The Development of an In Vivo Mobile Dynamic Microscopy System that Images the Hypoxic Microenvironments of Cancerous Tumors via Fluorescent and Phosphorescent Nanoparticles

by

Ashlyn Grace Rickard

Graduate Program in Medical Physics
Duke University

Date:_______________________

Approved:

___________________________
Gregory Palmer, Co-Supervisor

___________________________
Mark Dewhirst, Co-Supervisor

___________________________
Mark Oldham

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Graduate Program of Medical Physics in the Graduate School of Duke University

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ABSTRACT

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Abstract

Cancer is the second leading cause of death in the United States. Radiation therapy is an important and potentially lifesaving clinical intervention in the treatment of solid tumors. However, there is a wide variability in the relative radiosensitivity of individual solid tumors, and tumors with lower radiosensitivity correlated with diminished therapeutic response. A hypoxic tumor microenvironment substantially decreases tumor radiosensitivity. Unsurprisingly, hypoxia is also correlated with more negative patient outcomes in radiotherapy. In this thesis, we investigate this relationship has been observed and measured in tumor bearing mouse models using fluorescent and phosphorescent nanoparticles optical imaging.

Through ratiometric oxygen sensing, the hypoxic state of the solid tumors can be measured and characterized. Two substantive limitations of radiometric oxygen sensing in tumor-bearing mouse models has traditionally been that (i) the imaging systems require the subject to be imaged under anesthesia and (ii) that the data can only be recorded for a short period of time. Anesthesia creates difficulty in clearly defining the oxygen saturation levels in hemoglobin because of the effects on tumor vascular dynamics. Moreover, the time periods over which it would be desirable to track blood flow and oxygenation during the dynamic cycling of hypoxia during therapeutic
response in these subjects often exceeds what can easily be achieved through traditional anesthesia.

To overcome these two limitations, we designed a mobile imaging apparatus which directly attaches to the dorsal skinfold window chamber installed on nude murine models. This system uses dynamic microscopy to image the tumor’s hypoxic environment on un-anesthetized mice. The ability to avoid anesthesia will facilitate new and transformative insights into tumor biology and therapeutic response. Currently, we have quantified ratiometric oxygenation using boron nanoparticle solutions imaged under UV light with the mobile unit. The mobile unit can resolve vasculature and is sensitive enough to record nanoparticle emissions originating from tissue in a mouse window chamber model as shown preliminary in vivo studies.
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1. Introduction

With the increasing interest in nanoparticle oxygen sensing due to its large number of medical imaging applications, boron-based nanoparticles with duel-emissive properties have garnered attention [1]. Phosphorescent decay is highly sensitive to changing oxygen concentrations due to collisional quenching with oxygen, which can be quantified via either traditional imaging methods or our novel technique [1]. The second fluorescence emission is invariant to oxygen changes and thus can be used as a normalization factor between different samples. With the ratiometric response of fluorescence to phosphorescence, we can reliably measure and compare oxygen levels between samples as it minimizes error and variability via duel-emissive boron nanoparticles versus single-emissive nanoprobes that only emit phosphorescence [1,2].

Using mouse models with dorsal window chambers, breast cancer cells can be implanted under the window and subsequently grow into a solid tumor. To image and quantify the tumor microenvironment and vasculature in vivo, boron nanoparticles are injected and mice are imaged under anesthesia [3]. Traditional imaging under a fluorescent microscope has produced promising results; however, the required anesthetization of the subjects for this procedure gives cause for concern. Several studies have shown that under anesthetic conditions, oxygen concentrations change significantly over time and, in general, affect the hypoxic tumor conditions [3,4].
To overcome these limitations, a mobile imaging unit is being developed which allows subjects to remain conscious and active during imaging. Using a Raspberry PI computer with a small CCD-array camera the system can attach directly to the subject’s dorsal window chamber. The major benefit is that this will allow us to greatly increase the accuracy of our measured oxygen concentrations by removing any anesthesia associated artifacts. Furthermore, the intrinsic dynamics of the system will permit us to image the mouse for a longer period than previous anesthesia based methods allowed. This will allow blood flow and other novel functional endpoints to potentially be realized.

The end goal of this system is to use optical imaging to quantify and measure tumor hypoxia. We hope that through a greater understanding of tumor hypoxia that progress can be made to attenuate the negative patient outcomes associated with this physiological phenomenon. Over the years, interest in the temporal and spatial characteristics of hypoxia has increased due to greater association between hypoxia and negative pathological disease states [5]. Specifically, cycling hypoxia – the repeated temporal changes of hypoxia over time – is of special interest as it can be associated with increased probability of metastases and is a potential predictor of therapy response [6]. Therefore, the underlying interplay between cycling hypoxia and tumor response to therapy is of extreme interest.
1.1 Introduction to Molecular Sensing

It is not uncommon to find technologies such as magnetic resonance imaging, positron emission tomography, single-photon emission tomography, computed tomography, optical imaging and ultrasound developed and optimized specifically for small-animal imaging models. Their human scale counterparts are found in clinics across the world for diagnostic imaging and cancer-therapy planning. However, the small-animal research sphere has the added imaging several fluorescent and bioluminescent technologies that are not regularly used in clinical settings.

Despite their relatively low level of clinical integration currently, molecular imaging technologies have wide spread potential in cancer therapies, cell therapies, and many other clinical application described in Table 1 [12]. Taken together these reflect molecular imaging technologies to be a functional and physiological imaging technology that can provide in-depth information on specific biological constructs and processes. This paper will focus on the hypoxia application of imaging fluorescent boron nanoparticles.

Table 1: Description of molecular imaging technologies and applications

<table>
<thead>
<tr>
<th>Imaging Technology</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-protein interactions</td>
<td>The study of biological pathways</td>
</tr>
<tr>
<td>Apoptosis detection</td>
<td>Evaluation of new cancer therapies</td>
</tr>
</tbody>
</table>
Signal transduction | Real-time monitoring of signal pathways that change as a result of therapies
--- | ---
Bioluminescent imaging | Gene expression, cell and bacterial tracking and protein processing
Fluorescent imaging | Hypoxia imaging, vasculature modeling, evaluation of cancer therapies

### 1.2 Hypoxia in Cancerous Tumors

Hypoxia describes a biological environment in which oxygen is deficient. In general mammals possess a variety of internal oxygen sensors that maintain homeostasis in tissue, which allows for optimal oxygen to be delivered to the body’s systems [6,7]. Tissue oxygen saturation plays a crucial role in wound healing, myocardial and cerebral ischemia, as well as tumorigenesis. Oxygen sensing is therefore of extreme interest because of the wide gamut of potential applications. The role of tissue oxygenation is of importance in tumorigenesis and is the focus of this paper [14].

#### 1.2.1 Characteristics of Hypoxia

Hypoxic tumors, in comparison to normoxic tumors, have a diminished therapy response due to their aberrant oxygenation [7]. The primary characteristics that contribute to hypoxic tumor microenvironments are many but all result from a higher
demand for oxygen than what is supplied by the tumor vasculature. A lack of efficient arterial blood supply reduces the amount of oxygenated blood that is delivered to the tumor either because the hypoxic zone is distant from the arterial source or because the arterial source is exiguous [8]. A chaotic organization of vessels leads to a highly inhomogeneous blood supply scheme where some portions of the tumor are receiving an abundance of oxygen and others are lacking in supply. For example, the periphery of a solid tumor generally contains a high vascular density when compared to the tumor center [8]. This is exacerbated by large diameter shunts that drain blood away from the tumor.

Red blood cell flux also plays a major role in tumor hypoxia. Blood flow per unit time is highly variable in tumors and at a given time, it is not unusual for microvessels to be lacking in any red blood cells [8]. Furthermore, hypoxic red blood cells are stiffer than their normoxic counterparts, leading to an increased blood viscosity and uneven distributions at vessel bifurcations [8].

Finally, hypoxia features a temporal instability that leads to cyclical oxygenation [8]. Studies by Chaplin and Durand were some of the first to discover this. In the 1980s, they showed through a flow cytometry analysis of perfusion-marked dye in tumor cells that hypoxia has a period of several cycles in one hour in rodent tumors [9]. More recent studies have expanded this observation to include more complex hypoxia kinetics.
A study of vascular dynamics using a window chamber model showed significant changes in tumor architecture over a period of days [10]. The major cause of this change is the continual angiogenesis in tumors as they grow. These changes not only included variation in vessel organization but also of the oxygenation with a cyclical hypoxia period of several hours to days [8]. More information is needed to understand the full implication of cycling hypoxia.

1.2.2 Approaches to Oxygen Sensing

Oxygen sensors fall into two main categories: molecular and physiologic pathways and the external application of sensing mechanisms [13]. Molecular and physiologic pathways are utilized when cells detect changes in the oxygenation environment and respond accordingly using transcriptional and posttranscriptional mechanisms. Current research in this field has focused on the prolyl hydroxylase family of enzymes that require molecular oxygen for activity, the NAD(P)H oxidase family of enzymes that reduce reactive oxygen species, oxygen sensitive ion channels, and the electron transport chain determined through the oxidation of fluorescent probes and increased radicals detected by ESR spectroscopy [13].

External sensing methods involve applying sensors to a tissue environment that then react to the presence of oxygen. Fiachra B. Bolger and John P. Lowry (University of Dublin) conducted experiments using carbon paste electrodes to measure oxygenation
of brain tissue in and *in vivo* rat model [15]. They were successful in measuring the current changes from implantable amperometric enzyme-based biosensors.

More commonly found in external hypoxia sensors are fluorescence-based measurement. Jinjun Jiang, *et al* (Fudan University) are currently developing a biocompatible fluorescent sensor that attaches to the end of a fiber optic cable [16]. Their goal focuses on clinical applications for use in chronic obstructive pulmonary disease and acute lung injury/adult respiratory distress syndrome as it relates fluorescence with the partial pressure of oxygen. Both their *in vitro* and *in vivo* results thus far have shown that fluorescence emission is highly sensitive to changes in the oxygen environment – even in the presence of varying pH, partial pressure of carbon dioxide, and protein concentration [16].

Like fluorescent mechanisms, phosphorescence has also proven to be useful in oxygen sensing. Toshitada Yoshihara, *et al* (Gunma University) are developing molecular luminescent probes that can sense intracellular oxygen both *in vitro* and *in vivo* [17]. In their experiments, Iridium(III)-based small molecular probes are injected into murine models and have shown uptake in certain organs as well as in cancerous tumors. The phosphorescence in these tissues is then recorded to determine the oxygenation. The successful results suggest that there is a vast potential for using these probes for oxygen sensing, especially in cancer research.
The Palmer lab at Duke University in conjunction with the University of Virginia is developing a dual-emissive boron nanoparticle that senses oxygen through the ratio of phosphorescence to fluorescence at room temperature. Their experiments have shown this to be a sensitive method for determining the level of oxygen with a linear relationship between increasing phosphorescence intensity to decreasing oxygen levels from 0% oxygen to ~25% through the process of collisional quenching in spectroscopic studies. Using both in vitro cancer cell lines and in vivo mouse models, we have been successful in measuring hypoxia in response to various cancer therapies [2].

1.2.3 Factors that Affect Cancerous Tumor Hypoxia

There are several factors that can change the oxygen saturation of the tumor microenvironment. Beginning with the intrinsic causes, we examine the underlying physiology of tumorigenesis to determine which processes may be influencing or influenced by oxygen saturation within the tumor. Angiogenesis is present in cancerous tissue and requires nutrients and oxygen to advance tumor growth [17]. The formation of vessels within the tumor generally creates chaotic, leaky vasculature that causes inefficient oxygenation of the tumor, leading to hypoxia or heterogeneous oxygenation in some cases.

Many extrinsic factors affect the use of nanoparticles to measure these characteristic hypoxic regions within the tumor microenvironment. They include the
nanoparticle makeup, the location of the tumor, and the injection site of the nanoprobes. For the former, studies have shown that pegylated nanoparticles are necessary for attaching a polymer to a macromolecule via covalent and noncovalent bonding for \textit{in vivo} experiments which improved drug solubility, increased circulating life and stability, and decreased degradation [18]. As such all boron nanoparticles used experimentally for \textit{in vivo} and \textit{in vitro} applications are pegylated, which improves nanoparticle solubility, increases their stability, decreases cytotoxicity and decreases immunogenicity.

The location of the tumor can change the uptake rate of the nanoprobes as well as the ability to measure the oxygen saturation. Most studies that use fluorescent or phosphorescent nanoprobes will grow tumors in a window chamber that will allow the fluorescence to be detected unimpeded by skin and organs. Generally, because the tumor is visible, the uptake of nanoparticles can be monitored closely. Furthermore, it is possible to inject the nanoprobes into the tumor (or the tumor’s environment) directly to provide a highly-concentrated nanoparticle environment. Depending on the nanoparticle, it is likely that it will be cleared by the liver or spleen or degraded before it reaches the tumor if it is injected via a tail vein or an intraperitoneal injection; therefore, not allowing a proper measurement to be made. This degradation decreases the phosphorescence, causing hypoxic measurements to be undetectable as optical imaging
systems have a limiting sensitivity that cannot measure low-intensity phosphorescent emissions.

Of course, these extrinsic factors do not change the hypoxic state of the cancerous microenvironment; however, they do affect how accurate, precise and sensitive the measurements are, making an optimized protocol for administering and imaging nanoparticles imperative.

1.2.4 Therapeutic Effects on Cancerous Tumor Hypoxia

Radiation therapy from high linear-energy transfer x-rays or gamma-rays obtain their tumor-killing effects from two main interactions: direct and indirect action. Direct action is defined as ionizing radiation directly depositing its energy to a cell’s DNA, damaging it through single and double-strand DNA breaks and resulting in cell death. In indirect action radiation deposits its energy to the body breaking up water molecules into free radicals such as superoxide and hydroxyl ions. These free radicals interact with biomolecules such as DNA leading to oxidative damage that can facilitate mutagenesis, carcinogenesis and cell death [19].

This free radical effect of indirect action is highly correlated with the sensitivity and success of radiation therapy [20]. Due to the chaotic vessel structure in cancerous tissues, cells are often abnormally far from blood vessels. Should cells be beyond the diffusible distance of oxygen at 100-150µm, tissue experiences decreased respiration due
to extended periods without oxygen [20]. Not surprisingly it is common to find that many of the cells in tumor are chronically hypoxic or necrotic as a result of hypoxia. Oxygen, due to its high affinity for the unpaired electron on free radicals, causes further radiation injury from oxidized DNA damage. As such, cells that are acutely hypoxic or necrotic due to the lack of or fluctuating blood flow are protected from radiation. This is one of the reasons fractionation schemes are so successful at tumor control – it is theorized that they allow for tumor reoxygenation between radiation doses.

Radiation therapy is an example of how hypoxia adversely affects cytotoxicity; however, because hypoxia is hallmark of cancer itself, it can be a therapeutic target all its own. For example, drugs are currently being developed that target hypoxic cells. They are a non-viable version of cytotoxic compounds that require the reduction of one electron to form a radical [20]. In clinical trials, chemotherapy was co-administered with radiation therapy where the radiation kills a certain subset of cancerous cells, leaving behind a radioresistant tumor. The hypoxic-targeting drugs then destroy these remaining cells, efficiently increasing the therapeutic ratio. These trials failed; however, research continues with the goal to harness the hypoxic tumor effects in chemotherapy [20].

Immunotherapy and hypoxia also play a role in antitumor therapies. Cancers are highly immunosuppressive, and hypoxia exacerbates this effect. Several antibody
therapies including the T-cell immune checkpoint blockade and CTLA-4 and PD-1 antibody therapies have been successful in treating normoxic cancers. However, hypoxic tumors have been resistant to any effect. New research has found ways to overcome this challenge by targeting resistant cells with chemotherapy drugs that activate in the presence of hypoxia and can be co-administered with immunotherapy [21].

Combining radiation therapy with immunotherapy is an attractive concept because of the success of radiation therapy in curative and palliative care and the role that the immune system plays in cancer. A recent successful case study in a human patient co-treated with radiation and immunotherapy was reported by Postow et al [22]. The patient was diagnosed with melanoma and co-treated with radiotherapy and ipilimumab, a drug that targets CTLA-4 to activate the immune system [22]. The abscopal effect, in which a localized treatment successfully treats the primary target as well as distant metastasis outside of the scope of the applied treatment, was positively identified in this case. Postow also reported that the treatment increased antibody responses to tumor antigens [22]. This suggests that radiation therapy modulates immunotherapy in a way that activates the latter and increases anti-cancer effects.

1.3 The Physics Behind Ratiometric Sensing

In radiometric sensing the fluorescence and phosphorescence ratios respectively are proportional to the amount of oxygen in an environment. These two processes are
emitted through a duel-emissive nanoparticle that is selectively tuned such that its optical properties are optimized for \textit{in vivo} imaging \cite{2}. Ultra-violet (UV) light excites these nanoparticles to fluoresce and phosphoresce, and the phosphorescence increases in intensity as surrounding tissue oxygen concentration decreases. Since fluorescence is invariant to oxygen levels the ratio of fluorescence to phosphorescence can be used to describe oxygen concentrations between samples. This ratio is used to limit the amount of error and variability that is introduced across samples such as changes in emission intensity due to attenuation effects in varying tissue depths, natural degradation of phosphorescence in the model, and inhomogeneous nanoparticle distributions.

Fluorescence and phosphorescence both originate from a form of excitation such that emits light as it decays back into a ground state. Fluorescence relaxes from a singlet state when an orbital electron decays to its ground state. The electron can decay to various vibrational levels within the singlet state dissipating as heat. When it decays down to its ground state it fluoresces by releasing a photon that can be detected via high-speed imaging.
Figure 1: Energy diagram describing the process of fluorescence and phosphorescence. When an atom is excited to a high-energy state through the absorption of a photon, it can decay back into its ground energy state through fluorescence (shown in blue) or phosphorescence (in green).

Phosphorescence occurs when the excited electron decays from a triplet state. Similar to fluorescence the electron is excited and decays to its ground state releasing a phosphorescent photon. Generally, this decay occurs naturally as the ground state of the electron is also its lowest energy state; however, phosphorescence can also occur due to interactions with other molecules. When an excited nanoparticle interacts with oxygen, it relaxes to its ground state, phosphorescing through a process known as collisional quenching [2].
1.4 Nanoparticles

1.4.1 Synthesizing Nanoparticles

Nanoparticles have shown unique properties including sensitive ratiometric oxygen sensing, long-lived fluorescence and phosphorescence lifetimes, among other advantages, which further allow their utilization for a variety of applications. Generally, when nanoparticles are designed to fluoresce in biosensing applications they include a three-component polymer comprised of a dye loaded in a solid-gel matrix [23]. This can be done through entrapment or covalent linkage. The former’s usual method is through microemulsion polymerization where microemulsion droplets, ranging from 10nm-100nm, react with polymers to form nanoparticles [23]. Nanoparticles also can be synthesized through covalent linkage in a similar manner where the main difference is that the dye is covalently bonded with the polymer nanoparticle. The main advantage of this method is that it negates the risk of dye leaching, a common problem in biosensing applications [23]. Other advantages in covalent linkage include nanoparticles that have shown a higher fluorescence intensity as well as exceptional photostability. The final advantage – possibly the most valuable – is that covalently bonded dye-polymers can be selectively tuned for specific applications.
1.4.2 Properties of Boron Nanoparticles

It has been reported by Dr. Palmer at Duke University and his colleagues at the University of Virginia in a study on boron nanoparticle design that among the previously listed advantages of covalently-linked polymers, a single-component matrix instead of a three-component mixture allows for a minimal risk of dye leaching as well as a more homogeneous sample [2]. Another result of this study was that these single-component dye-polymer conjugates allow for both fluorescence and phosphorescence in a duel-emissive design.

It is the ability to modulate both fluorescence and phosphorescence from a single-dye matrix that makes the boron-based nanoparticle unique. By changing the length of the polymer chains, the PEGylation, and molecular weight both fluorescence and phosphorescence can be achieved at standard room temperature and pressure with long lifetimes on the order of milliseconds [2]. Furthermore, this fine-tuning allows for the phosphorescent and fluorescent peaks to be distinguished from one another. – an important feature in tumor-hypoxia sensing when changes in phosphorescent intensity will be quantified and divided by fluorescence to determine oxygen concentration [2].

This combination of high intensity fluorescence and phosphorescence in aqueous solutions and biocompatible polymers results in a highly sensitive method for evaluating hypoxic tumor environments. This study by Dr. Palmer at Duke University
and his colleagues at the University of Virginia have already shown successful results in both *in vitro* and *in vivo* mouse model experiments.

### 1.5 Murine Model

Current studies at Duke University with Dr. Palmer and their collaborators at the University of Virginia with boron nanoparticles utilize murine dorsal window chamber models [24]. Other imaging modalities might not require a window chamber. But due to the high absorption and scatter rate of fluorescence and phosphorescence it is necessary for there to be a short depth of tissue for the photons to traverse before interacting with the detector. Moreover, the dorsal window chamber model offers major advantages over other modalities in endpoints that involve cancerous tumor growth and metastasis, angiogenesis and vasculature modeling and gene-expressions [24]. Optical imaging methods are commonplace and intensity measurements that utilize them are reliably and easily visualized through a combination of emission measurements and physiologic imaging. These methods applied to solid tumor imaging allow their microenvironments to be directly and conveniently tracked.

#### 1.5.1 Dorsal Window Chamber

Our dorsal window chamber model utilizes athymic nude mice due to their ability to reliably and effectively grow solid tumors derived from human cancer cell lines. These athymic mice, in addition to a compromised immune system also naturally
lack hair and melanin. This is of additional benefit to this model as it leads to a lower absorption coefficient for nanoparticle emissions. A dorsal window chamber is surgically installed on a skinfold along the spine as shown in Figure 2. In this titanium chamber is a glass coverslip that protects a circular area of tissue where the skin has been cut away [24]. During surgery, an E0771 breast cancerous cell line were injected behind the glass coverslip for a tumor growth within the window. The tumor growth period is approximately one week.

![Mouse model with dorsal window chamber](image)

**Figure 2:** Mouse model with dorsal window chamber. A titanium window is surgically installed in mice on a dorsal skin flap. A circular section of skin is cut away at the viewing window and covered with saline and a glass coverslip. Tumor cells at high concentrations can be injected during this surgical process and grown in the exposed tissue over a period of approximately one week.

With only glass and a thin layer of saline covering the tumor, an ideal environment for optical imaging is be achieved. Moreover, nanoparticles can be injected
directly into the window chamber to minimize the amount that are metabolized or degraded before concentrating in the tumor.

1.5.2 Factors that Influence Hypoxia in Mice

As with any cancerous tumors the tumors in our in vivo mouse model experience a change in hypoxia after treatment. Generally, tumors become less hypoxic after radiation therapy due to the damage caused by indirect action. This is one reason why fractionation treatment schemes exist: they allow for tumor reoxygenation between doses. This allows the next radiation dose fraction to be more damaging to the tumor.

Another factor that influences hypoxia is the anesthesia used for mouse traditional imaging. Ketamine/xylazine and isoflurane are both primary anesthetic agents for mice. Studies by Kersemans, et al, and Mahling, et al, have both shown that these anesthetics affects the subject’s tumor’s oxygenation concentration when compared to other anesthetics and to mice breathing free air [3, 4]. Furthermore, oxygen saturation changed over time as the mice remained under anesthesia [3].

Kersemans, et al, performed a PET and SPECT study on mice anaesthetized under ketamine/xylazine and isoflurane with different carrier gases [3]. They compared their results with mice breathing free room air and found striking differences. Compared to mice breathing room air, all forms of anesthesia showed a marked decrease in the hypoxic state of the tumors [3]. Therefore, it is doubtless that studies whose
experimental outcomes involve the quantification of tumor hypoxia will be effected by anesthetic agents.

A similar PET radiotracer study performed by Mahling, et al, researched ketamine/xylazine and isoflurane anesthetic agents and compared them over time (see the figure below) [4]. After the radionuclide was injected, the hemodynamics were recorded over several hours showing a change in oxygen saturation. Their results also reflect a higher amount of oxygen present in tumors for mice dosed with either ketamine/xylazine or isoflurane vs room air [4]. Note that these effects are highly subjective to the anesthetic dose and imaging time, with the artifacts being minimized with both lower doses and shorter imaging durations. However, as our major goal is long-term tracking of cycling hypoxia, an ideal imaging system would still seek to remove any of these artifacts.
Figure 3: Data on hypoxia changes due to anesthesia as reported by Mahling, et al [4]. Mice injected with a radiotracer were anesthetized with either isoflurane or ketamine/xylazine with air or molecular oxygen carrier gases. Longitudinally imaging the mice over a period of approximately one hour shows changes in oxygen content based on the location of radiotracer uptake. The color scheme from blue to red represents hypoxia and normoxia, respectively [4].
1.6 Mobile Imaging Unit

Ratiometric oxygen sensing using duel-emissive boron nanoparticles can be used for hypoxic studies of cancerous tumor. The current standard procedure utilized in in vivo fluorescence microscopy (or other imaging modalities) requires that the mouse be anesthetized, which causes tumor hypoxia and changes in tumor oxygenation over time [2-4]. To overcome this limitation, a mobile imaging unit equipped with a CCD array and LED light sources is being developed to image awake mice. Despite the constraints of using a CCD array, our preliminary calibration studies have been successful in measuring various oxygen concentrations via fluorescence and phosphorescence. This current project will soon allow us to successfully obtain data directly from mouse window chambers that can accurately describe the tumor oxygen concentration.
2. Boron Nanoparticles

2.1 Characteristics of Iodine-Substituted Difluoroboron β-diketonate poly(lactic acid)

The specific boron nanoparticle used in our ratiometric oxygen sensing studies is iodine-substituted difluoroboron dibenzoylmethane-poly(lactic acid) (BF2dbm(I)PLA). It is multi-emissive through a single excitation wavelength, which allows both the fluorescent and phosphorescent decay to be imaged simultaneously [25]. This polymer is also useful in that it has overcome challenges in dye leaching and heterogeneity – common problems in multi-component nanoprobe as well as other sensing mechanisms like electrodes [2,17]. These nanoparticles generally show intense fluorescence between 440nm-485nm and exhibit long-lived phosphorescence around 530nm. These properties can be optimized and changed by altering the molecular weight of the polylactic acid. This can be useful when attempting to decrease the blue and green scatter that occurs through tissue interfaces by shifting the emission wavelengths towards red wavelengths [2,17].

All boron nanoparticles are pegylated, a process in which the nanoparticles are conjugated with polyethylene glycol. This improves nanoparticle solubility and decreases immunogenicity. It also increases nanoparticle stability without increasing toxicity [25].
The University of Virginia provided three boron nanoparticles for this project with different properties for testing with a red-green-blue channel camera system. All the nanoparticles were mixed with dye-PLA and PLA-PEG for water stability and concentrated at 5:1. The first of these, BNP 1 (BF$_2$dbm(I)PLA), has a chemical structure described by Figure 4.

![Figure 4: Chemical structure of BNP 1 (BF$_2$dbm(I)PLA). A combination of benzene rings, polylactic acid covalently bonded to oxygen, boron-oxygen bonds, BF$_2$ wedged bonds and iodine substitution allows for optimized dual-emissivity that minimized dye leaching and cytotoxicity.](image)

This skeletal structure (Figure 4) describes several carbon chains that increase dye conjugation and exhibit a red-shifted absorption, which is much less phototoxic to biological systems than pure UV excitation wavelengths [25]. Attached to these is a single covalent bond attaching polylactic acid to oxygen, a biodegradable polymer. It also shows boron-oxygen bonding, which allows for shorter phosphorescence lifetimes that are much more intense via increases the crossover efficiency to the triplet state by augmenting spin–orbit coupling [25]. The BF$_2$ wedged bonds produce the unique ability of metal-free phosphorescence in rigid environments as well as intense fluorescence by
limiting intramolecular twisting [25]. The iodine substitution allows for the ratiometric oxygen sensing [25].

The preliminary calibration performed at the University of Virginia shows an excitation wavelength of 375nm and emission wavelengths of fluorescence at 435nm and phosphorescence at 525nm. The figure below also describes the spectra of BNP 1 as it is exposed to different oxygen concentrations. The fluorescence peak does not vary as oxygen levels change; however, the phosphorescence intensities decrease as oxygen levels increase due to collisional quenching.

Figure 5: BNP 1 (BFdbm(I)PLA) emission spectra for varying oxygen concentrations performed at the University of Virginia. The excitation wavelength at 375nm is shown in the blue dotted line. The first peak at 435nm is fluorescence as it is invariant to oxygen changes. The phosphorescence peaks at 525nm show an intensity change that is inversely proportional to increased oxygen in the environment.
The second nanoparticle, BNP 2 (CD-5-21B), shows a similar chemical structure as shown in the Figure 6.

Figure 6: Chemical structure of BNP 2 (BFnbm(I)PLA). A combination of benzene rings, polylactic acid covalently bonded to oxygen, boron-oxygen bonds, BF$_2$ wedged bonds and iodine substitution allows for optimized duel-emissivity that minimized dye leaching and cytotoxicity. The additional benzene ring compared to the structure of BNP 1 changes the absorption and emission characteristics.

The fused benzene rings, also known as naphthalene, is often used to increase solubility.

This slightly altered structure also changes the absorption and emission characteristics of the nanoparticle, as shown in Figure 7 below.
Figure 7: BNP 2 (BFnm(1)PLA) emission spectra for varying oxygen concentrations performed at the University of Virginia. The excitation wavelength of 417nm is shown in the blue dotted line. The first peak is fluorescence at 443nm as it is invariant to oxygen changes. The phosphorescence peaks at 565nm show an intensity change that is inversely proportional to increased oxygen in the environment. Results from calibration performed with a filtered UV source at Duke University is shown in Figure 13.

The University of Virginia reports that the excitation wavelength is 417nm, the fluorescence emission is 443nm and the phosphorescence emission is 565nm. This is quite shifted compared to BNP 1, which was excited around 375nm. Moreover, the fluorescence emission in BNP 2 is far less intense than BNP 1.
Figure 8: Chemical structure of BNP 2 (BFnbm(I)PLA). A combination of benzene rings, polylactic acid covalently bonded to oxygen, boron-oxygen bonds, BF₂ wedged bonds and iodine substitution allows for optimized duel-emissivity that minimized dye leaching and cytotoxicity. The additional benzene ring compared to the structure of BNP 1 and BNP 2 changes the absorption and emission characteristics.

The third boron nanoparticle, BNP 3 (BFnbm(I)PLA), again shows different absorption properties. If we look to its chemical structure (Figure 8), there is another naphthalene ring, which further changes the BNP spectrum (Figure 9). The excitation wavelength is reported as 440nm and the fluorescence and phosphorescence emissions are at 506nm and 572nm, respectively.
Figure 9: BNP 3 (BFnbm(I)PLA) emission spectra for varying oxygen concentrations performed at the University of Virginia. The excitation wavelength of 440nm is shown in the blue dotted line. The first peak is fluorescence at 506nm as it is invariant to oxygen changes. The phosphorescence peaks at 572nm show an intensity change that is inversely proportional to increased oxygen in the environment. Results from calibration performed with a filtered UV source at Duke University is shown in Figure 16.

2.2 Calibration of Boron Nanoparticles via Spectroscopy

Our nanoparticles are synthesized at the University of Virginia, and are mailed to our laboratory. During the shipping process, the nanoparticles’ emission spectra and oxygen sensing properties have varied due to environmental factors that change their sensitivity. Generally, we store the nanoparticles in a cool environment at 3.8°C to increase the shelf life and in the dark, surrounded by foil, to minimize photobleaching.
Our standard protocol is to calibrate the nanoparticles upon arrival and again after several weeks of storage to ensure accurate data collection irrespective of natural nanoparticle degradation.

### 2.2.1 Experimental Setup of Calibration with UV LED Source

The first calibration was performed with a UV LED manufactured by BIVAR (part number UV3TZ-405-30) with a peak wavelength at 405nm and minimum power emission of 20mW. The specifications list the wavelength range to be between 402.5nm and 407nm; however, as the figure below shows, the wavelength ranges from approximately 375nm to 435nm.
Figure 10: UV LED emission spectrum showing a peak at 405nm. This UV source provides the excitation wavelengths for the boron nanoparticle emissions. It is essential that its spectrum is not too wide as the tails will contaminate the fluorescence emissions. As such, we include a bandpass filter that limits the bandwidth of the UV peak.

This causes one of the nanoparticles subtypes to be unsuitable for use with our UV excitation light source, as its excitation frequency does not lie within the UV LED’s spectrum.

The UV source was soldered in series with a 330ohm resistor and a 4.5V battery source. An excitation filter was placed in a holder directly above the UV LED to allow only wavelengths between 391 nm and 437 nm to pass. The nanoparticle sample is immersed in phosphate-buffered solution at a concentration of 1:1. Above the
nanoparticles is the longpass emission filter whose purpose is to block the UV excitation wavelength from being recorded. The UV source is more intense than the emissions; including it in our measurements would challenge our ability to resolve the low-intensity fluorescence and phosphorescence. As such, it blocked wavelengths below 458 nm. Finally, the spectra were recorded by a fiber optics cable attached to an Ocean Optics USB4000 spectrometer. This spectrum was recorded via SpectraSuite software. Note that all measurements were made in a dark room to minimize the background signal.

Three boron nanoparticle solutions were prepared with 0.5mL of nanoparticles combined with 0.5mL of phosphate-buffered saline in a transparent single-well plate. Covering the solution was a layer of Parafilm and a thin, transparent plastic cover to provide more stability (see Fig. 11). In this way, each well was covered and protected from the environment. A small tube attached to an oxygen gauge was inserted into a small hole through the Parafilm and used to change the oxygenation content within the wells.
Figure 11: Experimental set-up for spectra measurements. Boron nanoparticles are placed in a single-well plate at a 1:1 concentration with a phosphate buffer. Below the well is the UV source and the bandpass filter to narrow the UV bandwidth. In the well, oxygen is continuously bubbled to change the environment. The nanoparticle fluorescent and phosphorescent emissions are passed through a longpass filter to eliminate the UV source light. The emissions are measured with a fiber optic sensor and the data is sent to SpectraSuite to be recorded.

Oxygenation percentages (mixed with molecular nitrogen) were bubbled in the nanoparticle solution for five minutes each at 0% O₂, 5% O₂, 10% O₂ and 20% O₂ – after which the spectra were taken. These concentrations were bubbled through in series so
that the tube was not removed between oxygen percentages and the sample was not returned to normal atmospheric oxygen concentrations.

Spectra suite obtained 100 spectra per sample per oxygen concentration. Each of these measurements includes 5 averaged spectra with an integration time of 10ms. A MATLAB program was used to process the spectra by averaging the 100 spectra as well as subtracting the background signal for a final signal with less noise.

2.2.2 Results of Calibration Measurements with UV LED Source

As noted earlier, the first boron nanoparticle, BFNBM(I)PLA, has an excitation wavelength of 375nm, which the UV LED could not excite sufficiently since it is at the edge of its output. Therefore, no data was recorded for this nanoparticle.

The background spectra recorded ambient light from the computer monitor and dark noise and filter bleed-through were subtracted from the subsequent results in Matlab. The filter order and orientation were also checked with a measurement as seen in Figure 12. This confirms that the emission filter successfully narrows the UV LED's peak.
Figure 12: UV LED spectrum with emission filter in place. Previous figures illustrated a broad UV peak that could contaminate the emission spectra of the nanoparticles. With the bandpass filter in place, the UV peak is narrowed.

The other two nanoparticles, CD-5-21B and CD-5-21C, could be excited with the UV LED source. Of these two, CD-5-21B showed prominent, broad peaks for the fluorescence and phosphorescence at 472nm and 550nm, respectively. The fluorescence intensity measurement should not be changing significantly in the presence of varying levels of oxygen while the phosphorescence peaks should decrease as oxygen levels decrease. While it may appear that the fluorescence is changing in the figure below, it did not vary significantly with a standard deviation of 0.0046. Furthermore, this drop is
not entirely unexpected due to an overlap in the fluorescence and phosphorescence channels.

Figure 13: BNP 2 spectra over varying oxygen percentages. The fluorescence peak is at 472nm and the phosphorescence is the series of peaks at 550nm. The phosphorescence is exhibiting a change in intensity as the oxygenation changes. The most intense peak in blue is in an environment of 0% molecular oxygen and the least intense peak in purple is due to collisional quenching in a 20% molecular oxygen environment.

This calibration will be used to predict the levels of oxygen in a tumor environment based on the changing phosphorescent peaks. As seen in the figure below,
a linear relationship with oxygen is established.

![BNP 2 (CD-5-21B) Oxygen Concentration vs Phosphorescent Intensity](image)

Figure 14: This figure illustrates the expected linear relationship between increasing oxygen concentrations and decreasing phosphorescence. The data is in blue, the linear fit is in red and the 95% confidence intervals are in dashed red lines.

In Figure 14, the data is in blue, the linear fit is in red, and the 95% confidence interval is represented by red dashed lines. The linearity of this graph describes the relationship between oxygen and phosphorescence and has an adjusted $R^2$ value of 0.898 (Figure 14). The reported p-value is of this linear fit is 0.0345. When combined with the $R^2$ value, this
indicates a reasonably good linear relationship between phosphorescence intensity and oxygenation between 0% and 20%.

To compare different samples, we can take the ratio of the fluorescence measurements, as this will remain constant throughout the experiments. Figure 15 below shows the fluorescence/phosphorescence (F/P) plot as oxygen percentages increase. The blue line represents the F/P ratio, the red line is the linear fit and the dotted red lines are the 95% confidence intervals. The adjusted $R^2$ for this fit is 0.878 and the $p$-value is 0.0415 thus showing a good linear fit.
Figure 15: Percent oxygen versus fluorescence/phosphorescence intensity with a linear fit. We expect to see the F/P ratio increase as oxygen percentage increases. The data is in blue, the linear fit is in red and the 95% confidence intervals are in dashed red lines.

The third boron nanoparticle (CD-5-21C) shows fluorescence and phosphorescence at wavelengths closer to each other than the previous nanoparticle. According to the calibration measurements performed at the University of Virginia, the fluorescence peak is at 506nm and the phosphorescence at 572nm. In this calibration study, the fluorescence and phosphorescence maximums occur at 509nm and 560nm, respectively (see Figure 16).
Figure 16: BNP 3 spectra over varying oxygen percentages. The fluorescence peak is at 506nm and the phosphorescence is the series of peaks at 572nm. The phosphorescence is exhibiting a change in intensity as the oxygenation changes. The most intense peak in blue is in an environment of 0% molecular oxygen and the least intense peak in purple is due to collisional quenching in a 20% molecular oxygen environment.

We can see that the peak maximums are significantly closer in BNP 3 than BNP 2. This would make it more challenging to isolate the fluorescence and phosphorescence peaks for in vivo studies. Furthermore, the overall intensity of the peaks is much less than those of BNP 2. If we look at the samples with 0% O2 and plot both BNP 2 and BNP 3 on the same graph as shown in Figure 17, we can see that this significant intensity...
difference. When taken together, these differences BNP 2 and BNP 3 indicate that BNP 2 is a better potential candidate for our study.

Figure 17: Spectra comparison of BNP 2 and BNP 3 at 0% oxygen. Note the difference in wavelength emissions. Though we will use both nanoparticles for future calibration studies and the preliminary studies, BNP 2 in blue will likely prove the best to work with. Separating the fluorescent and phosphorescent peaks is necessary to perform a ratiometric analysis. As such, BNP 3, with its peaks separated by only a small distance might prove more difficult to resolve and separate.

Despite the closely-spaced fluorescence and phosphorescence peaks, there is still a linear relationship between both oxygen percentage and phosphorescence (Figure 18A) and oxygen percentage and the F/P ratio (Figure 18B). Figure 18A describes the
percent $O_2$ versus phosphorescence intensity where the data is blue and the linear fit is in red. The reported adjusted $R^2$ for this fit is 0.426. Figure 18B shows the linear fit of the percent oxygen versus the F/P intensity with the data is blue and the linear fit is in red. The adjusted $R^2$ is 0.993. The F/P $R^2$ values for the third nanoparticle are significantly lower than the second nanoparticle at $R^2_{\text{BNP2}} = 0.928$ and $R^2_{\text{BNP3}} = 0.993$, respectively. The p-values are $p_{\text{BNP2}} = 0.0415$ and $p_{\text{BNP3}} = 0.0025$.

![Figure 18A](image1.png)

**Figure 18A**: Describes the relationship between increasing oxygen concentration and decreasing phosphorescence. 18B describes the relationship between increasing oxygen concentration and increasing F/P ratio. The data is in blue, the linear fit is in red and the 95% confidence intervals are in dashed red lines.
2.2.3 Limitations

There are multiple potential sources of error in the measurements. When bubbling the oxygen in the single-well plate, it was not uncommon to find that some of the nanoparticles had adhered to the Parafilm and to the side of the dish. This led to a portion of the BNP not contributing to the spectral measurements because they were outside of the field of view. Additionally, these particles would not show oxygenation changes if they were not suspended in a solution. A closer look at Figure 17 shows a significant change in the fluorescence intensities between 0% and 5% oxygenation, which should be invariant under oxygenation changes. We would generally expect to see little variation across fluorescence as exhibited by BNP 2. However, the standard deviation of the fluorescence in BNP 3 across oxygen concentrations is 0.0130 – a 95% increase compared to the standard deviation of BNP 2.
3. Mobile Camera System

The mobile camera system’s purpose is to attach directly to mouse’s dorsal window chamber and gather data while the mouse is awake and active. The camera collects data on the vasculature as well as the oxygen concentration of the tumor microenvironment. It visualizes vasculature through a white light LED source and records oxygen measurements with a UV LED light excitation source and boron nanoparticle emissions.

Because the system must be attached directly to a 20-25g mouse, the system is limited in both size and weight. Figure 19 is an illustration of a window chamber model and the placement of each of the hardware components of the camera system. The LEDs are placed at the back of the window chamber where the light passes through the skin, the tissue, the glass coverslip, the filter and finally into the camera. This model represents our first iteration of the design. Our second iteration includes a second filter placed between the LEDs and the window chamber (not pictured below).
Figure 19: Mouse model with window chamber and mobile camera system components. An athymic mouse has a window chamber surgically installed. In the tumor-bearing experimental group, they will also have E0771, a breast cancer cell line, injected during surgery and grown through the subsequent week. On one side of the window chamber are the LED light sources. UV light is used to excite the nanoparticles so they emit fluorescence and phosphorescence. Not pictured is a bandpass filter to narrow the UV LED’s spectrum. The light passes through the window chamber and through a longpass filter on the opposite side. This eliminates UV light contamination. Finally, the light is recorded in the Raspberry PI camera and stored in the Raspberry PI computer for subsequent analysis.

Each of these components is placed in a holder that attaches directly to the chamber and are positioned as close as possible to the tissue (see Figure 20). The
limitation in both weight and size of the components and their respective holders is observed to minimize pain and difficulty for the subject. Additionally, an oversized system would increase the likelihood of injuring the delicate skin to which the window chamber is attached. Therefore, we designed the system to minimize these issues.

![Image of mobile unit components](image)

**Figure 20:** Image of mobile unit components. Included in this image is the Raspberry PI computer, the Raspberry PI camera connected via a ribbon cable, the LED circuit connected to the Raspberry PI, the camera holder and the LED light sources in their acrylic holder.

### 3.1 Equipment

The camera system is built from four essential components: A Raspberry PI computer (Figure 21), the Raspberry PI camera, the LED light sources and the filters. The
Raspberry PI computer system provides a Linux environment as well as general-purpose input/output (GPIO) pin on an integrated circuit. This system can control the light sources and the camera through software coding. The camera can attach directly to the circuit via a 1m long ribbon cable and the LED circuit is controlled via pins in the GPIO. The Raspberry PI can be connected through Bluetooth and wireless Internet.

Although its processing speed and RAM are both lower than conventional computers, it can carry out the simple function of gathering and storing data efficiently. Its Internet capabilities are especially helpful in expanding its mobility. While it can be connected to monitors through an HDMI port, we control it through a virtual private network. This enables us to easily move the system and transfer data directly onto a computer for analysis.
Figure 21: Raspberry PI 3 Model B computer. It has ports for power, Ethernet, HDMI, USB and a ribbon cable for connecting the camera. In the upper left-hand corner are the GPIO pins that allow circuit components to be connected and controlled.

The 40 GPIO pins allows us to connect two LED light sources directly to the Raspberry PI and control them via a software interface. There are two LEDs, one emitting white light and the other emitting UV light. Each has their own circuits and their own GPIO pins. The pins each output 3.3V. Due to limits in the current, the white LED circuit contains a ballast resistor of 330 ohms in series with the LED. Despite having their own input pins, they share a ground pin on the GPIO.

The system contains two filters: one to narrow the excitation range of the UV LED and the other to limit the wavelengths recorded by the camera. The excitation filter
passes the excitation frequency ranges of the UV LED. We determined through a pilot spectroscopy experiment that the UV LED spectrum contains long tails and a broad peak that interfered with our fluorescence measurement. These tails are not needed for excitation purposes, so we introduced a bandpass to pass only the wavelengths around 405nm (see Figure 22). The filter has a diameter of 10mm, which makes it ideal for a system that is limited in size. This filter is attached to the acrylic holder that houses the LEDs and attaches to the window chamber.

Figure 22: Excitation filter transmission spectrum. Manufactured by Chroma, the ET405/20x 12.5mm bandpass filter is used to narrow the bandwidth of the UV light source to around 405nm. The UV LED spectrum is fairly wide and contaminates the fluorescence emission of the nanoparticles if it is not filtered.

The second filter is a longpass filter designed to transmit only the fluorescence and phosphorescence while blocking the UV excitation frequencies. Figure 23 shows the spectra of the emission filter. It transmits all wavelengths above 450nm and is placed
directly between the mouse window chamber and the camera as shown in Figure 19. We choose a filter with a diameter of 10mm to minimize its size and weight.

![Emission filter spectrum. Manufactured by Chroma, the AT450lp longpass filter. This is placed such that the UV excitation light source’s wavelengths are blocked and the emission spectra of the boron nanoparticles is passed. It is placed between the emissions and the camera and passes wavelengths above 450nm.](image)

The final components of the device are the two holders. One houses the LEDs and the excitation filter and the other holds the emission filter and camera. The LED holder is an acrylic piece into which we drilled holes to fit directly onto the bolts protruding from the back of the window chamber. The LEDs fit in another set of holes aligned with the target tissue and were kept in place through an epoxy application. The window chamber sits between the LED and the camera. Each greater in dimension than the window chamber and are affixed directly to one another.
The challenge with this design is that it does not allow for the excitation filter to be placed between the window chamber and the LEDs. We are currently working to redesign the holder to allow for additional room to house the filter.

The camera itself has a minimum thickness due to the size of the lens as seen in the figure below. The holder that houses both the camera and the filter is larger than the LED holder and has gone through several design iterations to minimize its size and weight. Currently, a lightweight foam houses the hardware which is attached to the LED holder with bolts.

Figure 24: Raspberry PI camera model 1538 is used for image acquisition. It is small – only 24 x 25 x 10 mm and capable of resolving small vessels in window chamber models.

3.2 Python Coding

The Raspberry PI controls the LEDs and camera via a Python interface. Python is a high-level programming language that has a wide variety of applications and can be
run on nearly every operating system. A compiler was the only outside installation needed on the Raspberry PI to run the Python programs.

The Python interface is simple with its main function controlling the camera, LEDs and storing the data in certain directories. We added a primitive user interface so that each sample is named and saved as the program runs. The camera’s warm-up time, exposure time, sensitivity, and image format are all included in the program. The exposure time and sensitivity can easily be changed to accommodate different experimental situations. We added the Python image library module to the Raspberry PI to save the data in uncompressed data formats. As such, all images were recorded as both .data and .tiff.

The LED controls are incorporated in the same program that controls the camera. This allows us to turn on the light source, acquire an image and turn the LED off through a few lines of code. This is made possible by allowing Python to act as a voltage switch for the GPIO pins.

**3.3 System Specifications**

The system specifications can be found in Table 2. This includes the capabilities reported by the various manufactures for each piece of hardware as well as the settings used in the complete unit as applicable.
Table 2: Hardware specifications for Raspberry PI mobile unit for mouse window chamber models

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<p>| Weight        | 3g               |</p>
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<tr>
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<td>Excitation Filter</td>
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<td>Power Output: High Intensity</td>
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<table>
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<th>C513A-WSS-CV0Y0151</th>
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<td>2g</td>
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<tr>
<td>LED Holder</td>
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<td></td>
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<tr>
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<td></td>
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<tr>
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<td>5g</td>
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<tr>
<td>Camera Holder</td>
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<td></td>
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<tr>
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<td>Weight</td>
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<td>10g (with camera and filter)</td>
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<td>Window Chamber</td>
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</tr>
<tr>
<td>Weight</td>
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<td>4g</td>
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3.4 Unit Analysis

The mobile unit performed well in calibration and preliminary *in vivo* studies. The resolution is high enough to resolve tumor vasculature under the white LED. The sensitivity is high enough to detect fluorescence/phosphorescence for quantification. However, there are several challenges that are being addressed in the mobile unit as it is being optimized.

The first issue is the camera’s focal length. The Raspberry PI camera has a permanent focal length of 3.6mm that is out of focus for our purposes. However, we require the camera to be focused between 1 and 1.5mm. To obtain this result, the camera lens was forcibly removed and a small, acrylic washer was inserted between the lens and the camera. This increased distance between the sensor and the camera lens to be within our required range (see Figure 25).
Figure 25: The Raspberry PI camera’s lens system is a permanent fixture and incapable of focusing at different points. Therefore, it is necessary to remove the lens entirely and insert an acrylic washer to increase the focal length, which moves the focus closer to the camera.

Another challenge is the UV source itself. The UV LED, as shown in Table 2, has a constant power output of 30μW. While this intensity output is sufficient for calibration studies where the nanoparticles are suspended in a saline fluid, it is not intense enough for in vivo applications. Therefore, for the studies reported in this paper, an outside UV source was used.

This outside UV source when utilized in the fluorescent microscope, has produced successful results in both in vitro and in vivo studies. Additionally, the outside UV source has a dynamic output which is ideal for our purposes. We quantified phosphorescence in our preliminary calibration studies with a UV power output of ~14mW. During in vivo studies where a higher intensity is necessary to transmit through tissue, we used an output of ~150mW and successfully detected nanoparticle emissions.
However, the UV source described above is not mobile and cannot be attached to the window chamber. Therefore, we require a new mobile, high-power, dynamic UV LED source. To address the need for a dynamic UV source, we can design a circuit that connects the mobile UV LED in series with a potentiometer or variable resistor. This is one of our immediate next steps in optimizing the mobile unit.
4. Boron Nanoparticle Calibration with Camera

4.1 Method

Our experiments use a camera system to excite samples of diluted boron nanoparticles under varying oxygen concentrations. This experiment uses the camera system to excite samples of diluted boron nanoparticles under varying oxygen concentrations. The nanoparticles were diluted 1:1 in a phosphate buffer solution and placed in a single well in a standard 6-well plate. The plate’s top and bottom were layered with black tape to minimize scattering and reflection of UV light. A small hole was left open through which the camera imaged the nanoparticles. A changing oxygen environment was maintained via continuously pumping oxygen through a tube and into the well via oxygen and nitrogen gases mixed through an apparatus that includes a gauge in which to change the mixture of gases.

Samples were prepared at oxygen levels of 0% O₂, 5% O₂, 10% O₂, 20% O₂ and 100% O₂ were tested. The oxygen was mixed with molecular nitrogen and pumped continuously in the well for three minutes and turned off for the subsequent imaging. The exposure time and sensitivity of the camera was kept constant throughout the experiment at 10ms and 300, respectively. The camera setup is described in Chapter 3 with information on how the data is recorded. The analysis was performed in Matlab by placing an ROI around the area of highest intensity and recording the mean values for the red, green and blue channels. More information is found in Section 4.2.
4.2 Results

4.2.1 Boron Nanoparticle 2

The results from BNP 2 are shown in the figure below. The three digital color channels are split and shown in red, green and blue. As expected, there is a decreasing trend in intensity as oxygenation levels increase. Furthermore, the majority of signals is in the green and blue channels, which follows what the spectra for this nanoparticle reported previously.

Figure 26: Phosphorescence intensity changes in the presence of varying oxygen for BNP 2. On the y-axis, we have intensity measurements for each of the red, green and blue (RGB) color channels. On the x-axis is the oxygen concentration from 0% to 100%. Here, the fluorescence wavelengths are represented in the blue channel and the phosphorescence is represented in the green and red channels. Their different
trends in the presence of different oxygen concentrations allows for quantification of the ratiometric fluorescence/phosphorescence ratio. 4.2.2 Boron Nanoparticle 3

The third boron nanoparticle results are reported below. There is a general decreasing trend in all three channels (red, green and blue) as oxygenation increases. In fact, each channel is following essentially the same overall trend at different intensities that coincide with the emission spectra of the nanoparticle.

![BNP 3: Mean Pixel Values for Varying Oxygen Concentrations](image)

Figure 27: Phosphorescence intensity changes in the presence of varying oxygen for BNP 3. On the y-axis, we have intensity measurements for each of the red, green and blue (RGB) color channels. On the x-axis is the oxygen concentration from 0% to 100%. Here, the florescence wavelengths are represented in the blue channel and the phosphorescence is represented in the green and red channels. Their different trends where the green and red channels decrease much more quickly than the blue channel as oxygen concentration increases allows for quantification of the ratiometric fluorescence/phosphorescence ratio.
4.3 RGB Channel Analysis

The analysis of the data requires that the fluorescence and phosphorescence data be split from the overall data. This is accomplished by splitting the red, green and blue (RGB) channels of the .tiff data. Ideal nanoparticles would fluoresce in the blue channel and phosphoresce in either the green or the red channel. In reality, there is an overlap in the channels.

A region of interest is placed around the brightest portion of the image to acquire a lower standard deviation and an accurate mean pixel value. This is repeated for each color channel and each observation after a dark image is subtracted to reduce noise. Five observations were taken of each sample, and the mean of their mean pixel value was taken. This final value is used for subsequent analysis of changing intensity under varying oxygenation conditions.

4.3.1 Boron Nanoparticle 2

For BNP 2, we isolated the fluorescence and phosphorescence. In Figure 26 each channel decreases in intensity as phosphorescence decreases with increasing oxygen exposure. The most relevant channels are blue for fluorescence and green for phosphorescence as the latter has a higher intensity than the red channel despite the same trend. There may be some overlapping in the intensities where the fluorescence and phosphorescence are both represented in the same channel due to large channel
bandwidths; however, because the blue channel does not decrease as quickly as the green channel suggests that we can represent phosphorescence in green and fluorescence in blue. We can see that the blue channel between 5% and 20% stays within an intensity range of +\(-3. For the same O2 range the green channel’s intensity changes by approximately +\(-15.

For BNP 3, there is not as clear a change in intensity between the green and blue channels. If we look at its spectra in Figure 15, we can see that the fluorescence and phosphorescence peaks are both in the green, with only 50nm separating their maxima. We can still see a trend (shown in Figure 27) of increasing oxygenation and decreasing intensity, which is the phosphorescence. While this cannot be used for radiometric analysis, it is still useful in proving that our camera is sensitive enough to pick up these weak signals. Additionally, it validates the use of BNP 3 for experiments in which phosphorescence alone would be needed. Note that the increasing blue intensity between 20% oxygen and 100% oxygen is due to light scatter in the well. At higher phosphorescence intensities, this signal is minimal; however, the scatter intensities approach fluorescent and phosphorescent intensities at 100% oxygen.
5. *In Vivo* Pilot Mouse Study

5.1 Method

As a pilot study, our goal was to determine whether our system can adequately resolve vasculature structures and quantify fluorescence and phosphorescence in *in vivo* mouse models. We had two experimental groups: one tumor bearing and the other non-tumor bearing experimental control. Each mouse subject had a dorsal window chamber surgically installed. For the tumor-bearing mice, the E0771 breast cancer cell line was cultured and 5 million cells/µL were suspended in a phosphate buffered solution and prepared for injection. The recovery period and tumor growth time is approximately one week.

The subjects were anesthetized with ketamine/xylazine at a dose of 10µL/g when imaging the vasculature and isoflurane was used to during nanoparticle imaging.

Thirty minutes prior to imaging, the mice were injected with 50µL of boron nanoparticles. Using a fine needle, the nanoparticles are injected directly into the chamber through the skin at the back. This waiting time allows for the nanoparticles to diffuse and interact with the tissue environment. This injection protocol keeps the nanoparticles from degrading and losing their phosphorescence as they would if injected through a tail vein or intraperitoneally.
The vasculature was imaged under the white LED with an ISO of 300 and an exposure time of 100µs. The fluorescence and phosphorescence was excited with the UV source attached to the fluorescent microscope, as the UV LED was not intense enough to transmit through the tissue. The camera settings are recorded in Table 3.

**Table 3: Camera setting for the boron nanoparticle emissions *in vivo* mouse study**

<table>
<thead>
<tr>
<th></th>
<th>ISO</th>
<th>Exposure Time [µs]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BNP 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>900</td>
<td>360</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>100</td>
<td>2400</td>
</tr>
<tr>
<td><strong>BNP 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>900</td>
<td>360</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>1600</td>
<td>3600</td>
</tr>
</tbody>
</table>

**5.2 Results**

The vasculature is shown in the image below for a negative control mouse without a tumor. The focus was set to ~1.5mm. Note that the vasculature can clearly be distinguished from the surrounding tissue.
Figure 28: Vasculature of negative control mouse imaged under white light LED. This subject was not tumor-bearing and boron nanoparticle emissions are not evident. As we are illuminating the tissue with a white-light LED, this is expected as we are interested in imaging the tissue and vasculature.

The nanoparticle emissions are reported below. Note that the image is out of focus, as the setup for an external UV source required a different imaging distance than the vasculature imaging. However, as we are solely interested in the fluorescence and phosphorescence quantification, the image is sufficient for our proof of concept.
Figure 29: Negative control mouse imaged with BNP 2. This subject was not tumor-bearing and was injected with BNP 2 30 minutes prior to imaging. While the subject is out of focus due to a change in imaging setup, we are only concerned with recording the nanoparticle emissions and not analyzing the vasculature. Therefore, this image is sufficient for our current proof-of-concept study.

Figure 30: Negative control mouse imaged with BNP 3. This subject was not tumor-bearing and was injected with BNP 3 30 minutes prior to imaging. While the subject is out of focus due to a change in imaging setup, we are only concerned with
recording the nanoparticle emissions and not analyzing the vasculature. Therefore, this image is sufficient for our current proof-of-concept study.

Both boron nanoparticle types (BNP 2 and BNP 3) were used in this study with the first mouse as the negative control in Figures 29 and 30 (no tumor) and the second mouse with a tumor (Figures 31 and 32).

Figure 31: Mouse with cancerous tumor imaged with BNP 2. This subject was tumor-bearing with an E0771 breast cancer solid tumor. The subject was injected with BNP 2 30 minutes prior to imaging. The emission spectra of the nanoparticles is evident as we are exciting the tissue with UV light.
Figure 32: Mouse with cancerous tumor imaged with BNP 3. This subject was tumor-bearing with an E0771 breast cancer solid tumor. The subject was injected with BNP 3 30 minutes prior to imaging. The emission spectra of the nanoparticles is evident as we are exciting the tissue with UV light.

5.3 Analysis

Beginning with the vasculature image in Figure 28, the white-light LED is intense enough to transmit through the tissue. The green hue is due to the excitation and emission filters. The resolution and sensitivity of the camera are high enough to clearly differentiate vasculature in the image. Notice that there is some vignetting in the bottom of the image where there is a discrepancy in the brightness compared to the center of the image. This is due to the curvature of the tissue. Any slight changes in tissue depth are going to be apparent when working with a small field-of-view and at such a close
working distance. As long as the camera is centered on the vasculature of interest, this should not be problematic.

The nanoparticle emission images were also successful. Ignoring the blur due to the fixed focus, the images still show some of the stronger features, especially in Figures 31 and 32. However, the field of view is clearly too large at this setup where the UV source is not exciting more than a portion of the sample adequately. Nevertheless, with a UV LED (as seen with the white LED), a more uniform image might be achievable.

There is a clear change in color between, not only the different boron nanoparticles, but also the negative control mouse and mouse with a tumor. This is not surprising as we consider their contrasting environments. Furthermore, their acquisition parameters were not kept constant as an attempt to determine the optimal settings for each environment.

With that said, we attempted to use our previous calibration curves to quantify the oxygen in the mice; however, the results made it clear that the system needs to be fully optimized and variable reduced before this is feasible. In our next in vivo experiment with the proper UV source we intend to change the oxygenation environment that the mice breathe to determine if we can quantify the ratiometric change accordingly.
6. Future Work

Our immediate goal is straightforward: to develop a high-resolution mobile imaging unit that can measure ratiometric oxygen sensing on active mouse models via fluorescence and phosphorescence. We have successfully built the system and obtained favorable results. The system optimization is the direct next step with a dynamic UV source and new holders to accommodate this source and the additional excitation filter. Further nanoparticle calibration experiments are also necessary as we determine the optimized nanoparticles for RGB channel analysis. We would also like to characterize any nanoparticle dependence on biological factors such as temperature and pH. After these steps are completed, we will focus on the long-term goals of this system and its useful endpoints in cancer studies such as dynamic cycling hypoxia and vasculature modeling.

From a physiological standpoint, the vasculature is of great interest in cancerous tumors because it forms chaotic, unpredictable pathways before therapy that change and reform after therapy. The angiogenesis of cancerous tumors describes how it grows, progresses, and regenerated from injury. Recording the growth and development of the vasculature in a longitudinal study combined with functional information can yield invaluable insight on the effectiveness of treatment response. The Raspberry PI camera system has video capability. While this resource was not exploited in our current
optimization scheme the uncompressed video files at user-selected frame rates could obtain valuable information on cancerous angiogenesis as well because we could analyze the real-time vasculature changes after treatment and model the dynamic changes.

From a functional standpoint, oxygenation has already been described as an important endpoint as hypoxia is a strong predictor of patient outcomes. Additionally, because hypoxic tumors are less radiosensitive, their therapy responses are diminished. This longitudinal study of hypoxia and radiation therapy can also be applied for chemotherapy, immunotherapy and radiosensitizing drugs. As with analysis of the ongoing angiogenesis and cycling hypoxia, video capability in addition to the photos provides a fruitful avenue for the continued research and development of our system. We also know that hypoxia can change quickly during and after therapy and has an intrinsic periodicity of several hours to days [8]. But it is also true that the effectiveness and thus the ideal treatment regimen at any given time is highly affected by tumor oxygenation. Thus, the ability to obtain near-continuous data as tumor hypoxia changes over time could lead to new breakthroughs in understanding the nature of cycling hypoxia and its effects in cancer therapy. Furthermore, the fact that the system does not require the subjects to be anesthetized means that on top of the breakthroughs in dynamic hypoxia measurement, the system will bring a kind of accuracy to its hypoxia
measurements in general that have not yet been seen in this kind of research and could have wide ranging potential for the improvement of cancer therapies.
7. Conclusion

Duel-emissive nanoparticles are paving the way in ratiometric oxygen sensing studies. By studying the hypoxic states and how they vary in tumors in in vivo mouse models we can describe the microenvironment, hemodynamics, temporal instability of hypoxia and response for a variety of therapeutic applications [2]. The current method of using in vivo fluorescence microscopy (or other imaging modalities) requires that the mouse be anesthetized, which causes tumor hypoxia and changes in tumor oxygenation over time [2-4]. To overcome this limitation, a mobile imaging unit was developed to image active awake mice breathing room air over long periods of time. Despite the initial limitations of using the CCD array to obtain high resolution and sensitivity measurements, our calibration studies have been successful in measuring various oxygen concentrations via fluorescence and phosphorescence for two types of boron nanoparticles. This has allowed us to experimentally determine the ideal nanoparticle emission spectra to enable RGB analysis, which is an important first step in optimizing this system for in vivo hypoxia studies.

Our in vivo studies have also been successful at visualizing vasculature and detecting boron nanoparticle emissions. By injecting nanoparticles directly into dorsal window chambers in mice, we detected phosphorescence and fluorescence for mice with and without the presence of cancerous tumors. Using the white light function of the
camera, we were also successful in resolving the vasculature in the window chambers. Our next steps involve optimizing the mobile camera system as well as the boron nanoparticles such that it can provide a novel imaging technique for quantifying hypoxia in cancerous tumors, visualizing the changing vasculature and modeling cycling hypoxia while the mice are not anesthetized. All in all, our initial success and the obvious avenues of continued development represent potentially transformative development in cancer therapy.
Appendix A

This appendix includes the Python code that controls both the camera and the LEDs.

```python
import time
camera = PiCamera()
magic Semiconductor
import os
import picamera
import numpy as np
from PIL import Image
GPIO.setmode(GPIO.BCM)
GPIO.setup(7, GPIO.OUT)
GPIO.setup(29, GPIO.OUT)

# Update the date before performing the study in the "if not" statement and in
# image_data
# The folder with the date does not need to be made beforehand
image_data = raw_input("Enter study information: ")
if not os.path.exists('/home/pi/Image_Data/3-23-17/\nimage_data = os.makedirs('/home/pi/Image_Data/3-23-17/\n
# Prompt user for input
iterations = raw_input("Enter image number: ")
i = int(iterations)

with Camera() as camera:
    with Camera.Array.PiBayerArray(camera) as streamUV:
        # Turn on UV LED
        cameraISO = 300
        camera.shutter_speed = 10000
        camera.capture(streamUV, 'jpeg', bayer=True)
        outputUV = (streamUV.demosaic() >> 2).astype(np.uint8)
        with open('/UVimage%d.data' % i, 'wb') as dataUV:
            outputUV.tofile(dataUV)
            im = Image.fromarray(outputUV)
            im.save('UVimage%d.tiff' % i)
        GPIO.output(7, False)

with Camera() as camera:
    with Camera.Array.PiBayerArray(camera) as stream_white:
        # Camera warm-up
        time.sleep(2)
        cameraISO = 800
        camera.shutter_speed = 2500
        camera.capture(stream_white, 'jpeg', bayer=True)
        time.sleep(2)
        output_white = (stream_white.demosaic() >> 2).astype(np.uint8)
        with open('whiteimage%d.data' % i, 'wb') as data_white:
            output_white.tofile(data_white)
            im = Image.fromarray(output_white)
            im.save('whiteimage%d.tiff' % i)
        GPIO.output(29, False)
        GPIO.cleanup()
```

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References


