## Intelligent Choice of a Laser for Two-Photon Scanning Microscopy

Finding the right combination of a probe and a laser for a desired class of biological samples is the defining step in two-photon microscopy. As lasers prices vary significantly, it is of a particular importance to choose the best laser for the money without substantial loss in resolution.

In this note, we show how to pre-test lasers, when looking for an alternative to a usually expensive Titanium:Sapphire (Ti:Sapphire) laser, by performing SimphoSOFT numerical calculations. We calculated the number of fluorescent photons generated from the dye Alexa 488 by single pulses from two well known models of Ti:Sapphire lasers and one fiber laser, TOPTICA Photonics FemtoFErb 780, to compare their performance in two-photon microscopy. The results indicate that one can use a cheaper fiber laser to reach approximately the same image quality as in the case of a typical expensive Ti:Sapphire. Check out the last column of the table below to compare quantitatively the fluorescence signals from the Alexa 488 probe predicted by SimphoSOFT for different excitation lasers.

| Excitation laser type | Excitation wavelength (Alexa 488) | Laser pulse length (fs) | Average laser power (mW) | Laser pulse energy (nJ) | Fluorescence output (# photons per 100 pulses) |
|---|---|---|---|---|---|
| FemtoFErb 780 (fiber) | 780 | 90 | 10 | 0.10 | 102,800 |
| MaiTai BB (Ti:Sapphire) | 780 | 80 | 10 | 0.125 | 179,600 |
| Chameleon Ultra I (Ti:Sapphire) | 780 | 140 | 10 | 0.125 | 103,200 |

Two-photon scanning microscopy, based on natural or artificial fluorescent probes, is now one of the primary means for monitoring and quantifying biomedical processes in living or preserved tissue (Ref 1-5). In order to increase light penetration depth in biological tissue, two-photon scanning microscopy typically uses high-intensity infrared lasers rather than the visible light sources that are normally used for one-photon microscopy. Following two-photon absorption, the probe molecules can emit visible wavelength fluorescence and phosphorescence that can be easily detected. A schematic diagram of a two-photon scanning microscope is shown in Fig. 1a.

The two-photon cross-sections (in units of GM named after Maria Goeppert Mayer) are shown in Fig. 1b for some organic dyes in the wavelength range from 700 nm to 1040 nm (Ref 6). Although Ti:Sapphire lasers are widely used for two-photon microscopy due to their high output power, high intensity and tunability, Ti:Sapphire lasers are very expensive (typically around $150,000) and take up considerable space in a characterization laboratory. In addition, the high output power of these lasers can generally not be fully utilized due to potential tissue damage. As shown in Fig. 1b, many common fluorescing molecules can be excited by a wide range of infrared wavelengths between 740 nm and 840 nm, allowing the use of less expensive lasers such as 780 nm fixed-wavelength femtosecond fiber lasers.

Two-photon microscopy requires high laser pulse intensity. The highest intensity pulses are generated by femtosecond lasers with pulses of 80-400 fs or less. The variable wavelength Ti:Sapphire lasers, MaiTai BB and Chameleon Ultra I, and the 780 nm fixed wavelength fiber laser, TOPTICA Photonics FemtoFErb 780, all generate pulses in the 80-140 fs time range. Is it possible to make an intelligent choice of a laser without obtaining these lasers for onsite measurements? With SimphoSOFT – it is!

Fig. 1a: Schematic diagram of a two-photon scanning microscope

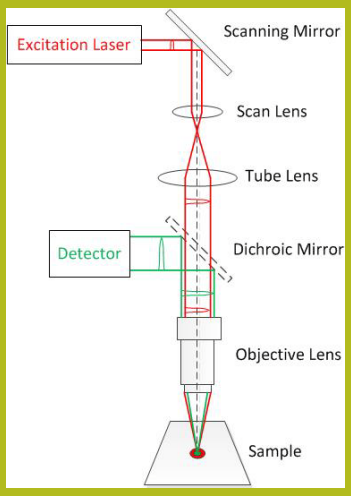
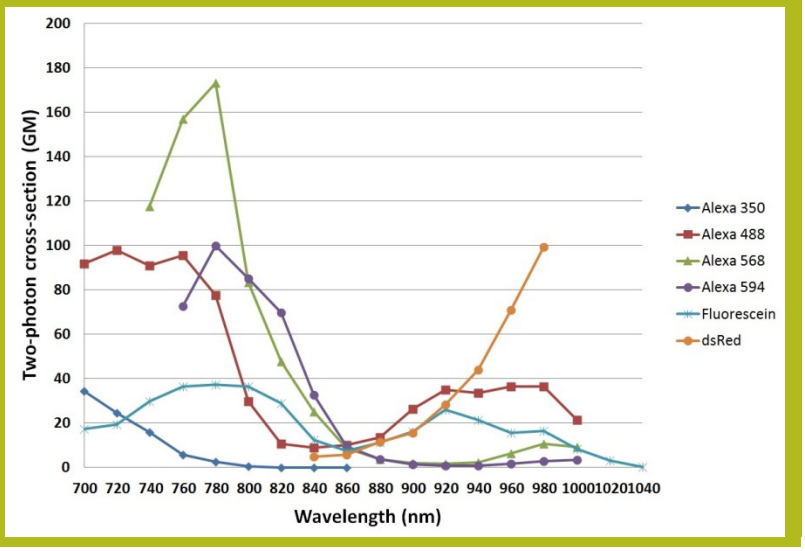



Fig. 1b: Organic dye two-photon cross-sections vs. wavelength




## SimphoSOFT simulations of light emission from single laser pulses

SimphoSOFT numerically solves both the rate equations, which describe the dynamics of the probe molecules' energy levels, and simultaneously the propagation equations for light traversing the sample filled with probe molecules. Its mathematical model can accurately predict the fluorescence and phosphorescence emission spectra at any position in a sample, starting at the light incidence surface. The user provides the beam radius at the input surface, which in these examples is 0.5 μm. The minimum beam radius is determined by the numerical aperture of the microscope objective lens.

SimphoSOFT calculates the light emission for a sample volume element based on the user-provided emission spectra. In principle, one can define a separate spectrum for each radiative relaxation transition of the probe molecule. A spectrum is set by either a table of values or Gaussian-shaped or (Lorentzian-shaped) function. We used a published source (Ref 7) to get the table of values for for Alexa 488. A schematic diagram of a laser beam directed at a cylindrical volume element of the sample that we specify for calculating the number of emitted photons is shown in Fig. 2a.

The rate of photon emission is related to the population of the singlet excited state divided by the fluorescence lifetime $\tau$ ($\tau$ = 4.1 ns for Alexa 488). Probe molecules, in general, can be excited to higher excited states (or even triplet states) – emission from these states can be also calculated by SimphoSOFT. To get the total emitted fluorescent photons, one must integrate the photon emission rate over the volume element width and radius and over the specific time range (10 ns in this case). Shown in Fig. 2b are the results simulating a single pulse from a TOPTICA Photonics FemtoFErb 780 fiber laser at 780 nm that excites Alexa 488 molecules at the sample depths 0 mm and 0.5 mm. At 0 mm, 1028 photons were emitted in the volume element (blue curve). At 0.5 mm, 437 photons were emitted (orange curve). The results are lower at 0.5 mm due to high host material linear absorption (assumed to be 10 $cm^{-1}$ in these calculations). The results (not shown) for Spectra Physics MaiTai and Coherent Chameleon Ultra I were calculated in the same way using the appropriate parameters for each laser.

Fig. 2a: Schematic diagram of a Gaussian-shaped laser pulse directed at a sample volume element

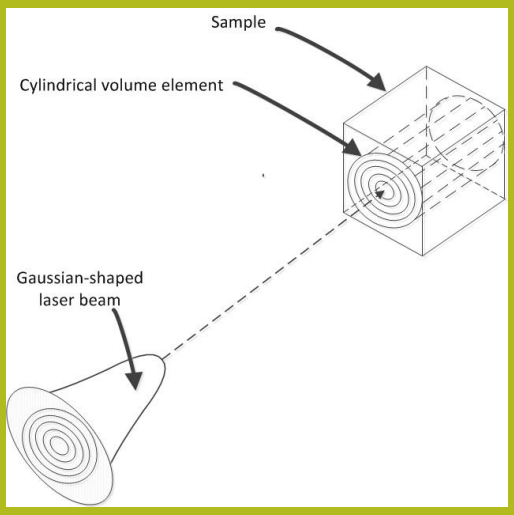
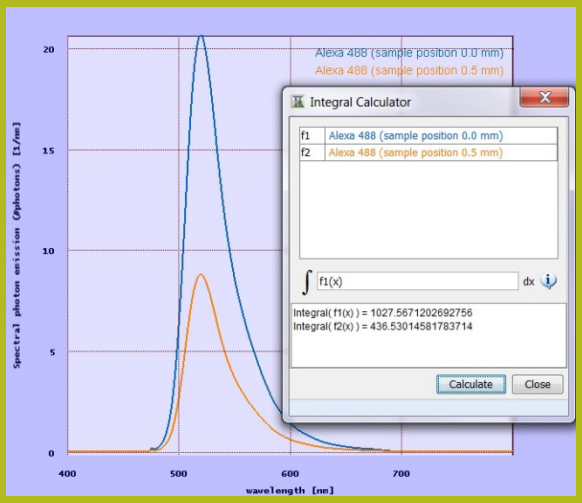



Fig. 2b: Alexa 488 emission at sample depth 0 mm (blue) and sample depth 0.5 mm (orange) using TOPTICA FemtoFErb 780 fiber laser at 780 nm




## Using multiple laser pulses per image pixel improves the resolution

A 100 MHz laser emits a laser pulse every 10 ns or 100 pulses every 1000 ns (1 µs). If the 100 MHz laser is scanned across the sample at a rate corresponding to 1 µs per image pixel (i.e. 100 pulses per pixel), a 1000 pixel by 1000 pixel image can be acquired in the reasonably short time of 1 second. It is quite reasonable to utilize 100 or more laser pulses per image pixel for scanning two-photon microscopy. For example, the 1028 photons emitted in one pulse become approximately 102,800 photons for 100 pulses, which is more than sufficient for high quality imaging.

SimphoSOFT simulations make it possible to predict the efficiency of two-photon microscopy devices without doing lengthy onsite experiments. SimphoSOFT can save tremendous amounts of time choosing the laser(s) to purchase for two-photon microscopy in order to get high performance at reasonable cost.

References:
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