



# Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo

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**Multiple roles of reactive oxygen species (ROS) and their consequences for health and disease are emerging throughout biological sciences. This development has led researchers unfamiliar with the complexities of ROS and their reactions to employ commercial kits and probes to measure ROS and oxidative damage inappropriately, treating ROS (a generic abbreviation) as if it were a discrete molecular entity. Unfortunately, the application and interpretation of these measurements are fraught with challenges and limitations. This can lead to misleading claims entering the literature and impeding progress, despite a well-established body of knowledge on how best to assess individual ROS, their reactions, role as signalling molecules and the oxidative damage that they can cause. In this consensus statement we illuminate problems that can arise with many commonly used approaches for measurement of ROS and oxidative damage, and propose guidelines for best practice. We hope that these strategies will be useful to those who find their research requiring assessment of ROS, oxidative damage and redox signalling in cells and in vivo.**

Reactive oxygen species (ROS) (Box 1) are intimately involved in redox signalling but in some situations can also lead to oxidative damage. Hence, they have both physiological and pathophysiological roles in biology<sup>1–4</sup>. Consequently, researchers from diverse fields often need to measure ROS, to assess oxidative events and to investigate their biological importance using antioxidants (Box 1) or inhibitors to modulate the phenomena observed. There are many assays and commercial kits available, but their use and interpretation are challenging and open to artefacts. There is a well-established field of biophysics/biochemistry/chemistry focusing on the identification of ROS, their chemical reactions and products of oxidative damage. However, as with many specialized fields, this literature can be hard to interpret by those working outside the area. Frequently problems arise due to reliance on commercial kits that claim to measure ‘ROS’ or ‘oxidative damage’, or from the use of

‘antioxidants’ in general terms, when progress requires understanding of specific molecular mechanisms.

To address these points, this international group has set out guidelines on the nomenclature and measurement of ROS, oxidative reactions and oxidative damage. Our focus is on the techniques used to measure ROS and oxidative damage. These can be applied to their role in pathology, but it is also important to note that changes in the levels of ROS and consequent changes in the activity of redox-sensitive cellular processes are central to the field of redox signalling<sup>1–4</sup>. We hope that these guidelines will be useful for researchers who find themselves carrying out experiments in this area. These topics, and indeed the approaches we advocate, have been covered by many reviews in the past<sup>1–11</sup> and which researchers are strongly encouraged to read. Here we distill the key points underlying this consensus statement.

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**Box 1 | Definitions**

Reactive oxygen species (ROS) is a collective term for species derived from O<sub>2</sub> that are more reactive than O<sub>2</sub> itself. The term includes not only the superoxide radical anion (O<sub>2</sub><sup>•-</sup>) and some other oxygen radicals, but also some non-radical derivatives of O<sub>2</sub> such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hypochlorous acid (HOCl) and peroxyxynitrite/ peroxyxynitrous acid (ONOO<sup>-</sup>/ONOOH). Hence all oxygen radicals are ROS, but not all ROS are radical species (the latter being defined as a species with one or more unpaired electrons). ‘Reactive’ is a relative term; O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> are selective in their reactions with biological molecules, leaving most of them unscathed, whereas •OH attacks everything (Table 1).

Antioxidant is a term often used but difficult to define clearly.

When ROS are generated in vivo, many antioxidants come into play. Their relative importance depends upon:

- which ROS is generated, in what amounts and over what time course
- how and where it is generated
- what target of damage by ROS is measured

One definition of an antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule”<sup>11</sup>. There is no universal ‘best’ antioxidant: different antioxidants react with different ROS at variable rates, act in various locations and protect different molecular targets. An alternative definition is “a substance that reacts with an oxidant to regulate its reactions with other targets, thus influencing redox-dependent biological signalling pathways and/or oxidative damage”.

Oxidative damage: the biomolecular damage caused by the attack of ROS upon the constituents of living organisms. Increased levels of oxidative damage can occur from increased ROS production but also from decreased repair or removal processes—for example, failure to clear oxidized proteins or repair oxidized DNA sufficiently rapidly: both can happen in certain diseases.

Biomarker: can be defined as any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease<sup>54</sup>.

**What are ROS, antioxidants and oxidative damage?**

One problem that underlies the measurement of ROS and oxidative damage and the use of ‘antioxidants’ is the lack of precision in the use of these terms. ROS is an abbreviation that covers a wide range of chemical species with different properties, reactivities and interactions (Box 1, Table 1). For example, one important reactive species found in biology, the superoxide radical anion (O<sub>2</sub><sup>•-</sup>), is formed by the one-electron reduction of oxygen (O<sub>2</sub>). In itself, O<sub>2</sub><sup>•-</sup> is not very reactive, except with another radical nitric oxide (•NO) to form peroxyxynitrite<sup>11</sup>, or with Fe–S clusters in proteins<sup>12</sup>. Similarly, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), formed by various oxidase enzymes<sup>14</sup> and by the action of superoxide dismutase (SOD), is poorly reactive, which allows its use as an important signalling molecule in vivo<sup>2,4</sup>. Nevertheless, in the presence of ferrous or cuprous ions, H<sub>2</sub>O<sub>2</sub> forms the extremely reactive hydroxyl radical (•OH) by Fenton chemistry: •OH reacts non-specifically and essentially instantaneously with any nearby biomolecule (Table 1)<sup>1,13</sup>. The availability of transition metal ions to catalyse Fenton chemistry is carefully controlled in vivo<sup>1</sup>, but these can be released by tissue injury or when certain proteins with Fe–S clusters encounter O<sub>2</sub><sup>•-</sup> (refs. <sup>1,12,14</sup>). Their importance in vivo has recently been underscored by the growing literature on ferroptosis, a form of cell death involving ‘catalytic’ iron ions<sup>15</sup>. H<sub>2</sub>O<sub>2</sub> is a substrate for haem peroxidases such as myeloperoxidase, generating

further reactive species such as HOCl (Table 1). Despite its poor overall reactivity, H<sub>2</sub>O<sub>2</sub> can selectively oxidize some methionine (Met) and cysteine (Cys) residues<sup>16,17</sup> in certain proteins.

A (far from complete) list of the physicochemical properties of the most common ROS encountered in biology is given in Table 1, which provides insights into what reactions might be plausible in vivo when these species are generated. What should also be evident is that ‘reactive’ is highly context dependent, because the reactivity of different ROS varies over a wide scale, as do their lifespans, ability to diffuse and potential to generate further downstream reactive species. In short, not all ROS are the same. The generalization ‘ROS’, although widely used (including in this paper!) does not give information about the actual chemical species causing the observed effect. *Recommendation 1:* wherever possible, the actual chemical species involved in a biological process should be stated and consideration given to whether the observed effect is compatible with its reactivity, lifespan, products generated and fate in vivo. If this is not possible, caveats about use of the term ‘ROS’ should be discussed.

A wide range of antioxidants is present in biology. These include enzymes and small molecules that react with individual ROS to decrease oxidative damage and/or modulate redox signalling<sup>12</sup>. As with ‘ROS’, the use of ‘antioxidant’ as a general term can be imprecise and misleading (Box 1). Often the effect of a putative antioxidant on a biological outcome is used to infer a role for a ROS, as if all antioxidants were equivalent. However, each antioxidant has its own specific chemistry and reactivity with different ROS. Furthermore, the major antioxidants in vivo are enzymatic systems such as SOD for O<sub>2</sub><sup>•-</sup>, peroxidases for H<sub>2</sub>O<sub>2</sub>, and metal ion sequestration<sup>1,14</sup>. Most low-molecular-mass compounds commonly employed as ‘antioxidants’ are stoichiometric scavengers of certain ROS and often have modest (if any) reactivity with O<sub>2</sub><sup>•-</sup> or H<sub>2</sub>O<sub>2</sub>. For example, N-acetylcysteine (NAC) is a widely used ‘antioxidant’ but it has other (and sometimes more important<sup>18</sup>) modes of action. NAC can indeed scavenge some ROS in vitro, but not others, most notably not H<sub>2</sub>O<sub>2</sub> (ref. <sup>18</sup>). It can also increase the cellular Cys pool and thereby enhance glutathione (GSH) levels, generate H<sub>2</sub>S, and directly cleave protein disulphides<sup>18</sup>. Low-molecular-mass compounds that do act as antioxidants in vivo include vitamin E, which scavenges lipid peroxyl radicals<sup>19</sup>. Sometimes ‘•OH scavengers’ are used to infer a role for this ROS yet they can rarely, if ever, achieve a sufficiently high concentration to prevent the effectively instantaneous reaction of •OH with biomolecules<sup>1,7,13</sup>. Consequently, many of the biological effects assigned to ‘antioxidants’, especially NAC, are due to other effects. Other agents often used as ‘antioxidants’, such as TEMPO/TEMPOL, mito-TEMPO and porphyrin-based ‘SOD mimetics’, undergo complex redox reactions in vivo and are better described as ‘redox modulators’ rather than ‘antioxidants’ or ‘O<sub>2</sub><sup>•-</sup> scavengers’<sup>1,20,21</sup>. *Recommendation 2:* for an intervention to be attributed to an antioxidant activity, the particular chemical species targeted by the ‘antioxidant’ needs to be made explicit. It should be recognized that low-molecular-mass ‘antioxidants’ are unlikely to act by scavenging H<sub>2</sub>O<sub>2</sub>. The specificity, rate constant, location and concentration of the antioxidant within the cell should render an antioxidant effect chemically plausible. Wherever possible the activity of the antioxidant should be confirmed by measuring a decrease in oxidative damage.

A key procedure to attributing oxidative damage, or activation of a redox signalling pathway, to a particular ROS can be by selective generation of the ROS in a biological context. This can be done by using redox cycling compounds such as paraquat (PQ) or quinones to generate O<sub>2</sub><sup>•-</sup>, or MitoPQ to generate O<sub>2</sub><sup>•-</sup> within mitochondria<sup>1,22</sup>. Of course, increased O<sub>2</sub><sup>•-</sup> generation will also increase H<sub>2</sub>O<sub>2</sub> production by O<sub>2</sub><sup>•-</sup> dismutation. Glucose oxidase can be used to generate H<sub>2</sub>O<sub>2</sub> directly in vitro, while the regulated generation of H<sub>2</sub>O<sub>2</sub> within cells can be achieved using genetically expressed

**Table 1 | Common ROS encountered in biological systems**

ROS	Chemical formula	Reactivity lifespan
<b>Superoxide radical anion</b>	$O_2^{\bullet-}$	Selectively reactive, does not attack most biological molecules Can reduce transition metals ( $Fe^{3+}$ , $Cu^{2+}$ ), reaction rate depends on the metal ion ligand Reacts very rapidly with nitric oxide ( $k_2 > 10^9 M^{-1} s^{-1}$ ) to yield peroxynitrite: $O_2^{\bullet-} + NO \rightarrow ONOO^-$ and with other radicals to form hydroperoxides: $O_2^{\bullet-} + R^{\bullet} + H^+ \rightarrow ROOH$ Can damage certain enzymes that contain Fe-S clusters
<b>Hydrogen peroxide</b>	$H_2O_2$	Unreactive with most biomolecules Reacts slowly with most thiols—for example, $k \approx 1 M^{-1} s^{-1}$ for GSH—but more rapidly with certain protein Cys residues, particularly those with low $pK_a$ Reacts with certain transition metal ions to give $\bullet OH$ (rate constants $10^2$ – $10^7 M^{-1} s^{-1}$ , depending on the metal and ligands to the metal ion) Main biological reactions are with haem, thiols and peroxidase enzymes Reacts with $CO_2$ to form the more reactive peroxymonocarbonate ( $HCO_4^-$ ) <sup>50</sup>
<b>Hydroxyl radical</b>	$\bullet OH$	Indiscriminately reactive; reacts with whatever is adjacent to it at near diffusion-controlled rates
<b>Peroxynitrite</b> (the physiological mixture of peroxynitrite, $ONOO^-$ and its more reactive protonated form, peroxynitrous acid, $ONOOH$ ; $pK_a$ 6.8)	$ONOO^-/ONOOH$	Direct reactions with thiols and transition metal centres up to $10^7 M^{-1} s^{-1}$ ; reacts with $CO_2$ to give nitrosoperoxycarbonate ( $ONOOCO_2^-$ ) Peroxynitrite itself can oxidize several biomolecules or homolysis of peroxynitrous acid can generate $\bullet OH$ $ONOOH \rightarrow NO_2^{\bullet} + \bullet OH$ , although a more prominent reaction is $ONOOH \rightarrow NO_3^- + H^+$ $ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow$ minor ( $CO_3^{\bullet-} + NO_2^{\bullet}$ ) + major ( $CO_2 + NO_3^-$ )
<b>Carbonate radical anion</b>	$CO_3^{\bullet-}$	Formed from reaction of $CO_2$ with peroxynitrite (see above) and also from reaction of $HCO_3^-$ with $\bullet OH$ . Fairly reactive, oxidizes guanine in DNA, cysteine, tyrosine and tryptophan
<b>Hypohalous acids</b> (hypochlorous, hypobromous acids)	$HOCl$ $HOBr$	Strong oxidants, major reactions are with thiols and methionine Reactions with amines generate secondary chloramines/bromamines, which retain less (but still considerable) oxidizing ability React rapidly with thiocyanate ( $SCN^-$ ), present at high levels in many body fluids, to generate $HOSCN$ (which is also generated by peroxidases); $HOSCN$ is less reactive and highly specific for thiols
<b>Singlet oxygen</b>	$^1O_2$	Two singlet states of $O_2$ exist, although only the $^1\Delta_g$ state (not a free radical) is of major biological relevance. The singlet electron configuration makes this state much more reactive than ground-state triplet $O_2$ . Can be formed by photosensitization reactions in which molecules such as porphyrins, riboflavin, bilirubin and chlorophyll absorb light and transfer this energy to ground-state $O_2$ , and also via chemical reactions of peroxy radicals and $HOCl$ , amongst others
<b>Nitrogen dioxide radical</b>	$NO_2^{\bullet}$	A major atmospheric pollutant. Also generated from peroxynitrite (see above) and oxidation of nitrite ( $NO_2^-$ ) by peroxidase enzymes. Rapidly oxidizes electron-rich compounds (for example, ascorbate and thiols). Undergoes addition reactions with radicals derived from tyrosine, tryptophan, lipids and DNA bases (for example, guanine) to give nitrated products (for example, 3-nitrotyrosine, nitrotryptophans, nitrolipids and nitrated DNA bases). Some nitrated products have signalling functions

D-amino acid oxidase, an enzyme that generates  $H_2O_2$  as it oxidizes D-amino acids<sup>23</sup>. It can be targeted to different sites in the cell, and the flux regulated by varying the added concentration of its substrate, D-alanine<sup>23</sup>. NADPH oxidase (NOX) enzymes are important sources of  $O_2^{\bullet-}$  and  $H_2O_2$  for redox signalling, as well as oxidative damage<sup>24</sup>, and modulation of their activity is an important approach to understanding these processes. A number of fairly specific inhibitors of NOX enzymes have been described<sup>24</sup>. However, the use of compounds such as apocynin and diphenyleneiodonium as ‘NOX inhibitors’ is still widespread, even though their lack of specificity is well established<sup>1,24</sup>. **Recommendation 3:** we recommend the use of PQ, quinones and MitoPQ for selective generation of  $O_2^{\bullet-}$  and the cellular expression of D-amino acid oxidase for controlled generation of  $H_2O_2$ . Avoid the use of inhibition of a phenomenon by apocynin or diphenyleneiodonium as sole evidence for a role of NOX enzymes, or at least discuss their lack of specificity. Specific

inhibitors<sup>24</sup> or deletion or knockdown of NOX components should be used to identify their roles.

### General principles of measurement of ROS and oxidative damage

When investigating ROS in biological systems, it is important to detect and quantify the ROS of interest. This can be done using electron paramagnetic (spin) resonance (EPR/ESR), various probe molecules or by measuring oxidative modifications (‘oxidative damage’, Box 1) caused by the ROS<sup>1</sup>. Most ROS probes capture only a small percentage of any ROS formed. Indeed, if the probe reacted with most of the ROS generated this would perturb the system and affect experimental results (for example, inhibition of oxidative damage or interference with redox signalling). However, it is important that the percentage capture remains approximately constant over different rates of production of the ROS in question.

Oxidative damage can take many forms; the chemical processes by which it arises from a particular ROS and how it is assessed and quantified are complex. Furthermore, the final level of any oxidative damage biomarker measured is the difference between its rate of production and its removal by repair, degradation, excretion or diffusion. *Recommendation 4:* when oxidative damage levels to any biomolecule are presented, the chemical processes by which they arise and the methods used to quantify them should be made explicit. The impact of repair and clearance on the final levels measured should be considered and discussed.

### Guidelines and limitations of the detection and measurement of ROS

Consideration of ROS, antioxidants and oxidative damage as monolithic concepts limits the precision and interpretation of experiments and glosses over the need to establish precise molecular mechanisms. To put these precepts into practice requires measurement of specific ROS and/or oxidative damage products, as well as the effects of antioxidants. This is a major practical challenge, because most ROS are short lived (lifespans of milliseconds or less) and their steady-state levels are low (picomolar to low micromolar) and alter rapidly, because they are affected by continuously varying rates of generation, chemical reaction and diffusion.

In simple *in vitro* systems it is possible to detect several ROS (Table 2). For example,  $O_2^{\bullet-}$  production can be monitored by the reduction of cytochrome *c*, and its selectivity assessed by inhibition by added SOD. However, even such a 'simple' system can be surprisingly complex. For example, semiquinones can reduce cytochrome *c* in a reaction inhibited by SOD<sup>25</sup>. The bottom line is that all methods used to assess ROS are susceptible to artefact, and appropriate controls are required to be certain of the species and amounts measured. Hence, it is important to corroborate measurements with 'orthogonal techniques' that rely on an alternative approach using a different detection method to avoid method-specific artefacts. These complexities are magnified when one attempts to measure ROS in cells. Commonly used cell culture conditions promote oxidative damage due to both limited antioxidants in the medium and high  $O_2$  concentrations relative to those *in vivo*<sup>26</sup>. Consequently, cultured cells generate more ROS than these cells would *in vivo*.

*Recommendation 5:* use commercial kits only if the actual species being measured and the method of detection are explained in the kit materials, are chemically plausible and the limitations are understood. The use of commercial kits without such information is strongly discouraged. To avoid method-specific artefacts, confirm results using techniques that rely on different principles of detection.

Small-molecule fluorescent probes are frequently used to assess ROS within cells. In some cases, often involving kits, a lack of description of the chemical reactivity or structures of these probes makes it difficult to interpret results and therefore such probes should be avoided. Even for probes of known structure there can be concerns. Consider the widely used fluorescent probe 2',7'-dichlorodihydrofluorescein (DCFH), usually administered in its diacetate (DCFH-DA) form, which enters cells readily. DCFH is oxidized to the fluorescent product 2',7'-dichlorofluorescein (DCF) by several ROS, and so it is not specific for any particular ROS<sup>67</sup>. DCFH is not oxidized directly by  $H_2O_2$  (which it is often claimed to detect), but only after  $H_2O_2$  is converted to more reactive species by redox-active metals or by haem proteins such as cytochrome *c* or peroxidases. Furthermore, the oxidation of DCFH and fluorescence of DCF are sensitive to local  $O_2$  levels and pH, and fluorescence yield may not be linear with increased ROS levels<sup>27–29</sup>. This is not to say that DCFH, and other non-specific fluorescent probes such as dihydrorhodamine, should never be used, but their limitations (selectivity, problems of quantification, linearity of response and susceptibility to artefact) should be understood and results interpreted cautiously<sup>28</sup>. In particular, their response should not

be attributed to a specific ROS without detailed controls to validate this, and their use should be restricted to an initial assessment of a change in cellular redox state, to be followed up by a more detailed investigation into mechanism. While many small-molecule and protein fluorescent probes are more selective than DCF, it is always important to validate data by a number of simple controls: does the response change over time and with the amount of biological sample in a plausible manner? Can the effect be replicated by generating the ROS of interest (for example, using PQ for  $O_2^{\bullet-}$  or D-amino acid oxidase for  $H_2O_2$ )? Do negative controls that should abolish the ROS-generating process (for example, gene knockouts, knockdowns, inhibitors, radical scavengers) respond as expected? *Recommendation 6:* when using fluorescent ROS probes (especially DCFH-DA), the chemistry involved, the selectivity for particular chemical species and potential artefacts should be made clear and discussed. Wherever possible, controls to show that the response is due to the proposed species should be carried out and orthogonal techniques used to corroborate the conclusion.

Extending measurements of ROS from cells in culture to tissues *in vivo* or *ex vivo* is vital. However, in some cases this gap has been addressed by the addition of 'ROS probes' to fresh or previously frozen tissue slices or homogenates *ex vivo*. These measurements may be meaningless, because the very short lifespan of ROS means that any present *in vivo* will have long disappeared by the time the material is assayed. Furthermore, freezing or homogenization disrupts membranes and alters substrate and ion concentrations (for example, raising levels of  $Ca^{2+}$  or 'catalytic'  $Fe^{2+}$ )<sup>1</sup>, such that any ROS production in the tissue slice or homogenate bears no relation to the levels that would have been generated *in vivo*. There are valid methods available to assess ROS *in vivo* or in perfused organs, but in these situations the process is either monitored *in vivo* (for example, see Table 2 for the use of catalase compound I to measure  $H_2O_2$ ) or the system is quenched to stabilize the probe for analysis *ex vivo*. *Recommendation 7:* measurements of ROS should be carried out in cells, tissues or organs under physiologically relevant conditions *in vivo* or *ex vivo*. ROS should not be 'measured' in tissue homogenates or cryosections, unless the probe or sensor employed is able to irreversibly capture the reactive species when the cells/tissues/organs are under biologically relevant conditions.

### Direct measurement of ROS

Here we outline what we consider to be, currently, the best approaches to the measurement of commonly encountered ROS.

**Superoxide.** In simple systems  $O_2^{\bullet-}$  can be measured in a number of ways, such as by the SOD-inhibitable reduction of cytochrome *c* (ref. 25). The generation of  $O_2^{\bullet-}$  can also be assessed by spin trapping followed by EPR, which has the benefit of direct detection of the radical<sup>1</sup>. The Fe-S cluster in aconitase is inactivated by  $O_2^{\bullet-}$ , and by other ROS, but its interaction with  $O_2^{\bullet-}$  is fast, reasonably specific and reversible, making it a good indicator for  $O_2^{\bullet-}$  in mitochondria<sup>30</sup>. The chemiluminescent 'superoxide probes' luminol and lucigenin are widely used to 'detect  $O_2^{\bullet-}$ ', but interpretation of such data is difficult because these probes generate radicals that produce  $O_2^{\bullet-}$  themselves; they do not react with  $O_2^{\bullet-}$  directly<sup>31,32</sup>.

*Recommendation 8:* the use of luminol and lucigenin to 'detect  $O_2^{\bullet-}$ ' should be discouraged, but they can be used as general indicators of increased ROS production. SOD-sensitive reduction of cytochrome *c* *in vitro* and aconitase inactivation within mitochondria are better strategies.

In cells,  $O_2^{\bullet-}$  is often detected by measuring the fluorescence arising from oxidation of dihydroethidium (sometimes called hydroethidine (HE)), or mitochondria-targeted HE (MitoSOX). Unfortunately, detection by fluorescence is misleading because these probes form both ethidium ( $E^+$ ), a non-specific oxidation product, and the  $O_2^{\bullet-}$ -specific product 2-hydroxyethidium.

**Table 2 | Some recommended approaches for detection and quantification of ROS in different biological contexts**

Oxidant	Approach	Method	Context	Detection (D)/ Quantification (Q)	Controls required to discriminate between different ROS
Superoxide radical anion ( $O_2^{\bullet-}$ )	Fe release from aconitase	Aconitase enzymatic activity measurement	TT, C, O	D, Q	Inhibited by SOD
	Cytochrome c reduction	Optical spectroscopy	TT, C	D, Q	Inhibited by SOD, absence of semiquinones/quinones
	Dihydroethidium oxidation to 2-hydroxyethidium	LC separation, detection by fluorescence or MS	TT, C, O	D, Q	Verification of product as 2-hydroxyethidium required; inhibited by SOD
	Spin trapping	ESR/EPR	TT, BF, C, A, H	D, Q	Low sensitivity; signal inhibited by SOD; needs careful controls <sup>1</sup>
Hydrogen peroxide ( $H_2O_2$ )	Formation of compound 1 from catalase	Optical difference spectroscopy at 600 nm	TT, C, O, BF	D, Q	Titration with hydrogen donor (for example, methanol) and allows flux measurements (see ref. <sup>103</sup> for details)
	Oxidation of boronate probes (for example, PO1, MitoB, MitoPY1, boronate-caged luciferin)	Fluorescence/luminescence detection or MS (mitoB).	TT, C, BF, O, A	D, Q	Inhibited by enzymes that remove $H_2O_2$ ; contribution from other ROS needs to be considered: especially need to rule out involvement of ONOO <sup>-</sup> (for example, using NOS inhibitors)
	Genetically encoded thiol-based probes (for example, HyPer7, roGFP2-Orp1, roGFP2-Tsa2)	Fluorescence detection	TT, C, O, A	D, Q	Use mutant (that is, oxidation insensitive) probes in parallel to recognize artefacts
	Peroxidase-catalysed oxidation of Amplex Red	Optical spectroscopy	BF, C ( $H_2O_2$ release only)	D, Q	Not applicable for intracellular measurements or complex systems; inhibited by $H_2O_2$ -removing enzymes; reductants and peroxidase substrates interfere
Peroxynitrite / peroxynitrous acid (ONOO <sup>-</sup> /ONOOH) Nitrogen dioxide radical ( $NO_2^{\bullet}$ )	Nitrated products from endogenous targets (for example, 3-nitroTyr from Tyr, 6-nitroTrp from Trp, 8-nitroguanosine from guanosine, nitrated lipids) or added exogenous probes (for example, boronates)	LC-MS	TT, C, BF, O, A, H	D, Q	Authentic standards required; quantification by heavy-isotope standards preferable; $NO_2^{\bullet}$ also formed by peroxidase activity from $NO_2^-$
		Antibodies (for example, in ELISA, immunoblot, immunocytochemistry)	TT, C, BF, O, A, H	D	Needs well-characterized and validated antibodies
Carbonate radical anion ( $CO_3^{\bullet-}$ )	Direct measurement	EPR/ESR spectroscopy	TT, BF	D, Q	Low sensitivity
Hypochlorous acid (HOCl)	Chlorinated products from endogenous targets (for example, 3-chloroTyr from Tyr, chlorinated lipids) or added probes (for example, chlorinated ethidium from dihydroethidium or hypocrates)	LC-MS	TT, C, BF, O, A, H	D, Q	Careful controls required; quantification by heavy-isotope standards preferable
Singlet oxygen ( $^1O_2$ )	Direct probe oxidation (for example, Singlet Oxygen Sensor Green) or chemical addition of oxygen to probe molecule (for example, anthracenes)	Weak phosphorescence at -1,270 nm detected by near-infrared spectrofluorimetry	TT, BF, C	D, Q	Signals enhanced using $D_2O$ buffers; scavengers/quenchers, such as azide and histidine, also react with radicals (reviewed in ref. <sup>1</sup> )
		Fluorescence or LC-MS	TT, BF, O	D, Q	

TT, test tube; C, cells; O, isolated organs; A, animals; H, humans; BF, biological fluids. References to methods are contained in the main text, except where indicated.

Because these two products have overlapping fluorescence spectra, it is hard to differentiate the contribution of non-specific oxidation and  $O_2^{\bullet-}$ -dependent oxidation (if any) to the overall fluorescence<sup>33</sup>. Accurate quantification of the 2-hydroxyethidium product can be achieved using liquid chromatography–mass spectrometry (LC–MS)<sup>33</sup>. Another factor that should be considered is the extent of cellular uptake of HE/MitoSOX and the intracellular concentrations

of these and their multiple products. Furthermore, HE oxidation products intercalate into DNA, greatly enhancing their fluorescence and creating another artefact. NeoD and MitoNeoD contain a modified HE that does not intercalate into DNA<sup>34</sup>.

Mitochondrially accumulated  $O_2^{\bullet-}$ -probes, such as MitoSOX, are often used to 'detect  $O_2^{\bullet-}$ ' within mitochondria. When using these probes, and others that have positive charges or generate positively

charged species (including 2-hydroxyethidium and ethidium), it is important to remember that probe accumulation is dependent on plasma and mitochondrial membrane potentials and mitochondrial size, shape and mass<sup>35</sup>. Furthermore, fluorescence can be quenched when these are present at high concentrations in mitochondria<sup>36</sup>.

**Recommendation 9:** use only HE or MitoSOX probes to detect O<sub>2</sub><sup>•-</sup> by simple fluorescence measurements when the product has been independently validated as 2-hydroxyethidium. Fluorescence measurements with probes such as dihydroethidium and MitoSOX<sup>33</sup> should be conducted using the lowest probe concentration possible, and must include controls for changes in plasma and mitochondrial membrane potentials and mitochondrial mass and morphology, such as normalization to a similar membrane-potential-responsive, but redox-insensitive, probe. LC-MS methods, which measure all modified species<sup>33</sup>, should be performed when possible.

**Hydrogen peroxide.** In simple systems, H<sub>2</sub>O<sub>2</sub> can be measured by horseradish peroxidase (HRP)-oxidizing substrates, one frequently used being Amplex Red. These methods can be interfered with by other HRP substrates (for example, ascorbate and NAC)<sup>1</sup> and by O<sub>2</sub><sup>•-</sup> (which can inactivate HRP), the latter preventable by the addition of SOD<sup>37</sup>. Since H<sub>2</sub>O<sub>2</sub> can cross membranes directly or via aquaporins, this system can also be used to measure H<sub>2</sub>O<sub>2</sub> release from cells. However, please be aware that this release reflects the balance between H<sub>2</sub>O<sub>2</sub> production, removal by intracellular enzymes and the rate of diffusion out of the cell.

Within cells, H<sub>2</sub>O<sub>2</sub> detection by phenylboronate-based probes is more reliable<sup>38</sup> although these may lack sufficient sensitivity because they react only slowly with H<sub>2</sub>O<sub>2</sub>, which can make it difficult to detect small or localized changes in H<sub>2</sub>O<sub>2</sub> levels<sup>39</sup>. However, recent studies suggest that borinic acids, which react more rapidly with H<sub>2</sub>O<sub>2</sub>, may be more sensitive detectors<sup>40</sup>. The mechanism of oxidation of phenylboronates to phenols requires a two-electron oxidant, such as H<sub>2</sub>O<sub>2</sub>. Because H<sub>2</sub>O<sub>2</sub> is typically generated at higher concentrations than other ROS, boronate probes can be selective for H<sub>2</sub>O<sub>2</sub> detection subject to proper controls<sup>39,40</sup>. However, boronate probes react with ONOO<sup>-</sup>/ONOOH or HOCl much more rapidly than they do with H<sub>2</sub>O<sub>2</sub> which can sometimes complicate measurements, and orthogonal approaches or the use of inhibitors can aid validation<sup>41</sup>. For example, H<sub>2</sub>O<sub>2</sub>- and peroxynitrite-dependent signals can be distinguished using nitric oxide synthase (NOS) inhibitors and catalase<sup>38,39,41,42</sup>.

Genetically encoded fluorescent protein sensors have provided major advances in cellular H<sub>2</sub>O<sub>2</sub> detection<sup>43–46</sup>. These probes contain a dithiol switch that changes the overall fluorescence of the probe depending on its oxidation status. High sensitivity and specificity for H<sub>2</sub>O<sub>2</sub> have been achieved by coupling a redox-sensitive green fluorescent protein (GFP) mutant to a H<sub>2</sub>O<sub>2</sub>-sensitive thiol protein, such as oxyR (HyPer series), or to a peroxidase such as Orp1 or TSA2 (roGFP2-based probes). HyPer7 and roGFP2 coupled to a peroxidase provide the highest sensitivity<sup>44,45</sup>. While HyPer7- and roGFP2-based probes are pH stable, earlier versions of HyPer are not and require expression of a control probe (SypHer) to control for signal changes due to variation in pH<sup>43</sup>. Imaging analysis by fluorescence microscopy is normally employed, but fluorescence plate readers can also be used. The redox status measured represents a balance between the rate of oxidation and re-reduction of the probes by cellular reductants, including glutaredoxin/GSH and thioredoxin, permitting real-time, live-cell assessments of redox state. Because excitation wavelengths of both reduced and oxidized probes are used, the probes are ratiometric and the output is not dependent on the level of protein probe expression. By incorporation of appropriate targeting gene sequences, these probes can be directed to different cell compartments, including mitochondria, microtubules, endoplasmic reticulum, nucleus and cytoplasm<sup>43–46</sup>. Hence, subcellular regions of interest can be studied and the probe then

calibrated at the end by full reduction (2 mM dithiothreitol), washout and full oxidation (2 mM *t*-butylhydroperoxide)<sup>44,45</sup>. This calibration yields a measure of oxidation percentage, permitting comparisons across experiments and among subcellular compartments<sup>44,45</sup>. These probes have been expressed in transgenic animals to provide useful assessments of in vivo H<sub>2</sub>O<sub>2</sub> generations<sup>46,47</sup>. Plasmid transfection of viral vectors can be used with cultured cells, and targeted roGFP2 probes are available commercially ([www.addgene.com](http://www.addgene.com)).

In most experiments the H<sub>2</sub>O<sub>2</sub> probes are expressed as free proteins that distribute within the cell. Nevertheless, given uncertainties about intracellular H<sub>2</sub>O<sub>2</sub> diffusion distances, it is still unclear what resolution is needed to understand subcellular H<sub>2</sub>O<sub>2</sub> distribution. Therefore, tethering H<sub>2</sub>O<sub>2</sub> probes to sub-compartmental locations such as protein complexes or organelle contact sites is an important approach.

**Recommendation 10:** genetically encoded fluorescent probes (some of which are commercially available) are currently the most sensitive detectors of H<sub>2</sub>O<sub>2</sub> and we recommend their use in cells and animals if expression is possible. Boronate probes (some of which are also commercially available) are the preferred small-molecule probes, but controls to determine specificity for H<sub>2</sub>O<sub>2</sub> are required and sensitivity is limited for physiological H<sub>2</sub>O<sub>2</sub> levels. Amplex Red with HRP can measure H<sub>2</sub>O<sub>2</sub> release from cells if other reducing agents or peroxidase substrates are absent.

**Peroxynitrite.** Peroxynitrite (ONOO<sup>-</sup>) exhibits complex chemistry<sup>42,48,49</sup> and itself can oxidize certain biomolecules. A major physiological reaction is with CO<sub>2</sub> (Table 1), and hence the CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> content of biological systems plays a role in determination of the biological impact<sup>50</sup> of ONOO<sup>-</sup>. Products of this reaction include reactive species such as the carbonate radical anion (CO<sub>3</sub><sup>•-</sup>) and the nitrating agent nitrogen dioxide (NO<sub>2</sub><sup>•</sup>) (Table 1), both of which react with many of the general 'ROS probes'. Peroxynitrite oxidizes boronate-based probes nearly a million times more rapidly than H<sub>2</sub>O<sub>2</sub> and, under the right conditions, these probes can be used to assess ONOO<sup>-</sup>/ONOOH production<sup>42,49</sup>. Peroxynitrite has been measured in tissues ex vivo using boronate probes<sup>51</sup>.

**Hypochlorous acid and other reactive halogen species.** HOCl, hypobromous acid (HOBr) and some of the chloramines and bromamines derived from them (Table 1) react with most of the general probes used to detect ROS, including DCFH and luminol. However, many of these probes are also substrates of the peroxidases that generate HOCl or HOBr, confounding their use. More specific fluorescent probes for reactive halogen species have been reported and some are commercially available<sup>52</sup>. A genetically encoded probe for reactive halogen species has been developed, enabling dynamic monitoring of these species both in cell culture and in vivo<sup>53</sup>.

### Measurement of oxidative damage

The presence of ROS can be inferred by their effects on protein, carbohydrates, nucleic acids and lipids to generate specific compounds which, so long as they cannot be formed by other mechanisms, can be used as 'biomarkers' of oxidative damage (Box 1)<sup>1,54,55</sup>. However, do note that the measured levels of biomarkers represent a balance between the generation and removal of the biomarker (for example, by degradation, diffusion or excretion), plus any artefactual increased levels caused by oxidative damage during isolation or analysis.

**Lipid peroxidation.** Polyunsaturated fatty acids (PUFAs), are readily oxidized, and hence lipid peroxidation products are widely used to characterize oxidative damage<sup>56–58</sup>. Lipid peroxidation can be initiated by certain ROS and proceed as a random, non-enzymatic (often chain) radical process. However, there are also enzymatic mechanisms (for example, lipoxygenases) available for peroxidation

of free PUFAs or PUFA-phospholipids that produce specific signalling products with biological roles. Thus, when measuring lipid peroxidation the focus might be placed on either (1) establishment of increased lipid peroxidation as an example of oxidative damage or (2) identification of individual oxidatively modified lipid molecules acting as signals by selective interaction with certain cellular targets.

In PUFAs, the presence of a double bond adjacent to a methylene group makes the methylene C–H bond weaker and therefore the *bis*-allylic hydrogen is more susceptible to H<sup>•</sup> abstraction<sup>56–58</sup>. The carbon-centred radical (L) generated by H<sup>•</sup> abstraction is stabilized by delocalization over the double bonds. Subsequent reaction with O<sub>2</sub> gives a peroxy radical (LOO) with the formation of a conjugated diene system and a range of peroxides (LOOH). LOO<sup>•</sup> can react further to yield highly oxidized secondary products, including epoxy-, oxo- or cyclic peroxides<sup>56–58</sup>. Hence, there are multiple end products of lipid peroxidation that show vast chemical heterogeneity and variable stability and polarity, and thus measurement of only a single oxidation product in no way represents the whole process of lipid peroxidation.

Several methods are available to assess ‘general’ lipid peroxidation. In simple model systems (for example, isolated lipoproteins), diene conjugation can be measured by ultraviolet (UV) absorbance but this method is not suitable for use in cells or body fluids because of the presence of interfering UV-absorbing molecules that do not result from lipid peroxidation<sup>1</sup>. In cells, ‘lipid peroxidation’ can be assessed by changes in the fluorescence of BODIPY conjugated to a peroxidation-sensitive undecanoic acid moiety<sup>59</sup>. This assay is technically simple but should be interpreted cautiously because BODIPY’s rate of reaction with peroxy radicals is slower than that of radical-scavenging antioxidants, and hence suppression of BODIPY fluorescence by antioxidants need not always reflect their ability to suppress lipid peroxidation<sup>59</sup>. Another fluorometric assay for lipid peroxidation employs *cis*-parinaric acid (PnA), a fatty acid with four conjugated double bonds. Oxidation of PnA disrupts its conjugated system and hence fluorescence. Because PnA may be incorporated into different classes of phospholipid, high-performance liquid chromatography (HPLC) separation provides information on the oxidation of different phospholipids<sup>60</sup>. However, extrapolation of PnA-based results to endogenous phospholipid oxidation is difficult due to the higher oxidation rate of PnA, its vulnerability to photobleaching and variable metabolic incorporation of PnA into different phospholipids<sup>60</sup>.

Lipid peroxidation is frequently assessed by the measurement of end products such as  $\alpha,\beta$ -unsaturated hydroxyalkenals<sup>61</sup>, ideally by MS-based techniques. In particular, 4-hydroxynonenal (HNE) formation has been widely used. Antibodies against the protein adducts formed by HNE are widely available and frequently used in immunostaining of tissues, but it should be realized that different antibodies can detect different epitopes and so give different answers, depending on what amino acid residues the HNE binds to in proteins<sup>61–64</sup>.

One minor end product of lipid peroxidation is malondialdehyde (MDA)<sup>61</sup>, which can also be a useful biomarker if measured by MS techniques. However, the widely used ‘MDA assays’ utilizing thiobarbituric acid-reactive substances (TBARS) are unspecific since TBA generates chromogens from many biomolecules other than MDA<sup>1,65</sup>. Use of HPLC to separate the ‘real’ TBA–MDA adduct from false chromogens increases specificity but does not eliminate all problems<sup>1</sup>.

**Recommendation 11:** application of the simple TBA test (TBARS), or kits based on its use, to cells, tissues or body fluids is not recommended as the only test used for evaluation of oxidative lipid damage because of the low specificity that can result in false-positive results. HPLC-based TBA tests are less prone to artefacts.

The detection of lipid oxidation products has been revolutionized by the development of LC–MS for detailed analysis of oxidized

lipid mixtures<sup>66</sup>. Collection and storage of samples to avoid artefactual peroxidation is key to any lipid peroxidation study, and samples for later analysis should be immediately frozen in liquid nitrogen. Biofluids may require the addition of chemicals (for example, butylated hydroxytoluene) to prevent auto-oxidation during storage<sup>1,67</sup>. The internal standards used for quantification should be added to samples before solvent extraction. Such LC–MS-based methods have the advantages of high sensitivity, small sample volume requirements and the ability to detect multiple end products of lipid peroxidation. This makes LC–MS protocols the methods of choice for assessment of general lipid peroxidation and identification of individual products, including those with specific signalling functions. However, limitations of available standards may sometimes preclude quantitative analysis of certain products. Scrupulous attention to methodology is required in such studies<sup>68</sup>.

Prominent among lipid oxidation products that have been quantified by MS-based approaches are the F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs)<sup>69</sup>. Sixty-four F<sub>2</sub>-IsoP stereoisomers can be generated from the free-radical-induced, non-enzymatic oxidation of arachidonic acid and can be separated from those that arise from the enzymatic oxidation of arachidonic acid by cyclo-oxygenase enzymes (COX-1/-2). F<sub>3</sub>- and F<sub>4</sub>-isoprostanes arise from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively, but have been less well characterized than the F<sub>2</sub>-isoprostanes. ELISA methods have been developed to quantify one F<sub>2</sub>-IsoP isomer, 8-iso-PGF<sub>2</sub> $\alpha$  (also referred to as 15-F<sub>2t</sub>-IsoP or iPF<sub>2</sub> $\alpha$ -III), and compared with gas chromatography–MS and LC–tandem MS (LC–MS/MS) methods<sup>69–73</sup>. In all these studies there was poor agreement between commercially available ELISA kits and MS methods; 8-iso-PGF<sub>2</sub> $\alpha$  is one of 64 different F<sub>2</sub>-IsoP isomers generated during arachidonic acid peroxidation, and antibody cross-reactivity between 8-iso-PGF<sub>2</sub> $\alpha$  and related isomers is challenging. Pre-analysis sample clean-up may allow for more precise measurement of 8-iso-PGF<sub>2</sub> $\alpha$  by ELISA<sup>72,73</sup>, but by far the most accurate method for quantification of F<sub>2</sub>-IsoPs is by LC–MS/MS and is very strongly recommended.

**Recommendation 12:** F<sub>2</sub>-IsoPs are a generally accepted biomarker of lipid peroxidation, but it should be realized that they are one of many end products and that the levels of various types can be affected by experimental conditions<sup>69</sup>. Quantification using ELISA is susceptible to artefact, but sample clean-up may allow measurement of 8-iso-PGF<sub>2</sub> $\alpha$  by ELISA<sup>73</sup>. LC–MS/MS with appropriate internal standards is the preferred approach.

**Protein damage.** Amino acid residues in proteins are sensitive to oxidative modification, some forms of which provide useful biomarkers<sup>74,75</sup>. Detailed protocols for measurement of multiple products can be found in refs. <sup>76,77</sup>. A common protein modification is the formation of ‘protein carbonyls’ due to oxidation of specific amino acid residues to carbonyl group-bearing products; carbonyls can also be formed by the reaction of aldehydes with nucleophilic sites on proteins or by glycation<sup>75,77</sup>. Many assays involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine to form a dinitrophenylhydrazone (DNP). This product can be detected spectrophotometrically, although this approach can suffer from high background and low reproducibility; to circumvent this, DNP adducts can be separated by LC before measurement. Alternatively, carbonyls can be detected using an antibody against DNP products by ELISA or immunoblotting<sup>78</sup>. Changes in protein carbonyls can be measured in tissue homogenates treated with fluorescein-5-thiosemicarbazide (FTC) to generate fluorophore-labelled proteins that can be separated by gel electrophoresis<sup>79</sup>. Enrichment methods using biotin-tagged derivatization coupled with LC–MS detection have been developed<sup>80</sup>. Protein carbonyls,  $\alpha$ -amino adipic semialdehyde and glutamic semialdehyde have also been assayed individually by stable isotopic dilution analysis LC–MS/MS<sup>77</sup>. Of course, data from a single time point reflect the

difference between the rates of formation and removal (for example, by repair or proteolysis) of these products.

Protein analysis using MS allows detection and identification of modifications with characteristic mass increases (for example, hydroxylation, nitration and chlorination)<sup>75,76</sup>. This has been particularly useful in studies of oxidative damage to brain proteins in patients with dementia by 'redox proteomics'<sup>81</sup>. Peptide-level mapping after proteolytic cleavage allows detection of the nature of the modification, its location within the protein sequence and concomitant loss of the parent peptide, allowing relative quantification. Amino acid analysis after complete digestion allows determination of types and absolute concentrations of particular species (determined by the use of isotope-labelled standards) together with the parent species, allowing determination of a 'mass balance'<sup>75–77,82</sup>. Care must be taken in sample handling to prevent artefactual oxidation of Cys or Met, and also during protein hydrolysis, because some products of oxidative protein damage are labile. LC–MS analyses have many advantages including high specificity, high sensitivity, the capacity to detect many different modifications and parent species concurrently, as well as the capacity to detect products that are diagnostic of the ROS involved, such as chlorination from HOCl<sup>83</sup> and nitrated species arising from the action of myeloperoxidase in the presence of NO<sub>2</sub><sup>-</sup> and/or by reactions of ONOO<sup>-</sup>/ONOOH<sup>49,84</sup>. These LC–MS approaches can be carried out on materials ranging from isolated proteins to tissue samples. Quantification relative to non-modified amino acids or peptides, and preferably against added heavy-isotope-labelled materials, is recommended to overcome potential artefacts arising from sample handling and preparation. However, do bear in mind that plasma and urinary levels of oxidized amino acids might have contributions from absorption of oxidized amino acids from proteins in food or from increased tissue proteolysis as a result of pathology. Neither of these have yet been studied in detail.

Cysteine is a major target for modification due to its ease of oxidation (particularly in its thiolate form, RS<sup>-</sup>) and its nucleophilicity, which results in ready adduct formation with electrophiles. Oxidation can be irreversible—for example, to a sulphinic or sulphonic acid, which can be useful biomarkers of oxidative protein damage<sup>4,85</sup>. Reversible oxidation of Cys residues in proteins is a prominent mechanism of redox signalling<sup>2,4</sup>. Reversible products include disulphides, sulphenic acids, S-nitrosothiol and persulphide species<sup>2,4,85–87</sup>. These modifications can be reversed by the GSH/glutaredoxin or thioredoxin systems<sup>87</sup>. A common approach in detection of the pool of reversibly modified Cys residues is to first block reduced thiols with a reactive reagent and then reduce and derivatize the previously oxidized residues with a tag that can be identified by LC–MS of tryptic digests<sup>88</sup>. These approaches can be extended to the use of modification-specific chemistry to tag only a particular oxidation product, such as an S-nitrosothiol, sulphenic acid or persulphide<sup>88</sup>. Until recently, major limitations to these approaches were low coverage of the total Cys pool and lack of quantitation of the modification at individual residues<sup>87–89</sup>. The latter is of particular importance when interpreting the biological importance of reversible modifications. Substantial improvements in quantification have been achieved by isobaric tagging, in which reduced Cys residues are first labelled with one tag, reversibly modified residues are reduced and then labelled with a chemically identical, but heavy-isotope-modified, tag that enables quantification of the proportion oxidized for each particular Cys. These methods have been extended to tags that incorporate moieties such as biotin, which enable enrichment of the labelled peptides, greatly enhancing Cys coverage. The most recent iteration of this approach, as exemplified by the OxiMOUSE study<sup>89</sup>, has superseded previous methods.

Methionine is also a major site of redox post-translational modifications. Oxidation to methionine sulphoxide can be reversed enzymatically by methionine sulphoxide reductase enzymes, potentially

facilitating redox signalling by the installation/removal of a single oxygen atom<sup>17</sup>. Reagents have been developed for methionine bioconjugation that can identify and characterize redox-sensitive methionine sites in proteomes<sup>90</sup>.

*Recommendation 13:* ELISA, FTC and immunoblotting are useful tools in the detection of protein carbonyls as a biomarker of general oxidative protein damage, although it must be realized that not all protein oxidation products contain carbonyls. LC–MS approaches, using carefully prepared samples, are the best available techniques for assessment of protein oxidation due to the sensitivity, selectivity and quantitation available with these methods. The use of orthogonal approaches, such as specific and validated antibodies (see below) against individual oxidation products, is also encouraged.

**Nucleic acids.** Oxidative modifications of DNA and RNA are often used as biomarkers of oxidative damage<sup>1,13,91</sup>. One method used to assess 'general' oxidative damage to DNA in cells is the comet assay, which detects DNA strand breaks. Such breaks can arise by several mechanisms, not necessarily via oxidative damage, but the use of repair enzymes that 'nick' DNA at the sites of oxidation increases the specificity for oxidative DNA damage. The simplest measurement is the length of the DNA 'ghost' following electrophoresis of cells embedded on a gel on a microscope slide<sup>92</sup>.

Oxidative damage to DNA usually focuses on oxidation of guanine to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8OHdG, or 8-oxodG). Data on modifications at other bases are limited, although these are likely to be biologically important<sup>1,13</sup>. These measurements require the isolation of the DNA and its digestion to release modified bases, and there can be spurious oxidation during sample handling and analysis. Multi-laboratory initiatives<sup>93</sup> have established protocols to avoid this and have determined 'normal' levels of 8OHdG. The amount of 8OHdG (or any other product of oxidative DNA damage) measured in DNA is the balance between the rate of oxidation and that of repair. The best methodology is ultra-performance LC–MS/MS (UPLC–MS/MS)<sup>94</sup>. Caution should be exercised in using ELISA methods, which lack sensitivity and specificity and can give variable results between batches, and there is sometimes cross-reaction between 8-hydroxyguanosine (8OHG) and 8OHdG. However, immunohistochemistry can be useful in identification of cells that have higher amounts of 8-OHdG in vivo, if applied appropriately<sup>95</sup>.

Oxidized nucleosides from both DNA and RNA can be detected in various body fluids. Originally they were believed to arise from DNA repair, particularly nucleotide excision repair. However, they also arise from oxidation of the DNA and RNA nucleotide precursor pools 'sanitized' by removal of oxidized products<sup>94</sup>. The relative contributions of DNA repair and nucleotide pool sanitization to the levels of oxidized nucleosides detected in body fluids are currently unclear. Urine collected over 24 h will represent the number of guanines in DNA/RNA and/or the respective nucleotide precursor pools oxidized during that period<sup>96</sup>. Urine sampling represents formation within the entire body, and is best suited to situations where all tissues are assumed to be affected, but it could be inadequate in the detection of changes that occur only in certain organs. Measurement in specific tissues will be a snapshot of the balance between generation and repair, and may not represent processes in other organs.

*Recommendation 14:* when measuring oxidative modifications of nucleic acids from extracted cells or tissue samples, great care must be taken to avoid spurious oxidation in the preparative and analytical steps. Methods such as the comet assay (using DNA repair enzymes) on isolated cells and UPLC–MS/MS for 8OHdG and 8OHG determination in body fluids or nucleic acids extracted from tissues are presently the best available. ELISA-based methods, especially in kit form, are usually insufficiently validated and their use is not recommended.



### Some general comments on antibodies

As discussed above, antibodies have been widely used to detect oxidation products (and also adducts) formed on proteins (for example, carbonyls and 3-nitro- and 3-chlorotyrosine), DNA (for example, 8-oxodG) and lipids (F<sub>2</sub>-Isoprostanes). They have been used, for example, in ELISA, immunohistochemistry and immune precipitation formats, but often suffer from background reactivity, cross-reactivity and lack of specificity. To address this, the epitope used to generate the antibody should be documented (for example, as for HNE)<sup>62–64</sup> and controls to eliminate background should be included. Blocking by authentic samples of the epitope is recommended to determine selectivity. Relative quantification is possible but absolute quantification can be difficult—for example, due to poor epitope accessibility (for example, in proteins the oxidation product may be buried). In addition, antibodies are typically generated against unstructured, chemically modified peptides and the epitope(s) recognized may not always have been determined.

**Recommendation 15:** well-validated antibodies against specific products are useful detection tools when used with appropriate care and controls, including those for non-specific interactions. Competition data with authentic epitopes should be included whenever possible.

### Measurement of ROS and oxidative damage in vivo

Measurement of ROS in vivo is a challenge. EPR methods have been developed but are not yet widely used. Bioluminescent approaches to ROS detection include peroxy-caged luciferin-1 which, upon oxidation, forms luciferin in situ that is oxidized in luciferase-transfected systems to generate bioluminescence<sup>97</sup>. As noted earlier, genetically encoded redox biosensors have been used in animal studies. With the development of improved sensitivity and detection modalities, positron emission tomography is now being used to image ROS in vivo<sup>98</sup> but is still in its infancy. In mitochondria of cells and tissues, changes in H<sub>2</sub>O<sub>2</sub> can be assessed using the mitochondria-targeted boronate MitoB, which accumulates in these organelles and is converted by H<sub>2</sub>O<sub>2</sub> into MitoP. The ratio of MitoP to MitoB can then be determined by MS<sup>99</sup>.

### Measurement of ROS and oxidative damage in clinical trials

Because oxidative damage plays a central role in many human pathologies, there is considerable interest in developing therapeutic interventions to decrease this damage<sup>1–3</sup>. A corollary is that in clinical trials we should be able to demonstrate how these interventions affect oxidative damage. For example, many double-blinded randomized clinical trials have been conducted using ‘antioxidants’ such as beta-carotene, vitamin C and vitamin E. These generally failed to influence disease activity. Unfortunately, in most cases the effect of the intervention on oxidative damage was not measured, making it uncertain whether the putative therapy was actually effective at decreasing oxidative damage: if it was not, lack of effect is predictable<sup>1,55</sup>.

To address this, it is essential to assess the impact of these interventions on levels of oxidative damage in the patients in clinical trials. Currently, methods are limited to measuring end points of oxidative damage in either biopsies (for example, skin or muscle) or clinically accessible body fluids such as plasma, saliva, sputum or urine, and sometimes cerebrospinal fluid. These biomarkers have included those for oxidation of nucleic acids such as 8OHG and 8OHdG<sup>100</sup> and F<sub>2</sub>-isoprostanes as a biomarker of lipid peroxidation<sup>69,101</sup>. To date, limited use has been made of biomarkers of protein oxidation in clinical trials. However, there is evidence for strong associations of alterations in protein thiol/disulphide ratios, and increased protein carbonyls and other modifications with pathologies<sup>75–77</sup>.

More generally, clinical trials should include internationally validated biomarkers: the biomarker should ideally have undergone

#### General recommendations

- 1 If possible, avoid the term ‘ROS’ and define the actual chemical species involved and its properties. If not, discuss caveats about the term ROS.
- 2 Any putative effects of antioxidants should be chemically plausible, and confirmed by measurements of oxidative damage.
- 3 Selective generation of O<sub>2</sub><sup>•−</sup> and H<sub>2</sub>O<sub>2</sub>, and specific inhibition/deletion of redox-active enzymes, should be used to confirm the roles of these species.
- 4 How oxidative damage to a biomolecule arises, is repaired and cleared and is measured should be discussed when presenting levels of oxidative damage markers.

#### Measurement of ROS

- 5 Use commercial kits only if the species being measured and the detection method are explained and are chemically plausible.
- 6 When using fluorescent ROS probes, the selectivity and potential artefacts should be made clear (especially for DCFH-DA). Controls should be done to show that the response is due to the proposed species, and orthogonal techniques used to corroborate the conclusion.
- 7 ROS should not be ‘measured’ in tissue homogenates or cryosections.
- 8 To detect O<sub>2</sub><sup>•−</sup> in vitro use the SOD-sensitive reduction of cytochrome c, while aconitase inactivation within mitochondria can be used in vivo. Be cautious when using luminol or lucigenin.
- 9 Hydroethidine or MitoSOX probes cannot be used to detect O<sub>2</sub><sup>•−</sup> production by simple fluorescence measurements. Use specific identification of 2-hydroxyethidium products instead.
- 10 Genetically encoded fluorescent probes are sensitive detectors of H<sub>2</sub>O<sub>2</sub> in vivo. If their expression is not possible, boronate probes are the preferred technique.

#### Measurement of oxidative damage

- 11 Application of the TBARS assay to cells, tissues or body fluids as the sole measure of lipid peroxidation is not recommended.
- 12 Measurement of F<sub>2</sub>-IsoPs by LC–MS/MS with appropriate internal standards is the preferred biomarker of lipid peroxidation.
- 13 Analysis of protein carbonyls by ELISA, FTC and immunoblotting can detect general oxidative protein damage. Orthogonal approaches to quantify individual oxidation products are encouraged.
- 14 Measurement of oxidative modifications of nucleic acids can be done using the comet assay on isolated cells and by UPLC–MS/MS for 8OHdG and 8OHG determination in body fluids or tissues.
- 15 Use of antibodies to measure specific oxidation products must incorporate controls for non-specific interactions and competitive data with authentic epitopes.
- 16 Biomarkers must be measured to confirm that any antioxidants used decrease oxidative damage to the relevant biomolecules.

**Fig. 1 | Summary of recommendations for measurement of ROS.** Here we have summarized and abbreviated the recommendations for best practices developed in this manuscript.

interlaboratory comparison. Many biomarkers rely on concentration measurement in body fluids such as plasma, but these reflect only the balance between formation and elimination rates and therefore cannot readily be interpreted as ‘oxidative stress’. However,

models have been developed to estimate the 24 h production of certain biomarkers<sup>100</sup>. Ideally a panel of biomarkers should be used<sup>54,55</sup> since end products of oxidative damage to lipids, proteins and nucleic acids do not necessarily correlate with each other, nor would we expect them to since they are different molecular targets of different ROS.

**Recommendation 16:** if intervening with antioxidants, first use biomarkers in preliminary dose-ranging studies to determine whether the intervention does indeed decrease oxidative damage to the relevant biomolecules. They should include well-defined biomarkers analysed with a validated methodology and/or orthogonal approaches. We do not recommend in clinical (or other!) studies the use of the d-ROMS assay (for reasons explained in ref. <sup>1</sup>), TBARS, determinations of total antioxidant activity<sup>1,102</sup> or kit-based methods where the methodology behind the kit is not clear and/or has not been validated.

### Concluding remarks

The goal of this consensus statement is to generate a useful resource for researchers from diverse fields who find themselves needing to measure ROS and to assess oxidative events to investigate their biological importance. We have discussed the limitations of many of the procedures currently used and suggested the best currently available approaches. Inevitably, new techniques will be developed and applied in the future, but the principles of our cautious philosophy, illustrated by our 16 recommendations (summarized in Fig. 1), will remain valid.

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### Author contributions

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### Competing interests

The authors declare no competing interests.

### Additional information

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