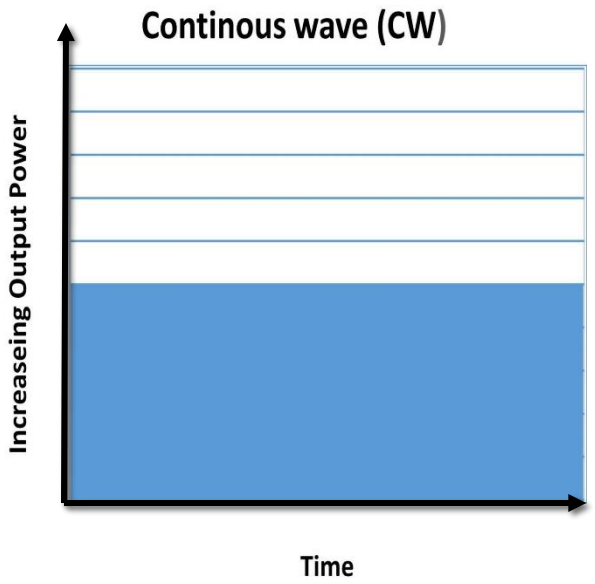
1P, 2P, 2PE, MP, NLO, SHG, and Light Sheet

A PRIMER

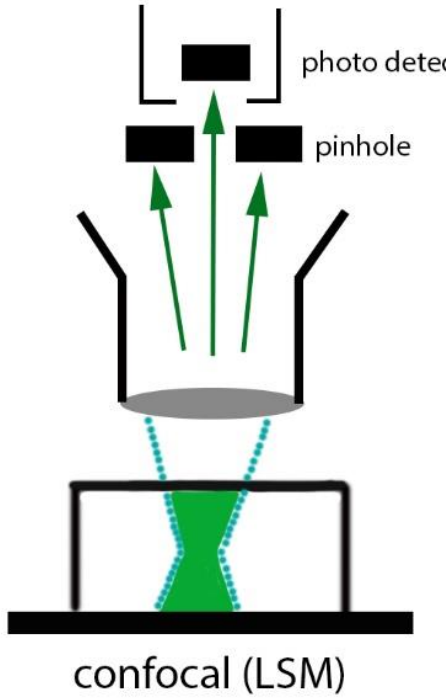
Confocal Imaging

Confocal (also referred to as **1P or laser scanning microscopy**) uses a continuous wave (CW) laser (A) to excite a dye which then emits a longer wavelength photon at lower energy when it returns to the ground state (B). A pinhole is used to collect emitted photons returning in a direct path from the focal plane, while blocking scattered light from coming back to the detector (C). This allows one to optically section, which is an advantage over wide field epifluorescence. However, the sample volume above and below the focal plane is still illuminated by the laser light and thus subject to photobleaching during the scan (D).

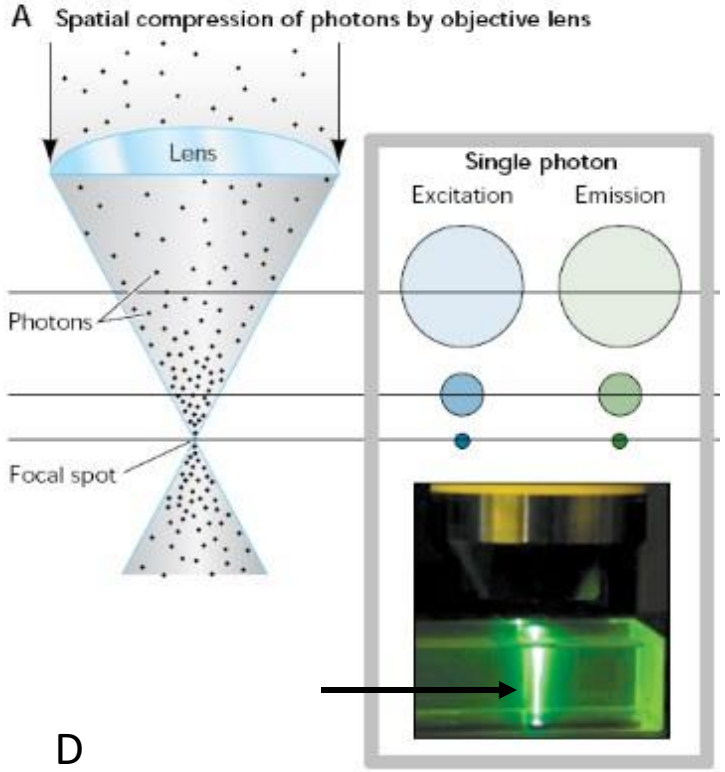


A

B



C



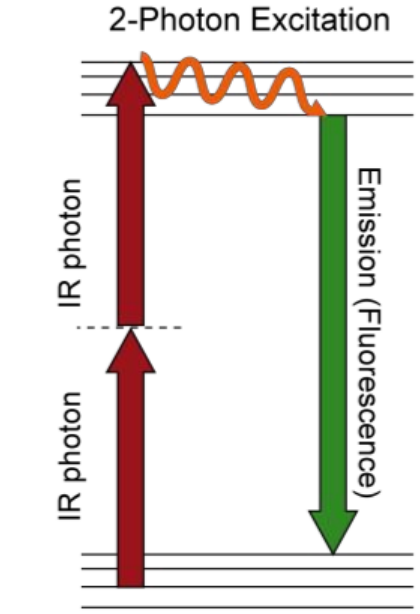
D

Kurtz, Bright solutions to get sharp Images: confocal and two-photo fluorescence microscopy and the pros and cons of new multifocal approaches, in Modern Research and Educational Topics in Microscopy, 2007

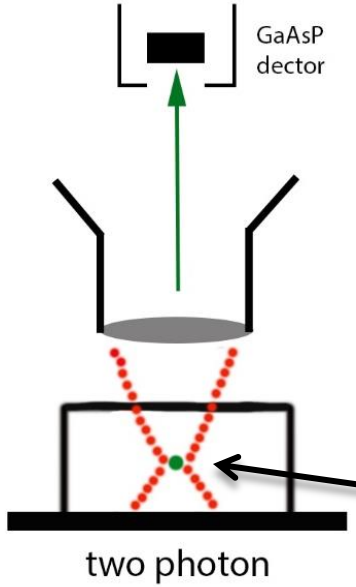
Stutzmann, Seeing the brain in Action: how multiphoton imaging has advanced our Understanding of neuronal function, Microscopy and Microanalysis. 2008, Vol 14, p482-491

Two Photon Imaging

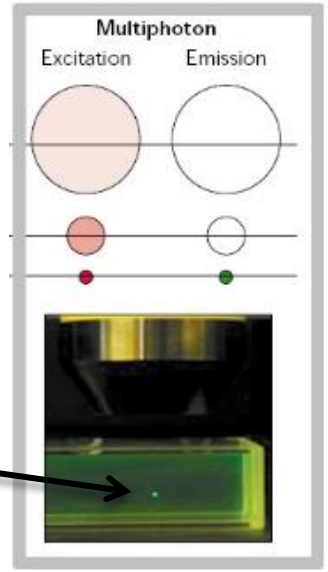
Two-photon microscopy (also referred to as non-linear optics (**NLO**), **2PE**, **multiphoton (MP)**, or **two-photon laser scanning microscopy**) is an alternative to confocal microscopy. *The concept of two-photon excitation is based on the idea that two photons, of comparably lower energy than needed for one photon excitation (ie of longer wavelength), can also excite a fluorophore in one quantum event (A). Each photon carries approximately half the energy necessary to excite the molecule. An excitation results in the subsequent emission of a fluorescence photon. The probability of the near-simultaneous absorption of two photons is extremely low.* Therefore, a high flux of excitation photons is typically required from a pulsed femtosecond laser. Due to the low probability of excitation, only molecules in the focal plane are excited, reducing photobleaching in the tissue above and below the focal plane. Thus no pinhole is needed, and all light is collected. The use of infrared light, which scatters less than lower wavelength lasers, allows for deeper penetration of the excitation beam, and the lack of pinhole allows for more resulting emission to be collected (B and C).



A



B



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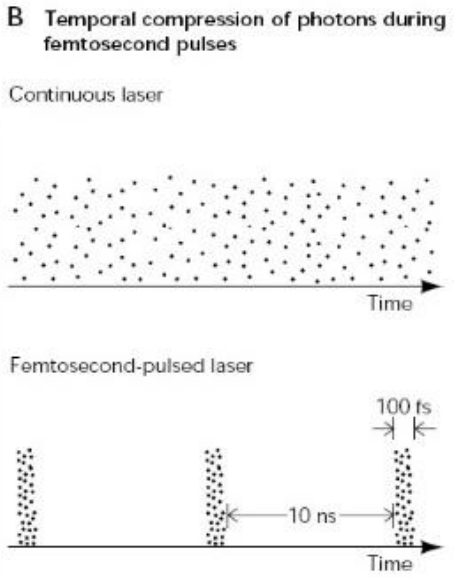

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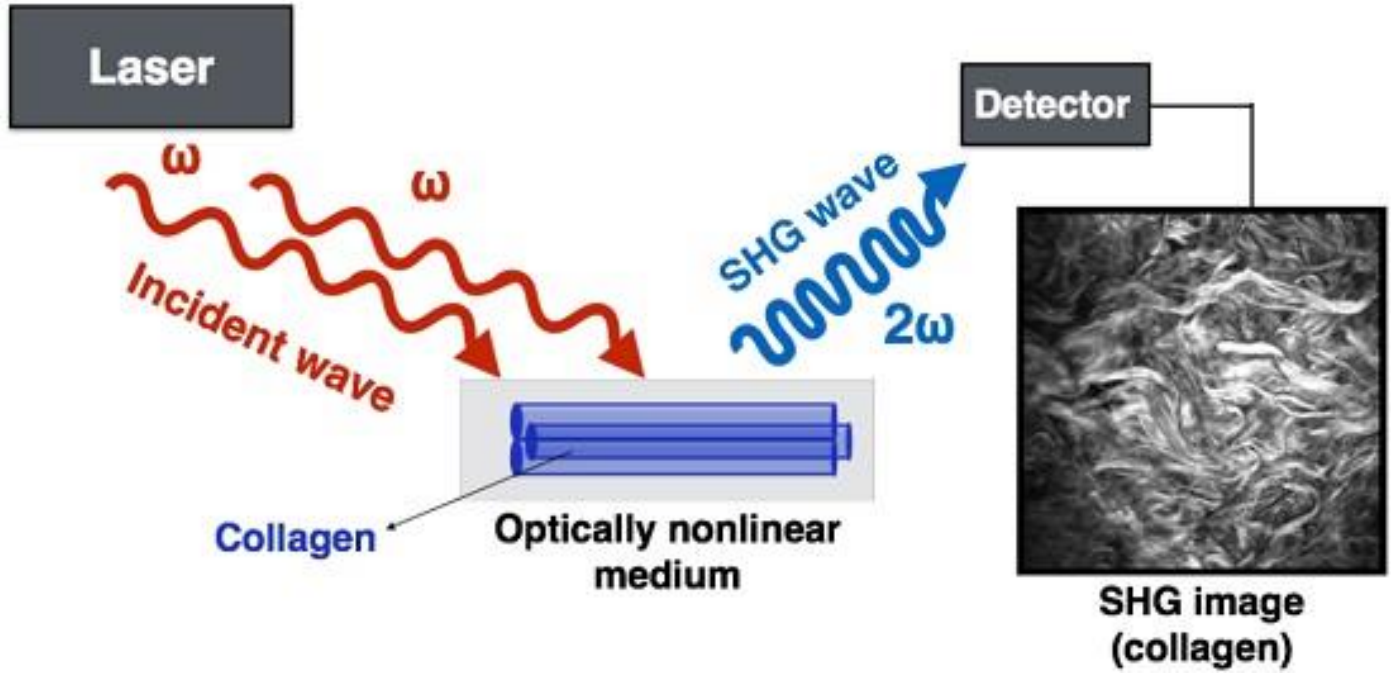
Stutzmann, Seeing the brain in Action: how multiphoton imaging has advanced our Understanding of neuronal function, Microscopy and Microanalysis. 2008, Vol 14, p482-491

SHG

Second harmonic generation microscopy (also known as **SHG**) is another category of NLO imaging. The same pulsed femtolaser is used, but only tissue structures featuring *highly non-centro-symmetric molecular assemblies* will emit this signal, which occurs at exactly ½ of the excitation wavelength. SHG is typically excited at 800 +/- 20nm, although it can be more weakly excited up to 900nm. In addition to the banded collagens, microtubules and myosin will emit this signal. Orientation is important – the strongest emission occurs when the excitation beam is 90 degrees to the orientation of the filaments. SHG also emits in two directions – a transmitted (through) direction, which most of the signal goes to, or a backwards direction. Due to sample thickness, we typically look at the backwards (reflected) signal on our system, sending the emission to the highly sensitive two channel BiG (**2 PMT GaAsP**) detector, however we can image thinner preps in the forward direction using the another PMT.



Mostaco-Guidolin, et al. Int J Mol Sci. 2017, vol 18, 1772
https://doi.org/10.3390/ijms18081772

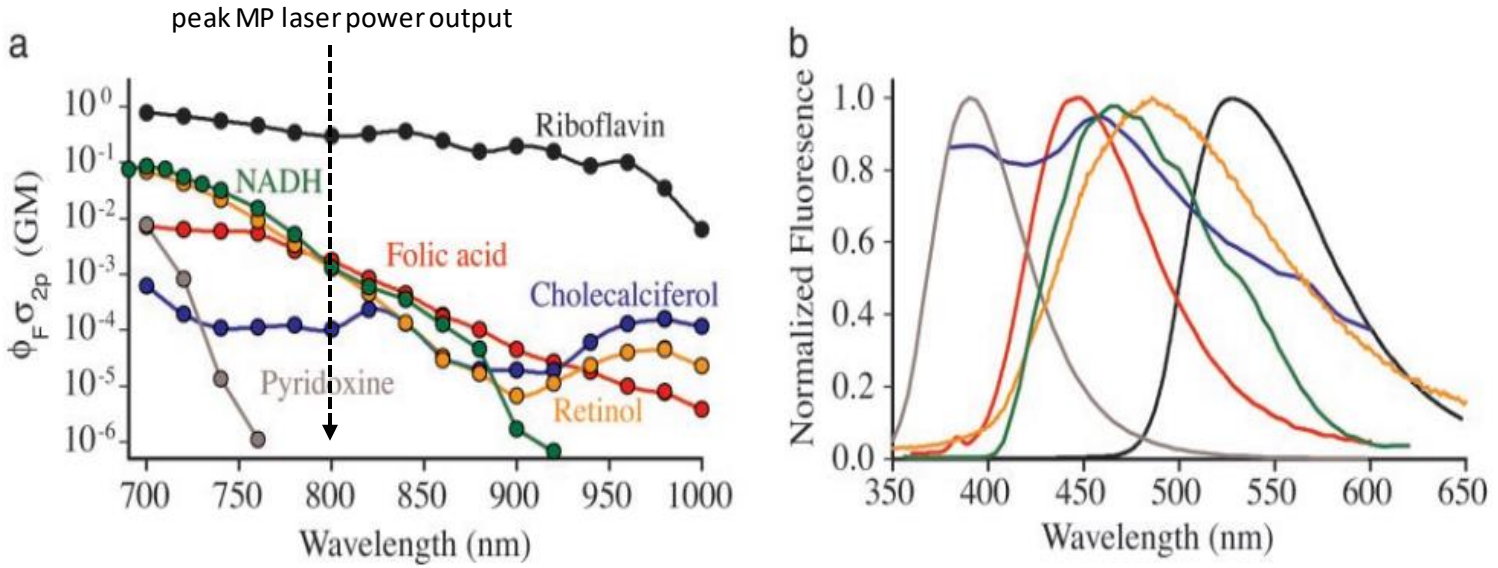
2P vs 2PE – are they the same thing? Actually, yes.

Two photon (2P) and two photon excitation (2PE) microscopy refer to excitation of the following: transgenic fluorescent protein markers (eGFP, eCFP, etc.), traditional fluorescent stains such as DAPI, fluorescent secondary antibodies or markers like dendrimers, and intrinsic fluorophores.

Intrinsic fluorophores are primarily derived from the aromatic amino acids such as Trp, Tyr, and Phe (UV emission) and from vitamin derivatives which emit at longer wavelengths (400–600 nm). Examples of the latter include retinol, riboflavin, the nicotinamide ring of NAD(P)H derived from niacin, or the pyridolamine crosslinks found in elastin and some collagens, which are based on pyridoxine (vitamin B6).

Examples of use: Changes in NAD(P)H fluorescence have been used to measure the metabolic activity in cells over time. We have used imaging of collagen and the 2PE emission to measure biomechanics of the human and mouse ONH.

Multiphoton intrinsic emitters



A. Two photon cross section = excitation curve in MP
B. Corresponding emission spectra

Table 1. Multiphoton excitation (MPE) characteristics of intrinsic emitters in the 700- to 1,000-nm range

Molecule	Process	λ_{ex} 50% max, nm*	Cross section	MPE, refs.†
Tyrosine	3PE	<700	1×10^{-84}	10
Tryptophan	3PE	700–740	1×10^{-84}	10
Serotonin	3PE	700–720	4×10^{-84}	10
Melatonin	3PE	700–720	7×10^{-84}	
5-HIAA	3PE	700–720	2×10^{-84}	
5-HTOL	3PE	700–720	5×10^{-84}	
Retinol	2PE	700–830	7×10^{-52}	
Flavins	2PE	700–730	$1–8 \times 10^{-51}$	9, 13
NADH	2PE	690–730	9×10^{-52}	6–9, 13
Pyridoxine	2PE	690–710	8×10^{-53}	
Folic acid	2PE	700–770	7×10^{-53}	
Cholecalciferol	2PE	<700	6×10^{-54}	
Elastin	2PE	700–740	—	
NFTs	2PE	700–780	—	
Lipofuscin	2PE	700–850	High‡	
Collagen	SHG	700–740	—	41
Microtubules	SHG	—	—	13
Skel. muscle	SHG	—	—	13

2PE-action cross sections given for 700-nm excitation, units are cm^4 s; 3PE cross sections measured at 720 nm, units are $cm^6 \cdot s^2$. (For comparison to Fig. 1: $10^{-50} \cdot cm^4$ s = 1 GM for 2PE cross sections. There is no unit equivalent to the GM for 3PE cross sections.)

*The Ti:S wavelength range where the cross section is >50% of the peak value.

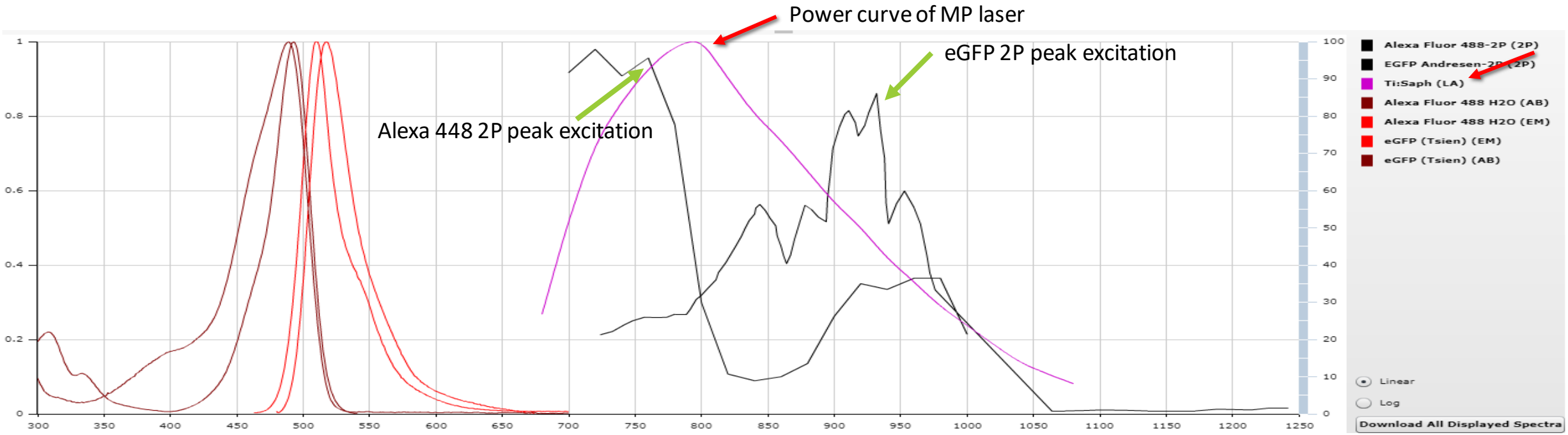
†Listed values include results from previously published work. We note, however, that our collagen SHG efficiency spectrum differs significantly from ref. 41.

‡Varies according to type.

Zipfel et al, PNAS. 2003. Vol 100, p7075-7080
https://doi.org/10.1073/pnas.0832308100

Breaking the rules

The oft quoted rule of thumb is that MP excitation will be twice the 1P emission for a given fluorescent molecule or protein, but this proves to be the exception, not the rule. Example below compares Alexa 488 and eGFP, which have similar 1P profiles, but different 2P profiles. Also keep in mind that the excitation output is also affected by the efficiency of the laser, aka power curve. Hence we "walk" the laser line when developing a new protocol to find the ideal excitation wavelength for a given sample.



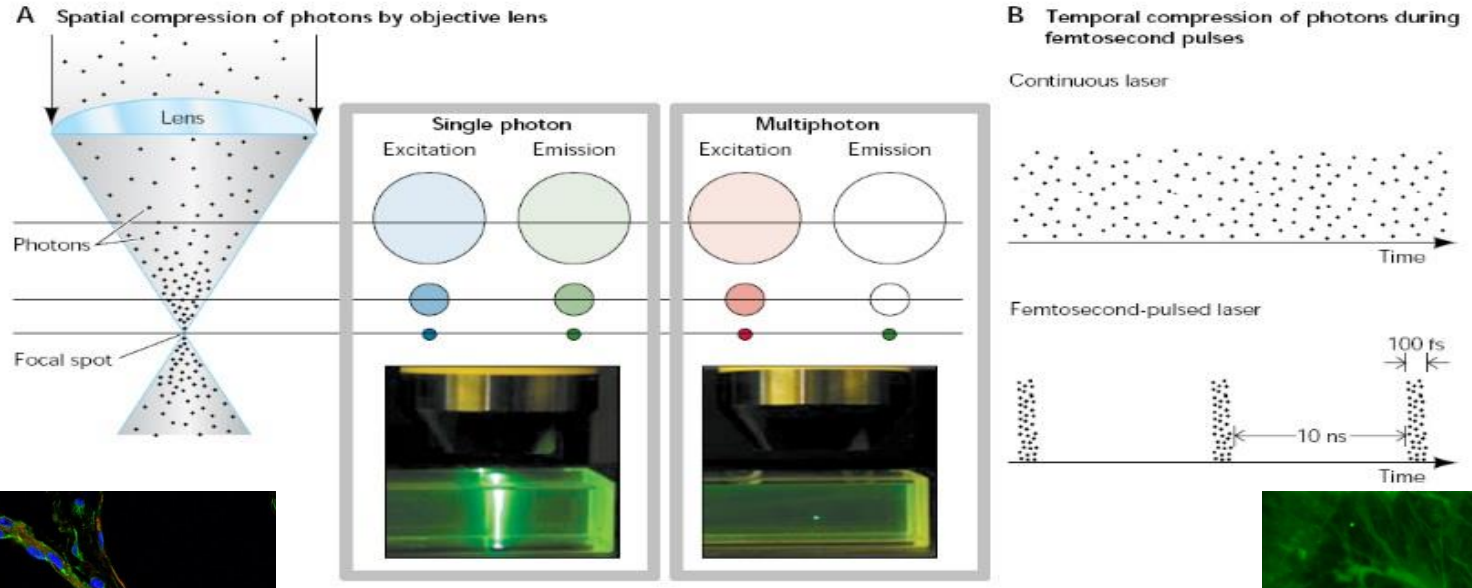
1P Alexa 488 and eGFP excitations on left and emissions on right are very similar.

2P Alexa 488 and eGFP excitation curves are very different.

Above graphics were created using the University of Arizona Spectra Database, http://www.spectra.arizona.edu/

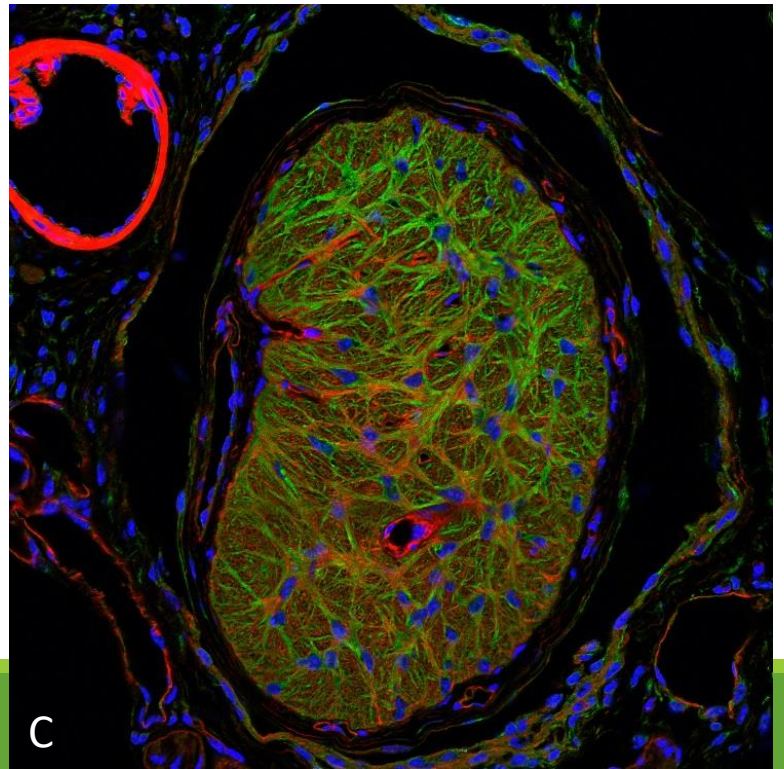
Confocal and multiphoton imaging direct comparison

Confocal is ideal for samples up to 100um and is capable of imaging multiple color dyes in the same sample



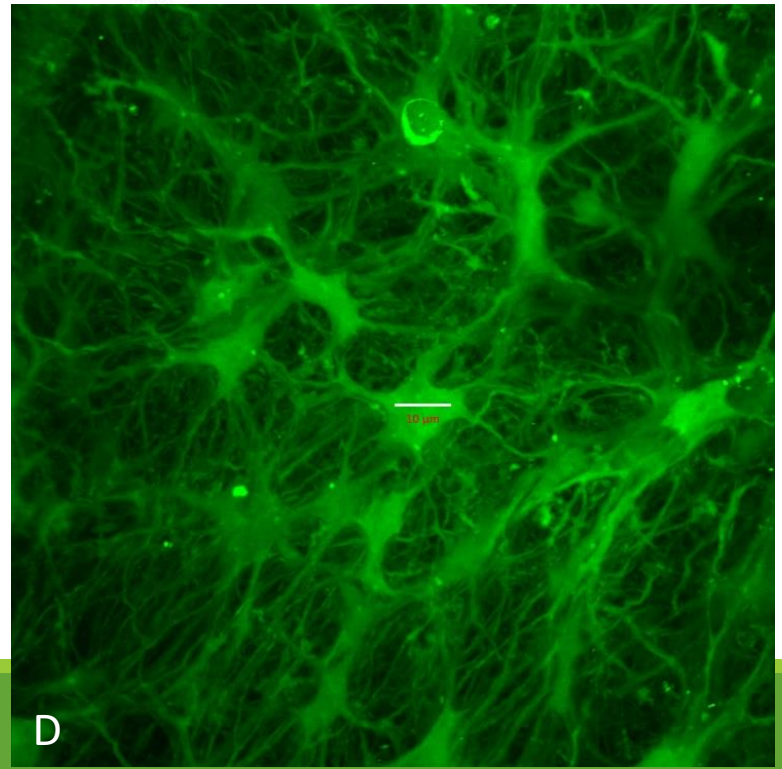
Stutzmann, Seeing the brain in Action: how multiphoton imaging has advanced our Understanding of neuronal function, Microscopy and Microanalysis. 2008, Vol 14, p482-491.

Multiphoton is ideal for thicker samples >50um (vibratome, explants, zebra fish, organoids), imaging with the highly sensitive 2 channel GaAsP detector



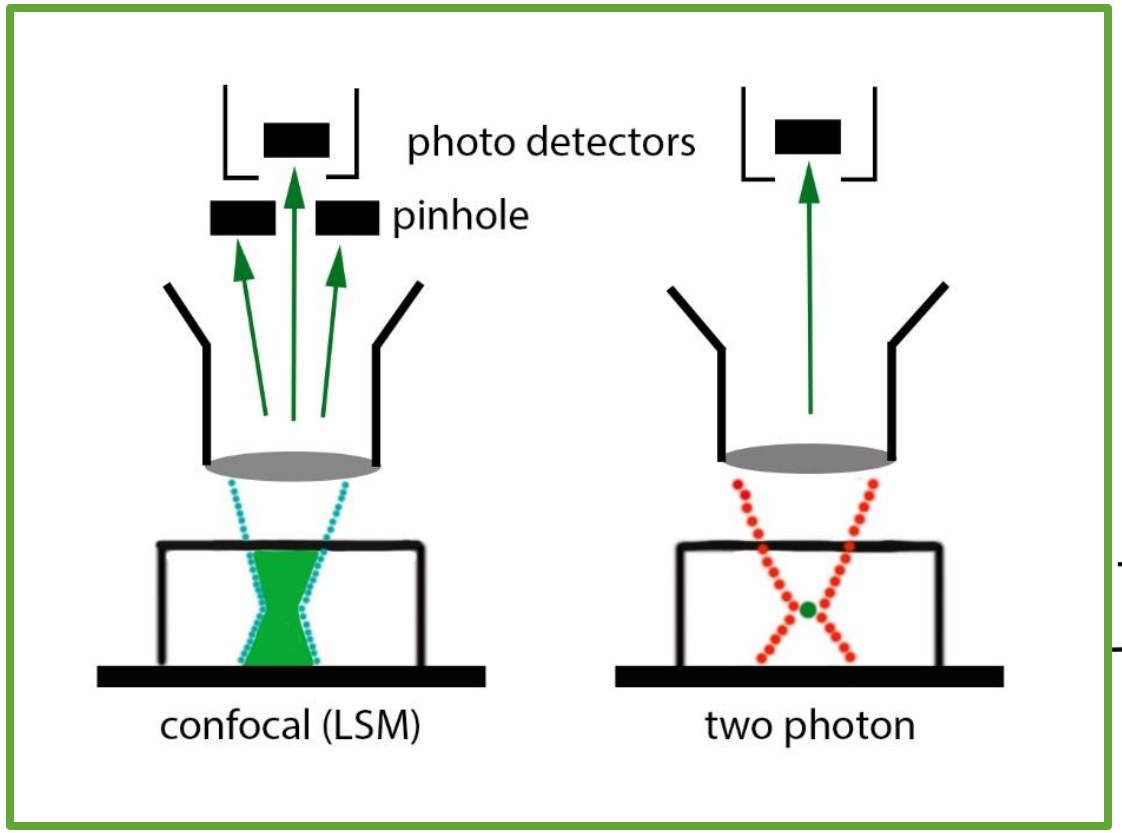
C. 8um section of mouse optic nerve head labelled for GFAP (green), phalloidin (red) and DAPI (blue). Imaged at 63x as a 2x2 tile. Quigley Lab, unpublished

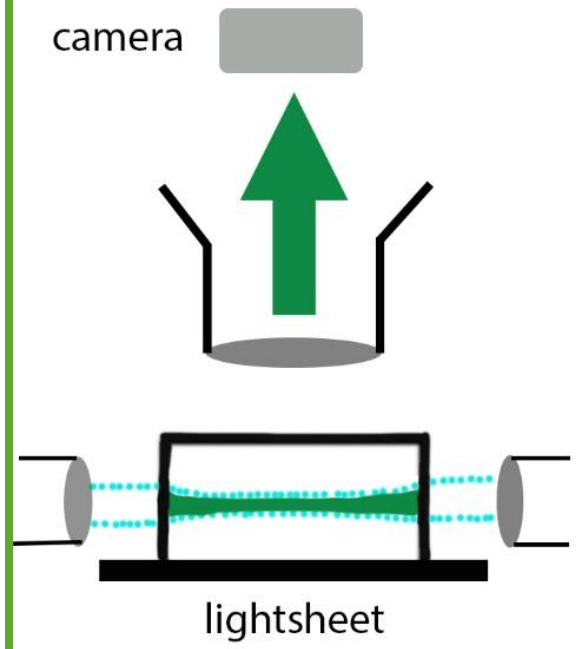
D. 75um vibratome slice of mouse optic nerve head expressing eGFP in the astrocytic lamina cribrosa. Images were captured as a Z stack using the multiphoton laser at 895nm excitation and 63x dipping lens prior to image processing as a maximum projection. Quigley Lab, unpublished.

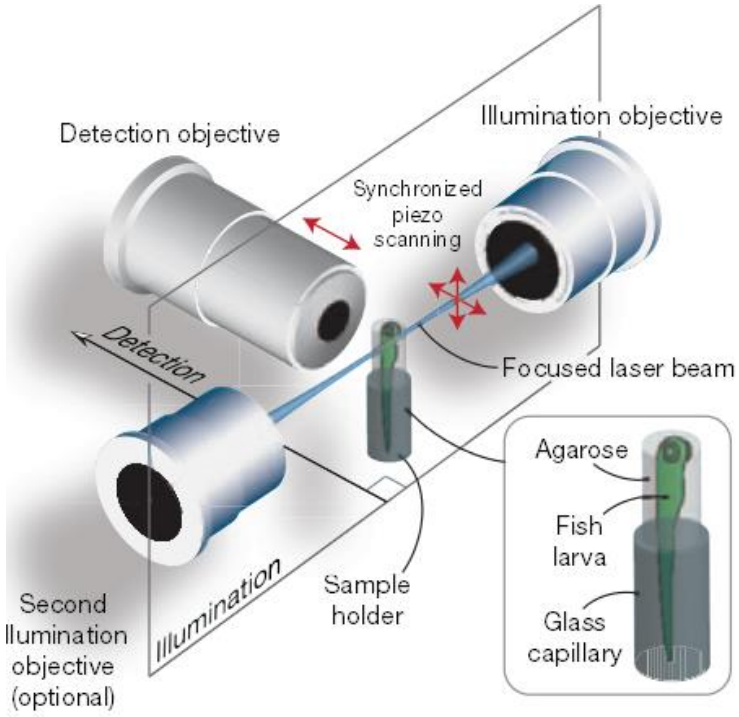


Confocal vs multiphoton vs the light sheet

What will work best for you? Our facility can help you with your confocal and multiphoton imaging needs. *Contact us for a consultation.*







Ahrens et al, Nature Methods Vol 10, p414, 2013