Subcellular Redox Targeting: Bridging *In Vitro* and *In Vivo* Chemical Biology

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Abstract

Networks of redox sensor proteins within discrete microdomains regulate the flow of redox signaling. Yet, the inherent reactivity of redox signals complicates the study of specific redox events and pathways by traditional methods. Herein, we review designer chemistries capable of measuring flux and/or mimicking subcellular redox signaling at the cellular and organismal level. Such efforts have begun to decipher the logic underlying organelle-, site-, and target-specific redox signaling *in vitro* and *in vivo*. These data highlight chemical biology as a perfect gateway to interrogate how Nature choreographs subcellular redox chemistry to drive precision redox biology.

Graphical abstract
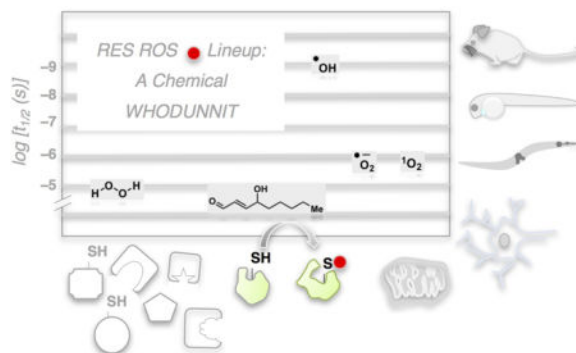
Introduction

Biological systems must respond rapidly to both external and internal stimuli to maintain homeostasis, prevent disease, and repair damage. Protein expression level changes are one way to mount dynamic responses. But to orchestrate a rapid response without relinquishing upstream control, biology often relies on posttranslational modifications (PTMs) of specific

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proteins to effect changes in activity, function, locale, or stability of preexisting proteins. The resulting signaling cascades can influence decision making on an organelle, cellular, and whole-organism level.

Since there are many possible sources of stress, a complex series of signaling subsystems has evolved.[6] The chemical modifications that comprise these systems underpin cellular "signal exchange" (Key words) in which a specific chemical signal is transferred between different proteins or regulates PTMs on other proteins, enabling exquisite control of downstream responses. Traditional signaling events—in which hydrolytically stable adducts are formed between target proteins and the signal—are typically enzyme-catalyzed. Removal of the signal is also enzyme-catalyzed. In an emerging non-canonical signaling mechanism called "redox signaling", reactive chemicals, including electrophilic and oxygen species (RES/ROS)—the focus of this review—serve as signaling mediators modifying discrete signaling proteins, thereby initiating signaling cascades, *largely* without enzyme catalysis. In contrast to canonical enzyme-catalyzed signaling, most RES/ROS are inherently reactive and diffuse rapidly, but have relatively short diffusion distances. Thus, these reactive signals likely use proximity, kinetic privilege, and privilege of occupancy (phenotypic dominance; Key words) to initiate specific downstream events with exquisite specificity.[5–7] Lack of enzymatic control as well as high context dependence has made understanding redox signaling uniquely challenging.[4, 8, 9] Additionally, RES/ROS show hormesis and are toxic at supraphysiological concentrations.

The broader lack of enzyme regulation *at the point of RES/ROS signaling initiation and target adduction* means this area is ideal for study through chemical biology. This review thus focuses on non-enzymatic redox modifications as signal-initiating/transfer points. We concentrate on customized chemical biology toolsets—mostly developed over the last 5–7 years—aimed at deciphering Nature's design principles of redox signaling subsystems in cultured mammalian cells and higher eukaryotic models. Emphasis is placed on endogenous RES/ROS. These studies show that redox-modified sensor proteins behave differently from their unmodified state and have unique functions and interactomes.

Since RES/ROS signaling is nuanced, we focus on mechanisms that operate against or exploit the heterogeneous distribution across distinct subcellular or tissue-scale compartments[10], and time. Accordingly, the majority of this review is concerned with kinetically-controlled processes that help to couple RES/ROS flux to downstream signaling cascades. Given this constraint, as well as the privileged role cysteine adopts in higher organisms,[11] the resurgence of cysteine-targeting covalent drugs,[12] and appreciation of acquired cysteine mutations in disease,[13] we limit our discussion to cysteine-specific, redox-linked modifications. Other nucleophilic residues (e.g., Histidine, Lysine) are targeted by RES/ROS,[14] but to a lesser extent and are primarily detected under bolus RES/ROS treatment or after equilibration.[15]

Unusual suspects

Both RES and ROS are toxic and cause deleterious effects when misregulated, overproduced, or administered for a prolonged time. Only ROS are actual oxidants; RES

influences redox balance through modulation of specific protein activity/function and depleting reducing agents in a redox-independent process. There are relatively few chemotypes of ROS. Endogenous ROS are mostly generated by enzymes with overlapping function/regulation,[16] and classified based upon the oxidation state of molecular oxygen (Table 1). Unlike RES, ROS can form metal-bound complexes. These complexes may show different physiochemical properties to their parent compounds and can themselves form other ROS.[17] These pathways have been implicated in numerous physiological processes, including a recently discovered cell-death pathway, ferroptosis.[18]

ROS engage in both radical and two-electron chemistry, and specific forms can interconvert. For example, $^1O_2$ forms organic peroxides through ene/Diels–Alder chemistry with unsaturated lipids; superoxide is converted to peroxide enzymatically. Thus it can be hard to ascertain which ROS is responsible for a specific bioactivity. The half-lives of ROS also vary, although these calculations are approximations. $^1O_2$, $OH^•$ and $O_2^{•−}$ are universally acknowledged to be short-lived with particularly short diffusion distances; peroxides are at least an order of magnitude longer-lived than the other species discussed here (Table 1).[21] However, even assuming the upper estimate of half-life for peroxide (1 ms), the resulting diffusion length for peroxide is no more than a tenth of the length of a mammalian cell,[23] meaning that even peroxide is localized in cells. Many ROS forms are metabolized enzymatically through direct enzyme–ROS reaction, concurrently modifying these enzymes largely reversibly. Interestingly, these modified enzymes can orchestrate specific signaling events as we discuss in subsequent sections.

There are many types of endogenous bioactive RES. Most of these form aliphatic or aromatic adducts to nucleophilic residues that are kinetically stable. There are some RES-based acylating agents that kinetically react with cysteine, but can be transferred to other residues like lysine in a context-specific manner.[15] Common examples of *endogenous* RES that form kinetically-stable adducts include: enals [e.g., 4-hydroxynonenal (HNE)]; enones (e.g., 15-deoxy- ^{12,14}-prostaglandin-J$_2$); α-chloro aldehydes (e.g., 2-chlorohexadecanal);[24] and quinones (e.g., vitamin K). There are also dietary plant-based RES of which the most common are isothiocyanates.[25] Isothiocyanates form thiocarbamylated enzyme adducts that initially form cysteine adducts that may transfer to lysine, depending on conditions/ protein.[15] There are also well-known synthetic RES including electrophilic drugs, such as dimethyl fumarate (Tecfidera®) approved for the treatment of multiple sclerosis, and bardoxolone methyl in Phase 2/3 trials for various conditions. These compounds likely form irreversible enzyme adducts as they are chemically similar to HNE. Both enzymatic and non-enzymatic lipid peroxidation pathways for the production of RES such as HNE from polyunsaturated fatty acids are known.[26] RES are metabolized through enzymatic redox chemistry[27] and by carnitine/carnosine and glutathione conjugation—the latter catalyzed by glutathione-S-transferase (GST) in cells.[28, 29] Glutathione conjugation to RES does not modify GST, although cellular redox balance is affected due to glutathione depletion. The redox:glutathione adduction pathways partition roughly equally (40 % : 30 %) 2 minutes post exposure.[30] Interestingly, the percentage of HNE-protein adducts formed following acute HNE exposure varies significantly depending on cell type [1% (intestinal enterocytes), 3% (hepatocytes), 8.5% (Ehrlich ascites tumor cells) of total HNE] and subcellular locale (30% HNE adducts are mitochondrial).[31]

General roles of RES and ROS in information transfer

Peroxide interacts with enzymes initially by forming chemically/enzymatically *reversible* cysteine sulfenic acids (cysteine-SOH). Subsequent oxidations are slower, producing sulfinic acid (cysteine-SO_2H, mostly irreversible, although reduced by certain enzymes[32]) and sulfonic acid (cysteine-SO_3H, irreversible).[33] These modifications are redox signals.[34–36] Sulfenic acid formation exerts a reverse effect on cysteine: sulfenic acids can react with nucleophiles, and are often trapped out by another thiol (*signal exchange*, Key words). The resulting disulfide bond can form a crosslink between two spatially distinct residues on a protein, inducing a conformational change. These disulfides can also "*change hands*" (Key words) and oxidize other specific protein thiols to propagate a redox signal through "disulfide relays". Erv1/ALR and Mia40 engage in such a pathway that ultimately stimulates mitochondrial translocation of p53.[37] It has recently been reported that a kinetically controlled ROS-mediated enzyme oxidation event at a *signaling node* (Key words) can usher an enzyme-orchestrated redox-signaling cascade through disulfide exchange.[38] This shows how a "non-targeted," initiating redox signal can be efficiently exchanged into an alternative signal that can be propagated with specificity.

RES modification can elicit protein conformational changes, presumably to a larger extent in cases in which a protein reacts with bifunctional RES such as HNE (e.g., Michael addition with cysteine and Schiff base formation with lysine[6]). Since many RES bind proteins irreversibly, they cannot change hands. Thus, degradation of the target protein is usually required to turn off RES signaling. A RES-modulated signaling node thus likely requires *signal exchange* to propagate cellular information.[39] An exception is acylating RES agents, such as plant-derived isothiocyanates,[25] that can themselves undergo signal exchange.[15]

We will discuss below many cellular pathways that are regulated by RES/ROS. Although some redox-dependent processes have not yet been studied using the chemical biology techniques discussed herein, known RES/ROS-sensitive pathways include several essential transcriptional processes mediated by the redox-sensitive transcription factors FOXO,[40] NFκB[41] and hypoxia inducible factor,[42] among others. Numerous mRNA binding proteins have also been shown to be RES/ROS sensitive, including GAPDH.[43]

(A) Subcellular Redox Target Capture: Molecular Finger Print Analysis

Capture of cysteine thiols/thiolates—Functional cysteines typically have high nucleophilicity. Although an approximation, nucleophilicity generally correlates with low pKa.[44] Reported pK_a values for cysteines in proteins range from 2.5–12, representing at least a ±4 unit change in pK_a with respect to cysteine.[45] Methods to identify redox-functional cysteines typically use electrophilic probes to irreversibly modify reactive cysteines. These probes usually contain an affinity tag for enrichment of modified proteins. Early probes were acyloxymethyl- and chloromethyl-ketones. These reactive probes work well in lysates but are not readily compatible with intact cells. These reagents uncovered the activation and function of caspases during apoptosis.[46,47] Iodoacetamide (**1**)/maleimide-functionalized affinity agents (e.g., **2–3**) are also used and show some preference for labeling specific enzymes, but do not remedy compatibility issues (Figure 1).[48,49, 50] *o*-Nitrobenzyl acetal-caged electrophiles (e.g., **4**) that are compatible with live cells have been developed.[51]

However, the requirement for high probe concentrations and liberation of a reactive nitrosoketone side product limits this technology and its use in cells.

Organelle-specific variants—Some "organelle-specific" capture probes use affinity reagents tailored to a specific class of organelle-specific enzymes or reactive probes fused to a subcellular localization marker. Profiling activity changes in lysosomal cathepsins during autophagy stimulation has been achieved using epoxide-functionalized peptides that react selectively with cathepsins[52]. To assist targeting to the lysosome's acidic interior, the peptides were also functionalized with a weakly basic element [3-(2,4-dinitroanilino)-3′-amino-N-methyldipropylamine]. Global profiling of mitochondrial proteins has recently been achieved using a nonspecific reactive group fused to a mitochondrial targeting sequence.[53] Some (~75%) specificity for the intended organelle was achieved with simple reactive functions. The authors attempted to use photoactivatable reactive groups (phenyl azide and benzophenone) to localize the capture to the mitochondria, but both reactive groups proved unable to identify any labeled peptides by mass spectrometry (MS). Improvements will likely need photocaged electrophiles that are compatible with MS techniques.

Identification of reversible modifications to Cys—A variation on the above methods is used to identify cysteine sulfenic acids. Free thiols are blocked using an unfunctionalized alkylating agent followed by chemoselective reduction of unreactive cysteine-SOH into corresponding thiols by reducing agents (such as arsenite[54]). Thiols thus formed are captured with biotin-maleimide (**5**). MS following affinity capture, electrophoresis, and digestion allows proteins containing cysteine-SOH to be identified (Figure 2a).[54] Similar methods detect cystine, cysteine-SNO and other univalent sulfur modifications.[55–57] Chemical probes for cysteine-SOH harness the dual reactivity of the sulfur within sulfenic acids.[58] The nucleophilicity of cysteine-SOH is utilized by probes like 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (**6**),[59] and more recently reversible arylboronic acids (**7**) and benzoxaborole (**8**).[60] The electrophilicity of cysteine-SOH has been exploited using functionalized dimedones (or analogous 1,3-dicarbonyls[61]) (**9**) that selectively displace water from sulfenic acids either in lysates or in intact cells.[62–64] This approach has been converted to an MS-based method to differentiate between sulfenyl-thiol- and thiol-containing proteins by treating a lysate with D_6-dimedone (**10**) (nucleophilic; reacts with sulfenyl-thiol) and iododimedone (**11**) (reacts with thiols).[63, 65] These two probes produce isotopomeric products contingent only upon the oxidation state of the specific sulfur modified, assuming labeling is independent of redox state of both probe and cysteine (Figure 2b). Interestingly, bicyclo[6.1.0]nonyne traps cysteine sulfenyl acid more than 100 times faster than 1,3-dicarbonyls providing an alternative affinity probe.[66] The rate of adduction is important because lysis is typically carried out under weakly reducing conditions in which cysteines are prone to oxidation. These conditions may give false positives due to competing post-lysis oxidation, especially since *bolus oxidative challenges are often used to upregulate ROS in one sample relative to an unoxidized sample, rendering downstream variables hard to control*. One approach to side-step many of these issues uses yeast transcription factor Yap1, which reacts selectively with cysteine-SOH to form a mixed disulfide.[67] Direct MS analysis has been used in either a bottom up or top down approach.

Detection of RES-modified cysteine—The above strategies are not applicable to RES modifications. Activity-based protein profiling (ABPP) is most commonly used to identify RES-sensitive proteins.[68] In competitive ABPP (Figure 2c), an intact cell, or more commonly a lysate, is treated with the desired RES, then lysates (either derived from cells or directly from the first step as appropriate) are treated with a cysteine-specific labeling agent, such as a functionalized iodoacetamide. A control group is prepared in parallel from lysates treated with the activity probe but with no RES pretreatment. The activity probe contains a handle that allows click attachment of biotin for enrichment and an isotopomeric barcode specific to the control group or the test sample. After enrichment of the labeled proteins using streptavidin-agarose, digestion and liberation from the beads, cysteines labeled by the reagent of choice are enriched in the sample not treated with test compound.[68] This method is high-throughput and has opened a new lens on redox signaling. However, ABPP is capable of profiling only a small percentage (<5%) of cysteines within the proteome, often does not capture active-site cysteines[6] and cannot *prove* that the specific residue profiled is modified by the ligand of interest (only that a functional impediment/loss of signal has occurred). ABPP mostly uses whole cell lysates and is prone to artifacts due to disruption of subcellular redox homeostasis post-lysis. Secondary validations for identified hits are necessary for experimental confidence. Furthermore, ABPP typically gives no information about compartmentalization, although mitochondria-specific profiling during oxidative stress using stable-isotope-labeled cells has been reported.[53]

Complementary alternatives to ABPP rule in specific modifications. Chemical derivatization approaches take advantage of the carbonyl group residual in many RES modifications (such as the aldehyde that is retained after Michael addition to HNE by a cysteine). Typical derivatization reagents include (*a*) hydrazine probe [such as dinitrophenylhydrazine (DNPH)] containing an affinity tag (e.g., **12**) to create a hydrazone, and (*b*) tagged hydroxylamine (e.g., **13**) to form an oxime. Hyrdazones require reduction by borohydride to form a stable hydrazide for identification of modified proteins following enrichment, digestion, and MS.[69, 70] Oximes are sufficiently stable for direct MS analysis and the method has identified carbonyl-modified proteins in cardiac mitochondria.[71] These protocols rely only on the presence of a carbonyl group residual from electrophilic modification so they detect many different electrophilic modifications. Importantly, the identity of the modification can be ascertained from MS. Antibodies detecting various lipid-derived electrophiles (LDEs) are available.[72, 73] Some have affinity for particular modified residues, although several authors have questioned their specificity.[73] Such antibodies are used in immunohistochemistry,[74] western or dot blot, and ELISA[75]. Anti-HNE allowed visualization of HNE-protein adducts due to ischemia in mouse heart[76] and Alzheimer's disease.[77] MS has also been used to identify directly modified proteins. Antibodies to DNPH are also available (so-called "oxy-blot" analysis) but their use is more appropriate for the analysis of non-specific carbonylation within the oxidatively-damaged proteome, as opposed to residual carbonyls from RES conjugation to cysteine or lysine.[78]

Modified lipid electrophiles (e.g., dye-conjugated derivatives) are available, but these reagents are non-native and results should be analyzed with caution.[79] Alkyne-modified HNE (**14**) has also been used. The first application of this strategy involves treatment of

RKO cells with **14**, click chemistry with biotin-azide to append biotin to HNEylated proteins for enrichment, and MS (Figure 2d). This approach has identified many novel cellular HNE-targets.[80] An extension of this method utilizes heavy- and light-isotope-labeled photo-cleavable linkers to which biotin is appended. Following treatment of cells with **14** under different conditions, heavy and light-labeled biotin reagents are conjugated to HNEylated proteins using click chemistry. Following enrichment, exposure to UV light liberates HNEylated targets tagged with a heavy or light barcode from the streptavidin beads. Subsequent MS analysis allows mapping of the HNEylated proteome under different conditions.[81]

Complexities—RES/ROS reactions with proteins are complex. Ignoring the many forms of RES/ROS (some of which interconvert or can convert to other RES/ROS), each RES/ROS can yield various chemical structures upon protein adduction.[82] Some of these adducts may be bioactive, others potentially not. Chemical detection methods may only recognize a subset of possible protein adducts. For instance, worries have been raised that dimedone (**9**) may also (preferentially) probe for sulfenyl amides, the dehydration product of cysteine-SOH.[83] Activity profiling (ABPP) can also lead to false positives due to functional coupling/degradation/adventitious oxidation. We thus urge researchers to validate specific modifications from proteins enriched from extracts by MS wherever possible, use a functional downstream assay under controlled conditions or use the methods below to trigger specific signals in the appropriate context.

(B) Subcellular Redox Imaging: a Biological Stakeout

Typical sensors employ a chromophore activated by a specific redox signal. On/off systems give good signal to noise, but have no internal standard, making comparison of different steady states/cells difficult. Intrinsic variation must be considered because transient expression (that gives a diverse expression profile between different cells) is often used to introduce genetically-encoded reporters (e.g., fluorescent proteins). A common strategy to sidestep problems with on/off sensors is ratiometric measurements, where the chromophore shuttles between *two fluorescent* states. Thus the readout is independent of expression level and can be calibrated using standards.

Genetically encoded sensors for H_2O_2—Development of genetic sensors typically involves mutagenesis of a known protein. Fluorescent proteins are ideal because mutants with <25% homology to wild-type (WT) can retain activity.[84] The most common sensors are roGFP[85] (redox-sensitive GFP) and HyPer (Figure 3a).[86] These sensors couple redox-induced chemical modifications to fluorescence. However, these protein-based probes *react* with H_2O_2 and may short circuit cellular redox couples and/or misdirect signaling. The most recent probes claim to be free of this artifact, although redox balance is finely tuned and it may be hard to detect all physiologically relevant fluctuations.[87] Furthermore, many sensors are not ideal; for instance both roGFP and HyPer are sensitive to pH.

GFP exhibits two excitation bands, originating from neutral and anionic forms of the chromophore.[88] roGFP was engineered by installation of cysteines at S147 and Q204 in p-strands 7 and 10. These two residues serve as a cysteine/disulfide redox switch to form a

unique *ratiometric* sensor by opposing changes in the two excitation A and B bands through a single isobestic point (Figure 3a).[89] roGFP2 is a variant that contains an additional S65T mutation.[90] This protein has several advantages over roGFP1, including greater brightness and more favorable emission properties. roGFP2 is pH-sensitive but this affects the brightness of both emissions equally and hence only affects sensitivity.[91]

Next-generation sensors feature roGFP directly fused with H_2O_2-scavenging peroxidases like Orp1.[91] Orp1 *non-enzymatically* reacts with H_2O_2 to form intramolecular disulfides that near-quantitatively *change hands* (Key words) with redox-sensor cysteines within fused roGFP2 in a disulfide relay process. The oxidized roGFP can be reduced *in vivo* affording reversibility and continual detection.[92] Similarly, a rxYFP-glutaredoxin1 (Grx1) reporter measures GSH/GSSG redox couple ratio specifically.[93]

HyPer is an insertion of circularly permuted YFP (cpYFP) between amino acids 205 and 206 of the H_2O_2-sensitive regulatory domain of OxyR, a redox-sensor protein *from E. coli.*[94] Upon exposure to H_2O_2, OxyR undergoes a conformational change as a result of the peroxidatic cysteine, Cys-199, undergoing a direct, *non-enzymatic* reaction with H_2O_2 followed by the resolving cysteine, Cys-208, forming a C199–C208 disulfide.[95] An induced structural change around the cpYFP chromophore simultaneously reduces excitation at 420 nm (F_{420}) and proportionally increases excitation at 500 nm (F_{500}). The detection range of H_2O_2 for 1st generation HyPer was low-mid nM H_2O_2 and the dynamic range (F_{500}/F_{420}) was only 3 fold.[86] Using Hyper, the intracellular tolerance for H_2O_2 before the onset of cell death was estimated to be less than 7.5 nM.[96] The latest version, "HyPer-3," has an improved, albeit still limited, 6-fold dynamic range.[97] Redox sensitive RFPs have been developed, possibly allowing dual molecule/organelle sensing in the future.[98] The emission ratio of HyPer is particularly pH-sensitive. One way to account for this variable is to express a redox-insensitive mutant or to measure pH fluctuations under the specific conditions tested.[99]

Organelle-specific stakeouts—By genetically targeting the redox-sensor FPs to a specific organelle, microenvironment-specific redox perturbations can be measured. These sensors should have a redox couple that equilibrates with the predominant redox-active enzyme in that compartment. For example, an ER lumen-localized roGFP probe, roGFPiE, was designed to be in equilibrium with protein disulfide isomerase (PDI), a redox-mediating chaperone in the endoplasmic reticulum (ER).[100] *In vivo* fluorescence lifetime imaging using this probe revealed surprising redox stability during unfolded protein response (UPR), but an unexpected shift to a reducing ER in response to loss of lumen calcium.[101] Another ER-specific probe led to discovery of peroxiredoxin 4 (PRDX4)-mediated restoration of the ER's oxidative environment in endoplasmic reticulum oxidation 1 [ERO1 (an enzyme that maintains oxidizing conditions with the ER)]- deficient cells.[102] Similar strategies measured mitochondria-specific redox response to TGF-β[103] and hypoxia,[104] as well as redox environments of endosomes, lysozomes[105], and peroxisomes[106]. Localized H_2O_2 sensing was also achieved using "HyPer-Tau"—wherein HyPer is tethered to microtubules.[107]

Small-molecule-based H_2O_2 sensors—These probes tend to be on/off sensors, which is less of a problem for small-molecules because their concentration is easier to control than

ectopic proteins. Common issues associated with the use of small molecules, such as variable uptake, excretion, and distribution/localization have been reported.[108–110] Probes can function either by nucleophilic addition/bond migration or radical chemistry.[111] 2′,7′-Dichlorodihydrofluorescein (DCFH), the earliest H_2O_2 probe, gains fluorescence via radical chemistry (Figure 3b).[112] In addition to pioneering work in mammalian cells, these probes also work in plant cells.[113] Later probes built upon addition-migration chemistry use an element like boron (Figure 3c), which is a good acceptor of electrons and facilitates migration to appended weak bonds. Peroxide exposure prompts nucleophilic addition followed by bond migration, converting the non-fluorescent phenyl boronic ester to a fluorescent phenol.[114, 115] These probes have seen widespread applications to detect redox flux in cells/organelles and have been expanded to ratiometric variants.[116] One further stumbling point with chemical sensors is their low specificity compared to enzymatic counterparts: DCFH is activated by hydroxyl radical,[117] NO,[118] peroxynitrite,[119] and depletion of ROS.[120, 121] Boron-based probes sense HOCl and peroxynitrite >1000 times faster than peroxide.[122] Another issue is irreversibility, although a reversible on/off cyanine-based sensor has been reported and was applied to H_2O_2 detection during L-buthionine sulphoximine (inhibitor of glutathione synthesis)-induced apoptosis of HepG2 cells and at the site of injury of zebrafish.[123]

Detection of endogenous RES—The first reported HNE/enal probe was DNPH (**15**), which forms an adduct with aldehydes with absorption at 360–380 nm.[124] Similarly, the reaction between aldehydes and 1,3-cyclohexanediones produces fluorescent decahydroacridines.[125] Treatment of biological samples with fluorescent variants of these probes, and subsequent HPLC analysis, demonstrated the presence of aldehyde adducts in the livers of vitamin E-deficient rats.[125] A GC-MS-based method employing carbonyl-reactive pentafluorobenzyl-hydroxylamine (**16**) identified free HNE as a biomarker for neuronal retinal ceroidosis.[126]

RES versus ROS detection in terms of chemistry and biology—Probes for direct and selective in-cell detection of RES and resulting modifications are underdeveloped. There are no methods to measure free RES flux *in real time*, or even to measure free levels in live cells. This can be traced to: (1) the sheer number of structurally similar LDEs compared to the relatively few forms of ROS; (2) the fact that (unlike ROS) few proteins react *diagnostically* with RES; and (3) the fact that, even post-enzyme conjugation, many RES still contain a reactive functional group[6] (e.g., a reactive carbonyl, *vide supra*). The remaining reactive motif likely would be detected by the RES-detection probe [whereas ROS, on the other hand, either form chemically distinct species upon reaction (e.g., disulfides) or form secondary ROS chemically distinct from the first species]. The irreversibility/long residence of RES modifications also limits the likelihood of ever being able to measure flux *in real time*. Unsurprisingly, many end-point RES probes are nonspecific, detecting all LDEs and their adducts. Clearly the relative merits of global versus specific profiling are context dependent, but different LDEs have different signaling properties.

(C) Uncontrolled Redox Release: Neighborhood Canvassing

Treatment of cells with H_2O_2 to elicit redox signaling is a widely-employed general strategy to model oxidative stress. But this approach cannot mimic endogenously-generated H_2O_2 in terms of pulse duration, localization, and timing.

Bolus generation of reactive oxygen species—Traditional approaches that restrict ROS generation to a specific locale include chemical tethering of a fluorophore (or genetically encoding its binding site) to a protein of interest (POI), e.g., (*a*) FLAsH/ReAsH;[127] (*b*) ligand-specific protein tags such as Halotag (a protein that reacts stoichiometrically and irreversibly with chloroalkane-tethered ligands);[128] (*c*) antibody-conjugated chromophore (e.g., malachite green)[129] (*d*) fluorophore (e.g., fluorescein)[130]; (*e*) expression of ROS-producing enzymes; or (*f*) photosensitizing fluorescent proteins (FPs).[131] Traditional FPs show weak photosensitization properties. KillerRed is a modified FP with a more open β-barrel structure promoting oxygen photosensitization.[132] KillerRed, the subsequently developed KillerOrange,[133] the monomeric miniSOG (mini singlet-oxygen generator),[134] and supernova[135] have been used to kill cells.[131, 132, 136]

Inactivation of specific proteins through spatially-restricted bolus ROS generation has also been achieved using c̲hromophore/f̲luorofore-a̲ssisted l̲ight i̲nactivation (CALI/FALI) (Figure 2e). For example, cell division arrest was achieved by chromatin-directed CALI targeting histone H2B.[137] Organelle-specific ROS release has also been examined by targeting the chromophore/fluorophore to particular organelles/subcellular compartments. These experiments illustrate the complex way by which the cell responds to ROS: mitochondria-localized ROS triggers apoptosis, whereas necrosis is elicited by plasma-membrane-specific ROS.[131]

Uncontrolled RES generation—Bolus dosing can mimic RES-induced stress and signaling. Bolus dosing of electrophiles has given new insights into redox signaling, but reservations must be placed on the validity of bolus dosing as a physiologic redox *signaling* model. No RES-sensitization methods equivalent to CALI/FALI have been reported.

(D) Controlled Release: Sting Operations

Controlling ROS signaling—Methods for the controlled generation of ROS to regulate redox signaling remain limited mainly because ROS are highly diffusible, their release is hard to control, and they readily interconvert. ROS are also typically generated through sensitization of cellular oxygen (i.e., via CALI), rendering efficacy of ROS generation context dependent. The primary radius of influence of many CALI probes is small (< 5 nm), but there are examples of off-target modification/cross-linking.[138] It is also hard to assess other proteins *indirectly* affected through reversible ROS-mediated signaling (i.e., disulfide change of hands). None-the-less, CALI-type probes have helped deconvolute redox signaling: controlled generation of superoxide/HO• through photosensitization of Pd-bacteriochlorophyll-serine changes phosphorylation of JN kinase, p38, extracellular signal-regulated kinase (ERK), and Akt.[139] A photocaged polyphenol is also used for on-demand generation of H_2O_2 (via superoxide) in HEK293 cells. This probe elicits cofilin-actin rod formation, which also occurs upon H_2O_2 (50–200 μM) treatment.[140] Although probe

concentrations used were high (200 μM), the authors show this concentration is not toxic. Of equal importance, they show that the byproducts of photouncaging are not active. An alternative method to generate ROS that does not generate superoxide employs enzyme D-amino acid oxidase from *Rhodotorula graciles*, which produces H_2O_2 dependent upon D-amino acids.[141] This strategy successfully induces antioxidant response protecting neurons from oxidative stress.[142]

Controlled generation of RES as a signal: T-REX—Recently, Class II proximity-enhancement through pseudo-intramolecular delivery[1] has been used to modify a specific protein of interest (POI). The POI is genetically fused to Halotag[143] that recognizes a photocaged precursor to a specific bioactive lipid-derived electrophile (LDE) such as HNE. The method is called targetable reactive electrophiles and oxidants (T-REX) (Figure 2f).[2–5, 7] Treatment of cells with a custom-designed chloroalkane-containing photocaged LDE-precursor yields complete binding of the photocaged probe to the Halotag. Following photoactivation that rapidly ($t_{1/2} < 1$ min) releases a maximum of *one equivalent* of LDE, the POI fused to Halotag will be labeled with LDE, provided the POI's reaction with LDE is rapid. Because the amount of LDE liberated is maximally stoichiometric to the POI and localized to the microenvironment of the POI, this technique mirrors "endogenous signaling" and has a proven ability to elicit downstream signaling through single-protein-target modification.[3] Several proteins have proven amenable to T-REX, including Keap1,[2–5] Akt3[7] and PTEN.[2, 5] triggering downstream responses in the antioxidant response pathway and FOXO transcriptional activation, and regulation of membrane-bound PIP2/PIP3 phosphoinositides, respectively).

The key differences between RES and ROS sensitization methods are stoichiometry and specificity. One chromophore can generate many ROS, meaning that CALI/FALI etc., approximate overload approaches. Although "selective" target protein perturbation has been demonstrated, a significant percentage of ROS sensitization strategies is applied to killing/ablation/overstressing cells. There is currently no sensitization method to form RES, mainly because a chemical reaction, as opposed to photosensitization/intersystem-crossing/electron transfer, is required to convert RES precursors to active RES. T-REX is broadly analogous to CALI, but the need for a photocaged precursor limits the amount of RES produced. Thus T-REX is particularly well-suited for studying physiological RES signaling. Time will tell how useful T-REX-type probes are to study localized, non-targeted stress.

Additional considerations—Many of the methods that use target-specific redox signaling to modulate a protein's function overexpress proteins bearing a genetically-encoded protein tag, such as Halo. This approach is useful for proof-of-concept studies, or to study phenotypically-dominant (Key words) modifications. But especially for loss of function (e.g., in CALI/FALI systems), endogenous POI can mask/complement the desired phenotype).[144] Thus, knocking in tags to endogenous POI genetic loci or expression of a transgene in a knockout/null background should be used wherever possible.

(E) Redox Model Systems: Means, Motives, and Opportunities

Adapting these chemical biology techniques to whole organisms is challenging and underdeveloped. Many sensing techniques employ fluorescent sensors/photosentitization, which is challenging in many multicellular organisms because they are made up of tissues (which may be less permeant to light than cultured cells) and many are pigmented. Fortunately, there are multiple methods to surmount these obstacles, such as transparent organisms (*C. elegans*, *Xenopus*/zebrafish larvae), choosing translucent organs, using genetic backgrounds that have reduced pigmentation[145] or using near-IR (tissue-penetrating) light (e.g., multiphoton) (Table 2). Some reports indicate that it is possible to use visible light-initiated photouncaging in mice xenografts.[146] However, the magnitude of the resulting responses is relatively low. Furthermore, whole organisms are much less compatible with small molecules than cultured cells, and adsorption/distribution/metabolism/excretion/toxicity (ADMET) must be considered. Zebrafish and Xenopus have an almost fully-functioning set of organs 3-day post-fertilization.[147] *C elegans* is surrounded by a cuticle that shields it from many chemicals, meaning that chemical biology applications are largely underdeveloped.[148] Tissue/structural complexity can make even simple experiments highly complex and require intimate familiarity with specific anatomy/physiology. Nevertheless, we highlight some of the elegant examples where chemical biology was applied to study redox physiology in whole organisms.

H_2O_2 overload strategies in whole organisms—Numerous photochemical methods to induce injury in transparent organisms are known, including enzymatic sensitization of prodrugs[149] and direct laser ablation.[150] Unfortunately, these strategies are technically complex, require intricate knowledge of anatomy, and cannot be done easily on a large scale. CALI/FALI approaches are useful alternatives. CALI/FALI-mediated ablation has been applied to *Xenopus laevis* embryos,[151] *C. elegans*[152], and tumor xenografts in mice.[153] One interesting application is inhibition of synapses with CALI (InSynC) (Figure 3d).[148] VAMP2 is a mammalian analog of the *C. elegans* neuronal protein synaptobrevin that localizes to synaptic vesicles. A miniSOG–/– citrine fusion of the human synaptobrevidin analog, VAMP2, rescues motility deficiency shown by synaptobrevin null worms, validating that VAMP2 is functional in *C. elegans*. Upon 480-nm light irradiation, worms were paralyzed but recovered over the next day (Figure 3d upper). Similar loss of function was observed when miniSOG-VAMP2-citrine was expressed in wild-type worms, showing that ROS inhibition of VAMP2 behaves in a dominant-negative manner. The latter effect is likely due to heterodimerization of VAMP2 with analogous *C. elegans* proteins.[154] However, the authors also demonstrate off-target effects both within the synaptic vesicle and at the plasma membrane. Similar experiments have been performed in *D rerio*.[155] This system employed a fluorescence activating protein—targeted and activated photosensitizer (FAP-TAP), a domain that binds a specific otherwise inefficient fluorophore, enhancing its fluorescence. The protein/fluorophore pair was optimized in vitro to produce high levels of singlet oxygen, when exposed to near-IR light. When fish expressing this FAP-TAP specifically in cardiac cells were injected with the fluorophore and exposed to tissue-penetrating near-IR radiation cardiac ablation was observed (Figure 3d lower). Müller glia cells in mice have also been used as a model system.[156] The latter approach ingeniously uses the transparency of the eye to great effect.

Mitochondrial redox homeostasis in model organisms—Mitochondria are implicated in age-dependent increase in oxidative stress as well as cardiac damage in mice.[157] Overexpression of mitochondria-specific peroxiredoxin-3 or small molecule antioxidants like MitoQ in mice have mitigated neuorodegenerative diseases.[158] Generation of ROS by disruption of the mitochondrial electron transport chain and subsequent mitohormesis leads to lifespan expansion in mice.[159] Similar lifespan expansion by mitohormesis or through mitochondria-selective antioxidant enzyme has been noted in yeast,[160] *C. elegans*,[161] and *drosophila*.[162]

Subcellular connectomics—Given the increasing appreciation for H_2O_2 diffusion through membranes[163], redox signals may be one way to transfer information between different organelle/cellular compartments. Indeed, redox crosstalk between peroxisomes and mitochondria, for instance, has been reported.[164, 165] Inhibition of peroxisomal catalase activity (increasing H_2O_2 cellular concentrations) enhances mitochondrial ROS presumably through transport of H_2O_2 from the peroxisome.[166, 167] On the other hand, transient generation of H_2O_2 by expression of peroxisomal catalase rescues glucose metabolism in insulin-resistant cells by increasing mitochondrial respiration.[168] Investigations into ER/cytosol crosstalk have used chemical probes to understand redox signaling. Dimedone treatment of lysates of oxidatively-stressed *C elegans* identifies IRE-1 sulfenylation as an evolutionarily-conserved mechanism to upregulate antioxidant response and promote longevity.[169] This pathway is suppressed by mutating the target cysteine identified by dimedone, further documenting that redox signaling functions through specific modifications to form defined states with biological significance. Perhaps most surprisingly, mounting evidence indicates that this organelle homeostasis is regulated across tissues.[170]

Outlook

Reactive species are an often-overlooked component of cellular information transfer. This is not surprising given that redox messages are transient, have intrinsically low specificity and are context dependent. But since pioneers began to scrutinize these unusual signals, it has become clear that biology will harness all means at its disposal to control decision making. Classically biology has been a field that measures flux and response to specific perturbations. These measurements or perturbations were usually achieved using specific inhibitors or genetic manipulations, when studying canonical signaling pathways. The study of redox signaling likewise measures flux and employs specific perturbation, but since chemistry rather than enzymology dominates redox-signaling initiation, chemical biology is the ideal tool to use. Chemical biology has indeed risen to this challenge and many innovative tools have been developed. Using these new techniques, progress has been made in measuring flux of specific chemical signals. But there are still many challenges left. In our opinion focus should shift to the nuanced way in which biology uses compartmentalized RES/ROS signaling to influence its decisions at the organelle, cell/tissue, or whole-organism scale. To do this, it is our belief that we need to identify and investigate specific proteins whose modifications alone are sufficient to trigger signaling cascades in cells and whole animals under endogenous signaling conditions. At an organismal level, we need to gain precision control in terms of specificity and timing to successfully mimic endogenous redox

signaling. We hope that this review will stimulate new interdisciplinary research and push researchers from different fields to tackle the challenging yet rewarding field of redox signaling, ideally in whole organisms.

Acknowledgments

Abbreviations

ABPP	activity-based protein profiling
Ac	acetyl
ADMET	absorption, distribution, metabolism, excretion, toxicity
CALI/FALI	chromophore/fluorophore-assisted light inactivation
cpYFP	circularly-permuted yellow fluorescent protein
Cys	cysteine
DCFH	dichlorodihydrofluorescein
DNPH	dinitrophenylhydrazine
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
FAP-TAP	fluorescence activating protein—targeted and activated photosensitizer
FOXO3	forkhead box O3
FP	fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GS-MS	gas chromatography-mass spectrometry
His	histidine
HNE	4-hydroxynonenal
HPLC	high-performance liquid chromatography
IR	infrared
LDE	lipid-derived electrophile
Lys	lysine
mCer3	monomeric cerulean 3

MitoQ	mitoquinone
MS	mass spectrometry
myl7	myosin light chain promoter
NFκB	nuclear factor κB
PIP2/3	phosphoinisitol bis/trisphosphate
POI	protein of interest
PTM	posttranslational modification
RES	reactive electrophilic species
roGFP	redox-sensitive green fluorescent protein
ROS	reactive oxidative species
SOG	singlet oxygen generator
T-REX	targetable reactive electrophiles and oxidants
UPR	unfolded protein response
VAMP2	vesicle-associated membrane protein 2
WT	wild-type
Yap1	Yes-associated protein 1

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Key Words

Change (of) Hands: **exchange of one redox modification on a specific protein to the same redox modification on another protein/site (e.g., interconversion of disulfide bonds)**.

Class II Proximity Enhancement: a "virtual tethering strategy" in which a reactive ligand/signal is **delivered to a target protein of interest through on-demand precision redox-signal targeting guided by proximity**[1] **(e.g., CALI/FALI and T-REX)**.

Signal Exchange: **conversion of one form of signaling to a gain or loss of another (e.g., a reactive oxygen species (ROS) signal regulating phosphorylation, ubiquitylation, etc.). This occurs at a** signaling node.

Signaling Node: **a protein at which signal exchange occurs**.

Kinetic Privilege: **highly reactive proteins under favorable conditions will intercept reactive species faster than less reactive proteins**.

Phenotypically Dominant: **a signaling event that elicits gain of function or dominant loss of function such that low-occupancy modification(s) on a single protein likely elicit(s) a phenotypic response**.

Privilege of Occupancy: **phenotypically-dominant modifications require intrinsically low occupancy and so will exert their influence earlier than recessive effects (such as loss of function).** Redox Sensor: **a protein that senses reactive electrophilic/oxygen species (RES/ROS) (e.g., Keap1,**[2–5] **PTEN**[2, 5]**)**.

Redox Signal: **a reactive electrophile or oxidant that can covalently modify a sensor protein eliciting downstream responses (a broad denomination of signaling)**.
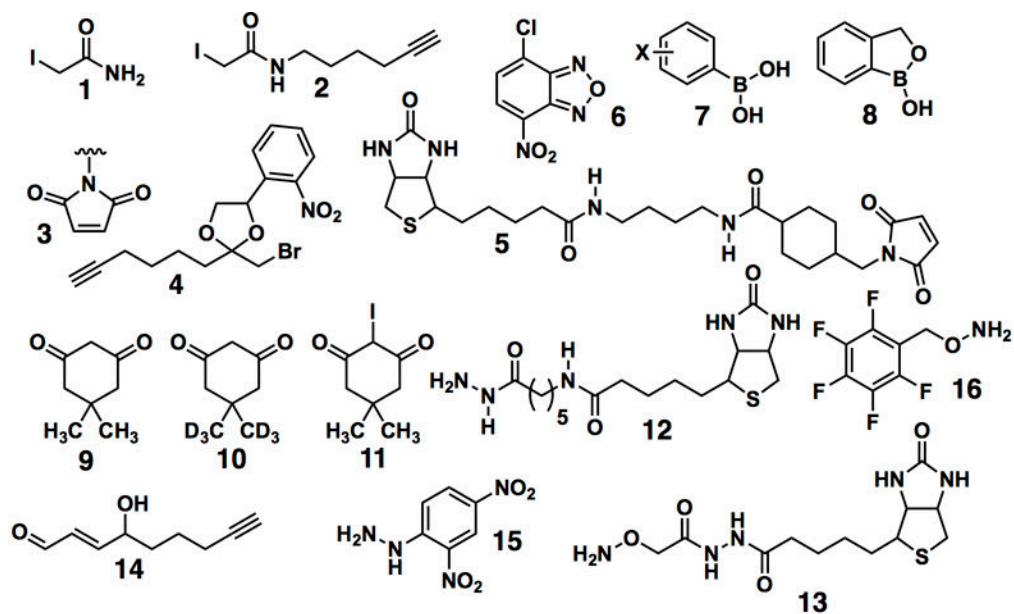
Figure 1.
Glossary and chemical structures of representative probes highlighted in this review. (**3 illustrates maleimide-functionalized affinity agents. X in 7: H, 4-NO₂, 3-NO₂, 3-Br, 4-Br, 4-F, 4-OCH₃**).
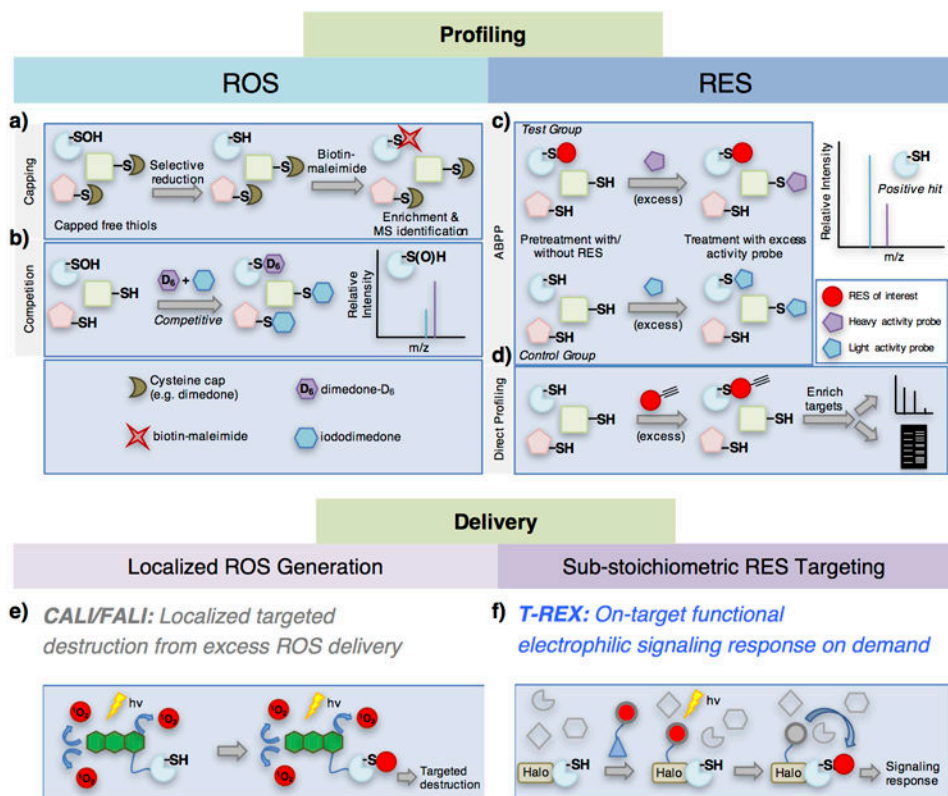
Figure 2.
Representative ROS/RES target profiling and delivery strategies. **ROS target profiling can be broken down into (a) "capping" strategies, wherein free thiols are capped before labeling of modified Cys (in this example, Cys-SOH), and (b)** "chemoselective" strategies that use a chemoselective reagent to "pick out" a specific **modification, such as Cys-SOH and label it selectively. Combinations of these** strategies can be used in "competition," **wherein simultaneous chemoselective reactions are run on the same sample. RES target profiling can similarly be broken down into (c) activity-based proteomic profiling (ABPP) which utilizes a stepwise competition approach or (d)** "direct profiling," in which cells/lysates are treated with a **functionalized probe and directly detected by MS analysis. Delivery strategies for ROS have generally been limited to (e) CALI/FALI approaches, which approximate bolus dosing, although they can be restricted to particular proteins or cellular compartments. (f) By contrast, the only available RES delivery strategy, T-REX, more closely mimics** endogenous electrophile signaling and is capable of evoking a specific signaling response triggered by on-target electrophilic modifications.
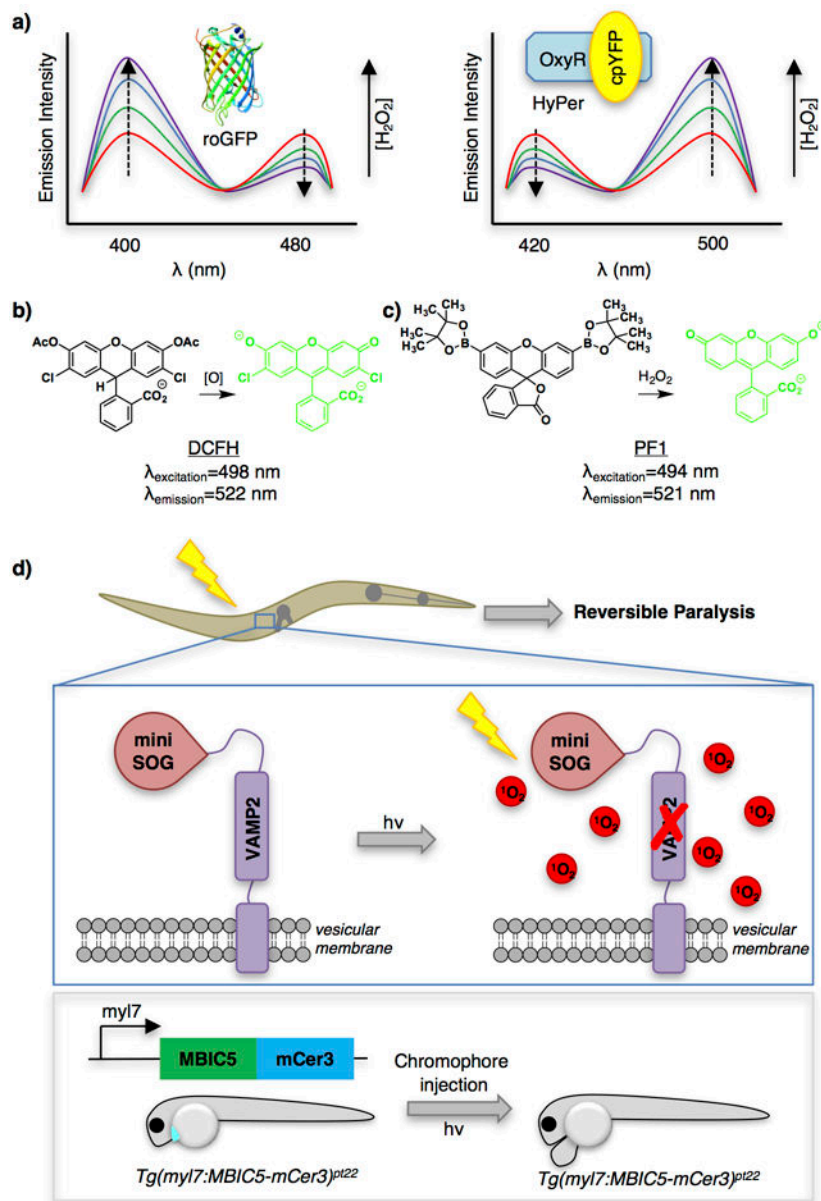
Figure 3.
Selected examples of protein- or small-molecule-based ROS sensors (a–c) and an example application of excess ROS delivery tools in model organisms (d). **(a) Fluorescent protein-based tools to measure ROS flux (left, roGFP; right, HyPer) and their respective excitation spectra across varying concentrations of H_2O_2. (b and c) Dichlorodihydrofluorescein (DCFH) and Peroxyfluor 1 (PF1), two turn-on small molecule probes for detecting ROS. (d) Two examples of applications in model organisms making use of chemical tools for localized generation of excess ROS. Top panel: Inhibition of synapses with CALI (InSynC) was demonstrated in** C. elegans **by fusing miniSOG (a genetically encodable singlet oxygen generator) with the synaptic protein VAMP-2. Worms showed reversible paralysis following light activation of miniSOG. Lower panel: A similar approach has been demonstrated in zebrafish**

through expression of "Fluorescence Activating **Protein—Targeted and Activated Photosensitizer (FAP-TAP)"** under a cardiomyocyte-**specific promoter** (myl7)**. Zebrafish exposed to light showed ablation of cardiomyocytes with prolonged damage of cardiac tissue. cpYFP, circularly-permuted yellow fluorescent protein; DCFH, dichloro-dihydro-fluorescein; mCer3, monomeric cerulean 3;** myl7**, myosin light chain promoter; PF1, peroxyfluor-1; roGFP, redox-sensitive GFP; SOG, singlet oxygen generator; VAMP2, vesicle-associated** membrane protein 2. Also see the "Abbreviation" list at end of the manuscript. **Zebrafish construct nomenclature is as follows:** Tg(regulatory sequence:coding sequence) **where** Tg **indicates transgene, regulatory sequence is a promoter (or enhancer), and coding sequence is the POI.**

Table 1

Overview of ROS

Entry	ROS	Oxidation State	Description	Sources	Half life (s)
1[19,20]	Singlet oxygen ($^1O_2$)	0	Unfavored spin state	Many	10^{-6}
2[21]	Superoxide ($O_2^{\bullet -}$)	−1/2	Reactive radical	Electron transfer from NADPH to O_2 by NADPH oxidases	10^{-6}
3[21]	Peroxide (H_2O_2)	−1	Two-electron oxidant	Many	10^{-3}–10^{-5}
4[21]	Hydroxyl radical ($HO^{\bullet}$)	−1	Reactive radical	Reaction of superoxide with peroxide (Fenton reaction)	10^{-9}
5[22]	Peroxynitrite		Endogenous pseudohalogen	Reaction of superoxide with NO	10^{-3}

Table 2

Advances in studying ROS and RES signaling in model organisms.[171]

Entry	Model Organism	Advantages	Limitations	Examples
1[172]	*C. elegans*	• Simple culture conditions/rapid life cycle • Transparent • Many transgenic and reporter lines readily available • Hermaphrodites can generate many cloned copies rapidly; but genetic crosses possible. • High-throughput compatible	• Cuticle prevents small-molecule penetrance • Lacks many organs/systems analogous to humans. • The average protein homology to humans is 41% (20%~71%)	• InSynC (CALI)
2[173]	*D. rerio*	• Transparent embryos • Pigmentation can be reduced using mutant lines or small-molecule treatment during development • Rapid, observable development • Relatively simple genetic manipulation • Fully functioning set of organs 3 days post-fertilization • High-throughput compatible	• Genome duplication can make genetics challenging • Lack some organs/structures present in humans	• T-REX • CALI
3[171]	*X. laevis/tropacalis*	• Transparent embryos • Rapid, observable development • High brood size • Functioning organs 3 days post-fertilization • *X. tropicalis* is diploid • Physiology similar to humans • Knockdown possible • High-throughput compatible	• Difficult transgenesis • *X. laevis* is allotetraploid	• CALI
4[171]	*M. musculus*	• Highly homologous to humans	• Low light penetrance for optical tools • High cost per embryo • Early development is not observable • Genetic manipulation difficult/development slow • Low throughput	• CALI (in eyes) • CALI (xenograft)