Biological Oxygen Sensing *via* Two-photon Absorption by an Ir(III) Complex using a Femtosecond Fiber Laser

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Near-infrared two-photon absorption of the phosphorescent iridium (III) complex (2,4-pentanedionato-κO^2, κO^4)bis[2-(6-phenanthridinyl-κ*N*)benzo[*b*]thien-3-yl-κ*C*]iridium (BTPHSA) was characterized. It exhibited a 800–1200 nm two-photon absorption band, and thus could be electronically excited by 1030-nm femtosecond Ti:sapphire and Yb-doped fiber lasers. By using BTPHSA, oxygen concentrations in human embryonic kidney 293 (HEK293) cells were imaged. These results demonstrate two-photon oxygen sensing of live tissues *via* easily operable excitation sources.

1. Introduction

Two-photon excitation microscopy (TPM) involves simultaneous two-photon absorption (TPA) by a luminescent probe.[1, 2] Because the absorption rate depends on the square of the intensity of the excitation source, a tightly focused laser beam enables three-dimensional spatial selectivity.[3,4] For this reason, TPM is a powerful tool for the three-dimensional imaging of cells, tissues and organs.[5-7] This spatially selective imaging is maximized within the relatively transparent "tissue optical window"[8,9] of 600–1300 nm, where absorption coefficients of biological materials such as water, hemoglobin, and melanin are low. Thus light in this range will penetrate more deeply into tissue than that from conventional microscopy light sources.

A femtosecond (fs) Yb-doped fiber laser operating at 1030–1070 nm is thus an attractive excitation light source for TPM imaging. The fiber laser oscillator is much smaller than that of a Ti:sapphire laser, which is typically used for TPM. In addition, the fs fiber laser is stable over wide temperature and humidity ranges, and requires less maintenance than a Ti:sapphire laser. However, there have been only a few reports on *in vivo* red-emitting probes excited by fs fiber lasers. For example, Resan *et al.* have visualized mouse intestines,[10] and Kim *et al*. have obtained two-dimensional and three-dimensional images of dorsal root ganglions.[11] The Ir(III) complex (2,4-pentanedionato-κO^2, κO^4)-bis-[2-(6-phenanthridinyl-*N*)benzo[*b*]thien-3-yl-κ*C*]iridim (BTPHSA, Fig. 1) is an oxygen-sensing probe that emits near-infrared phosphorescence at 720 nm, which is in tissue optical window.[12] BTPHSA has an one-photon absorption (OPA) peak at 530 nm; thus, it should exhibit TPA at around 1060 nm and could be a probe for a fiber-laser TPM imaging. If excitation of BTPHSA by a fs Yb-doped fiber laser was feasible, then both luminescence and excitation will be in the tissue optical window.

Here, we characterize the two-photon induced luminescence (<- fluorescence になつてました) of BTPHSA. A TPA band was observed over 800–1200 nm. In addition, we demonstrate two-photon oxygen sensing in human embryonic kidney 293 (HEK293) cells using a fs Yb-doped fiber laser.

2. Experimental methods

2.1 Materials

BTPHSA was synthesized according to the previous report[12]. HEK293 cells were

used for microscopy imaging. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich Japan) supplemented with penicillin, streptomycin and 10% (*v*/*v*) fetal bovine serum (FBS, Sigma Aldrich Japan) at 37 °C in 5% CO_2. Cells were treated overnight with a medium-supplemented DMSO solution of BTPHSA with a concentration of 1×10^{-3} mol dm^{-3}, giving a final BTPHSA concentration of 1 mol dm^{-3}. Cells were washed twice with a phenol-red-free medium supplemented with 10% (*v*/*v*) FBS after treatment with BTPHSA.

2.2 One-photon absorption spectra

One-photon absorption spectra of dimethyl sulfoxide (DMSO) solutions of BTPHSA at a concentration of 3.94×10^{-5} mol dm^{-3} were recorded on a V-670-UV-VIS-NIR spectrophotometer (Jasco Co.) using 10 mm quartz cuvettes.

2.3 Two-photon absorption spectra

Two-photon absorption spectra were acquired *via* induced fluorescence.[13] The two-photon absorption cross-section was estimated by:

$$\sigma_s^{(2)} = \frac{n_s I_s C_r \Phi_r}{n_r I_r C_s \Phi_s} \sigma_r^{(2)}$$

where *n, I*, *C,* and σ are the refractive index of the solvent, the luminescence intensity, the concentration, the luminescence quantum yield, and the two-photon absorption cross-section, respectively. Subscripts *s* and *r* refer to the experimental and reference samples. As a reference sample, 4,4′-((1*E*,1′*E*)-(3,8-dibutylpyrene-1,6-diyl)bis(ethene-2,1-diyl))bis(1-methylpyridin-1-ium) iodide (PY) was used[14]. A femtosecond (fs) pulsed beam from an optical parametric amplifier (OPA-800C, Spectra-Physics) pumped by a beam from a regenerative amplifier (Spitfire, Spectra-Physics) was used as the light source. The pulse duration was typically 150 to 200 fs, at a repetition rate of 1 kHz. The average incident power was 0.1–0.5 mW. The incident beam was focused by a plano-convex lens (*f*=80 mm), and the emitted phosphorescence was detected with a liquid-nitrogen-cooled CCD (LN/CCD-1100PB, Princeton Instruments). BTPHSA and PY were dissolved in DMSO at concentrations of 1.12×10^{-4} and 8.10×10^{-5} mol dm^{-3}, respectively.

2.4 Measurements of phosphorescence quantum yield[15]

Phosphorescence quantum yields were obtained using an absolute photoluminescence quantum yield measurement system (C9920-02G, Hamamatsu

Photonics). The excitation wavelength was 375 nm, which was the one-photon absorption maximum. DMSO was used as the solvent and the concentration of the experimental solution was 1×10^{-5} mol dm^{-3}. The solution was de-oxygenated by N_2 bubbling for 30 minutes to ensure maximum quantum yields.

2.4 Measurements of luminescence lifetime

Luminescence lifetimes were determined with a fs Ti:sapphire laser and a streak camera. DMSO was used as the solvent and the solution concentration was 1×10^{-4} mol dm^{-3}.

2.5 One-photon and two-photon induced luminescence spectra

One- and two-photon induced luminescence spectra were excited using UV light (365 nm) or a fs pulsed beam from an optical parametric amplifier (OPA-800C, Spectra-Physics), pumped by a beam from a regenerative amplifier (Spitfire, Spectra-Physics). The BTPHSA luminescence was detected with a USB spectrometer (USB 4000, Ocean Optics). Prior to measurements, the solution was deoxygenated by N_2 bubbling for 30 minutes. DMSO was used as the solvent and the solution concentration was 1×10^{-6} mol dm^{-3}.

2.6 One-photon luminescence microscopy

One-photon luminescence microscopy was performed with Eclipse 50i (Nikon Instruments Inc.) equipped with digital camera DS-Ril (Nikon Instruments Inc.). Phosphorescence from BTPHSA and fluorescence from DAPI were collected through G-2A filter cube (excitation filter 510-560 nm, dichroic mirror 565 nm, barrier filter 590 nm) and UV-2A filter cube (excitation filter 330 – 380 nm, dichroic mirror 400 nm, barrier filter 590 nm).

2.7 Two-photon luminescence microscopy

Two-photon luminescence microscopy was performed with either a fs Ti:sapphire laser (Mira, Coherent) or a fs fiber laser (L12948, Hamamatsu Photonics). A galvano scanner (C10516, Hamamatsu Photonics) was used as the laser-scanning unit. Cells were placed in a 35-mm glass base dish. BTPHSA was excited by an 850 nm beam from the Ti:sapphire laser or a 1030 nm beam from the fs fiber laser through a water immersion objective (UApo 340 40X, Olympus). The sample was placed on a power stage and scanned along the optical axis. A photon counting head (H7421-40, Hamamatsu Photonics) was employed for signal

detection.

3. Results and discussion

The one- and two-photon absorption spectra of BTPHSA in dimethyl sulfoxide (DMSO) are shown in Fig. 2. The TPA spectrum was acquired by a two-photon induced fluorescence-based technique.[13] The OPA band was observed in the wavelength region of 300 – 700 nm, and the absorption band located between 450-600 nm was assigned to metal-to-ligand charge transfer (MLCT).[12] The TPA band was observed over 800–1200 nm. This wavelength region is almost double that of the OPA band, suggesting that the TPA transitions are not forbidden by selection rules in the primary one-photon allowed absorption band. This is common for asymmetrical molecules.[16] The peak of the TPA cross- section ($\sigma^{(2)}_{peak}$) at 1060 nm was 18 GM (1 GM = 10^{-50} cm^4 s $photon^{-1}$ $molecule^{-1}$). This peak is close to double the wavelength of the MLCT band in the one-photon absorption spectrum. The value of the TPA cross-section ($\sigma^{(2)}$) rapidly increased below 900 nm, which is attributed to double resonances.[17-19] The maximum value was 31 GM at 840 nm. Much larger values might occur below 840 nm, but they were not determined because of the very weak OPA band. The TPA data indicate that BTPHSA should undergo two-photon excitation by fs Ti:sapphire lasers and fs Yb-doped fiber lasers. Biological probes that are two-photon excitable with fs Yb-doped fiber lasers are very limited in number; hence BTPHSA should have widespread applications.

One-photon and two-photon induced luminescence spectra of BTPHSA in DMSO were shown in Fig. 3. The luminescence maxima for one- and two-photon excitation were observed at 712 nm, and the shapes of spectra were essentially the same for both excitations. It indicated that emission of BTPHSA occurred from the same excited state even when the excitation processes were different. This is the same tendency for typical luminescence molecular materials.[20] Luminescence lifetime was measured to clarify the luminescence process. Luminescence decay curve of BTPHSA in DMSO is shown in Fig. 4. Luminescence from BTPHSA was gradually decreased on the order of microseconds. For this measurement, luminescence lifetime was determined to be 0.8 μs. Luminescence with the lifetime on the order of microseconds can be attribute to phosphorescence.[21-23] Iridium complexes generally exhibit phosphorescence through the excited triplet-state.[24, 25] BTPHSA exhibited phosphorescence in the same way for typical Iridium complexes.

TPA is the excitation process of luminescence probes in TPM imaging, thus we investigated oxygen sensing ability of BTPHSA under two-photon excitation condition. The

two-photon induced phosphorescence spectra of BTPHSA in DMSO under standard condition and that under de-oxygenated condition by N_2 bubbling for 30 minutes to the solution were measured. Obtained phosphorescence spectra were shown in Fig. 5. The phosphorescence intensity from de-oxygenated condition solution was 4 times as larger as that from standard condition. BTPHSA exhibited strong phosphorescence in de-oxygenated condition compared to standard condition even under two-photon excitation process. The one-photon phosphorescence quantum yields of BTPHSA in DMSO under standard condition and de-oxygenated condition were 0.08 and 0.31, respectively. This indicated that BTPHSA exhibited phosphorescence under de-oxygenation condition four times stronger than under standard condition. This trend was essentially the same as that under two-photon excitation condition.

Figure 6 shows one-photon induced luminescence microscopy images of HEK293 cells stained with DAPI and BTPHSA. Under an N_2 atmosphere, red phosphorescence from BTPHSA was observed at the cell and did not overlapped with blue fluorescence from DAPI. This merged image indicated that BTPHSA localized on cytoplasm and endoplasmic reticulum, not nuclei in the cells. Similar localization behavior was also observed in the probe with similar molecular structure of BTPHSA, bis(2-(2'-benzothienyl)-pyridinato-*N*, C^3')iridium(acetylacetonate), which was reported to reference 12. In contrast, under a standard O_2 atmosphere, phosphorescence from BTPHSA was quenched and thus it was detected only in hypoxic environments.

TPM images of HEK293 cells stained with BTPHSA are presented in Fig. 7. These TPM images were essentially the same as the one-photon luminescence microscopy images. Thus, oxygen mapping was achieved by employing BTPHSA as the probe for not only one-photon luminescence microscopy but also TPM. Furthermore, TPM images were essentially identical when acquired with either a fs Ti:sapphire laser or a fs Yb-doped fiber laser. This study is thus the first example of oxygen mapping using 1030 nm irradiation from a fs fiber laser. To date, Ir(III) complexes for TPM probes have been designed to be excited by conventional Ti:sapphire lasers.[26-28] However, since BTPHSA has a MLCT band between 450 and 600 nm, it can be excited by fs lasers in the wavelength region from 800 – 1200 nm.

It should be noted that BTPHSA has additional attractive characteristics as oxygen monitoring TPM probe. TPM is well suited to the observation of the dynamics of biological systems. For this reason, porphyrin derivatives exhibiting efficient TPA have been synthesized.[29, 30] Generally, a low molecular weight is required for this purpose, and the molecular weight of BTPHSA (969) is almost half that of these porphyrin derivatives (over

2000). BTPHSA also exhibits a suitable TPA cross-section without any special molecular modification. Furthermore, cell activity was not changed following staining with BTPHSA and so the toxicity of BTPHSA is thought to be sufficiently low so as to allow its use as a TPM probe.

4. Conclusions

In summary, the TPA of the Ir(III) complex BTPHSA was characterized. It exhibited a peak at 1060 nm and a maximum at 840 nm. Oxygen-sensitive BTPHSA phosphorescence enabled oxygen sensing in HEK293 cells using both fs Yb-doped fiber lasers and Ti:sapphire lasers as excitation sources. Other phosphorescent Ir(III) complexes having MLCT transitions are expected to exhibit TPA characteristics similar to BTPHSA, and could be additional oxygen-sensing TPM probes.

Acknowledgments

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14
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Figure Captions

Fig. 1. Chemical structure of BTPHSA.

Fig. 2. One-photon absorption (OPA) spectrum (left vertical and bottom axes) and two-photon absorption (TPA) spectrum (right vertical and top axes) of BTPHSA in DMSO.

Fig. 3. One-photon (blue line) and two-photon (red line) induced luminescence spectra of BTPHSA in DMSO. The one-photon and two-photon excitation wavelengths were 375 and 1030 nm, respectively.

Fig. 4. Luminescence decay curve of BTPHSA in DMSO.

Fig. 5. Two-photon induced luminescence spectra. Red line and blue line indicate deoxygenation condition and standard condition, respectively.

Fig. 6. One-photon luminescence microscopy images and merged image of HEK293 cells stained with BTPHSA and DAPI under an N_2 atmosphere (a-c) and an O_2 atmosphere (d-f).

Fig. 7. TPM images of HEK293 cells stained with BTPHSA under an N_2 atmosphere (top) and an O_2 atmosphere (bottom). Phosphorescence over 670.5–745.5 nm was imaged.

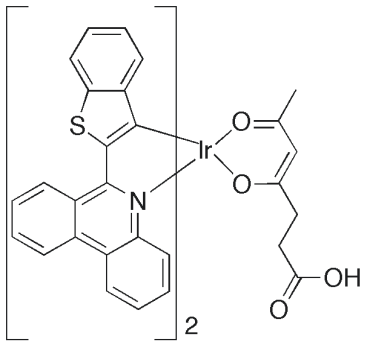

Fig. 1

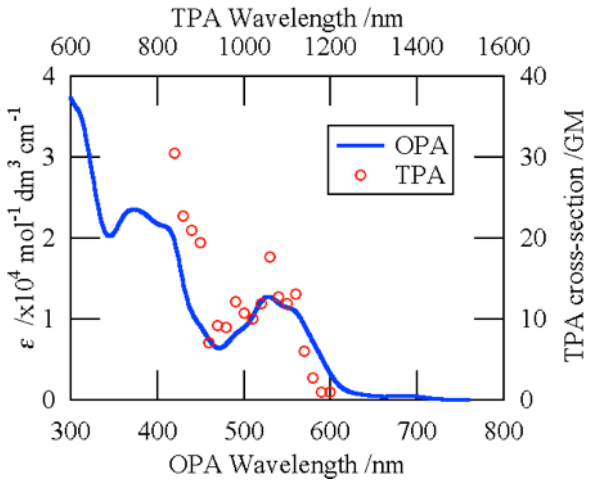
TPA Wavelength /nm
600
800
1000
1200
1400
1600
4
3
2
1
0
40
30
20
10
0
ε /x10^4 mol^-1 dm^3 cm^-1
TPA cross-section /GM
OPA
TPA
300
400
500
600
700
800
OPA Wavelength /nm

Fig. 2

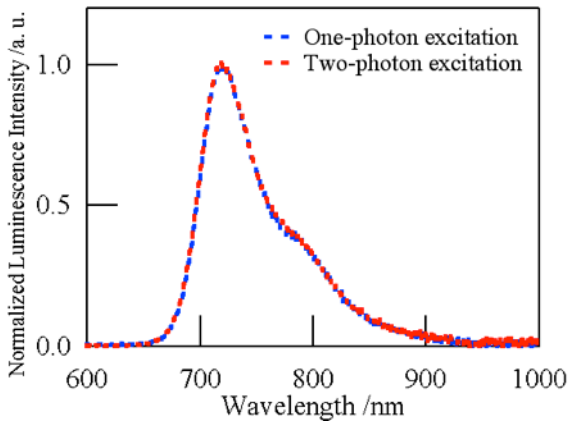
One-photon excitation
Two-photon excitation
Normalized Luminescence Intensity /a. u.
1.0
0.5
0.0
600
700
800
900
1000
Wavelength /nm

Fig. 3

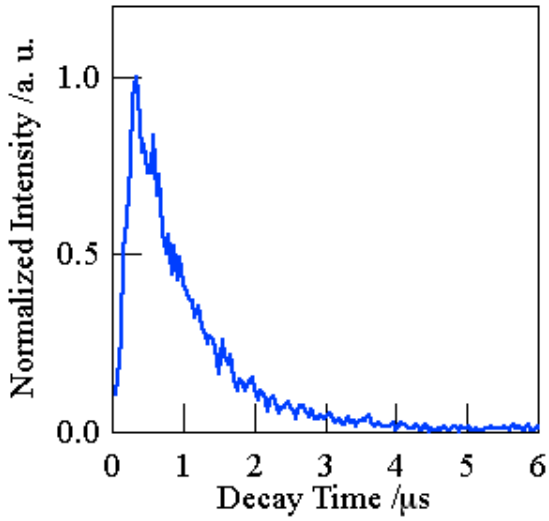

Fig. 4

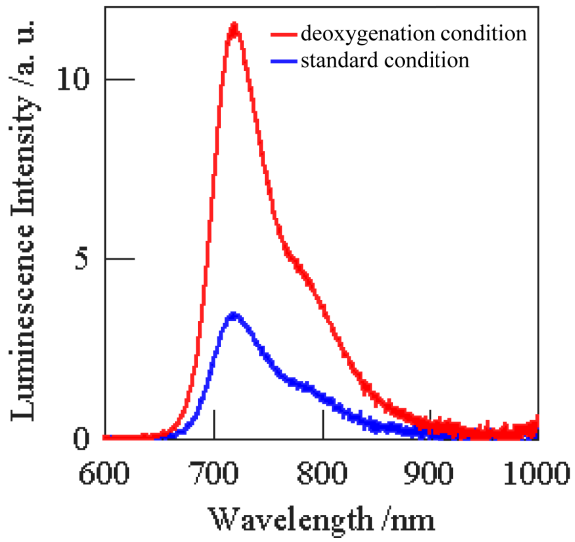
deoxygenation condition
standard condition
Luminescence Intensity /a. u.
10
5
0
600
700
800
900
1000
Wavelength /nm

Fig. 5

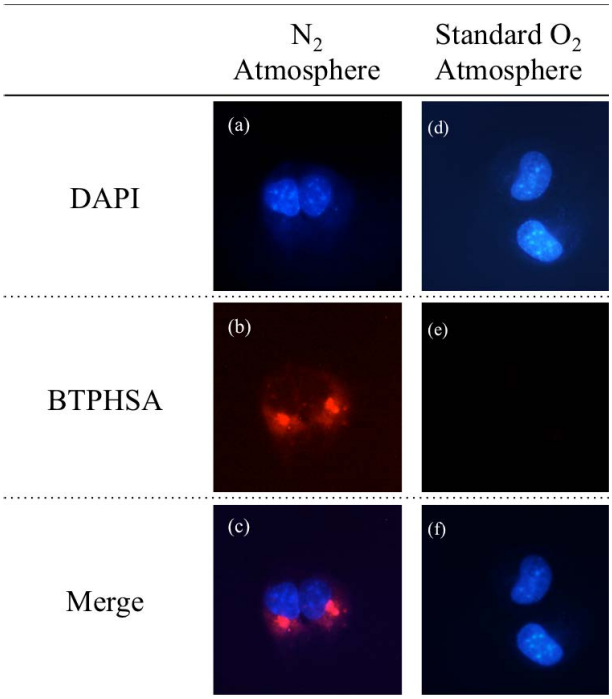
N2 Atmosphere
Standard O2 Atmosphere
DAPI
BTPHSA
Merge
(a)
(b)
(c)
(d)
(e)
(f)

Fig. 6

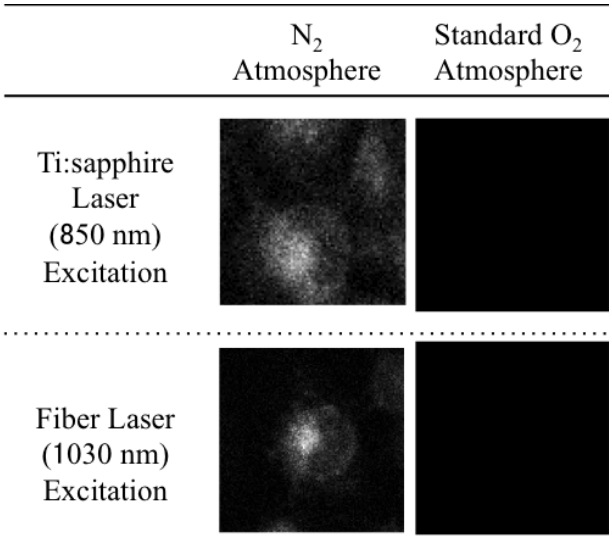
N_2 Atmosphere
Standard O_2 Atmosphere
Ti:sapphire Laser (850 nm) Excitation
Fiber Laser (1030 nm) Excitation

Fig. 7