

Introduction and Background

Two-photon microscopy is a type of fluorescence microscopy using two-photon excitation. It has been widely used in neuroscience because it can image internal structures of the brain at fine resolution.

Principle of fluorescence microscopy

Molecules that emit fluorescence are called fluorophores. These molecules have multiple electron orbits, which are approximately spaced by the energy of a photon of visible light. The electrons of fluorophores are usually in low-energy orbits, but they go into high-energy orbits by absorbing a photon. The probability of absorption depends on the wavelength, which is called the excitation spectrum. After absorbing a photon, first the electron relaxes to other orbits with slightly lower energy, and then goes back to the lower-energy orbits by emitting light. The wavelength of emission is probabilistic, and follows the emission spectrum. Importantly, the emission spectrum has a longer wavelength than the excitation spectrum because of the relaxation before going back to the lower-energy orbits. Using dichroics to dissociate excitation and emission wavelengths, fluorescence microscopy can achieve higher contrast than conventional microscopy using the same wavelength of light. In addition, the excitation and emission spectrum is specific to a fluorophore, which also leads to higher contrast.

Two-photon microscopy

Instead of using a light within the excitation spectrum, two-photon microscopy utilizes a light with twice as long a wavelength. Two photons with half the energy can be simultaneously absorbed by a fluorophore to elicit fluorescence. As the probability of this phenomenon is proportional to the square of the light intensity, the intensity of fluorescence emission drops biquadratically to the distance from the focal plane, which is faster than the quadratic decrease of single-photon excitation. With this smaller focal volume, an image with finer resolution can be obtained. Furthermore, a light with a longer wavelength is less scattered, which enables imaging deeper planes of the tissue.

Methods

System overview

We followed closely the design principles previously described (Tsai and Kleinfeld, 2009, overview shown in Figure 1). Briefly, a Ti:sapphire laser was pumped and mode-locked to emit a spectrum of light centered at 780nm. An optical path was designed that split the 500mW beam into two beams so that another group could construct a similar system in tandem. The beam was then wrapped around a vertical tower via a system of mirrors. A set of scanning mirrors directed the beam down through a series of lenses to expand the beam in order to completely fill the back aperture of the objective. Light was collected from a sample through the objective, reflected off a dichroic, passed through lenses, and directed into a photomultiplier tube (PMT). The PMT signal was converted to a voltage signal, and read out by a data acquisition board into a PC computer running custom imaging software.

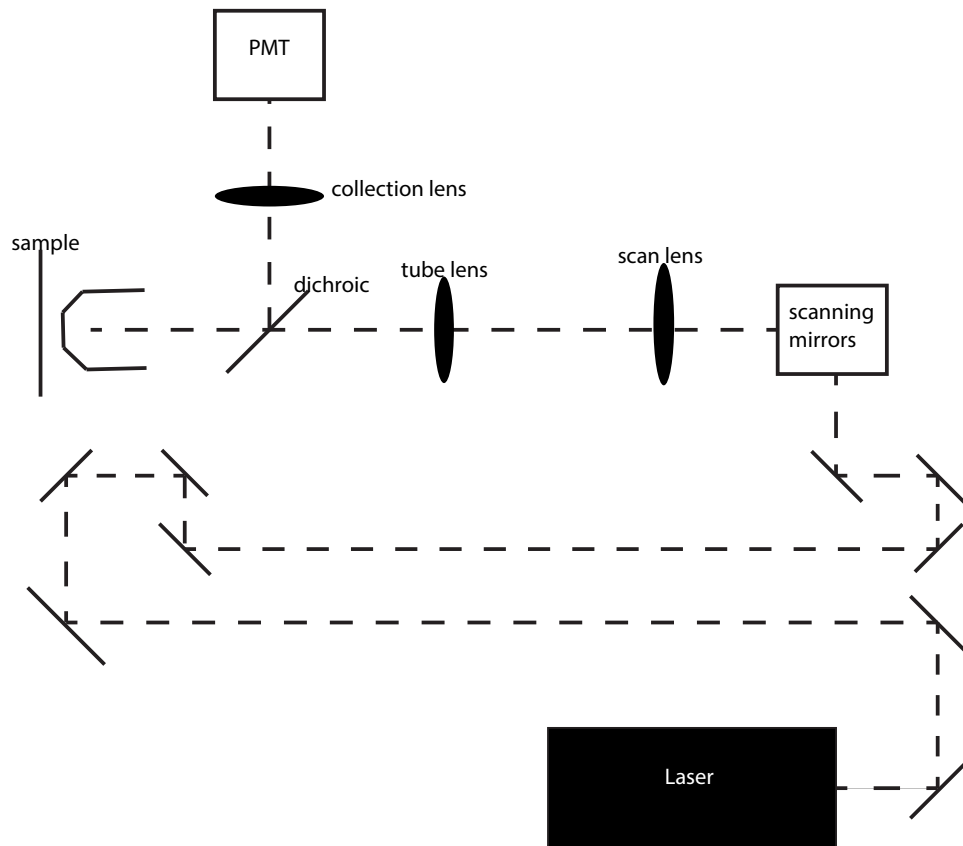


Figure 1 System overview. Dashed lines indicate optical path. Solid lines indicate reflective surfaces (mirrors or dichroics).

Laser and optical path

We used a Spectra Physics Millenia V diode-dumped, CW visible laser to pump a spectra Physics Mode-locked Ti:sapphire laser. The difference in intensity profile between a mode-locked and non-mode locked laser is significant (see *Troubleshooting* section for more information on mode-locking and Figure 2).

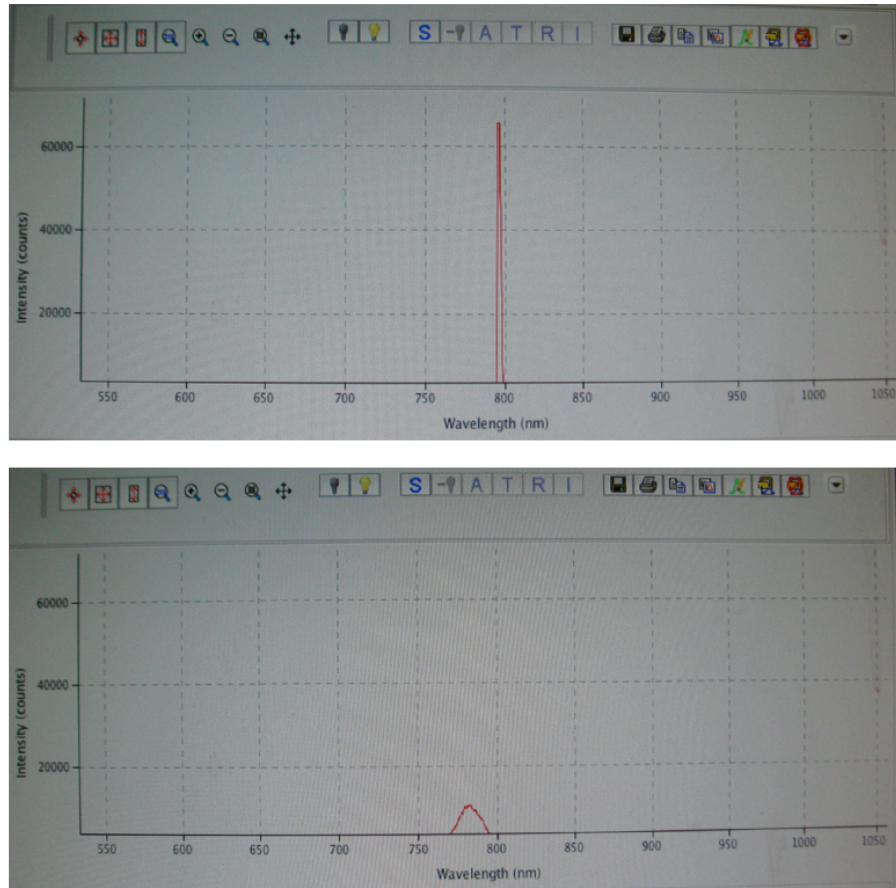


Figure 2 Laser mode-locking. (Top) Laser not mode-locked. Intensity distribution is peaked. (Bottom) Mode-locked laser displaying significantly broadened intensity distribution.

The power of the laser drops off as it traverses the optical path (see *Troubleshooting* section for more information on losing beam power across the scanning mirrors, and Figure 3). The path was sequentially aligned using a two-mirror two-aperture method to center the beam on the succeeding point in the pathway. The proximal mirror was used to fit the beam through the proximal pinhole and the distal mirror was used to fit the beam through the distal pinhole. After bringing the beam to the top of a vertical tower it was bent at 90 degrees by the scanning mirror system. It then passed through a scan lens (focal length 50.8mm) and a tube lens (focal length 76.2mm), the purpose of which were to collimate the beam for an infinity-corrected 20X Ph1 Zeiss water-immersion objective. The constraints on path length and the placement of the objective and lenses is previously described (Tsai and Kleinfeld, 2009) but briefly, the scan lens is placed to optimally expand the beam, the tube lens collimates the beam coming out of the scan lens, and together they create an imaging condition between the scan mirror and the back aperture of the objective. Optimal expansion of the beam depends on the diameter of the back aperture and the characteristics of the beam, which is Gaussian in its intensity profile. Ideally the

full width at half maximum is taken as the diameter of the beam, which is then expanded to slightly overfill the back aperture.

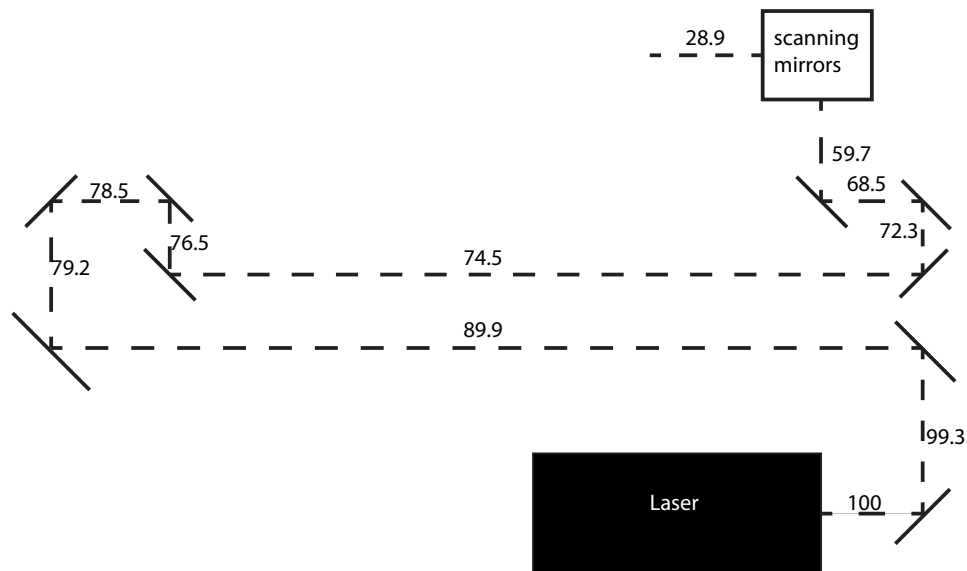


Figure 3 Percentage of maximal intensity of laser beam at different points along the optical path (normalized to 100 at the laser outlet). Measured by a handheld Coherent FieldMate Laser Power Meter.

Epi-fluorescence collection is used to direct light from the sample to the PMT. Collected light is collimated coming out of the objective, and reflected off of a dichroic that passes excitation wavelengths down to the sample and reflects emission wavelengths towards the detector apparatus. It passed through a collection lens (focal length 50.2mm), the purpose of which is to create an imaging condition between the back aperture of the objective and the active surface of the PMT, as well as to magnify the beam to fit the active surface of the PMT. In our system, given our lens setup, beam profile, and detector sizes, the path length from back aperture to collection lens was 101.6mm and from collection lens to detector was 6.5mm.

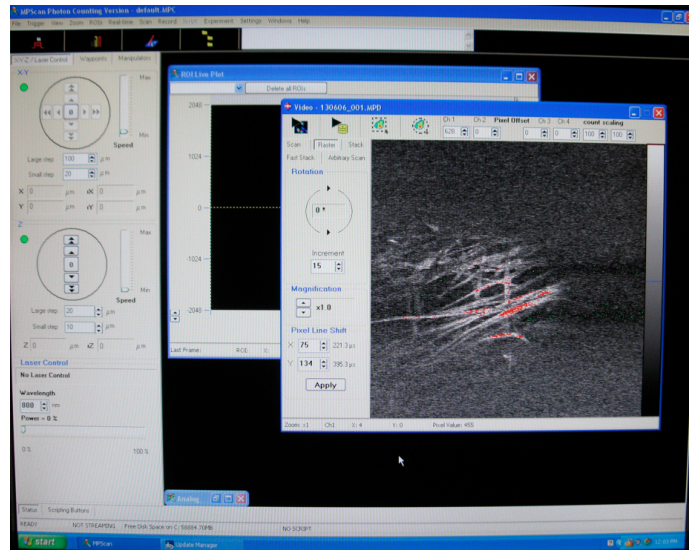
Scanning system

We used a set of open-loop X-Y scanners from Midwest Laser to complete X-Y scanning. Initially we assumed that these were silvered mirrors, but later learned that they are dielectric mirrors (see *Troubleshooting* section for more information). Each mirror had its own control board, which was powered by a custom power box Figure 4A), and received analog inputs from a National Instruments (NI) BNC-2090 attached to a PC. Output control from the PC was done through the custom program MPScope 2.0 (Figure 4B). Adjusting the “Maximal Mirror Command Voltage” changed the amplitude of the scan, and adjusting the frame rate or magnification changed the speed of the scan.

A



B



C



D

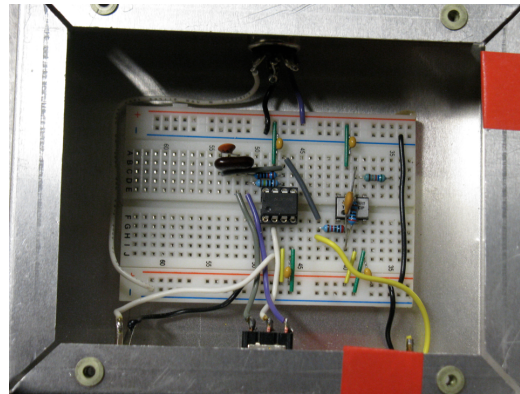


Figure 4 Custom electronics and software. (A) Power supply to drive the actuators in the scanning mirrors. (B) Screen shot of MPIScope 2.0 showing an example of a real-time image. (C) Power supply for the PMT. (D) I to V converter and pre-amplifier that sits between the PMT and the data acquisition board.

Detection electronics and software

We used a Hamamatsu GaAsP H7422 PMT for epi-fluorescence detection and powered it with a custom power supply (Figure 4C). A laser-blocking filter was mounted on the detector to reduce background from scattered photons. The PMT output was fed into a custom I-to-V converter box (see *Troubleshooting* section for more information and Figure 4D), where it was also amplified, and then into the same NI BNC-2090 and PC computer. The output was read out by MPScope and displayed in a window that allowed real-time manipulation of the image (Figure 4A).

Sample preparation

We prepared a sample for testing the microscope using thin lens paper, which has a fine microstructure clearly visible under 20X and 40X objectives. The paper was dipped in fluorescein, which should emit fluorescence with two-photon excitation in the 750-800nm range. The fluorescein paper was then placed under a 1.5 coverslip on a glass slide and sealed with nail polish. The paper must be dry after dipping in fluorescein so that the fluorescein molecules are forced to adhere to the paper and not remain dissolved in water.

Results

Two-photon fluorescence from fluorescein

We succeeded in taking an image of a lens paper with fluorescein dye (Figure 5).

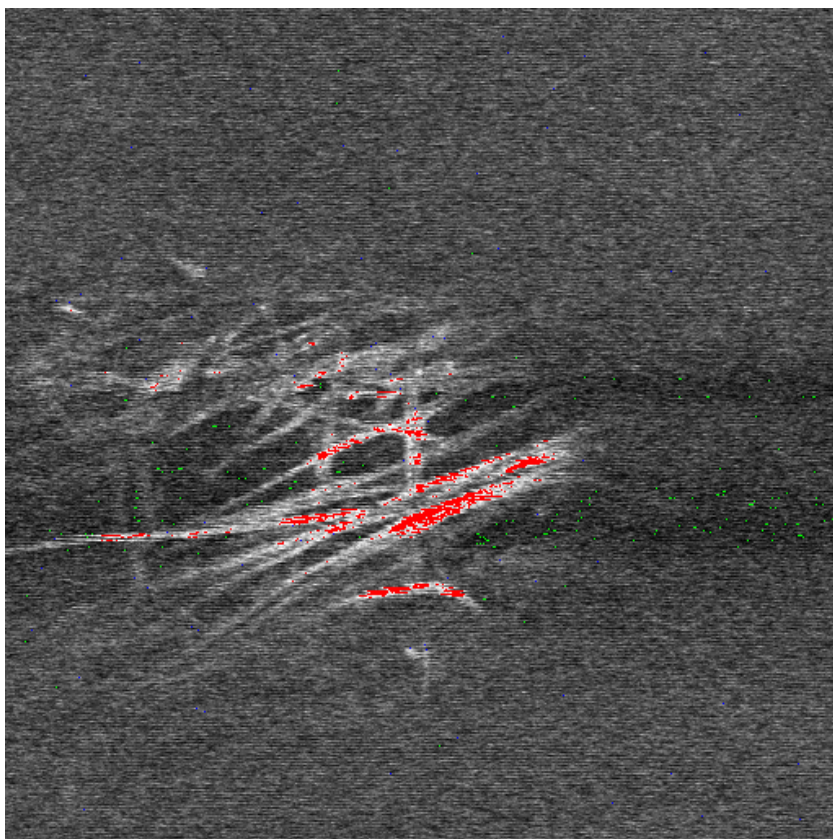


Figure 5 Image of a lens paper with fluorescein dye taken with two-photon excitation microscopy.

Discussion/Troubleshooting

Finally we could image the fibers of a paper with our two-photon microscope. At least at the center of the image, we could identify each fiber. However, the signal intensity and the contrast dropped rapidly at the peripheral of the image. We could not completely solve this problem, but this could be partly because of misalignment in the optical path. For example, if the back aperture of the objective is not exactly at the imaging plane of the scanning mirrors, the laser moves around and does not fill the back aperture properly when imaging peripheral pixels. The same could happen when the PMT is not at the image of the back aperture.

Here are some other problems and solutions we came up with during setting up the system:

1. Mirrors/pinholes were not fixed properly.

Optical path alignment requires sequential alignment of mirrors. One day after finished the alignment, we noticed the first few mirrors and pinholes were loose. We had to tighten them and align the rest again.

2. Battery of laser pointer was not easy to remove.
The laser pointers used for alignment require two AA batteries. The second one was not easy to replace. We taped the two batteries together so that they come out together.
3. Some parts required metric screws.
We had to be careful not to use different screws.
4. Scanning mirror was broken.
It worked properly at the beginning, but failed to work at high frequencies. We had to replace the scanning mirror. The broken one was labeled as broken.
5. I-V converter had too long time constant.
First, the image taken had horizontal lines (Figure 6). This might be because the I-V converter had too long time constant and blurred the image along the axis of scanning. We replaced the I-V converter box.

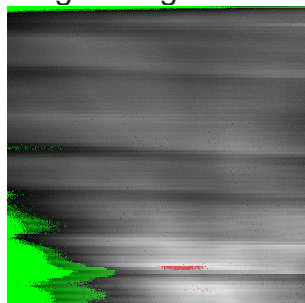


Figure 6 Example image taken with a improper I-V converter.

6. Dichroic mirror was not reflecting fluorescence.
After we switched from single-photon setup with green-laser pointer to two-photon setup with Ti:Sapphire laser, we had to switch to a different dichroic mirror because the wavelength was different.
7. Scanning mirror was not reflecting laser.
Because the scanning mirrors were not silvered mirrors but dielectric mirrors, they did not reflect the infrared laser properly, and large portion of the laser power was lost through the mirrors. To compensate that, we took out the beam splitter and used all the laser power in this setup rather than sharing a laser with the other group.
8. Laser was not mode-locked properly.
Sometimes the laser turned to be not mode-locked. We had to monitor the spectrum regularly to make sure it is mode-locked.

References

In vivo two-photon laser scanning microscopy with concurrent plasma-mediated ablation: Principles and hardware realization. P. S. Tsai and D. Kleinfeld. In Methods for In Vivo Optical Imaging, Second Edition, R. Frostig, editor, 2009, CRC Press, 3:59-115.

Appendix I

Pictures of the completed system

