Finding the right combination of a probe and a laser for a desired class of biological samples is a defining step in 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 Ti:Sapphire laser, by performing SimphoSOFT numerical calculations. We calculated the number of fluorescent photons generated from a given set of probes for a series of lasers – Spectra Physics MaiTai, Coherent Chameleon Ultra I, and Toptica Photonics FemtoFErb 780 – to see their performance in two-photon microscopy. Below is one sample from the results acquired for Alexa 488 probe. The results indicate 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 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)
Toptica FemtoFErb 780 (fiber)	780	90	10	0.10	102,800
Spectra Physics MaiTai BB (Ti:Sapphire)	780	80	10	0.125	179,600
Coherent Chameleon Ultra (Ti:Sapphire)	780	140	10	0.125	103,200

Two-Photon Scanning Microscopy

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 1-7). 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. Although Titanium:Sapphire (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 and take up considerable space in a characterization laboratory. In addition, the high output power of Ti:Sapphire lasers can generally not be fully utilized due to potential tissue damage.

Many common fluorescing molecules can be excited by a wide range of infrared wavelengths, allowing the use of fixed wavelength lasers that are much less expensive and much smaller in physical size than Ti:Sapphire lasers. Examples of such lasers include femtosecond fiber lasers with fixed wavelengths of 780 nm or approximately 1045 nm.

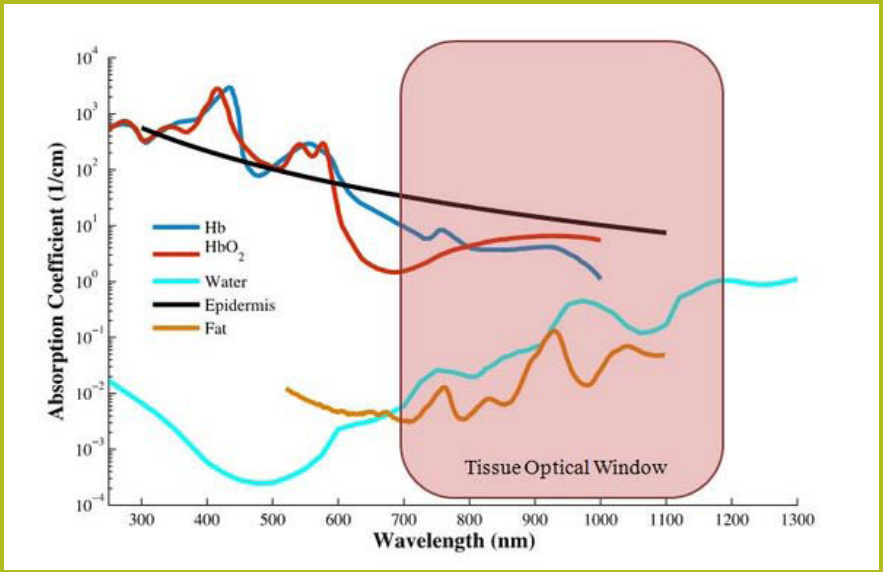
In this application note, we will show that the less expensive fixed wavelength laser can be just as effective as Ti:Sapphire lasers for many digital imaging applications. We picked two molecules, Alexa 488 and fluorescent protein mEGFP, modeled fluorescent signals by using SimphoSOFT numerical calculations and compared the number of photons emitted by each of the molecule when excited by 780 nm or 920 nm single pulses of Ti:Sapphire lasers or by 780 nm single pulses of a fixed wavelength laser. The advantages of using multiple laser pulses for each image pixel are also discussed.

We will show that the SimphoSOFT mathematical model can calculate the fluorescence and phosphorescence emission spectra at any position in a sample. The calculations include the number of fluorescence and phosphorescence photons emitted as a function of wavelength and the total number of photons emitted by a single laser pulse in a user-specified sample volume element during a user-specified time interval.

Life science two-photon microscopy

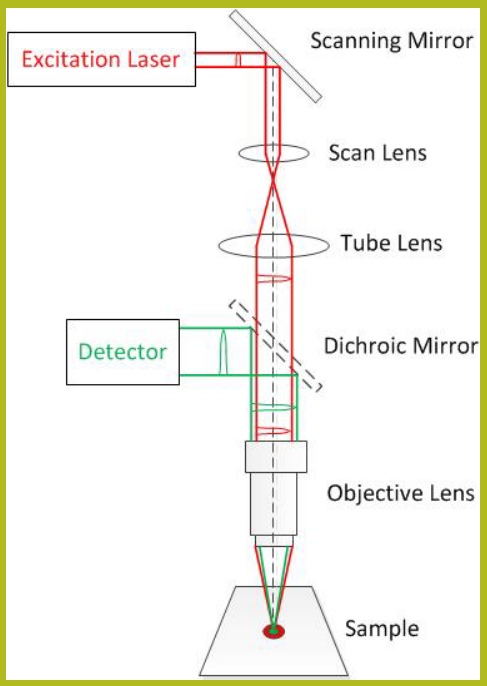
Living tissue both highly absorbs and highly scatters light, especially in the visible region from 400-700 nm. However, there is an optical window from about 700-1200 nm (Ref 8) where the absorption is about 10 cm^{-1} or less (see figure below). This is the region where two-photon absorption is most advantageous due to the greater penetration depth than is possible in the 400-700 nm region.

Wavelength range of tissue optical window



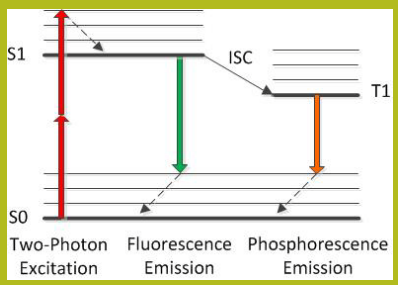
A simplified schematic diagram of a two-photon scanning microscope is shown below. An infrared excitation laser is directed to a scanning mirror that raster scans the beam over a portion of the sample area. A high NA microscope objective lens focuses the beam onto the sample, causing probe molecules to undergo two-photon absorption followed by emission of fluorescence and phosphorescence. A portion of the emitted light is collected by the same microscope objective lens and directed to a detector.

Schematic diagram of a two-photon scanning microscope



The processes of excitation and de-excitation of a photo-active probe molecule can be described by a Jablonski energy level diagram (shown below). For simplicity, the diagram has been reduced to two singlet electronic energy levels, a ground state level S0 and an excited state level S1, and one triplet level T1. Probe molecules, in general, can be excited to higher excited states and emission from these states can also be calculated by SimphoSOFT. If the light intensity is great enough at the focus of the excitation laser beam, two-photon absorption (double arrow shown in red) can occur followed by fluorescence (green arrow). Phosphorescence (orange arrow) may also occur if intersystem crossing (ISC) is present. Two-photon absorption only occurs in the highest intensity portions the focused laser beam. The resulting fluorescence and phosphorescence are emitted in all directions. Some of the emitted light can be absorbed by the tissue. Only a portion (for example 20%) is collected by the microscope objective and directed back to the detector(s). The fluorescence and phosphorescence signals can be separated by use of appropriate bandpass filters or a spectrometer (not shown in the microscope diagram above).

Jablonski energy level diagram



The resolution of a focused laser beam can be less than 1 μm. The maximum diffraction-limited resolution of a focused laser spot, Δd, is given by:

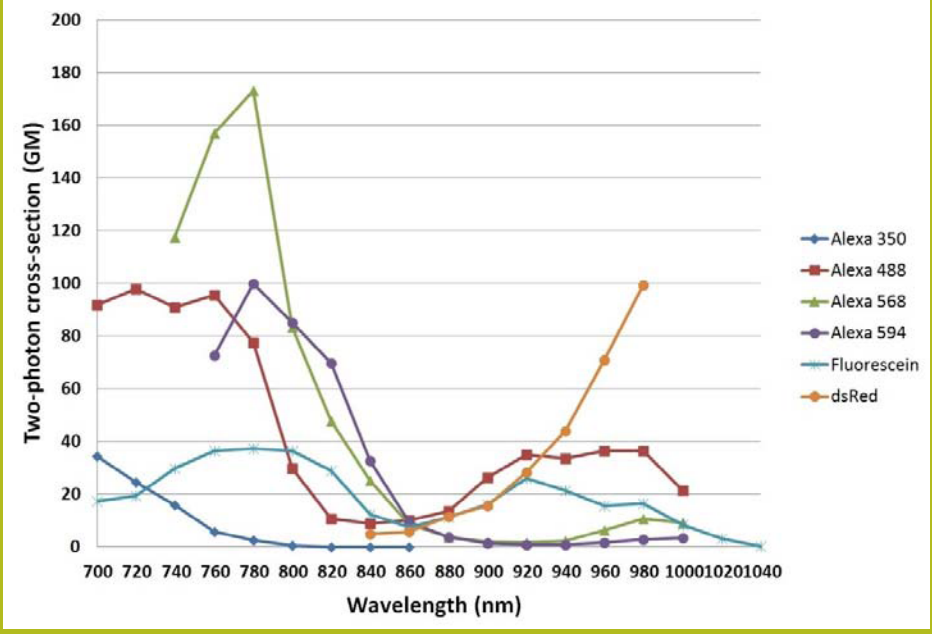
$$\Delta d = \frac{\lambda}{2n\sin\alpha} = \frac{\lambda}{2NA}$$

where λ is the wavelength, n is the refractive index of the light-transmitting medium, the angle α is the half-angular aperture of the focusing lens and NA is the numerical aperture of the focusing lens. As an example, if $\lambda = 780\ nm$ and $NA = 1$, then $\Delta d = 390\ nm$ or $0.39\ \mu m$. By using two-photon absorption processes, higher resolution than specified by the above equation can be achieved.

Two-photon cross-sections

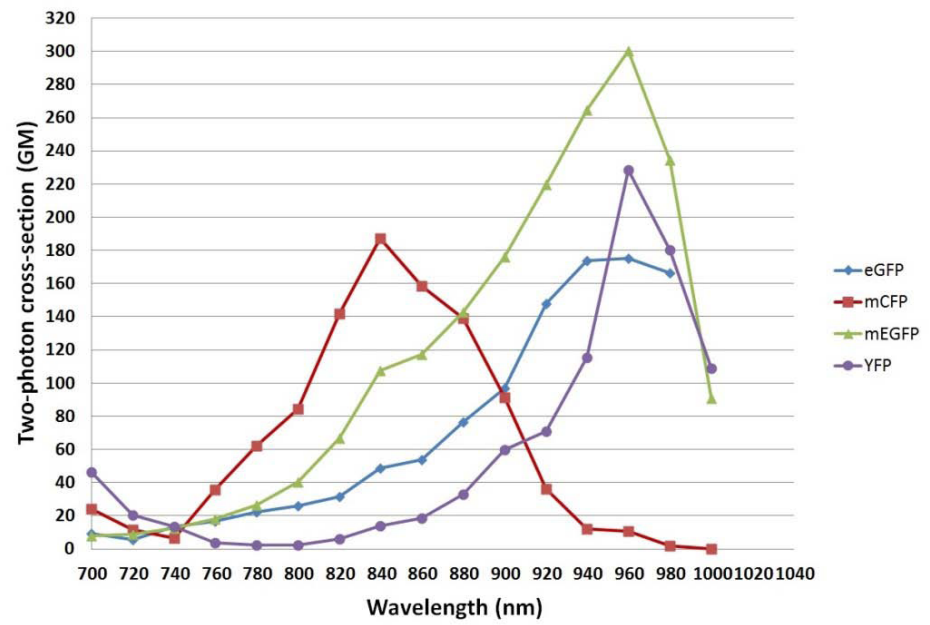
The two-photon cross-sections (in units of GM named after Maria Goeppert Mayer) are shown below for some organic dyes in the wavelength range from 700 nm to 1040 nm (Ref 9). For this set of dyes, many have maximum two-photon cross-sections in the wavelength range from about 700 nm to 840 nm. However, one dye, dsRed, has high cross-sections for wavelengths greater than 900 nm and may have significant two-photon absorption at 1045 nm when excited using Yb-doped fiber or disk lasers. Note that many cross-section measurements are done with tunable Ti:Sapphire lasers that have little power for wavelengths greater than about 1000 nm. This results in fewer measurements for the longer wavelengths.

Organic dye two-photon cross-sections vs. wavelength



Below are shown some additional two-photon cross-sections for selected fluorescent protein molecules (Ref 9; abbreviations: GFP - green fluorescing protein; CFP - cyan fluorescing protein; YFP - yellow fluorescing protein). Some have high two-photon cross-sections for wavelengths greater than 850 nm and may also have significant cross-sections for wave-lengths greater than 1000 nm that are not shown in the figure. Yellow fluorescent protein (YFP), for example, may be a good candidate for two-photon excitation at 1045 nm using Yb-doped fiber or disk lasers.

Fluorescing protein two-photon cross-sections vs. wavelength



Types of lasers for two-photon scanning microscopy

Titanium:Sapphire (Ti:Sapphire) lasers are widely used for two-photon microscopy due to their wide tuning range (e.g. 690 nm to 1040 nm) and high output power (> 1W in some cases). However, the high output power of Ti:Sapphire lasers can generally not be fully utilized due to potential tissue damage. The maximum useful average power for scanning biological samples is 10 mW or less. In this application note, we will therefore assume that the example lasers all have 10 mW of average power at the light incident surface of the samples.

Two-photon microscopy requires high laser pulse intensity. The highest intensity pulses are generated by femtosecond lasers with pulse widths of 80-400 fs or less. A few example lasers are listed in the table below along with the operating wavelength, pulse rate, pulse length, maximum average power, pulse energy at maximum average power and pulse energy at 10 mW average power. As mentioned previously, Ti:Sapphire lasers are expensive and consume significant physical space. Three models of Ti:Sapphire lasers are listed below, Coherent Chameleon Ultra I, Spectra-Physics MaiTai HP and Spectra-Physics MaiTai BB.

New types of fixed wavelength fiber lasers are now available that may replace Ti:Sapphire lasers in many routine two-photon microscopy applications. Two examples of fiber lasers are lasers that operate at 780 nm or at about

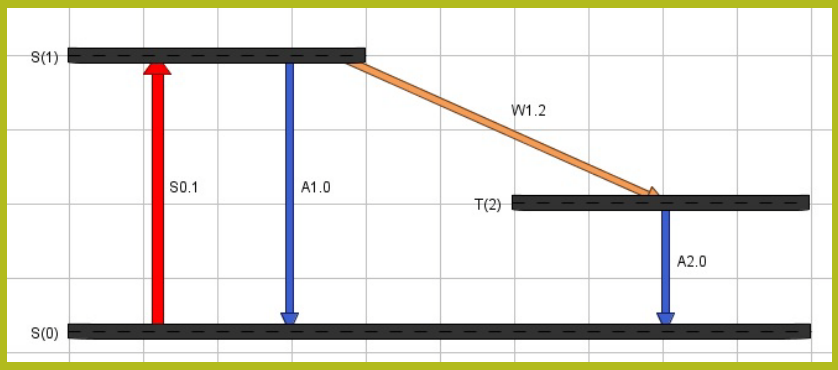
1020-1055 nm (1045 nm is typical). Light at 780 nm is generated by frequency-doubled erbium (Er) doped fiber lasers such as TOPTICA Photonics FemtoFErb 780 laser, CALMAR LASER Carmel CFL and IMRA Femtolite Ultra AX-20. The 780 nm lasers are suitable for many fluorescent organic dyes and fluorescent proteins as shown by the example spectra above. Light of about 1020-1055 nm (depending on laser properties) is generated by ytterbium (Yb) doped fiber lasers or disk lasers. Some fluorescent proteins and dsRed can probably be excited by such lasers although the excitation spectra shown above do not extend to the 1020-1055 nm range. Example Yb-doped lasers are manufactured by IMRA, Coherent, ALPHALAS, JENOPTIK and Spectra-Physics. The femtosecond lasers operate at pulse repletion rates of 50-100 MHz. At 100 MHz, the pulse-to-pulse time separation is 10 ns. The table below shows properties of various types of lasers.

Laser company	Model	Laser type	Excitation wavelength	Laser pulse rate	Laser pulse length	Maximum average power	Laser pulse energy (at max average power)	Laser pulse energy (at 10 mW power)
Not tunable								
TOPTICA Photonics	FemtoFErb 780	Freq-doubled Er-doped fiber	780 nm	100 MHz	90 fs	50 mW	0.5nJ	0.1 nJ
TOPTICA Photonics	FemtoFiber pro NIR	Freq-doubled Er-doped fiber	780 nm	80 MHz	100 fs	150 mW	1.88 nJ	0.125 nJ
CALMAR LASER	Carmel CFL	Freq-doubled Er-doped fiber	780 nm	50 MHz	80 fs	500 mW	10 nJ	0.2 nJ
IMRA	Femtolite Ultra AX-20	Freq-doubled Er-doped fiber	780 nm	50 MHz	100 fs	20 mW	0.4 nJ	0.2 nJ
IMRA	Femtolite FX-150	Freq-doubled Er-doped fiber	810 nm	75 MHz	140 fs	150 mW	2 nJ	0.13 nJ
IMRA	FCPA uJewel DE	Yb doped fiber	1045 nm	200 kHz	50 fs	10 W	50000 nJ	50.0 nJ
Coherent	Fidelity 1055-2	Yb-doped fiber	1055 nm	70 MHz	70 fs	2 W	29 nJ	0.14 nJ
ALPHALAS	FEMTOLAS-1000	Yb doped; DPSS	1022-1050 nm	100 MHz	200 fs	1 W	10 nJ	0.1 nJ
JENOPTIK	JenLas D2.fs 5W	Yb:KYW thin disk	1025 nm	500 kHz	400 fs	5 W	10000 nJ	20.0 nJ
Spectra-Physics	HighQ-2	Yb doped	1045 nm	63 MHz	250 fs	1.5 W	24 nJ	0.16 nJ
Tunable								
Coherent	Chameleon Ultra I	Ti:Sapphire	690-1040 nm	80 MHz	140 fs	1.5 W (710 nm) 1.45 W (920 nm)	19 nJ	0.125 nJ
Spectra-Physics	MaiTai HP	Ti:Sapphire	690-1040 nm	80 MHz	100 fs	1.35 W (710 nm) 1.35 W (920 nm)	17 nJ	0.125 nJ
Spectra-Physics	MaiTai BB	Ti:Sapphire	710-990 nm	80 MHz	80 fs	650 mW (710 nm) 650 mW (920 nm)	8 nJ	0.125 nJ

Setting up Alexa 488 simulations in SimphoSOFT

In the example below, we assume that Alexa 488 molecules having two singlet states, S0 and S1, and one triplet state, T1, are dispersed in a host material. We will assume that the host material has an absorption coefficient of 10 cm^{-1}. Note that many biological materials will have effective absorption coefficients (absorption plus scattering) significantly greater than 10 cm^{-1}. The photo-physical properties of the probe molecule are set in the SimphoSOFT M-CAD panel. A screenshot with Alexa 488 energy level diagram is shown below which includes two-photon absorption (2PA) at 780 nm (S0.1), fluorescence emission (A1.0) with a relaxation time of 4.1 ns, intersystem crossing (W1.2) with a relaxation time of 46.0 ns and phosphorescence emission (A2.0) with a much slower relaxation time of 6.66 µs. Vibrational states are not shown since vibrational relaxations are very fast and usually do not affect the overall results. However, a user may add vibrational relaxations to the simulation if desired. The user may also add additional electronic energy levels for more complex molecules if needed.

Screenshot of energy level diagram shown in SimphoSOFT M-CAD for Alexa 488



SimphoSOFT energy level simulation parameters values for Alexa 488:

From level(s):	To level(s):	Cross-section:	Relaxation time:
S(0)	S(1)	3.05×10^{-21} cm^4/GW (2PA) or 77.7 GM	
S(1)	S(0)		4.1 ns (radiative)
S(1)	T(2)		46.0 ns (non-radiative)
T(2)	S(0)		6.66 µs (radiative)

[a]Note that over 1100 two photon materials are available from Simphotek's INFO+, which is a searchable information-base of chemical structures and ~20,000 parameters**.** A description and examples of INFO+ is available on the web: www.simphotek.com.

The actual experimental setup is defined in the SimphoSOFT E-CAD panel. For Alexa 488 it includes one laser beam at 780 nm and a sample 0.5 mm thick. SimphoSOFT simulates the laser-material interactions starting at the sample input surface and does not calculate the entire scanning microscope optics.

The pump (excitation) beam has a Gaussian radial dependence and Gaussian time dependence. The formula for the intensity of the Gaussian pump beam is:

$$I(r,t) = I_0 e^{-\left(\frac{t}{T_0}\right)^2} e^{-\left(\frac{r}{R_0}\right)^2} \quad \text{where} \quad T_0 = t_{FWHM}/2\sqrt{\ln 2} \quad R_0 = w_{HW1/e^2M}/\sqrt{2} \quad I_0 = \frac{E_{in}}{\pi\sqrt{\pi}R_0^2 T_0}$$

E_{in} is the input pulse energy. T_0 and R_0 are the *1/e* values for pulse length and radius, respectively, and are related to user inputs t_{FWHM} and radius $w_{HW1/e2M}$.

SimphoSOFT E-CAD input beam and sample parameters summary:

Pump laser parameters	Value
Radial shape	Gaussian
Pulse energy	0.100 or 0.125 nJ
Pulse radius (HW1/e^{2}M)	0.5 µm
Pulse radius (R_0)	0.354 um
Pulse FWHM	80, 90 or 140 fs
Pulse T_0	48, 54 or 84 fs
Laser wavelength	780 nm

Sample properties	Value
Fluorescent molecule dopant density (concentration) in the host material	1.0×10^{17} molecules/cm^3
Host material linear refractive index	$n_0 = 1.5$ (780 nm and 920 nm)
Host material linear absorption	10 cm^{-1}
Sample length	0.5 mm (500 µm)

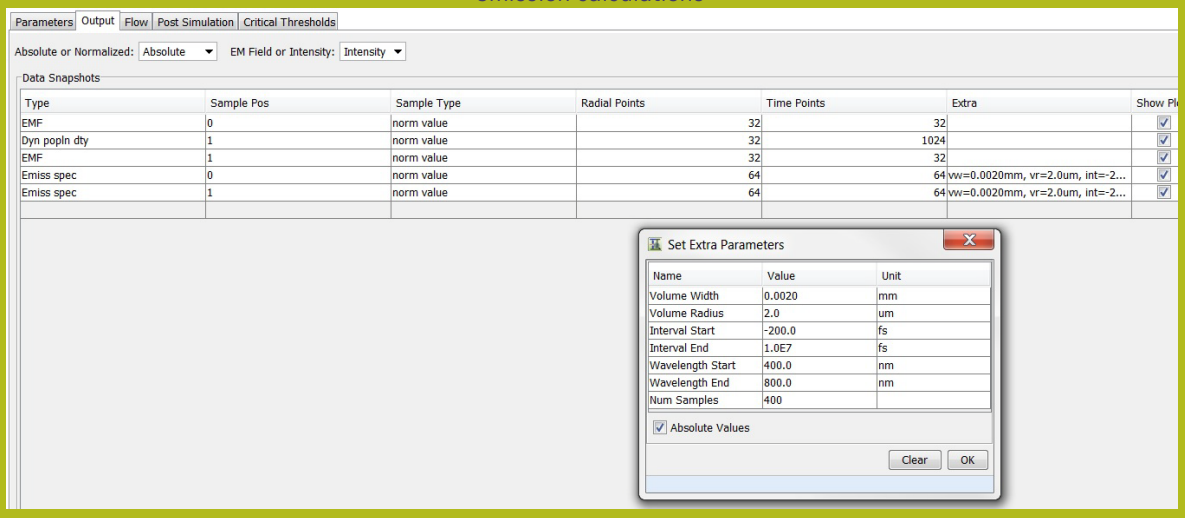
SimphoSOFT program feature – emission spectra

SimphoSOFT solves numerically the rate equations, which describe the dynamics of the sample energy levels, simultaneously with the propagation equations for light traversing the sample. For estimating light emission, an emission spectrum is generated using either a user-provided table of values or Gaussian-shaped or Lorentzian-shaped functions. For Alexa 488 a table of values was obtained from a published source (Ref. 10).

To direct SimphoSOFT to generate emission spectra, the user provides one or more emission spectrum lines (labeled "Emiss spec') in the Output tab of the Run Configuration panel. In the example screenshot below, we have selected to generate two emission spectra, one at normalized sample position 0 (at the sample input surface) and at normalized sample position 1 (at the sample output surface). By selecting the appropriate 'Extra' cell in the table, the 'Set Extra

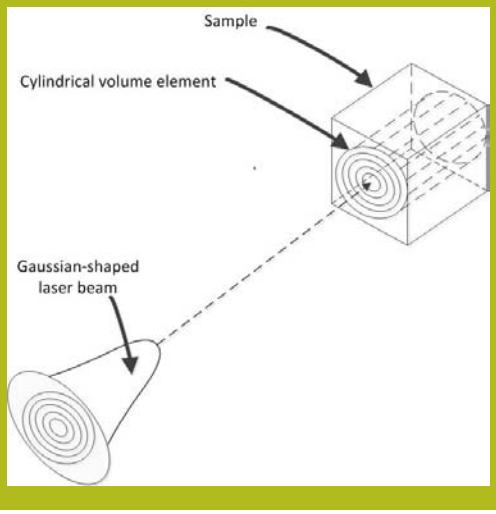
Properties' dialog opens where one can enter the volume element parameters (width or thickness and the radius) and time parameters for calculating the number of photons emitted in the volume element in the wavelength range during the selected time interval.

Screenshot of SimphoSOFT 'Output' tab of 'Run Configuration' panel with 'Set Extra Parameters' dialog open to set emission calculations



A schematic diagram of the laser beam directed at the cylindrical volume element that we specified for calculating the number of emitted photons is shown below.

Schematic diagram of a Gaussian-shaped laser pulse directed at a sample volume element



The rate of photon emission by the volume element within a time interval is related to the population of the S1 state times divided by the fluorescence lifetime τ (τ = 4.1 ns for Alexa 488).

Photon emission rate = (population of state S1)/τ

Since one electron in state S1 can be converted to one fluorescent photon, the units of the above equation are photons per second per unit volume. SimphoSOFT calculates the S1 population in each simulation propagation slice, radial slice and time slice. In 'Set Extra Parameters' shown above, the volume width (thickness) is set to 0.002 mm (2 μm), the volume element radius is set to 2 μm, the time range is set from -200 fs to +1E7 fs (in SimphoSOFT, time = 0 is at the center of the 90 fs pulse). Note that 1E7 fs is 10 ns, which is the pulse-to-pulse separation for a pulse repletion rate of 100 MHz. To get the total emitted fluorescent photons one must integrate the photon emission rate shown above over the volume element width and radius and over the specific time range. The result is the total fluorescence emitted in the volume element after one laser pulse and before the next laser pulse.

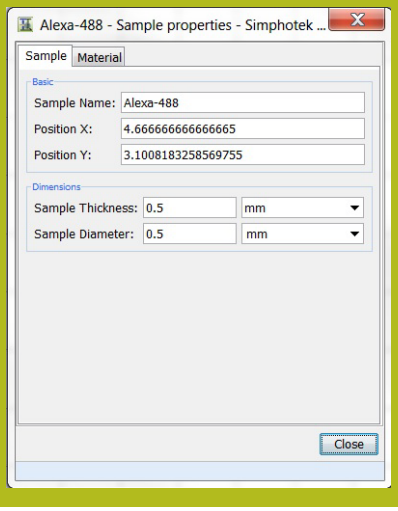
Using the same procedure, SimphoSOFT will also calculate total phosphorescence emitted after one 90 fs pulse and before the next 90 fs pulse if specified.

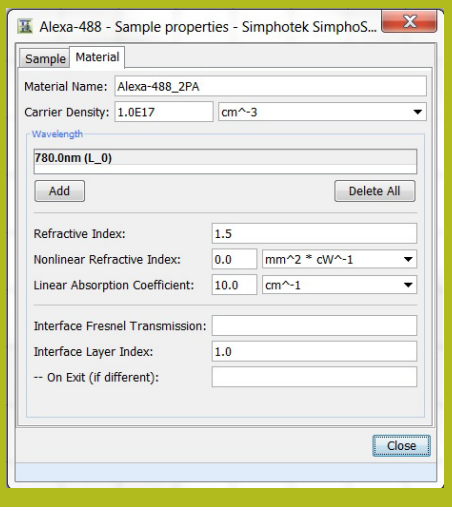
Proper grid sizes for numerical calculations should also be set before simulation. Appendix A contains helpful hints for defining optimal numerical parameters provided for successful calculation of emission output.

SimphoSOFT simulations: Alexa 488 and mEGFP using three different fs lasers

Below we will show simulation results that calculate the number of photons emitted within 10 ns by Alexa 488 or mEGFP using either single pulses from a 780 nm fiber laser or single pulses from two models of Ti:Sapphire lasers. To prevent sample damage, we keep the average laser power at the sample surface at 10 mW for each laser, which is much less than the maximum output of the lasers. In each case, we will keep the 'Sample properties' the same and we need to vary inputs to the 'Absorption TM' cross-section properties, laser 'Beam Properties' and 'Numerical Setup' to correspond to different laser specifications. The fixed 'Sample properties' are shown in the two screenshots below. The sample thickness (in the direction of the beam propagation) is 0.5 mm or 500 μm. The 'Carrier Density' of the fluorescent molecules is 1E17 cm^{-3}. The refractive index of the host material is 1.5 and the linear absorption of the host material is 10 cm^{-1}.

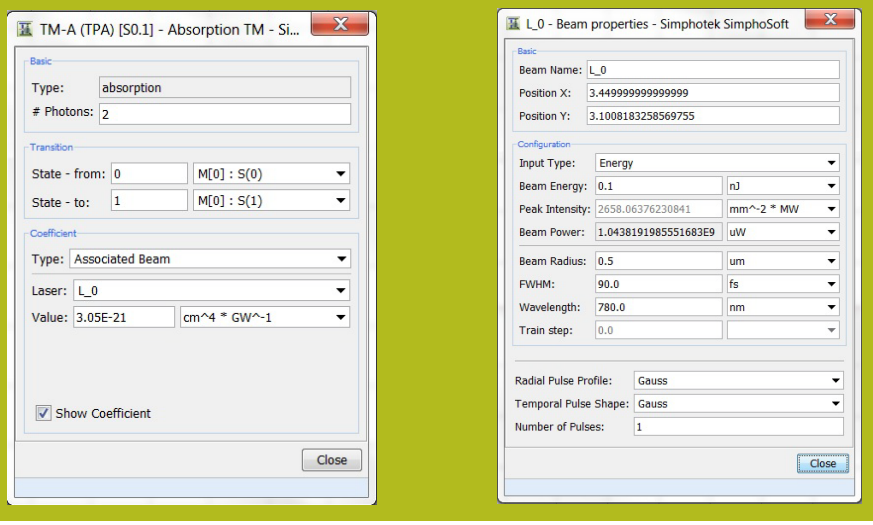
SimphoSOFT screenshots of sample properties





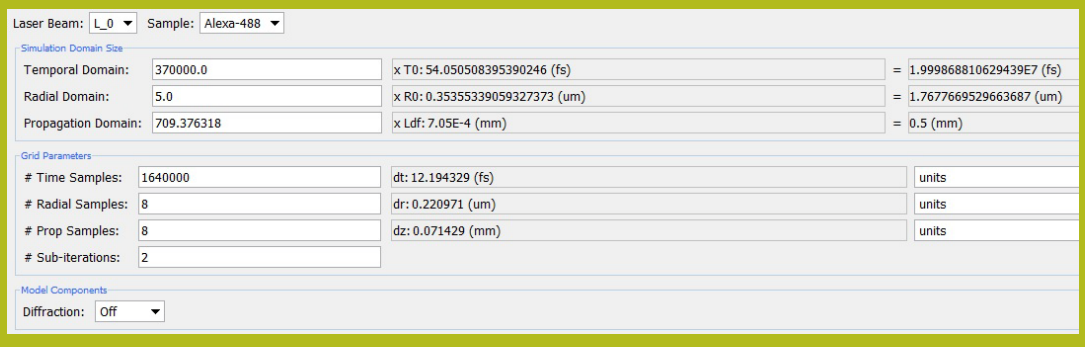
The 'Absorption TM' properties and laser 'Beam properties' are shown in the screenshots below. The cross-section 'Value' will be different for the different molecules, Alexa 488 and mEGFP, and for different wavelengths of the same molecule. In 'Beam properties', 'Beam Energy' and the pulse width (FWHM) will be different for different lasers.

SimphoSOFT screenshots of absorption TM properties and laser beam properties



In 'Numerical Setup', we want the set the full time domain to be about 20 ns. The time domain will then extend from -10 ns to +10 ns since, in SimphoSOFT, the center of the laser pulse is at t = 0. The time domain is defined as some multiplication factor times T_0. An example is shown in the screenshot below where the multiplication factor is 370,000 and T_0 is about 54 fs (when FWHM is 90 fs). We also want the number of time computational slices for 20 ns to be large enough so that each time computational slice is much less than the laser pulse FWHM. In the example below the number of time slices (# Time Samples) is 1,640,000 and each time slice dt is 12.2 fs. The number of radial samples (# Radial Samples) is 8 and the number of propagation samples (# Prop Samples) is 8. Note that the number of propagation slices is 8 – 1 = 7.

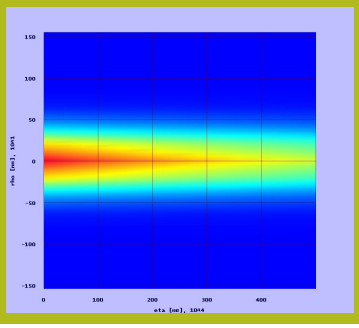
SimphoSOFT screenshot of Numerical Setup



CASE 1: Alexa 488 using TOPTICA Photonics FemtoFErb 780 fiber laser:

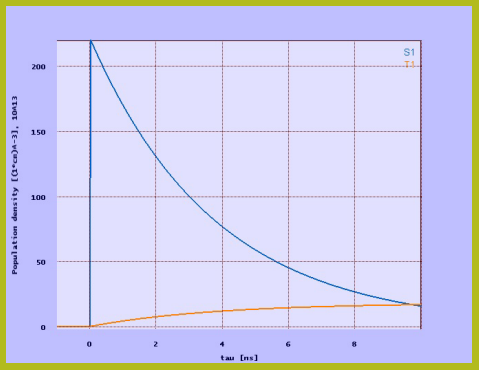
Absorption TM properties: cross-section 3.05E–21 cm^4/GW (i.e. 77.7 GM)
Beam properties: Beam Energy 0.1 nJ; Beam Radius 0.5 µm; FWHM 90 fs; Wavelength 780 nm
Numerical Setup parameters: Temporal Domain 370,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8
Sample properties: thickness 0.5 mm; carrier density 1E17 cm^{-3}; linear absorption 10 cm^{-1}
Due to the relatively high linear absorption of 10 cm^{-1}, the calculated intensity of the laser pulse drops as it passes through 0.5 mm of the sample material. This dropoff is illustrated in the SimphoSOFT screenshot below showing the pulse intensity as the pulse passes through the sample.

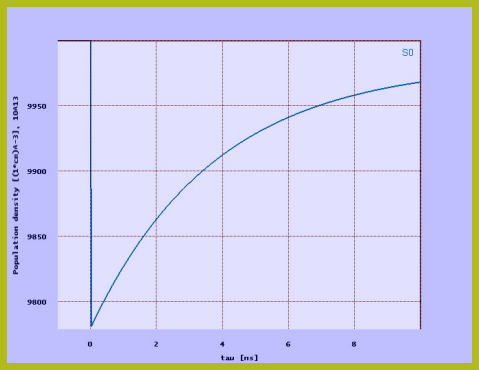
Calculated pulse intensity vs. sample distance using TOPTICA Photonics FemtoFErb 780 fiber laser



SimphoSOFT calculates the populations of all the specified energy levels as functions of time, radius and distance through the sample. The plots below show the populations of states S1 and T1 (left) as well as S0 (right) at the sample input at radius = 0 after the pulse has passed through the first propagation slice. The 90 fs laser pulse is at time tau = 0 in the plots. The fluorescence decay, S1 (left; blue curve), has a time constant of about 4 ns and is not complete in the 10 ns time period before the next laser pulse occurs. In addition to the fluorescence decay, a portion of the electrons transfer by intersystem crossing to state T1 (left; orange curve) during the 10 ns time period. At time 10 ns, the time at which the next laser pulse will occur, ground state S0 is depleted from 10000E13 (or 1E17) to about 9960E13, a difference of 40E13. About half of the depletion is due to transfer to the long-lived triplet state. The other half is due to the incomplete S1 florescence relaxation, which will be nearly complete in a few laser pulses. The transfer to the triplet state T1 will be much longer lived.

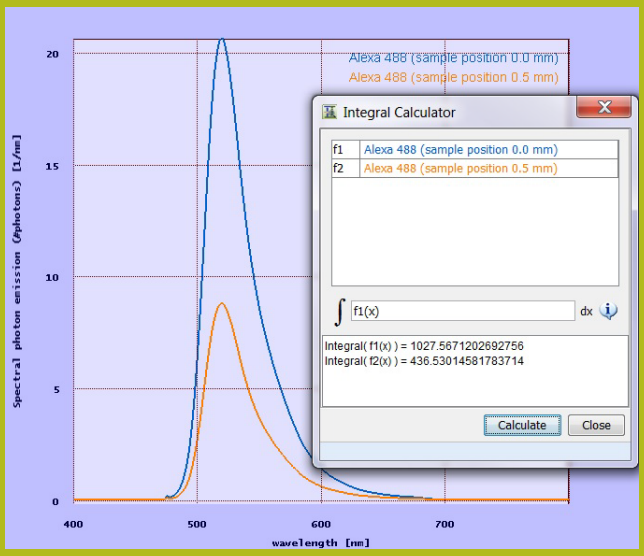
Screenshots of S0, S1 and T1 populations for Alexa 488 using TOPTICA Photonics FemtoFErb 780 fiber laser





The fluorescence emission spectra in the volume element at sample position 0 mm and at sample position 0.5 mm are shown below, along with integral calculations of the total number of photons in each spectrum. At sample position 0 mm, 1028 photons were emitted in the volume element (blue curve). At sample position 0.5 mm, 437 photons were emitted in the volume element (orange curve). Phosphorescence emission was also calculated by SimphoSOFT, but the number of phosphorescent photons emitted in 10 ns was less than 1 and is not shown.

Alexa 488 emission results using TOPTICA Photonics FemtoFErb 780 fiber laser at 780 nm



Using multiple laser pulses per image pixel

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 (100 pulses per pixel), a 1000 pixel by 1000 pixel image can be acquired in the reasonably short time of 1 second. If 1000 laser pulses are utilized per pixel, the image acquisition time is still only 10 seconds.

It is quite reasonable to utilize 100 or more laser pulses per image pixel for scanning two-photon microscopy. The maximum number of fluorescent photons emitted in 100 laser pulses is 100 times the numbers calculated above; assuming the ground state S0 is not depleted by the laser pulses. For example, the 1028 photons emitted in one pulse become approximately 102,800 photons for 100 pulses. In this example, however, a single laser pulse caused approximately 20E13 electrons (fraction 0.002 of the total electrons) to transfer to the triplet state T1. The ground state is depleted to the fraction 0.998 of the original number of electrons 10 ns after the first pulse due to intersystem crossing. One can do a rough estimate of the ground state depletion after several pulses. After 100 pulses, for example, the ground state will be depleted to approximately the fraction $0.998^{100} = 0.82$ of the starting value. After 1000 pulses, the ground state will be depleted to approximately the fraction $0.998^{1000} = 0.14$. These are rough estimates and indicate that at least 100 pulses per pixel can easily be utilized for image acquisition without greatly reducing the fluorescence emission per pulse. Using 100 or more pulses of the TOPTICA FemtoFErb 780 fiber

laser should be sufficient for high-quality imaging. Note that with SimphoSOFT, the user can also use the multi-pulse option to calculate the above result more accurately if needed.

Note that SimphoSOFT calculates the total number of fluorescence (or phosphorescence) photons emitted in all directions by the volume element. Only a portion, for example 20%, will be collected by the microscope objective and directed to the detector. The SimphoSOFT calculations show that a 780 nm fiber laser can generate sufficient numbers of fluorescence photons to form a very good image of the sample. Furthermore our results are in agreement with experimental data using Alexa dyes and 780nm femtosecond lasers in multiphoton microscopy (Ref. 11).

CASE 2: Alexa 488 using Spectra-Physics MaiTai BB at 780 nm:

Absorption TM properties: cross-section 3.05E-21 cm^4/GW (i.e. 77.7 GM)
Beam properties: Beam Energy 0.125 nJ; Beam Radius 0.5 μm; FWHM 80 fs; Wavelength 780 nm
Numerical Setup parameters: Temporal Domain 417,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8

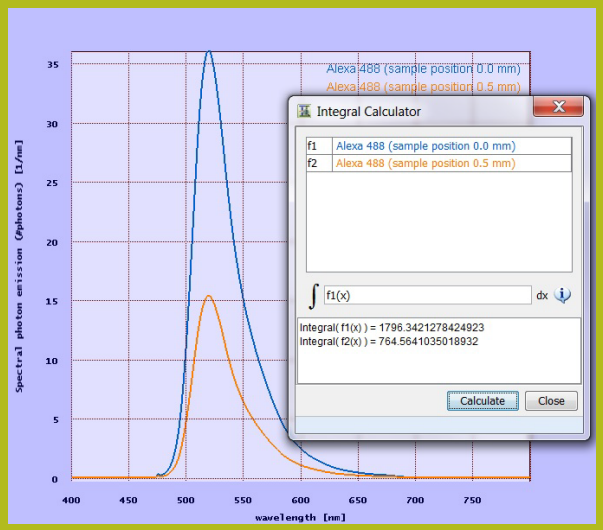
The results are shown below (left figure) for an 80 fs pulse. At sample position 0.0 mm, 1796 photons fluorescence photons are emitted in the volume element. At sample position 0.5, 765 fluorescence photons are emitted in the volume element. Due to the shorter pulse width of 80 fs and higher pulse energy of 0.125 nJ of the Spectra-Physics MaiTai BB compared to the TOPTICA FemtoFErb 780 fiber laser pulse width of 100 fs and pulse energy of 0.100 nJ, using the Spectra-Physics MaiTai BB laser results in higher fluorescence emission. However, the fluorescence emission of the TOPTICA FemtoFErb 780 fiber laser should be sufficient for imaging.

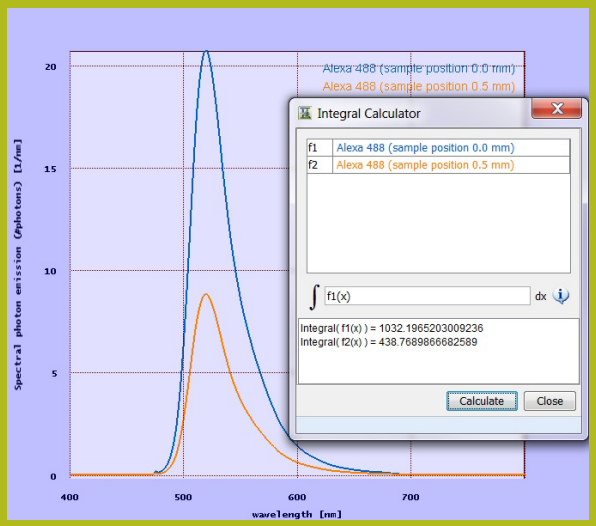
CASE 3: Alexa 488 using Coherent Chameleon Ultra Ti:Sapphire laser at 780 nm:

Absorption TM properties: cross-section 3.05E-21 cm^4/GW (i.e. 77.7 GM)
Beam properties: Beam Energy 0.125 nJ; Beam Radius 0.5 μm; FWHM 140 fs; Wavelength 780 nm
Numerical Setup parameters: Temporal Domain 240,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8

The results are shown below (right figure) for a 140 fs pulse. At sample position 0.0 mm, 1032 photons fluorescence photons are emitted in the volume element. At sample position 0.5, 439 fluorescence photons are emitted in the volume element. Due to the higher pulse energy of 0.125 nJ of the Coherent Chameleon Ultra I compared to the TOPTICA FemtoFErb 780 fiber laser pulse pulse energy of 0.100 nJ, using the Coherent Chameleon Ultra I laser results in higher fluorescence emission, but the increase is relatively small.

Alexa 488 emission results for Spectra-Physics MaiTai BB (left) and Coherent Chameleon Ultra (right)





SimphoSOFT simulation results for mEGFP

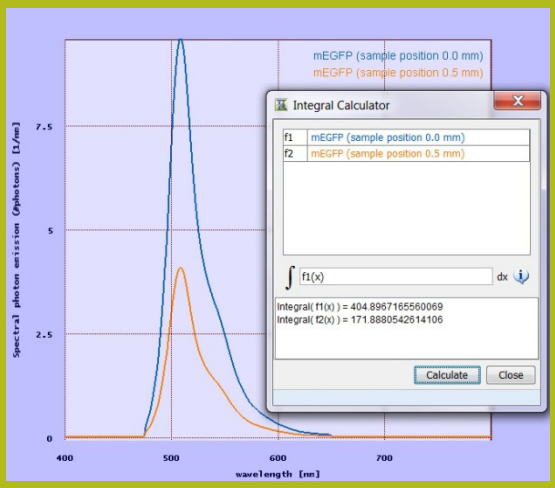
The protein mEGFP is one of many proteins that are used for fluorescence imaging. Due to a lack of information about the triplet state of mEGFP, the following calculations for mEGFP will assume just two energy levels, S0 and S1, and no triplet state, T1. The fluorescence lifetime is assumed to be 2.5 ns. We will compare using the TOPTICA FemtoFErb 780 fiber laser at 780 nm to the two Ti:Sapphire lasers at 920 nm. Since the two-photon cross-section of mEGFP is much higher at 920 nm than at 780 nm, the Ti:Sapphire lasers will produce significantly higher fluorescence per laser pulse than the 780 nm laser.

CASE 4: mEGFP using TOPTICA Photonics FemtoFErb 780 fiber laser:

Absorption TM properties: cross-section 1.04E-21 cm^4/GW (i.e. 26.5 GM)
Beam properties: Beam Energy 0.1 nJ; Beam Radius 0.5 μm; FWHM 90 fs; Wavelength 780 nm
Numerical Setup parameters: Temporal Domain 370,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8

The fluorescence emission spectra at sample position 0 mm and at sample position 0.5 mm are shown below, along with integral calculations of the total number of photons in each spectrum. At sample position 0 mm, 405 photons were emitted in the volume element (blue curve). At sample position 0.5 mm, 172 photons were emitted in the volume element (orange curve). Phosphorescence emission was also calculated by SimphoSOFT, but the number of phosphorescent photons emitted in 10 ns was less than 1 and is not shown. The emission from a single pulse of the TOPTICA FemtoFErb 780 fiber laser may be too small for adequate imaging, but the user can easily use 100 to 1000 laser pulses per image pixel to get high quality fluorescence images.

mEGFP emission results using TOPTICA Photonics FemtoFErb 780 fiber laser at 780 nm



CASE 5: mEGFP using Spectra-Physics MaiTai BB at 920 nm:

Absorption TM properties: cross-section 1.02E-20 cm^4/GW (i.e. 219.8 GM)
Beam properties: Beam Energy 0.125 nJ; Beam Radius 0.5 µm; FWHM 80 fs; Wavelength 920 nm
Numerical Setup parameters: Temporal Domain 417,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8

The results are shown below (left figure) for an 80 fs pulse. At sample position 0.0 mm, 7850 photons fluorescence photons are emitted in the volume element. At sample position 0.5, 3380 fluorescence photons are emitted in the volume element. Due to the higher two-photon cross-section for mEGFP at 920 nm than at 780 nm and due to the shorter pulse width of 80 fs and the higher pulse energy of 0.125 nJ of the Spectra-Physics MaiTai BB compared to the TOPTICA FemtoFErb 780 fiber laser, using the Spectra-Physics MaiTai BB laser results in much higher fluorescence emission than using the 780 nm fiber laser.

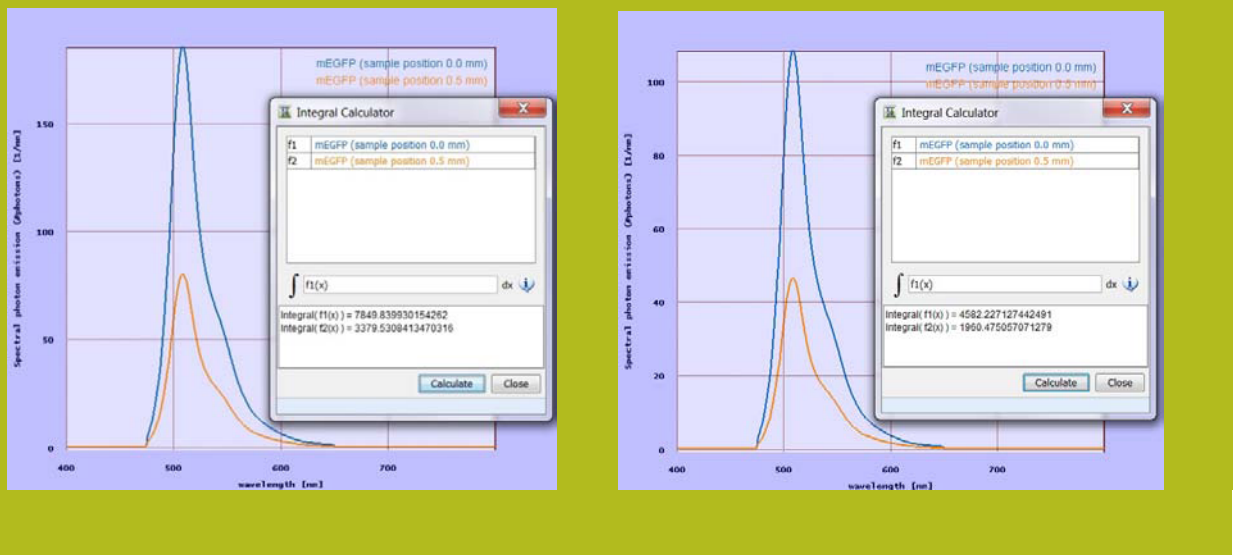
CASE 6: mEGFP using Coherent Chameleon Ultra Ti:Sapphire laser at 920 nm:

Absorption TM properties: cross-section 1.02E-20 cm^4/GW (i.e. 219.8 GM)
Beam properties: Beam Energy 0.125 nJ; Beam Radius 0.5 µm; FWHM 140 fs; Wavelength 920 nm
Numerical Setup parameters: Temporal Domain 240,000; # Time Samples 164,000; # Radial Samples 8; # Prop Samples 8

The results are shown below (right figure) for a 140 fs pulse. At sample position 0.0 mm, 4582 photons fluorescence photons are emitted in the volume element. At sample position 0.5, 1960 fluorescence photons are emitted in the volume element. Due to the higher two-photon cross-section for mEGFP at 920 nm than at 780 nm and due to the

higher pulse energy of 0.125 nJ of the Coherent Chameleon Ultra I compared to the TOPTICA FemtoFErb 780 fiber laser, using the Coherent Chameleon Ultra I laser results in much higher fluorescence emission than using the 780 nm fiber laser.

mEGFP results for Spectra-Physics MaiTai BB (left) and Coherent Chameleon Ultra I (right) at 920 nm



Instrument Cost versus Performance

As an example, consider two cases. According to manufacturers, the typical price of a Ti:Sapphire laser is about $130,000-$150,000; whereas the typical price for a fiber laser is about $30,000-$50,000. Of course the Ti:Sapphire laser is tunable so this needs to be considered by the user. But SimphoSOFT allows one to make the trade-off without the cost of buying every laser.

Table of values for the above simulations

The following table lists all the emission results described above for Alexa 488 and mEGFP using the three lasers plus additional results for the TOPTICA FemtoFiber pro NIR laser. Although the Ti:Sapphire lasers give higher photon emission than the 780 nm fiber laser, using 100 to 1000 pulses per pixel from the 780 nm fiber laser should be sufficient for high quality imaging.

Molecule	Excitation laser type	Excitation wavelength (nm)	Laser pulse length (fs)	2PA cross-section (GM)	2PA cross-section (cm4/GW)	Assumed laser average power (mW)	Laser pulse rate (MHz)	Laser pulse energy (nJ)	Fluorescence at position 0.0 mm (photons for 1 pulse)	Fluorescence at position 0.5 mm (photons for 1 pulse)	Maximum fluorescence at position 0.0 mm (photons for 100 pulses)	Collection efficiency	Collected fluorescence at position 0.0 mm (photons per 100 pulses)
Alexa 488	TOPTICA FemtoFErb 780 (fiber)	780	90	77.7	3.05E-21	10	100	0.10	1,028	437	102,800	0.20	20,560
Alexa 488	TOPTICA FemtoFiber pro NIR (fiber)	780	100	77.7	3.05E-21	10	80	0.125	1441	613	144,100	0.20	28,820
Alexa 488	Spectra Physics MaiTai BB (Ti:Sapphire)	780	80	77.7	3.05E-21	10	80	0.125	1,796	765	179,600	0.20	35,920
Alexa 488	Coherent Chameleon Ultra (Ti:Sapphire)	780	140	77.7	3.05E-21	10	80	0.125	1,032	439	103,200	0.20	20,640
mEGFP	TOPTICA FemtoFErb 780 (fiber)	780	90	26.5	1.04E-21	10	100	0.10	405	172	40,500	0.20	8,100
mEGFP	TOPTICA FemtoFiber pro NIR (fiber)	780	100	26.5	1.04E-21	10	80	0.125	569	242	56,900	0.20	11,380
mEGFP	Spectra Physics MaiTai BB (Ti:Sapphire)	920	80	219.8	1.02E-20	10	80	0.125	7,850	3,380	785,000	0.20	157,000
mEGFP	Coherent Chameleon Ultra (Ti:Sapphire)	920	140	219.8	1.02E-20	10	80	0.125	4,582	1,960	458,200	0.20	91,640

Summary:

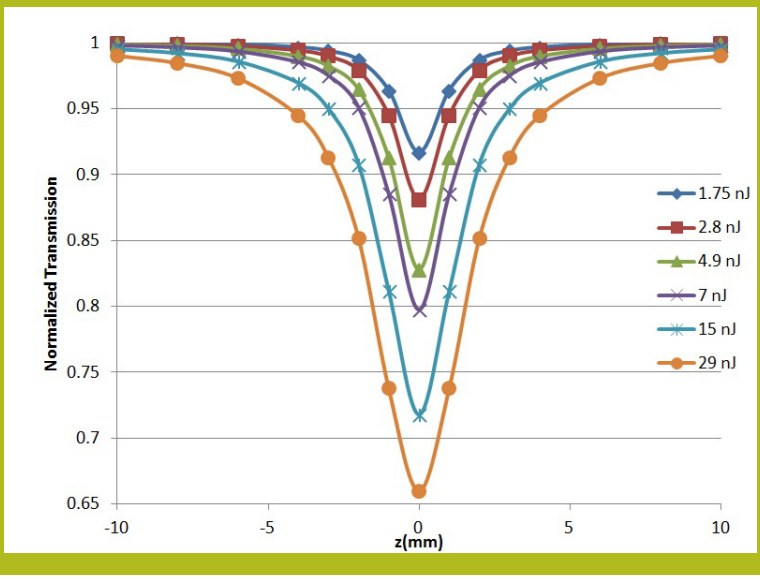
SimphoSOFT simulations make it possible to predict the efficiency of two-photon microscopy devices without doing lengthy experiments and can save optical engineers tremendous amounts of time designing new microscopes. Published in this note description and the results of using SimphoSOFT built-in feature 'Emission spectra' show how to use SimphoSOFT to determine the fluorescence and phosphorescence emission for different fluorescent molecules and for different lasers. SimphoSOFT can also be used by microscope users to compare the performance of fluorescence emission of different lasers and to determine the best fluorescent materials. Users can predict the intensity of fluorescence and phosphorescence emission before doing time-consuming experiments. The laser can have any duration from continuous wave to ~50 fs and any wavelength. We can also model broadband or "white" light sources.

A separate Simphotek product INFO+ allows users to quickly search thousands of two photon materials and obtain all the photo-physical parameters necessary for SimphoSOFT simulation. For example, users can select specific values or a range of absorption wavelength(s), emission wavelength(s), chemical structure(s), chemical name(s), quantum yield(s) and about 20 other physical parameters.

Footnote:

SimphoSOFT's numerical algorithm has been validated against experimental data and analytical solutions many times. Below is one example using published data from a laser transmission study. The solid curves are experimental data and the marks (dots,x, squares,…) are the calculated values using SimphoSOFT. The values are in agreement with the published results (Ref 12).

Comparison of SimphoSOFT z-scan simulations and z-scan experimental data



APPENDIX A

Helpful hints for defining parameters in 'Numerical Setup' and 'Set Extra Parameters':

The Numerical Setup parameters are not the same as the parameters in 'Set Extra Parameters' that are specified for emission spectra. In Numerical Setup, the user defines the Time Domain, Radial Domain, the number of radial slices and the number of propagation slices for the full simulation. In this particular calculation, we want the time domain in Numerical Setup to be about 20 ns in order to cover the entire time of 10 ns between laser pulses (the simulation will go from -10 ns to +10 ns since the center of the pulse is at 0). We want the radial domain to be significantly larger than the laser beam radius. For accuracy, we want the 90 fs pulse to be divided into several time slices (e.g. 10 fs slices) for the calculation. We will need over 1,000,000 time slices to cover the time range from 10 fs to 1E7 fs (10 ns). For these calculations, we set the number of radial samples to 8 and the number of propagation samples to 8 (the number of propagation slices is actually 8 -1 =7). Since the sample is 0.5 mm (500 μm) thick, the thickness of each of the 7 slices is 71.4 μm. The number of samples can be increased if greater accuracy is desired, but this will increase the computer memory needed for the calculation.

In 'Set Extra Parameters' for the emission spectra, the volume width (thickness) should be less than or equal to the thickness of a propagation slice for the simulation (71.4 μm in this example). For the following examples, we set the volume width to be 2 μm, which is approximately a typical depth of focus for the laser beam in a two-photon microscopy setup and is much less than the propagation slice width of 71.4 μm. In general, SimphoSOFT calculates the populations of each energy level for each propagation slice, for each radial slice and for each time slice. For the emission spectrum calculations, only a portion of the total calculated information is used. We specify for these examples that we want emission spectra at normalized positions 0 and 1. For normalized position 0, the program will do the emission calculation using the radial and time populations of slice 1 (from the total of 7 slices). For normalized position 1, the program will do the emission calculation using the radial time populations of slice 7 (from the total of 7 slices).

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(8) See, for example, the spectra at http://nanohybrids.net/pages/contrast-agents-for-better-imaging-results.
(9) www.drbio.cornell.edu/cross_sections.html
(10) See http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html
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