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New Chemical Tools for Fluorescent Detection of Hydrogen Peroxide in Living Cells

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New Chemical Tools for Fluorescent Detection of Hydrogen Peroxide in Living Cells

by

Duangkhae Srikun

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Chemistry
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:

Professor Christopher J. Chang, Chair
Professor Richmond Sarpong
Professor Michelle C. Chang
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New Chemical Tools for Fluorescent Detection of Hydrogen Peroxide in Living Cells

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Abstract

New Chemical Tools for Fluorescent Detection of Hydrogen Peroxide in Living Cells

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Duangkhae Srikun

Doctor of Philosophy in Chemistry

University of California, Berkeley

Professor Christopher J. Chang, Chair

As one of the toxic by-product of aerobic metabolism, hydrogen peroxide (H₂O₂), at uncontrolled levels and distributions, is a sign of oxidative stress, aging and disease. However, H₂O₂ also plays an essential part in normal physiological system. H₂O₂ levels are regulated by many enzymes and metabolites that generate or break-down H₂O₂. In macrophages, the presence of invading pathogens activates the production of microbicidal levels of H₂O₂ by NADPH oxidase (Nox). Isoforms of Nox are expressed in many non-phagocytic cells and tissues. Nox-generated H₂O₂ is a secondary messenger involved in signaling for growth, proliferation, differentiation and controlled cell death; these variations in downstream biological effects are regulated by both the spatial and temporal production of H₂O₂. Small molecule fluorescent probes bearing boronate ester moieties have been developed for chemoselective detection of H₂O₂ in both oxidative stress levels and cellular signaling events. This dissertation describes the design, synthesis, characterization and application of new boronate-based fluorescent probes with added functionality. Peroxy-Lucifer-1 (PL1) and Peroxy-Naphthalene-1 (PN1) are ratiometric fluorescent probes that can detect oxidative bursts in immune response events. Ratiometric probes allow simultaneous detection of two signals from the reacted and unreacted probes in the same sample, providing a built–in correction for variations such as uneven probe loading, sample environment and detection efficiency. PN1 also has a high two-photon cross section. The increased penetration depth of near-infrared excitation light allows the detection of H₂O₂ in tissue specimens with PN1. SNAP-Peroxy-Green-1 (SPG1) and SNAP-Peroxy-Green-2 (SPG2) are capable of detecting local concentration of H₂O₂ in subcellular compartments such as mitochondria, endoplasmic reticulum, nucleus, and plasma membrane. The precise localization of probes to the targeted organelle is facilitated by highly specific recognition of the SNAP ligand bound to the probe by the SNAP fusion protein. Furthermore, simultaneous detection of H₂O₂ at two different locations is feasible by using a SNAP tag with an orthogonal CLIP tag; such combined use of SNAP and CLIP tags is assisted by the expanding color palette of SNAP and CLIP peroxo probes. Multi-modal probes using PAMAM-G5 dendrimer platform was developed for the real-time imaging of the interplay between H₂O₂ and other physiological events. Coordination of the oxidative burst and progressive acidification in phagosomes of macrophages was elucidated with G5-SNARF2-PF1-Ac, a nanoprobe decorated with the H₂O₂ sensing module PF1 and pH sensor SNARF2.
For my parents
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Chapter 1
An ICT-Based Approach to Ratiometric Fluorescence Imaging of Hydrogen Peroxide Produced in Living Cells

Portions of this work were published in the following scientific journal:

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Evan Miller and Dylan Domaille assisted in confocal fluorescence imaging.
Abstract

We present the synthesis, properties, and biological applications of Peroxy Lucifer (PL), a new series of fluorescent probe for imaging H₂O₂ produced in living cells by a ratiometric response. PL probes utilize a chemoselective boronate-based switch to detect H₂O₂ by modulation of internal charge transfer (ICT) within a 1,8-naphthalimide dye. PL features high selectivity for H₂O₂ over similar reactive oxygen species, including superoxide and nitric oxide, and a 65 nm shift in emission from blue-colored fluorescence to green-colored fluorescence upon reaction with H₂O₂. Confocal microscopy experiments in live macrophages show that PL probes can ratiometrically visualize localized H₂O₂ bursts generated in living cells at immune response levels.

Introduction

The chemistry of hydrogen peroxide (H₂O₂) in living systems is receiving increasing attention owing to its diverse contributions as a marker for oxidative stress and damage events associated with aging and disease,¹ as a second messenger for cellular signal transduction,²⁻⁴ or as a killing agent generated by immune cells to combat microbial invasion.⁵ A key step towards elucidating the complex biological roles of this reactive oxygen metabolite is the development of new methods to monitor dynamic changes in peroxide concentrations generated within localized regions of the cell, as spatial and temporal variations in cellular H₂O₂ flows can lead to dramatically different physiological or pathological consequences. Small-molecule reporters offer one approach to meet this need⁶ and indicators that give a turn-on emission increase in response to H₂O₂ have been reported and applied for the study of peroxide biology.⁷⁻¹⁵ Despite advances in the development of new chemical probes, a limitation of intensity-based probes is that variability in excitation and emission efficiency, sample environment, and probe distribution pose potential problems for use in quantitative measurements. In contrast, ratiometric probes provide the practical advantage of built-in corrections for such variants by allowing simultaneous detection of two signals resulting from reacted and unreacted forms of the probe in the same sample.¹⁶ Our approach to ratiometric fluorescence detection of cellular H₂O₂ relies on controlling internal charge transfer (ICT) within a dye platform to promote a change in its emission color upon reaction with H₂O₂. Specifically, modulation of the electron-donating amine at the 4-position on a 1,8-naphthalimide (e.g., Lucifer Yellow) affects both ICT and emission color, as making this substituent more electron deficient results in ICT-induced blue shifts in emission maxima. We reasoned that modifying the 4-amino donor into a more electron-withdrawing carbamate group that could be specifically decaged by H₂O₂ back to the amine would provide a switch for ratiometric detection of H₂O₂, a strategy that is inspired by indicators for pH,¹⁷ anions,¹⁸ metal ions,¹⁹,²⁰ and sugars.²¹

We now present the synthesis and application of Peroxy Lucifer (PL), a new series of ratiometric fluorescent reporters for imaging H₂O₂ produced in living systems. Owing to its dual emission readout, PL probes are capable of visualizing highly localized changes in H₂O₂ concentrations generated by live cells in response to phagocytic stimulation while retaining the ability to spatially monitor relative [H₂O₂] fluxes throughout the rest of the sample.

Results and Discussion

Design and Synthesis of Peroxy Lucifer (PL). Previously, our lab has developed Ratio-Peroxyfluor-1 (RPF1), a ratiometric fluorescent sensor for H₂O₂ based on a fluorescence
resonance energy transfer (FRET) mechanism between a coumarin donor and a boronate-protected fluorescein receptor. RPF1 possess a large dynamic range; the green to blue emission ratio changes from 0.45 in the absence of H2O2 to 3.7 after treatment with 200 µM H2O2 for 1 h. However, RPF1 has limitations in live-cell imaging due to its poor membrane permeability resulting from the rigidity of the compound structure. In the PL series of probes, we utilized the manipulation of the ICT state as the mechanism for ratiometric fluorescence response (Scheme 1). The ICT probes require only one fluorophore which allows for a smaller and less rigid probe. In the uncaged naphthalimide probe, Lucifer Yellow (LY), the fluorophore contains both an electron donating and an electron withdrawing group, which results in greater stabilization and lower energy of the internal charge transfer excited state. However, when the amino group is caged by the carbamate moiety, the push-pull mechanism of the ICT state is disrupted, giving a blue-shifted fluorescence emission from the higher energy excited state.

We synthesized Peroxy-Lucifer-0 (PL0) starting from the commercially available 4-amino-1,8-naphthalic anhydride (Scheme 2). Lucifer-Yellow-0 (LY0) was obtained from condensation of naphthalic anhydride and alkylamine under microwave irradiation. The H2O2 responsive 4-(dihydroxyboryl)benzoxycarbonyl (Dobz)22 was installed by the reaction of isocyanate generated in situ by refluxing LY0 with triphosgene in dry toluene, with compound 4.

Observing that LY0 and PL0 display a tendency for aggregation in aqueous buffer, we proceeded to synthesize LY1 and PL1, replacing butylamine with more hydrophilic 2-(2-aminoethoxy)ethanol. In diversifying the alkylamine substitution (PL2 - PL5), we have revised the synthetic route (Scheme 3-5). The overall yield is greatly improved by masking the 4-amino group as 4-nitro until the very last step before carbamate formation. The 4-nitro substitution eliminates the hydrogen bonding found in the 4-amino substitution, resulting in better solubility and ease of purification in organic solvent.

**Spectroscopic Properties and Optical Responses to H2O2.** We assessed the spectroscopic properties of PL dyes and their uncaged LY dyes under physiological-like conditions (20 mM HEPES, pH 7.4) (Table 1). In the absence of H2O2, PL displays one major absorption band centered at 375 nm with a corresponding blue-colored fluorescence maximum at 475 nm. The relative blue-shift of these absorption and emission features compared with the uncaged LY dyes (λabs = 475 nm, λem = 545 nm) is consistent with ICT involving the relatively electron-poor carbamate donor. Alkyl chloride and carboxylic acid ester functional group did not affect the spectroscopic properties of PL and LY dyes. However, maleimide functionality in PL5 and LY5 results in a marked fluorescence quenching (Φ < 0.1). Addition of glutathione (1 mM) results in an instantaneous increase in fluorescence emission in both PL5-GSH (Φ = 0.47), and LY5-GSH (Φ = 0.33). The thiol-ene reaction of PL5 with GSH23,24 can be used for trappability of probes, in addition to the improved signal-to-noise due to the low quantum yield of PL5 with unreacted maleimide.

Reaction of PL probes with H2O2 triggers chemoselective cleavage of the boronate-based carbamate protecting group to deliver the green-fluorescent LY dyes as characterized by its absorption (λabs = 435 nm) and emission (λem = 540 nm) spectra, respectively (Figure 2). All PL probes showed similar responses to H2O2 except PL3 that displayed slower response to H2O2 in both absorption and fluorescence emission profile. In this case, it is likely that the hydrophobic butylchloride moiety in PL3 induces microscopic aggregation, decreasing the interface area between PL3 and H2O2. Under pseudo-first-order conditions (1 µM PL1 and 1 mM H2O2), the
observed rate constant for H$_2$O$_2$ deprotection is $k_{\text{obs}} = 8.8 \times 10^{-4}$ s$^{-1}$ (Figure 3). The ratiometric emission response of PL1 is highly selective for H$_2$O$_2$ over other reactive oxygen species (Figure 4). Compared to reactions with H$_2$O$_2$, the ratio of carbamate- to amine-substituted naphthalimides does not change appreciably with tert-butylhydroperoxide, hypochlorite, superoxide, singlet oxygen, nitric oxide, hydroxyl radical, or tert-butoxy radical. Moreover, we demonstrated that the fluorescence emission profile of PL1 and LY1 was stable across a biologically relevant pH range (Figure 5).

**Fluorescence Detection of H$_2$O$_2$ in Living Cells using Confocal- and Two-Photon Microscopy.** PL and LY dyes, like other ICT based fluorescent dyes display solvatochromism (Figure 6), therefore the fluorescence emission profile can be affected by subcellular localization of chromophore. This phenomena is demonstrated by the uptake of PL3 into 3T3-L1 adipocytes (Figure 7). The fluorescence emission from PL3 in lipid droplets showed higher $F_{\text{green}}/F_{\text{blue}}$ ratio correlated with the blue-shifted emission maxima of PL3 in the more hydrophobic environment. Treatment of PL3-labeled adipocytes with 200 µM H$_2$O$_2$ for 30 min resulted in the overall increase $F_{\text{green}}/F_{\text{blue}}$ emission ratio (Figure 8). The ability of PL probes to detect rising H$_2$O$_2$ concentration in cellular environments was demonstrated in the ratiometric change in fluorescence emission when HEK293T loaded with PL probes were treated with 200 µM H$_2$O$_2$ for 30 min (Figure 9). $F_{\text{green}}/F_{\text{blue}}$ varied among the PL probes, the result of the different subcellular localization induced by the functional side chain. $F_{\text{green}}/F_{\text{blue}}$ is governed by the hydrophobicity of the side chain. PL3 and LY3, with their butylchloride group, displayed the lowest $F_{\text{green}}/F_{\text{blue}}$, and mostly localized to membrane-bound structures such as golgi apparatus and endoplasmic reticulum (Figure 10). On the other hand, PL5 and LY5 displayed the highest $F_{\text{green}}/F_{\text{blue}}$ because PL5 and LY5 can react with glutathione rapidly to generate tripeptide conjugates which help retaining the probes within cytoplasm. The difference in $F_{\text{green}}/F_{\text{blue}}$ of PL3 and PL5 were elaborated by images obtained from lambda mode scanning. The normalized emission spectra showed PL3 and PL5 sharing the same emission maximum at 478 nm, however with less intensity in green emission wavelength from PL3 (Figure 11). The difference between LY3 and LY5 is more dramatic with LY3 showing emission maximum at 517 nm and LY5 showing red-shifted emission maximum at 536 nm (Figure 12).

Having established the ratiometric response of PL probes to bath application of H$_2$O$_2$ in cell cultures, we then tested the ability of PL probes to detect endogenous bursts of H$_2$O$_2$ produced within living cells. Treatment of RAW24.7 macrophage cells with phorbol myristate acetate (PMA) rapidly induces phagocytosis; the event is associated with a dramatic change in cell morphology from round to amoeboid shape packed with phagosomes, which are also visible in brightfield images. To detect the oxidative burst in phagosome, the probe must be hydrophilic enough to remain in solution phase, otherwise the rapid insertion of hydrophobic probe into plasma membrane will give a population of phagosomes without fluorescence emission signal. RAW264.7 cells were incubated with PMA (1 µg/mL) and either PL1 or PL4 (5 µM, in DMEM) for 15 min; then washed twice with fresh buffer. Confocal fluorescence images show a heterogenous population of dormant round cells and stimulated amoeboid-like cells. Both PL1 (Figure 13) and PL4 (Figure 14) were able to detect the oxidative burst within phagosome, reflecting the higher green emission from phagosome compared to the rest of the cell body as shown in images obtained from channel mode and lambda mode.

The ratiometric fluorescence imaging with PL1 is also demonstrated using two-photon excitation at 820 nm. The ratio image constructed from 535 - 600 nm (green) and 430 - 495 nm (blue) fluorescence collection windows using ImageJ software gave $F_{\text{green}}/F_{\text{blue}}$ of 0.6 in PL1-
loaded RAW264.7 cells (Figure 15a). Treatment cells with 100 μM H$_2$O$_2$ for 60 min resulted in an increase F$_{\text{green}}$/F$_{\text{blue}}$ to 1.0 (Figure 15b), consistent with H$_2$O$_2$-mediated boronate cleavage occurring within these cells. Stimulating PL1-loaded macrophages with PMA induced phagocytosis-associated H$_2$O$_2$ generation and showed an increase in F$_{\text{green}}$/F$_{\text{blue}}$ localized within the phagocytic vesicles compared to other intracellular regions (Figure 15c). Flow cytometry of RAW264.7 incubated with PL1 for 6 h showed no toxicity as there was negligible change in percentage of dead cells stained by SYTOX green compared to control sample (Figure 16).

**Concluding Remarks**

In summary, we have presented the synthesis, properties, and live-cell evaluation of a new type of ratiometric fluorescent probe for H$_2$O$_2$. PL features a chemospecific H$_2$O$_2$ switch to modulate ICT with a marked blue-to-green emission color change. Experiments in macrophages show that PL probes are capable of live-cell imaging of H$_2$O$_2$ at natural immune response level. Moreover, the ratiometric read-out provided by this probe allows for detection of highly localized changes in H$_2$O$_2$ concentrations within phagosomes while at the same time visualizing [H$_2$O$_2$] variations throughout the rest of the cytoplasm.

**Experimental Section**

**Synthetic Materials and Methods.** Silica gel P60 (SiliCycle) was used for column chromatography. Microwave reactions were performed using a CEM Intelligent Explorer/Discover (Matthews, NC). 4-Amino-1,8-naphthalic anhydride and catalase from bovine liver were purchased from Sigma Aldrich (St. Louis, MO). NOC5 (3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-1-propanamine) was purchased from Calbiochem (La Jolla, CA). 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde was purchased from Oakwood (West Columbia, SC). Live/Dead cell vitality assay kit with C$_{12}$-resazurin and SYTOX green was purchased from Invitrogen/Molecular Probes (Carlsbad, CA). 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 DMSO-$d_6$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on a Bruker AVQ-400 or a Bruker AV-300 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. Low resolution mass spectral analyses were carried out with Agilent Technology 1200 series with 6130 Quadrupole LC/MS (Santa Clara, CA).

A: General procedure for synthesis of 4-nitro-N-alkynaphthalimide (7, 11, 12, 18) through condensation of 4-nitro-1,8-naphthalic anhydride with primary amine under microwave irradiation. A heavy-walled microwave tube equipped with a small magnetic stir bar was charged with 4-nitro-1,8-naphthalic anhydride (200 - 400 mg), followed by ethanol (maximum volume 4 mL for 20 mL microwave tube), and primary amine (1.1 eq). The reaction tube was then sealed by a plastic snap cap. Naphthalic anhydride in ethanol was a heterogeneous mixture at room temperature and became soluble during the reaction. However, if there were many big chunks of solid, the reaction tube was sonicated for several minutes. The reaction tube was placed into a microwave reactor which programmed to run at 120 °C for 60 min. The
naphthalimide product normally precipitated out once the reaction tube was cooled to room temperature. The reaction tube was further cooled in an ice bath; the precipitate of the first crop was collected by vacuum filtration and washed with ice-cold methanol. The filtrate was concentrated by a rotary evaporator; the second crop normally needed purification with flash chromatography.

B: General procedure for synthesis of 4-amino-\(\text{N}\)-alkynaphthalimide (9, 12, 15) by hydrogenation of 4-nitro-\(\text{N}\)-alkynaphthalimide. A round-bottomed Schlenk flask equipped with a magnetic stir bar was charged with solution of 4-nitro-\(\text{N}\)-alkynaphthalimide in ethanol, followed by palladium doped carbon (Pd/C, 0.1 eq). The flask was sealed with a rubber septum, and put under vacuum briefly. A hydrogen-filled balloon was connected to the reaction flask through a needle vault. The reaction was stirred overnight. As the reaction progressed, the mixture developed more intense yellow-green color of the product. TLC showed product as yellow spot with green fluorescence emission under 365 nm UV lamp compared to the non-fluorescent and less polar starting material. Pd/C was filtered off and the filtrate was concentrated to dryness giving product as yellow solid. The product obtained was > 95 % clean by NMR and was used in the next step without further purification.

C: General procedure for synthesis of PL series (5, 6, 10, 16, 17, 21) from LY through in situ formation of isocyanate with triphosgene. To a mixture of LY (1 eq) and DMAP (3 eq) in toluene or acetonitrile (5 - 10 mL/50 mg LY) in an oven-dried two-necked flask equipped with a rubber septum and a condenser with nitrogen inlet was added a solution of triphosgene (0.35 eq) in toluene, dropwise. The solution was heated to reflux for 3 h. The formation of isocyanate was monitored by TLC; a drop of reaction mixture was taken into methanol to generate methoxy carbamate that emitted blue fluorescence under 365 nm UV compared to the green fluorescence LY starting material. After cooling to room temperature, the reaction mixture was diluted with dry CH\(_2\)Cl\(_2\) (6 mL). Insoluble materials were removed by passing the solution through a glass pipette plugged with a ball of glass wool. To the filtrate was added \(\text{4} \) (1 eq), and the solution was stirred at room temperature for 3 h. The reaction mixture was concentrated by a rotary evaporator and purified by flash chromatography to give product as a yellow solid.

4-Amino-\(\text{N}\)-butynaphthalimide (1), LY0. This compound was prepared by the modification of a literature procedure.\(^{18}\) A mixture of 4-amino-1,8-naphthalic anhydride (0.50 g, 2.3 mmol), \(n\)-butylamine (1 mL, 1.0 mol), triethylamine (1 mL, 0.7 mol) and DMF (5 mL) was heated at 150 °C in a heavy-walled flask for 24 h. After cooling to room temperature, the solution was poured into 5% KHSO\(_4\) to precipitate the desired product. Purification by flash chromatography (1:1, EtOAc/CH\(_2\)Cl\(_2\)) gave product as a yellow solid (0.53 g, 2.0 mmol, 84%). \(^1\)H NMR (DMSO-\(d_6\), 300 MHz): \(\delta\) 0.88 (3H, t, \(J = 6.9\) Hz), 1.31 (2H, m), 1.55 (2H, m), 3.98 (2H, t, \(J = 6.9\) Hz), 6.81 (1H, d, \(J = 8.4\) Hz), 7.41 (2H, s), 7.62 (1H, t, \(J = 8.1\) Hz), 8.16 (1H, d, \(J = 8.4\) Hz), 8.39 (1H, d, \(J = 8.1\) Hz), 8.58 (1H, d, \(J = 8.1\) Hz).

4-Amino-\(\text{N}\)-(2-(2-hydroxyethoxy)ethyl)naphthalimide (2). A mixture of 4-amino-1,8-naphthalic anhydride (400 mg, 1.9 mmol) and 2-(2-aminoethoxy)ethanol (250 μL, 2.5 mmol) in DMF (3 mL) in a heavy-walled test tube was heated by microwave irradiation for 90 min at 150 °C. After cooled to room temperature, the solution was poured into 5% KHSO\(_4\) to precipitate the desired product. Purification by flash chromatography (1:1, EtOAc/CH\(_2\)Cl\(_2\)) gave product as a yellow solid (502 mg, 1.7 mmol, 89% yield). \(^1\)H NMR (DMSO-\(d_6\), 400 MHz): \(\delta\) 8.58 (1H, d, \(J = 8.0\) Hz), 8.39 (1H, d, \(J = 8.0\) Hz), 8.15 (1H, d, \(J = 8.4\) Hz), 7.63 (1H, t, \(J = 8.1\)Hz), 7.44 (2H, s), 6.80 (1H, d, \(J = 8.4\) Hz), 4.55 (1H, s-br), 4.16 (2H, t, \(J = 6.0\) Hz), 3.57 (2H, d, \(J = 6.0\) Hz), 3.42 (m, 4H).
4-Amino-N-(2-(2-acetoxyethoxy)ethyl)naphthalimide (3), LY1. A mixture of 2 (100 mg, 0.33 mmol) and acetic anhydride (40 μL, 0.39 mmol) in pyridine (2 mL) was stirred at room temperature overnight. The solvent was removed under reduced pressure. Purification by flash column chromatography (1:1, EtOAc/CH₂Cl₂) gave product as an orange solid (60 mg, 0.18 mmol, 53% yield). \(^1\)H NMR (DMSO-\text{d}_6, 400 MHz): \(\delta 8.58 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.39 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.15 (1\text{H}, \text{t}, \text{J} = 8.1 \text{ Hz}), 7.44 (2\text{H}, \text{s}), 6.80 (1\text{H}, \text{d}, \text{J} = 8.4 \text{ Hz}), 4.16 (2\text{H}, \text{t}, \text{J} = 6.0 \text{ Hz}), 4.02 (2\text{H}, \text{d}, \text{J} = 6.0 \text{ Hz}), 3.60 (4\text{H}, \text{m}), 1.87 (3\text{H}, \text{s}).

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzylalcohol (4). 27 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (5 g, 21 mmol) was dissolved into methanol (50 mL), and the solution was cooled in an ice-bath. Sodium borohydride (850 mg, 22 mmol) was added in small portions to the reaction mixture over 10 min. The reaction was allowed to warm to room temperature in 30 min. Once the reaction was completed by TLC, ice-cold water (2 mL) was added to the reaction mixture, dropwise. The reaction was concentrated by a rotary evaporator, taken into EtOAc (100 mL), washed with water (2 x 100 mL) and brine. The organic layer was dried over Na₂SO₄ and concentrated to give product as a clear oil which solidified as white waxy solid at room temperature (4.5 g, 19 mmol, 89% yield). \(^1\)H NMR (CDCl₃, 400 MHz): \(\delta 7.80 (2\text{H}, \text{d}, \text{J} = 7.8 \text{ Hz}), 7.36 (2\text{H}, \text{d}, \text{J} = 7.8 \text{ Hz}), 4.71 (2\text{H}, \text{s}), 1.34 (12\text{H}, \text{s}).

4-(p-Dihydroxylborylbenzyloxycarbamyl)-N-butynaphthalimide (5), PL0. Procedure C: LY0 (50 mg, 0.18 mmol), DMAP (55 mg, 0.56 mmol), toluene (5 mL), triphosgene (55 mg, 0.18 mmol), and 4 (42 mg, 0.18 mmol). Flash chromatography (1:1, EtOAc/CH₂Cl₂) gave product as a yellow solid (34 mg, 0.064 mmol, 35%). \(^1\)H NMR (CDCl₃, 400 MHz): \(\delta 8.61 (1\text{H}, \text{d}, \text{J} = 7.6 \text{ Hz}), 8.57 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.36 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.17 (1\text{H}, \text{d}, \text{J} = 8.4 \text{ Hz}), 7.84 (2\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 7.73 (1\text{H}, \text{t}, \text{J} = 8.0 \text{ Hz}), 7.58 (1\text{H}, \text{s}), 7.43 (2\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 5.28 (2\text{H}, \text{s}), 4.14 (2\text{H}, \text{t}, \text{J} = 7.6 \text{ Hz}), 1.69 (2\text{H}, \text{m}), 1.46 (2\text{H}, \text{m}), 1.42 (12\text{H}, \text{s}), 0.97 (3\text{H}, \text{t}, \text{J} = 7.6 \text{ Hz}). 13C NMR (CDCl₃, 400 MHz): \(\delta 164.1 (\text{C}=\text{O}), 163.6 (\text{C}=\text{O}), 152.9 (\text{C}=\text{O}), 141.8, 138.8, 138.1, 135.1, 132.5, 131.2, 128.9, 127.7, 126.6, 125.8, 123.5, 122.9, 117.9, 116.7, 83.9, 67.8, 40.2, 30.2, 24.8 (4), 20.5, 13.8. HRFAB-MS: calculated for [MH⁺] 529.24, found 529.25.

4-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxycarbamyl)-N-(2-(2-acetoxyethoxy)ethyl)naphthalimide (6), PL1. Procedure C: LY1 (60 mg, 0.17 mmol), DMAP (64 mg, 0.52 mmol), toluene (5 mL), triphosgene (52 mg, 0.17 mmol), and 4 (42 mg, 0.18 mmol). Flash column chromatography (1:1, EtOAc/CH₂Cl₂) gave PL1 as a yellow solid (40 mg, 0.066 mmol, 38% yield). \(^1\)H NMR (CDCl₃, 400 MHz): \(\delta 8.62 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.60 (1\text{H}, \text{d}, \text{J} = 8.4 \text{ Hz}), 8.40 (1\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 8.17 (1\text{H}, \text{d}, \text{J} = 8.4 \text{ Hz}), 7.86 (2\text{H}, \text{d}, \text{J} = 7.6 \text{ Hz}), 7.77 (1\text{H}, \text{t}, \text{J} = 8.0 \text{ Hz}), 7.43 (2\text{H}, \text{d}, \text{J} = 8.0 \text{ Hz}), 4.44 (2\text{H}, \text{t}, \text{J} = 6.0 \text{ Hz}), 3.84 (2\text{H}, \text{t}, \text{J} = 6.0 \text{ Hz}), 3.75 (2\text{H}, \text{t}, \text{J} = 4.4 \text{ Hz}), 1.98 (3\text{H}, \text{s}), 1.36 (12\text{H}, \text{s}). 13C NMR (CDCl₃, 400 MHz): \(\delta 171.3, 164.5, 164.0, 153.2, 139.2, 138.4, 138.6, 135.1, 132.5, 131.2, 128.9, 127.7, 126.6, 125.8, 123.5, 122.9, 117.9, 116.7, 84.2, 77.5, 68.7, 68.2, 64.0, 39.2, 25.1, 21.2. HRFAB-MS: calculated for [MH⁺] 603.24, found 603.25.

4-Nitro-N-(2-(2-hydroxyethoxy)ethyl)naphthalimide (7). Procedure A: 4-nitro-1,8-naphthalic anhydride (1.0 g, 4.1 mmol), 2-(2-hydroxyethoxy)ethylamine (430 mg, 4.1 mmol). Purification by flash column chromatography (1:1, EtOAc/CH₂Cl₂) gave product as a pale yellow solid (1.0 g, 3.0 mmol, 75% yield). \(^1\)H NMR (CDCl₃, 300 MHz): \(\delta 8.80 (1\text{H}, \text{d}, \text{J} = 8.7 \text{ Hz}), 8.71 (1\text{H}, \text{d}, \text{J} = 7.2 \text{ Hz}), 8.66 (1\text{H}, \text{d}, \text{J} = 7.8 \text{ Hz}), 8.37 (1\text{H}, \text{d}, \text{J} = 7.8 \text{ Hz}), 7.96 (1\text{H}, \text{dd}, \text{J}_1 = 8.7 \text{ Hz}, \text{J}_2 = 7.2 \text{ Hz}), 4.43 (2\text{H}, \text{t}, \text{J} = 5.4 \text{ Hz}), 3.84 (2\text{H}, \text{t}, \text{J} = 5.4 \text{ Hz}), 3.63 (4\text{H}, \text{s}).

4-Nitro-N-(2-(2-chloroethoxy)ethyl)naphthalimide (8). To a mixture of 7 (200 mg, 0.61 mmol) and DMAP (220 mg, 1.8 mmol) in toluene (4 mL) in a dried two-necked flask
equipped with a rubber septum and a condenser with nitrogen inlet was added a solution of triphosgene (179 mg, 0.60 mmol) in one portion. The solution was heated to reflux for 3 h. After cooling to room temperature, the reaction mixture was concentrated. The crude product was purified by flash chromatography (2:1, CH₂Cl₂/EtOAc) to give product as a pale yellow solid (69 mg, 0.20 mmol, 33% yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.86 (1H, d, J = 8.8 Hz), 8.75 (1H, d, J = 6.4 Hz), 8.71 (1H, d, J = 8.0 Hz), 8.42 (1H, d, J = 8.0 Hz), 8.00 (1H, dd, J₁ = 8.8 Hz, J₂ = 7.2 Hz), 4.47 (2H, t, J = 6.0 Hz), 3.89 (2H, t, J = 5.6 Hz), 3.79 (2H, t, J = 6.0 Hz), 3.58 (2H, t, J = 5.6 Hz).

4-Amino-N-(2-(2-chloroethoxy)ethyl)naphthalimide (9), LY2. Procedure B: Product was obtained as a yellow solid. ¹H NMR (DMSO-d₆, 400 MHz): δ 8.58 (1H, d, J = 8.0 Hz), 8.39 (1H, d, J = 6.8 Hz), 8.15 (1H, d, J = 8.4 Hz), 7.63 (1H, t, J = 8.0 Hz), 6.80 (1H, d, J = 8.4 Hz), 4.17 (2H, t, J = 6.4 Hz), 3.65 (8H, m).

4-(p-Dihydroxylborylbenzyloxycarbamyl)-N-(2-(2-chloroethoxy)ethyl)naphthalimide (10), PL2. Procedure C: LY2 (40 mg, 0.12 mmol), DMAP (30 mg, 0.25 mmol), toluene (5 mL), triphosgene (20 mg, 0.067 mmol), and 4 (30 mg, 0.12 mmol). Flash chromatography (1:3, EtOAc/CH₂Cl₂) gave product as a yellow solid (30 mg, 0.052 mmol, 43 % yield). ¹H NMR (CDCl₃, 400 MHz): δ 8.63 (1H, d, J =7.6 Hz), 8.60 (1H, d, J =8.4 Hz), 8.40 (1H, d, J = 8.4 Hz), 8.17 (1H, d, J = 8.4 Hz), 7.86 (2H, d, J = 8.0 Hz), 7.77 (1H, dd, J₁ = 8.4 Hz, J₂ = 7.6 Hz), 7.47 (1H, s), 7.45 (2H, d, J = 8.0 Hz), 5.32 (2H, s), 4.44 (2H, t, J = 6.0 Hz), 3.86 (2H, t, J = 6.0 Hz), 3.59 (2H, t, J = 6.0 Hz), 1.36 (12H, s). HRFAB-MS: calculated for [MH⁺] 579.19, found 579.20.

4-Nitro-N-(3-chloropropyl)naphthalimide (11). Procedure A: 4-nitro-1,8-naphthalic anhydride (250 mg, 1.1 mmol), 3-chloro propylamine hydrochloride (140 mg, 1.1 mmol), triethylamine (200 µL). Purification by flash column chromatography (1:10, EtOAc/CH₂Cl₂) gave product as a pale yellow solid (250 mg, 0.78 mmol, 71% yield). ¹H NMR (CDCl₃, 300 MHz,): δ 8.84 (1H, d, J = 8.7 Hz), 8.74 (1H, d, J = 7.8 Hz), 8.69 (1H, d, J = 8.1 Hz), 7.99 (1H, dd, J₁ = 8.7 Hz, J₂ = 7.5 Hz), 4.36 (2H, t, J = 6.9 Hz), 3.58 (2H, t, J = 6.6 Hz), 2.24 (2H, m).

4-Nitro-N-(4-carboxybutyl)naphthalimide (12). Procedure A: 4-nitro-1,8-naphthalic anhydride (250 mg, 1.1 mmol), 4-aminobutanoic acid (110 mg, 1.1 mmol). Purification by flash column chromatography (silica gel, 1:1 EtOAc/CH₂Cl₂) gave product as a pale yellow solid (230 mg, 0.70 mmol, 64% yield). ¹H NMR (CDCl₃, 300 MHz,): δ 8.84 (1H, d, J = 8.4 Hz), 8.74 (1H, d, J = 7.2 Hz), 8.40 (1H, d, J = 7.8 Hz), 7.99 (1H, dd, J₁ = 8.4 Hz, J₂ = 7.8 Hz), 4.27 (2H, t, J = 6.9 Hz), 2.48 (2H, t, J = 7.5 Hz), 2.08 (2H, m).

4-Nitro-N-(4-methoxy-4-oxobutyl)naphthalimide (13). A round-bottomed flask equipped with a magnetic stir bar and a condenser was charged with 12 (100 mg, 0.30 mmol) and thionyl chloride (3 mL). The mixture was refluxed for 1 h then allowed to cool to room temperature. Methanol (3 mL) was added to the reaction mixture, dropwise. After cooling in an ice-bath, the desired product precipitated out as a pale yellow solid. The precipitate was collected by vacuum filtration, washed with ice-cold methanol, and dried (90 mg, 0.26 mmol, 88% yield). ¹H NMR (CDCl₃, 300 MHz,): δ 8.84 (1H, d, J = 7.8 Hz), 8.73 (1H, d, J = 7.5 Hz), 8.69 (1H, d, J = 8.1 Hz), 8.41 (1H, d, J = 8.1 Hz), 8.01 (1H, t, J = 7.8 Hz), 4.26 (2H, t, J = 6.9 Hz), 3.64 (3H, s), 2.46 (2H, t, J = 7.5 Hz), 2.10 (2H, m).

4-Amino-N-(3-chloropropyl)naphthalimide (14), LY3. Procedure B: product was obtained as a yellow solid. ¹H NMR (DMSO-d₆, 400 MHz): δ 8.58 (1H, d, J = 8.8 Hz), 8.40 (1H, d, J = 7.2 Hz), 8.16 (1H, d, J = 8.4 Hz), 7.62 (1H, t, J = 8.0 Hz), 7.43 (2H, s), 6.81 (1H, d, J = 8.4 Hz, 6.4 Hz), 8.71 (1H, d, J = 8.0 Hz), 8.42 (1H, d, J = 8.0 Hz), 8.00 (1H, dd, J₁ = 8.8 Hz, J₂ = 7.2 Hz), 4.47 (2H, t, J = 6.0 Hz), 3.89 (2H, t, J = 5.6 Hz), 3.79 (2H, t, J = 6.0 Hz), 3.58 (2H, t, J = 5.6 Hz).
Hz), 4.11 (2H, t, J = 6.8 Hz), 3.67 (2H, t, J = 6.4 Hz), 2.03 (2H, m).

4-Amino-N-(4-methoxy-4-oxobutyl)naphthalimide (15), LY4. Procedure B: product was obtained as a yellow solid.\(^1\) H NMR (DMSO-\(d_6\), 400 MHz): \(\delta 8.58 (1H, d, J = 8.0 \text{ Hz})\), 8.39 (1H, d, J = 7.2 Hz), 8.15 (1H, d, J = 8.4 Hz), 7.62 (1H, dd, \(J_1 = 8.0 \text{ Hz}, J_2 = 7.2 \text{ Hz}\)), 7.42 (2H, s), 6.80 (1H, d, J = 8.4 Hz), 4.01 (2H, t, J = 6.8 Hz), 3.48 (3H, s), 2.30 (2H, t, J = 7.8 Hz), 1.85 (2H, m).

4-(\(p\)-Dihydroxylborylbenzyloxycarbamyl)-N-(3-chloropropyl)naphthalimide (16), PL3. Procedure C: LY3 (50 mg, 0.17 mmol), DMAP (30 mg, 0.25 mmol), toluene (5 mL), triphosgene (25 mg, 0.084 mmol), and 4 (40 mg, 0.17 mmol). Flash column chromatography (1:3, EtOAc/CH\(_2\)Cl\(_2\)) gave product as a yellow solid (35 mg, 0.064 mmol, 37 % yield). 1H NMR (CDCl\(_3\), 400 MHz): \(\delta 8.64 (1H, d, J = 7.2 \text{ Hz})\), 8.61 (1H, d, J = 8.4 Hz), 8.41 (1H, d, J = 8.0 Hz), 8.18 (1H, d, J = 8.4 Hz), 7.86 (2H, d, J = 7.6 Hz), 7.78 (1H, t, J = 8.0 Hz), 7.49 (1H, s), 7.46 (2H, d, J = 8.0 Hz), 5.32 (2H, s), 4.34 (2H, t, J = 6.4 Hz), 3.65 (2H, t, J = 6.4 Hz), 2.24 (2H, m), 1.36 (12H, s). HRFAB-MS: calculated for [MH\(^+\)] 549.19, found 549.19.

4-(\(p\)-Dihydroxylborylbenzyloxycarbamyl)-N-(4-methoxy-4-oxobutyl)naphthalimide (17), PL4. Procedure C: LY4 (34 mg, 0.11 mmol), DMAP (25 mg, 0.21 mmol), toluene (5 mL), triphosgene (32 mg, 0.10 mmol), and 4 (25 mg, 0.11 mmol). Flash column chromatography (1:3, EtOAc/CH\(_2\)Cl\(_2\)) gave product as a yellow solid (30 mg, 0.052 mmol, 47 % yield). 1H NMR (CDCl\(_3\), 400 MHz): \(\delta 8.63 (1H, d, J = 7.2 \text{ Hz})\), 8.61 (1H, d, J = 8.4 Hz), 8.42 (1H, d, J = 8.0 Hz), 8.17 (1H, d, J = 8.0 Hz), 7.86 (2H, d, J = 8.0 Hz), 7.80 (1H, dd, \(J_1 = 8.4 \text{ Hz}, J_2 = 7.2 \text{ Hz}\)), 7.46 (2H, d, J = 8.0 Hz), 5.32 (2H, s), 4.25 (2H, t, J = 7.2 Hz), 3.65 (3H, s), 2.45 (2H, t, J = 7.6 Hz), 2.05 (2H, m), 1.35 (12H, s). HRFAB-MS: calculated for [MH\(^+\)] 573.23, found 573.24.

4-Nitro-N-(2-(\(\text{tert}\)-butylcarbamate)ethyl)naphthalimide (18). Procedure A: 4-nitro-1,8-naphthalic anhydride (250 mg, 1.1 mmol), and \(\text{tert}\)-butyl-2-aminoethylcarbamate (180 mg, 1.1 mmol). Purification by flash column chromatography (1:5, EtOAc/CH\(_2\)Cl\(_2\)) gave product as a pale yellow solid (400 mg, 1.0 mmol, 94% yield). 1H NMR (CDCl\(_3\), 400 MHz): \(\delta 8.85 (1H, d, J = 8.0 \text{ Hz})\), 8.75 (1H, d, J = 8.0 Hz), 8.71 (1H, d, J = 8.0 Hz), 8.41 (1H, d, J = 8.0 Hz), 7.99 (1H, dd, \(J_1 = 8.8 \text{ Hz}, J_2 = 7.5 \text{ Hz}\)), 4.38 (2H, t, J = 5.6 Hz), 3.55 (2H, t, J = 5.6 Hz), 1.24 (9H, s).

4-Nitro-N-(2-(2,5-dione-pyrrol-1-ly)ethyl)naphthalimide (19). A round-bottomed flask equipped with a magnetic stir bar and a condenser was charged with 18 (200 mg, 0.52 mmol), maleic anhydride (51 mg, 0.52 mmol), and acetic acid (5 mL). The reaction mixture was heated to reflux overnight. Once cooled down the reaction mixture was neutralized with NaHCO\(_3\) (aq), and extracted with CH\(_2\)Cl\(_2\) (2 x 100 mL). Flash column chromatography of the concentrated crude product (1:1, CH\(_2\)Cl\(_2\)/EtOAc) gave product as a white solid (140 mg, 0.38 mmol, 73% yield). 1H NMR (CDCl\(_3\), 400 MHz): \(\delta 8.84 (1H, d, J = 8.8 \text{ Hz})\), 8.71 (1H, d, J = 8.0 Hz), 8.66 (1H, d, J = 8.0 Hz), 8.40 (1H, d, J = 8.0 Hz), 7.98 (1H, dd, \(J_1 = 8.8 \text{ Hz}, J_2 = 7.6 \text{ Hz}\)), 6.63 (2H, s), 4.44 (2H, t, J = 5.2 Hz), 4.01 (2H, t, J = 5.2 Hz).

4-Amino-N-(2-(2,5-dione-pyrrol-1-ly)ethyl)naphthalimide (20), LY5. To a 20-mL vial equipped with a small magnetic stir bar was added 19 (100 mg, 0.28 mmol), SnCl\(_2\) (75 mg, 0.33 mmol), and ethanol (5 mL). The mixture was stirred at 60 °C for 1 h. Solvent was removed by a rotary evaporator. The concentrated crude product was purified by flash column chromatography (1:1, EtOAc/CH\(_2\)Cl\(_2\)) to give product as a yellow solid (55 mg, 0.16 mmol, 59% yield). 1H NMR (DMSO-\(d_6\), 400 MHz): \(\delta 8.57 (1H, d, J = 8.8 \text{ Hz})\), 8.34 (1H, d, J = 7.2 Hz), 8.10 (1H, d, J = 8.0 Hz), 7.59 (1H, dd, \(J_1 = 8.8 \text{ Hz}, J_2 = 7.2 \text{ Hz}\)), 7.42 (2H, s), 6.87 (2H, s), 6.78 (1H, d, J = 8.0 Hz), 4.15 (2H, t, J = 4.8 Hz), 3.73 (2H, t, J = 4.8 Hz). HRFAB-MS: calculated for [MH\(^+\)] 336.09, found 336.09.
4-(p-Dihydroxylborylbenzylxycarbamyl)-N-(2,5-dione-pyrrol-1-ly)ethyl naphthalimide (21), PL5. Procedure C: LY5 (25 mg, 0.074 mmol), DMAP (25 mg, 0.21 mmol), acetonitrile (5 mL), triphosgene (25 mg, 0.084 mmol), and 4 (19 mg, 0.081 mmol). Flash column chromatography (1:4, EtOAc/CH2Cl2) gave product as a yellow solid (24 mg, 0.040 mmol, 55 % yield). 1H NMR (CDCl3, 400 MHz): δ 8.59 (1H, d, J = 7.2 Hz), 8.56 (1H, d, J = 8.4 Hz), 8.38 (1H, d, J = 8.4 Hz), 8.16 (1H, d, J = 8.4 Hz), 7.86 (2H, d, J = 8.0 Hz), 7.75 (1H, dd, J1 = 8.4 Hz, J2 = 7.2 Hz), 7.47 (2H, d, J = 8.0 Hz), 7.45 (1H, s), 6.62 (2H, s), 5.32 (2H, s), 4.42 (2H, t, J = 4.8 Hz), 3.99 (2H, t, J = 4.8 Hz), 1.36 (12H, s). HRFAB-MS: calculated for [MH+] 595.21, found 596.22.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded using 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 (1.5-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by reference to fluorescein in basic ethanol (Φ = 0.97).28

Reactive oxygen species (200 μM unless otherwise stated) were administered to PL1 in 20 mM HEPES (pH 7.4, 25 °C) as follows. H2O2, tert-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. Hydroxyl radical (•OH), and tert-butoxy radical (•OtBu) were generated by reaction of 1 mM Fe2+ with 200 μM H2O2 or TBHP, respectively. Nitric oxide (NO) was generated from NOC5 (stock solution 1 mM in 0.1 M NaOH); degradation of 280 μM NOC5 in aqueous solution will generate 200 μM NO after 60 min. Superoxide (O2−) was delivered from the enzymatic reaction of xanthine oxidase (0.02 unit/mL) and hypoxanthine (1 mM in phosphate buffer pH 7.8) in the presence of 5 units catalase as a scavenger for any H2O2. The rate of O2− production observed from the reduction of cytochrome c was 1.5 μM/min. Singlet oxygen (1O2) was generated by UV irradiation of Sensitox II (1 mg, kindly provided as a gift from Prof. Kris McNeill, University of Minnesota) in dye solution using 450 W mercury arc lamp powered by Aceglass power supply for 2 min. Caution: Reactive oxygen species such as singlet oxygen are highly oxidizing and should be handled with care.

Preparation and Staining of Cell Cultures. RAW264.7 and HEK293T cells were cultured in DMEM (Invitrogen) containing high glucose with GlutaMAX™ (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS, Hyclone). One day before imaging, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine (50 μg/mL, Sigma, St. Louis, MO). PL and LY were delivered from concentrated 5 mM stock solutions in DMSO. Phorbol myristate acetate (PMA) was delivered from 1 mg/mL stock solution in DMSO. H2O2 was delivered from 50 mM stock solution in Millipore water. Labeling buffer contained 5 μM PL or LY in HEPES. Labeling of HEK293T cells were accomplished by incubating cells in labeling buffer for 30 min at 37°C under 5% CO2 atmosphere then washing twice with fresh buffer. Phagocytosis stimulation in RAW264.7 was accomplished by incubating cells in labeling buffer containing 1 μg/mL PMA for 15 min at 37°C under 5% CO2 then washing
twice with fresh buffer. [5x HEPES buffer pH 7.4: 750 mM NaCl, 100 mM HEPES, 5 mM CaCl₂, 25 mM KCl, 5 mM MgCl₂]

**Fluorescence Imaging Experiments.** Confocal fluorescence imaging was performed with Zeiss 710 Axiovert laser scanning microscope and 20x or 40x air objective lens. Excitation of PL or LY loaded cells was carried out with 405 nm laser. Blue emission was collected with a detection window 440-490 nm. Green emission was collected with a detection window 540 - 650 nm. Lambda mode scanning was performed with 9.7 nm step size over 449 - 643 nm range. Two-photon confocal fluorescence imaging was performed with a Zeiss LSM510 NLO Axiovert 200 laser scanning microscope and 40x or 63x water-immersion objective lens with 2x optical zoom. Excitation of PL1-loaded cells was carried out with MaiTai two-photon laser at 820 nm pulses (mode-locked Ti:sapphire laser, Tsunami Spectra Physics). Blue emission was collected using a META detector with a 430 - 495 nm window, and green emission was collected with a 535 - 600 nm window. Data were analyzed using ImageJ software (author: Wayne Rasband, NIH) and Ratio Plus plug-in (programmer: Paulo Magalhães, University of Padua, Italy).

**Cell Viability Assays.** The viability of cells loaded with PL1 was evaluated both by the Trypan Blue exclusion test and Live/Dead cell vitality assay with C₁₂-resazurin and SYTOX green. RAW 264.7 cells were incubated with 10 µM PL1 for 6 h. Cell suspensions were obtained by trypsinizing, then pelleted and resuspended in PBS containing 1% FBS. For the Trypan Blue exclusion test, equal volumes of cell suspension and 0.4% Trypan Blue Stain were mixed and allowed to stand for 10 min before cell counting. Cells incubated with PL1 were alive and viable. For the live/dead vitality assay, cell suspensions were incubated with 500 nM C₁₂-resazurin and 40 nM SYTOX green for 15 min at 37°C and kept in ice bath prior to cell counting with a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems) using 488 nm Ar laser excitation. The fluorescence emission from C₁₂-resazurin and SYTOX green was collected using 585 nm bandpass filter (FL2), and 530 nm bandpass filter (FL1), respectively. Dead cells controls were obtained by incubation of RAW 264.7 cells with 30% ethanol for 2 h.
Figures and Schemes

Previously developed ratiometric sensors - FRET

New ratiometric sensors - Internal Charge Transfer (ICT)

Peroxy Lucifer (PL)

Scheme 1. Design of ICT-based fluorescent probes for H₂O₂.
Scheme 2. Synthesis of PL0 and PL1.
Scheme 5. Synthesis of PL5.
Table 1. Spectroscopic properties of PL and LY series.

<table>
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<tr>
<th>Compound</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
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Figure 1. The PL series of ratiometric fluorescent probes for H$_2$O$_2$. 
Figure 2. Absorption and fluorescence emission ($\lambda_{\text{exc}} = 410$ nm) responses of 5 μM PL to 1 mM H$_2$O$_2$. Spectra were taken 0, 10, 20, 30, 40, 50 and 60 min after addition of H$_2$O$_2$. 
Figure 3. Kinetic plot of fluorescence emission intensity at 475 nm of the pseudo-first-order reaction of 1 μM PL1 to 1 mM H₂O₂, using excitation wavelength at 380 nm. The slope of the plot corresponds to the observed reaction rate of $k_{obs} = 8.8 \times 10^{-4} \text{ s}^{-1}$. 
Figure 4. Fluorescence responses of 5 μM PL1 to various reactive oxygen species (ROS) at 200 μM. Bars represent emission intensity ratios $F_{540}/F_{475}$ 0, 15, 30, 45, 60, 90 and 120 min after addition of each ROS. Data were acquired at 25°C in 20 mM HEPES, pH 7.4, $\lambda_{exc} = 410$ nm.
Figure 5. The ratio of fluorescence emission at 500 and 550 nm (F_{550}/F_{500}) of PL1 and LY1 is stable across the biologically relevant pH. (0.1 M phosphate buffered solution, $\lambda_{exc} = 410$ nm)
Figure 6. Solvatochromism of PL and LY fluorophores.
Figure 7. Confocal fluorescence images of 3T3-L1 adipocytes labeled with PL3. Cells were incubated with PL3 (5 µM) for 15 min then washed twice with HEPES. (c) Overlay image and (d) pseudo color image of $F_{\text{green}}/F_{\text{blue}}$ shows the difference in fluorescence emission profile between cytoplasm and lipid droplet; PL3 localized in lipid droplets show higher blue color emission. Scale bar = 20 µm. ($\lambda_{\text{exc}} = 405$ nm, blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm)
Figure 8. Confocal fluorescence images of 3T3-L1 adipocytes labeled with PL3 showing ratiometric fluorescence emission response to H$_2$O$_2$. Cells were incubated with PL3 (5 µM in HEPES buffer) for 15 min then washed twice with fresh buffer. For the control experiment (a), cells were further incubated in buffer for another 30 min before images were taken. (b) Incubation of PL3-labeled cells with H$_2$O$_2$ (200 µM, 30 min) caused increase F$_{green}$/F$_{blue}$ emission ratio. (c) Quantification of emission ratio, error bars represent standard deviation of average F$_{green}$/F$_{blue}$ obtained from 5 images. Images were taken using $\lambda_{exc} = 405$ nm; blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm. Scale bar = 20 µm.
Figure 9. Pseudo color images representing $F_{\text{green}}/F_{\text{blue}}$ in HEK293T labeled with PL probes in response to added H$_2$O$_2$. Cells were incubated with PL probes (5 µM in HEPES buffer) for 30 min then washed twice with fresh buffer. For the control experiment (a), cells were further incubated in buffer for another 30 min before images were taken. (b) Incubation of PL-labeled cells with H$_2$O$_2$ (200 µM, 30 min) caused an increase in $F_{\text{green}}/F_{\text{blue}}$ emission ratio. (c) Quantification of emission ratio, error bars represent standard deviation of average $F_{\text{green}}/F_{\text{blue}}$ obtained from 5 images. Images were taken using $\lambda_{\text{exc}} = 405$ nm; blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm. Scale bar = 20 µm.
Figure 10. Lambda coded images obtained from confocal fluorescence imaging in lambda scanning mode. Rainbow scale represents emission wavelength from 400 to 700 nm. $\lambda_{\text{exc}} = 405$ nm; blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm. Scale bar = 20 µm.
Figure 11. Confocal fluorescence images in lambda mode scanning of HEK293T labeled with (a) PL3 or (b) PL5. The normalized emission intensity plot of the average emission obtained from three experiments is shown in (c). $\lambda_{\text{exc}} = 405$ nm.
Figure 12. Confocal fluorescence images in lambda mode scanning of HEK293T labeled with (a) LY3 or (b) LY5. The normalized emission intensity plot of the average emission obtained from three experiments is shown in (c). $\lambda_{\text{exc}} = 405$ nm.
Figure 13. Confocal fluorescence imaging using (a) channel mode and (b) lambda mode of PMA-induced phagocytosis in PL1-labeled RAW264.7 cells. Oxidative burst in phagosomes was detected by increasing $F_{\text{green}}/F_{\text{blue}}$ emission from $\text{H}_2\text{O}_2$-mediated deprotection of PL1 to LY1. $\lambda_{\text{exc}}$ = 405 nm; blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm. Scale bar = 20 µm.
Figure 14. Confocal fluorescence imaging using (a) channel mode and (b) lambda mode of PMA-induced phagocytosis in PL4-labeled RAW264.7 cells. Oxidative burst in phagosomes was detected by increasing $F_{\text{green}}/F_{\text{blue}}$ emission from H$_2$O$_2$-mediated deprotection of PL4 to LY4. $\lambda_{\text{exc}}$ = 405 nm; blue collection window = 440 - 490 nm, green collection window = 540 - 650 nm. Scale bar = 20 µm.
Figure 15. Two-photon confocal ratiometric fluorescence images of live RAW264.7 macrophage cells. Images displayed in pseudo color represent the ratio of emission intensities collected in optical windows between 535 - 600 (green) and 430 - 495 nm (blue), respectively, upon two-photon excitation at 820 nm. (a) Cells incubated with 5 µM PL1 for 15 min at 37 °C. (b) PL1-loaded cells after treatment with 100 µM H₂O₂ for 60 min at 37 °C. (c) Brightfield images of cells in panel (b) with 20 µm scale bar. (d) PL1-loaded cells after treatment with PMA (1 µg/mL) for 30 min at 37 °C. (e) Cells in panel (d) showing overlay of blue and green channel. (f) Brightfield image of cells in panels (d) and (e) with 20 µm scale bar. (g) Relative F_{green}/F_{blue} ratios displayed by cells loaded with PL1 only (control) and PL1-stained cells treated with H₂O₂ or PMA (cytoplasm vs vesicle).
Figure 16. Flow cytometry test for viability of RAW264.7 cells using SYTOX green and C_{12}-resazurin for labeling of dead cells. (a) Viable cells. (b) Dead cell controls. (c) Cells incubated with PL1 for 6 hours, confirming their viability.
References


Chapter 2
A Two-Photon Fluorescent Probe for Ratiometric Imaging of Hydrogen Peroxide in Live Tissue

Portions of this work were published in the following scientific journal:

Portions of this work were performed in collaboration with the following people:
Chul Chung and Chang Su Lim performed two-photon spectroscopic analysis, confocal two-photon fluorescent imaging of PMA-stimulated macrophage RAW264.7, and two-photon imaging of hippocampal slices.
Introduction

Hydrogen peroxide (H$_2$O$_2$) is a prominent member of the reactive oxygen species (ROS) family that includes the superoxide anion radical (O$_2^•$), hydroperoxy radical (HO$_2^•$), hydroxyl radical (HO$^•$), peroxy radical (RO$_2^•$), singlet oxygen (’O$_2$), and hypochlorous acid (HOCl), and plays important roles in physiology, aging, and disease in living organisms. Whereas unregulated increases in cellular levels of H$_2$O$_2$ have been linked to DNA damage, mutation, and genetic instability, controlled bursts of H$_2$O$_2$ are utilized for cell signalling. In this context, a key challenge for elucidating the biological roles of H$_2$O$_2$ in living systems is the ability to monitor this specific ROS at the level of the cell, tissue, and organism. For this purpose, a growing number of fluorescent probes for H$_2$O$_2$ have been developed; one of our labs has focused on H$_2$O$_2$ reporters based on a boronate deprotection mechanism, which offers selectivity for H$_2$O$_2$ over other ROS and is suitable for imaging H$_2$O$_2$ fluxes in living cells. However, the majority of these probes has been evaluated using one-photon microscopy (OPM) and with relatively short excitation wavelengths (< 525 nm), which limits their use in deep tissue imaging due to the shallow depth of penetration (< 80 μm). Methods for imaging H$_2$O$_2$ in thicker samples, such as tissues and whole organisms, are in comparison, less common.

An attractive approach to the detection of H$_2$O$_2$ deep inside living tissues is the use of two-photon microscopy (TPM). TPM, which employs two near-infrared photons as the excitation source, offers a number of advantages over one-photon microscopy, including increased depth of penetration (> 500 μm), localized excitation, and prolonged observation time due to the use of less-damaging lower energy light. To our knowledge, there have been no reports on the targeted design and evaluation of two-photon (TP) probes for H$_2$O$_2$ that are applicable for deep tissue imaging. To this end, an effective TP probe for H$_2$O$_2$ should exhibit the following features: sufficient solubility in aqueous buffers, selective reactivity to H$_2$O$_2$, a high TP cross section, and stable spectroscopic properties over a biologically relevant pH range.

With these aims in mind, we now report Peroxy Naphthalene (PN), a new series of ratiometric TP fluorescence probe for H$_2$O$_2$. PN1 was constructed by introducing a boronate-based carbamate leaving group to 2-acetyl-6-aminonaphthalene (AN1), a TP fluorophore that has been successfully utilized in live tissue imaging. We anticipated that the H$_2$O$_2$-triggered boronate cleavage of the electron-poor carbamate linkage would liberate the more electron-rich AN1, giving rise to a red-shifted fluorescence emission. PN1 was sensitive enough to detect the increase cellular H$_2$O$_2$ in macrophage RAW264.7 undergoing PMA-induced phagocytosis. The real application of PN1 is in deep tissue imaging, in which we have demonstrated that PN1 can be utilized in imaging oxidative stress in hippocampal slices with the penetration depth up to 180 μm. Further, we also install a handle for bioconjugation in PN2 and AN2 for future use in constructing multi-functional or targetable probes.

Results and Discussion

AN1 was synthesized using a modification methodology reported by Cho et al (Scheme 1). Previously, AN1 was synthesized by amination of 2-acetyl-6-hydroxynaphthalene using sodium metabisulfite (Na$_2$S$_2$O$_5$) and ammonium hydroxide in a steel-bomb reactor at 140 °C. With the aim to install a functional handle for bioconjugation, we sought a milder synthetic route. We were able to obtain AN1 in a good yield (50%, two steps) from the Buchwald-Hartwig amination of the aryltriflate with tert-butylcarbamate using Pd$_2$(dba)$_3$ and the xantphos ligand as the catalysts, followed by deprotection of the Boc group with TFA. Using the same
methodology, we were able to obtain AN2, a derivative with a carboxylic acid ester handle (Scheme 2). Installation of the H$_2$O$_2$ responsive trigger [$p$-dihydroxyborylbenzoylloxycarbonyl (Dobz)]$^{14,16,36}$ by the reaction of the isocyanate of AN1 and AN2, which were generated in situ using triphosgene in refluxing toluene, with (4-pinacolboryl)benzylalcohol, gave PN1 and PN2, respectively.

PN1 exhibits an absorption maximum at 321 nm ($\varepsilon = 12,200$ M$^{-1}$ cm$^{-1}$) while the uncaged AN1 features red-shifted absorption maximum at 338 nm ($\varepsilon = 13,200$ M$^{-1}$ cm$^{-1}$). PN1 exhibits a fluorescence maximum at 453 nm ($\Phi = 0.70$), which is almost 50 nm blue-shifted from that of AN1 ($\lambda_{em} = 500$ nm, $\Phi = 0.40$). The larger Stokes' shift observed in AN1 compared to PN1 is due to greater stabilization of the charge-transfer excited state of the former fluorophore that contains both electron-donating and electron-withdrawing groups. PN2 and AN2, with the functional group handle, preserve the same absorption and fluorescent emission spectra as in PN1 and AN1 (Table 1).

Reaction of PN1 with H$_2$O$_2$ resulted in formation of AN1 as seen in both the absorption and fluorescence emission spectra. PN1 treated with 1 mM H$_2$O$_2$ in HEPES buffer shows a gradual increase absorbance at 343 nm with a concomitant decrease at 323 nm (Figure 1a). The corresponding fluorescent emission spectra showed a decrease in PN1 emission at 450 nm and gradual increase of AN1 emission at 490 nm (Figure 1b), following pseudo-first-order kinetics, with $k_{obs} = 9.6 \times 10^{-4}$ s$^{-1}$ (Figure 1c). Similarly, PN2 shows the ratiometric absorption and fluorescent emission response to H$_2$O$_2$ with $k_{obs} = 1.1 \times 10^{-3}$ s$^{-1}$ (Figure 2).

PN1 demonstrates high selectivity for H$_2$O$_2$ over competing ROS, as revealed by the unperturbed $F_{green}/F_{blue}$ ratios upon addition of 200 μM concentrations of various ROS, including tert-butylhydroperoxide, hypochlorite, superoxide, singlet oxygen, nitric oxide, hydroxyl radical, and tert-butoxy radical (Figure 3). Moreover, the $F_{green}/F_{blue}$ ratio is independent of solution pH over a biologically relevant pH range (Figure 4). The combined results reveal that PN1 can detect H$_2$O$_2$ by ratiometric fluorescence imaging with minimum interference from pH or from other ROS.

The TP action spectra of PN1 and AN1 in HEPES buffer at pH 7.1 indicate $\delta_{max}$ values of 12 and 45 GM at 720 and 740–750 nm, respectively (Figure 5), which are high enough for two-photon imaging application. Using 740 nm TP excitation in a scanning lambda mode, HEK293T cells labeled with PN1 show an emission maximum at 450 nm while cells labeled with AN1 show a red-shifted emission maximum at 480 nm (Figure 6). The blue-shift in AN1 emission in cells compared to that observed in aqueous buffer is due to the solvatochromic response of AN1 to the more hydrophobic environment in which AN1 intercalates in membrane layers. Upon TP excitation, the ratio image constructed from blue and green collection windows give average emission ratios $F_{green}/F_{blue}$ of 0.79 and 1.02 for PN1 and AN1, respectively. $F_{green}/F_{blue}$ ratio increased to 0.85 after cells were treated with 100 μM H$_2$O$_2$ for 30 min (Figure 7). PN1 is nontoxic to cells over the course of imaging experiments, as determined by a WST-1 assay. Similarly, TPM images of PN2-labeled HEK293T showed ratiometric fluorescence emission response to H$_2$O$_2$, establishing that appended conjugation site is appropriate for further functionalization (Figure 8).

Having established the ratiometric response of PN1 to exogenous H$_2$O$_2$, we then tested the ability of PN1 in detecting endogenously produced H$_2$O$_2$. Using 750 nm TP excitation, RAW264.7 cells labeled with PN1 or AN1 have average $F_{green}/F_{blue}$ of 0.89 and 2.03, respectively (Figure 9). When the cells were pretreated with 100 μM H$_2$O$_2$ for 30 min before labeling with PN1, the ratio increased to 1.51. More importantly, PN1 is responsive to the
endogenous burst of H$_2$O$_2$ produced in a living cell, showing a patent increase in the F$_\text{green}$/F$_\text{blue}$ ratio to 1.28 after stimulation of the PN1-labeled cells for 30 min with phorbol myristate acetate (PMA), which induces phagocytosis-associated H$_2$O$_2$ generation.\textsuperscript{37-39}

To further establish the utility of PN1 for bioimaging applications, we also investigated the ability of this new probe to detect changes in H$_2$O$_2$ levels deep within live tissue. The hippocampus was isolated from a 2-day old rat. We acquired 10 TPM images at depths of 90–180 µm through hippocampal slices labeled with PN1 (Figure 10), with PN1 after the pretreatment with H$_2$O$_2$ (Figure 11), or with AN1 (Figure 12). The ratiometric image constructed from the accumulation of TPM images along the z-direction at depths of 90–180 µm of the slice incubated with 20 µM PN1 for 1 h at 37 °C revealed that the basal H$_2$O$_2$ levels in the CA1 and CA3 regions are higher than in the DG region (Figure 13), with average emission ratios of 0.71 and 0.90 in the CA3 and CA1 regions, respectively. The F$_\text{green}$/F$_\text{blue}$ of CA3 and CA1 increased to 1.05 and 1.16, respectively when the hippocampal slice was preincubated with 1 mM H$_2$O$_2$ for 30 min. Moreover, the images of the CA1 region at a higher magnification showed F$_\text{green}$/F$_\text{blue}$ of individual cells following the similar trend, with F$_\text{green}$/F$_\text{blue}$ of the tissue treated with H$_2$O$_2$ and PN1 lying between those measured in PN1 and AN1-labeled tissues (Figure 11 d-f).

**Concluding Remarks**

To conclude, we have developed a new TP probe, PN1, which shows a significant TP cross section, a marked blue-to-green emission color change in response to H$_2$O$_2$, good ROS selectivity, and low cytotoxicity. This new probe can visualize H$_2$O$_2$ levels in live cells, as well as in living tissues at depths of 90–180 µm without interference from other biologically relevant ROS. In an attempt toward creating multifunctional and targetable PN1 derivative, we were able to install a carboxylic acid handle in PN2 and AN2 with conserving reactivity to H$_2$O$_2$.

**Experimental Section**

**Synthetic Materials and Methods.** Silica gel P60 (SiliCycle) was used for column chromatography. The WST-1 cell viability assay kit was purchased from Roche Applied Science (Mannheim, Germany). ProliNONOate was obtained from Cayman Chemical (Ann Arbor, MI, USA). 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$_3$OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on a Bruker AVQ-400 or a Bruker AV-300 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS).

**2-Acetyl-6-naphthol (1)** was prepared by demethylation of commercially available 6-methoxy-2- acetophenone following a literature method.\textsuperscript{27} $^1$H NMR (CDCl$_3$, 300 MHz): δ 8.40 (1H, s), 7.99 (1H, dd, $J_1$ = 8.7 Hz, $J_2$ = 1.8 Hz), 7.88 (1H, d, $J$ = 8.7 Hz), 7.71 (1H, d, $J$ = 8.7 Hz), 7.18 (1H, s), 7.15 (1H, d, $J$ = 8.7 Hz), 2.70 (3H, s). GC-MS: calculated for [M$^+$] 186.07, found 186.1
2-Acetyl-6-trifluoromethanesulfonyl naphthalene (2). A 25-mL single-necked round-bottomed flask equipped with a magnetic stir bar was charged with 1 (200 mg, 1.1 mmol) in acetonitrile (5 mL), followed by N-phenyl bis-trifluromethanesulfonamide (400 mg, 1.1 mmol) and triethylamine (500 µL, 3.6 mmol). The flask was capped with a nitrogen inlet adapter, and the mixture was stirred at room temperature for 3 h. Volatile solvent was removed by a rotary evaporator. Purification of the crude oil by flash column chromatography (1:4, EtOAc/hexane) gave product as yellow oil (256 mg, 0.80 mmol, 75% yield). 1H NMR (CDCl₃, 400 MHz): δ 8.48 (1H, s), 8.12 (1H, d, J = 8.4 Hz), 8.05 (1H, d, J = 9.2 Hz), 7.92 (1H, d, J = 8.4 Hz), 7.77 (1H, d, J = 2.4 Hz), 7.45 (1H, dd, J₁ = 9.2 Hz, J₂ = 2.4 Hz), 2.72 (3H, s).

2-Acetyl-6-tert-butylcarbamyl naphthalene (3). The reaction was set up in an inert atmosphere glovebox. To a dry 25-mL Schlenk tube was added 2 (160 mg, 0.50 mmol), Cs₂CO₃ (217 mg, 0.67 mmol), tert-butylcarbamate (79 mg, 0.67 mmol), xantphos (30 mg, 0.050 mmol), Pd₂(dba)₃ (16 mg, 0.020 mmol), and dioxane (5 mL). The sealed flask was heated at 100 °C for three days. After the flask cooled to room temperature, the reaction mixture was diluted into EtOAc (50 mL). In a 100-mL separatory funnel, the mixture was washed with DI water (2 x 25 mL) and brine (25 mL). The organic layer was dried over Na₂SO₄, and concentrated by a rotary evaporator. Purification with flash column chromatography (1:1, EtOAc/hexane) gave product as a white solid (80 mg, 0.29 mmol, 58% yield). 1H NMR (CDCl₃, 400 MHz): δ 8.36 (1H, d, J = 1.6 Hz), 8.04 (1H, d, J = 2.4 Hz), 7.97 (1H, dd, J₁ = 8.8 Hz, J₂ = 1.6 Hz), 7.85 (1H, d, J = 9.2 Hz), 7.77 (1H, d, J = 8.8 Hz), 7.38 (1H, dd, J₁ = 9.2 Hz, J₂ = 2.4 Hz), 6.73 (1H, s), 2.68 (3H, s), 1.51 (9H, s).

2-Acetyl-6-aminonapthalene (4), AN1. To a 20-mL vial equipped with a magnetic stir bar was added 3 (80 mg, 0.29 mmol) and 2:1 CH₂Cl₂:TFA (2 mL). The mixture was stirred at room temperature for 1 h. Solvent was removed by a rotary evaporator. Purification with flash column chromatography (1:1, EtOAc/hexane) gave product as pale yellow solid (46 mg, 0.25 mmol, 86% yield). 1H NMR (CD₃OD, 300 MHz): δ 8.45 (1H, s), 7.90 (1H, d, J = 8.7 Hz), 7.87 (1H, d, J = 8.7 Hz), 7.65 (1H, d, J = 8.7 Hz), 7.18 (1H, s), 7.16 (1H, d, J = 8.7 Hz), 2.66 (3H, s).

GC-MS: calculated for [M⁺] 185.08, found 185.1.

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzalalcohol (5) was prepared following a literature protocol. 1H NMR (CDCl₃, 400 MHz): δ 7.80 (2H, d, J = 7.8 Hz), 7.36 (2H, d, J = 7.8 Hz), 4.71 (2H, s), 1.34 (12H, s).

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl 6-acetylnaphthalen-2-yl carbamate (6), PN1. To a 10-mL two-necked flask equipped with a condenser fitted with a nitrogen inlet was added DMAP (40 mg, 0.32mmol), and AN1 (30 mg, 0.16 mmol) in 5 mL toluene. The flask was capped with a rubber septum and flushed with nitrogen for 10 min. The mixture was heated to 50 °C until all solid was dissolved. A solution of triphosgene (30 g, 0.10 mmol) in toluene was added to the reaction mixture via a syringe in one portion, and the mixture was heated to reflux at 110 °C for 2 h. Insoluble materials were removed by passing the reaction mixture through a glass pipette plugged with a ball of glass wool. The filtrate was added to a solution of 5 (40mg, 0.17 mmol) in CH₂Cl₂. The mixture was stirred at room temperature for 1 h. Solvent was removed by a rotary evaporator. Purification by flash column chromatography (10:1, CHCl₃/EtOAC) afforded product as a white solid (37 mg, 0.083 mmol, 51%) 1H NMR (CDCl₃, 300 MHz) δ: 8.39 (1H, s), 7.83 (2H, d, J = 8.4 Hz), 7.79 (1H, d, J = 9.0 Hz), 7.45 (1H, dd, J₁ = 9.0 Hz, J₂ = 2.0 Hz), 7.42 (2H, d, J = 8.4 Hz), 6.90 (1H, s), 5.26 (2H, s), 2.70 (3H, s), 1.34 (12H, s). 13C NMR (CDCl₃, 400 MHz): δ 198.1, 153.3, 138.9, 137.9, 136.7, 135.3, 133.6, 130.9, 130.1, 129.3.
tert-Butyl-4-(6-methoxynaphthalene-2-yl)-4-oxobutanoate (7). To a flame-dried two-necked round-bottom flask under N₂ atmosphere was added a solution of 6-methoxy-2-acetophenone (2 g, 10 mmol) in dried THF (50 mL). The solution was equilibrated to -78°C in acetone/dry ice bath, followed by dropwise addition of lithium diisopropylamine (2 M in THF, 6 mL, 12 mmol) was added dropwise, and the solution was allowed to warm up to room temperature. The solution was quenched with cold water in ice bath, and then extracted with EtOAc (2 x 100 mL). The organic layer was dried over Na₂SO₄, concentrated to dryness (2.2 g). GC-MS showed greater than 80% conversion. Due to the difficulty of separation the product and the remaining starting material, the dried solid was used in the next step without further purification.

4-(6-Hydroxynaphthalen-2-yl)-4-oxobutanoic acid (8). To a round-bottomed flash equipped with a magnetic stir bar and a condenser was added 7 (2.2 g, 7.0 mmol), acetic acid (20 mL) and HBr (10 mL). The mixture was heat to reflux overnight. The reaction mixture was concentrated by a rotary evaporator, and then diluted into EtOAc (200 mL). The organic phase was washed with water (2 x 50 mL) and brine. The concentrated crude product was purified by flash column chromatography (silica gel, 1:1 CH₂Cl₂/EtOAc) to give product as a white solid (1.2 g, 4.9 mmol, 70% yield). ^1H NMR (CD₃OD, 300 MHz): δ 8.45 (1H, s), 7.97 (1H, d, J = 9.0 Hz), 7.87 (1H, d, J = 9.0 Hz), 7.71 (1H, d, J = 9.0 Hz), 7.20 (1H, d, J = 9.0 Hz), 7.18 (1H, s), 3.43 (2H, t, J = 6.6 Hz), 2.82 (2H, t, J = 6.6 Hz).

Methyl 4-(6-hydroxynaphthalen-2-yl)-4-oxobutanoate (9). To a round-bottomed flash equipped with a magnetic stir bar and a condenser was added 8 (1 g, 4.1 mmol), methanol (50 mL) and H₂SO₄ (0.2 mL). The mixture was heat to reflux overnight. The reaction mixture was concentrated by rotary evaporator, and then diluted into EtOAc (100 mL). The organic phase was washed with water (2 x 50 mL) and brine, then dried over Na₂SO₄. Solvent was removed by rotary evaporator to give product as white solid (950 mg, 3.7 mmol, 90% yield). ^1H NMR (CD₃Cl, 300 MHz): δ 8.41 (s, 1H), 7.97 (dd, 1H, J₁ = 1.8 Hz, J₂ = 8.7 Hz), 7.84 (d, 1H, J = 8.7 Hz), 7.68 (d, 1H, J = 8.7 Hz), 7.15 (m, 2H), 3.72 (s, 3H), 3.42 (t, 2H, J = 6.6 Hz), 2.81 (t, 2H, J = 6.6 Hz).

Methyl 4-oxo-4-(6-(trifluoromethylsulfonyloxy)naphthalene-2-ly)butanoate (10). A 25-mL single-necked round-bottomed flask equipped with a magnetic stir bar was charged with 9 (200 mg, 0.77 mmol) in acetonitrile (5 mL), followed by N-phenyl bis-trifluoromethanesulfonylamide (300 mg, 0.80 mmol) and NEt₃ (200 µL). The flask was capped with a nitrogen inlet adapter, and the mixture was stirred at room temperature for 3 h. Volatile solvent was removed by a rotary evaporator. Purification of the crude oil by flash column chromatography (1/2, EtOAc/hexane) gave product as yellow oil (256 mg, 0.66 mmol, 85% yield). ^1H NMR (CD₂Cl₂, 300 MHz): δ 8.55 (1H, s), 8.13 (1H, d, J = 8.7 Hz), 8.07 (1H, d, J = 8.7 Hz), 7.94 (1H, d, J = 8.7 Hz), 7.79 (1H, d, J = 2.4 Hz), 7.45 (1H, dd, J₁ = 8.7 Hz, J₂ = 2.4 Hz), 3.72 (3H, s), 3.45 (2H, t, J = 6.6 Hz), 2.83 (2H, t, J = 6.6 Hz).

Methyl 4-(6-(tert-butoxycarbonylamino)naphthalene-2-yl)-4-oxobutanoate (11). The reaction was set up in an inert atmosphere glovebox. To a dry 25-mL Schlenk tube was added 10 (70 mg, 0.18 mmol), Cs₂CO₃ (82 mg, 0.25 mmol), tert-butylcarbamate (25 mg, 0.21 mmol), xantphos (6 mg, 0.010 mmol), Pd₂(dba)₃ (3 mg, 0.0037 mmol), and dioxane (5 mL). The sealed flask was heated at 100 °C for 48 h. After the flask cooled down to room temperature, the reaction mixture was diluted into EtOAc (50 mL). In a 100-mL separatory funnel, the mixture...
was washed with DI water (2 x 25 mL) and brine (25 mL). The organic layer was dried over Na₂SO₄, and concentrated by rotary evaporator. Purification with flash column chromatography (1:1, EtOAc/hexane) gave product as white solid (30 mg, 0.084 mmol, 46% yield). 

**Methyl 4-(6-aminonaphthalen-2-ly)-4-oxobutanoate (12), AN2.** To a 20-mL vial equipped with a magnetic stir bar was added 11 (30 mg, 0.08 mmol) and 2:1 CH₂Cl₂:TFA (2 mL). The mixture was stirred at room temperature for 1 h. Solvent was removed by rotary evaporator. Purification with flash column chromatography (1:1, EtOAc:hexane) gave product as pale yellow solid (20 mg, 0.07 mmol, 95% yield). 

**Spectroscopic measurements.** Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded using 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 (1.5-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by using quinine sulfate as the reference (Φ = 0.54 in 0.5 M H₂SO₄). Reactive oxygen species (200 μM unless otherwise stated) were administered to PN1 in 20 mM HEPES (pH 7.1, 25 °C) as follows. H₂O₂, tert-butyldihydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. Hydroxyl radical (•OH), and tert-butoxy radical (•OtBu) were generated by reaction of 1 mM Fe²⁺ with 200 μM H₂O₂ or TBHP, respectively. Nitric oxide (NO) was generated from proliNONOate (stock solution 70 mM in 0.1M NaOH, the concentration of intact compound was determined from its characteristic UV absorption at 254
nm (ε = 8,400 M\(^{-1}\) cm\(^{-1}\)). Superoxide (O\(_2^-\)) was delivered from the enzymatic reaction of xanthine oxidase (0.02 unit/mL) and hypoxanthine (1 mM in phosphate buffer pH 7.8) in the presence of 5 units catalase as a scavenger for any H\(_2\)O\(_2\). The rate of O\(_2^-\) production observed from the reduction of cytochrome c was 1.5 μM/min. Singlet oxygen was generated from the thermodissociable endoperoxide of disodium 3,3'-[(1,4-naphthalene)bispropionate (400 μM in phosphate buffer, pH 7.4 at 37 °C).\(^{42}\)

**Measurement of Two-Photon Cross Sections.** The two-photon cross section (δ) of dyes were determined by using a femto second (fs) fluorescence measurement technique as described.\(^{43}\) PN1 and AN1 were dissolved in 20 mM HEPES buffer (pH 7.1) at concentrations of 2.0 \(\times\) 10\(^{-6}\) M (PN1) and 2.0 \(\times\) 10\(^{-6}\) M (AN1) and then the two-photon induced fluorescence intensity was measured at 720–940 nm by using Rhodamine 6G in MeOH as the reference, whose two-photon properties have been well characterized in the literature.\(^{44}\) The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using \[ \delta = \delta_r(\Phi_r/c_r)/(\Phi_s/c_s), \] where the subscripts s and r stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as \(S\). \(\Phi\) is the fluorescence quantum yield. \(\phi\) is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as \(c\). \(\delta\) is the TPA cross section of the reference molecule.

**Cell Culture and Imaging.** HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before imaging, cells were passaged and plated on 24 well plates with coverslips coated with poly(lysine) at a density of 2\(\times\)10\(^5\)/well. For labeling, the growth medium was removed and replaced with DPBS containing calcium and magnesium and 5 μM PN or AN. Cells were incubated with the staining solution for 30 min. The coverslips of cells loaded with dye were transferred into a fresh DPBS solution. Hydrogen peroxide was delivered from a 50 mM stock solution in MilliQ water. PMA was added from stock solution of 1 mg/mL in DMSO. For proliferation assays, one day before experiments, HEK293T cells were passaged and plated on 96 well plates at a density of 3\(\times\)10\(^4\)/well. Cells were incubated with WST-1 solution in DMEM containing DMSO, AN1, or PN1 at indicated concentrations for 2 h at 37 °C with 5% CO\(_2\). The formation of the formazan product was measured by absorption at 440 nm using SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). AN and PN in DMEM have no background absorption at 440 nm. Confocal fluorescence imaging of HEK293T cells was performed with a Zeiss 510 Axioimager laser scanning microscope and 40x Acroplan water-immersion objective lens. Excitations of AN and PN were carried out with a MaiTai two-photon laser (mode-locked Ti:sapphire laser, Spectra-Physics, 80 MHz, 80 fs) using 700 - 750 nm pulses and output power 2400 mW. Lambda mode scanning was acquired with META spectral detector with 10.7 nm steps. Data were analyzed using ImageJ software (NIH) and Ratio Plus plug-in (programmer: Paulo Magalhes).

**Fresh Rat Hippocampal Slices Preparation and Imaging.** Rat hippocampal slices were prepared from the hippocampi of 2-days-old rat (SD) according to an approved institutional review board protocol. Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO\(_3\), 1.25 mM NaH\(_2\)PO\(_4\), 10 mM D-glucose, 2.4 mM CaCl\(_2\), and 1.3 mM MgSO\(_4\)). Slices were
incubated with 20 μM PN1 in ACSF bubbled with 95% O₂ and 5% CO₂ for 2 h at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. Two-photon (TP) fluorescence microscopy images of probe-labeled tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with 100x (NA = 1.30 OIL) and 20x (NA = 0.30 DRY) objective lens. The TP fluorescence microscopy images were obtained using a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 750 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 390 – 465 nm and 500 – 550 nm range, internal PMTs were used to collect the signals in an 8 bits unsigned 512 × 512 pixels at 400 Hz scan speed.
Figures and Schemes

Scheme 1. Synthesis of 2-acetyl-6-aminonaphthalene (AN1), and Peroxy-Naphthalene-1 (PN1).
Scheme 2. Synthesis of AN2 and PN2.
### Table 1: Spectroscopic properties of PN and AN fluorophores

<table>
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<th>Compound</th>
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<th>$\lambda_{\text{em}}$ (nm)</th>
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Figure 1. Reaction of PN1 (5 µM in HEPES buffer pH 7.4) to H₂O₂ (1 mM) monitored by (a) absorption spectra (0, 10, 20, 30 min), and (b) fluorescence emission spectra (0, 5, 10, 20, 30 min, λ_exc = 350 nm). (c) Plot of changes in fluorescence intensity at 500 nm (λ_exc = 375 nm) under pseudo-first-order conditions (1 µM PN1, 1 mM H₂O₂) gave k_obs = 9.6 x 10⁻⁴ s⁻¹.
Figure 2. Reaction of PN2 (5 µM in HEPES buffer pH 7.4) to H₂O₂ (1 mM) monitored by (a) absorption spectra (0, 10, 20, 30 min), and (b) fluorescence emission spectra (0, 5, 10, 20, 30 min, λ_{exc} = 350 nm). (c) Plot of changes in fluorescence intensity at 500 nm (λ_{exc} = 375 nm) under pseudo-first-order conditions (1 µM PN2, 1 mM H₂O₂) gave k_{obs} = 1.1 \times 10^{-3} \text{ s}^{-1}.
Figure 3. Fluorescence responses of 3 µM PN1 to various reactive oxygen species (ROS) at 200 µM. Bars represent emission intensity ratios $F_{\text{green}}/F_{\text{blue}}$ 0, 15, 30, 45, 60, 90, and 120 min after addition of each ROS. Data were acquired at 25 °C in 20 mM HEPES, pH 7.0, $\lambda_{\text{exc}} = 370$ nm
Figure 4. Effect of pH on $F_{\text{green}}/F_{\text{blue}}$ ratio of AN1 and PN1 in HEPES buffer (20 mM, pH 7.0). $\lambda_{\text{exc}} = 370$ nm.
Figure 5. Two-photon action spectra of 2 μM PN1 and 2 μM AN1 in HEPES buffer ([HEPES] = 20 mM, pH 7.1). The estimated uncertainties for the two-photon action cross section values (δΦ) are ±15%.
Figure 6. Two-photon confocal microscopy cells in lambda mode scanning of HEK293T labeled with (a) PN1 and (b) AN1 using $\lambda_{\text{exc}} = 740$ nm. (c) Normalized fluorescence emission spectra obtained from lambda mode scanning in (a) and (b).
Figure 7. (a-c) Two-photon confocal images of HEK293T displaying ratiometric fluorescence response of PN1-labeled cells to H$_2$O$_2$. Cells were loaded with PN1 (a,b), or AN1 (c) then washed twice with fresh buffer. In control experiment (a,c), PN1 or AN1-labeled cells were further incubated in DPBS for 30 min. (b) Cells treated with 100 µM H$_2$O$_2$ for 30 min showed increase in F$_{\text{green}}$/F$_{\text{blue}}$ ratio displayed in pseudocolor with 16 colors look-up table presenting ratio of 0 to 2. Images were acquired using 700 nm excitation and fluorescence emission windows: Blue = 435 - 485 nm, Green = 500 - 550 nm. Scale bar = 20 µm. (d) Quantification of imaging experiment, error bars represent standard deviation of the average F$_{\text{green}}$/F$_{\text{blue}}$ obtained from 5 images. (e) WST-1 assay affirmed that incubation of cells with 5 - 10 µM AN1 or PN1 for 2 h in DMEM induce no cytotoxicity.
Figure 8. Two-photon confocal images of HEK293T displaying ratiometric fluorescence response of PN2-labeled cells to H₂O₂. Cells were loaded with PN2 (5μM in DPBS, 30 min) then washed twice with fresh buffer. In control experiment (a), PN2-labeled cells were further incubated in DPBS for 30 min. (b) Cells treated with 100 μM H₂O₂ for 30 min showed increase in F_{green}/F_{blue} ratio displayed in pseudocolor with 16 colors look-up table presenting ratio of 0 to 2. Images were acquired using 710 nm excitation and fluorescence emission windows: Blue = 435 - 485nm, Green = 500 - 550 nm. Scale bar = 20 μm. (c) Quantification of imaging experiment, error bars represent standard deviation of the average F_{green}/F_{blue} obtained from 5 images.
Figure 9. Pseudo-colored TPM ratiometric images ($F_{\text{green}}/F_{\text{blue}}$) of RAW264.7 cells labeled with (a) PN1 and (d) AN1. (b,c) Cells were pretreated with (b) PMA ($1 \mu$g/mL) and (c) 100 µM $H_2O_2$ for 30 min before labeling with PN1. (e) Average $F_{\text{green}}/F_{\text{blue}}$ in images (a-d). (f) Lambda mode scanning of PN1, PMA, $H_2O_2$ and AN1 in figures (a-d). Images were acquired using 750 nm excitation and fluorescence emission windows: Blue = 390 - 465 nm, Green = 500 - 550 nm. Scale bar = 30 µm.
**Figure 10.** TPM images of a fresh rat hippocampal slice stained with 20 μM PN1. Fluorescence emission was collected at 390 - 465 (blue) and 500 - 550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of ~ 90 - 180 μm with 10x magnification. Ratiometric images were constructed from images in blue and green row. Scale bar = 300 μm.
Figure 11. TPM images of a fresh rat hippocampal slice stained with 20 μM PN1 after treatment with 1 mM H₂O₂ for 30 min. Fluorescence emission was collected at 390 - 465 (blue) and 500 - 550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of ~ 90 - 180 μm with 10x magnification. Ratiometric images were constructed from images in blue and green row. Scale bar = 300 μm.
Figure 12. TPM images of a fresh rat hippocampal slice stained with 20 μM AN1. Fluorescence emission was collected at 390 - 465 (blue) and 500 - 550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of ~ 90 - 180 μm with 10x magnification. Ratiometric images were constructed from images in blue and green row. Scale bar = 300 μm.
Figure 13. Ratiometric TPM image of a fresh rat hippocampal slice. (a-c) Ratiometric TPM images were accumulated along the z-direction at the depths of 90 - 180 µm (10 images) with 10x magnification: (a) a hippocampal slice labeled with PN1 (20 µM, 30 min), (b) a hippocampal slice pretreated with H₂O₂ (1mM, 30 min) before PN1 labeling (20 µM, 30 min), and (c) a hippocampal slice labeled with AN1 (20 µM, 30 min). (d-f) Enlarged images of (a-c) at 120 µm depth with 100x magnification. (g) Approximate positions (dotted circles) used for measurements of emission ratios in the CA3 and CA1. (h) Average F_{green}/F_{blue} in figure (a-c). The TPEF were collected at two channels (Blue = 390 - 465 nm, Green = 500 - 550 nm) upon excitation at 750 nm with fs pulse. Scale bars: (a) 30 µm and (d) 300 µm.
References


Chapter 3
Organelle-Targetable Fluorescent Probes for Imaging Hydrogen Peroxide in Living Cells via SNAP-Tag Protein Labeling

Portions of this work were published in the following scientific journal:

Portions of this work were performed in collaboration with the following people:
Aaron E. Albers synthesized SPG1 and STG1.
Christine Nam assisted in plasmid cloning.
Anthony T. Iavarone performed LC-MS characterization of SNAP-Tag protein.
Abstract

Hydrogen peroxide (H$_2$O$_2$) is a potent small-molecule oxidant that can exert a diverse array of physiological and/or pathological effects within living systems depending on the timing and location of its production, accumulation, trafficking, and consumption. To help study the chemistry and biology of this reactive oxygen species (ROS) in its native cellular context, we now present a new method for monitoring local, subcellular changes in H$_2$O$_2$ levels by fluorescence imaging. Specifically, we have exploited the versatility of the SNAP-tag technology for site-specific protein labeling with small molecules on the surface or interior of living cells with the use of boronate-capped dyes to selectively visualize H$_2$O$_2$. The resulting SNAP-Peroxy-Green (SNAP-PG) probes consist of appropriately derivatized boronates bioconjugated to SNAP-tag fusion proteins. Spectroscopic measurements on the SNAP-PG constructs confirm their ability to detect H$_2$O$_2$ with specificity over other biologically relevant ROS. Moreover, these hybrid small-molecule/protein reporters can be used in live mammalian cells expressing SNAP-tag fusion proteins directed to the plasma membrane, nucleus, mitochondria, and endoplasmic reticulum. Imaging experiments using scanning confocal microscopy establish organelle-specific localization of the SNAP-tag probes and their fluorescence turn-on in response to changes in local H$_2$O$_2$ levels. This work provides a general molecular imaging platform for assaying H$_2$O$_2$ chemistry in living cells with subcellular resolution.

Introduction

Reactive oxygen species (ROS) molecules derived from the metabolism of oxygen by living organisms are classically known as indicators for oxidative stress and potential contributors to aging$^{1-6}$ and diseases ranging from cancer$^{7-12}$ to diabetes$^{13-15}$ to neurodegeneration.$^{16-18}$ However, organisms that live in aerobic environments can also harness and incorporate ROS chemistry into their normal physiology by means of regulated production, release, and compartmentalization of these oxygen metabolites.$^{19-26}$ A prime example of ROS chemistry used for beneficial purposes is the oxidative burst within phagosomes of macrophages and neutrophils that serves as a defense against invading pathogens.$^{27-29}$ In this context, a major ROS produced in living systems is hydrogen peroxide (H$_2$O$_2$), which is a newly recognized messenger in cellular signal transduction but also a participant in oxidative stress and damage. Whereas high concentrations of H$_2$O$_2$ can trigger apoptotic or necrotic cell death, controlled bursts of H$_2$O$_2$ produced in response to stimulation by various growth factors, cytokines, and neurotransmitters can act as secondary messengers to signal cell growth, proliferation, and differentiation.$^{19-26,30-39}$

Because variations in the spatial and temporal production of H$_2$O$_2$ lead to disparate downstream biological effects, new methods that allow detection of this ROS in living systems with subcellular resolution can help decipher its complex cellular chemistry. To this end, an elegant example of a fluorescent protein sensor based on a circularly permuted YFP with an OxyR insert has been reported for cellular H$_2$O$_2$ detection.$^{40}$ We envisioned developing an alternative chemical biology approach that combines the versatility of small-molecule reporters with the targetability of genetically encodable protein scaffolds for subcellular localization. Currently, selective protein labeling in living cells with synthetic compounds is feasible in either a covalent and non-covalent manner.$^{41-45}$ Non-covalent labeling methods utilizing the specific binding of ligands to short peptide or protein domains include the binding of biarsenic compounds to tetracysteine hairpins (FLAsH, ReAsH),$^{46}$ the binding of metal complex to oligo-
His or oligo-Asp motifs, the recognition of trimethoprim by dihydrofolate reductase, and a high affinity binding interaction of protein FKBP12(F36V) with its ligand FK-SLF. Examples of covalent self-labeling mediated by fusion enzymes include attachment of alkyl chlorides to dehalogenase enzymes (HaloTag), formation of covalent adduct between cutinase and p-nitrophenyl phosphonate (pNPP) derivatives, labeling of AGT (O^-alkyguanine-DNA alkyltransferase) fusion proteins with SNAP/CLIP substrates, and covalent labeling by enzymes involved in post-translation modifications such as phosphopantetheine transferase, transglutaminase, biotin ligase BirA, lipoic acid ligase, sortaseA, protein farnesyltransferase, and formylglycine generating enzyme. In particular, we were attracted to the SNAP tag technology, which within short time after its invention by Johnsson and coworkers, has found broad utility in a variety of biological applications. Use of the SNAP tag allows precise targeting of bioactive reagents or probes to specific subcellular locales; recent examples include photosensitizers for chromophore-assisted laser inactivation (CALI) of fusion proteins and fluorescent sensors for intracellular metal ions including zinc(II) and calcium(II). Simultaneous labeling of two different targets can also be achieved using tandem SNAP and CLIP tags. The SNAP tag has been used for protein tracking with spatial and temporal resolution, where a fusion protein at a given time point is initially marked by a fluorescent or non-fluorescent SNAP tag substrate label, and subsequent newly synthesized proteins can be differentiated by a second label. Other applications of the SNAP tag technology include its use in combination with fluorescent proteins as FRET-based reporters for studying protein-protein interactions and the development of semisynthetic fluorescent sensors or photoactivable switches.

In this report, we present the design, synthesis, properties, and cellular applications of a new class of organelle-targetable fluorescent probes for H_2O_2 based on SNAP-AGT bioconjugation chemistry. We have prepared and characterized a pair of boronate-caged, Peroxy Green-type fluorescent peroxide indicators bearing SNAP substrates, where one is a membrane-permeable version for intracellular use and another is a membrane-impermeable version for cell surface labeling, and coupled them to AGT proteins expressed in various cellular compartments. The resulting SNAP-Peroxy-Green (SNAP-PG) hybrid small-molecule/protein conjugates are capable of detecting H_2O_2 over a variety of ROS and can be targeted to various subcellular locales, including the plasma membrane, nucleus, mitochondrial inner membrane, and endoplasmic reticulum, to sense changes in H_2O_2 levels within or on the surface of living cells. This method provides a general platform for studying the chemistry of H_2O_2 by molecular imaging in living biological specimens with subcellular resolution.

Results and Discussion

Design and Synthesis of SNAP-Peroxy-Green Bioconjugates as Organelle-Targetable Fluorescent Reporters for Cellular Hydrogen Peroxide. Scheme 1 depicts our overall strategy for creating subcellular-targetable fluorescent H_2O_2 probes by combining the SNAP-tag methodology for site-specific labeling with the chemoselective H_2O_2-mediated deprotection of boronate esters to phenols for reaction-based detection of H_2O_2. Fusion of AGT with proteins containing a signaling sequence allows expression of AGT in defined subcellular compartments. These localized AGT scaffolds can then be tagged by covalent labeling at Cys145 with a boronate Peroxy Green (PG) fluorescent probe bearing an appended SNAP substrate. The resulting SNAP-PG conjugates are hybrid small-molecule/protein reporters that give a fluorescent turn-on response upon H_2O_2-mediated boronate cleavage.
To this end, we previously reported the synthesis and application of Peroxy-Green-1 (PG1), a cell-permeable and selective H$_2$O$_2$ probe that can be activated by a single boronate deprotection. Along these lines, we sought to create PG1 analogs for organelle-specific labeling by replacing its 4-methoxy moiety with benzylguanine or benzyl-2-chloro-6-aminopyrimidine SNAP tag substrates. Scheme 2 depicts the synthesis of SNAP tag derivatizes for coupling to a PG1 scaffold. Benzylguanine was synthesized according to literature protocols. In a similar manner, benzyl-2-chloro-6-aminopyrimidine 3 was synthesized by nucleophilic substitution of 2,6-dichloropyrimidin-4-amine with trifluoro-N-(4-hydroxymethyl-benzyl)-acetamide 1. The trifluoroacetamide protecting group was subsequently removed with 33% methylamine in ethanol.

Scheme 3 details the synthesis of SNAP-PeroxyGreen-1 (SPG1) and SNAP-PeroxyGreen-2 (SPG2) probes by coupling the aforementioned SNAP tag substrates with a carboxylate-derivatized PG1; the latter is prepared in five steps from the fluorescein-like dye Tokyo Green. Treatment of Tokyo Green 5 with boron tribromide at -78 °C in dry CH$_2$Cl$_2$ furnishes phenolic Tokyo Green 6. O-alkylation of 6 in anhydrous DMF with excess cesium carbonate and a slow addition of tert-butylbromoacetate gives tert-butyl ester Tokyo Green 7. Reaction of 7 with bis(trifluoromethane)sulfonamide affords triflate 8. Palladium-mediated borylation of triflate 8 with bis(pinacolato)diboron, cyclohexyl JohnPhos, and diisopropylethylamine in anhydrous 1,4-dioxane provides boronic ester 9. Subsequent treatment with trifluoroacetic acid in anhydrous CH$_2$Cl$_2$ delivers carboxyl PG 10. Amide bond formation between carboxyl PG 10 and SNAP substrate 4 or 3 mediated by N,N,N′,N′-tetramethyl-O-(7-azabenzotriazol-1-yl)uroniumhexafluorophosphate (HATU) affords SPG1 (11) or SPG2 (12), respectively.

Finally, we also synthesized Tokyo Green (TG) analogs bearing SNAP tags 3 or 4 as control compounds for cell labeling and imaging as shown in Scheme 4. Treatment of tert-butyl ester Tokyo Green 7 with TFA furnishes carboxyl Tokyo Green 13. The HATU-mediated amide coupling of 13 with either 4 or 3 provides SNAP-TokyoGreen-1 (STG1, 14) and SNAP-TokyoGreen-2 (STG2, 15), respectively.

**Spectroscopic Properties, In Vitro Protein AGT labeling, and Peroxide Responses of SNAP-PG and SNAP-TG Fluorophores.** Table 1 summarizes the spectroscopic properties of the SNAP-PG and SNAP-TG fluorophores in aqueous buffer at neutral pH (20 mM HEPES, pH 7). SPG1 and SPG2 both show visible absorption bands centered at 465 nm (SPG1: $\varepsilon = 10,200$ M$^{-1}$ cm$^{-1}$, SPG2: $\varepsilon = 9,800$ M$^{-1}$ cm$^{-1}$) and weak fluorescence with an emission maximum at 515 nm. In contrast, the STG1 and STG2 fluorophores exhibit a prominent absorption at 495 nm (STG1: $\varepsilon = 36,000$ M$^{-1}$ cm$^{-1}$, STG2: $\varepsilon = 37,000$ M$^{-1}$ cm$^{-1}$) and fluorescence emission maximum at 513 nm. The quantum yield of STG1 ($\Phi = 0.12$) is much lower than that of STG2 ($\Phi = 0.87$), presumably due to the greater quenching effect of guanidine moiety; a similar effect has been observed in other SNAP fluorophores. We thus proceeded to test the H$_2$O$_2$ reactivity of these free dyes with the SPG2 analog. Reaction of SPG2 with 1 mM H$_2$O$_2$ triggers a ca. 32-fold fluorescence turn-on for the dye (Figure 1) confirming the peroxide response of these SNAP tag reporters. Kinetics measurements of the SPG2 boronate deprotection performed under pseudo-first order conditions (1 µM dye, 1 mM H$_2$O$_2$) give an observed rate constant of $k_{obs} = 1.4(1) \times 10^{-3}$ s$^{-1}$.

We next proceeded to test the ability of the SPG and STG dyes to label purified AGT protein samples and to evaluate the spectroscopic properties and peroxide responses of the isolated fluorophore-protein conjugates. For these *in vitro* protein labeling assays, hexahistidine-
tagged AGT (His-AGT) was overexpressed in *E. coli* and purified with a standard Ni-NTA affinity column. After incubation of His-tagged AGT with SPG1, SPG2, STG1, or STG2 dyes in 20 mM HEPES at pH 7 for 30 min at 37 °C, we confirmed the formation of the desired covalently labeled products, AGT-PG and AGT-TG, by both 488 nm excitation in-gel fluorescence measurements (Figure 2,3) and LC-MS (Figure 4-6). Specifically, AGT-TG and AGT-PG show mass increases of 478 and 506 Da, respectively, from His-AGT; these values are consistent with the calculated mass of the protein tagged with TG or the boronic acid form of PG, respectively. We purified the AGT-PG and AGT-TG bioconjugates for further spectroscopic evaluation using MWCO spin columns and size-exclusive gel chromatography to remove the free organic fluorophores. AGT-TG possesses a dominant absorption maximum in the visible region centered at 500 nm, which is slightly red-shifted relative the free TG dye. The AGT-TG complex shows prominent absorption at 280 nm (standard protein absorption) and 500 nm (TG dye), consistent with labeling of the AGT protein with the small-molecule TG payload (Figure 7a). Furthermore, formation of AGT-TG conjugate is also reflected in the observed fluorescence quantum yield, as binding of STG1 to AGT ejects guanidine from the dye and eliminates its quenching effect, resulting in a 6-fold fluorescence increase (Figure 7b). The absorption and emission spectra of AGT-PG are similar to SPG1 and SPG2, with an additional 280 nm absorption due to the AGT protein.

Importantly, the SPG dyes are able to retain their fluorescence response to H$_2$O$_2$ upon protein labeling, as purified AGT-PG conjugates exhibit a marked turn-on emission response to added H$_2$O$_2$ (Figure 8). The H$_2$O$_2$-induced fluorescence increases occur with concomitant growth of the characteristic TG absorption at 500 nm, consistent with boronate cleavage of PG to produce the phenol TG product (Figure 9). Finally, we observe a linear correlation between various H$_2$O$_2$ concentrations added and observed fluorescence emission responses (Figure 10).

**Fluorescence Detection of H$_2$O$_2$ in Various Compartments of Living Cells with SNAP-AGT Peroxy Green Reporters.** With spectroscopic data establishing the formation of AGT-PG conjugates and their fluorescence turn-on response to H$_2$O$_2$, we next sought to apply SPG1 and SPG2 for optical detection of H$_2$O$_2$ in living biological samples. Specifically, we focused on directing these probes to a broad range of subcellular compartments that are either capable of locally generating H$_2$O$_2$ in response to various stimuli or to sites where their function and regulation are sensitive to H$_2$O$_2$ flux. In this study, we targeted SPG reporters to a broad spectrum of locations. Included are the plasma membrane, mitochondria, endoplasmic reticulum, and nucleus. To this end, plasmids for expression of the SNAP-tag in live mammalian cells were obtained from Covalys and then subjected to further modification as necessary. The parent SNAP-tag without a signaling sequence (pSNAP), encoding a genetically optimized AGT protein, gives a uniform intracellular expression of SNAP-tag in the cytoplasm and the nucleus. Localization of the SNAP-tag to nucleus is achieved by fusion of SNAP-tag to the C-terminus of the histone H2B protein (SNAP-H2B). Fusion of the SNAP-tag to the C-terminus of cytochrome c oxidase subunit 8 (Cox8A) enables its localization to the mitochondrial inner membrane (SNAP-Cox8A). For plasma membrane targeting, SNAP-tag is expressed as a fusion to the N-terminus of neurokinin-1 receptor (NK1R) and the C-terminus of 5HT3A serotonin receptor signaling sequence, resulting in a SNAP-tag exposed to the extracellular space (SNAP-NK1R). Addition of KDEL, a signaling peptide for retention of protein in endoplasmic reticulum, to the C-terminus of SNAP results in an ER localized SNAP-tag (SNAP-KDEL).
We first tested the site-specific labeling of various SNAP fusion proteins with control STG1 and STG2 dyes in live HEK293T cells. To our surprise, we found that STG1 and STG2 exhibit marked differences in cell permeability that can be exploited to obtain more selective conjugation to extracellular and intracellular spaces. Specifically, whereas both STG1 and STG2 gave excellent membrane labeling with SNAP-NK1R, intracellular tagging of pSNAP and SNAP-H2B was observed only with STG2 (Figure 11). The same trend applies to SPG1 and SPG2, where only the latter is membrane permeable. Our findings are consistent with previous work that reports that benzylguanine can hamper the cell permeability of its conjugated partners in a fluorophore-dependent manner. STG2 shows desirable properties for intracellular site-specific labeling applications, as the dye is cell permeable and unbound probe can be readily washed out. This latter property is crucial to minimizing background fluorescence from non-specific or off-target labeling. Indeed, in HEK293T cells transiently expressing SNAP-KDEL and SNAP-Cox8A to target the endoplasmic reticulum and mitochondria, respectively, we were able to successfully tag these organelles with STG2. The subcellular localization of this dye to these compartments was confirmed by fluorescence overlay of green emissive STG2 with red emission from mCherry-KDEL and mCherry-Cox8A constructs (Figure 12).

Recognizing these SNAP-tag-dependent differences in membrane permeability, we then proceeded to evaluate the boronate SPG1 and SPG2 reporters for site-specific subcellular H$_2$O$_2$ detection, employing SPG1 for extracellular plasma membrane targeting and SPG2 for intracellular labeling. For extracellular measurements, HEK293T cells transiently expressing SNAP-NK1R display weak but detectable green fluorescent emission localized to the plasma membrane after SPG1 conjugation. The relatively poor membrane permeability of this probe allows for rapid and selective labeling of cell surfaces with washing. Treatment of SPG1-labeled cells with 100 µM H$_2$O$_2$ triggers a marked increase in fluorescence from the plasma membrane region as shown by time lapse image acquisitions (Figure 13a). Importantly, negligible emission comes from non-transfected cells in the same image frame even after treatment with H$_2$O$_2$, establishing that specific SPG1 binding to the SNAP-NK1R fusion protein scaffold is required for imaging changes in H$_2$O$_2$ levels localized to the plasma membrane. As a further control, we do not observe fluorescence turn-on responses for SPG1-loaded cells in the absence of H$_2$O$_2$, and a combination of nuclear staining and brightfield transmission measurements confirm that the cells are viable throughout the imaging experiments.

To label intracellular compartments, we subjected cells to 5 µM SPG2 for 30 min followed by a 30 min incubation in fresh complete medium (DMEM +10% FBS) to wash out any unbound probe. HEK293T cells transiently expressing pSNAP display weak intracellular fluorescent emission after labeling with SPG2. Addition of 100 µM H$_2$O$_2$ to the cells results in a patent increase in overall intracellular fluorescence from this protein/small-molecule reporter (Figure 13b). Likewise, SPG2-loaded HEK293T cells transiently expressing SNAP-H2B show nuclear-localized fluorescence that becomes more intense after stimulation with H$_2$O$_2$ (Figure 12c). Moreover, SPG2 can be precisely immobilized onto the mitochondrial inner membrane and ER regions of HEK293T cells transiently expressing SNAP-Cox8A (Figure 14) and SNAP-KDEL (Figure 15), respectively. In both cases, the turn-on green fluorescence emission from the SNAP-PG probes after H$_2$O$_2$ treatment display good co-localization with the red mCherry emission of the corresponding SNAP protein target, and the staining pattern can persist for many hours. Because the mitochondrial and ER structures are relatively small compared to the overall volume of the cell, fluorescence emission from these organelles could be easily overwhelmed by noise from any non-specific staining in cytoplasm. These experiments clearly demonstrate that
non-specific binding is not an issue with the SPG2 probe, as unbound dye can be effectively removed from cells with a simple washing step. Taken together, these data show that SPG1 and SPG2 can be used to label and sense changes in H$_2$O$_2$ levels in specific subcellular compartments via selective bioconjugation with a variety of localizable SNAP-tag fusion constructs (Figure 16).

**Concluding Remarks**

In summary, we have described the synthesis, spectroscopic properties, H$_2$O$_2$ responses, and live-cell imaging applications of a new class of organelle-targeted reporters for cellular H$_2$O$_2$. SPG1 and SPG2 combine boronate-caged Tokyo Green derivatives for H$_2$O$_2$ detection with SNAP tags for site-specific protein labeling at extracellular and intracellular locations, respectively. When SPG1 and SPG2 are exposed to AGT or an AGT fusion protein, these dyes form covalent AGT-PG adducts that respond to H$_2$O$_2$ by a turn-on fluorescence increase upon H$_2$O$_2$-mediated boronate deprotection. These hybrid small-molecule/protein reporters can be used to label a variety of subcellular locations, including the plasma membrane, nucleus, mitochondria, and endoplasmic reticulum, and detect changes in local H$_2$O$_2$ fluxes in living cells by confocal microscopy. The methodology presented here combines the precision of site-specific labeling using genetically encodable tags with the chemical versatility of small-molecule probes. Ongoing efforts are focused on expanding the expanding color palette of targetable H$_2$O$_2$ probes, optimizing their sensitivities and dynamic turn-on responses to H$_2$O$_2$, creating targetable reporters with ratiometric readouts, and developing additional labeling methodologies for multichannel imaging. We anticipate that simultaneous measurements of H$_2$O$_2$ and other ROS fluxes in multiple locales using hybrid small-molecule/protein conjugates will contribute to a better understanding of oxidation biology in states of health, aging, and disease, with particular interest in elucidating cellular redox regulation and signaling in multiple cellular compartments.

**Experimental Section**

**Materials and Methods.** All reactions were carried out under a dry nitrogen atmosphere. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. Palladium(II) acetate [Pd(OAc)$_2$], Dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) [Pd(dppf)Cl$_2$], and 2-(Dicyclohexylphosphino)biphenyl [Cyclohexyl JohnPhos] were purchased from Strem Chemicals (Newburyport, MA). Bis(pinacolato)diboron was purchased from Boron Molecular (Research Triangle Park, NC). 2,2,2-Trifluoro-N-(4-hydroxymethyl-benzyl)-acetamide (1) and O6-(4-Amino-methyl-benzyl)guanine (4) were synthesized following literature procedures. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. $^1$H NMR and $^{13}$C NMR spectra were collected in CDCl$_3$, DMSO-$d_6$, or CD$_3$OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using a Bruker 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Restriction endonuclease and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Hoechst 33342, monoclonal Anti-FLAG M2 antibody and Alexa Fluor 488 Goat
anti-mouse IgG antibody were obtained from Invitrogen (Carlsbad, CA). pCEMS-CLIP10m-NK1R, pSEMS-SNAP26m-ADBR, pCEMS1-H2B-CLIP10m, and pSEMS1-Cox8A-SNAP26m plasmids were purchased from Covalys Biosciences (Witterswil, Switzerland). pmCherry and DsRed Golgi were obtained from Clontech Laboratories (Mountain View, CA). pET28a (+) was obtained from Novagen (EMD Chemicals, Gibbstown, NJ). Primers for PCR amplification were synthesized by Intergated DNA Technologies (San Diego, CA).

N-(4-((2-Amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-2,2,2-trifluoroacetamide (2). Compound 1 (1.0 g, 4.3 mmol) in anhydrous DMF (7 mL) was added to a dry 50 mL Schlenk flask under a nitrogen atmosphere. NaH (60% in mineral oil, 515 mg, 12.8 mmol) was then added slowly to the reaction flask. After stirring at room temperature for 15 min, the solution appeared blue-green in color. A solution of 4,6-dichloropyrimidin-4-amine (700 mg, 4.3 mmol) in anhydrous DMF (5 mL) was added dropwise to the reaction flask over a 5 min period, giving a brown-colored solution. The reaction was then heated to 90 °C for 6 h. The reaction was cooled to room temperature, quenched with slow addition of cold water (20 mL), extracted with EtOAc (3 × 30 mL), and dried over Na2SO4. Purification by column chromatography (2:1, EtOAc/hexanes) gave 2 as clear viscous oil (300 mg, 0.83 mmol, 19% yield). 1H NMR (DMSO-d6, 400 MHz): δ 9.99 (1H, s-br), 7.39 (2H, d, J = 8.4 Hz), 7.26 (2H, d, J = 8.4 Hz), 7.09 (2H, s), 6.10 (1H, s), 5.26 (2H, s), 4.36 (2H, s). GC-MS: calculated for [M+] 360.06, found 360.1.

4(4-(Aminomethyl)benzoyloxy)-6-chloropyrimidin-2-amine (3). Methylamine (33% in EtOH, 2.5 mL) was added to the solution of 2 (250 mg, 0.69 mmol) in MeOH (5 mL). The mixture was stirred at room temperature for 24 h. The solvent was removed in vacuo, providing product 3 as a white powder (175 mg, 0.66 mmol, 95% yield) 1H NMR (DMSO-d6, 400 MHz): δ 7.33 (2H, d, J = 8.0 Hz), 7.31 (2H, d, J = 8.0 Hz), 7.10 (2H, s-br), 6.10 (1H, s), 5.25 (2H, s), 3.68 (2H, s). LC-MS: calculated for [MH+] 265.08, found 265.1.

6-Hydroxy-9-(4-hydroxy-2-methylphenyl)-3H-xanthen-3-one (6). Tokyo Green 5 (6.3 g, 18.9 mmol) was added to a dried 500 mL round bottom flask. Anhydrous CH2Cl2 (300 mL) was added by cannula and the resulting insoluble mixture stirred at room temperature for 5 min. The flask was cooled to -78 °C in a dry ice/acetone bath and boron tribromide (14.2 g, 5.4 mL, 56.7 mmol) was added dropwise over 15 min. The reaction was allowed to warm to room temperature overnight and added slowly to 500 mL of vigorously stirred ice/H2O slurry, causing precipitation of a reddish orange solid. The mixture was stirred for an additional 24 h and the solid collected by vacuum filtration. Purification by flash column chromatography (1:10, MeOH/CH2Cl2) afforded 6 as a brick red solid (5.3 g, 16.6 mmol, 87% yield). 1H NMR (DMSO-d6, 400 MHz): δ 10.00 (1H, s), 7.35 (2H, d, J = 9.2 Hz), 7.08 (2H, d, J = 8.0 Hz), 7.07 (1H, s), 7.03 (2H, dd, J1 = 9.2 Hz, J2 = 2.4 Hz), 6.87 (1H, d, J = 2.4 Hz), 6.83 (2H, dd, J1 = 8.0 Hz, J2 = 2.4 Hz), 1.90 (3H, s). LC-MS: calculated for [MH+] 319.09, found 319.1.

tert-Butyl 2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)acetate (7). Phenolic Tokyo-Green 6 (3.13 g, 10.0 mmol) was added to a dried 500 mL Schlenk flask. Anhydrous DMF (300 mL) and cesium carbonate (22.2 g, 68.1 mmol) were added, and the mixture stirred at room temperature for 60 min. tert-Butylbromoacetate (1.95 g, 1.46 mL, 10.0 mmol) was added by micropipettor and the resulting reaction mixture stirred at room temperature overnight. The reaction was filtered and solvent concentrated under reduced pressure, giving a dark brown mass. H2O (100 mL) was added and the solution neutralized with 1M HCl, giving a red orange precipitate. The solid was collected by vacuum filtration, washed with ddH2O (100 mL), and dried in air. Purification by flash column chromatography (1:20, MeOH/CH2Cl2) furnished 5 as a brick red solid (1.54 g, 3.56 mmol, 36% yield). 1H NMR (CDCl3, 400 MHz): δ
7.10 (2H, d, J = 9.2 Hz), 7.09 (1H, d, J = 8.4 Hz), 6.93 (1H, d, J = 2.4 Hz), 6.89 (1H, s), 6.88 (2H, s), 6.83 (2H, dd, J1 = 9.2 Hz, J2 = 2.4 Hz), 4.61 (2H, s), 2.03 (3H, s), 1.54 (9H, s). LC-MS: calculated for [MH+]+ 433.16, found 433.2.

**tert-Butyl-2-(3-methyl-4-(3-oxo-6-(trifluoromethylsulfonyloxy)-3H-xanthen-9-yl)phenoxy)acetate (8).** tert-Butyl Tokyo-Green 7 (1.06 g, 2.44 mmol) was added to a dried 25 mL Schlenk flask. Anhydrous DMF (5 mL) and DIPEA (1.57 g, 2.1 mL, 12.2 mmol) were added, and the resulting mixture stirred at room temperature for 60 min. N-Phenyl bis-trifluoromethane sulfonylimide (1.37 g, 3.84 mmol) was dissolved in anhydrous DMF (3 mL) and added dropwise to the stirring basic solution over 5 min. The mixture was stirred at room temperature overnight, diluted in toluene (200 mL), washed with H2O (3 × 100 mL), and dried over MgSO4. Purification by flash column chromatography (1:4, EtOAc/hexanes) gave 8 as a puffy, orange crystalline solid (930 mg, 1.64 mmol, 67% yield). 1H NMR (CDCl3, 400 MHz): δ 7.39 (1H, d, J = 2.4 Hz), 7.18 (1H, d, J = 8.8 Hz), 7.09 (2H, d, J = 8.8, 2.4 Hz), 7.01 (1H, d, J = 10.0 Hz), 6.95 (1H, d, J = 2.4 Hz), 6.91 (1H, dd, J = 10, 2.4 Hz), 6.60 (1H, dd, J1 = 10.0 Hz, J2 = 2.0 Hz), 6.44 (1H, d, J = 2.0 Hz), 4.61 (2H, s), 2.05 (3H, s), 1.54 (9H, s). LC-MS: calculated for [MH+]+ 565.11, found 565.1.

**tert-Butyl-2-(3-methyl-4-(3-oxo-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-xanthen-9-yl)phenoxy)acetate (9).** Compound 8 (641 mg, 1.14 mmol), Pd(OAc)2 (84 mg, 0.12 mmol), Cyclohexyl JohnPhos (201 mg, 0.57 mmol), and bis(pinacolato)diboron (446 mg, 1.76 mmol) were added to a dried 25 mL scintillation vial in a glove box. Anhydrous 1,4-dioxane (10 mL) was added and the solution stirred at room temperature for 5 min. Anhydrous DIPEA (742 mg, 1.00 mL, 5.74 mmol) was added dropwise by syringe and the reaction mixture was stirred at room temperature overnight. The reaction vial was removed from the glove box, poured into saturated aqueous NH4Cl (30 mL), extracted with EtOAc (3 × 30 mL), and dried over MgSO4. Purification by flash column chromatography (1:4, EtOAc/hexanes) gave 9 as an orange crystalline solid (615 mg, 1.13 mmol, 99% yield). 1H NMR (CDCl3, 400 MHz): δ 7.90 (1H, s), 7.82 (1H, d, J = 8.0 Hz), 7.07 (1H, d, J = 8.4 Hz), 7.01 (1H, d, J = 9.6 Hz), 6.94 (1H, d, J = 2.4 Hz), 6.89 (1H, dd, J1 = 8.4 Hz, J2 = 2.4 Hz), 6.59 (1H, dd, J1 = 9.6 Hz, J2 = 2.0 Hz) 6.43 (1H, d, J = 2.0 Hz), 4.61 (2H, s), 2.03 (3H, s), 1.54 (9H, s), 1.37 (12H, s). LC-MS: calculated for [MH+]+ 543.25, found 543.3.

**2-(3-Methyl-4-(3-oxo-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-xanthen-9-yl)phenoxy)acetic acid (10).** tert-Butyl Peroxy-Green 9 (542 mg, 1.0 mmol) was dissolved in CH2Cl2 (3 mL) and added dropwise by Pasteur pipette to a stirring mixture of CH2Cl2:TFA (1:1, 25 mL). The solution was allowed to stir for 4 h and the solvent removed under reduced pressure. Purification by flash column chromatography (1:10, MeOH/CH2Cl2) gave 10 as an orange film (419 mg, 0.86 mmol, 86% yield). 1H NMR (CDCl3, 400 MHz): δ 8.23 (1H, s), 7.82 (1H, d, J = 8.0 Hz), 7.41 (1H, d, J = 8.0 Hz), 7.40 (1H, d, J = 8.8 Hz) 7.15 (3H, m), 7.04 (1H, s), 7.00 (1H, d, J = 8.8 Hz), 4.83 (2H, s), 2.01 (3H, s), 1.39 (12H, s). LC-MS: calculated for [MH+]+ 487.18, found 487.2.

**N-(4-((2-Amino-9H-purin-6-yl)oxy)methyl)benzyl)-2-(3-methyl-4-(3-oxo-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-xanthen-9-yl)phenoxy)acetamide (11), SPG1.** Compound 10 (99 mg, 0.20 mmol) was added to a dried 25 mL Schlenk flask. Anhydrous DMF (3 mL) was added by syringe and the solution was stirred at room temperature for 5 min. HATU (89 mg, 0.23 mmol), 6-(4-(aminomethyl)benzoxyl)-9H-purin-2-amine 4 (63 mg, 0.23 mmol), and DIPEA (266 mg, 0.359 mL, 2.06 mmol) were added, and the reaction stirred at room temperature overnight. The reaction was poured into an ice-water slurry (30 mL) and stirred to
precipitate an orange solid. The solid was collected by vacuum filtration and dried in vacuo. Purification by flash column chromatography (1:10, MeOH/CH₂Cl₂) provided SPG1 11 as an orange film (17.1 mg, 0.023 mmol, 11% yield). ¹H NMR (DMSO-d₆, 400 MHz): δ 12.41 (1H, s), 8.75 (1H, s), 7.78 (1H, s), 7.71 (1H, s), 7.55 (1H, d, J = 7.6 Hz), 7.45 (2H, d, J = 7.2 Hz) 7.31 (2H, d, J = 7.6 Hz), 7.24 (1H, d, J = 8.4 Hz), 7.11 (1H, s), 7.04 (2H, d, J = 8.4 Hz), 6.94 (1H, d, J = 9.6 Hz), 6.50 (1H, d, J = 9.6 Hz), 6.29 (2H, s), 6.25 (1H, s), 5.44 (2H, s), 4.67 (2H, s), 4.39 (2H, d, J = 6.4 Hz), 1.98 (3H, s), 1.31 (12H, s). HRFAB-MS: calculated for [MH⁺] 739.30, found 739.30.

N-(4-((2-Amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-2-(3-methyl-4-(3-oxo-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-xanthen-9-yl)phenoxy)acetamide (12), SPG2. Compound 10 (25 mg, 0.051 mmol) dissolved in anhydrous DMF (2 mL) was added to a dried 5 mL round-bottomed flask and the solution was stirred at room temperature for 5 min. HATU (25 mg, 0.066 mmol), compound 3 (18 mg, 0.068 mmol), and DIPEA (25 uL) were added, and the reaction stirred at room temperature overnight. DMF was removed under reduced pressure and the orange oil was redissolved in 10% MeOH in CH₂Cl₂. Purification by flash column chromatography (1:10, MeOH/CH₂Cl₂) afforded SPG2 12 as an orange film (20 mg, 0.027 mmol, 53% yield). ¹H NMR (20% MeOD in CDCl₃) δ 7.92 (1H, s), 7.55 (1H, d, J = 6.4 Hz), 7.33 (2H, d, J = 6.4 Hz), 7.28 (2H, d, J = 6.4 Hz), 7.07 (1H, d, J = 6.8 Hz), 7.02 (1H, d, J = 6.4 Hz), 6.9-7.0 (3H, m), 6.58 (1H, d, J = 7.6 Hz), 6.43 (1H, s), 6.06 (1H, s), 5.26 (2H, s), 4.61 (2H, s), 4.53 (2H, d, J = 3.6 Hz), 1.98 (3H, s), 1.32 (12H, s). HRESI-MS: calculated for [MH⁺] 733.25, found 733.26.

2-(4-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)acetic acid (13). Compound 7 (200 mg, 0.46 mmol) was stirred in a mixture of CH₂Cl₂: TFA (1:2, 20 mL) for 1 h. Removal of the solvent under reduced pressure gave a viscous orange oil. EtOAc (10 mL) was added, and the mixture was sonicated for 30 min. Slow addition of the EtOAc mixture into a beaker of vigorously stirring hexane (30 mL) gave carboxyl Tokyo Green 13 as orange solid precipitate (120 mg, 0.32 mmol, 69% yield). ¹H NMR (CD₃OD, 400MHz) δ 7.32 (2H, d, J = 8.6 Hz), 7.19 (1H, d, J = 8.4 Hz), 7.08 (1H, d, J = 2.0 Hz), 7.03 (1H, dd, J₁ = 8.4 Hz, J₂ = 2.0 Hz), 6.96 (2H, s), 6.92 (2H, d, J = 8.6 Hz), 4.80 (2H, s), 1.97 (3H, s). LC-MS: calculated for [MH⁺] 377.09, found 377.0.

N-(4-((2-Amino-9H-purin-6-yloxy)methyl)benzyl)-2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)acetamide (14), STG1. Compound 13 (235 mg, 0.67 mmol) was added to a dried 25 mL Schlenk flask. Anhydrous DMF (5 mL) was added by syringe and the solution was stirred at room temperature for 5 min. HATU (256 mg, 0.67 mmol), 6-(4-(aminomethyl)benzyloxy)-9H-purin-2-amine 4 (182 mg, 0.67 mmol), and DIPEA (0.87 mL) were added, and the reaction stirred at room temperature overnight. The reaction was poured into an ice-water slurry (30 mL) and stirred to precipitate an orange solid. The solid was collected by vacuum filtration and dried in vacuo. Purification by flash column chromatography (1:10, MeOH/CH₂Cl₂) delivered STG1 14 as an orange solid (197 mg, 0.31 mmol, 46% yield). ¹H NMR (DMSO-d₆, 400 MHz): δ 12.42 (1H, s-br), 8.74 (1H, s), 7.82 (1H, s), 7.45 (2H, d, J = 7.2 Hz), 7.29 (2H, d, J = 7.2 Hz), 7.20 (1H, d, J = 8.4 Hz), 7.09 (1H, s), 7.02 (1H, d, J = 8.0 Hz), 6.87 (2H, d, J = 8.8 Hz), 6.50-6.80 (4H, m), 6.29 (2H, br s), 5.44 (2H, s), 4.66 (2H, s), 4.38 (2H, d, J = 5.2 Hz), 3.16 (1H, s), 1.98 (3H, s). HRESI-MS: calculated for [MH⁺] 629.21, found 629.21.

N-(3-((2-Amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-2-(4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-3-methylphenoxy)acetamide (15), STG2. Compound 13 (30 mg, 0.079 mmol) in anhydrous DMF (2 mL) and HATU (30 mg, 0.078 mmol) were added to a dried 5 mL
round-bottomed flask and the solution was stirred at room temperature for 5 min. Compound 3 (21 mg, 0.079 mmol), and DIPEA (100 µL) were added, and the reaction was stirred at room temperature overnight. DMF was removed in vacuo and the orange oil was redissolved in 10% MeOH in CH₂Cl₂. Purification by flash column chromatography (1:10, MeOH/CH₂Cl₂) afforded STG2 as an orange film (45 mg, 0.072 mmol, 91% yield). ¹H NMR (20% CD₃OD in CDCl₃) δ 7.35 (1H, t, J = 6.0 Hz), 7.25 (2H, d, J = 8.2), 7.21 (2H, J = 8.2 Hz), 6.98 (1H, d, J = 8.0), 6.91 (2H, d, J = 8.8 Hz), 6.85 (1H, s), 6.82 (1H, d, J = 8.0), 6.41 (2H, d, J = 2.0 Hz), 6.60 (2H, dd, J₁ = 8.8 Hz, J₂ = 2.0 Hz), 5.97 (1H, s), 5.16 (2H, s), 4.52 (2H, s), 4.44 (2H, d, J = 6.0 Hz), 1.97 (3H, s). HRESI-MS: calculated for [MH⁺] 623.16, found 623.17.

Expression and Purification of His-AGT for SNAP Labeling. The sequence encoding SNAP was PCR amplified from pSEMS-SNAP-ADBR (Covalys) and subcloned into PET28a(+) (Novagen) using NheI and XhoI restriction sites. The resulting plasmid, after verified by DNA sequencing, was transformed into E. Coli strain BL-21(DE3). A bacterial culture was grown at 37 °C in TB medium (50 mL) with 50 µg/mL kanamycin for 6-8 h until an optical density (OD₆₀₀nm) of 0.8 was reached. Expression of His-AGT was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was grown at 16 °C for 12 h and was harvested by centrifugation at 8000 rpm for 10 min at 4 °C. Cell lysates were prepared by resuspending the pellet in buffer (PBS with 0.5 mM phenylmethylsulfonyl fluoride) followed by cell disruption with French press (Thermo Scientific). Insoluble protein and cellular debris were removed by centrifugation at 10,000 rpm for 30 min. For purification of His-AGT, Ni-NTA (Qiagen) was used according to the supplier’s protocol. The fractions with His-AGT were combined and concentrated using an Amicon Ultra-15 Ultracel®-10K centrifugal unit (Millipore). The concentrated His-AGT solution was then desalted using G25 Sephadex (Sigma Aldrich) with the following elution buffer: 150 mM NaCl, 25 mM Tris-Cl, 1mM DTT pH 7.5. The purified His-AGT protein was concentrated using an Amicon Ultra-4 Ultracel®-10K and stored at -20 °C. The protein concentration was determined using the BCA assay, yielding 8.6 mg of purified His-AGT (1.5 mM in 250 µL elution buffer).

In vitro SNAP Labeling and Analysis by ESI-MS and In-Gel Fluorescence Scanning. Purified His-AGT (0.5 uM) was incubated in reaction buffer (PBS pH 7.4, 1 mM DTT) at 37 °C with 0.5 µM of either SPG1, SPG2, STG1, or STG2 for 30 min at 37 °C. Binding of the fluorescent dyes to His-AGT was analyzed by SDS-PAGE, followed by in-gel fluorescence scanning (488 nm Argon excitation, 520 BP 40 emission filter, Typhoon 9410 imaging system, GE Healthcare) and Coomassie protein staining. For mass spectrometric analysis and spectroscopic measurement, His-AGT (1 µM) was incubated in 20 mM HEPES buffer pH 7 (1 mL) with 5 µM of either SPG1, SPG2, STG1, or STG2 for 30 min at 37 °C. The reaction was concentrated to 50 µM with an Amicon® 4 10K column (Milipore) and subjected to size-exclusive gel filtration with Bio-Rad Micro Bio-Spin 6 chromatography column to remove the excess dye. The deconvoluted MS data were collected by Dr. Anthony T. Iavarone at UC Berkeley QB3 Mass Spectrometry Facility.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.0, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). Fluorescein in 0.1 M NaOH (Φ = 0.85) was used as standard for quantum yield measurements.

**Cell Culture and Labeling Procedures.** HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before transfection, cells were passaged and plated on 4-wells Lab-Tek borosilicate chambered coverglass (Nunc). Transient expression of SNAP fusion protein or mCherry was performed by following the standard protocol of Lipofectamine 2000 (Invitrogen). SNAP-tag labeling was achieved by incubating cells in DPBS containing 5 µM dye and 1 µM Hoechst 33342 for 30 min at 37 °C. For membrane labeling with cell-impermeable SPG1 or STG1, cells were washed with fresh DPBS (2 × 1 mL) before image acquisition. Labeling of intracellular targets with STG2 or SPG2 required incubation of cells in fresh DMEM with 10% FBS (2 × 1 mL) for 30 min after dye loading to remove any unbound fluorophores. All confocal fluorescence images were acquired in DPBS media.

**Fluorescence Imaging Experiments.** Confocal fluorescence imaging studies were performed with a Zeiss LSM510 NLO Axiovert 200 Laser scanning microscope and a 40x oil-immersion objective lens. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO₂ humidified atmosphere. Excitation of the SNAP tag probe at 488 nm was carried out with an Argon laser and emission was collected using a 500 - 550 nm filter. Excitation of mCherry was carried out with a Helium-Neon 543 nm laser and emission was collected using a LP560 filter. Excitation of Hoechst 33342 was carried out using a MaiTai two-photon laser at 770 nm pulses (mode-locked Ti:sapphire laser, Tsunami Spectra Physics) and emission was collected through 385 - 425 nm filter. Image analysis was performed in ImageJ (National Institute of Health).

**Mass Spectrometric Analysis of intact AGT, AGT-PG, and AGT-TG.** Protein samples were analyzed using an Agilent 1200 series liquid chromatograph (LC; Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA). The LC was equipped with C₈ guard (Poroshell 300SB-C8, 5 µm, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100 µL sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials sealed with rubber septa caps (Wheaton Science, Millville, NJ) were loaded into the Agilent 1200 autosampler compartment prior to analysis. For each sample, approximately 50 to 100 picomoles of protein analyte was injected onto the column. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 µL/min. The elution program consisted of a linear gradient from 30% to 95% B over 24.5 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 0.5 min, and then isocratic conditions at 0.5% B for 9.5 min, at a flow rate of 90 µL/min. The column and sample compartments were maintained at 35 °C and 10 °C, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-contamination between samples.
The connections between the LC column exit and the mass spectrometer ion source were made using PEEK tubing (0.005” i.d. × 1/16” o.d., Western Analytical, Lake Elsinore, CA). External mass calibration was performed prior to analysis using the standard LTQ calibration mixture containing caffeine, the peptide MRFA, and Ultramark 1621 dissolved in 51% acetonitrile/25% methanol/23% water/1% acetic acid solution (v/v).91 The ESI source parameters were as follows: ion transfer capillary temperature 275 ºC, normalized sheath gas (nitrogen) flow rate 25%, ESI voltage 2.3 kV, ion transfer capillary voltage 33 V, and tube lens voltage 125 V. Mass spectra were recorded in the positive ion mode over the range \( m/z = 500 \) to 2000 using the Orbitrap mass analyzer, in profile format, with a full MS automatic gain control target setting of \( 5 \times 10^5 \) charges and a resolution setting of \( 6 \times 10^4 \) (at \( m/z = 400 \), FWHM).92 Raw mass spectra were processed using Xcalibur software (version 4.1, Thermo) and measured charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ), using default “small protein” parameters and a background subtraction factor of 1.5.

**Plasmid Constructs.** pSEMS-SNAP-COX8A-FLAG was constructed by insertion of FLAG tag (annealed from oligonucleotides Aesc1-aFLAG-F and Xhol-aFLAG-R) to pSEMS-COX8A plasmid. pCEMS-H2B-SNAP-FLAG was constructed by replacing CLIP sequence in pCEMs-H2B-CLIP with a PCR amplified SNAP-FLAG (using primer Nhel-SNAP-F and Xhol-FLAG-R). pSNAP, encoding SNAP-tag with no signaling sequence was made by replacing GFP in pMaxGFP (Amaxa) with a PCR amplified SNAP-FLAG (using primer Nhel-kSNAP-F and Xhol-FLAG-R). pCEMS-SNAP-NK1R was made by replacing ADBR sequence of pCEMS-SNAP-ADBR with NK1R-FLAG sequence cut from pCEMS-CLIP-NK1R using Shfl and BamHI restriction sites. pCEMS-SNAP-FLAG-KDEL was made by cloning a PCR amplified SNAP-FLAG-KDEL (using primer Nhel-SNAP-F and BamHI-KDEL-FLAG-R) into pCEMS-CLIP-NK1R that was cut with Nhel and BamHI to remove CLIP-NK1R sequence. Similarly, pCEMS-mCherry-KDEL was made by cloning the PCR amplified mCherry (using primer Nhel-mcherry-F and Xhol-KDEL-mCherry-R) with pCEMS-CLIP-NK1R that was cut at Nhel and Xhol restriction site to remove CLIP-NK1R sequence. DsRed-Golgi and pmCherry from Clontech was employed in the making of Cox8A-mCherry. mCherry-Golgi was obtained by replacing DsRed with mCherry sequence cut from pmCherry at BamHI and NotI site. Using Nhel and AgeI restriction site, golgi signaling sequence was then replaced by mitochondria targeting Cox8A sequence (PCR primer: Nhel-Cox8A-F and AgeI-Cox8A-R). All constructs are verified by DNA sequencing. Expression and correct localization of the fusion proteins in HEK293T are verified by immunofluorescence staining of FLAG tag in fixed cells using monoclonal Anti-FLAG M2 antibody (Sigma Aldrich) and Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen).

**Restriction Enzymes:**

- **Aesc1-aFLAG-F:** 5’ CGC GGA CTA CAA GGA CGA CGA TGA CAA GTG AC
- **Xhol-aFLAG-R:** 5’ TCG AGT CAC TTG TCA TCG TGC TCC TTG TAG TC
- **Nhel-SNAP-F:** 5’ GCT AAG GCT AGC ATG GAC AAA GAC TGC GAA
- **Xhol-FLAG-R:** 5’ GCC TCA CTC GAG TCA CTT GTC ATC GTC GTC CTT
- **Nhel-kSNAP-F:** GCT AAG GCT AGC GCC ACC ATG GAC AA GAC TGC GAA
- **BamHI-KDEL-FLAG-R:** 5’ TCA GGATCC TTA CAG CTC GTC GTC CTT CTT CTC GTC
- **Nhel-mCherry-F:** 5’ AGA TCC GCT AGC GCC ACC ATG GTG AGC AAG GCC GAG
- **Xhol-KDEL-mCherry-R:** 5’ ATC GAG CTC GAG TCA TTA CAG CTC GTC CTT GTC GTA CAG CTC GTC

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*Nhel*-Cox8A-F: 5’ AGA TCC GCT AGC GCC ACC ATG TCC GTC CTG ACG CCG
*Agel*-Cox8A-R: 5’ GTG GCG ACC GGT GGC TCT GGC CTC CTG TAG GT
Scheme 1. Design strategy for organelle-specific hydrogen peroxide reporters using the SNAP tag methodology.
Scheme 2. Synthesis of SNAP-tag substrates.
Scheme 3. Synthesis of SNAP-Peroxy-Green-1 (SPG1) and SNAP-Peroxy-Green-2 (SPG2).
Scheme 4. Synthesis of SNAP-Tokyo Green-1 (STG1) and SNAP-Tokyo Green-2 (STG2).
Table 1. Spectroscopic properties of SNAP dyes and their AGT bioconjugates.

<table>
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<th>Compound</th>
<th>$\lambda_{\text{exc}}$ (nm)</th>
<th>(M$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
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<td>36000</td>
<td>513</td>
<td>0.12</td>
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<td>STG2</td>
<td>495</td>
<td>37000</td>
<td>513</td>
<td>0.87</td>
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<tr>
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<td>500</td>
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<td>515</td>
<td>0.57</td>
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<td>10200</td>
<td>515</td>
<td>0.10</td>
</tr>
<tr>
<td>SPG2</td>
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<td>9800</td>
<td>515</td>
<td>0.09</td>
</tr>
<tr>
<td>AGT-PG</td>
<td>465</td>
<td>11500</td>
<td>515</td>
<td>0.07</td>
</tr>
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Table 2. Observed mass peaks for ESI-MS of His-AGT with or without SNAP tag dyes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\text{MW}_1$ (Da)</th>
<th>$\text{MW}_2$ (Da)</th>
<th>Mass Difference (Da)</th>
</tr>
</thead>
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<tr>
<td>His-AGT</td>
<td>23071</td>
<td>23249</td>
<td></td>
</tr>
<tr>
<td>His-AGT + STG1</td>
<td>23549</td>
<td>23727</td>
<td>478</td>
</tr>
<tr>
<td>His-AGT + STG2</td>
<td>23549</td>
<td>23727</td>
<td>478</td>
</tr>
<tr>
<td>His-AGT + SPG1</td>
<td>23577</td>
<td>23755</td>
<td>506</td>
</tr>
<tr>
<td>His-AGT + SPG2</td>
<td>23577</td>
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<td>506</td>
</tr>
</tbody>
</table>
Figure 1. (a) Absorption spectra of 3 µM SPG2 response to added 1 mM H₂O₂ in 20 mM HEPES, pH 7 at 25 °C. Spectra were taken at 0, 10, 20, 30, 40, 50, 60, and 90 min after H₂O₂ was added. (b) Fluorescence emission spectra of 1 µM SPG2 after addition of 1 mM H₂O₂ at 0, 10, 20, 30, 60, and 120 min. Spectra were collected in 20 mM HEPES, pH 7 at 25 °C (λ<sub>exc</sub> = 488 nm).
Figure 2. Conjugation of SNAP tag dyes to His-AGT. Shown above are (a) Coomassie stain and (b) in-gel fluorescence ($\lambda_{exc} = 488$ nm) of (1) His-AGT, (2) His-AGT + STG1, (3) His-AGT + STG2, (4) His-AGT + SPG1, (5) His-AGT + SPG2. Molecular weight is marked by PageRuler™ Plus prestained protein ladder (Fermentas).
Figure 3. SNAP-Tag labeling in oxidizing environment. Shown above are (a) Coomassie stain and (b) in-gel fluorescence ($\lambda_{exc} = 488$ nm) of His-AGT (1 µM) with STG2 (5 µM) in HEPES pH 7 together with various concentration of H$_2$O$_2$. Molecular weight is marked by PageRuler™ Plus prestained protein ladder (Fermentas).
Figure 4. Deconvoluted mass spectrum of purified His-AGT.
Figure 5. Deconvoluted mass spectrum of AGT-TG formed by covalent labeling of His-AGT with (a) STG1 and (b) STG2.
Figure 6. Deconvoluted mass spectrum of AGT-PG formed by covalent labeling of His-AGT with (a) SPG1 and (b) SPG2.
Figure 7. a) Normalized UV-Vis absorption spectra of STG1 (---) and AGT-TG (–––). b) Fluorescence emission spectra of STG1 (0.5 µM) after addition of ATG-His (1 µM) at 0, 1, 2, 3, 6, 8 and 10 min. Spectra were collected in 20 mM HEPES, pH 7 at 25 °C ($\lambda_{exc} = 488$ nm).
Figure 8. Fluorescence responses of 1 µM AGT-PG, a conjugate formed from the reaction of AGT with SPG2 to 100, 250, 500, and 1000 µM H₂O₂ in 20 mM HEPES, pH 7 at 25 °C. The plot shows emission responses at 0, 10, 20, 30, 40, 50, 60, and 120 min after H₂O₂ addition. The complete conversion is achieved after 12 h incubation in 1 mM H₂O₂, giving 15 folds increase in emission intensity. The collected emission was integrated between 500 - 650 nm (λ<sub>exc</sub> = 488 nm).
Figure 9. Absorption spectra of 1 µM AGT-PG in 20 mM HEPES, pH 7 upon addition of 1 mM H₂O₂. Spectra were acquired at one hour intervals for 8 hours. An isosbestic point was observed at 460 nm.
Figure 10. Fluorescence responses of 1 µM AGT-PG to various concentrations of added H₂O₂. Spectra were acquired in 20 mM HEPES, pH 7 at 25 °C after incubation of the probe with H₂O₂ for 30 min. The collected emission was integrated between 500 - 650 nm (λ<sub>exc</sub> = 488 nm).
Figure 11. Targeted localization of STG1 and STG2 in living HEK293T cells by conjugation to SNAP-AGT fusion proteins. Cells were incubated with 5 µM STG1 or STG2 for 30 min, and washed with fresh DMEM + 10% FBS for 30 min (2 x 1 mL) before image acquisition. Rows (a) and (b) show HEK293T cells transiently expressing SNAP-NK1R. Rows (c) and (d) display HEK293T cells transiently expressing pSNAP for non-specific intracellular tagging, and row (e) presents HEK293T cells transiently expressing SNAP-H2B. For each series: (1) emission from labeling with STG1 or STG2, (2) nuclear staining with Hoechst 33342, and (3) DIC image. Scale bar = 20 µm.
Figure 12. Targeted labeling of endoplasmic reticulum and mitochondria organelles with STG2. Row (a) shows HEK293T cells expressing SNAP-KDEL in the ER lumen, and row (b) depicts HEK293T cells expressing SNAP-Cox8A for mitochondrial tagging. For each series: (1) emission from labeling with STG2, (2) emission from (a) mCherry-KDEL or (b) mCherry-Cox8A, (3) overlay of STG2 and mCherry, (4) nuclear staining with Hoechst 33342, and (5) DIC image. Scale bar = 20 µm.
Figure 13. Fluorescence detection of H$_2$O$_2$ on the surface of or within living HEK293T cells. Row (a) shows cells transiently expressing SNAP-NK1R tagged with SPG1, row (b) displays cells transiently expressing pSNAP tagged with SPG2, and row (c) presents cells transiently expressing SNAP-H2B tagged with SPG2. H$_2$O$_2$ was added from 50 mM solution in Mili-Q water. Time-lapse image acquisition was assisted by a motorized stage equipped with incubator and humidifier maintaining 37 °C and 5% CO$_2$ atmosphere. For each series: (1-4) pseudo-color coding of fluorescent emission at 0, 10, 20, and 30 min after addition of 100 µM H$_2$O$_2$, (5, 6) fluorescent emission before (5) and after (6) treatment of cells with 100 µM H$_2$O$_2$ for 30 min ($\lambda_{\text{exc}} = 488$ nm, $\lambda_{\text{em}} = 500 - 550$ nm), (7) nuclear staining with Hoechst 33342, (8) DIC image. Scale bar = 20 µm.
Figure 14. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing SNAP-KDEL and mCherry-KDEL. Panels (a) and (b) show fluorescent emission from cells labeled with SPG2 before (a) and after (b) treatment with 100 µM H$_2$O$_2$ for 30 min ($\lambda_{\text{exc}} = 488$ nm, $\lambda_{\text{em}} = 500$ - 550 nm), panel (c) presents emission from mCherry-KDEL, panel (d) displays the overlay of (b) and (c), panel (e) shows nuclear staining with Hoechst 33342, and panels (f-i) display pseudo-color coding of fluorescence emission at 0, 10, 20, and 30 min after addition of 100 µM H$_2$O$_2$. Panel (j) presents the DIC image of cells in (i). Scale bar = 20 µm.
Figure 15. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing SNAP-Cox8A and mCherry-Cox8A. Panels (a) and (b) show fluorescent emission from cells labeled with SPG2 before (a) and after (b) treatment with 100 µM H$_2$O$_2$ for 30 min ($\lambda_{\text{exc}} = 488$ nm, $\lambda_{\text{em}} = 500 - 550$ nm), panel (c) presents emission from mCherry-Cox8A, panel (d) displays the overlay of (b) and (c), panel (e) shows nuclear staining with Hoechst 33342, and panels (f-i) display pseudo-color coding of fluorescence emission at 0, 10, 20, and 30 min after addition of 100 µM H$_2$O$_2$. Panel (j) presents the DIC image of cells in (i). Scale bar = 20 µm.
Figure 16. Relative fluorescence emission from time-lapse confocal images of SPG1 or SPG2 labeled HEK293T cells in response to added 100 µM H₂O₂ at 0, 10, 20, 30 min. Error bars represent standard error measurement (s.e.m).
References


Chapter 4
Expanding the Palette of Fluorescent Probes for Multi-site Detection of Hydrogen Peroxide with SNAP and CLIP Tag in Living Cells

Portions of this work were performed in collaboration with the following people:
Brian C. Dickinson synthesized Fmoc-piperazine rhodol boronate.
Aaron A. Albers synthesized SPF1.
Introduction

Hydrogen peroxide, like the other members of the reactive oxygen species (ROS) has long been believed to be the sign of oxidative stress, aging, and disease. However, mounting evidence suggests that hydrogen peroxide also plays an important role as a signaling messenger in the normal cellular system through the reversible sulfenylation of proteins, a control switch for the modulation of protein activity similar to phosphorylation. H$_2$O$_2$ has been found to be involved in multiple signaling pathways, including cell growth, proliferation, differentiation, and controlled cell death in apoptosis and necrosis.

The downstream biological effect of this ROS is regulated by both the spatial and temporal production of H$_2$O$_2$, reflected in the abundance cellular machineries for production, sensing, and elimination of H$_2$O$_2$. A by-product of aerobic metabolism, H$_2$O$_2$ can be generated from the incomplete reduction of O$_2$ during mitochondrial respiration. Other enzymatic sources of cellular H$_2$O$_2$ include phagocytic oxidase and NADPH oxidase (Nox) in the plasma membrane; superoxide dismutase (SOD2), mitochondrial p66 and amine oxidase in mitochondria; peroxisomal oxidase in peroxisomes; sulphydryl oxidase in the endoplasmic reticulum; and amino-acid oxidase, cyclooxgenase, lipid oxygenase, xanthine oxidase and superoxide dismutases (SOD1) in the cytosol. Although H$_2$O$_2$ has a relatively long lifetime and is able to diffuse across plasma membrane, in the cellular environment, H$_2$O$_2$ has a limited effect in a compartmentalized manner due to the presence of abundant enzymatic and non-enzymatic cellular antioxidants. Exogenously-added H$_2$O$_2$ has been shown to be less effective in stimulating signaling cascades than endogenously produced H$_2$O$_2$. Following the discovery of isoforms of NOX and their distribution in many cell types and tissues other than phagocytic cells, investigators have found the connection between H$_2$O$_2$-mediated signal transduction and colocalization with NOX in specific subcellular compartments. For example, oxidation and inactivation of PTP1B as a downstream effect of epidermal growth factor signaling requires colocalization of PTB and Nox4 in the endoplasmic reticulum. Moreover, each isoform of Nox seems to have a unique role in signaling. In human endothelial cells, the expression of Nox4 was found to be much higher than that of Nox2 during cell proliferation. Detailed investigation affirmed that Nox4 directly regulates Akt, the central signaling hub of cell growth, while Nox2 places a role in maintaining the cytoskeleton, supporting cell survival by preventing apoptosis. In neurons, NMDA receptor (NMDAR) activation results in increases in cellular H$_2$O$_2$ levels. However, stimulation of synaptic and non-synaptic NMDAR exerted opposite effects on extracellular signal-regulated kinase (ERK) activity. Extra-synaptic NMDAR activation also promoted damage to cell bodies and dendrites, leading to speculation of different ROS-mediated signaling between synaptic and extra-synaptic NMDAR.

Detection of ROS with subcellular resolution would help elucidate the complexity of ROS-mediated signaling. Small-molecule fluorescent probes for H$_2$O$_2$ have been developed and optimized for biocompatibility, chemospecificity and optical response. Moreover, targeting small-molecule probes to specific subcellular locations has become more feasible with the new technology of genetically-encodable protein tags. We have previously reported SPG1 and SPG2, targetable H$_2$O$_2$ probes that can be precisely delivered to subcellular compartments that express the SNAP fusion protein. In this study, we aim to simultaneously deliver H$_2$O$_2$ probes to two different targets using tandem SNAP and CLIP tags. Both SNAP and CLIP fusion protein are derived from O$_6$-alkyguanine-DNA alkyltransferase (AGT), but they react with high specificity to orthogonal substrates. Successful applications of SNAP and CLIP multi-site targeting include the study of ligand-induced internalization of oroxin OX1 and the cannabeno
CB1 receptor, determination of subunit composition in the heterodimeric metabotropic glutamate receptor, cross-linking of an interacting pair of protein with bifunctional tags containing SNAP and CLIP substrates, and a FRET-based semi-synthetic fluorescent sensor.

In this report, we present the design, synthesis, properties, and cellular applications of a series of organelle-targetable fluorescent probes for H₂O₂ based on SNAP-AGT and CLIP-AGT bioconjugation chemistry. We demonstrated double labeling of plasma membrane and nucleus with CLIP and SNAP probes with no overlap in turn-on fluorescence emission in response to treatment with exogenous H₂O₂.

Results and Discussion

Design and Synthesis of Orthogonal SNAP and CLIP Probes as Organelle Targetable Fluorescent Reporters for Multicolor Imaging of Cellular Hydrogen Peroxide.

Scheme 1 depicts our overall strategy for multi-site labeling of cells expressing subcellular targeting AGT(SNAP) or AGT(CLIP) fusing proteins with fluorescent probes containing SNAP and CLIP substrates. The SNAP/CLIP tag was attached to boronate-caged fluorescein or rhodamine probes by amide bond formation at the 5’ or 6’ carboxy position on the lactone ring or at the piperazine handle (Scheme 2). We have previously reported the application of boronate-caged fluorescein (PF1) for the detection of H₂O₂ in the phagosomal oxidative burst in macrophages, and oxidative stress in yeast with damaged electron transport chains. CPF1 and SPF1 were synthesized by coupling of a CLIP tag or SNAP tag to 5’ carboxy of PF1-COOH (1) (Scheme 3). The boronate-caged rhodol-based probes (PY1 and PO1) were previously demonstrated to have higher sensitivity and ability to report H₂O₂ at signaling events, such as the H₂O₂ burst in EGF stimulation. The xanthone cores of PY1 and PY2 probes was synthesized by one-pot acid-catalyzed condensation of 3-bromophenol, 3-carboxyphthalic anhydride, and 3-(dimethylamino)phenol (Scheme 4). Mixtures of 5’ and 6’ carboxy isomers were obtained in this step; subsequent reaction with NHS generated the N-hydroxysuccinimide ester, enabling separation of the two isomers by flash column chromatography. Reaction of the N-hydroxysuccinimide ester (7, 8) with CLIP or SNAP tag, followed by palladium-catalyzed borylation of arylbromide gave CLIP-Peroxy-Yellow (CPY1, CPY2, Scheme 4) and SNAP-Peroxy-Yellow (SPY1, SPY2, Scheme 5), respectively. The xanthone core of SNAP-Peroxy-Orange (SPO1) was obtained by a similar synthetic procedure with the PY probe. The 5’ and 6’ carboxy isomers were effectively purified by flash column chromatography, allowing direct amide bond coupling with SNAP-tag (Scheme 6). For CPY3, CLIP-tag with carboxy handle was obtained from the reaction of CLIP-tag with glutaric anhydride; the resulted product was then used in the subsequent step of HATU-assisted amide bond formation with Rhodol-piperazine boronate.

Spectroscopic Properties and Responses to Hydrogen Peroxide.

We evaluated the spectral properties and H₂O₂ responses of the SNAP/CLIP probes in aqueous media buffered to physiological pH (20 mM HEPES, pH 7.4); data are provided in Table 1, and full spectra are shown in Figure 1-2. CPF1 and SPF1 are non-fluorescent and have no absorption in the visible region. The addition of H₂O₂ triggers a marked increase in fluorescence intensity with emission maxima at 514 nm (λexc = 488 nm, ΦCPF1/OH = 0.60, ΦSPF1/OH = 0.33). Kinetic measurement of the H₂O₂-mediated boronate deprotection performed under pseudo-first-order conditions (1 µM dye, 10 mM H₂O₂) gave observed rate constant of kobs = 1.1 x 10⁴ s⁻¹ for CPF1 and SPF1, a value similar to that observed for other PF1 derivatives. The mono-boronated semi-rhodol probes are
more reactive to H$_2$O$_2$, with $k_{\text{obs}} = 2.2 - 2.6 \times 10^{-3}$ s$^{-1}$. The phenol-form of CPY2 and SPY2 ($\Phi = 0.10$) exhibited lower quantum yields than that of CPY1 and SPY1 ($\Phi = 0.17$) due to the higher donor-excited photoinduced electron transfer (d-PeT) processes with the 6’ amide isomers.$^{61}$ Reaction of CPY probes and SPY probes with H$_2$O$_2$, monitored with 514 nm excitation wavelength, showed concomitant increases in fluorescence emission with emission maxima at 540 - 548 nm. SPO1, with julolidine rhodol, has more red-shifted absorption and emission. Boronate-caged SPO1 is weakly fluorescent ($\Phi = 0.17$). Conversion of SPO1 to the phenol form results in increase of both absorption at 542 nm and fluorescence emission at 572 nm ($\Phi = 0.44$). The CLIP/SNAP probes were able to covalently attach to purified AGT protein; in-gel fluorescence scanning and Coomassie staining confirmed that the fluorescence emission intensity of AGT-bound probes correlated to the concentration of environmental H$_2$O$_2$ (Figure 3). We have therefore successfully synthesized CLIP- and SNAP-tagged fluorophores that retain their fluorescence response to H$_2$O$_2$.

**Fluorescence Detection of H$_2$O$_2$ in Living Cells Expressing Subcellular Targeting SNAP/CLIP Fusion Protein.** With spectroscopic data establishing the formation of AGT-probes conjugates and their fluorescent turn-on in response to H$_2$O$_2$, we next sought to investigate the ability of the newly synthesized probes for the detection of H$_2$O$_2$ in living biological samples at the targeted subcellular region. CPF1 and SPF1 are poorly permeable to cells, therefore CPF1 and SPF1 can be effectively applied for plasma membrane labeling with reduced noise from intracellular space. HEK293T cells transiently-expressing membrane-localized CLIP-NK1R and SNAP-NK1R were labeled with CPF1 and SPF1 by incubation in labeling buffer for 30 min, followed by a 30 min wash with DMEM containing 10% FBS. CPF1 and SPF1-labeled HEK293T cells exhibited a weak fluorescence from plasma membrane; treatment with 100 µM H$_2$O$_2$ triggers a marked increase in fluorescence with high signal to noise from the target site (Figure 4).

CPF probes (CPY1 - CPY3) are membrane permeable and can, therefore, be used to label intracellular targets. However, we encountered difficulties in using CPY probes for intracellular labeling; the lipophilic cationic property of the probes drives mitochondrial localization. We also observed higher background fluorescence from non-specific staining of the cytoplasm with CPY probes compared to the previously-reported SPG2. The unspecified background noise cannot be completely removed despite prolonged incubation and repeated washing. Similar problems were also experienced for other semi-rhodol SNAP probes: SPY1, SPY2 and SPO1. The high background noise is problematic for the labeling of intricate organelle such as the ER and mitochondria, but the problem is minimized with relatively large targets such as the nucleus. Labeling with semi-rhodol probes required at least 60 min wash time. CPF probes were able to detect rises in H$_2$O$_2$ within the plasma membrane and nucleus of HEK293T transiently expressing CLIP-NK1R or CLIP H2B (Figure 5-7). Similary, SPY1 can be used for detection of H$_2$O$_2$ in cells expressing SNAP-NK1R and SNAP-H2B (Figure 8). We were less successful in plasma membrane labeling with SPO1; presumably the hydrophobicity of probe increases its affinity for the phospholipid layer, and limit the interaction time between SPO1 and extracellular SNAP-NK1R. SPO1 can correctly localize to nucleus of cells expressing SNAP-H2B fusion protein, with increased fluorescence emission upon treatment with H$_2$O$_2$ (Figure 9).

**Multicolor Simultaneous Detection of H$_2$O$_2$ in Living Cells Using Orthogonal SNAP and CLIP Probes.** Having demonstrated the ability of each SNAP/CLIP probe to report local concentrations of H$_2$O$_2$ at targeted subcellular compartments, we proceeded to test the
application of the probes for the simultaneous detection of H_{2}O_{2} in the nucleus and plasma membrane. HEK293T cells transiently expressing CLIP-NK1R and SNAP-H2B were labeled with CPF1 and SPO1. Fluorescent detection of CPF1 and SPO1 was performed by multi-track scanning with excitation wavelengths of 488 nm and 543 nm respectively. Treatment with H_{2}O_{2} (500 µM) gave fluorescent turn-on from both the plasma membrane and the nucleus; the signal 30 min after addition of H_{2}O_{2} showed 4 fold turn-on in CPF1 and 5 folds turn-on in the SPO1 channel (Figure 10). CPF1 and SPO1 can, therefore, detect H_{2}O_{2} in their targeted compartment independently without signal overlap between the two channels (Figure 11).

Concluding remarks

To conclude, we have performed an initial study of multi-site detection of H_{2}O_{2} in subcellular locations with SNAP/CLIP-conjugated boronate-caged fluorescein and semi-rhodol probes. We have demonstrated simultaneous detection of H_{2}O_{2} in the nucleus and on the extracellular face of plasma membrane using CPF1 and SPO1. Labeling with semi-rhodol probes suffered from unspecific staining with high signal from mitochondria and cytoplasm. Future directions will focus on expanding the color palette of the targetable probe to red and near-IR emission as well as modification of probes for optimal labeling. Ultimately, the optimized protocol and selection of probes would be applied in study of ROS mediated signaling in biological context.

Experimental Section

Materials and Methods. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. PF1-COOH (1), SNAP-Peroxyfluor-1, SPF1 (5) and Fmoc-piperazine rhodol boronate (22) were synthesized as reported. Palladium(II) acetate [Pd(OAc)2], Dichloro[1,1’-bis(diphenyl phosphino)ferrocene]palladium (II) [Pd(dppf)Cl2], and 2-(Dicyclohexylphosphino)biphenyl [Cyclohexyl JohnPhos] were purchased from Strem Chemicals (Newburyport, MA). Bis(pinacolato) diboron was purchased from Boron Molecular (Research Triangle Park, NC). 2-(4-(aminomethyl)benzyloxy)pyrimidin-4-amine (2, CLIP tag), 6-(4-(aminomethyl)benzyloxy)-9H-purin-2-amine (4, SNAP tag) and 4-(4-(aminomethyl)benzyloxy)-6-chloropyrimidin-2-amine (13, SNAP tag) were synthesized following literature procedure. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. 1H NMR and 13C NMR spectra were collected in CDCl3, DMSO-‐d6, or CD3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using a Bruker 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Restriction endonuclease and T4 DNA ligase and restriction endonuclease were purchased from New England Biolabs (Ipswich, MA). Hoechst 33342 was obtained from Invitrogen (Carlsbad, CA). pCEMS-CLIP10m-NK1R, pSEMS-SNAP26m-ADBR, pCEMS1-H2B-CLIP10m, and pSEMS1-Cox8A-SNAP26m plasmids were purchased from Covalys Biosciences (Witterswil, Switzerland). pCEMS-H2B-SNAP-FLAG and pSEMS-SNAP-NK1R were synthesized as reported. pET28a(+) was obtained from Novagen (EMD Chemicals, Gibbstown, NJ). Primers for PCR amplification were synthesized by Integrated DNA Technologies (San
A: General procedure for Pd-catalyzed borylation of arylbromide of xanthene fluorophore (Compound 11, 12, 16, 17, 20). To an oven-dried 25-mL Schlenk tube was added Pd(dppf)Cl$_2$ (0.05 eq.), KOAc (3 eq.), and bis(pinacolato)diboron (1.5 eq.). The reaction tube was put under vacuum for 1 min and refilled with nitrogen atmosphere, and a solution of arylbromide (1 eq.) in dioxane (5 - 10 mL) was delivered through a syringe in one portion. The reaction mixture was subjected to three cycles of vacuum and N$_2$ refill. The reaction tube was sealed and heated at 110 °C for 12 h. EtOAc (10 mL) was added to reaction mixture, that was cooled to room temperature. The mixture was passed through a 1-inch layer of silica gel. The filtrate was concentrated by rotary evaporator and dissolved in a minimal amount of CH$_2$Cl$_2$ under sonication. An equal volume of hexane was added slowly to the reaction mixture with vigorous stirring. The precipitated product was collected by vacuum filtration.

CLIP-Peroxyfluor-1, CPF1 (3). To a round-bottomed flask equipped with a magnetic stir bar was added PF1-COOH (30 mg, 0.050 mmol), HATU, (19 mg, 0.050 mmol), DMF (2 mL), and NEt$_3$ (10 µL). After 10 min stirring under N$_2$ atmosphere, solution of 2 (12 mg, 0.052 mmol) in DMF was added and the reaction was continued at room temperature for 3 h. DMF was removed by distillation, the remaining oil was taken into EtOAc (10 mL) and filtered through a glass pipette plugged with a ball of glass wool. The filtrate was concentrated by a rotary evaporator. The crude product was purified by flash column chromatography (EtOAc with 5% MeOH) to give product as a white solid (20 mg, 0.025 mmol, 50% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.60 (1H, s), 8.28 (1H, d, $J = 8.4$ Hz), 8.12 (2H, m), 7.80 (1H, d, $J = 6.4$ Hz), 7.68 (2H, s), 7.59 (1H, s), 7.41 (2H, d, $J = 7.6$ Hz), 7.41 (1H, s), 7.32 (2H, d, $J = 8.0$ Hz), 7.22 (2H, d, $J = 8.0$ Hz), 6.85 (2H, d, $J = 7.6$ Hz), 6.21 (1H, d, $J = 6.4$ Hz), 5.31 (2H, s), 4.43 (2H, s), 1.33 (24H, s). HRFAB-MS: calculated for [MH$^+$] 809.35, found 809.35

Isomeric mixture of 3'-bromo-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid and 3'-bromo-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (6). To a pressure flask was added 3-(dimethylamino)phenol (1.4 g, 10 mmol), 3-bromophenol (2 mL, 10 mmol), and 1,3-dioxo-1,3-dihydroisobenzofuran-5-carboxylic acid (2.0 g, 10 mmol). MeSO$_3$H (10 mL) was added, and the reaction mixture was sonicated for 30 min. Heating at 120 °C for 3 h gave a viscous brown solution which subsequently gave a brown solid upon dilution into ice-water. The sticky precipitate was taken into EtOAc (200 mL), washed with brine, and dried over Na$_2$SO$_4$. Purification by flash column chromatography (CH$_2$Cl$_2$ with 5% MeOH) gave the product as a pale pink solid (1.2 g, 2.6 mmol, 26% yield). Separation of the two isomers was difficult and could be obtained only in small amount for each pure isomer. $^1$H NMR (CD$_3$OD, 400 MHz) 6-carboxy: δ 8.42 (1H, d, $J = 8.0$ Hz), 8.21 (1H, d, $J = 8.0$ Hz), 7.90 (1H, s), 7.55 (1H, s), 7.23 (1H, d, $J = 8.4$ Hz), 6.76 (1H, d, $J = 8.4$ Hz), 6.60-6.58 (3H, m), 3.00 (6H, s). 5-carboxy: δ 8.72 (1H, s), 8.46 (1H, d, $J = 8.0$ Hz), 7.55 (1H, d, $J = 2.0$ Hz), 7.44 (1H, d, $J = 8.0$ Hz), 7.22 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz), 6.77 (1H, d, $J = 8.4$ Hz), 6.62-6.57 (3H, m), 3.11 (6H, s). LC-MS: calculated for [MH$^+$] 466.02, found 466.1.

2,5-Dioxopyrrolidin-1-ly 3'-bromo-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylate (7). And 2,5-dioxopyrrolidin-1-ly 3'-bromo-6'-(dimethyl amino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (8). To a round-bottomed flask equipped with a magnetic stir bar was added 6 (1g, 2.1 mmol), N,N'-diisopropylcarbodiimide (1.3 mL, 8.5 mmol), DMAP (150 mg, 1.1 mmol), N-hydroxy succinimide (370 mg, 3.2 mmol), and DMF (5 mL). The reaction mixture was stirred overnight.
under N₂ atmosphere at room temperature. The reaction mixture was diluted into EtOAc (200 mL), washed with water (2 x 100 mL), and brine. The concentrated organic phase was purified by flash column chromatography (1:1, hexane/EtOAc) to give a pale pink solid (overall yield 75%, 300 mg for each isomer). \(^1\)H NMR (CDCl₃, 400 MHz): 5’carboxy (7): δ 8.81 (1H, d, J = 0.8 Hz), 8.39 (1H, dd, J₁ = 8.0 Hz, J₂ = 1.6 Hz), 7.47 (1H, d, J = 2.0 Hz), 7.32 (1H, d, J = 8.4 Hz), 7.16 (1H, dd, J₁ = 8.4 Hz, J₂ = 1.5 Hz), 6.63 (1H, d, J = 8.4 Hz), 6.58 (1H, d, J = 9.2 Hz), 6.50 (1H, d, J = 2.4 Hz), 6.45 (1H, dd, J₁ = 9.2 Hz, J₂ = 2.4Hz), 3.02 (6H, s), 2.96 (4H, s). 6’carboxy (8): δ 8.37 (1H, dd, J₁ = 8.0 Hz, J₂ = 1.2 Hz), 8.16 (1H, d, J = 8.0 Hz), 7.89 (1H, s), 7.48 (1H, d, J = 2.0 Hz), 7.17 (1H, dd, J₁ = 8.4 Hz, J₂ = 2.0 Hz), 6.63 (1H, d, J = 8.4 Hz), 6.57 (1H, d, J = 8.8 Hz), 5.50 (1H, d, J = 2.4 Hz), 6.47 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.4 Hz), 3.01 (6H, s), 2.90 (4H, s).

\(-\text{N-(4-((4-aminopyrimidin-2-yloxy)methyl)benzyl)-3’-bromo-6’-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-5-carboxamide (9).}\) To a 15-mL tube equipped with a small magnetic stir bar was added 7 (50 mg, 0.089 mmol), 2 (20 mg, 0.087 mmol), and DMF (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (EtOAc with 2% MeOH) to give the product as a pale pink solid (45 mg, 0.066 mmol, 74% yield). \(^1\)H NMR (CDCl₃, 400 MHz): δ 8.40 (1H, s), 8.23 (1H, d, J = 8.0 Hz), 7.94 (1H, d, J = 5.6 Hz), 7.45 (1H, s), 7.4-7.36 (4H, m), 7.26 (1H, s), 7.22 (1H, d, J = 8.4 Hz), 7.11 (1H, d, J = 8.4 Hz), 6.59 (1H, d, J = 8.4 Hz), 6.55 (1H, d, J = 8.8 Hz), 6.48 (1H, s), 6.41 (1H, d, J = 8.8 Hz), 6.11 (1H, d, J = 5.6 Hz), 5.60 (2H, s), 4.47 (2H, s) 2.99 (6H, s). LC-MS: calculated for [M⁺] 677.10, found 676.2.

\(-\text{N-(4-((4-aminopyrimidin-2-yloxy)methyl)benzyl)-3’-bromo-6’-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9’-xanthene]-6-carboxamide (10).}\) To a 15-mL tube equipped with small magnetic stir bar was added 7 (60 mg, 0.11 mmol), 2 (30 mg, 0.13 mmol), and DMF (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (EtOAc with 2% MeOH) to give product as a pale pink solid (50 mg, 0.074 mmol, 66% yield). \(^1\)H NMR (CD₂OD, 400 MHz): δ 8.10 (1H, d, J = 9.2 Hz), 8.03 (1H, d, J = 8.0 Hz), 7.93 (1H, s), 7.79 (1H, d, J = 6.0 Hz), 7.62 (1H, s), 7.44 (1H, d, J = 2.0 Hz), 7.33 (2H, d, J = 8.0 Hz), 7.26 (2H, d, J = 8.0 Hz), 7.13 (1H, dd, 1H, J₁ = 8.4 Hz, J₂ = 2.0 Hz), 6.63 (1H, d, J = 8.4 Hz), 6.55 (1H, d, J = 8.8 Hz), 6.48 (1H, d, J = 2.4 Hz), 6.47 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.4 Hz), 6.11 (1H, d, J = 6.0 Hz), 5.25 (2H, s), 4.46 (2H, s), 2.97 (6H, s).

**CLIP-Peroxy-Yellow-1, CPY1 (11).** Procedure A: 9 (36 mg, 0.053 mmol), Pd(dppf)Cl₂ (5 mg, 0.005 mmol), KOAc (30 mg, 0.32 mmol), Bpin₂ (27 mg, 0.106 mmol). Product is pale pink solid (20 mg, 0.027 mmol, 51% yield).

\(^1\)H NMR (CD₂OD, 400 MHz): δ 8.45 (1H, d, J = 1.2 Hz), 8.20 (1H, dd, J₁ = 8.4 Hz, J₂ = 1.2 Hz), 7.82 (1H, d, J = 6.0 Hz), 7.66 (1H, s), 7.38 (2H, d, J = 8.4 Hz), 7.35 (1H, d, J = 8.4 Hz), 7.23 (1H, d, J = 8.0 Hz), 6.72 (1H, d, J = 8.0 Hz), 6.55 (1H, d, J = 8.8 Hz), 6.51 (1H, d, J = 2.4 Hz), 6.43 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.4 Hz), 6.11 (1H, d, J = 6.0 Hz), 5.28 (2H, s), 4.60 (2H, s), 2.97 (6H, s), 1.32 (12H, s). HRESI-MS: calculated for [MH⁺] 727.30, found 727.32.

**CLIP-Peroxy-Yellow-2, CPY2 (12).** Procedure A: 10 (36 mg, 0.053 mmol), Pd(dppf)Cl₂ (5 mg, 0.005 mmol), KOAc (30 mg, 0.32 mmol), Bpin₂ (27 mg, 0.11 mmol). Product is a pale pink solid (24 mg, 0.033 mmol, 62% yield).

\(^1\)H NMR (CD₂OD, 400 MHz): δ 8.12 (1H, dd, J₁ = 8.0 Hz, J₂ = 1.6 Hz), 8.05 (1H, d, J = 7.6 Hz), 7.80 (1H, d, J = 5.6 Hz), 7.64 (1H, s), 7.61 (1H, s), 7.36 (1H, d, J = 8.0 Hz), 7.33 (2H, d, J = 8.0 Hz), 7.25 (2H, d, J = 8.0 Hz), 6.75 (1H, d, J = 8.0 Hz).
Hz), 6.58 (1H, d, J = 8.8 Hz), 6.53 (1H, d, J = 2.4 Hz), 6.48 (1H, dd, J₁ = 8.8 Hz, J₂ = 2.4 Hz), 6.10 (1H, d, J = 5.6 Hz), 5.24 (2H, s), 4.45 (2H, s), 2.98 (6H, s), 1.33 (12H, s). LC-MS: calculated for [MH⁺] 727.30, found 727.3.

\(N-(4-((2\text{-Amino-6-chloropyrimidin-4-yloxy})\text{methyl})\text{benzyl})-3'\text{-bromo-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]}-5\text{-carboxamide (14).}\) To a 10-mL tube equipped with a small magnetic stir bar was added 7 (40 mg, 0.071 mmol), 13 (20 mg, 0.075 mmol), and DMF (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (EtOAc) to give product as a pale pink solid (30 mg, 0.042 mmol, 59% yield).

\(^1\text{H NMR (CD}_3\text{OD, 400 MHz):} δ 9.24 (1\text{H, s-br}), 8.59 (1\text{H, s}), 8.36 (1\text{H, d, J = 8.0 Hz}), 7.69 (1\text{H, s}), 7.58 (1\text{H, s}), 7.50 (4\text{H, m}), 7.38 (1\text{H, d, J = 8.4 Hz}), 7.25 (1\text{H, d, J = 8.4 Hz}), 6.74 (1\text{H, d, J = 8.8 Hz}), 6.67 (1\text{H, d, J = 8.8 Hz}), 6.62 (1\text{H, s}), 6.57 (2\text{H, d, J = 8.8 Hz}), 6.20 (1\text{H, s}), 5.38 (2\text{H, s}), 4.67 (2\text{H, s}), 3.11 (6\text{H, s}). LC-MS: calculated for [MH⁺] 712.09, found 712.2.

\(N-(4-((2\text{-Amino-6-chloropyrimidin-4-yloxy})\text{methyl})\text{benzyl})-3'\text{-bromo-6'-(dimethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]}-5\text{-carboxamide (15).}\) To a 10-mL tube equipped with small magnetic stir bar was added 8 (30 mg, 0.053 mmol), 13 (21 mg, 0.079 mmol), and DMF (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed under reduced pressure and the crude product was purified by flash column chromatography (EtOAc) to give product as pale pink solid (35 mg, 0.059 mmol, 93% yield).

\(^1\text{H NMR (DMSO-}d_6\text{, 400 MHz):} δ 9.20 (1\text{H, s-br}), 8.19 (1\text{H, d, J = 8.0 Hz}), 8.09 (1\text{H, d, J = 8.0 Hz}), 7.69 (1\text{H, s}), 7.62 (1\text{H, s}), 7.32 (2\text{H, d, J = 8.0 Hz}), 7.26 (1\text{H, s}), 7.23 (2\text{H, d, J = 8.0 Hz}), 7.07 (2\text{H, s-br}), 6.76 (1\text{H, d, J = 8.4 Hz}), 6.54-6.51 (3\text{H, m}), 6.08 (1\text{H, s}), 5.22 (2\text{H, s}), 4.36 (2\text{H, s}), 2.92 (6\text{H, s}). LC-MS: calculated for [MH⁺] 760.26, found 760.2.

SNAP-Peroxy-Yellow-1, SPY1 (16). Procedure A: 14 (30 mg, 0.042 mmol), Pd(dppf)Cl₂ (3 mg, 0.004 mmol), KOAc (13 mg, 0.12 mmol), Bpin₂ (16 mg, 0.063 mmol). Product is a pale pink solid (17 mg, 0.022 mmol, 53% yield).

\(^1\text{H NMR (DMSO-}d_6\text{, 400 MHz):} δ 9.40 (1\text{H, s}), 8.50 (1\text{H, s}), 8.25 (1\text{H, d, J = 8.0 Hz}), 7.58 (1\text{H, s}), 7.39 (2\text{H, d, J = 8.0 Hz}), 7.33 (4\text{H, m}), 7.08 (2\text{H, s-br}), 6.80 (1\text{H, d, J = 7.6 Hz}), 6.57 (1\text{H, d, J = 8.8 Hz}), 6.51 (1\text{H, s}), 6.50 (1\text{H, d, J = 8.8 Hz}), 6.10 (1\text{H, s}), 5.27 (2\text{H, s}), 4.50 (2\text{H, s}), 2.93 (6\text{H, s}), 1.27 (12\text{H, s}). LC-MS: calculated for [MH⁺] 760.26, found 760.2.

SNAP-Peroxy-Yellow-2, SPY2 (17). Procedure A: 15 (30 mg, 0.042 mmol), Pd(dppf)Cl₂ (3 mg, 0.004 mmol), KOAc (13 mg, 0.12 mmol), Bpin₂ (16 mg, 0.063 mmol). Product is a pale pink solid (24 mg, 0.031 mmol, 76% yield).

\(^1\text{H NMR (DMSO-}d_6\text{, 400 MHz):} δ 9.20 (1\text{H, s-br}), 8.18 (1\text{H, d, J = 8.0 Hz}), 8.09 (1\text{H, d, J = 8.0 Hz}), 7.65 (1\text{H, s}), 7.52 (1\text{H, s}), 7.34 (1\text{H, s}), 7.31 (2\text{H, d, J = 8.0 Hz}), 7.22 (2\text{H, d, J = 8.0 Hz}), 7.07 (2\text{H, s-br}), 6.57-6.51 (3\text{H, m}), 6.07 (1\text{H, s}), 5.22 (2\text{H, s}), 4.36 (2\text{H, s}), 2.93 (6\text{H, s}), 1.27 (12\text{H, s}). LC-MS: calculated for [MH⁺] 760.26, found 760.2.

3'-Bromo julolidine-1,9-xanthene-5-carboxylic acid (18). To a heavy-walled pressure flask was added 3-hydroxyjulolidine (400 mg, 2.1 mmol), 3-bromophenol (400 mg, 2.3 mmol), 1,3-dioxo-1,3-dihydroisobenzofuran-5-carboxylic acid (400 mg, 2.1 mmol), and MeSO₃H (3 mL). The reaction mixture was heat at 100 °C for 3 h. Once cooled to room temperature, the reaction mixture was poured into ice-water, and the product precipitated out as a red solid. The precipitate was collected by vacuum filtration, redissolved into EtOAc, and dried over Na₂SO₄. Purification with flash column chromatography (EtOAc) gave product as a red solid (80 mg, 0.15 mmol, 0.5% yield).

\(^1\text{H NMR (CD}_3\text{OD, 300 MHz):} δ 8.67 (1\text{H, s}), 8.32 (1\text{H, d, J = 7.8 Hz}), 7.53 (1\text{H, s}), 7.23 (1\text{H, d, J = 7.8 Hz}), 7.14 (1\text{H, d, J = 8.4 Hz}), 6.63 (1\text{H, d, J = 8.4 Hz}), 6.19 (1\text{H, s}),
3.24 (4H, s-br), 2.90 (2H, s-br), 2.52 (2H, s-br), 2.00 (2H, s-br), 1.86 (2H, s-br). LC-MS: calculated for [MH⁺] 518.05, found 518.0.

**N-(4-((2-Amino-6-chloropyrimidin-4-yloxy)methyl)benzyl)-3'-bromo julolidine-1,9-xanthene-5-carboxylic acid (19).** To a 10-mL tube equipped with small magnetic stir bar was added 18 (100 mg, 0.20 mmol), DMAP (12 mg, 0.10 mmol), HATU (75 mg, 0.020), DMF (2 mL) and NEt₃ (20 µL). After stirring for 10 min, 13 (53 mg, 0.20 mmol) was added. The reaction mixture was stirred overnight at room temperature. Solvent was removed in vacuo and the crude product was purified by flash column chromatography (EtOAc  with 5% MeOH) to give product as pale pink solid (120 mg, 0.16 mmol, 78% yield). 1H NMR (DMSO-d₆, 400 MHz): δ 9.40 (1H, s-br), 8.48 (1H, s), 8.28 (1H, d, J = 7.8 Hz), 7.63 (1H, s), 7.39 (3H, m), 7.35 (2H, d, J = 8.0 Hz), 7.22 (1H, d, J = 8.8 Hz), 7.18 (2H, s-br), 6.74 (1H, d, J = 8.8 Hz), 6.10 (1H, s), 6.08 (1H, s), 5.27 (2H, s), 4.52 (2H, s), 3.13 (4H, m), 2.85 (2H, s-br), 2.45 (2H, s-br), 1.95 (2H, s-br), 1.76 (2H, s-br). LC-MS: calculated for [M+H⁺] 763.12, found 762.1.

**SNAP-Peroxy-Orange-1, SPO1 (20).** Procedure A: 19 (30 mg, 0.039 mmol), Pd(dppf)Cl₂ (3 mg, 0.004 mmol), KOA(c) (13 mg, 0.12 mmol), Bpin₂ (12 mg, 0.047 mmol). Product is a pale pink solid (15 mg, 0.018 mmol, 47% yield). 1H NMR (DMSO-d₆, 400 MHz): δ 9.39 (1H, s-br), 8.48 (1H, s), 8.25 (1H, d, J = 8.0 Hz), 7.55 (1H, s), 7.38 (2H, d, J = 8.0 Hz), 3.36 (3H, m), 7.30 (1H, d, J = 8.8 Hz), 6.72 (1H, d, J = 8.8 Hz), 6.10 (1H, s), 6.09 (1H, s), 5.27 (2H, s), 4.50 (2H, s), 3.15 (4H, m), 2.86 (2H, s-br), 2.45 (2H, s-br), 1.92 (2H, s-br), 1.74 (2H, s-br), 1.27 (12H, s). LC-MS: calculated for [M+H⁺] 812.29, found 812.2.

**5-(4-((4-Aminopyrimidin-2-yloxy)methyl)benzylamino)-5-oxopentanoic acid (21).** To a 10-mL tube equipped with small magnetic stir bar was added 2 (100 mg, 0.43 mmol), glutaric anhydride (50 mg, 0.43 mmol), and DMF (1 mL). The reaction mixture was stirred overnight at room temperature. Solvent was removed in vacuo and product was precipitated out from 1:1 EtOAc/hexane (2 mL) as a white solid (140 mg, 0.40 mmol, 95% yield). 1H NMR (CD₃OD, 300 MHz): δ 7.96 (1H, s-br), 7.82 (1H, d, J = 6.0 Hz), 7.40 (2H, d, J = 8.1 Hz), 7.26 (2H, d, J = 8.1 Hz), 6.13 (1H, d, J = 6.0 Hz), 5.30 (2H, s) 4.34 (2H, s), 2.36-2.25 (4H, m), 1.92 (2H, m). LC-MS: calculated for [MH⁺] 344.15, found 345.1.

**CLIP-Peroxy-Yellow-3, CPY3 (23).** To a 10-mL tube equipped with a small magnetic stir bar was added 22 (50 mg, 0.064 mmol) in acetonitrile (3 mL), followed by piperazine (7 mg, 0.08 mmol). After stirring at room temperature for 30 min, the solvent was removed by a rotary evaporator. The orange solid was dissolved into DMF (1 mL) and then added to a mixture of 21 (30 mg, 0.064 mmol), HATU (24 mg, 0.065 mmol), NEt₃ (10 µL), and DMF (2 mL) which has been stirring at room temperature under a N₂ atmosphere for 30 min. After 3 h, DMF was removed by vacuum distillation. The remained solid was dissolved in to CH₂Cl₂ (5 mL) and passed through a glass pipette with a glass wool plug. The filtrate was concentrated and subjected to purification by flash column chromatography (3:1, EtOAc/MeOH) to give product as a pale pink solid (8 mg, 0.01 mmol, 15% yield). 1H NMR (CD₂OD, 400 MHz): δ 8.03 (1H, d, J = 6.8 Hz), 7.75 (1H, d, J = 5.6 Hz), 7.62 (2H, m), 7.42 (1H, d, J = 7.6 Hz), 7.39 (1H, d, J = 8.0 Hz), 7.30 (2H, d, J = 8.0 Hz), 7.12 (1H, d, J = 7.2 Hz), 6.80 (1H, d, J = 7.6 Hz), 6.72 (1H, s), 6.69 (1H, d, J = 8.8 Hz), 6.51 (1H, d, J = 8.8 Hz), 6.05 (1H, d, J = 5.6 Hz), 5.26 (2H, s), 4.34 (2H, s), 3.60 (2H, s-br), 3.55 (2H, s-br), 3.23 (4H, s-br), 2.38 (2H, t, J = 7.6 Hz), 2.29 (2H, t, J = 6.8 Hz), 1.95 (2H, m), 1.35 (12H, s). LC-MS: calculated for [MH⁺] 837.37, found 837.3.

**Expression and Purification of His-AGT for SNAP and CLIP Tag Labeling.** Expression of His-SNAP fusion protein was described in Chapter 3. The sequence encoding the
CLIP tag was PCR amplified from pCEMS-CLIP10m-NK1R (Covalys) and subcloned into PET28a(+) (Novagen) using *N*heI and *Xho*I restriction sites. The resulting plasmid, after verification by DNA sequencing, was transformed into *E. Coli* strain BL-21(DE3). A bacterial culture was grown at 37 °C in TB medium (50 mL) with 50 µg/mL kanamycin for 6 - 8 h until an optical density (OD$_{600nm}$) of 0.8 was reached. Expression of His-CLIP was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The culture was grown at 16 °C for 12 h and was harvested by centrifugation at 8000 rpm for 10 min at 4 °C. Cell lysates were prepared by resuspending the pellet in buffer (PBS with 0.5 mM phenylmethylsulfonyl fluoride) followed by cell disruption with a French press (Thermo Scientific). Insoluble proteins and cellular debris were removed by centrifugation at 10,000 rpm for 30 min. For purification of His-CLIP, Ni-NTA (Qiagen) was used according to the supplier’s protocol. The fractions with CLIP tag were combined and concentrated using an Amicon Ultra-15 Ultracel®-10K centrifugal unit (Millipore). The concentrated His-CLIP solution was then desalted using G25 Sephadex (Sigma Aldrich) with the following elution buffer: 150 mM NaCl, 25 mM Tris-Cl, 1mM DTT pH 7.5. The purified His-CLIP protein was concentrated using an Amicon Ultra-4 Ultracel®-10K and stored at -20 °C. The protein concentration was determined using the BCA assay, yielding 2.3 mg of purified His-CLIP (0.5 mM in 200 µL elution buffer).

**In vitro SNAP and CLIP Labeling Analyzed by In-Gel Fluorescence Scanning.** Purified His-CLIP or His-SNAP (5 µM) was incubated in reaction buffer (HEPES pH 7.4) with the corresponding CLIP or SNAP probe (5 µM) for 30 min at 37 °C (Total volumn 40 µL). H$_2$O$_2$ was added from stock solution of 1 mM or 5 mM to give the final concentration of 0, 25, 50, 125, and 250 µM, and the reaction was continued for another 30 min at 37 °C. Binding of the probes to His-CLIP or His-SNAP and their response to added H$_2$O$_2$ were analyzed by SDS-PAGE, followed by in-gel fluorescence scanning (488 nm Argon excitation, 520 BP 40 emission filter, Typhoon 9410 imaging system, GE Healthcare) and Coomassie protein staining.

**Spectroscopic Materials and Methods.** Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). Fluorescein in 0.1 M NaOH (Φ = 0.90) and Rhodamine B in PBS (Φ = 0.34) were used as standards for quantum yield measurements.

**Cell Culture and Labeling Procedures.** HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before transfection, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine in a 24-wells culture plate. Transient expression of SNAP or CLIP fusion protein was performed by following the standard protocol for Lipofectamine 2000 (Invitrogen). SNAP-tag and CLIP-tag labeling was achieved by incubating cells in HEPES containing 5 µM dye and 1 µM Hoechst 33342 for 30 min at 37 °C. Cells were washed with fresh DMEM with 10% FBS (2 × 1 mL) for 60 - 90 min after dye loading to remove any unbound fluorophore. All
confocal fluorescence images were acquired in HEPES buffer. [5x HEPES buffer pH 7.4: 750 mM NaCl, 100 mM HEPES, 5 mM CaCl2, 25 mM KCl, 5 mM MgCl2]

**Fluorescence Imaging Experiments.** Confocal fluorescence imaging studies were performed with a Zeiss LSM510 NLO Axiovert 200 Laser scanning microscope with a 40x oil-immersion objective lens or a Zeiss LSM710 Axiovert Laser scanning microscope with a 20x air objective. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO2 humidified atmosphere. Image analysis was performed in ImageJ (National Institute of Health). Imaging of nucleus with Hoechst 33342 was carried out using 405 nm excitation and emission with collected at 430 – 480 nm. Imaging of CPF1 and SPF1 was carried out using 488 nm excitation and emission was collected at 500 – 550 nm. Imaging of CPY and SPY was carried out using 514 nm excitation and emission was collected through 530 - 700 nm. Imaging of SPO1 was carried out using 543 nm excitation and emission was collected at 555 - 700 nm.
Scheme 1. Multi-site detection of H₂O₂ with CLIP and SNAP tags.
Scheme 2. Structure of boronate-based $\text{H}_2\text{O}_2$ probes conjugated to SNAP/CLIP tags.
Scheme 3. Synthesis of CPF1 and SPF1.
Scheme 4. Synthesis of CPY1 and CPY2.
Scheme 5. Synthesis of SPY1 and SPY2.
Scheme 7. Synthesis of CPY3.
### Table 1: Spectroscopic properties of CLIP/SNAP dyes

<table>
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<th>Compound</th>
<th>Boronate-form</th>
<th>Phenol-form</th>
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<tr>
<td></td>
<td>(\lambda_{\text{abs}}) (nm)</td>
<td>(\lambda_{\text{em}}) (nm)</td>
</tr>
<tr>
<td>CPF1</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>CPY1</td>
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<tr>
<td>CPY3</td>
<td>514</td>
<td>539</td>
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<tr>
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<td>n/a</td>
</tr>
<tr>
<td>SPY1</td>
<td>524</td>
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<tr>
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Figure 1. Absorption and fluorescence emission response of 1 µM CLIP probes to 2 mM H₂O₂. Spectra were taken 0, 10, 20, 30, 40, 50, and 60 min after addition of H₂O₂. (CPF1: $\lambda_{\text{exc}} = 488$ nm, CPY: $\lambda_{\text{exc}} = 514$ nm).
Figure 2. Absorption and fluorescence emission response of 1 µM SNAP probes to 1 mM H₂O₂. Spectra were taken 0, 10, 20, 30, 40, 50, and 60 min after addition of H₂O₂. (SPY: λ<sub>exc</sub> = 514 nm, SPO1: λ<sub>exc</sub> = 543 nm).
Figure 3. Conjugation of CLIP/SNAP probes to His-AGT. Purified His-AGT(CLIP) or His-AGT(SNAP) were incubated with CLIP/SNAP probes for 30 min at 37 °C in HEPES buffer pH 7.4. H$_2$O$_2$ was added to the AGT-conjugated probes to final concentrations of 0, 25, 50, 125, and 250 µM. After 30 min incubation, 4x reducing Laemmli loading buffer was added, followed by denaturation at 97 °C for 5 min. Binding of probes to AGT and their response to added H$_2$O$_2$ were verified by SDS-PAGE and in-gel fluorescence scanning. (SPO1: $\lambda_{ex} = 532$ nm, filter LP560, others: $\lambda_{ex} = 488$ nm, filter 520 BP40).
Figure 4. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing (a) CLIP-NK1R or (b) SNAP-NK1R. Cells were labeled with CPF1 or SPF1 (5 µM, 30 min in HEPES). Unbound probes was removed by incubation in DMEM with 10% FBS for 30 min. Images were taken with and without 30 min addition of 100 µM H$_2$O$_2$. Scale bar = 20 µm.
Figure 5. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing (a) CLIP-NK1R or (b) CLIP-H2B. Cells were labeled with CPY1 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. Images were taken with and without 30 min addition of 100 µM H$_2$O$_2$. Scale bar = 20 µm.
Figure 6. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing (a) CLIP-NK1R or (b) CLIP-H2B. Cells were labeled with CPY2 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. Images were taken with and without 30 min addition of 100 µM H$_2$O$_2$. Scale bar = 20 µm.
Figure 7. Fluorescence detection of $\text{H}_2\text{O}_2$ in living HEK293T cells transiently expressing (a) CLIP-NK1R or (b) CLIP-H2B. Cells were labeled with CPY3 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. Images were taken with and without 30 min addition of 100 µM $\text{H}_2\text{O}_2$. Scale bar = 20 µm.
Figure 8. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing (a) SNAP-NK1R or (b) SNAP-H2B. Cells were labeled with CPY3 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. Images were taken with and without 30 min addition of 100 µM H$_2$O$_2$. Scale bar = 20 µm.
Figure 9. Fluorescence detection of $H_2O_2$ in living HEK293T cells transiently expressing SNAP-H2B. Cells were labeled with SPO1 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 60 min. Images were taken with and without 30 min addition of 100 µM $H_2O_2$. Scale bar = 20 µm.
Figure 10. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing SNAP-H2B and CLIP-NK1R. Cells were labeled with CPF1 and SPO1 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. a) Addition of H$_2$O$_2$ (500 µM) resulted in increased fluorescence emission from both plasma membrane and nucleus. b) Quantification of fluorescence emission intensity during time-course imaging. Error bars represent standard deviation of average fluorescence intensity obtained from five images.
Figure 11. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells transiently expressing SNAP-H2B and CLIP-NK1R. Cells were labeled with CPF1 and SPO1 (5 µM, 30 min in HEPES). Unbound probe was removed by incubation in DMEM with 10% FBS for 90 min. Overlay image shows no overlap of signal from CPF1 and SPO1. Scale bar = 20 µm. (CPF1: $\lambda_{\text{exc}}$ = 488 nm, collection window 500 - 550 nm, SPO1: $\lambda_{\text{exc}}$ = 543 nm, collection window 560 - 700 nm)
References


Chapter 5
A Dendrimer-Based Platform for Simultaneous Dual Fluorescence Imaging of Hydrogen Peroxide and pH Gradients Produced in Living Cells

Portions of this work were published in the following scientific journal:

Portions of this work were performed in collaboration with the following people:
Aaron E. Albers synthesized PF1-COOH.
Abstract

We present a modular dendrimer-based platform for simultaneous dual fluorescence imaging of hydrogen peroxide (H$_2$O$_2$) and pH gradients produced in living cells. Acetyl-capped G5 PAMAM dendrimers functionalized with boronate-caged Peroxyfluor-1 (PF1) fluorophores for H$_2$O$_2$ detection and semi-naphthorhodafluor (SNARF2) dyes for pH sensing provide a single probe system that is capable of sensing multiple analytes at a time by multicolor fluorescence imaging. Spectroscopic measurements of the doubly-labeled dendrimer conjugates establish their ability to simultaneously monitor changes in both H$_2$O$_2$ and pH using different excitation/emission profiles. Moreover, this dual-probe platform allows for selective discrimination between H$_2$O$_2$ and pH changes in live RAW264.7 macrophage cells when stimulated by an immune insult. Further imaging experiments show that pharmacological inhibition of NADPH oxidase (Nox) proteins triggers a decrease in both oxidative burst and in pH regulation within phagocytic compartments and leads to disruptions of endocytic activity, suggesting that Nox-derived H$_2$O$_2$ signaling is critical to the maintenance of multiple components of the immune response. This work establishes a general molecular platform for simultaneous, real-time imaging of multiple analytes associated with redox biology in living systems and should be applicable to a wide range of chemosensor constructs.

Introduction

Hydrogen peroxide (H$_2$O$_2$) and related reactive oxygen species (ROS) generated during aerobic metabolism can have diverse consequences for human health, aging, and disease. Oxidative stress stemming from imbalances in ROS production is connected to aging\textsuperscript{1-4} and terminal diseases ranging from cancer\textsuperscript{5} to neurodegeneration,\textsuperscript{6-9} as well as to metabolic disorders such as diabetes.\textsuperscript{10-14} Despite their potential toxicity, ROS are increasingly appreciated to play essential roles in the normal physiology of life.\textsuperscript{15-29} The classic example of a beneficial role for ROS is in immunology, where sentinel cells like macrophages and neutrophils employ ROS to kill pathogens or other harmful antigens during phagocytosis.\textsuperscript{18,30,31} Indeed, the absence or deficiency of ROS production in the immune system can be fatal as in the case of chronic granulomatous disease (CGD), in which patients suffer concurrent and severe bacterial and fungal infections due to an inability to produce sufficiently high levels of ROS in phagocytes due to genetic mutations in the NADPH oxidase (Nox) enzyme.\textsuperscript{32-34}

The interplay between redox biology and cellular immune response is, however, more sophisticated than the simple killing of pathogens by ROS bursts.\textsuperscript{35-38} This complexity is well-illustrated by the dendritic cells, which are another class of phagocytes that participate in the initiation of cytotoxic immuneresponses through antigen cross-presentation. Foreign particles engulfed by dendritic cells are processed through milder degradation pathways, where the synergistic use of ROS and pH gradients modulates lysosomal protease activity in order to preserve an identifiable fragment for subsequent T-cell recognition.\textsuperscript{39-41} As such, many details of the phagocytosis regulatory pathway remain elusive.

Molecular imaging provides an attractive approach to address mechanistic questions surrounding phagocytic immune response. In this context, genetically encoded fluorescent biosensors are effective tools for understanding physical aspects of phagocytotic processes, such as mechanisms of protein trafficking,\textsuperscript{42-44} membrane surface potential, and lipid dynamics.\textsuperscript{45,46} Small-molecule fluorophores offer a complementary set of tools for monitoring chemical changes within phagocytic vesicles, such as pH\textsuperscript{47} and ROS fluxes.\textsuperscript{48} With regard to the latter, we
recently performed dual probe imaging of H₂O₂ and HOCl in PMA-stimulated macrophages, and
discovered three distinct types of phagosome populations that produce either or both of these
oxidants for pathogen killing.⁴⁹ To further advance our ability to simultaneously track multiple
analytes in living systems, with specific interest in studying phagosomes during immune
response, we turned our attention to dendrimers as a platform for carrying multiple types of
compounds, which can range from imaging agents to targeting molecules to therapeutic agents.
Depending on a given dendrimer architecture, these active compounds can be encapsulated
within the dendrimers scaffold or covalently attached to its surface,⁵⁰-⁵⁸ along with acylation or
addition of PEG chains to increase the biocompatibility of these branched particles.⁵⁹-⁶¹ Recent
eamples of acetylated PAMAMs in biological applications include dendrimer-based pH
sensors⁶² and folate-functionalized PAMAMs for targeted anti-cancer drug delivery.⁶³,⁶⁴

In this report, we present the design, synthesis, spectral properties, and live-cell imaging
studies of a new type of dendrimer-based multifunctional imaging probe that can simultaneously
report both pH and H₂O₂. We describe a simple protocol for decorating PAMAM-G5 dendrimers
with both Peroxyfluor-1 (PF1),⁶⁵ a H₂O₂-responsive fluorescent probe, and SNARF2, a pH-
sensitive dye. PF1 is a member of boronate-caged reporters for H₂O₂; this reaction-based
approach offers selectivity for detecting H₂O₂ over other ROS produced in immune signaling
such as superoxide (O₂⁻), nitric oxide (NO), hydroxyl radical (•OH), and hypochlorous acid
(HOCl).⁴⁹,⁶⁶-⁷¹ This platform is capable of detecting both pH changes and H₂O₂ bursts in
macrophages upon immune stimulation using multicolor fluorescence imaging, and can be
applied to situations in which pharmacological inhibition of Nox leads to decrease in ROS fluxes
and pH regulation of the phagosome. In addition to providing a potentially powerful new tool for
reporting two critical chemical species involved in immune response and in redox biology, we
anticipate that the modularity of this dendrimer platform and related conjugates will afford a
versatile approach to multifunctional imaging in living systems.

Results and Discussion

Design and Synthesis of a Multifunctional PAMAM Dendrimer Platform for
Simultaneous Dual Imaging of Hydrogen Peroxide and pH. Our overall design strategy for
developing new reporters for simultaneous dual imaging of hydrogen peroxide and pH is to
conjugate multiple types of responsive small-molecule fluorescent probes to a modular chemical
scaffold. Scheme 1 depicts our strategy for creating PAMAM-based imaging agents with
conjugated H₂O₂ and pH probes. For H₂O₂ detection, we prepared a new carboxy-functionalized
version of PF1, an established fluorescent indicator sensitive to oxidative stress levels of H₂O₂.
H₂O₂-mediated deprotection of the boronate esters of PF1 and subsequent opening of the bottom
ring lactone yields the highly fluorescent green fluorescein (Scheme 2). For pH detection we
chose SNARF2, a semi-naphthorhodafluor that is sensitive to pH with pKa 7.5.⁷² Changes in
proton concentration [H⁺] shift the equilibrium between acidic and basic forms of the dye, with
corresponding changes in absorption and emission ratio values.

PF1 and SNARF2 were covalently attached to PAMAM-G5 particles via amide linkages,
and the dual-conjugated PAMAMs were subsequently acetylated to produce low-toxicity,
bioconpatible nanoprobe (Scheme 3). Unreacted small-molecule fluorophores were separated
from the dendrimers by Amicon 10K centrifugal units (regenerated cellulose membrane MWCO
10,000) and by size-exclusive chromatography using a Sephadex LH-20 column. The average
number of functional groups on the surface of the dendrimers’ surfaces was determined by
MALDI-TOF analysis (Figure 1) and UV-Vis spectroscopy (Figure 2); the results are summarized in Table 1. G5-SNARF2-Ac is decorated, on average, with two SNARF2 dyes per dendrimer, whereas G5-SNARF2-PF1-Ac displays an average number of two and six SNARF2 and PF2 dyes per dendrimer, respectively. Dynamic light scattering (DLS) experiments show that G5-SNARF2-Ac and G5-SNARF2-PF1-Ac reporters are narrowly distributed spherical nanoparticles with average hydrodynamic diameters of 3.63 and 3.56 nm, respectively (Figure 4). The modified dendrimers have a more compact structure than PAMAM-G5, which has an average hydrodynamic diameter of 5 nm, owing to the decrease in charge repulsion from fewer numbers of protonated primary amines in the parent unmodified dendrimer.

Spectroscopic Properties and Responses to pH and Hydrogen Peroxide. G5-SNARF2-Ac shows red shifts in absorption and emission maxima compared to SNARF2 alone (Table 2). The dendrimer-conjugated basic form of SNARF2, (SNARF2[B]), displays an absorption maximum at 600 nm and an emission maximum at 650 nm, whereas the dendrimer-conjugated acidic form of SNARF2, SNARF2[A], exhibit absorption maxima at 525 and 563 nm and an emission maximum at 590 nm (Figure 5). The pKa value of G5-SNARF2-Ac is 7.76 as determined by absorbance measurements in phosphate buffered solution.

The absorption spectrum of G5-SNARF2-PF1-Ac in phosphate buffer at pH 7.4 is virtually identical to that of G5-SNARF2-Ac absorption in the visible region for the colorless PF1 dye in its closed lactone form. Reaction of the G5-SNARF2-PF1-Ac probe with H2O2 triggers the appearance of a fluorescein-derived absorption peak near 500 nm (Figure 6a). We observe a 4-fold turn-on in fluorescence at 520 nm with 488 nm excitation after 30 min incubation of the probe with 100 µM H2O2 (Figure 6b). The observed rate constant of the H2O2-mediated deprotection of G5-SNARF1-PF1-Ac under pseudo-first order conditions (10 µg/mL G5-SNARF2-PF1-Ac, ~ 1.5 µM PF1, 1 mM H2O2) is \( k_{obs} = 2.8 \times 10^{-4} \text{ s}^{-1} \) (Figure 7). The quantum yield of G5-SNARF2-PF1-Ac when PF1 is fully deprotected to fluorescein is 0.16; which is lower than the quantum yield of fluorescein in solution (\( \Phi = 0.93 \)).\(^{73} \) The observed decrease in quantum efficiency is likely due to interactions of the fluorophore with the densely packed amide and amine groups on the dendrimer surface. Further evidence that the G5-SNARF2-PF1-Ac probe is responsive to H2O2 is provided by fluorescence images of SDS-PAGE samples of G5-SNARF2-PF1-Ac treated with various concentrations of H2O2 (Figure 8). Finally, the reaction of G5-SNARF2-PF1-Ac with H2O2 has no effect on the fluorescence emission profile of the SNARF2 pH reporter and this probe maintains the ability to sense pH changes in aqueous solution, confirming that the dual-conjugated probe can independently and simultaneously detect changes in both H2O2 levels and pH using different excitation wavelengths (Figure 9).

Fluorescence Imaging of Phagosomal Acidification in RAW264.7 Macrophages with G5-SNARF2-Ac. We proceeded to test the dendrimer-based fluorescent reporters in live-cell imaging experiments. First, WST-1 assays showed no significant cytotoxicity after 3 hour incubations of live RAW264.7 macrophages with 500-1000 µg/mL levels of G5-SNARF2-Ac or G5-SNARF2-PF1-Ac (Figure 10). We then established that G5-SNARF2-Ac is able to report the progressive acidification of the phagosomal lumen in RAW 264.7 cells stimulated to an immune response with phorbol 12-myristate 13-acetate (PMA) (Figure 11). For these experiments we employed the fluorescent filter set: Ch1-SNARF2[B] (\( \lambda_{exc} = 600 \text{ nm}, \lambda_{em} = 660 – 710 \text{ nm} \)) and Ch2-SNARF2[A] (\( \lambda_{exc} = 550 \text{ nm}, \lambda_{em} = 570 – 640 \text{ nm} \)) to allow spectral separation of the acidic and basic forms of SNARF2, respectively, without spectral overlap with the optical window for
subsequent PF1-derived H$_2$O$_2$ detection. Images of a 50 µg/mL solution of G5-SNARF2-Ac in 50 mM phosphate buffer enabled the color-coded pH calibration as shown in Figure 5. Fifteen minutes after PMA stimulation, the RAW264.7 macrophage cells are largely populated with early phagosomes, which maintain slightly basic to neutral pH, and hence give a strong signal from the Ch1-SNARF2[B] channel. Apart from these neutral phagosomes, the live-cell images also show a population of smaller but more acidic vesicles, which we assign to cytosolic endosomes that have obtained G5-SNARF2-Ac either by pinocytosis or via a kiss-and-run interaction with phagosomes.$^{74,75}$ During endocytic trafficking, phagosomes also acquire V-ATPase, a transmembrane proton pump through fusion with endomembrane compartments. V-ATPase is responsible for the acidification of phagosomes and provides an optimal pH for the activation and function of other microbicide agents, including hydrolytic enzymes and cationic peptides.$^{76}$ Forty-five minutes after PMA stimulation, we observed the acidification of mature phagosomes, as indicated by a prominent emission from the Ch2-SNARF2[A] channel. These experiments demonstrate that the dendrimer-based probes are capable of imaging changes in pH in living cells and show a progression of pH-dependent events during a stimulated phagocytic immune response.

Simultaneous Multicolor Imaging of H$_2$O$_2$ and pH Fluxes During Phagocytosis with G5-SNARF2-PF1-Ac. Phagocytosis triggers the assembly of NADPH oxidase (Nox), a family of multi-subunit enzymes that possess both membrane-bound and cytosolic components. By facilitating electron transfer to molecular oxygen, Nox generates fluxes of superoxide (O$_2^-$) within the phagosome that can reach millimolar levels and then undergo rapid dismutation to give H$_2$O$_2$ as the primary microbicide.$^{77}$ Using G5-SNARF2-PF1-Ac, we were able to directly observe H$_2$O$_2$ generation in early phagosomes upon PMA-stimulation of RAW264.7 macrophages based on the colocalization of emission signals derived from PF1 and basic SNARF2 signatures (Figure 12a). Similar results were obtained when the cells were stimulated in the presence of superoxide dismutase (5,000 U/mL), which catalyzes the dismutation of O$_2^-$ to H$_2$O$_2$. This result confirms the selectivity of G5-SNARF2-PF1-Ac for H$_2$O$_2$ over O$_2^-$ in living cells (Figure 12b). A further control experiment shows that addition of both superoxide dismutase (5,000 U/mL) and catalase (5,000 U/mL), which catalyzes the decomposition of H$_2$O$_2$, led to a marked decrease in the PF1 signal corresponding to diminished phagosomal H$_2$O$_2$ production in these stimulated cells (Figure 12c).

Dendrimer-Based Multiplex Imaging Reveals the Effects of Nox Activity on Phagosomal ROS and pH Fluxes. We next applied the G5-SNARF2-PF1-Ac probe to fluorescence imaging of PMA-stimulated RAW264.7 macrophages treated with various doses of diphenylene iodonium (DPI), a broad-spectrum small-molecule Nox inhibitor. Unlike superoxide dismutase and catalase enzymes that scavenge ROS without largely perturbing the physiological environment of phagosome, inhibition of Nox activity disrupts various equilibria within and surrounding the phagosomal lumen and simultaneously influences ROS levels, local pH, and membrane potential. By mediating electron transfer to molecular oxygen from the cytosol to the phagosome, O$_2^-$ production by Nox is accompanied by a transient depolarization of the plasma membrane.$^{78,79}$ In addition, dismutation and/or reduction of O$_2^-$ also consumes protons leads to temporary alkalinization of the phagosomal lumen; this process is most prominent in neutrophils, which produce phagocytes with the highest ROS fluxes.$^{80}$ Furthermore, phagosomal pH is regulated by a complex system involving the interplay of many membrane-bound proteins, including Nox, V-ATPase, voltage-gated channels, and other unidentified H$^+$ channels.$^{81}$
Live-cell imaging with G5-SNARF2-PF1-Ac in PMA-stimulated RAW264.7 cells shows a marked decrease in the H$_2$O$_2$-responsive, PF1-derived fluorescein signal, indicating lower H$_2$O$_2$ production, upon addition of DPI in a dose-dependent manner (Figure 13(3)). In addition to reducing levels of H$_2$O$_2$ production during oxidative bursts, inhibition of Nox activity also results in more rapid acidification of phagosomes due to a loss of pH regulation (Figure 13(5)). Fifteen minutes after the onset of phagocytosis, phagosomes of stimulated macrophages with normal Nox activity are mostly neutral in pH with a concomitant robust H$_2$O$_2$ flux. However, cells treated with the Nox inhibitor DPI produce fewer phagosomes; these early phagosomes possess more acidic lumen than cells without DPI treatment. The effects of DPI on the frequency of phagosome formation becomes particularly evident in macrophages treated with 5 µM DPI, where there is an apparent elimination of phagocytosis. Our findings are in line with previous reports that DPI-treated macrophages show suppression in phagocytosis of myelin proteins and apoptotic neutrophils. Evidently, Nox plays an important role in the process of programmed cell clearance in macrophages. We speculate that high doses of DPI might disrupt the PI3K signaling pathway, which is involved in membrane remodeling and trafficking required for pseudopod extension, during both phagocytosis and pinocytosis, as H$_2$O$_2$ is a regulator of PI3K signaling through redox-mediated inhibition of the opposing phosphatase PTEN. In complementary experiments, flow cytometry analysis of PMA-stimulated macrophages treated with DPI also show a dose-dependent decrease in the H$_2$O$_2$-responsive, PF1-derived fluorescein signal. Finally, in accordance with the imaging data, 5 µM DPI inhibition of endocytosis results in a lower average fluorescein intensity in comparison to control cells in which G5-SNARF2-PF1-Ac uptake is facilitated by pinocytosis (Figure 14).

**Concluding Remarks**

In summary, we have described the synthesis, properties, and biological applications of G5-SNARF2-PF1-Ac, a new dendrimer-based, dual-responsive fluorescent reporter for the simultaneous imaging of both H$_2$O$_2$ and pH gradients in living cells. This work demonstrates the utility of PAMAM dendrimers as modular and versatile scaffolds for the loading of multiple...
chemically-responsive probes for multiplex imaging. Using this new G5-PAMAM imaging platform, we studied the contributions of ROS and pH fluxes to phagocytosis during immune insult using PMA-stimulated RAW264.7 macrophages as a model system. G5-SNARF2-PF1-Ac affords a method for the direct imaging of confined H$_2$O$_2$ bursts in early endosomes, as well as a way to monitor the progression of acidification during endosome trafficking and maturation. Additional imaging experiments show that the regulation of ROS through discrete molecular sources plays a critical role in the physiological immune response, as DPI-induced inhibition of Nox, the major source of phagocytic H$_2$O$_2$ bursts, not only affects transient ROS levels but also alters the course of phagosomal trafficking. Moreover, decreased Nox activity also results in a loss of pH regulation and more rapid rate of phagosomal acidification, with V-ATPase acting as another primary regulator for proper phagosome maturation.

This work contributes to the mounting evidence that the roles of H$_2$O$_2$ in immunology are more sophisticated and extend beyond the simplistic, primary view of ROS as microbicides. Our data also highlights the value of multifunctional fluorescent probes as powerful tools that can help to elucidate complex and transient changes in multiple molecular species with spatial and temporal resolution. Ongoing efforts include the application of this dual-imaging probe to studies of Nox chemistry in a variety of biological models, the development of second generation probes with improved optical brightness and photostability properties, and an expansion of the range of dendrimers and other molecular scaffolds for attachment of multiple responsive sensors and dosimeters.

Experimental Section

**Synthetic Materials and Methods.** SiliaFlash P60 (230 – 400 mesh, Silicycle, Quebec City, Canada) was used for column chromatography. Analytical thin layer chromatography was performed using SiliaPlate TLC (0.25 mm thickness, Silicycle). Dichloro[1,1′-bis(diphenylphosphino)ferrocene]palladium (II), Pd(dppf)Cl$_2$ was purchased from Strem Chemicals (Newburyport, MA). Bis(pinacolato)diboron was purchased from Boron Molecular (Research Triangle Park, NC). PAMAM-G5 (ethylenediamine core, molecular weight 28824.8, 128 amine end groups, solution 5 wt. % in methanol), phorbol 12-myristate 13 acetate (PMA), diphenylene iodonium (DPI) and concanamycin A were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. Sephadex™ LH-20 was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). The WST-1 cell proliferation reagent was purchased from Roche Applied Science (Indianapolis, IN). Amicon Ultracel-10K centrifugal units (regenerated cellulose 10,000 MWCO) were purchased from Millipore. SpectralPor regenerated cellulose MW3500 was used as a dialysis membrane (Spectrum Laboratories, Inc, Rancho Dominguez, CA). $^1$H NMR spectra were collected in CDCl$_3$, DMSO-$d_6$, or (CD$_3$)$_2$CO (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. Mass spectral analyses were carried out using Agilent Technology 1200 series with 6130 Quadrupole LC/MS (Santa Clara, CA). Matrix assisted laser desorption-ionization-time of flight mass spectrometry (MALDI-TOF-MS) was collected on a Voyager DE MALDI-TOF (Perseptive Biosystems, Framingham, MA) equipped with a 337 nm pulsed nitrogen laser. MALDI-TOF spectra were obtained in linear positive mode with the following settings: accelerating voltage of 25,000 V, grid voltage 92 % of accelerating voltage,
extraction delay time of 500 ns. 2,5-Dihydrobenzoic acid (DHB) was used as the matrix. The DHB matrix solution was 20 mg/mL DHB in 1:1 H2O/DMF. A solution of the analyte (PAMAM ~10 mg/mL) was mixed with the matrix solution at the volume ratio of 1:5, 1:10 and 1:20. The resulting solution was spotted on the MALDI plate (1 µL/spot) and vacuum dried. Dynamic light scattering (DLS) was performed on Zetasizer Nano-ZS (Malvern Instruments, estborough, MA) equipped with 4.0 mW, 633 nm He-Ne laser. DLS measurement was done at 25 °C with buffered solution containing 2 mg/mL dendrimers. All samples was filtered through 0.2 µM PVDF membrane before DLS measurement.

Isomeric Mixture of 2-(4-(Diethylamino)-2-hydroxybenzoyl)terephthalic Acid (1) and 4-(4-(Diethylamino)-2-hydroxybenzoyl)isophthalic Acid (2). 3-(Diethylamino)phenol (6.6 g, 40 mmol) and 1,2,4-benzenetricarboxylic anhydride (7.7 g, 40 mmol) were added to a 1L round bottom flask filled with 500 mL toluene. The mixture was heated to reflux overnight and allowed to cool down to room temperature. The solid precipitate was collected by vacuum filtration and washed with CH2Cl2 to give a yellow-brown solid that can be used in the next step without further purification (8.2 g, 23 mmol, 57% yield). Purification with flash column chromatography (4:1 EtOAc: hexane) gave a yellow solid. 1H NMR ((CD3)2CO, 400 MHz): δ 8.69 (1H, d, J = 1.6 Hz), 8.31 (1H, dd, J1 = 8.0 Hz, J2 = 1.6 Hz), 8.22 (1H, dd, J1 = 8.0 Hz, J2 = 1.6 Hz), 8.16 (1H, d, J = 8.0 Hz), 7.98 (1H, d, J = 1.6 Hz), 7.55(1H, d, J = 8.0 Hz), 6.91(1H, d, J = 9.2 Hz), 6.87 (1H, d, J = 8.8 Hz), 6.21-6.17 (2H, m), 6.09 (s, 2H), 3.44 (8H, q, J = 7.2 Hz), 1.16 (12H, t, J = 7.2Hz). LC-MS: calculated for [MH+] 358.12, found 358.1.

Isomeric Mixture of C.SNARF2: 10-(Diethylamino)-3-hydroxy-3'-oxy-3'H-spiro[benzo[c]xanthenes-7,1'-isobenzofuran]-6'-carboxylic acid (3) and 10-(diethylamino)-3-hydroxy-3'-oxy-3'H-spiro[benzo[c]xanthenes-7,1'-isobenzofuran]-5'-carboxylic Acid (4). The isomeric mixture of compounds 1 and 2 (170 mg, 0.47 mmol), 1,6-dihydroxynaphthalene (80 mg, 50 mmol), and methanesulfonic acid (2 mL) were added to a heavy-walled reaction flask. The mixture was sonicated for 3 hours. Dilution of the reaction mixture into ice water (100 mL) gave a purple-colored precipitate, which was collected by vacuum filtration. Purification by flash column chromatography (EtOAc) gave C.SNARF2 as a purple solid (120 mg, 0.25 mmol, 53% yield). 1H NMR ((CD3)2CO, 400 MHz): δ 8.55 (1H, s), 8.48 (1H, dd, J1 = 9.2 Hz, J2 = 2.4 Hz), 8.38 (1H, dd, J1 = 8.0, J2 = 1.6 Hz), 8.33 (1H, dd, J1 = 8.0 Hz, J2 = 1.6 Hz), 8.14 (1H, d, J = 8.0 Hz), 7.82 (1H, s), 7.34 (2H, d, J = 8.8 Hz), 7.28 (2H, dd, J1 = 9.2 Hz, J2 = 2.0 Hz), 7.21 (2H, t, J = 2.4 Hz), 7.00 (1H, dd, J1= 8.0 Hz, J2= 1.6 Hz), 6.78 (2H, t, J = 2.0 Hz), 6.72 (2H, dd, J1 = 8.8 Hz, J2 = 2.8 Hz), 6.68 (2H, dd, J1 = 8.8 Hz, J2 = 2.8 Hz), 6.67 (2H, dd, J1 = 8.8 Hz, J2 = 2.0 Hz), 3.45 (8H, q, J = 7.2 Hz, 1.26 (12H, t, J = 7.2 Hz). LC-MS: calculated for [MH+] 482.15, found 482.0.

SNARF2-CONHS: 2,5-Dioxopyrrolidin-1-yl 10-(diethylamino)-3-hydroxy-3'-oxy-3'H-spiro[benzo[c]xanthenes-7,1'-isobenzofuran]-5'-carboxylate (5). C.SNARF2 (50 mg, 0.10 mmol) and N-hydroxysuccinimide (15 mg, 0.13 mmol) and DMF (1 mL) were added to a 4 mL vial. The mixture was sonicated briefly until all solids were dissolved. Then, EDC-HCl (25 mg, 0.12 mmol) was added and the mixture was allowed to stir at room temperature for 3 hours. The product was isolated by flash column chromatography (4:1 EtOAc:Hex) giving isomerically pure SNARF2-CONHS as a purple solid (15 mg, 0.026 mmol, 25% yield). 1H NMR ((CD3)2CO, 400 MHz): δ 8.62 (1H, s), 8.47 (1H, d, J = 8.0), 8.45 (1H, d, J = 8.8), 7.58 (1H, d, J = 8.0), 7.35 (1H, d, J = 8.8 Hz), 7.29 (1H, dd, J1 = 8.8 Hz, J2= 2.4 Hz), 7.21 (1H, d, J = 2.4 Hz), 6.80 (1H, d, J = 8.8 Hz), 6.79 (1H, d, J = 2.4 Hz), 6.76 (1H, d, J = 8.8 Hz), 6.55 (1H, dd, J1= 8.8 Hz, J2 = 2.4 Hz)}
(4H, q, J = 7.2 Hz), 2.99 (4H, s), 1.25 (6H, t, J = 7.2 Hz). LC-MS: calculated for [MH$^+$] 579.17, found 579.2.

3',6'-Dibromo-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (6). 3-Bromophenol (10.5 g, 0.050 mol), 1,2,4-benzenetricarboxylic acid (17.3 g, 0.10 mol), and methanesulfonic acid (50 mL) were added to a 150 mL heavy-walled reaction flask and heated at 135 ºC for 72 h. The reaction was cooled to 25 ºC, poured into 400 mL of an ice/ddH$_2$O slurry, and stirred vigorously to precipitate a greenish solid. The solid was collected by vacuum filtration and treated with 300 mL of acetic anhydride and 100 mL of pyridine to give a pinkish white solid that was recrystallized three times from a mixture of acetic anhydride and pyridine (2/1). The white solid was treated with 1 M HCl to give isomerically pure carboxylic acid 6 as a bone white powder (6.3 g, 0.013 mol, 25% yield). $^1$H NMR (DMSO-$d_6$, 400 MHz): δ 13.67 (1H, s), 8.26 (1H, d, J = 8.0 Hz), 8.17 (1H, d, J = 8.0 Hz), 7.86 (1H, s), 7.70 (2H, d, J = 2.0 Hz), 7.33 (2H, dd, J$_1$ = 8.4 Hz, J$_2$ = 2.0 Hz), 6.90 (2H, d, J = 8.4 Hz). LC-MS: calculated for [M - H] 500.9, found 501.

PF1-COOH: 3-Oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylic acid (7). Compound 6 (2.01 g, 4.0 mmol), Pd(dppf)Cl$_2$ (989 mg, 1.2 mmol), KOAc (3.94 g, 40.1 mmol), and bis(pinacolato)diboron (4.00 g, 15.8 mmol) were added to a dried 100 mL Schlenk flask under nitrogen. Anhydrous, degassed DMF (50 mL) was added and the solution stirred at room temperature for 5 min. The reaction was heated to 80 ºC for 12 h, cooled to 25 ºC and the solvent removed under reduced pressure, giving a dark brown mass. Purification by flash column chromatography (silica gel, EtOAc) provided a brown viscous oil which was precipitated in minimal Et$_2$O, washed with Et$_2$O (3 x 25 mL), and dried in vacuo to give 7 as a peach solid (1.47 g, 61% yield). $^1$H NMR (DMSO-$d_6$, 400 MHz): δ 13.68 (1H, s), 8.24 (1H, d, J = 8.0 Hz), 8.19 (1H, d, J = 8.0 Hz), 7.67 (1H, s), 7.40 (2H, d, J = 7.6 Hz), 6.95 (2H, d, J = 7.6 Hz), 1.30 (24H, s). LC-MS: calculated for 597.24, found 597.2.

PF1-CONHS: 2,5-Dioxopyrrolidin-1-yl 3-oxo-3',6'-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3H-spiro[isobenzofuran-1,9'-xanthene]-6-carboxylate (8). PF1-COOH (50 mg, 0.085 mmol), N-hydroxysuccinimide (11 mg, 0.086 mmol) and DMF (2 mL) were added to a 4 mL vial. EDC-HCl (17 mg, 0.085 mmol) was then added and the mixture was allowed to stir at room temperature for 3 hours. The product was isolated by flash column chromatography (1:1 EtOAc:Hex) to give PF1-CONHS as a pale yellow solid (30 mg, 0.043 mmol, 51 % yield). $^1$H NMR (CDCl$_3$, 400 MHz): δ 8.36 (1H, d, J = 8.0 Hz), 8.16 (1H, d, J = 8.0 Hz), 6.83 (2H, d, J = 7.6 Hz), 2.87 (4H, s), 1.36 (24H, s). LC-MS: calculated for [MH$^+$] 694.26, found 694.2.

G5-SNARF2. SNARF2-NHS ester (4 mg, 6.9 µmol) in DMSO (1 mL) was added to a solution of PAMAM-G5 in MeOH (40 mg, 1.4 µmol, 5 mg/mL) after which stirring continued for 1 h in the dark at room temperature. This solution of G5-SNARF2 was then ready for further functionalization in the next step. For analysis of the number of SNARF2 dyes on the dendrimer, 200 µL of the reaction mixture was taken into 3 mL H$_2$O and concentrated with an Amicon 10K centrifugal unit to a final volume of 100 µL. Small organic molecules were removed by size-exclusive gel chromatography using Sephadex LH-20 equilibrated with MeOH. The collected dendrimer fraction was diluted with H$_2$O to approximately 20 % MeOH and concentrated with an Amicon 10K to a final volume of 50 µL. The solution was kept at 4 ºC for analysis with MALDI-TOF
**G5-SNARF2-Ac.** Triethylamine (20 µL, 143 µmol) followed by acetic anhydride (7 µL, 74 µmol) were added to a solution of G5-SNARF2 (4.4 mL, 0.7 µmol). After stirring for 2 h at room temperature, the reaction mixture was dialyzed against 20% MeOH for 1 h to dilute its DMSO content. The dendrimer solution was concentrated to a final volume of 2 mL with an Amicon 10K centrifugal unit and applied to a Sephadex-LH20 column equilibrated with MeOH. The collected dendrimer fraction was diluted with H2O to approximately 20% MeOH and concentrated with an Amicon 10 K to a final volume of 1 mL and then stored in the dark at -20 °C. The concentration of dendrimer solution, determined from the dried mass of a lyophilized sample of known volume, was 11 mg/mL.

**G5-SNARF2-PF1.** A solution of PF1-NHS ester (5 mg, 7.2 µmol) in DMSO (0.5 mL) was added to a solution of G5- SNARF2 (4.4 mL, 0.7 µmol). After 1 h, 200 µL of reaction mixture was subjected to purification and analysis in a similar procedure as described for G5-SNARF2.

**G-SNARF2-PF1-Ac.** Acylation of G5-SNARF2-PF1 was achieved by following a similar protocol to the synthesis of G5-SNARF-Ac to give 1 mL of a 12 mg/mL dendrimer solution.

**SDS-PAGE In-Gel Fluorescence.** G5-SNARF2-PF1-Ac was diluted in to PBS pH 7.4 to the concentration of 200 µg/mL. To a PCR tube was added 15 µL G5-SNARF2-PF1-Ac solution and 15 µL H2O2 solution (0, 50, 100, 200, 400, 800 µM in PBS). After 30 minute incubation at 37 °C, 10 µL loading buffer (50% glycerol, 10% SDS, bromophenol blue) was added to the reaction mixture. Samples were loaded into 12% polyacrylamide gel (20 µL/well) in Tris-Glycine running buffer at 200 V gradient. In-gel fluorescence scanning was obtained on a Typhoon 9410 imaging system (GE Healthcare). The dendrimer bands were visualized with Coomassie Blue staining.

**Spectroscopic Materials and Methods.** Milipore water was used to prepare all aqueous solutions. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm x 1-cm 1.25 mL volume quartz cuvettes (Starna, Atascadero, CA). Spectroscopic properties of G5-SNARF2-Ac at various pH values were measured in 50 mM phosphate buffer. pKa values of G5-SNARF2-Ac were obtained from analysis of the absorption spectra at 597 and 545 nm to fit the following equation,

\[ \text{pH} = \text{pK}_a + c \left[ \log \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right] + \log \frac{I^a}{I^b} \]

where \( I^a \) and \( I^b \) are the absorbances in acid and base respectively at the wavelength chosen for the denominator of R; this correction vanishes at the isosbestic wavelength (545 nm). C is the slope. R is the ratio of absorbance at two selected wavelengths, with maximum and minimum values of \( R_{\text{max}} \) and \( R_{\text{min}} \), respectively.

**Preparation of Cell Cultures and Cytotoxic Assays.** RAW264.7 macrophage cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose with GlutaMAX™ (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS,
Thermo Scientific/Hyclone, Logan, UT). Cells were split 1/30 twice a week. One day before imaging, cells were passaged and plated, and seeded at a density of $3 \times 10^5$ cells/well, in 4-well Lab-Tek Chamber slides (Thermo Scientific/ Nalgene Nunc, Rochester, NY) pre-coated with poly-L-lysine (50µg/mL, Sigma, St. Louis, MO). For cytotoxic assays, RAW 264.7 cells were seeded (50,000 cells/well) in a 96-well cell culture plate and maintained for 24 h. The cells were washed once with Hank’s buffered salt solution (HBSS) and incubated with 100 µL of dendrimer probe (500 -1000 ug/mL in DMEM) or in DMEM alone as a control medium. After 3 h, the cells were washed twice with fresh DMEM to remove the dendrimer probe and replaced with 100 µL of WST-1 solution (1:10 dilution) in DMEM. After incubation for 2 h, absorbance was measured at 440 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Five measurements were taken for each concentration of PAMAM dendrimers.

**Fluorescence Imaging Experiments.** Fluorescence imaging was performed with a Zeiss Axiovert 200M microscope (Zeiss, Jena, Germany) equipped with a mercury light source X-Cite 120 series (Exfo Life Science Divisions, Ontario, Canada) and a 63X/1.4 oil Plan-Apochromat objective lens. During image acquisitions, samples were kept at 37 °C in a 5% CO₂ using a Chemlide TC incubator system, consisting of a chambered cover glass connected to a CU-109 temperature controller, FC-7 gas mixture and a humidifier (Live Cell Instrument, Seoul, Korea). The filter sets used in image acquisition are listed in Table 3 and in the data captions. All imaging was performed with fixed acquisition times of 1000 ms for Cy5, 500 ms for Cy3, 250 ms GFP and automatic mode for DAPI and DIC channel. Stimulation of phagocytosis was achieved by the addition of PMA at 4 µg/mL delivered from a 1 mM stock solution in DMSO. The NADPH oxidase inhibitor DPI was delivered from a stock solution of 5 mM in DMSO. Hoechst 33342 was delivered from a stock solution of 1 mM in water. Concanamycin A was delivered from a 0.1 mM stock solution in DMSO. G5-SNARF2-Ac and G5-SNARF2-PF1-Ac were delivered from stock solutions in water, and imaging was done with 300 µg/mL working concentrations of dendrimer in DMEM. Dendrimers, Hoechst 33342, PMA, DPI and concanamycin A were premixed in DMEM by vortexing. For phagocytosis imaging, the culture medium of RAW264.7 cells was replaced by the labeling DMEM solution. After 15 min incubation at 37 °C with 5 % CO₂, cells were washed twice with DMEM to remove the dendrimer, and placed in HBSS buffer for imaging of early phagosomes. Cells were further incubated in HBSS for another 30 min for late endosome images. Image analysis was performed on an Axiovision Rel 4.8 (Zeiss). Ch1, Ch2, and Ch3 were adjusted to the same brightness/contrast setting. Ch4 and DIC were adjusted to the min/max value. Exported TIF color images were cropped by using ImageJ (National Institute of Health).
Scheme 1. PAMAM as a core structure for multifunctional nanoprobes for monitoring pH and H₂O₂.
Scheme 2. Synthesis of SNARF2-NHS ester (5), and PF1-NHS ester (8).
Scheme 3. Synthesis of functionalized PAMAMs.
**Table 1.** Molecular weight of functionalized PAMAM-G5 dendrimers

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>m/z</th>
<th>Functionality</th>
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<tr>
<td>PAMAM-G5</td>
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<td></td>
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<td>G5-SNARF2</td>
<td>27200</td>
<td>SNARF2(2)</td>
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<td>G5-SNARF2-Ac</td>
<td>31000</td>
<td>SNARF2(2), Ac(88)</td>
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<tr>
<td>G5-SNARF2-PF1</td>
<td>30660</td>
<td>SNARF2(2), PF1(6)</td>
</tr>
<tr>
<td>G5-SNARF2-PF1-Ac</td>
<td>32500</td>
<td>SNARF2(2), PF1(6), Ac(44)</td>
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</table>

**Table 2.** Spectroscopic properties of G5-SNARF2-Ac

<table>
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<tr>
<th>Compound</th>
<th>$\lambda_{A}^{\text{abs}}$ (nm)</th>
<th>$\lambda_{B}^{\text{abs}}$ (nm)</th>
<th>$\lambda_{A}^{\text{Em}}$ (nm)</th>
<th>$\lambda_{B}^{\text{Em}}$ (nm)</th>
<th>$\Phi_{A}$</th>
<th>$\Phi_{B}$</th>
<th>pH</th>
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<tr>
<td>SNARF2</td>
<td>518, 550</td>
<td>576</td>
<td>584</td>
<td>633</td>
<td>0.022</td>
<td>0.110</td>
<td>7.50</td>
</tr>
<tr>
<td>G5-SNARF2-Ac</td>
<td>527, 563</td>
<td>596</td>
<td>597</td>
<td>650</td>
<td>0.013</td>
<td>0.028</td>
<td>7.76</td>
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<tr>
<td>G5-SNARF2-PF1-Ac</td>
<td>527, 564</td>
<td>599</td>
<td>598</td>
<td>650</td>
<td>0.051</td>
<td>0.043</td>
<td>7.78</td>
</tr>
</tbody>
</table>

The superscript $^A$ and $^B$ designate acidic (pH 5-6) and basic (pH 10-12) aqueous solutions, respectively. Quantum yields are relative to Rhodamine 101 in ethanol ($\Phi = 0.92$)

**Table 3.** Filter sets used in the imaging experiments.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Channel</th>
<th>Excitation</th>
<th>Beamsplitter</th>
<th>Emission</th>
<th>Specification</th>
</tr>
</thead>
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<tr>
<td>Hoechst 33342</td>
<td>Ch4</td>
<td>G 365</td>
<td>FT 395</td>
<td>BP 445/50</td>
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</tr>
<tr>
<td>PF1</td>
<td>Ch3</td>
<td>BP 470/40</td>
<td>FT 495</td>
<td>BP 525/50</td>
<td>Filter set 38 HE (489038)</td>
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<tr>
<td>SNARF2 (acid)</td>
<td>Ch2</td>
<td>BP 550/25</td>
<td>FT 570</td>
<td>BP 605/70</td>
<td>Filter set 43 HE (489043)</td>
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<tr>
<td>SNARF2 (base)</td>
<td>Ch1</td>
<td>BP 575-625</td>
<td>FT 645</td>
<td>BP 660-710</td>
<td>Filter set 326 (488026)</td>
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</table>
Figure 1. MALDI-TOF spectra of functionalized PAMAM dendrimers. (a) PAMAM-G5 with an average MW of 26,300 Da. (b) G5-SNARF2 with an average MW of 27,200 Da. (c) G5-SNARF2-Ac with an average MW of 31,000 Da. (d) G5-SNARF2-PF with an average MW of 30,660 Da. (e) G5-SNARF2-PF1-Ac with an average MW of 32,500 Da.
Figure 2. Absorption spectra of G5-SNARF2-PF1-Ac (20 µg/mL ~ 0.6 µM) of which PF1 was fully converted to fluorescein by reaction with 1 mM H$_2$O$_2$ for 60 min in PBS pH 7.4. The solution was adjusted to pH 10 with 0.1 M NaOH to give the absorption spectrum of basic SNARF2. Calculation from the known extinction coefficients (dianion fluorescein, $\varepsilon = 76,900$ M$^{-1}$cm$^{-1}$; SNARF2, 46,400 M$^{-1}$cm$^{-1}$) gave six molecules of fluorescein and two molecules of SNARF2 per dendrimer.
Figure 3. PAMAM-G5 is stable under oxidative conditions. The hydrodynamic diameter of PAMAM-G5 measured by dynamic light scattering (DSL) shows no difference between (a) PAMAM-G5 (average diameter 4.95 nm), and (b) PAMAM-G5 after 2 h with 10 mM H₂O₂ in PBS buffered to pH 7 (average diameter 5.10 nm).
Figure 4. Size distributions of functionalized PAMAM dendrimers as determined by dynamic light scattering (DLS) measurements. The average hydrodynamic diameters of (a) G5-SNARF2-Ac and (b) G5-SNARF2-PF1-Ac are 3.63 and 3.56 nm, respectively. Measurements were done in acidic buffer, 0.1 M citric acid buffered to pH 2.5, in order to avoid 633 nm absorption from basic SNARF2.
Figure 5. (a) Absorption spectra of G5-SNARF2-Ac (20 µg/mL) in 50 mM phosphate-buffered solutions at a range of pH values. (b) Fluorescence emission spectra of G5-SNARF2-Ac, $\lambda_{\text{exc}} = 543$ nm, and (c) $\lambda_{\text{exc}} = 600$ nm.
Figure 6. (a) Absorption spectra of G5-SNARF2-PF1-Ac (10 µg/mL) in response to 100 µM H₂O₂ in PBS buffered to pH 7.4. Spectra were taken at 0, 10, 20, 30, and 60 min after H₂O₂ was added. (b) Fluorescence emission spectra of G5-SNARF2-PF1-Ac in panel (a) with λ<sub>exc</sub> = 488 nm.
Figure 7. Kinetic plot of the fluorescence emission intensity at 520 nm of the pseudo-first order reaction of G5-SNARF2-PF1-Ac (10 µg/mL, ~1.5 µM PF1) with 1 mM H$_2$O$_2$. The slope of the plot corresponds to the observed rate of reaction $k_{obs} = 2.8 \times 10^{-4}$ s$^{-1}$. 

\[ y = 14.469 - 0.00027649x \quad R^2 = 0.99835 \]
Figure 8. SDS-PAGE of G5-SNARF2-PF1-Ac after reaction with various concentration of added H₂O₂. (a) Coomassie stain. (b) Fluorescence emission from PF1 on the dendrimer. (c) Fluorescence emission from SNARF2. (d) Merged image of PF1 and SNARF2 channel. (λ_{exc} = 532 nm)
Figure 9. (a) Absorption spectra of G5-SNARF2-PF1-Ac (10 µg/mL) at 30 min after 100 µM H$_2$O$_2$ was added. The reaction in phosphate-buffered solution pH 7.4 was adjusted to pH 5.0 and pH 9.0 using 1N HCl and 1M NaOH, respectively. (b) Fluorescence emission spectra of G5-SNARF2-PF1-Ac in (a) using λ$_{exc}$ = 543 nm.
Figure 10. WST-1 cell viability assay of RAW264.7 cells after 3 h incubation with 500 – 1,000 µg/mL dendrimer in DMEM: PAMAM-G5 (light gray), G5-SNARF2-Ac (dark gray), and G5-SNARF2-PF1-Ac (black). Error bars represent standard deviation.
Figure 11. Fluorescence imaging of pH changes in phagosomal lumen with G5-SNARF2-Ac. RAW264.7 cells were at rest in DMEM containing 300 µg/mL G5-SNARF2-Ac before PMA (4 µg/mL) was added to stimulate phagocytosis. Fluorescence emission from basic and acid form of SNARF2 are displayed in Ch1-SNARF2[B] and Ch2-SNARF2[A], respectively. A pH profile is generated from merging signal from Ch1 and Ch2. Row (a) shows image taken 15 min after the onset of phagocytosis. Cells are populated by early phagosomes which have a basic to neutral pH. Row (b) shows progressive acidification in phagosomal lumen after cells in row (a) were incubated in fresh media for another 30 min. Column (1) Ch1 filter set (600: 670 - 710), (2) Ch2 filter set (550: 570 - 640), (3) DIC image, scale bar = 10 µm, and (4) overlay image of Ch1 and Ch2, nuclear staining with Hoechst 33342.
Figure 12. Fluorescence imaging of oxidative burst and pH change in early phagosome with G5-SNARF2-PF1-Ac (300 µg/mL in DMEM). Row (a) shows RAW264.7 cells stimulated with PMA for 15 min. Generation of H₂O₂ in early phagosomes is displayed by the colocalization of SNARF2[B] with fluorescein, a product of the reaction of PF1 with H₂O₂. Row (b) displays PMA stimulated RAW264.7 in the presence of superoxide dismutase (SOD 5,000 U/mL). Row (c) shows PMA stimulated cells in the presence of SOD (5,000 U/mL) and catalase (5,000 U/mL). Column (1) Ch1 filter set (600: 670-710), (2) Ch2 filter set (550: 570 - 640), (3) Ch3 filter set (470: 500 - 550), (4) DIC image, scale bar = 10 µm, and (5) overlay image of Ch1 and Ch2, nuclear staining with Hoechst 33342.
Figure 13. Inhibition of Nox activity in PMA-stimulated RAW264.7 cells with various doses of DPI. (a) no DPI, (b) 200 nM, (c) 500 nM, (d) 1 µM, (e) 5 µM, and (f) unstimulated cells with no PMA and no DPI. Phagosomal H$_2$O$_2$ decreases in a dose-dependent manner with DPI. DPI treated cells also show faster acidification of phagosome. At 5 µM DPI, endocytic activity is disrupted, showing the absence of pinocytosis compared to the unstimulated cells. Column (1) Ch1 filter set (600: 670 - 710), (2) Ch2 filter set (550: 570 - 640), (3) Ch3 filter set (470: 500 - 550), (4) DIC image, scale bar = 10 µm, and (5) overlay image of Ch1 and Ch2, nuclear staining with Hoechst 33342.
Figure 14. Flow cytometry detection of fluorescent emission intensity of PF1-derived fluorescein in PMA-stimulated RAW264.7 macrophages. Cells were harvested after 30 min incubation in media containing G5-SNARF2-PF1-Ac and the indicated concentration of PMA and DPI. ($\lambda_{\text{exc}} = 488\, \text{nm}, \lambda_{\text{em}} = 500 - 550\, \text{nm}$). Error bars represent s.e.m.
Figure 15. Fluorescence images of concanamycin A-treated macrophages that lead to the inhibition of the phagosomal acidification. RAW264.7 cells were incubated with G5-SNARF2-PF1-Ac (300 µg/mL in DMEM), PMA (4 µg/mL), with or without concanamycin A (400 nM) and DPI (500 nM). After 15 min, the labeling solution was removed. Cells were further incubation in label-freed media for another 30 min before imaging. Row (a) shows PMA-stimulated RAW264.7 cells 45 min after PMA stimulation. Overlay image of SNARF[A] and SNARF[B] shows marked acidic environment in phagosomal lumen. Row (b) displays concanamycin A-treated cells after 45 min PMA stimulation showing mostly neutral phagosome. Row (c) displays PMA stimulated cell treated with both PMA and DPI, in which mixed population of acidic and neutral pH phagosomes can be observed. Column (1) Ch1 filter set (600: 670 - 710), (2) Ch2 filter set (550: 570 - 640), (3) Ch3 filter set (470: 500 - 550), (4) DIC image, scale bar = 10 µm, and (5) overlay image of Ch1 and Ch2, nuclear staining with Hoechst 33342.
References


Appendix 1

Fluorescent Reporters for Hydrogen Peroxide using Styryl Dyes
**Synopsis**

This appendix describes the synthesis, spectroscopic characterization, and confocal fluorescence imaging of reporters for H$_2$O$_2$ based on a family of styryl dyes: quinolium styryl, indolium styryl, and dicyanomethylenehydrofuran (DCDHF) styryl. Most styryl dyes have internal charge transfer excited state (ICT), and thus their fluorescence emission spectra are sensitive to the dipole moment of the molecule in the excited state.\(^1\)\(^2\) We envisioned creating a ratiometric H$_2$O$_2$ probes based on the difference in ICT energy between styryl dyes with an electron-withdrawing boronate ester and those with an electron-donating phenol.

**Results and Discussion**

Quinolinium styryl dyes were synthesized by piperazine-catalyzed Knoevenagel condensation of aldehydes with quinolinium salts (Scheme 1). Spectroscopic characterization of styryl dyes 5 - 8 was performed in ethanol due to partial aggregation in aqueous buffer. Styryl dyes with a boronate ester group possess lower excitation and emission wavelengths than their hydroxy derivatives (Figure 1), in agreement with the lower energy of the internal charge transfer excited state (ICT) of fluorophores containing both electron donating and electron withdrawing group, known as the push-pull effect. To improve the solubility of the probes in aqueous buffers, we replaced the N-methyl substitution with a propanesulfonate group, obtained from reaction of quinoline with 1,3-propanesultone. In HEPES buffer at pH 7.4, compound 10 has an absorption maximum at 390 nm and an emission maximum at 480 nm. Compound 11, with the electron donating 4-hydroxy group, has higher absorption and emission wavelengths, with an absorption maximum and emission maximum at 420 and 560 nm, respectively. Reaction of 10 with H$_2$O$_2$ displayed a colorimetric response with decreasing absorption at 390 nm and a concomitant increase in absorption at 420 nm, corresponding to the absorption profile of 11 (Figure 1a). Excitation at the isosbestic wavelength (400 nm) gave a ratiometric fluorescence emission response; as the reaction progressed, it was possible to observe the decrease in emission at 480 nm with a concomitant emission increase at 560 nm (Figure 1b). The observed reaction rate under pseudo-first-order reaction (5 µM 11, 1 mM H$_2$O$_2$) was $k_{obs} = 5.3 \times 10^{-3}$ s$^{-1}$ (Figure 1c).

Indolium styryl dyes with a 4-hydroxy group (16, 17) displayed broad fluorescence emission spectra centered at 550 – 560 nm, which is in the same range as that observed for quinolinium styryl 11. Compound 16 has two absorption maxima at 425 nm and 520 nm, which correspond to the acidic and basic form of the dye respectively. Compound 17 showed only one, prominent absorption at 540 nm due to the more favorable equilibrium shift to the basic form from the presence of an electron-withdrawing methoxycarbonyl group on the indole ring. Boronate ester derivatives (18, 19) of indolium styryls have broad absorption spectra centered at 400 nm, and are non-fluorescent. Reactions of 18 and 19 with H$_2$O$_2$ gave turn-on fluorescence emission accompanied by colorimetric changes in absorption ($k_{obs} = 4.5 - 5.4 \times 10^{-3}$ s$^{-1}$, Figure 2a, 2b). Indolium styryl dye 21 with benzylether self-immolative linkers are also non-fluorescent; reaction of 21 with H$_2$O$_2$ to release 16 has $k_{obs} = 2.6 \times 10^{-3}$ s$^{-1}$ (Figure 2c).

HEK293T cells loaded with 18, 19, or 21 showed localized emission from mitochondria, which is expected for the chromophores that are lipophilic cations. Although, 18, 19 and 21 showed fast responses to H$_2$O$_2$ in spectroscopic measurements, we observed no difference in emission intensity between control and H$_2$O$_2$ treated cells. We speculated that the proton gradient or membrane potential inside the mitochondria could influence the change in fluorescence.
emission properties of the boronate ester probes or their hydroxy analogs. We later found success in indolium styryl dyes with 4-(dihydroxyboryl)benzoyloxycarbonyl (DobZ) protecting group. Compound 25 and 26 exhibited fluorescent turn-on response to H$_2$O$_2$ with $k_{obs} = 1.6 – 2.1 \times 10^{-3}$ s$^{-1}$ (Figure 2d, 2e). Fluorescence imaging experiments in HEK293T cells treated with 100 µM showed turn-on emission from mitochondria of cells labeled with 25 and 26 (Figure 5 - 6). However, these probes are less useful compared to the brighter fluorescent probes of the previously reported xanthenes dyes scaffold.$^{3,4}$

DCDHF styryl dyes with 4-hydroxy group (29, 30) have marked red-shifted emission (600 nm) compared to the quinolinium styryl and indolium styryl counterparts. Compound 29 has two absorption maxima at 450 nm and 571 nm which correspond to the acidic and basic form of the dyes respectively. Installation of fluorine at the ortho position to the hydroxyl group contributes to a lower pKa and more favorable equilibrium towards the basic form in compound 30. However, the boronate ester derivative with fluorine substitution, compound 32, displayed a much slower rate of reaction with H$_2$O$_2$ compared to the non-substituted compound 31 (Figure 3). This is because the rate-limiting migration step of boronate ester deprotection favors a more electron-rich benzene ring. Fluorescence imaging experiments with compound 31 in control and H$_2$O$_2$-treated HEK293T cells gave a very low fluorescence emission read-out.

**Summary:** We have synthesized styryl dyes that are capable of colorimetric detection of H$_2$O$_2$ in aqueous solution. Compound 18 was later developed independently by another group and used for colorimetric detection of trace H$_2$O$_2$ in rainwater.$^5$ However, these dyes are not suitable for application as fluorescent probes for detection of H$_2$O$_2$ in cellular environment because of their low fluorescence quantum yield and their sensitivity to other factors such as pH and electrical potential.$^{1,2,6,7}$ Fluorescence quantum yields of styryl dyes can be as high as 0.2 when the dyes are trapped into a hydrophobic confined space, such as within DNA or protein binding grove.$^{8-10}$ The best scenario would be to screen for a non-emissive probe that exhibits fluorescent turn-on when bound to the target sites, and then reports the presence of analytes by a ratiometric emission response.

**Experimental Section**

**Materials and Methods.** Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. 4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (4) was purchased from Oakwood (West Columbia, SC). Dicyanomethylenedihydrofuran (DCDHF) was synthesized following literature protocol.$^{11-14}$ All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. $^1$H NMR and $^{13}$C NMR spectra were collected in CDCl$_3$, DMSO-$d_6$, or CD$_3$OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVQ-400 or AV300 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.
General procedure for Knoevenagel condensation of quinilinium salt, indolium salt, or DCDHF with benzaldehyde. A mixture of quinolinium salt or indolium salt or DCDHF (1 eq, 50–100 mg), and benzaldehyde (1.2 eq.) in methanol (1 – 2 mL) was added to a reaction tube equipped with a small magnetic stir bar. A catalytic amount of piperazine (10 - 20 µL) was added to the reaction mixture. After stirring at room temperature overnight, the precipitate formed was collected by vacuum filtration, washed with ice-cold methanol, and air dried.

1,2-Dimethylquinolinium iodide (1) was synthesized following a literature procedure. $^9$ $^1$H NMR (CDCl$_3$, 300 MHz): δ 8.71 (1H, d, $J = 8.4$ Hz), 8.21 (1H, d, $J = 9.0$ Hz), 8.01-8.11 (2H, m), 7.79 (1H, d, $J = 8.4$ Hz), 4.32 (3H, s), 2.98 (3H, s).

1,4-Dimethylquinolinium iodide (2) was synthesized following a literature procedure. $^9$ $^1$H NMR (CD$_3$OD, 300 MHz): δ 9.19 (1H, d, $J = 6.0$ Hz), 8.56 (1H, d, $J = 8.4$ Hz), 8.48 (1H, d, $J = 9.0$ Hz), 8.26 (1H, t, $J = 7.8$ Hz), 8.06 (1H, t, $J = 8.4$ Hz), 7.95 (1H, d, $J = 6.0$ Hz), 4.63 (3H, s), 3.05 (3H, s).

1-(3-Propanesulfonyl)-4-methylquinolinium (3). To a round-bottomed flask equipped with a magnetic stir bar was added lepidine (0.5 mL, 3.8 mmol), and 1,3-propanesultone (0.39 mL, 4.4 mmol). The mixture was heated at 110 °C for 1 h. The mixture solidified once it cooled to room temperature. The solid was dispersed in diethylether (5 mL) and collected by vacuum filtration. The solid paste was washed twice with ice-cold methanol (2 x 3 mL) and dried to give product as a pale pink solid (900 mg, 3.4 mmol, 90% yield). $^1$H NMR (D$_2$O/CD$_3$OD 2:1, 400 MHz): δ 9.01 (1H, d, $J = 6.0$ Hz), 8.41 (1H, d, $J = 8.0$ Hz), 8.37 (1H, d, $J = 8.0$ Hz), 8.14 (1H, t, $J = 8.0$ Hz), 7.92 (1H, t, $J = 8.0$ Hz), 7.95 (1H, d, $J = 6.0$ Hz) 5.05 (2H, t, $J = 7.6$ Hz), 2.92 (3H, s), 2.90 (2H, t, $J = 7.2$ Hz), 2.39 (2H, m).

2-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)-N-methyl-quinolinium iodide (6). Reaction mixture: 1 (120 mg g, 0.42 mmol), 4 (150 mg g, 0.63 mmol). Product was obtained as an orange solid (0.17 g, 0.34 mmol, 81% yield). $^1$H NMR (DMSO-d$_6$, 400 MHz): δ 9.10 (1H, d, $J = 9.2$ Hz), 8.58 (1H, d, $J = 9.2$ Hz), 8.57 (1H, d, $J = 8.0$ Hz), 8.35 (1H, d, $J = 8.0$ Hz), 8.19 (1H, t, $J = 8.0$ Hz), 8.17 (1H, d, $J = 16.0$ Hz), 8.02 (1H, d, $J = 16.0$ Hz), 7.96 (2H, d, $J = 8.0$ Hz), 7.94 (1H, t, $J = 8.0$ Hz), 7.82 (1H, d, $J = 6.0$ Hz) 5.05 (2H, t, $J = 7.6$ Hz), 2.92 (3H, s), 2.90 (2H, t, $J = 7.2$ Hz), 2.39 (2H, m).

2-(4-Hydroxystyryl)-N-methyl-quinolinium iodide (7). Reaction mixture: 1 (100 mg, 0.35 mmol), 5 (60 mg, 0.50 mmol). Product was obtained as a red solid (0.17 g, 0.34 mmol, 81% yield). $^1$H NMR (DMSO-d$_6$, 400 MHz): δ 8.94 (1H, d, $J = 9.2$ Hz), 8.53 (1H, d, $J = 9.2$ Hz), 8.50 (1H, d, $J = 7.6$ Hz), 8.30 (1H, d, $J = 7.6$ Hz), 8.20 (1H, d, $J = 16.0$ Hz), 8.14 (1H, t, $J = 7.6$ Hz), 7.90 (1H, t, $J = 7.6$ Hz), 7.86 (2H, d, $J = 8.8$ Hz), 7.68 (1H, d, $J = 16.0$ Hz), 6.90 (2H, d, $J = 8.8$ Hz), 4.50 (3H, s). 13C NMR (DMSO-d$_6$, 400 MHz): δ 155.5, 148.1, 139.9, 134.0, 133.9, 129.8, 127.3, 126.3, 120.5, 119.9, 118.3, 38.4. HRFAB-MS: calculated for [M$^+$] 262.12, found 262.12.

4-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)-N-methyl-quinolinium iodide (8). Reaction mixture: 2 (0.12 g, 0.42 mmol), 4 (0.15 g, 0.63 mmol). Product was obtained as a yellow solid (0.17 g, 0.34 mmol, 81% yield). $^1$H NMR (DMSO-d$_6$, 400 MHz): δ 9.37 (1H, d, $J = 7.6$ Hz), 9.04 (1H, d, $J = 8.8$ Hz), 8.50 (1H, d, $J = 7.6$ Hz), 8.44 (1H, d, $J = 8.8$ Hz), 8.40 (1H, d, $J = 16.0$ Hz), 8.26 (1H, t, $J = 7.6$ Hz), 8.13 (1H, d, $J = 16.0$ Hz), 8.04 (1H, t, $J = 7.6$ Hz), 7.98 (2H, d, $J = 8.0$ Hz), 7.77 (2H, d, $J = 8.0$ Hz), 4.53 (3H, s), 1.29 (12H, s). 13C NMR (DMSO-d$_6$, 400 MHz): δ 152.7, 148.7, 142.7, 139.1, 138.5, 135.5, 129.8, 128.5, 127.0, 126.8, 121.5, 119.8, 117.1, 84.3, 25.1. HRFAB-MS: calculated for [M$^+$] 372.21, found 372.21.
4-(4-Hydroxystyryl)-N-methyl-quinolinium iodide (9). Reaction mixture: 2 (60 mg, 0.21 mmol), 5 (30 mg, 0.24 mmol). Product was obtained as a brown solid (60 mg, 0.12 mmol, 57% yield). $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 9.22 (1H, d, $J = 6.6$ Hz), 9.00 (1H, d, $J = 8.4$ Hz), 8.40 (2H, d, $J = 6.6$ Hz), 8.37 (1H, d, $J = 8.4$ Hz), 8.21 (1H, t, $J = 7.5$ Hz), 8.13 (1H, d, $J = 16.2$ Hz), 8.07 (1H, d, $J = 16.2$ Hz), 7.98 (1H, t, $J = 7.5$ Hz), 7.84 (2H, d, $J = 8.7$ Hz), 6.86 (2H, d, $J = 8.7$ Hz), 4.47 (3H, s). $^{13}$C NMR (DMSO-$d_6$, 400 MHz): $\delta$ 151.9, 145.4, 145.0, 139.2, 134.3, 133.5, 128.0, 126.4, 125.6, 119.6, 118.8, 112.1, 110.9, 43.5. HRFAB- MS: calculate for [M+] 262.12, found 262.12.

4-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)-N-(3-propanesulfonyl)quinolinium iodide (10). Reaction mixture: 3 (50 mg, 0.18 mmol), 4 (46 mg, 0.20 mmol). Product was obtained as an orange solid (50 mg, 0.10 mmol, 58% yield). $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 9.38 (1H, d, $J = 6.4$ Hz), 9.05 (1H, d, $J = 8.8$ Hz), 8.71 (1H, d, $J = 8.8$ Hz), 8.56 (1H, d, $J = 6.4$ Hz), 8.42 (1H, d, $J = 16.0$ Hz), 8.25 (1H, t, $J = 8.8$ Hz), 8.14 (1H, d, $J = 16.0$ Hz), 8.08 (1H, t, $J = 7.2$ Hz), 2.53 (2H, t, $J = 7.2$ Hz), 2.25 (2H, m), 1.30 (12H, s). LC-MS: calculated for [M+] 479.19, found 477.1.

4-(4-Hydroxystyryl)-N-(3-propanesulfonyl)quinolinium iodide (11). Reaction mixture: 3 (50 mg, 0.18 mmol), 5 (25 mg, 0.20 mmol). Product was obtained as an orange solid (54 mg, 0.14 mmol, 81% yield). $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 9.22 (1H, d, $J = 6.8$ Hz), 9.00 (1H, d, $J = 8.4$ Hz), 8.58 (1H, d, $J = 8.4$ Hz), 8.38 (1H, d, $J = 6.8$ Hz), 8.20 (1H, m), 8.10 (2H, s), 7.99 (1H, m), 7.84 (2H, d, $J = 8.4$ Hz), 6.87 (2H, d, $J = 8.4$ Hz), 5.05 (2H, t, $J = 6.8$ Hz), 2.53 (2H, t, $J = 7.2$ Hz), 2.25 (2H, m). LC-MS: calculated for [MH+] 370.10, found 370.1.

2,3,3-Trimethyl-3H-indole (12). To a round-bottom flask equipped with a magnetic stir bar and a condenser was added phenylhydrazine (5 mL, 51 mmol), 3-methylbutanone (5.5 mL, 51 mmol) and ethanol (20 mL). The reaction mixture was heated to reflux for 30 min. Cooled to room temperature gave hydrazone precipitate, which was collected by vacuum filtration. The solid was then added to glacial acetic acid (20 mL), and heated to reflux under N$_2$ atmosphere for 3 h. Solvent was removed by a rotary evaporator. Crude product was purified by flash column chromatography (CH$_2$Cl$_2$ with 5% MeOH) to give orange oil product (7.0 g, 44 mmol, 80% yield). $^1$H NMR (CD$_3$OD, 400 MHz): $\delta$: 7.41 (1H, d, $J = 7.6$ Hz), 7.36 (1H, d, $J = 7.2$ Hz), 7.21 (1H, dd, $J_1 = 7.6$ Hz, $J_2 = 7.2$ Hz), 7.21 (1H, dd, $J_1 = 7.6$ Hz, $J_2 = 7.2$ Hz), 1.92 (3H, s), 1.31 (6J, s). GC-MS: calculated for [M+] C$_{11}$H$_{13}$N 159.10, found 159.1.

Methyl 2,3,3-trimethyl-3H-indole-5-carboxylate (13). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added methyl 4-hydrazinylbenzoate (400 mg, 2.4 mmol), 3-methylbutanone (1 mL, 9.3 mmol) and ethanol (10 mL). The reaction mixture was heated to reflux for 30 min. Cooled to room temperature gave hydrazone precipitate which was collected by vacuum filtration. The collected solid was then added to glacial acetic acid (10 mL). The mixture was heated to reflux under N$_2$ atmosphere for 3 h. Solvent was removed by a rotary evaporator. Crude product was purified by flash column chromatography (CH$_2$Cl$_2$ with 5% MeOH) to give orange oil (450 mg, 2.1 mmol, 86% yield). $^1$H NMR (CD$_3$OD, 400 MHz): $\delta$: 7.41 (1H, d, $J = 7.6$ Hz), 7.36 (1H, d, $J = 7.2$ Hz), 7.28 (1H, dd, $J_1 = 7.6$ Hz, $J_2 = 7.2$ Hz), 7.21 (1H, dd, $J_1 = 7.6$ Hz, $J_2 = 7.2$ Hz), 1.92 (3H, s), 1.31 (6J, s). GC-MS: calculated for [M+] C$_{11}$H$_{13}$NO$_2$ 217.11, found 217.1.
mL) and dispersed by sonication for 5 min. White insoluble solid was collected by vacuum filtration, and air dried (1.2 g, 3.9 mmol, 62% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 7.84 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 5.6$ Hz), 7.74 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 5.6$ Hz), 7.66 (1H, d, $J = 6.0$ Hz), 7.64 (1H, d, $J = 5.6$ Hz), 4.05 (3H, s), 2.81 (2H, s), 1.60 (6H, s).

5-(Methoxycarbonyl)-1,2,3,3-tetramethyl-3H-indolium iodide (15). A round-bottomed flask equipped with a magnetic stir bar and a condenser was charged with 13 (300 mg, 1.4 mmol), methyl iodide (300 µL, 4.8 mmol), and acetonitrile (10 mL). The mixture was heated to reflux overnight. Solvent was removed by a rotary evaporator. The concentrated paste was taken into 1:1 CH$_2$Cl$_2$:EtOAc (15 mL) and dispersed by sonication for 5 min. White insoluble solid was collected by vacuum filtration, and air dried (330 mg, 0.92 mmol, 65% yield). $^1$H NMR (DMSO-d$_6$, 300 MHz): δ 8.40 (1H, s), 8.20 (1H, d, $J = 8.1$ Hz), 8.02 (1H, d, $J = 8.1$ Hz), 4.01 (3H, s), 3.91 (3H, s), 2.78 (3H, s), 1.54 (6H, s).

(E)-2-(4-Hydroxystyryl)-1,3,3-trimethyl-3H-indolium iodide(16). Reaction mixture: 14 (100 mg, 0.33 mmol), 5 (40 mg, 0.33 mmol). Product was obtained as an orange solid (90 mg, 0.22 mmol, 66% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.16 (1H, d, $J = 14.8$ Hz), 7.79 (2H, d, $J = 8.8$ Hz), 7.54 (1H, d, $J = 7.2$ Hz), 7.46 (1H, d, $J = 7.2$ Hz), 7.41 (1H, t, $J = 7.2$ Hz), 7.35 (1H, t, $J = 7.2$ Hz), 6.88 (1H, d, $J = 14.8$ Hz), 6.58 (2H, d, $J = 8.8$ Hz), 3.79 (3H, s), 1.75 (6H, s).

HRFAB-MS: calculated for [M$^+$] 278.37, found 278.15.

(E)-2-(4-Hydroxystyryl)-5-(methoxycarbonyl)-1,3,3-trimethyl-3H-indolium iodide (17). Reaction mixture: 15 (50 mg, 0.13 mmol), 5 (17 mg, 0.13 mmol). Product was obtained as an orange solid (30 mg, 0.065 mmol, 50% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.46 (1H, d, $J = 16.4$ Hz), 8.32 (1H, s), 8.26 (1H, d, $J = 8.4$ Hz), 8.01 (2H, d, $J = 8.8$ Hz), 7.82 (1H, d, $J = 8.4$ Hz), 6.97 (2H, d, $J = 8.8$ Hz), 4.10 (3H, s), 3.96 (3H, s), 1.85 (6H, s), 1.36 (12H, s). HRFAB-MS: calculated for [M$^+$] 336.40, found 336.16.

(E)-1,3,3-Trimethyl-2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)-3H-indolium iodide (18). Reaction mixture: 14 (100 mg, 0.33 mmol), 4 (80 mg, 0.33 mmol). Product was obtained as an orange solid (90 mg, 0.17 mmol, 53% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.40 (1H, d, $J = 16.4$ Hz), 8.03 (2H, d, $J = 8.0$ Hz), 7.90 (2H, d, $J = 8.0$ Hz), 7.84 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 5.6$ Hz), 7.75 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 5.6$ Hz), 7.72 (1H, d, $J = 16.4$ Hz), 7.65 (1H, d, $J = 6.0$ Hz), 7.64 (1H, d, $J = 5.6$ Hz), 4.20 (3H, s), 1.97 (6H, s), 1.36 (12H, s). HRFAB-MS: calculated for [M$^+$] 388.24, found 388.24.

(E)-5-(Methoxycarbonyl)-1,3,3-trimethyl-2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)styryl)-3H-indolium iodide (19). Reaction mixture: 15 (50 mg, 0.13 mmol), 4 (30 mg, 0.13 mmol). Product was obtained as an orange solid (40 mg, 0.070 mmol, 54% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.50 (1H, d, $J = 16.4$ Hz), 8.39 (1H, s), 8.30 (1H, d, $J = 8.4$ Hz), 8.06 (2H, d, $J = 8.4$ Hz), 7.94 (1H, d, $J = 8.4$ Hz), 7.91 (2H, d, $J = 8.4$ Hz), 7.75 (1H, d, $J = 16.4$ Hz), 4.22 (3H, s), 3.97 (3H, s), 1.88 (6H, s), 1.36 (12H, s). HRFAB-MS: calculated for [M$^+$] C$_{27}$H$_{33}$BNO$_4$ 446.37, found 446.25.

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (20). To a 20-mL vial equipped with a small magnetic stir bar was added 5 (60 mg, 0.49 mmol), 2-(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolan (145 mg, 0.48 mmol), Cs$_2$CO$_3$ (150 mg, 0.46 mmol) and DMF (2 mL). The reaction mixture was heat at 70°C overnight. The mixture was poured in to EtOAc (50 mL), and washed with water (2 x 25 mL) and brine. The organic layer was dried over Na$_2$SO$_4$. Removal of solvent by a rotary evaporator gave product as a white solid (150 mg, 0.44 mmol, 95% yield). $^1$H NMR (CDCl$_3$, 400 MHz): δ 9.88 (1H, s), 7.85
(2H, d, J = 8.0 Hz), 7.82 (2H, d, J = 8.8 Hz), 7.43 (2H, d, J = 8.0 Hz), 7.07 (2H, d, J = 8.8 Hz), 5.19 (2H, s), 1.35 (12H, s).

(E)-1,3,3-Trimethyl-2-(4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)styryl)-3H-indolium iodide (21). Reaction mixture: 14 (50 mg, 0.16 mmol), 20 (56 mg, 0.16 mmol). Product was obtained as an orange solid (60 mg, 0.080 mmol, 50% yield). 1H NMR (CD3OD, 400 MHz): δ 8.38 (1H, d, J = 16.0 Hz), 8.04 (2H, d, J = 8.8 Hz), 7.74 (2H, m), 7.60 (2H, m), 7.49 (1H, d, J = 16.0 Hz), 7.44 (2H, d, J = 7.2 Hz), 7.18 (2H, d, J = 8.8 Hz), 7.04 (2H, d, J = 7.2 Hz), 5.24 (2H, s), 4.12 (3H, s), 1.82 (6H, s), 1.38 (12H, s). HRFAB-MS: calculated for [M+] 494.29, found 494.29.

2,5-Dioxopyrrolidin-1-yl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (22). To a 20-mL vial equipped with a small magnetic stir bar was added (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol (720 mg, 3.07 mmol), bis(2,5-dioxopyrrolidin-1-yl)carbonate (787 mg, 3.07 mmol), DMAP (30 mg, 0.24 mmol), and CH2Cl2 (5 mL). The reaction mixture was stirred for 3 h at room temperature. Solvent was removed by a rotary evaporator. Purification with flash column chromatography (4:1, CH2Cl2/EtOAc) gave product as a white solid (950 mg, 2.73 mmol, 89% yield). 1H NMR (300 MHz, CDCl3): δ 7.83 (2H, d, J = 8.1 Hz), 7.38 (2H, d, J = 8.1 Hz), 5.32 (2H, s), 2.83 (4H, s), 1.34 (12H, s).

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl 4-(hydroxymethyl)phenyl carbamate (23). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added (4-aminophenyl)methanol (35 mg, 0.29 mmol), 22 (100 mg, 0.29 mmol), NEt3 (100 uL), and THF (5 mL). The reaction mixture was heated at 60 °C overnight. Solvent was removed by a rotary evaporator. Purification by flash column chromatography (3:1, CH2Cl2/EtOAc) gave product as colorless oil (90 mg, 0.23 mmol, 81% yield). 1H NMR (CDCl3, 300 MHz): δ 7.81 (2H, d, J = 8.1 Hz), 7.39 (2H, d, J = 8.1 Hz), 7.36 (2H, d, J = 8.7 Hz), 7.29 (2H, d, J = 8.7 Hz), 6.67 (1H, s-br), 5.21 (2H, s), 4.63 (2H, d, J = 8.0 Hz), 1.33 (12H, s).

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl 4-formylphenylcarbamate (24). To a 20-mL vial equipped with a small magnetic stir bar was added 23 (90 mg, 0.23 mmol), pyridinium chlorochromate (75 mg, 0.34 mmol) and CH2Cl2 (5 mL). The reaction was stirred at room temperature for 3 h. Insoluble solid was removed by vacuum filtration. The filtrate was concentrated. Purification with flash column chromatography (1:1, hexane/EtOAc) gave product as a white solid (75 mg, 0.20 mmol, 87% yield). 1H NMR (CDCl3, 400 MHz): δ 9.91 (1H, s), 7.85 (2H, d, J = 8.8 Hz), 7.82 (2H, d, J = 8.0 Hz), 7.56 (2H, d, J = 8.8 Hz), 7.41 (2H, d, J = 8.0 Hz), 6.95 (1H, s-br), 5.24 (2H, s), 1.35 (12H, s).

(E)-1,3,3-Trimethyl-2-(4-((4-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)carbonylamino)styryl)-3H-indolium iodide (25). Reaction mixture: 14 (20 mg, 0.066 mmol), 24 (25 mg, 0.065 mmol). Product was obtained as an orange solid (15 mg, 0.022 mmol, 34% yield). 1H NMR (CDCl3, 400 MHz): δ 8.15 (1H, d, J = 16.0 Hz), 8.11 (2H, d, J = 8.8 Hz), 7.83 (2H, d, J = 8.0 Hz), 7.68 (1H, d, J = 16.0 Hz), 7.66 (2H, d, J = 8.8 Hz), 7.60-7.55 (4H, m), 7.41 (2H, d, J = 8.0 Hz), 7.14 (1H, s), 5.26 (2H, s), 4.40 (3H, s), 1.86 (6H, s), 1.37 (12H, s). HRFAB-MS: calculated for [M+] 537.29, found 537.29.

(E)-5-(Methoxycarbonyl)-1,3,3-trimethyl-2-(4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)carbonylamino)styryl)-3H-indolium iodide (26). Reaction mixture: 15 (15 mg, 0.066 mmol), 24 (25 mg, 0.065 mmol). Product was obtained as an orange solid (25 mg, 0.035 mmol, 54% yield). 1H NMR (CDCl3, 400 MHz): δ 8.23-8.28 (2H, m), 8.19 (1H, s), 7.94 (2H, d, J = 8.8 Hz), 7.75 (2H, d, J = 8.0 Hz), 7.65-7.70 (3H, m), 7.43 (1H, d, J =
(E)-1,3,3-Trimethyl-2-(4-nitrostyryl)-3H-indolium iodide (27) Reaction mixture: 14 (80 mg, 0.26 mmol), 4-nitrobenzaldehyde (40 mg, 0.26 mmol). Product was obtained as an orange solid (60 mg, 0.15 mmol, 57% yield). \(^1\)H NMR (DMSO-\(d_6\), 400 MHz): \(\delta\) 8.34 (1H, d, \(J = 16.4\) Hz), 8.32 (2H, d, \(J = 8.8\) Hz), 8.26 (2H, d, \(J = 8.8\) Hz), 7.84 (1H, d, \(J = 8.4\) Hz), 7.77 (1H, d, \(J = 8.4\) Hz), 7.70 (1H, d, \(J = 16.4\) Hz), 7.61 (2H, m), 2.47 (3H, s), 1.72 (6H, s). LC-MS: calculated for [M\(^+\)] 307.14, found 307.2.

(Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). Fluorescence quantum yield was measured in relative to the reference standard: fluorescein (Φ = 0.90 in 0.1N NaOH) or rhodamine B (Φ = 0.34 in PBS).15

**Fluorescence Imaging Experiments.** HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before imaging, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine in 24-wells plates. For labeling, cells were incubating in HEPES containing 5 µM probes, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red for 30 min at 37 °C. Cells were washed twice with HEPES buffer and further incubated for another 30 min in either a) HEPES buffer or b) HEPES buffer with 100 uM H2O2 before images were taken. Confocal fluorescence imaging studies were performed with a Zeiss LSM710 laser scanning microscope and a 40x objective lens. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO2 humidified atmosphere. Image analysis was performed in ImageJ (National Institute of Health).
Figures and Schemes

Scheme 1. Synthesis of quinolinium styryl probes.
Scheme 2. Synthesis of indolium styryl probes.
Scheme 3. Synthesis of indolium styryl probes with 4-(dihydroxyboryl)benzyloxycarbonyl (DobZ) protecting group.
Table 1. Spectroscopic properties of styryl dyes

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1. Measured in ethanol.
2. HEPES pH 7.4. Quantum yields are relative to fluorescein ($\Phi = 0.90$ in 0.1 N NaOH)
3. HEPES pH 7.4. Quantum yields are relative to rhodamine B ($\Phi = 0.34$ in PBS)
Figure 1. Reaction of 10 (5 µM in HEPES pH 7.4) with 1 mM H₂O₂, monitored by (a) absorption and (b) fluorescence emission spectra (λ<sub>exc</sub> = 400 nm). (c) Observed rate constant calculated from changes in absorbance at 450 nm, k<sub>obs</sub> = 5.3 x 10⁻³ s⁻¹.
Figure 2. Reaction of indolium styryl probes (5 µM in HEPES pH 7.4) with 1 mM H₂O₂, monitored by absorption and fluorescence emission spectra (λ<sub>exc</sub> = 514 nm).
Figure 3. Under pseudo-first-order conditions (5 µM dyes, 1 mM H₂O₂), observed rate constant, $k_{obs}$, is calculated from the changes in absorbance at 500 nm.
Figure 4. Reaction of DCDHF styryl dyes (5 µM in HEPES pH 7.4) with 1 mM H$_2$O$_2$, monitored by (a) absorption and (b) fluorescence emission spectra ($\lambda_{exc} = 543$ nm).
Figure 5. Confocal fluorescence images of H$_2$O$_2$ in live HEK293T cells. HEK293T cells were incubated with 5 µM 25, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red in HEPES for 30 min. Cells were wash twice with HEPES buffer. Cells were further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with 100 µM H$_2$O$_2$ before images were taken. Scale bar = 20 µm. (Hoechst, $\lambda_{\text{exc}}$ = 405 nm, collection window = 430 - 480 nm; 25, $\lambda_{\text{exc}}$ = 514 nm, collection window = 530 - 630 nm; MitoTracker Deep Red, $\lambda_{\text{exc}}$ = 633 nm, collection window = 650 - 700 nm)
Figure 6. Confocal fluorescence images of H$_2$O$_2$ in live HEK293T cells. HEK293T cells were incubated with 5 µM 26, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red in HEPES for 30 min. Cells were wash twice with HEPES buffer. Cells were further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with 100 µM H$_2$O$_2$ before images were taken. Scale bar = 20 µm. (Hoechst, $\lambda_{\text{exc}} = 405$ nm, collection window = 430 - 480 nm; 26, $\lambda_{\text{exc}} = 514$ nm, collection window = 530 - 630 nm; MitoTracker Deep Red, $\lambda_{\text{exc}} = 633$ nm, collection window = 650 - 700 nm)
References

Appendix 2
Attempts Toward Ratiometric Fluorescent Detection of Hydrogen Peroxide on BODIPY Platform
Synopsis

This appendix describes an attempt towards ratiometric fluorescent probes for H$_2$O$_2$ using 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY®) as a reporter. We anticipated a ratiometric change in fluorescence emission profiles of BODIPY dyes undergoing H$_2$O$_2$-mediated conversion of an electron-withdrawing boronate ester to an electron-donating phenol. We also envisioned a ratiometric H$_2$O$_2$ detector based on the difference in spectroscopic properties between the “open” and “closed” form of 3,5-diaryl BODIPY, in which the ring constraint from the intramolecular nucleophilic displacement was reported to facilitate an emission red-shift by more than 50 nm.$^{1,2}$

Results and Discussion

Styryl BODIPY was synthesized by condensation of bodipy 1 with benzoaldehyde (Scheme 1). In ethanol, 2 has an absorption maximum at 585 nm and an emission maximum at 590 nm (Figure 1a). With electron donating 4-hydroxystyryl, 3 has an absorption maximum at 590 nm, and an emission maximum at 600 nm (Figure 1b). The absorption and emission profiles of 2 and 3 in aqueous buffer are similar to that in ethanol, except very low fluorescence emissions were observed for both 2 and 3. Most styryl BODIPY dyes reported in the literature are tested in organic solvents, or in a mixture of polar solvent and water.$^{3-6}$ Hydrophilic styryl BODIPY, with conjugated dipeptide, were reported to have 30-50% lower quantum yield in 0.1% TritonX-100 compared to methanol.$^7$ Together with the very small shift in emission maxima between 2 and 3 (10 nm), this scaffold of styryl BODIPY was not further pursued.

We sought to increase the fluorescence emission shift between the boronate-form and phenol-form of BODIPY by adding conformational strain (Scheme 2). We envisioned a BODIPY probe with arylboronate moieties, of which reaction with H$_2$O$_2$ give the uncaged phenol groups that can subsequently undergo nucleophilic replacement of BF$_2$ core to form a cyclic BODIPY. Diaryl BODIPY was synthesized following the standard protocol of TFA-catalyzed condensation of benzaldehyde with aryl pyrrole. 2-(2-bromophenyl)pyrrole can be prepared in high yield from a Au(I)-catalyzed intramolecular acetylenic Schmidt reaction.$^{8,9}$ 2-(2-Methoxyphenyl)pyrrole was prepared by palladium-catalyzed cross-coupling of a pyrrole anion with 1-bromo-2-methoxybenzene. Oxidation with $p$-chloranil and subsequent reaction with BF$_3$·OEt$_2$ in the presence of (i-Pr)$_2$NEt in CH$_2$Cl$_2$ gave the fluorescent diaryl BODIPY derivative. Miyaura borylation$^{10}$ of 10 gave 12 in 20% yield. Treatment of 11 with BBr$_3$ provided the ring strained BODIPY 13 in 40% yield.

In MeOH, 13 has an emission maximum at 640 nm ($\Phi = 0.35$), which is red-shifted by 70 nm from 12 ($\lambda_{em} = 570$ nm, $\Phi = 0.12$), and 50 nm from 11 ($\lambda_{em} = 590$ nm, $\Phi = 0.08$) (Figure 2). Unfortunately, 13 is not fluorescent in buffered aqueous solution ($\Phi < 0.001$). Meanwhile, 12 aggregated in aqueous solution, which can be identified by a broad UV absorption$^{14a}$ and broad red-shifted emission bands at 600-700 nm.$^{11-13}$ After the solution of 12 is allowed to equilibrate at room temperature, the hydrolysis of pinacol ester to boronic acid facilitates formation of the monomer, observed in the emission spectrum, with formation of a peak at 560 nm (Figure 8), which corresponds to the emission of monomer 12 observed in MeOH (Figure 3). In a 2:1 HEPES:EtOH solution, both 12 and 13 are fluorescent with no excimer emission observed. Addition of H$_2$O$_2$ to the solution of 12 in 2:1 HEPES:EtOH gave no significant change in emission wavelength (Figure 4).
Keeping in mind the problem of probe aggregation in aqueous solution, we proceeded to synthesize mono-aryl BODIPY 17 and 19 (Scheme 3). In HEPES buffer, 17 and 19 showed an absorption maximum at 515 nm, with a corresponding emission maximum at 535 nm. BODIPY 17 and 19 showed good solubility in aqueous buffer with high quantum yield of 0.53 and 0.65, respectively. Addition of H$_2$O$_2$ to a solution of 17 and 19 resulted in decreased emission intensity. Reaction of 19 with H$_2$O$_2$ was also accompanied by a 10 nm red-shift in fluorescence emission (Figure 4). Data from the styryl BODIPY and diaryl BODIPY (11, 12) predicts the fluorescence emission maximum of phenol-form to be 10 nm red-shifted from that of boronate ester-form. Therefore, in the case of 17 and 19, the small shift in fluorescence emission spectra suggested that reaction with H$_2$O$_2$ only gave the “open” BODIPY with a phenol moiety with no formation of “closed” derivatives.

**Summary:** BODIPY 2 and 12 are not a suitable platform for a ratiometric H$_2$O$_2$ fluorescent probe because the difference in fluorescence emission between the boronate ester- and phenol-form is very small (10 nm). BODIPY dyes with extended aromatic Π conjugates exhibit more red-shifted fluorescence emission, but the dyes also tend to form non-fluorescent aggregate species in aqueous solution.

**Experimental Section**

**Materials and Methods.** All reactions were carried out under a dry nitrogen atmosphere. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. $^1$H NMR and $^1$C NMR spectra were collected in CDCl$_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using a Bruker AVQ-400 or AV300 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)styryl BODIPY (2). To a microwavable heavy-walled tube was added 1 (65 mg, 0.21 mmol), and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (194 mg, 0.85 mmol), toluene (1 mL), and piperazine (20 μL, 0.25 μmol). The microwave reaction was carried out with constant heating at 150 °C for 3 h. Solvent was removed under reduced pressure, the residue was purified by flash chromatography (CH$_2$Cl$_2$) to give a blue solid (5 mg, 0.01 mmol, 5% yield). $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.78 (2H, d, $J$ = 7.8 Hz), 7.65 (1H, d, $J$ = 16.5 Hz), 7.55 (2H, d, $J$ = 7.8 Hz), 7.25 (1H, d, $J$ = 16.5 Hz), 6.96 (1H, s), 2.67 (2H, q, $J$ = 7.5 Hz), 2.54 (3H, s), 2.39 (2H, q, $J$ = 7.5 Hz), 2.20 (3H, s), 2.18 (3H, s), 1.34 (12H, s), 1.22 (3H, t, $J$ = 7.5 Hz), 1.07 (3H, t, $J$ = 7.5 Hz). HRFAB-MS: calculated for [M+H]$^+$ 518.31, found 518.31.

(4-Hydroxy)styryl BODIPY (3). To a microwavable heavy-walled tube was added 1 (30 mg, 0.10 mmol), and 4-hydroxybenzaldehyde (48 mg, 0.39 mmol), toluene (1 mL), and piperazine (20 μL, 0.25 μmol). The microwave reaction was carried out with constant heating at 150 °C for 3 h. Solvent was removed under reduced pressure, the residue was purified by flash chromatography (CH$_2$Cl$_2$) to give a blue solid (2 mg, 0.005 mmol, 5% yield). $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.78 (2H, d, $J$ = 7.8 Hz), 7.65 (1H, d, $J$ = 16.5 Hz), 7.55 (2H, d, $J$ = 7.8 Hz), 7.25 (1H, d, $J$ = 16.5 Hz), 6.96 (1H, s), 2.67 (2H, q, $J$ = 7.5 Hz), 2.54 (3H, s), 2.39 (2H, q, $J$ = 7.5 Hz), 2.20 (3H, s), 2.18 (3H, s), 1.34 (12H, s), 1.22 (3H, t, $J$ = 7.5 Hz), 1.07 (3H, t, $J$ = 7.5 Hz). HRFAB-MS: calculated for [M+H]$^+$ 518.31, found 518.31.
300 MHz) δ 7.80 (1H, d, J = 16.5 Hz), 7.45 (2H, d, J = 8.4 Hz), 7.28 (2H, d, J = 16.5 Hz), 6.94 (1H, s), 6.82 (2H, d, J = 8.4 Hz), 2.65 (2H, q, J = 16.5 Hz), 7.45 (2H, d, J = 8.4 Hz), 2.53 (2H, q, J = 8.4 Hz), 2.19 (3H, s), 2.17 (3H, s), 1.20 (3H, q, J = 7.5 Hz), 1.06 (3H, q, J = 7.5 Hz). HRFAB-MS: calculated for [M+H] 408.22, found 408.22.

4-(2-Bromophenyl)but-3-yn-1-ol (4). Under N₂ inert atmosphere, a Schlenk flask was charged with 1-bromo-2-iodobenzene (0.70 mL, 9.3 mmol), PdCl₂(PPh₃)₂ (0.27 g, 5 mol%), CuI (73 mg, 5 mol%), NEt₃ (3.2 mL, 23 mmol), and MeCN (25 mL). Following this, but-3-yn-1-ol (1.0 mL, 7.8 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred for 1 h at room temperature, and quenched by the addition of saturated NH₄Cl solution (20 mL). Extraction with ether (3 x 20 mL) followed by concentration under reduced pressure gave the crude product as brown oil. Purification by flash column chromatography (4:1, hexane/EtOAc) gave 4 as a yellow oil (1.2 g, 5.5 mmol, 70% yield). ¹H NMR (400 MHz, CDCl₃): δ 2.75 (2H, t, J = 7.5 Hz), 3.86 (2H, t, J = 7.5 Hz), 7.15 (1H, t, J = 8.0 Hz), 7.25 (1H, t, J = 8.0 Hz), 7.44 (1H, d, J = 8.0 Hz), 7.57 (1H, d, J = 8.0 Hz).

4-(2-Bromophenyl)but-3-ynyl 4-methylbenzenesulfonate (5). To a solution of 4 (0.79 g, 3.5 mmol) in CH₂Cl₂ (10 mL) was added pyridine (0.56 mL, 7.0 mmol) and TsCl (1.0 g, 5.2 mmol). The reaction mixture was stirred at room temperature overnight. Water (10 mL) was then added. The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 x 10 mL). The organic phases were combined, dried over Na₂SO₄, and concentrated. Purification by flash chromatography (4:1 hexanes, EtOAc) gave 5 as a yellow oil (1.1 g, 3.0 mmol, 85% yield). ¹H NMR (CDCl₃, 300 MHz): δ 7.81 (2H, d, J = 8.1 Hz), 7.54 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.35 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.32 (2H, d, J = 8.1 Hz), 7.22 (1H, dt, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.15 (1H, dt, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 4.21 (2H, t, J = 7.2 Hz), 2.83 (2H, t, J = 7.2 Hz), 2.40 (3H, s).

1-(4-Azidobut-1-ynyl)-2-bromobenzene (6). To the solution of 5 (0.76 g, 2.0 mmol) in DMSO (5 mL) was added NaN₃ (195 mg, 3.0 mmol). The reaction mixture was stirred overnight at room temperature. Water (10 mL) was added, and the mixture was extracted with Et₂O (3 x 10 mL). The organic layer was concentrated and purified by flash chromatography (4:1, hexane/EtOAc) to give 5 as a colorless oil (0.39 g, 1.6 mmol, 78%). ¹H NMR (CDCl₃, 300 MHz): δ 7.49 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.43 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.24 (1H, dt, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 7.14 (1H, dt, J₁ = 7.5 Hz, J₂ = 1.8 Hz), 3.52 (2H, t, J = 7.5 Hz), 2.76 (2H, t, J = 7.5 Hz).

2-(2-Bromophenyl)-1H-pyrrole (7). This compound was prepared by modification of a literature procedure. To a solution of 6 (0.22 g, 1.0 mmol) in CH₂Cl₂ (5 mL) was added (AuCl)₂(dppm) (42 mg, 5 mol%), and AgSbF₆ (8 mg, 2.5 mol%) in the respected order. The reaction mixture was stirred for 0.5 h at room temperature, and another 5 mol % of (AuCl)₂(dppm) and 2.5 mol % of AgSbF₆ was added. After stirring for another 0.5 h, the reaction mixture was filtered through basic alumina. The filtrate was concentrated and dried to give analytically pure product as a yellow oil (0.19 g, 0.84 mmol, 84% yield). ¹H NMR (CDCl₃, 300 MHz): δ 9.00 (1H, s-br), 7.60 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.51 (1H, dd, 1H, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.31 (1H, dt, 1H, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.09 (1H, dt, 1H, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 6.92 (1H, m), 6.56 (1H, m), 6.30 (1H, dd, J₁ = 6.3 Hz, J₂ = 2.7 Hz).

2-(2-Bromophenyl)-5-((1Z)-5-(2-bromophenyl)-2H-pyrrol-2-ylidene)(phenyl)methyl)-1H-pyrrole (8). Benzaldehyde (45 mg, 0.42 mmol) and 7 (180 mg, 0.84 mmol) were dissolved in nitrogen-flushed dry CH₂Cl₂. One drop of trifluoroacetic acid (TFA) was added through a syringe under nitrogen and the mixture was stirred overnight at room temperature. A
solution of p-chloranil (100 mg, 0.42 mmol) in dry CH₂Cl₂ was added. The reaction mixture was stirred for 0.5 h, quenched with water, and extracted with CH₂Cl₂. The organic phases were concentrated and dried. The crude product was purified by flash chromatography (Al₂O₃ basic, 4:1, hexane/EtOAc) to give product as a deep red solid (0.19 g, 0.36 mmol, 86% yield). ¹H NMR (CDCl₃ 300 MHz): δ 13.50 (1H, s-br), 7.77 (2H, d, J = 7.5 Hz), 7.66 (2H, d, J = 7.5 Hz), 7.57-7.60 (2H, m), 7.38 (2H, t, J = 7.5 Hz), 7.20 (2H, t, J = 7.5 Hz), 6.99 (2H, d, J = 7.5 Hz), 6.88 (2H, d, J = 4.2 Hz), 6.67 (2H, d, J = 4.2 Hz).

2-(2-Methoxyphenyl)-5-((1Z)-(5-(2-methoxyphenyl)-2H-pyrrol-2-ylidene)(phenyl)methyl)-1H-pyrrole (9). Benzaldehyde (50 mg, 0.47 mmol) and 2-(2-bromophenyl)-1H-pyrrole (160 mg, 0.94 mmol) were dissolved in nitrogen-flushed dry CH₂Cl₂. One drop of trifluoroacetic acid (TFA) was added through a syringe under nitrogen and the mixture was stirred overnight at room temperature. A solution of p-chloranil (230 mg, 0.47 mmol) in dry CH₂Cl₂ was added. The reaction mixture was stirred for 0.5 h, quenched with water, and extracted with CH₂Cl₂. The organic phases were concentrated and dried. The crude product was purified by flash column chromatography (Al₂O₃ basic, 4:1, hexane/EtOAc) to give product as a deep purple solid (98 mg, 0.23 mmol, 48% yield). ¹H NMR (CDCl₃, 300 MHz): δ 13.74 (1H, s-br), 8.05 (2H, d, J = 7.5 Hz), 7.52-7.57 (2H, m), 7.43-7.47 (3H, m), 7.37 (2H, t, J = 7.5 Hz), 7.02 (2H, t, J = 7.5 Hz), 6.99 (2H, d, J = 7.5 Hz), 6.92 (2H, d, J = 4.2 Hz), 6.61 (2H, d, J = 4.2 Hz), 3.85 (6H, s).

4,4-Difluoro-3,5-bis-(4-bromophenyl)-4-bora-3a,4a-diaza-s-indacene (10). To a solution of dipyrromethene 8 (190 mg, 0.36 mmol) in CH₂Cl₂ was added (i-Pr)₂NEt (0.22 mL, 1.8 mmol). The solution was stirred for 30 min, after which BF₃·OEt₂ (0.34 mL, 3.6 mmol) was added, and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by gradient flash column chromatography with CH₂Cl₂ and a gradually increasing amount of MeOH (up to 10%) to give a bright pink solid (0.17 g, 0.29 mmol, 78% yield). ¹H NMR (CDCl₃, 300 MHz): δ 7.2-7.57 (9H, m), 7.30 (2H, t, J = 7.0 Hz), 7.20 (2H, t, J = 7.0 Hz), 6.92 (2H, d, J = 4.5 Hz), 6.57 (2H, d, J = 4.5 Hz). HRFAB-MS: calculated for [M⁺] 575.98, found 575.99.

4,4-Difluoro-3,5-bis-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene (11). To a solution of dipyrromethene 9 (98 mg, 0.23 mmol) in CH₂Cl₂ was added (i-Pr)₂NEt (0.39 mL, 2.2 mmol). The solution was stirred for 30 min, after which BF₃·OEt₂ (0.57 mL, 4.5 mmol) was added, and the reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by gradient flash column chromatography with CH₂Cl₂ and a gradually increasing amount of MeOH (up to 10%) to give a bright pink solid (80 mg, 0.16 mmol, 72%). ¹H NMR (CDCl₃, 300 MHz): δ 7.72 (2H, d, J = 7.5 Hz), 7.57-7.63 (2H, m), 7.48-7.56 (3H, m), 7.33 (2H, d, J = 7.5 Hz), 6.98 (2H, t, J = 7.5 Hz), 6.91 (2H, d, J = 7.5 Hz), 6.83 (2H, d, J = 3.9 Hz), 6.58 (2H, d, J = 3.9 Hz), 3.76 (6H, s). HRFAB-MS: calculated for [M⁺] 480.18, found 480.18.

4,4-Difluoro-3,5-bis-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-4-bora-3a,4a-diaza-s-indacene (12). An oven dried Schlenk flask was charged with 10 (32 mg, 0.055 mmol), KOAc (16 mg, 0.16 mmol), bis(pinacolato) diboron (84 mg, 0.33 mmol), Pd(dppf)Cl₂ (10 mg, 25 mol%), and anhydrous DMF (2 mL). The reaction mixture was heated at 100 °C under N₂ for 4 h, following which the DMF was removed under reduced pressure. The residue was diluted with CH₂Cl₂, washed with water, concentrated and dried. Purification by flash chromatography (3:1, CH₂Cl₂/hexane) gave 12 as a purple solid (10 mg, 0.014 mmol, 27%). ¹H NMR (CDCl₃, 300 MHz): δ 7.59-7.63 (6H, m), 7.54-7.57 (3H, m), 7.34-7.38 (6H, m), 7.28(2H, s-br), 1.15
(2H, s). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 164.7, 139.5, 136.3, 134.2, 132.7, 130.5, 128.9, 128.3, 127.0, 83.4, 25.0. HRFAB-MS: calculated for [M$^+$] 672.33, found: 672.34.

“Closed” 3,5-diaryl BODIPY (13). A solution of 11 (60 mg, 0.12 mmol) in CH$_2$Cl$_2$ (5 mL) under N$_2$ atmosphere was cooled to 0 °C. Next, BBr$_3$ (0.10 mL, 1.1 mmol) was added dropwise, after which the solution was warmed to 25 °C and stirred for 5 h. The reaction mixture was filtered through Celite, concentrated and purified by flash chromatography (4:1, hexane/EtOAc) to give product as a blue solid (20 mg, 0.048 mmol, 40%). $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.78 (2H, dd, $J_1 = 6.9$ Hz, $J_2 = 1.5$ Hz), 7.74 (2H, m), 7.53-7.60 (3H, m), 7.34 (2H, dt, $J_1 = 6.9$ Hz, $J_2 = 1.5$ Hz), 7.08 (2H, d, $J = 4.5$ Hz), 7.05 (2H, t, $J = 6.9$ Hz), 6.97 (2H, d, $J = 6.9$ Hz), 6.90 (2H, d, $J = 4.5$ Hz). $^{13}$C NMR (CDCl$_3$, 400 MHz): δ 154.1, 149.9, 134.3, 133.8, 132.2, 130.5, 130.1, 128.7, 120.4, 119.7, 116.5. HRFAB-MS: calculated for [M$^+$] 412.14, found: 412.14.

5-(2-Bromophenyl)-1H-pyrrole-2-carbaldehyde (14). A two-necked round-bottomed flask equipped with a magnetic stir bar was charged with solution of 7 (140 mg, 0.67 mmol) in dichloroethane (5 mL), then put under N$_2$ atmosphere and cooled in an ice bath. To the reaction mixture was added DMF (50 uL, 0.94 mmol), followed by POCl$_3$ (90 µL, 0.94 mmol). After 1 h of stirring in an ice bath, saturated NaOH (0.5 mL) was added slowly to the reaction mixture, and the solution was warmed up to room temperature over 1 h. Extraction with CH$_2$Cl$_2$ and purification with flash column chromatography (4:1, hexane/EtOAc) gave product as a white solid (73 mg, 0.29 mmol, 46% yield). $^1$H NMR (CDCl$_3$, 300 MHz): δ 10.20 (1H, s-br) 9.51 (1H, s), 7.64 (1H, d, $J = 7.8$ Hz), 7.51 (1H, t, $J = 7.8$ Hz), 7.34 (1H, t, $J = 7.8$ Hz), 7.19 (1H, t, $J = 7.8$ Hz), 7.01 (1H, dd, $J_1 = 3.9$ Hz, $J_2 = 2.1$ Hz), 6.65 (1H, dd, $J_1 = 3.9$ Hz, $J_2 = 2.1$ Hz).

4,4-Difluoro-(4-bromophenyl) BODIPY (16). A two-necked round-bottomed flask equipped with a magnetic stir bar was charged with 14 (73 mg, 0.29 mmol), 3-ethyl-2,3-dimethyl-1H-pyrrole (39 µL, 0.29 mmol) and CH$_2$Cl$_2$ (5 mL). The reaction flask was flushed with N$_2$ atmosphere, followed by addition of POCl$_3$ (40 µL, 0.44 mmol). The reaction was stirred at room temperature for 1 h, after that volatile solvent was removed by a rotary evaporator to give 15 as a red solid. A round-bottomed flask was charged with solution of 15 in CH$_2$Cl$_2$, followed by (iPr)$_2$NEt (180 µL, 1.46 mmol). After stirring for 15 min, BF$_3$·OEt$_2$ (280 µL, 2.92 mmol) was added to the reaction mixture and the reaction was continued for 3 h. Solvent was removed in vacuo. Purification by flash column chromatography (4:1, hexane/EtOAc) gave product as a red solid (58 mg, 0.14 mmol, 29% yield). $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.66 (1H, d, $J = 7.8$ Hz), 7.62 (1H, d, $J = 7.8$ Hz), 7.38 (1H, t, $J = 7.8$ Hz), 7.23 (1H, t, $J = 7.8$ Hz), 7.13 (1H, s), 6.91 (1H, d, $J = 3.9$ Hz), 6.42 (1H, d, $J = 3.9$ Hz), 2.52 (3H, s), 2.37 (2H, q, $J = 7.5$ Hz), 2.18 (3H, s), 1.04 (3H, t, $J = 7.5$ Hz).

4,4-Difluoro-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl) BODIPY (17). A oven dried Schlenk flask was charged with 16 (58 mg, 0.14 mmol), KOAc (71 mg, 0.72 mmol), bis(pinacolato)diboron (368 mg, 1.44 mmol), Pd(dppf)Cl$_2$ (31 mg, 0.04 mmol) and anhydrous DMF (2 mL). The reaction mixture was heated at 100 °C under N$_2$ for 8 h, following which DMF was removed under reduced pressure. The residue was diluted with CH$_2$Cl$_2$, washed with water, concentrated and dried. Purification by flash chromatography (4:1, hexane/EtOAc) gave 17 as a red solid (27 mg, 0.060 mmol, 43%). $^1$H NMR (CDCl$_3$, 300 MHz): δ 7.63 (1H, d, $J = 6.5$ Hz), 7.63 (1H, d, $J = 6.5$ Hz), 7.37 (1H, d, $J = 3.9$ Hz), 7.26 (1H, d, $J = 3.9$ Hz), 7.09 (1H, s), 2.46 (2H, s), 2.36 (2H, q, $J = 7.5$ Hz), 2.19 (3H, s), 1.27 (12H, s), 1.04 (3H, t, $J = 7.5$ Hz). HRFAB-MS: calculated for [M$^+$] 450.25, found 450.25.
4,4-Dimethoxy-(4-bromophenyl) BODIPY (18). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added solution of 16 (25 mg, 0.06 mmol) in CH₂Cl₂ (5 mL), followed by AlCl₃ (80 mg, 0.06 mmol). The reaction mixture was heated at 40 °C for 15 min. MeOH (3 mL) was added to the reaction flask, then the reaction was continued for 30 min. Solvent was removed by a rotary evaporator. Purification by flash column chromatography gave product as a red solid (12 mg, 0.028 mmol, 45% yield). ¹H NMR (CDCl₃, 300 MHz): δ 7.65 (1H, d, J = 7.8 Hz), 7.58 (1H, d, J = 7.8 Hz), 7.34 (1H, t, J = 7.8 Hz), 7.18 (1H, t, J = 7.8 Hz), 7.09 (1H, s), 6.90 (1H, d, J = 3.9 Hz), 6.45 (1H, d, J = 3.9 Hz), 2.85 (6H, s), 2.45 (3H, s), 2.38 (2H, q, J = 7.5 Hz), 2.16 (3H, s), 1.06 (3H, t, J = 7.5 Hz). LRFAB-MS: calculated for [M⁺] 426.11, found: 426.1.

4,4-Dimethoxy-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl) BODIPY (19). A dry Schlenk flask was charged with 18 (65 mg, 0.15 mmol), KOAc (72 mg, 0.74 mmol), bis(pinacolato)diboron (375 mg, 1.48 mmol), Pd(dppf)Cl₂ (32 mg, 0.04 mmol) and anhydrous DMF (2 mL). The reaction mixture was heated at 100 °C under N₂ for 8 h, following which DMF was removed under reduced pressure. The residue was diluted with CH₂Cl₂, washed with water, concentrated and dried. Purification by flash chromatography (1:1 hexane/EtOAc) gave 18 as a red solid (13 mg, 0.027 mmol, 18%). ¹H NMR (CDCl₃, 300 MHz) δ:7.60-7.66 (2H, m), 7.33 (2H, d, J = 7.5 Hz), 7.30 (1H, s), 7.28 (1H, s), 7.05 (1H, s), 2.76 (6H, s), 2.47 (3H, s), 2.38 (2H, q, J = 7.5 Hz), 2.20 (3H, s), 1.18 (12H, s), 1.06 (3H, t, J = 7.5 Hz). LRFAB-MS: calculated for [M⁺] 474.29, found: 474.3.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). Fluorescence quantum yield was measured in relative to the reference standard: fluorescein (Φ = 0.90 in 0.1N NaOH)¹⁴ or rhodamine 101 (Φ = 1.00 in EtOH).¹⁵
Scheme 1. Synthesis of styryl BODIPY 2 and 3.
**Table 1.** Spectroscopic properties of BODIPY dyes.

<table>
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<th>Compound</th>
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<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Phi$</th>
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<td>590</td>
<td>0.05</td>
<td>1</td>
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<td>3</td>
<td>590</td>
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<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
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<td>590</td>
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<td>1</td>
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<tr>
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<td>570</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
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<td>640</td>
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<td>1</td>
</tr>
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<td>535</td>
<td>0.53</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>515</td>
<td>535</td>
<td>0.65</td>
<td>2</td>
</tr>
</tbody>
</table>

1. Measured in MeOH. Quantum yields relative to Rhodamine 101, $\Phi = 1.00$ in EtOH.
2. Measured in HEPES buffer, pH 7.0. Quantum yields relative to Fluorescein, $\Phi = 0.90$ in 0.1 N NaOH.
Figure 1. Normalized absorption and fluorescence emission spectra of styryl BODIPY (a) 2 and (b) 3 in methanol.
Figure 2. Normalized absorption and fluorescence emission spectra of BODIPY (a) 12, (b) 11 and (c) 13 in methanol.
Figure 3. (a) Fluorescence emission spectra of BODIPY 12 (5 µM in HEPES buffer pH 7.4). $\lambda_{\text{exc}} = 530$ nm. (b) Reaction of BODIPY 12 (5 µM in 2:1 HEPES buffer pH 7.4:EtOH) with 150 µM H$_2$O$_2$, monitored by fluorescence emission spectra ($\lambda_{\text{exc}} = 530$ nm).
Figure 4. Reaction of BODIPY (a) 17 and (b) 19 with 150 µM H₂O₂, in HEPES buffer pH 7.0. ($\lambda_{exc} = 530$ nm)
Appendix 3
Green and Yellow Fluorescent Probes for Hydrogen Peroxide using
Rhodamine Fluorophores
**Synopsis**

This appendix describes synthesis, spectroscopic characterization and confocal fluorescence microscopy of Rhodamine-based fluorescent probes for hydrogen peroxide. Chemoselective triggers, \( p \)-dihydroxyborylbenzylxy carbonyl (Dobz) groups, have been utilized in the synthesis of ratiometric fluorescent probes.\(^1\) Here, we sought to expand the utility of Dobz group by incorporating it onto a family of Rhodamine fluorophores, which is best known for its high extinction coefficient, high quantum yield, high photostability, pH insensitivity and relatively long emission wavelength.

**Results and Discussion**

In this study, we have synthesized Rho110-Dobz by reaction of commercially available Rhodamine 110 (Rho110) with benzyl \( N \)-succinimidyl carbonate 1. Following the report by Raines et. al. on the usefulness of a urea moiety as a handle for target-molecule conjugation to Rhodamine with relatively low suppression on the extinction coefficient and quantum yield,\(^2\) we also synthesized the \( N \)-carbonylmorpholine derivative of Rho110-Dobz as shown in Scheme 1. In tuning the probe toward longer emission wavelength, we synthesized asymmetric Rhodamine Rho110B using a two step protocol. First, half rhodol 5 was formed by condensation of phthalic anhydride with 3-(diethylamino)phenol in toluene at refluxing temperature. Stirring mixture of half rhodol 5 and 3-amino phenol in sulfuric acid for 2 days at room temperature gave mixture of fluorescent products.\(^5\) Analysis of the reaction mixture indicated that the major products were the symmetric Rhodamine 110 and Rhodamine B, with formation of Rho110B as a minor product (6.8%). Rho110B has an emission maximum at 555 nm in aqueous solution, which is an intermediate point between the emission maximum of Rhodamine 110 (\( \lambda_{em} = 525 \) nm) and Rhodamine B (\( \lambda_{em} = 570 \) nm).\(^6\)

In a HEPES buffer solution, Rho110-Dobz is moderately fluorescent (\( \Phi = 0.21 \)) with an emission maximum at 530 nm, while Rho110M-Dobz and Rho110B-Dobz are non-fluorescent. Reaction of Rho110-Dobz with \( \text{H}_2\text{O}_2 \) led to a dramatic increase in absorption and fluorescence emission which corresponds to the formation of Rho110 (Figure 1). Similarly, Rho110M-Dobz (Figure 2) and Rho110B-Dobz (Figure 3) also showed a fluorescent emission turn-on upon reaction with \( \text{H}_2\text{O}_2 \). Under the pseudo-first-order conditions (1 \( \mu \)M dye, 1 mM \( \text{H}_2\text{O}_2 \)), the observed rate constant of \( \text{H}_2\text{O}_2 \)-mediated deprotection of Dobz group was fastest with Rho110-Dobz, and slowest with Rho110M-Dobz. The slow reaction rate of Rho110M-Dobz can be explained by the change in equilibrium between the closed lactone form and the opened zwitterionic form of the dye. The urea moiety prefers the closed lactone form, whereas the deprotection of Dobz group is driven by resonance in the zwitterionic form.

We proceeded to test the application of Rho110-Dobz and Rho110B-Dobz in live cell imaging. Rho110-Dobz and Rho110B-Dobz are membrane permeable with an affinity for mitochondria due to their lipophilic cation character. HEK293T cells labeled with Rho110-Dobz showed a two-fold fluorescence enhancement in response to the addition of 100 \( \mu \)M \( \text{H}_2\text{O}_2 \). Likewise, cells labeled with Rho110B-Dobz showed a 1.3 fold fluorescence enhancement in response to oxidative assault. Rho110-Dobz and Rho110B-Dobz are the complement addition to the series of \( \text{H}_2\text{O}_2 \) probes with direct borylation of the xanthone ring.\(^7\)

**Summary:** Although successful for \( \text{H}_2\text{O}_2 \) detection in stressed cells, this Rhodamine-based \( \text{H}_2\text{O}_2 \) probes with Dobz group are slower than probes with direct borylation of the xanthone ring.
This strategy of the Dobz group trigger, however, can be useful in the case where the aniline moiety is a crucial component for desirable optical properties of the reporter fluorophores.

**Experimental Section**

**Materials and Methods.** MitoTracker Deep Red and Hoechst 33342 were obtained from Invitrogen (Carlsbad, CA) Rho110 was obtained from Exciton (Dayton, OH). Other chemicals were obtained from Sigma Aldrich and used as received. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. $^1$H NMR spectra were collected in CDCl$_3$, or CD$_3$OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using a Bruker AVQ-400 or AV300 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. Low-resolution mass spectral analyses were carried out using Agilent Technology 1200 series with 6130 Quadrupole LC/MS (Santa Clara, CA). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

**2,5-Dioxopyrrolidin-1-yl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (1).** To a 20-mL vial equipped with a small magnetic stir bar was added (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol (720 mg, 3.07 mmol), bis(2,5-dioxopyrrolidin-1-yl)carbonate (787 mg, 3.07 mmol), DMAP (30 mg, 0.24 mmol), and CH$_2$Cl$_2$ (5 mL). The reaction mixture was stirred for 3 h at room temperature. Solvent was removed by a rotary evaporator. Purification with flash column chromatography (4:1, CH$_2$Cl$_2$/EtOAc) gave the product as white solid (950 mg, 2.73 mmol, 89% yield). $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 7.83 (2H, d, $J$ = 8.1 Hz), 7.38 (2H, d, $J$ = 8.1 Hz), 5.32 (2H, s), 2.83 (4H, s), 1.34 (12H, s).

**Rho110-Dobz (2).** To a solution of Rhodamine 110 (112 mg, 0.30 mmol) in DMF (1.5 mL) was added NaH (23 mg, 0.60 mmol). The reaction mixture was stirred at room temperature under N$_2$ for 1 h. Compound 1 (112 mg, 0.30 mmol) was then added, and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. Purification by flash chromatography (4:1, CH$_2$Cl$_2$/EtOAc) gave product as white solid (950 mg, 2.73 mmol, 89% yield). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 8.01 (1H, d, $J$ = 7.2 Hz), 7.80 (1H, d, $J$ = 8.0 Hz), 7.67 (1H, t, $J$ = 7.2 Hz), 7.61 (1H, t, $J$ = 7.2 Hz), 7.52 (1H, s-br), 7.37 (2H, d, $J$ = 8.0 Hz), 7.16 (1H, d, $J$ = 7.2 Hz), 6.87 (1H, d, $J$ = 8.2 Hz, $J_2$ = 2.4 Hz), 6.68 (1H, d, $J$ = 8.2 Hz), 6.66 (1H, s), 6.55 (1H, d, $J$ = 8.2 Hz), 6.53 (1H, d, $J$ = 2.4 Hz), 6.34 (1H, d, $J$ = 8.2 Hz, $J_2$ = 2.4 Hz), 5.18 (2H, s), 1.32 (12H, s). HRFAB-MS: calculated for [MH$^+$] 591.22, found 591.23.

**Rho110M (3).** was synthesized following a literature protocol.$^4$ $^1$H NMR (CD$_3$OD, 300 MHz): $\delta$ 7.99 (1H, d, $J$ = 7.2 Hz), 7.68-7.74 (2H, m), 7.52 (1H, d, $J$ = 2.1 Hz), 7.19 (1H, d, $J$ = 7.2 Hz), 7.03 (1H, dd, $J_1$ = 8.7 Hz, $J_2$ = 2.1 Hz), 6.64 (1H, d, $J$ = 8.7 Hz), 6.55 (1H, d, $J$ = 2.1 Hz), 6.49 (1H, d, $J$ = 8.4 Hz), 6.42 (1H, dd, $J_1$ = 8.4 Hz, $J_2$ = 2.1 Hz), 3.69 (4H, t, $J$ = 4.5 Hz), 3.50 (4H, t, $J$ = 4.5 Hz).

**Rho110M-Dobz (4).** To a solution of Rhodamine 110M (25 mg, 0.056 mmol) in DMF (1.5 mL) was added NaH (6 mg, 0.015 mmol). The reaction mixture was stirred at room temperature under N$_2$ for 1 h. Compound 1 (25 mg, 0.072 mmol) was then added, and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. Purification by flash chromatography (1:1, CH$_2$Cl$_2$/EtOAc) gave product as a pale yellow solid (10 mg, 0.014 mmol, 24% yield). $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.97 (1H, d, $J$ = 7.2 Hz), 7.79 (2H, d, $J$ = 8.0 Hz), 7.72 (1H, d, $J$ = 7.6 Hz), 7.57-7.65 (2H, m), 7.41 (1H, s), 7.37 (1H, d, $J$ = 8.0 Hz), 7.25 (1H, s), 7.08 (1H, d, $J$ = 7.2 Hz), 7.01 (1H, d, $J$ = 8.4 Hz), 6.94 (1H, d, $J$ = 8.0 Hz).
= 8.4 Hz), 6.67 (1H, d, J = 8.4 Hz), 6.60 (1H, d, J = 8.4 Hz), 5.19 (2H, s), 3.70 (4H, t, J = 4.4 Hz), 3.48 (4H, t, J = 4.4 Hz), 1.34 (12H, s).

2-(4-Diethylamino-2-hydroxybenzoyl)benzoic acid (5). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added 3-(diethylamino)phenol (1.0 g, 6.0 mmol), phthalic anhydride (900 mg, 6.0 mmol), and toluene (25 mL). The mixture was refluxed overnight to give pink-purple solution. Cooled to room temperature gave pale pink precipitate that was collected by vacuum filtration, washed with cold methanol, and air dried (1.1 g, 3.2 mmol, 53% yield). 1H NMR (400 MHz, CD3OD): δ 13.04 (1H, s -br), 12.53 (1H, s), 7.93 (1H, d, J = 7.6 Hz), 7.65 (1H, t, J = 7.6 Hz), 7.58 (1H, t, J = 7.6 Hz), 7.35 (1H, d, J = 7.6 Hz), 6.76 (1H, d, J = 8.2 Hz), 6.15 (1H, d, J = 8.2 Hz), 6.04 (1H, s), 3.34 (4H, q, J = 6.4 Hz), 1.06 (6H, t, J = 6.4 Hz).

Rho110B (6). A solution of 3-aminophenol (0.19 g, 1.7 mmol) and 5 (0.50 g, 1.7 mmol) in concentrated sulfuric acid (3 mL) was stirred at room temperature for 2 days. The reaction mixture was poured into ice water (50 mL) and the red sticky solid was collected by filtration. The residue was re-suspended in 1 M NaOH (50 mL). Red solid was collected by vacuum filtration and air dried. Purification with flash chromatography (100:20:3, CH2Cl2/MeOH/acOH) gave 6 as a bright pink solid (45 mg, 0.12 mmol, 7% yield). 1H NMR (CD3OD, 400 MHz): δ 8.08 (1H, d, J = 7.2 Hz), 7.63 (1H, t, J = 7.2 Hz), 7.58 (1H, t, J = 7.2 Hz), 7.41 (1H, d, J = 9.6 Hz), 7.21 (1H, t, J = 7.2 Hz), 7.15 (1H, d, J = 9.6 Hz), 6.95 (1H, d, J = 9.6 Hz), 6.86 (1H, m), 3.61 (4H, q, J = 6.8 Hz), 1.25 (6H, t, J = 6.8 Hz). 13C NMR (400 MHz, CD3OD): δ 172.1, 161.8, 159.3, 158.3, 157.8, 155.3, 140.2, 131.8, 129.4, 129.3, 129.0, 128.9, 115.7, 113.8, 113.4, 113.3, 96.8, 95.5, 45.2, 11.3. HRFAB-MS: calculated for [MH+] 387.16, found: 387.17.

3-Methyl-1-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoyl)carbonyl)-1H-imidazol-3-ium trifluoromethanesulfonate (7). This compound was prepared by the modification of a literature procedure.8 To a solution of (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)methanol (300 mg, 1.3 mmol) and DMAP (31 mg, 0.25 mmol) in dry CH2Cl2 (5 mL) was added carbonyl–diimidazole (420 mg, 2.5 mmol). The reaction mixture was stirred at room temperature overnight, following which it was washed with water, saturated NaHCO3, and brine. The organic phases were dried, concentrated, and purified by flash chromatography (4:1, hexane/EtOAc) to give imidazole derivative as a white solid (0.17 g, 0.52 mmol, 40%). The solution of imidazole derivative obtained in the previous step in CH2Cl2 was cooled to 0 °C, and MeOTf (85 mg, 0.52 mmol) was added dropwise. After 30 min, the solution was diluted with Et2O (20 mL). The white precipitate that formed was collected by filtration, washed with Et2O, and dried (0.21 g, 0.42 mmol, 82% yield). 1H NMR (CDCl3, 300 MHz): δ 9.60 (1H, s), 7.83 (2H, d, J = 7.5 Hz), 7.71 (1H, s), 7.47 (2H, d, J = 7.5 Hz), 7.41 (1H, s), 5.53 (2H, s), 4.07 (3H, s), 1.26 (12H, s).

Rho110B-Dobz (8). To a solution of Rhodamine 110B (25 mg, 0.064 mmol) in DMF (1.5 mL) was added NaH (5 mg, 0.12 mmol). The reaction mixture was stirred at room temperature under N2 for 1 h. Compound 7 (35 mg, 0.071 mmol) was then added, and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. Purification by flash chromatography (1:1, CH2Cl2/EtOAc) gave product as a pale yellow solid (2 mg, 0.003 mmol, 4% yield). 1H NMR (CDCl3, 400 MHz): δ 9.60 (1H, s), 7.83 (2H, d, J = 7.5 Hz), 7.71 (1H, s), 7.47 (2H, d, J = 7.5 Hz), 7.41 (1H, s), 5.53 (2H, s), 4.07 (3H, s), 1.26 (12H, s).
Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ). Samples for absorption and fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). Fluorescein in 0.1 M NaOH (Φ = 0.90) was used as standard for quantum yield measurements.9

Fluorescence Imaging Experiments. HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before transfection, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine in a 24-wells culture. Labeling is achieved by incubating cells in HEPES containing 5 µM Dobz probes, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red for 30 min at 37 °C. Cells were washed twice with HEPES buffer and further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with 100 µM H2O2 before images were taken. Confocal fluorescence imaging studies were performed with a Zeiss LSM710 laser scanning microscope and a 40x objective lens. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO2 humidified atmosphere. Excitation of the Rho110-Dobz probe at 488 nm was carried out with an Argon laser and emission was collected using a 500 - 630 nm filter. Excitation of Rho110B-Dobz was carried out with a Helium-Neon 514 nm laser and emission was collected at 530 - 630 nm. Excitation of Hoechst 33342 was carried out using a 405 nm and emission was collected at 430 - 480 nm. Excitation of MitoTracker Deep Red was carried out using a 633 nm and emission was collected at 650 - 700 nm. Image analysis was performed in ImageJ (National Institute of Health).
Figures and Schemes

Scheme 1: Synthesis of Dobz derivatives of Rhodamine 110.
**Table 1**: Spectroscopic properties of Rhodamine dyes and their Dobz protected forms in HEPES buffer pH 7.4

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<th>Compound</th>
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<th>( \varepsilon ) (M(^{-1})cm(^{-1}))</th>
<th>( \lambda_{\text{em}} ) (nm)</th>
<th>( \Phi )</th>
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Figure 1. Reaction of Rho110-Dobz with 1 mM H$_2$O$_2$. (a) Absorption spectra at 0, 10, 20, 30, 40, 50, and 60 min. (b) Fluorescence emission spectra ($\lambda_{exc} = 488$ nm) at 0, 10, 20, 30, 40, 50, and 60 min. (c) Rate constant obtained from changes of the fluorescence emission at 520 nm, $k_{obs} = 1.24 \times 10^{-3}$ s$^{-1}$. 
Figure 2. Reaction of Rho110M-Dobz with 1 mM H₂O₂. (a) Absorption spectra at 0, 10, 20, 30, 40, 50, and 60 min. (b) Fluorescence emission spectra (λ exc = 488 nm) at 0, 10, 20, 30, 60, and 600 min. (c) Rate constant obtained from changes of the fluorescence emission at 520 nm, k obs = 0.11 x 10⁻³ s⁻¹.
Figure 3. Reaction of Rho110B-Dobz with 1 mM H$_2$O$_2$. (a) Absorption spectra at 0, 10, 20, 30, 40, 50, and 60 min. (b) Fluorescence emission spectra ($\lambda_{exc} = 514$ nm) at 0, 10, 20, 30, 40, 50, and 60 min. (c) Rate constant obtained from changes of the fluorescence emission at 540 nm, $k_{obs} = 0.61 \times 10^{-3}$ s$^{-1}$.
Figure 4. Confocal fluorescence images of H$_2$O$_2$ in live HEK293T cells. HEK293T cells were incubated with 5 µM Rho110-Dobz, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red in HEPES for 30 min. Cells were washed twice with HEPES buffer. Cells were further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with 100 µM H$_2$O$_2$ before images were taken. Scale bar = 20 µm. (Hoechst, $\lambda_{exc} = 405$ nm, collection window = 430 - 480 nm; Rho110-Dobz, $\lambda_{exc} = 488$ nm, collection window = 500 - 630 nm; MitoTracker Deep Red, $\lambda_{exc} = 633$ nm, collection window = 650 - 700 nm).
**Figure 5.** Confocal fluorescence images of H$_2$O$_2$ in live HEK293T cells. HEK293T cells were incubated with 5 µM Rho110B-Dobz, 1 µM Hoechst 33342, and 50 nM MitoTracker Deep Red in HEPES for 30 min. Cells were washed twice with HEPES buffer. Cells were further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with 100 µM H$_2$O$_2$ before images were taken. Scale bar = 20 µm. (Hoechst, $\lambda_{\text{exc}} = 405$ nm, collection window = 430 - 480 nm; Rho110B-Dobz, $\lambda_{\text{exc}} = 514$ nm, collection window = 530 - 630 nm; MitoTracker Deep Red: $\lambda_{\text{exc}} = 633$ nm, collection window = 650 - 700 nm).
References


Appendix 4
A Naphthalimide-Based Fluorescent Probe for Hydrogen Peroxide with
Pyridinone-Methide self-Immolative Linker
Synopsis

This appendix describes the synthesis and characterization of a napthalimide-based fluorescent probe with 1,6-pyridinone-methide as a self-immolative spacer linking the reporter fluorophore to the H₂O₂ responsive boronate ester trigger. We have previously reported the PL and PN series of ratiometric fluorescence reporters for H₂O₂, utilizing 1,6-quinone-methide elimination reaction. Although with success in detecting H₂O₂ at oxidative stress level, these probes exhibited slower response to H₂O₂ compared to the probes with one step detection mechanism. In the development of penicillin-G amidase (PAG) responsive dendrons, Shabat et al. found probes that utilize pyrimidone-methide elimination exhibited a faster response than their quinone-methide analogs. Based on this report, we designed a pyridinone-methide analog of PL probes with an anticipation that the new probe could exhibit a faster sensitivity for the detection of H₂O₂ in cellular signaling events.

Results and Discussion

We synthesized a new napthalimide based fluorescent reporter that incorporates the pyridinone-methide linker in six steps. Briefly, formylation of commercially available 2,5-dibromopyridine with BuLi and DMF gave picolinaldehyde, which was subsequently reduced to pyridinyl methanol by NaBH₄. Pd-catalyzed Miyaura borylation of failed to give the desired product. With a TBDMS protecting group, the cross-coupling product can be obtained in moderate yield (43%). Treatment with 1% HCl in ethanol gave pyridinylmethanol. Nucleophilic addition of to the isocyanate, generated in situ from reaction of with triphosgene, gave the new probe. Compound exhibited similar spectroscopic properties to other PL probes, with an absorption maximum at 375 nm (ε = 14,500 M⁻¹ cm⁻¹) and an emission maximum at 475 nm (Φ = 0.75). Reaction of compound 7 with H₂O₂ released LY3, showing increased absorbance at 440 nm, and increased fluorescence emission at 540 nm. Under pseudo first-order conditions (1 µM 7, 1 mM H₂O₂), the observed rate constant was kₜₐₚₙ = 3.7 x 10⁻⁴ s⁻¹ (Figure 1), which is much slower than other probes in the PL series. While the electron poor pyridine ring helps accelerate the elimination reaction, it has an opposite effect to the Bayer Villegger type deprotection of boronic acid ester by H₂O₂, in which the rate-limiting migration step prefers more electron-rich benzene ring (Figure 2).

Summary: We found that the new probe with a pyrimidone-methide linker exhibited similar absorption and fluorescence emission profile to that of other PL probes. The new probe, however, showed overall slower reaction toward H₂O₂ because the electron-poor pyridine hinders the rate-limiting migration step of boronate oxidation.

Experimental Section

Materials and Methods. 5-bromopicolinaldehyde was obtained from Oakwood (West Columbia, SC). Other chemicals were obtained from Sigma Aldrich and used as received. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. ¹H NMR spectra were collected in CDCl₃, (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AV-300 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. Low-resolution mass spectral analyses were carried out using Agilent Technology 1200 series with 6130 Quadrupole LC/MS (Santa Clara, CA).
5-Bromopicolinaldehyde (1). To a dried Schlenk flask under N₂ atmosphere was added 2,5-bromopyridine (4 g, 17 mmol), then the flask was submerged in an acetone-dry ice bath for 5 min. BuLi (11 mL, 1.4 M in toluene, 15.4 mmol) was slowly added to the reaction flask via syringe over 10 min. The solution was stirred at -78 °C for 2 h. DMF (1.5 mL) was added to the reaction mixture via syringe in one portion, and the solution was allowed to slowly warm to room temperature. Solution of saturated NH₄Cl (4 mL) was slowly added to the reaction mixture cooled in an ice-bath. The reaction mixture was diluted in EtOAc (200 mL) and washed with water (2 x 100 mL) and brine. The organic phase was dried over Na₂SO₄, and concentrated by rotary evaporator. The crude product was purified by flash column chromatography (9:1, hexane/EtOAc) to give the product as a white powder (1.5 g, 8.0 mmol, 47% yield). ¹H NMR (CDCl₃, 300 MHz): δ 10.03 (1H, s), 8.85 (1H, d, J = 1.8 Hz), 8.02 (1H, dd, J₁ = 8.1 Hz, J₂ = 1.8 Hz), 7.85 (1H, d, J = 8.1 Hz). GCMS: calculated for [M⁺] 184.95, found 185.0.

(5-Bromopyridin-2-yl)methanol (2). To the solution of 1 (700 mg, 3.7 mmol) in MeOH (10 mL) cooling in ice bath was added NaBH₄ (140 mg, 3.8 mmol) in small portion over 10 min. MeOH was removed by a rotary evaporator. The mixture was taken into EtOAc (200 mL), washed with water (2 x 100 mL) and brine. The organic phase was dried over Na₂SO₄. Purification by column chromatography (1:2, hexane/EtOAc) gave product as a white powder (650 mg, 3.5 mmol, 94% yield). ¹H NMR (CDCl₃, 300 MHz): δ 8.62 (1H, s), 7.80 (1H, d, J = 8.4 Hz), 7.18 (1H, d, J = 8.4 Hz), 4.72 (2H, s), 3.27 (1H, s-br). GC-MS: calculated for [M⁺] 186.96, found 187.0.

5-Bromo-2-((tert-butyldimethylsilyloxy)methyl)pyridine (3). A round-bottomed flask equipped with a magnetic stir bar was charged with 2 (200 mg, 1.1 mmol), NEt₃ (200 µL), DMF (4 mL). TBDMS-Cl (1.5 mL, 1M in THF, 1.5 mmol) was added to the reaction mixture. After 3 h stirring at room temperature, the reaction mixture was diluted in to EtOAc (100 mL), washed with water (2 x 50 mL) and brine. The organic phase was dried over Na₂SO₄. Purification by column chromatography (3:1, hexane/EtOAc) gave the product as a clear oil (300 mg, 1.0 mmol, 91% yield). ¹H NMR (CDCl₃, 300 MHz): δ 8.54 (1H, d, J = 1.8 Hz), 7.81 (1H, dd, J₁ = 8.4 Hz, J₂ = 1.8 Hz), 4.76 (2H, s), 0.94 (9H, s), 0.10 (6H, s).

2((tert-Butyldimethylsilyloxy)methyl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (4). To an oven dried 25-mL Schlenk tube was added bis(pinacolato) diboron (300 mg, 1.2 mmol), KOAc (300 mg, 3.0 mmol), Pd(dppf)Cl₂ (40 mg, 0.05 mmol). The reaction mixture was purged with N₂ for 5 min. Solution of 3 (300 mg, 1.0 mmol) in anhydrous dioxane (4 mL) was added to the reaction tube through syringe in one portion. The reaction tube was subjected to three cycle of vacuum/N₂ purge. The reaction tube was sealed and heated to 110 °C in an oil bath overnight. The reaction mixture was diluted into EtOAc (100 mL), washed with water (2 x 50 mL) and brine. The organic phase was dried over Na₂SO₄. Purification by column chromatography (2:1, hexane/EtOAc) gave the product as a clear oil (150 mg, 0.43 mmol, 43% yield). ¹H NMR (CDCl₃, 300 MHz): δ 8.82 (1H, d, J = 7.6 Hz), 7.49 (1H, d, J = 7.6 Hz), 4.84 (2H, s), 1.34 (12H, s), 0.95 (9H, s), 0.10 (6H, s). GC-MS: calculated for [M⁺] 349.22, found 349.3.

(5-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine-2-yl)methanol (5). A round-bottomed flask equipped with a magnetic stir bar was charged with solution of 4 (200 mg, 0.57 mmol) in EtOH (10 mL), followed by HCl (70 µL). The mixture was heat to 80 °C for 30 min. Solvent was removed by a rotary evaporator. The mixture was taken into CH₂Cl₂ (50 mL) and dried over Na₂SO₄. Concentration by rotary evaporator gave the product as a white solid (120 mg, 0.51 mmol, 89% yield). ¹H NMR (CDCl₃, 300 MHz): δ 8.96 (1H, s), 8.57 (1H, d, J = 7.5 Hz).
Hz), 7.76 (1H, d, $J = 7.5$ Hz), 5.07 (2H, s), 1.35 (12H, s). GC-MS: calculated for [M$^+$] 235.14, found 235.1.

6-Amino-2-(3-chloropropyl)-1$H$-benzo[de]isoquinoline-1,3(2H)-dione (6) was synthesized as described in chapter 1.

(5-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)methyl 2-(3-chloropropyl)-1,3-dioxo-2,3-dihydro-1$H$-benzo[de]isoquinolin-6-ylcarbamate (7). To a mixture of 6 (18 mg, 0.060 mmol) and DMAP (15 mg, 0.12 mmol) in toluene or acetonitrile (10 ml) in an oven dried two-necked flask equipped with a rubber septum and a condenser with nitrogen inlet was added a solution of triphosgene (12 mg, 0.040 mmol) in toluene dropwise. The solution was heated to reflux for 3 h. The formation of isocyanate was monitored by TLC; a drop of reaction mixture was taken into methanol to generate methoxy carbamate that emitted blue fluorescence under 365 nm UV compared to the green fluorescence of the starting material. After cooled to room temperature, the reaction mixture was diluted with dried CH$_2$Cl$_2$ (6 mL). Insoluble materials were removed by passing the solution through a glass pipette plugged with a ball of glass wool. To the filtrate was added 5 (20 mg, 0.085 mmol), and the solution was stirred at room temperature for another three hours. The reaction mixture was concentrated with a rotary evaporator and purified by flash chromatography (EtOAc, 5% MeOH) to give product as a yellow solid (22 mg, 0.040 mmol, 64%). $^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 8.96 (1H, d, $J = 1.5$ Hz), 8.64 (1H, d, $J = 7.2$ Hz), 8.60 (1H, d, $J = 8.4$ Hz), 8.39 (1H, d, $J = 8.4$ Hz), 8.21 (1H, d, $J = 7.8$ Hz), 8.11 (1H, dd, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz), 7.78 (1H, dd, $J_1 = 7.5$ Hz, $J_2 = 8.4$ Hz), 7.69 (1H, s-br), 7.41 (1H, d, $J = 7.8$ Hz), 5.41 (2H, s), 4.32 (2H, t, $J = 6.9$ Hz), 3.64 (2H, t, $J = 6.6$ Hz), 2.31 (2H, m), 1.35 (12H, s). LC-MS: calculated for [MH$^+$] 550.18, found 550.4

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA).
Figures and Schemes

Figure 1. Reaction of 2 µM 7 to 1 mM H₂O₂ monitored by (a) absorption and (b) fluorescence emission (λ_{exc} = 410 nm). Spectra were taken 0, 10, 20, 30, 40, 50, and 60 min after addition of H₂O₂. (c) Kinetic plot of fluorescence emission intensity at 470 nm gave an observed reaction rate k_{obs} = 3.7 \times 10^{-4} \text{ s}^{-1}.
Figure 2. H$_2$O$_2$-mediated deprotection of the arylboronate ester is slower in the probe with the pyridinone-methide linker than in that with the quinone-methide.
References


Appendix 5
Attempts Toward Red Fluorescent Probes for Hydrogen Peroxide Based on Acridinone Fluorophores
**Synopsis**

This appendix describes the synthesis, spectroscopic characterization, and confocal fluorescence microscopy of 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)/DDAO-derived red fluorescent probes for H$_2$O$_2$. Fluorescent probes with longer excitation and emission wavelengths have an advantage of improved signal to noise because of decreased background from cellular auto-fluorescence and backscattered excitation light. DDAO-based sensors have been demonstrated to have application in both cellular and whole animal imaging. We envisioned H$_2$O$_2$ probes in which DDAO reporters are masked with a boronate-ester of benzylether, a self-immolative trigger that was previously utilized in the development of bioluminescence probe for H$_2$O$_2$.

**Results and Discussion**

H$_2$O$_2$ probes, DDAO-B1 and DDAO-B2, were obtained from the nucleophilic substitution reaction of DDAO with 2(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,4-dioxaborolane (Scheme 1). For purification, DDAO-B1 and DDAO-B2 were separated by column chromatography and the structures were verified by 2D NMR spectroscopy (Figure 1-2). In HEPES buffer pH 7.4, DDAO-B1 has a broad absorption band centered at 475 nm, while DDAO-B2 has a broad absorption at 400 nm. Reaction of DDAO-B1 and DDAO-B2 with H$_2$O$_2$ resulted in formation of DDAO, with characteristic broad absorption at 600-640 nm, and a fluorescence emission maximum at 650 nm. Under a pseudo first-order conditions (1 µM probes, 1mM H$_2$O$_2$), the observed rate constants were $k_{obs} = 1.2 \times 10^{-4}$ s$^{-1}$ and $4.1 \times 10^{-4}$ s$^{-1}$ for DDAO-B1 and DDAO-B2, respectively (Figure 3). We proposed that the faster rate for DDAO-B2 was the result of faster elimination of the quinone methide which is facilitated by the electron poor aromatic ring with two choline atoms. The acridinone core of DDAO is a good leaving group. While DDAO-B1 and DDAO-B2 are stable in HEPES buffer at room temperature, we observed significant increase fluorescence emission upon incubation of DDAO-B1 or DDAO-B2 in phosphate buffer. The hydrolysis is more prominent at 37°C incubation in which release of DDAO occurs in both HEPES and phosphate buffer. Romieu et al. observed similar hydrolysis of the benzylether derivative DDAO in 8:2 mixtures of phosphate buffer and acetone. DDAO-B1 and DDAO-B2 are membrane-permeable and can respond to changes in intracellular H$_2$O$_2$ concentration. HEK293T cells loaded with DDAO-B1 or DDAO-B2 displayed a significant increase in intracellular fluorescence after incubation with exogenous H$_2$O$_2$ (100 µM) for 30 min (Figure 4 - 5).

**Summary**: The DDAO platform shows a promising result for development of red fluorescent probe for H$_2$O$_2$. Future investigation to improve the stability of probes in aqueous buffer is needed. One approach would be to replace the benzylether group with self-immolative alkylether linkers, which literature precedents have shown to be stable in aqueous buffer. Additionally, screening a substitution pattern on the acridinone core could possibly give a solution to the stability problem as well as a lead for near-IR fluorescent dyes (Figure 6). For example, DDAO with a (dimethyl)amino substitution, DDAO-NMe$_2$, shows an absorption maximum at 670 nm and a fluorescence emission maximum at 678 nm, which is 20 nm red-shifted from that of DDAO (Figure 7).
**Experimental Section**

**Materials and Methods.** Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. $^1$H NMR, $^{13}$C NMR, 2D-HSQC, and 2D-HMBC spectra were collected in CDCl$_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVB-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard $\delta$ notation of parts per million. Low-resolution mass spectral analyses were carried out using LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

DDAO was synthesized using modified protocol from literature$^7$ (Scheme 1). We found that cyclization using TFA at room temperature gave cleaner products than the original method of refluxing in 2 M HCl.

**DDAO-B1 and DDAO-B2.** A mixture of DDAO (100 mg, 0.32 mmol), Cs$_2$CO$_3$ (200 mg, 0.62 mmol), and 4-(bromomethyl) benzene boronic acid pinacol ester (150 mg, 0.50 mmol) in dioxane (10 mL) was stirred for 48 h at ambient temperature in a closed flask protected from light. The organic reaction mixture was diluted with EtOAc (300 mL), washed with 0.1 M citric acid and brine. The organic layer was dried over Na$_2$SO$_4$, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (1:3, EtOAc/hexane) to give the products as orange solids (25 mg DDAO-B1 and 30 mg DDAO-B2, 33% total yield).

**DDAO-B1.** $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.86 (2H, d, $J = 8.0$ Hz), 7.64 (1H, s), 7.60 (1H, d, $J = 8.8$ Hz), 7.45 (2H, d, $J = 8.0$ Hz), 7.08 (1H, d, $J = 2.4$ Hz), 6.96 (1H, dd, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 5.19 (2H, s), 1.86 (6H, s), 1.35 (12H, s). $^{13}$C NMR (CDCl$_3$, 400 MHz): $\delta$ 171.1, 162.1, 147.5, 140.6, 139.3, 138.8, 136.7, 135.8, 135.2 (2), 134.5, 134.0,126.6 (2), 114.0, 113.8, 83.9, 70.4, 39.1, 26.8, 24.8. HRMS-ESI: calculated for [M]+ 524.24, found 524.1560.

**DDAO-NMe2.** A mixture of 2-(3-dimethylamino)phenyl)propan-2-ol (500 mg, 2.8 mmol) and 2,6- dichloro-4-(chloroimino)cyclohexa-2,5-dienone (530 mg, 2.6 mmol) in 1:1 H$_2$O/THF (5 mL) was cooled in an ice bath. Aqueous 5 mM NaOH (2.5 mL) was added over 10 min. After 1 h reaction in the ice bath, the reaction mixture was extracted with EtOAc (2 x 25 mL). The organic layer was dried over Na$_2$SO$_4$ and concentrated. Flash column chromatography (1:1, hexane/EtOAc) gave a dark purple solid (100 mg), TLC showed a mixture of a blue and a purple substance. The dark purple solid was dissolved in TFA (3 mL) and the reaction was stirred at room temperature for 3 h. TFA was removed under reduced pressure, and the remained viscous substance was taken into EtOAc (50 mL). Solution of NaIO$_4$ (1 g in 50 mL H$_2$O) was added to a separatory funnel containing the reaction mixture, followed by vigorous mixing for 5 min. The dark blue organic layer was dried over Na$_2$SO$_4$ (10 mg, blue solid). LC-MS: calculated for [M]+ 335.06, found 335.1.

**Spectroscopic Materials and Methods.** Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4.
Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded using 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 (1.5-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by reference to Rhodamine B in PBS ($\Phi = 0.34$).8

**Preparation and Staining of Cell Cultures.** HEK293T cells were cultured in DMEM (Invitrogen) containing high glucose with GlutaMAX™ (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS, Hyclone). One day before imaging, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine (50 µg/mL, Sigma, St. Louis, MO). H₂O₂ was delivered from 50 mM stock solution in Millipore water. Labeling buffer contained 5 µM DDAO-B1 or DDAO-B2 in HEPES. Labeling of HEK293T cells were accomplished by incubating cells in labeling buffer for 30 min at 37°C under 5% CO₂ atmosphere then washing twice with fresh buffer. [5x HEPES buffer pH 7.4: 750 mM NaCl, 100 mM HEPES, 5 mM CaCl₂, 25 mM KCl, 5 mM MgCl₂]. Cells were further incubated for 30 min in either HEPES buffer or HEPES buffer with added exogenous H₂O₂.

**Fluorescence Imaging Experiments.** Confocal fluorescence imaging was performed with Zeiss 710 Axiovert laser scanning microscope and 20x or 40x air objective lens. Excitation of DDAO-B1 or DDAO-B2 loaded cells was carried out with 633 nm laser, emission collection 650 - 700 nm. Data were analyzed using ImageJ software (author: Wayne Rasband, NIH) and Ratio Plus plug-in (programmer: Paulo Magalhes, University of Padua, Italy).
Table 1. Spectroscopic properties of DDAO dyes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\Phi$</th>
<th>$k_{\text{obs}}$ (x 10$^{-3}$ s$^{-1}$)</th>
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<td>DDAO</td>
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<td>n/a</td>
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<tr>
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<td>678</td>
<td>-</td>
<td>680</td>
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</tr>
</tbody>
</table>
Figure 1. (a) HSQC and (b) HMBC 2D-NMR spectra of DDAO-B1
Figure 2. (a) HSQC and (b) HMBC 2D-NMR spectra of DDAO-B2.
Figure 3. Reaction of DDAO-B1 and DDAO-B2 (0.5 µM in HEPES buffer, pH 7.4) with 1 mM H₂O₂, monitored by absorption and fluorescence emission spectra (λ<sub>exc</sub> = 600 nm). Spectra were taken at 0, 10, 20, 30, 40, 50, and 60 min after addition of H₂O₂.
Figure 4. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells with DDAO-B1. Cells were loaded with DDAO-B1 (5 µM in HEPES, 30 min). Cells were washed twice with fresh buffer, followed by 30 min incubation in (a) HEPES buffer or (b) HEPES buffer with 100 µM H$_2$O$_2$ before image acquisition ($\lambda_{exc} = 633$ nm, emission collection 650 – 700 nm, scale bar = 20 µm).
Figure 5. Fluorescence detection of $\text{H}_2\text{O}_2$ in living HEK293T cells with DDAO-B2. Cells were loaded with DDAO-B2 (5 µM in HEPES, 30 min). Cells were washed twice with fresh buffer, followed by 30 min incubation in (a) HEPES buffer or (b) HEPES buffer with 100 µM $\text{H}_2\text{O}_2$ before image acquisition ($\lambda_{\text{exc}} = 633$ nm, emission collection 650 – 700 nm, scale bar = 20 µm).
Figure 6. Proposed strategy for improving stability of DDAO probes in aqueous buffer by utilizing self-immolative alkylether linker.
Figure 7. Normalized absorption and fluorescence emission spectra of (a) DDAO and (b) DDAO-NMe2. ($\lambda_{exc} = 600$ nm, HEPES, pH 7.4)
References


Appendix 6
Red Fluorescent Probes for Detection of Hydrogen Peroxide Based on Seminaphthorhodafluors (SNARFs) and Naphthofluorescein
Synopsis

This appendix describes the synthesis, spectroscopic properties, and application for fluorescence imaging of H₂O₂ responsive fluorescent probes for H₂O₂ based on naphthofluorescein and seminaphthorhodafluors (SNARFs) scaffolds. This family of naphthoxanthone dyes is best known for its application as fluorescent pH sensors.¹⁻³ We have previously developed Naphthoperoxyfluor-1 (NPF1), a chemoselective boronic ester probe that releases the fluorescent reporter naphthofluorescein upon reaction with H₂O₂.⁴ Here, we report a family of naphthoxanthone probes with pKa lower than 7.4, which increases the ratio of the more emissive basic form of fluorophores at physiologically relevant pH.

Results and Discussion

Naphthofluorescein-4F (NF-4F) was synthesized by condensation of 5-fluoronaphthalene-1,6-diol and 3-carboxyphthalic anhydride in methanesulfonic acid. The acidic and basic form of NF-4F has absorption maxima at 590 and 620 nm, respectively (Figure 1a). The acidic form is non-fluorescent while the basic form has an emission maximum at 685 nm (Φ = 0.005). Fluorination of the naphthol ring lowers the pKa to 7.2 (naphthofluorescein, pKa = 7.6). Reaction of NF-4F with bis(trifluoromethane)sulfonamide affords triflate, in which installation of a boronate ester by Miyaura’s palladium-catalyzed cross coupling reaction gave the desired probe Naphthoperoxyfluor-2, NPF2 (Scheme 1). Reaction of NPF2 with H₂O₂ features a turn-on fluorescence emission with a maximum at 685 nm, which is 15 nm red-shifted from NPF1/naphthofluorescein. Under pseudo first-order conditions, reaction of NPF2 with H₂O₂ has an observed rate constant kₐₜ = 0.4 x 10⁻³ s⁻¹, which is in the same order of magnitude as that of NPF1 (Figure 2a).

Seminaphthorhodafluor-X-4F (SNARF-X-4F) was synthesized by condensation of 5-fluoronaphthalene-1,6-diol with julolidine-phthalic acid in methanesulfonic acid. In acidic buffer, SNARF-X-4F features absorption maxima at 530 and 570 nm with a corresponding fluorescence emission maximum at 600 nm (Φ = 0.050). The basic form of SNARF-X-4F has an absorption maximum at 590 nm with a corresponding emission maximum at 635 nm (Φ = 0.105, Figure 1b). SNARF-X-4F has pKa = 6.9 (SNARF-X, pKa = 7.9). Naphthoperoxyrhodol-1, NPR1, was obtained from the palladium-mediated coupling of SNARF-X-4F triflate with bis(pinacolato)diboron. The spectral properties of NPR1 have striking resemblance to the acidic form of SNARF-X-4F. Indeed, NPR1 displayed ratiometric change in fluorescence emission upon reaction with H₂O₂, kₐₜ = 1.5 x 10⁻³ s⁻¹ (Figure 2b). Confocal fluorescence imaging in lambda mode scanning showed HEK29T cells labeled with NPR1 and SNARF-X-4F in orange and red color, respectively (Figure 3). However, the spectra shift in conversion of NPR1 to SNARF-X-4F (30 nm) is not significant in cell cultures treated with sub-millimolar dose of H₂O₂ (Figure 4). Therefore, despite having ratiometric response, which is the first case of a boronate-based xanthone fluorophore, NPR1 is not applicable for detecting H₂O₂ in physiological level. NPR2 features a self-immolative linker benzylether caged derivative of SNARF-X-4F. Unlike NPR1, NPR2 is non-fluorescent, with a slower rate of fluorescence emission turn-on with H₂O₂, kₐₜ = 0.6 x 10⁻³ s⁻¹ (Figure 2c).

SNARF-NMe2 is an isomer of the commercially available pH sensor SNARF-1, but there is a striking different in their spectral properties (Figure 1c). SNARF-NMe2 has pKa = 5.7, compared to 7.5 and 6.4 reported for SNARF-1 and its fluorinated SNARF-4F. The acidic form of SNARF-NMe2 has more red-shifted absorption than the basic form (λₐₜ = 565 nm, λₑ₅₅ =
546 nm), which is in opposite direction to trend observed in other SNARF dyes. The acid form is almost non-fluorescent while the basic form has an emission maximum at 650 nm (Φ = 0.041). Installation of boronate ester gave the H₂O₂ responsive probe NPR3. NPR3 showed fluorescence emission turn-on with kᵢₒsb = 0.9 x 10⁻³ s⁻¹ under pseudo first-order conditions (Figure 2d). NPR3 is capable of detecting an increased level of H₂O₂ in HEK293T cells under oxidative stress condition, 100 µM H₂O₂ (Figure 5).

**Summary:** The seminaphthorhodafluors platform shows promising result for further development of red and near-IR probes. Although symmetric naphthofluorescein has longer emission wavelength, it has low quantum yield with pKa higher than desirable range for working in physiological pH. NPR1 and NPR3 can be developed to near-IR sensors with Si-substitute xanthone scaffold (Figure 6). The large Stokes’ shift of seminaphthorhodafluors platform also makes them a good candidate for FRET pair sensors.

**Experimental Section**

**Materials and Methods.** Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. 5-Fluoronaphthanlene-1,6-diol (1) was synthesized following a literature protocol. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. ¹H NMR and ¹³C NMR spectra were collected in CDCl₃ or CD₃OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVQ-400 or AV300 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. Low-resolution mass spectral analyses were carried out using GC-MS (Agilent Technology 5975C, inert MSD with triple axis detector) or LC-MS (Agilent Technology 6130, Quadrupole LC/MS). High-resolution mass spectral analyses (ESI-MS, FAB-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Isomeric mixtures of 4,10-difluoro-3,11-dihydro-3’oxo-3’H-spiro[dibenzo[c,h]xanthenes-7,7’-isobenzofuran]-5’-carboxylic acid and 4,10-difluoro-3,11-dihydro-3’oxo-3’H-spiro [dibenzo[c,h]xanthenes-7,1’-isobenzofuran]-6’-carboxylic acid (2), NF-4F. To a heavy-walled pressure flask was added 1 (178 mg, 1.0 mmol), 3-carboxyphthalic anhydride (192 mg, 1.0 mmol), and methanesulfonic acid (3 mL). After sonication for 3 h, the reaction was allowed to continue overnight at room temperature. Pouring the viscous reaction into ice water gave purple precipitate, which was collected by vacuum filtration and then redissolved into EtOAc. Purification with flash column chromatography (EtOAc, with 5% MeOH) gave product as a purple solid (100 mg, 0.19 mmol, 19% yield). ¹H NMR (CD₃OD, 400 MHz): δ 8.71 (1H, s), 8.28-8.35 (6H, m), 8.11 (1H, d, J = 8.0 Hz), 7.34 (1H, s), 7.58 (4H, d, J = 8.8 Hz), 7.35 (4H, t, J = 10.8 Hz), 7.17 (1H, d, J = 8.0 Hz), 6.73 (4H, d, J = 8.8 Hz). LC-MS: calculated for [M+H]⁺ 513.07, found 513.1.

Isomeric mixtures of bis(trifluoromethylsulfonyloxy)-NF-4F-5’-carboxylic acid and 6’-carboxylic acid (3). To a 20-mL vial equipped with a magnetic stir bar was added NF-4F (70 mg, 0.14 mmol), N-phenylbis(trifluoromethane)sulfonamide (98 mg, 0.27 mmol), acetonitrile (5 mL), and NE₃ (100 µL). After stirring at room temperature overnight, the reaction mixture was taken in to EtOAc and washed with 1 N HCl and brine. Purification with flash chromatography (2:1, EtOAc/hexane) gave product as colorless oil (50 mg, 0.06 mmol, 45% yield). LC-MS: calculated for [M⁺] C₃₁H₁₂F₆O₁₁S₂ 775.97, found 776.8.
Isomeric mixtures of bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-NF-4F-5'-carboxylic acid and 6'-carboxylic acid (4). To an oven-dried 25-mL Schlenk tube was added Pd(dppf)Cl₂ (5 mg, 0.006 mmol), KOAc (40 mg, 0.40 mmol), and bis(pinacolato)diboron (49 mg, 0.19 mmol). The reaction tube was put under vacuum for 1 min and refilled with nitrogen atmosphere, and solution of 3 (50 mg, 0.06 mmol) in dioxane (5 mL) was delivered through a syringe in one portion. The reaction mixture was subjected to three cycles of vacuum and N₂ refill. The reaction tube was sealed and heated at 110 °C for 12 h. EtOAc (10 mL) was added to reaction mixture that was cooled to room temperature. The mixture was passed through a 1-inch layer of silica gel. Flash column chromatography of the concentrated crude product (2:1, EtOAc/hexane) gave 4 as a white powder (10 mg, 0.013 mmol, 22% yield). LC-MS: calculated for [M+] C₄₁H₃₆B₂F₂O₉ 732.25, found 732.2.

2-(8-Hydroxy-1,2,3,5,6,7-hexahydropyrido[3,2,1-ij]quinoline-9-carbonyl)benzoic acid (5). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added 3-hydroxyjulolidine (1 g, 5.3 mmol), phthalic anhydride (1 g, 6.7 mmol) and toluene (20 mL). The reaction mixture was heated to reflux overnight. Cooling to room temperature gave a pale pink solid precipitate that was collected by vacuum filtration, washed with cold methanol, and air dried to yield 5 (800 mg, 2.3 mmol, 45% yield). ¹H NMR (CD₃OD, 400 MHz): δ 7.05 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.63 (1H, td, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.55 (1H, td, J₁ = 7.5 Hz, J₂ = 1.5 Hz), 7.30 (1H, dd, J₁ = 7.5 Hz, J₂ = 1.2 Hz), 6.41 (1H, s), 3.24 (4H, t, J = 5.7 Hz), 2.66 (2H, t, J = 6.3 Hz), 2.42 (2H, t, J = 6.3 Hz), 1.92 (2H, m), 1.81 (2H, m).

SNARF-X-4F (6). To a heavy-walled pressure flask was added compound 5 (128 mg, 0.40 mmol), 1 (70 mg, 0.39 mmol), and methanesulfonic acid (3 mL). After sonication for 3 h, the reaction was allowed to continue overnight at room temperature. Pouring the viscous reaction into ice water gave purple precipitated solid. The solid was collected by vacuum filtration and then taken into EtOAc. Purification with flash column chromatography (EtOAc, with 5% MeOH) gave product as a purple solid (50 mg, 0.10 mmol, 25% yield). ¹H NMR (CD₃OD, 400 MHz): δ 8.40 (1H, d, J = 7.6 Hz), 8.30 (1H, d, J = 9.2 Hz), 7.84-7.91 (2H, m), 7.80 (1H, d, J = 9.2 Hz), 7.45 (1H, d, J = 7.2 Hz), 7.32 (1H, t, J = 8.4 Hz), 7.10 (1H, d, J = 8.8 Hz), 6.91 (1H, s), 3.66 (4H, s-br), 3.29 (2H, s-br), 2.79 (2H, s-br), 2.11 (2H, s-br), 2.01 (2H, s-br). LC-MS: calculated for [MH+] C₃₁H₂₂F₄NO₆S 612.10, found 612.1.

SNARF-X-4F-OTf (7). To a 20-mL vial equipped with a magnetic stir bar was added NF-4F (50 mg, 0.10 mmol), N-phenylbis(trifluoromethane)sulfonamide (43 mg, 0.12 mmol), acetonitrile (5 mL), and NEt₃ (100 µL). After stirring at room temperature overnight, the solvent was removed by a rotary evaporator. Purification with flash chromatography (EtOAc, with 5% MeOH) gave product as a pale pink solid (55 mg, 0.09 mmol, 90% yield). ¹H NMR (CD₃OD, 400 MHz): δ 8.38 (d, 1H, J = 9.2 Hz), 8.07 (d, 1H, J = 7.2 Hz), 7.64-7.70 (3H), 7.54 (dd, 1H, J₁ = 9.2 Hz, J₂ = 6.8 Hz), 7.16 (d, 1H, J = 7.2 Hz), 6.92 (d, 1H, J = 9.2 Hz), 6.23 (s, 1H), 3.25 (t, 2H, J = 5.2 Hz), 3.19 (t, 2H, J = 5.2 Hz), 3.13 (t, 2H, J = 5.2 Hz), 2.60 (m, 2H), 2.13 (s-br, 2H), 1.91 (s-br, 2H). LC-MS: calculated for [MH⁺] C₃₃H₂₀F₄NO₆S 642.10, found 642.1.

SNARF-X-4F-Bpin (8), NPR1. To an oven-dried 25-mL Schlenk tube was added Pd(dppf)Cl₂ (2 mg, 0.002 mmol), KOAc (20 mg, 0.20 mmol), and bis(pinacolato)diboron (20 mg, 0.08 mmol). The reaction tube was put under vacuum for 1 min and refilled with nitrogen atmosphere, and a solution of 7 (35 mg, 0.06 mmol) in dioxane (5 mL) was delivered via syringe in one portion. The reaction mixture was subjected to three cycles of vacuum and N₂ refill. The reaction tube was sealed and heated at 110 °C for 12 h. EtOAc (10 mL) was added to reaction mixture after cooled to room temperature. The mixture was passed through a 1-inch layer of
silica gel. Flash column chromatography of the concentrated crude product (2:1, EtOAc/hexane) gave product as a pink powder (20 mg, 0.03 mmol, 50% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.24 (2H, d, $J = 9.2$ Hz), 8.06 (1H, d, $J = 7.8$ Hz), 7.86 (1H, dd, $J_1 = 9.2$ Hz, $J_2 = 8.8$ Hz), 7.70 (1H, d, $J = 8.8$ Hz), 7.60-7.68 (2H, m), 7.18 (1H, d, $J = 7.8$ Hz), 6.78 (1H, d, $J = 9.2$ Hz), 6.23 (1H, s), 3.23 (2H, t, $J = 5.2$ Hz), 3.17 (4H, m), 2.57 (2H, m), 2.12 (2H, s-br), 1.91 (2H, s-br), 1.42 (12H, s). LC-MS: calculated for [MH$^+$$] 590.24, found 590.3.

SNARF-X-4F-Bpin(benzylether) (9), NPR2. To a round-bottomed flask equipped with magnetic stir bar was added 6 (20 mg, 0.04 mmol), 2-(4-(bromomethyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (13 mg, 0.04 mmol), Cs$_2$CO$_3$ (32 mg, 0.10 mmol), and DMF (3 mL). The reaction mixture was heated at 70 °C for 2 h. The reaction mixture was taken into EtOAc (50 mL), washed with water (2 x 50 mL) and brine. The organic layer was dried over Na$_2$SO$_4$ and concentrated by a rotary evaporator. Purification with flash column chromatography (2:1, EtOAc/hexane) gave product as a pink powder (18 mg, 0.03 mmol, 75% yield). $^1$H NMR (CD$_3$OD, 400 MHz): δ 8.17 (1H, d, $J = 9.2$ Hz), 8.05 (1H, d, $J = 7.8$ Hz), 7.83 (2H, d, $J = 7.8$ Hz), 7.62-7.67 (2H, m), 7.61 (1H, d, $J = 8.8$ Hz), 7.48 (2H, d, $J = 7.8$ Hz), 7.34 (2H, t, $J = 8.8$ Hz), 7.16 (1H, d, $J = 6.8$ Hz), 6.75 (1H, d, $J = 9.2$ Hz), 6.22 (1H, s), 5.34 (2H, s), 3.21 (2H, t, $J = 5.2$ Hz), 3.16 (2H, t, $J = 5.2$ Hz), 3.10 (2H, t, $J = 5.2$ Hz), 2.58 (2H, m), 2.09 (2H, s-br), 1.90 (2H, s-br), 1.35 (12H, s). LC-MS: calculated for [MH$^+$$] 696.29, found 696.2.

2-(2,4-Dihydroxybenzoyl)benzoic acid (10). To a round-bottomed flask equipped with a magnetic stir bar and a condenser was added resorcinol (0.5 g, 4.5 mmol), phthalic anhydride (1 g, 6.7 mmol) and toluene (20 mL). The reaction mixture was heated to reflux overnight. Cooling to room temperature gave a pale yellow solid precipitate that was collected by vacuum filtration, washed with cold methanol, and air dried to yield 10 (750 mg, 2.9 mmol, 64% yield). $^1$H NMR (CD$_3$OD, 300 MHz): δ 8.07 (1H, dd, $J_1 = 7.5$ Hz, $J_2 = 0.9$ Hz), 7.71 (1H, td, $J_1 = 7.5$ Hz, $J_2 = 1.2$ Hz), 7.60 (1H, td, $J_1 = 7.8$ Hz, $J_2 = 1.2$ Hz), 7.35 (1H, dd, $J_1 = 7.5$ Hz, $J_2 = 1.2$ Hz), 6.91 (1H, d, $J = 9.0$ Hz), 6.30 (1H, d, $J = 2.4$ Hz), 6.21 (1H, dd, $J_1 = 9.0$ Hz, $J_2 = 2.4$ Hz).

SNARF-NMe2 (11). To a test tube was added compound 10 (65 mg, 0.25 mmol), 6-dimethylamino)naphthalene-1-ol (50 mg, 0.25 mmol), and methanesulfonic acid (1 mL). The reaction mixture was heated at 100 °C for 1 h. Pouring the cooled viscous reaction into ice water gave dark green precipitation. The solid was collected by vacuum filtration and then taken into EtOAc. Purification with flash column chromatography (EtOAc, with 5% MeOH) gave product as dark green solid (20 mg, 0.050 mmol, 20% yield). LC-MS: calculated for [MH$^+$$] C$_{26}$H$_{19}$NO$_4$ 410.13, found 410.2.

SNARF-NMe2-OTf (12). To a 20-mL vial equipped with a magnetic stir bar was added 11 (20 mg, 0.050 mmol), N-phenylbis(trifluoromethane)sulfonamide (25 mg, 0.070 mmol), acetonitrile (2 mL), and NEt$_3$ (20 µL). After stirring at room temperature overnight, the solvent was removed by a rotary evaporator. Purification with flash chromatography (1:1, EtOAc/hexane) gave product as a pale pink solid (25 mg, 0.045 mmol, 90% yield). LC-MS: calculated for [MH$^+$$] C$_{26}$H$_{16}$NO$_4$ 410.13, found 410.2.

SNARF-NMe2-Bpin (13), NPR3. To an oven-dried 25-mL Schlenk tube was added Pd(dppf)Cl$_2$ (2 mg, 0.002 mmol), KOAc (13 mg, 0.13 mmol), and bis(pinacolato)diboron (13 mg, 0.050 mmol). The reaction tube was put under vacuum for 1 min and refilled with nitrogen atmosphere, and a solution of 12 (25 mg, 0.045 mmol) in dioxane (5 mL) was delivered via syringe in one portion. The reaction mixture was subjected to three cycles of vacuum and N$_2$ refill. The reaction tube was sealed and heated at 110 °C for 12 h. EtOAc (10 mL) was added to reaction mixture that was cooled to room temperature. The mixture was passed through a 1-inch
layer of silica gel. Flash column chromatography of concentrated crude product (2:1, EtOAc/hexane) gave product as a pink powder (8 mg, 0.015 mmol, 66% yield). LC-MS: calculated for [MH\(^{+}\)] 520.22, found 520.3.

**Spectroscopic Materials and Methods.** Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.4, 25 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ). Samples for absorption and fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4 mL volume, Starna, Atascadero, CA). C.SNARF2 in 0.1 M NaOH (Φ = 0.11) was used as standard for quantum yield measurements.\(^2\)

**Fluorescence Imaging Experiments.** HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before transfection, cells were passaged and plated on 18-mm glass coverslips coated with poly-L-lysine in a 24-wells culture plates. Cells were incubated in HEPES containing 5 μM probes for 30 min at 37 °C. Cells were washed twice with HEPES buffer and further incubated for another 30 min in either (a) HEPES buffer or (b) HEPES buffer with H\(_2\)O\(_2\) before images were taken. Confocal fluorescence imaging studies were performed with a Zeiss LSM710 laser scanning microscope and a 40x objective lens. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO\(_2\) humidified atmosphere. Image analysis was performed in ImageJ (National Institute of Health).
Scheme 2. Synthesis of NPR1 and NPR2.
### Table 1. Spectroscopic properties of dyes

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Figure 1. Absorption spectra of (a) NF-4F, (b) SNARF-X-4F and (c) SNARF-NMe2 in 50 mM phosphate-buffered solution at a range of pH values.
Figure 2. Fluorescence emission spectra of reaction of (a) NPF2 ($\lambda_{\text{exc}} = 600\text{nm}$), (b) NPR1, (c) NPR2 and (d) NPR3 probes (NPR1-3, $\lambda_{\text{exc}} = 550\text{nm}$) with 5 mM H$_2$O$_2$. 
Figure 3. Confocal fluorescence images in lambda mode scanning of HEK293T labeled with NPR1 (a, c) or SNARF-X-4F (b, d).
Figure 4. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells with NPR1. Cells were loaded with NPR1 (5 µM, 30 min in HEPES buffer), washed twice with fresh buffer. Cells were further incubated for 30 min in HEPES buffer or HEPES + 200 µM H$_2$O$_2$. b) Average fluorescence emission intensity of yellow channel and red channel. c) Quantification of emission ratio $F_{\text{red}}/F_{\text{yellow}}$. Scale bar = 20 µm. ($\lambda_{\text{exc}}$ = 543 nm, yellow channel = 560 – 600 nm, red channel = 600 – 700 nm).
Figure 5. Fluorescence detection of H$_2$O$_2$ in living HEK293T cells with NPR3. Cells were loaded with NPR3 (5 µM, 30 min in HEPES buffer), washed twice with fresh buffer. Cells were further incubated for 30 min in HEPES buffer or HEPES + 100 µM H$_2$O$_2$. Scale bar = 20 µm. ($\lambda_{\text{exc}} = 543$ nm, collection window = 560 – 700 nm).
Figure 6. Proposed structure for near-IR SNARF.
References


Appendix 7

Development of a Genetically-Encoded Red Fluorescent Protein Sensor for Hydrogen Peroxide Imaging

Portion of this work was performed by the following people:
Steve Wilson synthesized cpRuby-OxyR-X/X.
Jasper Akerboom provided resourceful advice.
Synopsis

This appendix describes an attempt toward generating a red-emitting H$_2$O$_2$ responsive genetically-encoded fluorescent protein. Our strategy is to replace cpYFP reporter of Hyper, a H$_2$O$_2$-responsive fluorescent protein developed by Belousov et al., with circularly permuted red-emitting fluorescent protein. cpRuby was developed by Looger et al, and has been successfully utilized as a cpRFP based calcium sensor in cells, displaying fluorescence turn-on with emission maxima at 605 nm (Scheme 1). Our constructs of cpRuby-OxyR with various amino acid linkers showed no fluorescence emission response to H$_2$O$_2$, as well as changes in redox state. Expression and maturation of cpRuby is also very sensitive to the sequence of the linkers connecting the sensor region and cpRuby. Future investigation is needed to optimize the linker lengths between cpRuby and the OxyR regions.

Results and Discussion

Genetically encoded fluorescent protein sensors are an indispensable tool for real-time visualization and tracking of various cellular events. In the development of passive markers, GFP-like proteins from both jelly fish *Aequorea victoria* and *Anthozoa* corals are now available spanning from blue to near-IR emission wavelength. Active markers for measuring biochemical events consist of two subclasses: the FRET-based sensors and the monomeric sensors in which the barrel structures are engineered to be directly sensitive to the molecule of interest. A major improvement in fluorescent protein sensors comes from the generation of circularly permuted fluorescent protein (cpFP). In a cpFP, the original N and C termini are joined by a flexible linker. New termini are introduced into a barrel region close to the chromophore, which result in an increased optical responsiveness to stress or conformational changes induced by the analytes. The cpFP improves the dynamic response of the monomeric sensors as well as the dynamic emission ratio when incorporated into FRET-base sensors. Ca$^{2+}$ indicators such as GCaMP (cpEGFP) or Pericams (cpYFP) are created by insertion of cpFP between calmodulin (CaM) and M13 (a peptide which binds to CaM in a Ca$^{2+}$ dependent manner). In similar manner, Hyper, a H$_2$O$_2$ indicator, was constructed by insertion of cpYFP into a regulatory domain of prokaryotic H$_2$O$_2$-sensing protein, OxyR. In response to H$_2$O$_2$, a disulfide bond is formed between between C199 and C208, inducing a huge conformational change in OxyR. After extensive screening of series of constructs, it was determined that maximum sensing was obtained when cpYFP is inserted between residue 205 and 206 of OxyR. cpYFP utilized in Hyper and ratiometric Pericam has two absorption maximum at 400 and 500 nm corresponding to the protonate and deprotonate state of the chromophore; both excitation gave an emission maxima at 520 nm. Disulfide formation in Hyper and binding to Ca$^{2+}$ in Pericam promoted the ionization of chromopore which results in increasing absorbance in blue light over violet light.

mRuby is a monomeric red fluorescent protein variant of eqFP611, a tetrameric fluorescent protein isolated from *Entacmaea quadricolor*, a member of *Anthozoan* family. Circular permutation was conducted on mRuby by using a peptide linker GTGGS to connect the natural N and C termini. New termini were introduced into surface-exposed loop regions of the B-barrel at N143 and P142. Ca$^{2+}$ sensor RCaMP1.019 developed by Looger lab contains cpRuby between M13 and CaM sequence with short amino acid linkers AI and TR. In our hand, HEK293T cells transiently expressing RCaPM1.019 exhibits a 1.5 fold turn-on upon treatment with a Ca$^{2+}$ ionophore/ionomycin in Ca$^{2+}$ containing buffer (Figure 1).
To obtain cpRuby-OxyR, sequence overlap extension PCR was performed on cpRuby sequence amplified from RCaMP1.019 and OxyR1/OxyR2 amplified from Hyper-cyto. cpRuby and OxyR1/OxyR2 were connected directly without linker or with short amino acid linkers similar to that found in Hyper-cyto or RCaMP1.019 (Scheme 2). We found that proper folding and maturation of cpRuby-OxyR constructs were highly sensitive to the linker sequences, as only the cpRuby-OxyR-Al/TR which contains linkers mimicking the original sequence of RCaMP1.019 displayed optimal expression and maturation (Figure 2). HEK293T transiently expressing cpRuby-OxyR-Al/TR showed no changes in fluorescence emission intensity upon treatment with either H$_2$O$_2$ (100 µM) or DTT (1 mM) (Figure 3). Similar results were obtained from other cpRuby-OxyR constructs. It is possible that the difference in barrel structure and the site of termini between cpRuby and cpYFP affects the disulfide formation between OxyR1 and OxyR2. Either there is no disulfide formation or the disulfide bond does not substantially change the cpRuby conformation. To further investigate the OxyR portion of this protein, purified cpRuby-OxyR can be used to determine the number of reduced cysteine in reducing and oxidizing condition. Site-directed mutagenesis of OxyR1 and OxyR2 sequence in proximity to the Al/TR linker could potentially lead to insights for optimizing the sensor construct. Switching to other circularly permuted red fluorescence protein such as cp(mCherry) and cp(mKate)$^9$ could also lead to some improvement because of the different permutation site in each clone.$^{10,11}$

**Summary:** Replacing cpYFP in Hyper with cpRuby did not give a hit clone for red-emitting fluorescent protein sensor for H$_2$O$_2$. Future investigation is needed on optimizing the linkers between cpRuby and OxyR to improve the sensing dynamic range as well as the protein expression and maturation.

**Experimental Section**

**Materials and Methods.** pHyper-Cyto was obtained from Evrogen (Moscow, Russia). pMax-GFP was obtained from Amaxa-Lonza (Switzerland). mRuby-CaMP was a gift from Looger Lab (HHMI). DNA polymerase Phusion HF, restriction endonuclease *NheI* and *XhoI* and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Primers for PCR amplification were synthesized by Integrated DNA Technologies (San Diego, CA). Chemically competent TOP10 was obtained from Invitrogen (Carlsbad, CA).

**Synthesis of cpRuby-OxyR Constructs and Sub-Cloning.** OxyR1, 5’region of OxyR gene, corresponding to the N terminal, was amplified from pHyper-Cyto template using primer P1 and the appropriate reverse primer containing sequence for linkers. OxyR2, 3’region of OxyR gene, corresponding to the C terminal was amplified using primer P2 and the appropriate forward primer containing sequence for linkers. The coding sequence for cpRuby was obtained from mRuby-CaMP by amplifying using primers containing sequence for N and C termini linker. Amplified regions were purified after separation on a 1% agarose gel. Sequence overlap extension PCR was used to combined OxyR1, cpRuby, and OxyR2. Following the completion of the PCR cycle, the product was purified by separation on 1% agarose gel, and doubly digested with *NheI* and *XhoI* for 4 h at 37°C. The doubly-cut cpRuby-OxyR constructs were combined in a 1:2 (template: insert) molar ratio with *NheI/XhoI* cut pMax and ligated using T4 DNA ligase at 15 °C for 12 h. Ligation reactions were transformed into chemically competent TOP10 cells and plated onto LB/agar/kanamycin and grown overnight. Colonies were screened for the presence of
desired insert using colony PCR using P1 and P2 primers. Positive hits were identified by the presence of a band corresponding to the molecular weight of the cpRuby-OxyR. Positive hits were confirmed by sequencing.

Expression of cpRuby-OxyR Constructs in HEK293T and Fluorescence Imaging Experiments. HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before transfection, cells were passaged and plated on 4-wells Lab-Tek borosilicate chambered coverglass (Nunc). Purified plasmid from the confirmed clones were transformed into HEK293T cells using Lipofectamine 2000 (Invitrogen) following the standard protocol. At 24 h after transfection, fluorescence imaging was performed with a Zeiss LSM710 Axiovert laser scanning microscope and a 40x water-immersion objective lens. The motorized stage on the microscope was equipped with an incubator, maintaining the sample at 37 °C in a 5% CO₂ humidified atmosphere. Excitation of mRuby was carried out with a Helium-Neon 543 nm laser and 560 – 700 nm emission collection window. The culture media was replaced with 250 µL DPBS, and then imaged using fast scanning. Four fields of view containing high population of cells expressing mRuby were marked. H₂O₂ was delivered on-stage (250 µL, 200 µM in DPBS). Images were taken before and after addition of H₂O₂ for 5, 10, and 15 min. Image analysis was performed in ImageJ (National Institute of Health).
Figures and Schemes

Scheme 1. Design and synthesis of cpRuby-OxyR, cpYFP on Hyper-cyto is substituted by cpRuby obtained from RCaMP1.019.
| Scheme 2. cpRuby-OxyR obtained from sequence overlap extension PCR. |
Figure 1. Detection of subcellular Ca\(^{2+}\) by cpRuby-CaMP. (a) HEK293T transiently expressing cpRuby-CaMP. (b) Treatment with ionomycin caused influx of calcium, resulting in 1.5 fold increase in fluorescence emission intensity. (c) Brightfield image with 20 µm scale bar. (\(\lambda_{\text{exc}} = 543\) nm, collection window 560 - 700 nm).
Figure 2. Expression and maturation of variants of cpRuby-OxyR which differ in the short amino acid linkers between OxyR and cpRuby. Images were taken 24 h after transfection using the same setting for image acquisition. ($\lambda_{exc} = 543$ nm, collection window 560 - 700 nm).
Figure 3. cpRuby-OxyR-AI/TR showed no response to added H₂O₂ or DTT. HEK293T was transiently expressing cpRuby-OxyR-AI/TR. (a) Images of cells before and after 15 min treatment with 100 µM H₂O₂. (b) Images of cells before and after 15 min treatment with 1 mM DTT. Scale bar = 20 µm. (λ<sub>exc</sub> = 543 nm, collection window 560 - 700 nm)
<table>
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<tr>
<th>PCR Fragment</th>
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*Underline = aminoacid linker, Italic = restriction site, Bold = OxyR sequence, Bold in red = cpRuby sequence*
References


