A Fluorescent Sensor for Imaging Reversible Redox Cycles in Living Cells

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Synthetic Materials and Methods. Silica gel P60 (SiliCycle) was used for column chromatography. Analytical thin layer chromatography was performed using SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick). Naphthalene 1,8-disulfide **1** was synthesized according to literature procedures.[1] Cesium carbonate was purchased from Alfa Aesar (Ward Hill, MA) and was used as received. Resorcinol was purchased from MC&B and was used as received. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. ^{1}H NMR spectra were collected in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on 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 using the peak of residual proton signals of $CDCl_3$ or CD_3OD as an internal reference. Mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Naphthalene-1,8-disulfide-2-carboxaldehyde (2a) and Naphthalene-1,8-disulfide-4-carboxaldehyde (2b). The 2- and 4-formyl naphthalene disulfides were synthesized by adding $POCl_3$ (2.0 g, 1.2 mL, 12.9 mmol, 1.75 equiv) dropwise to a stirred solution of disulfide **1** (1.4 g, 7.4 mmol) in dry DMF (7 mL) at 0 ºC under a nitrogen atmosphere. The resulting dark red solution was allowed to warm to room temperature, stirred overnight under nitrogen, and quenched with water to give an orange solid. The residue was dissolved in 300 mL EtOAc and washed three times with brine. The organic layer was dried over Na_2SO_4, filtered, and dry-loaded onto silica gel. Purification by flash column chromatography (silica gel, toluene) provided compound **2a** (872 mg, 54%) as a yellow-orange powder and compound **2b** (420 mg, 26% yield) as an orange powder. Characterization data for **2a**: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.08 (1H, s), 7.70 (1H, d, J = 8.4 Hz), 7.49 (4H, m). FAB-MS: calculated for [M^+] 218, found 218. Characterization data for **2b**: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.17 (1H, s), 8.81 (1H, d, J=6.3 Hz), 7.73 (1H, d, J = 5.9 Hz), 7.53 (1H, t, J = 5.8 Hz), 7.33 (1H, d, J = 5.7 Hz), 7.25 (1H, d, J = 5.7 Hz). FAB-MS: calculated for [M^+] 218, found 218.

6-Hydroxy-9-(4-naphtho-1,8-disulfide)-3*H*-xanthen-3-one, RedoxFluor-1 (RF1) (3). Aldehyde **2** (50 mg, 0.23 mmol) and resorcinol (55 mg, 0.51 mmol, 2.2 equiv) were combined with 4 mL TFA in a heavy-walled reaction tube. The reaction was sealed, heated at 110 ºC for 12 h, and cooled to room temperature. After removing the TFA solvent by rotary evaporation, the residue taken up in 10% methanol/dichloromethane and dry-loaded onto silica gel. Flash column chromatography (silica gel, 10% methanol/dichloromethane) furnished sensor **3** as a dark orange/brown solid (23 mg, 12% yield). ^{1}H NMR (CD_3OD, 300 MHz): δ 8.90 (1H, s), 7.94 (2H, m), 7.27 (1H, m), 7.10 (2H, m), 6.99 (3H, m), 6.60 (2H, m). MALDI-MS: calculated for [MNa^+]: 422, found 422. The membrane-permeable, acetoxymethyl ester of RF1 (RF1-AM) was prepared directly from RF1 by reaction with acetoxymethyl bromide and cesium carbonate in DMF solution. RF1 (12 mg, 0.03 mmol) and Cs_2CO_3 (42 mg, 0.129 mmol) were combined in a hot Schlenk flask and cooled under vacuum. Under a nitrogen atmosphere, 2 mL of anhydrous DMF was added. Acetoxymethyl bromide (5.5 mg, 0.036 mmol) was added to the basic RF1 solution

in one portion, and the reaction was stirred at room temperature overnight in the dark under nitrogen. The mixture was filtered and lyophilized to give RF1-AM as a dark orange powder that was used without further purification. ESI-MS: calculated for [M^+] 472.5, found 472.5.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7. Absorption spectra were recorded on a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded on 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. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (3.5-mL volume, Starna, Atascadero, CA). The fluorescence quantum yield for oxidized RF1 was determined in 20 mM HEPES, pH 7, and referenced to fluorescein in 0.1 N NaOH ($\Phi = 0.95$).[2] For *in vitro* redox cycling experiments, 5 μM solutions of RF1 were treated with 25 μM TCEP followed by 100 μM H_2O_2 for the indicated times. The observed rate constant for oxidation of RF1 by H_2O_2 represents the average of at least three independent experiments.

Preparation and Staining of Cell Cultures. HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), and glutamine (2 mM). One day before imaging, cells were passed and plated on 25-mm glass coverslips. Immediately before the experiments, cells were washed with PBS buffer, incubated with the probe in PBS, and imaged.

Fluorescence Imaging Experiments. Confocal fluorescence imaging was performed with a Zeiss LSM510 NLO Axiovert 200 laser scanning microscope and a 40×/0.8 NA water-immersion objective lens. Cells were incubated with membrane-permeable RF1-AM, which is subsequently converted to RF1 by intracellular esterases. Excitation of RF1-stained cells at 488 nm was carried out with an Argon laser at 0.5% power, and emission was collected using a META detector from 495-559 nm with the gain set at 600. The pinhole was set at 108 μm, corresponding to 1 Airy unit and the pixel dwell time was 1.60 μs. Images for all experiments were acquired using identical settings on the microscope to ensure reproducibility. H_2O_2 was added by bath application on the stage. For semi-quantitative analysis of the fluorescence intensity of the cells, the average fluorescence intensity within the cells was determined by selecting an ROI and analyzing the corresponding histogram using Adobe Photoshop 6.0. Temperature was maintained using a Warner Instruments (Hamden, CT) chamber, platform and temperature controller (RC-21BR, PH-2, and TC-344). Relative fluorescence intensities are the average of at least three experiments.

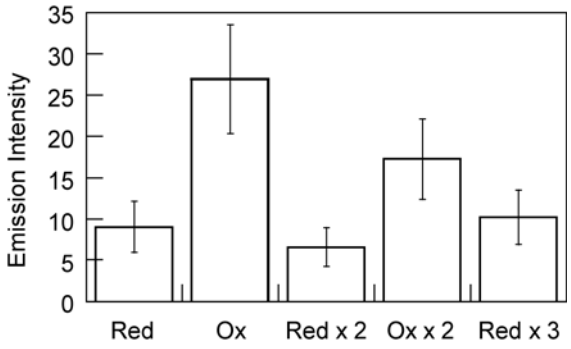

Figure S1. Relative fluorescence intensities of RF1-labeled HEK 293T cells undergoing multiple oxidation-reduction cycles. Each bar and its corresponding error bar represents confocal fluorescence microscopy data collected from at least three separate imaging experiments (n = 3). Initial fluorescence turn off of RF1 in cells by the reducing intracellular environment (Red) within 30 min at 37 °C was monitored followed by oxidation of intracellular RF1 (Ox) by 100 μM H_2O_2 within 5-10 min. Subsequent restoration of the cellular reducing environment after an additional 5-10 min ensues (Red x 2). Adding a second aliquot of 100 μM H_2O_2 triggers oxidative stressand re-oxidation of the probe within 5-10 min (Ox x 2), followed by restoration of the reducing cellular environment after 5-10 additional min (Red x 3). Excitation was provided at 488 nm and emission intensity was collected from 495-559 nm using a META detector.

References

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