Multi-photon fluorescence microscopy: behavior of biological specimens under high intensity illumination

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ABSTRACT

Recent development in multi-photon fluorescence microscopy, second and third harmonic generation microscopy (SHG and THG) and CARS open new dimensions in biological studies. Not only the technologies allow probing biological specimen both functionally and structurally with increasing spatial and temporal resolution, but also raise the interest in how biological specimens respond to high intensity illumination commonly used in these types of microscopy. We have used maize leaf protoplast as a model system to evaluate the photo-induced response of living sample under high intensity illumination. It was found that cells can be seriously damaged by high intensity NIR irradiation even the linear absorption coefficient is low in these wavelengths. Micro-spectroscopy of single chloroplast also allows us to gain insight on the possible photo-damage mechanism. In addition to fluorescence emission, second harmonic generation was observed in the maize protoplasts.

Keywords: Multi-photon fluorescence microscopy, photon damage, cell damage, high intensity illumination, maize

1. INTRODUCTION

Multi-photon fluorescence microscopy has been cited for its advantage in the intrinsic axial resolution and in increased depth penetration due to low linear absorption coefficient of biological specimen and many organic polymers in the near infrared (NIR) range[1,2]. Using a pulsed laser, it is possible to efficiently excite two -photon fluorescence with a high peak power while keeping the average power low to minimize thermal and photochemical damages to the specimen. Currently, mode-locked Ti:sapphire and Cr-Forsterite lasers[3] that generate sub-picosecond pulses are used as light source for multi-photon fluorescence microscopy. Because of the need of high peak power for efficiently exciting two-photon fluorescence, the relationship between cell damage and peak power has become an interesting and much debated topic in the applications of multi-photon fluorescence microscopy [4-7]. It is conceivable that at high illumination intensity, non-linear photochemical processes have impacts on cell physiology and viability in ways much different from low illumination in the linear domain. Therefore, it has become a frequently asked question whether there is an optimal peak intensity and pulse width for biological and material multi-photon fluorescence microscopy.

2. MATERIALS AND METHODS

1. Biological specimen preparation

Leaf protoplasts of maize (*Zea mays* L., var. Ohio 43) were isolated from growth chamber-grown seedlings at 4th leaf emerging. Huang and Chen's [8] protocol was followed in the protoplast isolation. The protoplast culture of maize consists of a mixture of mesophyll, epidermal and bundle sheath cells. For microscopic observation, the protoplast was placed in a chambered coverglass (Lab-Tek, Illinois, USA). To evaluate cell survival under high intensity illumination, protoplasts were loaded with 2mM Calcein AM (Molecular Probes, OR) for 15min prior to NIR irradiation.

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In *Optical Sensing, Imaging, and Manipulation for Biological and Biomedical Applications*, Robert R. Alfano, Ping-Pei Ho, Arthur E. T. Chiou, Editors, Proceedings of SPIE Vol. 4082 (2000) • 0277-786X/00/$15.00

2. Optical set-up

Two-photon fluorescence microscopy was performed on a modified Olympus Fluoview confocal microscope equipped with a Coherent Verdi pumped Spectra-Physics Tsunami mode-locaked Ti:sapphire laser operated at 780nm with 100fs pulse at 82MHz. An Olympus water immersion objective (UPLANApo 60x W-PSF, NA=1.2) was used in this experiment. A 650nm short-pass dichroic beam splitter (Chroma Technology, 650DCSP) was used in the illumination beam. The average and peak power densities at the focal point approximate $3x10^6 W/cm^2$ and $3.9x10^{11} W/cm^2$, respectively.

Two-photon induced fluorescence spectra were measured with a SpectraPro-500 spectrometer (Acton Research) equipped with a TE-cooled PMT. The excitation laser beam is derived from a Spectra Physics Tsunami Ti:sapphire laser pumped by a Coherent Verdi solid-state laser at 532nm. The Ti:sapphire laser is mode-locked at 780nm and has a repetition rate of 82 MHz with a pulse width approximately 100fs. A ChromaTech dichroic beam splitter (650DCSP) was used to achieve epi-illumination and on-axis fluorescence detection in a modified Olympus BX microscope. In addition, two IR cut-off filters (Edmond Scientific, Cat. K53-710) were installed in front of the entrance slit of the monochromator to reject scattered IR from the sample. Using this set-up, site-specific spectral information was obtained from the samples[9]. Excitation intensity as high as $10^{12} W/cm^2$ was reached at the focal point. A second set-up using a Spectra-Physics Millennia IR (1064nm) pumped Chromium-doped Forsterite laser (built by CKS), operated at 120MHz and 130fs pulse, was used for 1234nm infrared (IR) excitation.

3. RESULT AND DISCUSSION

The intrinsic optical sectioning capability of two-photon excitation is demonstrated in Fig. 1. Under a microscope objective lens, the fluorescing volume in a dye solution appears only in the vicinity of the focal spot. In addition, the use of NIR and IR as excitation source in two-photon excitation opens the entire visible spectrum for fluorescence detection. Figure 2 shows simultaneous excitation of four fluorescence dyes by 780nm NIR.

Maize protoplasts exhibit a strong red fluorescence peaked at 663nm and a green fluorescence peak at 570nm[10]. The 663nm emission is the result of chlorophyll fluorescence while the origin of the 570nm emission is as yet unidentified. Upon high intensity near infrared (NIR, 780nm) irradiation, maize protoplast shows an initial increase in both green and red auto-fluorescence followed by a rapid decrease in red and green fluorescence (Figure 5). The green fluorescence intensity has a higher rate of increase initially, resulting in a green-shift of the fluorescence image when visualized (i.e. the protoplasts initially fluorescing in red, then rapidly shifted to yellow and finally green). If the ratio of red and green fluorescence intensity is plotted against NIR irradiation, an exponential decade function is evident (Figure 6). When correlated with Calcein dye retention (as a live cell indicator), cell survival can be identified by measuring the red/green fluorescence ratio. Figure 3 shows a sequence of NIR irradiation to Calcein AM loaded maize protoplasts. Micro-spectroscopy study of single chloroplast (C) from maize protoplast (Figure 4a) reveals detailed information on spectral changes as photo-damage proceeds. Figure 4b and 4c shows time-lapse study of the red fluorescence spectrum as a function of irradiation dose. Since we have recently characterized the linear absorption and multi-photon fluorescence properties, maize protoplasts can be used as a model system in the study of cell response to high intensity illumination.

Figure 7 shows emission spectrum of the cortex of a maize stem under high intensity IR (1234nm) illumination. In addition to the 682nm red fluorescence resulting from chlorophylls[11], a small 617nm peak is evident. The 617nm peak is the result of second harmonic generation from maize tissue.

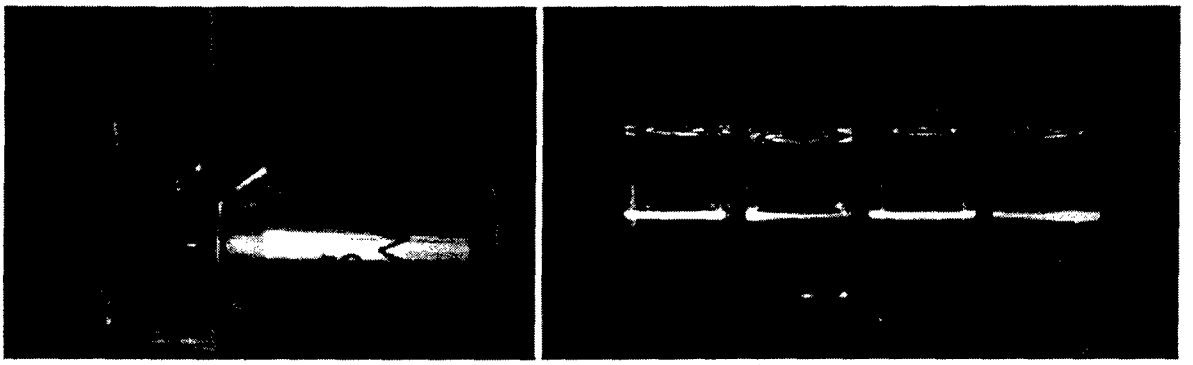

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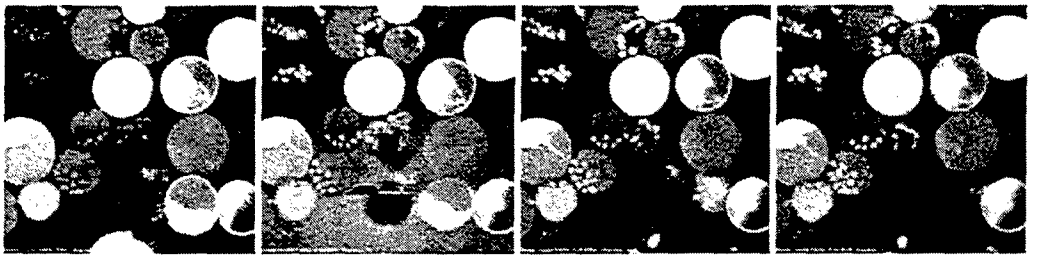
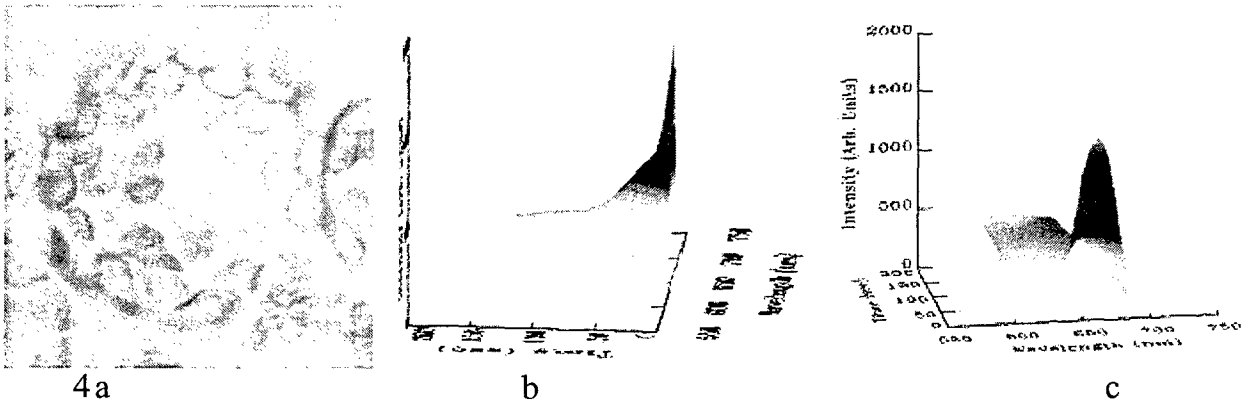
Figure 1. Two-photon excited fluorescence spot in a dye solution. Note the fluorescence generation is restricted to the vicinity of the focal spot, hence provides intrinsic optical sectioning capability. The dye solution was APSS[1] in EtOH. Excitation wavelength was 780nm from a mode-locked Ti:sapphire laser.

Figure 2. Two-photon excited fluorescence in a series of four vials containing dye-solution. The unfocused NIR (780nm) beam enters from the left. From left to right: Calcofluor White, BBTDOT[1], APSS[1] and Rodamine 6G.



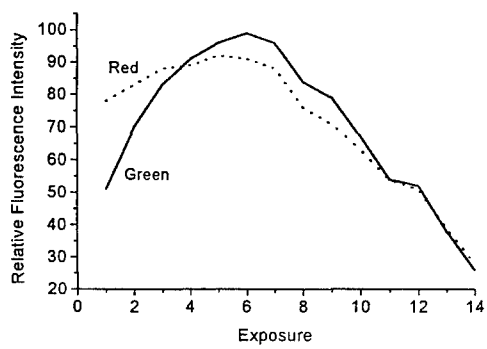
3a b c d

Figure 3. Sequence of NIR exposure to Calcein AM loaded protoplasts. 3a represents the first exposure similar to the dose indicated in Fig 5, 3b: the 3rd exposure, 3c: the 5th exposure, 3d: 7th exposure. Note cell #1 in Fig. 3a expelled its Calcein dye in the 3rd scan, and cell #2 in Fig. 3b busted in the 5th scan.



4a b c

Figure 4. (a) maize protoplast showing numerous chloroplast (c). (b) and (c) different prospective of a 3D plot of two-photon excited red fluorescent spectrum against NIR irradiation dose. The data was obtained from a single chloroplast.



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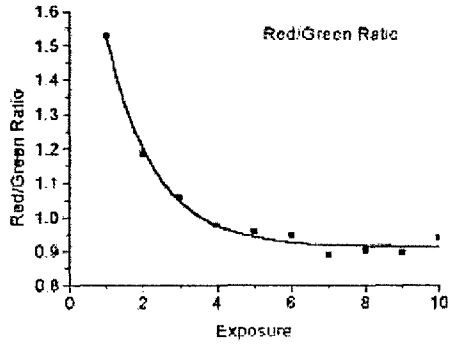

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Figure 5. Normalized auto-fluorescence intensity plot against NIR exposure. The total energy exert on each pixel is 54nJ x number of exposures.

Figure 6. Fluorescence intensity ratio (red/green) plot against NIR exposure showing an exponential decay function.

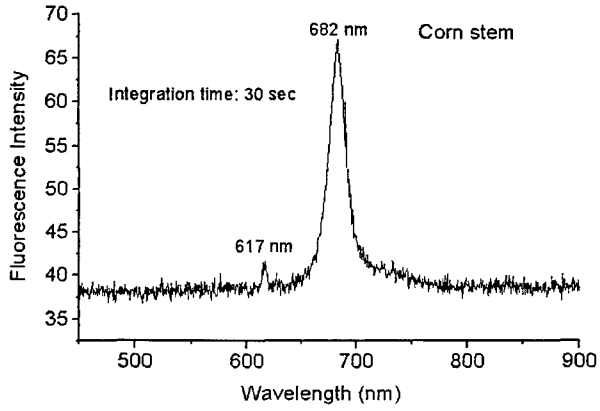

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Figure 7. Emission spectrum of the cortex region of a maize stem showing red fluorescence (682nm) and second harmonic generation singal at 617nm. The illumination wavelength is 1234nm from a Chromium-doped Forsterite laser, operated at 120MHz and 130fs pulse.

ACKNOWLEDGEMENTS

Supported by Academia Sinica (BLL), National Science Council, Republic of China [NSC-88-2311-B-001-087 (BLL), NSC-89-2112-M-110-016, NSC89-2216-E-110-003 (FJK), NSC-88-2811-B-001-0023 (PCC)], the Academic Excellence Program of the Ministry of Education (89-B-FA08-1-4), and Mr. Jin-Mu Huang and Mrs. Li-Ling Huang of Aurum Belle Investment Co., (on behalf of the Ge-An Charity).

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