Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 188 (2018) 135–140

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Multimodal nonlinear microscope based on a compact fiber-format laser source

Francesco Crisafi [a,1], Vikas Kumar [a,1], Antonio Perri [a], Marco Marangoni [a], Giulio Cerullo [a], Dario Polli [a,b,*]

[a] IFN-CNR, Dipartimento di Fisica, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milano, Italy
[b] Center for Nano Science and Technology @Polimi, Istituto Italiano di Tecnologia, via Giovanni Pascoli 70/3, 20133 Milano, Italy

ARTICLE INFO

Article history:
Received 4 February 2017
Received in revised form 26 May 2017
Accepted 30 June 2017
Available online 5 July 2017

Keywords:
Nonlinear optical microscopy
CARS
SRS
TPEF
Multimodal
Coherent Raman spectroscopy

ABSTRACT

We present a multimodal non-linear optical (NLO) laser-scanning microscope, based on a compact fiber-format excitation laser and integrating coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS) and two-photon-excitation fluorescence (TPEF) on a single platform. We demonstrate its capabilities in simultaneously acquiring CARS and SRS images of a blend of 6-μm poly(methyl methacrylate) beads and 3-μm polystyrene beads. We then apply it to visualize cell walls and chloroplast of an unprocessed fresh leaf of *Elodea* aquatic plant via SRS and TPEF modalities, respectively. The presented NLO microscope, developed in house using off-the-shelf components, offers full accessibility to the optical path and ensures its easy re-configurability and flexibility.

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1. Introduction

Optical microscopy is an extremely powerful investigation tool in life sciences, thanks to its ability of visualizing morphological details in cells and tissues on the sub-micrometer spatial scale [1]. It provides a much higher spatial resolution compared to magnetic resonance imaging, and, at the same time, it does not require the sample to be fixed, as in electron microscopy. Nonlinear optical (NLO) microscopy techniques, in particular, offer additional advantages, such as inherent 3D-sectioning capability and greater penetration depth, due to the use of infrared wavelengths. The most widespread NLO technique is two-photon-excitation fluorescence (TPEF) [2–4]. It provides very high sensitivity, but in most cases it requires the addition of markers to the sample to be studied, either exogenous (staining dyes or semiconductor quantum dots) or endogenous (fluorescent proteins). Other NLO microscopy techniques have the advantage of being label-free, allowing the use of pristine samples. Some of these techniques, such as second-harmonic generation (SHG) [5–7], sum-frequency generation (SFG) [8,9] and third-harmonic generation (THG) [4,10] microscopy, do not provide chemical contrast, i.e. they are not able to selectively differentiate between specific components of a cell or a tissue. SHG observes contrast in non-centrosymmetric structures like collagen fibers in tissue, while THG is mainly sensitive to local changes of nonlinear refractive index within the sample occurring at interfaces, such as lipid cell membranes or lipid droplets. Chemical selectivity is provided by coherent Raman scattering (CRS) techniques [11], which employ the vibrational spectrum of a molecule to provide an endogenous signature that can be used for its identification. CRS exploits the third-order nonlinear optical response of the sample to set up and detect a vibrational coherence within the ensemble of molecules inside the laser focus. When the difference between pump and Stokes frequencies matches a characteristic vibrational frequency, then all the molecules in the focal volume are resonantly excited and vibrate in phase; this vibrational coherence enhances the Raman response by many orders of magnitude with respect to the incoherent spontaneous Raman process.

CRS microscopy has two popular forms: coherent anti-Stokes Raman scattering (CARS) [12,13] and stimulated Raman scattering (SRS) [14–17]. In both CARS and SRS, two synchronized narrowband pulses, the pump (at frequency ω_p) and the Stokes (at frequency ω_s), are focused on a sample and their frequency difference is tuned to a Raman-active vibrational mode Ω of the targeted molecule, i.e. $\Omega = \omega_p - \omega_s$. When it happens, in the case of CARS, a strong anti-Stokes signal at frequency $\omega_{as} = 2\omega_p - \omega_s$ is generated, which is utilized to probe the molecules under study. Since CARS signal is at higher frequency with respect to input pump-Stokes frequencies, it can be easily spectrally separated and is also immune to one-photon fluorescence signals which fall at lower frequencies. This makes CARS signal naturally free from any kind of linear background. On the other hand, the CARS process has the serious drawback that it suffers from nonlinear non-resonant background (NRB). CARS signal is given by $I_{CARS} \propto |\chi^{(3)}|^2 = |\chi_R^{(3)}(\Omega) + \chi_{NR}^{(3)}|^2$,

* Corresponding author at: IFN-CNR, Dipartimento di Fisica, Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milano, Italy.
E-mail address: dario.polli@polimi.it (D. Polli).
[1] Authors contributed equally to work.

http://dx.doi.org/10.1016/j.saa.2017.06.055
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where $\chi_R^{(3)}(\Omega)$ is the complex resonant response of the targeted vibration and $\chi_{NR}^{(3)}$ is the real non-resonant response of the background, which does not deliver any chemically specific information. In SRS, on the other hand, the coherent interaction with the sample induces stimulated emission from a virtual state of the sample to the investigated vibrational state, resulting in a Stokes-field amplification (stimulated Raman gain, SRG) and in a simultaneous pump-field attenuation (stimulated Raman loss, SRL). SRS is inherently free from NRB, however it is technically demanding as it requires the detection of a weak signal (the SRG/SRL) on top of a large and fluctuating linear background. This typically requires sophisticated modulation-transfer techniques.

Multimodal NLO microscopy can capitalize the potential of different NLO modalities by combining two or more of them in a single imaging platform. Hence, it can provide richer microscopic information by imaging different kinds of molecules or structures in a sample. Multimodal CARS/TPEF/SHG microscopes have already proven for the capability of biological imaging and identification of cancerous tissues in brain [18], lung [19] and kidney [20]. Cheng et al. have utilized the combination of TPEF/SFG/CARS modalities to investigate the central nervous system in diseased and healthy states [21], in the study of the progression of arterial diseases [22] and in imaging and quantitative analysis of atherosclerosis, a major cause of cardiovascular diseases [6]. The combination of THG/SHG/TPEF microscopies has been used e.g. to visualize the microstructure of human cornea [4].

The main stumbling block which prevents widespread adoption of multimodal NLO microscopy techniques in the biological and medical communities is the complication and cost of the experimental apparatus, both for the excitation laser and the microscope. Regarding the excitation source, the most critical technique is CRS. A compromise must be found between high peak laser power to enhance the nonlinear signal, which points towards shorter pulses, and the frequency resolution to preserve molecular selectivity, which points towards narrow laser bandwidth. In the condensed phase, Raman transitions exhibit linewidths of the order of tens of cm^{-1}, so that the optimal pulse duration lies in the 1–3 ps range for pump/Stokes pulses. Moreover, their frequency difference should be tunable, to access a large vibrational bandwidth and the laser repetition rate should be high ($\approx$100 MHz) to reach the shot-noise limit in detection and avoid multi-photon absorption sample damage caused by the high peak power of the pulses. An output power of $\approx$100 mW per branch is also typically required to compensate for losses in the optical chain of the microscope. The complexity of the excitation laser is one reason why, after its early demonstration in 1982 [23], the development of CRS microscopy has stopped for nearly two decades. Following its revival, initial CRS implementations were based on two electronically synchronized picosecond Ti:sapphire oscillators [24–26], while the current "gold standard" is represented by an optical parametric oscillator synchronously pumped by a picosecond $Nd:YVO_4$ oscillator [27–29]. Such systems are complex, expensive, and they all critically require a synchronization between two independent laser sources, which must be maintained over time. Drastically simplified excitation architectures, with lower cost and smaller footprint, are thus greatly in demand, as they would lower the technological entrance barriers to CRS microscopy. In addition, the multimodal NLO microscope platform cannot easily rely on commercially available solutions. The detection of the different nonlinear signals, in fact, requires full accessibility to the optical paths, in order to place different components and detectors required to implement the various techniques. This is difficult in commercial microscope systems that, being designed for the end user, are typically not accessible and modifiable.

In this paper, we describe a multimodal NLO laser-scanning microscope with a highly simplified architecture, based on a compact fiber-based laser, which enables the TPEF, CARS and SRS modalities. The fiber laser generates two synchronized beams of picosecond pulses, the pump at 780 nm and the Stokes tunable between 950 and 1050 nm, with sufficient spectral coverage to implement CARS and SRS microscopy in the C—H stretching region. A single excitation beam can also be used for other NLO microscopy modalities, such as TPEF, SHG, THG and SFG. The excitation source is coupled to a home-built multimodal scanning microscope, based on off-the-shelf components and allowing maximum accessibility to the beam paths. We demonstrate CARS/SRS imaging of polymer beads and of leaves of the *Elodea* aquatic plant.

2. Materials and Methods

Fig. 1(a) shows the architecture of the compact multi-branch fiber-format laser source that generates the multi-colour pulses required for the different NLO microscopy modalities. It is based on a mode-locked Erbium:fiber oscillator working at 40-MHz repetition rate, which feeds three independent Er-doped fiber amplifiers (EDFAs), each producing 350-mW average power at 1560-nm central wavelength. In this way, all three EDFA outputs are inherently synchronized and phase coherent [30]. Further, they are compressed to nearly transform-limited sub-100-fs duration by individual pairs of silicon prisms. Two of the EDFA outputs (referred to as 'Arm 1' and 'Arm 2' in Fig. 1(a)) are coupled to highly nonlinear fibers (HNLFs) that considerably broaden the laser spectrum, generating side-lobes at both longer and shorter wavelengths with respect to the input fundamental one. The longer wavelength peak is a soliton, produced mainly through self-frequency shift due to intrapulse Raman scattering. On the other hand, the shorter wavelength peak is propagating in the normal dispersion regime, also phase-matched to the soliton. The spectral positions of the two peaks sensitively depend on the overall dispersion of the pulse coupled to the HNLF. Acting on the silicon prism-pair, the position of soliton peak can be continuously tuned and precisely controlled between 1700 and 2100 nm.

The third EDFA output [referred to as 'Arm 0' in Fig. 1(a)] at 1560 nm is sent to a non-linear crystal to generate the pump pulses for the CRS processes at a fixed wavelength of 778 nm. We employed a 10-mm-long MgO-doped periodically poled lithium niobate (PPLN) crystal with a poling period of 19.3 μm. It generates a narrow-bandwidth (15-cm^{-1} linewidth) second harmonic, according to the spectral compression technique [31], with 120-mW average power [see green spectrum in Fig. 1(b)]. In the same manner, the redshifted soliton output of one of the HNLFs (in 'Arm 1') is frequency doubled in a 10-mm-long PPLN crystal having a fan-out grating design, spanning the poling period range of 26–33 μm. It generates tunable Stokes pulses for the CRS modalities from 950 to 1050 nm [see red spectra in Fig. 1(b)] with power up to 10 mW and linewidth ranging between 18 and 30 cm^{-1}. Tuning of the Stokes wavelength is achieved simply by transversely translating the fan-out crystal using a motorized stage, calibrated to guarantee a rapid (within a fraction of a second) and reproducible selection of the vibrational Raman shift. The pump-Stokes frequency detuning in the range 2330–3330 cm^{-1} fully covers the CH-vibrational region, which is the most commonly used in CRS microscopy. Before entering the microscope, the pump and Stokes pulses are temporally matched by a mechanical delay line and then collinearly combined by a dichroic beam splitter (Semrock, LP02-785RS-25). For the SRS modality, the pump beam is modulated at 1 MHz by an acousto-optic modulator placed in the beam before the combiner. The narrowband pump or Stokes pulses can individually be utilized as an excitation pulse for TPEF or SHG/THG experiments as per demand of the sample. Furthermore, a part of the compressed sub-100 fs output of the EDFA at 1560 nm just before the PPLN (in Arm 0) can be split and used as an excitation pulse for THG microscopy.

The third branch [referred to as 'Arm 2' in Fig. 1(b)] is used, by properly adjusting the silicon prism pair before the HNLF, to generate a broadband spectrum in the 840- to 1100-nm wavelength region. After compression in a SF-10 prism pair, we obtain sub-20-fs pulses, which can be used either as Stokes pulses to implement broadband CARS/SRS configurations [32,33] or as excitation pulses for TPEF or SHG experiments performed on the microscope. In the following, we will present experimental results obtained coupling only 'Arm 0' and 'Arm 1' beams to the multimodal NLO microscope.

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