Biochimica et Biophysica Acta 1840 (2014) 757–767

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

# Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases ☆

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ARTICLE INFO

*Article history:*
Received 8 February 2013
Received in revised form 29 April 2013
Accepted 30 April 2013
Available online 7 May 2013

*Keywords:*
NADPH oxidase
NOX protein family
Superoxide anion
Hydrogen peroxide

ABSTRACT

*Background:* The recent recognition that isoforms of the cellular NADPH-dependent oxidases, collectively known as the NOX protein family, participate in a wide range of physiologic and pathophysiologic processes in both the animal and plant kingdoms has stimulated interest in the identification, localization, and quantitation of their products in biological settings. Although several tools for measuring oxidants released extracellularly are available, the specificity and selectivity of the methods for reliable analysis of intracellular oxidants have not matched the enthusiasm for studying NOX proteins.

*Scope of review:* Focusing exclusively on superoxide anion and hydrogen peroxide produced by NOX proteins, this review describes the ideal probe for analysis of $O_2^{\bullet-}$ and $H_2O_2$ generated extracellularly and intracellularly by NOX proteins. An overview of the components, organization, and topology of NOX proteins provides a rationale for applying specific probes for use and a context in which to interpret results and thereby construct plausible models linking NOX-derived oxidants to biological responses. The merits and shortcomings of methods currently in use to assess NOX activity are highlighted, and those assays that provide quantitation of superoxide or $H_2O_2$ are contrasted with those intended to examine spatial and temporal aspects of NOX activity.

*Major conclusions:* Although interest in measuring the extracellular and intracellular products of the NOX protein family is great, robust analytical probes are limited.

- Several reliable methods for measurement of extracellular $O_2^{\bullet-}$ and $H_2O_2$ by NOX proteins are available.
- Chemiluminescent probes for both extracellular and intracellular $O_2^{\bullet-}$ and $H_2O_2$ detection have shortcomings that limit their use.
- Options for quantitation of intracellular $O_2^{\bullet-}$ and $H_2O_2$ are very limited.
- However, non-redox sensitive probes and genetically encoded reporters promise to provide spatial and temporal detection of $O_2^{\bullet-}$ and $H_2O_2$.

*General significance:* The widespread involvement of NOX proteins in many biological processes requires rigorous approaches to the detection, localization, and quantitation of the oxidants produced. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.



## 1. Introduction

Oxidants generated by cellular NADPH oxidases (NOX proteins) participate in many biological processes, serving both as critical elements of signaling pathways as well as important effector molecules [1–6]. The recognition that human neutrophils utilize oxidants to kill ingested microbes and to promote biochemical events in sterile inflammation inspired decades of work to elucidate the components, organization, regulation, and much of the biochemistry of the phagocyte NADPH oxidase (reviewed in [3,7]). Insights from that body of work have provided the context for subsequent work on non-phagocyte oxidases, although important differences in tissue distribution and

*Abbreviations:* CGD, chronic granulomatous disease; cpYFP, circularly permutated form of the yellow fluorescent protein; $DCFH_2$, dihydrofluorescein; $DH_2R$, dihydrorhodamine; DUOX, dual oxidase (1 or 2); DUOXA, dual oxidase maturation factor (1 or 2); fMLF, formyl methionylleucylphenylalanine; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HE, hydroethidine; HOCl, hypochlorous acid; HRP, horseradish peroxidase; HVA, homovanillic acid; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; NOX, NADPH oxidase protein; 2-OH-$E^+$, 2-hydroxythidium; PMA, phorbol myristate acetate; SOD, superoxide dismutase

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http://dx.doi.org/10.1016/j.bbagen.2013.04.040

activity of the NOX isoforms make extrapolation from the phagocyte system sometimes challenging. Appreciation of the wide distribution of NOX isoforms throughout biology has stimulated great interest in oxidants and inspired studies in a variety of scientific disciplines. On the down side, however, enthusiasm for studying the physiology and pathophysiology of NADPH oxidases has in many cases fostered dependence on assays that are not specific, selective or quantitative and have spawned proposed mechanisms that are frequently fanciful and occasionally implausible. Recognition of the challenges to measuring products of the NOX proteins in biological settings and the limitations of available reporter systems [8] may help avoid the pitfalls of magical thinking.

## 2. The ideal probe

Probes used to measure products of NOX proteins include both those subject to redox modification (*i.e.* change fluorescence or chemiluminescence when oxidized) as well as those without a redox-based mechanism for reporting. For comprehensive discussion of individual probes, the reader is referred to any of the many excellent recent reviews of the most frequently used probes that highlight the chemistry underlying their ability to detect oxidants, their shortcomings, and their applications to the measurement of reactive oxygen and reactive nitrogen species generated in biological systems [9–15]. In addition, Winterbourn provides elsewhere in this issue an updated review of the challenges of measuring $O_2^{\bullet-}$ and $H_2O_2$. Although the cellular NADPH oxidases initiate production of a variety of oxidants, the comments that follow focus on only approaches for the detection and quantitation of superoxide anion and hydrogen peroxide generated by NOX proteins. Detection of hypochlorous acid, a major product of the phagocyte NADPH oxidase, will be reviewed in detail by Kettle elsewhere in this issue. The methods discussed in this review will be limited to those that require relatively routine analytical equipment. For that reason, methods to measure directly electron transfer such as patch clamping [16] or oxidant production using electron spin resonance [17] are not included.

To link specific products of NADPH oxidase activity with posttranslational modifications in downstream targets and specific physiologic or pathophysiologic pathways, it is essential to identify precisely the oxidant generated, which in this discussion is limited to superoxide anion and hydrogen peroxide. Ideal probes to target $O_2^{\bullet-}$ and $H_2O_2$ should exhibit several features that are desirable irrespective of the site of oxidant production. However, reporters for intracellular oxidants require additional, specialized attributes (Table 1). The optimal probe should respond to low concentrations of superoxide anion or $H_2O_2$ and be sensitive, in that it is responsive over several orders of magnitude of $O_2^{\bullet-}$ or $H_2O_2$ concentrations that span physiologic and pathophysiologic levels. Reactions between probe and target should be specific for the oxidant of interest and insensitive to pH, other reactive oxygen or nitrogen species, oxidized glutathione, or antioxidant agents. Probes should be cell-permeable, nontoxic to cells, and operate reliably at concentrations low enough to leave the cellular redox balance unaltered. With the chemistry for its reaction with the oxidant of interest defined, a probe should provide precise quantitation with very low background signal. The product of oxidant and probe should be non-reactive, thereby avoiding spurious signals from secondary downstream reactions. For optimal application, use of the probe would require neither specialized equipment nor expertise, and its output would be simple to quantify accurately.

**Table 1**
Attributes of an ideal probe for $O_2^{\bullet-}$ and $H_2O_2$.

1. Responsive to low concentrations of the oxidant of interest
2. Sensitive to relevant concentrations of oxidant
3. Responds to oxidant more rapidly than do competing reactants
4. Specific and selective for the oxidant of interest
5. Quantitative
6. Permeable to cells and can be modified to target specific organelles
7. Nontoxic to cells and does not perturb cellular redox state
8. Chemistry well-defined and stoichiometry of reaction with oxidant of interest known
9. Product nonreactive; no secondary reactions and no secondary signals
10. Easy to use, requiring neither special expertise nor dedicated equipment

Some properties (*e.g.* 6 and 7) would be relevant only when monitoring $O_2^{\bullet-}$ and $H_2O_2$ in intracellular compartments. The relative importance of specific attributes depends on the goal of the study, whether it is to quantitate precisely the products of NADPH oxidase activity, to identify cellular compartments in which oxidants are generated, or to monitor kinetics of oxidant production in the context of signaling cascades.

No single probe has all of these attributes, but many studies of NADPH oxidase biology require reporters that need to possess only some of these properties and goals can be achieved with the currently available analytical tools. For example, dissecting signaling properties of specific NOX proteins may rest on detection of the spatial and temporal aspects of specific oxidant production, with quantitation a much less important parameter. Regardless of the particular experimental setting, however, it is prudent to employ two or more assays, each relying on a different biochemical principle, to detect or measure $O_2^{\bullet-}$ and $H_2O_2$ and to complement measurements with other experimental approaches, such as pharmacologic inhibitors, inhibitory RNA technology, or cells genetically deficient in the specific NOX isoforms or components.

## 3. Anatomy of a NOX protein

Authors frequently ascribe the detection of increased cytoplasmic probe activity ("ROS production") to the presence of products of plasma or endosomal membrane NADPH oxidase activity. Correct interpretation of experimental findings and construction of plausible models for underlying mechanisms for NOX proteins require an appreciation of the topology of electron transfer by NADPH oxidases. NOX proteins transport electrons from NADPH on the cytoplasmic face of plasma, endosomal, or phagosomal membranes to $O_2$ at the extracellular space or in the lumen of the endosome or phagosome, respectively (Fig. 1). Consequently, for $O_2^{\bullet-}$ or $H_2O_2$ produced by NOX proteins to engage detectors present in the cytoplasm, the oxidant must move from its site of origin, across a membrane, and into the cytoplasm. As a charged species, $O_2^{\bullet-}$ would require passage through an anion channel to reach the cytoplasm [18,19]. Uncharged but nonpolar, $H_2O_2$ diffuses across membranes in mammalian cells to a very limited extent [20] but could readily enter cytoplasm through isoforms of aquaporin [21]. As discussed elsewhere ([22] and Winterbourn elsewhere in this issue) $O_2^{\bullet-}$ and $H_2O_2$ differ in reactivity. However, the rapidity of the cellular responses attributed to NADPH oxidase-derived oxidants, often in milliseconds, and the presence of antioxidant species in cytoplasm that can readily consume $H_2O_2$ require: (1) signaling targets to be close to the oxidant source, or (2) the oxidants to initiate a cascade of posttranslational modifications that both relay signals intracellularly and prompt downstream intracellular oxidant production. Schematics that overlook these intermediate steps and depict oxidants generated by the plasma membrane-associated NADPH oxidase *directly* driving intracellular signaling represent implausible mechanisms.

### 3.1. The human neutrophil paradigm

Historically, the patriarch of the NOX protein family, the phagocyte NADPH oxidase, illustrates many structural and functional features that are shared by other NOX proteins. However, important differences exist in composition, subcellular distribution, and activity of non-phagocyte oxidases that likely reflect their cell- or tissue specific functions. Essentially all the $O_2$ consumed and oxidants generated by stimulated neutrophils reflect the activity of the NADPH oxidase, with negligible contributions from mitochondria. Unassembled in resting phagocytes, the enzymatically active multicomponent

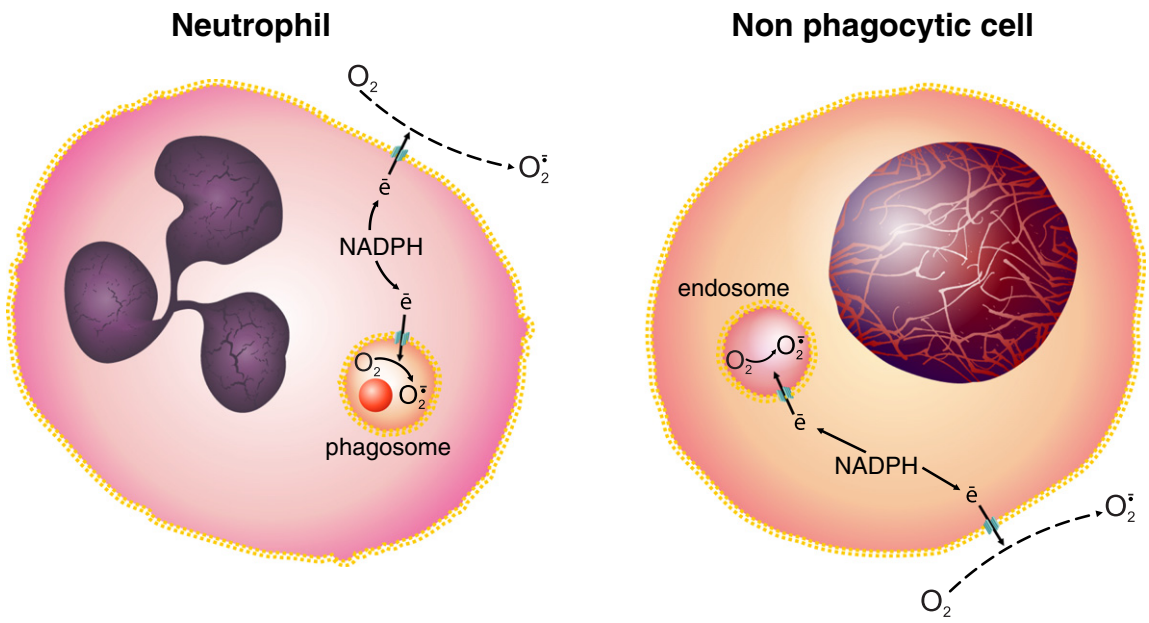


**Fig. 1.** Topology of NOX proteins in phagocytes and nonphagocytes. Operating as electron transferases, all NOX protein family members transport electrons from NADPH, generated by the hexose monophosphate shunt, across the plasma or phagosomal membranes in neutrophils and other phagocytes and across the plasma membrane or endosomal membranes in non-phagocytic cells. In neither case do NOX proteins generate $O_2^{\bullet-}$ directly into the cytoplasm.

phagocyte NADPH oxidase includes proteins that reside in plasma or granule membranes as well as those recruited from cytoplasm to dock with membrane components after stimulation (Fig. 2) (reviewed in [3]). The membrane component is flavocytochrome $b_{558}$, a heterodimeric membrane protein composed of gp91*phox* and p22*phox*, and serves as an electron transferase with $O_2$ as the electron acceptor. Structural stability, heme acquisition, and transport from the endoplasmic reticulum to target membranes require heterodimer formation [23–26]. Gp91*phox* (aka NOX2), containing one molecule of flavin adenine dinucleotide (FAD) [27] and two molecules of heme, serves as the catalytic center of the phagocyte oxidase. Two electrons from NADPH are transferred to FAD, followed by two sequential single-electron reductions of two inequivalent heme groups to $O_2$ to form two molecules of $O_2^{\bullet-}$ [28]. Electron transfer by the phagocyte oxidase is robust, with more than $10^{10}$ electrons translocated within 5 min in response to formylated peptides [29] and this magnitude of electron redistribution would depolarize the plasma or phagosomal membrane > 200 mV within milliseconds and thereby terminate

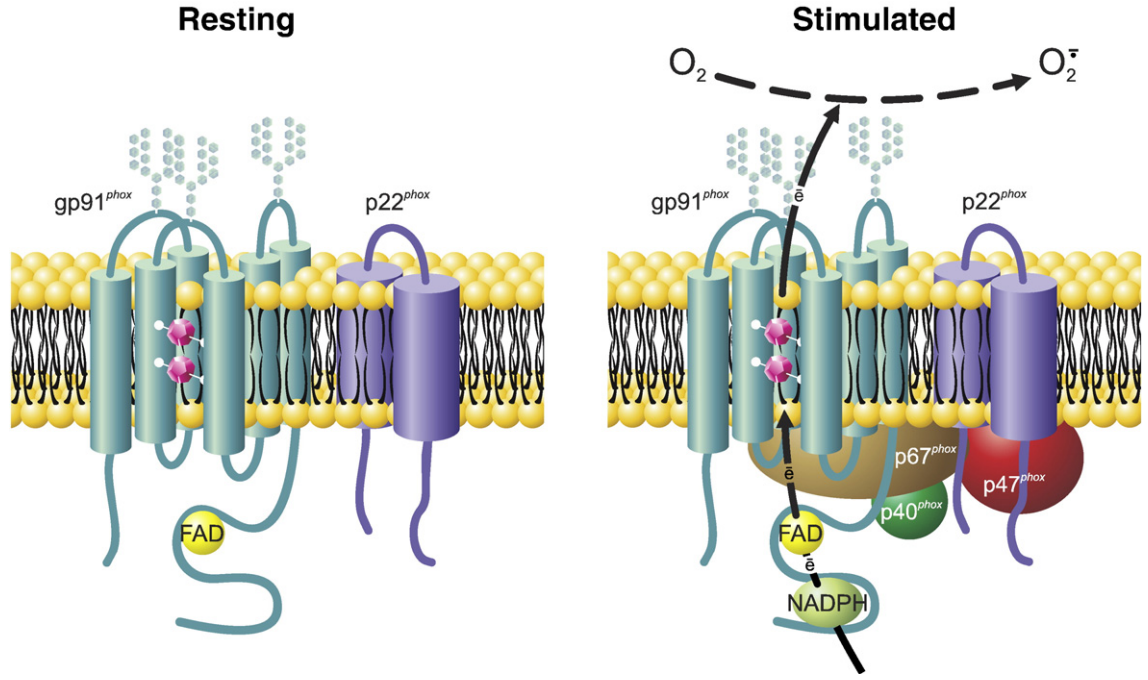


**Fig. 2.** Anatomy of the phagocyte NADPH oxidase. The membrane component of the phagocyte NADPH oxidase, flavocytochrome $b_{558}$, is a heterodimeric heme-containing flavoprotein comprised of NOX2 (aka gp91*phox*) and p22*phox* located in unstimulated neutrophils in the plasma membrane and membranes of secretory vesicles and specific granulesIn stimulated neutrophils, cytoplasmic components are recruited to the membrane (see text), and NADPH binds to NOX2 and shuttles electrons to FAD and across the membrane *via* the two inequivalent hemes (magenta dodecahedrons) linked between transmembrane helices in NOX2. Consequently, oxygen undergoes single electron reduction to form $O_2^{\bullet-}$.

oxidase activity if uncompensated. However, the action of a voltage-gated proton channel, Hvn1, compensates for >95% of the negative charge created by the translocation of electrons [29,30].

Transformation of the unassembled and inactive state to an active enzyme assembled on phagosomal or plasma membranes reflects agonist-dependent conformational changes in the cytoplasmic components that result in their translocation. Too extensive to review here, elegant studies have elucidated many features of phagocyte oxidase assembly, including conformational changes in flavocytochrome $b_{558}$ and surrounding membrane, phosphorylation of multiple sites on p47*phox*, phosphorylation of p67*phox* and p22*phox*, and contributions of the PX domains of p47*phox* and p40*phox* [31–36]. Whereas sustained activity requires a source of cytoplasmic NADPH [37], the mechanisms underlying termination of the phagocyte oxidase are not defined [38].

The initial product of the phagocyte oxidase is $O_2^{\bullet-}$, which dismutates spontaneously or *via* reactions involving myeloperoxidase [39], to yield $H_2O_2$. As much as 40% of the $O_2$ consumed by human neutrophils can be recovered as HOCl, the product of myeloperoxidase and $H_2O_2$ in the presence of chloride anion (reviewed in [40]). Methods for quantitation of HOCl and its derivatives are presented by Kettle elsewhere in this issue.

### 3.2. Beyond phagocytes, the NOX protein family

Identification of mox1 (now known as NOX1) as a homologue of human gp91$^{phox}$ by the Lambeth lab [41] heralded the recognition that rather than being a property unique to phagocytic immune cells, the phagocyte NADPH oxidase is a member of a family of NADPH-dependent oxidases, the NOX protein family. NOX protein family members are conserved throughout both plant and animal kingdoms, with many cells possessing more than one isoform [5,6,42,43]. Likewise impressive is the remarkably broad range of biological processes served by NOX proteins, including not only host defense, as best exemplified by NOX2, but also thyroid hormone synthesis, respiratory and gastrointestinal mucosal host defense, regulation of gene expression and cell growth, cell death, angiogenesis, cardiomyocyte differentiation, regulation of blood pressure, maintenance of normal pancreatic beta cell function, and maturation of sperm (reviewed in [6]).

Like the phagocyte oxidase, all NOX proteins are flavoproteins that operate as electron transferases, transporting electrons derived from cytoplasmic NADPH across a membrane to the electron acceptor oxygen (Fig. 3). However, with regard to both structural composition and organization, dependence on cofactors, and function, the non-phagocyte NOX proteins exhibit notable exceptions to the human neutrophil paradigm. The structural organization of NOX proteins has been reviewed in detail recently [2,3], although a brief overview of some key points relevant to their oxidant production merits mention in the context of this review.

With regard to the structure of the NOX protein itself, NOX5, DUOX1, and DUOX2 differ from other family members in possessing EF hands that bind calcium and regulate activation [44–47]. Duox has as an additional unique structural feature an additional transmembrane loop and extended extracellular domain at its amino terminus. The >540 amino acid extracellular sequence shares 19–20% identity at the amino acid level with myeloperoxidase, prompting its frequently being called the "peroxidase-like domain". Although the *C. elegans* DUOX protein supports peroxidase-mediated chemistry [48], the human DUOX proteins lack biochemical evidence for peroxidase activity, either as holoprotein or isolated extracellular domains [49,50]. Pertinent to the discussion regarding the oxidants released extracellularly by DUOX, the extracellular domain of DUOX lacks superoxide-dismutase activity [49].

Only NOX1, NOX3, and NOX4 share with NOX2 a required association with p22*phox*, which contributes to proper trafficking to the target membrane and provide a potential docking site for cytoplasmic oxidase components [51–54]. NOX proteins differ as well with respect to essential cytoplasmic components. Like NOX2, NOX1 and NOX3 require recruitment of cytosolic proteins that organize the multicomponent oxidase at the plasma membrane and that are part of the catalytic center of the assembled enzyme. NOX organizing protein 1 (NOXO1) and NOX activating protein 1 (NOXA1) are functional and structural homologues of p47*phox* and p67*phox*, respectively [55,56]. NOX4 maybe be negatively regulated by NOXA1 [57], and no cytoplasmic elements are required for the activity of NOX5 and DUOX. Whereas DUOXes do not require cytoplasmic proteins for activity *per se*, they do depend on maturation factors DUOXA1 and DUOXA2 for proper targeting to membranes during biosynthesis of DUOX1 and DUOX2, respectively [58].

In contrast to the phagocyte oxidase, NOX4, NOX5, and DUOX are constitutively active. With respect to the oxidants generated by NOX proteins, nonphagocyte NOX proteins NOX1, NOX3 and NOX5 produce $O_2^{\bullet-}$ (reviewed in [6]). However, oxidant production by NOX4 and DUOX presents incompletely understood behavior. In the case of NOX4, transfected 293 HEK cells, COS-7 cells, or renal tubular cells produce $H_2O_2$ extracellularly [59–63] and no detectable extracellular $O_2^{\bullet-}$, except from mesangial cells [64,65]. Mutations in the first intracellular loop (B loop) eliminate activity, whereas those in the terminal extracellular loop (E loop) reduce $H_2O_2$ and increase $O_2^{\bullet-}$ detected extracellularly [61]. Data from studies of intracellular oxidant production by NOX4 have been interpreted as demonstrating $O_2^{\bullet-}$ production

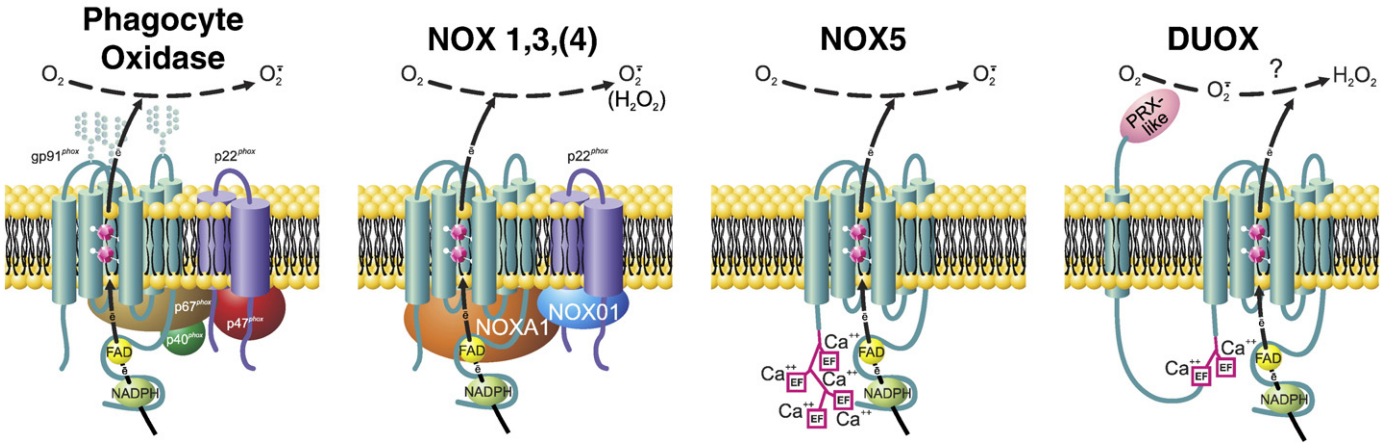


**Fig. 3.** Composition of activated NOX protein family members in membranes. Individual NOX proteins vary in their requirements for associated p22*phox*, cytosolic cofactors, and calcium-binding EF hands. In addition, DUOX1 and 2 have an extracellular peroxidase-like (PRX-like) domain and associated transmembrane helix not present in other NOX protein family members. Whereas the phagocyte oxidase (NOX2) and NOX1, 3, and 5 produce $O_2^{\bullet-}$ extracellularly, only $H_2O_2$ is recovered extracellularly from NOX4, DUOX1 or DUOX (see text for details).

[61,63–70], but the assays used lack specificity for measurement of $O_2^{\bullet-}$ (see later). However, Serrander et al. reported intracellular reduction of nitroblue tetrazolium (NBT) in 293 HEK cells expressing an inducible NOX4 construct without any evidence for $O_2^{\bullet-}$ detected in the cytoplasm [62]. Similarly, NOX4-dependent NBT reduction has been detected in airway smooth muscle cells exposed to TGF-β [71]. It is plausible that $O_2^{\bullet-}$ production in those transfectants occurs in a membrane-bound cytoplasmic compartment that contains NBT but is inaccessible to the other probes used. Subcellular localization of many of the nonphagocyte NOX proteins has been challenging because of the paucity of reliable and validated antibodies, a shortcoming that adds to the ambiguity and uncertainty engendered by limitations of probes to measure intracellular oxidants.

As noted for NOX4, $H_2O_2$ is the oxidant detected extracellularly by cells expressing transfected DUOX or by cells expressing endogenous DUOX (*e.g.* thyroid, airway or colonic epithelium, and lung cancer cell lines) [50,57,72–75]. Heterodimer formation, proper targeting, and extracellular $H_2O_2$ production depend on the integrity of the associated DUOX (*i.e.* DUOXA1 with DUOX1 and DUOXA2 with DUOX2) [50,74,76]. When DUOX is trapped intracellularly, oxidant production is disrupted, with studies suggesting that $O_2^{\bullet-}$ rather than $H_2O_2$ is produced and leaked extracellularly [50]. A comprehensive study employing chimeric constructs of DUOXA1 and DUOXA2 expressed in COS-7 cells to identify structural determinants of oxidant production in the DUOX system [76] suggests that the second intracellular loop and cytoplasmic terminus of DUOXA1 are responsible for $H_2O_2$ production by DUOX1, whereas the amino terminus of DUOXA2 dictates oxidant production by DUOX2; wild-type DUOXA2 supports $H_2O_2$ production and amino terminal mutants result in $O_2^{\bullet-}$ by DUOX2. As with the analysis of intracellular $O_2^{\bullet-}$ by NOX4, the shortcomings inherent in the probes used to detect $O_2^{\bullet-}$ undermine confidence that structure–function relationships between NOX4 and DUOX and their products are fully understood.

## 4. Monitoring activities of cellular NADPH oxidases

Both the specific activity of the phagocyte NADPH oxidase and the ease with which large numbers of normal neutrophils can be obtained make detailed analysis and quantitative examination of the phagocyte NADPH oxidase possible. One can quantitate activity of the phagocyte NADPH oxidase by measuring hexose monophosphate shunt activity (source of NADPH and electrons driving the oxidase), consumption of the electron recipient $O_2$, or products released into the extracellular space. However, quantitation of products generated within phagosomes or intracellularly by nonphagocyte NOX proteins is challenging. The merits and shortcomings of currently available probes have been comprehensively presented recently in several excellent reviews (see [9–15]).

### 4.1. Oxygen consumption

Measuring oxygen consumption is free of competing reactions or confounding influences caused by intermediate reporter substrates or their associated activity. In addition, $O_2$ consumption reflects oxidase activity whether oxidants are generated intra- or extracellularly. Cells suspended in buffer are placed in the sample chamber (37 °C) of a Clark electrode, and the decrease in current is monitored continuously after addition of buffer or agonist. Current flow is directly proportional to the concentration of dissolved oxygen equilibrating across the electrode membrane. Since the system is calibrated with each run, the concentration of dissolved oxygen can be determined by the current at any given time, thereby providing a continuous measurement of oxygen consumption.

Measurement of oxygen consumption with a traditional Clark electrode is not without its shortcomings. Inherently, the response of the Clark electrode is sluggish, as the oxygen concentration in the cell suspension needs to equilibrate across the probe membrane with the electrolyte solution, the compartment in which the oxygen content is directly measured. Vigorous mixing of the cell suspension promotes rapid equilibration and optimal measurements, and the standard Clark electrode requires relatively large numbers of cells in suspension and a robust cellular response, as achieved by neutrophils but perhaps not when cells expressing isoforms of the nonphagocyte oxidase are studied. In principle, NOX protein activity in non-phagocytes should be amenable to study by quantitating oxygen consumption by stimulated cells. However, the level of NADPH oxidase activity in many cells of interest is lower than that in neutrophils, and mitochondria contribute to the overall cellular oxygen consumption to a much greater extent in non-phagocytes than they do in neutrophils. However, microplate-based respirometry can be performed with adherent cells, providing measurements that mirror those obtained with a Clark electrode [77,78]. Although not yet applied specifically to the study of NOX proteins, this technology has been used to measure mitochondrial oxygen consumption by stimulated murine embryonal fibroblasts [79] and murine macrophages [80], and should be easily adapted to the study of NADPH oxidase activity in adherent cells, both phagocytic and non-phagocytic.

### 4.2. Extracellular $O_2^{\bullet-}$ and $H_2O_2$

Extracellular $O_2^{\bullet-}$ and $H_2O_2$ can be quantified precisely in several ways. For measurement of extracellular $O_2^{\bullet-}$, we favor using superoxide dismutase-inhibitable reduction of ferricytochrome C, as initially applied to human neutrophils by the Babior laboratory [81] and used as well to quantitate extracellular superoxide production by nonphagocyte NOX proteins (*e.g.* [50,53,59–62,64,65,82]). The $O_2^{\bullet-}$ released extracellularly reduces ferricytochrome C to ferrocytochrome C with 1:1 stoichiometry, the amount of reduced ferricytochrome C can be measured spectrophotometrically at 550 nm. For every experimental condition, an identical sample that also contains superoxide dismutase (SOD) must be assayed in parallel. Using the millimolar extinction coefficient at 550 nm for the difference between reduced and oxidized ferricytochrome C (21.1 $mM^{-1}$ $cm^{-1}$ [83]), nmoles of $O_2^{\bullet-}$ produced can be calculated from the difference in absorbances in the absence and presence of SOD. The very sharp peak of reduced ferricytochrome C at 550 nm requires special attention. Unless using a spectrophotometer with a monochromator that is precisely tuned to the correct wavelength or an instrument with a very narrow bandpass filter will underestimate the amount of $O_2^{\bullet-}$ detected. It is essential to perform the SOD-containing samples in parallel for all conditions, as other biological substances may reduce ferricytochrome C, including ascorbate, glutathione, and several reductases, a confounding issue particularly problematic with broken cell systems. Although reoxidation of ferrocytochrome C back to ferricytochrome C by $H_2O_2$ is possible and does not occur to a significant extent in most settings, the addition of catalase will eliminate the activity if problematic. The SOD-inhibitable reduction of ferricytochrome C can be used in either continuous [84] or discontinuous [81] assays, and can be adapted to a microtiter plate format [85]. Use of the latter requires adjustment for the light path in the calculation of the amount of $O_2^{\bullet-}$, in accordance with Beer's law; whereas the light path is 1 cm in the standard spectrophotometric cuvette format, it is 0.6 cm for the microtiter plate assay. Others probes can be used to detect $O_2^{\bullet-}$ using the operational definition of SOD-inhibitable response. Chemiluminescence-based probes are discussed later, but extracellular $O_2^{\bullet-}$ production can be measured spectrophotometrically as SOD-inhibitable reduction of WST-1, a cell impermeable sulfonated tetrazolium salt [86]. Its greater reactivity in comparison with ferricytochrome C may reflect signal amplification secondary to the contribution of intermediate radicals rather than greater sensitivity to $O_2^{\bullet-}$. Hydropropidine, a membrane impermeant fluorogenic probe derived from propidium iodide, has recently been employed to detect extracellular $O_2^{\bullet-}$ by stimulated murine macrophages [87]. Use of

SOD-inhibitable fluorescence of hydropropidine compares favorably with $O_2^{\bullet-}$ detection using ferricytochrome C and merits further study.

Extracellular $H_2O_2$ can be quantitated in two general ways. Using a hydrogen peroxide electrode, $H_2O_2$ in solution can be measured polarigraphically, using the same principles as were discussed earlier with regard to measuring oxygen consumption. The sensitivity of the electrode allows precise and rapid measurement of extracellular $H_2O_2$ with very little consumption of the $H_2O_2$ and without the use of targets to react with or capture the $H_2O_2$ [88]. Cells are suspended in a chamber maintained at 37 °C with continuous stirring and the change in current in solution, reflecting changes in $H_2O_2$ concentration, is monitored continuously. Alternatively, one can exploit the ability of $H_2O_2$ to oxidize susceptible probes, including scopoletin [89], homovanillic acid (HVA) [90], phenol red [91,92] or Amplex red (N-acetyl-3,7-dihydroxyphenoxazine) [93], in the presence of horseradish peroxidase (HRP). Whereas the fluorescence of scopoletin decreases after oxidation, oxidized products of HVA, phenol red, and Amplex all increase, thereby providing a more sensitive assay. Developed initially to measure NOX2 activity, all four probes have been used to measure extracellular $H_2O_2$ production by NOX4 [59,60,62] and DUOX [50,57,72–74,76]. Note that accurate detection of $H_2O_2$ by these assays requires addition of SOD, because $O_2^{\bullet-}$ reacts with the probe, resulting in underestimation of $H_2O_2$ produced [94].

The oxidation of Amplex red is irreversible, has a stoichiometry of 1:1 [93], and is sensitive to low levels of $H_2O_2$, with a 5 pmol lower limit of detection and 30 pmol lower limit of quantitation [13,95]. In a 100 μl reaction volume in a microtiter plate format, 5 pmol of $H_2O_2$ from $2 \times 10^3$ phorbol myristate acetate-stimulated neutrophils can be reliably quantitated [93]. Using absorbance readings from experimental samples and a standard curve of defined concentrations of $H_2O_2$ in the presence of Amplex red and HRP, the amount of $H_2O_2$ generated extracellularly can be calculated.

In many ways, $H_2O_2$ detection using HRP and Amplex red mirrors $O_2^{\bullet-}$ measurements using SOD-inhibitable reduction of ferricytochrome C as a method to quantitate extracellular products of the NADPH oxidase. However, one important difference in use of the two assays needs to be kept in mind if using a broken cell system to study any of the NOX family members. The broken cell system is an experimental approach that combines enriched, purified, or recombinant subcellular oxidase components in the presence of NADPH and an amphiphile to recapitulate the activity of the NADPH oxidase of phagocytes ([96–100] and reviewed [101]). Its development and application to the study of phagocytes made possible the identification of cytosolic oxidase components [102–105] and the dissection of many other features of the phagocyte NADPH oxidase. Whereas the ferricytochrome C assay can be used to measure $O_2^{\bullet-}$ production by the broken cell system, NADPH and other reduced pyridine nucleotides can directly oxidize Amplex Red in an HRP-dependent fashion [106]. Although the reaction of NADPH and Amplex Red is slow and limited [95], caution should be exercised when interpreting data from studies utilizing this probe in broken cell NADPH oxidase systems. Use of lower concentrations of $NADP^+$ and inclusion of an NADPH-generating system can minimize this problem (personal communication).

### 4.3. Intracellular $O_2^{\bullet-}$ and $H_2O_2$

Intracellular oxidants produced by NOX proteins have been associated with a wide range of biological process, both in phagocytes and nonphagocytic cells, although the specific compartments in which oxidant generation occurs differ in these two cell types. Nearly all the oxidant generation is within phagosomes when neutrophils ingest opsonized particles at low particle-to-neutrophil ratios (1:1 to 5:1) in the absence of agents that poison the cytoskeleton (*e.g.* cytochalasins). Commercially available redox-sensitive probes that are coupled to carrier substrates are frequently used to monitor intraphagosomal oxidant production following phagocytosis. For example, Oxyburst Green (Molecular Probes) represents a complex of dihydrofluorescein ($DCFH_2$), bovine serum albumin (BSA), and rabbit antibody to BSA that engages the phagocyte Fc receptor, thereby accessing the phagosome when particles are ingested. Although such oxidation-sensitive conjugates can detect events in neutrophil phagosomes that are dependent on the NADPH oxidase and myeloperoxidase (MPO), the biochemistry in the phagosomes of human neutrophils is remarkably complex and the identity of the reactive species generated therein incomplete (reviewed [40,107,108]). Furthermore, there are significant shortcomings to using $DCFH_2$ to detect products of NADPH oxidases (see below). No probes that are currently available allow precise or specific quantitation of $O_2^{\bullet-}$ or $H_2O_2$ generated within phagosomes.

Oxidants generated by nonphagocyte oxidases in endosomes have been implicated in a variety of signaling cascades. Consequently, there is a need to access intracellular compartments in order to quantitate oxidants generated therein. Substrates used to detect oxidants within intracellular compartments include both oxidant-sensitive and non-redox probes.

#### 4.3.1. Oxidant-sensitive probes

*4.3.1.1. Chemiluminescence.* Stimulated neutrophils emit light in a fashion that requires an intact NADPH oxidase and enzymatically active MPO for maximum chemiluminescence [109–111]. Many different luminescent substrates have been used to amplify the light emitted from stimulated neutrophils, including lucigenin (bis-N-methyl acridinium nitrate), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), and isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) in order to improve detection. For example, use of luminol allows detection of responses from $2 \times 10^4$ stimulated neutrophils [112], thus providing a valuable tool in situations when neutrophils are scarce, as when studying cells from children or patients with neutropenia. The cell-permeable agents luminol and lucigenin are commonly used to detect oxidant production both intracellularly and extracellularly, whereas isoluminol, which does not penetrate into cells, can serve to probe the extracellular space (reviewed in [113]). The addition of HRP makes isoluminol-enhanced chemiluminescence by stimulated neutrophils solely dependent on the activity of the NADPH oxidase and able to detect responses of as few as 250 cells [114]. Because neutrophils require a functional NADPH oxidase and active MPO to emit optimal luminol-enhanced chemiluminescence [111,115], abnormalities in either the oxidase (*e.g.* chronic granulomatous disease) or MPO (*e.g.* MPO deficiency) can result in abnormal neutrophil responses when luminol is used. Lucigenin-enhanced chemiluminescence is not dependent on MPO [110].

Lucigenin, luminol, and related or enhanced substrates, including Diogenes (National Diagnostics) and L-012 [116] have been employed in a variety of settings to measure $O_2^{\bullet-}$ derived from non-phagocyte NOX proteins, both extracellularly as SOD-inhibitable responses and intracellularly [50,59,63,69,70,76]. Despite their capacity to detect and amplify signals associated with activation of the NADPH oxidase, the use of chemiluminescent probes to monitor or measure $O_2^{\bullet-}$ or $H_2O_2$ has serious limitations that undermine use as analytical tools. The chemistry underlying the emission of luminescence in the presence of oxidants is complicated and incompletely unraveled. Because of uncertainties about the identify of reactive species detected and concerns about the inherent behavior of substrates used to amplify the chemiluminescence (see Winterbourn elsewhere in this issue), many with expertise in free radical biology are skeptical about the use of these probes (reviewed in [10,11,14,15,110,117–119]). None of these probes is specific, selective, or quantifiable and all have significant shortcomings. For example, lucigenin interacts with a variety of nucleophiles and reducing agents that can generate the single electron product of lucigenin reduction ($LC^{\bullet+}$), which in turn generates $O_2^{\bullet-}$

[10,117,118] and produces luminescence. After detailed examination of its chemistry (review [120]), luminol fares no better than lucigenin as a probe for $O_2^{\bullet-}$ and hydrogen peroxide production by NADPH oxidases. In biological systems, the multiplicity of interfering substrates in intracellular compartments is too great to allow luminol to serve as a specific probe for $O_2^{\bullet-}$ or $H_2O_2$ production by stimulated neutrophils or in broken cell superoxide generating systems derived from neutrophils [121,122]. However, luminol-enhanced chemiluminescence may yield useful information. Detection of products of the NADPH oxidase released extracellularly can be monitored as isoluminol-enhanced chemiluminescence, which requires $O_2^{\bullet-}$ production [114]. However, neither the specific reactive species responsible nor the stoichiometry of the reactions with isoluminol is known, leaving the assay of limited utility. Luciferin (coelenterazine) derivatives, including the methoxy derivative MCLA, show promise as a substrate for $O_2^{\bullet-}$ detection [123,124]. In contrast to lucigenin, MCLA does not undergo redox cycling [125]. However, it is cell impermeable, thus limiting application of SOD-inhibitable MCLA chemiluminescence to the detection of extracellular $O_2^{\bullet-}$.

*4.3.1.2. Redox responsive fluorescent probes.* In addition to the luminescent probes, substrates that become fluorescent when oxidized, just as discussed earlier with using Amplex red, are widely used to monitor oxidant production (reviewed in [9–12,14,15,126–130]). Most commonly used among these redox-sensitive fluorescent probes are dihydrofluorescein ($DCFH_2$), dihydrorhodamine ($DH_2R$), and hydroethidine (HE). Because cells are loaded with a cell-permeable form of the probe, the efficiency of uptake and the rate of leakage of the reporter become additional variables to consider when using these probes to compare cellular responses under different experimental conditions.

$DCFH_2$ reacts very slowly with $O_2^{\bullet-}$ and $H_2O_2$ [10] and inefficiently with HOCl [131] but can be oxidized by $H_2O_2$ in the presence of catalysts, including metals (*e.g.* iron and copper), peroxidases, and species with peroxidase-like activity (*e.g.* catalase, hemoglobin, myoglobin, and superoxide dismutase) [10]. Thus, the susceptibility of $DCFH_2$ to oxidation by constituents in cytoplasm can obfuscate interpretation of data from intracellular measurements. For example, DCF fluorescence during neural cell death was initially interpreted as evidence linking reactive oxidant production with apoptosis [132]. However, cytochrome c released from mitochondria during apoptosis [133,134] can directly oxidize $DCFH_2$ [135], providing a mechanism for increasing DCF fluorescence independent of oxidant production. In similar ways, competing reactions between $DCFH_2$ and cytoplasmic contents have proved troublesome when using this probe to monitor oxidant production in cardiovascular tissue (reviewed in [13,14]).

DHR shares many of the properties of $DCFH_2$, most notably the lack of reactivity with $O_2^{\bullet-}$ and $H_2O_2$ in the absence of a catalyst [127,131]. Both HOCl and chloramines can oxidize DHR, and the latter reaction is particularly efficient in the presence of iodide [136]. Consequently, DHR has a special application in studies of neutrophil function. As neutrophils from patients with CGD fail to promote DHR oxidation [137], flow cytometry of DHR oxidation by stimulated human neutrophils can be used to screen subjects for the presence of CGD [138]. The assay can be performed on as little as 400 μl of anticoagulated whole blood, making it very useful for evaluating the small volumes of blood often available from very young children. The assay can identify both affected patients and carriers of X-linked disease, as the latter have two populations of circulating cells, normal and defective. It is critical to recognize that DHR oxidation in this assay depends on the activity of endogenous MPO [139]. Consequently, neutrophils from patients with complete MPO deficiency will have abnormal DHR oxidation that resembles that of patients with CGD [140]. Neutrophils from subjects with partial MPO deficiency have sufficient peroxidase activity to support oxidation of DHR and appear as normal neutrophils in flow cytometry.

Interest in delineating the contribution of NOX proteins to signaling provides a strong incentive for identifying and quantitating intracellular superoxide and $H_2O_2$ in nonphagocytic cells. The redox probe hydroethidine (HE) has several properties distinct from those of $DCFH_2$ and DHR that make it suitable for use as a quantitative analytical tool. In contrast to lucigenin, luminol, and $DCH_2F$, there are no known reactions between the product of HE and $O_2^{\bullet-}$ with oxygen, thereby eliminating a mechanism for spontaneous $O_2^{\bullet-}$ generation [94]. $O_2^{\bullet-}$ reacts more readily with HE than with $DCFH_2$ and DHR, with a rate of ~$2 \times 10^6$ $M^{-1}$ $s^{-1}$ [129], and generates a specific product, 2-hydroxythidium (2-OH-$E^+$), that can be quantitated [128,141,142]. Like other radical probes, HE can produce background fluorescence secondary to amine oxidation by air and light. Deuteration at the α-amine carbon–hydrogen bond appears to alleviate this problem [143], although the modified radical oxidant probes are not currently commercially available.

HE can also react with $H_2O_2$ in the presence of peroxidases [144] or nonenzymatically with non-peroxidase heme proteins (*e.g.* cytochromes, hemoglobin, and myoglobin [145]), thereby competing for $O_2^{\bullet-}$ and complicating quantitation of $O_2^{\bullet-}$ by HE fluorescence alone. Although such reactions undermine the application of confocal fluorescence microscopy to monitor specifically $O_2^{\bullet-}$ product as changes in HE fluorescence [130,146], methods have been developed to recover and quantitate 2-OH-$E^+$ in lysates or stimulated cells. Because ethidium ($E^+$), the two-electron oxidation product of HE unrelated to $O_2^{\bullet-}$ generation [141], and 2-OH-$E^+$ have overlapping spectra, HPLC analysis and quantitation are requisites for the selective determination of $O_2^{\bullet-}$ -generated products [128,147]. It is important to recognize that HE will be competing with intracellular proteins, such as SOD, that can interact readily with $O_2^{\bullet-}$, likely resulting in 2-OH-$E^+$ quantitation underestimating the amount of $O_2^{\bullet-}$ produced *in situ* [147]. For this and other theoretical concerns [147], it is best to consider 2-OH-$E^+$ measurement as a semiquantitative determination of intracellular $O_2^{\bullet-}$ production. However, results using this assay parallel those obtained when $O_2^{\bullet-}$ is detected as SOD-inhibitable reduction of ferricytochrome C, as clearly demonstrated in studies of endothelial cells in intact vascular tissue [142]. Furthermore, 2-OH-$E^+$ is stable, allowing samples to be stored frozen and analyzed at a later time [13]. Thus, the detection and quantitation of 2-OH-$E^+$ by HPLC provide a specific and selective means to compare relative $O_2^{\bullet-}$ levels in cells under varied experimental conditions.

*4.3.2. Non-redox probes*

With the exception of electrodes to monitor oxygen consumption and hydrogen peroxide production, the probes discussed thus far depend on their redox properties for use as detectors of $O_2^{\bullet-}$ and $H_2O_2$. Whereas modification of redox-sensitive probes by oxidants depends on catalysts such as HRP, generation of radicals as intermediates, and oxidation of the reporter molecule, non-redox probes exploit very different properties.

*4.3.2.1. Boronate-based probes.* In addition to these redox-sensitive probes, small molecules containing fluorophores and a boronate ester have been used to detect $H_2O_2$ in biological systems, exploiting the reactivity of $H_2O_2$ with arylboronates to generate phenols [148]. The strategy underlying the design of these probes rests on positioning the boronic ester coupled to a fluorophores in such a conformation that the structure is a colorless, non-fluorescent lactone until it reacts with $H_2O_2$, which transforms the arylboronate to a phenol and generate a fluorescent product [149]. Modifications in the specific fluorescent probes included in the boronate structure provide a variety of reporting molecules from which to choose for specific applications [150]. A variety of boronate-based, water-soluble fluorochromes have been generated and applied to detect $H_2O_2$ at micromolar concentrations [151] as well as NO and $H_2S$ [152], both in standard experimental settings as well as high throughput assays

(reviewed in [150,151,153–155]). However, some of the probes react slowly with $H_2O_2$ (k ~ 1 to 2 $M^{-1}$ $s^{-1}$) [156] compared to their reactivity with $ONOO^-$ or HOCl, oxidant species relevant in the context of biological systems with NADPH oxidases [120,151,153,157]. For example, reactivity of some boronates with HOCl is 1000-fold faster than that with $H_2O_2$, with both oxidants producing the same fluorescent product [122]. Furthermore, the probes do not provide quantitative data, although new approaches measuring spectral ratios by mass spectrometry are promising, as recently applied to measuring $H_2O_2$ from mitochondria in living *Drosophila* [120].

The Chang lab has extended use of the boronate derivatives to include caged luciferin, Peroxy Caged Luciferin-1 (PCL-1) to detect and image $H_2O_2$ in the context of acute inflammation in response to endotoxin challenge [158]. As presented earlier, $H_2O_2$ oxidizes an aryl boronic acid to phenol, but the result with PCL-1 is the release of part of luciferin. D-cysteine, the other half of luciferin, is released in response to a caspase 8 activity probe, thereby providing a sensitive method to monitor endotoxin-elicited oxidant production. Applications to study cell *ex vivo* as well as intact animals hold special promise for such tools.

*4.3.2.2. Genetically based reporters.* Challenges facing the utility of the boronate-based probes, namely selectivity in distinguishing between reactive oxygen and reactive nitrogen species, and reversibility are addressed in part by genetically encoded probes (review [15,126]). Probes such as roGFP use site-directed mutants of GFP that undergo a conformational change when oxidants promote disulfide bond formation in the chromophore [159]. However, roGFP is not selective for $H_2O_2$ and, like many of these probes, more accurately reflects the redox state of the cellular compartment in which it resides than it does the presence or amount of a specific oxidant. A variation on fluorescent redox-sensitive probes such as roGFP is HyPer, a fluorescent probe that is sensitive, reversible, and selective for $H_2O_2$ [160]. HyPer exploits the properties of oxyR, a peroxide sensor that responds to local changes in $H_2O_2$ concentration to initiate transcription of antioxidant genes in *Escherichia coli* [161]. A circularly permutated form of the fluorescent protein YFP (cpYFP) was introduced into the regulatory domain of oxyR, such that oxidation of critical cysteines in cpYFP results in conformational changes that alter its two excitation maxima in opposing directions; the excitation at 420 nm decreases while that at 500 nm increases, thus enabling HyPer to function as a ratiometric probe [160]. Although sensitive to pH, HyPer oxidation-dependent changes in fluorescence are reversible and are selective for $H_2O_2$, as the probe is unaffected by $O_2^{\bullet-}$, NO, ONOO, or oxidized glutathione [160]. As an example of an application of this methodology to the study the generation of intracellular oxidants by non-phagocyte NOX proteins, investigators used HyPer-ER, a modified reporter that targets endoplasmic reticulum (ER), to examine $H_2O_2$ production mediated by NOX4 in the ER of human umbilical vein endothelial cells [162].

Concerned that the sensitivity of HyPer to pH limits reliable detection of oxidants in intracellular compartments in which pH would change during cell activation, the Geiszt group developed two novel, genetically-encoded probes that utilize fluorescence resonance energy transfer (FRET) to report the presence and changes in intracellular $H_2O_2$ [163]. The sensor utilizes two redox-sensitive proteins from *Saccharomyces cerevisiae*, oxidant receptor 1 (Orp1) and yeast activator protein 1 (Yap1), that together constitute a two-component $H_2O_2$-sensing system; the peroxidase Orp1 catalyzes oxidation of the transcription factor Yap1 in the presence of $H_2O_2$, thereby providing the yeast with the capacity to respond transcriptionally to oxidant stress [164]. The Geiszt lab engineered two fusion proteins, using variants of Cerulean and Venus fluorescent proteins and domains of Orp1 and Yap1, to create constructs that exhibit inverse FRET when oxidized. Concurrent determination of ratiometric FRET measurements and use of confocal fluorescence microscopy permit both qualitative detection of $H_2O_2$ production as well as identification of the cellular compartment from which it originates. The authors applied the method to detect $H_2O_2$ intracellularly after NOX2 and DUOX1 activation as well as $H_2O_2$ that diffused into cells from an extracellular source, as would be relevant to paracrine signaling by oxidants [165]. This approach shows promise for spatial and temporal localization of $H_2O_2$ within cells, although it is not a quantitative assay.

*4.3.2.3. Other probes.* Probes based on deprotection of fluorescein derivatives have been developed to measure $O_2^{\bullet-}$ [166]. The bis(2,4-dinitrobenzenesulfonyl) fluoresceins increase fluorescence in the presence of $O_2^{\bullet-}$ generated by xanthine–xanthine oxidase or by stimulated neutrophils. However, unstimulated neutrophils as well as glutathione alone reduce the probe, both undesirable features. Additional modification of this family of protected fluorescein derivatives will be needed before they can be applied to study NOX protein activity.

Nanotechnology has been applied to measuring intracellular reactive oxygen species in biological systems, using fluorescein conjugates immobilized on gold nanoprobes. For example, hyaluronic acid that has been modified to resist reduction by glutathione can be immobilized to gold nanoprobes and introduced into cells to monitor intracellular production of reactive oxygen species [167,168], exploiting oxidant-dependent degradation of hyaluronic acid [169]. As sensitive as DCF, the gold nanoprobes were used to measure intracellular oxidant generation by macrophages challenged with nanogram amounts of endotoxin [167]. However, details of the specific oxidants detected ($O_2^{\bullet-}$, OH, $H_2O_2$, $ONOO^-$, or $^-OCl$) in biological settings await further definition.

## 5. Summary

Cellular NADPH oxidases participate in a wide range of important biological processes. Furthermore, they are distributed in nearly all cell types and tissues, and many cells express more than one NOX protein isoform. Together, their wide distribution and scientific importance have stimulated interest in the detection and quantitation of products of stimulated NOX proteins in biological systems. However, the quality of the available analytical tools to measure NOX protein products does not match the enthusiasm for their study. Approaches to quantitate oxygen consumption, extracellular release of $O_2^{\bullet-}$ (using SOD-inhibitable reduction of ferricytochrome C) or $H_2O_2$ (using HRP plus Amplex Red), and intracellular $O_2^{\bullet-}$ production (using HPLC to quantitate 2-OH-$E^+$) provide reliable assessment of NADPH oxidase activity in a given population of cells. Spatial and temporal localization in individual cells or tissue depends on probes that often lack specificity, selectivity, or the potential for precise quantitation. However, their use in conjunction with other probes and their interpretation with the recognition of their shortcomings can provide valuable qualitative insights.

It is certain that probes better able to provide quantitation as well as localization of intracellular $O_2^{\bullet-}$ and $H_2O_2$ will be available in the future. Until that time, if limited to the use of fluorescent or luminescent reporters, it is best to employ more than one method to assess NOX protein activity, where the underlying principles of the assay differ and to complement measurements with the use of well-characterized pharmacologic inhibitors or genetic approaches that target the specific NOX protein of interest. Until we have probes that precisely meet our needs, we must rely on sound understanding of the principles and topology of NOX proteins, rigorous experimental design, and careful interpretation of results to unravel the contributions of cellular NADPH oxidase to biology.

## Acknowledgement

I thank Dave Lambeth for informative and helpful suggestions during the review process and both Dave and Yukio Nishimoto for the tip on employing Amplex Red in the broken cell assay system. Work in

the Nauseef laboratory is supported by funding from the National Institute of Health grants AI70958 and AI044642, and a Merit Review grant from the Veterans Administration.

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