Application Note

Coherent Anti-Stokes Raman Scattering (CARS) Imaging Complements Other Multi-modal Imaging Techniques

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Introduction

Coherent Anti-Stokes Raman Scattering (CARS) Imaging has emerged in recent years as a powerful imaging technique, complementing traditional multiphoton imaging techniques such as multiphoton excited fluorescence (MPEF) and second harmonic generation (SHG) microscopy. The CARS method already described elsewhere[1-8] enables label free imaging of biological samples exhibiting certain natural vibrational resonances. Specifically, CARS involves using two pulses of different wavelengths where the difference in frequency between the two pulses matches a molecular vibrational transition within the specific tissue of interest. Since the linewidth of most Raman active transitions is typically around 10 cm^{-1}, a picosecond pulse is optimum for a CARS-only setup. However, in many cases, spectrally broader femtosecond pulses are preferred as they allow multiple imaging techniques to be used in a single multi-modal setup.[3-8] This paper explores the features of a femtosecond optical parametric oscillator (OPO), which make it the ideal choice for multimodal biological imaging including CARS.

Multi-Modal Imaging

Figure 1 shows the Jablonski diagrams for CARS, Second and Third Harmonic Generation (THG) Imaging and multiphoton excited fluorescence. For CARS Imaging, the Pump and Pump pulses are set at the same wavelength, while the wavelength of the Stokes pulse is red-shifted by the frequency corresponding to the vibrational mode of interest. This results in a scattered beam at the Anti-Stokes wavelength. The Pump and the Stokes beams are collinearly directed into the microscope so that the pulses overlap in space and time. As they are raster-scanned across the focal plane, the relative intensity of the Anti-Stokes Signal is captured to develop a two-dimensional image. As in other multiphoton imaging techniques, the focus can then be moved to a different plane to form a three-dimensional image. For practical purposes, most Raman active transitions have fairly narrow linewidths. One important exception is the symmetric CH_2 stretch at 2,840 cm^{-1}, which has a linewidth of around 50-80 cm^{-1}, very close to the bandwidth of a femtosecond OPO. This transition allows imaging of lipids without the need for added fluorophores.[6]

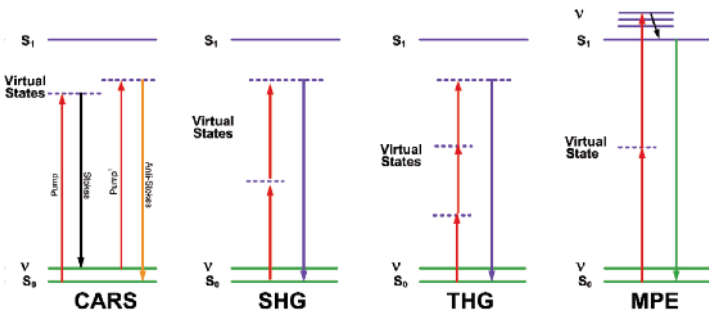

Figure 1: Energy Level Diagrams of Imaging Techniques. Coherent Anti-Stokes Raman Scattering (CARS), Second Harmonic Generation (SHG), Third Harmonic Generation (THG), and Multiphoton Excited Fluorescence (MPEF, here: two-photon excited fluorescence).

SHG Imaging[9] is similar to the more popular MPEF Imaging but shares with CARS Imaging the key benefit of not requiring the sample of interest to be tagged with a fluorophore in order to produce an image. In this case, two photons interact through a virtual state two form one photon of twice the energy generating emission at twice the frequency of the original Pump. The selection rules for the SHG process require the tissue to contain well-ordered protein assemblies, such as collagen, microtubuli or muscle myosin. THG Imaging is similar to SHG Imaging, but requires three photons to generate the signal with emission at three times the frequency of the original Pump. Finally, MPEF Imaging, the most common of the above techniques, has become the preferred choice for live tissue imaging. In most experiments, MPEF Imaging utilizes a fluorophore or fluorescent protein to enhance the features of interest.[10]

In order for these techniques to be used with a single setup, several key laser parameters must be carefully considered. SHG, THG and MPEF benefit from short femtosecond pulses due to the non-linear processes involved, whereas CARS may benefit from a narrow bandwidth. In addition, the requirement to have two synchronized pulses at the correct wavelength separation mandates that the laser system has a wide spectral tunability in specific spectral regions. Therefore, for a single setup to allow the most flexibility, the best choice is a femtosecond Ti:Sapphire pumped OPO where either laser is used for SHG, THG and MPEF, and the combination of OPO and Ti:Sapphire is utilized for CARS imaging.

Synchronized Pulses

As mentioned above, the pulses used in CARS Imaging must be overlapped in both space and time at the sample's image plane in order to generate a signal. In the past, techniques such as CARS and Pump-Probe spectroscopy relied on electronic synchronization of pulses from two different laser sources. Recently, OPO technology advances have allowed perfect synchronization since the pump, signal and idler pulses of the OPO are inherently optically synchronized. However, since these three beams exit the OPO housing following slightly different beam paths, they require being re-timed optically to ensure temporal overlap at the sample (see Figure 2). This is typically accomplished with a finely tunable optical delay inserted between the OPO and the microscope.

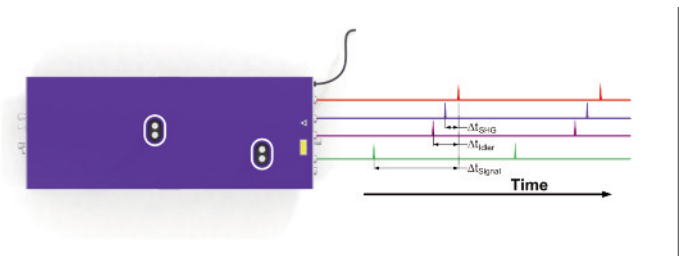

Figure 2: Inspire OPO Timing. The various output beams of the Inspire OPO exit the housing synchronized, but with fixed delays relative to the main Pump pulse.

The Inspire™ Advantage

The strengths of the Spectra-Physics® Inspire™ OPO are directly realized from its unique design and architecture. The OPO cavity is pumped by the frequency doubled Mai Tai® HP femtosecond Ti:Sapphire output at 410 nm, rather than with the typical Ti:Sapphire fundamental at 800 nm, making it possible to generate synchronized outputs at wavelengths that are much closer in frequency. This translates into a broader range of accessible vibrational transition frequencies down to 1,300 cm^{-1} (see Figures 3 & 4) compared to a lower limit of only 2,650 cm^{-1} for other OPOs, which are pumped with the Ti:Sapphire fundamental. This range of excitation frequencies allows imaging of lower vibrational frequency modes. It also makes the Inspire OPO ideal for other pump-probe spectroscopy applications.

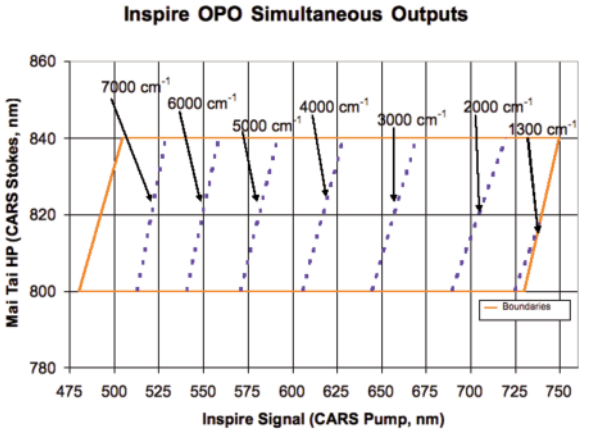

Figure 3: Simultaneous Outputs from the Mai Tai HP and the Inspire OPO Signal. The area within the orange parallelogram indicates wavelength choices that are available for simultaneous output.

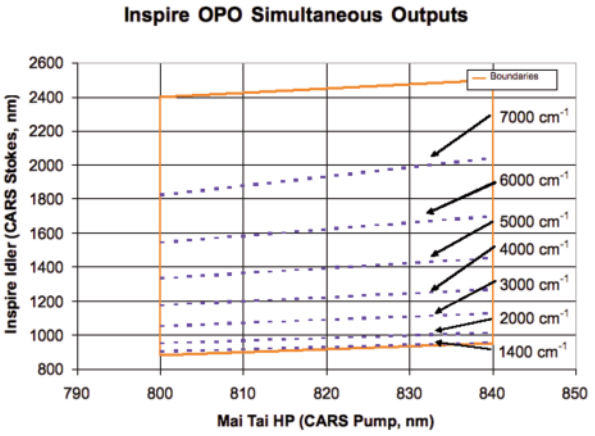

Figure 4: Simultaneous Outputs from the Mai Tai HP and the Inspire OPO Idler. The area within the orange parallelogram indicates wavelength choices that are available for simultaneous output.

In addition to allowing access to a wide range of possible vibrational modes, the unique architecture of the Inspire OPO also provides the largest gap-free tuning range of any standard femtosecond OPO system without the need to reconfigure the main optical cavity. The Inspire OPO's wide tuning range (345-2500 nm, see Figure 5) makes it a powerful workhorse in a multi-user facility where experiments might change weekly, or even daily.

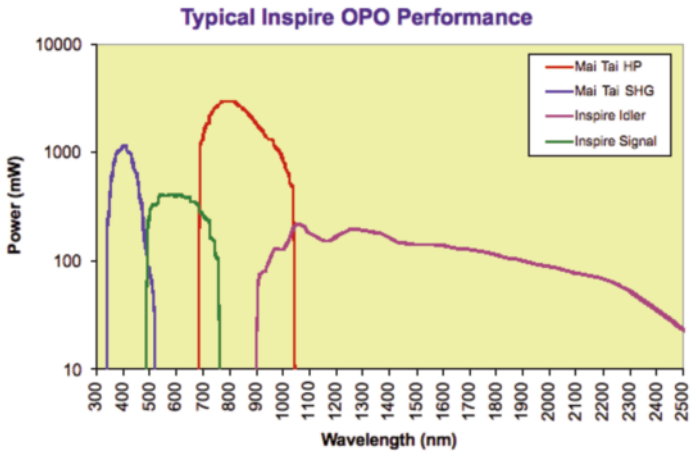

Figure 5: Tuning Curves for the Inspire OPO.

In the majority of imaging techniques, the laser beam quality is an important parameter to allow the level of optical control needed to generate a high quality image. For example, getting the beam successfully routed through the microscope objective with a tight focus is mandatory. CARS Imaging, where two beams (e.g. the Pump and Signal) are overlapped in both space and time, requires good beam quality for both beams. The Inspire OPO has been specifically designed to deliver excellent beam quality for the Signal and Idler beams (see Figure 6). The beam quality benefits not only CARS imaging and long wavelength MPEF Imaging, but also other pump-probe spectroscopy applications where spatial overlap of two pulse trains is also required.

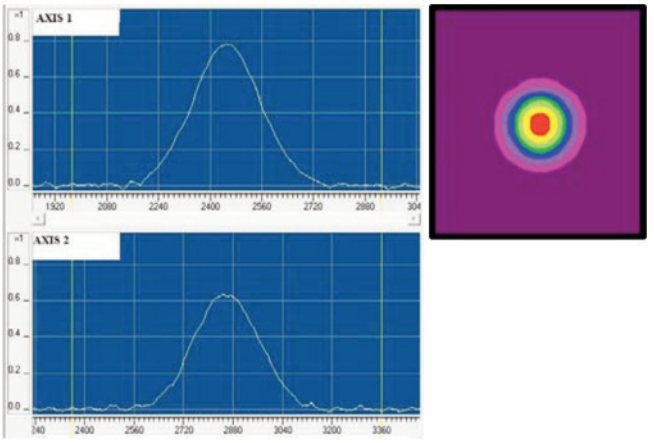

Figure 6: Inspire OPO Idler beam profile at 1,030nm

Turnkey Operation and Flexibility of Use

The Inspire OPO features several different models with varying levels of automation, depending on the user's needs. The Hands-Free (HF) version offers full automation, where necessary adjustments are software-controlled. The HF models are intended to be used in laboratories where imaging is the primary focus. The Auto version provides automated wavelength tuning, but allows the end user to manually optimize key laser parameters, including pulse width and average power. These models are intended to be used in laboratories where development of the next cutting edge applications is the primary focus.

As mentioned above, when the Inspire OPO is pumped by the

Mai Tai HP, users also benefit from the direct Ti:Sapphire output for other imaging applications as well as various non-imaging applications. The Mai Tai HP provides ample average power and with the DeepSee™ automated dispersion compensation option, delivers more than 380KW of peak power to the sample. The Mai Tai family also provides the industry leading beam pointing specification of 50μRad/100nm, meaning no realignment is ever needed after wavelength or dispersion compensation changes.

Results

Figure 7 demonstrates the Inspire/Mai Tai HP powerful multimodal imaging capabilities, with simultaneous CARS, SHG and MPEF Imaging. The image reveals an unstained mouse kidney tissue section, showing a region near a blood vessel. The elastin autofluorescence (green) demarcates the arterial wall, which is surrounded by collagen (blue). Fat accumulation (CARS, red) in the kidney is observed, a result of a high fat/high cholesterol diet and compromised kidney function (5/6 nephrectomy). The cellular structure of the nearby tubules surrounding the blood vessel can be clearly seen in the autofluorescence channel.

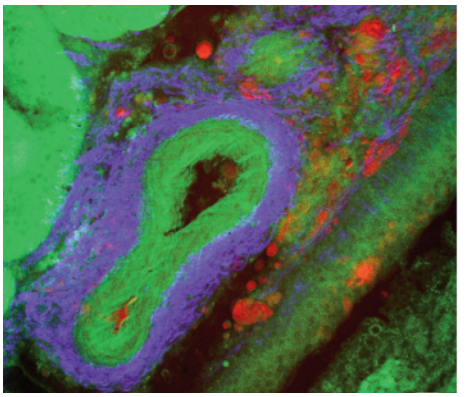

Figure 7: Multi-Modal images of mouse kidney featuring CARS (lipids, red) SHG (collagen, blue) and MPE (elastin autofluorescence, green). Image provided by Professor Eric Potma at the University of California at Irvine, sample provided by Professor Moshe Levi at the University of Colorado, Denver.

As mentioned above, the lipid transition at 2850cm^{-1} can be effectively used in femtosecond CARS imaging. Figure 8 shows a comparison of CARS images of lipids in a mouse small intestine using a femtosecond Mai Tai HP pumped Inspire and two synchronized picosecond Tsunami® oscillators. In the picosecond setup, 5ps, 300 mW at 707 nm, 300 mW at 885 nm and a 600/50 filter were used while in the femtosecond setup, 200fs, 50 mW at 665 nm, 50 mW at 820 nm and 560/40 filter. Comparable CARS signal intensities were achieved in both cases, with the femtosecond setup requiring 6X less average power from the laser source. This clearly demonstrates the benefit of the high peak power delivered by the combination of Mai Tai HP and Inspire.

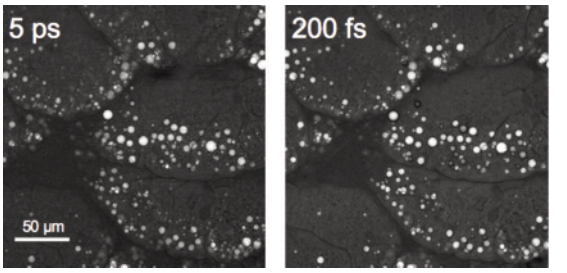

Figure 8: CARS Images of mouse small intestine tissue taken with a picosecond source (left) and femtosecond source (right). Image provided by Ji-Xin Cheng, Associate Professor, Weldon School of Biomedical Engineering and Department of Chemistry Purdue University.

Summary

The Inspire OPO is the ideal source to complete a multi-modal imaging setup which combines techniques such as CARS, SHG, THG, and MPEF microscopy. With its unique combination of a wide, gap-free tuning range, excellent beam quality, and automation the Inspire is the most versatile OPO available today.

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