Line-Shaped-Illumination Two-Photon Microscopy

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Line-shaped illumination is a useful configuration to obtain a multifocal pattern to be used in two-photon microscopy: the light beam emitted by the illuminating laser is first shaped by means of cylindrical lenses and then is focused inside the sample as a continuous line. The simultaneous excitation of several points in the specimen is expected to reduce the acquisition time with respect to the usual point-scanning two-photon microscopes, as a two-dimensional image is obtained by scanning the line along a single direction.

Keywords: two-photon microscopy ; two-photon absorption ; imaging technique ; nonlinear spectra ; dyes

1. Introduction

Nonlinear fluorescence microscopy is widely recognized as an important and consolidated technique to investigate biological samples. In most cases, this approach relies on two-photon excited fluorescence (TPEF), which exploits two-photon absorption (TPA) and consequent fluorescence emission to obtain images, especially of biological samples [1]. This method has several advantages, such as great penetration depth, high longitudinal resolution, suitability for three-dimensional image reconstruction, low phototoxicity, and low photobleaching effects [2][3][4][5]. However, a specific drawback of this technique is that generally, real-time imaging (at least 30 frames per second) is not available: the acquisition of a two-dimensional image usually requires several seconds, so that the possible reconstruction of a three-dimensional image from a stack of in-plane images may take minutes, a time range which is too long for several applications [1]. Moreover, quite expensive and tricky single-photon counters are often required as detectors, with the consequent need for complex driving electronics [6][7]. Although many approaches have been devised to solve this issue, up until now, no satisfying solutions have achieved the necessary reliability to be exploited in commercial instruments [6]. In principle, the increase of the acquisition rate can be obtained by exploiting a multifocal configuration, in which several focal points are generated in the sample at a time. Line-shaped illumination is the most straightforward configuration to obtain this multifocal pattern: the light beam emitted by the illuminating laser is first shaped by means of cylindrical lenses and is focused inside the sample with a continuous line. The simultaneous excitation of several points in the specimen is expected to reduce the acquisition time, as a two-dimensional image is obtained by scanning the line along a single direction. As a consequence, a N × N-pixel image would require a number of acquisitions reduced by a factor of N. The time for the reconstruction of a three-dimensional image would then be strongly decreased as well. Nevertheless, past experimental works showed that such an approach would strongly worsen the axial resolution with respect to point-scanning microscopes. Namely, Guild et al. pointed out that, contrarily to the TPEF in a point-scanning configuration, the total line-focused two-photon excited fluorescence (TPEF) increases logarithmically with the thickness of the sample, so that the additional spurious fluorescence reaches twice the signal level generated in the focal volume for a thickness of about 100 μm [8]. On the other hand, Brakenhoff et al. evaluated the worsening of the sectioning capability in comparison to point-focused TPEF as 5 μm with respect to 1 μm [4][9]. Although some solutions have been devised to overcome this drawback, which in principle could provide roughly the same resolution of a point-scanning system, none of them have spread out among applications yet [10]. Particularly, in [10], a 1.5 μm resolution has been obtained by means of temporal focusing.

In recent years, an innovative line-scanning TPEF microscopy configuration has been proposed [11]. Actually, theoretical calculations have shown that a line-shaped illumination is able to yield an axial resolution comparable to that obtained by a point-shaped illumination, provided that the optical elements are properly arranged [12]. Indeed, the proposed setup exploits a relatively simple optical configuration, only using standard spherical and cylindrical lenses, nonetheless obtaining an optimized performance in terms of resolution. Such approach for a *line-scanning two-photon microscope* (LSTPM) allows to obtain both the acquisition of images at video rate and the suppression of the resolution degradation, which is usually found in line-scanning systems. A specific feature of the proposed setup is the large flexibility in applications. Indeed, until now, it has been experimentally demonstrated to be a good tool for measurements of two-

photon absorption spectra and cross-sections [13][14], as well as for imaging of several kinds of biological samples and for their in-depth image reconstruction [14][15][16][17].

2. Overview of Applications

The LSTPM has been used for different purposes, demonstrating the reliability of the proposed experimental approach in different fields of research. As mentioned above, the technique has been tested for the characterization of dyes, namely two-photon spectral and cross-section measurements, and for imaging, namely investigation of biological samples and in-depth sample image reconstruction. Here, an overview of such works is provided.

2.1. Two-Photon Spectral Measurements

Several procedures have been devised for the measurement of TPA spectra. The technique exploiting the LSTPM setup belongs to the class of the so-called indirect methods [7][18]. The use of a line-shaped excitation light beam enables to obtain a high signal level, which is usually very difficult to obtain in two-photon fluorescence measurements (Sutherland2003).

2.2. Two-Photon Cross-Sections

The above-described technique exploiting LSTPM to measure nonlinear fluorescence spectra of dyes has been extended to obtain absolute measurements of the two-photon cross-section as a function of wavelength. In a pioneering work, two dyes which are commonly used for staining in standard white-light microscopy, eosin and hematoxylin, have been investigated [14]. First, their relative TPA spectra were measured following the procedure referenced in the previous section. Then, the absolute values of the cross-sections provided in GM units were evaluated by comparison to a dye with a known cross-section, following the procedure detailed in [14]. The TPA spectrum of Alexa 488 displayed and the corresponding numerical values of the cross-section, which are available [19], are assumed as the reference data. Particularly, the cross-section of eosin was estimated from the ratio of the values of the measured fluorescence intensity. The data were rescaled by the ratio of the quantum yields and concentrations of the two dyes, which were assumed to be equal for the one-photon and the two-photon processes [20]. On the other hand, as far as hematoxylin is considered, its specific quantum yield is not known, so the same procedure provides the values of the so-called action cross-section [21]. The final results provide the absolute TPA cross-sections of eosin and hematoxylin at several wavelengths and concentrations in water solution**.**

These data demonstrate the reliability of the LSTPM setup to suitably assess the TPA properties of a given sample. Particularly, the proposed technique is able to overcome the low signal-to-noise ratio of TPA measurements, which is often regarded as one of the main issues of such a kind of measurements [7]. This makes the proposed method very promising for the development of relatively easy and cheap setups for nonlinear absorption characterization.

2.3. Imaging of Biological Samples

As far as imaging is concerned, the performance of the microscope setup has been tested with several biological samples. As a first example, it was reported results about murine macrophage-like RAW 264.7 cells incubated with lipoprotein(a), whose related morphological changes are studied. As lipoprotein(a) is well-known to be a genetic risk factor for atherosclerotic diseases, but its pathogenic mechanism is not completely clear, it is very interesting to highlight its internalization by cells. For this investigation, a 60× objective with a 1.4 numerical aperture have been employed so that the field of view is 150 × 112 μm^2. The excitation wavelength was 830 nm and the average power impinging on the sample was 60 mW. The cells were stained with Nile red and they generated no fluorescence signal when they were not treated. On the other hand, a strong signal upon two-photon excitation could be easily detected following incubation.

2.4. In-Depth Sample Reconstruction

One of the key features of two-photon microscopy is the capability of imaging at deep positions inside highly scattering media, which is when investigating biological samples. In this regard, the performance of the LSTPM setup has been tested by the three-dimensional reconstruction of a 100 μm-thick sample of a nevus obtained by a stack of 70 two-dimensional images which have not been processed. For this investigation, a 60× objective with a 1.4 numerical aperture have been employed. The excitation wavelength was 800 nm and the average power impinging on the sample was 50 mW. The very good quality of such raw image even down to several tens of micrometers is clear proof not only of the penetration depth of the microscope setup, but also of its axial resolution, which turned out to be comparable to the resolution of point-scanning systems. The measured axial resolution was 1.2 μm for such experimental parameters. By

drawing a comparison with the results reported in [10], it was noted that the LSTPM yields a better resolution with no need for control of the temporal profile of the excitation pulse.

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