Four-color three-dimensional live cell imaging by two-photon excitation spinning disk confocal microscopy

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Two-photon excitation fluorescence laser scanning microscopy (TPLSM) is a powerful tool to visualize microstructures in living specimens. This is because of its superior penetration depth and less-invasiveness in specimens owing to its near-infrared (NIR) excitation laser wavelength. Since most of TPLSM systems employs 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 from near the beginning of the 21st century [1]. Recently, TPLSM systems equipped with a spinning-disk confocal scanning unit (TPLSM-SD), which incorporates a micro-lens array disk and a Nikpow disk containing a set of confocal pinholes, have been developed [2, 3].

Biologists, who have interests in interactions and relative localizations of biomolecules, organelles and cells, have used fluorescence microscopy since its superior ability in multi-color visualization. Conventional TPLSM system use one wavelength for excitation, resulting that the number of excitable fluorophores is limited. In addition, 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. In this study, we first added in TPLSM-SD a fast-switching system to select either of two NIR laser light pulses (910 nm pulses from mode-locked Titanium-Sapphire laser or 1040 nm pulses from Yitterbium-based laser). We applied developed system to four fluorescently-labelled organelles in tobacco BY-2 cells. Here, histones, microtubules, centromeres, and lipid bilayers were labelled with msGFP, mCitrine, mCherry, and FM1-43, respectively. As the result, three-dimensional movements of four individual organelles during cytokinesis were visualized without photo-damages. Moreover, we improved a linear-unmixing method to separate these fluorescent signals more clearly by utilizing differences in absorption and emission spectra. From the 3D data sets, this method reconstructed finer views of each organelles successfully.

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