and MD Anderson Cancer Center makes the original plan much more difficult. Therefore, we have decided that the clinical studies will be performed at the Dartmouth-Hitchcock Medical Center. This center houses the Geisel School of Medicine as well as the Norris Cotton Cancer Center which ensures adequate patient population and appropriate collaborators. Furthermore, Dr. Luke's new lab is located in the medical center, which greatly facilitates the development of the clinical system. While the transition to the new location will obviously cause some administrative and project delays, we believe that the elimination of travel between Austin and Houston will put the project back on track for on-time completion.

- **Changes that had a significant impact on expenditures**

Nothing to Report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

▪ **Journal publications.**

Geoffrey P. Luke and Stanislav Y. Emelianov; Label-free Detection of Lymph Node Metastases with US-guided Functional Photoacoustic Imaging; Radiology; Volume 277, No. 2; 2015; pp 435-442; published; federal support **was** acknowledged.

- **Books or other non-periodical, one-time publications.**

  Nothing to Report.

- **Other publications, conference papers, and presentations.**

S. Y. Emelianov, A. Hannah, and G. P. Luke, "Detection and Characterization of Sentinel Lymph Node using Contrast-Enhanced Ultrasound and Photoacoustic Imaging," IEEE Ultrasonics Symposium, Taipei, Taiwan, Oct. 23, 2015.

G. P. Luke, K. V. Sokolov, and S. Y. Emelianov, "Spectroscopic Photoacoustic Imaging for the Detection of Lymph Node Metastases," *OSA Frontiers in Optics and Laser Science*, San Jose, CA, Oct 19, 2015

G. P. Luke A. S. Hannah, and S. Y. Emelianov, "Comprehensive Approach to Localization of Sentinel Lymph Node and Detection of Micrometastases using Sound, Light and Molecular Contrast nanoAgents," *World Molecular Imaging Congress*, Honolulu, HI, Sept. 4, 2015.

G. P. Luke and S. Y. Emelianov, "Label Free Ultrasound-Guided Spectroscopic Photoacoustic Imaging of Lymph Node Micrometastases," *Photons Plus Ultrasound: Imaging and Sensing Proc. SPIE*, San Francisco, CA, Feb. 9, 2015.

G. P. Luke and S. Y. Emelianov, "Ultrasound and Photoacoustic Imaging of Anatomical and Functional Indicators of Lymph Node Metastasis," *Biomedical Engineering Society Annual Meeting*, San Antonio, TX, Oct 25, 2014.

- **Website(s) or other Internet site(s)**

  http://fmilab.com: This is the new lab website for Geoffrey P. Luke. Major research findings and links to all publications will be posted here to help disseminate results.

- **Technologies or techniques**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report.

- **Other Products**

During this reporting period we have made significant progress towards the creation of two important products: 1) a clinical sPA/US imaging system, and 2) the software which enables real-time acquisition and visualization of the data. We anticipate that during the next year these two products will be highly polished. Once they are, we will do our best to make them widely available to the research community as is appropriate (e.g., code, CAD designs, etc.). Of course, the project

is still centered on the creation of a complete clinical diagnostic tool; this will be the most worthwhile product to emerge from this research.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

***Example**:*

Name:	Geoffrey Luke
Project Role:	PI
Researcher Identifier	ORCID: 0000-0002-1486-3398
Nearest person month worked:	6
Contribution to Project:	Dr. Luke oversaw the research and designed, programmed, and tested the clinical imaging system. He also helped initiate the animal studies.

Name:	Stanislav Emelianov
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Emelianov contributed to the experimental design and development of ultrasound image processing methods.

Name:	Carolyn Bayer
Project Role:	Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Dr. Bayer has performed the bulk of the animal imaging experiments.
Funding Support:	Breast Cancer Research Foundation

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Geoffrey P. Luke has transitioned to a tenure-track position at Dartmouth College. As part of this new position, Dr. Luke has received a start-up package that will support the formation of a new laboratory, where this research project will continue.

- **What other organizations were involved as partners?**
  - **Organization Name:** MD Anderson Cancer Center
  - **Location of Organization:** Houston, TX
  - **Partner's contribution to the projec**t
  - **Collaboration:** Consulting by Dr. Wei Yang on the design of the clinical system and on the clinical applications of the project.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

9. APPENDICES:

Nothing to Report.