Advanced Drug Delivery Reviews xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

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

Beyond the borders — Biomedical applications of non-linear Raman microscopy

Martin Josef Winterhalder, Andreas Zumbusch *

Department of Chemistry, University of Konstanz, 78457 Konstanz, Germany

ARTICLE INFO

Available online xxxx

Keywords:
Non-linear Raman
CARS
SRS
Optical microsopcy
Biomedical imaging

ABSTRACT

Raman spectroscopy offers great promise for label free imaging in biomedical applications. Its use, however, is hampered by the long integration times required and the presence of autofluorescence in many samples which outshines the Raman signals. In order to overcome these limitations, a variety of different non-linear Raman imaging techniques have been developed over the last decade. This review describes biomedical applications of these novel but already mature imaging techniques.

Contents

1. Introduction

Optical microscopy and cellular biology share a common historical development. In fact, the development of early microscopes was prerequisite to the discovery of the cellular organization of biological material by Robert Hooke in the 17th century. From this early start on, scientific questions from both fields have mutually stimulated their respective progress. With respect to the spatial resolution achievable with light microscopy, the physical limits posed by diffraction have been reached already in the late 19th century by Abbe. This physical barrier has only very recently been broken by two different types of super-resolution microscopy, which were rewarded with the Nobel prize in Chemistry in 2014 [1,2]. Since Abbe's time, the main focus of the further development of optical microscopy concerned the introduction of new modes of contrast generation. In the beginning, this involved various staining approaches leading to specific absorption of light by different parts of the sample. Examples are the hematoxylin and eosin (H&E) stains, perhaps the most widely used staining in histology. One should note that already the generation of images using these simple stains involves a combination of absorption spectroscopy with microscopy in that sample regions are discerned based on their different properties with respect to the absorption of visible light. The principle of using a wide range of other well-established spectroscopic techniques has subsequently been a basis for a wide range of different microscopy modalities.

* Corresponding author.
E-mail address: andreas.zumbusch@uni-konstanz.de (A. Zumbusch).

http://dx.doi.org/10.1016/j.addr.2015.04.024
0169-409X/

During the last three decades, especially fluorescence excitation in combination with optical microscopy has become one of the most important practical techniques for the investigation of biological samples. There are several reasons for its success: i) Fluorescence spectroscopy is exquisitely sensitive. It has been shown in the late 1980s by Orrit and coworkers that fluorescence detection is sensitive enough to detect single molecules [3]. In practice, it is much easier to detect a weak fluorescence signal than a weak absorption signal, because the Stokes shifted fluorescence can be separated from the excitation light by means of a simple optical filter. For a single molecule, this means that a fluorescence signal of typically 10^8 photons/s can be detected against essentially a zero background, whereas a similar absorption measurement requires detection of a change in intensity of 10^8 photons/s against the background of the excitation light level of typically 10^{14} photons/s [4]. ii) Fluorescence imaging can be performed with a very high three-dimensional resolution. Standard wide-field fluorescence microscopy offers a diffraction limited spatial resolution of typically 200–300 nm in the plane perpendicular to the excitation beam propagation direction if high numerical aperture objectives are used. Along the beam propagation direction, the spatial resolution can be improved to be slightly below 1 μm if a confocal microscope is used. In this case, the detection volume is restricted by virtue of a pinhole in the beam path [5]. iii) The importance of fluorescence microscopy greatly increased with the advent of fluorescent proteins as genetically encoded fluorescence labels [6–8], since labeling with fluorescent proteins offers the best target selectivity imaginable.

Despite the enormous success of fluorescence microscopy, specific applications still require the development of new microscopic approaches which cannot be tackled with existing methods. Especially medical applications are a realm of such approaches, since here staining techniques can most often not be employed. This has served as a strong motivation for the development of label-free microscopy techniques which should otherwise perform similar to fluorescence microscopy. Especially vibrational spectroscopy techniques have attracted a lot of attention, since in many cases, molecular selectivity is desired. In vibrational microscopy, contrast is generated on the basis of the vibrational spectra of the sample molecules. Since even small molecules exhibit many different vibrational resonances and because in many cases these are spectrally very narrow, an unequivocal identification of pure compounds based on their vibrational spectra is possible. The downside of this multitude of vibrational bands, however, is that mixtures of compounds as they are typically found in most biological applications, will lead to a plethora of vibrational bands which is hard to analyze. While in principle, direct infrared absorption and Raman scattering can be employed for microscopic approaches, for a number of practical reasons the overwhelming majority of experiments especially aiming at investigations of biological samples is based on Raman scattering. Raman scattering is the inelastic scattering of excitation light, i.e., laser light impinging onto the sample is scattered with a transfer of energy between the excitation light and the sample. The scattered light can be higher (anti-Stokes) or lower (Stokes) in energy than the illumination light. In practice, Raman microscopy is nearly exclusively based on the analysis of Stokes-scattered light, since these bands have a much higher intensity. Raman spectroscopy is readily adapted to microscopy, because the excitation light can be chosen to be in the visible or near infrared spectral range, such that high quality objectives can be used. The use of confocal pinholes for high 3D resolution is also possible and widely applied [9].

The major drawback of spontaneous Raman microscopy is the low Raman scattering cross section of molecules. Therefore, Raman microscopy is a rather slow technique which requires long integration times, even if progress towards faster imaging by using line illumination has recently been made [10]. Equally important, the low scattering efficiency together with a weak autofluorescence, which is present in many samples, often prevents the detection of a Raman signal. In order to overcome these limitations, non-linear optical microscopy Raman techniques, namely Coherent anti-Stokes Raman Scattering (CARS) and Stimulated Raman Scattering (SRS) microscopy have been developed in the last two decades. After an initial period of mainly methodological development, both schemes have meanwhile found a lot of applications. Recently published review articles give an excellent overview over the methodology and basic research in this field [11–16]. The focus of this article, by contrast, will be to review the current state of the art of biomedical applications of CARS and SRS microscopy.

2. Technical background

In the following, we will give a very basic description of the technical background of non-linear Raman microscopy with the main aim of highlighting the specific features of CARS and SRS microscopy which need to be known in order to apply them properly. A more extensive practical guide to the implementation of these experiments has recently been published [17].

The basic idea of both techniques is that vibrational spectroscopy is used to generate contrast for microscopic images. In both approaches this is typically achieved by coupling two laser beams with intensities I_{pump} and I_{Stokes} into a multiphoton fluorescence microscopy experiment (Fig. 1). The generated contrast is strongest, if a resonance condition between the excitation light and the sample molecules under investigation is met. Ideally, a simple relation connects the signal intensity with the number N of molecules of interest which are probed.

Both SRS and CARS are non-linear optical effects. This means that the signal intensity does not depend linearly on the excitation intensity, but quadratically (SRS) or cubically (CARS):

$$I_{SRS} \propto N \sigma_{Raman} I_{pump} I_{Stokes}$$

and

$$I_{CARS} \propto \left|\chi^{(3)}\right|^2 I_{pump}^2 I_{Stokes}.$$

Here, σ_{Raman} is the Raman scattering cross section and $\chi^{(3)}$ is the third order non-linear optical susceptibility. Since $\chi^{(3)}$ is proportional to the number of molecules, the two equations point out an important difference between SRS and CARS: whereas SRS has a linear dependence on the concentration of probed molecules, CARS has a quadratic dependence. In addition, $\chi^{(3)}$ contains a non-resonant component, which gives rise to a background signal. In practice this has two consequences. Firstly, a quantitative interpretation of SRS signals as the number of molecules in the probe volume is much easier than that for CARS signals. Secondly, CARS signals can only be detected easily where the resonant signal is much stronger than the non-resonant background. To recover weak resonances in CARS microscopy, sophisticated excitation and data analysis schemes have to be employed [18,19]. In practice this means that CARS microscopy has mostly been limited to imaging in the spectral range of CH-stretch vibrations, whereas SRS increasingly is also applied in the vibrational fingerprint region between 600 and 1800 cm^{-1}.

Yet, CARS microscopy in many cases is still the preferred choice. The reason for this is that it offers the advantage that the detection of CARS signals is much easier than that of SRS signals. In a CARS process, the signal has the frequency $\omega_{CARS} = 2\omega_{pump} - \omega_{Stokes}$ which is different from that of the excitation light. By contrast SRS signals are detected as a loss or gain in the intensity of one of the two exciting lasers. This means that whereas, just as in the case of fluorescence microscopy, the CARS signal can be separated from the excitation light by means of a simple optical filter [20], the detection of a SRS signal requires more demanding lock-in detection [21,22]. The detection in CARS microscopy is therefore similar to other multiphoton techniques such as two-photon excited fluorescence emission and/or second (third) harmonic generation signals. For multimodal implementations, only the addition of the respective detection channels is required, since the lasers used for CARS signal generation can also be used to drive the other excitation processes [23]. This

Please cite this article as: M.J. Winterhalder, A. Zumbusch, Beyond the borders — Biomedical applications of non-linear Raman microscopy, Adv. Drug Deliv. Rev. (2015), http://dx.doi.org/10.1016/j.addr.2015.04.024