VARIATION OF FLUORESCENCE WITH TEMPERATURE IN HUMAN TISSUE

By

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

May, 2010

Nashville, Tennessee

Approved:

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ACKNOWLEDGEMENTS

I would like to thank Alex Walsh for assisting me in almost all of these experiments. I would also like to thank Raiyan Zaman and Dr. A.J. Welch at the University of Texas at Austin for the initial work on this project and their continued expertise in this area.

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CHAPTER I

BACKGROUND AND PRELIMINARY EXPERIMENTS

Introduction

The interaction of fluorescence, optical properties, and temperature in human tissue is a fundamental scientific topic in biomedical optics. The field of biomedical optics uses lasers and other optical techniques for scientific experimentation, treatment, diagnosis, and the guidance of therapy. Thermal effects during these applications can often present confounding experimental factors. Changes in tissue due to heat generated by absorption of light is of vital importance during ablative procedures using lasers, but the effect of temperature variation in optical diagnosis has received much less scrutiny.

The exact mechanism by which temperature affects fluorescence in tissue is not completely understood or defined. It is known that temperature past a certain threshold can cause coagulation, which creates a change in optical properties. Specifically, coagulation causes an increase in scattering and increased opacity in some tissues [1]. These optical properties modulate fluorescence, and comprise part of the mechanism by which temperature affects fluorescence. It is also known that increased temperature can cause quenching, a change in molecular energy levels, a breakdown of fluorophores, and other optical property changes distinct from coagulation. The understanding of these

mechanisms in tissue fluorescence would augment the effectiveness and accuracy of fluorescence spectroscopy in biomedical applications.

Of the many variables which affect fluorescence in tissue, temperature is often ignored. As fluorescence continues to gain popularity as a tool for diagnosis and therapeutic guidance, it is being implemented in applications in which temperature is often not constant. Fluorescence guidance has been applied to thermotherapies such as radiofrequency ablation [2] and has potential for guidance in emerging thermotherapies such as microwave ablation. In addition, fluorescence techniques examining specimens *in vitro* are carried out at a different temperature than a potential *in vivo* application. Thus, temperature effects are important in experimental planning and predictions when transitioning from *in vitro* to *in vivo.*

Fluorescence

Fluorescence occurs when a molecule in its ground state absorbs energy and is excited to an electronically excited state. From there, the molecule undergoes vibrational relaxation and/or internal conversion in which energy is generally emitted as heat. Then, from the lowest vibrational level in an excited electronic state, the molecule returns to its ground state by emitting a photon. Since the molecule has undergone a non-radiative decay (heat generation), the emitted photon has less energy than the incident photon. This is known as a Stokes shift. Thus, fluorescence is only present at longer wavelengths than the excitation, or incident, light. A diagram of fluorescence in terms of energy levels is displayed in Figure 1.1, known as a Jablonski diagram. As can be seen in the Jablonski

diagram, fluorescence is not the only phenomenon possible when a photon is absorbed. Other paths include elastic scattering, inelastic scattering, phosphorescence, and non-radiative decay (vibrational relaxtion).

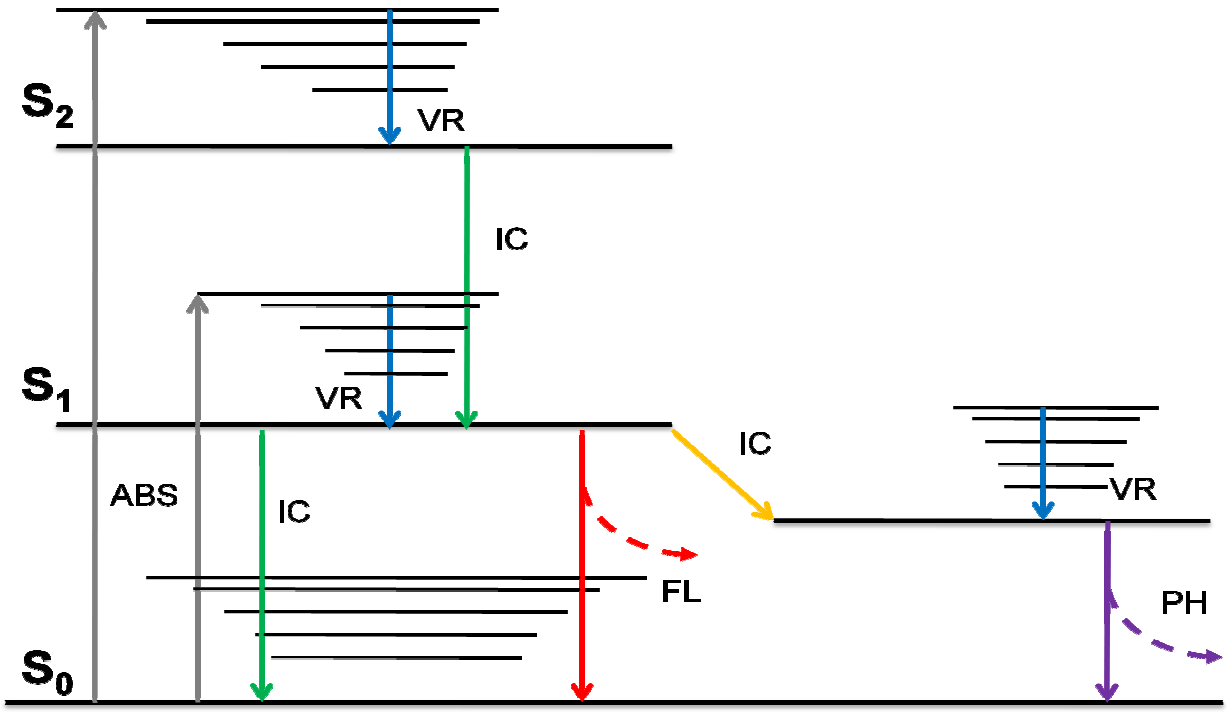


Figure 1.1 Jablonski diagram. Fluorescence occurs when a photon is absorbed, exciting a molecule to an excited electronic state. After a non-radiative decay to the lowest vibrational level of an electronic state, the molecule returns to its ground state by emitting a fluorescent photon. Key: ABS, absorption; IC, internal conversion; VR, vibrational relaxation; FL, fluorescence emission; IC, intersystem crossing; PH, phosphorescence.

The timescale of fluorescence depends on the exact substance and incident energy, but is much longer than the absorption time course. The time course of absorption is on the order of 10^{-15} s, while the fluorescent lifetime is on the order of 10^{-8} s. The short lifetime of absorption, according to the Franck-Condon principle, does not allow for any

molecular interactions during the process, while the longer relative lifetime of fluorescence emission allows for a variety of other interactions [3].

Fluorescence Quenching

Fluorescence quenching refers to any process that decreases the intensity of emitted fluorescent light. The most common form of quenching in fluorescence is collisional quenching. This occurs when a certain molecule, or quencher, comes into contact with the fluorophore in its excited state. This collision results in a non-radiative decay back to the ground state of the fluorophore. This decrease in fluorescence due to collisional quenching is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + \kappa_q \tau_0 [Q] \quad (1)$$

Where F is the measured fluorescence, F_0 is the initial, unquenched fluorescence, κ_q, is the biomolecular quenching constant (s^{-1}), τ_0 is the unquenched fluorescence lifetime (s), and [Q] is the quencher concentration. The biomolecular quenching constant (s^{-1}) can be calculated based on temperature and viscosity using the Stokes-Einstein relationship, or experimentally determined. Quenching is able to occur due to the usual lifetime of fluorescence (10^{-8} to 10^{-10} s), which is ample time for quenchers to collide with excited-state fluorophores. Many substances can behave as a quencher, but the most prominent in biological tissue is oxygen [3].

Optical Properties

When light interacts with tissue, a variety of events can occur. It is useful to define light as a particle, or photon, for most tissue optics applications. When a photon is incident upon a piece of tissue it can reflect off the surface, known as specular reflectance, or enter into the tissue. Whether a photon is specularly reflected or enters into the tissue is governed by Fresnel's laws, which depend on the incident angle, incident index of refraction, tissue index of refraction, and the polarization state of the incident light. If the photon enters the tissue, it can be absorbed, elastically scattered, inelastically scattered, or transmitted.

Due to the relatively complex molecular nature of tissue, these events are best understood on a probabilistic and empirical level. The probability of a photon being absorbed while traveling over an infinitesimal distance, *dz* (cm), is described by the product of the absorption coefficient, μ_a (cm^{-1}), and the distance, *dz* (cm). The absorption coefficient is related to light irradiance in a non-scattering medium through Beer's Law:

$$E(z) = E_0 e^{-\mu_a(\lambda)z} \tag{2}$$

Where E_0 is the incident irradiance (W/m^2), μ_a is the wavelength dependent absorption coefficient (cm^{-1}), z is the depth in the medium (cm), and E(z) is the irradiance at that depth (W/m^2). Similarly, the probability of a photon being scattered while traveling over an infinitesimal distance, *dz* (cm), is described by the product of the scattering coefficient, μ_s (cm^{-1}), and the distance, *dz* (cm). The angle at which the scattering occurs is described by the anisotropy factor, g. The anisotropy represents the average cosine of

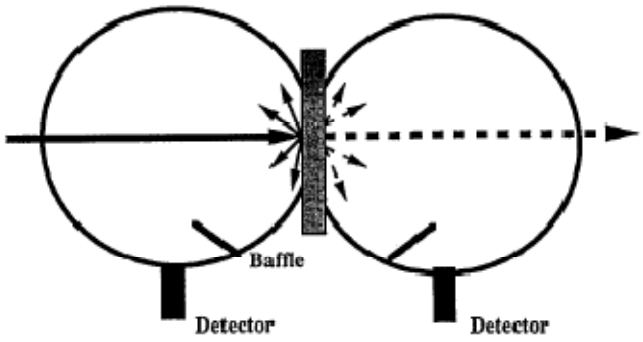
the scattering angle, where -1 represents total backscattering, 0 represents isotropic scattering, and 1 represents total forward scattering. Tissue is highly forward scattering, with g values between 0.7-0.99. The anisotropy and scattering coefficient can be described by a single term known as the reduced scattering coefficient, and is defined as:

$$\mu_s' = \mu_s(1 - g) \tag{3}$$

This is often used if the exact values of μ_s and g are coupled, and their individual values are not known.

Methods to Determine Optical Properties

Although several methods exist to determine μ_a, μ_s, and g in tissue, two methods are particularly relevant to the work described here. A popular method includes the use of two integrating spheres and an inverse-adding doubling algorithm (I.A.D.).



[4]

Figure 1.2 Double integrating sphere diagram. The sample is placed in between the two spheres, and collimated light enters through a port in the first sphere and exits the port in the second sphere. Portions of the light are reflected from the sample and gather by the first detector. Light that is transmitted through the sample and scattered is detected by the second detector.

A complete description of the double integrating sphere method can be found in a paper by Pickering *et al.* [4]. In short, the sample for which the optical properties are to be determined is placed between two integrating spheres, the insides of which have a highly diffuse and reflective coating. Collimated and monochromatic light enters the spheres through a port in the first integrating sphere. Then, reflected and transmitted light is measured by detectors in separate ports in each of the two spheres. Two detectors will allow for the calculation of two optical properties, μ_a and μ_s'. Measurements are wavelength dependent, and must be separately determined for each wavelength of incident light. This method is also sensitive to light lost through the sides of the sample, so an optically thin sample produces the most accurate results. In addition, the coating of the spheres is not perfectly reflective, usually on the order of 99%, and must be corrected for in the final calculations.

Detectors are calibrated with no sample present to determine baseline levels (0%) of light present. Maximum reflectance values are measured by placing a reflective standard (R=100%), similar to the sphere's coating in the place of the sample and measuring the value in the first detector. Maximum transmittance is measured by placing the reflective standard (R=100%) at the exit port of the second sphere with no sample in between the two spheres. When the sample is placed between the spheres, the intensities are measured for each detector, and normalized as a percent based on the 0% and 100% measurements. The final result yields a percent transmission (%T), percent reflectance (%R), and possibly an unscattered percent transmission. These values, along with the thickness of the sample, serve as an input into an inverse-adding doubling algorithm.

The inverse-adding doubling algorithm is described in complete detail by Prahl et al. [5]. This method uses the adding-doubling method, which juxtaposes two identical slabs of given optical properties with known reflectance (R) and transmittance (T). The R and T values are added to obtain the reflectance and transmittance of the new slab, which is twice as thick. The R and T values of the initial, thin slab, are calculated using single scattering and Fresnel's laws. This process is repeated until the thickness of the slab is equivalent to the desired thickness. Thus, the adding-doubling method receives the tissue's optical properties and thickness, and yields the reflectance and transmittance of the incident light.

In short**,** the inverse adding-doubling algorithm guesses a set of optical properties and generates reflectance and transmission values from these using the adding-doubling method. If μ_a, μ_s, and g are desired, the unscattered transmission from the integrating spheres must be supplied in addition to the diffuse reflectance and transmittance values. If only diffuse reflectance and transmittance values are given, the inverse adding-doubling method yields μ_a and μ_s'. The calculated reflectance (R) and transmittance (T) values are compared to those measured in the integrating spheres, and the process is repeated until it converges to a unique solution for μ_a and μ_s'. If the scattering anisotropy, g, is known, or the unscattered transmission is measured, a unique solution exists using this method. In order to achieve accurate results, corrections must be made for integrating sphere imperfections and the presence of glass slides. This method works for almost any combination of optical properties, but is not guaranteed to converge. For instance, the sample measured in the integrating spheres should be optically thin enough

to get a transmission measurement that is sufficiently greater than the noise of the detectors.

Once the optical properties have been obtained, a probabilistic method such as Monte Carlo simulation can be used to predict the propagation of light in tissue. Monte Carlo uses a random walk method to trace the path of single photons in tissue. The optical properties of the tissue are used to create probability distributions of what happens to a photon at each point in the tissue. At any given point, a photon can pass through the point with no interaction, undergo a scattering event, or be absorbed. The tissue is organized into discrete grids, and a photon is launched into the tissue. The path of the photon through each point and its termination point (i.e. where it is absorbed) are recorded. Large numbers of photons are launched and recorded in this manner, usually on the order of 10^8 photons. A sufficient number of photons yields information about how light is reflected and transmitted within the tissue. Monte Carlo is advantageous, since it works for any combination of optical properties, and can handle multiple layers with different optical properties. Monte Carlo also assumes homogenous optical properties within the layers. The disadvantage is that it can require long computation times to generate enough photons for statistical certainty. This is generally not a problem with modern computing capabilities.

In order to apply the I.A.D. method to obtain the tissue optical properties, both reflectance and transmittance must be available to generate optical properties. Transmittance measurements are not always available if the sample is too thick or if only reflectance data is measured. The latter is often the case in probe-based fluorescence

measurements, where only reflectance measurements are available. In this case, the inverse-adding doubling method may not be used due to the lack of transmission measurements. However, an inverse Monte Carlo can be used to calculate the optical properties based on a diffuse reflectance measurement. This consists of guessing a set of optical properties, running a Monte Carlo simulation, and comparing the results to the measured diffuse reflectance spectrum. This is continued until the generated diffuse reflectance matches the measured diffuse reflectance. The optical properties used to produce the final reflectance curve are provided as the optical properties of the tissue. The accuracy of this method depends heavily on the initial guess, as the final calculated optical properties are not unique. Thus, an educated guess of the initial optical properties is essential for an accurate result. This method has been applied in tissue and is described in detail by Palmer et al. [6, 7].

Effect of Optical Properties on Fluorescence

Fluorescence emission intensity is primarily a function of the concentration of a certain fluorophore, the excitation wavelength, and the emission wavelength. In turbid media, however, absorption and scattering modulate the fluorescence emission intensity. This presents a problem for the use of fluorescence for diagnosis and therapeutic guidance in tissue due to strong absorbers such as blood. The presence of scatterers and absorbers can modulate fluorescent output for identical tissues, thus hindering the reproducibility and reliability of fluorescence spectroscopy as a diagnostic tool. The elimination of optical property modulation of fluorescence yields a fluorescence spectrum

that is solely a result of the intrinsic fluorophores and their concentrations, known as intrinsic fluorescence. Two methods to extract instric fluorescence, which are applicable in tissue in the UV-visible range, are discussed.

Gardner et al. developed an algorithm for extracting the intrinsic fluorescence from tissue phantoms based on fluorescence measurements and known optical properties [8]. This method was able to determine fluorophore concentration to within 15% in a tissue phantom. Zhang et al. also developed an algorithm for the extraction of intrinsic fluorescence, but their method did not require the prior knowledge of optical properties. Instead, it required the assumption that fluorescence and reflectance would be modulated similarly by optical properties, which is accurate if diffuse reflectance and fluorescence are measured simultaneously using the same probe. Their algorithm is summarized by the equation below.

$$f_{exc,em} = \frac{F_{exc,em}}{\frac{1}{\mu_{s,exc} l}\left(\frac{R_{0,exc}R_{0,em}}{\epsilon_{exc}\epsilon_{em}}\right)^{\frac{1}{2}}\frac{R_{exc}}{R_{0,exc}}\left(\frac{R_{em}}{R_{0,em}}+\epsilon_{em}\right)} \quad (4)$$

[9]

The intrinsic fluorescence ,$f_{exc,\,em}$, is a function of the measured fluorescence, $F_{exc,\,em}$, the scattering coefficient at the excitation wavelength, $\mu_{s,\,exc}$ (cm^{-1}), sample thickness, l (cm), measured reflectance , R, and reflectance in the case of no absorption, R_0. R_0 is determined by analytically using a reflectance model described by Zonios *et al* [10]. ϵ is a constant that depends on the anisotropy parameter, g, and the probe geometry. The subscripts *exc* and *em* represent the excitation and emission wavelengths, respectively.

Equation 3 shows the obvious dependence on scattering due to the μ_s term in the denominator, but the reflectance terms (R_{em}, R_{exc}) represent a combined effect of both scattering and absorption on intrinsic fluorescence. This method is not accurate in the case of excess absorption, in which the intrinsic fluorescence is masked by absorption beyond the point of recovery.

Effect of Heat on Optical Properties

Optical properties exhibit a known dependence due to thermal damage and coagulation. The deposition of laser energy in tissue generally results in heating of the tissue. This can result in vasodilation, which allows blood to act as a heat sink. If a large enough amount of thermal energy is deposited or it is deposited over a very short time, the heat can cause damage to the tissue, coagulation of the blood, dehydration, denaturation, conformational changes, or any combination of these. Changes in scattering and absorption are mediated by these different types of thermal damage [1, 4, 11, 12]. These changes are mostly irreversible, but show some reversible characteristics [12]. These studies, like most examining optical property changes due to thermal effects, focused on coagulation and dehydration caused by an ablative laser. These parameters are important for the effectiveness of laser ablation, but are mostly drastic, irreversible changes.

Optical properties have been shown to be temperature dependent over a physiological temperature range in the near-infrared region [13, 14] in certain types of tissue. The effect of temperature on optical properties is less defined in the UV-visible

region, and has not been examined simultaneously with fluorescence. Optical properties and their thermal dependencies are expected to vary from the near-infrared to the UV, and expected to vary across different types of tissue, such as skin and fat. If optical properties are temperature dependent in the UV-visible range, their temperature dependency could be used to manipulate fluorescence in tissue or measure temperature changes. It would also have important practical consequences for extracting intrinsic fluorescence from tissues *in vitro* and *in vivo.*

Other Thermal Considerations

Thermal energy can affect the fluorophore itself. Certain proteins, such as collagen, are important biological fluorophores. Proteins undergo denaturation and conformational changes at temperatures above body temperature (37°C), and these conformational changes can cause a change in fluorescence. Menter et al. considered the change in collagen conformation due to temperature, as well as its eventual denaturation, as a major factor in the change in fluorescence due to temperature [15]. Lipids also undergo documented phase changes that affect optical properties and fluorescence at temperatures above body temperature [14].

Preliminary Experiments: "Effect of Temperature on Fluorescence: An Animal Study."

In order to examine the effects of temperature on fluorescence, experiments on animal tissue were performed. Porcine eyes, porcine cornea, and rat skin were all used in these experiments. The first goal was to characterize the relationship between fluorescence intensity and temperature in biological tissue *in vitro*. The second goal was to determine if a change in optical properties was the only mechanism causing a temperature dependent change in fluorescence intensity. Fluorescence and diffuse reflectance measurements were made with varying temperature of 5 porcine eyes, 6 porcine corneas, and 5 rat skin samples. Changes in fluorescence intensity, emission peak location, and overall fluorescence lineshape were examined. Diffuse reflectance was also examined at every temperature to assess any changes in optical properties. Optical property measurements were also made before and after each experimental run using a spectrophotometer to obtain information about the starting and end points of the tissue.

Materials and Methods

A spectroscopic system was used to make fluorescence and diffuse reflectance measurements. The probe-based system uses a 337 nm nitrogen laser for fluorescence excitation (VSL 337, High Pressure Nitrogen Dye Laser; Oriel Corporation, Stratford, Connecticut) pulsed at 20 Hz and a white light source (150 W halogen lamp; Fiber Lite, Model180; Edmund Industrial Optics, Barrington, New Jersey) for reflectance measurements. A fiber optic probe was used for light delivery and collection. The probe

consists of seven 300 μm diameter fibers in a six around one configuration. Illumination was delivered by two fibers at the 6 o'clock and 12 o'clock positions on the outer ring, while the other five were devoted to light collection. A 385 nm long-pass filter was placed in the spectrometer to filter out the excitation light of the nitrogen laser. Input from a thermal camera (FLIR A20, FLIR Systems Inc., Boston, Massachusetts) was incorporated into the spectroscopic system through an analog to digital converter (A/DC) used to measure the surface temperature of the sample. The temperature of the samples submerged in PBS could not be measured directly with the thermal camera, but were assumed to be in thermal equilibrium with the surrounding liquid due to their relatively small volume. A LabVIEW program developed in house was used to control the system and acquire both spectroscopic and thermal data during all experiments. A diagram of the experimental setup is given in Figure 1.3.

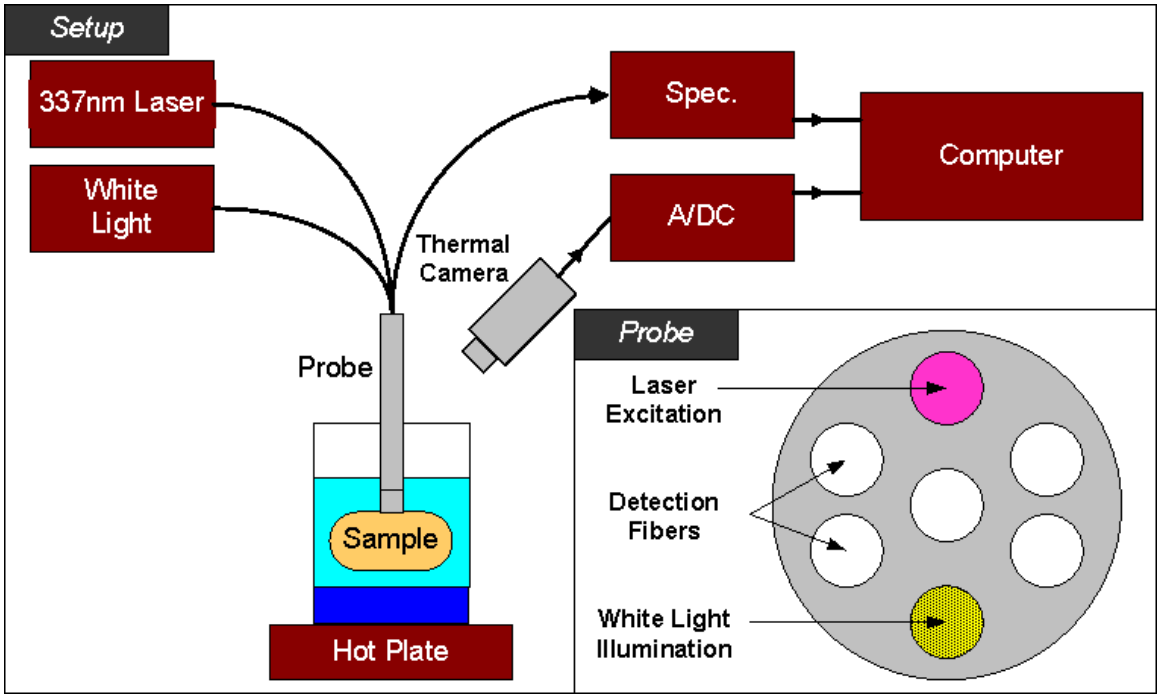


Figure 1.3 Setup for fluorescence and temperature experiments. A 337 nm N2 laser is used for fluorescence excitation, and a white light source is used for reflectance illumination, each delivered by a single, separate fiber. Five collection fibers deliver the collected light to a spectrometer, which delivers the spectra to LabVIEW program on a laptop. The thermal camera delivers a temperature reading to an analog to digital converter (A/DC) which delivers the reading to the program on the computer. The sample is held in place in the beaker of saline solution using a custom holder. A separate holder keeps the probe in place for the duration of the experimental run. Inset: a diagram of the tip of the probe and its fiber configuration.

The spectroscopic system and thermal camera described previously were positioned on an optical table. The sample was placed on a custom sample holder and held in place such that the thermal camera could measure the temperature on a spot adjacent to the optical probe. The optical probe was held in place at a fixed point on the sample for the duration of the experiment.

Porcine eye tissue and rat skin was excised immediately upon the animals' sacrifice and flash frozen in liquid nitrogen. All tissue samples were stored in a freezer at -80°C. Samples were removed from the freezer and placed above the normal saline solution. The probe was placed lightly at the approximate center of the surface of the

sample, and locked into place for the remainder of the experiment. For excised porcine eyes, the probe was placed at the center of the cornea's exterior. A spot was chosen for the thermal camera recordings such that the point was as close to the probe as possible without encompassing the probe's image. Spectroscopic measurements were made as soon as the probe and thermal recording spot were set, usually beginning at about -20°C. Each measurement acquired the temperature at that point, a baseline spectrum, a reflectance spectrum, and a fluorescence spectrum. The temperature was allowed to increase by no more than 5°C, and another measurement was taken. This process continued until the sample passively heated to room temperature (23°C), which was equal to the temperature of the normal saline below the sample. Additional saline at room temperature was added until the sample was submerged. The PBS temperature in the beaker was confirmed to be at room temperature before and after sample submersion using the thermal camera before proceeding. Neither the probe nor the sample was moved in this process. The normal saline solution with the submerged sample was then actively heated using a hot plate to approximately 50°C or greater than 70°C. Measurements were taken at approximately 5°C intervals while heating. Once the sample reached the target temperature, the hot plate was turned off and the sample was allowed to passively cool to room temperature and measurements were made at 5°C intervals as it passively cooled.

Results

The results indicate that fluorescence intensity decreases with increasing temperature for all three tissue sample types. Figure 1.4 displays fluorescence curves at

selected temperatures for each tissue type. By 60°C the fluorescence peak has decreased by 50-64%, depending on the tissue type.

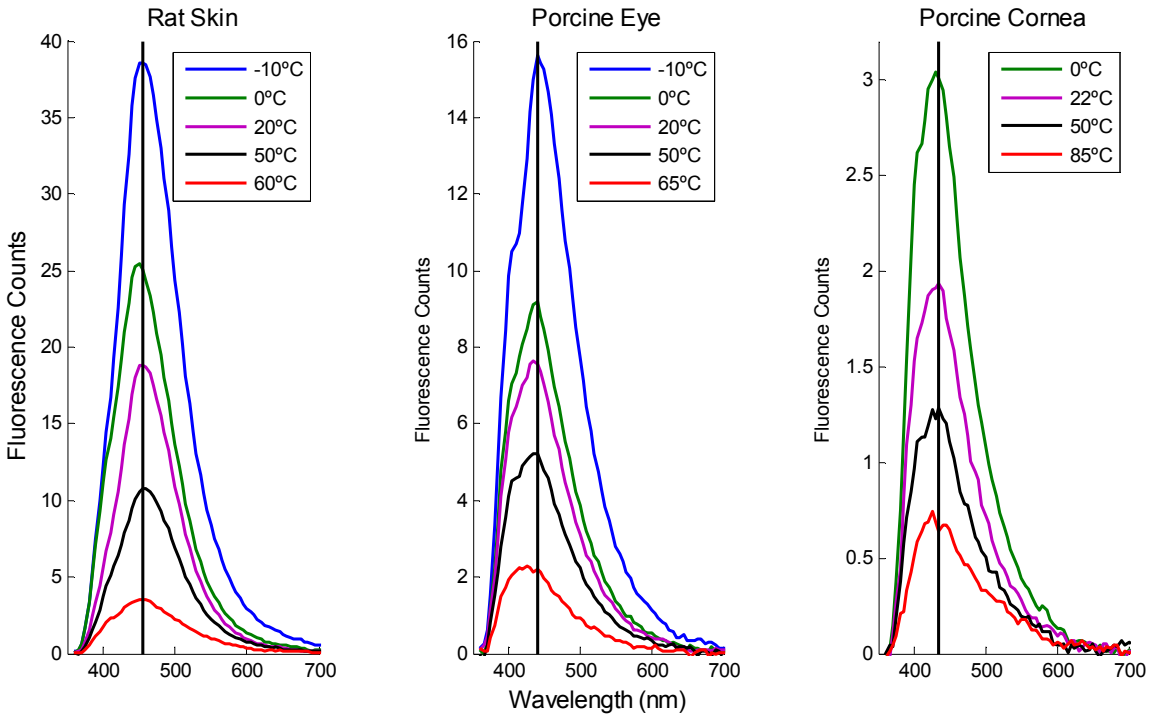


Figure 1.4 Fluorescence curves for all 3 tissue types at selected temperatures

Although all tissues showed a decrease in fluorescence intensity, peak location changes were specific to the porcine eyes and excised corneas. In the eyes and corneas the peak shifted to lower wavelengths around 60 ºC. Figure 1.4 shows the peak location of the three different tissue types. Since the cornea was the point of examination in both the intact eyes and the excised corneas, this peak shift is thought to be a result of protein denaturation in the cornea, which is comprised mostly of collagen.

The change in fluorescence peak intensity for the three different tissue types is shown in Figure 1.5. Fluorescence intensity trends were normalized such that the

fluorescence peak intensity at 0°C equals 1 for each sample. All samples showed a decrease in peak intensity with increasing temperature up to approximately 60°C. The variance seen around 0°C in Figure 1.5 is attributed to the conformational and optical property changes due to ice crystals and thawing effects. The excised cornea samples exhibited a secondary fluorescence intensity increase around 60°C. This abrupt change is due to protein denaturation, which results in a visible change in optical properties when the cornea becomes opaque. This effect is not seen in the porcine eyes since they are not heated to such a high temperature, but are expected to be similar to that of the excised cornea samples. However, this study was not able to confirm this, as our experimental methods were unable to effectively measure the porcine eyes above approximately 60°C. The rat skin samples are not expected to display this effect, as they represent a more complex combination of fluorophores in addition to collagen, but their behavior is not confirmed due to the same limitations experienced measuring the enucleated porcine eyes.

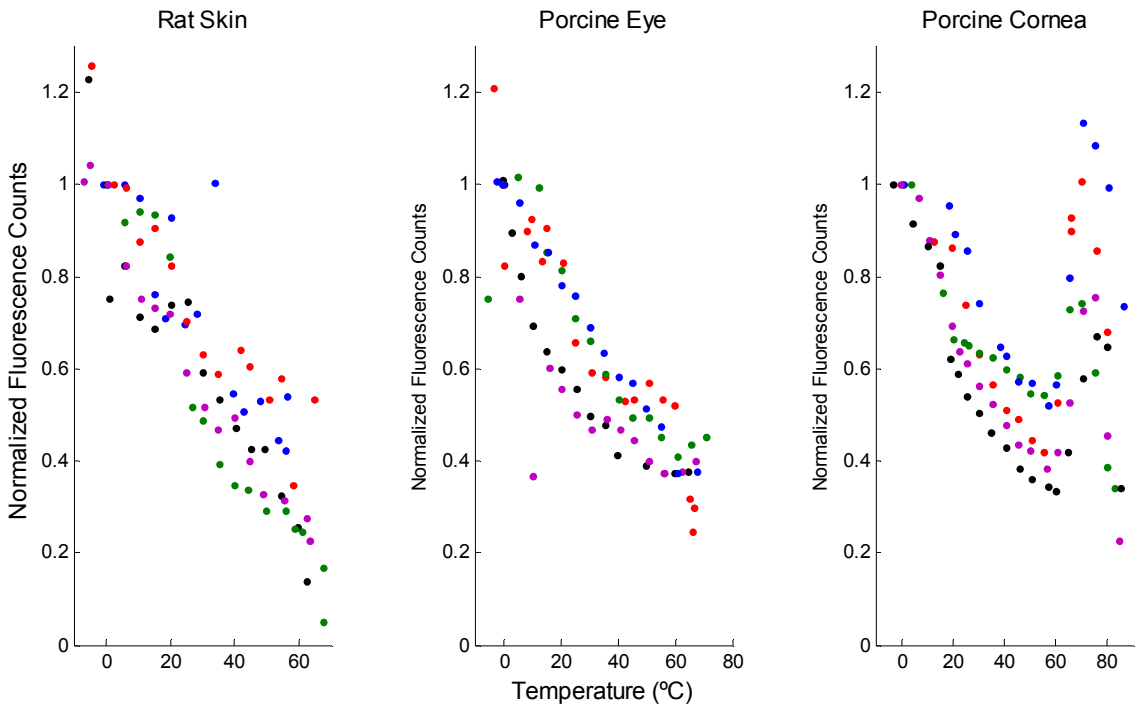


Figure 1.5 Relative fluorescence peak intensity as a function of temperature for every sample for all three tissue types. Each color represents a different sample.

The diffuse reflectance values at 625 nm were also examined to assess possible changes in optical properties. A wavelength of 625 nm was chosen as a point of interrogation due to the negligible effects of blood absorption. The scattering intensities at 625 nm as a function of temperature are presented in Figure 1.6 for all three tissue types.

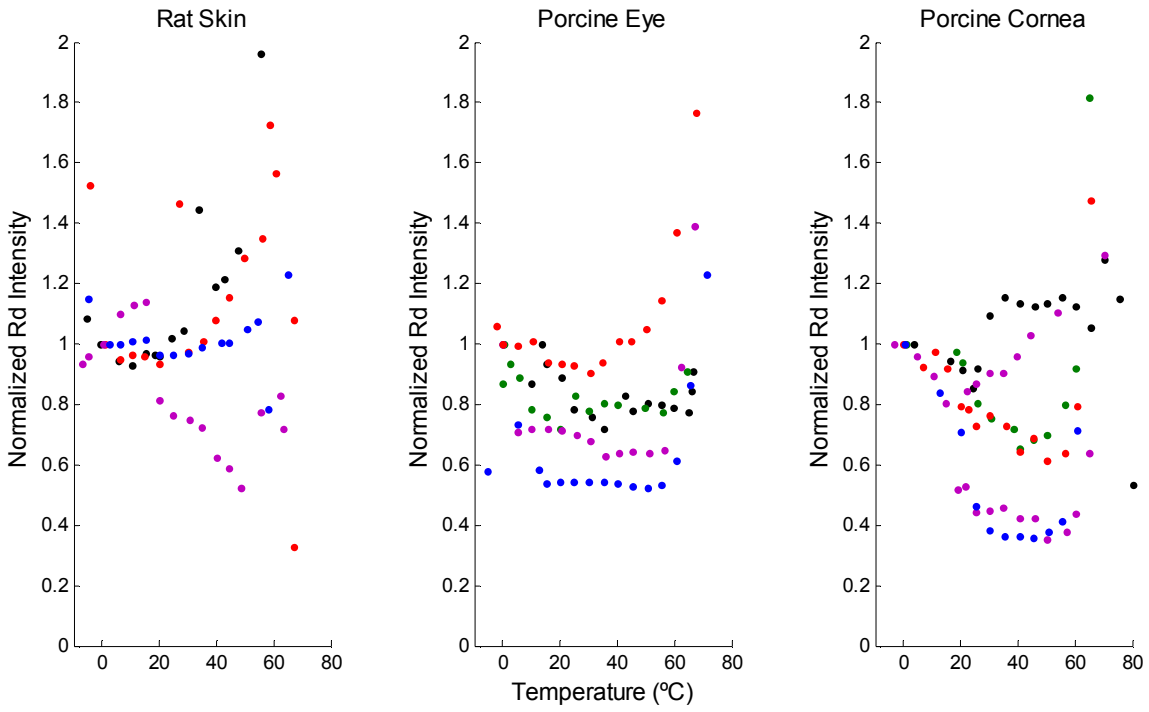


Figure 1.6 Reflectance values at 625 nm as a function of temperature for every sample for all three tissue types. Each color represents a different experimental sample.

The porcine eyes and porcine corneas show an expected increase in scattering around approximately 60°C. The skin shows less consistent data, but exhibits some increase in scattering with increased temperature. These data suggest that the increase in scattering could be decreasing the overall fluorescence intensity, especially at higher temperatures where coagulation occurs. Specifically, higher levels of scattering and absorption in tissue compete with fluorescence generation. Thus, tissue with large absorption and scattering coefficients will generate less fluorescence [16, 17].

The reversibility of the fluorescence loss was examined by allowing the temperature to passively cool back to room temperature while making spectroscopic measurements. The results of the reversibility measurements are presented in Figure 1.7.

50°C was guessed as a maximum temperature at which thermal damage would be completely reversible. In contrast, tissue in which we intended to produce irreversible thermal damage was heated to 70°C. An example of each is shown in Figure 1.7.

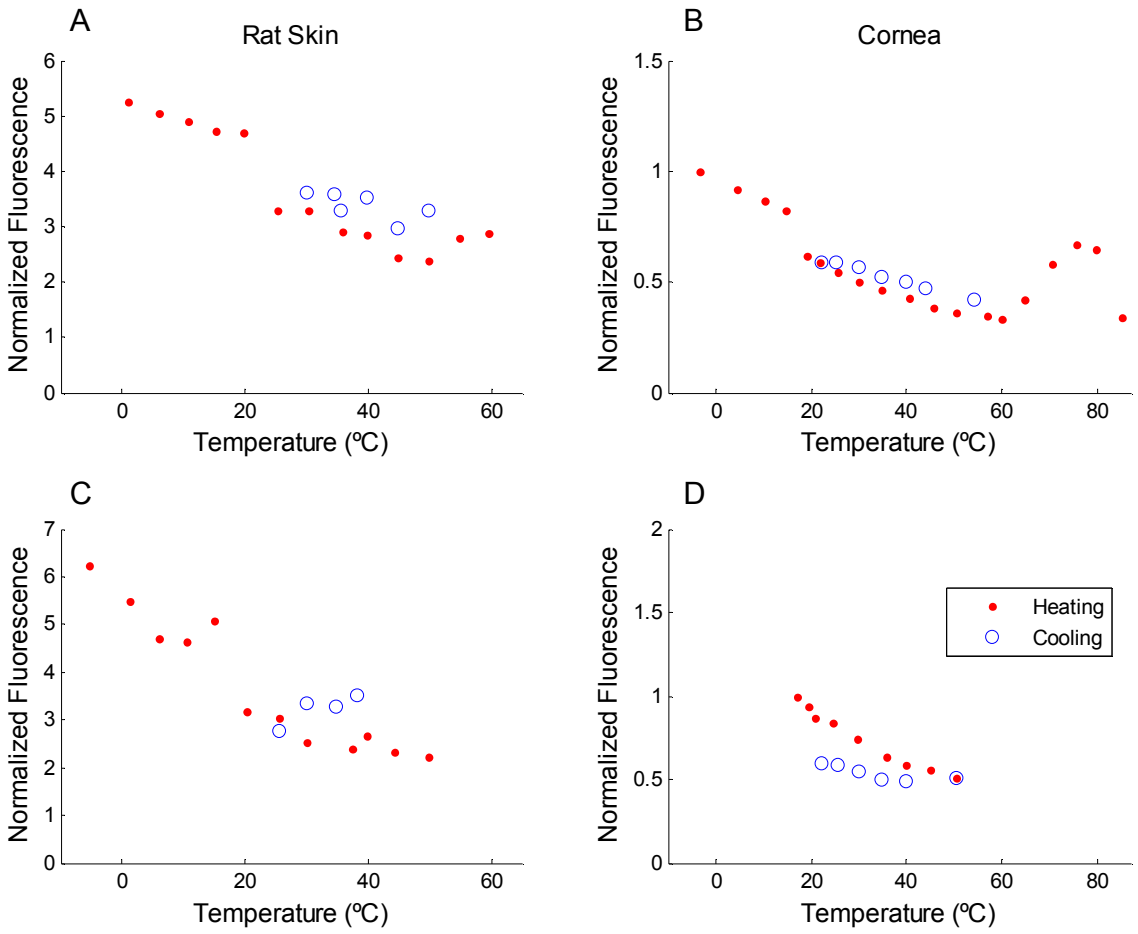


Figure 1.7 Reversibility of fluorescence loss for rat skin (A,C) and excised porcine cornea (B,D). A sample heated to greater than 60°C is shown for each tissue type (A,B), as well as a sample heated to approximately 50°C (C,D). The red dots represent the heating phase, and the blue circles represent the passive cooling phase.

The data in Figure 1.7 show that all the samples, regardless of maximum temperature or tissue type, showed some reversibility. The reversibility of the rat skin did

not show substantial differences between maximum temperatures of 60°C and less than 50°C. The rat skin samples showed mostly reversible fluorescence loss in both situations. The cornea behaved similarly to the rat skin. The cornea heated to 50°C and the cornea heated above 80°C both showed some level of reversibility. In this instance, the cornea heated above 80°C actually showed more reversibility than the sample heated to 50°C, contrary to our expectations.

Discussion

The expected decrease of fluorescence with increasing temperatures was observed in every animal tissue sample tested *in vitro*. In order to characterize these changes, peak location, scattering, and reversibility were also observed. The intent of these analyses was to determine what mechanisms might be responsible for the fluorescence increase. The examination of peak intensity served to confirm the inverse relationship between temperature and fluorescence in tissue. The examination of peak location as a function of temperature was necessary to see if any changes in the fluorophore or conformational changes were contributing to a change in fluorescence. The extraction of diffuse reflectance values was intended to yield information on scattering in the tissue, and thus implicate any changes in optical properties that may contribute to fluorescence changes. Finally, the reversibility was examined to see if permanent thermal damage was contributing to the decrease in measured fluorescence intensity, and to see if it was possible to create a completely reversible fluorescence decrease.

Although most of the results and analyses are straightforward, the use of diffuse reflectance to make inferences about optical properties requires further consideration. Diffuse reflectance can be used to determine optical properties, but is not a pure measure of optical properties. The diffuse reflectance spectra yield information about the scattering and absorption properties that are coupled. The mixture of these two optical properties is impossible to separate without making assumptions or using complex analytical methods. The raw reflectance information, however, can be useful if analyzed carefully. For instance, this study examined diffuse reflectance at 625nm, a region in which most of the absorption in tissue (due primarily to blood at 420 nm, 540 nm, and 580 nm) is not present. In the case of the cornea, where no blood is present, 625 nm was chosen to be consistent with the skin and enucleated eyes. Thus, we are able to assume that the scattering contributes to the majority of the information provided in that region of the diffuse reflectance spectrum.

In order to understand how optical properties affect fluorescence, optical properties must be understood at the excitation wavelength (337 nm) and emission wavelength of interest. The reflectance at 625 nm provides information about the overall scattering in the sample, but the exact modulation of fluorescence by optical properties would require information from both the peak location wavelength and excitation wavelength. Diffuse reflectance from the fluorescence peak location is available from the data in this study, but no data is available at the excitation wavelength due to the 385 nm longpass filter in the spectrometer.

The reversibility trends do not show the expected results. Our hypothesis was that the fluorescence loss would be irreversible beyond 50°C, or at least expected to be irreversible beyond 70°C. However, the discrepancy between expectations and results may be due to the fact that thermal effects in tissue rely on both temperature and time. Thermal damage in tissue has been described using an Arrhenius damage model. This model uses the Arrhenius integral to quantify thermal damage as a function of temperature and time. Using this method, thermal damage is described using the following equation:

$$\Omega(\tau) = A\int_0^{\tau} exp\left[\frac{-E_a}{RT(t)}\right] dt \quad (5)$$

Where Ω is the degree of thermal damage, A is a frequency factor (s^{-1}), τ is the total heating time (s), E_a is the activation energy barrier, R is the universal gas constant, and T is the absolute temperature (°K). Values for A and E_a must be empirically determined [18]. Since we heated the tissue in such a way that the temperature was always increasing, there was very little time spent at a constant temperature. Thus, temperature alone was examined as an independent variable in these experiments.

Conclusion

These experiments have shown that fluorescence intensity is inversely related to temperature in animal tissue *in vitro*. In the porcine cornea, whether in an intact eye or excised, a peak location change is seen due to increasing temperature, suggesting a change in the primary fluorophore conformation of these tissues. In this study, the

respective contributions of optical properties and fluorophore denaturation are not entirely clear, but they both contribute to the fluorescence decrease in some way as a function of temperature, prompting further investigation. The loss in fluorescence was at least partially reversible in all the tissue types examined up to maximum temperatures of 60-80°C. The fluorescence change in temperature ranges below denaturation temperatures in which the diffuse reflectance values are roughly constant suggest that other processes may be involved.

Hypothesis and Objectives

In order to more robustly understand the effects of temperature on fluorescence spectroscopy, this study will examine the effects of temperature on fluorescence and optical properties in human tissue both *in vitro* and *in vivo*. We hypothesize that the inverse relationship between temperature and fluorescence intensity can be shown in human tissue both *in vivo* and *in vitro.* We further hypothesize that the fluorescence loss due to temperature is not caused solely by a change in optical properties due to coagulation and dehydration.

The results of the preliminary study suggest that the fluorescence change due to temperature is a combination of optical properties, fluorophore degradation, and a non-radiative decay mechanism. In order to further investigate these possibilities, similar experiments will be performed in human tissue *in vitro,* and additional experiments will be performed in human tissue *in vivo*. The diffuse reflectance results of the previous study also suggest that optical properties are temperature dependent, but the optical

properties are not extracted from the reflectance curves. This study will calculate the absorption coefficient and reduced scattering coefficient from the reflectance curves to explicitly examine the temperature dependence of optical properties and how they affect fluorescence. Thus, there are 4 main objectives to this study. The first is to demonstrate the inverse relationship between fluorescence intensity and temperature *in vitro* in two different tissue types: skin and adipose tissue. The second objective is to show how the optical properties change as a function of temperature and how these correlate to the simultaneous changes in fluorescence. We also intend to examine the reversibility of the fluorescence loss by taking spectroscopic measurements in both heating and cooling stages. Variable maximum temperatures will also be used for the samples to see if a threshold for reversibility is present. Finally, we will see if the fluorescence changes observed *in vitro* can be reproduced *in vivo*.

The overall goal of this study is to understand how temperature affects fluorescence both *in vitro* and *in vivo*. Once understood, this phenomenon could aid in the use of fluorescence spectroscopy for guidance in thermotherapies such as radiofrequency and microwave ablation. In addition, this phenomenon could possibly be manipulated to yield better fluorescence signals if tissue temperature could be intentionally varied. From a scientific standpoint, this study will also increase our knowledge of the mechanism causing fluorescence changes due to temperature. Specifically, this study should determine if fluorescence changes due to temperature are more sensitive to thermal variation than coagulation induced optical property changes.

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CHAPTER II

VARIATION OF FLUORESCENCE WITH TEMPERATURE IN HUMAN TISSUE

Introduction

Fluorescence spectroscopy has been demonstrated in a variety of applications to discriminate diseased tissue and provide therapeutic guidance [1-4]. Despite its prevalence in biomedical research, the relationship between fluorescence and temperature in tissue is not explicitly defined. In many instances, the temperature at which fluorescence is measured in tissue is held constant, usually at body temperature *in vivo* or room temperature *in vitro*. In contrast, there are instances in which temperature varies greatly as a result of therapy or surgery, such as radio-frequency [5-7] or laser ablation [8-10]. A thorough understanding of the effect of temperature on fluorescence in tissue is required in order to consistently apply fluorescence techniques to these types of procedures.

It has long been known that fluorescence varies with temperature in a variety of substances. Bowen and Sahu showed that the fluorescence yield of some fluorescent solutions varied inversely with temperature [11]. The fluorescence dependence on temperature has been attributed to intersystem crossing, or temperature dependent internal conversion, a process that competes with fluorescence to return excited molecules back to their ground state [12]. Romano *et al.* used the temperature

dependence of fluorescence as a type of thermometry, using a fluorescent film to measure temperature to within 1°C. In addition, they found that the film required heating above 55°C immediately before being used as a temperature sensor. They explained that the initial, brittle state of the film was dominated by quenching similar to that seen in crystals, but after heating to 55°C, the film transitioned to a softer state in which viscosity quenching dominated. This resulted in a hysteretic fluorescence behavior in the film, with its characteristics changing after being heated to 55°C [13]. Additional studies have shown the inverse relationship between fluorescence intensity and temperature in non-biological samples [14-17]. In tissue, a linear decrease in fluorescence intensity with increasing temperature was found in retinal pigment epithelium, suggesting that fluorescence could be used to detect the interior temperature of the eye [18]. This relationship is sometimes described as a function of increased internal conversion and decreased fluorescence lifetime at higher temperatures [19-21].

The effects of temperature on fluorescence depend on the material being observed [22]. Although an inverse relationship between temperature and fluorescence intensity is most common [19], this is not always the case. For example, laser induced fluorescence in nitric oxide shows temperature dependence when quenched by H_2O, but not when quenched by O_2 , presumably due to differences in long range attractive and repulsive forces between the quencher and the fluorophore [23]. When fluorescence dependence on temperature exists, the cause is not always clear [24, 25]. Due to the different possible dependencies on temperature, each material must be individually examined. However, examining human tissue further complicates the process, as it represents a complex combination of many different molecules. Due to a variety of possible fluorescence

dependencies on temperature, the dependence of fluorescence on temperature in human tissue must be experimentally determined.

In addition to temperature dependent changes in fluorescence quantum yield or quenching, heating human tissue results in a change in optical properties, both reversible and irreversible. Optical properties heavily influence the spatial distribution of fluorescence excitation and emission in tissue. When a photon enters tissue, it can travel through the tissue unaffected, be absorbed, or undergo one or more scattering events. When a molecule absorbs a photon its electrons can be brought to an excited state and decay back to the ground state. The absorption and decay process can yield a photon (fluorescence) or may result in the conversion of potential energy into heat. If a photon is absorbed and followed by internal conversion, or scattered, it does not generate fluorescence. Thus, tissue with high absorption or scattering coefficients will generate less fluorescence [26, 27]. The optical properties themselves (μ_a, μ_s',n) are altered by changes in temperature [28, 29]. Fluorescence and diffuse reflectance spectroscopy has been shown to detect changes in optical properties in tissue due to thermal damage *in vitro* [28] and *in vivo* [29]. A recent study by Zaman *et al.* examined the fluorescence dependence on temperature in rhodamine dye, enucleated porcine eyes, *in vivo* hamster skin, and *in vivo* forearm skin up to 42°C. They found an inverse relationship between fluorescence intensity and temperature in all cases. These findings were explained as a degradation of the primary fluorophores and a disturbance of their natural states. Since their study only examined temperatures up to 42°C, their analysis did not consider optical property changes [30].

In a different study, we investigated the effects of temperature on fluorescence in animal tissue *in vitro*. Enucleated porcine eyes, porcine cornea, and excised rat skin were examined using fluorescence and diffuse reflectance over a temperature range of -20°C to over 85°C. Generally, the inverse relationship between fluorescence intensity and temperature was verified. Diffuse reflectance was also measured concurrently with fluorescence to assess changes in optical properties. It was found that the diffuse reflectance changed due to temperature variation, suggesting temperature dependent changes (reversible and irreversible) in tissue optical properties. We also found that fluorescence changed even when reflectance remained relatively constant. Also, the fluorescent lineshape changed due to temperature increase in tissues composed mostly of collagen, such as excised porcine cornea. The change in fluorescence was suggested to be a combination of fluorophore denaturation and some other temperature dependent decay mechanism competing with fluorescence [31].

A number of questions arose from this study concerning the mechanism of the change in fluorescence due to temperature. Specifically, this study did not directly examine the role of optical properties in the fluoresecence dependence on temperature. Rather, inferences were indirectly made about optical properties from reflectance data. The exact mechanism that mediates the change in fluorescence may include optical property changes, which can occur due to coagulation. Coagulation or thermal damage can also cause fluorophore degradation, possibly in the form of denaturation if the fluorophore is a protein. Collisional quenching or other non-radiative decay mechanisms are also possible. The combined presence of these mechanisms and their interaction in tissue at physiological temperatures is yet to be determined. The goal of this study is to

observe the effect of temperature on fluorescence and optical properties in human tissue. *In vitro* samples include human skin and adipose tissue. *In vivo* samples include human skin from the lateral forearm. We demonstrate the overall decrease of fluorescence intensity with temperature and examine optical property changes. *In vitro* temperatures range from -20°C to greater than 70°C in order to capture any trends over a broad range. *In vivo* experiments encompass a temperature range of 15°C to 40°C.

Materials and Methods

Instrumentation

A portable spectroscopic system was used to measure fluorescence and diffuse reflectance from *in vitro* and *in vivo* samples. This system has been described in previous publications [32-34]. The probe-based system uses a 337 nm nitrogen laser for fluorescence excitation (VSL 337, High Pressure Nitrogen Dye Laser; Oriel Corporation, Stratford, Connecticut) pulsed at 20 Hz and a white light source (150 W halogen lamp; Fiber Lite, Model 180; Edmund Industrial Optics, Barrington, New Jersey) for diffuse reflectance measurements. A fiber optic probe was used for light delivery and collection. The probe consists of seven 300 μm diameter, fibers in a six around one configuration. White light illumination was delivered by one fiber at the 6 o'clock position on the outer ring, and laser excitation was delivered by one fiber at the 12 o'clock position on the outer ring. The other five fibers were devoted to light collection. A 385 nm long-pass

filter (CVI Melles Griot, Albuquerque, NM) was placed in the spectrometer to filter out the excitation light of the laser. A thermal camera (FLIR A20, FLIR Systems Inc., Boston, Massachusetts) was used to measure the temperature of each sample, or the PBS in which it was submerged. A custom LabVIEW program was used to control the system and acquire both spectroscopic and thermal data during all experiments. A diagram of the experimental setup is given in Figure 2.1.

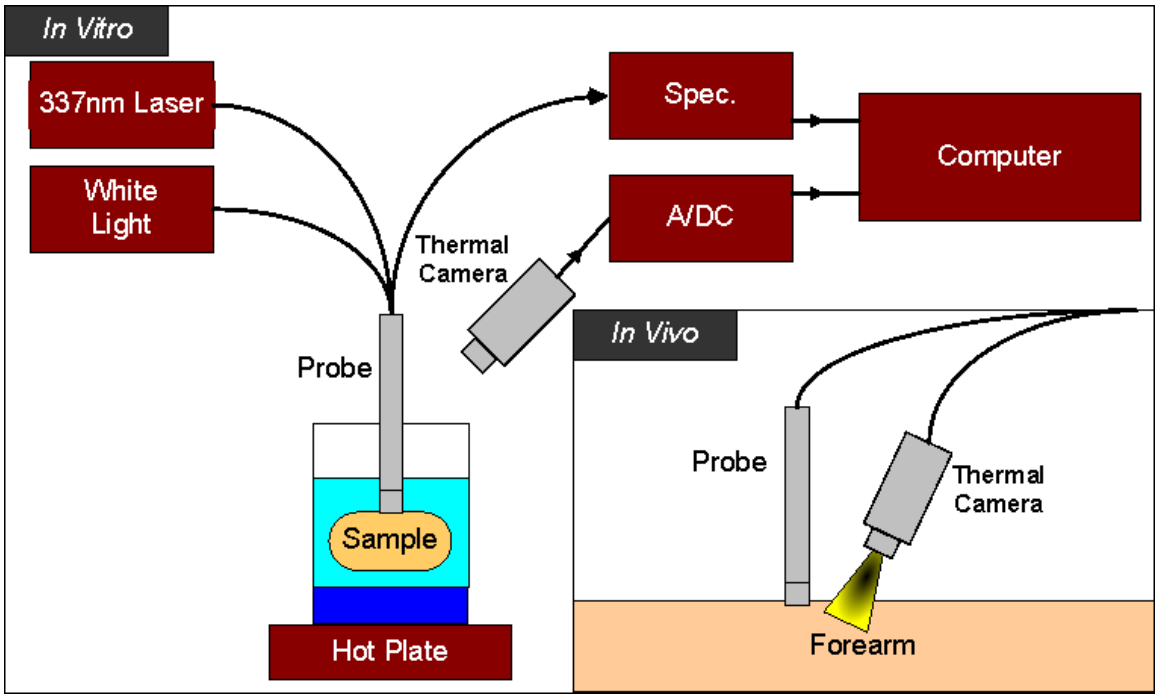

Figure 2.1 Setup for *in vitro* fluorescence and temperature experiments. A 337 nm N_2 laser is used for fluorescence excitation, and a white light source is used for reflectance illumination, each delivered by a single, separate fiber. Five collection fibers deliver the collected light to a spectrometer, which delivers the spectra to LabVIEW program on a laptop. The thermal camera delivers a temperature reading to an analog to digital converter (A/DC) which delivers the reading to the program on the computer. The sample is held in place in the beaker of PBS. The probe is locked in place for the duration of the experimental run.

In Vitro Methods

For the *in vitro* experiments, the spectroscopic system and thermal camera were positioned above the sample, which was placed on a sample holder. The thermal camera was positioned to measure the sample's temperature on a spot adjacent to the optical probe, as close to the probe as possible without encompassing the probe's image. The optical probe was held in place at a fixed point on the sample for the duration of the experiment. A total of 8 skin samples and 4 adipose tissue samples were measured.

Samples were obtained from the Cooperative Human Tissue Network (CHTN) with prior IRB (Internal Review Board) approval. Skin and adipose tissue samples were obtained from liposuction or breast-reduction surgeries. Immediately upon the samples' surgical excision, they were flash frozen in liquid nitrogen, transported to a -80°C freezer, and kept there until time for the experiments. Samples were removed from the -80°C freezer and placed in a custom probe holder above a room temperature phosphate buffered saline (PBS) solution. The probe was placed at the center of the sample's surface, and locked into place for the remainder of the experiment. Spectroscopic measurements were made as soon as the probe and thermal recording spot were set, usually beginning at about -20°C. At each measurement point, the temperature, a baseline spectrum, a reflectance spectrum, and a fluorescence spectrum were measured. Every 2.5°C, a measurement was taken. This process continued until the sample passively heated to the temperature of the PBS temperature below the sample. Additional PBS at room temperature was added until the sample was submerged. After submersion of the sample, the thermal camera was only able to provide temperature readings from the

surface of the PBS. Due to the small size of the samples, the samples were assumed to be at the same temperature as the PBS for the remainder of the experiment. Neither the probe nor the sample was moved in this process. The PBS solution containing the sample was then actively heated using a hot plate to a maximum temperature. Once the sample reached the target temperature, the hot plate was turned off and the sample was allowed to passively cool to room temperature while measurements were made at 5°C intervals.

In Vivo Methods

This *in vivo* study was approved and performed in accordance with the regulations of the Institutional Review Board of Vanderbilt University. Measurements were made on the lateral forearm of 7 different volunteers. Before any measurements were made, a circle was drawn with a felt tipped pen in order to obtain consistent measurements at a single spot. A cold pack was removed from a -20°C freezer and placed on the volunteer's lateral forearm and held there for 3 minutes. The volunteer then placed his or her forearm in position under the thermal camera and probe. The probe was placed at the marked spot and the thermal camera position was adjusted appropriately. As the skin passively warmed, spectroscopic and thermal measurements were made until the skin was at normal body temperature (approximately 33°C for forearm skin). Then, a heat pack was placed on the forearm and held in place for 3 minutes. This was removed, and the probe was repositioned at the marked spot. Spectroscopic and thermal measurements were made as the skin cooled back to an equilibrium temperature. Practically, this method allowed for the measurement of skin temperatures from 10°C up to 40°C.

Analysis

Spectral Processing

Each reflectance and fluorescence spectrum was background subtracted. Fluorescence spectra were corrected for the spectral response of the detection system using calibration factors determined using a National Institute of Standards and Technology (NIST) calibrated tungsten-ribbon filament lamp. Reflectance spectra were calibrated using factors obtained from the reflectance measurement of a known standard (Spectralon 20% Reflectance Standard, Labsphere, North Sutton, NH). The spectra were median filtered and binned in 5 nm intervals over the spectral range of 400-800nm.

Data Extraction

For the fluorescence spectra, peak height was examined as a function of temperature. For each individual sample *in vitro*, the fluorescent values were normalized such that the peak height at 0°C would be equal to one. For the *in vivo* experiments, the intensity value at 0°C was calculated using a linear fit of the data. Fluorescence emission peak position was also examined as a function of temperature. The reflectance data was used to assess the change in optical properties in the *in vitro* experiments. An inverse Monte Carlo program was used to calculate the optical property changes as a function of

temperature. This algorithm was adapted to our collection geometry from a program used by Palmer *et al.* [35, 36] to predict μ_a and μ_s'. The measured diffuse reflectance was used as input for the inverse Monte Carlo algorithm, and optical property measurements of similar samples from a spectrophotometer were used to produce initial guesses in order to ensure model convergence and a unique inverse solution. In order to quantify the fluorescence change, linear regression was performed on the fluorescence peak height data as a function of temperature. The *in vitro* regression was restricted to the temperature range of 15°C to 40°C. This temperature range was chosen to correspond to the *in vivo* data collected. The means of these slopes and their standard deviations were compared using a Mann-Whitney U-test to determine if the trends were significantly different.

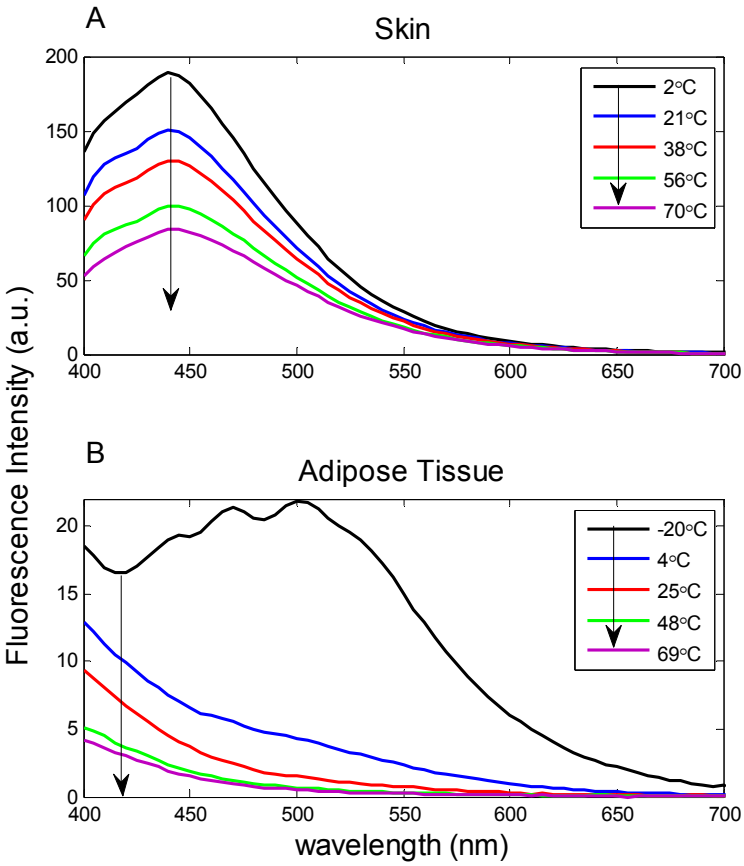

Figure 2.2 Representative fluorescence spectra as a function of temperature for the skin (A) and adipose tissue (B). All spectra shown are decreasing in intensity with increasing temperature.

Results

In Vitro

Figure 2.2 shows a decrease in fluorescence intensity with increasing temperature for both tissue types in this *in vitro* experiment. The fluorescence spectra in Figure 2.2 show no change in lineshape in the skin samples. In addition to the decrease in fluorescence intensity, the adipose fluorescence spectra exhibit a lineshape change as the

samples approach 0°C. As the adipose tissue samples approached 0°C, the fluorescent peak values around 500 nm disappeared. This fluorescent lineshape change was not seen in any of the skin samples.

In order to examine the relationship between optical properties and fluorescence, the reduced scattering and absorption coefficients, calculated using the inverse Monte Carlo simulations, are displayed with the fluorescence peak intensities as a function of temperature. The results of two skin samples (Figure 2.3) and two adipose tissue samples (Figure 2.4) are shown.

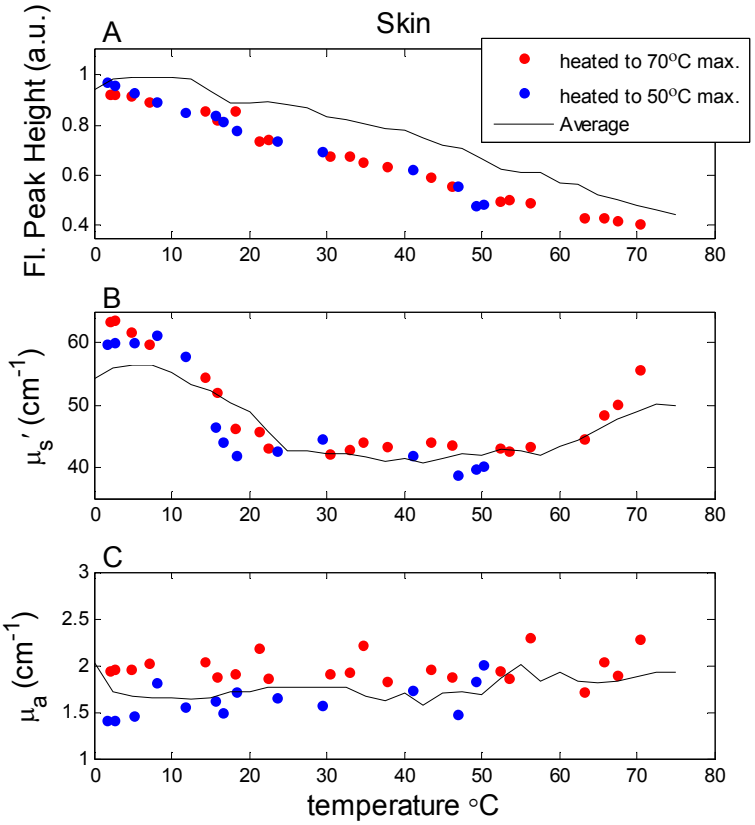

Figure 2.3 Fluorescence and optical property trends of two skin samples, one heated to a maximum of 70°C (red) and one heated to a maximum of 50°C (blue). The maximum fluorescence intensity (A), μ_s' (B), and μ_a (C) are displayed for each temperature. Averages of all samples (n=8) for the skin are shown by a solid line for each type of data.

Two representative skin samples and the averages of all the skin samples are displayed in Figure 2.3. They show a monotonic decrease in fluorescence intensity with increasing temperature. The reduced scattering coefficient in the skin tends to decrease initially starting at 0°C, and then increase at approximately 60°C. The absorption coefficient in the skin showed a slight increase due to temperature, but the change was on a much smaller scale than the reduced scattering coefficient changes.

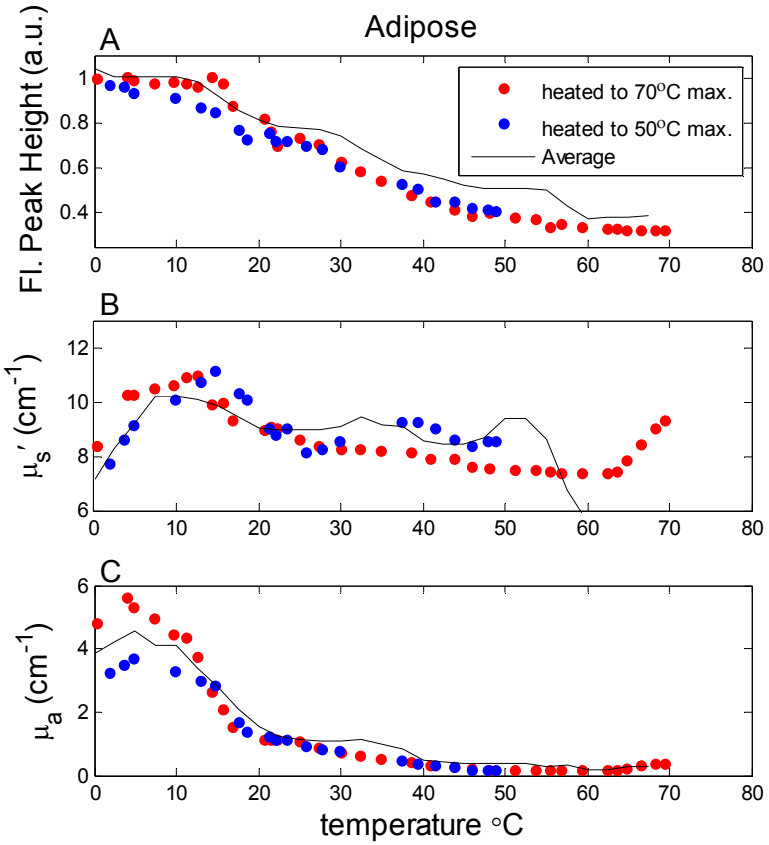

Figure 2.4 Fluorescence and optical property trends of two adipose tissue samples, one heated to a maximum of 70°C (red) and one heated to a maximum of 50°C (blue). The maximum fluorescence intensity (A), μ_s' (B), and μ_a (C) are displayed for each temperature. Averages of all samples (n=4) for the adipose tissue are shown by a solid line for each type of data.

Two representative adipose tissue samples and the averages of all the adipose samples are displayed in Figure 2.4. The adipose tissue samples show a consistent decrease in fluorescence intensity with increasing temperature. An initial increase in the reduced scattering coefficient is seen around 10°C, followed by a decrease up to around 60°C. The adipose samples heated to 70°C show an increase in the reduced scattering coefficient around 60°C. The absorption coefficient initially increased to approximately 5°C before steadily decreasing to approximately 40°C. The adipose tissue showed virtually no absorption at temperatures higher than 40°C.

All of the *in vitro* data display a decrease in maximum fluorescence intensity as a function of increasing temperature as shown in Figures 2.2-2.4. The fluorescence intensity versus temperature monotonically decreases over the range of 20-70°C. Some samples show a slight increase in fluorescence intensity over the temperature range of -20°C to 20°C. There is also no shift in emission peak location in the *in vitro* skin data.

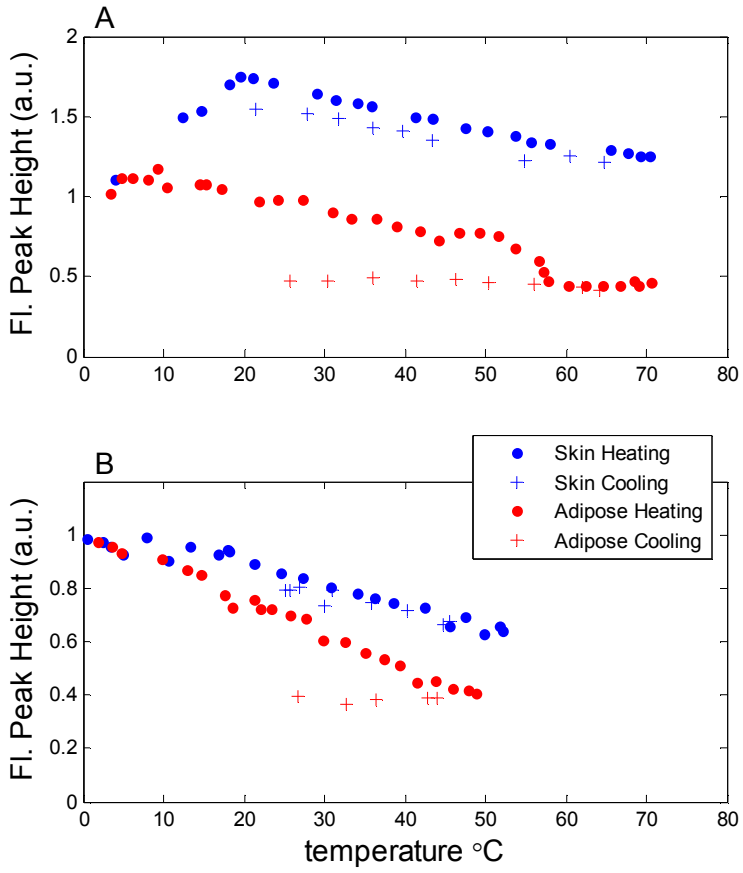

Figure 2.5 Reversibility trends for two skin samples and two adipose tissue samples. Each tissue type has a sample heated to a maximum of 70°C (A) and one heated to a maximum of 50°C (B). The heating phase (filled circles) and cooling phase (crosses) are displayed for each sample.

Representative heating and cooling fluorescence trends for the samples are displayed in Figure 2.5. Fluorescent peak heights of two skin samples heated to 50°C and

70°C, respectively, and two adipose tissue samples heated to 50°C and 70°C, respectively, are displayed. The skin samples heated to 70°C show a partially reversible trend upon cooling of the tissue. The skin samples heated to 50°C shows an almost completely reversible change in fluorescence intensity due to temperature increase. The adipose tissue shows irreversible fluorescence changes due to temperature for both maximum temperatures. The fluorescence intensity of the adipose tissue heated to 70°C reaches a minimum around 58°C, as seen in Figure 2.5, from which it does not recover.

In Vivo

The results of the experimental runs on 2 different volunteers are displayed in Figure 2.6. This figure represents passive heating to body temperature from a colder state, and passive cooling to body temperature from a heated state. The homeostatic, normal temperature for forearm skin was approximately 33°C for every volunteer before any thermal variation. The expected decrease in fluorescence with increasing temperature was seen for all 7 *in vivo* forearm samples. Because of practical limitations in the temperature range *in vivo*, there is less overall change in the fluorescence intensity compared to the *in vitro* results.

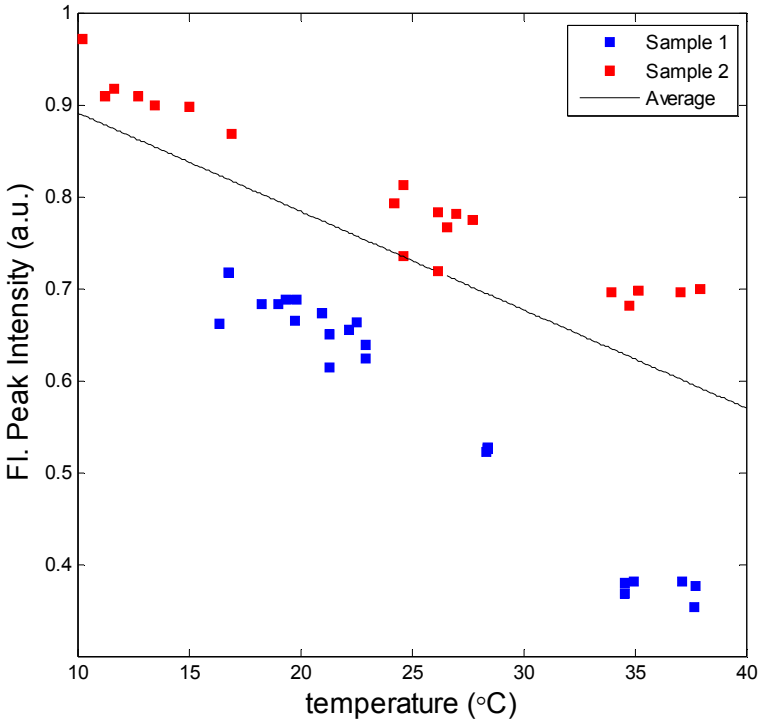

Figure 2.6 Two representative *in vivo* experimental runs are displayed, representing two different volunteers. Fluorescence peak height is plotted versus temperature for the forearm skin region. The average line obtained from the linear regression of all samples is also shown (solid line).

The fluorescence trends of the *in vitro* skin samples were compared to the fluorescence trends of the *in vivo* skin samples. For every sample, the slopes of the fluorescence decrease as a function of temperature were calculated between 15°C and 40°C using linear regression. This temperature range was chosen to represent the approximate range of *in vivo* temperatures examined. The average slope of the fluorescence versus temperature trend from 15°C to 40°C was slightly larger for *in vivo* skin than for *in vitro* skin. A Mann-Whitney U-test was performed on the slopes of the skin samples, and no significant difference was found ($p=0.15$) between the *in vitro* skin trends and the *in vivo* skin trends, suggesting that we successfully reproduced the *in vitro* fluorescence changes *in vivo*. The average slopes with standard deviations are presented

in Figure 2.7. The vertical axis represents the magnitude of the mean slope between 15°C and 40°C obtained from linear regression.

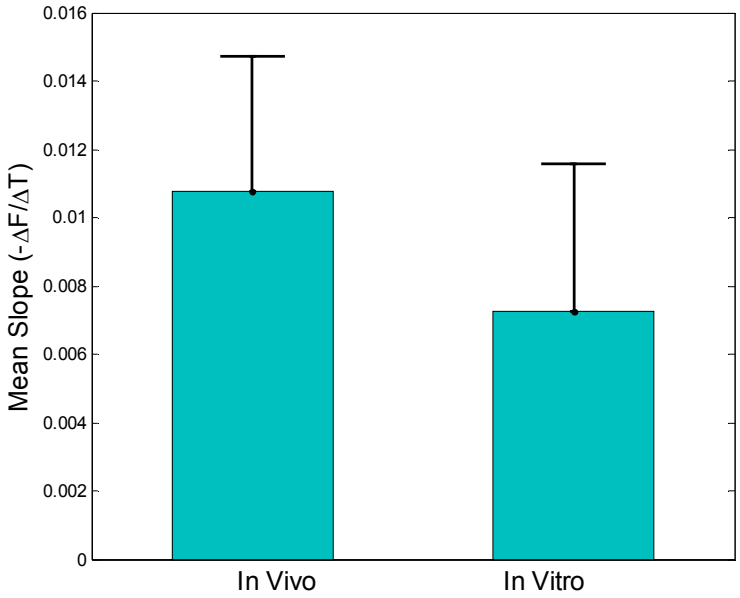

Figure 2.7 Mean slopes and standard deviations are displayed for the collective *in vitro* skin samples and the *in vivo* experimental runs. The y-axis represents the magnitude of the negative slope obtained from linear regression on all the samples in the appropriate category. The bars represent the mean slope magnitudes and the error bars represent 1 standard deviation. The difference is not statistically significant (p=0.15).

Discussion

The fluorescence intensity in all of the samples decreased with increasing temperature from 20°C to 70°C, but a variety of mechanisms could be causing this relationship. In previous studies, the fluorescence dependence on temperature has been attributed to intersystem crossing or temperature dependent internal conversion [12]. The relationship is sometimes described as a function of decreased fluorescence lifetime at higher temperatures [19-21]. In addition, optical properties are known to be temperature dependent and affect fluorescence. The effects of temperature on optical properties in

tissue have previously been examined in the near-infrared region. Optical properties in *ex vivo* human dermis and sub-dermis were found to be temperature dependent in the spectral region of 650 – 1000 nm. Specifically, the reduced scattering coefficient was found to increase with increased temperature from 25°C to 40°C for the dermis, and decrease for the sub-dermis. The absorption coefficient showed no significant dependence on temperature [37]. *In vivo* experiments on the human forearm have reported similar temperature dependencies for the reduced scattering coefficient at 590 nm. The absorption coefficient's dependence on temperature *in vivo* was not entirely clear, but it was suggested that changes in absorption were due to the pooling of blood in the skin's capillaries [38].

In Vitro

Although the reduced scattering coefficient *in vitro* is temperature dependent, it does not account for the fluorescence intensity decrease. For instance, the skin samples in Figure 2.3 show relatively constant reduced scattering coefficient values from 20°C to about 50°C, but the fluorescence intensity continues to decrease. The initial decrease in the reduced scattering coefficient in skin at lower temperatures is not entirely understood, but may be due to the thawing of the tissue. While the adipose tissue in Figure 2.4 shows reduced scattering coefficient values that are variable from 20°C to 50°C, they do not correspond to changes that would result in a fluorescence decrease. That is, a reduction in the reduced scattering coefficient should yield more fluorescence, but the opposite is seen in Figure 2.4. The only reduced scattering coefficient trend that could cause a

decrease in fluorescence is seen above 60°C in both tissue types, when scattering increases, presumably due to coagulation. The regions from 20°C to 50°C, despite the relative low variability in optical properties, still exhibit a fluorescence decrease consistent with the overall fluorescence trend. This suggests that while the reduced scattering coefficient may be contributing to the fluorescence decrease, the mechanism is a combination of optical property changes and fluorescence effects sensitive to temperature.

The absorption coefficient *in vitro* does not account for the fluorescence intensity increase in the skin or the adipose tissue. Although the absorption coefficient shows temperature dependence in the adipose tissue, it does not correspond to a decrease in fluorescence intensity. The absorption coefficient decreases with increasing temperature in the adipose tissue while the fluorescence intensity also decreases. Similar to the reduced scattering coefficient trends in the adipose tissue, if the absorption coefficient was causing the fluorescence decrease, an increase in the absorption coefficient would be expected, but the opposite is seen in Figure 2.4. The absorption coefficient in the skin samples shown in Figure 2.3 shows a slight trend with temperature, but this trend is on a much smaller scale than the reduced scattering coefficient changes. Since the primary absorber in tissue is blood [26], the small absorption coefficient values for our *in vitro* tissue samples previously rinsed with saline are expected. In addition, the placement of the samples in PBS further removed blood from the sample as time in the PBS increased. Thus, the slight increase in the absorption coefficient in skin is likely not physically relevant, and does not contribute to any fluorescence changes.

The reversibility trends in Figure 2.5 yield information about fluorophore degradation due to temperature. The major endogenous fluorophores in biological tissue at 337 nm excitation are collagen, elastin, NADH (reduced nicotinamide dinucleotide), NADPH (reduced nictinamide dinucleotide phosphate), and lipids [4]. It is important to note, however, that some changes in optical properties and fluorophore degradation are reversible. For instance, while coagulation causes an irreversible change in optical properties, collagen conformational changes can be both reversible and irreversible, depending on the temperature, heating rate, and type of collagen [20]. In Figure 2.5, the adipose tissue fluorescence seems to degrade in an irreversible fashion, while the skin samples' show more reversible fluorescence changes.

Based on the reversibility trends seen in Figure 2.5, it appears that the fluorophores degrade in skin at temperatures above 50°C. It also appears that the fluorescent content of adipose tissue breaks down at or below 50°C, much lower than that of skin. Previous work has attributed optical property changes in lipids to phase changes occurring up to 45°C, as they transition to a liquid state [37]. Our previous work showed that *in vitro* animal tissue heated to a maximum of 50°C showed reversible changes in fluorescence, while maximum temperatures closer to 60°C produced more irreversible changes [31]. Thus, maximum temperatures of 50°C and 70°C were chosen for this study, even though the exact threshold for reversibility is unknown. The results seen in Figure 2.5 are generally expected, but the exact contribution of the degradation of the fluorophores to the fluorescence decrease is not entirely clear. The irreversibility of fluorescence loss past a certain threshold suggests that the breakdown of fluorophores is a contributing factor to the decrease of fluorescence due to temperature.

In Vivo

The *in vivo* data show an overall decrease in fluorescence due to temperature as can be seen in Figure 2.6. These results show that the decrease in fluorescence due to increased temperature *in vitro* can be reproduced *in vivo* over a physiologically relevant temperature range. Since no permanent tissue damage was done in the *in vivo* study due to the physiological temperatures used, it can be inferred that the loss in fluorescence is completely reversible over this temperature range. The temperature range of the *in vivo* portion precludes coagulation as possible mechanisms for the fluorescence decrease. The reversibility and absence of permanent damage also limit the possible causes of the fluorescence decrease. If fluorophore breakdown or optical property changes are causing the fluorescence change, they are doing so in a completely reversible manner. Other factors, such as a non-radiative decay mechanism, may be responsible for changes in fluorescence *in vivo*.

In general, the *in vivo* fluorescence trends show greater inter-sample and intra-sample variability than the *in vitro* experiments. A variety of factors may contribute to the increased variability *in vivo.* Primarily, it is more difficult to accurately control temperature *in vivo* than *in vitro*. For the *in vitro* samples, the exact PBS solution temperature could be actively controlled while making spectroscopic and thermal measurements. For the *in vivo* experiments, instrumental requirements restricted the spectroscopic and thermal measurements to be made during the passive heating and cooling phases, when the thermal pack was not covering the interrogation spot. The human body actively controls temperature, while our method of thermal variation was

passive. Thus, the window of passive heating and cooling passed very quickly and was not easily controlled. Despite these challenges, the fluorescence decrease due to increased temperature was reproduced in all 7 volunteers *in vivo.*

Overall

Although the average slopes for the *in vitro* skin samples and the *in vivo* skin samples differ slightly, this difference is not statistically significant in this study ($p=0.15$). The non-parametric Mann-Whitney U-test was used on the skin *in vitro* ($n=8$) and the *in vivo* forearm skin ($n=7$). This suggests that although a variety of factors influence the change in fluorescence, these factors are mostly similar *in vitro* and *in vivo* over the small temperature range surrounding normal body temperature. The results of the *in vivo* experiments suggest that the fluorescence of human tissue can be manipulated using temperature *in vivo* without causing any damage to the tissue. A larger sample size of both *in vivo* and *in vitro* samples would allow for a more powerful statistical test if these trends are similar.

All of these experiments relied on the relationship between temperature and fluorescence in tissue, but the time maintained at a certain temperature is also a variable influencing tissue's response to heat. Specifically, thermal damage in tissue depends exponentially on temperature and linearly on time [28, 39]. Achieving a consistent heating rate is especially difficult if samples vary in size, as they did in this study. Despite variable heating rates and optical property changes, the fluorescence intensity change was consistent for all samples due to temperature changes.

Other mechanisms should also be considered when observing fluorescence and temperature relationships. These experiments suggest that the fluorescence decrease due to temperature is not a function of optical property changes and coagulation over the physiological temperature range. The presence of a temperature dependent non-radiative decay mechanism seems plausible in the *in vivo* experiments, since the fluorescence decrease occurs without any permanent changes to the tissue. Even the *in vitro* samples show fluorescence decreases in the physiological temperature range similar to those *in vivo*. Therefore, collisional quenching is a likely non-radiative decay mechanism that is temperature dependent. Collisional quenching would contribute to the fluorescence decrease with increased temperature by causing more molecules to quench the excited fluorophores. This results in the return of the molecule to its ground state before the radiative, fluorescent decay can occur, resulting in a shorter fluorescence lifetime and lower fluorescence emission. In biological tissue, the most prevalent quencher is oxygen [40]. Further work including time-resolved fluorescence spectroscopy would be necessary to demonstrate the presence of collisional quenching.

These results suggest that fluorescence may be an accurate indicator of tissue temperature, with little dependence on optical properties in the physiological temperature range. A fluorescence mechanism that is more sensitive to temperature than a change in optical properties would have significant implications. The understanding of such a mechanism would enable fluorescence to be used as an accurate indicator of tissue temperature. In addition to being able to distinguish between coagulated tissue and native tissue, fluorescence could be used to indicate relative tissue temperature, the state of the fluorophore, or thermally-mediated cell death [28, 29]. The ability to detect cell death

prior to coagulation could reduce the time needed for thermal ablative therapies and reduce the unnecessary vaporization of tissue.

As fluorescence is increasingly used for guidance and diagnosis in medical applications, the effect of temperature on such techniques must be considered. Thermal ablation is a frequently used surgical technique that involves drastic changes in temperature. If fluorescence is used in conjunction with this type of technique, the effects of temperature must be taken into account. In addition, those using fluorescence in laboratory and animal research must also consider the effects of temperature if there is any thermal variability present. For example, a research study attempting to reproduce results from an animal model in a human subject should consider any differences in body temperature, and carefully maintain body temperature in anesthetized small rodents. Studies attempting to reproduce *in vitro* fluorescence results *in vivo* should also consider the change from room temperature to body temperature. Further investigation of the precise mechanism influencing the effects of temperature on fluorescence could yield both a deeper scientific understanding of light-tissue interaction and practical applications to photonics in medicine.

Conclusions

The results of the *in vitro* experiments show that fluorescence decreases with increasing temperature in human tissue. The change in fluorescence due to temperature is partly reversible and partly irreversible, depending on the maximum temperature reached in the tissue and tissue type. The *in vivo* results confirm that reversible changes in

fluorescence can be obtained in human tissue using temperature variation over a small range surrounding body temperature. The inverse relationship between fluorescence intensity and temperature is due to a combination of factors. Based on the changes in fluorescence when the reduced scattering coefficient is mostly unchanged, it appears that optical properties have minimal influence over fluorescence changes due to temperature in the physiological temperature range. The results of this study suggest that fluorescence may be more sensitive to temperature changes than optical properties, and could be utilized to measure tissue temperature.

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CHAPTER III

SUMMARY AND FUTURE DIRECTIONS

Summary

This study has shown that the inverse relationship between fluorescence intensity and temperature is present in human tissue both *in vivo* and *in vitro*. Combined with the preliminary study described in Chapter I, the exact dependence of fluorescence on temperature depends on the tissue type (skin, adipose, cornea, eye), but exhibits an overall inverse relationship with temperature over the temperature range of 20°C to 70°C.

This study shows strong evidence that optical properties are not the only mechanism, evidenced by fluorescence changes in thermal regions where there is little or no optical property variation. This study also shows fluorescence changes at temperatures well below coagulation or fluorophore degradation, implicating an additional mechanism. One possible mechanism includes collisional quenching due to the presence of oxygen or other quenchers. Further work would be needed to prove this.

The *in vivo* portion of this study has shown that the fluorescence decrease due to temperature recorded *in vitro* can be reproduced *in vivo* over a smaller temperature range. This shows that fluorescence can be varied using thermal manipulation of *in vivo* tissue

without causing coagulation or damage. It also shows that the fluorescence decrease due to heat is completely reversible in *in vivo* human tissue over a small temperature range. The statistical test used in Chapter II demonstrated that there was no significant difference between the decrease in fluorescence *in vitro* and *in vivo* over the temperature range of 15°C to 40°C for the samples in this study. In order to conclude that the trends are fundamentally similar, larger numbers of both *in vitro* and *in vivo* experiments would need to be performed in order to apply a more powerful, parametric statistical test.

Future Directions

In order to confirm or refute the presence of collisional quenching as a mechanism of fluorescence decrease, specific experiments targeting this phenomenon would need to be conducted. The only way to prove or disprove the presence of collisional quenching is to perform time-resolved fluorescence measurements. Since collisional quenching works by decreasing the fluorescent lifetime and thus decreasing the overall fluorescence [1], a time-resolved method would detect this occurrence if it is present. In addition, the samples could be placed in a non-polar solvent instead of water, saline, or PBS. Polar solvents are known to increase the occurrence of quenching. Since oxygen is the most ubiquitous known quencher, a solvent that contained no oxygen could also be used in further experiments to see if the same amount of fluorescence decrease occurs.

Although the data showed clear trends in most cases, the spectroscopic system has an inherent variability in fluorescence excitation that could be improved. This is due to the lack of synchronization between the laser pulses (20 Hz) and the acquisition of the spectrometer. This is usually remedied by taking three or more measurements at a time and averaging them to obtain a single spectrum. This method of averaging is mostly effective in *in vitro* situations, but in *in vivo* situations where the induced temperature returns to body temperature very quickly, there is not enough time to take three measurements for every desired data point. Previous work with this system (unpublished) has shown that proper timing between the laser pulses and the spectrometer can decrease the fluorescence intensity variance by as much as an order of magnitude. Making these instrumental corrections would greatly increase the confidence and consistency of any future *in vivo* experiments.

In order to extract optical properties, an inverse Monte Carlo algorithm was used. This method required diffuse reflectance spectra as input and predicted the reduced scattering coefficient and absorption coefficient over the available spectral range. The available spectral range for the system we used was approximately 400-800 nm. Wavelengths below 400 nm begin to undergo powerful attenuation due to the presence of a 385 nm longpass filter placed in the spectrometer. This filter is intended to filter out the laser excitation light for fluorescence measurements, but it also filters the diffuse reflectance light since it is inside the spectrometer. In order to determine exactly how optical properties modulate fluorescence, optical properties must be known at both the excitation wavelength (337 nm) and the emission wavelength (400-800 nm). Our current study only extracts information about optical properties from 400-800 nm, but the

behavior at the excitation wavelength is unknown. If a different method of filtering was used that filtered the excitation laser for fluorescence, but not during diffuse-reflectance, a complete picture of the optical properties affecting fluorescence could be obtained.

Larger numbers of samples for both the *in vitro* and *in vivo* portions are needed to form any definite conclusions about the similarity between *in vivo* and *in vitro* experimental runs. With less than 10 samples in each group of skin examined, a non-parametric test was required to test for significance. Larger sample numbers would not only increase confidence about reproducibility, but allow for more powerful statistical tests to be performed.

Finally, a major hindrance to the *in vivo* experiments was the thermal influence of probe contact on the skin. The thermal properties of the probe are different from those in the skin, and contact between the two causes the temperature of the skin being interrogated to return to body temperature more rapidly than the tissue surrounding it. The method of temperature measurement further augments this problem, as the thermal camera interrogates a spot directly adjacent to, but not underneath, the probe. Thus, the skin being spectroscopically interrogated may be at a different temperature than the skin being thermally interrogated. The best solution to this problem would be to employ a non-contact method of spectroscopic measurement. A fluorescence imaging system could be coupled to the thermal camera in much the same way as the spectroscopic system used in this study. This approach would not cause any undesirable temperature changes due to probe contact.

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