Enabling Safe, High-Resolution Two- and Three-Photon Imaging at Microwatt Powers

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1. Main Text

Multiphoton microscopy has become one of the most powerful imaging tools by enabling deep, three-dimensional visualization of living tissues with high spatial resolution and reduced photodamage [1]. Two- and three-photon excitation, in particular, extend penetration depth and provide intrinsic optical sectioning [2], making them indispensable in neuroscience [3], developmental biology [4], and ophthalmology [5]. Their abilities to minimize out-of-focus excitation and harness longer wavelengths open powerful opportunities for in vivo studies where tissue integrity must be preserved.

However, many of these applications are constrained by the need to achieve strong nonlinear excitation at very low average powers. This requirement is especially critical in scenarios such as long-term neuronal imaging, embryonic development studies, or ophthalmoscopy, where excess light exposure can cause irreversible photodamage, bleaching, or thermal injury [6]. Conventional femtosecond sources, such as Ti:Sapphire lasers [7], often fail to meet these conditions efficiently, forcing compromises between image quality and sample safety. Here, we introduce a compact, all-fiber system that employs a gain-managed nonlinear (GMN) amplifier with integrated pulse picking, which resolves this limitation. By combining ultrashort pulses with high peak power and tunable repetition rates, our system enables robust two- and three-photon imaging at microwatt-level average powers, unlocking new possibilities for safe and effective low-power imaging in delicate biomedical tissues.

2. Methods and results

Our imaging system, schematically shown in Fig. 1(a), integrates five main elements: a fiber oscillator, a pulse picking unit, a GMN amplifier, a pulse compressor, and a custom multiphoton microscope. The oscillator (described in [8,9]) produced ~10 ps pulses with the energy of ~10 nJ at the central wavelength of 1030 nm and the repetition rate (f_{rep}) of 15.2 MHz. The pulse picking unit enabled flexible reduction of the repetition frequency. Next, amplification in the GMN stage generated pulses with energies up to ~42 nJ and a spectral width of ~90 nm, centered at around 1070 nm [Fig. 1(b)]. These pulses were subsequently compressed to a 39 fs pulse duration [Fig. 1(c)] in a simple, two diffraction grating compressor. This combination of high peak power, broad spectrum, and sub-40 fs pulse duration yields a robust source for multiphoton excitation. Moreover, our architecture is fully fiber-based up to the amplifier output, ensuring compactness, ease of alignment, and enhanced stability.

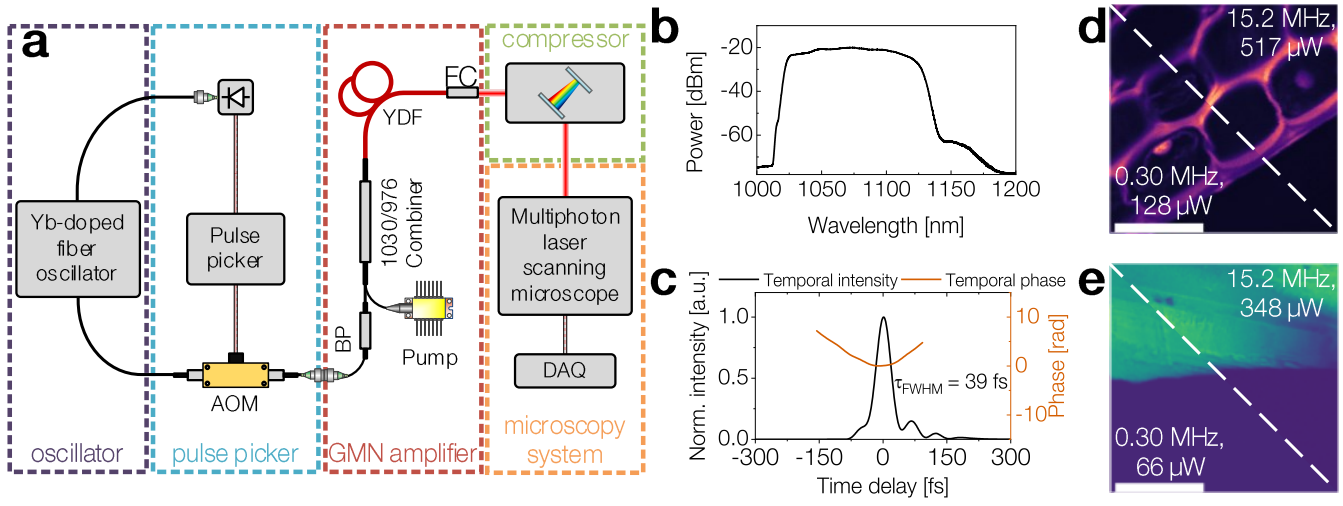

Fig. 1. (a) Schematic of the experimental system. AOM – acousto-optic modulator, BP - bandpass filter, YDF – Yb:doped fiber, FC - fiber collimator. Characterization of the pulse at the output of the compressor: (b) optical spectrum, (c) FROG-retrieved temporal intensity (black) with temporal phase (red). (d) 2PE fluorescence images of *convallaria majalis* obtained at the f_{rep} 15.2 MHz and P_{avg} at the sample 517 µW, and f_{rep} 0.30 MHz and P_{avg} at the sample 128 µW (e) SHG microscopy images of urea microcrystals obtained at the f_{rep} 15.2 MHz and P_{avg} at the sample 348 µW, and f_{rep} 0.30 MHz and P_{avg} at the sample 66 µW. Scale bars: 80 µm.

In multiphoton excitation, the detected fluorescence depends on both the average power (P_{avg}) and the relationship between pulse duration and repetition rate. By lowering the pulse repetition rate, it was possible to decrease the average power at the sample while preserving a comparable fluorescence response. Figure 1(d) illustrates this effect with two-photon excitation (2PE) microscopy images of a *convallaria majalis* root cross-section stained with acridine orange. The panel

compares two images: one taken at 15.2 MHz with an average power of 517 µW and the other at 0.30 MHz with an average power of 128 µW, arranged as complementary sections within a single image. The seamless transition between the two halves highlights that reducing the repetition frequency 50 times enabled a more than fourfold decrease in excitation power without loss of image quality. To further validate this approach, Fig. 1(e) presents second-harmonic generation (SHG) microscopy images of urea microcrystals acquired under comparable conditions. At the repetition rate of 15.2 MHz, clear images were obtained with an average excitation power of 348 µW. When the repetition rate was reduced to 0.30 MHz, the same image quality was preserved at only 66 µW average power at the sample. This remarkably low power level highlights the efficiency of our system in enabling high-quality nonlinear imaging under conditions that minimize the risk of photodamage.

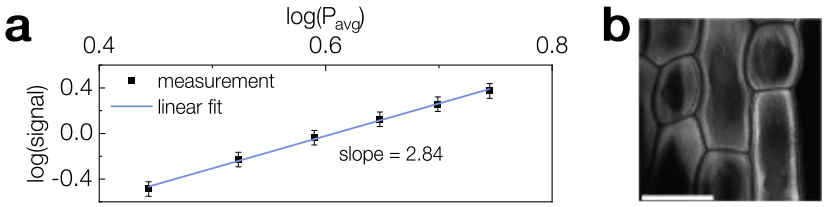

Fig. 2. (a) Log-log plot of the power-dependent fluorescence intensity with a linear fit. (b) 3PE fluorescence image of outer epidermal cells obtained at the f_{rep} 580 µW and P_{avg} 0.30 MHz.

Our system also supports three-photon excitation (3PE) imaging at remarkably low average powers. As a demonstration, we imaged the outer epidermal cell layer of an onion. The dependence of fluorescence intensity on excitation power, shown in Fig. 2(a), follows the cubic trend expected for 3PE (fitted slope of 2.84). Figure 2(b) presents an image acquired at a repetition rate of 0.30 MHz with only 580 µW average power at the sample, confirming the capability of our source for efficient low-power 3PE imaging. Importantly, achieving stable three-photon excitation at sub-milliwatt levels opens the door to label-free, minimally invasive imaging of endogenous structures in living tissues.

In summary, we have developed a compact, almost fully-fiber and robust laser system that enables multiphoton imaging across two-, three-photon, and SHG modalities at exceptionally low excitation powers. The integration of a GMN amplifier with a pulse picking unit provides precise control over pulse energy and repetition rate, allowing the average power at the sample to be minimized without sacrificing image quality. This capability is particularly important for reducing photodamage and photobleaching in long-term studies of living specimens, as well as for protecting delicate materials in biomedical and materials science applications.

3. Acknowledgement

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4. References

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