



Commentary

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

Balaraman Kalyanaraman^{a,*}, Victor Darley-USmar^b, Kelvin J.A. Davies^{c,d}, Phyllis A. Dennerly^e, Henry Jay Forman^{c,f}, Matthew B. Grisham^g, Giovanni E. Mann^h, Kevin Mooreⁱ, L. Jackson Roberts II^j, Harry Ischiropoulos^e

^a Department of Biophysics and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA

^b Center for Free Radical Biology and Department of Pathology, University of Alabama, Birmingham, AL 35294, USA

^c Ethel Percy Andrus Gerontology Center, Davis School of Gerontology, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA 90089, USA

^d Division of Molecular & Computational Biology, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA 90089, USA

^e Department of Pediatrics, University of Pennsylvania Division of Neonatology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

^f University of California at Merced, Merced, CA 95344, USA

^g Immunology and Inflammation Research Group and Department of Molecular and Cellular Physiology, Louisiana State University Health Science Center, Shreveport, LA 71130, USA

^h Cardiovascular Division, BHF Centre of Research Excellence, King's College London, London, UK

ⁱ Department of Medicine, University College London Medical School, London, UK

^j Department of Pharmacology, Vanderbilt University, Nashville, TN 37240, USA

ARTICLE INFO

Article history:

Received 16 May 2011

Revised 8 August 2011

Accepted 24 September 2011

Available online 2 October 2011

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.

© 2011 Elsevier Inc. All rights reserved.

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-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 H₂O₂ and other oxidants?

DCFH-DA is the most widely used probe for detecting intracellular H₂O₂ 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 H₂O₂ 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 H₂O₂ measurement

* Corresponding author. Fax: +1 955 456 6512.

E-mail address: balarama@mcw.edu (B. Kalyanaraman).

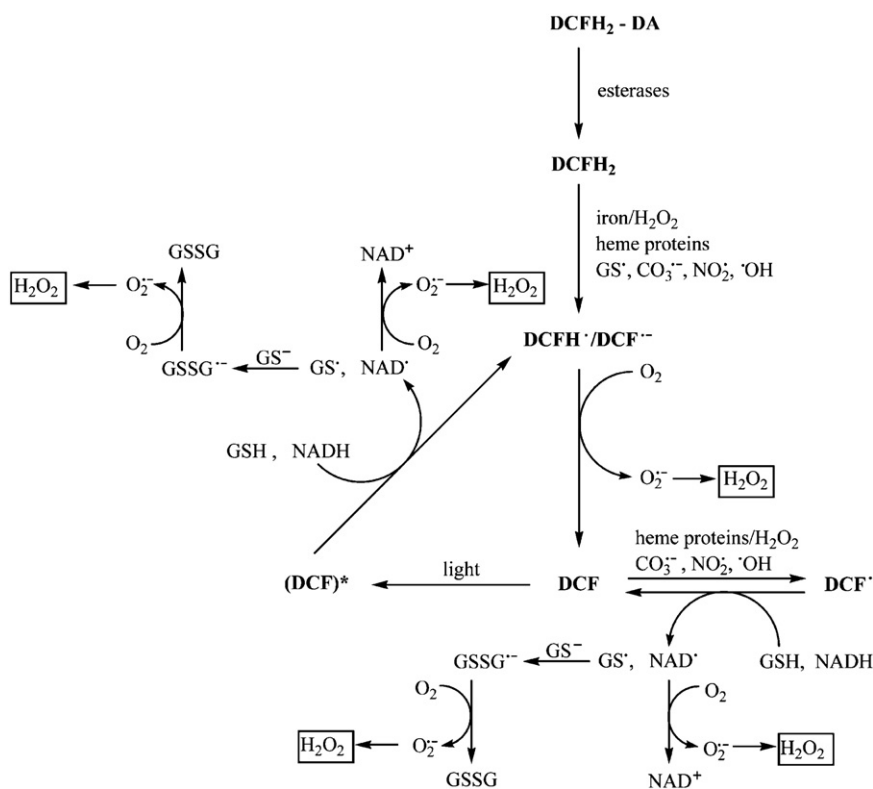


Fig. 1. Oxidative reactions mediated by the dichlorodihydrofluorescein radical (DCF•⁻) in the presence of oxygen, heme proteins, and glutathione (reproduced from Ref. [35]).

[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 H₂O₂ to form the fluorescent product, DCF. Therefore, DCF fluorescence cannot be used as a direct measure of H₂O₂.
- (2) Several one-electron-oxidizing species will oxidize DCFH to DCF (Fig. 1). These include hydroxyl radicals (•OH), compounds I and II formed from peroxidase or heme interaction with H₂O₂, •NO₂ formed from the myeloperoxidase/H₂O₂/NO₂⁻ system, hypochlorous acid (HOCl), and reactive species formed from peroxynitrite (ONOO⁻/ONOOH) decomposition. Peroxynitrite decomposition also forms •OH or carbonate anion radicals (CO₃•⁻) in the presence of bicarbonate.
- (3) The intermediate radical, DCF•⁻, formed from the one-electron oxidation of DCFH, rapidly reacts with O₂ ($k = 10^8 \text{ M}^{-1} \text{ s}^{-1}$) to form superoxide (O₂•⁻) (Fig. 1). The dismutation of O₂•⁻ yields additional H₂O₂[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., Fe²⁺) promote DCFH oxidation in the presence of oxygen or H₂O₂[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 H₂O₂ 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 H₂O₂- 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 H₂O₂?

Very few small organic molecules stoichiometrically react with H₂O₂ to form a detectable intracellular fluorescent product. However, aromatic boronates (boronic acids and esters) react with H₂O₂ to

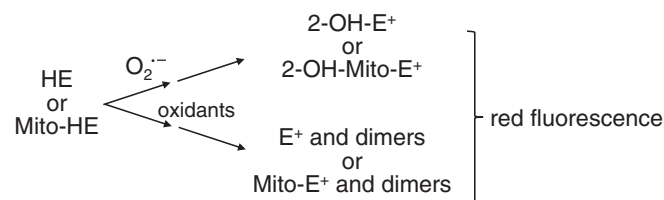
form a single major product, the corresponding phenol (reaction yield is nearly 100% [18]). Recently, the boronate moiety was attached to a fluorophore (e.g., fluorescein) that, upon reaction with H_2O_2 , forms a highly fluorescent molecule [19]. The boronate esters attached to a fluorophore are cell-permeable 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 ($0.1\text{--}1.0\text{ M}^{-1}\text{ s}^{-1}$). 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 $\text{O}_2^{\cdot-}$ 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 ratio-metric 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 $\text{O}_2^{\cdot-}$ [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 $\text{O}_2^{\cdot-}$ [27,28]. Instead, another product, 2-hydroxyethidium (2-OH- E^+), with similar fluorescence characteristics, is formed from the HE/ $\text{O}_2^{\cdot-}$ reaction [29]. To the best of our knowledge, 2-OH- E^+ is not formed during the reaction between HE and other oxidants (ONOO^- , $\cdot\text{OH}$, 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 $\text{O}_2^{\cdot-}$ [30] (Scheme 1). However, it will not be possible to use fluorescence-



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

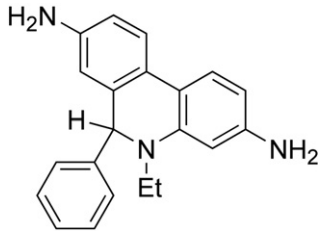
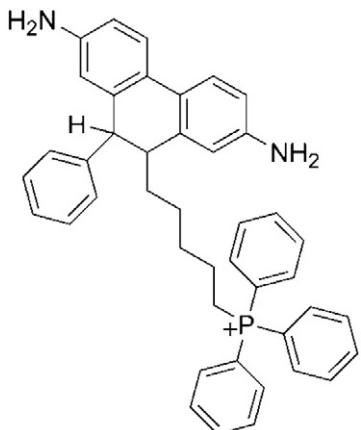
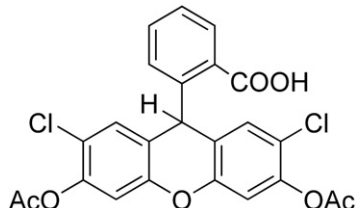
based intracellular techniques alone to assess $\text{O}_2^{\cdot-}$ formation because intracellular red fluorescence detection, using HE, does not automatically translate to $\text{O}_2^{\cdot-}$ measurement. One has to confirm the formation of 2-OH- E^+ using other analytical techniques (HPLC-fluorescence 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 $\text{O}_2^{\cdot-}$ [31]. The chemistry of Mito-SOX with $\text{O}_2^{\cdot-}$ is similar to that of HE and $\text{O}_2^{\cdot-}$ [32] and the same caveats apply as above. Being a positively charged molecule, Mito-SOX reacts slightly faster with $\text{O}_2^{\cdot-}$ compared to HE [31]. Mito-SOX reacts with $\text{O}_2^{\cdot-}$ 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 $\text{O}_2^{\cdot-}$ 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 $\text{O}_2^{\cdot-}$ formation. Mito-SOX red fluorescence could arise from the $\text{O}_2^{\cdot-}$ product, from a composite of $\text{O}_2^{\cdot-}$ 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].

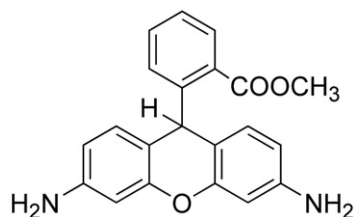
Is dihydrorhodamine a specific probe for measuring intracellular peroxynitrite?

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 ($\cdot\text{OH}$, compounds I and II, and $\cdot\text{NO}_2$), 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 $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ is influenced by the free radical chemistry of DHR radical, showing a bell-shaped response [34]. The oxidation of DHR by cogenerated $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ is mediated by oxidants ($\cdot\text{NO}_2$ and $\cdot\text{OH}$) formed from the rapid and spontaneous decomposition of ONOO^- , and DHR oxidation is not induced directly by ONOO^- . The intermediate radical, DHR \cdot , formed from the one-electron oxidation of DHR, also rapidly reacts with oxygen ($k = 7 \times 10^8\text{ M}^{-1}\text{ s}^{-1}$) [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 $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$ 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].

Table 1
Guidelines for proper use of fluorescent probes.

Fluorogenic probe	Advantages	Disadvantages	Recommendations
<p>HE</p> 	<p>Reacts with O_2^- to form a diagnostic marker product, 2-OH-E⁺. Reacts with other oxidants (e.g., $\cdot OH$, $ONOO^-$) to form E⁺ and dimers. Intermediate HE-derived radical does not react with molecular oxygen to form O_2^- and H_2O_2.</p>	<p>2-OH-E⁺ and E⁺ have similar fluorescence spectral characteristics. Further extraction and HPLC analyses are needed for proper identification.</p>	<p>Suitable for detection of intracellular O_2^-. 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.</p>
<p>Mito-SOX or Mito-HE</p> 	<p>Localizes into mitochondria. Reacts with O_2^- to form a diagnostic marker product, 2-OH-Mito-E⁺. Reacts with other oxidants ($\cdot OH$, $ONOO^-$) to form Mito-E⁺ and dimers. Intermediate Mito-HE-derived radical does not react with O_2 to form O_2^- and H_2O_2.</p>	<p>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.</p>	<p>Suitable for detection of mitochondrial O_2^-. 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.</p>
<p>DCFH-DA</p> 	<p>Cell-permeable Easy to use. Responds to changes in intracellular iron signaling or enhanced peroxidase activity (see Ref. [36]).</p>	<p>Artificial amplification of the fluorescence intensity via a redox-cycling mechanism involving an intermediate radical, $DCF^{\cdot -}$.</p>	<p>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.</p>

DHR

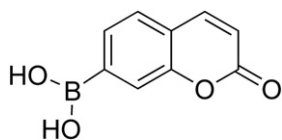


Cell-permeable
Easy to use.
Responds to cogenerated NO and O_2^- via a predictable radical chemistry.

Artificial amplification of the fluorescence intensity via a redox-cycling mechanism involving an intermediate radical, DHR $^{\cdot}$.

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

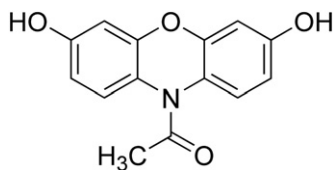


Reacts very rapidly and nearly stoichiometrically with ONOO^- to form a fluorescent product.

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^- and H_2O_2 . The intermediate Amplex red-derived radical will react with O_2^- 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 artificial generation of H_2O_2 . Amplex red-derived radical can rapidly react with O_2^- and inhibit product formation.

Suitable for measuring extracellular H_2O_2 under conditions limiting the secondary radical reactions induced by resorufin.

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.

References

- Halliwell, B., Gutteridge, J.M.C. (Eds.), 1999. *Free Radicals in Biology and Medicine*, 3rd edition. Oxford Univ. Press, New York; 1999.
- D'Autreaux, B.; Toledano, M. B. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* **8**:813–824; 2006.
- Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **4**:278–286; 2008.
- Murphy, M. P.; Holmgren, A.; Larsson, N. G.; Halliwell, B.; Chang, C. J.; Kalyanaraman, B.; Rhee, S. G.; Thornally, P. J.; Partridge, L.; Gems, D.; Nyström, T.; Belousov, V.; Schumacker, P. T.; Winterbourn, C. C. Unraveling the biological roles of reactive oxygen species. *Cell Metab.* **13**:361–364; 2011.
- Tampo, Y.; Kotamraju, S.; Chitambar, C. R.; Kalivendi, S. V.; Keszler, A.; Joseph, J.; Kalyanaraman, B. Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. *Circ. Res.* **92**:56–63; 2003.
- Kotamraju, S.; Tampo, Y.; Keszler, A.; Chitambar, C. R.; Joseph, J.; Haas, A. L.; Kalyanaraman, B. Nitric oxide inhibits H₂O₂-induced transferrin receptor-dependent apoptosis in endothelial cells: role of ubiquitin–proteasome pathway. *Proc. Natl. Acad. Sci. U. S. A.* **100**:10653–10658; 2003.
- Kotamraju, S.; Kalivendi, S. V.; Konorev, E.; Chitambar, C. R.; Joseph, J.; Kalyanaraman, B. Oxidant-induced iron signaling in doxorubicin-mediated apoptosis. *Methods Enzymol.* **378**:362–382; 2004.
- Karlsson, M.; Kurz, T.; Brunk, U. T.; Nilsson, S. E.; Frennesson, C. I. What does the commonly used DCF test for oxidative stress really show? *Biochem. J.* **428**:183–190; 2010.
- Zmijewski, J. W.; Moellering, D. R.; Le Goffe, C.; Landar, A.; Ramachandran, A.; Darley-Usmar, V. M. Oxidized LDL induces mitochondrially associated reactive oxygen/nitrogen species formation in endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **289**:H852–H861; 2005.
- Lee, S.; Gharavi, N. M.; Honda, H.; Chang, I.; Kim, B.; Jen, N.; Li, R.; Zimman, A.; Berliner, J. A. A role for NADPH oxidase 4 in the activation of vascular endothelial cells by oxidized phospholipids. *Free Radic. Biol. Med.* **47**:145–151; 2009.
- Burkitt, M. J.; Wardman, P. Cytochrome c is a potent catalyst of dichlorofluorescein oxidation: implications for the role of reactive oxygen species in apoptosis. *Biochem. Biophys. Res. Commun.* **282**:329–333; 2001.
- Bonini, M. G.; Rota, C.; Tomasi, A.; Mason, R. P. The oxidation of 2',7'-dichlorofluorescein to reactive oxygen species: a self-fulfilling prophesy? *Free Radic. Biol. Med.* **40**:968–975; 2006.
- Hempel, S. L.; Buettner, G. R.; O'Malley, Y. Q.; Wessels, D. A.; Flaherty, D. M. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5 (and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine. *Free Radic. Biol. Med.* **27**:146–159; 1999.
- Folkes, L. K.; Patel, K. B.; Wardman, P.; Wrona, M. Kinetics of reaction of nitrogen dioxide with dihydrorhodamine and the reaction of the dihydrorhodamine radical with oxygen: implications for quantifying peroxynitrite formation in cells. *Arch. Biochem. Biophys.* **484**:122–126; 2009.
- Qian, S. Y.; Buettner, G. R. Iron and dioxygen chemistry is an important route to initiation of biological and free radical oxidations: an electron paramagnetic resonance spin trapping study. *Free Radic. Biol. Med.* **26**:1447–1456; 1999.
- LeBel, C. P.; Ischiropoulos, H.; Bondy, S. C. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5**:227–231; 1992.
- Kooy, N. W.; Royall, J. A.; Ischiropoulos, H. Oxidation of 2',7'-dichlorofluorescein by peroxynitrite. *Free Radic. Res.* **27**:245–254; 1997.
- Sikora, A.; Zielonka, J.; Lopez, M.; Joseph, J.; Kalyanaraman, B. Direct oxidation of boronates by peroxynitrite: mechanism and implications in fluorescence imaging of peroxynitrite. *Free Radic. Biol. Med.* **47**:1401–1407; 2009.
- Miller, E. W.; Abers, A. E.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. Boronate-based fluorescent probes for imaging cellular hydrogen peroxide. *J. Am. Chem. Soc.* **127**:16652–16659; 2005.
- Dickenson, B. C.; Huynh, C.; Chang, C. J. A palette of fluorescent probes with varying emission colors for imaging hydrogen peroxide signaling in living cells. *J. Am. Chem. Soc.* **132**:5906–5915; 2010.
- Cochemé, H. M.; Quin, C.; McQuaker, S. J.; Cabreiro, F.; Logan, A.; Prime, T. A.; Abakumova, I.; Patel, J. V.; Fearnley, I. M.; James, A. M.; Porteous, C. M.; Smith, R. A.; Saeed, S.; Carré, J. E.; Singer, M.; Gems, D.; Hartley, R. C.; Partridge, L.; Murphy, M. P. Measurement of H₂O₂ within living *Drosophila* during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. *Cell Metab.* **13**:340–350; 2011.
- Zielonka, J.; Sikora, A.; Joseph, J.; Kalyanaraman, B. Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide. *J. Biol. Chem.* **285**:14210–14216; 2010.
- Mishin, V.; Gray, J. P.; Heck, D. E.; Laskin, J. D. Application of the Amplex red/horseradish peroxidase assay to measure hydrogen peroxide generation by recombinant microsomal enzymes. *Free Radic. Biol. Med.* **48**:1485–1491; 2010.
- Zhao, B.; Rangelova, K.; Jian, J. J.; Zielonka, J.; Kalyanaraman, B.; Mason, R. Studies on the photosensitized reduction of resorufin and implications for the detection of oxidative stress with Amplex red. *Free Radic. Biol. Med.* **49** (Suppl. 1): S105–S106; 2010.
- Belousov, V. V.; Fradkov, A. F.; Lukyanov, K. A.; Staroverov, D. B.; Shakhbazov, K. S.; Terskikh, A. V.; Lukyanov, S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods* **3**:281–286; 2006.
- Zhao, H.; Kalivendi, S.; Zhang, H.; Joseph, J.; Nithipatikom, K.; Vásquez-Vivar, J.; Kalyanaraman, B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic. Biol. Med.* **34**:1359–1368; 2003.
- Zhao, H.; Joseph, J.; Fales, H. M.; Sokolowski, E. A.; Levine, R. L.; Vasquez-Vivar, J.; Kalyanaraman, B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **102**:5727–5732; 2005.
- Dikalov, S.; Grienling, K. K.; Harrison, D. G. Measurement of reactive oxygen species in cardiovascular studies. *Hypertension* **49**:717–727; 2007.
- Zielonka, J.; Kalyanaraman, B. Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. *Free Radic. Biol. Med.* **48**:983–1001; 2010.
- Zielonka, J.; Vasquez-Vivar, J.; Kalyanaraman, B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat. Protoc.* **3**:8–21; 2008.
- Robinson, K. M.; Janes, M. S.; Beckman, J. S. The selective detection of mitochondrial superoxide by live cell imaging. *Nat. Protoc.* **3**:941–947; 2008.
- Zielonka, J.; Srinivasan, S.; Hardy, M.; Ouari, O.; Lopez, M.; Vasquez-Vivar, J.; Avadhani, N. G.; Kalyanaraman, B. Cytochrome c-mediated oxidation of hydroethidine and mito-hydroethidine in mitochondria: identification of homo- and heterodimers. *Free Radic. Biol. Med.* **44**:835–846; 2008.
- Wardman, P. Methods to measure the reactivity of peroxynitrite-derived oxidants toward reduced fluoresceins and rhodamines. *Methods Enzymol.* **441**:261–282; 2008.
- Jourd'heuil, D.; Jour'dheuil, F. L.; Kutchukian, P. S.; Musah, R. A.; Wink, D. A.; Grisham, M. B. Reaction of superoxide and nitric oxide with peroxynitrite. *J. Biol. Chem.* **276**:28799–28805; 2001.
- Zielonka, J.; Kalyanaraman, B. "ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis"—a critical commentary. *Free Radic. Biol. Med.* **45**:1217–1219; 2008.
- Bass, D. A.; Parce, J. W.; Dechatelet, L. R.; Szejda, P.; Seeds, M. C.; Thomas, M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **130**:1910–1917; 1983.
- Kettle, A. J.; Carr, A. C.; Winterbourn, C. C. Assays using horseradish peroxidase and phenolic substrates require superoxide dismutase for accurate determination of hydrogen peroxide production by neutrophils. *Free Radic. Biol. Med.* **17**:161–164; 1994.