Chinese Chemical Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Original article

A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging

Yong Dai [a,c], Bao-Kuo Lv [b,*], Xin-Fu Zhang [c], Yi Xiao [c,*]

[a] Department of Criminal Science and Technology, Sichuan Police College, Luzhou 646000, China
[b] Liaoning Water Environment Monitoring Center, Shenyang Branch, Shenyang 110005, China
[c] State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, China

ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 26 April 2014
Accepted 29 April 2014
Available online xxx

Keywords:
Mitotracker
Mitochondria
Namphthalimide
Two-photon
Fluorescence imaging

ABSTRACT

PAHPN, a naphthalimide-based mitotracker with reasonable two-photon excitation emission activity and polarity-sensitive fluorescence properties has been efficiently synthesized and studied in two-photon, co-localization, and FLIM imaging.

1. Introduction

Mitochondria, as cell's power producers, are important organelles for cellular respiration that ultimately generates fuel for the cellular activities [1]. In addition, mitochondria are involved in other tasks such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth [2]. In particular, mitochondria's key role in activating apoptosis has attracted much attention [3]. Mitochondria are highly dynamic organelles that continuously move, divide and fuse in a highly regulated fashion during various cellular processes [4]. For example, mitochondrial fission accompanies apoptotic cell death and appears to be important for the progression of the apoptotic pathways [5].

Mitotrackers [6], namely mitochondria targeted fluorescent probes, have proved valuable tools to visualize mitochondria's dynamic changes during apoptosis and other cellular processes. However, the common mitotrackers, *e.g.*, Rhodamine 123 and tetramethylrhodamine methyl ester (TMRM), are not efficient two-photon fluorophores, which restricted their application in TPEE (two photon excitation emission) microscopy. This newly emerging imaging technique using pulsed NIR excitation can be a superior alternative to confocal microscopy (one photon imaging) due to its deeper tissue penetration (>500 μm), efficient light detection, and reduced phototoxicity [7,8]. Due to its imaging mechanism, it also provides advantages such as highly localized excitation and prolonged observation time. These are desired imaging properties for trackers and the development of two-photon mitotrackers represents a critical priority.

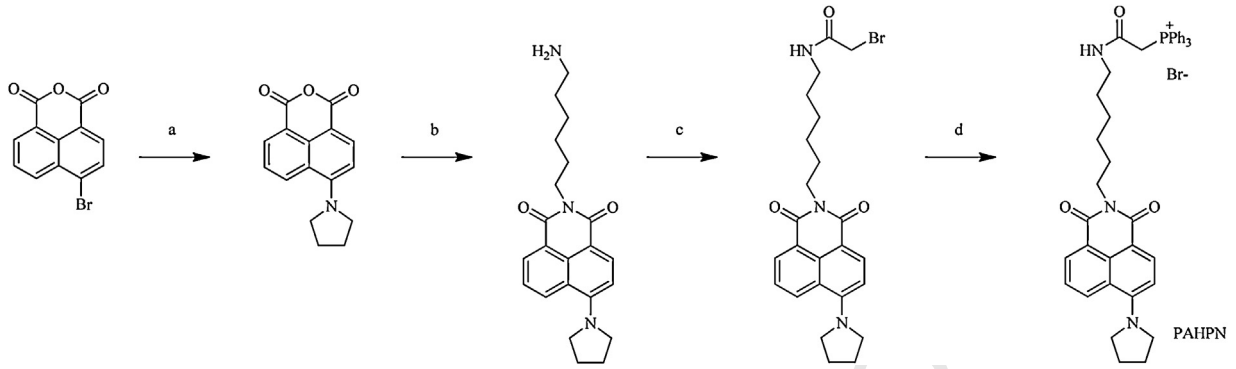
Herein, we have developed PAHPN, a new mitotracker with reasonable TPEE activity. There are two key aspects in our design. Firstly, we chose 4-pyrrolidino-1,8-naphthalimide as the fluorophore because its TPEF activity is foreseeable since another 4-amino naphthalimide probe showing strong TPEE has been reported by ourselves very recently [9]. Secondly, a triphenylphosphonium (TPP) moiety has been attached to the naphthalimide fluorophore *via* a flexible long alkyl chain, which generates a positively charged but highly lipophilic molecule that tends to accumulate in mitochondrial inner membranes at negative potentials [10,11].

As shown in Scheme 1, the synthesis of PAHPN can be achieved from 4-Br-1,8-naphthalic anhydride in four steps, namely, nucleophilic substitution, condensation, acylation and quarternization. Each reaction is facile under mild conditions, thanks to Qian's pioneering work on the modifications of the naphthalimide platform [12].

* Corresponding authors.
E-mail addresses: lvbaokuo1987@163.com (B.-K. Lv), xiaoyi@dlut.edu.cn (Y. Xiao).

http://dx.doi.org/10.1016/j.cclet.2014.05.020
1001-8417/

Please cite this article in press as: Y. Dai, et al., A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging, Chin. Chem. Lett. (2014), http://dx.doi.org/10.1016/j.cclet.2014.05.020



Scheme 1. Synthesis of PAHPN. (a) Pyrrolidine, 2-methoxyethanol, reflux, 72%. (b) Hexamethylenediamine, ethanol, reflux, 80%. (c) Bromoacetic acid, DCC, dichloromethane, r.t., 94%. (d) Triphenylphosphine, dichloromethane, r.t., 35%.

2. Experimental

Culture of cells and fluorescent imaging: MCF7 (human breast carcinoma) cells, RAW 264.7 (macrophages cells) and COS-7 cells were obtained from Institute of Basic Medical Sciences (IBMS) of Chinese Academy of Medical Sciences (CAMS). All cell lines were maintained under standard culture conditions (atmosphere of 5% CO_2 and 95% air at 37 °C) in RPMI 1640 medium, supplemented with 10% FBS (fetal calf serum).

Cells were grown in the exponential phase of growth on 35 mm glass-bottom culture dishes (Ø20 mm) for 1–2 days to reach 70–90% confluency. These cells were used in co-localization experimentation. The cells were washed three times with RPMI 1640, and then incubated with 2 mL of RPMI 1640 containing probes (1 μmol/L) in an atmosphere of 5% CO_2 and 95% air for 3 min at 37 °C. Cells were washed twice with 1 mL of PBS at room temperature and observed under a confocal microscopy (Olympus FV1000).

The 400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were collected at room temperature and were given in supporting information. Melting points were obtained with a capillary melting point apparatus in open-ended capillaries and were uncorrected. Chromatographic purifications were conducted using silica gel. All solvent mixtures are given as volume/volume ratios.

Compound **1**: 4-Bromo-1,8-naphthalic anhydride (10 g, 36.2 mmol) was dissolved in ethylene glycol monomethyl ether under reflux. Pyrrolidine (5 mL, 66.4 mmol) was added in four portions in 2 h. After the addition of pyrrolidine, the mixture was refluxed for one more hour. After the reaction mixture was cooled to room temperature, the yellow solid was collected (7 g, 72.4%).

Compound **2**: Compound **1** (1 g, 3.74 mmol) and hexamethylenediamine (1.3 g, 11.23 mmol) were refluxed in 10 mL ethanol. The reaction mixture was cooled to room temperature. After the ethanol was removed under reduced pressure, the residue was recrystallized from ethanol to give the compound **2** (1 g, 80%). Mp. 60–61.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 (m, 6H), 1.74 (s, 2H), 2.1 (d, 6H), 2.71 (t, 2H), 3.77 (s, 4H), 4.16 (t, 2H), 6.80 (d, 1H), 7.52 (t, 1H), 8.41 (d, 1H), 8.58–8.54 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.2, 26.7, 27, 28.2, 29.8, 33.2, 40.1, 42.1, 53.3, 108.7, 111, 122.8, 123.2, 131.1, 131.3, 132, 133.5, 152.8, 164.2, 165.0. HRMS (MALDI-TOF) (m/z): Calcd. for $C_{22}H_{27}N_3O_2$: 365.2103, found: 366.2192 ($[M+H^+]^+$).

Compound **3**: Compound **2** (0.2 g, 0.55 mmol), bromoacetic acid (47.24 μL, 0.66 mmol) and DDC (160 mg, 0.66 mmol) were stirred in CH_2Cl_2 at room temperature for 6 h. The insoluble materials were filtered off and the filtrate was evaporated to provide the compound **3** (0.25 g, 94.0%). Mp 79–80.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (m, 2H), 1.74 (m, 4H), 1.93 (d, 2H), 2.11 (s, 4H), 3.29 (m, 2H), 3.79 (s, 4H), 3.9 (s, 2H), 4.18 (t, 2H), 6.69 (s, 1H), 6.82 (d, 1H, J = 8.8 Hz), 7.54 (t, 1H), 8.42 (d, 1H), 8.58 (t, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.1, 25.7, 26.3, 28, 29.1, 29.5, 34, 39.9, 40.2, 49.4, 53.3, 108.3, 110.6, 122.7, 123.2, 131.2, 132.1, 133.2, 152.6, 164.3, 165, 165. HRMS (MALDI-TOF) (m/z): Calcd. for $C_{24}H_{28}BrN_3O_2$: 485.1314, found: 486.1394 ($[M+H^+]^+$).

PAHPN: a mixture of compound **3** (0.12 g, 0.247 mmol) and triphenylphosphine was stirred in CH_2Cl_2 at room temperature for 5 h. After the CH_2Cl_2 was removed under reduced pressure, the residue was purified by silica gel column chromatography using eluent CH_2Cl_2/MeOH (20/1, v/v). A yellow solid was obtained (0.065 g, 35.2%). Mp 94–96 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.31–1.16 (m, 6H), 1.62 (m, 2H), 2.09 (s, 4H), 3.05 (d, 2H, J = 6 Hz), 3.76 (s, 4H), 4.09 (t, 2H), 5.02 (d, 2H, J = 14 Hz), 6.79 (d, 1H, J = 8.8 Hz), 7.50 (t, 1H), 7.64 (m, 6H), 7.82 (m, 9H), 8.38 (d, 1H, J = 8.8 Hz), 8.55 (m, 2H), 9.29 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.1, 26.8, 26.9, 28.1, 29, 31.9, 32.5, 40.1, 40.2, 53.2, 108.6, 110.7, 118.1, 119, 122.5, 123.1, 130.1, 130.2, 131.0, 133.4, 134.1, 134.3, 135.0, 152.7, 162.1, 164, 164.8. HRMS (MALDI-TOF) (m/z): Calcd. for $C_{42}H_{43}BrN_3O_3P$: 747.2225, found: 668.2996 ($[M-Br^-]^+$).

3. Results and discussion

PAHPN exhibits polarity-sensitive fluorescence properties. Its absorption and emission spectra in various solvents are shown in Fig. 1, and the basic data are listed in Table 1. Briefly, with the increase in polarity, the fluorescence spectra red-shift to longer wavelength range and the fluorescence quantum yields decrease sharply. For example, in toluene, the emission maximum is at 500 nm and the quantum yield is 0.96, while in acetonitrile, the emission peak moves to 541 nm and the quantum yield declines to 0.14. However, the absorption properties are less dependent on solvent polarity. Although the absorption spectra also shift in various solvents, the difference in molar extinction coefficients is not as significant as that of fluorescence quantum yields. The sharply different fluorescence is an advantage for PAHPN's application in mitochondria imaging since those PAHPN molecules localized in mitochondrial inner membrane would emit strong fluorescence due to the nonpolar lipophilic environment while the background noise from some PAHPN distributed in other aqueous intracellular compartments would be low. Its fluorescence life times (τ) in various solvents are given in Fig. 1, and the average τ values are listed in Table 1. Fluorescence life time shows a decrease with the increase in polarity, which can be used to measure the polarity of mitochondria.

The sensitivity of fluorescence toward polarity could be explained by TICT (twisted intramolecular charge transfer, or twisted ICT) mechanism [13]. PAHPN's fluorophore, 4-pyrrolidino-1,8-naphthalimide accords with the standard TICT structure: the strong electron donor (pyrrolidino) is connected to the strong

Please cite this article in press as: Y. Dai, et al., A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging, Chin. Chem. Lett. (2014), http://dx.doi.org/10.1016/j.cclet.2014.05.020

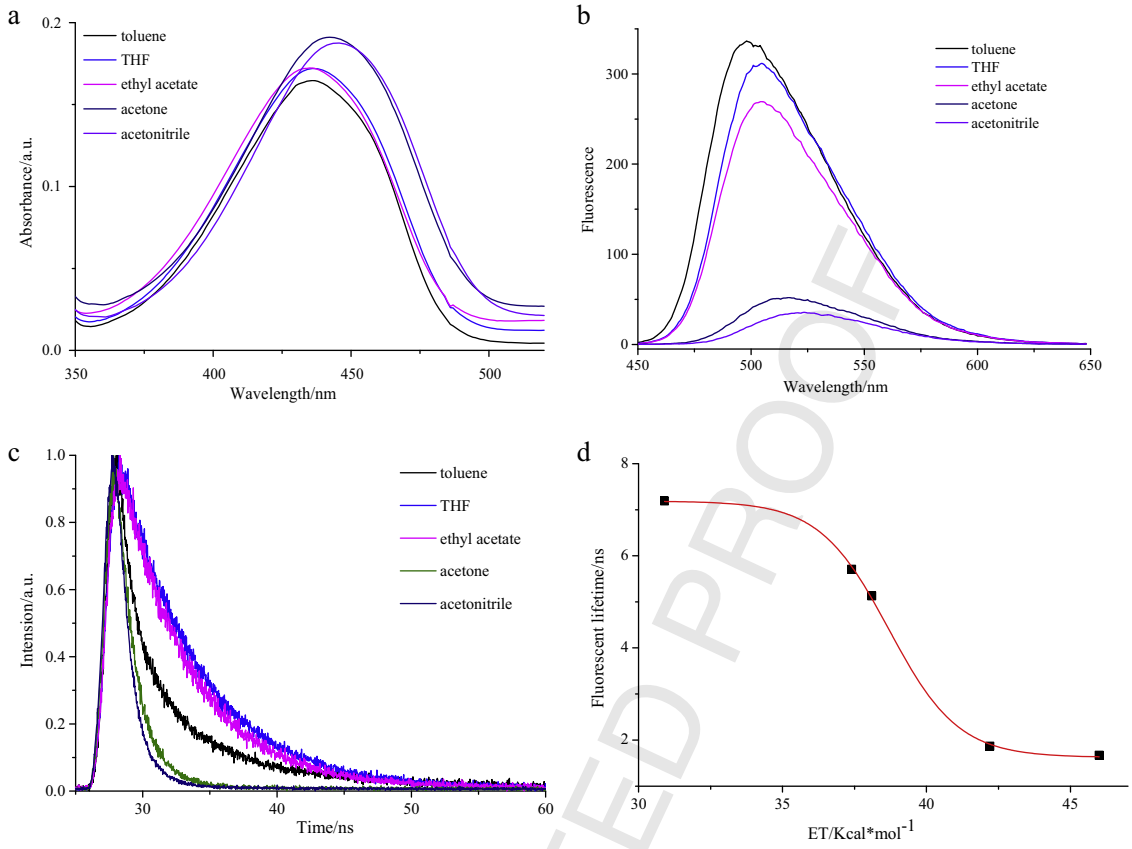


Fig. 1. Absorption (a) and emission (b) spectra of PAHPN in solvents of different polarities; fluorescence life time of PAHPN in solvents of different polarities (c) and (d).

electron acceptor (naphthalimide) by a single bond. Upon excitation of such a fluorophore, the single bond will be twisted quickly so that the donor plane and the acceptor plane become perpendicular to each other. Such a twisted configuration in excited state means a charge separation between the donor and acceptor, or a complete charge transfer. This movement of the single bond consumes the excitation energy and thus quenches the fluorescence sharply. As we know, highly polar solvents can stabilize and thus promote TICT state, while nonpolar solvents destabilize and disfavor TICT.

PAHPN demonstrates a reasonable two-photon excitation activity. As shown in Fig. 2, the two-photon excitation spectrum of PAHPN in toluene exhibits a broad band from 700 to 1000 nm and the maximum is at 820 nm, which is approximately the double of the one-photon absorption maximum. The TPEE activity across section ($\Phi \times \delta$, the product of Φ and δ) at 820 nm is around 90 GM, which is a reasonable value and higher than that of several common fluorophores. In other word, the intrinsic brightness of PAHPN's TPEE is sufficient for two-photon microscopic applications.

Table 1
Spectral data of PAHPN.

Solvent	λ_{ab} (nm)	λ_{em} (nm)	Φ[a]	ε	τ (ns)
Toluene	436	500	0.96	15,400	7.19
THF	436	505	0.98	15,900	5.71
Ethyl acetate	435	506	0.97	15,900	5.13
Acetone	443	517	0.20	17,700	1.87
CH3CN	446	523	0.14	17,400	1.67

[a] Quinine sulfate (Φ = 0.55) as reference.

The applicability of PAHPN as a two-photon fluorescent cell marker has been confirmed by two-photon microscopy imaging experiments. As shown in Fig. 3, strong TPEE has been observed in MCF-7 cells stained with PAHPN. The distribution of fluorescence clearly indicates that the PAHPN molecules are not uniformly dispersed but localized in certain areas. Mitochondria usually assemble into networks, although single ones have an ellipsoid shape [6]. In Fig. 3, fluorescent networks are easily visualized with high resolution because the areas surrounding these networks are almost 'dark' (without background noise). Thus, the quality of two-photon imaging is high.

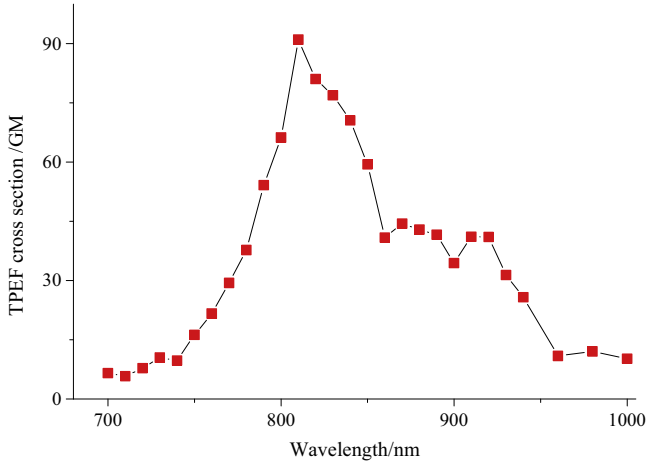


Fig. 2. Two-photon excitation spectrum of PAHPN in toluene.

Please cite this article in press as: Y. Dai, et al., A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging, Chin. Chem. Lett. (2014), http://dx.doi.org/10.1016/j.cclet.2014.05.020

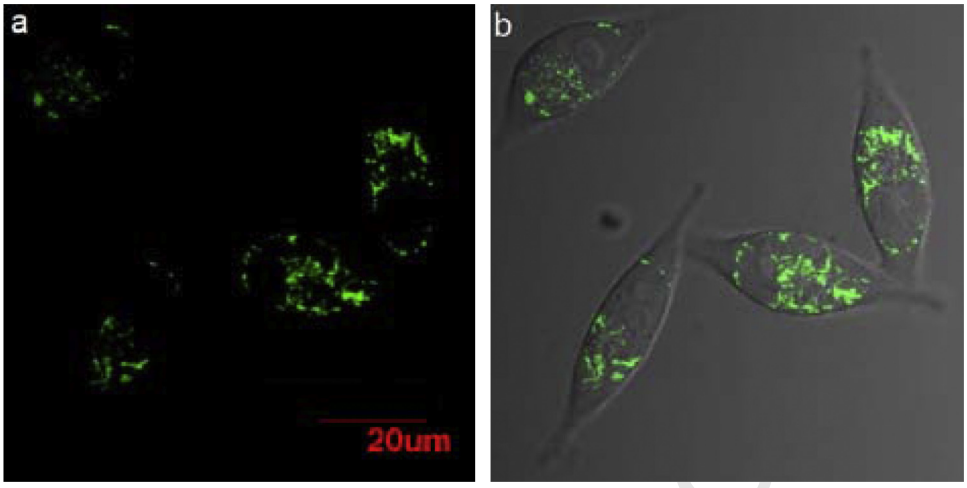


Fig. 3. Two photon fluorescence image of MCF-7 cells incubated with PAHPN (1 μmol/L) for 3 min at 37 °C. (a) Fluorescence image (λ_{ex} = 840 nm, λ_{em} = 520–560 nm). (b) Overlay of fluorescence image and brightfield image.

Actually, we have also applied PAHPN in one-photon confocal imaging (Fig S1 in supporting information) and have found that there is no difference in imaging quality between two-photon and one-photon microcopies.

Furthermore, PAHPN proves to be a specific mitotracker. A colocalization imaging experiment has been performed. PAHPN and two commercial mitrotrackers (tetramethylrhodamine methyl ester, TMRM and mitotracker deep red) are adopted to co-stain different cell lines including MCF-7 cells, RAW264.7 cells and COS-7 cells. As TMRM and mitotracker deep red emits at considerably longer wavelengths than PAHPN, their different fluorescence signals can be collected in two channels without crosstalk. As shown in Fig. 4, in MCF-7 cells, the green fluorescence in the PAHPN channel overlaps well with the red fluorescence in the TMRM (or mitotracker deep red) channel, which produces a high colocalization coefficient up to 0.92 (or 0.85 in the case of mitotracker deep red) and indicates that PAHPN and TMRM (or mitotracker deep red) are localized in similar intracellular areas, namely, mitochondria. Similar colocalization results are also obtained in RAW264.7 and COS-7 cells, as shown in the supporting information (Figs. S2 and S3) In other words, PAHPN shows stable localization in mitochondria of different cell lines. Thus, it can be concluded that PAHPN is mitochondria-targeted.

Finally, PAHPN is used in fluorescence life time imaging (FLIM) to reflect polarity in mitochondria. Fig. 5 shows heterogeneous polarity distribution in mitochondria in a single cell or in different cells, which indicates that polarity of mitochondria under different physiology status could be different. Thus, PAHPN can be used as an indicator for polarity in mitochondria. In future, the combination of PAHPN's polarity-sensitivity and FLIM technique will be further applied in order to monitor the mitochondrial micro-environmental variations under different physiological, pharmacological, and toxicological conditions.

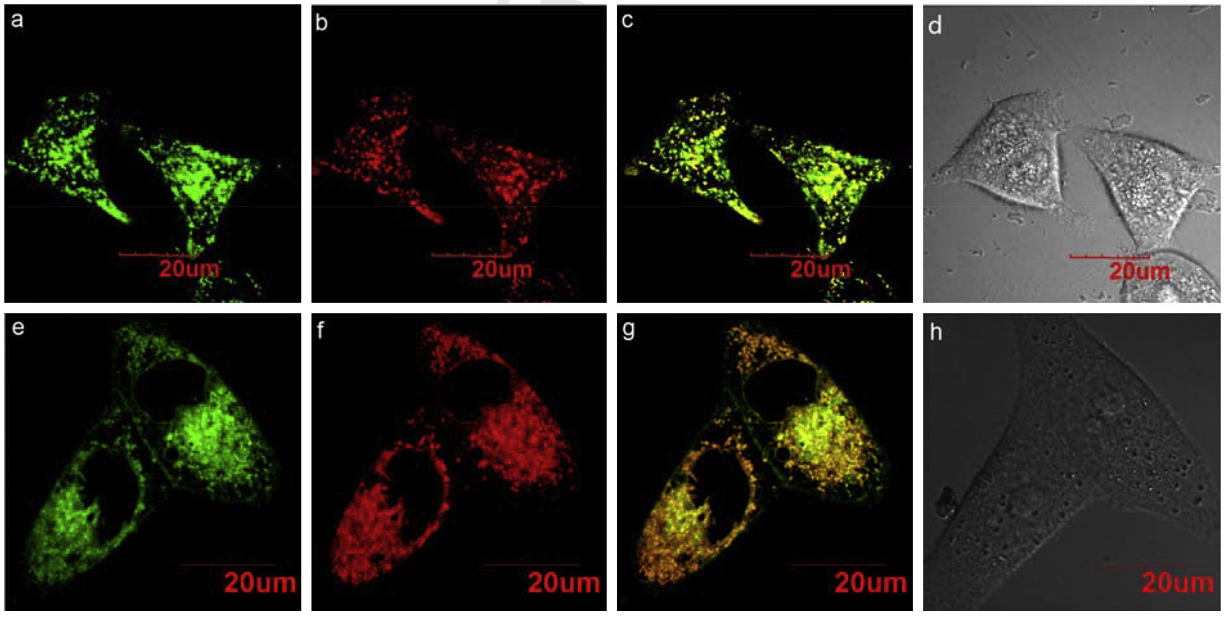


Fig. 4. Colocalization imaging of MCF-7 cells costained by PAHPN and commercial mitrotracker TMRM (a–d) mitotracker deep red (e–h) for 3 min at 37 °C. (a and e) Fluorescence image of PAHPN (λ_{ex} = 405 nm, λ_{em} = 470–530 nm). (b) Fluorescence image of TMRM, λ_{ex} = 559 nm, λ_{em} = 600–630 nm. (c) Overlay image of a and b, colocalization coefficient 0.92. (f) Fluorescence image of mitotracker deep red, λ_{ex} = 635 nm, λ_{em} = 655–755 nm. (g) Overlay image of e and f, colocalization coefficient 0.85. (d and h) Brightfield image.

Please cite this article in press as: Y. Dai, et al., A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging, Chin. Chem. Lett. (2014), http://dx.doi.org/10.1016/j.cclet.2014.05.020

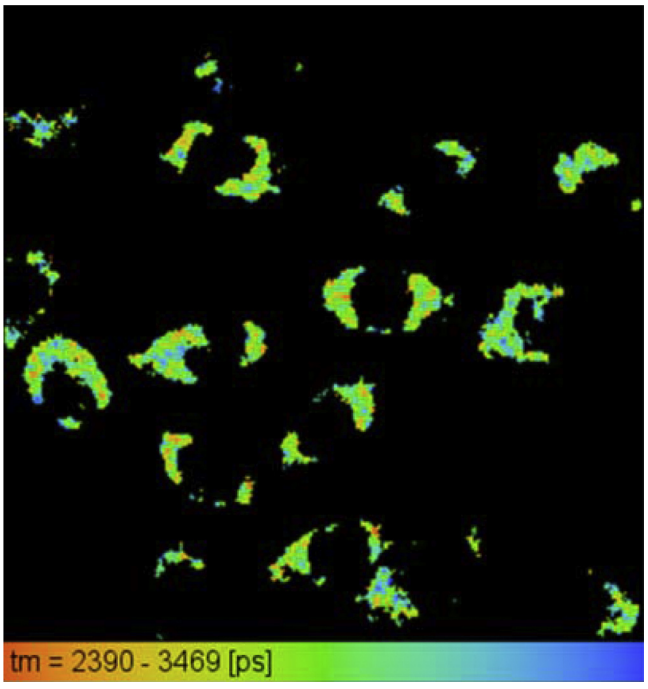


Fig. 5. Fluorescence life time imaging of MCF-7 cells (1 mol/L PAHPN for 3 min at 37 °C). The excited light is 405 nm, observing emission wavelength at 535 ± 15 nm.

4. Conclusion

In conclusion, PAHPN, a new two-photon mitotracker has been efficiently synthesized through the attachment of a triphenylphosphonium moiety to the naphthalimide fluorophore. PAHPN exhibits strong fluorescence in nonpolar solvents but extremely weak fluorescence in water, which favors the reduction of background fluorescence from the aqueous environment outside the mitochondrial membrane. This new molecular probe shows reasonable two-photon excitation emission activity and presents high-quality fluorescence images under two-photon microscopy. PAHPN's specificity toward mitochondria has been confirmed by the colocalization imaging studies on three different cell lines with two commercial mitotrackers TMRM and mitotracker red as the references. FLIM imaging using PAHPN has mapped the different polarity of mitochondria. The applications of PAHPN in the investigations on cellular processes related to mitochondrial dynamics are now being studied.

Acknowledgments

We thank National Natural Science Foundation of China (Nos. 21174022, 21376038), National Basic Research Program of China (No. 2013CB733702), Key Project of the education department of Sichuan Province (No. 12ZA087), and Specialized Research Fund for the Doctoral Program of Higher Education (No. 20110041110009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2014.05.020.

References

[1] K. Henze, W. Martin, Evolutionary biology: essence of mitochondria, Nature 426 (2003) 127–128.
[2] H.M. McBride, M. Neuspiel, S. Wasiak, Mitochondria: more than just a powerhouse, Curr. Biol. 16 (2006) 551–560.
[3] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.
[4] J. Bereiter-Hahn, M. Vösth, Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria, Microsc. Res. Tech. 27 (1994) 198–219.
[5] R.J. Youle, M. Karbowski, Mitochondrial fission in apoptosis, Nat. Rev. Mol. Cell Biol. 6 (2005) 657–663.
[6] B. Chazotte, Labeling mitochondria with mitotracker dyes, Cold Spring Harb. Protoc. 8 (2011) 990–992.
[7] F. Helmchen, W. Denk, Deep tissue two-photon microscopy, Nat. Methods 15 (2005) 932–934.
[8] C.S. Lim, B.R. Cho, Two-photon probes for biomedical applications, BMB Rep. 46 (2013) 188–194.
[9] H. Yu, Y. Xiao, L. Jin, A lysosome-targetable and two-photon fluorescent probe for monitoring endogenous and exogenous nitric oxide in living cells, J. Am. Chem. Soc. 134 (2012) 17486–17489.
[10] M.F. Ross, G.F. Kelso, F.H. Blaikie, et al., Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology, Biochemistry (Mosc.) 70 (2005) 222–230.
[11] B.C. Dickinson, C.J. Chang, A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells, J. Am. Chem. Soc. 130 (2008) 9638–9639.
[12] X.F. Guo, X.H. Qian, L.H. Jia, A highly selective and sensitive fluorescent chemosensor for Hg^{2+} in neutral buffer aqueous solution, J. Am. Chem. Soc. 126 (2004) 2272–2273.
[13] W. Rettig, R. Lapouyade, Fluorescence probes based on twisted intramolecular charge transfer (TICT) states and other adiabatic photoreactions, Top. Fluoresc. Spectrosc. 4 (1994) 109–149.

Please cite this article in press as: Y. Dai, et al., A two-photon mitotracker based on a naphthalimide fluorophore: Synthesis, photophysical properties and cell imaging, Chin. Chem. Lett. (2014), http://dx.doi.org/10.1016/j.cclet.2014.05.020