Fall 12-1-2017

Two-Photon Excitation, Fluorescence Microscopy, and Quantitative Measurement of Two-Photon Absorption Cross Sections

Fredrick Michael DeArmond
Portland State University

Let us know how access to this document benefits you.

Follow this and additional works at: https://pdxscholar.library.pdx.edu/open_access_etds

Part of the [Physics Commons](#)

Recommended Citation

DeArmond, Fredrick Michael, "Two-Photon Excitation, Fluorescence Microscopy, and Quantitative Measurement of Two-Photon Absorption Cross Sections" (2017). *Dissertations and Theses*. Paper 4036.

[10.15760/etd.5920](https://pdxscholar.library.pdx.edu/open_access_etds/10.15760/etd.5920)

This Dissertation is brought to you for free and open access. It has been accepted for inclusion in Dissertations and Theses by an authorized administrator of PDXScholar. For more information, please contact pdxscholar@pdx.edu.

Two-Photon Excitation, Fluorescence Microscopy,
and Quantitative Measurement of Two-Photon Absorption Cross Sections

by

Fredrick Michael DeArmond

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Applied Physics

Dissertation Committee:
Erik J. Sánchez, Chair
Erik Bodegom
Ralf Widenhorn
Robert Strongin

Portland State University
2017

ABSTRACT

As optical microscopy techniques continue to improve, most notably the development of super-resolution optical microscopy which garnered the Nobel Prize in Chemistry in 2014, renewed emphasis has been placed on the development and use of fluorescence microscopy techniques. Of particular note is a renewed interest in multiphoton excitation due to a number of inherent properties of the technique including simplified optical filtering, increased sample penetration, and inherently confocal operation. With this renewed interest in multiphoton fluorescence microscopy, comes increased interest in and demand for robust non-linear fluorescent markers, and characterization of the associated tool set.

These factors have led to an experimental setup to allow a systematized approach for identifying and characterizing properties of fluorescent probes in the hopes that the tool set will provide researchers with additional information to guide their efforts in developing novel fluorophores suitable for use in advanced optical microscopy techniques as well as identifying trends for their synthesis.

Hardware was setup around a software control system previously developed ^[1]. Three experimental tool sets were set up, characterized, and applied over the course of this work. These tools include scanning multiphoton fluorescence microscope with single molecule sensitivity, an interferometric autocorrelator for precise determination of the bandwidth and pulse width of the ultrafast Titanium Sapphire excitation source,

and a simplified fluorescence microscope for the measurement of two-photon absorption cross sections.

Resulting values for two-photon absorption cross sections and two-photon absorption action cross sections for two standardized fluorophores, four commercially available fluorophores, and ten novel fluorophores are presented as well as absorption and emission spectra.

To my parents Fredrick and Cynthia DeArmond.

You are missed dearly.

ACKNOWLEDGMENTS

I have now spent many years at Portland State University working in the Sánchez Nano-Development Lab. During my tenure I have participated in a host of research projects ranging from scanning probe microscopy, physics education, thin films, charged-beam microscopies, and eventually non-linear optical phenomena and applications.

I have obtained a great deal of experience and satisfaction during this time designing, building, testing, fixing, and using various tools and techniques as well as teaching others in those same areas.

As a result of working with my advisor Erik Sánchez, I've been presented with numerous and unique opportunities to work with graduate students, professors, and industry engineers and scientists to solve complex experimental problems.

No body of experimental work is completed in isolation and the work presented herein is no exception. As such I would very much like to expressly thank the following organizations for their support during the course of my work: Intel, Sapling Learning, Liquid Wire LLC, National Instruments, Keithley Instruments, Delmar Photonics, Edmund Optics, Swamp Optics, Princeton Instruments, Budget Sensors, Molecular Vista, and Free Geek.

I would additionally like to thank a host of individuals for their time, feedback, guidance, and general support: Derek Nowak, AJ Lawrence, Rich Swinford, Phillip

Witham, Chris Halseth, Rodolfo Fernandez Rodriguez, Kyle Juedes, Mohan Vattipalli, Micah Eastman, Jeff Doughty, Austin Shigemoto, Abigail Thompson, Luke Lutkus, Peter Steyger, Summer Gibbs, Mark Lowry, Jorge Escobedo, Anthony Sánchez, Alex Chally, Jeff Black, Alex Smith, Mike Hopkins, Rick Trebino, Kim Doty-Harris, and Laurie Tull.

Thanks also to the Department of Physics and all of its faculty for its support in all forms, not least of which in the form of teaching assistantships.

I cannot offer enough thanks to my advisor Erik Sánchez for his support and the opportunities he has allowed me during my time with him. The knowledge and experience he possesses as well as an ability to communicate it has proven invaluable.

Finally, I'd like to thank my partner Alisha Hanks for her love, support, and Saint-like patience as I completed the non-linear journey to my PhD.

Table of Contents

ABSTRACT	i
DEDICATION	iii
ACKNOWLEDGMENTS	iv
LIST OF TABLES	ix
LIST OF FIGURES	x

Contents

1 Introduction	1
2 Fluorescence	4
2.1 Multiphoton Excitation	5
2.2 Applications and Advantages	8
3 Multi-Photon Fluorescence Microscopy.....	10
3.1 Background	10
3.2 Experimental Setup	10
3.3 Microscope Alignment Note	13
3.4 Continuous Wave (CW) Excitation	14
3.5 Simultaneous Excitation of Fluorophores with Different Absorption Spectra	15
3.6 Diffraction Limited Imaging.....	18
3.7 Z-Discrimination	19
3.8 Comparison with Commercial Confocal System	22
3.9 Single Molecule Sensitivity.....	23
3.10 Future Work	26
4 Ultrafast Laser Source	29

4.1 Background: Pulsed Sources	29
4.2 Principle of Ultrafast Laser Generation	31
4.3 Day to Day Operation	35
4.4 Laser Output Monitoring.....	37
4.5 Pulsed Operation	39
4.6 Spectral Distribution.....	40
4.7 Power Output	41
5 Ultrafast Pulse Measurements	42
5.1 Ultrafast Pulse Measurement	42
5.2 Experimental Setup	44
5.3 Control.....	47
5.4 Results: Coherence Time	49
5.5 Results: Pulse Length.....	51
5.6 Difficulties.....	52
5.7 Future Work	55
6 Fluorophores	58
6.1 Group One: Standards.....	58
6.2 Group Two: Commercial Dyes.....	60
6.3 Group Three: Novel Dyes	61
7 Measurement of Two-Photon Absorption Cross Sections	63
7.1 Background.....	63
7.2 Experimental Setup	65
7.3 Methods: Signal Collection.....	67

7.4 Methods: Sample Preparation	71
7.5 Methods: Considerations of Optical Detection Efficiency	73
7.6 Methods: Validation	76
7.7 Calculating 2PA and Action Cross Sections	78
7.8 Results	77
7.9 Difficulties.....	81
7.10 Future Work	82
8 Summary.....	83
8.1 Importance	83
8.2 Limitations and Suggestions.....	84
Bibliography.....	86
Appendices	
Appendix A: 3D Printed Optical Components	91
Appendix B: Optics and Detector Data Sheets.....	102
Appendix C: Compiled Spectra, Structures, and 2PA.....	111

List of Tables

Table 6.1 Rhodamine B and 6G structures	59
Table 6.2 Commercial dyes Rhodamine 110, Cal Fluor Red 635 (referred to as 074 herein), Cy3B, and Atto488 structures	60
Table 6.3 Chemical structures for novel dyes described in US Patent: US2017015652A1 and the annulated Xanthene synthesized by the Strongin lab at Portland State University	61
Table 7.1 2PA in GM with the solution concentrations, quantum yields, and raw signal level. Uncertainties represent 18% of the measured values.....	80
Table 7.2 Two photon action cross sections in $\text{cm}^4\text{s}/\text{photon}$ with solution concentration and raw signal level. Uncertainties represent 18% of the measured values	81

List of Figures

Figure 2.1 A simplified Jablonski diagram showing the absorption of a photon with a frequency of ω_0 . The excitation is represented by the purple arrow, a non-radiative transition then to singlet state S_1 followed by a fluorescent transition back to the ground state S_0 represented by the yellow arrow	4
Figure 2.2 Absorption (blue) and emission (orange) spectrum of 037X, a derivative of Coumarin 314. The difference in peak wavelengths is referred to as the Stokes' Shift	5
Figure 2.3 Left to right: absorption of one, two, and three photons to affect a transition to an excited state and ultimately result in a fluorescent transition	6
Figure 2.4 Detailed molecular Jablonski diagram with Lenard-Jones potentials replaces the discrete energy levels seen before	7
Figure 2.5 Transmission windows (shaded) observed in biological tissues	9
Figure 3.1 Block diagram of optical pathways of the fluorescence microscope. SP-APD is a single photon counting avalanche photo diode used for low signal level collection but can be replaced with less sensitive detectors such as PMTs.....	11
Figure 3.2 Comparison of open-loop (left) and closed-loop (right) imaging performance. Both images were collected with the same microscope platform on which the fluorescence microscope was built. Images are of 10 μm pitch calibration grids with pillar height of 119 nm.....	12
Figure 3.3 Aluminum vacuum chuck for petri dish sample holders	13
Figure 3.4 (Left) Misaligned excitation beam as indicated by the asymmetric pattern. (Right) Pattern resulting from corrected alignment	14

Figure 3.5 PIC dye excited by 850 nm CW from a New Focus 7000 diode laser. Left: Raw image as displayed in the GUI with two line traces. Right: Results of the line traces showing signal to noise on the order of 1000:1	15
Figure 3.6 Left to right: Gentamicin Texas red conjugate, PICI, Annulated Xanthene, and Rhodamine 6G. All images display a 15 μ m scale bar and were acquired using pulses with a central wavelength of 830 nm. The absorption peaks for each dye is indicated directly under the corresponding image	16
Figure 3.7 Fluorophore and excitation spectrum and filter characteristics example pairing. The relevant fluorescence spectral pair are the two furthest left and appear blue and green. (Chroma)	17
Figure 3.8 Spectrum of the dichroic mirror used in this experiment. The dashed blue lines represent the emission peaks of more than 10 fluorophores studied in this work. The red rectangle represents the excitation bandwidth	18
Figure 3.9 Diffraction limited images. Edge contrast of $\sim$ 400 nm measured in the image on the left. Top right is a 3D representation of a large area diffraction limited image ..	19
Figure 3.10 Left and right show the difference between excitation volumes for one and two photon excitation respectively. Excitation volume is highlighted in the two photon image in the center of the yellow circle. Samples are mM concentrations of Rhodamine B	20
Figure 3.11 Left: Traditional confocal microscope with a pinhole aperture to eliminate the out of focal plane light signal (dotted lines). Right: Two-photon excitation microscopy achieving the same principle but in the absence of the pinhole near the detector, simplifying the setup	21
Figure 3.12 Left: Images numbered 1-4 represent individual slices of a sample. This series was imaged sequentially by stepping the focal plane up through the sample by	

translating the imaging objective with a linear translator in roughly one micron increments. All images are 74 μm by 74 μm . Right: 3-dimensional representation of the same image set to illustrate the orientation of the images relative to one another within the sample volume **22**

Figure 3.13 The images above are 65 μm by 65 μm scans of MDCK cells with gentamicin-Texas red conjugate. The image on the left was acquired using our home built multi-photon system while the image on the right was acquired using a commercial confocal system. (Steyger) **23**

Figure 3.14 Collection of individual annulated Xanthene molecules imaged on cover glass. A solution of 0.2 nM was spin coated onto the slide for imaging. Scan area is 25 μm by 25 μm **24**

Figure 3.15 Single isolated molecule as identified by the diffraction limited Gaussian profile **25**

Figure 3.16 From left to right: the polarization is aligned with the dipole, it is then rotated 90° using a half-wave plate, and then back again. The brightness scale is the same on all images..... **26**

Figure 3.17 Modified sample bed with cap (right) and the 3D printed light shield as mounted on the microscope (left) with a rendering drawn in Autodesk Inventor inset. **28**

Figure 4.1 Schematic of the Trestles-50 Ti:SAPPH oscillator cavity. (Delmar Photonics) **32**

Figure 4.2 Overlapping intensity envelopes (left) where the black represents the entire broadband laser generation consisting of both short (blue) and long (red) wavelengths. As this generation passes from left to right through the lasing medium the shorter wavelengths see a higher index of refraction and thus experience a longer optical pathlength. This leaves the blues retarded with respect to the reds as the pulse becomes stretched **34**

Figure 4.3 Geometric principle of negative GVD and the resulting relationship between the reds and blues of the broadband laser generation. Note how they shift relative to each other as they pass through the compensator and then through the dispersive element labelled “objective.”	35
Figure 4.4 Sampling of transverse modes of laser generation and propagation. (RP Photonics)	36
Figure 4.5 Block diagram of optical pathways and test points upstream from the experiments described in this work.....	38
Figure 4.6 The reflection % of various metal surfaces as a function of wavelength.....	39
Figure 4.7 Pulsatile behavior of laser output as measured by fast photo-diode. Note the repetition rate of 78 MHz. The time between pulses is equal to the round trip time of the pulse.....	40
Figure 4.8 Spectrum of pulsed Ti:SAPPH with CW component of output measured as a large, narrow-band peak acquired with a SpectraPro spectrometer and intensified CCD (inset) with a simplified representation for easier viewing.....	41
Figure 5.1 Intensity autocorrelator. Consisting of a beam splitter, variable delay line with retro reflecting roof mirror, focusing lens, second harmonic generating crystal (SHG), aperture, filter for the fundamental frequency, and slow detector	42
Figure 5.2 Interferometric autocorrelator. Consisting of a beam splitter, variable delay line, focusing lens, second harmonic generating crystal (SHG), aperture, filter for the fundamental frequency, and slow detector	44
Figure 5.3 Schematic of our interferometric autocorrelator. Consisting of a beam splitter, variable delay line, focusing lens, and a GaAsP photodiode (PD)	45

Figure 5.4 Spectral response of GaAsP. (Hamamatsu) Note that the response falls to zero long before the excitation wavelength of ~850 nm used here, thus ensuring that photocurrent generated is the result of two photon absorption	46
Figure 5.5 Interference pattern observed at time zero using the digital camera on a Samsung Galaxy 3	47
Figure 5.6 LabVIEW GUI for delay line control. Top right is the control to power the encoders and turn on the motor. Moving to the right the “Position Limit” is a user set value dictating the linear range of the delay line, followed by a numeric indicator of the value. The “reset” button serves to reset the encoder value to zero when it is positioned at time zero. The “Case Number” is a diagnostic indicator with a value of either 1 or 2 representing forward or backward motion of the delay line. Finally there are two indicators on the H-Bridge cartoon represent positive logic states for real time monitoring by the user	48
Figure 5.7 LabVIEW block diagram for control and monitoring of the delay line	49
Figure 5.8 Linear interferogram trace. The time scale for the delay is self-calibrating as the spacing between adjacent fringes is physically restricted by the properties of the electric field and therefore must be equal to the central wavelength divided by the speed of light. FWHM measured to be 78 fs corresponding to a bandwidth of about 30 nm	50
Figure 5.9 Interferometric autocorrelation trace. Full width half maximum value is indicated as ~100 fs corresponding to a pulse length of ~65 fs for a sech^2 pulse	51
Figure 5.10 Schematic of SpectraPhysics 409 Intensity Autocorrelator. (SpectraPhysics)	52
Figure 5.11 Trace acquired from autocorrelator with rotating glass block. Note the central artifact as well as the wings flanking it	53

Figure 5.12 Sequential traces showing a reduction in dispersion as seen by the decrease in magnitude of the signal offset to either side of the central trace. A) Thick beam splitting cube and thick rotating block. B) Thin beam splitter and thick rotating block. C) Thin beam splitter and thin rotating block	54
Figure 5.13 Asymmetric trace caused by non collinear beams. Amplitude is shown in arbitrary units and the time (delay) axis is self-calibrating with the spacing between adjacent fringes equal to the central wavelength divided by the speed of light. FWHM is 78 fs	55
Figure 5.14 Schematic of GRENNUOILLE geometry. (Swamp Optics)	56
Figure 7.1 Photo of setup. Red arrows represent the IR excitation light and the blue arrow the fluorescence signal. Pictured here with an APD and with no true sample holder. Detail changes were made to this setup including an enclosure, some new mounting hardware, and a legitimate sample holder for the measurements presented in this chapter and is shown below in figure 7.5	66
Figure 7.2 PMT signal with labelled constituents. (Hamamatsu) Where I_d is the mean value of the noise component i_d (rms) is the AC component of the noise, I_{p+d} is the mean value of the signal, and i_{p+d} (rms) is the AC component of the signal	68
Figure 7.3 Quadratic relationship of fluorescence for Rhodamine B is shown to be quadratic and therefore governed by a second order process	71
Figure 7.4 Enclosure used for all 2PA measurements. (A) Cuvette holder (B) Lid (C) 3D printed mounting hardware	72
Figure 7.5 Results for Rhodamine 6G over a 20 nm tuning range. Bright yellow markers represent interpolated results for use as reference values	77

1 Introduction

Gentamicin is an aminoglycoside antibiotic and is clinically important for treating bacterial sepsis, meningitis, burns, combat related injuries, and tuberculosis. Gentamicin has been a mainstay in pediatric care for decades. Although new antibiotics are constantly under development, gentamicin continues to play an important role in clinical medicine^[2]. Worldwide, aminoglycosides are the most commonly used antibiotics^[3]. About 10% of patients receiving aminoglycosides experience acute nephrotoxicity and/or permanent ototoxicity as a side effect^[2]. Nephrotoxicity is a poisonous effect on the kidneys while ototoxicity is a toxic effect resulting in damage to the cochlea, or auditory nerve and sometimes the vestibular system. The modern era of evidence-based ototoxicity emerged in the 1940s following the discovery of streptomycin in 1944 and the subsequent discovery of their ototoxic side effects^[4-7]. Aminoglycosides, like other ototoxic drugs, are frequently nephrotoxic, as both organs regulate fluid and ion concentration. The most prominent side effects of aminoglycoside treatments—cochlear, vestibular, and renal impairment—are a limiting factor in the utility of these drugs. The necessary usage of aminoglycosides adds importance and urgency to the task of identifying mechanisms of cellular uptake and clearance. Despite efforts, the mechanisms of uptake and clearance of gentamicin, which lead to the aforementioned debilitating effects, are still not understood.

The most frequently utilized tool for studying cellular uptake and clearance of gentamicin is scanning fluorescence microscopy, where the gentamicin is tagged with a

fluorophore and a series of images are acquired over a period of time to reveal the position of the drug near and within a collection of cells as a function of time. While these studies have done much to elucidate information about the systems they have also shed light on potential problems and limitations of the technique. The latter efforts have shown that gentamicin uptake does not require endocytosis (uptake via engulfing the drug), suggesting cation channels are a means for gentamicin to enter the cell. Having identified ion-permissive channels as a suspect for clearance/uptake, it is now necessary for researchers to identify the regulatory protein responsible for allowing the gentamicin to enter the cell. Regulatory proteins reside in the cell membrane and allow passage into the cell.

The need for increased resolution and novel fluorophores to address research problems like that described above is a pressing issue. Especially so as the time progresses and the length scales investigated correspondingly shrink, requiring higher and higher resolution. This issue of resolution has recently resulted a leap forward in the form of the development of a number of so called super-resolution techniques including Photoactivated Localization Microscopy (PALM), Stochastic Optical Reconstruction Microscopy (STORM), Stimulated Emission Depletion (STED), and Structured Illumination Microscopy (SIM) achieving far better than diffraction limited imaging with contrast on the order of $\lambda / 30$ where λ is the wavelength of the light source. These efforts have led to the award of the Nobel Prize in chemistry in 2014 and have opened new avenues of inquiry for researchers to explore.

The secondary problem faced in the investigation of these small scale biological mechanisms is the availability of suitable fluorophores that can be attached to the structures of interest for observation. Fluorophores, to be useful, must have a number of properties including:

- Photostability, emitting many photons before finally photobleaching
- Suitable spectral properties to match excitation and collection systems available
- The ability to fluoresce in the sample environment. Fluorescent properties are dependent on pH, lipid environments, solvents, and linking methods.
- The efficient absorption of excitation energy.

The importance of advancements in fluorescence microscopy is difficult to overstate as optical techniques are still among the most flexible tools for investigating biological systems, especially live ones. Great advancements have of course been made in the study of the biological structures using electron microscopy but such approaches demand the sample be located in a vacuum, thus (mostly) eliminating any potential for in vivo studies as the samples must undergo any number of fixation processes which greatly limit the research questions that can be accessed. New advances in environmental electron microscopy has gone some way in addressing these shortcomings but it's unlikely that the access, affordability, and flexibility of optical techniques will be displaced any time in the near future.

2 Fluorescence

Fluorescence refers to emission of light resulting from an atom or molecule absorbing light to affect a transition from the ground state to an excited state. A simplified diagram representing this process in terms of electronic structure is shown below.

Figure 2.1 A simplified Jablonski diagram showing the absorption of a photon with a frequency of ω_o . The excitation is represented by the purple arrow, a non-radiative transition then to singlet state S_1 followed by a fluorescent transition back to the ground state S_0 represented by the yellow arrow.

The colors were chosen purposely to invoke the visible light spectrum where violet light has higher energy per photon than yellow light. This difference in energy is always present, not in quantity but in general that the emitted energy is less than that absorbed (some exceptions exist but for the purposes of this work they do not warrant consideration). This can be alternatively visualized by directly observing the absorption and emission spectrum of a fluorophore, an example of which is shown below.

Figure 2.2 Absorption (blue) and emission (orange) spectrum of 037X, a derivative of Coumarin 314. The difference in peak wavelengths is referred to as the Stokes' Shift.

2.1 Multiphoton Excitation

The above description of the excitation process, built around an introductory understanding of the quantum mechanical description of atomic and molecular structure, tells us that in order to reach an excited electronic state, an atom or molecule needs to absorb a single discrete packet of energy (a photon) with an energy equal to the difference between the two states involved in the transition. The energy of a photon can be written as $E = h \cdot \omega$, where h is Planck's constant and ω is the frequency of the radiation.

However, in her PhD dissertation in 1930, Maria Goeppert-Mayer proposed that the same transition could be made by absorbing multiple photons of lesser energy. In principle the absorption of n photons, each of energy $h \cdot \omega / n$, could be achieved so long

as the photons arrive nearly simultaneously, much shorter than the lifetime of excited state S_i . This time window incidentally can be approximated as the transit time of light over the length of the molecule. Below are simplified Jablonski diagrams of excitations by one, two, and three photons from the ground state to an excited state, followed by a non-radiative transition and then finally a transition back to the ground state accompanied by the emission of fluorescence.

Figure 2.3 Left to right: absorption of one, two, and three photons to affect a transition to an excited state and ultimately result in a fluorescent transition.

While such schemes obviously have a dramatic effect on the absorption spectrum^[8,9], it has been shown that no shift in the emission spectrum occurs as a result of multi-photon excitation^[8-10,12]. N-photon absorption only changes how a fluorophore gets to an excited state, but not how it transitions back down to ground state. The coincidence between the one and two photon emission spectra arises from the favorability of

internal conversion between singlet states S_1 and S_2 resulting from vibrational coupling of S_1 and S_2 ^[8].

A further note about this process is that a naïve view might suggest that if a fluorophore had a peak one photon absorption of 300 nm, the two and three photon absorption peaks would occur at 600 nm and 900 nm respectively. As a general rule however this is not the case and the multiphoton absorption peaks are blue shifted, that is to say that perhaps in the case just described the actual two and three photon absorption peaks might be closer to 500 nm and 700 nm. To understand this, we need to take a slightly more detailed look at the excitation process in terms of the electronic structure.

Figure 2.4 Detailed molecular Jablonski diagram with Lenard-Jones potentials replaces the discrete energy levels seen before.

Here we get a hint as to why the spectrum is blue shifted. The n -photon absorption is accessing a rich structure of vibrational energy levels as opposed to the much oversimplified version shown in figure 2.3 which requires overly precise energy matching that has little practical relevance.

2.2 Applications and Advantages

The application of multiphoton excitation brings with it a number of advantages in accessing fluorescent properties. While these will be discussed in further detail in the subsequent chapters, they warrant explicit identification.

Chief among the advantages and following closely upon the point of the previous section is that because the excitation is blue-shifted and sometime significantly so, a single spectral distribution can be used to excite dyes with absorption peaks covering a large range, thus increasing the pool of potential targets to study without the need for additional excitation sources.

Because the excitation wavelengths differ from the emission peak by something approaching a factor of two, the collection of signal is also simplified and again can offer access to a variety of dyes without the use of additional filter sets.

The use of near-IR excitation to study dyes in the visible regime, often in biological samples, has the added benefit of increased sample penetration as a result of both being a much longer wavelength but, as will be discussed later, also because excitation is only achieved at the points of highest photon flux. This latter point brings with it the added benefits of simplified spatial filtering requirements and limiting photo-

damage to the focal plane^[11,12]. The former point of increased sample depth penetration is stated in the context of biological tissues which display low absorption in the near-IR^[13,14] allowing penetration depths of up to 800 μm ^[14].

Figure 2.5 Transmission windows (shaded) observed in biological tissues^[13].

3 Multiphoton Fluorescence Microscopy

Multiphoton fluorescence microscopy refers to any fluorescence microscopy that relies on the simultaneous absorption of two or more photons to excite the fluorophores in the sample of interest.

3.1 Background

Multiphoton microscopy was developed in 1990 by Denk et al.^[15] based on the principles described by others regarding the simultaneous absorption of n-photons. Since its invention it has proven to be an excellent tool with a number of distinct advantages offered over traditional fluorescence microscopy techniques. It has since 1990 not only grown in application but has not been limited to two-photon absorption processes but in fact both three^[16,17]- and four^[18]-photon microscopy have been demonstrated. However, these techniques have not been widely adopted due to a number of factors, not least of which each additional photon absorbed requires an immensely greater photon flux to generate a reasonable probability of absorption. Additionally, as these powers increase, additional interactions begin competing which further reduces the likelihood of generating useful signal.

3.2 Experimental Setup

The details of the experimental setup have been described in previous work by Nowak et al. The core microscope, its components, and control software are all based upon that design and so will only be briefly described to provide the relevant details as

they relate to this work. Below is a simplified block diagram of the optical pathways.

Figure 3.1 Block diagram of optical pathways of the fluorescence microscope. SP-APD is a single photon counting avalanche photo diode used for low signal level collection but can be replaced with less sensitive detectors such as PMTs.

The optical pathways for the multiphoton fluorescence microscope begins with a beam expander to ensure overfill for the back aperture of the focusing objective and thereby maximize the numerical aperture. A periscope mirror pair M1 and M2 then direct the excitation onto a dichroic mirror that in turn redirects the excitation light to the imaging objective and onto the sample. The sample stage is mounted to a closed-loop piezo electric x-y scan bed providing sub nm resolution and ability to navigate with great control and reproducibility in finding user selected region of interest. Closed loop refers to the use of a feedback mechanism, capacitive in this instance, to correct for

piezo hysteresis. A comparison of open-loop and closed-loop scans is shown below in the context of an atomic force microscopy image collected with this same microscope platform.

Figure 3.2 Comparison of open-loop (left) and closed-loop (right) imaging performance. Both images were collected with the same microscope platform on which the fluorescence microscope was built. Images are of 10 μm pitch calibration grids with pillar height of 119 nm.

Fluorescence is collected back down through the dichroic and a series of two-photon excitation filters before ultimately be collected by either an APD or a PMT.

In order to image cell cultures for biological studies, a special vacuum chuck sample holder was made from aluminum and shown below. The O-ring grooves and O-rings are made to custom dimensions to fit commercially available sample dishes from Ted Pella. The O-rings were cut and glued together from chord stock. The sample

holders are held by a low vacuum provided by a Digital Instruments Vacuum Supply Module.

Figure 3.3 Aluminum vacuum chuck for petri dish sample holders.

3.3 Microscope Alignment Note

Alignment of the excitation beam is assisted by the CCD camera shown above in figure 3.1 and is also used to image the sample surface. For alignment purposes however, the image plane of the camera is purposely located such that it is not in the conjugate plane to the focal plane. The reason for this is so that the focused beam can be checked for alignment. When the beam is off axis to the imaging objective the pattern seen in the camera image is asymmetric, see below. Were the camera's image plane aligned with the focal plane the user would see only a tight spot with no metric by which to determine the quality of the alignment.

Figure 3.4 (Left) Misaligned excitation beam as indicated by the asymmetric pattern. (Right) Pattern resulting from corrected alignment.

3.4 Continuous Wave (CW) Excitation

Although it's advantageous to use pulsed excitation for the greatly increased photon flux, images can be collected using CW excitation as well. The first images collected using the setup described were collected using a CW source. The optical pathways were identical to that already described with the exception of an anamorphic prism pair used to circularize the output of New Focus 7000 diode laser. With a wavelength of 850 nm and time averaged power at the sample surface, excellent signal to noise was achieved as shown below.

Figure 3.5 PIC dye excited by 850 nm CW from a New Focus 7000 diode laser. Left: Raw image as displayed in the GUI with two line traces. Right: Results of the line traces showing signal to noise on the order of 1000:1.

3.5 Simultaneous Excitation of Fluorophores with Different Absorption Spectra

As described in chapter two, the pathway from ground state to the excited state via the absorption of two photons is not a discrete absorption of two photons of precisely half energy but instead the two photons excite to any one of a number of a complex vibration structure. As such, a single excitation spectrum can excite a wide variety of dyes. Seen below is a small collection of dyes with different absorption properties that were all excited with the same incident pulse.

Figure 3.6 Left to right: Gentamicin Texas red conjugate, PICl, Annulated Xanthene, and Rhodamine 6G. All images display a 15 μm scale bar and were acquired using pulses with a central wavelength of 830 nm. The absorption peaks for each dye is indicated directly under the corresponding image.

This ability for a single pulse to excite a number of dyes greatly simplifies the filtering of both excitation and emission and eliminates the need for unique filter sets for each fluorophore. Consider for instance a typical absorption and emission spectrum along with the associated filter set and its spectral characteristics shown below.

Figure 3.7 Fluorophore and excitation spectrum and filter characteristics example pairing. The relevant fluorescence spectral pair are the two furthest left and appear blue and green. (Chroma)

Notice the greatly reduced excitation power as a result of filtering as well as the reduction in fluorescence signal for the same reason. Now consider the use of the single dichroic mirror used in the experiments presented in this work in comparison to the emission peaks of 13 fluorophores studied here and in a later chapter represented by blue dashed lines and the excitation bandwidth shown as a red rectangle. Only nine emission peaks are noted because multiple dyes either share peak values or are too closely spaced to clearly illustrate.

Figure 3.8 Spectrum of the dichroic mirror used in this experiment. The dashed blue lines represent the emission peaks of more than 10 fluorophores studied in this work. The red rectangle represents the excitation bandwidth.

The above figure illustrates the flexibility of a two-photon excitation fluorescence microscope and represents one of the reasons for the renewed interest in the technique.

3.6 Diffraction Limited Imaging

Resolution can be described in terms of edge contrast and when images are analyzed in detail as below the resolution is determined to be diffraction limited. For multiphoton excitation with a high numerical aperture this corresponds to something better than the back of envelope half lambda.

Figure 3.9 Diffraction limited images. Edge contrast of ~ 400 nm measured in the image on the left. Top right is a 3D representation of a large area diffraction limited image.

3.7 Z-Discrimination

Because any two photons must arrive within such a short timeframe ($\sim 10^{-15}$ s or roughly the light transit time over the length of a molecule), excitation is restricted to regions with high photon density i.e. within the focal volume. Below shows this difference graphically.

Figure 3.10 Left and right show the difference between excitation volumes for one and two photon excitation respectively. Excitation volume is highlighted in the two photon image in the center of the yellow circle. Samples are mM concentrations of Rhodamine B.

This spatial restriction eliminates the need to use spatial filtering such as apertures found in typical confocal systems, like that seen below, to reduce background signal because the signal only originates from the focal plane. The practical result of this is twofold, firstly the aforementioned elimination and alignment of an aperture in the image plane and secondly to allow for easy image stacking. That is, collecting an image in one plane of the sample before sequentially moving the focal volume up through the sample to obtain 3-dimensional imaging.

Figure 3.11 Left: Traditional confocal microscope with a pinhole aperture to eliminate the out of focal plane light signal (dotted lines). Right: Two-photon excitation microscopy achieving the same principle but in the absence of the pinhole near the detector, simplifying the setup.

Figure 3.12 Left: Images numbered 1-4 represent individual slices of a sample. This series was imaged sequentially by stepping the focal plane up through the sample by translating the imaging objective with a linear translator in roughly one micron increments. All images are 74 μm by 74 μm . Right: 3-dimensional representation of the same image set to illustrate the orientation of the images relative to one another within the sample volume.

In the above series of images, each is 70 μm by 70 μm , images were collected and then the objective lens was translated in z to increase the depth at which of focus was located. Note the appearance and disappearance of features as penetration depth is increased.

3.8 Comparison with Commercial Confocal System

The restriction of excitation to the focal plane makes a two-photon fluorescence microscope inherently confocal in its behavior. As such it is worthwhile to directly

compare image quality between this lab-built system and a commercially available confocal fluorescence microscope.

Figure 3.13 The images above are 65 μm by 65 μm scans of MDCK cells with gentamicin-Texas red conjugate. The image on the left was acquired using our home built multi-photon system while the image on the right was acquired using a commercial confocal system. (Steyger)

Note the similar performance despite lacking a spatial filter as the inherently small excitation volume reduces the background signal and thus provides a natural spatial filter

3.9 Single Molecule Sensitivity

A final demonstration of the sensitivity and resolution of the microscope is the collection of single molecule images. To collect single molecule images, samples are

prepared by spin coating a dilute solution of dye (0.2 nM) onto cover glass leaving very few molecules per unit area.

Figure 3.14 Collection of individual annulated Xanthene molecules imaged on cover glass. A solution of 0.2 nM was spin coated onto the slide for imaging. Scan area is 25 μm by 25 μm.

Verification that what's being observed are in fact single molecules can be done a number of ways.

It is also possible to verify that the observed signals are from single molecules by estimating the number of molecules per unit area based on the concentration of the solution deposited along with the volume dispensed and area covered. Additionally,

you can measure the spot size. If it is indeed a single source of light then it should be a diffraction limited spot, which is shown directly below.

Figure 3.15 Single isolated molecule as identified by the diffraction limited Gaussian profile.

The most convincing way to correctly identify the presence of single molecules is to take advantage of the polarization dependence on absorption of the excitation light. As a first approximation, a molecule on a surface can be thought of as a dipole. As such, only light polarized along the direction of the dipole will result in excitation. In fact the primary interaction between a molecule and the incident field is via the dipole moment^[19]. Therefore, if we image the molecule and then rotate the polarization using a half wave plate by 90° we should see the signal fall close to zero and then return after rotating the polarization back. Exactly this was done and is shown below in figure 3.16.

Figure 3.16 From left to right: the polarization is aligned with the dipole, it is then rotated 90° using a half-wave plate, and then back again. The brightness scale is the same on all images.

It is clear that the sample does not fall to exactly zero. This happens for a number of reasons. The laser radiation is not purely linear polarization, polarization states are not perfectly conserved after focusing through a high NA objective, and the approximation of a dipole interaction is just that: an approximation.

3.10 Future Work

Future development of the microscope may include a number of simple modifications or addition of accessories and other developments could be much more complex.

Beginning with the more complex changes, it may be useful to mount the focusing objective on a motorized translation stage to provide not only remote focus adjustment, and finer adjustment, but also the possibility to add the capability of doing automated z-stack images by stepping a fixed difference between images and saving them as a stack.

A simple upgrade may be the addition of light shielding to allow the microscope to be used with the room lights on or at least without a large and bulky enclosure. In the current configuration imaging can be done using a PMT with the lights dimmed. This includes turning off nearby sources of light such as computer monitors and oscilloscopes. However, the use of the APD still requires a completely darkened room. As part of these shielding efforts, I have already modified the sample stage and added a cap to block out light from the top of the microscope. Additionally, I have designed and 3D printed an accessory to replace a cover on the dichroic mirror housing with integrated light shields to surround the cage system under the sample bed to block out even more light. With a second version of this plus the addition of a few extra measures would likely accomplish the goal of operating with the room lights on.

Figure 3.17 Modified sample bed with cap (right) and the 3D printed light shield as mounted on the microscope (left) with a rendering drawn in Autodesk Inventor™ inset.

4 Ultrafast Laser Source

All experiments conducted for this work utilize the same Delmar Photonics Trestles-50 KLM ultrafast Titanium Sapphire laser.

4.1 Background: Pulsed Sources

Due to the nonlinear nature of multiphoton processes along with a need to avoid sample damage from sustained exposure to excitation radiation, a very high photon fluence is required to produce enough excitation for imaging or fluorescence measurements. The two primary means of generating sufficiently high photon flux are the use of high-powered continuous wave (CW) laser sources or with ultra-short pulses. In the former case, a time averaged power on the order of 10s of mW is required whereas a train of ultrafast pulses, a time averaged power of only 10s of μ W is required. The underlying reason behind this is the instantaneous power and therefore the instantaneous photon flux.

A laser producing 50 fs pulses at 78 MHz, while sharing the same time averaged power and energy per photon, spends most of it's time off with nearly 13 ns between pulses. This translates to spending nearly 6 orders of magnitude more time not producing light than pulsing. Practically, this translates to an extraordinarily high photon fluence while reducing the exposure time to the sample.

A quick comparison between the CW and pulsed beam photon flux might be useful to provide context. Compare for instance two near-IR beams at 850 nm producing

a time-averaged 20 mW, one CW beam and one 50 fs, 78 MHz repetition rate pulsed beam. Now compare the peak powers and number of photons per second.

First consider the CW beam. This is straight forward because the peak power is equal to the time-averaged power. The pulsed system however requires us to divide the time-averaged power by the duty cycle.

$$P_{peak} = \frac{P_{ave}}{D}$$

Where D is the duty cycle and,

$$D = \frac{\Delta t}{T}$$

Here Δt is the pulse width and T is the period.

Plugging in values for the previously described pulse gives us our peak power.

$$P_{peak} = 5.2 \text{ kW}$$

Power is the time rate of energy deposition. The energy carried by the laser beams is done so by photons and so the total energy E can be written as the number of photons n times the energy of each photon E_p .

$$E = n * E_p$$

$$E_p = \frac{h * c}{\lambda}$$

$$E_p = 2.3 * 10^{-19} \text{ J}$$

Here h is Planck's constant, c is the speed of light and λ is the wavelength associated with the photon, in this case it is taken to be the central wavelength of 850 nm. This then leads to the number of photons per second n ,

$$P_{peak} = n * \frac{E}{t}$$

$$n_{pulsed} = 2.2 * 10^{22} \text{ photons/second}$$

This same calculation for the CW beam yields the following.

$$n_{CW} = 8.5 * 10^{16} \text{ photons/second}$$

It is this difference of 6 orders of magnitude that gives pulsed operation such an advantage over CW.

4.2 Principle of Ultrafast Laser Generation

All lasers require a population inversion to generate laser radiation. Population inversion is achieved when the lasing medium is populated by a greater number of excited states than ground states. This inversion is what allows for the stimulated emission of radiation. As a transition is made from an excited state to the ground state and a photon is emitted it is then reflected back through the medium by a cavity end mirror. It then passes through the medium and, if there is a population inversion, the

passing photon stimulates an additional transition (one photon in and two out: light amplification) and the two photons travel out of the medium in phase with one another.

Shown below in figure 4.1 is the cavity design of the Trestles-50 Kerr effect mode-locked (KLM) Titanium Sapphire laser. The first major component of this system is the pump beam, a 5W CW Coherent Verdi™ V5 Neodymium Vanadate laser producing 532 nm light. This is used to longitudinally pump the water-cooled titanium sapphire crystal why doping with titanium ions gives the sapphire the needed electronic properties for laser generation.

Figure 4.1 Schematic of the Trestles-50 Ti:SAPPH oscillator cavity. (Delmar Photonics)

The generation of ultrashort pulses relies on the properties of the Ti:SAPPH crystal behaving as a saturable absorber and achieving saturation requires the complete

depletion of the ground state. Once the absorber (lasing medium) is saturated, the entire ensemble returns to the ground state emitting a burst of photons. Achieving saturation is in and of itself a nontrivial task and requires very high electric field intensities. This particular system is a Kerr lens mode-locking (KLM) system using a physical aperture and a Kerr self-focusing effect to achieve ground state depletion and thereby produce ultrafast pulses.

Limiting the intensity of the fields within the Ti:SAPPH crystal is a self-broadening phenomenon that occurs when the broadband non-pulsed laser generation circulates within the cavity and is directed through the lasing medium. The wavelength dependence of the index of refraction serves to broaden the pulse in space and time reducing the field strength and preventing ground state depletion. This effect is known as positive group velocity dispersion (GVD) and is represented in Figure 4.2 below where the long and short spectral components are represented by red and blue Gaussian envelopes respectively. From this point forward these long and short wavelengths will be referred to as reds and blues for brevity.

Figure 4.2 Overlapping intensity envelopes (left) where the black represents the entire broadband laser generation consisting of both short (blue) and long (red) wavelengths. As this generation passes from left to right through the lasing medium the shorter wavelengths see a higher index of refraction and thus experience a longer optical pathlength. This leaves the blues retarded with respect to the reds as the pulse becomes stretched.

Compensating for this pulse broadening requires the introduction of negative GVD. This is accomplished by the addition of an intra-cavity prism pair consisting of P1 and P2 seen in Figure 4.1 above. With these prisms removed from the beam path, the laser operates in CW mode (dotted line) M5 and M6 acting as the final mirrors in the cavity. Because you can't advance the blues relative to the reds, you must retard the reds sufficiently to accomplish the same thing and the geometric principle of negative GVD is seen below in figure 4.3. By allowing the reds to travel through more glass you increase their optical pathlength which is equal to the index of refraction times the linear pathlength.

Figure 4.3 Geometric principle of negative GVD and the resulting relationship between the reds and blues of the broadband laser generation. Note how they shift relative to each other as they pass through the compensator and then through the dispersive element labelled “objective.”

The linear spacing between the prisms serves as the coarse adjustment for –GVD while the translation of P2 along its axis provides the fine adjustment. Negative GVD is also referred to as pre-compensation as it ultimately serves to prepare the pulse for the dispersive lasing medium in the case of a saturable absorber or a system of dispersive optics in the case of a fluorescence microscope as described earlier. Now, as this pre-compensated pulse encounters the lasing medium the blues are again retarded but, because of the intra-cavity pre-compensation, this only brings them back in line with the reds and preserves the high photon density required for ground state depletion.

4.3 Day to Day Operation

The term "mode-locking" or "mode-locked" can mean several things and the term mode in and of itself can generate confusion. There are two general families of modes to consider when encountering the term: 1) transverse modes and 2) longitudinal modes. The former is a description of the intensity profile of the output

beam (shown below in figure 4.4) whereas the latter refers to a conditional phase matching relationship present between longitudinal modes.

Figure 4.4 Sampling of transverse modes of laser generation and propagation.

(RP Photonics)

Mode-locked operation in the context of this work refers to the repetition and locking on to a longitudinal mode of laser generation also referred to as "resonator modes," producing a train of pulses. These pulses are spaced in time by the round-trip time of a pulse within the cavity and are thus dependent on the positions of any cavity mirrors that serve to apparently change the cavity length. M2 is mounted on a translation stage that can be moved along the axis of beam propagation and thus serves as the primary point of adjustment for mode-locked operation. Once mode-locked operation is

achieved by the translation of M2, day to day operation requires the user to simply maximize the output power by tuning the output couple OC and the folding mirror M3.

Prism P2 is mounted on a motorized platform to function as a starter for the laser when it doesn't start mode-locked. Starting mode-locked operation requires quickly extending P2 into the beam path and then retracted back to its initial position. This shift in $-GVD$ is enough to achieve ground state depletion and thus begin pulsed operation.

Wavelength tuning of the output is achieved by translating a mechanical slit along the beam profile in a region between P1 and P2 where spatial separation is present as a result of the GVD tuning. Similarly, the bandwidth can be limited by tuning the width of the slit.

4.4 Laser Output Monitoring

Basic monitoring of the laser output is necessary to understand the excitation mechanisms at the sample and ensure stable operation of the ultrafast oscillator. To this end, 3 basic properties are measured continuously: pulsed operation, spectral distribution, and time averaged power. The monitor points are indicated in figure 4.5 below.

Figure 4.5 Block diagram of optical pathways and test points upstream from the experiments described in this work.

The pick-offs used in this experiment are one of two types: 1) a microscope cover-glass slip that has undergone plasma cleaning or 2) a cover glass slip with a thin film of aluminum or copper. The former is the primary type as it re-directs only a small fraction of the beam power, 8% is a general rule of thumb for the loss per reflective surface. The latter is used if, for instance, a greater proportion of the beam needs to be redirected. For instance, at least one 50/50 beam splitter was produced using an aluminum coating deposited via DC magnetron sputtering in the lab. This process involves first plasma cleaning the substrate (cover glass) and then depositing 10s of nms of Al to achieve ~50% reflection. Aluminum provides good broadband spectral response as seen below in figure 4.6 but shows a slight dip in reflectance at just the point of operation for the

laser and so the mirror labelled "M-Cu" in figure 4.5 was made, as the name suggests, by coating a surface with copper.

Figure 4.6 The reflection % of various metal surfaces as a function of wavelength.
(photonics.com/Handbook)

Replacing the Al mirror with a Cu mirror resulted in an increase in reflection from ~88% to >93% reflection at 840 nm central wavelength as measured by a commercial power meter.

4.5 Pulsed Operation

The first and primary line of monitoring the laser output is with the use of an ultrafast photodiode. By directing a small portion of the beam into the photodiode and monitoring the signal on an oscilloscope reveals whether or not the system is pulsing, see figure below.

Figure 4.7 Pulsatile behavior of laser output as measured by fast photo-diode. Note the repetition rate of 78 MHz. The time between pulses is equal to the round trip time of the pulse.

The appearance and magnitude of the observed pulses can be used as immediate feedback when tuning the intracavity mirrors.

4.6 Spectral Distribution

The next point of monitoring is one of two pick-off points used to monitor the spectral distribution of the laser's output. For a Fourier limited (or near-transform limited) pulse, a broad-band spectrum should be observed and the transition from narrow bandwidth to broadband can be used in much the same way as the pulsed signal during tuning. Monitoring the spectrum also allows real time feedback for tuning the central wavelength.

As described above, a pulsed system results in a greatly reduced time-averaged power as compared with a CW system. As such, any CW component in the output will

show up disproportionately when viewing the spectral distribution of the output. Figure 4.8 below shows such a spectrum.

Figure 4.8 Spectrum of pulsed Ti:SAPPH with CW component of output measured as a large, narrow-band peak acquired with a SpectraPro spectrometer and intensified CCD (inset) with a simplified representation for easier viewing.

4.7 Power Output

Finally, the output power is measured using one of two commercial power meters. Users should note that either meter is calibrated using correction factors to compensate for differences in spectral sensitivity and one should always check that the registration wavelength has been set appropriately. The time-averaged power is measured before during and after all experiments to normalize observed signals if power fluctuates and to identify potential instabilities in the lasers that need to be tuned out.

5.1 Ultrafast Pulse Measurement

Measurement of the details of an ultrafast pulse can be rather difficult to perform and interpret. The most common device for the measurement of pulse length is the intensity autocorrelator also referred to as a background-free autocorrelator, commercially available for prices at and around \$20k. Shown below in figure 5.1, this type of device consists of an interferometer with one arm providing a variable delay before recombining the two non-collinear pulses inside a nonlinear medium such as a frequency doubling crystal.

Figure 5.1 Intensity autocorrelator. Consisting of a beam splitter, variable delay line with retro reflecting roof mirror, focusing lens, second harmonic generating crystal (SHG), aperture, filter for the fundamental frequency, and slow detector.

Despite the prevalence of such devices they are currently viewed among the leaders in ultrafast pulse characterization as an obsolete technology^[20]. This technique is criticized for its inability to provide, even in principle, any detail about the pulse other than something roughly equal to the pulse length though even this ability has been shown to produce results that can differ from actual pulse length by nearly a factor of two.

Interferometric autocorrelation (IAC) on the other hand is capable of producing accurate and detailed information about the pulse. In fact, IAC can in principle allow you to reconstruct the entire pulse^[21] less the spatial distortions such as pulse-front tilt though this has not yet been realized in its entirety experimentally. Phase retrieval has however been demonstrated in some instances^[22]. The technique is nearly identical to the background free autocorrelator but with one important difference: the beams exiting the Michelson Interferometer, shown below, are co-linear. See figure 5.2.

Figure 5.2 Interferometric autocorrelator. Consisting of a beam splitter, variable delay line, focusing lens, second harmonic generating crystal (SHG), aperture, filter for the fundamental frequency, and slow detector.

5.2 Experimental Setup

The experimental setup for this work is a modified interferometric autocorrelator which replaces the frequency doubling crystal with a wide band gap GaAsP photodiode as proposed and carried out by others^[23,24]. This also eliminates the need for an optical filter for the fundamental frequency.

Figure 5.3 Schematic of our interferometric autocorrelator. Consisting of a beam splitter, variable delay line, focusing lens, and a GaAsP photodiode.

We are able to substitute the PD for the frequency doubling crystal because what is physically required is the use of some second harmonic generator. The use of this particular PD has been shown to measure pulse length as short as 6 fs with some modifications^[23], while others have shown that 2-photon absorption in photomultiplier tubes (PMT) can also be used to acquire autocorrelation signals^[25]. Below is the spectral sensitivity of the PD from which it can be seen that no one photon current generation can occur with incident beams at or around 850 nm as is used in this work.

Figure 5.4 Spectral response of GaAsP. (Hamamatsu) Note that the response falls to zero long before the excitation wavelength of ~ 850 nm used here, thus ensuring that photocurrent generated is the result of two photon absorption.

Aligning the autocorrelator is assisted by placing an expansion lens at the entrance of the interferometer. By expanding the beam, the user can observe an interference pattern on a screen placed at the output only when the two pulses are overlapping. The resulting pattern is shown below. The interference pattern is present over a difference in arm length equal to the spatial extent of the pulse which is given by the product of the speed of light and the pulse width. The center of this range is referred to as time zero. The spherical expanding lens results in a pattern consisting of concentric circles.

Figure 5.5 Interference pattern observed at time zero using the digital camera on a Samsung Galaxy 3.

In addition, it is necessary that the two halves of the beam are collinear as they exit the interferometer and so a series of irises are placed on the output and the end mirrors are adjusted until the both beams pass through the centers over a lone (20-40 cm) linear distance.

5.3 Control

Mirror 2 is mounted on a linear motor with encoders and controlled by LabVIEW software in conjunction with an analog H-Bridge circuit utilizing BJT transistors and a hex inverter in conjunction with a 5 V DC power supply for motor drive. Control of logic states, encoder power, monitoring of encoder values, and PD signal are all accomplished using the mixed in/out (MIO) bus on a National Instruments 7833R FPGA card.

User control is done through a LabVIEW graphical user interface (GUI) shown below.

Figure 5.6 LabVIEW GUI for delay line control. Top right is the control to power the encoders and turn on the motor. Moving to the right the “Position Limit” is a user set value dictating the linear range of the delay line, followed by a numeric indicator of the value. The “reset” button serves to reset the encoder value to zero when it is positioned at time zero. The “Case Number” is a diagnostic indicator with a value of either 1 or 2 representing forward or backward motion of the delay line. Finally there are two indicators on the H-Bridge drawing represent positive logic states for real time monitoring by the user.

The range driven in either direction from time zero is controlled by the user input. Encoder values are displayed as a function of time. Logic states switch once encoder values reach and exceed the user-set limit. This crossing condition can be seen below in the LabVIEW block diagram.

Figure 5.7 LabVIEW block diagram for control and monitoring of the delay line.

5.4 Results: Coherence Time

Measuring the output of the interferometer using the GaAsP photodiode by terminating into a 1 M-ohm resistance on a Rigol DS1204B 200 MHz digital oscilloscope and also into an analog IO on the FPGA

Figure 5.8 Linear interferogram trace. The time scale for the delay is self-calibrating as the spacing between adjacent fringes is physically restricted by the properties of the electric field and therefore must be equal to the central wavelength divided by the speed of light. FWHM measured to be 78 fs corresponding to a bandwidth of about 30 nm.

This measurement represents a precise determination of the coherence time τ_c . The coherence time is the reciprocal of the pulse's bandwidth.

The practical effect of this is that the FWHM of both the linear interferogram and the interferometric autocorrelation are equal to or nearly equal to one another. The linear interferogram measurements were not intentionally made but were in fact the result of a slight offset in the alignment of the interferometer and only after those measurements was it recognized as a linear interferogram and therefore a representation of the coherence time^[22, 26, 27].

5.5 Results: Pulse Length

The pulse length is measured in exactly the same way only with slight changes to the for better pulse overlap. In doing so a change in the ratio of the signal magnitude above and below the background level is observed with is indicative of an interferometric autocorrelation trace. While it is argued that a true autocorrelation trace has a characteristic signal to noise of 8^[22], many have reported interferometric autocorrelation traces that do not meet this criterion^[25,26,28] and it has been pointed out that the factor of 8 is a theoretical upper limit to the signal to background^[29]. Indicated full width half maximum (FWHM) value is obtained by multiplying the observed 100 fs by a scale factor of 1.54 assuming a sech^2 intensity profile.

FWHM 68 fs

Figure 5.9 Interferometric autocorrelation trace. Full width half maximum value is indicated as ~100 fs corresponding to a pulse length of ~65 fs for a sech^2 pulse.

5.6 Difficulties

Aside from the predictable difficulty of overlapping a femtosecond pulse in space and time, a number of other problems were encountered. Initial attempts to measure autocorrelation utilized a hybrid experimental setup, combining aspects of a commercial SpectraPhysics 409 intensity autocorrelator (shown below), where the pulse delay was introduced using a rotating glass block, and the collinear propagation used in the interferometric family of autocorrelators.

Figure 5.10 Schematic of SpectraPhysics 409 Intensity Autocorrelator. (SpectraPhysics)

In contrast to the 409 design however, in which both beams traverse the rotating block, our design was such that only one of the arm included the glass block.

This was the result of a simple failure to account for the difference in dispersion and path length introduced in such an arrangement. By introducing such large dispersion in only one arm of the apparatus means that anything measured will at best be a cross-correlation and that dispersion will appear in the resulting trace. The result is shown below.

Figure 5.11 Trace acquired from autocorrelator with rotating glass block. Note the central artifact as well as the wings flanking it.

Two major problems are present in the above trace. Firstly, there is a dip at the point of zero delay between the two arms where as it must be a peak. Secondly, there are prominent features on either side of the central pattern. The dip in the center is a result of the beam only passing through the block in one arm. What this arrangement fails to do is to both advance and delay the pulse in that arm relative to the other. It

instead only retards the pulse relative to the other half and so the two path lengths are never equal to one another.

The two raised features either side of the main pattern are interesting and illustrate one property of pulses that this type of measurement is good at identifying which is the presence of significant dispersion or chirp. It has been shown in a number of works that these features are signals riding over the rest of the signal and result from large dispersion^[28,30]. To test this the original beam splitting cube was replaced with a much thinner beam splitter (>1 cm to ~5 mm) and the glass block originally used was replaced with a much thinner piece of glass as well (6.4 mm to 1 mm) and a reduction in this signal was observed and is shown below.

Figure 5.12 Sequential traces showing a reduction in dispersion as seen by the decrease in magnitude of the signal offset to either side of the central trace. A) Thick beam splitting cube and thick rotating block. B) Thin beam splitter and thick rotating block. C) Thin beam splitter and thin rotating block. Amplitude scale is in arbitrary units.

Misalignment can also lead to asymmetries in the trace as two beams overlapping one another at an angle will have a time delay across the beam and thus generate unequal signal on the positive and negative delays relative to the central peak.

Figure 5.13 Asymmetric trace caused by non collinear beams. Amplitude is shown in arbitrary units and the time (delay) axis is self-calibrating with the spacing between adjacent fringes equal to the central wavelength divided by the speed of light. FWHM is 78 fs.

5.7 Future Work

The above technique, while perfectly adequate for the purpose of this work, is ultimately limited in its ability to fully characterize the pulse and requires difficult alignment. In order to get a complete picture of the pulses in both space and time, new paths will need to be pursued. Frequency resolved optical gating (FROG) and the more recently developed of Grating Eliminated No-nonsense Observation of Ultrafast Incident Laser Light E-fields (GRENOUILLE) are prime candidates for these future efforts.

The cutting-edge technique is GRENOUILLE, which claims to be alignment-free and consists of no moving components^[20]. A schematic of the device is shown below in figure 5.14

Figure 5.14 Schematic of GRENUOILLE geometry. (Swamp Optics)

The BBO crystal replaces the diffraction grating present in the closely related FROG device by being "thick" enough to provide the required for mapping of spectral separation. The governing equation of GRENOUILLE is given below and is related to the ratio of the group velocity dispersion (GVD) and the group velocity mismatch (GVM) relative to the length of the crystal.

$$\frac{GVM}{GVD} \gg TBP$$

Where GVM is the group velocity mismatch, GVD is the group velocity dispersion, and TBP is the time-bandwidth product. All quantities are defined as follows in terms of the

group velocity v_g , the central wavelength λ_o , the pulse duration τ_t , and the coherence time τ_c .

$$GVM = \frac{1}{v_g(\frac{\lambda_o}{2})} - \frac{1}{v_g(\lambda_o)}$$

$$GVD = \frac{1}{v_g(\lambda_o - \frac{\delta\lambda}{2})} - \frac{1}{v_g(\lambda_o + \frac{\delta\lambda}{2})}$$

$$TBP = \frac{\tau_t}{\tau_c}$$

Most of the components have been purchased for this setup including both AR-coated cylindrical lenses and the Fresnel bi-prism. The Sanchez Lab is well equipped with high-end cameras suitable for the image acquisition and the software is freely available as a MATLAB interface from Rick Trebino and Swamp Optics. The single missing component at this time is the "thick" frequency doubling crystal. An attempt was made to use a crystal already possessed by the lab but at 7.5 mm it was much too thick to use and the traces it produced (as projected onto an image screen after expansion) displayed significant distortion. A rough calculation has been done by the Trebino group in determining the maximum thickness of the crystal and the results of their calculations and my own show that the 7.5 mm is much too thick, with 5 mm represented something close to the upper limit^[31,32].

6 Fluorophores

The fluorophores studied can be split into three groups. The first group consists of two well characterized rhodamines to provide validation of the methods and results described in chapter 7. The second group consists of four commercially available but uncharacterized dyes.

The third group is a series of novel dyes, mostly coumarin derivatives previously un-characterized along with a novel xanthene molecule. Full absorption spectra, collected using a Shimadzu UV-Vis spectrophotometer. A complete summary of properties is contained in Appendix C.

6.1 Group One: Standards

In order to validate the methods described in this work a pair of standards were chosen for their availability and the prominence with which they appear in the literature characterized in the same manner. These two standards were Rhodamine B and Rhodamine 6G.

Rhodamine B	Rhodamine 6G

Table 6.1 Rhodamine B and 6G structures.

6.2 Group Two: Commercial Dyes

Commercially available dyes Rhodamine 110, Cy3B, Atto488, and Cal Fluor Red 635 are also included in the set of dyes examined.

Rhodamine 110	Cal Fluor Red 635 (074)

Cy3B	Atto488

Table 6.2 Commercial dyes Rhodamine 110, Cal Fluor Red 635 (referred to as 074 herein), Cy3B, and Atto488 structures.

6.3 Group Three: Novel Dyes

Finally, a large group of novel dyes was studied. Comprised primarily of a series of coumarin derivatives provided by Anthony Sánchez and described in US Patent: US2017015652A1 along with a single annulated xanthene molecule synthesized in the Strongin lab at Portland State University.

001X (derivative of Coumarin 2)	031X

033X (derivative of Coumarin 102)	037X (derivative of Coumarin 314)

040X (derivative of Coumarin 102)	054X

067X (derivative of Coumarin 102)	084X

086AX2 (derivative of Coumarin 102T)	Annulated Xanthene (MSV 08-134F6-7)

Table 6.3 Chemical structures for novel dyes described in US Patent Application: US2017015652A1 and the annulated xanthene synthesized by the Strongin lab at Portland State University.

7 Measurement of Two-Photon Absorption Cross Sections

This work has led to the quantitative measurement of two-photon absorption cross sections, 2PA herein, for eleven previously uncharacterized dyes and the closely related quantity known as the two-photon action cross section for three others. 2PAs have been measured for a number of both commercial and novel dyes, in a wide variety of environments, and over a large wavelength regime. This has resulted in an ever-increasing catalog of dyes and this work adds to that catalog^[34].

7.1 Background

2PA is measured and reported in units of Goeppert Mayers (GM) reflecting named after the Maria Goeppert Mayer who first proposed multiphoton absorption. The GM is not a fundamental unit but is defined as a $\text{cm}^4 \text{ s/photon}$. The cm^4 reflects the product of two areas, and the s/photon reflects the dependence on photon flux.

The brightness of a fluorophore is of prime concern of any microscopist. A basic description of the brightness can be closely approximated as the product of the quantum yield and the 2PA^[35], a quantity known as the two-photon action cross section. Quantum yield is defined as the number of photons emitted per photon absorbed and is a dimensionless quantity. Although the quantum yield is dimensionless, the two-photon action cross section is typically reported in units of $\text{cm}^4 \text{ s/photon}$ instead of GM as it does represent a different physical quantity. While the brightness and the action cross section track very closely, they should not be considered one and the same^[35], though

as a first pass this is a perfectly fine way to think about and qualitatively describe brightness. Ultimately, in order to assist researchers in developing better dyes, a measure inherent to the dye on its own is necessary. 2PA measurements achieve this.

A number of methods for the determination of 2PA have been proposed and carried out, including an absolute measurement carried out in isolation^[36], measurements derived from the brightness^[35], measurements obtained through use of a calibrated standard^[37,38], hyper-Rayleigh scattering^[39], nonlinear transmission methods^[40], and density functional theory modelling^[41].

The action cross section on the other hand more heavily depends on the environment due to the presence of the quantum yield term. 2PA measurements may prove particularly useful in the development of more robust density functional theory (DFT) descriptions of fluorescence properties. The current state of DFT for predicting such a quantity while sometimes in good agreement can differ from experimental results by as much as a factor of four^[50] while differences between reported nonlinear transmission methods and fluorescence methods can be as great as a factor of eleven when conditions and nonlinear effects are not sufficiently considered^[40].

7.2 Experiment Setup

The experimental setup is shown below in figure 7.1. It is essentially the same as the fluorescence microscope described in chapter 3 only oriented horizontally in the plane of the optical table. It consists of a neutral density filter wheel box to control excitation power, beam pick-off for power monitoring, a beam expander to maximize the numerical aperture of the focusing lens, a long pass excitation filter R785LP, a dichroic mirror 725 DCSPXR, to a Melles Griot plano-convex 50 mm focus lens into an IR and UV transparent 2 mL cuvette. The light is then collected back through this system beginning with the same focusing lens, through the dichroic filter, and then through a set of two-photon filters before being focused via another Melles Griot lens (1" diameter 25 mm focal length) onto a Hamamatsu R928 PMT typically biased at -600 V though it can be increased to approximately 1 kV. However, the signal level generated from this sort of bulk measurement at relatively high concentrations (10^{-5} M) results in saturation of the detector above -690 V. This latter fact must be carefully considered when carrying out measurements to avoid spuriously low count rates resulting in systematic error.

Figure 7.1 Photo of setup. Red arrows represent the IR excitation light and the blue arrow the fluorescence signal. Pictured here with an APD and with no true sample holder. Detail changes were made to this setup including an enclosure, some new mounting hardware, and a legitimate sample holder for the measurements presented in this chapter and is shown below in figure 7.4.

A note about alignment: the neutral density filter wheels (not shown above) and the sample cuvette are both mounted at slight angles such that the excitation beam is not incident normal to the surfaces. Normal incidence should be avoided whenever possible to mitigate feedback into the Ti:SAPPH. Direct feedback back into the laser can result in instability and loss of mode-locked operation. Multiphoton filters like those used in this experiment, labelled as "IR Filter Set" in front of the PMT, are designed with this in mind and are mounted at shallow angles within their housings. Lenses with a planar surface should be oriented such that the beam is incident on the curved surface for the same

reason of avoiding direct reflection feedback into the laser. Incidentally, this orientation is a best practice for focusing system as it reduces spherical aberration.

Concentration is controlled by the experimenter by serial dilution of 20 mL volumes of high concentration (10^{-2} M) stock solution to be on the order of 10s of μM .

7.3 Methods: Signal Collection

Signal is collected using a Hamamatsu PMT, but the Perkin Elmer single photon counting avalanche photodiode (SP-APD) can be used as a substitute. This substitution of detector includes the addition of at least one additional two-photon filter while the 1" lens is replaced by a 0.5" diameter, 20 mm focal length lens and used in conjunction with a ThorLabs CVH100-COL fiber coupler. The lens is mounted on a Thorlabs spacer to ensure focusing on the entrance aperture of the fiber coupling to the SP-APD. Ultimately the SP-APD offers more sensitivity than is required for signal levels of this magnitude. This state of affairs combined with the risk of damage to and cost of replacement precludes it from being used in any practical sense for these measurements.

We report fluorescence signal magnitude as the mean value of the signal, and taking the uncertainty in the measurement to be $\frac{1}{2}$ the range of the AC component of the noise. Figure 7.2 illustrates how these values are determined from the observed PMT signal. The uncertainty is determined to be roughly 18% of the value of the counts for all of the dyes and so error bars are equal to 18% of the value. This is in line with Xu

and Makarov who present 15% as a standard error^[36,38], though they do not provide justification for that value.

Figure 7.2 PMT signal with labelled constituents. (Hamamatsu) Where I_d is the mean value of the noise component i_d (rms) is the AC component of the noise, I_{p+d} is the mean value of the signal, and i_{p+d} (rms) is the AC component of the signal.

Counts rates were taken to be this mean signal level minus the mean signal with only a cuvette containing methanol. This control sample was placed into the sample holder are measured after every 3 dyes to ensure no change in the background signal to ensure the integrity of the measurements. There is also some mean value of the noise as shown above but this value is constant for a given gain voltage applied between dynodes and so the magnitude of the signal between dyes are compared as arbitrary units.

Potential nonlinear effects arising from the high peak powers generated by the ultrafast pulses is a constant and major concern. For this reason, care is taken to ensure that individual components behave linearly. Checks for the non-linear behaviors should be made before any data collection to avoid problems. In an attempt to verify as many components as possible the following linear relationships were checked: 1) power into the beam expander vs reading on the power meter 2) power into the beam expander vs power at the sample 3) power into a cuvette with solvent only (MeOH) vs power out 4) excitation power with no sample and PMT background signal. All relationships were linear.

A commercial power meter is used for all experiments and measures the power via a beam pick-off that re-directs ~10% of the excitation power to the power meter. The power is then again, measured at the sample location. If any fluctuation were observed, the signal could be normalized to correct for that change. No such fluctuation was observed over the course of any data set collection and the power into the microscope was recorded for each measurement.

Additionally, it is necessary to continuously monitor the excitation wavelength to ensure that the central wavelength does not drift over the course of a day or indicates the presence of CW modes. This is accomplished through the use of another beam pick-off and a fiber-coupled spectrometer.

The collected signal is then collected using the existing FPGA code developed and described previously by Nowak et al.^[1] However, that particular code was configured to read the PMT signal on a digital IO with a minimum logic level of +2.0 V. The consequence of this is that if the mean signal level from the PMT reaches and or exceeds the nominal 2 V the count rate will always be equal to the clock cycle and thus record inaccurate count rates. To avoid signal clipping I added to the FPGA code utilizing one of the otherwise unused analog channels. Within the FPGA code, high throughput math functions wired to the FPGA resource serve to convert the 16-bit integer input to a signal in units of mV so that it can be directly compared to signals observed on an ordinary oscilloscope during alignment and testing procedures.

A final, and necessary step in verifying the method of data collection is to check for the quadratic nature of fluorescence signal vs excitation. This quadratic relationship is clearly present and shown below in figure 7.4.

Figure 7.3 Quadratic relationship of fluorescence for Rhodamine B is shown to be quadratic and therefore governed by a second order process.

The presence of a quadratic relationship indicates a mechanism governed by a two-photon process.

7.4 Methods: Sample Preparation

All samples were prepared from solid powders weighed on a precision balance with 100 µg precision. All powders were placed in 20 ml sample bottles and filled with methanol obtained from the chemistry department stockroom. These bottles were then labelled with the corresponding concentrations on the order of mM and used to then perform serial dilution. Stock bottles were sealed using a parafilm wrap around the caps and stored in a sample refrigerator.

Samples were all prepared in μM concentrations (exact concentrations are listed below in table 7.1) and placed in identical disposable cuvettes. The cuvettes are held in a commercial holder taken from a UV-Vis spectrophotometer. A crude enclosure was constructed around the sample holder to reduce background signal with a flat black plastic lid that was placed on top during measurements. A photo of the setup is shown below. This arrangement provides sufficient light shielding from surrounding sources to prevent saturation of the detector when the lid is removed.

Figure 7.4 Enclosure used for all 2PA measurements. (A) Cuvette holder (B) Lid (C) 3D printed mounting hardware.

Details regarding the 3D printed parts shown in the figure above as well as others are contained in Appendix A.

All measurements made were done at 845 nm central wavelength with a spectral distribution showing a full width half max of 17 nm. This value was chosen for the stable operation observed by the laser, roughly constant value for 2PA of Rhodamine B at and near this point, and the proximity to the 2PA peak of Coumarin 307 (800nm, Xu), the peak of Coumarin 485 (800 nm, Makarov), and Coumarin 540A (820 nm, Makarov). The coumarins were chosen as most of the dyes investigated are coumarin derivatives and therefore share many structural characteristics.

7.5 Methods: Considerations of Optical Detection Efficiency

The full expression of the time averaged fluorescence signal shown below and used by others for their absolute 2PA measurements^[38].

$$\langle F(t) \rangle \approx \frac{1}{2} \phi n' C \sigma \frac{g_p}{f_\tau} \frac{8n \langle P(t) \rangle}{\pi \lambda} \quad (1)$$

Where $\langle F(t) \rangle$ is the time averaged photon flux detected, ϕ is fluorescence detection efficiency, η' is the fluorescence quantum yield, C is the dye concentration, σ two-photon cross section, g_p is a dimensionless quantity determined by the shape of the laser pulse, f_τ is the duty cycle, n is the index of refraction of the sample, $\langle P(t) \rangle$ is the time averaged excitation power, and λ is the central wavelength of excitation source.

This expression requires however full pulse retrieval for the ultrafast oscillator in order to determine the pulse shape and the detection efficiency is a particularly difficult quantity to determine. The overall collection efficiency of the system is estimated to be 3-4% and is typical for fluorescence microscopes. However, estimating this value has been recognized as one of the largest contributors to uncertainty in absolute measurements.

If we limit ourselves, as is the purpose of this work, to the inherent properties of the dyes as opposed to the properties of the excitation and collection systems then the above expression becomes a simple proportionality.

$$\langle F(t) \rangle \propto n' \sigma \quad (2)$$

That is the product of the quantum yield and the 2PA. This product is known as the two-photon action cross section and while it shares units with the 2PA because the quantum yield is a dimensionless quantity, units are instead reported as cm⁴s/photon instead of the abbreviated GM.

While this proportionality may be useful as a qualitative description of the brightness of a fluorophore it is of no use on its own for the purposes of measuring 2PA. If however we note that expression (1) contains the quantities g , f_τ and λ , each of which is a property of the excitation source and thus independent of the dye we can perhaps use expression (1) but avoid some potential sources of uncertainty and simplify the process. If for instance we had a dye with a known 2PA at the experiment's registration

wavelength we could, as suggested by Makarov et al. take the ratio of signals between a known and an unknown dye such that all of the dye independent terms drop out. This leaves the following.

$$\sigma_{2,s} = \frac{F_{2,s}(\lambda_{reg})C_r\eta_r(\lambda_{reg})}{F_{2,r}(\lambda_{reg})C_s\eta_s(\lambda_{reg})}\sigma_{2,r} \quad (3)$$

Where F_2 is the fluorescence intensity, λ_{reg} , is the fluorescence emission peak. Indices r and s stand, respectively, for the reference and the sample under study, and $\eta(\lambda_{reg})$ is the quantum yield. This expression takes the spectral response of the detector to be constant over a sufficiently small interval, however, in characterizing such a spectral distribution as that represented by the fluorophores in this work I have kept the spectral response q of the detector in the above expression and so used that slightly modified relationship for all the calculations included here.

This, provided that a standard exists to compare with, can be used to calculate the 2PA for an otherwise uncharacterized dye. The index of refraction can be considered a constant value because all samples use the same cuvette, solvent, and approximate concentration.

Note that this expression does away with the collection efficiency term as well as the same collection system is used for both samples. This requires however the assumption that the emission wavelengths are sufficiently close that the spectral response of the collection optics and detectors are approximately equal. While it holds that over a sufficiently short spectral separation the response of any given detector is in

fact constant, the wide range of emission peaks and therefore detected signals present in this work meant that the term representing the collection efficiency was not cancelled. So the expression (3), with the collection efficiency included and written as q , representing only the efficiency of the detector and not the entire collection system, is written below expression (4). Subscripts of r and s represent the reference and sample respectively.

$$\sigma_{2,s} = \frac{F_{2,s}(\lambda_{reg})C_r\eta_r(\lambda_{reg})q_r}{F_{2,r}(\lambda_{reg})C_s\eta_s(\lambda_{reg})q_s}\sigma_{2,r} \quad (4)$$

Detector efficiency can be obtained using the manufacturer's data sheet for spectral response using a free program called "Data Thief" which allows the user to set numerical scales to images and determine precise values by dragging a cursor in the user interface over the spectral response curve. The data sheets for all optics discussed in this document are compiled and presented for easy reference in Appendix B.

7.6 Methods: Validation

As suggested by others^[8,38], the use of standards may be a valuable tool in evaluating 2PE cross sections. The parameter space is simple too large to consistently produce results across independent experiments. However, by using Rhodamine B (Rh-B) as a standard and making comparisons to those values, results can be obtained for unknown samples. Makarov suggested his results be used as standards by other researchers and that's what has been done here.

Initial attempts to measure 2PA were performed with Rhodamine 6G (Rh-6G) as the sample. The value obtained was somewhat larger than that reported by Makarov and errors in the experimental setup were immediately suspected. However, it was noticed that Rh-6G 2PA is linear over a range of wavelengths from 820 nm – 860 nm while Rh-B is nearly constant over a similar range of 830 nm – 850 nm. It was then considered that demonstrating the methods and setup being deployed were sound that tuning the laser over a similar range and obtaining a series of measurements for Rh-6G and observing the same linear trend would vindicate the method.

Doing exactly this and taking measurements in 5 nm increments for yielded the results shown below in figure 7.5.

Figure 7.5 Results for Rhodamine 6G over a 20 nm tuning range. Bright yellow markers represent interpolated results for use as reference values.

The linear trend obtained by Makarov is extended beyond the range investigated in this work to make the linear trend over the tuning range more clear. As seen from the results, the method produces results consistent with the observed trend over a tuning range though the numerical values are systematically larger than reported by Makarov^[38]. These systematically high values are consistent once again with the values obtained here for Rhodamine 110 of 40.6 ± 7 GM compared with 32 ± 5.6 GM obtained by Mutze^[35].

This author would like to propose a general approach to making such comparative measurements, that is to say duplicating a reported trend over some tuning range as the more important factor in justifying results than the numerical values as others have reported experimental values different from those obtained by others by factors of 2. Once the Rh-B has been measured at a selected wavelength within the 820 – 860 nm regime Rh-6G should follow. The relative measurement as a function of wavelength should show the same linear dependence that should be in agreement with the literature values to reduce the potential of spurious results misleadingly indicating sound methodology.

7.7 Calculating 2PA and Action Cross Sections

Quantum yields are either taken as values found in the literature if they were determined in the same solvent and at similar concentrations. Values of quantum yields

for the coumarin derivatives were provided as estimates based on values from parent molecules.

Once counts have been measured and tabulated, they are entered into a spreadsheet that calculates values for 2PA using equation (4). When quantum yields were unknown, action cross sections are reported instead.

7.8 Results

The values obtained by these experiments are summarized in the data table below along with concentrations each dye was prepared to.

Fluorophore	Cross Section (GM) @ 845 nm	Quantum Yield	Concentration (μM)	Time Averaged Fluorescence Signal (AU)
Rhodamine B	195.0 ± 35.1	0.8	20	488.68
Rhodamine 6G	47.9 ± 8.6	0.95	20	174.54
Rhodamine 110	40.6 ± 7.3	0.92	20	152.00
Cy3B	208.9 ± 37.6	0.67	20	433.06
Atto 488	40.6 ± 7.3	0.8	20	126.39
074 (Cal Fluor Red)	147.9 ± 26.6	0.8	20	239.85
001x	9.2 ± 1.7	0.68	15	24.43
086AX2	40.7 ± 7.3	0.58	15	80.57
054x	63.8 ± 11.5	0.7	70	839.78
067x	141.7 ± 25.5	0.58	15	328.68
040x	198.5 ± 35.7	0.58	20	503.38
037x	313.6 ± 56.4	0.68	20	865.93
Annulated Xanthene	343.8 ± 61.9	0.2	20	160.10

Table 7.1 2PA in GM with the solution concentrations, quantum yields, and raw signal level. Uncertainties represent 18% of the measured values. All samples prepared in methanol.

Fluorophore	Action Cross Section (cm ⁴ s/photon) @ 845 nm	Concentration (μM)	Time Averaged Fluorescence Signal (AU)
033X	2.4 ± 0.4	400	253.26
084X	3.8 ± 0.7	100	96.43
031X	12.1 ± 2.2	80	254.18

Table 7.2 Two photon action cross sections in cm⁴s/photon with solution concentration and raw signal level.

7.9 Difficulties

The systematic error observed in the values of 2PA for Rhodamine 6G could be due to any number of things. Unfortunately, the parameter space for fluorescence measurements is quite large. Consider that the quantum yield has been shown to vary significantly with solvent, concentration, pH, and temperature. The last one is of particular interest as the temperature in the lab was monitored using a VWR temperature and humidity sensor and swings of as much as 6° C (10° F) were observed. As temperature increases, the quantum yield falls off which would drive the observed 2PA higher and result in systematically large values.

As shown in Bairu^[41] the environment can greatly affect the 2PA further broadening the parameter space, with the 2PA depending on many of the same parameters as quantum yields. Also, as quantum yields and 2PA are so closely intertwined it is difficult even in principle to isolate them from one another given the scope of the parameter space.

7.10 Future Work

Experimentally, a new sample holder is already being designed for better light shielding to ultimately replace the small enclosure. Much like the problems described in chapter 3, it is a goal to reduce the extraneous light collected to a point where the system can be operated with the room lights on. Additional automation could also be done with the addition of a sample tray or turret for sample exchange as well as an integrated power meter for automatic normalization.

Procedurally, it would be interesting to show a trend for each of a handful of dyes over a slightly larger tuning range than that used here for Rh-6G. Alternatively a single wavelength could be chosen for excitation and then the 2PA could be studied as a function of concentration, solvent, and polarization in an attempt to map out an at least somewhat more substantial portion of the overall parameter space.

8 Summary

The construction, application, and characterization of two-photon fluorescence microscopy has been demonstrated with both CW excitation and pulsed excitation. These efforts successfully achieved diffraction limited, z-resolved imaging with image quality of commercially available systems while possessing single molecule levels of sensitivity.

The ultrafast laser was tuned and characterized using a number of methods to describe its output. These efforts included the construction, control, and characterization of an interferometric autocorrelator for the measurement of pulse details.

Finally, a tool set was constructed, validated, and used to measure detailed properties of previously uncharacterized fluorophores.

8.1 Importance

The usefulness of two-photon fluorescence microscopy cannot be understated and as the cost of pulsed light sources comes down it is sure to increase in popularity. Curiously, it has been my experience that despite the number of advantages offered by two-photon microscopy and the fact that it has been used for more than 25 years I regularly meet researchers at conferences who are largely or even entirely unaware of the technique.

The efforts previously published, while covering significant parameter space, largely serve to generate and then expand what amounts to a catalog of fluorophores and the associated 2PA or action cross sections. These efforts have created a substantial and expanding list of fluorophores and properties and this work serves to add no less than fourteen values to that collective body of work.

8.2 Limitations and Suggestions

As has been mentioned, the parameter space involved in 2PA measurements is quite large. 2PA, quantum yield, and even the absorption and emission peaks of a fluorophore depend on concentration, solvent, pH, temperature, and even the properties of the ultrafast pulse. As such it seems that this may be one of the reasons for common disagreement of values when comparing results from one paper (experiment) to the next.

The practical implication of this is simply that each study must be considered in isolation and as the number of fluorophores characterized rises, care should be taken when attempting to apply lessons learned from previous work done by others to one's own.

Instead, it would seem that perhaps the most useful way to use these methods and the data they generate might be to investigate a wide parameter space for a single "family" of fluorophores, dyes with closely related structural properties derived from the same parent molecule. In doing so it may prove more useful to those synthesizing

these dyes as well as the theoreticians attempting to model their behaviors to proactively identify pathways most likely to bear fruit.

Bibliography

- [1] Nowak, D.B., Lawrence, A.J., Sanchez, E.J. *Applied Optics* **49**, 6766-6771 (2010)
- [2] Pillars, D.A.M., Schleiss, M.R. Gentamicin in the clinical setting. *Volta Review* **105** 205-210 (2005)
- [3] Schacht, Jochen, *Otolaryngology Head Neck Surgery*; 118:674-7 (1998)
- [4] Kahlmeter, G., Dahlager, J.I., Aminoglycoside toxicity - a review of clinical studies published between 1975 and 1982. *J Antimicrob Chemother.* **13**, Suppl A:9-22. Jan (1984)
- [5] Miller J.J., Handbook of ototoxicity. *CRC Press*, Boca Raton, FL, (1985)
- [6] Barza, M., Lauermann, M., Why monitor serum levels of gentamicin? *Clin. Pharmacokinet*, **3**, 202–215 (1978)
- [7] Lerner, S.A., Matz, G.J., Suggestions for monitoring patients during treatment with aminoglycoside antibiotics. *Otolaryngology Head Neck Surgery* **87**, 222–228 (1979)
- [8] Albota, M., Xu, C., Webb, W. Two-photon fluorescence excitation cross sections of biomolecular probes from 690 to 960 nm. *Applied Optics* Vol. **37** No. 31, Nov. (1998)
- [9] Bestvater, F., Spiess, E., Stobrawa, G., Hacker, M., Feurer, T., Porwol, T., Berchner-Pfannschmidt, U., Wotzlaw, C., Acker, H., Two-photon fluorescence absorption and emission spectra of dyes relevant for call imaging. *Journal of Microscopy*, **208**, 108-115 (2002)
- [10] Nie, S., & Zare, R.N. Optical detection of single molecules. *Annu. Rev. Biophys. Biomol. Struct.* **26**, 567-596 (1997)
- [11] Zipfel, W., Williams, R., Webb, W., Nonlinear magic: multiphoton microscopy in the biosciences. *Nature Biotechnology* **21** No. 11, (2003)

- [12] König, K., Multiphoton microscopy in life sciences. *Journal of Microscopy* **200**, 83-104 (2000)
- [13] Smith, A., Mancini, M., Nie, S., Second window for in vivo imaging. *Nature Nanotechnology* **4(11)**, 710-711, (2009)
- [14] Helmchen, F., Denk, W., Deep tissue two-photon microscopy. *Nature Methods* **2** No. 12, (2005)
- [15] Denk, W., Strickler, J., Webb, W., Two-photon laser scanning fluorescence microscopy. *Science* **248** Issue 4951, 73-76 (1990)
- [16] Hell, S., Bahlmann, K., Schrader, M., Soini, A., Malak, H., Gryczynski, I., Lakowicz, J., Three-photon Excitation in Fluorescence Microscopy. *Journal of Biomedical Optics* **1(1)**, 71-74 (1996)
- [17] Horton, N., Wang, K., Kobat, K., Clark, C.G., Wise, F., Schaffer, C., Xu, C., In vivo three-photon microscopy of subcortical structures within an intact mouse brain. *Nat. Photonics* **7(3)**, 205–209 (2013)
- [18] Sinefeld, D., Paudel, H., Ouzounov, D., Bifano, T., Xu, C., Adaptive optics in multiphoton microscopy: comparison of two, three and four photon fluorescence. *Optics Express* **23** No. 24, (2015)
- [19] Backlund, M., Lew, M., Backer, A., Sahl, S., Moerner, W., The Role of Molecular Dipole Orientation in Single-Molecule Fluorescence Microscopy and Implications for Super-Resolution Imaging. *Chemphyschem* **15(4)**, 587–599 (2014)
- [20] O'Shea, P., Kimmel, M., Gu, X., Trebino, R., Highly simplified device for ultrashort-pulse measurement. *Optics Letters* **26** No. 12, (2001)
- [21] Naganuma, K., Mogi, K., Yamada, H., "General method for ultrashort light pulse chirp measurement," *IEEE J. Quantum Electron* **25(6)**, 1225-1233 (1989)

- [22] Kleimeier, N., Harlammert, T., Witte, H., Schühle U, Hochedez, J., BenMoussa, A., Zacharias, H., Autocorrelation and phase retrieval in the UV using two-photon absorption in diamond pin photodiodes.
- [23] Ranka, J., Gaeta, A., Baltuska, A., Pshenichnikov, M., Wiersma, D., Autocorrelation measurement of 6-fs pulses based on the two-photon-induced photocurrent in a GaAsP photodiode. *Optics Letters* **22**, No. 17 (1997)
- [24] Chong, E., Watson, T., Festy, F., Autocorrelation measurement of femtosecond laser pulses based on two-photon absorption in GaP photodiode. *Applied Physics Letters* **105**, (2014)
- [25] Hattori, T., Kawashima, Y., Daikoku, M., Inouye, H., Autocorrelation Measurement of Femtosecond Optical Pulses Using Two-Photon-Induced Photocurrent in a Photomultiplier Tube.
- [26] Parys, F., Allard, J., Desmullier, F., Houde, D., Cornet, A., Coherence analysis of diffused femtosecond laser pulses. *Journal of Optics A: Pure and Applied Optics* **6** (2004)
- [27] Hartmann, S., Elsasser, W., A novel semiconductor-based, fully incoherent amplified spontaneous emission light source for ghost imaging. *Nature Scientific Reports* **7** (2017)
- [28] Diels, J.C., Fontaine, J., McMichael, I., Simoni, F., Control and measurement of ultrashort pulse shapes (in amplitude and phase) with femtosecond accuracy. *Applied Optics* **24** No. 9, (1985)
- [29] Avesta Lasers and Optical Systems. What is the Interferometric Autocorrelation
- [30] Sorokina, I., Dvoyrin, V., Tolstik, N., Sorokin, E., Mid-IR Ultrashort Pulsed Fiber-Based Lasers. *IEEE Journal of Selected Topics in Quantum Electronics* **20** No. 5, (2014)
- [31] Silva, A., GRENOUILLE-Practical issues: quick manual. *arXiv:physics/0312134v1[physics.optics]* (2003)

- [32] O'Shea, P., Akturk, S., Kimmel, M., Trebino, R., Practical issues in ultra-short-pulse measurements with 'GRENOUILLE.' *Applied Physics B* **79**, 683-691, (2004)
- [33] Lim, S., Escobedo, J.O., Lowry, M., & Strongin, R.M. Detecting specific sacharides via a single indicator. *Chem. Communication* **47**, 8295-8297 (2011)
- [34] DeArmond et al, In Preparation
- [35] Mutze, J., Iyer, V., Macklin, J., Colonell, J., Karsh, B., Petra, Z., Schwille, P., Looger, L., Lavis, L., Harris, T. Excitation Spectra and Brightness Optimization of Two-Photon Excited Probes. *Biophysical Journal* **102**, 934-944 (2012)
- [36] Xu, C., & Webb, W.W. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J. Opt. Soc. Am. B* **13**, 481-491 (1996)
- [37] Albota, M., Beljonne, D., Bredas, J., Ehrlich, J., Fu, J., Heikal, A., Hess, S., Kogej, T., Levin, M., Marder, S., McCord-Maughon, D., Perry, J., Rockel, H., Rumi, M., Subramaniam, G., Webb, W., Wu, X., Xu, C., Design of Organic Molecules with Large Two-Photon Absorption Cross Section. *Science* **281**, (1998)
- [38] Makarov, N., Drobizhez, M., Rebane, A. Two-Photon absorption standards in the 550-1600 nm excitation wavelength range. *Optics Express* **16**, No. 6 (2008)
- [39] Kaatz, P., Shelton, P., *Journal of the Optics Society of America B* **16**, 998–1006 (1999)
- [40] D. A. Oulianov, D., Tomov, I., Dvornikov, A., Rentzepis, P., *Optics Communications* **191**, 235–243 (2001)
- [41] Bairu, S., Ramakrishna, G., Two-Photon Absorption Properties of Chromophores in Micelles: Electrostatic Interactions. *The Journal of Physical Chemistry* **117**, 10484-10491 (2013)

- [42] Svoboda, K., Yasuda, R., Principles of Two-Photon Excitation Microscopy and Its Applications to Neuroscience. *Neuron* **50**, 823-839 (2006)
- [43] Drobizhev, M., Tillo, S., Makarov, N., Hughes, T., Rebane, A., Absolute Two-Photon Absorption Spectra and Two-Photon Brightness of Orange and Red Fluorescent Proteins. *The Journal of Physical Chemistry B Letters* **113**, 855-859 (2009)
- [44] Albota, M., Xu, C., Webb, W., Two-photon fluorescence cross sections of biomolecular probes from 690 to 960 nm. *Applied Optics* **37**, No. 31 (1998)
- [45] Rebane, A., Makarov, N., Drobizhev, M., Spangler, B., Tarter, E., Reeves, B., Meng, M., Suo, Zhiyou, S., *Quantitative Prediction of Two-Photon Absorption Cross Section Based on Linear Spectroscopic Properties. J. Phys. Chem. C* **112**, 7997-8004 (2008)
- [46] Suarez, M., Ting, A., Fluorescent probes for super-resolution imaging in living cells. *Nature Reviews Molecular Cell Biology* **9**, (2008)
- [47] Saxon, R., Eichler, J., Theoretical calculation of two-photon absorption cross sections in atomic oxygen. *Physical Review A* **34**, No. 1 (1986)
- [48] Liu, X., Guo, J., Ren, A., Huang, S., Feng, J., Theoretical Prediction of One- and Two-Photon Absorption Properties of N-Annulated Quaterrylenes as Near-Infrared Chromophores. *The Journal of Organic Chemistry* **77**, 585-597 (2011)
- [49] Clay, G., Schaffer, C., Kleinfeld, D., Large two-photon absorptivity of hemoglobin in the infrared range of 780-880 nm. *The Journal of Chemical Physics* **126**, (2007)
- [50] Jha, P., Wang, Y., Agren, H., Two-Photon Absorption Cross-Sections of Reference Dyes: A Critical Examination. *ChemPhysChem* **9**, 111-116 (2008)
- [51] A. Karotki, M. Khurana, J. R. Lepock, and B. C. Wilson, "Simultaneous two-photon excitation of photofrin in relation to photodynamic therapy," *Photochem. Photobiol.* **82**, 443-452 (2006)

Appendix A: 3D Printed Optical Components

The listed components are used in the experimental setup either in place of Thorlabs cage type components or when no such component is available. All designs were made in Autodesk Inventor Professional which is freely available for download and install for students. All parts are shown with general dimensions As 3D printed components however, liberties can be taken when designing features that while easy to print may be difficult or impossible to create in a lathe or mill but full dimensioning is needed for manufacturing metal components. Test components and final designs used in the experiment were printed and tested using PLA. Full details, Autodesk Inventor drawings, full dimensions, and print files are available for download along with all associated details and applications notes at thingiverse.com/Freds_Optix_Hardware.

0.5" Post Holder with Pedestal Base

0.5" Post Holder with Slotted Base - Needs Redone

0.5" Post Clamp

3" Table Clamp

2" Table Clamp

1" Lens Tube Clamp Top Half

1" Lens Tube Clamp Bottom Half

1" Lens Tube Cap

Clip-On Light Shield

Dynamic Cube Light Shield

Cuvette Holder

Appendix B: Optics and Detector Data Sheets

Spectral response curves for all optics used in the experiments described in this document are compiled here for easy reference to assist experimental calibration.

- ET 750SP Two Photon Filter (Chroma)
- ET 700SP Two Photon Filter (Chroma)
- 725 DCSPXR Dichroic Short Pass Mirror (Chroma)
- R785LP Long Pass Excitation Filter (Chroma)
- 69220 Dichroic Short Pass Mirror (Edmund Optics)
- G1115 GaAsP Photodiode (Hamamatsu)
- R928 Photomultiplier Tube (PMT) (Hamamatsu)
- SPCM-AQR Single Photon Counting Module (Perkin Elmer)

Figure B.1 Transmission spectrum of ET 750SP two-photon emission filter. (Chroma)

Figure B.2 Transmission spectrum of ET 700SP two-photon emission filter. (Chroma)

Figure B.3 Transmission spectrum of 725 DCSPXR dichroic mirror. (Chroma)

Figure B.4 Transmission spectrum of 69220 dichroic mirror. (Edmund)

Figure B.5 Transmission spectrum of R785 long-pass. (Chroma)

GaAsP photodiode

▣ Spectral response

Figure B.6 Spectral response of G1115 gallium arsenide phosphor photodiode.
(Hamamatsu)

Figure B.7 Hamamatsu R928 PMT sensitivity.

Figure 4. Photon detection efficiency (pd) vs. wavelength

Figure B.8 Perkin Elmer single photon counting module response curve.

Appendix C: Compiled Spectra, Structures, and 2PA

The emission spectra were acquired using a Photon Technology International Quanta 300. All values for cross sections and action cross sections were obtained using a 25 fs pulse generated by a Ti:SAPPH laser with a central wavelength of 845 nm. All samples were prepared by serial dilution of 20 ml of stock solution of 10s of mM concentrations down to μM concentrations at or around 20 μM . Uncertainties in cross sections are 18%. All absorption curves are displayed in blue while the emission is displayed in orange. All data is normalized. The sole exception to these rules is the Xanthene which was measured in 7.4 pH solution, and the traces shown are not normalized to compare the multiple traces. Those data were provided by the Strongin lab.

Cal Fluor Red 635 (074)

Absorption Peak (nm)	590
Emission Peak (nm)	630
Stokes Shift (nm)	40
Cross Section (GM)	147.9 ± 26.6
Quantum Yield	.8

Description: Commercially available Cal Fluor Red 635 Pyridinium salt.

037X

Absorption Peak (nm)	418
Emission Peak (nm)	486
Stokes Shift (nm)	68
Cross Section (GM)	313.6 ± 56.4
Quantum Yield	.68

Description: Derivative of Coumarin 314

Rhodamine B

Absorption Peak (nm)	540
Emission Peak (nm)	570
Stokes Shift (nm)	130
Cross Section (GM)	195 ± 35.1
Quantum Yield	.8

Used as a calibration standard.

Rhodamine 6G

Absorption Peak (nm)	520
Emission Peak (nm)	560
Stokes Shift (nm)	50
Cross Section (GM)	47.9 ± 8.6
Quantum Yield	.95

Used as a validation of the method by comparing to Rhodamine B

001X

Absorption Peak (nm)	365
Emission Peak (nm)	450
Stokes Shift (nm)	85
Cross Section (GM)	9.2 ± 1.7
Quantum Yield	.68

Description: Derivative of Coumarin 2

086AX2

Absorption Peak (nm)	385
Emission Peak (nm)	465
Stokes Shift (nm)	80
Cross Section (GM)	40.7 ± 7.3
Quantum Yield	.58

Description: Derivative of Coumarin 102T

Xanthene (MSV 08-134F6-7)

Absorption Peak (nm)	550
Emission Peak (nm)	650
Stokes Shift (nm)	100
Cross Section (GM)	343.8 ± 61.9
Quantum Yield	0.2

Data Courtesy of the Strongin Lab, Department of Chemistry, Portland State University

033X

Absorption Peak (nm)	300
Emission Peak (nm)	455
Stokes Shift (nm)	155
Action Cross Section (cm ⁴ s/photon)	2.4 ± 0.4

Description: Derivative of Coumarin 2

084X

Absorption Peak (nm)	355
Emission Peak (nm)	455
Stokes Shift (nm)	100
Action Cross Section (cm ⁴ s/photon)	3.8 ± 0.7

031X

Absorption Peak (nm)	355
Emission Peak (nm)	465
Stokes Shift (nm)	110
Action Cross Section (cm ⁴ s/photon)	12.1 ± 2.2