Non-degenerate multiphoton microscopy for deep brain imaging

Mu-Han Yang[1], Maxim Abashin[1], Payam A Saisan[2], Anna Devor[2,3], Yeshaiahu Fainman[1]
[1]*Department of Electrical and Computer Engineering, UCSD, San Diego, CA*
[2]*Departments of Neurosciences and Radiology, UCSD, San Diego, CA*
[3]*Martinos Center for Biomedical Imaging, Harvard Medical School, Charlestown, MA*
muy003@eng.ucsd.edu

Abstract: We achieve non-degenerate 2-photon excitation (ND2PE) using a pair of IR and NIR beams and show that the emission intensity is proportional to the power of each beam. ND2PE provides an alternative to 3-photon excitation.

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1. Introduction

The advent of 2-photon microscopy has enabled *in vivo* imaging of cerebrocortical structure and function with micron resolution [1,2]. Yet, penetrating deep into the cortex remains a challenge due to the scattering and absorption of light by cerebral tissue. Overcoming these effects requires an increase in the illumination intensity eventually resulting in significant increase in probability of 2-photon excitation (2PE) at the cortical surface (a.k.a. "out-of-focus" excitation) decreasing the image quality and damaging the surface tissue. The combined unwanted effects of scattering and absorption can be reduced by employing longer illumination wavelengths around 1400 nm or 1700 nm. These wavelengths, however, are not suitable for the conventional degenerate 2PE (i.e., absorption of 2 photons of the same energy or color) of visible spectrum probes (e.g, with red or green emission). Recently, it was demonstrated that degenerate 3PE – using absorption of 3 photons from the same laser in the infrared (IR) spectral range – improves the depth penetration [3]. However, the efficiency of this excitation mode is orders of magnitude lower than that for 2PE resulting in low photon count (under non-harmful illumination intensity) thus slowing down image acquisition. To address this challenge, we are exploring an alternative approach – 2PE by the simultaneous absorption of different energy photons [4] using 2 different laser frequencies (or colors) (Fig. 1). The non-degenerate approach offers independent control of the photon flux (i. e., the intensity) and polarization for each beam. We expect that by increasing the intensity of the longer wavelength beam while lowering the intensity of the shorter wavelength beam, we would be able to achieve the depth penetration of 3-photon microscopy while circumventing the low probability of 3PE. We also expect to decrease the unwanted "out-of-focus" excitation on the brain surface [5]. This is because the shorter wavelength beam will not have enough photon density at the surface while the longer wavelength beam will lie outside the 2PE range (and 3-photon absorption cross-section is very low).

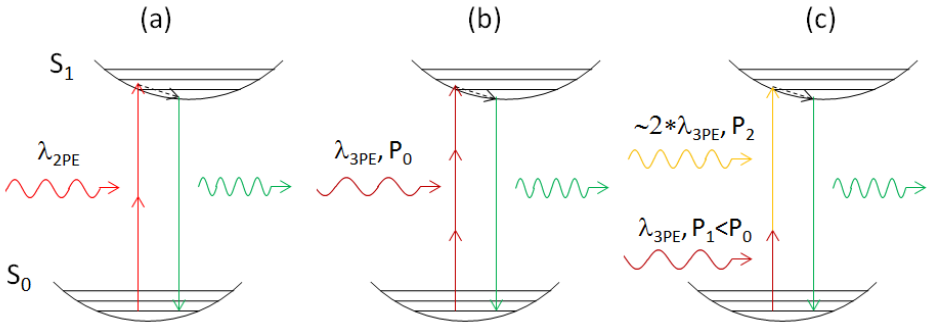

Fig. 1. Schematic energy diagram demonstrating degenerate and non-degenerate multi-photon excitation of a molecule: (a) degenerate 2-photon excitation; (b) degenerate 3-photon excitation; and (c) non-degenerate 2-photon excitation.

2. Methods

To study non-degenerate 2PE, we created a light source with 2 spatially-aligned and synchronized beams that we call "pump" and "probe" [6]. For the probe beam, we employed a Ti:Sapphire mode-lock laser (Coherent Mira 900 Ti:Sap femtosecond laser) as a master laser tuned to 825 nm and separated it into two beams by a polarizing beam

splitter. One of the beams was launched into the Optical Parametric Oscillator (Coherent Mira OPO). The OPO generated the pump beam (~1300-1550 nm). This allowed choosing our fluorescence pump wavelength within the desired IR window of 1300-1400 nm. The probe and pump beams were temporally synchronized because OPO was pumped by the same Ti:Sapphire laser. A variable delay line was used to correct the differences in propagation lengths between the pump and probe pulses. The probe and pump beams differed also in the beam size. Therefore, the beam size for both beams was adjusted before combining the two using telescopes. Two half-wave plates were inserted for polarization analysis.

Specific considerations: For imaging of brain activity (e.g., with calcium indicators) one of the requirements is high acquisition speed. This requirement becomes even more stringent when large-scale sampling is desired. In fluorescence intensity imaging (the leading application), the speed requirement translates into maximizing the number of emitted photons *n* within a given "dwell time" of the beam per pixel. For pulsed laser sources, used in multiphoton excitation, *n* depends both on the peak power and the repetition rate. These considerations were taken into account in the design of the non-degenerate excitation source with the requirement for fast imaging as a major constraint. E.g., this is the reason that we did not consider using Optical Parametric Amplifier (OPA) that provides more power but offers relatively low repetition rate (250 KHz) ultimately slowing down the speed of data acquisition [7].

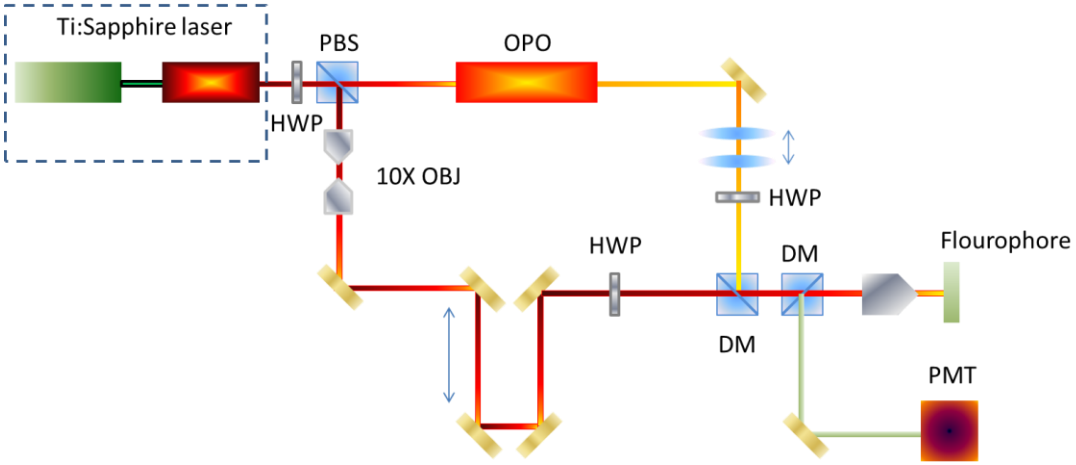

Fig. 2. Experimental setup for non-degenerate 2-photon excitation. PBS-polarizing beam splitter; OPO-optical parametric oscillator; OBJ- objective lens; HWP-half wave plate; PMT- photomultiplier tube.

3. Results and Discussion

The probe and pump beams were spatially aligned using adjustable mirror mounts. The temporal alignment was optimized by scanning the optical delay line. The fluorescence signal was detected by a photomultiplier tube (PMT). The solid line in Fig. 3A indicates the background noise that includes the dark PMT current and the signal due to ambient photons. Irrespective of the position of the delay line, we observed a fluorescence signal presumably generated by degenerate 2PE due to the probe beam. We moved the delay line in steps of 10 μm using a stepper motor. This step size was chosen considering the pulse width of the probe and pump beam around 150 fs and 200 fs, respectively. We observed an increase in the detected fluorescence signal as two beams overlapped. This presumably indicates the additive effect of the non-degenerate 2PE occurring due to the simultaneous absorption of the pump and probe photons.

To investigate the nature of the observed fluorescence signals, we examined their dependence on the power of the probe (Fig. 3B) and pump beams (Fig. 3C). In the absence of proper alignment, the fluorescence signal scaled quadratically with the power of the probe beam with no dependence on the power of the pump (green curves in Fig. 3B and 3C, respectively). This clearly indicates degenerate 2PE. With the two beams temporally aligned, we observed higher fluorescence signal (blue curves in Fig. 3B and 3C, respectively). The increase was assumed due to the non-degenerate 2PE on top of the degenerate effect. Subtracting the contribution of the degenerate 2PE we obtained the dependence of this additional signal on the power of the probe and pump beams (red curves in Fig. 3B and 3C, respectively). The signal increased linearly in both experiments (i.e., while increasing the power of the probe or pump beam). This clearly indicates that the signal was due to the non-degenerate 2PE that is proportional to the power in each beam. It is worth noticing that the experimentally obtained power dependences in Fig. 3C do not start from zero because of the fluorescence due to degenerate 2PE at the fixed power of the probe beam. We should

notice that 3PE was not observed here due to insufficient power of the pump beam. This also emphasizes low efficiency of 3PE compared with 2PE (due to low 3-photon absorption cross section).

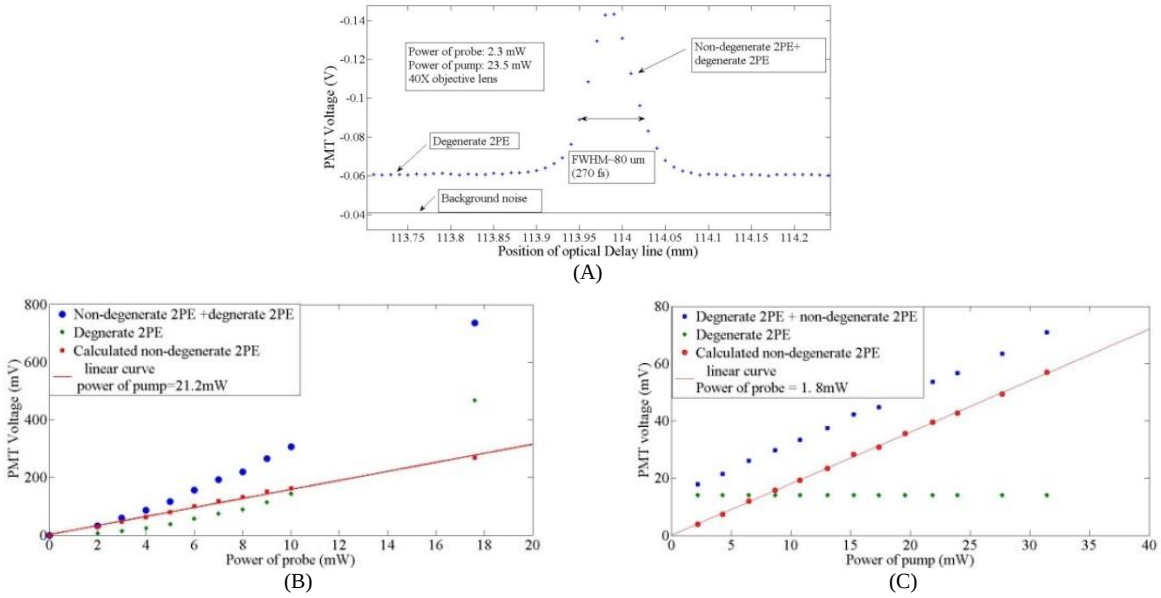

Fig. 3 (a). The beam profile of the fluorescence obtained by scanning the time delay line. (b). Power dependence of the fluorescence with varied power of probe beam and fixed power of pump beam. (c) Power dependence of the fluorescence with varied power of pump beam and fixed power of probe beam.

4. Conclusions

We have demonstrated that non-degenerate 2PE of the fluorophores relevant for brain imaging can be achieved with a pair of infrared (IR, pump) and near infrared (NIR, probe) beams and that the power of each beam can be independently modulated. In the brain, the IR beam would experience reduced losses to scattering and absorption and would be able to deliver high photon flux to the focal volume deep inside the cortical tissue. The NIR beam would experience more scattering. However, the flux requirement for this beam under the non-degenerate excitation is relaxed (compared to that in the conventional 2-photon microscopy). Thus, non-degenerate 2PE may be a competitive alternative to 3PE for achieving deep high-resolution imaging taking advantage of IR illumination but circumventing the low probability of 3-photon absorption.

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5. References

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