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COMMUNICATION

Development of a mitochondrial-targeted two-photon fluorescence turn-on probe for formaldehyde and its bio-imaging applications in living cells and tissue

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A mitochondrial-targeted two-photon fluorescent formaldehyde probe (MT-FA) with a large emission enhancement was developed. MT-FA could image formaldehyde in mitochondria of living cells for the first time, and also could image formaldehyde in living liver tissue slices.

Formaldehyde (FA), a common environmental toxin, identified as one of most the dangerous carcinogens by the World Health Organization (WHO). Prolonged exposure to FA will increase the risk of developing some malignant diseases, such as respiratory diseases, cancer, Alzheimer's disease.[1] Although FA is a harmful substance, endogenous FA exists in organisms. The metabolism of methylated amines by the enzymatic reaction of Semicarbazide-sensitive amine oxidases (SSAO) may produce FA in living systems.[2] The endogenous FA plays an important role in cells,[3] especially organelles, such as the one carbon metabolic cycle in Mitochondrion.[4] However, the role of FA in living organisms is still not well-defined. It is urgent to develop a method to effectively detect FA in living organisms.

In eukaryotic cells, mitochondria, as the main energy supply organelles, play the pivotal role in the metabolic processes and cell death pathways of living cells. When the mitochondrial dysfunction caused by oxidative stress, it may lead to a series of diseases, including neurodegenerative and cardiovascular diseases.[5] Excessive FA may induce mitochondria mediated caspase-released apotosis.[6] The dualism of the role (signal/toxicology) that FA played in mitochondria is still ambiguous. Therefore, it is highly essential to develop a mitochondrial-target fluorescence probe for constantly monitoring the fluctuations of the FA level in mitochondria to further disentangle physiological and pathological effects of FA.

Nowadays, there are lots of traditional methods for monitoring FA with some complicated operation process and destructive irreversible damage, such as electronic method, high performance liquid chromatography (HPLC), gas chromatography and colorimetric method.[7] Compared with these methods, the fluorescence imaging techniques serve as a powerful tool to monitor target biomolecules in organisms.[8] The techniques provide real-time and in situ monitoring of biological activated molecules with the non-invasive mode. In recent years, although a few FA fluorescent probes have been developed,[9] the organelle-targeted fluorescent FA probes are still scarce. And most of these probes are limited by one-photon (OP) excitation with short wavelengths which are not able to detect FA in living tissue. By contrast, the two-photon microscopy (TPM) has the deep tissue penetration ability and could provide improved three-dimensional imaging long wavelength excitation.[10] To our best knowledge, there is still no mitochondrial-target two-photon fluorescence probe for FA has been revealed up to date. Therefore, it is necessary to develop the fluorescent probes for detecting FA in mitochondria of living cells and living tissue.

There are some ideas for targeting mitochondria, such as using of carrier to migrate to mitochondria,[11a] Compared with this indirect targeting method, the positively charged lipophilic compounds, such as triphenylphosphine (TPP), based on membrane potential are more frequently applied for targeting mitochondria.[11b, 11c] Herein, we linked hydrazine group, the FA response site, and TPP, the mitochondrial-target functional group, directly to 1,8-naphthalimide fluorescence platform, the classical two-photon (TP) fluorescence platforms,[12] to construct the first mitochondrial-target TP fluorescent probe **MT-FA**. Due to the photo-induced electron transfer (PET) pathway from hydrazine group to 1,8-naphthalimide fluorescence platform, the probe displayed almost no fluorescence. When FA was introduced, the PET pathway was suppressed by the condensation reaction between hydrazine group and FA, and the strong fluorescent signal was detected (Scheme 1).[9b]

The probe **MT-FA** was conveniently synthetized in a four-step reaction (Scheme S1). Triphenylphosphine (TPP)

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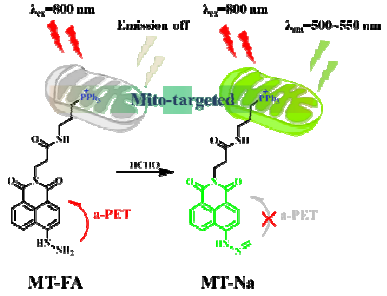
underwent a nucleophilic addition reaction with 3-bromopropylamine hydrobromide yielding the compound **1**. 4-Bromo-1,8-naphthalic anhydride and β-alanine underwent nucleophile substitution reaction yielding the compound **2**. Compound **1** and compound **2** underwent a condensation reaction obtaining the compound **3**, which reacted with hydrazine hydrate to get the finally product **MT-FA**. The detailed synthetic processes and the characterization data (standard ^{1}H NMR, ^{13}C NMR and mass spectrometry) of all new compounds were presented in supporting information (see the ESI†).



Scheme 1. The fluorescence response mechanism of the mitochondrial-target TP fluorescent FA probe **MT-FA** based on condensation reaction between hydrazine group and FA.

To evaluate the response ability of **MT-FA** to FA, the absorption and fluorescence titration experiments was conducted in 10 mM PBS buffer (pH 7.4, contain 5% DMSO as co-solvent) at room temperature. As shown in the Fig. 1a, the free probe **MT-FA** (5 μM) showed the maximum absorption at 439 nm (ε=8020 M^{-1} cm^{-1}). However, upon addition of increasing concentrations of FA (0-150 μM), the absorption gradually increased. As designed, due to the PET pathway, **MT-FA** showed almost non-fluorescent. Satisfactorily, upon addition of amount of FA (0-150 μM) to the **MT-FA** solution, the fluorescent intensity was gradually increased (up to 43.5-fold, Fig. 1b and inset of it) at about 539 nm, and a consistent results was shown in the two-photon (TP) fluorescence spectrum experiments of **MT-FA** in presence and absence of FA (Fig. S1). The TP fluorescence spectrum of only **MT-FA** displayed a relatively weak fluorescent signal due to the PET pathway. Upon addition of FA, a strong TP fluorescence signal was observed at around 550 nm upon excitation at 800 nm, and the shape of the spectrum closely resembles that of the one-photon (OP) fluorescence spectrum. These results indicate **MT-FA** is capable of detecting FA in one and two-photon mode.

In addititon, the detection limit of **MT-FA** was calculated to be 4.9×10^{-6} M (Fig. S2), and an excellent linear relationship between the emission intensity and concentrations of FA in range of 0-50 μM was shown in Fig. S3 It is worthy to note that the combination of the large switch-on signal and the low detection limit of the probe **MT-FA** may render it highly desirable for quantitative detecting the basal level of FA in the living samples. We used fluorescence spectral (Fig. S4) and HR-MS (Fig. S5) to verify the identification mechanism of **MT-FA** for FA. When **MT-FA** was treated with FA, a strong emission signal was found at around 539 nm, which closely resembles that of compound **MT-Na** (the product of the react between **MT-FA** and FA), and the results of HR-MS was consistent with compound **MT-Na** proposed in scheme 1.

It is well known that a slightly basic condition exists in mitochondria (pH=8.05±0.11).[13] In order to evaluate whether **MT-FA** is applicable to the detection of FA in mitochondria, the pH effect tests about **MT-FA** in the absence or presence of FA were carried out. As shown in Fig. 1c, **MT-FA** was stable at wide range of pH value from 4.0 to 10.0. With the introduction of FA, the strong fluorescent signal enhancement change was observed, and even within the mitochondrial pH environment, the fluorescence intensity was about 31.1-fold enhancement. These results illustrated **MT-FA** is applicable to detection of FA in mitochondria. The response rate experiments of **MT-FA** at 539 nm with the different concentrations (0 and 150 μM) of FA were investigated (Fig. 1d). Upon addition of FA, the emission intensity reached the equilibrium state at about 40 min at ambient temperature. In contrast, the fluorescent signal of only **MT-FA** almost had no changed under identical condition, suggesting that **MT-FA** has potential real-time imaging application value in living systems.

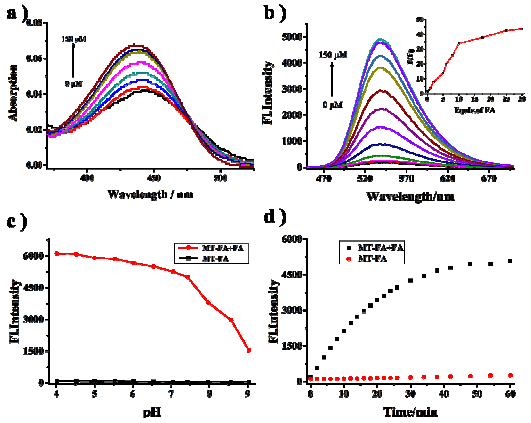


Fig. 1. (a) The absorption titration spectra were recorded upon treatment of **MT-FA** (5 μM) with FA (0-150 μM) in PBS buffer (pH 7.4, 5% DMSO) for 40 min; (b) The fluorescence titration spectra were recorded upon treatment of **MT-FA** (5 μM) with FA (0-150 μM) in PBS buffer (pH 7.4, 5% DMSO) for 40 min, Inset: Fluorescence intensity ratio (F/F_0) changes at 539 nm of **MT-FA** with the amount of FA (0-150 μM); (c) Fluorescence intensity changes of **MT-FA** (5 μM) treated or untreated with FA (150 μM) at different pH solutions for 40 min; (d) Reaction-time profiles of **MT-FA** (5 μM) treated with FA (0 and 150 μM). λ_{ex} = 440 nm.

To test the selectivity of **MT-FA** for FA, **MT-FA** was treated with various aldehydes and biological relevant analytes in PBS buffer (pH 7.4, 5% DMSO) at room temperature, including amino acids, 1 mM for GSH, Hcy, Cys; inorganic salts, 1 mM for $CaCl_2$, $MgCl_2$, KNO_3, $NaHSO_3$, Na_2SO_4, $NaNO_2$, Na_2S; reactive oxygen and nitrogen species, 100 μM for NaClO, H_2O_2, *di-tert*-butyl peroxide (DTBP), *tert*-butyl hydroperoxide (TBHP), NO; ketone and aldehydes, 150 μM for FA, glyoxal, methylglyoxal, sodium pyruvate, 4-dimethylamino bezaldehyde, acetone, trichloro-acetaldehyde, acetaldehyde, 4-nitro-benzaldehyde. The above species induced nearly no fluorescent signal changed except of glyoxal (2.9-fold enhancement), Ca^{2+} (2.8-

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fold enhancement), TBHP (3.9-fold enhancement), while upon addition of FA, a 43.5-fold enhanced fluorescent signal was obtained (Fig. 2). In the anti-interference experiments, only $NaHSO_3$ caused a violent decrease of fluorescence intensity due to FA was consumed by HSO_3^- via nucleophilic addition reaction, other interfering species has a small influence on **MT-FA** response to FA. (Fig. S6) The property of high selectivity and anti-interference of the probe indicated the probe is critical to its potential biological applications. Furthermore, we also investigated the photo-stability of the probe. After **MT-FA** was irradiated or non-irradiated by the short wavelength light source (UV light, the wavelength is 365 nm), the fluorescence intensity of the probe were almost no change (Fig. S7), indicating that **MT-FA** would not be affected by the UV light and it may have potential imaging application value.

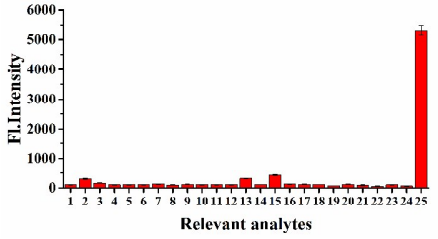

Fig. 2. Selectivity profiles: the fluorescence intensity of **MT-FA** (5 μM, at 539 nm) responding to FA in presence of various interfering species: amino acids, 1 mM; inorganic salts, 1 mM; reactive oxygen and nitrogen species, 100 μM; ketone and aldehydes, 150 μM. Legend: (1) **MT-FA**; (2) glyoxal; (3) methylglyoxal; (4) sodium pyruvate; (5) 4-dimethyl-aminobezaldehyde; (6) trichloroacetaldehyde; (7) acetaldehyde; (8) 4-nitro-benzaldehyde; (9) acetone; (10) NaClO; (11) H_2O_2; (12) DTBP; (13) TBHP; (14) NO; (15) $CaCl_2$; (16) $MgCl_2$; (17) KNO_3; (18) $NaHSO_3$; (19) Na_2SO_4; (20) $NaNO_2$ (21) Na_2S; (22) GSH; (23) Hcy; (24) Cys; (25) FA. λ_{ex} = 440 nm.

As an imaging reagent, **MT-FA** should have no significant cytotoxicity. Then the cytotoxicity of the probe was investigated by the MTT assay. As shown in the Fig. S8, the MTT results showed that **MT-FA** has little cytotoxicity at low dose level (0-10 μM), indicating that **MT-FA** could be used in imaging experiments.

The exogenous FA fluorescence imaging experiments were carried out by OP and TP modes, and the results were shown in the Fig. S9. The HeLa cells that treated with 5 μM **MT-FA** for 40 min only showed almost invisible fluorescence whether by the OP mode or TP mode (Fig. S9, a2 and a4). While the HeLa cells were pre-treated with 150 μM FA for 20 min, then treated with 5 μM **MT-FA** for another 40 min, the marked enhanced green fluorescence was observed at both OP and TP modes (Fig. S9, b2 and b4). We used $NaHSO_3$ for negative control experiments because it is a commonly used FA inhibitor.[9b] When the cells pre-treated with 150 μM FA and 300 μM $NaHSO_3$ for 20 min, then treated with 5 μM **MT-FA** for more 40 min, the green fluorescence became very faint and almost invisible (Fig. S9, c2 and c4). In addition, we had reached the consistent conclusion from the spectrum (Fig. S10). These studies showed that **MT-FA** has good membrane permeability and may monitor exogenous FA in living cells.

Encourage by exogenous imaging experiments, we continued to conduct endogenous imaging experiments. We chose thapsigargin (TG) as the endogenous formaldehyde stimulator[9l] to conduct endogenous FA imaging experiments. As shown in the Fig. 3, the HeLa cells that were treated with 15 μM TG for 30 min only showed no fluorescence whether by OP mode or TP mode (Fig. 3, a2 and a4). While the HeLa cells were pre-treated with 15 μM TG for 30 min, then treated with 5 μM **MT-FA** for another 40 min, the strong fluorescent signals was observed by OP and TP modes (Fig. 3, b2 and b4). While the cells were pre-treated with 15 μM TG and 300 μM $NaHSO_3$ for 30 min, then treated with 5 μM **MT-FA** for more 40 min, there was almost no fluorescence in green channel was observed (Fig. 3, c2 and c4). These studies showed that **MT-FA** is capable of tracking endogenous FA in living cells.

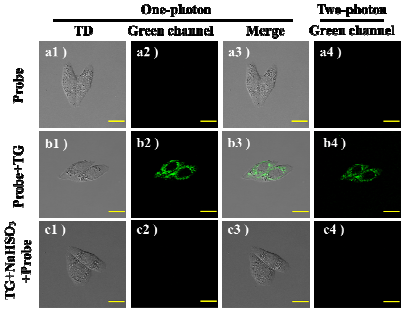

Fig. 3. Fluorescence imaging of the endogenous FA in the HeLa cells by one and two photon mode. a1)-a4) The image of the HeLa cells treated with TG (20 μM); b1)-b4) The image of the HeLa cells treated with TG (20 μM) for 30 min and **MT-FA** (5 μM) for further 20 min; c1)-c4) The image of the HeLa cells treated with TG (20 μM), $NaHSO_3$ (300 μM) for 30 min and **MT-FA** (5 μM) for further 20 min. OP-excitation was at 488 nm, TP-excitation was at 800 nm and emission collection was from 500 - 550 nm. Scale bar: 20 μm.

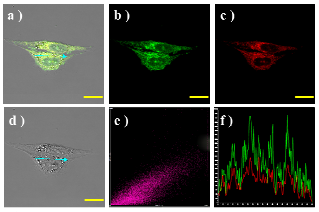

Fig. 4. The images of the living HeLa cells treated with TG (15 μM) 30 min, **MT-FA** (5 μM) for further 40min and then treated with Mito-Tracker Deep Red (500 nM) for 5 min. a) Bright-field image of the HeLa cells; b) The fluorescence image of the green channel; c) The fluorescence image of the red channel; d) The merged image of a, b, and c; e) Intensity scatter plot of the green and red channels. f) Intensity profile of linear region of interest across the cell costained with red channel of Mito Tracker Deep Red and green channel of **MT-FA** of a). The green channel: λ_{ex} = 440 nm, λ_{em}= 500-550 nm; The red channel: λ_{ex} = 647 nm, λ_{em}= 663-738 nm Scale bar: 20 μm.

The co-localization experiment was carried out to investigate the mitochondrial-target ability of **MT-FA**. Colocalization results obtained using a confocal laser microscope with 5 μM **MT-FA** and 500 nM Mito-tracker deep red (the commercial mitochondrial location dye, *Thermo Fisher Scientific*.) in presence of 15 μM TG and 150 μM FA separately. As shown in Fig. 4 and Fig. S11, the red channel of Mito-tracker deep red overlapped well with the green channel of **MT-FA** treated with endogenous and exogenous FA separately. The intensity profile of the linear regions of interest in green channel of **MT-FA** and red channels of Mito-tracker deep red displayed a tendency toward synchrony. The corres-ponding Pearson's correlation coefficient was calculated as 0.91 and 0.90 separately, indicating the location of **MT-FA** may

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accumulate in the mitochondria and could detect the FA in the mitochondria.

Encouraged by the satisfactory results of cell imaging experiments, the tissue imaging experiments were also investigated. Consistent with expectations, the results of the tissue imaging experiments were consistent with the results of cell imaging. When the mice liver tissue slices were soaked in FA solution (300 μM) for 1 h, and then soaked in the **MT-FA** solution (10 μM) for another 40 min, the strong fluorescent signals was observed with the penetration depth up to about 80 μm (Fig. 5). By contrast, the mice liver tissue slices were only soaked in **MT-FA** solution (10 μM) for 40 min showed no fluorescence signal in green channel (Fig. S12). These data implied that the probe is capable of detecting FA in the liver tissue slides.

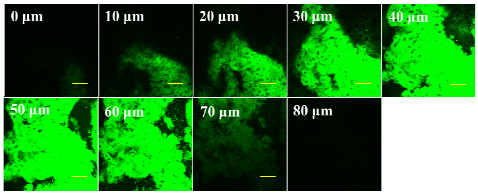

Fig. 5. Two-photon fluorescence imaging of FA in the liver slides. Fluorescence images of the liver slides incubated with FA (300 μM), and then incubated with **MT-FA** (10 μM). Excitation was at 800 nm by the femtosecond laser and the emission collection was from 500-550 nm. Scale bar: 50 μm.

In conclusion, the first mitochondrial-targeted TP fluorescent probe, named as **MT-FA**, has been rationally designed and synthesized. Upon reaction with FA, the PET pathway which inhibits the fluorescence emission of the probe is suppressed, and the probe shows significant fluorescence enhancement than the other analytes. Importantly, the co-localization experiments indicate that **MT-FA** may effectively accumulate in the mitochondria for monitoring of FA in living cells. Moreover, **MT-FA** could also successfully detect FA in living mice liver tissue slices with the penetration depth up to about 80 μm. We believe the **MT-FA** is conducive to further disentangle physiological and pathological effects of FA in living system, especially in mitochondria.

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Conflicts of interest

There are no conflicts to declare.

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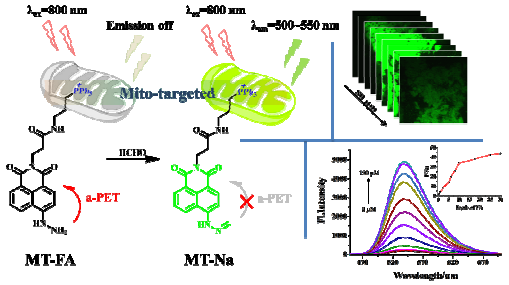

The first mitochondrial-targeted two-photon fluorescent probe (**MT-FA**) for formaldehyde (FA) was engineered for monitoring FA in mitochondria of the living cells and liver tissue by one- and two-photon modes.

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