

**LUMINESCENCE LIFETIME INSTRUMENTATION
DEVELOPMENT FOR MULTI-DYE ANALYSIS**

A Senior Scholars Thesis

by

ADAM HARBI SHADFAN

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biomedical Engineering

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Approved by:

Research Advisor:
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ABSTRACT

Luminescence Lifetime Instrumentation Development for Multi-Dye Analysis.
(April 2011)

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An efficient and accurate luminescence based instrument capable of determining differences in lifetime decay emissions of multiple dyes by using a new multi-luminophore lifetime calculation method was developed for eventual use in dual sensing applications. Current methods for monitoring multiple luminescent dyes, such as dual lifetime determination (DLD), do not accurately calculate actual lifetimes. In this work, a mathematical model of the system was produced by using two different computer programs in order to simulate variables and to develop an efficient and accurate method for simultaneously calculating the lifetimes. The calculation method was based on a new correction algorithm recently developed in the research group. Using these models and optical hardware, an instrument was created to be driven by a personal computer equipped with custom LabVIEW software, which also analyzed the recorded data. During testing, the system was able to accurately calculate the lifetimes of two distinct luminophores. It was determined that this system is advantageous over current multi-dye analysis techniques by providing accurate and computationally-efficient calculations

with the potential of implementing low-cost materials in the future. This system could eventually be implemented for many dual-sensing applications, where two parameters must be monitored at once. For example, patients suffering from diabetes could use a non-invasive monitor based on this system to detect varying tissue oxygen levels to compensate for enzymatic glucose sensor response.

ACKNOWLEDGMENTS

This work could not have been completed without the assistance of Dr. Michael J. McShane of the BioMINDS laboratory in the Biomedical Engineering Department at Texas A&M University. Specifically, work could not have been performed without the help of Bradley Collier, a research assistant of the BioMINDS laboratory. The BioMINDS lab as well as Honors & Undergraduate Research funded this work.

NOMENCLATURE

TD	Time-Domain
FD	Frequency-Domain
NLR	Non-Linear Least Squares Regression
RLD	Rapid Lifetime Determination
DLD	Dual Lifetime Determination
MLD	Multi-Luminophore Lifetime Determination
I_0	Initial Luminescence Intensity
Γ	Radiative Emission
k_{nr}	Non-Radiative Emission
Φ_ω	Phase
m_ω	Modulation
ω	Angular Frequency
Q	Quencher Concentration
F_0	Luminescence Intensity in Absence of Quencher
F_0	Luminescence Intensity in Presence of Quencher
K	Stern-Volmer Quenching Constant
k_q	Bimolecular Quenching Constant
τ_0	Unquenched Lifetime
τ	Lifetime in Single Dye System
I	Time-Dependent Intensity

I_0	Initial Intensity
t	Time
W_1	The Integral of Values Within Window 1
W_2	The Integral of Values Within Window 2
W_3	The Integral of Values Within Window 3
W_4	The Integral of Values Within Window 4
t_1	Start Time of Window 1
t_2	Start Time of Window 2
t_3	Start Time of Window 3
t_4	Start Time of Window 4
τ_1	Shorter Lifetime in Two Dye System
τ_2	Longer Lifetime in Two Dye System
LED	Light Emitting Diode
PMT	Photomultiplier Tube
DAQ	Data Acquisition Board
SD	Standard Deviation
95% CI	95% Confidence Interval
SNR	Signal-to-Noise Ratio

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CHAPTER I

INTRODUCTION

This work follows the development of a luminescence instrument capable of determining difference in lifetime emissions of multiple dyes by using a new multi-luminophore lifetime calculation method for eventual use in dual sensing applications. With this system, concentrations of several chemical analytes could be observed simultaneously. For example, patients suffering from diabetes could use a non-invasive monitor based on this system to detect varying tissue oxygen levels with a luminescence sensor to compensate the response of a second enzymatic glucose sensor.

Background

Monitoring glucose levels is crucial for people suffering from diabetes. According to the 2011 National Diabetes Fact Sheet released by the Centers for Disease Control and Prevention, Diabetes affects nearly 25 million Americans, with 7 million undiagnosed individuals [1]. The fundamental issue involves how the body metabolizes glucose. For type I diabetes, the pancreas does not produce enough insulin to facilitate glucose storage. Type II is where the body produces insulin, but the cells do not use it correctly. Both types can potentially lead to many complications including blindness and kidney failure if blood glucose levels are not maintained at a healthy level [1]. Traditional

This thesis follows the style of Analytical Biochemistry.

monitoring methods utilize a lancet to draw blood onto a test strip that is read by a meter. This method is painful, invasive, and can become quite expensive after taking several measurements a day. Dr. Michael J. McShane's BioMINDS laboratory in the Biomedical Engineering department of Texas A&M University has been developing a method for *in vivo* glucose monitoring by using luminescence based instrumentation to non-invasively observe luminescent changes due to enzymatic oxygen consumption using microscale glucose sensors, known as "smart tattoos" [2]. There is concern, however, that varying ambient oxygen within an implanted tissue site could cause erroneous glucose readings [3]. For this reason, a second luminescent sensor is needed to monitor ambient oxygen levels in order to effectively compensate glucose sensor response.

Operating principles

Over the last several decades, luminescence spectroscopy has become a standard for optical based biomedical measurements [4,5,6]. For example, enzymatic glucose sensors can be implanted within tissue and interrogated by light through several luminescence means [2]. Fundamentally, luminescence measurements involve observing the light emitted from an electronically excited luminescent material [7]. Luminescence is divided into two sub-categories: fluorescence and phosphorescence. In fluorescence, light is

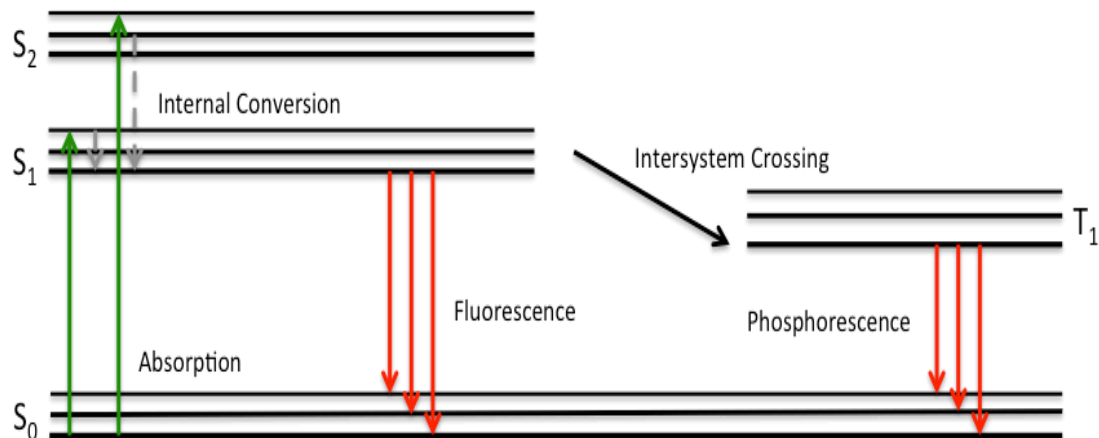


Fig 1: Jablonski Diagram. This diagram depicts luminescence excitation and emission: The ground-state and first and second singlet states are represented by S₀, S₁, and S₂, respectively, and the triplet state T₁.

emitted from the electrons returning from the singlet-excited state after absorption. This refers to the rapid emission of energy due to the return to the ground-state orbital by an electron that temporarily occupied the excited state orbital and is paired with an opposite spin to a ground-state electron (Fig. 1). Emission rates, as a result, are very fast ($\sim 10^8 \text{ s}^{-1}$), leading to a fluorescence lifetime (τ), or the amount of time a fluorophore remains in the excited state following excitation, of approximately 10 ns [7]. Phosphorescent light is emitted from the triplet-excited states, where the spin of the electron in the excited state is the same as the spin of the paired ground-state electron. The alignment of the same spin orientation occurs during intersystem crossing, and impedes transition back to the ground state, resulting in slower emission rates (milliseconds to seconds) and longer

lifetimes. In all cases, the electron can return to the ground state without releasing light, as non-radiative decay [7].

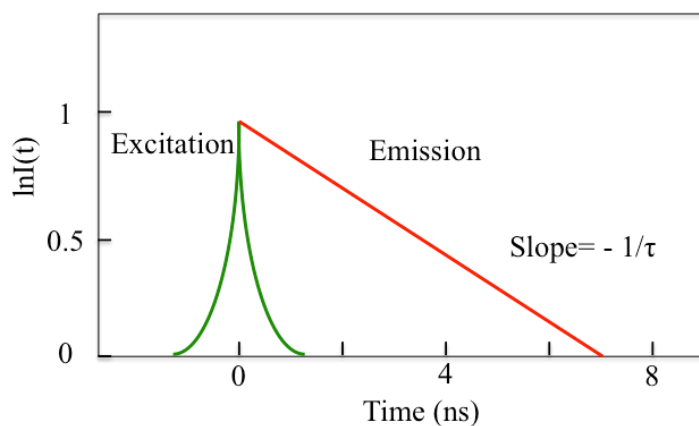


Fig 2: Time domain lifetime diagram. The green excitation pulse induces red emission. In this example, a dye lifetime of $\tau = 7\text{ns}$ was modeled.

Luminescence is a useful measurand because it is affected by solvent interactions, quenching and energy transfers [7]. These variables are helpful in monitoring chemical reactions and changes in concentration. Luminescence can be analyzed by observing the number of photons emitted (intensity), the energy distribution (spectra), the temporal distribution (lifetime) or the orientation (anisotropy) [4]. Measuring methods are often divided into two categories: steady-state and time-resolved [7]. Steady-state, or intensity-based measurements rely on constant excitation and observance of emission, without requiring expensive equipment. However, the measurement is only an average of time resolved measurements over the intensity decay of the sample – resulting in a

loss of much of the molecular information. Time resolved or lifetime based measurements, rely on calculations of the intensity decay over time [7]. The equipment is more complex and expensive but is not prone to the same errors as intensity based measurements [3-6]. Properties of a dye's environment (e.g. concentrations of a chemical that interacts with the dye) can be quantitatively analyzed by monitoring the lifetime of the dye. Lifetime analysis is often measured using time-domain or frequency-domain methods [7].

Time-domain lifetime measurements

In time-domain (TD) measurements, a short pulse of light is used to excite a luminophore sample [8]. Following excitation, the decay of the sample is then measured over a period of time by counting the number of photons. The single exponential time decay rate is defined by the following equation:

$$I(t) = I_0 e^{-t/\tau}$$

where I_0 is the initial intensity and τ is the lifetime, such that:

$$\tau = (\Gamma + k_{nr})^{-1}$$

where Γ and k_{nr} represent the radiative and non-radiative emission, respectively [8]. The lifetime can be calculated from the slope of a plot of $\log I(t)$ versus t (Fig 2), but more commonly by fitting the data to assumed decay models (finding the time at which the intensity decreases to I/e of the intensity at $t = 0$).

Frequency-domain lifetime measurements

Frequency domain (FD) lifetime measurements rely on excitation by an intensity-modulated (typically a sine wave) light source. Depending on the lifetime of the sample, the phase, Φ_ω , will shift and modulation, m_ω , will change (Fig 3). The modulation is defined as: $m = \frac{B/A}{b/a}$, where a is the DC component and b is the amplitude of the excitation signal, and A is the DC component and B is the amplitude of the emitted signal [9]. In the FD approach, Φ_ω and m_ω are measured. By assuming a single exponential decay, the lifetime of the system can be calculated with either the phase shift or modulation:

$$\tan \Phi_\omega = \omega\tau \text{ and } m_\omega = (1 + \omega^2\tau^2)^{-1/2}$$

where ω is the angular frequency [9]. These measurements do not require detection as rapid as TD methods, but most have equipment capable of discerning the change in Φ_ω and m_ω . FD sampling of multiple probes involves measuring and comparing phase shifts of the probes after excitation of multiple frequencies [9].

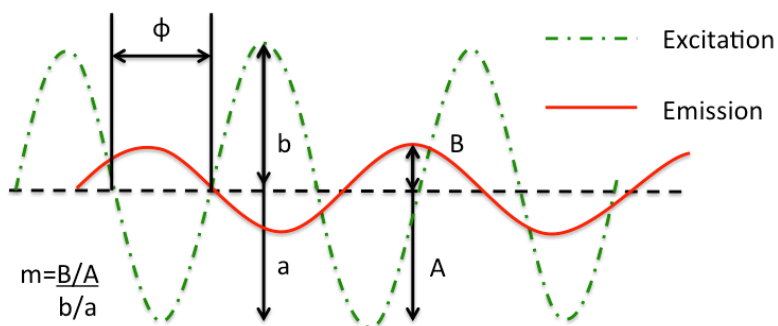


Fig 3: Frequency-Domain Lifetime Diagram. Lifetime calculations are performed by measuring Φ_ω , m_ω , or both.

Lifetime analysis

Lifetime analysis allows for observation and quantitative comparisons of the interactions of solvent molecules with luminophores, the rotational diffusion of biomolecules, the distances between sites on biomolecules, conformational changes, and binding interactions [7]. The majority of applications utilize luminescence lifetime analysis to quantify changes in concentrations. Luminophores can be sensitive to molecules or atoms, resulting in a change in how fast the electrons return to the ground state and subsequently, luminescence lifetime. Quenching, the change in intensity due to the deactivation of the excited-state luminophore in solution through non-radiative transitions to the ground state, is a useful tool for measuring concentration changes [7]. A quencher is the molecule or atom causing the deactivation, allowing the concentration to be determined. O_2 , I^- , Cs^+ and acrylamide are commonly used quenchers [6]. The

Stern-Volmer equation is used to normalize the exponential decay changes in lifetime due to the changing concentration values:

$$\frac{F_0}{F} = 1 + K[Q] = q + k_q \tau_0 [Q]$$

where F_0 and F are the fluorescence intensities observed in the absence and presence, respectively, of the quencher, K is the Stern-Volmer quenching constant, k_q is the bimolecular quenching constant, τ_0 is the unquenched lifetime, and $[Q]$ is the quencher concentration [7]. Utilizing the linear relationship between lifetime and concentration, the amount of quencher can be determined at different points in time [7]. Viscosity, pH, temperature, and polarity are other examples of variables that be accounted for via lifetime analysis [9].

Dual sensing application

In the BioMINDS lab, phosphorescent dyes sensitive to molecular oxygen dyes are co-immobilized with glucose oxidase in microparticles with mass transport limiting nanofilms [2]. As oxygen levels increase, the properties of the dye change, reducing the luminescence intensity. Glucose oxidase consumes glucose and oxygen that diffuses into the microsphere sensors as a result of the properties of the nanofilms, depleting oxygen levels at a comparable rate to glucose oxidation, and increasing the intensity and lifetime of the dye [2]. Local oxygen levels in the tissue around the implanted sensors, however, could vary, skewing the enzymatic measurements of the sensors [3]. For this reason, it is necessary to compensate by measuring the varying local oxygen levels with another

probe. Unfortunately, the luminescence probes available often have overlapping spectra, which can cause difficulties when measuring multiple dyes simultaneously with intensity based measurements [10-15]. By instead measuring the lifetimes of the different probes with the same absorbance spectra, quantitative comparisons can be made using an appropriate method [15]. To reiterate, luminescence lifetime is not as prone to instrumentation drift or calculation errors, and has been used to monitor multiple dyes [15, 16]. Several research groups have attempted to monitor multiple fluorescent probes at once utilizing either FD or TD methods [15-23]. However, many of these methods use expensive equipment and do not calculate the actual lifetime rapidly.

In general, low cost TD instrumentation utilizes a LED for excitation, optical filters, fiber optics, and a detector, often in the form of a photomultiplier tube (PMT) [15,16,19]. For determining the lifetime of multiple probes, TD methods require the lifetimes to be distinct in order to allow a shorter-lived probe to fully decay and not affect the measurement of the longer-lived one [15,16]. To calculate the two lifetimes, measurements must be taken at two different times: immediately after excitation, and following the decay of the shorter-lived probe. [15]. Lifetimes calculated in this fashion represent a fairly accurate calculation of the different lifetimes, which can then be used with the Stern-Volmer equation to monitor changes in glucose and oxygen simultaneously [15]. FD measurements utilize similar equipment, often with the aid of a

lock-in amplifier to perform phase and modulation measurements [16-23]. The change in amplitude and phase of each signal can then be used to calculate the lifetimes.

The problem

Several systems capable of making multiple lifetime measurements have been proposed using traditional lifetime techniques, but none have been able to calculate the actual lifetimes. The closest method does not calculate the shorter-lived lifetime rapidly, but uses a rough estimate that varies proportionally to the actual lifetime [15]. A non-linear least squares regression (NLR) method avoids the miscalculation, but is more computationally complex, taking too long for rapid measurements [24]. To get around this, a new method must be created and implemented to accurately and quickly calculate lifetimes.

CHAPTER II

METHODS

In order to prove the concept of a rapid, accurate dual lifetime determination method, a computer-based model to mathematically emulate the setup and calculations was created. A second software program was created to control the hardware and analyze the data. Two oxygen sensitive probes with greater than 10x difference in maximum lifetime were used to test the system. Modeling was performed with MATLAB (Mathworks, Natick, MA) and LabVIEW (National Instruments, Austin, TX). MATLAB modeling provided proof of concept by utilizing and testing several different methods with control over many variables, while the custom LabVIEW program was implemented to perform calculations and control hardware.

MATLAB Simulations

The MATLAB model can be broken into three main parts – generating a square wave signal, simulating a sample with decay properties, and back calculation of the lifetime (see Fig 4.). The square wave was generated by calling a function to produce a signal similar to a train of pulses. An offset was introduced to ensure that a negative voltage would not be used. The model allows for simple adjusting of the frequency, amplitude, and offset of the square wave. For simplicity, these parameters along with the amount of

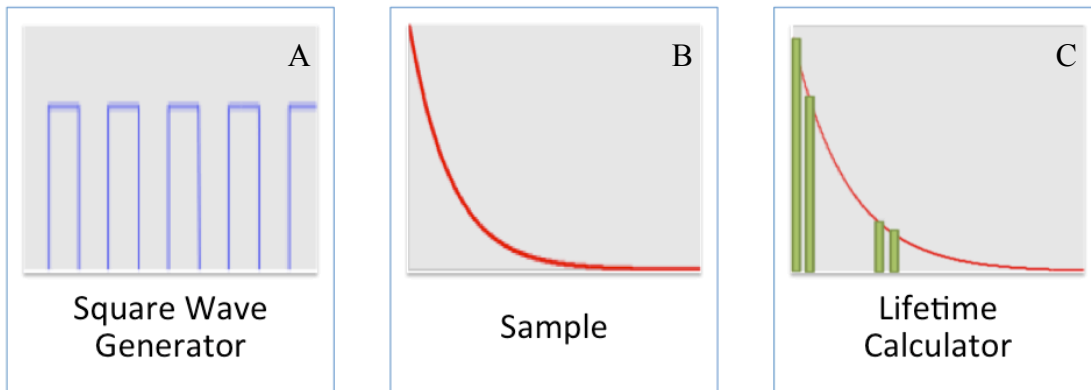


Fig 4: Chart of Modeling Goal. a) A square wave is generated to excite the sample. b) The sample represents the luminescence lifetime decay expected of an actual sample. c) Two lifetimes are back calculated with the lifetime calculator.

time that the signal is produced are based on the lifetime (τ) of the sample, which is entered as an input at the start of the program. The sampling rate, which is the rate at which samples are taken, is also adjustable in order to insure enough values are acquired to accurately calculate the lifetime without oversampling. Under or over-sampling increases computer processor overhead, potentially slowing the program. Under-sampling will lead to low signal-to-noise ratio and inaccurate lifetime calculations. Initial testing was performed with a single decay rate in order to simplify the model. Original testing values included a square wave of 2 kilohertz frequency (Fig 5.) and lifetimes of 70 and 500 μ s were acquired with a nanosecond sampling rate. The length of the test allows for five pulses at an arbitrary constant intensity.

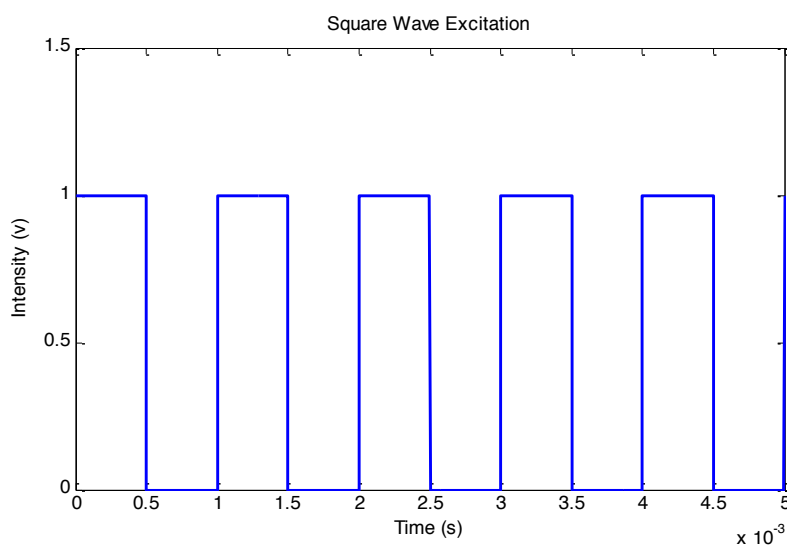


Fig 5: MATLAB Generated Square Wave.

The sample

To simulate the sample, a well-defined intensity decay equation was used:

$$I(t) = I_0 \exp\left(-\frac{t}{\tau}\right)$$

where I is the time dependent intensity, I_0 is initial intensity, t is time, and τ is lifetime.

Known as the impulse response function, this equation describes the decay of a single luminophore probe after excitation (Fig. 6).

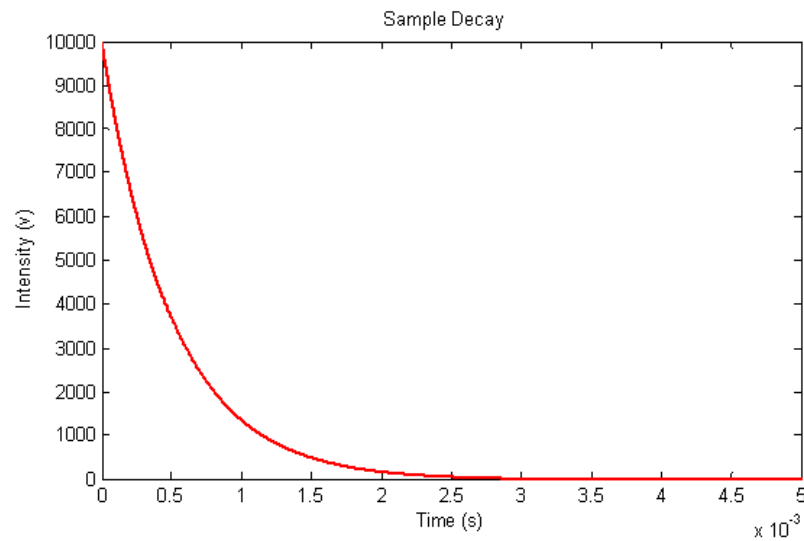


Fig 6: Example of MATLAB Rendering the Sample Decay.

In order to simulate the time dependent intensity response, the convolution of the excitation square function and impulse response function is performed. For dual lifetime samples, two impulse response functions are added together either before or after convolution with the square wave (Fig. 7).

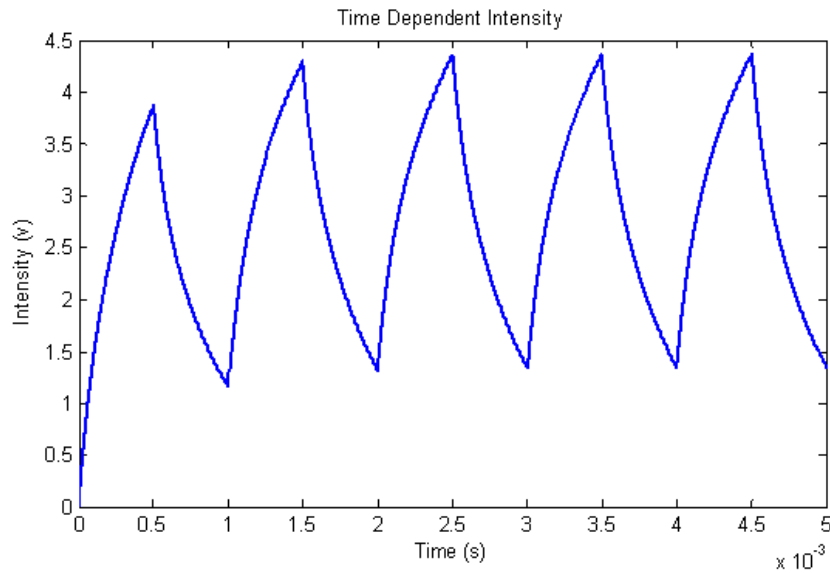


Fig 7: MATLAB Chart of Time Dependent Intensity.

Back calculation

Before τ could be calculated, a single decay had to be separated from the train of pulses. This was accomplished by creating a loop to index the different decays after the end of excitation. To calculate a single lifetime using a windowing technique, the decay signal must be broken up into at least two windows. The previously mentioned loop creates the two windows to be used for the different calculation methods. In Fig. 8, the two windows are represented as W_1 and W_2 with t_1 and t_2 as the starting times of each, respectively. Each lifetime calculating method repeats the calculation for each excitation pulse, and eventually the five calculations are averaged.

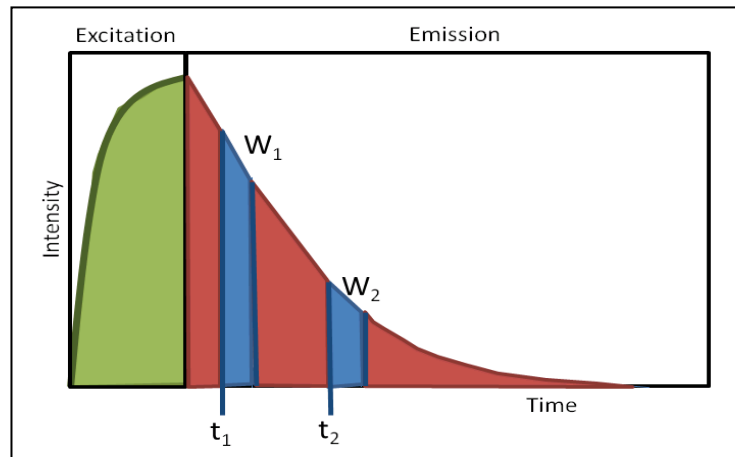


Fig 8: Single Decay Profile with Excitation. W_1 and W_2 are the integral of the signal in the windows 1 and 2, respectively. t_1 and t_2 are the start times for each window.

Non-linear least-squares fitting

Using a built in non-linear least-squares fitting function (NLR), the lifetime of two overlapping decay signals can be calculated provided that additional accurate information is supplied. Specifically, the function calls for the equation it is supposed to fit and an initial guess for values of the variables. With known lifetimes, as utilized in this model, the guesses were very accurate. However, with unknown values, the function is not as precise [24]. Furthermore, this function is relatively computationally complex, requiring more time and power than simpler functions. For these reasons, this fitting function is used only as a comparison to other methods; however, it did allow for testing of the effects of changing variables such as lifetime, sampling rate, window width and square wave characteristics.

Rapid lifetime determination and dual lifetime determination

Rapid Lifetime Determination (RLD) is a method that uses less intensive functions to accurately calculate a single lifetime with two windows. RLD relies on the equation

$$\tau = \frac{t_2 - t_1}{\ln \frac{W_1}{W_2}}$$

where τ is lifetime, W_1 and W_2 are the integrals of the values within windows 1 and 2, respectively, while t_1 and t_2 are the start times of the windows. Dual Lifetime

Determination (DLD) utilizes two iterations of RLD to calculate the lifetime of a shorter and longer-lived decay. This requires the use of four distinct windows, with the first set of windows (W_1 and W_2) consisting of both decays, and the last set (W_3 and W_4) only containing the longer-lived τ_2 (Fig. 9). The last two windows must be taken long enough after the end of excitation to allow the shorter-lived lifetime (τ_1) signal to decay such that the signal can be assumed to be zero before data is collected. While DLD can accurately calculate the longer-lived probe, the method does not produce a good representation of τ_1 because the calculation does not compensate for the fact that the first two windows contain both τ_1 and τ_2 decays. DLD requires the values of the two lifetimes to be different enough to allow the shorter lifetime to decay completely before W_3 is started. This method remains in use because this calculated τ_1 value still changes proportionally to the actual τ_1 [16]. The DLD method was utilized in the MATLAB model to compare with other methods. DLD modeling allowed for testing of the effects of changing variables such as lifetime, sampling rate, window width and square wave characteristics.

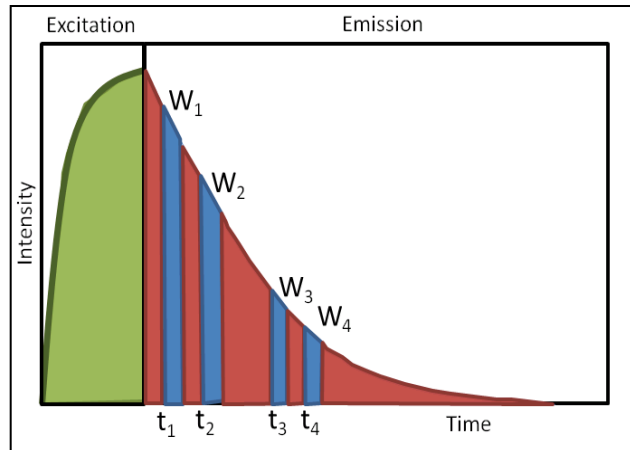


Fig 9: Time Decay Profile with Four Windows (W_1 - W_4).

Multi-luminophore lifetime determination

Starting with the original equation of the impulse response function, a modified DLD method was developed by a colleague to produce a more accurate representation of τ_l (results not shown). Simply put, the modified method, known as multi-luminophore lifetime determination (MLD), uses the same RLD equation to calculate τ_2 from the final two windows and then uses that value to account for and remove the τ_2 component in the first two windows – resulting in window integrals representative of the τ_l decay. The equation is:

$$\tau_1 = \frac{t_2 - t_1}{\ln \frac{W_{1,\tau_1}}{W_{2,\tau_1}}},$$

where W_{1,τ_1} is corrected (no τ_2 component) window 1:

$$W_{1,\tau_1} = W_1 - W_4 e^{\frac{t_4 - t_1}{\tau_2}}$$

and W_{2,τ_1} is the corrected window 2:

$$W_{2,\tau_1} = W_2 - W_4 e^{\frac{t_4-t_2}{\tau_2}} .$$

As with DLD however, the lifetimes must be different enough to allow the shorter lifetime to decay completely before window 3 is started. Window width values must be equal for RLD, DLD, and MLD calculations. The MLD method was implemented into the model as well, as a less computationally complex and more accurate method for back calculating two lifetimes. As with DLD and fitting, Modified DLD modeling allowed for testing of the effects of changing variables such as lifetime, sampling rate, window width and square wave characteristics.

Instrumentation development

LabVIEW software was used for taking measurements and analyzing the data because it can be easily implemented with hardware. The software was also created by separating into three major components: square wave generation, acquisition of decay signal from the sample, and calculation of the lifetime. Again, this separation allows for simple addition of hardware. All programming was created to utilize a National Instruments Data Acquisition (DAQ) Board (NI USB-6259) controlled by a personal computer via USB to interact with the hardware. A block diagram of the system can be seen in Fig 10.

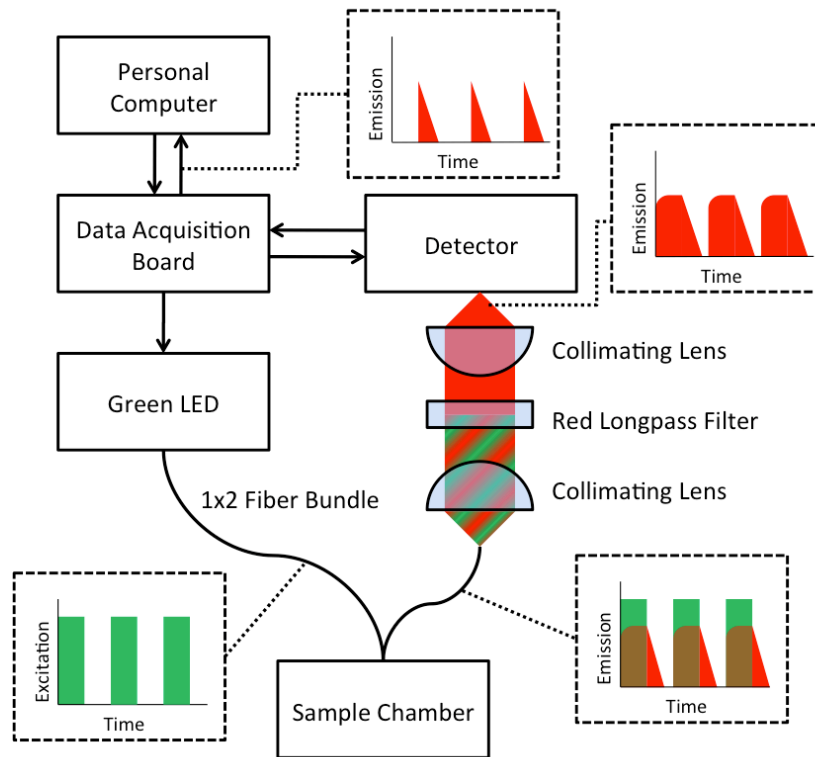


Fig 10. Block Diagram of the System.

Square wave generator

To minimize computational overhead, the DAQ board controlled the square wave independently using an internal clock and parameters sent through the PC. The signal, with a controllable frequency and duty cycle alternates between 5 and 0 volts. The voltage drives the green LED (IF-E93 Industrial Fiber Optics, Inc) to pulse at the user's discretion.

Sample

For the physical sample, two luminescence probes sensitive to oxygen were used. These probes, or dyes, were platinum octaethyl porphyrin (PtOEP) and palladium (II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PdTFPP). When oxygen has been purged from the dyes, the lifetimes of the dyes are approximately 90 μs for PtOEP and 1000 μs for PdTFPP. Each dye was dried onto a glass slide individually, and combined, for three testing slides.

Optical configuration

The pulsed light travels from the LED through an optical fiber bundle to the sample. A testing chamber allowed for a gas to be exposed to the sample while the fiber bundle allows excitation of the sample (pulsed LED) and acquisition of the luminescence decay. Three levels of oxygen were used to test the system, 0%, 21% (atmospheric level), and 100%, by using compressed nitrogen, air, and oxygen, respectively. The fiber optic passes the light through a series lenses. The first lens, a LA1951-A, collimates the light to a filter. The red long pass filter removes the excitation wavelengths, allowing only the longer wavelength luminescence from the sample to pass. A second collimating lens (LA1951-A) focuses the light onto the PMT detector (H8249e Hamamatsu). The PMT was powered by a power supply and a user defined control voltage that increases detection and signal noise. The outputted voltage of the PMT, which is proportional to

the amount of light it receives, was sent to the DAQ board to be interpreted by the software. A timing mechanism was used to acquire signals at the fastest possible sampling rate determined by the DAQ board specifications (1.25MHz).

Lifetime calculation

Unlike the MATLAB modeling, only the MLD method was used for the LabVIEW software. This was done to save on computing overhead, and because MATLAB results showed MLD to be a superior calculator of lifetimes compared to DLD. Before excitation begins, the PMT acquires data to account for background noise seen by the system. These dark reads were subtracted from the acquired decay signals. The decay was divided by four user chosen windows that integrated the signal for use by the MLD method to calculate the lifetimes. The software recorded the two lifetimes (τ_1 , τ_2) for each excitation pulse of the sample. Lifetime values were calculated for each dye independently and combined using the three sample slides previously described. The lifetimes and decay data were recorded. Following system testing, MATLAB was then used to perform non-linear least squares regression on the data to find the lifetimes as a comparison to the MLD calculations. Five iterations of the lifetime calculations were used to find a standard deviation and a 95% confidence interval. The signal-to-noise ratio (SNR) was also calculated.

CHAPTER III

RESULTS

The outputs of the MATLAB model were used to set the initial parameters for the LabVIEW software. The lifetimes of two combined decay were outputted by the MATLAB for five iterations for three calculation methods. The LabVIEW outputted lifetimes for each sample alone and combined at three different oxygen levels. Five iterations were performed and a second MATLAB function was used to find the regression values as a comparison. A 95% confidence interval was the basis of significance of the comparisons.

MATLAB simulation results

The program was designed to output two average lifetime values for each of the three calculation methods: non-linear least squares regression (NLR), DLD, and MLD. With predefined lifetimes of 70 and 500 microseconds, which were chosen based on previous studies, calculated lifetime values were calculated and are listed in Table 1 [16].

Table 1. Output of MATLAB. Dual lifetime produced by the three calculation methods, 5 Iterations. Parentheses after each lifetime refers to the percent difference between the determined lifetime and the actual.

	Actual (μs)	NLR (μs)	NLR St. Dev. (μs)	DLD (μs)	DLD St. Dev. (μs)	MLD (μs)	MLD St. Dev. (μs)
Shorter Lifetime	70	70.0	2.76e- 12	31.8 (54.6%)	2.27e-1	65.8 (6.02%)	8.43e-3
Long Lifetime	500	500	2.07e- 12	502 (.43%)	4.90e-3	502 (.43%)	4.90e-3

The results of the three different lifetime determination methods indicate that the non-linear least squares regression accurately predicts the actual lifetimes. DLD and MLD average a difference of only 0.43% for the long-lived lifetime element. However, the short-lived lifetime prediction derived from DLD is even more different (54.6%) from the actual lifetime. MLD on the other hand averages a difference of only around 6%. The supplied standard deviations serve to support the accuracy of the readings when repeated for five iterations. The regression method was the most robust, producing the smallest standard deviations. Changing the lifetimes noticeably reduced the accuracy of the short lifetimes calculated by the MLD method. For these reasons, two luminescence probes were chosen with known lifetimes of at least a 10-fold difference.

Instrumentation results

The graphical output of the LabVIEW data collected for the two dyes exposed to 0% oxygen can be seen in Fig. 11. The intensity decays were normalized for comparison because the quantum yields of the two dyes were different.

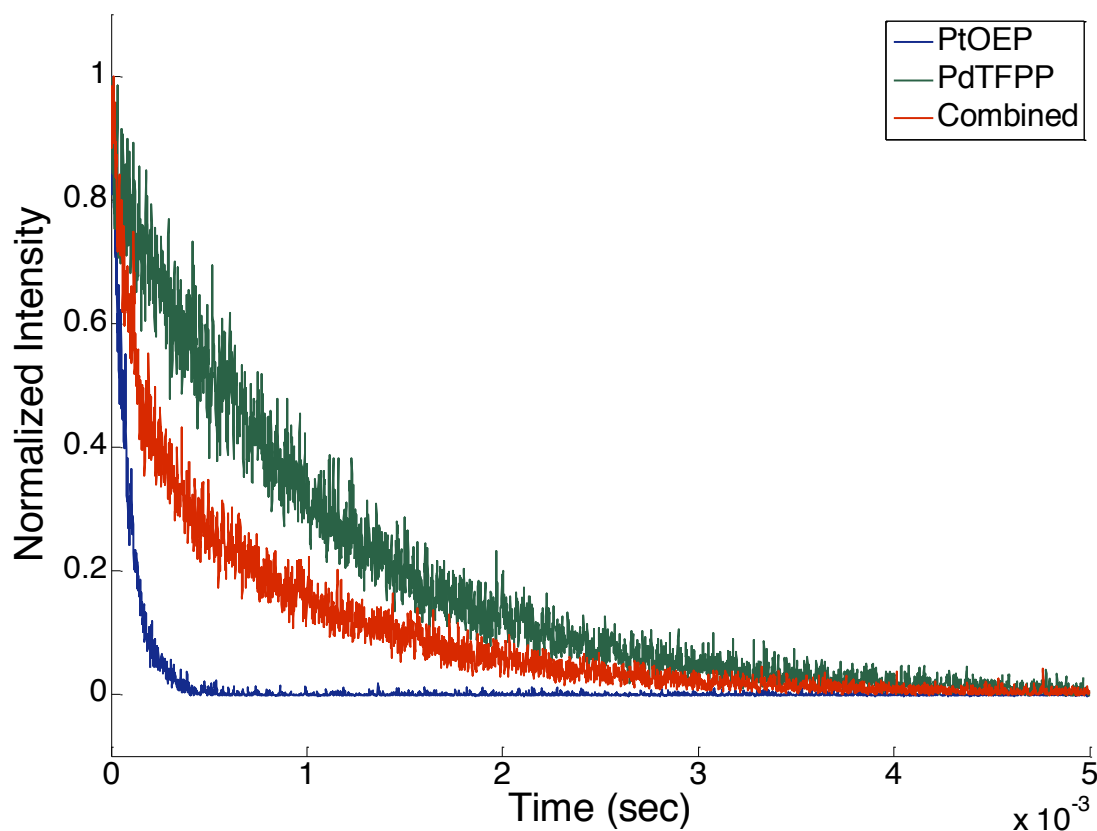


Fig 11. Normalized Decays of the Dyes. Includes individually and combined at 0% oxygen.

Individual dye results

The LabVIEW returned a single lifetime for each dye based on the calculations of the MLD method. The data were then used by MATLAB to calculate the lifetime based on a

non-linear least squares regression. From the data, Table 2 was created to show the calculated values after 5 samples were taken.

Table 2. LabVIEW Output of Single Probes.

% O ₂	Dye	Control Voltage (V)	Window Width (μs)	t ₁ (μs)	t ₂ (μs)	RLD Mean ±95CI (μs)	NLR Mean ±95CI (μs)	SNR
0	PtOEP	0.8	250.4	0	250.4	94.5 ±1.20	84.7 ±2.03	78.8
	PdTFPP	0.8	250.4	500.8	751.2	1043.4 ±60.3	1028.2 ±10.3	17.3
21	PtOEP	1.1	10.4	0	10.4	18.2 ±2.95	24.0 ±3.66	5.4
	PdTFPP	1.1	10.4	0	10.4	19.4 ±8.38	21.5 ±4.03	2.3
100	PtOEP	1.1	10.4	0	10.4	9.04 ±1.70	8.02 ±1.56	5.3
	PdTFPP	1.1	10.4	0	10.4	11.7 ±11.0	2.36 ±0.69	1.1

The table shows the results of the LabVIEW at the three oxygen levels. The PMT control voltage had to be increased to account for the higher levels of oxygen, which dramatically decreased the intensity as the dyes became quenched. Similarly, the width of the calculation windows had to be decreased due to the shortened lifetimes. This also required the window start times (t_1, t_2) to be moved closer to the start of the decay.

Window size and start time strongly influence the lifetimes calculated. Because only one dye was tested at a time, the RLD method was used to calculate the lifetimes. The 95% CI of the lifetimes found by the system overlapped with the regression CI for all cases except PtOEP at 0% O₂ and PdTFPP at 100% O₂. The larger CI of the PdTFPP was due to the low SNR of the signal. The non-overlap of the PtOEP was most likely due to uneven weighting of the decay curve during regression analysis. Because the majority of the intensity of PtOEP decayed in less than 500 μs, and the acquired signal length is 5000 μs, the majority of the acquired data points are background noise. The regression method attempts to fit this data, which is weighed heavily with zero values, reducing the calculated lifetime values.

With higher oxygen levels, the lifetimes of the dyes decreased, as did the ability of the instrument to accurately calculate the lifetimes. This was due to the low SNR and the inability of the DAQ board to sample at a higher rate. It believed that the high SNR of PtOEP at 0% oxygen is due to the high quantum yield of the dye. With the current limitations of the instrument due to the DAQ board, only dyes with high quantum yields can be used to keep the SNR low.

Combined dye results

The two dyes were combined into a single sample to be measured by the system. At 0% oxygen, dual lifetime calculations were made for MLD and regression (Table 3).

Table 3. LabVIEW Dual lifetime calculation. Displays MLD compared to the individual dye results.

Dye	Control Voltage (V)	Window Width (μs)	t_1 (μs)	t_2 (μs)	t_3 (μs)	t_4 (μs)	MLD Dual Mean \pm 95%CI (μs)	RLD Mean \pm 95%CI (μs)
PtOEP	0.8	250.4	0	250.4	--	--	111.4 \pm 29.5	94.5 \pm 1.20
PdTFPP	0.8	250.4	--	--	500.8	751.2	972.5 \pm 61.9	1043.4 \pm 60.3

In the table, t_1 - t_4 were used as the start times for each window 1-4, respectively.

Windows 1 and 2 were used for calculating τ_1 (PtOEP) and windows 3 and 4 were used for calculating τ_2 (PdTFPP). MLD was used to calculate the two lifetime components (PtOEP and PdTFPP). The 95% CI of the MLD and RLD methods overlapped, indicating that the MLD method can accurately calculate two lifetimes simultaneously at 0% oxygen. The system was not tested at the higher oxygen levels due to the low SNR observed in the individual tests, and the inability of the system to distinguish the decays for windowing purposes. Again, window width length and start times strongly affect the lifetime calculations, and have yet to be optimized.

CHAPTER IV

CONCLUSIONS

The data show that the Multi-Luminophore Lifetime Determination method employed by the built instrument can accurately and repetitively calculate the lifetime of multiple dyes simultaneously if the signal-to noise-ratio is high enough (>10) and the lifetime values are distinguishable. With the introduction of higher levels of oxygen, the lifetime of the dyes drops to almost below detection. Due to the low sampling ability of the DAQ board, the SNR falls too low, and repetitive values cannot be calculated. Although the system could somewhat determine the lifetime of a single dye at higher oxygen levels, the lack of samples amplified the noise. It was also observed that window widths and start times must be chosen carefully in order to accurately calculate the lifetime values. The first two windows should be chosen carefully so that accurate calculations can be made for the entire expected range of lifetimes. A larger window width allows for more samples to be taken, increasing the SNR. For these reasons, only distinguishable, long lived samples can be measured simultaneously. The limit, again, is the DAQ board as well as the optics of the system. Both must be upgraded to improve acquisition and determination of smaller decay signals.

Future work will investigate the ability of the instrument to continuously monitor lifetime changes in real time due to varying oxygen levels. Furthermore, the signal to

noise ratio must be improved by increasing both the sample rate and the detector sensitivity. Investigation into maximizing the intensity of the excitation LED and the affect on SNR should also be performed. Lastly, implementing optical filters to separate the spectra of the two dyes could result in more accurate measurements.

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