

# BIOPHOTONICS

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## WHITE PAPERS & APPLICATION NOTES



### Sub 50 femtosecond pulse lasers for gentler multiphoton microscopy

Finding the ideal ultra-fast laser source for a multiphoton imaging setup is not trivial. It is a fine balance between peak power, pulse energy and laser wavelength. In this white paper we discuss important laser parameters and the impact those laser parameters have on the quality of the results.

#### Background

Multiphoton and higher-order harmonic generation microscopy is particularly suited for high-resolution three-dimensional and live-cell imaging, where single-photon techniques are limited by out-of-focus fluorescence background and penetration depth. Imaging techniques based on multiple-photon interactions, such as two- or three-photon excitation and higher-order harmonic generation such as second or third-harmonic generation (SHG or THG) imaging, have become very attractive tools in many biomedical research or clinical diagnosis applications, as they provide high contrast imaging capabilities with reduced tissue damage while not necessarily requiring artificially induced fluorescent dyes.

For multiphoton excitation to occur, incident photons must arrive at the sample at the same time and place, in order to increase the probability of simultaneous multiphoton absorption events by a single fluorescent compound, which means that the efficiency in multiphoton excitation is strongly dependent on the peak intensity of the incident light during the pulse. Therefore, the shorter the pulse, the higher the peak intensity and the stronger the generated signal.

As a consequence of the nonlinear dependence on the light intensity in multiphoton microscopy and higher-order harmonic imaging, the signal is already collected from within the plane of focus and the scattered light problem almost ceases to exist in these systems. This resulting confined multiphoton excitation volume is especially suited for live-cell imaging, as it results in higher depth resolution and significantly less photodamage through the sample when performing cell viability.

In addition, the longer wavelengths used in multiphoton imaging increase the penetration depth in the sample thanks to the lower scattering. Thus, multiphoton microscopy is often preferred over other techniques for experiments requiring deep penetration and imaging of large cells and tissues. Multiphoton excitation and higher-order harmonic imaging require high peak intensities, which demands the use of mode-locked lasers with ultrashort femtosecond pulses. To trigger a significant

number of multiphoton absorption events, the photon density during the pulse must be orders of magnitude larger than in single-photon absorption. Previously published results [1, 2] suggest that even shorter pulses than the commonly used pulse durations of 100-500 fs, enable lower cell lethality rates and allow for longer term live-cell imaging. In this white paper we show that the use of sub-50 fs pulsed lasers produces very bright multiphoton and higher-order harmonic images while keeping the average laser power low, and that this presents a distinct advantage in extending live-cell imaging.

#### Imaging depth - longer wavelength?

One of the initial considerations in three-dimensional imaging is sample transparency at the selected laser wavelength. In most cases, the penetration depth is limited by light scattering due to the inhomogeneous nature of cells.

Light is scattered in anisotropic media, but longer wavelengths undergo less scattering than shorter wavelengths and therefore allow for deeper optical sectioning, a greater penetration depth and three-dimensional imaging into thick samples. Light scattering (Rayleigh scattering scales with  $\lambda^{-4}$ ), meaning that scattering is about 3 times less at 1.0  $\mu\text{m}$  compared to at 0.6  $\mu\text{m}$ . Although even longer NIR wavelengths (e.g. 1.2  $\mu\text{m}$  or 1.7  $\mu\text{m}$ ) will further reduce light scattering effects, smaller deep tissue microscopy results can be obtained using wavelengths around 1.0  $\mu\text{m}$ .

#### Absorption - shorter wavelengths?

As shown in Figure 1, another challenge for penetration depth and harmonic generation for longer NIR wavelengths, is absorption within the sample, especially by water. For imaging water with 0.2  $\mu\text{m}$  can help attenuate this to some degree, but the laser power will need to be reduced to avoid heating of the sample. The main chemical component of tissue is water and the absorption cross-section of water in Figure 2, can be used to help identify a suitable excitation wavelength. As can be seen, the water absorption is lowest in the visible region. With a shot of the light absorbed per cm, there is also a clear absorption minimum around 0.6  $\mu\text{m}$ .

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