A FRET-Based Approach to Ratiometric Fluorescence Detection of Hydrogen Peroxide

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Synthetic Materials and Methods. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. Analytical thin layer chromatography was performed using Fisher 60 F254 silica gel (precoated sheets, 0.25 mm thick). Dichloro[1,1´-bis(diphenylphosphino) ferrocene]palladium (II), Pd(dppf)Cl_2, was purchased from Strem Chemicals (Newburyport, MA), anhydrous N, N'-dimethylformamide (DMF) was purchased from Sigma-Aldrich (St. Louis, MO), DriSolv® DMF was purchased from EMD Pharmaceuticals (Durham, NC), 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid was purchased from Maybridge Limited (Cornwall, UK), and each reagent was used as received. Dulbecco's phosphate buffered saline (DPBS) was purchased from Invitrogen (Carlsbad, CA) and was used as received. Antimycin A from *Streptomyces* sp. (mixture of components A_1, A_2, A_3, and A_4) and β-nicotinamide adenine dinucleotide reduced disodium salt (β-NADH) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Acros Organics (Morris Plains, NJ) and were used as received. ^{1}H NMR spectra were collected in $CDCl_3$, CD_3OD, or DMSO-d_6, (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AV-300 or AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million. High-resolution mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

3',6'-Dibromo-6-carboxy-fluoran pyridinium salt (1). This compound was prepared according to a slight modification of the literature procedure.[1] 3-Bromophenol (3.5 g, 20 mmol), 1,2,4-benzenetricarboxylic acid (2.1 g, 10 mmol), and methanesulfonic acid (10 mL) were added to a 75-mL heavy-walled reaction flask and heated at 140 ºC for 72 h. After cooling to room temperature, the dark purple solution was poured into 200 mL of an ice/water slurry and stirred vigorously to precipitate a greenish yellow solid. The solid was collected by vacuum filtration and dried in air to give 4.47 g of the crude carboxyfluoran as a mixture of 5 and 6 isomers. Crystallization from 30 mL of acetic anhydride and 10 mL of pyridine provided a white solid that was recrystallized three times from a 2:1 mixture of acetic anhydride and pyridine to give isomerically pure **1** as a white powder (1.4 g, 26% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.68 (2H, dd, J_1 = 2.8 Hz, J_2 = 1.6 Hz), 8.26 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz), 8.17 (1H, d, J = 8.0 Hz), 8.03 (1H, m), 7.86 (1H, s), 7.71 (2H, t, J = 4.0 Hz), 7.59 (2H, m), 7.33 (2H, dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz), 6.87 (2H, d, J = 8.4 Hz). ESI-MS: calculated for [M^-] 500.9, found 500.9.

3',6'-Dibromofluoran-6-carboxy succinimidyl ester (2). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl, 254 mg, 1.3 mmol), and *N*-hydroxysuccinimide (155 mg, 1.4 mmol) were dissolved in dry DMF (4 mL) in a 25-mL round bottom flask. Fluoran **1** (304 mg, 0.5 mmol) in dry DMF (2 mL) was added dropwise to the solution. The reaction was stirred at room temperature for 48 hours in the dark. The resulting pale yellow solution was poured into 80 mL of a stirring ice/water slurry to precipitate a white solid that was collected by

vacuum filtration. The solid was dissolved in 1% methanol/dichloromethane and dried over $MgSO_4$. Purification by flash column chromatography (silica gel, 1% methanol/dichloromethane) gave **2** as a white, crystalline solid (187 mg, 61% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.40 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz), 8.19 (1H, d, J = 8.0 Hz), 7.86 (1H, s), 7.53 (2H, d, J = 2.0 Hz), 7.25 (2H, dd, J_1 = 8.4 Hz, J_2 = 1.6 Hz), 6.69 (2H, d, J = 8.8 Hz), 2.90 (4H, s). HRFAB-MS: calculated for [MH^+] 597.9144, found 597.9137.

Coumarin amine derivative (4). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (278 mg, 1.5 mmol), and *N*-hydroxysuccinimide (166 mg, 1.4 mmol) were dissolved in dry DMF (4 mL) in a 25-mL round bottom flask. 7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (257 mg, 0.98 mmol) was dissolved in dry DMF (4 mL) and added dropwise to the EDC solution. The reaction was stirred at room temperature for 48 hours in the dark. The resulting yellow mixture was poured into 150 mL of an ice/water slurry and stirred to precipitate a yellow solid. The solid was then collected by vacuum filtration, washed with 200 mL water, and dried in air overnight to give 7-(diethylamino)-2-oxo-2H-chromene-3-succinimidyl ester **3** as a yellow solid (319 mg) that was taken forward in the synthesis without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.58 (1H, s), 7.38 (1H, d, J = 9.0 Hz), 6.65 (1H, dd, J_1 = 9.3 Hz, J_2 = 2.7 Hz), 6.46 (1H, d, J = 2.4 Hz), 3.49 (4H, q, J = 7.2 Hz), 2.90 (4H, s), 1.27 (6H, t, J = 6.9 Hz). HRFAB-MS: calculated for [M^+] 358.1156, found 358.1165.

With the exclusion of light, a solution of 7-(diethylamino)-2-oxo-2H-chromene-3-succinimidyl ester (302 mg, 0.84 mmol) in dry DMF (20 mL) was added dropwise by addition funnel over 24 hours, to a solution of *trans*-1,4-cyclohexanediamine (1.94 g, 17.0 mmol) in dry DMF (7 mL) in a 50-mL round bottom flask. The reaction was stirred at room temperature for 24 hours in the dark. A large quantity of yellow precipitate was observed in the reaction flask. The mixture was concentrated to 10 mL and poured into 150 mL of a stirring ice/water slurry to precipitate a yellow solid. The solid was then collected by vacuum filtration, washed with 200 mL water, and dried in air to give **4** as a yellow solid (239 mg, 74% yield). The resultant product was taken forward in the synthesis without further purification. ^{1}H NMR (CD_3OD, 300 MHz): δ 8.52 (1H, s), 7.45 (1H, d, J = 9.0 Hz), 6.73 (1H, dd, J_1 = 9.0 Hz, J_2 = 2.1 Hz), 6.48 (1H, d, J = 2.1 Hz), 3.72 (1H, m), 3.44 (4H, q, J = 7.2 Hz), 2.59 (1H, m), 1.90 (4H, q, J = 11.1 Hz), 1.30 (4H, q, J = 10.2 Hz), 1.14 (6H, t, J = 6.9 Hz). HRFAB-MS: calculated for [MH^+] 358.2128, found 358.2131.

RPF1 dibromo precursor (5). A solution of fluoran succinimidyl ester **2** (95 mg, 0.16 mmol) in dry DMF (1 mL) was added dropwise to a solution of **4** (43 mg, 0.12 mmol) in dry DMF (6 mL) in a 25-mL round bottom flask. The reaction was stirred at room temperature for 24 hours in the dark. A yellow-colored precipitate was observed in the reaction flask. The opaque mixture was poured into 200 mL of a stirring ice/water slurry to precipitate a yellow solid, which was then collected by vacuum filtration, washed with 200 mL water, and dried in air overnight. The resulting solid was dissolved in 5% methanol/dichloromethane, dried over $MgSO_4$, and purified by flash column chromatography (silica gel, 5% methanol/dichloromethane), to afford **5** as a canary yellow solid (79 mg, 77% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.75 (1H, d, J = 8.1 Hz), 8.65 (1H, s), 8.10 (1H, d, J = 8.1 Hz), 8.00 (1H, d, J = 8.1 Hz), 7.53 (2H, s), 7.46 (1H, s), 7.43 (1H, d, J = 9.0 Hz), 7.21 (2H, d, J = 10.2 Hz), 6.69 (2H, d, J = 8.4 Hz), 6.65 (1H, d, J = 8.4 Hz), 6.51 (1H, s), 5.96 (1H, d, J = 8.4 Hz), 3.95 (2H, m), 3.46 (4H, q, J = 6.9 Hz), 2.14 (4H, m), 1.45 (4H, m), 1.25 (6H, t, J = 6.6 Hz). HRFAB-MS: calculated for [MH+] 553.2569, found 553.2579.

Ratio-Peroxyfluor 1, RPF1, (6). Dibromo precursor **5** (39 mg, 0.05 mmol), bis(pinacolato) diboron (72 mg, 0.28 mmol), potassium acetate (30 mg, 0.31 mmol), and Pd(dppf)Cl_2 (11 mg, 0.013 mmol) were dried *in vacuo* overnight in a 25-mL Schlenk tube before adding anhydrous DMF (5 mL) by syringe. The reaction was heated at 80 ºC for 5 hours under a nitrogen atmosphere. The resulting dark brown reaction was cooled to room temperature and poured into 80 mL of a stirring ice/water slurry to precipitate a greenish gray solid, which was then dissolved in dichloromethane and dried over $MgSO_4$. The resulting solution was concentrated and eluted through a 1-cm plug of silica gel with 1% methanol/dichloromethane to give pure **6** as a bright yellow solid (12 mg, 28% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.73 (1H, d, J = 7.8 Hz), 8.65 (1H, s), 8.10 (1H, d, J = 7.8 Hz), 8.03 (1H, d, J = 9.3 Hz), 7.76 (2H, s), 7.45 (2H, dd, J_1 = 6.9 Hz, J_2 = 0.9 Hz), 7.41 (1H, d, J = 9.0 Hz), 7.33 (1H, s), 6.84 (2H, d, J = 7.8 Hz), 6.64 (1H, dd, J_1 = 9.3 Hz, J_2 = 2.4 Hz), 6.50 (1H, s), 5.95 (1H, d, J = 7.8 Hz), 3.92 (2H, m), 3.45 (4H, q, J = 7.8 Hz), 2.10 (4H, m), 1.40 (4H, m), 1.36 (24H, s), 1.24 (6H, t, J = 8.4 Hz). HRFAB-MS: calculated for [MH^+] 936.4440, found 936.4414.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. Spectroscopic measurements were performed in Dulbecco's phosphate buffered saline (DPBS, Invitrogen, Carlsbad, CA) supplemented with 1% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using either a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver or a Jobin Yvon/Spex FluoroMax-2 spectrofluorometer (Edison, NJ) equipped with a xenon lamp. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 and 3.5-mL volume, Starna, Atascadero, CA).

Various reactive oxygen species (ROS) were administered to RPF1 as follows. Experiments employed 10 mM O_2^-, 2 mM for $^1O_2$, and 200 μM for all other ROS. Superoxide (O_2^-) was added as solid KO_2. Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (OCl^-) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) and *tert*-butoxy radical (•O^{t}Bu) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or 200 μM TBHP, respectively. Nitric oxide (NO) was added using NO gas (Matheson) and NO^+ was delivered using *S*-nitrosocysteine (SNOC).[2] Ozone (O_3) was generated by photolysis of O_2 using a Welsbach Ozonator (Philadelphia, PA). Singlet oxygen ($^1O_2$) was generated by photolysis of Sensitox II (polymer-supported Rose Bengal).[3] Briefly, 1 mg of Sensitox II (kindly provided as a gift from Prof. Kris McNeill, University of Minnesota) was suspended in a 1 μM solution of dye in DPBS buffer with 1% FBS, pH 7.4. The mixture was irradiated for 5 min at 25 °C with a 450 W mercury arc lamp powered by an Aceglass power supply. Production of $^1O_2$ under these conditions was calibrated using a colorimetric histidine assay according to a literature protocol.[51] Assuming that each molecule of $^1O_2$ generated oxidizes one molecule of histidine, a lower limit of 2 mM $^1O_2$ is produced within 5 min of irradiation. *Caution: Reactive oxygen species such as singlet oxygen and ozone are highly oxidizing and should be handled with care.*

Mitochondrial Purification from *Saccharomyces cerevisiae*. Mitochondria were isolated from wild-type *S. cerevisiae* using a published protocol.[4] Briefly, 3 L of cells were grown at 30 °C to log-phase (A_{600} = 0.5/mL) in medium containing 1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol. Cells were pelleted by centrifugation at 1500 × g for 5 min and washed once with water. The cell wall was partially destabilized by treating cells at 20 OD_{600}/mL with 100 mM Tris-HCl (pH 9.4) containing 50 mM β-mercaptoethanol for 15 min at 30 °C. Cells were washed once with 100 mL sorbitol buffer (1.2 M sorbitol, 5 mM $MgCl_2$, 20 mM potassium phosphate, pH 7.5) and resuspended in sorbitol buffer with 3 mg/mL Zymolyase 20T (MP Biomedicals, Irvine, CA) to 50 OD_{600}/mL. Cell walls were digested for 60-90 min at 30 °C while spheroplast formation was assayed using phase-contrast light microscopy. Cells were pelleted at 1500 × g for 5 min at 4 °C, washed once with 100 mL ice-cold sorbitol buffer, and resuspended in ice-cold mitochondrial isolation buffer (0.6 M sorbitol, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 20 mM HEPES, pH 7.5) to 50 OD_{600}/mL. Cells were lysed in a dounce homogenizer (Wheaton Scientific, Millville, NJ) with 15 strokes using the tight pestle. The resulting cell lysate was diluted two fold with ice-cold mitochondrial isolation buffer and centrifuged at 3000 × g for 5 min at 4 °C to pellet unlysed cells and other cell debris. Mitochondria were enriched by spinning the cleared lysate at 9500 × g for 10 min at 4 °C. The resulting pellet was resuspended in mitochondrial isolation buffer and snap frozen in liquid nitrogen for storage. Mitochondrial yield was estimated using Bio-Rad protein assay kit (# 500-0006).

Mitochondrial Assays. Aliquots of purified mitochondria (2 mg in 250 μL mitochondrial isolation buffer) from *S. cerevisiae* were thawed for 3-5 min in a 37 °C water bath before use. DPBS (9.0 μL) was added to each microcentrifuge tube, followed by addition of ethanol (40.0 μL, control samples) or Antimycin A (40.0 μL of a 10 mM stock solution in ethanol calibrated to A_2, inhibited samples). All tubes were mixed by gentle inversion (10 times) and incubated for 5 min in a 37 °C water bath. β-NADH (100.0 μL of a 40 mM aqueous solution) was then added to each sample, followed by RPF1 (1.0 μL of a 1 mM stock solution in ethanol). Each microcentrifuge tube was mixed by gentle inversion (10 times) and incubated for 2, 4, 6, or 8 hours in a 37 °C water bath with the exclusion of light. Upon completion of the incubation step, samples were centrifuged at 13,000 rpm (16, 060 x *g*) in a Sorvall BioFuge Pico centrifuge for 15 min at 4°C. A 325.0 μL aliquot of the pink supernatant was removed, diluted with 1% FBS/DPBS (pH 7.4, 1175.0 μL), and used directly for fluorescence assays.

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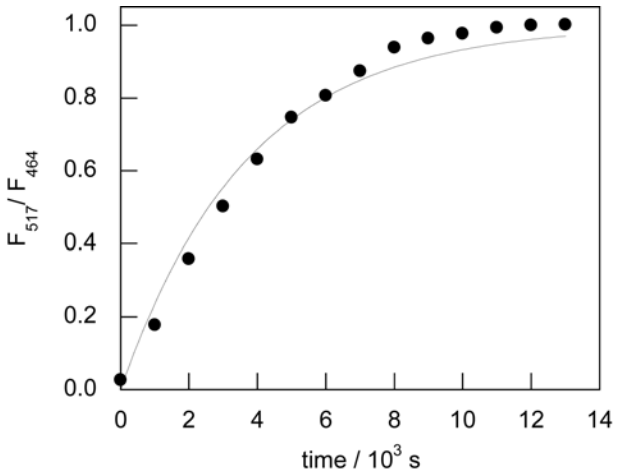

Figure S1. Time-course kinetic measurement of the ratiometric fluorescence response of RPF1 to H_2O_2. Data were collected under pseudo first-order conditions (1 μM RPF1, 1 mM H_2O_2). Spectra were acquired at 25 °C in DBPS buffer with 1% FBS, pH 7.4. Excitation was provided at 420 nm, and the ratio of emission intensities at 517 nm and 464 nm was measured over various time points.