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ARTICLE

# A new water-soluble two-photon fluorescent probe for detection of trace benzoyl peroxide in wheat flour and living cells and tissues imaging

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Haiyuan Ding‡, Gangqiang Yuan‡, Liyi Zhou*

Benzoyl peroxide (**BPO**), a member of small-molecule reactive oxygen species (**ROS**), it has attracted wide attention because of its impact on human health and industrial importance. Herein, to quantitatively detect **BPO** in real samples and fluorescence imaging in living cells and tissues, a new water-soluble two-photon (**TP**) fluorescent probe (**CM-1**) was constructed. Specifically, **CM-1** adopted the carboxyl (-COOH) as the water-soluble unit, cyano (**-CN**) forms the D-π-A-structure as an acceptor to increase the two-photon action absorption cross-section and phenylboric acid (**PBA**) as the **BPO** reaction moiety. After the **PBA** moiety reaction with **BPO**, **CM-1** displayed a ~82-fold fluorescence intensity enhancement in 485nm and high sensitivity with a low detection limit of 33nM. Selectivity experiment demonstrated that probe can detect **BPO** with high selectivity over other common substances, which makes it of great potential use in quantitative and simple detection of **BPO** in wheat flour. Moreover, **CM-1** could be employed for imaging **BPO** in living HeLa cells and rat liver tissues with large tissue-image depth (40-150μm) under two-photon excitation (800nm), thus demonstrating its practical application in biological systems for the study of physiological and pathological functions of **BPO**.

## Introduction

Benzoyl peroxide (**BPO**), a member of small-molecule reactive oxygen species (**ROS**) has attracted wide attention due to its key role in human health and disease. On one hand, **BPO** has been widely used in our daily life for bleaching flour [1], treating acne [2], and initiating polymerization [3]. On the other hand, **BPO** can easily enter the human body by food intake or skin absorption, resulting in potential risks. It can act as a tumor promoter and is able to degrade into deleterious substances (e.g., biphenyl, benzoic acid), inevitably leading to tissue damage and diseases [4-5]. Besides, once it used as flour additives to improve the color and lustre of wheat flour and corn starch would cause the destruction of the flour nutrients [6]. Therefor, in order to get deeper understanding of the chemical and biological properties of **BPO**, highly sensitive and highly selective detection techniques for tracking **BPO** are urgently desirable, since the complex manifestations of **BPO** in both physiological and pathological states, as well as its underlying molecular events are still not fully understood.

In the past few years, some methods have been developed for monitoring **BPO**, such as chemiluminescence [7], electrochemistry [8], UV-spectrophotometry [9], HPLC [10] and so on. However, most of these methods require a time-consuming sample pretreatment and separation, and thus are inconvenient for the fast and *in situ* detection of **BPO**. Compared with them, fluorescence-based methods could maintain comparable efficiency and accuracy, offer convenience, high sensitivity, noninvasive, as well as real-time imaging [11a,11b]. Especially, two-photon (**TP**) probe-based fluorescent imaging, which is an emerging technique and employing near-infrared (**NIR**) light source excitation that can provide improved spatial resolution and theoretically remarkably increased imaging depth than traditional one-photon (**OP**) imaging, might be the most attractive one for *in vivo* detection of bio-related species [12]. in the past few years, very few fluorescent probes for detection of **BPO** in real samples and biosystems for **OP** excitation [13-15]. Unfortunately, to the best of our knowledge, no water-soluble and **TP** fluorescent probe have been reported for **BPO** detection so far. Herein, for the first time, we present the design, synthesis, and characterization of **TP** fluorescent probe **CM-1** (Scheme 1) as a new water-soluble **TP** fluorescent probe for detection of **BPO** in real samples and fluorescence imaging in living HeLa cells and tissues.

Herein, we choose a coumarin derivative (**CM-CN**) as a signaling unit for **BPO**. As a result, the new probe **CM-1** (**Scheme 1**) was synthesized with a good yield (81%) *via a* two-step reaction, whichupon reaction with **BPO**, will induce the cleavage of **PBA** moiety to be left, resulting in the forming of the

[a] College of Food Science and Technology, Central South University of Forestry and Technology, Changsha, Hunan 410004, P. R. China. E-mail: zhouly0817@163.com.

[b] Hunan Key Laboratory of Processed Food for Special Medical Purpose, Changsha, Hunan410004, P. R. China.

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‡Contributed equally.

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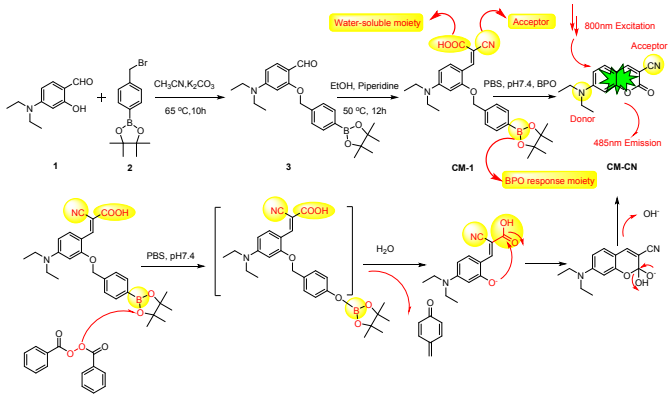
fluorophore and "turn-on" of the fluorescent signal. After reaction with **BPO**, the probe **CM-1** exhibited a more than 82-fold fluorescence intensity enhancement at an emission wavelength of 485nm. It also showed the probe displayed high selectivity toward **BPO** over other analytes. Subsequently the probe was successfully applied to detect **BPO** for real samples and bioimaging.

## Results and discussion

### Design and synthesis of fluorescent probe CM-1

Previously, few of **BPO** probes have been designed [13-15]. However, they are all water-insoluble and require a large amount of organic solvent as a cosolvent. And they are constructed by one-photon, could not be effectively used to analyze deep tissue imaging studies. In order to acquire a better *in vitro* detection, biological image resolution, and penetration depth, we decided to develop a new water-soluble fluorescent probe for detection of **BPO** in wheat flour, live cells, and tissues. As a proof-of-concept, a D-π-A-structured **CM-CN** was chosen as the fluorophore for its outstanding two-photon property, while a boronic acid unit was serviced as the recognition moiety due to its reaction with **BPO** rapidly by some reference reported [13-15]. All compounds were characterized by $^{1}H$, $^{13}C$ NMR, and ESI-MS (see the Supporting Information).




**Scheme 1.** The synthetic route of the fluorescent probe **CM-1** and its possible reaction with **BPO**.

### Optical property, selectivity and effect of pH value on fluorescent probe CM-1

As expected, **CM-1** is non-fluorescent (**Fig. 1b**) due to the strong electron-withdrawing boronic acid unit that prevented the **CM-1** changed to fluorophore **CM-CN**. Once upon reaction with **BPO**, the **PBA** moiety converting into an electron-donor -$O^-$ group, which results quickly converting into **CM-CN** and a significant off-on fluorescence enhancement at 485nm (with a~82-fold enhancement), and its intensity increased linearly with the concentration of **BPO** ranging from 0μM to 0.7μM (**Fig. 1d**). Moreover, once under excitation of 365nm UV light, the changes in the fluorescence of the probe **CM-1** before and after reaction with **BPO** had obviously changed (**Fig. 1e and Fig. 1f**), which allow to distinguish it with the naked eye. The detection limit (utilizing the 3σ/k method) for **BPO** was determined to be 3.3nM, which is enough for direct detection of **BPO** in the wheat flour and *in vivo* as its concentrations are usually in submicromolar range. To explore the spectroscopic response mechanism, the reaction products of **CM-1** with **BPO** were subjected to MS and $^{1}H/^{13}C$ NMR analyses, see the **Scheme 1** for its possible reaction with **BPO** and see the Supporting Information for MS and $^{1}H/^{13}C$ NMR results, the results exhibit that the reaction produced the fluorophore **CM-CN**.

High selectivity is an important parameter to evaluate a newly designed fluorescent probe performance. For this purpose, **CM-1** was treated with a series of analytes such as tert-butyl hydroperoxide (*t*-BuOO·, TBHP), $O_2^-$, NO, $OCl^-$, vitamins, $NaClO_4$, $NaClO_3$, **BPO**, amino acids (Cys, Hcy and GSH), $NaNO_2$, $KBrO_3$, $H_2O_2$, $^1O_2$, $ONOO^-$, $KIO_3$, $KMnO_4$, $S^{2-}$, $HSO_3^-$, $SO_3^{2-}$, and CuOOH to examine its selectivity. The results showed in **Fig. 2a**, the probe showed almost unchanged fluorescence intensity responses before and after addition of other analytes to the probe resolution. More interestingly, compared to **BPO**, the other oxidants tested produce a much weaker fluorescence except $H_2O_2$ and ONOO-. These results demonstrated that probe **CM-1** could meet the selective requirements for practical applications. Next, we have studied the effect of pH on **CM-1** in the absence and presence of **BPO** (**Fig. 2b**). Without **BPO**, no obvious characteristic fluorescence could be observed from pH 4.0-9.0. Upon reaction with **BPO**, the best response towards **BPO** could be achieved with a pH range of 6.0-8.5. Thus, the **PBS** solution (pH 7.4) was utilized throughout the experiment. These results indicated that the probe was favourable for applications in practical samples at different pH values.

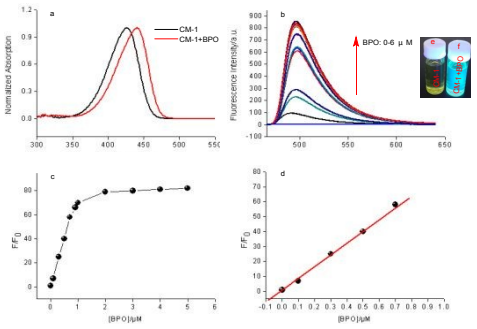



**Fig.1.** (a) UV-vis normalized absorption, black line: 1μM **CM-1**; red line: 1μM **CM-1**+6μM **BPO**; (b) The fluorescence spectra of probe **CM-1** (1μM) in the presence of various concentrations of **BPO** (0-6μM); (c) Calibration curve of **CM-1** to **BPO**. The curve was plotted with fluorescence intensity *vs* **BPO** concentration (0-6μM); (d) Linear relationship between $F/F_0$ and BPO concentration (0-0.7μM); (e) and (f) Change in the fluorescence of the probe **CM-1** before and after adding 5μM **BPO** in 1μM **CM-1** under excitation of 365 nm UV light.

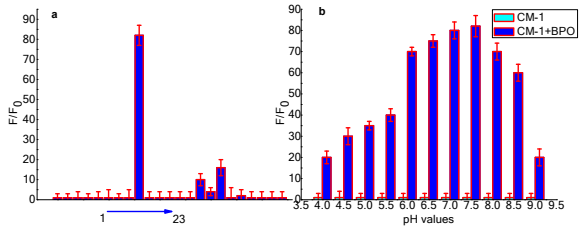


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**Fig.2.** (a) Selectivity experiments, fluorescence response of 1μM **CM-1** to 6μM **BPO** or 50μM other analytes. The numbers from 1 to 23 correspond to black (**CM-1**) to TBHP, $O_2^-$, NO, $OCl^-$, vitamins, $NaClO_4$, $NaClO_3$, **BPO**, amino acids (Cys, Hcy and GSH), $NaNO_2$, $KBrO_3$, $H_2O_2$, $^1O_2$, $ONOO^-$, $KIO_3$, $KMnO_4$, $S^{2-}$, $HSO_3^-$, $SO_3^{2-}$, and cumene hydroperoxide; (b) Effects of pH (4-9) on **CM-1** in the absence or presence of **BPO**.

**Table 1. Determination of BPO in wheat flour samples with probe CM-1.**

| Sample | **BPO** spiked (M) | **BPO** determined (M) meana ± SDb | Recovery (%) |
|---|---|---|---|
| Wheat flour 1 | 0.0 | not detected | |
| Wheat flour 2 | $1.0 \times 10^{-6}$ | $(9.58 \pm 0.05) \times 10^{-7}$ | 95.8 |
| Wheat flour 3 | $3.0 \times 10^{-6}$ | $(3.15 \pm 0.06) \times 10^{-6}$ | 105 |
| Wheat flour 4 | $5.0 \times 10^{-6}$ | $(4.65 \pm 0.03) \times 10^{-6}$ | 93.0 |
| Wheat flour 5 | $7.0 \times 10^{-6}$ | $(7.21 \pm 0.03) \times 10^{-6}$ | 103 |

[a]Mean of three determinations. [b]SD: standard deviation



**BPO Detection in wheat flour**

**BPO** has been extensively used as flour bleaching agents, while excessive **BPO** could induce allergic reactions and potential carcinogenicity and exert an effect on human peripheral lymphocytes. To evaluate the practical applicability of **CM-1** for real samples such as wheat flour is of great significance. The wheat flour of the pH was adjusted to 7.4 prior to use. At first, wheat flour was added to **CM-1** resolution and fluorescence signal intensity at 485nm was recorded. However, wheat flour may contain little seven fluorescent whitening agents (FWA135, FWA140, FWA162, FWA184, FWA185, FWA367 and FWA393)[17-18], they absorb invisible ultraviolet light and emit visible blue or blue-violet fluorescence. Due to the excitation light employed in the experiment was 400nm and not their best excitation light. Moreover, background interference has been deducted during the measurement. Thus, the fluorescent whitening agents could be not excited to affect the detection results. The calculated **BPO** concentration in the wheat flour sample was 48mg/Kg. The **BPO** stock solution at different concentrations was spiked in these wheat flour samples, and the probe **CM-1** was then added to detect its concentration. The recovered **BPO** concentrations were shown in **Table 1**. The results show good recovery values, which confirmed that the other coexisting species hardly interfere with the **BPO** assay. Furthermore, the determination does not require a time-consuming separation. This indicates that probe **CM-1** has good capacity to quantify **BPO** in real samples.

**Detection of BPO in live cells and tissues**

In order to evaluate the imaging performance of **CM-1**, we used this probe to detect **BPO** in living cells and tissues. HeLa cells were chosen as the model cell line. Before imaging, the cytotoxicity of the **CM-1** was tested. The results showed that it was nearly nontoxic for living cells under experimental conditions (**Fig. S1**). Then, HeLa cells were incubated with **CM-1** (1μM) at 37 °C for 30min, followed by excitation at 800nm for **TP** image, the HeLa cells showed weak fluorescence intensity in the green channel by **TP** image (**Fig. 3b**). According to some literature reports, **BPA** moiety was responsive to hydrogen peroxide, and hydrogen peroxide is a major endogenous ROS, so, the green channel showed weak fluorescence intensity[16]. In contrast, treating **CM-1**-incubated cultured cells with 5μM **BPO** for 30min, the fluorescence intensity enhanced in the green channel obviously (**Fig. 3e**) by TP image. Taking together, these results showed that **CM-1** was cell membrane-penetrable and could be used for **TP** images in live cells.

Finally, **CM-1** was further applied for **TP**-excited fluorescence image of **BPO** in liver tissue slices from rat with images at different tissue depths recorded by **TP** image in the Z-scan mode. The imaging results showed that the probe could be successfully applied for image of **BPO** in liver tissues at a depth of 40-150μm in green fluorescent channel (**see Fig. 4**). These results demonstrated that the probe possessed a good staining capability and high penetrating ability in tissues for image of **BPO**.

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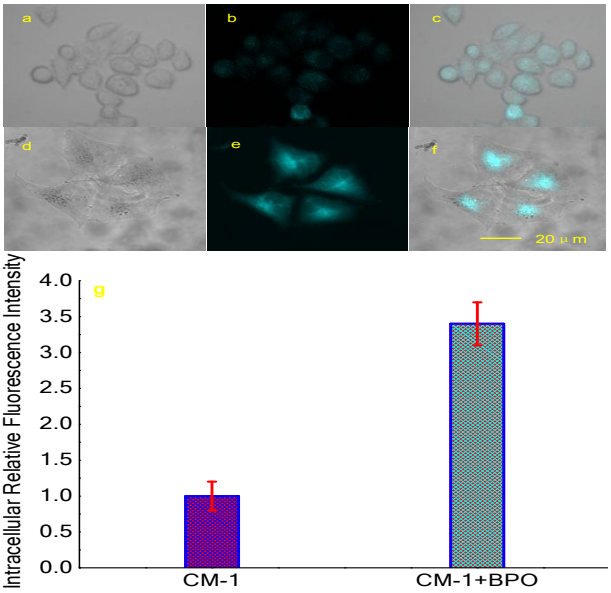


**Fig.3.** TP fluorescence microscope (**TPFM**) images of HeLa cells: (a) Bright field image; (b) HeLa cells incubated with 1μM **CM-1** for 30min; (c) Merged image of (b) and bright field image (a); (d) Bright field image; (e) HeLa cells pre-treated with 5μM **BPO** for 15min and then incubated with 1μM **CM-1** for 30min image; (f) Merged image of (e) and bright field image (d). (g) Intracellular relative fluorescence intensity of (b: **CM-1**) and (e: **CM-1**+BPO). TP images: $\lambda_{ex}$=800nm, $\lambda_{em}$=470-550nm. All images were acquired with a 40×oil immersion objective, scale bar: 20μm.

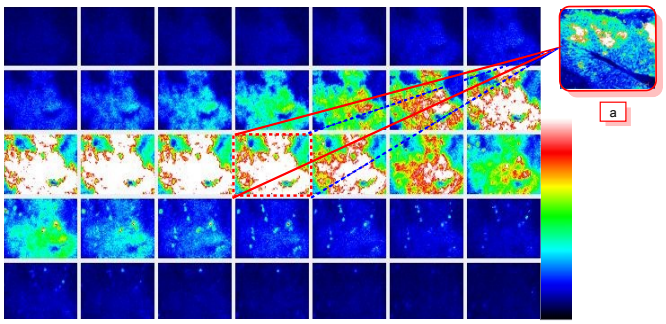


**Fig.4. TP** fluorescence images of a frozen liver tissue slice for the Spec3 mode imaging. Rat liver tissue slice was stained with 1μM **CM-1** for 60min at 37 °C, followed by treatment with 5μM **BPO** and incubated for another 60min. (a) The Spec3 mode imaging at a depth of 92μm. TP images: $\lambda_{ex}$=800nm, $\lambda_{em}$=470-550nm. All images were acquired with a 40×oil immersion objective, scale bar: 10 μm.

## Conclusions

In summary, we rational designed and synthesized a novel water-soluble **TP** fluorescent probe **CM-1** to detect **BPO** in wheat flour and living cells and tissues. The probe is based on a **TP** excitation D-π-A-structure of coumarin derivatives fluorophore (**CM-CN**), which is obtained upon removal of a trigger moiety by the **BPO** of interest. The probe was demonstrated to efficiently image **BPO** produced in live cells, and tissues, we believe that probe **CM-1** will be a useful tool for in biological systems for the study of physiological and pathological functions of **BPO**.

## Experimental section

### Reagents and apparatus

Unless or otherwise specified, all chemicals were obtained from commercial suppliers and used without further purication. Water used in all experiments was double-distilled and purified by a Milli-Qsystem (Millipore, USA). LC-MS analyses were performed using an Agilent 1100 HPLC/MSD spectrometer. $^{1}$HNMR and $^{13}$CNMR spectra were recorded on a BrukerDRX-400 spectrometer operating at 400 and 100MHz. All chemical shifts are reported in the standard notation of parts per million. UV-*vis* absorption spectra were recorded in 1.0cm path length quartz cuvettes on a Shimadzu 2450 UV-visible Spectrometer. All fluorescence measurements were carried out on a G-9800A fluorescence spectrometer (Agilen) with both excitation and emission slits set at 2.5nm, respectively. The pH was measured with a Mettler-Toledo Delta 320 pH meter. Fluorescence imaging of HeLa cells and Tissues was conducted on a confocal laser scanning microscope (Olympus, Japan) with 800 nm excitation.

### Synthesis of water-soluble TP fluorescent probe CM-1

**Synthesis and characterization of compound 3:** 0.193g (1.00mmol) 4-(diethylamino)-salicylaldehyd, 0.297g 2-(4-(bromomethyl) phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (1.00mmol), 0.294g (3mmol) $K_2CO_3$ and 40mL $CH_3CN$ were added into 100mL flask with a reflux condenser, and the mixture stirred at 65°C for 10 hours under argon protection. And then, the mixture was filtrated, the solvent was evaporated by rotary evaporator. At last, the target compound was obtained by the quickly column chromatography (petroleum ether/ethyl acetate = 4:1, v/v) to yield **3** as a yellow solid (0.389g, 95.1%). $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ: 8.5 (s,1H), 8.24-8.22 (d, *J*=8Hz, 1H), 7.73-7.71 (d, *J*=8Hz, 1H), 7.49-7.47 (d, *J*=8Hz, 1H), 6.52-6.50 (d, *J*=8Hz, 1H), 6.26 (s, 1H), 5.30 (s, 1H), 3.55-3.43 (dd, *J*=24Hz, 4H), 1.11-1.08 (t, *J*=6Hz, 6H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) *δ:* 164.92, 161.57, 154.19, 147.16, 140.75, 135.00, 130.41, 126.71, 118.48, 115.12, 109.03, 106.24, 95.26, 90.38, 83.99, 70.23, 62.58, 61.71, 44.87, 12.78; ESI-MS: $[M]^+$ calcd: 359.3, found: 360.1.

**Synthesis and Characterization of CM-1:** Cyanoacetic acid (0.085g, 1mmol) and compound 3 (0.205g, 0.5mmol) were dissolved in EtOH (40mL) with piperidine (0.2mL) and acetic acid (0.2mL) under argon protection at 50°C for 12hours. Followed by the solvent was evaporated in vacuo, and the crude solid was purified by column chromatography on silica gel eluting ($CH_2Cl_2$/MeOH = 100:1, v/v) to afford a yellow solid in 85.3% yield. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ: 9.67 (s, 1H), 8.88 (s, 1H), 8.43-8.40 (d, *J*=12Hz, 1H), 7.76-7.74 (d, *J*=8Hz, 1H), 7.15-7.12 (d, *J*=12Hz, 1H), 6.80-6.77 (d, *J*=12Hz, 1H), 6.59 (s,1H), 3.48-3.43 (q, *J*=10Hz, 4H), 1.14-1.10 (t, *J*=8Hz, 6H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) *δ:* 185.94, 156.60, 156.25, 154.00, 141.47, 139.18, 134.13,

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130.17, 122.29, 118.72, 115.31, 109.83, 103.55, 44.71, 12.71; ESI-MS: $[M]^-$ calcd: 426.4, found: 426.8.

**Spectroscopic materials and methods**

The fluorescence intensity of fluorescent probe **CM-1** measurement experiments were conduct in 10mM PBS buffer (pH7.4). The fluorescent emission spectra was recorded at 460 to 650nm using 400nm excitation.

**Selectivity experiments**

NO was generated from of 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene(NOC-5), $^1O_2$ was produced by the reaction of $H_2O_2$ with NaOCl, $O_2^-$ was generated from $KO_2$[19], ·OH was formed from Fenton reaction between $Fe^{2+}$(EDTA) and $H_2O_2$ quantitatively, $Fe^{2+}$(EDTA) concentrations represented ·OH concentrations [20], and tert-butylhydroperoxide (*t*-BuOOH), cumene hydroperoxide could also use to induce ROS in biological systems [21].

**BPO detection in wheat flour**

Wheat flour was purchased from a local supermarket (Changsha, P.R. China). By the following procedure to prepare the **BPO** samples. Firstly, the phosphate-buffered saline (**PBS**) solutions (10mM, pH 7.4, 10% ethanol (because of the **BPO** is water-insoluble)) containing various concentrations of **BPO** (0, 0.1, 0.3, 0.5, 0.7, 0.9, 1, 2, 3, 4, 5, and 6 μ M) were mixed with wheat flour (1g). Secondly, samples were sonicated for 3min and filtered with organic membrane (0.22 μ m). At Last, the resulting samples were prepared with probe **CM-1**, and the fluorescence spectrum was detected by G-9800A fluorescence spectrophotometer.

**Preparation and staining of cell and tissue cultures**

Prior to the image experiments, the HeLa cells were washed with PBS, and incubated with 1μM probe **CM-1** for 30minutes at 37 °C, then it washed with **PBS** for three times and incubated with 10μM **BPO** for another 30munites at 37 °C, finally, the Hela cells were washed with **PBS** three times again before image. Like the cell cultures and staining, the rat liver tissue sections were prepared by frozen section machine. The sections were incubated with 1μM probe **CM-1** for 1hour at 37 °C, then it washed with **PBS** for three times and incubated with 5μM **BPO** for another 1hour at 37 °C, finally, the sections were washed with **PBS** three times again before image. Confocal fluorescence image of **BPO** in HeLa cells and liver tissues was observed under an Olympus FV 1000 laser confocal microscope. Two-photon image: λex=800nm, λem=470-550nm. All images were acquired with a 40×oil immersion objective, scale bar: 10μm or 20μm.

## Conflicts of interest

The authors declare no competing financial interest.

## Acknowledgements

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