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Abstract

The functional group of cysteine is a thiol group (SH) that, due to its chemical reactivity, is able to undergo a wide array of modifications each with the potential to confer a different property or function to the molecule harboring this residue. Most of these modifications involve the reversible oxidation of the thiol to sulfenic acid (SOH), and disulfide, including intra- and intermolecular disulfides between polypeptides and glutathione-polypeptide (glutathionylation). The reversibility of these oxidations allows thiol groups to serve as versatile chemical and structural transducing elements in several low molecular mass metabolites and proteins. A plethora of cellular functions such as DNA and protein synthesis, protein secretion, cytoskeleton architecture, differentiation, apoptosis and anti-oxidant defense, are recognized to be modulated, at certain stage, by thiol-disulfide exchange mechanisms of redox active thiol groups. All organisms are equipped with enzymatic systems composed by NADPH-dependent reductases, redoxins and peroxidases that provide kinetic control of global thiol-redox homeostasis as well as target selectivity. These redox systems are distributed in different subcellular compartments and are not in equilibrium with each other. In consequence, measuring cellular thiol-disulfide status represents a challenge for studies aimed to obtain dynamic and spatio-temporal resolution. This review provides a summary of the methods and tools available to quantify the thiol redox status of cells.

Measurement and meaning of cellular thiol:disulphide redox status

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Abstract

The functional group of cysteine is a thiol group (SH) that, due to its chemical reactivity, is able to undergo a wide array of modifications each with the potential to confer a different property or function to the molecule harboring this residue. Most of these modifications involve the reversible oxidation of the thiol to sulfenic acid (SOH), and disulfide, including intra- and intermolecular disulfides between polypeptides and glutathione-polypeptide (glutathionylation). The reversibility of these oxidations allows thiol groups to serve as versatile chemical and structural transducing elements in several low molecular mass metabolites and proteins. A plethora of cellular functions such as DNA and protein synthesis, protein secretion, cytoskeleton architecture, differentiation, apoptosis and anti-oxidant defense, are recognized to be modulated, at certain stage, by thiol-disulfide exchange mechanisms of redox active thiol groups. All organisms are equipped with enzymatic systems composed by NADPH-dependent reductases, redoxins and peroxidases that provide kinetic control of global thiol-redox homeostasis as well as target selectivity. These redox systems are distributed in different subcellular compartments and are not in equilibrium with each other. In consequence, measuring cellular thiol-disulfide status represents a challenge for studies aimed to obtain dynamic and spatio-temporal resolution. This review provides a summary of the methods and tools available to quantify the thiol redox status of cells.

Introduction

Thiol-disulfide redox pairs in biology

An evolutionary trait of all organisms inhabiting the surface of this planet is that they are equipped with redox systems in charge of maintaining a reducing environment at the intracellular level. The major driving force behind the appearance and further specialization of redox systems has been the increasingly more oxidizing atmosphere that begun to dominate the world million of years ago. In this context, the ancient task of the redox systems was probably confined to provide defense against oxidants. By doing so, a manifold of metabolic and cellular processes originally developed to work under reducing conditions were protected from inactivation or malfunctioning. On the other hand, making use of discrete redox sensing and controlling mechanism represents an evolutionary advantage on the basis that adapting (*e.g.* mutagenesis) complete metabolic pathways and several macromolecules to a new redox environment would have been significantly costly for the organisms.

The sulfur atom present in different low molecular mass metabolites (*e.g.* glutathione, mycothiol, trypanothione, lipoic acid, coenzyme A) and proteins (*e.g.* cysteine) has been playing a starring role in redox biochemistry because of its chemical reactivity [1]. The thiol group (SH; sulfur oxidation state -2) of these molecules is prone to undergo a wide array of oxidations and modifications, some of which are fully reversible. The deprotonated form of SH, namely the thiolate (S^-), is a stronger nucleophile that readily reacts with oxidants and electrophilic species but that can also serve as structural element, ligand for metal binding or catalyst in proteins. The reactivity of the thiols, in other words, their propensity to exist in the S^- form, depends on several chemical, physical and structural features of the microenvironment where they are placed [2]. The most relevant and well characterized reversible oxidations of thiols involve: (i) sulfur oxidation state -1: formation of intra- or inter-molecular disulfides and mixed-disulfides between protein thiols and low molecular mass monothiols (glutathione: glutathionylation; mycothiol: mycothiolation) [3, 4], and formation of *S*-nitrosothiol [5]; (ii) sulfur oxidation state 0: formation of sulfenic acid (SOH). There is an increasing number of works showing that different cysteine oxidations act as distinct redox signals [4, 6-10].

On the other hand, the exceptional chemical reactivity of thiols suggests that organisms are forced to make a rational use of cysteine residues in their proteome in order to avoid unwanted effects such as protein inactivation by (over) oxidation or loss of specificity in

function. Miseta and Csutora studied the occurrence and relative positions of cysteine residues in proteins from evolutionary distant organisms and concluded that this residue is underrepresented in various organisms but its abundance correlates positively with the complexity of the species (e.g. the content of cysteine in mammalian *vs.* Archaea proteins is 92% *vs.* 50%) [11]. The authors also revealed that the increase in cysteine content in the proteome of higher organisms is likely not a random event since the distribution of the residue in protein homologues is highly conserved, a feature typical for functionally important amino acid residues. In all organisms studied except for plants and yeasts, cysteine was found as an integral part of dithiol motifs CXXC >> CxC > CXXXC. Recent studies have also revealed the presence of a highly conserved subset of ubiquitous proteins containing a monothiol CXXS motif that are redox silent but participate in iron-binding/usage [12]. The CXXC motif is also found in metal-binding proteins and is a hallmark of oxidoreductases such as thioredoxin, glutaredoxin and protein disulfide isomerases. More than 20% of the human and archaeal proteins containing Cys harbor the CXXC signature [1, 11]. Taking all these evidences together, it seems rational to speculate that the thiol group originally fulfilled an electrochemically passive role as ligand for metal coordination that later evolved into a redox active element with capacity to transfer reducing equivalents to different molecular targets and play fine regulatory roles due to its susceptibility to be posttranslationaly modified.

Indeed, oxidoreductases with a CXXC motif fulfill a central role in thiol-based redox systems by acting as general redox hubs that kinetically transduce perturbations in thiol/disulfide pool to a variety of molecular targets, including redox signaling pathways [13-17]. Most living organisms are endowed with two major thiol-redox systems, which are named according to the oxidoreductase that acts as hub for the distribution of redox potential: the glutaredoxin- and thioredoxin-system [13, 15]. The systems consist of: i) flavoenzymes –such as thioredoxin reductase, TrxR, and glutathione reductase, GR– that transfer the reducing power of NADPH to thioredoxins or glutathione disulfide and, ii) multipurpose thiol-disulfide oxidoreductases, namely Trx and Grx, that shuttle the reducing equivalents received from TrxR or GSH to different protein targets. Variations of these systems are present in several prokaryotic (mycobacteria, bacillus, archaea and *Staphylococcus* species; reviewed in [18]) and lower eukaryote organisms (plasmodium [19], platyhelminths [20], trichomona [21], trypanosomatids [22]), though the function and the mechanistic aspects that characterize the Trx- and Grx-system are preserved. For the examples provided above, the low molecular mass thiol that acts as redox adaptor molecule is the major driving force for structural and

functional adaptations in protein partners of the corresponding redox systems. For instance, in some organisms GSH is surrogated by related or non-related sulfur-containing molecules such as cysteine (trichomonas [21]), bis-glutathionylspermidine or trypanothione (trypanosomatids [22]), mycothiol (*N*-acetylcysteine glucosamine inositol [4]) and bacillithiol (a glycoside of L-cysteinyl-D-glucosamine with L-malic acid [23]) (Fig. 1). In consequence, these organisms are equipped with NADPH-dependent reductases and redoxins specifically adapted to utilize these thiol-containing molecules as substrates. Other deviations from the canonic systems are represented by the oxidoreductase plasmoredoxin [24] and the TrxR/Grx hybrid [25] unique to plasmodial and platyhelminth parasites, respectively.

Glutaredoxins (Grx) and thioredoxins (Trx) are small proteins (10-15 kDa) that present a CPYC and CGPC active site motif, respectively [15, 26]. At structural level both proteins are closely related since they share a common arrangement composed by a core of four-stranded β-sheets surrounded by three α-helices. Some variations to this canonical fold occur in isoforms of Grx and Trx and include the insertion of an α-helix at the N- and/or C-terminus (Grx) or an additional β-strand at the N-terminus (Trx) [27, 28]. In Grx and Trx, the first thiol from the active site is always located at the N-terminus of an α-helix, facing the solvent, whereas the second cysteine is more buried albeit accessible to react with the oxidized form of the N-terminal cysteine. Trx are recognized as reductases of intra- or inter-chain protein disulfides whereas Grx, although capable to reduce selected protein disulfides, are specialized in the reduction of protein-GSH mixed disulfides. The reduction mechanism catalyzed by the redoxins follows a series of thiol-disulfide exchange reactions. For both type of oxidoreductases, the N-terminal cysteine from the active site provides the nucleophilic thiol that attacks the disulfide on the target protein leading to the formation of a transient redoxin-GSH (Grx) or -protein (Trx and, to a minor extent, Grx) disulfide bond. The intermolecular disulfides can then be solved by the C-terminal cysteine of the redoxin, which renders the disulfide form of the redoxin and releases the molecular target in reduced form or, in the special case of Grx, by a second GSH molecule that regenerates the thiol on Grx with the concomitant formation of GSH disulfide (GSSG). Depending whether the nucleophilic or both cysteines from the active site participate in the thiol-disulfide exchange reaction, catalysis is referred to proceed *via* a mono- or dithiol mechanism.

Electron transfer through these redox systems implies the formation of different thiol-disulfide couples and a thermodynamically favored direction for electron flow. Provided that cells are a metastable system, the ratio thiol/disulfide for each couple is not static along time but varies according to, at least, the concentration of the molecules and the kinetic parameters

that describe the reactions where each species participate. Overall, this determines a regulated metabolic flux of reducing power that, despite thermodynamic preferences, is rationally administered by kinetic laws and the physiological demand of the cell [28-30]. The intracellular context provides additional levels of complexity to the regulation and function of these systems. For instance, it is well know that the Trx- and Grx-system are not in equilibrium between each other [31-33]. Therefore, the redox state of a particular thiol/disulfide couple cannot be used to infer the corresponding parameter of the counterpart from other system, even less a global thiol/disulfide redox status. In addition, these systems are distributed non-uniformly in different cellular compartments and domains, where they fulfill specific metabolic demands [34]. For compartments containing only a subset of the system components, the transport of redox cofactors (e.g. pyridine nucleotides, either in reduced or oxidized forms, are unable to permeate cellular membranes by simple difussion) across cell membranes and their turn-over are additional variables that contribute to the heterogeneity of redox states inside a cell. In some cases, additional redox metabolites (lipoic acid, ascorbate) [35, 36] or uncharacterized redox proteins may take over the function of the canonical ones. An enigmatic example is the mitochondrial matrix of trypanosomatids, which contains indispensable proteins that *in vitro* have been demonstrated to use very efficiently trypanothione as redox cofactor but lack enzymes in charge of its synthesis and reduction [22]. Although not yet identified, it is tempting to speculate that putative transporters in the inner membrane exchange unidirectionally the reduced and oxidized species of trypanothione. Another example where the redox systems appear uncoupled from the pyridine pool is the endoplasmic reticulum (ER) of mammalian cells and the periplasmic space of bacteria. The major activity taking place in these subcellular domains involves the oxidative folding of membrane and/or secretory proteins. The machinery devoted to thiol oxidation and disulfide isomerization consists of small thiol oxidases (e.g. the prokaryotic **Dis**ulfide **b**ond family of enzymes and the eukaryotic **P**rotein **D**isulfide **I**somerases) containing a Trx domain with a CXXC motif critical for activity, GSH as low molecular mass thiol that assists disulfide isomerization, and an electron transfer cascade (*e.g.* the flavoenzyme Ero1 with a CXXC motif and the thioredoxin-like proteins Sep15 and SelM) [37-39] that regenerates the redox state of the effector proteins. In agreement with this function, the lumen of the ER (and also de the periplasmic space) features a more oxidative milieu than, for instance, the cytosol [40]. On the other hand, the ER hosts the pathway for the reductive biosynthesis of steroids and is characterized for possessing a prevalently reduced pyridine nucleotide pool. This apparent inconsistency, i.e. low thiol/disulfide ratio *vs.*

high NADPH/$NADP^+$ ratio, is explained because the lumen of the ER is devoid of TrxR [41] and GR activity [42, 43]. Hence the reducing power of NADPH is not connected to the thiol/disulfide metabolism and almost fully available to support reductive biosynthesis. Adding value to the uneven distribution of redox systems at intracellular level, the peroxisomes and the mitochondrial matrix, despite having a high pool of reduced GSH, were shown to be highly sensitive to perturbations of their redox milieu [44, 45]. Another interesting finding was the discovery that yeast actively accumulates GSSG in a cytosolic vacuole devoid of GR activity, which has raised alarms about determinations of GSH/GSSG ratios based on methods that disrupt cell integrity [46]. The influence of mixing compartmentalized pools of thiol/disulfide couples when whole cell extracts are used for quantitative purposes has been clearly explained by Schwärzlander et al. [47]. A difference of ~100 mV in the GSH redox potential is observed when data from invasive (-230 mV) and non-invasive methods (-320 mV) are compared, and the less reducing potential obtained from the first can be ascribed to the contribution of the GSSG pool present in the ER and related secretory vacuoles that is released upon membrane lysis. An additional aspect revealed by previous works [46, 48] is that Trx and Grx can support the regeneration of GSH from GSSG or glutathionylated proteins in the absence of GR. Moreover, the initial assumption that Grx activity is predominantly associated to the de-glutathionylation of proteins, has been challenged by the use of genetically encoded biosensors containing Grx as transducer element [49, 50]. In such arrangement, Grx was able to catalyze very efficiently the glutathionylation of a redox sensitive fluorescent protein, providing evidence that *in vivo* this oxidoreductase may actively contribute to the redox-mediated regulation of protein function under physiological conditions that alter the GSH/GSSG pool. At extracellular level, the pair cysteine/cystine constitutes the major low molecular mass thiol/disulfide couple of mammals whose turnover is fully integrated to intracellular metabolism *via* the transcellular GSH/cysteine cycle [34, 51].

Overall, this shows how intricate can be the regulation of thiol/disulfide balance in the cells, where multiple synthetic, transport and effector pathways of reducing equivalents can compete or synergize providing kinetic control according to the metabolic needs of specific compartments and highlights how important it is to pursue spatial resolution and probe specificity when addressing such questions.

Methods for measuring thiol-disulfide status

Chemical probes for detection of thiols

A series of excellent articles reviewing the battery of chemical probes available for detection and quantitation of biological thiols have recently been published [52-56].

All chemical probes exploit the nucleophilicity of thiols to react reversibly or irreversibly with disulfides, eletrophilic compounds, sulfonamides, sulfonate esters, selenium-N bonds or metal complexes. The thiol-modifying agents are designed so that the covalent bond between the thiol and the chemosensor changes the optical properties of the sensor, e.g. fluorescence, phosphorecence or UV-visible absorption, allowing quantitative measurements. Despite the relative low abundance of cysteine in proteins, it is very difficult to achieve a selective labeling of protein targets with the currently available probes. Nevertheless, protein specific thiol-probes may still be developed by optimizing the chemical properties of the reactive dye after a careful kinetic and structural analysis of the local environment of the protein thiol. Thus, and because low molecular mass thiols are present in physiological fluids or are easily extracted from cells, most of these probes were developed for the detection of non-protein thiols such as free cysteine, GSH and homocysteine.

It is worth to stress that when aiming to measure thiol-disulfide status with thiol probes, the experiments must be designed including the detection of total and reduced thiol content. The level of reduced thiols can be directly assessed in the study sample whereas determination of total thiols requires the conversion of the native pool of disulfides into thiols by chemical- or enzyme-mediated reduction. The (bio)chemical reagent used for reduction should not interfere with the chemosensor or detection system, or, at least, the interference should be quantified to cancel out its contribution to the signal. By subtracting the value of total thiols to the value of reduced thiols, the level of oxidized thiols (disulfide, mixed-disulfide or sulfenic acid; due to the low abundance/stability of the last, it is expected to be a minor contributor) is obtained. The ratio thiol/disulfide can then be calculated. Alternatively, the pool of oxidized thiols can be measured directly by pre-treating a fraction of the sample with a thiol-alkylating agent to block free thiols followed by reduction of the native disulfides before reaction with the probe. The major drawback of this technique, as any other based on disruption of cell integrity, is the impossibility to monitor the dynamics of the thiol/disulfide ratio on real time and in a compartment-specific fashion. In addition, the thiol-probes cannot distinguish between protein targets and hence provide an estimation of the global thiol/disulfide redox state of cellular proteins. The inclusion of mass spectrometry analysis

during the quantitative procedure allows identifying and assessing the redox state of single proteins as discussed in the section *Redox Proteomics*.

The different types of chemosensors classified according to the mechanism for thiol detection are presented below.

Probes (in)activated by thiol-mediated disulfide and Se-N cleavage

Several disulfide-based reagents were developed for detection of thiols. 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) or Ellman's reagent is one of the oldest and widely used probe to detect protein and non-protein thiols [57]. The transsulfuration reaction between DTNB and thiols is favored by the low pKa of the leaving group TNB (5-mercapto-2-nitrobenzoate). The reaction is stoichiometric and the released TNB is a strong chromogene that absorbs at 412 nm (Fig. 2). Besides the chemoselectivity of DTNB, the reaction product TNB is not reactive towards protein disulfides, which lowers the risk of interfering with biochemical processes when used in complex biological samples. At neutral pH TNB readily ionizes to TNB^{2-}, which is able to react with protein sulfenic acids [58-60]. However, this species is fairly unstable under aerobic conditions hence the occurrence of this modification in biological systems is quantitatively negligible. The major drawbacks of DTNB are its low stability at basic pH and double charge nature that limits the range of thiol targets that can be detected. In addition, DTNB is not cell-permeable, thus quantitation of intracellular protein thiols requires cell disruption. Nevertheless, DTNB proved useful to quantify the redox status of exofacial proteins from the membrane of human peripheral blood mononuclear cells [61]. Novel derivatives of DTNB where one TNB group was replaced by a 2-amino-ethyl (ADNB) and *n*-octyl (ODNB) group were synthesized. These compounds are equally reactive as DTNB but, due to the lack of the second TNB moiety and less activated disulfide, display a lower background signal and are significantly more stable at alkaline pH [62-64]. In addition, the chemical nature of the aliphatic chain of ODNB and ADNB renders these alkylating agents more reactive towards protein thiols located in hydrophobic and hydrophilic environments, respectively.

Thiopyridyl disulfides (*e.g.* 2,2'-dipyridyl and 4,4'-dipyridyl disulfide; DPS) are reduced by GSH and protein thiols yielding the corresponding thiopyridones that absorbs at the near-UV (e.g. 320-340 nm) [65, 66] (Fig. 2). The higher potency of these compounds to react with thiols at lower pH can be ascribed to the protonation of the nitrogen on the pyridyl ring that activates the disulfide [67]. Based on the reactivity of DPS with thiols at low pH and that the potent reductant sodium borohydride rapidly decomposes under acidic conditions, Hansen et al. [68] proposed the simultaneous use of these reagents for the quantification of thiols

without the need of further purification steps. If the detection is performed with an HPLC-system, the authors reported a sensitivity in the pM range for protein thiols (*e.g.* 1 μg of a 50 kDa protein with a single thiol). This technique complemented with the use of another thiol labeling agent was implemented for determining the global thiol/disulfide redox status of cells [69, 70] and is discussed later.

Based on the same concept of thiol/disulfide exchange-based (in)activation of chromogenic compounds, a series of FRET-based probes were recently developed. This type of thiol probes consists of a fluorescence donor linked to a fluorescence acceptor or quencher by a disulfide bond that is susceptible to reduction by thiols. One example is the probe based on the pair fluorescein (Ex/Em 494/521 nm) and rhodamine (Ex/Em 530/566 nm) linked by an aliphatic or aromatic disulfide, where disulfide reduction frees fluorescein from the quenching effect of rhodamine [71] (Fig. 2). The chemosensors taken up by *Escherichia coli* - with the conjugate containing the aromatic linker being more permeable than the aliphatic one – detected differences in the thiol content of wild type and TrxR or GR defective strains. Due to their low redox potential (-0,6 V), the applicability of the probe is limited to the detection of strong reducing environments. In this respect, administration of the probe to zebrafish embryo resulted in staining of the chorion and lack of signal in the yolk and embryo, suggesting the presence of a highly reducing environment in the outermost membrane of the embryo. Also the chorion from eggs of the caterpillar *Bombyx mori* has been reported to contain a high level of cysteine-rich proteins, nonetheless, it is assumed that their thiol groups are fully engaged on protein crosslink rendering this structure resistant to external (bio)chemical and physical challenges [72]. The reactivity of the probe towards isolated thiol-containing proteins deserves to be tested before elaborating on its specificity.

A variant of this probe was synthesized by Piggot and Karuso [73]. The chemosensor FSSF consists of two fluorescein moieties connected by a disulfide-containing linker that upon reductive cleavage releases the FRET-mediated quenching between the fluorophore molecules that emit light at 522 nm. FSSF proved to be 2 orders of magnitude more sensitive than DTNB for detecting GSH and presented an impressive dynamic range of four Log_{10} units. Although the linker included four amides to improve FSSF solubility, the biological application of the probe was so far tested *in vitro* as colorimetric reagent for a GR assay.

In the probe Ratio-HPSSC (for ***ratio***metric probe - ***h***ydroxyphenyl ***p***orphyrin disulfide bond ***c***oumarin), the donor fluorophore coumarin (Ex/Em 350/459 nm) is tethered by a disulfide bond to a hydroxyphenyl porphyrin (absorption at 421 nm) that acts as FRET acceptor

quenching fluorescence emission by the donor [74]. The emission intensity of the porphyrin at ~650 nm (Q4 band), which was not affected by thiols, allows to obtain ratiometric measurements (Em 459/658 nm) (Fig. 2). The probe displayed specificity to react with free Cys (detection limit 0.73 μM) and to a minor extent with GSH, and featured a high dynamic range (the range of analyte concentrations a probe can detect). The compound added at 10 μM in the culture medium rapidly permeated into Hela cells and pre-treatment of the cells with the thiol-blocking agent *N*-ethylmaleimide resulted in a decrease in fluorescence intensity ratio for coumarin/porphyrin compared to the non-treated control. Despite the promising potential of this probe for *in vivo* applications, there are still some aspects that should be investigated such as reversibility of the reaction, reactivity towards protein thiols and cytotoxicity.

Another type of probes activated by thiols relies on a disulfide bond joined to a fluorophore by a carbamate linkage. Reduction of the disulfide makes the thiol of the latent fluorophore available to attack the carbonyl carbamate, upon this cyclization reaction the free amine and active fluorophore is released. RhoSS, ASS and SSH-Mito are representative of this series (Fig. 3). In RhoSS, rodhamine 110 presents two disulfide-bond extensions that are reduced by GSH *in vitro* and at intracellular level resulting in a fluorescence increase at 535 nm [75]. In cell-based assays, RhoSS did not impair cell viability when added up to 80 μM and was able to report the redox status of Hela and MCF7 mammalian cell lines using confocal microscopy and/or flow cytometry. As the parental fluorophore, the distribution of RhoSS was confined to the mitochondria, which limits its range of biological application.

Zhang et al. 2007 reported the first generation of a ratiometric thiol-probe for deep-tissue bioimaging by two-photon microscopy [76]. A 8-oxo-acenaphthopyrrole derivative allowed the selective detection of Cys and homoCys over GSH, likely due to steric hindrance that precludes the thiol group from the tripeptide to reach the unsaturated carbon. Thiol addition of Cys or homoCys to the naphthalene moiety shifted the absorption maxima of the compound from 430 nm to 580 nm with peak emission at 588 nm. The suitability of the probe for cell imaging applications was validated in mammalian cell lines from different origin (metastatic adenoid cystic carcinoma cells, pancreatic cancer cells, Caov-3 ovarian carcinoma cells and Hela cells) that were treated or not with *N*-ethylmaleimide and subjected to confocal and two-photon microscopy analysis. Notably, the subcellular distribution of the signal varied between cell lines probably indicating the existence of cell-specific pools of Cys/homoCys at intracellular level. As drawback, this probe demanded a high laser power for deep-tissue

imaging (which may damage cells), rendered a low depth penetration and, although not investigated, the naphthopyrrole moiety may contribute to cytotoxicity.

As superior fluorescence sensors for deep-tissue imaging, 2-methylamino-6-acetylnaphthalene-based probes were recently developed [77, 78]. Upon cyclization, ASS and SSH-Mito, the last containing an additional tryphenylphosphonium group, become excitable at 514 nm (one-photon microscopy) and at 740-780 nm femtosecond pulse (two-photon microscopy) (Fig. 3). Both compounds were targeted to the mitochondria of Hela cells and displayed a similar reactivity towards Cys and GSH without interference from other biologically relevant species. Using preparations of rat hippocampus, ASS and SSH-Mito allowed the measurement of thiol distribution at depths of 90-190 μM with a 10-fold increase in the two-photon excited fluorescence intensity. SSH-Mito presented a low cytotoxicity and was insensitive to pH changes within the physiological range [77]. At variance with ASS, SSH-Mito allows ratiometric measurements.

By linking galactose to naphthalimide *via* a thiol-cleavable disulfide bond, Lee et al. generated a probe (named ASGP/R) for hepatic thiol imaging in living cells and animals [78]. The sugar moiety mediated the receptor-dependent endocytosis of the probe by human hepatocarcinoma cells (HepG2 cells) and reduction of the thiol linker unmasks the phthalamide fluorophore leading to a ratiometric increase in the fluorescence emission ratio 540/473 nm (Fig. 3) that is enhanced or fully inhibited by pre-treatment of cells with a thiol-alkylating or a lipotoxic agent, respectively. *In vitro*, ASGP/R showed a concentration- and time-dependent reduction/activation by GSH even at subphysiological concentrations (< 50 μM). The probe was also reduced by Trx (5 μM), although thorough kinetic studies with this oxidoreductase and Grx were not performed.

Ex vivo analysis of organs from male Sprague-Dawley rats that received an intravenous dose of ASGP/R confirmed the tissue-specificity of the probe but also revealed the accumulation of signal in kidney. ASGP/R was very stable and active at a wide pH-range while its cytotoxicity/clearence remains to be assessed to a more wide use *in vivo*.

The generation of the first fluorescent probe for mammalian thioredoxin reductase (TrxR), TRFS-green, was recently reported by Zhang et al. [79]. The probe features a five-membered cyclic disulfide scaffold (1,2-dithiolane scaffold bound to naphthalimide), which can be selectively reduced by TrxR in the presence of NADPH. Intramolecular cyclization by the liberated thiolate leads to the formation of a five-membered cyclic carbamate and the consequent release of the quenched fluorophore naphthalimide that presents an emission peak

at 538 nm (Ex 438 nm). TRFS-green proved to be almost refractory to other related reductase and dehydrogenase enzymes as well as to various low molecular mass thiols and biomolecules with reducing power. In cell-based assays, the signal intensity of TRFS-green correlated well with the endogenous TrxR activity of different cell types, and the inhibition or depletion of TrxR by 2,4-dinitrochlorobenzene or immunoprecipitation, respectively. TRFS-green represents an interesting probe prototype for development of oxido-reductase specific fluorogenic substrates for biological studies.

A series of selenyl-sulfides have also been developed to selectively functionalize cysteine side chains [80, 81]. These molecules combines the chemoselectivity of Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a compound harboring a Se-N bond that is targeted by GSH, and a rodhamine moiety as fluorophore that upon removal of the ebselen group increases fluorescence emission at 550 nm. The first generation of thiol-probes used rhodamine 6G as signaling molecule and displayed a higher (2- to 3-fold) sensitive towards protein thiols from Trx, GR and metallothionein than for GSH [80] (Fig. 2). *In vitro*, the detection limit for GSH was in the one-digit nM level. Cell imaging of human liver cell lines from normal (HL-7702) or tumoral origin (HepG2) incubated with only 0.5 μM probe was successful and, intriguingly, revealed a marked difference in the thiol content of both cell lines. An improved second generation of this probe, named Rh-Se-2, was designed to achieve a lower background/signal ratio [81]. The probe presented a spirocyclic form that yielded a 170-fold increase in fluorescence intensity upon Se-N bond cleavage. As for the related former compound, Rh-Se-2 sensed a different thiol status in HL-7702 and HepG2 cells and detected intracellular thiol depletion induced by NEM treatment.

Probes based on nucleophylic substitution by thiol

Another class of probes relies on the irreversible modification of the thiol group. Most of these alkylating agents involve alkyl halides or Michael acceptors (see next section), which should be used under controlled reaction conditions to avoid non-specific labeling of proteins or metabolites. The probes were developed to label low molecular mass thiols present in biological fluids or released from lysed cells, and in most cases a separation step by HPLC or capillary electrophoresis is required to identify and quantify the modified thiol-species. Some of the most commonly used thiol-labeling agents include: monobromobimane (mBB), 4-fluoro-7-sulfamoylbenzofuran (ABD-F), 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic ammonium salt (SBD-F), 5-iodoacetamidofluorescein (5-IAF), 2-chloro-1-methylpyridinium iodide (CMPI) and 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT). Substitution of

the halogen atom by the thiol group yields conjugates that emit fluorescence (mBB, ABD-F, SBD-F and 5-IAF) (Fig. 4) or absorb UV-light (CMPI and CMQT). The first class of probes is preferred for its higher sensitivity (pM range), although interference is an issue in excess of probe. By means of a well designed experimental approach, Hansen et al. were able to quantify the thiol-disulfide redox status of low molecular mass thiols and of soluble and integral membrane proteins from two mammalian cell lines of extended use that were exposed to diamide [69]. Starting from cell lysis under acidic conditions (10% TCA), which protects free thiols from undergoing non-specific thiol/disulfide exchange, the soluble fraction containing low molecular mass components and the protein pellet were processed differentially to quantify the pool of oxidized and reduced species. Free thiols from both fractions are reacted at low pH and quantified with 4-dithiopyridine. For measuring the pool of oxidized thiols, free thiols in the sample are blocked with NEM, then the (mixed)disulfides are reduced with sodium borohydride or tris(hydroxypropyl)phosphine followed by quantitative labeling of thiols with 4-dithiopyridine or SBD-F. SBD-F derivatized thiols of low molecular mass (GSH) are then separated and quantified by HPLC. The strong solubilizing conditions (e.g. 5% SDS or 6 M urea) used to resuspend proteins from the TCA-pellet allows to recover membrane proteins. The study confirmed that the majority (>90%) of the protein sulfhydryl groups are reduced in non-stressed cells and disclosed differences in the absolute content of GSH (30% higher in Hela than in HEK cells). Almost 4.5% of cellular GSH was found in the oxidized form, a value relatively high compared to previous reports on the cytosolic GSH/GSSG ratio [82, 83], but indicative that a substantial amount of GSSG may be present in secretory compartments and/or the mitochondrial intermembrane space [47]. An interesting finding of this study was that conversely to early assumptions, protein thiols constitute a redox active pool that counteracts thiol-disulfide stress. More recently, this method was implemented to the study of the intracellular thiol-disulfide status of proteins and GSH that accompanies the differentiation of B-cells into antibody-secreting plasmatic cells [70]. The work showed that during the differentiation process the ratio thiol/disulfide decreased more markedly at the protein (3.3-folds) than at the GSH (1.6-folds) level, and that the content of glutathionylated proteins and GSSG remained almost constant whereas intracellular GSH was depleted, the last explaining the decrease in the GSH/GSSG ratio.

The strong electro-withdrawing nitro group makes haloarenes extremely reactive toward thiols, although other sulfur species may also be modified in the presence of similar compounds, and capable to quench a nearby fluorophore [54]. Most of these probes include a sulfonamide or sulfonate ester that links the fluorophore to the dinitrophenyl ring and is target

of nucleophilic attack by the thiol, releasing the non-quenched form of the fluorophore. Fluorescein derivatives of 2,4-dinitrophenyl sulfonyl showing a null-background signal are available [84]. A pH- and temperature-stable analogue compound conjugated to a red-emitting fluorophore (Ex/Em 560/623 nm) proved to be selective against Cys and suitable for cell-based assays [85] (Fig. 4). Analogues of this probe displaying selectivity for selenoCys were designed by Maeda et al. [86]. A red-emitting styryl boron-dipyrromethene (BODIPY)/2,4-dinitrobenzenesulfonyl (DNBS) dyad specific for Cys detection was generated [87]. The fluorescence of the probe at 590 nm (Ex 520 nm) was increased by 46-fold upon reduction by biological thiols, preferentially Cys, and was independent of H^+ concentration in the physiological pH range. The reaction of the probe with protein thiols was not evaluated. The good permeability of the probe allowed the detection of changes in thiol status (*i.e.* Cys) in the cytoplasm of mammalian cells.

Two cyanine-based fluorescent thiol-specific probes, containing a dinitrobenzenesulfonamide group (DNBS) or a 5-(dimethylamino)naphthalene-sulfonamide group (DMANS), were prepared. The cyanine moiety confers the probe with the capacity to emit light in the near infrared range (e.g. 655-755 nm), which is ideal for in vivo imaging because it precludes biological damage by high-intensity radiation and allows deeper tissue penetration. Both probes permeated cell membranes and reacted with GSH, Cys and homoCys in Hela cells, although, DMANS showed a higher preference for GSH over Cys and homoCys. DMANS reported changes in intracellular thiol content in response to H_2O_2 and LPS treatment. It should be noted that also in non-endothelial cells, LPS is known to trigger cell death by a mechanism involving oxidative stress [88]. A single dose of DMANS injected intravenously to C57BL/6 mice followed by in vivo imaging analysis 20 min after administration revealed strong fluorescence signal in the abdominal and supradiaphramatic cavities that was reverted by pre-treatment of the animals with NEM. No major interactions of DMANS with SH-group-containing serum proteins and biomolecules in the blood were observed, hence *ex vivo* analysis of organs showed a non-selective distribution of the probe in different organs (e.g. spleen, liver, lung and kidney). The potential of this probe for toxicological studies *in vivo* was provided by overdosing of mice with the analgesic and antipyretic agent acetaminophen, which revealed a significant and massive decrease in signal (or thiol depletion) in different organs.

Probes based on thiol-Michael addition click reaction

Thiolates are good Michael donors and readily reacts to an α,β-unsaturated system, such as that present in maleimide (2,5-pyrroledione). On the other hand, it must be recalled that the

reaction of maleimide is only selective towards the thiol group at pH 7 [89]. *N*-ethyl maleimide has good cell permeability and the ethyl group can be easily replaced by other substituents. Thus, fluorophores spanning a wide range of the emission spectra (ThioGlo series, Em 466-536 nm) were conjugated to maleimide generating extremely sensitive thiol-probes (*e.g.* detection limit for GSH of 50 fM with TioGlo3) [90] (Fig. 4). Other maleimide-based probes rely on conjugation to BODIPY or tetraphenylethene, which yielded highly sensitive thiol-probes that detected nM or 1 ppb thiol, respectively [91]. More recently, a but-3-yn-2-one-based 7-diethylaminocoumarin fluorescent dye that is activated by thiol-mediated Michael addition was synthesized [92]. The probe detected very specifically Cys over homoCys and GSH, due to an isomerization of the compound that is favored by single aminoacid structures. The use of the probe for cell imaging was validated by confocal microscopy of human renal cell carcinoma 786-0 cells loaded with probe. Different Michael acceptors have been exploited for the development of fluorescent sensors of thiols [93, 94]. Some of the probes that were developed presented a high sensitivity (low to sub μM range) and fluorescence intensity upon thiol addition. The electron-deficient squaraine moieties have been used to produced thiol probes that presented fluorescence emission in the red (Em 640 nm) to near infrared (Em 730-802 nm) spectrum and were reactive towards Cys and GSH [95, 96]. The spectroscopic features of the probes make them potentially suitable for deep-tissue *in vivo* imaging.

Metal-based thiol probes

Metal-based complexes were also developed as thiol sensors [53]. Among those that were tested in biological systems are: a cerium/quinine [97, 98] or cerium/$Ru(phen)_3^{2+}$ couple [99], a Cd/Te quantum dots-Hg^{2+} system [100], a Ru(II) poly(1,10-phenanthroline)-DNBS complex [101], a Ir(III)-DNBS complex [102]. The reduction of Ce(IV) by Cys or GSH produces the chemiluminiscent form of cerium, namely Ce(III). FRET between the weak luminiscence of Ce(III) and quinine (Ex 300-400 nm, Em 450 nm) allows amplification of the signal. The system was highly sensitive and specific to detect concentrations of Cys in the low nM range and in a 1000-fold diluted serum sample. In the quantum dots method, the fluorescence of the nanoparticles is to a great extent quenched by Hg^{2+}. The high affinity of Hg^{2+} to react covalently with thiols releases the quenching. The reactivity of the system followed the order GSH > homoCys > Cys, with detection limits of 0.1 and 0.6 μM for GSH and Cys, respectively. The applicability of the system, or optimized versions thereof, for intracellular detection of thiols remains to be investigated. When exposed to thiols from a

lysate of Hela cells, the sensor displayed a response according to the type and magnitude of the treatment (*e.g.* incubation with thiols or NEM). Phosphorescence of a Ru-phenathroline/DNBS complex is switched on selectively by Cys over other aminoacids. The DNBS-exscinded sensor showed a 90-fold enhanced phosphorescence (Ex/Em 455/598 nm) and a long luminescence lifetime, which can eliminate the interference from the short-lived background fluorescence and improve the signal-to-noise ratio (Fig. 5). Luminescent imaging of intracellular thiols was successfully tested in NCI-H446 cells [101]. The same principle of "off-on" activation and read-out advantages apply for the Ir(III)-DNBS complex that emit phosphorescence upon reaction with Cys or homoCys. On the other hand, this was the first work reporting imaging of biothiols (Cys/homoCys) in living cells with a heavy metal-based probe [102]. Using the same prototypic probe, a Ir(III) complex conjugated to a 2,2'-biquinoline and two-functionalized 2-phenylpyridine ligands with an α,β-unsaturated ketone moiety as the thiol reaction site was designed and synthesized for one- and two-photon microscopy applications. A nanoprobe was prepared by absorbing the probe to silica nanoparticles, which allowed the ratiometric detection of biothiols in absolute water solution and living cells [103]. Another thiol-probe relies on Michael addition of biothiols to analogues of the 7-nitro-2,3-dihydro-1H-cyclopenta[b]chromen-1-one (non fluorescent) that generate the fluorescent 4-nitrophenolate (Ex 405nm) [104]. The probe reacts very fast (within 10 sec) with low molecular mass thiols, displaying a detection limit in the sub-μM range and capacity to detect thiol distribution in zebrafish organs and structures.

Enzyme-based method to detect Cys

An enzymatic method to quantify the total amount of free/reduced and disulfide/oxidized L-cysteine is based on the biosynthetic pathway of D-luciferin that uses L-cysteine to generate the firefly bioluminescent substrate luciferin. The bioluminescence method has a lower detection limit of 0.26 μM and was suitable to determine Cys in serum samples [105].

Redox Western blots

The thiol/disulfide redox status of proteins containing redox active cysteines can be quantified using Western blot techniques that allow the separation, labeling and immune detection of the redox species based in their differential migration pattern on gel electrophoresis ran under native or denaturing conditions and selective tagging (Fig. 6). To distinguish the reduced from the oxidized species, the active site thiols are reacted with an alkylating agent that should modify the physicochemical properties of the protein (*e.g.* charge, mass). In some cases, the migration shift of the reduced and oxidized species is

evident enough that no further modification of the molecular target is required, albeit free-thiols must be blocked to prevent artifactual oxidation of protein thiols. The most common Cys-modifying reagents used are iodoacetamide (IAA), 4-acetoamido-4´-maleimidylstilbene-2,2´-disulfonic acid (AMS) and different types of pegylating reagents (*e.g.* maleimide-polyethylene glycol MW 1000-20000 Da, mal-PEG). IAA adds one negative charge per thiol, which increases the electrophoretic migration of the modified protein (corresponding to the previously reduced form) with respect to the unmodified (oxidized) form. The incorporation of AMS to a thiol increases its mass in ~500 Da allowing the separation of the modified reduced species (higher mass, lower electrophoretic migration) from the non-modified or oxidized form. Similarly, the addition of mal-PEG to the protein increases it mass according to the size of the PEG used. Due to the high solvation of PEG derivatives, the PEG-modified proteins commonly present aberrant migrations on polyacrylamide gels. Therefore, low molecular mass mal-PEG are preferred and, if available, controls with the recombinant protein are recommended. Because cell lysis is performed in the presence of a buffer containing an excess of the alkylating agent or under strongly acidic conditions (10% trichloroacetic acid), non-specific thiol/disulfide exchange reactions are prevented to a great extent. Moreover, alkylation takes place under denaturing conditions, which allows the modification of thiols independently of their reactivity and accessibility to the solvent. After electrophoretic separation under native or denaturing conditions, the bands containing the target protein are identified in the blotted membrane using specific antibodies. If antibodies against the target protein are missing, expression of a tagged-version can help to overcome this problem. Quantitative measurements are performed by densitometric analysis of the blot bands.

Just to mention a few, this immune-based method has been successfully applied to investigate the redox state of Trx isoforms located in different subcellular compartments (*e.g.* cytosol, mitochondria and nucleus) [106-110], of PDI and MlnI, a protein with redox active Cys participating in the ER-mediated degradation of proteins [111], and of oxidoreductases that control the thiol-disulfide state of proteins in the periplasmic space of gram (-) bacteria [112, 113].

Such subtle changes in mass or charge are difficult to detect on larger proteins by this method. An alternative, though laborious, technique to overcome this problem is based on the use of biotin-conjugated iodacetamide to selectively label mammalian TrxR [114, 115]. The modified protein is pulled-down from the cell extract using anti-TrxR antibodies, separated

by SDS-gel electrophoresis, blotted and revealed using stretptavidin-HRP and anti-TrxR antibodies. With this technique, Kim *et al.* demonstrated that stimulation of Hela cells with TNF-α induced the oxidation of mitochondrial TrxR [115].

Redox proteomics

Proteomic-based approaches offer the advantage of performing a large scale, thorough, quantitative and high throughput sampling of the protein repertoire of cells. In addition, mass spectrometry analysis allows distinguishing specific posttranslational modifications in single proteins. This approach has been applied in multiple studies aimed at identifying and quantifying proteins with reactive thiols and, when combined with specific probes or enzymatic systems, for the identification of selective posttranslational modifications and partners of master thiol/disulfide oxidoreductases (*i.e.* Trx and Grx) [116-132]. For an updated review on mass spectrometry techniques applied to study thiol chemistry and signaling please refer to [133-136].

One of the first studies that were conducted on a redox-proteomic basis aimed at identifying protein with thiols undergoing *S*-glutathionylation. One of the methods consisted on the specific reduction of mixed disulfides by Grx, their reaction with *N*-ethylmaleimide-biotin, affinity purification of tagged proteins, and identification by proteomic analysis [116]. The other method used (35)*S*-radiolabeled GSH followed by non-reducing 2D-electrophoresis and detection of labeled proteins by phosphorimaging and their identification by mass spectrometry techniques [117]. Insult with different oxidants modified the overall redox proteome of the cells and although the precise redox status of the target proteins was not quantified, these works highlighted the potential of the technique for quantitative proteomic approaches. Few years later, a differential thiol trapping method that relied on alkylation of protein thiols in non-reduced and reduced cell extracts with IAM or its C^{14}-labelled variant, was used to identify proteins of *E. coli* containing cysteine residues susceptible to oxidation [121]. The authors demonstrated the suitability of this technique for quantitative determinations of redox state using lipoamide dehydrogenase and the alkylhydroperoxidase C as example. The ratio of oxidized/reduced proteins was estimated by densitometric analysis of protein spots from the differential 2D-gels and was used to extrapolate the corresponding redox potential of the proteins at intracellular level. The redox potentials for the dehydrogenase and peroxidase inferred from this method agreed well with values obtained by other techniques. Lemoan *et al.* combined the use of *N*-(6-(biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide to purify oxidized protein thiols and *N*-[14C]ethylmaleimide to

quantify this oxidation in the cytoplasmic proteome of *Saccharomyces cerevisiae* [122]. The redox status of several proteins could be estimated in wildtype, Trx-defective and wildtype H_2O_2-treated yeasts. The comparative analysis of the thiol-proteome of these cells led to two important findings that illuminated future work in the field of thiol-mediated redox regulation: the Trx and Grx system operates in different pathways and H_2O_2 does not cause a massive oxidation of proteins but targets a subset of them.

Hagglund *et al*. employed iodoacetamide-based isotope-coded affinity tag (ICAT) coupled to quantitative liquid chromatography and mass spectrometry (LC-MS) to disclose the identity and quantify the extent of protein-disulfides that are targeted by Trx in plants [123] (Fig. 7). Leichert and coworkers designed a thiol-trapping technique, termed OxICAT, to identify, quantify, and monitor oxidative thiol modifications *in vivo* [124]. The technique consists on the sequential labeling of thiols with nonradioactive (C^{12}) IAM followed by reduction of oxidized thiols and alkylation with a 9-Da-heavier isotopically heavy IAM (C^{13}). After trypsin digest of the sample, the biotin labeled peptides are purified by affinity and subjected to analytic and quantitative mass spectrometry analysis. The reducing agent used in the intermediary labeling step can be chosen to detect specific Cys oxidations, for instance, replacing TCEP for Grx or ascorbate would allow the detection of glutahionylated or nitrosylated proteins, respectively. Since the OxICAT technique allows measuring the absolute ratio of reduced and oxidized protein in the same sample, changes in protein expression or stability will have no influence in the analysis. In agreement with previous studies, OxICAT revealed that only a minor pool of proteins was partially (<20%) oxidized in *E. coli* and the degree of oxidation of alkyl hydroperoxidase C, thiol peroxidase and glyceraldehyde dehydrogenase was quantified. The technique probed also useful to reveal oxidant-specific response of the tiolome and identified novel redox regulated proteins that allow dissecting the yeast "redoxome" [125]. The OxICAT method was further applied to study thiol modifications originating from nitrosative stress, therefore it was named NOxICAT [126]. In an attempt to improve the resolution of the technique, Held *et al.* developed a highly sensitive method denominated OxMRM, that integrates protein purification, differential alkylation using a stable isotope version of NEM with 5 deuteriums, and multiple reaction monitoring (MRM) [128]. OxMRM displayed a remarkable sensitivity for detecting oxidative modifications present in proteins of low abundance and/or with multiple Cys residues. The superior performance of OxMRM with respect to OxICAT can be ascribed, to a great extent, to the small size of the probe that provides minimal interference with the alkylation reaction and antibody recognition during immuneprecipitation. Using cell

extracts of MCF7 and HCA2, the potency of the technique was validated with p53 as target molecule, a low abundant protein with a very short half-life (6-30 min), and then further extended to characterize oxidative modifications in PTP1B.

In another approach, the difference gel electrophoresis (DIGE) technique was adapted to identify and quantify redox changes in proteins by the use of thiol-reactive fluorescent tags [137, 138] (Fig. 7). The new approach was named redox-DIGE and consists on labeling protein thiols in control and study samples with two different fluorescent dyes (Cy3 and Cy5) containing thiol-reactive maleimide groups, followed by detection of changes in the relative fluorescence on a single two dimensional gel and subsequent protein identification by mass spectrometry. Similarly to other techniques, the free thiols are first blocked by NEM and the oxidized cysteines can then be reduced by TCEP, DTT or specific oxidoreductases prior to fluorescent thiol-tagging. The method was validated using preparations of rat heart mitochondria and allowed the identification of significant changes on protein thiol oxidation even at the lowest oxidant concentrations (2.5 μM H_2O_2 and 10 μM *S*-nitroso-*N*acetyl-DL-penicillamine). The major advantage of this technique lies on the possibility to compare multiple samples on the same gel, which improves reproducibility and quantification. Nevertheless, this work underscored the importance of a careful optimization of the procedure, which includes labeling time, concentration of reagents, numbers of replicates and proper controls, to maximize statistical significance when the method is intended for quantitative purposes.

Fluorescent protein-based redox-biosensors

The discovery of the green fluorescent protein (GFP) from *Aequorea victoria* together with the boom of the recombinant DNA technology and the increased need in the field of cell biology for gaining spatiotemporal resolution of biochemical events has prompted the development of genetically encodable fluorescent biosensors (Table 1).

Following this trend, almost a decade ago, Østergaard and coworkers reported the engineering of the first genetically-encoded redox biosensor, a yellow fluorescent protein (YFP) capable to detect perturbations in the intracellular thiol/disulfide redox status of *E. coli* [48] and yeast [31]. The so called "redox YFP" (rxYFP) is a mutant to which a redox sensitive molecular switch consisting of a pair of carefully positioned (surfaced exposed and in proximity to the chromophore) cysteine residues has been introduced. The cysteines undergo reversible thiol/disulfide exchange with the medium, which translates into an also fully reversible conformational change that affects the physicochemical properties of the

chromophore (Ex/Em 480/510 nm) [48]. Structural analysis of rxYFP suggested that the redox-induced conformational change modify the hydrogen bonding network around the fluorophore (originally a Ser-Tyr-Gly peptide) with a concomitant shift in the equilibrium between its neutral (phenolate) and protonated (phenolic) form, which translates into a decrease or increase in fluorescence under oxidizing or reducing conditions, respectively [48]. The biosensor was validated using bacterial and yeast strains defective in Trx, Grx and GR as well as different redox stimuli and complementary quantitative techniques (HPLC-based assessment of GSH/GSSG pool and redox-Western blot of rxYFP). The main conclusions obtained from the use of the biosensor included the confirmation that: i) it was possible to monitor dynamic redox changes on real-time without disrupting cell integrity; ii) rxYFP equilibrates with the GSH/GSSG pool, and not the Trx-system, *via* Grx activity, iii) at intracellular level, rxYFP responded dynamically to redox stimuli; iv) under physiological conditions, the cytosol is a highly reducing environment with low levels of GSH- and protein-disulfides. Another interesting finding from these studies is the fact that Grx catalyzed very efficiently the oxidation of the biosensor, suggesting that under certain conditions Grx may operate as thiol oxidase (*i.e.* glutathionylation of protein targets).

Few years later, the laboratories from S. J. Remington and R. Y. Tsien created a series of redox-sensitive variants of GFP (named roGFP) with improved features [82, 139]. For the sake of this review, hereon will be discussed the results obtained for the most widely used GFP redox biosensor, roGFP2. In contrast to rxYFP, roGFP2 is significantly more refractory to changes in the pH of the medium and upon oxidation undergoes changes in its spectral properties that allows for ratiometric measurements (Ex/Em 405/510 nm oxidized form, Ex/Em 488/510 nm reduced form). The ratiometric behavior of roGFP2 improves the dynamic range for analyte detection and cancel-out any difference in fluorescence intensity arising from different expression levels of the biosensor, photobleaching or quenching, endogenous (*e.g.* pigments) or exogenous (*e.g.* light source, filters, etc) signal interference and variations on cell/tissue thickness. Similarly to rxYFP, *in vitro* roGFP2 reacted at very slow, physiologically negligible, rate with different oxidants (including GSSG) but readily reported reversible changes in the intracellular redox milieu of mammalian cells in response to redox stimuli, supporting the idea that an enzyme-mediated process contributes to the rapid equilibration of the probe. Another added value of these works was to demonstrate that roGFP2 can be targeted to distinct subcellular compartments wherefrom the reporter communicate signals, albeit with kinetics and magnitudes that differed between compartments.

Based on the direct [31, 48] and indirect [82] observations that rxYFP and roGFP equilibration with the GSH/GSSG pool relies on Grx catalysis and in order to improve the kinetic response of the biosensor, a second generation of biosensors fused to Grx was generated (Fig. 8). As expected, fusion of yeast Grx1p or human Grx1 to rxYFP [140] or roGFP2 [50, 141], respectively, increased by almost three orders of magnitude the kinetic response of the biosensors enabling determinations to be done in the msec range. The reactions catalyzed by Grx on roGFP2 involved the glutathionylation during the oxidative phase and deglutathionylation during the reductive phase. Formation of the GSH-roGFP2 mixed disulfide is thermodynamically unstable and rapidly solved by the formation of the intramolecular disulfide on roGFP2 and release of GSH. An additional advantage offered by the hybrid biosensor is that redox equilibration does not depend on the presence or relative concentration of the endogenous Grx. In this respect, the use of Grx-roGFP2 contributed to clarify that the GSH/GSSG ratio in peroxisomes and mitochondrial intermembrane space, two subcelullar domains with no or limited amounts of native Grx, is comparable to that of the cytosol [44, 45]. On the other hand, re-enforcing the idea that roGFP2 is insensitive to Trx, fusion of this oxidoreductase to the N- or C-terminus of the biosensor did not translate into a better response towards redox challenges [50]. roGFP2 (– 280 mV) [50] presents a midpoint redox potential more negative than that of rxYFP (– 265 mV) [140], which makes the first more sensitive to detect minor increases in the GSSG level. Applying the Nernst equation it results that 50% oxidation of roGFP2 is achieved with 44 nM GSSG when [GSH] = 1 mM and 17% oxidation of the sensor will be observed with 0.9 μM GSSG at 1 mM GSH [141]. In contrast, rxYFP will be responsive at far lower GSH/GSSG ratios at 10 mM GSH [48].

The main application of GFP has been its use as protein tag for localization studies. Exploiting this capacity, Pal *et al.* fused roGFP2 to $p47^{phox}$, an integral component of the NADPH-oxidase 2 complex located in the plasma membrane of cells from the immune system (*e.g.* neutrophils, macrophages) and endothelium, with the aim to get insights into the activation process of the oxidase [142-144]. This highlighted the great potential of the biosensors to be used as taggable redox indicators that, in such assemble, can provide valuable information on the site-specific redox environment that surrounds the protein or process under study. An important requirement is that the tagged-biosensor should not interfere with the activity and/or with interactions of the molecular target with putative partners or biological structures. Another interesting application of the redox biosensor has

been its use as topological reporter of an ER membrane protein [145]. Tagging roGFP2 to the N- and C-terminal of the target transmembrane protein is followed by ratiometric measurements of fluorescence intensity that allows discriminating the localization of the protein termini. Such application can be extended to proteins located in membranes from other cellular compartments as long as the GSH/GSSG ratio differs between both side of the membrane.

Soon after these validation experiments were conducted, redox-reporter cell lines and transgenic organisms from several species were generated [146-157] (for a full compilation please see [47]). In each case, the reporter cell lines have been very valuable to address different questions related to redox regulation of cellular processes in model organisms.

Some drawbacks associated to the use of the currently available redox biosensors are: i) despite biosensor variants with less reducing mid potential have been generated [158-160], the range of redox potentials that is currently covered is still narrow and precludes measurements under highly oxidizing or reducing environments/conditions; ii) transiently or stably transfected cell lines are required to conduct the studies; iii) some cell lines may be refractory to express in stable form the redox biosensor; iv) each new transgenic cell line has to be subjected to a careful phenotypic analysis to rule out any genetic or epigenetic interference of the sensor with critical cellular activities; v) low expression levels may compromise the dynamic range of the biosensor and, under this condition, the cellular pigments may become an major interference; vi) limited portfolio of biosensors (*e.g.* restricted to detect GSH/GSSG, H_20_2) and spectral variants thereof.

With respect to potential perturbations of the intracellular thiol-redox status originated from the expression of an exogenous redox-active biosensor, at least for wildtype yeast this has been shown not to occur [46]. However, Grx-roGFP2 is expected to compensate, to certain extent, the lack of endogenous Grx in cells or subcellular compartments devoid of this activity, as demonstrated for yeast mutants of Grx [43, 46]. The magnitude of this compensatory activity will certainly depend on both the expression level of the biosensor and the concentration of GSH in the corresponding compartment.

As for the midpoint potentials, a more varied color palette of redox biosensors will enable the simultaneous measurements of the GSH thiol/disulfide ratio in different compartments and/or coupled to other metabolic probes or fluorescence-based markers for high content analysis. In this regard, multiparametric measurement of Src kinase activity with a FRET-based fluorescence biosensor and GSH/GSSG ratio by Grx-roGFP2 has recently been attainable [161]. This work provided direct evidence that epidermal growth factor (EGF)-induced Src

signaling was negatively regulated by H_2O_2 *via* perturbation of the GSH/GSSG homeostasis, and highlights the feasibility to dissect the complex orthogonal control of signal transduction systems by redox-sensing mechanism [1].

This year, the first generation of red- and blue-shifted redox biosensors has been reported, though these sensors are far from optimal and present several limitations [160, 162]. The red-shifted redox-biosensor (rxRFP) uses the scaffold of a circularly permutated red fluorescent protein bearing one cysteine at the N- and C-termini of the protein that upon oxidation form a disulfide bond that stabilizes the chromophore leading to a 4-fold increase in fluorescence intensity at physiological pH (Ex/Em 576/600 nm) [162]. As other sensors based on circularly permutated green fluorescence related-proteins, the chromophore is accessible to solvent in the open conformation of the protein (*i.e.* reduced). In consequence, the pH markedly affected the fluorescence of rxRFP with low relative fluorescence intensities at pH < 8 and certain promiscuity to react with $ONOO^-$ and $O_2^{\cdot-}$. This phenomena, together with the fact that the sensor is not ratiometric, forces a complementary analysis of the parental cell line expressing a redox insensitive RFP (called pHRFP by the authors) to cancel out the influence of proton concentration or direct oxidative modification of the chromophore in quantitative measurements. As for rxYFP, the more negative midpoint potential of rxRFP makes this probe only sensitive to detect low to mid μM [GSSG] in compartments with a high [GSH] (*e.g.* >5 mM, cytosol). The blue-shifted biosensors consisted of Sirius (Oba-Qs; Ex/Em 370/425 nm), cyan fluorescence protein (Oba-Qc; Ex/Em 430/480 nm) and enhanced blue fluorescence protein (Oba-Qb; Ex/Em 380/440 nm) that were mutagenized to introduce redox active cysteines and additional residues that favored conformational changes in the biosensor and entail spectral changes in fluorescence [160]. A novelty of these sensors is that oxidation triggered an **o**xidation **b**alance **s**ensed **q**uenching of the fluorescence from which the acronym Oba-Q stems. Redox titration of Oba-Qc at different pH did not significantly alter the fluorescence intensity of the sensor whereas Oba-Qs emission was markedly affected at increasing pH. The authors predicted that similarly to other GFP-based biosensors, the Oba-Q series will increase their kinetic response to oxidants, in particular to GSSG, if fused to Grx. Indeed, *in vitro* Grx1-Oba-Qc showed remarkable changes in fluorescence intensity that were proportional to the concentration of oxidized glutathione and reverted by treatment with DTT. Oba-Qs and Oba-Qc present midpoint redox potentials of -232 mV and -249 mV, respectively, which make them more suited for oxidizing environments. The major

disadvantage of these sensors is the impossibility to perform ratiometric and *in vivo* (whole animal) measurements.

Other class of redox biosensors based on fluorescent proteins relies on the FRET-based mechanism. In these sensors, the FRET acceptor and FRET donor fluorescence proteins are linked by a module that contains the receptor structure that is responsive to changes in the thiol/disulfide state of low molecular mass thiols or oxidoreductases from the medium. Oxidation of the receptor triggers a conformational change in the linker that spatially approaches the donor and acceptor allowing FRET (Fig. 8). Kolossov *et al.* developed and optimized FRET-based redox biosensors [163-165] that use enhanced the cyan- and yellow-fluorescent protein as FRET pair connected by α-helical linkers of different length [*i.e.* $A(EAAAK)_nA$, with n= 2–8] and containing the Trx active site sequence WCGPCK, adjacent or dispersed vicinal cysteine residues. During oxidation and reduction the redox linkers (RL) undergo a dynamic helix-coil transition that confers the flexibility required to approach the fluorophores each other. *In vitro*, all constructs responded to redox stimuli with those containing dispersed vicinal cysteine presenting the higher values of FRET efficiency (70-90 %). The most efficient biosensors were expressed in CHO cells and responded reversible to diamide and DTT treatment. To allow quantitative measurements, the FRET signal was normalized to the fluorescence spectra performed for each fluorophore. Further improvement of the biosensor performance was achieved by a careful exploration of the factors enhancing signal and dynamic response [164]. This work yielded a probe with a 6-fold increase in FRET and oxidizing midpoint redox potential of -143 mV, suitable for monitoring redox changes in oxidizing environments. In fact, the new probe was employed in combination with roGFP1-iL to investigate the ER redox environment of mammalian cells [165]. Applying a similar concept, Yano *et al.* [166] developed Redoxfluor, a FRET-based redox biosensor that consist of the cerulean (a version of the enhanced CFP) and citrine (a version of the enhanced YFP) proteins linked by the cysteine rich domain of the redox-sensitive transcription factor YAP1 from yeast [167]. Redfluor was employed to investigate the redox state of the peroxisomes from CHO cells defective in peroxisome assembly. The probe indicated that this compartment is, unexpectedly, more reductive than the cystosol, as previously observed in plants [83], and, conversely, cells lacking assembled peroxisomes present a more reducing cytosol.

Finally, a novel redox biosensor specific for the main low molecular mass thiol of actinomycetes (e.g. *Mycobacterium tuberculosis*), namely mycothiol, has recently been developed [156]. The sensor (Mrx1-roGFP2) is based on a fusion of roGF2 to mycoredoxin 1

from *M. tuberculosis*, a mycothiol-dependent oxidoreductase with a CGYC active site that, as Grx with GSH/GSSG, actively equilibrates the pool of reduced/oxidized mycothiol with target proteins. The catalysis of the oxidoreduction of roGFP2 by the couple mycothiol/mycoredoxin follows the same mechanism described for the Grx-roGFP2 biosensor and only requires the N-terminal cysteine from the oxidoreductase. The bioprobe shows high specificity and sensitivity (nM range) for mycothiol. Mrx1-roGFP2 revealed that the intramycobacterial mycothiol/mycothiol disulfide ratio does not differ significantly between drug-resistant clinical isolates and drug-sensitive laboratory strains but differences were observed between fast- (midpoint redox potential of -300 mV) and normo-growing (midpoint redox potential of -273 to -280 mV) species. To monitor redox changes on the mycothiol pool in bacteria located at the intrafagosomal compartment of macrophages, the authors conducted the studies on NEM-treated and paraformaldehyde-fixed samples followed by flow cytometry or confocal microscopy analysis. The technique has been previously shown to yield data as reliable as those conducted with living cells [168, 83] albeit it does not provide real-time information. The sensor allowed to distinguish an heterogenous behavior of the redox state of intracellular mycothiol that was time- and strain-dependent. The time-dependence was associated to the internalization process that involved the passage of the engulfed bacteria through different vacuolar compartments (*e.g.* endosomes, lysosomes and authophagosomes) that generated redox gradients. As expected, immune stimulation of macrophages and drug treatment triggered oxidation of mycothiol, whereas the ratio of reduced to oxidized mycothiol correlated positively with drug tolerance. An additional outcome of clinical relevance from these studies is that inducing macrophage authopagy with rapamacyn led mycobacteria to accumulate in authophagosomes and thus become more sensitive to drugs due to the synergistic depletion of the pool of reduced mycothiol. More recently, this laboratory conducted infection assays with HIV on macrophages expressing the Grx-roGFP2 and found a major recalibration of cellular redox homeostatic pathways during persistence and active replication of the human virus [157].

Redox biosensors for Trx are missing, in part due to steric hindrance between regions of the oxidoreductase and the sensor that prevents the correct interaction between the partners that should render the sulphur atoms from the corresponding active sites mutually aligned to allow the S2N substitution [141]. A rationale, structure-based design of the biosensor will be required to make accessible the regions surrounding the cysteines of GFP2 to the dithiol from Trx.

Several thorough revisions on redox biosensors have been published and are recommend for readers seeking for additional information about the properties, mechanistic and kinetics aspects, protocols, applications and biological impact on the use of these genetically encoded fluorescent probes [47, 141, 168-171].

Concluding remarks

Measuring the oxidation state of thiols within live cells is challenging. As stated earlier, different redox systems provide kinetic control on the thiol/disulfide balance of low molecular mass thiols and macromolecules, they are not in equilibrium and present compartment-specific distributions. Nonetheless, many useful reagents, tools and methods have been developed for the quantitative assay of thiols and disulfides. The biochemical approaches that rely on chemosensors are methodologically easy and sensitive, although in some cases the reaction conditions must be carefully controlled to avoid non-specific side reactions. In this respect, though the bioorthogonality (*i.e.* the capacity of the reagent to react inside living systems without interfering with endogenous biochemical processes) of the chemosensors has steadily been optimized, the development of compartment- and protein-specific redox sensors is lagging behind. With few exceptions, these methods are for end-point measurements due to the irreversible reaction of the probe with the analyte, which precludes obtaining information of thiol/disulfide redox states at the dynamic equilibrium. In many cases, these methods requires cell lysis to separate and quantify the labeled species, which prevents the spatial resolution of the redox changes, may lead to artifactual signals and only pictures the global state of the species quantified. Thus, the thiol/disulfide status of particular redox pairs in a specific compartment cannot and should not be inferred from whole cell measurements.

The assessment of the redox status of individual redox pairs by quantitative redox Western blot is only possible if the reduced and oxidized form of the protein presents differential migration patterns during electrophoresis or any of them is susceptible to selective modification with thiol-reactive reagents. The technique is quite informative in reporting the thiol/disulfide state of single proteins whatever the subcellular domain they occupy. As drawback, the technique does not allow online monitoring of redox changes, because sample processing requires disruption of cell integrity, and for low abundance proteins and subtle redox changes, sensitivity may be an issue if the molecular target cannot be isolated or enriched.

Redox proteomic-based methods demonstrated to be quite powerful in identifying with extreme sensitivity and specificity proteins that undergo redox changes in relevant biologic conditions as well as to reveal with accuracy the type of oxidative modifications that took place and, eventually, the oxidoreductase responsible for reducing the oxidized target. In addition, quantitative measurements of thiol-redox status in target proteins that stem from cells exposed to different conditions are possible and were boosted by the introduction of isotopic labeling of thiols. However, precision is difficult to achieve because of moderate assay reproducibility, hence the values are reported as range, cut-off or relative to control conditions. Another limitation of the proteomic-based approaches lies on the time demanded for sample preparation and data analysis, which usually includes the processing of several controls and replicates, the accessibility to analytical instrument (only available at specialized core facilities) and the impossibility to perform real-time measurements of dynamic redox changes. Similarly to the redox Western blot, protein underrepresented or integral to membranes may not be detected.

Genetically encoded biosensors offer several advantages over the techniques above that only convey a "static" view of the thiol/disulfide status. First, the redox biosensors based on fluorescent proteins can be expressed in whole organisms and single cells or tissues, and be targeted to specific subcellular compartments. Thus, spatial resolution of intracellular redox changes is guaranteed so far the expression of the sensor is robust and its midpoint redox potential is within the range of concentrations and redox potential of the thiol/disulfide species to be detected. Spatial resolution of redox changes can be addressed using high resolution microscopy techniques and complementary high throughput analysis of cell populations can be performed by flow cytometry. The new "imaging flow cytometers" that combine the statistical power of flow cytometry with microscopic resolution appear as an attractive technological tool to explore the redox biology of cells with fluorescent biosensors. Because the methods based on biosensors do not require cell lysis, cells are kept alive during the experiment and can later be subjected to additional studies. In this respect, a cell sorting approach can be useful to isolate cells presenting peculiar phenotypes from a heterogenous population. In contrast to whole cell-based determinations, the non-invasive nature of the genetically-encoded biosensors allows compartment-specific measurements. Moreover, since the basis of the mechanism employed by the receptor and the fluorescence sensor involves thiol/disulfide exchanges and the concomitant conformational changes in the sensor are fully reversible, it is possible to monitor dynamic changes in the redox state of the thiol/disulfide

pair of interest. Two important features that a good receptor must have are: i) selectivity for the redox stimulus/signal and ii) kinetic competence to compete with the endogenous systems that shut-off the signal (e.g. GR or Grx) and to transfer efficiently the signal (redox state) to the sensor molecule. The selectivity has been attained by coupling Grx or Mrx to redox-sensitive fluorescent proteins, and kinetic efficiency of the signaling process has been improved by linking the receptor to the sensor module, which increases the chances of molecular collision. In analogy to the redox chemosensors, the biosensors are compatible with the simultaneous and multiparametric assessment of additional cell features (*i.e.* cell cycle, viability, apoptosis, receptors, metabolites, kinase activation, etc.) as long as the probes or biochemical methods employed do not display signal cross-interference. High content phenotypic screening of gene function, drug activity (applied to the screening of chemical libraries and studies on drug mechanism of action), and global metabolic profiling are promising areas of application for redox biosensors. In support of its inert effect in cells, so far, fluorescent-based redox biosensors were not reported to be cytotoxic. Some disadvantages associated with the use of genetically encoded biosensors is that, if not available from other laboratories, the reporter cell lines or animals have to be generated and phenotypically characterized, which is time-consuming. The characterization of the parental *vs*. transgenic organism is a must before the studies of interest are to be conducted, because any metabolic, growth or structural/morphological alteration in the novel cell line or organism may be indicative of an unwanted side effect caused by integration or expression of the ectopic copy of the biosensor. Such quality control will prevent a misinterpretation of the results. The number of redox biosensors available is rather limited. So far, the GSH/GSSG and the mycothiol/mycothiol disulfide are the only redox pairs for which fluorescent biosensors have been developed. Redox biosensors for Trx (or homologue redoxins from other organisms) are missing and would be needed to gain insight into the biological role of the second major thiol/disulfide redox system of cells. On the other hand, additional receptor modules can be added to the fluorescent sensor with the aim to obtain novel thiol/disulfide protein couples for investigation. A good example of the feasibility of this principle is provided by the chimera Orp1 (a glutathione peroxidase with a midpoint redox potential of -315 mV) -roGFP2 that enables monitoring H_2O_2 levels. A favorable redox potential and structural compatibility between the receptor and the sensor couple deems relevant to achieve an efficient thiol/disulfide exchange. Obviously, the number of thiol/disulfide redox pairs to be analyzed inside the cell with fluorescent proteins is finite and restricted to the availability of sensor modules with Ex/Em spectra compatible with simultaneous measurements.

The selection of the method to be employed for measuring thiol/disulfide ratios will be clearly dominated by the scientific question or hypothesis to be addressed and how ambitious it is in terms of technical resolution (*e.g.* from single protein studies to full proteomic approaches, from static to dynamic monitoring, from local to global assessments) and, not least, by the availability of reagents and equipments. An important point to consider is that the different techniques described above are not mutually exclusive but instead fully complementary between each other. Hence valuable and integral information can be obtained about thiol/disulfide mediated processes by combining different analytical methods on the basis of experiments properly designed. If so, novel and impacting discoveries are foreseen in the short-term whereas several biochemical assumptions and old dogmas will likely fell off while not withstanding the consistent power of these techniques. Deepening our understanding on the redox biochemistry that governs cells and organisms will foster metabolic engineering interventions in bioprocesses, drug discovery programs and translational research aimed to combat transmitted and non-transmitted diseases where redox perturbations and signaling play important pathological roles.

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Declaration of interest

The author reports no declarations of interest. The author alone is responsible for the content and writing of the paper.

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Figure Legends

Figure 1. Biological thiols of low molecular mass. The chemical structure of cysteine- (upper panel) and non-cysteine (lower panel) containing thiols is depicted. The disulfide form of trypanothione is shown with the spermidine moiety highlighted with an oval. The organosulphur metabolite lipoic acid (disulfide form) is commonly found covalently bound to proteins *via* lysine residues, where it plays an important role as prosthetic group (*e.g.* pyruvate dehydrogenase complex).

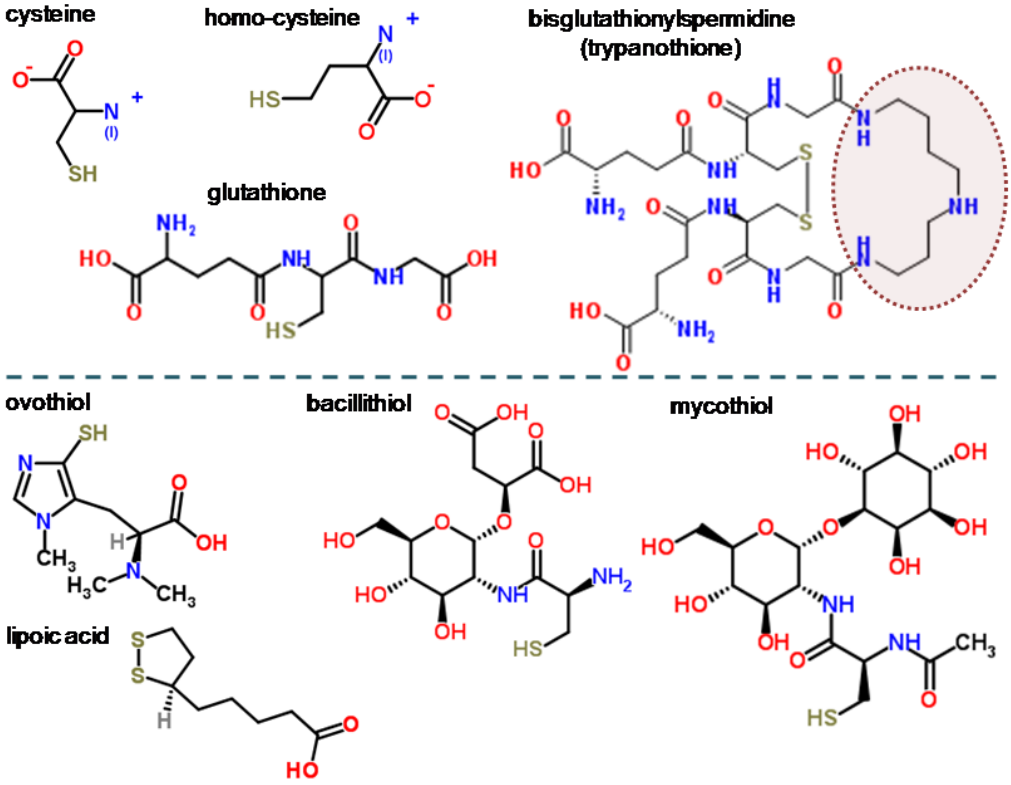


Figure 2. Chemosensors activated by thiol-mediated disulfide or Se-N cleavage. Upper panel: Representative chromogenic- and fluorescence-based probes are shown. Upon cleavage of thionitrobenzoic acid (DTNB) and 2,2´-dipyridyl (DPS) disulfides by thiols, reaction (1), the released molecule present a high absorption coefficient that allows the stoichiometry quantitation of thiols. In the selenium-containing probe, the phenyl ring quenches the fluorescence of the neighbor rhodamine 6G that is thetered to the aromatic group by a Se-N bond. Cleavage of this bond releases the fluorophore that emits light at 550 nm upon excitation at 523 nm. This probe shows preference for protein thiols. Lower panel: Ratiometric chemosensors. For the fluorescein-rhodamine (left) and Ratio–HPSSC (right) probes, reduction of the disulfide linker frees the fluorescence donor (fluorescein: Ex/Em 494/521 nm and coumarin: Ex/Em 350/459 nm, respectively) from the quenching effect of the acceptor molecule (rhodamine and porphyrin, respectively). Measurement of fluorescein-rhodamine FRET (Em 566 nm) and prophyrin (Em 650 nm) allows performing ratiometric determinations (e.g. ratio Em 521/566 nm and ratio Em 459/658 nm, respectively).

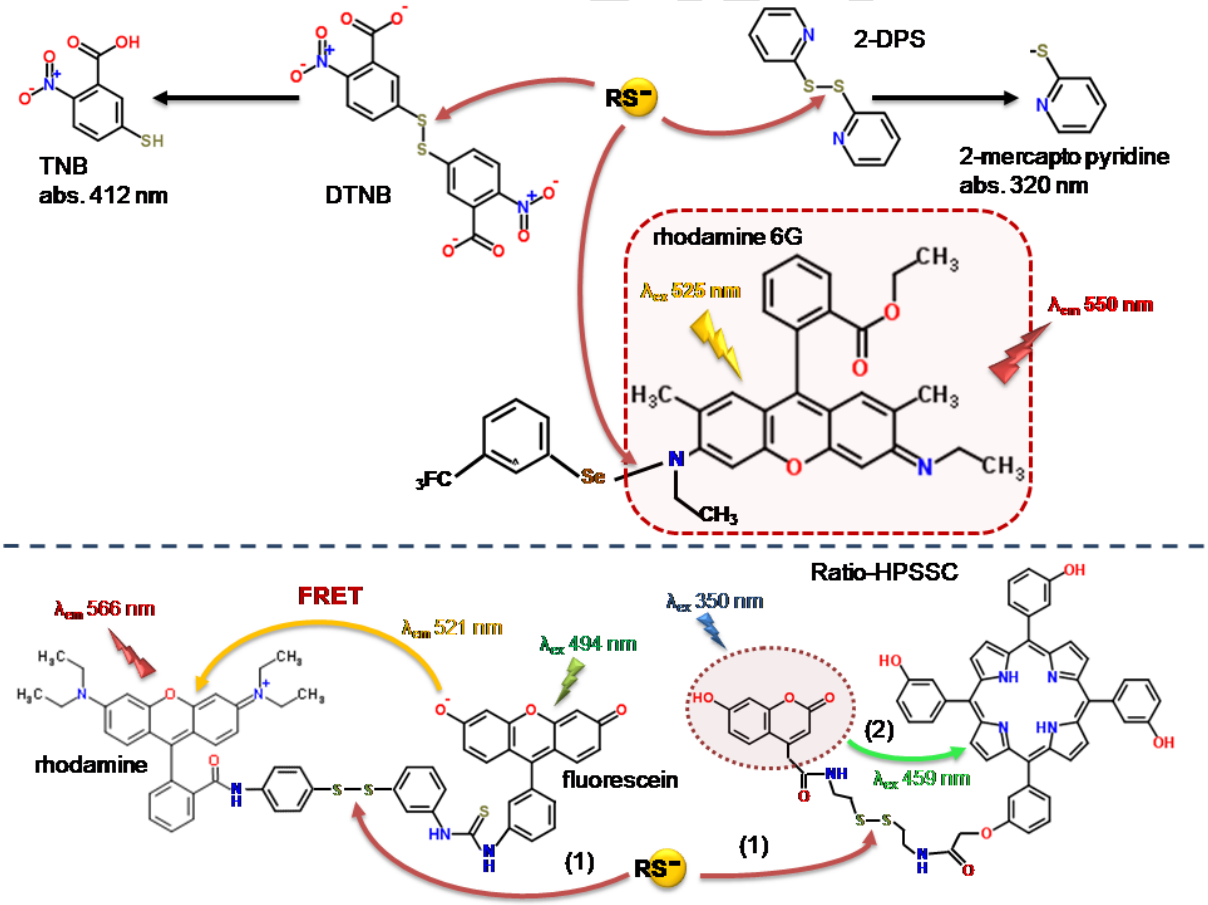


Figure 3. Chemosensors activated by thiol-mediated disulfide cleavage followed by cyclization release. Three representative thiol probes (ASS, RhoSS and ASGP-R) where the latent fluorophore is activated upon cleavage of a disulfide bond, reaction (1), followed by attack of the free thiol to the carbonyl carbamate, reaction (2). In ASS, the freed methylamino naphthalene group (highlighted in a box) emits fluorescence upon one- (514 nm) or two-photon (740-780 nm femtosecond pulse) excitation. The probe RhoSS contains a rhodamine 110 moiety that emits fluorescence at 530 nm upon excitation at 485 nm. The probe ASGP-R consists of a naphthalimide (highlighted in a box) fluorophore that has been additionally linked to a sugar moiety (galactose) to allow its receptor-mediated endocytosis by hepatocytes.

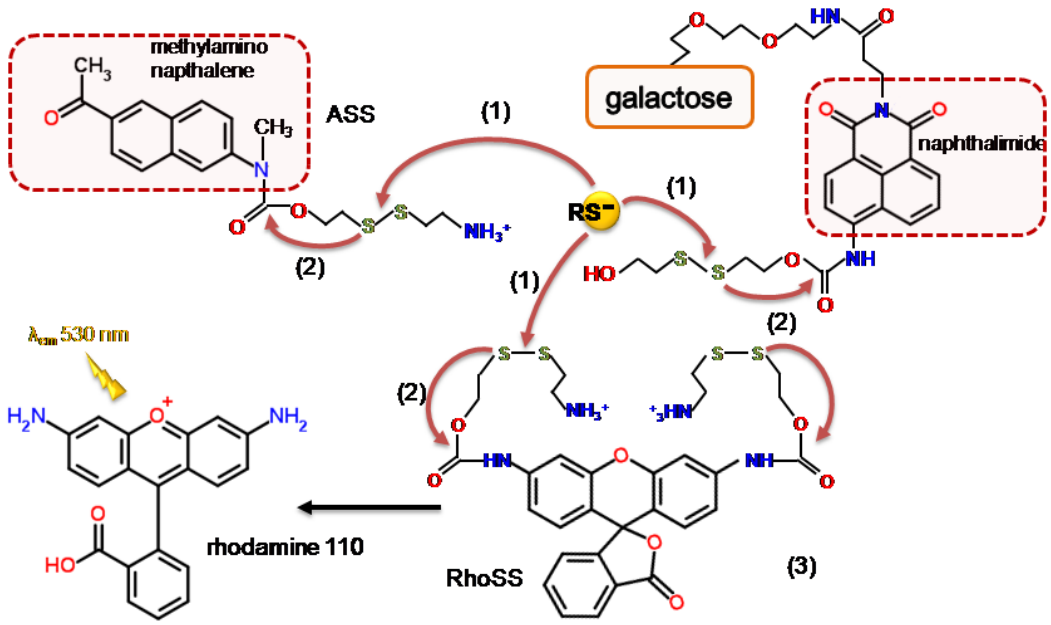


Figure 4. Chemosensors based on nucleophylic substitution and Michael addition by thiols. The halogen atom of monobromobimane (mBB) and 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic ammonium salt (SBD-F) undergoes nucleophylic substitution with thiol groups with the concomitant formation of a thiol-probe adduct that emit fluorescence at 490 nm and 510 nm, respectively. For the probes DNBS-Bodipy and ThioGlo 5, a Michael addition reaction of thiol groups with strong electron withdrawing molecules take places, which releases fluorescence quenching on the previously covalently bound fluorophore: Bodipy (Em/Ex 527/570 nm) and benzocumarin (Em/Ex 275/515 nm), respectively.

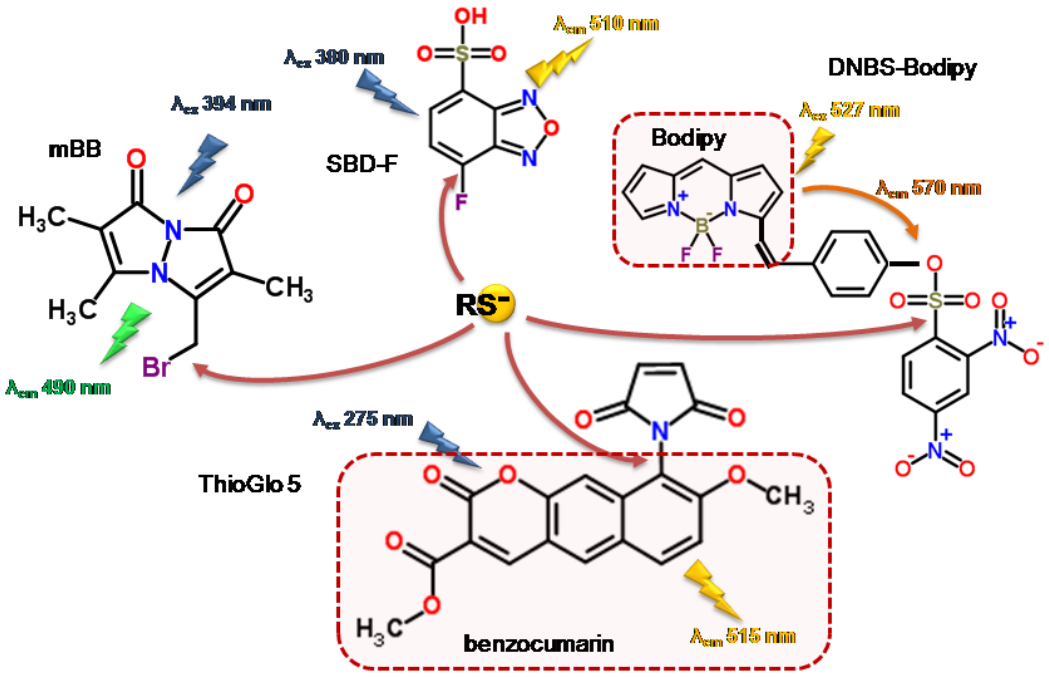


Figure 5. Metal-based thiol chemosensor. Off-on phosphorescent sensor consisting of a DNBS moiety that is covalently linked to a Ru(II) poly(1,10-phenanthroline) ring. Thiol-mediated cleavage of DNBS releases its strong electron-acceptor effect that quenches the phoshorecence of the Ru-phenanthroline complex.

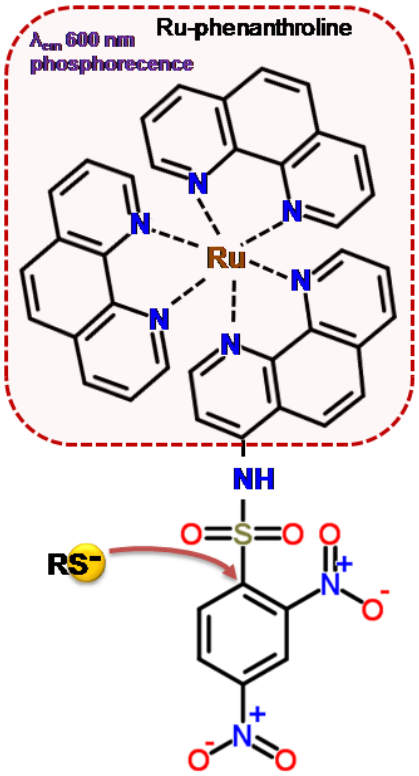


Figure 6. Strategies to detect thiol-redox state of proteins by redox Western blotting. Proteins from a biological sample are extracted (see text for further details on extraction conditions to freeze sample redox state) and subjected or not to different treatments that allow the detection of their different redox species. After electrophoretic separation on a polyacryalmide gel run under denaturing (SDS-PAGE) and non-reducing conditions and blotting into membranes, the target protein, irrespective of the treatment applied, is identified using specific antibodies. (A) The reduced and oxidized form of certain proteins presents different migration patterns on a SDS-PAGE that are observed without special labeling procedures (lane 1). (B) Free-thiols of proteins can be tagged with a reagent that increases their mass (e.g. 0.5 kDa for AMS: 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid, or several kDa for maleimide polyethylenglycol derivatives, not shown). (C-E) Oxidized species can be selectively detected by a procedure that consists of: (C) blocking thiol-free groups with an alkylating agent (e.g. N-ethyl maleimide, NEM), followed by (D) reduction of disulfides with a reducing agent and (E) tagging of nascent thiols with the labeling reagent (e.g. AMS). Note: the figure depicts the thiolate form of cysteine (S^-) as the reactive species undergoing modification.

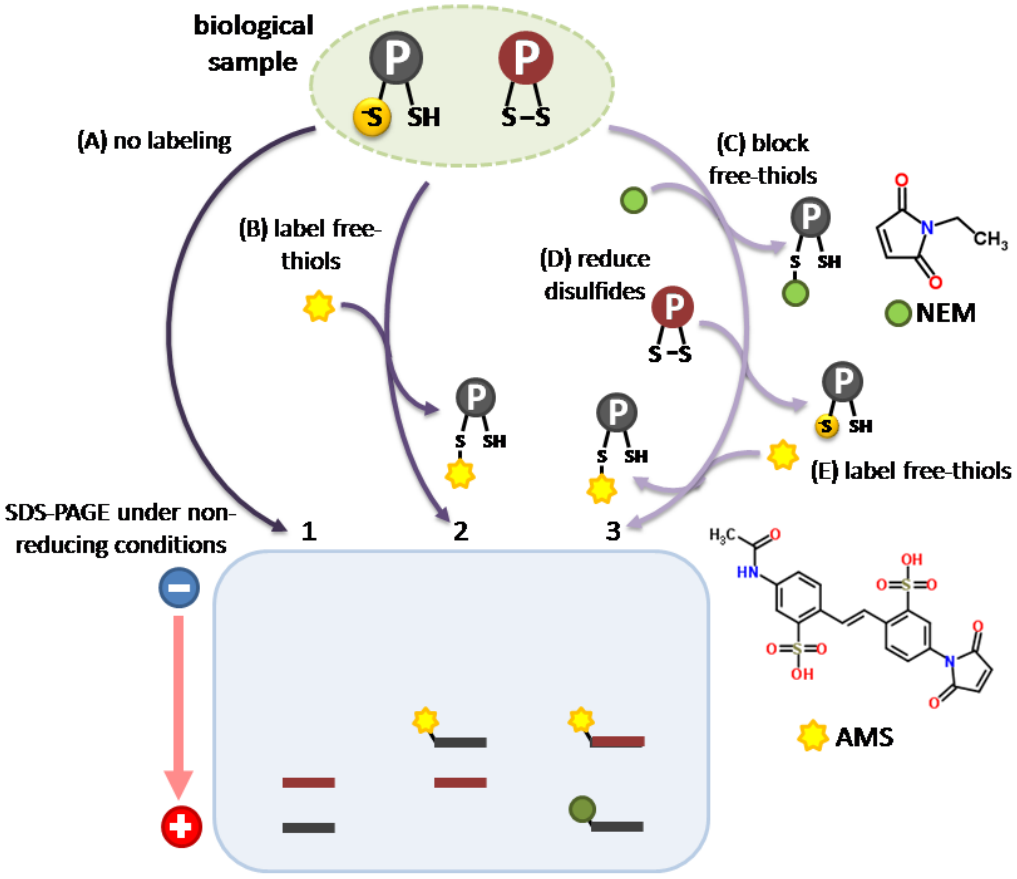


Figure 7. Redox Proteomics. The redox proteomic approaches rely on selective labeling of free thiols followed by two-dimensional and/or liquid chromatography (LC) mass spectrometry (MS) to detect and quantify thiol-redox status of proteins. The reagents commonly used to label thiols include non-radioactive C^{12} and C^{13} isotopes of iodoacetamide, deuterated- or C^{14}-NEM derivatives and maleimide conjugated to Cy3 and Cy5 fluorophores. After labeling the free-thiols of (reduced) proteins, the oxidized species are reduced and the nascent thiols are derivatized with a second labeling reagent. The molecules tagged to thiols differ in mass or spectroscopic properties, which allow distinguishing the differentially labeled protein species. Low abundance protein species can be enriched from the sample using biotin-streptavidin affinity purification on previously maleimide-biotin derivatized thiols. Several samples (e.g. subjected to different treatment conditions) can be analysed simultaneously using the redox proteomic techniques. See text for more details.

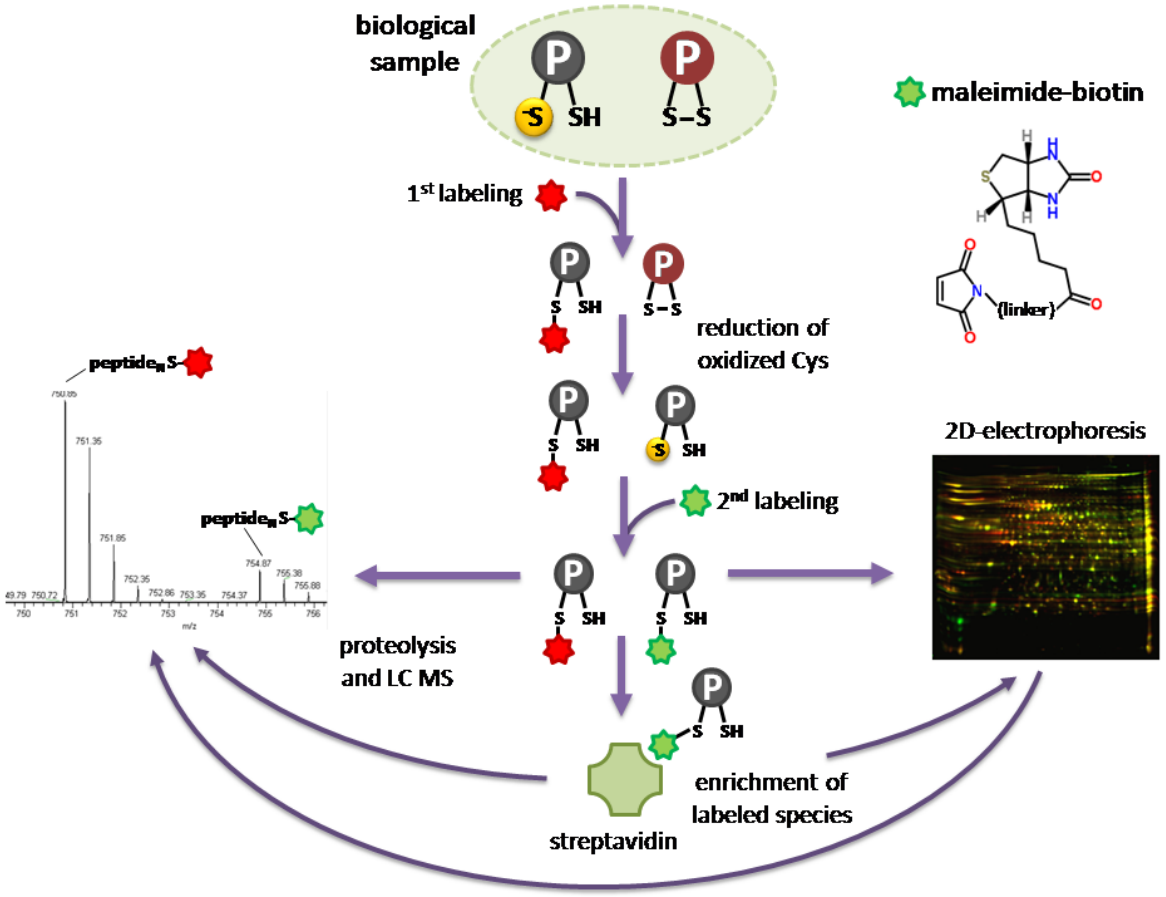
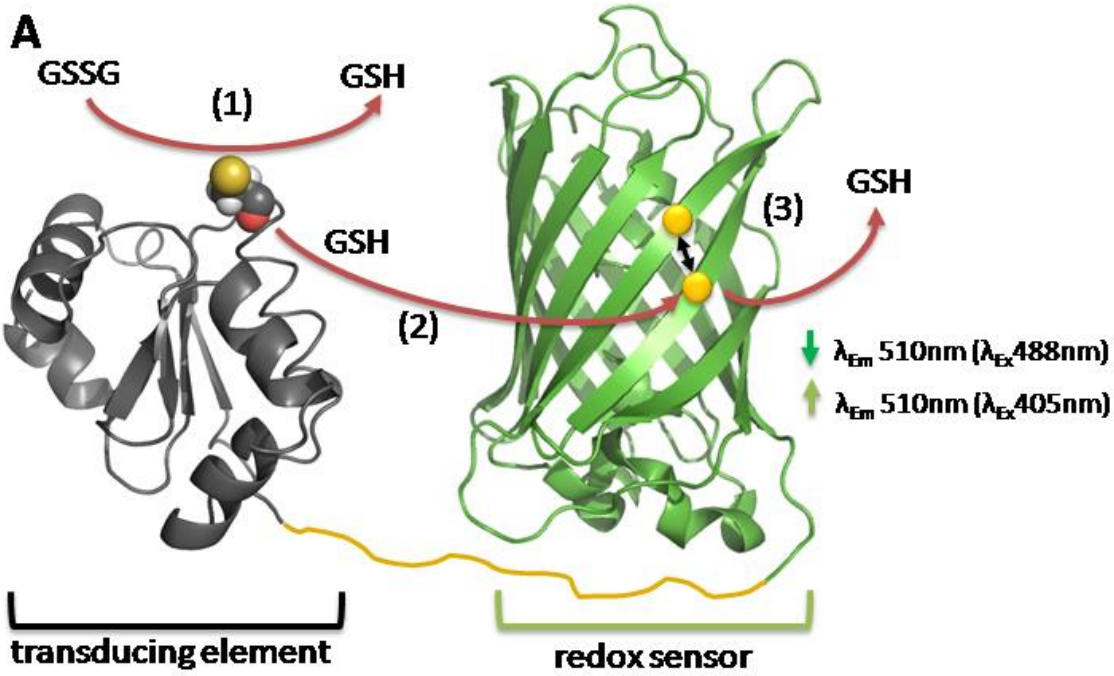


Figure 8. Redox biosensors based on fluorescent proteins. A) hGrx-roGFP2 consists of: the oxidoreductase glutaredoxin (hGrx, 3D structure shown in dark grey cartoon) that acts as transducing element conferring specificity for the thiol/disulfide couple GSH/GSSG, roGFP2 (3D structure shown in green cartoon) as the sensor element and a linker region (depicted with a yellow line) that connects both modules. Reaction of the N-term cysteine (residue shown as spheres) from the active site of hGrx leads to a transient glutathionylation of the oxidoreductase (hGrx-SG) and releases one mol of GSH (1). Thiol/disulfide exchange between hGrx-SG and roGFP2 (2) entails glutathionylation of a Cys residue (shown as yellow dot) from the sensor and the release of reduced hGrx. Finally, the thiol group of a vicinal residue attacks the glutathionylated Cys yielding an intramolecular disulfide on roGFP2 (oxidized form) and free GSH (3). The oxidation of roGFP2 triggers conformational changes in the environment surrounding the chromophore that cause a ratiometric decrease and increase in fluorescence intensity (Em 510 nm) upon excitation at 488 nm (absorption maximum for reduced roGFP2) and at 405 nm (absorption maximum for oxidized roGFP2), respectively. B) The CFP-YFP FRET-based biosensor consists of the cyan fluorescence protein (CFP, 3D structure shown in cyan cartoon) as FRET donor, the yellow fluorescence protein (YFP, 3D structure shown as yellow cartoon) as FRET acceptor and a linker region (depicted as orange line) containing redox active cysteine residues (yellow balls). In reduced state, the FRET phenomenon is negligible since fluorescence donor and acceptor are far away from each other to allow energy transfer. Upon an oxidative stimulus (e.g. GSSG), the cysteine residues of the transducing element are oxidized to intramolecular disulfides, which induces a helical folding of this region and a concomitant approximation of donor and acceptor modules allowing efficient FRET (Ex/Em 430/530 nm). Non-FRET signal serves as a normalizing factor.



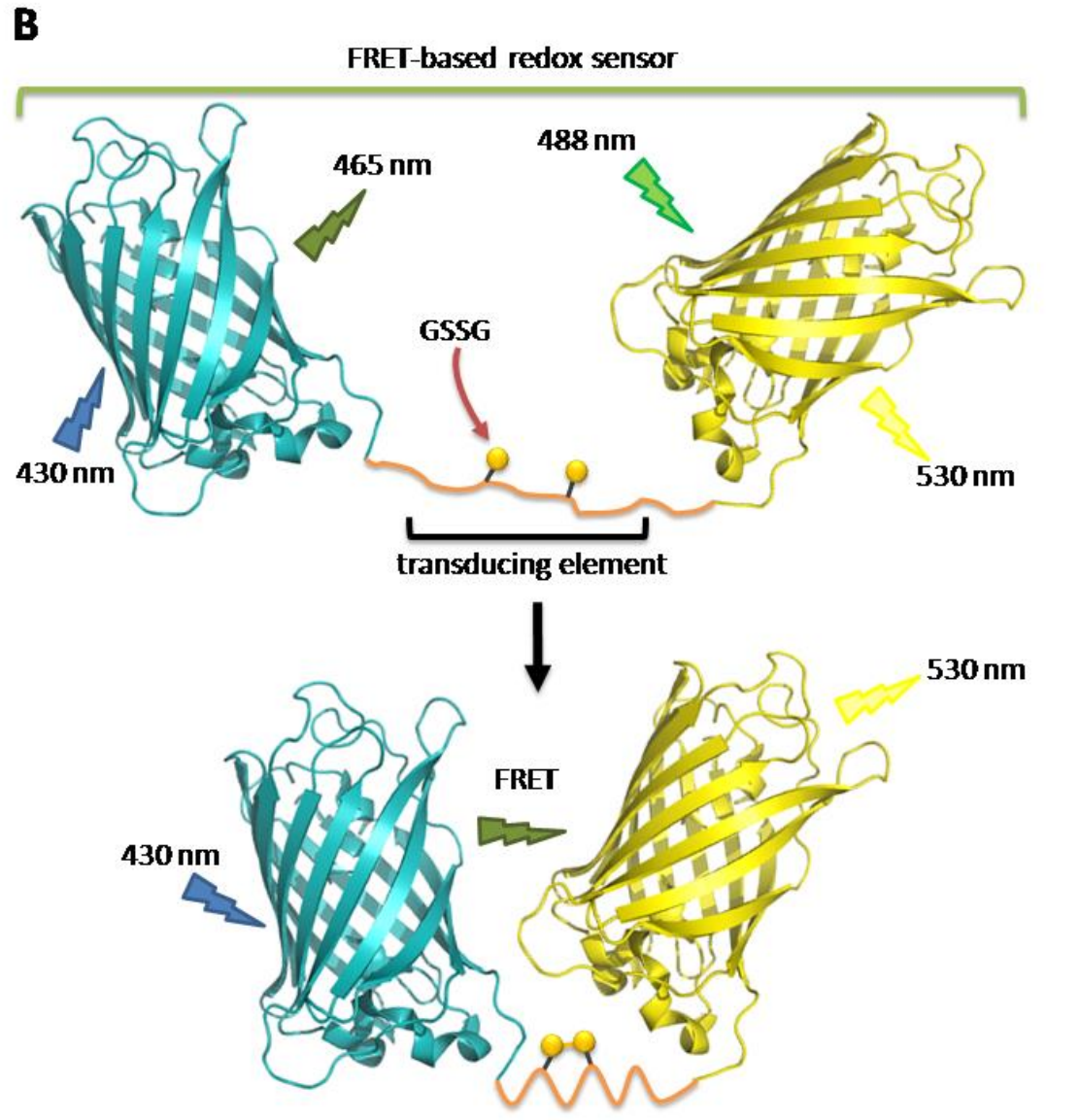


Table 1 Fluorescent-protein based redox biosensors.

	Oba-QS	Oba-Qb	Oba-Qc	roGFP			rxYFP		CFP-YFP	rxRFP
Transducing element	-	-	Grx1	-	hGrx (human Grx)	Mrx1 (*M. tuberculosis* mycoredoxin)	-	Grp1 (yeast Grx)	Cys-containing α-helical linker	-
Redox sensor (mutants redox sensitive)	Sirius	enhanced blue fluorescence protein (eBFP)	cyan fluorescence protein (CFP)	enhanced green fluorescence protein (GFP)			yellow fluorescence protein (YFP)		CFP-YFP pair	circular permutated red fluorescence protein (cpRFP)
λ Ex/Em (nm)	370/425	380/440	430/480	405/510, 488/510			480/510		430/530	576/600
Signal upon oxidation	Reversible fluorescence quenching			Reversible , ratiometric roGFP2: ↓ λ_{Em} 510 nm, for λ_{Ex} 488/405 nm roGFP1: ↑ λ_{Em} 510 nm, for λ_{Ex} 488/405 nm			Reversible ↓ λ_{Em} 510nm redox insensitive λ Ex/Em 450/510 nm is used for normalization		Reversible ↑ FRET ratiometric: λ Ex/Em 430/530nm vs. λ Ex/Em 488/530 nm	Reversible ↑ λ_{Em} 600nm
E^0 redox potential	-232 mV -251 mV for S147CG mutant	-263 mV	-249 mV	roGFP1-4: -280–299 mV roGFP1-R12 and roTurbo: -275 mV roGFP1iE, roGFP1iL, roGFP2iL : -229–236 mV			-265 mV		-143 mV	-290 mV
In vivo kinetic	≥ min	≥ min	≤ sec	≥ min	≤ sec		≥ min	≤ sec	ND	≥ min
Selectivity and sensitivity	*in vitro*: GSH/GSSG[a]	*in vitro*: GSH/GSSG[a]	GSSG μM GSSG	GSSG and several oxidants μM range	GSH nM GSSG	mycothiol (MSH) nM MSH_{ox}	GSH[a] μM GSSG	GSH nM GSSG	*in vitro*: GSH/GSSG[a]	*in vitro*: GSH/GSSG, and to minor extent $O2^{-}$ and $ONOO^{-}$
Advantages	Oba-QS suited for oxidizing compartments	Suited for reducing compartments	Signal stable at pH 6-8, rapid response	Works a wider pH ranges and redox potentials	Coupling to transducing element increases selectivity, sensitivity and kinetic response to low molecular mass thiols.		Redox sensor with highest quantum yield	High selectivity and sensitivity for GSSG	High dynamic range, suitable for highly oxidizing environments	Suitable for deep-issue imaging
Disadvantages	No ratiometric and not suited for deep-tissue imaging . Oba-QS is sensitive to pH. Oba-QS and Oba-Qb are susceptible to direct oxidation by diamide.			Response *in vivo* dependent on endogenous Grx availability .	Potential disturbance of GSH/GSSG ratio in compartments lacking Grx	Mrx1-roGFP2 is not suited for detecting MSH in oxidizing compartments	Response *in vivo* dependent on endogenous Grx availability. pH sensitive	Potential disturbance of GSH/GSSG ratio in compartments lacking Grx.	The response to physiological oxidants and reducing agents/proteins is unknown	Low dynamic range, pH sensitive, promiscuous activity against oxidants, no ratiometric, suitable for compartments with high [GSH]