One Plus One Equals Three

Multi-line fiber lasers for nonlinear microscopy

Marion Lang

Any biomedical technique needs to prove its applicability in day-to-day research and diagnosis, not only in "hero-experiments". And the success of a new imaging method significantly depends on the cost and complexity of the employed instrument with the laser playing a major part in both aspects. This is especially true when ultrashort laser pulses are required which is the case for all nonlinear imaging techniques. In this article, we will discuss the advantages of fiber lasers for integrated instruments and present a new cost-effective and maintenance-free fiber laser concept that provides femtosecond pulses at three fixed wavelengths. This allows exciting all conventional fluorescent markers and enables simultaneous multi-color 2-photon imaging with short acquisition times.

Nonlinear optical microscopy

For many biological questions, researchers want to see the object of their interest with three-dimensional (3D) resolution on a cellular level. In a confocal microscope this 3D resolution is achieved by a pinhole, which rejects the out-of-focus fluorescence and achieves a resolution of about 200 to 250 nm laterally and 500 nm axially. Nonlinear microscopy techniques like 2-photon excitation imaging (first demonstrated in 1990 [1]), Second Harmonic Generation (SHG), Third Harmonic Generation (THG) or Coherent Antistokes Raman Scattering on the other hand have the big advantage, that they intrinsically provide 3D resolution and all the generated signal can be used for image formation. 2-photon excitation (2PE) microscopy for example makes use of the fact that a fluorophore can simultaneously absorb two photons of about twice the 1-photon excitation wavelength (i. e. half the energy necessary to excite the fluorophore). This process depends on the square of the intensity of the illumination light and will therefore only occur in the very focus of the objective, generating so-called "optical sections" of the imaged sample (with a resolution comparable to confocal microscopy). The same applies to SHG, with the difference that this is a coherent process and requires non-centrosymmetric structures. Such structures are able to convert the incident light to half the initial wavelength (e. g. from 900 nm to 450 nm). In biological samples, mainly collagen, microtubules or myosin generate sufficiently strong SHG signals for imaging. Fig. 1 shows the energy levels of the nonlinear imaging techniques mentioned above.

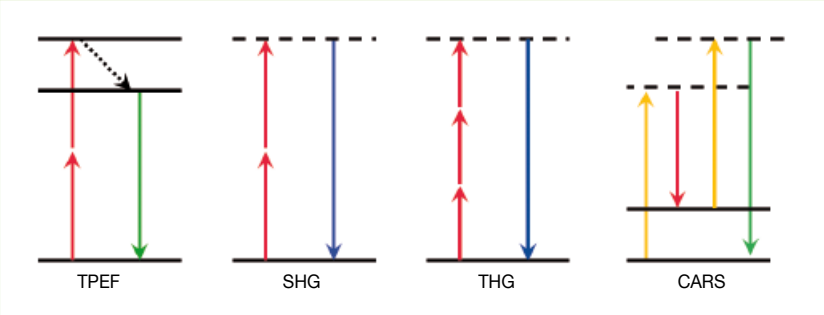

Fig. 1 Energy level schemes of 2-photon excitation, SHG, THG and CARS.

Most commonly used fluorescent markers exhibit a single-photon excitation maximum between 400 and 500 nm and therefore require a 2PE-wavelength that lies in the near infrared. General advantages of these longer excitation wavelengths are the reduced photodamage of the sample and the higher penetration depth (of more than 1 mm) due to less scattering at longer wavelengths (the amount of scattered light is inversely proportional to the fourth power of the wavelength). Furthermore, biological tissue exhibits a so-called "optical window" between 600 nm and 1300 nm, where the penetration depth is maximal. In this window, water and hemoglobin, the most dominant light absorbers in tissue possess only a weak absorption coefficient and the light/tissue interaction is dominated by scattering.

These advantages make nonlinear imaging techniques to high-potential candidates for biological research and (pre-)clinical diagnosis, especially for (long-term) in-vivo imaging.

Femtosecond laser pulses

The imaging of biological samples with nonlinear microscopy requires extremely short laser pulses. The reason is that all these processes have very low probabilities and therefore need very high photon densities to generate a sufficiently high signal-to-noise ratio. In order to keep the average power at a level that cells and specimens can tolerate short laser pulses with pulse durations in the 100-fs region need to be employed. The laser is "off" most of the time and emits photons only for a fraction of a second (10^{-13} s), however with very high peak powers of several tens of kilowatt. These femtosecond pulses are frequently generated by Ti:sapphire

lasers which are quite expensive, bulky and technically challenging systems and require active cooling. Virtually maintenance-free, compact and rugged alternatives are ultrafast fiber lasers that provide one or more fixed wavelengths and are the ideal solution for 24/7-use in research labs and system integration.

Fiber lasers

Fiber lasers use an optical fiber doped with rare-earth elements (e.g. erbium, ytterbium, neodymium, dysprosium, praseodymium, thulium) as active gain medium. They can be very compact, as the fiber can be coiled and bent in order to save space. Inherently they feature a high optical quality and are very rugged, reliable and insensitive to changing environmental conditions. They offer basically zero-maintenance turnkey operation as they run without free-beam optics and are also highly cost efficient. Compared to Ti:sapphire lasers, fiber lasers have much less power consumption and need only passive air cooling. With these properties a fiber laser is the ideal tool for research, and also potential (pre-)clinical applications.

Wavelengths for 2-photon microscopy

2-photon fluorescence excitation typically requires excitation wavelengths ranging from 750 nm to about 1050 nm. The fundamental wavelength of an erbium-doped fiber laser can be easily and efficiently frequency-converted to 780 nm. This wavelength is suitable for 2-PE of many commonly used synthetic dyes [2]. The reason lies in the spectroscopic properties of the fluorophores. Many fluorophores that exhibit no overlapping 1-photon excitation spectra can be efficiently excited at the same 2-PE wavelength. Most 2-PE spectra are rather broad, so many dyes that need different excitation wavelengths in the 1-photon case can be conveniently 2-photon excited with just one (fixed) wavelength. Additionally, the 2-PE maximum is frequently shifted towards shorter wavelengths compared to two times the 1-photon excitation wavelength. The 2-PE maximum of Alexa 568 for example might be expected around 1140 nm (two times the 1-photon excitation maximum of 570 nm). In fact, it is found to be at 780 nm. It turns out that many commonly used synthetic dyes have a 2-PE maximum at or near 780 nm, just like some dyes of the well-established Alexa-family Alexa 488, Alexa 568 and Alexa 594 [c. f. 3].

The green fluorescent protein (GFP) and derivates play a major role in (multiphoton) microscopy. These genetically encoded fluorescent probes have revolutionized the imaging of living organisms (Nobel Prize in Chemistry 2008, Shimomura, Chalfie, Tsien). They can specifically mark structures without preparing the sample with toxic staining procedures and synthetic dyes. Researchers can use the fluorescent proteins to image the transport, localization and interaction of different molecules in-vivo. GFP being the first isolated fluorescent protein is now widely used in research, thanks to stable cell lines and established protocols. GFP has a 2-PE maximum around 920 nm, so this wavelength, too, plays a major role in live-cell microscopy.

New GFP-like variants with different excitation and emission wavelengths allow for multi-color imaging. Just recently a new generation of red-fluorescent proteins has emerged, with long-wavelength excitation- and emission-spectra, offering less absorption and scattering and thus higher penetration depths. At these excitation wavelengths the fluorescence signal is also less impaired by unwanted autofluorescence. These newly developed fluorescent proteins exhibit 2-PE maxima between 1000 nm and 1100 nm [4]. This wavelength regime is very attractive for SHG microscopy of collagen or microtubules in living specimens, too, generating an SHG signal in the visible range and offering imaging depths of several hundred microns.

In conclusion, three excitation wavelengths covering the different regimes should be sufficient to address the majority of all 2-PE applications: one wavelength below or around 800 nm, a second wavelength around 900 nm (especially for GFP) and a third wavelength > 1000 nm for far-red fluorescent markers.

A new laser concept for nonlinear microscopy

A completely new laser approach for nonlinear microscopy is the FemtoFiber dichro. This all-fiber system provides two wavelengths from one aperture: 780 nm and 1030 nm. 780 nm is generated by frequency doubling

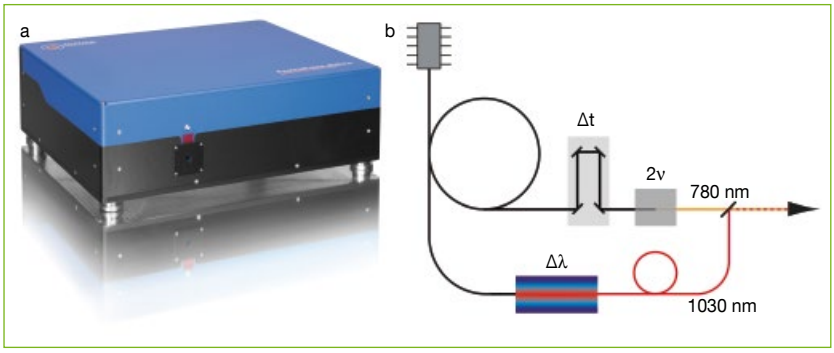

Fig. 2 The dual-color fiber laser FemtoFiber dichro delivers 780 nm and 1030 nm and additionally a virtual wavelength (888 nm) for 2-photon microscopy. As both wavelengths come from the same oscillator, the pulses are perfectly synchronized. A delay-line allows shifting one wavelength with respect to the other, in order to adjust the temporal overlap of the pulses at the sample.

Company

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the fundamental wavelength of the Er-fiber oscillator. A frequency shift generates 1030 nm, the second output wavelength. Fig. 2 shows the schematic of the laser. As one oscillator generates both outputs, they are perfectly synchronized in time. With a delay-line in one of the two arms the user can shift the pulses of one of the wavelengths with respect to the other output. This allows adjusting the temporal overlap of the pulses, so that both pulses can be set to arrive simultaneously or time-delayed at the sample. The laser emits both wavelengths collinear, so both foci will overlap perfectly in *x* and *y* at the sample. Due to chromatic aberrations in the microscope however, both foci may be shifted in the *z*-direction (most conventional microscope optics correct for chromatic aberrations in the visible range only and not in the infrared). This aberration can be easily corrected by adjusting the divergence for one of the two wavelengths. After this one-time adjustment for each microscope both foci have perfect spatial overlap.

With these properties the new fiber laser is a promising light source for 2-photon microscopy. Both wavelengths can be used individually for conventional 2-PE of suitable fluorophores. Additionally, this special laser configuration allows exciting fluorophores with a 2-PE maximum between the two output wavelengths. When the pulses of both wavelengths overlap in space and time, a process called 2-color 2-PE can occur (Fig. 3). This process has already been demonstrated with a different technical setup, see e. g. [5]. The fluorophor simultaneously absorbs one 780 nm and one 1030 nm photon. This corresponds to a virtual excitation wavelength of $\lambda_3 = 2/(1/\lambda_1+1/\lambda_2) = 888$ nm, a wavelength that allows for instance to excite GFP.

A major benefit of the presented configuration is that all three wavelengths are available at the same time. This allows performing live-cell multicolor 2-photon imaging with two or three excitation wavelengths simultaneously with fast acquisition times comparable to single-channel imaging. In contrast, when using a Ti:sapphire laser all wavelengths need to be imaged sequentially, with an additional delay required for tuning from one wavelength to the other.

Results: Neurons and neutrophils

Fig. 4 shows first results of the new fiber laser on an Intravital2P microscope (FEI, Munich). The sample is a section of the Hippocampus area of a mouse brain (courtesy Prof. Herms, LMU, Munich). About 10 % of the neurons express the yellow fluorescent protein (YFP) (b, yellow). The neutrophil white blood cells are marked with GFP (b, green circle). Figs. 4(c)-(f) show the fluorescence signals for different excitation wavelengths: 780 nm (c), 1030 nm (d) both wavelengths with (e) and without (f) temporal overlap at the sample. 1030 nm excites the YFP fluorescence efficiently as the YFP 2-photon excitation spectrum shows a shoulder around 1030 nm. If both pulses reach the focus synchronously (e), they can excite also the GFP-expressing neutrophil white blood cells. This result with the new fiber laser is comparable to exciting the sample with a Ti:sapphire laser set to 900 nm (g).

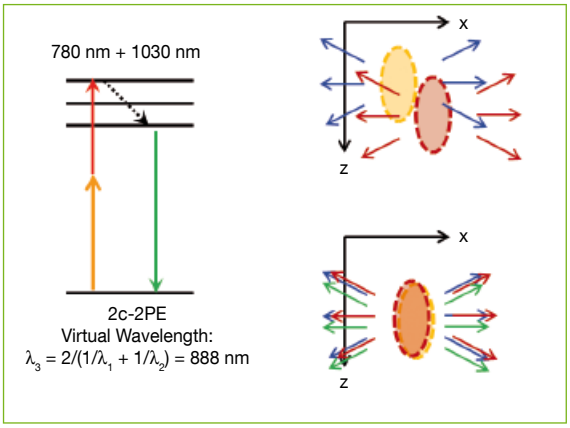

Fig. 3 Energy level scheme of 2-color 2-photon excitation (2c-2PE). This process provides the virtual 2-PE wavelength 888 nm and is maximized when both pulses exhibit perfect spatial and temporal overlap at the sample.

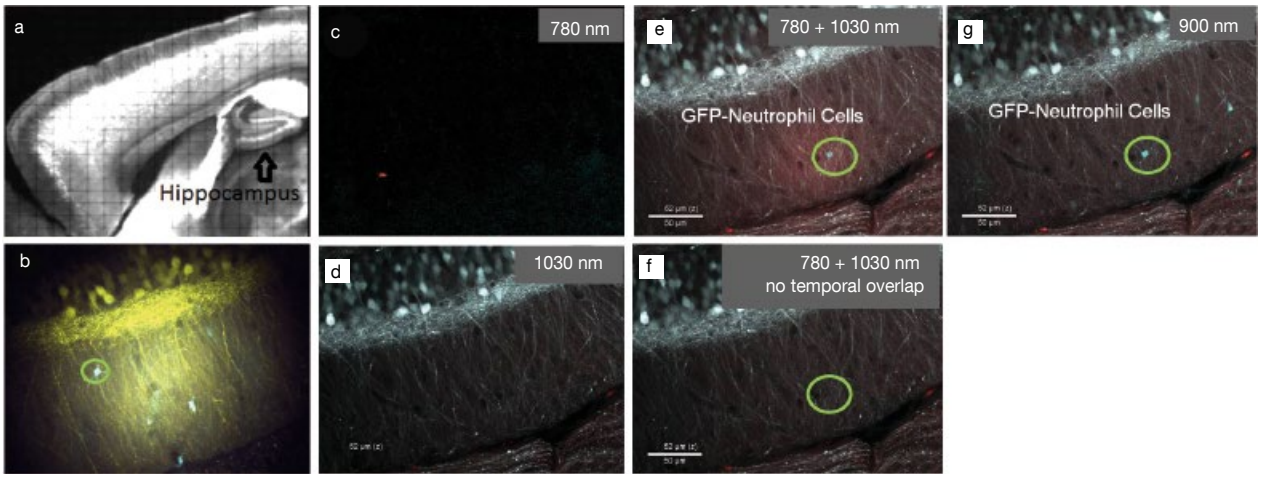

Fig. 4 The Hippocampus-area (mouse) (overview images (a), (b)) shows YFP-expressing neurons and neutrophil white blood cells marked with GFP. The images display 2-PE of the sample at 780 nm (c), 1030 nm (d), 780 + 1030 nm simultaneously with (e) and without (f) temporal overlap and a comparison with a Ti:sapphire laser at 900 nm (g). When pulses at 780 nm and 1030 nm reach the sample simultaneously (e), GFP is efficiently excited via 2-color 2-photon excitation.

Conclusion

Fiber lasers are compact, reliable and easy-to-use alternatives to complex Ti:sapphire lasers with well-established fixed wavelength systems at 780 nm and/or 1560 nm that are so far frequently employed for single-purpose applications (e.g. Calcium imaging with Fluo-4). A new fiber laser system now provides three wavelengths simultaneously. These three wavelengths allow exciting all conventional fluorescent markers used for 2-photon microscopy: a variety of synthetic dyes can be excited at 780 nm and many red fluorescent proteins at 1030 nm. The virtual wavelength 888 nm allows exciting markers with a 2-PE maximum around 900 nm, which is especially relevant for GFP-expressing samples. As all three wavelengths are available simultaneously this is the first commercial system that allows running 2-PE multi-color live-cell experiments with short acquisition times (comparable to 1-color 2PE-imaging). The robust all-fiber system is a convenient solution for integrated instruments and thus will support the further success of nonlinear imaging techniques in the research lab and beyond.

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[5] *P. Mahou* et al., Multicolor two-photon tissue imaging by wavelength mixing, Nat. Methods **9** (2012) 815–818

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