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An update on methods and approaches for interrogating mitochondrial reactive oxygen species production

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ABSTRACT

The chief ROS formed by mitochondria are superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). Superoxide is converted rapidly to H_2O_2 and therefore the latter is the chief ROS emitted by mitochondria into the cell. Once considered an unavoidable by-product of aerobic respiration, H_2O_2 is now regarded as a central mitokine used in mitochondrial redox signaling. However, it has been postulated that $O_2^{\cdot-}$ can also serve as a signal in mammalian cells. Progress in understanding the role of mitochondrial H_2O_2 in signaling is due to significant advances in the development of methods and technologies for its detection. Unfortunately, the development of techniques to selectively measure basal $O_2^{\cdot-}$ changes has been met with more significant hurdles due to its short half-life and the lack of specific probes. The development of sensitive techniques for the selective and real time measure of $O_2^{\cdot-}$ and H_2O_2 has come on two fronts: development of genetically encoded fluorescent proteins and small molecule reporters. In 2015, I published a detailed comprehensive review on the state of knowledge for mitochondrial ROS production and how it is controlled, which included an in-depth discussion of the up-to-date methods utilized for the detection of both superoxide ($O_2^{\cdot-}$) and H_2O_2. In the article, I presented the challenges associated with utilizing these probes and their significance in advancing our collective understanding of ROS signaling. Since then, many other authors in the field of Redox Biology have published articles on the challenges and developments detecting $O_2^{\cdot-}$ and H_2O_2 in various organisms [1–3]. There has been significant advances in this state of knowledge, including the development of novel genetically encoded fluorescent H_2O_2 probes, several $O_2^{\cdot-}$ sensors, and the establishment of a toolkit of inhibitors and substrates for the interrogation of mitochondrial H_2O_2 production and the antioxidant defenses utilized to maintain the cellular H_2O_2 steady-state. Here, I provide an update on these methods and their implementation in furthering our understanding of how mitochondria serve as cell ROS stabilizing devices for H_2O_2 signaling.

1. Introduction

The production and significance of ROS in biological systems has been a topic of research since the discovery of catalase and its ubiquitous cellular expression over 100 years ago. This was followed by several other significant events in the study of ROS. In 1956, Denham Harmen formulated the "Free Radical Theory of Aging", which postulated that free radicals, molecules with one or more unpaired electrons, and, non-radicals, like H_2O_2, cause aging and the pathogenesis of various diseases [4]. This posit stipulated that oxyradicals, other free radicals, and non-radicals were an unfortunate and unavoidable consequence of life existing in an atmosphere rich in molecular oxygen (O_2). These radicals accumulate over time causing irreversible damage to macromolecules culminating with cell death and tissue damage. A few years later, unequivocal evidence was generated demonstrating that mitochondria are significant sources of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) following the one and two-electron reduction of O_2 [5]. Thus, mitochondria were labeled the major source for a cell's free radical burden and therefore thought to be responsible for the finite existence of mammalian cells [6].

While over production of ROS in living systems and its link(s) to pathogenesis and aging were being intensely researched, there was a foment in studying its physiological functions as well. Interest in the physiological function(s) of ROS can be traced back to 1976 when it was found that macrophages produce $O_2^{\cdot-}$ to eliminate pathogens [7]. This was later attributed to NADPH oxidase (NOX), which produces $O_2^{\cdot-}$ through an electron transfer reaction from NADPH to O_2 [8]. This physiological feature was originally thought to be unique to immune cells until it was found that $O_2^{\cdot-}$ can stimulate division in non-immune cells [9]. NOX isozymes were also found to be ubiquitously expressed,

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indicating ROS may fulfill many physiological functions [1]. In 1998, mitochondria were identified as the source of ROS for hypoxic signaling [10]. The origin of this ROS was complex III and hypoxic conditions induce a burst in $O_2^{\cdot-}$ production and its conversion to H_2O_2 resulting in the stabilization of hypoxic inducible factor-1α (HIF-1α). Now, it is evident that mitochondrial H_2O_2 emission is vital for adipocyte differentiation, T-cell activation, induction of cell proliferation and growth, insulin signaling and release, satiety signaling and circadian/ultradian rhythms, muscle wound healing and growth, adaptive signaling (e.g. HIF-1α and NF-E2p45-related factor2 (Nrf2) signaling), and many more functions [1,2,11,12].

Documenting the cellular and physiological function(s) of ROS is a relatively new development when compared to overall historical interest in studying free radical chemistry in biological systems. This can be attributed, in part, to the lack of tools for the specific and sensitive detection of physiological concentrations of $O_2^{\cdot-}$ and H_2O_2. Commonly used molecular probes for ROS have supplied important information on the (path)physiological function(s) of $O_2^{\cdot-}$ and H_2O_2. Unfortunately, these probes suffer from issues such as specificity, sensitivity, impermeability to membranes, auto-oxidation, capacity to catalyze ROS formation, and inability to accurately detect ROS *in vivo*, which severely limited their use in fully characterizing the physiological functions of $O_2^{\cdot-}$ and H_2O_2 [3]. However, progress over the last decade has led to the development of novel chemical and genetically encoded probes that have allowed for the quantification of physiological $O_2^{\cdot-}$ and H_2O_2 levels in cellular compartments. These probes were reviewed in 2015 and included novel detectors such as mitochondria-targeted boronate compounds and protein-based reporters, such as the H_2O_2 detecting HyPer and OxyR and the glutathione detector roGFP-GRX1 [13]. However, these probes still suffered from several limitations [13,14]. Additionally, when this 2015 review was published, a reliable $O_2^{\cdot-}$ detector still did not exist [15]. Here, I provide an update on the novel probes that have been developed since then to accurately quantify $O_2^{\cdot-}$ and provide more sensitive H_2O_2 estimates in cells and live animals. This includes the novel roGFP2-Tsa2 probe and its variants and several small molecules that have been developed to measure and visualize $O_2^{\cdot-}$ using positron emission tomography (PET), electron paramagnetic spin resonance (EPR), and fluorimetry [14,16,17]. I will also discuss experimental approaches that can be utilized to study the twelve individual ROS sources in mitochondria and their contribution towards overall mitochondrial H_2O_2 production.

2. Principles of mitochondrial ROS production and signaling

2.1. How mitochondria generate ROS

Fuel oxidation, chemiosmotic coupling, and oxidative phosphorylation (OXPHOS) rely on electron transferring redox active centers embedded in mitochondrial dehydrogenases and multi-subunit complexes inserted in the mitochondrial inner membrane (MIM). Electron donating and accepting centers include iron-sulfur (Fe–S) clusters, heme, covalently bound flavins, copper, nicotinamide adenine dinucleotide (NAD^+), and ubiquinone (UQ). Redox centers in mitochondrial dehydrogenases and the electron transport chain (ETC) are surrounded by polypeptide chains and the hydrophobic interior of the MIM and therefore electron transfer cannot occur by the simple donation or acceptance of electrons. Transfers between two redox centers are instead governed by a phenomenon called "electron tunneling" [18]. Tunneling predicts the statistical probability of an electron's location and whether it will move from one redox center to another. The probability that an electron will move from one a donor to an acceptor molecule is influenced by: 1) distance between the two, 2) redox potential of the donor and acceptor, and 3) response of both the donor and acceptor to a change in charge [18]. Electron transfer can vary by several orders of magnitude based on the distance between donor and acceptor molecules in the respiratory chain. The maximum distance for electron transfer between centers is 14 Å and occurs at a rate of $\sim 10^4\ s^{-1}$ [18]. The rate increases by several magnitudes as the distance between a donor and acceptor decreases [18].

The factors that influence the probability for an "electron tunneling" to an acceptor molecule apply to ROS production as well. Molecular oxygen would need to be within 14 Å of a redox center and its capacity to accept one or more electrons is influenced by redox potential and charge of a donor. Molecular oxygen is also a small molecule and thus can readily contact flavin centers, or other electron donors in the matrix, to generate ROS. Therefore, the rate of ROS production also depends on the number of times O_2 can collide with an electron donor. The concentration of O_2 in air-saturated solution is ~200 μM whereas it has been estimated to occur at ~3 μM inside mitochondria [19]. This discrepancy has caused some to question if mitochondria truly are important ROS sources given that $[O_2]_{cytoplasm} > [O_2]_{matrix}$. However, the standard reduction potential for the univalent transfer of an electron to O_2 to form $O_2^{\cdot-}$ (−160 mV) and the concentration of the latter is in the pM range [19]. Thus, the formation of $O_2^{\cdot-}$ in mitochondria is always thermodynamically favorable.

Another consideration is the concentration and availability of the electron donor. Flavin groups occur at a high concentration in mitochondria and produce ROS through the formation of flavin hydroperoxide moiety following its activation of O_2 [20]. This results in the generation of a flavin radical and $O_2^{\cdot-}$, which can then react to form H_2O_2 at a rate of $10^8\ M^{-1}\ s^{-1}$ [20]. Mitochondria contain up to 16 sources of ROS, 12 of which are associated with fuel oxidation and electron transfer pathways involved in OXPHOS (discussed further below). Of these 12 sites, several are flavin-dependent respiratory complexes and dehydrogenases that utilize either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN; unique to complex I) to activate O_2 for ROS production [21]. Complex III and potential several dehydrogenases that donate electrons directly to the UQ pool do not use a flavin group to make ROS, but instead rely on the donation of an electron from semiquinone radical ($UQ^{\bullet}$) to O_2 [18]. Superoxide was, and still is, considered by some to be the proximal ROS formed by these sites of production [22]. However, several groups have demonstrated that ROS sources such as pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (KGDH), complex I, and complex II, produce a mixture of $O_2^{\cdot-}$ and H_2O_2 [23–25]. This property is related to the activation of O_2 by flavins and the generation of flavin hydroperoxides and flavin radical intermediates [20]. Additionally, these studies have shown that H_2O_2 accounts for over 70% of the ROS formed by these sites [23–25]. Coupled with this, any $O_2^{\cdot-}$ made by any of the 12 sites of production is rapidly converted to H_2O_2 at a rate of $\sim 10^9\ M^{-1}\ s^{-1}$ by superoxide dismutase-1 (SOD1) in the intermembrane space (IMS) or SOD2 in the matrix [19]. Taken together, the proximal ROS formed and emitted from mitochondria to the cell is H_2O_2.

2.2. Classifying the 12 sources of ROS production into sub-families

Complexes I and III are often considered the most important, and sometimes only, sources of $O_2^{\cdot-}/H_2O_2$ in mitochondria. However, over the past two decades it has become evident that mitochondria harbor many other ROS generators that are associated with fuel oxidation and OXPHOS pathways. KGDH was identified as a source as early as 2004 in synaptosomes and other α-keto acid dehydrogenases like PDH were shown to generate ROS afterwards [24,26,27]. The source of $O_2^{\cdot-}/H_2O_2$ in KGDH and PDH was identified to be the FAD prosthetic group in the NAD^+ binding E_3 subunit of both enzyme complexes [28]. It was also shown that the α-keto acid decarboxylase subunit, E_1, can also form $O_2^{\cdot-}/H_2O_2$ through the formation of a thiamine pyrophosphate radical within the subunit [28,29]. Additionally, the same group also found that KGDH can generate $O_2^{\cdot-}/H_2O_2$ by reverse electron transfer (RET) from NADH [30]. This led to the development of the postulate that a defect in

complex I activity could potentially drive high rates of ROS production by KGDH due to the accumulation of NADH, which was hypothesized to contribute to the pathogenesis of neurological disorders [30]. Later research also identified PDH as a source of $O_2^{\cdot-}/H_2O_2$ during RET and that the over production of ROS by this enzyme complex may be associated with the development of insulin resistance and metabolic disorders [23,27]. KGDH and PDH make up over 40% of the total ROS emitted by liver mitochondria, with the former enzyme complex accounting for ~35% of the $O_2^{\cdot-}/H_2O_2$ formation [31]. Additionally, KGDH and PDH produce 8x and 4x more ROS than complex I in rat muscle [32]. It remains unknown if KGDH and PDH contribute to mitochondrial H_2O_2 signaling and the role of both enzyme complexes in the induction of oxidative distress in dysfunctional mitochondria remains understudied. However, the evidence collected so far has shown that both enzymes are important $O_2^{\cdot-}/H_2O_2$ sources that may influence cell signals and contribute to oxidative distress.

KGDH and PDH are not the only "unconventional" $O_2^{\cdot-}/H_2O_2$ sources that make a significant contribution to overall mitochondrial ROS production. Complex II was also identified to be an important ROS generator in several studies [33], as were *sn*-glycerol-3-phosphate dehydrogenase (G3PDH), proline dehydrogenase (PRODH), branched chain keto acid dehydrogenase (BCKDH), dihydroorotate dehydrogenase (DHODH), 2-oxoadipate dehydrogenase (OADH), and electron transferring flavoprotein oxidoreductase (ETFQO) (reviewed in Ref. [34]). A significant advance in our understanding of mitochondrial $O_2^{\cdot-}/H_2O_2$ production was made in 2014 when the individual contributions of these sites, including complexes I and III, to overall mitochondrial $O_2^{\cdot-}/H_2O_2$ production was made [32]. It was found that KGDH, PDH, and BCKDH produce more $O_2^{\cdot-}/H_2O_2$ than complex I when mitochondria are oxidizing Krebs cycle-linked substrates [32]. By contrast, complex I is a significant source during RET and complex II can generate significant amounts during reverse electron flow from fatty acid oxidation pathways or the metabolism of proline or glycerol-3-phosphate [35–37]. Similar observations were made in cardiac and liver mitochondria where it was found that the chief sources of production depended on the substrate(s) being oxidized by mitochondria (reviewed in Ref. [38]). Based on this, it was suggested that the different ROS generators be categorized based on the electron donor and acceptor molecule required to produce $O_2^{\cdot-}/H_2O_2$; the NADH/NAD^+ and ubiquinol (UQH_2)/UQ isopotential groups [34]. KGDH, PDH, BCKDH, OADH, and complex I are part of the NADH/NAD^+ isopotential group since $O_2^{\cdot-}/H_2O_2$ generation depends on the oxidation and reduction of nicotinamides [34]. Complexes I, II, and III and G3PDH, PRODH, ETFQO, and DHODH comprise the UQH_2/UQ isopotential group [34]. The classification of these ROS generators and the impact of different fuels and physiological conditions and sex on the individual rates of production has been reviewed several times (reviewed in Ref. [38]). It is being briefly discussed here since methods for interrogating these ROS generators are being discussed later.

2.3. How are H_2O_2 signals mediated?

Hydrogen peroxide signals are often suggested to be mediated through the direct oxidation of proteinaceous cysteine thiols (P-SH) to a corresponding sulfenic acid moiety (Pr-SOH) [1]. Following its production, the Pr-SOH group is resolved either through the formation of a disulfide bridge with a neighboring protein thiol or glutathionylation. The disulfide bridges are then reduced by thioredoxin (TRX) and thioredoxin reductase (TR) or glutaredoxin (GRX) and GSH [39]. However, most cysteine thiols react with H_2O_2 at 1-10 M^{-1} s^{-1}, which is far too slow for any signaling molecule to elicit rapid changes in cell behavior in response to stimuli [14]. There are certain exceptions to the reactivity of proteinaceous cysteine thiols towards H_2O_2. This includes the TRX and GSH antioxidant pathways and enzymes with a catalytic cysteine with a low pKa, such as glyceraldehyde-3-phosphate dehydrogenase. The TRX pathway first relies on atypical and typical peroxiredoxins (PRX) for the elimination of H_2O_2. This is achieved with a catalytic peroxidatic cysteine residue (C_P), which harbors a low pKa and thus ionizes rapidly to facilitate nucleophilic attack on H_2O_2 (reviewed in Ref. [13]). The resulting C_P-SOH is resolved by a neighboring cysteine, called the resolving cysteine (C_R), forming a disulfide bridge [13]. The intra (atypical PRX) or intermolecular (typical PRX) is then resolved by TRX and TR in the presence of NADPH. PRX eliminates H_2O_2 in ~10^7 M^{-1} s^{-1} [14].

Several studies have provided evidence that H_2O_2 signals may be conveyed through thiol peroxidases via two different mechanisms: the floodgate and redox relay models (Fig. 1). The floodgate model relies on the prolonged, yet temporary, deactivation of thiol peroxidases (Fig. 1A) [40,41]. This is achieved through the hyper-oxidation of the C_P moiety from Pr-SOH to a sulfinic acid (Pr-SO_2H) [40,41]. Pr-SO_2H groups are reduced by sulfiredoxins (SRX) in the presence of ATP [40,41]. However, the reaction occurs slowly resulting in the prolonged deactivation of PRX [42]. This allows H_2O_2 to accumulate and oxidize protein cysteine thiols [40,41]. Several drawbacks remain for this mechanism; this includes the innate inability of most cellular thiols to react with H_2O_2 and the rapid clearance of H_2O_2 by the glutathione system. The second and more plausible mechanism is the redox relay model. In this case, the intra or intermolecular disulfide bridges in atypical and typical PRXs formed after H_2O_2 removal is transferred to target proteins through a thiol disulfide exchange reaction (Fig. 1B). The first peroxidase-dependent redox relay system was identified in *S. cerevisiae* and involves transferring a cysteine oxidation from peroxidase Orp1 to the transcription factor Yap1 [43]. Several similar redox relay systems have been identified in mammalian cells and include passing a disulfide bridge from typical PRX to signal transducer and activator transcription-3 (STAT3) and apoptosis signaling regulated kinase-1 (ASK1) [44,45].

The GSH system also rapidly quenches H_2O_2 at ~10^7 M^{-1} s^{-1} [46]. This is mediated by glutathione peroxidase (GPX), which utilizes a catalytic selenocysteine to react with H_2O_2 forming a selenol that is resolved by two GSH [46]. The resulting oxidized glutathione molecule (GSSG) is reduced by glutathione reductase (GR) and NADPH [46]. The GSH system is integral for the elimination of H_2O_2, xenobiotics, and lipid hydroperoxides but is also required to control protein function. This is achieved through protein S-glutathionylation, a ubiquitous and reversible redox modification that involves adding and removing GSH from a proteinaceous cysteine (Fig. 1C) [47]. So far, ~883 and over 2000 modifiable cysteines have been identified in rodent liver and muscle tissue, respectively [48,49]. The reactions are also rapid and site specific, serving as a rheostat to modulate protein function in response to fluctuations in GSH/GSSG [50]. The reversible glutathionylation of cellular proteins plays an integral role in chemotaxis, phagocytosis, pathogen elimination, energy sensing, ion homeostasis, muscle contraction, and the regulation of many mitochondrial functions (reviewed in Ref. [51]). It is for this reason that protein S-glutathionylation has been suggested to serve as the redox interface between the exposome and changes in cell behavior [52]. Additionally, recent work has also shown that glutathionylation is a critical negative feedback regulator for mitochondrial ROS production and integral for restoring the redox buffering capacity of antioxidant networks through the diversion of metabolites into pathways that generate NADPH [52]. My group also recently generated evidence showing that cytoplasmic ROS sources like xanthine oxidoreductase (XOR) are also inhibited by glutathionylation (*unpublished data*). Taken together, the accumulated evidence does indicate that the redox relay system and glutathionylation are likely how H_2O_2 signals are conveyed to proteins. But, overall, the latter mechanism seems to play more of a global role in mediating these signals.

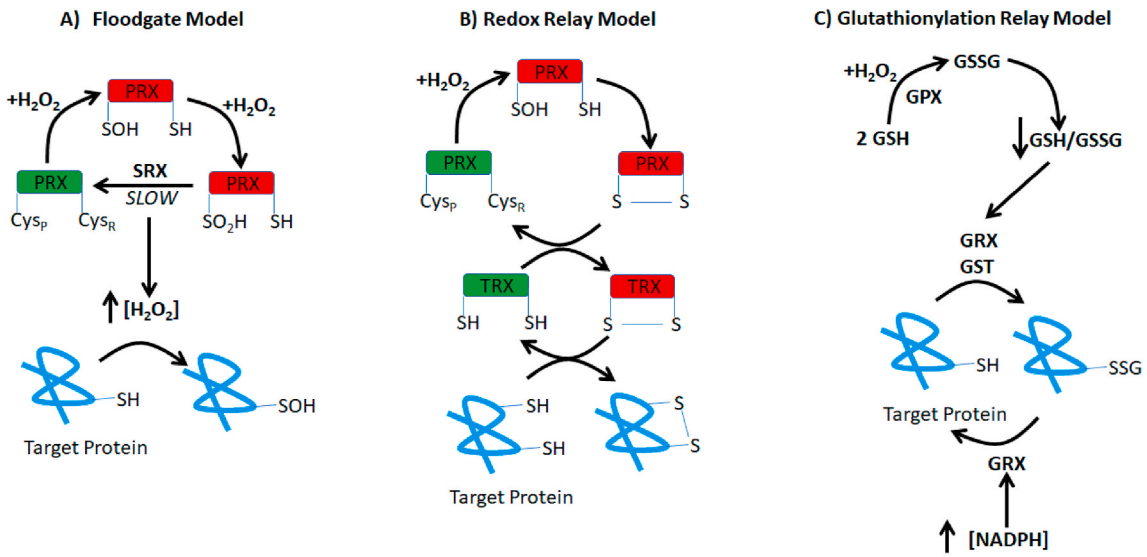

Fig. 1. The three proposed mechanisms for the amplification of H_2O_2 signals. **A.** The floodgate model. Cell signals (e.g. the binding of growth factors to their cognate receptors on the cell surface) triggers the production of H_2O_2 by cytoplasmic and mitochondrial sources resulting in the oxidation of active site peroxidatic cysteine (Cys_P) in peroxiredoxin (PRX). Instead of the resulting sulfenic acid (-SOH) being resolved by the neighboring resolving cysteine (Cys_R), it is oxidized to a corresponding sulfinic acid (-SO_2H). The -SO_2H is resolved by sulfiredoxin (SRX) in the presence of ATP, but the kinetics are slow. This prolongs PRX deactivation, allowing for H_2O_2 to accumulate and elicit changes in the function/activities of target proteins through protein cysteine thiol (-SH) oxidation. **B.** The redox relay model. Cellular signals induce H_2O_2 generation, which is quenched by PRX. The -SOH is resolved through the formation of an intra- or intermolecular disulfide bond. PRX is reactivated through a thiol disulfide exchange reaction with thioredoxin (TRX). The disulfide bridge from TRX is then transferred to a target protein, altering its function. **C.** The glutathionylation relay model. Here, H_2O_2 production is induced by cell signals resulting in the rapid oxidation of reduced glutathione (GSH) by glutathione peroxidase (GPX). This decreases the GSH/GSSG ratio resulting in the spontaneous glutathionylation of the catalytic cysteine in glutaredoxin (GRX). GRX transfers the glutathionyl moiety to a target protein through a thiol disulfide exchange reaction which alters the activity/function of the target. Restoration of the reducing capacity of the GSH pool by NADPH-dependent glutathione reductase (GR) drives the deglutathionylation of the target protein, restoring its function. This is also driven by GRX and is mediated through thiol disulfide exchange reactions with the target protein and GSH.

3. The methodological toolkit for interrogating individual rates of ROS production

The interrogation of individual ROS sources in the NADH/NAD^+ and UQH_2/UQ isopotential groups can be achieved using a toolkit of enzyme inhibitors and substrate combinations. However, interpretation of results needs to be done with caution since this approach has several drawbacks, which are discussed below. It also requires other considerations such as disabling $O_2^{\cdot-}/H_2O_2$ production by complexes I and III and preventing protonic back pressure on the respiratory chain from increasing ROS production. Indeed, there is a non-Ohmic relationship between the membrane potential and ROS production and a small increase in protonic back pressure can augment $O_2^{\cdot-}/H_2O_2$ production by several orders of magnitude [53]. These factors can interfere with the direct quantification of the native rates of ROS production by sources outside of complex I and III. Eliminating the membrane potential can be easily achieved by permeabilizing the mitochondrial inner membrane with repeated freeze-thaw cycles, a Triton X-100 solution of low concentration, or alamethicin [54]. Note that successful permeabilization should be verified, which can be easily done by measuring the activity of malate dehydrogenase [54]. The added benefit of permeabilizing mitochondria is that hydrophilic inhibitors of the different ROS sources in the matrix can be utilized (discussed in more detail below). Other considerations that should be made when measuring the native rates for ROS production by these individual sites include correcting for the quenching of $O_2^{\cdot-}/H_2O_2$ by matrix antioxidant defense systems. The presence of fully functional antioxidant defenses can lead to the underestimation of the native rates for ROS production by the individual sites. Therefore, it is advised that estimations of $O_2^{\cdot-}/H_2O_2$ are conducted in the presence of inhibitors for the GSH, TRX, and catalase systems. Effective inhibitors for all three systems include 2,4-dinitrochlorobenzene (CDNB), auronofin (AF), and 3-Amino-1,2,4-triazole (3-AT), respectively [25,55]. Each individual inhibitor should be titrated into reaction chambers until maximal inhibition is reached, which is when the maximal rate of $O_2^{\cdot-}/H_2O_2$ production has been reached. Once determined, assays should be conducted in the absence and presence of all three inhibitors to accurately estimate the maximal rate of $O_2^{\cdot-}/H_2O_2$ by the individual sites.

A complete list of inhibitors and substrate combinations that can be utilized to interrogate the 12 sites for ROS production can be found in Fig. 2. Inhibitors for the different sites of production, either administered alone or in combination with other inhibitors, are required for the accurate quantification of the rates of $O_2^{\cdot-}/H_2O_2$ production by the individual sites. These substrate and inhibitor combinations have been successfully used to demonstrate $O_2^{\cdot-}/H_2O_2$ sources outside of complex I and II can also generate significant amounts of ROS [34,56]. These approaches have been implemented to study ROS production in various disease models including Barth's syndrome, heart disease, insulin resistance, and diet-induced obesity [27,56–58]. Additionally, this toolkit was used to show there are sex and mouse-strain differences in the native rates of production for the twelve sites [56,59]. Most of these inhibitor interventions revolve around disabling complex I or III ROS production, however: there are also inhibitors for the other ROS sources, which are discussed below. Commonly utilized inhibitors include rotenone (complex I), stigmatellin (complex III), and myxothiazol (complex III) [34,56,60]. Rotenone is a competitive inhibitor for the UQ binding site in the Q-module of complex I. Stigmatellin and myxothiazol are potent inhibitors of the UQH_2 oxidation site of the cytochrome bc1 complex. Use of these inhibitors, in isolation or in combination, has proven useful when interrogating $O_2^{\cdot-}/H_2O_2$ production when UQH_2/UQ isopotential group enzymes are being studied. For example, these inhibitor combinations were utilized to study the native rates of $O_2^{\cdot-}/H_2O_2$ by α-keto acid dehydrogenases and complex II, PRODH, and G3PDH in liver and cardiac mitochondria collected from C57BL6N and C57BL6J mice [56]. The authors were able to successfully demonstrate that these sites make significant contributions to the overall amount of ROS

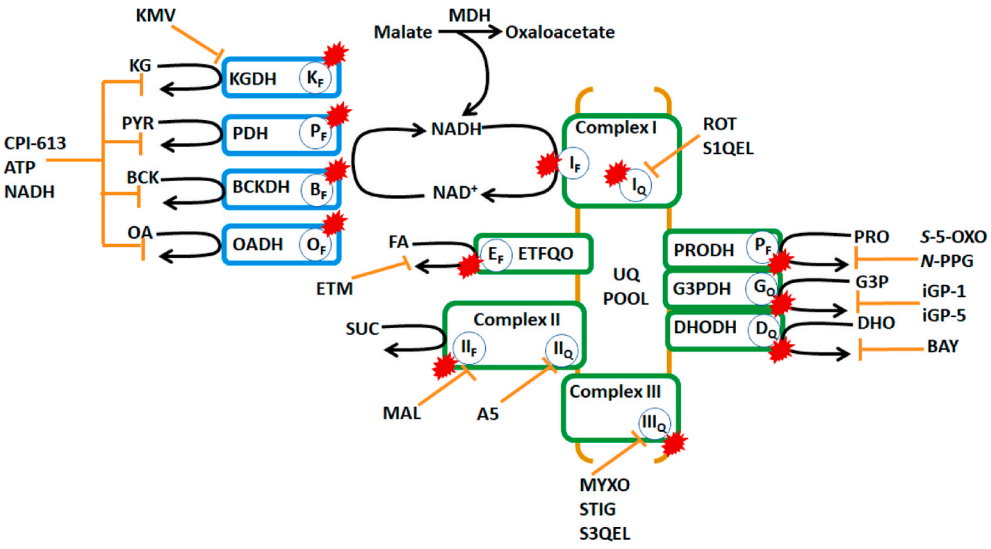

Fig. 2. Schematic representation of a toolkit for the interrogation of the native rates for $O_2^{\cdot-}/H_2O_2$ production by the 12 individual sites associated with nutrient metabolism and electron transfer reactions in mitochondria. Sites for ROS production are denoted by red stars. The individual site for production is labeled in the circle for each enzyme (Upper case letter denotes the first letter of the enzyme name and the lower case letter indicates the factor involved in ROS production: F = flavin, Q = ubiquinone). Substrates: KG; α-ketoglutarate, PYR; pyruvate, BCK; branched chain keto acids, OA; 2-oxoadipate (note that TPP and CoASH should be included when interrogating the α-keto acid dehydrogenases), SUC; succinate, FA; fatty acids, PRO; proline, G3P; glycerol-3-phosphate, DHO; dihydroorotate. Inhibitors: KMV; 3-methyl-2-oxovaleric acid, CPI-613; 6,8-Bis(benzylthio)-octanoic acid, ETM; etomoxir, MAL; malonic acid, A5; atpenin A5, ROT; rotenone, S1QEL; N1-(3-acetamidophenyl)-N2-(2-(4-methyl-2-(p-tolyl)thiazol-5-yl)ethyl)oxalamide, S-5-oxo; S-5-oxo-2-tetrahydrofurancarboxylic acid, N-PPG: N-propargylglycine, iGP-1 and iGP-5; *sn*-Glycerol-3-Phosphate Dehydrogenase Inhibitor, BAY; BAY 2402234, MYXO; myxothiazol, STIG; stigmatellin, S3QEL; 1-(3,4-Dimethylphenyl)-*N*,*N*-dipropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

produced by liver and cardiac mitochondria [56]. Furthermore, important mouse strain-dependent differences were identified with some sites making more ROS than others [56]. Utilizing this approach to study ROS production by members of the UQH_2/UQ isopotential group also led to the following significant observations: 1) complex II is an important ROS source in liver and muscle mitochondria and 2) most of the ROS formed during the oxidation of UQ-linked substrates like proline, glycerol-3-phosphate, or fatty acids is generated by RET to complexes I and II and forward electron transfer (FET) to complex III [33,36,37,56]. The contribution of complex II towards overall mitochondrial ROS production has been known for some time and over generation of $O_2^{\cdot-}/H_2O_2$ by this site has been linked to several pathologies such as cancer and ischemia-reperfusion injury in the myocardium [61]. Identification of complex II as a significant ROS source during RET was achieved using the inhibitors atpenin A5 and malonic acid [33,56]. The source of ROS in complex II is the FAD group located in the succinate binding site in the SDHA subunit. Malonate is competitive inhibitor for the succinate binding site and is thus a good tool for studying ROS production in isolated mitochondria oxidizing succinate. However, use of atpenin A5 proved to be valuable for demonstrating complex II is an important ROS source during RET. Atpenin A5 is a competitive inhibitor for the UQ binding site in the SDHD subunit [62]. Inclusion of atpenin A5 in reaction mixtures oxidizing UQ-linked substrates resulted in a significant decrease in ROS generation in skeletal muscle mitochondria. Additionally, atpenin A5 should also be included in reactions if one wishes to interrogate ROS production by other UQH_2/UQ isopotential group enzymes other than complex II. For example, a recent study added atpenin A5 in combination with rotenone and myxothiazol in permeabilized mitochondria to exclusively study the mouse strain-dependent differences in $O_2^{\cdot-}/H_2O_2$ production by PRODH and G3PDH [56]. There are also inhibitors for the different UQH_2/UQ isopotential group enzymes that can be implemented to verify rates for ROS production. Examples include etomoxir (prevents ROS production by fatty oxidation pathways by inhibiting carnitine palmitoyl transferase-1) and several novel inhibitors for G3PDH, DHODH, and PRODH (Fig. 2) [63–66].

Examining the NADH/NAD^+ isopotential group enzymes can be more challenging due to the position of the $O_2^{\cdot-}/H_2O_2$ source in complex I (Fig. 2). The FMN in the NADH binding site of the hydrophilic N-module that contacts the matrix is the main ROS source in complex I [67]. The challenge associated with this is the lack of a site-specific inhibitor for this site. Electron flow from NADH to complex II and III of the respiratory chain can be negated using either rotenone or complex I knockout models [34,68]. The caveat to this approach is that it inhibits NADH oxidation and electron flow from FMN, leading to increased ROS production by complex I and several α-keto acid dehydrogenases. Indeed, KGDH and PDH can generate high amounts of ROS by RET from NADH [23]. Additionally, NADH can serve as a feedback inhibitor for PDH and KGDH, resulting in the blockage of electron flow through the enzyme complexes [69]. Overall, this can result in the over-estimation of $O_2^{\cdot-}/H_2O_2$ production by sites outside of complex I. There are several ways to negate over-estimating ROS production by the NADH/NAD^+ isopotential group. The first is consideration of substrate type and concentration. Most studies invested in measuring mitochondrial $O_2^{\cdot-}/H_2O_2$ production utilize supraphysiological millimolar concentrations, which would only be seen under pathological conditions *in vivo*. However, it was recently reported that low-to-mid micromolar amounts of substrate is sufficient to stimulate ROS production by the NADH/NAD^+ and UQH_2/UQ isopotential group enzymes in permeabilized and intact mitochondria [56]. Furthermore, max rates for ROS production by liver, muscle, and cardiac mitochondria can be achieved in the low-to-mid micromolar range [56,59]. Additionally, using low amounts of substrate would negate the accumulation of NADH and thus limit ROS production by complex I or RET to α-keto acid dehydrogenases. Other substrate considerations include limiting the accumulation of enzyme products that can feed back and inhibit α-keto acid dehydrogenases. Thiamine pyrophosphate, coenzyme A, and NAD^+ need to be included in the reaction mixtures when studying ROS production by the α-keto acid dehydrogenases. However, this also results in acyl-CoA production, feedback inhibitors that can artificially increase ROS production by these enzymes. This can be prevented by promoting their clearance. For example, acetyl-CoA or succinyl-CoA can be cleared by inducing acetyl-CoA/carnitine transferase (ACAT) or succinyl-CoA synthetase activity [27,32]. Other approaches that can prevent the over estimation of ROS production by the individual components of the NADH/NAD^+ isopotential group include the use of α-keto acid dehydrogenase inhibitors and estimating the rate of ROS production of complex I. There are several excellent α-keto acid dehydrogenase inhibitors such as

3-methyl-2-oxovaleric acid (KMV) and CPI-613 [31,32]. The former is a competitive inhibitor for the α-ketoglutarate binding site in the E1 decarboxylase subunit of KGDH and can almost completely abolish ROS production by the E3 subunit [32]. CPI-613 is a dihydrolipoic acid analog and was originally employed to sensitize cancer cells towards chemotherapeutics [70]. It has now been successfully implemented in ROS studies and serves as a pan-inhibitor for all α-keto acid dehydrogenases [31]. Both KMV and CPI-613 have been successfully utilized to estimate contributions of PDH and KGDH towards total mitochondrial ROS production in muscle and liver [31]. Overall, using these inhibitors in combination with different substrates led to the observation that both PDH and KGDH are significant ROS sources and produce more $O_2^{\cdot-}/H_2O_2$ than complex I. The final consideration is estimating complex I activity and then subtracting its rate for ROS production from the rates measured for the other NADH/NAD^+ isopotential group enzymes. This can be achieved using samples oxidizing malate alone. Malate dehydrogenase is not a ROS source and the oxidation of malate generates NADH [32]. Exclusion of pyruvate or glutamate from reaction mixtures avoids priming the Krebs cycle assuring that ROS formed by PDH or KGDH is minimized [32].

Estimates for ROS production that rely on the use of the respiratory chain inhibitors listed above and in Fig. 2 or by knocking out one of the complexes also results in the inhibition of mitochondrial respiration. This is a major limitation since electron flow through the chain is impeded which may result in the over or under estimation of ROS production, regardless of the precautions taken in the experimental design. Thus, there is a need to identify novel molecules or approaches that can inhibit ROS production by the complexes without compromising mitochondrial respiration. To this end, recent work has identified several novel compounds, called S1QELS and S3QELS, that inhibit $O_2^{\cdot-}/H_2O_2$ production by complexes I and III, respectively, without affecting their activities or mitochondrial respiration [71]. The value of these new molecules has been discussed in several articles and will thus only be summarized here [71]. S1QELS and S3QELS have been successfully utilized to demonstrate that mitochondria are quantifiably the most important ROS sources in cultured cells and have been successfully implemented in the study $O_2^{\cdot-}/H_2O_2$ production by the twelve sites of generation [72]. Surprisingly, even though these chemicals are commercially available, S1QELS and S3QELS are still not routinely utilized by many groups in the study of mitochondrial ROS production.

Although the approaches listed above have successfully shown mitochondria can contain many sites for ROS production, these methods still have several limitations. Firstly, most of the studies have focused exclusively on using isolated mitochondria. This approach limits the physiological relevance of the results collected since estimation of $O_2^{\cdot-}/H_2O_2$ production is being conducted in the presence of atmospheric O_2 levels. As noted above, the rate of production depends on the number of times O_2 collides with an electron donating center, which is influenced by its concentration. Therefore, it is plausible that the rates of ROS production by the twelve sites are being over estimated. Secondly, the $O_2^{\cdot-}/H_2O_2$ production inhibitors listed in Fig. 2, except for the S1QELS and S3QELS, also inhibit the activity of these enzymes. This can result in the over-reduction of electron donating sites resulting in an artificial increase or decrease in ROS production. ROS production by these sites has also not been studied using cultured cells, making it difficult to justify the physiological relevance of findings collected with isolated mitochondria. Studies using permeabilized muscle fibers and the Oroboros O2K system have been conducted, providing some important physiologically relevant information on ROS production by the "unconventional" sources. Indeed, using permeabilized muscle fibers from rodents, it was demonstrated that PDH is a significant ROS source and over production by this site is associated with the development of metabolic diseases [27]. Another drawback is the use of antioxidant defense inhibitors to estimate the maximal rate of ROS production by the 12 different sites. Notably, inhibitors like CDNB have off-target effects and may inhibit sulfur-rich dehydrogenases, like the α-keto acid dehydrogenases. Collectively, studying isolated mitochondria using this methodological toolkit has provided valuable information on how this double membraned organelle produces ROS. However, caution must be exercised when interpreting the results and appropriate controls need to be implemented to avoid over or under estimating ROS production. Additionally, new approaches need to be developed to measure the rate of ROS production by the 12 sites in a cellular context. This could either be with using permeabilized fibers or tissues in the O2K system or developing genetically modified cell lines that allow for the interrogation of ROS production by these sites.

4. Examining the H_2O_2 quenching capacity if mitochondria

It should not be surprising, but the observation that mitochondria can serve as sinks for extramitochondrial hydroperoxides is a relatively new one. Indeed, Zoccarato et al. observed that the H_2O_2 clearing capacity of mitochondria was ~0.3–6.7 nmol min^{-1} mg $protein^{-1}$ [73]. Furthermore, it was estimated to reach 9–12 nmol min^{-1} mg $protein^{-1}$ in brain mitochondria, which exceeds the rate of ROS production by one-hundred times [73]. Finally, the highest rates of extramitochondrial H_2O_2 removal by brain mitochondria are achieved when NAD^+ - linked substrates are fueling metabolism [73]. Overall, these findings implied that mitochondria are not only a ROS source but also a *bona fide* sink for H_2O_2. These studies were followed up by Starkov et al. where it was shown that brain mitochondria can serve as both a source and sink for H_2O_2 [74]. Here, the authors were able to carefully estimate that mitochondria can generate large amounts of H_2O_2 when fueled with NAD^+-linked substrates or succinate [74]. However, they also demonstrated that mitochondria simultaneously quench extramitochondrial H_2O_2 until a stead-state concentration is reached that is balanced by the rate of production and clearance [74]. This led to the development of the hypothesis that mitochondria serve as "cellular ROS stabilizing devices" [74]. This concept was later utilized to produce the posits that the ROS clearing capacity of mitochondria may participate in quenching cytoplasmic H_2O_2 signals and the use of H_2O_2 by mitochondria as a second messenger requires that the rate of production is faster than the rate of clearance [42].

Several studies have interrogated the hydroperoxide clearing capacity of mitochondria isolated from liver, skeletal muscle, and brain tissue from rodents and fish [55,74–76]. A complete list of inhibitors and substrate combinations that can be used to profile the clearance of hydroperoxides by isolated mitochondria is provide in Fig. 3. The inhibitors are utilized site specifically disable the different arms of the matrix antioxidant defense machinery. Auranofin is utilized to disable the thioredoxin-2 (TRX2) system. This achieved by the transfer of the Au from AF to the active site of thioredoxin reductase-2 (TR2). TR2 relies on a catalytic protein cysteine thiol to catalyze the reduction of disulfide bridges formed by TRX2 following the re-activation of peroxiredoxin-2 (PRX2) or PRX5 after the H_2O_2 mediated oxidation of Cys_P (Fig. 3) [75]. The Au from AF is transferred to this catalytic cysteine through a thiol disulfide exchange reaction, blocking its activity [77]. As noted above, CDNB can be used to deactivate the GSH pathway. CDNB deactivates the glutathione pathway by forming irreversible covalent adducts with GSH, a reaction that is catalyzed by glutathione S-transferase (Fig. 3).

Notably, measuring the H_2O_2 clearing capacity of mitochondria requires a fuel supply to power the production of NADPH, a key reducing factor that is required to reactivate the TRX2 and GSH systems after a round of ROS degradation (Fig. 3). There are several sources of NADPH in mitochondria including isocitrate dehydrogenase-2 (IDH2), malic enzyme (ME), and glutamate dehydrogenase (GDH). Intriguingly, several studies have shown that all three enzymes do contribute to maintaining the NADPH supply but are not the chief sources in mitochondria [78,79]. It is now established that nicotinamide nucleotide transhydrogenase (NNT), which is embedded in the mitochondrial

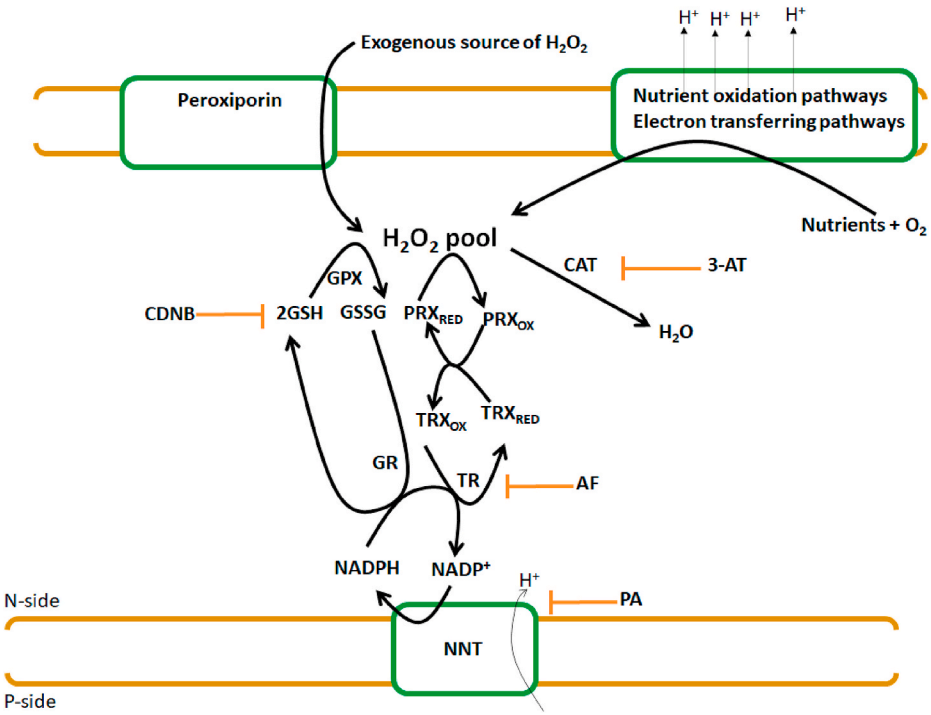

Fig. 3. Schematic representation of a tool kit for the interrogation of the H_2O_2 clearing capacity by isolated mitochondria and intact cells. Inhibitors: AF; auranofin, CDNB; 1-chloro-2,4-dinitrobenzene, 3-AT; 3-amino-1,2,4-triazole, PA; palmitoyl-CoA.

membrane, is the main source of NADPH in the matrix [79]. NNT generates NADPH by coupling the transfer of a hydride from NADH to $NADP^+$ through proton return to the matrix. It is logical that NNT would be the chief NADPH supplier since it can produce NADPH using a universal energy source, the protonmotive force. Interrogating the contribution of NNT towards H_2O_2 clearance can be easily achieved using inhibitors or transgenic mouse models. Palmitoyl-CoA is an effective NNT inhibitor and has been successfully utilized to quantify its contribution towards H_2O_2 degradation (Fig. 3) [55]. The second option is the C57BL6J (6J) mouse strain, which harbors a loss-of-function mutation in the *Nnt* gene (Fig. 3) [78]. This model, in combination with palmitoyl-CoA, has been successfully implemented to measure H_2O_2 and *t*-butyl hydroperoxide clearance by liver mitochondria. It is advised that measuring H_2O_2 clearance by mitochondria from the 6J strain is carried out in tandem with the 6NJ, which harbors a knock-in for *Nnt*. Another consideration is which substrate to supply. Even though IDH2 is not as significant of an NADPH generator overall, it can produce large amounts if Krebs cycle substrates are present. Therefore, assessing the contribution of NNT towards H_2O_2 should be carried out using Krebs cycle-linked and UQH_2-linked substrates in combination (Fig. 3). The latter by-passes the Krebs cycle but also powers the production of a PMF for NNT activity. Adding a UQH_2 substrate like succinate could also be complemented with adding malate to preserve NADH production for NNT activity, however: this could also trigger ME-mediated NADPH production.

Investigations into the contribution of the NADPH-dependent TRX2 and GSH systems using liver mitochondria revealed that both antioxidant networks were not solely responsible for clearing H_2O_2 from the extramitochondrial environment [31]. Inclusion of 3-AT revealed that catalase also makes a considerable contribution towards clearing H_2O_2 (Fig. 3). It was revealed that catalase is expressed in liver mitochondria from mice and fish and that it is required to remove H_2O_2 when it is at a higher than normal concentration (>400 nM) [31]. Catalase clears ~85–90% of the external H_2O_2 within 10–30 s [31]. Once the [H_2O_2] is in the mid-nM concentration, liver mitochondria maintain a steady-state concentration of ~400 nM using the GSH and TRX2 systems [31]. The value of using the 6J and 6NJ models is underscored by the finding that liver mitochondria from 6J mice increase catalase by several fold to maintain its H_2O_2 clearing capacity [55]. Indeed, liver mitochondria from 6J mice produce significantly less ROS and increase catalase by ~7-fold to clear extramitochondrial H_2O_2 [55]. Both the GSH and TRX2 systems made much smaller contributions to removing H_2O_2 due to the lack of NNT [55]. Overall, these methodological approaches demonstrated that 3-AT should be administered in combination with CDNB and AF when studying mitochondrial H_2O_2 clearance (Fig. 3).

Few studies have been invested in studying H_2O_2 clearance by isolated mitochondria and fewer have utilized cultured cells as a model system. However, in 2016, Dey et al. investigated the compartment-specific degradation of H_2O_2 in H9c2 cardiac cells and the contribution of cytoplasmic and matrix-specific antioxidant defenses towards cell ROS homeostasis [80]. The authors utilized several knockdown/knockout models for the cells including multiple knockdowns for genes encoding sources of NADPH and the different arms of the antioxidant networks in the cytoplasm and mitochondria [80]. Using these genetic approaches coupled with protein-based fluorescent probes, they were able to demonstrate that cellular H_2O_2 homeostasis largely depends on mitochondrial substrate catabolism, the provision of NADPH with NNT, and that mitochondria are a sink for cytoplasmic H_2O_2 [80]. Taken together, by genetically manipulating H9c2 cells, Dey and colleagues validated findings that relies on using isolated mitochondria and a panel of inhibitors and substrates. This shows that mitochondria are important sinks for ROS and are required to maintain the H_2O_2 steady-state in cells, a phenomenon that is worthy of further scrutiny and investigation.

5. Novel protein-based probes for H_2O_2 detection

5.1. *A brief history on genetically encoded H_2O_2 sensors*

A complete list of the H_2O_2 sensors that are discussed is provided in Table 1. The first genetically encoded intracellular H_2O_2 sensor to be developed was HyPer [81]. This probe utilizes the bacterial H_2O_2 sensor,

Table 1
Different chemical and genetically-encoded probes intended to detect H_2O_2 and $O_2^{\cdot-}$ and the drawbacks and advantages of each detector.

Name	ROS	Limitations	Advantages	Ref
Amplex Ultra Red	H_2O_2	• AUR can react with peroxynitrite and give a fluorescent signal. • Adding excess exogenous SOD can diminish the fluorescent signal. • Reagents cannot cross lipid bilayers • Sensitive to light and carboxylesterases.	• Sensitive to H_2O_2 down to the pM range. • Provides direct linear relationship between H_2O_2 production and the fluorescent signal. • Kinetic measurements can be performed simultaneously with other measurements (e.g. measuring dehydrogenase activity using the auto-fluorescence of nicotinamides).	[13, 101, 102]
Green fluorescent protein-based probes (HyPer, roGFP2, roGFP2-Orp1, GRX1-roGFP2) and the TScGP probe		• Reaction kinetics are ~10^5 M^{-1} s^{-1}, which is several magnitudes lower when compared to endogenous peroxidases. • Changes in fluorescence may be sensitive to pH. • May not measure absolute concentrations or allow for *in situ* calibrations.	• Genetically encoded and can be localized to specific subcellular compartments. • Can provide real time measures of localized changes in H_2O_2. • Can be reduced by endogenous antioxidant systems allowing for the measure of ROS production oscillations over time. • Ratiometic detection makes change in fluorescence independent of GFP availability.	[14,88, 103]
New roGFP2-Tsa1, roGFP2-Tsa2		• Tsa1 has a higher affinity for TRX, which compromises its sensitivity towards H_2O_2	• Mutation of conserved cysteines improves sensitivity of the probes. • Mutated probes could detect basal H_2O_2. • Genetically encoded and can be localized to specific subcellular compartments. • Can provide real time measures of localized changes in H_2O_2. • Can be reduced by endogenous antioxidant systems allowing for the measure of ROS production oscillations over time. • Ratiometic detection makes change in fluorescence independent of GFP availability.	[14]
DHE and MitoSOX	$O_2^{\cdot-}$	• Reaction with superoxide is several magnitudes lower than the dismutation activity of SOD. • Can form non-fluorescent dimers. • Can intercalate in DNA and auto-fluoresce or form non-specific oxidation adducts. • Non-specific oxidation adducts can be disproportionately higher than the hydroxylation adduct making it difficult to correlate changes in fluorescence with superoxide levels.	• MitoSOX selectively accumulates in mitochondria. • Cell permeable.	[104]
cpYFP		• Sensitive to pH changes and not $O_2^{\cdot-}$. • No clear mechanism for how $O_2^{\cdot-}$ induces rapid changes in fluorescence.	• Genetically encoded and can be targeted to subcellular compartments.	[15, 89]
New small molecule detectors (Benzothiazoline-based probes, CRET-based probes, Te and Se-containing probes)		• Probe specificities have not been verified. • Reactivity with other free radicals and electron donating groups has not been thoroughly tested. • Changes in signal have only been conducted in a handful of studies. • No mechanism for targeting probes to specific cell compartments or tissues.	• Can detect $O_2^{\cdot-}$ down to the nM and pM range in cultured cells and *in vivo*. • Uses fluorescent and chemiluminescent technologies that are readily available in laboratories.	[16]
[^{18}F]ROStrace		• Probe relies on DHE, which has the same limitations as described above. • Access to a PET and cyclotron to generate the probe may be limiting. • Probe specificity was never thoroughly verified and may thus react with other oxyradicals.	• Uses positron emission tomography, a sensitive instrument that allows for precise detection of signal changes *in vivo*.	[17]

OxyR, tagged to a green fluorescent protein (GFP) [81]. OxyR senses H_2O_2 levels through the oxidation-reduction of several cysteine residues [81]. Oxidation of these cysteine residues followed by the formation of disulfide bridges in OxyR activates the transcription activity of the sensor inducing the expression of genes involved in H_2O_2 clearance and antioxidant defense [81]. In the case of HyPer, oxidation of the OxyR by H_2O_2 results in a conformational change in the protein altering GFP fluorescence. The development of HyPer represented a breakthrough for the successful quantification of intracellular H_2O_2 since it allowed its selective measurement in real time. Additionally, the HyPer probe could be modified with intracellular localization peptide sequences for the selective subcellular quantification of H_2O_2 [82]. Efforts to enhance the sensitivity of the probe towards H_2O_2 led to the development of other probes such as roGFP2, GFP with enhanced fluorescence due to the insertion of two cysteines next to the chromophore in the protein [83]. Essentially, roGFP2 operates in the same manner as HyPer where H_2O_2 oxidizes one of the two cysteines resulting in the formation of a disulfide bridge and the alteration of its fluorescence [14]. Also, disulfide bridge formation results in a shift in its intrinsic fluorescence from ~405 nm to ~488 nm, allowing for the ratiometric detection of H_2O_2, which is advantageous since fluorescent changes are independent of roGFP2 concentration [84]. Additionally, changes in pH do not alter its fluorescence. Efforts to increase the sensitivity of genetically encoded probes led to the development of the roGFP2-Orp1 sensor [85]. Orp1 is a thiol peroxidase expressed in *S. cerevisiae* that is required to activate the transcriptional regulator Yap1 in response to an increase in H_2O_2 [85]. The mechanism involves passing the thiol oxidation from Orp1 to Yap1, which activates this transcription factor following disulfide bridge formation [85]. The mechanism for induction of fluorescent changes in the roGFP2-Orp1 sensor involves the formation of a disulfide bridge in Orp1 and its subsequent transfer to roGFP2 (reviewed in Ref. [82]). Finally, the GRX1-roGFP2 probe was also developed over the past decade, which allows for the quantification of H_2O_2 indirectly by reacting with GSSG (reviewed in Ref. [13]). This probe also allows for the real time

quantification of changes in the GSH/GSSG, which provides valuable information on subcellular changes in redox status. Overall, the development of genetically encoded H_2O_2 sensors represents a breakthrough in understanding the relationship between mammalian cells and H_2O_2 and how the latter is utilized as a second messenger.

5.2. Promising new genetically encoded H_2O_2 sensors

The caveat to the roGFP2-Orp1 sensor is that it reacts with H_2O_2 at a rate of $\sim 10^5$ M^{-1} s^{-1} [86]. This is in comparison to intracellular peroxidases that remove H_2O_2 at $\sim 10^7$ M^{-1} s^{-1}. Therefore, the roGFP2-Orp1 probe is likely not detecting smaller physiological changes in H_2O_2. It is possible to use roGFP2-Orp1 in tandem with the inhibitors for antioxidant defenses (Fig. 3) to more accurate quantify intracellular H_2O_2 changes in real time. However, this also may limit the physiological relevance of findings since it may artificially augment cellular H_2O_2 levels due to the loss of antioxidant defense machinery. To get around the limitations associated with the roGFP2-Orp1 probe, genetically encoded sensors tagged to peroxiredoxins have been developed recently (reviewed in Ref. [14]). This includes roGFP2-Tsa1, roGFP2-Tsa2, and roGFP2-PRDX2 and several mutants where Cys_P and/or Cys_R have been modified (roGFP2-Tsa2ΔC_P, roGFP2-Tsa2ΔC_R, and roGFP2-Tsa2$\Delta C_P \Delta C_R$) [14]. The mechanistic details surrounding how each probe reacts with H_2O_2 has been reviewed extensively in Ref. [14]. The developers of these new probes unexpectedly found that both the roGFP2-Orp1 and roGFP2-Tsa2 probes reacted with H_2O_2 at similar rates, a property attributed to the higher affinity of Tsa1 for TRX [87]. In effort to augment the sensitivity of the sensor and mitigate sensitivity towards TRX, the authors mutated Cys_R of Tsa2 (roGFP2-Tsa2ΔC_R) [82,87]. This increased the sensitivity probe by 20-fold [14]. More importantly, the roGFP2- Tsa2ΔC_R probe was able to measure basal H_2O_2 levels [14]. This is promising since subtle spatiotemporal changes in H_2O_2 in the nM range plays an integral role in oxidative eustress signaling. Furthermore, although it has not been done yet, tagging the roGFP2-TSa sensors with a mitochondria localization sequence would provide incredibly valuable information on how these organelles use ROS in cell signaling. Considerable work still needs to be done to continue increasing the sensitivity of the roGFP2 probes and understand the complex interactions of the roGFP2-Tsa1 and Tsa2 probes with endogenous peroxidases. However, the development of these sensors represents are considerable breakthrough in redox biology that will enhance our collective understanding of how cells use H_2O_2 as a second messenger in response to environmental cues. This includes potential *in vivo* measurements of H_2O_2 concentration changes using various animal models.

Yang et al. also recently generated a genetically encoded peroxiredoxin-based sensor for the detection of H_2O_2 called TScGP [88]. This sensor employs cpYFP sandwiched between a fungal PrxA and TrxA mutant (TrxA-C39S) and takes advantage of the redox relay system between the peroxiredoxin and thioredoxin proteins [88]. PrxA Cys_P is oxidized by H_2O_2 yielding -SOH which is then resolved by Cys_R [88]. TrxA then forms an intermolecular disulfide bridge with PrxA to reduce Cys_R and Cys_P [88]. However, the mutation of Cys39 to a Ser inhibits TrxA from fully reducing the disulfide bridge in PrxA resulting in the formation of a stable disulfide bridge between the two proteins [88]. This causes a significant conformational change in cpYFP resulting in a shift in its fluorescence and the rationmetic detection of changes in H_2O_2 availability [88]. The authors reported that the probe can detect H_2O_2 within the 0.5–5 μM range and does not react with other ROS such as $O_2^{\cdot-}$ or nitric oxide (NO). Additionally, it was reported that it does not detect changes in S-nitrosothiols, disulfides, or cysteine thiols [88]. However, TScGP also displayed a ratiometric increase in fluorescence in response to *tert*-butyl hydroperoxide, indicating it is not selective for just H_2O_2, but may react with many hydroperoxides. This is problematic given that cells experiencing oxidative distress or ferroptosis generate high amounts of lipid hydroperoxides indicating that TScGP may not be suitable for assessing H_2O_2 in stressed cells. Another limiting factor is that the authors did not examine the effect of pH on the probe. This is critical since cpYFP was shown to alter its fluorescence in response to pH changes with a similar sensitivity and amplitude as Sypher [15,89]. Finally, the detection range for the TScGP would hinder its capacity to H_2O_2 concentration changes in the low-to-mid nM range during oxidative eustress, an adaptive response where nM amounts of H_2O_2 are used to change cell behavior in response to environmental cues. Despite these shortcomings, this peroxiredoxin-based sensor does show a lot of promise in terms of engineering it further to increase its sensitivity and selectivity for H_2O_2 detection.

6. Interrogating $O_2^{\cdot-}$; challenges, stochastic "flashes", and some new chemical probes

6.1. Limitations of traditional chemical probes and the controversial cpYFP $O_2^{\cdot-}$ detector

The limitations and advantages of each $O_2^{\cdot-}$ detector discussed below can be found in Table 1. The accurate quantification of mitochondrial $O_2^{\cdot-}$ is vital given its role in oxidative distress and potential role in mammalian cell signaling. However, this has proven to be very challenging given its low cellular concentration (low-to-mid pM range) and high activity and concentration of superoxide dismutase (SOD), which converts $O_2^{\cdot-}$ into H_2O_2 at a rate of 1.8×10^9 M^{-1} s^{-1} [19]. Chemical probes such as dihydroethidine (DHE) and DHE tagged with a triphenylphosphonium (MitoSOX) have been used in many studies to quantify cellular and mitochondrial $O_2^{\cdot-}$. Unfortunately, these probes suffer several limitations that diminish their capacity to detect physiological changes in $O_2^{\cdot-}$ and are only useful when assessing oxidative distress (reviewed in Ref. [13]). First, the ethidine probes react with $O_2^{\cdot-}$ at $\sim 10^6$ M^{-1} s^{-1} [90]. Unfortunately, the rapid kinetics and high concentration of SOD in mitochondria make it a significant challenge to accurately quantify basal changes in $O_2^{\cdot-}$, making it only suitable for oxidative distress studies. Secondly, the probe can intercalate with DNA and fluoresce or form non-fluorescent dimers, giving either false fluorescent signals or decrease the availability of ethidine for $O_2^{\cdot-}$ detection. Additionally, HPLC analyses has demonstrated that the non-specific oxidation of the probes occurs at higher amounts than the hydroxylated product formed following a reaction with $O_2^{\cdot-}$. The ethidine probes are most commonly used in fluorescent microscopy; however, these limitations make it difficult to correlate changes in the fluorescent signal with alterations in $O_2^{\cdot-}$. The non-specific oxidation adducts and the hydroxylated product can be separated and quantified by HPLC, providing more accurate data on $O_2^{\cdot-}$ availability. Overall, however, the ethidine probes, which are still widely used, cannot supply information on physiological and basal changes in $O_2^{\cdot-}$ availability.

The significant limitations with the use of these traditional chemical sensors left a significant technological gap for the accurate quantification of mitochondrial $O_2^{\cdot-}$ level changes in response to physiological stimuli. To get around these limitations, many groups have relied on indirectly quantifying $O_2^{\cdot-}$ levels by measuring H_2O_2 using Amplex UltraRed (AUR). The premise here is that cells or isolated mitochondria are exposed to an AUR detection solution supplemented with or without exogenous SOD [23]. AUR is highly selective for H_2O_2 down to the low nM range and taking the difference in AUR fluorescence in reactions with or without exogenous SOD can be used to calculate the amount of $O_2^{\cdot-}$ formed [23]. However, there are limitations to this approach such as AUR and the other reagents are not membrane permeable and thus fluorescent detection of H_2O_2 relies on its diffusion into the surrounding cell culture medium or buffer. Second, AUR can auto-oxidized in the presence of peroxinitrite (ONOO). Finally, it has been demonstrated that excess amounts of exogenously added SOD can actually underestimate $O_2^{\cdot-}$ levels. This is because the dismutation of one molecule of $O_2^{\cdot-}$ produces 0.5 molecules of H_2O_2 [91]. Therefore, caution must be taken

when calculating the amount of $O_2^{\cdot-}$ is measured using AUR. Another approach has been used to assess $O_2^{\cdot-}$ availability using the activity of aconitase. Here, $O_2^{\cdot-}$ disassembles the cubane Fe–S cluster in aconitase, rendering it inactive. The lower the activity of aconitase, the more $O_2^{\cdot-}$ is available. However, this approach does not quantify the amount $O_2^{\cdot-}$ available and thus supplies very limited information on its concentration and rate of production. This led to investigations into the use of cyclic permuted yellow fluorescent protein (cpYFP) for the detection of changes in $O_2^{\cdot-}$ [92]. Use of cpYFP was viewed as being highly advantageous since it is a genetically encoded probe that can either be expressed in cultured cells or *in vivo*. It can also be targeted to the matrix or intermembrane space using a mitochondria localization sequences [93]. The probe was used initially to detect small fluctuations in mitochondrial $O_2^{\cdot-}$ production and to demonstrate that these "stochastic flashes" can induce mitochondrial permeability pore opening [93]. cpYFP was then used routinely thereafter to examine the role of these $O_2^{\cdot-}$ flashes in physiological processes. However, it was later revealed in two studies by Schwarzlander et al. and in a commentary by Demaurex et al. that cpYFP does not change its fluorescence in response to oxidation by $O_2^{\cdot-}$ but is rather sensitive changes in pH [15,89,94]. It was reported that cpYFP utilizes several cysteines to react with $O_2^{\cdot-}$ [15]. Unfortunately, $O_2^{\cdot-}$ reacts very slowly with protein thiols and Schwarzlander et al. reported that these cysteines are not accessible to solutes [15]. Taken together, cpYFP is not detecting these $O_2^{\cdot-}$ flashes through a cysteine oxidation reaction.

6.2. Novel fluorescent and luminescent probes for $O_2^{\cdot-}$ detection

Over the last several years, several promising chemical sensors for $O_2^{\cdot-}$ detection have been developed by several groups. The underpinnings of the chemical selectivity of these probes and sensitivity for $O_2^{\cdot-}$ and their applicability for conducting measurements *in situ* or *in vivo* was recently reviewed and will thus only be summarized here [16]. Benzothiazoline-based probes have shown a lot of promise due to their selective dehydrogenation by $O_2^{\cdot-}$ [16]. These chemical sensors were shown to be highly selective and sensitive towards $O_2^{\cdot-}$ and were successfully utilized to quantify bursts in $O_2^{\cdot-}$ production in cultured macrophages stimulated with 4b-phorbol 12- myristate 13-acetate (PMA) [16,95]. This led to the development of the quinoline-benzothiazoline-conjugated two-photon fluorescent probe, which allows the detection of $O_2^{\cdot-}$ deep within animal tissues. Indeed, using its high tissue penetrability, Liu et al. showed that $O_2^{\cdot-}$ levels increase in a rodent lung inflammation mouse model [96]. Several other benzothiazoline sensors that selectively accumulate in mitochondria have also been developed, which show promising results in the selective interrogation of $O_2^{\cdot-}$ levels cellular subcompartments. Additionally, given the deep tissue penetration that can be achieved two photon fluorescent probes, it is possible to apply sensors that accumulate in mitochondria and other organelles for the detection on $O_2^{\cdot-}$ changes *in vivo*.

Some other exciting chemical $O_2^{\cdot-}$ sensors that have been developed include probes that take advantage of chemiluminescence resonance energy transfer (CRET). For example, Li et al. developed a CRET system composed of an energy donating imidazopyrazinone and an energy accepting polymer [97]. The authors reported that this CRET system could measure $O_2^{\cdot-}$ down to a limit detection of 19 pM, which is highly advantageous given that $O_2^{\cdot-}$ has been estimated to fluctuate in the low-to-mid pM range in cells [97]. What is more promising however is the authors were able to utilize this CRET probe to detect $O_2^{\cdot-}$ changes in cancer tissues *in vivo* [97]. Using imidazopyrazinone, Niu et al. coupled this chemiluminescent detector to a fluorescent tetraphenylethene group to enhance the sensitivity for $O_2^{\cdot-}$ detection using aggregation-induced emission [98]. The authors successfully detected $O_2^{\cdot-}$ down to the ~0.21 nM concentration and implemented it to assess $O_2^{\cdot-}$ changes in a rodent model treated with lipopolysaccharide (LPS) and LPS with the scavenger Tiron [98].

Several Te and Se-containing probes and carbon dots have also been synthesized for the accurate quantification of cellular $O_2^{\cdot-}$ levels (reviewed in Ref. [16]). Te-containing carbon dot molecular probes were found to detect $O_2^{\cdot-}$ down to ~8 pM, which is a promising outcome given that its physiological levels were estimated to fluctuate in the pM range [99]. The probe was successfully utilized in the measurement of low amounts of $O_2^{\cdot-}$ in several cell types. However, what is most impressive was its utilization to quantify abdominal changes in $O_2^{\cdot-}$ in rodents subjected to exercise, noise, or depression [99]. Findings with exercise are exciting given that it was utilized to confirm that $O_2^{\cdot-}$ levels do increase in response to an increase in physical activity, confirming that small increases in ROS plays a vital role in the adaptations of muscles to exercise. It is worth noting here that this group has synthesized a number of small fluorescent probes for the selective and sensitive detection of $O_2^{\cdot-}$ (reviewed in Ref. [16]). Readers are therefore encouraged to consult this review since the list of compounds developed is too large to be summarized here. Collectively, these small chemical probes represent a potential alternative to the more commonly utilized ethidum probes or cpYFP since these sensors can detect $O_2^{\cdot-}$ down to the low pM range in cultured cells or *in vivo*. Of note is that the specificity and selectivity of these small chemical probes needs to be verified to ensure that these sensors are not reacting with other free radicals formed by living systems and do not elicit unfavorable effects that may induce cell toxicity and oxidative distress. Indeed, one noted deficiency with these studies is that it remains unknown if the probes react with other free radicals or non-radicals *in vivo*, such as peroxynitrite or other electron donating groups. Historically, the major drawback for the successful detection of small changes in $O_2^{\cdot-}$ using HPLC, fluorescence, or PET (discussed below) has been the sensitivity and selectivity of the probes. The probes listed here should be met with similar scrutiny given that there selectivity and specificity have not been verified using various model systems and controls. For instance, the *in vivo* study of these probes and their capacity to detect $O_2^{\cdot-}$ was generally performed on rodents treated with LPS in the absence or presence of the $O_2^{\cdot-}$ quencher, Tiron. Although the finding that Tiron decreases the signal is promising, it should be verified that these small chemical probes do not undergo unwanted side reactions or react with other oxidants to provide false signals.

6.3. Detecting superoxide using positron emission tomography (PET)

As noted above, commercially available chemical dyes such as the ethidium compounds have proven to be valuable in the evaluation of $O_2^{\cdot-}$ in the contexts of oxidative distress. However, these probes can undergo side reactions that can limit the amount of probe available for $O_2^{\cdot-}$ detection or provide false fluorescent signals. More importantly, these sensors cannot be utilized for the *in vivo* quantification of $O_2^{\cdot-}$, which severely limits defining the physiological function of $O_2^{\cdot-}$ in living systems responding to environmental cues. In the section above, I briefly reviewed important breakthroughs in the development of small chemical probes that could potentially be implemented for the quantification of $O_2^{\cdot-}$ in the pM range using deep tissue two photon fluorescence and chemiluminescence *in vivo*. Recent research has also led to the development of a molecular probe called [^{18}F]ROStrace that can release positrons following its reaction with $O_2^{\cdot-}$ [17]. This is an exciting finding since it can allow for the real time measurement of $O_2^{\cdot-}$ using PET [17]. This same group had previously generated an ethidium probe analog tagged with a positron emitting 18-fluoro group [100]. Although it was shown to be effective at detecting $O_2^{\cdot-}$ *in vivo*, one major drawback was that it was unable to cross the blood-brain-barrier (BBB) [100]. However, the same group was able to later generate a new analog for this compound called [^{18}F]ROStrace that can cross the BBB for the detection of $O_2^{\cdot-}$ changes in the neural tissue of live rodents subjected to neuroinflammation [17]. The authors reported that the [^{18}F]ROStrace

displayed a similar specificity to $O_2^{\cdot-}$ as DHE and was not reactive against H_2O_2, hydroxyl radical, and several two electron donating non-radicals [17]. Rodents treated with LPS displayed detectable increases in $O_2^{\cdot-}$ availability in whole brain, the cerebellum, and cerebrum and were able to show that once oxidized by $O_2^{\cdot-}$ the probe remains trapped in the tissue [17]. However, there are several drawbacks with this probe and its selectivity and sensitivity for $O_2^{\cdot-}$ and therefore the results collected in this study should be interpreted with caution. First, the probe relies on use of the parent ethidium compounds discussed above, which suffer from several limitations including undergoing non-specific oxidation events or forming dimers. Hou et al. showed it has a similar reactivity to $O_2^{\cdot-}$ as DHE, indicating that [^{18}F]ROStrace may not be suitable for detecting basal or physiological changes in $O_2^{\cdot-}$ [17]. Indeed, the authors did not perform HPLC analysis on the [^{18}F]ROStrace probe and therefore it remains unknown if it undergoes non-specific oxidation or forms non-fluorescent dimers. Whether the potential non-specific oxidation product of [^{18}F]ROStrace exists and if it alters its positron emission properties should be investigated to eliminate the possibility that positron detection is not a false positive. The second issue is the authors only simply measured LPS-driven changes in positron emission. The rodents were not subjected to any other treatments and it is unclear if [^{18}F]ROStrace reacts other oxidants, electron donating groups, and oxy-radicals *in vivo*. Overall, the prospect of measuring $O_2^{\cdot-}$ by PET is an important step forward in the *in vivo* measurement of ROS. However, the [^{18}F]ROStrace likely suffers from the same deficiencies as DHE and MitoSOX and therefore its $O_2^{\cdot-}$ detecting capacity should be exercised with caution.

7. Concluding remarks

The role of $O_2^{\cdot-}$ and H_2O_2 in oxidative eustress signaling and oxidative distress form the bedrock of redox biology and the response of cells towards physiological cues and oxidative damage. The cell redoxome, which is composed of sulfur containing switches that become oxidized or reduced in response to fluctuations in $O_2^{\cdot-}$ and H_2O_2, communicates information transmitted from the intra and extracellular environment to the genome, transcriptome, and/or proteome, respectively, to elicit adaptive responses. Mitochondria form a vital part of the redoxome since they are quantitatively the most important source of ROS in most mammalian cells and utilize several redox switches to modulate cellular bioenergetics and adaptive signaling. The central role of ROS and the redoxome in regulating cellular functions demands access to sensitive and selective molecular probes and genetically encoded ROS sensors for their direct quantification *in vivo*. The quantification of physiological $O_2^{\cdot-}$ and H_2O_2 levels using isolated organelles, cultured cells or *in vivo* has been challenging given the short half-lives of both ROS and the limitations associated with various probes and techniques that allow for their detection. Furthermore, assessing the molecular pathways for mitochondria-to-cell communication (and vice versa) using H_2O_2 has been difficult due to the number of ROS sources in and outside of mitochondria (41 $O_2^{\cdot-}$ and H_2O_2 generating enzymes in human cells with 16 housed in mitochondria) [1]. Here, I have provided an update on the current techniques used for interrogating mitochondrial ROS production and H_2O_2 and the novel protein-based and small chemical probes that have been developed in recent years for the real time measurement of physiological changes in ROS. Given the availability of these advanced methods, it is anticipated that these approaches will lead to breakthroughs in understanding the physiological function of small bursts in ROS production in response to stimuli and the adaptive responses that $O_2^{\cdot-}$ and H_2O_2 in living systems. Additionally, these new approaches could lead to their future application in studies involving human volunteers and may for the first time provide information on real time changes in ROS availability in human tissues.

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