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A photostable fluorescent probe for rapid monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics*

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Yong Liu[a], Fangfang Meng[a], Yonghe Tang[a], Xiaoqiang Yu[b]*, Weiying Lin[a]*

Rapid monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics play critical roles for judging occurrence and development of the disease, and can give insights for studying apoptosis and cell degeneration. However, the existing probes are not capable of rapid monitoring and tracking the above dynamics process. To solve this problem, we develop a unique functional mitochondria probe containing long alkyl chains, 3, 5-bis((*E*)-2-(pyridin-4-yl)vinyl)-1*H*-indole monoiodide (**MT-PVIM**), which is capable of rapid real-time imaging and tracking mitochondrial fission and fusion dynamics. In addition, compared with commercially available mitochrondrial probe MTR, the probe **MT-PVIM** has excellent specificity to mitochondria with outstanding tolerance of micro-environmental changes, thus representing a potential candidate as a tracking agent for apoptosis studies. The good performance of our proposed approach demonstrates that this strategy might open up new opportunities for the development of rapid image mitochondria-targetable molecular tools for bioanalytical and biomedical applications.

Introduction

Mitochondria play a key role in various biological processes including energy production through the respiratory chains,[1-3] cell signaling via reactive oxygen species production,[4, 5] regulation of Ca^{2+} homeostasis,[6, 7] and the triggering of cell death.[8-11] Mitochondria are dynamic organelles that can quickly change in number and morphology within a cell during the development, the cell cycle, or when challenged by various toxic conditions.[12] They move from one location to another and undergo continuous fusion and fission events quickly. These behaviors are altogether classified as “mitochondrial dynamics”.[13] Mitochondrial dysfunction are associated with intrinsic apoptotic pathways and cause a variety of neurodegenerative diseases such as Alzheimer's disease, cancer and diabetes.[14] Mitochondrial fission and fusion are closely connected with mitochondrial dynamic changes. It plays role in maintaining protection of mitochondrial DNA (mtDNA).[15] And this process regulates mitochondrial morphology, number, location, and function.[16] Therefore, Rapid acrossing cell membrane and real-time tracking mitochondrial fission and fusion dynamics processes can give insight for studying occurrence and development of the disease and apoptosis and cell degeneration.

In the past decade, fluorescence probes have been an important detection means to image mitochondria and intracellular target.[17-20] To date, two classes of probes are mainly used. As shown in table S1, the first class of probes can real-time image mitochondria.[14, 21] But, most probes enter the cells and detect mitochondria are approximately 15-60 min. Thus, the probes cannot achieve rapid detect and real-time image mitochondria in a short period of time. Moreover, these probes unable to track mitochondrial fission and fusion dynamics processes. The second type of probes can track mitochondrial dynamics changes.[22, 23] For example, green fluorescent proteins (GFP),[24] CMT-red,[14] NPA-TPP,[23] Rhodamine 123[25] and JC-1,[26] which have been used to specifically target mitochondria to reveal that the mitochondria *in vivo* are highly dynamic. However, these fluorescent probes showed poor photostability so that could not rapid monitor and track trans membrane process and mitochondrial fission and fusion dynamics. For example, JC-1 is the most widely used fluorescent sensor for detecting mitochondrial membrane potential ($\Delta\Psi m$). However, though the probe JC-1 is highly sensitive to dye loading concentration and time, many literature studies reported false results of using JC-1 for studying mitochondria.[27] And it is not suitable for rapid monitoring and tracking rapid trans membrane and mitochondrial dynamics changes. All in all, the existing probes showed poor photostability and poor permeability so that could not monitor and track rapid trans membrane and mitochondrial fission and fusion dynamics process. Therefore,

[a] *Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Biological Science and Technology, University of Jinan, Jinan, Shandong 250022, P. R. China. E-mail: weiyinglin2013@163.com*
[b] *Center of Bio & Micro/Nano Functional Materials, State Key Laboratory of Crystal Materials, Shandong University, Jinan, 250100, PR China.*
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the search for a probe that can monitoring and tracking rapid trans membrane process and mitochondrial fission and fusion dynamics has always been an attractive and challenging goal.

In this work, as shown in Fig 1, we develop a unique mitochondria probe, 3,5-bis((*E*)-2-(pyridin-4-yl)vinyl)-1*H*-indole monoiodide (**MT-PVIM**). We predict that this probe is capable of monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics compared with MTR.

Experimental

Measurements equipments and materials

TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals. All chemicals mainly were analytical grade, and 4-picoline, 1-iododododecane and 5-bromo-1*H*-indole-3-carbaldehyde were purchased from Sinopharm Chemical Reagent Co., MitoTracker Red, Ltd (Shanghai, China), Palladium (II) acetate and tri-*o*-tolylphosphine were purchased from J&K Chemical (Shanghai, China). Tris and PBS were purchased from Seikagaku Corporation of Japan. All solvents maimly used in the spectral measurement are of chromatographic grade.

We used a Bruker Avanace 300/400 spectrometer to obtain Nuclear magnetic resonance spectra (^{1}H and ^{13}C) of all compounds. The HRMS spectra were obtained by LC/MS ThermoFisher LCQ FLEET or Agilent Technologies 6510 Q-TOF. The elemental analyses were performed on a Vario EI III instrument. The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a Cary 50 spectrophotometer using a quartz cuvette having 1 cm path length. The fluorescence fluorescence spectra were obtained by a HITACHI F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. All confocal microscopic photos were obtained with Carl Zeiss Microscopy LSM780 equipped with a high-resolution charge coupled device (CCD) (Pixel: 512x512) and 60×objective oil lens. The confocal microscopic image and differential interference contrast (DIC) image were taken with a 488 nm laser. Confocal microscopic images of MitoTracker Red (MTR) were collected between 569-691 nm upon excitation at 561 nm; Fluorescence images of **MT-PVIM** were collected between 490 -604 nm upon excitation at 488 nm.

Measurement of the fluorescence quantum yields

In this work, the fluorescence quantum yields (Φ) of the probe **MT-PVIM** were calculated by means of equation (1):

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)} \right) \left(\frac{n_s^2}{n_r^2} \right) \frac{F_s}{F_r} \quad (1)$$

where the subscripts *s* and *r* represent the sample and the reference materials, respectively. Φ stands for the quantum yield, *F* expresses the integrated emission intensity, *A* stands for the absorbance, and *n* is the refractive index. In this paper, Fluorescence quantum yields for **MT-PVIM** was determined by using fluorescein (Φ = 0.95 in water) as fluorescence standard.

Cell culture and image

Cancer cells SiHa were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 10% bovine calf serum in a 5% CO_2 incubator at 37°C. SiHa cells were grown overnight on a 35 mm petri dish with a cover slip or a plasma-treated 25 mm round cover slip mounted to the bottom of a 35 mm petri dish with an observation window. The living cells were stained with 3 μM **MT-PVIM** for 3 min (by adding 2 μL of a 1 mM stock solution of **MT-PVIM** in DMSO to 1 mL culture medium) or 250 nM MTR for 20 min (by adding 3 μL of a 200 μM stock solution of MTR in DMSO to 1 mL culture medium).

Tracking probe enter cells processes test

For the dye entering into cells process test, the cells in the 35 mm petri dishes without stain were loaded below the microscope; Next, using microsyringe join probe to petri use over time (starting from time 0, then at 5, 10 and 15 min) using confocal microscope (Zeiss Laser Scanning Confocal Microscope; LSM7 DUO) and ZEN 2009 software (Carl Zeiss). The probe **MT-PVIM** was excited at 488 nm and the fluorescence was collected at 490-604 nm. MTR was excited at 561 nm and fluorescence was collected at 569-691 nm.

Cell imaging with carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment

Living SiHa cells were grown overnight using a 35 mm petri dish and a cover slip. The cells were incubated with 10 μM CCCP (by adding 1 μL of a 200 mM stock solution of CCCP in DMSO to 2 mL culture medium) for 30 min. The CCCP treated cells were then stained by 3 μM **MT-PVIM** for 3 min or 250 nM MTR (by adding 0.5 μL of a 200 μM stock solution of MTR in DMSO to 2 mL culture medium) for 20 min.

Results and discussion

Preparation of probes

As we all know, a selective localization of hydrophobic fluorophore at the plasma membrane can be achieved by conjugating it with zwitterionic groups and long hydrophobic chains.[28] In our previous work, we found some structures containing indoles cationic salt were capable of sensing mitochondria.[29] Thus, in this work, we constructed a unique probe **MT-PVIM**, and the anchor group of the compound **MT-PVIM** was composed of long alkyl chains indoles cationic group, allowing strong interaction with the lipid membrane (Fig. 1a). First, this push-pull electronic system based on indole possess superior membrane permeability so that may rapidly enter cells and light up mitochondria in a short period of time. In addition, we anticipated that this system with linear alkyl chains may rapid across the cell membrane and track mitochondrial fission and fusion dynamics process (Fig. 1b).

The compound **MT-PVIM** was synthesized by Knoevenagel condensation reactions between *N*-(2-dodecane)-4 methylpyridinium iodide and a substituted indole-3-carboxaldehyde using piperidine as catalyst. The intermediate 3 was obtained by

Heck coupling reaction between 5-bromo-1*H*-indole-3-carbaldehyde with 4-vinylpyridine. The separated **MT-PVIM** were characterized by various spectroscopic methods, from which satisfactory analysis data were obtained (Scheme 1).

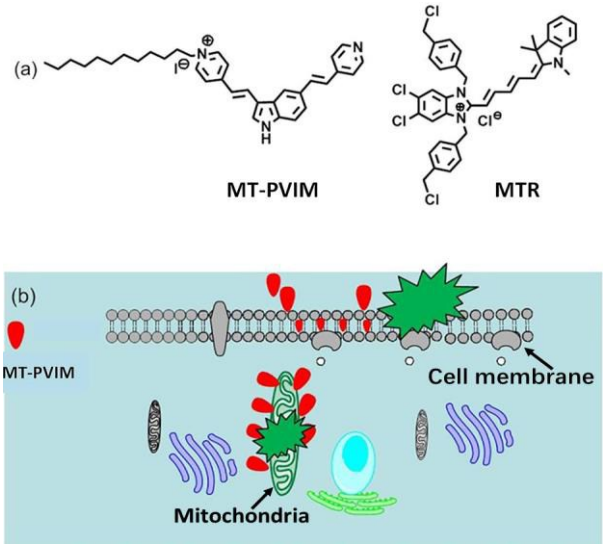
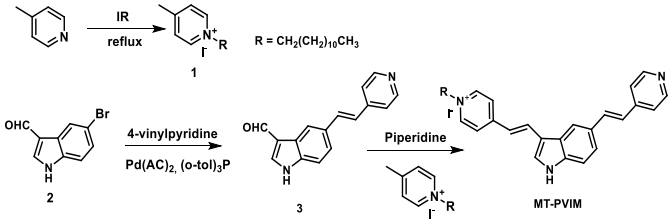


Fig. 1. (a) Structures of the probe **MT-PVIM** and commercially available mitochondrial probe MitoTracker Red (MTR); (b) Overall strategy of our switchable probe, **MT-PVIM** (red), that is capable of rapid monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics.



Scheme. 1. Synthesis of the probe **MT-PVIM**

Photophysical properties of the probe

With the probe **MT-PVIM** in hand, we proceeded to examine its photophysical properties. We find that the absorbance and fluorescence intensities are high in organic solvents and very low in tris-HCl buffer solution. Similarly, quantum yield (Φ) of the probe **MT-PVIM** in buffer solution only reach 1.2% (Fig. 2a and b). In organic solvent, maximum Φ of the probe **MT-PVIM** reached 19-40% (Table 1). As we all know, mitochondria are membrane organelles composing of different organic phase. Thus, we think that the **MT-PVIM** probe may occur strong fluorescence in mitochondria. At the same time, in pH 4.0–7.5, a wide physiological pH range, the **MT-PVIM** was weakly fluorescent (see Fig. S1 in the ESI†). This result showed that the probe **MT-PVIM** was not affect by pH.

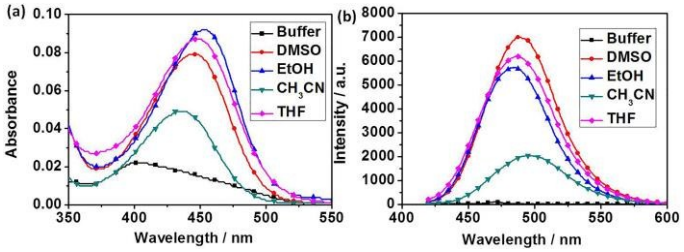


Fig. 2. (a) Absorption spectra and (b) fluorescence responses of **MT-PVIM** in various solvents. Buffer (■), DMSO (●), EtOH (▲), CH_3CN (▼), THF (◆). [**MT-PVIM**]: 2 μM.

Table 1. The photophysical properties of **MT-PVIM**

Probe	Solvents	λ^a	λ^b	Φ^c
MT-PVIM	Buffer	405	588	0.012
	DMSO	450	538	0.40
	EtOH	450	534	0.27
	CH_3CN	436	534	0.19
	THF	450	537	0.33

λ^a and λ^b are linear absorption and fluorescent maximum peak in nm, repectively; $^c\Phi$ is fluorescence quantum yield (Error limit: 8%) determined by using fluorescein (Φ = 0.95) in aqueous NaOH (pH 13) as the standard.

Colocalization experiment

Living SiHa cells were incubated with **MT-PVIM** and the commercially available mitochondrial indicator MTR to examine whether the probe could specifically stain the mitochondria (Fig. 3). The confocal image of **MT-PVIM** merged well with that of the MTR. Moreover, the Pearson's colocalization coefficient was determined as 0.90, demonstrating that **MT-PVIM** predominantly located in the mitochondria.[30-33]

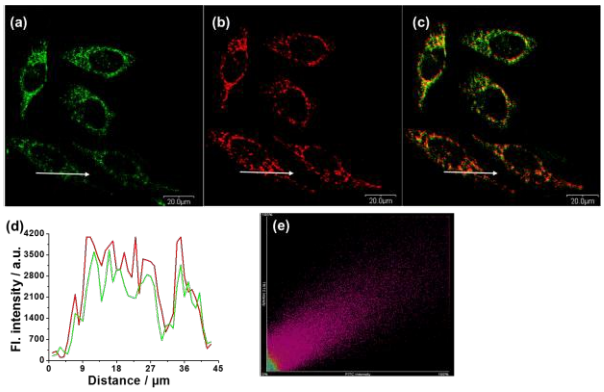


Fig. 3. (a) Fluorescence images of **MT-PVIM** (3 μM, 3 min) collected between 490 and 604 nm upon excitation at 488 nm; (b) Confocal fluorescence images of SiHa cells stained with MTR (250 nM, 10 min) collected between 569 and 691 nm upon excitation at 541 nm. (c) Merged image of a and b. (d) Intensity profile of linear region of interest (white arrow) across the SiHa cells costained with MTR and **MT-PVIM**; (e) Correlation plot of MTR and **MT-PVIM** intensities. Scale bar = 20 μm.

Rapid monitoring and tracking trans membrane and imaging mitochondria

We investigated whether the **MT-PVIM** probe can rapidly image and track mitochondrial morphological changes. First, we studied the process that the dye enters into the cells and illuminates the mitochondria. The **MT-PVIM** dye was added to a 35 mm Petri dish with an observation window containing 1mL culture medium consisting of SiHa cells and was imaged over time (initially, and then at 5 min intervals up to 15 min) using Zeiss laser scanning confocal microscope LSM780. As shown in Fig. 4a, during 30 scans with a total irradiation time of ~899 s, the fluorescence emission signal of **MT-PVIM** at 488 nm excitation first increased, then leveled off and finally gradually decreased in the cell membrane (See video. S1 in the ESI†). **MT-PVIM** clustered into the cell membrane in as little as 93 s. However, the intracellular fluorescence emission signals continue to increase until reached the saturation point under 488 nm continued laser excitation. The results showed that the probe possessed superior permeability to living cells and high sensitivity to intracellular mitochondria (Fig. 4b).

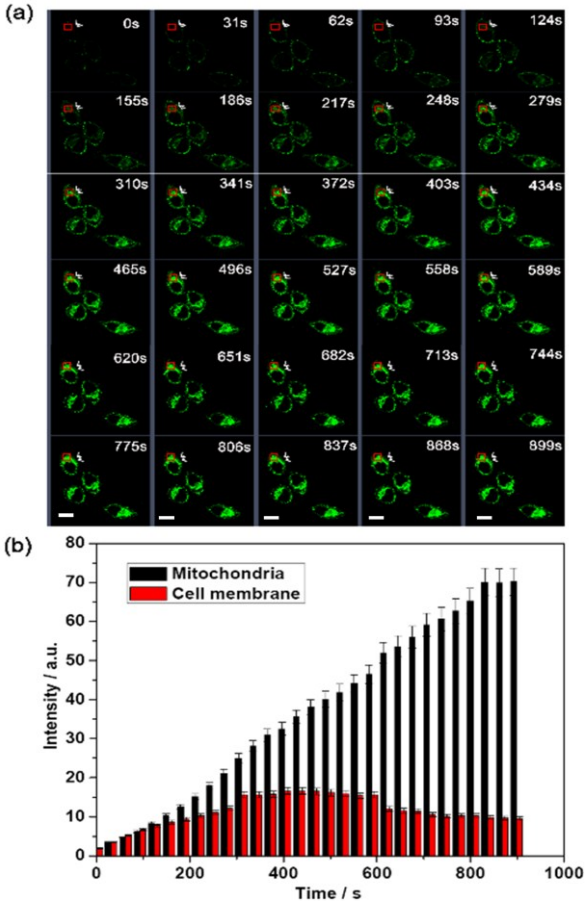


Fig. 4. (a) SiHa cells were stained with the probe **MT-PVIM** and imaged over time (starting from time 0, then at 5, 10 and 15 min, as shown in video S1) using confocal microscopy. (b) Intensity change of cell membrane (white arrow) and mitochondria at times from 0-899 s (red squares). Images and fluorescence spectra were acquired using 488 nm, excitation and emission windows of 490-604 nm; irradiation time: 30.98 s/scan. Scale bar = 10 μm.

Tracking mitochondrial fission and fusion dynamics

We have proved the probe **MT-PVIM** could rapid monitor and track trans membrane and image mitochondria. Next, we will track mitochondrial fission and fusion dynamics within 620-899 s. As shown in Fig. 5a, mitochondria appear to exist as a dynamic reticulum of long, thin, branched tubules that spread throughout the cytoplasm of the cell (Fig. 5d). In addition to high mobility and frequent shape changes, the fusion (arrows) and fission (arrowheads) of mitochondria can be detected (See video S2 in the ESI†). Changes of mitochondrial morphology are associated with a distinct increase in mitochondrial number, suggesting the participation of mitochondrial fission machinery. The number and morphology of mitochondria within a cell are controlled at precisely regulated rates of organelle fusion and fission. Under different growth conditions, fission or fusion rates may change and lead to an increase or decrease in mitochondrial number.

Until recently, little was known about the molecular mechanisms regulating mitochondrial number and morphology. However, combined genetic, biochemical and microscopic approaches have revealed the existence of a number of proteins that participate in mitochondrial fusion and fission.[34] The probe **MT-PVIM** enable us to have a first look at the molecular events governing the regulation of mitochondrial morphology in cells**.** By contrast, we cannot visualize the process how MTR enters cells or visualize the mitochondrial dynamic behavior in cultured mammalian cells. Because the fluorescence signals of MTR almost disappear after the second scan (Fig. 6).

The working concentration of **MT-PVIM** and MTR was 3 μM and 250 nM in our experiments, respectively. At first glance, one may think that MTR is more sensitive than **MT-PVIM**. In a sense, MTR can easily be photobleached by strong excitation fluorescence. At high concentrations, the probe MTR will lose the specificity and stain other cellular structures. [35]

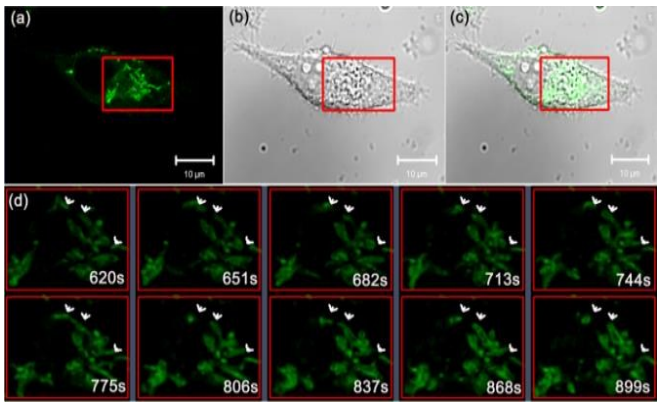


Fig. 5. Imaging of SiHa cells stained with **MT-PVIM** (a) fluorescent picture (b) DIC picture (c) merged picture. (d) Dynamic network-like behavior of mitochondria in cultured mammalian cells (red squares). In addition to high mobility and frequent shape changes, fusion (775 s) and fission (arrowheads 806 s) of mitochondria can be detected. Irradiation time: 30.98 s/scan. Scale bar = 10 μm.

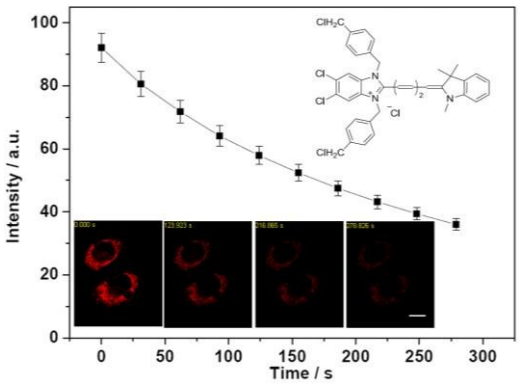


Fig. 6. SiHa cells were stained with the mitochondria fluorescent probe MTR and imaged over time (at time 0, followed by 279 s) using confocal microscopy. Images were acquired using 561 nm, excitation and emission windows of 569 and 691 nm; irradiation time: 30.98 s/scan. Scale bar = 20 μm.

Photostability and tolerance

Photostability is one of the most important criteria for developing fluorescence imaging agents.[36, 37] Continuous scanning by confocal microscope (Zeiss laser scanning confocal microscope LSM780) was used to quantitatively investigate the photostability of **MT-PVIM** (2 μM) and MTR (2 μM). Within 300 s, the fluorescence signals of **MT-PVIM** decreased slightly (~ 5%); In contrast, MTR exhibited significant photobleaching with only ~ 16% signal intensity remaining (see Fig. S2 in the ESI†).

Changes of mitochondrial membrane potential (ΔΨm) value will cause change about tolerance of probe. This part, we will use carbonyl cyanide CCCP to treat the cells prior to the staining procedure. The compound CCCP is an uncoupler that can result in decrease of ΔΨm value. It mainly caused by rapid acidification of the mitochondria and dysfunction of ATP synthase in cells.[36] Join of CCCP caused decreasing of intracellular pH.[38]

We study the influence of ΔΨm by cell imaging. The probe MTR and **MT-PVIM** incubated SiHa cells treated by 5 μM CCCP. The image results showed that MTR had no more specificity to mitochondria, and the sensitivity worsened (see Fig. S3 in the ESI†). Compared with MTR, the sensitivity of **MT-PVIM** to mitochondria is perfectly retained in the CCCP-treated cells (Fig. 7). Because the mitochondria imaging is closely related to ΔΨm, and decrease of ΔΨm will directly affect accumulation of cationic MTR in the mitochondria. Thus, compared with MTR, **MT-PVIM** allows a wider dynamic range for mitochrondrial targeting upon the change of ΔΨm.

The fluorescence intensity of the stained mitochondria can be used to estimate the mitochondrial ΔΨm. By monitoring the time-dependence of the fluorescence of the inner mitochondrial membrane, one can obtain qualitative information about the dynamics of the ΔΨm. The high tolerance of **MT-PVIM** to a decrease of ΔΨm enables the observation of changes in the mitochondrial morphology induced by CCCP (video S4 in the ESI†). Due to the photostability of **MT-PVIM**, the mitochondrial reticulum-like behavior was clearly observed (Fig. 7a, video S4 in the ESI†). However, compared to reticulum-like mitochondria, upon exposure to CCCP, the mitochondria exhibited small and dispersed fragmentation and different morphological changes over a long period of time. That is to say, before and after the cells were treated, **MT-PVIM** possessed outstanding photostability and tolerance of micro-environmental changes (Fig. 7c), thus representing a potential candidate as a tracking agent for apoptosis studies.

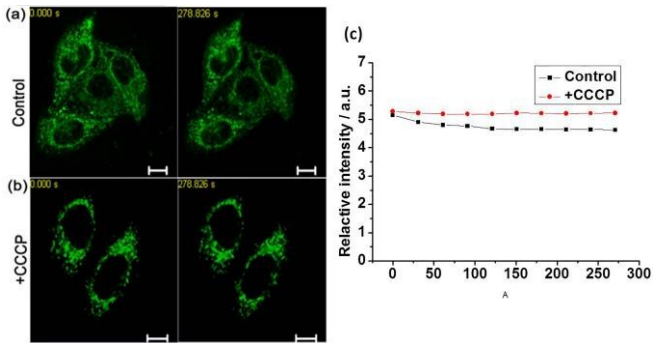


Fig. 7. Fluorescence images of living SiHa cells stained with **MT-PVIM** (3 μM) before (a) and after (b) treatment with CCCP (20 μM) as increasing scan time. (c) Fluorescent intensity changes of living SiHa cells stained with **MT-PVIM** (3 μM) without (a) and with (b) CCCP (20 μM) treatment as increasing scan time (the scan time shown in the upper left corner of the panel). Excitation wavelength: 488 nm; emission filter: 499−560 nm; irradiation time: 30.98 s/scan. Scale bar = 20 μm.

Conclusions

In summary, rapid acrossing cell membrane and tracking mitochondrial fission and fusion dynamics processes can give insight for studying development of the disease and cell degeneration. In this work, we designed and synthesized a unique mitochondria-targetable fluorescent probe **MT-PVIM** for monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics. Compared with commercially available mitochrondrial probe MTR, the probe **MT-PVIM** has excellent specificity to mitochondria and tolerance of micro-environmental changes. The good performance of our proposed approach demonstrates that this strategy might open up new opportunities for the development of efficient subcellular molecular tools for bioanalytical and biomedical applications.

Acknowledgements

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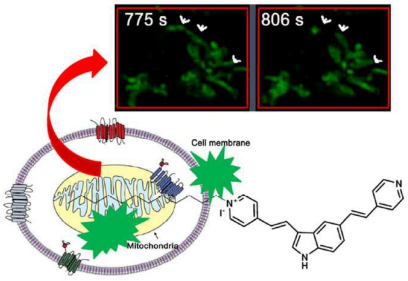

The probe **MT-PVIM** was capable of monitoring and tracking trans membrane process and mitochondrial fission and fusion dynamics.