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A Two-Photon Fluorescent Probe for Thiols in Live Cells and Tissues

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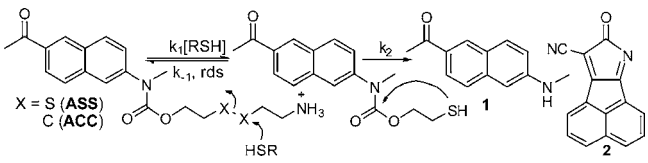
Thiols are a component of many amino acids and peptides such as cysteine (Cys) and glutathione (GSH). GSH is the most abundant cellular thiol whose concentration ranges from 1 to 15 mM.[1] It reduces disulfide bonds within cytoplasmic proteins to thiols while being oxidized to GSSG and can be regenerated from GSSG by the enzyme glutathione reductase. In healthy cells and tissues, more than 90% of the total glutathione exists in the reduced form (GSH) and an increased GSSG-to-GSH ratio is considered to be indicative of oxidative stress.[2] To detect thiols in a living system, various fluorescent probes have been developed.[3] Most of them utilized fluoroscein, rhodamine, or green fluorescent protein (GFP) as the fluorophore and functional groups that react with the thiols to produce strongly emitting products. However, use of these probes with one-photon microscopy (OPM) requires a rather short excitation wavelength (<525 nm) that limits their use in tissue imaging due to the shallow penetration depth (<80 μm).

To detect thiols deep inside the live tissues, it is crucial to use two-photon microscopy (TPM). TPM, which employs two near-infrared photons as the excitation source, has the advantages of increased penetration depth (~500 μm), localized excitation, and prolonged observation time, thereby allowing tissue imaging.[4] Recently, a TPM image of cells labeled with an 8-oxo-acenaphthopyrrole derivative (**2**) has been reported.[3f] This probe allowed visual detection of thiols with a change of the λ_{max} from 430 to 580 nm and a 75-fold increase in the fluorescence intensity, respectively, upon addition of cystein and homocystein (40 equiv). However, the two-photon (TP) cross section of **2** was so small (~10 GM) that a high laser power (50 mV) was required to excite the fluorophore. Moreover, there has been no report on the TP probe that can detect thiols deep inside the intact tissue. Herein, we report the synthesis and biological imaging application of a TP probe (ASS) derived from 2-methylamino-6-acetylnaphthalene (**1**) as the reporter and a disulfide group as the thiol reaction site that can detect thiols in live cells and living tissues at a 90−180 μm depth (Scheme 1).

Scheme 1. Structures of ASS, ACC, **1**, and **2**, and Mechanism of the Thiol-Induced Reduction of ASS



The preparation of ASS and ACC is described in the Supporting Information. The water solubility of ASS as determined by the fluorescence method was 6.0 μM,[6] which is sufficient to stain the cells. The absorption and emission spectra of ASS were insensitive to the solvent polarity (Figures S1b and S1c and Table S1), whereas those for **1** showed large red shifts in the order 1,4-dioxane < EtOH < H_2O (Figure S2 and Table S1). The effect was greater for the emission (76 nm) than absorption spectra (12 nm), thus indicating the utility of **1** as a polarity probe.

When 2-aminoethanethiol (2-AET, 10 mM) was added to ASS (5 μM) in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution (30 mM, 100 mM KCl, 10 mM EGTA, pH 7.2), the one- and two-photon fluorescence intensity increased gradually with time (Figures 1a and S2a). The reaction produced **1** as the only product as revealed by the HPLC analysis (Figure S3). Moreover, the plots of $\ln(F_\infty - F)$ vs time were straight lines, where F_∞ and F are the fluorescence intensity measured at infinity (>10 half-lives) and at a given time, respectively (Figures 1b and S2b and S2c). The slopes of the plots are the observed pseudo-first-order rate constants (k_{obs}). The plot of k_{obs} vs [2-AET] is a straight line passing through the origin. This indicates that the reaction is overall second-order, first-order to the ASS, and first-order to the 2-AET, with $k_2 = 2.2 \times 10^{-5}$ M^{-1} s^{-1} (Figure 1b). Therefore, the observed result can most reasonably be attributed to the rate-limiting attack of the thiol at the disulfide bond followed by the cleavage of the C−N bond to afford **1** as outlined in Scheme 1.

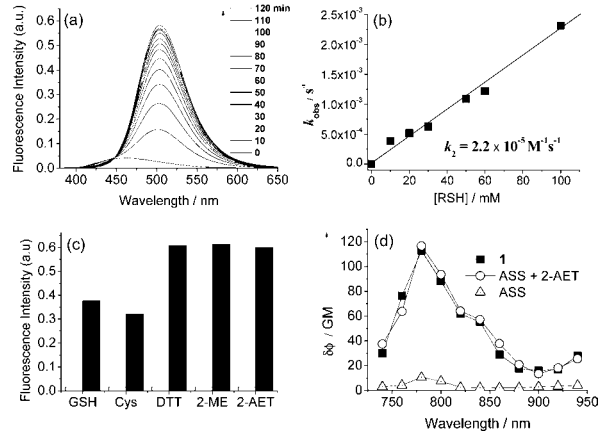


Figure 1. (a) Change of one-photon fluorescence intensity with time for the reactions of ASS (5 μM) with 2-AET (10 mM) in the MOPS buffer (3 mL, pH, 7.4). (b) Plot of k_{obs} vs 2-AET concentration. (c) Fluorescence responses of ASS toward GSH, Cys, DTT, 2-ME, and 2-AET. The white and black bars represent the fluorescence intensity of ASS before and 1 h after addition of the thiols, respectively. (d) Two-photon excitation spectra for ASS, ASS + 2-AET, and **1** in MOPS buffer.

ASS showed strong responses toward GSH, Cys, dithiothreitol (DTT), 2-mercaptoethanol (2-ME), and 2-AET and a negligible response toward amino acids without thiol groups (glu, ser, val, met, ala, ile), metal ions (Ca^{2+}, K^+, Na^+, Fe^{3+}, Zn^{2+}, Mg^{2+}), and H_2O_2 (Figure 1c and Figure S4) and was pH insensitive at a biologically relevant pH (Figure S5). Therefore, this probe can detect the intracellular thiols without interference from other biologically relevant analytes and pH.

The TP action spectra of ASS and the reaction product between ASS and 2-AET in MOPS buffer solution showed the two-photon

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action cross section ($\Phi\delta$) values of 11 and 113 GM at 780 nm, respectively (Figure 1d). This indicates a 10-fold increase in the two-photon excited fluorescence (TPEF) intensity, as ASS is reduced to **1**. As expected, the two-photon action spectrum of the reaction product is nearly identical to that of **1**. Further, the $\Phi\delta$ value of **1** is 11-fold larger than that of **2**,[3f] predicting that the TPM images for ASS-labeled cells should be much brighter than those stained with **2**.

We then tested the ability of ASS to detect thiols in live cells. The TPM image of the ASS-labeled HeLa cells was bright, presumably because of the easy loading, convenient rate of thiol-induced reduction, and significant $\Phi\delta$ value of the reaction product (Figure 2a). The TPEF intensity increased significantly when the cells were preincubated for 1 day with α-lipoic acid (Figure 2b), which increases GSH production,[7] and decreased dramatically upon treatment with *N*-ethylmaleimide (NEM) (Figure 2c), a well-known thiol-blocking agent.[8] In contrast, little TPEF was detected from the cells labeled with ACC (Figure S6d). Moreover, the bright field image confirms that the cells are viable throughout the imaging studies (Figure 2d). Hence, ASS is clearly capable of detecting the thiols in live cells. Further, the TPM images of HeLa cells costained with ASS, MitoTracker, and Hoechst 33342, well-known one photon fluorescent probes for the mitochondria and nucleus, respectively,[9] merged well with the OPM image of mitochondria (Figure 3e). This result indicates that the thiols exist predominantly in the mitochondria.

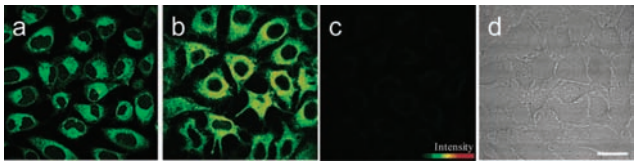

Figure 2. TPM (a−c) and bright field (d) images of HeLa cells incubated with ASS (3 μM) (a) for 30 min. (b,c) TPM images of HeLa cells pretreated with lipoic acid (500 μM) for 1 day (b) and NEM (100 μM) for 30 min (c) before labeling with ASS. Two-photon excitation was provided at 780 nm with fs pulses, and the TPEF were collected at 500−620 nm. Scale bar, 30 μm. Cells shown are representative images from replicate experiments ($n = 5$).

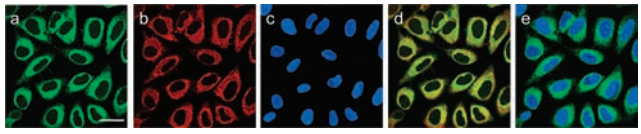

Figure 3. TPM and OPM images of HeLa cells colabeled with ASS (3 μM), Mitotracker (1 μM), and Hoechst (1 μM). The TPEF was collected at 500−620 nm (ASS) (a), and one-photon fluorescence was collected at 600−660 nm (Mitotracker) (b) and 450−550 nm (Hoechst) (c), respectively. (d,e) Colocalized images. The wavelengths for one- and two-photon excitation were 514 and 780 nm, respectively. Scale bar, 30 μm. Cells shown are representative images from replicate experiments ($n = 5$).

We further investigated the utility of this probe in tissue imaging. The bright field image reveals the CA1 and CA3 regions as well as the dentate gyrus (DG, Figure 4a). The TPM image of a part of a fresh rat hippocampal slice incubated with 10 μM ASS shows that thiols are abundant in the CA1 and DG regions at a 120 μm depth (Figure 4b). The image at a higher magnification clearly reveals the thiol distribution in the CA1 region at a 100 μm depth (Figure 4c). Moreover, the TPM images at the depths 90, 120, 150, and 180 μm show the thiol distribution in each *xy* plane along the *z* direction (Figure S7). Further, the TPEF intensity decreased dramatically when the tissue slice was pretreated with NEM (Figure 5d). These findings demonstrate that ASS is capable of detecting thiols at a 90−180 μm depth in live tissues using TPM.

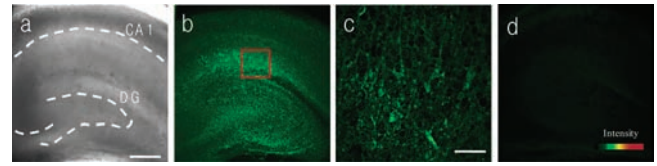

Figure 4. (a) Bright field and (b) TPM images of a fresh rat hippocampal slice stained with 20 μM ASS at a depth of ∼120 μm with magnification at 10×. Scale bar, 300 μm. (c) TPM image in the CA1 region (red box) at a depth of ∼100 μm by magnification at 100×. Scale bar, 30 μm. (d) TPM image of (b) pretreated with NEM (200 μM) before labeling with 20 μM ASS. The TPEF were collected at 500−620 nm upon excitation at 780 nm with fs pulse.

To conclude, we have developed a TP probe (ASS) that shows 10-fold TPEF enhancement in response to thiols, is pH-insensitive at a biologically relevant pH, and emits 11-fold stronger TPEF than the existing probe (**2**). This probe can detect thiols in live cells and living tissues at a 90−180 μm depth without interference from other biologically relevant species.

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Supporting Information Available: Synthesis, photophysical study, cell culture, and two-photon imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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