A specific probe for two-photon fluorescence lysosomal imaging

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ABSTRACT

Lysosomes are vital organelles in physiological processes, as they receive and degrade macromolecules from the secretory and endocytic procedures. Evidences have shown that lysosomes were related to oncogenic activation and cancer progression, so lysosomes targeting and imaging probes make them convenient to be observed. In this study, a lysosome specific probe W-7 was designed and synthesized via convenient one-pot reaction and Heck reaction. This probe was derived from Tröger's base with a dimethylaminomethyl end group. The optical properties of this compound were measured. W-7 also showed two-photon absorption (TPA) effect by using laser excitation at the wavelength of infrared light. In vivo experiment, W-7 showed high specificity and selectivity for lysosomes in living cells (HeLa cells, MRC-5 cells and NRK cells), compared with LT Red, GT Red and MT Red ($R = 0.96$). Two-photon fluorescence images of HeLa cells stained by W-7 were obtained. And high resolution 3D reconstruction of lysosomes in one HeLa cell was provided by using two-photon confocal microscopy. The anantioseparation of racemic W-7 was carried out by chiral-HPLC, and the two enantiomers showed no significant difference in lysosomes imaging.

1. Introduction

Lysosomes are spherical-shaped, catabolic organelles that ubiquitously exist in eukaryotic cells and play essential roles in oncogenic activation and cancer progression, as they receive and degrade macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways. And many pathogens could use the endocytic pathways to enter cells have evolved mechanisms to avoid being degraded by the lysosomes [1–3]. So one important function of the lysosomes is protection protein from degradation. The lysosomes are different from other organelles, that the lysosomers' pH is maintained between 4.0 and 5.0 due to the proton-pumping function. This acidic environment provides optimal conditions for intracellular catalytic hydrolysis. Studies suggest that lysosomes involve in many physiological processes, such as cholesterol homeostasis, membrane repair, tissue remodelling, pathogen defence, cell

death and cell signalling [4]. Considering the significant biological function, probes which can target and label lysosomes are essential [5]. There are many other complicated organelles in living cells and they are dynamic in the physiological procedures, such as Golgi complex and Mitochondrion. And they all have important and specific functions in living cells. Up to now, it is a challenge to distinguish lysosomes from other organelles conveniently.

Fluorescence microscopy is a facile and convenient method for the imaging of organelles in living cells, due to the high sensitive and low agent dosage [6]. Lysosomes imaging could be achieved by using the commercial probe Lyso Tracker Red (LT Red), and it is expensive and not stable at room temperature. So the development of efficient and not costly probe for lysosomes imaging became a pressing issue. To date, only a handful of commercial probes for lysosomes imaging are available, such as Neutral Red and Acridine Orange derivatives, however their selectivity may not be satisfying and they cannot be used for two-photon fluorescence imaging [7]. Additionally, the short excitation wavelength often leads to both cell damage and limited penetration depth, limiting their use in tissue imaging. On the other hand, fluorophore-labelled antibodies, another efficient tool for subcellular organelles targeting could be candidate for

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lysosomer marker, however their high expense and time consuming experimental procedure lead to serious disadvantages to their overall usage. Moreover, pH sensitivity, low water solubility and stringent storage condition could also limit their utility.

Alternatively, two-photon fluorescence (TPF) becomes the suitable method for organelles imaging in living cells. TPF method usually employ the infrared laser excitation (>780 nm). This laser light source could decrease cell damage and gain better tissue penetration [8,9], because of the long wavelength excitation light source and highly localised excitation by laser [10,11]. Thus, developing a fluorescence lysosomal probe with both high specificity and two-photon absorption (TPA) properties becomes essential. Belfield et al. developed a two-photon probe for lysosmal imaging, which was symmetric and hydrophilic, and demonstrated its usage of lysosome labeling *in vivo*. This probe showed a high specificity for lysosomes in living cells ($R = 0.96$) [9].

We chose Tröger's base as the scaffold of the TPF probe for lysosomal imaging [12]. This Λ-shaped molecule is C_2-chiral due to the stereogenic nitrogen centers which occupy the bridge head positions of the bicyclical framework [13]. Its rigid concave structure has been used for the establishment of a chiral skeleton and the recognition of DNA and proteins [14–16]. A Tröger's base core and two benzyl-dimethylaminomethyl were combined by ethylenic linkages. One-pot reaction was employed to synthesize the Tröger's base core, and efficient Heck reaction was carried out for the construction of ethylenic linkages. The two-step synthetic procedure of this new lysosomal probe was much convenient. And this Tröger's base derived probe appeared stable at room temperature and in normal ambient. To the best of our knowledge, no TPF probe derived from Tröger's base has been reported, especially *in vivo* imaging of lysosomes. And this TPF probe could be potential using valuable as its easy preparation.

2. Materials and methods

2.1. Materials and equipments

Lyso Tracker Red and Golgi Tracker Red were purchased from Beyotime Institute of Biotechnology. Mito Tracker Red was purchased from Invitrogen. HeLa cells were friendly provided by Prof. Zhen Tan's group. All HeLa cells used were incubated at 37 °C in Dulbecco's modified Eagle's Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin streptomycin (Gibco) under 95% humidified atmosphere containing 5% CO_2. Chiral HPLC column was purchased from *astec* and anantioseparation was performed on LC-10 AP liquid chromatograph. UV absorption spectra were taken from UV 2550 UV–vis spectrophotometer and Fluorescence spectra were taken from LS 55 Fluorescence spectrometer. Two-photon emission spectrum was obtained by using femtosecond laser from Zeiss Corporation.

2.2. Synthesis and enantioseparation of W-7

The synthesis and the anantioseparation of the TPF molecule are shown in Scheme 1 (details see Supplemental data). And the structures of compound W-7 were confirmed by ^{1}H NMR, ^{13}C NMR (Mercury VX-300) and mass spectrometry (Figs. S9 and S10). Then we used a chiral column to separate the two enantiomers (W-7-1 and W-7-2) successfully through HPLC enantioseparation. The racemic mixture was loaded in HPLC in 1 mg/mL, 40% methanol in aqueous solution was used as mobile phase in the rate of 1 mL/min.
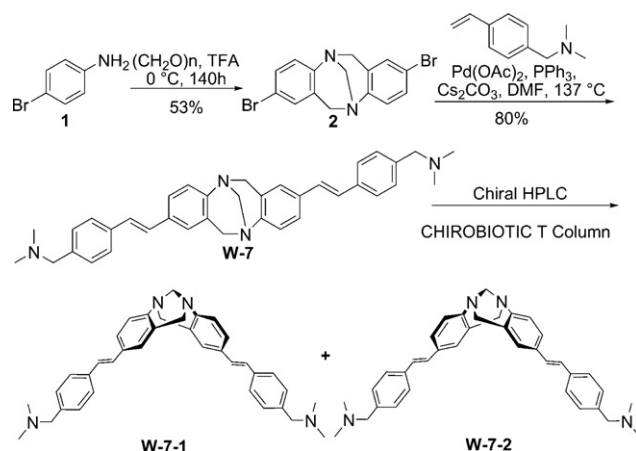
Scheme 1. Synthesis and anantioseparation procedure of W-7.

2.3. Cell culture and stained by W-7 and commercial markers

HeLa cells were adjusted to the density of 10,000/cm² and incubated for 48 h in complete DMEM, then the fluorescent compounds W-7-1, W-7-2 and racemic mixture W-7 dissolved in fresh DMEM without serum were added. Another 2 h incubation, cells were washed 3 times by PBS buffer, then the samples were readily to be detected by confocal microscopy. In colocalization assay, 60 nM LT Red was co-stained with synthetic probe for 2 h. For GT Red's co-staining, the concentration was 0.33 mg/mL, it was added 1.5 h after compounds' staining and kept for 0.5 h. For MT Red's co-staining, the concentration was 6 μM, it was added 1 h after compound's staining and kept for 1 h.
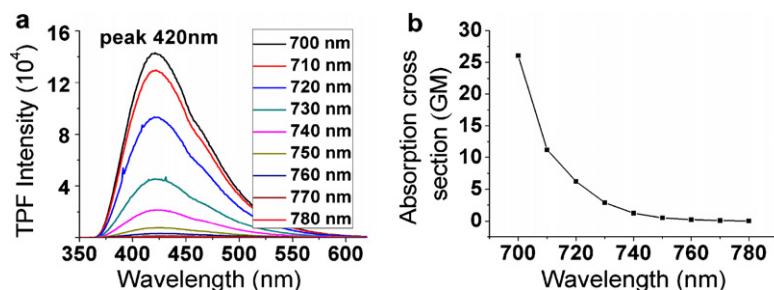
2.4. Study of optical properties

Absorption and one-photon emission spectra were primarily measured in DMSO, subsequently, methanol and aqueous buffer also used as solvent for comparison of optical properties. By using quinine sulfate as standard ($\Phi = 0.556$), quantum yield of W-7 in methanol (Φ_{W-7}) was obtained. Then two-photon emission spectra in methanol was measured using a femtosecond laser for excitation and TPA cross section was measured and calculated following literature methods, using Rhodamine B in methanol as standard. To determine whether the probe's optical properties were solvent and/or pH sensitive, we varied the solvent conditions from aqueous to organic and adjusted the pH to fall in the range of 4.0–8.0.

Fluorescence quantum yield (Φ) of both probe W-7 and LT Red (Φ_{W-7} and $\Phi_{LT\ Red}$) in methanol were measured with quinine sulfate as standard ($\Phi_{ref} = 0.556$) [19]. Two-photon fluorescence spectrum was monitored by two-photon laser in the wavelength range of 700–780 nm and absorption cross section (δ) was calculated according to the literature by Xu and Webb [17].

2.5. Colocalization study of living cells

In cellular imaging, the two enantiomers (W-7-1 and W-7-2) were adjusted to 10 μM in buffer, and then HeLa cells, NRK cells and MRC-5 cells were co-stained with the enantiomers and LT Red at 37 °C for 2 h. After incubation, cells were washed 3 times with PBS and fresh PBS was added, then the samples were readily to be detected under confocal microscope. Same conditions were employed for the racemic mixture (W-7).

Fig. 1. Two-photon optical properties of W-7 in the wavelength range of 700–780 nm. (a) Two-photon emission spectra in methanol with variety of excitation wavelengths. (b) Two-photon absorption cross section.
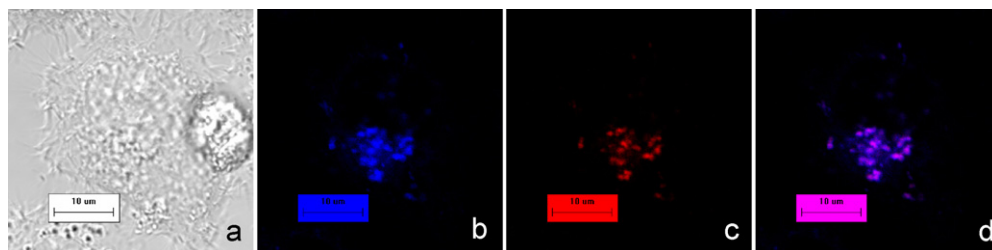
Fig. 2. Colocalization images of one HeLa cell incubated with 10 μM of W-7-1 for 2 h and Lyso Tracker Red. (a) Differential interference contrast (DIC) image; (b) one-photon confocal image of cell stained by W-7-1, Ex 405 nm, Em 461 nm/50 nm; (c) one-photon confocal image of cell stained by LT Red, Ex 559 nm, Em 618 nm/100 nm; (d) merged images.

3. Results and discussion

### 3.1. Synthesis procedure and the HPLC retention times of the two enantiomers

W-7 was conveniently obtained through a two-steps reaction with an overall 42% yield. In the procedure, compound 2 (Tröger's base core) was generated through a one-pot reaction, and W-7 was obtained through a classic Heck coupling reaction [18,19]. Previously, due to rigidity and hindrance of Tröger's base backbone, it is not easy for derivatization and application [20], especially in the field of biochemistry also remained to be discussed. Here we discovered a new lysosome probe derived from Tröger's base with convenient synthesis and economical starting material. The backbone of Tröger's base was modified with two dimethylaminomethyl groups at the end; they were connected to Tröger's base core through ethylene linkage. The dimethylaminomethyl groups are electron-rich, while the two bridgehead nitrogen atoms in the Tröger's base core are electron deficient. This general motif produces a D–π–A–π–D two-photon system. In addition, to possessing a two-photon system, the dimethylaminomethyl groups are also basic and demonstrate good biocompatibility [21]. These properties enable the probe to enter the lysosomal membrane and target the acidic hydrolyse, which were favourable for the compound's utilization in practice. This synthetic procedure and structural characteristics of the target molecule W-7 is quite straightforward and suitable, and the product is very stable at normal ambient, indicating that lysosome specific probe W-7 could be inexpensive.

Since chirality of the Tröger's base derivatives and the subsequent different characteristics in optical and biological properties, it was an attempt to anantioseparate W-7 by using chiral HPLC column. The corresponding retention times of the two enantiomers W-7-1 and W7-2 were respectively 31.52 min and 36.25 min (shown in Fig. S1). Although the mobile phase was not costly mixture of methanol and water, the retention times were considerable long and the consumption of chiral column could not be neglected, and the two enantiomers appeared no significant difference in vivo experiments (Section 3.3), it was reasonable to use W-7 as lysosome probe directly without further anantioseparation.

3.2. Analysis of the UV spectra and the fluorescence emission spectra

The absorption and emission spectra were measured and showed a maximum absorption and emission at 350 nm and 450 nm respectively in DMSO. The spectra showed that the absorption remained the same, while the fluorescence intensity decreased with the following sequence: DMSO > CH$_3$OH > aqueous buffer. Contrastingly, in different pH-buffered solutions, the emission
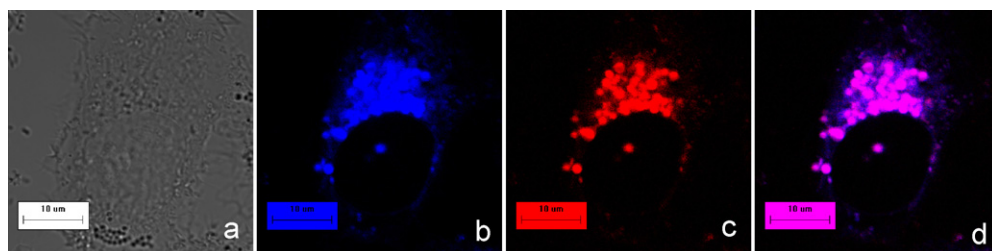

Fig. 3. HeLa cells were treated by 10 μM W-7-2 for 2 h. (a) DIC image; (b) one-photon confocal image of cell stained by W-7-2, Ex 405 nm, Em 461 nm/50 nm; (c) one-photon confocal image of cell stained by LT Red, Ex 559 nm, Em 618 nm/100 nm; (d) merged images.
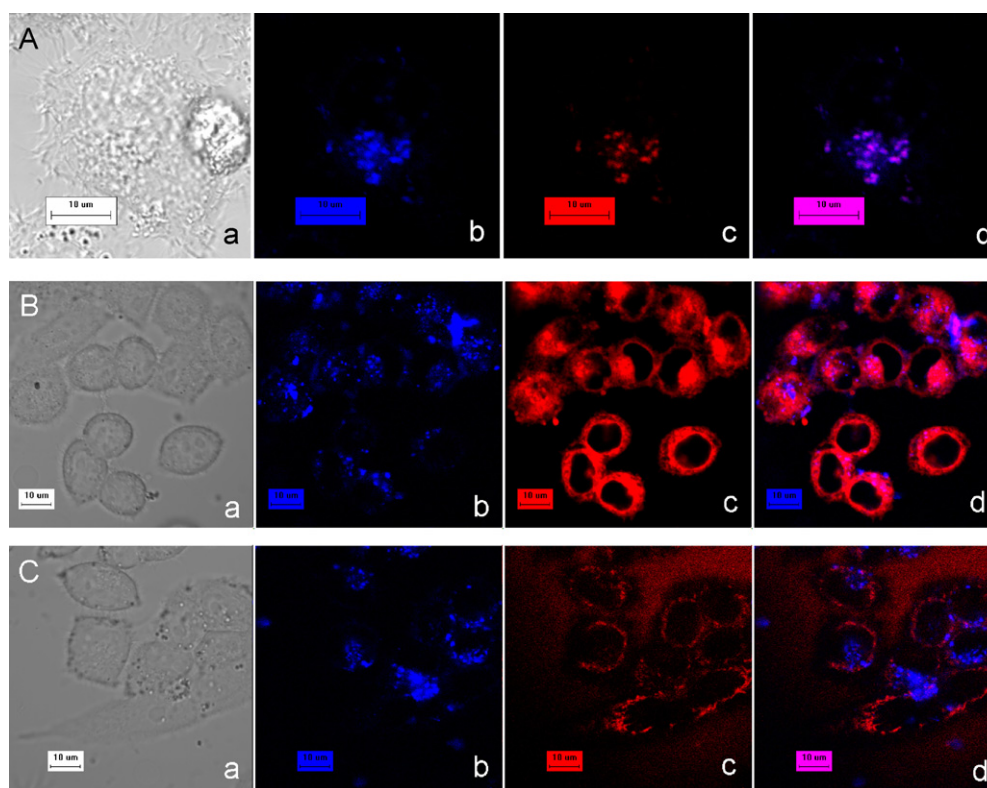
Fig. 4. Colocalization images of HeLa cells incubated with compound W-7-1 in 10 μM for 2 h and the three commercial dyes. Rows A, B and C were co-stained images with LT Red, GT Red, MT Red, respectively. Four images in each row standed for: (a) in A, B, C rows are differential interference contrast (DIC) images; (b) in A, B, C rows are cells stained by W-7-1, one-photon confocal images, Ex 405 nm, Em 461 nm/50 nm; (c) in A, B, C rows are cells stained by LT Red, GT Red and MT Red, respectively, one-photon confocal images, Ex 559 nm, Em 618 nm/100 nm; (d) in A, B, C rows are merged images respectively. All images were taken under 60× oil immersion objective.
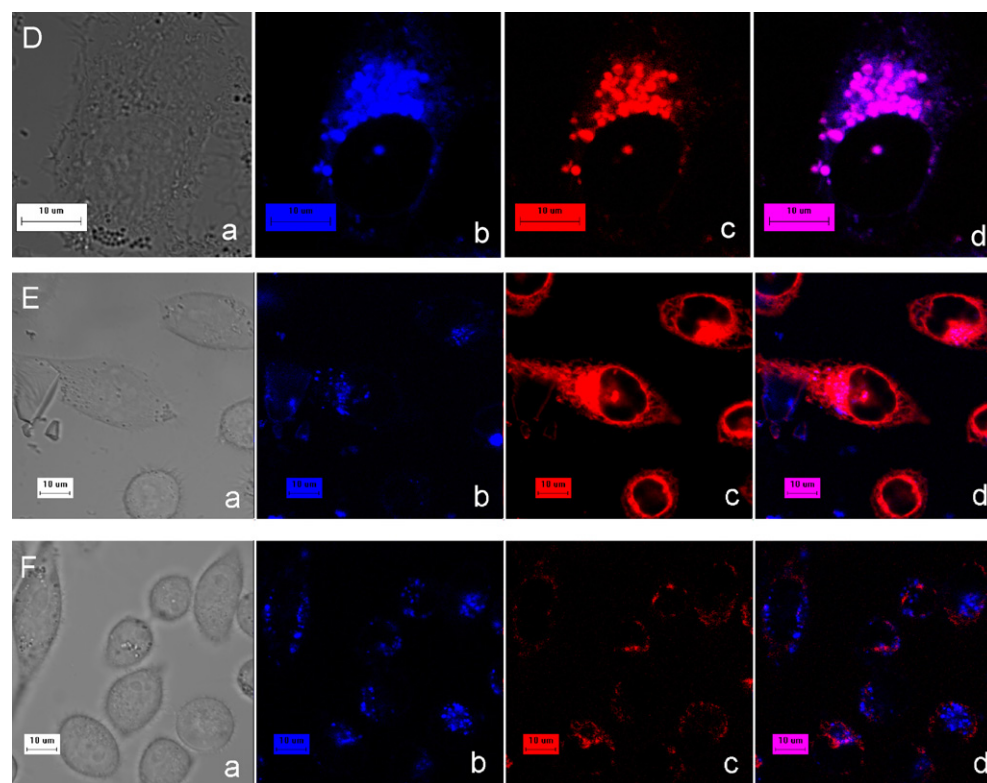
Fig. 5. Colocalization images of HeLa cells incubated with compound W-7-2 in 10 μM for 2 h and the three commercial dyes. Rows D, E and F were co-stained images with LT Red, GT Red and MT Red, respectively. Four images in each row standed for: (a) in D, E, F rows are differential interference contrast (DIC) images; (b) in D, E, F rows are cells stained by W-7-2, one-photon confocal images, Ex 405 nm, Em 461 nm/50 nm; (c) in D, E, F rows are cells stained by LT Red, GT Red and MT Red respectively, one-photon confocal images, Ex 559 nm, Em 618 nm/100 nm; (d) in D, E, F rows are merged images respectively. All images were taken under 60× oil immersion objective.
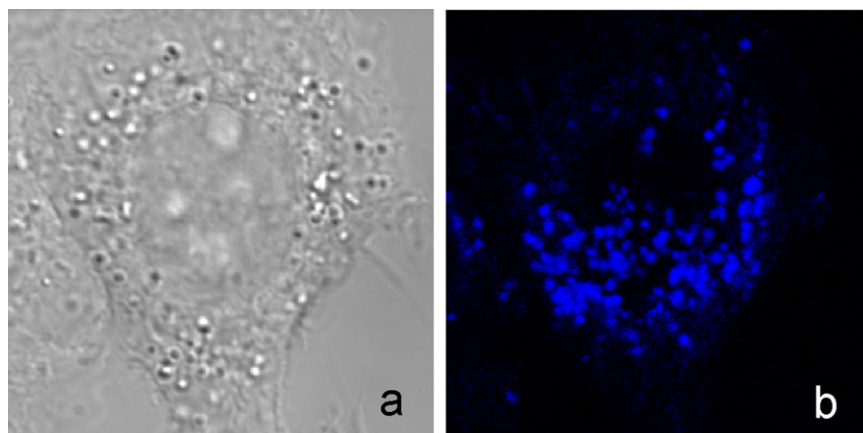
Fig. 6. Two-photon confocal microscopy image. HeLa cells were incubated with racemic mixture W-7 at 10 μM for 2 h. (a) DIC image and (b) two-photon fluorescence image, Ex 790 nm, Em 460 nm/50 nm.

spectra demonstrated limited changes (Figs. S2 and S3). The maximum absorption and emission of W-7 displayed a Stokes shift of 100 nm and the one photon excitation wavelength was in ultraviolet band, avoiding the disturbance of excited light source and natural light. The absorption spectra in different solvents and variety pH conditions showed that W-7 was quite stable in practice. These data also indicated that the synthetic TPF probe was sensitive to solvents but not to the pH of aqueous buffers, allowing the probe to maintain stable *in vivo* experiment. Although the fluorescence intensities in different aqueous buffers showed not intense enough, W-7 was successfully employed for the lysosome imaging as it showed well fluorescent property in living cells.

The fluorescence quantum yield of TPF probe is $\Phi_{W-7} = 0.62$, higher than that of commercial lysosomal marker LT Red ($\Phi_{LT\,Red} = 0.46$). Subsequently, we analyzed the TPF spectra of W-7 and calculated the TPA cross section (δ) according to literature method (Fig. 1) [22]. The calculated value of δ_{W-7max} is 26 GM ($1\,GM = 1 \times 10^{-50}\,cm^4\,s\,photon^{-1}$) at 700 nm. The advantage of W-7 was higher quantum yield than LT Red, and was important for an efficient probe. Two-photon excitation of W-7 made it potential applicable in practical lysosome imaging. Although the TPA cross section was measured at 700 nm and it seemed not good enough, the reliable TPF lysosomal images could be carried out excited at 790 nm with a concentration of 10 μM. These characters of W-7 indicated it was sensitive enough to be used in TPF imaging of living cells (Section 3.3). May be W-7 could bind with some proteins in lysosomes and lead to a change of optical aspect.

3.3. One-photon and two-photon fluorescence confocal microscopy images

One-photon laser confocal microscopy images indicated that all of the TPF probes could readily enter the cells. Moreover, this TPF probe in living cells provided enough fluorescence intensity and image resolution. HeLa cells were co-stained with commercial markers, Lyso Tracker Red (LT Red), Mito Tracker Red (MT Red), Golgi Tracker Red (GT Red) and each enantiomer W-7-1 and W-7-2, respectively. To our success, both enantiomers showed high lysosome specificity. Cellular images demonstrated that the lysosome localisation of both enantiomers and LT Red were almost completely identical (Figs. 2 and 3). However, for golgiosomal and mitochondrial colocalization, the compounds' localisation did not overlap with the individual commercial dyes used (Figs. 4 and 5). Specificity and excellent optical properties are two essential aspects for organelles marker design. Colocalization experiments demonstrated that W-7 possessed high lysosomal selectivity for

lysosomes in living cells. This results may be related to the Λ-shaped structure of Tröger's base and the dimethylaminomethyl end groups. The hydrophobic structure made it facile to be uptaken by living cells. The selectivity between different organelles in living cells may be respond to the slightly alkaline end groups, since it is weakly acidic inside the lysosomes [4]. Fluorescence probes which could be incepted by living cells were often filled all the cytoplasm and showed no selectivity for organelles. The Λ-shaped Tröger's base W-7 showed both good uptake by cells and excellent specificity and selectivity for lysosomes in living cells.

Pearson's method was used to quantitatively evaluate these colocalization effects. The correlation coefficient of each merged image was calculated by software, with data summarised in Table 1. Based on the calculated data, both enantiomers' colocalization with LT Red produced Pearson's correlation coefficients more than 0.9. Similarly, MT Red and GT Red resulted in correlation coefficients are not more than 0.4, suggesting the high specificity and selectivity toward lysosomes. Between two enantiomers (W-7-1 and W-7-2), no obvious difference was observed in colocalization studies. This suggested that racemic mixture (W-7) could be directly used as lysosome marker without any further anantioseparation and its colocalization property was also proved (Fig. S4). With the extended time to 8 h, the synthetic TPF probe W-7 remained localised within the lysosomes without any nonspecific diffusion and the colocalization efficient was even increased. The Pearson's correlation coefficients ran up to as high as 0.96 (Fig. S5). These findings suggested that the synthetic probe remained chemically and optically stable in lysosomes without any decomposition and diffusion, which could be very important and favorable for specific organelle probe design.

The probe W-7 could also be used as lysosomal marker in other cell lines. The MRC-5 cells (normal cells) and NRK cells (infinite cells) were employed for the colocalization studies. Results showed that W-7 could also localise lysosomes with good specificity in MRC-5 cells and NRK cells (Figs. S6 and S7). Therefore, this TPF probe was proved to be good marker for wide applications. Beside tumor cells, good lysosomal targeting for normal cells and infinite cell line, W-7 was proved to be good marker for wide use. Evidences suggested the enantiomers may not have specific interaction with

Table 1
Pearson's correlation coefficients of colocalization images.

	LT Red	MT Red	GT Red
W-7-1	0.92	0.14	0.40
W-7-2	0.91	0.29	0.37

membrane proteins or hydrolyses in lysosomes, therefore, endocytosis may be a possible cellular uptake pathway, which consistent with some reported basic amine containing lysosomal probes in previous reports [9].

3.4. 3D reconstruction

Finally, the superiority of the TPF probe in living cells was demonstrated by two-photon confocal microscopy. Herein, HeLa cells were used to be treated by W-7 at the same condition above, two-photon confocal images of them were taken (Fig. 6 and Fig. S8), which showed even higher resolution compared with one-photon confocal images above. 3D reconstruction was also created successfully by stack-scanning, which was shown as a 3D rotation movie (Video data). In the movie, even each lysosome could be clearly visualized. As a small molecule, W-7 could specifically target lysosomes in living cells, despite the complicated environment and many other organelles. W-7 could also be employed as an efficient TPF probe for lysosome imaging, much stable and not expensive compared with LT Red. These suggested that this TPF probe could be more useful than some commercial one-photon dyes in cell and tissue imaging.

4. Conclusions

Based on the Tröger's base scaffold, we successfully designed and synthesized a TPF probe for lysosomal imaging in living cells, through convenient synthetic procedure employed one-pot reaction and Heck reaction. The high specificity and selectivity for lysosomes was demonstrated by colocalization experiments, with the comparison of commercial organelles markers. This probe could also be used for lysosomes imaging in different kinds of cells. Furthermore, both high quality of TPF images and high resolution 3D reconstruction for lysosomes were obtained by using two-photon fluorescence microscope. This TPF probe could be a potential candidate for intracellular lysosome targeting and imaging.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.09.065.

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