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Compact diode laser source for multiphoton biological imaging

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Abstract: We demonstrate a compact, pulsed diode laser source suitable for multiphoton microscopy of biological samples. The center wavelength is 976 nm, near the peak of the two-photon cross section of common fluorescent markers such as genetically encoded green and yellow fluorescent proteins. The laser repetition rate is electrically tunable between 66.67 kHz and 10 MHz, with 2.3 ps pulse duration and peak powers >1 kW. The laser components are fiber-coupled and scalable to a compact package. We demonstrate >600 µm depth penetration in brain tissue, limited by laser power.

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1. Introduction

Multiphoton microscopy is a powerful technique for *in vivo* imaging of biological tissue. Long-wavelength excitation takes advantage of nonlinear optical processes in fluorophores and allows imaging deeper than 1 mm within intact tissue [1–4]. The depth penetration of multiphoton microscopy allows investigations of biological structure and function. Optical recording of the activation of neurons *in vivo* using fluorescent indicators such as GCaMP6 is an especially interesting area of research [5, 6].

In vivo imaging of freely moving animals provides a wealth of information compared to fixed-head experiments [6, 7]. However, studies of freely moving animal behavior require miniaturized microscope and laser systems implanted or attached to the animal [6, 7]. In addition, these devices can find use in human-prosthetic interfaces [8, 9]. One of the major roadblocks is the lack of a

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compact and efficient pulsed laser source. This paper presents a compact and miniaturizable diode laser solution.

The main challenge of nonlinear imaging in miniature microscopes is the high peak power required, typically on the order of several kW. For two-photon excitation, the number of fluorophores excited per pulse is [10]

$$N_{exc} \propto \frac{P_{ave}^2}{f\tau}, \tag{1}$$

where P_{ave}, f, and τ are the excitation source average power, repetition rate, and pulse duration, respectively. Two-photon excitation scales as the square of the laser power and inversely with both the repetition rate and pulse duration. Increasing the peak power increases the signal-to-noise ratio and therefore the image quality [1]. Table-top multiphoton laser scanning microscopes commonly use mode-locked Ti:sapphire laser systems [2]. While providing femtosecond pulses and >1 MW peak power [1], optically pumped solid-state lasers such as Ti:sapphire are challenging to miniaturize. In contrast, semiconductor and fiber lasers have potential to be compact and portable and have been demonstrated for two-photon imaging [11–16]. While mode-locking is an effective method for short pulse generation, the pulse parameters are constrained. The repetition rate is fixed by the laser cavity, while the pulse energy of mode-locked semiconductor lasers is limited by gain saturation and two-photon absorption [17, 18]. Amplified gain-switched semiconductor lasers provide an elegant alternative with arbitrary repetition rate, though the pulse durations are limited to ps [19–21]. Reaching fs duration using gain-switched pulses requires additional compression, such as using a time-lens [22].

A time-lens refers to parabolic temporal phase, which causes pulses to compress during propagation through a dispersive medium just as a beam of light is focused by a spatial lens [22]. In contrast to gain-switched lasers, time-lens compression enables electronic control over the pulse parameters, including the pulse repetition rate, duration, and chirp. Time-lens lasers can be entirely fiber coupled and alignment-free. In addition, the time-lens technique is applicable to any seed laser source and does not require specialized semiconductor lasers to generate short gain-switched pulses. Lasers based on the time-lens technique have achieved sub-ps pulses [23–26], ~1 W average power [26–28], and tunable repetition rate [24–26, 28, 29], combining the advantages of mode-locked and gain-switched lasers.

In this paper, we adapt time-lens pulse compression for two-photon fluorescence microscopy. Time-lens lasers are capable of fs pulse durations with arbitrary repetition rates, combining the advantages of mode-locked and gain-switched lasers. In addition, time-lens compression can generate electrically programmable chirp to compensate for dispersion in the microscope components, beyond the mechanical dispersion compensation using prism or grating compressors. Our source operates at 976 nm, which is ideal for two-photon microscopy of common fluorophores. While others have developed time-lens lasers with high power and short pulse duration, to the best of our knowledge, none have reported wavelengths compatible with two-photon excitation of eGFP (enhanced green fluorescent protein). In addition, the laser components are miniaturizable and capable of microchip-scale integration [30–32].

The gain-switched diode laser described in this paper generates 2.3 ps pulses after time-lens compression with peak power exceeding 1 kW and repetition rate tunable between 66.67 kHz and 10 MHz. A wavelength of 976 nm is chosen to match the peak two-photon absorption of common fluorescent markers, including eGFP and YFP (yellow fluorescent protein) [33]. Two-photon fluorescence imaging of GFP-labeled mouse brain tissue is demonstrated using the novel laser source and a standard laser-scanning microscope.

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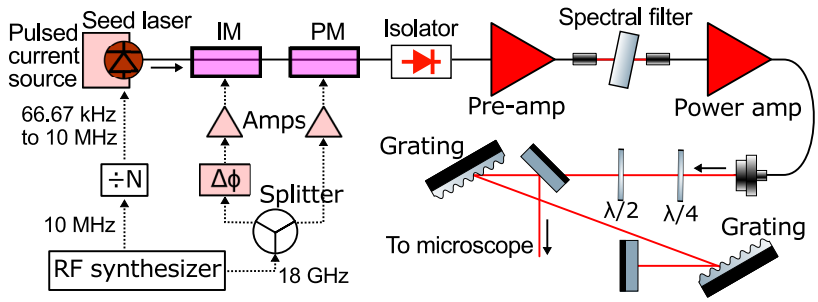

Fig. 1. Laser schematic. A gain-switched diode laser is used as the seed source. Electro-optic intensity and phase modulators (IM and PM) are driven at 18 GHz, generating bursts of chirped pulses. The pulse bursts are amplified in Yb:fiber amplifiers (Pre-amp: pre-amplifier; Power amp: power amplifier) and compressed with a double-passed grating compressor. A spectral filter reduces the amplified spontaneous emission from the pre-amp. Solid lines indicate the optical path; dashed lines indicate electrical paths.

2. Laser design and characterization

Multiphoton microscopy excitation efficiency increases with peak power [1, 10]. The peak power of time-lens laser systems can be increased by reducing the pulse duration from ps to fs [24–27] with additional amplification to Watt-level [26, 27]. Pulse durations for the time-lens technique can be reduced to fs with increased phase modulation amplitude [24, 26, 27] or additional nonlinear pulse compression stages [26]. Additionally, pulse picking to reduce the repetition rate can be used to increase the peak power [26] and average power can be boosted to Watt levels with fiber amplifiers [26, 27].

The laser design, shown in Fig. 1, uses a semiconductor seed laser followed by electro-optic phase and intensity modulators. The seed laser (Innovative Photonic Solutions I0976SB0500P) is gain-switched by a pulsed current source (T165, Highland Technology) producing ~100 ps pulses. Each laser pulse is carved into a burst of ~30 ps pulses using an electro-optic intensity modulator operating at 18 GHz. The individual pulses in the burst pass through an electro-optic phase modulator also operating at 18 GHz. The intensity modulator is driven at ~20 dBm to maximize the peak-to-background transmission, while the phase modulator is driven at the maximum power specification of the modulator, 30 dBm. The peak phase modulation is temporally aligned to the peak intensity using a radio frequency (RF) phase shifter.

The pre-amplifier and power amplifier are based on 10 cm and 16 cm lengths of single-mode Yb:fiber, respectively (YB164, Coractive). The pre-amplifier is pumped with 180 mW at 915 nm, while the power amplifier is pumped with ~300 mW at 912 nm. When operating with high gain, the Yb:fiber amplifiers also produce amplified spontaneous emission (ASE), which is reduced by placing a bandpass filter between the amplifiers.

In previous work [24–26], pulse picking of time-lens laser systems was performed using additional electro-optic modulators. In contrast, we use the seed laser to control the overall repetition rate and minimize loss by using only two electro-optic modulators. The seed laser pulses are triggered by the clock signal from the synthesizer, and a divide-by-N counter enables variation of the pulse repetition rate in submultiples of 10 MHz. For this work, we operated the laser between 66.67 kHz and 10 MHz (limited by the divide-by-N counter), spanning the range typically used for two-photon microscopy [1].

The strong temporal phase modulation broadens the laser spectrum and adds chirp to the pulses. Subsequent group velocity dispersion reduces the pulse duration. To avoid nonlinear effects during compression, we compensate dispersion with a double-pass grating compressor.

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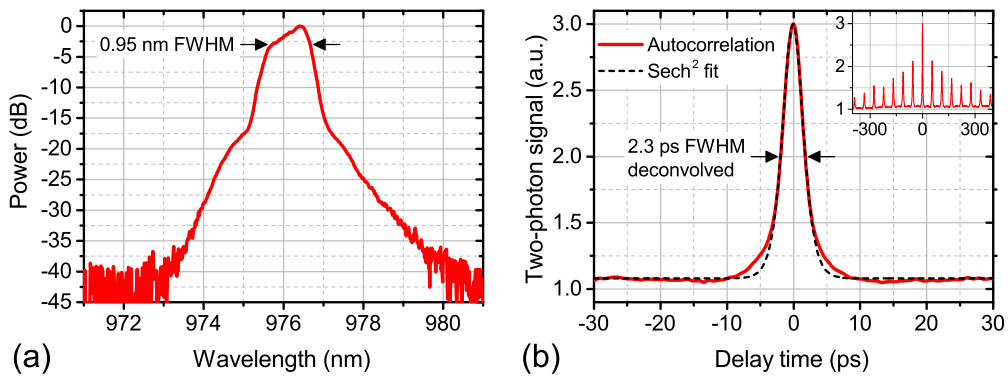

Fig. 2. (a) Optical spectrum of the laser source. The spectral bandwidth is 0.95 nm (-3 dB). The resolution bandwidth of the optical spectrum analyzer was 0.08 nm. (b) Autocorrelation of the laser source fitted to a sech^2 pulse shape (dashed line). The wings of the pulse indicate uncompensated higher order dispersion. The extended autocorrelation (inset) shows the burst duration of ~100 ps. Pulse bursts include several pulses of 2.3 ps duration.

The compressor is a pair of 1800 l/mm gratings separated by ~30 cm, which provides ~9 ps^2 dispersion. The transmission through the compressor is ~40%. The laser spectrum measured after the grating compressor is shown in Fig. 2(a), with a -3 dB bandwidth of 0.95 nm.

The laser produces short bursts of pulses. Figure 2(b) shows the autocorrelation of the primary pulse, while the inset shows the autocorrelation of the pulse bursts, measured after the grating compressor. The burst duration is equal to the duration of the seed laser pulses, ~100 ps, while the individual pulses have 2.3 ps duration. The pulses within the burst are separated by $(18\ \text{GHz})^{-1} \approx 55.56$ ps. The average power is 30 mW with 30 nJ of pulse energy in each burst and an estimated peak power of ~1.2 kW when operated at a 1 MHz repetition rate. At a 10 MHz repetition rate, the average power is 58 mW, with 5.8 nJ per burst and an estimated peak power of ~300 W. Pulse bursts reduce the peak power and are undesirable for nonlinear microscopy. Driving the electro-optic intensity modulator with a short (~50 ps) electrical pulse would reduce these bursts and concentrate more power in a single pulse.

The single electro-optic phase modulator used here limits the spectral bandwidth and the pulse duration. Sub-ps pulse duration can be achieved by increasing the phase modulation amplitude with additional phase modulators, compensating for higher order dispersion, and compressing the pulses in highly nonlinear optical fiber. The average power could be increased by reducing loss in the compressor or increasing the amount of amplification.

3. Two-photon fluorescence microscopy

To demonstrate the capabilities of the laser system, we imaged a ~1 mm thick coronal slice of fixed mouse brain tissue from a transgenic mouse line that expresses GFP driven by proteolipid protein (PLP) expression. The mouse was anesthetized with Nembutal (100 mg/kg) and perfused with 4% paraformaldehyde. The fixed brain was dissected out and sliced using a tissue chopper at approximately 1 mm intervals. The slices were mounted in Fluoromount G (SouthernBiotech) under a #1 coverslip. PLP is localized to oligodendrocytes, which are responsible for myelinating axons in the brain and are found with high density throughout the central nervous system [34].

The microscope setup is shown in Fig. 3. The laser output beam was expanded and sent into the Olympus IX71 microscope. The laser under-filled the back of a 20× objective with 1.0 numerical aperture (Zeiss 421452-9880-000, Plan-Apochromat, water immersion). The calculated resolution of the microscope at 976 nm is 410 nm laterally and 1.7 µm axially [33]. When the

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two repetition rates were compared with the same average power of 8.5 mW, larger fluorescence signal and faster image acquisition were obtained at 1 MHz repetition rate, due to higher peak power. However, the maximum laser power increases from 30 mW at 1 MHz repetition rate to 58 mW at 10 MHz. At this maximum power, the largest fluorescence signal, best image quality, and fastest frame rate were observed at 10 MHz repetition rate. For the demonstration in this paper, the laser was operated with these parameters. The total laser power after the objective was 26 mW, corresponding to peak power of ~135 W. The scan optics and objective transmitted 45% of the total power. Laser scanning was controlled with galvonometric mirrors, and depth scanning was performed with motorized axial movement of the microscope objective. Fluorescence was collected back through the same objective, reflected by a dichroic mirror (Chroma T670lpxr-UF3) in a non-descanned geometry, spectrally filtered (Chroma HQ575/250m-2p), and detected with a photon-counting detector (Hamamatsu H7422-PA-40).

Results are shown in Fig. 4. The full three-dimensional image resolution is 400 by 400 pixels in the lateral (x,y) dimensions and 350 pixels in the axial (z) dimension. The total field of view is ~320 µm (lateral) with ~0.8 µm pixel spacing and 700 µm (axial) with 2 µm pixel spacing. The dwell time was increased from 10 µs to 100 µs to compensate for attenuation in the tissue as the depth was increased. The frame rate increased correspondingly from 1.6 s to 16 s. Figure 4(a) shows a three-dimensional reconstruction of the tissue. The z-axis measures the depth into the tissue and the surface is at $z = 0$. Figure 4(b) shows three slices of the 3D volume in xy planes corresponding to the depths 200 µm, 400 µm, and 600 µm. Each image was individually normalized and processed with a median filter with 0.5 pixel radius.

Cells are still visible at 600 µm depth, and the fluorescence signal reduces to the background level beyond 600 µm. The scan depth is limited by the laser power, and increased peak power should enable deeper imaging depths [1]. The acquisition time for the image stack was 77 minutes, but can be improved with increased average power of the source.

The laser system occupies ~0.5 m^2 of table space and could be packaged to fit a standard equipment rack (~0.5 m by ~0.5 m). To reduce the laser size, the pulse compressor will be replaced with a fiber-coupled solution such as a Bragg grating or an optical fiber. Additional miniaturization is possible with compact laser diode mounts, RF components, and spectral filters. The eventual goal is a chip-scale source. Because two-photon excitation scales inversely with the product of the repetition rate and the pulse duration (Eq. 1), both ps and fs laser pulses can be effective for multiphoton microscopy [35]. For example, a laser with 1 ps pulses and 10 MHz repetition rate has the same two-photon efficiency per pulse [10] as a laser with 100 fs pulses and 100 MHz repetition rate at the same average power. In addition, ps lasers have narrow bandwidth and can be easily propagated through an optical fiber or optical fiber bundle without distortion. Dispersion compensation for ps pulses is not necessary for fiber-coupled multiphoton

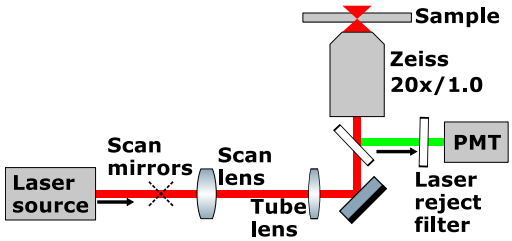

Fig. 3. Two-photon laser scanning microscope schematic. The laser beam is scanned across the sample using galvonometric mirrors and the combination of scan and tube lenses. The microscope objective focuses the the excitation laser and collects the fluorescence light. Fluorescence is separated from the excitation laser using a dichroic mirror and color filter and detected using a photon-counting photo-multiplier tube (PMT).

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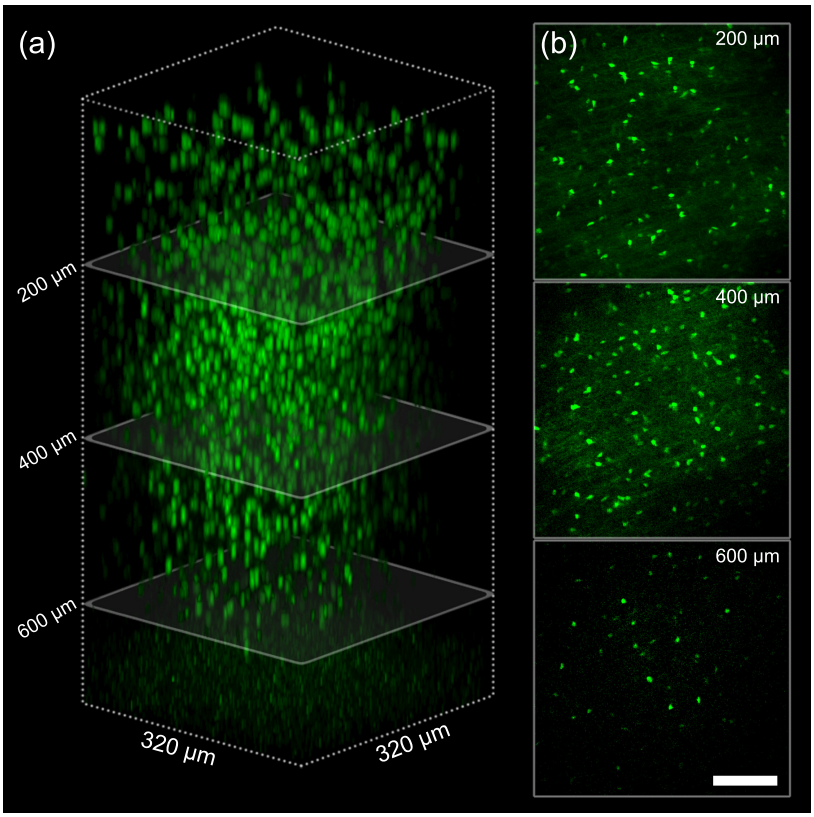

Fig. 4. Two-photon fluorescence images of GFP-labeled oligodendrocytes in *ex vivo* mouse brain slice. (a) Three-dimensional reconstruction. The z-axis corresponds to depth into the tissue. (b) Individual images at specified depths. The laser repetition rate was 10 MHz, and the power at the sample was 26 mW. The dwell time was increased to compensate for the attenuation of the tissue. The images were individually normalized and processed with a median filter of 0.5 pixel radius. The full scale is ~320 μm in x and y dimensions and 700 μm in the z-dimension. The scale bar is 80 μm.

microscopes, simplifying design. In this paper, we have demonstrated high quality two-photon imaging with a ps laser. The miniature pulsed laser source demonstrated here has applications for compact multiphoton microscopy systems. As a portable, fiber-coupled system, the laser could provide benefit for fiber-coupled multiphoton microscopes or endoscopes for optical biopsy in clinical settings. In addition, the technology has potential for neuroscience studies to image the brain in awake behaving animals, when the laser source is miniaturized to chip scale.

4. Conclusions

We have demonstrated two-photon fluorescence microscopy of brain cells beyond 600 µm depth in brain tissue with a pulsed semiconductor laser source at 976 nm. The GFP-compatible laser has tunable pulse parameters, including pulse duration (down to 2.3 ps) and repetition rate (66.67 kHz to 10 MHz). The chirp is also programmable by varying the drive voltage of the electro-optic phase modulator. The components are fiber-coupled, alignment-free, and miniaturizable for portable operation. Only one pair of electro-optic intensity and phase modulators are used for increased efficiency, and no specialized laser diodes are required to achieve short pulse durations. Future designs will use semiconductor amplifiers instead of fiber amplifiers to further improve

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the device efficiency and performance. The laser design can be extended to other wavelengths to excite other fluorescent molecules or implement three-photon excitation. Combined with miniaturized microscope components [36], we anticipate compact, portable, and efficient pulsed laser sources will revolutionize *in vivo* multiphoton fluorescence imaging.

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