Multiphoton scanning laser microscope based on a femtosecond fiber laser

Alicja Kwaśny,* Jakub Bogusławski, and Grzegorz Soboń

Laser and Fiber Electronics Group, Faculty of Electronics, Photonics and Microsystems, Wrocław University of Science and Technology, Wrocław, Poland,

Received November 10, 2022; accepted December 22, 2022; published December 31, 2022

Abstract—We present a multiphoton scanning laser microscope based on a femtosecond frequency-doubled erbium-doped fiber laser. The laser used in the epi-illumination microscope setup generated 95 fs pulses at the wavelength of 780 nm with 44.3 mW average power at 100 MHz pulse repetition rate. The imaging process was controlled by custom software developed in the NI LabVIEW environment. Detection of two-photon fluorescence was proven by acquiring a series of images from various biological samples.

Multiphoton microscopy uses multiphoton absorption to excite fluorophores and then captures the fluorescence emitted by the excited molecule. For two-photon fluorescence, the simultaneous absorption of two photons results in the excitation of a molecule, equivalent to single-photon absorption of half the wavelength [1]. Using longer, less energetic wavelengths allows us to excite molecules in biological tissues with absorption spectra in the UV without damaging the sample [2]. Moreover, multiphoton absorption occurs only in the focal spot, where the photon density is the highest. Consequently, there is no photobleaching in the beam path, and no pinhole prior to the detector is required to block the out-of-focus light. Given the benefits of this technique, multiphoton microscopy has become a widespread imaging tool in specialized scientific laboratories. However, further developments are still needed to facilitate translation to, e.g., healthcare facilities.

Multiphoton excitation requires femtosecond pulses in the near-infrared range. Currently, Ti:sapphire lasers are the most commonly used laser sources for this application. They produce short pulses (<200 fs), high average power (>2W), and provide a broad tunability range (690 to 1020 nm) required for the excitation of multiple molecules [3]. Unfortunately, such lasers are very expensive, have a large footprint, and require active water cooling. Furthermore, specialized maintenance is required from time to time, resulting in a high cost of ownership.

Femtosecond fiber lasers might be an alternative to Ti:sapphire lasers, especially in applications with high translational potential [4–6]. They are affordable, take up a lot less space, are passively air-cooled, and do not require any adjustments. However, compared with Ti:sapphire lasers, the fiber counterparts produce less average power and usually operate at a fixed wavelength range. However, tunable femtosecond fiber lasers are starting to appear [7].

In this work, we show a design and implementation of a compact multiphoton microscope featuring a femtosecond fiber laser as a light source.

The base of the laser source was a commercially available erbium-doped femtosecond fiber laser (Menlo T-Light). It generated 90 fs pulses with 110 mW of average power and a repetition rate of 100 MHz [8]. We performed frequency doubling to convert the laser optical spectrum from 1560 nm (central wavelength) to 780 nm. This way, we matched the laser spectrum to the two-photon absorption spectra of molecules, such as melanin or chlorophyll [9].

We performed the second harmonic generation (SHG) using a MgO:PPLN crystal (Covesion MSHG1550, 500 μm thickness). The crystal featured various poling periods for quasi-phase-matching (within 18.5 to 20.9 μm) and is mounted in a heating oven. Different focusing and collimating lenses were tested to optimize the setup for the highest possible power. Figure 1(a) demonstrates a comparison of multiple focusing lenses with focal lengths

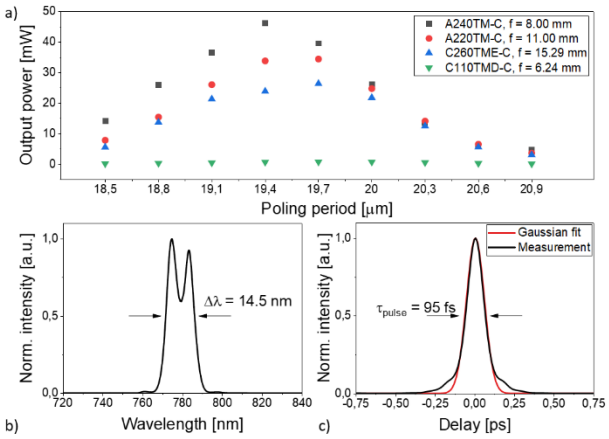

Fig. 1. Comparison of the output power of the generated second harmonic (λ = 780 nm) using a collimating lens of f=15.29 mm and focusing lenses with different focal lengths (top). Characteristics of the

* E-mail: alicja.kwasny@pwr.edu.pl

output pulse in the configuration with the highest output power: optical spectrum (b) and autocorrelation of the output pulse (c).

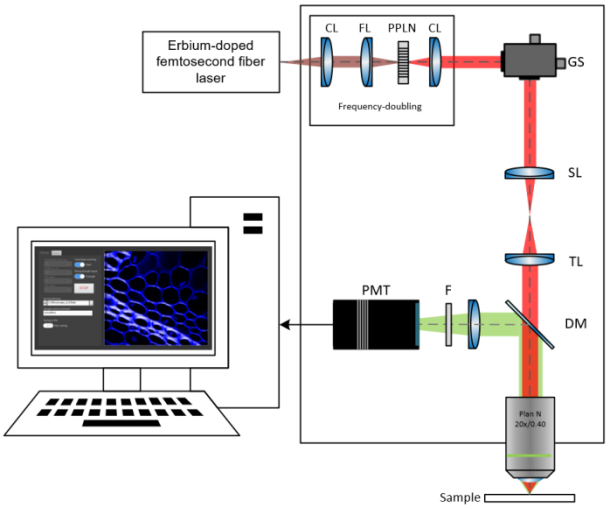

Fig. 2. Experimental setup of the developed laser source and constructed scanning laser microscope (CL – Collimating Lens, FL – Focusing Lens, SL – Scan Lens, TL – Tube Lens, DM – Dichroic Mirror, F – Filter, PMT – Photomultiplier Tube).

varying from 6.24 to 15.29 mm. Generally, the shorter the focal length, the higher the output power, although in the case of f=6.24 mm (C110TMD-C, Thorlabs) the resulting power is low because of a too short working distance. With an average output power of 44.3 mW we obtained final conversion efficiency of about 40.3% for poling period of 19.1 μm at 65°C crystal temperature. Figures 1(b) and 1(c) present the measurements of the optical spectrum and autocorrelation of the output pulse. The spectrum is centered at 778.84 nm with a full width at half maximum (FWHM) of 14.5 nm. The pulse duration is 95 fs, assuming a Gaussian pulse shape. After SHG stage, the laser was coupled to the multiphoton microscope.

Figure 2 shows the microscope system, designed to fit a compact breadboard (45×60 cm). Firstly, the collimated beam was directed to the galvanometric scanner (ScannerMAX Saturn-5), scanning the sample point-by-point. Then, the light passed through a scan lens (Thorlabs SL50-2P2) and tube lens (Thorlabs TTL200MP) to the objective (Nikon N20X-PF) and illuminated the sample.

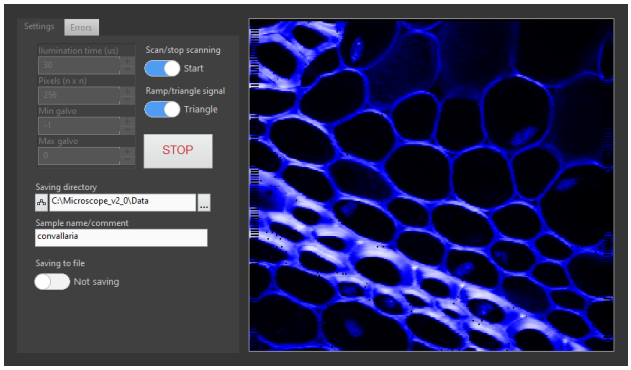

Fig. 3. Graphical User Interface (GUI) of the developed control application. The user can control multiple parameters, such as e.g., illumination time.

As we used an epi-illumination system, fluorescence light was collected using the same objective. It was separated from the residual illumination light by a dichroic mirror

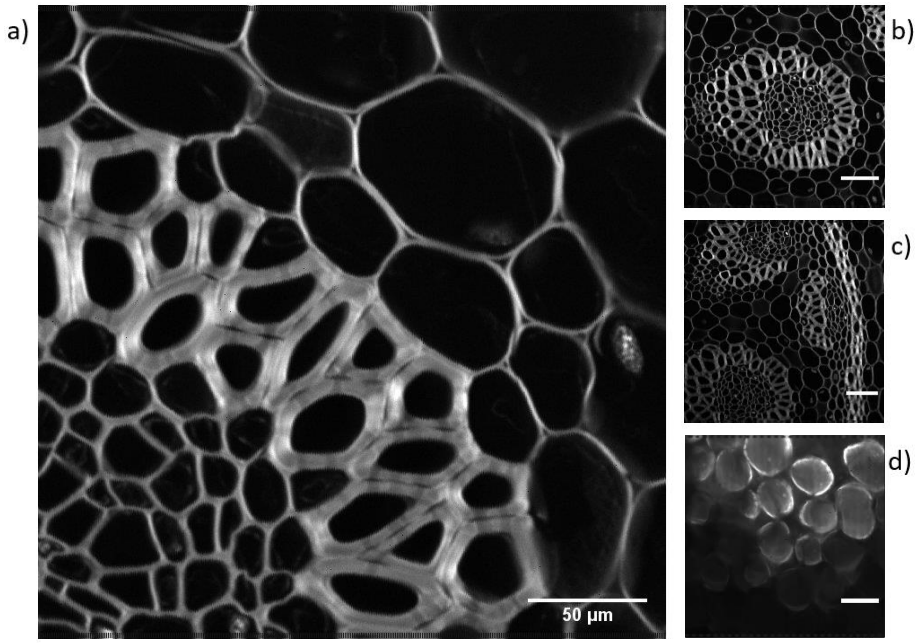

Fig. 4. Images acquired using the developed microscope: (a)–(c) Convallaria rhizome, (d) Matthiola incana. Scale bar in (b)–(d) indicate the length of 100 μm.

(Semrock HC 705 LP) and a shortpass filter (Thorlabs FESH0700) with a cut-off at 700 nm. The fluorescent light was then collected by a photomultiplier tube (Thorlabs PMT1001/M, with 230–920 nm range) and data acquisition card (NI-6363). We developed a custom control system using NI LabVIEW. Both galvanometric scanners, as well as PMT were connected via an NI DAQ card, providing simple integration with the computer. A structure based on producer-consumer architecture ensures fast operation, which is crucial for correct image acquisition in this application. The modular structure of the software leaves space for easy implementation of new features. In the graphical user interface (shown in Fig. 3), the user has control over multiple variables, such as the illumination time of one pixel, the size of the picture (in pixels), the range of the galvanometric scanners, and the type of scanning pattern. Moreover, the image can be saved to a file with the name and format specified by the user.

In Fig. 4, we present four images acquired by our microscope setup. Three of them [(a)–(c)] show Convallaria rhizome, stained with acridin orange for such imaging techniques. Figure 4(d) presents a freshly picked plant leaf of Matthiola incana. The sample was not prepared in any way, thus the observed signal comes only from autofluorescence. We suspect that chlorophyll is the fluorescence source in those particular samples.

Next, the fluorescence intensity was measured for different input power levels to prove that we obtained a two-photon excited fluorescence. As stated in [10, 11], the number of absorbed photon pairs per second in the two-photon excitation process (N_a) determines the fluorescence intensity:

$$N_a \propto \frac{1}{f_{rep}} \cdot \frac{\delta}{A^2 \tau} \cdot P_{avr}^2, \quad (1)$$

where: f_{rep} is the pulse repetition rate, δ is the two-photon absorption cross-section, A is the size of the focus beam spot, τ is the pulse duration, and P_{avr} is the average excitation power. We calculated a quadratic curve fit using the obtained points to check whether the results followed the dependency on the average power square. As shown in Fig. 5, the obtained $y = 1.9x^2–31.3$ fits well with the measurement points.

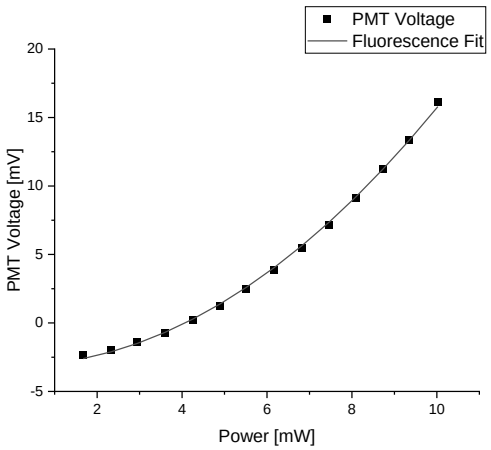

Fig. 5. PMT Voltage measured for different excitation power values. The accuracy of the fit ($y=ax^2+c$) proves that the obtained optical signal is two-photon excited fluorescence.

In summary, we presented a fully functional multiphoton microscope built from scratch from commercially-available components. Using a light source based on a fiber laser shows that those kinds of lasers are a great candidate to replace Ti:sapphire lasers in bioimaging applications. In the future, we plan to add a programmatically-controlled stage for the sample, allowing us to perform 3D imaging.

The presented work was funded by the National Centre for Research and Development (NCBR), project no.: LIDER/32/0119/L-11/19/NCBR/2020 and Research subsidy of the Department of Field Theory, Electronic Circuits and Optoelectronics (K35), Wrocław University of Science and Technology.

References

[1] J.W. Lichtman, J.A. Conchello, Nature methods **2**(12), 910 (2005).
[2] W. Zipfel, R. Williams, W. Webb, Nat. Biotechnol. **21**, 1369 (2003).
[3] Coherent, Chameleon Ultra Datasheet (2019).
[4] J. Boguslawski *et al.*, J. Clin. Invest. **132**(2), e154218 (2022).
[5] M.J. Marzejon *et al*., Biomed. Opt. Expr. **12**, 462 (2021).
[6] A. Fast *et al*., Sci. Rep. **10**, 18093 (2020).
[7] D. Stachowiak *et al*., Biomed. Opt. Expr. **13**, 1899 (2022).
[8] MenloSystems, T-light Femtosecond Fiber Laser 1560 nm (2013).
[9] J. Yao, L.V. Wang, Laser and Photonics Rev. **7**, 758 (2013).
[10] D. Stachowiak *et al.*, Biomed. Opt. Expr. **11**, 4431 (2020).
[11] B.R. Masters *et al.*, J. Biomed. Opt. **9**, 1265 (2004).

