A Photostable AIE Luminogen for Multifunctional Three-photon Bioimaging

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Abstract— In this study we realized multiphoton bioimaging, by utilizing a kind of fluorescent agent with aggregation induced emission (AIE) feature. The luminogen is called tetraphenylethene-triphenylphosphonium (TPETPP), and it is a highly specific mitochondria tracker. We studied its photostability under three-photon excitation, and found that even under long-term excitation, the three-photon fluorescence signals of TPETPP in the cells still kept very bright. Furthermore, under the same 1020 nm femtosecond excitation, TPETPP assisted three-photon imaging has higher spatial resolution than two-photon imaging of MitoTracker red FM (MT), which is a commercially available mitochondria imaging agent.

1. INTRODUCTION

With the advantages of sensitivity, selectivity, and biocompatibility, fluorescent probes have been a powerful tool for bioimaging and biosensing [1–4].

Multiphoton luminescence imaging, which takes the advantages of high spatial resolution, as well as large *in vivo* imaging depth [5], is playing an important role in both preclinical research and clinical diagnostic applications[6]. Multiphoton absorption/luminescence is a high order nonlinear optical process, which is closely related with the wavelength and intensity of the excitation light, as well as the luminescent efficiency of the fluorophore. Furthermore, fluorophore sample with high concentration can achieve nonlinear optical process more easily than that with low concentration.

However, most commercial organic fluorophores, which are highly emissive in solution, show weak or no emission in the solid state or aggregated state. This phenomenon is notoriously known as aggregation-caused quenching (ACQ) [7]. ACQ effect limits the applications of many fluorescent probes in high concentration. In 2001, a new phenomenon, which is opposite to ACQ effect and named as aggregation-induced emission (AIE), was discovered by Tang's group [8, 9]. In AIE system, the fluorescence of dye increased with the aggregation of the molecules instead of quenching.

Fluorescent probes which can specifically target to specific organelles are of significance. Very recently, Tang's group reported a type of AIE luminogen named tetraphenylethene-triphenylphosphonium (TPETPP), which is an excellent mitochondria tracker [10]. However, the absorption peak of TPETPP is around 320 nm, which is in ultraviolet spectral range. Excitation light with a wavelength in this range has high energy and may produce damage towards cells.

In this paper, we realized multiphoton fluorescence imaging of tumor cells, which were stained with TPPTPP, and further studied its photostability under three-photon excitation. Furthermore, under a 1020 nm-femtosecond laser excitation, three-photon imaging of TPETPP showed higher resolution than two-photon imaging of MitoTracker red FM (MT), which is a commercially available mitochondria targeting agent [11, 12]. We here demonstrate that TPETPP can light up mitochondria specifically with 1020 nm femtosecond laser in live cells with superior photostability as well as high spatial resolution, enabling the observation of mitochondrial morphological changes and study of these processes.

2. EXPERIMENTAL

2.1. Materials and Instruments

TPETPP is a tipical dye with AIE feature and was kindly provided by Prof. Tang's group [10]. Dimethylsulfoxide (DMSO) was purchased from Aldrich. Dulbecco Minimum essential medium

(MEM), fetal bovine serum (FBS), penicillin and streptomycin and MitoTracker Red FM were purchased from Invitrogen.

A Shimadzu 2550 UV-vis scanningspectrophotometer and a HITACHI F-2500 fluorescence spectrophotometer were used to measure the absorption and photoluminescence (PL) spectra of samples, respectively. An Olympus laser scanning confocal microscope (FV1000) equipped with various laser sources (e.g., CW laser, femtosecond laser) was used for one-photon and multiphoton imaging function.

2.2. Cell Culture

HeLa cells were cultivated in Dulbecco minimum essential media (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin, and 1% amphotericin B, at 37°C and 5% CO_2. After 24 h, the TPETPP or MT dissolved in DMSO solution was used to treat the HeLa cells. The working concentrations of TPE-TPP and MT in our experiments were 250 µM and 50 nM, respectively. After incubation, the cells were gently washed thrice by phosphate buffered saline (PBS, pH = 7.4, 10 mM) and imaged by the Olympus laser scanning confocal microscope.

3. RESULTS AND DISCUSSION

Figure 1(a) shows the chemical structure of TPETPP. It includes the group of TPE with AIE feature and the function group of TPP for the targeting of mitochondrial. Figure 1(b) is the absorption of TPETPP in DMSO solution, the absorption peak located around 320 nm.

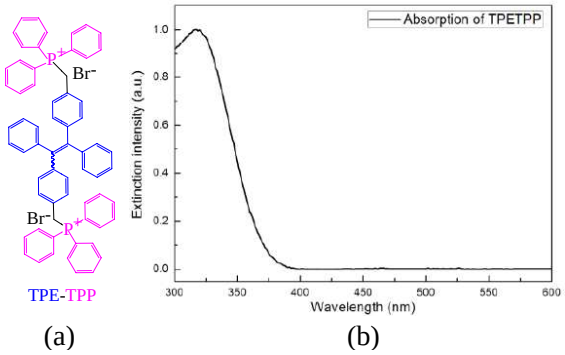


Figure 1: (a) Chemical structure of TPE-TPP, and (b) the absorption spectrum of TPETPP in DMSO.

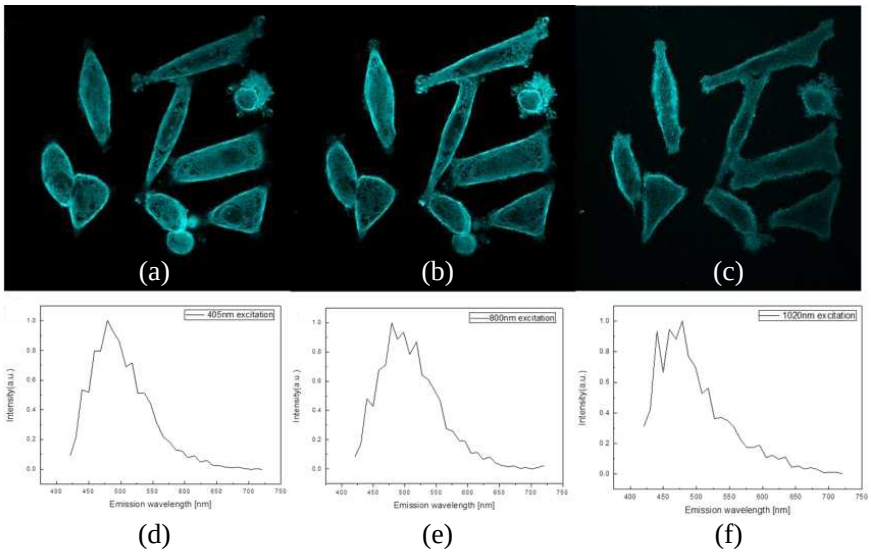


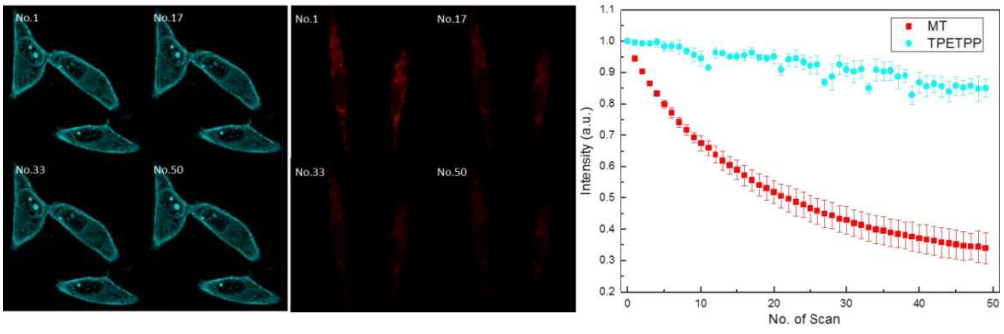
Figure 2: (a) One-photon, (b) two-photon and (c) three-photon fluorescence imaging of HeLa cell treated with TPETPP, and the excitation were (a) 405 nm CW laser, (b) 810 nm femtosecond laser, (c) 1020 nm femtosecond laser, respectively. The statistic (d) one-photon, (e) two-photon and (f) three-photon fluorescence spectra in the cells were shown accordingly.

Figures 2(a), (b), (c) show the one-photon, two-photon and three-photon fluorescence imaging of HeLa cells treated with TPETPP. Figures (d), (e), (f), show the statistic fluorescence spectra in the cells. They all have a luminescence spectrum in the range of 425–600 nm (with the maximum locating at 470 nm), and the envelopes of all the three spectra are very similar). It means under one-photon, two-photon and three-photon absorption, the excited TPETPP nanoparticles were finally relaxed to the same lowest excited electronic-vibrational state, from which the luminescence emission occurred. When the TPETPP sample was stimulated by 810 nm femtosecond laser, it needs to absorb two photons at the same time to get excited, and when the TPETPP sample was stimulated by 1020 nm femtosecond laser, it needs to absorb three photons at the same time to get excited.

Photostability is one of the most important requirements for fluorescence imaging. Tang's group have proved that in one-photon fluorescence imaging, TPETPP takes an obvious advantage over MT in photostability [10]. We then explore this property under three-photon excitation (by using a 1020 nm-femtosecond laser). Here, we still took MT as control. The initial three-photon fluorescence intensity referring to the first scan of TPE-TPP and MT stained cells was used for normalization, and the percentage of signal loss after each additional scan was calculated. As shown in Figure 3(c), under the excitation of 1020 nm laser, during 50 scans with total irradiation time of about 5 mins, the signal loss of TPETPP was less than 15%, and no significant difference was observed between the first and the 50th scan (Figure 3(a)). In contrast, the fluorescence signals of MT almost disappeared after 50 scans, with $< 40\%$ signal intensity remaining (Figure 3(b)).

The reason why TPETPP possesses high photostability under three-photon excitation can be explained as following. Due to the AIE feature, the nanoaggregates of TPETPP are much brighter emitters than its single molecular form, and in the mitochondrial, the TPE-TPP are in the state of aggregation. When exposed to excitation light, the outermost layer of the nanoaggregates may be photobleached. However, the condensed particles can prevent further photobleaching and photo-oxidation by avoiding oxygen diffusion into the particles. For MT, the low working concentration makes it present as an individual molecule which will be destroyed with ease by the strong excitation light.

High spatial resolution is another advantage of TPE-TPP-assisted three-photon bioimaging. Theoretically, under the same excitation wavelength, three-photon imaging is expected to achieve higher spatial resolution ($\lambda/2\sqrt{3}NA$) compared with one-photon imaging ($\lambda/2NA$) and two-photon imaging ($\lambda/2\sqrt{2}NA$). Here, we used the 1020 nm-femtosecond laser to excite TPETPP for three-photon fluorescence imaging and MT for two-photon fluorescence imaging. For MT, the theoretical spatial resolution under two-photon excitation is $1020/2\sqrt{2}NA = 360NA$. However, for TPETPP, the theoretical spatial resolution under three-photon excitation is $1020/3\sqrt{2}NA = 240NA$, which is higher than that of MT. Our experiments results further proved it. The areas of the multiphoton images of cells are magnified by 20 times, as shown in Figure 4. According to the multiphoton fluorescence signal in two different channels (cyan for TPETPP and red for MT) of the same part of the cell, we find that the spatial FWHM (full width at half maximum) in the cyan channel



(a) (b) (c)

Figure 3: Three-photon fluorescence images of living HeLa cells stained with (a) TPETPP (cyan cell) and (b) MT (red cell) with increasing number of scans (1–50 scans; the number of scans shown in upper left corner), (c) three-photon fluorescence loss (%) of TPE-TPP (cyan) and MT (red) in HeLa cells with increasing number of scans. Excitation source: 1020 nm femtosecond laser filter: 449–520 nm (for TPE-TPP) and 581–688 nm (for MT).

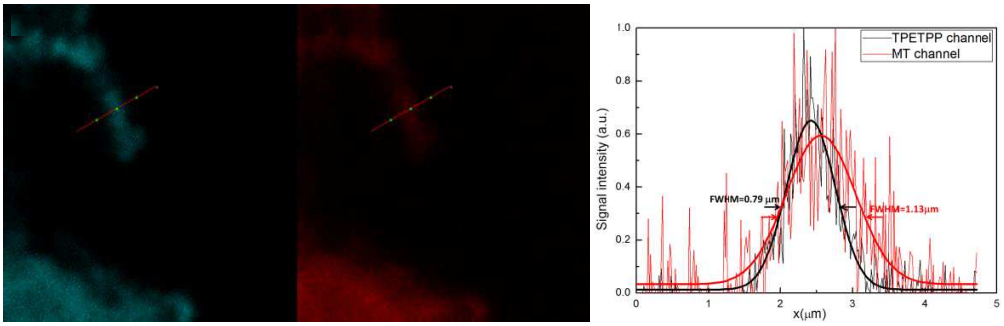


Figure 4: In vitro imaging of HeLa cells targeted by both TPETPP and MT, excitation wavelength:1020 nm, (a) channel 1 for the imaging of TPETPP, (b) channel 2 for MT, (c) line profile of the signal intensityfrom a (black), b (red line). Gaussianfits (smoothed line) are shown for clarity.

(0.79 μm) is narrower than that in the red one (1.13 μm), which illustrated TPETPP assisted three-photon imaging has higher spatial resolution than MT assisted two-photon imaging.

4. CONCLUSIONS

We studied multiphoton bioimaging, by utilizing TPETPP with AIE feature. Under the excitation of 1020 nm femtosecond laser, TPETPP achieved three-photon imaging and showed better photostability than a commercially available mitochondria tracker named MT. Besides, due to higher order nonlinear optical effect of TPETPP under 1020 nm excitation, TPETPP exhibited a higher spatial resolution than MT. TPETPP assisted three-photon bioimaging may have many promising biomedical applications in the future.

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