Development of two-photon microscope for insect brain imaging

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Introduction

Biomedical optical imaging technologies have been playing an irreplaceable role for both clinical diagnosis applications and scientific researches. The advancement of biomedical optical imaging techniques enables us to observe deeper and finer into biomedical tissues, cells and even subcellular structures. Two-photon microscopy is a nonlinear microscopy based on two-photon absorption where two photons are absorbed by the fluorophore simultaneously [1]. The probability of excitation for two-photon absorption is proportional to the square of optical intensity. Fluorescence emission will only happen in a small volume at the objective focus point, as shown in Fig. 1. Typically, infrared light source is used for two-photon excitation, which suffers less scattering in bio-tissue compared to visible light. Besides, unlike one-photon confocal microscopy where light is absorbed throughout the tissue, two-photon microscopy can penetrate deeper into the sample since light is only absorbed at the focus point. Therefore, two-photon microscopy has much deeper penetration depth (~1mm) compared to conventional one-photon confocal microscopy, making it a great tool for neuroscience researches.

Most commercial two-photon microscope uses Ti:sapphire laser as excitation source. Such solid-state laser can easily achieve high peak power with excellent reliability. However, it is usually bulky and expensive. Fiber laser offers a great alternative for low cost two-photon microscopy systems. Mode-locked fiber lasers have been successfully demonstrated for two-photon and three-photon microscopy [2,3]. Mode-locked lasers combined with wavelength

conversion techniques such as soliton self-frequency shift, optical parametric amplifier or fiber optical parametric oscillators have extended wavelength range to 1200-1700 nm for even deeper tissue imaging [3,4]. Remarkably, a low-cost custom-built ytterbium-doped mode-locked fiber laser has proven its excellent performance in deep tissue imaging [5].

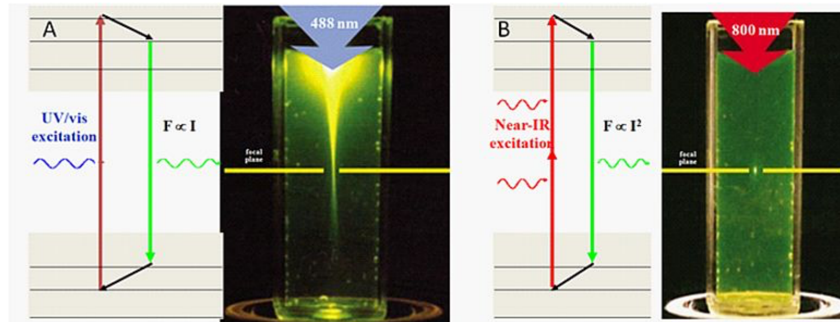

Fig. 1. Comparison of one-photon excitation (left) and two-photon excitation (right).

On the other hand, insect is a suitable subject for neuroscience research. Understanding how insect brain functions and how to simulate it also provide special insight into computer science research [6]. Furthermore, it is of great interest to be able to observe and monitor insect brain in real-time under external stimuli [7]. Concept of combining biomedical experiment and computer simulation is shown in Fig. 2. However, general commercial two-photon microscope has dedicated user software and therefore limited extensibility for such study of interest. For this purpose, it is necessary to construct our own microscope running with real-time capable operating system and high extensibility.

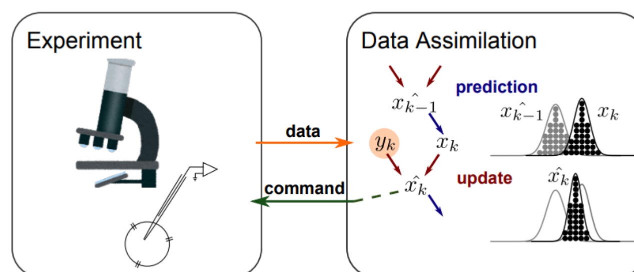

Fig. 2. Concept of combining biomedical experiment and computer simulation.

In this collaboration work, we studied and developed ultrafast fiber laser system with capable peak power for two-photon excitation. A two-photon microscope running with real-time capable operating system and high extensibility was also developed. Insect brain images were successfully taken. Image quality were comparable to commercial confocal microscope. We believe that this system will open up the door for more research opportunities to both labs.

Setup, results and discussion

1. Two-photon microscope

Figure 3 shows the picture of our constructed microscope. Light from the fiber laser system was collimated into free space by (1) collimator. The beam was then expanded from 0.82 mm to 8.2 mm by (2) a beam expander (ThorLabs CFP5-1064A). Next, the beam would pass through a (3) long pass dichroic mirror (semrock Di03-R635-t1) whose cut-on wavelength was 650 nm. Infrared laser light would pass through while excited fluorescence light would be reflected. Light from laser source was focused on (5) sample by (4) objective lens. Fluorescence light reflected by (3) dichroic mirror was directed to (6) tube lens, (7) emission filter and finally reaches to (8) photo multiplier tube (PMT, ThorLabs PMT2101/M). Objective lens was mounted on a Z axis motor. Sample was placed on a XY stage.

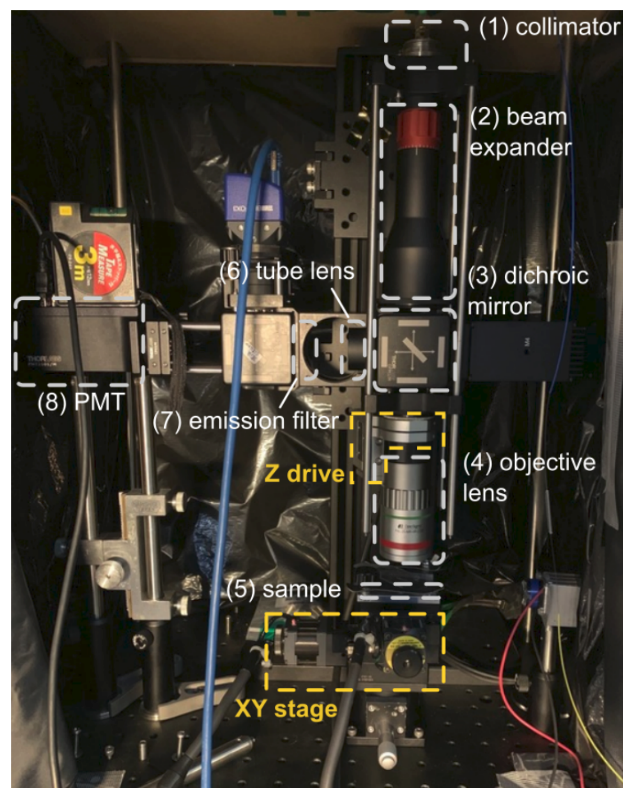

Fig. 3. Microscope optics and optomechanics.

Figure 4 shows the control system of the microscope setup. XY stage, Z axis motor and AOM were controlled by a BeagleBone AI via GPIO digital signal pins. Analog signal from PMT was received by an AD converter cape PRUDAQ and BeagleBone Enhanced. A second BeagleBone AI was used for controlling external stimuli to the insect.

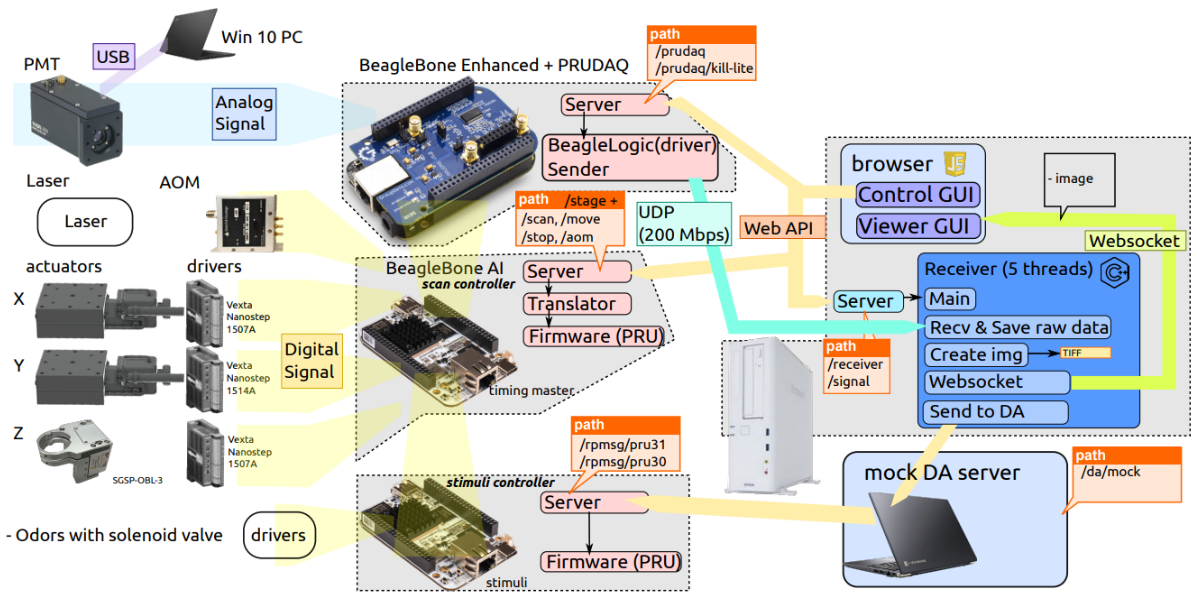

Fig. 4. Control systems of the microscope setup.

2. Fiber laser source

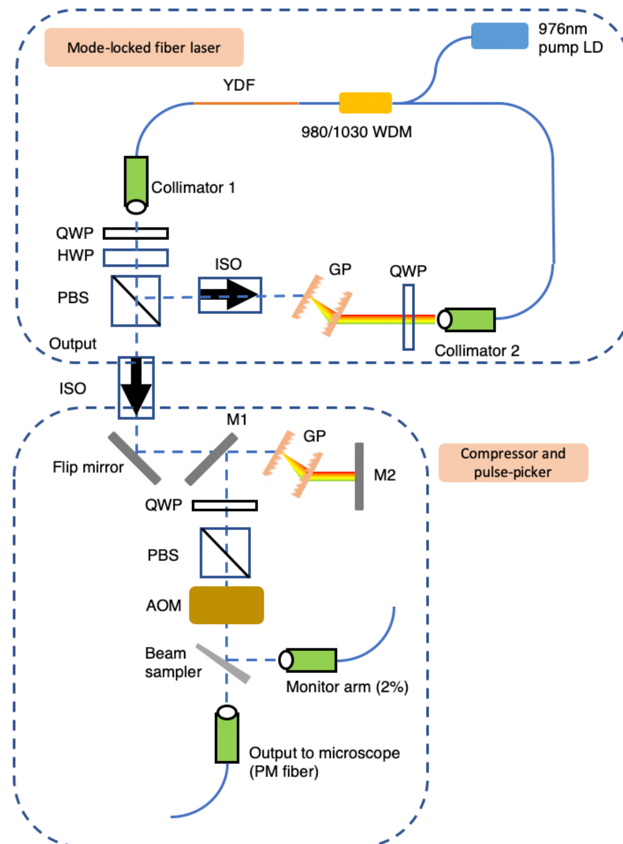

Fig. 5. Schematic of laser system.

Figure 5 shows the schematic of our laser system. It consists of two major part: (1) mode-locked fiber laser and (2) compressor and pulse-picker (power control).

(1). Mode-locked fiber laser was realized by the nonlinear polarization rotation (NPR) mode-locking of an ytterbium-doped fiber laser. A 25 cm long YDF (LIEKKI, YB1200-4/125) was pumped by a 976 nm single mode laser diode (LD). Pump light was coupled into the laser cavity by a 980/1030 nm WDM. The other end of YDF was spliced to a fiber pigtail collimator (HI1060 fiber) with fiber length of 15 cm. Total fiber length between YDF and collimator 2 was 49 cm. Two quarter waveplates (QWPs), one half waveplate (HWP) and a polarizing beam splitter (PBS) enabled the NPR scheme to realize mode-locking operation. An isolator (ISO) inside cavity ensured the unidirectional operation of the laser. A transmission type grating pair (LightSmyth, T-1000-1040) was used for providing anomalous dispersion, with one of the grating mounted on a one-axis stage for fine adjustment of cavity net dispersion. Optical length in free space was ~0.5 m. Overall cavity length corresponded to a fundamental frequency of 167 MHz. Part of the light was coupled out of the cavity via PBS as laser output. In order to generate the highest peak power possible, grating pair distance was set to ~3 mm so that the laser worked at dissipative soliton regime.

(2). Grating pair double-pass configuration was adopted for pulse compressor. The same grating pair as the ones in the laser cavity were used. Distance between the grating pair could be freely adjusted. Pulse compressor compressed our laser output to around ~100 fs pulse duration and allowed flexible dispersion control to compensate for the group velocity dispersion introduced by microscope optics and delivery fiber. An AOM controlled by a BeagleBone AI was placed after the compressor. With the AOM, laser was turned on for 50 μs and off for 25 μs periodically during the imaging process. It could lower down the average laser output power while maintaining high laser peak power to reduce sample damage. Both compressor and AOM introduced ~3 dB power loss. QWP and PBS were used to adjusting laser output to linear polarization state. A wedge shape beam sampler split 2% light as monitoring arm. Rest of the laser light was coupled into a PM fiber patchcord and delivered into the microscope. Typical optical power delivered to the microscope (after objective) was 25 mW under 400 mW pump power. Figure 6 shows the typical spectrum and autocorrelation trace of the laser output after compression. Spectrum had center wavelength of 1030 nm and bandwidth of ~60 nm. Pulse width was ~100 fs. Laser peak power was estimated to be ~1.5 kW at the sample.

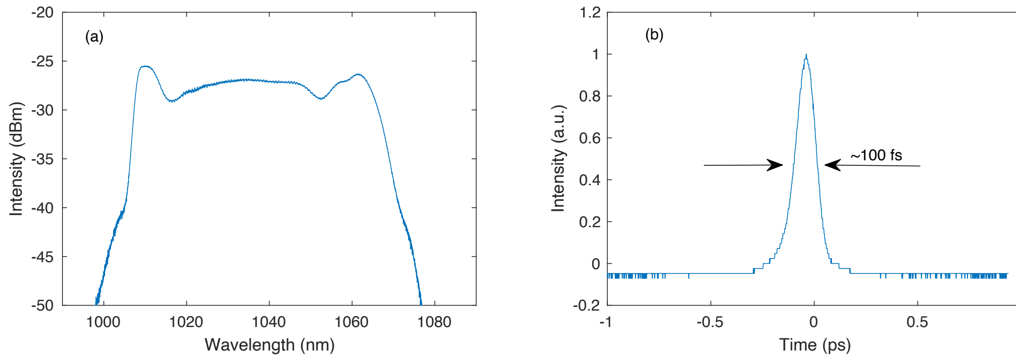

Fig. 6. (a) spectrum and (b) autocorrelation trace of typical laser output.

3. Imaging results and system evaluation

Insect brain images were taken using different samples and dyes. Here we show the images of honey bee brain dyed with Atto488 at different imaging depth (Fig. 7). The first column shows the histogram of two-photon microscopy images to their right side. 1% and 99% are indicated by orange dash lines (lower level means higher intensity). The second column shows the two-photon microscopy images with their corresponding relative depth on the upper right corner (unit: μm). Column three to seven shows the images taken by a commercial confocal microscopy (Zeiss LSM510). Column eight shows the histograms of the images in column three. Scale bar represents 100 μm.

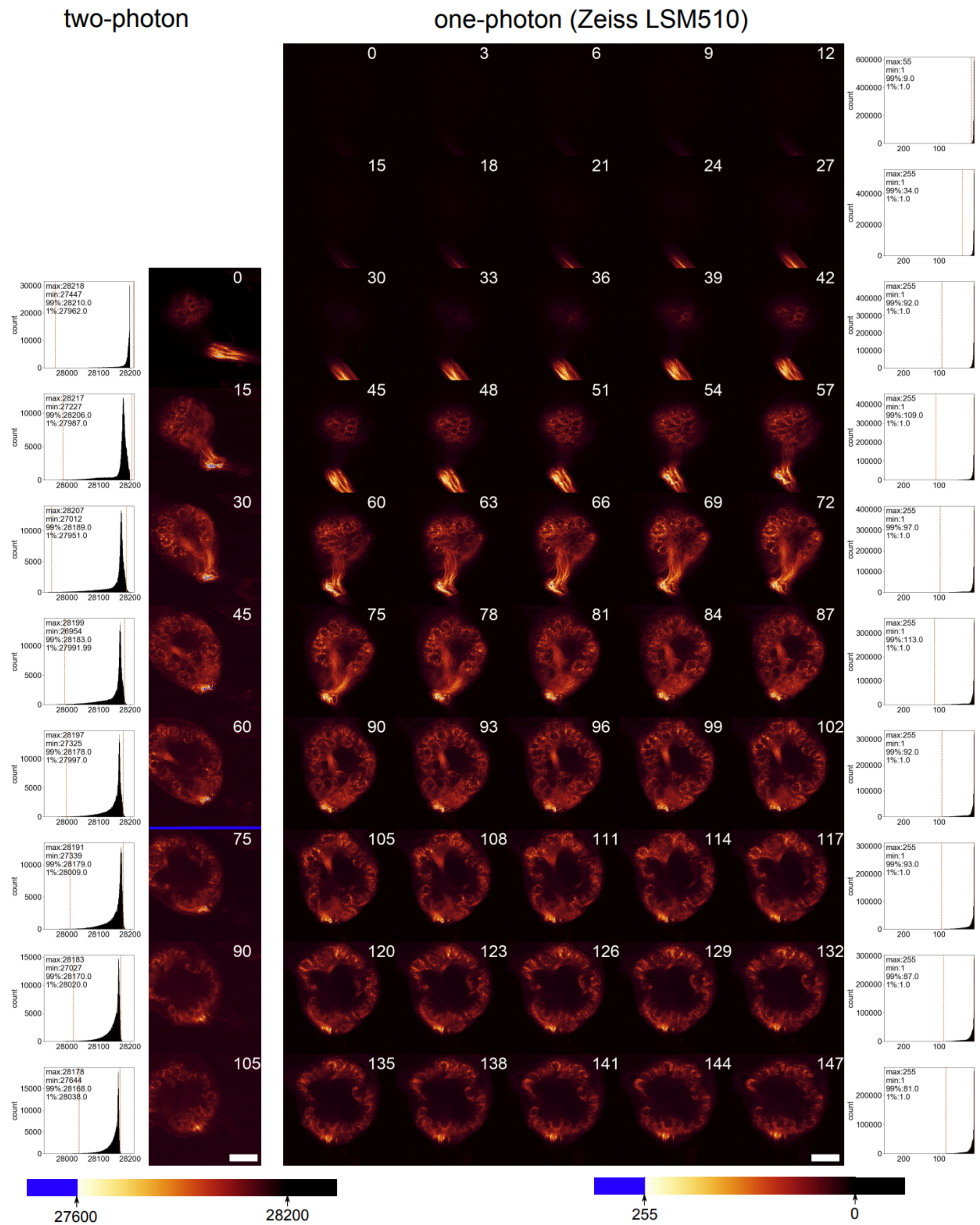

Fig. 7. Images of honey bee brain dyed with Atto488 at different imaging depth. Left: constructed two-photon microscope, right: commercial confocal microscope (Zeiss LSM510)

System evaluation:

(1) Laser source systems: the fiber laser was based on NPR mode-locking technique, which was sensitive to environmental changes such as vibration and temperature. During our

experiment, it was found that sometimes mode-locking could not be initiated, and the laser needed to be re-adjusted. The second issue was the power loss due to compressor and AOM. Original output power directly from laser source could reach over 100 mW. However, after various power losses, we could only get a typical power of ~25 mW after the objective lens. Finer adjustment can potentially help to reduce loss.

(2) Scanning speed: XY stages were used to scan the sample in the setup. Current imaging speed was limited by the scanning speed. For example, it would take 96 seconds to scan a 400x400 μm image. Galvoscanner is necessary in order to achieve higher or even real-time imaging speed.

(3) Wave-shape noise: The lower left part of Fig. 8. A shows the wave-shape noise. It would happen sometime after the X axis stage was started. Figure 8 B shows the noise comparison between different scanning speed. Faster scanning speed led to worse wave-shape noise. In the experiment, images were cropped to get rid of the noise on the left. Switching to galvoscanner can potentially remove this noise.

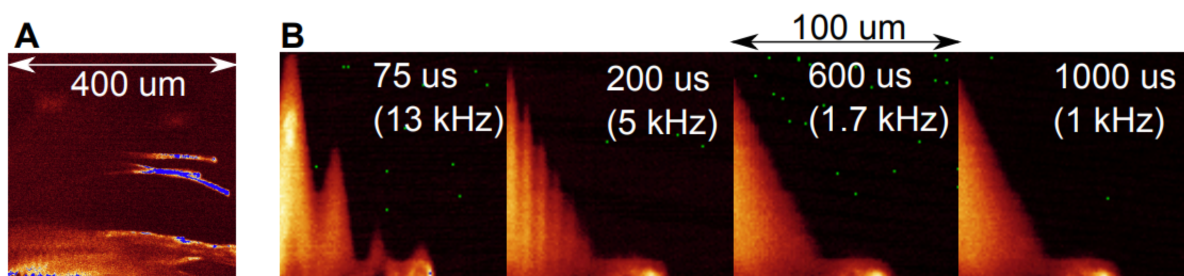

Fig. 8. Wave-shape noise.

(4) PMT trip: the PMT used in the microscope would be tripped when light intensity was too high. Restarting using the control software was necessary after being tripped. Therefore, some images exhibited a few cut lines in it as an example shown in Fig. 9.

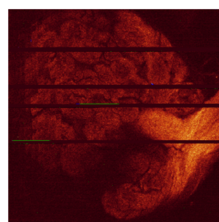

Fig. 9. Image with cut lines due to PMT trip.

Conclusion

In this project, we successfully constructed a two-photon microscope. It was running on real-time capable operating system and allowed high extensibility. We also built the laser system for two-photon excitation. Center wavelength of the laser was 1030 nm. Pulse width after compression was ~100 fs. Laser could provide ~1.5 kW peak power after objective. With this system, we successfully obtained two-photon images of honey bee brain. Results were comparable to commercial confocal microscope. We also evaluated and summarized the problems of the system. We believe that this system can open up the door for more research opportunities to both labs.

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