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Wavelength Dependent Damage in Biological Multi-Photon Microscopy: Ti:Sapphire vs. Cr:Forsterite Lasers

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Summary: Confocal laser scanning microscopy provides significant improvement in axial resolution over conventional epi-fluorescence microscopy by eliminating out-of-focus fluorescence using a confocal pinhole. However due to the linear absorption of the visible/ultraviolet excitation light, conventional single-photon confocal microscopy is not able to provide high penetration capability. The out-of-focus absorption in single-photon confocal microscope would not only cause out-of-focus photo bleaching but also cell damage [1]. With an IR excitation wavelength and two-photon absorption induced fluorescence (TPF), Denk *et al.* [1] demonstrated two-photon scanning fluorescence microscopy. With the quadratic dependence on the laser intensity and with an IR wavelength, TPF scanning microscopy showed high axial/depth discrimination even without a confocal pinhole. The out-of-focus absorption is strongly reduced with the IR wavelength with reduction of out-of-focus photo-bleaching and photo-damage.

However, this microscopy technique needs on-focus absorption and high optical illumination to stimulate nonlinear fluorescence for imaging purpose. This strongly focused laser light would still cause on-focus photo bleaching or even cell damage. Recently, König *et al.* [2] studied the influence of near-infrared (NIR) micro-irradiation from a femtosecond Ti:Sapphire laser at 730-, 760-, and 800-nm on cell vitality and cell reproduction with Chinese hamster ovary cells. With more than 6 mW average power, cells were found to be unable to form clones. Complete cell destruction occurs at an average power more than 10 mW (corresponding to peak intensity on the order of 10^{12} W/cm^2) due to intracellular plasma formation accompanied with intense luminescence. No obvious wavelength dependence among three NIR wavelengths was reported. P.-C. Cheng *et al.* [3] studied plant cell damage under high intensity micro-radiation also with a femtosecond Ti:Sapphire laser at a wavelength of 760 nm. With 6.4 mW average power, corresponding to a peak intensity of 3.9×10^{11} W/cm^2, cell damage was confirmed in mesophyll protoplast of *A. thaliana* with a cellular uptake of neutral red or lost of cytoplasmic calcium. It was observed that the red auto-fluorescence diminished more rapidly in comparison with the green autofluorescence as a function of irradiation time. Increased dosages

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resulted in decrease in overall fluorescence and visible structure damage. In leaves of *M. quadrifolia*, sample breakdown, characterized with intense white light emission, was also observed with similar illumination intensity. These recent reports on the on-focus photo-damage induced by Ti:sapphire lasers in multi-photon fluorescence microscope indicate the importance to reduce possible cell damage while performing this nonlinear fluorescence microscopy with an high intensity light source. One possible solution to reduce cell damage is to choose a different excitation wavelength. This is especially important due to the fact that most TPF microscopes are based on commercially available Ti:sapphire lasers with a laser emission wavelength in the NIR region between 700-1000 nm, which is longer than two-photon absorption threshold but shorter than three photon absorption threshold of protein (with single photon absorption threshold wavelength around UV). The high linear absorbance as well as nonlinear absorbance of plant material in the wavelength region of 700 nm also makes the use of NIR wavelength in multi-photon fluorescence not much different from single photon fluorescence microscopy.

In this paper, we report our study of wavelength dependent on-focus cell damage in multi-photon confocal microscopy with femtosecond IR laser sources. Experiments were performed in selected plant tissues. Two different laser sources were selected: a femtosecond Ti:sapphire laser and a femtosecond Cr:forsterite laser. We selected Cr:forsterite laser as the comparison laser source due to its emission wavelength (1230 nm in this study) longer than the three-photon absorption threshold of protein. Similar to previous studies [2,3], with more than 10 mW micro-irradiation at 800 nm from a femtosecond Ti:sapphire laser, multi-photon absorption induced cell damages due to plasma generation, accompanied with strong luminescence, can be frequently observed in our studies. The dosages (illumination time) required to produced plasma generation (characterized by strong luminescence) were found to decrease with increased illumination intensity. In contrast, with a longer output wavelength from a Cr:forsterite laser at 1230 nm, multi-photon absorption and auto-fluorescence were found to be significantly suppressed and the cell damage was found to be greatly reduced. Sustained multi-photon spectra can be observed in most plant specimens even with a tightly focused Cr:forsterite laser beam, with similar or higher intensity enough to produce plasma generation at 800 nm wavelength (with over 100 mW average power), under long term irradiation.

Reference:

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