All-fiber CARS microscopy of live cells

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Abstract: Using an all-fiber laser system consisting of a femtosecond Er/Yb fiber oscillator as the pump and an ultra-highly nonlinear fiber for Stokes generation, we demonstrate multimodal (TPF+SHG+CARS) nonlinear optical microscopy of both tissue samples and live cells. Multimodal imaging was successfully performed with pixel dwell times as short as 4 μs at low laser powers ($<$ 40 mW total).

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OCIS codes: (170.5810) Scanning microscopy; (180.4315) nonlinear microscopy; (300.6230) Spectroscopy; coherent anti-Stokes Raman scattering.

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1. Introduction

Coherent anti-Stokes Raman scattering (CARS) microscopy applies to important problems in biology and material sciences, as well as other fields [1, 2]. Wide use of the technique, however, has been limited in part by the expense and complexity of laser sources (e.g. synchronized picosecond (ps) mode-locked solid-state oscillators [3], or synchronously pumped ps OPOs [4]). One approach to simplifying the laser system was to use a high power picosecond oscillator with highly doped fibers for broadband Stokes generation [5]. Another successful approach was to use photonic crystal fibers (PCFs) and a single femtosecond (fs) oscillator [6, 7]. When such a system is augmented by the use of optimally chirped pulses, it permits a successful multimodal CARS microscope [8] that achieves optimal CARS performance while simultaneously recording two-photon fluorescence (TPF) [9] and second harmonic generation (SHG) signals [10]. Free space lasers generally require a stable environment or active feedback, whereas an all-fiber source could be used in less favorable environments while being more compact and reducing eye safety risks to surrounding personnel. Given these advantages, there is interest in using fiber lasers for CARS microscopy, for example by replacing the pump of an OPO [11]. All fiber pump and Stokes generation was successfully demonstrated for microspectroscopy [12] and for ps CARS imaging, when combined with free space components for doubling [13]. Fiber lasers are also of interest because of the ease with which they can be combined with endoscopy, which would greatly increase the utility of CARS microscopy for pre-clinical applications and tissue imaging [14]. Here we present a novel all-fiber multimodal CARS microscopy system which applies successfully to both tissue and live cell imaging.

In previous work, a two-zero-dispersion point (ZDP) PCF was used, as these are relatively immune to input noise [15]. Unfortunately, these PCFs require high pulse input energy (a few nJ/pulse). A new class of fibers, known as ultra-highly nonlinear fibers (UHNLF) [16], require much lower input energies and are therefore attractive for use with existing all-fiber oscillators. Furthermore, unlike some other schemes [17], UHNLFs do not require pulse shaping of
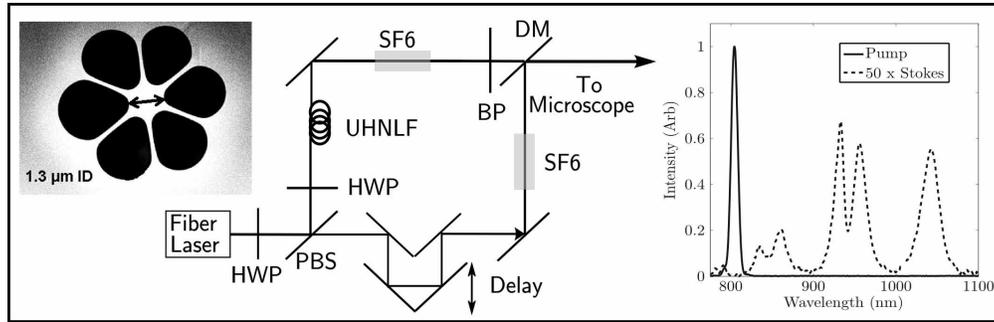
Fig. 1. All-fiber CARS microscopy arrangement. The fiber pump pulse is split using a half-wave plate (HWP) and polarizing beamsplitter (PBS). Part of the beam is sent to an UHNLF to generate the Stokes pulse. The Stokes and pump can both be chirped using blocks of SF6 glass before recombination on a dichroic mirror (DM). The combined beams are sent to the microscope. Left inset is a SEM image of the end face of the UHNLF. Right inset are the spectra of the pump and Stokes (Stokes scaled by 50 times) pulses.

the input pulse to achieve the desired output soliton. A drawback of UHNLFs is that they are susceptible to modulation instability (MI) causing the timing and spectrum of the output pulse to vary [18]. This noise is broadband, cannot be easily compensated for, and has deleterious effects on CARS performance. There have been significant efforts to characterize the source and behavior of this noise in supercontinuum and Raman shifting fibers [19–21]. In [19], pulses with slightly positive chirp and broader bandwidth were shown to reduce the relative intensity noise, and that it is minimized for Raman solitons in the infrared. Furthermore, this noise can be effectively eliminated by judicious choice of the input pulse and design of the fiber parameters such that the soliton fission length is less than the modulation instability length: $0.57\tau_{FWHM}(\beta_2\gamma P_0)^{-0.5} << 16(\gamma P_0)^{-1}$ where τ_{FWHM} is the input pulse temporal full width half maximum, β_2 is the dispersion of the fiber, γ is the nonlinear coefficient of the fiber and P_0 is the peak power of the pulse [21]. It is clear that the fiber design is an integral part of the overall system design for noise-free operation. Using an UHNLF in an arrangement similar to our PCF-based multimodal CARS laser system [8], we present a compact all-fiber based proof-of-concept laser system for multimodal CARS microscopy capable of imaging live cells at ~ 1 frame/sec.

2. Materials and methods

Figure 1 depicts the all-fiber arrangement used. A Femtolite FX-100 (IMRA America Inc.) with an output of 110 mW average power at 76 MHz repetition rate (810 nm center wavelength and 125 fs FWHM pulse duration) was used as the primary pump source. For efficient Stokes generation, an UHNLF was used in place of a standard PCF. The UHNLF was ~ 40 cm long and had a core diameter of ~ 1.27 μm, which was slightly elliptical. The core was suspended by glass webbing with strands of ~ 90 nm thickness. A SEM image showing the cross section of the fiber is presented as an inset in Fig. 1. The UHNLF had a loss of ~ 28 dB/km at 1550 nm and ~ 320 dB/km at 1390 nm (OH absorption). Over the relevant wavelength region (775-1050 nm) loss was < 0.1 dB/km. The zero dispersion wavelengths were at 664 nm and 1.628 μm and the nonlinear coefficient was $\gamma \approx 140$ $W^{-1}km^{-1}$ at 1.05 μm. The UHNLF was spliced to a single mode fiber at both the input and output in order to improve coupling and to safeguard the fiber face against damage.

The output of the fiber consisted of two redshifted solitons separated in time and frequency.

The soliton shifted further to the red was used as the Stokes pulse. The other soliton could be removed by a long pass filter. Alternatively, the desired Stokes soliton could be time-gated using the pump pulse. The center wavelength of the chosen soliton could be tuned from 1000-1100 nm by adjusting the input power to the UHNLF using a half waveplate and polarizing beamsplitter combination. For most experiments, the Stokes spectrum was tuned to $\sim$1053 nm so as to probe the lipid Raman shift around 2850 cm^{-1}; the spectra of the pump and Stokes are shown in Fig. 1. These operating conditions correspond to coupling 25-35 mW of power into the fiber. This achieves the necessary condition for stability, namely that the soliton fission length is less than the modulation instability length [21].

As was shown previously [8], temporally stretching (i.e. chirping) both fs pulses enables optimization of the performance for multimodal CARS microscopy. The output of the UHNLF is a near transform limited Raman soliton, however, it is passes through $\sim$ 10 mm of HI-1060 Corning single mode fiber followed by the collection optics, giving a slightly chirped Stokes. The pump and Stokes chirp were further adjusted to approach optimum characteristics using fixed blocks of SF6 glass. For the results presented here, both arms passed through 5 cm of SF6 to yield $\sim$ 80 cm^{-1} spectral resolution (optimal for lipid imaging in cells and tissue). This gave matched chirps for the pump and Stokes beams to within the limits of the glass blocks available. After recombination on a dichroic mirror, the power at the input to the microscope was $\sim$ 45 mW for the pump and $\sim$ 1.5 mW for the Stokes. The modified (improved IR transmission coatings on internal optics, with added detectors) Olympus FV300 microscope, the preparation of the WHHLMI (Watanabe heritable hyperlipidemic myocardial infarction) rabbit arterial tissue [22] and HuH-7 human Hepatoma cells has been previously described elsewhere [8].

3. Results

This all-fiber system was used for multimodal nonlinear imaging of tissue samples and live cells, with two representative results being shown in Fig. 2. Both of these images are the average of two scans, each using a 4 μs/pixel dwell time (512x512 pixels, 2 seconds per image). In Fig. 2(a) we show a multimodal image of unstained rabbit arterial tissue where the blue is SHG from collagen, green is TPF from elastin and red is CARS, tuned to 2850 cm^{-1} for imaging lipids. Figure 2(b) is an image of live Huh-7 human hepatocyte cells which have been stained with Bisbenzimide Hoescht 33342. The green is TPF from the stain and the red is from CARS, tuned to 2850 cm^{-1}. Both of these images clearly demonstrate the ability to perform multimodal imaging of biological samples.

To further characterize system suitability for live cell microscopy, we investigated both short and long term system stability by recording extended time course scans of live HuH-7 cells. The first and last frame of a sample time course are shown in Fig. 3; the entire time course is included as a video as supplementary material online. Here each frame was recorded with a 4 μs/pixel dwell time (no averaging, 256x256 pixels, 0.3 seconds per frame) and one frame was taken every minute for two hours. The red channel is the CARS signal, tuned to the lipid 2850 cm^{-1} stretch, and the green channel is due to two-photon autofluorescence. The cross-section included for the first frame shows that the contrast of the CARS channel is better than 8:1. To enhance the visibility of some details, the full color contrast range was reduced slightly in both images and the video. This time course demonstrates that both the long and short term stability of this all-fiber CARS microscopy system are sufficient for the imaging of biological samples.

Beyond optimal performance multimodal CARS imaging, temporally stretched (chirped) pulses permit rapid spectral scanning for probing different Raman levels [8, 23, 24]. This is achieved by varying the time delay between the pump and Stokes pulses which, in turn, changes the instantaneous difference frequency between the two colors and, hence, the Ra-
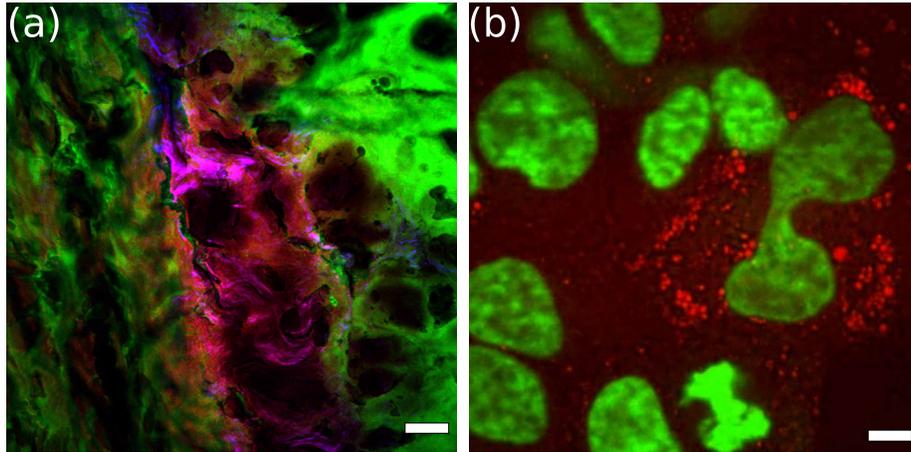
Fig. 2. All-fiber multimodal images of biological samples. All signals were collected simultaneously (4 μs/pixel, two frames averaged) (a) Aorta section from a 6 month old WHHLMI rabbit. Blue is SHG from collagen, green is TPF from elastin and red is CARS from lipids. Scale bar is 25 μm. (b) Live HuH-7 human hepatoma cells which have been stained with Hoescht 33342. Green is due to TPF from the Hoescht stain and red is due to CARS from lipids. Scale bar is 7 μm.
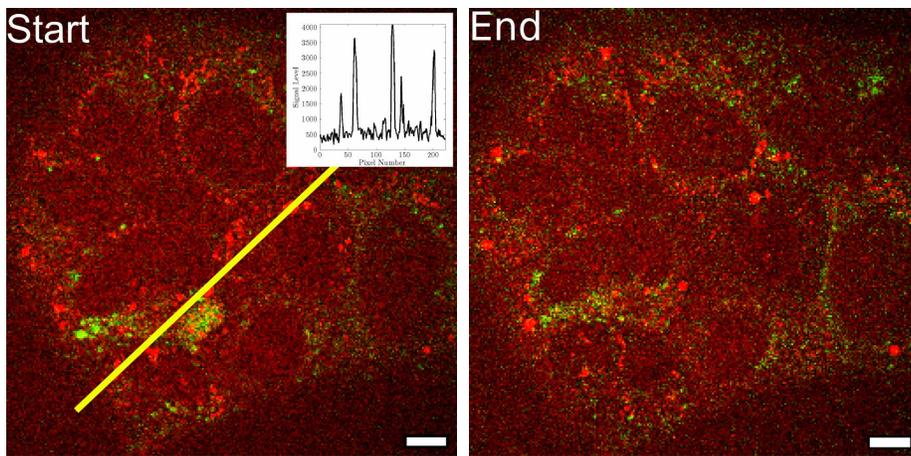
Fig. 3. First and last frame from a two hour video taken of live HuH-7 human hepatoma cells 4 μs/pixel, no averaging. Green is two-photon autofluorescence and red is CARS, tuned to the Raman lipid stretch (2850 cm^{-1}). A cross-section is included for the first frame showing the CARS signal and contrast. Both frames have a slightly contracted color range to allow more image detail to be viewed. Full video is included online (Media1). Scale bar is 7 μm

man level probed [8]. In the present case, the spectral scan range is limited due to the reduced bandwidth of the Stokes pulse. However, this limitation can be removed by adjusting the input power to the UHNLF, changing the Stokes center wavelength and thus extending the spectral scan range from ~ 2400 cm^{-1} to 3400 cm^{-1}. To extend this range even further, the dispersion profile of the UHNLF can be changed to support a different set of Raman solitons. For many cases of interest, however, sufficient Stokes bandwidth is achieved without changing the center wavelength. For example, (not shown here) we performed CARS spectral scans of methanol comparable to those presented in [8] by adjusting the temporal overlap of the pump and Stokes, obviating any variation of the Stokes central wavelength.

In order to further characterize noise sources in this all-fiber CARS imaging system, we used an RF spectrum analyzer (Agilent N9320A) and a fast photodiode (Thorlabs DET10A) to measure noise spectra of both pump and Stokes sources. If MI was the major source of noise, we would expect broadband white noise in the Stokes signal. In Fig. 4(a) we present an image of live HuH-7 cells (4 μs/pixel dwell time, no averaging of scans). The green is TPF from fluorescein, and the red is CARS imaging of lipids at $\sim$2850 cm^{-1}. By Fourier transforming each scan line of the image (512 pixels), a noise peak at 48 kHz was revealed. In Fig. 4(b) we present the spectrum of the Stokes noise measured with the RF spectrum analyzer and with the photodiode at the output of the UHNLF. Importantly, little broadband white noise was observed, indicating that MI is not a major noise source in this system. Two sharp peaks at 48 and 68 kHz are seen in the Stokes RF spectrum. These are assignable to the switching power supply in our version of the Femtolite FX-100. This appears in the Stokes pulse train as both spectral and timing shifts [18] which cause both the resonant and nonresonant CARS signal to fluctuate and makes the noise apparent while imaging. The noise source was intermittent and was not present for all experiments. As this noise source is readily and permanently removed by simple power supply modifications, we conclude that the noise sources in this all-fiber CARS system are well within the requirements of live cell microscopy. Finally, we note that both the pump laser and UHNLF are polarization maintaining, a characteristic relevant to long term environmental stability [25].

4. Discussion

PCFs are well known to exhibit a broadband white noise due to MI under certain operating conditions [19–21]. Given that any noise sources in microscopy can greatly degrade image quality and performance, the choice of fiber and operating conditions is of paramount importance. When using fs pulses and intensity levels compatible with multiphoton microscopy, PCFs are a viable option, since it is possible to operate far away from the regime where fundamental quantum noise is greatly amplified (it is worth noting that this is much more difficult to achieve when using ps pulses [21]). Although technical noise is apparent in Fig. 4, there is no evidence that MI noise amplification occurs significantly (which would otherwise prevent its use as a source for microscopy). The video presented in the supplementary material demonstrates that good stability was achieved over all time scales relevant to live cell microscopy. Further improvements to this system would be to replace the remaining free space coupling optics with all-fiber coupling (most notably reducing any in-coupling instability to the UHNLF). Another option for reducing technical noise coupling would be to choose a fiber which has mode characteristics that saturates the soliton red shift, as this has been shown to attenuate spectral power variations [26]. An all fiber system would present an attractive option for CARS endoscopy applications, however, maintaining the chirp as the pulses propagate through long stretches of fiber and limiting high order nonlinear effects from affecting system performance would be challenging and most likely call for fiber designed with a specific dispersion profile.

An optimized version of the proof-of-concept system demonstrated here would offer signifi-
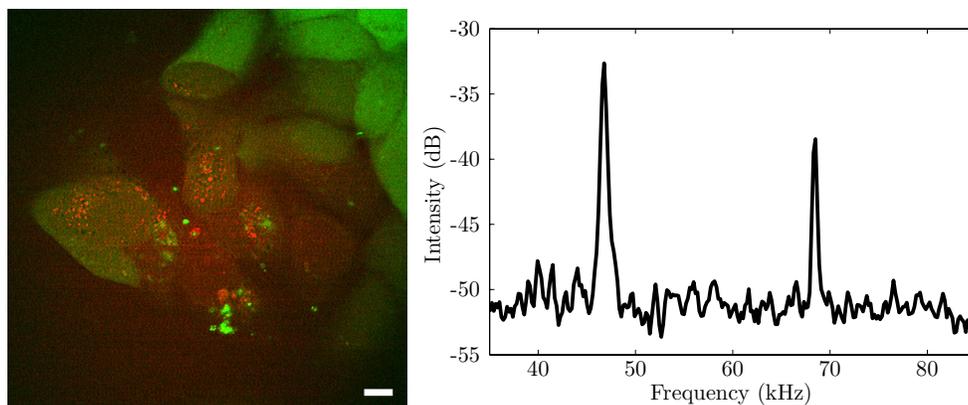
Fig. 4. (a) Multimodal CARS image with both signals collected simultaneously. Green is TPF from fluorescein and red is CARS tuned to observe the Raman stretch of lipids at 2850 cm^{-1}. There is a noticeable periodic noise source in the CARS image (TPF does not demonstrate the same noise). Scale bar is 5 μm. (b) RF spectrum of the Stokes output of the UHNLF measured with a photodiode and electrical spectrum analyzer. Two noise peaks are clearly visible at $\sim$ 48 kHz and $\sim$ 68 kHz. From analyzing the images (Fourier transform of each scan line), it appears the the noise source at 48 kHz corresponds to noise observed in the images. There is no evidence of broadband white noise due to MI.

cant advantages to end users, namely compact footprint, better environmental stability and low power consumption. While our proof-of-concept arrangement included some free space optics, these could be readily replaced with all-fiber components, permitting development of a mono-lithic package in a single compact box. The output power of the Stokes source could be the main limiting parameter of such a system. This, however, did not prevent our imaging of tissue samples up to 50 μm thick, and we speculate that using epi-detection, we would likely be able to image samples at least 100 μm thick.

5. Conclusion

We have demonstrated a proof-of-concept all-fiber multimodal CARS microscope based on a fs fiber pump and a UHNLF (for Stokes generation). By adjusting the chirp of both the pump and Stokes fs pulses to suit a particular problem, we performed simultaneous SHG, TPF and CARS imaging. RF spectral analysis of the Stokes showed that the UHNLF was operating in a regime where there is little broadband white noise present due to MI at the frequencies relevant to scanning microscopy. This demonstration points to a path for developing an all-fiber, compact, environmentally stable system useful for multimodal CARS imaging outside of typical laser laboratory situations.