Determination of tissue-level changes in tumors that are indicative of metastasis using optical spectroscopy

Sanidhya D. Tripathi
University of Arkansas, Fayetteville

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DETERMINATION OF TISSUE-LEVEL CHANGES IN TUMORS THAT ARE INDICATIVE OF METASTASIS USING OPTICAL SPECTROSCOPY

An Honors Thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biomedical Engineering

by

Sanidhya D. Tripathi

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Department of Biomedical Engineering
College of Engineering
University of Arkansas

Note: Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the funding agency.
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Abstract

Breast cancer, accounting for 12% of all new cancer cases, is one of the leading causes of death in women worldwide. Although patient survival has improved over the years, metastatic spread to other organ sites and not due to the primary tumor is the most common form of tumor recurrence, accounting for 90% deaths. Hypoxia is a common hallmark of solid tumors and is linked with metastasis, therapeutic resistance, and poor patient survival. Defined as a state of decreased oxygen availability, cells under hypoxia have an increased rate of genetic mutation, local invasion, and resistance to treatment such as radiation and chemotherapy. Previous studies have explored the connection between breast cancer receptor status to the eventual metastatic nature of a tumor; however, the functional characteristics of these tumors remain in question. In this study, diffuse reflectance spectroscopy (DRS) was used to evaluate functional changes in tumor xenografts originated from two breast cancer cell lines with variable metastatic potential. The murine mammary cell lines 4T1 and 67NR were injected into the right flanks of mice to grow tumor xenografts. Optical data from these tumor bearing animals was collected and using an empirical model, vascular oxygen saturation ($S_O^2$) and total hemoglobin concentration (cHb) was determined. Results indicated constant hemoglobin content in both cell lines across all time points of measurements and upward of vascular oxygen saturation in both metastatic and nonmetastatic tumors. Overall, this pilot study could aid in the understanding of the role that hypoxia has on breast tumors at a fundamental level and open the door for future studies in answering questions pertaining to specific pathways/tumor microenvironment that occur in response to such conditions both in vitro and in vivo.

**Keywords:** Diffuse reflectance spectroscopy, Hypoxia, Tumor microenvironment, Breast cancer, Metastasis
Introduction

Breast cancer, accounting for 12% of all new cancer cases, is one of the leading causes of death in women worldwide [1]. Characterized by a lack of progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor (HER2) expression, breast cancer is associated with an aggressive phenotype and poor prognosis. Although patient survival has improved over the years, metastatic spread to other organ sites and not due to the primary tumor is the most common form of tumor recurrence, accounting for 90% deaths [2]. Patients have shown to benefit to some degree from neoadjuvant chemotherapy, however they still face high recurrence and death rates compared to other cancer types especially within the first 2 years of diagnosis. Furthermore, breast tumors often develop resistance to chemotherapeutic drugs.

Clinically, physicians rely on anatomical markers, such as tumor stage and lymph node status for medical decisions related to neoadjuvant chemotherapy, and current methods to assess chemotherapy benefits. A 21-gene assay, known as Oncotype DX, is a popular molecular profiling test given to patients to determine the risk of recurrence after treatment [3]. Although this test can provide an individual risk assessment for patients who suffer from breast cancer, the Oncotype DX is highly expensive and not widely available except for those at high-volume clinical facilities. Furthermore, its application is limited by tumor size and established tumor receptor as it only considers tissue taken during initial biopsy or surgery. Due to the flaws that are presented by tests such as Oncotype DX, there remains an unmet clinical need to differentiate between indolent and aggressive disease. As a result, the development of a cost-effective prognostic tool that can be implemented to evaluate metastatic potential of breast tumors could significantly aid to the treatment of patients.
Regarding determining tumor fate, current understanding among physicians, researchers, and other professionals is quite limited. There are multiple factors contributed to the current state of breast cancer research including the inability to accurately predict which tumors are prone to metastatic progression as well as an incomplete knowledge of metabolic/molecular/physiological adaptations for cancer cells to survive. As a result, approximately 300,000 women are expected to suffer from breast cancer in 2022 and about 13% U.S. women are expected to develop invasive the disease in their lifetime [4]. Due to these alarming statistics and limited understanding of the disease, it is crucial to explore the vulnerabilities of breast cancer and discover potential targets for developing effective therapies for patients.

Among the countless causes for the onset of cancer in patients, hypoxia has been a popular discussion point in recent decades. Hypoxia is a common hallmark of solid tumors and is linked with metastasis, therapeutic resistance, and poor patient survival. Defined as a state of decreased oxygen availability, cells under hypoxia have an increased rate of genetic mutation, local invasion, and resistance to treatment such as radiation and chemotherapy [5]. The plethora of effects caused by hypoxia are highlighted in Figure 1. Previous studies have explored the connection between breast cancer receptor status to the eventual metastatic nature of a tumor; however, the functional characteristics of these tumors remain in question. Determining the functional characteristics of breast tumors in controlled studies of metastasis could help improve our understanding of specific tissue-level changes within the primary breast tumor that determine metastatic behavior as well serve as a guide to developing more personalized and effective therapies for women worldwide. To understand the features which drive cancer metastasis in a hypoxia environment, optical spectroscopy techniques are highly attractive due to their capacity to provide insights on tumor vascularity, tissue scattering, and oxygenation. Using these properties, researchers can be able to
determine if there are any microenvironmental features associated with metastasis in the primary tumor.

**Figure 1.** The spectrum of the effects of hypoxic stress on cancer cells [6].

Diffuse reflectance spectroscopy (DRS) is a non-invasive optical spectroscopy technique that uses visible and near-infrared light (NIR) to interrogate tissue. The reflected light is used to quantify tissue optical properties that provide information about tumor structure and function [7]. DRS is highly attractive to characterize the tumor microenvironment as it can be used to provide insights into tumor vasculature, tissue scattering, and oxygenation based on multiple scattering events in breast cancer tumor models. In a DRS setup, as seen in **Figure 2**, the source and detector are spatially offset for researchers to achieve greater sampling depth. Using DRS, previous studies have assessed surgical margins [8, 9], longitudinally monitored tumor response to immune checkpoint inhibitors [10], investigated radiation-induced changes in hemoglobin oxygenation saturation in tumors [11], and more however no studies have examined the changes in the tumor
microenvironment due to hypoxia and its connection to tumor metastasis. The discovery of key changes in the tumor microenvironment in breast cancer could aid in the development of viable treatments. In addition, this technique (DRS) could allow for physicians to be able to select patients who are likely to respond well prior to treatment and identify responses at an earlier time. Thus, the unnecessary treatment plans which are ultimately found to be ineffective can be mitigated.

Figure 2. Source-detector separation distance determines sampling depth.

Overall, the goal of this study was to determine the ability of probe-based optical spectroscopy (DRS) to identify functional changes in tumor xenografts that occupy the spectrum of metastatic progression. To accomplish the aims of this project, murine mammary cell lines 4T1 (metastatic) and 67NR (non-metastatic) were injected into the right flanks of mice to grow tumor xenografts. After growth, the tumor, that were derived from a single spontaneous breast tumor, were subjected to a period of hypoxia and reoxygenation. Optical data from these tumor bearing animals was collected and using an empirical model, vascular oxygen saturation ($S_\text{O}_2$) and total hemoglobin concentration (cHb) was determined to understand metabolic response of tumors to microenvironmental stress [12].
Project Aims

The goal of this study was to determine the relationship between tumor metastasis and hypoxia. Our specific objectives include:

- Generate two murine breast cancer tumor xenografts in a family of isogenic breast cancer cell lines
  - 4T1 (highly metastatic)
  - 67NR (non-metastatic)
- Determine the ability of probe-based optical spectroscopy (DRS) to identify functional changes in tumor xenografts that have varying metastatic potential

Given these objectives, it was thus hypothesized that DRS is sensitive to functional changes of murine breast tumors of varying metastatic potential that are subjected through hypoxia-reoxygenation stress.

Methods

Cell Culture & Tumor Xenografts

All studies and protocols were approved by the Institutional Animal Care & Use Committee (IACUC) of the University of Arkansas. 10 mice (5 per cell line) were housed at the South Campus Animal Facility (SCAF) and were maintained under standard 12-hour light/dark cycles with ad libitum access to water. The murine cells lines which consisted of 4T1 and 67NR were cultured in a mixture of Dulbecco’s Modified Eagle Medium (DMEM), 10% v/v fetal bovine serum (FBS), 2mM L-Glutamine, 1% v/v Penicillin–Streptomycin, 1% nonessential amino acids, and 1% v/v L-glutamine and kept in a humidified incubator that was set to 5% CO₂ at 37°C. These cell lines are chosen due to their difference in metastatic potential (highly metastatic or non-metastatic under normoxic conditions). Both cell lines were passaged until they reached 80%
confluency and were injected into the mice within the first 4 passages. Prior to injection, both the 4T1 and 67NR cells were trypsinized using 0.05% (v/v) trypsin and suspended in PBS. To form breast cancer tumor xenografts, each cell line was subcutaneously injected (200,000 cells/mL) into the right flank of the animals and allowed to reach an average tumor volume of 400 mm$^3$.

*Diffuse reflectance spectroscopy (DRS)*

The implemented DRS setup (seen in Figure 3) consisted of a tungsten halogen lamp (HL-2000, Ocean Optics; Dunedin, FL) for illumination, a fiber optic spectrometer for spectral acquisition (Flame, Ocean Optics; Dunedin, FL), and a fiber optic probe (FiberTech Optica, Ontario, Canada). The probe consisted of 4 source fibers (dia. = 200 μm, NA = 0.22) and 5 detector fibers located at 2.25 mm from the center of the source fibers. The diffusely reflected light was collected by the 5 peripheral fibers of the fiber optic probe, which surrounded 4 source central fibers. DRS spectra acquisition was performed at an average tumor volume of 400 mm$^3$ for 2 specific murine mammary cell lines, 4T1 and 67NR.

![Figure 3](image)

**Figure 3.** (A) Experimental setup of diffuse reflectance spectroscopy consisting of an optical fiber probe delivering light supplied from a halogen lamp and transferring the reflected light from tissue to a spectrometer. (B) Optical probe design with a source detector separation distance of 2.25 mm [13].
Optical spectroscopic measurements

Once tumor xenografts were formed, data acquisition using DRS occurred in 3 different phases (baseline, hypoxia, and reoxygenation). First, mice were anesthetized using isoflurane (1.5% v/v) and 100% O₂ to take baseline measurements. For hypoxic exposure, gas was switched to a hypoxic medical-grade 21% O₂ level using commercially available 21% O₂ tanks (Airgas) for a period of 30 minutes. During hypoxic exposure, measurements were performed by placing a pen-shaped probe at 10 spatially distinct locations for tumor and 5 spatially distinct locations for skin at 2 points in the 30 minute time period. Once the 30 minutes had passed, gas was switched to 100% O₂ level for a period of 30 minutes to collect reoxygenation data. These measurements were also performed by placing a pen-shaped probe at 10 spatially distinct locations for tumor and 5 spatially distinct locations for skin at 2 points in the 30 minute time period. These measurements did not exceed 5 minutes per mouse.

Quantification of tissue optical properties & statistical analysis

All spectra analysis were carried out using scripts written in MATLAB 2022a (Mathworks). Specifically, using an empirical lookup table (LUT)-based inverse model, reflectance, total hemoglobin concentration (cHb) and vascular oxygen saturation (sO₂) were determined [12]. After fitting the data and obtaining optical properties from all 10 mice using the LUT model, GraphPad Prism 9 was used to plot the relationship with error bars between the following: total hemoglobin content (cHb vs. time) and vascular oxygen saturation (sO₂) vs. time). Time referred to the various time points that spectra were acquired during the experiment (baseline, hypoxia, and reoxygenation).
Results

Representative in vivo Reflectance Spectra

Once raw spectra using DRS had been collected on all 10 mice, analysis was carried out using scripts written in MATLAB 2022a (Mathworks). Using the (LUT)-based inverse model, reflectance spectra were generated for all animals tested. These spectra consisted of data across all time points in the collection process including baseline, 2 hypoxia measurements, and 2 reoxygenation measurements. Representative DRS spectra from 2 mice (injected with either 4T1 and 67NR cell lines) are shown on Figures 4 and 5. Specifically, a spectra from each timepoint: baseline (Figures 4A and 5A), hypoxia (Figures 4B and 5B), and reoxygenation (Figures 4C and 5C) can be seen below. The solid blue line on each figure indicates the in vivo tumor measurements while and the solid red line the LUT model fit. All graphs were plotted from the 475 nm to 650 nm range.
Figure 4. (A) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of baseline measurement (100% O₂) from representative animal injected with 4T1 cells. (B) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of hypoxia measurement (21% O₂) from representative animal injected with 4T1 cells. (C) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of reoxygenation measurement (100% O₂) from representative animal injected with 4T1 cells.
**Figure 5.** (A) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of baseline measurement (100% O$_2$) from representative animal injected with 67NR cells. (B) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of hypoxia measurement (21% O$_2$) from representative animal injected with 67NR cells. (C) Diffuse reflectance spectra of tumor (blue line) and experimental-fit (red line) of reoxygenation measurement (100% O$_2$) from representative animal injected with 67NR cells.

**Spectroscopy Data Quantification**

After fitting all spectra and obtaining optical properties for each tumor, GraphPad Prism 9, a commercial scientific 2D graphing and statistics software accessible in the Laboratory for Functional Optical Imaging and Spectroscopy, was used to plot the relationship of total hemoglobin content and vascular oxygenation of murine breast tumors across the 3 prominent timepoints of the study. Relationships of these variables were plotted to examine any notable trends in metastatic (4T1) and non-metastatic tumors (67NR). Graphs with error bars were plotted between the following: total hemoglobin content (cHb vs. time) as seen in **Figure 6A** and vascular oxygen saturation (sO$_2$) vs. time) as seen in **Figure 6B**. Time referred to the various time points that spectra were acquired during the experiment (baseline, hypoxia, and reoxygenation).
Figure 6. (A) Comparison of total hemoglobin content of both metastatic (4T1) and non-metastatic (67NR) tumors after subjecting them through hypoxiareoxygenation stress. (B) Vascular oxygenation of both 4T1 and 67NR tumors continued to increase across all 3 phases (baseline, hypoxia, and reoxygenation) of measurements.

Discussion

The DRS spectra showed that both 4T1 and 67NR tumors have a less pronounced hemoglobin band, which is known as the section of the curve between 540 and 580 nm, indicating low oxygen saturation. Specifically, it was seen that in the baseline and hypoxia spectra for both 4T1 and 67NR tumors, there was a single bump/increase in the hemoglobin region (as seen in Figures 4A-B and 5A-B) while there was a double bump/increase in the reoxygenation spectra (as seen in Figures 4C and 5C). In addition, it was observed that in both 4T1 and 67NR tumors, the LUT model fit showed not as much consistency with the tumor spectra from 600 nm onwards. However, a consistent fit was seen prior to 600 nm. Regarding various timepoints from which spectra was gathered, in both 4T1 and 67NR tumors, it was observed that the DRS curve remained relatively constant/flat until 560 nm from where there was a slight increase in reflectance. This slight increase was more prominent during the reoxygenation phase of measurements rather than either baseline or hypoxia measurements.
After obtaining optical properties from mice that were injected with either 4T1 or 67NR cells, it was determined to observe the differences in hemoglobin content (cHb) and vascular oxygenation (sO₂) in tumors during the baseline, 2 hypoxia, and 2 reoxygenation measurements. These variables were specifically chosen out of all the other outputs from the LUT model due to the fact that they could possibly be the most relevant parameters to understand metabolic response of tumors to microenvironmental stress. From Figure 6A, it was seen that although 67NR tumors exhibited greater hemoglobin content consistent across all timepoints, there was not much significant difference compared to their metastatic counterpart (4T1 tumors). When examining data points in each timepoint, there is consistent trend between each measurement indicating that there is no difference in hemoglobin content from baseline measurements to the last reoxygenation time period measurement. Upon plotting the vascular oxygenation of both 4T1 and 67NR tumors, it was observed that both tumors had an increase in oxygenation despite being exposed to lower levels of oxygenation in the experiment. This was a unique trend that was observed since oxygenation levels were higher after the last reoxygenation measurement compared to the baseline measurement that occurred. The primary reason why this trend was observed was could be due to increase in deoxygenated hemoglobin intake. In addition, these results suggest, that perhaps inducing only 21% O₂ in mice was not enough to force a hypoxic state. Further testing with lower levels of oxygen would be needed to see expected changes in the data across all time points.

Conclusions & Future Directions

Breast cancer, affecting approximately 300,000 women, has become a major public health concern in recent decades. Although patient survival has improved over the years, metastatic spread to other organ sites and not due to the primary tumor is the most common form of tumor recurrence. In addition, tumor hypoxia is a common hallmark that has been linked with metastasis,
therapeutic resistance, and poor patient survival. To treat patients whose tumor has been metastasized, doctors rely on anatomical markers or highly expensive molecular profiling methods (such as Oncotype DX). However, a precise tumor fate for hypoxic tumors continues to be a challenge. In this study, the link between tumor metastasis and hypoxia was examined. From the results, it has been shown that DRS can be used to study the differences between metastatic 4T1 and non-metastatic 67NR tumors when subjected to hypoxia-reoxygenation stress. Additionally, results indicate an upward of vascular oxygen saturation in both metastatic and nonmetastatic tumors despite the fact that they were exposed to lower levels of oxygen in the study. The preliminary results of this study demonstrate the potential of using DRS in understanding the relationship between tumor metastasis and tissue-level changes in the primary tumor.

Future directions that could be pursue after the conclusion of this pilot study could be expansion into the full spectrum of metastasis (see Table 1) to examine functional changes in tumor xenografts that occupy the complete spectrum of metastatic progression. Pursuing a panel of breast cancer cell lines with varying metastatic potential could result in a more inclusive and accurate analysis of hypoxia’s role in various steps of the invasion-metastasis cascade as opposed to two opposite sides of the spectrum as was studied in this work. In addition, further testing of different gas sources (using 10% O$_2$ to induce higher levels of hypoxia) to investigate changes in tumor oxygenation can be tested. The use of 21% O$_2$ in this study and terming it “hypoxia” was a relative term compared to 10% O$_2$. The term “hypoxia” was used was due to the fact that 21% O$_2$ was a decrease from 100% O$_2$ as baseline. Finally, investigation of functional changes associated with knockout of metastasis-promoting genes (such as Twist which is the “master regulator” along with other genes in breast cancer metastasis process) could confirm the link of tumor metastatic changes to DRS observations [14]. Overall, this work could aid in the understanding of the role
that hypoxia has on breast tumors at a fundamental level and open the door for future studies in answering questions pertaining to specific pathways/tumor microenvironment that occur in response to such conditions both \textit{in vitro} and \textit{in vivo}.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Invasion-Metastatic Cascade</th>
<th>Metastatic Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>67NR</td>
<td>Primary tumor formation only</td>
<td>N/A</td>
</tr>
<tr>
<td>168FARN</td>
<td>+ Local invasion + Intravasation</td>
<td>Lymph nodes</td>
</tr>
<tr>
<td>4T07</td>
<td>+ Intravasation</td>
<td>Micrometastases in lung, lymph nodes</td>
</tr>
<tr>
<td>4T1</td>
<td>+ Metastatic growth</td>
<td>Lung, liver, bone</td>
</tr>
</tbody>
</table>

\textbf{Table 1.} Invasion – metastasis cascade for BALB/c tumor-derived breast cancer cell lines [15].

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