Multiplexed two-photon microscopy of dynamic biological samples with shaped broadband pulses

Rajesh S. Pillai,1 Caroline Boudoux,1,2 Guillaume Labroille,1 Nicolas Olivier,1 Israel Veilleux,1 Emmanuel Farge,2 Manuel Joffre,1 and Emmanuel Beaurepaire1

1Laboratoire d’Optique et Biosciences, Ecole Polytechnique, CNRS, and INSERM U696, 91128 Palaiseau, France, 2Ecole Polytechnique, Montreal, Canada, 3Institut Curie, CNRS, 75005 Paris, France

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.

© 2009 Optical Society of America

OCIS codes: (180.4315) Nonlinear microscopy; (320.5540) Pulse shaping; (230.6120) Spatial light modulators; (170.3880) Medical and biomedical imaging; (180.6900) Three-dimensional microscopy.

References and links
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 across 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/cm²) which would cause erasing of the phase profile from the readout beam.

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 diffraction...
Fig. 2. 2D masks as viewed in the beam propagation direction. In panel (a), a mask with uniform grating length is shown and the resulting angular chirp is shown in (c). The angular chirp was corrected by using a grating length directly proportional to the wavelength as shown in (b). Panel (d) schematically illustrates that the angular chirp is removed due to the scaled grating.

tion efficiency was set to its maximum by using a linear variation of the phase from 0 to $2\pi$ for each period of the grating. The sawtooth grating is used in a quasi Littrow configuration, so that the beam is sent back towards its original direction. After the second reflection on the scanning mirror, coupling out of the pulse shaper is performed with a slight vertical misalignment. The first of the two pulse shapes is obtained from the upper part of the 2D SLM (solid line), while for the second pulse shape the galvanometer (G) is rotated so that the beam is directed towards the lower part of the 2D SLM (dotted line), where a different phase profile can be programmed.

If the sawtooth grating were unchanged, the beam would be sent back with a different angle and thus could not be used for imaging. Our switchable pulse shaper therefore uses an inverted sawtooth grating on the lower part of the 2D SLM, so that the beam is also diffracted back in a way that the beams associated with the two pulse shapes are perfectly collinear after descanning through the second reflection on the scanning mirror.

Finally, we note that, unlike previous work associated with longer pulses [16], it is important here to use a sawtooth grating whose period is directly proportional to wavelength in order to have a constant diffraction angle. This issue is illustrated in Fig. 2(a) and (c). In Fig. 2(a), the 2D mask is shown with a uniform grating length. The resulting angular chirp is illustrated in Fig. 2(c), where the incoming spectrum will be angularly chirped after diffraction from the mask due to the same grating length experienced by different wavelengths. A 2D mask with variable grating length (proportional to the wavelength) as shown in Fig. 2(b) was used to correct for the angular chirp (Fig. 2(d)). The straightforward correction applied here is possible thanks to the non-pixelated control of the 2D SLM [16], so that the sawtooth grating period need not be an integer number of pixels.

3. Coherent control of two-photon excited fluorescence

In the absence of resonant intermediate level, which is typically the case for multiphoton microscopy, the fluorophores are effectively driven by the square of the incident field [22]. This
effective field, hereafter called the two-photon field, reads $\mathcal{E}^{(2)}(t) = \mathcal{E}(t)^2$ where $\mathcal{E}(t)$ is the complex field associated with the incident pulse. The 2PEF signal, $S$, can then be written as

$$S = \int g^{(2)}(\omega)|\mathcal{E}^{(2)}(\omega)|^2 \frac{d\omega}{2\pi}$$

(1)

where $g^{(2)}(\omega)$ is the 2PEF excitation spectrum and $\mathcal{E}^{(2)}(\omega)$ is related with $\mathcal{E}^{(2)}(t)$ through a Fourier transform

$$\mathcal{E}^{(2)}(\omega) = \int \mathcal{E}(t)^2 e^{i\omega t} dt$$

(2)

Since the Fourier transform of a product is a convolution product, we obtain

$$\mathcal{E}^{(2)}(\omega) = \int \mathcal{E}(\omega')\mathcal{E}(\omega - \omega') \frac{d\omega'}{2\pi}$$

(3)

or

$$\mathcal{E}^{(2)}(\omega) = \int |\mathcal{E}(\omega')\mathcal{E}(\omega - \omega')| \exp(i\Phi_{\omega}(\omega')) \frac{d\omega'}{2\pi}$$

(4)

where $\Phi_{\omega}(\omega') = \phi(\omega') + \phi(\omega - \omega')$ is the total phase associated with the pathway $\omega' + (\omega - \omega') \rightarrow \omega$. The final spectral amplitude in the two-photon field thus results from an interference between all possible pathways[8], a process known as multiphoton intrapulse interference (MII) [23]. Note that the two-photon spectrum, $|\mathcal{E}^{(2)}(\omega)|^2$, can be experimentally measured from the second-harmonic spectrum, to which it is simply related by a $\omega^2$ factor. Another method is to measure the second-order interferometric autocorrelation using two-photon absorption in a photodiode. The Fourier transform of the second-order interferometric autocorrelation then directly yields the two-photon spectrum at a frequency centered at twice the carrier frequency [25, 26]. Note that it follows immediately from eq. 3 that

$$|\mathcal{E}^{(2)}(\omega)| \leq \int |\mathcal{E}(\omega')\mathcal{E}(\omega - \omega')| \frac{d\omega'}{2\pi} = |\mathcal{E}^{(2)}(\omega)|$$

(5)

so that the spectral amplitude of the two-photon field at a given frequency is always maximized by that obtained for a transform-limited pulse ($\phi(\omega) = 0$). A remarkable property is that, if the spectral phase is antisymmetric with respect to a given frequency $\omega_1$, i.e. $\phi(\omega_1 + \Omega) = -\phi(\omega_1 - \Omega)$, then

$$\Phi_{2\omega_1}(\omega') = \phi(\omega') + \phi(2\omega_1 - \omega') = 0$$

(6)

and $|\mathcal{E}^{(2)}(2\omega_1)| = |\mathcal{E}^{(2)}(2\omega_1)|$, so that the maximum value is reached for $\omega = 2\omega_1$ [8]. In contrast, for frequencies other than $2\omega_1$, destructive interferences can result in a decrease of the spectral amplitude of the two-photon field. An appropriate antisymmetric phase can result in a two-photon spectrum which is a narrow peak around $2\omega_1$, so that selective excitation can be achieved through the frequency dependence of the 2PEF excitation spectrum, $g^{(2)}(\omega)$. Different fluorophores can then be simply addressed by changing the value of the center of antisymmetry, $\omega_1$.

Various spectral phase masks have been proposed or demonstrated, including sinusoids[8, 24, 27], quasi-random binary phase masks[28] or pseudorandom Galois fields[29]. In this work, we used a third-order phase

$$\phi(\omega') = \frac{1}{6}\phi'''(\omega_1)(\omega' - \omega_1)^3$$

(7)

which is indeed antisymmetric with respect to $\omega_1$ and will thus result in a peak of the two-photon field at frequency $2\omega_1$. Note that a third-order phase corresponds to a parabolic group delay, suggesting a time-domain interpretation of the coherent interference effect: at each time,
two frequency components symmetric with respect to $\omega_1$ can mix to produce a coherent excitation at frequency $2\omega_1$.

An appropriate choice of $\varphi''''$ will allow to control the bandwidth of the two-photon spectrum, while the choice of $\omega_1$ will allow to tune the position of the peak. In this work we used $\varphi''''(\omega_1) \sim 15000$ fs$^3$. For preferentially exciting the endogenous fluorophores and eGFP, $\omega_1$ was chosen to correspond to $\lambda = 780$ nm and $\lambda = 840$ nm respectively. Herein, these two pulse shapes will be referred to as “blue shifted” and “red shifted” respectively.

4. Results and discussion

4.1. Control of the spectral phase at focus

While propagating through the highly dispersive objective lens and other microscope optics (Fig. 3), the 10 fs transform limited pulses from the laser acquire a non-zero spectral phase, typically dominated by the second and third order phases [30]. Since the two-photon signal is given by the overlap integral of the two-photon spectrum of the laser and the two-photon absorption spectrum of the fluorophore (see above), the spectral phase has to be properly monitored and controlled at the focus of the microscope objective, where the two-photon fluorescence is excited. Characterizing and thereby controlling the spectral phase over the large bandwidth of the laser at the focus of the objective is critical for the selective microscopy experiment. A number of pulse characterization techniques utilising the pulse shaper [31, 32] can be used to characterize the spectral phase at the focus of an objective lens.

A partial precompensation of the dispersion due to the microscope was performed by translating the folding mirror of the pulse shaper slightly off the zero dispersion point. The residual spatial chirp originating from this arrangement was found to be acceptable, as the spectral measurements at the focus of the microscope objective indicated that all the spectral components were reaching the focus (see Fig. 4(c)). The residual spectral phase at the focus of the microscope objective was measured using a chirp scan method [31, 33]. The trace obtained from the chirp scan measurement for a 0.8NA 40× water immersion objective (back pupil overfilled) is shown in Fig. 4(a). The spectral phase extracted from the chirp scan measurement was compensated by the pulse shaper to obtain near transform-limited pulses at the focus of the...
Fig. 4. (a) Trace obtained from a chirp scan measurement for a 0.8NA 40× water immersion objective in the case of a residual third-order phase. (b) Second order interferometric autocorrelation trace (line) and the intensity autocorrelation trace (circles) corresponding to a near transform limited pulse (∼14 fs) at the focus of the microscope objective. (c) Laser spectrum (L.S.) at the focus of the microscope objective and Spectral phases (R.S.-red shifted and B.S-blue shifted) for the two different pulse shapes used in the imaging experiments. The "red shifted" spectral phase is anti-symmetric with respect to frequency corresponding to λ=840 nm and the "blue shifted" spectral phase is anti-symmetric with respect to frequency corresponding to λ=780 nm. (d) Two-photon spectra measured at the focus of the microscope objective for a near transform limited pulse (TL), "blue shifted" spectral phase and "red shifted" spectral phase in (c).

Objective. The pulse duration at focus measured using second order interferometric autocorrelation measurements was (assuming gaussian shape) ∼14 fs (Fig. 4(b)). We point out that the measured pulse duration at the focus of the pupil-filled objective indicates that the propagation time difference between the center and edge of this lens was only of a few fs.

The two-photon spectrum corresponding to the transform limited pulse is shown in Fig. 4d (black curve). The blue shifted and the red shifted two-photon spectra are shown in Fig. 4(d) (blue and red curves respectively). The laser spectra at the focus of the microscope objective and the spectral phases applied by the pulse shaper are shown in Fig. 4(c). The laser spectra were measured at the focus of the objective using linear autocorrelation measurements. Figure 4 demonstrates that by using the 2D-SLM, autocorrelation measurements and Fourier transform spectroscopy, it was possible to completely characterize and control the spectral phase and the electric field at the focus of the microscope objective.

4.2. Superposition of shaped beams

For the quasi-simultaneous imaging of different fluorophores to work effectively, it is equally important to ensure that beams diffracted from the two different positions of the 2D SLM overlap properly at the focus of the microscope objective. This spatial overlap was achieved by adjusting the driving voltage of the switching galvanometer (G). In addition, the spatial phase grating on the 2D SLM allows fine tuning of the direction of propagation of the diffracted beam simply by changing the period of the sawtooth grating, which, as previously mentioned, can

#108786 - $15.00 USD Received 17 Mar 2009; revised 4 May 2009; accepted 6 May 2009; published 13 Jul 2009
(C) 2009 OSA 20 July 2009 / Vol. 17, No. 15 / OPTICS EXPRESS 12748
Fig. 5. (a and b) Images of 100 nm beads located (a) at the center and (b) at the edge of the field of view respectively. The scale bars correspond to 1 μm. Each image is obtained by merging the two images acquired by the red-shifted (shown in green) and the blue-shifted (shown in blue) pulses. (c), (d): radial intensity point spread functions measured from the bead images shown in (a) and (b). FWHM is 0.49 μm. (e), (f): axial intensity point spread functions measured from the same beads. FWHM is 2 μm.

4.3. Measurement of spatial resolution

Having co-aligned the shaped beams using 10 μm beads, we recorded 3D images of ~100 nm fluorescent beads located at the center and at the edge of the field of view. Fig.5 displays the measured axial and lateral point spread functions for the beams diffracted from the two different positions on the 2D SLM. These data show that our pulse shaping scheme is compatible with microscopic imaging with an axial resolution of 2 μm and a lateral resolution of 0.49 μm.

4.4. Multiplexed imaging with two pulse shapes

The experimental setup for coherent-control imaging is shown in Fig. 3. The pulse train was sent through the switchable pulse shaper described in the previous section, and then injected into the scanning microscope (described in the Methods section). With the overall efficiency of 25% from the pulse shaper, an average laser power of ~15 mW was available at the focus of the overfilled microscope objective for the imaging experiments. The microscope dispersion was compensated as described above. The two different phase shapes required for the selective excitation of different fluorophores (eGFP and blue endogenous fluorescence) were displayed with sawtooth gratings with opposite grating angles on the top and bottom halves of the 2D-SLM. We acquired simultaneous image pairs with different pulse shapes by scanning each line twice and switching the position of galvanometer G between lines. The waveforms used for operating
Fig. 6. Multiplexed in vivo imaging of an eGFP-expressing Drosophila embryo using (a) blue-shifted pulse shaping for preferential excitation of the endogenous fluorescence at 780 nm and (b) red-shifted pulse shaping for preferential eGFP excitation at 840 nm. Dorsal side is up. (c), (d): linear combinations of images (a) and (b) for separating the two fluorescent components (spectral unmixing). (e), (f): images obtained by combining (c) and (d) at two different stages of embryo development. Tissue extension is visible at the embryo posterior pole during germ band extension (white arrow). (g), (h): Kymographs (space-time projections) along the dotted red lines indicated in (c) and (d). These projections reveal the correlated motion of cells (h) and underlying yolk structures (g) during extension movements. (i) and (Media 1) 3D+t movie of the posterior region of another embryo imaged from the dorsal side, during germ band extension. One 3D image was recorded every minute. Scale bars: 50 μm (X) and 5 min (time).

the three galvanometers (X, Y, and G) are depicted in the inset of Fig 3. Images corresponding to the two pulse shapes were then obtained by de-interlacing the data. This interlaced acquisition scheme provided simultaneous imaging capability at the millisecond scale with pixel-to-pixel spatial reproducibility between the two images. In turn, mathematical operations such as linear combinations between the images were possible.

4.5. Selective fluorescence microscopy of a developing embryo with shaped pulses

Finally, we demonstrated the ability of our system to provide multiplexed images of a rapidly evolving tissue with micrometer resolution by imaging live Drosophila embryos during gastrulation. We imaged a transgenic strain with eGFP-tagged cell nuclei [34]. These embryos exhibit a compartmented distribution of fluorophores which make them appropriate for calibration purpose. At the onset of gastrulation, cells form a single layer distributed at the periphery of the 500 μm × 200 μm ovoid embryo. The center of the embryo is filled with the yolk consisting of a high concentration of storage vesicles, some of which exhibiting strong blue endogenous flu-
orescence. Gastrulation then reorganizes the tissue (blastoderm) into a three-layered structure [35] through a complex ensemble of cell movements.

We recorded time-lapse 2D and 3D images of the posterior pole of the embryo during germ-band extension, a rapid process involving a large-scale spatial reorganization of the tissue and yolk in the posterior/dorsal regions of the embryos. Figures 6(a) and (b) show images recorded simultaneously with two different pulse shapes demonstrating the simultaneous preferential excitation of eGFP or yolk fluorescence. The image in Fig. 6(a) was acquired with an antisymmetric phase shape centered at 780 nm (shown in Fig. 4(b)), resulting in enhanced excitation of the yolk (blue) fluorescence. The image in Fig. 6(b) was simultaneously acquired using an antisymmetric phase shape centered at 840 nm (shown in Fig. 4(b)) resulting in enhanced excitation of eGFP localized in cell nuclei. For this particular experiment, images of 580 lines of 620 pixels (not including X-scanner flyback between lines) were acquired at a pixel rate of 75 kHz, and one image was recorded every 7 s during 40 min. The time per line of 12 ms (limited by the chosen number of pixels) defines the temporal resolution of the multiplexed excitation. We note that the acquisition time of approximately 6 seconds per image pair is an improvement of more than two orders-of-magnitude compared to the switching scheme based on the accousto-optic pulse shaper[12], and that this pixel rate is similar to that typically used in standard two-photon microscopy. Figures 6(c) and (d) present linear combinations of images (a) and (b) separating the two fluorescent components (spectral unmixing) based on their relative absorption spectra. These images show that an excellent overlap of the two beams was achieved throughout the field of view, and illustrate the selectivity achieved through spectral phase shaping. Figures 6(e) and (f) present images obtained by combining (c) and (d) at two different stages of embryo development. Kymographs (space-time projections) reveal the correlated motion of cells (h) and underlying yolk structures (g) during extension movements. Finally, Fig. 6(i) and (Media 1) present a 3D reconstruction of the posterior pole of another embryo imaged from the dorsal side. The acquisition time was 1 min per 2-channels XYZ stack. Overall these data illustrate that our system provides interlaced multiphoton imaging with two arbitrary pulse shapes, standard frame rates, and micrometer resolution in intact tissue.

5. Conclusion

The results reported here represent an important step towards the application of broadband pulse shaping methods to nonlinear microscopy of multiple signals. The quasi-simultaneous imaging scheme described here permits the visualization of dynamic biological changes in developing tissue with micrometer spatial resolution and millisecond simultaneity. By employing a 2D-SLM along with galvanometer switching we achieved two orders-of-magnitude faster imaging with two arbitrary pulse shapes compared to previously reported approaches. Adequate and precise pulse compression allowed sufficient signal-to-noise ratio for fast imaging of embryos. Importantly, the pixel dwell time in our experiments was comparable to that of a standard two-photon microscope. We note that the overall performance of the experimental setup described here (resulting in 15 mW power and 14 fs pulses at focus) could be improved by increasing the available laser power and bandwidth. Shorter pulses should make it possible to address additional relevant biological fluorophores, provided that the spectral phase is properly controlled in the focal plane. Higher available power will provide deeper imaging in scattering media, and/or faster frame rates. Along this line, we point out that the experiments described here relied on second order and third order phases for phase shaping. This could also readily be achieved using discrete optical elements such as glass pieces instead of a SLM, resulting in higher overall efficiency.

Regarding possible applications, we note that the broad pulse bandwidth resulted in very good chromophore selectivity. Pulse shaping is therefore an effective means to get additionnal
specificity compared to emission-only filtering, and two-dimensional spectral imaging may be used to separate resembling species. The millisecond-scale multiplexing ability will prove useful in ratiometric excitation measurements. Finally, shaping of broadband pulses can be used in CARS microscopy to isolate a specific vibration mode[7]. Our switching scheme can therefore be used to isolate a resonant signal from the nonspecific background, or to compare specific vibrational signatures. Coherent control of concurrent multiphoton processes generated by broadband pulses is therefore an effective means to achieve multiplexed imaging in nonlinear microscopy.

Annex: materials and methods

Drosophila embryo preparation. We used a transgenic *Drosophila melanogaster* strain expressing eGFP fused with a nuclear localization sequence (nls-GFP, Bloomington stock center), and therefore exhibit a strong fluorescent labeling of nuclei [34]. Embryos were collected during cellularization at developmental stage 5 (stages defined in [35]), dechorionated, and glued to a coverslip. During image acquisition, embryos were maintained in Phosphate Buffered Saline at room temperature (20±1°C). The large working distance objective prevented hypoxia.

Experimental setup. The experimental setup for coherent-control imaging is shown in Fig.3. The laser was a 10 fs oscillator (Synergy PRO, Femtolasers, Austria) associated with a broadband spectrum (130 nm FWHM, center wavelength 820 nm, 450 mW, 75 MHz) pumped with a 5-W solid state laser (Verdi, Coherent, USA). The pulse shaper consists of a diffraction grating (300 grooves/mm, Newport, USA), a folding mirror and a cylindrical mirror to focus the spectral components on the 2D SLM (programmable phase modulator X8267, Hamamatsu, Japan). A galvanometer mounted mirror (VM2500+, GSI Lumonics, USA) was used to rapidly switch between pulse shapes. A spectral interferometer based on a NIR-spectrometer (Ocean Optics, HR4000, USA) was used to characterize the spectral phase imparted by the pulse shaper, but otherwise bypassed during imaging (not shown). The pulse duration and the two-photon spectra at the focus of the microscope objective were obtained from second order interferometric autocorrelation measurements using two-photon absorption in a photodiode.

Laser scanning microscope. Imaging was performed using a custom-built scanning microscope incorporating two orthogonal galvanometer mounted mirrors (VM500, GSI Lumonics, USA), a 0.8 NA 40× water-immersion objective (LUMPlanFl/IR, Olympus, Japan) and NIR coated beam expanding optics. Fluorescent light was collected in the epi-direction using a dichroic mirror (695 dxru, Chroma Technology, USA), a laser-blocking filter (680/SP, Semrock, USA), a photon-counting photomultiplier module (PMT) (P25PC-02, ET Enterprises, UK), and lab-designed counting electronics. Scanning and signal acquisition were synchronized using LabView software and a multichannel I/O board (PCI-MIO-16E-1, National Instruments, USA). A square waveform was sent to the pulse shaper galvanometer to change the pulse shape between every line during flyback time. Image analysis was performed using ImageJ (National Institutes of Health, USA) and Amira (Mercury Computer Systems, USA).

Acknowledgements

We wish to thank A. Bonvalet, B. Chatel, A. Monmayrant, K. A. Nelson and K. W. Stone for fruitful discussions on pulse shaping. We also thank X. Solinas and J.-M. Sintes for expert technical assistance. This work was partially supported by Agence Nationale de la Recherche (ANR-06-PCVI-0013 and ANR-BLAN-0286-01), Délégation Générale pour l’Armement, Conseil Général de l’Essonne (Astre 2006), and Fondation Louis D. de l’Institut de France.