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White Paper

Longer Wavelengths Enable Deeper Tissue Microscopy with Minimized Photodamage

The push-button combination of a broadly tunable one-box Ti:Sapphire laser and an independently tunable compact OPO supports optimum excitation of new fluorophores and deeper imaging than ever before.

Overview

Non-linear imaging techniques like Multiphoton Excitation (MPE) or Second Harmonic Generation (SHG) Microscopy are unique tools to investigate cellular biology and physiology as well as neuronal structure and functionality. Different non-linear techniques can be applied simultaneously on the same sample to provide “information-rich” images, combining morphology, physiology and even chemistry. This “multimodal” approach requires increased flexibility and functionality from the ultrafast laser sources that is at the heart of the microscope system. Just as important, there is a growing interest towards longer wavelengths, specifically in the 1000 nm to 1400 nm range, to provide optimum excitation of new fluorescent probes and minimization of photodamage and scattering. Lower scattering translates directly in the capability to produce deeper in-vivo images, which is one of the main advantages of multiphoton microscopy.

Flexibility and extended operation should not compromise ease of use, as the biologist’s focus is getting images rather than spending time fiddling with the laser source. In addition, non-linear imaging microscopes are often shared resources and the associated laser equipment should operate as a turnkey tool even when operated by a laser novice. This whitepaper describes how these needs are fully satisfied by the Coherent Chameleon™ laser family, together with its novel and unique optical parametric oscillator – the Chameleon Compact OPO.

Why Longer Wavelengths?

Whether it’s to study some aspect of morphogenesis, model ischemic strokes, or the murine cortex, live tissue imaging is characterized by the need to minimize photodamage, maximize depth of penetration, and acquire images with higher signal-to-noise ratio. All of these goals can be better met by ultrafast laser sources that deliver longer wavelengths, from about 900 nm to beyond the limit of Ti:Sapphire lasers, and that produce relatively high powers at these long wavelengths. Of course it is also necessary that appropriate fluorophores, bright, non-toxic and excitable at these wavelengths be readily available. The use of longer wavelengths result in reduced scatter losses, enabling deeper imaging. Specifically, because of the homogenous nature of biological samples, the majority of the light attenuation is due to the Mie scattering which scales as 1/λ4. Even small increases (by tens or hundreds of nanometers) in the excitation wavelength can lead to significant reduction in scattering, which has proved to be a limiting factor in deep tissue imaging. It is important to note that tissue absorption of infrared light, especially in cases where the main absorber is water, has a local minimum around 1.06 micron and then increases steadily. However the main contributor to laser light attenuation is scattering and this – in turn – means that wavelengths longer than 1.06 micron can penetrate more deeply in most thick samples.

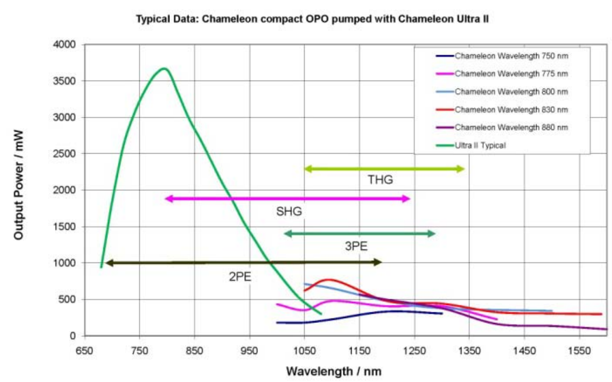

Figure 1: *Tuning curves of Chamelon Ultra II and Chameleon Compact OPO with overimposed the typical wavelength ranges used for two-photon excitation (2PE), three-photon excitation, second harmonic generation (SHG) and third harmonic generation (THG) microscopy.*

The latest generation of one-box Ti:Sapphire lasers for microscopy, such as the Coherent Chameleon Ultra and Vision families, provide tunable output from 680 nm to 1080 nm, comfortably reaching the point of lowest water absorption. The wavelength tuning is completely automated and takes only seconds via the graphical user interface. The keys to this extended wavelength range are simple: a more efficient Ti:Sapphire oscillator cavity with lower losses, and the availability of higher 532 nm pump power, with up to 18 Watts of usable power. These advances raise the entire power/wavelength output profile (see Figure 1), thus providing the twin benefits of longer wavelength and higher power, hence brighter images. The Chameleon also features a completely circular beam with little or no astigmatism which focuses to the smallest possible spot to produce superior image resolution and brightness.

Of course, longer wavelengths are only useful if there are fluorophores that can be optimally excited at these wavelengths, like the relatively new mFruit series of fluorescent proteins (e.g., mCherry, mTomato, mBanana) that have long wavelength two-photon excitation peaks. Fortunately, these are becoming increasingly more popular and available in brighter and stable versions and in 2009 Tsien reported even an Infrared Flourescent Protein (IFP1.4) [1].

The advantages of extending the laser wavelength range to 1080 nm and beyond are supported by several studies into the wavelength dependence of two-photon excited fluorescence intensity of mCherry, one of the most widely used members of the mFruit family. For instance, Figure 2 shows images from a study by Vadakkan et al, clearly indicating that maximum image brightness is obtained at a laser wavelength of 1070 nm [2]. Moreover, these researchers, using a Coherent OPO measured the two-photon absorption spectrum peak of mCherry and found a peak at 1160 nm. Two-photon absorption cross-section of various long-wavelength fluorescent proteins have been recently published [3] and IFP 1.4 is expected to have a two-photon absorption peak around 1300 nm to 1350 nm.

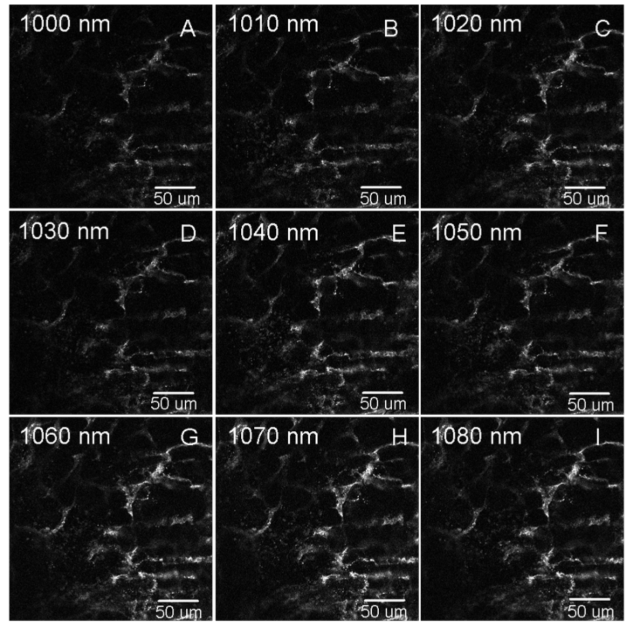

Figure 2: *Images of blood vessels in skin of neonate mice expressing mCherry.*

Push-Button Access to Even Longer Wavelengths
The 1080 nm wavelength is currently the practical limit for one-box Ti:Sapphire laser oscillators, but moving to even longer wavelengths should further reduce scatter, limit photodamage and increase the maximum imaging depth. This has been confirmed in studies by various groups [4-6]. For example, Kobat et al. [4] have conclusively shown that the decrease in scattering at longer wavelengths makes 1,200 nm to 1,300 nm an optimum region for deeper tissue penetration.

As stated earlier, acceptance of these longer wavelengths hinges on delivering them in a push-button fashion, with no additional complexity and with the same beam quality and consistency of current state-of-the-art Ti:Sapphire lasers.

Direct access to wavelengths beyond 1100 nm is not possible with Ti:Sapphire technology. Because of this limitation, Coherent has developed a sophisticated yet easy-to-use accessory that extends the wavelength of Chameleon lasers up to 1600 nm. The Chameleon Compact OPO is an optical parametric oscillator (OPO) designed for optical efficiency and simplicity of operation. In an OPO, a single input wavelength is converted into two longer output wavelengths via a nonlinear optical crystal. The shorter of these new wavelengths is called the signal wavelength, and the longer, the idler.

The relationship between these three wavelengths is given by:

1/input wavelength = 1/signal wavelength + 1/ idler wavelength

The exact value of the signal (and idler) can be seamlessly varied by small adjustments of the OPO cavity length through its microprocessor control. This simple tuning mechanism is enabled by a type of crystal that is "periodically poled" and that eliminates the need to change the pump wavelength or the crystal temperature, methods that are more cumbersome and slower.

Independent Tuning - Multimodal Imaging

An OPO based on a periodically poled crystal is optimized to operate at a single input wavelength. For OPOs used with Ti:Sapphire lasers, manufacturers usually set this wavelength at 780 nm to 830 nm. Using a novel growth process that produces so called "fan poled" crystals, this restriction is lifted: the same OPO can operate over a wide range of input wavelengths. This means that the Ti:Sapphire laser and OPO wavelengths can be separately tuned, thus providing two fully independent wavelength sets, when only part of the laser output beam is used to pump the OPO.

Moreover, both wavelengths can be varied in seconds through the software interfaces. Additional frequency doubling and tripling modules can double the laser and/or OPO wavelength, thus providing complete gap-free tuning all the way from 227 nm to 1600 nm. No other single microscopy source can approach this level of wavelength flexibility and ease of use. The graph in figure 3 summarizes the combination of simultaneous tuning ranges provided by the Chameleon OPO.

Having broad wavelength tuning together with two independently variable wavelengths enables the same compact laser setup to be used for complete multimodal imaging studies, using all the different types of nonlinear imaging techniques, including CARS, SHG/THG imaging, and multiphoton excitation (MPE). This is an advantage that greatly enhances the value of a single non-linear microscopy source since these techniques are complementary at imaging different types of structures. For example, CARS is often the best method for imaging lipids with a typical Raman shift of 2840 cm^{-1} to detect C-H stretch vibrations.

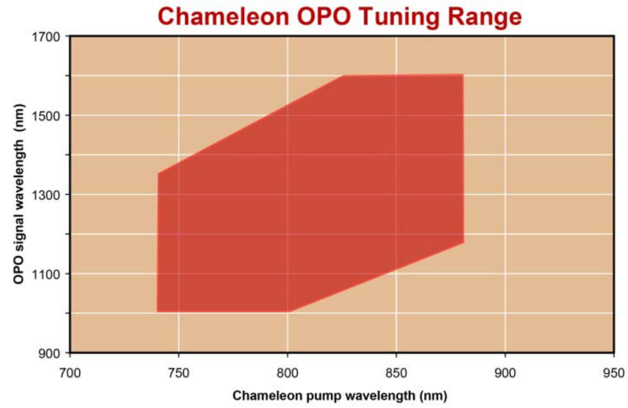

Figure 3: *The red area indicates the combination of wavelengths simultaneously available from Chameleon Ultra II (horizontal axis) and Chameleon Compact OPO (vertical axis).*

MPE on the other hand is ideal for exciting fluorophores as well as endogenous fluorescence, while SHG/THG methods are well-suited for imaging collagen, muscle sarcomeres, membranes and structures rich in lipids.

Power, Wavelength and Photodamage

The ability to perform live tissue imaging has always been recognized as one of the major advantages of nonlinear imaging techniques, compared to confocal microscopy using shorter, blue-green wavelengths, and deconvolution microscopy which has a significant speed limitation. A critical aspect of live cell imaging is to use laser operating parameters that maximize image brightness and minimize photobleaching and photodamage of the sample. The flexibility and long wavelength tuning of the Chameleon (and Chameleon OPO) is a substantial advantage here, too. That's because there are three factors that can influence the trade-off between image brightness and sample photodamage: average power, pulsewidth at the sample, and laser wavelength. Chameleon provides adjustability of these parameters over a greater range than any other laser microscopy source.

A number of studies showed that wavelength is a critical parameter in determining the amount of photodamage during imaging. For example, Chen et al [5] showed that sustained multi-photon spectra can be safely observed in most plant specimens with a tightly focused beam at a wavelength of 1280 nm under long term irradiation with more than 100 mW laser average power. In the same study, these researchers reported multi-photon absorption damage to occur within seconds when the power of a tightly focused 830 nm Ti:Sapphire laser beam exceeded 10 mW. In a study on embryos [6], Yakovlev concluded that "It is clear that there is a significant difference between 800 nm and 1250 nm high-intensity light irradiation. Longer wavelength radiation produces much less damage to embryos, and can be considered a safer radiation."

In contrast, the issue of the trade-off between average power and pulse duration (i.e., peak power) has been the subject of some debate. Indeed, this controversy has given rise to the question if the shortest pulses always lead to brighter images and how this correlates with photodamage. Most researchers seem to concur that in the case of two photon MPE excitation, the fluorescence signal depends on the probability of two-photon absorption, whereas the damage is a combination of two and three-photon absorption, possibly through an intermediate step.

Signal intensity $\alpha\ (P_{peak})^2$ x pulsewidth = P_{ave} x P_{peak}

whereas

Damage probability $\alpha\ (P_{peak})^{2.5}$ x pulsewidth = P_{ave} x $(P_{peak})^{1.5}$

What does this mean in practice? For a given average power, decreasing the pulsewidth increases the signal and damage – but the damage increases at a steeper rate than the signal. And for a given peak power, increasing the average power (and increasing the pulsewidth) will increase signal and damage at the same rate. Conversely, lower average power and longer pulsewidths decrease the probability of damage (and decrease the signal level). So, the bottom line is to use power sparingly and use shorter pulses only when really needed. If the contrast mechanism (fluorophore) permits or is designed for long-wavelength excitation, then use longer wavelength to minimize damage and increase penetration.

In addition to the Compact OPO, the Chameleon is available also with an integrated pre-compensation feature (Chameleon Vision Series). This is an optical assembly that introduces negative group velocity dispersion (GVD) to the laser output to exactly offset the dispersion in the downstream microscopy optics. The pulsewidth at the sample can thus be computer-controlled and smoothly varied from fully compensated (shortest) to a fully dispersed (longest) value. Chameleon Vision is the only laser to provide up to 47,000 fs^2 of pre-compensation, making it ideal for use with microscopes with highly dispersive optics, i.e. with one or more acousto-optic modulators (AOMs).

Conclusion

The advent of turnkey, one-box tunable lasers enabled and broadened nonlinear microscopy to move from a specialized, hardly-accessible and highly technical application to a family of user-friendly techniques. More recently, the emphasis of laser manufacturers has been on delivering second generation products that support deeper tissue imaging and the ability to observe live tissue over an extended interval without causing photodamage. The long wavelength capabilities of the Coherent Chameleon and its OPO accessory are unique in their ability to deliver dramatic improvements on both these fronts.

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