Efficient multiphoton microscopy with high-energy picosecond laser pulses

Katarzyna Kunio*#, Jakub Bogusławski, Grzegorz Soboń
Laser & Fiber Electronics Group, Faculty of Electronics, Photonics and Microsystems,
Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
#corresponding author email: katarzyna.kunio@pwr.edu.pl
**Presenting author*

Multiphoton microscopy [1] is an advanced imaging technique that uses multiple lower-energy photons to excite fluorophores, allowing for deeper tissue penetration and reduced photodamage compared to traditional fluorescence microscopy. This technique enables precise visualization of cellular structures and dynamic processes within intact biological samples by harnessing nonlinear optical processes. The most popular lasers for this use are Ti:sapphire lasers [2], which offer short pulse durations and high peak powers but, at the same time, are bulky, complex, and expensive. Moreover, short pulses are prone to broadening due to the dispersive optical elements in a microscope, necessitating careful dispersion management to maintain excitation efficiency. We propose an efficient multiphoton microscopy setup utilizing a 10 nJ Yb:fiber laser producing picosecond pulse trains, which can achieve a performance comparable to femtosecond pulse trains by reducing the pulse repetition rate (f_{rep}) while less affected by chromatic dispersion. This indicates that adding a compressor is not always necessary and can be replaced with a smaller and more user-friendly all-fiber setup.

Figure 1(a) shows the schematic of our multiphoton microscopy setup. We have built a figure-eight all-polarization maintaining Yb:fiber laser producing 10 nJ energy pulses with the central wavelength of the optical spectrum at 1026.5 nm [Fig. 1(b)]. The oscillator consists of the main loop and the nonlinear amplification loop mirror (NALM). Each loop is pumped with a 976 nm laser diode, which is always protected from back-reflection by an isolator and a pump protector. The NALM loop comprises a wavelength-division multiplexer (WDM) and an asymmetrically positioned active Yb-doped fiber. The two loops are connected with a 50/50 fiber coupler. The main loop consists of a YDF, an isolator, a WDM, a bandpass filter, and a 70/30 fiber coupler, which leads 70% of the optical signal out of the system. The duration of the oscillator's pulses was 10.1 ps [Fig. 1(c)], and the oscillator can be characterized by good stability [as shown in Fig. 1(d)] with the fundamental f_{rep} of 15.23 MHz. Its Relative Intensity Noise (RIN, measured in laboratory conditions, with no thermal stabilization) was equal to 0.53% root-mean-square measured in the range of 10 Hz to 3 MHz [Fig. 1(e)]. Next, we have placed a pulse picker unit consisting of an acousto-optic modulator (AOM) and an electronic driver. The pulse picker allows the user to change the f_{rep} of the pulse train by dividing its value by a positive integer. The photodiode is connected to the previously unused port of the WDM, allowing for the synchronized transmission of pulses. The beam leaving the pulse picker unit is collimated and led through the gradient filter (GF) into the multiphoton microscope connected to the lock-in amplifier with a reference frequency supplied from the photodiode. The GF allows for adjusting the power entering the microscope [3], and the lock-in amplifier ensures better image quality. Lastly, the output demodulated signal is sampled with a data acquisition card (DAQ).

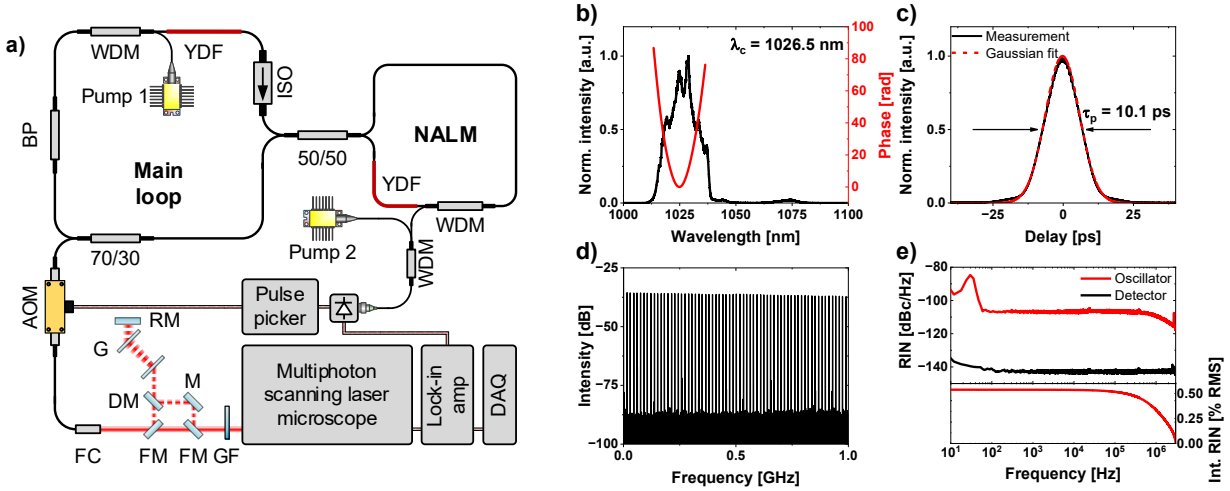

Fig. 1. a) Schematic of the experimental multiphoton microscopy setup. BP – bandpass filter, YDF – Yb-doped fiber, WDM – wavelength-division multiplier, ISO – isolator, AOM – acousto-optic modulator, FC – fiber collimator, FM – flip mirror, DM – D-shaped mirror, G – diffraction grating, RM- retro mirror, M – mirror, GF – gradient index filter, DAQ – data acquisition card. b) The optical spectrum of the Yb:fiber laser with the spectral phase. c) Autocorrelation of the pulse with the Gaussian fit. d) The radio frequency (RF) spectrum. e) RIN and integrated RIN of the oscillator.

Since we wanted to compare the efficiency and influence of chromatic dispersion of the picosecond pulse train with the reduced f_{rep} to the usually used femtosecond pulse train with the unchanged f_{rep}, we directed the output beam through a typical Treacy compressor employing flip-mounted mirrors comprising two parallel transmission diffraction gratings (estimated group delay dispersion of -1.17 ps^2 and transmission of 79%). The duration of the picosecond pulse was 10.3 ps [Fig. 2(a)], and it was barely affected by the chromatic dispersion of the microscopy setup. In contrast, the femtosecond pulse duration after compression was 205 fs [Fig. 2(b)], and it stretched to 227 fs with ~35% p_{peak} loss compared to the pulse before transmission through the microscopy system.

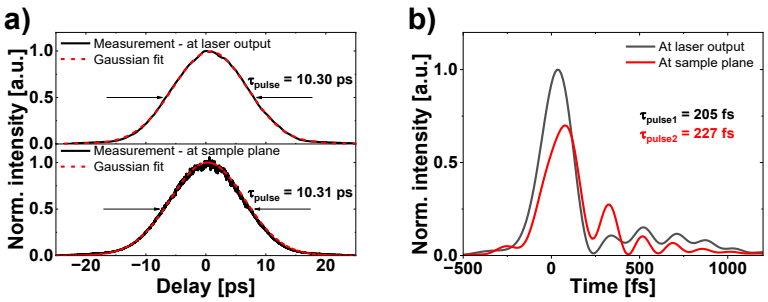

Fig. 2. Comparison of the pulse duration before and after propagation through the microscope system: a) autocorrelation of the picosecond pulse with a Gaussian fit at the laser output (top) and in the sample plane (bottom), b) reconstructed temporal profile of the pulse at the laser output and in the sample plane.

The fluorescence signal depends on the pulse train's duty cycle [4]. It is possible to achieve a comparable imaging result to shortening the pulse duration by using longer pulses with a lower repetition rate, provided they maintain the same duty cycle [5], which is the product of pulse duration (τ_p) and repetition rate (f_{rep}). Since the longer pulse can be thought of as a combination of multiple shorter pulses merged together, we adjusted the f_{rep} of the picosecond pulse train according to:

$$\mathbf{n} \sim \frac{\mathbf{p_{avr}^2}}{\boldsymbol{\tau_p \cdot f_{rep}}} = \mathbf{p_{peak}^2 \cdot \tau_p \cdot f_{rep}}, \quad \textbf{(1)}$$

where n is the average number of photons emitted by the fluorescing medium per second, p_{avr} and p_{peak} are the average and the peak power of the pulse, respectively. In our case, the f_{rep} of the picosecond pulse trains should equal 0.3 MHz, corresponding to the picking ratio of 1/50.

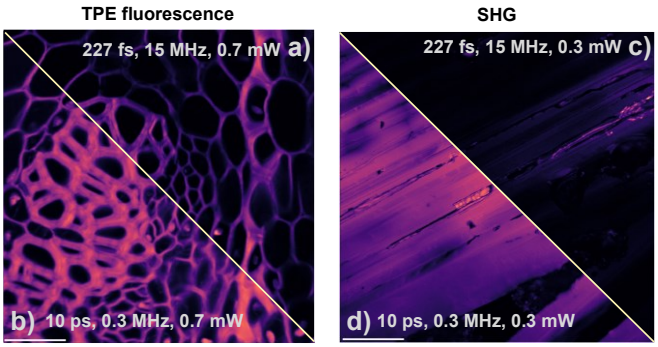

Fig. 3. TPE fluorescence images of a stained *convallaria majalis* sample obtained at 0.7 mW average power using: a) femtosecond, and b) picosecond pulse trains. Scale bar: 75 µm. SHG images of urea microcrystals obtained at 0.3 mW average power using: c) femtosecond, and d) picosecond pulse trains. Scale bar: 200 µm.

Figures 3(a)-(b) show the results of imaging a *convallaria majalis* root transverse section stained with acridine orange using pico- and femtosecond pulse trains with 0.3 MHz and 15.2 MHz, respectively. The average power at the sample in both cases was equal to 0.7 mW. Figures 3(c)-(d) show the results of imaging urea microcrystals (with an average power equal to 0.3 mW). The size of each image was set to 1024x1024 pixels, with the dwell time of one pixel set to 10 µs. It can be noticed that the images obtained using the picosecond pulse train had greater brightness and contrast. The mean pixel intensity of the TPE image captured using the picosecond pulse train was equal to 0.54 V, while for the femtosecond pulse train, it was equal to 0.25 V (54% higher fluorescence signal for the longer pulses). For the SHG imaging, these values were 0.685 V for the femto- and 0.072 V for the picosecond pulse trains (90% higher fluorescence signal for the longer pulses). The reason for the lower fluorescence intensity while using femtosecond pulses, despite maintaining a very similar duty cycle, is the imperfection in the compression of shorter pulses. They are also more susceptible to elongation caused by the chromatic dispersion of the microscope's internal optics (estimated to equal several thousands of fs^2). These findings demonstrated that temporal compression of fiber lasers isn't always effective and can be avoided by utilizing a more compact and user-friendly all-fiber setup.

In conclusion, we have highlighted the benefits of utilizing multiphoton microscopy with a high-energy Yb:fiber NALM oscillator operating at a reduced repetition rate (f_{rep}). Our research indicated that using the same average powers and duty cycles, the femtosecond pulse train resulted in lower-quality images compared to the picosecond pulse train. This portable, all-fiber system allows for flexible f_{rep} adjustment via the pulse-picking unit and is user-friendly due to its chromatic dispersion resistance. These results provide a fresh outlook on multiphoton microscopy.

Funding: National Science Centre, Poland (2021/43/D/ST7/01126)

References

[1] Denk, W., Strickler, J. H., and Webb, W.W., "Two-Photon Laser Scanning Fluorescence Microscopy," Science **248**, 73–76 (1990).
[2] König, K., Schenke-Layland, K., Riemann, I., and Stock, U. A., "Multiphoton autofluorescence imaging of intratissue elastic fibers," Biomaterials **26**, 495–500 (2005).
[3] Bogusławski, J., Kwaśny, A., Stachowiak, D., and Soboń, G., "Increasing brightness in multiphoton microscopy with a low-repetition-rate, wavelength-tunable femtosecond fiber laser," Opt. Continuum **3**, 22 (2024).
[4] Song, J., Kang, J., Kang, U., Nam, H. S., Kim, H. J., Kim, R.H., Kim, J. W., and Yoo, H., "SNR enhanced high-speed two-photon microscopy using a pulse picker and time gating detection," Sci Rep **13**, 14244 (2023).
[5] Karpf, S., Eibl, M., Sauer, B., Reinholz, F., Hüttmann, G., and Huber, R., "Two-photon microscopy using fiber-based nanosecond excitation," Biomed. Opt. Express **7**, 2432 (2016).