Preparation of a cyanine-based fluorescent probe for highly selective detection of glutathione and its use in living cells and tissues of mice

Jun Yin[1,2,5], Younghee Kwon[3–5], Dabin Kim[1], Dayoung Lee[1], Gyoungmi Kim[1], Ying Hu[1], Ji-Hwan Ryu[3,4] & Juyoung Yoon[1]

[1]Department of Chemistry and Nano Science, Ewha Womans University, Seoul, Korea. [2]Key Laboratory of Pesticide and Chemical Biology, Ministry of Education, College of Chemistry, Central China Normal University, Wuhan, China. [3]Research Center for Human Natural Defense System, Yonsei University College of Medicine, Seoul, Korea. [4]BK 21 Plus Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea. [5]These authors contributed equally to this work. Correspondence should be addressed to J.Y. (jyoon@ewha.ac.kr) or J.-H.R. (yjh@yuhs.ac).

Published online 15 October 2015; doi:10.1038/nprot.2015.109

Glutathione (GSH) is a major endogenous antioxidant that has a central role in cellular defense against toxins and free radicals. This protocol describes the preparation of CPDSA, a cyanine-based near-infrared (NIR) fluorescent probe for the detection of GSH in cells and *in vivo*. CPDSA is prepared with high yield through a simple two-step process. The first step is to react commercially available IR-780 iodide with excess anhydrous piperazine in anhydrous *N,N*-dimethyl formamide at 85 °C to form cyanine-piperazine (CP). The second step is the sulfonylation of CP with dansyl chloride in anhydrous dichloromethane. CPDSA selectively detects GSH in cells, and it has been shown to not react with other biothiols such as cysteine (Cys) and homocysteine (Hcy). This probe can also be used to monitor the GSH level of mouse bone marrow–derived neutrophils (BMDNs). The preparation of probe CPDSA takes 2 d, and experiments in cells and mice take 12–13 d.

© 2015 Nature America, Inc. All rights reserved.

INTRODUCTION

Biothiols such as Cys, Hcy and GSH have crucial roles in many physiological and pathological processes[1]. GSH is the most abundant intracellular thiol-based amino acid, and it has a central role in maintaining biological redox homeostasis in biological systems[2]. Changes in the levels of cellular thiols are directly linked to many serious diseases such as liver damage, cancer, AIDS, leukocyte loss, osteoporosis and Alzheimer's disease, as well as heart, inflammatory bowel and cardiovascular diseases[3]. Therefore, developing an efficient method to assess the levels of thiols in biological systems is of great significance to aid early clinical diagnosis and treatment of diseases[4].

In recent years, considerable attention has been directed toward thiol-based fluorescent probes for the purpose of detecting biothiols at low concentrations or with narrow ranges such as in cells and tissues[1,3,5]. Consequently, many fluorescent probes that possess the capability to detect or discriminate Cys, Hcy and GSH have been developed by using different fluorophores such as BODIPY[6–10], rhodamine[2,11], coumarin[12–16], pyrene[17,18] and nitrobenzothiadiazole[19]. Although these probes have displayed high selectivity to discriminate three amino acids, they often suffer from shorter emission, which restricts their application *in vivo*. In comparison with the shorter emission of fluorophores described above, NIR fluorescent probes within the wavelength range of 700–900 nm have the advantage of low absorption and autofluorescence from living systems in the NIR spectral range, which can minimize background interference, improve tissue depth penetration, and allow imaging sensitively and noninvasively. Furthermore, it is particularly important that NIR dyes can minimize photodamage to biological specimens, organisms and tissues as a result of the lower energy of NIR light, which can be significant when the objective of the experiment is to analyze a compound that responds to oxidative stress. However, developing highly selective NIR fluorescent probes that are suitable for the detection of biothiols in cells and *in vivo* is still a challenge.

Overview of the procedure

We recently reported a cyanine-based fluorescent probe, CPDSA (**Fig. 1**), with a NIR emission that is specific for GSH[20]. In this protocol, we describe how to prepare the CPDSA fluorescent probe (Steps 1–26) and successfully visualize the level of GSH in cultured cells (Step 27A–D) and cells derived from mouse models of sepsis (Step 27E or F; **Fig. 2**). The four example experiments in cultured cells are designed for monitoring changes in fluorescence on incubation of the probe with HeLa cells (Step 27A), for confirming the selectivity of the probe for GSH (Step 27B), for characterizing changes in the redox state of GSH on addition of H_2O_2 (Step 27C) and for investigating lipopolysaccharide (LPS) metabolism in conjunction with GSH oxidation (Step 27D). In addition, some crucial considerations for the probe's synthesis and application are discussed.

Comparison with other methods

Traditional analytical techniques for detecting GSH in cell extracts or biological samples include spectrophotometry, high-performance liquid chromatography (HPLC)/gas chromatography, high-performance capillary electrophoresis, enzymatic cycling Guaranteed reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (GR-DNTB), electrochemistry, flow cytometry, NMR spectroscopy, fluorescence spectroscopy methods, and so on[21–23]. Although some of them can be used to detect GSH under appropriate conditions *in vitro* or *in vivo*, they have various limitations that are summarized in **Supplementary Table 1**.

Other methods, e.g., the enzymatic reaction, are only suitable for *in vitro* detection, but not in cells or tissues. The enzymatic reaction can be performed using the total glutathione

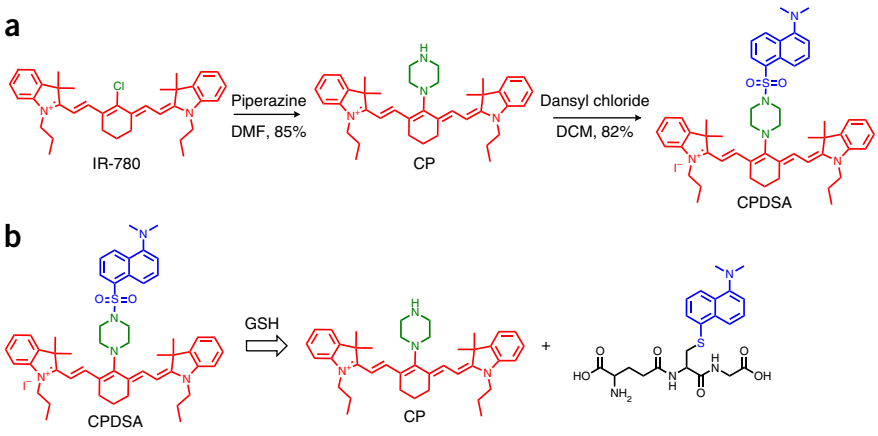

Figure 1 | The GSH-based fluorescence probe CPDSA. (**a**) The proposed reaction mechanism of CPDSA with GSH. (**b**) Scheme of the synthesis of CPDSA.

(glutathione disulfide (GSSG)/GSH) assay kit, as described in the **Supplementary Methods**. Even though the enzymatic reaction for measuring GSH concentration has high efficiency and is an excellent method for detecting total glutathione in an *in vivo* sample, it also suffers from additional disadvantages: sample preparation is complicated and the measurement process takes a longer time.

In recent years, fluorescent probes that are highly selective and that have good sensitivity for GSH have been developed and used in bioimaging analytical techniques[21–23]. It is especially worth mentioning that the ability to visualize fluorescent probes either in biochemical assays or in cells and tissues allows for much easier monitoring of signal changes in real time and space compared with other analytical techniques. Therefore, molecular imaging technologies based on the fluorescent response are considered to be some of the most powerful, economical and attractive approaches[5].

NIR fluorescent probes

The use of NIR fluorescent probes within the wavelength range of 700–900 nm is one of the most promising approaches for monitoring the biological activities and physiological events of GSH *in vitro* and *in vivo*, because there is little absorption and autofluorescence from living systems in the NIR spectral range. Compared with probes with fluorescence in the UV-visible range, these NIR probes have lower background interference and better tissue depth penetration, which allows for sensitive and non-invasive imaging. In addition, NIR dyes cause less photodamage to biological specimens, organisms and tissues because of the lower energy of NIR light[24].

Cyanine, a classical type of NIR fluorescent dye[25,26], is gaining popularity in applications for detecting various biologically relevant species in cells and organisms[27–42]. We were looking for a cyanine derivative that was fluorescent under physiological conditions that would be a good candidate for further derivatization such that the product would have either weak or no fluorescence, but would react with GSH in such a way as to result in the original, fluorescent molecule. We found that a piperazine derivative (CP) with the desired properties had been reported in previous literature[33] (**Fig. 3**). We coupled CP to a dansyl group via a sulfonamide bond. The sulfonamide bond is often used as a detecting group for thiols, and the dansyl group quenches the fluorescence from the cyanine fluorophore.

We found that this cyanine-based fluorescent probe, CPDSA (**Fig. 1**), had the necessary reactivity and 'turn-on' properties to make it capable of monitoring and imaging levels of GSH in living cells and *in vivo*[20]. This probe displays good water solubility, biocompatibility, cell penetration and high selectivity (**Supplementary Fig. 1**).

While this protocol was being developed, another research group applied the exact same design strategy, in which they used the CP analog as a cleavage product for a thiol-responsive fluorescent probe[43]. They performed control experiments using the thiol-blocking reagent *N*-methylmaleimide (NMM) in living cells and mouse tissues (such as liver, lung, kidney, spleen and so on), which were similar to the experiments we performed, and their results were in agreement with ours.

Although CPDSA has been confirmed to be an efficient biomarker, it also has limitations. It is well known that NIR dyes usually suffer from poor photostability because of their larger conjugated systems. CPDSA is no exception, and its solution in DMSO can be kept for only 2 d at room temperature (RT, 20–25 °C). Consequently, CPDSA is usually stored below 4 °C in a refrigerator, and a solution in DMSO used for measurement

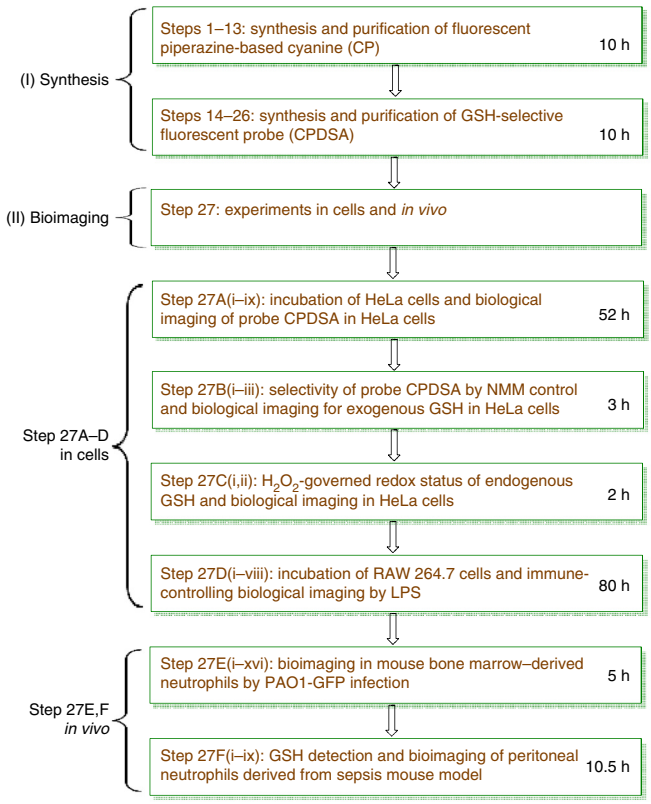

Figure 2 | Flowchart outlining the experimental procedures described in this protocol and expected timing for each option.

© 2015 Nature America, Inc. All rights reserved.

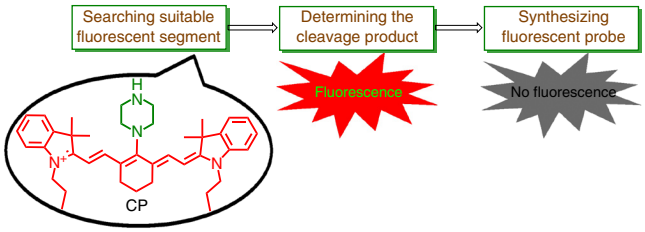

Figure 3 | Design strategy of the CPDSA fluorescent probe.

must be frozen at −20 °C or used quickly after the stock solution is made in order to ensure the activity of the fluorescent probe.

Despite this, we predict that this protocol will be commonly used by researchers in the field of fluorescent probes and bioimaging for the design and preparation of cyanine-based turn-on fluorescent probes to detect and image additional biological species in living cells and *in vivo*.

The specificity of CPDSA for GSH

It is possible that the high selectivity of the CPDSA probe for GSH is closely related to factors such as molecular structure, group confirmation, intermolecular weak interactions and so on. According to the investigation on theoretical calculations, the chair conformation of the piperazine ring and the distance of the S-N bond are different from those of other probes lacking selectivity for thiols. There are also structural differences between GSH and the other thiols that could account for the observed differences in reactivity. GSH contains two amide groups, and it has a longer flexible backbone compared with Cys and Hcy, which means that more intermolecular interactions with the CPDSA probe are possible. From the theoretically optimized structures, the amide units of GSH can form hydrogen bonds (such as C–H···N and C–H···O) with the piperazine ring unit of the CPDSA probe, and there may be an electrostatic interaction between the indolium cation of the CPDSA probe and the carbonate anion of GSH. These intermolecular interactions would result in the two reactive sites (SH group and sulfonamide group) being closer together, thereby explaining the selectivity of CPDSA for GSH. We also believe that this information will be useful for the design of highly selective fluorescent probes in the future.

Experimental design and crucial parameters

As described above, CPDSA is synthesized by treating dansyl chloride with CP, and CP is prepared by reacting commercially available IR-780 iodide with piperazine, as shown in **Figure 1a**. The two-step tractable synthetic procedure is performed on the 100-mg scale to produce CPDSA with ~65% yield, and one synthesis will result in enough CPDSA for many biological assays. Furthermore, this compound reveals excellent stability in the refrigerator, and it can be stored more than 1 year.

The procedure also includes example experiments using adherent mammalian cells (HeLa and RAW 264.7 cells) grown on coverslips and a series of bioimaging experiments in living cells that can be used to assess the cellular GSH fluorescence imaging capability of the CPDSA probe. Before the cell experiments, the activity of the probe should be checked by the measurement *in vitro*. We have confirmed experimentally that the activity of the probe is still very good after storage in a refrigerator for 1 year. Incubating probe CPDSA (20 μM) with living HeLa cells at 37 °C for 20 min induces an obvious red fluorescence emission inside the cells, which is visualized with a confocal fluorescence microscope and shown in **Figure 4a**,**b**. The next control experiment is performed using NMM as a thiol-blocking reagent. As shown in **Figure 5b**, much lower fluorescence is observed when the HeLa cells are first treated with NMM for 20 min, followed by incubation with CPDSA (20 μM) for 20 min. Subsequent addition of GSH reduced ethyl ester (GSH-MEE, 100 μM) in the solution and incubation for another 20 min results in a strong red emission in the same field of view shown in **Figure 4d**, whereas the addition of Cys or Hcy only shows minor changes (**Supplementary Fig. 2**). The result indicates that the CPDSA probe is fully cell-permeable and the excellent cell permeability is reflected in the selective GSH-detection experiment in HeLa cells.

It was important to determine the photostability of the CPDSA probe in living cells. The time-dependent fluorescence images of the cells were acquired with a Chamlide TC live-cell imaging system (Live Cell Instrument). This system creates the best conditions for imaging live cells, preserving the temperature (37 °C), humidity and 5% CO_2/95% O_2 air conditions. As we show in confocal microscopy images (**Supplementary Fig. 3**), the CPDSA probe still has a strong red emission 1 h later (**Supplementary Fig. 4**), indicating that it possesses good probe activity and that it is not subject to ambient interference. As mentioned above, the DMSO solution of the CPDSA probe can be kept for only 2 d at RT. However, this period is sufficient to perform the relevant bioimaging experiments.

The approaches described here have been demonstrated to be useful for monitoring the level of GSH in living cells[20]. As mentioned above, GSH has a crucial role in biological processes, such as in the maintenance of intracellular redox balance. Subsequent investigations were performed to monitor the oxidant-governed

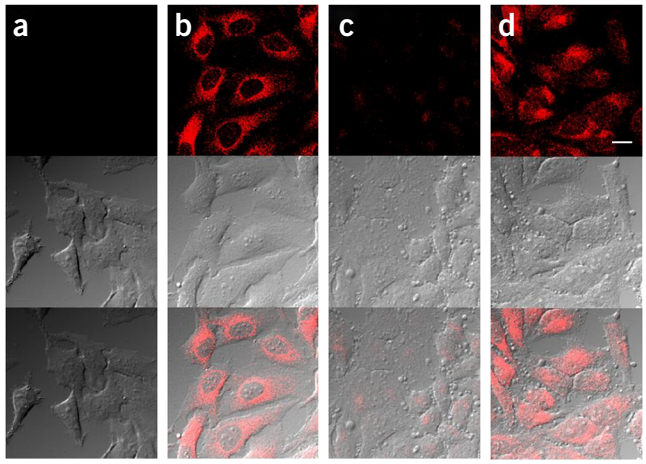

Figure 4 | Confocal microscopy images of CPDSA in HeLa cells. (The data were collected using a FV1200 instrument, Olympus) Cell images were obtained using an excitation wavelength of 635 nm and a band-pass (655–755 nm) emission filter. (**a**) Fluorescence image of HeLa cells. (**b**) Fluorescence image of HeLa cells incubated with the CPDSA probe (20 μM) for 20 min. (**c**) Fluorescence image of HeLa cells pretreated with NMM (1 mM) for 20 min and incubated with the CPDSA probe (20 μM) for 20 min. (**d**) Fluorescence image of HeLa cells pretreated with NMM (1 mM) for 20 min, treated with GSH-MEE (100 μM) for 20 min and then incubated with the CPDSA probe (20 μM) for 20 min. Scale bar, 15 μm. (**a**–**d**) Top, fluorescence images of HeLa cells; middle, bright-field images; bottom, merged images.

© 2015 Nature America, Inc. All rights reserved.

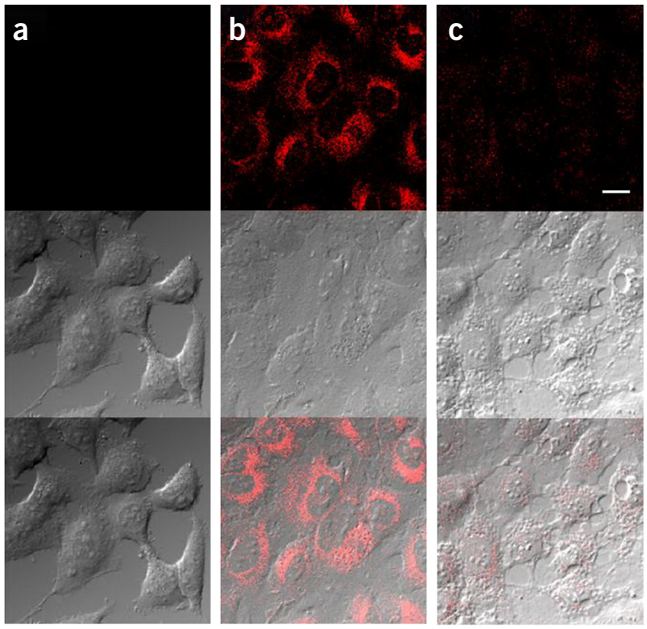

Figure 5 | Confocal microscopy images of the CPDSA probe in HeLa cells. (The data were collected using a FV1200 instrument, Olympus.) Cell images were obtained using an excitation wavelength of 635 nm and a band-pass (655–755 nm) emission filter. (**a**) Fluorescence image of HeLa cells. (**b**) Fluorescence image of HeLa cells incubated with the CPDSA probe (20 μM) for 20 min. (**c**) Fluorescence image of HeLa cells pretreated with H_2O_2 (100 μM) for 20 min and incubated with the CPDSA probe (20 μM) for 20 min. Scale bar, 15 μm. (**a**–**c**) Top, fluorescence images of HeLa cells; middle, bright-field images; bottom, merged images.

redox status of GSH in living cells. As shown in the confocal fluorescence microscopy images in **Figure 5**, the addition of H_2O_2 (100 μM) to a solution containing HeLa cells and probe CPDSA (20 μM) induces an obvious quenching of the fluorescence. This result seems to be the consequence of a decrease in the intracellular GSH concentration through GSH oxidation into GSSH promoted by H_2O_2. Therefore, this result suggests that the CPDSA probe might be capable of monitoring the redox state in living cells by specifically detecting the level of intracellular GSH.

LPS, the primary constituent of the outer membrane of Gram-negative bacteria, is reported to be responsible for immune stimulation by generating reactive oxygen species such as H_2O_2 in phagocytic cells[44,45]. Thus, we investigated whether the CPDSA probe could monitor alterations in GSH concentration caused by LPS treatment in phagocytic cells. As shown in **Figure 6**, a marked fluorescence decrease occurs when RAW 264.7 phagocytic cells are treated with LPS before treatment with CPDSA, suggesting that CPDSA might have the capacity to monitor the decrease in GSH concentration mediated by LPS treatment in immune cells.

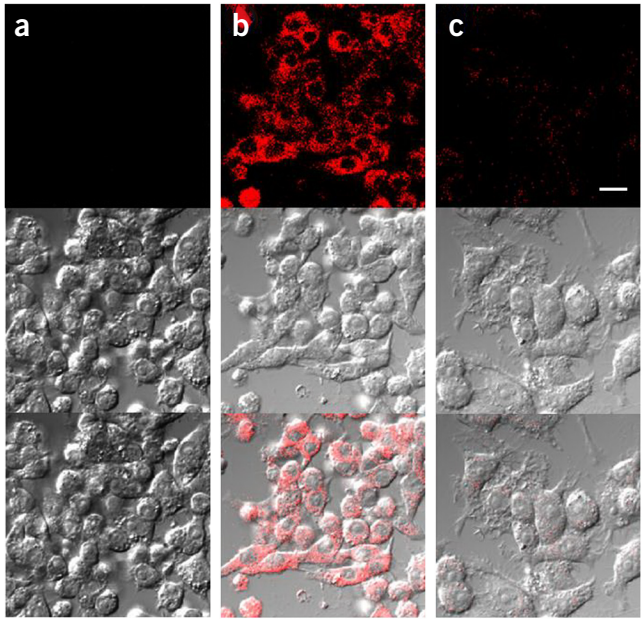

Figure 6 | Confocal microscopy images of probe CPDSA in RAW 264.7 cells. (The data were collected using a FV1200 instrument, Olympus.) Cell images were obtained using an excitation wavelength of 635 nm and a band-pass (655–755 nm) emission filter. (**a**) Fluorescence image of RAW 264.7 cells. (**b**) Fluorescence image of RAW 264.7 cells incubated with CPDSA (20 μM) for 20 min. (**c**) Fluorescence image of RAW 264.7 cells pretreated with lipopolysaccharide (1 μg/ml) for 16 h, IFN-γ (50 ng/ml) for 4 h and PMA (10 nM) for 30 min before incubating with the CPDSA probe (20 μM) for 20 min. Scale bar, 15 μm. (**a**–**c**) Top, fluorescence images of HeLa cells; middle, bright-field images; bottom, merged images.

Neutrophils, which are produced in the bone marrow and circulate in the blood, are the primary white blood cells that protect the host from bacterial infection[46]. Upon bacterial infection, neutrophils take up bacteria and produce reactive oxygen

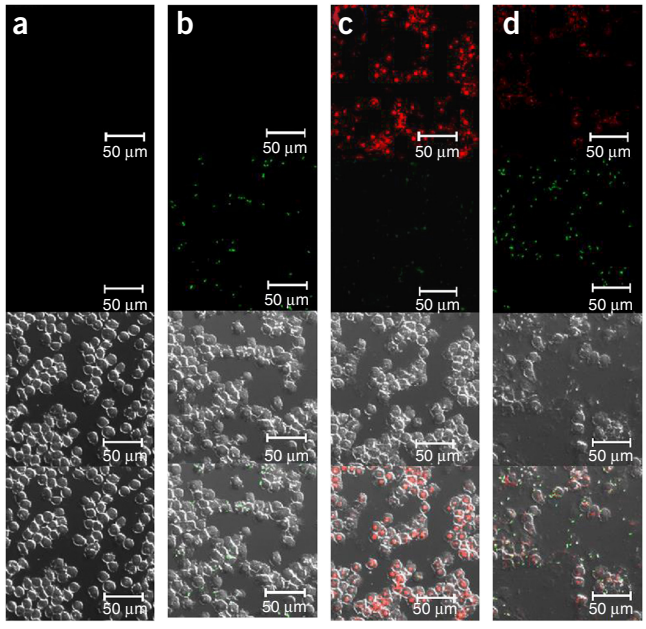

Figure 7 | Confocal microscopy images of probe CPDSA in mouse bone marrow–derived neutrophils. (The data were collected using a LSM780 instrument, Carl Zeiss.) Cell images for probe CPDSA (red) were obtained using an excitation wavelength of 633 nm and a band-pass (639–758 nm) emission filter. Cell images for PAO1-GFP bacteria (green) were obtained using an excitation wavelength of 488 nm, and the emission wavelength was 500–588 nm. (**a**) Fluorescence image of neutrophils without treatment. (**b**) Fluorescence image of neutrophils treated with 5×10^6 PAO1-GFP bacteria only for 30 min. (**c**) Fluorescence image of neutrophils treated with only CPDSA (10 μM) for 30 min. (**d**) Fluorescence image of neutrophils incubated with both PAO1-GFP 5×10^6 and CPDSA (10 μM) for 30 min. (**a**–**d**) First and second rows, fluorescence images of neutrophils; third row, bright-field images; fourth row, merged images.

© 2015 Nature America, Inc. All rights reserved.

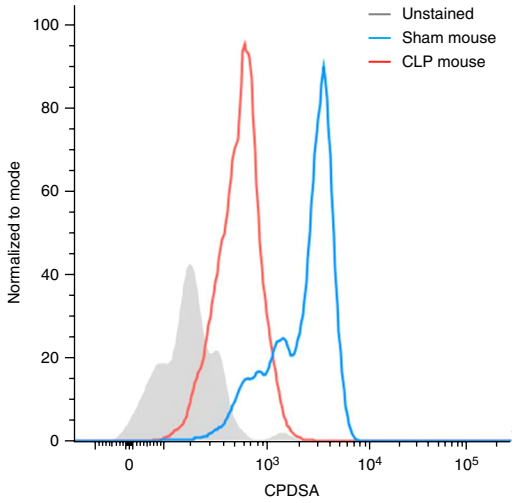

Figure 8 | Flow cytometry analysis images of the CPDSA probe in peritoneal neutrophils derived from the CLP mouse model. Flow cytometry image for the CPDSA probe was obtained using an excitation wavelength of 640 nm and a band-pass (690–750 nm) emission filter. Ly-6G–positive neutrophils of peritoneal cells derived from CLP mouse (red line); Ly-6G–positive neutrophils of peritoneal cells derived from sham mouse (blue line). The unstained group is filled in gray. The *x* axis shows the intensity of the detected CPDSA probe and the *y* axis means the number of neutrophils (normalized to mode). FlowJo software was used for the analysis of data. The graphs of the number of neutrophils were normalized to the mode to depict the data in terms of '% of max'. The % of max denotes the number of cells in each bin (the numerical ranges for the parameter on the *x* axis) divided by the number of cells in the bin that contains the largest number of cells. FlowJo uses 256 bins, and each graph was scaled to the percentage of its maximum bin.

species such as H_2O_2 and ClO^- to kill them[47]. Thus, we investigated whether the CPDSA probe could detect the alteration of GSH concentration in BMDNs caused by infection with GFP–tagged *Pseudomonas aeruginosa* (strain PAO1), a common pathogen associated with airway infection in clinical settings. Ingested GFP-tagged PAO1 was observed in BNDNs after infection in the absence of CPDSA (**Fig. 7b**). Confocal microscopy images revealed an obvious red emission when BMDNs were treated with the CPDSA probe without GFP-tagged PAO1 infection (**Fig. 7c**). Interestingly, the intensity of red emission from the CPDSA probe decreased substantially when GFP-tagged PAO1 was ingested in BMDNs (**Fig. 7d**). This result implies that CPDSA could monitor the decrease of GSH concentration caused by PAO1 infection in BMDNs.

The results obtained at the cellular level suggest that the CPDSA probe can be used to detect GSH in the *in vivo* mouse model. To evaluate this application, we examined the probability of using the CPDSA probe for GST detection in peritoneal neutrophils extracted from a sepsis mouse model such as cecal ligation puncture (CLP). Flow cytometry imaging of the CPDSA probe showed that GSH detection decreased in peritoneal neutrophils extracted from the CLP model, compared with peritoneal neutrophils from the control mouse (sham mouse; **Fig. 8**). These results suggest that the CPDSA probe may be used to monitor systemic inflammatory diseases such as sepsis through specific detection of GSH in neutrophils *in vivo*.

The GSH-selective probe described here is useful for monitoring the level of GSH in living cells and *in vivo*, which is helpful for exploring and understanding some of the biological processes and cellular functions related to GSH. More importantly, the cleavage product CP is a fluorescence turn-on fluorophore. Therefore, it is applicable to designing novel NIR fluorescent probes that are aimed toward other species for bioimaging in cells and tissues of mice. We believe that this protocol will also be useful to researchers wanting to prepare NIR cyanine-based fluorescent probes for other analytes[20,27–42]. Despite numerous data indicating that the CPDSA probe is an efficient tool for monitoring the level of GSH in cells and *in vivo*, it remains challenging to obtain quantitative information on the concentration of GSH in cells and *in vivo*.

© 2015 Nature America, Inc. All rights reserved.

MATERIALS

REAGENTS

! CAUTION All chemicals and reactions used for the synthesis of CPDSA in this protocol are potentially harmful. We recommend performing reactions in a fume hood, and thus gloves, a lab coat and other protective equipment should be used. The solid and liquid waste should be disposed of appropriately, according to relevant local and institutional guidelines.

- Distilled water (DW)
- IR-780 iodide (Sigma-Aldrich, cat. no. 425311)
- Anhydrous *N*,*N*-dimethyl formamide (DMF; Sigma-Aldrich, cat. no. 227056)
- Piperazine, anhydrous, ≥99.0% (Sigma-Aldrich, cat. no. 80621)
- Dansyl chloride (Sigma-Aldrich, cat. no. D2625)
- Anhydrous dichloromethane (Sigma-Aldrich, cat. no. 270997)
- Triethylamine (Sigma-Aldrich, cat. no. T0886)
- Dichloromethane for chromatography (Duksan Chemicals)
- Methanol for chromatography (Duksan Chemicals)
- HEPES (Invitrogen, cat. no. 15630-056)
- DMSO (Sigma-Aldrich, cat. no. 276855)
- L-Glutathione reduced BioXtra, ≥98.0% (Sigma-Aldrich, cat. no. G6529)
- Argon cylinder (Dong-A industrial gases)
- HeLa cell line (KCLB 10002) and RAW 264.7 cell line (KCLB 40071; HeLa cells and RAW 264.7 cells were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin and 100 U/ml streptomycin. All cells were maintained in an incubator at 37 °C and 5% CO_2)
- DMEM (high glucose; HyClone, cat. no. SH30243.01)
- FBS (HyClone, cat. no. SH30919. 03)
- Trypsin-EDTA solution (Life Technologies, cat. no. 25200-056)
- Penicillin/streptomycin, 100× (Life Technologies, cat. no. 15140-122)
- Dulbecco's PBS, 1× sterile solution (DPBS, pH 7.4; Welgene, cat. no. LB001-02)
- Lipopolysaccharide (Sigma-Aldrich, cat. no. L4391)
- Murine interferon-γ (PeproTech, cat. no. 315-05)
- Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, cat. no. P8139)
- *N*-Methylmaleimide (Acros Organics, cat. no. 12708)
- Hydrogen peroxide (Reagents Duksan, cat. no. 3059)
- Glutathione reduced ethyl ester (GSH-MEE, Sigma-Aldrich, cat. no. G1404)
- RPMI-1640 (with 25 mM HEPES and L-glutamine; Lonza, cat. no. 12-115F)
- FBS (Gibco, cat. no. 16000-044)
- PBS, pH 7.4 (Lonza, cat. no. 17-516F)
- Neutrophil isolation kit (Miltenyi Biotec, cat. no. 130-097-658)

- Anti-mouse Ly-6G (Gr-1) eFluor450 (eBiosciences, cat. no. 45-5931-32)
- LB agar plate
- LB broth
- Zoletil (Virbac)
- Rompun (Bayer Korea)

Animals

- C57B/L6N mice (male, 8 weeks): all mice used in this study were males 8–10 weeks old, and they were maintained in our animal facilities under specific, pathogen-free conditions. All animal experiments were performed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care using protocols approved by the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine **! CAUTION** All experiments involving animals must be performed in accordance with all institutional and governmental regulations and guidelines.

EQUIPMENT

- Rotary evaporator (Eyela)
- NMR spectrometer (Bruker AM-300 spectrometer)
- Fluorescence spectrophotometer (Shimada RF-5301/PC)
- Mass spectrometer (MALDI-TOF)
- Mass spectrometer (electrospray ionization (ESI))
- UV-visible spectrophotometer (Scinco 3000)
- Hot-plate magnetic stirrer with contact thermometer (Corning, PC-420)
- Balance (Shimadzu, AX 200)
- Handheld UV lamp (Tank, TN-4LC)
- Rubber septum (Sigma-Aldrich, cat. no. Z553964)
- Disposable glass Pasteur pipette (Hilgenberg, 150 mm)
- Disposable glass Pasteur pipette (Hilgenberg, 230 mm)
- Pipette bulb (Sigma-Aldrich, cat. no. Z136069)
- Pyrex one-necked round-bottomed flask, 50 and 100 ml
- Pyrex two-necked round-bottomed flask, 50 and 100 ml
- Conical beaker, 50 and 250 ml
- Reflux condenser
- Oil bath
- Pyrex separatory funnel
- Pyrex chromatography column (Changyoung)
- Disposable syringe (Top-point)
- Syringe needle (Hamilton, cat. no. 81320/81000)
- Mercury-free thermometer (Sigma-Aldrich, cat. no. Z561541)
- Teflon-coated magnetic stirring bar (Sigma-Aldrich, cat. no. Z126969)
- NMR tube (Sigma-Aldrich, cat. no. NORS55008)
- Silica gel, 230–400 mesh (Merck, cat. no. TA1363285)
- Silica gel 60 F254 thin-layer chromatography (TLC) plate (Merck, cat. no. OB549444)
- Confocal microscope (FV1200, Olympus)
- Cover glass bottom dish (35 mm; SPL, cat. no. 100350)
- Hemocytometer (Superior, cat. no. HSU-1401)
- CO_2 incubator for cell culture (Sanyo, MCO-18AIC)
- Clean bench (Kwang Dong Industrial, cat. no. KD-CB 144)
- Centrifuge (Hanil Science Industrial, no. FLETA5)
- Centrifuge tube (15 ml; SPL, cat. no. 50015)
- Cell culture dish (100 mm; Corning, cat. no. 430167)
- Serological pipette, 10 ml (Falcon, cat. no. 357551)
- Cell scraper (SPL, cat. no. 90020)
- Pipet-Aid (Falcon, cat. no. Express 357590)
- Confocal microscope (LSM780, Carl Zeiss)
- Tissue culture plate (12 well; Falcon, cat. no. 353043)
- Hemocytometer (Marienfeld-Superior, cat. no. PM0630030)
- CO_2 Incubator for cell culture (HERAcell 150, Thermo Scientific)
- Incubator for microbes (BioFree, cat. no. BF-150IN)
- Shaking incubator (BioFree, cat. no. BF-50SIR)
- Clean bench (Labconco, class II type A2)
- Centrifuge (TOMY, Flex-Spin LC-200)
- Centrifuge (Eppendorf, 5415R)
- Centrifuge tube (15 ml; SPL, cat. no. 50015)
- Centrifuge tube (50 ml; SPL, cat. no. 50050)
- Cytospin (Thermo Scientific, Cytospin 4)
- Cytospin accessory (Shandon Cytoclip Slide Clip, TPX sample chambers and filter cards; Thermo Scientific)
- Spectrometer (PerkinElmer, UV-visible spectrometer Lambda 25)
- Tube, 1.5 ml (Sarstedt, cat. no. 72.690)
- Serological pipette, 10 ml (SPL, cat. no. 91010)
- Pipet-Aid (Drummond Scientific, cat. no. 4-000-201-E)
- Syringe, 1 ml (Korea Vaccine)
- Syringe, 5 ml (Korea Vaccine)
- Nonabsorbable suture (AILEE, cat. no. sk434)
- BD FACSVerse (BD Biosciences)
- autoMACS pro separator (Miltenyi Biotec, cat. no. 130-092-545)

REAGENT SETUP

Culture medium Culture medium is DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. This medium should be stored at 4 °C until the expiration date (8–10 months).

NMM, 1 M Dissolve 111.1 mg of NMM in 1 ml of DMSO. NMM powder is stored at RT. Always freshly prepare NMM solution.

Hydrogen peroxide 100 mM Dissolve 10.9 µl of hydrogen peroxide solution in 1 ml of distilled water (DW). Always freshly prepare hydrogen peroxide.

LPS, 1 mg/ml Dissolve 1 mg of LPS in 1 ml of DW. It is stored at −20 °C for ~6 months.

Interferon gamma (IFN-γ), 50 µg/ml Dissolve 50 µg of IFN-γ in 1 ml of DW. It is stored at −20 °C for ~6 months.

Phorbol 12-myristate 13-acetate, 10 µM To make 10 mM phorbol 12-myristate 13-acetate (PMA), dissolve 6.2 mg of PMA in 1 ml of DMSO. To make 10 µM PMA, dilute 1,000× in DMSO and store it at −20 °C for ~6 months.

GSH-MEE, 100 mM Dissolve 33.5 mg of GSH-MEE in 1 ml of DW. This should be freshly prepared.

Cell isolation medium Cell isolation medium is RPMI-1640 supplemented with 2.5% FBS, without penicillin and streptomycin. This medium is stored at 4 °C for ~6 months.

Microbe infection medium Microbe infection medium is RPMI-1640 supplemented with 10% (vol/vol) FBS, without penicillin and streptomycin. This medium is stored at 4 °C for ~6 months.

Antibody-binding buffer Antibody-binding buffer is PBS supplemented with 3% (vol/vol) FBS. This buffer is stored at 4 °C for ~6 months.

Gey's solution Dissolve 4.15 g of ammonium chloride and 0.5 g of potassium bicarbonate in 500 ml of deionized water. Adjust the pH to 7.2, and filter the solution with a 0.22-µM filter. This solution is stored at 4 °C for ~6 months.

Preparation for microbial infection with PAO1-GFP (GFP-tagged *P. aeruginosa* PAO1) First, 2 d before the experiment, streak PAO1-GFP on an LB agar plate and incubate it overnight (12–14 h) at 37 °C in a no-CO_2 incubator. The next day, pick one colony and culture it in 10 ml of LB broth at 37 °C in a shaking incubator overnight. On the day of the experiment, transfer 100 µl of the cultured PAO1-GFP to a fresh 10-ml LB broth and culture it in a shaking incubator for 2 h. Measure the optical density (OD) using a UV-visible spectrometer at 600 nm. An OD_{600} of 1.0 indicates 1×10^9 PAO1 bacteria per 1 ml of LB broth.

EQUIPMENT SETUP

Confocal microscopy in live cells The fluorescence of the CPDSA probe in live cells can be visualized by confocal microscopy (FV1200, Olympus), with excitation at 635 nm using a diode laser (wavelength: 635 nm) and emission at 655–755 nm.

Confocal microscopy in BMDNs The fluorescence of the CPDSA probe in BMDNs can be visualized by confocal microscopy with excitation at 633 nm using a laser (wavelength: 633 nm) and emission at 639–758 nm.

Flow cytometry Peritoneal cells derived from sepsis mice can be gated into Ly-6G–positive neutrophils. The CPDSA probe in Ly-6G–positive neutrophils can then be detected by FACS analysis with excitation at 640 nm using a laser (wavelength: 640 nm) and emission at 690–750 nm.

© 2015 Nature America, Inc. All rights reserved.

PROCEDURE

Synthesis of compound CP ● TIMING 10 h

1| Connect a 25-ml two-necked round-bottomed flask containing an egg-shaped Teflon-coated magnetic stirring bar to a reflux condenser.

2| Weigh out 134 mg of IR-780 iodine (0.2 mmol, 1.0 equiv.) and 69 mg of piperazine (0.8 mmol, 4.0 equiv.), and add them to the flask.

3| Seal the apparatus with rubber septa, and fill the flask with argon gas using standard Schlenk techniques[20].

4| Add 10 ml of anhydrous *N*, *N*-dimethyl formamide to the mixture in the flask using a 12-ml syringe.

5| Securely attach the flask with a clamp into an RT oil bath placed on a temperature-controlled hot plate with a magnetic stirrer.

6| Heat the oil bath to 85 °C while stirring on the magnetic stirrer, and stir it for 4 h in the dark by covering it in aluminum foil and switching off the light in the fume hood, or by performing the reaction at night and switching off all the lights in the laboratory.

7| Turn-off the heating plate and allow the reaction to cool to RT.

8| Once the mixture is completely cool, remove the flask from the oil bath, carefully remove the cap and switch off the water supply to the condenser.

9| Remove the solvent via rotary evaporation by heating the mixture to 40 °C under reduced pressure. A blue solid should appear in the flask.

10| Purify the reaction product by silica column chromatography. Pack a silica gel column with a column diameter of 2 cm and a silica gel column height of 15 cm using dichloromethane.

11| Dissolve the crude product obtained from Step 9 in 0.5 ml of dichloromethane, and then load the resulting solution onto the silica gel bed (the height of the solution on the top of the silica gel bed will be ~0.5 cm).

12| Perform column chromatography purification using a 1:40 (vol/vol) mixture solution of methanol and dichloromethane as eluent. Monitor the collected fractions by silica gel TLC with a 1:40 (vol/vol) solution of methanol and dichloromethane eluent. The compound can be visualized as a blue spot (retardation factor R_f = 0.2) in silica gel TLC with UV absorbance at 254 nm. Collect those fractions containing the desired product and remove the solvent using a rotary evaporator at RT under reduced pressure to get 136 mg of compound CP as a blue solid (85% yield) as eluent.
? TROUBLESHOOTING

13| Establish the identity and purity of the compound CP by proton NMR spectroscopy (^{1}H NMR), carbon NMR spectroscopy (^{13}C NMR) and electrospray ionization MS (ESI-MS).
? TROUBLESHOOTING
■ **PAUSE POINT** CP can be stored in the dark at 4 °C for at least 12 months.

Synthesis of compound CPDSA ● TIMING 10 h

14| Assemble a 50-ml two-necked round-bottomed flask, a 10-ml pressure-equalized dropping funnel and an egg-shaped Teflon-coated magnetic stirring bar.

15| Weigh out 143 mg of compound CP (0.2 mmol, 1.0 equiv.) and add it to the two-necked round-bottomed flask.

16| Seal the apparatus with rubber septa, and fill the flask with argon gas using standard Schlenk techniques.

17| Weigh out 30 mg of triethylamine (0.3 mmol, 1.5 equiv.) and add it to 10 ml of anhydrous dichloromethane. Add the resulting solution to the flask using a 12-ml syringe.

© 2015 Nature America, Inc. All rights reserved.

18| Stir the mixture on a magnetic stirrer for 5 min at RT.

19| Weigh out 54 mg of dansyl chloride (0.2 mmol, 1.0 equiv.) and add it to 5 ml of anhydrous dichloromethane. Add the solution to a pressure-equalized dropping funnel using a 10-ml syringe.

20| Place the flask in an ice-water bath, and then add the dansyl chloride solution dropwise to the reaction mixture over 30 min.

21| Remove the flask from the ice-water bath and allow the reaction mixture to warm to RT.

22| Stir the reaction for an additional 4 h at RT, and then remove the solvent by rotary evaporation at RT under reduced pressure. A blue solid should appear in the flask.

23| Purify the reaction product by silica column chromatography. Pack a silica gel column with a column diameter of 2 cm and a silica gel column height of 15 cm using dichloromethane.

24| Dissolve the crude product obtained from Step 22 in 0.5 ml of dichloromethane, and then load the resulting solution onto the silica gel bed (the height of the solution on the top of the silica gel bed will be ~0.5 cm).

25| Perform column chromatography purification using a 1:30 (vol/vol) solution mixture of methanol and dichloromethane as eluent. Monitor the fractions by silica gel TLC with a 1:30 (vol/vol) solution of methanol and dichloromethane as eluent. Collect the fractions containing the desired product; CPDSA can be visualized as a blue spot (retardation factor R_f = 0.2) with UV absorbance at 254 nm. Remove the solvent by using a rotary evaporator at RT under reduced pressure to get 156 mg of CPDSA as a blue solid (82% yield).
? TROUBLESHOOTING

26| Establish the identity and purity of CPDSA by proton NMR spectroscopy (^{1}H NMR), carbon NMR spectroscopy (^{13}C NMR) and MALDI-TOF–MS.
? TROUBLESHOOTING
■ **PAUSE POINT** CPDSA can be stored in the dark at 4 °C for at least 6 months.

Experiments in cells and *in vivo*

27| CPDSA can now be used for experiments in cells and *in vivo* (**Fig. 2**). Perform the steps as described in the relevant options described below. Options A–D are procedures for bioimaging in cells, whereas options E and F list procedures for bioimaging *in vivo*.

(A) Intracellular GSH detection in HeLa cells with probe CPDSA ● TIMING 52 h

(i) Incubate HeLa cells in a 100-mm culture dish containing 10 ml of culture medium in an incubator with 5% CO_2 at 37 °C for 24 h.
(ii) Remove the culture medium from the cell culture and wash the cells with 5 ml of Dulbecco's PBS (DPBS).
(iii) Add 1 ml of trypsin-EDTA solution to the cells and incubate the cells at 37 °C until the cell layer is detached from the dish (usually 2–3 min).
(iv) Add 4 ml of culture medium to the dish to stop the trypsin digestion reaction, and then mix the cell solution and transfer it to a 15-ml centrifuge tube.
(v) Centrifuge the cells at 300*g* for 5 min at 25 °C, and then discard the supernatant.
(vi) Suspend the cell pellet in culture medium and count the number of cells using a hemocytometer.
(vii) Seed the cells in 35-mm glass-bottomed dishes at a density of 3 × 10^5 cells per dish in 2 ml of culture medium and incubate them inside an incubator containing 5% CO_2. Medium volume of 2 ml and tests are accomplished with three groups. At least three tests are recommended.
■ **PAUSE POINT** Incubate the cells for 24 h at 37 °C. Cells will attach to the glass surface during this time.
(viii) Treat the first group of HeLa cells with 20 μM CPDSA probe (final 0.6% (vol/vol) DMSO) for 20 min at 37 °C in a CO_2 incubator. Treat the second group of HeLa cells with 1 mM NMM (a compound that binds to thiols) for 20 min before washing them twice with DPBS and treating them with 20 μM CPDSA for 20 min at 37 °C. Treat the third group of HeLa cells with 1 mM NMM for 20 min before washing them twice with DPBS probe CPDSA for 20 min at 37 °C.
(ix) After washing with DPBS, fluorescence images can be acquired by confocal microscopy with excitation at 635 nm using a diode laser as a light source. The emission wavelength will be 655–755 nm.
? TROUBLESHOOTING

© 2015 Nature America, Inc. All rights reserved.

(B) Discrimination between GSH and other thiols in the cell ● TIMING 3 h

(i) Prepare cells as described in Step 27A(i–iii). For this experiment, the cells need to be divided into six treatment groups, and fluorescence images are obtained for each of these. Results for three separate experiments should be collected.

1	Untreated HeLa cells
2	HeLa cells incubated with CPDSA (20 μM) for 20 min
3	HeLa cells pretreated with NMM (1 mM) for 20 min and incubated with CPDSA (20 μM) for 20 min
4	HeLa cells pretreated with NMM (1 mM) for 20 min; Cys (100 μM) is then added and cells are incubated with CPDSA (20 μM) for 20 min
5	HeLa cells pretreated with NMM (1 mM) for 20 min; Hcy (100 μM) is then added and cells are incubated with CPDSA (20 μM) for 20 min
6	HeLa cells pretreated with NMM (1 mM) for 20 min; GSH-MEE (100 μM) is then added and cells are incubated with CPDSA (20 μM) for 20 min

(ii) To discriminate GSH from Cys and Hcy, treat HeLa cells with 1 mM NMM for 20 min, wash them twice with DPBS and treat them with 100 μM GSH-MEE, Cys and Hcy for 20 min. Wash them with DPBS and then treat them with 20 μM probe CPDSA for 20 min at 37 °C.

(iii) After washing with DPBS, acquire fluorescence images by confocal microscopy.

? TROUBLESHOOTING

(C) GSH difference by oxidative stress in the cell ● TIMING 2 h

(i) Prepare the cells as described in Step 27A(i–iii). For this experiment, the cells need to be divided into three treatment groups, and fluorescence images are obtained for each of these. Results for three separate experiments should be collected.

1	Untreated HeLa cells
2	HeLa cells incubated with probe CPDSA (20 μM) for 20 min
3	HeLa cells pretreated with H_2O_2 (100 μM) for 20 min and incubated with probe CPDSA (20 μM) for 20 min

(ii) To detect the difference in GSH with oxidative stress, treat HeLa cells with or without 100 μM H_2O_2 for 20 min. Wash the cells with DPBS and incubate them with 20 μM CPDSA for 20 min at 37 °C. After washing with DPBS, fluorescence images can be acquired by confocal microscopy.

? TROUBLESHOOTING

(D) Observation of endogenous GSH level in RAW 264.7 cells ● TIMING 80 h

(i) Incubate RAW 264.7 cells for 24 h in a 100-mm culture dish containing 10 ml of culture medium in an incubator containing 5% CO_2 at 37 °C.

(ii) Remove the culture medium from the cell culture and wash the cells with 5 ml of DPBS.

(iii) Add 5 ml of culture medium to the cells, detach the cells using a cell scraper, and then transfer the cell solution to a 15-ml centrifuge tube.

(iv) Centrifuge the cells at 300*g* for 5 min at 25 °C, and then discard the supernatant.

(v) Suspend the cell pellet in culture medium and count the cell number using a hemocytometer.

(vi) Seed cells in 35-mm glass-bottomed dishes at a density of 2×10^5 cells per dish in culture medium and incubate them inside an incubator containing 5% CO_2 for all experiments. Medium volume of 2 ml and tests are accomplished with three groups.

■ **PAUSE POINT** Incubate the cells for 24 h at 37 °C. Cells will attach to the glass surface during this time.

(vii) Treat RAW 264.7 cells with 1 μg/ml LPS for 16 h, 50 ng/ml IFN-γ for 4 h and 10 nM PMA for 30 min at 37 °C.

(viii) After washing with DPBS, treat the cells with 20 μM CPDSA probe for 20 min at 37 °C, wash them with DPBS and acquire fluorescence images using confocal microscopy.

? TROUBLESHOOTING

© 2015 Nature America, Inc. All rights reserved.

(E) GSH detection in mouse BMDNs by PAO1-GFP infection ● TIMING 5 h

(i) Acquire bone marrow from the long bones of mice. Cut out the four long bones (two femurs and two tibias) from one mouse, and then remove the muscles. Place the bones into cell isolation medium to prevent them from drying out. It typically takes at least 20 min to complete this step. The amount of bone marrow from the bones of a single mouse is sufficient to complete this protocol. However, it is recommended to have at least five replicates to obtain meaningful results.
! CAUTION All experiments involving animals must be performed in accordance with all institutional and governmental regulations and guidelines.

(ii) Cut the ends of the bones. Flush the bone marrow into a 50-ml centrifuge tube with cell isolation medium using a 1-ml syringe. Repeat this step two or three times.

(iii) Add cell isolation medium up to 50 ml, centrifuge the cells at 400*g* for 5 min and discard the supernatant.

(iv) Suspend the cell pellet in 3 ml of Gey's solution and 1 ml of PBS. After 1 min, add 45 ml of PBS to the tube to stop the red blood cell lysis reaction, and then filter the cell solution in a 40-μm cell strainer.

(v) Centrifuge the cells at 300*g* for 10 min at 25 °C, and then discard the supernatant.

(vi) (Use the neutrophil isolation kit for Step 27E(vi–x).) Suspend the cell pellet in 200 μl of antibody-binding buffer and transfer the cell solution to a 15-ml tube.

(vii) Treat the cells with 25 μl of neutrophil biotin-antibody cocktail for 10 min at 4 °C.

(viii) Add 10 ml of antibody-binding buffer, centrifuge the cells at 300*g* for 10 min at 25 °C and discard the supernatant.

(ix) Suspend the cell pellet in 200 μl of antibody-binding buffer and treat the cells with 50 μl of anti-biotin microbeads. Incubate the cells for 15 min at 4 °C (tapping every 2–3 min).

(x) Wash the cells by adding 10 ml of antibody-binding buffer and centrifuge them at 300*g* for 10 min at 4 °C. Aspirate the supernatant.

(xi) Resuspend the cells in 2 ml of antibody-binding buffer and separate the neutrophils using the autoMACS Pro (usually 20–30 min).

(xii) Count and seed the cells in microbe infection medium in a 12-well tissue culture plate at a density of 5×10^5 per 1 ml.

(xiii) Treat the first group of neutrophils with 5×10^6 PAO1-GFP for 30 min at 37 °C in a CO_2 incubator. Treat the second group of neutrophils with 10 μM CPDSA probe for 30 min. Treat the third group of neutrophils with 5×10^6 PAO1-GFP and 10 μM CPDSA probe for 30 min at 37 °C. Do not treat the control group.

(xiv) After incubation, collect the medium in a 1.5-ml tube and spin down the cells to a pellet at 200*g* for 10 min at 4 °C. Discard the supernatant.

(xv) Resuspend the cells in 500 μl of PBS. Centrifuge 200 μl of the resuspended cells using a Cytospin 4 at 100*g* for 5 min at 4 °C.

(xvi) Acquire fluorescence images by confocal microscopy with excitation at 488 nm using a laser as a light source. The emission wavelength for PAO1-GFP should be 500–588 nm. For the CPDSA probe, a 633-nm excitation laser can be used as a light source, and the emission wavelength should be 639–758 nm.

(F) GSH detection of peritoneal neutrophils derived from sepsis mouse model ● TIMING 10 h 30 min

(i) The first step is to make a CLP sepsis mouse model using an 8-week-old C57B/L6N mouse. In our laboratory, we use Zoletil and Rompun 2% for anesthesia. Zoletil contains 2.5 mg/ml tiletamine and 2.5 mg/ml zolazepam. We use 60 μl of this solution and 40 μl of 2% Rompun solution. Next, Zoletil and Rompun are diluted with 900 μl of PBS. Each mouse is anesthetized with 150 μl of the diluted solution. To make the sepsis model, open the abdomen and remove the cecum. About 1 cm from the end, ligate the mouse cecum and puncture it three times with a 1-ml syringe needle. Stow the cecum in the abdomen of the mouse and suture the mouse skin. For the control mouse (sham mouse), open the abdomen and remove the cecum, and then stow the cecum in the abdomen of the mouse and suture the mouse skin without puncturing the cecum.
! CAUTION All experiments involving animals must be performed in accordance with all institutional and governmental regulations and guidelines.

(ii) After 8 h, inject the sham mouse and CLP mouse with 4 ml of cell isolation medium, and collect peritoneal fluids, which include peritoneal cells, in a 15-ml centrifuge tube. Repeat this step two or three times.

(iii) Spin down to a pellet at 400*g* for 5 min at 4 °C, and then decant the supernatant.

(iv) Resuspend the cell pellet in 1 ml of antibody-binding buffer, and transfer the cell solutions to a 1.5-ml tube.

(v) Spin down the cells at 200*g* for 5 min at 4 °C, and then discard the supernatant.

(vi) Resuspend the cell pellets in 150 μl of buffer. Use 50 μl of cell solutions for the unstained group.

(vii) For the unstained group, peritoneal cells derived from CLP mouse are not treated. Treat peritoneal cells derived from the sham mouse or the CLP mouse with anti-mouse Ly-6G and 5 μM CPDSA. Treat with both Ly-6G antibody and CPDSA probe for 30 min at RT in the dark.

(viii) Wash the cells with 1 ml of antibody-binding buffer. Spin down the cells at 200*g* for 5 min at RT and discard the supernatant.

© 2015 Nature America, Inc. All rights reserved.

(ix) Resuspend the cell pellet in 200 µl of antibody-binding buffer. Analyze by flow cytometry using a 405-nm excitation laser as a light source and an emission wavelength of 443–533 nm for Ly-6G. For the CPDSA probe, a 640-nm excitation laser can be used as a light source and the emission wavelength should be 690–750 nm.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12,25	The product sticks to the column	Cyanine-based compounds are very polar	Run the column as quickly as possible
13,26	Reaction fails or there are many by-products	IR-780 iodine could have partially decomposed, or the photostability of CP is not good. There may also be oxygen or water in the reaction	The starting material IR-780 iodine should be stored in a refrigerator (<4 °C). Make sure that the reaction is set up in air-free conditions and make sure that all reagents are as dry as possible. Avoid photo-irradiation
27A(ix),27B(iii), 27C(ii),27D(viii)	Low fluorescence is observed	Decreased probe activity	The stock probe solution should be stored at –20 °C. Protect the probe from light. Use a freshly prepared solution. Avoid photo-irradiation
	High fluorescence is observed	CPDSA may partially decompose and form fluorescent CP	The stock probe solution should be stored at –20 °C. Protect the probe from light. Use a freshly prepared solution. Avoid photo-irradiation

● TIMING

With appropriate precursors in hand, the synthesis and purification of CPDSA is anticipated to require 2 d. Bioimaging experiments in cells and *in vivo* are anticipated to require 12–13 d
Steps 1–13, synthesis of CP: 10 h, plus purification
Steps 14–26, synthesis of CPDSA: 10 h, plus purification
Step 27, experiments in cells and *in vivo*
Step 27A, imaging experiments for GSH detection in cells: 52 h
Step 27B, imaging experiments for discrimination of GSH and other thiols in cells: 3 h
Step 27C, imaging experiments for oxidative stress in cells: 2 h
Step 27D, imaging experiments for endogenous GSH detection in cells: 80 h
Step 27E, imaging experiments for GSH detection in mouse bone marrow: 5 h
Step 27F, imaging experiments for GSH detection in sepsis mice: 10 h 30 min

ANTICIPATED RESULTS

Example results from experiments that we have performed using cultured cells and cells derived from mouse experiments are described in the INTRODUCTION and can be seen in **Figures 4–8**.

Analytical data

CP

Typical isolated yield 85%, blue solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (d, J = 15.0 Hz, 2H), 7.26–7.32 (m, 4H), 7.09 (t, J = 6.0 Hz, 2H), 6.97 (d, J = 6.0 Hz, 2H), 5.80 (d, J = 15.0 Hz, 2H), 3.88 (m, 8H), 3.26 (t, J = 6.0 Hz, 4H), 2.44 (t, J = 6.0 Hz, 4H), 1.84 (m, 6H), 1.68 (s, 12H), 1.02 (t, J = 6.0 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 169.1, 142.8, 141.0, 140.3, 128.3, 123.7, 123.5, 122.2, 109.4, 85.9, 55.3, 48.2, 47.2, 45.2, 29.2, 25.0, 21.8, 20.3, 11.8. ESI MS m/z = 589.6 $[M\text{-}I^-]^+$; calcd exact mass 716.3.

© 2015 Nature America, Inc. All rights reserved.

CPDSA

Typical isolated yield 82%, blue solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.76 (d, J = 9.0 Hz, 1H), 8.60 (d, J = 9.0 Hz, 1H), 8.32 (d, J = 9.0 Hz, 1H), 7.59–7.70 (m, 5H), 7.36 (m, 2H), 7.18–7.28 (m, 4H), 7.08 (d, J = 9.0 Hz, 2H), 5.95 (d, J = 15.0 Hz, 2H), 3.99 (t, J = 6.0 Hz, 4H), 3.55 (d, J = 6.0 Hz, 4H), 3.41 (d, J = 6.0 Hz, 4H), 2.96 (s, 6H), 2.47 (t, J = 6.0 Hz, 4H), 1.83 (m, 6H), 1.29 (s, 12H), 1.02 (t, J = 6.0 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 168.9, 152.0, 142.5, 141.7, 140.5, 132.0, 130.9, 130.1, 128.7, 128.6, 127.2, 124.4, 123.6, 122.0, 110.3, 98.8, 53.3, 48.3, 47.6, 45.8, 45.6, 28.2, 25.4, 21.5, 20.6, 11.7. MALDI-TOF MS m/z = 822.7 $[M\text{-}I^-]^+$; calcd exact mass 949.4.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS This research was supported by a grant from the National Creative Research Initiative programs of the National Research Foundation of Korea (NRF) funded by the Korean government (grant no. 2012R1A3A2048814), by the Basic Science Research Program through the NRF funded by the Ministry of Education (grant no. 2013R1A1A2008511), and by the Yonsei University College of Medicine (grant no. 6-2014-0135 to J.-H.R.) and the National Natural Science Foundation of China (grant no. 21402057).

AUTHOR CONTRIBUTIONS J. Yin, Y.K. and G.K. conducted experiments; D.K., D.L. and Y.H. measured partial data; and J. Yin, Y.K., G.K., J.-H.R. and J. Yoon designed experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Chen, X., Zhou, Y., Peng, X. & Yoon, J. Fluorescent and colorimetric probes for detection of thiols. *Chem. Soc. Rev.* **39**, 2120–2135 (2010).
2. Yang, X.-F. *et al.* A dual emission fluorescent probe enables simultaneous detection of glutathione and cysteine/homocysteine. *Chem. Sci.* **5**, 2177–2183 (2014).
3. Yin, C. *et al.* Thiol-addition reactions and their applications in thiol recognition. *Chem. Soc. Rev.* **42**, 6032–6059 (2013).
4. Yin, L.-L., Chen, Z.-Z., Tong, L.-L., Xu, K.-H. & Tang, B. Progress on fluorescent probes for thiols. *Chin. J. Anal. Chem.* **37**, 1073–1081 (2009).
5. Jung, H.S., Chen, X., Kim, J.S. & Yoon, J. Recent progress in luminescent and colorimetric chemosensors for detection of thiols. *Chem. Soc. Rev.* **42**, 6019–6031 (2013).
6. Niu, L.-Y. *et al.* BODIPY-based ratiometric fluorescent sensor for highly selective detection of glutathione over cysteine and homocysteine. *J. Am. Chem. Soc.* **134**, 18928–18931 (2012).
7. Işık, M. *et al.* Designing an intracellular fluorescent probe for glutathione: two modulation sites for selective signal transduction. *Org. Lett.* **16**, 3260–3263 (2014).
8. Zhai, D., Lee, S.-C., Yun, S.-W. & Chang, Y.-T. A ratiometric fluorescent dye for the detection of glutathione in live cells and liver cancer tissue. *Chem. Commun.* **49**, 7207–7209 (2013).
9. Shao, J. *et al.* A highly selective red-emitting FRET fluorescent molecular probe derived from BODIPY for the detection of cysteine and homocysteine: an experimental and theoretical study. *Chem. Sci.* **3**, 1049–1061 (2013).
10. Niu, L.-Y. *et al.* A turn-on fluorescent sensor for the discrimination of cysteine from homocysteine and glutathione. *Chem. Commun.* **49**, 1294–1296 (2013).
11. Wang, H., Zhou, G., Gai, H. & Chen, X. A fluorescein-based probe with high selectivity to cysteine over homocysteine and glutathione. *Chem. Commun.* **48**, 8341–8343 (2012).
12. Jung, H.S. *et al.* Molecular modulated cysteine-selective fluorescent probe. *Biomaterials* **33**, 945–953 (2012).
13. Hammers, M.D. & Pluth, M.D. Ratiometric measurement of hydrogen sulfide and cysteine/homocysteine ratios using a dual-fluorophore fragmentation strategy. *Anal. Chem.* **86**, 7135–7140 (2014).
14. Jung, H.S. *et al.* A cysteine-selective fluorescent probe for the cellular detection of cysteine. *Biomaterials* **33**, 8495–8502 (2012).
15. Liu, J. *et al.* Simultaneous fluorescence sensing of Cys and GSH from different emission channels. *J. Am. Chem. Soc.* **136**, 574–577 (2014).
16. Zhou, X., Jin, X., Sun, G., Li, D. & Wu, X. A cysteine probe with high selectivity and sensitivity promoted by response-assisted electrostatic attraction. *Chem. Commun.* **48**, 8793–8795 (2012).
17. Lee, H.Y. *et al.* Selective homocysteine turn-on fluorescent probes and their bioimaging applications. *Chem. Commun.* **50**, 6967–6969 (2014).
18. Hu, Y. *et al.* One-photon and two-photon sensing of biothiols using a bis-pyrene-Cu (II) ensemble and its application to image GSH in the cells and tissues. *Anal. Chem.* **87**, 3308–3313 (2015).
19. Lee, D., Kim, G., Yin, J. & Yoon, J. Aryl-thioether substituted nitrobenzothiadiazole probe for selective detection of cysteine and homocysteine. *Chem. Commun.* **51**, 6518–6520 (2015).
20. Yin, J. *et al.* Cyanine-based fluorescent probe for highly selective detection of glutathione in cell cultures and live mouse tissues. *J. Am. Chem. Soc.* **136**, 5351–5358 (2014).
21. Camera, E. & Picardo, M. Analytical methods to investigate glutathione and related compounds in biological and pathological processes. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **781**, 181–206 (2002).
22. Toyo'oka, T. Recent advances in separation and detection methods for thiol compounds in biological samples. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877**, 3318–3330 (2009).
23. Monostori, P., Wittmann, G., Karg, E. & Túri, S. Determination of glutathione and glutathione disulfide in biological samples: an in-depth review. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877**, 3331–3346 (2009).
24. Szaciłowski, K., Macyk, W., Drzewiecka-Matuszek, A., Brindell, M. & Stochel, G. Bioinorganic photochemistry: frontiers and mechanisms. *Chem. Rev.* **105**, 2647–2694 (2005).
25. Guo, Z., Park, S., Yoon, J. & Shin, I. Recent progress in the development of near-infrared fluorescent probes for bioimaging applications. *Chem. Soc. Rev.* **43**, 16–29 (2014).
26. Yuan, L., Lin, W., Zheng, K., He, L. & Huang, W. Far-red to near infrared analyte-responsive fluorescent probes based on organic fluorophore platforms for fluorescence imaging. *Chem. Soc. Rev.* **42**, 622–661 (2013).
27. Lim, S.-Y., Hong, K.-H., Kim, D.I., Kwon, H. & Kim, H.J. Tunable heptamethine–azo dye conjugate as an NIR fluorescent probe for the selective detection of mitochondrial glutathione over cysteine and homocysteine. *J. Am. Chem. Soc.* **136**, 7018–7025 (2014).
28. Wu, X. *et al.* *In vivo* and *in situ* tracking cancer chemotherapy by highly photostable NIR fluorescent theranostic prodrug. *J. Am. Chem. Soc.* **136**, 3579–3588 (2014).
29. Peng, X. *et al.* Fluorescence ratiometry and fluorescence lifetime imaging: using a single molecular sensor for dual mode imaging of cellular viscosity. *J. Am. Chem. Soc.* **133**, 6626–6635 (2011).
30. Karton-Lifshin, N. *et al.* A unique paradigm for a Turn-ON near-infrared cyanine-based probe: noninvasive intravital optical imaging of hydrogen peroxide. *J. Am. Chem. Soc.* **133**, 10960–10965 (2011).
31. Karton-Lifshin, N., Albertazzi, L., Bendikov, M., Baran, P.S. & Shabat, D. 'Donor–two-acceptor' dye design: a distinct gateway to NIR fluorescence. *J. Am. Chem. Soc.* **134**, 20412–20420 (2012).
32. Redy-Keisar, O., Kisin-Finfer, E., Ferber, S., Satchi-Fainaro, R. & Shabat, D. Synthesis and use of QCy7-derived modular probes for the detection and imaging of biologically relevant analytes. *Nat. Protocol.* **9**, 27–36 (2014).
33. Myochin, T. *et al.* Rational design of ratiometric near-infrared fluorescent pH probes with various pKa values, based on aminocyanine. *J. Am. Chem. Soc.* **133**, 3401–3409 (2011).
34. Takahashi, S. *et al.* Reversible off–on fluorescence probe for hypoxia and imaging of hypoxia–normoxia cycles in live cells. *J. Am. Chem. Soc.* **134**, 19588–19591 (2012).
35. Yang, Z. *et al.* Folate-based near-infrared fluorescent theranostic gemcitabine delivery. *J. Am. Chem. Soc.* **135**, 11657–11662 (2013).

© 2015 Nature America, Inc. All rights reserved.

36. Yu, F., Li, P., Wang, B. & Han, K. Reversible near-infrared fluorescent probe introducing tellurium to mimetic glutathione peroxidase for monitoring the redox cycles between peroxynitrite and glutathione *in vivo*. *J. Am. Chem. Soc.* **135**, 7674–7680 (2013).
37. Kundu, K. *et al.* Hydrocyanines: a class of fluorescent sensors that can image reactive oxygen species in cell culture, tissue, and *in vivo*. *Angew. Chem. Int. Ed.* **48**, 299–303 (2009).
38. Liu, Y. *et al.* A cyanine-modified nanosystem for *in vivo* upconversion luminescence bioimaging of methylmercury. *J. Am. Chem. Soc.* **135**, 9869–9876 (2013).
39. Edgington, L.E. *et al.* Functional imaging of legumain in cancer using a new quenched activity-based probe. *J. Am. Chem. Soc.* **135**, 174–182 (2013).
40. Tang, b. *et al.* Near-infrared neutral pH fluorescent probe for monitoring minor pH changes: imaging in living HepG2 and HL-7702 cells. *J. Am. Chem. Soc.* **131**, 3016–3023 (2009).
41. Fu, N., Xiong, Y. & Squier, T.C. Synthesis of a targeted biarsenical Cy3-Cy5 affinity probe for superresolution fluorescence imaging. *J. Am. Chem. Soc.* **134**, 18530–18533 (2012).
42. Guo, Z., Nam, S., Park, S. & Yoon, J. A highly selective ratiometric near-infrared fluorescent cyanine sensor for cysteine with remarkable shift and its application in bioimaging. *Chem. Sci.* **3**, 2760–2765 (2012).
43. Zhang, J. *et al.* A near-infrared fluorescence probe for thiols based on analyte-specific cleavage of carbamate and its application in bioimaging. *Eur. J. Org. Chem.* **2015**, 1711–1718 (2015).
44. Sanlioglu, S. *et al.* Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-α secretion through IKK regulation of NF-κB. *J. Biol. Chem.* **276**, 30188–30198 (2001).
45. Park, H.S. *et al.* Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-κB. *J. Immunol.* **173**, 3589–3593 (2004).
46. Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P. & Halbwachs-Mecarelli, L. Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.* **80**, 617–653 (2000).
47. Rosen, H. & Klebanoff, S.J. Bactericidal activity of a superoxide anion-generating system. *J. Exp. Med.* **149**, 27–39 (1979).

© 2015 Nature America, Inc. All rights reserved.