One wavelength to excite them all

Fiber lasers for multiphoton microscopy

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Multiphoton microscopy requires a pulsed infrared light source for excitation of the fluorophores. Titanium:Sapphire (Ti:Sa) lasers are frequently employed as workhorse in many multiphoton setups. They are appreciated for their tunability and output power, but they are also complex systems that require active cooling. As many experiments do neither require tunability nor high powers, compact Erbium-doped fiber lasers today are a convenient alternative. They are ready for many multi-photon applications, although they operate at a fixed wavelength (780 nm) and offer moderate output powers. Their benefits are their compactness, stability, ease-of-use and a competitive price.
In the following we will compare results obtained with a conventional Ti:Sa laser and a fiber laser operating at 780 nm. Our results will show that the FemtoFiber pro NIR is indeed capable of replacing Ti:Sa lasers as a light source in multiphoton microscopes.

The FemtoFiber pro NIR, a compact Er-fiber laser at 780 nm.

The iMIC multiphoton microscope (Till Photonics).

Experimental setup

The FemtoFiber pro NIR is a compact frequency-doubled Erbium-Fiber laser operating at 780 nm. We brought the portable laser into a Ti:Sa lab and placed it on the optical table along with the commercial Ti:Sa and an inverted microscope (iMIC, Till Photonics, equipped with an Olympus 60x 1.2 UPlan APO water immersion objective). Fig. 1 shows a schematic of the setup. The Ti:Sa and the FemtoFiber pro NIR were coupled into the microscope. A flip-mirror is used to switch between the two beam paths. With a telescope in each path, we ensured a beam diameter of 5 mm on the scan mirror for both lasers, which corresponds to a slight over-

illumination of the back aperture of the objective. The Ti:Sa laser was attenuated with an EOM, while the FemtoFiber pro NIR was attenuated with a graduated neutral density filter. We measured the laser power in the focal plane of the objective using a flat photodiode. Tab. 1 provides an overview of the characteristics of both lasers.

Tab 1: Laser systems compared in this study

Model	FemtoFiber pro NIR	Commercial Titanium:Sapphire
Design	Frequency-doubled Erbium-fiber laser	Diode-pumped Titanium:Sapphire laser
Wavelength	780 nm	710 – 990 nm
Average Power	140 mW	300 – 2500 mW[1)]
Repetition rate	80 MHz	80 MHz
Pulse length	100 fs	100 fs
Peak Power	8.9 kW	58 kW
Cooling	none	Water cooling
Size	280 x 229 x 151 mm^3	600 x 350 x 150 mm^3 (laser head)

1) Wavelength-dependent.

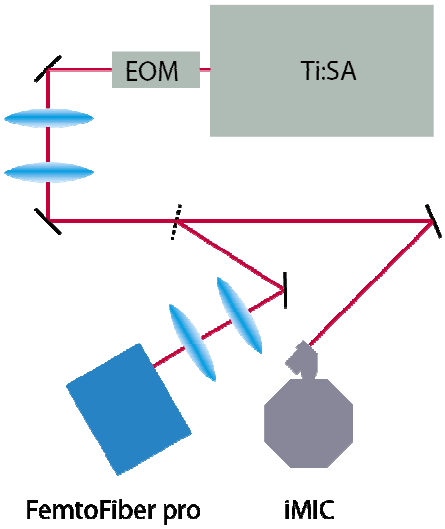

Fig. 1: Experimental setup. The Ti:Sa and the FemtoFiber pro NIR are coupled into an inverted microscope (iMic, Till Photonics). A flip-mirror (dotted line) is used to switch between the beam paths.

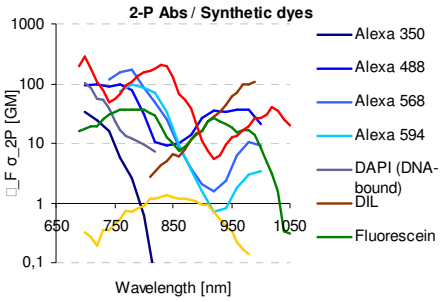

Fig. 2: 2-photon absorption spectra of typical cell and tissue stains (from [2]).

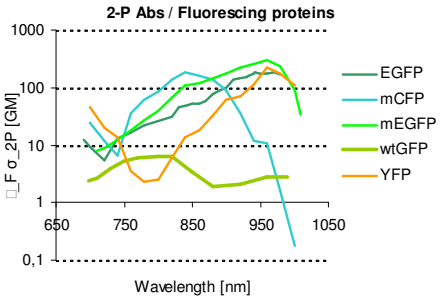

Fig. 3: 2-photon absorption spectra of fluorescent proteins (from [2]).

Power requirements for multiphoton microscopy

2-photon excitation is generally used for imaging of living cells, from single cell layers, to tissue slices and embryos up to whole animals. The nonlinearity of the process results in intrinsic optical sectioning, and the infrared light used for excitation has a higher penetration depth compared to shorter wavelengths, due to reduced scattering.

Reduced overall photodamage is one of the advantages compared to 1-photon excitation as the photodamage is confined to the focal region. But multiphoton microscopy and especially live-cell imaging is still limited by photodamage and phototoxicity at higher power levels. It has been reported that excitation intensities above 10 mW at the specimen plane lead to severe damage after only a few scans. Others state that only power levels of less than 2.5 mW at the specimen plane can be considered as “safe” for typical experiments [1]. Thus, the excessive power levels of Ti:Sa lasers are not necessarily an advantage as no biological tissue withstands these powers directly. The output power of the FemtoFiber pro NIR is still high enough to cause severe photodamage.

780 nm is ideal for 2-photon excitation of most dyes

Multiphoton absorption spectra are typically much broader than the corresponding 1-photon spectra. Usually the peak of the 2-photon absorption is not at two times the 1-photon absorption wavelength. Fig. 2 to 5 show 2-photon spectra of a selection of frequently used dyes and fluorescent proteins (Fig. 3) as well as endogenous auto-fluorescent molecules (Fig. 5). Most of these dyes can be efficiently excited at 780 nm, for instance the widely-used dyes Alexa 488, Alexa 568, and Alexa 594 (Fig. 2). Thus, the output wavelength of frequency-doubled Er-fiber lasers is suitable for exciting most dyes. DAPI and the fluorescent protein YFP are among the very few dyes that cannot be optimally excited with lasers operating at 780 nm.
In the following we will directly compare image pairs of samples stained with Alexa 488 (optimally excited at 780 nm) and YFP ("worst case") that were recorded with a Ti:Sa and the FemtoFiber pro NIR, respectively.

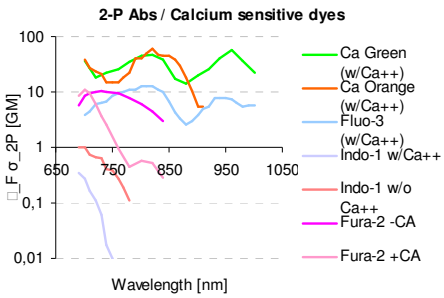

Fig 4: 2-photon absorption spectra of calcium sensing dyes (from [2]).

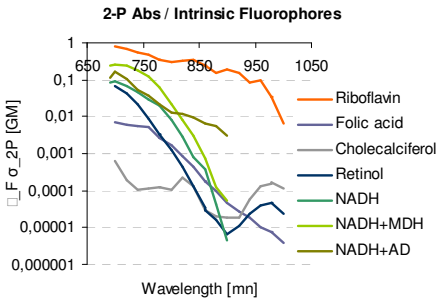

Fig. 5: 2-photon absorption spectra of auto-fluorescent cellular components (from [2]).

Results

The first test specimen was mouse olfactory bulb tissue with axons from receptor neurons stained with Alexa 488. For these measurements, the Ti:Sa was tuned to 780 nm, so that both lasers operated at a wavelength that is suitable to excite Alexa 488.
Fig. 6 and Fig. 7 are projections of three images acquired at a distance of 0.5 µm in the axial direction (FOV 100 µm x 100 µm). The image quality obtained with both lasers is identical. (Note that the brightness values of all displayed images are scaled linearly according to their individual min/max values.) The FemtoFiber pro NIR was set to 10 mW at the focal plane. To get identical image intensities for both lasers, the Ti:Sa had to be set to approx. 16 mW, revealing that the Ti:Sa laser reaches only ~60% of the excitation efficiency of the FemtoFiber pro NIR. This is due to the longer pulse length of the Ti:Sa at the sample which leads to a reduced excitation efficiency. In a separate experiment we quantified the pulse length in the focus of the objective and obtained 424 fs for the Ti:Sa and 210 fs for the FemtoFiber pro NIR. This finding explains the difference in fluorescence intensity.

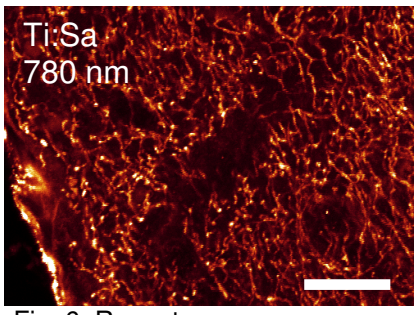

Fig. 6: Receptor neuron axons stained with Alexa 488, Ti:Sa at 780 nm, 15 mW at focal plane (scale bar 20 µm).

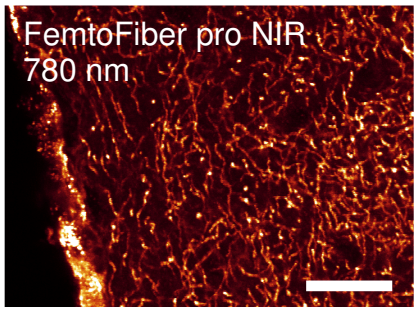

Fig 7: Same sample as in Fig. 6 but with FemtoFiber pro NIR at 780 nm, 10 mW at focal plane (scale bar 20 µm).

In a second experiment we imaged slices of mouse brain tissue in which ~1% of all nerve cells express YFP. YFP represents the "worst case scenario" for a laser with a fixed wavelength of 780 nm, when compared to a Ti:Sa that is tuned to the ideal wavelength for

YFP excitation. In this experiment we therefore compared the performance of the FemtoFiber pro NIR with the Ti:Sa tuned to the optimal wavelength which we determined to be at 950 nm. Both lasers were set to 10 mW in the focal plane of the objective.
Fig. 8 and Fig 9 show the results obtained with the Ti:Sa at 950 nm and the FemtoFiber pro NIR at 780 nm, respectively. The images show similar image quality, especially in the bright cell bodies. As expected, Fig. 9 shows a lower contrast because the excitation of YFP at 780 nm is less efficient than at 950 nm. Still, the fiber laser performs reasonably well.
The brighter background in Fig. 9 is due to auto-fluorescence. This signal is also present when the Ti:Sa is tuned to 780 nm. The absorption spectra of some typical auto-fluorescent cellular components (Fig. 5) indicate, that 780 nm is nicely suited for imaging of unstained samples.

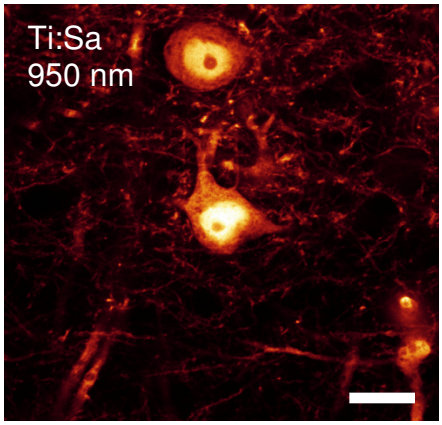

Fig 8: YFP-expression in mouse brain. Ti:Sa at 950 nm, 10 mW at focal plane (scale bar 20 µm).

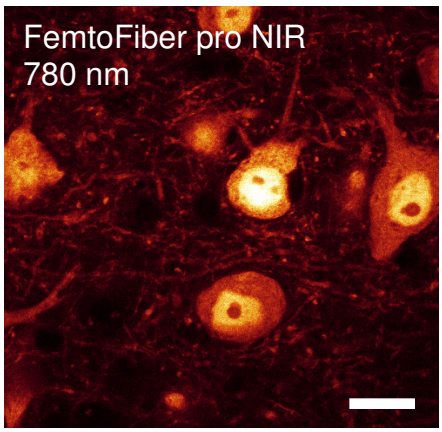

Fig 9: Same sample as in Fig. 8 but acquired with the FemtoFiber pro NIR at 780 nm, 10 mW at focal plane.

Fiber lasers offer a number of advantages:

In conclusion, a fiber laser performs as well as a Ti:Sa laser in many applications. With respect to handling and operation however, fiber lasers offer several advantages:

1. Fiber lasers are less complex; they do not require a pre-compensation.
2. Fiber lasers are stable, push-button systems. The inherent waveguide nature of the fiber makes them insensitive to changing environmental conditions (temperature drifts, vibrations) [3].
3. Fiber lasers are compact, silent and have a power consumption of less than 20 W.
4. Fiber lasers have a competitive price. Instead of sharing one Ti:Sa laser between several microscopes, microscope facilities can equip every microscope with a fiber laser and run these independently.

References

[1] *Highly Nonlinear Photodamage in Two-Photon Fluorescence Microscopy*, Alexander Hopt and Erwin Neher, Biophysical Journal, 80, Apr. 2001
[2] http://www.drbio.cornell.edu/cross_sections.html
[3] *FIBERS FOR FIBER LASERS: PC fibers make capable femtosecond-pulse amplifiers*, John Wallace, Laser Focus World, Feb. 2011