CARS Microscopy Made Simple

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ABSTRACT – We describe a very simple yet high performance version of multi-modal Coherent Anti-Stokes Raman Scattering (CARS) Microscopy, based upon a single fs Ti:Sapphire oscillator and a photonic crystal fibre. Simultaneous CARS, second harmonic and two-photon fluorescence microscopy of live cells and tissues are demonstrated at frame rates of $2s^{-1}$ and low light exposures (< 30 mW total).

1. INTRODUCTION

Coherent anti-Stokes Raman scattering (CARS) microscopy provides molecule specific yet label-free imaging of samples based on their Raman spectrum [1]. CARS has attracted significant interest for its applicability to imaging live cells and tissues. Most implementations of CARS microscopy are based on the use of a pair of transform-limited (TL) tunable ps pulses. The narrow linewidth of TL ps pulses ensures that the laser pulse linewidth falls within or matches the spectral linewidth of the Raman band of interest, thereby enhancing the contrast of resonant over nonresonant (background) CARS signals [2]. By contrast, nonresonant two-photon fluorescence (TPF) and second harmonic generation (SHG) signals benefit greatly from the use of shorter (fs) laser pulses. We recently showed that, for multimodal CARS microscopy, we could very successfully use fs laser pulses for high performance imaging of live cells and tissues [3]. Although fs pulses have poor spectral resolution, well known nonlinear optical methods exist for enhancing this, based upon the control over optical phase inherent to fs laser pulses. The simplest implementation is a quadratic spectral phase variation (linear chirp). By optimizing the degree of chirp in fs pulses, we obtained a very simple yet high performance CARS microscopy method that is robust and inexpensive yet stable enough for real time imaging of live cells over periods of days [3].

1.1 Optimally Chirped CARS Microscopy

In Fig.1, we show the chirped pulse CARS scheme. The degree of linear chirp is a variable parameter to be optimized under user control. The best performance (combination of signal and contrast) is achieved when the effective second order laser line width (the height $\Delta\Omega$ of the difference frequency ellipse) matches the width of the Raman resonance under study. As Raman line widths can vary considerably (5 - 400 cm^{-1}), the ability to easily tune the effective spectral width of the excitation pulses is a useful tool to put in the hands of the user.

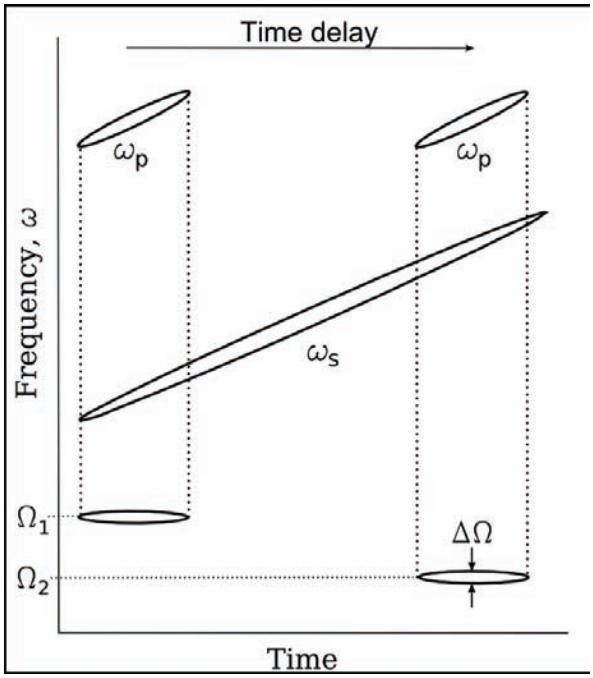

Figure 1: *Time-frequency plots of chirped Pump and Stokes pulses. The CARS spectral resolution $\Delta\Omega$ is determined by the height of the difference frequency ellipse $\omega_p - \omega_s$. Adjusting the chirp rates gives an adjustable instantaneous bandwidth and a user-variable spectral resolution. Changing the time delay of the Pump relative to the broadband Stokes scans this instantaneous difference frequency, probing different Raman modes (Ω_1 , Ω_2).*

We used a photonic crystal fiber (PCF) to generate the broadband synchronized Stokes light for broad Raman

tunability (~2500-4100 cm^{-1}). Rapid tuning of the Raman resonance is obtained by varying the time delay between chirped pump and Stokes pulses, whereas the effective Raman resolution and signal level are controlled by varying the chirp of the two pulses. In Fig.2, we show CARS spectra of a drop of liquid methanol, showing the effect of chirp rate on spectral resolution. The approach we describe is general to all fs sources and can also be used, for example, with synchronized fs oscillators or a fs OPO. Overall, the optimally chirped system is a high performance, versatile multimodal CARS microscope allowing for microspectral imaging of live cells and tissues. We provide sample illustrations of this capability by imaging myelin in rat dorsal nerves and an atherosclerotic arterial sample (rabbit aorta).

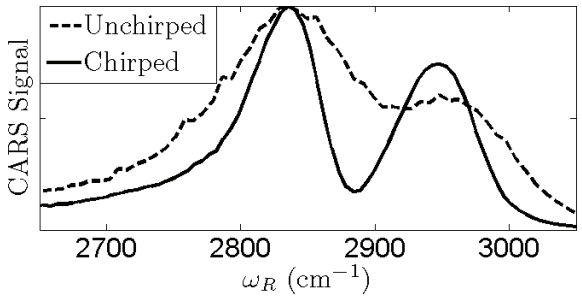

***Figure 2**: CARS spectra of methanol obtained by scanning the time delay between the pump and Stokes pulses shown in Fig.1. The dashed curve was for unchirped pulses, the solid for wich pulses width matched chirp rates.*

2. METHODOLOGY

2.1 A single Ti:Sa oscillator CARS source

An outline of the optical setup is given in Fig. 3. A Spectra Physics Tsunami Ti:Sapphire laser system produced pulses of 60 femtoseconds at 80 MHz with 550 mW total average power with a center wavelength of 800 nm. A Faraday isolator was used to avoid back reflections into the laser. To maximize performance of the PCF, the pulse train was sent through a prism compressor in order to have TL pulses at the input of the PCF. After the compressor, the beam was split by a 50:50 beam splitter into a pump and Stokes arm. The light in the Stokes arm was sent through the PCF. The pump was passed through a variable time delay stage and a variable neutral-density filter. To control the chirp in the simplest possible way, we used fixed length blocks of glass: one 3 cm block of SF6 glass was placed in the pump arm and a 5 cm block of SF6 glass was placed in the Stokes arm, to achieve nearly matched chirps. Typical powers before the microscope scan head were 7 mW in the Stokes and ~ 50 mW in the pump. These powers were attenuated by about a factor of two through the microscope system before reaching the back aperture of the objective lens inside the microscope.

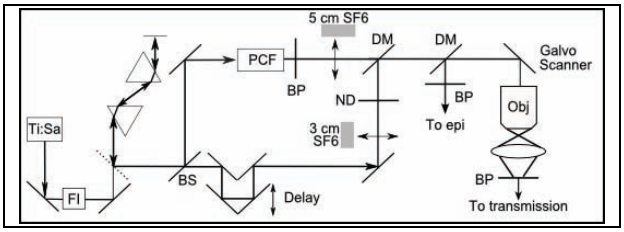

***Figure :** Simple CARS Microscope. A 50:50 beam splitter (BS) splits pulses from an isolated (FI), prism compressed Ti:Sa oscillator. One half went to a PCF and bandpass filter (BP) before being recombined on a dichroic mirror (DM). The other half was time delayed and attenuated (ND). Glass blocks (SF6) controlled the chirp rates. The recombined pulses were sent into the FV300 microscope for imaging.*

All imaging was performed on a specially modified Olympus Fluoview 300 (FV300) laser scanning system and IX71 inverted microscope using a 40X 1.15 NA objective lens, and a 0.55 NA condenser lens for forward collection (CARS and SHG). TPF signals were collected back through the objective lens (epi-detection). Filters were used to discriminate signals from each other and the input pump and Stokes beams. For imaging, light was directed to photomultiplier tubes (PMT).

3. RESULTS

In Fig. 4, we show measurements of rat spinal nerves, demonstrating the capability of using this system for CARS tissue imaging. The spinal nerves are approximately 300 μm in diameter and consist of bundles of roughly 100 myelinated axons, each about 15 μm diameter. The effective depth of field of the CARS signal was ~1.5 μm, necessary in order to see detail from this tissue. The pixel intensity profile of the indicated line is shown demonstrating the contrast achieved. It is clear that the signal intensity from the myelin is at least 60 fold greater than that from the axon and that fine detail in the myelin structure is clearly visible. The spectral response of the myelin (not shown) proved that the signals observed were due solely to resonant CARS and not due to changes in the non-resonant background.

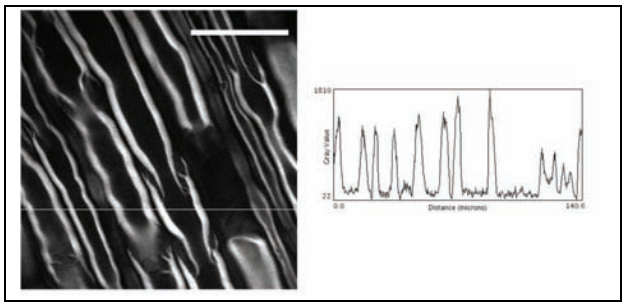

Figure 4: *Forward detected FV300 CARS imaging of fixed rat dorsal root nerves at 2850 cm^{-1} (lipid C-H stretch). The lipid-rich myelin sheath surrounds the neuronal axon and generates a strong CARS signal. The pixel intensity profile of the indicated line is shown, revealing high contrast. The scale bar is 50 μm. The pixel dwell time was 8 μs. [3]*

In Fig. 5, we show an atherosclerotic lesion from a rabbit aorta which was used as a test sample for label-free multimodal imaging. A 50 μm section of aorta was imaged showing lipids (CARS - red), collagen (SHG - blue) and smooth muscle elastin (TPF - green). Importantly, all three signals are endogenous to the sample and no dyes or stains were added to enhance contrast.

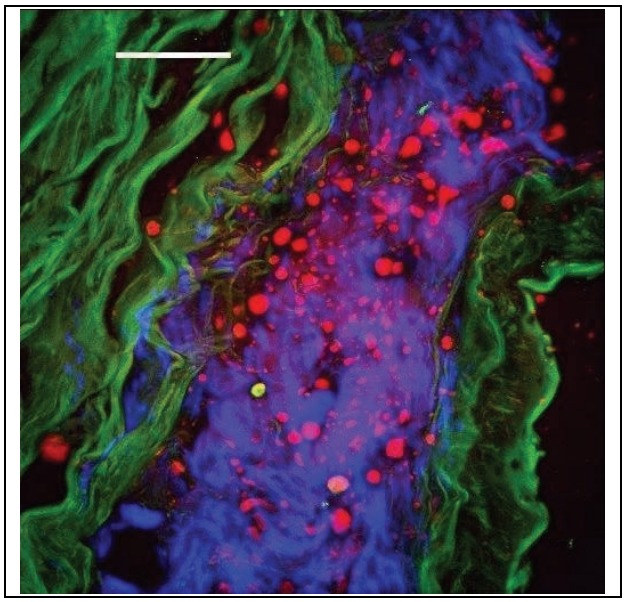

Figure 5: *Label-free multimodal CARS microscopy of atherosclerotic rabbit aorta. A 50 micron slice was imaged at 2850 cm^{-1} for lipids (CARS - red), collagen (second harmonic - blue) and smooth muscle elastin (fluorescence - green). This image is a z-projection of a 50 image data set (1 μm apart) of a 3D scan through the sample. The scale bar is 50 μm. The pixel dwell time was 8 μs. [3]*

This image is a projection of a 50 image data set recorded along the axial direction (1 μm interval between images). An extensive network of collagen surrounds the lipid rich tissues.

We have also performed extensive real time imaging (movies) of lipid trafficking in live human hepatocytes (liver cells), demonstrating that the arrangement shown in Fig.3 is stable on all time scales relevant to live cell microscopy [3].

4. CONCLUSIONS

We have presented an optimally chirped implementation of live cell CARS microscopy with the degree of linear chirp being an active user-controlled variable. The best performance is achieved when the effective pump and Stokes pulses spectral widths matches the Raman line width of interest. By using chirp as a control parameter, the microscope user can choose to optimize contrast in CARS imaging or enhance signals in various nonlinear optical processes (e.g. CARS, TPF, SHG, etc.) in a multimodal microscope. In our case, the Stokes pulse generated by the PCF has a much broader spectrum than the pump, permitting rapid multiplex CARS imaging by simply scanning the time delay between the pump and Stokes, avoiding the need to tune any lasers. The images and video presented here and elsewhere [3] demonstrate that this approach leads to a very simple, practical, yet high performance CARS microscope.

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