Two-photon excitation spinning-disk confocal microscopy and its biological applications

Kohei Otomo[1, 2], Takafumi Kamada[2], Ai Goto[2], Yumi Yamanaka[2], Tomomi Nemoto[1, 2]
([1] Exploratory Research Center on Life and Living Systems; National Institute for Physiological Sciences, National Institutes of Natural Sciences, Okazaki, Japan; [2] Research Institute for Electronic Science; Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Japan)
E-mail: otomo_at_nips.ac.jp

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Two-photon excitation laser scanning microscopy (TPLSM) is a powerful tool to visualize microstructures in living specimens, because of its superior penetration and less-invasiveness in specimens owing to the near-infrared (NIR) excitation wavelength. Since most of TPLSM systems employ a single-point laser scanning method using moving mirrors, their temporal resolution depends on the speed of the physical movement of these mirrors. On the other hand, multi-point scanning methods have been attempted to achieve high-speed TPLSM imaging. Among them, we have been developed TPLSM systems equipped with a NIR optimized spinning-disk confocal scanning unit (TPLSM-SD) [1]. This presentation will discuss recent technological developments and biological applications of our TPLSM-SD system.

1. TPLSM-SD system requires a high-peak-power laser light source in order to induce two-photon excitation processes at several hundred focal points simultaneously. We introduced high-peak-power 920 nm neodymium laser and 1040 nm ytterbium laser light source for various fluorophores, of which color was from green to red [2; K. Otomo *et al., in preparation*]. As a result of optimizations, the developed system achieved several tens-times brighter fluorescent intensity than using a conventional mode-locked titanium-sapphire laser light source.

2. Conventional TPLSM system uses one wavelength for excitation, resulting in that the number of excitable fluorophores is limited. Besides, to observe multi-color labeled specimens, overlapping of emission spectra of fluorophores caused a crosstalk of fluorescent signals among detection channels. Such a crosstalk causes a complication to distinguish individual targets. To achieve multi-color imaging, we introduced a fast-switching system to select either of two NIR laser light pulses. We applied the developed system to three fluorescently-labeled organelles in tobacco BY-2 cells and mammalian HeLa cells. As a result, the system visualized 3D movements of individual organelles during cytokinesis without severe phototoxicities. Moreover, we utilized a linear-unmixing method to separate these fluorescent signals more clearly, resulting in finer views of each organelle successfully [T. Kamada *et al., in preparation*].

3. TPLSM system enables to visualize fluorescent signals and second harmonic generation (SHG) signals, simultaneously. Recently, we introduced a polarization splitting detection system for our TPLSM-SD system and developed high-speed polarization-resolved SHG microscopy for living mouse tissues [3]. The developed system visualized complicated collagen fiber network structures with information of their lateral orientations. Finally, we demonstrated in vivo polarization-resolved SHG imaging of the collagen fibers in the mouse skeletal muscles at video-rate temporal resolution.

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