AWARD NUMBER: W81XWH-19-1-0187

TITLE: Development of Ultrasound-Guided Photoacoustic Imaging for Noninvasive Detection of Metastatic Lymph Nodes in Melanoma Patients

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REPORT DATE: August 2021

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
August 2021	Annual	01Aug2020-31Jul2021

4. TITLE AND SUBTITLE

Development of Ultrasound-Guided Photoacoustic Imaging for Noninvasive Detection of Metastatic Lymph Nodes in Melanoma Patients

5a. CONTRACT NUMBER
W81XWH-19-1-0187

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER
0011331863

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

1G474

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development 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

14. ABSTRACT

In melanoma skin cancer patients, determination of whether a malignancy has spread is the single most important factor used to develop a therapeutic plan and to predict prognosis. In most cases cancer cells initially spread through regional lymph nodes. The presence of malignant cells in the first lymph node to which a tumor drains - known as the sentinel lymph node (SLN) - is a harbinger of distant metastases and a low survival rate. Therefore, clinical evaluation for the presence of regional lymph node metastases is critical. We propose to develop an advanced, in-vivo, clinically translatable noninvasive imaging technology, i.e., integrated ultrasound (US) and photoacoustic (PA) imaging, capable of immediate and accurate assessment of SLN micro-metastases in real time. The central theme of this application is to design, build and test a prototype of the SLN ultrasound-guided photoacoustic (USPA) imaging tool.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	15	**19b. TELEPHONE NUMBER** ***(include area code)***

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1. INTRODUCTION:

In current clinical use, cancer staging evaluation involves invasive and ionizing methods, i.e. sentinel lymph node (SLN) biopsies guided by peritumoral injection of dye, a radioactive colloid, or both. There is no single imaging modality that is widely available, is simple to operate, is safe, and can reliably identify melanoma SLN and SLN micrometastases. We propose to develop an ultrasound-guided photoacoustic imaging system to reliably identify melanoma SLN and SLN micrometastases. The central theme of this application is to design, build and test a prototype of a clinically translatable non-invasive SLN ultrasound photoacoustic (SLN-USPA) imaging tool with integrated laser/ultrasound imaging system, capable of immediate and accurate assessment of SLN micro-metastases in real time. The proposed work, therefore, aims to accomplish the following tasks:

Aim 1: Develop laser/ultrasound imaging system for SLN-USPA imaging.

Aim 2: Validate the SLN-USPA imaging in a murine model of metastatic melanoma cancer.

The successful outcome of this study will enable design and development of clinical SLN+USPA imaging. This imaging technique will provide non-ionizing, non-invasive, reliable real-time diagnosis and be greatly helpful in planning patient treatment regimens, assessing patient response, with the ultimate goal of eradicating melanoma cancer.

2. KEYWORDS:

Skin cancer imaging, noninvasive micro-metastases assessment, ultrasound and photoacoustic imaging, melanoma diagnosis

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Develop a laser/ultrasound imaging system for sentinel lymph node – ultrasound photoacoustic (SLN-USPA) imaging.

- Major Task 1: Build and optimize an integrated system for SLN-USPA imaging
  1. Assemble hardware components for the SLN-USPA imaging system. The system will include a wavelength tunable pulsed laser, modern ultrasound imaging system for US and PA data acquisition and processing, optical fibers (light delivery) with an US imaging probe. (0-2 months, completed by 9/30/2019)
  2. Develop SLN-USPA imaging software. The imaging software will synchronize the ultrasound system and the laser system and enable USPA imaging and data acquisition for difficult wavelengths. (1-3 months, completed by 10/31/2019)

3. Establish protocol for SLN-USPA blood oxygen saturation (SO2) measurements. The study includes the investigation of imaging parameters and algorithm development that measures SO2 for each image pixel by comparing spectroscopic photoacoustic with known absorption spectrum from hemoglobin (Hb) and oxyhemoglobin (HbO2). (4-6 months, completed by 12/31/2019)
    - Milestone Achieved: We will build an operational SLN-USPA imaging system. (completed by 12/31/2019)
    - Local IACUC Approval (completed by 5/16/2019)
    - Milestone Achieved: ACURO Approval (completed by 9/10/2019)

- Major Task 2: Test and optimize the SLN-USPA imaging system for the real-time detection of SO2 measurement.
    1. SLN-USPA imaging experiments with various optical wavelengths using tissue-mimicking gel phantoms with blood inclusions inside for blood SO2 measurements. This study will validate the imaging protocol with known SO2. (6-8 months, originally planned to complete by 4/30/2020, works in progress, 50% completed)
    2. Characterize SLN-USPA imaging for various depths. This study involves varying the depth of the blood inclusion in the imaging plane and investigating the USPA signal change. Imaging algorithm and image processing algorithm will be developed for the optimization of the blood SO2 measurements. (8-16 months, originally planned to complete by 6/30/2020, works in progress, 70% completed)
        - Milestone Achieved: The SLN-USPA imaging system will be well determined and characterized, and thus ready for in vivo imaging. (originally planned to complete by 12/30/2020, works in progress, 90% completed)

Specific Aim 2: Validate the SLN-USPA imaging in vivo in a murine model of metastatic melanoma cancer.

- Major Task 3: Demonstrate that SLN-USPA imaging to detect SLN micro-metastases using an orthotopic murine
    1. Experiment on a few strains of mice (BALB/c, C57BL/6, B6N-Tyrc-Brd/BrdCrCrl) for the luciferase expressing melanoma cell line (B16F10 Red-FLuc) inoculation (1-2 x 10^5 cells) and determine the optimized strain, number of cells to be injected, and the monitor the growth of the tumor together with IVIS imaging for metastases tracking. Some in vitro study of melanoma cells may also be introduced to further validate the USPA imaging system. (16-20 months, originally planned to complete by 4/30/2021, works in progress, 80% completed)
    2. With the assumption that only 50% of the mice will develop metastases, 40 mice will be inoculated with the luciferase expressing melanoma cells and imaged by SLN-USPA imaging as well as IVIS imaging at the same day every 72 hours for 5 weeks, to track metastasis. At the conclusion of the last imaging session, histology of the exercised metastasized lymph nodes as well as the healthy nodes as control (maybe also the tumor) will be perform to serve as the ground truth. (20-36 months, originally planned to complete by 8/31/2022, 0% completed)
    3. IVIS imaging and 3D reconstructed histology analysis as ground truth to correlate with SLN-USPA imaging results. (20-36 months, originally planned to complete by 8/31/2022, 0% completed)

4. In case the luciferase expressing melanoma cell line (B16F10 Red-FLuc) induces different USPA signals, we may also use wild type B16F10 cells to verify. (24-36 months, originally planned to complete by 8/31/2022, 0% completed)
    - Milestone Achieved: Conclude the study with a correlation map between SLN-USPA SO2 measurement and SLN metastasis status based on SLN-USPA imaging as a clinically relevant diagnosis decision-making metric prototype. (planned to complete by 8/31/2022, 20% completed)

What was accomplished under these goals?

Aim 1: Develop a laser/ultrasound imaging system for sentinel lymph node – ultrasound photoacoustic (SLN-USPA) imaging

Major Activities

- Testing and optimization of Vevo LAZR for SLN-USPA imaging requirements. New hardware system components using a wavelength tunable pulsed Vevo LAZR laser, the Vevo 2100 platform, and a 43.5 MHz LZ550 photoacoustic imaging probe.
- Develop SLN-USPA imaging protocol: Single slice spectroscopic US/PA imaging for characterizing absorbers and for spectral unmixing of deoxyhemoglobin and oxyhemoglobin. 3D multispectral US/PA imaging of lymph node for volumetric blood oxygen saturation measurement.
- Testing of SLN-USPA blood oxygen saturation measurement methods, development of SO_2 algorithm. Dual wavelength 750 and 850 nm imaging for ratio-based SO_2 approximation provides a fast, live estimation of blood oxygen saturation. Post-processing MATLAB based SO_2 algorithm using additional wavelengths and spectral unmixing gives a more accurate blood oxygen saturation measurement.

Specific Objectives

- Build and optimize the USPA imaging system
- Develop the imaging protocol and software
- Establish image processing protocol

Key Outcomes

- We have successfully established an imaging and processing protocol for the imaging system with improved hardware and image quality, ready for *in vivo* imaging.

Discussion of Stated Goals Not Met

- Additional refinement of post-processing SO_2 measurements are needed, due to changes in imaging system and data acquisition format.

Aim 2: Validate the SLN-USPA imaging *in vivo* in a murine model of metastatic melanoma cancer.

Major Activities

- Developed melanoma mouse model after inoculation of B16F10 Red-Fluc melanoma cells in C57BL/6 mice.
- Longitudinal *In vivo* imaging of C57BL/6 mice with B16F10 Red-Fluc melanoma cancer. Imaging of 3 mice over 25 days to optimize *in vivo* imaging protocol and gather data for SO_2 measurement.
- Optimization of melanoma cell count, injection location, and IVIS imaging protocol. Lower cell count ($1x10^5$ cells) used for more time for metastatic spread before primary tumor growth. Right flank location is used for injection of primary tumor, making the right subiliac lymph node the sentinel lymph node of interest. Luciferin is injected intraperitoneally, with peak bioluminescence occurring 20-30 minutes after injection.

Specific Objectives

- Develop melanoma mouse model
- Optimize mouse strain and cell injection for metastatic growth.
- Develop IVIS bioluminescence imaging protocol
- Test US/PA imaging protocol *in vivo*

Key Outcomes

- We have successfully imaged our melanoma mouse model using our US/PA imaging protocol. This includes dual-wavelength SO_2 measurement (Fig 1.) and spectroscopic measurement for post-processing (Fig 2.). Lymph node outlined, with red overlay showing PA signal.
- Successfully visualized metastatic growth using bioluminescence imaging with IVIS.

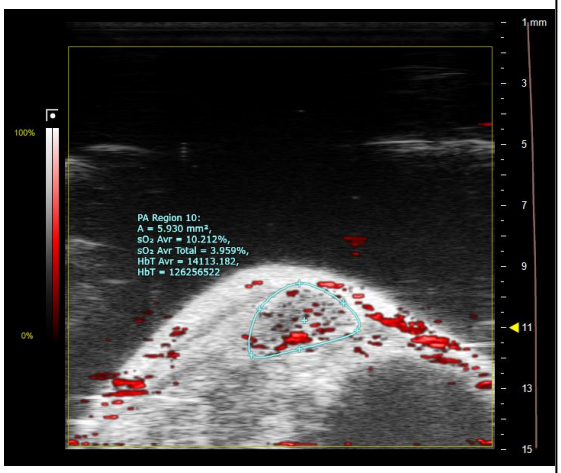

Figure 1. Oxy-Hemo Dual Wavelength SO_2 US/PA imaging in melanoma mouse model

Discussion of Stated Goals Not Met

- Further strain optimization needed – C57BL/6 strain chosen due to its syngeneic relationship with B16F10 melanoma cells, but allogeneic BALB/c mice have been suggested to have better metastatic growth.

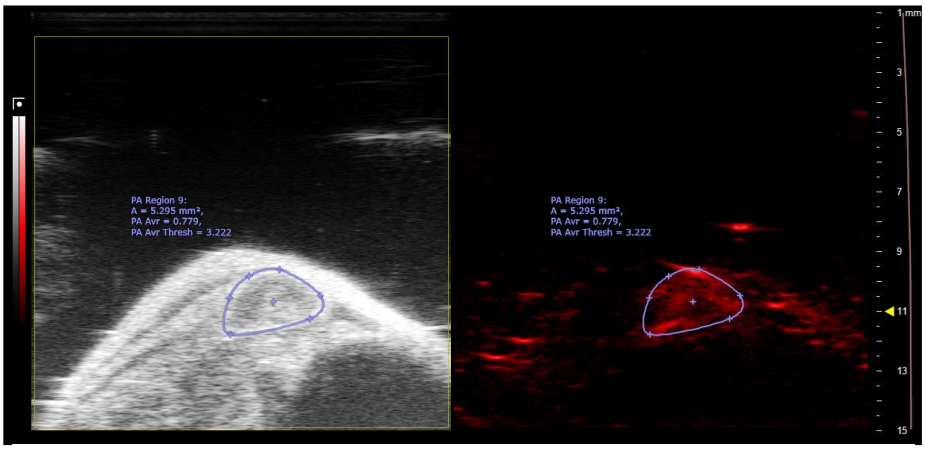

Figure 2. Spectroscopic US/PA imaging in melanoma mouse model

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

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

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

Additional optimization of animal strain is underway due to research suggesting that despite B16F10 being syngeneic in C57BL/6 mice, allogeneic BALC/c mice are significantly more susceptible to metastasis.

The post-processing blood oxygen saturation (SO2) algorithms are continuing to be refined.

Conduct the final animal experiments with histology to correlate SO2 and metastatic status to conclude the study.

4. IMPACT:

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

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The US/PA imaging system platform was changed from a Verasonics based platform to a Visualsonics Vevo LAZR platform. This system has improved hardware and image quality, is well-characterized with an established front-end imaging software, and can be integrated with external post-processing algorithms. This system is closer to clinical viability, with various transducers available to swap depending on application.

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

Ongoing studies were disrupted by a change in PI in the middle of this reporting period. This has since been resolved with data and other information being fully transferred.

Animal studies continued to be affected by the COVID-19 pandemic, as research continued to ramp up. Additionally, a scarcity of animal models persisted for an extended period. These issues have been resolved, with the institution being fully operational and animal models procured.

A malfunction with a cell dewar causing it to lose liquid nitrogen led to widespread cell death, including the B16F10-Fluc cells used in this protocol. These cells have been reprocured and stored, with additional precautions added to prevent a repeat malfunction.

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

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

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

Journal publications.

Nothing to Report

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

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

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

Nothing to Report

- **Technologies or techniques**

Nothing to Report

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

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Yiying Zhu
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-0567-1391
Nearest person month worked:	4 months
Contribution to Project:	Dr. Zhu has characterized the imaging system and started preliminary animal studies.
Name:	Anthony Yu
Project Role:	PI
Nearest person month worked:	8 months
Researcher Identifier (e.g. ORCID ID):	0000-0002-4830-6346
Contribution to Project:	Mr. Yu has continued developing imaging and processing protocols and has further conducted animal studies.

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: