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An Introduction to Reactive Oxygen Species

Measurement of ROS in cells

Abstract

Reactive oxygen species (ROS) have long been known to be a component of the killing response of immune cells to microbial invasion. Recent evidence has shown that ROS play a key role as a messenger in normal cell signal transduction and cell cycling. These reactive molecules are formed by a number of different mechanisms and can be detected by various techniques. This white paper briefly describes the biology behind some of these molecules and the means for their detection.

Introduction

Reactive oxygen species (ROS) describes a number of reactive molecules and free radicals derived from molecular oxygen. The production of oxygen-based radicals is the bane to all aerobic species. These molecules, produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal-catalyzed oxidation, have the potential to cause a number of deleterious events. It was originally thought that only phagocytic cells were responsible for ROS production as their part in host cell defense mechanisms. Recent work has demonstrated that ROS have a role in cell signaling, including apoptosis, gene expression, and the activation of cell signaling cascades.[1] It should be noted that ROS can serve as both intra- and intercellular messengers.

Types of reactive oxygen species

Most reactive oxygen species are generated as byproducts during mitochondrial electron transport. In addition, ROS are formed as necessary intermediates of metal-catalyzed oxidation reactions. Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell. This electron structure makes oxygen susceptible to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide (Figure 1).
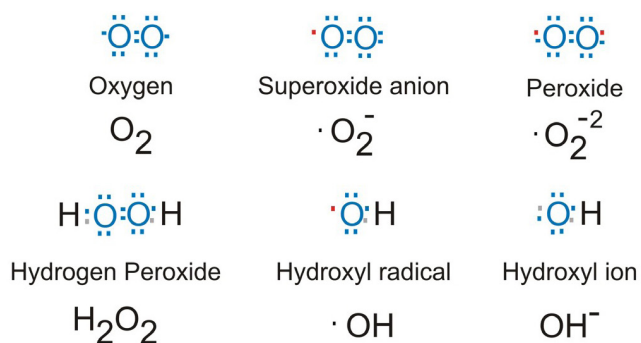

Figure 1. Electron structures of common reactive oxygen species. Each structure is provided with its name and chemical formula. The • designates an unpaired electron.

Cellular defense against ROS

Detoxification of reactive oxygen species is paramount to the survival of all aerobic life forms. As such, a number of defense mechanisms have evolved to meet this need and provide a balance between production and removal of ROS. An imbalance toward the pro-oxidative state is often referred to as oxidative stress.

Cells have a variety of defense mechanisms to ameliorate the harmful effects of ROS. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2)[3] (Equation 1). In the peroxisomes of eukaryotic cells, the enzyme catalase converts H_2O_2 to water and oxygen, and therefore completes the detoxification initiated by SOD (Equation 2). Glutathione peroxidase is a group of enzymes containing selenium, which also catalyze the degradation of hydrogen peroxide, as well as organic peroxides to alcohols.

Equation 1.　　$2 O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Equation 2.　　$2 H_2O_2 \rightarrow 2 H_2O + O_2$
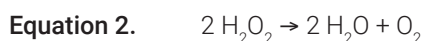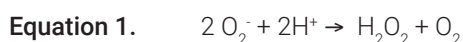
There are a number of nonenzymatic small molecule antioxidants that play a role in detoxification. Glutathione may be the most important intracellular defense against the deleterious effects of reactive oxygen species. This tripeptide (glutamyl-cysteinyl-glycine) provides an exposed sulphhydryl group, which serves as an abundant target for attack. Reactions with ROS molecules oxidize glutathione, but the reduced form is regenerated in a redox by an NADPH-dependent reductase. 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 to play a similar role in membranes.

The ratio of the oxidized form of glutathione (GSSG) and the reduced form (GSH) is a dynamic indicator of the oxidative stress of an organism.[2]

Reaction to oxidative stress

The effect of reactive oxygen species on cellular processes is a function of the strength and duration of exposure, as well as the context of the exposure. The typical cellular response to stress is to leave the cell cycle and enter into G_0. With continued exposure and/or high levels of ROS, apoptosis mechanisms are triggered. In cycling cells, p21 is activated in response to stress, such as oxidants or oxidative stress and blocks cell cycle progression.[4] Likewise, p27 production leads to G_1 arrest of cells. In cycling cells, p53 and p21 respond to

oxidants by inducing the dephosphorylation of retinoblastoma (RB). Exposure to oxidants such as H_2O_2 or nitric oxide also results in dephosphorylation of RB that is independent of p53 or p21. In either case cells are arrested in S-phase. Expression of p27 is controlled in part by the Foxo transcription factors, which are known to control the expression of genes involved in cell cycle progression, metabolism and oxidative stress response.[5] For example, mitogenic stimulation by the PI3K/Akt pathway maintains Foxo3a in the cytoplasm, but in the absence of stimulation Foxo3a enters the nucleus and upregulates genes for oxidant metabolism and cell cycle arrest, such as p27.[5] Under some conditions Foxo3a can directly activate *bim* gene expression and promote apoptosis.[6] Therefore, Foxo3a promotes cell survival of cycling cells under oxidative stress by enabling a stress response, but induces cell death when conditions warrant. Noncycling cells, such as neurons, also have coping mechanisms to oxidative stress that involve Foxo3a. Foxo3a induces expression of the manganese form of SOD in response to oxidative stress.[7]

Regulation of ROS production

Phagocytic cells

The stimulated production of reactive oxygen species by phagocytic cells was originally called "the respiratory burst" due to the increased consumption of oxygen by these cells.[8] This process is catalyzed by the action of NADPH oxidase, a multicomponent membrane-bound enzyme complex, and is necessary for the bactericidal action of phagocytes.[8]

While several enzymes are recognized as being able to produce ROS moieties, NADPH oxidase is the most significant.[9] NADPH oxidase activity is controlled by a complex regulatory system that involves the G-protein Rac (Figure 2).[10]

In resting cells a membrane-embedded heterodimer of two polypeptides (p22-phox and gp91-phox), which also contains two heme groups as well as a FAD group, enables the transfer of electrons from cytosolic NADPH across the membrane to molecular oxygen without NADPH oxidase activity.[9]

It is believed that the charge compensation occurs when gp91-phox polypeptide also acts as an H^+ ion channel. Upon stimulation, a number of polypeptides (p47-phox, p67-phox and p40phox) translocates to the inner face of the plasma membrane to form a fully active enzyme complex that possesses NADPH oxidase activity. A similar process is believed to take place in nonphagocytic cells as well.[11]
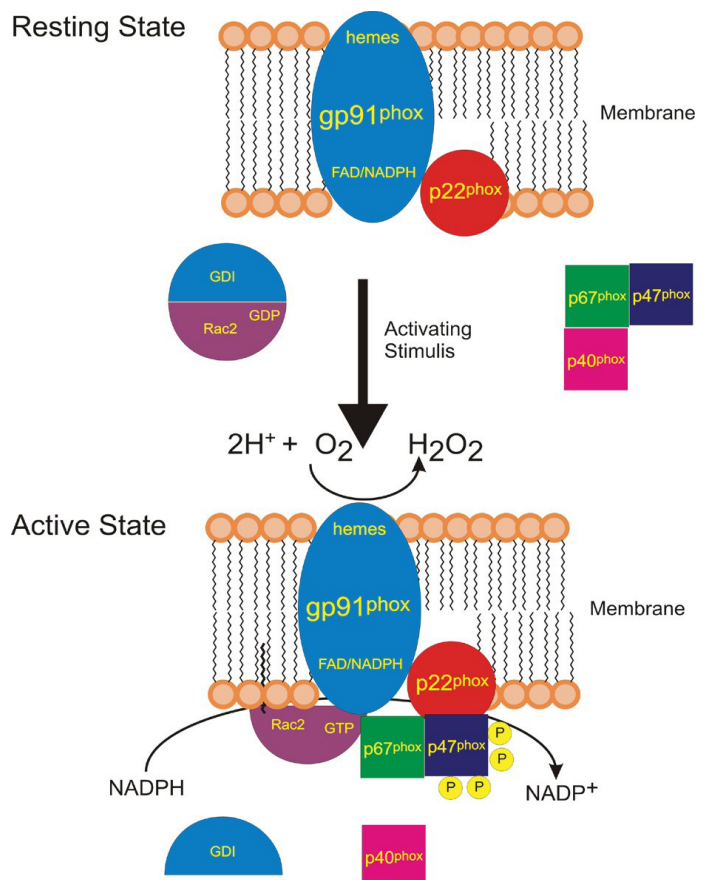

Figure 2. Schematic illustration of the activation of NADPH oxidase.

Signal transduction

Reactive oxygen species have a role in a number of cellular processes. High levels of ROS, which can lead to cellular damage, oxidative stress, and DNA damage, can elicit either cell survival or apoptosis mechanisms depending on severity and duration of exposure. Nitric oxide (•NO) has been shown to serve as a cell-to-cell messenger, being responsible for such effects as decreasing blood pressure.[12] Intracellularly, ROS species, in conjunction with antioxidant enzymes, are believed to play a role in turning enzymes on and off by redox signaling in a manner akin to that of the cAMP second messenger system.[12] Examples include superoxide anion and hydrogen peroxide. The steady state level of $•O_2^-$ is estimated to be so low, however, that its activity is spatially limited. Hydrogen peroxide (H_2O_2) is normally unreactive with thiols in the absence of catalyzing agents (e.g. enzymes," multivalent metals etc.), it does react with thiolate anion (S^-) to form sulfenic acid, which in turn ionizes to form sulfenate (SO^-). This intermediate can be reversed by the action of glutathione.[13]

Mitogenic signaling begins at the cell surface with the ligand-dependent activation of receptor tyrosine kinases, which activate important MAP kinase cascades necessary for proliferation. These cascades lead to the generation of H_2O_2 from several enzyme catalysts, including the NADPH oxidases.[14] It has been estimated that the production of H_2O_2 at nanomolar levels is required for proliferation in response to growth factors.[15] Hydrogen peroxide interacts with both the SOS-Ras-Raf-ERK and PI3K/Akt pathways through several mechanisms and in a dose-dependent manner (Figure 3). It has been suggested that small increases of H_2O_2 as a result of Nox1 expression result in increased reentry into the cell cycle, while sustained high levels of H_2O_2 lead to cell arrest and eventual apoptosis after prolonged arrest.

Peroxidoxins serve as important regulators of H_2O_2 and mitogenic signaling. These thiol-dependent peroxidases are activated and recruited to receptors as part of mitogenic stimulation and serve to limit the effect of ROS-associated stimulation on downstream targets of the mitogen cascade (Figure 3).[16]

Cell cycle control: redox signaling

As cells proliferate, they move through a coordinated process of cell growth, DNA duplication and mitosis referred to as the cell cycle. The cell cycle is a tightly regulated process with several checkpoints. Each one of these checkpoints is regulated by proteins and protein complexes that are influenced by the oxidative state of the cell. The relationship between the redox state and cell cycle control is described in great detail in a review by Heintz and Burhans.[5]

In multicellular animals most of the cells are not replicating and have withdrawn from the cell cycle either temporarily or permanently via terminal differentiation. The exit of G_0 and entry into G_1 in response to extracellular growth factors is controlled by oxidants. Redox-dependent signaling pathways promote the expression of Cyclin D1[17], the key protein for re-entry into the cell cycle. As such, cyclin D1 expression has been reported to be a marker for successful mitogenic stimulation.[15] The key regulatory point in G_1 is the restriction point (or R point), where cells become committed to entry into S-phase. At the R point, the retinoblastoma protein (pRB) becomes phosphorylated by Cyclin D/CDK complexes. Interestingly enough, studies have shown that there is a redox potential of approximately -207 mV for pRB phosphorylation, above which cells pRB is dephosphorylated and cells cease cycling.[18] It has been noted that in synchronized cells the production of ROS increases during the cell cycle, with peak levels occurring in the G_2/M phase.[19]

Reactive oxygen species play a role in apoptosis. NF-kB, which is a collective term to describe the Rel family of transcription factors, inhibits apoptosis by upregulating several antiapoptotic genes.[17] Conversely, the c-Jun N-terminal kinase (JNK) promotes apoptosis when activated for prolonged periods.[20] Prolonged activation has been shown to be caused by exposure to ROS directly, as well as by inactivating JNK inhibitors such as MAP Kinase phosphatases.[20] Suppression of TNF-α-induced ROS accumulation seems to be the mechanism by which NF-kB downregulates JNK activation.
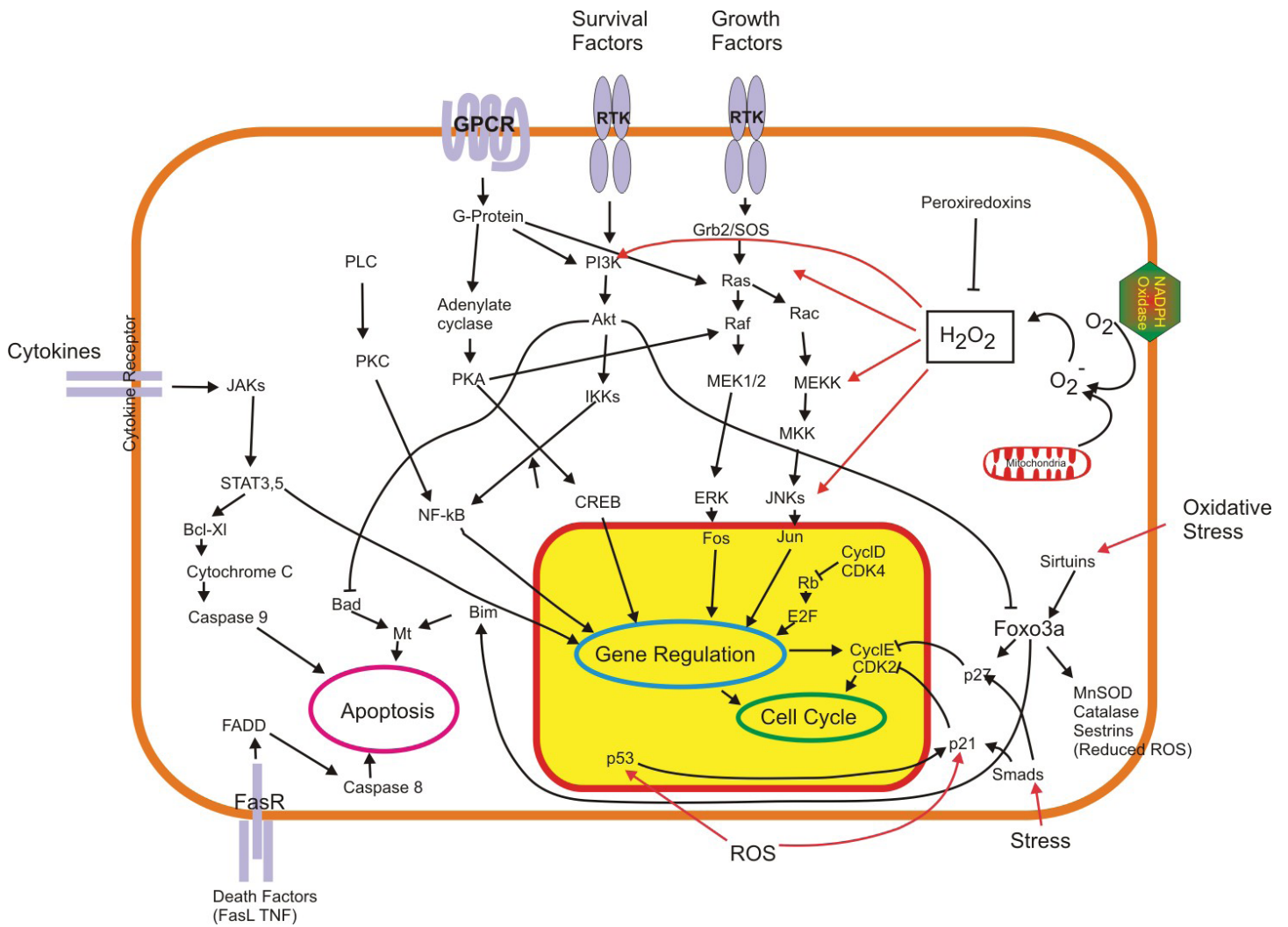
Figure 3. Schematic illustration of reported interactions of ROS and mitogenic cascades.

Measuring reactive oxygen species

The measurement of reactive oxygen species is dependent on the analytic target along with the reactive oxygen species in question. At the cellular level, specific ROS can be individually assessed from tissue culture, while at the animal level typically the effects of oxidative stress are measured from blood product (e.g. serum or plasma) or from urine samples.

Oxidative stress

Glutathione is the most significant nonenzymatic oxidant defense mechanism. It exists in relatively large amounts (mM levels) and serves to detoxify peroxides and regenerate a number of important antioxidants (e.g. α-tocopherol and ascorbic acid).[21]

Reduced glutathione (GSH) is regenerated from its oxidized form (GSSG) by the action of an NADPH-dependent reductase (Equation 3).

Equation 3. $\qquad$ GSSG + NADPH + H$^+$ → 2 GSH + NADP$^+$

Due to the rapid nature of the reduction of GSSG relative to its synthesis or secretion, the ratio of GSH to GSSG is a good indicator of oxidative stress within cells. GSH and GSSG levels can be determined by HPLC[22], capillary electrophoresis[23], or biochemically in microplates.[24]

Several different assays have been designed to measure glutathione in samples. By using a luciferin derivative in conjunction with glutathione S-transferase enzyme the amount of GSH would be proportional to the luminescent signal generated when luciferase is added in a subsequent step.[25] Total glutathione can be determined colorimetrically by reacting GSH with DTNB (Ellman's reagent) in the presence of glutathione reductase. Glutathione reductase reduces GSSG to GSH, which then reacts with DTNB to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 412 nm.

Subcellular detection and localization of GSH is important in understanding the modulation of cellular redox status, the effect of drugs, and the mechanisms of detoxification. Furthermore, differences in GSH levels in response to oxidative stress in subpopulations of cells have been reported, underscoring the importance of detection methods amenable to flow cytometry and automated fluorescence detection.

The bimane compounds monobromobimane and monochlorobimane, which are essentially nonfluorescent until conjugated, readily react with low molecular weight thiols, including glutathione to form fluorescent adducts with an excitation wavelength of 394 nm and an emission at 490 nm (Figure 4).[26] These reagents are useful for detecting the distribution of protein thiols in cells before and after chemical reduction of disulfides. Monochlorobimane, which is more thiol selective than monobromobimane, has long been the preferred thiol-reactive probe for quantitating glutathione levels in cells and for measuring GST activity.[27] Because the blue-fluorescent glutathione adduct of monochlorobimane eventually accumulates in the nucleus, it is not a reliable indicator of the nuclear and cytoplasmic distribution of cellular glutathione.[28]
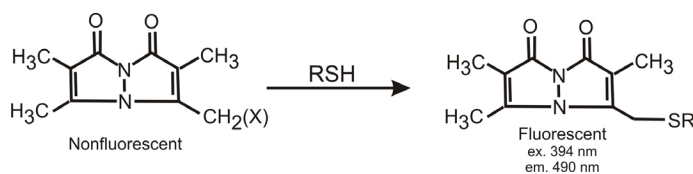

Figure 4. Bimane structure and reaction with thiols. Monobromobimane and monochlorobimane have either a Br or a Cl atom located at the 3-postion methyl group respectively and is nonfluorescent. This reactive group interacts with low molecular weight thiols to form fluorescent adducts, with an excitation maxima of 394 nm and an emission wavelength of 490 nm.

ThiolTracker Violet from Life Technologies (Woburn, MA) reacts with reduced thiols in intact cells for use in cellular analysis using flow cytometry and fluorescence microscopy. ThiolTracker Violet reagent can cross live cell membranes, becoming cell-impermeant after reacting with cellular thiols. Its 404 nm excitation and 526 nm emission wavelengths are suitable for imaging with DAPI filter sets. It is reported by its manufacturer to be 10 times as bright as bimane compounds.

Lipid peroxidation is one of the most widely used indicators of free radical formation, a key indicator of oxidative stress. Unsaturated fatty acids such as those present in cellular membranes are a common target for free radicals. Reactions typically occur as a chain reaction where a free radical will capture a hydrogen moiety from an unsaturated carbon to form water. This leaves an unpaired electron on the fatty acid that is then capable of capturing oxygen, forming a peroxy radical (Figure 5). Lipid peroxides are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde (MDA).
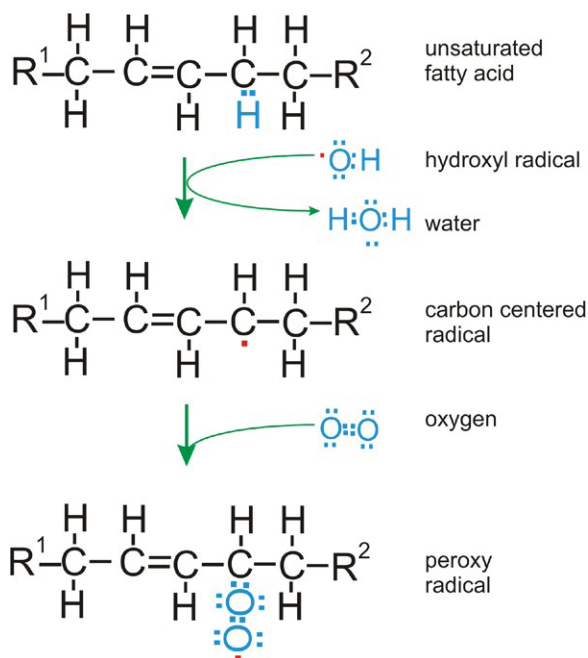
Figure 5. Illustration of lipid peroxidation.

Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA) reactive compounds such as malondialdehyde generated from the decomposition of lipid peroxidation products.[25] While this method is controversial in that it is quite sensitive, but not necessarily specific to MDA, it remains the most widely used means to determine lipid peroxidation. This reaction, which takes place under acidic conditions at 90 to 100 °C, results in an adduct that can be measured colorimetrically at 532 nm or by fluorescence using a 530 nm excitation wavelength and a 550 nm emission wavelength (Figure 6).[29] A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies.
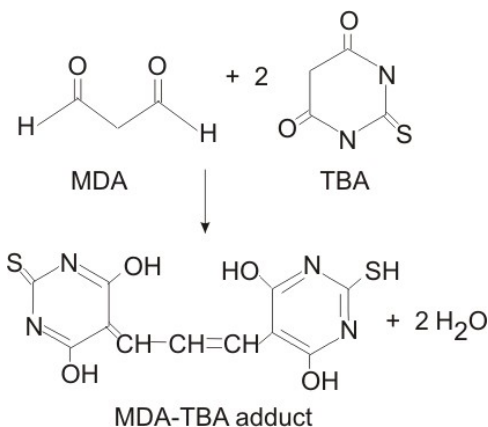

Figure 6. MDA-TBA adduct formation.

The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be specific for lipid peroxidation.[30] Unlike the TBA assay, measurement of IsoP appears to be specific to lipid peroxides, they are stable and are not produced by any enzymatic pathway, making interpretation easier.

There have been a number of commercial ELISA kits developed for IsoPs, but interfering agents in samples requires partial purification of samples prior to running the assay. The only reliable means for detection is through the use of GC/MS, which makes it expensive and limits throughput.[31]

Lipid peroxidation in live cells can be visualized using fluorescent derivatives that localize to membranes. For example, oxidation of the polyunsaturated butadienyl portion of BODIPY 581/591 undecanoic acid fluorophore results in a shift of the fluorescence emission from 590 to 510 nm (Figure 7).[32,33] This reagent commercially available as Image-iT Lipid Peroxidation Kit from Life Technologies (Woburn, MA) provides a simple ratiometric method for detecting the oxidative degradation of cellular lipids in live cells.[34] The ratio of red fluorescence to green fluorescence provides a measure of lipid peroxidation that is independent of factors such as lipid density that may influence measurement with single-emission probes. Because this reagent is compatible with live cells, measurements can take place in real time without fixation and staining. This reagent has also been used for demonstrating the antioxidant capacity of plasma[35] and lipid vesicles.[36]
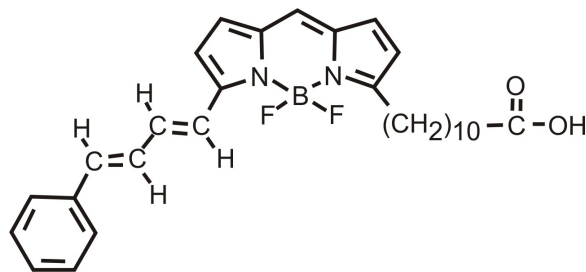

Figure 7. Structure of C11-BODIPY (581/591).

Lipid peroxidation-derived protein modifications in fixed cells can be detected using linoleamide alkyne (LAA) in conjunction with Click-iT chemistry from Life Technologies (Woburn, MA). Linoleic acid is the most abundant polyunsaturated fatty acid found in mammals and its lipid peroxidation products likely account for the majority of lipid-derived protein carbonyls.[37] When incubated with live cells, LAA incorporates into cellular membranes. Upon lipid peroxidation, the membrane-bound LAA is oxidized and produces 9- and 13-hydroperoxy-octadecadienoic acid (HPODE). These hydroperoxides decompose to α,β-unsaturated aldehydes that readily modify proteins surrounding them. Once cells are fixed, the resulting alkyne-containing proteins can be detected by reacting with Alexa Fluor 488 azide (Figure 8).
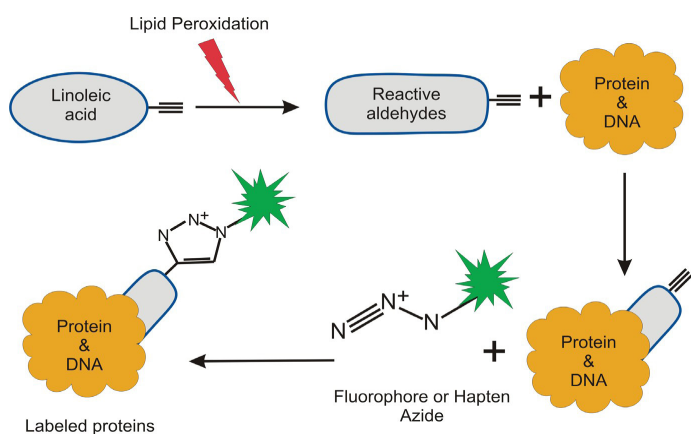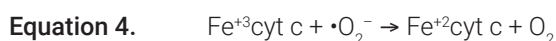

Figure 8. Mechansim of action of Click-iT LAA.

Superoxide

Superoxide detection is based on the interaction of superoxide with some other compound to create a measurable result. The reduction of ferricytochrome c to ferrocytochrome c has been used in a number of situations to assess the rate of superoxide formation (Equation 4).[38]

Equation 4. $Fe^{+3}cyt\ c + •O_2^- \rightarrow Fe^{+2}cyt\ c + O_2$

While not completely specific for superoxide this reaction can be monitored colorimetrically at 550 nm. The addition of enzyme inhibitors such as CN- or scavengers of reactive species such as catalase can minimize any reoxidation. Aconitase catalyzes the conversion of citrate to isocitrate. Superoxide inactivates this enzyme by oxidizing the Fe(II) moiety from its cubane {4Fe-4S} cluster. Therefore superoxide concentrations can be estimated by the degree of enzyme inactivation. The activity of the enzyme

can be monitored by following the conversion of 20 mM isocitrate to cicaconitate using absorbance at 240 nm. A coupled assay where the aconitase product, isocitrate, is then converted to α-ketoglutarate by NADP+ dependent isocitrate dehydrogenase can also be used. In this reaction the production of NADPH can be monitored colorimetrically at 340 nm.[21]

Chemiluminescent reactions have been used for their potential increase in sensitivity over absorbance-based detection methods. The most widely used chemiluminescent substrate is Lucigenin, but this compound has a propensity for redox cycling, which has raised doubts about its use in determining quantitative rates of superoxide production.[39] The use of low concentrations of this compound has been suggested as a means to minimize this problem. Coelenterazine has also been used as a chemiluminescent substrate. This lypophilic compound does not redox cycle and is brighter than Lucigenin. It is not however completely specific towards superoxide, as the presence of peroxynitrite will result in chemiluminescence.[40]

Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical. These dyes are synthesized by reducing the iminium cation of the cyanine (Cy) dyes with sodium borohydride. While weakly fluorescent, upon oxidation their fluorescence intensity increases 100 fold. In addition to being fluorescent, oxidation also converts the molecule from being membrane permeable to an ionic impermeable moiety.[41] The most characterized of these probes are Hydro-Cy3 and Hydro-Cy5.

Cellular production of superoxide can be visualized by dihydroethidium, also referred to as hydroethidine. This compound exhibits a blue fluorescence in the cytosol until oxidized primarily by superoxide and to a much lesser extent other reactive oxygen or reactive nitrogen species. Oxidation of dihydroethidium results in hydroxylation at the 2-position forming 2-hydroxyethidium (Figure 9). With oxidation the compound intercalated with cellular DNA, staining the nucleus a bright fluorescent red with reported excitation and emission wavelengths of 535 nm and 635 nm respectively.[42]
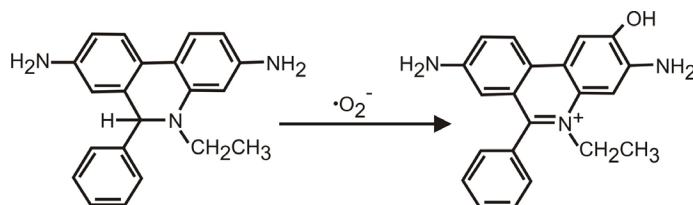

Figure 9. Oxidation of dihydroethidium to 2-hydroxyethidium by superoxide.

Superoxide specific to the mitochondria can be visualized using fluorescence microscopy with MitoSOX Red reagent from Life Technologies (Woburn, MA). MitoSOX Red reagent is a cationic derivative of dihydroethidium that permeates live cells where it selectively targets mitochondria. The cationic triphenylphosphonium substituent of MitoSOX Red indicator is responsible for the electrophoretically driven uptake of the probe in actively respiring mitochondria (Figure 10). As with dihydroethidium, this compound intercalates with mitochondrial DNA resulting in red fluorescence. While fluorescence measurements can be made using the peak excitation wavelength of 510 nm with an emission detection at 590 nm, it has been reported that a lesser excitation peak at ~400 nm that is absent in the excitation spectrum of the ethidium oxidation product generated by reactive oxygen species other than superoxide may provide better discrimination of superoxide.[42]
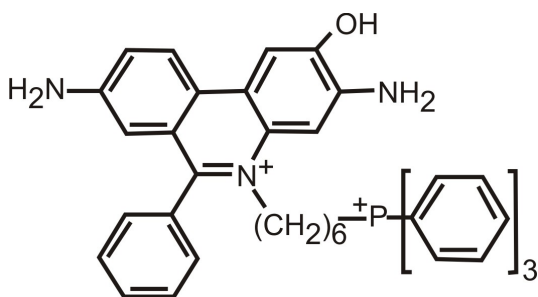

Figure 10. Structure of oxidized MitoSox Red mitochondrial superoxide indicator.

The generation of mitochondrial reactive oxygen species can be observed by imaging MitoSOX stained cells. As an example, the nonsteroidal anti-inflammatory drug diclofenac has been associated with hepatotoxicity through the induction of reactive oxygen species.[43] Mitochondrial oxidative stress was confirmed using the MitoSOX assay; following a three log titration of diclofenac dosing of liver microtissue over three days, a significantly increased signal from the red fluorescent probe was seen only in the highest dose (300 µM), suggesting that once a threshold dose is reached elevated superoxide levels can no longer be neutralized (Figure 11).
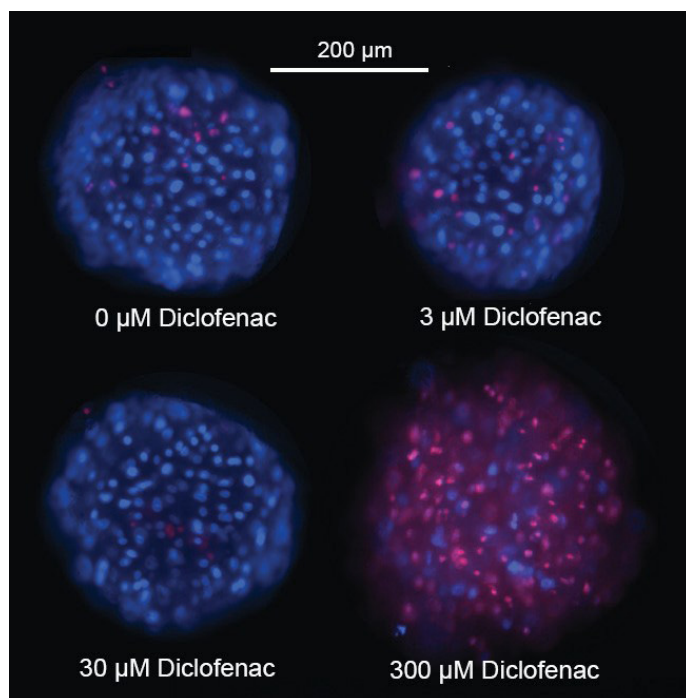

Figure 11. Mitochondrial oxidative stress assessment following a three-day diclofenac dosing. Overlaid images of 3D liver microtissues, stained with Hoechst 33342 (blue) and MitoSOX Red (red); captured using the DAPI or RFP Agilent BioTek Cytation 3 cell imaging multimode reader imaging channels, respectively. Autofocus performed on DAPI stained cells.[44]

The CellROX reagents are a series of proprietary reagents from Life Technologies. These cell-permeant dyes are weakly fluorescent while in a reduced state and exhibit photostable fluorescence upon oxidation by ROS. CellROX green only becomes fluorescent 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, making it amenable for imaging using the Green GFP filter sets. This reagent can be formaldehyde-fixed and its signal survives detergent treatment, allowing it to be 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 nm and an emission of 565 nm and can be imaged with a RFP filter cube, while CellROX Deep Red has an excitation peak of 640 nm and an emission peak of 665 nm and can be imaged with a CY5 cube.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is the most important ROS in regards to mitogenic stimulation or cell cycle regulation. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products.[21] While the list is quite extensive, the more commonly used substrates include diacetyldichloro-fluorescein[45], homovanillic acid[46], and Amplex Red.[47] In these examples, increasing amounts of H_2O_2 form increasing amounts of fluorescent product. For example, Amplex Red is oxidized by hydrogen peroxide in the presence of HRP and in doing so converted to resorufin.[47] Unlike Amplex Red, resorufin is a highly colored compound that can be detected colorimetrically at 570 nm or by fluorescence using excitation of 570 nm and emission of 585 nm (Figure 12).[48]
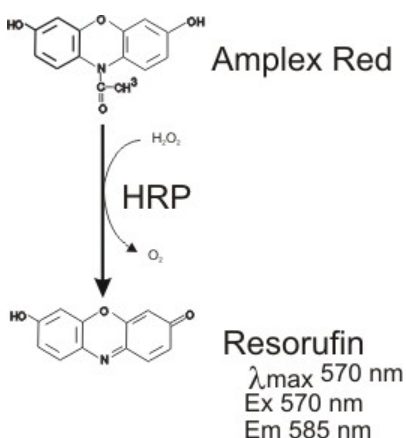

Figure 12. Conversion of Amplex Red to resorufin by HRP using H_2O_2.

Homovannilic acid dimerizes when oxidized by hydrogen peroxide through horseradish peroxidase catalysis. As with Amplex red, homovanillic acid monomer is nonfluorescent, but as a dimmer, it possesses a peak excitation wavelength of 315 nm, with an emission wavelength of 425 nm (Figure 13). Care should be taken when using this compound to assess hydrogen peroxide production. The near UV nature of the excitation and emission wavelengths for fluorescence measurements make this compound prone to inordinate background signal, particularly when polystyrene microplates are used. Several peroxidase-like metalloporphoryns have been shown to be catalytic for the reaction in addition to HRP.[49]
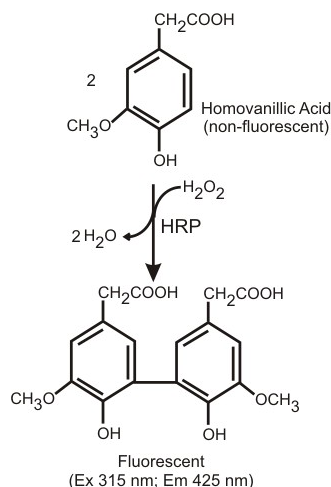

Figure 13. Dimerization of homovanillic acid by the action of HRP and hydrogen peroxide.

A number of colorimetric substrates such as tetramethylbenzidine (TMB) and phenol red have also been used in conjunction with HRP to measure hydrogen peroxide concentrations. In general, colorimetric means are less sensitive than fluorescence detection methods, but instrumentation costs are significantly lower than those required for fluorescence-based measurements when using tube- or microplate-based detection methodologies.

There are a number of issues that one should be aware of when using HRP catalyzed substrates to quantitate hydrogen peroxide. Cellular compounds such as thiols can serve as a substrate for HRP. Endogenous catalase activity can artificially reduce the amount of H_2O_2 present. Cellular components can affect the fluorescent signal depending on the excitation and emission wavelengths, as with homovanillic dimer, while other wavelengths may suffer from signal quenching.

Tarpley *et al.* summarized the utility of HRP-linked assays quite succinctly by stating that the methods are quite useful for the quantification of H_2O_2 levels "… cultured cells, organ cultures and isolated buffer perfused tissue preparations. However these methods are not suitable for determinations of H_2O_2 in plasma, or serum because many reducing agents are present in extracellular fluid…".[21]

The oxidation of 2'-7' dichlorofluorescin (H_2DCF) to 2'-7'dichlorofluorescein (DCF) has been used quite extensively for the quantitation of H_2O_2. The diacetate form, H_2DCFDA and its acetomethyl ester H_2DCFDA-AM are taken up by cells where nonspecific cellular esterases act upon it to cleave off the lipophilic groups, resulting in a charged compound believed to be trapped inside the cell. Oxidation of H_2DCF by ROS converts the molecule to 2', 7' dichlorodihydrofluorescein (DCF), which is highly fluorescent (Figure 14). The reported wavelengths for the measurement of DCF fluorescence are 498 nm for excitation and 522 nm for emission.
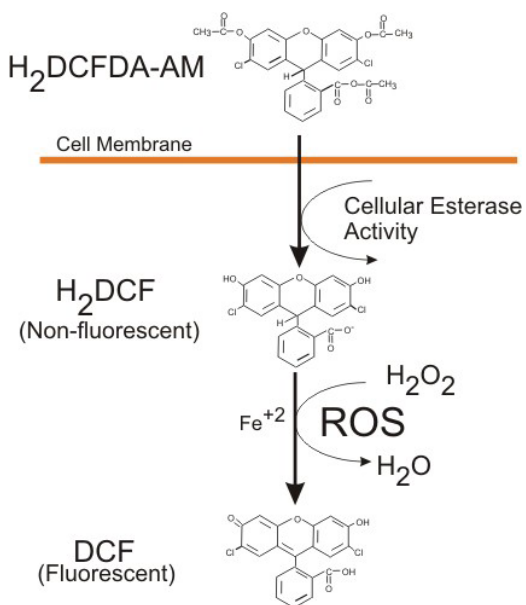

Figure 14. Formation of fluorescent compound DCF by ROS.

Originally, DCF was thought to be specific for hydrogen peroxide, but recent evidence has shown that other ROS such as nitrate and hypochlorous acid can oxidize H_2DCF.[18] Most importantly, H_2O_2-dependent oxidation of H_2DCF requires ferrous iron.[50] In addition, as H_2DCF is no longer ionic it is not precluded from migrating out of the cell and accumulating in the media, where it is free to interact with oxidants. In addition to PMT-based fluorescence measurements, oxidized DCF fluorescence can be imaged using FITC or GFP filter sets. For example, the cytotoxic quinoline alkaloid camptothecin, which inhibits DNA topoisomerase I, causes oxidative stress with cultured primary hepatocytes.[51] As demonstrated in Figure 15, micromolar concentrations of camptothecin typically induce ROS production in approximately 10 to 20% of the cells in the field of view.
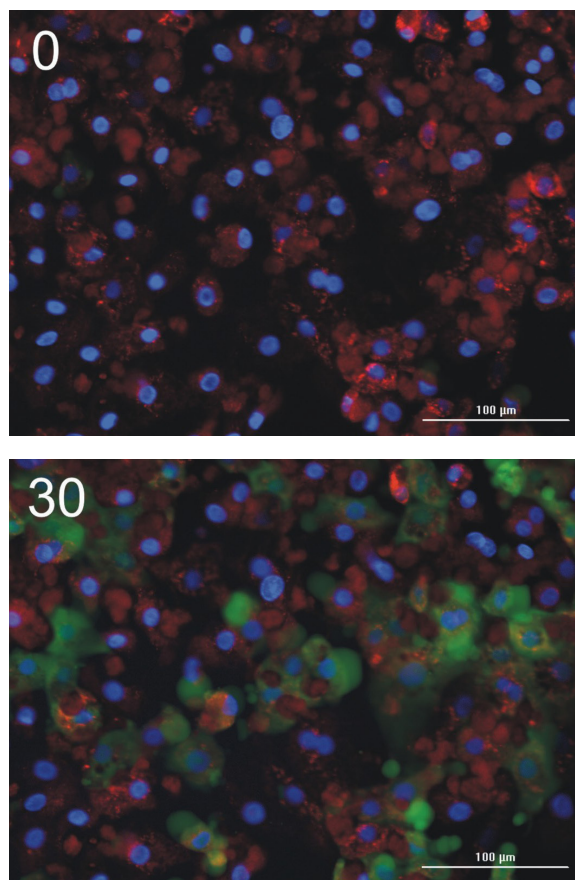

**Figure 15.** Oxidized DCF fluorescence in hepatocytes. Images of cultured hepatocytes captured after 0 and 30-minute treatments with 800 nM camptothecin. Cell nuclei were stained with Hoechst 33342 (blue), mitochondria were stained with MitoTracker Red (red); and oxidized DCF reagent is visualized in green.[68] Images were captured using an Agilent BioTek Cytation 3 cell imaging multimode reader using a 20x objective.

To address the weaknesses of DCF fluorescence several new fluorescent probes have been developed. Two such probes are Peroxy Green 1 (PG1) and Peroxy Crimson 1 (PC1). These boronate-based H_2O_2 probes have been reported to have high selectivity, membrane permeability, along with visible-wavelength excitation and emission wavelengths.[50] Reacting with hydrogen peroxide, results in a 10-fold and 40-fold increase in fluorescence for PG1 and PC1, respectively. PG1 features an excitation wavelength of 460 nm with emission maximum at 510 nm (Figure 16). PC1 demonstrates improved characteristics of red-shifted excitation and larger stokes shift which reduces autofluorescence (excitation: 480 nm; emission: 584 nm).[52]
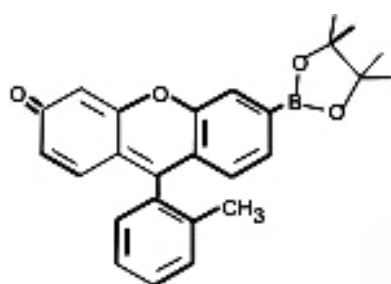
Peroxy Green 1
Ex 460 nm
Em. 510 nm
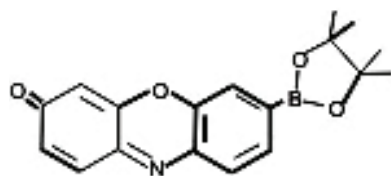
Peroxy Crimson 1
Ex 480 nm
Em. 584 nm

Figure 16. Structure of Peroxy Green 1 and Peroxy Crimson 1.

These molecules exhibit a direct reaction with hydrogen peroxide not observed with H_2DCF. In addition, these molecules are unreactive towards high-valent metal-oxo species derived from heme-proteins and H_2O_2. DCFH normally exhibits a greater response to this combination than it does towards H_2O_2.

Calcein-acetoxymethylester (Calcein-AM) has also been reported as a detector to intracellular oxidative activity.[53] Calcein-AM is a fluorogenic cell-permeable compound that is converted by intracellular esterases into the cell impermeant anion calcein, which is fluorescent (Figure 17). Historically, intracellular calcein production has been used in both microscopy and fluorometry as an indicator of viable cells. In regards to the detection of ROS, the kinetics of ROS reaction are favorable relative to esterease conversion to calcein.

The ROS-oxidized product of Calcein-AM has been shown to be chemically distinct from Calcein-AM through thin layer chromatography[53], but it still retains the ability to cross cell membranes and has similar spectral properties to the fluorescent calcein. Therefore it is important to keep the compound in the media rather than washing it away after cell loading, which is typically performed when using the compound for cell viability. Removal of the dye results in movement of the reacted compound back out of the cell on a concentration gradient. The physical properties of Calcein-AM are also different from either Calcein-AM or Calcein as the dye tends to aggregate and form intensely fluorescent localizations within the cell. While this dye has possibilities with confocal microscopy where cellular localization is possible, the inability to distinguish spectrally between ROS reacted calcein-AM and the esterase-reacted product makes the use of this dye in microplates problematic.
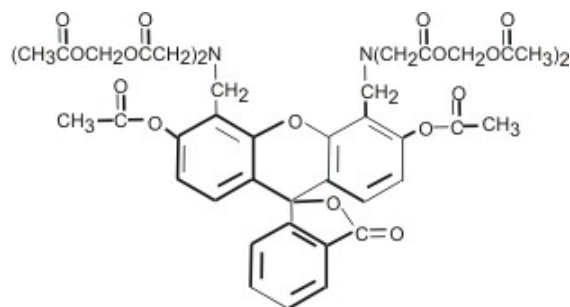

Figure 17. Calcein-AM structure.

Nitric oxide

The free radical nitric oxide (•NO) is produced by a number of different cell types with a variety of biological functions. Nitric oxide is a product of the oxidation of L-arginine to L-citrulline in a two-step process catalyzed by the enzyme nitric oxide synthase (NOS). Two major isoforms of nitric oxide synthase have been identified. The constitutive isoform found in neurons and endothelial cells, produces very low amounts of nitric oxide in a calcium- and calmodulin-dependent fashion. •NO activates soluble guanlyate cyclase in target cells, resulting in increased levels of cGMP, which in turn facilitates neuronal transmission and vascular relaxation, and inhibits platelet aggregation.[54]

The inducible isoform, found in macrophages, fibroblasts, and hepatocytes, produces •NO in relatively large amounts in response to inflammatory or mitogenic stimuli and acts in a host defensive role through its oxidative toxicity.[55] Regardless of the source or role, the free radical •NO has a very short half life ($t_{1/2}$ = 4 seconds), reacting with several different molecules normally present to form either nitrate (NO_3^-) or nitrite (NO_2^-)

A commonly used method for the indirect determination of •NO is the determination of its composition products nitrate and nitrite colorimetrically. This reaction requires that nitrate (NO_3) first be reduced to nitrite (NO_2), typically by the action of nitrate reductase (Figure 18).

Figure 18. Conversion of nitrate to nitrite by the action of Nitrate Reductase.

Subsequent determination of nitrite by a two-step process (Figure 19) provides information on the "total" of nitrate and nitrite. In the presence of hydrogen ions nitrite forms nitrous acid, which reacts with sulfanilamide to produce a diazonium ion. This then coupled to N-(1-napthyl) ethylenediamine to form the chromophore which absorbs at 543 nm.[56]

Nitrite-only determinations can then be made in a parallel assay where the samples were not reduced before the colorimetric assay. Actual nitrate levels are then calculated by the subtraction of nitrite levels from the total. This assay is relatively inexpensive and easy to perform. The reagents are easily obtained and there are numerous commercial kits available for this assay.
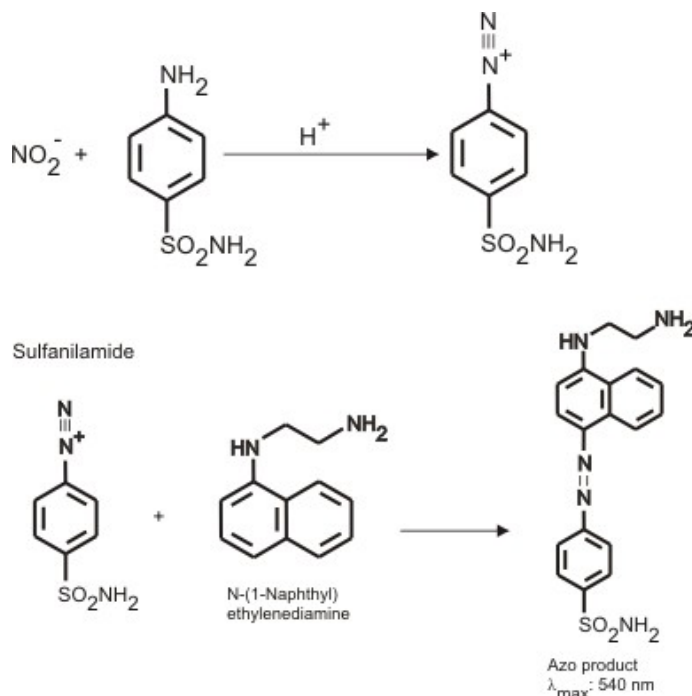
Figure 19. Greiss reaction for the determination of nitrate.

To improve upon the sensitivity of the Griess reagent assay for •NO a number of fluorometric assays have been developed. Like the Griess reaction they are dependent on dinitrogen trioxide or nitrous acid (N_2O_3), which is formed spontaneously by the acidification of nitrite (NO_2^-). The most commonly used method uses 2,3-diaminonaphthalene (DAN), which is relatively nonfluorescent, to react with nitrous acid to form 2,3-naphthotriazole (NAT), which is highly fluorescent with an excitation wavelength of 375 nm and an emission wavelength of 415 nm (Figure 20).

Figure 20. Fluorometric detection of nitrite using 2,3-diaminonaphthalene (DAN).

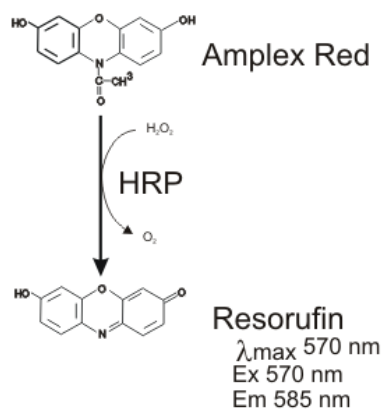## Genetically encoded sensors

Fluorescent protein-based biosensors have been developed for the investigation of the ROS *in situ* in real time. This new generation of live cell fluorescent sensors produces changes in fluorescence in response to alteration in the redox state or with fluctuations in specific target analyte. These sensors are genetically encoded, based on a single fluorescent protein and do not require the addition of any other reagents or cell lysis, making them very amenable to multiplexing.

The reduction-oxidation sensitive green fluorescent protein (roGFP) is an example of a redox sensitive biosensor. Two cysteines were introduced into the surface exposed residues of the β barrel structure in appropriate positions to form disulfide bonds of the GFP protein from Aequorea victoria. The oxidation state of the engineered thiols determines the fluorescence properties of the sensor.[57] Originally, different roGFP versions were presented to allow the *in vivo* imaging of reducing compartments such as the cytosol (roGFP2) in plants[58], with cysteines introduced at the amino acid positions 147 and 204 being found to produce the greatest change.[59] The specificity of roGFP2 for glutathione is further increased by linking it to the human glutaredoxin 1 (Grx1).[60] Glutaredoxins are small enzymes that are oxidized by substrates and reduced nonenzymatically by glutathione. By expressing the Grx1-roGFP fusion sensors in the organism of interest and/or targeting the protein to a cellular compartment, it is possible to measure the glutathione redox potential in a specific cellular compartment in real-time, which is a significant advantage over invasive static methods. Note that this probe does not directly measure ROS compounds. It does however detect the shift in oxidized/reduced (GSH/GSSG) glutathione equilibrium. In regards to detection, roGFPs have two fluorescence excitation maxima at approximately 400 and 490 nm with a common 515 emission which display rapid and reversible ratiometric changes in fluorescence in response to alterations in redox potential *in vitro* and *in vivo*. Disulfide formation results in the protonation of GFP with increases 405 nm excitation signals at the expense of 488 nm excitation signals when the emission output at 510 nm is determined. Therefore the ratio of fluorescence from excitation at 405 and 488 nm indicate the extent of oxidation. Because this measurement is ratiometric, variations in biosensor levels or matrix induced optical sensitivity is corrected for.
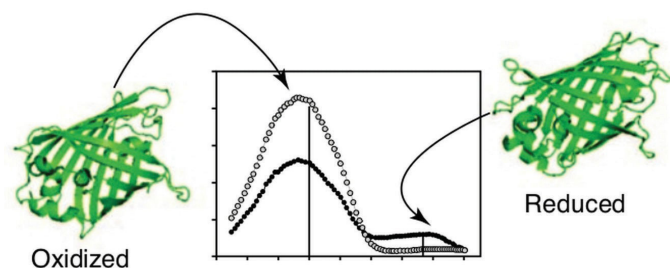

Figure 21. Principle of ratiometric redox-sensitive GFPs. The relative fluorescence intensity of the two excitation maxima of roGFPs shifts depending on the redox state: reduction causes a decrease in the excitation at 400 nm and an increase in the excitation at 480 nm (arrows).[61]

To directly sense H_2O_2 the roGFP construct has been linked to peroxidase Orp1, a yeast protein that forms disulfides upon reaction with H_2O_2, which are transferred to roGFP through a thiol-disulfide exchange mechanism.[62] The reaction is reversible through the action of cellular thioredoxin (Trx) or GRx/GSH systems. These probes are also available as BacMam 2.0 constructs under the Premo moniker (Life Technologies) to allow easy transfection.

Another method to sense H_2O_2 directly is to conjugate circularly permuted fluorescent proteins with redox-reactive protein domains, such that conformational changes brought about by redox activity are translated to the fluorescent protein. One such chimera, called HyPer, was designed in the laboratory of Sergey A. Lukyanov.[63] HyPer consists of a circularly permuted yellow fluorescent protein (cpYFP) inserted into the regulatory domain of the prokaryotic $H_2O_2^-$ sensing protein, OxyR.[64, 65] HyPer2, an improved version of the probe, was generated by a single point mutation A406V from HyPer corresponding to A233V in wild type OxyR.[64] Unlike the roGFP-Orp1 chimera, there is no transfer thiol-disulfide transfer, but rather two cysteines of OxyR form a reversible disulfide bond which induces a conformational change that is transferred to the cpYFP moiety.[65] HyPer demonstrates submicromolar affinity to hydrogen peroxide and has been shown to be insensitive to other oxidants, such as superoxide, oxidized glutathione, nitric oxide, and peroxinitrite.

Genetically encoded sensors can be targeted to specific cellular compartments by using cellular trafficking sequences as part of the chimeric molecule. For example, peptides can be targeted to the nucleus by inserting repeats of the nuclear localization signal (NLS), which consists of short sequences of positively charged lysines and arginines exposed on the protein surface. These sequences are recognized by cellular proteins (importin α and importin β), which mediate its transport into the nuclease. Similar sequences can be used to direct ROS sensors to mitochondria, peroxisomes or the cytoplasm.

Results and discussion

The interest in reactive oxygen species originally revolved around the pathology associated with the deleterious effects of aerobic respiration, the necessary evil caused by the leakage from the electron transport chain in mitochondria. In this context, research involved the role that these agents played in aging, chronic diseases, and cancer.

A new frontier was born with the discovery that the oxidative burst by phagocytic cells was actually the result of the intentional production of reactive oxygen species. This was thought to be a very precise application where specific cells produced what can only be described as toxic agents to kill invading microorganisms. Further recent work has shown that ROS are produced in all cell types and serve as important cellular messengers for both intra- and intercellular communications. It is now apparent that a very complex intracellular regulatory system involving ROS exists within cells. Cells respond to ROS moieties in different ways depending on the intensity, duration, and context of the signaling. In regards to intracellular signaling it appears that hydrogen peroxide (H_2O_2) is the most interesting candidate, while nitric oxide (•NO) is involved primarily with intercellular signaling.

The chemistry used initially for the detection of ROS was primarily absorbance measurement based. Research generally involved the measurement of glutathione levels to assess oxidative stress at the tissue or whole body level. With millimolar levels of analyte, absorbance-based measurements were more than adequate to be informative and quantitative. With the discovery that ROS are used as intracellular messengers and regulators, new chemistries were developed with the micromolar detection requirements in mind. These agents are primarily fluorescence based, but recently luminescence-based detections have been introduced.

The advent of image-based analysis using either small molecule fluorescent sensors or genetically encoded sensors has provided new insights in regards to ROS research. Image analysis can provide not only quantitate information, but also cellular localization. This can be accomplished through either reporter design or image analysis. Chemical ROS reporters or genetically encoded ROS sensors have been designed with chemical structures or peptide sequences, respectively, that result in the localization of the detector to specific cellular organelles (e.g. nuclei, mitochondria, lysosomes, etc.). Therefore any changes in imagery can be directly associated with a specific cellular location. Alternatively, localization of ROS changes with reporters that are not organelle specific can be accomplished colocalization with non ROS-related stains that are location specific. By overlaying separate color images cellular locations can be identified.

The biggest difficulty reported with much of the cellular ROS research has been with the lack of reporter agents specific for discrete molecules. ROS moieties by their nature are reactive with a number of different molecules; as such, designing reporter agents has been difficult. With more specific chemistries, particularly for hydrogen peroxide, the precise mechanisms for regulation will be elucidated.

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