CARS Microscopy with Spectral Focusing

With the help of spectral focusing, vibrational imaging modalities using multimodal CARS have become invaluable tools in neuroscience.

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Multiphoton/nonlinear laser scanning microscopy has become an important tool to investigate biological phenomena, where high resolution, 3-dimensional imaging with depth resolution is essential to uncover underlying biological functions. Two-photon or multiphoton imaging is preferred to single photon imagining techniques due to several intrinsic advantages of multiphoton techniques. One of the advantages is having a nonlinear intensity dependent absorption. For example, two-photon absorption is restricted to the focal volume so there is virtually no out-of-focus bleaching or signal contribution. As opposed to single photon imaging where the excitation wavelength is in the visible or near-UV range, multiphoton imaging with near-IR wavelengths is associated with reduced scattering. This helps with extended depth resolution from a few microns to several hundreds of microns. Additionally there is a physiological benefit from using near-IR wavelengths in multiphoton microscopy, as these wavelengths are safe in preserving cell viability due to reduced out-of-focus absorption restricting linear absorption in the surrounding area.

Coherent anti-Stokes Raman scattering (CARS) is a powerful imaging technique that continues to grow and draw attention of biologists due to its ability to provide label-free molecule-specific or functional group specific images of live cells and tissues. The different energies involved in the CARS set up are shown in **Figure 2**.

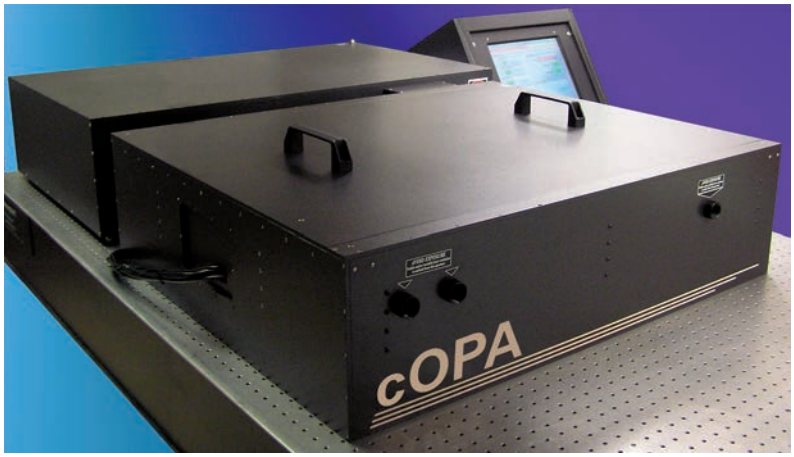

Fig. 1 The Model cOPA has been used for multimodal microscopy in the past years.

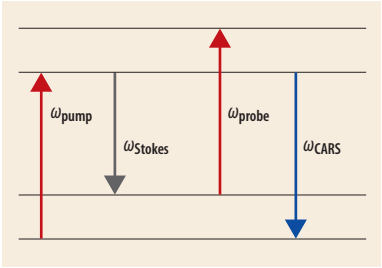

Fig. 2 In this CARS diagram both pump and probe wavelengths are the same. The CARS signal appears at the wavelength $\omega_{CARS} = 2\omega_p - \omega_s$.

CARS microscopy and microspectroscopy is generally combined with other multiphoton microscopy techniques such as two-photon fluorescence (TPF), second harmonic generation (SHG), third harmonic generation (THG) and other nonlinear techniques to generate a true multimodal microscopy. The versatility of CARS is to scan through the Raman resonance, providing molecule-specific information of the specimen in question.

For a true multimodal imaging system, the TPF, SHG, THG etc. needs shorter pulses as short pulses pack maximum energy within the pulse. This is desirable for these nonlinear microscopic methods but not for CARS microscopy as the non-resonant (background) signal can overpower the CARS signal. CARS imaging is traditionally done with a pair of picosecond (narrow bandwidth) pulses either electronically synchronized or generated with synchronously-pumped optical parametric amplifiers to match the vibrational resonance to enhance resonant contribution and suppress the non-resonance (background) CARS signal.

CARS with a spectral bandwidth of several to several hundred nanometers is becoming commonplace in order to achieve true multimodal imaging. On one hand, broad bandwidths allow the simultaneous probing of a large number of vibrations while on the other hand short pulses with large bandwidth may lead to a non-resonant signal contribution and poor degree of spatial resolution. One way to eliminate the large non-resonant background is to use a chirped pump or Stokes beam by purposely dispersing one pulse with a grating compressor, a pair of chirp mirrors or a highly dispersive glass block while the other beam remains near-transform limited. One drawback of this approach is that the interaction time between two pulses is restricted as one pulse is much shorter than the other so that the Stokes power is out of phase with most part of the pump and does not contribute to the CARS signal. As a result CARS

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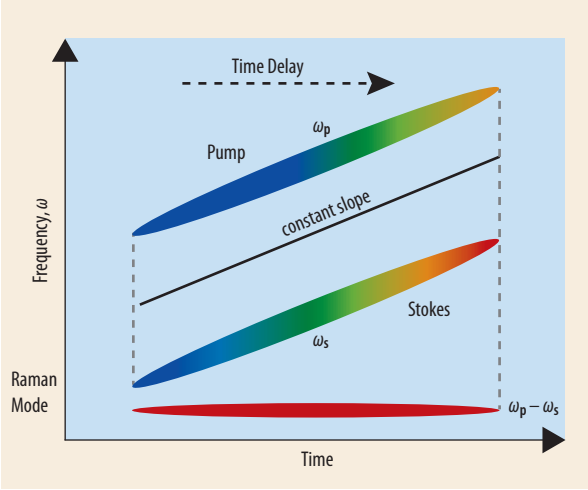

Fig. 3 Chirped CARS (spectral focusing) process. Constant slope gives narrow and constant frequency difference ($\omega_p - \omega_s$) and center tuning aids selection of vibrational modes, which can also be achieved by temporal delay.

signal levels are lower even though the background is minimized.

To overcome the poor spectral resolution with broad bandwidth pulses and increase the overlap of pulses, there are alternative methods that can be utilized yielding spectral resolution comparable to CARS imaging with picosecond pulses [1].

In order to overcome the difficulty of using short pulses for CARS imaging, spectral focusing, an innovative method of using spectrally dispersed or chirped pulses, was introduced by Zumbusch and coworkers in 2004 [2]. **Figure 3** shows the principle of this approach, in which both pump and Stokes pulses are intentionally chirped, with the result, that the frequency content of this pulse is spread out in time. In spectral focusing a broad-bandwidth pulse with controlled chirp can achieve Raman spectral resolution comparable to what can be achieved with picosecond pulses. The concept rests on the recognition that what is important in CARS is that the instantaneous difference between the pump and stokes pulse matches the Raman mode of interest. By far the simplest approach is to control linear (quadratic) chirp, a linear variation of frequency components within the pulse. When using matched linear dispersion (chirp), the interaction is maintained over a wide range of delays. Temporal delay with equally chirped beams can select different vibrations. Another important parameter is the repetition rate of the laser as photo damage and photo bleaching are crucial for biological samples.

Even though CARS is a third order nonlinear process, in most cases photo bleaching has a lower nonlinearity than the CARS process and can vary between 1.1 and 2.5 [3]. Even though the exact mechanism is unknown, linear absorption by surrounding molecules leading to heating plays a significant role. In this case high peak power and low rep rate lasers are better than high-rep rate lasers such as Ti:Sapphire oscillator (~80 MHz) as high rep rate and linear absorption can lead to excessive local heating resulting in photo damage [4]. Therefore it is advantageous to use a laser with lower repetition rate in the few MHz range.

A laser system which has been used in the last years in the field of multimodal microscopy as described above is the Model cOPA from Clark-MXR (**Fig. 1** and **4**). The whole system consists of an all-fibre based amplifier system (model Impulse), plus a set of optimized OPA-systems (optical parametric amplifier).

Impulse is a one-box system consisting of a Yb-doped femtosecond fibre oscillator seeding a fibre amplifier. The system delivers sub 250 fs pulses at adjustable repetition rates from 200 kHz up to 25 MHz at a wavelength of 1030 nm. In this particular set-up, the output of Impulse is used to pump two independently tunable NOPA (non-colinear optical parametric amplifiers). In the cOPA system two parallel OPAs are arranged to achieve two parallel synchronized output arms of wavelength tunable beams. Typical tuning ranges of the NOPA are given in **Figure 4**. With additional options it is also possible to get into the UV-range. Another very interesting capability of the NOPA is a substantial pulse shortening, potentially into the range of 20 fs or even lower [5]. This is a pulse length reduction of more than one order of magnitude as compared to the fundamental pump beam from Impulse. The Model cOPA, especially designed for microscopy applications, comes with a pulsewidth control mechanism that allows to adjust pulse duration from tens to hundreds of femtoseconds.

Beside the two arms from the NOPAs, Impulse delivers one additional output with a pulse energy of around 1 µJ at the fundamental wavelength of 1030 nm (**Fig. 4**).

One outstanding application for this system as a multimodal

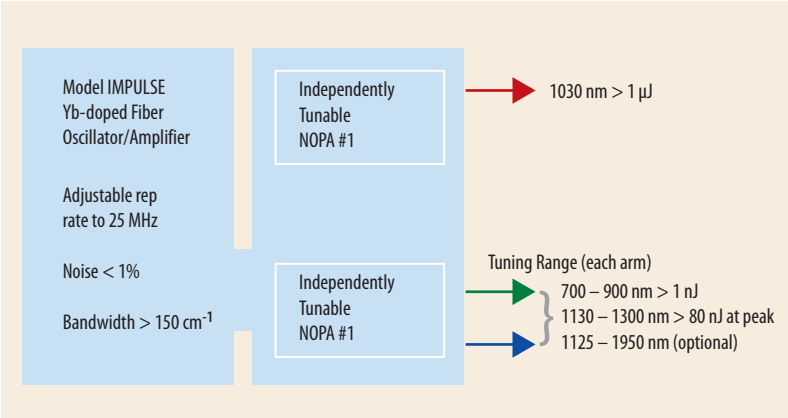

Fig. 4 Schematics of Model cOPA. Model IMPULSE pumps two NOPAs/OPAs giving two independently tunable, synchronized outputs. A synchronized, residual fundamental at 1030 nm with excess of 1 µJ is available from a third port that can be used to generate e.g. white light continuum.

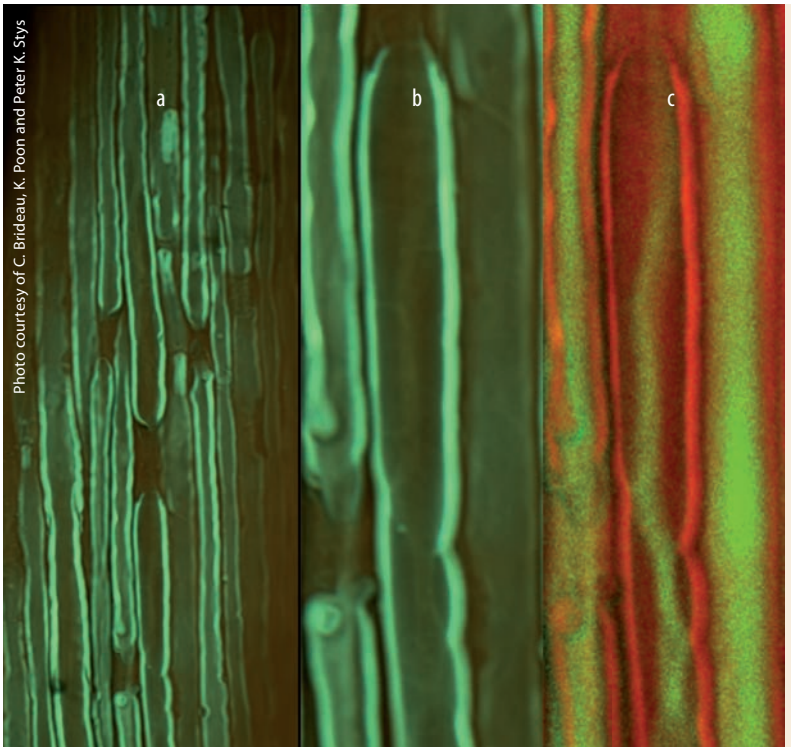

Fig. 5 Transgenic YFP mouse ventral root. Left: spectral CARS. Middle: Zoom on central axon. Right: Overlay of CARS and two-photon image.

microscopy tool has been realized by the group of Peter K. Stys at the Hotchkiss Brain Institute at the University of Calgary, Canada. In this group spectral CARS and two-photon microscopy were recorded simultaneously to visualize mouse ventral root (transgenic YFP).

Figure 5 shows an image of mouse ventral root, transgenic YFP, taken with spectral CARS (panel a), an expanded section of the same sample showing central axon (b), and the overlay of the spectral CARS image and the two-photon image taken simultaneously (c) [6]. These pictures were generated by a unique nonlinear data analysis package developed at the Hotchkiss Brain Institute.

In summary, with the help of spectral focusing, vibrational imaging modalities on tissue using Coherent anti-Stokes Raman Scattering (CARS) and TPF have been recognized as invaluable tools in neuroscience to probe intrinsic molecular biochemistry of neurological disease. It has been proven in the last few years that the Model cOPA from Clark-MXR is the ideal tool for this particular application.

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