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Review Article

Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects

Peter Wardman *

University of Oxford, Gray Cancer Institute, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK

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Abstract

Chemical probes for free radicals in biology are important tools; fluorescence and chemiluminescence offer high detection sensitivity. This article reviews progress in the development of probes for "reactive oxygen and nitrogen" species, emphasizing the caution needed in their use. Reactive species include hydrogen peroxide; hydroxyl, superoxide, and thiyl radicals; carbonate radical-anion; and nitric oxide, nitrogen dioxide, and peroxynitrite. Probes based on reduced dyes lack selectivity and may require a catalyst for reaction: despite these drawbacks, dichlorodihydrofluorescein and dihydrorhodamine have been used in well over 2000 studies. Use in cellular systems requires loading into cells, and minimizing leakage. Reactive species can compete with intracellular antioxidants, changes in fluorescence or luminescence possibly reflecting changes in competing antioxidants rather than free radical generation rate. Products being measured can react further with radicals, and intermediate probe radicals are often reactive toward antioxidants and especially oxygen, to generate superoxide. Common probes for superoxide and nitric oxide require activation to a reactive intermediate; activation is not achieved by the radical of interest and the response is thus additionally sensitive to this first step. Rational use of probes requires understanding and quantitation of the mechanistic pathways involved, and of environmental factors such as oxygen and pH. We can build on this framework of knowledge in evaluating new probes.

Keywords: Free radicals; Oxidative stress; Nitrosative stress; Fluorescent probes; Chemiluminescence; Hydroxyl radical; Carbonate radical; Nitrogen dioxide; Nitric oxide; Peroxynitrite

Contents

Abbreviations: $AscH^-$, ascorbate; DCF, 2′,7′-dichlorofluorescein; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; E^+, ethidium; ESR, electron spin resonance; FRET, fluorescence resonance energy transfer; HE, hydroethidine; HRP, horseradish peroxidase; LC^{2+}, lucigenin; LH^-, luminol; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-*a*]pyrazin-3-one; MPO, myeloperoxidase; RhH_2, dihydrorhodamine 123; RSH, thiols; SOD, superoxide dismutase; UH_2^-, urate.

* Corresponding author. Fax: +44 1923 835210.
E-mail address: wardman@gci.ac.uk.

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Introduction

With the rapid growth of interest in free radicals in biology has come a need to identify radicals being produced, to measure either their integrated production rates or the steady-state (instantaneous) concentrations, and to visualize their spatial distribution. The ideal chemical probe for a free radical, or a nonradical product such as hydrogen peroxide, would be highly reactive at low concentrations; specific; sensitive; without other reactivity; nontoxic; well-characterized chemically; easy to load into organelles, cells, or tissues without subsequent leakage or unwanted diffusion, excretion, or metabolism; readily available; easy to use without too specialized apparatus; and cheap. No probe matches this challenging specification, but probes generating either a fluorescent product or inducing chemiluminescence offer high sensitivity and can be applied using a variety of analytical techniques, from "plate readers" with fluorescence detection, through high performance liquid chromatography, to sophisticated optical microscopy involving multiphoton excitation with the potential to create three-dimensional images of radical generation in tissue.

A consequence of the rapid growth of the subject has been the widespread application of such probes as are available to diverse biological systems without a corresponding effort in developing new probes, or in characterizing appropriately existing ones. Many false conclusions may have been drawn. However, the balance is being restored with timely studies of the mechanisms involved in probe response, and better understanding of contraindications or pitfalls in their use, as well as wider effort to develop new probes to overcome the deficiencies. This article reviews the main areas of activity, sets out a logical framework for testing probe suitability in particular applications, and describes new probes not yet widely applied to biological systems but which have some potential. A detailed survey of many individual probes has been recently published [1], and there are earlier reviews [2–6]; to complement and update these, the present article focuses on more widely used probes, drawing attention to problems too often ignored, and presents a hopefully didactic overview of the important factors in probe application. A well-known commercial handbook of molecular probes has useful summaries, bibliographies, and tables of properties (see http://www.probes.invitrogen.com/handbook/).

Reactive oxygen and nitrogen (and sulfur, chlorine …) species potentially reactive toward the probes

Many readers of this Journal will be familiar with some individual routes to free radical generation in biology and the

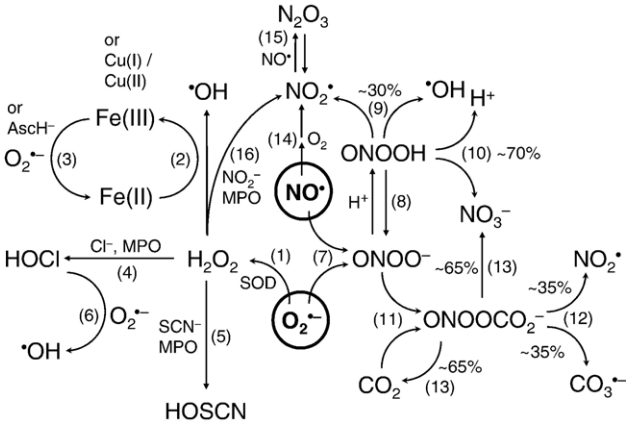

Fig. 1. Pathways to formation of other free radicals and reactive molecules in biology, via superoxide radicals and nitric oxide. Numbers in parentheses refer to the pathways discussed in the text.

"key players." However, a brief overview is presented here to emphasize the challenges in designing and selecting probes having any specificity, and to provide a framework for assessing probe reactivity. Fig. 1, adapted from a review discussing these species in the context of radiation damage [7], summarizes the main pathways to "reactive oxygen and nitrogen" species (actually also involving carbon, sulfur, and chlorine), illustrating the multiplicity of reactive radicals and nonradical molecules. While the use of terms such as "reactive oxygen species" is common, we should aim to reduce or even eliminate the use of such evidently imprecise terms as the behavior of individual radicals becomes better understood. Cellular antioxidants—particularly thiols, ascorbate, and urate—can interfere in these pathways for radical generation or reaction with probes, in several ways as discussed below.

Superoxide and nitric oxide (circled in Fig. 1) initiate the scenario. Broadly, the left-hand part of Fig. 1 groups well-known routes to "oxidative stress," while the right-hand grouping adds pathways, via nitric oxide, to "nitrosative stress." Here again terms are not always well-defined: chlorinating species are part of the first group, and two nonnitrosative species (hydroxyl and carbonate radicals) part of the second. Beginning with superoxide ($O_2^{\bullet-}$), disproportionation (path (1)) occurs to form hydrogen peroxide, whether or not superoxide dismutase (SOD) is present [8]. Routes to the formation in biology of highly reactive hydroxyl ($^{\bullet}OH$) radicals (or species with comparable reactivity) via pathway (2) involving Fenton chemistry catalyzed by Fe(II) or Cu(I) have been discussed extensively [9–14]; the catalytic metal is recycled by superoxide or ascorbate ($AscH^-$) (reaction (3)). In the presence of either chloride or thiocyanate, and myeloperoxidase (MPO), H_2O_2 forms hypochlorous or hypothiocyanous acids (pathways (4) and (5)). The former is a powerful oxidant and chlorinating agent [15,16] with characteristic "signatures" [17], and also capable in principle of generating $^{\bullet}OH$ on reaction with $O_2^{\bullet-}$ or Fe(II) (reaction (6)) [18,19]. In some biological milieu (e.g., saliva), high concentrations of thiocyanate (SCN^-) can compete with Cl^- to form HOSCN via MPO [20].

Reaction (7) of superoxide with nitric oxide to form peroxynitrite, in equilibrium (8) with peroxynitrous acid ($pK_a = 6.8$), introduces additional nitrosative and oxidative challenges [21–23]. The undissociated (acid) form (ONOOH), ~20% of the total at pH ~7.4, has a half-life of ~1–3 s at 37–25°C, decomposing via pathways (9) and (10) in the ratio ~30:70 to form hydroxyl radicals and nitrogen dioxide, and nitrate, respectively [24]. This does not necessarily mean that ~20% × 30% = 6% of peroxynitrite formed yields $^{\bullet}OH/NO_2^{\bullet}$, since ONOOH and $ONOO^-$ are in very rapid equilibrium and the overall outcome depends on other routes to remove either species, particularly reaction with HCO_3^-/CO_2 buffers [23,25–28]. Indeed, while many experiments in vitro involve media containing nonphysiological (low) levels of CO_2, it was argued [26] that the rapid reaction (11) of $ONOO^-$ with CO_2 largely outcompetes the homolysis pathways (9) and (10) in vivo (or in vitro using biomimetic buffers containing CO_2) because of the kinetics involved and the high levels of CO_2. The intermediate species ($ONOOCO_2^-$) formed in (11) decomposes in <1 μs, again in branching reactions, (12) and (13), to yield $NO_2^{\bullet}/CO_3^{\bullet-}$ or nitrate/CO_2 in the ratio ~1:2. This has important consequences in peroxynitrite chemistry [22–24,28,29]: both $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ are more selective radicals than $^{\bullet}OH$ in their reactions (and therefore also effectively more diffusive than $^{\bullet}OH$) [30]. (The rate constant database [30] can be accessed at http://www.rcdc.edu/.) Interest in the biological chemistry of $NO_2^{\bullet}$ and $CO_3^{\bullet-}$ is growing rapidly [31–34].

There are other possible routes to $NO_2^{\bullet}$ not involving peroxynitrite, although one still requires superoxide as a precursor to H_2O_2. Reaction (14) between $NO^{\bullet}$ and oxygen is a complex, multistep process with overall rate $\propto [NO^{\bullet}]^2[O_2]$, from which the half-life of $NO^{\bullet}$ for this reaction under physiological conditions where $[NO^{\bullet}] \ll [O_2]$ can be readily calculated for the relevant range of $[O_2]$ [31]. Even though both reactants will be at higher levels in lipid-rich compartments or in hydrophobic protein domains than in aqueous environments, the extravascular half-life of $NO^{\bullet}$ in vivo from other reactions is probably a fraction of a second, and the lifetime of $NO_2^{\bullet}$ is a few microseconds in the cytoplasm [35]. These short lifetimes imply very low steady-state or instantaneous cytosolic concentrations of both $NO^{\bullet}$ and (especially) $NO_2^{\bullet}$, and hence significant reaction of $NO^{\bullet}$ with O_2, and of $NO^{\bullet}$ with $NO_2^{\bullet}$, is unlikely in vivo [36,37], except perhaps under some extreme pathological conditions. In contrast, in nonbiomimetic models, bolus addition of convenient chemical sources of $NO^{\bullet}$ such as the "NONOates" [38] can result in such high concentrations of $NO^{\bullet}$ that, especially in (nonphysiological) air-equilibrated solutions, reaction between $NO^{\bullet}$ and O_2 occurs at a significant rate, possibly accompanied by N_2O_3 formation via (15). A number of studies of nitrosative reactions, including DNA modification, have used models involving unrealistically high concentrations of $NO^{\bullet}$ and/or O_2, where N_2O_3 formation via (14) and (15) can certainly occur [39], but more recent investigations have utilized more physiologically relevant conditions [40], at least of $NO^{\bullet}$ concentrations. Models, however, commonly lack physiological antioxidants reactive toward $NO_2^{\bullet}$, which normally maintain the concentration of the latter at very low levels, and extrapolation of such "nitration" studies to cellular and tissue conditions must be viewed with caution.

Formation of $NO_2^{\bullet}$ from nitrite via reaction (16) with H_2O_2 catalyzed by peroxidases (such as MPO [41–43] or lactoperoxidase [44]) is potentially a much more important route to $NO_2^{\bullet}$ than $NO^{\bullet}/O_2$. (Reactions consistent with peroxynitrous acid formation can result from H_2O_2 and nitrite under acidic conditions without catalyst [45]).

Potential reactions of probes with reactive radicals and oxidants must be considered alongside parallel reactions with cellular targets, mainly antioxidants, and the additional reactions thus introduced. This is discussed in more detail below, but the principles are outlined in Fig. 2. Reactions of $^{\bullet}OH$, $CO_3^{\bullet-}$, and $NO_2^{\bullet}$ with probes (multiple reactions abbreviated to pathway (a)) are in competition with reactions with abundant antioxidants such as thiols (pathway (b)) [30,35,46], urate (c) [35,47], and ascorbate (d) [30,48]. Another factor in considering the roles of antioxidants is that the products of oxidation, notably in the case of thiols, can be quite powerful oxidants

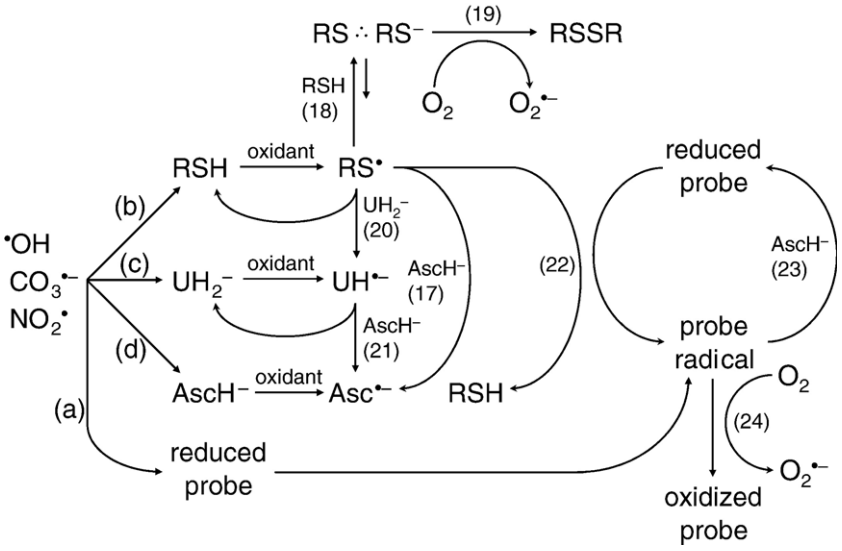

Fig. 2. Competition between reactions of oxidizing radicals (which may also include peroxidase intermediates) with reduced probes (path (a)) and antioxidants (paths (b)–(d)), and interactions between antioxidants and probes; RSH, thiols (e.g., GSH); UH_2^-, urate, $AscH^-$, ascorbate. Numbers in parentheses refer to the reactions discussed in the text.

reactive toward probes. Hence we should consider, in addition to what might be considered as "primary" radicals ($O_2^{\bullet-}$, $NO^\bullet$), "secondary" radicals ($^\bullet OH$, $NO_2^\bullet$, $CO_3^{\bullet-}$) and secondary nonradical products ($ONOO^-$/ONOOH, HOCl, etc.), the consequences of "tertiary" radical formation. The latter includes thiyl radicals formed directly from reactions of secondary radicals with thiols (Fig. 2, path (b)), and by "repair" of carbon-centered radicals formed from secondary radicals. Thiyl radicals rank as quite powerful biological oxidants, not dissimilar to $NO_2^\bullet$ [49] (see below). In tissues, thiyl radicals are likely to react mainly with ascorbate (Fig. 2, reaction (17)) as the dominant radical "sink" [50]; in cells cultured in vitro, commonly lacking ascorbate and hence not necessarily representative of tissues, conjugation (18) of thiyl and thiol(ate) to form superoxide via (19) [51] may compete effectively with oxidation of urate (20) (the latter is probably an important antioxidant in the vascular compartment [35]). If urate is oxidized, ascorbate can still serve as the ultimate "sink" via (21) [47]. When an oxidizable probe is present, the potential reaction of thiyl radicals with reduced probe, forming a probe radical via (22), must be considered in competition with the other "sinks" of thiyl radicals. The probe radical might interact with cellular antioxidants such as ascorbate (23), reversing oxidation, or with oxygen, reaction (24) (see below) to generate superoxide.

This survey highlights the main potential pathways of relevance for reactions with probes in the present context, although different probes exhibit different absolute reactivity. For brevity some topics have been omitted, especially where understanding is still emerging. These include the sources and reactions of the reduction product of $NO^\bullet$, nitroxyl (HNO): a review [52] and a recent article [53] point to the diversity of reactions and controversies. Singlet oxygen can best be detected directly from its emission at 1269 nm [54]; some probes for singlet oxygen have been reviewed recently [1]. A topic also excluded is of the use of probe luminescence to probe monitor lipid peroxidation, such as those based on energy transfer from excited carbonyl species formed during peroxidation (e.g., [55]), oxidation of phosphine derivatives [56], and lipid-targeted fluoresceins [57] or C11-BODIPY [58]).

Oxidation as a common activation route for probes

As an alternative to listing probes and discussing individually their reactivity, merits, and drawbacks in turn [1], it is instructive and complementary to develop a common framework in which groups of probes can be discussed and to evolve a rationale for evaluation more generally. Consideration of the chemical properties of most of the species of interest in Fig. 1 dictates that susceptibility to oxidation must be a key property for probe design or selection and, conversely, that oxidation is unlikely to be selective. Thus hydroxyl, carbonate, nitrogen dioxide, superoxide, and thiyl radicals are all quite powerful oxidants, with midpoint electrode (reduction) potentials vs NHE at pH ~7.4 of around 2.3 V ($^\bullet OH,H^+/H_2O$), 1.7 V ($CO_3^{\bullet-},H^+/HCO_3^-$), 1.0 V ($NO_2^\bullet/NO_2^-$), 0.9 V ($O_2^{\bullet-},2H^+/H_2O_2$), and 0.9 V ($GS^\bullet,H^+/GSH$), respectively [49,59]; peroxynitrite has a reduction potential at pH 7 of 1.6 V ($ONOO^-,2H^+/NO_2^\bullet$) [60]. (A didactic introduction and compilation of reduction potentials [59] can be accessed at: http://www.nist.gov/srd/PDFfiles/jpcrd372.pdf.) We should note that $^\bullet OH$ invariably adds to, or abstracts H from, organic molecules rather than simple electron transfer, and kinetic factors may slow down, or even reverse, apparently thermodynamically favorable electron transfer.

Typical redox dyes used as probes

Not surprisingly, then, by far the widest used probes for free radicals in the present context are dyes which are stable in a reduced state but can be oxidized by the species of

interest, oxidation being accompanied by a change in bonding to form highly resonant moieties which results in an increase in fluorescence. If fluorescein is the definitive fluorophor, then reduced fluoresceins (dihydrofluoresceins) are the prototypical probes for oxidizing radicals and H_2O_2 (care is needed in searching the literature since the term dichlorofluorescin is also used to describe dichlorodihydrofluorescein). The reduced dyes are often colorless ("leuco") and nonfluorescent, while the oxidized dyes are highly colored and fluorescent. Fig. 3 shows the four pairs of reduced and oxidized dyes probably most used. In fluoresceins and the phenoxazine, Amplex Red, the functional moiety for oxidation is phenolic, oxidized to a partially quinone-like structure; in rhodamines, amino substituents replace phenolic moieties, forming an imine/iminium on oxidation. Ethidium is reduced at the quaternary nitrogen to form hydroethidine (HE); while comparison of the latter with dihydropyridines such as NADH has been made [61], analogy with benzidine ([1,1′-biphenyl]-4,4′-diamine) is also possible since the site of deprotonation of the radical obtained on oxidation is not necessarily adjacent to the reaction center.

It is not possible to review the thousands of studies in free radical biology that have relied on these probes. Whether such reliance is justified, however, is an important question best addressed by considering the chemical properties of the probes, particularly chemical reactivity. More physical properties, such as solubility, biodistribution, absorption spectra (including two-photon cross sections), fluorescent emission

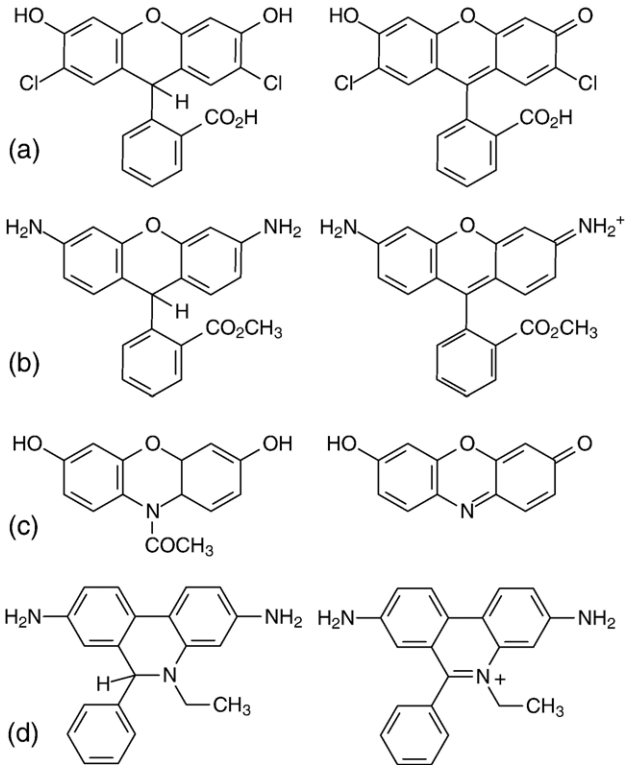

Fig. 3. Common probes for oxidants in biology. Left: reduced probe; right, oxidized (fluorescent) product. (a) Fluoresceins, e.g., $DCFH_2$; (b) rhodamines, e.g., RhH_2; (c) phenoxazines, e.g., Amplex Red; (d) ethidines, e.g. DHE.

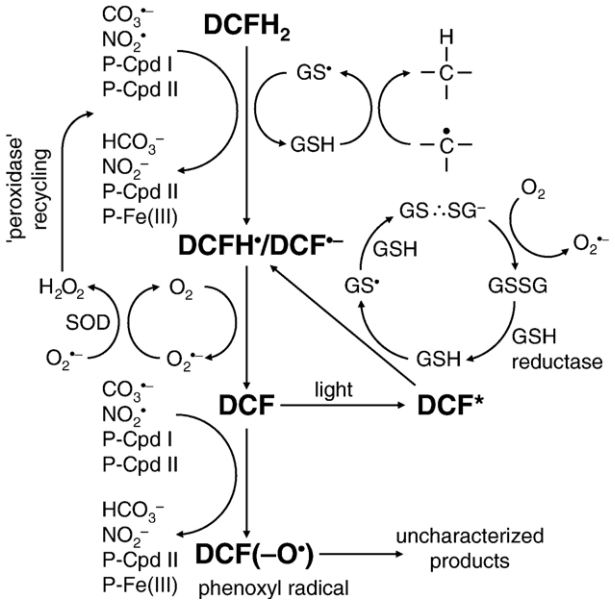

Fig. 4. Main reaction pathways possible in the use of reduced fluorescein dyes such as dichlorodihydrofluorescein to measure reactive species in cells. P, peroxidase-like catalysts.

spectra, quantum yields, fluorescence lifetimes, and susceptibility to photobleaching in microscopy, are all important but much can be gleaned from suppliers' data sheets and other reviews [1].

Reactivity of the key players in oxidative/nitrosative stress toward reduced redox dyes

Dihydrofluoresceins

Dichlorodihydrofluorescein ($DCFH_2$) is by far the commonest probe; the main reaction pathways involved in its use in biology are outlined in Fig. 4. We consider first the initial step, of oxidation of $DCFH_2$ to its radical $DCFH^{\bullet}$. $DCFH_2$ has very low reactivity toward superoxide radicals, or the dismutation product, hydrogen peroxide—although the latter can facilitate oxidation indirectly via a catalyst as described below: any "background" oxidation of $DCFH_2$ by $O_2^{\bullet-}$ or H_2O_2 probably reflects metal impurities in the buffer, etc. [62–65]. (Oxidation by Fenton chemistry ($Fe(II)/H_2O_2$) was characterized [66].) Superoxide dismutase had no effect on $DCFH_2$ oxidation when $O_2^{\bullet-}$ was generated enzymatically [64,66]; exceedingly weak fluorescence was observed when $O_2^{\bullet-}$ was generated by the radiolysis of oxygenated formate solutions (a highly specific, well-quantified and controllable source of superoxide) containing $DCFH_2$ [67]. Hence by itself (i.e., without a catalyst), $DCFH_2$ is not actually a useful probe for the two commonest "reactive oxygen species" in biology, despite still being widely viewed as such. It is also probably not effectively reactive directly with peroxynitrite (see below), although several studies have shown oxidation initiated by peroxynitrite (e.g., [68,69]). However, efficient conversion of $DCFH_2$ to DCF was reported using $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ as oxidants [67], species that can be formed via peroxynitrite as described above.

Hydroxyl radicals are very highly reactive toward aromatic molecules and, unsurprisingly, diffusion-controlled (i.e., maximal) reactivity of $^{\bullet}OH$ toward $DCFH_2$ was reported; however, the yield of fluorescent product was somewhat lower using $^{\bullet}OH$ as oxidant compared to using $CO_3^{\bullet-}$ or $NO_2^{\bullet}$ [67]. This probably reflects less specific reaction with $^{\bullet}OH$ than the other oxidants, such as some addition to the benzene ring substituent rather than oxidation of the phenolic moiety via ring addition/water elimination. Since $^{\bullet}OH$ radicals are so reactive toward a wide variety of biological substrates [30], probe reactivity toward $^{\bullet}OH$ is probably less important than reactivity toward other radicals: in cellular systems, $^{\bullet}OH$ will be intercepted ("scavenged") by $DCFH_2$ rather inefficiently unless cells are loaded with very high concentrations of $DCFH_2$ [70] (see below).

Carbonate radicals can be produced by reaction of $^{\bullet}OH$ with (bi)carbonate for chemical studies, and since $CO_3^{\bullet-}$ absorbs at 600 nm, its reactions can be easily followed if $^{\bullet}OH$ is generated by a short radiation pulse and microsecond spectrophotometry utilized. An estimate for the rate constant of $DCFH_2$ oxidation, $k_{25}=2.6\times10^8\ M^{-1}\ s^{-1}$ at pH 8.2 was reported [67], similar to that for oxidation of phenoxide ion by $CO_3^{\bullet-}$ [30]:

$$CO_3^{\bullet-} + DCFH_2 \rightarrow HCO_3^- + DCFH^{\bullet}. \quad (25)$$

It is technically difficult to use this method at pH 7.4 because HCO_3^- is much less reactive toward $^{\bullet}OH$ than CO_3^{2-} [30]; however, since the pK_as for dissociation of the phenolic moieties in $DCFH_2$, which might influence reactivity, are ~7.9 and 9.2 [71], reactivity of $CO_3^{\bullet-}$ toward $DCFH_2$ at pH 7.4 is probably little different from that at pH 8.2.) Although k_{25} is rather below the diffusion-controlled limit, it is still ~50-fold higher than the rate constant for reaction of $CO_3^{\bullet-}$ with glutathione or histidine, and ~6-fold higher than for reaction of $CO_3^{\bullet-}$ with tyrosine at pH 7 [30], but about the same as for reaction with tryptophan [30] or lipoic acid [72].

Nitrogen dioxide can be conveniently prepared from $^{\bullet}OH$ and nitrite but its absorbance is so weak that its production in real time must be monitored via a substrate that forms a colored, stable chromophore; the rate of formation of the chromophore is increased if a second, reactive substrate is included. Using this method, an estimate of the rate constant for reaction of $NO_2^{\bullet}$ with $DCFH_2$ at pH 7.5 of $1.3\times10^7\ M^{-1}\ s^{-1}$ was reported [67],

$$NO_2^{\bullet} + DCFH_2 \rightarrow NO_2^- + DCFH^{\bullet} + H^+, \quad (26)$$

a value only slightly lower than that characterizing reactivity of GSH with $NO_2^{\bullet}$ [35], with significant implications for the use of the probe (see below).

Thiyl radicals can be formed either by reaction of thiols with oxidizing radicals (Fig. 2), peroxidase intermediates [73], or nonspecific radical "repair", e.g., of carbon-centered radicals as shown in Fig. 4. Thiyl radicals derived from oxidation of glutathione oxidize $DCFH_2$ with a rate constant $k_{27}\ \sim4\times10^7\ M^{-1}\ s^{-1}$ at pH 7.4 (M. Wrona, personal communication):

$$GS^{\bullet} + DCFH_2 \rightarrow GSH + DCFH^{\bullet}. \quad (27)$$

Measuring the rate constants for reactions of $^{\bullet}OH$, $NO_2^{\bullet}$, and $CO_3^{\bullet-}$ with $DCFH_2$ is important not only in considering competition for reaction of these species between probe and endogenous scavengers (see below), but also in assessing the reactivity of the probe toward peroxynitrite. If reaction in a test system is limited by the rate of homolysis of ONOOH, and independent of the probe concentration, then reaction can be assumed to occur via the decomposition products ($^{\bullet}OH$, $NO_2^{\bullet}$, $CO_3^{\bullet-}$), since the rate constants for reaction of $DCFH_2$ with these radicals are so high; if reaction is faster than homolysis, then reaction is directly with ONOOH/$ONOO^-$. While it was originally thought that $DCFH_2$ is oxidized directly by peroxynitrite [65,74,75], a subsequent kinetic study [76] showed that probe oxidation mirrored the peroxynitrite homolysis rate and was independent of probe concentration, consistent with indirect reaction via the radical products of peroxynitrite decomposition, $^{\bullet}OH$, $NO_2^{\bullet}$, and $CO_3^{\bullet-}$. It would be desirable to confirm this applied to higher concentrations of $DCFH_2$ or RhH_2 than the 10 or 12.5 μM used in this study [76], to completely discount direct reaction of probes with peroxynitrite at high (e.g., 300 μM) cellular loading.

Hypochlorous acid oxidizes $DCFH_2$ to DCF but with low efficiency [65].

Dihydrorhodamines

Dihydrorhodamine (RhH_2) is, like $DCFH_2$, unreactive toward $O_2^{\bullet-}$ or H_2O_2 in the absence of catalyst [65,77]. However, RhH_2 forms fluorescent rhodamine efficiently on reaction with free $^{\bullet}OH$, $CO_3^{\bullet-}$, or $NO_2^{\bullet}$ radicals, with $k_{28}\sim6.7\times10^8\ M^{-1}\ s^{-1}$ [67], similar to that for reaction of $CO_3^{\bullet-}$ with aniline [30]:

$$CO_3^{\bullet-} + RhH_2 \rightarrow HCO_3^- + RhH^{\bullet}. \quad (28)$$

$NO_2^{\bullet}$ oxidized RhH_2 (~20 μM) on the submillisecond timescale; radical formation and disproportionation overlapped, preventing estimation of k_{29}, although it is likely to be $\sim10^8\ M^{-1}\ s^{-1}$ [67]:

$$NO_2^{\bullet} + RhH_2 \rightarrow NO_2^- + RhH^{\bullet} + H^+. \quad (29)$$

Dihydrorhodamine, like $DCFH_2$, is oxidized by peroxynitrite indirectly via the decomposition (radical) products [76]. Hypochlorous acid oxidizes RhH_2 to rhodamine with much higher efficiency than the corresponding reaction with $DCFH_2$ [65]. RhH_2 is also reactive toward chloramines (e.g., that produced on reaction of HOCl with taurine) in the presence of iodide, I^- forming ICl and then HOI, the oxidant reactive toward RhH_2 [78].

Hydroethidine

This probe behaves quite differently to the reduced fluoresceins or rhodamines. First, it is much more reactive than $DCFH_2$ or RhH_2 toward superoxide radicals, with the rate constant for reaction between $O_2^{\bullet-}$ and HE being $\sim2\times10^6\ M^{-1}\ s^{-1}$ [61]. Although early studies assumed ethidium (E^+) was produced from superoxide [79], a different product is formed [80]; this is now known to be 2-hydroxyethidium, with fairly similar, but distinguishable, fluorescence properties

compared to E^+ [81–83]. Other oxidants can generate this product [84,85], but in the present context it does appear to be one of the more specific fluorescent probes for superoxide reported to date. A similar conclusion, including similar reactivity toward $O_2^{\bullet-}$, was drawn for a variant of HE with a hexyl triphenylphosphonium cation as substituent, targeting the probe to mitochondria [86]. HE reacts with H_2O_2 via peroxidase catalysts [87], or with heme proteins [88], to produce products which may interfere with $O_2^{\bullet-}$ detection. Distinction can be made between extra-and intracellular production of superoxide [89]. The problem of uncontrolled fluorescence enhancement due to intercalation of 2-hydroxyethidium with variable amounts of mitochondrial DNA has been addressed [90].

Reduction of cytochrome *c* by HE, and catalysis of superoxide disproportionation, was reported [80]. Kinetic data for reactions of peroxynitrite-derived oxidants with HE are lacking. The ring-substituted product from $O_2^{\bullet-}$ and HE is formed in a two-stage process, as shown in Fig. 5 (cf. probes for nitric oxide, below). Unlike the case with $NO^{\bullet}$, superoxide can accomplish the initial oxidation step. It is probable (but not yet demonstrated) that the response of hydroethidine to superoxide can be altered by the flux of other oxidizing radicals capable of the initial oxidation step. Overall, hydroethidine appears to offer the attraction of some specificity for superoxide but there remain some important qualifications as to its use in quantitation.

Other reduced dyes

Comparable effort has not been directed toward other probes, but the comparison above of the reactivity of $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ toward $DCFH_2$ or RhH_2 with reactions of these radicals with phenol or aniline are important pointers suggestive that other reduced fluoresceins [91], Amplex Red [92], and dihydrocalcein [93] will behave qualitatively similarly to $DCFH_2$; other reactions of rhodamines, too, can be predicted by appropriate comparison with aromatic amines.

Importance and role of catalysts in probe oxidation

While superoxide is unreactive toward the common probes based on fluorescein or rhodamine, its dismutation product, H_2O_2, can participate in catalyzed oxidation cycles. Catalysts can include trace iron and copper (e.g., buffer impurities) via Fenton chemistry (Fig. 1, reaction (2) [66]), but the commonest catalysts are hemes. Peroxidase [62] or hematin [63] was used as a required component in early studies applying $DCFH_2$ to measure H_2O_2 (and a wide variety of organic hydroperoxides [63]), but this requirement seems to have been overlooked by many authors despite the importance of catalysts being demonstrated in these and several subsequent studies. Peroxidase-like catalysis of $DCFH_2$ and/or RhH_2 oxidation has been reported by the commonest peroxidase, from horseradish (HRP) [64,94–96], but also with cytochrome *c* [65,77,91,97–99], which is a general catalyst for oxidation of a variety of organic molecules [100] and nitrite [101] by H_2O_2.

It has been pointed out (V. Darley-Usmar, personal communication) that cytochrome *c* cannot function as a peroxidase until modified, because of the absence of a free coordination site for substrate. Indeed, there is often a lag phase in oxidation by cytochrome *c*/H_2O_2 [97,102], consistent with such a modification being required. Studies of oxidative modification of cytochrome *c* by H_2O_2 in cell-free systems have identified tyrosyl radicals [103,104] and dityrosine formation [105], indicators of electron transfer from an oxo-ferryl heme species similar to peroxidase Compound I [99]; in cells, cardiolipin is involved in controlling its peroxidase activity [106,107]. Oxidation of cytochrome *c* by HOCl [108] or nitration by peroxynitrite [109,110] or its radical decomposition products [111] markedly enhances its peroxidase activity, as does carboxymethylation [112].

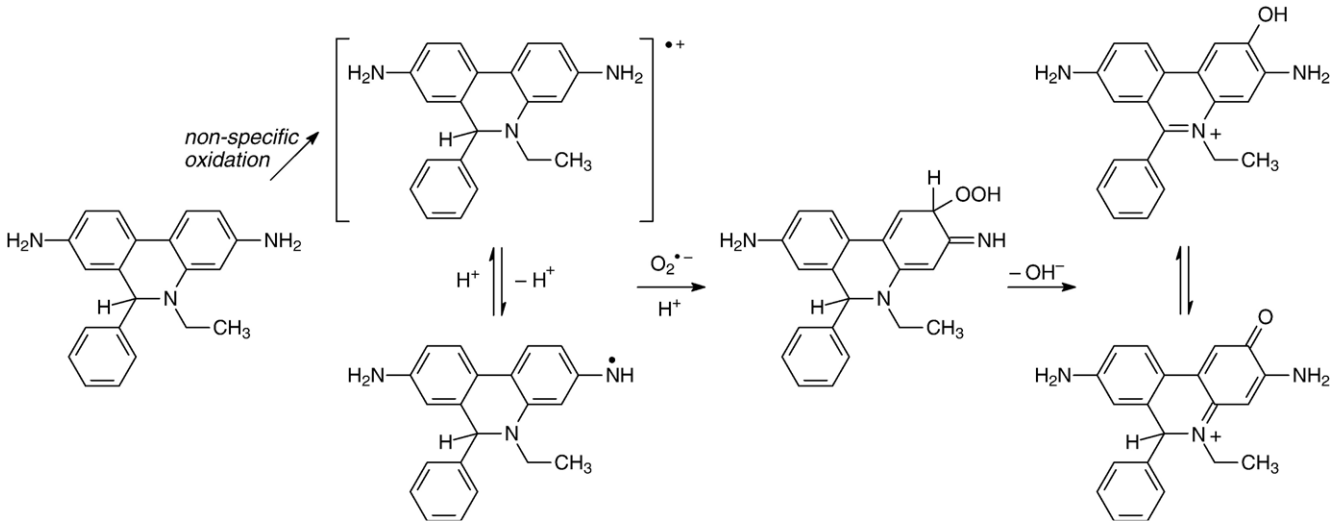

Fig. 5. Formation of 2-hydroxyethidium from superoxide radicals. Oxidation to a radical-cation is followed by addition of superoxide and rearrangement of the hydroperoxide intermediate. Only illustrative resonance forms are shown. Based on schemes by Zielnoka et al. [61,84]. In related work, Robinson et al. suggested hydroxylation on the alternative aminoaryl moiety [86].

Other catalysts reported having peroxidase-like activity with $DCFH_2$ include free iron [91,113]; hemoglobin and myoglobin [98]; catalase [91,94]; Cu/Zn SOD [91,114–116]; xanthine oxidase [66,91,117]; lipoxygenase [91]; and prostaglandin H synthase [118]. In Fig. 4 the "peroxidase" cycles are represented by Cpd I/Cpd II and Cpd II/native enzyme couples, but proteins such as SOD and xanthine oxidase effect their "peroxidase"-like action in different routes, e.g., involving $CO_3^{\bullet-}$ as an intermediate [117,119] or linked to trace iron [120]. The cellular labile iron pool has been shown to be important in this context [121]. Redox-active compounds such as pyocyanin oxidize $DCFH_2$ directly [122].

Sufficient H_2O_2 is produced by dye autoxidation to "drive" the peroxidase cycle with $DCFH_2$ and HRP even in the absence of added H_2O_2 [95], probably by the intermediate $DCFH^{\bullet}$ radical reacting with O_2 to yield $O_2^{\bullet-}$ and hence H_2O_2 (see below) [96]. In contrast, as noted by Ischiropoulos et al. [75], dihydrorhodamine oxidation is more H_2O_2 dependent.

Catalysis of oxidation by cytochrome *c*, in particular, has profound implications for the use as probes of reduced dyes such as $DCFH_2$ or RhH_2. This is because of its release into the cytosol during apoptosis [123–125]. Hence linking any phenomena observed during apoptosis (or a treatment that might initiate it) to enhanced generation of "reactive oxygen species," based solely on observations of increased production of DCF from $DCFH_2$, is not justified [97]. In model studies, only ~1 nM cytochrome *c* was sufficient to show catalysis of oxidation of $DCFH_2$ [97]; 10 nM cytochrome *c* was very effective with RhH_2 [77]. Although glutathione plays an inhibitory role depending on the oxidation state [99], further studies of $DCFH_2$ oxidation at higher concentrations, more representative of cellular loading, are needed to understand fully its role (see below).

Probe free radicals as obligate intermediates in probe oxidation

Oxidation of $DCFH_2$ (or RhH_2) to DCF (or rhodamine) is, overall, a two-electron process; yet as discussed above, potential two-electron oxidants such as H_2O_2 and peroxynitrite are not sufficiently reactive for two-electron oxidation to be of biological relevance. Indeed, the species reacting rapidly with the probes are the free radicals $^{\bullet}OH$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$, and thiyl radicals such as $GS^{\bullet}$, or one-electron oxidants from peroxidases and other proteins, reacting in a manner similar to peroxidase Compound I. These can abstract only single electrons from the reduced probes and thus, by definition, oxidation must involve a two-step process each involving one-electron steps. The radical oxidants must generate intermediate probe radicals, which we can designate $DCFH^{\bullet}$ and $RhH^{\bullet}$ in the case of the fluorescein or rhodamine probes. By analogy to semiquinone disproportionation to quinone and hydroquinone, formation of the oxidized, fluorescent probe can be envisaged via disproportionation of the radicals intermediate, e.g., by reaction (30):

$$2DCFH^{\bullet} \rightarrow DCF + DCFH_2. \quad (30)$$

The occurrence of such obligate radical intermediates has been generally neglected, except in a few studies, yet has major implications for the use of these probes, since these probe radicals are not inert: disproportionation is not their only possible fate (in fact, may be seldom their fate, as argued below).

Evidence for radical intermediates of probes in biomimetic systems

Changes in absorption spectra consistent with the reduction of HRP Compound I to Compound II (a one-electron step) on addition of $DCFH_2$ were observed by Rota et al. [95]. While one-electron oxidation of $DCFH_2$ to $DCFH^{\bullet}$ would be expected to generate a phenoxyl radical, which generally absorb around 400 nm with extinction coefficient ~2200 M^{-1} cm^{-1} [126], reactions (30) and (31) are so fast (see below) that the steady-state concentration of $DCFH^{\bullet}$ would be too low to detect optically against a background of HRP spectral changes. However, using spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), electron spin resonance (ESR) signals from the adduct of DMPO and superoxide radicals (DMPO/$^{\bullet}OOH$) were detected [95]. Because oxygen removal eliminated the signal, the authors proposed that this arose from reaction of the "DCF semiquinone radical ($DCF^{\bullet-}$)" with oxygen,

$$DCF^{\bullet-} + O_2 \rightarrow DCF + O_2^{\bullet-}, \quad (31)$$

with obvious and important implications: peroxidase-catalyzed oxidation of $DCFH_2$ generates the species that some users of the probe probably imagine they are measuring. (In this article the DCF "semiquinone" is usually represented as $DCFH^{\bullet}$ rather than $DCF^{\bullet-}$ for simplicity of balancing equations; the dissociation of $DCFH^{\bullet}$ to $DCF^{\bullet-}$ and H^+ is discussed below).

Direct observation of the "DCF semiquinone" radical by ESR was possible when visible light (>300 nm) was shone on solutions of DCF, DMPO, and the biologically important reducing agents, GSH and NADH [127]. The mechanism involves photo-excitation ($h\nu$) of DCF to excited state(s) (DCF*) which oxidize GSH (or NADH) to form both $DCFH^{\bullet}$ and $GS^{\bullet}$ (or $NAD^{\bullet}$) radicals:

$$DCF + h\nu \rightarrow DCF^* \quad (32)$$

$$DCF^* + GSH\ (NADH) \rightarrow DCFH^{\bullet} + GS^{\bullet}\ (NAD^{\bullet}). \quad (33)$$

In the absence of oxygen, pH-dependent ESR signals from $DCFH^{\bullet}/DCF^{\bullet-}$ were observed [127]; at a steady state, radicals from $NAD^{\bullet}$ would be difficult to detect since they disproportionate faster than $DCFH^{\bullet}/DCF^{\bullet-}$; $GS^{\bullet}$ radicals were spin-trapped with DMPO as DMPO/$^{\bullet}SG$ [127]. In the presence of oxygen, oxygen was consumed [127] since both $NAD^{\bullet}$ [128] and $GS^{\bullet}$ [129] react rapidly directly or indirectly, respectively, to form superoxide:

$$NAD^{\bullet} + O_2 \rightarrow NAD^+ + O_2^{\bullet-} \quad (34)$$

$$GS^{\bullet} + GSH \rightleftarrows GSSG^{\bullet -} \ (+H^{+}) \quad (35)$$

$$GSSG^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -}. \quad (36)$$

Properties of the intermediate radicals in probe oxidation

The same radical, "$DCFH^{\bullet}/DCF^{\bullet -}$", was formed either on oxidation of $DCFH^{\bullet}$ by the selective one-electron oxidant $N_3^{\bullet}$ or on reduction of DCF by $CO_2^{\bullet -}$, a highly reducing one-electron donor [71]. The absorption spectra were pH dependent, assigned to dissociation of the phenolic moieties. The fully reduced probe "$DCFH_2$" has two pK_as associated with dissociation of the phenol substituents, estimated at 7.9 and 9.2 [71]. (Even though the molecule is drawn with two apparently identical phenolic moieties, dissociation of one phenol function changes the pK_a of the other, and the two pK_as must differ by at least $\log_{10}(4)$ or ~0.6 [130,131].) On oxidizing $DCFH_2$ to the radical, the pK_as shift somewhat, with values of ~7.1 and 9.0 suggested [71], but not easy to measure because of overlap.

These values are of more than academic interest. First, as discussed below, intracellular trapping of $DCFH_2$ relies on the negatively charged, dissociated probe having impeded diffusion through lipid membranes. The proportion of charged to uncharged prototropic forms is a function of these pK_as and the extracellular or intracellular pH. Secondly, these equilibria confer pH-dependent rates of oxidation of $DCFH_2$ by oxidants (phenolates are usually more rapidly oxidized than phenols), and of both disproportionation of $DCFH^{\bullet}$ (reaction (30)) and reaction of $DCFH^{\bullet}/DCF^{\bullet -}$ with oxygen (reaction (31)). Hence if the pH changes in systems using these probes—including in a subcellular organelle, which can occur with some drugs—the amount of DCF formed may vary. There are also pH-dependent absorbance/fluorescence properties of DCF itself, arising from ionizations of the phenolic protons. It is no coincidence that fluoresceins are used as pH indicators [132].

The rate constant for reaction (31) varied from ~0.3 to $1.0 \times 10^9\ M^{-1}\ s^{-1}$ between pH 6 and pH 9; $k_{31} = 5.3 \times 10^8\ M^{-1}\ s^{-1}$ at pH 7.4 [71]. This implies conversion of $DCFH^{\bullet}/DCF^{\bullet -}$ to superoxide occurs with a half-life of ~5 μs in laboratory models in equilibration with ambient air ($t_{½} = (\ln 2)/(k_{31}[O_2])$). The competing reaction (30) (disproportionation) also increased with pH, with $2k_{30} \sim 1.6–3.6 \times 10^8\ M^{-1}\ s^{-1}$ between pH ~6 and pH 9.5; at pH 7.4; $2k_{30} = 2.8 \times 10^8\ M^{-1}\ s^{-1}$ [71]. The half-life of this radical–radical reaction varies with radical concentration ($t_{½} = (2k_{30}[DCFH^{\bullet}])^{-1}$), but for it to equal that for reaction (31) in air-equilibrated media, a steady-state $DCFH^{\bullet}$ radical concentrations of ~0.7 mM would be required. This is far higher than is reasonable in cellular systems. (It is easily shown, using the steady-state equation: radical formation rate=sum of radical decay rates, and solving the quadratic equation resulting, that if the radical generation rate is ~0.6 μM s^{-1}, as suggested, for example, for superoxide production in mitochondria [133], then the steady-state concentration of $DCFH^{\bullet}$ controlled by reactions (30) and (31) alone is ~5 pM.) Hence DCF formation via (30) is much less likely than (31) except when O_2 levels are very low. Unless there are alternative reaction pathways not yet characterized (or exogenous substances added, such as quinones, see below), generation of superoxide is an inevitable by-product accompanying fluorescence (DCF formation) in the use of $DCFH_2$ in biology.

As with lucigenin (see below), superoxide formation accompanying probe oxidation or other activation step is not inevitably a contraindication: it could be viewed positively, as a useful amplifying reaction, with (31) as the chain-propagating step. However, any reactant for superoxide has potential to influence the chain length of the oxidation process and thus the overall response. Changes in levels of SOD, cytochrome *c*, GSH, and ascorbate, are examples. On balance, ill-defined signal amplification from reactivity of $DCFH^{\bullet}$ toward O_2, generating further $O_2^{\bullet -}$, introduces major uncertainty into quantitative measurements using $DCFH_2$ and similar probes; the rhodamine radical reacts with O_2 about as fast as $DCFH^{\bullet}$ (M. Wrona, personal communication).

Involvement of antioxidants in probe chemistry

Four separate aspects of the involvement of antioxidants in probe chemistry must be considered: direct competition between probe and antioxidants for reactions with radicals such as $CO_3^{\bullet -}$ and $NO_2^{\bullet}$; reaction of antioxidant radicals, such as thiyl radicals, with probes; reactions of antioxidants with intermediates in catalyst chemistry such as in peroxidases; and "repair" of probe radicals by antioxidants.

Rate constants for reaction of oxidizing radicals with probes enable us to assess the possibility that probe response might reflect changes in levels of intracellular antioxidants rather than, or as well as, changes in the flux of "reactive oxygen/nitrogen" species. The key comparison is with the product of rate constant and concentration of probe or antioxidant. The main problems in such a comparison are twofold: first, few users of the probes measure the intracellular concentrations; secondly, even if measured, the values usually reflect averaged cytosolic concentrations whereas comparison in a particular organelle may be more appropriate.

However, to illustrate this point, a representative concentration of $DCFH_2$ of 0.3 mM was assumed [67]. With this value, if intracellular [GSH] is 5 mM, while ~67% of $CO_3^{\bullet -}$ will be intercepted by the probe in competition with reaction with GSH, only ~4% of $NO_2^{\bullet}$ radicals will be scavenged by the probe. Other antioxidants, e.g., urate (particularly in plasma) and ascorbate (in tissue samples), will also have to be considered. Both cysteine and urate inhibited peroxynitrite-mediate oxidation of dihydrorhodamine [77]. It was therefore stressed [67] that many studies probably involve nonsaturating concentrations of probes. Changes in probe loading or in antioxidant levels will result in a change in probe response even at constant oxidizing radical flux. Of course, if "oxidative stress" is defined as "a disturbance of the prooxidant–antioxidant balance in favor of the former" [134], then probe response might encompass this broad definition.

Simple competition for reaction is not the only factor: reaction of oxidizing radicals with thiols generates thiyl radicals,

also reactive toward $DCFH_2$ (reaction (27)). The influence of thiol and oxygen concentrations on $DCFH_2$ oxidation by $GS^{\bullet}$ (i.e., the effects of reactions (35) and (36) in parallel with (27)) is currently being investigated (M. Wrona, personal communication). Effects of GSH/GSSG on DCF formation in the presence of cytochrome *c* [110,135] or HRP [136] have been reported, but using very much lower concentrations of $DCFH_2$ than used in cellular systems. Thus oxidation of $DCFH_2$ by thiyl radicals would have been much less likely in these studies than under more biomimetic conditions, since other pathways for thiyl radical reaction would be favored at such low $DCFH_2$ levels. The vitamin E analog, Trolox, enhanced DCF formation in a cell-free system [137], although the kinetics of radical transfer between the two phenols have not been reported.

The situation concerning competition with antioxidants may not be nearly so unfavorable for reaction of probes with H_2O_2 via secondary reactions involving peroxidase Compound I/II-like intermediates. Data here are particularly lacking; while aliphatic thiols are very poor substrates for both HRP and myeloperoxidase [138,139], thiyl radicals are produced from HRP and GSH [73]. In contrast, phenols (cf. $DCFH_2$) and anilines (cf. RhH_2) are excellent peroxidase substrates [140]. Rate constants for peroxidase-catalyzed oxidation of both groups of compounds are particularly susceptible to electron-donating/-withdrawing substituents [141]; meta-chloro substitution in a phenol (cf. $DCFH_2$) might be expected to slow down oxidation, but para-chloro substitution has, unexpectedly, the opposite effect [140,142]. Whether the claimed advantages of dihydrofluorescein over $DCFH_2$ [91] reflect differences in peroxidase-like reactivity is not known. Ascorbate is absent from most normal cell cultures in vitro (in itself a note of caution regarding biomimetic criteria) but ascorbate is much less reactive toward HRP Compound I/II than phenols or anilines [141,143], so this antioxidant, too, may be less efficient in reacting with peroxidase intermediates.

We should, however, distinguish between competition from antioxidants reacting directly with redox-active hemes or other catalytic centers and direct reaction of the antioxidant with the probe radicals produced from peroxidase-catalyzed oxidation. The effects of GSH on oxidation of $DCFH_2$ catalyzed by cytochrome *c* or HRP noted above may well involve "repair" *equilibria*; i.e., reaction (27) can also be represented as the reverse reaction of the equilibrium:

$$DCFH^{\bullet} + GSH \rightleftarrows DCFH_2 + GS^{\bullet}. \quad (37)$$

Even if the equilibrium constant $K_{37} \ll 1$, reaction between $DCFH^{\bullet}$ and GSH can be "driven" to the right by removal of $GS^{\bullet}$ from the equilibrium via reactions (35) and (36) and the other "sinks" for thiyl radicals shown in Fig. 2. Both ascorbate and urate are highly reactive toward phenoxyl radicals such as that from tyrosine [144,145], and would be expected to react rapidly with $DCFH^{\bullet}$. Redox properties of the radical obtained on oxidation of rhodamines are less predictable but it would be surprising if the rhodamine radical was not reactive toward (repaired by) the key antioxidants. Indeed, it was suggested that both urate [74] and ascorbate re-reduces the rhodamine radical [146].

Susceptibility of oxidized probes to further reactions and to photosensitize radical production

It is generally overlooked that $DCFH_2$ still has one oxidizable phenolic function even after oxidation to the normally measured oxidized form, DCF. This renders the fluorescent product, DCF, susceptible to further reaction with oxidizing species. The rate constant k_{38} for reaction of $CO_3^{\bullet -}$ with DCF (producing a phenoxyl radical, also obtained on oxidation with HRP as shown in Fig. 4 [147]) is essentially the same as for reaction (25) with $DCFH_2$ [67]:

$$CO_3^{\bullet -} + DCF \rightarrow HCO_3^- + DCF(-O^{\bullet}). \quad (38)$$

Thus if circumstances are such that a significant fraction of $DCFH_2$ is oxidized to DCF, further exposure to oxidants could lead to a decrease in the fluorescence from DCF. Further oxidation of fluoresceins produces coupled (dimer) products, and loss of fluorescence has been used in an "oxygen radical absorbance capacity" assay [148]. However, DCF was ~80-fold less reactive toward $NO_2^{\bullet}$ than $DCFH_2$ [67]. The initial nonradical product of RhH_2 oxidation still has an aromatic amine moiety susceptible to further oxidation, but unlike DCF, the rhodamine product from RhH_2 oxidation is ~200-fold less reactive toward $CO_3^{\bullet -}$ than the reduced dye [67].

The oxidized probes might be susceptible to reduction back to reduced probes. A key predictive property for this possibility is the reduction potential of the oxidized probe/radical couple. There are often correlations between this property and the rates of reduction catalyzed by flavoproteins [149–153]. The reduction potential of the $DCF,H^+/DCFH^{\bullet}$ couple is pH dependent with a value at pH 7.4 of −0.75 V vs NHE [71]. This is much lower than that for typical quinone/semiquinone couples [59], and so DCF might be expected to be a poor substrate for common flavoprotein reductases. (The radical $DCFH^{\bullet}$ is best compared with ketyl radicals rather than as a semiquinone [71].) However, quinones with reduction potentials around −0.2 V are often used in cellular models to stimulate oxidative stress by redox cycling, e.g., 2,3-dimethoxy-1,4-naphthoquinone, and such quinones are likely to react very rapidly with $DCFH^{\bullet}$ radicals because of the large "energy gap" of ~0.5 V and could thus compete with oxygen in reacting with $DCFH^{\bullet}$, depending on relative concentrations.

The observations described above of generation of the $DCFH^{\bullet}$ radical by visible light illumination of DCF in the presence of reducing agents such as NADH or GSH, leading to superoxide formation via reactions (33), and (34) or (36) [127,154], have obvious practical importance. Illumination of biological samples, once any DCF is formed, either with ambient light in the laboratory or from light sources in measuring instrumentation or microscopy, can generate radicals involved in oxidative stress. While added antioxidants can suppress these reactions [155], they are obviously liable to interfere with other pathways. The potential drawback of photochemical sensitivity has been exploited in another study. A variant of $DCFH_2$, with a chloromethyl substituent in the benzene ring, reacts with cellular thiol groups and is thus trapped intracellularly by covalent binding; two-

photon excitation of the bound DCF moiety was then used to control the spatial generation of superoxide [156].

Intracellular loading of probes and efflux of oxidized probes

$DCFH_2$ and related probes are loaded into cells by incubating them with the diacetate ester, typically a few micromolar in the extracellular media, relying on cellular esterases to cleave the diacetate and form free $DCFH_2$. Two reviews describe the procedure in detail [75,157]. The most careful studies (e.g., [77]) have measured both intracellular uptake and subsequent leakage under a variety of conditions, but most users of such probes have ignored the need for such measurements.

It is often assumed the esterase-sensitive hydrolysis aids intracellular trapping by forming charged species, but this assumption requires knowledge of the relevant pK_as of the probe and its oxidized product. Passive diffusion of weak acids across lipophilic membranes with an extracellular:intracellular pH gradient will generate concentration gradients dependent on ΔpH and pK_as [158]; this will apply both to the cytoplasmic membrane and to individual organelles. While the pK_as for dissociation of the phenolic groups in $DCFH_2$ are ~7.9 and 9.2 [71]), and will be similar in 5(6)-carboxy-2′,7′-dichlorodihydrofluorescein, the corresponding values for dihydrofluorescein will be perhaps ~0.8 higher, lacking the meta chlorine substituent (chlorine substituents are pK_a decreasing [159]). The phenolic pK_a in DCF is much lower (~5.2) than those in $DCFH_2$ and ~0.7 lower than in fluorescein [160]. Whether differences in intracellular uptake of $DCFH_2$ and its des-chloro analogue [91] reflect such shifts in pK_a, or other parameters such as membrane potential (shown to be important in fluorescein efflux [161]), is not clear. What is clear is that changes in intracellular pH gradients, or esterase activity (which can vary even between normal and drug-resistant cells in the same cell line [162]), can potentially influence probe loading and leakage of oxidized probe.

Other factors can affect leakage of both reduced and oxidized probes, in addition to membrane potential. Multidrug resistance-associated protein was shown to influence efflux from cells of fluorescein and dihydrofluorescein [163], and rhodamine [164]; glutathione depletion inhibited efflux.

Cellular loading of dihydrorhodamine does not rely on esterases, and once produced, rhodamine leakage appears to be less of a problem than with DCF [77]. Binding of dihydrofluoresceins substituted with thiol-reactive functional groups (e.g., chloromethyl [165]) aids intracellular retention but obviously not without the possibility of perturbation of cellular functions, and this approach to cellular loading must be approached with caution. It is desirable to ascertain whether thiol selectivity of chloromethyl conjugation (toward GSH compared to protein thiols) is conferred via glutathione-*S*-transferase activity, and the extent of probe loading, GSH depletion, and protein-thiol binding.

The importance of knowing the intracellular concentration of probes has been noted above in the context of cellular antioxidants, but is so important that it is worth stressing. Most readers will be familiar with enzyme-based assays where reagents such as cofactors are included at "saturating" concentrations, and would not dream of using an assay where response is varying sharply with concentration of such a cofactor. Yet that is probably precisely the case with the common use of reduced dyes as probes. As noted above, the relevant free radical reactants ($^{\bullet}OH$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$) are all highly reactive toward cellular antioxidants, especially GSH, urate, and ascorbate; $^{\bullet}OH$ is of course highly reactive toward most biomolecules. Unless intracellular concentrations of $DCFH_2$ and RhH_2 exceed several millimolar, the probes cannot compete efficiently with endogenous antioxidants for reaction with these radicals (particularly in the case of $NO_2^{\bullet}$; less so for $CO_3^{\bullet-}$ [67]). Probe response will be often, perhaps always, under nonsaturating conditions and will vary directly with variations in probe loading or efflux, and/or antioxidant concentrations. Detailed comparison requires estimates of intracellular concentrations, which are seldom available.

Recently developed probes as alternative to reduced dyes

Probes reactive toward H_2O_2 or $O_2^{\bullet-}$

A variety of new approaches are being developed based on rational principles; thus emission from fluorescein can be finely modulated depending on redox substituents in the benzene ring [166,167]. Three groups [168–172] have reported probes for superoxide/hydrogen peroxide based on "nonredox" mechanisms, exploiting the nucleophilic character of superoxide. Thus profluorophors bearing substituted aryl or perfluoroalkyl sulfonyl ester linkages are cleaved to release the free fluorescein [169] or naphthofluorescein [172] moiety. One probe with a dinitrobenzenesulfonate "leaving group" (Fig. 6 (a)) was reported to have selectivity toward $O_2^{\bullet-}$ as a reactive nucleophile compared to H_2O_2 [169], while others using a pentafluorobenzenesulfonate substituent showed (apparently uncatalyzed) reactivity toward H_2O_2 with rate constants up to ~250 M^{-1} s^{-1} reported [168]. Recent progress in optimizing this approach has been described [173]. Naphthofluoresceins with perfluoroheptanesulfonate or *p*-tosylate (Fig. 6(b)) leaving groups were more reactive toward H_2O_2 than $O_2^{\bullet-}$, although treatment with some other reagents did result in some fluorescence [172]. Diphenylphosphinate leaving groups cleaved by superoxide to release a naphthofluorescein was the basis for imaging superoxide in macrophages [174].

A novel variation using chemoselective boronate deprotection of cell-permeable boronate dyes by hydrogen peroxide has been reported (Fig. 6(c)) [170,171]. While catalysts do not appear to be required for probe response, reactivity is not high: rate constants for reaction of the boronate dyes with H_2O_2 of ~0.5–1 M^{-1} s^{-1} were indicated [171]. This low reactivity must limit cellular applications to where catalase and glutathione peroxidase do not remove H_2O_2 efficiently.

Another new approach to H_2O_2 determination [175] involved exploiting the concept of photoinduced electron transfer. A rationally designed probe (Fig. 6(d)), in which a

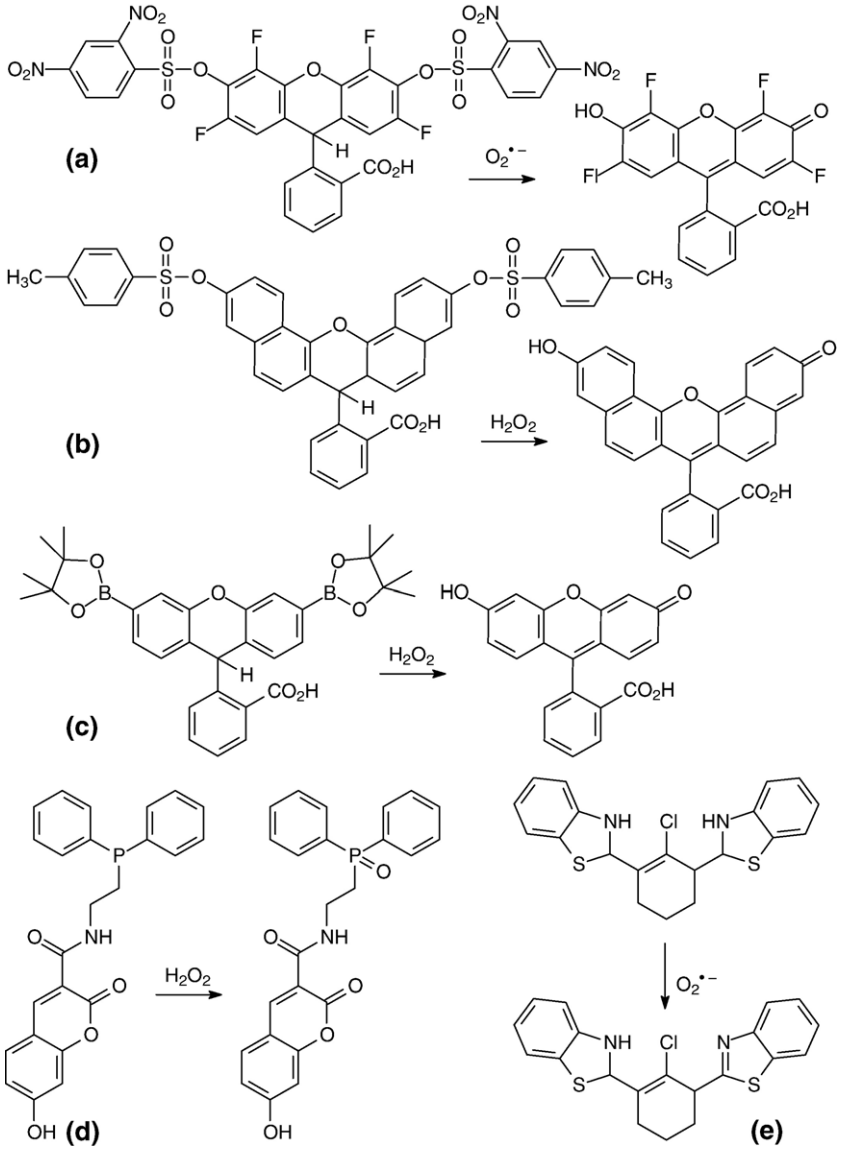

Fig. 6. Alternative approaches to generate fluorescent products by reaction of probes with $O_2^{\bullet-}$ or H_2O_2, based on displacement of a "leaving group" ((a)–(c)); photoinduced electron transfer (d) (probes on left, fluorescent product measured on right); or oxidation of a benzothiazolidine (e). From (a) Maeda et al. [169]; (b) Xu et al. [172]; (c) Miller et al. [171]; (d) Soh et al. [175]; (e) Gao et al. [178]; the reactants shown are those suggested by the authors.

diphenylphosphine moiety (oxidized on reaction with H_2O_2) was linked to a 7-hydroxycoumarin was evaluated, with off/on fluorescence switching accompanying oxidation. However, the reactivity toward H_2O_2 seems undesirably low, fluorescence appearing over tens of minutes at 50 μM H_2O_2. A probe for H_2O_2 based on a europium–tetracycline complex is similarly independent of the need for catalysts which characterizes the reduced dyes [176,177]; a ~1 μM detection limit in aqueous solution was reported, but phosphate interfered. Recently, other new probes for superoxide (e.g., Fig. 6(e)) have been described which show promise [178], although kinetic information is lacking. The "use of disulfides as redox reservoirs in biology" prompted the synthesis of a conjugate of fluorescein with naphthalene-1,8-disulfide [179], reversibly sensitive to oxidation by H_2O_2; again, full characterization is needed.

Probes reactive toward peroxynitrite, peroxyl radicals, and other oxidants

In somewhat related studies having potentially wider applicability but less selectivity, fluorescein esters with oxidizable phenolic or anilinyl substituents have been shown to react with $^{\bullet}OH$, $ONOO^-$, and ClO^- to release free fluorescein [180] (Fig. 7(a)). While this concept is, in fact, based on oxidation, it is a substituent in fluorescein that is oxidized, and not dihydrofluorescein, and thus the approach shares some conceptual basis with the above probes based on nucleophilic release of a leaving group. The work extends earlier related studies on probes for $^{\bullet}OH$ [181] (see below).

From observations of cyclization reactions of some ketones on treatment with peroxynitrite, a probe was developed (Fig. 7(b)) which is based on oxidation modifying a ketone substituent to

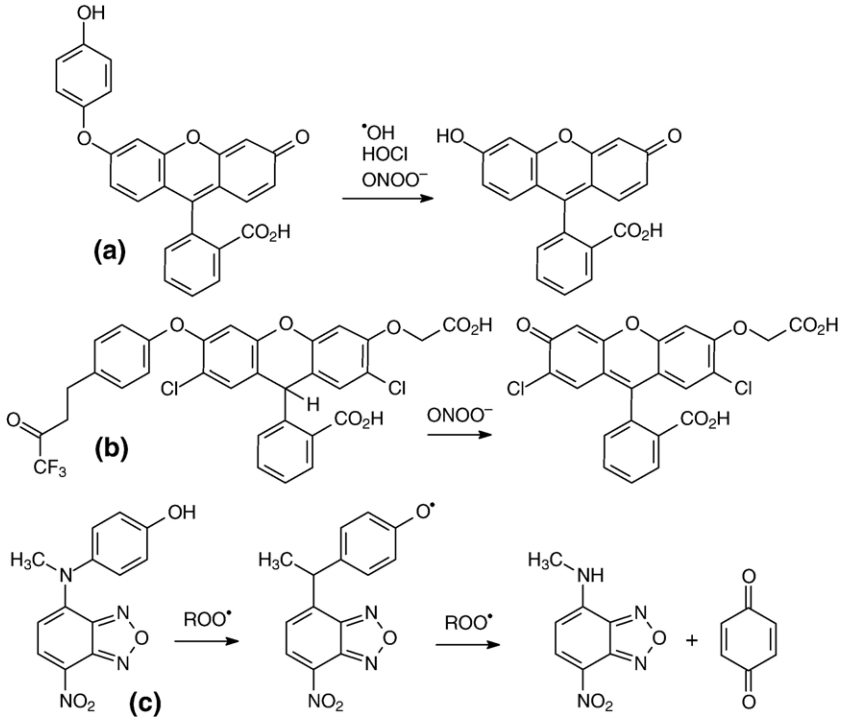

Fig. 7. Probes responsive toward peroxynitrite, •OH and HOCl ((a), from Setsukinai et al. [180]); peroxynitrite ((b), from Yang et al. [182]); or peroxyl radicals ((c), from Heyne et al. [183]).

release the free fluorescein derivative [182]. Selectivity toward peroxynitrite, and fluorescence from neuronal cells treated with the peroxynitrite donor, SIN-1, was reported, although whether reaction is actually via $NO_2^{\bullet}$ and/or $CO_3^{\bullet-}$ (cf. $DCFH_2/RhH_2$ [76]) is unknown. A probe reactive toward peroxyl radicals has been recently described [183] (Fig. 7(c); note the release of benzoquinone in this particular example). Oxidation of a phenol/fluorescein conjugate by peroxyl radicals stimulated release of free fluorescein in a related probe [184].

These studies illustrate both current interest in developing new probes which avoid some of the pitfalls of the reduced dyes, and the elegance of new rationally designed probes, but further studies are needed to characterize reactivity as well as the potential of interference by biomolecules.

Chemiluminescent probes for superoxide

Lucigenin: source and sink of superoxide

Chemiluminescence can be an extremely sensitive detection method for radical production. Lucigenin, bis-*N*-methylacridinium dinitrate (LC^{2+}, Fig. 8), has been used in well over a thousand studies as a luminescent indicator of superoxide production. However, there is no doubt whatsoever that, like $DCFH_2$, the probe has the potential to generate superoxide radicals. Luminescence occurs via addition of superoxide to the free radical obtained on reduction of lucigenin ($LC^{\bullet+}$), which forms an unstable dioxetane (Fig. 8) [185]. The dioxetane cleaves spontaneously to produce *N*-methylacridone in an excited state; it is this which emits light. Lucigenin is not specific for superoxide: numerous nucleophiles and reducing agents can induce luminescence (including alkaline H_2O_2 [186,187]). This is not surprising, since reducing agents can generate the radical $LC^{\bullet+}$; this generates superoxide via reaction (39), which can be observed either indirectly, e.g., by cytochrome *c* reduction [188,189], more specifically by spin trapping the superoxide produced [190], or directly with real-time observation of the absorbance of $LC^{\bullet+}$ [191], with forward rate constant $k_{39} \sim 3\times10^6\ M^{-1}\ s^{-1}$:

$$LC^{\bullet+} + O_2 \rightleftarrows LC^{2+} + O_2^{\bullet-} \quad (39)$$

It is not surprising that reaction (39) is rapid, and overwhelmingly in the forward direction (equilibrium constant $K_{39} \sim 50$), contrary to clearly erroneous claims that it is overwhelmingly in the reverse direction ($K_{39}=10^{-6}$) [192]. Thus the reduction potential of the couple $LC^{2+}/LC^{\bullet+}$ was estimated as −0.28 V vs NHE in aqueous solution [191], much less than O_2 (1 M)/$O_2^{\bullet-}$ (−0.18 V) [193,194]. (A higher value for the reduction potential of $LC^{2+}/LC^{\bullet+}$, −0.14 V, has been reported [195], inconsistent with the direct observations of decay of $LC^{\bullet+}$ in solutions containing oxygen [191]). The rate constant for reaction (39) is within an order of magnitude that expected for a 4,4′-bipyridinium radical-cation of such redox properties [196]. It is thus unlikely that superoxide can reduce LC^{2+} to form the required $LC^{\bullet+}$ intermediate unless probe concentrations are very high and SOD is absent. (If the reverse reaction has $k_{-39} \sim 3\times10^6/50$, i.e., $\sim 6\times10^4\ M^{-1}\ s^{-1}$, it would require ~0.1 M lucigenin for the reduction of LC^{2+} by $O_2^{\bullet-}$ to balance the rate of

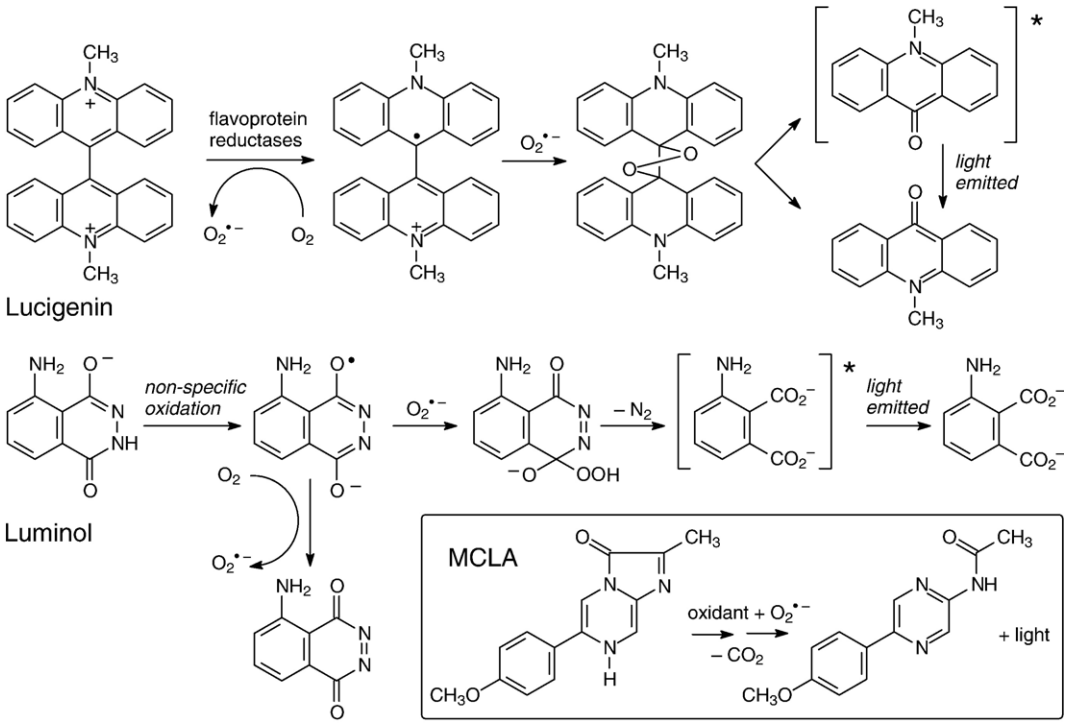

Fig. 8. Chemiluminescence pathways with lucigenin and luminol. Inset: Structure of MCLA and the final oxidation product; the reaction pathway is much less-well defined compared to lucigenin and luminol.

reaction of $O_2^{\bullet -}$ with ~3 μM Cu,Zn SOD, taking $k_{cat} \sim 2 \times 10^9$ M^{-1} s^{-1} [197]).

Hydrogen peroxide can act as a reductant of lucigenin, but the pH dependence shows that HO_2^- is the reactive species [186]. Since the pK_a for dissociation of H_2O_2 to HO_2^- is ~11.6, the reaction can be discounted at pH ~7 and physiologically relevant concentrations of H_2O_2: even at pH 9.5 the first-order rate constant for disappearance of lucigenin at 30 °C with ~10 μM LC^{2+} and 48 mM H_2O_2 was $\sim 2 \times 10^{-5}$ s^{-1} (half-life ~10 h) [186].

We must therefore look for reductants other than $O_2^{\bullet -}$ and H_2O_2 to generate the required $LC^{\bullet +}$ radical, an obligate intermediate in superoxide-dependent chemiluminescence. With the reduction potential of LC^{2+} about equal or slightly higher than that for typical flavin/flavosemiquinone couples at pH 7 [59], flavoprotein reductants are obvious candidates. Indeed, endothelial nitric oxide synthase [190], "several almost ubiquitory NAD(P)H reductases" [198], microsomal NADPH reductases [199], and cytochrome P450 reductase [200] have all been reported as able to reduce LC^{2+}. It was shown that LC^{2+} initiates a marked increase of superoxide formation in endothelial cell lysates in the presence of NADH [201].

Several articles have defended the use of lucigenin as a probe for superoxide, suggesting the use of very low concentrations of lucigenin to reduce the effects of redox cycling (reaction (39)) [202–204]. While redox cycling to generate $O_2^{\bullet -}$ will always occur with lucigenin, with low concentrations of the probe, the additional superoxide formed via (39) may become insignificant compared to basal or otherwise stimulated levels. How low a concentration of probe is needed to effectively eliminate this problem is likely to vary with biological system, reflecting differences in reductase activity. In one study involving vascular homogenates, 5 μM LC^{2+} was sufficient to induce a doubling of superoxide trapped by DMPO [205]; the same concentration of LC^{2+} had effects on endothelium-dependent relaxation of aortic endothelial cells consistent with superoxide generation [206,207].

However, even with low probe concentrations to reduce the effects of redox cycling, any reagent, in addition to superoxide, that can change the steady-state concentration of $LC^{\bullet +}$ has potential to influence probe response. In this regard an obvious contender would be, in some applications, quinones used as tools to stimulate superoxide generation, such as 2,3-dimethoxy-1,4-naphthoquinone [208]. Redox equilibration between viologen radicals and quinones (Q) is very rapid [209], so that equilibrium (40) is likely to be established quickly:

$$LC^{\bullet +} + Q \rightleftarrows LC^{2+} + Q^{\bullet -}. \quad (40)$$

Semiquinones ($Q^{\bullet -}$) disproportionate or react with electron acceptors (e.g., O_2) much more rapidly than $LC^{\bullet +}$. Removal of $Q^{\bullet -}$ from the equilibrium will pull equilibrium (40) to the right, reducing the "natural" lifetime of $LC^{\bullet +}$ and hence both its steady-state concentration and the rate of formation of the intermediate dioxetane via $O_2^{\bullet -}$. The author has observed directly by pulse radiolysis, in unpublished observations, accelerated decay of $LC^{\bullet +}$ in the presence of duroquinone, most likely via such routes. Equilibrium (40) must be taken into account when using the lucigenin probe in the presence of quinones, e.g., [210]. It would be interesting to measure the kinetics of reaction of $LC^{\bullet +}$ with cytochrome *c* and similar electron acceptors.

Luminol and related probes

The chemistry of this probe has been studied rather extensively [211,212]. A paper commencing with the sentence "Luminol is known to elicit chemiluminescence under almost an unlimited variety of conditions" [213] should alert us to approach its use in biology with caution. Indeed, an earlier review [214] concluded that "luminol-dependent chemiluminescence gives at present very little ability to discriminate between individual oxygen or radical species. Furthermore, luminol-dependent chemiluminescence used in biological systems is extremely prone to many interferences, which are very difficult to control." Other authors concluded that "luminol … can neither serve to study the formation kinetics nor be used as a valid continuous assay of ROS … generated by stimulated neutrophils … luminol speciously report on H_2O_2 or $O_2^{\bullet-}$ production even in a cell-free system" [215], and "Luminol is not well suited for the detection of $O_2^{\bullet-}$ within living cells " [185].

Despite these cautions, the probe remains in widespread use. It is therefore important to outline the basis for its limitations. Luminol also exhibits chemiluminescence via a two-step process to a precursor of the emitting species, the initial step being oxidative, in contrast to lucigenin. However, the luminol radical obtained on oxidation reacts with oxygen to generate $O_2^{\bullet-}$, so there are some parallels between the two probes. The route to chemiluminescence is shown in Fig. 8. The initial oxidation step, converting luminol (LH^-) to the radical ($LH^\bullet$) cannot be achieved by superoxide [211], but most other biologically relevant oxidants react with luminol.

Both $^\bullet OH$ and $CO_3^{\bullet-}$ are highly reactive toward luminol (rate constants $\sim 9\times10^9$ and 9×10^8 M^{-1} s^{-1}, respectively [213,216]). Carbonate radical was invoked to explain the effects of bicarbonate on luminol chemiluminescence in $^\bullet OH$-radical generating systems [217]. Nitrogen dioxide is a slightly more powerful oxidant than $ClO_2^\bullet$ [59,218], although $NO_2^\bullet$ is slightly less reactive toward phenolate than $ClO_2^\bullet$ [30]. The latter radical oxidizes luminol with rate constant $\sim 3\times10^6$ M^{-1} s^{-1} [219]); it is therefore probable that $NO_2^\bullet$ oxidizes LH^- to $LH^\bullet$ with a rate constant of at least 10^6 M^{-1} s^{-1} (G, Merényi, personal communication). (Luminol is widely used in atmospheric chemistry to measure $NO_2^\bullet$ [220], and has been used to measure nitric oxide in exhaled breath after oxidizing $NO^\bullet$ to $NO_2^\bullet$ [221].) Chemiluminescence occurs with luminol and peroxynitrite, modulated by bicarbonate/CO_2 [25,222], but the rate constant for reaction of peroxynitrite decay in the presence of luminol does not appear to have been reported; it is thus not clear whether a similar situation exists as with peroxynitrite and $DCFH_2$ and RhH_2 [76], i.e., homolysis to $^\bullet OH$ and $NO_2^\bullet$ and/or reaction with CO_2 occurs faster than peroxynitrite can oxidize luminol.

Glutathione thiyl radicals are about as oxidizing as $NO_2^\bullet$ (E_m ($GS^\bullet$,H^+/GSH)=0.92 V at pH 7.4 [49]); from the reduction potential of the $LH^\bullet/LH^-$ couple (0.87 V [219]) the equilibrium constant of (41) is of the order of 7 at pH 7.4:

$$GS^\bullet + LH^-(+H^+) \rightleftarrows GSH + LH^\bullet; \quad (41)$$

i.e., thiyl radicals are another potential source of $LH^\bullet$. Thiols can also be a sink of $LH^\bullet$, via (41) coupled to (35) and (36), which will pull equilibrium (41) to the left, again generating the superoxide required for chemiluminescence without direct reaction of $LH^\bullet$ with oxygen, so the overall likely effects of thiols are complex. While the kinetics of reaction (41) have not been reported, the energetics point to the possible involvement of thiol chemistry in luminol chemiluminescence, as has been reported in model systems, where thiyl radicals have been trapped [223], and enhanced luminescence observed on including cysteine or GSH [224].

There have been extensive studies of formation of $LH^\bullet$ from luminol via peroxidases [141,223]. Low concentrations (10 μM) of hypochlorite have a significant effect on luminol chemiluminescence driven by H_2O_2/Fe^{2+} [225]. Overall, then, there are numerous routes to the key intermediate $LH^\bullet$, but direct reaction of superoxide is not one of them.

The second stage in the chemiluminescence pathway is addition of $O_2^{\bullet-}$ to $LH^\bullet$, which is near diffusion-controlled (rate constant 3×10^9 M^{-1} s^{-1} [219]), forming an alpha hydroxy hydroperoxide, "LOOH" (Fig. 8):

$$LH^\bullet + O_2^{\bullet-} \rightarrow LOOH. \quad (42)$$

This reaction can potentially compete with reaction of $LH^\bullet$ with oxygen to generate $O_2^{\bullet-}$,

$$LH^\bullet + O_2 \rightarrow L_{ox} + O_2^{\bullet-}(+H^+), \quad (43)$$

where L_{ox} is the diazaquinone, 5-aminophthalazine-1,4-dione [213], but the rate constant for (43) is $\sim 6\times10^2$ M^{-1} s^{-1}, almost seven orders of magnitude lower than that for (42) [213]. If $O_2^{\bullet-}$ is generated from mitochondria at ~ 0.6 μM s^{-1}, in the presence of ~ 3 μM SOD the steady-state $O_2^{\bullet-}$ concentration calculated just from SOD-catalyzed disproportionation is ~ 100 pM. With this value, the rate of reaction (42) is about twice that of (43) in air-equilibrated media. If superoxide generation is averaged over the cytosolic volume, then reaction (43), generating superoxide, would be a major fate of $LH^\bullet$. More refined analysis is not possible without much more information, such as the rate of oxidation of luminol to form $LH^\bullet$ and data characterizing other "sinks" of $LH^\bullet$ (e.g., reaction (41) and the obviously rapid reaction of $LH^\bullet$ with ascorbate and/or urate), but such calculations illustrate the complexity underlying the use of this probe.

Antioxidants can, of course, both compete with radicals oxidizing luminol to $LH^\bullet$ and also react directly with this key intermediate; indeed, effects on luminescence have been used to assay antioxidants such as urate and ascorbate in biological fluids [226]. The (hypo)xanthine/xanthine oxidase system is a popular and convenient source of superoxide in model systems. The by-product, urate, has a marked inhibitory effect on luminol chemiluminescence [227], presumably because of reaction of urate with $LH^\bullet$. In addition to thiyl radicals, ascorbate radicals have been observed during oxidation of luminol by peroxidase [223], also probably via interaction of ascorbate with $LH^\bullet$. A further complication in biological applications of luminol is the possibility that the obligate intermediate radical $LH^\bullet$ can be routed down a "dark" pathway by reaction with nitric oxide [222].

The third stage of luminol chemiluminescence is decomposition of the hydroperoxide intermediate, eliminating nitrogen and forming aminophthalate in an excited state (Fig. 8). This is pH dependent (only one of the prototropic forms is involved in emission), occurs in microseconds [211], and seems unlikely to be influenced by cellular antioxidants. In the context of pH sensitivity, pH changes in stimulation of neutrophils and the need to consider buffering capacity of media have been noted [215]. In conclusion, the multiple stages involved in luminol luminescence, with several routes for involvement of several cellular oxidants and antioxidants, atmospheric oxygen, and pH-dependent steps, all point to the undesirable complexity inevitably accompanying the application of luminol as a probe in free radical biology.

Other chemiluminescent probes for superoxide

A number of alternatives to luminol have been explored, some quite closely related to luminol [228], but most based on luciferins of bioluminescent marine organisms such as coelenterazine [229] or "MCLA" (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-*a*]pyrazin-3-one, see Fig. 8) and its analogs [230]. Of the latter category, there have been some quite divergent kinetic studies (e.g., [231,232]), cautionary illustrations of lack of specificity [233], and impressive demonstrations of quantitation of superoxide in chemical [234] and biochemical [235] model systems. The most recent examples involve hybrid molecules in which energy transfer results in a red shift of the emitted light [236]. It is not impossible that chemiluminescence of these newer probes suffers from potential interference from other oxidants (and antioxidants), like luminol, and further work is needed—and justified in view of their promise—to characterize fully their reactivity and reaction pathways.

Probes for nitric oxide

Vicinal diamines requiring oxidative activation before reaction

A variety of very popular probes for nitric oxide share two common features: an aromatic moiety (usually a benzene ring) with two adjacent -NH_2 substituents; and a lack of direct reactivity toward nitric oxide itself. 2,3-Diaminonaphthalene is prototypical [237], but most work has utilized fluoresceins (e.g., DAF-2, Fig. 9) and rhodamines appropriately substituted in the benzene ring rather than the tricyclic system, especially from a range introduced from the group of Nagano and co-workers [238–245]. The diaminoaryl moiety is "electron rich" and can quench the fluorophor excited state by internal electron transfer; on transformation of the diamine to a triazole on adding nitric oxide after oxidation (Fig. 9, see below), the nonfluorescent probes become intensely fluorescent. The advantages of using rhodamine rather than fluorescein as a fluorophor are less pH sensitivity and higher quantum yield [242,244,246]. Very low extracellular concentrations of diaminofluorescein are usable in cellular systems [247,248]. Other aromatic systems with vicinal diamine functionality, including acridines [249], 9,10-anthraquinone [4], BODIPY derivatives [250,251], and carbocyanines

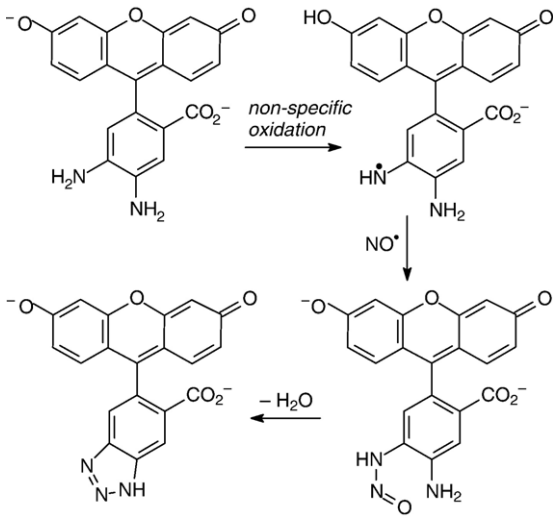

Fig. 9. Possible mechanism for formation of a fluorescent triazole from vicinal diamines by oxidation and nitric oxide addition. Example: DAF-2.

[252] have been explored. Two quite recent surveys of the use of this type of probe, as well as cheletropic traps (see below), in imaging or quantitation of $NO^{\bullet}$ in neuronal [4] or aortic [253] tissues describe progress and limitations. With care in calibration, in specific biological systems, they can provide useful information [254], but the basic chemical problems remain, as outlined below.

All such amine-based probes are unreactive toward $NO^{\bullet}$ without initial oxidation. In some model systems lacking antioxidants, fluorescent triazole formation might involve nitrogen trioxide or even nitrous acid [255]. N_2O_3 is easily formed in air-equilibrated media containing nitric oxide, but as argued above, formation of N_2O_3 in biology or in truly biomimetic models is not likely to be efficient (except perhaps in lipid membranes [256,257]) because of the high reactivity of the $NO_2^{\bullet}$ precursor toward antioxidants. It seems likely that in the cytosol or appropriate biomimetic models that the fluorescent product is formed in two stages as shown in Fig. 9: one-electron oxidation to an anilinyl radical, and subsequent addition of $NO^{\bullet}$ to this intermediate, although the steps have not been individually characterized to date. Hence the use of these probes shares some features of both lucigenin and luminol, in requiring an initial activation step before reaction with the radical of interest, and susceptibility toward interference by any reagent that serves to modify the steady-state concentration of the intermediate radical. Generation of N_2O_3 is not necessary because $NO_2^{\bullet}$ itself, or a multiplicity of other oxidants, including photosensitized oxidation (see below), can oxidize the amine to the anilinyl radical.

The second, *ortho* amino substituent will enhance oxidation rate (lowering the reduction potential of the anilinyl radical significantly [258]), but even aniline is oxidized by $CO_3^{\bullet-}$ with rate constant 5.4×10^8 M^{-1} s^{-1}; 1,4-phenylenediamine, likely to be similarly reactive as the vicinal diamines of interest, is oxidized by $NO_2^{\bullet}$ with rate constant 4.6×10^7 M^{-1} s^{-1} [30]. Thiyl radicals do not appear to have been studied as oxidants of aryl diamines, but thiyl radicals have reduction potentials at

physiological pH which are >0.3 V more positive than 1,4-phenylenediamine [49,59], so oxidation of the probes by, e.g., $GS^{\bullet}$, is probably energetically rather favorable. Note, however, that "repair" equilibria of the same form as reaction (41), when coupled to (35) and (36), can effectively reverse oxidation by thiyl radicals, particularly when [GSH] ≫ [probe]. Arylaminyl radicals participate in rapid hydrogen transfer reactions, significantly faster than corresponding reactions with phenols, which may be kinetically slow [259], also suggesting rapid oxidation reactions of the parent arylamines. While the redox properties of the probes have not been characterized, it would be very surprising if reversal of oxidation ("repair" of the probe radical) was not achieved rapidly by ascorbate. Indeed, thiols and ascorbate inhibited fluorescence from the diaminofluorescein probe, DAF-2, treated with "NONOates" to release $NO^{\bullet}$ in air-equilibrated buffer [253,260]. Both antioxidants will of course react with $NO_2^{\bullet}$ in competition with oxidation of the probe to the anilinyl radical; whether N_2O_3 is additionally involved cannot be deduced in the absence of appropriate kinetic and concentration data. It was shown that incubation of rat aortic tissue with 10 μM DAF-2 diacetate resulted in intracellular accumulation of ~750 μM DAF-2-associated species, that DAF-2 was compartmentalized around elastic fibers, and that high GSH concentrations influenced the fluorescence spectrum [253].

Ascorbate inhibited DAF-2 oxidation by the diethylamine NONOate "DEA/NO" much more efficiently than GSH [253,260], yet both antioxidants are about as reactive toward $NO_2^{\bullet}$ [30,35]; this might reflect the expected higher reactivity of the probe radical toward the former antioxidant. However, in an important study demonstrating the value of more specific analytical techniques, it was shown that DAF-2 itself reacts with both ascorbate and dehydroascorbic acid under physiological conditions, producing compounds with fluorescence spectra similar to the DAF-2 triazole [261]. (Note that $NO^{\bullet}$ itself reacts with ascorbate/dehydroascorbate via the ascorbate radical [262]). While physiologically relevant (~1 mM) concentrations of thiols abolished fluorescence from DAF-2 treated with an aerobic $NO^{\bullet}$ donor, as expected from competition with $NO_2^{\bullet}$, even 1 μM catecholamines was sufficient to reduce fluorescence [263], presumably reflecting kinetic factors.

Further, the two-step conversion of vicinal diaminoaryl probes to fluorescent product implies that the signal measured may reflect changes in the first (oxidation) step at constant concentrations/fluxes of nitric oxide. Superoxide will modulate not only the $NO^{\bullet}$ levels by peroxynitrite formation: the resulting $^{\bullet}OH/NO_2^{\bullet}/CO_3^{\bullet-}$ radicals are all capable of rapid reaction with the probe to generate the anilinyl radical, which can then add $NO^{\bullet}$. While moderate fluxes of superoxide can increase probe sensitivity to $NO^{\bullet}$ by increasing the steady-state concentration of the intermediate anilinyl radical in this way, a high flux of $O_2^{\bullet-}$ relative to $NO^{\bullet}$ can eliminate fluorescence from DAF-2 entirely [264]. Qualitatively similar conclusions were obtained using a related difluorofluorescein probe [265]. H_2O_2/HRP could also achieve the oxidative activation step, and hence nonspecific oxidants are extremely important variables in the response of such probes to $NO^{\bullet}$ [246,264]. Problems in differentiating between nitrosative and oxidative chemistry with the vicinal diamine probes have been recently summarized [266].

Susceptibility of probe chemistry to both oxidants and antioxidants via diverse potential pathways all points to the need for caution in drawing any quantitative mechanistic inferences using such probes, unless precautions are taken. One method to overcome interference from ascorbate has been suggested [267], using frozen samples where diffusion of $NO^{\bullet}$ occurs but not reaction with ascorbate.

A further possible contraindication to the use of the vicinal diamine probes is susceptibility to light (e.g., during experiments, measurements, or microscopy). We can draw analogy to the earlier discussion of a similar sensitivity with dichlorofluorescein, where the fluorescein excited state was quenched by reductants (GSH, NADH) to generate reducing radicals and hence superoxide (reactions (32)–(36)). It would therefore not be surprising that excitation by light, resulting in "internal" quenching of the fluorescein excited state by the easily oxidized diaminoaryl moiety in DAF-2 and similar probes, can generate the anilinyl radical. Potentiation of fluorescence from DAF-2 and $NO^{\bullet}$ by illumination was reported [268], presumably involving such a mechanism. Photolytic effects in other studies were ascribed to nitrate photolysis [253]. Other potential interfering substances include mercuric chloride [253] and Ca^{2+}/Mg^{2+} [245,268]; the latter interference has been questioned [269], and approaches to coimage $NO^{\bullet}$ and calcium have been described [4].

Extension of the vicinal diamine approach to permit near-infrared excitation and emission, using carbocyanine fluorophors, has been reported [252]. These are much more suitable than fluoresceins or rhodamines for use in imaging tissue because of less absorbance/scattering at longer wavelengths. While subject to the same problems as all diamine probes, such studies provide very valuable experience in applying probes to in vivo models.

Use of reduced fluoresceins and rhodamines as probes for "nitric oxide"

While it was claimed that "pure NO gas was capable of oxidizing $DCFH_2$" [270], it is obvious that oxygen is also required and that the oxidant is not $NO^{\bullet}$ but either $NO_2^{\bullet}$ and/or possibly N_2O_3 under some nonphysiological conditions. The nonspecificity of oxidation routes from $DCFH_2$ to DCF have been discussed above. Hence the use of dihydrofluoresceins (fluorescins) to measure $NO^{\bullet}$ in aerobic systems [270–272] is obviously subject to numerous caveats. All other oxidant and antioxidant challenges have to remain constant if probe oxidation is to reflect only changes in $NO^{\bullet}$. It was suggested that the interferences could be allowed for by comparing the effects of added scavengers of $NO^{\bullet}$ (hemoglobin), $O_2^{\bullet-}$ (SOD), and H_2O_2 (catalase) [271], but antioxidants, notably GSH, will still compete with the probe for oxidation by $NO^{\bullet}$-derived oxidants.

Under the same conditions used to detect oxidant production in L-arginine-supplemented activated macrophages by $DCFH_2$, dihydrorhodamine did not show increased oxidant production

[272]. This may reflect lower reactivity toward $NO_2^{\bullet}$ of RhH_2 compared to $DCFH_2$, lower intracellular concentrations of RhH_2, or both.

Other approaches to measuring or imaging nitric oxide

Fluorescence quenching of pyrene derivatives by nitric oxide was used to study diffusion of NO• in membranes, enabling comparison with O_2 [273], but limited to oxygen-free systems. Via fluorescence lifetime imaging, such an approach could be used to image nitric oxide distribution. A probe comprising a nitroxyl (TEMPO) labeled with acridine was shown to interact with an Fe(II) *N*-dithiocarboxysarcosine complex, becoming fluorescent; on exposure to NO•, binding to the Fe(II) center decreased fluorescence [274]. While offering quite high sensitivity, such "turn off" probes are less useful than the metal-based, "turn-on" approaches described below. Lifetime-based optical fiber sensors for nitric oxide, incorporating cytochrome c′ labeled with a fluorescein derivative which shows changes in fluorescence on binding NO•, have been reported, but the limit of detection was a few micromolar [275]. Fluorescence resonance energy transfer (FRET) has been used to visualize effects of NO• and an *S*-nitrosothiol on metallothionein. Yellow/green fluorescent protein (GFP) (EYFP) and a cyan GFP variant (ECFP) were fused to the-CO_2H and NH_2 termini, respectively, of human metallothionein MT-IIA and the effects of NO• on the emission ratio at the corresponding wavelengths measured [276–278]. This approach, while very useful in the specific applications involved in these studies, does not have the sensitivity of the amplified c-GMP-dependent FRET measurements described below.

Cheletropic detection of NO•, in which persistent, fluorescent nitroxyl radicals are produced, has been described (Fig. 10) [279]. Advantages include the ability, at least using

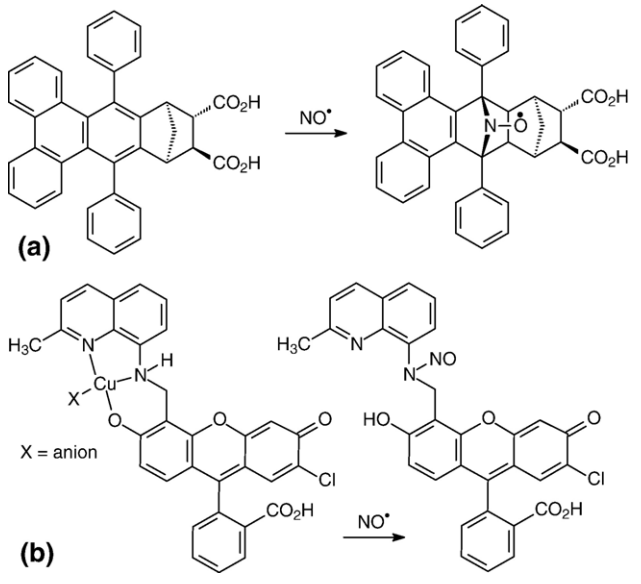

Fig. 10. Alternative probes for nitric oxide: (a) cheletropic trap, from Bätz et al. [279]; (b) copper-based probe, from Lim et al. [284,286,287].

high concentrations of NO•, to compare results using absorbance and fluorescence spectroscopy with EPR detection. A further increase in fluorescence intensity was observed in the presence of biological reductants such as GSH and ascorbate, corresponding to reduction of the nitroxyl radical. The initial reaction of the probe shown in Fig. 10 with NO• had a rate constant of 60 M^{-1} s^{-1} in water. This implies that high concentrations of probe are required to trap NO• anywhere near where it is produced: NO• probably diffuses around 10 μm in 10 ms in the cytoplasm, and for this half-life/rate constant of probe reaction, ~1 M probe is needed. Different cheletropic probes, but with similar reactivity toward NO•, were examined in related work [280,281]; a potential problem described was sensitivity toward peroxynitrite. It was pointed out that a low rate constant has the advantage that interference with NO•-dependent cellular responses would not be a problem, since only a fraction of NO• would be trapped [281]. Hence probes with low trapping efficiency but high sensitivity can be useful.

Metal-based, "turn-on" fluorescent probes for NO• look particularly interesting [282–287]. One approach is based on reduction of Cu(II) to Cu(I) by NO•; since paramagnetic Cu(II) is an efficient fluorescence quencher, the scope for probe design is considerable. Alternative but related approaches exploit reductive nitrosylation of Co(II) or Fe(III), or nonreductive metal–nitrosyl adduct formation on reaction of NO• with Fe, Ru, and Rh complexes, both with displacement of an attached fluorophor. The most promising probe to date is a Cu(II) complex with fluorescein substituted with an aminoquinoline ligand (Fig. 10(b)) [287], which has been used to visualize NO• in stimulated macrophages and neuroblastoma cells [286]. The possible consequences of the metal released on probe reaction need to be considered, e.g., as a catalyst in Fenton chemistry (Fig. 1), but the availability of alternate redox metals will aid in avoiding such complications.

A completely different, but equally exciting approach to visualize NO• is a novel cell-based indicator exploiting the amplified generation of guanosine-3′,5′-cyclic monophosphate (cGMP) following binding of NO• to soluble guanylate cyclase (sGC) [288,289]. Cells were transfected with a plasmid vector encoding a fluorescent indicator with donor and acceptor fluorescent tags enabling a cGMP-dependent FRET response. Outstanding sensitivity was expected because a single NO• molecule leads to ~6000 molecules/min cGMP: a detection limit of 20 pM was suggested. High reversibility was demonstrated, and high specificity is expected. By generating NO• via local photochemical release from a "caged" donor within a 20 μm diameter, it was possible to visualize NO• release from single, viable vascular endothelial cells. This cell-based indicator ("Piccell") offered advantages, particularly of enhanced reversibility, reproducibility, and applicability to a variety of cell types over a related, earlier method [290] in which a different, genetically encoded indicator was expressed.

Finally, a protein-based sensor for NO• has been recently described, presently involving absorbance measurements [291]. The H–NOX family of heme proteins has significant homology to sGC. A tyrosine residue (Y140) controls discrimination between NO• and O_2 binding; a mutant was developed without

affinity for O_2 but which binds NO$^\bullet$ in the stable Fe(II) oxidation state, with a change in the absorbance (extinction coefficient) in the Soret region of 3×10^4 M^{-1} cm^{-1} and therefore presently only useful for micromolar levels of NO$^\bullet$ (but note integrated or accumulated changes of this order are obviously of interest). It might be possible to adapt the approach to use fluorescence detection, and is noted here to emphasize the breadth of the new approaches now being developed.

Probes for hydroxyl radicals

Only brief, illustrative discussion of fluorescent probes for $^\bullet$OH is included here. Because of the high reactivity of $^\bullet$OH toward biomolecules, very high probe loading is normally required to intercept this radical in cells, unless the probe is concentrated near some important target, e.g., DNA. Hence much work in this area has mainly involved studies aimed to be useful in radiobiology, where radical damage to DNA is a key target, or in site-specific $^\bullet$OH generation via bound, Fenton-catalytic metals. Hydroxyl radicals add to aromatic rings rather than oxidize by electron transfer, so the formation of fluorescent hydroxy-aromatic moieties is the standard approach.

Most probes are based on hydroxylation of coumarins [292–295], or benzenoid molecules such as salicylate [296,297] or 1,4-benzenedicarboxylate (terephthalate) [298–300] (Fig. 11). The succinimidyl ester of coumarin-3-carboxylic acid binds to cellular macromolecules and so can probe in a site-directed manner, and has been used to probe $^\bullet$OH generation within core chromatic particles [295]; terephthalate was used to detect $^\bullet$OH radicals during radiolysis [301] or sonolysis [302]. The reactivity of a number of alternative aromatics was compared with fluorescence yields, with a view to develop probes which exploited fluorescence resonance energy transfer and required oxidation by two $^\bullet$OH radicals generated in close proximity (the interest here is in clustered radical damage from irradiation) [303]. 7-Aryloxycoumarins are nonfluorescent, but reaction of 7-(4′-hydroxyphenoxy)coumarin with $^\bullet$OH releases fluorescent 7-hydroxycoumarin [181]; whether the probe reacts (qualitatively) similarly with $NO_2^\bullet$ or $CO_3^{\bullet-}$ is unknown.

Trapping the product ($CH_3^\bullet$) of reaction of $^\bullet$OH with dimethyl sulfoxide, exploiting rapid reaction of alkyl radicals with nitroxides or nitrones bound to a fluorophor, is a less direct but highly sensitive method for detecting $^\bullet$OH [304] (cf. [305]). Another completely different approach was described [306], exploiting FRET detection of a probe with two fluorescent tags separated by an $^\bullet$OH-cleavable linker akin to DNA strand bonding (Fig. 11). The linker was attached to both fluorescein- and rhodamine-based tags; exciting the former chromophore results in energy transfer to, and emission from, the rhodamine; when cleaved by $^\bullet$OH, emission is from the fluorescein, as illustrated in Fig. 11.

A checklist for probe selection, application, and interpretation

Based on the above discussion, we can construct a simple questionnaire that can be used as an aid in evaluating the use of

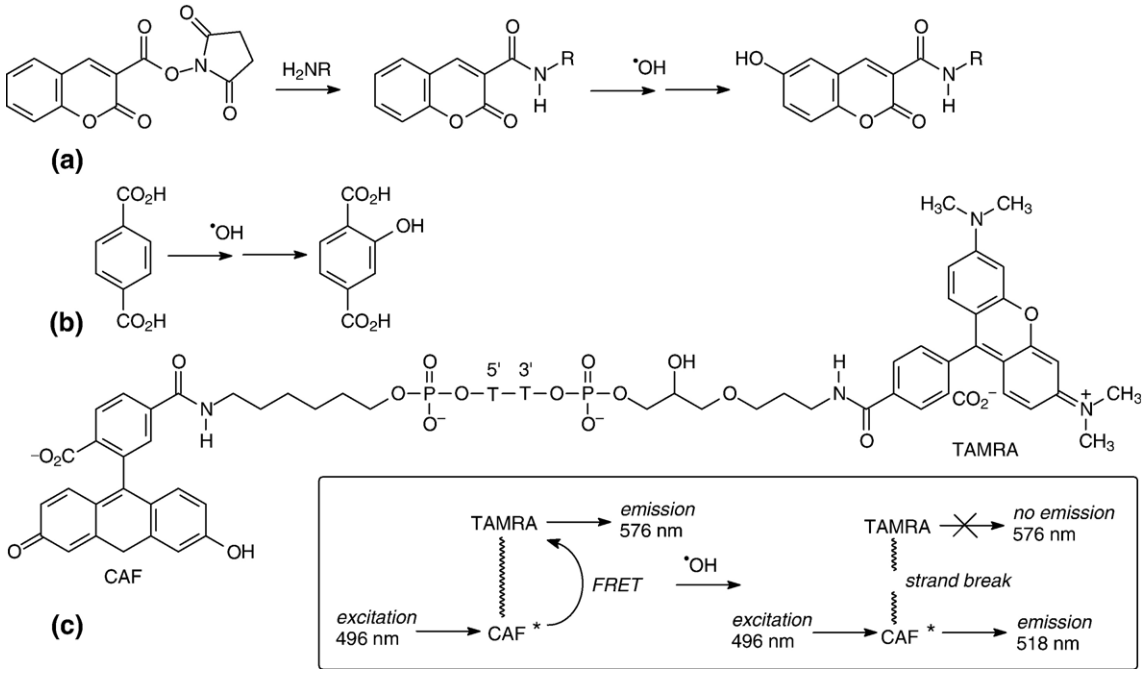

Fig. 11. Probes for hydroxyl radicals. (a) Succinimidyl ester of courmarin-3-carboxylic acid binds to biomolecules (H_2NR: proteins, nucleic acids) and is hydroxylated by $^\bullet$OH [292]. (b) Terephthalate is hydroxylated by $^\bullet$OH [301]. (c) Two fluorophors (CAF, carboxyfluorescein; TAMRA, tetramethylrhodamine) are linked by a 3′,5′-deoxythymidine dimer (T-T) which is cleaved by $^\bullet$OH in a manner similar to DNA strand breakage by $^\bullet$OH. Excitation of CAF results in energy transfer to TAMRA, with characteristic emission, provided the link is intact. On strand breakage, emission is from CAF, at shorter wavelength than from TAMRA. Adapted from Soh et al. [306].

fluorescent and luminescent probes in biological systems. In the main this is derived from experience with the commonest probes, and not all points apply to all reactants or all probe types, but hopefully the questions posed will serve as an aide memoire. Before using a probe we should attempt to answer the following questions:

- Probe reactivity. Which species do we think we are measuring? Does the probe react specifically with the species of interest, and how fast is reaction under biomimetic conditions? Is reaction sufficiently fast for the measured product to be found in the locality of radical generation, or is diffusion a factor?
- Catalyst. Is a catalyst needed for reaction, and will its concentration or localization change during the experiment?
- Probe intermediates required for response. If the probe requires initial activation to a reactive intermediate, which species can achieve the activating step? How fast can it be achieved? What controls the instantaneous concentration of the reactive intermediate, which will define probe response?
- Reactions of probe intermediates. Does an intermediate radical react with oxygen to produce superoxide, or with antioxidants? If so, what are the consequences?
- Probe distribution. What is the distribution of probe between extracellular media and intracellular space, and between organelles intracellularly? Is the intracellular/organelle concentration sufficiently high so that probe response is under "saturating" conditions? Can we reduce variations in probe loading as a factor, e.g., by lifetime-based rather than intensity imaging?
- Effects of antioxidants. Do cellular antioxidants react with the species being measured in competition with the reaction with the probe? Can we assess the competition quantitatively, comparing the products of rate constant × concentration? Is there similar competition with antioxidants after probes have been activated to a reactive intermediate?
- Photochemical reactions. Is the product being measured photostable under the conditions used, or can it initiate superoxide production via quenching of excited states with cellular reductants?
- Instrumental artefacts. Finally, basic precautions in the use of instruments need to be considered, such as avoiding inner filter effects in fluorescence measurements, and Rayleigh scattering and second-order diffraction artefacts.

Conclusions

Certainly the simplest, and arguably the single most important, point to make in the present context is that if the reactant is a free radical, and the probe not, then another free radical is initially formed on reaction, the properties of which must be considered. Despite repeated notes of caution (e.g., [5,75,77,91,96,97,307]), it is still appears not generally realized that the commonest probes for "reactive oxygen and nitrogen species" do not react directly at a significant rate with superoxide, hydrogen peroxide, peroxynitrite, or nitric oxide—although some individual studies, especially more recent [308], do demonstrate clear insight of the problems of selectivity. Some widely used probes actually generate superoxide radicals. Probe response often involves a two-step process, with different species involved in the two steps, so that assignment of response to one species is problematical. Our understanding of these facts relies, in the main, on a few tens of individual studies, in contrast to the thousands of investigations that have assumed properties of the probes which they simply do not have. Overall, the "triumph of hope over reality" [2] persists. However, understanding is underpinned by a broader base of free radical chemistry, which provides a logical framework for using the probes and predicting the likely properties and problems where specific information is lacking. There is also considerable scope for exploiting advances in nanotechnology to couple probes and/or activating enzymes to particles or sol–gels and so modify tissue or cellular distribution, as widely used in chemical sensors [309,310]; a recent example is coupling $DCFH_2$ to phagocytosable latex beads [308]. That probes react with radical products of peroxynitrite rather than directly, or a peroxidase-like redox intermediate activated by hydrogen peroxide rather than with H_2O_2 directly, or require activation before adding nitric oxide, need not necessarily detract from their use. If we have an appreciation of the mechanisms, reactivity of intermediates, likely competing or confounding reactions, intracellular concentrations and distribution between organelles, and susceptibility to environmental factors, then we can use some of these probes with a fair degree of confidence. We are on the way to that position, but have not yet arrived.

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References

[1] Gomes, A.; Fernandes, E.; Lima, J. L. F. C. Fluorescence probes used for detection of reactive oxygen species. *J. Biochem. Biophys. Methods* **65:**45–80; 2005.
[2] Tarpey, M. M.; Fridovich, I. Methods of detection of vascular reactive species. Nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ. Res.* **89:**224–236; 2001.
[3] Tarpey, M. M.; Wink, D. A.; Grisham, M. B. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286:** R431–R444; 2004.
[4] von Bohlen und Halbach, O. Nitric oxide imaging in living neuronal tissues using fluorescent probes. *Nitric Oxide* **9:**217–228; 2004.
[5] Halliwell, B.; Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* **142:**231–255; 2004.
[6] Soh, N. Recent advances in fluorescent probes for the detection of reactive oxygen species. *Anal. Bioanal. Chem.* **386:**532–543; 2006.
[7] Mikkelsen, R. B.; Wardman, P. Biological chemistry of reactive oxygen and nitrogen and ionizing radiation-induced signal transduction mechanisms. *Oncogene* **22:**5734–5754; 2003.

[8] Liochev, S. I.; Fridovich, I. The effect of superoxide dismutase on H_2O_2 formation. *Free Radic. Biol. Med.* **42:**1465–1469; 2007.

[9] Halliwell, B.; Gutteridge, J. M. C. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Lett.* **307:** 108–112; 1992.

[10] Goldstein, S.; Meyerstein, D.; Czapski, G. The Fenton reagents. *Free Radic. Biol. Med.* **15:**435–445; 1993.

[11] Wardman, P.; Candeias, L. P. Fenton chemistry: an introduction. *Radiat. Res.* **145:**523–531; 1996.

[12] MacFaul, P. A.; Wayner, D. D. M.; Ingold, K. U. A radical account of "oxygenated Fenton chemistry". *Acc. Chem. Res.* **31:**159–162; 1998.

[13] Dunford, H. B. Oxidations of iron(II)/(III) by hydrogen peroxide: from aquo to enzyme. *Coord. Chem. Rev.* **233-234:**311–318; 2002.

[14] Burkitt, M. J. Chemical, biological and medical controversies surrounding the Fenton reaction. *Prog. React. Kinet. Mech.* **38:**75–103; 2003.

[15] Peskin, A. V.; Winterbourn, C. C. Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Radic. Biol. Med.* **30:**572–579; 2001.

[16] Pattison, D. I.; Davies, M. J. Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. *Chem. Res. Toxicol.* **14:**1453–1464; 2001.

[17] Winterbourn, C. C.; Kettle, A. J. Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic. Biol. Med.* **29:**403–409; 2000.

[18] Candeias, L. P.; Stratford, M. R. L.; Wardman, P. Formation of hydroxyl radicals on reaction of hypochlorous acid with ferrocyanide, a model iron (II) complex. *Free Radic. Res.* **20:**241–249; 1994.

[19] Candeias, L. P.; Patel, K. B.; Stratford, M. R. L.; Wardman, P. Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. *FEBS Lett.* **333:**151–153; 1993.

[20] van Dalen, C. J.; Whitehouse, M. W.; Winterbourn, C. C.; Kettle, A. J. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem. J.* **327:**487–492; 1997.

[21] Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87:**1620–1624; 1990.

[22] Radi, R.; Peluffo, G.; Alvarez, M. N.; Naviliat, M.; Cayota, A. Unraveling peroxynitrite formation in biological systems. *Free Radic. Biol. Med.* **30:**463–488; 2001.

[23] Radi, R. Kinetic analysis of reactivity of peroxynitrite with biomolecules. *Methods Enzymol.* **269:**354–366; 1996.

[24] Radi, R.; Denicola, A.; Alvarez, B.; Ferrer-Sueta, G.; Rubbo, H. The biological chemistry of peroxynitrite. In: Ignarro, L.J. (Ed.), *Nitric Oxide Biology and Pathobiology.* Academic Press, San Diego, pp. 57–82; 2000.

[25] Radi, R.; Cosgrove, T. P.; Beckman, J. S.; Freeman, B. A. Peroxynitrite-induced luminol chemiluminescence. *Biochem. J.* **290:**51–57; 1993.

[26] Lymar, S. V.; Hurst, J. K. Rapid reaction between peroxynitrite ion and carbon dioxide: implications for biological activity. *J. Am. Chem. Soc.* **117:**8867–8868; 1995.

[27] Uppu, R. M.; Squadrito, G. l.; Pryor, W. A. Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327:**335–343; 1996.

[28] Denicola, A.; Freeman, B. A.; Trujillo, M.; Radi, R. Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch. Biochem. Biophys.* **333:**49–58; 1996.

[29] Augusto, O.; Radi, R. Peroxynitrite reactivity: free radical generation, thiol oxidation, and biological significance. In: Packer, L., Cadenas, E. (Eds.), *Biothiols in Health and Disease*. Dekker, New York, pp. 83–116; 1995.

[30] Ross, A. B.; Mallard, W. G.; Helman, W. P.; Buxton, G. V.; Huie, R. E.; Neta, P. *NDRL-NIST Solution Kinetics Database: Ver. 3*. Notre Dame Radiation Laboratory and National Institute of Standards and Technology, Notre Dame, IN and Gaithersburg, MD; 1998.

[31] Wardman, P. Nitrogen dioxide in biology: correlating chemical kinetics with biological effects. In: Alfassi, Z.B. (Ed.), *The Chemistry of N-Centered Radicals*. Wiley, New York, pp. 155–179; 1998.

[32] Augusto, O.; Bonini, M. G.; Amanso, A. M.; Linares, E.; Santos, C. C. X.; De Menezes, S. L. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic. Biol. Med.* **32:**841–859; 2002.

[33] Radi, R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. USA* **101:**4003–4008; 2004.

[34] Pryor, W. A.; Houk, K. N.; Foote, C. S.; Fukuto, J. M.; Ignarro, L. J.; Squadrito, G. L.; Davies, K. J. A. Free radical biology and medicine: it's a gas, man! *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291:**R491–R511; 2006.

[35] Ford, E.; Hughes, M. N.; Wardman, P. Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH. *Free Radic. Biol. Med.* **32:**1314–1323; 2002.

[36] Thomas, D. D.; Liu, X.; Kantrow, S. P.; Lancaster Jr., J. R. The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O_2. *Proc. Natl. Acad. Sci. USA* **98:**355–360; 2001.

[37] Lancaster Jr., J. R. Nitroxidative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chem. Res. Toxicol.* **19:**1160–1174; 2006.

[38] Keefer, L. K.; Nims, R. W.; Davies, K. M.; Wink, D. A. "NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. *Methods Enzymol.* **268:**281–293; 1996.

[39] Lewis, R. S.; Tannenbaum, S. R.; Deen, W. M. Kinetics of N-nitrosation in oxygenated nitric oxide solutions at physiological pH: role of nitrous anhydride and effects of phosphate and chloride. *J. Am. Chem. Soc.* **117:**3933–3939; 1995.

[40] Dong, M.; Wang, C.; Deen, W. M.; Dedon, P. C. Absence of 2′-deoxyoxyanosine and presence of abasic sites in DNA exposed to nitric oxide at controlled physiological concentrations. *Chem. Res. Toxicol.* **16:**1044–1055; 2003.

[41] van der Vliet, A.; Eiserich, J. P.; Halliwell, B.; Cross, C. E. Formation of reactive nitrogen species during peroxidase-catalysed oxidation of nitrite. A potential additonal mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272:**7617–7625; 1997.

[42] Byun, J.; Mueller, D. M.; Fabjan, J. S.; Heinecke, J. W. Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein. *FEBS Lett.* **455:**243–246; 1999.

[43] Burner, U.; Furtmüller, P. G.; Kettle, A. J.; Koppenol, W. H.; Obinger, C. Mechanism of reaction of myeloperoxidase with nitrite. *J. Biol. Chem.* **275:**20597–20601; 2000.

[44] Reska, K. J.; Matuszak, Z.; Chignell, C. F.; Dillon, J. Oxidation of biological electron donors and antioxidants by a reactive lactoperoxidase metabolite from nitrite (NO_2^-): an EPR and spin trapping study. *Free Radic. Biol. Med.* **26:**669–678; 1999.

[45] Oury, T. D.; Tatro, L.; Ghio, A. J.; Piantadosi, C. A. Nitration of tyrosine by hydrogen peroxide and nitrite. *Free Radic. Res.* **23:**537–547; 1995.

[46] Chen, S. N.; Hoffman, M. Z. Rate constants for the reaction of the carbonate radical with compounds of biochemical interest in neutral aqueous solution. *Radiat. Res.* **56:**40–47; 1973.

[47] Simic, M. G.; Jovanovic, S. V. Antioxidation mechanisms of uric acid. *J. Am. Chem. Soc.* **111:**5778–5782; 1989.

[48] Forni, L. G.; Mora-Arellano, V. O.; Packer, J. E.; Willson, R. L. Nitrogen dioxide and related free radicals: electron-transfer reactions with organic compounds in solutions containing nitrite or nitrate. *J. Chem. Soc., Perkin Trans.* **2:**1–6; 1986.

[49] Madej, E.; Wardman, P. The oxidizing power of the glutathione thiyl radical as measured by its electrode potential at physiological pH. *Arch. Biochem. Biophys.* **462:**94–102; 2007.

[50] Wardman, P. Evaluation of the 'radical sink' hypothesis from a chemical-kinetic viewpoint. *J. Radioanal. Nucl. Chem.* **232:**23–27; 1998.

[51] Winterbourn, C. C. Superoxide as an intracellular radical sink. *Free Radic. Biol. Med.* **14:**85–90; 1993.

[52] Fukuto, J. M.; Bartberger, M. D.; Dutton, A. S.; Paolocci, N.; Wink, D. A.; Houk, K. N. The physiological chemistry and biological activity of nitroxyl (HNO): the neglected, misunderstood, and enigmatic nitrogen oxide. *Chem. Res. Toxicol.* **18:**790–801; 2005.

[53] Lopez, B. E.; Shinyashiki, M.; Han, T. H.; Fukuto, J. M. Antioxidant actions of nitroxyl (HNO). *Free Radic. Biol. Med.* **42:**482–491; 2007.

[54] Gorman, A. A.; Rodgers, M. A. J. Current perspectives of singlet oxygen detection in biological environments. *J. Photochem. Photobiol. B* **14:** 159–176; 1992.

[55] Nakano, M.; Ito, T.; Arimoto, T.; Ushijima, Y.; Kamiya, K. A simple luminescence method for detecting lipid peroxidation and antioxidant activity *in vitro*. *Biochem. Biophys. Res. Commun.* **202:**940–946; 1994.

[56] Okimoto, Y.; Watanabe, A.; Niki, E.; Yamashita, T.; Noguchi, N. A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. *FEBS Lett.* **474:**137–140; 2000.

[57] Makrigiorgos, G. M.; Kassis, A. I.; Mahmood, A.; Bump, E. A.; Savvides, P. Novel fluorescein-based flow-cytometric method for detection of lipid peroxidation. *Free Radic. Biol. Med.* **22:**93–100; 1997.

[58] Drummen, G. P. C.; Gadella, B. M.; Post, J. A.; Brouwers, J. F. Mass spectrometric characterization of the oxidation of the fluorescent lipid peroxidation reporter molecule C11-BODIPY$^{581/591}$. *Free Radic. Biol. Med.* **36:**1635–1644; 2004.

[59] Wardman, P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **18:**1637–1755; 1989.

[60] Koppenol, W. H. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic. Biol. Med.* **25:**385–391; 1998.

[61] Zielonka, J.; Sarna, T.; Roberts, J. E.; Wishart, J. F.; Kalyanaraman, B. Pulse radiolysis and steady-state analyses of the reaction between hydroethidine and superoxide and other oxidants. *Arch. Biochem. Biophys.* **456:**39–47; 2006.

[62] Keston, A. S.; Brandt, R. The fluorimetric analysis of ultramicro quantities of hydrogen peroxide. *Anal. Biochem.* **11:**1–5; 1965.

[63] Cathcart, R.; Schwiers, E.; Ames, B. N. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.* **134:**111–116; 1983.

[64] LeBel, C. P.; Ischiropoulos, H.; Bondy, S. C. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5:**227–231; 1992.

[65] Crow, J. P. Dichlorofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite *in vitro*: implications for intracellular measurements of reactive nitrogen and oxygen species. *Nitric Oxide* **1:**145–157; 1997.

[66] Zhu, H.; Bannenberg, G. L.; Moldéus, P.; Shertzer, H. G. Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescin. *Arch. Toxicol.* **68:**582–587; 1994.

[67] Wrona, M.; Patel, K. B.; Wardman, P. Reactivity of 2′,7′-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms towards carbonate, nitrogen dioxide, and hydroxyl radicals. *Free Radic. Biol. Med.* **38:**262–270; 2005.

[68] Kooy, N. W.; Royall, J. A.; Ischiropoulos, H. Oxidation of 2′,7′-dichlorofluorescin by peroxynitrite. *Free Radic. Res.* **27:**245–254; 1997.

[69] Possel, H.; Noack, H.; Augustin, W.; Keilhoff, G.; Wolf, G. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. *FEBS Lett.* **416:**175–178; 1997.

[70] von Sonntag, C.; Schuchmann, H. P. Suppression of hydroxyl radical reactions in biological systems: consideration based on competition kinetics. *Methods Enzymol.* **233:**47–56; 1994.

[71] Wrona, M.; Wardman, P. Properties of the radical intermediate obtained on oxidation of 2′,7′-dichlorodihydrofluorescein, a probe for oxidative stress. *Free Radic. Biol. Med.* **41:**657–667; 2006.

[72] Trujillo, M.; Folkes, L.; Bartesaghi, S.; Kalyanaraman, B.; Wardman, P.; Radi, R. Peroxynitrite-derived carbonate and nitrogen dioxide radicals readily react with lipoic and dihydrolipoic acid. *Free Radic. Biol. Med.* **39:**279–288; 2005.

[73] Harman, L. S.; Carver, D. K.; Schreiber, J.; Mason, R. P. One- and two-electron oxidation of reduced glutathione by peroxidases. *J. Biol. Chem.* **261:**1642–1648; 1986.

[74] Kooy, N. W.; Royall, J. A.; Ischiropoulos, H.; Beckman, J. S. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic. Biol. Med.* **16:**149–156; 1994.

[75] Ischiropoulos, H.; Gow, A.; Thom, S. R.; Kooy, N. E.; Royall, J. A.; Crow, J. P. Detection of reactive nitrogen species using 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123. *Methods Enzymol.* **301:**367–373; 1999.

[76] Glebska, J.; Koppenol, W. H. Peroxynitrite-mediated oxidation of dichlorodihydrofluorescein and dihydrorhodamine. *Free Radic. Biol. Med.* **35:**676–682; 2003.

[77] Royall, J. A.; Ischiropoulos, H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302:**348–355; 1993.

[78] Dypbukt, J. M.; Bishop, C.; Brooks, W. M.; Thong, B.; Eriksson, H.; Kettle, A. J. A sensitive and selective assay for chloramine production by myeloperoxidase. *Free Radic. Biol. Med.* **39:**1477–1648; 2005.

[79] Carter, W. O.; Narayanan, P. K.; Robinson, J. P. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J. Leukoc. Biol.* **55:**253–258; 1994.

[80] Benov, L.; Sztejnberg, L.; Fridovich, I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic. Biol. Med.* **25:**826–831; 1998.

[81] 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.

[82] Fink, B.; Laude, K.; McCann, L.; Doughan, A.; Harrison, D. G.; Dikalov, S. Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. *Am. J. Physiol. Cell Physiol.* **287:**C895–C902; 2004.

[83] Zhao, H.; Joseph, J.; Fales, H. M.; Sokoloski, 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. USA* **102:**5727–5732; 2005.

[84] Zielonka, J.; Zhao, H.; Xu, Y.; Kalyanaraman, B. Mechanistic similarities between oxidation of hydroethidine by Fremy's salt and superoxide: stopped-flow optical and EPR studies. *Free Radic. Biol. Med.* **39:**853–863; 2005.

[85] Fernandes, D. C.; Wosniak Jr., J.; Pescatore, L. A.; Bertoline, M. A.; Liberman, M.; Laurindo, F. R.; Santos, C. X. Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems. *Am. J. Physiol. Cell Physiol.* **292:**C413–C422; 2007.

[86] Robinson, K. M.; Janes, M. S.; Pehar, M.; Monette, J. S.; Ross, M. F.; Hagen, T. M.; Murphy, M. P.; Beckman, J. S. Selective fluorescent imaging of superoxide *in vivo* using ethidium-based probes. *Proc. Natl. Acad. Sci. USA* **103:**15038–15043; 2006.

[87] Patsoukis, N.; Papapostolou, I.; Georgiou, C. D. Interference of non-specific peroxidases in the fluorescence detection of superoxide radical by hydroethidine oxidation: a new assay for H_2O_2. *Anal. Bioanal. Chem.* **381:**1065–1072; 2005.

[88] Papapostolou, I.; Patsoukis, N.; Georgiou, C. D. The fluorescence detection of superoxide radical using hydroethidine could be complicated by the presence of heme proteins. *Anal. Biochem.* **332:**290–298; 2004.

[89] Peshavariya, H. M.; Dusting, G. J.; Selemidis, S. Analysis of dihydroethidium fluorescence for the detection of intracellular and extracellular superoxide produced by NADPH oxidase. *Free Radic. Res.* **41:**699–712; 2007.

[90] Meany, D. L.; Thomson, L.; Arriaga, E. A. Simultaneously monitoring the superoxide in the mitochondrial matrix and extramitochondrial space by micellar electrokinetic chromatography with laser-induced fluorescence. *Anal. Chem.* **79:**4588–4594; 2007.

[91] 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′-dichlorodihydrofluoroscein diacetate, amd dihydrorhodamine 123. *Free Radic. Biol. Med.* **27:**146–159; 1999.

[92] Zhou, M.; Diwu, Z.; Panchuk-Voloshina, N.; Haugland, R. P. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of

phagocyte NADPH oxidase and other oxidases. *Anal. Biochem.* **253:**162–168; 1997.

[93] Keller, A.; Mohamed, A.; Dröse, S.; Brandt, U.; Fleming, I.; Brandes, R. P. Analysis of dichlorodihydrofluorescein and dihydrocalcein as probes for the detection of intracellular reactive oxygen species. *Free Radic. Res.* **38:**1257–1267; 2004.

[94] Henderson, L. M.; Chappell, J. B. Dihydrorhodamine 123: a fluorescent probe for superoxide generation? *Eur. J. Biochem.* **217:**973–980; 1993.

[95] Rota, C.; Chignell, C. F.; Mason, R. P. Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. *Free Radic. Biol. Med.* **27:**873–881; 1999.

[96] Bonini, M. G.; Rota, C.; Tomasi, A.; Mason, R. P. The oxidation of 2′,7′-dichlorofluorescin to reactive oxygen species: A self-fulfilling prophesy? *Free Radic. Biol. Med.* **40:**968–975; 2006.

[97] Burkitt, M. J.; Wardman, P. Cytochrome *c* is a potent catalyst of dichlorofluorescin oxidation: implications for the role of reactive oxygen species in apoptosis. *Biochem. Biophys. Res. Commun.* **282:**329–333; 2001.

[98] Ohashi, T.; Mizutani, A.; Murakami, A.; Kojo, S.; Ishii, T.; Taketani, S. Rapid oxidation of dichlorodihydrofluorescin with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species. *FEBS Lett.* **511:**21–27; 2002.

[99] Lawrence, A.; Jones, C. M.; Wardman, P.; Burkitt, M. J. Evidence for the role of a peroxidase compound-I type intermediate in the oxidation of glutathione, NADH, ascorbate, and dichlorofluorescin by cytochrome *c*/H_2O_2. Implications for oxidative stress during apoptosis. *J. Biol. Chem.* **278:**29410–29419; 2003.

[100] Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. Cytochrome *c*-catalyzed oxidation of organic molecules by hydrogen peroxide. *Arch. Biochem. Biophys.* **288:**112–117; 1991.

[101] Castro, L.; Eiserich, J. P.; Sweeney, S.; Radi, R.; Freeman, B. A. Cytochrome *c*: a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration. *Arch. Biochem. Biophys.* **421:**99–107; 2004.

[102] Radi, R.; Turrens, J. F.; Freeman, B. A. Cytochrome *c*-catalyzed membrane lipid peroxidation by hydrogen peroxide. *Arch. Biochem. Biophys.* **288:**118–125; 1991.

[103] Barr, D. P.; Gunther, M. R.; Deterding, L. J.; Tomer, K. B.; Mason, R. P. ESR spin-trapping of a protein-derived tyrosyl radical from the reaction of cytochrome *c* with hydrogen peroxide. *J. Biol. Chem.* **271:** 15498–15503; 1996.

[104] Qian, S. Y.; Chen, Y.-R.; Deterding, L. J.; Fann, Y. C.; Chignell, C. F.; Tomer, K. B.; Mason, R. P. Identification of protein-derived tyrosyl radical in the reaction of cytochrome c and hydrogen peroxide: characterization by ESR spin-trapping, HPLC and MS. *Biochem. J.* **363:**281–288; 2002.

[105] Kim, N. H.; Jeong, M. S.; Choi, S. Y.; Kang, J. H. Oxidative modification of cytochrome *c* by hydrogen peroxide. *Mol. Cells* **22:**220–227; 2006.

[106] Kagan, V. E.; Tyurin, V. A.; Jiang, J.; Tyurina, Y. Y.; Ritov, V. B.; Amoscato, A. A.; Osipov, A. N.; Belikova, N. A.; Kapralov, A. A.; Kini, V.; Vlasova, I. I.; Zhao, Q.; Zou, M.; Di, P.; Svistunenko, D. A.; Kurnikov, I. V.; G.G.B., GG. Cytochrome *c* acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat. Chem. Biol.* **1:**223–232; 2005.

[107] Basova, L. V.; Kurnikov, I. V.; Wang, L.; Ritov, V. B.; Belikova, N. A.; Vlasova, I. I.; Pacheco, A. A.; Winnica, D. E.; Peterson, J.; Bayir, H.; Waldeck, D. H.; Kagan, V. E. Cardiolipin switch in mitochondria: shutting off the reduction of cytochrome *c* and turning on the peroxidase activity. *Biochemistry* **46:**3423–3434; 2007.

[108] Chen, Y. R.; Deterding, L. J.; Sturgeon, B. E.; Tomer, K. B.; Mason, R. P. Protein oxidation of cytochrome *c* by reactive halogen species enhances its peroxidase activity. *J. Biol. Chem.* **277:**29781–29791; 2002.

[109] Cassina, A. M.; Hodara, R.; Souza, J. M.; Thomson, L.; Castro, L.; Ischiropoulos, H.; Freeman, B. A.; Radi, R. Cytochrome *c* nitration by peroxynitrite. *J. Biol. Chem.* **275:**21409–21415; 2000.

[110] Jang, B.; Han, S. Biochemical properties of cytochrome c nitrated by peroxynitrite. *Biochemie* **88:**53–58; 2006.

[111] Gebicka, L.; Didik, J. Mechanism of peroxynitrite interaction with cytochrome *c*. *Acta Biochim. Pol.* **50:**815–823; 2003.

[112] Prasad, S.; Maiti, N. C.; Mazumdar, S.; Mitra, S. Reaction of hydrogen peroxide and peroxidase activity in carboxymethylated cytochrome *c*: spectroscopic and kinetic studies. *Biochim. Biophys. Acta* **1596:**63–75; 2001.

[113] Myhre, O.; Andersen, J. M.; Aarnes, H.; Fonnum, F. Evaluation of the probes 2′,7′-dichlorofluorescein diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol.* **65:** 1575–1582; 2003.

[114] Liochev, S. I.; Fridovich, I. Copper, zinc superoxide dismutase as a univalent NO^- oxidoreductase and as a dichlorofluorescin peroxidase. *J. Biol. Chem.* **276:**35253–35257; 2001.

[115] Karunakaran, C.; Zhang, H.; Joseph, J.; Antholine, W. E.; Kalyanaraman, B. Thiol oxidase activity of copper, zinc superoxide dismutase stimulates bicarbonate-dependent peroxidase activity via formation of a carbonate radical. *Chem. Res. Toxicol.* **18:**494–500; 2005.

[116] Kim, Y.-M.; Lim, J.-M.; Kim, B.-C.; Han, S. Cu,Zn-superoxide dismutase is an intracellular catalyst for the H_2O_2-dependent oxidation of dichlorodihydrofluorescein. *Mol. Cells* **21:**161–165; 2006.

[117] Bonini, M. G.; Miyamoto, S.; Di Mascio, P.; Augusto, O. Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate. *J. Biol. Chem.* **279:**51836–51843; 2004.

[118] Larsen, L. N.; Dahl, E.; Bremer, J. Peroxidative oxidation of leucodichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. *Biochim. Biophys. Acta* **1299:**47–53; 1996.

[119] Zhang, H.; Joseph, J.; Felix, C.; Kalyanaraman, B. Bicarbonate enhances the hydroxylation, nitration, and peroxidation reactions catalysed by copper, zinc superoxide dismutase. Intermediacy of the carbonate anion radical. *J. Biol. Chem.* **275:**14038–14045; 2000.

[120] Lloyd, R. V.; Mason, R. P. Evidence against transition metal-independent hydroxyl radical generation by xanthine oxidase. *J. Biol. Chem.* **265:** 16733–16736; 1990.

[121] Balcerczyk, A.; Kruszewski, M.; Bartosz, G. Does the cellular labile iron pool participate in the oxidation of 2′,7′-dichlorodihydrofluorescein? *Free Radic. Res.* **41:**563–570; 2007.

[122] O'Malley, Y. Q.; Reszka, K. J.; Britigan, B. E. Direct oxidation of 2′,7′-dichlorodihydrofluorescein by pyocyanin and other redox-active compounds independent of reactive oxygen species production. *Free Radic. Biol. Med.* **36:**90–100; 2004.

[123] Ott, M.; Robertson, J. D.; Gogvadze, V.; Zhivotovsky, B.; Orrenius, S. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc. Natl. Acad. Sci. USA* **99:**1259–1263; 2002.

[124] Gogvadze, V.; Orrenius, S.; Zhivotovsky, B. Multiple pathways of cytochrome *c* release from mitochondria in apoptosis. *Biochim. Biophys. Acta* **1757:**639–647; 2006.

[125] Garrido, C.; Galluzzi, L.; Brunet, M.; Puig, P. E.; Didelot, C.; Kroemer, G. Mechanisms of cytochrome *c* release from mitochondria. *Cell Death Differ.* **13:**1423–1433; 2006.

[126] Land, E. J.; Ebert, M. Pulse radiolysis studies of aqueous phenol. *Trans. Faraday Soc.* **63:**1181–1190; 1967.

[127] Marchesi, E.; Rota, C.; Fann, Y. C.; Chignell, C. F.; Mason, R. P. Photoreduction of the fluorescent dye 2′-7′-dichlorofluorescein: a spin trapping and direct electron spin resonance study with implications for oxidative stress measurements. *Free Radic. Biol. Med.* **26:**148–161; 1999.

[128] Willson, R. L. Pulse radiolysis studies of electron transfer reactions in aerobic solution. *J. Chem. Soc., Chem. Commun.* 1005; 1970.

[129] Prütz, W. A.; Butler, J.; Land, E. J. The glutathione free radical equilibrium, $GS^{\bullet} + GS^- = GSSG^{\bullet -}$, mediating electron transfer to Fe(III)-cytochrome *c*. *Biophys. Chem.* **49:**101–111; 1994.

[130] Bell, R. P. *The Proton in Chemistry.* Methuen, London; 1959.

[131] Albert, A.; Serjeant, E. P. *The Determination of Ionization Constants. A Laboratory Manual*. Chapman and Hall, London; 1984.

[132] Mordon, S.; Devoisselle, J. M.; Maunoury, V. *In vivo* pH measurement and imaging of tumor tissue using a pH-sensitive fluorescent probe (5,6-carboxyfluorescein): instrumental and experimental studies. *Photochem. Photobiol.* **60:**274–279; 1994.

[133] Cadenas, E.; Davies, K. J. A. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic. Biol. Med.* **29:**222–230; 2000.

[134] Sies, H. Oxidative stress: introductory remarks. In: Sies, H. (Ed.), *Oxidative Stress*. Academic Press, London; 1985.

[135] Burkitt, M. J.; Jones, C.; Lawrence, A.; Wardman, P. Activation of cytochrome *c* to a peroxidase compound I-type intermediate by H_2O_2: relevance to redox signalling in apoptosis. *Biochem. Soc. Symp.* **71:** 97–106; 2004.

[136] Jakubowski, W.; Bartosz, G. 2,7-Dichlorofluorescin oxidation and reactive oxygen species: what does it measure? *Cell Biol. Int.* **24:**757–760; 2000.

[137] Kalinich, J. F.; Ramakrishnan, N.; McClain, D. E. The antioxidant trolox enhances the oxidation of 2′,7′-dichlorofluorescin to 2′,7′-dichlorofluorescein. *Free Radic. Res.* **26:**37–47; 1997.

[138] Burner, U.; Obinger, C. Transient-state and steady-state kinetics of the oxidation of aliphatic and aromatic thiols by horseradish peroxidase. *FEBS Lett.* **411:**269–274; 1997.

[139] Burner, U.; Jantschko, W.; Obinger, C. Kinetics of oxidation of aliphatic and aromatic thiols by myeloperoxidase compounds I and II. *FEBS Lett.* **443:**290–296; 1999.

[140] Job, D.; Dunford, H. B. Substituent effect on the oxidation of phenols and aromatic amines by horseradish peroxidase compound I. *Eur. J. Biochem.* **66:**607–614; 1976.

[141] Dunford, H. B. *Heme Peroxidases*. Wiley-VCH, New York; 1999.

[142] Folkes, L. K.; Candeias, L. P. Interpretation of the reactivity of peroxidase compounds I and II with phenols by the Marcus equation. *FEBS Lett.* **412:**305–308; 1997.

[143] Goodwin, D. C.; Yamazaki, I.; Aust, S. D.; Grover, T. A. Determination of rate constants for rapid peroxidase reactions. *Anal. Biochem.* **231:**333–338; 1995.

[144] Hunter, E. P. L.; Desrosiers, M. F.; Simic, M. G. The effect of oxygen, antioxidants, and superoxide radical on tyrosine phenoxyl radical dimerization. *Free Radic. Biol. Med.* **6:**581–585; 1989.

[145] Sturgeon, B. E.; Sipe Jr., H. J.; Barr, D. P.; Corbett, J. T.; Martinez, J. G.; Mason, R. P. The fate of the oxidizing tyrosyl radical in the presence of glutathione and ascorbate. Implications for the radical sink hypothesis. *J. Biol. Chem.* **273:**30116–30121; 1998.

[146] Kirsch, M.; de Groot, H. Ascorbate is a potent antioxidant against peroxynitrite-induced oxidation reactions. Evidence that ascorbate acts by re-reducing substrate radicals. *J. Biol. Chem.* **275:**16702; 2000.

[147] Rota, C.; Fann, Y. C.; Mason, R. P. Phenoxyl free radical formation during the oxidation of the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase. *J. Biol. Chem.* **274:**28161–28168; 1999.

[148] Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L.; Huang, D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J. Agric. Food Chem.* **49:** 4619–4626; 2002.

[149] Clarke, E. D.; Goulding, K. H.; Wardman, P. Nitroimidazoles as anaerobic electron-acceptors for xanthine oxidase. *Biochem. Pharmacol.* **31:**3237–3242; 1982.

[150] Orna, M. V.; Mason, R. P. Correlation of kinetic parameters of nitroreductase enzymes with redox properties of nitroaromatic compounds. *J. Biol. Chem.* **264:**12379–12384; 1989.

[151] Butler, J.; Hoey, B. M. The one-electron reduction potential of several substrates can be related to their reduction rates by cytochrome P-450 reductase. *Biochim. Biophys. Acta* **1161:**73–78; 1993.

[152] Cenas, N.; Anusevicius, Z.; Bironaité, D.; Bachmanova, G. L.; Archakov, A. I.; Öllinger, K. The electron transfer reactions of NADPH:cytochrome P450 reductase with nonphysiological oxidants. *Arch. Biochem. Biophys.* **315:**400–406; 1994.

[153] Cenas, N.; Nemeikaite-Ceniene, A.; Sergediene, E.; Nivinskas, H.; Anusevicius, Z.; Sarlauskas, J. Quantitative structure-activity relationships in enzymatic single-electron reduction of nitroaromatic explosives: implications for their cytotoxicity. *Biochim. Biophys. Acta* **1528:**31–38; 2001.

[154] Chignell, C. F.; Sik, R. H. A photochemical study of cells loaded with 2′,7′-dichlorofluorescin: implications for the detection of reactive oxygen species generated during UVA irradiation. *Free Radic. Biol. Med.* **34:**1029–1034; 2003.

[155] Afzal, M.; Matsugo, S.; Sasai, M.; Xu, B.; Aoyama, K.; Takeuchi, T. Method to overcome photoreaction, a serious drawback to the use of dichlorofluorescin in evaluation of reactive oxygen species. *Biochem. Biophys. Res. Commun.* **304:**619–624; 2003.

[156] King, B. A.; Oh, D. H. Spatial control of reactive oxygen species formation in fibroblasts using two-photon-excitation. *Photochem. Photobiol.* **80:**1–6; 2004.

[157] Zuo, L.; Clanton, T. L. Detection of reactive oxygen and nitrogen species in tissues using redox-sensitive fluorescent probes. *Methods Enzymol.* **352:**307–325; 2002.

[158] Roos, A.; Boron, W. F. Intracellular pH. *Physiol. Rev.* **61:**296–434; 1981.

[159] Perrin, D. D.; Dempsey, B.; Serjeant, E. P. *pK_a Prediction for Organic Acids and Bases*. Chapman and Hall, London; 1981.

[160] Mchedlov-Petrossyan, N. O.; Rubtsov, M. I.; Lukatskaya, L. L. Ionization and tautomerism of chloro-derivatives of fluorescein in water and aqueous acetone. *Dyes Pigments* **1992:**18; 1992.

[161] Prosperi, E. Intracellular turnover of fluorescein diacetate. Influence of membrane ionic gradients on fluorescein efflux. *Histochem. J.* **22:** 227–233; 1990.

[162] Loetchutinat, C.; Kothan, S.; Dechsupa, S.; Meesungnoen, J.; Jay-Gerin, J.-P.; Mankhetkorn, S. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. *Radiat. Phys. Chem.* **72:**323–331; 2005.

[163] Saengkhae, C.; Loetchutinat, C.; Garnier-Suillerot, A. Kinetic analysis of fluorescein and dihydrofluorescein effluxes in tumour cells expressing the multidrug resistance protein, MRP1. *Biochem. Pharmacol.* **65:** 969–977; 2003.

[164] Saengkhae, C.; Loetchutinat, C.; Garnier-Suillerot, A. Kinetic analysis of rhodamines efflux mediated by the multidrug resistance protein (MRP1). *Biophys. J.* **85:**2006–2014; 2003.

[165] Liu, S. X.; Athar, M.; Lippai, I.; Waldren, C.; Hei, T. K. Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc. Natl. Acad. Sci. USA* **98:**1643–1648; 2001.

[166] Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. Rational design principle for modulating fluorescence properties of fluorescein-based probes by photoinduced electron tranfer. *J. Am. Chem. Soc.* **125:**8666–8671; 2003.

[167] Ueno, T.; Urano, Y.; Setsukinai, K.-i.; Takukusa, H.; Kojima, H.; Ohkubo, K.; Fukuzumi, S.; Nagano, T. Radtional principles for modulating fluorescence properties of fluorescein. *J. Am. Chem. Soc.* **126:**14079–14085; 2004.

[168] Maeda, H.; Fukuyasu, Y.; Yoshida, S.; Fukuda, M.; Saeki, K.; Matsuno, H.; Yamauchi, Y.; Yoshida, K.; Hirata, K.; Miyamato, K. Fluorescent probes for hydrogen peroxide based on a non-oxidative mechanism. *Angew. Chem. Int. Ed.* **43:**2389–2391; 2004.

[169] Maeda, H.; Yamamoto, K.; Nomura, Y.; Kohno, I.; Hafsi, L.; Ueda, N.; Yoshida, S.; Fukada, M.; Fukuyasu, Y.; Yamauchi, Y.; Itoh, N. A design of fluorescent probes for superoxide based on a nonredox mechanism. *J. Am. Chem. Soc.* **127:**68–69; 2005.

[170] Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. A selective, cell-permeable optical probe for hydrogen peroxide in living cells. *J. Am. Chem. Soc.* **126:**15392–15393; 2004.

[171] Miller, E. W.; Albers, 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.

[172] Xu, K.; Tang, B.; Huang, H.; Yang, G.; Chen, Z.; Li, P.; An, L. Strong red fluorescent probes suitable for detecting hydrogen peroxide generated by mice peritoneal macrophages. *Chem. Commun.* 5974–5976; 2005.

[173] Maeda, H.; Yamamoto, K.; Kohno, I.; Hafsi, L.; Itoh, N.; Nakagawa, S.; Kanagawa, N.; Suzuki, K.; Uno, T. Design of a practical fluorescent probe for superoxide based on protection–deprotection chemistry of fluoresceins with benzenesulfonyl protecting groups. *Chem. Eur. J.* **13:** 1946–1954; 2007.

[174] Xu, K.; Liu, X.; Tang, B. A phosphinate-based red fluorescent probe for

imaging the superoxide radical anion generated by RAW264.7 macrophages. *ChemBioChem* **8:**453–458; 2007.

[175] Soh, N.; Sakawaki, O.; Makihara, K.; Odo, Y.; Fukaminato, T.; Kawai, T.; Irie, M.; Imato, T. Design and development of a fluorescent probe for monitoring hydrogen peroxide using photoinduced electron transfer. *Bioorg. Med. Chem.* **13:**1131–1139; 2005.

[176] Wolfbeis, O. S.; Dürkop, A.; Wu, M.; Lin, Z. A europium-ion based luminescent sensing probe for hydrogen peroxide. *Angew. Chem. Int. Ed.* **41:**4495–4498; 2002.

[177] Dürkop, A.; Wolfbeis, O. S. Nonenzymatic direct assay of hydrogen peroxide at neutral pH using the Eu3Tc fluorescent probe. *J. Fluoresc.* **15:**755–761; 2005.

[178] Gao, J. J.; Xu, K. H.; Tang, B.; Yin, L. L.; Yang, G. W.; An, L. G. Selective detection of superoxide anion radicals generated from macrophages by using a novel fluorescent probe. *FEBS J.*1725–1733; 2007.

[179] Miller, E. W.; Bian, S. X.; Chang, C. J. A fluorescent sensor for imaging reversible redox cycles in living cells. *J. Am. Chem. Soc.* **129:** 2458–3459; 2007.

[180] Setsukinai, K.-I.; Urano, Y.; Kakinuma, K.; Majima, H. J.; Nagano, T. Development of novel fluorescent probes that can reliably detect reactive oxygen species and distinguish specific species. *J. Biol. Chem.* **278:** 2170–3175; 2003.

[181] Setsukinai, K.-I.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. Fluorescence switching by O-dearylation of 7-aryloxycoumarins. Development of novel fluorescence probes to detect reactive oxygen species with high selectivity. *J. Chem. Soc., Perkin Trans.* **2:**2453–2457; 2000.

[182] Yang, D.; Wang, H.-L.; Sun, Z.-N.; Chung, N.-W.; Shen, J.-G. A highly selective fluorescent probe for the detection and imaging of peroxynitrite in living cells. *J. Am. Chem. Soc.* **128:**6004–6005; 2006.

[183] Heyne, B.; Beddie, C.; Sciano, J. C. Synthesis and characterization of a new fluorescent probe for reactive oxygen species. *Org. Biomol. Chem.* **5:**1454–1458; 2007.

[184] Heyne, B.; Maurel, V.; Sciano, J. C. Mechanism of action of sensors for reactive oxygen species based on fluorescein–phenol coupling: the case of 2-[6-(4¢-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid. *Org. Biomol. Chem.* **4:**802–807; 2006.

[185] Faulkner, K.; Fridovich, I. Luminol and lucigenin as detectors for $O_2^{\bullet-}$. *Free Radic. Biol. Med.* **15:**447–451; 1993.

[186] Maskiewicz, R.; Sogah, D.; Bruice, T. C. Chemiluminescent reactions of lucigenin. 1. Reactions of lucigenin with hydrogen peroxide. *J. Am. Chem. Soc.* **101:**5347–5354; 1978.

[187] Maskiewicz, R.; Sogah, D.; Bruice, T. C. Chemiluminescent reaction of lucigenin. 2. Reactions of lucigenin with hydroxide ion and other nucleophiles. *J. Am. Chem. Soc.* **101:**535–5364; 1978.

[188] Liochev, S. I.; Fridovich, I. Lucigenin (bis-*N*-methylacridinium) as a mediator of superoxide anion production. *Arch. Biochem. Biophys.* **337:**115–120; 1996.

[189] Liochev, S. I.; Fridovich, I. Lucigenin as mediator of superoxide production: revisited. *Free Radic. Biol. Med.* **25:**926–928; 1998.

[190] Vásquez-Vivar, J.; Hogg, N.; Pritchard Jr., K. A.; Martasek, P.; Kalyanaraman, B. Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study. *FEBS Lett.* **403:**127–130; 1997.

[191] Wardman, P.; Burkitt, M. J.; Patel, K. B.; Lawrence, A.; Jones, C. M.; Everett, S. A.; Vojnovic, B. Pitfalls in the use of common luminescent probes for oxidative and nitrosative stress. *J. Fluoresc.* **12:**65–68; 2002.

[192] Afanas'ev, I. B.; Ostrachovitch, E. A.; Korkina, L. G. Lucigenin is a mediator of cytochrome *c* reduction but not of superoxide production. *Arch. Biochem. Biophys.* **366:**267–274; 1999.

[193] Wood, P. M. The redox potential of the system oxygen-superoxide. *FEBS Lett.* **44:**21–24; 1974.

[194] Wardman, P. The reduction potential of benzyl viologen: an important reference compound for oxidant/radical redox couples. *Free Radic. Res. Commun.* **14:**57–67; 1991.

[195] Spasojevic, I.; Liochev, S. I.; Fridovich, I. Lucigenin: redox potential in aqueous media and redox cycling with O_2^- production. *Arch. Biochem. Biophys.* **373:**447–450; 1999.

[196] Farrington, J. A.; Ebert, M.; Land, E. J. Bipyridylium quaternary salts and related compounds. Part 6. Pulse radiolysis studies of the reaction of paraquat radical analogues with oxygen. *J. Chem. Soc., Faraday Trans.* **174:**665–675; 1978.

[197] Goldstein, S.; Fridovich, I.; Czapski, G. Kinetic properties of Cu,Zn-superoxide dismutase as a function of metal content—order restored. *Free Radic. Biol. Med.* **41:**937–941; 2006.

[198] Heiser, I.; Muhr, A.; Elstner, E. F. Production of OH-radical-type oxidant by lucigenin. *Z. Naturforsch.* **53c:**9–14; 1998.

[199] Schepetkin, I. A. Lucigenin as a substrate of microsomal NAD(P)H-oxidoreductases. *Biochemistry (Moscow)* **64:**25–32; 1999.

[200] Baker, M. A.; Krutskikh, A.; Curry, B. J.; McLaughlin, E. A.; Aitken, R. J. Identification of cytochrome P450-reductase as the enzyme responsible for NADPH-dependent lucigenin and tetrazolium salt reduction in rat epididymal sperm preparations. *Biol. Reprod.* **71:**307–318; 2004.

[201] Sohn, H.-Y.; Keller, M.; Gloe, T.; Crause, P.; Pohl, U. Pitfalls of using lucigenin in endothelial cells: implications for NAD(P)H dependent superoxide formation. *Free Radic. Res.* **32:**265–272; 1999.

[202] Li, Y.; Zhu, H.; Kuppusamy, P.; Roubaud, V.; Zweier, J. L.; Trush, M. A. Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enymatic and cellular systems. *J. Biol. Chem.* **273:**2015–2023; 1998.

[203] Li, Y.; Zhu, H.; Trush, M. A. Detection of mitochondria-derived reactive oxygen species production by the chemiluminescent probes lucigenin and luminol. *Biochim. Biophys. Acta* **1428:**1–12; 1999.

[204] Münzel, T.; Afanas'ev, I. B.; Kleschyov, A. L.; Harrison, D. G. Detection of superoxide in vascular tissue. *Arterioscler. Thromb. Vasc. Biol.* **22:**1761–1768; 2002.

[205] Janiszewski, M.; Souza, H. P.; Liu, Z.; Pedro, M. A.; Zweier, J. L.; Laurindo, F. R. M. Overestimation of NADH-driven vascular oxidase activity due to lucigenin artifacts. *Free Radic. Biol. Med.* **32:**446–453; 2002.

[206] Souchard, J.-P.; Barbacanne, M.-A.; Bayard, F.; Nepveu, F.; Arnal, J.-F. Detection of superoxide anion release by endothelial cells using reduced cytochrome c UV-visible absorption, ESR spin trapping, hydroethidine fluorescence, and lucigenin-enhanced chemiluminescence techniques. *Analysis* **28:**475–477; 2000.

[207] Barbacanne, M.-A.; Souchard, J.-P.; Darblade, B.; Iliou, J.-P.; Nepveu, F.; Pipy, B.; Bayard, F.; Arnal, J.-F. Detection of superoxide anion released extracellularly by endothelial cells using cytochrome c reduction, ESR, fluorescence and lucigenin-enhanced chemiluminescence techniques. *Free Radic. Biol. Med.* **29:**388–396; 2000.

[208] Watanabe, N.; Dickinson, D. A.; Liu, R.-M.; Forman, H. J. Quinones and glutathione metabolism. *Methods Enzymol.* **378:**319–340; 2004.

[209] Wardman, P.; Clarke, E. D. One-electron reduction potentials of substituted nitroimidazoles measured by pulse radiolysis. *J. Chem. Soc., Faraday Trans.* **1 72:**1377–1390; 1976.

[210] Afanas'ev, I. B.; Korkina, L. G.; Suslova, T. B.; Soodaeva, S. V. Are quinones producers or scavengers of superoxide ion in cells? *Arch. Biochem. Biophys.* **281:**245–250; 1990.

[211] Lind, J.; Merényi, G.; Eriksen, T. E. Chemiluminescence mechanism of cyclic hydrazides such as luminol in aqueous solutions. *J. Am. Chem. Soc.* **105:**7655; 1983.

[212] Merényi, G.; Lind, J.; Eriksen, T. E. Luminol chemiluminescence: chemistry, excitation, emitter. *J. Biolumin. Chemilumin.* **5:**53–56; 1990.

[213] Merenyi, G.; Lind, J.; Eriksen, T. E. The equilibrium reaction of the luminol radical with oxygen and the one-electron reduction potential of 5-aminophthalazine-1,4-dione. *J. Phys. Chem.* **88:**2320–2323; 1984.

[214] Vilim, V.; Wilhelm, J. What do we measure by a luminol-dependent chemiluminescence of phagocytes? *Free Radic. Biol. Med.* **6:**623–629; 1989.

[215] Samuni, A.; Krishna, C. M.; Cook, J.; Black, C. D.; Russo, A. On radical production by PMA-stimulated neutrophils as monitored by luminol-amplified chemiluminescence. *Free Radic. Biol. Med.* **10:**305–313; 1991.

[216] Baxendale, J. H. Pulse radiolysis study of the chemiluminescence from luminol (5-amino-2,3-dihydrophthalazine-1,4-dione). *J. Chem. Soc., Faraday Trans.* **1 69:**1665–1677; 1973.

[217] Oosthuizen, M. M. J.; Greyling, D. Hydroxyl radical generation: the effects of bicarbonate, dioxygen and buffer concentration on pH-dependent chemiluminescence. *Redox Rep.* **6:**105–115; 2001.
[218] Stanbury, D. M. Reduction potentials involving inorganic free radicals in aqueous solution. *Adv. Inorg. Chem.* **33:**69–138; 1989.
[219] Merényi, G.; Lind, J.; Shen, X.; Eriksen, T. E. Oxidation potential of luminol. Is the autoxidation of singlet organic molecules an outer-sphere electron transfer? *J. Phys. Chem.* **94:**748–752; 1990.
[220] Marley, N. A.; Gaffney, J. S.; White, R. V.; Rodriguez-Cuadra, L.; Herndon, S. E.; Dunlea, E.; Volkamer, R. M.; Molina, L. T.; Molina, M. J. Fast gas chromatography with luminol chemiluminescence detection for the simultaneous determination of nitrogen dioxide and peroxyacetyl nitrate in the atmosphere. *Rev. Sci. Instrum.* **75:**4595–4605; 2004.
[221] Robinson, J. K.; Bollinger, M. J.; Birks, J. W. Luminol/H_2O_2 chemiluminescence detector for the analysis of nitric oxide in exhaled breath. *Anal. Chem.* **71:**5131–5136; 1999.
[222] Castro, L.; Alvarez, M. N.; Radi, R. Modulatory role of nitric oxide on superoxide-dependent luminol chemiluminescence. *Arch. Biochem. Biophys.* **333:**179–188; 1996.
[223] Nakamura, M.; Nakamura, S. One- and two-electron oxidations of luminol by peroxidase systems. *Free Radic. Biol. Med.* **24:**537–544; 1998.
[224] Wheatley, R. A.; Sariahmetoglu, M.; Cakici, I. Enhancement of luminol chemiluminescence by cysteine and glutathione. *Analyst* **125:**1902–1904; 2000.
[225] Rost, M.; Karge, E.; Klinger, W. What do we measure with lumiol-, lucigenin-, and penicillin-amplified chemiluminescence? 1. Investigations with hydrogen peroxide and sodium hypochlorite. *J. Biolumin. Chemilumin.* **13:**355–363; 1998.
[226] Whitehead, T. P.; Thorpe, G. H. G.; Maxwell, S. R. J. Enhanced chemiluminescent assay for antoixidant capacity in biological fluids. *Anal. Chim. Acta* **266:**265–277; 1992.
[227] Radi, R.; Rubbo, H.; Thomson, L.; Prodanov, E. Luminol chemiluminescence using xanthine and hypoxanthine as xanthine oxidase substrates. *Free Radic. Biol. Med.* **8:**121–126; 1990.
[228] Daiber, A.; Oelze, M.; August, M.; Wendt, M.; Sydow, K.; Wieboldt, H.; Kleschyov, A. L.; Munzel, T. Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. *Free Radic. Res.* **38:**259–269; 2004.
[229] Lucas, M.; Solano, F. Coelentrazine is a superoxide anion-sensitive chemiluminescent probe: its usefulness in the assay of respiratory burst in neutrophils. *Anal. Biochem.* **206:**273–277; 1992.
[230] Lu, C.; Song, G.; Li, J.-M. Reactive oxygen species and their chemiluminecence-detection methods. *Trends Anal. Chem.* **25:**985–995; 2006.
[231] Suzuki, N.; Suetsuna, K.; Mashiko, S.; Yoda, B.; Nomoto, T.; Toya, Y.; Inaba, H.; Goto, T. Reaction rates for the chemiluminescence of *Cypridina* luciferin analogues with superoxide: a quenching experiment with superoxied dismutase. *Agric. Biol. Chem.* **55:**157–160; 1991.
[232] Akutsu, K.; Nakajima, H.; Katoh, T.; Kino, S.; Fujimori, K. Chemiluminescence of Cipridina luciferin analogues. Part 2. Kinetic studies on the reaction of 2-methyl-6-phenylimidazo[1,2-a]pyrazin-3(7H)-one (CLA) with superoxide: hydroperoxyl radical is an actual active species used to initiate the reaction. *J. Chem. Soc., Perkin Trans.* **2:**1699; 1995.
[233] Kambayashi, Y.; Ogino, K. Reestimation of Cypridina luciferin analogs (MCLA) as a chemiluminescence probe to detect active oxygen species—cautionary note for use of MCLA. *J. Toxicol. Sci.* **28:**139–148; 2003.
[234] Zheng, J.; Springston, S. R.; Weinstein-Lloyd, J. Quantitative analysis of hydroperoxyl radical using flow injection analysis with chemiluminescence detection. *Anal. Chem.* **75:**4696–4700; 2003.
[235] Tarpey, M. M.; White, C. R.; Suarez, E.; Richardson, G.; Radi, R.; Freeman, B. A. Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation. *Circ. Res.* **84:**1203–1211; 1999.
[236] Teranishi, K. Development of imidazopyrazinone red-chemiluminescent probes for detecting superoxide anions via a chemiluminecence resonance energy transfer method. *Luminescence* **22:**147–156; 2007.
[237] Miles, A. M.; Chen, Y.; Owens, M. W.; Grisham, M. B. Fluorometric determination of nitric oxide. *Methods* **7:**40–47; 1995.
[238] Kojima, H.; Nakatsubo, N.; Kibuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* **70:**2446–2453; 1998.
[239] Nakatsubo, N.; Kojima, H.; Kikuchi, K.; Nagoshi, H.; Hirata, Y.; Maeda, D.; Imai, Y.; Irimura, T.; Nagano, T. Direct evidence of nitric oxide production friom bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. *FEBS Lett.* **427:**263–266; 1998.
[240] Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Hirata, Y.; Nagano, T. Fluorescent indicators for imaging nitric oxide production. *Angew. Chem. Int. Ed. Engl.* **38:**3209–3212; 1999.
[241] Nagano, T. Practical methods for detection of nitric oxide. *Luminescence* **14:**283–290; 1999.
[242] Kojima, H.; Hirotani, M.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. Fluorescent indicators for nitric oxide based on rhodamine chromophore. *Tetrahedron Lett.* **41:**69–72; 2000.
[243] Itoh, Y.; Ma, F. H.; Hoshi, H.; Oka, M.; Noda, K.; Ukai, Y.; Kojima, H.; Nagano, T.; Toda, N. Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. *Anal. Chem.* **287:**203–209; 2000.
[244] Kojima, H.; Hirotani, M.; Nakatsubo, N.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Hirata, Y.; Nagano, T. Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore. *Anal. Chem.* **73:**1967–1973; 2001.
[245] Chatton, J.-Y.; Broillet, M.-C. Detection of nitric oxide production by fluorescent indicators. *Methods Enzymol.* **359:**134–148; 2002.
[246] Lacza, Z.; Horváth, E. M.; Pankotai, E.; Csordás, A.; Kollai, M.; Szabo, C.; Busija, D. W. The novel red-fluorescent probe DAR-4M measures reactive nitrogen species rather than NO. *J. Pharm. Toxicol. Methods* **52:**335–340; 2005.
[247] Leikert, J. F.; Räthel, T. R.; Müller, C.; Vollmar, A. M.; Dirsch, V. M. Reliable in vitro measurement of nitric oxide released from endothelial cells using low concentrations of the fluorescent probe 4,5-diaminofluorescein. *FEBS Lett.* **506:**131–134; 2001.
[248] Räthel, T. R.; Leikert, J. F.; Vollmar, A. M.; Dirsch, V. M. Application of 4,5-diaminofluorescein to reliably measure nitric oxide released from endothelial cells in vitro. *Biol. Proced. Online* **5:**136–142; 2003.
[249] Plater, J.; Greig, O.; Helfrich, M. H.; Ralston, S. H. The synthesis and evaluation of *o*-phenylenediamine derivatives as fluorescent probes for nitric oxide detection. *J. Chem. Soc., Perkin Trans.* **1:**2553–2559; 2001.
[250] Gabe, Y.; Urano, Y.; Kikuchi, K.; Kojima, H.; Nagano, T. Highly sensitive fluorescence probes for nitric oxide based on boron dipyrromethene chromophore—rational design of potentially useful bioimaging fluorescence probe. *J. Am. Chem. Soc.* **126:**3357–3367; 2004.
[251] Huang, K.-J.; Wang, H.; Ma, M.; Zhang, X.; Zhang, H.-S. Real-time imaging of nitric oxide production in living cells with 1,3,5,7-tetramethyl-2,6-dicarbethoxy-8-(3′,4′-diaminophenyl)-difluoroboradiaza-s-indacence by invert fluorescence microscope. *Nitric Oxide* **16:**36–43; 2007.
[252] Sasaki, E.; Kojima, H.; Nishimatsu, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. Highly sensitive near-infrared fluorescent probes for nitric oxide and their application to isloated organs. *J. Am. Chem. Soc.* **127:** 3684–3685; 2005.
[253] Rodriguez, J.; Specian, V.; Maloney, R.; Jourd'heuil, D.; Feelisch, M. Performance of diamino fluorophores for the localization of sources and targets of nitric oxide. *Free Radic. Biol. Med.* **38:**356–368; 2005.
[254] Ku, C.-J.; Karunarathne, W.; Kenyon, S.; Root, P.; Spence, D. Fluorescence determination of nitric oxide production in stimulated and activated platelets. *Anal. Chem.* **79:**2421–2426; 2007.
[255] Williams, D. H. L. *Nitrosation Reactions and The Chemistry of Nitric Oxide*. Elsevier, Amsterdam; 2004.
[256] Liu, X.; Miller, M. J. S.; Joshi, M. S.; Thomas, D. D.; Lancaster Jr., J. R. Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc. Natl. Acad. Sci. USA* **95:** 2175–2179; 1998.
[257] Möller, M. N.; Li, Q.; Vitturi, D. A.; Robinson, J. M.; Lancaster Jnr, J. R.; Denicola, A. Membrane "lens" effect: focusing the formation of reactive

nitrogen oxides from the $^{\bullet}NO/O_2$ reaction. *Chem. Res. Toxicol.* **20:**709–714; 2007.

[258] Jonsson, M.; Lind, J.; Eriksen, T. E.; Merényi, G. Redox and acidity properties of 4-substituted aniline radical cations in water. *J. Am. Chem. Soc.* **116:**1423–1427; 1994.

[259] Merényi, G.; Lind, J. Anilinyl radicals. In: Alfassi, Z.B. (Ed.), *N-Centered Radicals*. Wiley, Chichester; 1998.

[260] Espey, M. G.; Miranda, K. M.; Thomas, D. D.; Wink, D. A. Distinction between nitrosating mechanisms within human cells and aqueous solution. *J. Biol. Chem.* **276:**30085–30091; 2001.

[261] Zhang, X.; Kim, W.-S.; Hatchers, N.; Potgieter, K.; Moroz, L. L.; Gillette, R.; Sweedler, J. V. Interfering with nitric oxide measurements. 4,5-Diaminofluorescein reacts with dehydroascorbic acid and ascorbic acid. *J. Biol. Chem.* **277:**48472–48478; 2002.

[262] Kytzia, A.; Korth, H. G.; Sustmann, R.; de Groot, H.; Kirsch, M. On the mechanism of the ascorbic acid-induced release of nitric oxide from N-nitrosated tryptophan derivatives: scavenging of NO by ascorbyl radicals. *Chemistry* **12:**8786–8797; 2006.

[263] Nagata, N.; Momose, K.; Ishida, Y. Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. *J. Biochem. (Tokyo)* **125:** 658–661; 1999.

[264] Jourd'heuil, D. Increased nitric oxide-dependent nitrosylation of 4,5-diaminofluorescein by oxidants: implications for the measurement of intracellular nitric oxide. *Free Radic. Biol. Med.* **33:**676–684; 2002.

[265] Balcerczyk, A.; Soszynski, M.; Bartosz, G. On the specificity of 4-amino-5-methylamino-2′,7′-difluorofluorescein as a probe for nitric oxide. *Free Radic. Biol. Med.* **39:**327–335; 2005.

[266] Bryan, N. S.; Grisham, M. B. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med*. in press, doi:10.1016/j.freeradbiomed.2007.04.026.

[267] Ye, X.; Kim, W.-S.; Rubkhim, S. S.; Sweedler, J. V. Measurement of nitric oxide by 4,5-diaminofluorescein without interferences. *Analyst* **129:**1200–1205; 2004.

[268] Broillet, M.-C.; Randin, O.; Chatton, J.-Y. Photoactivation and calcium sensitivity of the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2): implications for cellular NO imaging. *FEBS Lett.* **491:**227–232; 2001.

[269] Suzuki, N.; Kojima, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. Orthogonality of calcium concentration and ability of 4,5-diaminofluorescein to detect $NO^{\bullet}$. *J. Biol. Chem.* **277:**47–49; 2002.

[270] Rao, K. M. K.; Padmanabhan, J.; Kilby, D. L.; Cohen, H. J.; Currie, M. S.; Weinberg, J. B. Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. *J. Leukoc. Biol.* **51:**496–500; 1992.

[271] Gunasekar, P. G.; Kanthasamy, A. G.; Borowitz, J. L.; Isom, G. E. Monitoring intracellular nitric oxide formation by dichlorofluorescin in neuronal cells. *J. Neurosci. Methods* **61:**15–21; 1995.

[272] Imrich, A.; Kobzik, L. Fluorescence-based measurement of nitric oxide synthase activity in activated rat macrophages using dichlorofluorescin. *Nitric Oxide* **1:**359–369; 1997.

[273] Denicola, A.; Souza, J. M.; Radi, R.; Lissi, E. Nitric oxide diffusion in membranes determined by fluorescence quenching. *Arch. Biochem. Biophys.* **328:**208–212; 1996.

[274] Soh, N.; Katayama, Y.; Maeda, M. A fluorescent probe for monitoring nitric oxide production using a novel detection concept. *Analyst* **126:**564–566; 2001.

[275] Barker, S. L. R.; Clarke, H. A.; Swallen, S. F.; Kopelman, R. Ratiometric and fluorescence-lifetime-based biosensors incorporating cytochrome *c*′ and the detection of extra- and intracellular macrophage nitric oxide. *Anal. Chem.* **71:**1767–1772; 1999.

[276] Pearce, L. L.; Gandley, R. E.; Han, W.; Wasserloos, K.; Stitt, M.; Kanai, A. J.; McLaughlin, M. K.; Pitt, B. R.; Levitan, E. S. Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. *Proc. Natl. Acad. Sci. USA* **97:**477–482; 2000.

[277] St Croix, C. M.; Stitt, M. S.; Leelavanichkul, K.; Wasserloos, K. J.; Pitt, B. R.; Watkins, S. C. Nitric oxide-induced modification of protein thiolate clusters as determined by spectral fluorescence resonance energy transfer in live endothelial cells. *Free Radic. Biol. Med.* **37:**785–792; 2004.

[278] St Croix, C.; Stitt, M. S.; Watkins, S. C.; Pitt, B. R. Fluorescence resonance energy transfer-based assays for the real-time detection of nitric oxide signaling. *Methods Enzymol.* **396**; 2005.

[279] Bätz, M.; Korth, H.-G.; Meineke, P.; Sustmann, R. Fluorescence detection of nitric oxide based on chelotropic spin traps. *Methods Enzymol.* **301:**532–539; 1999.

[280] Meinecke, P.; Rauen, U.; de Groot, H.; Korth, H.-G. Cheletropic traps for the fluorescence detection of nitric oxide (nitrogen monoxide) in biological systems. *Chem. Eur. J.* **5:**1738–1747; 1995.

[281] Meineke, P.; Rauen, U.; de Groot, H.; Korth, H.-G.; Sustmann, R. Cheletropic traps for the fluorescence spectroscopic detection of nitric oxide (nitrogen monoxide) in biological systems. *Chem. Eur. J.* **5:** 1738–1747; 1999.

[282] Hilderbrand, S. C.; Lim, M. H.; Lippard, S. J. Dirhodium tetracarboxylate scaffolds as reversible fluorescence-based nitric oxide sensors. *J. Am. Chem. Soc.* **126:**4972–4978; 2003.

[283] Smith, R. C.; Tennyson, A. G.; Lim, M. H.; Lippard, S. J. Conjugated polymer-based fluorescence turn-on sensor for nitric oxide. *Org. Lett.* **7:**3573–3575; 2005.

[284] Lim, M. H.; Wong, B. A.; Pitcock, J. W. H.; Mokshagundam, D.; Baik, M.-H.; Lippard, S. J. Direct nitric oxide detection in aqueous solution by copper(II) fluorescent complexes. *J. Am. Chem. Soc.* **128:**14364–14373; 2006.

[285] Lim, M. H.; Lippard, S. J. Metal-based turn-on fluorescent probes for sensing nitric oxide. *Acc. Chem. Res.* **40:**41–51; 2007.

[286] Lim, M. H.; Xu, D.; Lippard, S. J. Visualization of nitric oxide in living cells by a copper-based fluorescent probe. *Nat. Chem. Biol.* **2:**375–380; 2006.

[287] Lim, M. H. Preparation of a copper-based fluorescent probe for nitric oxide and its use in mammalian cultured cells. *Nat. Protoc.* **2:**408–415; 2007.

[288] Sato, M.; Nakajima, T.; Goto, M.; Unezawa, Y. Cell-based indicators to visualize picomolar dynamics pf nitric oxide release from living cells. *Anal. Chem.* **78:**8175–8182; 2006.

[289] Sato, M. Imaging molecular events in single living cells. *Anal. Bioanal. Chem.* **386:**435–443; 2006.

[290] Sato, M.; Hida, N.; Umezawa, Y. Imaging the nanomolar range of nitric oxide with an amplifier-coupled fluorescent indicator in living cells. *Proc. Natl. Acad. Sci. USA* **102:**14515–14520; 2005.

[291] Boon, E. M.; Marletta, M. A. Sensitive and selective detection of nitric oxide using an H–NOX domain. *J. Am. Chem. Soc.* **128:**10022–10023; 2006.

[292] Makrigiorgos, G. M.; Baranowska-Kortylewicz, J.; Bump, E.; Sahu, S. K.; Berman, R. M.; Kassis, A. I. A method for detection of hydroxyl radicals in the vicinity of biomolecules using radiation-induced fluorescence of coumarin. *Int. J. Radiat. Biol.* **63:**445–458; 1993.

[293] Makrigiorgos, G. M.; Bump, E.; Huang, C.; Baranowska-Kortylewicz, J.; Kassis, A. I. A fluorimetric method for the detection of copper-mediated hydroxyl free radicals in the immediate proximity of DNA. *Free Radic. Biol. Med.* **18:**669–678; 1995.

[294] Chakrabarti, S.; Makrigiorgos, M. G.; O'Brien, K.; Bump, E.; Kassis, A. I. Measurement of hydroxyl radicals catalyzed in the immediate vicinity of DNA by metal-bleomycin complexes. *Free Radic. Biol. Med.* **20:**777–783; 1996.

[295] Chakrabarti, S.; Kassis, A. I.; Slayter, H. S.; Bump, E. A.; Sahu, S. K.; Makrigiorgos, G. M. Continuous detection of radiation or metal generated hydroxyl radicals within core chromatin particles. *Int. J. Radiat. Biol.* **73:**53–63; 1998.

[296] Halliwell, B.; Kaur, H. Hydroxylation of salicylate and phenylalanine as assays for hydroxyl radicals: a cautionary note visited for the third time. *Free Radic. Res.* **27:**239–244; 1997.

[297] Coudray, C.; Favier, A. Determination of salicylate hydroxylation products as an in vivo oxidative stress marker. *Free Radic. Biol. Med.* **29:**1064–1070; 2000.

[298] Barreto, J. C.; Smith, G. S.; Strobel, N. H. P.; McQuillin, P. A.; Miller, T. A. Terephthalic acid: a dosimeter for the detection of hydroxyl radicals *in vitro*. *Life Sci.* **56:**89–96; 1995.

[299] Fang, X.; Mark, G.; von Sonntag, C. OH radical formation by ultrasound

in aqueous solutions. Part I: the chemistry underlying the terephthalate dosimeter. *Ultrason. Sonochem.* **3:**57–63; 1996.

[300] Saran, M.; Summer, K. H. Assaying for hydroxyl radicals: hydroxylated terephthalate is a superior fluorescence marker than hydroxylated benzoate. *Free Radic. Res.* **31:**429–436; 1999.

[301] Brandenburg, K.; Moll, H. The terephthalate dosimeter for γ-, X- and β-radiation: dependence on dose and dose rate. *Radiat. Phys. Chem.* **23:**431–434; 1984.

[302] Mark, G.; Tauber, A.; Laupert, R.; Schuchmann, H.-P.; Schulz, D.; Mues, A.; von Sonntag, C. OH-radical formation by ultrasound in aqueous solution. Part II. Terephthalate and Fricke dosimetry and the influence of various conditions on the sonolytic yield. *Ultrason. Sonochem.* **5:**41–52; 1998.

[303] Newton, G. L.; Milligan, J. R. Fluorescence detection of hydroxyl radicals. *Radiat. Phys. Chem.* **75:**473–478; 2006.

[304] Li, B.; Gutierrez, P. L.; Blough, N. V. Trace determination of hydroxyl radical in biological systems. *Methods Enzymol.* **300:**202–216; 1999.

[305] Pou, S.; Bhan, A.; Bhadti, V. S.; Wu, S. Y.; Hosmane, R. S.; Rosen, G. M. The use of fluorophore-containing spin traps as potential probes to localize free radicals in cells with fluorescence imaging methods. *FASEB J.* **9:**1085–1090; 1995.

[306] Soh, N.; Makihara, K.; Sakoda, E.; Imato, T. A ratiometric fluorescent probe for imaging hydroxyl radicals in living cells. *Chem. Commun.* 496–497; 2004.

[307] Bartosz, G. Use of spectroscopic probes for detection of reactive oxygen species. *Clin. Chim. Acta* **368:**53–76; 2006.

[308] Palazzolo-Ballance, A. M.; Suquet, C.; Hurst, J. K. Pathways for intracellular generation of oxidants and tyrosine nitration by a macrophage cell line. *Biochemistry* **46:**7536–7548; 2007.

[309] Pastor, I.; Esquembre, R.; Micol, V.; Mallavia, R.; Mateo, C. R. A reday-to-use fluorescent biosensor for superoxide radical using superoxide dismutase and peroxidase immobilized in sol–gel glasses. *Anal. Biochem.* **334:**335–343; 2004.

[310] Basabe-Desmonts, L.; Reinhoudt, D. N.; Crego-Calama, M. Design of fluorescent materials for chemical sensing. *Chem. Soc. Rev.* **36:** 993–1017; 2007.