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Review

The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells ☆

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ABSTRACT

Background: Small molecule fluorescent probes are vital tools for monitoring reactive oxygen species in cells.
Scope of review: The types of probe available, the extent to which they are specific or quantitative and complications in interpreting results are discussed.
Major conclusions: Most commonly used probes (e.g. dihydrodichlorofluorescein, dihydrorhodamine) have some value in providing information on changes to the redox environment of the cell, but they are not specific for any one oxidant and the response is affected by numerous chemical interactions and not just increased oxidant generation. These probes generate the fluorescent end product by a free radical mechanism, and to react with hydrogen peroxide they require a metal catalyst. Probe radicals can react with oxygen, superoxide, and various antioxidant molecules, all of which influence the signal. Newer generation probes such as boronates act by a different mechanism in which nucleophilic attack by the oxidant on a blocking group releases masked fluorescence. Boronates react with hydrogen peroxide, peroxynitrite, hypochlorous acid and in some cases superoxide, so are selective but not specific. They react with hydrogen peroxide very slowly, and kinetic considerations raise questions about how the reaction could occur in cells.
General significance: Data from oxidant-sensitive fluorescent probes can provide some information on cellular redox activity but is widely misinterpreted. Recently developed non-redox probes show promise but are not generally available and more information on specificity and cellular reactions is needed. We do not yet have probes that can quantify cellular production of specific oxidants. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

In order to understand how reactive oxygen species contribute to cellular function and dysfunction, it is essential to know what specific species is/are produced, at what time, in what locality, and in what quantity. Preferably this information needs to be acquired in real time with live cells. Yet this is one of the most challenging tasks confronting oxidative stress researchers and there are still many obstacles to achieving these objectives. Valuable information on the biological production of reactive oxidants can be obtained using high performance liquid chromatography, mass spectrometry or other analytical procedures to detect specific products generated either from exogenous probes [1] or from the oxidation of protein, DNA, lipid or other biomolecules [2–5]. In some cases these provide specific detection, for example for superoxide, hypochlorous acid or nitrating species [6–9]. However, this methodology does not provide real time monitoring and there is a strong push for fluorescent/luminescent imaging techniques. Two main approaches are currently available, using either small molecule probes or genetically encoded fluorescent proteins. The focus of this review is the small molecules, which can be categorised (Fig. 1) either as "oxidant sensitive" or "non-redox" probes. The former includes most of the widely used probes, and comprises aromatic compounds that undergo oxidation to a fluorescent product. The latter includes compounds containing a masked fluorophore that is released by attack of the oxidant on the masking group. Many of the newer probes fit into this category. Genetically encoded probes are not covered here but discussed in detail by Lukyanovov and Belousov in this issue [10] and by Meyer and Dick [11].

Numerous reactive oxidants are generated in biology, from both endogenous and exogenous substrates. This article focuses on major species, namely superoxide radicals, hydrogen peroxide, peroxynitrite, hypochlorous acid and free radicals in general. Detection of nitric oxide has been covered by others including [12–14] and will not be addressed here. There are many recent reviews and commentaries on the use of fluorescent probes for detecting oxidative species in cells and tissues [12,14–17]. These all stress problems and pitfalls and warn

Abbreviations: DCF, dichlorofluorescein; GFP, green fluorescent protein; SOD, superoxide dismutase

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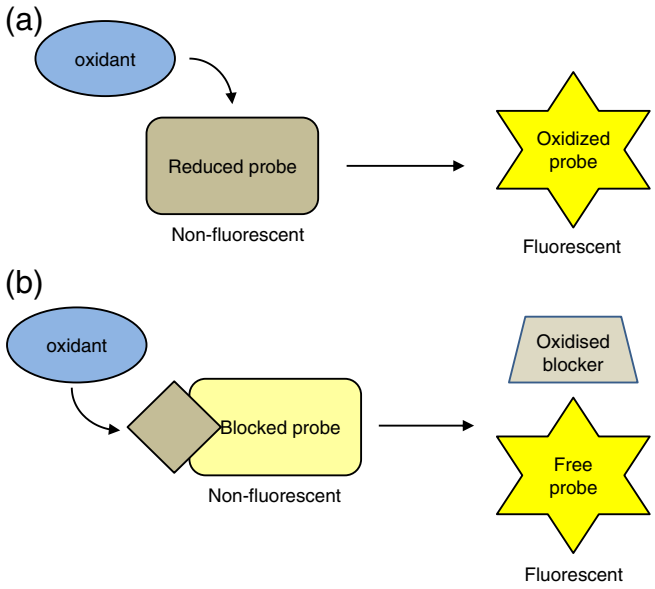

Fig. 1. Basic principles for oxidant detection using (a) oxidant-sensitive dyes and (b) "non-redox" probes.

about lack of specificity and over-interpretation of results. However, a measure of oxidant production is such an integral part of redox biology that these warnings are frequently ignored. There are many examples where otherwise excellent studies published in high profile journals are let down by conclusions drawn from simplistic and erroneous interpretation of probe data. It is not my intention to replicate the detailed critiques of the different methodologies, but provide an overview of what the data obtained with different types of probes can and cannot tell us, and what level of interpretation is justified. I will use references for much of the evidence and particularly recommend the excellent review by Wardman [12], who provides detailed analysis of probe mechanisms and critical assessment of how data can be interpreted or misinterpreted.

When contemplating the use of any probe, it is important to establish what type of information is required and whether it can be obtained with the chosen probe and experimental design. For live cells, the following might be wanted, in increasing order of difficulty:

1. Evidence for a disturbance in cellular redox state.
2. Detection of a specific oxidant.
3. Evidence for altered formation or destruction of a specific oxidant.
4. Identification of the site of production.
5. Quantification of how much of an oxidant is produced.

The ability of current techniques to deliver this information is discussed below, but to summarise, (1) is achievable, but generally provides limited mechanistic understanding, (2) is possible in some cases but is still a challenge, there are examples of (3) or (4) emerging, and (5) is yet to be achieved. However, even in apparently straightforward situations, there are many caveats to interpretation, many controls that are needed, and often the technique does not have the capability of answering the question asked.

2. Relevant properties of the oxidants

Before discussing how the different probes interact with specific oxidants, it is helpful to examine the relevant chemical properties of the oxidants. Fuller coverage of their biological chemistry is given elsewhere [18].

2.1. Superoxide radicals

Superoxide is both a weak oxidant and a weak reductant that reacts with relatively few biological molecules [19]. It can, however, oxidise some polyaromatic probe molecules directly. Superoxide is generated, often in large amounts, when organic radicals such as semiquinones react with oxygen, in a process called redox cycling [20]. A number of probes are oxidised or reduced to radicals that generate superoxide by this mechanism [12]. One facile reaction of superoxide is with other radicals to give non-radical products [21]. This can occur with many of the probe radicals and may result in reduction of the probe (thereby inhibiting the signal) or form an addition product that is in some cases responsible for probe fluorescence or luminescence [22,23].

2.2. Hydrogen peroxide

Hydrogen peroxide is a strong two electron oxidant, but because of high activation energy, most of its two electron oxidation reactions are too slow to be biologically relevant [24]. Important exceptions are reactions with selenocompounds and a minority of thiol proteins. Hydrogen peroxide does not directly oxidise the oxidant-sensitive probes in current use. It is much more reactive with transition metals, either in the form of low molecular weight complexes or metalloproteins. The resultant metal complexes can react with a wide range of substrates, including oxidant-sensitive probe molecules, to generate free radicals. Oxidation of these probes by hydrogen peroxide is therefore metal catalysed and subsequent reactions all involve free radical chemistry. Hydrogen peroxide can also act as a nucleophile and thereby act via a "non-redox" mechanism. The deprotonated (−OOH) form is the reactive species so with a pK_a of 11.6 these reactions can occur at neutral pH (where less than 1 in 10,000 hydrogen peroxide molecules are deprotonated) but tend to be slow. This property is exploited in some of the newer generation probes discussed in Section 4.2.

2.3. Peroxynitrite

Peroxynitrite exists in equilibrium with peroxynitrous acid (pK_a 6.8) which can directly oxidise various substrates including thiols [25] (Fig. 2). The acid also breaks down rapidly to give nitrogen dioxide and hydroxyl radicals [25]. However, with few exceptions, these reactions are of limited biological significance. Instead, peroxynitrite acts as a nucleophile and reacts predominantly with carbon dioxide to produce carbonate radicals and nitrogen dioxide [26,27]. The vast majority of the biological reactions of peroxynitrite, including with oxidant sensitive probes, are mediated by these radicals [25]. The nucleophilic nature of peroxynitrite enables it to react directly with some of the "non-redox" probes, as described in Section 4.2.

2.4. Hypochlorous acid

Hypochlorous acid is generated by the neutrophil enzyme, myeloperoxidase, and is particularly relevant in inflammatory conditions [28,29]. Chloramines, which are produced from hypochlorous acid and amines, are also important in this context as are hypobromous acid and bromamines. Discussion of probe reactivity here will be

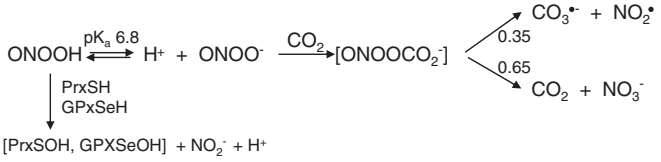

Fig. 2. Main biological reactions of peroxynitrite. As described by Ferrer-Sueta and Radi [25], breakdown of ONOOH to •OH and $•NO_2$ radicals is of little significance in the presence of CO_2. Reduced peroxiredoxins (PrxSH) and selenoglutathione peroxidases (GpxSeH) are the only known cell constituents capable of competing with the reaction with CO_2.

confined to hypochlorous acid, but many of the reactions will also occur (perhaps more slowly) with the other reactive halogen species. Detection of these oxidants is covered by Kettle in this issue [8]. In contrast to the species discussed above, the biologically relevant reactions of hypochlorous acid are almost exclusively two electron oxidations [18]. Thus it oxidises many of the oxidant sensitive probes by a non-radical mechanism. However, hypochlorous acid is generated by a peroxidase that also catalyses radical reactions, so distinguishing between it and peroxidation of a probe is often not straightforward. The high reactivity of hypochlorous acid is an advantage for designing probes that react poorly with radicals and other oxidants. However, it also means that it is readily scavenged, not only by numerous biological molecules [30], but also by components of organic buffers (Tris, HEPES, PIPES) and some solvents [31]. These can interfere with detection, especially if the probe reaction is not very fast. Excess hypochlorous acid can also further oxidise some detectors to a non-fluorescent product.

2.5. Free radicals

Free radicals warrant particular mention because the commonly employed oxidant-sensitive probes react by radical mechanisms (Section 4.1). A vast array of free radicals is generated in biological systems. They include highly reactive hydroxyl radicals and other inorganic radicals such as carbonate and nitrogen dioxide, as well as organic radicals derived from endogenous substrates (e.g. Cys, Tyr Trp, polyunsaturated fatty acids and many more) or exogenously added compounds (e.g. polyphenolic "antioxidants"). As described in Sections 2.2 and 2.3, metal-catalysed reactions of hydrogen peroxide and reactions of peroxynitrite proceed via free radical mechanisms. Radicals are also produced independently of these oxidants, for example by metal-catalysed autoxidation of phenolic compounds or reduction of quinone-like compounds to semiquinone radicals by flavoprotein reductases. Therefore, cells produce numerous free radical species that could oxidise probes directly. Likewise, there are many cell constituents that could act as scavengers of probe radicals.

3. Extracellular detection of reactive oxygen species

The strategy for detecting reactive oxidants differs depending on whether they are inside or outside the cell. Intracellular detection normally involves flow cytometry or fluorescence microscopy, whereas released oxidants can be monitored in real time by fluorescence or UV/visible spectrophotometry, or by sampling the medium at intervals, for example by HPLC or mass spectrometry. Although some of the complications with probes are the same in each case, extracellular detection is more straightforward as conditions are much more amenable to manipulation to optimise detection. A number of methods can be used to quantify release of specific oxidants. These will be discussed with a focus on superoxide and hydrogen peroxide; hypochlorous acid and peroxynitrite are discussed elsewhere [8,9].

3.1. Superoxide

A major advantage for extracellular detection is that superoxide dismutase (SOD) can be added to confirm specificity. The most established assay is spectrophotometric and measures SOD-inhibitable reduction of cytochrome c [32]. Provided sufficient cytochrome c is added to trap all the superoxide, the assay can reliably quantify all the superoxide released by cells such as neutrophils or monocytes that produce a robust burst of superoxide production when stimulated [33]. Adding catalase is also desirable to prevent re-oxidation of the product by any hydrogen peroxide that escapes from the cells. Reduced cytochrome c has a narrow absorbance maximum so accurate quantification using the quoted extinction coefficient requires a spectrophotometer slit width of 0.5 nm. Plate assays require independent calibration. Assays using the cell-impermeable formazan dye, WST-1 (which gives a water-soluble product) can also give quantitative information [34]. However, assays using formazans involve multistep radical reactions and are more open to interference. Detection of superoxide production by non-phagocytic cells is more problematic, as they produce order(s) of magnitude less than phagocytes and more specific and sensitive methods are needed. A fluorescence method using hydropropidine, a cell impermeable analogue of hydroethidine (see Section 4.1.3) has recently been described [35]. However, the product needs to be validated by LC/MS and the reaction rate is slower than for cytochrome c so it may not be as sensitive. Chemiluminescence assays, e.g. with MCLA (Section 4.1.4) may also be informative.

3.2. Hydrogen peroxide

Most assays for extracellular hydrogen peroxide involve peroxidase-mediated oxidation of a substrate to a fluorescent (or coloured) product. Any number of peroxidase substrates can be employed, including scopoletin (fluorescence decrease), homovanillic acid, phenol red or Amplex red (fluorescence or UV absorbance increase) [36–38]. Oxidation of Amplex red (10-acetyl-3,7-dihydroxyphenoxazine; Fig. 3) to resorufin [39] provides the greatest sensitivity and is currently the most widely used. Provided several important requirements are met, these assays can provide reliable quantitative data. Sufficient horseradish peroxidase must be present to scavenge all the peroxide as it is released, and SOD should be added to prevent the rapid reaction of the probe radical intermediate with superoxide [38]. Otherwise, this reaction can diminish the signal. There are other caveats. Free radical scavengers can inhibit the response by scavenging the probe radical (for example addition of apocynin to test for NADPH oxidase involvement can unintentionally have this effect). Care must be taken to minimise light-induced reduction of resorufin in the presence of reducing agents such as NAD(P)H or GSH [40] as this results in redox-cycling that artifactually amplifies the response. This is more of a problem with broken cells, but even without added reductants, continuous light exposure should be avoided. Variations of the Amplex red assay are available in kit form. These are subject to the same caveats, and it is just as important to establish that these (or any other) kits perform optimally and specifically under particular conditions as it is for non-kit assays.

The Amplex red and other peroxidase-based assays will measure externally generated hydrogen peroxide as well as any that is produced inside the cell and diffuses out. The extent to which hydrogen peroxide is released will depend on how efficiently it is consumed in the cell and is difficult to quantify. Release can be enhanced by adding azide to inhibit catalase, but in many cells hydrogen peroxide is metabolised mostly by glutathione peroxidases and peroxiredoxins, so it is not possible to assess what proportion is detected.

Extracellular hydrogen peroxide can be monitored by analysing samples taken at intervals from the medium. Without a peroxidase added as a trap, the peroxide can diffuse back into the cells, so such analyses give a steady state concentration that reflects a balance between generation and cellular consumption. Another, rather underutilised, way to monitor the steady state concentration of hydrogen peroxide is with a peroxide electrode (available from World Precision Instruments). Using this method, concentrations in the low micromolar range have been measured in the surroundings of stimulated neutrophils [41]. Whether the electrode is sensitive enough to detect peroxide release from other cells has not been tested.

4. Intracellular detection of reactive oxygen species

Fluorescence imaging and flow cytometry using sensitive and specific probes should in principle be ideal for monitoring cellular production of reactive oxygen species. Most laboratories have the required equipment and it is experimentally straightforward to add a detector to cells and monitor the effect of a treatment. The problem is that interpretation of the results is much more complex. A wide variety of probes

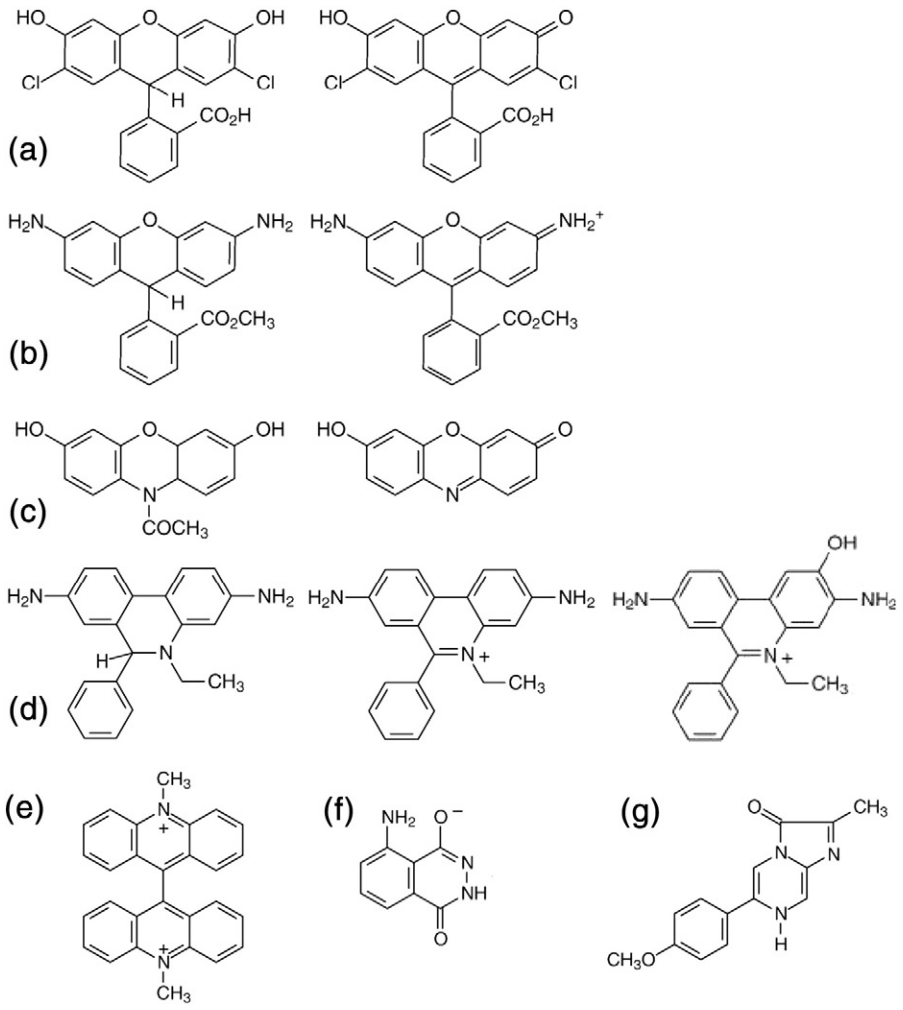

Fig. 3. Structures of commonly employed oxidant-sensitive fluorescent and chemiluminescent probes. (a) $DCFH_2$; (b) dihydrorhodamine; (c) Amplex red; (d) hydroethidine; (e) lucigenin; (f) luminol; (g) MCLA (luciferin analogue, 2-Methyl-6-(4-methoxyphenyl)imidazo[1, 2-*a*]pyrazin-3(7*H*)-one). For (a)–(d) the left hand column shows the structure of the reduced (non-fluorescent) probe, the right column the fluorescent oxidation product. For (d) the middle structure is the non-specific product, ethidium and the right hand structure is of 2-hydroxyethidium, which requires superoxide for formation. With all these probes, product formation is a multi-step reaction sequence. For the chemiluminescent probes (e–g) only the structures of the reduce forms are shown. These are oxidised by multistep radical reactions to give unstable peroxides that decompose to emit light.

is available. They are often used under the premise of detecting a particular species such as superoxide, hydrogen peroxide or peroxynitrite. However, this is commonly not the case and all to a greater or lesser extent have limitations of selectivity or sensitivity. Therefore, rather than structuring the following discussion around each oxidant, each class of probe and its oxidation mechanism will be discussed and specificity considered on this basis.

4.1. Oxidant-sensitive probes

These are oxidant-sensitive dyes that are able to penetrate cells (often as their esterified derivatives which undergo hydrolysis) and become oxidised to fluorescent products (a in Fig. 1). By far the most widely used is dihydrodichlorofluorescein ($DCFH_2$), which becomes oxidised to DCF. Also in this category are dihydrorhodamine, hydroethidine, Amplex red (although it is membrane impermeable and does not act intracellularly) as well as the chemiluminescence detectors lucigenin and luminol (Fig. 3).

A key feature of these probes is that they are all oxidised by a one electron, free radical mechanism, producing a probe radical intermediate which is subsequently oxidised to the fluorescent product [12]. They do not react directly with non-radical species such as hydrogen peroxide or peroxynitrite (although hypochlorous acid is an exception), but they are oxidised by a host of radical species or metal-dependent processes. The probe radical intermediate can react in a number of ways. This is illustrated for DCF in Fig. 4, which is reproduced from Wardman [12] not so much to go through the individual reactions (which are described in detail in his review), but to illustrate the many processes that influence the signal. Equally complex schemes can be written for the other detectors.

4.1.1. Dichlorofluorescein

Before discussing the implications of Fig. 4, it is interesting to recall the first use of $DCFH_2$ by Bass et al. [42]. They used it along with flow cytometry to measure the oxidative burst of neutrophils, and observed a 20-fold increase in fluorescence when the cells were stimulated. The method has since proved to be a simple and effective way of identifying patients with neutrophil defects such as chronic granulomatous disease (NADPH oxidase deficiency). Problems associated with the DCF assay are less of an issue with neutrophils as they generate large amounts of superoxide and their high content of myeloperoxidase ensures efficient catalysis of probe oxidation. This gives a much higher increase in fluorescence that is less prone to artefact compared with other cells (which typically change less than 2-fold). Nevertheless, variations in myeloperoxidase activity as well as peroxide production affect the response so obtaining mechanistic information is complex.

General use of DCF is much more problematic. The assay is most commonly used with the assumptions that it is detecting hydrogen

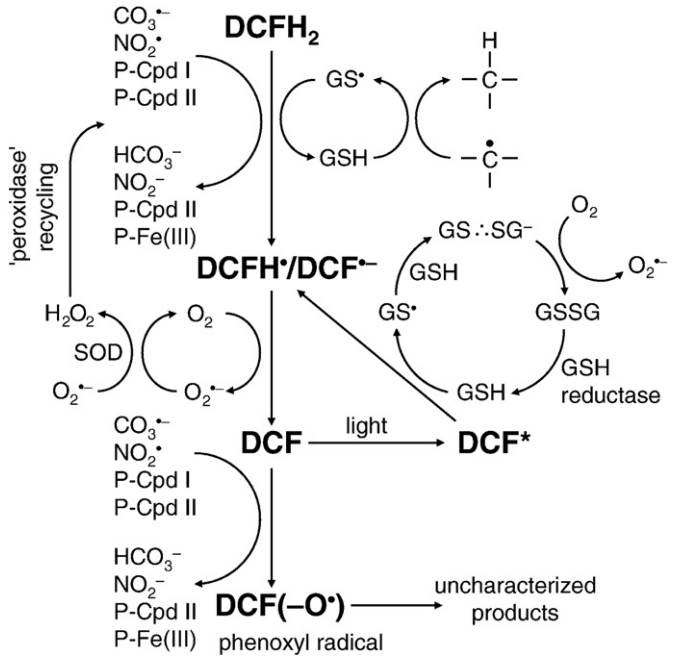

Fig. 4. Complexities of the mechanism of oxidation of reduced fluorescein dyes such as $DCFH_2$ and potential interacting pathways. P, peroxidase-like catalysts that act via Compounds I and II.
Reprinted with permission from Wardman [12].

peroxide and that any change in signal represents a change in hydrogen peroxide production. Although this may in some cases be true, the assay is not specific for hydrogen peroxide, and there are a number of factors that can change signal intensity without there being an increase or decrease in oxidant production.

Thus there are a number of limitations to the assay. These have been critically analysed by others [12,14–17,43] and the conclusions can be summarised as follows:

(a) Hydrogen peroxide itself does not react with $DCFH_2$. It requires a peroxidase or other low molecular weight or protein-bound transition metal catalyst. A negative response could therefore be misinterpreted if no catalyst were present. Likewise, an alternative explanation for an increase in signal is increased availability of a metal catalyst. Examples of this include cytochrome c release during apoptosis [44] or lysosomal iron release [45].
(b) Many free radicals, including those derived from peroxynitrite, tyrosine, thiols (see Fig. 2) can oxidise the probe [46]. These could be generated independently of hydrogen peroxide
(c) The probe radical is readily scavenged by added "antioxidants". For example compounds such as N-acetylcysteine or ascorbate are often added to a cell system and shown to inhibit fluorescence. This is most likely to be due to radical scavenging (either the probe radical or the initiating species) and not due to their reaction with hydrogen peroxide, which is extremely slow.
(d) The reaction itself generates hydrogen peroxide. The main route from $DCFH_2$ to DCF is via a reaction between the DCF radical and oxygen [47]. This generates superoxide and thence hydrogen peroxide, thus amplifying the reaction. Therefore, inhibition by catalase is not evidence that the reaction was initiated by hydrogen peroxide. Furthermore, a change in efficiency of oxidation of the probe radical to the final product could be due to a change in oxygen tension [47].
(e) Antioxidant enzymes that scavenge hydrogen peroxide or superoxide will compete with the probe, so a change in signal could represent an increase or decrease in enzyme activity.
(f) The probe can give an artifactual signal due to light-induced oxidation [48]. Care needs to be taken to avoid and control for extended light exposure.

The DCF assay must therefore be used cautiously, and interpreted with due attention to all these confounders. Some, such as light exposure, can largely be avoided or controlled for. However, the assay alone will not identify what reactive oxygen species is being detected and a change in signal cannot be interpreted as an increase or decrease in oxidant generation. It may be possible to overcome some limitations by accompanying the DCF assay with independent detection of specific oxidants, by including controls for changes in concentrations of catalytic metals, oxygen or radical scavengers, or by using it in conjunction with selective knockout of an oxidant generator, e.g. NADPH oxidase. However, it is then not a simple assay. Nevertheless, the simple DCF assay can be of some use in providing an indication of a disturbance in the redox state of a cell.

An often ignored corollary of the oxidation of $DCFH_2$ (and other oxidant-sensitive probes) being a free radical process, is that intracellular oxidation of the probe implies that the cells are generating free radicals of some form. This is pertinent to the redox signalling field, where the focus tends to be on hydrogen peroxide and two electron oxidations and is perhaps a reminder that radical reactions could play a role.

4.1.2. Dihydrorhodamine

Dihydrorhodamine and other oxidant sensitive probes have similar structural features to DCF and are oxidised by a similar radical mechanism [12]. Therefore, they are subject to the same limitations and are no more specific or quantitative. Furthermore, there is limited value in developing analogues with enhanced fluorescence sensitivity, if they react via a similar free radical mechanism. Dihydrorhodamine warrants specific mention as early studies suggested that it could be a sensitive detector of peroxynitrite [49] and many investigators have interpreted dihydrorhodamine oxidation as evidence for peroxynitrite formation. However, it is now well understood that dihydrorhodamine does not react directly with peroxynitrite but like DCF, with the nitrogen dioxide and carbonate radicals generated from it (Fig. 2) [50]. It also reacts with many other radicals and peroxidases [12] so is clearly not specific.

4.1.3. Hydroethidine

Whereas hydroethidine is oxidised non-specifically by a mechanism similar to $DCFH_2$, there are features of this reaction that make it useful for detecting superoxide. First, hydroethidine is unusual because superoxide is able to oxidise it to its radical. However, the reaction is relatively slow [35] and is more likely to be performed by other cellular oxidants. What is more important is that once formed, the hydroethidine radical combines rapidly with superoxide to generate a specific product, 2-hydroxyethidium (Fig. 3). In contrast, the main oxidation product from other routes is ethidium [23]. Although these two products have subtly different fluorescent spectra [51], they cannot be distinguished with confidence in a cellular matrix [23]. Therefore, a simple fluorescence assay with hydroethidine cannot detect superoxide formation. Evidence for superoxide can be obtained by analysis of 2-hydroxyethidium by HPLC or mass spectrometry, and dihydroethidine in combination with HPLC currently provides one of the better detection methods for superoxide production [1,52,53]. However, there are still caveats to interpreting changes in yield of 2-hydroxyethidium, as these will be affected not only by the superoxide flux but also by the rate of formation of the initial radical and by reactions of the radical that compete with superoxide addition.

4.1.4. Chemiluminescent probes

Due to high sensitivity and ease of detection with small cell numbers, chemiluminescence assays have been widely used to detect reactive oxygen species. These reactions also involve multistep radical

mechanisms, and have the same limitations as the fluorescence assays. Luminol (Fig. 3) has been most widely employed, especially with phagocytes where, like DCF, it can be useful for detecting cell stimulation or major defects in oxidative metabolism [54]. However, the luminol reaction cannot identify the reactive species or provide mechanistic information. The mechanism has been widely studied [22,55,56], and as described in detail elsewhere [12] involves an initial oxidation step that can be carried out by a peroxidase plus hydrogen peroxide or by a host of free radicals. Reaction of the radical product with oxygen generates superoxide, which combines with another luminol radical to generate an intermediate that breaks down with the evolution of light. Apart from being non-specific, there are numerous radical interactions that can influence the chemiluminescence response. Thus, the assay has very little ability to discriminate between individual reactive oxygen species in biological systems and is extremely prone to interferences that are very difficult to control [57]. More sensitive luminol analogues such as L-012 have been developed [58], but the same issues of specificity and interference remain. In spite of this lack of specificity, a luminol-based method has been reported to selectively detect myeloperoxidase activity in a mouse inflammation model [59].

Lucigenin has been widely used, in spite of clear evidence to the contrary [22,60], as a selective detector of superoxide. Lucigenin (Fig. 3) is also chemiluminescent via a radical mechanism but in this case, the parent compound is reduced to a radical that reacts with superoxide to give an intermediate that decomposes with the emission of light [60]. Thus the reaction is superoxide dependent. However the lucigenin radical also undergoes redox cycling with oxygen to generate superoxide so the assay is self-defeating. The initial reaction to generate the lucigenin radical is carried out by cellular reductases so the assay is sensitive to changes in reductase activity. It has been proposed that superoxide generation during the assay can be minimised by using a low lucigenin concentration [61], but this is debatabe [12] and in one example using vascular homogenate, even 5 μM was sufficient to double the superoxide detected by an independent method [62].

Luciferin analogues such as coelenterazine or MCLA (Fig. 3) show more promise for chemiluminescent detection of superoxide [63,64]. Their oxidation mechanism also involves an initial oxidation step and a subsequent radical reaction with superoxide, but generation of superoxide from the probe radical is less of a problem than with lucigenin [65]. These compounds do react with other radicals, so there are still specificity and interference issues [12,66], and MCLA detects only extracellular superoxide. However, where superoxide production has been validated by an alternative procedure, the assay could provide a sensitive monitoring function.

4.2. Newer generation "non-redox" fluorescent probes

The need to overcome the multiple problems associated with oxidant sensitive dyes has stimulated interest in developing alternative fluorescent probes that rely on a completely different type of chemistry. There has been a recent surge of publications by a number of groups who have used innovative design and elegant chemistry to produce such compounds. Most activity has centred on fluorophores protected by a blocking group that is released by reactive oxygen species. These are often referred to as "non-redox" probes, as the fluorescence of the probe is unmasked through nucleophilic attack of the reactive species on the blocking group (Fig. 5). Thus an oxidised form of the blocking group is released and the oxidation state of the fluorophore is unchanged. In principle any fluorophore can be used, and in a recent example, release of luciferin by this mechanism has enabled a chemiluminescent readout [67]. One major advantage of this approach is that it involves direct reaction of the oxidant with the probe without requiring a catalyst. Another is that the probe is not oxidised, free radical intermediates are not generated and many of the associated complications are avoided. Boronates have been mostly investigated as leaving groups, as well as substituted benzene sulfonyl groups and benzil derivatives, and probes for hydrogen peroxide, peroxynitrite and superoxide have been described.

4.2.1. Boronates

The Chang group first described the use of boronate derivatives to detect hydrogen peroxide [68–70]. They have synthesised a wide range of compounds with different fluorophores to give a palette of colours [71] and adapted structures to enable targeting to mitochondria and other cell compartments [72,73]. They have observed fluorescence enhancement in a number of cellular systems including stimulated macrophages and A431 cells treated with EGF [69,70,74]. Their in vitro testing indicated specificity for hydrogen peroxide over other oxidants (with the proviso that peroxynitrite was not tested and HEPES buffer, a good hypochlorous acid scavenger, was used), and the probes are being increasingly employed as detectors for hydrogen peroxide production. Kalyanaraman's group has also developed a boronate probe, and shown that it responds to peroxynitrite and hypochlorous acid as well as to peroxides [1,75,76]. Thus it would appear that boronate probes are an advance on oxidant-sensitive dyes but they are not necessarily specific for a particular species.

What can we deduce about the reactive species detected by boronate probes in cells? One consideration is how fast the different species react with the probe. This is described by the rate constant for the reaction, which for hydrogen peroxide (at neutral pH where there is very little HOO^-) is very low ($k \sim 1\ M^{-1}\ s^{-1}$) [70,75]. Consequently, it takes more than an hour for 100 μM reagent hydrogen peroxide to react with a few micromolar probe [68,77,78]. Peroxynitrite ($k \sim 10^6\ M^{-1}\ s^{-1}$) reacts a million times faster and hypochlorous acid ($k \sim 10^4\ M^{-1}\ s^{-1}$) lies between the two [1,70]. Equally relevant is what other reactions the reactive species can undergo. Thus, a slow reaction with a probe may still occur if there are few alternatives. The main reactions of peroxynitrite in most cells are with carbon dioxide and peroxiredoxins, and it can be calculated from the data of Ferrer-Sueta and Radi [25] that a boronate probe at 20 μM should be half as effective as physiological carbon dioxide at trapping peroxynitrite. The probe is therefore unlikely to scavenge all peroxynitrite but should detect its production.

The efficiency of hydrogen peroxide detection in cells would be very much less. Boronate probes have similar reactivity to GSH, so with 5–20 μM probe and a typical cell concentration of 5 mM GSH, more than 99% of added hydrogen peroxide would react with GSH. However, in cells, hydrogen peroxide is not scavenged by GSH directly but by enzymes such as glutathione peroxidases and peroxiredoxins that react almost ten million times faster. Even with conservative estimates of low micromolar concentrations of the reduced forms of these peroxidases, they should allow only 1 in a million hydrogen peroxide molecules to react with the probe (thus requiring molar peroxide for 10% probe oxidation). It can be argued that high fluorescent intensity can compensate for low reactivity, by giving a measurable signal even if only a small proportion of the hydrogen peroxide is trapped. Except perhaps in cellular compartments where there may be less competition, this would seem a daunting challenge. Clearly a greater understanding is needed of how these probes detect hydrogen peroxide in a cell.

Provided it has sufficient sensitivity, a potential advantage of a probe reacting with only a small proportion of the hydrogen peroxide in the cell is that it would have little influence on other reactions of the peroxide and could provide a measure of its steady state concentration (described as a bioorthogonal approach [69]). In this respect, the probe may be better suited to monitoring stable, steady state hydrogen peroxide concentrations (as discussed in [93]) rather than short term fluxes. It is also important to keep in mind that a change in signal does not necessarily represent a change in amount of oxidant generated. It could also be due a change in concentration of one of the competing substrates, e.g. carbon dioxide for peroxynitrite or the activity of a peroxiredoxin or glutathione peroxidase for hydrogen peroxide. The signal will also

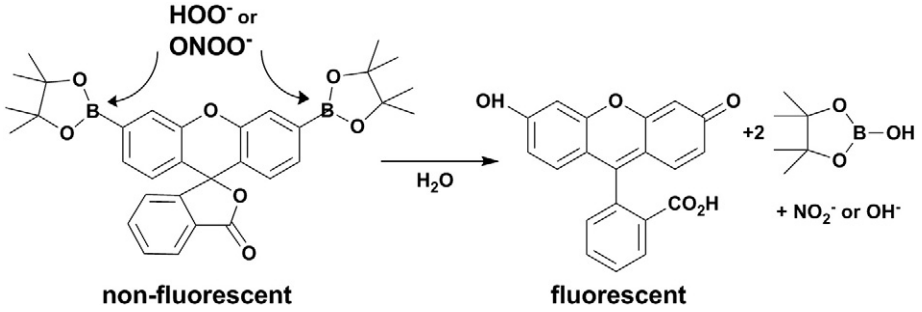

Fig. 5. Mechanism of fluorescence release from boronate probes. Reaction of PF1 (developed by Chang and coworkers [68]) with hydrogen peroxide or peroxynitrite is shown.

depend on how much probe is present, as at a higher concentration it will compete better for the oxidant and give a higher signal. Therefore, in any experimental study it needs to be established that the cellular concentration of probe does not vary depending on treatment. One way of accounting for such variation is to use a ratiometric probe. Such probes have been developed [78,79] and it will be interesting to see if they are sufficiently sensitive for hydrogen peroxide detection.

4.2.2. Other "non-redox" probes

Probes with rhodamine or fluoresceins protected with benzene sulfonyl ester [80,81] or benzil groups [82] have also been developed, in most cases for the detection of hydrogen peroxide. As with boronates, fluorescence is unmasked through a reaction with the blocking group. These compounds also react slowly with hydrogen peroxide and are subject to the same potential constraints. Initial testing showed selectivity for hydrogen peroxide over other oxidants, although peroxynitrite was not examined. The Maeda group has developed a doubly blocked fluorescein derivative with a preference for superoxide compared with hydrogen peroxide and other reactive species [83]. This has potential, especially for intracellular detection, although its relative low signal enhancement compared with cytochrome c in detecting superoxide from neutrophils suggests limited sensitivity. As with the boronates, these probes are promising developments but now need to be more widely available for extensive specificity testing and characterisation of what they detect in cells as against pure solution.

Another recently explored approach to oxidant detection is the use of cell-penetrating nanoparticles containing detectors such as benzyl-selenide-tricarbocyanine [84]. These authors report that their formulation shows selectivity for peroxynitrite, which reacts over seconds, and further development is warranted.

4.2.3. Probes for hypochlorous acid

Hypochlorous acid reacts with some of the oxidant sensitive and non-redox active probes described above, but they are too non-selective for specific detection. However, by taking advantage of its high reactivity, especially with sulfur or selenium centres, some success has been made with developing probes that appear to be specific (although reactivity with hypobromous acid and chloramines might be expected but not tested) [13]. These compounds have a highly reactive centre that once oxidised, releases the masked fluorescence of the probe [85–87]. They react fast enough to compete for at least some of the hypochlorous acid generated biologically. For example, real time detection of hypochlorous acid production inside phagosomes of stimulated neutrophils has been described [85,86]. As another approach, fluorescent CdSe–ZnS quantum dots that are quenched by HOCl have also been developed [88]. These and other [89,90] recently developed probes show promise and warrant further investigation.

4.2.4. Testing for and assigning specificity

Considerable advances in probe development have been made using novel approaches to synthesise molecules with excellent fluorescence properties and innovative blocking groups. It is important that this chemistry goes hand in hand with rigorous protocols to test specificity both with isolated oxidants and more complex biological matrices. To date most emphasis has been on chemical design and synthesis. In many of the published papers, specificity is addressed in one figure, in which a standard selection of oxidants is added, often in undefined media and each at an undefined single concentration, and changes monitored over an hour when the oxidant could have disappeared within seconds. Peroxynitrite, which is now known to react with boronate probes, has not commonly been included, potassium superoxide has been used as a source of superoxide but its rapid dismutation at the high concentrations added means it may not be ideal and HEPES buffer, which would convert hypochlorous acid to HEPES-derived chloramine, has often been used. With oxidant-sensitive probes, it has become clear that the more these compounds are studied, the less specific they become. Although the newer probes have fewer complications, there still needs to be caution in attributing a response to a particular oxidant. More extensive testing both in solution and in cellular settings is needed to ensure results from these probes are interpreted appropriately.

5. Localising oxidant production

To localise where an oxidant is produced, it is necessary to target the appropriate probe to different sites in the cell. Ideally, the probe should be highly reactive so that it traps the oxidant before it can diffuse away. To establish that the oxidant was generated at the site rather than diffusing into it, it also needs to be shown that an equivalent concentration of probe at other sites undergoes less oxidation. This can be achieved with the genetically encoded proteins, roGFP and Hyper, which react rapidly with peroxides, and by targeting Hyper to different sites, localised oxidant production during growth factor signalling has been observed [91,92]. The fluorescent non-redox probes react too slowly with hydrogen peroxide to prevent diffusion (see [18] for explanation) and the signal intensity at different sites would be highly dependent on how efficiently hydrogen peroxide was scavenged at each site. Even with targeted probes (e.g. to mitochondria) interpretation of signal localisation would be complex and achieving point 4 (Section 1) would be difficult.

6. Conclusions

Although assays using redox dyes such as DCF are simple to use and have given positive responses in numerous cell systems, their complex radical chemistry means that they are generally not specific to a particular oxidant and are affected by numerous chemical interactions. In particular, they do not react directly with hydrogen peroxide. Of the criteria listed in the Introduction, they have some value as indicators of redox changes in a cell (point 1) but as simple fluorescence assays (without accompanying analysis of a specific product such as 2-hydroxyethidium) they will not identify what oxidant is responsible (point 2). The complex mechanism of probe oxidation makes it

difficult to provide a mechanistic interpretation of changes in signal intensity (point 3) or to provide absolute quantification (point 5).

It is often argued that to attribute an effect to a particular oxidant, it is important to carry out more than one assay. While this is indeed desirable, there may be no advantage if the assays operate via similar mechanisms, e.g. evidence for superoxide or hydrogen peroxide is no better if both assays involve intermediates that generate these species. Also, as in this example, adding a scavenger such as catalase or superoxide dismutase provides no further proof. The assays need to be mechanistically different, such as using a boronate probe and measuring protein nitration to detect peroxynitrite.

The development of non-redox probes has been a major step forward. This continues to be an active field, with numerous recent reports describing elegant chemistry to design new and improved fluorophores and leaving groups. However, these probes tend to be selective rather than specific and their low reactivity raises questions about detecting hydrogen peroxide in cells. Advances in chemical design therefore need to be accompanied by further investigation of the biological properties of these probes, including more extensive testing of their specificity with a wider range of oxidants and nucleophilic species that they could encounter in a cell.

Compared with low molecular weight oxidant probes, genetically encoded Hyper and roGFP have some advantages. Their high reactivity and selectivity enable them to compete with other cell constituents for hydrogen peroxide, they can provide ratiometric detection, they can be targeted and there are fewer specificity issues. However, oxidation of these probes is reversed by cellular reductants and occurs in competition with enzymatic peroxide metabolism, and absolute quantification of intracellular peroxide is still complex. Also their use is constrained by having to express the protein. Therefore, there are advantages in having small molecules that can simply be added to a system and the availability of such probes that can quantify specific oxidants would be a major advance.

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