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Abstract: Coherent control can be used to selectively enhance or cancel concurrent multiphoton processes, and has been suggested as a means to achieve nonlinear microscopy of multiple signals. Here we report multiplexed two-photon imaging *in vivo* with fast pixel rates and micrometer resolution. We control broadband laser pulses with a shaping scheme combining diffraction on an optically-addressed spatial light modulator and a scanning mirror allowing to switch between programmable shapes at kiloHertz rates. Using coherent control of the two-photon excited fluorescence, it was possible to perform selective microscopy of GFP and endogenous fluorescence in developing *Drosophila* embryos. This study establishes that broadband pulse shaping is a viable means for achieving multiplexed nonlinear imaging of biological tissues.

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1. Introduction

Coherent control is attracting considerable interest as a way to enhance or cancel specific photoinduced multiphoton processes through quantum interference [1, 2, 3, 4]. Among the variety of possible application fields, increasing attention is being devoted to nonlinear microscopy [5, 6]. Indeed, pulse shaping makes selective excitation of specific species possible despite the

broad spectral bandwidth of the exciting pulse, thanks to the suppression of undesirable pathways through destructive interference. For example, Coherent Anti-Stokes Raman Scattering (CARS) microscopy performed with shaped broadband pulses has been shown to enhance the resonant CARS emission of a specific vibration mode with respect to the nonresonant background [7]. Similarly, based on the seminal experiment on coherent control of two-photon absorption [8], two-photon excited fluorescence (2PEF) selective microscopy with shaped broadband pulses has been reported for molecules in solution [9], for thick scattering tissues [10, 11], and more recently for live embryo imaging [12]. For biological imaging, coherent control shows indeed great potential for selective imaging of a collection of fluorophores, such as fluorescent protein-based constructs (GFP, YFP) or endogenous species (such as NADH) using a shaped broadband laser. As compared to using a tunable narrow-band femtosecond laser, the shaping approach has a unique potential for frequency agility, i.e. the possibility to rapidly switch the region of the 2PEF excitation spectrum that is effectively being addressed by the shaped pulse. The ability to simultaneously address several nonlinear signals under standard microscopy conditions of frame rate and resolution would be of considerable interest, since it would open the way to ratiometric or spectroscopic imaging of multiple signals in intact tissues. However the design of an experimental scheme combining efficient multiphoton imaging, fast switching and micrometer resolution remained challenging so far.

Earlier experiments on selective microscopy with shaped pulses [9, 11, 12] often used objective lenses with moderate NAs due to the difficulty of controlling the spectral phase of sub-15 fs pulses at the focus of a high NA lens. Furthermore, they either did not implement the frequency agility feature due to a slow update rate of the liquid-crystal-based pulse shapers [9, 11], or suffered from low overall efficiency resulting in long acquisition times [12]. In particular, this latter experiment used an acousto-optic programmable dispersive filter [13, 14] with high switch rate (10 kHz) so that the acquisition of two images could be performed quasi simultaneously; however, due to a limitation inherent to the acousto-optic technology in the case of broadband pulses, only a small fraction of the 80 MHz pulse train could be used for generating the signal. This low efficiency resulted in acquisition times greater than 5 min per image, so that only static samples could be observed[12].

In this article, we report on a multiplexed selective microscopy experiment where dynamic embryo tissue is imaged with frame rates and resolution similar to that of a standard two-photon microscope. Our approach relies on a recently proposed switching scheme based on scanning of the optical beam accross the vertical dimension of a programmable two-dimensional spatial light modulator (2D-SLM) [15], and on diffraction-based pulse shaping [16] using an optically addressed SLM. We additionally used phase measurements techniques for a precise control of the spectral phase at the focus of a 0.8-NA water immersion objective, providing high resolution imaging during rapid development stages of *Drosophila* embryos.

2. Pulse shaping with a fast switching rate

Our pulse shaping apparatus is inspired from two recent advances in pulse shaping technologies. The first one, reported by Frumker *et al.*, consists of a scanning-mirror based femtosecond pulse shaping technique able to switch at kHz rates between pulse shapes imprinted at different vertical locations on a static [17] or programmable [15] phase mask. The second advance, reported by Vaughan *et al.*, consists of diffraction based pulse shaping using an optically addressable 2D SLM placed in the Fourier plane of a folded zero dispersion line [16]. In this case, a sawtooth phase grating is written on the 2D SLM so that the incident beam is diffracted in the vertical direction. The spectral amplitude and phase are respectively controlled by the amplitude and phase of the sawtooth grating. This new scheme has several key advantages, including the ability to directly introduce large phase shifts [16] and the absence of pixelation due to

the optical addressing making the filtering of high spatial frequencies straightforward [18, 16]. This allows to limit the artefacts associated with pixelated devices [19], which are of particular concern in the case of microscopy [20].

Note, however, that the two shaping schemes discussed above - switching with a scanning mirror [17, 15] and diffraction-based shaping [16] - are not directly compatible. Indeed, the first scheme requires imaging with spherical optics, which is essential for descanning, whereas the second scheme requires cylindrical optics so that the light beam is vertically spread on at least several grooves of the sawtooth grating. Nonetheless, we succeeded in combining these two approaches (Fig. 1) by using cylindrical optics and by programming two inverted sawtooth gratings which diffracted the beams back in their original directions, for the two angular positions of the galvanometer-mounted mirror (G). An important advantage of using cylindrical optics is that the power density on the 2D SLM remains modest. The use of spherical optics would result in a power density one or two orders of magnitude greater than the maximum value specified for our device (2 W/cm^2) which would cause erasing of the phase profile from the readout beam.
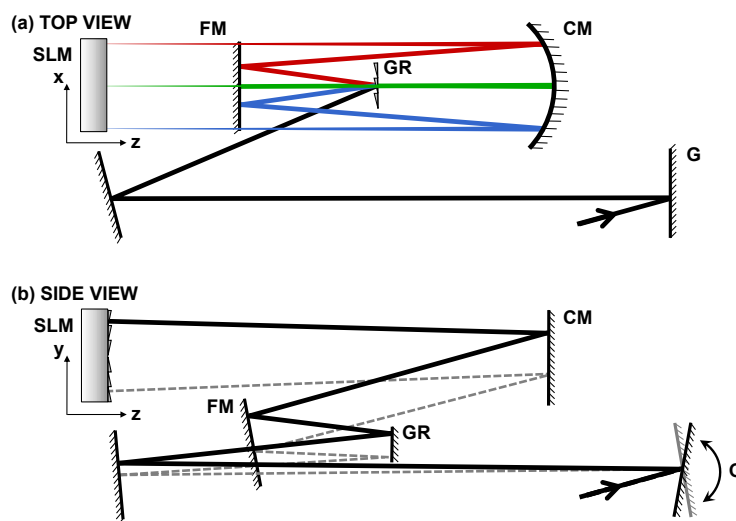

Fig. 1. Top (a) and side (b) views of the pulse shaper. A spatial light modulator (SLM) is placed in the Fourier plane of a grating (GR)-cylindrical mirror (CM) combination in a folded 4-f configuration. The 2D phase mask is used in the x-dimension as a phase shaper and in the y-dimension as a ruled grating used in Littrow configuration. A galvanometer mounted mirror (G) switches the light beam between the two areas of the SLM with two different phase shapes.

Figure 1(a) illustrates the top view of the pulse shaper, corresponding to the folded geometry discussed e.g. by Monmayrant and Chatel [21]. As in a conventional pulse shaper, the spectral components are angularly dispersed by a diffraction grating and a silver-coated cylindrical mirror then focuses each frequency in the Fourier plane where the phase is controlled with the SLM. The 2D SLM is an optically addressed phase modulator with 768×768 pixels permitting phase shifts up to 2.5π at 800 nm.

The side view (Fig 1(b)) shows the vertical alignment (exaggerated for clarity). The beam propagation is entirely determined by specular reflections on optics which are flat in this dimension, except on the phase mask where the beam is diffracted on a sawtooth grating imprinted on the 2D SLM. As all experiments reported in this article rely on phase-only shaping, the diffrac-