Synthesis and use of QCy7-derived modular probes for the detection and imaging of biologically relevant analytes

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This protocol describes the synthesis of modular turn-ON QCy7-based probes for the detection of biologically relevant analytes, such as hydrogen peroxide, ubiquitous sulfhydryl and β-galactosidase. The probes presented herein are prepared through a simple procedure that involves the preliminary alkylation of 4-hydroxy-isophthalaldehyde with a relevant analyte-responsive protecting group, followed by condensation of the resulting product with 2 equivalents of sulfo-indolium moieties. Evaluation of the turn-ON near-IR fluorescence response to their relevant analytes for the three different QCy7 probes is also reported. The preparation of a QCy7 diagnostic probe requires 1–2 d. Probes for other analytes can be prepared according to this modular procedure by incorporating a specific analyte-responsive group as a triggering substrate.

INTRODUCTION

Fluorescent dyes, constituted of small organic molecules, are widely used as part of molecular probes designed as medical and environmental diagnostic tools[1]. To detect a specific analyte or enzymatic activity[2], the diagnostic probe usually interacts with the analyte or enzyme of interest to form a fluorescent turn-ON response[3]. Several approaches to turn-ON a fluorescent probe are currently used, such as Förster resonance energy transfer (FRET), photoinduced electron transfer (PET) and internal charge transfer (ICT)[4–6].

We have recently reported a novel strategy for the design of long-wavelength fluorogenic probes with a turn-ON option[7]. The design is based on a donor-two-acceptor π-electron cyanine dye that can undergo an internal charge transfer to form a new fluorochrome with an extended π-conjugated system. The dye was synthesized through a simple two-step procedure, and it has a relatively high fluorescence quantum yield of 16% and a large extinction coefficient of 52,000 M^{-1} cm^{-1}. This synthetic strategy was translated into the preparation of a library of dyes with fluorescence emission in the near-infrared (NIR) region[8,9]. This optical range is particularly useful for *in vivo* imaging applications. As live tissues absorb and emit only minimally in the NIR range, NIR photons have the ability to penetrate tissues[10,11]. The concept was demonstrated by preparing a novel NIR turn-ON probe for the detection of hydrogen peroxide. The probe was successfully used to image induced inflammation in mice[7].

Our donor-two-acceptor dye system is composed of a protected phenol moiety (**I**) that functions as a latent donor conjugated with two indolium acceptors (**Fig. 1**). The protecting group is typically a substrate that can undergo a cleavage reaction in the presence of the analyte of interest. Analyte-triggered cleavage of the protecting group and subsequent deprotection of the phenol leads, therefore, to the formation of the phenolate active donor **II** that is able to donate a pair of π electrons to either of the conjugated indolium acceptors (structures **III** and **IV**) (**Fig. 1**). This intramolecular charge transfer generates a resonance species with a π-electron pattern similar to that of a cyanine fluorochrome. Accordingly, we have termed this new fluorochrome quinone-cyanine-7 (QCy7). The protected phenol **I** can thus be used as a molecular probe for the detection or imaging of specific analytes. Please note that an inherent limitation of QCy7-based probes is that, although some probes can be turned on by an analyte-binding event, the QCy7 system can only be used for the detection of analytes with chemical cleavage reactivity.

The QCy7-based probes have strong emission in the NIR region after reaction with their corresponding analytes. Although their maximum absorbance wavelength is not in the NIR region, it was demonstrated that NIR excitation can be achieved by implementing a two-photon excitation procedure[8].

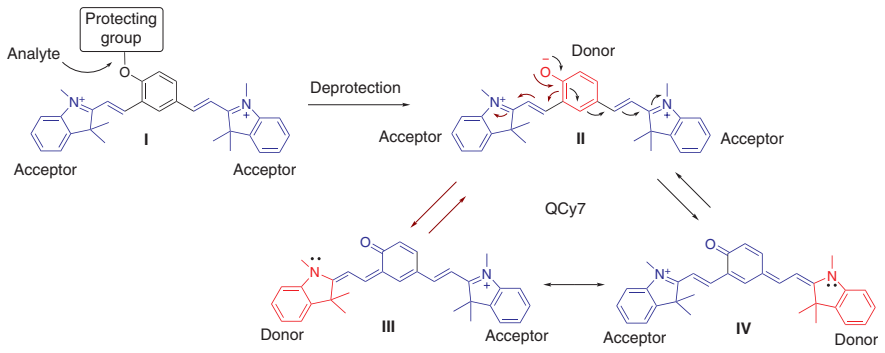

Figure 1 | Activation of a QCy7-modular probe by a specific analyte to produce a fluorescent turn-ON response.

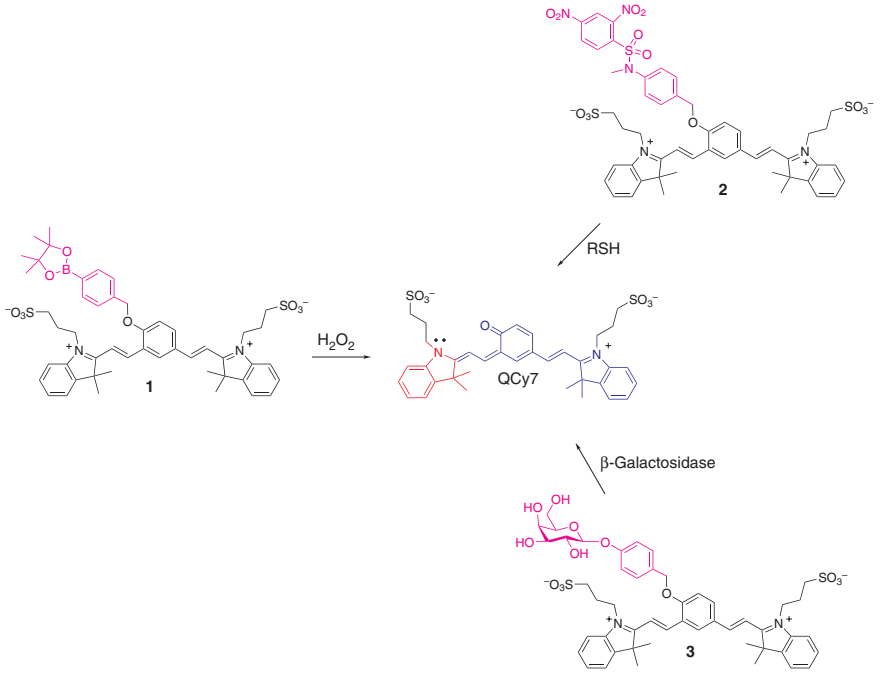

Figure 2 | Chemical structures and activation of three different QCy7-based probes. Probes are for the detection of hydrogen peroxide (**1**), ubiquitous sulfhydryl (**2**) and β-galactosidase (**3**).

The modular structure of the probe enables researchers to introduce various substrates, which can be tailored to react with a specific analyte, as protecting groups. In this protocol, we describe simple modular synthetic procedures for the preparation of three different QCy7-based probes for the detection and imaging of hydrogen peroxide, ubiquitous sulfhydryl groups and β-galactosidase. All of these activities are relevant for medical diagnosis of diverse pathologies. A relevant example would be the overproduction of H_2O_2 in cancer and inflammation, presented as oxidative stress, and in disorders such as cysteine deficiency. In addition, these modular probes can be used as biological research tools to evaluate transfection efficiency or qualitatively detect amounts of analytes or specific enzyme activities. For instance, β-galactosidase deficiency in humans can result in galactosialidosis or Morquio B syndrome. As enzyme replacement therapy is the medical treatment in patients in whom β-galactosidase is deficient or absent[12,13], real-time report of treatment efficiency could be achieved by administering our turn-ON probe.

Experimental design

The three QCy7-based probes are presented in **Figure 2**. Probe **1**, designed for the detection of hydrogen peroxide, is equipped with a phenyl-boronic-ester protecting group as a substrate[14]; probe **2**, designed for the detection of ubiquitous sulfhydryl, is equipped with a dinitro-benzene-sulfonyl protecting group[15]; and probe **3**, designed for the detection of the enzyme β-galactosidase, is equipped with a β-galactose protecting group[16,17]. Reaction of the specific analyte/enzyme with the appropriate probe results in the release of active QCy7 fluorochrome (see **Supplementary Figs. 1–3** for detailed activation mechanisms).

This protocol describes a simple general approach for the preparation of modular turn-ON NIR fluorescent probes. Recipe-style procedures are given for the three examples presented in **Figure 2**. The schemes for syntheses of probes **1** and **2** are presented in **Figure 3**. 4-Hydroxy-isophthalaldehyde is initially protected with the appropriate substrate (either phenyl-boronic-ester **1a** or dinitro-benzene-sulfone-amide **2a**) and then condensed with 2 equiv. of sulfo-indolium **4** to afford the corresponding probe.

The synthesis of probe **3** (**Fig. 4**) is initiated with alkylation of 4-hydroxy-isophthalaldehyde with β-galactose derivative **3a** to give ether **3b**. The latter is condensed with 2 equiv. of sulfo-indolium **4** to give intermediate **3c**, which is then deprotected of the acetate groups to afford probe **3**. The syntheses of phenyl boronic ester **1a**, dinitro-benzene-sulfonyl **2a** and β-galactose derivative **3a** are described in the **Supplementary Methods** and in **Supplementary Figures 4** and **5**.

Turn-ON response of the QCy7 probes to their analytes

Probes **1**, **2** and **3** were incubated with hydrogen peroxide, cysteine and β-galactosidase, respectively, in PBS (pH 7.4), and then NIR-fluorescent emission was recorded (**Fig. 5**)[7].

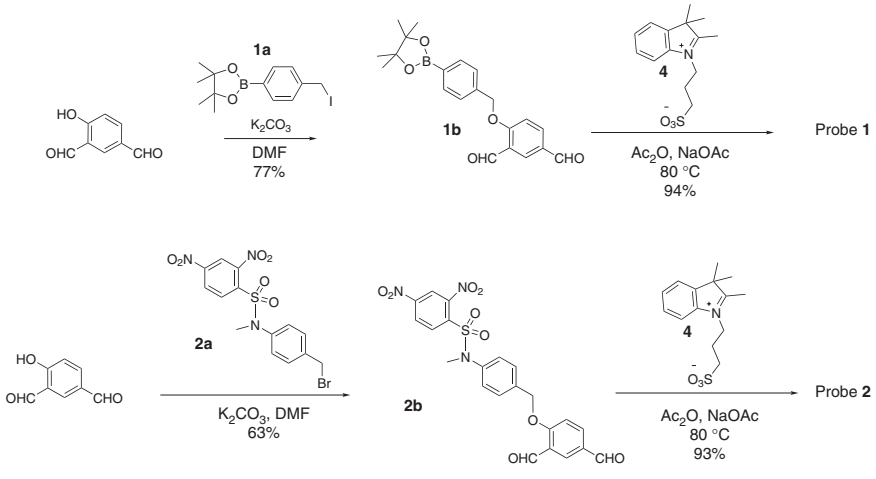

Figure 3 | Synthetic scheme for probe **1** and probe **2**.

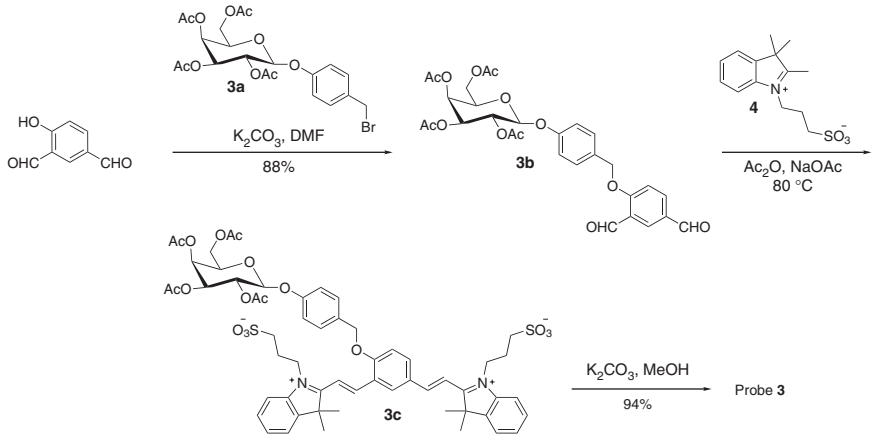

Figure 4 | Synthetic scheme for probe **3**.

probe and a probe with the analyte immediately after addition and imaged over time by using an intravital Cri Maestro imaging system (**Fig. 6**). Showing good *in vivo* compatibility with high signal-to-background ratio, probes injected with their corresponding analyte exhibit an increased NIR-fluorescent signal in mice. By contrast, when probes were injected without the analyte, we observed no fluorescence increase.

Probes sensitive to various analytes can be prepared according to this modular procedure simply by incorporating an analyte-specific responsive group on the 4-hydroxy-isophthalaldehyde before the condensation with the indolium moiety. Two independent research groups were 'fast' enough to report the incorporation of QCy7 in a probe system shortly before this protocol manuscript was edited for publication[18,19]. We predict that this protocol will be commonly used by others for the preparation of QCy7-based turn-ON probes for diagnosis and imaging additional analytes or enzymatic activities.

As expected, the NIR-fluorescent emission of each probe upon reaction with its corresponding analyte gradually increased over time. No fluorescence increase was observed in the absence of the analyte (**Supplementary Figs. 6–9**). To demonstrate the *in vivo* turn-ON imaging option, the probes were then injected subcutaneously into mice[7]. Each mouse was injected with the

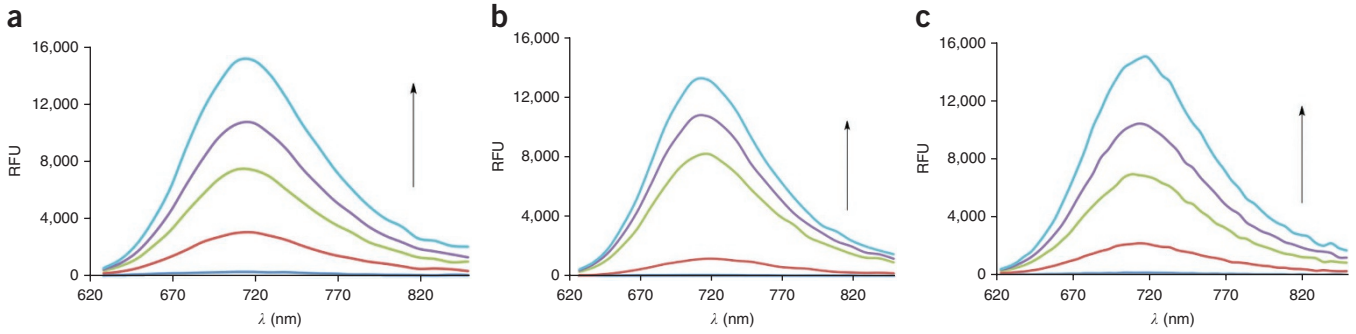

Figure 5 | Fluorescence turn-ON response of probe **1** to hydrogen peroxide, probe **2** to cysteine and probe **3** to β-galactosidase. (**a**) Time points represent 0, 3, 9, 15 and 40 min after the addition of 5 equiv. of H_2O_2 (1% (vol/vol) DMSO, PBS (pH 7.4); λ_{ex} = 590 nm, λ_{em} = 720 nm) to a 100 μM solution of probe **1**. (**b**) Time points represent 0, 1, 3, 9 and 30 min after the addition of 1 equivalent of cysteine (1% (vol/vol) DMSO, PBS (pH 7.4); λ_{ex} = 590 nm, λ_{em} = 720 nm) to a 100-μM solution of probe **2**. (**c**) Time points represent 0, 6, 18, 30 and 60 min after the addition of 1.37 enzyme units (1% (vol/vol) DMSO, PBS (pH 7.4); λ_{ex} = 590 nm, λ_{em} = 720 nm) to a 100-μM solution of probe **3**. RFU, relative fluorescence units.

MATERIALS

REAGENTS

- 2-(4-(Iodomethyl) phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (compound **1a**), prepared as described elsewhere[20] (see **Supplementary Methods** for details about the synthesis)
- N-(4-(Bromomethyl)phenyl)-N-methyl-2,4-dinitro-benzene-sulfonamide (compound **2a**) (**Supplementary Methods** and **Supplementary Fig. 4**)
- (2R,3S,4S,5R,6S)-2-(Acetoxymethyl)-6-(4-(bromomethyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (compound **3a**) (**Supplementary Methods** and **Supplementary Fig. 5**)
- 3-(2,3,3-Trimethyl-3*H*-indolium-1-yl) propane-1-sulfonate (compound **4**), prepared as described elsewhere[21] (see **Supplementary Methods** for details about the synthesis)
- 4-Hydroxy-isophthalaldehyde (TCI, cat. no. 4AOFG)
- Potassium carbonate (K_2CO_3, anhydrous; Bio-Lab, cat. no. 16300591)
- Sodium acetate (NaOAc; J.T. Baker, cat. no. Y06156)
- Acetic anhydride (Ac_2O; Acros, cat. no. 203-564-8)
- *N*,*N*-Dimethylformamide (DMF; Bio-Lab, cat. no. 04190501)
- Ethyl acetate (EtOAc; Bio-Lab, cat. no. 540056500)
- Hexanes (Hex; Bio-Lab, cat. no. 830056500)
- Acetonitrile for HPLC (ACN; LC gradient grade, Mercury, cat. no. 100305000)
- Methanol (MeOH; Bio-Lab, cat. no. 1368056500)
- Dichloromethane (DCM; Bio-Lab, cat. no. 1379026500)
- Trifluoroacetic acid for HPLC (TFA; HPLC grade, Bio-Lab, cat. no. 2023060100)
- Ammonium chloride (NH_4Cl; Bio-Lab, cat. no. 01260591)
- Sodium chloride (NaCl; Bio-Lab, cat. no. 19030591)
- Sodium sulfate (Na_2SO_4, anhydrous; Bio-Lab, cat. no. 948059)
- Hydrogen peroxide, 30% (wt/vol) in H_2O (H_2O_2; Sigma-Aldrich, cat. no. 216763)

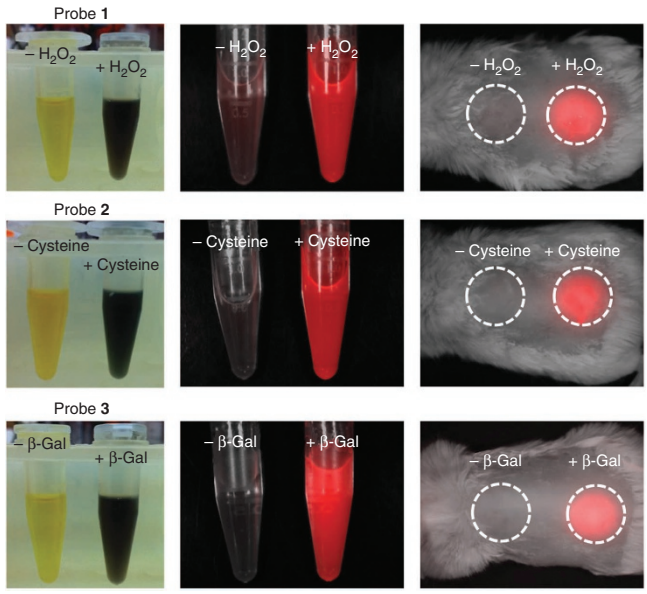

Figure 6 | Intravital fluorescence imaging of turn-ON response of the probes. Photograph taken by CR Maestro Imaging system of probes **1**, **2** and **3** (10 μM concentration). All three probes were incubated in saline (pH 7) containing 0.1% (vol/vol) DMSO in the presence (right-hand side) or absence (left-hand side) of their analyte. Filter set: excitation at 595 nm, emission cutoff filter of 715 nm. Eppendorf photographs of the probes' solutions [100 μM] were taken with a regular camera (left) and an NIR camera (middle); images of the mice (right) that underwent s.c. injection of either the probe alone or the probe alongside the analyte were obtained by using an intravital CRi Maestro imaging system. Please note that in the mouse experiments analytes were incubated with the probes *ex vivo* and then injected in the mice.

- L-Cysteine hydrochloride, anhydrous (Sigma-Aldrich, cat. no. 30120)
- β-galactosidase from *Escherichia coli* (Sigma-Aldrich, cat. no. G6008)
- Silica gel (Davisil, 40–63 micron)
- Deuterated solvents for NMR such as chloroform-D (Cambridge Isotope Laboratories)
- PBS buffer (10× concentrate for molecular biology, Bio-Lab, cat. no. 162323)
- DMSO (Sigma-Aldrich, cat. no. 472301)

EQUIPMENT

- Weighing balance
- Weighing boats
- Magnetic hot plate stirrer
- Oil bath
- Single-neck, round-bottomed flasks, 25–250 ml
- Stir bars of various sizes
- Rubber septa
- Erlenmeyer flasks, 250–500 ml
- Separatory funnels, 250–500 ml
- Reflux condenser
- Disposable glass Pasteur pipettes
- Analytical thin-layer chromatography (TLC) plates, 254-μm thickness
- Liquid funnel
- Columns for chromatography
- Disposable culture tubes
- Needles
- Syringes, 1–5 ml
- Metal spatulas
- Eppendorf tubes
- Pipettes
- Graduated cylinders, 10–200 ml
- Aluminum foil
- Cotton wool
- Dewar flask
- Rotary evaporator
- Vacuum pump
- Vacuum manifold with vacuum line
- NMR tubes, 5-mm width
- Analytical reverse-phase (RP)-HPLC instrument (Hitachi Elite LaChrom)
- Preparative RP-HPLC instrument (ECOM, Sapphire 600)
- Plate reader (TECAN, Infinite 200 PRO)

PROCEDURE

Synthesis of dialdehydes 1b, 2b or 3b ● TIMING 10–12 h, plus purification

1| Weigh out the following quantities of 4-hydroxy-isophthalaldehyde, depending on the probe you aim to synthesize:

Probe to synthesize	Amount of 4-hydroxy-isophthalaldehyde
1	175 mg (1.16 mmol)
2	115 mg (0.76 mmol)
3	190 mg (1.26 mmol)

2| Place an egg-shaped Teflon-coated magnetic stir bar and the 4-hydroxy-isophthalaldehyde in a 50-ml round-bottomed flask.

3| Add 3 ml of DMF to the flask by using a 5-ml syringe, and then stir the mixture.

▲ **CRITICAL STEP** Use the indicated amount of DMF, as using too much DMF will result in low product yield.

4| Cool the round-bottomed flask in an ice-water bath.

5| Weigh out the following quantities of K_2CO_3, depending on the probe you aim to synthesize:

Probe to synthesize	Amount of K_2CO_3
1	320 mg (2.32 mmol—2 equiv.)
2	127 mg (0.92 mmol—1.2 equiv.)
3	210 mg (1.52 mmol—1.2 equiv.)

6| Add K_2CO_3 to the cooled flask. Wait for 10 min. The color of the mixture at this point should be yellow.

7| For the synthesis of probe **1**, weigh out 481 mg (1.39 mmol, 1.2 equiv.) of **1a** (ref. 20). For probe **2**, weigh out 330 mg (0.76 mmol, 1.0 equiv.) of **2a**. For probe **3**, weigh out 656 mg (1.26 mmol, 1.0 equiv.) of **3a**. Add the relevant benzyl-halide (**1a**, **2a** or **3a**) to the cooled flask. Wait for 2 min.

8| Remove the flask from the ice-water bath and allow the reaction mixture to warm to room temperature (25 °C).

9| Stir the reaction mixture overnight (10–12 h) at room temperature.

10| Pour the reaction mixture into a separatory funnel. Add 100 ml of ethyl acetate and 100 ml of saturated aqueous ammonium chloride solution. Shake and separate the layers. Wash the organic layer with 100 ml of brine. Shake and separate the layers.

11| Dry the organic layer over ~5 g of sodium sulfate for 5–10 min, gravity-filter it through filter paper and remove the solvent by rotary evaporation (heat up the mixture to 30 °C if necessary).

12| Purify the reaction products by silica column chromatography according to option A, B or C, depending on which probe you are synthesizing.

(A) Purification of 1b

(i) Prepare a silica gel column with a column diameter of 3.5–5 cm and a silica gel column height of 17 cm.

(ii) Dissolve the mixture obtained in Step 11 in a minimum amount of DCM, and then load the resulting solution onto the top of the column. Begin to elute with 100 ml of hexanes, then with a mixture of 3:7 ethyl acetate:hexanes, and then increase the ethyl acetate content until a ratio of 4:1 ethyl acetate:hexanes is reached. The product will elute from the column at a ratio of 3:2 ethyl acetate:hexanes. Compound **1b** (~330 mg) is obtained as a white solid in 77% yield. R_f compound **1b** = 0.75 (60% ethyl acetate in hexanes).

(B) Purification of 2b

(i) Prepare a silica gel column with a column diameter of 3.5 cm and a silica gel column height of 15–17 cm.

(ii) Dissolve the mixture obtained in Step 11 in a minimum amount of DCM, and then load the resulting solution onto the top of the column. Begin to elute with 100 ml of hexanes, then with a mixture of 3:7 ethyl acetate:hexanes, and then increase the ethyl acetate content until a ratio of 4:1 ethyl acetate:hexanes is reached. This procedure yields 240 mg of compound **2b** as yellow oil in 63% yield. R_f compound **2b** = 0.45 (40% ethyl acetate in hexanes).

(C) Purification of 3b

(i) Prepare a silica gel column with a column diameter of 3.5 cm and a silica gel column height of 15–17 cm.

(ii) Dissolve the mixture obtained in Step 11 in a minimum amount of DCM, and then load the resulting solution to the top of the column. Begin to elute with 100 ml of hexanes, then with a mixture of 3:7 ethyl acetate:hexanes, and then increase the ethyl acetate content until a ratio of 9:1 ethyl acetate:hexanes is reached. The product will begin to elute from the column at a ratio of 4:1 ethyl acetate:hexanes. This protocol should yield 596 mg of compound **3b** as a white solid in 88% yield. R_f compound **3b** = 0.49 (40% ethyl acetate in hexanes).

13| Establish the identity and purity of the compounds by proton NMR spectroscopy (^{1}H NMR), carbon NMR spectroscopy (^{13}C NMR) and mass spectrometry (MS).

■ **PAUSE POINT** At this point, the product can be stored at 4 °C for at least 3–6 months.

Synthesis of probes 1–3 ● **TIMING 3–4 h**

14| For the synthesis of probe **1**, weigh out 30 mg of dialdehyde **1b** (0.08 mmol). For the synthesis of probe **2**, weigh out 50 mg of dialdehyde **2b** (0.1 mmol). For the synthesis of probe **3**, weigh out 50 mg of dialdehyde **3b** (0.09 mmol).

15| In a 25-ml round-bottom flask, place an egg-shaped Teflon-coated magnetic stir bar and add the relevant dialdehyde weighed in Step 14.

16| Use a 2.5-ml syringe to add 2 ml of Ac_2O.

17| Weigh out the following quantities of 3-(2,3,3-trimethyl-3*H*-indolium-1-yl) propane-1-sulfonate (compound **4**), depending on the probe you aim to synthesize:

Probe to synthesize	Amount of compound 4
1	58 mg (0.20 mmol—2.5 equiv.)
2	59 mg (0.21 mmol—2.1 equiv.)
3	55 mg (0.19 mmol—2.1 equiv.)

▲ **CRITICAL STEP** Compound **4** is highly hygroscopic. Ensure that it is not left in the weighing boat for too long.

18| Add compound **4** to the relevant mixture prepared in Step 15.

19| Weigh out the following quantities of NaOAc, depending on the probe you aim to synthesize:

Probe to synthesize	Amount of NaOAc
1	16.4 mg (0.20 mmol—2.5 equiv.)
2	17 mg (0.21 mmol—2.1 equiv.)
3	16 mg (0.19 mmol—2.1 equiv.)

20| Add NaOAc to the relevant reaction mixture from Step 19.

21| Place the flask in a room-temperature oil bath. Attach a reflux condenser to the reaction flask and heat the oil bath to 80 °C while stirring the flask.
▲ **CRITICAL STEP** The oil bath should not be heated above 80 °C; at higher temperatures, some decomposition of the product is observed.

22| Monitor the reaction progress by analytical RP-HPLC (column, RP-18; length, 25 cm; particle size, 5 µm; HPLC gradient, 10–90% ACN in 0.1% TFA in water—1 ml of TFA per 1 liter of water—20 min; flow rate, 1 ml min^{-1}). For probe 1, the product appears as two different peaks (the product in the form of a pinacol boronic ester and the product in the form of boronic acid) at retention times of 11.9 and 15.9 min, λ = 425 nm. For probe 2, the product should have a maximum absorbance at 425 nm and a retention time of ~15.7 min. For probe 3, product **3c** should have a maximum absorbance at 425 nm and a retention time of ~14.7 min.

23| After 30 min, turn off the heater and allow the reaction to cool to room temperature.
▲ **CRITICAL STEP** The reaction should reach completion in ~30 min. To avoid decomposition of the product, do not leave the reaction mixture at 80 °C for longer than is necessary. The reaction may be carried out at lower temperatures and even at room temperature; however, it will take longer to reach completion.

24| Upon completion, remove the acetic anhydride from the reaction mixture under reduced pressure by using a vacuum manifold with vacuum line while stirring and heating the flask to 50 °C.

25| (Optional) If you are synthesizing probe **3**, dissolve compound **3c** in 5 ml of MeOH. Add 25 mg of K_2CO_3 (0.18 mmol, 2.0 equiv.). Stir the mixture. Monitor the reaction progress by RP-HPLC, using a gradient of 10–90% ACN in water (0.1% (vol/vol) TFA in water) over 20 min. Under these conditions, the product should have a retention time of ~4 min shorter than that of the starting material. Please note that some residual Ac_2O from the previous step might consume a part of the base added. Therefore, if you do not observe any progress in the reaction, more K_2CO_3 should be added. Add 1 equiv. of base at a time. When the reaction is complete, a purple color is observed.

26| (Optional) Upon completion of the reaction described in Step 25, remove the solvent by rotary evaporation (heat up to 30 °C if necessary).

27| Purify the product by silica gel column chromatography according to option A, B or C, depending on the identity of the product.

(A) Purification of probe 1

(i) Mix the silica gel with MeOH for 5 min. Next, load the wet silica gel into the column and remove the MeOH (column diameter, 3 cm; silica gel column height, 10–15 cm). Afterward, run 100 ml of DCM through the column to remove residual MeOH, and then place the product mixture dissolved in 3 ml of DCM and a minimal amount of MeOH on top of the silica gel by using a Pasteur pipette. Wash the column with 100 ml of DCM.

(ii) Start the purification with 1:9 MeOH:DCM as an eluent and gradually increase the MeOH until a ratio of 1:1 DCM:MeOH is reached. Most of the product will exit the column at a 2:3 DCM:MeOH ratio, yielding 69 mg of probe **1** as a brown solid in 94% overall yield. R_f of probe **1** = 0.5 (30% MeOH in DCM); it appears yellow on a silica gel TLC plate.
▲ **CRITICAL STEP** The column should be run as quickly as possible, as the boronate group is prone to degradation when it is left on silica gel for too long.
? TROUBLESHOOTING

(B) Purification of probe 2

(i) Mix the silica gel with MeOH for 5 min. Next, load the wet silica gel into the column and remove the MeOH (column diameter, 3 cm; silica gel column height, 10–15 cm). Afterward, run 100 ml of DCM through the column to remove residual MeOH, and then place the product mixture dissolved in 3 ml of DCM and a minimal amount of MeOH on top of the silica gel by using a Pasteur pipette. Wash the column with 100 ml of DCM.

(ii) Start the elution step with a mixture of 1:9 MeOH:DCM and gradually increase the MeOH content until a ratio of 3:7 MeOH:DCM is reached. The product will begin to elute at a ratio of 3:7 MeOH:DCM, yielding 96 mg of probe **2** as an orange solid in 93% overall yield. R_f of probe **2** = 0.5 (20% MeOH in DCM); the product spot appears yellow on a silica gel TLC plate.

(C) Purification of probe 3

(i) Mix the silica with MeOH. Load the wet silica gel into the column and remove the MeOH (column diameter, 3 cm; silica gel column height, 15–17 cm). Run 100 ml of DCM through the column to remove residual MeOH. Dissolve the reaction mixture in ~3 ml of DCM and a minimal amount of MeOH. Place the product solution on the column.

(ii) Wash the silica through the column with 100 ml of hexanes followed by 100 ml of DCM.

(iii) Elute the product beginning with a mixture of 1:9 MeOH:DCM, and gradually increase the MeOH content until a ratio of 9:1 MeOH:DCM is reached. The product will begin eluting from the column at a ratio of 3:2 MeOH:DCM, yielding 80 mg of probe **3** as an orange solid in 94% overall yield. R_f of probe **3** = 0.5 (25% MeOH in DCM); the product spot appears yellow on a silica gel TLC plate.

28| Establish the identity and purity of the probes by ^{1}H NMR, ^{13}C NMR and MS.
▲ **CRITICAL STEP** Please note that you may find it difficult to evaluate the purity of the product using TLC. You should use RP-HPLC to evaluate fractions eluted from the column.
■ **PAUSE POINT** The probes can be stored in the dark at −20 °C for at least 1 month.

Validation of probe activity ● TIMING 1–2 h

29| Validate the activity of the probes according to option A, B or C, depending on the identity of the probe.

(A) Validation of probe 1 hydrogen peroxide activity ● TIMING 1 h

(i) Prepare a 10 mM stock solution of probe **1** (molecular weight 892 g mol^{-1}) in DMSO by dissolving 10 µmol of the probe in 1 ml of DMSO.
▲ **CRITICAL STEP** Once diluted, the probe **1** stock solution should be used the same day because of the potential decomposition of the boronate protecting group.

(ii) Prepare a 10 mM stock solution of H_2O_2 by adding 1.1 µl of 30% (wt/vol) H_2O_2 into 998.9 µl of water.

(iii) Add 1 µl of the probe **1** solution prepared in Step 29A(i) and 5 µl of the H_2O_2 solution prepared in Step 29A(ii) into 94 µl of 0.1 M PBS buffer, pH 7.4 (probe **1** final concentration 100 µM, and H_2O_2 final concentration 500 µM). This solution will serve as a positive control. Add 1 µl of probe **1** solution into 99 µl of 0.1 M PBS (pH 7.4) without adding H_2O_2. This solution will serve as a negative control.

(iv) Immediately begin reading the fluorescence intensity of both positive and negative control samples with a fluorometric plate reader. The excitation wavelength is 590 nm and the emission wavelength is 720 nm. The fluorescence of the H_2O_2-treated positive control sample should increase over time (**Fig. 5**), whereas the fluorescence of the negative control should remain unchanged.
? TROUBLESHOOTING

(B) Validation of probe 2 sulfhydryl activity ● **TIMING 1–2 h**

(i) Prepare a 10-mM stock solution of probe **2** (MW 1,026.16 g mol^{-1}) in DMSO by dissolving 10 μmol of the probe in 1 ml of DMSO.

(ii) Prepare a 10-mM stock solution of cysteine (using L-cysteine hydrochloride as the cysteine source) in DMSO.

(iii) Add 1 μl of the stock solution of probe **2** (prepared in Step 29B(i)) and 1 μl of the cysteine stock solution (from Step 29B(ii)) to 98 μl of 0.1 M PBS (pH 7.4). The final concentration of probe **2** will be 100 μM. This solution will serve as a positive control. Add 1 μl of probe **2** stock solution to 99 μl of 0.1 M PBS (pH 7.4). This solution will serve as a negative control.

(iv) Immediately begin reading the fluorescence intensity of both positive and negative control samples with a fluorometric plate reader. The excitation wavelength is 590 nm and the emission wavelength is 720 nm. The fluorescence of the thiol-treated positive control sample should increase in intensity over time (**Fig. 5**), whereas the fluorescence of the negative control should remain unchanged.

(C) Validation of probe 3 for β-galactosidase activity ● **TIMING 1–2 h**

(i) Prepare a 10-mM stock solution of probe **3** (MW 945 g mol^{-1}) in DMSO by dissolving 10 μmol of the probe in 1 ml of DMSO.

(ii) Prepare a stock solution of the β-galactosidase enzyme by adding 1 mg of the enzyme to 1 ml of 0.1 M PBS (pH 7.4). The resulting solution should have ~115 enzyme units per ml.
▲ **CRITICAL STEP** The exact amount of enzyme units per milligram can be found on the label attached to the enzyme flask by the supplier. Use it to determine the precise number of enzyme units in your stock solution.

(iii) Add 1 μl of the stock solution of probe **3** (prepared in Step 29C(i)) and 5 μl of the enzyme stock solution (from Step 29C(ii)) to 94 μl of 0.1 M PBS buffer, pH 7.4. The final concentrations will be 100 μM for probe **3** and 0.57 enzyme units per 100 μl. This solution will serve as a positive control. Add 1 μl of probe **3** stock solution to 99 μl of 0.1 M PBS (pH 7.4). This solution will serve as a negative control.

(iv) Immediately begin reading the fluorescence intensity of both positive and negative control samples with a fluorometric plate reader. The excitation wavelength is 590 nm and the emission wavelength is 720 nm. The fluorescence of the enzyme-treated positive control sample should increase in intensity over time (**Fig. 5**), whereas the fluorescence of the negative control should remain unchanged.

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
27A(ii)	The product sticks to the column	The silica can partially deprotect the pinacol yielding the boronic acid, which is very polar and hydrophilic, and is difficult to remove from the column	Run the column as quickly as possible, always keeping solvents flowing throughout the purification. After the product exits the column, wash the column with a polar solvent such as 1:4 DCM:MeOH to make sure that all the product exits the column
29A(iv)	High fluorescence background	Boronate may convert to the phenolate resulting in free QCy7	Probe **1** should be stored at −20 °C in the dark and under dry conditions. Deprotection of the boronate will yield a fluorescent product that will increase the background fluorescence signal and lower the sensitivity of the assay. Thus, probe **1** should be as pure as possible

● TIMING

Steps 1–13, synthesis of dialdehyde **1b**, **2b** or **3b**: 10–12 h, plus purification
Steps 14–28, synthesis of probe **1**, probe **2** or probe **3**: 3–4 h
Step 29, validation of H_2O_2 probe **1** activity: 1 h; validation of thiol probe **2** activity: 1–2 h; validation of β-galactosidase probe **3** activity: 1–2 h

ANTICIPATED RESULTS

***In vivo* use of probe 1 to visualize endogenously produced hydrogen peroxide**

As a proof of concept, our research group has used probe **1**, as prepared according to this protocol, to visualize hydrogen peroxide endogenously produced in the peritoneal cavity of mice[7]. In this *in vivo* imaging experiment, overproduction of

hydrogen peroxide was achieved by causing a lipopolysaccharide (LPS)-induced inflammatory response in mice. In detail, mice were injected with LPS (1 ml of 0.1 mg ml^{-1} solution) i.p. Six hours after this initial injection, a second i.p. injection was performed, through which 400 µl of probe **1** (1 mM) was transferred to the mouse peritoneal cavity. Thereafter, the production of free QCy7 was monitored with a CRI Maestro noninvasive fluorescence imaging system (CRI Maestro image; excitation at 595 nm, emission cutoff filter of 635 nm). Mice treated with LPS that had been injected with probe **1** were characterized by a NIR fluorescence signal of substantially greater intensity than mice not treated with LPS that had been injected with either probe **1** or PBS.

Analytical data

Compound 1b

Yield: 77%, white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 10.55 (1H, s), 9.93 (1H, s), 8.35 (1H, d, J = 2.2 Hz), 8.09 (1H, dd, J = 8.6, 2.2 Hz), 7.86 (2H, d, J = 8.0 Hz), 7.44 (2H, d, J = 8.0 Hz), 7.17 (1H, d, J = 8.7 Hz), 5.32 (2H, s), 1.34 (12H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 190.81, 189.19, 165.62, 138.68, 136.31, 136.05, 132.59, 130.59, 127.21, 125.90, 114.40, 84.72, 71.74, 25.59. MS (ES^+): *m/z* calc. for $C_{21}H_{23}BO_5$: 366.2; found 389.2 $[M + Na]^+$.

Compound 1

Yield: 94%, brown solid. ^{1}H NMR (CD_3OD, 400 MHz): δ = 9.21 (1H, s), 8.80 (1H, d, J = 16.4 Hz), 8.59 (1H, d, J = 16.3 Hz), 8.42 (2H, d, J = 9.08 Hz), 8.23 (1H, d, J = 16.5 Hz), 8.07 (1H, d, J = 16.2 Hz), 7.95–7.92 (2H, m), 7.86 (2H, d, J = 8.0 Hz), 7.80–7.76 (2H, m), 7.67–7.63 (4H, m), 7.60 (2H, d, J = 7.9 Hz), 7.49 (1H, d, J = 8.8 Hz), 5.46 (2H, s), 5.00 (4H, t, J = 6.3 Hz), 3.10–3.06 (4H, m), 2.44 (4H, m), 1.98 (6H, s), 1.80 (6H, s), 1.37 (12H, s). ^{13}C NMR (DMSO-*d6*, 100 MHz): δ = 182.41, 182.18, 162.02, 153.57, 153.35, 146.74, 144.44, 144.16, 141.25, 138.16, 137.77, 134.79, 133.37, 130.06, 129.66, 129.43, 128.74, 126.97, 124.35, 123.43, 123.39, 115.78, 115.50, 115.24, 114.91, 114.80, 112.76, 112.53, 71.37, 52.70, 52.47, 47.56, 46.20, 45.85, 26.50, 26.50, 26.68, 25.10. MS (ES^+): *m/z* calc. for $C_{49}H_{57}BN_2O_9S_2$: 892.3; found 915.6 $[M + Na]^+$. HPLC grad. 10–90% ACN in water 20 min: RT = 11.9 min (boronic acid) and 15.9 min (boronic ester), λ = 425 nm.

Compound 2b

Yield: 63%, yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.51 (1H, s), 9.96 (1H, s), 8.44 (1H, d, J = 2 Hz), 8.35 (1H, d, J = 2 Hz), 8.34 (1H, dd, J = 2.1, 8.7 Hz), 8.13 (1H, dd, J = 2.1, 8.7 Hz), 7.8 (1H, d, J = 8.6 Hz), 7.47 (2H, d, J = 8.3 Hz), 7.31 (2H, d, J = 8.3 Hz), 7.19 (1H, d, J = 8.6 Hz), 5.29 (2H, s), 3.44 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 190.69, 188.95, 150.59, 140.61, 137.37, 136.4, 133.98, 132.9, 130.9, 129.23, 128.68, 126.25, 125.97, 120.22, 114.21, 70.88, 30.39. MS (ES^+): *m/z* calc. for $C_{22}H_{17}N_3O_9S$: 499.07; found: 522.3 $[M + Na]^+$.

Compound 2

Yield: 93%, orange solid. ^{1}H NMR (400 MHz, MeOD): δ = 9.19 (1H, d, J = 1.8 Hz), 8.77 (1H, d, J = 16.4 Hz), 8.69 (1H, d, J = 2.2 Hz), 8.58 (1H, d, J = 16.2 Hz), 8.51 (1H, dd, J = 2.2, 8.7 Hz), 8.39 (1H, dd, J = 1.8, 8.8 Hz), 8.2 (1H, d, J = 16.4 Hz), 8.07 (1H, d, J = 16.2 Hz), 7.98 (1H, d, J = 8.6 Hz), 7.95–7.91 (2H, m), 7.78–7.75 (2H, m), 7.66–7.6 (6H, m), 7.47 (1H, d, J = 8.8 Hz), 7.39 (2H, d, J = 8.4 Hz), 5.47 (2H, s), 5.02–4.95 (4H, m), 3.44 (3H, s), 3.1–3.03 (4H, m), 2.44 (4H, m), 1.92 (6H, s), 1.8 (6H, s). ^{13}C NMR (100 MHz, MeOD): δ = 144.74, 143.49, 141.53, 140.55, 133.41, 132.87, 129.54, 128.3, 128.14, 126.54, 123.5, 122.97, 122.16, 121.63, 120.38, 115.56, 109.58, 109.05, 93.88, 63.8, 53.27, 41.85, 31.59, 30.07, 22.93. MS (ES^+): *m/z* calc. for $C_{50}H_{51}N_5O_{13}S_3$: 1,025.26; found: 1,048.5 $[M + Na]^+$. HPLC grad. 10–90% ACN in water 20 min: RT = 15.7 min, λ = 425 nm.

Compound 3b

Yield: 88%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.5 (1H, s), 9.94 (1H, s), 8.34 (1H, d, J = 2 Hz), 8.11 (1H, dd, J = 8.8, 2 Hz), 7.39 (2H, d, J = 8.6 Hz), 7.21 (2H, d, J = 8.7 Hz), 7.06 (2H, d, J = 8.6 Hz), 5.51–5.45 (2H, m), 5.23 (2H, s), 5.13 (1H, dd, J = 10.4, 3.4 Hz), 5.08 (1H, d, J = 7.9 Hz), 4.25–4.05 (3H, m), 2.18 (3H, s), 2.06 (3H, s), 2.05 (3H, s), 2.01 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 190.8, 189.15, 171.04, 170.83, 170.87, 170.13, 165.59, 157.84, 136.3, 132.7, 130.6, 130.53, 129.88, 125.87, 117.99, 114.28, 100.19, 71.82, 71.49, 71.38, 69.31, 67.53, 62.01, 21.38. MS (ES^+): *m/z* calc. for $C_{29}H_{30}O_{13}$: 586.17; found: 609.3 $[M + Na]^+$.

Compound 3

Yield: 94%, orange solid. ^{1}H NMR (400 MHz, MeOD): δ = 9.16 (1H, s), 8.68 (1H, d, J = 16.4 Hz), 8.55 (1H, d J = 16.2 Hz), 8.35 (1H, dd, J = 8.6, 2.2 Hz), 8.13 (1H, d, J = 16.4 Hz), 8.04 (1H, d, J = 16.2 Hz), 7.93–7.49 (11H, m), 7.23 (2H, d, J = 8.6 Hz), 5.37 (2H, s), 4.98–4.95 (4H, m), 3.97–3.56 (7H, m), 3.14– 2.86 (4H, m), 2.52–2.22 (4H, m), 2.14 (6H, s), 1.91 (6H, s). ^{13}C NMR (100 MHz, MeOD): δ = 183.47, 183.22, 163.58, 158.98, 154.54, 148.92, 144.70, 144.63, 141.44, 138.98, 133.61, 130.53, 130.36, 130.27, 130.02, 128.96, 128.38, 124.81, 123.44, 117.43, 115.69, 115.40, 114.86, 114.50, 102.05, 76.42, 74.19, 72.11, 71.64, 69.60, 61.87, 53.26, 46.35, 45.99, 30.04, 26.46, 26.22, 24.88, 23.56, 22.09. MS (ESI): *m/z* calc. for $C_{49}H_{56}N_2O_{13}S_2$: 945.1; found: 967.5 $[M + Na]^+$. HPLC grad. 10–90% ACN in water 20 min: RT = 10.6 min, λ = 425 nm.

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

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