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## Boronate Oxidation as a Bioorthogonal Reaction Approach for Studying the Chemistry of Hydrogen Peroxide in Living Systems

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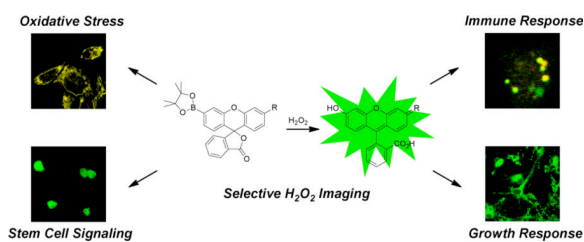
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### Conspectus

Reactive oxygen species (ROS), such as hydrogen peroxide, are important products of oxygen metabolism that, when misregulated, can accumulate and cause oxidative stress inside cells. Accordingly, organisms have evolved molecular systems, including antioxidant metalloenzymes (such as superoxide dismutase and catalase) and an array of thiol-based redox couples, to neutralize this threat to the cell when it occurs. On the other hand, emerging evidence shows that the controlled generation of ROS, particularly H<sub>2</sub>O<sub>2</sub>, is necessary to maintain cellular fitness. The identification of NADPH oxidase enzymes, which generate specific ROS and reside in virtually all cell types throughout the body, is a prime example. Indeed, a growing body of work shows that H<sub>2</sub>O<sub>2</sub> and other ROS have essential functions in healthy, physiological signaling pathways.

The signal–stress dichotomy of H<sub>2</sub>O<sub>2</sub> serves as a source of motivation for disentangling its beneficial from its detrimental effects on living systems. Molecular imaging of this oxygen metabolite with reaction-based probes is a powerful approach for real-time, noninvasive monitoring of H<sub>2</sub>O<sub>2</sub> chemistry in biological specimens, but two key challenges to studying H<sub>2</sub>O<sub>2</sub> in this way are chemoselectivity and bioorthogonality of probe molecules. Chemoselectivity is problematic because traditional methods for ROS detection suffer from nonspecific reactivity with other ROS. Moreover, some methods require enzymatic additives not compatible with live-cell or live-animal specimens. Additionally, bioorthogonality requires that the reactions must not compete with or disturb intrinsic cellular chemistry; this requirement is particularly critical with thiol- or metal-based couples mediating the major redox events within the cell.

Chemoselective bioorthogonal reactions—such as alkyne–azide cycloadditions and related click reactions, the Staudinger–Bertozzi ligation, and the transformations used in various reaction-based molecular probes—have found widespread application in the modification, labeling, and detection of biological molecules and processes. In this Account, we summarize H<sub>2</sub>O<sub>2</sub> studies from our laboratory using the H<sub>2</sub>O<sub>2</sub>-mediated oxidation of aryl boronates to phenols as a bioorthogonal approach to detect fluxes of this important ROS in living systems. We have installed this versatile switch onto organic and inorganic scaffolds to serve as ‘turn-on’ probes for visible and near-infrared (NIR) fluorescence, ratiometric fluorescence, time-gated lanthanide luminescence, and in vivo bioluminescence detection of H<sub>2</sub>O<sub>2</sub> in living cells and animals. Further chemical and genetic manipulations target these probes to specific organelles and other subcellular locales and can also allow them to be trapped intracellularly, enhancing their sensitivity. These novel chemical tools have revealed fundamental new biological insights into the production, localization, trafficking, and in vivo roles of H<sub>2</sub>O<sub>2</sub> in a wide variety of living systems, including immune, cancer, stem, and neural cell models.



## 1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ) play fundamental roles in health and disease but are difficult to study due to their reactive and transient nature (Figure 1).<sup>1–10</sup> In isolated mitochondria, 0.1–2% of all oxygen consumed is estimated to produce ROS,<sup>11</sup> noting that *in vivo* quantitation is challenging<sup>12</sup> and ROS measurements traditionally rely on analysis of downstream chemical products of oxidation.<sup>13</sup> When misregulated, these species can accumulate and cause oxidative damage to cellular protein,<sup>14</sup> nucleic acids,<sup>15</sup> and lipid<sup>16</sup> molecules, thereby contributing to aging<sup>17–19</sup> and age-related disease states ranging from neurodegeneration<sup>20–22</sup> to diabetes<sup>23–25</sup> to cancer.<sup>26–28</sup> Unsurprisingly, organisms have evolved molecular systems, including antioxidant metalloenzymes such as superoxide dismutase<sup>29</sup> and catalase,<sup>30–31</sup> as well as an array of thiol-based redox couples, to scavenge and/or respond to ROS in order to appropriately neutralize this threat to the cell.<sup>32–33</sup> On the other hand, emerging evidence shows that controlled generation of ROS, particularly  $H_2O_2$ , can also be beneficial to cell fitness. A prime example is the presence of active NADPH oxidase enzymes (Nox) that generate specific ROS and reside in virtually all cell types throughout the body.<sup>34</sup> Indeed, a growing body of work points to the fact that  $H_2O_2$  and other ROS play essential functions in healthy, physiological signaling pathways spanning growth,<sup>35–36</sup> differentiation,<sup>37–38</sup> migration,<sup>39–40</sup> and immune system function.<sup>41–42</sup>

The signal/stress dichotomy of  $H_2O_2$  provides motivation to disentangle its beneficial from its detrimental effects on living systems, and molecular imaging of this oxygen metabolite is a powerful method for real-time, non-invasive monitoring of  $H_2O_2$  chemistry in biological specimens. Two key challenges to studying  $H_2O_2$  in this context are chemoselectivity, as traditional methods for ROS detection<sup>43</sup> such as 2'-7'-dichlorodihydrofluorescein<sup>44</sup> and the amplex red/peroxidase system<sup>45</sup> suffer from non-specific reactivity with other ROS or require enzymatic additives that are not compatible with live-cell or live-animal specimens, and bioorthogonality, as not to compete with and/or disturb intrinsic cellular chemistry, which is particularly critical with thiol- or metal-based couples mediating the major redox events within the cell. In order to study the complex roles of  $H_2O_2$  in living systems and to elucidate its contributions to health, aging, and disease, we have initiated a program aimed at creating and applying new methods for chemoselective, real-time molecular imaging of  $H_2O_2$  in live cells, tissues, and whole organisms. This Account summarizes our efforts to date on the use of  $H_2O_2$ -mediated boronate oxidation as a bioorthogonal reaction-based approach to  $H_2O_2$  detection.

## 2. Application of Oxidative Boronate Cleavage as a Bioorthogonal Reaction for Imaging Hydrogen Peroxide in Living Systems

We considered two main strategies for creating probes to detect molecular entities in biological systems, which we term "recognition" and "reactivity" (Figure 2). Traditional methods for sensing biological analytes largely rely on former, an approach that has been particularly successful for imaging calcium and other biologically relevant metals using

small-molecule fluorescent chemosensors.<sup>46</sup> In this strategy, fluorophores linked to highly specific receptors are designed such that recognition and binding of the analyte of interest provides an observable optical response. However, because H<sub>2</sub>O<sub>2</sub> and other ROS are transient in nature, and many of these oxygen metabolites are similar in shape and size, building chemoselective hosts for these molecules by a traditional lock-and-key approach is particularly challenging. We reasoned that a reaction-based approach would be a more attractive detection strategy for H<sub>2</sub>O<sub>2</sub> in that it offers the opportunity for selective sensing based on the inherent chemical reactivity of a given species instead of its physical shape or size. Moreover, if the reaction is irreversible, this method affords the opportunity to accumulate signal from transient fluxes of reactive species over time.

In order to develop useful probes for H<sub>2</sub>O<sub>2</sub>, we were particularly motivated to identify chemical reactions that would be selective for H<sub>2</sub>O<sub>2</sub> over other biologically relevant ROS including, among others, superoxide (O<sub>2</sub><sup>-</sup>), hypochlorous acid (HOCl), alkyl peroxides (ROOH), and hydroxyl radical (•OH). The key insight to the success of this approach lies in understanding the inherent differences in the reactivity of these species, and in designing transformations that take advantage of the unique chemical properties of H<sub>2</sub>O<sub>2</sub>. In this context, H<sub>2</sub>O<sub>2</sub> possesses ambiphilic reactivity; its labile O–O bond allows it to react as a two-electron electrophilic oxidant, whereas H<sub>2</sub>O<sub>2</sub> can also be a good nucleophile owing to the  $\alpha$ -effect of adjacent non-bonding orbitals on its oxygen atoms.<sup>47–48</sup> To take advantage of these unique molecular features, we identified aryl boronates as species with complementary ambiphilic reactivity to H<sub>2</sub>O<sub>2</sub> (Figure 2). Upon initially reacting as an electrophile in a reversible manner with nucleophiles to form a negatively charged tetrahedral boronate complex, the C–B bond becomes subsequently capable of reacting as a nucleophile. We reasoned that we could capitalize on this dual-mode reactivity of H<sub>2</sub>O<sub>2</sub> with boronates to achieve selectivity over other biologically relevant ROS, as most of the other oxygen metabolites operate by one-electron transfers or purely electrophilic oxidation pathways. In addition, H<sub>2</sub>O<sub>2</sub> should react with boronates faster than the corresponding alkyl peroxides as water is a better leaving group than alcohols, giving specificity for free H<sub>2</sub>O<sub>2</sub> over lipid-derived peroxides, and the reaction is accelerated at higher pH values so caution is required in quantitating ROS bursts accompanied by large local pH changes. Having identified phenols as functional groups that could be released from masked boronates by the action of H<sub>2</sub>O<sub>2</sub>, we have exploited this single reaction to devise a wide array of fluorogenic and luminescent molecules that detect H<sub>2</sub>O<sub>2</sub> through boronate oxidation (Figure 2). We note that this approach offers generality for monitoring any molecular species of interest in living systems if appropriate combinations of reactivity and bioorthogonality can be suitably balanced.

### 3. First-Generation Boronate Probes for Detecting Hydrogen Peroxide at Oxidative Stress Levels in Living Cells

Fluorescein is a widely used dye for cellular imaging applications owing to its high fluorescence quantum yield, good water solubility, non-toxic nature, and established use in live and fixed cell specimens. We therefore synthesized the boronate masked fluorescein Peroxyfluor 1 PF1<sup>49</sup> as a first-generation H<sub>2</sub>O<sub>2</sub> probe prototype through a 3'–6'-diiodoxanthenone intermediate followed by a palladium-mediated boronate transmetalation (Table 1, Figure 3). Installation of boronates at the 3' and 6' positions drives the molecule into a closed lactone form, effectively eliminating its absorptive and emissive properties in the visible wavelength region. Reaction of PF1 with H<sub>2</sub>O<sub>2</sub> transforms the boronates into phenols with concomitant lactone opening resulting in a >1000-fold increase in fluorescence intensity upon generation of the green-fluorescent fluorescein product. Importantly, this response is selective towards H<sub>2</sub>O<sub>2</sub> over other biologically relevant ROS (Figure 4). Moreover, this first-generation probe could effectively image changes in high  $\mu$ M

concentrations  $\text{H}_2\text{O}_2$ , a concentration range is relevant to states of oxidative stress, in both live HEK293T cells and primary hippocampal neural cell cultures (Figure 5).<sup>50</sup> We further demonstrated the generality of this reaction-based approach for  $\text{H}_2\text{O}_2$  detection through the development of Peroxyxanthone 1 (PX1) and Peroxyresorufin 1 (PR1) probes, which are based on blue xanthone and red resorufin scaffolds, respectively. The success of these probes for visualizing changes in  $\text{H}_2\text{O}_2$  levels under oxidative stress conditions establishes that bioorthogonal boronate chemistry is a suitable approach for detection of this ROS in live biological samples.

#### 4. Second-Generation Boronate Probes for Studying Endogenous Peroxide Bursts Produced during Growth Factor and Immune Response Signaling

Although the first-generation family of PF1/PR1/PX1 probes were capable of imaging exogenously added  $\text{H}_2\text{O}_2$  in a variety of cell types at oxidative stress levels, attempts to use these reagents for visualizing endogenous production of  $\text{H}_2\text{O}_2$  were frustrated by their relatively low  $\text{H}_2\text{O}_2$  sensitivity. In order to address this challenge, we reasoned that switching from bis-boronate masked dyes to monoboronate caged fluorophores would result in enhanced sensitivity by requiring reaction of only a single equivalent of  $\text{H}_2\text{O}_2$  to yield the fluorescent product. To this end, we synthesized Peroxy Green 1 (PG1) based on Nagano's Tokyo Green scaffold.<sup>51</sup> Owing to its boronate cage, PG1 retains high selectivity for  $\text{H}_2\text{O}_2$  and we therefore moved on to apply this probe toward endogenous  $\text{H}_2\text{O}_2$  detection.

In this context, ROS are produced upon stimulations of cells with extracellular ligands such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), which then in turn can mediate intracellular kinase signaling.<sup>52–53</sup> However, the precise nature of the contributing ROS, the enzymatic source(s) of its generation, its trafficking, and cellular targets remain incompletely understood. With a sensitive and selective  $\text{H}_2\text{O}_2$  probe in our possession, we turned our attention to EGF signaling in A431 cells because this epidermoid carcinoma line possesses an unusually high copy number of the requisite EGF receptor.<sup>54</sup> After confirming the ability of PG1 to detect exogenously applied  $\text{H}_2\text{O}_2$  in the A431 line, we discovered that EGF stimulation of these cells also leads to a marked increase in PG1 fluorescence, directly establishing  $\text{H}_2\text{O}_2$  production in EGF signaling.<sup>51</sup> Furthermore, we utilized PG1 imaging in combination with a panel of pharmacological inhibitors to establish that a Nox enzyme was responsible for  $\text{H}_2\text{O}_2$  production and that the initial signal is dependent on phosphatidylinositol-3-OH kinase (PI3K) activity (Figure 6). In this same study, we also demonstrated that this growth factor signaling pathway is present in primary hippocampal cell cultures. Taken together, this work provides the first direct imaging evidence that  $\text{H}_2\text{O}_2$  is endogenously generated during cell signaling and establishes that the boronate reactivity approach can be used for elucidating the roles of  $\text{H}_2\text{O}_2$  in biological systems.

Encouraged by these results, we synthesized a panel of monoboronate probes for  $\text{H}_2\text{O}_2$  at physiological signaling levels with excitation and emission profiles that span the visible region.<sup>55</sup> We utilized three of these reagents, Peroxyfluor 3 (PF3), Peroxy Yellow 1 (PY1), and Peroxy Orange 1 (PO1), for chemoselective monitoring of  $\text{H}_2\text{O}_2$  signaling through growth factor stimulation as well as during phagocytic immune response. Moreover, we established the utility of this expanded color palette by performing two-color, dual-ROS imaging experiments with PO1 in combination with APF,<sup>56</sup> a probe for highly reactive oxygen species (hROS) that responds to hypochlorite ( $\text{HOCl}$ ), hydroxyl radical ( $\bullet\text{OH}$ ), and peroxynitrite ( $\text{ONOO}^-$ ). Interestingly, we discovered three different types of phagosomes as characterized by the distinct types of ROS produced - ones that produce predominately  $\text{H}_2\text{O}_2$ , ones that produce predominately hROS, and ones that produce both (Figure 7). This work highlights the advantage of using multiple bioorthogonal probes to disentangle which

discrete types of ROS molecules contribute to a given biological process, as each particular oxygen metabolite has its own distinct chemical reactivity within a more complex setting.

## 5. Dual-Wavelength Boronate Probes for Ratiometric Peroxide Imaging in Living Cells

Intensity-based turn-on fluorescent probes are of practical utility for many applications and can be employed for comparative discovery studies, but their use for precise quantitation of analytes is complicated by variations in sample thickness, cellular microenvironments, and local probe concentrations. To address these issues, we have explored ratiometric approaches in which one can simultaneously monitor two signals that change differentially with analyte concentration, such that the ratio of the signals will be independent of the probe concentration and environment and allow for more accurate and quantitative measurements.<sup>57</sup> Our first probe for ratiometric H<sub>2</sub>O<sub>2</sub> detection, Ratio Peroxyfluor 1 (RPF1), utilizes a two-dye cassette that undergoes changes in the ratio of fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores upon reaction with H<sub>2</sub>O<sub>2</sub>.<sup>58</sup> Whereas most FRET probes operate by a physical change in donor-acceptor distances and orientations to sense analytes, we opted to modulate FRET properties in an electronic fashion by triggering changes the donor-acceptor spectral overlap before and after reaction with H<sub>2</sub>O<sub>2</sub>. In the absence of H<sub>2</sub>O<sub>2</sub>, the FRET acceptor is a PF1 derivative that has negligible visible absorption, leading to poor FRET from the coumarin donor. However, upon reaction with H<sub>2</sub>O<sub>2</sub>, generation of the colored fluorescein product creates an efficient FRET acceptor for the coumarin donor. Experiments in isolated mitochondria from *Saccharomyces cerevisiae* show that RPF1 can detect H<sub>2</sub>O<sub>2</sub> generated by antimycin A inhibition of the electron transport chain (ETC). We have extended this ratiometric imaging approach to live-cell imaging by tuning the internal charge transfer (ICT) properties of a single dye to cause a shift in the observed emission maxima upon reaction with H<sub>2</sub>O<sub>2</sub>. Peroxy Lucifer 1 (PL1)<sup>59</sup> masks a pendant electron-rich amine as an electron-poor carbamate by using a *para*-boronate group that undergoes a selective H<sub>2</sub>O<sub>2</sub>-triggered self-immolative release.<sup>60</sup> PL1 can readily visualize localized increases in H<sub>2</sub>O<sub>2</sub> in the phagosomes of PMA-stimulated macrophages by ratiometric imaging (Figure 8).

## 6. Targetable and Trappable Boronate Probes for Understanding Peroxide Trafficking and Stem Cell Function

In addition to events at the whole single cell level, controlling the subcellular localization and trafficking of H<sub>2</sub>O<sub>2</sub> is a vital mechanism that the cell employs to attenuate its toxicity and offtarget function, allowing it mediate beneficial physiological processes. For example, organelles such as the mitochondria are equipped with numerous enzymes aimed at reducing levels of oxidative stress,<sup>12</sup> whereas the endoplasmic reticulum (ER) maintains a higher level of oxidative species in order to assure proper protein folding.<sup>61–62</sup> To better study the contributions of subcellular localization and trafficking of H<sub>2</sub>O<sub>2</sub> in physiological and pathological processes, we developed a family of boronate probes equipped with functional groups that are capable of targeting these tools to specific subcellular spaces. For example, Mitochondrial Peroxy Yellow 1 (MitoPY1) combines a monoboronate-masked fluorophore with Murphy's phosphonium cation for mitochondrial localization.<sup>63</sup> This probe can operate in a wide variety of cell types (HEK293T, HeLa, Cos7, and CHO.K1, etc) and can visualize discrete oxidative stress changes confined to this organelle, including those generated in a chemically-induced Parkinson's disease model.<sup>64</sup>

We have also recently explored a more general protein-based labeling approach that enables us to tag many parts of the cell<sup>65</sup> by exploiting Johnsson's elegant SNAP tag

methodology.<sup>66–68</sup> In this approach, any protein of interest can be fused with a mutant human O<sup>6</sup>-alkylguanine-DNA alkyltransferase (hAGT) protein that can be selectively tagged with benzyl guanine or benzyl-2-chloro-6-aminopyrimidin-4-amine containing dyes, allowing one to target a probe to any subcellular compartment of interest. Indeed, SNAP Peroxy Green 1 and 2 (SPG1 and SPG2) are two first-generation PG1 derivatives with linkages to benzyl guanine or benzyl-2-chloro-6-aminopyrimidin-4-amine, respectively, that afford a general method for directing H<sub>2</sub>O<sub>2</sub>-responsive dyes to the plasma membrane, mitochondria, ER, and nucleus (Figure 9).

Finally, we have developed two new types of probes, Peroxy Yellow 1 Methyl Ester (PY1-ME)<sup>69</sup> and Peroxyfluor 6 Acetoxymethyl Ester (PF6-AM),<sup>70</sup> that take advantage of multiple masked carboxylates to increase cellular retention and hence sensitivity to low levels of peroxide. In their ester-protected forms, the PY1-ME and PF6-AM dyes are more lipophilic than their carboxylate counterparts and can readily enter cells. Once inside cells, the protecting groups are rapidly cleaved by intracellular esterases to produce their anionic carboxylate forms, which are effectively trapped within cells as they cannot pass back through the plasma membrane. The increased retention of PY1-ME and PF6-AM leads to their enhanced sensitivity to H<sub>2</sub>O<sub>2</sub> by greater buildup of signal through irreversible boronate oxidation events. In particular, we utilized PY1-ME to interrogate the cellular mechanisms involved in the trafficking of H<sub>2</sub>O<sub>2</sub> during growth factor signaling (Figure 10) and discovered that certain classes of aquaporin water channels, the aquaglyceroporins and unorthodox aquaporins, but not classic aquaporins, can enhance the uptake of extracellularly-produced H<sub>2</sub>O<sub>2</sub> and regulate intracellular signal transduction. This work represents the first study revealing that aquaporins can mediate both H<sub>2</sub>O<sub>2</sub> transport and signaling in mammalian cells and has broad implications for H<sub>2</sub>O<sub>2</sub> biology in processes ranging from cell migration<sup>39</sup> to wound repair.<sup>40</sup> In parallel work using PF6-AM imaging, we have explored the roles of H<sub>2</sub>O<sub>2</sub> in the self-renewal of neural stem cells. Specifically, we discovered that adult hippocampal progenitor cells (AHPs) require basal generation of H<sub>2</sub>O<sub>2</sub> for their normal growth and proliferation in cell culture and *in vivo* and determined that the Nox2 enzyme and phosphatase PTEN are molecular sources and targets of H<sub>2</sub>O<sub>2</sub>, respectively (Figure 11). This study provides primary evidence that H<sub>2</sub>O<sub>2</sub> is a physiological regulator in living organisms and a molecular model for how H<sub>2</sub>O<sub>2</sub> can mediate beneficial events.

## 7. Boronate Reporters for Imaging Hydrogen Peroxide in Live Tissue and Animals

In addition to creating boronate probes for imaging in dissociated cell culture samples, we have also initiated several parallel approaches to expand H<sub>2</sub>O<sub>2</sub> imaging technologies to thicker tissue and whole animal specimens. In particular, we have explored optical H<sub>2</sub>O<sub>2</sub> detection in the near infrared spectroscopic window, where the absorption and autofluorescence of biological tissues is at a minimum, using Naphtho Peroxyfluor 1 (NPF1),<sup>71</sup> as well as lanthanide luminescence, which allows the use of time-gated imaging to decrease autofluorescence from native, short-lived organic species in biological specimens, through the synthesis and evaluation of Terbium Peroxy Reporters 1 and 2 (TPR1 and TPR2).<sup>72</sup>

Finally, we have recently succeeded in establishing boronate oxidation as a bioorthogonal reaction approach for imaging H<sub>2</sub>O<sub>2</sub> in living animals through the creation of the bioluminescent probe Peroxy Caged Luciferin 1 (PCL1).<sup>73</sup> Bioluminescence is a modality that drastically increases sensitivity for *in vivo* imaging owing to the absence of any autofluorescence background endemic to fluorescence techniques. By caging luciferin with a self-immolative boronic acid, we can use this reaction-based approach to selectively detect

H<sub>2</sub>O<sub>2</sub> in living luciferase-expressing mice (FVB-luc+) through H<sub>2</sub>O<sub>2</sub>-dependent luciferin generation (Figure 12). Moreover, PCL1 can visualize H<sub>2</sub>O<sub>2</sub> generated by a luciferase-expressing prostate cancer cell line (LNCaP-luc) as well as in an LNCaP-luc tumor xenograft model in immunodeficient SCID hairless outbred (SHO) mice following testosterone stimulation. By expanding the scope of boronate oxidation to living animals, these results offer a significant technical advance for exploring H<sub>2</sub>O<sub>2</sub> chemistry in complex biological settings, particularly for models of disease and aging that occur on the whole organism scale.

## 8. Concluding Remarks

In this Account, we have described the identification and application of H<sub>2</sub>O<sub>2</sub>-mediated boronate oxidation as a chemoselective reaction-based approach for studying the chemistry of H<sub>2</sub>O<sub>2</sub> in complex biological systems. This switch has been installed onto a diverse array of scaffolds for bioorthogonal optical detection of H<sub>2</sub>O<sub>2</sub> in living cells and animals. Monoboronate-bearing fluorophores have proved to be the most effective chemical tools for live-cell microscopy experiments and methods for reaction-based trapping inside cells have yielded probes useful for interrogating the production, trafficking, and targets of H<sub>2</sub>O<sub>2</sub> in growth factor signaling, immune response, and stem cell function. Both chemical and biological strategies have been successful in targeting probes to subcellular domains, providing a means to study the roles of H<sub>2</sub>O<sub>2</sub> in specific organelles, and continued efforts in the development of ratiometric, targetable, near-IR, and lanthanide-based luminescent probes are expected to provide new opportunities for the precise imaging of subcellular domains as well as the study of thicker biological specimens. Finally, the development of a boronate-caged luciferin has enabled the bioluminescent imaging of H<sub>2</sub>O<sub>2</sub> *in vivo* and opens up many new avenues for studying H<sub>2</sub>O<sub>2</sub> in animal models of health and disease.

In addition to these aforementioned studies, a growing number of methods exploiting boronate oxidation show the broader utility of this switch for probing and manipulating peroxide biology. Innovative examples include alternative H<sub>2</sub>O<sub>2</sub> indicators bearing blue-fluorescent,<sup>74,75</sup> dendrimer,<sup>76</sup> and near infrared<sup>77</sup> scaffolds, as well as mass spectrometry tags for tracking peroxide biology in whole organisms.<sup>78</sup> Oxidatively sensitive therapeutics such as masked metal chelators<sup>79,80</sup> and inhibitors<sup>81</sup> have also been devised using the boronate switch. Finally, recent data suggest that certain boronate derivatives may also find utility in probing peroxynitrite.<sup>82</sup> Finally, this boronate chemistry has inspired work into alternative bioorthogonal reactions for H<sub>2</sub>O<sub>2</sub>, including the oxidative decarboxylations of  $\alpha$ -ketoacids<sup>83</sup> and Baeyer-Villiger benzil oxidations.<sup>84</sup> The rapidly expanding toolbox for probing and manipulating H<sub>2</sub>O<sub>2</sub> in living systems presages new frontiers for discovering and understanding its fundamental roles in signaling and stress processes, and highlights the synergy between bioorthogonal chemistry and biology.

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## Biographies

Christopher J. Chang is an Associate Professor of Chemistry and HHMI Investigator at UC Berkeley. He received his B.S. and M.S. degrees from Caltech in 1997, working with Prof. Harry Gray. After spending a year as a Fulbright scholar in Strasbourg, France with Dr. Jean-Pierre Sauvage, Chris received his Ph.D. from MIT in 2002 under the supervision of Prof. Dan Nocera. He stayed at MIT as a postdoctoral fellow with Prof. Steve Lippard and

then began his independent career at UC Berkeley in Fall 2004. Research in the Chang lab is focused on chemical biology and inorganic chemistry, with particular interests in molecular imaging and catalysis applied to neuroscience, stem cells, cancer, infectious diseases, renewable energy, and green chemistry. His group's work has been honored by awards from the Dreyfus, Beckman, Sloan, and Packard Foundations, Amgen, Astra Zeneca, and Novartis, AFAR, Technology Review, the ACS Cope Scholar Fund, and the Society for Biological Inorganic Chemistry.

Alexander R. Lippert received his B.S. degree in 2003 from Caltech working with Prof. Linda Hsieh-Wilson. He then began his Ph.D. work with Prof. Jeffrey W. Bode at the University of California at Santa Barbara. In 2007, Alex moved with Prof. Bode to the University of Pennsylvania where he received his Ph.D. degree in 2008. He is currently a post-doctoral scholar with Prof. Chris Chang at the University of California, Berkeley, where he has applied organic chemistry to develop novel reaction-based approaches to studying biological systems.

Genevieve C. Van de Bittner was born and raised in Moraga, CA. She graduated with Honors in Chemistry (B.S. 2007) from Chapman University, where she performed research in the laboratories of Prof. Daniel Wellman and Prof. Kenneth Sumida. As an undergraduate, Genevieve also spent a summer at Cornell University working under the guidance of Prof. Dotsevi Sogah. She is currently completing her doctoral research in the laboratory of Prof. Chris Chang and was recently awarded a Klaus and Mary Ann Saegbarth Endowed Fellowship in Chemistry for her accomplishments in her graduate research.

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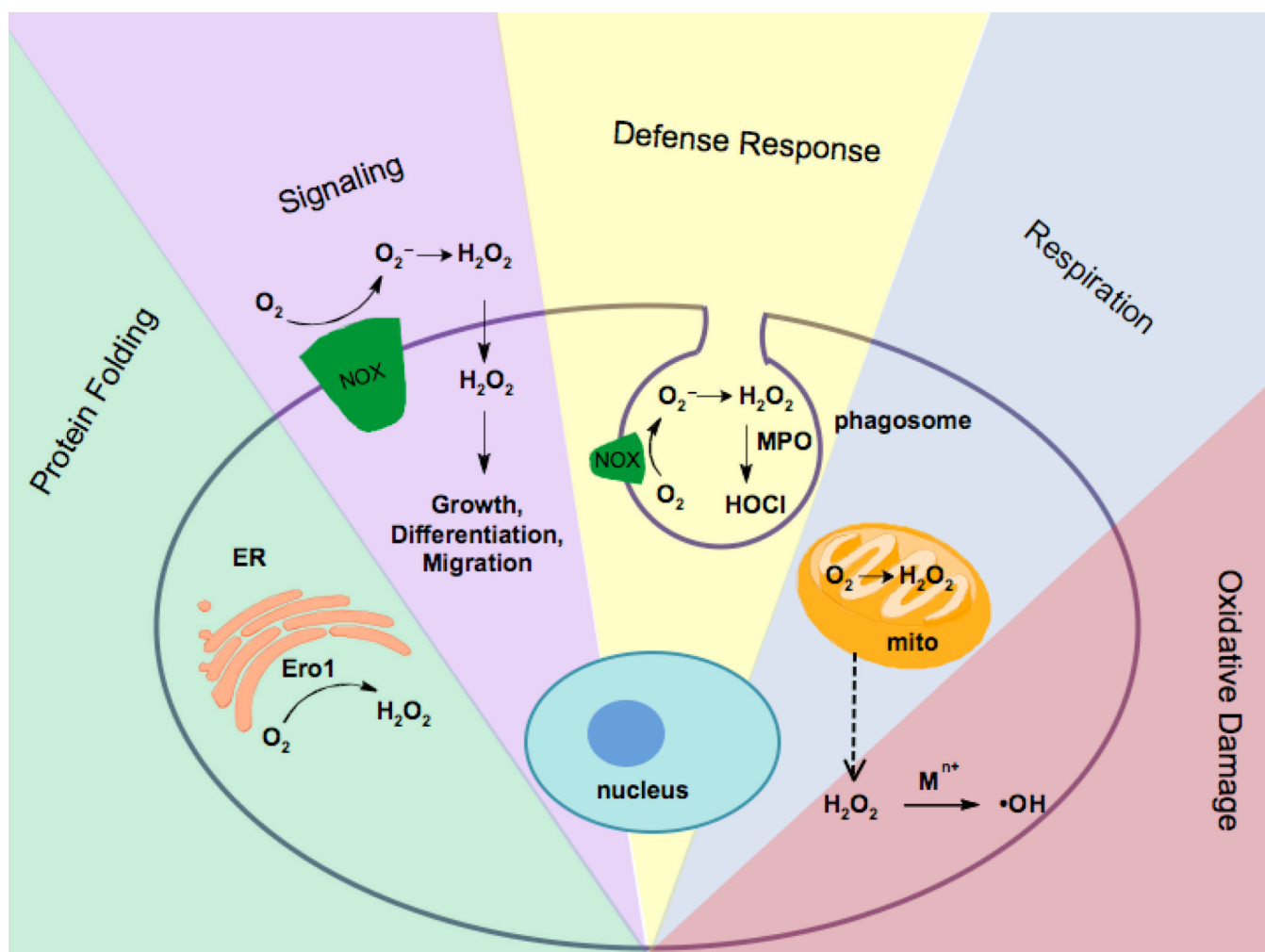


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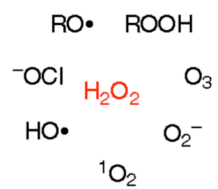
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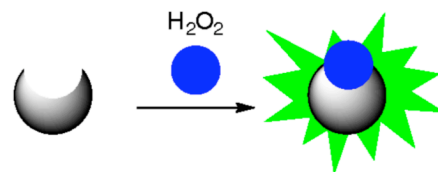
**Figure 1.** Unregulated production of ROS such as  $H_2O_2$  can result in oxidative damage, but these molecules also play central roles in protein folding, signaling, defense response, and respiration and metabolism.

### Solving the Selectivity Problem



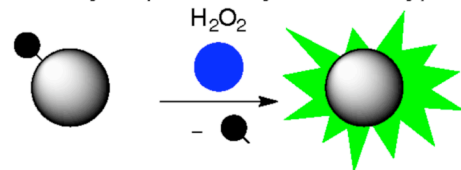
### Recognition

(difficult for small, reactive molecules)

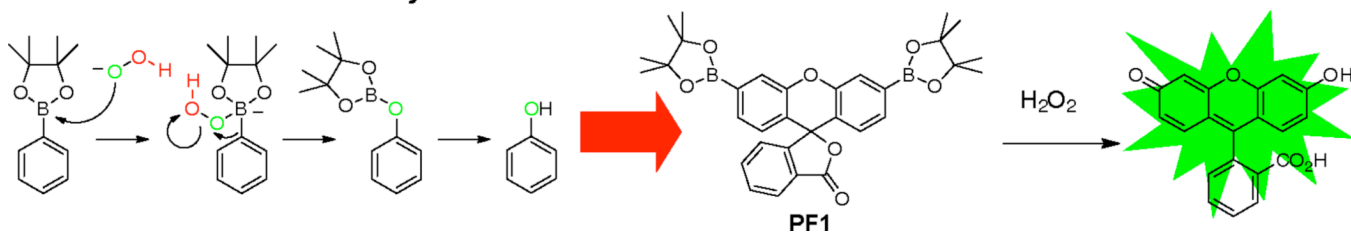


### Reactivity

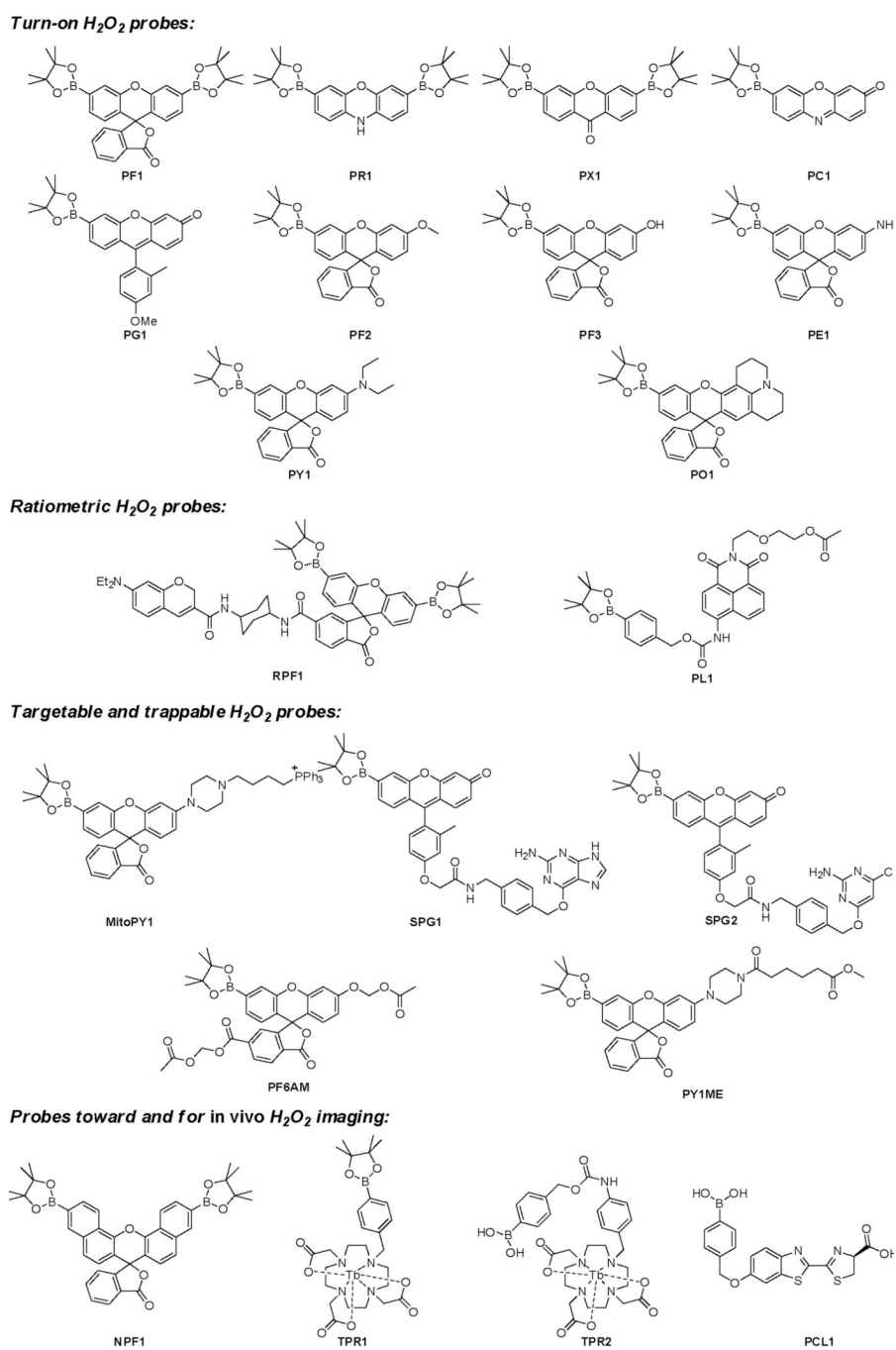
(selectivity imparted by chemistry)



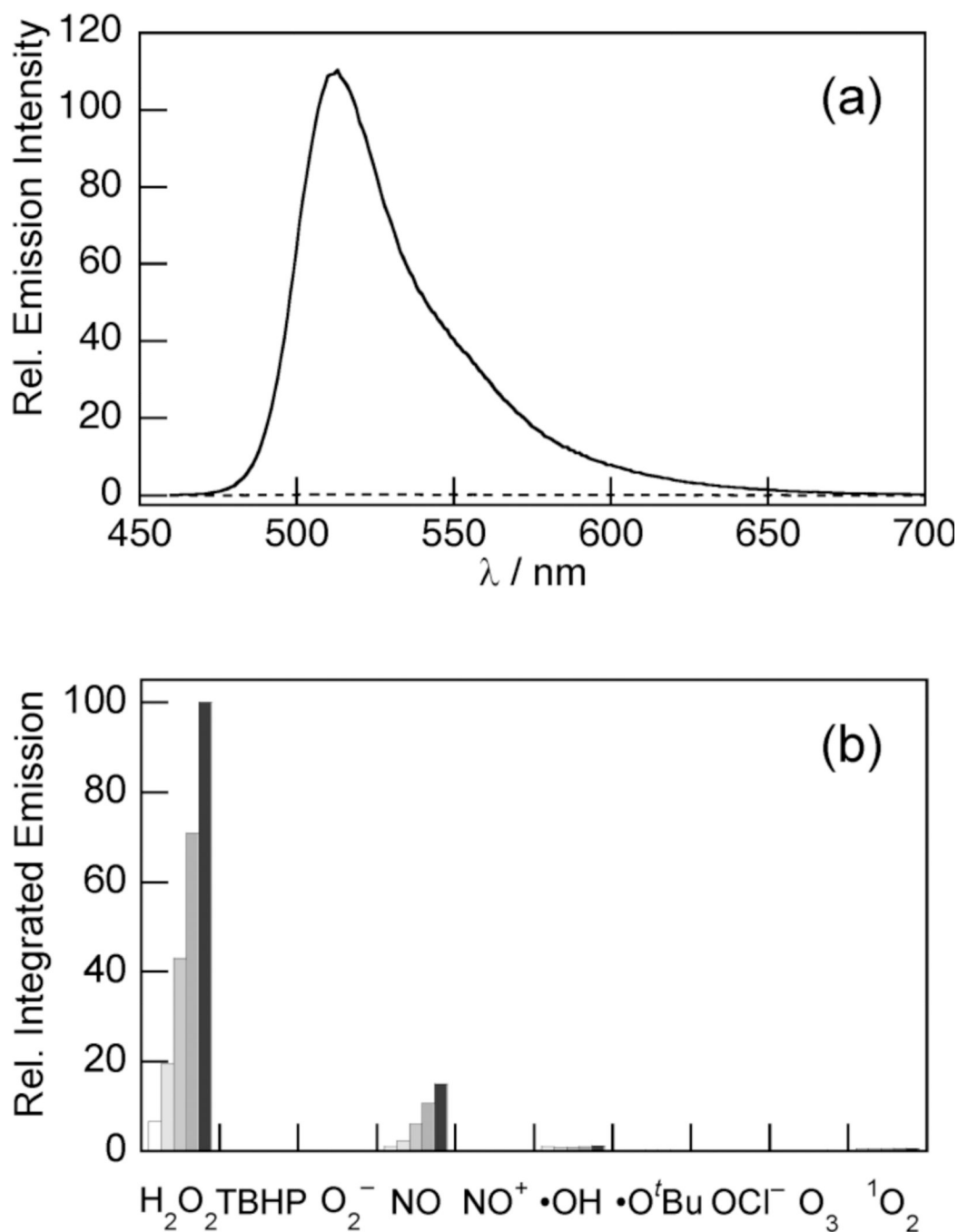
### Boronate Reactivity



**Figure 2.** Design of a bioorthogonal reactivity approach for selective  $\text{H}_2\text{O}_2$  detection via boronate oxidation.



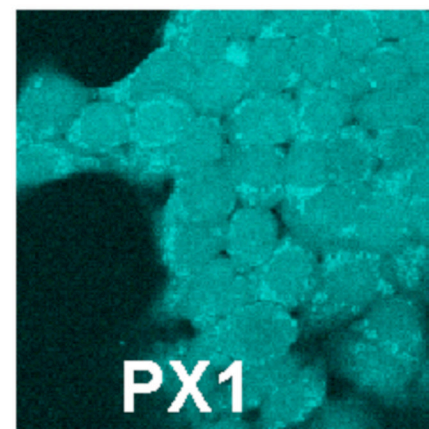
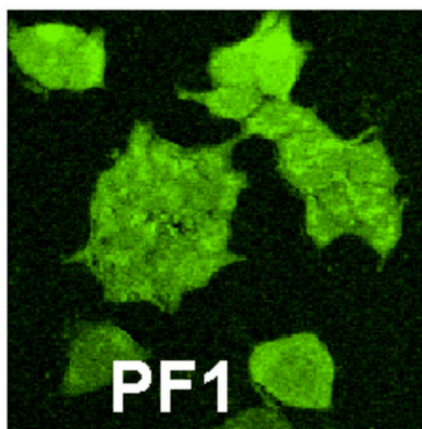
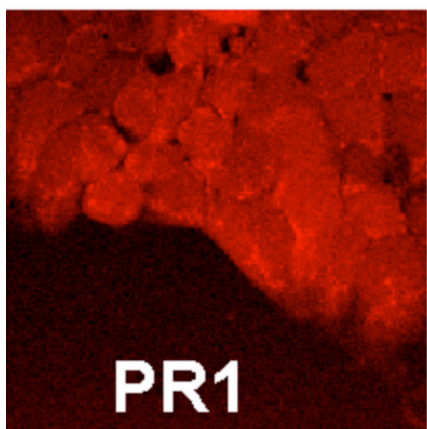
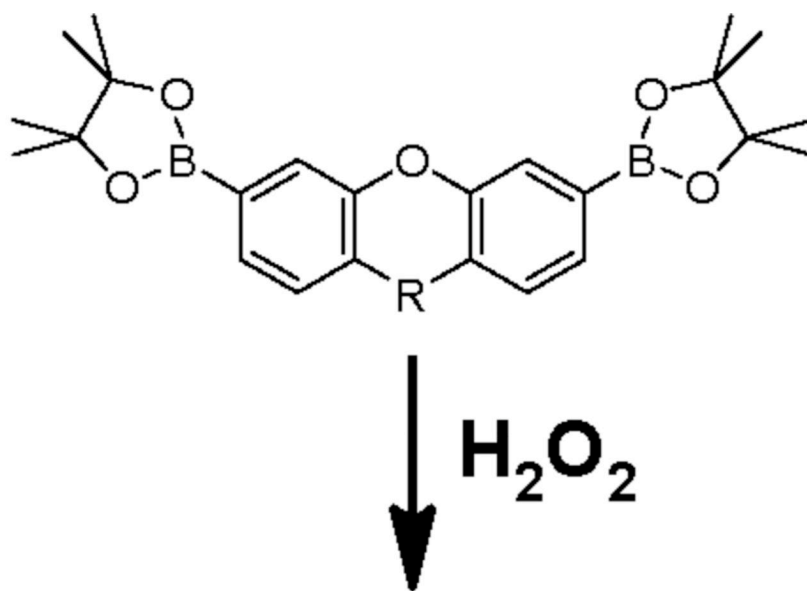
**Figure 3.**  
Boronate-based probes for  $H_2O_2$  detection and imaging.



**Figure 4.**

(a) Fluorescence response of Peroxyfluor 1 (PF1) to  $H_2O_2$ . The dashed and solid spectra were recorded before and after  $H_2O_2$  addition, respectively. (b) Fluorescence responses of PF1 to various ROS. Bars represent relative responses after 5, 15, 30, 45, and 60 min after addition of the given ROS.



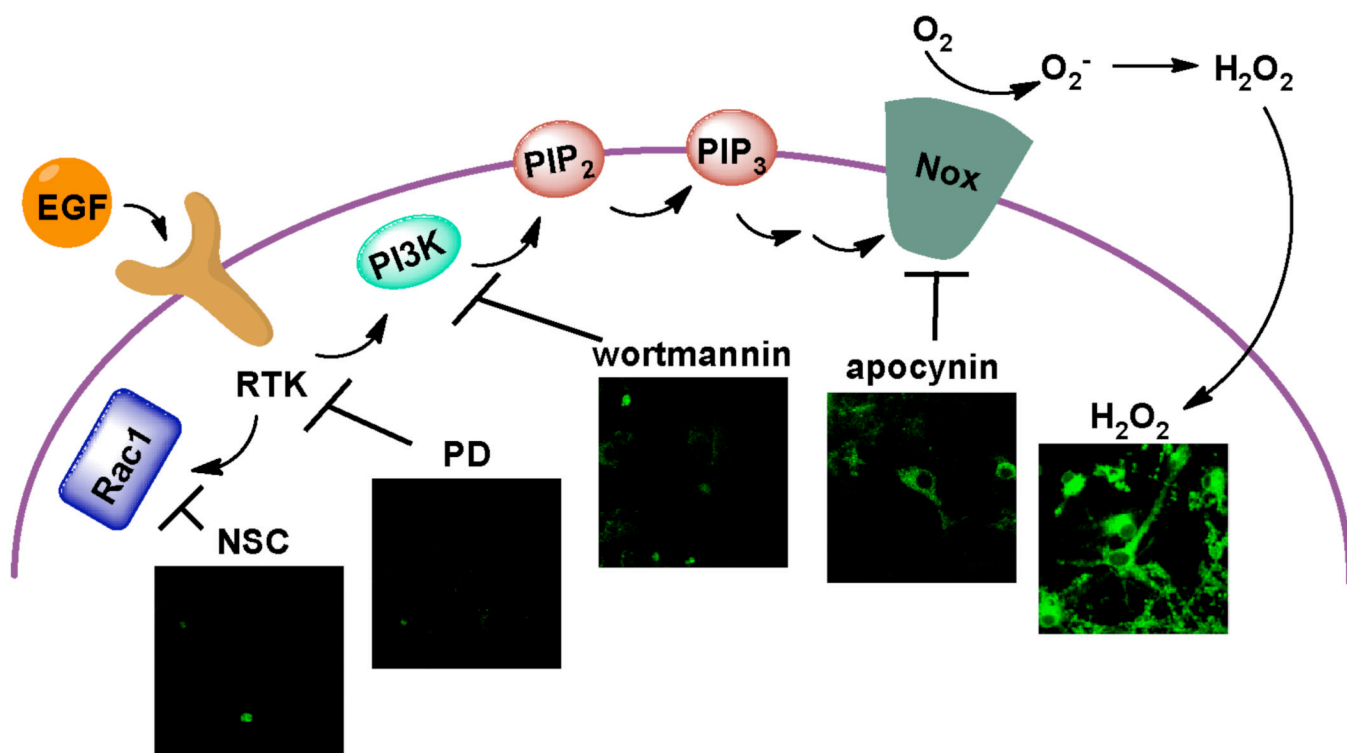


$\lambda_{\text{em}} = 590 \text{ nm}$

$\lambda_{\text{em}} = 512 \text{ nm}$

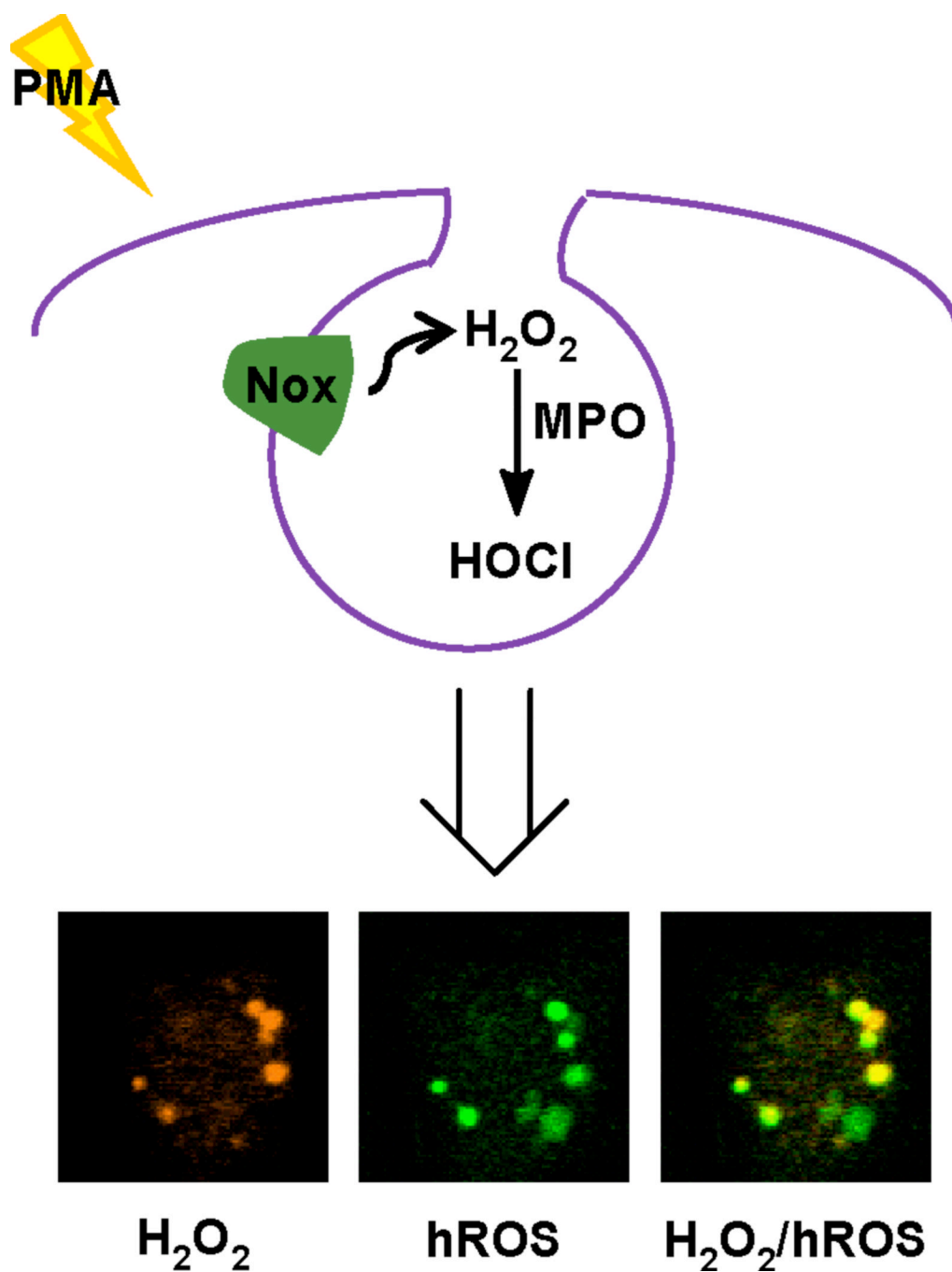
$\lambda_{\text{em}} = 450 \text{ nm}$

**Figure 5.** Images of Peroxyresorufin 1 (PR1), Peroxyfluor 1 (PF1), and Peroxyxanthone 1 (PX1) detecting  $\text{H}_2\text{O}_2$  fluxes in living cells.

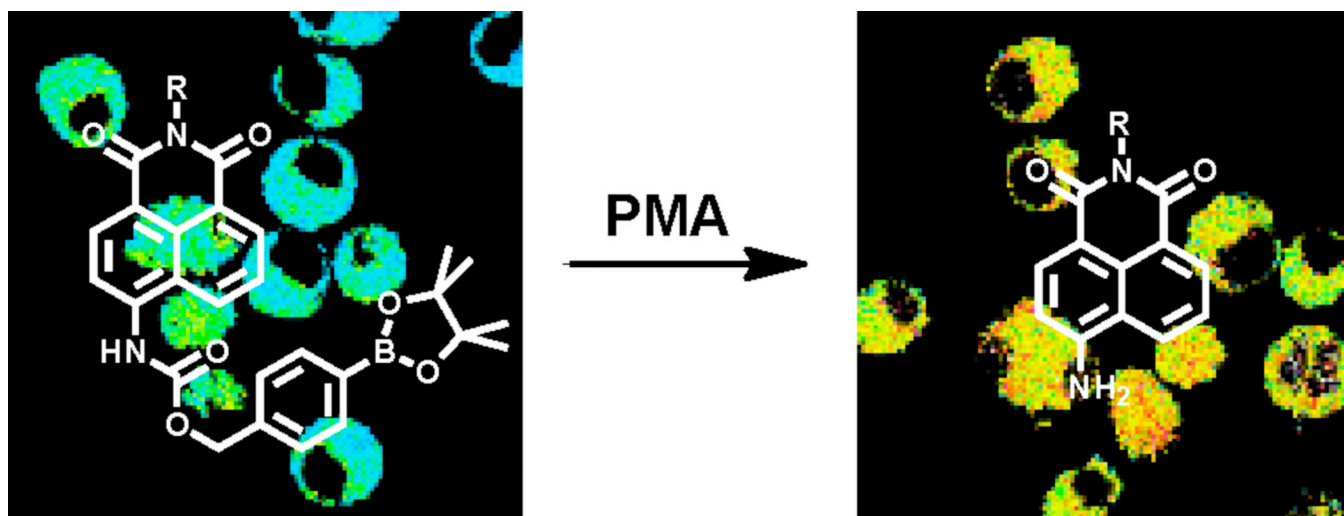


**Figure 6.**

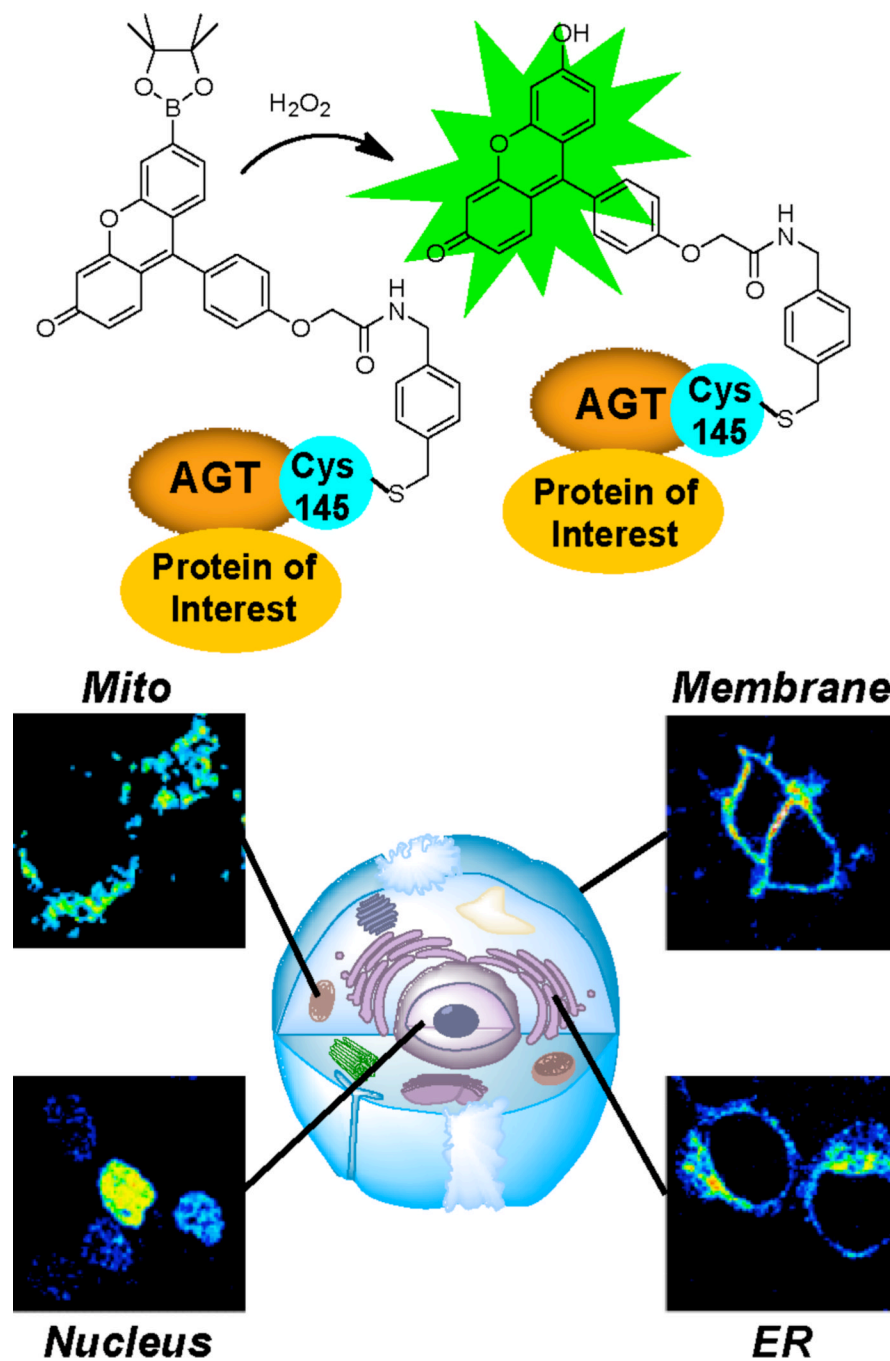
H<sub>2</sub>O<sub>2</sub> and growth factor signaling in living neurons. EGF stimulation produces an increase in the fluorescence response of Peroxy Green 1 (PG1). This response is attenuated by apocynin, a Nox inhibitor; wortmannin, an inhibitor of PI3K; PD153035, an inhibitor of the receptor tyrosine kinase domain of the EGF receptor; and NSC23766, a Rac1 inhibitor.



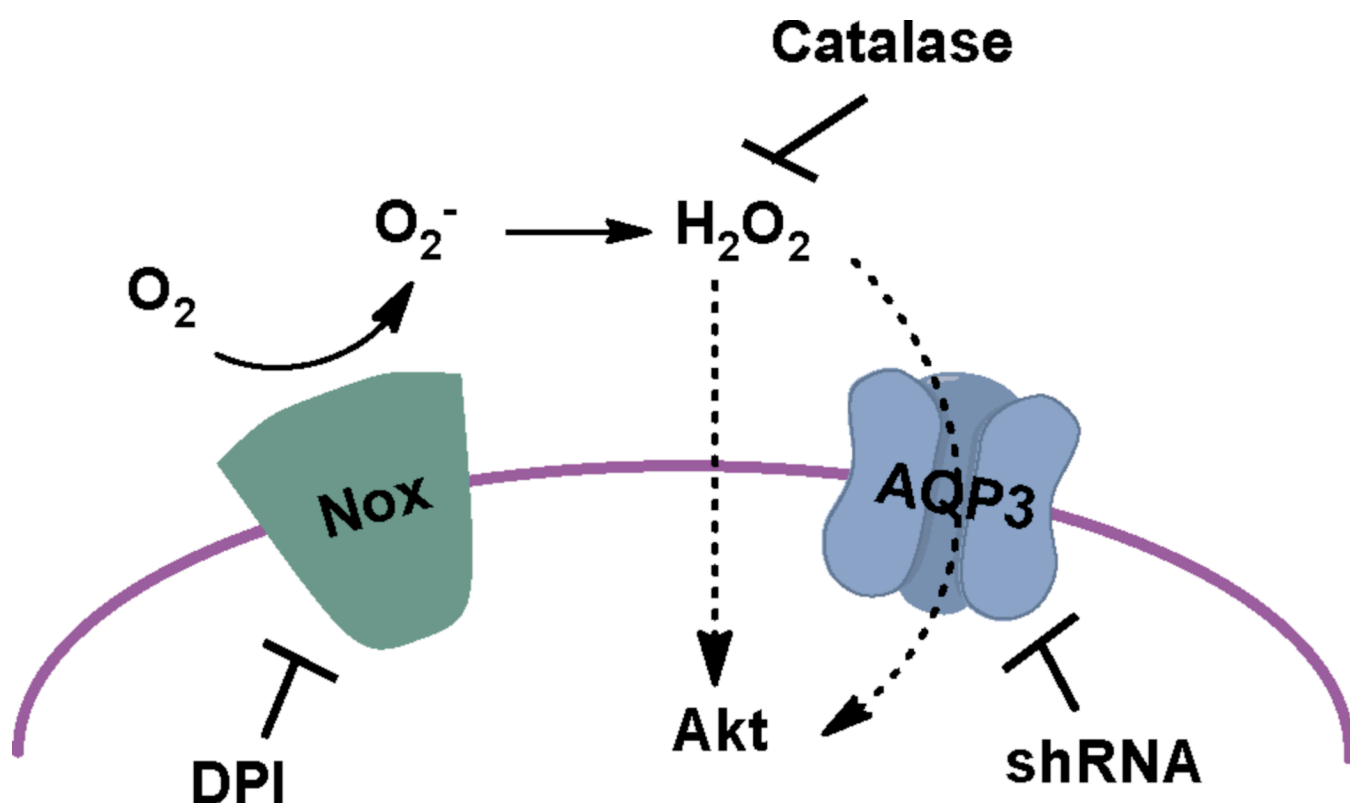
**Figure 7.** Confocal fluorescence images of  $H_2O_2$ -producing phagosomes, hROS-producing phagosomes, and dual  $H_2O_2$  and hROS-producing phagosomes in live RAW264.7 macrophages as distinguished by simultaneous imaging with Peroxy Orange 1 (PO1) and Aminophenyl Fluorescein (APF).



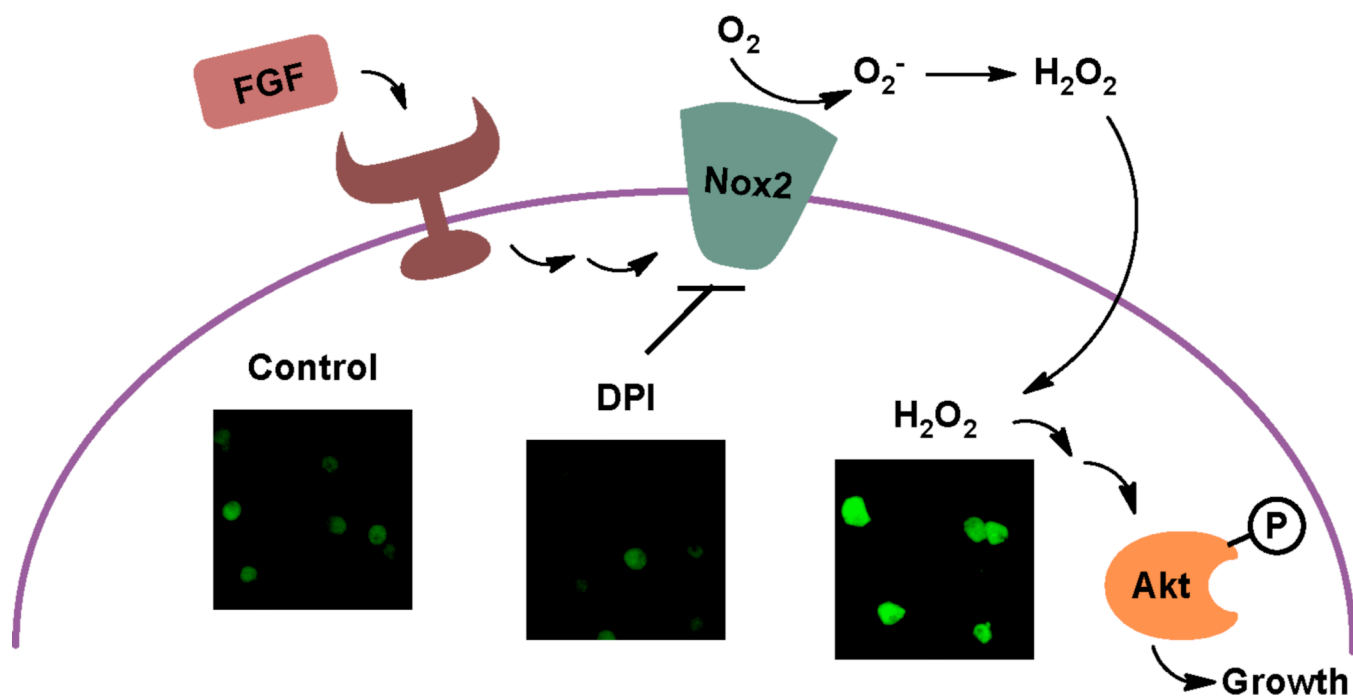
**Figure 8.** Ratiometric confocal fluorescence images of H<sub>2</sub>O<sub>2</sub> in stimulated live RAW 264.7 macrophages as visualized with Peroxy Lucifer 1 (PL1).



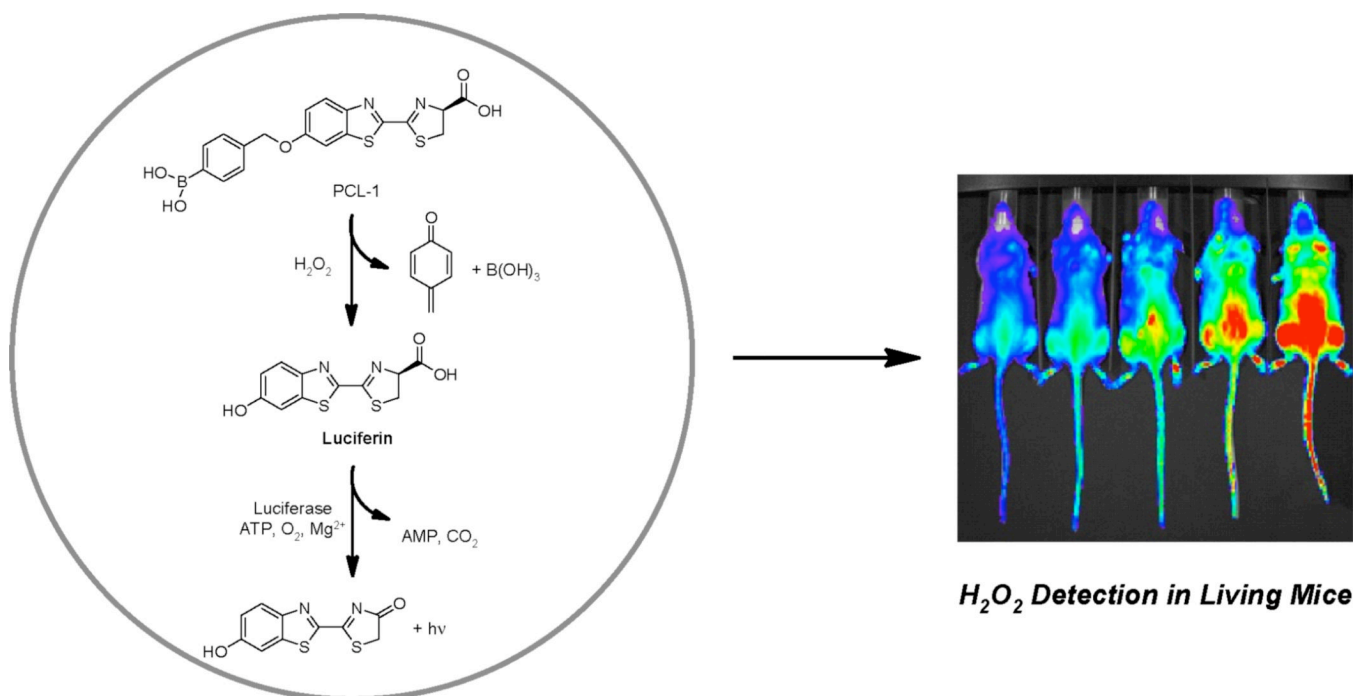
**Figure 9.** Images of SNAP Peroxy Green 2 (SPG2) localized to the plasma membrane, mitochondria, endoplasmic reticulum, and nucleus.



**Figure 10.** Aquaporins facilitate  $H_2O_2$  trafficking in growth factor signaling, as shown by the aquaglyceroporin family isoform Aquaporin 3 (AQP3).



**Figure 11.** FGF stimulation induces a Nox2-dependent increase in intracellular  $H_2O_2$  levels as imaged by Peroxyfluor 6 (PF6).



**Figure 12.** Peroxy Caged Luciferin 1 (PCL-1) detects  $H_2O_2$  *in vivo* using bioluminescence.



TABLE 1

Boronate probes for the detection of biological H<sub>2</sub>O<sub>2</sub>.

Probe	Probe $\lambda_{\text{abs}}/\lambda_{\text{em}}$ (nm)	Product $\lambda_{\text{abs}}/\lambda_{\text{em}}$ (nm)	Turn-on	Comments
Peroxyfluor 1 (PF1)	NA/NA	494/521	> 1000-fold	Exogenous H <sub>2</sub> O <sub>2</sub> detection in cells
Peroxyresorufin 1 (PR1)	NA/NA	530/584	> 1000-fold	Exogenous H <sub>2</sub> O <sub>2</sub> detection in cells
Peroxyxanthone 1 (PX1)	350/400	350/450	52-fold	Two-photon excitation
Ratio Peroxyfluor 1 (RPF1)	420/464	420/517,461	8-fold <sup>a</sup>	Ratiometric <i>in vitro</i> H <sub>2</sub> O <sub>2</sub> detection
Peroxy Green 1 (PG1)	460/510	460/510	10-fold	Detection of H <sub>2</sub> O <sub>2</sub> in cell signaling
Peroxy Crimson 1 (PC1)	480/584	530/584	40-fold	Detection of H <sub>2</sub> O <sub>2</sub> in cell signaling
Peroxy Lucifer 1 (PL1)	375/475	435/540	12-fold <sup>a</sup>	Ratiometric H <sub>2</sub> O <sub>2</sub> detection in cells
Mitochondria Peroxy Yellow 1 (MitoPY1)	489, 510/540	510/528	4.5-fold	Mitochondrially-targeted
Naphtho Peroxyfluor 1 (NPF1)	345/NA	598/660	25-fold	Far-red excitation and emission
SNAP Peroxy Green 1 and 2 (SPG1 and SPG2)	465/515	495/513	32-fold	Can localize dye to various cellular compartments
Peroxyfluor 2 (PF2)	NA/NA	475/511	50-fold	Multiple colors
Peroxyfluor 3 (PF3)	454/521	492/515	10-fold	Multiple colors
Peroxy Emerald 1 (PE1)	480/519	491/514	3-fold	Multiple colors
Peroxy Yellow 1 (PY1)	494/558	519/548	12-fold	Multiple colors
Peroxy Orange 1 (PO1)	507/574	540/565	8-fold	Multiple colors
Peroxy Yellow 1 Methyl Ester (PY1-ME)	489, 510/548	515/540	10-fold	Increased dye retention in cells
Peroxyfluor 6 Acetoxymethyl Ester (PF6-AM)	460/530	492/517	7-fold	Increased dye retention in cells
Terbium Peroxy Reporter 1 (TPR1)	226/545	226/545	6-fold	Utilizes lanthanide luminescence
Terbium Peroxy Reporter 2 (TPR2)	240/545	240/545	6-fold	Utilizes lanthanide luminescence
Peroxy Caged Luciferin 1 (PCL-1)	NA/NA	NA/612	7-fold	<i>In vivo</i> imaging

<sup>a</sup>Change in the ratio of the major peaks.