Solid state lasers for wide-field CARS microscopy

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ABSTRACT

We discuss the requirements for laser systems used in Coherent Anti-Stokes Raman Scattering (CARS) microscopy and particularly in its wide-field modification. While such laser parameters as wavelength, spectral width and frequency difference between pump and Stokes beams are similar for all CARS systems, requirements for pulse energy, repetition rate, pulse length and mode structure might be significantly different for scanning and wide-field approaches. We will present results obtained with a wide-field CARS microscope with non-phase matching illumination and compare its performance with other CARS microscopes. Objectives for the design of future laser systems for CARS microscopy will be outlined.

Keywords: Coherent anti-Stokes Raman scattering, Microscopy, Wide-field, Fast imaging, Chemically selective imaging, Multiphoton microscopy, Scanning microscopy

1. INTRODUCTION

The vast majority of modern microscopy methods are based on one of the two contrast mechanisms – refractive or chemical – or their combination. Gradient of the index of refraction within the sample alters direction of the illumination beam (bright-field and dark-field microscopy) or its phase (phase-contrast and differential interference microscopy) [1]. As a result, areas of the sample with different refractive indices exhibit different brightness on the image. For many biological samples, however, the small value of refractive index gradient makes it difficult to obtain images with sufficient contrast. Fluorescent dyes or other exogenous staining can greatly improve contrast in the biological samples. To achieve chemical selectivity multiplicity of agents were developed that selectively adhere to certain molecules or cellular organelles [2]. However, the applicability of these dyes to in-vivo imaging is limited since they often alter normal metabolism of the sample.

Noninvasive methods providing chemical contrast utilize intrinsic vibrational spectra of specimens. Most of the characteristic vibrational frequencies for various biological samples fall into the range 1000-3500 cm^{-1} (see Fig. 1). Infrared (IR) absorption microscopy provides direct way to characterize chemical structure of the sample with high spectral resolution, however, long wavelengths limit spatial resolution of this method and its applicability for aqueous samples is limited due to strong water absorption. Raman microscopy allows to probe vibrational spectrum of the sample in mid-IR range with visible and near-IR radiation. Spatial resolution achieved this way can be 3-10 times higher than that of IR absorption microscopy. Due to small Raman scattering cross-section this approach has low sensitivity and therefore requires high power excitation lasers and elaborate detection procedure. In addition, it is often difficult to separate generated Stokes signal from a fluorescent background.

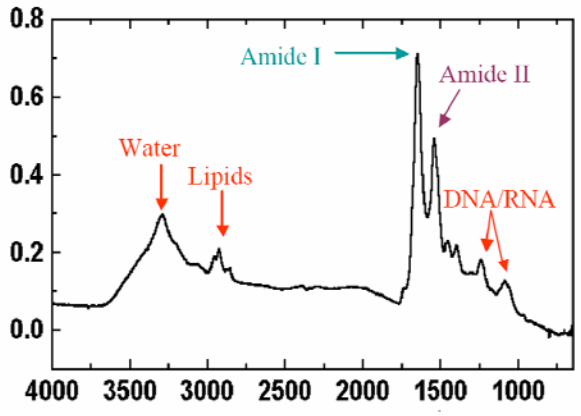

Fig. 1. Typical Raman spectrum of biological sample

CARS microscopy circumvents the main limitations of conventional Raman microscopy. In CARS process anti-Stokes photons are produced as a result of nonlinear interaction of two pump and one Stokes photons with the sample [3]. If the frequency difference between pump and Stokes radiation is close to the Raman resonance of the material CARS signal is efficiently generated, making this method several orders of magnitude more sensitive than spontaneous Raman scattering [4]. Choosing pump and Stokes wavelengths in near-IR range allows for high spatial resolution, minimizes Rayleigh scattering of incident beams, and eliminates the problem of water absorption. Since anti-Stokes frequency is higher than frequency of pump and Stokes it is relatively easy to separate CARS signal from the fluorescent background.

Similarly to multiphoton excitation microscopes, most of the CARS microscopes utilize tightly focused laser beams that are raster-scanned over the sample [5]. CARS signal is recorded by a highly sensitive detector e.g. photomultiplier tube and image is constructed point-by-point. Since CARS is a non-linear effect, the signal is generated only in a small focal volume (less than $1\mu m^3$), thus delivering high spatial resolution and limiting the amount of non-resonant background. Three-dimensional imaging capabilities of scanning approach were demonstrated with various samples and numerous techniques were suggested to further reduce level of non-resonant signal [6, 7]. Since scanning CARS is inherently multi-pulsed, it is time-consuming and only recently CARS microscope operating at video-rate regime was demonstrated [8].

Wide-field CARS microscopy is an alternative approach where the whole sample is imaged at once. Two wide-field microscopy methods were demonstrated so far. In the first approach pump and Stokes satisfy phase-matching condition in a narrow (5μm) layer within the sample [9]. Keeping the interaction layer thin limited the amount of non-resonant background signal generated by this method. Images of various biological samples were successfully obtained with acquisition time of 10s (100 pulses).

The second method of wide-field CARS microscopy is based on non-phasematching illumination geometry to avoid background generation in the bulk. As a result of scattering and/or refraction within the sample fraction of pump and Stokes radiation becomes phase-matched. If scattering/refracting center contains Raman active molecules CARS signal is generated. This approach delivers double contrast – refractive and chemical. The proof of concept was demonstrated using a tunable system based on Ti:Sapphire laser (1ps, 800nm, 1mJ, repetition rate up to 1kHz) and an optical parametric amplifier (OPA) [10].

In this work we further developed non-phasematching approach replacing the tunable laser source by a much more powerful fixed frequency laser and using more efficient illumination geometry. While having a tunable source is essential for proof of principle studies to demonstrate spectral selectivity of CARS microscope, practical applications may well utilized few fixed laser frequencies corresponding to the chemical components of interest. For instance lipids, which are very important for diagnostic of brain tumors, have resonance frequencies in the vicinity of 2900 cm^{-1}. This frequency range can be accessed using a Raman converter to shift frequency of the pump radiation to produce the appropriate Stokes beam. We report here technical implementation of such a system and demonstrate first results that conclusively demonstrate the advantages of this approach.

2. EXPERIMENTAL SETUP

While OPA provides tunability, it limits the amount of available Stokes energy (to ~10μJ), and also suffers from a low pulse-to-pulse stability (~40%). To resolve these issues we developed a new system for generating Stokes beam based on Raman conversion of the pump beam. The system was used on Ti:Sapphire regenerative amplifier which served as a source of pump radiation and also, after Raman frequency conversion as a source of Stokes beam. 30% of output energy of Ti:Sapphire laser were used as a pump beam, and remaining 70% were directed into Raman converter filled with methane (CH4) under 2MPa pressure (Fig. 2). We were able to obtain 10% conversion efficiency resulting in 70μJ of Stokes energy.

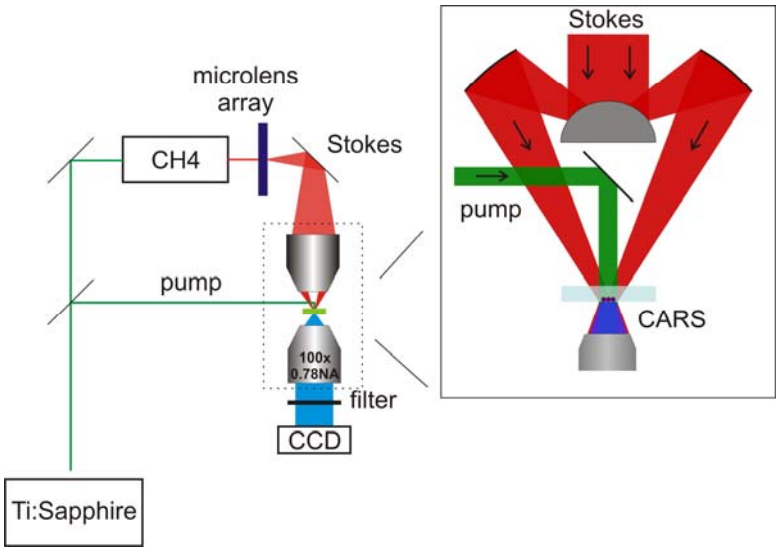

Fig. 2. Experimental setup. Ti:Sapphire laser is used for pump and to generate Stokes in Raman converter

Using methane as Raman active medium is an obvious choice for CARS targeting lipids. Their spectra exhibit several strong Raman peaks in the range 2800 – 3000 cm^{-1} owing to different C-H vibrations. In particular, aliphatic C-H vibration has frequency 2900 cm^{-1} that is practically the same as Raman shift generated in methane. Therefore, Raman converter ensures correct frequency difference between pump and Stokes even if the frequency of the laser is not stable. Another advantage of Raman converter is complete lack of temporal jitter between pump and Stokes.

Non-phasematching illumination required for the method was achieved by directing pump beam along the microscope objective axis and Stokes beam at an angle to it. In our system pump was collimated by a long focal length (f = 1m) lens to a 500μm diameter spot. The intensity variation over the central portion of it (~170μm diameter) corresponding to the microscope field of view, did not exceed 20%. To avoid damage to the sample and to the microscope objective, pump beam was attenuated so that its fluence in the sample plane did not exceed 100mJ/cm^2.

We used Cassegrain objective to focus Stokes beam onto the sample plane. Focused Stokes beam was spread from 9° to 16° with respect to the pump beam direction. The objective focuses parallel beam into a diffraction-limited spot of ~1μm diameter. In order to illuminate extended area (~200μm diameter) we had to introduce irregular divergence into the Stokes beam, which was achieved by means of using a microlens array. The drawback of this approach is that portions of the beam passing through different lenses of the array interfered with each other in the sample plane creating highly inhomogeneous illumination spot. We have quantified the quality of illumination by imaging a 10μm layer of fluorescent dye sandwiched between two coverslips. By analyzing the intensity of fluorescent signal emitted from each point we derived that the intensity of Stokes beam varied by a factor of 5 across the field of view. Introducing random motion of the array reduced the variation of the Stokes illumination intensity to ~10% after averaging over 100 pulses. Mean Stokes fluence in the sample plane after averaging was 18mJ/cm^2.

CARS images were acquired using an inverted microscope (Axiovert 35, Carl Zeiss) equipped with a 100x objective (NA = 0.78). The image was detected by a nitrogen cooled CCD camera (CCD-512-TKB/1/VISAR, Princeton Instruments). Residual pump and Stokes were blocked by a set of filters installed directly in front of the camera.

3. RESULTS AND DISCUSSION.

We have characterized the spatial resolution of our technique by imaging 1μm polystyrene beads placed on a 10μm Mylar film in two environments – surrounded by air and covered by 1mm layer of water (Fig. 3 a,b). The ratio of bead center brightness to the brightness of the surroundings in air was 10:1. This ratio reduced to 6:1 in water due to stronger background from a denser medium and lower refraction contrast between beads and surrounding material.

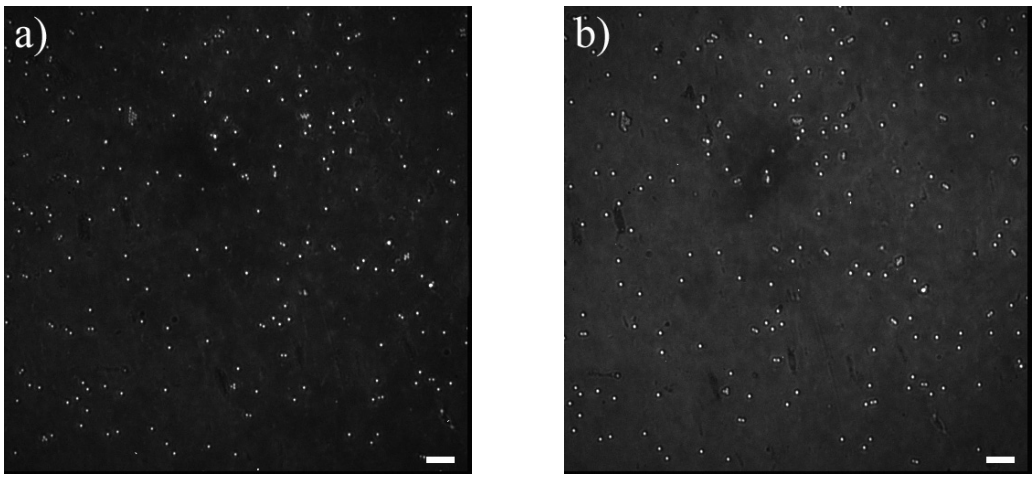

Fig. 3. a) CARS image of 1μm polystyrene beads on a 10 μm Mylar film in air; b) CARS image of 1μm polystyrene beads on a 10 μm Mylar film in water. Scale bar represents 10 μm on both images.

Using a Rayleigh resolution criterion for two beads in contact with each other, we obtained a resolution value of 0.5 μm which is practically equal to the theoretical maximum that can be achieved using imaging objective with NA = 0.78 at a signal wavelength of 650nm.

As a tissue sample we used a lipid-rich spinal cord tissue. (Fig. 4 a,b). It has been recently demonstrated that CARS imaging of myelin sheath can be useful for diagnosis of various neurological diseases [11]. We used RCS rat spinal cord to prepare 6μm thick samples of two types: with nerve bundles in the plane of the cut and perpendicular to it. Samples were immersed in phosphate buffer saline (PBS) and placed between 120μm coverslip and 10μm Mylar film. Images were acquired through the Mylar film to avoid aberrations that could be induced by a thicker substrate. For samples of both types lipid-rich myelin appears about 5 times brighter than the rest of the tissue.

In order to prove that acquired images have chemical contrast and the signal originates primarily from myelin we compared them with images of histological samples stained with either toluidine blue or myelin specific fluorescent dye FluoroMyelin green (Fig. 4 c-f). All three sets of images clearly show the same structure; therefore we conclude that CARS signal is selectively generated in lipid-rich tissue regions.

These results clearly demonstrate that wide-field CARS microscope with a non-phasematching illumination is capable of high resolution imaging with good chemical contrast. While lacking 3D sectioning capability it potentially allows for higher image acquisition rates and can use simpler laser systems than scanning microscopes do. While optimal pulse duration and laser wavelengths are essentially the same for both methods, pulse energies, repetition rates and beam quality requirements are significantly different.

Large illumination area in wide-field CARS requires much higher laser energy sources in a range of 100μJ – 1mJ. This is 4-5 orders of magnitude greater than energies typically used in scanning systems. However, much lower number of pulses required for single frame acquisition, and potential ability for single shot imaging allows using systems with repetition rate of few kHz and lower. It is 4-5 orders of magnitude lower than for scanning systems. Furthermore, wide-field illumination doesn't require high beam focusability, which is essential for the scanning approach. This allows using lasers with much higher M^2 values, which can also help to drive the cost of such system down.

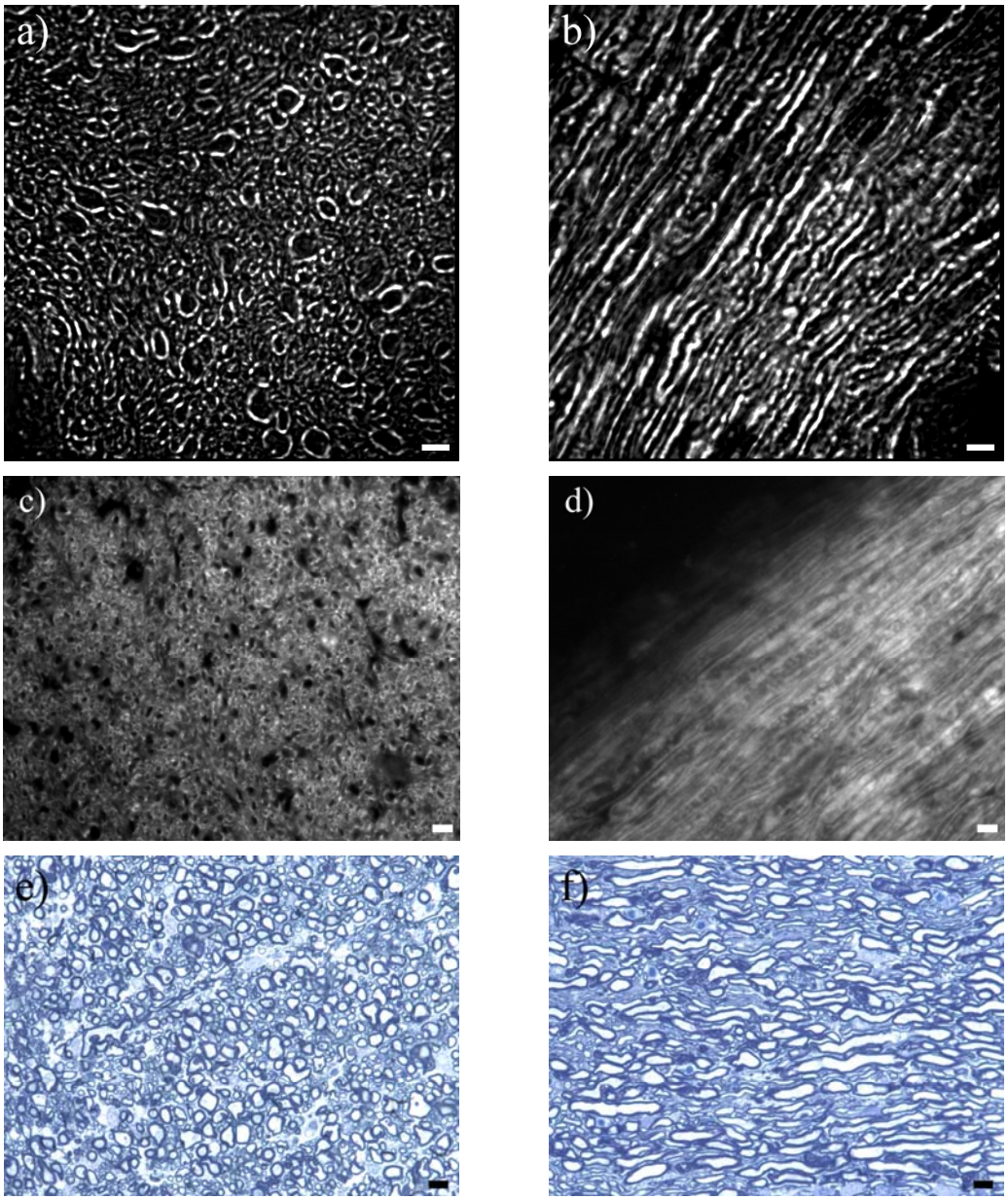

Fig. 4. a), b) CARS images of a cross- and longitudinal section of rat spinal cord; c), d) fluorescent images of the same tissue stained with myelin-specific stain (FluoroMyelin); e), f) of histological samples of the same tissue stained with toluidine blue. Scale bar represents 10 μm in all images.

4. CONCLUSIONS.

We have demonstrated that fixed frequency laser system can be successfully used for wide-field CARS microscopy. A 0.5 μm spatial resolution limited by the imaging optics has been achieved. High resolution images of biological samples with good chemical selectivity were obtained. Number of pulses as low as 100 was shown to be sufficient for single

image acquisition. Further optimization of the illumination geometry and laser beam parameters should make a single shot imaging possible.

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