DYNAMIC SPATIAL AND TEMPORAL CHANGES IN TUMOR MICROVESSEL HEMOGLOBIN SATURATION MEASURED WITH HYPERSPECTRAL IMAGING

By

NIKITA AGARWAL

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2008
ACKNOWLEDGMENTS

I want to thank my parents, my sister and brother-in-law for being so supportive of my work. I want to thank my advisor, Dr. Brian Sorg, for giving me the opportunity to work on this project and for his constant support and encouragement. This has been a life-changing experience and has taught me a lot about myself. I did not know I could meet project responsibilities that ranged from surgery, long imaging hours, to programming and analysis and a lot more. On a lighter note, I also did not know that it could be fun to catch the 2:00am bus, eat at Shands 24/7, and live in a lab with cool labmates, bad coffee and all kinds of germs.

Most importantly I want to thank my best friend Madhav. I would never have been able to do this without his support and strength to keep me going. I want to thank him for being my rock. I do not have enough words to describe everything he has done for me.

I thank the faculty and staff of the Biomedical Engineering Department, letting me borrow equipment and the van, and for always being supportive and helpful.

I was fortunate to meet several interesting people while working on this project. Working on this material has probably been the most difficult as well as one of the most rewarding experiences in my life. My education on the subject matter, as well as project management and teamwork skills have been enriched by this opportunity.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>3</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>6</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>8</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>10</td>
</tr>
<tr>
<td>Growth of Tumor Microvasculature</td>
<td>10</td>
</tr>
<tr>
<td>Study Overview</td>
<td>11</td>
</tr>
<tr>
<td>2 BACKGROUND</td>
<td>13</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>13</td>
</tr>
<tr>
<td>Biological Effects of Hypoxia in Tumors</td>
<td>13</td>
</tr>
<tr>
<td>Direct Effect of Hypoxia on Radiation and Chemotherapy</td>
<td>14</td>
</tr>
<tr>
<td>Effect of Hypoxia on Tumor Microvasculature Growth</td>
<td>15</td>
</tr>
<tr>
<td>Chronic and Acute Hypoxia: Emphasis on Acute Hypoxia</td>
<td>16</td>
</tr>
<tr>
<td>Structural Abnormalities of Tumor Microvasculature</td>
<td>17</td>
</tr>
<tr>
<td>Functional Abnormalities of Tumor Microvasculature</td>
<td>18</td>
</tr>
<tr>
<td>3 MAIN THEME AND MOTIVATION</td>
<td>19</td>
</tr>
<tr>
<td>Fluctuation Analysis</td>
<td>19</td>
</tr>
<tr>
<td>Whole Window Saturation Maps</td>
<td>20</td>
</tr>
<tr>
<td>Longitudinal Gradients in Tumor Induced Windows over Days</td>
<td>20</td>
</tr>
<tr>
<td>4 MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>Tumor Cells and Cell Culturing</td>
<td>21</td>
</tr>
<tr>
<td>Animal Model and Window Chamber Surgery</td>
<td>22</td>
</tr>
<tr>
<td>Imaging System</td>
<td>23</td>
</tr>
<tr>
<td>Image Acquisition</td>
<td>23</td>
</tr>
<tr>
<td>Animal Setup</td>
<td>23</td>
</tr>
<tr>
<td>Software Setup</td>
<td>24</td>
</tr>
<tr>
<td>Image Processing</td>
<td>24</td>
</tr>
<tr>
<td>Analysis Performed</td>
<td>25</td>
</tr>
<tr>
<td>Fluctuation Analysis</td>
<td>25</td>
</tr>
<tr>
<td>Whole Window Saturation Maps</td>
<td>26</td>
</tr>
<tr>
<td>Longitudinal Gradients in Tumor Induced Windows over Days</td>
<td>26</td>
</tr>
</tbody>
</table>
5 RESULTS ........................................................................................................................................27

Fluctuation Analysis ..................................................................................................................27
Whole Window Saturation Maps ...............................................................................................29
Longitudinal Gradients over Days ............................................................................................33

6 DISCUSSION ...........................................................................................................................60

LIST OF REFERENCES ...............................................................................................................68

BIOGRAPHICAL SKETCH ........................................................................................................71
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-1</td>
<td>Mouse with 4T07 tumor used for Fluctuation Analysis on Day 9 after surgery.</td>
<td>36</td>
</tr>
<tr>
<td>5-2</td>
<td>Mouse 1 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery.</td>
<td>37</td>
</tr>
<tr>
<td>5-3</td>
<td>Mouse 2 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery.</td>
<td>38</td>
</tr>
<tr>
<td>5-4</td>
<td>Mouse 3 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery.</td>
<td>39</td>
</tr>
<tr>
<td>5-5</td>
<td>Mouse 4 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery.</td>
<td>40</td>
</tr>
<tr>
<td>5-6</td>
<td>Non tumor window on Day 1 and 3 post surgery for Whole Window Analysis</td>
<td>41</td>
</tr>
<tr>
<td>5-7</td>
<td>Non tumor window on Day 5 and 17 post surgery for Whole Window Analysis</td>
<td>42</td>
</tr>
<tr>
<td>5-8</td>
<td>Tumor window 4T07 on Day 1 post surgery for Whole Window Analysis</td>
<td>43</td>
</tr>
<tr>
<td>5-9</td>
<td>Tumor window 4T07 on Day 3 post surgery showing Transmitted light image at</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>575nm for Whole Window Analysis</td>
<td></td>
</tr>
<tr>
<td>5-10</td>
<td>Tumor window 4T07 on Day 3 post surgery showing Hemoglobin saturation image</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>for Whole Window Analysis. Refer to Figure 5-5 for HbSat scale.</td>
<td></td>
</tr>
<tr>
<td>5-11</td>
<td>Tumor window 4T07 on Day 5 and 7 post surgery for Whole Window Analysis</td>
<td>46</td>
</tr>
<tr>
<td>5-12</td>
<td>Tumor window 4T07 on Day 9 and 11 post surgery for Whole Window Analysis</td>
<td>47</td>
</tr>
<tr>
<td>5-13</td>
<td>Tumor window 4T1 on Day 1 and 3 post surgery for Whole Window Analysis</td>
<td>48</td>
</tr>
<tr>
<td>5-14</td>
<td>Tumor window 4T1 on Day 5 and 7 post surgery for Whole Window Analysis</td>
<td>49</td>
</tr>
<tr>
<td>5-15</td>
<td>Tumor window 4T1 on Day 9 post surgery showing Transmitted light image at</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>575nm for Whole Window Analysis</td>
<td></td>
</tr>
<tr>
<td>5-16</td>
<td>Tumor window 4T1 on Day 9 post surgery showing Hemoglobin saturation image</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>for Whole Window Analysis. Refer to Figure 5-5 for HbSat scale.</td>
<td></td>
</tr>
<tr>
<td>5-17</td>
<td>Non tumor window on Day 11 post surgery for Longitudinal Gradient Analysis</td>
<td>52</td>
</tr>
<tr>
<td>5-18</td>
<td>Hemoglobin Saturation values for Non tumor window on Day 11 post surgery</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>shown in Figure 5-17.</td>
<td></td>
</tr>
<tr>
<td>5-19</td>
<td>Tumor window 4T1 on Day 5 post surgery for Longitudinal Gradient Analysis</td>
<td>54</td>
</tr>
<tr>
<td>5-20</td>
<td>Hemoglobin Saturation values for tumor window 4T1 on Day 5 post surgery</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>shown in Figure 5-19.</td>
<td></td>
</tr>
</tbody>
</table>
5-21 Tumor window 4T1 on Day 7 post surgery for Longitudinal Gradient Analysis..............56

5-22 Hemoglobin Saturation values for tumor window 4T1 on Day 7 post surgery shown in Figure 5-21..................................................................................................................57

5-23 Tumor window 4T1 on Day 9 post surgery for Longitudinal Gradient Analysis........58

5-24 Hemoglobin Saturation values for tumor window 4T1 on Day 9 post surgery shown in Figure 5-23................................................................................................................59
Solid tumors require growth of new microvessels in order to proliferate and grow beyond a size of about 1-2 mm. However the newly formed tumor vasculature is abnormal in structure and function compared to normal tissues and as a result blood flow and oxygen and nutrient delivery inside the tumor is heterogeneous. Recent research has demonstrated large magnitude, slow periodic fluctuations in tumor oxygenation that may have clinical relevance. Understanding the heterogeneous and dynamic nature of tumor blood flow and oxygen delivery may give insight into ways to improve treatment efficacy with therapies that require oxygen to be effective, such as radiation therapy and some chemotherapeutic drugs. In addition, a better understanding of tumor biology may be gained with added insight into how heterogeneous oxygen delivery alters tumor physiology. Hence high spatial and temporal resolution data on tumor microvascular oxygen delivery during tumor growth and development may aid in the design of better therapeutic treatment solutions.

Studies performed to investigate tumor microvasculature have employed oxygen microelectrodes, laser Doppler probes, MRI, and intravital microscopy. Each of these modalities yield data with varying amounts of spatial resolution and temporal resolution and each can measure different physiological parameters related to microvascular oxygen delivery. Recently,
it was demonstrated that hyperspectral imaging could be used to measure hemoglobin saturation in tumor microvessels grown in mouse dorsal skin-fold window chambers. In this study intravital microscopy was used in conjunction with hyperspectral imaging and image processing to obtain high spatial resolution information about hemoglobin saturation inside tumor microvasculature. Tumor growth progression was tracked with cells growing in an approximate 0.5 to 1 mm layer of skin in the mouse window chamber model. Dynamic changes in tumor microvascular oxygen transport function were tracked during tumor progression along a two dimensional area closest to the window glass.

Our study examined temporal and spatial alterations and fluctuations in tumor microvessel oxygenation in tumor and neighboring normal host vessels using this new unique imaging system.
CHAPTER 1
INTRODUCTION

Growth of Tumor Microvasculature

Solid tumors are metabolically very active and have a high demand for oxygen. Tumors initially grow by getting their nutrition from existing host vessels. (Holash et al. 1999). However, in order to grow beyond 0.5 mm in diameter, tumors need to undergo angiogenesis and develop their own vasculature (Folkman et al. 1986).

Neovascularisation in tumors (also known as angiogenic switch) can be sudden (Hanahan and Folkman 1996) and takes place through two methods known as vasculogenesis and angiogenesis. Vasculogenesis occurs due to the differentiation of vascular endothelial cells that form capillary-like tubes (Risau and Flamme 1995). Angiogenesis, on the other hand, takes place through sprouting of new capillaries and intussusception (non-sprouting angiogenesis) of existing capillaries (Risau et al. 1997, Patan et al. 1996).

Developing tumor vasculature does not grow in an organized fashion, and is abnormal in structure and function. This abnormal microvasculature cannot adequately meet the oxygen demands of the tissue (Manka et al. 2005) and results in the formation of hypoxic areas which affect tumor biology and hinder therapeutic treatments.

In tumors, hypoxia shares a cause and effect relationship with the developing microvasculature or the chicken and egg relationship, where one gives rise to the other. We can consider hypoxia to be the cause as well as the effect of an abnormal tumor microvasculature. It disturbs the tumor microenvironment and upregulates genes which encourage a deficient and unstable microvasculature. This structurally and functionally disturbed microcirculation in turn is unable to meet the demands of surrounding tissue and as a consequence the tissue further develops hypoxic areas which have biological and therapeutic consequences.
Study Overview

Motivation for this study stems from tumor hypoxia and its effect on tumor cells and neovasculature. Hypoxia directly affects radiation therapy and chemotherapy. It also affects genome and proteome changes in tumor cells and alters their behavior and their surrounding environment. Most importantly, hypoxia affects the growing tumor vasculature causing it to be structurally chaotic and functionally inefficient. Abnormal tumor microvasculature further acts as an indirect barrier to therapy.

Studies to measure tumor microvessel partial pressure of oxygen (pO2) and hemoglobin saturation are being carried using several techniques such as employed oxygen microelectrodes, laser Doppler probes, MRI, and intravital microscopy. In our study, we use intravital microscopy combined with hyperspectral imaging on non-tumor and tumor induced window chamber model on athymic nude mice. Resulting transmitted light images and hemoglobin saturation images for each mouse. Images obtained from this technique yield data with high spatial and relatively high temporal resolution (highest for this study is 120 datapoints in 40 minutes).

In this project, we specifically study fluctuation in hemoglobin saturation values in microvessels over a 40 minute period. Data for an area on the window is captured for 40 minutes (datapoint taken every 20 seconds) and resulting hemoglobin fluctuations in the frequency domain are compared for tumor vessels and neighboring host vessels. Another area studied in this project is tumor development. Tumor cell growth is followed from day one after surgery till approximately 15 days after surgery or when the tumor can no longer be imaged due to its size limitations. In this imaging, the whole window area is captured every alternate day and the transmitted light images show the size of the developing tumor while hemoglobin saturation images show the effect the tumor growth has on the neighboring vasculature as well as gives information about the tumor vessels itself. Last analysis carried out in this project shows that
vessels imaged in the same area serially every alternate day through the life of the window have a marked difference in their hemoglobin saturation values from one imaging session day to the next. Each such session was recorded over a 40 minute time frame. Data obtained from each imaging session was compared to the data from the previous session. Results obtained from this analysis indicate a measure of the effect of tumor growth on vessel structure and function.

Fluctuation analysis studied in this project is a continuation of the work published in (Sorg et al. 2008) and shows a larger set of animals confirming results from the paper. Hemoglobin saturation analysis is also a continuation from the work done previously by Dr. Sorg (Sorg et al. 2005) and in this project; this data is further studied in the same animal over days with a higher sampling rate. Data obtained from this project also confirms previous observations from the work done by other groups using different measurement modalities.

This study highlights the advantages of the hyperspectral imaging technique used with the window chamber model and Chapter 6, discusses future directions that can be explored with the new findings from the results of this project.
CHAPTER 2
BACKGROUND

Hypoxia

Hypoxia can be described as the reduced oxygen availability or decreased partial pressure of oxygen below critical levels that restrict normal cell function. Hypoxia can occur in normal or tumor tissue and is caused by irregular perfusion of the tissue, low partial pressure of oxygen (pO2) in blood, reduced ability of blood to carry oxygen (example, due to anemia), large diffusion distances between blood vessels and tissue and the inability of cells to use oxygenation due to intoxication (example, cyanide poisoning) (Höckel and Vaupel 2001). In normal vasculature, responsive regulatory mechanisms prevent hypoxia by increasing oxygen supply with the increase in demand and a hypoxic environment is created only under extreme or abnormal conditions.

Biological Effects of Hypoxia in Tumors

Oxygen deficient or hypoxic environment affects tissue metabolism. Low oxygen availability and thus consumption leads to a slow down of cellular metabolic activities and results in collapse of sodium and potassium gradients. Low uptake of chlorides, increased cytosolic calcium concentrations, cell swelling, membrane depolarization, and a more acidic cytosolic pH are further consequences of the biological changes taking place due to hypoxia. Proteome changes lead to inhibition or stimulation of regular gene expression and may lead to cell cycle arrest, apoptosis, growth retardation and dormant metastasis. Proteome and genome changes in hypoxic cells may enable them to adapt to nutritional deficiencies. They may also increase chances of metastasis by enabling cells to have increased tolerance to heat shock and therefore escape hostile environments. This may affect radioresistance for the cells and thus affect radiation therapy (Harrison et al. 2002).
Direct Effect of Hypoxia on Radiation and Chemotherapy

Hypoxia affects chemotherapy and radiation therapy—two of the most common forms of cancer treatment. Radiation therapy depends on the molecular oxygen content within the tumor. Interaction between radiation and macromolecules produces ionized radicals and the presence of oxygen prolongs the life of these radicals thus aiding in DNA damage of tumor cells. Hypoxia therefore affects radiation therapy results by affecting radiation sensitivity of the affected cells. Oxygen content of tumor is considered to be among the most important determinants of radiation response in single treatment therapy. Studies have shown that patients with tumor pretreatment pO2 values above 10 mmHg have higher chances of overall survival than patients with low pO2. Hypoxic cells below 10 mmHg pO2 are considered radioresistant and are a cause for concern since they are viable and clonogenic, having the potential to restore the tumor after therapy (Harrison et al. 2002).

Some chemotherapeutic agents, for example cytotoxic alkylating agents, require cells to be well oxygenated in order for the treatment to be effective. These agents work by transferring an alkyl group to cell DNA strand during division and prevent DNA synthesis. Hypoxia causes increase in the production of nucleophilic substances like glutathione that compete with DNA for alkylation. Another example of a chemotherapeutic agent limited by hypoxia is Bleomycin that requires free radicals which are reduced under hypoxic conditions. Hypoxia also affects chemotherapy by slowing down cell cycling and sometimes even inducing pre-S phase arrest in DNA synthesis. This may cause an increase in DNA repair enzymes and hinder function of chemotherapy agents as most anticancer drugs act during DNA synthesis by damaging the DNA and hence causing apoptosis. Hypoxia also increases the production of proteins like metallothioneins that show drug resistance (Shannon et al. 2003). All these examples show that hypoxia directly affects therapy and may contribute to treatment inefficacy.
Effect of Hypoxia on Tumor Microvasculature Growth

Hypoxia affects radiation and chemotherapy directly, as discussed above as well as indirectly by affecting developing tumor microvasculature. Inefficient and inadequate blood supply through a chaotic vasculature hinders the delivery oxygen and nutrient supply to tumor tissue thus affecting the efficacy of chemotherapeutic drugs and radiation.

Hypoxia has detrimental effects on tumor microvasculature growth. Hypoxia impacts microvasculature growth by affecting the regulation of hypoxia inducible transcription factor (HIF-1) which is important in the regulation of neovasculature. HIF-1 consists of HIF-1α and HIF-1β. In the presence of oxygen, HIF-1α binds to Von Hippel-Lindau (VHL) protein which neutralizes HIF-1α and causes it to degrade. However, under hypoxic conditions HIF-1α is phosphorylated and stabilized through oncogenic signaling pathways and combines with HIF-1β to form a composite that binds to hypoxia response elements (HREs).

This activates genes such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), transcription of nitric oxide synthase (NOS), activates glucose transporter (GLUT1) and lactate dehydrogenase (LDH-A) (Harris et al. 2002) This process consequently affects expression of growth factors that encourage cell proliferation. It is partially responsible for promoting the production of VEGF leading to increased angiogenesis.

Hypoxia mediated HIF-1 pathway also causes the metabolic cycle to switch to oxygen-independent glycolysis that aids in cell survival under stress, increases erythropoiesis and promotes angiogenesis and vasodialation.

Although hypoxia is not the sole cause of angiogenesis (Dewhirst et al. 2004), we can still conclude that hypoxia does play a very significant role in shaping tumor vascular morphology. Hypoxia therefore affects cell processes which in turn affect development of the tumor.
Chronic and Acute Hypoxia: Emphasis on Acute Hypoxia

Hypoxia remains a subject of ongoing research in an effort to better understand the tumor microenvironment. Hypoxia is classically described as being chronic (due to permanent limitations in oxygen supply) or acute (due to transient limitations in blood perfusion).

Both chronic and acute hypoxia promote spontaneous metastasis in tumors, however studies show tumors have a higher fraction of acute hypoxic cells than chronically hypoxic cells and that within a tumor environment, acute hypoxia encourages higher recurrence of tumor cells and increases their metastatic potential. (Rofstad et al. 2007). Therefore, further research on acute hypoxia could be beneficial in determining hypoxia-related predictive variables and in improving treatment outcome.

Vascular stasis was earlier believed to be the primary contributor to acute hypoxia however Dewhirst lab (Dewhirst et al.1998) have shown that instances of vascular stasis occurred 5% of the time, and usually in the same segments of vessels and for short periods (less than 20s). Also, vascular stasis was not necessarily accompanied by blood vessel collapse or clogging of the vessel. This could indicate the presence of flow through the vessel with the absence of erythrocytes. Hence efforts to better understand factors leading to acute hypoxia are required since intermittent hypoxia is unpredictable and is potentially more harmful to therapy than conventional or chronic hypoxia.

One of the prominent contributing factors of acute hypoxia is believed to be the instability in tumor blood flow. Disturbed blood flow is caused due abnormal tumor microvasculature structure which is chaotic and unorganized as compared to the regular branching orders of normal vessel network. This abnormal vasculature results in insufficient blood supply to the tumor tissue and causes the vasculature to be functionally inefficient. Structural and functional abnormalities are characteristic of tumor microvasculature.
Structural Abnormalities of Tumor Microvasculature

Tumor microvasculature shows morphological abnormalities such as irregular contour, tortuosity and increased microvessel diameter. Tumor vessels are also excessively large due to multiple fusion points with other vessels. Tumor vasculature differs from normal vasculature in terms of cellular composition, vascular permeability, regulation of cellular proliferation, and tissue integrity, (Folkman and Cotran 1976) and vascular endothelial growth factors (VEGF) upregulated by hypoxia, is shown to make the vessels more permeable. Arteriovenous (AV) anastomoses are another notable characteristic of tumor microvascular abnormality contributing up to 30% of shunting in blood flow (Sorg et al. 2008). Blind vessel endings, aberrant branching, interrupted endothelial linings (Vaupel et al. 2005), tortuous vessel length (Sevick and Jain 1991), leaky vessel walls, (Jain et al. 2003) and absence of vasomotion (Jain et al. 1988) are just some of the vascular malformations that contribute to the abnormal structure and hence function of tumor microvasculature.

Overall vascular network in tumors is heterogeneous and does not have regular organization or pattern (Jain et al. 1988). Architectural abnormalities include heterogeneous vessel distribution with increased inter-vessel distances and loss of hierarchy resulting in organizational or spatial heterogeneity. This spatial heterogeneity can be classified as peripheral or central vascularization. Peripheral tumor vasculature shows maximum vessel density at the periphery while the center remains poorly perfused, while central vasculature can be described as having central vessel density network that supports peripheral vessels (Jain et al. 1988). From literature, the peripheral arrangement can be further characterized by a necrotic avascular region, a semi-necrotic region with unbranched capillaries extending towards the necrotic area, a stable and well-perfused vascular region and lastly, an advancing front at the periphery (Hoon et al. 2003). The 4T1 and 4T07 tumors used in this study exhibit peripheral vascularization.
Functional Abnormalities of Tumor Microvasculature

Structural abnormalities in tumor vessels highlighted above give rise to functional inefficiencies. Normal vasculature morphology and its function is shaped by the needs of the surrounding tissue and is mainly affected by: response to local metabolic conditions of surrounding tissue, shear stress due to flow on vessel walls, intravascular pressure and information conduction upstream to regulate blood supply (Secomb et al. 1999).

In tumors, these processes are not regulated and the behavior of feeding vessels as well as tumor vasculature is not well documented. There are many functional abnormalities that exist in solid tumors. Some observed abnormalities are: increased permeability, geometric resistance to flow, viscous resistance, unstable flow velocity and direction, flow shunting, flow regurgitation (intermittent flow) and stasis, only plasma flow, formations of thrombus and RBC aggregates, inadequate lymphatic drainage causing increased interstitial fluid accumulation and hence high interstitial pressures (Secomb et al. 1999).

Documentation describing the above properties of tumor vessels is vast and borders with but is not integral to the scope of this thesis and therefore not explained in detail. In the following sections we discuss the microvascular heterogeneities of tumors studied in this thesis and the results obtained. Comparison of these results to known phenomenon are also made.

Our experiments describe spatial and temporal heterogeneities of solid tumors. Some of these have been studied with other methods while some are unique to this study due to the imaging methodology used.
CHAPTER 3
MAIN THEME AND MOTIVATION

Following three analyses were performed on the data for this project:

**Fluctuation Analysis**

Study of pO2 fluctuations in tumor vessels (Braun et al. 1999) and the effect of pO2 fluctuations of neighboring host vasculature on tumor vessels have been hypothesized. These quantities were individually studied using oxygen electrodes. However due to the limitations imposed by the method the relationship between pO2 of neighboring host vessels and the pO2 of tumor vessels could not be concretely established.

Hyperspectral imaging technique used on the window chamber model is used in this study. With this technique it is possible to obtain transmitted light images as well as images showing the relative hemoglobin saturation (HbSat) content in the blood vessels in dorsal skin fold on a mouse. The images show a comprehensive two dimensional hemoglobin saturation map of the area. The spatial resolution enables us to select areas on the vessels with precision. For this analysis, areas were selected on the host vasculature (arteries and veins) and on tumor vessels. Comparison between the fluctuation frequency of the HbSat values for tumor and for host vessels within that window were made.

Hyperspectral imaging has a lower sampling frequency as compared to oxygen electrodes, however with this technique it is possible to measure HbSat values inside blood vessels whereas the probes can only give regional oxygenation of the area. Also, with oxygen probes it is not possible to relocate the same area and readings from the probes themselves might differ slightly. Magnetic Resonance Imaging is also another method used to study HbSat values in tumors, however, hyperspectral imaging uses light microscopy and thus images give detailed functional information* as well as highest possible spatial resolution among techniques being used today.
Whole Window Saturation Maps

Whole window transmitted light and saturation maps are used to study tumor growth progression. These images show changes in tumor size as well as vascular changes taking place in the neighboring vessels as the tumor grows. Developing tumor vasculature can also be tracked through these images. Data comparison between non-tumor and tumor induced mice show the difference in windows due to deterioration and actual vascular changes affected by the tumor. This data at present is more observational rather than quantitative. Future work would involve finding ways to quantify the effect the tumor has on neighboring vessels and quantify this effect spatially for tumor growth analysis.

Longitudinal Gradients in Tumor Induced Windows over Days

Longitudinal gradients were recorded in tumor vessels for single imaging sessions. These gradients were then compared over days for the same vessels in the window. It was observed that although non-tumor vessels still show hemoglobin saturation fluctuations (see graphs), which is characteristic of normal vessels, tumor vessels are observed to gradually ‘flatten out’ or lose fluctuations and their hemoglobin values are much higher as compared to regular vessels or data from previous days.

*with some 3D limitations-as discussed in later sections
CHAPTER 4
MATERIALS AND METHODS

Imaging system, image acquisition, image processing techniques as well as the detailed animal model is described below.

**Tumor Cells and Cell Culturing**

The 4T1 and 4T-07 cells used in this study are metastatic and non-metastatic mouse mammary adenocarcinomas respectively. Cells were obtained from Duke University, Courtesy of Mark W. Dewhirst. The 4T1 tumor constitutively expressed green fluorescent protein which enabled tracking of single tumor cells.

Both cell lines, 4T1 and 4T-07 cells, were cultured using the following media: 500ml Dulbecco’s modified Eagle’s medium (DMEM) with 50ml 50% fetal bovine serum (FBS), 10ml L-Glutamine, and 10ml Penicillin streptomycin (antibiotic). Incubator settings were fixed to 5% CO2, 37°C, and 50% humidity.

Cells were stored in liquid nitrogen for long term storage. Frozen cells were thawed in a heated bath for 2 minutes. They were then transferred into 15ml tubes and 3-5ml fresh media was added very slowly to the newly defrosted cells. Cell suspension was then centrifuged at 1000rpm for 10 minutes. In a new flask, 10ml of cell culture media was added and made ready for the new cells. Strict sterile procedures were followed at all times. Once the centrifuge time was concluded, the supernatant from the tube was discarded and 5ml of fresh media was added to the cells. This suspension was then introduced in to the flask and cells were incubated for 3 days. Cells require approximately 3-5 days to proliferate. After this time cell confluence was visually checked under the microscope. Once the cells were 90% confluent, they were ready to be passaged. Culture medium was changed as needed when the pH became acidic as indicated by a color change to the phenol red pH indicator dye in the medium.
For cell passaging, old culture media was discarded from the flask and the cells were washed with 5ml of saline. Once the cells were washed and the saline discarded, 3ml of trypsin was added to detach the cells from the flask bottom. These trypsinized cells were incubated for 3 minutes. Cells were then observed under the microscope to ensure that most cells were in suspension. Mechanical agitation removed any cells that remained stuck to the flask bottom.

Next, 7ml of cell culture media was added to the flask, the density of cells was counted and accordingly 1-2ml of the cell suspension was used to further continue the flask population and the rest was discarded or frozen. Additional 13ml of cell culture media was added to the flask so the total quantity of medium in the suspension was approximately 15ml. These cells were incubated for further use.

**Animal Model and Window Chamber Surgery**

Animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee. Animal model for these experiments was the dorsal skin-fold window chamber (Sorg et al. 2005) used with female athymic (nu/nu) nude mice. Animals were weighed before surgery and those close to or greater than 25grams were selected for surgery. These animals were injected with anesthesia- ketamine 100mg/kg IP and xylazine 10mg/kg IP based on their weights. On the dorsal side of the mouse, skin from one area was removed. Custom-made titanium window chamber was fixed to the back of the mice.

Tumor cells are prepared by ‘lifting’ the cells with trypsin and washing them several times in DMEM solution. The cells are counted and a specific ratio is established to get a concentration of 5000 cells per 10μl. This cell suspension was injected superficially into the skin on the exposed side, towards the center of the window chamber. Total volume of suspension injected was about 10μl which approximated to inserting 5x10³ cells in the window.
After tumor cell implantation, the open skin was flooded with saline and sealed with a 12 mm diameter #2 round glass coverslip (Erie Scientific, Portsmouth NH). Retaining ring (part of window chamber) was put over the glass to hold the cover slip in place.

Animals were placed under recovery and then transferred to the environmental chamber (Thermotron) with controlled temperature and humidity of 33°C and 50%. These environmental conditions are necessary for proper growth of the tumor and to prevent desiccation of the window chamber. Animals were given twenty-four hour access to food and water and were subjected to standard 12-hour light/dark cycles.

**Imaging System**

Imaging system and its calibration was as described previously (Sorg et al. 2005). Zeiss AxioImager microscope served as the imaging platform (Carl Zeiss, Inc., Thornwood, NY). A 100W tungsten halogen lamp was used for trans-illumination of the window chamber. Images were acquired at 1388 x 1024 pixels and 12-bit dynamic range with a DVC 1412 CCD camera (DVC Company, Austin, TX) that was thermoelectrically cooled to -20°C. Objectives 2.5x and 5x Fluars, and a 10x Plan-NeoFluar with long working distances (Carl Zeiss, Inc., Thornwood, NY) were used. Bandlimited optical filtering for hyperspectral imaging was accomplished with a C-mounted liquid crystal tunable filter (CRI, Inc., Woburn, MA) with a 400 – 720nm transmission range and 10nm nominal bandwidth placed in front of the camera.

**Image Acquisition**

**Animal Setup**

Animals were placed under anesthesia using isoflurane (1-1.5%) in medical air. They were then prepared for imaging by cleaning the glass on the window chamber, and taking care of any other maintenance of screws or sutures that was required. They were then placed on a heating pad attached to a custom-built stage on the platform of the microscope for the imaging session.
**Software Setup**

Liquid tunable crystal filter and camera were controlled by custom-designed Labview software for data acquisition. This software was built to operate in two modes. In the viewing mode, real-time visualization of the image by the camera is possible. This mode was used mainly for microscope focus adjustments, exposure times, and gain settings. Acquisition mode was the second mode, used to acquire the hyperspectral data set. In the acquisition mode the gain and exposure times at each wavelength are pre-set in order to utilize the full dynamic range of the camera. These wavelength range and exposure times can be user specified. For this study, images were acquired at 16 wavelengths ranging from 500-575nm in 5 nm intervals. Images are stored with different numbers appended to filename for each wavelength. Minimum exposure time of 300ms was used to average out fluctuations due to random red blood cell motion. One data set was typically acquired in 13s, which included image acquisition, filter tuning, image transfer, and saving of the images to the computer hard drive. Image sets were acquired in the first phase of experiments every 1 minute for one hour and in the second phase every 20 seconds for 40 minutes.

**Image Processing**

Image processing was performed using Matlab software (The Mathworks, Inc., Natick, MA). Reference spectra for oxy- and deoxyhemoglobin were obtained similar to (Sorg et al. 2005) with this system. All images obtained were converted into double-precision arrays and a region free of blood vessels was selected as an estimate for unattenuated light. Vascular areas of interest representing oxygenated blood and de-oxygenated blood were selected and their hemoglobin value was calculated using linear least-squares regression of the data according to the method of Shonat. This information was stored as reference and later analysis use these values to calculate hemoglobin saturations for user selected Regions of Interest (ROI).
For each data set collected over a 60 minute or 40 minute period, the transmitted light images for 16 wavelengths for each datapoint are processed to create two .m files, namely RawData.m and ImageMap.m. RawData files contain the data where the base value is subtracted from the image, and each pixel is processed to solve for its hemoglobin saturation value by linear regression. So each pixel is given its hemoglobin saturation value from the reference HbSat array and this information is stored for this image in its RawData file. ImageMap.m files are used to generate images to view these Hemoglobin saturation maps.

In the above paragraph, processing and data information for a single datapoint is described. However, each imaging session contained several such datapoints. Images per datapoint were collected every 20 seconds for a 40 minute period (totally 120+ datapoints- higher sampling frequency) or they were collected every minute for 60 minutes (totally 60 datapoint-lower sampling frequency). Image-stacks for each datapoint were processed to give RawData.m and Imagemap.m files. Once these were obtained, analysis software programs were run to select Regions of Interest (ROI) on the image sets for each session to look for specific information.

Hemoglobin Saturation values for each region of interest (ROI) for each 40 minute or 60 minute data set, was stored in a three dimensional array described as- values for all datapoints in imaging session; Region of interest; mean value/standard deviation/median value and other information for selected pixels.

**Analysis Performed**

**Fluctuation Analysis**

Imaging was done at high sampling frequency of 20 second gaps over 40 minutes. After processing each datapoint for that imaging session, resulting data set contained Rawdata.m files and ImageMap.m files. These files together give hemoglobin saturation information for the imaged area. Also transmitted light images at 575nm as seen through direct light microscopy
were also available. Regions of interest were selected on the images for tumor vessels and for normal or host vasculature for 4T1 and 4T07 tumor datasets. Hemoglobin saturation values for selected regions over entire imaging session were converted into frequency domain and their normalized power spectrum was calculated to give fluctuations in hemoglobin saturations for selected vessels for that imaging session.

**Whole Window Saturation Maps**

In this analysis, hyperspectral imaging was performed for each area of the window and the hemoglobin saturation maps and light images at 575nm were manually ‘stitched’ together to give whole window maps. This data was collected every alternate day up to approximately 15 days after surgery. This data was collected to non-tumor mice, mice implanted with 4T1 and 4T07 tumors.

**Longitudinal Gradients in Tumor Induced Windows over Days**

For this analysis, ROI’s within vessel segments were selected for an imaging session for a mouse. These ROI’s were selected for the same vessel segment for that mouse over consecutive days. These ROI’s were selected for host vessels present nearby and overlaying the tumor tissue. This analysis was performed on non-tumor windows and on 4T1 and 4T07 tumor induced windows.
CHAPTER 5
RESULTS

In this chapter, results are divided into 3 sections as described before - Fluctuation analysis, whole window saturation maps, and longitudinal gradients over days.

Fluctuation Analysis

Figure 5-1 is an example of results obtained for fluctuation analysis. This figure shows the window chamber area for a 4T07 (non-metastatic) tumor, 9 days after surgery. Figure 5-1(A) shows the transmitted light image at 575nm. In the area, the upper-central darker region represents the thicker tumor tissue embedded in the surrounding normal tissue. Area in ROI 1 is selected in a vessel within the tumor tissue while ROI 3 and ROI 4 represent the venules in the normal tissue. Area in ROI 2 is selected inside an arteriole in the normal tissue region. Figure 5-1 (B) is the hemoglobin saturation map of Figure 5-1(A) and shows that ROI 3 and ROI 4 carry deoxygenated blood whereas ROI 2 carries oxygenated blood. Vessel inside the tumor, represented by ROI 1, also carries blood having high hemoglobin saturation values. Figure 5-1 (C) is a graph of the normalized power spectrum of the fluctuation frequency of the hemoglobin saturation values and shows the fluctuation data for all four regions of interest. In this graph we can see that ROI 2 and ROI 4 have similar peaks while ROI 1 and ROI 3 have almost identical peaks. Highest peaks for all data lines are below 0.2 cpm. Other peaks for some data lines, like ROI 3, were further ahead on the graph, between 0.19 and 0.28 cpm on the graph. However, the magnitude of such peaks was less than half the magnitude of the larger peaks.

Figure 5-2 is another example of fluctuation data analysis performed on a 4T1 (metastatic) tumor, 9 days after surgery. Figure 5-2(A) shows the transmitted light images at 575nm and the regions of interest selected for this area. Tumor tissue can be seen as the relatively circular thick mass in which the vessel endings seem to be distorted. Vessels inside tumor tissue are selected
with ROI 1 and 2. Vessels with ROI’s 3, 6 and 5 are selected in host vessels with more
deoxygenated blood close to the tumor area. Vessel shown by ROI 4 carries more oxygenated
blood and may be an arteriole for host vasculature. Fluctuation graph in Figure 5-1 (C) show all
six ROI’s have common fluctuation curves with the highest peak lying below 0.15. Similarities
in fluctuation data for all 6 ROI’s could be an indication of common regional flow
characteristics.

Similarly, in Figure 5-3 for this analysis, the transmitted light image is shown along with
hemoglobin saturation figures for 4T1 (metastatic) tumor. Data for tumor is also taken 9 days
after surgery (care was taken to see that all tumors selected were at the same development level).
Graphs shown in Figure 5-3 (C) represent the fluctuation data for that image-set. Tumor and
normal vessels show very similar changes in HbSat. All vessels other than the vessel represented
by ROI 5 show similar fluctuation characteristics. However, from the transmitted light images
and hemoglobin saturation images, this vessel can be considered to be an artery. Highest
fluctuation peak for all ROI’s in tumor vessels and for host vasculature is around 0.1.

Figure 5-4(C) shows a graph for the normalized power spectrum for regions of interest
selected in Figure 5-4(A). In this example, ROI 5 is selected in a host arteriole and the graph in
5-4(C) shows that the data for this ROI has frequency peaks at several points on the graph.
Similar data values for arterioles have been observed before (Sorg et al. 2008). Therefore, we can
say that arterioles may have fluctuations similar to regional fluctuations or may have
independent peaks over the whole frequency spectrum which shows that the hemoglobin
saturation supply might be varying and has different frequencies of delivery for arterioles.
Another example for 4T1 induced mouse is shown in Figure 5-5. Figure 5-5(A) and Figure 5-
5(B) show the transmitted and hemoglobin saturation images for the window respectively. Figure
5-5 (C) shows that for this window all tumor vessels as selected in ROI’s 1, 2, 3 have similar HbSat fluctuation patterns to ROI4 which is a host vessel, but the tumor vessels peak together at 0.07. The ROI’s 5 and 6 from Figure 5-5(A) were not included in graph Figure 5-5(C) to give better clarity to graph.

To summarize the above results, the normalized power spectrum graphs, for metastatic and non-metastatic tumors, 9 days after surgery show that host venules have low frequency fluctuations while arterial vessels show varying frequencies of fluctuation. Tumor vessels fluctuations seem to follow venules and also have low frequency fluctuations. Analyses for both, 4T1 and 4T07 tumors, show that the largest peaks in the power spectra for host venules and vessels in tumor tissue occur for frequencies below 0.2 cpm.

**Whole Window Saturation Maps**

Figure 5-6 shows transmitted light images at 575nm and hemoglobin saturation images for the whole window area. These images were taken individually by moving the microscope stage in a “Z” pattern. In all there were 12 images acquired and named as 1-1, 1-2, up to 4-3, where the first number represents the rows and the second number represents the columns. Images were manually ‘stitched’ together, that is, neighboring images were overlaid to find the best possible fit and once the two dimensional picture for the whole window was formed, the images were grouped together. Two dimensional maps for whole window were acquired for non-tumor, 4T07 (non metastatic) tumor induced windows and 4T1 (metastatic) tumor induced windows. Data for this analysis was collected every alternate day. For non-tumor windows this data was collected for 17-19 days after surgery while for tumor induced windows the data was collected till the tumor was 2-3 mm in size and imaging was possible for that mouse. This time frame was usually 10-15 days. Figure 5-6 (A) shows the transmitted light image for a non-tumor window one day after surgery. We can see that the window is mostly clear with some inflammation which is
common immediately after surgery. Figure 5-6(B) shows the hemoglobin saturation map for 5-6(A). This map shows the most of the vessels in the region have slow activity and only the artery and vein on the right side of the window are distinct features for this figure. Figure 5-6(C) and Figure 5-6(D) show the transmitted light image (575nm) and hemoglobin saturation images for the same mouse after 3 days after surgery. In this figure the inflammation clears out and there is increased blood vessel saturation level in the vessels of the window. It is important to note that ordinarily, without this type of imaging only (approximately) 1/12th the information would be available to us and it would not be possible to see the complete ongoing and changes in hemoglobin saturation levels for all the vessels in the window. Figure 5-7 shows changes taking place in the window for the same mouse on Day 5 and Day 17 after surgery. Figure 5-7 (B) shows higher levels of hemoglobin saturation values as compared to the previous day and sequentially the information for Day 1, 3 and 5 shows that the window remains well preserved. Figure 5-7 (C) and 5-7 (D) show the windows for Day 17 after surgery. By this time we can see that inflammation starts to take place at the edges of the non-tumor window. Also, even with the inflammation, the structure of the vessels in the window remains intact. There are a few or no changes in vessel position and the size of the vessels seems to enlarge only to a small extent.

For the 4T07 tumor, data Figure 5-8 (A) shows transmitted light image (575nm) and Figure 5-8 (B) shows the hemoglobin saturation images for the tumor, one day after surgery. This window shows inflammation on the sides but the tumor cannot be distinguished. Figure 5-9 shows the transmitted light images for the same mouse, 3 days after surgery. This image was increased in size as compared to other similar images to show that the transmitted light images do not provide as much information about the initial growth stages of a tumor as the hemoglobin saturation images. In the next figure, Figure 5-10 we can see the hemoglobin saturation image
for the window in Figure 5-9. In this image the increased hemoglobin saturation activity in the center of the window can immediately be seen. This is the site where the tumor cells were implanted and the figure clearly shows the area of the establishing cells. Difference between 5-9 and 5-10 show the benefit of images obtained with hyperspectral imaging and processed with the technique used for this project as compared to plain light microscopy. Figure 5-11 shows transmitted light images for the 4T07 tumor as it grows, on Days 5 and 7 after surgery. This tumor which is a benign tumor, grows as a mass or self contained ball, and does not have much effect on neighboring vessels. This can be seen very clearly with the whole window saturation maps, since the hemoglobin saturation activity for the growing tumor and the saturation and structure of the neighboring vessels in the window can be observed. Figure 5-12 shows transmitted light images (575nm) and hemoglobin saturation images for the 4T07 window on the same mouse on Day 9 and Day 11 after surgery. In Figure 5-12 (A) and Figure 5-12 (C) we can see that the tumor mass on Day 9 after surgery is much larger than Day 7. Figure 5-12 (B) and Figure 5-12 (D) show data for Day 11 after surgery for the same mouse. Transmitted light images show dark spots near the tumor area, these may be due to clots formations, and the corresponding hemoglobin saturation images show tumor hemoglobin saturation containing an outer layer of perfused vessels and an inner layer that seems necrotic. Even on Day 11, the tumor affects only those vessels that are directly connected to it by increasing the saturation values for these vessels while the neighboring branches remain unaffected.

Figure 5-13 shows transmitted light images and hemoglobin saturation images for Days 1 and 3 after surgery for a mouse implanted with 4T1 (metastatic) tumor. Figure 5-12 (A) and (C) show the data for Day 1 after surgery. We can see that the tumor cells start to establish themselves very quickly even at this stage and show more activity than can be seen when
comparing Day 3 of 4T07 tumor induced window (Figure 5-10). Figures 5-13 (B) and Figure 5-13 (D) show that the tumor already is well established and starts affecting the vessels in the neighboring area. In this window we can see the vein closest to the tumor starts to enlarge and become more saturated (from Figure 5-13 (D)). Figure 5-14 (A) and (C) show transmitted light images and hemoglobin saturation images for the 4T1 tumor induced window 5 days after surgery. It is interesting to observe that the tumor already divides itself into a form of peripheral vascularization and has a seemingly necrotic core with a highly saturated periphery. The 4T1 tumor form on Day 5 is comparable to the 4T07 tumor form from Day 11 in spite of its much smaller size. Figure 5-14 (B) and Figure 5-14 (D) show the 4T1 tumor window on Day 7. Growth rate of the tumor between Day 5 and Day 7 is extremely fast. On Day 7 it is already covering almost half the window and is bigger than the 4T07 tumor on Day 11. On Day 9 after surgery shown in Figure 5-15, transmitted light image and 5-16, hemoglobin saturation image, we can see that the tumor covers more than half the window and still has a strong necrotic core, but now with some blood vessels growing inwards.

The 4T1 tumor has a marked effect on its surroundings. On Day 3 after surgery, the rapidly growing tumor affects the saturation for all the vessels in the window. In the image, we can observe a prominent vein with two branches, one supplying the tumor and the other branch away from the tumor. Initially the vein is highly saturated and both branches are much thicker than before due to the effect of the growing tumor. In consecutive days we can see that the branch supplying the tumor is enlarged with highly saturated blood flowing through it while the other branch is very thin with low saturated blood. This changes as the tumor grows and advances towards the vessel. The vessel increases in size and saturation for the whole window area is very high on Day 9 after surgery, much before the tumor has reached these vessels, indicating that the
4T1 has a marked effect on the surrounding environment when compared to the 4T07 tumor.

To summarize the results in this analysis, the whole window saturation maps are obtained from twelve individual images stitched together to form area maps for transmitted light images (at 575nm) and hemoglobin saturation images for non-tumor, 4T1 and 4T07 tumor induced window over days. Data for non-tumor windows show that the windows are well preserved and the vessels in the window stay constant in structure and position over time. Non-tumor windows stay constant up to 17 days after surgery (imaging Day 9) when deterioration starts taking place at the edges of the window. For tumor induced windows, data is collected till the tumors are 2-3 mm thick and light can no longer pass through the tumors. Windows are healthy for up to 10-14 days after surgery. Data show 4T1 and 4T07 tumor induced windows from first day after surgery up to nine days after surgery, data collected every alternate day. Images show tumor progression and microvascular changes taking place as the tumor grows and the effect that the growing tumor has on neighboring vessels. This data emphasizes the spatial resolution and functional information obtained with the hyperspectral imaging technique for the early detection of establishing tumor cells and also shows the difference in growth for the 4T07, non-metastatic tumor and the 4T1, metastatic tumor types.

So far, the results of this analysis are very subjective, but attempts are being made to quantify them. However, the detail and information provided by the hyperspectral imaging and using this technique, while studying tumor physiological changes are very remarkable.

**Longitudinal Gradients over Days**

Figures 5-17 and 5-18 show the data for a non-tumor window on Day 11 after surgery for this analysis. Figure 5-17 (A) shows the transmitted light image at 575nm. In this image we can see that the data is collected towards the edge of the window and the image has normal tissue as well as some inflammation tissue towards the edge. Regions of interest are taken for both areas
and ROI’s 1, 2, 3, 4 and 5 show vessel areas that are not directly affected by the inflammation while ROI’s 6 and 7 show vessel areas within the inflamed tissue. Figures 5-18 show the graphs for the hemoglobin saturation values for these ROI’s taken for a time series. Data is collected at a higher sampling frequency of every 20 seconds over a period of 40 minutes. Graph in Figure 5-18(A) shows data values for ROI’s 1, 2, 3 and 4 with large fluctuation curves that last between 10-12 minutes for the largest curve (ROI 3) and between 3-4 minutes for the smallest curve (ROI 1). Hemoglobin saturation values for ROI 5 show similar data, but the magnitude of these fluctuation curves is much smaller in comparison to the other ROI’s. In Figure 5-18 (B) the vessels that lie in the inflammation area of the window that is ROI’s 6 and ROI 7 also show the large fluctuation curves that last between 10-12 minutes and maybe more. Data from this image at Day 11 after surgery shows that this large fluctuation in hemoglobin saturation values of vessels in non-tumor windows is retained days after the window is implanted and is not lost.

Figure 5-19 shows transmitted light images and hemoglobin saturation images for 4T1 window, on Day 5 after surgery. Figure 5-20 shows hemoglobin saturation curves for regions of interest selected in 5-19 (A). These regions are selected for areas in host vessels adjacent to the tumor and in branches of the vessels that directly overlay the tumor. Graph in Figure 5-20 shows that the vessel segments for one branch ROI’s 14,13,12 and 8 and the vessel segments for another branch ROI’s 18, 19 and 20 show the larger fluctuations over every 6-7 minutes The magnitude of fluctuations for these segments is between 30-40 hemoglobin saturation values. Figure 5-21 shows images for data taken at Day 7 after surgery for the same window. Figure 5-22 shows graphs for the ROI’s for this set (same ROI’s as selected on Day 5). The magnitude of fluctuations observed earlier is very small. Although the time period of fluctuations is almost the same, the magnitude is reduced and the fluctuation curves are of a different shape.
The last set of Figure 5-23 and Figure 5-24 show the hemoglobin saturation data for the same vessel segments, 9 days after surgery. Data for the ROI’s (same as selected for Day 5) show that there are negligible large fluctuations in the hemoglobin saturation values for the ROI’s.

To summarize the observations from this analysis, we can see strong fluctuation in hemoglobin saturation values for non-tumor windows even on Day 11, whereas in 4T1 windows these fluctuations exist initially but phase out as the tumor grows.

The name for this analysis is a misnomer as the analysis was initially named longitudinal gradients since it started out as measuring longitudinal gradients as a function of spatial separation between vessel segments for host vessels and for vessels directly supplying the tumor. This analysis was later modified to give information for the hemoglobin saturation values as a function of time in vessel segments. This data was collected serially over days for the same window.
Figure 5-1. Mouse with 4T07 tumor used for Fluctuation Analysis on Day 9 after surgery. A) Transmitted light image at 575nm. B) Hemoglobin saturation image. C) Normalized power spectrum for dataset for selected ROI’s from (A). Tumor vessel fluctuations (ROI 1) are very similar to HbSat supply of normal neighboring vein (ROI 3).
Figure 5-2. Mouse 1 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery. A) Transmitted light image at 575nm. B) Hemoglobin saturation image. C) Normalized power spectrum for dataset for selected ROI’s from (A). Overall changes in HbSat are very slow in this area—all vessels tumor and non-tumor vessels have a high peak below 0.1.
Figure 5-3. Mouse 2 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery. A) Transmitted light image at 575nm. B) Hemoglobin saturation image. C) Normalized power spectrum for dataset for selected ROI’s from (A). Tumor and normal vessels show very similar changes in HbSat (all except ROI5 which looks like an artery). Peak around 0.1.
Figure 5-4. Mouse 3 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery. A) Transmitted light image at 575nm. B) Hemoglobin saturation image. C) Normalized power spectrum for dataset for selected ROI’s from (A). All tumor vessels (ROI 1,2,3,4) follow similar HbSat fluctuation with ROI 6, but definitely seem to peak together around 0.1.
Figure 5-5. Mouse 4 with 4T1 tumor used for Fluctuation Analysis on Day 9 after surgery. A) Transmitted light image at 575nm. B) Hemoglobin saturation image. C) Graph showing normalized power spectrum for dataset for selected ROI’s from (A). All tumor vessels (ROI 1, 2, 3) have similar HbSat fluctuation patterns to ROI4, but peak together at 0.07. ROI’s 5 and 6 were not included to give better clarity to graph.
Figure 5-6. Non tumor window on Day 1 and 3 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse Day 1 after surgery. B) Hemoglobin saturation image for mouse on Day 1 after surgery. C) Transmitted light image at 575nm for mouse on Day 3 after surgery. D) Hemoglobin saturation image for mouse on Day 3 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-7. Non tumor window on Day 5 and 17 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 5 after surgery. B) Hemoglobin saturation image for mouse on Day 5 after surgery. C) Transmitted light image at 575nm for mouse on Day 17 after surgery. D) Hemoglobin saturation image for mouse on Day 17 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-8. Tumor window 4T07 on Day 1 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 1 after surgery. B) Hemoglobin saturation image for mouse on Day 1 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-9. Tumor window 4T07 on Day 3 post surgery showing Transmitted light image at 575nm for Whole Window Analysis
Figure 5-10. Tumor window 4T07 on Day 3 post surgery showing Hemoglobin saturation image for Whole Window Analysis. Refer to Figure 5-5 for HbSat scale.
Figure 5-11. Tumor window 4T07 on Day 5 and 7 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 5 after surgery. B) Transmitted light image at 575nm for mouse on Day 7 after surgery. C) Hemoglobin saturation image for mouse on Day 5 after surgery. D) Hemoglobin saturation image for mouse on Day 7 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-12. Tumor window 4T07 on Day 9 and 11 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 9 after surgery. B) Transmitted light image at 575nm for mouse on Day 11 after surgery. C) Hemoglobin saturation image for mouse on Day 9 after surgery. D) Hemoglobin saturation image for mouse on Day 11 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-13. Tumor window 4T1 on Day 1 and 3 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 1 after surgery. B) Transmitted light image at 575nm for mouse on Day 3 after surgery. C) Hemoglobin saturation image for mouse on Day 1 after surgery. D) Hemoglobin saturation image for mouse on Day 3 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-14. Tumor window 4T1 on Day 5 and 7 post surgery for Whole Window Analysis A) Transmitted light image at 575nm for mouse on Day 5 after surgery. B) Transmitted light image at 575nm for mouse on Day 7 after surgery. C) Hemoglobin saturation image for mouse on Day 5 after surgery. D) Hemoglobin saturation image for mouse on Day 7 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-15. Tumor window 4T1 on Day 9 post surgery showing Transmitted light image at 575nm for Whole Window Analysis
Figure 5-16. Tumor window 4T1 on Day 9 post surgery showing Hemoglobin saturation image for Whole Window Analysis. Refer to Figure 5-5 for HbSat scale.
Figure 5-17. Non tumor window on Day 11 post surgery for Longitudinal Gradient Analysis A) Transmitted light image at 575nm for non tumor window on Day 11 after surgery. B) Hemoglobin saturation image for non tumor window on Day 11 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-18. Hemoglobin Saturation values for Non tumor window on Day 11 post surgery shown in Figure 5-17. X-axis shows data points taken every 20 seconds for 40 minutes. A) Values for ROI’s 1, 2, 3, 4 and 5. B) Values for ROI’s 5, 6, 7 and 8.
Figure 5-19. Tumor window 4T1 on Day 5 post surgery for Longitudinal Gradient Analysis A) Transmitted light image at 575nm for 4T1 tumor window on Day 5 after surgery. B) Hemoglobin saturation image for non tumor window on Day 5 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-20. Hemoglobin Saturation values for tumor window 4T1 on Day 5 post surgery shown in Figure 5-19. X-axis shows data points taken every 20 seconds for 40 minutes. A) Values for ROI’s 14, 13, 12 and 8. B) Values for ROI’s 18,19 and 20.
Figure 5-21. Tumor window 4T1 on Day 7 post surgery for Longitudinal Gradient Analysis A) Transmitted light image at 575nm for 4T1 tumor window on Day 7 after surgery. B) Hemoglobin saturation image for non tumor window on Day 7 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-22. Hemoglobin Saturation values for tumor window 4T1 on Day 7 post surgery shown in Figure 5-21. X-axis shows data points taken every 20 seconds for 40 minutes. A) Values for ROI’s 14, 13, 12 and 8. B) Values for ROI’s 18, 19 and 20.
Figure 5-23. Tumor window 4T1 on Day 9 post surgery for Longitudinal Gradient Analysis A) Transmitted light image at 575nm for 4T1 tumor window on Day 9 after surgery. B) Hemoglobin saturation image for non tumor window on Day 9 after surgery. Refer to Figure 5-5 for HbSat scale.
Figure 5-24. Hemoglobin Saturation values for tumor window 4T1 on Day 9 post surgery shown in Figure 5-23. X-axis shows data points taken every 20 seconds for 40 minutes. A) Values for ROI’s 14, 13, 12 and 8. B) Values for ROI’s 18,19 and 20.
CHAPTER 6
DISCUSSION

The hyperspectral imaging system used with the window chamber model is a unique imaging tool that can provide functional information as well as high spatial resolution to study the developing tumor. Hemoglobin saturation values give information directly related to the tumor oxygenation supply (Rofstad et al. 2008) and are important to the understanding of tumor blood supply and function.

Disturbances in tumor blood supply lead to tumor hypoxia. Among the two forms of hypoxia, intermittent hypoxia has been shown to have higher potential in promoting tumor metastasis than chronic hypoxia (Rofstad et al. 2007). Acute hypoxia is believed to be mainly caused by fluctuating oxygenation in tumor vessels and instabilities in tumor blood flow, hence they are important factors in studying tumor physiology.

It has been shown using oxygen microelectrodes (Braun et al. 1999) that intussusceptive microvessel growth, arteriolar vasomotion and blood flow changes are partly responsible for pO2 fluctuations in tumor vessels. In this project, we take the pO2 values previously studied as an approximate reference to compare the hemoglobin saturation values in tumor vessels obtained in this work.

In this project, data and analysis for fluctuation measurements in hemoglobin saturation values were performed on tumor vessels and neighboring host vessels in the window chamber model using hyperspectral imaging shown in Figures 5-1 up to Figure 5-5. Figures show analysis on both 4T1 (metastatic) and 4T07 (non-metastatic) strains of mouse mammary adenocarcinomas. Regions of interest (ROI) were selected in each vessel segment and their hemoglobin saturation values were converted to the frequency domain and the normalized power spectrum for each ROI was calculated.
From the graphs in the figures it can be seen that, fluctuations in hemoglobin saturation for tumor vessels are very similar to fluctuations in normal veins. From the figures we observe that the fluctuation data line representing regions of normal vessels have their highest peak below 0.2 cpm. Also, fluctuation data for hemoglobin saturation values of tumor vessels selected are similar to the data for normal vessels and have their highest peak below 0.2 cpm. Vessels inside the tumor seem to peak at similar frequencies while host vessels might peak at similar or neighboring frequencies. For vessel segments considered to be arterioles for host vasculature, the hemoglobin saturation frequency data may peak similar to regional vasculature or may have independent peaks over the whole frequency spectrum which shows that the hemoglobin saturation supply might be varying and has different frequencies of delivery.

Results from this technique have the advantage of having a higher spatial resolution than oxygen electrodes. Oxygen electrodes can only measure regional pO2 and finding an area inside blood vessels is difficult due to the lack of spatial information also, it is hard to relocate the area measured previously since the area cannot be marked. Area measured with oxygen electrodes is restricted to the tip of the electrode and it is possible to measure an area of only a few microns at a time. Also, the data obtained might differ slightly from one electrode to the next because electrode calibrations differ slightly from one electrode to another.

With the hemoglobin saturation imaging technique, we get a comprehensive two dimensional map of the window chamber area and saturation in blood vessels can be completely observed. We can therefore make an accurate selection in regions of interest and due to high spatial information it is possible to relocate the area previously selected for measurement. This technique allows measurement of the entire area under the microscope up to a few millimeters. Calibrations for this technique remain constant even from one mouse to another.
The limitations with the hyperspectral imaging technique however are the inability to gather information about tissue pO2. Also, the three dimensional limitations of the technique enable us to only see the surface of the window chamber closest to the glass, and we are unable to see deeper tumor vessels once the tumor starts growing and thickening. However, hyperspectral imaging gives much higher spatial resolution and functional information when compared to imaging techniques being used to study tumor physiology today including MRI techniques.

With the analysis done through his work, we can make a definite comparison in fluctuation data between host vasculature, mainly venules, and tumor vessels. This data suggests that in 4T1 and 4T07 tumors, the mechanisms responsible for fluctuations in hemoglobin saturation in normal vessels may affect fluctuations in the hemoglobin saturation values of tumor vessels. Results from this study have implications in three dimensional modeling to study hemoglobin saturation (or oxygen supply) in tumor microenvironments and could be further extended to study how the rate of oxygen supply affects drug delivery.

The next section studies whole window saturation maps, in this project. This was an in-house technique developed using the original imaging method and taking images for the whole window and ‘stitching’ them manually together. Each section was imaged and the next section was selected in a ‘Z’-pattern. In all there were twelve images obtained to cover the entire window chamber area these images were stitched together by overlaying the best possible match between neighboring images and grouping all twelve images together.

The image maps help us observe the complete physiological ongoing inside the vessels of the window. Not only can the information for the tumor area be studied, but having the larger window area helps to find the reference sources of blood supply to the tumors. This data was
collected over days and the following the whole window saturation maps serially enables us to see the physiological changes taking place in a developing tumor. Change in hemoglobin saturation values of the surrounding vessels to meet the requirements of the tumor tissue can be studied in detail. Along with the effect the tumor has on the surrounding environment we can also study growth of tumor neovasculature.

Observing the vessels inside the window chamber as a complete picture is very interesting because we can see serial changes that take place in the surrounding area of the tumor over days which otherwise cannot be followed. For example, in this project, we can see as the days progress, a vein having two branches—one that supplies the tumor and the other that lies close by, has the first branch enlarge significantly in size with a higher hemoglobin saturation content than the other branch which remains relatively normal and even slightly reduces in size and had a lower hemoglobin saturation content.

The tumor itself can be observed, growing from a few cells to a large mass that is infused with blood vessels and later to a structure showing peripheral vascularization which has a necrotic center and a growing periphery, as with the example in this project. Tumor blood vessel growth can also be observed with this method and the vasculature can be followed from being extension of host vasculature to becoming tumor vessels.

Comparison made between non-tumor and tumor induced windows shows that the non-tumor windows remain well preserved over days. After seventeen days from surgery we can see some deterioration on the edges of the window. This indicates that the tumor windows are free from inflammation for at least the larger duration of the imaging period (thirteen to fifteen days). Non-tumor windows also show a direct comparison between blood vessels that remain constant over time compared to the vessels in the tumor environment.
Comparison between 4T1 (metastatic) and 4T07 (non-metastatic) windows show that the 4T07 tumor grows in a more self-contained manner, that is, it does not have much effect on the surrounding vasculature. We can see a rise in the saturation values for the neighboring vessels but the size remains the same whereas the 4T1 tumor seems to greatly affect the surrounding vessels. Saturation values for these vessels are much higher and their sizes are affected as the tumor grows.

The whole window saturation map offers advantages of extending the spatial resolution of the hyperspectral imaging to a larger area to give more information of the entire window environment. Results obtained from this technique need to be considered with the following limitations. Individual images taken were optimized to have the best spatial information for that area. Hence the focus and light transmission were adjusted for each image to give the best contrast and sharpness for that area. Reference area for each image was selected individually, and hence the reference varies from one image to the next. Stitching for the images was done manually and is not an exact representation of the actual area (slight shifts or area compressions might have taken place). Stitching was done to give the best possible match for significant vessels; shifts due to physiological changes, breathing artifacts and motion of stage, affects the images obtained between two neighboring regions and alignment for these images gets affected. The information from this imaging is still qualitative and efforts to quantify the effect of growing tumor on neighboring vessels spatially needs to be made for tumor growth analysis and to get better insight from these results.

Possible future applications for this imaging method are in the development of better detection techniques for tumors of the gastro-intestinal (GI) tract. Recent research (Heverhagen et al. 2007) shows that almost 25% of all GI tumors still go undetected and there is a need for
better imaging methods. Mapping together large areas that show hemoglobin saturation information obtained from hyperspectral imaging could help find pathology in the tissue in early stages of tumor development as shown in Figure 5-10.

The last analysis done in the project was the longitudinal analysis. This name is a misnomer because although this analysis started out as a longitudinal analysis to study a spatial gradient between vessel segments, it later developed to study the changes in hemoglobin saturation in vessel segments for the same mouse serially over days.

Observations for non-tumor windows show that the hemoglobin saturation measurements in vein vessel segments contain larger fluctuations in hemoglobin saturation values superimposed by smaller frequency changes. This can be observed in even on day eleven after surgery in the mouse. Data for 4T1 metastatic tumor type show that these larger fluctuation changes can be observed in the host venules during the initial days when the tumor is not well established. Vessel segments closer to the tumor cells also reflect these large fluctuation with slight differences in magnitude of hemoglobin saturation values. However, as the tumor grows, data shows that the hemoglobin saturation values seem to ‘flatten out’ with an increase in saturation levels. By the ninth day after surgery, these fluctuations seem to phase out completely. It is important to note that these changes were measured for the host vessels alone, ROI’s were taken for larger segments as well as segments supplying the tumor directly (overlaying the tumor). This data does not reflect tumor vessels.

The data obtained from this work can be used to indicate changes in hemoglobin saturation values in host vessels affected by tumor growth. Studies performed on other tumor types (Baudelet et al. 2002, 2005) using different techniques (MRI and light probe) show similar fluctuation in pO2 values. However, data is available for only for one day and the results from
this analysis cannot be compared to known references but could potentially give insight to the changes taking place in host vessels as the tumor develops. Functional changes in blood supply to the tumor can also be evaluated using this method as lack of fluctuations in hemoglobin saturation values for later days could be a result of degradation in the structure of blood vessels, that is, the blood vessels might have become leaky.

These results are very nascent and need further work to clearly explain their implications. We can see that compared to normal vessels in non tumor windows, the host vessels supplying a tumor have a change in pattern for the hemoglobin saturation data. This work needs results from more mice to further establish these observations. Data from 4T07 non metastatic tumor type could also be analyzed to support the results from the tumor vessels.

This analysis shows changes in blood supply to a tumor as a function of tumor growth and can have implications in drug therapy. It is important to be able to predict the behavior of blood supply to a growing tumor and this analysis could be a step in this direction. Also, in techniques for tumor detection using light microscopy, there are often false positives and false negatives where inflammation and nascent tumor growth is often confused. This method can be extended in use by contrasting data from these two areas and studying hemoglobin saturation values for inflamed tissue and tumor tissue get affected in the same manner. In inflammation although extravasations takes place, the vascular structure for vessels remains intact, whereas for tumors, the vessels are shown to become highly permeable and their structure is affected. Experiments on windows with tumors compared to windows with inflammation can be performed and their hemoglobin saturation values studied to see similarities or differences in these two tissue types.

In conclusion, all three analyses contribute to the study of tumor physiology and work to complete a part of the picture to study dynamic temporal and spatial changes in hemoglobin

66
saturation in a developing tumor and its effects on the surrounding environment. We now know that the supply to the tumor tissue from host vessels and tumor vessels are related. This supply has slow fluctuations and is not much affected by the higher metabolic tumor activity. Whole window saturation measurements show blood vessels supplying the tumor have a marked increase in the hemoglobin saturation values. Therefore, although the blood supply activity is slow, the value is much higher. This last analysis shows that the hemoglobin saturation data for host vessels and vessels supplying the tumor contain larger fluctuations in values which phase out as the tumor develops over days as compared to normal vasculature which retains these fluctuations. This could be a direct effect of the pathology introduced in host vasculature as a result of the tumor. Measuring this effect could help us quantify the differences in normal blood vessel hemoglobin saturation values and the difference in values for vessels that are affected by a developing tumor. Further analysis can be performed to combine the fluctuation analysis technique for individual imaging sessions and the pseudo longitudinal gradient analysis. Combining these two techniques could give detailed information about the fluctuations in hemoglobin saturation and nutrient supply to tumor tissue and the changes that take place as the tumor develops and how a non-tumor environment compares with these results.
LIST OF REFERENCES


J. Folkman, “How is blood vessel growth regulated in normal and neoplastic tissue?” Cancer Res. 46, 467-473 (1986)


BIOGRAPHICAL SKETCH

Nikita Agarwal is a masters student at the J. Crayton Pruitt Department of Biomedical Engineering at the University of Florida (UF), Gainesville. She obtained her undergraduate degree in biomedical engineering from Mahatma Gandhi Mission’s College of Engineering and Technology, University of Mumbai, India. Her enthusiasm for the field of biomedical engineering and commitment to the healthcare field consolidated her decision to pursue graduate education in the field. She’s been an active participant and organizer in the Biomedical Engineering Society at UF as Career Development Director for the society and was the chief editor and co-founder of her department newsletter (“Synapse”) in India.

Nikita is currently, working for a leading healthcare company, McKesson Corporation; and is employed as a Revenue Cycle Associate in McKesson Technology Solutions.