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TITLE: Development of Less Toxic Treatment Strategies for Metastatic and Drug-Resistant Breast Cancer Using Noninvasive Optical Monitoring

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CONTRACTING ORGANIZATION:
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14. ABSTRACT

This project involves the development of optically guided multi-agent therapies for metastatic and drug resistant breast cancer. Non-invasive Diffuse Optical Imaging technologies are able to monitor drug response and resistance through quantitative tracking of tumor metabolism and vascular supply, using clinic friendly and portable devices that can monitor deep lesions (multiple centimeters below the skin) in both breast tissue and at the site of bone metastases. During year 1 of the project we have received institutional IACUC approval as well as ACURO approval. We have successfully set up a new long wavelength intravital multiphoton microscopy system that will be important for future work on the project. We have conducted initial *in vivo* and *ex vivo* mouse imaging experiments with the system. We have also fabricated a new long wavelength Spatial Frequency Domain Imaging (SFDI) small animal imaging system and demonstrated imagining capability up to 1300 nm for the first time, which will allow for deep tissue extractions of hemoglobin, lipid, and collagen concentrations.

15. SUBJECT TERMS

metastases, drug resistance, imaging, optics, diffuse optical imaging, spatial frequency domain imaging, intravital microscopy, therapy monitoring

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Table of Contents

Page

1. **INTRODUCTION:** This project involves the development of optically guided multi-agent therapies for metastatic and drug resistant breast cancer. Non-invasive Diffuse Optical Imaging technologies are able to monitor drug response and resistance through quantitative tracking of tumor metabolism and vascular supply, using clinic friendly and portable devices that can monitor deep lesions (multiple centimeters below the skin) in both breast tissue and at the site of bone metastases. To demonstrate our strategy, we will show, for the first time, that non-invasive optical guidance can enhance the efficacy of combined cytotoxic and antiangiogenic therapy while simultaneously increasing the time until relapse and decreasing toxicities associated with high drug dosing. This will be accomplished without the use of exogenous contrast agents or dedicated imaging facilities. Additionally, in order to demonstrate the feasibility of translating optical signatures discovered in animal models to breast cancer patients, we will conduct a first of its kind clinical study of non-invasive optical monitoring of drug resistance in recurrent and metastatic disease.
2. **KEYWORDS:** *metastases, drug resistance, imaging, optics, diffuse optical imaging, spatial frequency domain imaging, intravital microscopy, therapy monitoring*
3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
  - For reference, the original SOW table is shown below, completed Tasks and Subtasks are shaded in dark blue, ongoing tasks are in lighter blue.

Specific Aim 1: Preclinical Investigation of Systemic Therapies with Optical Imaging	**Timeline**	**Site 1**
Major Task 1: Instrument Setup and Testing	Months	
Subtask 1: Local IACUC Approval	1-3	Dr. Roblyer
Subtask 2: Milestone Achieved: ACURO Approval	3-6	Dr. Roblyer
Milestone #1 ACURO approval obtained	6	Dr. Roblyer
Subtask 3: Custom SFDI Fabrication, order parts and construct device	1-9	Dr. Roblyer
Subtask 4: Setup Multiphoton Microscope, work with vendors to order and install/test microscope	1-9	Dr. Roblyer
Subtask 5: Perform initial SFDI and MPM animal testing with SHO mice and MDA-MB-231 cells. [12 mice x 1 groups = 12 mice]	9-12	Dr. Roblyer

Subtask 6: Evaluate growth rates and treatment response for SHO mice and MDA-MB-231 cells. [12 mice x 1 groups = 12 mice]	6-12	Dr. Roblyer
Milestone #2 SFDI and MPM ready for animal imaging	12	Dr. Roblyer
Major Task 2: Cytotoxic + Antiangiogenic Monitoring		
Subtask 7: Evaluate treatment timelines and response characteristics for SHO mice and MDA-MB-231 cells/ Monitor with SFDI. [18 mice x 3 groups = 54 mice]	12-24	Dr. Roblyer
Subtask 8: Correlate intravital MPM with SFDI [10 mice x 1 groups = 10 mice]	12-24	Dr. Roblyer
Subtask 9: Test growth rates of MMTV-PyMT mice [10 mice x 1 groups = 10 mice]	12-24	Dr. Roblyer
Milestone #3 Define optical signatures of cytotoxic and antiangionic therapy response	24	Dr. Roblyer
Milestone #4 Define quantitative correlates between MPM vascular imaging and SFDI	24	Dr. Roblyer
Major Task 3: Optically Defined Cytotoxic + Antiangiogenic Therapy		
Subtask 10: Test scheduling of cytotoxic + antiangiogenic based on optical signatures. (dependent on Major Task 2) [18 mice x 3 groups = 54 mice]	24-36	Dr. Roblyer
Subtask 11: Additional correlation of intravital MPM and SFDI in optical defined therapeutic window. [10 mice x 1 groups = 10 mice]	24-36	Dr. Roblyer
Subtask 12: Test SFDI and MPM imaging with MMTV-PyMT mice [10 mice x 1 groups = 10 mice]	24-36	Dr. Roblyer
Milestone #5 Improved survival/outcomes though optically defined therapy scheduling	36	Dr. Roblyer
Major Task 4: Early Response Monitoring		
Subtask 13: Test early (hrs) response with SFDI, MPM, IHC, determine immune modulators (dependent on Major Task 2 and 3 results) [18 mice x 3 groups = 54 mice]	36-48	Dr. Roblyer
Milestone #5 Characterize immune modulators of early optical response to systemic therapy.	48	Dr. Roblyer
Major Task 5: New Therapy Monitoring		
Subtask 14: Test optical signatures of response to her2/hormone and/or immunotherapies. (dependent on Major Task 2,3 and 4 results)	48-60	Dr. Roblyer

[18 mice x 3 groups = 54 mice]		
Milestone #6 Define optical response of new therapies.	60	Dr. Roblyer
Specific Aim 2: In-vivo Clinical Study of Progressive Resistance		
Major Task 6: dDOS fabrication		
Subtask 15: Design/Fabricate dDOS system and new custom dDOS probe	6-24	Dr. Roblyer
Milestone #7 Clinical Ready dDOS system	24	Dr. Roblyer
Major Task 7: Normal Volunteer Study		
Subtask 15: Local IRB Approval (for both normal volunteers and clinical study)	30-33	Dr. Roblyer
Subtask 16: HRPO Approval	33-36	Dr. Roblyer
Subtask 17: Measure 10 normal volunteers with appropriate updates to probe. Analyze and interpret imaging data. [10 normal volunteers]	36-48	Dr. Roblyer
Milestone #8 Normal Volunteer Study Completed and dDOS system/probe ready for clinical study.	48	Dr. Roblyer
Major Task 4:Breast Cancer Clinical Study		
Subtask 18: Measure 30 breast cancer patients. [30 breast cancer patients]	36-57	Dr. Roblyer
Subtask 19: Analyze and interpret imaging data.	36-60	Dr. Roblyer
Milestone #9 Clinical Study Completed, Results Published	60	Dr. Roblyer

- Completion dates for subtasks are listed here:
    - Subtask 1: Local IACUC Approval: 5/31/2016
    - Subtask 2: ACURO Approval: 8/17/2016
    - Subtask 3: Custom SFDI Fabrication: ongoing
    - Subtask 4: Setup Multiphoton Microscope: 8/1/2016
    - Subtask 5: Initial SFDI and MPM testing: ongoing
    - Subtask 6: Evaluate growth rates: ongoing

- **What was accomplished under these goals?**
    - **Major accomplishments and results are listed for each project goal for year 1 as stated in the subtasks in the SOW.**
    - **Subtask 1 and 2:** These subtasks were largely administrative.
    - **Subtask 3**: *Custom SFDI Fabrication, order parts and construct device*
        - **SFDI algorithm development:** Spatial Frequency Domain Imaging (SFDI) is an emerging label-free metabolic optical imaging modality that will be sued heavily

in this project for imaging small animals. Preliminary data suggested that edge artifacts (i.e. areas at the tumor boundary in a s.c. mouse tumor model) were problematic and that substantial corrections are required to salvage corrected data from these areas. We developed a new algorithm to correct SFDI images specifically for this purpose. The algorithm/method di described in details in the published paper attached in the appendix.

- **SFDI instrumentation development:** A new SFDI system has been fabricated utilizing long wavelength illumination supplied by a femtosecond tunable laser. The basic optical setup is shown in Figure 1 below.

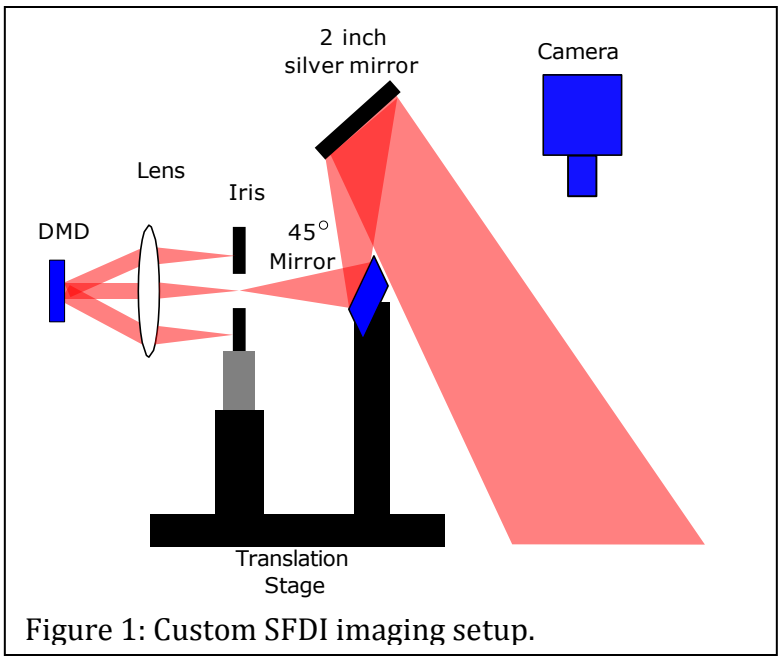


Figure 1: Custom SFDI imaging setup.

- The system is currently undergoing in-depth laboratory testing to quantify accuracy of optical property and chromophore extractions, but we have successfully shown projection of modulated signals up to 1300 nm for the first time (see Figure 2). Over the next few months we plan to develop an appropriate calibration phantom and quantify performance in solid and liquid phantoms, followed by publication of the technique.

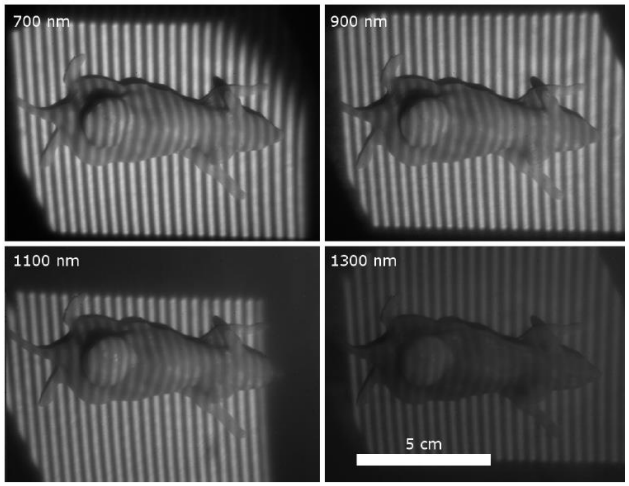
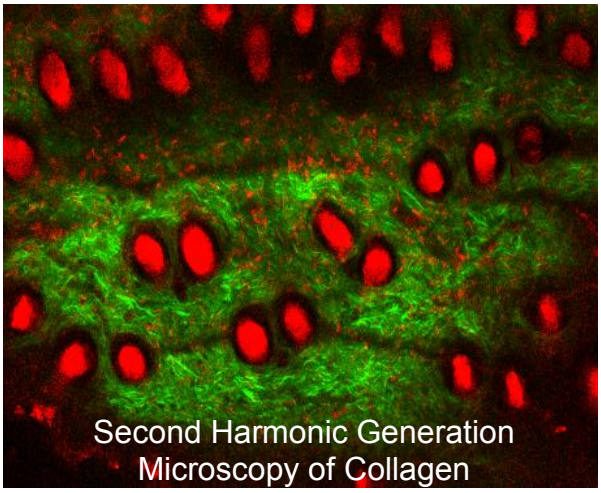


Figure 2. Spatially modulated projections to 1300 nm on a 3D printed mouse phantom.

- **Subtask 4:** *Setup Multiphoton Microscope, work with vendors to order and install/test microscope MPM microscope installed*
    - Both the new femtosecond laser and multiphoton microscope have been installed, students have undergone training, and we have constructed a custom light-tight enclosure for the system. One of the major new capabilities of this setup is enhanced tissue depth penetration and imaging due to longer wavelength illumination. We are currently conducting blood-phantom experiments to quantify tissue depth penetration of our multiphoton imaging setup.
- **Subtask 5**: *Perform initial SFDI and MPM animal testing with SHO mice and MDA-MB-231 cells*.
    - The multiphoton microscope (MPM) has been used to image four SHO mice to date. The initial mice have tumors from the PC3 cell line, although the specific tumor type is not relevant at this stage as initial feasibility testing is the goal of this subtask. Mouse imaging of the tumor through skin, after skin flap removal, and after tumor extraction have been performed to evaluate depth penetration and to determine which endogenous chromophores are measurable with the system. To date, we have identified layer skin structures by keratin and FAD autofluorescence, as well a collagen using Second Harmonic Generation (SHG) microscopy. The image below shows an image we captured of collagen fibrils (green) along with keratin in hair follicles in mouse skin. These endogenous

imaging parameters will be important as the project moves forward as we hypothesize the quantity of collagen and structural orientation will relate to therapy response and resistance during chemotherapeutic treatments, and these parameters will be correlated to SFDI parameters, included hemoglobin, lipids, and collagen bulk tissue measurements. Over the next few months we will continue to measure mouse tumors with MPM, with the goals of measuring exogenous dextran-conjugated AlexaFluor 680 dye, which will show tumor vascular patterns which can also be correlated to SFDI measurements, especially measurements of oxy and deoxyhemoglobin concentrations. .



- **Subtask 6:** *Evaluate growth rates and treatment response for SHO mice and MDA-MB-231 cells*.
    - Initial *in vitro* IC50 cytotoxicity testing has been conducted for two MMTV-PyMT breast cancer cell lines. As described in detail in section 5, we have now decided to use MMTV-PyMT derived cell lines rather than the MDA-MB-231 cell lines. Additional IC50 testing is required before attempting growth and treatment response experiments in SHO mice.
- **Additional Items not explicitly covered in Subtasks**:
    - Significant training and protocol optimization has been conducted for immunohistochemistry (IHC). These procedures will be important as the project goes forward as many imaging parameters will require validation with IHC and molecular markers tissue testing.

- Animal tail vein injection trainings have been conducted with the help of BU veterinary staff.

- **What opportunities for training and professional development has the project provided?**
  - All Ph.D. students and postdocs attend weekly lab meetings where they present their research results and have regular meeting with the Ph.D. Advisor (Roblyer)
  - All Ph.D. students and postdocs attend weekly Journal Club meetings where a different student presents a relevant paper in breast cancer and/or biomedical optics, accompanied by group discussions.
  - Several Ph.D. students attended or gave presentations at research conferences over the past year. Kavon Karrobi and Alyssa Torjesen attended the OSA Biomed Conference in April 2016, Alyssa Torjesen gave an oral presentation at the meeting. Fei Teng gave an oral presentation at the SPIE Photonics West conference in Feb 2016.
  - The PI (Roblyer), gave several invited seminars listed in section 6.
- **How were the results disseminated to communities of interest?**
  - I (Roblyer) gave a seminar talk to the Dana Farber/Harvard Caner Center Breast Cancer Patient Advocates Seminar on 3/2016.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - During the next reporting period, we plan to several goals including finalization and publication of the SFDI setup (Subtask 3), continue small animal imaging with MPM and begin initial testing of the new SFDI system with SHO mice (Subtask 5). We will also conduct additional *in vitro* cell cytotoxicity testing for the mammary cancer cell lines so that we can complete subtask 6. Completion of these tasks will allow us to begin animal testing and chemo response testing of SHO mice and initial MPM and SFDI measurements during treatment (Subtasks 7,8, and 9).

4. **IMPACT:** *Describe 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?**
  - The development of longer wavelength SFDI is likely to have a impact on the field of diffuse optical and preclinical oncology imaging, as deeper tissue penetration as well as the ability to quantify new chromophores, including lipids and collagen, may be highly relevant to chemotherapy and resistance monitoring.

- **What was the impact on other disciplines?**
  - As the project is still early, there is nothing to report at this time..
- **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**
  - This change was previously included in past quarterly reports: Additional literature review and discussions with other researchers have taken place relation to the choice of animal models. In the last progress report we had decided to alter the animal models from the MDA-MB-231 (cell line) SCID Hairless Outbred (SHO) mouse model to a syngeneic mammary mouse model (Balb/c mouse) with either 4T1 or EMT6 mouse mammary tumor cell lines. The cell lines are current being tested for growth rates and chemosensitivity using MTT assay. However, we have learned that both the 4T1 and EMT6 are fast growing and often develop scars on the skin covering the tumor, which is a major barrier for animal imaging. In addition to these two cell lines we have also now ordered two additional cell lines derived from the MMTV-PyMT spontaneous breast mouse model to determine if their growth characteristics are more favorable. Once we complete *in vitro* studies with these cell lines we will test growth rates in mice and make a determination about which mouse model(s) we will proceed with. If the MMTV-PyMT cell liens are promising, this change will effect subtasks 5, 6, 7, and 8 since the originally listed MDA-MB-231 and spontaneous MMTV-PyMT models will be replaced with the MMTV-PyMT derived cell lines. This is a change in the specific cell line, but not in the overall project goals.
  - Although not planned until year 3 of the study, based on current accrual trends with our clinical collaborators at the Boston Medical Center for different projects, the number of target subjects for the clinical study may need to modified, or an additional clinical site may need to be added to the study in order to reach the n=30 target accrual. More investigation into accrual limitations and other clinical sites will be explored over the next year to mitigate changes to the target accrual.
- **Actual or anticipated problems or delays and actions or plans to resolve them**

- There have been minor delays in several of the subtasks, but we believe this is well balanced by several unanticipated positive results and observations with probable additional publications likely to stem from the work, especially the development of the new SFDI small animal imaging studies for longer wavelengths.

- **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.**

Y Zhao, S Tabassum, S Piracha, M Sobhana Nandhu, M Viapiano, and **Darren Roblyer***, "Angle correction for small animal tumor imaging with spatial frequency domain imaging (SFDI),"* Biomedical Optics Express 7(6), 2373-2384 (2016), published, *acknowledgement of federal support (yes).*

- **Books or other non-periodical, one-time publications.** Nothing to Report.

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

The following invited seminar talks were given by the PI over the last year and data related to this project was featured:

09/2016	New England Section of the Optical Society of America, NES/OSA, Boston, MA
08/2016	University of Washington, Biomedical Optics Seminar, Seattle, WA
04/2016	Brown University, Dept. of Molecular Pharmacology, Physiology, and Biotechnology Seminar
03/2016	Dana Farber/Harvard Caner Center Patient Advocates Seminar

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

Nothing to Report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

- A new SFDI near infrared (NIR) and short wave infrared (SWIR) system was developed. This system is described in detail in section 3, under the research update for subtask 3.

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

  Nothing to Report.

- **Other Products**

  - *All relevant results and products have been described in previous sections.*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	*Darren Roblyer*
Project Role:	*PI*
Researcher Identifier (era Commons ID):	*droblyer*
Nearest person month worked:	*2*
Contribution to Project:	*Overall project management, data analysis, mentorship*
Funding Support:	DOD

Name:	*Irving Bigio*
Project Role:	*Collaborator*
Researcher Identifier (era Commons ID):	*ijbigio*
Nearest person month worked:	*1*
Contribution to Project:	*Mentor and collaborator. Provides technical support and feedback for optical instrumentation.*
Funding Support:	DOD

Name:	*David Waxman*
Project Role:	*Collaborator*
Researcher Identifier (era Commons ID):	*David_Waxman*
Nearest person month worked:	*1*
Contribution to Project:	*Mentor and collaborator for small animal studies and IHC.*
Funding Support:	DOD

Name:	*Fei Teng*
Project Role:	*Graduate Student*
Researcher Identifier (era Commons ID):	*na*
Nearest person month worked:	*8*
Contribution to Project:	*Working on clinical diffuse optical technologies*
Funding Support:	*DOD*

Name:	*Kavon Karrobi*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	na
Nearest person month worked:	10
Contribution to Project:	*Working on multiphoton intravital imaging as well as custom SFDI instrumentation.*
Funding Support:	DOD

Name:	*Alyssa Torjesen*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	na
Nearest person month worked:	*10*
Contribution to Project:	*Working on clinical diffuse optical technology development.*
Funding Support:	DOD

Name:	*Junjie Wu*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	na
Nearest person month worked:	*8*

Contribution to Project:	*Small animal chemotherapy monitoring studies, imaging studies, cell culture.*
Funding Support:	DOD

Name:	*Vivian Pera*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	na
Nearest person month worked:	*1*
Contribution to Project:	*Light propagation modeling in biological tissue.*
Funding Support:	DOD

Name:	*Asha Pant*
Project Role:	*Animal Technician*
Researcher Identifier (e.g. ORCID ID):	na
Nearest person month worked:	*5*
Contribution to Project:	*Small animal technician, animal care, cell studies, chemotherapy injections.*
Funding Support:	DOD

- **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?**
  - **Organization Name:** Modulated Imaging Inc.
  - **Location of Organization:** *Irvine, CA*
  - **Partner's contribution to the projec**t
    - **Collaboration** *Amman Mazhar and David Cuccia at Modulated Imaging Inc. provide technical support and feedback on imaging techniques throughout the project.*

8. **SPECIAL REPORTING REQUIREMENTS**
   - Not applicable
9. **APPENDICES:**

Biomedical Optics EXPRESS

Angle correction for small animal tumor imaging with spatial frequency domain imaging (SFDI)

Yanyu Zhao,[1] Syeda Tabassum,[2] Shaheer Piracha,[1] Mohan Sobhana Nandhu,[3] Mariano Viapiano,[3] and Darren Roblyer[1,*]

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[2]*Boston University, Department of Electrical & Computer Engineering, 8 Saint Mary's Street, Boston, Massachusetts 02215, USA*

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Abstract: Spatial frequency domain imaging (SFDI) is a widefield imaging technique that allows for the quantitative extraction of tissue optical properties. SFDI is currently being explored for small animal tumor imaging, but severe imaging artifacts occur for highly curved surfaces (e.g. the tumor edge). We propose a modified Lambertian angle correction, adapted from the Minnaert correction method for satellite imagery, to account for tissue surface angles up to 75°. The method was tested in a hemisphere phantom study as well as a small animal tumor model. The proposed method reduced μ_a and μ_s` extraction errors by an average of 64% and 16% respectively compared to performing no angle correction, and provided more physiologically agreeable optical property and chromophore values on tumors.

OCIS codes: (170.3880) Medical and biological imaging; (170.5280) Photon migration; (290.1990) Diffusion; (170.0110) Imaging systems.

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1. Introduction

Spatial Frequency Domain Imaging (SFDI) is a widefield imaging technique that can be used to quantify optical properties (absorption and reduced scattering) of diffusive media including biological tissue [1,2]. When optical properties at multiple wavelengths are measured, tissue chromophore concentrations can be extracted to help identify disease states, therapy response, and tissue metabolic function. SFDI is being explored for a number of preclinical and clinical applications, including skin flap viability, burn wound healing, and subsurface tomography [3–16].

Recently, our group and others have begun to investigate SFDI as a new tool to understand the *in vivo* tumor state in small animal oncology models. The application of SFDI to small animal imaging is complicated by the relatively small feature size of the tissues of interest, and the relative high surface curvature of subcutaneous tumors, which may protrude near-orthogonal to surrounding tissue for some models. Observationally, tumor edges, and other surfaces with a high surface normal angle in reference to the camera line of sight, suffer from extreme edge artifacts in SFDI, leading to physiologically implausible optical properties and chromophore concentrations in these regions. Typically, these artifacts manifest as underestimates of diffuse reflectance at low spatial frequencies. One potential method to mitigate these artifacts is to eliminate these steep surfaces from the data using a threshold method based on tissue angle. Unfortunately, this has the effect of censoring large parts of the tumor, which may be unacceptable for many applications.

Gioux *et al.* reported a Lambertian correction method for SFDI which could mitigate edge imaging artifacts for surface angles up to 40° [17]. For this method, a cosine divisor term was applied to SFDI data after image demodulation to increase diffuse reflectance values for surfaces at tilt angles. This method was shown to improve optical property extraction on tissue-simulating phantoms and human hand data, although corrections were limited to angles less than 40°. We expand on this work by applying the so-called Minnaert Correction, which was first proposed for lunar photometry and later developed to angle-correct satellite imagery from the effects of solar illumination angles and relative terrain angles [18,19]. In the context of SFDI measurements, we refer to this correction as the Modified Lambertian Correction (MLC). The MLC is a parameter optimization method that adds an additional correction factor to the Lambertian correction by empirically accounting for inter-object diffuse

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reflectance (e.g. light reflected off surrounding normal tissue onto the tumor), as well as other possible contributions to inaccurate diffuse reflectance values, especially near the tumor edge.

To validate the MLC method, SFDI measurements were taken on hemispheric tissue-simulating optical phantoms with a range of optical properties and different sizes, fabricated to mimic the geometry of subcutaneous xenografted tumors. The MLC method was compared against non-angle and the standard Lambertian correction for both lower angles (<40°) and higher angles (up to 75°). Additionally, MLC was applied to live mouse tumor data. Experimental results show that MLC yields similar improvements compared to standard Lambertian correction for low angles, and outperforms no-angle correction and standard Lambertian correction at higher angles, and MLC provides more physiologically reasonable optical property and chromophore values on live mouse tumor data, especially at the tumor edge, as will be reported here.

2. Methods

2.1. Spatial frequency domain imaging (SFDI)

The details of SFDI image acquisition and processing have been described in detail elsewhere [1,2]. Briefly, SFDI utilizes spatially modulated sinusoidal light patterns of visible or near-infrared light, projected at different spatial frequencies and wavelengths, to separate the relative contributions of absorption and scattering in diffusive media. Raw reflectance images are sequentially measured, demodulated, and calibrated to create diffuse reflectance maps, with pixel values normalized between 0 and 1. Diffuse reflectance (R_d) maps are created for each wavelength and each spatial frequency. The spatial frequency dependence of R_d at each pixel (i.e. the Modulation Transfer Function) then serves as the input to an inverse model, in this case a Monte-Carlo based look-up table method, which provides pixel-by-pixel optical absorption and reduced scattering values [20].

Key to the extraction of normalized R_d maps is the calibration of the demodulated raw image data (M_{ac}) against a calibration phantom with known optical properties. The calibration phantom is first measured with the SFDI system, and a forward model is used to determine the expected R_d values based on prior optical property knowledge. A second phantom or tissue-of-interest (with unknown optical properties), is then imaged using the same spatial frequencies and wavelengths, and calibrated R_d maps are produced using Eq. (1), which removes the instrument response.

$$R_{d,tis}(f_x) = \frac{M_{ac,tis}(f_x)}{M_{ac,ref}(f_x)} R_{d,ref}(f_x). \quad (1)$$

R_d and M_{ac} refer to diffuse reflectance and demodulated maps, respectively, and subscripts *tis* and *ref* refer to the tissue and calibration phantom, respectively [2].

2.2. Height correction

In order to account for reflectance intensity perturbations caused by height variation, a previously described height correction method was used [17]. Briefly, a calibration phantom is measured at multiple heights and the demodulated data (M_{ac}) at each height is extracted. Then, a height map of the object or tissue of interest is acquired using optical profilometry. A new $M_{ac,ref}$ map is then calculated by adjusting the M_{ac} values, pixel by pixel, according to the height versus M_{ac} relationship determined from the multi-height calibration measurements. This M_{ac} data is used to replace the calibration $M_{ac,ref}$ term in Eq. (1). The effect of height correction is to create a virtual calibration phantom such that it has the same pixel-wise height as the object.

2.3. Modified Lambertian correction (MLC)

In prior work by Gioux *et. al.* [17], a Lambertian angle correction was applied as a cosine term to the demodulated raw data of the tissue-of-interest, as shown in Eq. (2).

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

$$M_{AC,corrected} = M_{AC,uncorrected} \times \frac{1}{\cos(\theta)}. \quad (2)$$

The angle, θ, refers to the tilt angle of a flat phantom, or more generally, the angle of the tissue/phantom surface normal relative to the camera axis as shown in Fig. 1. θ is determined for each pixel in the image using an optical profilometry methodology previously described [17]. The Lambertian correction increases the demodulated image intensity for surfaces at higher surface normal angles.

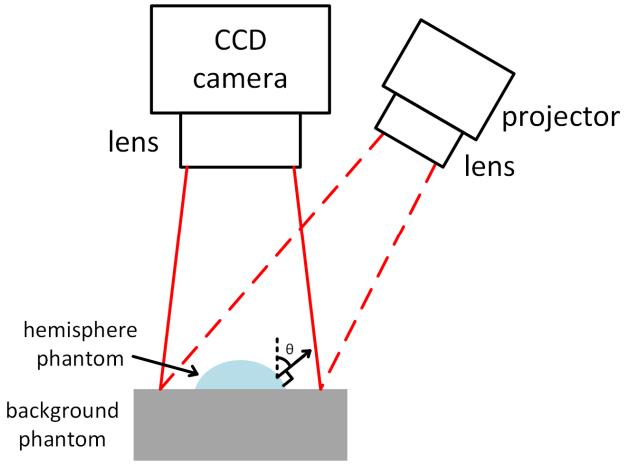

Fig. 1. Diagram of angle θ in SFDI imaging geometry.

The proposed MLC method adds a coefficient k to the cosine term, as shown in Eq. (3). This coefficient accounts for the object-to-object diffuse reflectance (i.e. reflectance from an object's background onto the object), and potentially other phenomenon not accounted for by the standard Lambertian method. When k is equal to 0, no angle correction occurs, and when k is 1, the MLC is equivalent to the standard Lambertian correction.

$$M_{AC,corrected} = M_{AC,uncorrected} \times \frac{1}{\cos(\theta)^k}. \quad (3)$$

In order to find an appropriate coefficient k for each M_{ac} map of an object-of-interest, we propose a parameter optimization method (Eq. (4). Note that for SFDI, there is a demodulated M_{ac} map for each spatial frequency and wavelength, and the coefficient k is different for each demodulated map.

$$k = \arg\min_{k\in[0,1]} \sum_{i=1}^{n} \left(M_{ac}(f_x)_i \frac{1}{\cos(\theta_i)^k} - M_{ac,ref} \right)^2 \quad (4)$$

In Eq. (4), f_x, θ, i, and n refer to spatial frequency, surface angle at the pixel location, pixel index, and total number of pixels in the optimization region of interest (ROI), respectively. The ROI (i.e. the tumor) is manually selected on the uncorrected M_{ac} map. $M_{ac,\ ref}$ is the average M_{ac} value of the low-angle areas with ≤10° thresholding within the ROI. It is used as a "gold standard" M_{ac} for the minimization. In a practical sense, the low-angle area is a region of the ROI where surface angle effects are minimal. The parameter optimization will find the k value that minimizes the difference between MLC-corrected M_{ac} and $M_{ac,\ ref}$ for the ROI. The optimization is solved using Newton's method [21]. The determined k value is then applied to all pixels on the object to get the corrected M_{ac} map. A different k value is determined for each spatial frequency and wavelength. The corrected M_{ac} maps are used to

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

calculate diffuse reflectance maps (Eq. (1), from which optical properties and chromophores are determined using Monte-Carlo look-up table method and linear fitting, respectively.

2.4. Experimental validation

The OxImager RS SFDI system (Modulated Imaging Inc., Irvine, CA) was used for all optical measurements in this study. This system provides LED illumination at up to 11 wavelengths spanning the visible to NIR and images with a 15 cm × 20 cm field of view. Height correction is applied in data processing [17]. SFDI measurements were taken at 526 nm and 659 nm for phantom studies, and a series of spatial frequencies were used: 0, 0.05, 0.1, 0.15, 0.2, 0.3, and 0.5 mm^{-1}. The integration time of each image is adjusted from tens to hundreds of milliseconds to utilize the dynamic range of the camera.

Non-angle corrected, standard Lambertian, and MLC angle corrected SFDI measurements were compared using a set of hemisphere tissue-simulating optical phantoms. The hemispheres were fabricated using silicone as base solvent, Nigrosin as absorber, and titanium dioxide (TiO_2) as scatterer. Hemispheres were made with diameters of 1 cm, 2 cm, or 3 cm. These diameters were chosen to mimic the expected range of preclinical xenograft tumors. The maximum surface normal angle of the hemispheres was 75°. Hemispheres were made with a range of optical properties; each phantom was homogenous. The optical properties of the phantoms were adjusted by varying the amount of absorbers and scatterers.

First, the effect of different k coefficients on demodulated M_{ac} values for measurements on the hemispheres were compared over a line profile taken through the center of the hemisphere. Then, the relationship between spatial frequency and optimized k values was explored for hemispheres of different sizes, optical properties, and for different background phantoms. Then, the root-mean-square-errors (RMSE) of extracted μ_a and $\mu_s`$ were compared for non-angle, standard Lambertian, and MLC correction methods for all hemispheres. Errors were compared over a small angle range (<40°), which matches the reported range for the standard Lambertian correction [17], as well as for the full angle range (up to 75°). For RMSE calculations, calculations were done using all pixels within the angle range being analyzed.

The angle correction methods were also compared on a mouse tumor model. A malignant glioma cell line (GBM34-Lum) were injected subcutaneously on the flank of a nude mouse. The tumor was treated with combination of temozolomide and the anti-angiogenic bevacizumab. The mouse was measured with SFDI under isofluorane anesthesia, 4 days after the end of a treatment session, with a tumor size of 11.6 mm × 10.5 mm. The mouse was measured at 659 nm, 691 nm, 731 nm, and 851 nm illumination and tumor optical absorption and reduced scattering was extracted at these wavelengths. Tissue-level chromophore concentrations, including oxy and deoxyhemoglobin, were calculated using the extracted optical absorption by linear fitting. All animal procedures were approved by the Brigham and Women's Hospital Animal Care and Use Committee.

3. Results

3.1. Optical properties of fabricated hemisphere phantoms

Figure 2 shows white light images and optical properties (at 526nm) of the 2 cm diameter hemisphere phantoms. Their optical properties at 526 nm and 659 nm are shown in Table 1. The 1 cm and 3 cm diameter hemispheres were made from the same phantom batch and had closely matching optical properties.

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

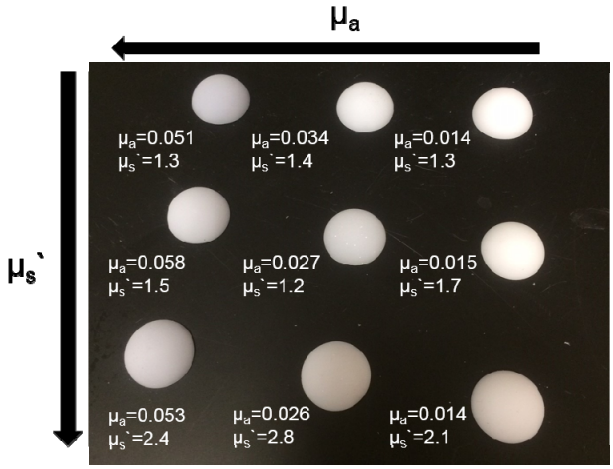

Fig. 2. White light image of the 2 cm diameter hemisphere phantoms. Optical properties are shown for 526 nm.

Table 1. Optical properties of the 2 cm diameter hemisphere phantoms. 1 cm and 3 cm hemispheres were fabricated from the same batch and had closely matching optical properties.

λ	Hemisphere	#1	#2	#3	#4	#5	#6	#7	#8	#9
526 nm	μ_a (mm^{-1})	0.014	0.015	0.014	0.027	0.034	0.026	0.051	0.058	0.053
	μ_s` (mm^{-1})	1.3	1.7	2.1	1.2	1.4	2.8	1.3	1.5	2.4
659 nm	μ_a (mm^{-1})	0.019	0.020	0.019	0.037	0.032	0.036	0.066	0.069	0.068
	μ_s` (mm^{-1})	0.90	1.4	1.6	0.88	1.0	2.2	0.97	1.3	1.9

3.2. M_{ac} line profiles for varied k coefficients

The effect on angle corrected demodulated image data with different MLC *k* coefficients was first explored on hemispheric phantoms. Figure 3 shows the line profiles of demodulated image data through the center of a 2 cm diameter optically diffusive homogeneous hemisphere phantom measured at 526 nm with optical properties of μ_a = 0.014 mm^{-1} and μ_s` = 1.3 $mm^{-1}$. The hemisphere was imaged on a flat background phantom with optical properties of $\mu_a$ = 0.053 $mm^{-1}$ and $\mu_s$` = 1.2 mm^{-1}. The left subfigure corresponds to SFDI data collected at 0 mm^{-1} spatial frequency (i.e. DC), whereas the right subfigure corresponds to 0.15 mm^{-1}. The dashed black line in the figure represents the expected M_{ac} value (i.e. the "gold standard" value) determined by the average M_{ac} of the low-angle areas with ≤10° thresholding. The colored lines correspond to angle-corrected data with different *k* coefficient values ranging from 0 to 1.

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

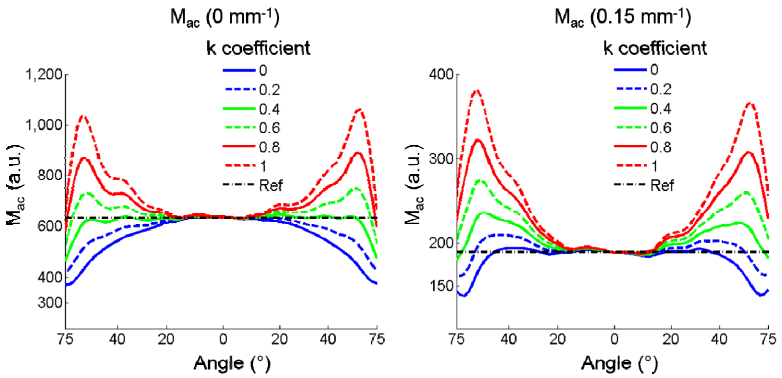

Fig. 3. Angle-corrected M_{ac} versus coefficient k.

The solid blue line represents the demodulated intensity without any angle correction (k = 0). For the 0 mm^{-1} spatial frequency (Fig. 3. Left), M_{ac} values are increasingly under-reported as the hemisphere surface normal angle increases from 0° (the center of the hemisphere) to 75° (the distal edge of the hemisphere). The red dashed line shows Lambertian correction (k = 1), which substantially over-corrects the demodulated intensity. For the higher spatial frequency, 0.15 mm^{-1} (Fig. 3. Right), non-angle corrected (k = 0) provides a better match to the expected M_{ac} than Lambertian correction (k = 1) or other non-zero k values. In general, Fig. 3 visually supports the idea that some ideal k coefficient exists, which is spatial frequency dependent, which can minimize angle artifacts for homogenous hemispheric phantoms.

3.3. k coefficient determined by parameter optimization

The effect of spatial frequency and object (hemisphere) and background optical properties on the choice of k coefficient was explored. Figure 4 shows the MLC coefficient k for a range of spatial frequencies determined by the parameter optimization. For the left subfigure, a 2 cm diameter hemisphere phantom (μ_a = 0.068 mm^{-1}, μ_s` = 1.9 mm^{-1}) was placed on five background phantoms with different optical properties. Each combination was measured with SFDI at 659 nm. Each line in the left subfigure represents a distinct background optical phantom whose optical properties are listed in the legend. The solid blackline represents a black background phantom, which has strong absorption and little scattering. The left subfigure shows that the coefficient k approaches 0 at higher spatial frequencies regardless of the optical properties of the background phantom. With a black background, k approaches 1 (Lambertian correction) for low spatial frequencies. For the right subfigure, six 2 cm diameter hemisphere phantoms with different optical properties were measured on the same background phantom (μ_a = 0.053 mm^{-1}, μ_s` = 1.2 mm^{-1}). Figure 4 shows that higher overall hemisphere optical attenuation requires higher k values and that k values are dependent on object optical properties and imaging spatial frequency.

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

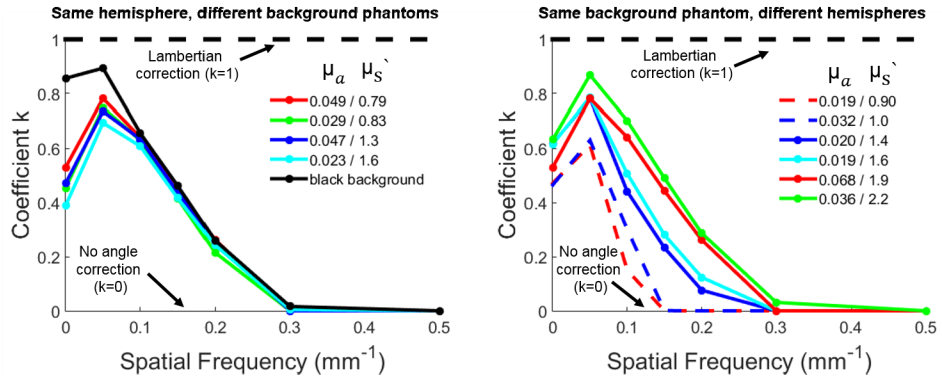

Fig. 4. Coefficient *k* versus spatial frequency.

3.4. Comparison of non-angle, standard Lambertian, and MLC on hemisphere phantoms

Comparisons were made between errors in optical property extractions for non-angle, standard Lambertian, and the MLC method. Figure 5 shows the line profiles of the absorption and reduced scattering through the center of a 2 cm diameter hemisphere placed on a background phantom (μ_a = 0.053 mm^{-1}, μ_s` = 1.2 mm$^{-1}$) measured at 526 nm. The dashed black line represents the expected $\mu_a$ / $\mu_s$` values ("gold standard"). The dashed blue line, dashed green line, and solid red line correspond to non-angle, standard Lambertian, and MLC corrected μ_a / μ_s` data, respectively. For absorption, non-angle correction leads to overestimation of μ_a, while the Lambertian correction underestimates μ_a, causing it to be near 0 at the edges of the hemisphere (high angle areas). The MLC corrected μ_a is very close to the gold standard, outperforming the other two methods. For the reduced scattering, the non-angle correction and MLC are nearly identical, and both outperform standard Lambertian.

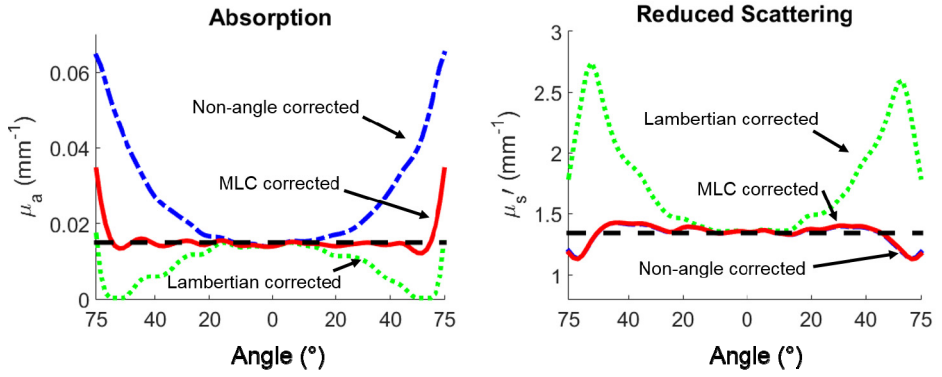

Fig. 5. Line profiles of absorption and reduced scattering through the center of a 2 cm diameter hemisphere using different angle correction methods.

Figure 6 shows 3D absorption and reduced scattering maps rendered for the same hemisphere and background phantom. The black arrows indicate the "gold standard" μ_a / μ_s` values. The 3D visualizations and histograms demonstrate that non-angle corrected data skews towards $\mu_a$ values higher than the known value at high angles, and the standard Lambertian correction skews towards lower $\mu_a$ values at high angles. Lambertian correction also overestimates $\mu_s$` at high angles, whereas MLC gives μ_a and μ_s` values that are close to the "gold standard". It is of note that standard Lambertian correction produces a substantial number of pixels with $\mu_a$ values close to 0 mm$^{-1}$, and $\mu_s$` values close to 2 mm^{-1}, the upper limit of displayed values. In contrast, the MLC data have a tighter distribution around the gold standard values, with modest μ_a underestimates at the extreme hemisphere edge.

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

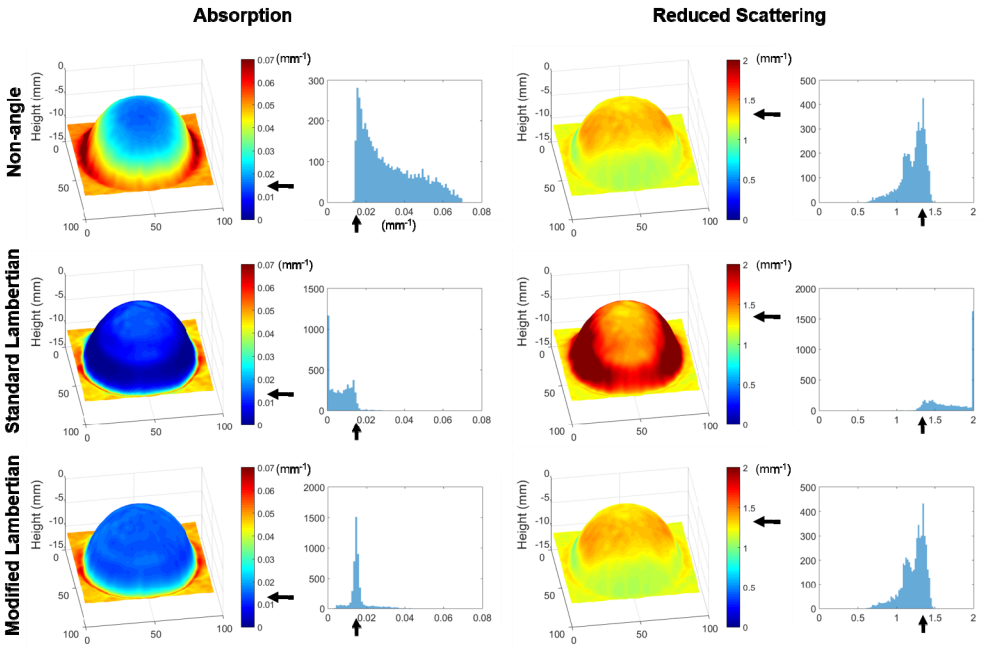

Fig. 6. Absorption and reduced scattering plots rendered with 3D heights, and histograms of the hemisphere region. Optical properties are shown for a 2 cm diameter hemisphere at 526 nm.

To quantitatively compare non-angle, standard Lambertian, and MLC correction methods, groups of hemisphere phantoms with different sizes and optical properties were measured at 526 nm and 659 nm. Their μ_a and $\mu_s`$ values were extracted, the three correction methods were applied, and the root-mean-square-errors (RMSE) were calculated for both low angles (<40°), and higher angles (up to 75°). Representative RMSE values for the larger angle range (up to 75°) are shown in Fig. 7. Values are plotted as bar plots representing the error between the known optical properties and the corrected optical properties over the entire hemisphere. This group has nine middle size hemisphere phantoms (2 cm diameter), measured at 659 nm. The average μ_a RMSE of non-angle, standard Lambertian, and MLC of this group was 0.026, 0.017, and 0.008 mm^{-1}, respectively. For $\mu_s`$ the average RMSE for the three methods was 0.295, 0.387, and 0.256 $mm^{-1}$, respectively. The MLC method reduces the $\mu_a$ RMSE by 68.9% and 52.4% respectively over non-angle and standard Lambertian correction, and reduces the $\mu_s`$ RMSE by 13.2% and 33.9% respectively.

Comparing the three methods for angles less than 40° across all hemispheres (n = 27) and background phantoms (n = 5), the overall average μ_a RMSE of non-angle, standard Lambertian, and MLC was 0.0094, 0.0063, and 0.0052 mm^{-1}, respectively. For $\mu_s`$ the overall average RMSE for the three methods for angles less than 40° was 0.16, 0.20, and 0.15 mm^{-1}, respectively. These results demonstrate improved but comparable error levels for the standard Lambertian and MLC methods for smaller angles.

The overall average μ_a RMSE for angles up to 75° across all hemispheres was 0.029, 0.019, and 0.010 mm^{-1}, respectively. For $\mu_s`$ the overall average RMSE for the three methods was 0.250, 0.454, and 0.209 $mm^{-1}$, respectively. On average, the MLC method reduces the $\mu_a$ RMSE by 63.7% and 49.9% respectively over non-angle and standard Lambertian correction, and reduces the $\mu_s`$ RMSE by 15.9% and 51.9% respectively. In general, data from all measured hemispheres revealed that the MLC method greatly improved μ_a extractions over the other two methods for larger angles. For $\mu_s`$, MLC did little to improve non-angle correction, but as expected, outperformed standard Lambertian correction which is not valid at angles higher than 40°.

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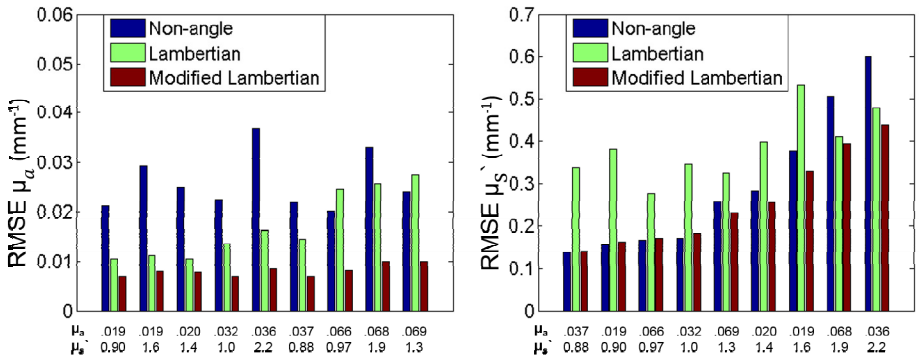

Fig. 7. RMSE bar plots of 2 cm diameter hemisphere phantoms, measured at 659 nm.

3.5. Comparison of non-angle, standard Lambertian, and MLC on live mouse tumors

Figure 8 compares non-angle, standard Lambertian, and MLC on a live mouse tumor model. The μ_a and total hemoglobin concentration were extracted using the three methods respectively. The extracted tumor data distributions are also presented in the histograms. For non-angle correction, the tumor absorption values skew higher on the edges (high angles), with corresponding higher estimated total hemoglobin concentrations. For standard Lambertian correction, a perimeter at the base of the tumor has 0 mm^{-1} μ_a values, with corresponding 0 μM total hemoglobin values at these areas. Although "gold standard" values are not available for the mouse tumor, the extracted data distributions are tighter with the MLC method, and μ_a and total hemoglobin values are physiologically plausible throughout the tumor ROI.

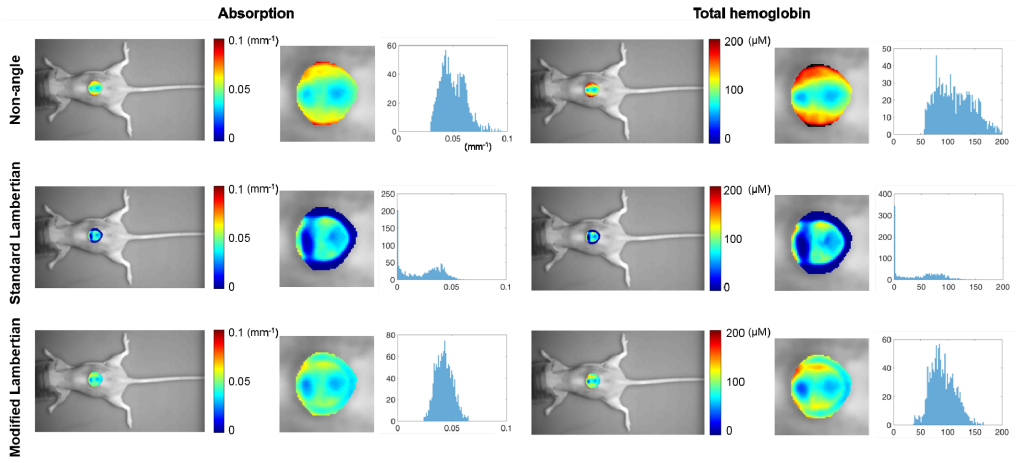

Fig. 8. Comparison of three methods on mouse tumor data and their distributions shown in histograms, measured at 659nm, 691 nm, 731 nm, and 851 nm. The plotted absorption data was measured at 659 nm. Optical properties are only shown for the tumor ROI. Zoomed-in figures are shown for the tumor optical absorption and total hemoglobin.

4. Discussion and conclusion

MLC correction for SFDI reduced μ_a extraction errors over a range of phantom hemisphere dimensions and optical properties by an average of 63.7% compared to performing no angle correction. Additionally, in contrast to non-angle correction and standard Lambertian correction, MLC produced optical property and chromophore extractions that better match

#260851 Received 9 Mar 2016; revised 12 May 2016; accepted 16 May 2016; published 24 May 2016

physiologically reasonable values on a xenograft mouse tumor model, especially at the tumor edge. The empirical approach taken here to determine k requires measurement of surface angle, but does not require explicit knowledge, measurement, or input of background optical properties or instrument geometry, allowing it to be practically implemented in tumor monitoring studies regardless of specific instrument used and with little additional analysis effort.

The experiments performed in this study revealed that angle related effects in SFDI are dependent on the optical properties of the object of interest and background, the spatial frequency, the wavelength, and the imaging surface normal angle. For example, it was demonstrated that lower k values are required at high spatial frequencies, and almost no angle correction ($k = 0$) was required for spatial frequencies of 0.3 mm^{-1} and above. This suggests that data collected at higher spatial frequencies are less affected by both Lambertian effects and inter-object reflections. Since higher spatial frequencies are preferentially sensitive to tissue optical scattering [2,22], $\mu_s`$ data is preserved even without angle correction (i.e. $k = 0$), which was demonstrated by the relatively low RMSE values observed in scattering data when no angle correction was used. At DC, greater correction was required and k approached 1 (i.e. Lambertian correction) when the diffusive phantom was imaged on a dark, highly absorbing background. When hemisphere phantoms were imaged on a diffuse background, intermediate correction (i.e. $0 < k < 1$) was required as the standard Lambertian correction significantly overcorrected R_d values for surfaces at higher angles. This is likely due to the inter-object reflections occurring between the background phantom and the hemisphere, which increase measured light intensity at the hemisphere edge. These angle dependent inter-object reflections have previously been described in the context of correcting satellite imagery [19]. At low AC spatial frequencies (e.g. 0.05 mm^{-1}), higher k-values were needed, suggesting that Lambertian effects dominate. Although not explicitly explored in this work, effects related to the increase in projected imaging spatial frequency on the highly curved surfaces likely contribute to edge effects.

There are several limitations of the MLC correction method related to the assumptions required for its implementation. For example, the MLC method assumes that the M_{ac} value of low-angle areas are representative of the entire region or object-of-interest, and large heterogeneities are likely to introduce errors. Specifically, under scenarios where low angle zones are not available or there are large inherent heterogeneities, the optimization could be invalid. Despite this limitation, MLC provided better optical property and chromophore extractions on actual tumors compared to non-angle or standard Lambertian corrections at the tumor edge. Another limitation is that the hemisphere phantoms tested here were fabricated with a maximum 75° surface normal relative the vertical since the angle dependence relationship of R_d above 75° deviated from the relationship observed below 75°, possible because of noise in the optical profilometry data or due to more complex interobject effects. It is of note that the mouse tumors imaged for this study had almost no pixels >75°, suggesting this may not be a limiting factor for small animal tumor imaging. Finally, MLC was only tested for a limited set of geometric shapes, and the method was tested only for corrections on a limited region-of-interest within the field-of-view (i.e. the tumor).

In the future, MLC will be tested for its ability to correct optical property extractions from more complex object geometries with spatially varying optical properties (i.e. an entire mouse). This is likely to require spatially varying k values, although the general trends observed in this study relating k to object and background optically properties and spatial frequency may allow for simpler implementations under constrained conditions, such as a limited range of optical properties throughout the field-of-view and known background optical properties.

The MLC method described here is likely to be useful for small animal tumor imaging as it provides a relatively simple method to recover high-angle data that would otherwise have to be censored from the data set. The application of MLC to SFDI for longitudinal drug response studies in subcutaneous mouse tumor models may allow for the identification of prognostic

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optical biomarkers of therapy response and resistance that can then be translated to *in vivo* human imaging using SFDI and other diffuse optical imaging technologies.

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