Fourier-transform coherent anti-Stokes Raman scattering microscopy

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We report a novel Fourier-transform-based implementation of coherent anti-Stokes Raman scattering (CARS) microscopy. The method employs a single femtosecond laser source and a Michelson interferometer to create two pulse replicas that are fed into a scanning multiphoton microscope. By varying the time delay between the pulses, we time-resolve the CARS signal, permitting easy removal of the nonresonant background while providing high resolution, spectrally resolved images of CARS modes over the laser bandwidth ($\sim$1500 cm^{-1}). We demonstrate the method by imaging polystyrene beads in solvent. © 2006 Optical Society of America

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Multiphoton microscopy is an important tool that is increasingly used in biological research[1] owing to its ability to produce three-dimensional images of complex samples. To probe particular species, fluorescent labels are most commonly used but have several disadvantages, including photobleaching and possible modification of the system under study. Coherent anti-Stokes Raman scattering (CARS) microscopy offers the advantage of providing endogenous contrast based on the inherent vibrations of different chemical species. Because of the nonlinear nature of the involved light–matter interaction, CARS microscopy shares many of the benefits of multiphoton microscopy while avoiding problems associated with fluorescent labeling.[2] In this Letter we present a Fourier-transform-based method of CARS microscopy. The technique employs a single broadband laser source and provides a simple approach to obtaining high-resolution spectrally resolved CARS images. We demonstrate the method by spectrally resolving images of polystyrene beads immersed in solvent.

CARS is a nonlinear scattering process in which light of different frequencies interacts through the third-order susceptibility of the medium. Two photons, of frequencies ω_p and ω_s, such that the difference frequency $\omega_p-\omega_s$ matches a vibrational resonance in the sample, cause the scattering of a third photon, of frequency $\omega_{p'}$, to produce a higher-energy anti-Stokes photon of frequency $\omega_p-\omega_s+\omega_{p'}$, as shown in Fig. 1(a).[3] Other nonlinear processes that are not related to any vibrational resonances can also occur, producing a nonresonant background that can obscure the CARS signal [see Fig. 1(b)].

Since its first demonstration in 1982,[4] most CARS microscopy implementations to date have employed picosecond pulses and have reduced the nonresonant background through the use of near-IR excitation and polarization selection.[2,5] While using picosecond pulses reduces the nonresonant background, it also necessarily restricts the spectral range of the CARS method, such that single CARS modes are imaged at a time. Obtaining CARS spectral images over the entire fingerprint region, where molecular spectra are most distinguishable, is important for simultaneous imaging of multiple species and thereby significantly enhances the capabilities of CARS microscopy. Picosecond implementations of CARS microscopy can produce broadband CARS spectra by the time-consuming process of tuning one of the excitation frequencies.[2] A more rapid approach has been to combine picosecond and femtosecond excitation, providing CARS images with up to $\sim$200 cm^{-1} of bandwidth.[6,7]

The use of femtosecond pulses provides yet larger spectral bandwidth but requires more elaborate methods for dealing with the nonresonant back-

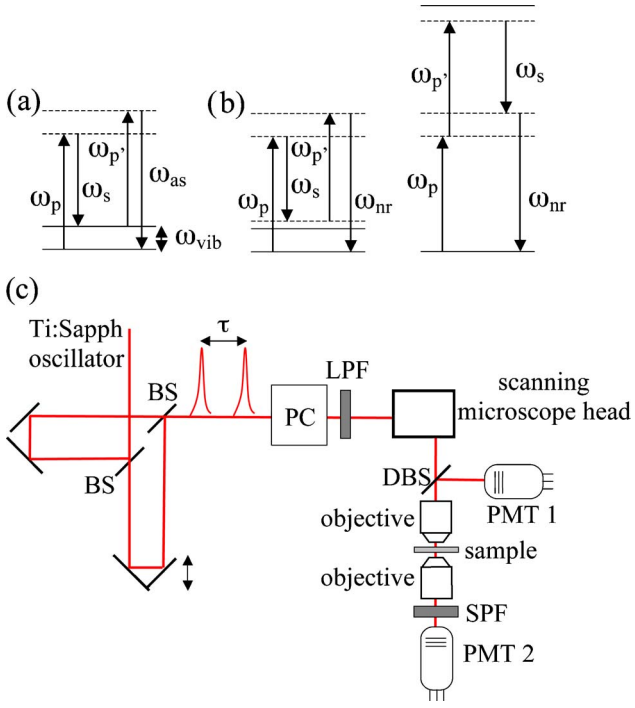

Fig. 1. (Color online) (a) Resonant CARS excitation, (b) nonresonant excitation. (c) Experimental setup for FT CARS microscopy: BS's, beam splitters; PC, prism compressor for dispersion compensation; LPF, long-pass filter, cutoff at 780 nm; DBS, dichroic beam splitter; SPF, short-pass filter, cutoff at 760 nm; PMT 1, photomultiplier tube for epifluorescence detection; PMT 2, photomultiplier tube for CARS detection.

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ground, which increases with decreasing pulse duration.[2] Recently CARS images were obtained using broadband femtosecond pulses,[8–10] which can potentially excite simultaneously all vibrational modes of frequencies smaller than the laser bandwidth, as in impulsive stimulated Raman scattering.[11] In this approach, mode selectivity is achieved using various techniques such as phase-only pulse shaping,[8,9] spectral focusing with chirped pulses,[12,13] and selecting a narrowband pump beam by use of amplitude-only pulse shaping associated with spectral broadening of the Stokes beam and frequency-resolved detection of the CARS emission.[10] In the case of phase-only pulse shaping,[8,9] a sinusoidal spectral phase was applied such that the exciting pulse actually consisted of a quasi-periodic pulse sequence that allows mode selectivity as in multiple-pulse impulsive stimulated Raman scattering.[14] Frequency-resolved Raman spectra were then obtained through a Fourier transform of the data measured as a function of the sequence pseudoperiod. However, although multiple pulse sequences are ideal for driving a given Raman mode, it is well known that the entire Raman spectrum can also be obtained in a more straightforward way through a Fourier transform of time-domain impulsive stimulated Raman scattering data.[14] Our approach to single-laser CARS microscopy is thus to use a broadband laser source to record the CARS emission as a function of the time delay between only two identical collinear excitation pulses and then to obtain the CARS spectrum through a simple Fourier transform. Furthermore, windowing out the contribution around zero time delay allows a straightforward removal of the nonresonant background, similarly to a previous time-domain CARS experiment performed at a fixed time delay[15] and an interferometric CARS experiment that demonstrated a difference in time evolution between resonant and nonresonant signals.[16]

In the research reported in this Letter we generate spectrally resolved CARS images over a bandwidth of $\sim$1500 cm^{-1}, using a single broadband Ti:sapphire oscillator. In our time-domain Fourier-transform CARS (FTCARS) method, two broadband pulse replicas are created in a Michelson interferometer. The first pulse provides the pump and Stokes fields to impulsively excite a vibrational coherence, while the second, time-delayed pulse probes this coherence. The bandwidth obtainable is determined by the spectral width of the excitation source, which must impulsively excite the Raman modes to be imaged.

We demonstrate FTCARS microscopy by using a home-built laser-scanning microscope and a $\sim$20 fs Ti:sapphire oscillator (Ti:sapph; center wavelength, 820 nm) excitation source. Before it reached the microscope, the 100 MHz pulse train was sent into a Michelson interferometer, as shown in Fig. 1, producing two collinear pulses that could be variably time delayed with respect to each other. This delay was accurately monitored with a He–Ne laser (633 nm).[17,18] Precompensation of the microscope dispersion was performed with a fused-silica prism compressor,[19] which was optimized via second-order autocorrelations in a GaAsP two-photon photodiode at the sample position.[20] The combined excitation power of the two pulses was 15 mW at the sample, which was focused with an Olympus LMPLAN IR 20×, 0.4 N.A. objective (the actual beam diameter reduced this value to an effective N.A. of 0.3). The CARS signal was detected in the forward direction, while simultaneous fluorescence images were recorded in the backward direction by a photon-counting photomultiplier tube (Electron Tubes PC25). The CARS signal was isolated by spectral filtering,[8] which was achieved by a combination of a long-pass filter (Melles Griot; cutoff, 780 nm) placed before the microscope and two short-pass filters (Melles Griot; cutoff, 760 nm) located before the detection photomultiplier tube (Hamamatsu R636-10). In addition, the fluorescence in the forward direction was filtered out. The analog time-resolved CARS signal was digitized at 5 MHz (National Instruments, PCI-6110E DAQ board). Subsequent data processing to remove the nonresonant background and obtain the spectrally resolved image was performed with LabView 7.0 Software (National Instruments).

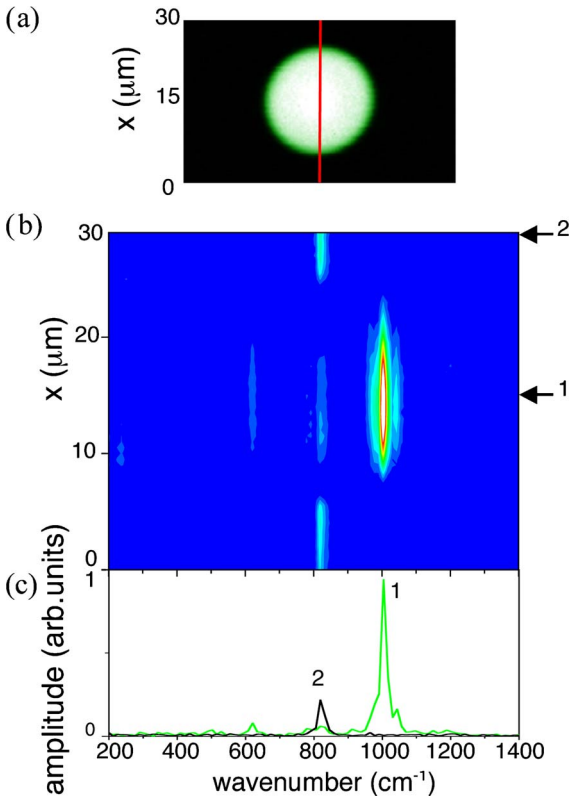

Fig. 2. (Color online) (a) Two-photon fluorescence image of a 20 μm polystyrene bead immersed in 2-propanol. (b) Spectrally resolved FTCARS image of the line in (a) [see (c) for scale]. (c) CARS spectra at positions 1 and 2 in (b).

We demonstrate FTCARS imaging in a sample of 20 μm diameter fluorescent polystyrene beads (Bangs Labs, FS07F) immersed in 2-propanol. Figure 2(a) shows a two-photon fluorescence image of a polystyrene bead. Figure 2(b) gives the corresponding FTCARS spectral image of a line scanned through the sample at the location indicated in Fig. 2(a). In 2-propanol the dominant C—C stretching mode at

816 cm^{-1} is clearly resolved,[21] as is the dominant mode in polystyrene at 1005 cm^{-1}, as well as smaller-amplitude modes at 783 and 620 cm^{-1} [Fig. 2(c)].[21,22] The peak near 800 cm^{-1} in the polystyrene spectrum may have contributions from both the 2-propanol and the polystyrene as a result of limited axial resolution ($\sim$30 μm). The CARS signal exhibits attenuation at the solvent–bead interface, in agreement with other reports.[23] The spectral line image in Fig. 2(b) was obtained by slowly scanning the time delay while simultaneously scanning the microscope's galvanometric mirrors at $\sim$200 Hz. A time series was then reconstructed for each pixel in the line, from which the CARS spectrum for each pixel was determined after averaging over 100 time-delay scans. Figure 3 shows the direct time-domain CARS signal that corresponds to location 1 in Fig. 2(b), where the $\sim$1000 cm^{-1} mode in polystyrene dominates the signal, as shown in the inset of Fig. 3. The peak at zero delay reflects the nonresonant response, which is easily windowed out before the Fourier transform, yielding the CARS spectra shown in Fig. 2(c). The maximum time delay actually used was 2.5 ps, yielding a spectral resolution of 13 cm^{-1}, a value that can easily be reduced by use of longer delays. Furthermore, scanning the time delay more rapidly and improving data processing should significantly reduce the total acquisition time (currently 10 min).

In summary, we have proposed a Fourier-transform technique for CARS microscopy that employs a single broadband laser source and a Michelson interferometer. We have demonstrated the technique by spectrally imaging a polystyrene bead in 2-propanol. This approach offers a straightforward method for removing the nonresonant background from CARS images while sharing the advantages of other Fourier-transform spectroscopies (no need for a grating spectrometer, high throughput, arbitrarily high spectral resolution limited only by the maximum time delay). Compared with methods based on pulse shaping, FTCARS microscopy offers a more compact optical setup and higher spectral resolution. Furthermore, when the possibility of recording two-photon absorption spectra as well is taken into account,[18] Fourier-transform microscopy should make possible the simultaneous acquisition of frequency-resolved two-photon and CARS images.

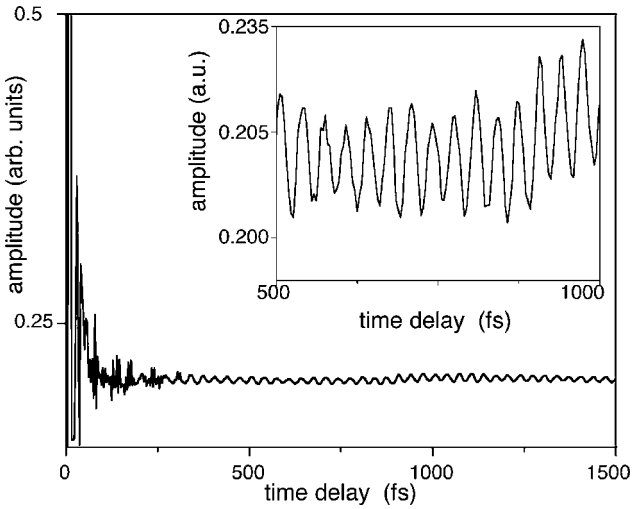

Fig. 3. Time-domain CARS signal in polystyrene at location 1 of Fig. 2(b). The corresponding CARS spectrum is obtained through the Fourier transform after windowing out the nonresonant background at zero delay. The resultant spectrum is shown in Fig. 2(c). Inset, clear oscillations corresponding to the 1005 cm^{-1} mode of polystyrene.

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