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

PRINCIPAL INVESTIGATOR: Anthony Yu

CONTRACTING ORGANIZATION: Georgia Tech Research Corporation

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Fort Detrick, Maryland 21702-5012

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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			USAMRDC
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

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

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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, completed using animal data)
    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, completed using animal data)
        - Milestone Achieved: The SLN-USPA imaging system will be well determined and characterized, and thus ready for in vivo imaging. (completed by 9/31/2021)

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, completed by 1/31/2022)
    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 excised metastasized lymph nodes as well as the healthy nodes as control will be performed to serve as the ground truth. (20-39 months, completed by 10/31/2022)
    3. IVIS imaging and 3D reconstructed histology analysis as ground truth to correlate with SLN-USPA imaging results. (20-39 months, IVIS imaging analysis completed by 10/31/2022)

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. (24-39 months, did not need to perform as B16F10 Red-FLuc did not extraneous USPA signal)
    - 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. (completed by 10/31/2022)

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

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

Major Activities

- Finalize SLN-USPA imaging protocol: Spectroscopic US/PA imaging of both the sentinel (left subiliac) and contralateral lymph nodes for characterizing absorbers and for spectral unmixing of deoxyhemoglobin and oxyhemoglobin. 3D US imaging of both lymph nodes for accurate lymph node volume measurement.
- Finalize bioluminescent imaging protocol: Performed a Luciferin kinetic curve to determine the ideal time (15 minutes) for bioluminescent imaging after luciferin administration.
- Fully developed SO2 algorithm and additional image processing for US/PA imaging. Dual wavelength 750 and 850 nm imaging for ratio based SO2 approximation via Vevo LAZR provides a fast, live estimation of blood oxygen saturation. The spectral unmixing algorithm implemented in MATLAB uses non-negative matrix factorization to determine the relative concentrations of oxy and deoxyhemoglobin at every pixel within each image.
- Developed MATLAB based bioluminescence imaging analysis to estimate metastatic flux (photons/s)

Specific Objectives

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

Significant Results

- All imaging protocols and developed processing algorithms were tested on preliminary animal data to be optimized and verified for in vivo blood SO2 measurement. An example of the SO2 image processing is shown below where a whole lymph node SO2 measurement is made. SO2 = $[HbO_2]$ / ([Hb] + $[HbO_2]$). The surrounding background SO2 in a 2-mm-thick layer of tissue around the lymph node was also averaged and subtracted from the nodal SO2 in order to account for mouse to mouse variations in background SO2.

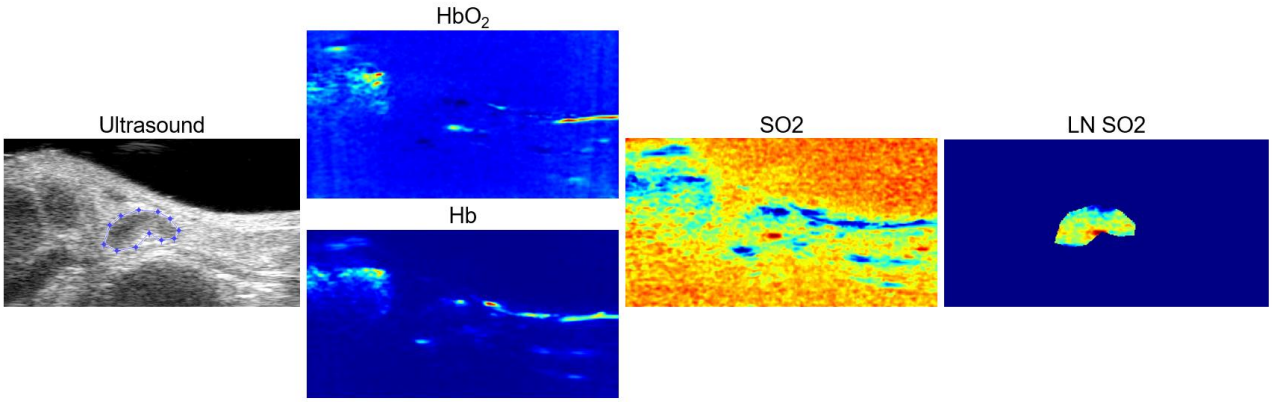

Figure 1: Overview of spectral unmixing workflow

Discussion of Stated Goals Not Met

- All stated goals met.

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

Major Activities

- Large scale *in vivo* imaging of 15 C57BL/6 mice with B16F10 Red-Fluc melanoma cancer, imaged with US/PA and BLI (bioluminescence imaging) over 25 days.
- SO2 analysis conducted on all animals and compared with BLI analysis and lymph node volume measurements from 3D US imaging.

Specific Objectives

- Execute large animal study and collect all SO2, BLI, and US data.
- Apply the developed image processing protocol to all collected data.
- Compile all processed data and correlate SO2 with metastatic status metrics to build a map.

Significant Results

- Bioluminescence imaging with IVIS shows consistently increasing tumor burden and metastatic flux (Figure 2, Figure 3).
- The volume of the sentinel lymph node was found to consistently increase over time which is another indicator of metastatic status (Figure 4).
- Background-subtracted SO2 increased over time, showing a correlation with the increasing metastatic indicators of bioluminescent flux and nodal volume.

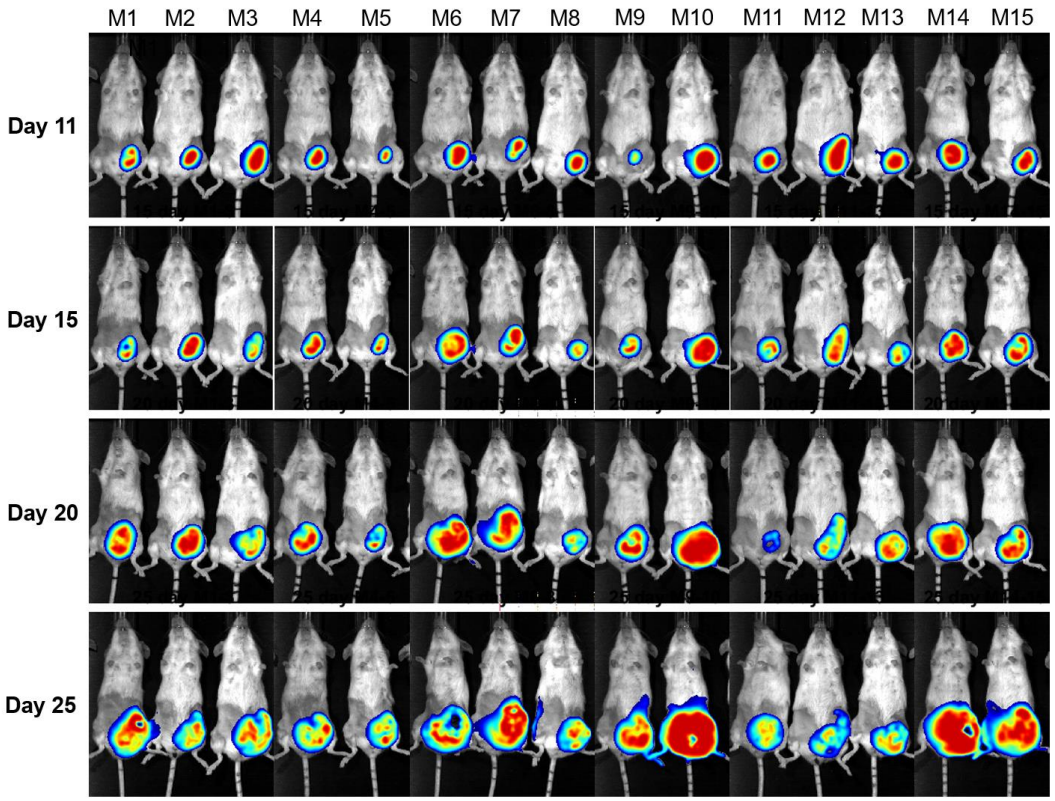

Figure 2: Bioluminescence imaging of all animals over time

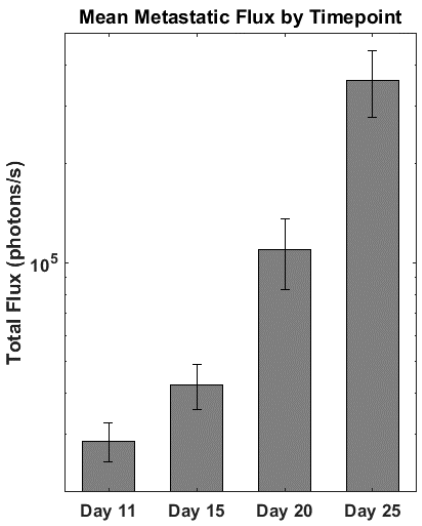

Figure 3: Mean metastatic flux measured by bioluminescence imaging

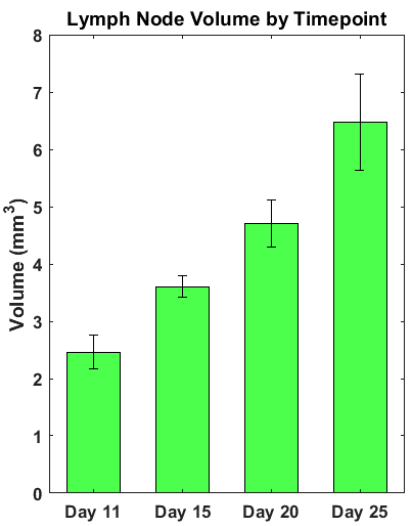

Figure 4: Sentinel lymph node volume measured by 3D US imaging

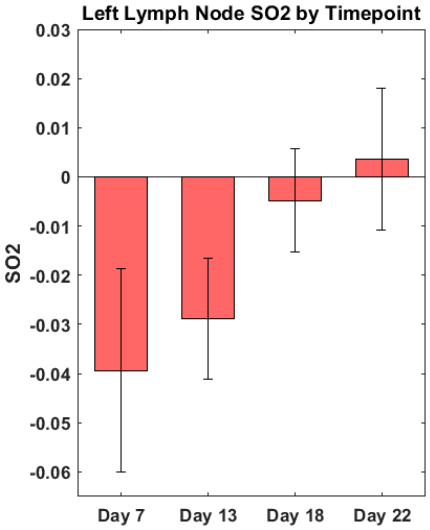

Figure 5: Sentinel lymph node background subtracted SO2 measured by spectroscopic PA imaging

- Scatterplots of average background subtracted SO2 versus lymph node volume and metastatic flux shows that they are positively correlated, with a wider spread of SO2 values when lymph nodes are non-metastatic / small (healthy).

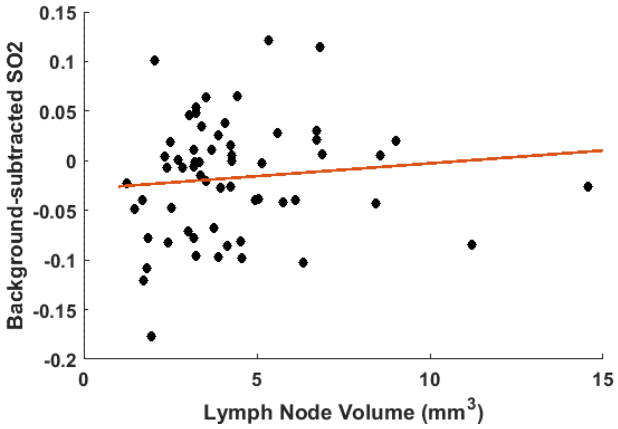

Figure 6: Scatterplot of average SO2 versus lymph node volume across all animals and timepoints.

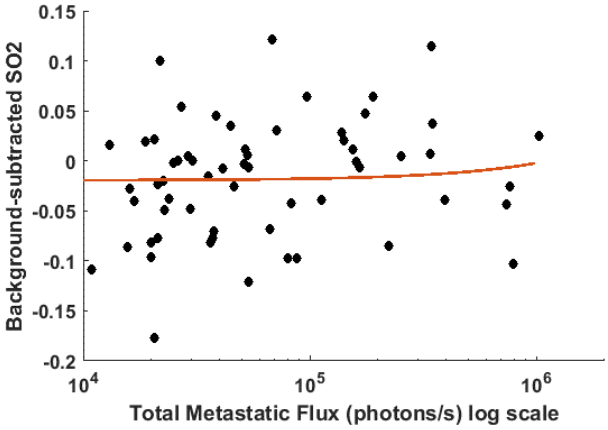

Figure 7: Scatterplot of average SO2 versus metastatic flux across all animals and timepoints.

Discussion of Stated Goals Not Met

- Animal number was reduced to 15 as melanoma uptake was 100% not the assumed 50%. Additionally, both the sentinel and contralateral lymph nodes were imaged doubling the number of lymph node samples.
- Imaging was conducted over 25 days with each imaging session alternating between US/PA and Bioluminescence imaging in order to meet animal anesthesia regulations, instead of the stated 5 weeks every 72 hours.
- Histology was not relied upon for measuring metastatic status as it cannot be used longitudinally. Lymph nodes were excised for histology at the conclusion of the study on day 25 to be correlated with bioluminescence imaging and lymph node volumes.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This project extends upon previous exploratory studies using photoacoustic imaging for SO2 estimation in oral cancer lymph nodes by expanding the work to melanoma cancer while adding a significantly larger animal sample. The use of a highly metastatic cell line in combination with the larger animal sample allows for a wide sampling of lymph nodes in various stages of metastasis, allowing us to go beyond basic detection of metastatic presence and build a correlation of SO2 to metastatic status. Additionally, the use of bioluminescent melanoma cells enables longitudinal monitoring of metastatic status using bioluminescent imaging and SO2 without needing to extract lymph nodes for histology. Together, these results have reinforced the feasibility of previous approaches in a new cancer model while introducing additional metrics with which we can correlate SO2 to metastatic status longitudinally.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee

(or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Submitted: S.T. Martirosyan, X. Huang, D. Qin, A. Yu, S. Emelianov, "Optical Inversion and Spectral Unmixing of Spectroscopic Photoacoustic Images," IEEE Transactions on Medical Imaging (2023)
  Acknowledgement of federal support : Yes

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*

- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

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:	35 months
Researcher Identifier (e.g. ORCID ID):	0000-0002-4830-6346
Contribution to Project:	Mr. Yu has continued developing the imaging system and image processing protocols and has further conducted all 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*