Microsc. Microanal. 21 (Suppl 3), 2015
doi:10.1017/S1431927615004390 Paper No. 0360

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

Weijian Zong[1, 2,] Zuosheng Liu[3], Fuzeng Niu[3], Shaowa Li[1], Jia Zhao[1], Aimin Wang[3*] and Liangyi Chen[1*]

[1.] The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Molecular Medicine, Peking University, Beijing 100871
[2.] China Department of Cognitive Sciences, Institute of Basic Medical Sciences, Beijing 100850
[3.] State Key Laboratory of Advanced Optical Communication System and Networks, School of Electronics Engineering and Computer Science, Peking University, Beijing 100871, China
[*]Corresponding author: lychen@pku.edu.cn, wangaimin@pku.edu.cn

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 5×5 μm2 and more than 200×200 μm2 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 ~1 μm in spite of the change in illumination area between 5×5 μm^2 and more than 200×200 μm^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 labled with EGFP in the Tg (kdrl:EGFP) transgenic fish line, and vascular epithelial cells are labeled with RFP in Tg (kdrl: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 duel-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.

This research was supported in part by the National Natural Science Foundation of China grants (31327901, 81222020, 31221002, 61475008) and the Beijing Natural Science Foundation (7121008).

References:

1. P. A. Santi, "Light Sheet Fluorescence Microscopy: A Review," J Histochem Cytochem **59**, 129 (2011).
2. T. Truong, W. Supatto, D. Koos, J. Choi, S. Fraser, "Deep and fast live imaging with two-photon scanned light-sheet microscopy," Nat Methods **8**, 757-760 (2011).
3. P.J. Keller and E. H. K. Stelzer, "Digital Scanned Laser Light Sheet Fluorescence Microscopy," Cold Spring Harb Protoc.**5** (2010).
4. T.A.Planchon, L.Gao, D.E.Milkie , M.W.Davidson, J.A.Galbraith, C.G.Galbraith, and E Betzig, "Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination," Nat. Methods **8**,417–423 (2011).
5. Zong W, Zhao J, Chen X, Lin Y, Ren H, Zhang Y, Fan M, Zhou Z, Cheng H, Sun Y, Chen L. Large-field high-resolution two-photon digital scanned light-sheet microscopy. Cell Res. 2014. doi: 10.1038/cr.2014.124. [Epub ahead of print].
6. P. Mahou, J. Vermot, E. Beaurepaire, W. Supatto, "Multicolor two-photon light-sheet microscopy," Nat Methods **11**, 600-601 (2014).

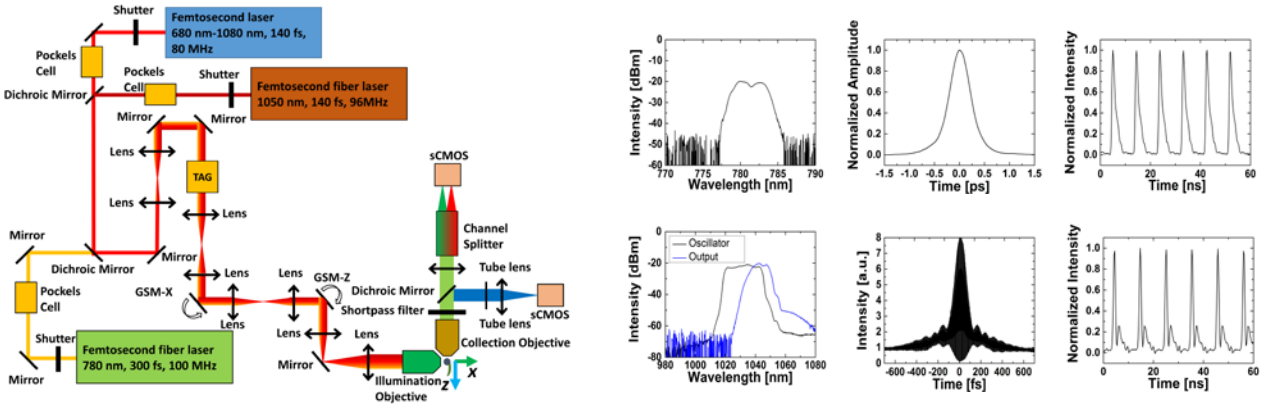

Fig. 1. (a) Schematic illustration of the 3C2P3A-DSLM setup. (b) The spectrum, pulse width and pulse sequence diagram of 780 nm fiber laser. (c) The spectrum, pulse width and pulse sequence diagram of 1050 nm fiber laser.

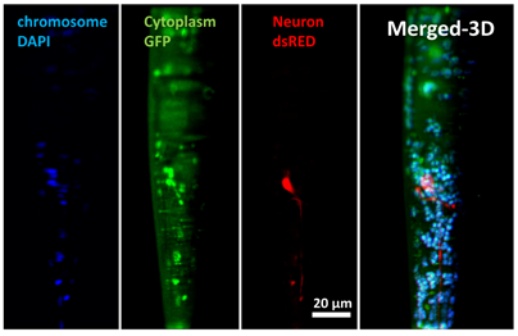

Fig. 2. 3C2P3A-DSLM three-color imaging of C. elegant. (a)−(c) Single-channel images of DAPI labeled chromosome, GFP-transgenic cytoplasm and dsRED-transgenic motor neuron, respectively. (d) The 3D reconstruction and three-channel merged image of (a)-(c). Scale bar: 20 μm.

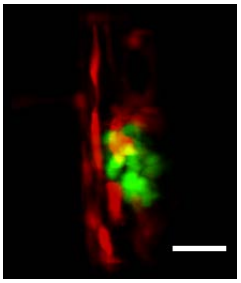

Fig. 3. Two-color 3D fast deep-tissue imaging of pancreas islet and blood vessel in zebrafish. Vascular epithelial cell is transgenic with transgenic with Tg (*kdrl:RFP*) and showed in Red color. β-cell is transgenic with Tg (*kdrl:EGFP*) and showed in Green color. Scan bar: 20 μm