Antioxidants and Redox Signaling

DOI: 10.1089/ars.2018.7540

Forum Rewiew Article

In vivo imaging of hydrogen peroxide with HyPer probes.

Dmitry S. Bilan[1,2], Vsevolod V. Belousov[1,2,3*]

[1]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia, 117997.

[2]Pirogov Russian National Research Medical University, Moscow, Russia, 117997.

[3]Institute for Cardiovascular Physiology, Georg August University Göttingen, Göttingen, Germany, D-37073.

*send correspondence to V.V.B.: phone +7-499-7248466, fax +7-495-3307056, e-mail belousov@ibch.ru

8200 words, 126 references, Color Figures 6 (online 6)

Antioxidants and Redox Signaling
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Abstract

Significance: Hydrogen peroxide (H_2O_2) is a key signaling molecule involved in the regulation of both physiological and pathological cellular processes. Genetically encoded HyPer probes are currently among the most effective approaches for monitoring H_2O_2 dynamics in various biological systems because they can be easily targeted to specific cells and organelles. Since its development in 2006, HyPer has proved to be a robust and powerful tool in redox biology research. **Recent Advances**: HyPer probes were used in a variety of models to study the role of H_2O_2 in various redox process. HyPer has been increasingly used in the last few years for *in vivo* studies, which has already led to many important discoveries, for example, that H_2O_2 plays a key role in the regulation of signaling cascades involved in development and aging, inflammation, regeneration, photosynthetic signaling, and other biological processes. **Critical Issues**: In this review, we focus on the main achievements in the field of redox biology that have been obtained from *in vivo* experiments using HyPer probes. **Future Directions**: Further *in vivo* studies of the role of H_2O_2 largely depend on the development of more suitable versions of HyPer for *in vivo* models: those having brighter fluorescence and a more stable signal in response to physiological changes in pH.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Introduction

The important role of hydrogen peroxide (H_2O_2) in the regulation of cellular signal transduction pathways has been well established in recent years. Initially, similar to other reactive oxygen species (ROS), including the superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), H_2O_2 was viewed only from a negative standpoint. Indeed, these compounds by their nature are extremely reactive and cause oxidation of various cellular molecules such as proteins, lipids, and nucleic acids. A ROS burst usually leads to oxidative stress in cells and tissues, an event that accompanies the development of many pathological conditions and aging (1,45,67,116). However, at low concentrations, ROS serve as important redox regulators. In particular, H_2O_2 is now considered a second messenger alongside such messengers as cAMP and Ca^{2+}. Hundreds of studies have been published to date confirming various biological functions of H_2O_2 and its participation in the regulation of signal transduction cascades (32,42,43,51,60,77,99,103,109,110,112,117).

In cells, H_2O_2 is formed from short-lived $O_2^{\bullet -}$, which in turn is produced mainly by seven identified sites in mitochondria (17) or by NADPH oxidases (Nox) (6,69). Previously, it was suggested that NADPH oxidases only exist in immune phagocytic cells, which use superoxide to oxidize molecules of pathogens (4). However, it turns out that this type of proteins is also present in many types of nonphagocytic cells (6). Various growth factors, cytokines, and intracellular proteins are involved in the regulation of NADPH oxidase activity (6,41,61,75), demonstrating once again that the role of superoxide and H_2O_2 in cellular function is much more diverse than previously thought. In addition, a wide range of other enzymes also participate in the formation of intracellular $O_2^{\bullet -}$, mainly as by-products of their reactions (60,94). Generated $O_2^{\bullet -}$ is rapidly converted into H_2O_2 spontaneously or mostly by the action of specialized enzymes called superoxide dismutases. These enzymes are involved in antioxidant protection in almost all cells. Eukaryotic cells are characterized by the presence of two different superoxide dismutase isoforms localized in the cytosol and mitochondrial matrix (47,48,119), where they restrict the intracellular lifetime of $O_2^{\bullet -}$ and thereby prevent damage to proteins containing iron–sulfur clusters. In addition, superoxide dismutases catalyze H_2O_2 formation, which can regulate signaling cascades.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

H_2O_2 functions as a second messenger due to its ability to modify cysteine residues in some proteins. Most cysteine residues in cytosolic proteins are protonated (Cys-SH) at physiological pH, and these thiols react weakly with H_2O_2. However, H_2O_2 effectively oxidizes a thiolate anion ($Cys–S^-$) to a sulfenic acid (Cys–SOH) on some cysteine residues with a low pKa. For example, H_2O_2 reacts very slowly with glutathione and free cysteine, with a rate constant of $2 \times 10^1\ M^{-1}\ s^{-1}$. However, this rate constant can reach values of $1 \times 10^6\ M^{-1}\ s^{-1}$ for specific cysteine residues on certain proteins (29,44,68). The ability of a given cysteine residue to be oxidized by H_2O_2 is determined not only by its pKa value but also by the pH inside cellular compartments. However, not only pKa determines cysteine reactivity with H_2O_2. Other factors include, but not limited to, steric orientation of H_2O_2 and thiol in the reaction site, thiol accessibility, stabilization of the reaction transition state structure which lowers the activation barrier of the reaction (92).

Sulfenic acid (Cys–SOH) is highly reactive and immediately forms disulfide bonds in the presence of an accessible -SH group. The formation of inter/intramolecular disulfide bonds often leads to global conformational rearrangements in protein molecules that alter their functions. However, this reaction is fully reversible, and disulfides can be reduced by the glutathione- and thioredoxin-dependent reduction systems (3,26,29,40,72). Thus, the reversible and specific H_2O_2-mediated oxidation of certain cysteine residues within different proteins is a clear example of a signal transduction mechanism carried out by ROS. Such cysteine residues serve as redox-dependent switches that can regulate the activities and functions of many proteins in both prokaryotic and eukaryotic cells. This group of proteins includes certain kinases and phosphatases, structural proteins, metabolic enzymes, transcriptional factors, and numerous others (110,117), and the redox regulation of so many proteins inevitably affects intracellular processes via signaling pathways. The most striking example is H_2O_2 generation in response to some growth factors, as a result of which H_2O_2 acts as a signaling molecule and regulates the activity of protein tyrosine phosphatases (24,114). To date, the participation of H_2O_2 has already been demonstrated in such global biological processes as aging and development, inflammation, regeneration, proliferation, differentiation, immune modulation, and circadian rhythms (60,110,117).

H_2O_2 is a nonpolar molecule, so it is capable of diffusing through biological membranes in contrast to the charged $O_2^{\bullet -}$, for example. Although it was previously

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

assumed that H_2O_2 transport is carried out by simple diffusion, it was later shown that this molecule mainly uses water channels, the aquaporins, and such transport is much more effective (9,57,83,87). The term "peroxiporins" refers specifically to those aquaporins that play an important role in H_2O_2 translocation (109). Inside cells, H_2O_2 is distributed non-uniformly and acts quite locally, and under normal conditions, an H_2O_2 gradient can be formed even within single cellular compartments (90,110). Similar to other signaling molecules, the level of H_2O_2 is finely regulated by cellular systems. Catalases, peroxiredoxins, and glutathione peroxidases are the main enzymes involved in the degradation of H_2O_2, thereby limiting its effects (19,23,59,122).

Generally, the physiological concentrations of H_2O_2 fall within the nanomolar range (110). Oxidative stress, for example, is caused by an imbalance in H_2O_2 production/degradation, which leads to a significant increase in its concentration in cells. Elevated H_2O_2 leads to excessive accumulation of sulfenic (–SOH), sulfinic ($–SO_2H$), and sulfonic ($–SO_3H$) acids in proteins. Cys–SO_2H is a reversible modification of cysteine residues; it can be reduced by ATP-dependent enzymes such as sulfiredoxin and sestrins (14,20). In contrast, the formation of Cys–SO_3H is irreversible and the highest degree of thiolate anion oxidation, which leads to protein damage.

In addition to cysteine residues, some others can also be modified by H_2O_2, such as methionine residues (31,71,111). The physiological functions of these modifications are still poorly investigated. Thus, the interrelated and complex operation of antioxidant- and ROS-generating systems is a delicate intracellular balance between redox regulation and oxidative stress.

Increased interest in the study of the role of H_2O_2 has stimulated the development of methodological approaches that allow such studies. To date, a broad range of synthetic dyes for H_2O_2 detection have been developed, including dihydrorhodamine 123 (DHR), 2,7-dichlorodihydrofluorescein (DCFH), Peroxy Green 1 (PG1), and Peroxy Crimson 1 (PC1) (18,28,46,50,100,121). Some of these indicators are able to penetrate into living cells. Nevertheless, there are some difficulties in their application. In addition to H_2O_2, other types of ROS can also affect the fluorescence signal of many such indicators. Moreover, the reactions of them are irreversible. For a long time, the use of these synthetic indicators

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

was the only available tool for visualizing H_2O_2 generation in cell cultures, and *in vivo* studies in more complex biological systems were impossible until recently.

Genetically encoded redox biosensors based on fluorescent proteins have opened a new era in the field of redox biology (12). Each such biosensor is a protein molecule that, similar to a conventional fluorescent protein, can be localized within any biological system, from cellular compartments to the tissues of living organisms. The fluorescence signal is specifically dependent on the redox state of a particular redox pair or reactive molecule. As a result, the dynamics of redox processes can be observed directly in living systems in real time. To date, several types of biosensors have already been developed to investigate glutathione (52,105), thioredoxin (39), NAD(H) (10,125), and NADP(H) (22,113) redox states.

Investigation of the role of H_2O_2 in living systems has also become possible due to the development of specific genetically encoded biosensors. There are two families of such biosensors, differing in their principle of action. One type of biosensor is based on redox-sensitive GFP (roGFP), which differs from the original variant of GFP by the presence of a surface-exposed cysteine pair capable of forming an intramolecular disulfide bond. As a result, roGFP is involved in the cellular reactions of thiol-disulfide exchange. The reversible formation of the disulfide bond between these redox-active cysteines located on adjacent β-sheets affects the spectral characteristics of the fluorescent protein. In addition to determining the redox status of the glutathione pool, roGFP-like proteins can also be used as H_2O_2 biosensors. For this purpose, roGFP2 was fused with the yeast thiol peroxidase Orp1, which is extremely sensitive to H_2O_2 (53). In the roGFP2-Orp1 fusion, Orp1 is oxidized by H_2O_2 and involved in the thiol-disulfide exchange reaction with roGFP2, oxidizing it in a practically stoichiometric manner. In another example, roGFP2 was fused with yeast 2-Cys peroxiredoxin Tsa2. These chimeric proteins are the most sensitive indicators of H_2O_2 currently available and can detect low concentrations of endogenous H_2O_2 at basal levels (91). However, because the roGFP domain in these H_2O_2 sensors retains its intrinsic sensitivity to the cellular glutathione redox state, appropriate controls are needed.

Whereas in roGFP-peroxidase fusions the redox relay mechanism is involved in roGFP oxidation, in HyPer, the first genetically encoded indicator for H_2O_2 detection (7,11),

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

difulfide formation directly leads to conformational rearrangements resulting in spectral changes. Over the years, the HyPer family probes have become one of the most popular tools in redox biology used to investigate the pathological and physiological function of H_2O_2 in a wide variety of biological models *in vivo*. This review focuses on HyPer use *in vivo*.

HyPer family probes

Bacteria have evolved special sensory mechanisms to ensure their defense against oxidative stress. This kind of protection is provided by activating regulons controlled by specific transcription factors, such as SoxR, OxyR, and PerR, that are sensitive to either superoxide or H_2O_2 (64). It is noteworthy that intracellular bacterial systems can strictly distinguish between different types of ROS, thus acting as natural biosensors. OxyR is widely distributed in bacteria and acts as a H_2O_2 sensor due to the specific mechanism of conformational changes caused by direct oxidation. Upon reaction with H_2O_2, the thiol of the cysteine residue at position 199 (Cys199) is oxidized to sulfenic acid, as a result of which this modified residue moves from a hydrophobic pocket and becomes spatially close to Cys208, with which it forms a disulfide bond (25). Conformational rearrangements of OxyR caused by oxidation affect its binding to DNA. Oxidized proteins can be reduced by cellular thiol exchange systems (25,70,126). As a result, the bacterial cells actively control the H_2O_2 concentration by regulating the expression of a number of antioxidant genes.

The H_2O_2-sensitive regulatory domain of OxyR from *E.coli* was used as a basis for a genetically encoded biosensor for H_2O_2 detection. A circularly permuted fluorescent protein (cpFP) was integrated via short peptide linkers into the OxyR region of high conformational mobility (between amino acids 205 and 206). Within the resulting chimeric protein, the OxyR domain retains selectivity and kinetic properties toward H_2O_2, resulting in a conformational change upon formation of a Cys199–Cys208 disulfide bond. Conformational rearrangements of OxyR are then transmitted to the fluorescent protein, resulting in a spectrum change. Thus, the oxidized and reduced state of this molecule can be distinguished by its spectral characteristics. This biosensor was named HyPer (7) and is now widely used as a tool for real-time H_2O_2 monitoring in living systems.

The HyPer family probes are now represented by five variants. Four versions, HyPer (7), HyPer-2 (79), HyPer-3 (13), and TriPer (85), are based on the circularly permuted

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

yellow fluorescent protein (cpYFP) **(Fig. 1 A)**. Another version, HyPerRed, is based on the red fluorescent protein cpmApple (35). All cpYFP-based versions are characterized by the presence of two peaks in the fluorescence excitation spectrum with maxima at 420 nm and 500 nm and one peak in the emission spectrum with a maximum at 516 nm. Upon oxidation of the biosensors, the intensity of fluorescence excitation at 420 nm (F_{420}) decreases, while the fluorescence excitation at 500 nm (F_{500}) increases proportionally. Thus, most often, the fluorescence (emission) measurement is carried out using two separate channels corresponding to the excitation maxima. The HyPer signal is defined as the ratio of fluorescence intensities in the two excitation channels (F_{500}/F_{420}), and this value increases with increasing H_2O_2 concentration in any system under study **(Fig. 1 B,C)**.

HyPer-2 and HyPer-3 are improved versions of the original HyPer, as their dynamic range is at least 2-fold higher compared to the parental HyPer. Using a cell culture model, it was demonstrated that an excess of exogenous H_2O_2 results in an approximately 6-fold F_{500}/F_{420} ratio change in HyPer-2 and HyPer-3, while the HyPer signal under the same conditions increases by only 3-fold (13). This expanded dynamic range is an important advantage of these biosensors especially at low H_2O_2 concentrations, for example, when H_2O_2 is endogenously generated in response to growth factors (79). HyPer-3 has higher oxidation and reduction rates compared to HyPer-2 while retaining the same high dynamic range (13). HyPer-2 and HyPer-3 differ from the original variant of the biosensor by only single point mutations in the OxyR domain: Ala406Val for HyPer-2 and His34Tyr for HyPer-3 (13,79). This illustrates how the properties of biosensors and likely other proteins can differ markedly due to the introduction of one amino acid substitution. It is noteworthy that both mutations affect not only the dynamic range and kinetic characteristics of HyPer probes but also their oligomeric states. HyPer and HyPer-3 are monomeric, whereas HyPer-2 forms a stable dimer (13,79).

HyPer probes have some limitations in their application. For example, in the high thiol-oxidizing environment of the endoplasmic reticulum (ER) lumen, HyPer is almost completely oxidized. This phenomenon likely occurs because of both enhanced levels of H_2O_2 and a high local glutathione redox potential, which favors the formation of disulfide bonds in proteins (107). Moreover, experimental data suggest that oxidized HyPer in the endoplasmic reticulum reflects general oxidizing conditions inside this compartment to a

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

greater extent than H_2O_2 production (84). The version named TriPer was specially developed for such conditions. TriPer (tri-cysteine probe) differs from HyPer by the introduction of an additional cysteine (Ala187Cys) to the OxyR structure near the functional Cys208. In the oxidizing conditions of the endoplasmic reticulum, this additional cysteine can form a disulfide with Cys208. Thus, the fraction of molecules with redox active Cys199 are retained in their reduced state even under such conditions so that H_2O_2 can be detected. In the case of TriPer, Cys199 interacts in *trans* to form an intermolecular disulfide. ER-localized TriPer can be successfully used for monitoring changes in luminal H_2O_2 (85).

HyPer-Red is characterized by a single peak in the fluorescence excitation spectrum with a maximum at 575 nm and an emission peak at 605 nm (35). This fluorescence intensity increases twice during the transition from its fully reduced to H_2O_2-mediated fully oxidized form (35) **(Fig. 1 D-F)**.

Since all versions of HyPer are based on the same OxyR, they have identical sensitivity to H_2O_2. Approximately 20-30 nM H_2O_2 produces a minimally detectable response in isolated and fully reduced HyPer probes. A similar effect can be achieved in the cytoplasm of eukaryotic cells by adding 5 μM H_2O_2 (7,11). This difference is explained by the presence of up to 650-fold gradient of H_2O_2 across the plasma membrane of mammalian cells, which was confirmed experimentally (62). HyPer can be reversibly reduced by cellular antioxidant systems, as well as by wild-type OxyR. Importantly, all biosensors of the HyPer family are highly specific for H_2O_2, while other oxidants, such as $O_2^{\bullet -}$, NO, $ONOO^-$, GSSG (7,35), and ClO^- (unpublished data), do not affect the signal. Another important feature of any biosensor is its brightness, which should especially be taken into account when working *in vivo*. HyPer-Red is the brightest version. Despite the fact that all green versions of HyPer are based on the same fluorescent protein cpYFP, for unknown reasons, HyPer-2 is brighter than HyPer and HyPer-3 (13).

pH-dependency of HyPer probes: need for an appropriate control

The main drawback of all currently available HyPer probes is their high sensitivity to pH changes in the physiological range. Because the protonation state of Tyr in many GFP-like proteins depends on the pH of the environment, fluorescent proteins can be used, for example, as probes of intracellular pH (34,56). Circularly permuted proteins are usually

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

even more sensitive to physiological pH changes compared to proteins with intact β-barrel structures. HyPer probes based on cpYFP (with pKa 8.6 at 490 nm) (7) and HyPer-Red (with pKa 8.7) (35) can thus reflect not only H_2O_2 concentration dynamics but also intracellular pH changes. This problem can be addressed by using an appropriate pH probe as a control in HyPer experiments.

Many synthetic dyes (54) or GFP-based probes (8) can be successfully used as pH indicators. However, a H_2O_2-insensitive version of the HyPer probe with the same pKa value is the most reliable pH control for these biosensors. The simplest way to render OxyR insensitive to H_2O_2 is to mutate one of the redox active cysteines (Cys199 or Cys208). Initially, a version of HyPer with the Cys199Ser mutation was generated in order to confirm that this amino acid residue plays a key role in the mechanism of H_2O_2 recognition by the biosensor. Indeed, HyPer-Cys199Ser does not respond to H_2O_2 and mimics the fully reduced state of HyPer. Both versions have identical spectral characteristics and pH titration curves, thereby making their direct comparison possible in parallel experiments. HyPer-Cys199Ser was named SypHer and is now used as a reliable pH indicator for various cellular organelles (98). SypHer-2 is the improved version that is three times brighter compared with SypHer when expressed in mammalian cells (81). Based on the same principle, a H_2O_2-insensitive version of HyPer-Red was developed to monitor pH changes in parallel experiments (35).

It should be noted that other modifications in addition to point mutation of a redox active cysteine in the OxyR part can be used to make HyPer probes insensitive to H_2O_2. For example, in the reduced form of wild type OxyR, Arg-266 increases the reactivity of the Cys-199 thiol group by lowering its pKa (25). For this reason, the introduction of an Arg266Ala mutation abolishes the H_2O_2 responsiveness of HyPer and TriPer (85). However, the HyPer versions containing the Cys199Ser mutation are the most straightforward pH controls, and their use is highly recommended.

Where and how HyPer probes can be used

The fluorescence signal of the HyPer probes can be detected in living cells and tissues using conventional confocal or widefield microscopy. Since the fluorescence of green versions of HyPer is excited at two separate peaks (F_{420} and F_{500}), it must be detected in separate channels of a microscope. This yields a ratiometric biosensor readout

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

that provides some advantages; for example, the signal does not depend on protein expression levels in different cells, object movement, or changes in its shape. However, signal dynamics can also be monitored in one channel, as, for example, in the case of single-wavelength HyPer-Red. Furthermore, HyPer can be employed to obtain relative quantitative data using a plate reader assay (63).

Two-photon excitation microscopy is better suited for working with complex objects such as living tissue, and HyPer has been successfully tested using this fluorescence imaging technique (120). Another technique, fluorescent lifetime imaging microscopy (FLIM), was applied to HyPer in several studies (13,85,120). As the fluorescence intensity of cytosolic HyPer in response to H_2O_2 increases, its fluorescence lifetime decreases. It is noteworthy that HyPer-3 has a higher dynamic range compared to HyPer and exhibits more pronounced changes in fluorescence lifetime upon oxidation (13). Exposure of cells expressing TriPer localized in the endoplasmic reticulum to H_2O_2 results in highly reproducible increases in the fluorescence lifetime (85). FLIM has several advantages over fluorescence intensity measurement; for example, it is relatively indifferent to photobleaching and provides a quantitative readout. Finally, HyPer-2 was used in live-cell stimulated emission depletion (STED) microscopy (89), which allowed the imaging of H_2O_2 dynamics with subdiffractional resolution in parallel with STED imaging of cellular intermediate filaments.

Similar to any other gene, HyPer probes can be expressed in any cell type, whether prokaryotic or eukaryotic. For example, HyPer has been successfully used in experiments with cultured endothelial, epithelial, embryonic, and neuronal cell types, among others, during the years of this biosensor`s existence (11). For this, one must insert the HyPer gene into a vector containing tissue-specific functional elements to drive its expression in the desired system. Similar to conventional fluorescent proteins, the use of special signal sequences makes it possible to localize HyPer probes in any cellular compartment (11,76). Considering that HyPer biosensors are represented by red- and green-emitting versions, they can be localized to different compartments within single live cells for use in multiparametric imaging (35).

In recent years, more complex molecular tools based on HyPer have appeared that, in addition to monitoring H_2O_2, combine additional functions. For example, the fusion of

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

HyPer with the PH domain from Bruton's tyrosine kinase resulted in a probe that senses two independent parameters, namely, phosphatidylinositol 3,4,5-trisphosphate (PIP3) and H_2O_2. Translocation of the resulting probe (named PIP-SHOW) to the cellular plasma membrane reflects an increase in PIP3 concentration, which in turn indicates the activity of phosphatidylinositol 3-kinase (PI3K). At the same time, the HyPer portion reports H_2O_2 changes. A typical example of the application of this biosensor was demonstrated in fibroblasts, in which stimulation by platelet-derived growth factor (PDGF) caused H_2O_2 generation and PI3K activation. Both of these parameters were simultaneously visualized with the use of the PIP-SHOW probe (88).

In another work, HyPer was fused with the yeast D-amino acid oxidase (DAO), an enzyme that produces H_2O_2 upon the oxidation of D-amino acids, which are relatively uncommon in mammalian cells. Thus, the intracellular production of H_2O_2 in this model can be induced by the extracellular addition of D-amino acids, for example D-alanine, while the presence of HyPer makes it possible to visualize the H_2O_2 generation (80).

Ideas for the development of such biosensors with combined functionalities are, in fact, limitless. First, instead of conventional fluorescent protein any ratiometric biosensor can be combined with any translocation-based probe. Second, H_2O_2 generators such as DAO, can be combined not only with HyPer, but also with other probes in order to study how local H_2O_2 production influences other signaling and metabolic pathways.

In vivo imaging of H_2O_2 with HyPer probes

In vivo studies at the level of the whole organism are rapidly gaining popularity in the field of redox biology. This is partly due to the development of more reliable research tools, including genetically encoded redox biosensors (12), which can be stably or transiently expressed in different organisms and targeted to specific organs and tissues of interest. HyPer family probes have been successfully used in many *in vivo* models to study the role of H_2O_2 in various biological processes (**Fig. 2**). In this review, we will focus on the most striking discoveries.

Development and aging

The nematode *Caenorhabditis elegans* has long been one of the favorite objects of *in vivo* studies, especially in the field of developmental biology and aging. These animals with a short lifecycle (approximately 3 days under optimal conditions) are best suited for

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

this purpose. In addition, *C. elegans* is transparent, which greatly facilitates *in vivo* imaging of fluorescent reporters localized in any part of transgenic animals. In recent years, *C. elegans* has been extensively used to investigate biological redox processes, including through the use of genetically encoded biosensors (16).

HyPer has been used in several *in vivo* studies to visualize changes in H_2O_2 levels in different organs of *C. elegans* (5,21,36,65,66,118). Dr. Fisher and colleagues clearly demonstrated the application of HyPer to nematodes and discussed in detail important issues including the generation of transgenic animals, microscopy equipment, and settings for ratiometric readout imaging of HyPer and subsequent analysis of the images (118). In two other independent studies, HyPer was used to detect H_2O_2 levels in the tissues of *C. elegans* during its development cycle and aging (5,66). A team of researchers led by Ursula Jakob (66) discovered high oxidant levels in nematodes at the larval development stage, which rapidly decreased as they matured and entered into reproductive age. A second wave of oxidation during the worm life span was found in aging animals. Several approaches were used in this work to clarify the role of oxidative stress during *C. elegans*` life span. First, the authors applied the quantitative redox proteomic technique OxICAT using animals of different ages from early stages of development to late adulthood. In total, this team determined the thiol redox status of almost 140 different proteins, which revealed that the oxidation of protein thiols generally increases with age. However, high levels of thiol oxidation were also observed in developing larvae, whereas reproductive ages were characterized by reducing conditions. Interestingly, not all proteins that were determined to be more oxidized during development were also more oxidized in aging animals (66). Endogenous changes in H_2O_2 levels were directly monitored with HyPer in individual transgenic animals at different stages. In agreement with the redox proteomics results, HyPer confirmed H_2O_2 generation during larval development. H_2O_2 levels then decreased in the reproductive period and remained low during the fertile phase, followed by increased concentrations again in the aging population (66).

Age-dependent H_2O_2 fluctuations were confirmed in an independent study conducted by Braeckman and colleagues (5). Remarkably, in both mentioned studies, in animals with an extended life span caused by either dietary restriction (5) or mutation in the insulin/IGF signaling receptor (66), the age-related H_2O_2 increase was weakened.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

However, the study of Braeckman et al. did not reveal any significant changes in the HyPer signal during larval development in contrast to the experiments conducted by the team of Jakob.

H_2O_2 concentrations are unevenly distributed throughout the *C. elegans* body, resulting in the formation of local H_2O_2 hot spots. The highest H_2O_2 levels were detected in canal-associated neurons, the hypodermal syncytium, and body wall muscles in young animals on their first day of adulthood (5). Regarding the latter, it is possible that the high H_2O_2 signal observed in wall muscles might be an artifact associated with experimental procedures. All animals were immobilized for microscopy with levamisole, which is an acetylcholine receptor agonist of body wall muscle cells and causes their contraction. The increased H_2O_2 levels in wall muscles might be caused by levamisole (16). This could be easily verified by using other anesthetics and microscopy approaches for living subjects. However, the same localizations of high H_2O_2 level were not found in six-day-old animals (5).

In another study, the redox biosensors roGFP and HyPer were expressed in the endoplasmic reticulum and the cytosol to investigate the organellar redox state in *C. elegans* muscle and neuronal tissue during development and aging (65). Despite the fact that HyPer has limited use in the endoplasmic reticulum, as previously reported, HyPer expressed in the ER of nematode cells responded to exogenous H_2O_2. SypHer was used as a control in this model. This study showed that the redox state of different cellular compartments varies in different ways during aging. As demonstrated previously, the cytosol becomes more oxidized in aged animals. However, the redox state of the endoplasmic reticulum changes in the opposite direction and becomes more reduced with age. Similar trends were observed for aggregation-prone protein expression and proteasome inhibition in both *C. elegans* and mammalian cell culture. Although the underlying mechanism for this is not yet known, the original article contained an extensive discussion of these results with several possible explanations for the effects found (65).

Vertebrate animals are more closely related to humans compared with *C. elegans* or another popular model system, the fly *Drosophila melanogaster.* Among vertebrate model organisms, the zebrafish *Danio rerio* is the most popular model for studying development due to several important advantages, including its optical

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

transparency at early stages and the large number of offspring that develop and grow at an extremely fast rate. In addition, the fish genome has been fully sequenced, opening up broad possibilities for genetic manipulation. Moreover, genetically encoded biosensors can be easily expressed in different tissues. In experiments where transient expression is sufficient, mRNA injection at the one-cell stage is the easiest way to accomplish this.

Dr. Vriz and colleagues conducted research using a *D. rerio* line with stable expression of HyPer under the control of a ubiquitous promoter (49). They demonstrated that the H_2O_2 concentration varies significantly during embryonic development. Moreover, local zones with high H_2O_2 were identified. H_2O_2 levels had already increased at the early stage of gastrulation on the side opposite to the shield and became highest during somitogenesis, especially at the 20 somite stage. Subsequently, in most tissues, HyPer gradually reduced, remaining more oxidized only in the heart and neuronal tissues. At 48 hours post-fertilization, the biosensor revealed high levels of H_2O_2 only in some organs (optic tectum, spinal cord, and notochord), whereas 24 hours later, H_2O_2 was reduced in all tissues and remained low in adults. For each of these time points, the authors determined the activity of the main enzymes participating in H_2O_2 homeostasis, superoxide dismutase and catalase. Whereas superoxide dismutase activity remained unchanged throughout development, catalase activity decreased, and this time interval coincided with the increase of H_2O_2 in the tissues. Upon development of the embryo, catalase activity increased again with a simultaneous decrease in H_2O_2. Therefore, the degradation system determines H_2O_2 levels during development (49).

It was reported that H_2O_2 promotes peripheral sensory axon regeneration in wounded zebrafish larvae (101) and during regeneration in adults (82). The high H_2O_2 level in the nervous system during *D. rerio* development suggests that in this process, H_2O_2 also plays an important signaling function. To verify this, H_2O_2 levels were artificially decreased in larval tissues by incubating animals with a selective NADPH oxidase inhibitor **(Fig. 3 A,B)**. Indeed, retinal ganglion cell axonal projections in developing zebrafish with reduced H_2O_2 levels had diminished length and branching compared to the axons of control animals **(Fig. 3 C-E)**, an effect that could be partially rescued by exogenous H_2O_2 treatment or ectopic activation of the Hedgehog pathway. Thus, during the development of *D. rerio* under

normal physiological conditions, H_2O_2 levels are tightly controlled, and this is required for the regulation of many important processes, including axonal growth (49).

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

Inflammation

Wounding of the zebrafish larval tail fin is one of the most popular *in vivo* models for the study of inflammation and regeneration, and HyPer probes have been effectively used to visualize H_2O_2 in this model in real time, starting from the earliest stages of inflammation (**Fig. 4**). For the first time, HyPer was used in the wound model of zebrafish larva by Niethammer and colleagues (95), who introduced HyPer into zebrafish embryos by RNA injection and showed that the biosensor was evenly expressed throughout the animal tissues at 3 days post-fertilization. Local injury of the tail fin led to a rapid and significant increase in the H_2O_2 concentration in the wound area, which reached a maximum value ~20 min after wounding with a gradient extending ~100–200 μm inward from the wound margin (95). In parallel, these researchers monitored leukocyte recruitment to the wound using specific fluorescent tags. These experiments revealed that H_2O_2 in the wound is generated by epithelial cells rather than leukocytes as was previously believed (106), which was evidenced by the fact that H_2O_2 production always preceded recruitment of the first leukocyte to the wound region (95). Since NADPH oxidases are the main physiological source of superoxide and H_2O_2, the role of particular enzymes in the formation of a H_2O_2 gradient in the wound was assessed using pharmacological and genetic inhibition. Subsequently, it was determined that increased H_2O_2 levels in the wounded zebrafish larval tail fin are caused by the activity of dual oxidase (DUOX) in epithelial cells. *duox* knockdown significantly weakened H_2O_2 generation and strongly attenuated the recruitment of leukocytes to the wound. Thus, H_2O_2 acts like a chemotactic signal, which is required for leukocyte recruitment to the area of inflammation (95). It was later revealed that the Src-family kinase Lyn also mediates neutrophil recruitment to the wound (124).

The successful use of HyPer probes in this model of inflammation was also corroborated independently by other laboratories (13,74,97,113,123,124). In their study, Dr. Yang and colleagues (113) clearly demonstrated that the H_2O_2 gradient in the wound region of a zebrafish larval tail fin is caused by NADPH oxidase activity. For this, they utilized HyPer-Red in a multi-parameter imaging mode together with the green biosensor iNap for NADPH imaging, which enabled the simultaneous imaging of H_2O_2 and NADPH

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

dynamics in real time **(Fig 5)**. Upon local injury of the tail fin, HyPer-Red revealed increased H_2O_2 levels in the wound, while the iNap biosensor was used to visualize a reduction in the NADPH concentration in the same area caused by its consumption in the NADPH oxidase-mediated reaction. These changes were not observed when NADPH oxidase inhibition was applied to this model (113).

Another interesting study headed by the Lieschke team (97) found that leukocytes are not the source of H_2O_2 in the inflammatory region; instead, they participate in H_2O_2 degradation, decreasing its gradient in the wound via the myeloperoxidase reaction. HyPer has revealed similar increases in H_2O_2 levels in injured tissues of wild-type and leukocyte-depleted zebrafish larvae. However, in the wounded area of leukocyte-depleted animals, the high H_2O_2 level was maintained for a long time, while in wild-type fish, it declined over time. In addition, the H_2O_2 burst in wild-type animals was limited to the wound area, while in leukocyte-depleted individuals, the H_2O_2 gradient spread much farther from the wound margin. The use of a myeloperoxidase-deficient zebrafish mutant, *durif*, revealed that this enzyme plays a key role in H_2O_2 reduction, as neutrophils lacking myeloperoxidase enzymatic activity were unable to reduce the H_2O_2 concentration in the inflammatory region. Moreover, populating *durif* embryos with wild-type neutrophils by transplantation at the blastula stage restored the ability to decrease the H_2O_2 concentration (97). As previously stated, changes in a HyPer signal in response to H_2O_2 can also be imaged using FLIM (13). Using the same zebrafish tail fin wounding protocol, it was possible to follow increased levels of H_2O_2 in the wound area via lifetime changes of HyPer-3 (13). As in the case of *D. rerio* larvae, epidermal wounding of *Xenopus laevis* tadpoles also caused an increase in H_2O_2 levels at the injury site (74).

The pH sensitivity of all HyPer probes raises the following question: what does HyPer detect in the zebrafish wound region, H_2O_2 or pH changes? In the original article (95), the authors used YFP and the synthetic dye BCECF as pH reporters. Neither approach indicated any significant pH change in the system. However, it must be acknowledged that these are not optimal pH controls for HyPer probes in contrast to the SypHer biosensor, which was not used in these experiments. Nevertheless, there is reason to believe that HyPer reflects, at least in part, H_2O_2 dynamics in this model. Although HyPer-3 (with an improved dynamic range) and HyPer have identical pH titration profiles, HyPer-3

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

demonstrated elevated signal changes compared to HyPer in the area of inflammation, suggesting a substantial contribution of H_2O_2 to this response (13). Further evidence comes from FLIM experiments: the fact that the HyPer3 lifetime is decreased in the wound site compared to the body suggests H_2O_2 production (13), whereas elevated pH (based on ratiometric imaging) typically leads to an increased lifetime for green *Aequorea victoria* GFP derivatives (93,104).

Neutrophils are recruited not only to physically wounded areas but also to sites of infection. It turns out that distinct signaling mechanisms are involved in sensitizing neutrophils to pathogen- and non-pathogen-induced tissue damage. For example, H_2O_2 is not required for neutrophil recruitment to tissue areas damaged by bacterial infection. Deng and colleagues in their work used a localized bacterial infection model that is optimal for monitoring pathogen–leucocyte interactions (30). For this, bacteria expressing a fluorescent marker were injected into the inner ear of zebrafish larvae. These experiments were conducted using transgenic fish expressing a HyPer biosensor together with a neutrophil-specific fluorescent protein that allowed visualization of their movement. Another option is expression of any redox biosensor such as HyPer. It was found in the experiments with HyPer that the infection (*Pseudomonas aeruginosa* PAK strain) does not cause H_2O_2 generation in zebrafish tissue, and inhibition of NADPH oxidase enzymatic activity did not affect neutrophil recruitment to the infection area. In contrast, a phosphoinositide 3-kinase inhibitor had a pronounced effect in both mechanical tissue injury and infection (30).

Regeneration

H_2O_2 serves as an important signaling molecule during the process of tissue regeneration. Increased H_2O_2 production following *Xenopus laevis* tadpole tail amputation and during tail regeneration was observed in HyPer probe experiments carried out in Amaya's lab (74). H_2O_2 levels increased several minutes after amputation and remained at a high level for several days. At the same time, the use of a pH-sensitive probe, pHluorin, revealed that during the tail regeneration process, pH does not change significantly. The H_2O_2 source was also not associated with inflammatory cells, as is the case of wounding of the zebrafish larval tail fin. The formation of a H_2O_2 gradient in the tissue is due to NADPH oxidase enzymatic activity, and the H_2O_2 burst precedes the recruitment of inflammatory

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
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cells to the injured area. These features are clearly similar to the zebrafish wounding model. Different approaches to reducing H_2O_2 levels, including NADPH oxidase inhibition and incubation with the free radical scavenger MCI-186, caused impaired tail regeneration of animals, as evidenced eventually by a shorter tail length in comparison with control groups. Interestingly, NADPH oxidase inhibitors and antioxidant scavengers affect the regeneration process differently. NADPH oxidase complex activity is required for the initiation of the regeneration program in the first 3 days after tadpole tail amputation. After this, regeneration is suppressed even in the absence of inhibitors, whereas the effect of the antioxidant scavenger is maintained only if it is constantly present in the medium. This study also revealed that sustained high levels of H_2O_2 during regeneration are required for Wnt/β-catenin signaling activity (74).

In another study, the role of H_2O_2 was studied in the context of *D. rerio* heart regeneration (55). A marked increase in the concentration of H_2O_2 was observed using HyPer probes in intact injured hearts at 3, 7, and 14 days post-amputation. The H_2O_2 level decreased to the initial concentration in the tissue after nearly complete regeneration at 30 days post-amputation. It is noteworthy that wounding of the zebrafish tail fin and ventricular resection of its heart induce the generation of H_2O_2, but the dynamics of gradient formation are very different in these organs. In the fin epithelium, H_2O_2 rises within the first few minutes after injury, whereas in injured adult hearts, no changes in HyPer signal were observed even during the first hour. The enzymatic activity of the NADPH oxidase complex is also the primary source of H_2O_2 generation during cardiac regeneration, which was confirmed in experiments using various inhibitors. Moreover, lowering the H_2O_2 concentration by NADPH oxidase inhibition or catalase overexpression leads to weakening of cardiac regenerative capacity. The role of H_2O_2 in this process is mediated through destabilization of the redox-sensitive phosphatase Dusp6, thus providing a derepression of the canonical MAP kinase signaling cascade (55).

H_2O_2 signaling in plants

Chloroplasts are organelles with a high rate of electron flow, which provides an additional source of oxidant production in plants. Electron leakage can occur at the levels of photosystem I and photosystem II, and superoxide generation by these sources is enhanced in plant cells subjected to adverse conditions limiting CO_2 fixation. Other general

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
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sources of ROS generation in plants are similar to those in mammalian cells, including the mitochondrial electron transport chain, several mitochondrial matrix enzymes, and NADPH oxidases. However, some differences exist; for example, in photosynthetic cells, the mitochondrial electron transport chain contributes less to superoxide production compared to mammalian cells. Nonetheless, ROS production and scavenging in a plant cell, as in any other cell type, is a finely adjustable balance between redox signaling and oxidative stress (96,108,115), and plant cells are also characterized by the presence of highly efficient antioxidant systems. Some interesting discoveries in the field of redox biology in plants have also been made with the use of HyPer biosensors (2,15,27,37,38,58).

Exposure of photosynthetic cells of plants to high light intensities can cause a rapid rise in H_2O_2 related to photosynthetic metabolism. According to one hypothesis, H_2O_2 plays an important role as a signaling molecule that contributes to acclimation of cells under high light intensities. However, oxidative stress can occur if antioxidant systems cannot cope with elevated H_2O_2 levels. Seedlings of *Arabidopsis thaliana* were transformed with HyPer to monitor cellular H_2O_2 under these conditions. It is noteworthy that the H_2O_2 level increased even in nonphotosynthetic cotyledon epidermal cells after 20 minutes of exposure to high light intensity. This was evidenced by an increase in the HyPer signal by approximately 45 % compared to the zero time point (37). Recently, by using HyPer-2, it was found that exposure to high light intensity increases H_2O_2 production in the chloroplast stroma, cytosol, and nuclei of photosynthetic *Nicotiana benthamiana* epidermal cells (38). It is important to note that stromal H_2O_2 was reduced by over-expression of ascorbate peroxidase in this compartment and that treatment with an inhibitor of photosynthesis attenuated nuclear H_2O_2 accumulation and high light-responsive gene expression. Additionally, over-expression of cytosolic ascorbate peroxidase had a much smaller effect on nuclear H_2O_2 levels and high light-responsive gene expression **(Fig. 6)**. Thus, H_2O_2 is transferred directly to the nucleus from the chloroplasts closely associated with it, bypassing the cytoplasm, and performs photosynthetic control over gene expression (38).

In another study, HyPer was used to visualize dynamic changes in H_2O_2 in *Arabidopsis* roots induced by aluminum treatment (58). It is known that micromolar concentrations of aluminum significantly limit plant growth by disrupting root hair

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
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development, although the precise mechanism by which aluminum inhibits root elongation has not yet been established. HyPer experiments revealed a clear H_2O_2 distribution in growing roots of 5-day-old transgenic *Arabidopsis* seedlings, with the highest concentration observed in the root tip region. However, incubation with 800 nM aluminum for 5 minutes caused a decrease in the overall H_2O_2 level, with the most significant reduction observed in the elongation region. The mature region (where the root hairs have ceased growing) was the most resistant to aluminum exposure. After 15 min of such conditions, root growth was arrested and did not reinitiate, even after several hours, although generation of short secondary roots was stimulated to compensate for this effect. Findings indicate that increased H_2O_2 level is a necessary and tightly regulated event during optimal root growth in plants (58).

In the tip-growing pollen tube, NADPH oxidases produce tip-localized H_2O_2, which is required to maintain a steady, tip-focused Ca^{2+} gradient that is essential for growth; this has also been shown with the use of genetically encoded indicators (15). HyPer can also be expressed in different compartments of the plant cell. For example, HyPer targeted to peroxisomes confirmed the modulation of the H_2O_2 level by Ca^{2+} signaling in this compartment. Intraperoxisomal Ca^{2+} rises after the increase of Ca^{2+} in the cytoplasm, as shown by the use of a GFP-based Cameleon Ca^{2+} indicator. This, in turn, stimulates catalase activity in this compartment (27).

H_2O_2 is involved in signaling processes during the establishment of legume-*Rhizobium* symbioses. As a result of complex molecular interactions between the plant and the symbiont, root nodules are formed, in which nitrogen fixation occurs. NADPH oxidase enzymatic activity appears to play an important role in H_2O_2 generation during the process of symbiosis formation (78), and HyPer probes have been used for imaging H_2O_2 accumulation in nodules. For this purpose, *Medicago truncatula* plants expressing HyPer were inoculated with the *Sinorhizobium meliloti* DsRed strain. Then, H_2O_2 production was observed in 12-day-old root nodules. In this work, the effect of H_2O_2 on certain gene expression levels was studied in the context of the *Medicago truncatula–Sinorhizobium meliloti* symbiotic interaction (2). H_2O_2 generation also occurs during plant–pathogen interactions. For example, in the rice blast fungus, *Magnaporthe oryzae*, it plays an important role in the development of special structures called appressoria that are used to

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

infect host plants. Importantly, appressoria cannot form without the production of H_2O_2. Deletion of NADPH oxidases prevents appressorial development (33). HyPer expression in transformed *M. oryzae* has enabled real-time monitoring of H_2O_2 dynamics during spore germination and appressorial development. Here, the barley leaf surface was infected with conidia, after which HyPer signals were imaged by confocal microscopy at different time intervals during important stages of the plant infection process (63). HyPer-2 was used to analyze H_2O_2 dynamics in mycelia of *Fusarium graminearum in vitro* and in the early infection stages of wheat (86). In another study, HyPer was expressed in the necrotrophic maize pathogen *Cochliobolus heterostrophus* to detect changes in H_2O_2 concentration after exposure to plant phenolic compounds in the wild-type strain and a mutant in the redox-sensitive transcription factor chap1 (102).

HyPer in bacteria

HyPer probes can also be used successfully in prokaryotic systems. All versions of these biosensors have been successfully tested in *E. coli* in order to determine the minimum concentration of exogenous H_2O_2 that leads to changes in fluorescence, which is approximately 5 μM for HyPer (7) and 10 μM for HyPer-Red (35). These studies were aimed only at demonstrating HyPer probe utility in bacterial cells. However, bacteria exposed to external oxidative stress are excellent *in vivo* models for describing events that occur as a result of the immunological response of higher organisms against invasive pathogens. Sikes and colleagues performed detailed characterization of the behavior of the biosensor signal in *E. coli* exposed to external H_2O_2 (73). For example, it was found that HyPer expression decreases the cell's peroxide scavenging capacity. It is also interesting to note that for any experiment, the choice of bacterial strain, timing of measurement, and even growth conditions may significantly affect the HyPer signal. Therefore, all these parameters should be taken into account before carrying out comparative analysis (73).

Conclusions

HyPer family probes are a reliable and proven tool for studying the role of H_2O_2 in various physiological and pathological processes. The main advantage of HyPer, similar to any other genetically encoded indicator, stems from its protein nature so that this probe can be easily expressed in a biological system of any complexity. In recent years, biosensors have been successfully used in various *in vivo* models. This is especially

important because complex processes such as aging and development cannot be studied *in vitro* or even in cell cultures. Modern approaches using indicators based on florescent proteins allow such studies to be carried out in intact living organisms in real time.

A few important points should be taken into account when working with HyPer probes. i) In all eukaryotic systems, HyPer is a foreign protein. Since the H_2O_2-sensitive OxyR part of the biosensor has a bacterial origin, the probability of HyPer interactions with other biomolecules in eukaryotic cells is minimal. An exception is the thiol reduction antioxidant systems through which HyPer can be reversibly reduced after H_2O_2-induced oxidation. Nevertheless, proper control experiments should be performed in any new study. For example, Ursula Jakob and colleagues in their investigation of oxidative stress during the *C. elegans* life span verified that HyPer expression does not affect the life span of the animals (66). ii) Changes in pH in the physiological range affect the HyPer signal. Therefore, pH monitoring in the same system is highly recommended. The most suitable pH control for any HyPer version is a SypHer or SypHer-2 probe. iii) The brightness of HyPer family probes may not be sufficient for use in some subjects, for example, in mammalian tissues.

HyPer probes have great potential for future studies. An important achievement is the development of the red HyPer version, which has expanded the capabilities of multiparametric H_2O_2 imaging. New bright pH stable permuted fluorescent proteins will inevitably appear in the near future and will lead to the development of a set of universal HyPer probes ideally suited for *in vivo* studies.

Acknowledgements

The work was supported by Russian Science Foundation grant 17-15-01175.

Author Disclosure Statement

No competing financial interests exist.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

References

1. Adler V, Yin Z, Tew KD, and Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. *Oncogene* 18: 6104-6111, 1999.
2. Andrio E, Marino D, Marmeys A, de Segonzac MD, Damiani I, Genre A, Huguet S, Frendo P, Puppo A, and Pauly N. Hydrogen peroxide-regulated genes in the Medicago truncatula-Sinorhizobium meliloti symbiosis. *New Phytol* 198: 179-189, 2013.
3. Arner ES and Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267: 6102-6109, 2000.
4. Babior BM, Kipnes RS, and Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest* 52: 741-744, 1973.
5. Back P, De Vos WH, Depuydt GG, Matthijssens F, Vanfleteren JR, and Braeckman BP. Exploring real-time in vivo redox biology of developing and aging Caenorhabditis elegans. *Free Radic Biol Med* 52: 850-859, 2012.
6. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245-313, 2007.
7. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, and Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods* 3: 281-286, 2006.
8. Bencina M. Illumination of the spatial order of intracellular pH by genetically encoded pH-sensitive sensors. *Sensors (Basel)* 13: 16736-16758, 2013.
9. Bienert GP, Schjoerring JK, and Jahn TP. Membrane transport of hydrogen peroxide. *Biochim Biophys Acta* 1758: 994-1003, 2006.
10. Bilan DS and Belousov VV. Genetically encoded probes for NAD+/NADH monitoring. *Free Radic Biol Med* 100: 32-42, 2016.
11. Bilan DS and Belousov VV. HyPer family probes: state of the art. *Antioxid Redox Signal* 24: 731-751, 2016.
12. Bilan DS and Belousov VV. New tools for redox biology: From imaging to manipulation. *Free Radic Biol Med* 109: 167-188, 2017.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

13. Bilan DS, Pase L, Joosen L, Gorokhovatsky AY, Ermakova YG, Gadella TW, Grabher C, Schultz C, Lukyanov S, and Belousov VV. HyPer-3: a genetically encoded H(2)O(2) probe with improved performance for ratiometric and fluorescence lifetime imaging. *ACS Chem Biol* 8: 535-542, 2013.

14. Biteau B, Labarre J, and Toledano MB. ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin. *Nature* 425: 980-984, 2003.

15. Boisson-Dernier A, Lituiev DS, Nestorova A, Franck CM, Thirugnanarajah S, and Grossniklaus U. ANXUR receptor-like kinases coordinate cell wall integrity with growth at the pollen tube tip via NADPH oxidases. *PLoS Biol* 11: e1001719, 2013.

16. Braeckman BP, Smolders A, Back P, and De Henau S. In vivo detection of reactive oxygen species and redox status in Caenorhabditis elegans. *Antioxid Redox Signal* 25: 577-592, 2016.

17. Brand MD. The sites and topology of mitochondrial superoxide production. *Exp Gerontol* 45: 466-472, 2010.

18. Brewer TF, Garcia FJ, Onak CS, Carroll KS, and Chang CJ. Chemical approaches to discovery and study of sources and targets of hydrogen peroxide redox signaling through NADPH oxidase proteins. *Annu Rev Biochem* 84: 765-790, 2015.

19. Brigelius-Flohe R and Maiorino M. Glutathione peroxidases. *Biochim Biophys Acta* 1830: 3289-3303, 2013.

20. Budanov AV, Sablina AA, Feinstein E, Koonin EV, and Chumakov PM. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304: 596-600, 2004.

21. Cabreiro F, Ackerman D, Doonan R, Araiz C, Back P, Papp D, Braeckman BP, and Gems D. Increased life span from overexpression of superoxide dismutase in Caenorhabditis elegans is not caused by decreased oxidative damage. *Free Radic Biol Med* 51: 1575-1582, 2011.

22. Cameron WD, Bui CV, Hutchinson A, Loppnau P, Graslund S, and Rocheleau JV. Apollo-NADP(+): a spectrally tunable family of genetically encoded sensors for NADP(+). *Nat Methods* 13: 352-358, 2016.

23. Chelikani P, Fita I, and Loewen PC. Diversity of structures and properties among catalases. *Cell Mol Life Sci* 61: 192-208, 2004.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

24. Cho SH, Lee CH, Ahn Y, Kim H, Kim H, Ahn CY, Yang KS, and Lee SR. Redox regulation of PTEN and protein tyrosine phosphatases in H(2)O(2) mediated cell signaling. *FEBS Lett* 560: 7-13, 2004.

25. Choi H, Kim S, Mukhopadhyay P, Cho S, Woo J, Storz G, and Ryu SE. Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105: 103-113, 2001.

26. Collet JF and Messens J. Structure, function, and mechanism of thioredoxin proteins. *Antioxid Redox Signal* 13: 1205-1216, 2010.

27. Costa A, Drago I, Behera S, Zottini M, Pizzo P, Schroeder JI, Pozzan T, and Lo Schiavo F. H2O2 in plant peroxisomes: an in vivo analysis uncovers a Ca(2+)-dependent scavenging system. *Plant J* 62: 760-772, 2010.

28. Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide* 1: 145-157, 1997.

29. D'Autreaux B and Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8: 813-824, 2007.

30. Deng Q, Harvie EA, and Huttenlocher A. Distinct signalling mechanisms mediate neutrophil attraction to bacterial infection and tissue injury. *Cell Microbiol* 14: 517-528, 2012.

31. Drazic A and Winter J. The physiological role of reversible methionine oxidation. *Biochim Biophys Acta* 1844: 1367-1382, 2014.

32. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47-95, 2002.

33. Egan MJ, Wang ZY, Jones MA, Smirnoff N, and Talbot NJ. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proc Natl Acad Sci U S A* 104: 11772-11777, 2007.

34. Elsliger MA, Wachter RM, Hanson GT, Kallio K, and Remington SJ. Structural and spectral response of green fluorescent protein variants to changes in pH. *Biochemistry* 38: 5296-5301, 1999.

35. Ermakova YG, Bilan DS, Matlashov ME, Mishina NM, Markvicheva KN, Subach OM, Subach FV, Bogeski I, Hoth M, Enikolopov G, and Belousov VV. Red fluorescent genetically encoded indicator for intracellular hydrogen peroxide. *Nat Commun* 5:

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

5222, 2014.

36. Ewald CY, Hourihan JM, Bland MS, Obieglo C, Katic I, Moronetti Mazzeo LE, Alcedo J, Blackwell TK, and Hynes NE. NADPH oxidase-mediated redox signaling promotes oxidative stress resistance and longevity through memo-1 in C. elegans. *Elife* 6: e19493, 2017.
37. Exposito-Rodriguez M, Laissue PP, Littlejohn GR, Smirnoff N, and Mullineaux PM. The use of HyPer to examine spatial and temporal changes in H2O2 in high light-exposed plants. *Methods Enzymol* 527: 185-201, 2013.
38. Exposito-Rodriguez M, Laissue PP, Yvon-Durocher G, Smirnoff N, and Mullineaux PM. Photosynthesis-dependent H2O2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. *Nat Commun* 8: 49, 2017.
39. Fan Y, Makar M, Wang MX, and Ai HW. Monitoring thioredoxin redox with a genetically encoded red fluorescent biosensor. *Nat Chem Biol* 13: 1045-1052, 2017.
40. Fernandes AP and Holmgren A. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid Redox Signal* 6: 63-74, 2004.
41. Ferreira LF and Laitano O. Regulation of NADPH oxidases in skeletal muscle. *Free Radic Biol Med* 98: 18-28, 2016.
42. Finkel T. Signal transduction by reactive oxygen species in non-phagocytic cells. *J Leukoc Biol* 65: 337-340, 1999.
43. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol* 194: 7-15, 2011.
44. Finkel T. From sulfenylation to sulfhydration: what a thiolate needs to tolerate. *Sci Signal* 5: pe10, 2012.
45. Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247, 2000.
46. Freitas M, Lima JL, and Fernandes E. Optical probes for detection and quantification of neutrophils' oxidative burst. A review. *Anal Chim Acta* 649: 8-23, 2009.
47. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64: 97-112, 1995.
48. Fridovich I. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem* 272: 18515-18517, 1997.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

49. Gauron C, Meda F, Dupont E, Albadri S, Quenech'Du N, Ipendey E, Volovitch M, Del Bene F, Joliot A, Rampon C, and Vriz S. Hydrogen peroxide (H2O2) controls axon pathfinding during zebrafish development. *Dev Biol* 414: 133-141, 2016.

50. Gomes A, Fernandes E, and Lima JL. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods* 65: 45-80, 2005.

51. Groeger G, Quiney C, and Cotter TG. Hydrogen peroxide as a cell-survival signaling molecule. *Antioxid Redox Signal* 11: 2655-2671, 2009.

52. Gutscher M, Pauleau AL, Marty L, Brach T, Wabnitz GH, Samstag Y, Meyer AJ, and Dick TP. Real-time imaging of the intracellular glutathione redox potential. *Nat Methods* 5: 553-559, 2008.

53. Gutscher M, Sobotta MC, Wabnitz GH, Ballikaya S, Meyer AJ, Samstag Y, and Dick TP. Proximity-based protein thiol oxidation by H2O2-scavenging peroxidases. *J Biol Chem* 284: 31532-31540, 2009.

54. Han J and Burgess K. Fluorescent indicators for intracellular pH. *Chem Rev* 110: 2709-2728, 2010.

55. Han P, Zhou XH, Chang N, Xiao CL, Yan S, Ren H, Yang XZ, Zhang ML, Wu Q, Tang B, Diao JP, Zhu X, Zhang C, Li CY, Cheng H, and Xiong JW. Hydrogen peroxide primes heart regeneration with a derepression mechanism. *Cell Res* 24: 1091-1107, 2014.

56. Hanson GT, McAnaney TB, Park ES, Rendell ME, Yarbrough DK, Chu S, Xi L, Boxer SG, Montrose MH, and Remington SJ. Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry* 41: 15477-15488, 2002.

57. Henzler T and Steudle E. Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H(2)O(2) across water channels. *J Exp Bot* 51: 2053-2066, 2000.

58. Hernandez-Barrera A, Velarde-Buendia A, Zepeda I, Sanchez F, Quinto C, Sanchez-Lopez R, Cheung AY, Wu HM, and Cardenas L. Hyper, a hydrogen peroxide sensor, indicates the sensitivity of the Arabidopsis root elongation zone to aluminum treatment. *Sensors (Basel)* 15: 855-867, 2015.

59. Hofmann B, Hecht HJ, and Flohe L. Peroxiredoxins. *Biol Chem* 383: 347-364, 2002.

60. Holmstrom KM and Finkel T. Cellular mechanisms and physiological consequences

of redox-dependent signalling. *Nat Rev Mol Cell Biol* 15: 411-421, 2014.

61. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 98: 453-462, 2006.

62. Huang BK and Sikes HD. Quantifying intracellular hydrogen peroxide perturbations in terms of concentration. *Redox Biol* 2: 955-962, 2014.

63. Huang K, Caplan J, Sweigard JA, Czymmek KJ, and Donofrio NM. Optimization of the HyPer sensor for robust real-time detection of hydrogen peroxide in the rice blast fungus. *Mol Plant Pathol* 18: 298-307, 2017.

64. Imlay JA. Transcription factors that defend bacteria against reactive oxygen species. *Annu Rev Microbiol* 69: 93-108, 2015.

65. Kirstein J, Morito D, Kakihana T, Sugihara M, Minnen A, Hipp MS, Nussbaum-Krammer C, Kasturi P, Hartl FU, Nagata K, and Morimoto RI. Proteotoxic stress and ageing triggers the loss of redox homeostasis across cellular compartments. *Embo j* 34: 2334-2349, 2015.

66. Knoefler D, Thamsen M, Koniczek M, Niemuth NJ, Diederich AK, and Jakob U. Quantitative in vivo redox sensors uncover oxidative stress as an early event in life. *Mol Cell* 47: 767-776, 2012.

67. Kohen R and Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 30: 620-650, 2002.

68. Krumova K and Cosa G. Chapter 1 Overview of Reactive Oxygen Species. In: *Singlet Oxygen: Applications in Biosciences and Nanosciences, Volume 1*. The Royal Society of Chemistry; 2016. pp. 1-21.

69. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4: 181-189, 2004.

70. Lee C, Lee SM, Mukhopadhyay P, Kim SJ, Lee SC, Ahn WS, Yu MH, Storz G, and Ryu SE. Redox regulation of OxyR requires specific disulfide bond formation involving a rapid kinetic reaction path. *Nat Struct Mol Biol* 11: 1179-1185, 2004.

71. Liang X, Kaya A, Zhang Y, Le DT, Hua D, and Gladyshev VN. Characterization of methionine oxidation and methionine sulfoxide reduction using methionine-rich cysteine-free proteins. *BMC Biochem* 13: 21, 2012.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)
This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

72. Lillig CH, Berndt C, and Holmgren A. Glutaredoxin systems. *Biochim Biophys Acta* 1780: 1304-1317, 2008.

73. Lim JB, Barker KA, Huang BK, and Sikes HD. In-depth characterization of the fluorescent signal of HyPer, a probe for hydrogen peroxide, in bacteria exposed to external oxidative stress. *J Microbiol Methods* 106: 33-39, 2014.

74. Love NR, Chen Y, Ishibashi S, Kritsiligkou P, Lea R, Koh Y, Gallop JL, Dorey K, and Amaya E. Amputation-induced reactive oxygen species are required for successful Xenopus tadpole tail regeneration. *Nat Cell Biol* 15: 222-228, 2013.

75. Ma MW, Wang J, Zhang Q, Wang R, Dhandapani KM, Vadlamudi RK, and Brann DW. NADPH oxidase in brain injury and neurodegenerative disorders. *Mol Neurodegener* 12: 7, 2017.

76. Malinouski M, Zhou Y, Belousov VV, Hatfield DL, and Gladyshev VN. Hydrogen peroxide probes directed to different cellular compartments. *PLoS One* 6: e14564, 2011.

77. Marinho HS, Real C, Cyrne L, Soares H, and Antunes F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol* 2: 535-562, 2014.

78. Marino D, Andrio E, Danchin EG, Oger E, Gucciardo S, Lambert A, Puppo A, and Pauly N. A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning. *New Phytol* 189: 580-592, 2011.

79. Markvicheva KN, Bilan DS, Mishina NM, Gorokhovatsky AY, Vinokurov LM, Lukyanov S, and Belousov VV. A genetically encoded sensor for H2O2 with expanded dynamic range. *Bioorg Med Chem* 19: 1079-1084, 2011.

80. Matlashov ME, Belousov VV, and Enikolopov G. How much H(2)O(2) is produced by recombinant D-amino acid oxidase in mammalian cells? *Antioxid Redox Signal* 20: 1039-1044, 2014.

81. Matlashov ME, Bogdanova YA, Ermakova GV, Mishina NM, Ermakova YG, Nikitin ES, Balaban PM, Okabe S, Lukyanov S, Enikolopov G, Zaraisky AG, and Belousov VV. Fluorescent ratiometric pH indicator SypHer2: Applications in neuroscience and regenerative biology. *Biochim Biophys Acta* 1850: 2318-2328, 2015.

82. Meda F, Gauron C, Rampon C, Teillon J, Volovitch M, and Vriz S. Nerves control redox levels in mature tissues through schwann cells and hedgehog signaling.

Antioxid Redox Signal 24: 299-311, 2016.

83. Medrano-Fernandez I, Bestetti S, Bertolotti M, Bienert GP, Bottino C, Laforenza U, Rubartelli A, and Sitia R. Stress regulates aquaporin-8 permeability to impact cell growth and survival. *Antioxid Redox Signal* 24: 1031-1044, 2016.

84. Mehmeti I, Lortz S, and Lenzen S. The H2O2-sensitive HyPer protein targeted to the endoplasmic reticulum as a mirror of the oxidizing thiol-disulfide milieu. *Free Radic Biol Med* 53: 1451-1458, 2012.

85. Melo EP, Lopes C, Gollwitzer P, Lortz S, Lenzen S, Mehmeti I, Kaminski CF, Ron D, and Avezov E. TriPer, an optical probe tuned to the endoplasmic reticulum tracks changes in luminal H2O2. *BMC Biol* 15: 24, 2017.

86. Mentges M and Bormann J. Real-time imaging of hydrogen peroxide dynamics in vegetative and pathogenic hyphae of Fusarium graminearum. *Sci Rep* 5: 14980, 2015.

87. Miller EW, Dickinson BC, and Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci U S A* 107: 15681-15686, 2010.

88. Mishina NM, Bogeski I, Bolotin DA, Hoth M, Niemeyer BA, Schultz C, Zagaynova EV, Lukyanov S, and Belousov VV. Can we see PIP(3) and hydrogen peroxide with a single probe? *Antioxid Redox Signal* 17: 505-512, 2012.

89. Mishina NM, Mishin AS, Belyaev Y, Bogdanova EA, Lukyanov S, Schultz C, and Belousov VV. Live-cell STED microscopy with genetically encoded biosensor. *Nano Lett* 15: 2928-2932, 2015.

90. Mishina NM, Tyurin-Kuzmin PA, Markvicheva KN, Vorotnikov AV, Tkachuk VA, Laketa V, Schultz C, Lukyanov S, and Belousov VV. Does cellular hydrogen peroxide diffuse or act locally? *Antioxid Redox Signal* 14: 1-7, 2011.

91. Morgan B, Van Laer K, Owusu TN, Ezerina D, Pastor-Flores D, Amponsah PS, Tursch A, and Dick TP. Real-time monitoring of basal H2O2 levels with peroxiredoxin-based probes. *Nat Chem Biol* 12: 437-443, 2016.

92. Nagy P. Kinetics and mechanisms of thiol-disulfide exchange covering direct substitution and thiol oxidation-mediated pathways. *Antioxid Redox Signal* 18: 1623-1641, 2013.

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

93. Nakabayashi T, Wang HP, Kinjo M, and Ohta N. Application of fluorescence lifetime imaging of enhanced green fluorescent protein to intracellular pH measurements. *Photochem Photobiol Sci* 7: 668-670, 2008.

94. Nathan C and Cunningham-Bussel A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat Rev Immunol* 13: 349-361, 2013.

95. Niethammer P, Grabher C, Look AT, and Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* 459: 996-999, 2009.

96. Ortega-Villasante C, Buren S, Baron-Sola A, Martinez F, and Hernandez LE. In vivo ROS and redox potential fluorescent detection in plants: Present approaches and future perspectives. *Methods* 109: 92-104, 2016.

97. Pase L, Layton JE, Wittmann C, Ellett F, Nowell CJ, Reyes-Aldasoro CC, Varma S, Rogers KL, Hall CJ, Keightley MC, Crosier PS, Grabher C, Heath JK, Renshaw SA, and Lieschke GJ. Neutrophil-delivered myeloperoxidase dampens the hydrogen peroxide burst after tissue wounding in zebrafish. *Curr Biol* 22: 1818-1824, 2012.

98. Poburko D, Santo-Domingo J, and Demaurex N. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. *J Biol Chem* 286: 11672-11684, 2011.

99. Reczek CR and Chandel NS. ROS-dependent signal transduction. *Curr Opin Cell Biol* 33: 8-13, 2015.

100. Rhee SG, Chang TS, Jeong W, and Kang D. Methods for detection and measurement of hydrogen peroxide inside and outside of cells. *Mol Cells* 29: 539-549, 2010.

101. Rieger S and Sagasti A. Hydrogen peroxide promotes injury-induced peripheral sensory axon regeneration in the zebrafish skin. *PLoS Biol* 9: e1000621, 2011.

102. Ronen M, Shalaby S, and Horwitz BA. Role of the transcription factor ChAP1 in cytoplasmic redox homeostasis: imaging with a genetically encoded sensor in the maize pathogen Cochliobolus heterostrophus. *Mol Plant Pathol* 14: 786-790, 2013.

103. Schieber M and Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol* 24: R453-462, 2014.

104. Schmitt FJ, Thaa B, Junghans C, Vitali M, Veit M, and Friedrich T. eGFP-pHsens as a highly sensitive fluorophore for cellular pH determination by fluorescence lifetime

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

imaging microscopy (FLIM). *Biochim Biophys Acta* 1837: 1581-1593, 2014.

105. Schwarzlander M, Dick TP, Meyer AJ, and Morgan B. Dissecting redox biology using fluorescent protein sensors. *Antioxid Redox Signal* 24: 680-712, 2016.

106. Sen CK and Roy S. Redox signals in wound healing. *Biochim Biophys Acta* 1780: 1348-1361, 2008.

107. Sevier CS, Qu H, Heldman N, Gross E, Fass D, and Kaiser CA. Modulation of cellular disulfide-bond formation and the ER redox environment by feedback regulation of Ero1. *Cell* 129: 333-344, 2007.

108. Sharma P, Jha AB, Dubey RS, and Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* 2012: 26, 2012.

109. Sies H. Role of metabolic H2O2 generation: redox signaling and oxidative stress. *J Biol Chem* 289: 8735-8741, 2014.

110. Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biol* 11: 613-619, 2017.

111. Stadtman ER, Moskovitz J, and Levine RL. Oxidation of methionine residues of proteins: biological consequences. *Antioxid Redox Signal* 5: 577-582, 2003.

112. Stone JR and Yang S. Hydrogen peroxide: a signaling messenger. *Antioxid Redox Signal* 8: 243-270, 2006.

113. Tao R, Zhao Y, Chu H, Wang A, Zhu J, Chen X, Zou Y, Shi M, Liu R, Su N, Du J, Zhou HM, Zhu L, Qian X, Liu H, Loscalzo J, and Yang Y. Genetically encoded fluorescent sensors reveal dynamic regulation of NADPH metabolism. *Nat Methods* 14: 720-728, 2017.

114. Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* 121: 667-670, 2005.

115. Tripathy BC and Oelmuller R. Reactive oxygen species generation and signaling in plants. *Plant Signal Behav* 7: 1621-1633, 2012.

116. Uttara B, Singh AV, Zamboni P, and Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol* 7: 65-74, 2009.

117. Veal EA, Day AM, and Morgan BA. Hydrogen peroxide sensing and signaling. *Mol Cell* 26: 1-14, 2007.

118. Wang H, Karadge U, Humphries WH 4th, and Fisher AL. Analyzing cell physiology in C. elegans with fluorescent ratiometric reporters. *Methods* 68: 508-517, 2014.

119. Weisiger RA and Fridovich I. Superoxide dismutase. Organelle specificity. *J Biol Chem* 248: 3582-3592, 1973.

120. Weller J, Kizina KM, Can K, Bao G, and Muller M. Response properties of the genetically encoded optical H2O2 sensor HyPer. *Free Radic Biol Med* 76: 227-241, 2014.

121. Winterbourn CC. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochim Biophys Acta* 1840: 730-738, 2014.

122. Wood ZA, Schroder E, Robin Harris J, and Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28: 32-40, 2003.

123. Yan B, Han P, Pan L, Lu W, Xiong J, Zhang M, Zhang W, Li L, and Wen Z. IL-1beta and reactive oxygen species differentially regulate neutrophil directional migration and Basal random motility in a zebrafish injury-induced inflammation model. *J Immunol* 192: 5998-6008, 2014.

124. Yoo SK, Starnes TW, Deng Q, and Huttenlocher A. Lyn is a redox sensor that mediates leukocyte wound attraction in vivo. *Nature* 480: 109-112, 2011.

125. Zhao Y and Yang Y. Real-time and high-throughput analysis of mitochondrial metabolic states in living cells using genetically encoded NAD+/NADH sensors. *Free Radic Biol Med* 100: 43-52, 2016.

126. Zheng M, Aslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718-1721, 1998.

Abbreviations used

BCECF = 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein

cAMP = cyclic adenosine monophosphate

cpFP = circularly permuted fluorescent protein

DAO = D-amino acid oxidase

Duox = dual oxidase

FLIM = fluorescence lifetime imaging microscopy

FPs = fluorescent proteins

ICAT = isotope-coded affinity tag

IGF = insulin growth factor

MAP kinase = mitogen-activated protein kinase

PDGF = platelet-derived growth factor

PI3K = phosphatidylinositol 3-kinase

PIP3 = phosphatidylinositol 3,4,5-trisphosphate

roGFP = redox green fluorescent protein

ROS = reactive oxygen species

STED microscopy = stimulated emission depletion microscopy

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

Figure legends

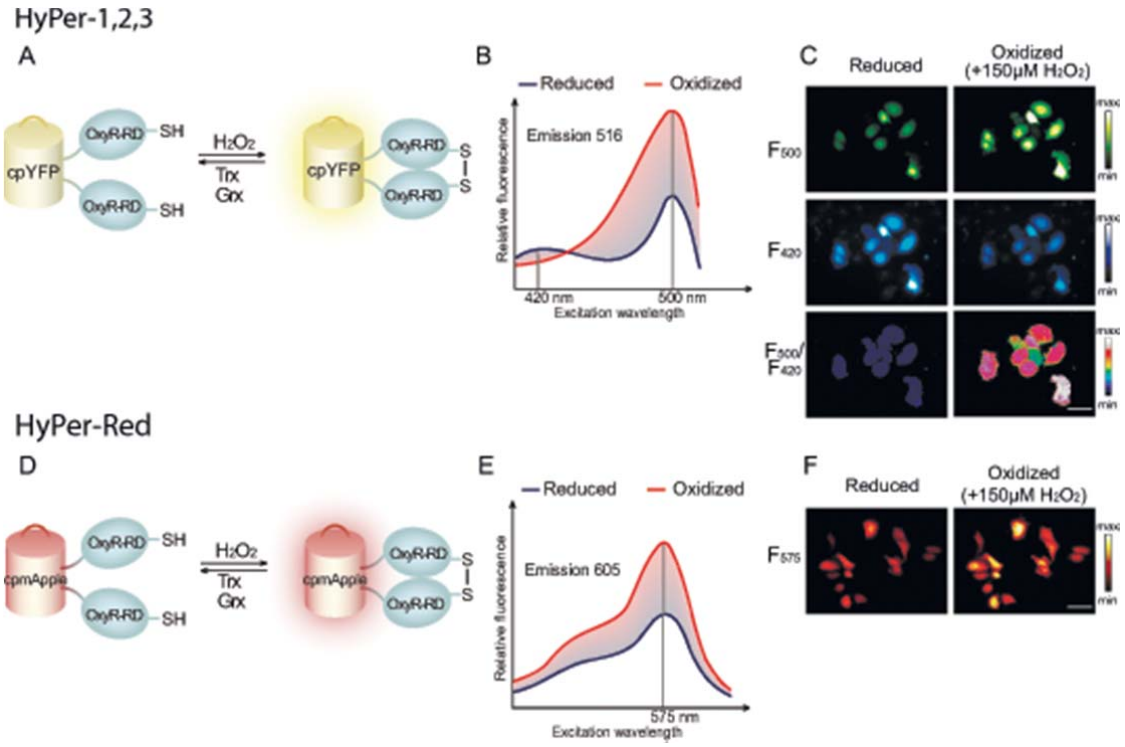

FIG. 1. Graphical schemes and spectral characteristics of the HyPer family probes. The scheme of HyPer-1,2,3 (A) and HyPer-Red (D) structures and their oxidation and reduction reactions. Excitation spectrum of reduced (blue line) and full oxidized (red line) HyPer probes based on cpYFP (B) and cpmApple (E). Images of HyPer-3 (C) and HyPer-Red (F) before and after addition of 150 μM of H_2O_2. HyPer-3 was excited in two channels at 420 nm and 500 nm (F_{420} and F_{500}), respectively. HyPer-Red excited at 575 nm (F_{575}). Scale bar, 40 μm. Lookup tables indicate HyPer probes brightness changes in corresponding channels and F_{500}/F_{420} ratio for HyPer-3. **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

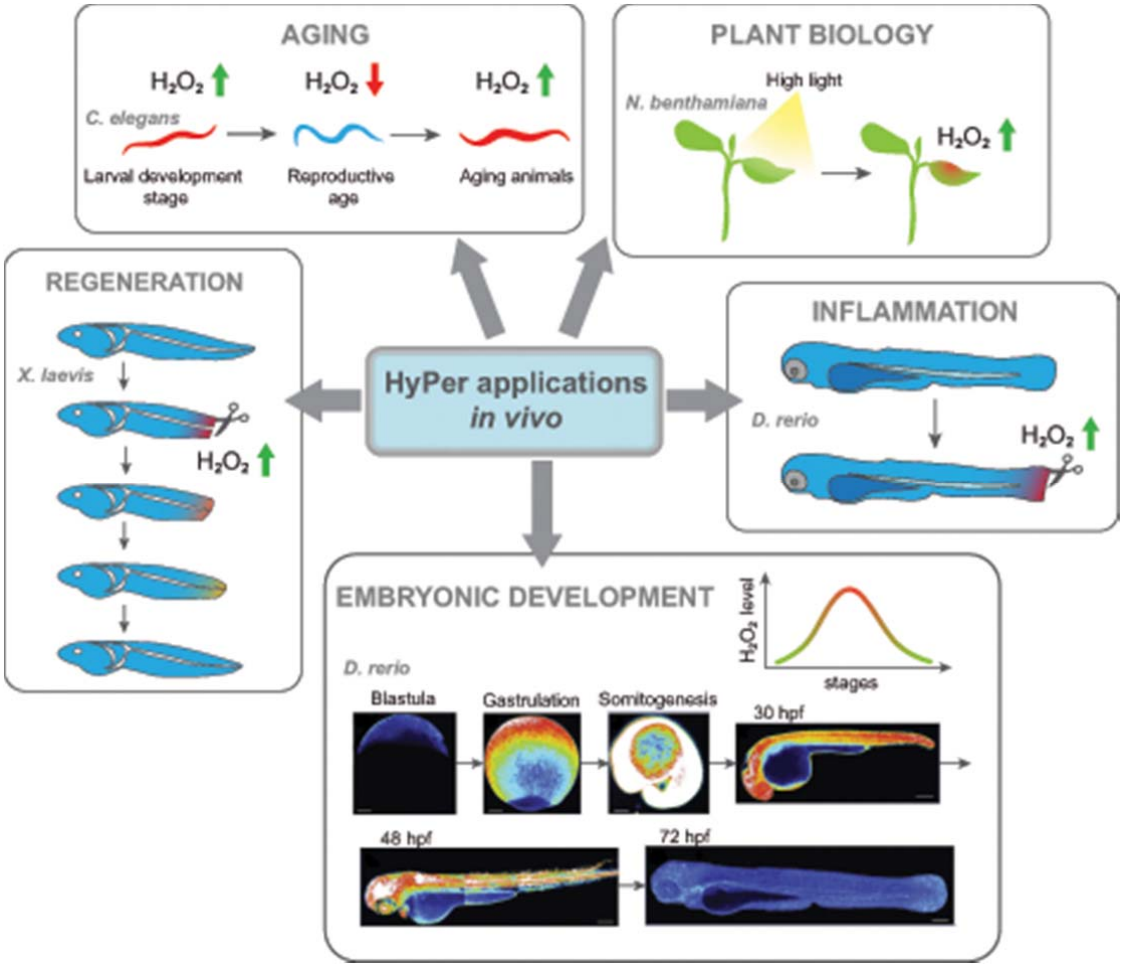

FIG. 2. Selected examples of HyPer use *in vivo*. HyPer probes can be used in a wide variety of model organisms. These and other examples detailed in the text. (Panel "Embryonic development" was reprinted with permission from Gauron et al., (49)). **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

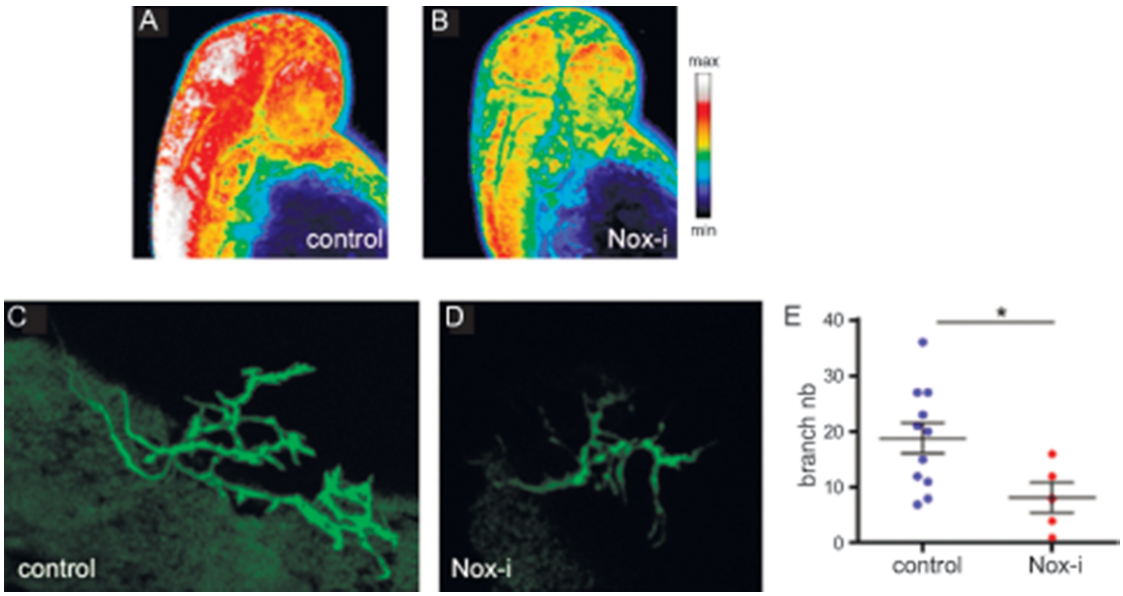

FIG. 3. A reduction of the H_2O_2 levels impairs the retinal ganglion cells projections (Reprinted with permission from Gauron et al., (49)). (A, B) HyPer imaging at 2 dpf in the control (n=3), or NADPH oxidase inhibitor (Nox-i)-treated larvae (n=3). Lookup table indicates HyPer F500/F420 ratio. (C, D) GFP expression in the [pou4f3:GAL4, UAS:GAP-GFP] larvae with or without Nox-i treatment. (E) Branch number per axon in this transgenic line. The error bars represent the standard error of the mean (SEM) (*p<0.05). **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

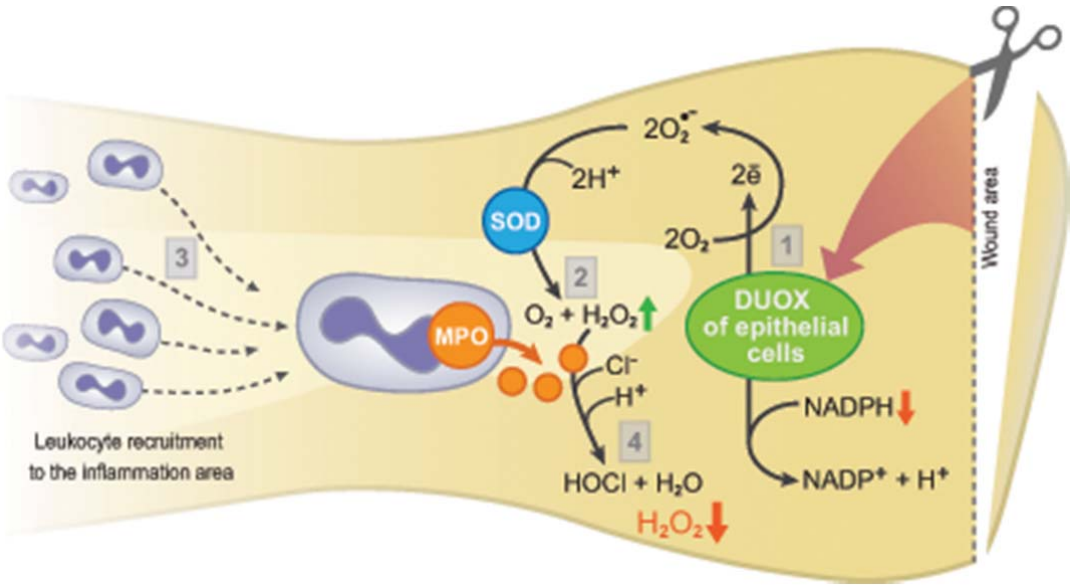

FIG. 4. Schematic illustration of redox events in the inflammation area caused by wounding of the tail fin of zebrafish *D. rerio* larva. 1 – The wound in the tail fin causes the activation of dual oxidase (DUOX) in epithelial cells. 2 – DUOX-driven $O_2^{\bullet-}$ rapidly dismutates into H_2O_2 due to the reaction catalyzed by superoxide dismutase (SOD). 3 – H_2O_2 gradient formed in the tissue is required for leukocyte recruitment to the area of inflammation. 4 – Ultimately, leukocytes participate in H_2O_2 degradation, decreasing its gradient via the myeloperoxidase (MPO) reaction. **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

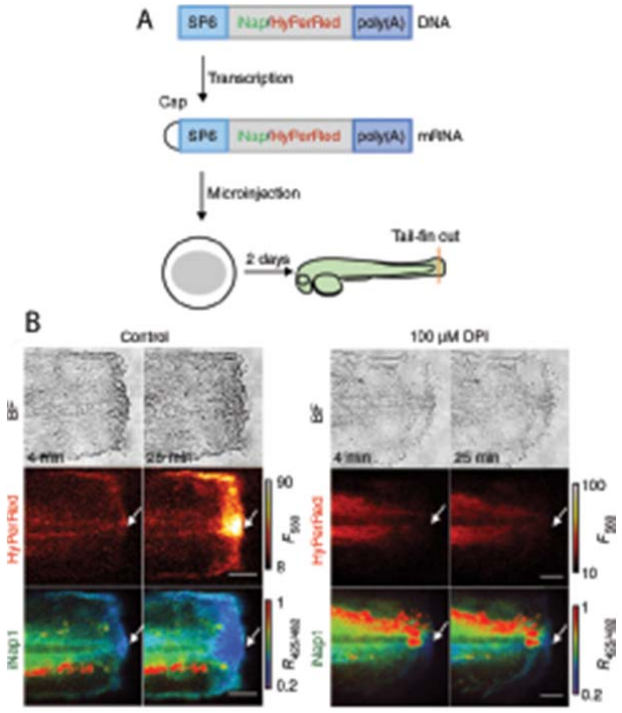

FIG. 5. Simultaneous visualization of NADPH and H_2O_2 dynamics in wound margin by coexpression of iNap1 and HyPerRed in zebrafish larvae (Reprinted with permission from Tao et al., (113)). (A) General transgenic zebrafish procedure. (B) Simultaneous visualization of NADPH and H_2O_2 dynamics in wound margin by coexpression of iNap1 and HyPerRed in zebrafish larvae with or without 100 μM DPI (diphenyleneiodonium, the NADPH oxidase inhibitor). Tail-fin tip amputation was performed at the 0-min time point. Scale bars, 50 μm. **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)

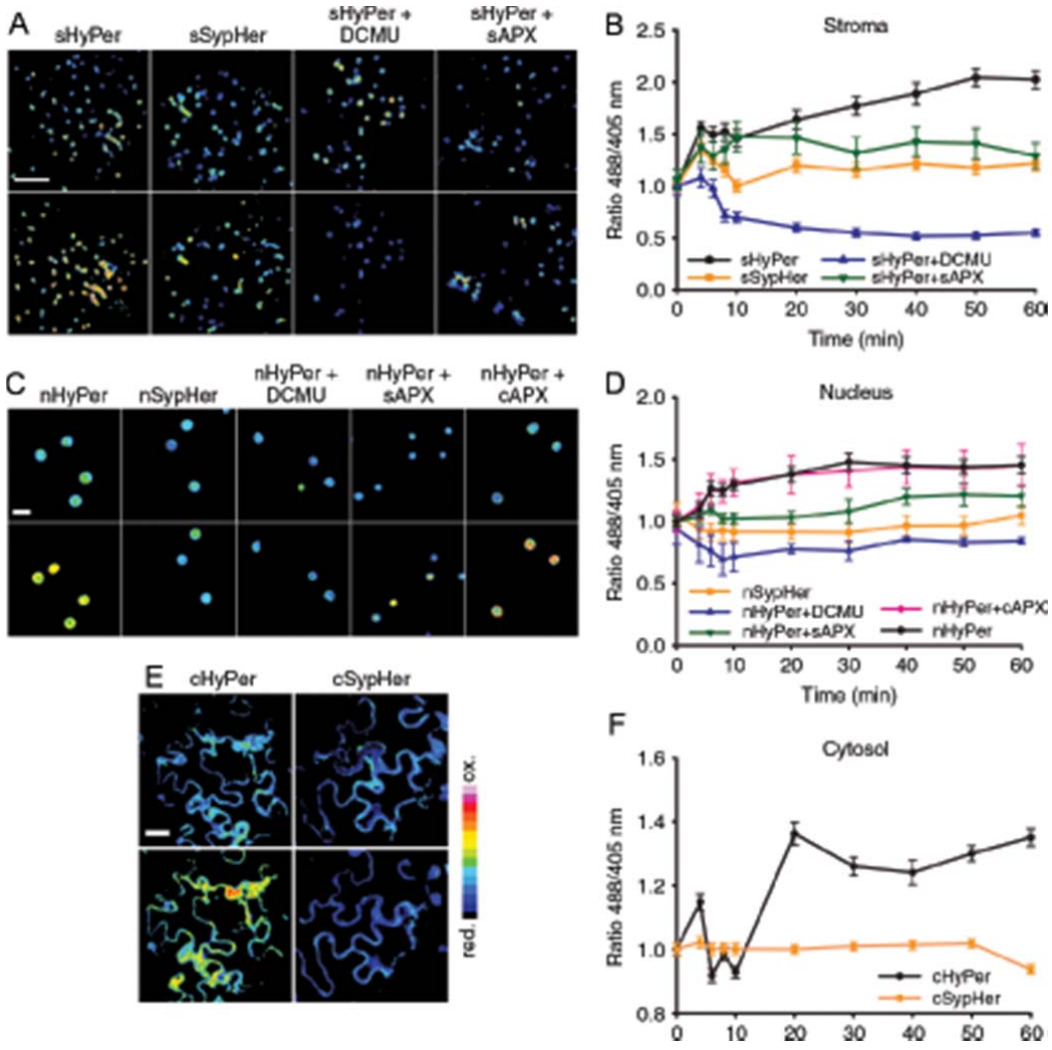

FIG. 6. Time course of HyPer2 and SypHer2 responses to high light (HL) expressed in different subcellular compartments of *N. benthamiana* epidermal cells (Reprinted with permission from Exposito-Rodriguez et al., (38)). (A, B) *N. benthamiana* abaxial epidermal cells expressing stromal sHyPer2 and sSypHer2. (A) 488/405 nm ratio images of resting state in the dark (upper row) and after 60 min HL (lower row). From left to right, the panels show the fluorescence of chloroplasts of cells from leaves expressing sHyPer2, sSypHer2, sHyPer2 treated with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, the photosynthetic electron transport inhibitor) (10 μM for 20 min prior to illumination) and over-expressing sAPX (ascorbate peroxidase), respectively. (B) The effect of exposure to HL over 60 min on the 488/405 nm fluorescence ratio of chloroplasts in epidermal cells expressing sHyper2 and sSypHer2. The data points were normalized to the mean initial value for each treatment and represent the mean ± SEM values from 40–70 chloroplasts

from 3 independent experiments per treatment. (C, D) Equivalent to panels (A) and (B) except that the cells expressed nuclear nHyPer2 and nSypHer2. From left to right, the panels show the fluorescence from nuclei expressing nHyPer2, nSypHer2, sHyPer2 treated with DCMU (10 µM for 20 min prior to illumination), over-expressing sAPX and cAPX in stroma and cytosol, respectively. The data points were normalized to the mean initial value for each treatment and represent the mean ± SEM from 8–18 nuclei from 3 independent experiments per treatment. (E, F) *N. benthamiana* abaxial epidermal cells expressing cytoplasmic cHyPer2 and cSypHer2. (E) 488/405 nm ratio images of resting state in the dark (upper row) and after 60 min HL (lower row) of cHyPer2 and cSypHer2 right and left column, respectively. (F) Quantification of the HL-dependent changes in cHyPer2 and cSypHer2 fluorescence over 60 min HL. The data points were normalized to the mean initial value for each treatment and represent the mean ± SEM from 12–15 cells from 3 independent experiments per treatment. Scale bar = 50 µm. **To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.**

Antioxidants and Redox Signaling
In vivo imaging of hydrogen peroxide with HyPer probes (DOI: 10.1089/ars.2018.7540)