Fluorescent Probes

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Development of a Two-Photon Fluorescent Probe for Imaging of Endogenous Formaldehyde in Living Tissues

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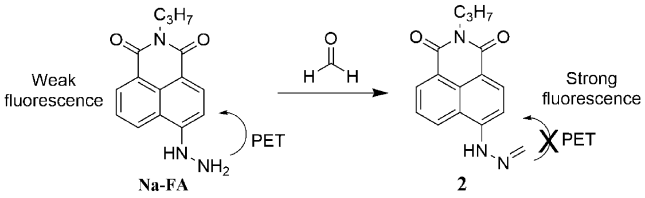
Abstract: *Investigation of the physiological and pathological functions of formaldehyde (FA) are largely restricted by a lack of useful FA imaging agents, in particular, those that allow detection of FA in the context of living tissues. Herein, we present the rational design, synthesis, and photophysical property studies of the first two-photon fluorescent FA probe,* ***Na-FA****. Importantly, the highly desirable attributes of the probe* ***Na-FA*** *(such as a very large turn-on signal (up to 900-fold), a low detection limit, and a very fast onset imparted by the unique design aspects of the probe), make it possible to monitor endogenous FA in living tissues for the first time. Furthermore, sodium bisulfite was identified as a simple and convenient inhibitor of FA within biological environments.*

FA has found widespread use in the chemical industry as a synthetic material for plastics, cosmetics, wood processing, food, and drugs.[1] Furthermore, FA is employed as a cell fixative and tissue embalming species owing to its strong DNA and protein binding abilities. However, FA has been confirmed by the World Health Organization as the third largest indoor chemical pollutant. Exposure to FA may cause a wide variety of diseases.[2] Endogenous FA in living systems may be generated during the course of histone demethylation,[3,4] or methylation of DNA.[5] Moreover, degradation of methylamine and polyamine with semicarbazide sensitive amine oxidase may also produce endogenous FA.[6] Endogenous FA exists in many living tissues and cells in concentrations ranging from the low to high micromolar range.[7,8] Nevertheless, up to now the biological roles of FA have not been well-defined, which is presumably because of a lack of robust molecular tools for investigating FA in the context of living systems.

Fluorescence imaging has emerged as a powerful means to track biomolecules in living systems, thanks to significant advances in spectroscopic techniques and development of fluorescent molecular probes.[9] However, the development of fluorescent probes suitable for detecting FA in living samples is very challenging.[10] Current fluorescent FA probes are activated by a one-photon excitation. Thus, it is very difficult to employ them for tracking the endogenous FA in living tissues, which are obviously much thicker than living cells. To the best of our knowledge, imaging of endogenous FA inside living tissues has not yet been realized.

One of the advantages of two-photon technology[11] over one-photon excitation is that it offers much deeper penetration of biological samples, and is thus favorable for imaging of biomolecules in living tissues. With this in mind, we were interested in engineering a two-photon fluorescent FA probe. To this end, we developed the fluorescent probe **Na-FA**, which enables imaging of endogenous FA in living tissues for the first time.

The fluorescence behavior of **Na-FA** is illustrated in Scheme 1. The condensation of aldehydes with amines or



Scheme 1. Design of the two-photon fluorescent FA probe **Na-FA**.

hydrazides has been employed to construct fluorescent probes for aldehydes, including FA and methylglyoxal.[12] However, as pointed out by Chang et al., probes may exhibit low sensitivity to formaldehyde because these condensation reactions are in equilibrium under physiological conditions.[10a] To circumvent this problem, we envisioned a condensation reaction between hydrazine and FA that rapidly generates a stable methylenehydrazine product. Thus, a hydrazine was selected as the reaction trigger, which was further incorporated into 1,8-naphthalimide, a classic two-photon dye scaffold,[13] to form **Na-FA**. We anticipated that **Na-FA** would display little fluorescence because of a photoinduced electron transfer (PET) pathway from hydrazine to the dye. However, reaction with FA affords compound **2**, for which the PET pathway is suppressed and fluorescence recovered, and thus a turn-on signal should be detected. This hypothesis is supported by theoretical calculations (Supporting Information, Figure S1).

The synthesis of compound **Na-FA** is straightforward (Supporting Information, Scheme S1). With the compound **Na-FA** in hand, we first evaluated its absorption profile (Figure S2). The free probe is almost non-fluorescent (Figure 1). However, when titrated with FA, an intense fluorescence peak at around 543 nm evolves. Notably, the turn-on response is very rapid and large. For example, upon incubation of 5 μM of probe with 2000 μM of FA, we observed an immediate 325-fold fluorescence enhancement (Fig-

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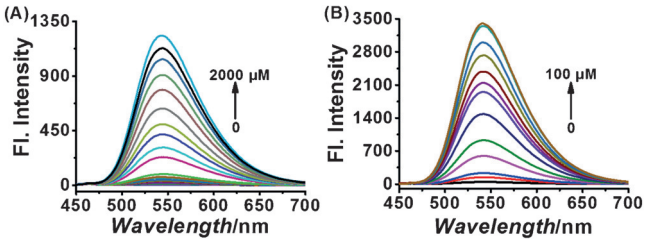

Figure 1. The fluorescence response of the probe **Na-FA** (5 μM) to FA at varied concentrations in PBS buffer (pH 7.4, 1% DMSO). $\lambda_{ex} = 440$ nm. A) Spectra were recorded immediately upon treatment of the probe with FA (0–2000 μM); B) Spectra were recorded after treatment of the probe with FA (0–100 μM) followed by a 30 min incubation period.

ure 1A). Significantly, after treatment of 5 μM of probe with 100 μM of FA for 30 min, a very large 900-fold increase in fluorescence was detected (Figure 1B). Furthermore, at a much lower probe concentration (1 μM), strong turn-on signals were also observed immediately, or after 30 min of incubation of the probe with FA (Figure S3). Based on the titration studies, the detection limit for **Na-FA** was calculated to be 7.1×10^{-7} M using the assay conditions shown in Figure S4. It is worth noting that the combination of very large switch-on signal and low detection limit of the probe **Na-FA** render it highly desirable for detecting basal levels of FA present in living samples.

Since detection of the probe **Na-FA** is reaction-based, it is important to investigate its kinetic profiles in the presence of FA. When 5 μM of probe was incubated with 20 μM of FA in 10 mM PBS buffer (pH 7.4, 1% DMSO), a strong fluorescence increase was noted, and the signal reached a maximum at 30 min (Figure S5). By contrast, the signal reached a maximum within 5 min after treatment of the probe with a large excess of FA (200 μM). Under these pseudo-first-order conditions, the rate constant for the probe was determined to be $k = 0.39\ \text{min}^{-1}$ (Figure S6).

To further verify the design strategy of the probe, the reaction product of the probe and FA was isolated. Analysis of the ^{1}H NMR (Figure S7), ^{13}C NMR (Figure S8), and HR-MS spectra (Figure S9) of the product confirmed that it is indeed compound **2**, as proposed in Scheme 1. Interestingly, the fact that the stable product could be generated rapidly (within 10 min) is consistent with the fast response characteristic of the probe.

Selectivity studies demonstrate that the probe **Na-FA** has a higher selectivity for FA over other species of biological origin (Figures S10 and S11), suggesting that the probe **Na-FA** is favorable for investigation of FA in biological systems. pH-Dependent kinetic studies show that the response of the probe to FA is more favorable in weakly acidic to neutral conditions (Figures S12 and S13). Photostability studies suggest that the probe is highly stable, even under excitation by short wavelength UV light for 30 min (Figure S14). Furthermore, the probe demonstrates minimal cytotoxicity to HeLa cells, as revealed by standard microculture tetrazolium assays (MTT) (Figure S15).[14]

Taken together, the desirable attributes of the probe include working appropriately at physiological pH, excellent photostability, low cytotoxicity, in particular a very large signal-to-noise ratio, a low detection limit, and fast onset, which render the probe highly favorable for monitoring the endogenous FA in living systems.

The stage was set for monitoring added FA introduced into living cells using the probe **Na-FA**. Control experiments indicate that isolated probe **Na-FA** in culture medium (Figure S16b), untreated living HeLa cells (Figure S16e), or HeLa cells treated only with FA (Figure S16h), display almost no fluorescence. By contrast, cells incubated with both the probe and FA exhibit bright fluorescence in the one-photon mode (Figure S16k), suggesting that the probe is capable of imaging added FA by one-photon excitation. Notably, bright-field images (Figures S16g,j) of the cells treated with FA (40 μM) show that the cells remain in the normal morphology, implying that they are alive under the experimental conditions. As 1,8-naphthalimide is a typical two-photon dye, we further applied the probe to imaging of added FA in living cells in a two-photon mode. The results were in good agreement with those obtained in the one-photon mode; essentially no fluorescence was observed in the untreated living HeLa cells (Figure S17b), or the HeLa cells treated with only FA (Figure S17e). However, cells incubated with both the probe and FA show significant fluorescence (Figure S17h). The corresponding two-photon excited fluorescence spectrum is displayed in Figure S18. Photostability studies of **Na-FA** with added FA in live HeLa cells indicate that the probe has relatively good stability (Figures S19 and S20). These studies demonstrate that the probe is suitable for detecting added FA in living cells in both the one- and two-photon modes.

Prior to undertaking fluorescence detection of endogenous FA in living systems, it was necessary to find a simple and effective FA inhibitor. We turned our attention to sodium bisulfite ($NaHSO_3$), as it can react effectively with FA to destroy the central carbonyl structure (Scheme S2). We performed a number of control experiments to demonstrate the applicability of the inhibitor. Firstly, we investigated pH change after addition of $NaHSO_3$ to a pH 7.4 PBS buffer. The results indicate that addition of 200 μM of $NaHSO_3$ to a pH 7.4 PBS buffer elicits almost no marked change in pH. Secondly, because $NaHSO_3$ is often used as a source of SO_2, which is also regarded as an endogenous gas messenger in living systems, we examined the possibility of $NaHSO_3$ interference to the emission profile of the probe. As shown in Figure S21, $NaHSO_3$ does not induce any significant fluorescence variations. Thirdly, the probe treated with FA displays a strong emission peak. However, a sample of FA pre-treated with $NaHSO_3$ and subsequently incubated with the probe produced almost no fluorescence (Figure S22). These controls indicate that $NaHSO_3$ is a valid inhibitor of FA in fluorescence assays containing the probe **Na-FA**.

Encouraged by the above results, we decided to further apply **Na-FA** to tracking of endogenous FA in living cells. HeLa cells incubated with $NaHSO_3$ inhibitor display essentially no emission in the two-photon mode (Figure 2b). By comparison, cells treated with probe **Na-FA** exhibit green fluorescence (Figure 2e). However, almost no emission was observed from cells that were pre-treated with inhibitor and

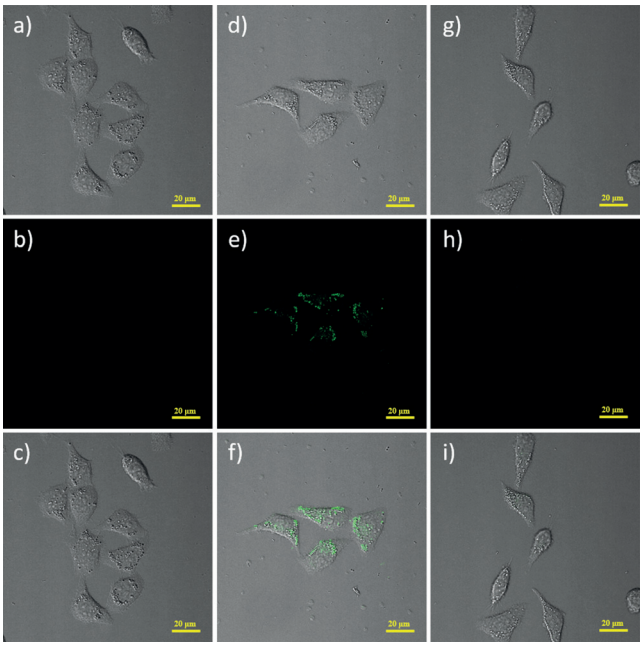

Figure 2. Two-photon fluorescence imaging of endogenous FA in living HeLa cells. a) Bright-field image of HeLa cells treated with $NaHSO_3$ (200 μM); b) Two-photon fluorescence image of (a); c) The merged image of (a) and (b); d) Bright-field image of HeLa cells treated with probe **Na-FA** (5 μM); e) Two-photon fluorescence image of (d); f) The merged image of (d) and (e); g) Bright-field image of HeLa cells incubated with $NaHSO_3$ (200 μM) and then with the probe **Na-FA** (5 μM); h) Two-photon fluorescence image of (g); i) The merged image of (g) and (h). Excitation was at 880 nm and emissions were collected from 500–550 nm. Scale bar: 20 μm.

then incubated with the probe (Figure 2h). These studies demonstrate that the probe can monitor endogenous FA in living cells in the two-photon mode. This conclusion is further substantiated by the data from the imaging studies in one-photon mode (Figure S23). The results of time-dependent confocal fluorescence imaging of endogenous FA inside live HeLa cells suggest that the fluorescence of cells increased with incubation time (Figures S24 and S25).

To further examine the sub-cellular distribution of probe **Na-FA**, Hela cells were co-incubated with probe and an organelle indicator, such as ER-TrackerTM Red (BODIPY TR Glibenclamide), LysoTracker Red DND-99, MitoTracker Red CMXRos, or Golgi-Tracker Red. The data reveals that the probe **Na-FA** is primarily located in the endoplasmic reticulum, the lysosome, and the Golgi apparatus, but very little is present in the mitochondria (Supporting Information, Table S1). Representative images are shown in Figure S26. The results are consistent with previous reports.[10,15]

The favorable results of the living cell studies prompted us to further apply the probe to tracking of FA inside living tissues. As shown in Figure S27C, liver tissue slides treated with FA and then with the probe **Na-FA** show green fluorescence up to a depth of 70 μm. By contrast, no apparent emission was observed in the control experiments; in either the untreated liver slides, (Figure S27A) or liver slides incubated with FA (Figure S27B). Thus, the data implies that the probe is capable of detecting added FA in liver tissue slides. This fact is further corroborated by two-photon imaging studies using lung tissue slides (Figure S28).

We proceeded to investigate the application of probe to monitoring of endogenous FA in living tissues. A control sample containing only liver tissue showed no obvious emission (Figure 3A). In another control experiment, liver slides pre-treated with $NaHSO_3$ inhibitor followed by probe still exhibit no fluorescence (Figure 3B). In sharp contrast, liver slides treated with probe **Na-FA** display green emission (Figure 3C). These studies demonstrate for the first time that a chemical probe can detect basal levels of FA in living tissues.

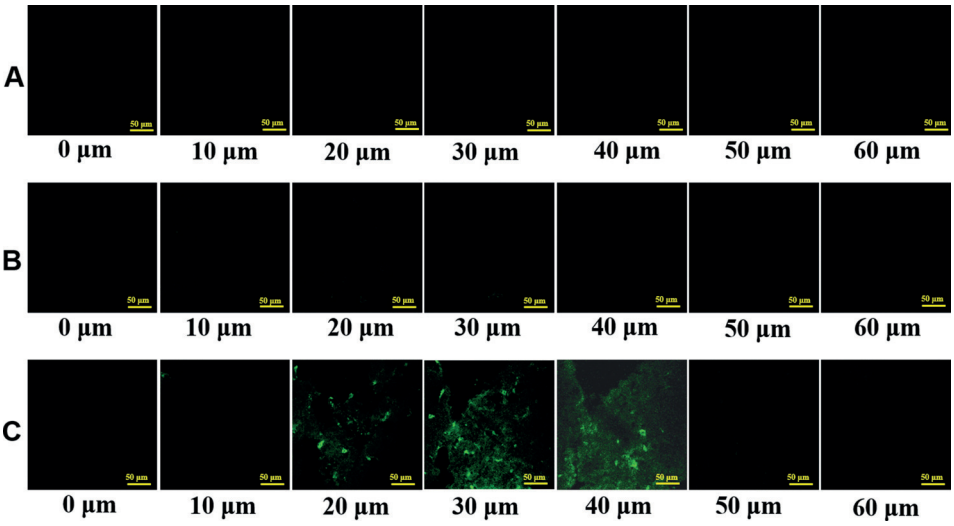

Figure 3. Two-photon fluorescence imaging of endogenous FA in liver slides. A) Fluorescence images of liver slides. B) Fluorescence images of liver slides incubated with the inhibitor $NaHSO_3$ for 30 min, and then with the probe (10 μM) for another 1 h. C) Fluorescence images of liver slides incubated with probe (10 μM) for 1 h. Excitation was at 880 nm by femtosecond laser, and the emission collection was from 500–550 nm. Scale bar: 50 μm. Labels from 0–60 μm indicate scanning depths of the tissue slices.

In summary, we have engineered the first two-photon fluorescent FA probe, **Na-FA**, which displays highly favorable properties; notable among these, a very large turn-on signal, a low detection limit, and a very fast onset. These critical attributes, and the desirable two-photon properties of the probe, enable tracking of endogenous FA in living tissues for the first time. Thus, we expect that the probe will be a powerful molecular tool for studying both the physiological and pathological roles of FA in the context of living tissues. Moreover, the robust approach for the rational design of the fluorescent FA probe introduced herein, and the exploitation of the hydrazine moiety as the reaction switch, will be

adapted to develop a wide variety of fluorescent FA probes. Finally, the discovery that sodium bisulfite is a simple and effective inhibitor of FA in solution, living cells, and living tissues, may facilitate application of the fluorescent probe **Na-FA** in biological and medical settings.

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