

Fluorescence detection of protoporphyrin IX in living cells: a comparative study on single- and two-photon excitation

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1 Introduction

Photodynamic therapy (PDT) has been established as a new modality for some medical indications during the last two decades. The principle of this modality is that the photosensitizing drugs preferably accumulate in a lesion and produce active oxygen species when excited with the appropriate wavelength light to destroy the lesion.¹ Several photosensitiz-

Abstract. Photodynamic therapy (PDT) involves a combination of a lesion-localizing photosensitizer with light and has been established as a new modality for some medical indications. Much evidence has shown the correlation between subcellular localization of a photosensitizer with its photodynamic efficiency. However, the fluorescence of most photosensitizers in cells is weak and easily photobleached. We compare the effect of single-photon excitation (SPE) with that of two-photon excitation (TPE) on fluorescence detection of protoporphyrin IX (PpIX), a potent photosensitizer, in the PLC hepatoma cells *in vitro*. By using laser scanning confocal fluorescence microscopy, both fluorescence images and spectra of intracellular PpIX are studied with SPE of 405- and 488-nm lasers, and TPE of 800-nm femtosecond laser. The 405-nm laser is more efficient at exciting PpIX fluorescence than the 488-nm laser, but causes a considerable photobleaching of the PpIX fluorescence and induces weak autofluorescence signals of native flavins in the cells as well. The 800-nm TPE is found to significantly improve the quality of PpIX fluorescence images with negligible PpIX photobleaching and minimized endogenous autofluorescence, indicating the potential of 800-nm TPE for studying cellular localization of porphyrin photosensitizers for PDT. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2907316]

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ers of porphyrins (Photofrin) and their precursors (5-aminolevulinic acid and its esters) have been officially approved for uses in clinical treatments,²⁻⁵ but a better understanding of the exact mechanism can further improve the efficiency of this therapy. Increasing evidence has shown that PDT efficacy is largely dependent on the intracellular localization pattern of a photosensitizer.⁶ Sensitizers that localize at sensitive and vital sites of cells can destroy the cells efficiently after light activation. However, studying such dis-

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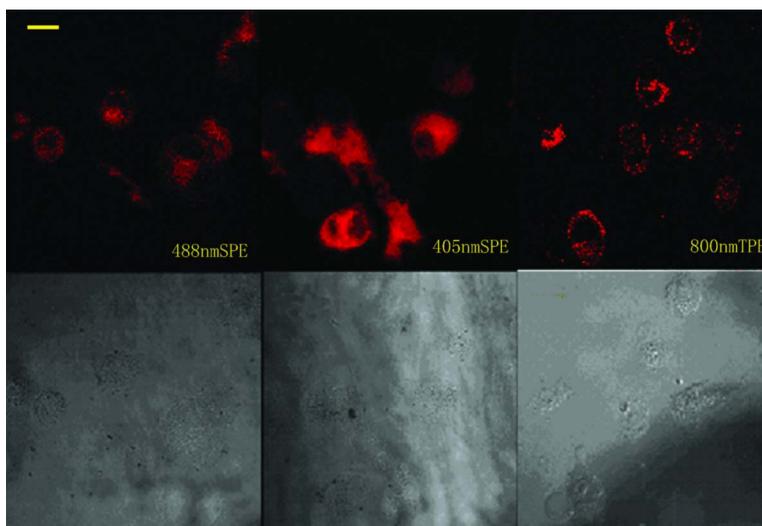


Fig. 1 Fluorescence of cellular PpIX (upper panel) and corresponding DIC (lower panel) images excited at various wavelengths (left to right: 488-nm SPE, 405-nm SPE, and 800-nm TPE, scale bar: 20 μm). Scanning intensities are kept the same for 488 and 405 nm.

tribution pattern by microscopy with conventional light sources (lamps, single-photon lasers) is largely limited by both photobleaching of the sensitizer and autofluorescence of endogenous fluorophores in cells.⁷ There is, thus, a need for a reliable method to study intracellular localization of photosensitizers for effective⁸ PDT. In this study a laser scanning confocal microscope (LSCM) equipped with a spectrophotometer was used to compare single-photon excitation (SPE) by a 405- or 488-nm laser with two-photon excitation (TPE) by an 800-nm femto-second laser in the fluorescence detection of protoporphyrin IX (PpIX), a potent porphyrin photosensitizer for PDT, in the PLC hepatoma cells.

2 Experiments

2.1 Chemicals

Protoporphyrin IX was obtained from Sigma (MI, USA). It was dissolved in 0.1-mM NaOH to make a stock solution of 50 $\mu\text{g}/\text{mL}$ and stored at 4°C at dark for less than 3 days before use. Serum-free Dulbecco modified Eagle's medium (DMEM) with glucose was used to dilute the PpIX stock solution to desired concentrations in experiments. All solutions were freshly prepared in a dark room just prior to experiments.

2.2 Cell Culture

PLC hepatoma cells were purchased from ATCC (ATCC #CRL-8024). The cells were cultured in DMEM supplemented with high-glucose, 2-mM glutamine, penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a humidified 5% CO₂ incubator.

Cells were seeded on the glass slice, which was placed in the middle of 10-cm² culture dishes in the incubator. Thirty-six hours after the seeding, the cells were incubated with PpIX at the final concentration of 5 $\mu\text{g}/\text{mL}$ for 90 min. This relatively short incubation may provide initial sites of intracellular PpIX with less photodynamic damage to cells during

microscopic studies. After drug incubation, the cells were washed with fresh medium 3 times before fluorescence measurements.

2.3 Experimental Measurements

Fluorescence images of intracellular PpIX were acquired with a LSCM (Olympus, FV300, IX71) equipped with a photomultiplier tube (PMT) and a bandpass filter of 585 to 640 nm in a detection channel. Corresponding differential interference contrast (DIC) images were recorded simultaneously in a transmission channel to exhibit the cell morphology. To obtain the best contrast of DIC images, a pair of Wollaston prisms was carefully adjusted. Three beams of lasers were coupled into the LSCM for fluorescence images: 405- (coherent, semiconductor) and 488-nm (Melles Griot, Argon ion) lasers for SPE and 800-nm femtosecond laser (Coherent, Ti:Sapphire) for TPE. To compare the photobleaching effects of the three lasers on cellular PpIX, a series of fluorescence images with time were acquired with each laser. The light intensity of both 488- and 405-nm SPE was 0.37 mW, while that of 800 nm was kept at around 10 mW to generate TPE.

Based on the obtained fluorescence images, some areas of individual cells were chosen to measure microregion fluorescence spectra of the cellular PpIX using the laser point-stay mode of the LSCM system. Instead of scanning, this mode enables, the laser to continuously irradiate the selected spot for taking the spectral measurements. The spectra were measured using a spectrometer (Acton, Spectropro 2150i) equipped with a liquid-nitrogen-cooled CCD (Princeton, Spec-10:100B LN). The fluorescence output from the side exit of the microscopic system was directly focused onto the entrance slit of the spectrometer.⁹

3 Results and Discussion

3.1 Excitation Sensitivity and Resolution

Figure 1 shows fluorescence images of PpIX in the PLC cells excited with the laser of 405, 488, or 800 nm. The corre-

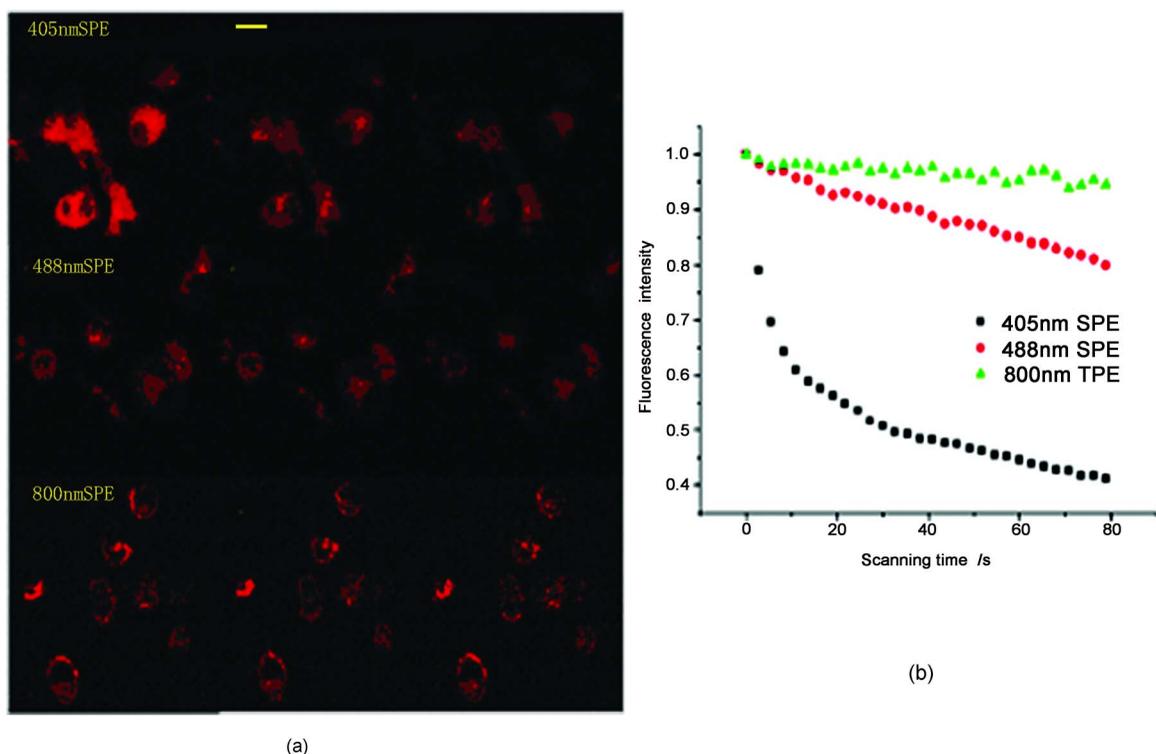


Fig. 2 Photobleaching of cellular PpIX fluorescence. Thirty consecutive fluorescence images were made with the excitation of 405-nm SPE, 488-nm SPE, or 800-nm TPE, respectively: (a) 1st, 10th, and 30th fluorescence images from their respective image series (scale bar: 20 μm) and (b) the normalized average fluorescence intensities of each image from the three image series as a function of exposure time over a period of 80 s.

sponding DIC images were also made to show the morphology of the same cells. The scanning intensities for 405- and 488-nm lasers were adjusted to the same intensity of 0.37 mW for all fluorescence measurements. For the 800-nm femtosecond laser, a higher scanning intensity (10 mW) was necessary to generate TPE fluorescence, as the two-photon absorption of the molecules is generally smaller than the single-photon absorption. These images demonstrate that the 405-nm SPE is more efficient than 488-nm SPE to produce the fluorescence signals of cellular PpIX. This may be due to the fact that 488-nm locates outside the Soret band of PpIX, making a lower excitation efficiency of this wavelength, even though the 488-nm laser can still be used in the PpIX detection.¹⁰ PpIX has been reported¹¹ to have a two-photon absorption cross section of 0.7 to 2.0 GM (1 GM = $10^{-50} \text{ cm}^4 \text{ s}$) around 800 nm. This is much larger than cellular native fluorophores such as flavins¹² and TPE with the 800-nm femtosecond laser is thus also suitable for the PpIX fluorescence measurement. As can be seen in Fig. 1, the PpIX fluorescence image excited with the 800-nm TPE has a granular pattern rather than a diffuse one, suggesting a higher resolution than those excited with SPE of 405 or 488 nm. This is probably due to the fact that 800-nm TPE confines the fluorescence excitation to a smaller volume at the focus and also that the photon flux is insufficient in the out-of-focus layers to excite the fluorescence.

3.2 Photobleaching

When excited with SPE, the photobleaching of PpIX fluorescence causes problems in detecting the distribution of

intracellular¹³ PpIX. However, TPE may reduce the photobleaching effect, as it can produce a smaller excitation volume than SPE in a focusing area and also the photobleaching, if any, does not occur beyond the focus.¹⁴

To compare photobleaching effects of cellular PpIX, 30 consecutive fluorescence images were made with the excitation of 405-nm SPE, 488-nm SPE, or 800-nm TPE, respectively. Figure 2(a) shows the 1st, 10th, and 30th fluorescence images from their respective image series; while Fig. 2(b) demonstrates the average fluorescence intensities of each image for these three image series plotted as a function of exposure time. Although the 405-nm excitation efficiently excited the PpIX fluorescence, it caused a significant photobleaching effect because of a maximal absorption of PpIX at this wavelength. Thus, to acquire the initial distribution of cellular PpIX, the 405-nm excitation may be the good choice; but it is probably not suitable for long-time PpIX measurements. After scanning for 80 s with 800-nm TPE, the fluorescence intensity of cellular PpIX remained 95% of its original level, demonstrating that 800-nm TPE produced a negligible photobleaching effect.

In the LSCM with the SPE of 405 nm, the PpIX molecules in both the in and out focusing layers of the samples are excited and the emitted fluorescence photons are then directed through a detector pinhole to produce a confocal image. However, only the PpIX molecules in the focusing layer give the contributions to the confocal image, while the out-of-focus molecules not only fail to contribute to the useful signals but also cause photobleaching. As a result, the SPE of 405 nm gave rise to a rapid loss of both intensity and contrast in the

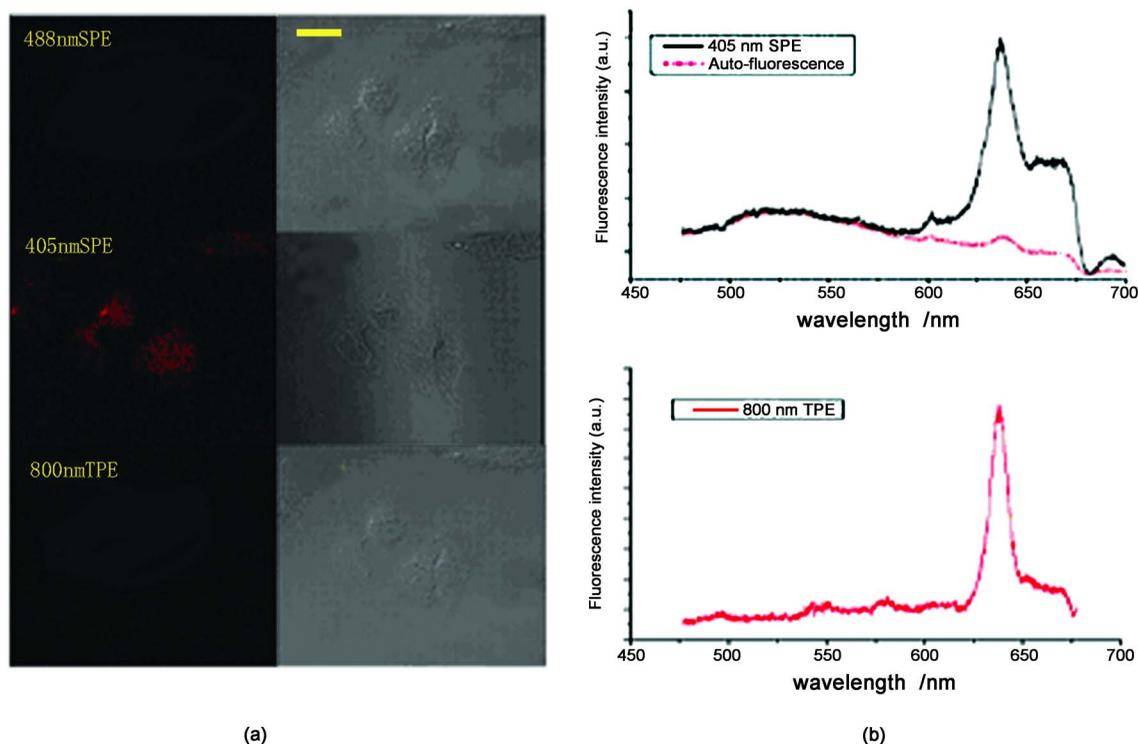


Fig. 3 (a) Autofluorescence (left) and corresponding DIC (right) images of the control cells excited with 488-nm SPE (top), 405-nm SPE (middle), and 800-nm TPE (bottom). Scale bar: 20 μm . (b) (upper): Microregion fluorescence spectra of the control cell and the PpIX incubated cell, excited with 405-nm SPE and (lower): the fluorescence spectrum of the PpIX incubated cell excited with 800-nm TPE. The spectra were captured on selected parts of the fluorescence images with the laser point-stay mode of the microscopic system, as described in Sec. 2.

confocal images. In the case of 800-nm femtosecond excitation, the PpIX molecules absorb the energy of two photons of 800 nm, equivalent to that of 400 nm in the Soret band of porphyrins with a similar extinction coefficient of 405 nm. Since 800-nm TPE occurs only in the center of the focusing layer where the excitation strength density is higher than the threshold,¹⁵ the photobleaching effect from the out-of-focus excitation of PpIX molecules can be avoided.

3.3 Influence of Autofluorescence

Autofluorescence from endogenous fluorophores can interfere with fluorescence measurements of photosensitizers in living cells, particularly in the detection of weak signals. The broad fluorescence spectra of certain native fluorophores such as flavins may have a portion extended into the channel of PpIX detection,¹⁶ overlapping with the real signals of cellular PpIX. It has been reported¹² that native fluorophores generally have a low two-photon excitation cross section (TPECS). For example, TPECS of flavins and NADH under 800 nm are 0.035 and 10^{-4} GM, respectively; while TPECS of PpIX is at least 20 times larger than that of flavins.¹¹ Thus, in principle, TPE can be used to detect cellular PpIX while suppressing the autofluorescence.

To determine the effects of autofluorescence of native fluorophores on the PpIX detection, the autofluorescence images were measured in the control cells with the excitations of these three lasers, respectively. The morphology of these cells were also recorded in the DIC channel. As can be seen in Fig. 3(a), only the 405-nm laser produced some weak auto-

fluorescence signals that may interfere with the PpIX detection. The other two lasers of 488 and 800 nm did not induce any detectable signals. Further, microregion fluorescence spectra of the control and PpIX-incubated cells were compared with the 405-nm excitation to evaluate the extent of autofluorescence interference with the PpIX detection. As shown in Fig. 3(b), there is a broad autofluorescence band of native fluorophores in the control cells peaked around 525 nm, consistent with the fluorescence spectrum of flavins, and also a small peak of 635 nm, probably from endogenous porphyrins. The main autofluorescence band also exists in the fluorescence spectrum of the PpIX incubated cells, and extends to the region around 600 nm that overlaps partly with the fluorescence spectrum of cellular PpIX, resulting in a disturbance for the PpIX detection. The extent of such disturbance depends on the concentration of cellular PpIX. In this study, the effect is about 20% by comparing the spectral area of autofluorescence curve in the 585- to 640-nm region with that of the fluorescence curve of the PpIX [Fig. 3(b), upper]. Generally, if the amount of porphyrin photosensitizer in cells is high enough, the influence of autofluorescence may not be important to study the initial intracellular distribution pattern of the photosensitizer with 405-nm SPE. However, to measure the kinetics of cellular distribution of porphyrins with time, the disturbance of the autofluorescence caused by 405-nm SPE would become a serious problem because, in this case, the PpIX signals have been photobleached during the measurements (Fig. 2). Interestingly, 800-nm femtosecond excitation did not induce any autofluorescence of native fluoro-

phores in the PpIX incubated cells [Fig. 3(b), lower], representing the best SNR.

4 Summary

In conclusion, much evidence has shown that the intracellular localization pattern of a photosensitizer is a determinant for its photodynamic efficiency. However, studying such distribution pattern by SPE light sources is largely limited by both photobleaching of the sensitizer and disturbance of autofluorescence from endogenous fluorophores. The results obtained from this study show several advantages of using 800-nm TPE over 405-nm SPE and 488-nm SPE to generate fluorescence images of cellular PpIX with a higher resolution due to negligible photobleaching of PpIX as well as to induction of no autofluorescence from native fluorophores in the PLC hepatoma cells.

Acknowledgments

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