

Three-Color Two-Photon Three-Axis Digital Scanned Light-Sheet Microscopy (3c2p3a-DSLM)

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Light-sheet fluorescent microscopy (LSFM) or selective plane illumination microscopy (SPIM) is a very powerful tool for biologists to do *in vivo* imaging[1–4]. With low photo-bleaching rate, fast acquisition speed and good axial resolution, LSFM is the optimal choice for long-term observation of model animal development, such as *Drosophila* embryos, *Zebrafish* embryos and *C. elegans*. By combining two-photon fluorescence excitation with LSFM, Truong *et al.* have created two-photon digital scanned light-sheet microscopy (2P-DSLM), allowing for deep-tissue imaging of highly scattering *Drosophila* embryos and of fast beating hearts of *Zebrafish* [2]. To achieve high axial resolution (thin light sheet) and large field of view simultaneously, we have developed a novel two-photon three-axis digital scanned light-sheet microscope (2P3A-DSLM) based on ultrafast axial scanning of illumination focal spot with a tunable acoustic gradient (TAG) index device[5].

Instead of using wave-mixing to create three excitation wavelengths[6], we used two home-made femtosecond fiber lasers with wavelength centers at 780 nm and 1050 nm, respectively. The 780 nm femtosecond pulses were obtained by frequency doubling of a high power 1560 nm mode-locked Er fiber laser. The final spectrum width of about 10 nm, a repetition rate of 100 MHz and a maximum output power of 880 mW (Fig. 1 (b)). A mode-locked Yb-fiber laser with a fiber amplifier was used to generate the 1050 nm pulses with 10 nm spectrum width, 100 MHz repetition rate, and 1.5 W output power (Fig. 1 (c)). The third laser is a commercial Ti-sapphire laser (Coherent), and its wavelength can be tuned between 680 nm and 1080 nm. Therefore, we can simultaneous excite three fluorophores with different colors. Moreover, with the 2P3A configuration [5], we can tailor our lightsheet to any shape between $5 \times 5 \mu\text{m}^2$ and more than $200 \times 200 \mu\text{m}^2$ with constant thickness limited by diffraction and fast imaging rates limited by the detector. The tailorable illumination area allows multi-scale field of view (FOV), and is capable of imaging cells, tissue and live model animals. Overall, our system is flexible in wavelength combination, excitation intensity tuning and variable sizes of field-of-view selection.

The thickness of the lightsheet was maintained at $\sim 1 \mu\text{m}$ in spite of the change in illumination area between $5 \times 5 \mu\text{m}^2$ and more than $200 \times 200 \mu\text{m}^2$, which is optimal to resolve subcellular structure in live organisms. We firstly tested its performance by doing three-color three-dimension imaging of *C. elegans*. DAPI-labeled chromosome, GFP-labeled cytoplasm and dsRED-labeled motor neuron could be excited and acquired simultaneously(Fig.2). Single nuclear and dendrite could easily be resolved (Fig.2). Next we imaged the pancreas islet and blood vessel of a three-day-old zebrafish to evaluate the capability of our system to do fast deep-tissue multiple-color imaging (Fig. 3). β -cells are labeled with EGFP in the Tg (kdr1:EGFP) transgenic fish line, and vascular epithelial cells are labeled with RFP in Tg (kdr1:RFP) transgenic fish line. At this early stage of zebrafish embryo, pancreas islet is small and consists only a few cells. They are buried about 250 μm inside the body of a 3-day-old zebrafish, which is difficult to visualize using one-photon light-sheet microscopy. Two-photon point-scanning microscopy could work but is too slow to catch fast dynamics such as blood flow and calcium waves. Moreover, its high photon-bleaching rates renders long-term imaging impossible. Using our 3C2P3A-DLSM with 920 nm and 1050 nm dual-color excitation, the structure of β -cells and the blood vessels and their migration and vascularization can be well resolved.

An up-to-date light sheet microscopy arrangement (3C2P3A-DSLM) is demonstrated. The 3C2P3A-DSLM combines multiple-color excitation, tailorable light-sheet area and fast three-dimension imaging in one setup. This will facilitate highly flexible two-photon lightsheet imaging within cells, tissue and live model animals of different labels.

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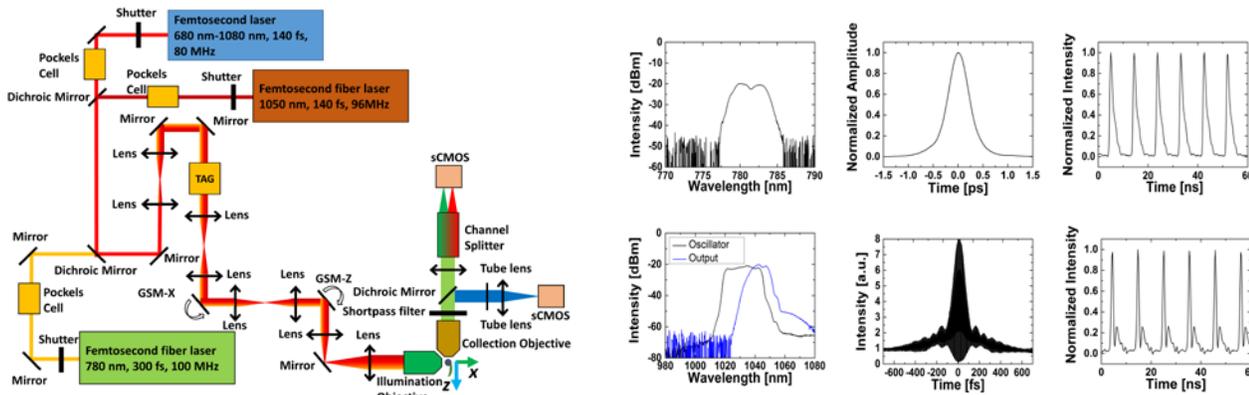


Fig. 1. (a) Schematic illustration of the 3C2P3A-DSLM setup. (b) The spectrum, pulse width and pulse sequence diagram of 780 nm fiber laser. (c) The spectrum, pulse width and pulse sequence diagram of 1050 nm fiber laser.

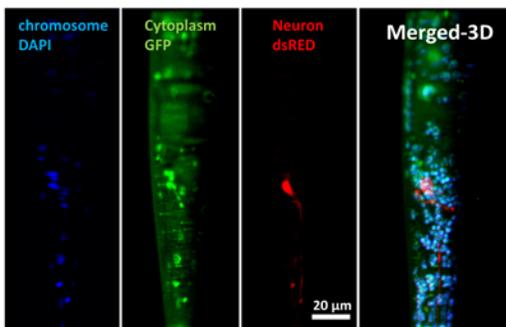


Fig. 2. 3C2P3A-DSLM three-color imaging of *C. elegans*. (a)–(c) Single-channel images of DAPI labeled chromosome, GFP-transgenic cytoplasm and dsRED-transgenic motor neuron, respectively. (d) The 3D reconstruction and three-channel merged image of (a)–(c). Scale bar: 20 μm.

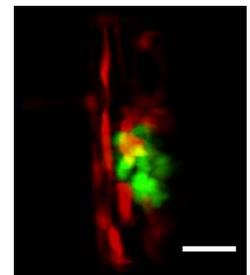


Fig. 3. Two-color 3D fast deep-tissue imaging of pancreas islet and blood vessel in zebrafish. Vascular epithelial cell is transgenic with Tg (*kdrl:RFP*) and showed in Red color. β-cell is transgenic with Tg (*kdrl:EGFP*) and showed in Green color. Scan bar: 20 μm