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Short communication

Real-time and in-situ cell imaging of thiol compounds in living cells using maleimide BODIPY labeling

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ABSTRACT

In this paper, a simple analytical method for the visualization of thiol compounds including glutathione (GSH), *N*-acetylcysteine (NAC), cysteine (Cys) and coenzyme A (CoA) in living cells is developed based on 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene (TMPAB-*o*-M) labeling. By using of this fluorogenic reagent, in-situ imaging of thiol compounds in cells could be achieved in only 90 s, which is much faster than that mentioned in other reports. The fluorescence of derivative products in living cells could be stable for atleast 15 min under irradiation, and can be quantified by HPLC easily in only 6 min.

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1. Introduction

Thiol containing low molecular weight biological substances, such as glutathione (GSH), *N*-acetylcysteine (NAC), cysteine (Cys) and coenzyme A (CoA), play key roles in living organism. For instance, GSH, NAC and Cys are important reactants of intracellular reductive–oxidative metabolic cycles, substrates for protein synthesis and enzyme cofactors [1–5], and the quantitative modifications of them are widely considered as indices of oxidative damage. CoA is a coenzyme which is critical to the processes of decomposition of sugar and amino acids, fatty acid oxidation, acid degradation and tricarboxylic acid cycle [6]. Therefore, many methods for thiol determination have been reported [3,7]. However, the poor stability of thiol containing compounds, the easy autoxidation and other reactions of them are still the major problems in thiol determination [8,9]. Thus, it is suggested to use real-time and in-situ analysis to investigate levels of thiol compounds in cells and other living systems.

Fluorescent bioimaging techniques provide feasible ways for in vivo and in-situ visualization of transient target molecules in bodies and cells by means of trapping these molecules with fluorescent probes [10]. Theoretically, in order to mirror the distributive information of target molecules, their reaction with fluorescent probes should be ultrafast to avoid the stress response from living systems themselves and interruption from the external environment. Till now, various classes of probes have been exploited for different transient targets, such as thiol compounds [11–14], nitric oxide [15,16], and reactive oxygen species [17]. Nevertheless, in these literatures the incubation always takes more than 30 min, which is too long to achieve real-time analysis.

1,3,5,7-Tetramethyl-8-phenyl-(2-maleimide) difluoroboradiaza-s-indacene (TMPAB-*o*-M, Fig. 1) is a fluorescent probe for thiols developed recently [18]. In our previous work, it has been applied for detection of GSH selectively and analysis of CoA, GSH, Cys and NAC simultaneously under different conditions [19,20]. It was found that TMPAB-*o*-M could react with thiols in physiological conditions rapidly. Therefore, it can be speculated that this probe could be used in background free cell imaging with immediate incubation. Accordingly, the potential of TMPAB-*o*-M in cell imaging for thiols has been evaluated in this paper.

2. Materials and methods

2.1. Apparatus

The Agilent 1100 series HPLC system (Agilent Technologies, Böblingen, Germany) was used in the experiments. It comprised the following modules: a high-pressure gradient quaternary pump, an online vacuum degasser, a manual injection (20 μL), and a fluorescence detection (FLD) system. FLD wavelengths were set at $\lambda_{ex}/\lambda_{em}$ = 507/520 nm. Chromatographic separations were achieved with a Kromasil C18 column (250 mm × 4.6 mm, i.d., 5 μm; Eka Chemicals, Bohus, Sweden) at room temperature (25 °C).

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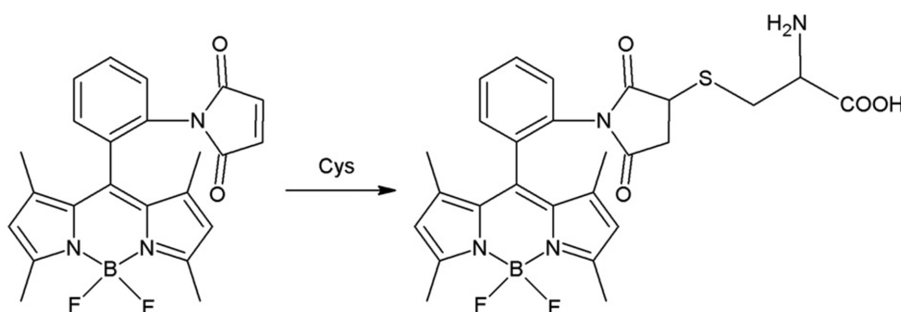

Fig. 1. The derivatization reaction of TMPAB-*o*-M and Cys.

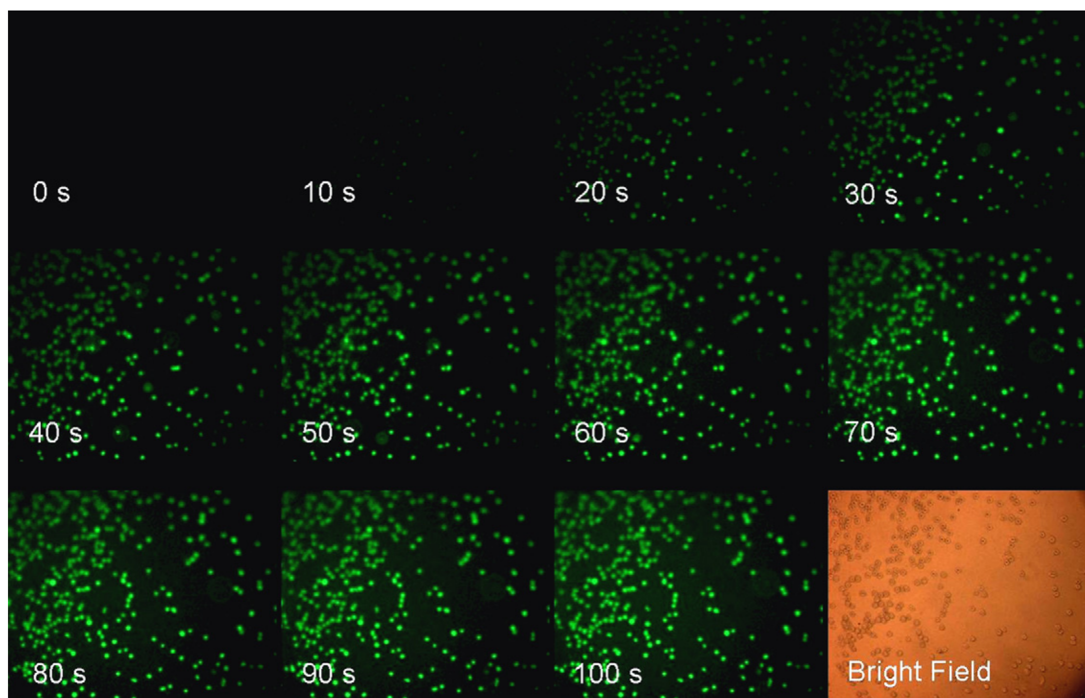

Fig. 2. Bright-field and the change of fluorescence intensity of living Hela cells in PBS containing 1×10^{-5} M TMPAB-*o*-M at 37 °C.

Imaging was performed on an Aviovert 200 M inverted fluorescence microscope (Zeiss, Germany), using a 10 × objective lens and a high resolving power digital camera for imaging (3900 × 3090 pixel, AxioCam, Zeiss). The imaging was obtained at the excitation wavelength of 470 nm, filtering the emission at 515 nm. The temperature of the stage was maintained at 37 °C with a tempcontrol-37 (Zeiss, Germany). Images were taken at intervals of 10 s. Optical signals were recorded with an Axiocan HR (Zeiss, Germany), which controlled the image acquisition and display.

2.2. Reagents

All reagents except for coenzyme A (CoA) and 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene (TMPAB-*o*-M) were of analytical reagent grade, and used without further purification. CoA (with a purity higher than 85%) was purchased from Sigma (Shanghai, China). Cys, NAC and GSH were purchased from Shanghai Chemicals (Shanghai, China). TMPAB-*o*-M was synthesized in our laboratory according to Ref. [18].

Unless otherwise specified, all solutions were prepared with double distilled water. A TMPAB-*o*-M stock solution was prepared by dissolving TMPAB-*o*-M in DMSO to obtain 2.0×10^{-3} M solution. 1.0×10^{-3} M of CoA, GSH, NAC and Cys standard solutions were prepared daily. Dilution of these stock solutions to appropriate concentrations was performed immediately before use. The physiological buffer solution was prepared by dissolving 4.00 g NaCl, 0.10 g KCl, 0.10 g KH_2PO_4 and 1.39 g $Na_2HPO_4 \cdot 12H_2O$ in 500 mL water and the buffers were adjusted to the required pH value using 1.0 M HCl and NaOH. H_3Cit–NaOH buffer was prepared by mixing 0.1 M of NaOH solution with 0.1 M of H_3Cit solution to the required pH value.

2.3. Cell culture and treatments

The cell line U937 (human histiocytic lymphoma) was cultured in RPMI 1640 (with L-glutamine), supplemented with 5% heat-inactivated fetal bovine serum, and 1% L-glutamine, 1% penicillin–streptomycin. K562 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine and 1% penicillin–streptomycin in an incubator humidified with 95% air and 5% CO_2. Hela cells were cultured in a 10 cm (diameter) tissue culture dish in DMEM, supplemented with 10% FBS, 1% penicillin–streptomycin, 0.15% $NaHCO_3$ and 1 mM sodium pyruvate (Sigma, Shanghai, China) at 37 °C in a humidified 5% CO_2 atmosphere. The cells grew to 80–90% confluence and were detached by trypsin.

After washing with PBS for 3 times, cells were suspended in PBS containing 1×10^{-5} M TMPAB-*o*-M and mounted on the inverted

fluorescence microscope immediately. The temperature of all the solutions and the stage of microscope was maintained at 37 °C.

After cell imaging was taken, about 200 ± 10 cells and 25 μL mobile phase were transfered into an Eppendorf tube. Then the mixture was sonicated for 1 min and centrifugated at 12,000 rpm for 2 min. 20 μL of the supernatant was injected to the chromatographic system.

2.4. Chromatography

Before analysis, the C_{18} column was pre-equilibrated for 30 min with the mobile phase consisting of methanol–water–buffer (84:6:10, v/v/v, buffer pH 5.75 H_3Cit–NaOH) at room temperature. An aliquot of 20 μL of sample solution was injected using an autosampler into the C_{18} column and the derivatives were eluted at a flow rate of 0.5 mL/min with isocratic elution. The detection wavelengths were set at $\lambda_{ex}/\lambda_{em}$=507/520 nm. Peak areas were measured for quantitative calculations.

3. Results and discussion

3.1. Feasibility experiment for cell imaging

A feasibility experiment for in-situ cell imaging was performed on Hela cell, an immortal and widely used cell line in cancer research. In our previous work, it was reported that TMPAB-*o*-M could be derivatized with thiols under physiological conditions (pH 7.40, 20 mM phosphate buffered saline (PBS), 37 °C) in only 5 min when the concentration of TMPAB-*o*-M is higher than 9×10^{-6} M [20]. However, the concentration of the reagent in cells and in PBS solution may be different. Therefore, the concentration of TMPAB-*o*-M for cell imaging should be investigated. Hela cells were incubated at 37 °C in PBS containing 1×10^{-7}–2×10^{-5} M of TMPAB-*o*-M for 5 min, and then the cells were mounted on the inverted fluorescence microscope without any washing because TMPAB-*o*-M had no autofluorescence and could not be observed under experimental conditions. Under the same apparatus parameters and exposure time, it could be found that hela cells became brighter owing to the increase of TMPAB-*o*-M concentration and appeared the brightest when the concentration reached 1.0×10^{-5} M.

Incubation time is another factor for cell imaging. After being suspended in PBS containing 1.0×10^{-5} M TMPAB-*o*-M, hela cells were mounted on the microscope instantly and images were taken at intervals of 10 s. The results are interesting since the fluorescence intensity of the cell imaging reached its maximum within only 90 s (Fig. 2), which is much faster than our expectation (5 min). It may be attributed to the fact that the reactivity of TMPAB-*o*-M to thiols is much higher under the real physiological conditions compared to those in simulated physiological conditions. The results also indicated that TMPAB-*o*-M could be readily loaded into the cells. The great hydrophobicity ensured its strong membrane-penetrative ability. After permeating into the cells, it reacted with thiols quickly and formed hydrophilic derivatization products which carry amino and carboxyl groups (Fig. 1), and was retained in the cells owning to its relatively poor permeating ability. Moreover, the fluorescence intensity of cells was stable atleast for 15 min under irradiation and the shape of cells was shown to be intact under microscope during this time. This phenomenon means that TMPAB-*o*-M and the thiol derivatives should be slightly toxic to cells and stable against

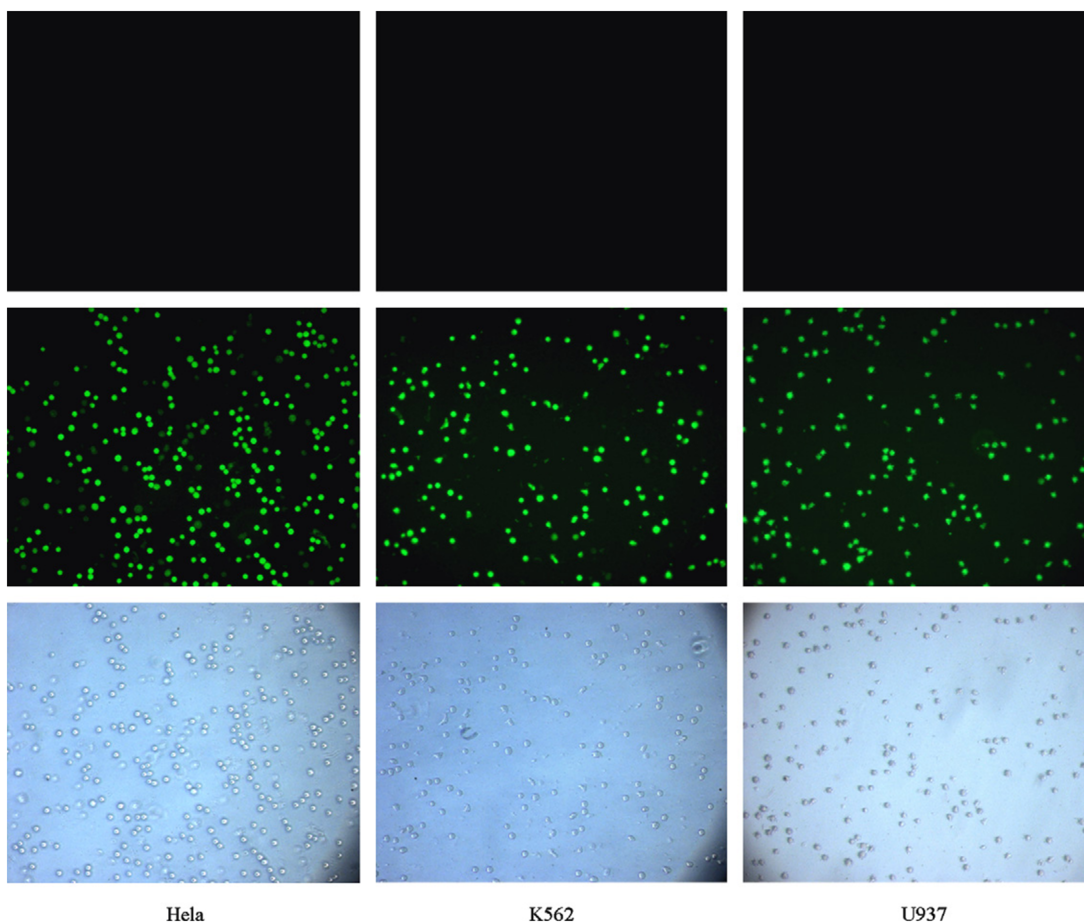

Fig. 3. Bright-field and the fluorescence images of different kinds of living cells. $C_{TMPAB\text{-}o\text{-}M}=1 \times 10^{-5}$ M.

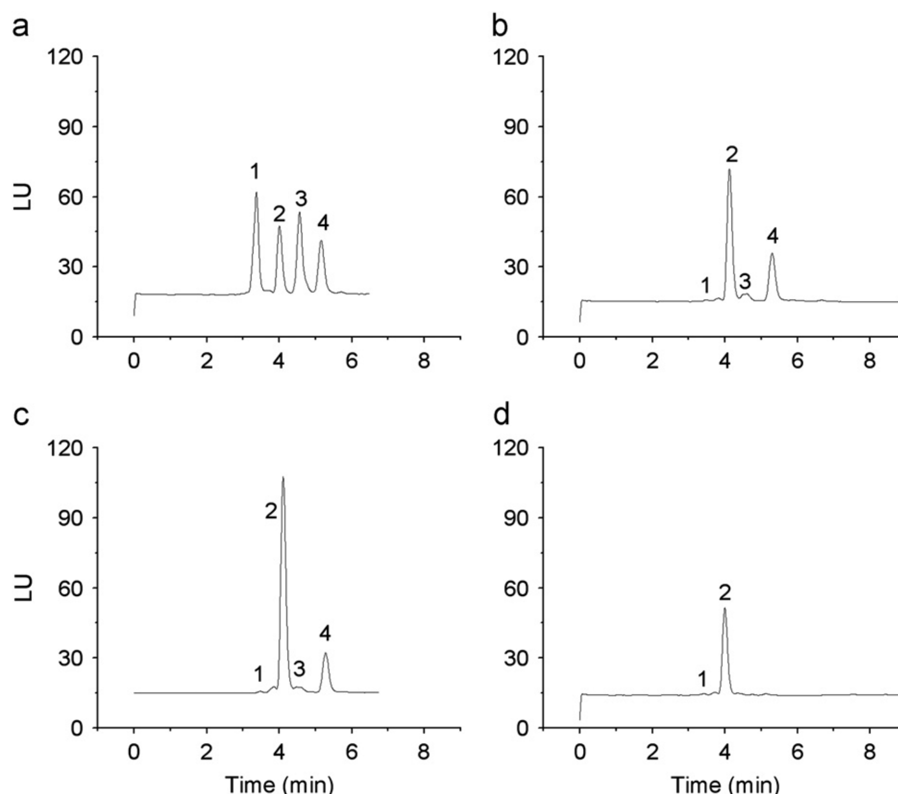

Fig. 4. Chromatograms obtained from different kinds of cells. Peaks—(1) CoA, (2) GSH, (3) NAC, and (4) Cys: (a) Typical chromatogram of 54.5 nM thiol derivatives; (b) Hela; (c) K562; and (d) U937.

photobleaching, which are greatly preferred for continuous tracking studies over a long period of complex biological processes, comparable to quantum dots.

3.2. *In-situ cell imaging and quantitative analysis of thiol compounds*

The potential of TMPAB-*o*-M in cell imaging had been validated by three classes of cells, Hela, K562 and U937. U937 cells are important models of human macrophage function and K562 cells are the first human immortalized myelogenous leukemia line to be established. As shown in Fig. 3, the brightness of the resulting images from different kinds of cells is not the same as others and that of U937 cells is the lowest, which should have been caused by the variety of the total concentration of thiol containing compounds. To confirm this conjecture and obtain the amount of thiols in these living cells, HPLC followed by fluorescence detection was performed and the results are given in Fig. 4 and Table 1 (200 ± 10 cells each for use). The peak areas of GSH, NAC and Cys in U937 cells were smaller than those of Hela and K562 cells, which is in accordance to the results of cell imaging. Also, as shown in Fig. 4 and Table 1, the fluorescence intensity of cells was mainly contributed from GSH, and the level of thiol compounds in different cell lines differed from each other. In four thiol containing compounds, only the amount of intracellular GSH was reported constantly, whose concentrations detected in Hela, K562 and U937 cells using the proposed method were agreeable with other established protocols [21–25].

Table 1
Analytical results of samples using HPLC.

Samples	Hela cell (200 ± 10)		K562 cell (200 ± 10)		U937 cell (200 ± 10)	
	Found (pmol)	RSD (%, n=6)	Found (pmol)	RSD (%, n=6)	Found (pmol)	RSD (%, n=6)
CoA	0.0183	4.7	0.0205	4.6	0.0146	4.3
GSH	2.21	3.4	3.59	3.4	1.51	3.6
NAC	0.0913	4.3	0.0546	3.8	–	–
Cys	1.09	2.1	0.849	4.0	–	–

4. Conclusions

In summary, a simple real-time and in-situ analytical method for the visualization of thiol compounds in living systems was developed using cell imaging based on TMPAB-*o*-M labeling. The in-situ labeling could be achieved in only 90 s and could provide the imaging of thiols closely approaching real state of their distribution. Owing to the stability of TMPAB-*o*-M and its derivatives of thiols under irradiation, visualized monitoring of cell activity may be realized by means of TMPAB-*o*-M labeling. Furthermore, the excellent photostability of the derivatives allows acquisition of many consecutive focal-plane images and their reconstruction into a high-resolution three-dimensional projection. Further application about this technology is in progress.

Acknowledgments

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