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Review

Nonlinear vibrational microscopy applied to lipid biology

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ABSTRACT

Optical microscopy is an indispensable tool that is driving progress in cell biology. It still is the only practical means of obtaining spatial and temporal resolution within living cells and tissues. Most prominently, fluorescence microscopy based on dye-labeling or protein fusions with fluorescent tags is a highly sensitive and specific method of visualizing biomolecules within sub-cellular structures. It is however severely limited by labeling artifacts, photo-bleaching and cytotoxicity of the labels. Coherent Raman Scattering (CRS) has emerged in the last decade as a new multiphoton microscopy technique suited for imaging unlabeled living cells in real time with high three-dimensional spatial resolution and chemical specificity. This technique has proven to be particularly successful in imaging unstained lipids from artificial membrane model systems, to living cells and tissues to whole organisms. In this article, we will review the experimental implementations of CRS microscopy and their application to imaging lipids. We will cover the theoretical background of linear and non-linear vibrational micro-spectroscopy necessary for the understanding of CRS microscopy. The different experimental implementations of CRS will be compared in terms of sensitivity limits and excitation and detection methods. Finally, we will provide an overview of the applications of CRS microscopy to lipid biology.

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1. Introduction

For centuries, developments in optical microscopy have led to new insights in cell biology which in turn stimulated the evolution of these optical tools. In particular, the advent of the laser providing a bright light source with a well defined spatial and temporal mode has had a profound impact on the contrast techniques available in optical microscopy. Today, most prominent among the laser-based microscopy techniques are confocal and two-photon fluorescence (TPF) microscopy [1], which together with the development of highly specific fluorescent labeling techniques have shaped modern cell biology. In confocal microscopy, continuous wave lasers are employed to excite the fluorophore of interest via one-photon absorption. An image is generated by raster scanning the focused beam across the sample and the emitted fluorescence is recorded sequentially with a single-channel detector. Three-dimensional (3D) spatial resolution is achieved by filtering the detected light by a confocal pinhole, suppressing out-of-focus light. An alternative way to obtain 3D resolution is realized in TPF microscopy where two near-infrared photons, for which one-photon absorption is negligible, are absorbed simultaneously by the fluorophore. As in confocal fluorescence, an image is obtained by raster scanning the focused beam over the sample while collecting the emitted fluorescence into a single-channel detector. Since two-photon absorption is non-linear and occurs dominantly in the focal volume where high photon densities are reached, TPF has an intrinsic 3D resolution without the need of a detection pinhole. In turn the collection efficiency is improved, since light generated in the focal volume and subsequently scattered within the sample is not rejected. An important advantage of TPF is the use of near-infrared light which enables deeper penetration into thick specimens due to the reduced Rayleigh scattering at longer wavelengths and the reduced one-photon absorption from endogenous cellular material. Importantly, TPF requires pulsed lasers to generate sufficient photon densities at the pulse peak while maintaining moderate average powers. Typical laser sources for TPF microscopy, which are now found in many bio-medical laboratories, provide pulses with durations of approximately 100 fs and repetition rates on the order of 100 MHz.

The success of TPF microscopy paved the way for research into the use of other non-linear optical phenomena for the generation of contrast in high-resolution optical microscopy. It was quickly realized that non-linear processes are especially well suited for the investigation of unlabeled samples. While fluorescence labeling is a mature technique to specifically tag and hence visualize biomolecules in living cells, it also has inherent drawbacks. Firstly, fluorescence labeling requires a sample preparation procedure, conditioning the types of samples which can be studied, and adding time delays and cost. Secondly, the labeling changes the system under investigation. Although in many cases these changes are acceptable, there are numerous examples of labeling artifacts. In the field of lipid biology, this concerns for example the observation of fusion of lipid droplets which can be induced by standard labeling protocols [2–4]. A third problem of fluorescence microscopy is the photobleaching of organic fluorophores. Electronic excitation of organic fluorophores leads with a certain probability to chemical reactions towards products which do not absorb at the excitation wavelength and/or do not fluoresce. This often severely limits the observation times in fluorescence microscopy and is accompanied by cytotoxic effects.

One should note that many linear label-free optical microscopy techniques are widely used already for decades. Examples are differential interference contrast (DIC) microscopy and phase contrast microscopy. The contrast in these techniques relies on refractive index differences in the sample which gives little information on the chemical composition. On the other hand, the ability to distinguish chemically specific structures in cells and tissues (e.g. lipid vesicles from other cellular vesicles) is crucial to the understanding of their functional behavior within the cellular machinery. One way to generate chemical contrast without the need for external labeling is to exploit the vibrational spectra of biomolecules. Vibrational resonances depend on the masses of the constituting atoms and their respective bond strengths. A typical vibrational spectrum therefore contains a large number of resonances relating to the set of vibrational modes of the molecule reflecting the chemical composition of the sample. Conventional techniques for vibrational micro-spectroscopy are the absorption of infrared (IR) light and Raman scattering (see Section 2.1). IR absorption microscopy is a well established technique. Yet, it is much more widely used in material science than in biological applications. The main two reasons for this are the poor spatial resolution achievable with IR wavelength and the strong IR absorption of water, which makes IR investigations of biological samples difficult. Raman microscopy, by contrast, utilizes light sources in the visible wavelength range. For this reason, it delivers spatial resolutions comparable to those achievable in confocal fluorescence microscopy. The application of Raman microscopy is not hampered by the presence of water in the sample. However, Raman scattering is weak, such that it can be overwhelmed even by weak autofluorescence in the sample. In addition, the measurement times for the generation of a well-resolved Raman image often are prohibitively long.

By exploiting nonlinear optical effects, novel microscopy techniques have been developed over the last ten years to rapidly image living cells in a chemically specific label-free way. As a matter of fact, most non-linear optical processes do not rely on the presence of a chromophore since a nonlinear signal is generated, with varying efficiencies of the process, in any polarizable medium, i.e. in any sample. This said, it is important to note that different symmetry restrictions and resonance conditions apply to different nonlinear effects. For example, second harmonic generation (SHG), that is the generation of light at exactly half the wavelength of the exciting light, has been used extensively for imaging collagen fibers [5,6] since the formation of SHG signal reflects the local symmetry and order of the sample. Specifically, SHG is forbidden in isotropic media, which causes the SHG signal to be suppressed in most of the liquid cell interior due to the random orientation of the molecules. Structures like collagen fibers, however, are ordered, allowing the coherent addition of the SHG from the individual non-inversion symmetric molecules. Another non-linear process used for label-free optical microscopy is third-harmonic generation (THG), producing light with exactly a third of the wavelength of the exciting light. THG is created also in inversion symmetric structures, but due to the significantly different refractive index at the THG wavelength, the coherent superposition oscillates in space and is suppressed away from interfaces or structures having sizes comparable to the excitation wavelength [7,8]. Since lipids have a much higher nonlinear susceptibility [9,10] for THG than water, this microscopy modality is useful for imaging lipid-rich structures [11,12]. On the other hand, SHG and THG do not provide vibrational contrast, since the involved light frequencies are well above the vibrational frequency range. This situation changes when laser fields containing frequency differences in the vibrational frequency range are used for excitation. By tuning the frequency *difference* in the exciting light field to match the frequency of a molecular vibrational resonance, this resonance is driven coherently, such that the resulting Raman scattering of each molecule is mutually coherent. This leads to coherent Raman scattering (CRS), in which the Raman scattering fields of all molecules constructively interfere, which results in a quadratic scaling of the scattered intensity with the number of molecules, as opposed to

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