Multiphoton Imaging with a Nanosecond Supercontinuum Source

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ABSTRACT

Multiphoton microscopy is a well-established technique for biological imaging of several kinds of targets. It is classically based on multiphoton processes allowing two means of contrast simultaneously: two-photon fluorescence (TPF) and second harmonic generation (SHG). Today, the quasi exclusive laser technology used in that aim is femtosecond titanium sapphire (Ti: Sa) laser. We experimentally demonstrate that a nanosecond supercontinuum laser source (STM-250-VIS-IR-custom, Leukos, France; 1 ns, 600-2400 nm, 250 kHz, 1 W) allows to obtain the same kind of image quality in the case of both TPF and SHG, since it is properly filtered. The first set of images concerns the muscle of a mouse. It highlights the simultaneous detection of TPF and SHG. TPF is obtained thanks to the labelling of alpha-actinin with Alexa Fluor® 546 by immunochemistry. SHG is created from the non-centrosymmetric organization of myosin. As expected, discs of actin and myosin are superimposed alternatively. The resulting images are compared with those obtained from a standard femtosecond Ti: Sa source. The physical parameters of the supercontinuum are discussed. Finally, all the interest of using an ultra-broadband source is presented with images obtained *in vivo* on the brain of a mouse where tumor cells labeled with eGFP are grafted. Texas Red® conjugating Dextran is injected into the blood vessels network. Thus, two fluorophores having absorption wavelengths separated by 80 nm are imaged simultaneously with a single laser source.

Keywords: Multiphoton microscopy, nanosecond supercontinuum laser source, Multicolor imaging

1. INTRODUCTION

Today, two-photon fluorescence microscopy (TPFM) is an indispensable tool for the analysis of biological samples. Step by step, it replaces usual confocal microscopy systems thanks to significant advantages of multiphoton microscopy well established for deep tissue imaging with subcellular resolution and with limited impact of photobleaching effect [1]. It was first demonstrated by Denk et al. in 1990 [2]. Its advantages compared to confocal microscopy are numerous and uncontestable. For example, the multiphoton process occurs exclusively at the focal point of the excitation beam, allowing reduced photobleaching and obviating the need for a confocal pinhole. Moreover, the use of excitation wavelengths in the near infrared (NIR) range enables a deeper penetration and two types of contrast by two-photon fluorescence (TPF) and second harmonic generation (SHG). This opened the way for endogenous fluorescence of biological substances such as elastin, nicotinamide adenine dinucleotide (NADH) or tryptophan, and at the same time for SHG of non-centrosymmetric structures as collagen.

Unfortunately the probability of inducing multiphoton processes remains low compared to linear fluorescence phenomenon (confocal microscopy). To increase the level of multiphoton signal, a high number of laser pulses with sufficient peak power must be delivered in a short period of time at the target. Hence femtosecond pulses in the NIR with a high repetition rate of several tens of MHz and few kW of peak power are primarily used for multiphoton imaging and presented as indispensable and irreplaceable. In this context, mode locked Ti: Sa laser sources are widely used, typically characterized by 150 fs pulse duration, 10 nm spectral bandwidth at the full width half maximum (FWHM) with wavelength tunability between 700 and 1000 nm, 80 MHz repetition rate and few watts of average power. However, these laser sources have some important limitations. First, the 10 nm spectral bandwidth is not sufficient to

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simultaneously excite more than two fluorophores with different central wavelengths of absorption. Second, the 300 nm tunability range is not accessible with a constant level of average power. Finally, this spectral range does not permit the excitation of all the interesting endogenous fluorophores of a biological sample having a two-photon absorption spectrum in the middle of the visible range, for example tryptophan or phenylalanine.

During the past ten years, other solutions have been proposed to solve these issues. One consists of the generation of a supercontinuum (SC) thanks to the spectral broadening of femtosecond pulses in a photonic crystal fiber (PCF) [3-5]. The broadband pulses temporally enlarged due to chromatic dispersion in the PCF are then recompressed to generate femtosecond pulses with a spectrum of hundreds of nanometers. This Ti: Sa-based technology requires a high level of complexity in optics for its experimental implementation both in terms of SC generation in the PCF and temporal post-compression. At present there is not a stable, turnkey solution of such a source that can be coupled with a two-photon microscope scan head for routine biological studies.

The aim of this work is to identify a simplified SC-based laser technology and demonstrate its compatibility with multiphoton microscopy, as an alternative to femtosecond solutions as a whole. This obviously raises many issues regarding the feasibility of generating multiphoton processes. The key point stands in the peak power delivered to the target. Typically, few kW are used in a multiphoton microscope with Ti: Sa laser excitation. Considering that parameter as the main factor governing the nonlinear mechanism, no argument appears against the use of pulses having a similar level of peak power but different duration and repetition rate [6, 7]. Thus TPF and SHG imaging based on nanosecond microchip laser excitation at 1064 nm has been reported by our group [8], while the SC radiation has previously only been used as the broadband Stokes wave in coherent Raman microspectroscopy. In this presentation, we experimentally show that nanosecond SC laser pulses can be used as the sole pump to generate multiphoton processes. For the first time, TPF and SHG images of a biologic sample are simultaneously obtained from a very easy-to-use and compact SC laser source coupled to a standard multiphoton microscope. These results are compared to those obtained using a conventional Ti: Sa laser [9]. The strong interest of SC sources is then illustrated by *in vivo* multiphoton imaging of tumor cells and blood vessel network in mouse brain[1] thanks to the combination of two fluorophores having their excitation wavelengths separated by 80 nm. Finally the peak power available is discussed in the case of nanosecond SC illumination.

2. EXPERIMENTAL SETUP, SAMPLE PREPARATION AND FIRSTS RESULTS

The experimental setup is based on a commercial two-photon microscope from Olympus (BX61WI) with a standard galvanometer-based scanning system, dichroic mirrors, filters and photomultiplier tubers (PMTs). The whole is driven by a computer where images are reconstructed. The nanosecond SC excitation source is composed of a pulsed pump laser and 1-m-long of solid-core PCF. The pump laser delivers nanosecond pulses (1 ns, 10 µJ) centered at 1550 nm with a repetition rate of 250 kHz. The PCF has a Ge-doped silica core with a zero-dispersion wavelength close to 1400 nm. It is thus pumped in the anomalous dispersion regime. The SC spectrum is broadened from roughly 500 to 2000 nm.

The wideband spectrum is filtered between 700 and 1000 nm thanks to a combination of high pass and low pass filters. Figure 1 left relates the corresponding scheme. The resulting images are compared to the one when obtained with a standard Ti: Sa laser source (Chameleon Ultra II, Coherent, USA).

[1] The use of animals for this study has received the approval of the Ethical and Animal Care Committee (registration number: 15-2014-15). All animal cares and experimental procedures were conducted in conformity with the French Décret n° 2013–118 1st February 2013 NOR: AGRG1231951D in accordance with European Community guidelines (directive 2010/63/UE for the Care and Use of Laboratory Animals).

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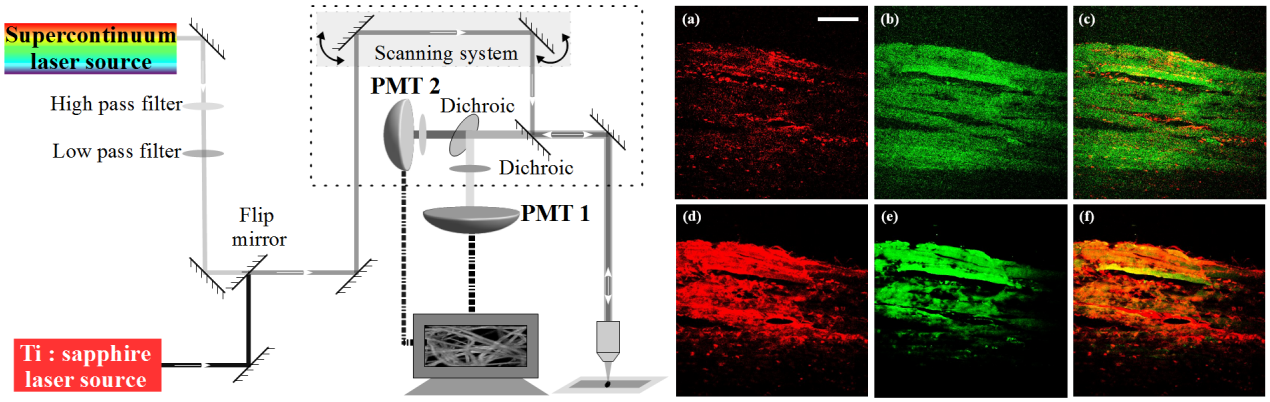

Figure 1. Left: Experimental setup. Right: sample images; a-c. Supercontinuum excitation. d-f. Ti: Sa excitation. Red color: TPF of Alexa Fluor; green color: SHG of myosin. Scale bar: 100 µm. 40 µs/pix; 512x512 pixels.

The sample is resected from a leg mouse muscle having a fibrous structure [14]. Muscle fibers are composed of discs of myosin and actin superimposed alternatively. Myosin organization is known to give rise to SHG [15]. We have identified the presence of alpha-actinin, a cytoskeletal actin-binding protein, by immunohistochemistry and labelling with a secondary antibody coupled to Alexa Fluor® 546. The target is ideal for two different means of contrast: TPF of Alexa Fluor® 546 revealing the presence of alpha-actinin simultaneously with SHG from the myosin structure. The sample has been fixed on microscope glass slides. Figs 1a and 1d correspond to the image of alpha-actinin; figs 1b and 1e are related to the myosin organization; figs 1c and 1f are representing there global and relative positioning.

Figure 1 right displays 500×500 μm^2 images of mouse muscle, obtained from SC and Ti: Sa illumination in the case of TPF, SHG and both combined. As expected, the fibrous structure of the muscle is revealed by the TPF (alpha-actin) and the SHG (myosin). It is immediately apparent that the SC source provides satisfactory multiphoton images. The surface of the sample excited and visualized with the SC source (Fig. 1a) is larger than that of the Ti: Sa laser (Fig. 1d), especially regarding TPF. Interestingly, TPF imaging under SC excitation presents a greater analysis depth and is thus less sensitive to the angle of the sample surface. This difference results from the chromaticity of the microscope objective whose focal point is spread in deepness due to the 300 nm spectral bandwidth of the SC. We have attempted to evaluate the depth of field (DOF) obtained with the SC source in the case of TPF imaging. To do so, under Ti: Sa laser excitation, the microscope objective is translated axially until visualizing satisfactorily all the parts constituting a single image obtained with the SC. The DOF is given by the total translation of the objective and measured to be 2 µm. It should be emphasized that the higher DOF provided by SC illumination is an attractive feature even if it results in a decrease of TPF image contrast, as noticeable in the top right-hand corner of Fig. 1a. In this case, the axial stretching of the focal volume is significant, insofar as the whole 300 nm bandwidth of the SC contributes to the excitation of Alexa Fluor® 546. On the contrary, only 60 nm of the SC (780-840 nm range) participate to the generation of the SH signal, which is detected between 390 and 420 nm. Because of this, the SHG image obtained with the SC source (Fig. 1b) has much more contrast.

3. THEORETICAL CONSIERATION JUSTIFYING THE EXPERIMENTAL RESULTS

Two-photon absorption is the main nonlinear process occurring in TPF. This second-order process consists in the simultaneous absorption of two photons. This nonlinear process has been demonstrated with several kinds of laser sources, from a continuous wave one [6, 7] until femtosecond oscillators [Lefort 2014 JBO]. The generation of two-photon absorption by the fluorophore is easily obtained with a high flux of photons in the range between 1020 and 1030 photons per cm^2 and per second [11]. A mode-locked laser source, having a high fluw of photon compared to a continuous wave laser, increases highly the presence of the two-photon absorption phenomenon by a factor of several tens of thousands.

ϕ describes the fluorescence emission rate resulting from two-photon excitation being function of the excitation source parameters. For a mode-locked laser with defined average power (P) and central wavelength (λ) and for defined fluorophore and microscope objective, the fluorescence emission rate ϕ is inversely proportional to the repetition rate (τ) and pulse duration (Δt) of the excitation source. ϕ is defined as [6, 7, 11]:

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$$\phi \propto \kappa \frac{1}{(\frac{h}{2\pi}\omega)^2} \frac{P^2}{(\tau \times \Delta t)} \frac{\sigma_2}{F^2} \quad (1)$$

where κ is a constant parameters depending on the central wavelength of the excitation source, F corresponds to the proprieties of the microscope objective and σ_2 is related to the two-photon cross section of the fluorophore. Considering this equation (1), when the pulse duration decrease, simultaneously with the decrease of the repetition rate, the fluorescence emission rate increases. In this context, we can compare theoretically TPF when obtained with a classical mode-locked Ti: Sa laser source and with a pulsed-ns SC one. To generate the same fluorescence rate σ from a fluorophore, the average power required is 2.2 mW for the Ti: Sa source and 10 mW for the SC; these values are in the same rage. Experimentally, to obtain the images presented in Fig. 1, the average power measured just after the microscope objective is set at 5.5 mW and 92 mW respectively for the SC and Ti: Sa lasers.

4. INTEREST OF WIDEBAND MULTIPHOTON IMAGING

The interest of using a wideband source for multiphoton imaging stays in the simultaneously excitation of several fluorophores in the same time and having their excitation wavelengths separated by more than 10 nm, which is the spectral bandwidth of the Ti: Sa laser [12, 13]. To illustrate this experiment, we have imaged *in vivo* the brain of a mouse anaesthetized with ketamine/xylazine, where glioma tumor cells (GL261) stably expressing eGFP were previously grafted thanks to the implementation of a cranial window as described in [14]. On the one hand, the tumors cells are detected; on the second hand, the second fluorophore, Texas Red® conjugated Dextran (70 kD), is injected in a tail vein allowing to visualize cortical blood vessels. The general objective of this combination of fluorophores is to visualize the evolution of the organization of the blood vessels all around the tumor cells during several weeks. The two-photon absorption spectrum of Texas Red® is centered at 840 nm, versus 920 nm for the eGFP; each spectrum of emission or two-photon absorption of the fluorophores used are presented in Figure 2a. The TPF is respectively detected in the ranges 580-700 nm and 490-540 nm. These spectral areas, represented in Figure 2a, are detecting the maximum of the emission of each fluorophores and are not superimposed from one to the other one. Figure 2b shows the resulting images obtained 200 µm under the surface of the brain.

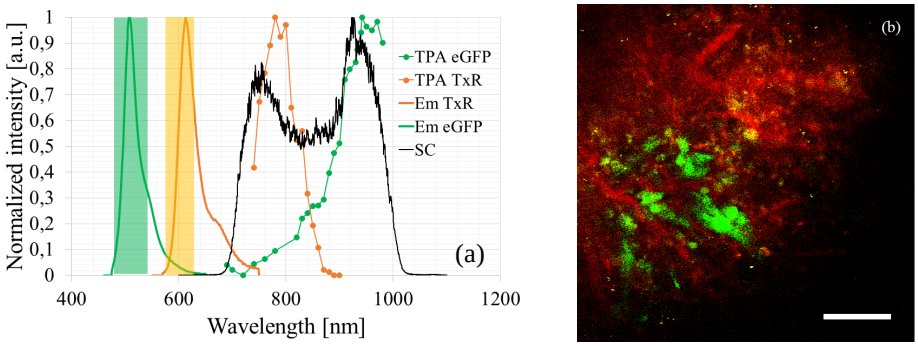

Figure 2. a. Superimposition of the absorption/emission spectra of the fluorophores used (EGFP and Texas Red) and the SC excitation spectrum. b. In vivo image of tumor cell labelled with EGFP grafted into the brain of a mouse with Texas Red (TxR) injected into the blood vessels network.

The ability of the SC laser source to generate an excitation spectrum adapted to the fluorophores used and to their number is of huge interest in the field of multiphoton fluorescence microscopy. Here, Figure 2a highlights an excitation spectrum adapted to each of the two-photon absorption spectra, extracted from [15]. Figure 2b presents the localization of the tumor cells detected, with the blood vessels network.

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5. CONCLUSION

For the first time, a pulsed-ns SC laser source was used for two-photon fluorescence microscopy. It demonstrates the utility of this ultra-wideband source for biological imaging. Two-photon excitation fluorescence was established on a fixed sample of mouse muscle where the myosin (non-centrosymmetric structure) was image thanks second harmonic generation and alpha-actin was labelled with Alexa Fluor 546. The capability of a pulsed-ns SC laser source for imaging living biological samples was demonstrated in vivo in the brain of a cranial window-implanted mouse grafted with EGFP expressing tumors cells and with vasculature labelled with the fluorophore Texas Red. This work demonstrated the potential of a pulsed-ns SC laser source for in vivo imaging with TPFM.

The limitation of the number of means of contrasts is thus not linked to the laser source but exclusively from the number of detectors in a case of a system with photomultiplier tubes as we have used in our setup. A way to obtain a complete system might be the use of a spectrometer with a spectral reconstitution of the image as it is usually done in classical Raman microscopes.

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