Multiphoton excitation imaging via an actively mode-locked tunable fiber-cavity SOA laser around 800 nm

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Abstract: In this study, an active mode-locked tunable pulsed laser (AML-TPL) is proposed to excite picosecond pulsed light with a rapid wavelength tunability of approximately 800 nm for multiphoton microscopy. The AML-TPL is schematically based on a fiber-cavity semiconductor optical amplifier (SOA) configuration to implement a robust and align-free pulsed light source with a duration of 1.6 ps, a repetition rate of 27.9271 MHz, and average output power of over 600 mW. A custom-built multiphoton imaging system was also built to demonstrate the imaging performance of the proposed AML-TPL by comparing with the commercial Ti:Sapphire femtosecond laser. Two-photon excited fluorescence images were successfully acquired using a human breast cancer cell line (MDA-MB-231) stained with acridine orange.

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1. Introduction

Nonlinear imaging methods, including multiphoton microscopy (MPM), second-harmonic generation, and third-harmonic generation, have become important tools for high-resolution and non-invasive imaging of thick biological tissues [1–4]. Compared to conventional linear optical imaging, MPM techniques can be operated in various wavelength regions owing to the nonlinear mechanism. Because longer wavelength lasers have lower light scattering and absorption coefficient in biological samples, MPM has been successfully used for the deep tissue in vivo imaging by exciting wavelengths twice or thrice longer than that of single-photon microscopy [5–7]. In addition, MPM techniques can be applied to obtain various functional images using various nanomaterials for multiphoton-excited photoluminescence (PL), such as quantum dots [8], nanorods [9], nanowires [10], and nanosheets [11,12]. Because the multiphoton-excited PL of nanoparticles has unique properties, such as excitation and emission wavelength dependence, polarization dependence, and chirality dependence, it can provide functional information in addition to the conventional morphologic information [8,13,14]. For this reason, MPM has become a powerful tool for biological and clinical imaging; thus, it has been extensively involved in neuroscience [15–21], research on tumor/cancer, oncogene [22–27], and photothermal therapy [11,12,28].

The success of deep-tissue MPM imaging fundamentally depends on the invention of novel lasers for pulsed excitation light sources. Generally, solid-state Ti:Sapphire lasers have been utilized as the excitation source for conventional MPM because of their wide spectral tunability, sufficient output power, and ultrashort pulse duration (approximately 100 fs). Under the threshold energy level of optical damage for living tissue samples, Ti:Sapphire lasers can be used as a reliable laser source for multiphoton imaging [29,30]. However, conventional Ti:Sapphire laser
sources have several limitations, including bulky configuration, high price, immobility, and requirements for precise alignment. These shortcomings of the Ti:Sapphire laser sources have become the main reason for slow access to MPM in the laboratory as well as in the medical field.

As a result, intensive efforts have been made to develop compact multiphoton systems for in vivo imaging and clinical applications by adopting novel laser sources. Among the numerous laser types, fiber lasers have been commonly applied to non-linear imaging modalities instead of Ti:Sapphire laser, owing to their compactness, low-loss, and robust configuration [31–35]. In addition, because the alignment in the fiber laser cavity is not necessary compared to the troublesome and unstable process in solid-state lasers, fiber lasers are less susceptible to external influences such as vibration and room temperature fluctuation, which is quite beneficial in terms of maintenance [6,36–38].

Because there are many popular endogenous and exogenous fluorophore materials around 400 nm for single-photon excitation, they are also used as light sources of approximately 800 nm for two-photon excitation, and around 1200 nm for three-photon excitation. For this reason, many researchers have widely been developed fiber lasers in different ways such as Yb-doped fiber with photonic crystal fiber [39], frequency-doubled Er-doped fiber laser [40–42], a gain-switched laser diode (LD) [43], or external cavity mode-locked LD [44] to generate around 800 nm. Wavelength-tunable fiber laser sources around the 800 nm region for MPM have also been proposed with soliton self-frequency shift [45], coherent anti-Stokes Raman scattering [46], or self-phase modulation [47] techniques. However, these technologies are still based on complex configuration, slow tunability, or inefficient non-linearity.

In this study, we propose a novel active mode-locked tunable pulsed laser (AML-TPL) based on a semiconductor optical amplifier (SOA) gain medium for MPM applications. The direct gain modulation technique was implemented to generate a pulsed output under AML conditions. AML lasers are a novel lasing technique for building a swept-source, which has been conventionally used to scan the output wavelength for optical coherence tomography and fiber Bragg grating sensor applications [48–50]. Among the various types of swept-source configurations, almost all swept sources have been operated under continuous mode output in the time domain, but only our AML-type swept sources can generate a pulsed mode laser output in time. This means that the output light from AML lasers can be tuned in both the wavelength and time domains.

To apply AML-TPL as an excitation light source for MPM, the most critical issue is to overcome the threshold to induce multiphoton-excitation. A tapered amplifier (TA) is cascaded to the output port of the developed laser to enhance the average output power of approximately 20 mW from a picosecond-order pulsed output. Owing to the master oscillator power amplifier (MOPA) configuration, the amplified output power from the AML-TPL shows a high average output power (over 600 mW). To demonstrate the MPM imaging performance of the AML-TPL, we obtained two-photon excited fluorescence (TPEF) images from acridine orange (AO)-stained human breast cancer cells using a custom-built MPM system. Our results suggest that the proposed laser can be used as a new alternative pulsed excitation source for MPM.

2. Laser design and characterization

2.1. Theoretical elements of a laser source for MPM

The number of photon absorptions in TPEF with a single laser pulse can be described by the formula reported in [7]:

$$n_a = \frac{P_{\text{avg}}^2}{\tau_p f_{\text{rep}}^2} \left( \frac{NA^2}{2hc\lambda} \right)^2 = \frac{P_{\text{avg}}^2}{\tau_p f_{\text{rep}}^2} S^2$$

(1)

where $P_{\text{avg}}$ is the average output power of the laser, $\tau_p$ is the duration of a single laser pulse, $f_{\text{rep}}$ is the pulse repetition rate of the laser, and $S$ is a system parameter that includes the numerical aperture, speed of light, wavelength, etc. According to Eq. (1), to achieve high photon absorption...
efficiency, both narrow pulse duration and low pulse repetition rate are required. It was reported that a lower pulse repetition rate of 2.5 MHz [4] and 1 MHz [51] allows much deeper imaging depth because a low repetition rate for the fixed average power and pulse duration increases the number of photon absorption, as shown in Eq. (1). It can be explained that high peak power due to a low repetition rate is helpful to enhance signal intensity. It has also been reported that 9.1 times higher TPEF signal can be obtained by reducing a pulse repetition rate from 9 to 1 MHz for the fixed average power [40]. However, there are other reports that extensive high pulse energy due to low pulse repetition rate may induce the photobleaching effect [52,53]. For this reason, various pulse repetition rates were compared in the experiment, and it was reported that a higher pulse repetition rate of 750 MHz can reduce the photobleaching effect compared to lower pulse repetition rates of 21.8 and 94 MHz [54]. According to a previous study considering not only the TPEF image quality but also sample safety, the optimal pulse repetition rates are approximately 20–30 MHz or 20–40 MHz [55]. Therefore, we designed the AML-TPL to have a pulse repetition rate of approximately 30 MHz by adjusting the cavity length. The $n_a$ described above only considers the photon absorption from a single laser pulse. However, in general, each pixel of the TPEF image is not constructed by a single laser pulse. The pixel dwell time and illumination time of the laser per pixel allow photon absorption with multiple laser pulses; therefore, the total number of photon absorptions in TPEF can be described as

$$N_a = \sum n_{\text{group}} n_a = n_a \times f_p \times \tau_{\text{dwell}} = S \times \frac{P_{\text{avg}}^2}{DC} \times \tau_{\text{dwell}}$$

where $\tau_{\text{dwell}}$ is the pixel dwell time, and $DC$ is the duty cycle of the laser. Thus, to check the signal intensity of the TPEF image, it is necessary to analyze not only the characteristics of a single laser pulse but also the total number of pulses applied during the pixel dwell time.

2.2. Active mode-locked tunable pulsed laser

A schematic of the proposed laser is shown in Fig. 1. The laser has an all-fiber ring cavity configuration and consists of an SOA, 50:50 coupler, two polarization controllers (PCs), an acousto-optic tunable filter (AOTF), a fiber optic isolator, and a delay line. The SOA (SOA-352–830, Superlum) was selected to obtain the near-infrared gain around the 800 nm region. The SOA generates a wide gain bandwidth of 55 nm at the center of 830 nm. The AOTF (L2986-01, Gooch & Housego) with a broad transmission bandwidth of 5 nm was also used to select a specific wavelength lasing point within the gain bandwidth. A fiber optic isolator (IS83P23111, Fiberer) and delay line were set between the AOTF and SOA to isolate the backward beam propagation and reduce the pulse repetition rate, respectively. In this setup, the fiber optic delay line was specifically determined to match the pulse repetition rate of approximately 30 MHz. A wideband 50:50 coupler (TW850R5A2, Thorlabs) was used for the output light coupling. Owing to the polarization dependency of the AOTF, two PCs were incorporated into the fiber ring cavity and placed before and after the AOTF for spectral bandwidth maximization.

The SOA and AOTF were independently operated with two function generators (AFG3252, Tektronix). The repetition rate of the AML-TPL was directly determined by the frequency of the SOA control signal, which is the same as the harmonic order of the mode-locking condition of the laser cavity.

$$f_m = N \times \text{FSR} = N \frac{c}{nL}$$

where $f_m$, $N$, and FSR are the mode-locking frequency, integer order of the mode-locking condition, and free spectral range of the laser cavity, respectively. As represented in Eq. (3), FSR can be expressed by the speed of light $c$, the refractive index of the fiber optic cavity $n$, and the total cavity length of the laser $L$. Therefore, the pulse period ($\tau_r = \frac{nL}{c}$) of the actively mode-locked laser is inversely proportional to FSR.
The principle of active mode-locking (AML), the synchronization of the round trips of a photon in the resonator and the amplitude loss of the resonator, leads to the generation of ultrashort pulses. Therefore, the pulsed output can be achieved by modulating the SOA operation in time as the photon passes through the entire laser cavity, involving all fiber optic components.

The pulsed output with AML may be achieved at the integer term of 1, which is called a fundamental mode-locking condition. The integer term of 2 is called a second-harmonic order mode-locking condition. It is well known that it is more difficult to obtain a stable pulsed output using a higher-order, including the second-harmonic order in mode-locking [56–58]. To stabilize the output in the second-harmonic order mode-locking, the timing jitter and phase noise of the fundamental order should be suppressed. Additional equipment, such as a phase-locked loop feedback circuit or self-feedback fiber ring architecture, could be helpful for the suppression process [59,60].

It is also well known that a lower number of mode-locking orders is useful for a wider linewidth AML laser output in the wavelength domain [48,50]. Because the pulse duration of the mode-locking laser is inversely proportional to the linewidth of the laser, a wider linewidth laser is advantageous for acquiring shorter pulses. To make it suitable for MPM applications, we used the fundamental mode-locking condition, which enables a short pulse train and stable pulse generation without any additional pulse stabilization equipment.

Figure 2 shows the pulse characteristics of the AML-TPL. An output pulse train of the developed laser was received by a 400 MHz bandwidth avalanche photodiode (APD430A/M, Thorlabs) and monitored by an oscilloscope (TDS3054C, Tektronix) and a spectrum analyzer (R3267, Advantest). The frequency bandwidth and sampling rate of the oscilloscope were 500 MHz and 5 GS/s, respectively. The frequency bandwidth of the spectrum analyzer was 8 GHz. Figure 2(a) shows the time series pulse train of the laser monitored with an oscilloscope. Figure 2(b) shows the measurement of the spectrum analyzer with a resolution of 300 Hz. The harmonic orders of the fundamental order, 27.9271 MHz, are clearly shown in Fig. 2(b); no additional modes were detected between the harmonic frequencies of 27.9271 MHz. The signal-to-noise ratio at the fundamental order is approximately 63 dB, as shown in Fig. 2(c). The autocorrelation trace of
the developed fiber laser was measured by in situ autocorrelation to represent the pulse duration of the laser, and the results are plotted in Fig. 2(d). The measured pulse duration was 1.57 ps.

**Fig. 2.** The output response of the AML-TPL at fundamental order of mode-locking. (a) Time series and (b) frequency domain responses of the developed laser. (c) The amplitude of fundamental mode-locking frequency and (d) pulse duration.

### 2.3. AOTF

In this study, we incorporated the AOTF into the fiber optic cavity to attain the spectral tunability of the laser in the wavelength domain. The non-mechanical operation of the AOTF results in several attractive characteristics such as accurate wavelength selection and better stability, compared to mechanically tunable filters [61]. The transmission spectrum of the AOTF can be changed according to the frequency of the AOTF control signal, and the center wavelength of the spectrum is inversely proportional to the frequency of the AOTF control signal [62,63]. During the operation of the AOTF, the transducer physically glued to the crystal generates an acoustic wave and acts as a diffraction grating with a specific groove spacing, which is proportional to the frequency of the AOTF control signal. The relationship between the frequency of the AOTF control signal and the wavelength of the diffracted light can be derived as follows:

\[
k = \frac{2\pi}{\lambda} = \frac{2\pi}{\Delta n \alpha V_a} \cdot f_a
\]

where \(\Delta n\) is the birefringence of the acousto-optic crystal, \(\alpha\) is a complex parameter depending on the design of the AOTF, \(V_a\) is the speed of the ultrasound wave, and \(f_a\) is the frequency of the AOTF control signal. According to Eq. (4), the filtered wavelength can be controlled by adjusting the frequency of the AOTF control signal.

Figure 3(a) shows the output spectra of the developed laser at different AOTF control signal frequencies. The spectra were measured using an optical spectrum analyzer (MS9740A, Anritsu).
As the frequency of the AOTF control signal was changed from 65.30 to 68.70 MHz, the output wavelength shifted from 854.24 to 817.04 nm. Figure 3(b) shows the static output spectrum at 65.64 MHz. As shown in Fig. 3(b), the measured 3-dB linewidth of the laser is 1.12 nm at 850.6 nm.

![Fig. 3. (a) Static output spectra of the AML-TPL with different AOTF control signal frequencies. (b) Output spectrum at 65.64 MHz. (Center wavelength: 850.6 nm; 3-dB linewidth: 1.12 nm).](image)

2.4. Tapered amplifier

The AML-TPL, which is based on a single SOA, has an output average power of less than approximately 20 mW. The output is insufficient for exciting fluorophores and acquiring TPEF images from biological samples in vivo and in vitro, considering the optical attenuation of multiple optical components of the MPM, especially the attenuation of the objective lens. To obtain a high average power of several hundreds of mW, a post-amplification system with a tapered amplifier (TPA850P10, Thorlabs) was adopted by cascading to the output port of the proposed laser. It is known that tapered amplifiers (TAs) are useful for the amplification of various spectral ranges of continuous-wave external-cavity diode lasers. They can be applied to a wideband ranging gain while preserving the spectral properties of the seed laser [64,65]. TAs are also widely used for femtosecond or picosecond pulse laser amplification based on a MOPA structure because they have significant advantages in ultrafast carrier dynamics [66]. When a seed laser with pulsed output is amplified using the MOPA method, it is expected that the output of the amplified laser has a synchronized pulse duration and repetition rate with the seed laser.

Commercial SOA fiber lasers with a spectral range of 780–850 nm is still limited to an average power of less than 20 mW. To enhance the output power, TA is the best choice, as it allows single-mode SOA fiber lasers to attain the power of over a hundred mW output power [64]. In this study, we present a TA-based MOPA system around 850 nm, which is matched to the center wavelength of the AML-TPL. A schematic of the MOPA system based on the TA is shown in Fig. 4(a). The overall MOPA system consisted of two isolators, a TA and two relay optics. The first isolator, a fiber optic isolator (IS83P23111, Fiberer), was located between the AML-TPL and TA to avoid back-reflection light from the TA. Another isolator, the free-space isolator (ISO-05-800-BB, Newport), was placed after TA to remove back-reflection light from the relay optics and microscopy system. Placing isolators in appropriate positions is very important for preventing unwanted damage to the laser system. Owing to the strong polarization dependency of the TA, a PC was inserted between the fiber optic isolator and the TA. Two relay optics were incorporated into the free-space beam path to collimate and reduce the asymmetry of the beam profile. Figure 4(b) shows the schematic setup of relay optics 1. As shown in Fig. 4(b), relay optics 1 and lens pair for beam collimation consist of an aspheric lens and cylindrical lens for
The TA and average output power while the average output power of the seed laser, AML-TPL, amplification efficiency in advance. Fig. 5(a) shows the dependency of the applied current on can cause thermal degradation of the TA; therefore, it is extremely important to determine the was fixed at 6.5 mW. The black dotted lines indicate the amplified laser output according to reduce the asymmetry of the beam profile. Fig. 4(b) shows the schematic setup of relay optics 1. As shown in Fig. 4(b), relay optics 1 and lens pair for beam collimation consist of an aspheric lens and cylindrical lens for fast- and slow-axis beam collimation, respectively. Beam after relay optics 1 was well collimated; however, there is still a requirement for reducing ellipticity. Figure 4(c) shows the measured beam profile after relay optics 1, and the derived ellipticity of the beam is 2.36. Figure 4(d) represents relay optics 2 and a cylindrical lens pair for ellipticity reduction. The beam profile after relay optics 2 is shown in Fig. 4(e). In Fig. 4(e), owing to relay optics 2, the beam profile becomes more quasi-Gaussian-like than that in Fig. 4(c), and the measured ellipticity is 1.23.

Fig. 4. (a) Schematic setup of the MOPA system. (b,d) Schematic setup of the relay optics 1 and 2 and (c,e) measured beam profiles after relay optics 1 and 2, respectively.

Fig. 5. (a) Laser output power after the MOPA according to applied current to TA. (b) Measured pulse duration after MOPA.

fast- and slow-axis beam collimation, respectively. Beam after relay optics 1 was well collimated; however, there is still a requirement for reducing ellipticity. Figure 4(c) shows the measured beam profile after relay optics 1, and the derived ellipticity of the beam is 2.36. Figure 4(d) represents relay optics 2 and a cylindrical lens pair for ellipticity reduction. The beam profile after relay optics 2 is shown in Fig. 4(e). In Fig. 4(e), owing to relay optics 2, the beam profile becomes more quasi-Gaussian-like than that in Fig. 4(c), and the measured ellipticity is 1.23.
Amplified spontaneous emission amplification without a seed laser or low seed laser power can cause thermal degradation of the TA; therefore, it is extremely important to determine the amplification efficiency in advance. Figure 5(a) shows the dependency of the applied current on the TA and average output power while the average output power of the seed laser, AML-TPL, was fixed at 6.5 mW. The black dotted lines indicate the amplified laser output according to the applied current. As shown in Fig. 5(a), an average output power of 600 mW is achieved at a current of 2500 mA. Figure 5(b) shows the measured pulse duration after the MOPA system. The measured pulse duration after the MOPA was 1.6 ps, and no significant change was detected. Therefore, the peak power of the amplified laser, calculated considering a pulse duration of 1.6 ps, pulse repetition rate of 27.9271 MHz, and average power of 600 mW, was 13 kW.

3. MPM design and performance analysis

A custom-built MPM system was used with the AML-TPL to demonstrate the imaging performance of the developed laser for MPM. Figure 6 shows a schematic of the custom-built MPM. The laser beam generated from the MOPA system was delivered to a two-dimensional (2D) galvanometer scanner, which was used for 2D raster scanning. The laser beam was then expanded using a beam expander designed with a 4f lens configuration. The 4f lens configuration has been widely used for scanning microscopy to reduce image degradation caused by a focal point shift during beam scanning. Then, the expanded laser beam was delivered to a short-pass dichroic mirror (ZT775sp-2p, Chroma Technology). Finally, the reflected beam from the short-pass dichroic mirror was focused onto the sample using an objective lens (UPlanFL n 40x, Olympus) with an NA of 1.3. The TPEF signals were produced by the focused beam, and the signals from the sample were recollected in the backward direction by the same objective lens and further guided to a photomultiplier tube (PMT). For green color imaging, a band-pass filter (ET525/50M, Chroma Technology) was placed before the PMT. Photons of the TPEF signals were converted to electric signals using a PMT (H10682-210, Hamamatsu Photonics) through photon counting methods. The custom-written LabVIEW code allowed the visualization of the acquired TPEF signals and control of the entire device. The LabVIEW code-based field-programmable gate array module processed several simultaneous tasks, including control of the galvanometer scanner for 2D raster scanning, Z-axis precision stage for focus tuning, and processing of photon counting from the TPEF signals.

Fig. 6. (a) Photograph of the custom-built MPM and the AML-TPL (red box). (b) Schematic of the MPM imaging system.

Capability for TPEF imaging with the proposed laser was assessed by acquiring images of AO-stained human breast cancer cells. The human breast cancer cell line (MDA-MB-231) was kindly provided by the Korean Cell Line Bank (Seoul, South Korea), and the AO dye was obtained
from Sigma-Aldrich Co. (St. Louis, MO, USA). AO dye is a nucleic acid-selective binding dye that emits green fluorescence when bound to the DNA of living cells. Therefore, AO staining is commonly used to visualize nuclear changes and apoptotic cell formation. As can be seen from the excitation and emission spectra of AO in Fig. 7(a), the single-photon absorption peak of the AO dye is located at approximately 502 nm, and the emission peak is mainly located in the green region, 525 nm [67]. The center wavelength of the bandpass filter placed in front of the PMT was specifically selected to match the emission peaks located at approximately 525 nm. Figure 7(b) shows a log–log plot of the TPEF signal against the excitation laser power. As the average excitation power of the laser increased from 5 to 14 mW, the TPEF signal shows a quadratic dependence on the excitation laser power. The linear fitting (red solid line) of the data has a slope of 2.09. This means that the AML-TPL, picosecond pulsed fiber laser, can generate the TPEF signal with commonly used visible dyes; thus, it can be partially used as a substitute for a commercial Ti:Sapphire femtosecond laser for a specific limited material.

![Excitation and emission spectra of AO.](image1)

![Log–log plot of the laser power dependency of the AO fluorescence signal and linear fitting.](image2)

To evaluate the TPEF imaging performance using living cellular targets for the developed laser, MDA-MB-231 cells were incubated using the standard cell culture procedure. These cells were maintained in Dulbecco’s modified Eagle’s medium and cultured in a humidified atmosphere in an incubator containing 5% CO₂ at 37 °C. The cells were then stained with the AO dye to visualize the performance of the TPEF imaging. In brief, the cell specimen preparation procedure was as follows. The incubated cells were washed with phosphate-buffered saline (PBS) and stained by adding 100 mL AO (10 μg/mL) for 5 min. The residual dye was then removed by washing the cells three times with PBS. After staining, the cells were visualized under a custom-built MPM system using two types of laser sources: the developed laser and commercial Ti:Sapphire femtosecond laser.

Figure 8 compares the TPEF images obtained by homemade AML-TPL and commercial Ti:Sapphire laser (Chameleon Ultra II, Coherent). The table below the images indicates several parameters of the lasers used for imaging. According to the table, the number of photon absorptions in TPEF of two laser sources, homemade AML-TPL and commercial Ti:Sapphire laser, are $321 \times 10^{-6} \times S$ and $112 \times 10^{-6} \times S$, respectively. This means that the developed laser has three times higher photon absorption than the Ti:Sapphire laser. However, as shown in Eq. (2), the total number of photon absorptions in the TPEF is determined not only by the output characteristics of the laser but also by the pixel dwell time. The total numbers of photon absorptions in TPEF, which correspond to the signal intensity of the image, of two lasers have the almost same value of around $530 \times 10^{-3} \times S$. Therefore, the homemade AML-TPL was calculated numerically from the table in Fig. 8, and using the average power twice that of the Ti:Sapphire
laser, we can obtain TPEF images of similar brightness. This was also proved experimentally through the acquisition of TPEF images shown in Fig. 8(a) and (b).

\begin{table}
\begin{tabular}{|c|c|c|}
\hline
& Developed AML-TPL & Commercial Ti:Sapphire laser \hline
Average output power ($P_{\text{avg}}$) & 20 mW & 10 mW \hline
Pulse repetition rate ($f_{\text{rep}}$) & \sim 28 MHz & 80 MHz \hline
Pulse duration ($\tau_p$) & 1.6 ps & 140 fs \hline
Peak power ($P_{\text{peak}}$) & 0.448 W & 0.893 W \hline
# of photon absorption in TPEF ($n_a$) & $321 \times 10^{-6} \times S$ & $112 \times 10^{-6} \times S$ \hline
Duty cycle (DC) & 4.47% & 1.12% \hline
Pixel dwell time ($\tau_{\text{dwell}}$) & 60 $\mu$s & 60 $\mu$s \hline
Total # of photon absorption in TPEF ($N_a$) & $537 \times 10^{-3} \times S$ & $536 \times 10^{-3} \times S$ \hline
Image size & 256 x 256 & 256 x 256 \hline
\end{tabular}
\end{table}

**Fig. 8.** (a) TPEF images obtained using homemade AML-TPL and (b) commercial Ti:Sapphire femtosecond laser. The table below the images includes several parameters for TPEF images; $S$: system parameter.

4. Discussion and conclusion

We demonstrated a novel pulsed excitation source with AML-TPL for MPM imaging. Direct gain modulation of the SOA was implemented for tunable AML pulsed output generation at approximately 800 nm. A TA-based MOPA system was proposed to obtain a high-power output to overcome the excitation threshold power. To obtain a well-collimated laser output with low ellipticity, two relay optics were used, which allowed a quasi-Gaussian beam profile with an ellipticity of 1.23. The developed laser was confirmed to have a short pulse duration of 1.6 ps, a repetition rate of 27.9271 MHz, and an average output power of 600 mW, which is sufficiently high for MPM imaging. High-contrast TPEF images of AO-stained MDA-MB-231 cancer cells were acquired to compare images obtained using the commercial Ti:Sapphire laser and AML-TPL sources.

There are a bunch of limitations owing to the use of the developed laser as a substitution of the commercial Ti:Sapphire laser. First, insufficient spectral bandwidth is the main limitation of the developed fiber laser, but extended wavelength accessibility can be achieved by using
SOA of different wavelengths or multiple SOA configurations \cite{68}. Since those approaches increase system complexity and cost, it will be necessary to predetermine the target material and laser wavelength to perform MPM using the developed fiber laser more efficiently. Second, the developed fiber laser shows a pulse duration of 1.6 ps, which is much longer than conventional Ti:Sapphire laser. For this reason, twice higher average power than that of Ti:Sapphire laser was required, and it may cause photodamage of the sample. However, we obtained a sufficient TPEF signal through average power of 20 mW in this experiment, and no significant photobleaching effect was observed during image acquisition. Thus, it will be helpful to use an additional pulse compressing process to improve TPEF efficiency and reduce sample damage.

Despite of those limitations, there is a tremendous demand for the development of compact, portable, and efficient pulsed laser sources for in vivo medical applications of MPM. The proposed MPM was experimentally verified to be a promising alternative laser source for MPM. Moreover, the developed AML-TPL can also be used for various other clinical applications that require pulsed output and spectral tunability.

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**Disclosures.** The authors declare no conflicts of interest.

**Data availability.** The data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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