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Starch-based backwards SHG for *in situ* MEFISTO pulse characterization in multiphoton microscopy

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Summary

We report a simple methodology to provide complete pulse characterization at the sample plane of a two-photon excited fluorescence (TPEF) microscope. This is achieved by using backward propagating second-harmonic generation (SHG) from starch granules. Without any modification to the microscope, SHG-autocorrelation traces were obtained by using a single starch granule that was placed alongside the biological specimen being imaged. A spectrally resolved SHG autocorrelation was acquired by placing a spectrometer at the output port of the microscope. Complete *in situ* pulse information is then directly retrieved in an analytical way using the measurement of electric filed by interferometric spectral trace observation (MEFISTO) technique.

Introduction

Multiphoton excited fluorescence microscopy (Denk *et al.*, 1990) has become an important tool for biomedical imaging. This success is owed to its ability to capture high-resolution, three-dimensional images while at the same time minimizing photobleaching and an overall reduced photo-damage to the specimen. To optimize the efficiency of the generated fluorescence it is now common to shape the pulse entering the microscope to produce transform-limited pulses at the sample plane of the microscope. This is normally achieved by adding negative dispersion to compensate for the positive dispersion introduced by the high numerical aperture (NA) objective lens (Guild *et al.*, 1997; Müller *et al.*, 1998). Recently, active pulse shaping has also been carried out to enhance resonant multiphoton transitions beyond the level obtained by maximizing the peak intensity of the pulse (Dudovich *et al.*, 2001). This has been achieved by selectively removing some spectral bands from the pulse. Other results have shown that phase-modulated femtosecond pulses can selectively excite one type of probe molecules whereas leaving the others in their ground state (Pastrick *et al.*, 2003; Ogilvie *et al.*, 2006). Phase-optimized pulses are also used to attenuate photobleaching without decreasing the fluorescence signal intensity (Kawano *et al.*, 2003). It has been shown that even small changes in the spectral wings can significantly affect the efficiency of non-linear processes. This has implications for biological multiphoton imaging where it may be desirable to minimize sample exposure to radiation and maximize fluorescence or harmonic efficiency (Bardeen *et al.*, 1999).

To gain a better insight of the physics involved in all these processes, these techniques require characterizing the pulse at the sample plane. A common practice is to characterize pulses before entering the microscope. For relatively long pulses (>200 fs) this will give a reasonable approximation of the pulse at the sample plane. However, as pulse durations decrease the pulse will become increasingly different to the pulse at the sample plane of the microscope, making the measurement effectively meaningless. For this reason there is a need to develop techniques that allow for a fast, easy and reliable way to characterize pulses exactly where they interact with the sample in a multiphoton microscope.

The required accuracy and complexity of the characterization technique depends primarily on the experiment being carried out. For example, changing the dispersion to obtain a near transform-limited pulse only requires an autocorrelation measurement (Tang *et al.*, 2006). The autocorrelation trace has, in the past, been achieved by measuring the TPEF signal from the specimen itself (Cannone

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et al., 2003). This technique has the advantage that it can be easily adapted into a TPEF microscope but the fluorescence is subject to photobleaching, making the measurement unreliable over a period of time. Other applications require more accurate knowledge of the pulse parameters. For example, in pulse shaping, information about the temporal intensity and phase profiles is essential. The pioneering research is based on the well-known Frequency Resolved Optical Gating (FROG) (Kane & Trebino, 1993; Trebino *et al.*, 1997) showed how to characterize pulses at the sample plane by means of type II phase- matching collinear geometry (Fittinghoff *et al.*, 1998). Multiphoton intrapulse-interference phase scan (MIIPS) (Lozovoy *et al.*, 2004; Xu *et al.*, 2006), based on the use of a Spatial Light Modulator (SLM) to phase modulate each frequency component of the pulse, is another technique that has been successfully demonstrated to measure and correct the phase of femtosecond pulses. These are examples of techniques that can be implemented in a two-photon excited fluorescence (TPEF) microscope. However, the accuracy of these techniques comes at the expense of complexity, requiring forward collecting optics in all of them. In addition to this, the choice of the non-linear medium is crucial because of the large acceptance angles introduced by the high-NA objective lens, the large bandwidth of the ultra short pulses and the polarization sensitivity of the non-linear medium. Starch has been shown to have a naturally high χ^2 coefficient that can be used over a broad spectral range (Chen *et al.*, 2002). Its properties have successfully been utilized to completely characterize the ultra short pulses at the focal plane of a high-NA objective lens using Collinear SHG-FROG (CFROG) technique (Amat-Roldán *et al.*, 2004a) in the forward direction.

In this paper, we report the significant SHG signal generated from starch granules in the backward direction and its use to obtain information of the ultrashort pulses interacting with the specimen inside a high-resolution (high NA) multiphoton microscope. Backward SHG provides an extremely simple way to measure the SHG-autocorrelation trace from an unmodified TPEF microscope. With the simple addition of a spectrometer to the output port of the microscope, it is possible to spectrally resolve the autocorrelation trace. This frequency-resolved SHG autocorrelation can then be processed to fully characterize the pulse. This can be done using several techniques such as CFROG (Amat-Roldán *et al.*, 2004b) or as Interferometric FROG (Stibenz & Steinmeyer, 2005). However, these need an iterative retrieval algorithm to obtain the pulse information, adding complexity to the methodology. We decided to use the MEFISTO technique (Amat-Roldán *et al.*, 2005; Amat-Roldán *et al.*, 2006), which allows for the direct extraction of the pulse in an analytical way.

The use of backward propagating SHG from starch granules is especially important in multiphoton microscopy because the same epi-detection scheme can be used to collect both the TPEF and SHG signals making starch ideally suited to work with in a TPEF microscope. Additionally, there are several significant practical advantages of using starch as a non-linear medium. Apart from being cheap, and non photobleaching, it is also non-soluble and non-toxic, so it can be added into the culture medium where the specimen is.

Experimental setup

In order to show that *in situ* pulse information can be obtained while imaging, two different situations were examined. In the first one, we added a very small quantity of starch granules into a dish of live HeLa cancer cells (GFP labelled). In the second, starch granules were placed alongside a more complex organism, *Caenorhabditis elegans* that was prepared for *in vivo* neuron imaging (GFP labelled on D-type neurons). The granules embedded in this way were approximately 5–10 μm in size. The non-toxic and non-soluble nature of starch allows it to be among living cells or the organisms without affecting them in any way. A schematic diagram of the optical arrangement used in this experiment is shown in Fig. 1.

Imaging was performed on an adapted inverted microscope (Nikon TE2000-U) with the optical scanning being performed by a pair of galvanometric mirrors (Cambridge Technology, 6215H). A X60 oil immersion objective lens (NA 1.4, Nikon Plain Apo-Achromatic) was used throughout the experiment. The ultrashort laser pulses were produced from a mode-locked Ti: Sapphire laser (MIRA 900 f), which was set at a central wavelength of 840 nm and a repetition rate of 76 MHz. The pulses were sent through a Michelson interferometer before entering the microscope. A telescope arrangement was used to ensure that the beam filled the entire aperture of the objective lens.

The generated TPEF signal from the cells and the backward SHG from the starch granules were collected in the backward direction via the same objective lens. The signals were then passed through the hot mirror, a BG39 filter and onto the photomultiplier tube (PMT, Hamamatsu H9305-04).

Results and discussion

SHG microscopy of starch granules

In order to compare the SHG signal from starch granules emitted in the forward and backward (epi-) directions we used an additional mount for forward direction as well. This mount was equipped with a collecting objective (1.25 NA), a band-pass filter centered at 420 nm and a photomultiplier tube. Figure 2 shows the combined forward and backward collected SHG images of the starch granules for different polarizations of the fundamental beam. The images correspond to a plane passing through the centre of the granule. In this plane, the signal in the backward direction originates only from the edges of the granule and does not overlap with the forward signal originate from inside the granules. Interestingly, both, the forward and backward SHG signals can be generated

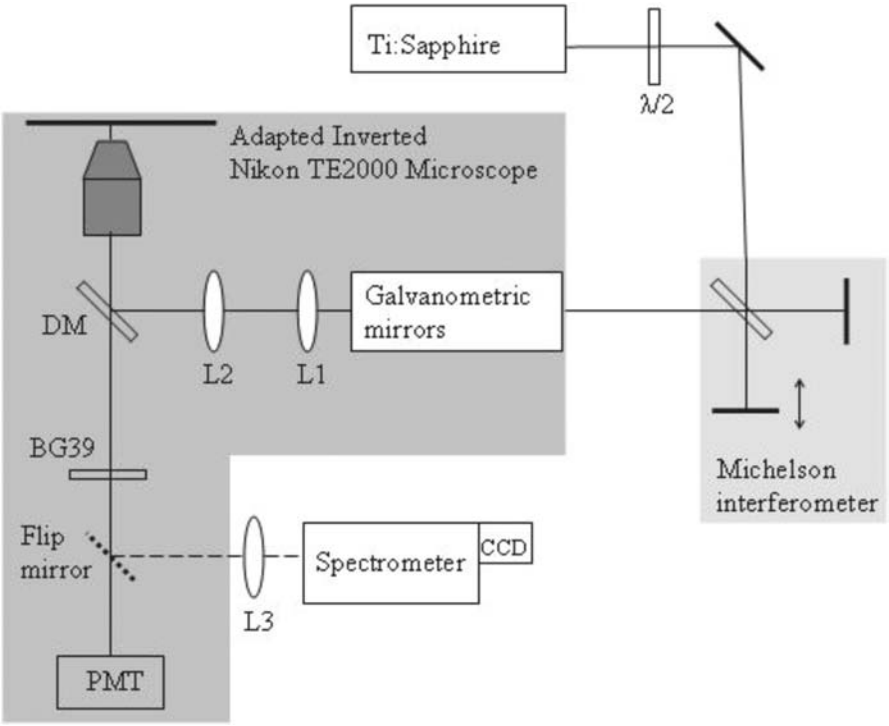

Fig. 1. A schematic diagram of the experimental setup

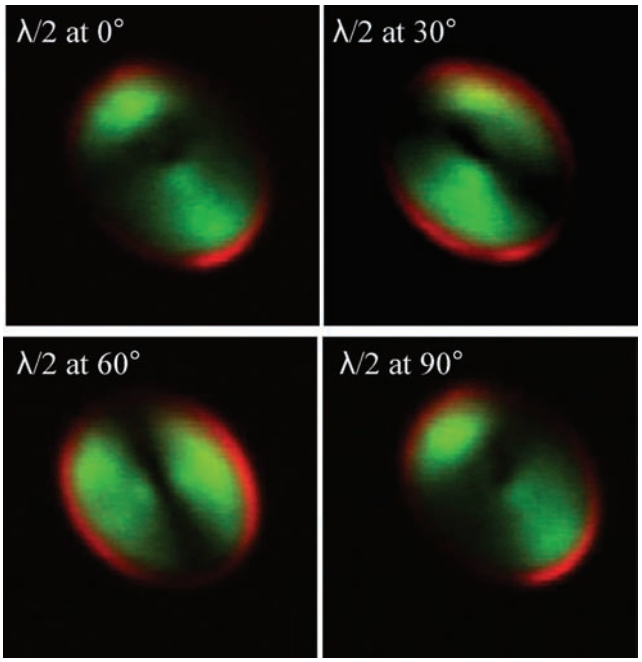

Fig. 2. SHG images of starch granules detected in the forward (green) and backward (red) directions for different polarizations.

in starch for any laser polarization. Compared to the use of crystals, which would require perfect alignment for satisfying phase-matching conditions, this polarization independence makes starch ideal for pulse characterization inside a microscope.

This observation of backward SHG signal from starch granules could be due to backward scattering, since starch is a highly scattering medium. In addition, the possibility of backward phase matching in starch (possessing semi-crystalline shells of approximately 10 nm of thick amylopectin side chain clusters (Gallant *et al.*, 1997)) cannot be neglected as it has been shown that nearly equal forward and backward phase matching occurs when objects have an axial size less than $\lambda_{SHG}/10$ (approximately 40 nm) (Moreaux *et al.*, 2000; Mertz & Moreaux, 2001; Williams *et al.*, 2005). However, these fail to explain the lack of a signal generated in the forward direction. The exact nature of the backward SHG signal from starch granules is not within the scope of this contribution and needs further investigation (see for example, Psilodimitrakopoulos *et al.*, 2007).

In situ pulse characterization using backward SHG from starch

Having a better understanding of the capabilities of starch to generate SHG signal in the backward direction, we proceeded to perform *in situ* pulse characterization and imaging. Removing the forward collecting mount (experimental arrangement as depicted in Fig. 1) and blocking one of the interferometric arms, it was possible to simultaneously acquire both the backward SHG and the TPEF to obtain image of both the starch and the cells. Cancer cells are highly prone to contaminations but we found that the addition of starch granules do not affect them in anyway, strongly supporting the use of starch for this kind

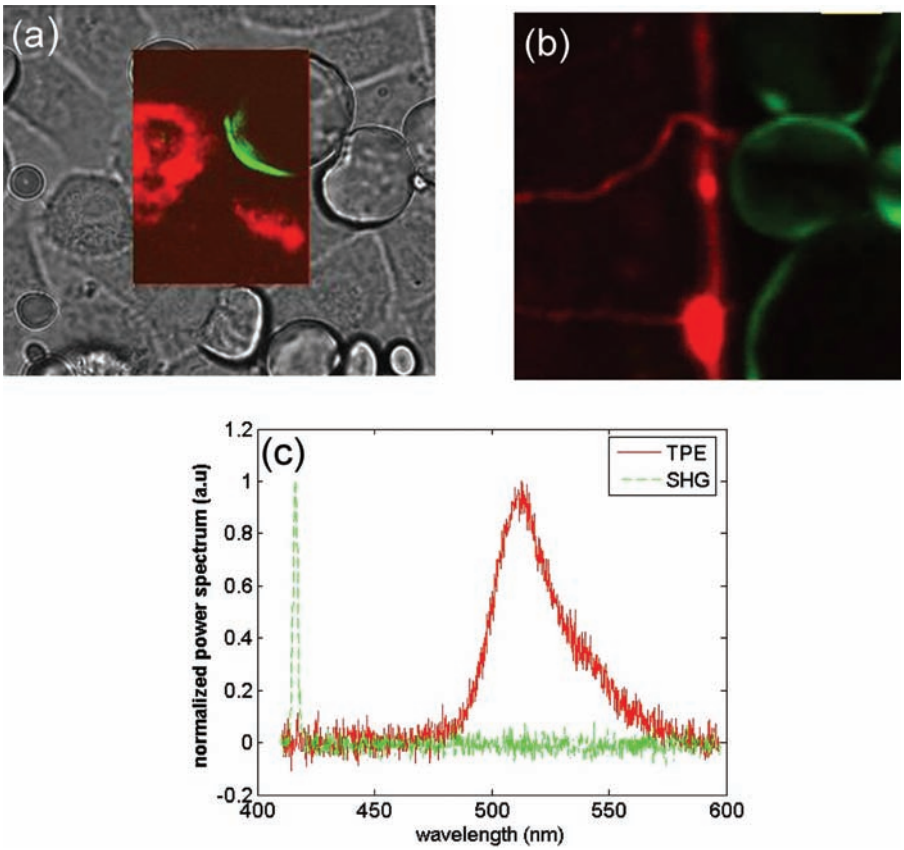

Fig. 3. (A) Image of the HeLa cells together with the starch granules. The inset shows the TPEF signals from cancer cells (red) and the backward-directed SHG signals (green) from a starch granule. (B) The epi-detected TPEF image of the *C. elegans* neurons (red) and SHG image of the starch granules (green). To show the contour of the granules, images taken with different polarizations had been added. (C) The spectra of the observed signals; TPEF (red) and SHG (green).

of applications. Figure 3A shows a photograph of the cell and starch mixture, which has been overlaid with the summed SHG and TPEF image. Similarly, we simultaneously epi-detected the TPEF signal from the neurons of *C. elegans* and SHG signal from starch granules (Fig. 3B). Here again, presence of starch granules in the culture medium of the *C. elegans* do not affect the development of the organism. To check that we were indeed observing the combined signals we replaced the PMT with a spectrometer (Jobin Yvon, Triax 180) and a back-thinned charge coupled device (CCD) linear array (Hamamatsu, HC 230–1007) to measure the different spectra that were being generated. We confirmed that the SHG signal (at 420 nm) was only generated when the laser was focused upon a granule of starch, and the TPEF signal from the cancer cells was observed with a central wavelength of 510 nm (Fig. 3B). Importantly, in both cases, the two signals were well separated from one another.

To characterize the pulses, we focused the beam onto one of the starch granules that lies on top of the living cells or besides the *C. elegans*. SHG autocorrelation can be directly measured by rapidly scanning one of the interferometric arms while recording the output voltage of the PMT. To fully characterize the pulse, we used a spectrometer at the output port of the microscope to obtain the interferometric spectrally resolved autocorrelation. This trace is then treated in order to be able to use the MEFISTO technique to retrieve the pulses (Amat-Roldán *et al.*, 2005). To support the effectiveness of the MEFISTO technique we compared the results with the more conventional CFROG technique. The same experimental data are used in both cases. Figure 4 shows the numerical interferometric autocorrelations obtained from MEFISTO and the CFROG techniques and are compared with the experimentally acquired one. In both cases there is an excellent overlap showing the validity of both retrievals.

The retrieved temporal intensity, spectral intensity and phase profiles of the pulse at the sample plane of the microscope are displayed in Fig. 5. The results obtained using both characterization techniques are in a good agreement except for the spectral intensity and phase profiles. The retrieved spectral intensity profiles are validated by comparing them with the experimentally measured data (Fig. 5B). The spectral intensity profile obtained by using MEFISTO technique

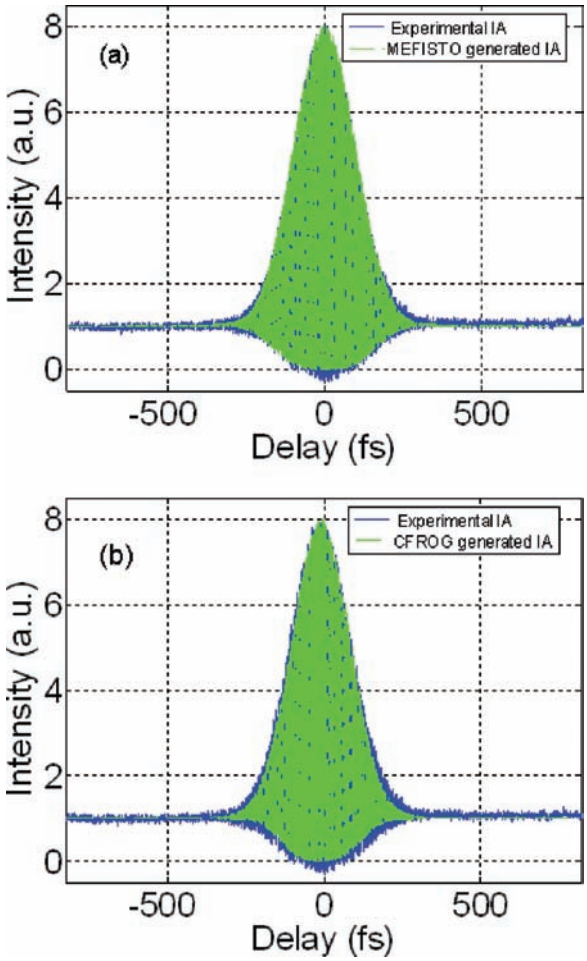

Fig. 4. Interferrometric autocorrelations. (A) from MEFISTO and (B) from CFROG.

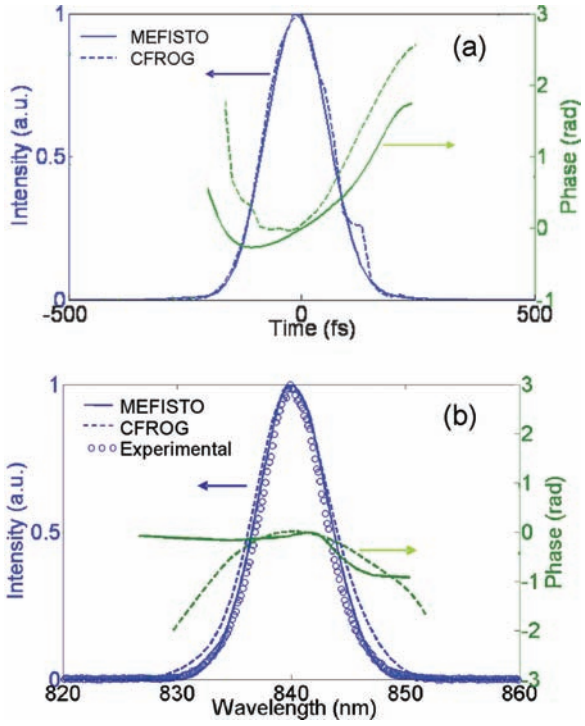

Fig. 5. Retrieved phase and intensity profiles of the pulse at the sample plane of the microscope obtained with MEFISTO (solid line) and standard CFROG procedure (dashed line): (A) temporal intensity and phase. The temporal width (FWHM) of the pulse at the sample plane is 163 fs. (B) The measured spectrum (circles) and the retrieved spectral intensity and phase profiles.

shows a better agreement with the measured spectrum. The small disagreement of the spectrum obtained with CFROG is attributed to the averaging effect introduced by the iterative algorithm.

Conclusions

In conclusion, we report an ideal approach for pulse characterization within a TPEF microscope using the backward SHG signals from starch granules. Both the fluorescence and SHG signals can be collected using the same epi-detection scheme, helping to considerably simplify the experimental arrangement. Furthermore, the structural nature of starch allows efficient phase matching of the input beam at any incident polarization. The addition of starch to the sample of study, being a non-toxic and non-soluble material, does not affect living cells or organisms allowing the pulse characteristics to be measured *in situ*, without the need to move the sample. We have demonstrated that with no alteration to a standard TPEF microscope a complete characterization of the pulse can be made. We highlight the use of MEFISTO technique as a simple (non iterative) method specially suited for characterizing pulses in a non-linear microscope. The proven characteristics of starch will allow the technique to characterize pulses with far larger bandwidths than described here, even with the large acceptance angles that a high-NA objective lens possesses.

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