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CONSENSUS STATEMENT

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

Michael P Murphy*
University of Cambridge, UK

Hülya Bayir
University of Pittsburgh, USA

Vsevolod Belousov
Federal Center of Brain Research and Neurotechnologies, Moscow, Russian Federation

Christopher J. Chang
University of California, Berkeley, USA

Kelvin J. A. Davies
University of Southern California, Los Angeles, California, USA

Michael J. Davies
University of Copenhagen, Denmark

Tobias P. Dick
German Cancer Research Center (DKFZ), Heidelberg, Germany

Toren Finkel
University of Pittsburgh, USA

Henry J. Forman
University of California, Merced and University of Southern California, USA

David Gems
University College London, UK

Yvonne Janssen-Heininger
University of Vermont, USA

Valerian E. Kagan
University of Pittsburgh, USA

Balaraman Kalyanaraman
Medical College of Wisconsin, USA

Nils-Göran Larsson
Karolinska Institutet, Sweden

Ginger L. Milne
Vanderbilt University Medical Center, USA

Thomas Nyström
University of Gothenburg, Sweden

Henrik E. Poulsen
University Hospital Copenhagen, Denmark

Rafael Radi
Universidad de la República, Montevideo, Uruguay

Holly Van Remmen
Oklahoma Medical Research Foundation, USA

Paul T. Schumacker
Northwestern University, USA

Paul J. Thornalley
Qatar Biomedical Research Institute, Qatar

Shinya Toyokuni
Nagoya University Graduate School of Medicine, Japan

Christine C. Winterbourn
University of Otago, Christchurch, New Zealand

Huiyong Yin
Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, Shanghai, China

Barry Halliwell*
National University of Singapore, Singapore

* Corresponding authors

Abstract

Multiple roles of reactive oxygen species (ROS)* and their consequences for health and disease are emerging throughout biological sciences. This has led researchers unfamiliar with the complexities of such species 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, oxidative damage and role as signalling molecules. In this consensus statement we illuminate problems that can arise with many commonly used approaches 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*.

Introduction

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[1-4]. Consequently, researchers from diverse fields often need to measure ROS, to assess oxidative events and to investigate their biological significance by using antioxidants* 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 focussing on the identification of ROS, their chemical reactions and products of oxidative damage. However, as with many specialised 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 of *measuring* 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 the

** Footnote*
These terms are defined in box 1

consequent changes in the activity of redox sensitive cellular processes is central to the field of redox signalling[1-4]. 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[1-11] which researchers are strongly encouraged to read. Here we distil the key points underlying this consensus statement.

What are reactive oxygen species, 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**). For example, one important reactive species found in biology, the superoxide radical anion ($O_2^{\bullet-}$), is formed by the one electron reduction of oxygen (O_2). In itself, $O_2^{\bullet-}$ is not very reactive, except with another radical nitric oxide (•NO) to form peroxynitrite[11], or with [FeS] clusters in proteins[12]. Similarly, hydrogen peroxide (H_2O_2), formed by various oxidase enzymes[1,4] and by the action of superoxide dismutase, is poorly reactive, which allows its use as an important signalling molecule *in vivo*[2,4]. Yet in the presence of ferrous or cuprous ions H_2O_2 forms the extremely reactive hydroxyl radical ($^{\bullet}OH$) by Fenton chemistry; $^{\bullet}OH$ reacts non-specifically and essentially instantaneously with any nearby biomolecule (**Table 1**)[1,13]. The availability of transition metal ions to catalyse Fenton chemistry is carefully controlled *in vivo*[1] but they can be released by tissue injury or when certain proteins with [Fe-S] clusters encounter $O_2^{\bullet-}$ [1,12,14]. Their importance *in vivo* has been recently underscored by the growing literature on ferroptosis, a form of cell death involving "catalytic" iron ions[15]. H_2O_2 is a substrate for haem peroxidases such as myeloperoxidase, generating further reactive species such as hypochlorous acid (HOCl) (**Table 1**). Despite its poor reactivity, H_2O_2 can selectively oxidise certain methionine (Met) and cysteine (Cys) residues[16,17] in proteins.

A far from complete list of the physico-chemical 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 since the reactivity of different ROS varies over a wide scale, as do their lifetimes, ability to diffuse and potential to generate further downstream reactive species. In short, not all ROS are the same. The generalisation "ROS", although widely

used (including in the present 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, lifetime, 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 to modulate redox signalling[1,2]. 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 $O_2^{\bullet-}$, peroxidases for H_2O_2 and metal-ion sequestration[1,14]. Most low molecular mass compounds commonly employed as "antioxidants" are stoichiometric scavengers of certain ROS and often have modest (if any) reactivity with $O_2^{\bullet-}$ or H_2O_2. For example, *N*-acetylcysteine (NAC) is a widely used "antioxidant", but it has other (and sometimes more important[18]) modes of action. NAC can indeed scavenge some ROS *in vitro*, but not others, most notably not H_2O_2[18]. It can also increase the cellular Cys pool and thereby enhance glutathione (GSH) levels, generate H_2S, and directly cleave protein disulphides[18]. Low molecular mass compounds that do act as antioxidants *in vivo* include vitamin E, which scavenges lipid peroxyl radicals[19]. Sometimes "$^{\bullet}OH$ scavengers" are used to infer a role for this ROS. Yet, they can rarely, if ever, achieve a high enough concentration to prevent the effectively instantaneous reaction of $^{\bullet}OH$ with biomolecules[1,7]. Consequently, many of the biological effects assigned to "antioxidants", especially NAC, are due to other effects. Other agents often used as "antioxidants" such as TEMPOL and porphyrin-based "SOD mimetics" undergo complex redox reactions *in vivo* and are better described[1] as "redox modulators" rather than "antioxidants" or "$O_2^{\bullet-}$ scavengers". ***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 recognised that low molecular mass "antioxidants" are unlikely to act by scavenging H_2O_2. 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 assign damage, or activation of a redox signalling pathway, to a particular ROS can be by selectively generating the ROS in a biological context. This can be done by redox cycling compounds such as paraquat (PQ) or quinones to generate $O_2^{\bullet-}$, or MitoPQ to generate $O_2^{\bullet-}$ within mitochondria[1,20]. Glucose oxidase can be used to generate H_2O_2 *in vitro,* while the regulated generation of H_2O_2 within cells can be achieved using genetically expressed D-amino acid oxidase, an enzyme that generates H_2O_2 as it oxidises D-amino acids. It can be targeted to different sites in the cell and the flux regulated by varying the added concentration of its substrate, D-alanine[21]. NADPH oxidase (NOX) enzymes are important sources of $O_2^{\bullet-}$ and H_2O_2 for redox signalling as well as oxidative damage[9,22] and modulating their activity is an important approach to understanding these processes. A number of fairly specific inhibitors of NOX enzymes have been described[22]. However, the use of compounds such as apocynin and diphenyleneiodonium as "NOX inhibitors" is still widespread, even though they lack specificity. ***Recommendation 3****: We recommend the use of paraquat, 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 (see*[22]*) or deletion or knockdown of NOX components should be used to identify their roles.*

General Principles: Measuring ROS and Oxidative Damage

In 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 the oxidative modifications ("oxidative damage" **Box 1**) caused by the ROS[1]. 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 (e.g. inhibiting oxidative damage or interfering 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 marker 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.*

Detecting and Measuring ROS: Guidelines and Limitations

Considering 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 (lifetime milliseconds or less), and their steady state levels are low (picomolar to low micromolar) and alter rapidly, as 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. 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[23]. The bottom line is that *all* methods 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 in order 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*[24]. 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, employ techniques using different principles of detection.*

Small molecule fluorescent probes are frequently used to assess ROS within cells. In some cases, a lack of description of the chemical composition or structures of these probes makes it difficult to interpret results and these should be avoided. Even for known probes there can be concerns. Consider the widely used fluorescent probe 2',7'-dichlorodihydrofluorescein (DCFH), usually administered as its diacetate (DCFH-DA) form, which enters cells readily. While DCFH is oxidised to the fluorescent product 2',7'-dichlorofluorescein (DCF) by several ROS, these reactions are non-specific[6,7]. DCFH is not oxidized directly by H_2O_2 (which it is often claimed to detect), but only after the H_2O_2 is converted to more-reactive species by redox-active metals, or by haem proteins. Furthermore, the oxidation of DCFH and the fluorescence of DCF are sensitive to local O_2 levels and pH, and the fluorescence yield may not be linear with increased ROS levels[25-27]. 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[26]. 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 is DCF, it is always important to validate data by a number of simple controls: does the response change over time and with amount of biological sample in a plausible manner? Can the effect be replicated by generating the ROS of interest (e.g. using PQ for $O_2^{\bullet-}$, D-amino acid oxidase for H_2O_2)? Do negative controls that should abolish the ROS-generating process (e.g. 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 adding "ROS probes" to fresh or previously frozen tissue slices or homogenates *ex vivo*. These measurements may be meaningless, because the very short lifetime of ROS means that any present *in vivo* will have

long gone by the time the material is assayed. Furthermore, freezing or homogenisation disrupts membranes and alter substrate and ion concentrations (e.g. raising levels of Ca^{2+} or "catalytic" Fe^{2+}), 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 to assess ROS *in vivo* or in perfused organs, but in these situations the process is either monitored *in vivo* (e.g. see Table 3 for H_2O_2), or the system is quenched so as to stabilise 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 was able to irreversibly capture the reactive species when the cells/tissues/organs were under biologically relevant conditions.*

Direct measurements of ROS

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

Superoxide. In simple systems $O_2^{\bullet-}$ can be measured in a number of ways, such as by the SOD-sensitive reduction of cytochrome c[23]. The generation of $O_2^{\bullet-}$ can also be assessed by spin trapping followed by EPR, which has the benefit of detecting the radical directly[1]. 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[28]. The chemiluminescent "superoxide probes" luminol and lucigenin are widely used to "detect $O_2^{\bullet-}$" but interpreting such data is difficult because these probes generate radicals that produce $O_2^{\bullet-}$ themselves; they do *not* react with $O_2^{\bullet-}$ directly[29,30].

Recommendation 8*: 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 (sometime called hydroethidine; HE), or mitochondria-targeted hydroethidine (MitoSOX). Unfortunately, detection by fluorescence is misleading, as these probes form both ethidium (E^+), a non-specific oxidation product, and the $O_2^{\bullet-}$–specific product 2-hydroxyethidium (2-OH-E^+) which have overlapping fluorescence spectra, so it is hard to

differentiate the contribution of non-specific oxidation and $O_2^{\bullet-}$–dependent oxidation (if any) to the overall fluorescence[31]. Accurate quantitation of the 2-hydroxyethidium product can be achieved using liquid chromatography-mass spectrometry (LC-MS)[31]. Another factor that should be considered is the extent of cellular uptake of hydroethidine/MitoSOX and the intracellular concentrations of them and their multiple products. Furthermore, hydroethidine oxidation products intercalate into DNA, greatly enhancing their fluorescence and creating another artefact. NeoD and MitoNeoD contain a modified hydroethidine that does not intercalate into DNA[32].

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 which have positive charges or generate positively-charged species (including 2-hydroxyethidium and ethidium), it is important to remember that probe accumulation is dependent on the plasma and mitochondrial membrane potentials, mitochondrial size, shape and mass[33]. Furthermore, fluorescence can be quenched when they are present at high concentrations in mitochondria[34].

Recommendation 9*: Only use hydroethidine or MitoSOX probes to detect $O_2^{\bullet-}$ by simple fluorescence measurements when the product has been independently validated as 2-hydroxyethidium.* ***Fluorescence*** *measurements with probes such as dihydroethidium and MitoSOX[33] should be conducted using the lowest probe concentration possible, and must include controls for changes in the 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 the modified species[31], should be performed when possible.*

Hydrogen peroxide. In simple systems, H_2O_2 can be measured by horseradish peroxidase (HRP), oxidizing substrates such as Amplex Red. These methods can be interfered with by other HRP substrates (e.g. ascorbate, NAC)[1] and by $O_2^{\bullet-}$ (which can inactivate HRP), the latter can be prevented by adding SOD[35]. Since H_2O_2 can cross membranes directly or via aquaporins, this system can also be used to measure H_2O_2 release from cells. However, please be aware that this release reflects the balance between H_2O_2 production, removal by intracellular enzymes and the rate of diffusion out of the cell.

Within cells, H_2O_2 detection by phenylboronate-based probes is more reliable[36], although they may lack sufficient sensitivity as they react only slowly with H_2O_2, which can make it difficult to detect small or localized changes in H_2O_2 levels[37]. However, recent studies suggest that borinic acids, which react faster with H_2O_2, may be more sensitive detectors[38]. The mechanism of oxidation of phenylboronates to phenols requires a two-electron oxidant, such as H_2O_2. Because H_2O_2 is typically generated at higher concentrations than other ROS, boronate probes can be selective for H_2O_2 detection, subject to proper controls[37,38]. However, boronate probes react with $ONOO^-$/ONOOH or HOCl much faster than they do with H_2O_2 which can sometimes complicate measurements, and orthogonal approaches or the use of inhibitors can aid validation[39]. For example, H_2O_2- and peroxynitrite-dependent signals can be distinguished using nitric oxide synthase inhibitors and catalase[37,40].

Genetically-encoded fluorescent protein sensors have provided major advances in cellular H_2O_2 detection[41-45]. 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_2O_2 has been achieved by coupling a redox-sensitive green fluorescent protein (GFP) mutant to an H_2O_2-sensitive thiol protein, such as oxyR (HyPer series), or to a peroxidase, such as Orp-1 or TSA2 (roGFP2 based probes). HyPer7 and roGFP2 coupled to a peroxiredoxin provide the highest sensitivity[42,43]. While the 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 pH changes[41]. Imaging analysis by fluorescence microscopy is normally employed, but fluorescence plate readers can also be used. Redox status represents a balance between the rate of oxidation and re-reduction of the protein by cellular reductants, including glutaredoxin/GSH and thioredoxin, permitting real-time live cell assessments of redox state. Because excitation wavelengths of both the reduced and oxidised forms are used, the probes are ratiometric and the output is not dependent on the level of protein expression. By incorporating appropriate targeting gene sequences, these probes can be directed to different cell compartments, including mitochondria, microtubules, endoplasmic reticulum, nucleus, and cytoplasm[41-45]. Hence, subcellular regions-of-interest can be studied and the probe then calibrated at the end by fully reducing (2 mM dithiothreitol), washout, then fully oxidizing (2 mM t-butylhydroperoxide)[42,43]. This calibration yields a measure of oxidation percentage, permitting comparisons across experiments and among subcellular compartments[42-44]. These protein sensors have been expressed *in vivo* in transgenic animals to provide useful assessments

of *in vivo* H_2O_2 generations[45,46]. Plasmid transfection of viral vectors can be used with cultured cells, and targeted roGFP2 probes are available commercially (www.addgene.com).

In most experiments the H_2O_2 probes are expressed as free proteins that distribute across the cell. Nevertheless, given uncertainties about intracellular H_2O_2 diffusion distances, it is still unclear what resolution is needed to understand subcellular H_2O_2 distribution. Therefore, tethering H_2O_2 probes to sub-compartmental locations such as protein complexes or organelle contact sites is an important task for the future.

Recommendation 10: *Genetically encoded fluorescent probes (some of which are commercially available) are currently the most sensitive detectors of H_2O_2 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_2O_2 are required and sensitivity is limited for physiological H_2O_2 levels. Amplex Red with HRP can measure H_2O_2 release from cells if other reducing agents or peroxidase substrates are absent.*

Peroxynitrite ($ONOO^-$) exhibits complex chemistry[40,47,48] and itself can oxidise few biomolecules. A major physiological reaction is with CO_2 (Table 1) and hence, the CO_2/ $HCO_3^{\bullet-}$ content of biological systems plays a role in determining the biological impact of $ONOO^-$. Products of this reaction include the highly reactive species carbonate radical anion ($CO_3^{\bullet-}$) and the nitrating agent nitrogen dioxide ($NO_2^{\bullet}$) (Table 1), both of which interact with many of the general "ROS probes". Peroxynitrite oxodises boronate-based probes nearly a million times faster than H_2O_2 and under the right conditions these probes can be used to assess $ONOO^-$/ONOOH production[40,48]. Peroxynitrite has been measured in tissues *ex vivo* by using boronate probes[49].

Hypochlorous acid and other reactive halogen species. HOCl, HOBr, (hypobromous acid) 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[50]. A genetically encoded probe for

reactive halogen species has been developed, enabling dynamic monitoring of these species in cell culture and *in vivo*[51].

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 that cannot be formed by other mechanisms and thus can be used as “biomarkers” of oxidative damage (**Box 1**)[1,52,53]. However, do note that the measured levels of biomarkers represent a balance between the generation and turnover of the biomarker, plus any artefactual increased levels caused by oxidative damage during isolation or analysis.

Lipid peroxidation. Polyunsaturated fatty acids (PUFAs), are readily oxidised, hence lipid peroxidation products are widely used to characterize oxidative damage[53-55]. Lipid peroxidation can be initiated and proceed as a random non-enzymatic (often chain) radical process. However, there are also selective enzymatic mechanisms (e.g. lipoxygenases) 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: i) establishing increased lipid peroxidation as an example of oxidative damage or ii) on identification of individual oxidatively modified lipid molecules acting as signals by selectively interacting 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• abstraction. The carbon-centred radical ($L^{\bullet}$) generated by $H^{\bullet}$ abstraction is stabilized by delocalization over the double bonds. Subsequent reaction with O_2 gives a peroxyl radical ($LOO^{\bullet}$) with formation of a conjugated diene system and a range of peroxides (LOOH). $LOO^{\bullet}$ can react further to yield highly oxidised secondary products, including epoxy-, oxo-, or cyclic peroxides[54,55]. There are multiple end products of lipid peroxidation that show vast chemical heterogeneity, variable stability and polarity[54,55]. Thus, measurement of only a single oxidation product in no way represents the whole process of lipid peroxidation.

There are several methods to assess "general" lipid peroxidation. In simple model systems (e.g. isolated lipoproteins), diene conjugation can be measured by UV-absorbance, but this method is not suitable for use in cells or body fluids because there are interfering UV-absorbing molecules that do not result from lipid peroxidation[1]. In cells "lipid peroxidation" can be assessed by changes in the fluorescence of BODIPY conjugated to a peroxidation-sensitive undecanoic acid moiety[56]. This assay is technically simple but should be interpreted cautiously because BODIPY's rate of reaction with peroxyl radicals is slower than that of radical-scavenging antioxidants, hence suppression of BODIPY fluorescence by antioxidants need not always reflect their ability to suppress lipid peroxidation[56]. 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. As PnA may be incorporated into different classes of phospholipids, HPLC separation provides information on the oxidation of different phospholipids[57]. 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[57].

Lipid peroxidation is frequently assessed by the measurement of end products such as α,β-unsaturated hydroxyalkenals[58], ideally by MS-based techniques. In particular, 4-hydroxynonenal (HNE) formation has been widely used. Antibodies against the protein adducts that HNE forms are widely available and frequently used in immunostaining of tissues, but it should be realised that different antibodies can detect different epitopes and so give different answers, depending on how the HNE binds to proteins[58-61].

One minor end-product of lipid peroxidation is malondialdehyde (MDA)[58], which can also be a useful biomarker if measured by MS techniques. However, the widely used "MDA assays" using thiobarbituric acid reactive substances (TBARS) are unspecific since TBA generates chromogens from many biomolecules[1,62]. Use of HPLC to separate the TBA-MDA adduct increases specificity, but does not eliminate all problems[1].

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 to evaluate oxidative lipid damage because of their low specificity that can result in false-positive results.*

The detection of lipid oxidation products has been revolutionised by the development of LC-MS for the detailed analysis of oxidized lipid mixtures[63]. Collection and storage of samples to avoid artefactual peroxidation are key and samples should be immediately frozen in liquid nitrogen. Biofluids may require addition of chemicals (e.g. butylated hydroxytoluene, BHT) to prevent autooxidation or metabolism before storage[1,64]. The internal standards used for quantification should be added to the samples prior to 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 assessments of general lipid peroxidation and identification of individual products, including those with specific signalling functions. However, limitations of available standards may complicate accurate quantitative analysis.

Most prominent among the lipid oxidation products that have been quantified by MS-based approaches are the F_2-Isoprostanes (F_2-IsoPs)[65]. Sixty-four F_2-IsoP stereoisomers can be generated from the free radical catalysed, non-enzymatic oxidation of arachidonic acid and can be separated from those that arise from the enzymatic oxidation of arachidonic acid by the cyclooxygenase enzymes (COX-1/-2) F_3- and F_4- isoprostanes can arise from EPA and DHA respectively, but have been less well characterised than the F_2-isoprostanes. ELISA methods have been developed to quantify one F_2-IsoP isomer, 8-iso-PGF$_{2\alpha}$ (also referred to as 15-F_{2t}-IsoP or iPF$_{2\alpha}$ -III), and compared with GC/MS and LC/MS/MS methods[65-69]. In all these studies, there was poor agreement between commercially available ELISA kits and MS methods. 8-iso-PGF$_{2\alpha}$ is one of 64 different F_2-IsoP isomers generated during arachidonic acid peroxidation, and antibody cross-reactivity between 8-iso-PGF$_{2\alpha}$ and related isomers is challenging. Pre-analysis sample clean up may allow for more precise measurement of 8-iso-PGF$_{2\alpha}$ by ELISA[69,70], but by far the most accurate method to quantify F_2-IsoPs is by LC-MS/MS and is very strongly recommended.

Recommendation 12: *F_2-IsoPs are a generally-accepted biomarker of lipid peroxidation but it should be realised that they are one of many end-products and the levels of various types can be affected by experimental conditions. Quantification using ELISA is susceptible to artifact* but sample clean up may allow measurement of 8-iso-PGF$_{2\alpha}$ by ELISA[70]. *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[71,72]. Detailed protocols for measuring multiple

products can be found in[74]. 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[72,74]. Many assays involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) to form a dinitrophenylhydrazone (DNP). This product can be detected spectrophotometrically, although this approach can suffer from a 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 the DNP products by ELISA or immunoblotting[75]. Global changes in oxidized proteins can be measured in tissue homogenates treated with fluorescein-5-thiosemicarbazide (FTC) to generate fluorophore-labelled proteins, that can be separated by gel electrophoresis[76]. Enrichment methods using biotin-tagged derivatization coupled with LC-MS detection have been developed[77]. Protein carbonyls, α-aminoadipic semialdehyde (AASA) and glutamic semialdehyde (GSA), have also been assayed individually by stable isotopic dilution analysis LC-MS/MS[74]. Of course, data from a single time point reflect the difference between the rates of formation and removal (e.g., by repair or proteolysis) of these products.

Protein analysis using MS allows detection and identification of modifications with characteristic mass increases (e.g., hydroxylation, nitration, chlorination)[72,73]. This has been particularly useful in studies of oxidative damage to brain proteins in dementia patients by "redox proteomics"[78]. 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 use of isotope-labelled standards) together with the parent species, allowing a 'mass balance' to be determined[72-74,79]. Care must be taken in sample handling to prevent artefactual oxidation of Cys or Met, and also during protein hydrolysis as some products 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[80] and nitrated species arising from the action of myeloperoxidase in the presence of NO_2^- and/or by reactions of $ONOO^-$/ONOOH[48,81]). 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 possible artefacts arising from sample handling and preparation. However, do bear in mind that plasma and urinary levels of oxidized amino acids may have contributions from absorption of oxidized amino acids from proteins in food and increased tissue proteolysis as a result of pathology.

Cysteine is a major target for modification due to its ease of oxidation (particularly in its thiolate form, RS^-) and its nucleophilicity which results in ready adduct formation with electrophiles. Oxidation can be irreversible, for example to a sulphinic (RSO_2H) or sulphonic acid (RSO_3H) which can be useful biomarkers of oxidative protein damage[4,82]. Reversible oxidation of Cys residues in proteins is a prominent mechanism of redox signalling[2,4]. Reversible products include disulphides, sulphenic acids, S-nitrosothiol and persulphide species[2,4,82-84]. These modifications can be reversed by the glutathione/glutaredoxin or thioredoxin systems[84]. A common approach to detect the pool of reversibly modified Cys residues is to first block reduced thiols with a reactive reagent and then reduce and derivatise the previously oxidised residues with a tag that can be identified by LC-MS of tryptic digests[85]. These approaches can be extended to use modification-specific chemistry to tag only a particular oxidation product, such as an S-nitrosothiol, sulphenic acid or persulphide[85]. Until recently, significant limitations to these approaches were the low coverage of the total Cys pool and the lack of quantitation of the modification at individual residues[84-86]. The latter is of particular importance when interpreting the biological significance 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 oxidised for each particular Cys. These methods have been extended to tags that incorporate moieties such as biotin that enable enrichment of the labelled peptides, greatly enhancing Cys coverage. The most recent iteration of this approach, as exemplified by the OxiMOUSe study[86] has superseded previous methods.

Methionine, another sulphur-containing amino acid is also a major site of redox post-translational modifications. Oxidation to methionine sulphoxide can be reversed enzymatically by methionine sulphoxide reductase enzymes, potentially allowing redox signalling by the installation / removal of a single oxygen atom[17]. Reagents have been developed for methionine bioconjugation that can identify and characterize redox-sensitive methionine sites in

proteomes[87]. ***Recommendation 13:*** *ELISA, FTC and immunoblotting are useful tools to detect protein carbonyls as a biomarker of general oxidative protein damage, although it must be realised that not all protein oxidation products contain carbonyls. LC-MS approaches, using carefully prepared samples, are the best available techniques to assess protein oxidation, due to the sensitivity, selectivity and quantitation available from 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[1,13,88]. One method to assess "general" oxidative damage to DNA 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 site 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[89].

Oxidative damage to DNA usually focusses 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 they are likely to be biologically important[1,13]. 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[90] have established protocols to avoid this and 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 the rate of repair. The best methodology to use is ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS)[91]. 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 8OHG and 8OHdG. However, immunohistochemistry can be useful to identify cells that have higher amounts of 8-OHdG *in vivo*, if applied appropriately[92].

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 which are "sanitized" by removal of oxidised products[91]. The relative contributions of

DNA repair and nucleotide pool sanitization to the levels of oxidised nucleosides detected in body fluids are currently unclear. Urine collected over 24 hours will represent the number of guanines in DNA/RNA and/or the respective nucleotide precursor pools that are oxidized during that period[93]. Urine sampling represents formation in the entire body, and is best suited to situations where all tissues are assumed to be affected, but it could be inadequate to detect changes that only occur in some 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 like the comet assay (using DNA repair enzymes) on isolated cells and UPLC-tandem mass spectrometry for 8OHdG and 8OHG determination 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 (e.g. carbonyls, 3-nitro- and 3-chlorotyrosine), DNA (e.g. 8-oxodG) and lipids (F_2-Isoprostanes). These 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 (e.g. as for HNE)[59-61], 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 is difficult, due to poor epitope accessibility (e.g. 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. Competitive data with authentic epitopes should be included whenever possible.*

Measuring reactive oxygen species and oxidative damage *in vivo*

Measuring 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, that upon oxidation, forms luciferin *in situ* that is oxidized in luciferase-transfected systems to generate bioluminescence[94]. 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 (PET) is now being used to image ROS *in vivo*[95] but is still in its infancy. In mitochondria of cells and tissues, changes in H_2O_2 can be assessed using the mitochondria-targeted boronate MitoB, which accumulates in these organelles and is converted by H_2O_2 into MitoP. The ratio of MitoP to MitoB can then be determined by mass spectrometry[96].

Measuring reactive oxygen species and oxidative damage in clinical trials

As oxidative damage plays a central role in many human pathologies, there is considerable interest in developing therapeutic interventions to decrease this damage[1-3]. 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. They generally failed to influence disease activity. Unfortunately, in most cases the effect of the intervention on oxidative damage was not measured, making it uncertain if the putative therapy was actually effective at decreasing oxidative damage: if it wasn't, lack of effect is predictable[53].

To address this, it is essential to assess the impact of these intervention on levels of oxidative damage in the patients in the clinical trials. Currently, methods are limited to measuring end points of oxidative damage in biopsies (e.g. skin, 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[97] and F_2-isoprostanes as a biomarker of lipid peroxidation[65,98]. So far, 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[72-74].

More generally, clinical trials should include internationally validated biomarkers; the biomarker should ideally have undergone interlaboratory comparison. Many biomarkers rely on concentration measurement in body fluids such as plasma but these only reflect the balance between the formation and elimination rates and therefore cannot readily be interpreted as "oxidative stress", but models have been developed to estimate the 24-hour production of an oxidized product[97]. Ideally a panel of biomarkers should be used[52,53] 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 decide if 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 1), TBARS, determinations of total antioxidant activity[1,99] 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 in order to investigate their biological significance. 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 **Table 3**), will remain valid.

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Author contributions

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Box 1: Definitions

Reactive oxygen species (ROS) is a collective term for species derived from O_2 that are more reactive than O_2 itself. The term includes not only the superoxide radical anion ($O_2^{\bullet-}$) and some other oxygen radicals, but also some non-radical derivatives of O_2, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxynitrite/peroxynitrous acid ($ONOO^-$/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; $O_2^{\bullet-}$ and H_2O_2 are selective in their reactions with biological molecules, leaving most of them unscathed, whereas $^{\bullet}OH$ 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**[1]. 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 (courtesy of Prof. Christine Winterbourn) 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 decreased repair or removal processes, e.g. failure to clear oxidised proteins or repair oxidised 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[52].

Table 1. Common ROS encountered in biological systems.

ROS	Chemical formula	Reactivity Lifetime
Superoxide radical anion	$O_2^{\bullet -}$	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 fast with nitric oxide ($k_2 > 10^9 M^{-1}s^{-1}$) to yield peroxynitrite. $O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^-$ and with other radicals to form hydroperoxides $O_2^{\bullet -} + R^{\bullet} + H^+ \rightarrow ROOH$ Can damage some enzymes that contain [Fe-S] clusters.
Hydrogen peroxide	H_2O_2	Unreactive with most biomolecules Reacts slowly with most thiols e.g. $k \sim 1 M^{-1}s^{-1}$ for GSH), but more rapidly with selected Cys residues, particularly those with a low pK_a Reacts with some transition metal ions to give $^{\bullet}OH$ (rate constants $10^2 – 10^7 M^{-1}s^{-1}$ depending on the metal and the 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^-).
Hydroxyl radical	$^{\bullet}OH$	Indiscriminately reactive. Reacts with whatever is adjacent to it at near diffusion controlled rates.
Peroxynitrite (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 ($ONOOCO_2^-$). $ONOO^-$ itself can oxidise a few biomolecules or can protonate to peroxynitrous acid, which 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 ($CO_2 + NO_3^-$)
Carbonate radical anion	$CO_3^{\bullet-}$	Formed from reaction of CO_2 with peroxynitrite, (see above) also from reaction of HCO_3^- with $^{\bullet}OH$. Fairly reactive, oxidises guanine in DNA, cysteine, tyrosine and tryptophan.
Hypohalous acids (hypochlorous, hypobromous acids)	HOCl HOBr	Strong oxidants, major reactions with thiols/methionine. Reactions with amines generate secondary chloramines/bromamines which retain less (but still considerable) oxidising 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.
Singlet oxygen	$^1O_2$	Two singlet states of O_2 exist though 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 peroxyl radicals and HOCl, amongst others.
Nitrogen dioxide radical	$\bullet NO_2$	A major atmospheric pollutant. Also generated from peroxynitrite decomposition (see above) and oxidation of nitrite (NO_2^-) by peroxidase enzymes Rapidly oxidises electron rich compounds (e.g. ascorbate and thiols). Undergoes addition reactions with radical s derived from tyrosine, tryptophan, lipids and DNA bases (e.g. guanine) to give nitrated products (e.g. 3-nitrotyrosine, nitrotryptophans, nitrolipids and nitrated DNA bases). Some nitrated products have signalling functions.

Table 2. Some recommended approaches to detect and quantify ROS in different biological contexts. Abbreviations used: TT, test tube; cells, C; isolated organs, O; animals, A; humans, H; biological fluids, BF. References to methods are contained in the main text, except where indicated.

Oxidant	Approach	Method	Context	Detection (D) / quantification (Q)	C d o
Superoxide radical anion ($O_2^{\bullet -}$)	Fe-release from aconitase	Aconitase enzymatic activity measurements	TT, C, O, A	D, Q	I
	Cytochrome c reduction	Optical spectroscopy	TT, C	D, Q	I s
	Dihydroethidine oxidation to 2-hydroxyethidium	Liquid chromatography separation, detection by fluorescence or mass spectrometry	TT, C, O	D, Q	V h I
	Spin trapping	ESR/EPR	TT, BF, C, A	D	F S
Hydrogen peroxide (H_2O_2)	Formation of Compound 1 from catalase	Optical difference spectroscopy at 600 nm	TT, C, O, BF	D,Q	T n n
	Oxidation of boronate probes (e.g. PO1, MitoPY1, Boronate-Caged Luciferin))	Fluorescence/luminescence detection	TT, C, BF, O, A	D,Q	I H o i C t
	Genetically encoded thiol-based probes (e.g. HyPer7, roGFP2-Orp1, roGFP2-Tsa2)	Fluorescence detection	TT, C, O, A	D,Q	U i r b
	Peroxidase-catalyzed oxidation of Amplex Red	Optical spectroscopy	BF, C (H_2O_2 release only)	D	N n I e p

Peroxynitrite /peroxynitrous acid ($ONOO^{-}$ / ONOOH) Nitrogen dioxide radical ($NO_2^{\bullet}$)	Nitrated products from endogenous targets (e.g. 3-nitroTyr from Tyr, 6-nitroTrp from Trp, 8-nitroguanosine from guanosine, nitrated lipids) or added exogenous probes (e.g. boronates)	Liquid chromatography - mass spectrometry	TT, C, BF, O, A, H	D,Q	A Q s f N
		Antibodies (in ELISA, immunoblot, immunocytochemistry)	TT, C, BF, O, A, H	D	N v
Carbonate radical anion ($CO_3^{\bullet-}$)	Direct	EPR/ESR spectroscopy	TT, BF	D,Q	L
Hypochlorous acid (HOCl)	Chlorinated products from endogenous targets (e.g. 3-chloroTyr from Tyr, chlorinated lipids) or added probes (e.g. chlorinated ethidium from dihydroethidium or hypocrates)	Liquid chromatography - mass spectrometry	TT, C, BF, O, A, H	D,Q	C Q s
Singlet oxygen ($^1O_2$)	Direct probe oxidation (e.g. Singlet Oxygen Sensor Green) or chemical addition of oxygen to probe molecule (e.g. anthracenes)	Weak phosphorescence at ~1270 nm detected by near-infrared spectrofluorimetry	TT, BF, C	D,Q	S b a w
		Fluorescence or liquid chromatography-mass spectrometry	TT, BF	D,Q	

Table 3. Summary of recommendations

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, lifetime, products generated and fate in vivo. If this is not possible, caveats about use of the term "ROS" should be discussed.

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 recognised that low molecular mass "antioxidants" are unlikely to act by scavenging H_2O_2. 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.

Recommendation 3: We recommend the use of paraquat, 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 (please see[22]) or deletion or knockdown of NOX components should be used to identify their roles.

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.*

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, engage techniques using different principles of detection.

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.

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 was able to irreversibly capture the reactive species when the cells/tissues/organs were under biologically relevant conditions.

Recommendation 8: 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.

Recommendation 9: Only use hydroethidine or MitoSOX probes to detect $O_2^{\bullet -}$ by simple fluorescence measurements when the product has been independently validated as 2-hydroxyethidium. Fluorescence measurements with probes such as dihydroethidium and MitoSOX[33] should be conducted using the lowest probe concentration possible, and must include controls for changes in the 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 the modified species[31], should be performed when possible.

***Recommendation 10*:** Genetically encoded fluorescent probes (some of which are commercially available) are currently the most sensitive detectors of H_2O_2 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_2O_2 are required and sensitivity is limited for physiological H_2O_2 levels. Amplex Red with HRP can measure H_2O_2 release from cells if other reducing agents or peroxidase substrates are absent.

***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 to evaluate oxidative lipid damage because of their low specificity that can result in false-positive results.

Recommendation 12**:** F_2-IsoPs are a generally-accepted biomarker of lipid peroxidation but it should be realised that they are one of many end-products and the levels of various types can be affected by experimental conditions. Quantification using ELISA is susceptible to artifact but sample clean up may allow measurement of 8-iso-PGF$_2\alpha$ by ELISA[70]. LC-MS/MS with appropriate internal standards is the preferred approach.

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

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 like the comet assay (using DNA repair enzymes) on isolated cells and UPLC-tandem mass spectrometry for 8OHdG and 8OHG determination are presently the best available. ELISA-based methods, especially in kit form, are usually insufficiently validated, and their use is not recommended.

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. Competitive data with authentic epitopes should be included whenever possible.

Recommendation 16: If intervening with antioxidants, first use biomarkers in preliminary dose-ranging studies to decide if 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 1), TBARS, determinations of total antioxidant activity[1,99] or kit-based methods where the methodology behind the kit is not clear and/or has not been validated