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Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations

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Abstract

The purpose of this position paper is to present a critical analysis of the challenges and limitations of the most widely used fluorescent probes for detecting and measuring reactive oxygen and nitrogen species. Where feasible, we have made recommendations for the use of alternate probes and appropriate analytical techniques that measure the specific products formed from the reactions between fluorescent probes and reactive oxygen and nitrogen species. We have proposed guidelines that will help present and future researchers with regard to the optimal use of selected fluorescent probes and interpretation of results.

Introduction

The generation of reactive oxygen and nitrogen species has been implicated in the onset and progression of several diseases (e.g., atherosclerosis, cancer, diabetes, neurodegeneration) [1]. At a molecular level, reactive oxygen and nitrogen species exhibit signaling and cell-

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function-modifying roles [2]. As pointed out in a recent review [3], reactive oxygen and nitrogen species are not single entities but represent a broad range of chemically distinct reactive species with diverse biological reactivities [3,4]. To clearly attribute a particular cell signaling event to a specific reactive oxygen or nitrogen species, it is essential to detect and characterize these species accurately. Several analytical approaches (EPR, chemiluminescence, fluorescence) have been used to detect reactive oxygen and nitrogen species. Many reviews on this subject have been published in this journal. In this position paper, we discuss the relative merits and weaknesses of the fluorescent probes (e.g., dichlorodihydrofluorescein, hydroethidine, and dihydrorhodamine) that are being used frequently for measuring hydrogen peroxide, superoxide, and peroxynitrite in biological systems.

Is dichlorodihydrofluorescein diacetate (DCFH-DA) a suitable probe for measuring intracellular H_2O_2 and other oxidants?

DCFH-DA is the most widely used probe for detecting intracellular H_2O_2 and oxidative stress. This probe is cell-permeable and is hydrolyzed intracellularly to the DCFH carboxylate anion, which is retained in the cell. Two-electron oxidation of DCFH results in the formation of a fluorescent product, dichlorofluorescein (DCF), which can be monitored by several fluorescence-based techniques (e.g., confocal microscopy, flow cytometry). This is a relatively easy and user-friendly assay that has become immensely popular. Investigators have routinely used DCFH-DA to measure intracellular generation of H_2O_2 and other oxidants or monitor redox signaling changes in cells in response to intra- or extracellular activation with oxidative stimulus [5–10].

However, as shown in Fig. 1 and outlined below, the intracellular redox chemistry of DCFH is complex and there are several limitations and artifacts associated with the DCF assay for intracellular H_2O_2 measurement [11–13]. It is essential to keep these limitations in mind for proper interpretation of data obtained with the DCFH-DA probe. Specifically:

1. DCFH does not directly react with H_2O_2 to form the fluorescent product, DCF. *Therefore, DCF fluorescence cannot be used as a direct measure of H_2O_2.*
2. Several one-electron-oxidizing species will oxidize DCFH to DCF (Fig. 1). These include hydroxyl radicals ($^{\bullet}OH$), compounds I and II formed from peroxidase or heme interaction with H_2O_2, $^{\bullet}NO_2$ formed from the myeloperoxidase/H_2O_2/NO_2^- system, hypochlorous acid (HOCl), and reactive species formed from peroxynitrite ($ONOO^-$/ONOOH) decomposition. Peroxynitrite decomposition also forms $^{\bullet}OH$ or carbonate anion radicals ($CO_3^{\bullet-}$) in the presence of bicarbonate.
3. The intermediate radical, $DCF^{\bullet-}$, formed from the one-electron oxidation of DCFH, rapidly reacts with O_2 (k=10^8 M^{-1} s^{-1}) to form superoxide ($O_2^{\bullet-}$) (Fig. 1). The dismutation of $O_2^{\bullet-}$ yields additional H_2O_2[14], which can establish a redox-cycling mechanism leading to artifactual amplification of the fluorescence signal intensity.
4. Cytochrome *c*, a heme protein that is released from mitochondria to the cytosol during apoptosis, is capable of oxidizing DCFH directly or indirectly via a peroxidase-type mechanism, forming DCF [8, 11]. The increase in DCF fluorescence that occurs during apoptosis of cells loaded with DCFH-DA has frequently been associated with enhanced oxidant production.
5. Redox-active metals (e.g., Fe^{2+}) promote DCFH oxidation in the presence of oxygen or H_2O_2 [15].

6. In addition, one cannot always assume that control and experimental samples exhibit the same efficiency in DCF radical generation and the same linear dependence of self-propagating redox-cycling reactions induced by the DCF radical (Fig. 1).

Therefore, even if differences in DCF fluorescence are documented under control and experimental conditions, one has to consider the various caveats listed above for proper interpretation of the data. Failure to recognize these limitations often leads to erroneous interpretations and diverts the researchers' attention away from the more significant findings (e.g., increased peroxidase activity induced by the experimental conditions, or a new redox signaling/redox modification mechanism). Our recommendation to researchers in this field is that the DCFH-DA probe cannot be reliably used to *measure* intracellular H_2O_2 and other reactive oxygen species.

Now the important question: *What type of useful information can one obtain using the fluorescent probe DCFH-DA?* On a positive note, it is conceivable that useful redox-signaling mechanisms can be explored using this probe [6–8]. There are numerous examples in the literature that support the role of oxidant-induced iron signaling in DCFH oxidation, of which a few are discussed here. Previous studies implicated a role for redox-active iron in intracellular oxidation of DCFH to DCF [16], although the origin of the cellular iron was not known. In earlier studies, it was shown that H_2O_2- and lipid hydroperoxide-induced intracellular DCFH oxidation to DCF was mediated by transferrin receptor (TfR)-dependent uptake of iron [5–7]. Intracellular DCF fluorescence was inhibited by the monoclonal IgA-class anti-TfR antibody that blocked TfR endocytosis and the iron uptake. These studies also showed that peroxide-dependent DCF fluorescence was critically dependent on intracellular glutathione levels [5]. DCFH-DA was also used to monitor doxorubicin-induced iron signaling and the associated mitochondrial damage in endothelial cells [7]. DCFH-DA was used as an indicator for mitochondrial generation of oxidants and peroxynitrite in endothelial cells treated with oxidized low-density lipoprotein (oxLDL) [9]. A role for iron, endothelial nitric oxide synthase activity, and complex II activity in oxLDL-dependent DCF fluorescence was proposed [9,10,17]. DCFH-DA may be used as a redox indicator probe that responds to changes in intracellular iron signaling or peroxynitrite formation.

Are there other probes for measuring intracellular H_2O_2?

Very few small organic molecules stoichiometrically react with H_2O_2 to form a detectable intracellular fluorescent product. However, aromatic boronates (boronic acids and esters) react with H_2O_2 to form a single major product, the corresponding phenol (reaction yield is nearly 100% [18]). Recently, the boronate moiety was attached to a fluorophore (e.g., fluorescein) that, upon reaction with H_2O_2, forms a highly fluorescent molecule [19]. The boronate esters attached to a fluorophore are cell-permeable and interact directly with intracellular H_2O_2 to form fluorescent products in cells. However, the rate constant for the reaction between boronates and H_2O_2 is relatively low (0.1–1.0 M^{-1} s^{-1}). It is unlikely to compete with H_2O_2-detoxifying enzymes such as the peroxiredoxins, which react 7 orders of magnitude faster with H_2O_2[3,4]. Nevertheless, even a small fraction of the reaction between the fluorescein boronate molecule and H_2O_2 would result in detectable intracellular fluorescence. This assay has been used to detect intracellular H_2O_2 formation [19,20]. Mitochondrial-targeted boronate (aromatic boronate conjugated to an alkyl triphenylphosphonium group) was recently used in vivo to quantitate H_2O_2 in fly mitochondria [21]. Mass spectrometry was used to quantitate the product [21]. However, aromatic boronates also react nearly stoichiometrically with $ONOO^-$ a million times faster than they do with H_2O_2[18,22], which should be taken into consideration (see Is dihydrorhodamine a specific probe for measuring intracellular peroxynitrite?). The use of

inhibitors (L-NAME, catalase, PEG–catalase, SOD, or PEG–SOD) should help distinguish $ONOO^-$ from H_2O_2 in boronate-based fluorescence assays.

Another probe that is frequently used to measure H_2O_2 is Amplex red [23]. Amplex red is oxidized by horseradish peroxidase (HRP) and H_2O_2 to a fluorescent product, resorufin [23]. Although other one-electron oxidants are capable of oxidizing Amplex red to resorufin, the oxidation catalyzed by HRP in the presence of H_2O_2 is highly efficient and vastly increases the yield of resorufin. Therefore this assay is a viable method to continuously measure the extracellular formation of H_2O_2. A major complicating factor is light-mediated photochemical oxidation of resorufin in the presence of biological reductants (glutathione, NADH) that induce resorufin radical-mediated formation of $O_2^{\bullet-}$ and H_2O_2[24]. Because of the confounding radical reactions induced by resorufin, the use of Amplex red or cell-permeable Amplex red analog to measure intracellular H_2O_2 could pose problems. Under conditions where H_2O_2 is released from the cell or in isolated mitochondrial preparations, the Amplex red probe can be used to measure H_2O_2 in the presence of HRP.

Other approaches for monitoring intracellular H_2O_2 levels include the use of a redox-active biosensor, HyPer, whose design is based on OxyR, a natural bacterial H_2O_2 sensor [25]. Recent developments include HyPer targeted to various cellular compartments. This ratiometric assay for H_2O_2 is potentially promising, although its range of applications in biology has not yet been critically examined.

Measurement of intracellular and mitochondrial superoxide using hydroethidine (HE) and Mito-SOX

HE, or dihydroethidium, is another widely used probe for detecting intracellular $O_2^{\bullet-}$[26]. The red fluorescence formed from the two-electron oxidation product, ethidium (E^+), is usually equated to intracellular superoxide formation. Previous research suggests that E^+ is not formed from the direct oxidation of HE by $O_2^{\bullet-}$[27,28]. Instead, another product, 2-hydroxyethidium (2-OH-E^+), with similar fluorescence characteristics, is formed from the HE/ $O_2^{\bullet-}$ reaction [29]. To the best of our knowledge, 2-OH-E^+ is not formed during the reaction between HE and other oxidants ($ONOO^-$, $^{\bullet}OH$, H_2O_2, compounds I and II), although they oxidize HE to E^+ and other dimeric products. However, one cannot rule out the direct hydroxylation of HE to form 2-OH-E^+ by cytochrome P-450 or by a similar enzymatic metabolism. Hydroethidine-derived radicals do not react with oxygen to form superoxide. Because of other oxidative reactions of HE, we suggest that 2-OH-E^+ formation is only a qualitative and not a quantitative indicator of intracellular and/or extracellular $O_2^{\bullet-}$ [30] (Scheme 1). However, it will not be possible to use fluorescence-based intracellular techniques alone to assess $O_2^{\bullet-}$ formation because intracellular red fluorescence detection, using HE, does not automatically translate to $O_2^{\bullet-}$ measurement. One has to confirm the formation of 2-OH-E^+ using other analytical techniques (HPLC-fluorescence or electrochemical detection or LC–MS). Another advantage of measuring E^+ and other dimers of HE (HE–HE, HE–E^+, E^+–E^+) is that they can be used as an indicator for one-electron oxidant formation in cells.

Mitochondrial-targeted HE or Mito-SOX, a triphenylphosphonium cation conjugated to HE via a linker carbon–carbon alkyl chain, has been used to measure mitochondrial $O_2^{\bullet-}$[31]. The chemistry of Mito-SOX with $O_2^{\bullet-}$ is similar to that of HE and $O_2^{\bullet-}$[32] and the same caveats apply as above. Being a positively charged molecule, Mito-SOX reacts slightly faster with $O_2^{\bullet-}$ compared to HE [31]. Mito-SOX reacts with $O_2^{\bullet-}$ and forms a red-fluorescent product, 2-hydroxymitoethidium (2-OH-Mito-E^+), and not Mito-E^+ (Scheme 1).

2-OH-Mito-E^+ (specific reaction product of $O_2^{\bullet -}$ with Mito-SOX) and Mito-E^+ (nonspecific oxidation product of Mito-SOX) have overlapping fluorescence spectra. Thus, the red fluorescence formed from Mito-SOX localized in mitochondria is not a reliable indicator of mitochondrial $O_2^{\bullet -}$ formation. Mito-SOX red fluorescence could arise from the $O_2^{\bullet -}$ product, from a composite of $O_2^{\bullet -}$ product and an oxidation product, or solely from an oxidation product of Mito-SOX induced by one-electron oxidants (cytochrome *c*, peroxidase, and H_2O_2). As shown for the HE probe, it is necessary to separate and identify 2-OH-Mito-E^+ by HPLC. For details, see previous publications [29–31].

Is dihydrorhodamine a specific probe for measuring intracellular peroxynitrite?

Dihydrorhodamine (DHR) is the most frequently used probe for measuring $ONOO^-$[33,34]. This assay is based on the oxidative conversion of DHR to its corresponding two-electron-oxidized fluorescent product, rhodamine. In many respects, the oxidative mechanisms of DHR and DCFH are very similar [15]. DHR oxidation to rhodamine is triggered by several oxidants ($^{\bullet}OH$, compounds I and II, and $^{\bullet}NO_2$), similar to DCFH oxidation by oxidants as shown in Fig. 1. Thus, DHR oxidation to rhodamine is also nonspecific. The oxidative conversion of DHR to rhodamine is mediated by an intermediate DHR radical. The overall reaction profile of DHR to rhodamine in the presence of cogenerated $^{\bullet}NO$ and $O_2^{\bullet -}$ is influenced by the free radical chemistry of DHR radical, showing a bell-shaped response [34]. The oxidation of DHR by cogenerated $^{\bullet}NO$ and $O_2^{\bullet -}$ is mediated by oxidants ($^{\bullet}NO_2$ and $^{\bullet}OH$) formed from the rapid and spontaneous decomposition of $ONOO^-$, and DHR oxidation is not induced directly by $ONOO^-$. The intermediate radical, $DHR^{\bullet}$, formed from the one-electron oxidation of DHR, also rapidly reacts with oxygen (k=7×10^8 M^{-1} s^{-1}) [15,33], setting up a redox cycling mechanism leading to artifactual amplification of the fluorescence signal intensity (see Fig. 1). The limitations and caveats associated with DCF assay apply to DHR assay as well. The roles of $^{\bullet}NO$ and $O_2^{\bullet -}$ or iron in intracellular DHR oxidation should be independently confirmed with appropriate inhibitors (e.g., L-NAME, PEG–SOD, desferrioxamine). Thus, DHR can be used only as a nonspecific indicator of intracellular $ONOO^-$ and HOCl formation [33].

Emerging probes for measurement of peroxynitrite

Recent research shows that aromatic boronates can be rapidly oxidized by $ONOO^-$ yielding the corresponding phenols as a major product (85% yield) [18,22]. Boronate-containing fluorophores (e.g., coumarin boronate) react in a similar fashion with $ONOO^-$ giving rise to the corresponding fluorescent products. Some of these boronate-based fluorophores are cell-permeable and can be effectively used to measure intracellular $ONOO^-$. With increased availability of these probes, significant advances and new understanding in reactive oxygen and nitrogen research are likely. Synthesis of newer cell-permeable probes, coupled with enhanced understanding of their radical/nonradical chemistry, will provide additional research tools with which the complex roles of reactive oxygen and nitrogen species in cell signaling, cell growth, and cell differentiation can be unraveled.

We hope that this position paper clarifies some aspects of the appropriate use of fluorescent probes in the detection and characterization of reactive oxygen and nitrogen species in biology, and that it also highlights the many challenges and limitations of detection, characterization, and measurement of intracellular reactive oxygen and nitrogen species. The advantages, disadvantages, and recommendations of selected fluorescent probes listed in Table 1 should help alleviate some confusion with regard to the choice of suitable probes for detecting reactive oxygen and nitrogen species.

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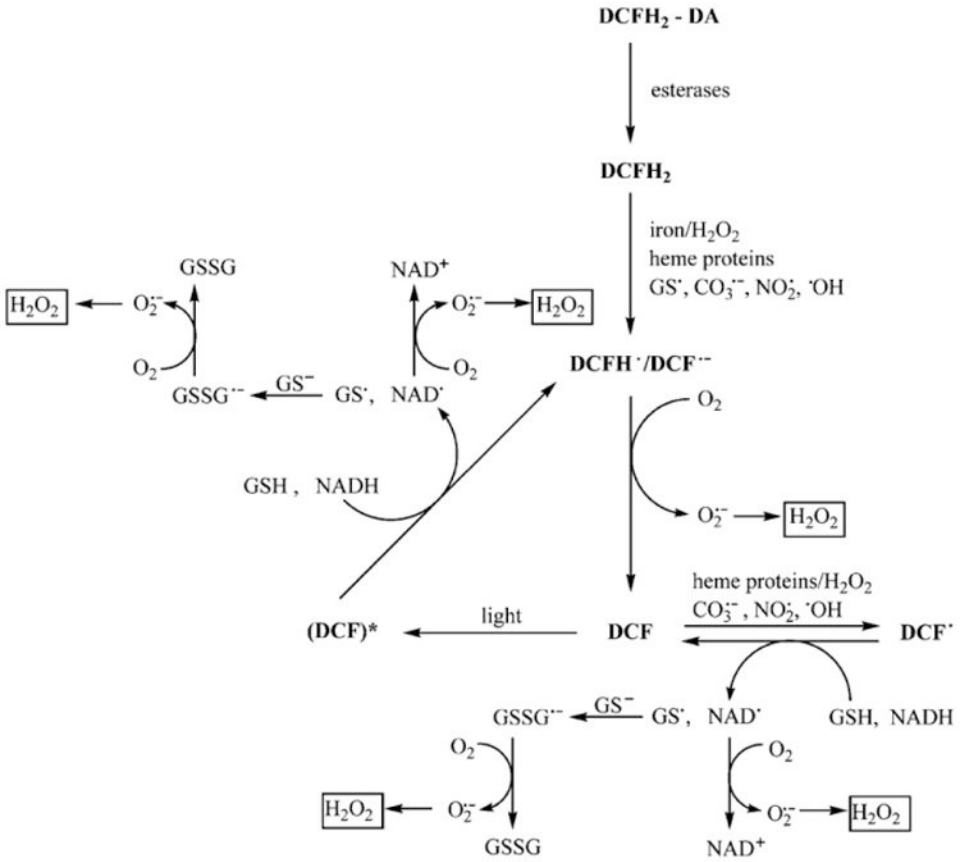
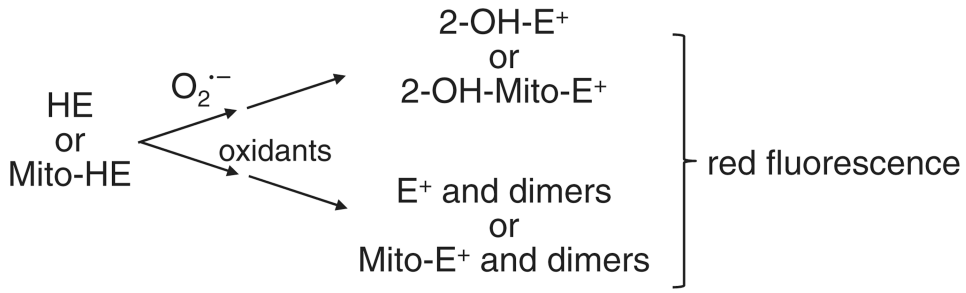


Fig. 1.

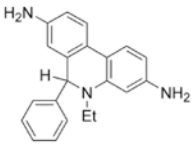
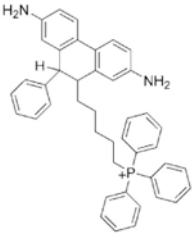
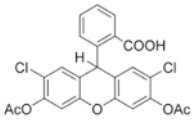
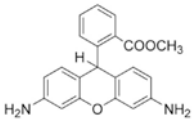
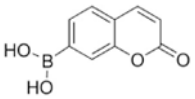
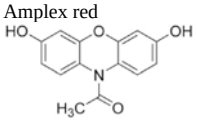
Oxidative reactions mediated by the dichlorodihydrofluorescein radical ($DCF^{\bullet -}$) in the presence of oxygen, heme proteins, and glutathione (reproduced from Ref. [35]).



Scheme 1.
Oxidative reactions of HE and Mito-HE.

Table 1

Guidelines for proper use of fluorescent probes.

Fluorogenic probe	Advantages	Disadvantages	Recommendations
HE 	Reacts with $O_2^{\bullet-}$ to form a diagnostic marker product, 2-OH-E^+. Reacts with other oxidants (e.g., $^{\bullet}OH$, $ONOO^-$) to form E^+ and dimers. Intermediate HE-derived radical does not react with molecular oxygen to form $O_2^{\bullet-}$ and H_2O_2.	2-OH-E^+ and E^+ have similar fluorescence spectral characteristics. Further extraction and HPLC analyses are needed for proper identification.	Suitable for detection of intracellular $O_2^{\bullet-}$. Suitable for detection of intracellular oxidants (e.g., iron and H_2O_2, cytochrome *c* and H_2O_2). However, red fluorescence measurements using fluorescence or confocal microscopy will not be able to distinguish between E^+ and 2-OH-E^+. HPLC or other analytical measurements of products are crucial.
Mito-SOX or Mito-HE 	Localizes into mitochondria. Reacts with $O_2^{\bullet-}$ to form a diagnostic marker product, 2-OH-Mito-E^+. Reacts with other oxidants ($^{\bullet}OH$, $ONOO^-$) to form Mito-E^+ and dimers. Intermediate Mito-HE-derived radical does not react with O_2 to form $O_2^{\bullet-}$ and H_2O_2.	2-OH-Mito-E^+ and Mito-E^+ have similar fluorescence spectral parameters. HPLC analyses of products are necessary for proper identification probe. Mitochondrial toxicity is a concern.	Suitable for detection of mitochondrial $O_2^{\bullet-}$. Suitable for detection of intracellular oxidants (e.g., iron and H_2O_2, cytochrome *c* and H_2O_2). However, the "red fluorescence" measurements alone will not allow distinction between the hydroxylated and the oxidized Mito-HE. HPLC or other analytical measurements of products are crucial.
DCFH-DA 	Cell-permeable Easy to use. Responds to changes in intracellular iron signaling or enhanced peroxidase activity (see Ref. [36]).	Artifactual amplification of the fluorescence intensity via a redox-cycling mechanism involving an intermediate radical, $DCF^{\bullet-}$.	Not suitable for measuring intracellular H_2O_2 or other oxidants. May be used as a redox indicator probe for uncovering new redox signaling mechanisms keeping in mind the various caveats.
DHR 	Cell-permeable Easy to use. Responds to cogenerated $^{\bullet}NO$ and $O_2^{\bullet-}$ via a predictable radical chemistry.	Artifactual amplification of the fluorescence intensity via a redox-cycling mechanism involving an intermediate radical, $DHR^{\bullet}$.	Not suitable for measuring intracellular $ONOO^-$ or other oxidants. May be used as a nonspecific indicator for intracellular $ONOO^-$ or oxidants derived from it.
Coumarin boronate 	Reacts very rapidly and nearly stoichiometrically with $ONOO^-$ to form a fluorescent product.	Further metabolism of the product (7-hydroxycoumarin) and possible excretion out of cells may be a hindrance.	Suitable for measuring extracellular $ONOO^-$ formation. As this probe also reacts with H_2O_2 (albeit slowly), proper controls using appropriate inhibitors should be performed.
Amplex red 	HRP/H_2O_2-dependent oxidation of Amplex red to resorufin is very efficient. The intermediate Amplex red-derived radical does not react with O_2 for form additional $O_2^{\bullet-}$ and H_2O_2. The intermediate Amplex red-derived radical will react with $O_2^{\bullet-}$ to decrease resorufin formation (see Ref. [37]). Reducing agents (e.g., ascorbate) and peroxidase substrates (nitrite anion) will inhibit resorufin formation.	Room light-mediated photochemical oxidation of resorufin in the presence of GSH, NAD(P)H, or ascorbate will greatly increase artifactual generation of H_2O_2. Amplex red-derived radical can rapidly react with $O_2^{\bullet-}$ and inhibit product formation.	Suitable for measuring extracellular H_2O_2 under conditions limiting the secondary radical reactions induced by resorufin.