Cytomics of Oxidative Stress: Probes and Problems

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Abstract Oxidative stress has been implicated in cellular senescence and aging, as well as in the onset and progression of many diverse genetic and acquired diseases and conditions. However, reactive oxygen (ROS) and nitrogen (RNS) species initiating oxidative stress also serve important regulatory roles, mediated by intercellular and intracellular signaling, adaptation to endogenous and exogenous stress, and destruction of invading pathogens. Fluorescence-based analysis of oxidative stress and related processes is an important cytomic application; almost 4000 papers were published between 1989 and 2016. To ascertain the specific role of ROS and RNS in oxidative stress studies by cytomic methodologies, it is essential to detect and characterize these species accurately. Unfortunately, the detection and quantitation of individual intracellular ROS and RNS remains a challenge, but different, complementary cytometric strategies directed toward other endpoints of oxidative stress may also be considered. In this chapter we present and briefly discuss the limitations and perspectives of such approaches.

Keywords Cytomics · Fluorescence · Flow cytometry · Image cytometry · Reactive oxygen species · Reactive nitrogen species · Antioxidants

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J.P. Robinson and A. Cossarizza (eds.), *Single Cell Analysis*, Series in BioEngineering, DOI 10.1007/978-981-10-4499-1_4

1 Introduction to Reactive Oxygen (ROS) and Nitrogen (RNS) Species

Life on Earth has evolved by creating organisms that need oxygen to live. Most living beings depend on oxygen to obtain large amounts of metabolic energy from the oxidation of biomolecules [1, 2]. Paradoxically, the oxygen functions essential to living things depend on a chemical property dangerous to them: the structure of the oxygen molecule (O_2) has two unpaired electrons, and O_2 can accept individual electrons to generate highly unstable and highly reactive molecular forms known as reactive oxygen species (ROS). The term ROS may be applied to a variety of molecules not derived from O_2 alone and includes both free radicals and species derived from free radicals [3, 4].

Main ROS include singlet-oxygen radical, superoxide anion radical, hydrogen peroxide (H_2O_2), hydroxyl radical, hypochlorous acid (HOCl), lipid peroxides (ROOH), and ozone (O_3) [3, 4]. There are also free radicals and reactive nitrogen-containing molecules, the reactive nitrogen species (RNS), including nitric oxide (NO) and peroxynitrite [5, 6]. Because they also contain oxygen and their generation is connected to ROS generation, they are often considered as ROS. Thus, ROS and RNS are not single entities but represent instead a broad range of chemically distinct reactive species with diverse biological reactivities.

The generation of ROS and RNS has been implicated in cellular senescence and aging [7–9], as well as in the onset and progression of genetic [10, 11] and acquired diseases and conditions, including, but not limited to, inflammatory conditions [12–14], cardiovascular diseases [15–18] and thrombosis [19], cancer [20–23] and anticancer chemotherapy [24], HIV progression [25, 26], neurodegenerative diseases [27–29], and metabolic disorders [30]. However, ROS and RNS also serve important regulatory roles, mediated by intercellular and intracellular signaling [31–34], and cell function–modifying processes involved both in the destruction of invading pathogens [35] and in the fine tuning of cellular adaptation to endogenous and exogenous stress [36–38]. Phagocytes use ROS and NOS as a powerful antimicrobial weapon (oxidative burst), and in low concentrations, ROS and NOS serve also as second messengers of signal transduction.

2 The Physiological Side of ROS and NOS

2.1 Sources of ROS and RNS

The generation of ROS and RNS can be endogenous, associated with oxidative processes (such as the mitochondrial chain of electronic transport, NADPH oxidase, xanthine oxidase and various flavoproteins) [39] or exogenous, derived from inflammatory pathologies, exposure to xenobiotics, ionizing radiation, etc. [40]

Most ROS and RNS arise physiologically in specific subcellular compartments. The intracellular location of ROS and RNS is of great importance, as microenvironment will affect both the intrinsic functions of the reactive species and the population of molecules and structures that will be eventually affected by their interaction with ROS and RNS. In higher organisms, the major generation of ROS takes place in the mitochondria, during the tetravalent reduction of O_2 occurring in the electron-transport chain associated with oxidative phosphorylation. In prokaryotic cells, this mechanism takes place on the plasma membrane. This process, directed to the production of ATP, gives rise to H_2O as the final product, via a sequence of univalent reductions that generate ROS [5]. Other organelles with localized ROS generation include the phagosomes, where ROS and RNS are focused on pathogen killing, and the peroxisomes, where many catabolic oxidation reactions are confined [39, 40].

Nitric oxide (NO) is synthesized from L-arginine in a reaction catalyzed by NO synthase [32, 33]. NO reacts readily with superoxide to form the peroxynitrite anion, a RNS with strong oxidant properties [34]; activated macrophages and neutrophils produce NO and superoxide, and thus peroxynitrite, at similar rates.

2.2 ROS in Phagocytosis

The stimulated production of ROS and RNS by phagocytic cells is known as the respiratory burst, because of the increased consumption of O_2 by these cells during phagocytosis necessary for the bactericidal action of phagocytes [41]. This process is initiated by NADPH oxidase, a multicomponent membrane-bound enzyme complex that generates superoxide anion radicals, which in turn give rise to further ROS. Similarly, activated nitric oxide synthase 2 catalyzes the production of nitric oxide radicals, which leads to the formation of reactive nitrogen intermediates. ROS and RNS can interact to form further reactive species. While each of these antimicrobial systems operates independently, they are synergistic in destroying invading pathogens [42].

2.3 ROS and RNS in Cellular Signaling

Most cytoplasmic proteins contain free SH groups, which may undergo oxidation/reduction cycles. In coordination with antioxidant proteins and molecules, ROS may turn functional proteins on and off by redox cycling [43]. A large number of such proteins are involved in signal transduction or in the regulation of gene expression in eukaryotes and prokaryotes [44].

ROS signaling is involved in cell survival and adaptation to stress. Signaling through mitogen-activated protein kinases (MAPKs) leads to the generation of H_2O_2 from several enzymes, including NADPH oxidases [45]; production of H_2O_2 at nanomolar levels is required for proliferation in response to growth factors [46]. In synchronized cells ROS increase along the cell cycle, peaking at the G2/M phase [47]; it has been suggested that small increases of H_2O_2 result in increased reentry into the cell cycle, while sustained high levels of H_2O_2 lead to cell arrest and apoptosis [36]. Apoptosis induced by prolonged activation of c-Jun N-terminal kinase (JNK) has been shown to be caused by exposure to ROS [48, 49]. Conversely, autophagy is also triggered as an adaptive response, among other stressors, to intracellular ROS [38].

ROS have also recently been related to signaling in platelets [50]. ROS produced after platelet stimulation with collagen are responsible for a series of platelet-activating events owing to oxidative inactivation of SHP-2, which promotes tyrosine phosphorylation–based signal transduction.

NO plays a critical role as a molecular mediator of a variety of physiological processes, including blood-pressure regulation and neurotransmission [32, 33]. NO that diffuses into smooth muscle cells binds to the heme group of guanylate cyclase. Peroxynitrite, a RNS considered as an inflammatory mediator in various cardiovascular pathologies, has more recently been recognized as a modulator of signal-transduction pathways owing to its ability to nitrate tyrosine residues, thereby influencing responses dependent on tyrosine phosphorylation [34].

3 Oxidative Stress: Definition, Causes, and Consequences

3.1 Definition of Oxidative Stress

Despite the powerful and complex antioxidant machinery of higher organisms, when the capacity of these protective mechanisms is overcome by the intensity or duration of oxidative processes, a situation occurs called oxidative stress, which is defined as an alteration in the equilibrium between ROS production and antioxidant defenses, producing oxidative damage [39, 40].

Oxidative stress can result from two separate but not exclusive processes. On the one hand is the decrease in the levels or the activity of enzymes of the antioxidant defense by mutation or destruction of the active center, induced by the ROS themselves [40]. Deficiencies in the dietary supply of soluble antioxidants can also cause oxidative stress. On the other hand, increased production of ROS, exposure of cells or organisms to elevated levels of exogenous ROS or their metabolic precursors, and even excessive induction of protective (immunological, detoxifying) systems that produce ROS can lead to the situation of oxidative stress [5].

3.2 Causes of Oxidative Stress

To prevent the harmful effects of the in vivo production of ROS and RNS, evolution has provided prokaryotes and higher organisms with complex and effective antioxidant systems that include enzymatic antioxidant mechanisms and antioxidant molecules, broadly understood as those molecules that protect a biological target against oxidative stress [5, 39, 40, 51].

The first line of antioxidant enzymes is the superoxide dismutase family of enzymes (SOD) that catalyze the dismutation of superoxide to H_2O_2. Catalase converts H_2O_2 to water and O_2, and thus completes the detoxification initiated by SOD. Glutathione (GSH) peroxidase includes a group of Se-containing enzymes that also catalyze the decomposition of H_2O_2, as well as of organic peroxides. In the GSH peroxidase process, GSH is consumed by oxidation, so that GSH reductase is required to transform oxidized GSH (GSSG) into GSH [40].

The non-enzymatic antioxidants are a large group of molecules that exert various protective antioxidant mechanisms, and include molecules that react with ROS, such as GSH, tocopherol and β-carotene, or proteins such as transferrin and ceruloplasmin, capable of chelating transition metals. GSH is the most important intracellular defense against the toxic effects of ROS. Vitamin C or ascorbic acid is a water-soluble molecule capable of reducing ROS, while vitamin E (α-tocopherol) is a lipid-soluble molecule that has been suggested as playing a similar role in membranes [40].

3.3 Consequences of Oxidative Stress

Oxidative damage to cells and tissues produced by ROS is associated with free-radical chain reactions with all kinds of biomolecules, such as carbohydrates, lipids, proteins, and DNA.

3.3.1 Protein Damage

Protein oxidation plays an important role in many of the effects of oxidative and nitrosative stress. The modifications produced may be irreversible, such as carbonylation of lysine (Lys) and arginine (Arg), formation of di-tyrosine bonds, protein-protein bonds, and nitration of Tyr and tryptophan [52]. These changes generally result in loss of permanent function of damaged proteins. In complex enzymes, free-radical interaction can also be damaging at the level of the prosthetic group, leading to functional inactivation [53].

3.3.2 Lipid Peroxidation

Lipid peroxidation is a process that occurs in three phases: initiation, propagation, and termination. The initiation phase involves the reaction of free radicals with cellular lipids, generating peroxyl radicals. In the propagation phase, the reaction of these newly formed peroxyl radicals with intact lipid triggers a chain reaction that may be terminated by the action of antioxidants. It is a phenomenon detrimental to the cell, since changes in the physico-chemical properties of the membrane, as its fluidity, as well as the inactivity of transporters and membrane enzymes can occur [53, 54].

3.3.3 Oxidative Lesions to DNA

More than 100 different free radical–induced DNA modifications have been described, either in nitrogenated bases or in deoxyribose. The hydroxyl ion seems to be the main cause of these lesions, an effect that is facilitated by the polyanionic character of the phosphodiester bond, since it is attracted by metals such as Fe^{2+}, favoring the Fenton reaction. NO and its derivatives have the capacity to induce lesions in the DNA, mainly through the deamination of bases, although other processes are involved [55].

Modifications produced by ROS in the bases may be mutagenic, leading to incorrect pairing of the bases, or cytotoxic if an arrest of replication occurs. Of special relevance are oxidation reactions with purines that generate different products with high mutagenic capacity, such as 8-oxo-7, 8-dihydroguanine (8oxoG), widely used as an important marker of oxidative DNA damage [56, 57]. Cross-linking reactions between nucleotides of the same chain may be cytotoxic or mutagenic, the best known case being the formation of pyrimidine dimers.

4 Strategies and Reagents for Cytomic Analysis of Oxidative Stress

Given the participation of ROS and RNS in physiological and pathological issues, active search for biomarkers of oxidative stress has become relevant to many biomedical fields [58], and many different methods are applied to assess the redox state of the body or of specific tissues and cells [59–65]. Fluorescence-based analysis of oxidative stress and related processes is an extended application of flow cytometry [66, 67]; more than 3700 papers on this topic have been published between 1989 and 2016, according to PubMed Central (Fig. 1). Imaging approaches, including confocal microscopy [68], high-content analysis by automated microscopy [69], and the more recent imaging flow cytometry [70, 71] allow one in addition to visualize and quantify topographical issues of intracellular ROS production and action (Fig. 2).

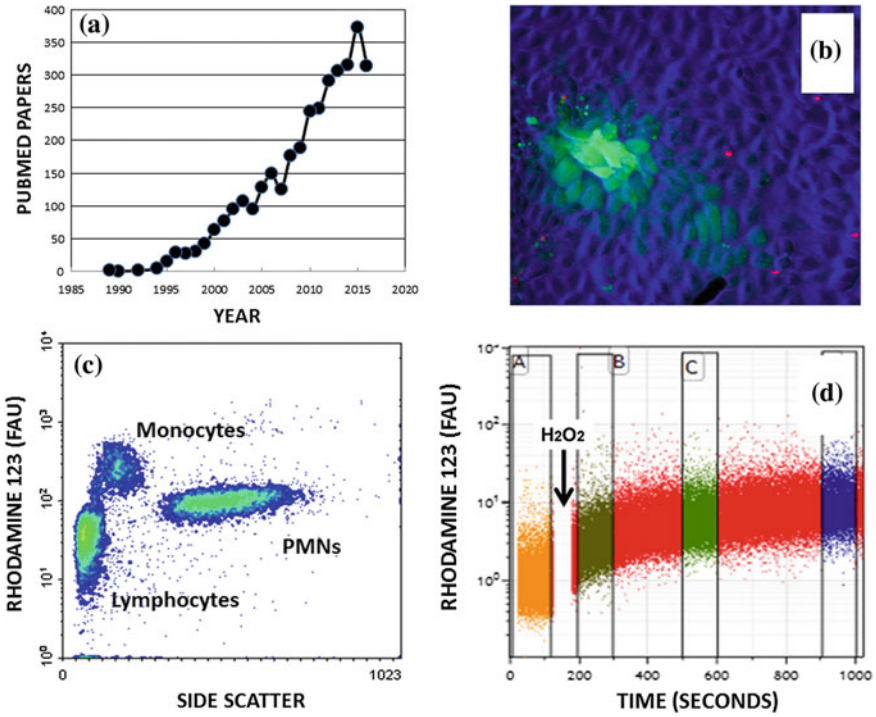

Fig. 1 Examples of relevant cytometric techniques for the study of ROS, RNS and oxidative stress. **a** PubMed-indexed papers containing the general terms "Cytometry" AND "oxidative stress" published between 1985 and 2016. Panels b-d show three complementary cytometric applications having in common the same fluorogenic substrate, dihydrorhodamine 123 (DHR123), which detects mainly H_2O_2 and peroxynitrite, as explained in the paper. **b** Example of analysis by fluorescence microscopy of ROS generation. A confluent monolayer of MDCK cells was stained with DHRH123 and treated with 25 μM $CdCl_2$ for 1 h, before being photographed with a standard fluorescence microscope. General experimental conditions are similar to those described in [181]. **c** Example of a whole-blood assay by flow cytometry of ROS/RNS generation by resting leukocytes. A sample of whole blood in heparin from a healthy volunteer was stained with CD45-PC5 antibody, to exclude erythrocytes from the analysis, and with DHRH123. Leukocyte populations are distinguished by their side-scatter properties. The fluorescence intensity of rhodamine 123, the oxidation product of DHR123, shows that monocytes at rest generate more ROS/RNS than do neutrophils and lymphocytes. General experimental details can be found in [111]. **d** Example of a whole-blood assay by real-time cytometry of ROS/RNS generation by resting leukocytes. Experimental conditions were similar to those in panel c, but data acquisition was started before H_2O_2 was added to the sample stained with CD45, CD14, and DHRH123, already running in the flow cytometer. The generation of ROS/RNS in monocytes was followed by means of a kinetic plot of rhodamine 123 fluorescence intensity versus time. Analytical regions allow quantification of the rate of fluorescence variation. FAU: Fluorescence Arbitrary Units

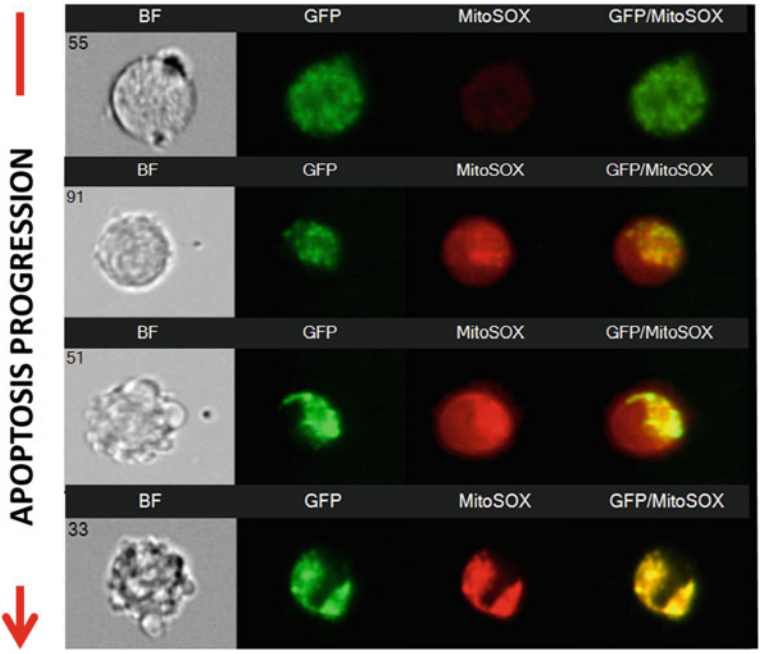

Fig. 2 Imaging flow cytometry applied to the analysis of ROS generation and oxidative stress in vitro. Colocalization by imaging flow cytometry of mitochondria and the superoxide radical–sensitive probe MitoSox Red dye during apoptosis. The human liposarcoma SW872/GFP cell line obtained by transfection with a retroviral vector expresses the GFP-tagged mitochondrial LON protease [182]. Apoptosis was induced by treatment with camptothecin and at appropriate times cells were trypsinized and stained with the mitochondrial superoxide sensor MitoSox Red dye. Cell suspensions were analyzed in an Amnis ImageStream flow cytometer that collects multispectral images of single cells at high speed. In each row of images, channels show from left to right, bright field illumination, expression of GFP-Lon protease (i.e., mitochondrial compartment), MitoSox Red dye fluorescence (i.e., generation of superoxide radical), and the merged image wherefrom colocalization may be observed and quantified. The series of images from top to bottom show that the morphological progression of apoptosis (brightfield channel) is accompanied by generation of superoxide that is associated with the mitochondrial compartment

However, to ascertain the specific role of ROS and RNS in oxidative stress studies by cytomic methodologies, it is essential to detect and characterize these species accurately. Unfortunately, the detection and quantitation of individual intracellular ROS and RNS remains a challenge [60–65], but different, complementary cytometric strategies aimed to other endpoints of oxidative stress may also be considered. In this chapter we present and discuss briefly the limitations and perspectives of such approaches.

4.1 Cytomic Strategies in the Analysis of Oxidative Stress

The complex processes involved in the generation of ROS and RNS, their control by the antioxidant system, and the physiological or pathological consequences of their action may be approached by cytomic analysis at different levels or stages, using complementary methodologies based upon fluorescence, in multiple cell types and clinical situations or experimental models. Thus, the most common cytomic strategies to the study of oxidative stress include:

(a) Direct detection of ROS and RNS, the initiators of the oxidative stress process. This task is complex owing to the low concentration, short half-life, and extensive interactions of ROS and RNS, as well as by intrinsic limitations of both probes and experimental conditions.
(b) Detection of more stable products of ROS and RNS reaction with cell components or with exogenous probes, including the analysis of lipid peroxidation and oxidative damage to DNA.
(c) Assessment of antioxidant defenses, mostly GSH and SH-containing proteins. This indirect approach to oxidative stress may be limited by issues related to the complexity of the antioxidant defense by itself and to the specificity of enzymes required for fluorescent reporting of the process.

4.2 Detection of ROS and RNS Using Fluorogenic Substrates

The use of fluorescent probes and fluorogenic substrates (Fig. 3) appears a simple and easy approach for the detection and quantification of ROS production in cellular systems. However, there are many limitations and artifacts in this methodology. In this section we mention the principal fluorescent probes and fluorogenic substrates used in cytometric analysis of ROS and RNS. Their main limitations and potential sources of artifacts will be considered further along in this chapter.

4.2.1 2',7'-Dichlorodihydrofluorescein Diacetate (H_2DCF-DA) and Related Probes

The cell-permeant H_2DCF-DA is one of the most commonly fluorogenic substrates used in studies related to ROS and RNS generation [62–64]. Upon cleavage of the acetate groups by intracellular esterases, the intracellular oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) produces 2′,7′-dichlorofluorescein (DCF), a fluorescent compound (λ excitation = 498 nm; λ emission = 522 nm). Initially, H_2DCF was widely accepted as a specific indicator for H_2O_2 [72] but as discussed later in this chapter, H_2DCF is oxidized by other ROS, such as hydroxyl and

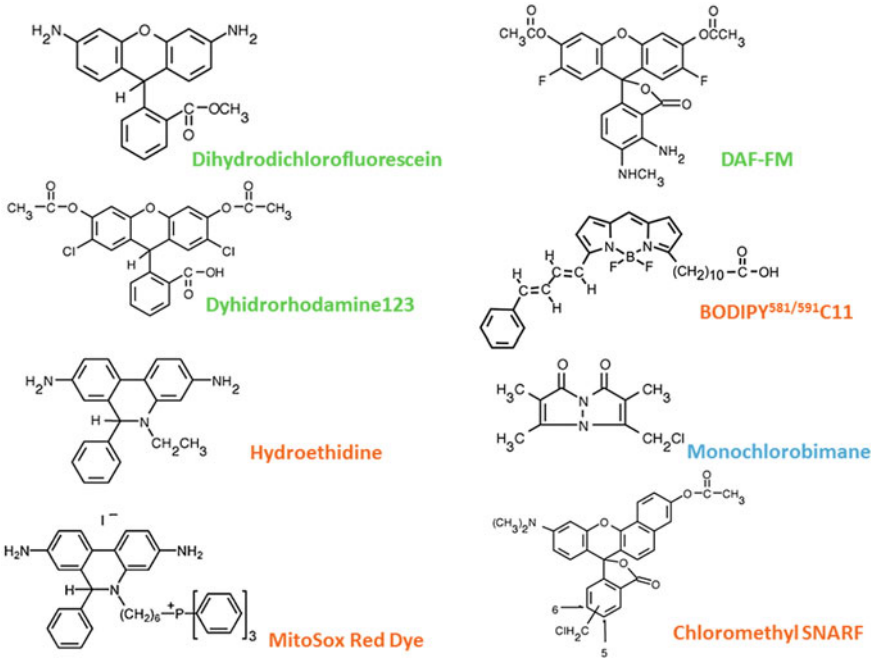

Fig. 3 Some examples of fluorescent probes and fluorogenic substrates frequently used in the cytometric analysis of ROS, RNS and oxidative stress. The properties, applications, and limitations of these reagents are described in the corresponding sections of the chapter. The letter colors of reagent names indicate the spectral region of their fluorescence emission, according to [80]

peroxyl radicals, and also by RNS like peroxynitrite [62–64]. On the other hand, it seems well established that H_2DCF is not oxidized by superoxide anion, hypochlorous acid, or NO [62]. With these caveats, H_2DCF has been successfully used for studies of oxidative burst in phagocytes [73, 74] and to follow the generation of prooxidants in many cell models [75–79].

Intracellular oxidation of H_2DCF tends to be accompanied by leakage of the product, DCF. To enhance retention of the fluorescent product, several analogs with improved retention have been designed, such as carboxylated H_2DCFDA (carboxy-H_2DCFDA), which has two negative charges at physiological pH, and its di-(acetoxymethyl ester) [80]. The halogenated derivatives 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), and 5-(and 6-) carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) exhibit much better retention in live cells; they have been used for following oxidative burst in inflammatory and infectious processes and have been applied to different experimental settings related to oxidative stress [80].

4.2.2 Dihydrorhodamine 123 (DHR123)

DHR123 is a non-fluorescent molecule that upon oxidation generates rhodamine 123, a fluorescent cationic and lipophilic probe (λ excitation = 505 nm; λ emission = 529 nm) [62, 80]. The lipophilicity of DHR123 facilitates its diffusion across cell membranes. Upon oxidation of DHR123 to the fluorescent rhodamine 123, one of the two equivalent amino groups tautomerizes into an imino group, effectively trapping rhodamine 123 within cells [81]. Like H_2DCF, DHR123 is oxidized by H_2O_2 in the presence of peroxidases, but this probe has low specificity, since it can also be oxidized by other reactive oxidants, namely peroxynitrite, Fe^{2+}, Fe^{3+} in the presence of ascorbate or EDTA, cytochrome c, or HOCl [62, 81]. DHR123 is not directly oxidizable by H_2O_2 alone, nor by superoxide anion or by the system xanthine/xanthine oxidase [62, 81].

4.2.3 New Fluorescent Probes for H_2O_2 Detection

New chemoselective fluorescent indicators are being developed to provide improved selectivity for H_2O_2 over other ROS [82]. A very promising approach is based on the selective H_2O_2-mediated transformation of arylboronates to phenols [83]. Arylboronates are linked to fluorogenic moieties, such that reaction with H_2O_2 generates a fluorescent product.

Arylboronate probes include peroxyfluor-2 (PF2), peroxy yellow 1 (PY1), peroxy orange 1 (PO1), peroxyfluor-6 acetoxymethyl ester (PF6-AM), and mitochondria peroxy yellow 1 (MitoPY1) [82–84]. This family of probes can detect physiological changes in endogenous H_2O_2 levels, allowing various combinations for multicolor imaging experiments. The addition of acetoxymethyl ester groups gives rise to the dye peroxyfluor-6 acetoxymethyl ester (PF6-AM), which increases cellular retention and further increases sensitivity to H_2O_2 [84, 85]. In addition, the recently developed Ratio Peroxyfluor 1 (RPF)-1 provides a ratiometric change of two fluorescent signals upon reaction with H_2O_2, allowing normalization of fluorescence ratio to probe concentration [86].

Combining the boronate–based probe design with appropriate functional groups has resulted in organelle-specific probes that can measure H_2O_2 levels with spatial resolution [87]. In particular, several mitochondria-targeted probes have been generated, including MitoPY1 and SHP-Mito [84, 88, 89] for mitochondrial targeting and Nuclear Peroxy Emerald (NucPE), for nuclear targeting [90].

4.2.4 Hydroethidine or Dihydroethidium (HE)

HE is widely used as a fluorogenic substrate for detecting superoxide anion [62–64, 80, 91]. HE is membrane-permeant and cytosolic HE exhibits blue fluorescence, but once oxidized by superoxide, it generates 2-hydroxy-ethidium (E^+), a fluorescent

compound (λ excitation = 520 nm; λ emission = 610 nm). E^+ is retained in the nucleus, intercalating with the DNA, a fact that increases its fluorescence [62].

HE has been repeatedly used in studies related to the oxidative burst in leukocytes [92, 93] and to inflammation [94–97]. HE has been used also for mitochondrial superoxide detection [80, 98, 99] although the more recently developed MitoSOX Red indicator provides more specific mitochondrial localization, as discussed later in this section [80, 100]. Moreover, since mitochondria play a fundamental role in apoptosis, which can be triggered by ROS and RNS, through mitochondrial membrane permeabilization and release of proapoptotic factors, HE and Mito-Sox have been also used to detect changes in mitochondrial superoxide generation associated with the induction and execution of apoptosis [98, 99].

HE may have important limitations when used for analysis of intracellular superoxide. It has been shown that cytochrome c is able to oxidize HE, an aspect that might be important in situations where the detected superoxide is mainly of mitochondrial origin or in conditions leading to apoptosis, where cytochrome c is released to cytosol [97]. Owing to the interconnection between oxidative stress and the apoptotic processes, it will be difficult, in these situations, to assume that HE oxidation to E^+ depends only on superoxide. Furthermore, HE can also be oxidized by a variety of reactive species, including peroxynitrite. Thus, HE should be considered as an indicator of ROS and RNS production [62–64, 97].

4.2.5 MitoSOX Red Mitochondrial Superoxide Indicator (MitoSox Red)

MitoSOX Red, a cationic derivative of HE, was introduced for selective detection of superoxide in the mitochondria of live cells [64, 80, 101]. MitoSOX Red contains a cationic triphenylphosphonium substituent that selectively targets this cell-permeant probe to actively respiring mitochondria, where it accumulates as a function of mitochondrial membrane potential and exhibits fluorescence upon oxidation and subsequent binding to mitochondrial DNA [80]. MitoSOX Red has been used for detection of mitochondrial superoxide production in a wide variety of cell types and conditions [80, 100, 101].

Oxidation of MitoSOX Red by superoxide results in hydroxylation of the ethidium moiety at the 2-position, to yield a 2-hydroxyethidium substituent. Therefore, the fluorescence spectral properties of oxidized MitoSox Red are identical to those of HE [80]. On the other hand, since the chemical reactivity of MitoSOX Red with superoxide is similar to the reactivity of HE with superoxide, all the limitations of HE apply to MitoSOX Red as well [64, 102].

4.2.6 CellROX® Reagents as General Probes for ROS

The CellROX® reagents are a series of proprietary reagents from Life Technologies-Thermofisher. These cell-permeant dyes are weakly fluorescent in the

reduced state and exhibit photostable fluorescence upon oxidation by ROS [103–105].

CellROX® green becomes fluorescent only with subsequent binding to DNA, limiting its presence to the nucleus or mitochondria. This compound has an excitation wavelength of 485 nm and an emission wavelength of 520 nm. This reagent can be formaldehyde-fixed and its signal survives detergent treatment, allowing it to be it multiplexed with other compatible dyes and antibodies. CellROX® Orange and CellROX® Deep Red do not require DNA binding for fluorescence and are localized in the cytoplasm. CellROX® orange has an excitation wavelength of 545 and an emission of 565, while CellROX® Deep Red has an excitation peak of 640 nm and an emission peak of 665 nm [104].

4.2.7 4,5-Diaminofluorescein Diacetate (DAF-2 DA)

The NO radical is short-lived and physiological concentrations are very low. NO is readily oxidized to the nitrosonium cation (NO^+), which is moderately stable in aqueous solutions but highly reactive with nucleophiles or other nitrogen oxides. Under aerobic conditions, these reactive nitrogen oxides, but not nitric oxide itself, can be bound by aromatic 1,2-diamines to form fluorescent benzotriazoles [80, 106].

DAF-2 was the first fluorogenic probe for NO [106]. DAF-2 DA is a membrane-permeant substrate that can be hydrolyzed to DAF-2 and trapped within the cell [107]. The fluorescent chemical transformation of DAF is based on the reactivity of the aromatic vicinal diamines with NO in the presence of molecular oxygen. DAF-2, which shows low fluorescence, reacts with NO-derived NO^+ to produce the highly fluorescent triazolofluorescein (DAF-2T). The fluorescence quantum efficiency increases more than 100 times after the modification of DAF-2 by NO^+. DAF-2 DA has been used to detect intracellular NO by fluorescence microscopy and flow cytometry with high sensitivity [108].

4.2.8 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM DA)

DAF-FM DA is a cell-permeant diacetate derivative with properties similar to DAF-2 [80]. DAF-FM DA is cleaved by esterases to generate intracellular DAF-FM, which is then oxidized by NO to a triazole product much more fluorescent. Indeed, the fluorescence quantum yield of DAF-FM increases about 160-fold after reacting with NO [80].

DAF-FM has been used in many studies related to NO generation under a variety of experimental conditions [80, 109, 110], including the kinetic analysis of NO generation and consumption in whole-blood monocytes using real-time cytometry [111].

4.2.9 Dihydrorhodamine 123 (DHR123) for Detecting Peroxinitrite

Although DHR123 was described initially as a fluorogenic susbstrate for H_2O_2 [64], currently it is the most frequently used probe for measuring peroxynitrite [62–64], based on the oxidative conversion of DHR123 to its corresponding two-electron oxidized fluorescent product, rhodamine 123, (λ excitation = 505 nm; λ emission = 529 nm) mediated by an intermediate DHR123 radical [64]. The oxidation of DHR123 by peroxynitrite is not induced directly by this ROS, but is mediated by intermediate oxidants formed from the rapid and spontaneous decomposition of peroxynitrite [64, 112].

4.3 Detection of Lipid Peroxidation

Peroxyl radicals are formed by the decomposition of various peroxides and hydroperoxides, including lipid hydroperoxides. The hydroperoxyl radical is also the protonated form of superoxide, and approximately 0.3% of the superoxide in the cytosol is present as this protonated radical [80].

4.3.1 *cis*-Parinaric Acid

cis-Parinaric acid is a fluorescent 18-carbon polyunsaturated fatty acid, containing four conjugated double bonds in positions 9, 11, 13, and 15 [62, 80]. *cis*-Parinaric acid can be metabolically integrated into membrane phospholipids of cultured cells, where its conformation and mobility are comparable to endogenous phospholipids. Moreover, its fluorescent and peroxidative properties are combined in the conjugated system of unsaturated carbon–carbon bonds. The fluorescence of *cis*-parinaric acid (λ excitation = 320 nm; λ emission = 432 nm) is lost upon oxidation [62, 80]

This probe has been repeatedly used to measure lipid peroxidation in a multiplicity of cell systems and conditions [113, 114]. However, there are some problems associated with the use of *cis*-parinaric acid in living cells, such as its absorption in the UV region, a wavelength still absent in most routine cytometers, and where most test compounds may absorb. In addition, *cis*-parinaric acid is most sensitive to air and undergoes photodimerization under illumination, which results in loss of fluorescence and overestimation of the extent of lipid peroxidation [80].

4.3.2 4,4-Difluoro-5-(4-Phenyl-1,3-Butadienyl)-4-Bora-3a,4a-Diaza-S-Indacene-3-Undecanoic Acid (BODIPY$^{581/591}$C11) and Related BODIPY Probes

BODIPY$^{581/591}$ C11 is a fluorescent probe (λ excitation = 510 nm; λ emission = 595 nm) used for evaluating lipid peroxidation and antioxidant efficacy in

different experimental models [62, 80, 115]. BODIPY$^{581/591}$ C11 has a long-chain unsaturated fatty acid (C11) of non-polar character, which makes this probe liposoluble, while the conjugated double bonds in the fluorophore make it susceptible to oxidation by peroxyl radicals [62, 80]. BODIPY $^{581/591}$ C11 undergoes a shift from red to green fluorescence emission upon oxidation [80]. This oxidation-dependent emission shift enables fluorescence ratiometric analysis of free radical–mediated oxidation in the lipophilic domain of the membranes. The primary target for ROS is the diene interconnection, leading to the formation of three different oxidation products that are responsible for the shift from red to green fluorescence [62].

BODIPY$^{581/591}$ C11 is sensitive to multiple oxidizing species. It has been demonstrated that this probe is oxidized by peroxyl, hydroxyl radicals, and peroxynitrite, while being insensible to H_2O_2, singlet oxygen, superoxide, NO radical, transition metals, and hydroperoxides in the absence of transition metals [116].

Lipid peroxidation has been detected in cell membranes using BODIPY$^{581/591}$ C11 [117–119] and other similar BODIPY derivatives, such as BODIPY$^{493/503}$ [120], BODIPY FL EDA (a water-soluble dye) [80], or BODIPY FL hexadecanoic acid [80].

4.3.3 Lipophilic Fluorescein Derivatives

The probe 5-(N-dodecanoyl) aminofluorescein (C11-Fluor), a lipophilic derivative of fluorescein, has been used in flow cytometry for determining membrane-lipid peroxidation [60, 121]. This probe remains associated with cellular membranes in a stable and irreversible way. Other lipophilic derivatives of fluorescein include 5-hexadecanoylaminofluorescein (C16-Fluor), 5-octadecanoyl-aminofluorescein (C18-Fluor), and di-hexadecanoyl-glycerophosphoethanolamine (Fluor-DHPE) [122].

4.4 Detection of Metabolic Derivatives of Peroxidized Lipids

4.4.1 Immunofluorescent Detection of 4-Hydroxy-2-Nonenal (4-HNE)

As a final consequence of the peroxidation process, a variety of aldehydes may be formed. 4-HNE is an unsaturated aldehyde arising from peroxidation of ω-6 unsaturated fatty acids. 4-HNE has been found to be a reliable biomarker of lipid peroxidation, as it is highly reactive towards free SH groups of proteins, producing thioether adducts that further undergo cyclization to form hemiacetals. HNE induces heat-shock protein, inhibits cellular proliferation, and is highly cytotoxic and genotoxic to cells [123, 124].

Monoclonal antibodies recognizing adducts of 4-HNE with histidine, lysine, and cysteine in proteins are now commercially available [125]. These antibodies have

been conjugated with distinct fluorochromes and can be used for in situ detection of advanced stages of lipid peroxidation in different cell types with high specificity [126].

4.5 Immunofluorescent Detection of Oxidized Bases in DNA

The oxidized DNA base 8-oxodeoxyguanine (8-oxoDG) is a major form of oxidative DNA damage derived from the attack by hydroxyl radical on guanine at the C8-position, resulting in a C8-OH-adduct radical. Thus, 8-oxoDG is formed during free radical damage to DNA and is a sensitive and specific indicator of DNA oxidation [56, 57].

8-oxoDG can be quantified with the OxyDNA assay, based on the specific binding of a monoclonal antibody conjugated with FITC to 8-oxoDG moieties in the DNA of fixed and permeabilized cells [127]. This assay has been used to detect oxidative genotoxicity in vitro [128], including environmental studies [129]. Of particular interest, the OxyDNA assay has been used in a number of fertility studies related to oxidative stress during cryopreservation of sperm cells [130] and the relation of oxidative DNA damage to fertility in humans [131–133] and animals [134].

4.6 Assessment of Antioxidant Defenses: GSH and Thiol Groups

Cellular thiols, especially GSH, act as nucleophiles and can protect against toxicity, mutagenicity, or transformation by ionizing radiation and many carcinogens [40]. The availability of many thiol-reactive fluorescent probes allowed development since the early 1980s of cytometric assays for GSH [135, 136] and free thiol groups [137] in living cells. Currently, the analysis of intracellular levels of GSH and activity of GSH S-transferase (GST) is a relevant application of functional cytometry in oxidative stress and drug resistance [138], as the more than 1800 papers indexed in PubMed between 1981 and 2016 attest. Cytometric assays for GSH and intracellular SH groups have been critically reviewed on several occasions [139–142].

The probes most used for cytometric analysis of GSH and GST have been the UV-excited, cell-permeant bimanes, particularly monobromobimane (mBrB) and the more selective monochlorobimane (mClB). Both probes are essentially nonfluorescent until conjugated to GSH [138–141]. o-Phthaldialdehyde, another UV reagent, reacts with both the thiol and the amine functions of GSH, yielding a cyclic derivative with excitation and emission maxima shifted from those of its protein adducts, improving the specificity of GSH detection [138–141].

ThiolTracker Violet is up to 10-fold brighter than the bimanes, when excited at 405 nm, yielding emission at 525 nm. An advantage of this cell-permeant probe is that it resists formaldehyde fixation and detergent extraction, allowing analysis of fixed cells [138, 142].

GSH can be determined using visible light–excitable probes, including 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA), and chloromethyl SNARF-1 acetate. Both probes form adducts with intracellular thiols that are well retained by viable cells. CellTracker Green CMFDA is brighter than MClB and is highly specific for GSH over free SH groups [138]. The GSH adduct of chloromethyl SNARF-1 emits beyond 630 nm, allowing multicolor protocols and reducing the impact of cellular autofluorescence.

5 Problems and Limitations in the Determination of ROS and RNS

As commented above, detection of ROS and RNS, the initiators of the oxidative stress process, is a complex task owing to the low concentration, short half-life, and extensive interactions of ROS and RNS, as well as by intrinsic limitations of both probes and experimental conditions. Such limitations and potential sources of artifacts make quantitative measurements of intracellular generation of ROS and RNS a difficult challenge and require careful design of the experiments and cautious interpretation of data.

5.1 Short Half-Life and Intracellular Location of ROS and RNS

Because of their reactivity, most ROS and RNS are short-lived molecules. For example, the half-life of hydroxyl radical within a cell is only about 10^{-9} s, compared to about 1 ms for H_2O_2 [39]. This means that hydroxyl radical will react at or very near its origin, whereas H_2O_2 can diffuse away from its source [39].

The variability in ROS half-life and the complexity of the microenvironments where they are produced and consumed make ROS and RNS quantification almost impossible in cellular systems [63, 64]. While ROS and RNS of low reactivity may accumulate with time, the more reactive ROS will reach a steady state in which the rate of their generation will be equal to the rate of disappearance, the rate of disappearance being the sum of reaction rates of this ROS with various components of the system, plus the rate of self-reaction, plus the rate of reaction with the fluorescent probe.

To attenuate this complication, flow cytometric techniques based on real-time measurements [143, 144] and imaging cytometry of intracellular location of ROS can be used [111], as exemplified in Figs. 1 and 2.

5.2 Complex Interactions Among and Between ROS, RNS, and Fluorescent Probes

A clear example of ROS interplay is mitochondrial respiration, where superoxide anion, H_2O_2, and hydroxyl radical are sequentially produced by a series of partial reductions. Incorporation of an electron into O_2 gives rise to superoxide anion, which is a poorly reactive radical but which can oxidize thiols and ascorbic acid [5, 10, 40]. Superoxide gives rise to H_2O_2 by spontaneous reaction or by the action of superoxide dismutase. H_2O_2, in turn, can react with different organic compounds to produce peroxyl radicals that will eventually release hydroxyl radicals during their metabolism. Moreover, by way of the Fenton reaction, hydroxyl radicals are produced when H_2O_2 and a transition metal, such as Fe^{2+}, react together, yielding Fe^{3+} that consumes superoxide for recycling Fe^{2+}. In the Haber-Weiss reaction, superoxide and H_2O_2 react together to produce hydroxyl radicals [5, 10].

The interaction of ROS with nitrogen derivatives can generate RNS. NO, a gas that is synthesized from L-arginine in many cell types by various isoforms of the enzyme NO synthetase, is a weak reductor and reacts with O_2 to form NO_2, but reacts much faster with superoxide to produce peroxynitrite (ONOO-), a powerful oxidant [33, 34, 111].

5.3 Influence of the Probes on the Experimental System

All reduced fluorogenic substrates are subject to auto-oxidation, which usually produces singlet oxygen, superoxide, and by its dismutation, H_2O_2. If the auto-oxidation rate is significant, it may result in artifactual detection of ROS and higher background, a problem especially important for probes such as HE or MitoSox Red [62–65].

The concentration of the probe is also relevant, as it may affect the stoichiometry of the process under study. For instance, the stoichiometry of the reaction between HE and superoxide depends on the ratio of superoxide flux and HE concentration. Owing to HE-catalyzed superoxide dismutation, the efficiency of HE oxidation decreases at higher rates of superoxide generation, and high HE concentrations might lead to fluorescence increase independent of superoxide [62].

Fluorescent probes at high concentration may perturb cells and be toxic. For example, when irradiated with UVA, H_2DCF auto-oxidizes and photo-sensitizes cells [63]. In addition, probes may affect the activity of ROS-producing enzymes.

For instance, H_2DCF can be a source of electrons for the oxidation of arachidonic acid by prostaglandin H synthase [63], while dihydrocalcein was reported to inhibit the activity of mitochondrial complex I [64].

5.4 Experimental Artifacts

Artifactual generation of ROS may result from photochemical reactions of components of culture media [60, 145]. The presence of ROS has been detected even in natural environments, such as seawater [146, 147]. Xenobiotics and endogenous compounds such as catechols, dopamine, hydralazine, and molecules with SH groups may also produce significant ROS upon interaction with media [60].

On the other hand, binding to macromolecules in the medium may lead to quenching of fluorescent probes. For example, quenching of DCF fluorescence has been reported by binding to native or glyoxal-modified human serum albumin [60, 148].

5.5 Cell Integrity and Functional Competence and Intracellular Localization of Probes

As previously commented in this chapter, ROS and RNS are usually produced and act in discrete intracellular locations. This situation is successfully approached by chemical modifications in the probes that allow them to cross the plasma and, eventually, the organelle membranes to be targeted to specific intracellular environments [62–65]. However, artifacts may arise when these assumptions are not realized. For instance, dihydrocalcein accumulates in mitochondria, in contrast to H_2DCF, which usually localizes in the cytoplasm [149], but preferential localization of H_2DCF in the mitochondria of rat cardial myocytes has been reported [150].

A much more common problem involves extracellular leakage of fluorogenic probes or their oxidation products. Passive probe leakage will always be present, to an undetermined extent, in necrotic or apoptotic cells, owing to enhanced plasma membrane permeability, leading to artifacts or erroneous interpretation of results (Fig. 4).

The presence of active multidrug transporters in the plasma membrane of cells may result in probe extrusion and underestimation of oxidative stress [151], as multidrug-resistant cells with elevated level of expression of some transporters can appear to produce less ROS. Substances such as rhodamine 123 and ethidium are good substrates for P glycoprotein, while substances such as fluorescein and dihydrofluorescein are substrates for MRP1 [152]. Dihydrocalcein has been preferred to H_2DCF because its oxidation product calcein is believed to not leak out of cells; however, calcein is also a good substrate for MRP1 and MRP2 transporters [60].

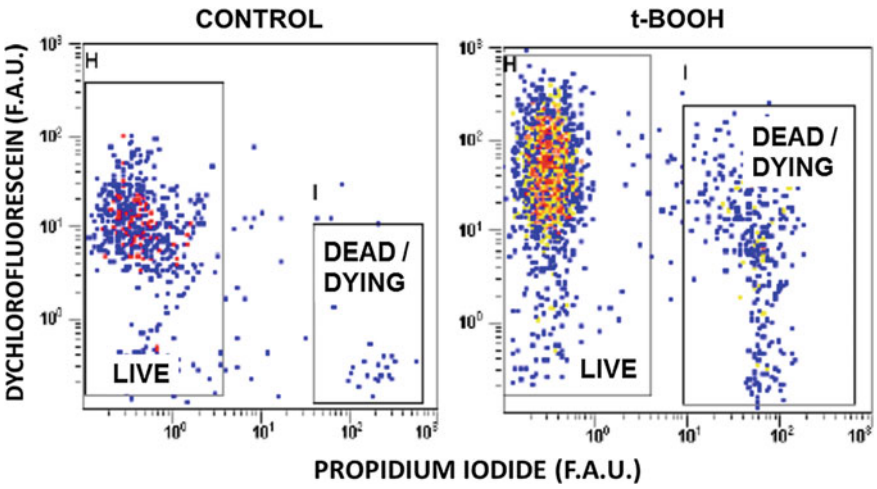

Fig. 4 Example of passive H_2DCF leakage from necrotic or apoptotic cells due to enhanced plasma membrane permeability. Human kidney adenocarcinoma A.704 cells were resuspended from monolayer culture by tripsinization and treated for 1 h with 150 μM *t*-butyl hydroperoxide (t-BOOH), a prooxidant model compound, or with DMSO as vehicle (control). Cells were stained for 30 min with 5 μg/mL H_2DCF-DA; 2.5 μg/mL propidium iodide was added immediately before analysis with a standard flow cytometer. Treatment with *t*-BOOH increases in live cells the fluorescence of DCF, the product of H_2DCF-DA oxidation. Dead or dying cells (positive for propidium iodide), exhibit decreased intracellular DCF fluorescence. FAU: Fluorescence Arbitrary Units

5.6 Intrinsic Limitations of Fluorogenic Substrates and Probes

5.6.1 Probes Used for Detection of H_2O_2 and Organic Peroxides

H_2DCF-DA is possibly the probe most widely used for detecting intracellular H_2O_2 and oxidative stress. Traditionally, H_2DCF-DA and DHR123 are believed to be oxidized by H_2O_2 and organic peroxides, and have been used for assaying peroxides [72, 80]. However, these probes do not react directly with H_2O_2 in the absence of peroxidases [63, 64], and the fluorescence of DCF or rhodamine 123 is not a direct measure of H_2O_2. Even if H_2DCF oxidation also occurs by action of H_2O_2 or O_2 in the presence of Fe^{2+}, the hydroxyl radical is the species responsible for such oxidation [62].

Since the oxidation of H_2DCF and DHR123 by H_2O_2 under physiological conditions requires peroxidase-dependent systems, enzyme activity may become a limiting factor; thus measurement of probe oxidation might be rather considered a measure of peroxidase activity. However, H_2DCF and DHR123 can be oxidized not only by the peroxidases, but also by other related enzymes, such as xanthine oxidase, superoxide dismutase, and cytochrome c [62].

H_2DCF and DHR123 are not oxidized by NO or superoxide to any significant extent, but they are very efficiently oxidized by peroxynitrite via the radicals generated during peroxynitrite decomposition [153, 154].

DCF may undergo photoreduction by visible light or by UVA radiation [155]. This mechanism may generate a semiquinone radical from DCF that produces superoxide by reaction with O_2. Sequentially, the dismutation of superoxide generates H_2O_2, which leads to an artificial increase of H_2DCF oxidation and to amplification of DCF fluorescence.

Mito PY-1 and other aromatic boronate derivatives have been proposed for analysis of intramitochondrial generation of H_2O_2 [82–84]. However, aromatic boronates also react nearly stoichiometrically with peroxynitrite a million times faster than they do with H_2O_2 [156]. Because of this reactivity, it is critical to perform proper controls when using a boronate-based fluorescent probe, such as expression of catalase, or using a peroxynitrite-specific probe.

5.6.2 Probes Used for Detection of Superoxide

Measurement of intracellular and mitochondrial superoxide using HE and Mito-SOX Red is also a widely used strategy for studying oxidative stress [62–64]. The red fluorescence of the two-electron oxidation product of HE, ethidium (E^+), is usually considered proof of intracellular superoxide formation. However, it has been demonstrated that E^+ is not formed from the direct oxidation of HE by superoxide [157, 158]. Instead, 2-hydroxyethidium (2-OH-E^+), a different product with similar fluorescence characteristics, is the reaction product of HE with superoxide [102]. E^+ and other dimeric products, but not 2-OH-E^+, are generated during the reaction between HE and other oxidants such as peroxynitrite, hydroxyl, H_2O_2, and peroxidase intermediates. Thus, 2-OH-E^+ is only a qualitative indicator of intracellular superoxide [64, 102].

The chemistry of Mito-SOX with superoxide is similar to that of HE and the same caveats apply [64]. Because of its positive charges, Mito-SOX reacts slightly faster with superoxide compared to HE [101]. Mito-SOX reacts with superoxide and forms a red fluorescent product, 2-hydroxymitoethidium (2-OH-Mito-E^+), and not Mito-E^+. 2-OH-Mito-E^+, the specific product of superoxide with Mito-SOX, and Mito-E^+, the nonspecific product of Mito-SOX, have overlapping fluorescence spectra. Thus, the red fluorescence formed from Mito-SOX localized in mitochondria is not a reliable indicator of mitochondrial formation of superoxide, as it might arise also from an oxidation product of Mito-SOX induced by one-electron oxidants (such as cytochrome c, peroxidase, and H_2O_2) [64, 101, 102, 157, 158].

HE is oxidized directly by ferricytochrome c [91] and by other heme proteins. Oxidation of the probe by cytochromes c, c1, b_{562}, b_{566}, and aa3 is oxygen-independent, whereas oxidation by methemoglobin and metmyoglobin is strictly oxygen-dependent, with products consisting of a mixture of species resulting from 1- to 4-electron abstraction from HE. Although they are different

from the superoxide oxidation product, their excitation/emission peaks are close to those generated by superoxide [60, 159].

5.6.3 Probes Used for Detection of NO and Peroxynitrite

Diaminofluoresceins were initially reported to be specific for NO, but DAF-2 reacts mainly with peroxynitrite rather than with nitric oxide [60, 160]. DAF-FM also reacts with peroxynitrite, but under conditions of physiological concentrations of NO and peroxynitrite it is fairly specific for NO.

Oxidants also interfere with the reaction of DAF-2 with NO [161], while reducing compounds such as catecholamines, ascorbate, dithiothreitol, mercaptoethanol, and glutathione attenuate the fluorescence of the reaction product [162]. Peroxidases in the presence of H_2O_2 oxidize DAF-2 to a relatively stable nonfluorescent intermediate that reacts directly with NOS, thus increasing fluorescence yield. Therefore, intracellular oxidation of DAF-2 may result in increase of DAF-2 fluorescence, erroneously indicating increased NO production [163].

DHR123 is the most frequently used probe for measuring peroxynitrite [62–64], but oxidation to rhodamine 123 is actually mediated by the radicals $\bullet NO_2$ and •OH formed from the rapid and spontaneous decomposition of peroxynitrite, and is not induced directly by peroxynitrite itself. In addition, the intermediate radical, $DHR^{\bullet}$, formed from the one-electron oxidation of DHR123, also reacts rapidly with O_2 and Fe^{2+} [112, 164], triggering a redox cycling mechanism leading to artifactual amplification of the fluorescence signal intensity. Thus, DHR123 can be used only as a nonspecific indicator of intracellular peroxynitrite and HO radical formation [64].

5.6.4 Probes Used for Detection of Lipid Peroxides

The presence of four double bonds in *cis*-parinaric acid makes this probe very susceptible to oxidation if not rigorously protected from air [62, 80]. During experiments, *cis*-parinaric samples should be handled under inert gas and the solutions prepared with degassed buffers and solvents. *cis*-Parinaric acid is also photolabile and undergoes photodimerization when exposed to intense illumination, resulting in loss of fluorescence [80].

$BODIPY^{581/591}$ C11 is photosensitive, degrading under high-intensity illumination conditions, such as those typical of laser confocal microscopy [165]. In addition, $BODIPY^{581/591}$ C11 is more sensitive to oxidation than are endogenous lipids, and therefore tends to overestimate oxidative damage and underestimate antioxidant protection effects [80].

5.6.5 Probes Used for the Determination of GSH

Several fluorescent probes are used to determine intracellular GSH, but all of them may have limitations for quantitative studies. In many cases, the fluorescent reagents designed to measure GSH may react with other free or protein-bound intracellular thiols [137, 140, 166]. An important aspect in the use of GSH reagents is the large interspecies and tissue variability of cellular GSH content and the presence of GST isozymes, which may complicate enzyme-based measurements under saturating substrate conditions [166]. For instance, mClB, which is highly selective for GSH in rodents, should not be applied with quantitation purposes to human cells because of its low affinity for human GST [140].

5.7 *Controls in the Cytometric Analysis of ROS, RNS, and Oxidative Stress*

According to the limitations and caveats presented above, including appropriate positive and negative controls is very important when performing cytometric experiments or analyses related to ROS, RNS, and oxidative stress. When possible, direct visualization of intracellular ROS and RNS generation by co-localization techniques is highly recommendable [70] (Fig. 2); detailed discussion of possible controls in such studies is beyond the scope of this chapter, as the biochemical complexity of experimental oxidants and antioxidants parallels that of their biological counterparts [40, 167].

In general, the most frequent controls are positive controls, molecules or complex systems that directly or indirectly increase the intracellular level of ROS or RNS or mimic the cellular effects of oxidative stress. Prooxidants are chemicals that induce oxidative stress, either by generating reactive oxygen species or by inhibiting antioxidant systems [40, 167]. To mimic mitochondrial H_2O_2 production, cells can be treated with the complex I respiratory-chain inhibitor rotenone [84]. Peroxyl radicals, including alkylperoxyl and hydroperoxyl radicals, can be generated from compounds such as 2,2'-azobis(2-amidinopropane) and from hydroperoxides such as *t*-butyl hydroperoxide or cumene hydroperoxide [84]. The hydroxyl radical can be generated from superoxide donors (e.g., plumbagin or menadione) [111] or by exogenous H_2O_2 in a Fenton reaction catalyzed by Fe^{2+} or other transition metal, as well as by the effect of ionizing radiation [80]. Superoxide can be most effectively produced by the hypoxanthine/xanthine oxidase–generating system [168]. Many xenobiotics, including anticancer agents such as anthracyclines and cis-platin [169], and natural redox-active toxins, like pyocyanin, [170] generate ROS and can be used as positive controls.

Intracellular levels of ROS can also be increased by attenuating or inhibiting antioxidant defenses. A convenient strategy involves depletion of intracellular GSH stores by inhibiting GSH biosynthesis or by accelerating GSH oxidation [171].

Inhibitors of antioxidant enzymes, such as superoxide dismutase [172] and catalase [173], have also been used to increase intracellular ROS and induce oxidative stress.

The intracellular content of RNS can be increased by using NO donors, a heterogeneous group of chemicals (including ester nitrates, furoxans, benzofuroxans, NONOates, S-nitrosothiols, and metal complexes) that cross the cell membrane and generate intracellular NO [111, 174, 175] or peroxynitrite [176].

In addition, negative controls may be designed to reduce the levels of ROS or RNS or attenuate their biological effects. If possible, controls should be specific with respect to which particular reactive species or enzyme system is involved, but in most cases, controls do not attempt that degree of specificity [63–65]. Antioxidants can be categorized as enzymatic and nonenzymatic [167]. Enzymatic antioxidants work by transforming oxidative products to H_2O_2 and then to H_2O, in a sequential process. Cell-permeable forms of antioxidant enzymes, such as polyethyleneglycol–superoxide dismutase [177] can be also used to decrease specifically intracelllular ROS. Non-enzymatic antioxidants work by interrupting free radical–initiated chain reactions. Such antioxidants can be classified depending on whether they are hydrophilic (e.g., ascorbic acid, N-acetyl cysteine, GSH-esters) or lipophilic (e.g., α-tocopherol and Trolox) [40]. In general, water-soluble antioxidants react with oxidants in the cytosol while lipid-soluble antioxidants protect cell membranes from lipid peroxidation [40]. In addition, chelators of transition metals [178] also exert antioxidant effects, based upon the attenuation of Fenton-type reactions [40, 167].

Regarding the use of chemical antioxidants as negative controls, it should be kept in mind that reducing agents may become prooxidants. For instance, ascorbate has antioxidant activity when it reduces oxidizing substances such as H_2O_2, but it can also reduce metal ions, leading to the generation of free radicals through the Fenton reaction [40, 167]. When considering the specificity of antioxidants, all organic compounds react with hydroxyl radicals with rate constants approaching the diffusion limitation. Thus, in solution, no compound really has any more significant hydroxyl radical–scavenging activity than other compounds (proteins, lipids, nucleic acids, amino acids, numerous metabolites, etc.) already present in any biological system [40]. On the contrary, α-tocopherol, owing to its specific uptake into membranes and relatively rapid kinetics of reaction with lipid hydroperoxyl radicals compared with their propagation reaction, may be an effective chain breaker in lipid peroxidation [40].

In recent years, novel approaches to design positive and negative controls in studies of oxidative stress have involved genetically modified organisms. For instance, Guo et al. [85] used an enzymatic method to generate cytoplasmic H_2O_2 in astrocytes. Primary astrocytes were transduced with adenoviruses containing the cDNA for cytoplasmic D-amino acid oxidase (DAAO). DAAO oxidatively deaminates D-amino acids using FAD as the electron acceptor. At the same time, DAAO uses O_2 to oxidize FAD, thus generating H_2O_2 in a dose-dependent manner relative to the concentration of D-alanine.

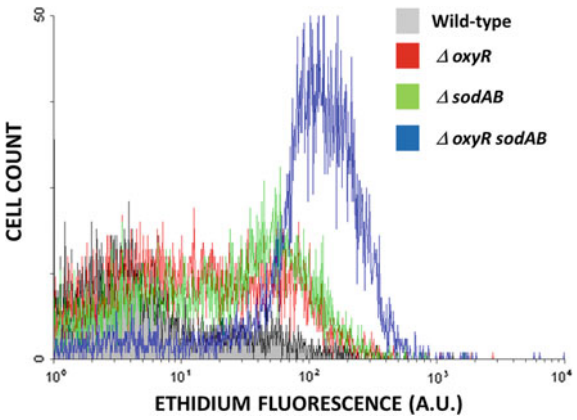

Fig. 5 Example of enhanced sensitivity to oxidative stress in oxyR-deficient strains of *Escherichia coli* B WP2. The WP2 strain of *Escherichia coli* B is characterized by increased membrane permeability to low-molecular compounds, including fluorescent probes. In this genetic background, inactivation of key genes involved in the sensing of ROS (oxyR) and/or the antioxidant defense (sodA, sodB) provoked increased accumulation of intracellular ROS as compared to the wild-type WP2 strain when incubated for 30 min with 10 μM plumbagin, a superoxide donor. Bacteria were stained with hydroethidine and analyzed by flow cytometry, in experimental conditions similar to those described in [180, 181]. Δ oxyR: strain deficient in the oxyR operon; Δ sodAB: strains deficient in the sodA and sodB genes, codifying for superoxide dismutases. Δ oxyRsodAB: triple mutant strain. A. U.: Fluorescence Arbitrary Units

To provide biosensors of oxidative stress our own group [179] has developed a collection of genetically modified strains of *Escherichia coli* B WP2, based on the inactivation of the oxyR operon, a main sensor of oxidative stress [44]. *Escherichia coli* B WP2 strains possess an altered cell-wall lipopolysaccharide that results in increased membrane permeability; we have previously shown that flow cytometric analysis of WP2 strains is a convenient alternative for cytometric assays of bacterial function [180]. Such oxyR-deficient bacterial strains show enhanced sensitivity to oxidative stress and increased accumulation of intracellular ROS when examined by flow cytometry using fluorogenic susbstrates (Fig. 5).

Acknowledgements The authors wish to acknowledge the financial support of the University of Valencia (UVEG) through grants UV-INV-AE15-349700 (Convocatòria Accions Especials 2015) and MOGDETECT (Programa VLC-BIOMED).

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