

Nonlinear optics and two-photon microscopy

Sam Whiteley and Seth Parker

PHYS 173/BGGN 266

July 13, 2014

Table of contents

1. Introduction
2. Optical setup
3. Initial images and troubleshooting
4. Determining scalebar for microscope
5. Imaging other fluorescent medium
6. Point spread function
7. Tips for future two-photon projects
8. References

Introduction

Absorbance is a process by which photons are absorbed by molecules causing electrons to excite to a higher electronic energy state. Electrons can absorb varying amounts of energy depending on the energy of incident photons. Due to the vibrational degeneracy of energy states, electrons that absorb higher amounts of energy will excite to a higher degenerate state than those that absorb lower energy. One means through which the energy absorbed by excited electrons can be dissipated is through non-radiative vibrational relaxation, which occurs on the order of femto- to pico- seconds. This dissipation causes electrons to decrease in energy to the lowest quantum state of the excited electronic energy state. Fluorescence is caused by an excited electron releasing a photon and decreasing in energy to one of the quantum states of the ground electronic state, which occurs on the order of nanoseconds. This process is demonstrated by the Jablonski diagram, below (Figure 1).

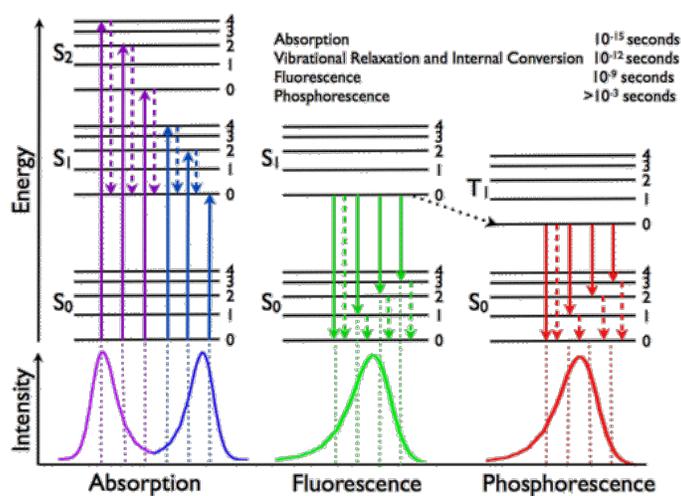


Figure 1. The Jablonski Diagram (adapted from Visser A.J.W.G. and Rolinski O.J.).

Fluorescence is commonly used in microscopy as a means to visualize specific biological molecules or proteins in cells and tissues. The most common type of fluorescence microscopy is epifluorescence microscopy, which utilizes a light source and excitation filter to allow light of a specific wavelength range to excite a biological sample. Fluorescent light emitted by the sample is reflected by a dichroic mirror to direct light through an emission filter and either an eyepiece or camera. Emission filters are used to remove light of wavelengths other than the fluorescent wavelength through to the imaging apparatus. One downside of this approach is that single photon absorption is linear with incident light intensity; therefore, the observed fluorescence is accumulated from several positions along the z-axis. Several more advanced techniques have been developed that enable optical sectioning in the z-axis, including laser scanning confocal and two-photon microscopy. Two-photon microscopy utilizes a process whereby two photons of higher wavelength have an equivalent energy to a single photon sufficient to excite an electron to a higher electronic energy state. Thus, similar fluorescence emission is obtained

when using one, two, or even three photons of differing energies (Figure 2). Two photon absorption is non-linear, causing the probability that fluorescent events occur outside of the focal plan to decrease dramatically (Figure 3). In this project, we aim to utilize a Ti:Sapphire laser to design and build a two-photon microscope. We will image fluorescein on filter paper in order to evaluate the quality of our laser alignment and optical setup. Then, we will image DAPI-stained cheek cells and attempt to obtain a z-stack of fluorescein on filter paper. Additionally, we will characterize both the scale and point spread function of our microscope.

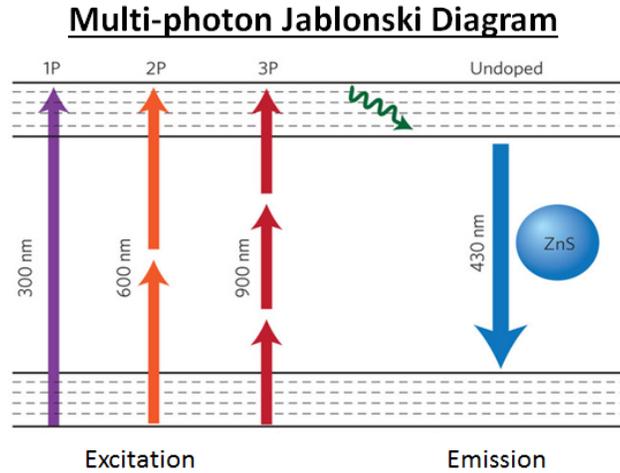


Figure 2. Jablonski diagram for one, two, and three photon absorption and emission. (Adapted from Zagorovsky K. and Chan W.C.W.).

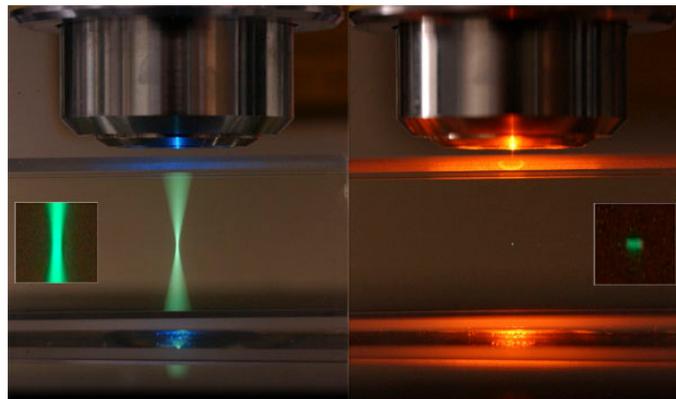


Figure 3. Two-photon (right) optical sectioning along the z-axis compared to conventional epifluorescence (left). Image by Steve Ruzin and Holly Aaron at UC Berkeley.

Optical setup

Initially, a handheld green laser was mounted to the optical bench and used to align the mirrors (Figure 4). Silver mirrors were used to guide the beam through two irises, used to verify optical alignment, and along a vertical stand towards the scanning mirrors and objective. With the green laser correctly aligned, the Ti:Sapphire laser was initially mode-locked with the help of Dr. Phil Tsai and aligned through the irises to follow the same optical path as the green laser. Mode locking is one method through which laser energy is delivered as short pulses of approximately 10-100 fs. This allows very high peak intensities, increasing the probability for two photon absorption events to occur. Similar intensities can be delivered by increasing the output power of the laser, but this will drastically increase the chance of sample ablation and photobleaching; therefore, pulsed lasers are preferable to achieve sufficient two photon absorption for fluorescent microscopy. A flip mirror was used to switch between Ti:Sapphire laser and handheld green laser. The optical setup was carried out using the green laser for safety.

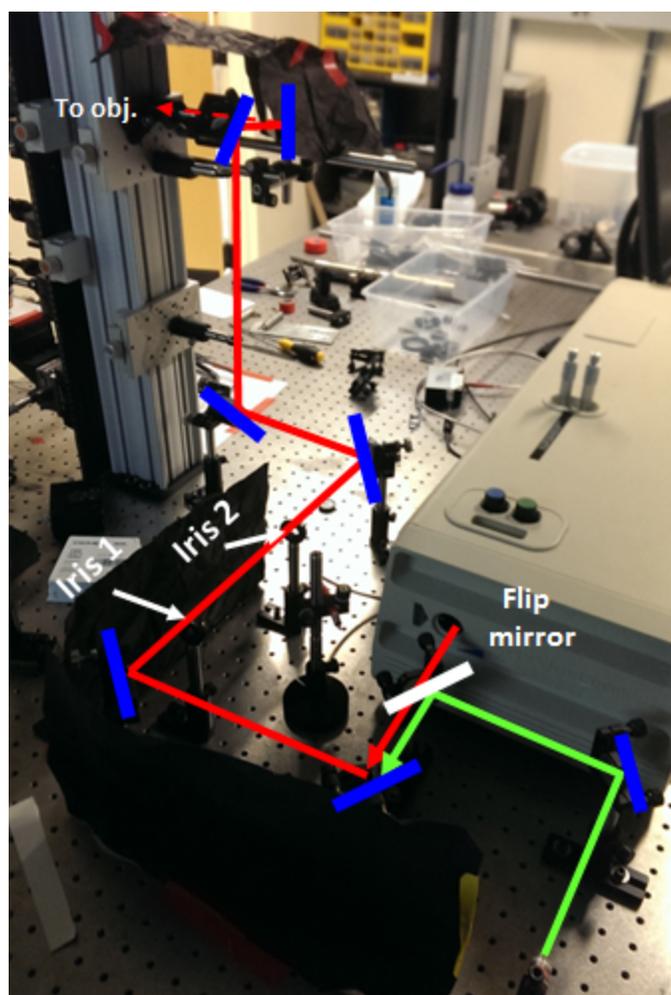


Figure 4. Optical alignment of green and Ti:Sapphire laser. Blue denotes mirrors; green is the light path of a green laser pointer used for safety; red is the light path from the Ti:Sapphire laser system.

Laser light was aligned to the scan mirrors and through a set of lenses to expand the laser beam (Figure 5). The factor by which the laser beam was expanded is determined by the ratio of the tube to scan lens focal lengths.

$$\frac{D_{out}}{D_{in}} = \frac{f_{tube}}{f_{scan}}$$

Focal lengths were chosen such that the beam was expanded by approximately 3.5 fold in order to fill the entire back aperture of the objective (Figure 6). A 40x water immersion objective (0.8NA, infinity corrected) was aligned to the laser path. A stage was constructed that allowed for fine adjustment in the z-axis and rough adjustment along both x- and y- axes. A dichroic mirror was placed above the objective to reflect fluorescent light towards the photomultiplier tube (PMT). A benchtop spectrophotometer was used to characterize the dichroic to ensure the transmittance wavelength range was correct for the desired applications. Light reflected towards the PMT was concentrated by a collecting lens and passed through an emission filter to remove background light. Current generated by the PMT was converted to voltage and sent to the computer for image collection.

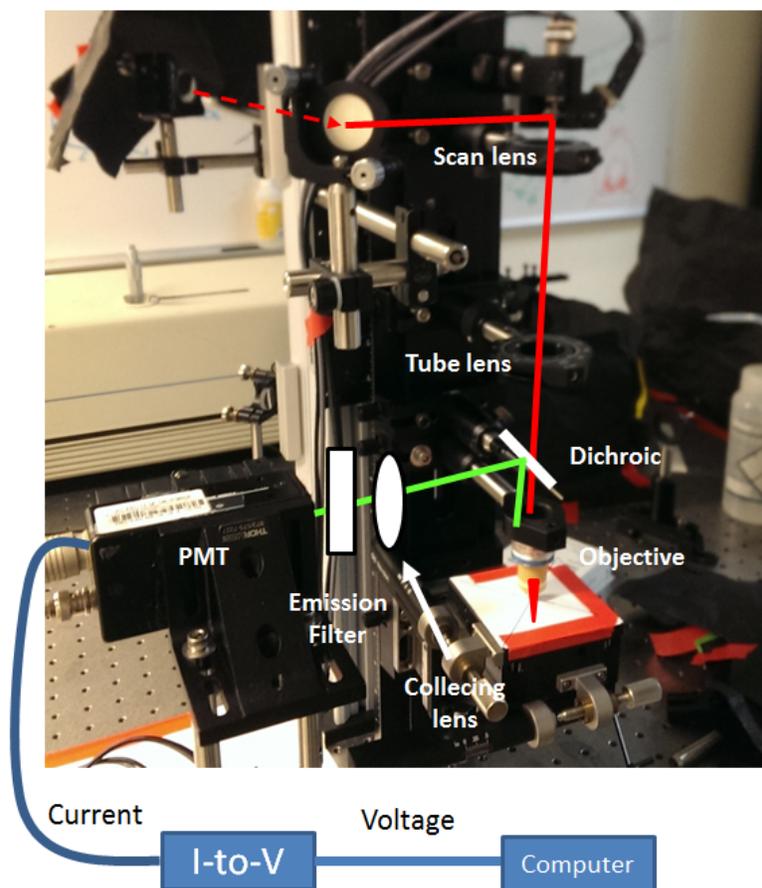


Figure 5. Optical setup including scanning mirrors, beam expander, objective, dichroic mirror, collecting lens, and photomultiplier tube (PMT).

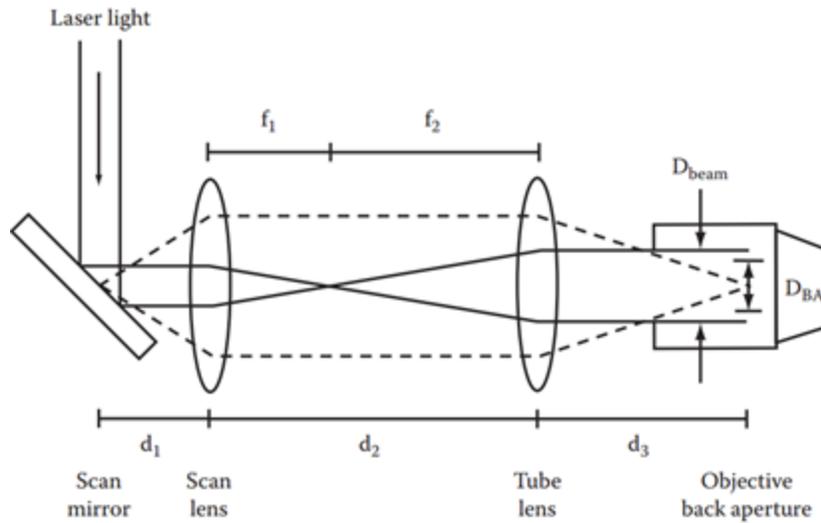


Figure 6. Cartoon of beam expander setup. d_1 was set f_1 away from the scan mirror, and d_3 was set f_2 away from the objective back aperture. d_2 was set to the sum of f_1 and f_2 . Taken from Tsai and Kleinfeld, 2009.

Initial images and troubleshooting

Images were obtained for fluorescein on filter paper (Figure 7). Long lateral smearing of imaged individual fibers indicate that the time constant of the IV converter was too long. Upon inspection, the RC pair was such that the time constant was 20 milliseconds. The IV converter was replaced with one with a time constant of 2 μ s and better images were obtained (Figure 8).

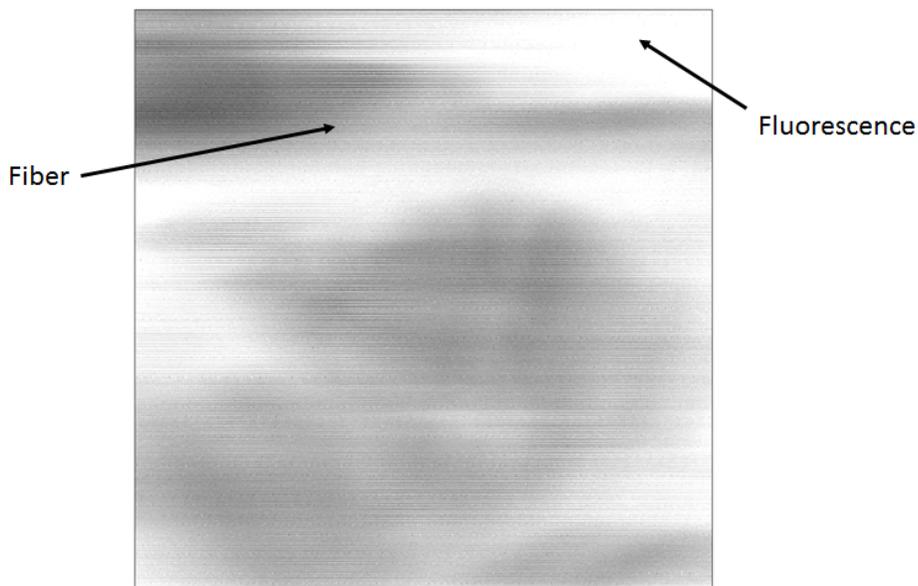


Figure 7. Initial image of filter paper with fluorescein indicating problem with IV converter.

A circular dark spot was observed in the following images indicative of a problem with the alignment somewhere along the optical path (Figure 8). Upon investigation, the dichroic was off alignment and fluorescent light reflected off the mirror was partially hitting the edge of the collecting lens. The dichroic was correctly aligned and images were improved (Figure 9).

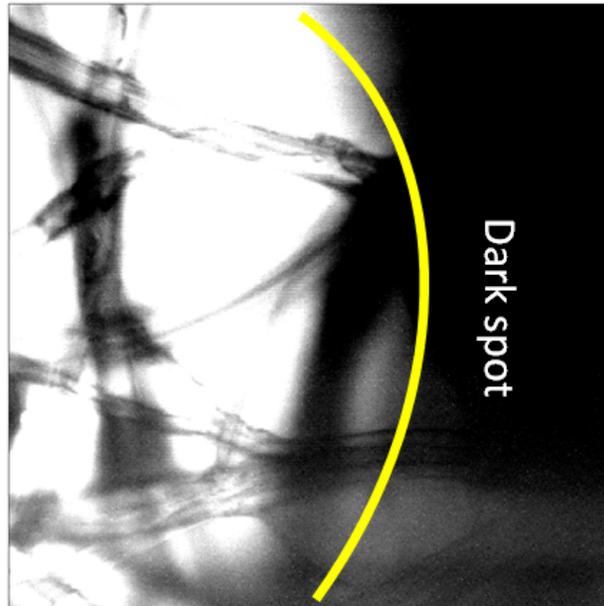


Figure 8. Image of filter paper with fluorescein. Dark spot indicative of problem with optical alignment.

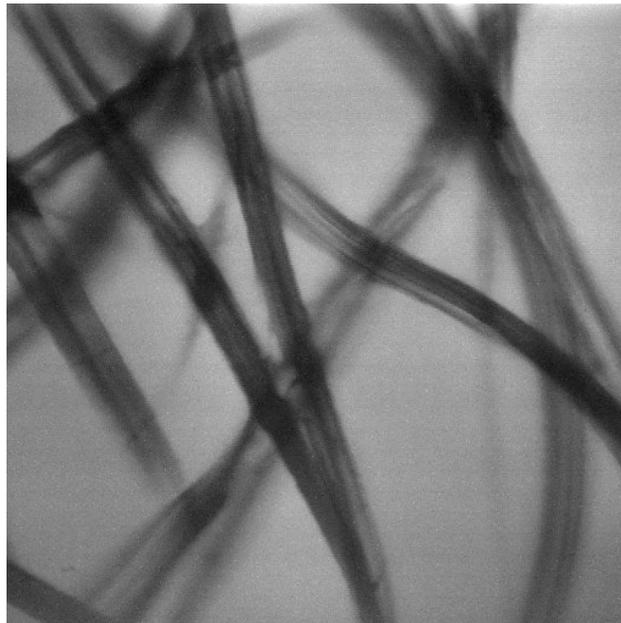


Figure 9. Resulting image of filter paper with fluorescein with correct IV converter and proper dichroic alignment.

Determining scale bar for microscope

In order to determine the scale of our microscope (in $\mu\text{m}/\text{pixel}$), images of filter paper soaked with fluorescein were taken before and after small (50 and 100 μm) displacement using the fine x-adjustment (Figure 10). Several points were measured manually using ImageJ and Adobe Photoshop CS5 to determine pixel shifts after displacement. Samples were slightly off center resulting in diagonal displacement. The scale at 1x, 2x, and 2.5x scan mirror voltages were measured and 5x was estimated from a nonlinear fit of measured data (Table 1).

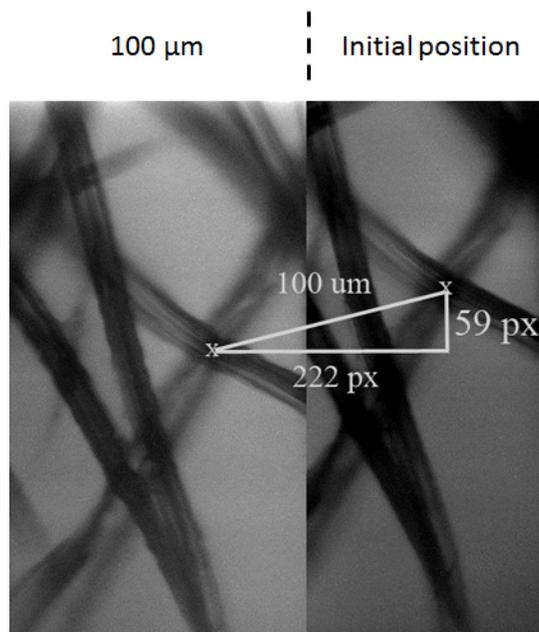


Figure 10. Determining scale of our microscope at varying scan mirror voltages. Images before and after 100 μm displacement for 1x scan mirror voltage shown above.

Table 1. Scale in $\mu\text{m}/\text{pixel}$ at varying scan mirror voltages.

Scan Mirror	Scale ($\mu\text{m}/\text{px}$)	Std. Dev. ($\mu\text{m}/\text{px}$)
1x	0.43	0.01
2x	0.21	0.01
2.5x	0.17	0.005
5x	0.08	-

Imaging other fluorescent medium

In addition to fluorescein on filter paper, we also labeled cheek cells with 4,6-diamidino-2-phenylindole (DAPI) (Figure 11). DAPI is a commonly used stain in fluorescent microscopy ($\lambda_{\text{emission}} = 461 \text{ nm}$) that is capable of crossing the cellular membrane in live or fixed cells and binds to DNA. The one-photon absorption is 358 nm and was readily excited by our two-photon microscope.

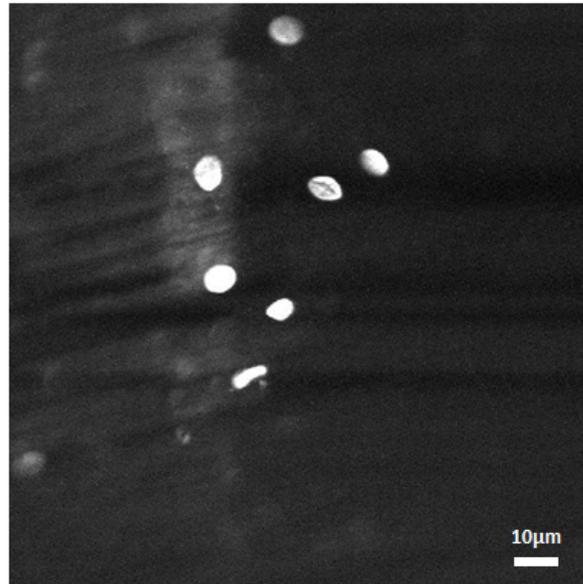


Figure 11. Cheek cells stained with DAPI (1x).

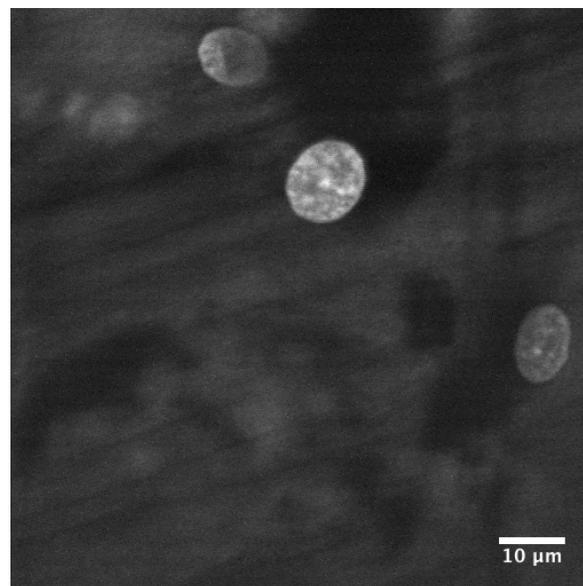


Figure 12. Cheek cells stained with DAPI (2.5x). Internal cellular structure is visible.

Point spread function

There is a fundamental limit for the resolution of light microscopes due to diffraction. This limit is estimated using the Abbe's resolution limit equation, given below (Li, Q. et al.).

$$\Delta x = \frac{\lambda}{2NA}$$

The Ti:Sapphire laser used was mode-locked with a maximum emission wavelength at approximately 780 nm; therefore, the resolution limit of our microscope is $\sim 0.5 \mu m$. While in theory a point stimulus should always return a gaussian-shaped PSF, non-isotropic PSFs can occur from low pass filters (smearing in one dimension), poor optical alignment, and quality.

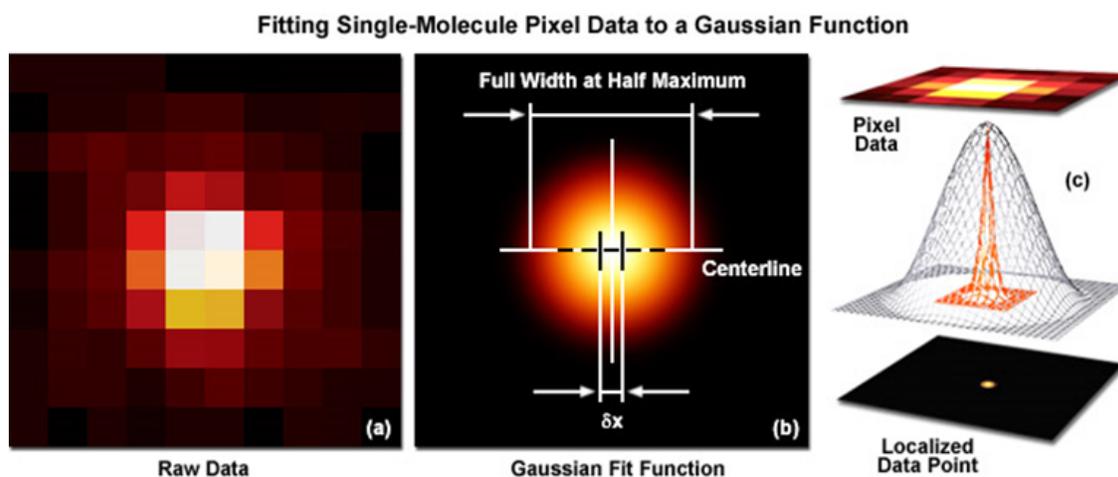


Figure 3

Figure 13. A microscope's point spread function in 2-dimensions is the result of a diffraction limited focus scanning over a point source. (a) The point spread function is visible in the image. (b) Any approximate point source, of size δx , will produce a point spread function.

The point spread function of our two photon microscope was measured with $0.2 \mu m$ fluorescent beads. The beads were fixed to a slide with ethanol, protected with a coverslip, and imaged with multiple magnifications (1x, 2x, 2.5x) (Figure 14).



Figure 14. Images of individual $0.2 \mu m$ fluorescent beads at varying scan mirror voltages.

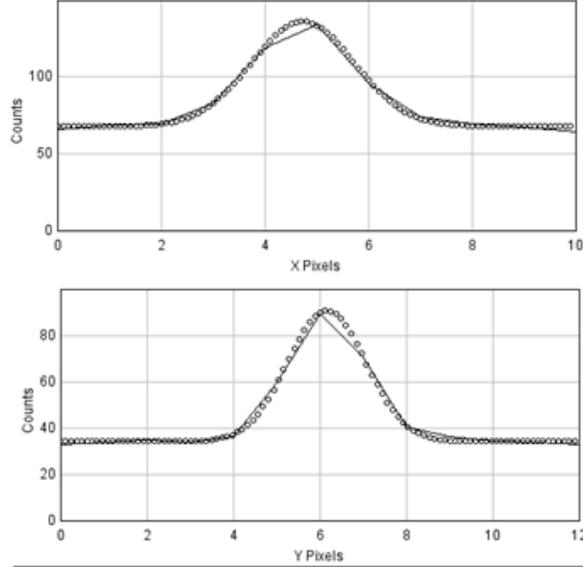


Figure 15. Example of FWHM gaussian fits for x- and y- PSFs.

Table 2. Point spread function in x- and y- direction at 1x, 2x, and 2.5x scan mirror voltages. The FWHM for three independently imaged beads were used to quantify the standard deviation.

Scan (N=3)	PSF _x (μm)	SD _x (μm)	PSF _y (μm)	SD _y (μm)
1x	0.95	0.07	0.44	0.07
2x	0.70	0.08	0.47	0.03
2.5x	0.53	0.05	0.34	0.07

Interestingly, the PSF in the x-direction was larger than in the y-direction. Initially, we believed this was caused by aberrations in our optical setup; however, after discussing with Dr. Tsai we now believe it is due to the frequency of the IV converter. As the laser rasters in the x-direction, the IV converter is integrating the signal collected over 2 μs; therefore, some of the signal from the previous pixel is smeared across other pixels in the x-direction. By the time the laser reaches the next pixel in the y-direction, the signal from the previous x-slice is dumped causing this smearing not to affect the PSF in the y-direction. As the scan mirror voltages increase, the x- and y- PSFs begin to converge supporting this hypothesis.

Tips for future two-photon projects

In this project, we were able to quantify the 2-dimensional point spread function for our microscope. Future projects should try to quantify the 3-dimensional point spread function. For this, fine control of the z-axial position of the stage using a piezoelectric motor is needed. Furthermore, proper air cushioning of the optical bench is required in order to eliminate small vibrational movements. Additionally, the X-Y point spread functions should be measured with 2.5x or 5x magnification to obtain the most accurate full width at half maximum.

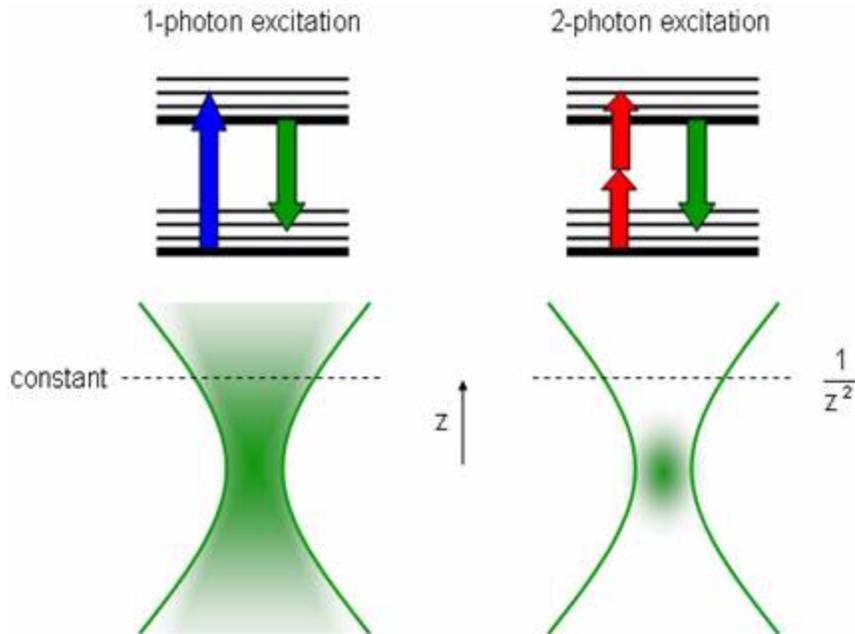


Figure 16. Axial illumination and point spread functions of single photon excitation (left) versus multi-photon excitation (right). The total luminosity in single photon imaging techniques is constant over the axial dimension (z) whereas with two photon excitation luminosity decreases as $1/z^2$.

Additional project topics with a constructed two photon include but are not limited to second harmonic generation, more biological data, in vivo imaging, and two-photon cross sections for new dyes.

References

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