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Improved multiphoton imaging in biological samples by using variable pulse compression and wavefront assessment

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ABSTRACT

A variable prism-pair-based pulse compressor with wavefront aberration sensing was used to enhance multiphoton imaging in biomedical samples. This was incorporated into a custom-made microscope to reduce pulse temporal length, improve the quality of images of different layers of thick tissues and increase penetration depth. The laser beam aberrations were found to hardly change with the different experimental configurations of the pulse compressor. The optimum pulse compression state was maintained with depth within the tissue, independently of its thickness. This suggests that for each sample, a single experimental configuration is able to provide the best possible image at any depth location; although this needs to be experimentally obtained. Furthermore, a simple method based on laser average power reduction is presented to minimize the risk of photo-damage in biological samples. The use of pulse compression in multiphoton microscopy might have a potential for accurate and improved biomedical imaging.

1. Introduction

Over the last decades multiphoton (MP) microscopy has become a useful tool due to its inherent confocal properties, free-marker imaging conditions and minimized photo-damage effects [1]. Despite these optical sectioning capabilities, MP imaging at deeper layers within a sample is affected by aberrations and scattering that lead to blurred images with reduced contrast and resolution. Adaptive optics techniques have been used to improve MP imaging through the correction of the aberrations of the femtosecond (fs) laser beam, the microscope optics and the specimen itself [2–5]. However, these implementations only optimize the spatial properties of the beam to obtain a smaller focal spot.

Temporal properties of the illumination fs-laser are also of importance in MP imaging performance. Short pulses responsible for the generation of MP phenomena have a broad spectrum and they might suffer from chromatic dispersion due to the microscope optics and the sample itself. That is, the different frequency components travel at different speed and then the pulses are broadening in time. This limits MP efficiency and reduces imaging performance. To increase the effectiveness of MP processes, higher average laser power is needed. However, this increases the risk of non-controlled thermal side effects and photo-damage that should be avoided when imaging biological samples.

Pulse compression techniques are often used to restore "short laser pulses". Pulse compression devices use prisms [6], diffraction gratings [7] or chirped mirrors [8]. Most strategies are based on pre-compensation by measuring or estimating the temporal dispersion and apply the opposite so that the total dispersion is zero at the focal plane [9]. The implementation of pulse compression techniques into MP microscopy has been reported to increase imaging performance [10–13].

Müller and co-workers compared two-photon excitation fluorescence (TPEF) images of a fish retina using a double prism-pair dispersion pre-compensation unit [9]. They reported an increase in TPEF signal higher than 70% when comparing 170 and 340 fs laser pulses. A pulse compressor based on photonic crystal fibers was also combined with a MP microscope [13]. TPEF signal increased up to 7 times (in guinea pig intestine tissue and rat pulmonary artery cells) when using pulses of less than 35 fs (compared to 250 fs). A similar improvement (5.6× increase in TPEF) was reached with a grating-based pulse compressor when reducing the pulse duration from 190 to 38.7 fs [11]. With a two-prism pulse compressor used to reduce the pulse duration from 215 to 96 fs, second harmonic generation (SHG) signals from different collagen samples (liver, collagen gel, muscle) were improved 2×–3× [12]. Other authors reported up to 11-fold improvement in TPEF signal from cells and tissues, and up to 19-fold improvement in SHG images of a rat tendon specimen using sub-20 fs laser pulses [10].

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An alternative approach to control chromatic dispersion includes pulse shapers. These are based on spatial light modulators and have also been reported to increase TPEF and SHG generation efficiency [14–16]. More recently a spatial light modulator and a deformable mirror have been combined with a MP microscope [17,18].

However, this previous literature on MP image improvement through pulse compression dealt with single plane imaging and results on thick samples are scarce [19,20]. An increase in TPEF imaging penetration in a labeled section of guinea pig detrusor was reported when using a grating-based pre-dispersion compensator [19]. TPEF and SHG signals from a human skin sample recorded with two compression states (120 and sub-20 fs) were compared [20]. Shorter pulses provided higher signal at different depth locations.

In the past, the limitations on MP imaging imposed by temporal pulse properties and the presence of aberrations (mainly from the sample) have been analyzed as independent issues. The usual adaptive optics MP microscopes only dealt with changes in aberrations [2–5]. On the other hand, improvements obtained after pulse compression did not have into account the effects of changes in the aberration pattern when modifying the optics of the compression device [9–13,19,20].

In this paper, we further analyze the effects of pulse compression on MP microscopy of biomedical samples. Thick samples from ocular tissues providing both TPEF and SHG signal were used in the experiment. We report on the use of a variable pulse compressor and a wavefront sensor introduced in the illumination pathway of the MP microscope. The temporal duration of the pulses is sequentially varied during MP imaging to obtain the optimum conditions for image improvement. The influence of the sample's depth location on the choice of the optimum compression conditions, and the corresponding improvement with depth are analyzed in detail. Moreover, the possible changes in the laser beam aberration are controlled through the wavefront sensor. If these are negligible, the spatial stability of the beam would allow isolating the effects of the pulse duration. Finally, the benefit of using pulse compression in order to reduced photo-damage is also discussed.

2. Methods

2.1. Experimental setup

The experimental setup combines a custom-built MP microscope and a two-prism-based pulse compressor (Fig. 1). The MP microscope is composed of a fs-laser source (760 nm), a XY scanning unit and an inverted microscope [5]. The pulse compressor (FemtoControl, APE, Berlin, Germany) was included in the illumination pathway and it was used to pre-compensate for the pulse dispersion induced by the microscope optics and the different imaged specimens. The different pulse compression states (PCSs) generated by the device were controlled through the user interface and depended on the prism positions and the used wavelength (see calibration procedure below). The non-linear signal (TPEF and/or SHG) emitted by the samples under study was detected through the same objective (in-air, long-working distance, 20×, $NA = 0.5$). This type of objective has been reported to be suitable for imaging ex-vivo ocular tissues and more recently to acquire SHG images from living human eyes (both cornea and sclera) [21]. A photomultiplier tube and a photon-counting unit were used in the registration pathway, together with TPEF/SHG spectral filters.

Additionally, the stability of the laser beam in terms of aberrations was also measured to analyze possible changes induced by the different PCSs due to the displacements of the prisms. For this, a Hartmann–Shack (HS) sensor was also placed in the experimental system as indicated in Fig. 1. From each HS image the wavefront aberration (WA) was calculated and expressed in Zernike polynomials up to 4th order for a diameter of 5 mm (see [22,23] for further details).

2.2. Image acquisition and samples

After the pulse compressor device was calibrated (see Results below), the different PCSs were used to improve the quality of the acquired MP images. The effectiveness of the pre-compensation technique was tested at randomly chosen planes within different samples and also for entire thick samples. For the former, a regular XY MP image was acquired for each PCS. The total intensity of each image was calculated and the PCS corresponding to the maximum value was chosen as the optimum PCS. To analyze the effect of sample's depth on the optimum PCS, a stack of images (for different depth locations) for the entire set of PCSs was recorded. It is interesting to notice that this action requires high acquisition times.

As biological specimens (not all shown here), different non-stained ocular tissues were imaged: a human epiretinal membrane, a rat retina, a piece of bovine sclera (fixed in paraformaldehyde), ex-vivo non-stained porcine, rabbit and chicken corneas, and a histological section of a rabbit cornea embedded in paraffin wax. Each sample has a different thickness, ranging between ~40 μm (epiretinal membrane and histological section) and ~500 μm (porcine cornea). Moreover, the specimen in paraffin presents reduced transparency. For a better discrimination, TPEF and SHG images are displayed in green and blue (false colors), respectively.

The use of tissue samples from animals and human donors in this study was approved by the Universidad de Murcia ethics committee and all procedures were carried out in accordance with the approved guidelines.

2.3. Calibration of the pulse compressor and laser pulse duration measurement

The pulse compressor was calibrated for the wavelength used in this work. Once the wavelength was introduced in the control panel, the positions of the prisms (in internal units) for the four basic settings were automatically set by the device. These were named as PCS0, PCS4, PCS8 and PCS12. A decrease in the prism position is associated with a reduction in the amount of prism glass introduced in optical pathway of the laser beam. Then, the prism positions corresponding to the basic PCSs were linearly interpolated to get 12 different PCSs. The next step was to measure the actual temporal duration of the laser pulses. An autocorrelator (Mini, APE, Berlin, Germany) was used for this. The pulse duration was measured at two different locations in the experimental system (see Fig. 1): at the exit of the pulse compressor (point A) with the configuration PCS0 (used as a reference) and at the entrance of the microscope (point B) for all PCSs from PCS0 to PCS12. This operation permits to investigate the temporal broadening of the pulse due to the optics, as well as to estimate the prism configuration providing the minimum pulse duration at the microscope entrance.

According to the manufacturer, the pulses emitted by the Ti–Sapphire laser (Mira 900f, Coherent) should have a pulse duration of 200 fs or less. The autocorrelator placed at the exit of the pulse compressor (point A in Fig. 1) set to PCS0 (i.e. no compression in operation) measured pulses of 230 fs (red dot in Fig. 2). As expected, due to the travel of the light beam through the optics of the experimental setup, the laser pulses at the microscope entrance (point B in Fig. 1) were broadened in time up to 400 fs (blue dot at PCS0 in Fig. 2). The PCS was then sequentially changed from PCS0 to PCS12 and, as shown in Fig. 2, a minimum in the pulse duration of ~150 fs was found for PCS7.

Since the minimum pulse duration shown in Fig. 2 might not lead to the best image at every depth location within the sample, we decided to use the term PCS throughout this paper. This might be due not only to the possible depth-dependent properties of the sample, but also to the non-negligible effects of the microscope objective. Although it is known that the PCS term is not a physical parameter, it will be used to facilitate the graphical representation of the results (i.e. the seeking