

Aminolevulinic Acid based Photodynamic Therapy and Diffuse Optical Reflectance Spectroscopy

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Dedication

To my parents,
Patricia and Paul Engelking.

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I would like to thank all of those that helped me through the learning process of designing, writing and following through on all aspects of a PhD thesis.

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Abstract

Aminolevulinic Acid based Photodynamic Therapy and Diffuse Optical Reflectance Spectroscopy

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In this thesis, aminolevulinic acid, ALA, induced photodynamic therapy is explored as a possible treatment of both equine sarcoids and canine acanthomatous epulis. ALA is relatively inexpensive, can be applied locally (either topically or injected) or systemically and does not leave the patient photosensitive for extended periods of time. This is because ALA is converted into protoporphyrin IX, PpIX, which is made in the heme pathway. Since cells normally create this PpIX in smaller amounts than is used for a PDT treatment, the cells already are good at minimizing the photosensitivity from this drug.

Currently sarcoids are the most common equine tumor. Unfortunately none of the methods to treat sarcoids are effective at treating these sarcoids. Some of the side effects of the current treatments can be scarring or a tumor can become aggravated and begin to grow rapidly afterwards making veterinarians hesitant to treat these tumors for fear of making them more aggressive.

Acanthomatous epulis is a cancer in the canine mandible that is very aggressive and often progressing into the bone of the mandible. Under normal circumstances

the animal has to have a pieces of the mandible removed if the cancer is not caught at an early stage. If it was possible to treat this tumor with another method other than surgical excision of the bone then this would be a great advantage to the afflicted animal.

One portion of these photodynamic therapy treatments was to develop a optical method to measure the changes in the tissue, such as a change in the amount of blood in the local tissue volume. A diffuse optical reflectance spectrometer was assembled and calibrated using tissue phantoms. These tissue phantoms were created to simulate the absorption and scattering ranges of tissue. These phantoms have known spectral absorption coefficients, from india ink, and reduced scattering coefficients, from intralipid, all suspended in acrylimide gels. Properties of the tissue such as the absorption of the oxygen saturation, fraction of blood and the protoporphyrin IX and the scattering in the tissue can be monitored by an iterative function based on the root mean square error from the measured and predicted tissue reflectance program written in Matlab. The absorption coefficient range for these gels was from 0 to 6.5cm^{-1} and the reduced scattering coefficient varied from 1 to 28.3 cm^{-1} . By fitting for the original phantoms the measurements showed a variation $\pm 0.68\text{ cm}^{-1}$ in the middle of our range and at the extreams up to $\pm 2.2\text{ cm}^{-1}$. But these two spectra, the absorption of india ink and the scattering coefficient, were so similar the for more unique spectra the accuracy is increased.

Using this Matlab program the build up of the concentration of the photosensitive drug, protoporphyrin IX, can be measured using its absorption at 635nm. Other properties of the tissue that can be measured are the amount of blood, oxygenation of the blood and the scattering of the tissue. In this document, one canine afflicted with acanthomatous epulis and fourteen equine with sarcoids that have been treated with one or multiple photodynamic therapy treatments are reviewed.

The minimum amount of PpIX and light in the local tissue volume for an effective treatment is dependant on the type of tissue that is treated. The photodynamic threshold dose found for the canine acanthomatous epulis tissue was $7.3(\pm 0.7) \times 10^{18}$ photons per gram of tissue. For equine sarcoid tissue, the photodynamic threshold

dose ranged from $6 - 8 \times 10^{18}$ photons per gram of tissue. For most treatments, the treatment depth was about 2 mm. The extinction coefficient of PpIX at 635 nm was found to measure in the tissue to be about $21.6 \text{ mm}^{-1} \text{ mM}^{-1}$.

A component model describing the rates that ALA and PpIX build up and leave the local treatment tissue was used. In this model, ALA was applied to the surface of the tissue at a known concentration. After the ALA had converted to PpIX the absorption of the PpIX could be measured. This rate was found to be about 1.2×10^{-3} to $1.6 \times 10^{-4} \text{ [hr}^{-1}\text{]}$. The rate that the PpIX was found to leave the tissue with no treatment light on was about 0.4 to $1.4 \text{ [hr}^{-1}\text{]}$. The rate that the PpIX was photobleach or converted to photoproducts was found to be about $0.1 \text{ [hr}^{-1}\text{]}$.

For the canine acanthomatous epulis, the photodynamic treatment was not aggressive enough for this tissue type. The current treatment light will not treat deep enough into the tissue to treat the full extent of the tumor down to the bone. For equine sarcoids this treatment seems an effective method of maintenance since surgery is known to aggravate the tumors in to aggressive growth but in the combination with a PDT treatment the growth seems to be kept under control. Combined with surgery, aesthetic results that may last up to two years can be achieved. Once the sarcoid starts returning another photodynamic therapy treatment will be needed to keep the tumor under control and limiting the size. This combination of surgery and photodynamic therapy was very effective at treating an equine with a tumor over the eye where other methods of treatment may have lost the eye itself, the photodynamic therapy treatment with surgical excision gave back the use of the eye.

In conclusion, ALA-induced photodynamic therapy is not the best method for curing such deeply penetrating tumors. Though ALA-induced photodynamic therapy is effective as a maintenance treatment for sarcoids and may be useful for other shallow ailments.

Chapter 1

Introduction

1.1 Background

In this study, aminolevulinic-acid-based photodynamic therapy was used to treat equine sarcoids and a canine acanthomous epulis. Many of these treatments were designed to find the dosimetry necessary to treat the tissue and were not designed to cure the animal of the tumor. During these treatments, the tissue was monitored optically using a diffuse optical reflectance probe.

1.1.1 Photodynamic Therapy Based on 5-Aminolevulinic Acid

Photodynamic therapy (PDT) is a light-activated chemotherapy. The delivery of light activates a photosensitizing drug to yield oxidizing molecules, singlet oxygen, that cause lethal injury to cells. This treatment is selective since the treatment will only work when all three things are available: (1) light, (2) photosensitizer and (3) oxygen.

The treatment area is illuminated on the surface with a laser or light emitting diode (LED). Each of the PDT drugs used for PDT have optimal wavelengths that they absorb at. One of the most important aspects to a PDT treatment is getting the drugs and the correct wavelength of light to the depth necessary for an effective treatment.

In this thesis, the drug, 5-aminolevulinic acid (ALA), was administered to the

treatment area. The ALA was converted in the cells to a photosensitive drug, protoporphyrin IX (PpIX), by a process described in section 3.3. Ideally the PpIX will only accumulate in the sarcoid tissue and not in the normal tissue for a treatment.

The oxygen necessary for a treatment is often found in the tissue itself. A treatment may become ineffective if there is not enough oxygen in the local area. This was not a problem for the tissue types studied here since they are vascular.

During a PDT treatment, both light and photosensitizer must combine to send the cells into either apoptosis or necrosis. This dosimetry is dependent on the drug, tissue type and light source used. The minimum amount of light and drug combination that will damage the tissue is called the threshold PDT dose [2, 3]. Depending on the tissue type this PDT dose can vary due to the vascularity, amount of water and other absorbers or scatterers in the tissue. Chapter 5 will cover the specifics of a PDT treatment in depth.

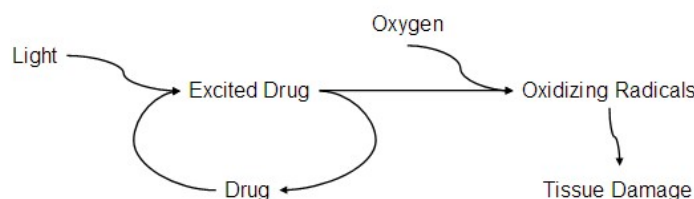


Figure 1.1: This figure shows the three key factors for an effective PDT treatment. The drug is added to the tissue of interest. Once light is absorbed by the drug the drug becomes activated. When oxygen is in the presence of the activated drug, singlet oxygen may be formed that can cause damage to the cells. If any one of these factors are missing the treatments becomes ineffective.

Photodynamic therapy based on ALA has been used to treat many skin conditions including Bowen's disease, actinic keratosis, basal cell carcinoma, psoriasis and acne.

Topical application of ALA is common for the treatment of these skin conditions though a systemic administration of the ALA could also be performed. Once ALA is administered it begins to convert to PpIX which fluoresces. The PpIX builds up

more in the abnormal tissue than the normal tissue. This has made it possible to use the fluorescence of the PpIX as a diagnostic tool to locate and image the skin conditions. There are not many side effects from a PDT treatment. Two of the major side effects of a PDT treatment are erythema and photosensitivity. Photofrin is one of the most commonly used drugs for PDT treatments though it has a much more extreme photosensitivity side effect. Photofrin can leave a human photosensitive for weeks to a month while PpIX will only photosensitize the body for hours to days when given systemically.

1.1.2 Sarcoids

Sarcoids are the most common neoplasm in horses, donkeys and mules [4–8]. These tumors are invasive and recurrent yet not classified as malignant. Location and size of the sarcoid are factors that can dictate the severity and need for treatment. They can occur singly or in clusters [9]. Sarcoids can occur anywhere in the equine skin yet are most common in the abdomen and limbs [4, 6]. The appearance of the tumor can range from benign verrucous tumors to locally aggressive ulcerative fibroblastic lesions [10]. There are five main classifications for the gross appearance of sarcoids: occult, verrucous, nodular, fibroblastic and mixed. Occult sarcoids are hairless sometimes roughened, circular skin areas. Verrucous sarcoids are wart-like lesions. Fibroblastic sarcoids are fleshy and ulcerated. Nodular sarcoids are firm masses lying under apparently normal skin. Mixed sarcoids are a combination of verrucous, nodular and fibroblastic sarcoids [11].

All types of these sarcoids present a thickening of the skin and may be ulcerated and/or infected. Sarcoids are usually not life threatening in their nature but do develop in locations that cause problems. In animals afflicted with sarcoids the areas of a horse that are irritated or sweat frequently may induce a sarcoid to grow. If the sarcoid is on the back of a horse it may prevent saddling and riding the horse. The sarcoids often will grow around the halter and can blind a horse by growing around the eye. Show horses are unable to perform due to the unsightly sarcoid growths.

These skin tumors may increase in size to become as large as a man's fist or larger in some cases.



Figure 1.2: This is a donkey that has lost use of its left eye due to the growth of a sarcoid on her left eyelid. Eventually this sarcoid grew so large to obstruct the eye itself.

Histologically these different sarcoid types look very similar [11]. Histological diagnosis is based on the presence of a capillary-poor fibroblast proliferation of moderate to high cell density [4, 9]. Individual cells are fusiform or spindle shaped, forming whorls, interlacing bundles and haphazard arrays with one another [9]. Biopsies can be very dangerous unless treated directly after and can often precipitate a considerable proliferation both on the surface and in the adjacent dermis and epidermis [12].

Sarcoids are made up mostly of equine dermal fibroblasts with varying amounts of collagen. The mitotic rate is very low [4, 9]. About 20% of all equine tumors have been sarcoids [9]. The sarcoids can grow to be larger than the size of a man's fist with thick or ulcerated epidermis.

There is some debate as to the cause of the sarcoids and how they are spread. In 1948 studies from Olson suggested an infectious origin, a hypothesis subsequently examined by several others [4, 8, 13, 14]. Bovine papilloma virus (BPV) when injected

into horses created sarcoid-like tumors that regressed on their own while antibodies to the virus are formed [14, 15]. Polymerase chain reaction, (PCR), has been used to determine the presence of BPV-DNA. In a study by Martens et al., all of their tested sarcoid tumors were positive for BPV-DNA. BVP1 and BVP2 were detected at 74% and 26% respectively.[8] Sarcoids also express the BPV types 1 and 2 major transforming protein, E5 [10, 16].

The sarcoid often has the majority of its mass in one location with fingers of tumor tissue that reach into the normal tissue. The depth of these fingers is not found in the literature but we have found they are usually on the order of 5 millimeters. Often surgery will not remove all of the sarcoid tissue leaving tumor cells from one of the fingers to seed a new growth that usually reappears as a more aggressive tumor. Due to this reason many veterinarians will not treat small sarcoids for fear of aggravating them. When treating cancers in humans, it is always best to catch the tumors at early stages. Veterinarians also need an effective method of treatment for sarcoids at an early stage before the tumors become large and invasive. PDT may be a method to noninvasively treat sarcoid tumors at an early stage.

There are many different types of treatments for sarcoids yet none of the current treatments for equine sarcoids are effective for all cases [9]. Among these methods are surgical excision, cryotherapy, carbon dioxide laser surgery, immunotherapy, radiation therapy, hyperthermia, and chemotherapy. The cryotherapy used after surgical excision has given a 70% success rate with a 30% recurrence rate [4].

Though none of these methods are consistently effective at treating sarcoids for example: cryotherapy can not be used near the eye tissue without damaging the eye and can often cause scarring. Some of these methods of treatment can become very expensive, and some can not be repeated due to toxicity.

Photodynamic therapy has not been used on equine sarcoids previously save for one case. Martens et al. performed a treatment using hypericin, a photosensitizer originating from Saint John's wart [11]. Horses that are allowed to feed in a field containing this plant may ingest enough to become photosensitive and are easily burnt by the sun. Martens' group reduced the size of a sarcoid to 81% on a donkey

by using hypericin based PDT.

Due to the nature of sarcoids, aggravation by biopsy or treatment can cause it to start growing quickly. Many veterinarians will not take a biopsy unless they are going to treat the sarcoid [7, 12, 17]. They will wait until it becomes a large tumor or starts growing on its own. The optimal time to treat these sarcoids would be when they are small and in a method that does not aggravate them and initiate rapid growth.

1.1.3 Diffuse Optical Reflectance Spectroscopy

Diffuse optical reflectance spectroscopy is a method to get optical information from a medium by directing a light source into the substrate and collecting the light that is reflected. This method can yield optical information of the substrate. The concentration of both the optical absorption and scattering of the substrate will change the path the light takes once incident upon the surface of the substrate to result in returning more or less light. The wavelength dependence of the absorption and scattering can also be explored.

An Ocean Optics spectrometer (S-2000) with an Ocean Optics tungsten halogen light source (LS-1) and an optical fiber probe was used to measure changes in the diffuse reflectance from the tissue. The geometry of the fiber probe is six fibers around one all of them with a diameter of 200 microns. This diffuse optical reflectance spectroscopy probe is held up to the tissue with light pressure. The light reflected from the tissue is recorded with a laptop computer with an integration time of 15 ms.

There are many different components in tissue that absorb light. Oxy-hemoglobin, deoxy-hemoglobin, water, and melanin all have their own absorption spectra. Linear combinations of the absorption spectra found in the tissue are fit to the recorded reflectance spectra using an iterative fitting algorithm. As the ALA converts to PpIX, it begins absorbing more light with large peaks at 400, 502, 538, 573 and 627 nm absorption spectra in methanol from Sigma. Though the major PpIX absorption peak observed in tissue is the 627 nm peak and it is observed at 635 nm. This could be due to the PpIX in tissue instead of methanol and the transmission of light through

tissue. The PpIX absorption spectrum from the tissue is represented in the model as a Gaussian centered at 635 nm. The model for the reflectance spectra is designed in Matlab and based on a linear combination of absorption spectra fit using a least squares iterative fitting routine.

1.2 Motivation...Why PDT on sarcoids?

Photodynamic therapy (PDT) is the use of a photosensitizer and its activating wavelength of light to achieve destruction of target tissue through a photochemical reaction involving oxygen [18]. When the photosensitizer absorbs the energy from the light, its reaction is either a Type-I or Type-II photochemical reaction [19, 20]. Most photosensitizers used for PDT follow a Type-II photoreaction, for example the photosensitizer protoporphyrin IX (PpIX). PpIX is created in the heme pathway [1, 21]. A large reservoir of aminolevulinic acid (ALA) is applied to the tissue and the cells may actively uptake the ALA by active transport into the mitochondria where it is converted to heme [22, 23]. Once the iron in the tissue is used up the ALA converts to PpIX instead of heme and the tissue becomes photosensitive. This photosensitivity can occur naturally in the form of a porphyria but normally there is a negative feedback to stop the production of ALA if there is not enough iron in the tissue [21].

The damage the photosensitizer does to the cell can have various effects depending on the location in the cell that the damage is done. The cell can become necrotic or apoptotic. Optimally, if the damage is done immediately after the PpIX is created, and the photosensitizer is still in the mitochondria, the damage in the mitochondria could induce apoptosis [24–26].

The photosensitizers used in photodynamic therapy have shown selectivity to different types of tissues, though some have more selectivity than others. The increased amount of build up of the photosensitizer in the malignant compared to the benign cells has been used to treat the malignant cells right next to the benign cells without treating the benign cells [27]. This gradient in fluorescence around the tumor boundaries is often how the porphyrins making up the photosensitizers can be used for

fluorescence diagnosis. These porphyrins were imaged using fluorescent microscopy from photocutaneous disorders in 1972 [28]. Fluorescence is the most commonly used method for measuring relative concentrations of photosensitizers, though absorption from the photosensitizer has been used as another method to measure its concentration [3, 29, 30].

The photosensitizer most commonly used is porfimer sodium (Photofrin). Photofrin is a first generation drug with poor tumor selectivity [31, 32]. ALA is a second generation drug which means that it is the precursor to the photosensitizer. Photofrin also has a significant side effect leaving patients photosensitive for several weeks after the treatment [32, 33].

There are several ideas as to why ALA builds up more in the malignant cells than the benign cells but the precise mechanism has not yet been determined. The factors responsible for this change in photosensitizer concentration are postulated to be the higher activity of the transport system in tumor tissue, a higher cell proliferation rate, and reduced iron concentration in tumors [23].

The amount of light absorbed by the photosensitizer per gram of tissue is considered the PDT dose [ph/g] [3].

$$PDTdose(z) = \mu_a^{(PpIX)}(z)H(z)\frac{\lambda}{hc}\frac{1}{\rho} \quad (1.1)$$

where $H(z) = H_oT(z)$ and $\mu_a^{(PpIX)}(z)$ are the spatial distributions of radiant exposure and PpIX absorption coefficient. The density, ρ , is assumed to be that of water which is $1\frac{g}{cm^3}$, c is the speed of light and h is Plank's constant. The value for the threshold PDT dose for PpIX has been $10^{(17)} - 10^{(18)}\frac{ph}{g}$ in the report by Lilge et al. on rat brain tissue [34].

After a photodynamic therapy treatment it has been shown that there is a decrease in the blood flow and the oxygen concentration [35, 36]. During an ALA based PDT treatment photoproducts are produced. The major photoproduct produced is photoprotoporphyrin (Ppp) which is a photosensitizer itself, absorbing at 670 nm. In theory, it seems a more effective treatment could be achieved if this photosensitizer was also excited but this additional effect is negligible [37].

There are compartment models used to show the kinetics during a PDT treatment. A three compartment model was used to simulate the fluorescence kinetics of PpIX after topical ALA application using first order kinetics [23]. A three compartment model can also show the pharmacokinetics of PpIX both before and after photoirradiation [38].

The kinetics of the synthesis of PpIX in the skin has been examined after oral ingestion of ALA using fluorescence techniques. The maximum fluorescence intensities in the skin appeared between 6.5 and 9.8 hours after ingestion [39]. The oxygen in the tissue is also very important for PDT though high fluence rates photochemically deplete ambient tumor oxygen while lower fluence rates increased tumor oxygenation is found [40].

The production of one PpIX molecule is based on getting eight ALA molecules into the tissue at the same place [1]. This makes the PpIX very dependent on the concentration of the ALA in the tissue. The ALA has a diffusion coefficient close to the diffusion coefficient of glucose in plasma $10^{(-6)} \frac{cm^2}{s}$ [41]. Using the diffusion coefficient the rate it takes for 50% of the applied ALA to diffuse into the tissue 1 millimeter is 21 minutes. Knowing that the drug penetration into the tissue is the limiting factor it may be necessary to debulk large tumor masses before a PDT treatment. Debulking of basal cell carcinomas prior to a 20% topical ALA-based PDT has proven to be a easy, effective and safe therapy with excellent cosmetic effects [42].

The dosimetry of a PDT treatment is affected by many factors such as photobleaching and effective fluence. Photobleaching of PpIX during a PDT treatment decreases the amount of PpIX in the treatment area which has been shown in cell cultures [43]. It has also been shown in mouse models that after photobleaching of PpIX more PpIX is made that could be used for a second treatment [44]. The effective fluence rate, E_d at a depth d , as described by Moseley, takes in to account the absorption, $A(\lambda)$, of the photosensitizer, transmission, $T_d(\lambda)$, of light through tissue to a depth, d , and the incident spectral irradiance, $I(\lambda)$, from a light source at wavelength, λ [2].

$$E_d = \int I(\lambda)T(\lambda)A(\lambda)d\lambda \quad (1.2)$$

The photosensitizer used for a PDT treatment should not kill the cells with out exposure to light. If it does this is called dark toxicity. In culture, $2\frac{mmol}{L}$ of ALA on human fibroblast cells kills less than 8% of the cells through dark toxicity [45].

As stated above, the dosimetry is very dependent on the amount of light that reaches the treatment depth. There are many models currently used to simulate the transport of light through tissue such as diffusion theory, Monte Carlo, photon migration and Kubelka-Munk models [46–51]. Noninvasive optical measurements are used to determine the optical properties of tissue [29]. From these optical properties phantoms can be made with absorbers, such as India ink, and scatterers to simulate tissue [52]. Computer simulations have been made of the skin’s reflectance spectra [53]. Dosimetry of a specific type of tissue is understood better through these methods.

1.3 Outline of Thesis

Chapter 2 reviews the transport of light through tissue. Each major absorption component in the tissue and its absorption spectra have been interpreted as to how they effect the PDT treatment. Chapter 2 shows how the diffuse optical reflectance spectrometer is calibrated and used to measure the amount of each absorption coefficient there is in the tissue using a fitting algorithm. From this system the optical properties of the normal and sarcoid equine tissue have been found.

Chapter 3 describes the conversion from aminolevulinic acid to protoporphyrin IX. The pathways for ALA and PpIX are described along with the mechanism PpIX uses to kill tissue during a PDT treatment. Many methods remove the PpIX when it forms in the tissue. These methods are described in chapter 3. Since the PpIX absorbs some of the treatment light chapter 3 will also describe the effect PpIX has on the light penetration depth. The absorption of the PpIX will also be converted to a concentration of the PpIX in the tissue.

Chapter 4 covers both a kinetic model and a treatment model to identify the rate constants that may be involved when the PpIX is building up in the tissue and also the rates discovered for the drug being taken out of the local area.

Chapter 5, first, describes the protocols used for the PDT treatments. The PDT dose is described and defined for a canine treatment. A component model is used to describe the rate constants for the build up and decay of the PpIX absorption signal due to drug application, blood flow and light treatment.

Chapter 6 covers many of the treatment topic previously covered in this document but it is instead applied to a canine that was given ALA orally instead of locally. The smaller size of a canine when compared to an equine makes this type of treatment much less expensive since much less ALA is necessary.

1.4 Animals

Animals were clinical patients in the School of Veterinary Medicine, Oregon State University, Corvallis, OR, presenting cutaneous sarcoids. All surgical excisions and PDT treatments were performed by Dr. Scott Gustafson DVM with the owners consent.

Chapter 2

Light

2.1 Introduction

When light is incident upon tissue one of two things can occur. The photons can either be (1) scattered or (2) absorbed. Scattering can send a photon deeper into the tissue or away from the it completely. The absorption primarily occurs in the tissue due to one or many primary absorbers.

Knowing the optical properties, both absorption and scattering coefficients, of tissue can not only be useful in designing a photodynamic therapy, PDT, treatment but can also be used to measure the major contributing optical components within the tissue. In this chapter, the optical properties from both normal and sarcoid tissue have been determined. In order to discuss the technique of tissue spectroscopy it is important to explore the theory and methods used to describe the way light travels through tissue. Some components of the tissue will be defined as major optical influences for either absorbing or scattering the light.

There are several components in the tissue that contribute to the depth that light will travel. These components can be considered to absorb or scatter light as it travels through the tissue. The theory of light interactions with tissue and the major biological components responsible for absorption and scattering will be defined in this chapter along with methods used to simulate the light reflection measurement from tissue.

2.2 Optical Components in the Tissue

The major components in the tissue that absorb light are water, oxygenated and deoxygenated hemoglobin and melanin. Protoporfrin IX also can be found in the normal tissue but often in tumor tissue. It absorbs in the ultraviolet but has an absorption peak in the red, figure 3.6. Hemoglobin and melanin play a much larger role in the absorption of light over the visible spectrum while water starts absorbing in the near infrared. Bilirubin absorbs in the ultraviolet and near the ultraviolet in the visible spectrum. Structures in the tissue are a large contributor to the scattering in the tissue.

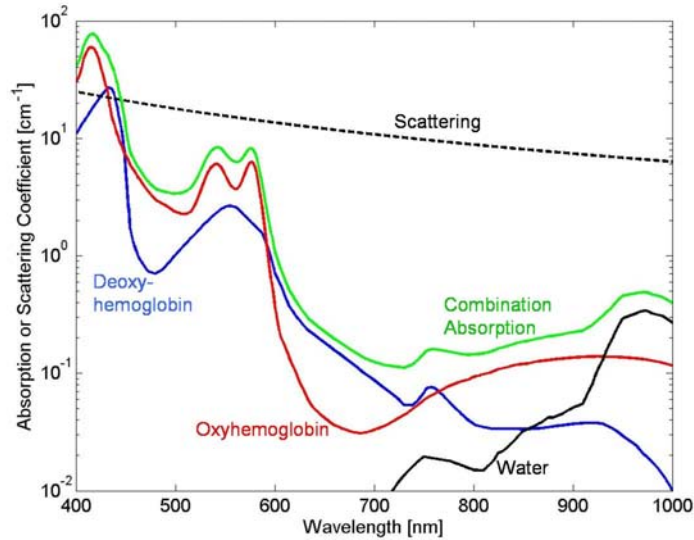


Figure 2.1: Absorption and Scattering: The combination absorption is made up of 75% water, 3% fraction of blood and 70% oxygen saturation in the blood. The scattering coefficient represented is similar to what would be found in tissue.

Water

Tissue is composed mostly of water, though the absorption of light due to the water is minimal below 800 nm. Above 1000 nm water absorption is the dominant absorber in tissue.

$$\mu_a = f^{water} \cdot \mu_a^{water} \quad (2.1)$$

where:

$$f^{water} = \text{fraction of water in the measured volume}$$

$$\mu_a^{water} = \text{absorption coefficient of water}$$

Hemoglobin

Hemoglobin has different optical absorption spectra characteristics depending on whether it is oxygenated or deoxygenated. Varying oxygen levels in hemoglobin changes the shape of the optical absorption. When blood leaves the lungs it is mostly oxygenated. As the blood is pumped through the body, the oxygen contained in the hemoglobin is consumed causing the blood to become deoxygenated. This change can be monitored optically using oxygenated and deoxygenated hemoglobin spectra [41].

$$\mu_a^{blood} = f^{blood} \cdot (S \cdot \mu_a^{oxy} + (1 - S) \cdot \mu_a^{deoxy}) \quad (2.2)$$

where:

$$f^{blood} = \text{dermal volume fraction of blood at 45\% hematocrit}$$

$$S = \text{average oxygen saturation of tissue}$$

$$\mu_a^{oxy} = \text{oxygenated hemoglobin absorption}$$

$$\mu_a^{deoxy} = \text{deoxygenated hemoglobin absorption}$$

Melanin

Melanin can be thought of as an optical filter at the surface of the tissue that the light would have to travel through. This filter's optical density decreases with an increase of wavelength and is more complex than a neutral density filter [54]. Most of the tumors in this study have lost the layer of skin which contains melanin hence

negating the need to fit for this variable. The normal skin measurements were often taken of light colored skin to negate the absorption effect due to the melanin.

$$\mu_a^{melanin} = f^{melanin} \cdot \mu_{ao}^{melanin} \cdot (frac\lambda{1100})^{-3.48} \quad (2.3)$$

where:

$$\begin{aligned} f^{melanin} &= \text{fraction of melanin in the measured volume} \\ \mu_a^{melanin} &= \text{absorption of melanin} \end{aligned}$$

Bilirubin

When blood is broken down, one of its by-products is bilirubin. Bilirubin appears yellow to the eye and absorbs light in the lower wavelengths such as 400 nm. This absorption peak is broad and it may be approximated by a gaussian function with a peak at 460 nm and a $frac{1}{e^2}$ of 80 nm as shown in equation 2.4.

$$\mu_a^{bilirubin} = f^{bilirubin} \cdot \mu_{ao}^{bilirubin} \cdot e^{-\left(\frac{\lambda - 460 \text{ nm}}{80 \text{ nm}}\right)^2} \quad (2.4)$$

where:

$$\begin{aligned} f^{bilirubin} &= \text{fraction of bilirubin in the measured volume} \\ \mu_a^{bilirubin} &= \text{absorption of bilirubin at 460 nm} \end{aligned}$$

Protoporphyrin IX

Normal tissue is usually good at preventing the build up of this heme precursor but it can be found to naturally build up in tumor cells [55]. Protoporphyrin IX, PpIX, will be discussed at a greater depth in chapter 3. For now the PpIX spectrum will be approximated by the gaussian function in equation 2.5 with a peak at 635 nm and a $frac{1}{e^2}$ of 35 nm.

$$\mu_a^{PpIX} = f^{PpIX} \cdot \mu_{ao}^{PpIX} \cdot e^{-\left(\frac{\lambda - 635 \text{ nm}}{35 \text{ nm}}\right)^2} \quad (2.5)$$

where:

$$\begin{aligned} f^{PpIX} &= \text{fraction of PpIX in the measured volume} \\ \mu_a^{PpIX} &= \text{absorption of PpIX at 635 nm} \end{aligned}$$

Scattering

Cell membranes, collagen, changes in indices of refraction in many structures add to light scattering by tissue. The overall scattering found in tissue has been approximated by the equation 2.6 [56].

$$\mu'_s(\lambda) = a\lambda^{(-b)} \quad (2.6)$$

There have been people that have tried to assign meaning to the values for the variables a and b [56].

Equation 2.6 can be rewritten for a specific wavelength, λ_0 , and solved for the variable a .

$$\mu'_s(\lambda_0) = a\lambda_0^{-b} \quad (2.7)$$

$$a = \mu'_s(\lambda_0)\lambda_0^b \quad (2.8)$$

Now substituting this value for a into equation 2.6 we get:

$$\mu'_s(\lambda) = \mu'_s(\lambda_0) \left(\frac{\lambda}{\lambda_0} \right)^{-b} \quad (2.9)$$

The wavelength $\lambda_0 = 1100 \text{ nm}$ was chosen.

$$\mu'_s = \mu'_s(1100 \text{ nm}) \left(\frac{\lambda}{1100 \text{ nm}} \right)^{-b} \quad (2.10)$$

2.3 Light Sources

Three light emitting diode, LED, devices were used for PDT treatments. The initial device consisted of ten LEDs in a parallel circuit and in two physical rows

of five. The LED emission wavelengths had a gaussian distribution centered at 641 nm with $21 \text{ nm } \frac{1}{e^2}$. The LEDs were purchased from Laughing Rabbit Inc. with a maximum output of 8 milliwatts. This initial device produced about $5 \frac{mW}{cm^2}$ total light emission. Power was supplied from a 3-volt source compatible with a standard outlet.

The second device consisted of 88 LEDs but these LEDs were all filed down on the sides and tops to fit flush to one another side by side. This device is shown in figure 2.2. The filing of the tops decreased the amount of light able to emit from the LED dramatically. Even with the close spacing of 3 mm center to center the output was only about $7 [\frac{mW}{cm^2}]$ over a 3 cm diameter. The power supply was identical to that in the first device. The device is enclosed in plexiglass on the front and back with PCV pipe around the outside.



Figure 2.2: The second light source: LED device for treatment of circular treatment zones

The third device is set up as two rows of 16 for a total of 32 LEDs in the device. This device is shown in figure 2.3. Each of the LEDs are 3 mm apart from each other and the device has an output of $9 - 11 [\frac{mW}{cm^2}]$. This device was built with the need to

treat inside the ear of a horse so the device is a rectangle 6 cm by 1 cm. This is the first device to be powered by batteries. Four "D" sized batteries are held in a pack that can be strapped to the back of the horse. Epoxy prevents the circuits on the back from being exposed. The front has a clear flexible plastic over the front of the LEDs.

2.4 Diffuse Optical Reflectance Spectroscopy

The setup for the diffuse optical reflectance spectroscopy consisted of an optical fiber spectrometer (Ocean Optics, S2000) with a white light source (Ocean Optics LS-1 tungsten halogen light source) and a fiber bundle probe (Ocean Optics probe six fibers around one) as shown in figure 2.4. This probe can be held similarly to a pencil and the metal casing is about 5 cm long.

The fiber bundle consisted of six optical fibers for delivery of white light around one central optical fiber for collection of reflected light. The fibers were $200\ \mu\text{m}$ in core diameter and centers were separated by $200\ \mu\text{m}$ as shown in figure 2.5. The probe was placed in contact with the sample for measurements using water to match the index of refraction between the optical fibers and the tissue. The measurements were taken with an integration time of either 5 or 15 milliseconds depending on the reflectivity of the tissue.

Before tissue measurements were taken, the probe was placed against an epoxy standard as a stable reference which is much more stable than a white card. M_{epoxy} All subsequent measurements that day were normalized to this reference measurement. This was done to account for any variations in the intensity of the light source from one day to another. A second measurement M_{card} of a white card was taken in a dark room. M_{card} was measured at a distance of about one foot away from the probe. This signal gave the spectral shape of the signal. The final reference signal M_{ref} was the spectral reference normalized by the intensity signal. This was necessary since M_{card} was not reproducible.



Figure 2.3: Top: The third light source. This light source was developed to fit inside a horses ear and effectively treat a sarcoid using batteries. Shown with room lights on and device off. Bottom: LED device designed to treat the inside of the equine ear. The ear can be wrapped around the device during a treatment. Shown with the device on in a dark room.

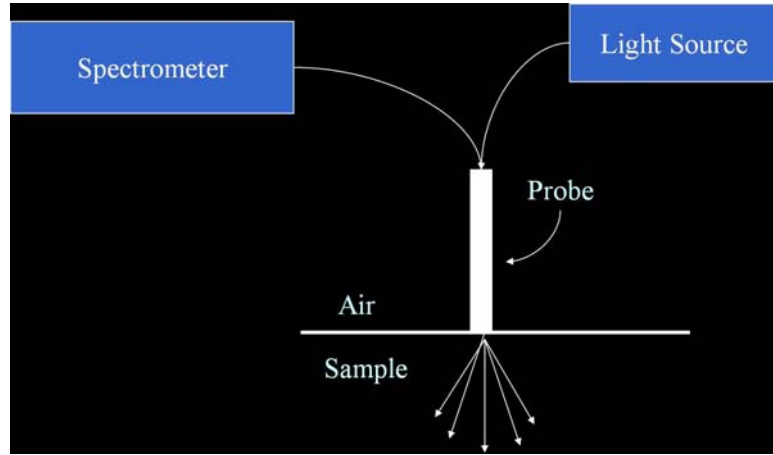


Figure 2.4: Schematic of the diffuse optical reflectance spectrometer system. Light is emitted from the light source through the optical fiber probe and transmits into the sample. Some light will be reflected and travel back up through the fiber probe to the spectrometer. Most of the light will absorb in the tissue or escape out the tissue and air boundary.

$$M_{ref}(\lambda) = M_{card}(\lambda) \cdot \left(\frac{\max(M_{epoxy}(\lambda))}{\max(M_{card}(\lambda))} \right) \quad (2.11)$$

The sample spectral measurement ($M(\lambda)$ [dimensionless]) was defined as the tissue measurement ($M_s(\lambda)$ [counts]) divided by the reference measurement ($M_{ref}(\lambda)$ [counts]).

$$M = \frac{M_s(\lambda)}{M_{ref}(\lambda)} \quad (2.12)$$

Such normalization controlled for day to day variations in the light source and system. Spectra were acquired over the 350-1000 nm where the signal-to-noise was acceptable. The wavelength range used for analysis was 470-850 nm.

2.4.1 Calibration of the Diffuse Optical Reflectance Spectrometer using phantoms

Phantoms

An array of optical phantoms with known absorption and scattering coefficients was created with a range of optical properties above and below that of skin. These

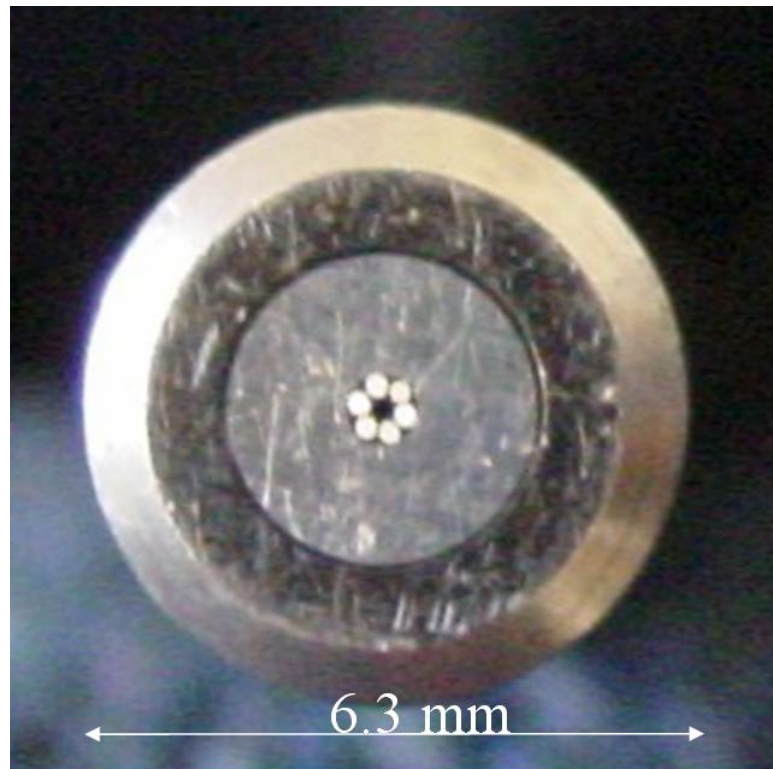


Figure 2.5: Fiber probe: This figure shows the end of the fiber probe. There are six illuminated fibers around one dark fiber. Each fiber has a diameter of $200\ \mu\text{m}$

phantoms are shown in figure 2.6. This range was chosen so that the optical probes can be calibrated to the range of natural optical properties. The probes in turn will be used to determine the optical properties of the tissue to be treated. With these optical phantoms, other optical probes could be calibrated. If theory only was used to calibrate these probes it may prove inaccurate. There are many interfaces, changes in indices of refraction, mismatched boundary conditions and geometries particular to a physical device that make using the optical phantoms a much more accurate way of calibrating the probes. Experimentally calibrating the optical probe is a much easier method to interpret the signal than trying to optically simulate all the subtleties of a physical system. Fortunately, calibration of optical probes as done in this section can prove to be a method that can be performed on many diffuse optical reflectance probes.

A matrix of polyacrylamide gels was made to calibrate the optical probes. A total of 64 phantoms were prepared with increasing amounts of absorber (India ink) and scatterer (lipid emulsion, Intralipid) as shown in figure 2.6. The optical properties of the gels were determined by both $R(r)$ experiments and integrating sphere measurements [57]. The rationale for this approach was that μ_a is robustly specified by added-absorber experiments and the μ_a values were not expected to be affected by gel or intralipid. Usually the properties of the ink in each gel dominated the absorption. In contrast, the addition of gel was expected to affect the μ'_s values due to differences in the medium surrounding the lipid droplets from water to gel. An integrating sphere allowed convenient measurement of the final gels without concerns about how the fiber contacted the gel. A single sphere reflection measurement then provided μ'_s since the absorption coefficient was already known.

The first experiment used a pair of optical fibers: one as a light source and one as a collector. The fibers were separated by varying distances, in liquid mixtures of scatterer with ink. These liquid phantoms had the same composition of ink and intralipid as the acrylimide gel phantoms. Adding 18% of the acrylimide was not expected to change the absorption properties. The reflectance, $R(r)$, was detected by the collection fiber and recorded as a function of the distance, r , between the

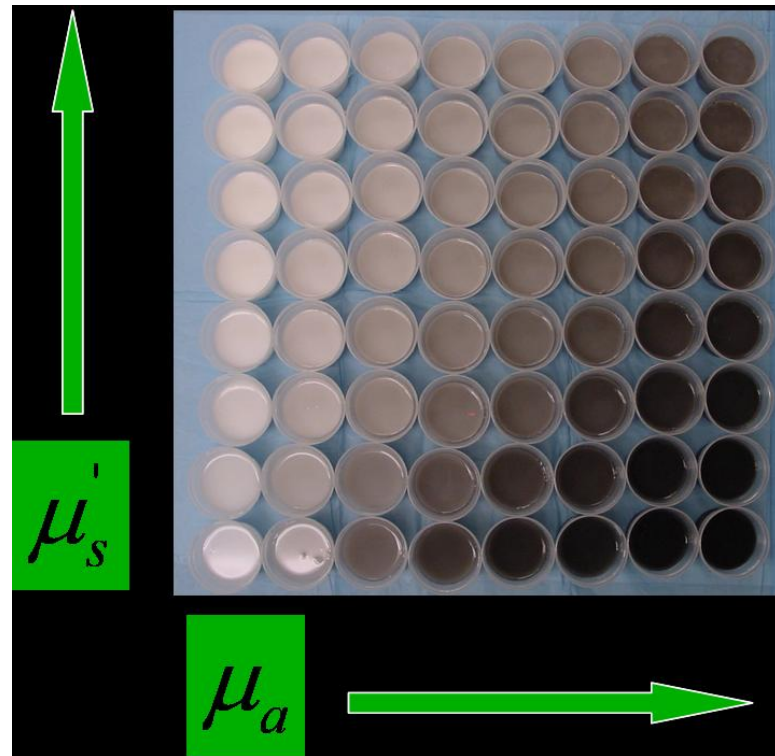


Figure 2.6: Polyacrylamide gels made with India ink and Intralipid. The absorption coefficient increases from left to right. ($\mu_a = 0, 0.1, 0.4, 0.9, 1.6, 2.5, 4.9, 6.5 [cm^{-1}]$) The scattering coefficient increases bottom to top. ($\mu'_s = 1.0, 3.2, 6.1, 9.0, 13.3, 17.6, 22.3, 28.3 [cm^{-1}]$)

two fibers. Analysis by diffusion theory [46] yielded the absorption spectra for the intralipid absorption due to water and lipid droplets. The ink absorption was measured previously in a standard transmission spectrometer. Since the absorption for each phantom was known, the scattering could then be determined from the $R(r)$ data.

The second experiment used an integrating sphere to measure the total reflectance of the 64 gels, placing each gel directly at the open port of the sphere and illuminating the gel with white light from an optical fiber. Analysis of the integrating sphere data was converted to transmission and reflection of the sample. The reflection and transmission spectra of the sample were analyzed using inverse adding-doubling [58] [59] to obtain the reduced albedo:

$$a' = \frac{\mu'_s}{\mu_a + \mu'_s}$$

The absorption coefficients from the first experiment allowed the reduced scattering coefficients to be found for each gel.

$$\mu'_s = \frac{a' \mu_a}{1 - a'}$$

Hence, the absorption coefficient, μ_a , and the reduced scattering coefficient, μ'_s , of each of the 64 gels at each of the wavelengths between 470-850 nm were measured.

Analysis

Measurements of the optical phantoms were made with the diffuse optical reflectance spectrometer probe system of section 2.4. The known absorption and reduced scattering coefficients of each phantom were used to calibrate the device as described in this section.

Each measurement, M [dimensionless], consisted of a measurement of the sample, M_s [counts], normalized by the lamp spectrum, M_{ref} [counts] as shown in equation 2.12.

The logarithms of the measured reflectance values, $\log_{10}(M)$ [dimensionless], are plotted in a matrix relating the absorption and scattering coefficients with the intensity of the measurement shown in figure 2.7.

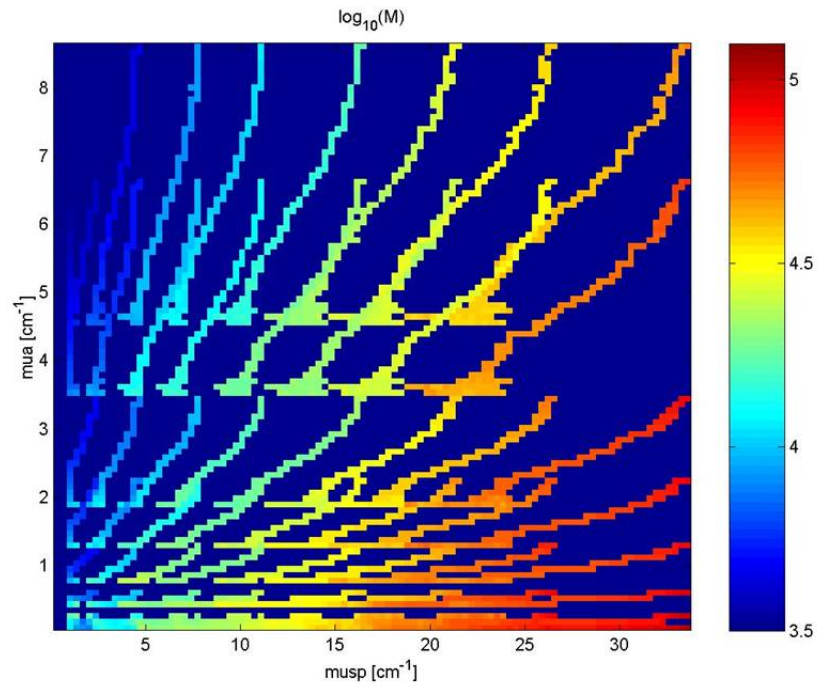


Figure 2.7: Logarithmic measured reflectance $\log(M)$ plotted as a function of the known absorption, μ_a , and reduced scattering coefficients, μ'_s . This image shows the measurements space that was measured. Using theory and the measured values a full map of this space can be approximated to account for measurements of samples with absorption and scattering coefficients in this range.

Figure 2.7 has many areas that have not been filled in represented as a dark blue. The rest of this graph needs to be interpolated before it becomes useful. To start, the scattering coefficient is held constant and then the intensities that were measured are plotted as a function of the absorption coefficient as shown in figure 2.8.

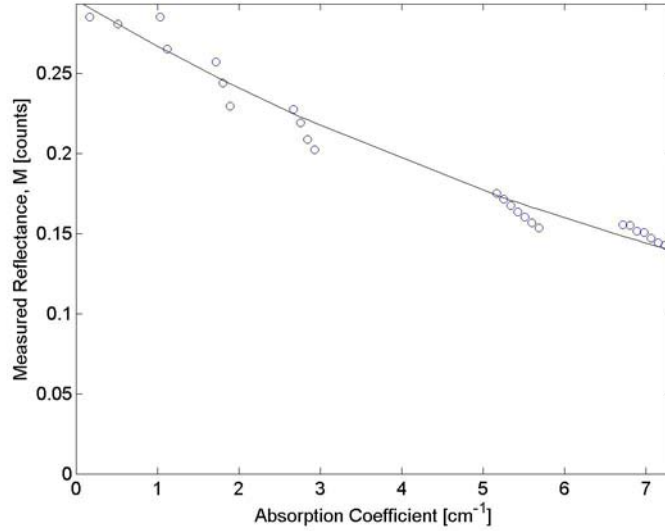


Figure 2.8: Measured reflectance plotted as a function of the known absorption coefficients as a function of the absorption coefficient, μ_a , for one scattering coefficient.

The intensity of reflected light as a function of the absorption coefficient follows Beer's Law and can be fit using Beer's Law given as:

$$M = M_0 \cdot e^{-\mu_a \cdot L} \quad (2.13)$$

Since the $\log_{10}(M)$ was plotted we will fit for:

$$\log_{10}(M) = (-\mu_a \cdot L) + \log_{10}(M_0) \quad (2.14)$$

Equation 2.14 follows the form of:

$$y = C_1 x + C_2 \quad (2.15)$$

Where:

$$x = \mu_a \quad (2.16)$$

$$y = \log(M) \quad (2.17)$$

$$C_1 = -L \quad (2.18)$$

$$C_2 = \log(M_0) \quad (2.19)$$

In figure 2.8 the log of the reflected intensity is fit as a function of the absorption coefficient. For each reduced scattering coefficient a pair of C_1 and a C_2 can be found. For each C_1 and C_2 there is an M_0 and an L . Arrays of M_0 and L as a function of the scattering coefficient are developed after a slope, C_1 , and an intercept of C_2 are found for each scattering coefficient. Figures 2.9 and 2.10 are a plots of each of these values as a function of the reduced scattering coefficient. A line can be fit to each term L and M_0 as a function of the reduced scattering coefficient.

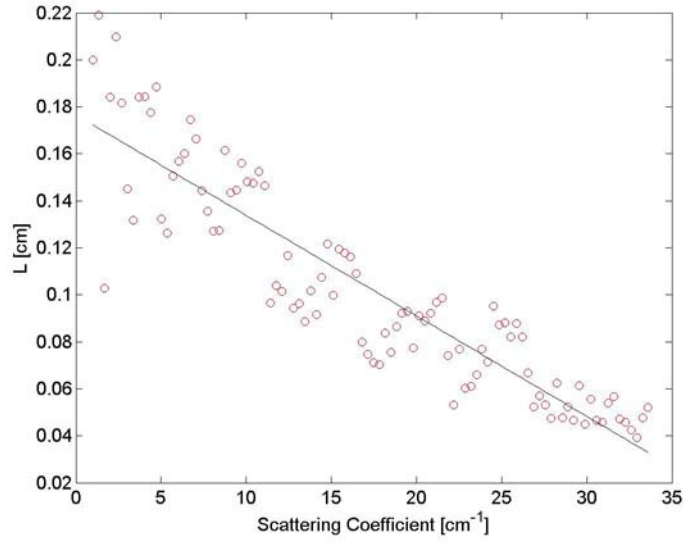


Figure 2.9: The value L is plotted as a function of the reduced scattering coefficient.

By linearly fitting for the values of L and M_0 as a function of μ'_s , four coefficients can be found that are characteristic to the system, a, b, c and d .

$$L = a\mu'_s + b \quad (2.20)$$

$$M_0 = c\mu'_s + d \quad (2.21)$$

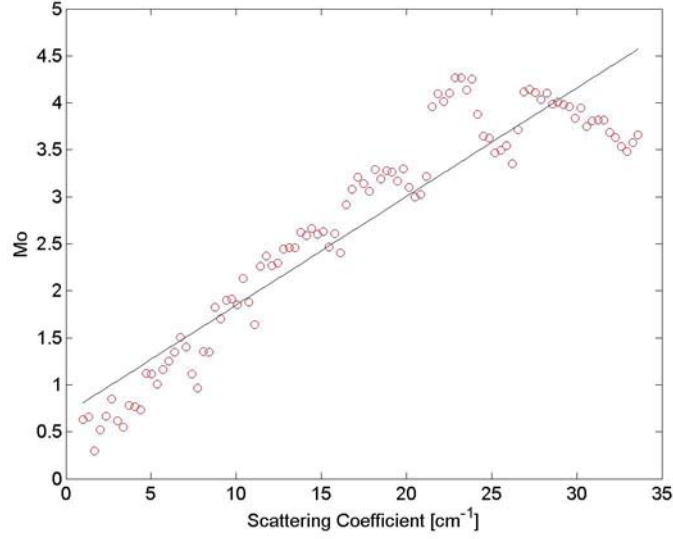


Figure 2.10: The value M_0 is plotted as a function of the reduced scattering coefficient.

The measured spectra, M , is now a function of the absorption coefficient and the reduced scattering function only given that a, b, c and d were found to be:

$$a = 0.005 \quad (2.22)$$

$$b = -0.184 \quad (2.23)$$

$$c = 0.160 \quad (2.24)$$

$$d = 0.223 \quad (2.25)$$

Now if we were given an absorption and a scattering coefficient we could reproduce what the measurements would look like using equation 2.13.

$$M = e^{\log(M_0)} \cdot e^{-\mu_a \cdot L} = \frac{M_0}{\ln(10)} \exp(-\mu_a \cdot L) \quad (2.26)$$

Next we expand this equation using equations 2.17-2.19 through 2.4.1 and we get:

$$M = e^{C_2} \cdot e^{\mu_a \cdot C_1} \quad (2.27)$$

$$M = e^{\log(c\mu'_s+d)} \cdot e^{\mu_a \cdot (a\mu'_s+b)} \quad (2.28)$$

$$M(\mu_a, \mu'_s) = e^{(0.160\mu'_s+0.223)} \cdot e^{\mu_a \cdot (-0.005\mu'_s+0.184)} \quad (2.29)$$

Now by using equation 2.29 the absorption and reduced scattering coefficients can be fit for as shown in the next section.

2.4.2 Fitting Algorithm

This fitting algorithm is used to find the intrinsic absorption and scattering coefficients of the tissue. For example, oxygenated and deoxygenated hemoglobin are two of the dominant absorbers in tissue. The total absorption in the tissue can be accounted for by fitting for the concentrations of each of the tissue components.

To find the absorption and scattering coefficients for the measured sample there must be an initial estimation of these values. Using equation 2.29, a mathematically derived test measurement is created once guesses for the absorption and scattering coefficients are substituted into this equation. To determine the initial values for the absorption and scattering coefficients, the spectra of the components found in the tissue are used. The total absorption coefficient is a linear composition of the individual component absorptions as shown in equation 2.31. These components consist of oxygenated hemoglobin, deoxygenated hemoglobin, water, bilirubin and for PDT treatments protoporphyrin IX. The equations for these were described in section 2.2.

$$\mu_a = \mu_a^{blood} + \mu_a^{water} + \mu_a^{bilirubin} + \mu_a^{PpIX} \quad (2.30)$$

$$\mu_a = f^{blood} \cdot (S \cdot \mu_a^{oxy} + (1 - S) \cdot \mu_a^{deoxy}) + f^{water} \mu_a^{water} + \mu_a^{bilirubin} + \mu_a^{PpIX} \quad (2.31)$$

The absorption spectra due to the bilirubin can be approximated by a Gaussian function with the peak centered at 460 nm.

$$\mu_a^{bilirubin} = f^{bilirubin} \cdot \mu_{ao}^{bilirubin} \cdot e^{-\left(\frac{\lambda-460}{80}\right)^2} \quad (2.32)$$

The absorption spectra due to the protoporphyrin IX can be approximated by a Gaussian function with the peak centered at 635 nm.

$$\mu_a^{PpIX} = f^{PpIX} \cdot \mu_{ao}^{PpIX} \cdot e^{-\left(\frac{\lambda-635}{35}\right)^2} \quad (2.33)$$

The scattering coefficient is given by equation 2.10.

Figure 2.11 is a graphical representation of the steps taken to find the sample's optical properties from the measured data. Initially an estimate is made as to the

amount of each component that is in the tissue; for example the fraction of blood. Once an initial estimate has been made, a sample measurement, $M_{fit}(\lambda)$, is created. Next, the sample measurement $M_{fit}(\lambda)$ is compared to the real measurement, $M(\lambda)$. If the error is larger than a set threshold then the error is minimized. The error is then minimized between the two spectra over all measured wavelengths by using an iterative method by changing the amounts of the components of the tissue. When the error becomes less than the threshold then the values for the amounts of each component are reported.

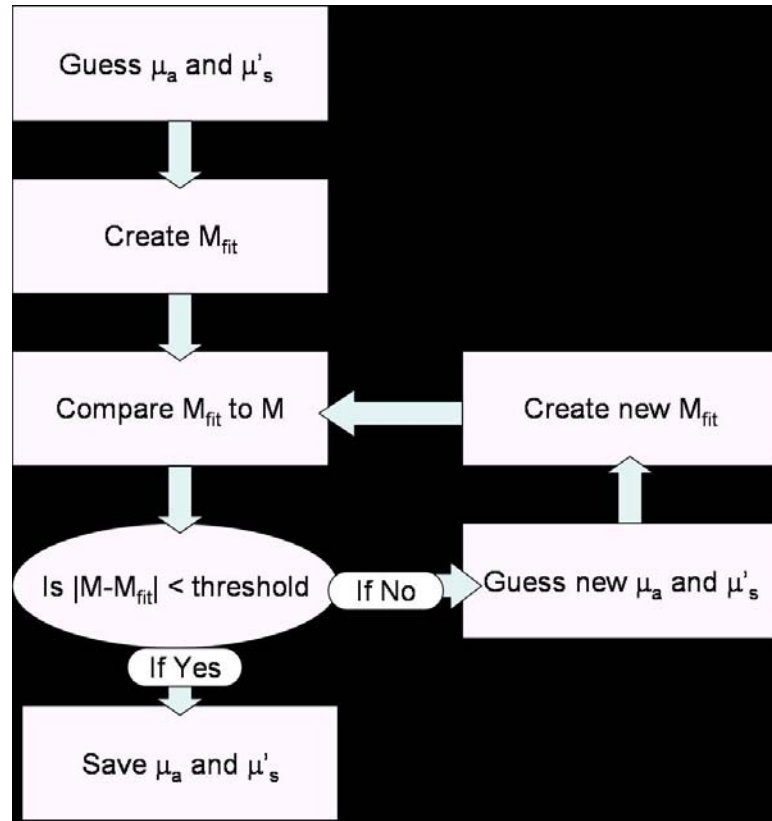


Figure 2.11: Flow chart for the iterative process of fitting to a measured spectra.

2.4.3 Fit to the Original Phantoms

The reflectance from the phantoms that were used to create the fitting algorithm can be fit with the fitting algorithm to show that it returns the correct absorption and

scattering coefficients. This is important since we already know the absorption and scattering coefficients of the media. Using the known absorption of the ink and the equation 2.10 used previously for scattering the reflectance spectra for each phantom was fit. Figure 2.12 shows the fits for eight of these phantoms between 518 and 750 nm.

There were sets of eight phantoms that all had the same scattering coefficient and this knowledge was used during the fitting. Each sets of eight phantoms were fit together. This was necessary since the absorption and scattering spectra have such a similar shape. If this was not done then both the absorption and scattering would compete and play off each other. Figure 2.13 shows the absorption coefficients compared to the known absorption coefficients for each phantom.

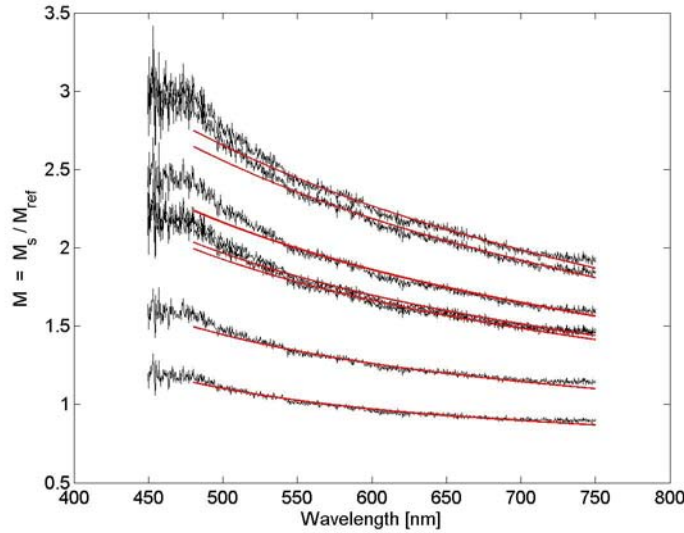


Figure 2.12: This is an example of the fitting for the phantoms. This graph represents eight phantoms that all have the same scattering coefficient and eight different absorption coefficients. The data was fit from 518 to 750 nm.

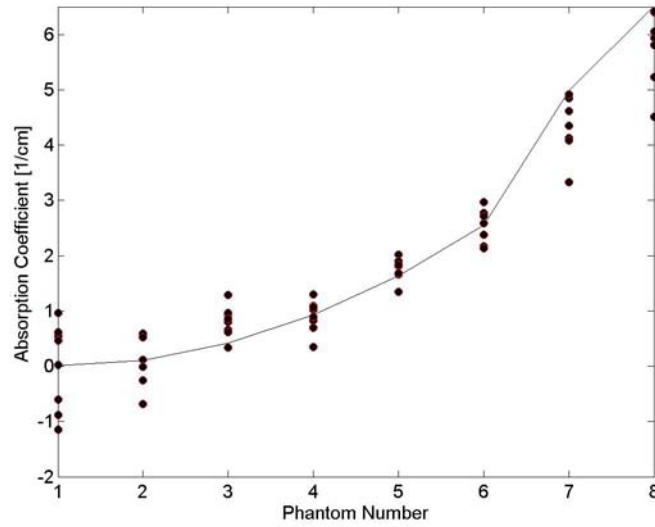


Figure 2.13: The line in this graph represents the absorption coefficient that the phantoms were made to. The data points are what the fitting algorithm fit the data to.

2.5 Variable Dependence within the fitting parameters

The fitting algorithm that was used finds optimal values for the variables shown in table 2.5 of the equation used to fit the data. Based on the constraints in the program and the shapes of the spectra there will be a value of these variables that returns a minimum error between the measured spectra and the simulated spectra. Most of these constraints are dependent on the physical realm they are based on. For example, the oxygen saturation is limited to some value greater than or equal to 0% and less than or equal to 100%. Table 2.5 shows the constraints given to the variables while fitting the spectra.

From Siadi's thesis $\frac{1mg}{100g}$ bilirubin in tissue corresponds to $2.52[\frac{1}{cm}]$ absorption [60]. Jaundice is defined by bilirubin concentration that is equal to or greater than $2.5[\frac{mg}{dl}]$ [bilirubin/tissue] [61].

The values of a and b that describe scattering can not have real physical values

Variable	Min Value	Max Value
Fraction of Blood, f^{Blood}	0	1
Oxygen Saturation, S	0	1
Fraction of Protoporfrin IX, f^{PpIX}	0	no max
Fraction on Bilirubin, $f^{Bilirubin}$	0	no max
a	0	no max
b	0	no max

that they describe unless more information is given. There are many pairs of a and b that will result in the same reduced scattering coefficient at a specific wavelength.

One example is from the experiment below in section 2.6.1. The values for a and b were fit for each set of data so for each measurement there was a pair of a and b . During the experiment the reduced scattering coefficient should not have changed but strangely the values for a and b varied drastically as seen in figure 2.15. This pattern kept occuring for other experiments too. There was a relationship between a and b where multiple pairs of a and b gave the same reduced scattering coefficient. A reminder of the relationship between these variables is given in the equation of the reduced scattering coefficient shown in equation 2.34.

$$\mu'_s = a * \lambda^{-b} \quad (2.34)$$

This equation can be rewritten as:

$$a = \mu'_s * \lambda^b \quad (2.35)$$

The relationship between a and b can be found by fixing both λ and μ'_s . If these variables are fixed then b can be plotted as a function of a as in figure 2.14. In figure 2.14 lambda is fixed equal to 550 nm and the relationship between a and b is shown for the change in value of μ'_s from 10 to 30 $[\frac{1}{cm}]$ by 2. (i.e $\mu'_s = 10, 12, 14...$)

$$\lambda = 550nm \quad (2.36)$$

$$\mu'_s = 10 - 30[\frac{1}{cm}](by2) \quad (2.37)$$

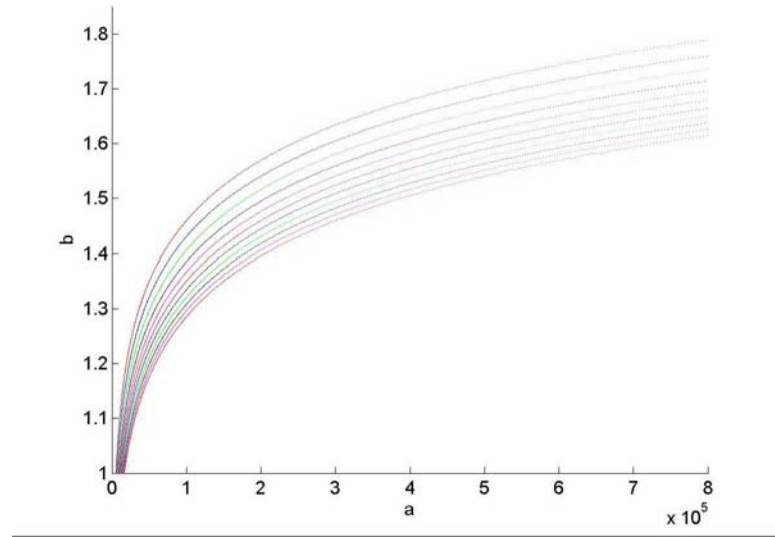


Figure 2.14: These curves represent the relationship between the variables a and b . Each curve has a fixed wavelength and reduced scattering coefficient. The top line is $10[\frac{1}{cm}]$ while the bottom, closest to the x-axis, is $30[\frac{1}{cm}]$. The wavelength λ is set to 550 nm.

2.6 Examples of the system

This section gives examples of physical changes in the tissue that can be measured by the diffuse optical reflectance spectrometer system.

2.6.1 Cutaneous Measurements of Induced Erythema

The purpose of this experiment was to determine the reproducibility of the local measurements and if physical changes could be detected. In this experiment one location (less than a 5 mm diameter area) on a caucasian forearm was repeatedly measured. One location was chosen that had minimal color variation and looked to be away from any veins. This location was then slapped and more measurements were taken directly after the slap and 10 minutes afterwards.

The measurements were taken by placing the probe on the forearm in a mostly dark room. Between each of the measurements the probe was lifted from the measurement location and then placed back down on the chosen location. An attempt

was made to measure the exact same spot each time.

Materials and Methods

Several measurements were taken of one spot on a forearm using the diffuse optical reflectance system with a white light source (Ocean Optics LS-1 tungsten halogen light source), a spectrometer (Ocean Optics S2000) and a fiber probe (Ocean Optics, six 200 micron fibers around one). There were four time points that were measured and they were (1) the arm before slap with no water at the measurement site, (2) the arm before slap with water at the measurement site, (3) the arm right after the arm was slapped and (4) the arm ten minutes after the arm was slapped. At each time point, ten measurements were taken at a rate of about 10 seconds per measurement. Light pressure was applied to the tissue with the probe.

The theory was that slapping the arm would increase blood flow and oxygen saturation to the local area. These changes in the tissue should produce differences in the measurements larger than the errors from the probe placement and from the fitting of the program together.

Results

The physical parameters were fit for each measurement taken. For each measurement there was a reduced scattering coefficient found with associated variables a and b . In figure 2.15 these pairs of a and b are plotted against one another. Comparing the shape of this curve to figure 2.14 shows that the scattering coefficient has been held constant for all of these measurements. All of these pairs of a and b must correspond to the same reduced scattering coefficient since during this experiment it was not expected to change.

After the tissue had been slapped it was expected that this would cause erythema and both the oxygen saturation and the fraction of blood would increase as a result of the capillaries dilating. The mean value and standard deviation for the oxygen saturation are given in table 2.6.1 and shown graphically in figure 2.16. It is easily seen that the error in the system for a tissue measurement can be differentiated from

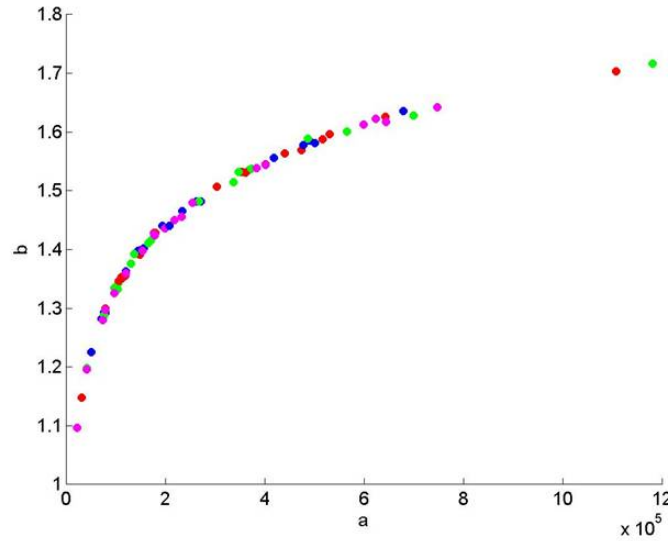


Figure 2.15: This figure plots the variable b as a function of a . The reduced scattering coefficient should not have changed for all of these measurements. The shape of this curve demonstrates the dependence of a on b for a fixed reduced scattering coefficient.

the physical changes in the tissue.

Time Point	Mean Oxygen Saturation	Standard Deviation
1	0.44	0.05
2	0.49	0.06
3	0.76	0.05
4	0.72	0.05

Table 2.1: This is a table of the mean oxygen saturation and standard deviation at each point for time points 1-4.

The mean value and standard deviation for the fraction of blood are shown in table 2.6.1 and figure 2.17. It is easily seen that the error in the system for a tissue measurement can be differentiated from the physical changes in the tissue.

After reviewing this data, it can be shown that changes in the oxygen saturation and fraction of blood are outside of the error of multiple measurements of tissue with this diffuse optical reflectance spectrometer system.

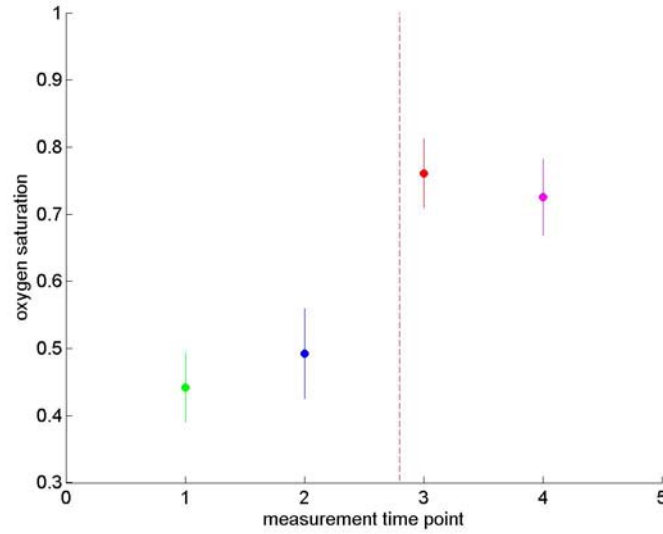


Figure 2.16: This is the mean and standard deviation of the oxygen saturation as a function of the time point the measurements were taken. There were four time points that were measured and they were (1)the arm before slap with no water at the measurement site, (2)the arm before slap with water at the measurement site, (3)the arm right after the arm was slapped and (4)the arm ten minutes after the arm was slapped. The dotted line represents the time that the arm was slapped.

Time Point	Mean Fraction of Blood	Standard Deviation
1	0.006	0.001
2	0.005	0.001
3	0.010	0.002
4	0.008	0.002

Table 2.2: This is a table of the mean fraction of blood and standard deviation at each point for time points 1-4.

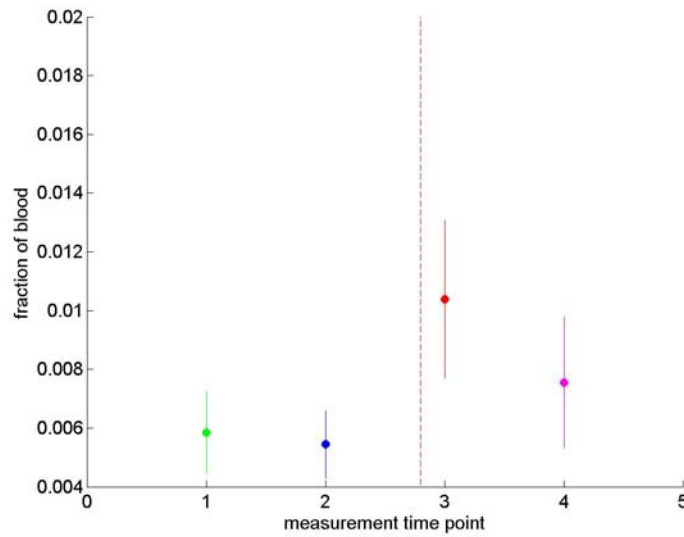


Figure 2.17: This is the mean and standard deviation of the fraction of blood as a function of the time point the measurements were taken. There were four time points that were measured and they were (1)the arm before slap with no water at the measurement site, (2)the arm before slap with water at the measurement site, (3)the arm right after the arm was slapped and (4)the arm ten minutes after the arm was slapped. The dotted line represents the time that the arm was slapped. The amount of blood at the surface of the skin was assumed to be low since the experiment was performed in a cool room.

2.6.2 Cutaneous Measurements of Local Erythema

The diffuse optical reflectance spectrometer system was used to observe a site of interest on the skin of a human patient located on the forearm. This site was induced the previous day from a bug bite. The type of bug was unknown. Obvious erythema had occurred as seen in figure 2.18. The system was used to document the resulting changes between the site of interest and the local tissue around it.

Six points were measured in a line across the erythema location. Position one was on the outer part of the arm outside of the perceived erythema zone. The second was on the boarder just inside the erythema zone. The third site was located in the center of the irritation. The fourth was on the inside of the erythema zone between the middle and the edge of the zone. The fifth and sixth points were both located outside of the erythema zone in a line toward the inside of the arm.



Figure 2.18: This is a picture of erythema due to a bug bite. The black line indicated the location of a series of six measurements. Position one was located at the top of this line while the sixth measurement was at the bottom of this line and each measurement linearly between in order. (1) the outer part of the arm outside of the perceived erythema zone. (2) the boarder just inside the erythema zone. (3) the center of the irritation. (4) the inside of the erythema zone between the middle and the edge of the zone. (5,6) both located outside of the erythema zone in a line toward the inside of the arm.

Each of these spectral images were processed for the fraction of blood and oxygen

saturation in the local area. The goal was to be able to see a change in these values with the diffuse optical reflectance system. Figure 2.19 shows the fraction of blood change in the local area based on the location of the optical probe. Figure 2.20 demonstrates an increase in the oxygen saturation in the site of interest.

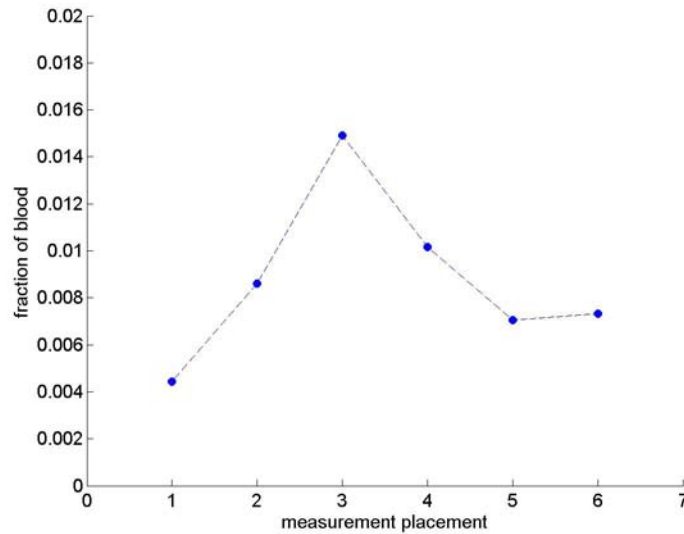


Figure 2.19: This figure shows the fraction of blood found from each measurement as a function of the location of the measurement across the erythematic area in figure 2.18.

2.7 Conclusion

This chapter delved into the details of calibrating and taking a measurement using a device for optical monitoring of tissue. The device described in this chapter was a diffuse optical reflectance spectrometer. Initially, the major optical components in the tissue were identified. Second, the process used to calibrate the device using an array of optical phantoms was described in detail. The third major item this chapter covered was how to convert the data taken into optical properties of the tissue from the raw reflectance measurement. Finally these measurements were shown to be able to identify physical changes in tissue. Accurate calibration will be essential in

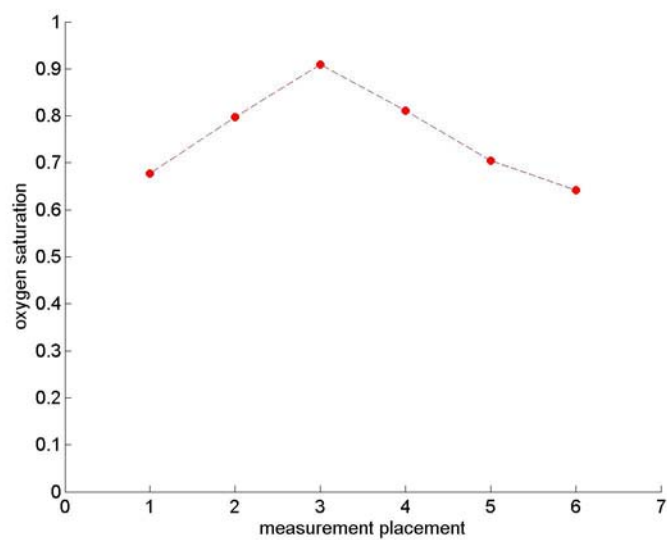


Figure 2.20: This figure shows the oxygen saturation found from each measurement as a function of the location of the measurement taken across the erythematic area in figure 2.18.

collection of accurate and reproducible data sets when interpreting data from local monitoring and PDT treatments.

Chapter 3

Drug

3.1 Summary

The propagation of light in tissue was discussed in the previous chapter. Physical changes in the tissue can effect the optical properties of the tissue and hence effect the way light will migrate in the tissue. For example, changes in the concentration of oxygen and blood in the tissue vary the way light travels through the tissue. The optical properties in the tissue can be measured by using the diffuse optical reflectance spectroscopy system that was calibrated. The depth and distribution of the light in the tissue can be modeled as a function of wavelength once the optical properties of the tissue are known. Modeling the light distribution in the tissue is important for dosimetry of a photodynamic therapy treatment.

Photodynamic therapy is a selective treatment, needing light, a photosensitive drug and oxygen in the local treatment area. For each tissue type, a photosensitive drug must be chosen that is both effective in the tissue and builds up more in the abnormal tissue than the normal tissue. So before treatments are performed, experiments must be pursued to show that the drug chosen will be both effective and selective during a PDT treatment. This chapter focuses on the optical properties of the photosensitizer chosen, protoporphyrin IX, and the distribution of the drug into the treatment zone.

3.2 Introduction

When considering a drug for use as the photosensitizer for a photodynamic treatment there are at least five major things to consider. First, the drug needs to be able to reach the treatment location. Second, the drug must absorb a wavelength that can travel through the tissue to the location of the drug. Third, the drug should preferably damage the abnormal cells leaving the normal healthy cells undamaged. Fourth, the drug must be able to be eliminated by the body without causing more damage. Fifth, the drug must be accessible to the patient.

Several things can effect the ability of the drug to get to the treatment zone. Some of these things to consider are the method of application, the size of the molecule and how quickly it may travel through the tissue. The drug may be able to be given systemically by an intravenous or oral method. Another option would be to apply the drug locally by multiple injections or by application to the surface and allowing it to diffuse through the skin. No matter which application method is chosen, the drug diffusion will need to be considered. The size of the molecule must be considered since it will drastically change the diffusion rates through the tissue.

The second item to consider is the absorption of the treatment light. Not only does the light have to be absorbed by the drug, but the light has to be able to reach the drug in the tissue. This limits the treatment sources to wavelengths found in the optical window discussed in chapter 2. There are many photosensitizers that strongly absorb short wavelengths, around 400 nm, but these wavelengths do not penetrate very far into the tissue before being absorbed by the tissue itself. Drugs often have a second absorption peak at a longer wavelength that is not nearly as strong.

The third item when choosing a drug is the selectivity of the drug. First off there should be very little or no dark toxicity from the drug. The only time the chosen drug should be destructive to tissue is when it is combined with light. Some drugs have been shown to preferentially build up in abnormal tissue such as tumors. Based on the tissue type some drugs will build up more than others. When treating a tissue type it is important to characterize the difference in drug build up between normal

tissue and the abnormal tissue to be treated. Often this change in drug concentration in certain tissue types measured by its fluorescence has been used to aid diagnosis and find borders for abnormal tissues.

During a photodynamic therapy treatment, it is important to minimize the side effects that may occur from the drug lingering in the body. Some of the photosensitive drugs are very difficult for the body to eliminate from the tissue, such as Photofrin. This drug can leave a patient photosensitive for about a month after a treatment. During this time it is very painful and often damaging to be exposed to sunlight. Other drugs can more easily be eliminated by the body. For an example, protoporphyrin IX, PpIX, is photosensitive but can also be found in the heme pathway. To treat with this drug, a very large amount is introduced to the tissue before treatment. After the treatment, it only takes about 8 hours to return to normal levels of PpIX in the body. The body is much better at dealing with this drug since there is already a method set up to eliminate it through the heme pathway to prevent the body from becoming photosensitive [1]. This will be discussed more in this chapter.

The fifth item to consider when choosing a drug for treatments is its accessibility. The drug needs to be both available to the patient and inexpensive enough for the treatment to be an option for most patients. Also the FDA needs to approve any treatment as safe that may be used on human patients. If the procedure has not been approved then it will not be accessible to most patients.

For many photodynamic therapy treatments, Photofrin is often the drug used as the photosensitizer. Photofrin was approved by the FDA for clinical use in humans in 1998 [62]. One major disadvantage to using Photofrin is that the patient remains photosensitive for several weeks after a photodynamic therapy, PDT, treatment [62]. Using aminolevulinic acid, ALA, instead of Photofrin for the drug during a PDT treatment gives the distinct advantage of decreased photosensitivity after the treatment. ALA was approved by FDA in 1999 [62]. Another benefit to ALA is that it is much less expensive than Photofrin.

This chapter will cover the delivery, and the results of monitoring ALA as it is applied to and absorbed by the tissue. By the processes described in this chapter the

ALA will be converted to Protoporphyrin IX, PpIX, in the tissue. The diffuse optical reflectance system described in chapter 2 was used to monitor the tissue. This device is not able to detect the ALA but can detect the absorption from the PpIX. This detection process will be described later in the chapter.

For a PDT treatment, the photosensitizer, light and oxygen must all be in the treatment area. Depending on the mechanism of the photosensitizer, the drug may start off as photosensitive or the drug may start off as non-photosensitive and convert to a photosensitive drug in the tissue.

3.2.1 History of and uses for Aminolevulinic Acid

ALA has been used to treat many conditions such as Bowen's disease, basal cell carcinoma, actinic keratosis, psoriasis and acne.

3.3 Getting Aminolevulinic acid to the Treatment Location

Aminolevulinic acid, ALA, must be kept under specific conditions. Next the chemical structure of the drug, how it converts into protoporphyrin IX, PpIX, and how that molecular size change effects the diffusion of the drug into the tissue will be explored further in this section. Finally when considering a treatment, the method of the drug administration and the dose of the drug are both very important and will be covered in this section.

3.3.1 Chemical structure of ALA and PpIX

Aminolevulinic acid must be stored at 4°C and maybe better to store at -20°C [63]. For each treatment, a new solution should be made for either topical or injectable application. Storing the ALA at higher temperatures or in solution may render it ineffective [63]. Crystalline ALA readily mixes with a lipid emulsion, saline or mepivacaine.

Aminolevulinic acid ALA, may convert to PpIX in tissue. This conversion will be covered in the section 3.3.2. Eight ALA molecules are needed to create one PpIX molecule. As a result the PpIX molecule is 3.3 times larger than the ALA molecule.

The drug 5-aminolevulinic acid hydrochloride was attained from Sigma-Aldrich (product number: A3785). This product is about 98% pure (tested by thin film chromatography) and has a molecular weight of 167.59. Figure 3.1 shows the structural representation of the ALA molecule. The linear molecular formula is [64]:

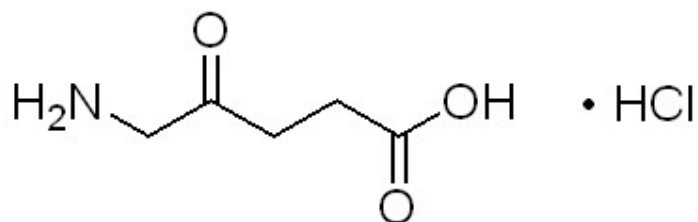
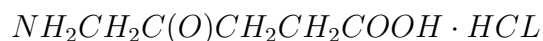
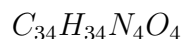


Figure 3.1: The chemical structure of aminolevulinic acid.

In the tissue, eight ALA molecules combine to create a PpIX molecule. Figure 3.2 shows the chemical structure of PpIX. The drug protoporphyrin IX has a molecular weight of 562.66 and its molecular linear formula is [64]:



3.3.2 Conversion of Aminolevulinic Acid to Protoporphyrin IX

Aminolevulinic acid be found the body naturally. ALA is a chemical intermediate in the heme pathway and arises from the citric acid cycle following a series of enzymatic reactions [1]. Succinyl coenzyme A, CoA, is one of the major intermediates in the citric acid cycle. The production of heme begins by combining glycine with

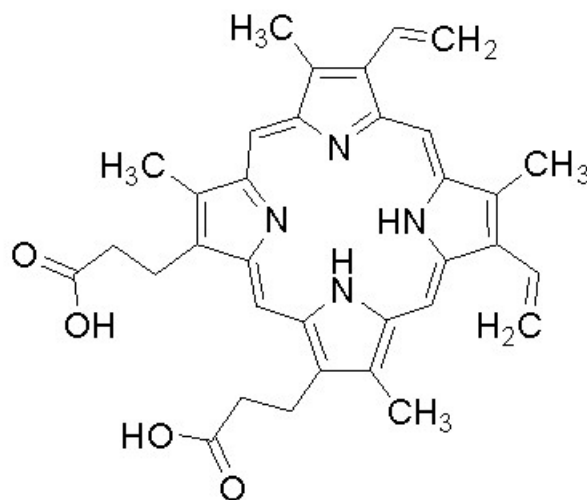


Figure 3.2: The chemical structure of Protoporphyrin IX.

CoA, which diverts the reaction from the citric acid cycle and into the heme pathway [1]. The combination of glycine and CoA with the enzyme ALA synthase creates ALA [65]. This combination occurs inside the mitochondria of the cell. The ALA moves out of the mitochondria and into the cytosol of the cell. In the cytosol the ALA becomes porphobilinogen and then uroporphyrinogen III which moves back into the cell's mitochondria. Inside the mitochondria, the uroporphyrinogen III becomes protoporphyrinogen IX and then protoporphyrin IX, PpIX. At this point the PpIX normally combines with iron to make heme. If there is insufficient iron to make heme then production of ALA is downregulated as shown in figure 3.3. The most optimal time to activate the PpIX is while the PpIX is in the mitochondria of the cell [66,67]. This can send a cell into apoptosis instead of necrosis.

If the ALA is given topically or systemically then ALA could enter the cells by a passive diffusion or by an active transport (such as the BETA transporter GAT-2 [68].) Once in the cells, the ALA is converted to PpIX as described above. By infusing the cells with large amounts of ALA, large amounts of PpIX are produced. Eight ALA molecules are needed to build one PpIX molecule (figure 3.4). Once the PpIX builds

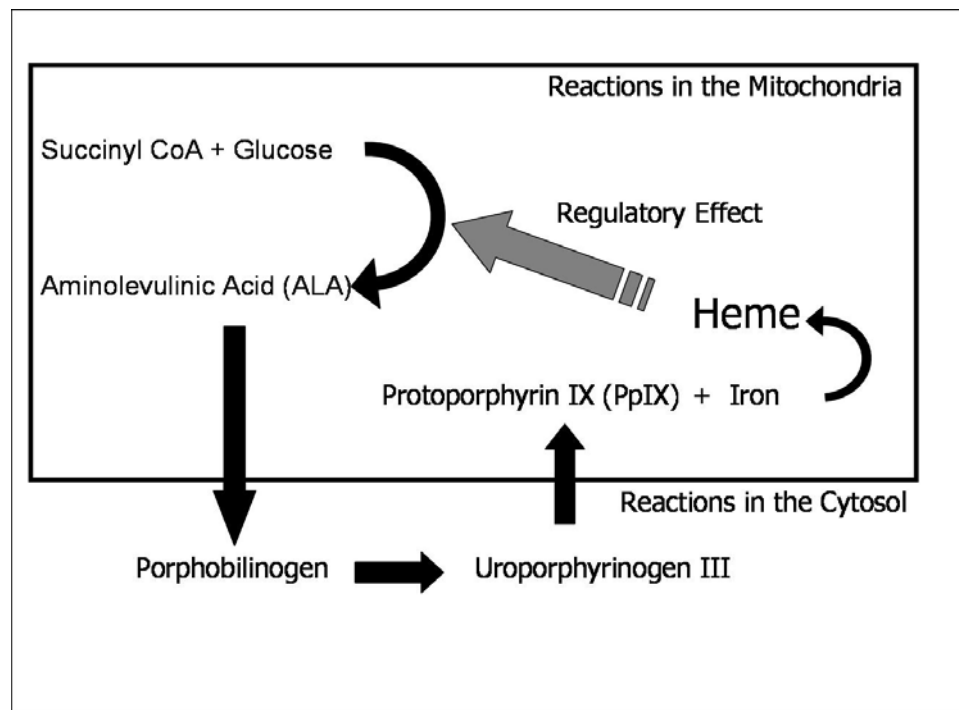


Figure 3.3: A graphical representation of the heme pathway originating the protoporphyrin IX creation from the citric acid cycle in side of the cell's mitochondria [1]. Notice that the regulatory effect can be negated once a large amount of ALA is introduced into the cell.

up in the tissue, the PpIX may leave the local tissue through the circulatory system. This will be covered more in section 4.3.

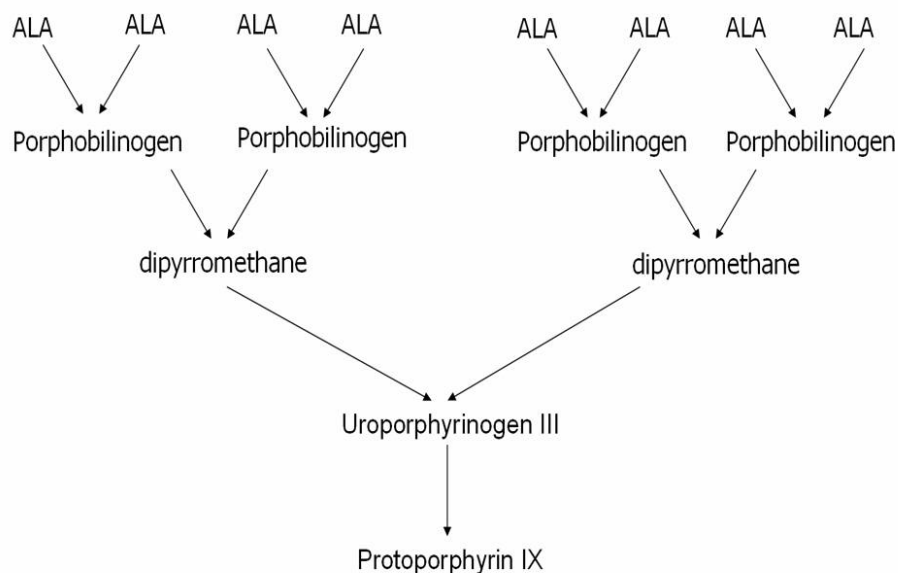


Figure 3.4: Eight ALA molecules are needed to create one protoporphyrin IX molecule. This process happens in the cell.

3.3.3 Delivery methods for ALA (injection vs. topical)

From diffuse optical reflectance spectroscopy techniques, it will be shown that the ALA starts to convert to PpIX in the tissue within 15 minutes. Diffusion after 15 minutes will be limited by movement of the larger PpIX molecule (since the smaller ALA molecule has been consumed.) Both topical application of ALA and injections of ALA were used in this research to apply the drug to a local area. This was a practical treatment method since the sarcoid neoplasms are locally invasive in the skin and subdermal tissue.

After applying aminolevulinic acid to the surface of the tissue or by local injection, the ALA will diffuse into the tissue. The diffusion coefficient of ALA reported by

Svaasand *et al.* is $10^{-6}[\frac{cm^2}{s}]$ [69]. The concentration as a function of the depth into the tissue can be approximated using the diffusion equation. If there is an infinite source of ALA at the surface of a semi-infinite tissue then the diffusion process behaves as,

$$C(x, t) = C_0 * \operatorname{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) \quad (3.1)$$

$$(3.2)$$

where x is the depth into the tissue, t is the time, D is the diffusion coefficient, and erfc is the complementary error function.

In figure 3.5, the fraction of ALA diffused into the tissue as a function of the depth in the tissue is graphed for different times.

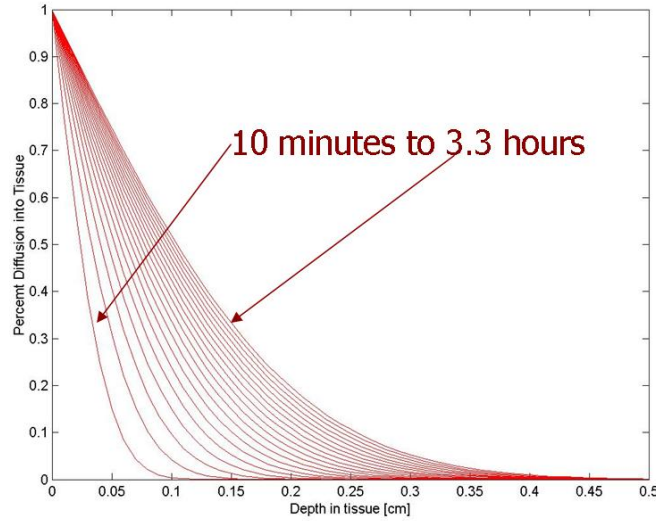


Figure 3.5: Diffusion of the aminolevulinic acid molecules into the tissue. The diffusion coefficient is $10^{-6}[\frac{cm^2}{s}]$. The separation in time between each curve is 10 minutes.

Once the ALA molecules combine to form a PpIX molecule the size of the molecule increases. It is expected that the molecule will slow its diffusion as it changes in size from an ALA molecule to a PpIX molecule.

Based on the slow diffusion of the PpIX in the tissue, it was decided to inject ALA directly into the treatment sites when treating a sarcoid. Many injections up to one centimeter from the surface were given. This reduced the time needed for the drug to diffuse into the tissue and allowed deeper treatments.

3.3.4 Dosage of ALA

Topical applications used 20% ALA in lipoderm [70]. For injecting into the tissue, we used an ALA concentration of 10% in saline. After a few of treatments 10% ALA in mepivacaine was used instead of saline to locally numb the treatment area. This was necessary since the local area became very sensitive after multiple needle injection sites.

3.4 Getting Light to the Drug and Treatment Location

As discussed previously in this thesis, it is important to have all three factors in one location to have an effective PDT treatment: (1) light, (2) drug and (3) oxygen. Problems can arise preventing one of these factors from getting to the desired location. The treatment may be modeled before a treatment occurs if a few things are known about the system. These things to consider will be covered in this section such as the absorption and effective spectrum of the protoporphyrin IX.

3.4.1 Absorption Spectrum of Protoporphyrin IX

If the absorption spectrum for protoporphyrin IX is known then it is possible to predict how much light the protoporphyrin IX will absorb. This section will describe an experiment and its results to find the absorption of protoporphyrin IX.

Also this section will describe an experiment that gives the relationship between the concentration of the drug as a function of the absorption at one specific wavelength.

Wavelength Dependent PpIX Absorption Spectrum

The purpose of this experiment was to measure the spectral shape of the protoporphyrin IX absorption spectrum in methanol.

Materials and Methods

The powdered drug protoporphyrin IX, PpIX, was purchased from Sigma-Aldrich. A solution of PpIX in methanol was made up at a concentration of $17.6[\frac{\mu g}{mL}]$. The solution was sonicated to ensure that the powder was not clumping. There was an attempt to make this same solution with PpIX and water instead of methanol and the PpIX would precipitate out of the solution quickly.

The PpIX solution was poured into a 3 milliliter quartz cuvette with 1 cm pathlength. The transmission through the sample was measured with an HP spectrometer. The optical density of the sample OD_S was recorded from 190 to 820 nanometers every 2 nanometers. A reference measurement was also taken of a quartz cuvette filled with methanol. The optical density of this reference measurement, OD_{Ref} , was also recorded. To get the optical density of just the PpIX OD_{PpIX} , the reference measurement was subtracted from the sample measurement.

$$OD_{PpIX} = OD_S - OD_{Ref} \quad (3.3)$$

Results

The optical density of protoporphyrin IX as a function of the wavelength is corrected for the cuvette pathlength and molar concentration and is shown in figure 3.6.

Concentration Dependent PpIX Absorption at a Fixed Wavelength

To make this optical model useful not only do we need the absorption spectrum of the PpIX but we will also need the relationship between the concentration of the PpIX and the optical absorption. Previously the spectral shape of the PpIX optical

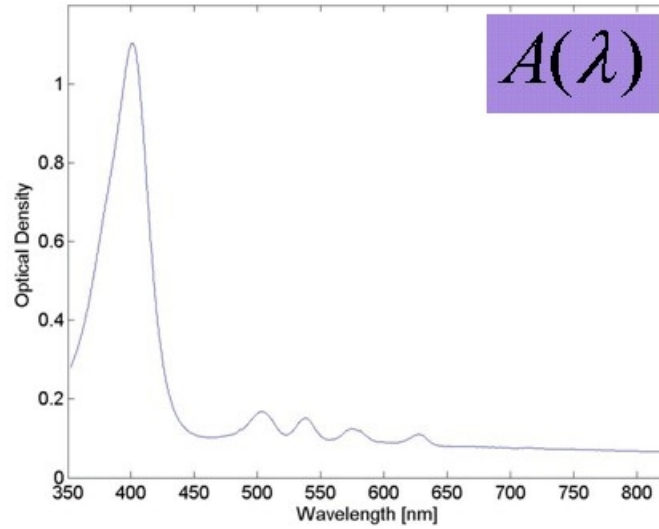


Figure 3.6: Absorption Spectrum of PpIX in methanol at a concentration of $17.6[\frac{\text{micrograms}}{\text{mL}}]$ of PpIX in methanol as a function of the wavelength.

density for one concentration was found. Now to determine the concentration of the PpIX based on its optical density the relationship between the two needs to be experimentally found.

Materials and Methods

To approximate the absorption of PpIX in tissue the PpIX was suspended in a protein solution containing concentrated 25 human serum albumin. Albumin was chosen since it is easily accessible and is a protein found in blood. Twenty samples were made. These samples ranged in concentration from 0 to 200 micrograms per milliliter $[\frac{\mu\text{g}}{\text{mL}}]$ PpIX in albumin, (Albumin, human, USP 25% solution Buminate 25%, Baxter Healthcare Corporation, Hyland Immuno, Glendale, CA, 91203, USA).

The light transmitted through each of these samples was measured using 3 milliliter cuvettes in a spectrophotometer (Beckman Coulter DU Series 500). The absorbance, A^{635} at 635 nm was recorded for each sample. Next the transmission, T^{635} , through the sample was calculated in equation 3.4.

$$T^{635} = 10^{-A^{635}} \quad (3.4)$$

Then the absorption coefficient at 635 nm, μ_a^{635} , was calculated using equations 3.5 and 3.6 where L is the pathlength through the sample. In this case L was one centimeter.

$$T^{635} = e^{-L\mu_a^{635}} \quad (3.5)$$

$$\mu_a^{635} = \left(\frac{-\ln T^{635}}{L} \right) \quad (3.6)$$

The extinction coefficient ϵ^{PpIX} is

$$\epsilon^{PpIX} = \frac{\mu_a^{PpIX} W}{\ln(10) C} \quad (3.7)$$

Where W is the molecular weight of PpIX, ϵ^{PpIX} is the extinction coefficient of PpIX and C is the concentration of PpIX.

Results

The concentration of PpIX is linearly related to the absorption coefficient at 635 nm, figure 3.7. The molar extinction coefficient at 635 nm was about $21.6 \text{ mm}^{-1} \text{ mM}^{-1}$ or equivalently $C^{PpIX} = 113\mu_a^{635}$ if C^{PpIX} is in ug/ml and μ_a^{635} is in cm^{-1} .

The relationship between the optical absorption and the concentration of PpIX can be approximated as a linear relationship in this concentration range. Because the the concentration of PpIX is not expected to increase above 200 microgram per milliliter, this linear relationship should hold for all of our experiments.

$$C^{PpIX} = 113\mu_a^{635} \quad (3.8)$$

3.4.2 Total Effective Fluence of Protoporphyrin IX

For a photodynamic therapy treatment to be effective, both the light and the drug must be localized to the same treatment area. The drug must be able to absorb the light reaching the treatment location.

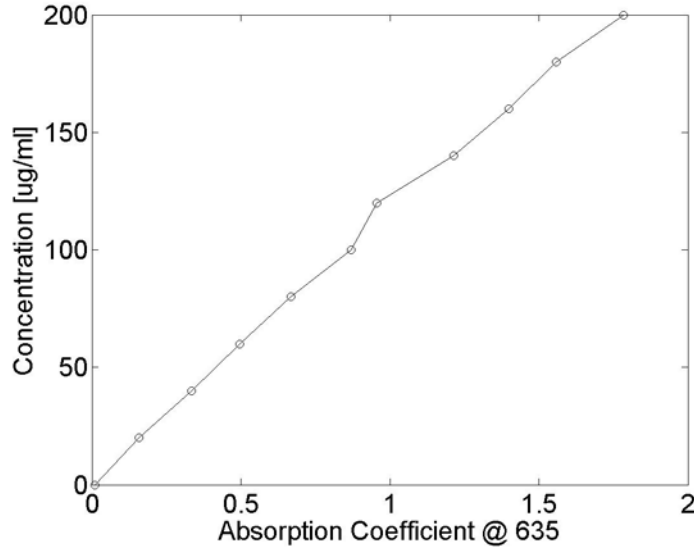


Figure 3.7: The concentration of PpIX in albumin as a function of the absorption coefficient at 635 nm in 25% albumin. The conversion factor between the absorption coefficient and the concentration of PpIX is $113[\frac{cm\mu g}{mL}]$ and the line that fits this data is $C^{PpIX} = 113\mu_a^{635}$.

Active Spectrum

The active spectrum is the spectral shape of the light source multiplied by the wavelength dependent absorption of the drug, PpIX. The absorption spectrum of PpIX is shown in figure 3.6.

The active spectrum also takes into account the spectral shape of the source light. During a photodynamic therapy treatment the light must travel through tissue to reach the treatment location. The tissue will attenuate some wavelengths more than others and so the source spectrum will change with depth.

Light transmission through tissue can be approximated using optical properties from section 2.2. The absorption coefficient was assumed to be 75% water, 1% blood and 80% oxygen saturation in the blood at depths, d , of 0.25, 0.50, 0.75 and 1 centimeter. The change in the transmission as a function of wavelength is shown in figure 3.8.

$$f_b = 0.01 \quad (3.9)$$

$$f_w = 0.75 \quad (3.10)$$

$$S = 0.80 \quad (3.11)$$

$$d = [0.25, 0.50, 0.75, 1] \quad (3.12)$$

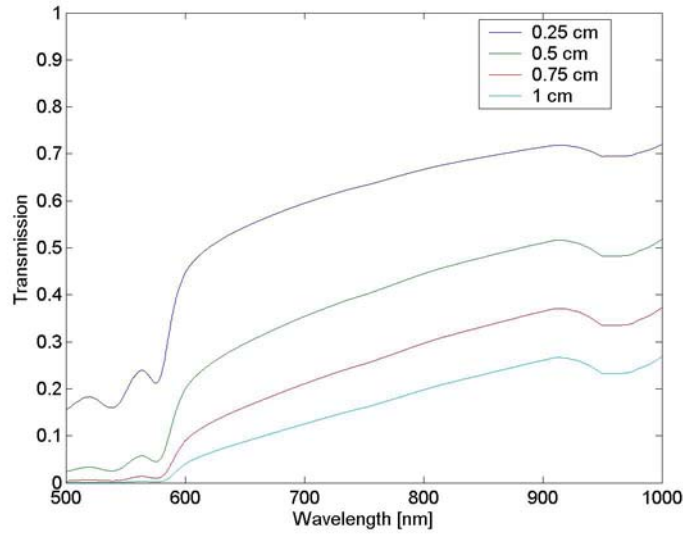


Figure 3.8: Notice that the transmission as a function of depth below 600 nanometers drops significantly with depth into the tissue. Fraction of water was 0.75, the fraction of blood was 0.01 and the oxygen saturation was 0.80. The depth d ranged the values 0.25, 0.50, 0.75 and 1 centimeter into the tissue. Both absorption and scattering of the tissue were taken into account for the transmission.

The absorption of the tissue is not always a constant and during a PDT treatment the photosensitizer is building up in the tissue. This means that there is more drug present during the treatment. One consequence is that absorption of light by superficial drug may block light from reaching deep or locations in the tissue.

The diffuse optical reflectance spectrometer system from section 2.4 is able to detect the PpIX concentrations in the tissue. All of the measurements were analyzed after treatment but could potentially be done during treatment to find real-time feedback to the operator.

Total Effective Fluence

For the drug, PpIX, to be effective during a PDT treatment it needs to absorb light. One might choose a wavelength near 405 nm since there is a strong absorption peak (3.6). This would make sense if the light did not have to travel through tissue first. Notice that in figure 3.8 most of the light at 405 nm is blocked. The total effective fluence, E_D , is the product of both of these important factors and also taking into account the treatment light incident on the tissue, $I(\lambda)$ [2,3].

For now back to focusing on the product of the transmission, $T_d(\lambda)$ through the tissue and the absorbance $A(\lambda)$ of the drug. Figure 3.9 displays the active spectrum as a function of wavelength for multiple depths which is this product $T_d(\lambda)A(\lambda)$. This spectrum is assuming a gaussian shape of the PpIX absorption at 635 nm as described in the fitting algorithm section 2.4.2. Looking at this figure it is obvious that 635 nm light would be much more consistant for all depths into the tissue.

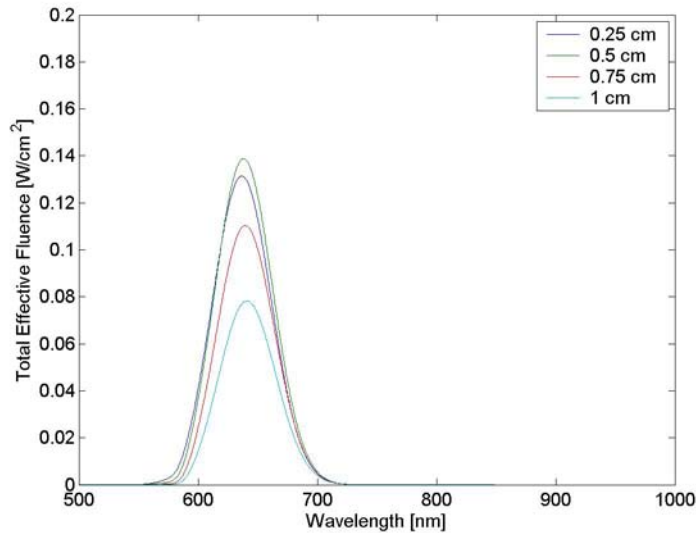


Figure 3.9: An example of the active spectrum of PpIX in typical tissue. The peak at 635 nm was chosen for deep treatments based on this active spectrum importance.

As hinted at above, the incident treatment light, intensity and wavelengths, $I(\lambda)$, will make a huge difference as to whether the drug can absorb the light before the

tissue does. Equation ?? gives the integral that describes the total effective fluence during a PDT treatment.

$$E_d = \int I(\lambda)T_d(\lambda)A(\lambda)d\lambda \quad (3.13)$$

Where: $I(\lambda)$ = incident spectral irradiance from the light source at λ $T_d(\lambda)$ = optical transmission through tissue to depth d $A(\lambda)$ = absorption of the sensitizer at λ

3.5 Conclusion

In this chapter it has been shown that the drug, PpIX, can be delivered to and measured in the local treatment area for a sarcoid. Once the drug is in the tissue, the treatment light has been shown to be optimized to both the drug absorption and the tissue transmission by considering the active spectrum of the drug-tissue combination. Finally a model has been created that gives the rate constants for the drug building up in the tissue as the blood is taking part of it away. In the next chapter a similar model to this will be considered during a treatment where the light is also changing the drug and effectively removing it.

Chapter 4

Component Model

4.1 Summary

In chapter 3, it was shown that the PpIX absorbs with a peak at 635 nm and that there was a linear correlation between the PpIX absorption and the concentration of the PpIX. Using the diffuse optical reflectance system in chapter 2, the PpIX absorption can be measured and monitored as it builds up.

4.2 Introduction

In this chapter, a model can be shown to fit the concentration of the drug in the tissue before and during a treatment. Having a model to fit the concentration of the drug is very useful during a treatment to predict if the drug is effective anymore. Also the rate constants for both the build up and the fall off of the concentration of the PpIX in the tissue are fit for.

4.3 Compartmental Model (Kinetic)

A compartmental model has been developed for a topical application of aminolevulinic acid, ALA. This experiment was performed to be able to predict the build up of PpIX based on a given application of ALA. This is necessary to know and be able to predict when determining the dosimetry of a photodynamic therapy treatment.

During a topical treatment 20% ALA in Lipoderm was applied to the tissue surface. Through a diffusion process the ALA penetrates into the tissue. Once in the tissue the cells convert the ALA to PpIX as discussed in section 3.3.2. During this conversion process eight ALA molecules combine in the cell's mitochondria to form one PpIX molecule with the molecular weight of 562.66. Without a way for the cells to prevent this reaction from occurring at this step in the pathway, the ALA continues to convert to PpIX and build up in the tissue as long as the source of ALA remains on the surface. As long as iron was in the tissue, hemoglobin was made but eventually the supply of iron ran out and PpIX built up as discussed in section 3.3.2. Using the reflectance spectra from the diffuse optical reflectance spectrometer, the absorption due to the PpIX could be detected as described in section 2.4. The rate that ALA penetrated into the tissue and was converted to PpIX could be approximated by a rate constant k_0 .

Now as the PpIX was building up in the tissue there was also blood flow in the local region that takes some of the PpIX away from the local application area. The rate the the PpIX was taken away by the blood was approximated by a rate constant of k_1 . These rate constants can be thought of as a bunch of little rates bundled into one larger overall trend. For example the rate that the PpIX leaves the tissue is not just taken at the rate of blood. The rate that the PpIX moves into the extra cellular fluid and then at a separate rate it moves into the capillaries but these rates are included in the rate constant of k_1 .

This system can be approximated by the figure 4.2. The boxes in this diagram represent locations either ALA or PpIX can be. The arrows between the boxes represent the rate the drug can move from one box to the next.

Next an experiment was designed to determine the values of each of the rate constants, k_0 and k_1 . To start, diffuse optical reflectance spectrometer measurements of the sarcoid area were taken. After the initial measurements, 20% ALA in lipoderm was applied topically to the tissue. At this time the concentration of ALA in the surface reservoir was known and went from zero to $250[\frac{mg}{g}]$ ALA in Lipoderm. After 32 minutes the topical was removed to approximate an impulse function. A model of



Figure 4.1: Schematic of the local system. At the top of the surface there is a reservoir of topical ALA. In this kinetic study blood flow is a significant factor that takes the ALA out of the tissue.

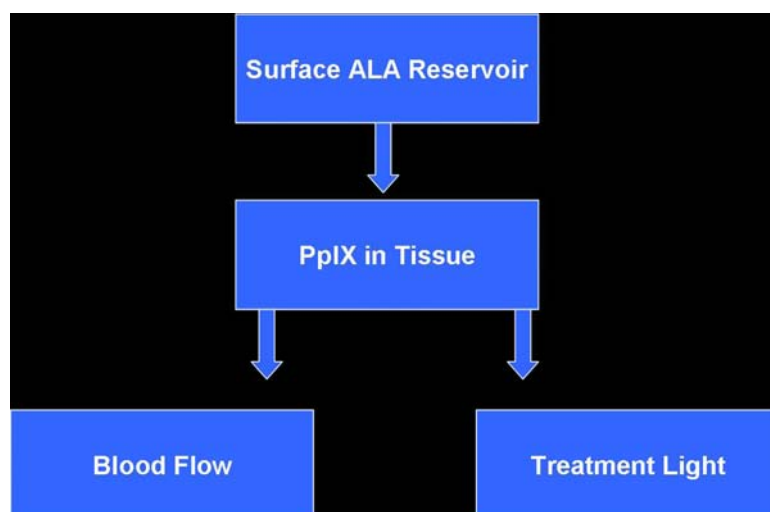


Figure 4.2: Diagram of the component model. Each arrow represents the rate constant between each group. The middle box, PpIX in Tissue, is measurable by the diffuse reflectance spectrometer.

this application of ALA as an impulse function is shown in figure 4.3.

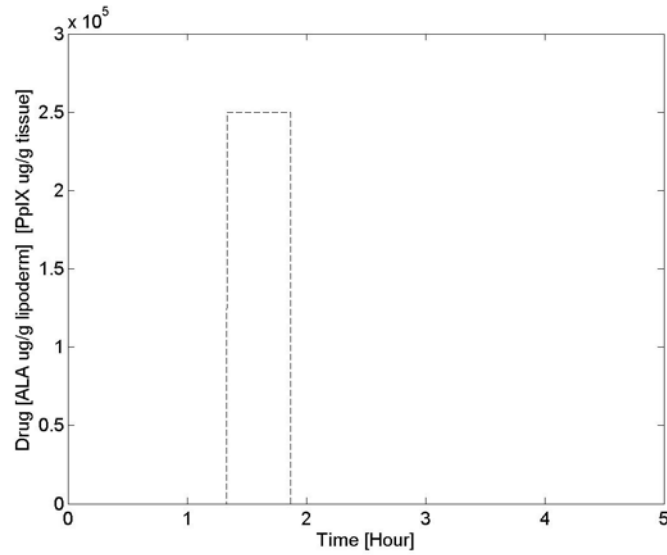


Figure 4.3: This figure is a graphical representation of the concentration of topocal ALA at the surface of the treatment tissue site. The application can be represented as an impulse function. Before application there was no ALA at the surface of the tissue. After a little over an hour the topocal ALA was applied with a concentration of $250 \left[\frac{mg}{g}\right]$ ALA in Lipoderm. After 32 minutes the topical was wiped off and the concentration of ALA at the surface went back to zero.

An animal with very little melanin was chosen for these measurements so melanin would not have to also be fit for. The sarcoid was easily accessible on the hind leg.

Before, during and after ALA application, ten measurements per time point were taken. Each of these measurements were fit for the fraction of blood, oxyhemoglobin, deoxyhemoglobin, PpIX, bilirubin and scattering as described in section 2.4. The absorption due to PpIX at 635 nm is found from the fitting program and then is converted to the concentration of PpIX in the tissue using the calibration factor found in equation 3.8. For each time point the average concentration was found. Using the equation 4.1 a Matlab program was written to find the rates constants, k_0 and k_1 that fit the data in figure 4.4. ALA and PpIX correspond to concentrations.

$$\frac{d[PpIX]}{dt} = k_0 * (ALA) - k_1 * (PpIX) \quad (4.1)$$

Rates that fit the data are:

$$k_0 = 3.4e^{-7}[\frac{1}{s}] \quad (4.2)$$

$$= 1.2e^{-3}[\frac{1}{hr}] \quad (4.3)$$

$$k_1 = 4.1e^{-4}[\frac{1}{s}] \quad (4.4)$$

$$= 1.4[\frac{1}{hr}] \quad (4.5)$$

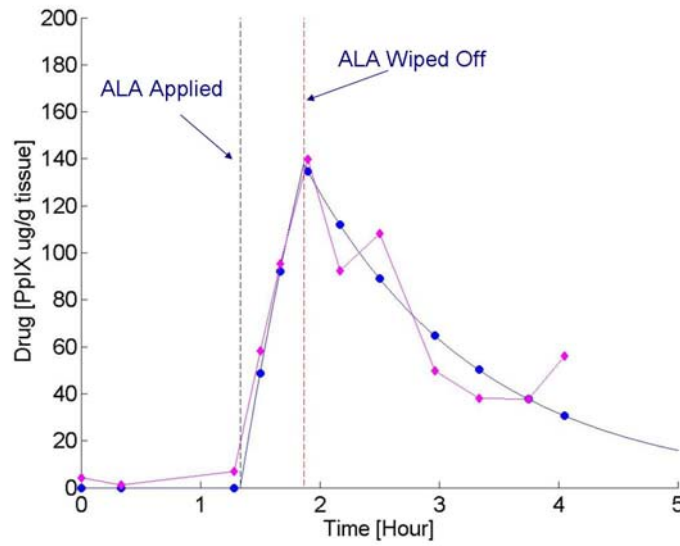


Figure 4.4: This is a plot of the mean of the measured concentration of PpIX as a response to the topical ALA applied to the tissue surface and a curve fit to the data. The data is represented by pink diamonds and line and the fit is represented by the blue circles and line. This is the data that was used to determine the rates k_0 and k_1 where the rate that ALA penetrated into the tissue and was converted to PpIX could be approximated by a rate constant k_0 and the rate the the PpIX was taken away by the blood was approximated by a rate constant of k_1 .

Now that the rate constants are known and a model has been designed, it is then possible to determine the amount of PpIX that has left the treatment site through the blood flow. The rate constants are shown in figure 4.5. Figure 4.6 is a graph of the amount of PpIX lost by the blood flow.

So now there is a model for the build up of PpIX in the sarcoid tissue based on the application of topical ALA. An impulse of ALA was applied to the tissue and

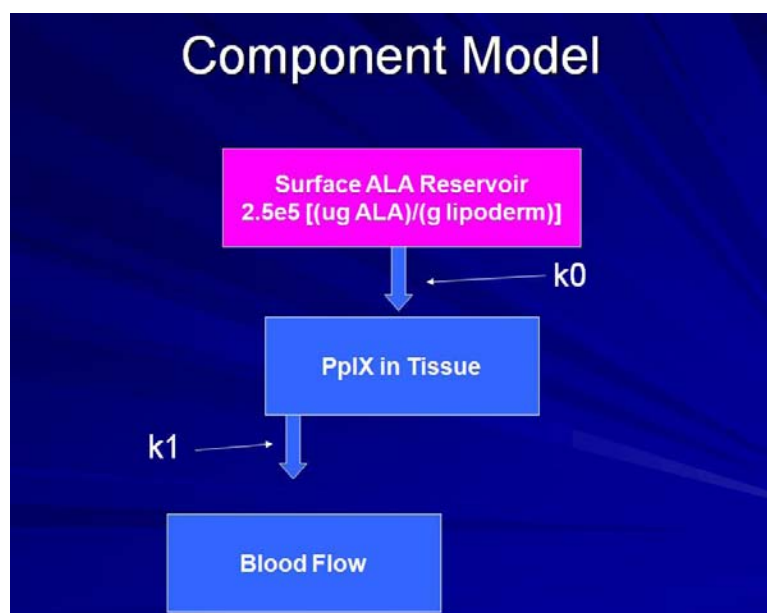


Figure 4.5: This is a graphical representation of the rate constants that were found from this experiment.

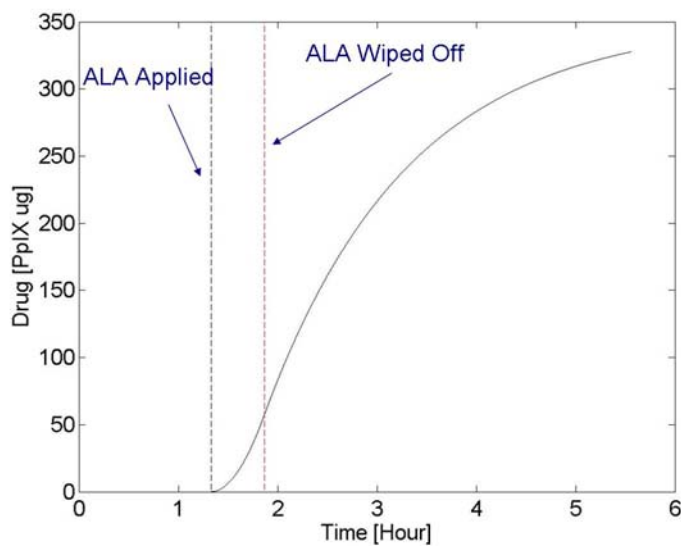


Figure 4.6: This is a graph illustrating the total micrograms of PpIX that has been removed from the tissue by the blood flow.

simulated. Measurements before and after this impulse were then taken. Based on these measurements the amount of PpIX building up in the tissue was found and modeled based on the knowledge of the local tissue. In the next section, the effect of a treatment light on the tissue containing PpIX will be modeled based on treated tissue.

4.4 Component Model (During a PDT Treatment)

In chapter 3, a kinetic model was designed to determine the rate, k_0 , that the ALA would diffuse into the tissue and convert to PpIX and the rate, k_1 , that the PpIX is taken away from the local tissue by the blood flow as shown in figure 4.7. During a PDT treatment the PpIX is exposed to the treatment light which will convert the PpIX to another molecule, decreasing the concentration of PpIX in the tissue. The PpIX is converted at a rate of k_2 as shown in figure 4.7. The rate of photobleaching can be considered to be included in this rate constant k_2 . There is just not enough information to be able to distinguish between the photobleaching and the conversion to photoproducts during a treatment.

4.4.1 Materials and Methods

To determine the rate constants during a PDT treatment an animal with a sarcoid in the right tear duct was used. A PDT treatment had been performed once before in this location. Diffuse optical reflectance spectrometer measurements were taken before the PDT treatment began.

After initial measurements were taken the PDT treatment began. Topical ALA was applied to the surface of the tissue at a 20% concentration in Lipoderm, or 250 $[\frac{mg}{g}]$ ALA in Lipoderm. This was left on the tissue for a total of about 5 hours unlike the kinetic study in section 4.3 where the application of topical ALA was treated as an impulse function. To assure that there was always a constant ALA supply at the surface of the tissue, the topical was reapplied every half hour. Figure 4.8 is a graphical representation of the application of topical ALA to the surface of

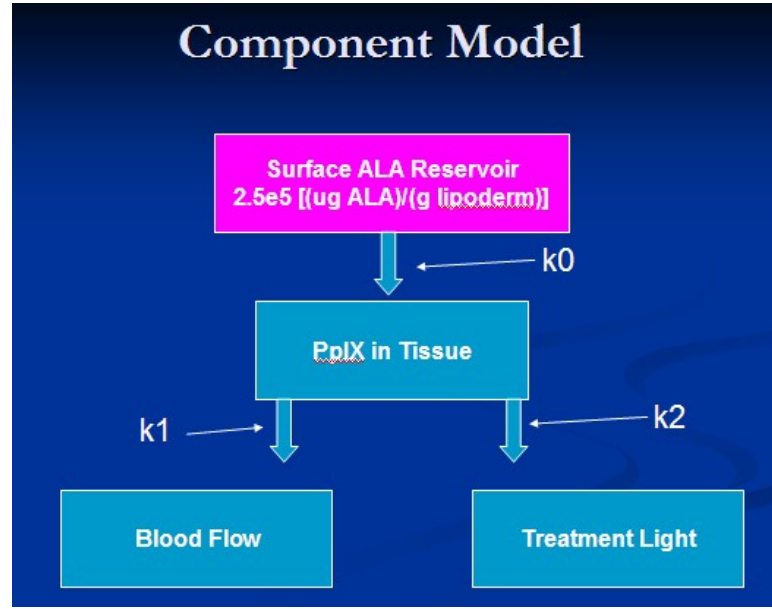


Figure 4.7: Diagram of the component model. Each arrow represents the rate constant between each group. The middle box, PpIX in Tissue, is measurable by the diffuse reflectance spectrometer. In this section the rate k_2 is determined.

the tissue.

Diffuse optical reflectance spectrometer measurements were taken before, during and after the application of the treatment light to the tissue. For this treatment the 635 nm diode laser light was used to treat for a total of $304 \left[\frac{J}{cm^2} \right]$. For each of these measurements, a fitting program described in section 2.4.2 was run which reported physical characteristic changes in the tissue. These properties were then averaged for each time point and then fit for the rate constants using the equation 4.6.

$$\left(\frac{d(PpIX)}{dt} \right) = k_0 * (ALA) - k_1 * (PpIX) - k_2 * (PpIX) \quad (4.6)$$

Where the (ALA) is the concentration of ALA at the surface and $(PpIX)$ is the concentration of PpIX in the tissue treatment location.

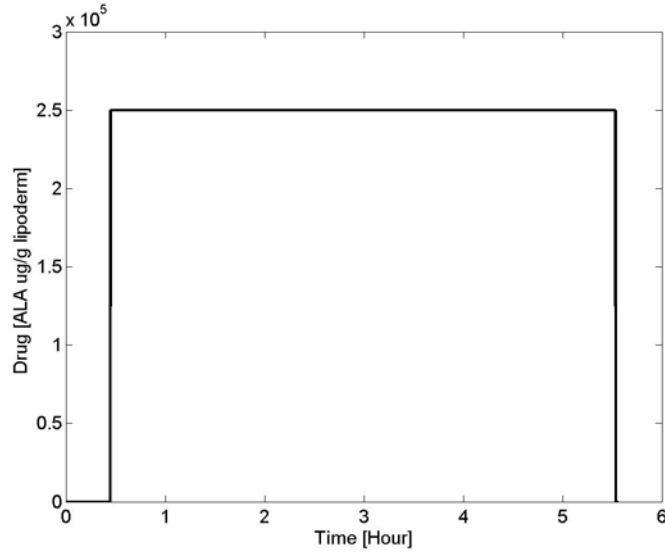


Figure 4.8: Input function of the concentration of the drug, ALA, in a topical diffusing into the tissue.

4.4.2 Results

Figure 4.9 shows the mean values of PpIX in the tissue as a function of time during the PDT treatment. Indicated in the picture are the times that the ALA and light began and ended. The mean PpIX concentration was fit by equation 4.6 and resulted in a curve that fit the data shown in figure 4.10. The rate constants shown in equation 4.7 are what were used to make the fit to the data in figure 4.10.

$$\left(\frac{d(PpIX)}{dt} \right) = k_0 * (ALA) - k_1 * (PpIX) - k_2 * (PpIX) \quad (4.7)$$

Where:

$$k_0 = 4.67e^{-8} \left[\frac{1}{sec} \right] \quad (4.8)$$

$$= 1.6e^{-4} \left[\frac{1}{hr} \right] \quad (4.9)$$

$$k_1 = 1.29e^{-4} \left[\frac{1}{sec} \right] \quad (4.10)$$

$$= 0.4 \left[\frac{1}{hr} \right] \quad (4.11)$$

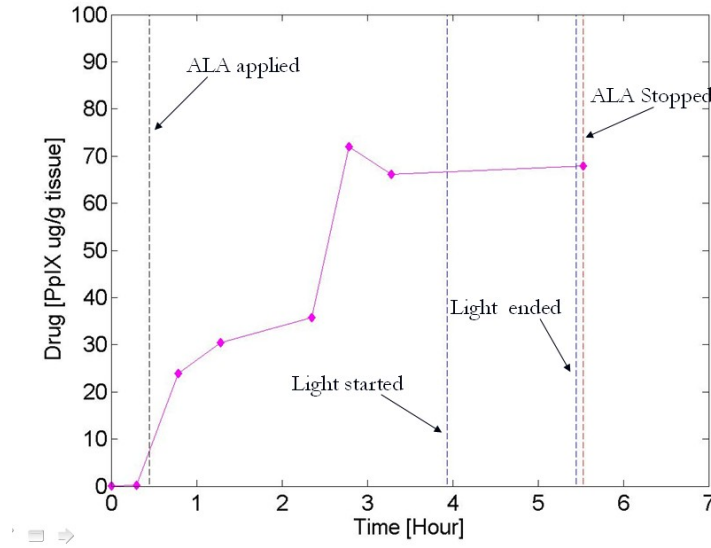


Figure 4.9: This figure shows the mean values of PpIX in the tissue as a function of time during the PDT treatment. Indicated in the picture are the times that the ALA and light began and ended.

$$k_2 = 5.33e^{-5} \left[\frac{1}{sec} \right] \quad (4.12)$$

$$= 0.1 \left[\frac{1}{hr} \right] \quad (4.13)$$

If the light was not included in this treatment figure 4.11 demonstrates what the concentration of the PpIX in the tissue may do.

Figure 4.12 is a graphical representation of the rate constants for a PDT treatment.

Figure 4.13 shows the amount of PpIX that is lost to the blood and the light during application of the ALA at the surface.

Type of treatment	k_0	k_1	k_2
Kinetic	1.2e-3	1.4	
PDT treatment	1.6e-4	0.4	0.1

Table 4.1: This table describes the rate constants of the build up and fall off of PpIX in the tissue. The units are $[hr^{-1}]$.

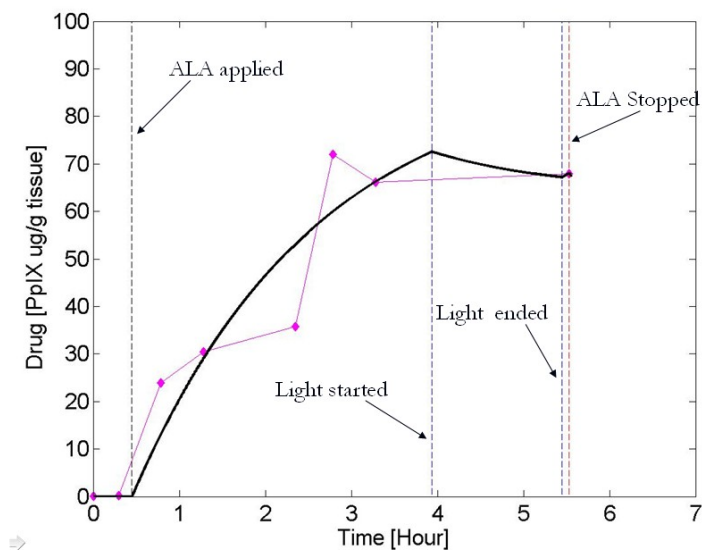


Figure 4.10: This figure shows the curve fit to the mean PpIX in the tissue. The pink diamonds and line are the data and the black line is the fit.

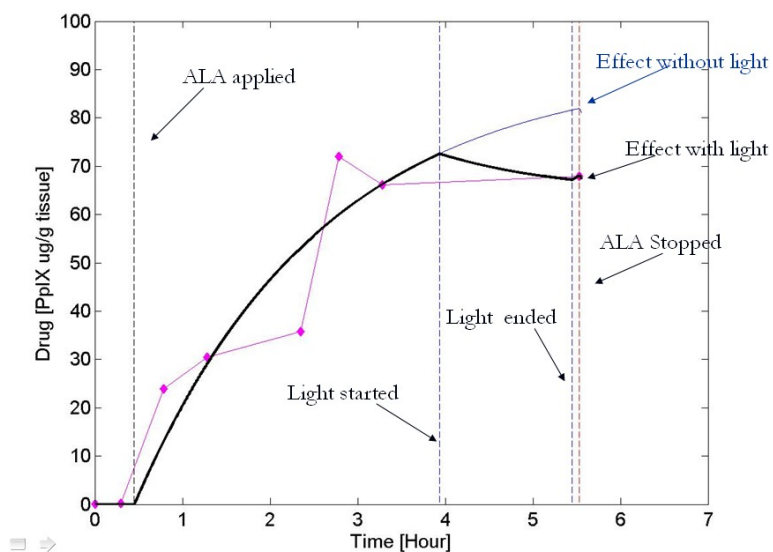


Figure 4.11: If the rate constant k_2 were set to zero or if the light were not used during this application of ALA then this figure demonstrates the resulting curve and increase in concentration of PpIX in the tissue.

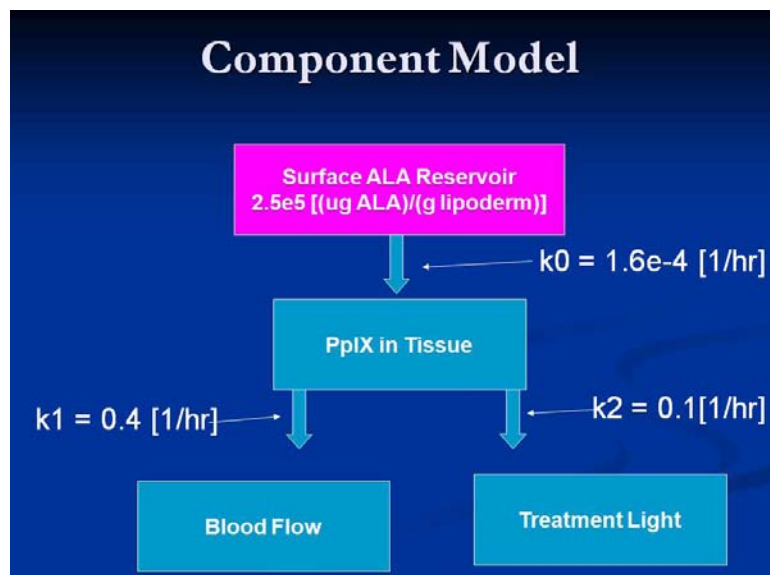


Figure 4.12: This figure shows the rate constants for each step in the PDT treatment process.

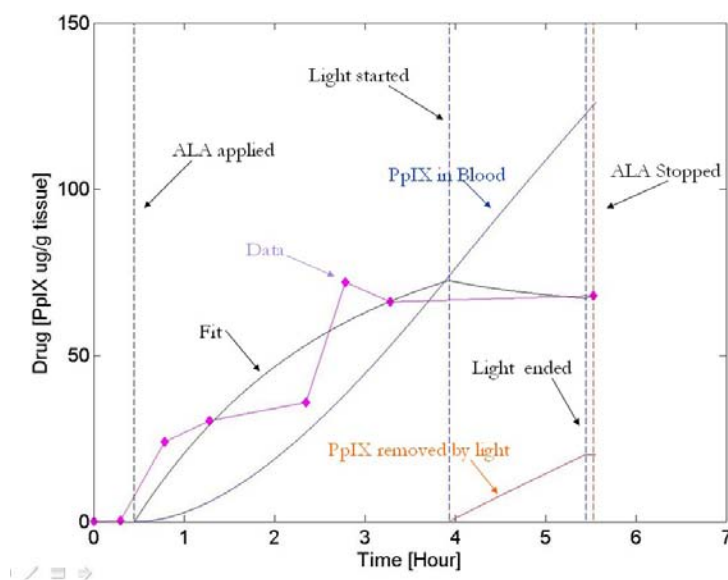


Figure 4.13: This figure shows the amount of PpIX that is lost to the blood and the light during application of the ALA at the surface.

4.5 Conclusion

In this chapter the concentration of PpIX in the tissue was measured using diffuse optical reflectance measurements. These measurements were used to determine the rate constants k_0 , k_1 and k_2 . These rate constants are: 1) k_0 predicts the amount of ALA diffusing into the tissue and how quickly it converts to PpIX, 2) k_1 is the rate that the PpIX is lost through the blood flow and 3) k_2 is the rate that the PpIX is lost by a treatment light.

Chapter 5

PDT Effect

5.1 Summary

In this chapter the combination of both light and drug working together for a photodynamic therapy treatment will be explored. The amount of drug in the tissue can be measured but the minimum amount of light and drug needed to treat a specific tissue, or the photodynamic dose threshold, remains unknown. If the photodynamic dose is known for a specific tissue type then the dosimetry necessary for future treatments can be predicted.

5.2 Introduction

Several animals were treated with PDT. The dose of the drug and the light were recorded. These experiments were performed to determine the dosimetry of a photodynamic therapy treatment necessary to treat specific tissue types.

We expect to find the threshold dose of both the light and drug together necessary to cause damage to the tissue. This threshold will be referred to as the photodynamic therapy threshold dose. This PDT threshold dose defines the depth in the tissue that the treatment will be effective up to. Photodynamic therapy is much less damaging to the tissue and may cause less scarring than other treatments such as cryotherapy. This makes PDT a prime candidate for an effective treatment to surface lesions as long as the PDT threshold dose is achieved at a depth greater than the thickness of the tumor. An effective method for treating larger tumors may be to repeat the treatment

until the desired treatment depth has been achieved. Problems with current sarcoid treatments are that they are ineffective, cause scarring and may not be repeatable.

5.3 Standard Protocol of PDT

Before a photodynamic therapy treatment, the baseline diffuse optical reflectance was measuremented. Aminolevulinic acid, ALA, was applied to the local treatment area either applied topically or injected. The equine are such large animals that just local treatments were performed on them, due to the cost of the quantity of the ALA, but the canine was much smaller and an oral administration of the drug was performed. During local application of the drug, an estimated concentration of 1 milligram ALA to one gram treatment tissue was attempted to be held constant but for the oral administration 60 mg ALA per kilogram body weight was administered. For the topical application 20% ALA was mixed with lipoderm and for an injection 10% ALA was mixed with carbocaine as described in section 3.3.4. The topical was then applied with a gloved finger saturating the surface of the treatment zone. The injectable ALA was injected into the local area using a 27 gauge needle, down to a depth of 1 centimeter and multiple points of entry. The solution was injected as the needle was pulled out of the tissue to ensure the solution was saturated throughout the tissue mass.

Once the ALA was in the tissue it was converted to protoporphyrin IX, PpIX. More diffuse optical reflectance measurements were taken to monitor the increase in PpIX in the tissue. After the PpIX was allowed to build up in the tissue, the area was irradiated by a treatment light for a predetermined amount of time. Once the photosensitizer and the light combined in the tissue, they created singlet oxygen that damaged the cells. After the light treatment was completed, more diffuse optical reflectance measurements were taken.

Differences in the protocols for each patient are more specifically defined later in this chapter but below is the general protocol used in this study.

5.4 Equine case treatments

Some of the patients had many sarcoids, not all of which were treated with PDT. Lesions were chosen for treatment based on accessibility, tumor aggressiveness, lack of other good treatment options, and whether or not the tumor was clinically significant. Some tumors were selected for treatment to determine if either a partial clinical improvement or a histologic change, such as cellular apoptosis or necrosis, could be elicited, with no expectations of curing the tumor. The patients and information about the lesions and tumor sizes treated are shown in table 5.1. All owners were informed of the experimental nature of the treatment and signed consent forms.

Patient	Lesion number	Tumor size	Treatment Location
A	1	1.5 cm diameter	Base of Left Ear
	2	1.5×0.7 cm	Left pinna margin
	3	1×1.5 cm	Left medial thigh
	4	2×2.5 cm	Left lateral fore limb
B	5	6×3.5 cm	Left Ear
	6	7×4 cm	Right Ear
C	7	6×3×3 cm	Left eye
D	8	1.5×1.5×1 cm	Medial canthus left eye
E	9	4×1×1 cm	Left pinna
F	10	2×2 cm	Left of midline on cranial chest
	11	2×3 cm	Left ventrolateral abdomen
G	12	3×8 cm	Right side of face
H	13	0.5×0.5 cm	Left side of back
I	14	3×2.5 cm	Left side of face
	15	9×8×5 cm	Left ventral flank
	16	2×1.5×0.5 cm	Midline just cranial to the manubrium
J	17	3×3 cm	Top of Right Leg
	18	5×4×1 cm	Right side of chest
K	19	1×2 cm	Right of nose
L	20	9×8×3 cm	Top of left leg
	20	2×1.5×0.5 cm	Chest
	21	3×2.5 cm	Left of nose
M	22	4.5×4.5 cm	Right side stomach
N	23	1×1 cm	Right side of middle back

Table 5.1: This table describes the size and location of the sarcoids that were treated.

The photosensitizing agent used was 5-aminolevulinic acid (ALA) compounded either as a 20% topical solution in a specially compounded, rapidly absorbed carrier or as a 1% solution in carbocaine. Solutions were prepared immediately prior to each treatment due to the instability of ALA in solution. Three light sources were used. The primary source was a laser diode operating at 635 nm pigtailed to an optical fiber with a collimating lens that delivered a uniform beam of 170 mW over a 3-cm-diameter area to yield an irradiance of $25 \left[\frac{mW}{cm^2} \right]$. The second source was an array of 72 light emitting diodes (LEDs) in a clear plastic disk operating at 641 nm, 21-nm full-width half-maximum, which yielded an irradiance of $7 \left[\frac{mW}{cm^2} \right]$ over a 3-cm-diameter area. The third was also an LED array with two rows of 16 light emitting diodes in a long clear rectangular shape operating at 641 nm, 21-nm full-width half-maximum, which yielded an irradiance of $7 \left[\frac{mW}{cm^2} \right]$. Calculations were made to adjust for the fact that the LED source was not monochromatic. The irradiance of the LED array at 641 nm will be considered equivalent to a 635-nm laser diode that delivers an irradiance of $5 \left[\frac{mW}{cm^2} \right]$. The LED light exposures have been converted to equivalent exposure using a 635-nm diode laser. Fluence, $J \left[\frac{J}{cm^2} \right]$, was calculated by using the equation 5.1 where T is treatment time in seconds, $[s]$, and $F \left[\frac{W}{cm^2} \right]$ is the fluence rate.

$$J = T * F \quad (5.1)$$

5.4.1 Patient: Horse A

Horse A was a 7-year-old Arabian gelding with lesions 1 through 4. Initially, multiple sarcoids (PDT-treated lesions as well as some lesions not treated with PDT) were debulked/excised with a CO_2 laser and a scalpel. Three days after surgery, PDT was performed under sedation using injections of ALA and the LED array as the light source. Biopsy results of the lesions were consistent with incomplete excision of lesions 1-3 and complete excision of lesion 4.

Lesion 1 was a 1.5 cm diameter fibroblastic sarcoid at the base of his left ear. A line block was performed on this ear. Two milliliters of ALA in sterile water were

injected into the ear. The line block was released and then treated with PDT. This lesion was treated again about a month later with PDT.

Lesion 2 was a 1.5×0.7 cm verrucous sarcoid on the left pinna margin. Two milliliters of ALA in sterile water were injected into the ear and light applied for a PDT treatment. A second PDT treatment occurred about a month later.

Lesion 3 was a 1×1.5 cm mixed verrucous/fibroblastic sarcoid on his left medial thigh. Four milliliters of ALA in sterile water were injected into the thigh and then was treated with PDT. A second PDT treatment occurred about two months later.

Lesion 4 was a 2×2.5 cm verrucous sarcoid on his left lateral forelimb just proximal to the carpus. Two milliliters of ALA in sterile water were injected into the forelimb. This horse had many other sarcoids (more than 10) on his limbs, ventral abdomen, axilla, and genitalia. He had been treated with multiple treatments including surgery, immunotherapy, and topical treatments with 100% recurrence and the continuing development of new lesions.

PDT was repeated using the same protocol at week 2 on all 4 lesions and at 6 weeks for the first 3 lesions. Lesion 4 appeared fibroblastic and ulcerated following treatment 1. A biopsy was collected of all lesions at the time of treatment 2. Histologic examination of lesions 1-3 was consistent with sarcoid tissue. The histological examination of lesion 4 revealed focal proliferation of vascular channels and in situ squamous cell carcinoma. Exuberant endothelial proliferation has been observed in some lesions of caprine viral papillomatosis[16]. Lesion 4 was removed from the trial and the other lesions were treated at weeks 6 and 9 with PDT using the same protocol. Biopsies taken of lesions 1-3 three days after treatments 3 and 4 were all consistent with sarcoid tissue. Although all lesions appeared to have an initial treatment effect, at approximately 10 days post-treatment the recurrence of tumor was evident and confirmed by histopathologic examination. Due to lack of sustained response, the owner elected not to pursue further treatment in the clinical trial. This horse has since received various different treatments, with no response. There were no optical measurements taken of horse A.

5.4.2 Patient: Horse B

Horse B was a 9-year-old Thoroughbred gelding with lesions 5 and 6. Both lesions were clusters of multiple fibroblastic sarcoids ranging in size from 0.5 cm in diameter to 3.5×6 cm on the concave surface of each pinna, shown in figure 5.1. There were areas of ulceration and bleeding in both ears. The owner reported 13 prior treatments by 7 different veterinarians with continuing progression of the lesions. No other sarcoids were present.



Figure 5.1: Horse B: This shows the sarcoids before excision and treatment using PDT.

The masses were debulked under general anesthesia using a combination of 20-watt CO_2 laser, electrosurgery, and scalpel dissection, shown in figures 5.2 and 5.4 for the left and right pinna respectively. PDT was performed immediately following the debulking, with the ALA administered by injection by injection into the ear vein after tourniquet application. The tourniquet was left in place for 20 minutes, then removed and exposure to the LED light source was started 20 minutes after removal of tourniquet. A second PDT treatment using the same protocol was performed 1 week later. A biopsy was performed at the time of the second PDT treatment. There

was histopathologic evidence of necrosis and capillary thrombosis. In the 2 months following treatment, the lesions became much smaller, flatter, and quiescent. The owners elected not to continue treatment because of the positive outcome.



Figure 5.2: Horse B: This shows the left pinna sarcoids during excision.

Follow-up evaluations occurred approximately every 3 months by the author. Occult and nodular sarcoids remain in both ears but have not changed in the 20 months since their maximum regression, shown in figures 5.3 and 5.5. They are epithelialized and are not causing the horse or owners any problems.

The light source 2.3 was designed to treat these sarcoids specifically since it was a challenge to get the light into an equine ear without constantly holding the ear open. Optical measurements were taken after a PDT treatment and for five days consecutively after that.



Figure 5.3: Horse B: This shows the left pinna 20 months after the PDT treatments.



Figure 5.4: Horse B: This shows the right pinna sarcoids during excision.

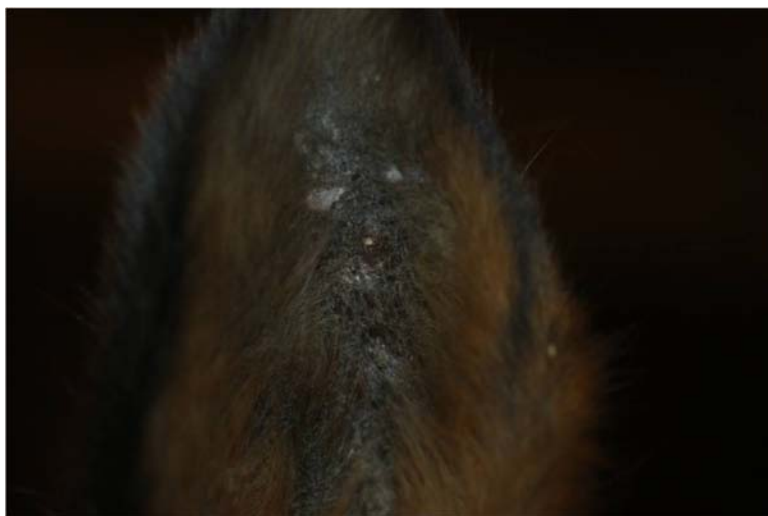


Figure 5.5: Horse B: This shows the right pinna 20 months after the PDT treatments.

5.4.3 Patient: Patient C

Patient C was a 5-year-old donkey with lesion 7, a $6 \times 3 \times 3$ cm ulcerated fibroblastic sarcoid along the entire dorsal lid margin and medial canthus of the left eye. The tumor prevented the donkey from opening her eye. The sarcoid had first been noticed by the owner approximately 1 year earlier. No previous treatments had been performed and no other sarcoids were present. Initial treatment consisted of debulking with a 20-watt CO_2 laser and limited electrocautery for hemostasis. The surgery and all subsequent treatments were done under general anesthesia. Three days postoperatively, PDT was performed with topical application and intralesional injection of ALA. PDT was performed using the same protocol at 2 and 6 weeks postoperatively. Biopsies of the affected area were taken 3 days following PDT 1 and 2, and at the time of PDT 3. The tissue biopsied after PDT 1 had severe tissue damage evidenced by necrosis and vascular thrombosis. Biopsy results following PDT 2 were consistent with granulation tissue. No sarcoid tissue was evident. The final biopsy report described sarcoid tissue present at the margins of the biopsy. The owners declined further treatment due to financial reasons. Follow-up was performed by phone with recurrence of the tumor developing at one year post-treatment and a new sarcoid

tumor developing on the right eyelid two months later. The owner had started topical treatment with bloodroot extract with no positive results.

Below are images of patient C before, during and after her treatments. Figure 5.6 shows her before any PDT treatments. Figure 5.7 shows the surgical excision of the bulk of the tumor. Figure 5.8 is an image of patient C during a PDT treatment with the treatment light active. Figure 5.9 is an image of patient C after three PDT treatments. There were no OPTical measurements taken on this animal.



Figure 5.6: Patient C: This is an image of patient C's 6×3×3 cm ulcerated fibroblastic sarcoid tumor over the left eye. This image was taken previous to any PDT treatments. The tumor was blocking the use of that eye.



Figure 5.7: Patient C: Immediately after the excision of the bulk of patient C's sarcoid tumor.

5.4.4 Patient: Horse D

Horse D was a 5-year-old female Miniature Horse with lesion 8, a $1.5 \times 1.5 \times 1$ cm fixed nodular lesion at the medial canthus of the right eye. The nodule was completely epithelialized. Figure 5.10 shows horse D before any treatments.

Initial treatment consisted of debulking the tumor with sedation and local anesthesia. Fibrous fingers of tissue extending deep to the excision site were visible after debulking. Histopathology confirmed the presence of neoplastic cells at the deep excisional border. Histopathologic examination of the excised tissue was consistent with sarcoid tissue. The lesion was reported to have a history of several months duration. PDT was performed immediately following debulking with topical application of ALA over a 3 hour period followed by irradiation with a 635-nm diode laser. PDT was repeated using the same protocol 3 weeks later. Figure 5.11 is a picture taken during a PDT treatment.

Figure 5.12 shows the wound one month after the last PDT treatment. The wound healed normally with no evidence of tumor formation at 6 months. At approximately



Figure 5.8: Patient C: This is the image of patient C during a PDT treatment with the treatment light.



Figure 5.9: Patient C: This is patient C after three PDT treatments. She has regained full use of her left eye.



Figure 5.10: Horse D: This is an image of patient D before a PDT treatment.



Figure 5.11: Horse D: This is an image taken during a PDT treatment.

10 months post-treatment a nodule "smaller than a pea" was noted by the owner dorsal to the site of the original lesion. There were optical measurements that were taken on this treatment.



Figure 5.12: Horse D: This is a picture of horse D one month after the last PDT treatment.

5.4.5 Patient: Horse E

Horse E was a 12-year-old warmblood mare with a history of sarcoid on the margin of her left pinna (lesion 9). Figure 5.13 shows the sarcoid before a PDT treatment. The shape of the ear has been altered due to previous aggressive surgical treatments.

No other sarcoids were present. Aggressive surgical excision of the tumor had been attempted twice. Confirmed recurrence of sarcoid resulted after the first surgery and recurrence was suspected based on the clinical signs and progression following the second surgery. After the second surgery, the pinnal margin was appearing to heal uneventfully by primary intention healing. Three to four weeks following surgery, the owner reported early ulceration and dehiscence consistent with the previous recurrence. At approximately 2 months postoperatively, the mare was presented to the author and the pinnal margin was proliferative and ulcerated with thickening of the pinna extending 1 cm proximally. The lesion was measured at $4 \times 1 \times 1$ cm. The mare was extremely resistant to any manipulation of the ear.

Initial treatment consisted of excision of visible abnormal tissue with no attempt to remove a margin of normal tissue using a CO_2 laser set at 35 watts. Excision was



Figure 5.13: Horse E: This is a picture of horse E before a PDT treatment.



Figure 5.14: Horse E: This image is of horse E in a surgery suite before at PDT treatment.

immediately followed by PDT with ALA injected into the excision site. Figure 5.14 is of Horse E in a surgery suit before a PDT treatment. Figure 5.15 shows the excision of the sarcoid using the CO_2 laser.



Figure 5.15: Horse E: This is a picture of horse E during CO_2 laser excision of the bulk of the sarcoid.

Figures 5.16 and 5.17 show the injection of ALA into the base of the excised sarcoid and the light treatment during a PDT treatment.

Two more PDT treatments were done under general anesthesia at 3 and 6 weeks postoperatively and a final PDT treatment was done under heavy sedation at approximately 10 weeks postoperatively. At each treatment, examination of the ear revealed decreased thickening and less ulceration. Figure 5.18 is of 3 weeks after the first PDT treatment. No thickening of the pinna was palpated by the author 4 weeks after treatment 4 and the mare had no objection to manipulation of the ear. Ten weeks after last treatment is shown in figure 5.19. 4 months postoperatively, no further recurrence had been noted by the owner or referring veterinarian. There were optical measurements taken of this animal.



Figure 5.16: Horse E: The tissue remaining from the excised sarcoid is injected with ALA in preparation for a PDT treatment.

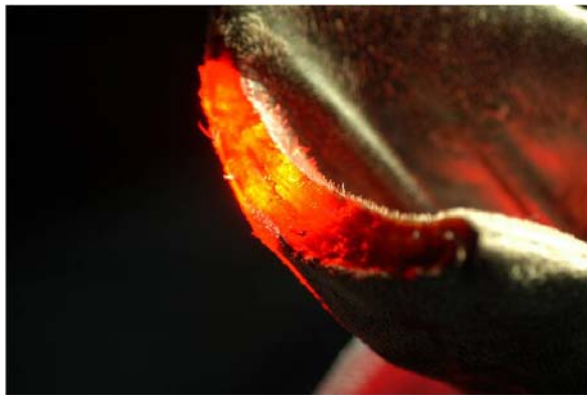


Figure 5.17: Horse E: This is the treatment light for the base of the sarcoid excision.



Figure 5.18: Horse E: Three weeks after the first PDT treatment.



Figure 5.19: Horse E: This is a picture of the treatment location 10 weeks after the last PDT treatment. There was no sign that the sarcoid was returning.

5.4.6 Patient: Horse F

Horse F was a 10-year-old Morgan mare with lesions 10 and 11. Lesion 10 was a fibroblastic, ulcerated mass approximately 2 cm in diameter and 2 cm deep at the left of midline on the cranial chest, shown in figure 5.20. Lesion 11 was an occult sarcoid approximately 2x3cm on the left ventrolateral abdomen just cranial to the flank. Both lesions had a history of several years duration and no prior treatment.

Initial treatment of lesion 10 was excision with a CO_2 laser using sedation and local anesthesia. Excision was immediately followed with PDT. The ALA was applied to lesion 10 topically and injected into the margins of the excision, shown in figure 5.21. Application time was 2 hours prior to irradiation with the 635-nm diode laser.

Lesion 11 had ALA applied topically 4 hours prior to irradiation with the diode laser. The treatments were repeated 4 weeks later. At the time of the second treatment, lesion 10 was healing well with an open granulating area of approximately 1 cm diameter surrounded by a flaking hairless area approximately 1.5- 2 cm in diameter, shown in figure 5.22. Lesion 11 had shrunk to approximately 2x2cm. At 4 months



Figure 5.20: Horse F: This is lesion 10 before treatment.



Figure 5.21: Horse F: Lesion 10 during ALA administration.

post-treatment, lesion 10 is not visible or palpable according to the owner. Lesion 11 is quiescent and approximately 2×1 cm.



Figure 5.22: Horse F: Lesion 10 after treatments and healing.

5.4.7 Patient: Horse G

Horse G was an 8-year-old Quarter Horse palomino mare with lesion 12, a verrucous sarcoid on the right side of the face approximately 4 cm dorsal to the most rostral aspect of the facial crest. The lesion was somewhat rectangular in appearance, approximately 3 cm ventral to dorsal and 8 mm wide, shown in figure 5.23. The most dorsal aspect was ulcerated, while the ventral 2/3 was hyperpigmented. Multiple other verrucous and occult sarcoid lesions were present but this one was irritated by the noseband of the halter and bridle. Initial treatment consisted of repeated topical applications of 20% ALA for 5 hours prior to the application of light. The hair around the lesion was clipped to facilitate drug application and diffuse reflectance spectroscopy measurements. Over the first 3 days, redness and edema were visible over the entire clipped area. The epidermal surface became moist, with the center most significantly affected. A full-thickness epidermal and partial dermal burn was present over the entire area. At 5 weeks post-treatment, the tissue was still erythematous and peeling over the entire clipped area. The area of the tumor that had previously been ulcerated was still visible and had a scab present but it was smaller and less raised. The owner reported 4 months after treatment that the area appeared normal. The horse was then sold and lost to subsequent follow-up. There were no optical measurements taken on this animal.

5.4.8 Patient: Horse H

Horse H was a 12-year-old warmblood with a verrucous and nodular sarcoid (lesion 13) on his back. The lesion was raised and thickened with a nodule palpable deep to the epidermis. There was some hair loss over the area, but no ulceration was present. The horse was uncomfortable from saddle pressure on the lesion. The duration of the sarcoid was less than 1 year and no other sarcoids were present.

Initial treatment consisted of PDT with repeated topical applications of ALA for the three hours prior to 20 minutes of light application, shown in figure 5.24 after the ALA was applied. Identical PDT protocols were repeated at weeks 3 and 7.



Figure 5.23: Horse G: This is lesion 12 during a PDT treatment. The larger of the two lesions were treated.



Figure 5.24: Horse H: This picture was taken after topical ALA had been applied for the first PDT treatment.

Following the third treatment, the owner reported several days of soreness and mild swelling. Four weeks after the third treatment, a follow-up examination revealed no thickening and no palpable nodule, with darkened hairless epidermis at the site where the lesion had been. Four months following treatment, there is still no evidence of recurrence. Figure 5.25 was taken 3 months after the initial PDT treatment. Diffuse optical reflectance measurements were attempted but there was too much melanin to get a reflectance back.

5.4.9 Patient: Horse I

Horse I was a 12-year-old Quarter Horse mare with multiple sarcoids. The sarcoids had been present for years and appeared to the owner to be static. Three were selected for treatment. Lesion 14 was a 3×2.5cm occult sarcoid on the left side of the face immediately rostral to the facial crest. Lesion 15 was a 9x8x5cm ulcerated, fibroblastic sarcoid with 2 prominent nodules on the left ventral flank. Lesion 16 was a 2×1.5x0.5cm verrucous sarcoid on midline just cranial to the manubrium. Lesion



Figure 5.25: Horse H: This picture was taken 3 months after the initial PDT treatment.

15 was the first lesion treated. ALA was applied both topically and by injection to the most central nodule for the initial PDT with 1 hour of light exposure. A biopsy sample was collected from the lesion to confirm the sarcoid diagnosis at the time of initial treatment. A second biopsy sample was collected 5 days post-treatment. Histopathologic evidence of necrosis was evident across the entire sample depth (0.6 cm). 10 weeks after the first treatment, a second PDT was performed on the same area of lesion 15 with ALA introduced by injection and 20 minutes of light exposure. The area that had been previously treated appeared to be reduced in volume although the overall surface area of lesion 15 appeared unchanged. On the same day, lesions 14 and 16 were also treated with PDT. Lesion 14 had repeated topical applications of ALA for the 4 hours prior to 20 minutes of light exposure. Lesion 16 had ALA introduced by injection and 20 minutes of light exposure. Biopsies were taken of lesion 15 three days after PDT 2. There was histologic evidence of cellular necrosis from the epidermis to the deep dermis. At week 13, all lesions were treated with the same protocol as week 10. All lesions appeared to have a treatment effect compared to week 10. A final biopsy was taken from lesion 15. A more superficial kill zone was found compared to the previous biopsies. At 5 months post-PDT, lesion 15 appeared

the same as it did after treatment, lesion 14 had continued to regress but was still present, and lesion 16 had returned to pre-treatment status. There were no optical measurements taken on this animal.

5.4.10 Patient: Horse J

Horse J was a 17-year-old Arabian mare with numerous (more than 30) sarcoid tumors. The sarcoids had been present for more than 10 years. Most of her tumors were verrucous and covered large areas of her body. The owner described areas of verrucous tumor periodically becoming fibroblastic and developing a reddened, ulcerated, often pedunculated mass that would be hemorrhagic and appear infected. Lesion 17 was such a mixed verrucous and fibroblastic tumor on the lateral aspect of the right stifle, shown in figure 5.26. The verrucous area was approximately $7 \times 7 \times 2$ cm in size.



Figure 5.26: Horse J: Lesion 17 before treatment.

A proliferative, ulcerated, fibroblastic mass approximately 2×3 cm in size was in the center portion of the verrucous sarcoid. Lesion 17 was treated twice (treatment a) using the LED array as the light source, and then again 16 months later with injection of ALA followed by exposure to a 635-nm diode laser (treatment b), the injections are shown in figure 5.27. Histologic examination of a biopsy taken following the second LED treatment was consistent with superficial necrosis and vascular thrombosis of the lesion. Some superficial changes were noted clinically after all treatments. According

to the owner, 10 weeks after the final treatment, lesion 17 remained unchanged. Optical measurements were taken on this lesion.



Figure 5.27: Horse J: The injection of ALA into lesion 17.

Lesion 18 was also a verrucous and fibroblastic, proliferative, pedunculated tumor on the right ventral fold of the flank. The pedunculated portion was approximately $5 \times 4 \times 3$ cm in size, shown in figure 5.28.

Lesion 18 was initially treated with injection of ALA followed by exposure to a 635-nm diode laser. Figure 5.29 shows what lesion 18 looked like after the PDT treatment. Results of the biopsy taken 3 days later were consistent with necrosis with intravascular thrombi present throughout. The bulk of the tumor was excised to view the extent of the treatment in the tissue. A cross section of this tumor is shown in figure 5.30. At 7 months post-treatment, lesion 18 was still present, but the treated proliferative mass had regressed after PDT and had not recurred. Optical measurements were not taken on this lesion.



Figure 5.28: Horse J: Lesion 18 before treatment.

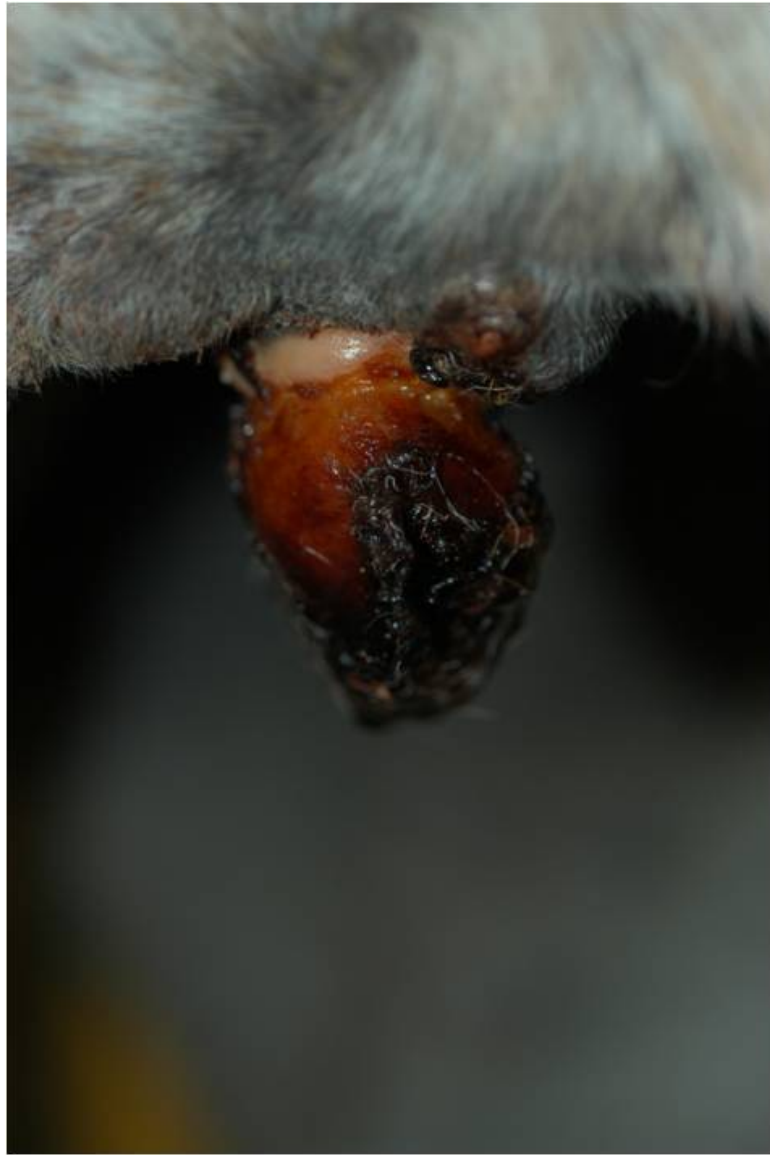


Figure 5.29: Horse J: Lesion 18 after a PDT treatment.

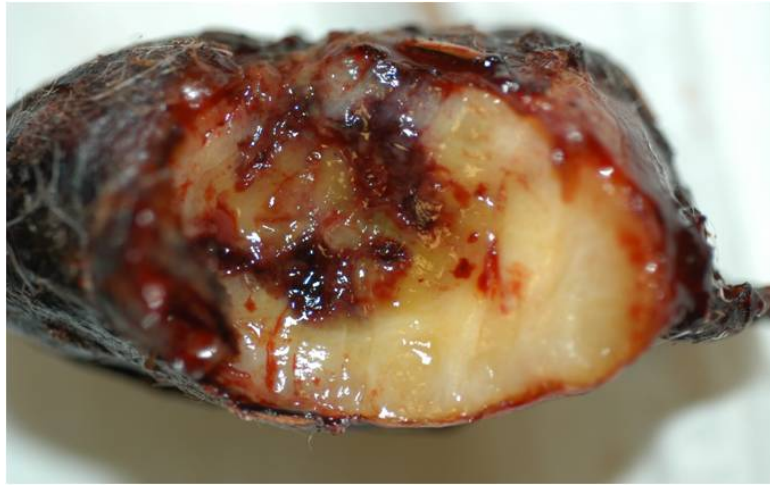


Figure 5.30: Horse J: A cross section of the tumor after a PDT treatment.

5.4.11 Patient: Horse K

Horse K was a mare with lesion 19. Lesion 19 was an occult sarcoid on the right side of her nose, shown in figure 5.31. The lesion was initially 0.8×3 cm. This was the only lesion on this animal and had not been treated previously by any other method. This animal came in twice, (1) once to treat the lesion and (2) for a second time to measure the kinetics of the PpIX build up in the tissue.

The initial visit was for a PDT treatment using a 20% ALA in Lipoderm topical. The hair was clipped around the treatment area in preparation for the treatment, shown in figure 5.32. The 635 nm laser diode was used for the treatment light. After this initial treatment, an adverse effect similar to a sunburn occurred from the treatment, shown in figure 5.33. The second visit was for a kinetic study of the build up of the PpIX in the tissue without the treatment light. The kinetic diffuse optical reflectance measurements were taken and compared between both sarcoid tissue and normal tissue. Optical measurements were taken on this animal.



Figure 5.31: Horse K: Lesion 19 before treatment.



Figure 5.32: Horse K: Lesion 19 after the hair was clipped around the treatment area and the thickened epidermis flaked off.



Figure 5.33: Horse K: Lesion 19 three days after the PDT treatment. This picture shows the sunburn type side effect of the PDT treatment.

5.4.12 Patient: Horse L

Horse L was a mare with lesions 20 through 22. Lesion 20 was a fibroblastic sarcoid on the left side of the nose and 3 cm by 2.5 cm and was treated topically twice. Lesion 21 was a fibroblastic sarcoid on the chest with dimensions 2×1.5 cm and 0.5 cm deep. Lesion 21 was treated by injections twice. The lesion 22 is a fibroblastic lesion on the top of the left flank with a large dimension of 9 by 8 cm and 3 cm deep, shown in figure 5.34.



Figure 5.34: Horse L: Lesion 22 before treatment.

There were two comparable large nodules in this lesion. The left nodule was chosen to be treated. During treatment the size of the two nodules could be compared. The left part of lesion 22 was treated three times by injection. Five days after the last PDT treatment figure 5.35 shows the results. Notice the the left nodule has decreased in size. The left nodule was then excised to view the treatment depth and is shown in figure 5.36.



Figure 5.35: Horse L: Lesion 22 five days after a PDT treatment.

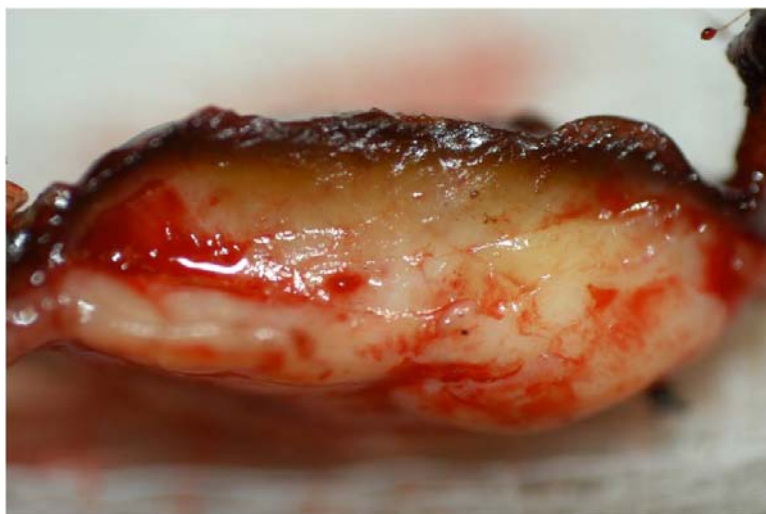


Figure 5.36: Horse L: The excision of lesion 22 after the PDT treatments.

5.4.13 Patient: Horse M

Horse M was a mare with a fibroblastic sarcoid, lesion 23, located on the right side of the abdomen with dimensions of 4.5 by 4.5 cm. This sarcoid was not treated with PDT but instead injected with ALA and had five biopsies taken. These biopsies were then sectioned and imaged under a confocal microscope. There were no large differences noticed by the confocal microscope. There were no optical measurements taken.

5.4.14 Patient: Horse N

Horse N was a mare with lesion 24. This lesion was a flat occult sarcoid located in the middle, right of the back. This animal underwent one topical PDT treatment with the 635 nm diode laser. There was little change due to the treatment. There were no optical measurements taken.

5.5 Optical Properties of the Equine Tissue

Animal	Sarcoid Tissue	Sarcoid Tissue
	Mean Absorption Coefficient μ_a	Mean Scattering Coefficient μ_s'
Horse K	2.5	9.9

Table 5.2: Optical properties of equine sarcoid tissue. The measurement of horse K was taken with the tumor unaltered. Horse D had the surface of the tumor removed and the measurements for these measurements were taken on the bulk of the tumor.

5.6 Conclusion

During a PDT treatment the threshold dose can be found to help aid in the prediction of the light and drug dose necessary for future effective PDT treatments. Many methods have been used in the past to treat equine sarcoids though none of them have worked effectively to fully treat the sarcoids. Using PDT to treat the

sarcoids was an attempt to find a method to effectively cure the animals of the tumors. These studies show that PDT may be a useful treatment to maintain sarcoids but has not been shown to effectively cure an animal of these tumors. One reason sarcoids are hard to treat is due to the metabolism of the tumors. Lactic acid builds up in tumors and this may be due to using up all of the oxygen in the local area. This would decrease the effectiveness of PDT since oxygen is necessary for a PDT treatment to be effective.

Chapter 6

Canine PDT Treatment of Acanthomatous Epulis

6.1 Summary

The previous chapter covered equine photodynamic therapy treatments. All of these treatments the drug, aminolevulinic acid (ALA), was given locally. The ALA was given locally due to the cost of the drug needed to treat a large animal with an oral dose. This chapter covers an animal that was treated with oral administration of ALA. A canine was chosen to be treated orally due to the smaller size of the animal. With this treatment the build up of ALA in the tumor will be detected using the fluorescence of the tumor and the absorption of the drug. This experiment was performed to show that the ALA will effectively build up in this type of canine tumor using an oral dose of ALA.

6.2 Introduction

Photodynamic therapy, PDT, is a treatment that uses light and a photosensitizer to damage unwanted tissue. Both the drug and the light need to be in the tissue together to create tissue damaging singlet oxygen from the oxygen found in the tissue [19]. Because of the dependence of the treatment on both light and drug, PDT can be a selective treatment. During PDT treatments, many of the photosensitizers

build up their concentration in tumor tissue more quickly than in normal tissue. Unfortunately, some of these drugs can leave the patient photosensitive for weeks after the treatment, such as the currently FDA approved drug Photofrin.

Protoporphyrin IX, PpIX, is a photosensitizer that is made from aminolevulinic acid, ALA, in the heme pathway. Normally, iron is combined with PpIX to create heme in cells. Administering ALA produces greater than normal concentrations of PpIX in the body. The PpIX combines with and then depletes the available iron. The PpIX then builds up in the tissue and increases the photosensitivity of the patient.

During a PDT treatment, damage will be done to the tissue depending on the PDT threshold dose. This PDT threshold is dependent on the drug and light concentrations delivered to the tissue. The PDT threshold dose is about $10^{17} - 10^{19}$ [ph/g] for PpIX [3].

Diffuse optical reflectance spectroscopy measurements have been used in simplified models of tissue to determine the components and their amounts in tissue [71]. Fluorescence is often used to locate a PDT drug in the tissue and to measure the relative changes in concentration. Diffuse optical reflectance spectroscopy measurements are used as a non-invasive method for determining the concentration PpIX in the tissue by its optical absorption [29].

It has been shown by Dalton *et al.* that the concentration of ALA in the canine plasma after oral administration initially increases the ALA concentration and then decreases below the normal level within minutes [72]. As the ALA is converted to PpIX in the cells, the body stops producing ALA and the remaining ALA is converted to PpIX. As the ALA decreases in concentration, the PpIX concentration should increase. This work monitors the PpIX absorption changes with time at the surface of the tumor. The PpIX concentrations in tumor is compared to normal tissue.

6.2.1 Materials and Methods

Animal

The canine used in this study was privately owned. The patient had oral cancer diagnosed as acanthomatous epulis. The tumor was located on the lower mandible under the nose. Within the past year the tumor had been surgically excised but grew back to a size of $7 \times 3 \times 2.5 \text{ cm}^3$.

PDT Treatment

Aminolevulinic acid hydrochloride was purchased from Sigma. Prior to the PDT treatment, the patient was given acepromazine and butorphanol to sedate the animal. Aminolevulinic acid, ALA, was administered orally to the patient. The dose of ALA administered, $60 \left[\frac{\text{mg}}{\text{kg}} \right]$, was mixed with 2 grams of wet cat food. This dose was chosen to maximize the PpIX in the tumor without the negative side effects of higher doses work [70]. The patient readily took the ALA orally. Before and after the ALA was administered, diffuse optical reflectance measurements were taken 4 hours and 40 minutes elapsed after ALA administration before part of the tumor was treated with 100 Joules of 635 nm light from a diode laser at a fluence rate of $55.7 \left[\frac{\text{mW}}{\text{cm}^2} \right]$. At three and twelve days after the treatment, the patient was brought back to the clinic for evaluation. Three biopsies were taken at twelve days after the treatment, since a treatment effect was noticed. Three 4 mm punch biopsies were taken: 1) in the treated area, 2) on the edge of the treated area, 3) outside the treated area.

Diffuse Optical Reflectance Spectroscopy Measurements

Diffuse optical reflectance measurements were taken in the treatment location approximately every 20 minutes after the ALA was administered orally. These measurements were taken before and after the PDT treatment. More measurements were taken in normal tissue both before and after administration of ALA.

The reflectance measurements were taken over the visible spectrum from 500-850 nm. The data was then normalized by a standard giving the transmission of the

tissue sampled. A linear combination of absorption spectra found in the tissue and a scattering spectrum were fit to the data. Among these spectra were the absorption of oxy- and deoxygenated hemoglobin, water, the drug in the tissue, PpIX and others.

Optical Fiber Spectrometer

The study used an optical fiber spectrometer (Ocean Optics, S2000) with a white light source (Ocean Optics LS-1 tungsten halogen light source) and a fiber bundle probe (Ocean Optics probe) consisting of six optical fibers for delivery of white light around one central optical fiber for collection of reflected light. The fibers were 200 μm in core diameter and were separated by 200 μm . The probe was placed in contact with the tissue for measurements. The probe was placed against an epoxy standard as a stable reference. The final spectral measurement (M [dimensionless]) was defined as the tissue measurement (M_s [counts]) divided by the reference measurement (M_{ref} [counts]):

$$M = \frac{M_s}{M_{ref}} \quad (6.1)$$

Such normalization controlled for any variation in the light source and system response from day to day. Spectra were acquired over the visible to near-infrared range, but the signal was acceptable only in the 500-850 nm range which was used for analysis.

Concentration PpIX

The optical density at 635 nm was found for PpIX in 25% albumin using a Beckman Coulter spectrophotometer, DU Series 500. Knowing the concentration of the PpIX the extinction coefficient can be found.

$$\left(\frac{OD}{L \cdot C} \right) \quad (6.2)$$

Where OD is the optical density, L is the pathlength of the light and C is the concentration of the PpIX. The extinction coefficient at 635 nm is then $2000 \pm 100 [\frac{L}{cm \cdot mole}]$. Solving this equation for the concentration of the PpIX in the tissue shows how the concentration can be found once the absorption coefficient is measured:

$$C = \left(\frac{-\log(e^{(-\mu_a^{PpIX} \cdot L)})}{L \cdot Ext} \right) \quad (6.3)$$

Where L is the separation between the fiber centers and μ_a is the absorption coefficient due to the PpIX in the tissue at 635 nm.

The PDT Dose

The PDT dose is defined as the number of photons absorbed by photosensitizer per gram of tissue $[\frac{ph}{g}]$, which is calculated:

$$PDTdose(z) = \mu_a^{PpIX}(z) \cdot H(z) \frac{\lambda}{hc\rho} \quad (6.4)$$

Where:

$$H(z) = H_0 T(z) \quad (6.5)$$

$$\mu_a^{PpIX}(z) \quad (6.6)$$

Where equations 6.5 and 6.6 are the spatial distributions of radiant exposure and PpIX absorption coefficient, respectively. The density, ρ , is assumed to be that of water which is $1 [\frac{g}{cm^3}]$, c is the speed of light and h is Planks constant. To kill tissue the PDT threshold dose is about $10^{17} - 10^{19} [\frac{ph}{g}]$ for PpIX seen in our own unpublished work on cell cultures and other publications [3].

Fluorescence

Fluorescence is often used to determine if the PpIX is present in the tissue. The absolute concentration of the drug is hard to determine from the fluorescence but the presence of the PpIX can be confirmed. During this experiment five 405 nm LEDs were used to illuminate the tumor temporarily in a dark room. The camera used to take the digital images had a filter to block the excitation light. Images were taken of the fluorescence to show it was present.

6.2.2 Results

Optical measurements on the surface

The scattering coefficients of these two tissue types are very different. In figure 6.1, the normal tissue is shown in open circles while the filled diamonds represent the tumor tissue. The scattering coefficient of the two tissue types is significantly different by anova. Currently there is not enough information, $n=1$, to determine how typical this scattering difference is between normal and cancerous acanthomus epulis tissue. If this change in scattering is characteristic of these tissue types then the scattering coefficient may be a useful tool to use diagnostically to discriminate tissue type. If the scattering coefficient turned out to be useful in discriminating tissue type, then an ROC curve can be created to find the best threshold point for discriminating the tissue types. Figure 6.1 displays the distribution of the scattering coefficient as a function of the absorption coefficient. Figure 6.2 is an ROC curve based on the scattering distribution of the data. For this animal, the threshold would be placed at a reduced scattering coefficient of $11 \left[\frac{1}{cm} \right]$ where the specificity and sensitivity are maximized at 0.92 and 0.91 respectively.

The absorption coefficient at 635 nm in the cancerous tissue is much more spread out due to the build up of the PpIX in the cancerous tissue. After the ALA administration the PpIX built up selectively in the cancerous tissue more than the normal tissue. Figure 6.3 examines the heterogeneous build up of the PpIX in the cancerous tissue over time. Notice there is a small amount of PpIX in the tumor tissue before the administration of ALA. This PpIX natural build up in tumors has been documented by other groups.

Optical measurement of PpIX build up on the surface

Figure 6.3 shows the heterogeneous absorption from PpIX on the surface of the tumor. Ten data points have been taken at each time point at random places on the left half of the tumor. The graph may seem to represent less than 10 points for each time point but especially for the initial values the concentration of PpIX was zero.

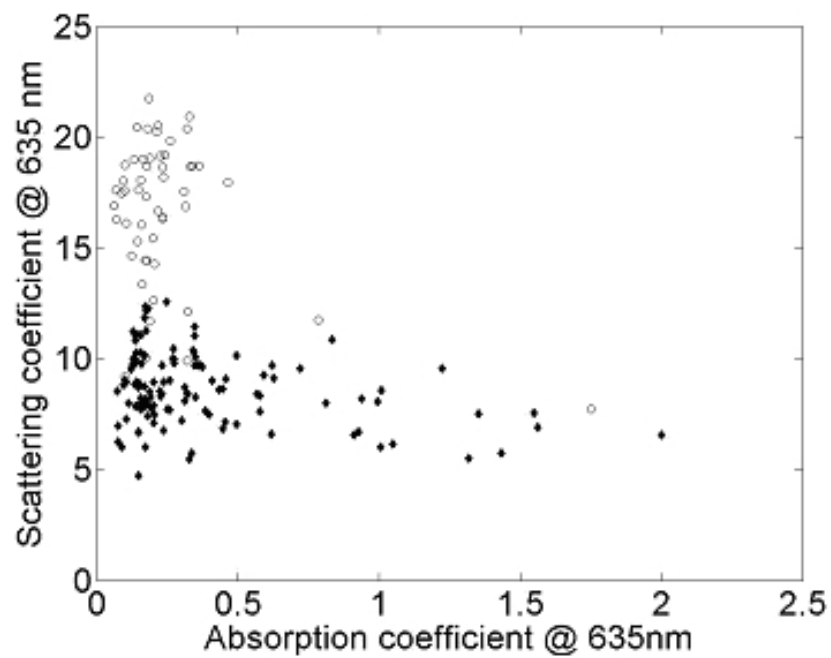


Figure 6.1: Fitting results of normal and cancerous tissue before, and after ALA administration. Normal tissue is the open circles and black diamonds are the results for the tumor.

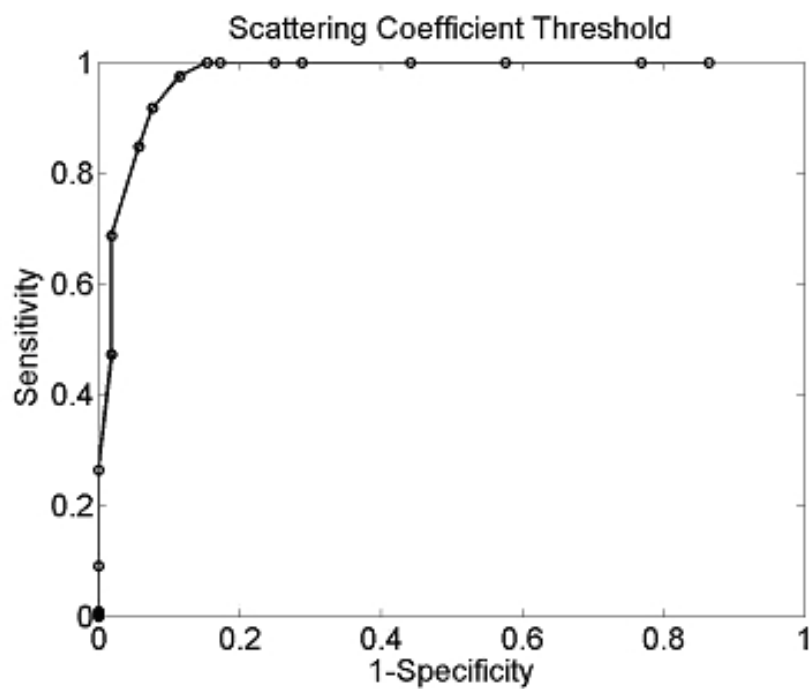


Figure 6.2: ROC curve for tissue types using the scattering coefficient as a discriminator.

Vascularity, hydration and iron concentrations could be reasons the PpIX does not build up uniformly over the tumor. Over five hours after ALA administration, the PpIX levels in the tissue continue to increase.

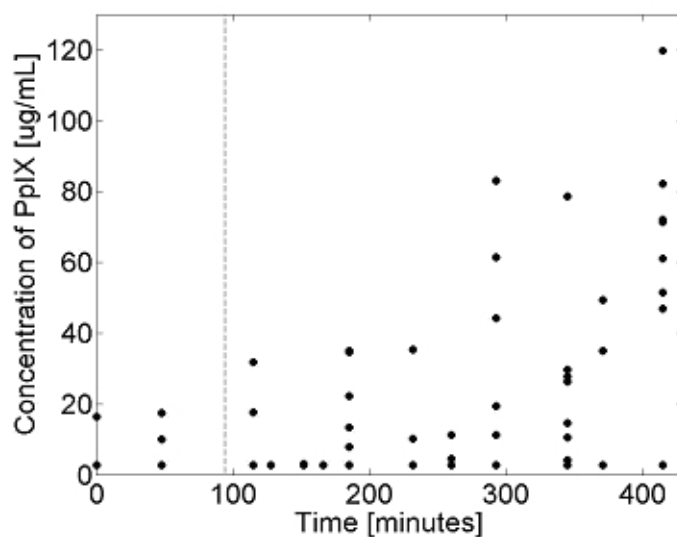


Figure 6.3: PpIX concentration at 635 nm in cancerous tissue after oral administration. The dotted line shows the time the ALA was administered orally.

Optical absorption changes due to increase of drug in cancerous and normal tissue

Table 6.1 compares the difference the PpIX built up in normal and cancerous tissue. Initially the cancerous tissue contains some PpIX while the normal tissue does not. After four hours the amount of PpIX has increased in both tissue types

but the increase in the cancerous tissue was about 1.6 times that of the increase of normal tissue. The total amount of PpIX in the tissue is 2.3 times that in the normal tissue four hours after the ALA was administered. This demonstrates that after four hours the PpIX build up is selective to this tumor tissue when compared to normal tissue.

	Cancerous Tissue [$\mu\text{g/mL}$]	Normal Tissue [$\mu\text{g/mL}$]
Before ALA Admin	6.1	0.0
4 hours After ALA Admin	19.7	8.4

Table 6.1: Mean concentration of PpIX in cancerous and normal tissue [$\mu\text{g/mL}$].

The fluence into the tissue as a function of depth is shown, figure 6.4, using the optical properties of the cancerous tissue before and four hours after ALA administration. The fluence of the tissue four hours after ALA administration is less than the tissue before ALA. The fluence shown in figure 6.4 is derived from a collimated source at the surface of the tissue simulated by Monte Carlo. Notice that the increase in absorption due to the buildup of PpIX results in a decrease of light at all depths.

PDT Threshold dose

Once the concentration of light and drug that are necessary to kill the tissue are known for the specific tissue type the photodynamic dose can be predicted. In figure 6.5, the dotted line in the graph represents the threshold dose. All of the tissue above the depth of the PDT dose was killed. The biopsies and the depression in the tissue in the treated area showed that the depth of treatment was about 2mm total. The PDT threshold dose is the photodynamic dose that the tissue received at 2mm, or determined to be 7.3×10^{18} photons per gram of tissue.

Fluorescence of the tumor after the treatment light

Fluorescence was used to confirm the presence of PpIX in the tissue. The fluorescence also was not uniform over the whole tumor. There were areas with more intense

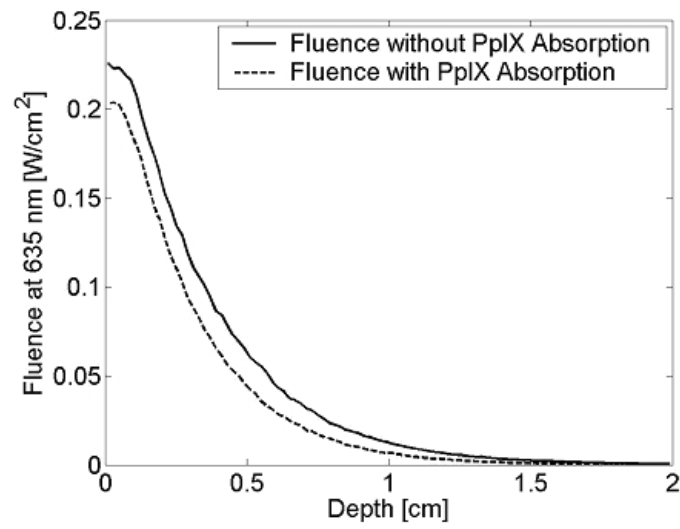


Figure 6.4: Simulation of the 635 nm light distribution in the tissue due to the optical properties for the tumor tissue four hours after ALA was administered and the same optical properties with the PpIX absorption removed.

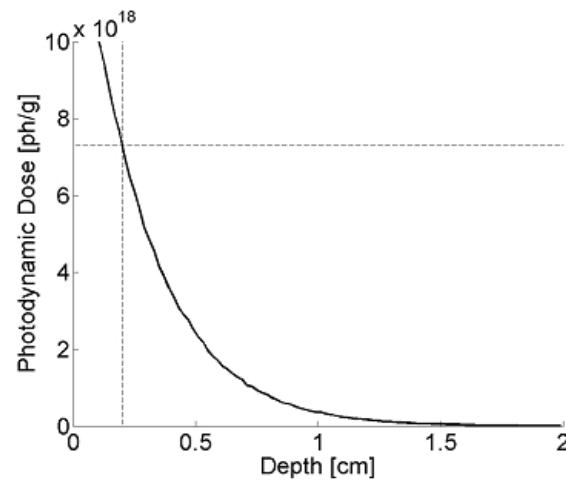


Figure 6.5: Expected depth the tissue will be killed due to the light and PpIX concentration, the PDT threshold. In this case it was found to be 7.3×10^{18} photons per gram of tissue.

fluorescence than others. Figure 6.7 is an image of the fluorescence of the tumor. It demonstrates the variation in intensity seen from the tumors fluorescence.



Figure 6.6: This is a picture of the acanthomus epulis tumor immediately after light treatment. The tumor is on the lower mandible under the nose with a size of $7\text{ cm} \times 3\text{ cm} \times 2.5\text{ cm}$.

6.2.3 Discussion

Previous papers have reported vomiting after the ALA was administered orally [70]. During the PDT treatment, the patient responded well and was able to hold down the ALA. There were no noticeable negative effects to the treatment.

This study has shown that the concentration of the PpIX in the tissue could be

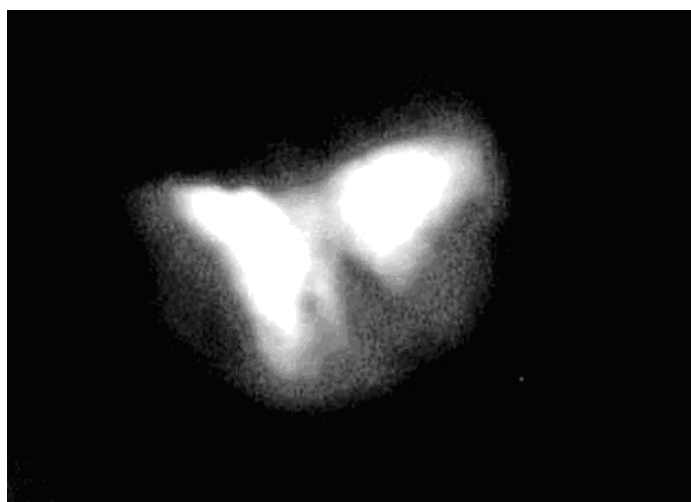


Figure 6.7: Fluorescence from the tumor. The image was converted to greyscale and both the contrast and brightness were enhanced.

measured both non-invasively and optically. The concentration of PpIX in the tumor tissue was 2.3 times more than in the normal tissue. This demonstrates the selectivity of the PpIX in the different types of tissue. The fluorescence from the tumor in figure 6.7 is representative of the selectivity of the tumor compared to the normal tissue.

The PDT threshold for damaging the tissue was found to be 7.3×10^{18} photons per gram of tissue. This is approximately the concentration that has been expected for tissue damage from previous work. During this clinical case the PpIX was allowed to build up in the tissue before treating with the light. The treatment may be more effective if the light was administered at the same time as the PpIX was forming in the mitochondria of the cell [24,25]. The free radicals produced by the light and drug interaction in the mitochondria of the cell may have more of an effect on the cell death and causing apoptosis.

Chapter 7

Conclusion

In this thesis, aminolevulinic acid, ALA, induced photodynamic therapy is explored as a possible treatment of both equine sarcoids and canine acanthomatous epulis. ALA is relatively inexpensive, can be applied locally (either topically or injected) or systemically and does not leave the patient photosensitive for extended periods of time.

Currently sarcoids are the most common equine tumor. Unfortunately there is no method of treatment that is consistently effective at treating these sarcoids. Also during some of these treatments the tissue can be scarred or a tumor can become aggravated and begin to grow rapidly afterwards making veterinarians hesitant to treat some of the smaller tumors.

Acanthomatous epulis is a cancer in the canine mandible that is very aggressive. Under normal circumstances the animal has to have a significant amount of the mandible removed if the cancer is not caught at an early stage. If it was possible to treat this tumor with another method other than surgical excision of the bone then this would be a great advantage to the afflicted animal.

This problem was approached by first developing a method to measure the changes in the tissue. A diffuse optical reflectance spectrometer was assembled and calibrated using tissue phantoms. Many properties of the tissue can be monitored using a fitting program written in Matlab. With this device the build up of the concentration of the photosensitive drug, protoporphyrin IX, can be measured using its absorption at 635 nm. Other properties of the tissue that can be measured are the amount of blood,

oxygenation of the blood and the scattering of the tissue. Next one canine afflicted with acanthomatous epulis and fourteen equine with sarcoids were treated.

The photodynamic threshold dose found for the canine tissue was 7.3×10^{18} photons per gram of tissue. For the variety of equine sarcoid treatments the photodynamic threshold dose ranged from $6 - 8 \times 10^{18}$ photons per gram of tissue. For most treatments the treatment depth was about 2 mm. The extinction coefficient at 635 nm was found to measure in the tissue to be about $21.6 \left[\frac{1}{(mm * mM)} \right]$.

For the canine acanthomatous epulis this photodynamic threshold dose was not aggressive enough for this tissue type. The treatment will not treat deep enough into the tissue. For equine sarcoids this treatment seems an effective method of maintenance. Combined with surgery, vary aesthetic results that may last up to two years can be achieved. Once the sarcoid starts returning another photodynamic therapy treatment will be needed to keep the tumor under control. This combination of surgery and photodynamic therapy was very effective at treating an equine with a tumor over the eye where other methods of treatment may have lost the eye itself, the photodynamic therapy treatment with surgical excision gave back the use of the eye.

In conclusion, ALA- induced photodynamic therapy is not the best method for curing such deeply penetrating tumors. Though ALA-induced photodynamic therapy is effect as a maintenance treatment for sarcoids and may be useful for other shallow ailments.

Appendix A

Programs

These programs were written to calibrate the diffuse optical reflectance spectrometer. The goal was if given an absorption and a reduced scattering coefficient a simulated reflectance could be produced.

.1 Organize.m

```
% organize.m
% PC version
% Reads all the directories, and prints out a list of DIRECTORY TREE
% Also, SAVES list of paths to each spectrum as list(:, :) to list.mat
clear
pA    ='Spectra\'; % target directory
A     = dir(pA);
NA    = length(A);

disp(' ')
disp('-----')
disp('List the SPECTRA directory')
disp('-----')
disp(' ')
disp(pA)

ii = 0; % pointer to spectral files
flag = 0;
```

```

% LEVEL A
% list spectral files A
for i=1:length(A)
    if (A(i).isdir == 0)
        sA = A(i).name;
        M = length(sA);
        if sA(M-5:M) == '.scope'
            ii = ii + 1;
            disp(sprintf('%d\t%s', ii, sA))
            if flag == 0
                list = [pA sA]; % first time need to start list
                flag = 1;
            else
                list = char(list, [pA sA]);
            end
        end
    end
end
end
end

% list directories A
for i=1:length(A)
    if (A(i).isdir == 1)
        sA = [A(i).name ':'];
        disp(sprintf('\t%s', sA))
    end
end

% LEVEL B
pB = [pA sA];
B = dir(pB);
% files B
for iB=1:length(B)
    if (B(iB).isdir == 0)
        sB = B(iB).name;
        M = length(sB);

```

```

if sB(M-5:M) == '.scope'
ii = ii + 1;
disp(sprintf('%d\t\t%s', ii, sB))
list = char(list, [pB sB]);
end
end
end
% directories B
for iB=1:length(B)
if (B(iB).isdir == 1)
sB = [B(iB).name ':'];
disp(sprintf('\t\t%s', sB))

% LEVEL C
pC = [pB sB];
C = dir(pC);
% files
for iC=1:length(C)
if (C(iC).isdir == 0)
sC = C(iC).name;
M = length(sC);
if sC(M-5:M) == '.scope'
ii = ii + 1;
disp(sprintf('%d\t\t\t%s', ii, sC))
list = char(list, [pC sC]);
end
end
end
% directories
for iC=1:length(C)
if (C(iC).isdir == 1)
sC = [C(iC).name ':'];
disp(sprintf('\t\t\t%s', sC))

```


.2 MasterCreate.m

```
% master.m
clear
global nm T filenames

%%%%%%%%%%
% Load EXPERIMENTAL SPECTRA
%%%%%%%%%%
global nm T filenames
%%%%%
% Calls function that loads spectra
% from desired directory.
% You must specify path to directory that holds spectra.
% % ---> nm(:,1), T(:,Nj), filenames(Nj,:)
%%%%%
load list
Nj = length(list);
blank = '';
for ii = 1:Nj
    % remove trailing spaces in filename
    [Ni Nj] = size(list);
    i = 1;
    while (i <= Nj) & ~(list(ii,i) == blank); % check (i < Nj) first !!
        i = i+1;
    end
    file = list(ii, 1:i-1);

    spectrum = getspectrum2(file);
    nm = spectrum(:,1);
    T(:,ii) = spectrum(:,2);
end
filenames = list;
%%%%%%%%%%
```

```
figure(1);clf
for i=1:Nj
plot(nm, T(:,i),'r-')
hold on
end

% save output nm T filenames
```

.3 MasterLook.m

```
% MasterLook.m
clear
close all

%%%%%%%%%%%%%%
% light source
%%%%%%%%%%%%%%
spectrum = getspectrum2('Spectra\epoxy150_fil1_grey.Master.scope');
source = spectrum(:,2);

%%%%%%%%%%%%%%
load output % --> nm T filenames from mastercreate.m
%%%%% Filters %%%%%%%%%%%%%%%

%%%%%%%%%%%%%%
% Filter A
for k= 1:2048
    fil1(k,1) = -log(T(k,26)/T(k,27))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,2) = -log(T(k,34)/T(k,35))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,3) = -log(T(k,43)/T(k,44))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,4) = -log(T(k,52)/T(k,53))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,5) = -log(T(k,61)/T(k,62))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,6) = -log(T(k,69)/T(k,70))/log(10); % --> filter 1 to fil 2 and 3
    fil1(k,7) = -log(T(k,78)/T(k,79))/log(10); % --> filter 1 to fil 2 and 3
end

figure(2)
plot(nm, fil1(:, :))
axis([300 1000 0 2])
title('Filter 1 to filters 2 and 3')
```

```

for k=1:2048
    filA(k,1) = mean(mean(fil1(k,:)));
    filAstd(k,1) = std(std(fil1(k,:)));
end

% Filter B
for k= 1:2048
    filB(k,1) = -log(T(k,81)/T(k,82))/log(10); % --> filter 2 and 3 to no fil
end

figure(3)
plot(nm, filB(:, :))
axis([300 1000 0 1])
title('Filter 2 and 3 to no filter')

% Filter C
filC = filA+filB; % brings filter 1 back to 0 OD

fil(:,1) = filA;    % --> filter 1 to fil 2 and 3
fil(:,2) = filB;    % --> filter 2 and 3 to 0 OD
fil(:,3) = filC;    % --> filter 1 to 0 OD
fil(:,4) = zeros(2048,1); % no filter

for i = 1:3
    fil(:,i) = abs(fil(:,i));
end

uu = (281:1682);
figure(4)
hold on
plot(nm(uu), fil(uu,1), 'b')
plot(nm(uu), fil(uu,2), 'k')
plot(nm(uu), fil(uu,3), 'r')
axis([450 900 0 2])

```

```

xlabel('Wavelength [nm]')
ylabel('Filter Optical Density [OD]')
title('Filters')
legend('filter 2 and 3 to 1', '0 filter to fil2 and 3', 'filter from 0 od to filte

```

```

Mfiles(1:15,1:34) = filenames(11:25,1:34);
Mfiles(16:22,1:34) = filenames(27:33,1:34);
Mfiles(23:30,1:34) = filenames(35:42,1:34);
Mfiles(31:38,1:34) = filenames(44:51,1:34);
Mfiles(39:46,1:34) = filenames(53:60,1:34);
Mfiles(47:53,1:34) = filenames(62:68,1:34);
Mfiles(54:61,1:34) = filenames(70:77,1:34);
Mfiles(62:63,1:34) = filenames(79:80,1:34);
Mfiles(64,1:34) = filenames(82,1:34);

```

```

%Tfinal
Tfinal(:,1:15) = T(:,11:25);
Tfinal(:,16:22) = T(:,27:33);
Tfinal(:,23:30) = T(:,35:42);
Tfinal(:,31:38) = T(:,44:51);
Tfinal(:,39:46) = T(:,53:60);
Tfinal(:,47:53) = T(:,62:68);
Tfinal(:,54:61) = T(:,70:77);
Tfinal(:,62:63) = T(:,79:80);
Tfinal(:,64) = T(:,82);

```

```

fmask = [ 3 3 3 3 3 3 3 3
          3 3 3 3 3 3 3 2
          3 3 3 3 3 3 2 2
          3 3 3 3 3 3 2 2

```

```

        3 3 3 3 3 3 2 2
        3 3 3 3 3 3 2 2
        3 3 3 3 3 2 2 2
        3 3 3 3 3 2 2 4 ]; % fmask(ink,il)

nmK = nm;
clear nm
% load optical properties
load muail4_Rr_inf_matrixV
load muspil2_intsph_matrixV
% 8 scatterer concentrations and 8 absorber concentrations
total_vol = 100; % [ml]
Vil       = [35 25 17.5 12.5 7.5 5 2.5 1.25]; % [ml]
Vink      = [0 0.025 0.1 0.225 0.4 0.625 1.225 1.6]; % [ml]
vol_water = 50; % [ml]

% musp
figure(5)
plot(nm, muspRx1)
hold on
plot(nm, muspRx2)
plot(nm, muspRx3)
plot(nm, muspRx4)
plot(nm, muspRx5)
plot(nm, muspRx6)
plot(nm, muspRx7)
plot(nm, muspRx8)

v(:,1) = muspRx1;
v(:,2) = muspRx2;
v(:,3) = muspRx3;
v(:,4) = muspRx4;
v(:,5) = muspRx5;

```

[illegible]


```

disp(sprintf('%d \t%d \t %s \t %s', j, u, clr(u), Mfiles(j,:) ))
filter = 10.^-(fil(:,fmask(iink(j),jil(j))));
for mm = 1:2048
TK(mm,j) = (Tfinal(mm,j)./(filter(mm,1).*source(mm,1))); %
    TK2(mm,j) = (Tfinal(mm,j)./filter(mm,1));%./source(mm,1);% <----- !!!!
end
plot(nmK, TK2(:,j), [clr(u) '-'])
hold on
    axis([400 950 0 6])
    cnt = cnt +1
    xlabel('Wavelength [nm]')
    ylabel('Corrected Measurement --> Measure/(source*filter)')
end
break
clear filenames nm T
filenames = Mfiles;

nm=nmK;
T = TK;
break

% save output2 nm T mua musp filenames iink jil

% save Transport T nm iink jil mua musp

```

.4 MapIt.m

```
% mapit.m

clear
disp(sprintf('\n\nbegin ----- \n\n'))

load output2 % <--- nm T mua musp filenames

Ns = 100;
Na = 100;

[Nnm Nf] = size(T);
Nf = 64;
muamax = 8.5201;
muspmax = 33.2695;
dma = muamax/(Na-1);
dms = muspmax/(Ns-1);

M = zeros(Na,Ns);
cnt = zeros(Na,Ns);

for j=1:Nf % for each spectrum
%disp(sprintf('%d',j))
for i=380:1750 %1:Nnm % for each wavelength 380:1750

ma = mua(i, iink(j), jil(j));
ms = musp(i, jil(j));

ima = round(ma/dma) + 1;
ims = round(ms/dms) + 1;
```

```

if ima>Na; ima=Na;end
if ims>Ns; ims=Ns;end

M(ima, ims) = M(ima, ims) + T(i, j);
cnt(ima, ims) = cnt(ima, ims) + 1;
end
end
clear T
M = M./cnt;
M(isnan(M)) = 0;

```

```

figure(2);clf
xmusp = (1:Na)'*dms;
ymua = (1:Ns)'*dma;
imagesc(xmusp, ymua, log10(M))
axis xy
colorbar
% caxis([3.5 5.1])
colorbar
xlabel('musp [cm-1']')
ylabel('mua [cm-1']')
title('log10(M)')
%break
%INTERPOLATE M vs mua @ each musp
for j = 1:Ns % for each musp(j)
    cnt = 0;
    xx = 0;
    MM = 0;
    for i = 1:Na
        if M(i,j)>0
            cnt = cnt+1;
            MM(cnt) = M(i,j);
            xx(cnt) = ymua(i);
        end
    end
end

```

```

end
fit(j,1:2) = polyfit(xx,log(MM),1); % y = fit(1)*xx^2 + fit(2)*xx + fit(3)
yy = exp(polyval(fit(j,:), xx));

if cnt > 0
    figure(3); hold off
    plot(xx, MM,'o')
    hold on
    plot(xx, yy,'k-')
    title(sprintf('%d',j))
    axis([0 max(xx) 0 max(yy)])
    drawnow
%     pause
end %if
end %j

if 0
for j=1:100
    for i=1:100
        T(i,j) = exp(polyval(fit(j,:), i*dma));
    end
end
T = T.*(T>0);
T(isnan(T)) = 0;
T(1,:) = T(3,:);
T(2,:) = T(3,:);

figure(4);clf
imagesc(xmusp, ymua, log10(T) )
axis xy
colorbar
caxis([3.5 5.1])
colorbar

```

```

xlabel('musp [cm-1'])
ylabel('mua [cm-1'])
end

figure(5)
subplot(2,1,1)
plot(xmusp(3:Ns), (fit(3:Ns,1)), 'ro')
title('L(musp) slope')
subplot(2,1,2)
plot(xmusp(3:Ns), exp(fit(3:Ns,2)), 'ro')
title('Mo(musp) exp(intercept)')
xlabel('musp')

a_b = polyfit(xmusp(3:Ns), fit(3:Ns,1),1);
figure(5)
subplot(2,1,1)
hold on
plot(xmusp(3:Ns), polyval(a_b, xmusp(3:Ns)), 'k-')

c_d = polyfit(xmusp(3:Ns), exp(fit((3:Ns),2)),1);
figure(5)
subplot(2,1,2)
hold on
plot(xmusp(3:Ns), (polyval(c_d, xmusp(3:Ns))), 'k-')

break

%save Ind2Values a_b c_d

break

%save map dma dms M T

```

.5 getM.m

```
function M = getM(mua, musp)
% these values are from a calibration on acrylimide gels
% scattering was intralipid
% absorption was india ink
% a = -0.0050;
% b = 0.184;
% c = 0.160;
% d = 0.223;

a = -0.0050;
b = 0.184;
c = 0.160;
d = 0.223;

L = a*musp + b;
Mo = c*musp + d;

M = Mo.*exp(-mua.*L);
```



```

%

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
muaoxy   = interp1(nm,muaoxy,   nmK);
muadeoxy = interp1(nm,muadeoxy, nmK);
muawater = interp1(nm,muawater, nmK);
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
load tempNM
load tempspectra
load IntegrationTime
load Nx % loads the number of nm in each spectra = Ni and the length of the list=
load source

nmosp = tempNM;
spectra = tempspectra;
itime = IntTime;
clear tempspectra
clear tempNM
clear IntTime

[Nnm Nsp] = size(spectra);
%Results = zeros(Nj, 7);
load Results  %( isp, fb, S, a, P1, b, P2)

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
tilefigs
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

for isp = 1:Nsp
    Ms    = spectra(:,isp);

```

```

nmK = nmsp;
Mref = source(:,isp);

isp
theerror=0;
Components
tilefigs
Results(isp,1) = isp;
Results(isp,2:7) = result;
save Results Results
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
nm = nmK;
ss = strvcats(ss,'err');

save output nm out ss Results
datestr(now)

```

.7 Components.m

```
% Components.m
% changed for post thesis work
% call with nmK, Ms, and Mref specified

warning off
global cnt theerror %cntb

N = length(nmK);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Load standard spectrum
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
offset1 = - mean(Mref(1:10));
offset2 = - mean(Mref(2038:2048));
fit = polyfit([nm(1) nm(2048)], [offset1 offset2],1);
Mref = Mref + polyval(fit, nm);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Load spectrum
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
offset1 = - mean(Ms(1:10));
offset2 = - mean(Ms(2038:2048));
fit = polyfit([nm(1) nm(2048)], [offset1 offset2],1);
Ms = Ms + polyval(fit, nm);

M = Ms./(max(Mref)/max(std99)*std99);

figure(1);clf
plot(nmK, Ms,'r-')
hold on
plot(nmK, Mref,'m-')
plot(nmK, std99,'k-')
```

```
hold on
plot([350 1000],[0 0],'k-')
```

```
% starting values
```

```
fb = 0.04;
S = 0.8;
a = 1e6;
b = 2;
nmMS = 1100;
MS = a*(nmMS)^-b;
```

```
P1 = 3;%2;
```

```
w1 = 80;
```

```
nm1 = 460;
```

```
P2 = 0.5;
```

```
w2 = 35;
```

```
nm2 = 635;
```

```
fw = 0.75;
```

```
i630 = 1;while nmK(i630) < 630; i630 = i630 + 1; end
```

```
musp = MS*(nmK./nmMS).^b;
```

```
pMw = getM3(fw*muawater, musp);
```

```
% dM = pMw(i630) - M(i630);
```

```
% if dM > 0; P2 = 0.1; end
```

```
% if dM > .1; P2 = 0.5; end
```

```
% if dM > .2; P2 = 1; end
```

```
% if dM > .25; P2 = 1.5; end
```

```
start = [fb S a P1 b P2];%
```

```

ss = 'f_b';
ss = strvcat(ss,'S');
ss = strvcat(ss,'a');
ss = strvcat(ss,'P1');
ss = strvcat(ss,'b');
ss = strvcat(ss,'P2');

options = optimset('MaxFunEvals',1e5,'MaxIter',1e8, 'TolFun', 1e-4);%, 'TolX', 1e-
uu = [200:1600];%280:1348]; %[520:1200]; % to 800 nm

cnt = 0;
cntfit =0;
result = fminsearch('SpectraFit', start, options, ...
muaoxy(uu), muadeoxy(uu), muawater(uu), ...
fw, w1, nm1, w2, nm2, ...
nmK(uu), Ms(uu), Mref(uu), std99(uu), ss, nmMS);
fb      = result(1);
S       = result(2);
a       = result(3);
P1      = result(4);
b       = result(5);
P2      = result(6);

nm = nmK;

muablood = fb*(S*muaoxy + (1-S)*muadeoxy);
bilirubin = P1*exp(-((nm - nm1)/w1).^2);
muaPpIX   = P2*exp(-((nm - nm2)/w2).^2);
mua = fb*(S*muaoxy + (1-S)*muadeoxy) + fw*muawater + bilirubin + muaPpIX;
MS     = a*(nmMS)^-b;
musp   = MS*(nmK./nmMS).^b;

```

```

if ((theerror >= 0.01) & (cntfit <= 10))
    for cntfit = 0:10
        start = [fb S a P1 b P2];
        cntfit = cntfit+1
        result = fminsearch('SpectraFit', start, options, ...
muaoxy(uu), muadeoxy(uu), muawater(uu), ...
fw, w1, nm1, w2, nm2, ...
nmK(uu), Ms(uu), Mref(uu), std99(uu), ss, nmMS);
        fb      = result(1)+0.01;
        S        = result(2);
        a        = result(3);
        P1       = result(4);
        b        = result(5);
        P2       = result(6);

        nm = nmK;

        muablood = fb*(S*muaoxy + (1-S)*muadeoxy);
        bilirubin = P1*exp(-((nm - nm1)/w1).^2);
        muaPpIX  = P2*exp(-((nm - nm2)/w2).^2);
        mua = fb*(S*muaoxy + (1-S)*muadeoxy) + fw*muawater + bilirubin + muaPpIX;
        MS      = a*(nmMS)^-b;
        musp    = MS*(nmK./nmMS).^b;
    end
end
theerror
cntfit

sz = 18;

ideoxy = 1;      while nm(ideoxy) < 480; ideoxy = ideoxy + 1; end
iw      = ideoxy; while nm(iw) < 580;      iw = iw + 1;      end
iPPIX   = iw;     while nm(iPPIX) < 635;   iPPIX = iPPIX + 1;   end
ioxy    = iPPIX;  while nm(ioxy) < 650;   ioxy = ioxy + 1;     end

```

```

ipp    = ioxy;    while nm(ipp) < 710;    ipp = ipp + 1;    end

figure(2);hold off
set(figure(2),'color','w','position',[200 150 500 550])
semilogy(nm, mua,'k-','linewidth',2)
hold on
plot(nm, fw*muawater,'k-')
plot(nm, fb*S*muaoxy,'k-')
plot(nm, fb*(1-S)*muadeoxy,'k--')
plot(nm(iPPIX), mua(iPPIX), 'ko','markersize',14)
set(gca,'fontsize',sz)
xlabel('Wavelength [nm]','fontsize',sz)
ylabel('_a [cm^-1]','fontsize',sz)
axis([350 1000 1e-4 1e2])
text(600, 40, sprintf('_a    = %0.2f cm^-1 @ 635 nm', mua(iPPIX) ),'fontsize',sz)
text(600, 10, sprintf('_s'' = %0.2f cm^-1 @ 635 nm', musp(iPPIX) ),'fontsize',sz)

text(380, .001, 'water','fontsize',sz)

text(485, .01, 'oxyHGb','fontsize',sz)
plot([580 nm(ioxy)], [0.013 fb*S*muaoxy(ioxy)], 'k-')

text(370, 0.1, 'deoxyHGb','fontsize',sz)
plot([460 nm(ideoxy)], [0.13 fb*(1-S)*muadeoxy(ideoxy)], 'k-')

text(800, 0.18, 'total','fontsize',sz)

figure(4);hold off
set(figure(4),'color','w','position',[200 120 500 350])
plot(nm, musp,'k-','linewidth',2)
hold on
set(gca,'fontsize',sz)
xlabel('Wavelength [nm]','fontsize',sz)
ylabel('_s'' [cm^-1]','fontsize',sz)

```

```

%%%%%%%%%%%%%%
%%%%%%%%%%%%%%
set(gcf(3),'color','w','position',[10 20 700 500])
set(gcf(2),'color','w','position',[10 20 700 700])
set(gcf(4),'color','w','position',[10 20 700 500])

disp(' ')
disp('RESULT = ')
for i=1:length(ss)
disp(sprintf('%10.3f\t%s', result(i), ss(i,:) ))
end
disp(' ')

out(1:6, isp) = result';
out(7, isp)   = theerror;

```

.8 SpectraFit.m

```

function err = SpectraFit(start, ...
    muaoxy, muadeoxy, muawater, ...
    fw, w1, nm1, w2, nm2, ...
    nm, Ms, Mref, std99, ss, nmMS)

global cnt theerror
cnt = cnt + 1;

fb      = start(1);
S       = start(2);
a       = start(3);
P1      = start(4);
b       = start(5);
P2      = start(6);

M = Ms./(max(Mref)/max(std99)*std99);
Mx = Ms./(max(Mref)/max(std99)*std99);

muablood = fb*(S*muaoxy + (1-S)*muadeoxy);
bilirubin = P1*exp(-((nm - nm1)/w1).^2) ;
muaPpIX   = P2*exp(-((nm - nm2)/w2).^2);
MS        = a*(nmMS)^-b;
musp      = MS*(nm./nmMS).^b;

%%%
% instrument
%%%
pM0 = getM3(0, musp);

%%%

```

```

% ADD dry, scatter, and water has negligible effect
% no blood, no PPIX
%%%
mua = fw*muawater;
pMw = getM3(mua, musp);

%%%
% ADD blood
% no PPIX
%%%
mua = fw*muawater + muablood;
pMwb = getM3(mua, musp);

%%%
% ADD bilirubin
%%%
mua = fw*muawater + muablood + bilirubin + muaPpIX;
pMwbb = getM3(mua, musp);

%%%
i860 = 1;while nm(i860) < 860; i860 = i860 + 1; end
i750 = 1;while nm(i750) < 750; i750 = i750 + 1; end
i800 = 1;while nm(i800) < 800; i800 = i800 + 1; end

uu = [280:i800];%[280:i750];
v = [1:i860];
err = (sum( (pMwbb(uu) - M(uu)).^2 ));
%%%

if fb<0; err = err*10; end
if S<0; err = err*100; end
if fw<0; err = err*10; end
if a<0; err = err*10; end
if b<0; err = err*10; end

```

```

if P1 < 0; err = err*100; end
if P2 < 0; err = err*100; end

if fb>1; err = err*10; end
if S>1; err = err*10; end
if fw>1; err = err*10; end

theerror = (sum( (pMwbb(uu) - M(uu)).^2 ));%err;

if mod(cnt,10000)==0

iPPIX = 1;while nm(iPPIX) < 635; iPPIX = iPPIX + 1; end
iw = 1;while nm(iw) < 580; iw = iw + 1; end

%%%%%%%% Fig 3 %%%%%%
figure(3)
set(figure(3),'color','w','position',[10 20 500 350])
sz = 18;
sz2 = 14;

plot(nm(uu), M(uu), 'k-')
hold on
plot(nm(uu), pM0(uu), 'k--','linewidth',1)
plot(nm(uu), pMw(uu),'b-','linewidth',1)
plot(nm(uu), pMwb(uu),'g-','linewidth',1)
plot(nm(uu), pMwbb(uu),'r-','linewidth',1)
set(gca,'fontsize', sz2)
title(sprintf('%d', cnt))
xlabel('Wavelength [nm]','fontsize', sz)
ylabel('M = M_s / M_r_e_f','fontsize', sz)

ymax = 3;
Nst = length(ss);

```

```

N = Nst + 2;
for i=1:Nst
    if start(i)>1
        text(800, (1 - i/N)*ymax, sprintf('%s = %0.1f', ss(i,:), start(i)), 'fontsize', sz2)
    else
        text(800, (1 - i/N)*ymax, sprintf('%s = %0.3f', ss(i,:), start(i)), 'fontsize', sz2)
    end
end

text(400, 0.8*ymax, sprintf('_s''(635) = %0.1f cm-1', MS*(635./nmMS).^-b ), 'font'
text(400, 0.9*ymax, sprintf('_a(635) = %0.2f cm-1', mua(iPPIX) ), 'fontsize', sz2)
plot([nm(iw) 600], [pMw(iw), 0.6*ymax], 'k-')
text(600, 0.6*ymax, '+w', 'fontsize', sz2)
plot([635 660], [pMwb(iPPIX), 0.7*ymax], 'k-')
text(660, 0.7*ymax, '+w+b', 'fontsize', sz2)

axis([350 1100 0 ymax])
hold off
drawnow

end

```

.9 getspectrum3.m

```

function spectrum = getspectrum3(file)
% where file = path + filename
% PC version
% FOR LOADING SPECTRAL DATAFILES FROM OCEAN OPTICS SPECTROMETER
% ---> nm(:,1) = wavelengths
% ---> T(:,j) = measured transmission for j = # of .SOC files in directory
% ---> filenames(j, :) = filenames of .SOC files
% Assumes spectra have 2048 wavelengths
% Subtracts baseline = mean of first 2:200 wavelengths
% Sets T(1) = 0;
% Replaces '_' with '-' in filenames used in graphing

disp(file)

% read spectrum
fid = fopen(file,'r');
for i=1:14 %19%14
line = fgetl(fid);
end

A = fscanf(fid, '%lf %lf\n', [2, 2048]);
fclose(fid);
A = A';

nm = zeros(2048,1);
T = zeros(2048,1);

nm(:,1) = A(:,1);
T(:,1) = A(:,2);% - mean(A(2:200,2));
T(1,1) = 0;

spectrum = zeros(2048,2);

```

```
spectrum(:,1) = nm;  
spectrum(:,2) = T;
```

.10 GetIntTime.m

```

function T = GetIntTime(list);
% Finds the integration time recorded for each spectra.
% Given a list from Organize.m, GetData(list) will retrieve the data from
% each file name
% Returns a data set M, first column is the wavelength
% the second col is the first data intensities,
% the third is the second data set
% The number of rows that M has are the length of the data files. (2048)
% The number of columns that M has is the number of files read + 1

blank = ' ';

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% CYCLE THROUGH ALL SPECTRA
%%%
[Ni Nj] = size(list);
L = length(list)
for ii=1:Ni %L
% remove trailing spaces in filename
[Ni Nj] = size(list);
i = 1;
while (i <= Nj) & ~(list(ii,i) == blank) % check (i < Nj) first !!
i = i+1;
end
file = list(ii, 1:i-1);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Load EXPERIMENTAL SPECTRA
%%%%%%%%
%%%
% Calls function that loads spectra

```

[illegible]

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