Layout: T1 Standard Unicode | Book ID: 318422_1_En | Book ISBN: 978-3-319-13241-9
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Simultaneous Selective Two-Photon Microscopy Using MHz Rate Pulse Shaping and Quadrature Detection of the Time-Multiplexed Signal

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Abstract We demonstrate a method for simultaneous fast selective two-photon excited fluorescence (TPEF) microscopy imaging of two different fluorophores using quadrature detection of the signal from a single PMT detector.

1 Introduction

The broad bandwidth and high peak intensity of femtosecond lasers enable the two-photon excitation of many different fluorophores with high efficiency using a single laser setup. Selective excitation of particular fluorophores can be achieved by a computer controlled pulse shaper taking advantage of multiphoton intrapulse interference (MII) [1–4]. Phase shaping, in particular, can maintain the high efficiency, but excite a particular frequency within the two-photon bandwidth with high spectral resolution. Unfortunately, pulse shapers appropriate for microscopy have slow refresh rates which limit the speed with which multiple fluorophores can be measured with a single detector. Here we achieve 162 MHz rate pulse shaping on a near octave spanning laser using passive components. With a single photomultiplier tube (PMT) detector, we are able to selectively image two fluorophores with excitation separated by 50 nm.

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K. Yamanouchi et al. (eds.), *Ultrafast Phenomena XIX*,
Springer Proceedings in Physics 162, DOI 10.1007/978-3-319-13242-6_200

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2 Experimental

An ultra-broad bandwidth oscillator and pulse shaper (femtoAdaptiv, BioPhotonic Solutions Inc.) with central wavelength at 812 nm (Fig. 1) is capable of exciting two-photon transitions in the 380–500 nm range. Selective excitation in the blue and red portions of the spectrum is achieved by a combination of second order dispersion (SOD) and third order dispersion (TOD) [2, 3]. As it is shown in Fig. 2a, the output from the laser was split into two arms with different second order dispersion (SOD). The recombined beams create a train of pulses with spectral phase switching at 162 MHz rate. Each pulse induces selective TPEF on the sample at wavelengths determined by the amount of SOD and TOD in the beam [6].

Fluorescence is detected by a single photomultiplier tube (PMT) detector. Signal from the PMT detector contains fluorescence signals from two different selectively-excited fluorophores. The two separate signals are isolated by quadrature detection using a lock-in amplifier as shown in Fig. 2b. Images are obtained from two different fluorophores simultaneously. This allows fast frame-rate imaging.

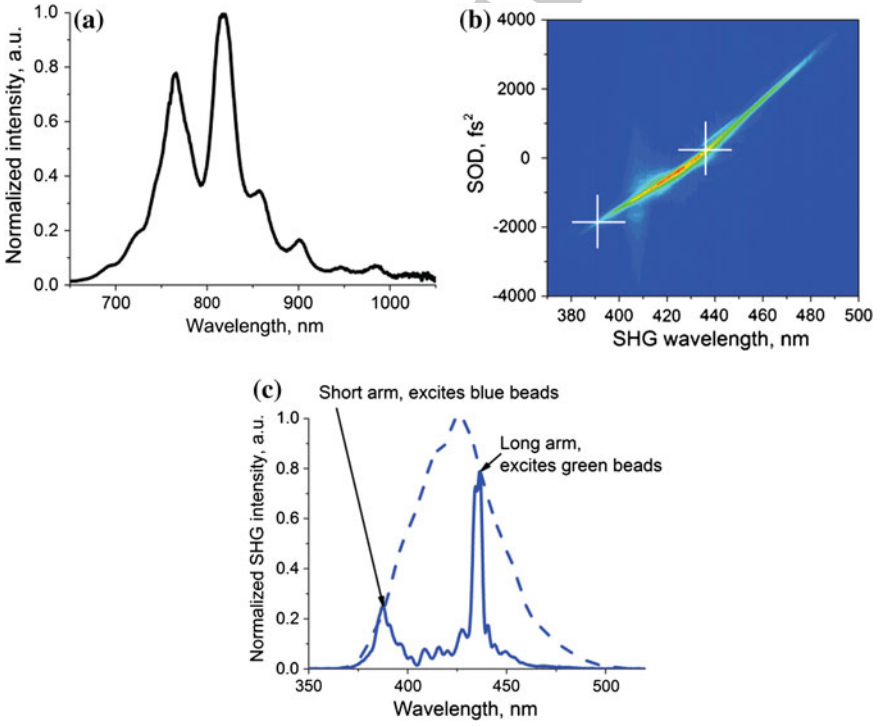

Fig. 1 **a** Emission spectrum of laser oscillator; **b** MIIPS scan [5] of the laser pulse without compensation mask applied. **c** SHG spectrum of laser pulse with applied phase compensation (*dashed*), pre-chirped and delayed in different arms (*solid*). All measurements made at the focal plane of a Zeiss LD C-APOCHROMAT 40×/1.1 objective

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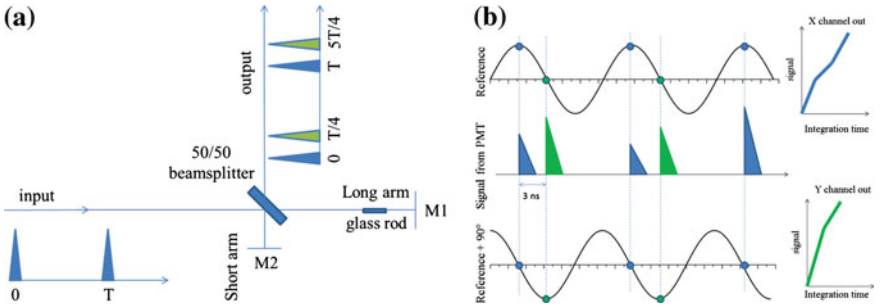

Fig. 2 **a** Schematic representation of excitation beam quarter-period delay line with different amount of SOD in arms. **b** Simultaneous acquisition of signals from two different fluorophores at single photo-detector by quadrature detection using lock-in amplifier

3 Results and Discussion

Excitation laser pulses with different shapes (typical energy ~110 pJ per pulse at the focus of the objective) are temporally delayed by ~3 ns. The microscopy image (shown in Fig. 3) provides selective signal from 10 μm blue microspheres when detecting signal from the X channel of the lock-in amplifier. The image from the 6 μm beads is obtained when detecting signal from the Y channel of the lock-in amplifier (with 90° phase difference). The combination of both signals provides the full image.

Wide tunability of the two-photon excitation wavelength, fast switching rate between the selective excitation, and low photodamage (due to the higher peak intensity of shorter pulses [7]) enables application of this method for in vivo dynamic imaging in biological samples.

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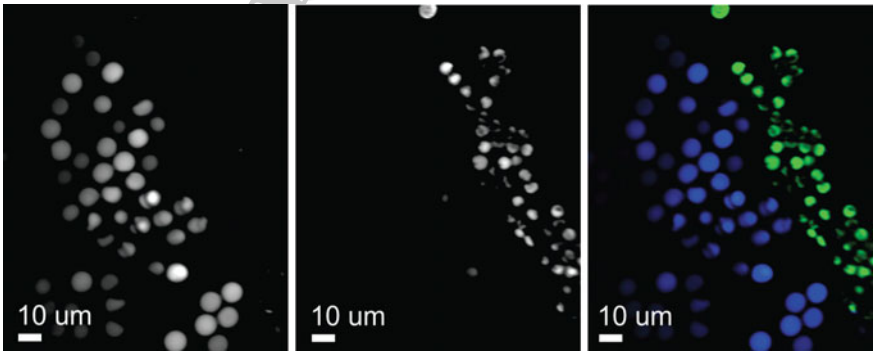

Fig. 3 Microscopy images of fluorescent beads, using the signal from X channel (*left image*) and Y channel (*center image*) of the lock-in amplifier. False colored image obtained by adding the X and Y channel signals (*right image*)

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