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Review article

# Toward selective detection of reactive oxygen and nitrogen species with the use of fluorogenic probes – Limitations, progress, and perspectives

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ABSTRACT

Over the last 40 years, there has been tremendous progress in understanding the biological reactions of reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is widely accepted that the generation of ROS and RNS is involved in physiological and pathophysiological processes. To understand the role of ROS and RNS in a variety of pathologies, the specific detection of ROS and RNS is fundamental. Unfortunately, the intracellular detection and quantitation of ROS and RNS remains a challenge. In this short review, we have focused on the mechanistic and quantitative aspects of their detection with the use of selected fluorogenic probes. The challenges, limitations and perspectives of these methods are discussed.


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## Introduction

It has been more than 40 years since McCord and Fridovich [1] discovered that erythrocuprein, known as superoxide dismutase (SOD), catalyzes the dismutation of the superoxide radical anion ($O_2^{\bullet -}$). This discovery attracted the attention of the scientific community to the role of reactive molecular oxygen metabolites in biological processes. The group of reactive oxygen species (ROS) and reactive nitrogen species (RNS) consists of hydrogen peroxide ($H_2O_2$), protein and lipid peroxides (ROOH), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and radical oxidants, including

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$O_2^{\bullet-}$, hydroxyl $^{\bullet}OH$ ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and thiyl radicals ($RS^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and carbonate radical anion ($CO_3^{\bullet-}$).

Since the discovery of SOD, tremendous progress in understanding the biological reactions of ROS and RNS and their physiological significance has been made, yet their intracellular detection and quantitation remain a challenge [2–4]. Several ROS-sensitive probes producing easily detectable and relatively stable products have been developed. In addition to spin trapping techniques [5,6], luminescent probes have also become widely used tools in the studies on oxidative stress.

A variety of small molecule fluorescent probes are available for detecting ROS and RNS in cells. Their rational use requires a deep understanding of the mechanism of their action. In most cases, the probe is oxidized to the corresponding fluorescent product. Determination of the reactivity pattern of the primary products of this process (*i.e.*, probe-derived radicals) is of great importance for understanding of reaction mechanisms and proper interpretation of experimental data [2].

In this article, we discuss the mechanisms of oxidative transformation of selected fluorogenic probes: hydroethidine (detection of $O_2^{\bullet-}$), Amplex® Red (detection of $H_2O_2$), and boronate-based fluorogenic probes (detection of peroxynitrite and hydroperoxides).

## Hydroethidine and the detection of superoxide radical anion

Superoxide radical anion is formed by the process of one-electron reduction of molecular oxygen. It can be produced *in vivo* by a number of oxidases (NADPH oxidases family [7] and xanthine oxidase [8]), by the mitochondrial electron transport chain [9], or by redox cycling agents (*e.g.*, paraquat and menadione) in the presence of electron donors [10,11]. In a protic environment, $O_2^{\bullet-}$ undergoes spontaneous ($k = 2 \times 10^5\ M^{-1}\ s^{-1}$) [12] or SOD-catalyzed dismutation ($k = 1.6 \times 10^9\ M^{-1}\ s^{-1}$) [13] to form $O_2$ and $H_2O_2$. Superoxide radical anion is the primary ROS produced *in vivo* and a precursor of $H_2O_2$.

Two major groups of probes are used for the detection of $O_2^{\bullet-}$. The first group comprises chemiluminescent probes (*e.g.*, lucigenin, luminol and its derivative, L-012, Fig. 1A) that react with ROS to form a product in the excited state, which relaxes to ground state with the emission of photons (Fig. 1B and C). Chemiluminescent probes have been widely utilized due to their high sensitivity. The mechanism of probes oxidation involves several intermediates, including probes-derived radicals. The major limitation of their use is that the radical intermediates react with $O_2$ leading to the formation of $O_2^{\bullet-}$ (Fig. 1B and C) [2,14–16]. This reactivity should be considered when interpreting chemiluminescence data. Moreover, those probes do not react directly, or react slowly with superoxide, while other biologically relevant oxidants (or reductants) can react with them, producing radical intermediates, which further complicate the system. The second major group of probes consists of fluorogenic probes: dichlorodihydrofluorescein (DCFH), dihydrorhodamine (DHR) (Fig. 2A), hydrocyanines (Fig. 2B), and hydroethidine (HE) along with its analogs hydropropidine (HPr) and MitoSOX™ Red (Fig. 2C). In the case of dichlorodihydrofluorescein and dihydrorhodamine, one has to keep in mind that the radical intermediates formed upon one-electron oxidation react rapidly with oxygen to generate $O_2^{\bullet-}$ (Fig. 3A and B) [17,18].

Hydrocyanines, proposed recently as a tool for the imaging of ROS and $O_2^{\bullet-}$ production [19], still await better characterization of their specificity and chemical reactivity toward biologically relevant oxidants.

Hydroethidine is a cell-permeable probe that reacts with superoxide to form a unique marker product, 2-hydroxyethidium (2-OH-$E^+$), whereas another red fluorescent product, ethidium ($E^+$), is formed in the reaction with other cellular oxidants (Fig. 4) [20]. HE has been used for the detection of $O_2^{\bullet-}$ in a variety of biological systems, ranging from intracellular organelles to whole organs in live animals [21,22]. The reaction of HE and superoxide involves a radical mechanism. In the first step, hydroethidine reacts with hydroperoxyl radical $HO_2^{\bullet}$ or other one-electron oxidants to form a HE radical cation. Because the oxidation of HE by superoxide at pH 7.4 is rather slow ($k = (6.2 \pm 0.8) \times 10^3\ M^{-1}\ s^{-1}$) [23], the first step of HE oxidation in cells seems more likely to be achieved by other oxidants. Importantly, the radical intermediate does not react with $O_2$, thus the generation of superoxide by probe-derived radicals is avoided. HE radical cation combines rapidly with superoxide ($k = 2 \times 10^9\ M^{-1}\ s^{-1}$) to generate a specific oxidation product, 2-hydroxyethidium [24]. Although superoxide-specific (2-OH-$E^+$) and non-specific ($E^+$) oxidation products have slightly different fluorescent spectra, the distinction between them is difficult using currently available fluorescence techniques [25]. The formation of 2-OH-$E^+$ has to be confirmed and quantitated with the use of other analytical techniques (*e.g.*, HPLC with fluorescence detection or LC–MS) [26,27]. The HE-derived radical can also produce an HE–HE dimer, which can be further oxidized to the HE–$E^+$ and $E^+$–$E^+$. The measurement of $E^+$ and the dimers (HE–HE, HE–$E^+$ and $E^+$–$E^+$) provides useful information about the cellular oxidation of HE [28]. It has been suggested that 2-OH-$E^+$ formation should be considered only as a qualitative indicator of intracellular superoxide production [29–31].

Hydroethidine analogs (*e.g.*, hydropropidine and MitoSOX™ Red, Fig. 2C) can be used for the detection of superoxide with the same limitations as assigned to HE [28]. Hydropropidine, a water-soluble analog of HE, possesses a highly localized positive charge that prevents its cellular uptake, making this probe a convenient tool for measurements of extracellular superoxide. It seems possible that in the presence of horseradish peroxidase (HRP), the sensitivity of detection for extracellular superoxide with hydropropidine can be significantly increased. Recently, we have shown that the addition of HRP to HE and xanthine/xanthine oxidase system dramatically enhances 2-OH-$E^+$ formation [31].

The requirement of HPLC separation for 2-OH-$E^+$ from other HE oxidation products is the major limitation of superoxide detection in cells. The rational design and synthesis of new hydroethidine analogs is needed to make real-time monitoring and/or imaging of superoxide in biological systems possible [32].

## Amplex® Red and the detection of hydrogen peroxide

Hydrogen peroxide can be generated *in vivo*, directly by an enzymatic two-electron reduction of $O_2$ or from dismutation of $O_2^{\bullet-}$ [13,33]. $H_2O_2$ can be produced by several enzymes, such as L-amino acid oxidase, urate oxidase, glycolate oxidase and monoamine oxidase [34,35]. $H_2O_2$ is assumed to be an important signaling molecule regulating enzymes such as protein kinases and phosphatases [36–38].

Hydrogen peroxide is a neutral molecule that can diffuse through lipid membranes. It can slowly react with thiols, which can lead to the formation of sulfenic acids or sulfoxides [39]. High $H_2O_2$ toxicity is ascribed to $^{\bullet}OH$ ($HO^{\bullet}$) formed through the Fenton reaction. To protect cells from $H_2O_2$ toxicity, the iron pool in cells is tightly controlled, and cells possess endogenous enzymatic $H_2O_2$ scavengers: peroxiredoxins, glutathione peroxidases, and catalase [40–42].

Currently, there are very few methods that yield reliable quantitative data on $H_2O_2$ production in cells. One method is the Amplex® Red (AR, 10-acetyl-3,7-dihydroxyphenoxazine) assay, which is based on the enzymatic oxidation of a resorufin derivative [43]. Amplex® Red is a colorless and non-fluorescent compound that upon oxidation by $H_2O_2$, in the presence of HRP, is transformed into a highly fluorescent resorufin [43]. The

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