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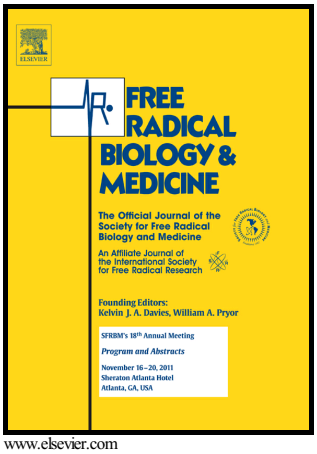

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Assessment of glutathione/glutathione disulphide ratio and *S*-glutathionylated proteins in human blood, solid tissues, and cultured cells

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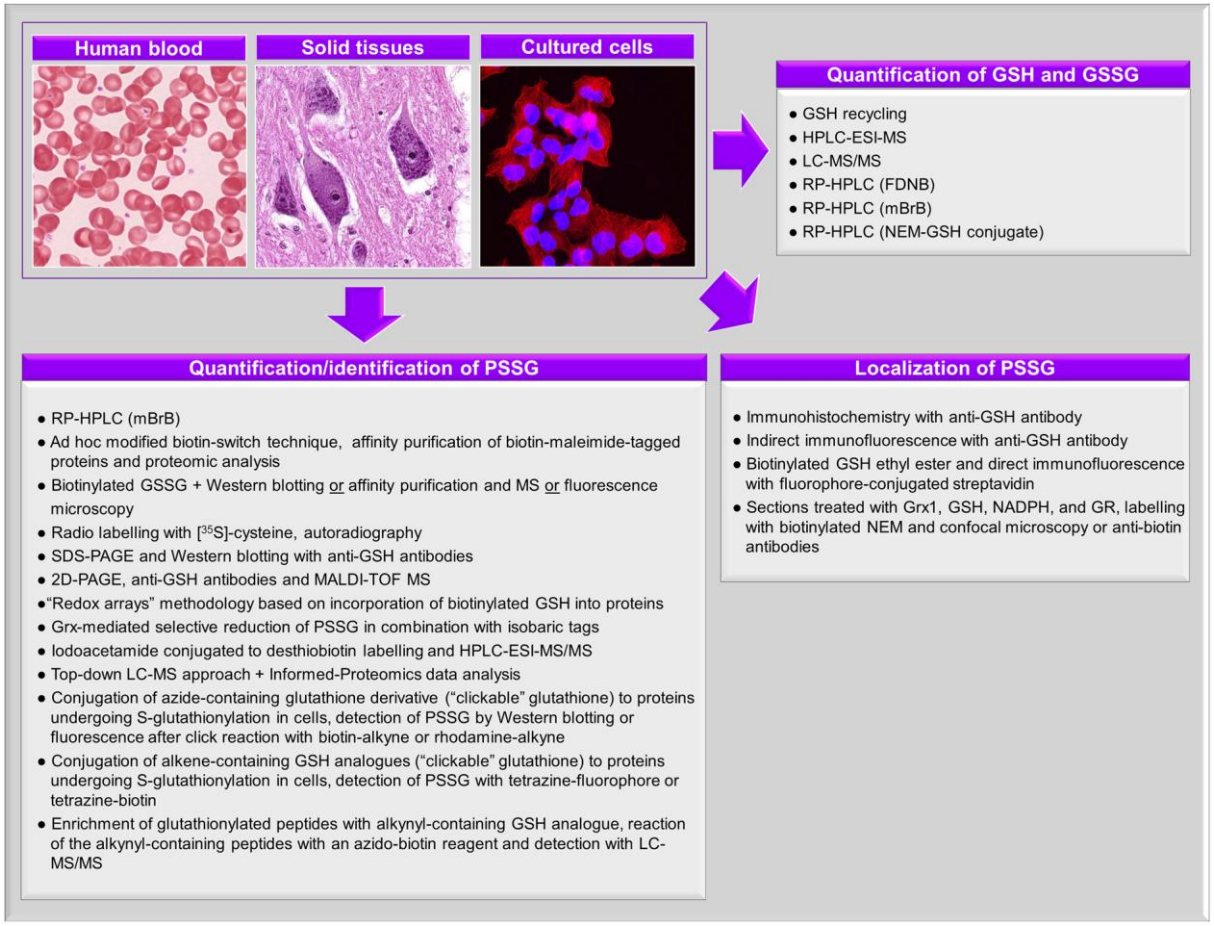
Running Title: Assessment of GSH/GSSG ratio and *S*-glutathionylated proteins

Abstract

Glutathione (GSH) is the major non-protein thiol in humans and other mammals, which is present in millimolar concentrations within cells, but at much lower concentrations in the blood plasma. GSH and GSH-related enzymes act both to prevent oxidative damage and to detoxify electrophiles. Under oxidative stress, two GSH molecules become linked by a disulphide bridge to form glutathione disulphide (GSSG). Therefore, assessment of the GSH/GSSG ratio may provide an estimation of cellular redox metabolism. Current evidence resulting from studies in human blood, solid tissues, and cultured cells suggests that GSH also plays a prominent role in protein redox regulation via *S*-glutathionylation, i.e., the conjugation of GSH to reactive protein cysteine residues. A number of methodologies that enable quantitative analysis of GSH/GSSG ratio and *S*-glutathionylated proteins (PSSG), as well as identification and visualization of PSSG in tissue sections or cultured cells are currently available. Here, we have considered the main methodologies applied for GSH, GSSG and PSSG detection in biological samples. This review paper provides an up-to-date critical overview of the application of the most relevant analytical, morphological, and proteomics approaches to detect and analyse GSH, GSSG and PSSG in mammalian samples as well as discusses their current limitations.

Graphical Abstract



Keywords: glutathione; *S*-glutathionylated proteins; thiol alkylation; proteomics; biotinylation; anti-GSH antibodies; fluorescent probes; clickable glutathione.

1. Introduction

Oxidative stress was firstly defined as an imbalance between pro-oxidants and antioxidants in favour of the former, leading to potential damage [1]. Therefore, an increased oxidation of some cellular (macro)molecules (protein amino acidic residues, lipids, nucleic acids, carbohydrates) may represent a mechanism of damage for the cellular functioning. Indeed, oxidative stress has been reasonably hypothesized to be a pathogenic and/or etiological factor of several serious human diseases, such as Alzheimer's disease [2−4], cystic fibrosis [5,6], and end-stage renal disease [7−9]. Moreover, oxidative stress is associated with ageing [10,11] and life-style factors, such as excessive alcohol consumption [12] and cigarette smoking [13,14]. However, there is growing evidence that reactive oxygen species (ROS) may play a possible physiological role as redox signalling messengers for some regulatory functions [15]. The basic mechanism for this is that ROS regulate specific and reversible post-translation modifications (PTMs) on target protein thiols [16]. The question is still open and deserves further investigation. In order to better evaluate the Janus face of ROS, the study of the oxidative modifications of thiols has a central role.

Glutathione (γ-glutamyl-l-cysteinylglycine, GSH) is part of a well-organized antioxidant defence system, which constantly counteracts the increased ROS production, thus minimizing oxidative damage to tissues and cells. In the human body, the liver is the primary organ for the *de novo* synthesis of GSH and is responsible for supplying 90% of circulating GSH [17]. The GSH concentration within hepatocytes (~10 mM) is the result of equilibrium between its synthesis and its efflux into blood plasma. Liver GSH varies as a function of diet, time of day, and body needs [18]. Skeletal muscle and red blood cells (RBCs) cooperate with liver to the synthesis of GSH. In human blood, most of GSH is located inside RBCs (~3 mM per RBC), since leukocytes, although they contain 2-4 mM GSH, represent only a small volume compared to RBCs. Human RBCs possess the enzymatic machinery to synthesize GSH and are important as biological carriers of GSH by *de novo* synthesis, contributing up to 10% of whole body GSH synthesis [19] and thus providing an

important detoxifying system within the blood circulation. A sustained export of GSH (about 21 nmol/h/ml RBCs) has been measured in human RBCs [20].

GSH is present within human/mammalian cells at millimolar concentrations (~1-2 mM in most cells) and, along with its oxidised counterpart, glutathione disulphide (GSSG), is the most abundant intracellular redox couple [21−23]. Its concentration varies not only depending on the cell type, but also during cell proliferation or in cancer cells in comparison with quiescent or not dividing cells [24,25]. The situation is somewhat different in extracellular matrices, such as human plasma, where a substantial amount of sulfhydryl groups is present as free cysteine, whereas GSH concentration is in the range of 2-20 μM [10]. In response to oxidative stress, two GSH molecules are oxidized to glutathione disulphide (GSSG), which is reduced back to GSH via GSSG reductase, at the expense of oxidation of NADPH, which in turn is re-generated via the pentose phosphate pathway. Under physiological conditions, the GSH/GSSG ratio in the cytosol of most cells is ~100:1, or higher [26]. In contrast, in the endoplasmic reticulum, which has a more oxidizing environment to permit oxidative protein folding, the GSH/GSSG ratio is ~3:1 [27]. The redox steady-state of GSH and GSSG (i.e., the GSH/GSSG ratio) is crucial for normal cellular physiology and is involved in cellular signalling processes, proliferation, differentiation, and apoptosis [28,29]. In clinical studies, the GSH/GSSG ratio is most often measured in whole blood or in isolated RBCs based on the assumption that, although indirect, this minimally invasive type of analysis provides valuable information on the redox metabolism of less accessible tissues and organs and of the whole organism. A decrease in intracellular GSH concentration and/or in the GSH/GSSG ratio are often interpreted as evidence of cellular redox imbalance and have been associated to a variety of human diseases, amongst which diabetes mellitus, renal failure, malignancy, and neurodegenerative diseases [26,30,31]. However, changes in the GSH/GSSG ratio are unlikely to have any causal role for biological events; rather, they might have a diagnostic and prognostic significance, indicating whether something is going wrong in redox metabolism [32].

During oxidative stress, a notable amount of cellular glutathione can be reversibly bound to the –SH group of protein cysteinyl residues (*S*-glutathionylation), which generates *S*-glutathionylated proteins (PSSG). Most PSSG are intracellular because GSH is present at high concentrations within cells and intracellular proteins have thiols predominantly in the reduced state and thus are available to form mixed disulphides with GSH, likely through a protein thiolate or sulphenate or nitro-*S*-cysteine formation [21,26,33]. In contrast, in extracellular proteins most thiols form disulphide bridges or mixed disulphides with cysteines (i.e., cysteinylated proteins). PSSG might form in response to changes in the GSH/GSSG ratio or through a thiol/disulphide exchange between protein thiols (PSH) and GSSG, a reaction that removes GSSG, thus restoring the physiological redox conditions. However, reactions of protein disulphide exchange with a thiol are quite slow unless catalysed by an enzyme such as protein disulphide isomerase, which is particularly abundant in the endoplasmic reticulum, and the ratio of GSH/GSSG remains usually very high inside the cytosol, even during oxidative stress. Indeed, the formation of PSSG can also occur by a variety of other mechanisms [26,33]. *S*-glutathionylation is reversible and may have a dual role: protection of sensitive PSH from irreversible oxidative damage and/or regulation of protein structure and activity [33,34]. The latter function is particularly crucial for proteins involved in redox signalling processes, suggesting a role for *S*-glutathionylation in physiological signalling [35−37]. *S*-glutathionylation can also serve as a means to maintain GSH inside the cell, whereas GSH oxidized to GSSG is rapidly exported. PSSG have also been investigated as possible biomarkers of oxidative stress in human diseases characterized by deregulation of redox homeostasis, thereby acquiring a prognostic/diagnostic value [26,33,38]. For instance, it has been shown that PSSG are increased in Alzheimer disease [39] and Friedreich's ataxia [40], in non-alcoholic fatty liver disease [41], in cardiovascular diseases such as myocardial infarction, cardiac hypertrophy, and atherosclerosis [42], and in maintenance haemodialysis patients [43]. Differently, sputum PSSG levels were found to be decreased in patients with asthma [44].

In view of the important patho-physiological implications of GSH/GSSG ratio and PSSG concentration, a variety of different methodologies have been developed for the detection and/or quantification of glutathione and PSSG in cells, tissues, and body fluids. This review is an updated overview of the applications, drawbacks, and advantages of the most relevant analytical, morphological and proteomics approaches used to analyse GSH, GSSG and PSSG in mammalian/human samples.

2. Measurement of GSH/GSSG ratio

Accurate analysis of the GSH/GSSG ratio in biological samples requires separate analytical measurements for GSH and GSSG for two main reasons: i) the free thiol of GSH must be immediately blocked to prevent any unwanted reaction (either oxidation or reduction); ii) the concentration of cytosolic GSSG is about three order of magnitude lower than that of GSH and, in addition, it lacks the high reactive -SH group; therefore, specific methodological procedures are needed to detect it.

A very important pre-analytical step, frequently neglected, common for both GSH and GSSG detection, is represented by the treatment of sample, as soon as possible after its collection, with substances able to prevent any artificial increase and decrease in GSSG concentration [45–47]. In fact, based on previous observations and confirmed by our experimental work, once the sample is collected the GSH/GSSG ratio can be artificially altered in both directions (i.e., increase in GSH or in GSSG) depending on several variables. On the one hand, cellular reductases may continue to work after sample collection, thus reducing artificially the GSSG concentration, proportionally to the time elapsed (from seconds to minutes) until the analytical measurement [45,46,48–50]. On the other hand, we have demonstrated that sample acidification (a pre-analytical step that is common to almost all the procedures used to detect GSH and GSSG) *per se* results in an artefactual oxidation of GSH, at a certain extent, which depends on several factors, such as the kind and dilution of

sample, type and concentration of acid [51]. We have studied this phenomenon in blood samples, where we observed that the binding of both the molecular oxygen and the ferrous ion to the haeme group may be responsible for the GSH oxidation during sample acidification. In fact, the levels of GSSG were shown to increase after acidification, mostly in arterial blood rather than in venous blood and proportionally to the amount of O_2 added to de-oxygenated erythrocytes [51]. Afterwards, we observed that this methodological problem also occurs during GSH and GSSG detection in other biological samples, such as platelets, blood plasma, cultured cells, and solid tissues [47].

According to these observations, the main steps of GSH and GSSG analysis are: 1) sample treatment with proper agents able to prevent both artifactual –SH oxidation and GSSG reduction during sample handling; 2) splitting the sample into two sub-samples and analysis of GSH in one sample aliquot; 3) reduction of GSSG in the remaining sample aliquot and analysis of the released GSH. These steps are described in more detail below.

2.1 Prevention of artifactual GSH thiol oxidation and GSSG reduction during sample handling

Notwithstanding the evident susceptibility of –SH groups to oxidation during sample manipulation, this step is frequently by-passed, even by many skilled scientists worldwide. Instead, according to our experience, this issue needs a particular attention in order to obtain an accurate measure of the GSH/GSSG ratio. This problem may be especially relevant for analyses of the cytosol, where this ratio is very high in favour of GSH under basal conditions (*i.e.*, 500: 1 or even more) and, therefore, the concentration of GSSG is quite low. Consequently, even a minimal oxidation of GSH can induce a dramatic increase in GSSG in terms of percentage: for instance, if only 1% of GSH oxidizes, it would result in a ~200% bias of the measured GSSG than the actual concentration, with an unacceptable analytical accuracy [47]. However, thiol reactive agents are

frequently used only with the aim to "remove" GSH from the sample and to avoid any cross-reaction with the "new GSH" released from GSSG during the analysis of the latter.

Some different thiol alkylating agents are routinely used to bind GSH, such as iodoacetic acid (IAA), *N*-ethylmaleimide (NEM), 2-vinyl pyridine (2-VP), *S*-methyl methanethiosulfonate (MMTS), which are not equivalent and, in most cases, are used with different aims [49,52]. In fact, these reagents are often used only to remove the GSH from the sample for the GSSG analysis, and, therefore, they are added to the aliquot of sample used for this kind of measurement (i.e., after an indefinite delay from sample collection). However, thiol blocking must precede any sample manipulation if the aim of the investigator is to measure the GSH/GSSG ratio, otherwise the artifactual oxidation of GSH is not avoided.

Actually, only NEM and 2-VP have been proposed as eligible reactants to be used to avoid GSH artifactual oxidation. Maleimides have a double bond that binds GSH by a Michael addition reaction. The notable advantages of NEM use are: i) the reaction is very fast at neutral or slightly acidic pH; ii) cells are permeable to NEM; iii) NEM is also able to inhibit GSSG reductase [49,51,53]. This means that, when NEM is added to any biological sample, it rapidly (within a few seconds) enters the cells and efficiently masks GSH. The last advantage is fundamental, since it avoids any artifactual reduction of GSSG to GSH during sample handling as a consequence of the shift toward the right of the following reaction catalysed by GSSG reductase (GR):

$$GSSG + NADPH \xleftarrow{GR} 2\ GSH + NADP^+$$

Therefore, the reaction of NEM with –SH groups is so rapid and effective that any perturbation of the GSH/GSSG ratio is avoided. Several chemical issues should be taken into consideration when using NEM. First of all, the reaction of thiols with NEM is reversible in the presence of other thiol compounds at physiological pH and temperature; therefore, a possible migration of the maleimide among different thiols may occur [54]. In addition, maleimide adducts are prone to ring-open by

hydrolysis in alkaline buffers [55]. Nevertheless, these chemical features of NEM can be faced considering that the binding of NEM to GSH at physiological pH is very fast; therefore, the reaction can be stopped after 1-2 minutes, whereas the measured half-lives of conversion for the texted maleimide-thiol adducts varied from 20 to 80 hours and the hydrolysis required more than 30 minutes at pH 8.0 [54,55]. We previously evaluated the reliability of NEM use for GSH and GSSG detection in comparison with other commonly used thiol alkylating agents and reported that both IAA and monobromobimane react slowly with –SH groups, yielding too high values of GSSG [51]. The same was also reported for iodoacetamide [49]. Additionally, only in samples treated with NEM before acidic deproteinization we obtained a good recovery of GSSG added to haemolysate [51].

Conversely, 2-VP was introduced for GSSG detection by the GSH recycling method just by virtue of its inability to inhibit GR [56]. However, this implies that other oxidoreductases occurring in cells can work during sample manipulation, so the perturbation of the GSH/GSSG ratio cannot be avoided. In analogy to NEM, 2-VP reacts with GSH at the double bond, but this reaction is about 500-fold slower than that of NEM [49]. Therefore, long reaction times are required for complete reaction. In addition, cells are not permeable to 2-VP and this problem is frequently solved by adding it to acid-treated samples. Therefore, 2-VP cannot be so effective in preventing the acid-induced GSH artifactual oxidation. To confirm this, it has been reported that the levels of GSSG measured in blood by the use of 2-VP are significantly higher than those measured by the use of NEM in comparative experiments [57,58].

For all these reasons, NEM should be considered the most suitable alkylating agent to protect thiol groups from oxidation [45,46,59]. Whenever possible, NEM should be added directly in the collection tube or, alternatively, it should be used as soon as possible after sample collection. We generally add it directly to collection tubes for blood sample analysis and to homogenization buffers for solid tissue analysis. To prevent artifactual GSH oxidation in cultured cells, we perform washings with PBS containing NEM before cell lysis [59,60].

2.2 Analysis of GSH

Analysis of GSH should be performed on aliquots of samples pre-treated with NEM before the acidification step. In these samples, the easiest way to measure GSH is to detect the GS-NEM conjugate. As we recently reported, the conjugate can be measured by UV/vis-HPLC in supernatants [61]. The main advantage of this method is that it does not require any further derivatization of the sample. In fact, the conjugate is revealed by measuring the absorbance of supernatant at 265 nm. As reported in Fig. 1, from the analysis of GS–NEM conjugate in acidified blood samples by reversed-phase HPLC we obtained two peaks at the retention times of 4.23 and 4.97 min. Two peaks are formed because the reaction of NEM with thiols generates two diastereomers that have different chemical and physical properties and, consequently, are resolved as two peaks under achiral chromatographic conditions [62]. The peak at 4.23 min of retention time was selected for quantitative analysis since it resulted to be always free from interfering reactions in all the analysed samples.

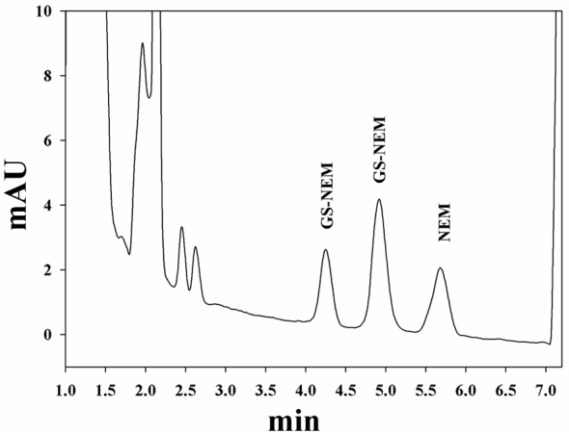


Fig. 1. Representative chromatogram obtained from a blood sample derivatized for GSH analysis. Blood from a healthy donor was collected in the presence of NEM. Sample was then deproteinized and supernatant was analysed by reversed-phase HPLC at 265 nm wavelength [61]. The GS-NEM

separation resulted in two almost symmetrical peaks at 4.23 and 4.97 min of retention time, respectively, whereas the unbound NEM eluted at 5.87 min of retention time.

Alternatively, the GS-NEM conjugate can be detected by liquid chromatography/mass spectrometry (LC/MS) separation. For example, Steghens et al. [63] used the positive electrospray ionization (ESI) mode after chromatographic separation to this aim. More recently, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed [64], by which samples are subjected to chromatographic separation using a Hypercarb column and analysed by MS/MS in the positive-ion mode. Notwithstanding both methods are worth to be used, in both cases NEM is added to samples together with acid in a precipitating solution. Therefore, under these conditions GSH auto-oxidation is not completely prevented and reductases could work until are denatured by the acidic treatment, which can influence, at an indefinite and variable amount, the results.

The GSH recycling assay is the most popular method to detect GSH [45]. The main advantages of this method are that it can be performed by spectrophotometer, it is specific for GSH and it is very sensitive. Basically, it measures total GSH (tGSH, i.e., GSH + GSSG) since the principle of the method is based on the reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form the mixed disulphide (GS-TNB) and the chromophore 5-thio-2-nitrobenzoic acid (TNB), followed by the reduction of GS-TNB by GR and NADPH. Actually, GR also reduces the GSSG occurring in the sample. The reaction proceeds in a cyclic way and the constant production of TNB is measured by spectrophotometry at 412 nm wavelength. Generally, the GSH recycling method is performed on blood samples not pre-treated with alkylating agents precisely because it measures both GSH and GSSG and the eventual oxidation of GSH to GSSG does not influence the results. Under several conditions (e.g., in control samples) the measurement of tGSH can be assumed to correspond to that of GSH, since GSSG levels are about three orders of magnitude

lower. Differently, in other kinds of samples GSSG levels must be subtracted by tGSH to obtain the GSH values.

Quantification of intracellular GSH levels can also be made using fluorescence activated cell sorting (FACS) [65]. FACS (and, in general, flow cytometry) methods are based on the use of fluorescent dyes that form fluorescent conjugates with GSH via its –SH group. FACS analysis has the advantage of not requiring the lysis of cells and, therefore, the artefactual oxidation of GSH during sample deproteination is avoided. However, FACS analysis suffers from several other technical problems, in particular regarding the choice of the fluorescent dye that forms adduct with GSH (*e.g.*, mercury orange, *o*-phthaldialdehyde, monochlorobimane). In fact, the specificity of these fluorescent probes is variable, so they can possibly label other intracellular thiols, such as PSH. Monochlorobimane can bind specifically to GSH since the reaction requires catalysis by glutathione *S*-transferase. However, human glutathione *S*-transferase has a low affinity for monochlorobimane; therefore, in human cells, GSH is not adequately labelled or a perturbation of the GSH/GSSG ratio can occur. Additionally, background labelling represents a further problem, mostly for quantification [65]. Therefore, the development of new more specific fluorescent probes for GSH labelling in human cells is a *sine qua non* of reliable GSH quantification by FACS. In this regard, the first fluorescent probe (ThiolQuant Green, TQ Green) for quantitative imaging of GSH in live (mouse and human) cells can be conveniently used in bulk cell measurements to measure GSH level changes by FACS [66].

2.3 Analysis of GSSG

The easiest way to measure GSSG in samples pre-treated with NEM is by the GSH recycling method [45]. Once protected by NEM, samples can be deproteinized and then GSSG can be measured in the supernatant. As NEM can inhibit GR at the neutral pH of the reaction, an extraction step is needed in order to remove it from the sample. This step can be carried out either

with dichloromethane or ether, both the solvents work fine [45,59]. It is worth to be mentioned here that the GSH recycling method is not easy to perform, since it is based on an enzymatic reduction and it is not an end-point measurement. Therefore, several variables can influence the results: in particular, the acid used to deproteinize the sample and traces of NEM, if not completely extracted. We have analysed these problems in depth [59] and suggest to perform a two-step analysis for each sample, which includes adding a known quantity of GSSG to the cuvette after the sample reaction has achieved the steady state, in order to take into consideration the effect of these variables for quantitative analysis.

As an alternative to the recycling method, GSSG can be measured by HPLC after its reduction to GSH. In this case too, NEM should be extracted from the sample in order to avoid interfering reactions with the released GSH. Then, GSSG is reduced to GSH. Several reducing agents are routinely used to this aim. One class of these is represented by thiol reagents that reduce GSSG by thiol-disulphide exchange reactions, such as dithiothreitol (DTT) and 2-mercaptoethanol. Alternatively, phosphines can be used. The main difference between these two classes of reagents is that the reduction reaction by thiol reagents is reversible, whereas that by phosphines is irreversible, since it occurs by a nucleophilic attack on one of the two sulphur atoms forming a phosphonium ion sulphur adduct, which is subsequently hydrolysed [52]. This means that thiols must be used at high concentration and it may be necessary to remove them from the reaction mixture before sample derivatization. In addition, reactions with thiols proceed at neutral pH, a condition where oxidation of new generated thiols may occur. This is the reason why we generally perform reduction with DTT in the presence of the metal chelating agent EDTA [67].

Given the expected low concentrations of GSSG, at least in control samples, the use of fluorescent probes coupled with chromatographic separation is particularly suitable for this kind of measurement. Monobromobimane (mBrB) is one of the most widely used molecules for this purpose. It becomes fluorescent after reaction with thiols (λ_{ex} = 380 nm, λ_{em} = 480 nm) [68]. We generally use mBrB coupled with separation by reversed-phase HPLC. We do not remove excess

DTT from the sample since the conjugate with mBrB does not interfere with the separation of GSH [67]. The main problem when working with mBrB under these conditions is represented by the formation of non-specific peaks in the chromatogram (Fig. 2) Therefore, a blank run consisting of mBrB treated with DTT under the same conditions of the samples is necessary. Other fluorescent probes frequently used for thiol detection are benzofurazans (*e.g.*, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, SBDF), *o*-phthalaldehyde, dansyl chloride [69–71]. GSSG can be measured without the reduction step by MS. For example, Moore et al. [64] used the LC-MS/MS method described above for GSH analysis (section 2.2) also for GSSG detection.

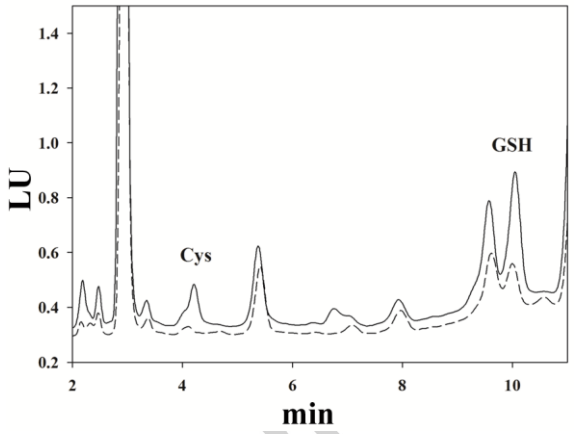


Fig. 2. Representative chromatogram obtained from rat liver homogenate derivatized for GSSG analysis. Liver was homogenized and rapidly treated with NEM. GSH was measured on the supernatant after reduction of the disulphide bridge by DTT and thiol labelling with mBrB [67]. GSH eluted at 10.05 min of retention time. A peak corresponding to cysteine (Cys) was also observed at 4.211 min of retention time. The dotted line shows the chromatogram obtained from a blank run.

The use of 1-fluoro-2,4-dinitrobenzene (FDNB) as derivatizing agent for both GSH and GSSG has the advantage of allowing contemporary evaluation of both these molecules in the same sample aliquot [72]. FDNB reacts with the amine group to produce a dinitrophenyl-derivative. In

the original method, IAA was used to pre-treat samples, followed by derivatization with FDNB and separation by HPLC with an anion exchange column. Detection is performed by a UV/Vis detector set at an excitation wavelength of 355 nm [72]. We have proposed a modification of this method making it applicable to samples treated with NEM [73]. Therefore, by this modified method, it is possible to measure both GSH and GSSG with a single HPLC run and, at the same time, pre-treat the sample in order to prevent artifactual oxidation of GSH.

2.4 Reference values for GSH and GSSG

By application of the above mentioned methods for measurement of GSH, GSSG and GSH/GSSG ratio, coupled to pre-treatment of samples with NEM, we measured the levels of these parameters in several biological samples (Table 1) [43,60,61,67,73,74]. In blood, the GSH/GSSG ratio was quite high, about 600 in healthy people and mouse RBCs. A GSH/GSSG ratio of about 400 was measured in rat RBCs. This ratio was shown to significantly decrease in RBCs of patients affected by end stage renal disease undergoing to haemodialysis. These values are in agreement with the levels measured by some other research groups using NEM to protect GSH from oxidation [46,64,75,76], whereas other research groups measured even higher GSH/GSSG values [45,63]. In some cases, a significant alteration of the GSH/GSSG ratio between controls and diseased people was observed. Specifically, Srivastava and Beutler measured GSH and GSSG in RBCs of people affected by deficiency of glucose-6-phosphate dehydrogenase (G6PDH) [46]. The content of GSSG in healthy people was 3.6 ± 1.4 μM, whereas in G6PDH-deficient patients it was 10.3 ± 3.0 μM. Also the GSH/GSSG ratio resulted to be significantly lower in G6PDH-deficient people (142 vs 500) [46]. A significant decrease in the GSH/GSSG ratio was also measured in hypertensive pregnant women and in neonatal infants affected by retinopathy of prematurity [75,76]. We also recently found a significant decrease of GSH/GSSG ratio (661 vs 320) in maintenance haemodialysis patients [43]. However, these values greatly differ from those measured by most of

other research groups [see articles cited in 31,47,48] making the comparison of results very hard. This is particularly true for GSSG analysis, the concentration of which is very low within cells and more difficult to measure without artifactual oxidation. Analogously to what has been observed for blood, also when studying the GSH/GSSG ratio in cultured cells we faced a similar heterogeneity about the reference values [60].

The GSH/GSSG ratio is a promising biomarker of oxidative stress. We demonstrated that it is very sensitive also in response to slight oxidative perturbations [48]. However, the next necessary step, in order to increase the knowledge about its prognostic value in several human pathologies, is to reach more agreement among researchers about methodologies and reference levels.

3. Measurement of *S*-glutathionylated proteins (PSSG)

As awareness of protein *S*-glutathionylation as a relevant mechanism of redox signalling and a possible biomarker of oxidative stress in human diseases has grown, several different methodologies have been developed for quantification or identification of PSSG. Some methodologies allow the quantification of the total PSSG content in cells and tissues, other ones allow the in situ identification of individual proteins undergoing *S*-glutathionylation.

3.1 Quantitative analysis of the total content of PSSG in cells and tissues

In many cases, the measurement of PSSG is carried out by detecting the released GSH, after its cleavage from the mixed disulphide bond. Therefore, the main steps for the measurement of total PSSG are: i) separation of proteins; ii) treatment of sample to release protein-bound GSH; iii) detection of GSH.

3.1.1 Separation of proteins

Sample proteins are separated by acidification/centrifugation or by gel filtration to remove soluble GSH. The use of acids, such as trichloroacetic, perchloric, sulfosalicylic, metaphosphoric acid, is very frequent, given the rapidity and efficacy of the procedure. However, analogously to what we have highlighted for the measurement of the GSH/GSSG ratio (section 2.1), also for this analysis we have to face with the methodological problems related to the artifactual oxidation of GSH. This problem mostly manifests during the acidification step and occurs primarily when working with cells, where the concentration of GSH is very higher (from 1 to 10 mM [21−23]) than the concentration of PSSG, whose steady-state level is <0.1% of the total protein cysteines and <1% of the total protein disulphide (*i.e.*, PSSP + PSSG) value in HEK and HeLa cells, for instance [77]. In fact, we have demonstrated that artifactually high levels of PSSG can form at a variable extent in several kinds of biological samples, such as blood, RBCs, solid tissues and cultured cells [47]. Without protecting –SH groups before sample acidification, PSSG levels in blood samples obtained from healthy people were found to be about 100-fold higher than the real value [48]. We also observed that the extent of this artifactual increase was influenced by several variables, such as the type of acid used for sample deproteinization, sample dilution before deproteinization, the partial pressure of O_2 bound to haemoglobin [48]. Therefore, also for this kind of analysis samples should be treated with NEM as soon as possible after sample collection, *i.e.*, before the deproteinization step (as described in section 2.1 for measurement of the GSH/GSSG ratio) in order to protect the –SH group from oxidation. Excess NEM can be then removed from samples by performing several washings under slight acidic conditions. At the end of this procedure, we usually obtain a purified protein pellet, ready to undergo the proper treatment for the release of protein-bound GSH. Differently, to measure *S*-glutathionylated haemoglobin we prefer to separate the protein by gel-filtration in order to remove at once excess NEM, low molecular weight thiols and disulphides [78].

3.1.2 Treatment of sample to induce release of protein-bound GSH

Several reagents can be used to cleave the disulphide bridge in PSSG, some of which have been already described in section 2.1, such as the thiol-containing reductants (DTT, 2-mercaptoethanol) and phosphines. Another reducing agent widely used for reduction of PSSG is sodium borohydride. In general, reduction of protein disulphides with sodium borohydride requires strong alkaline conditions (pH 12) and high temperatures (50°C or more): such conditions can induce foam formation. Therefore, to avoid this phenomenon octanol or hexanol should be added to sample [49,79]. One advantage of the use of sodium borohydride is that it can be easily removed by acidification of sample [80]. However, at the elevated pH value used to reduce the disulphide bridge some side-reactions may also occur, such as peptide bond cleavage. We generally use a solution of DTT/EDTA at neutral pH to reduce PSSG and do not remove DTT from sample because we avoid any interfering reaction by analysing samples by HPLC: in fact, DTT has a different retention time than that of GSH. Therefore, according to our experience, DTT is the eligible reducing agent to induce release of GSH from PSSG.

3.1.3 Detection of released GSH

The released GSH can be measured by one of the methods described in the sections dedicated to GSH and GSSG analysis (*i.e.*, sections 2.2 and 2.3). As commented previously, given the expected low basal levels of PSSG, the most suitable technique to detect the released GSH is its labelling with fluorescent probes (such as mBrB, *o*-phthalaldehyde), followed by chromatographic separation. By the use of mBrB coupled to reversed-phase HPLC, we could detect the content of PSSG in RBC cytosol and membranes, platelets, blood plasma, cultured cells, and solid tissues. Nevertheless, other methods can be used to measure PSSG at low level. For example, HPLC-coupled to ESI-MS can be applied to detect *S*-glutathionylated haemoglobin [81]. In that case, the levels of normal (unmodified) and *S*-glutathionylated haemoglobin β-chain were obtained by monitoring the +18 (880·8 and 897·8 *m/z*, respectively) and +19 (834·5 and 850·6 *m/z*,

respectively) charge states. We wish to emphasize that these methods make it possible to detect the total content of PSSG, but they do not enable the identification of specific PSSG.

3.1.4 Levels of PSSG in some biological fluids

By using HPLC and fluorescent labelling with mBrB, we measured 50 to 500-fold lower levels of *S*-glutathionylated haemoglobin in healthy people (0.013 ± 0.003 nmol/mg haemoglobin) than other research groups [see 31,43,48 and citations therein], but in agreement with those found by Gladwin et al. [81]. We also found that levels of *S*-glutathionylated haemoglobin increase significantly in haemodialysis patients [43]. We think that the main reason for this discrepancy about levels of *S*-glutathionylated haemoglobin in healthy humans is due to the fact that we were the first to protect the –SH group of GSH from oxidation by masking it with NEM. We measured similar levels of *S*-glutathionylated haemoglobin in rat and mouse blood samples [78,82]. PSSG values in RBC membranes of both healthy people and rats were even lower (0.736 ± 0.159 nmol/g haemoglobin and 0.682 ± 0.259 nmol/g haemoglobin, respectively) [78]. The values of PSSG in other biological fluids and cultured cells measured in our laboratory are reported in Table 2. PSSG levels resulted to be very low under basal conditions in all samples analysed. This observation is in agreement with results obtained by Hansen et al. [77], who found very low levels of PSSG also in HEK and HeLa cells, with values of a factor of 1,000 below the value of total protein disulphides (*i.e.*, PSSP + PSSG). Analogously, very low levels of PSSG (*i.e.*, 57 ± 42 nmol/mg protein) were measured in rat liver by means of LC–MS/MS [83], whereas we observed a significant increase in PSSG levels in the organs from aged animals [67]. The reference levels for PSSG in human plasma are widely accepted [84]. It should also be underlined that, although extracellular fluids like blood plasma, unlike most intracellular compartments, are characterized by the prevalence of the oxidized than the reduced forms of thiol/disulphide pools, the major extracellular low molecular weight thiol/disulphide redox couple is cysteine/cystine [85]; therefore, plasma PSSG levels are quite low.

3.2 Methodologies for identification of PSSG in cells and tissues

The total amount of PSSG in cells or tissues, measured by one of the methods described in section 3.1, may be an index of oxidative stress. However, identification of specific PSSG, both under physiological and pathophysiological conditions, requires more specific methodologies (Table 3).

The original biotin-switch technique developed to study protein *S*-nitrosylation [86] also can be adapted to study protein *S*-glutathionylation, by changing the specific reducing agent ascorbate with a recombinant glutaredoxin system. Affinity purification of biotin-maleimide-tagged proteins and proteomic analysis allows characterization of cellular PSSG (also denoted as "glutathionylome") [87].

3.2.1 Radiolabelling of GSH

A direct methodology for the identification of PSSG within cells is based on the use of radio-labelled GSH [88]. Cells are pre-incubated with cycloheximide to block protein synthesis, incubated with [^{35}S]-cysteine to radio-label GSH, exposed to oxidative stress or a glutathionylating agent (*e.g.*, diamide). After removal of free radio-labelled GSH, radio-labelled PSSG are detected and quantified by autoradiography. When coupled with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation and MS, the site of *S*-glutathionylation can be identified. The main advantages of this methodology are that it is sensitive, efficient, and robust and can be used under various oxidative stress conditions. The main disadvantages are that it requires the use of cycloheximide and, therefore, perturbs cell physiology and does not permit discrimination between PSSG and *S*-cysteinylated proteins. However, in eukaryotic organisms/cells, *S*-cysteinylation occurs rarely within cells (where GSH is by far the most abundant low molecular weight thiol), whereas it occurs more frequently in extracellular compartments, such as human plasma [10,89,90] and it is a general mechanism for protein thiol protection after oxidative stress in

Gram-positive bacteria, which lack GSH (with the exception of a few strains [35,91]). Moreover, radiolabel tagging – for instance, according to the very reliable procedure published by Hill and colleagues [88] − followed by immunoprecipitation with anti-GSH antibodies (see below) could be used to purify PSSG for further analysis by ESI-MS or matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and aid in identifying the specific cysteines modified.

3.2.2 Conventional proteomic methodologies with anti-GSH antibodies

Both the burst of improvements in proteomic methodologies and the commercial availability of antibodies that specifically reacts with glutathione bound to proteins (for an exhaustive list of the commercially available anti-GSH antibodies see [92]) allow researchers to detect *S*-glutathionylation at protein level in cells or tissues without pre-treatments with radiolabelled GSH precursors or tagged GSH analogues, thus making experimental conditions more close to physiological ones. Western blotting with anti-GSH antibodies can be performed after one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) 93−97] or 2D-PAGE separation, in the absence of reducing agents. However, the number of proteins susceptible to *S*-glutathionylation is small compared with the whole intracellular proteome. The lability/reversibility of the protein-glutathione bond is also a problem, which accentuates the previous one. Moreover, these methodologies may suffer from sensitivity; therefore, only highly abundant PSSG are detected.

In 1D SDS-PAGE analysis, the thiol alkylating reagent NEM is added to both the lysis/homogenization buffer and the Laemmli sample buffer to block all free thiols [98]. It is also critical that NEM be present during the blocking step of a Western blotting procedure, after transfer of the proteins to polyvinylidene fluoride (PVDF) or nitrocellulose membranes. This step increases the detection of PSSG several-fold by preventing the reduction of GSH adducts by PSH in the milk or albumin Cys34. For this, NEM is added to the blocking buffer. The anti-GSH antibody is then

diluted in PBS-Tween20 and incubated overnight at 4°C. An example of PSSG detection using this methodology is shown in Fig. 3 [93].

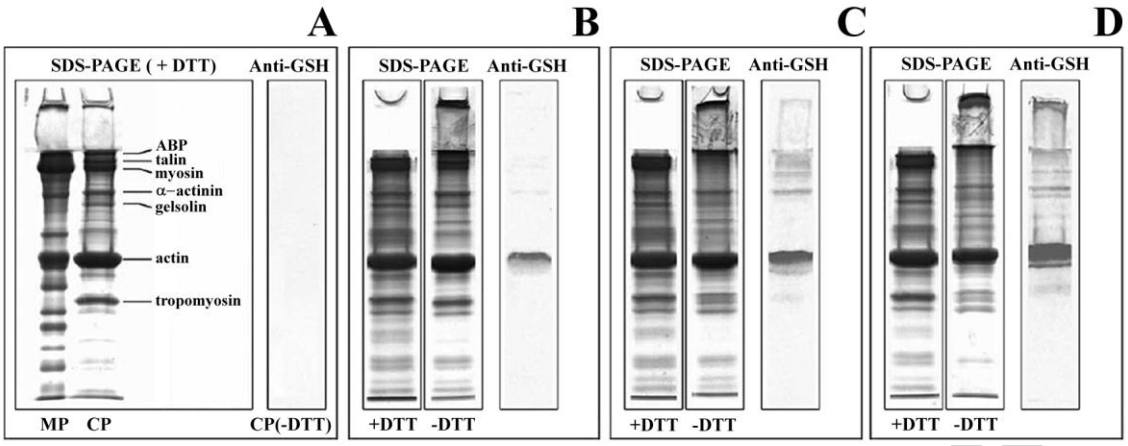


Fig. 3. *S*-glutathionylation of cytoskeletal proteins in human platelets. Human platelets were challenged with (B) 0.2, (C) 0.4, and (D) 0.75 mM diamide for 60 min. Cytoskeletal proteins were analysed by SDS-PAGE (12% gel) run under either reducing (+DTT) or non-reducing (-DTT) conditions. Samples under non-reducing conditions were then blotted to PVDF membrane and probed with anti-GSH antibody (right strip in each panel). Data are representative of three independent experiments. (A): CP, platelet cytoskeletal proteins; MP, bovine cardiac myofibrillar proteins. Western blot probed with anti-GSH antibody shows that there are no *S*-glutathionylated cytoskeletal proteins in unstressed (control) platelets [CP (-DTT)]. Reprinted by permission of Elsevier from ref. [93] with slight modifications.

A very reliable 2D-PAGE method has been developed by Hill and colleagues [88]. Cells or tissues can be lysed or homogenized in low salt buffer containing Triton X-100 or NP-40 and NEM. The proteins can then be loaded on immobilized pH gradient (IPG) strips and electrofocused using standard protocols. If the sample to be analysed by 2D-PAGE contains too much salt, a clean-up step may be required. Before the second dimension, IPG strips must be equilibrated in equilibration buffer containing NEM. To obtain peptides for MALDI-TOF MS, protein spots that were

immunoreactive with anti-GSH antibodies are excised from parallel Sypro Ruby-stained gels and digested with trypsin using the method described by West and colleagues [99]. For example, redox proteomics studies targeting PSSG have been performed by Butterfield's research group on the inferior parietal lobule of patients suffering from Alzheimer's disease compared with control subjects [39]. As shown in Fig. 4, this study revealed the increased *S*-glutathionylation of deoxyhaemoglobin, α-crystallin B, glyceralde-3-phosphate dehydrogenase, and α-enolase in individuals with Alzheimer's disease; the last two proteins were also shown to have reduced activity in the diseased inferior parietal lobule [39].

It is important to note that, in total protein extracts, the anti-GSH antibody only detects a few abundant proteins. Hence, the anti-GSH antibodies currently commercially available do not appear to be appropriate for large-scale detection of PSSG by conventional proteomic approaches, which suffer from a common limitation of proteomic methods, where the presence of high-abundance proteins make the identification of low-abundance ones very difficult. However, immunoprecipitation followed by immunoblotting with anti-GSH antibodies can enhance detection [96,100].

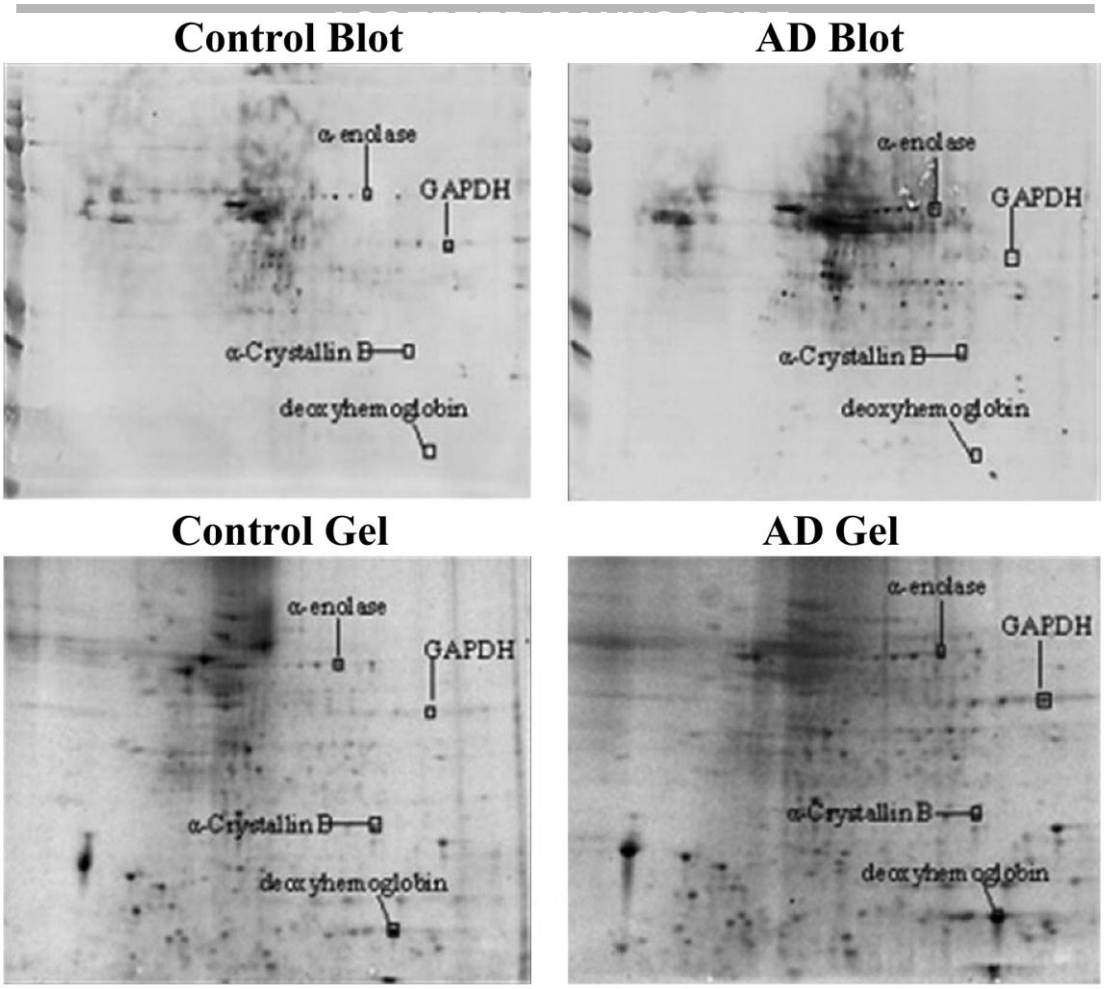


Fig. 4. The proteins from Alzheimer's disease patients (inferior parietal lobule) and age-matched controls were separated by 2D-PAGE. The gels were then stained with Sypro Ruby stain. An example control and Alzheimer's disease gel are displayed at the bottom. 2D Western blots demonstrate the immunochemical detection of PSSG. An example age-matched controls and Alzheimer's disease blot are presented at the top. Labelled protein spots are proteins identified in this study. Reprinted by permission of John Wiley and Sons from ref. [39] with slight modifications.

3.2.3 Chemoselective probe-based labelling methodologies

Development of chemoselective probes has enabled new, direct labelling methods to the analysis of thiol redox chemistry, including protein *S*-glutathionylation [101]. Several biotinyl

analogues of GSH or GSSG have been developed to bypass limitations of radiolabelling and conventional proteomics methods based on anti-GSH antibodies [88,102−105]. In the biotinylation methodology, cells or tissues are incubated with biotinylated GSSG, which is membrane permeable and traverses the cell [103]. The biotinylated GSSG reacts via thiol-disulphide exchange with PSH that are prone to *S*-glutathionylation when the level of GSSG markedly increases (as during oxidative stress) to form PSSG-biotin adducts, which can be detected by Western blotting with streptavidin or by other avidin-based techniques. An excellent protocol has been published by Hill and colleagues [88]. PSSG-biotin adducts can be affinity purified on streptavidin columns and identified by nano LC-MS/MS [104,106]. Sites of *S*-glutathionylation can be identified after digestion of biotinylated proteins with trypsin, peptide affinity purification and analysis by tandem mass spectrometry (nano LC-MS/MS) [104,106]. PSSG-biotin adducts can also be localized within cells by fluorescence microscopy [103]. In addition, a methodology based on membrane-permeable biotinylated GSH ethyl ester has also been reported [105]. Tagging GSH with biotin enable detection of purified PSSG through avidin-conjugated agarose beads followed by immunoblotting with anti-biotin antibodies or avidin-based affinity chromatography. Biotinylated GSH ethyl ester, like biotinylated-GSSG, can be used to extract and analyse intracellular PSSG by immunoprecipitation and MS. The primary advantages of using biotinylated GSH ethyl ester over biotinylated GSSG include its increased cell permeability and its use for detecting *S*-glutathionylation due not only to increased GSSG, but also to increased *S*-oxidation and *S*-nitrosylation [88].

Albeit biotinyl analogues of GSH or GSSG have been successfully applied to several biological systems to identify PSSG or *S*-glutathionylation sites, they too have some limitations. In particular, biotin steric hindrance may limit access to some Cys residues undergoing *S*-glutathionylation. Therefore, to bypass such limitations, a selective and versatile approach has been developed to detect *S*-glutathionylation by metabolically tagging intracellular GSH with small clickable functionality [107]. This approach relies on the use of a mutant of GSH synthetase, which

efficiently and selectively catalyses coupling of azido-Ala in place of Gly to γGlu-Cys to form GSH. Cells were transfected with the GSH synthetase mutant and incubated with azido-Ala, thereby generating the azide-containing glutathione derivative, γGlu-Cys-azido-Ala. Following cell exposure to oxidizing conditions (0.1 - 1 mM H_2O_2 for 15 min), clickable glutathione was conjugated to proteins undergoing *S*-glutathionylation in cells, which allowed for selective and sensitive detection of PSSG by Western blotting or fluorescence after click reaction (a Cu(I)-catalysed azide-alkyne cycloaddition, CuAAC) with biotin-alkyne or rhodamine-alkyne, respectively. In the first case, cell lysates were subjected to a click reaction with biotin-alkyne and probed with streptavidin-peroxidase. Alternatively, the click reaction with rhodamine-alkyne provided similar results while facilitating in-gel fluorescence detection**.** γGlu-Cys-azido-Ala coupled with biotin-alkyne or fluorophore-alkyne (*e.g.*, Alexa-Fluor 647-alkyne) can also be used for chemoselective purification or cellular imaging of PSSG, respectively [107]. This clickable glutathione approach has recently been used for detection and identification of protein *S*-glutathionylation in human embryonic kidney (HEK) 293 cells in response to glucose starvation [108]. The same authors have recently increased the chemical tools for labelling GSH and detecting protein *S*-glutathionylation by using tetrazine-alkene chemistry for biosynthesis of clickable glutathione and analysis of protein *S*-glutathionylation [109]. They show that allyl-Gly and allyl-Ser can be efficiently used for the biosynthesis of clickable glutathione containing allyl-Gly (allyl-glutathione) or allyl-Ser (allyl-*O*-glutathione) in cells expressing GSH synthetase mutant. Both alkene-containing GSH analogues can be the chemical probes for detecting *S*-glutathionylation under ROS-inducing conditions in cells. The subsequent reaction of a small terminal alkene in allyl-glutathione (which has the smallest size of the alkene group and, therefore, may be less interfering with intracellular GSH function) with tetrazine-fluorophore or tetrazine-biotin allows for enrichment and identification of PSSG [109].

A further novel method with an alkynyl-containing GSH analogue based on click reaction has been designed to enrich *S*-glutathionylated peptides in order to profile the glutathionylomes of *E. coli* and *Drosophila* by LC-MS/MS analysis [110].

3.2.4 Highly sensitive proteomic methodologies

Recently, Mullen and colleagues described a "redox arrays" methodology based on incorporation of biotinylated GSH into proteins to identify proteins undergoing *S*-glutathionylation and have applied it to the secretome and the proteome of human monocytic THP-1 leukaemia cells [111]. Comparison of the redox array with conventional proteomic methods showed that the redox array is much more sensitive, and can be performed using more than 100-fold less protein than is required for methods based on MS [111].

A highly sensitive proteomic methodology for quantification and identification of PSSG in mouse liver has recently been described [112]. This methodology combines high-accuracy proteomics with isobaric tandem mass tags (TMTs) to provide precise, extensive coverage of PSSG. This method is based on the specificity of glutaredoxin-1 (Grx1) to deglutathionylate PSSG. A previous publication demonstrated Grx-mediated selective reduction of PSSG in combination with isobaric tags for relative and absolute quantification (iTraq) to identify the *S*-glutathionylated cysteine residues in a cell culture system under stress conditions [113]. More recently, a highly sensitive proteomic methodology for identification of PSSG in monocytes and macrophages has been described [114], which involves a novel enrichment technique based on iodoacetamide conjugated to desthiobiotin labelling, followed by HPLC-ESI-MS/MS, to identify and quantify PSSG. This methodology allowed researchers who developed the method to identify over 130 PSSG in lysates of macrophages isolated from dyslipidaemic and mildly hyperglycaemic atherosclerosis-prone mice, an animal model of metabolic stress and human chronic inflammatory disease [114].

MS is an ideal platform for analysing PTMs of proteins because it provides a detailed characterization at molecular level. Among different MS strategies, top-down approach is a method that allows to preserve the *in vivo* PTMs because it is able to analyse intact proteins rather than investigating peptides produced by *in vitro* proteolysis [115,116]. Protein *S*-glutathionylation is a relatively new PTM occurring on mouse histone H3 [117,118]. Following a top-down strategy, intact histones purified from mouse (strain C57BL/6J) brains were recently analysed without enzymatic digestion and applying a workflow that uses Informed-Proteomics for data analysis and visualization from raw data files [119]. This multistep software package employs ProMex for intact mass deconvolution, MSPath-Finder as search engine, and LcMsSpectator as a data visualization tool. The application of top-down LC-MS approach followed by Informed-Proteomics data analysis led to the identification of multiple major histone proteoforms. Among them, a protein mass shift of +305 Da was observed and matched with histone H3 *S*-glutathionylation [119]. Considering that histone H3 *S*-glutathionylation is elevated in proliferating and cancer cells, it was previously suggested that it could regulate chromosomal structure by modulating nucleosome stability [117,118]. In addition, histone *S*-glutathionylation could also represent the redox sensor by which chromatin can sense and modulate its accessibility in response to cellular and tissue redox status as suggested by its description in a highly differentiated and not proliferating tissue such as the brain [120]. This example highlights the power of high-resolution MS for identifying unknown protein PTMs in top-down MS experiments although, at present, it is not yet an available approach in most research laboratories.

3.2.5 Visualization of PSSG in tissue sections or in cultured cells

The methodologies mentioned above allow the quantification and identification of single PSSG, but they provide no insight into the PSSG location in tissues or cells. By immunohistochemistry with anti-GSH antibody, Sparaco and colleagues showed constitutive protein *S*-glutathionylation in paraffin-embedded sections of normal human cerebellum, spinal cord,

and frontal cortex [94]. Anti-GSH antibodies showed a faint immunoreactivity uniformly distributed throughout the molecular layer of the cerebellum, the grey matter of the spinal cord, and all cortical layers of the frontal cortex. The degree of immunocytochemical staining was clear in neurons, mild in oligodendrocytes, and weaker in astrocytes [94]. By immunohistochemistry with anti-GSH antibody, PSSG were also detected in Friedreich's ataxia, an autosomal recessive disorder caused by mutations in the gene encoding frataxin [121]. In particular, in the cervical sections of spinal cord derived from autopsies of individuals with Friedreich's ataxia, immunostaining with anti-GSH antibodies was significantly stronger than in control sections, suggesting a significant increase in PSSG amount in all grey matter neurons, particularly in motor neurons of the anterior horns (Fig. 5), as well as in the axonal spinal tracts (mostly in posterior white columns, spinocerebellar and corticospinal tracts). These findings suggest the occurrence of oxidative stress in the spinal cord of patients with Friedreich's ataxia as a consequence of reduced frataxin expression [121].

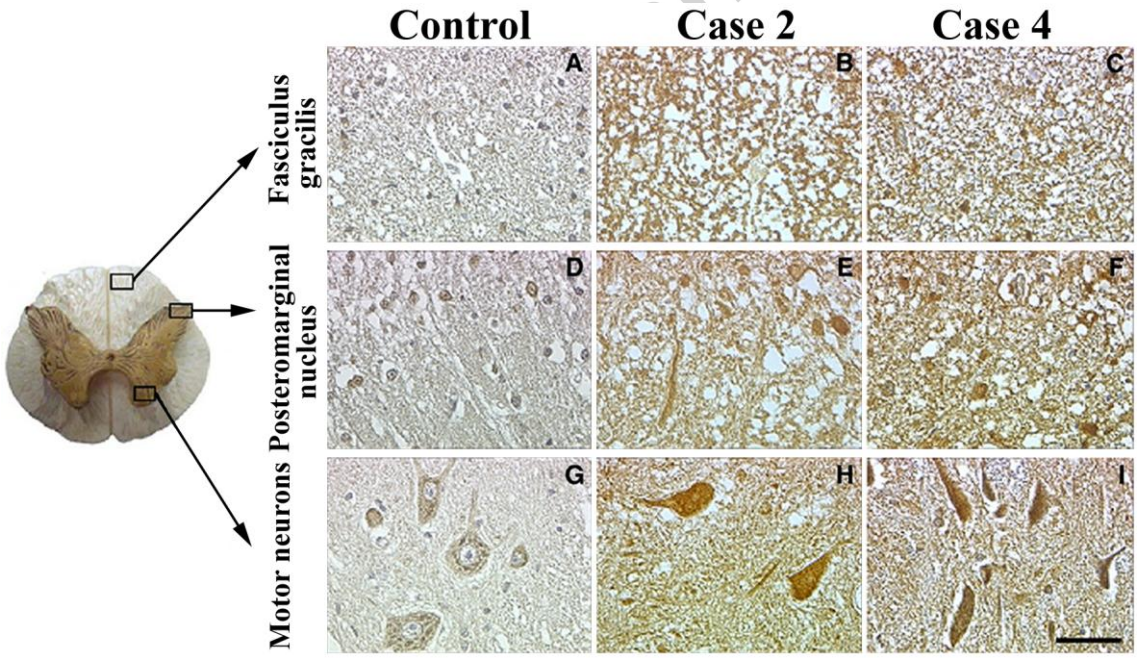


Fig. 5. Immunostaining of cervical sections of spinal cord from a control and two patients with Friedreich's ataxia localizing PSSG. The patients show a strong immunoreactivity for PSSG, suggesting an increase of protein *S*-glutathionylation in cells and fibres of fasciculus gracilis (B, C),

posteromarginal nucleus (E, F), and anterior horns (H, I). The images are representative of all the patients tested. Scale bar: 50 μm. Reprinted by permission of Elsevier from ref. [121] with slight modifications. To see this illustration in colour, the reader is referred to the web version of this article at https://www.journals.elsevier.com/free-radical-biology-and-medicine/

Anti-GSH antibodies allow researchers to detect PSSG also in cultured cells and intracellular compartments by indirect immunofluorescence techniques. Undoubtedly, the location and distribution of a protein are key aspects to understanding its physiological function and its role in biological processes. Moreover, the ability to visualize the compartmentalization of PSSG in cells will represent an additional tool to study protein *S*-glutathionylation in physiological/normal conditions and in diseases. For example, by indirect immunofluorescence with anti-GSH antibodies, constitutively PSSG have been shown to co-localize with the nuclear lamina, endoplasmic reticulum, and cytoskeleton in primary human dermal fibroblasts from skin biopsies from normal subjects [122]. To better characterize the distribution of PSSG, the authors used specific markers for microfilaments, microtubules, and intermediate filaments (vimentin). The results displayed a strong co-localization between PSSG and cytoskeleton components. As shown in Fig. 6, PSSG overlapped with microfilaments in correspondence to stress fibres, microtubules exhibited a complete overlap in all distribution points, whereas intermediate filaments showed co-localization with PSSG only in central and perinuclear areas. An efficient protocol for indirect immunofluorescence that permits the detection of PSSG localization in intracellular compartments using anti-GSH antibodies has been recently published by Piemonte and colleagues [123].

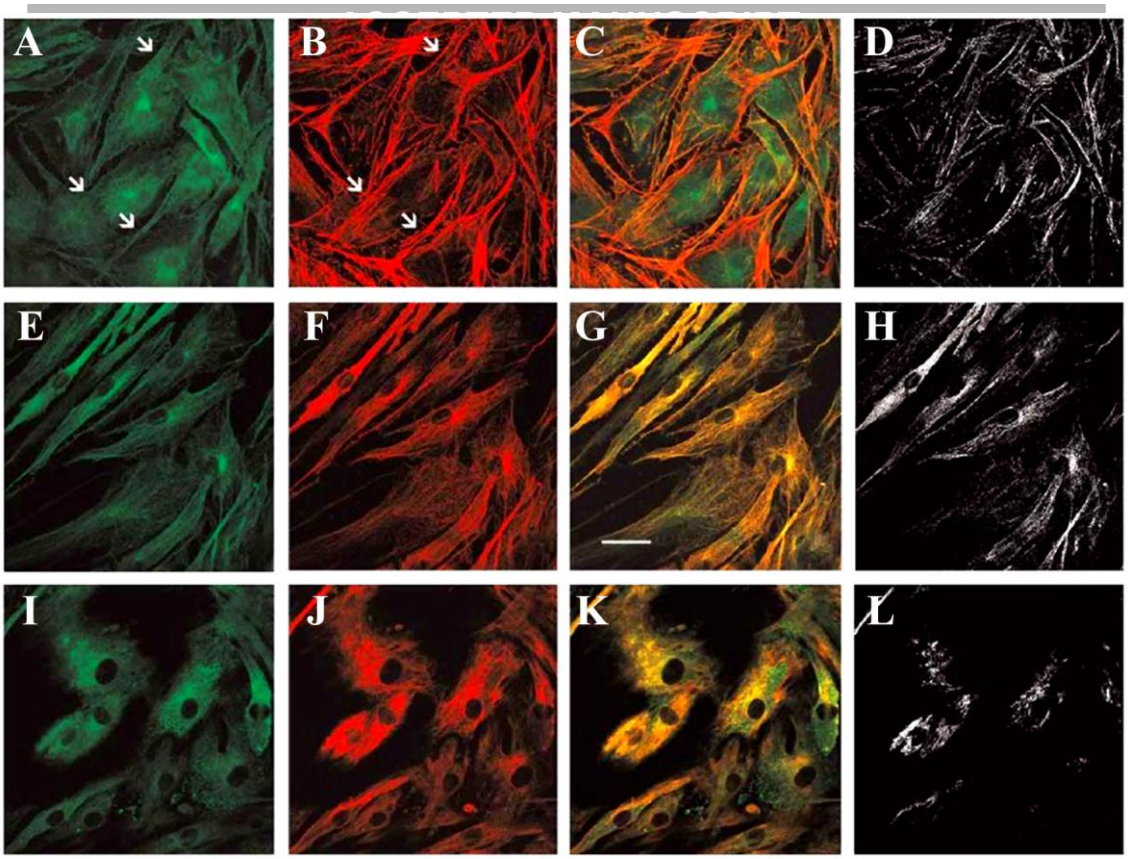


Fig. 6. Immunofluorescence analysis of the cytoskeleton *S*-glutathionylation in human primary dermal fibroblasts. Anti-GSH antibodies (primary antibody) (A) and phalloidin (B) double staining showed a codistribution (C) at stress fibres (arrows in A and B), with a significant colocalization pattern as visualized by their colocalization mask (D). Double immunolabelling of PSSG (E) in association with microtubules (F) exhibited a complete overlap degree between PSSG and microtubules (yellow signal in G) in all distribution points (colocalization mask in H). PSSG (I) and intermediate vimentin filaments (J) showed a partial codistribution, particularly in central and perinuclear areas (K), as pointed out by their colocalization mask (L). Scale bar, 40 μm. Reprinted by permission of Taylor & Francis from ref. [102] with slight modifications. To see this illustration in colour, the reader is referred to the web version of this article at https://www.journals.elsevier.com/free-radical-biology-and-medicine/

A very reliable methodology for visualization of protein *S*-glutathionylation *in situ* in cells or tissues utilizes the catalytic specificity of Grx1 [124,125], which carries out de-glutathionylation reaction (*i.e*., the detachment of GSH from PSSG) [33]. In this reaction, the *N*-terminal Cys of Grx1 undergoes a thiol disulphide exchange with the PSSG, which results in reduction of the protein thiol and a glutathionylated intermediate of Grx1 (Grx1-SSG). Grx1-SSG, in turn, is reduced by GSH, giving rise to GSSG, which can be reduced via GR [126]. Under physiological conditions, where the GSH/GSSG ratio is high, the action of Grx1 is to de-glutathionylate PSSG, while under oxidative stress conditions, where the GSH/GSSG ratio is low, Grx1 can also catalyse *S*-glutathionylation reactions [127]. Briefly, the first step in this methodology is to deparaffinize tissue sections, followed by rehydration using a graded series of alcohol. Sections are next permeabilized with Triton X-100 in the presence of NEM. After washes in PBS, sections are then incubated with PSSG derivatization buffer containing recombinant Grx1, GSH, NADPH, and GR, in order to decompose the PSSG bond, leading to a newly formed thiol group. The latter is then labelled with biotinylated NEM. Patterns of PSSG can subsequently be visualized by detection of the biotin moiety with fluorophore-conjugated streptavidin reagents or anti-biotin antibodies by confocal microscopy [125]. It is important to note that the use of confocal microscopy is critical for optimal detection of PSSG over potential background signals and to detect hidden PSSG that could not be revealed using wide field fluorescence [124]. Using Grx1-catalysed cysteine derivatization and confocal microscopy, Aesif and colleagues demonstrated changes in *S*-glutathionylation patterns in mice models of lung disease [128].

3.2.6 Measurement of GSH, GSSG and PSSG in different intracellular compartments

The analysis of GSH and its oxidized forms in the different intracellular compartments represents a further challenge for researchers due to their artificial auto-oxidation and/or export from a specific compartment during the manipulation of sample for fractionation. Methods have been progressively improved to overcome these problems. In particular, the use of fluorescent

probes has been applied to measure redox potential in living cells. In addition, redox Western blot techniques utilizing specific constructs to target accumulation of proteins in different subcellular compartments have been developed and other ones are being developed. It is remarkable that, by the use of these new techniques, both qualitative information on the thiol distribution and the GSH redox potentials can be acquired, whereas the exact quantification is still far to be obtained. Some examples of measurements performed in different intracellular compartments and related problems are reported below. As a general concern, total cellular concentrations of GSH and its redox forms are considered to correspond to the cytosolic content. One exception is represented by hepatocytes, where mitochondria are 15% of cell volume. Here, it is considered that 15% of total GSH is in mitochondria [129].

In mitochondria, glutathione is considered to be predominant in the reduced form, with the ratio GSH:GSSG being greater than 100:1 and also proteins are in the reduced thiol state [130]. However, mitochondria seem not to possess GSSG transporters [131], although ABC transporters of mitochondria (ATM) from model organisms *Arabidopsis thaliana* (ATM3) and *Saccharomyces cerevisiae* (Atm1) were recently shown to transport radiolabelled GSSG [132]. Therefore, under oxidative stress conditions, GSSG could accumulate here and also promote the formation of PSSG. Interestingly, it was demonstrated that quantification of both GSH and GSSG in mitochondria greatly differs depending on the method used for isolation (differential centrifugation combined with discontinuous Percoll density gradient centrifugation being currently the most used) [133]. In this work, both GSH and GSSG were measured by HPLC with electrochemical detection and, in experiments where rat mitochondria were isolated by differential centrifugation, the GSH:GSSG ratio for liver mitochondria was 38:1. Immunoblotting and LC/MS/MS were used to detect and identify PSSG: ATP synthase and succinyl-CoA transferase were found to be S-glutathionylated in rat brain mitochondria [133]. HPLC with electrochemical detection was also used in another study to quantify GSH and GSSG in mitochondria separated by differential centrifugation from lymphoblastoid cell lines derived from autistic children. In these cells, the GSH: GSSG ratio was

only 11:6 and reflected the ten-fold lower GSH level in mitochondria relative to the whole-cell extracts [134]. Therefore, it is evident that an agreement about the GSH/GSSG ratio in mitochondria still lacks.

The analysis of GSH and its oxidized forms in the nucleus gave contradictory results too, because of the lack of standardization during sample manipulation. By the use of monochlorobimane and glutathione *S*-transferase as GSH markers in primary cultured rat hepatocytes, it was suggested that the nuclear GSH is about three times more concentrated than the cytosolic one [135]. However, because of the permeability due to nuclear pores, it was suggested that analysis of GSH should require a non-aqueous cell fractionation. Under these experimental conditions, the total glutathione (GSH+GSSG) content in the nucleus of rat hepatocytes was found to be similar to that of cytosol [136]. In another work, where nuclei from HeLa cells were isolated by a silicon oil layer, GSH concentration was found to be about 6 mM [137]. Whereas data on GSSG levels in the nucleus are still poorly investigated, there is some information about the nuclear concentration of PSSG. For example, in human colonic epithelial (HT-29) cells the nuclear PSSG content, quantified by HPLC with fluorescence detection, was measured to be about 0.5 nmol/mg protein, with the concentration in cytosol being about 1.5 nmol/mg protein [138]. The study of the GSH content in the nucleus is also influenced by the different phases of the cell cycle. In fact, cells that are preparing to divide have high nuclear GSH levels [139], whereas confluent cells do not show any difference in GSH levels between the cytoplasm and the nucleus as shown by triple staining (5-chloromethylfluorescein diacetate -Hoechst-propidium iodide or 5-chloromethylfluorescein diacetate -Hoechst-MitoTraker) in association with confocal microscopy analysis in 3T3 fibroblasts used as cellular model [139]. Data for GSH are expressed as the ratio between cytosol and the nucleus; therefore, no information is available about its concentration. Nuclear PSSG were also detected by Western blot and their abundance varied in accordance with GSH levels [139]. These data are relevant since may explain the apparent discrepancies in the GSH distribution previously reported [135−136].

For several years it was accepted that the endoplasmic reticulum (ER) was more oxidizing than other intracellular compartments in order to ensue protein processing. However, there is recent evidence that *ex vivo* thiol oxidation can occur during microsomal isolation, which may have influenced the results. By applying a new method able to prevent such artefactual thiol oxidation and fluorometric HPLC determination, the ER GSH/GSSG ratio was found to be significantly higher than it was assessed before [140]. Specifically, the GSH/GSSG ratio in ER from rat liver microsomes was about 4. It is to note that IAA was used to preserve the GSH redox state. According to our previous observations about the different efficacy of different thiol masking agents in preventing thiol oxidation, we can infer that this ratio may be actually even higher. The same authors also measured less than 5% of total GSH bound to proteins (PSSG).

Lysosomes too are considered to be relatively oxidized in comparison with other subcellular organelles. This observation was obtained by treating PC3 cells with a redox sensitive variant of *Aequorea victoria* green fluorescent protein (GFP), consisting in rhodamine GFP (roGFP1). It has two cysteine residues on either side of its chromophore and is pH-insensitive. The fluorescence excitation maxima at 400 and 490 nm vary depending on the presence or absence of a disulphide bond between these cysteines. By the binding of roGFP1 to various endocytic compartment marker proteins, it was estimated that the endocytic pathway compartments are at least as oxidizing as –240 mV, compared with a much more reducing value of –318 mV for the mitochondria [141]. Research on specific thiol probes for detection of thiols in lysosomes is in progress. For example, a novel ruthenium(II) complex, Ru-2, has recently been developed, which contains a morpholine moiety driving Ru-2 molecules into lysosomes [142]. The specific intracellular localization of Ru-2 was confirmed by lysosome co-localization imaging of HeLa cells, where the Ru-2-loaded cells were further stained with LysoTracker Green, a commercially available lysosome-specific fluorescent indicator. However, its use for quantification has not been proven yet and, especially, Ru-2 showed high selectivity and sensitivity for the detection not only of GSH but also of cysteine and

homocysteine under physiological conditions; therefore, it does not allow the assessment of GSH redox state.

Redox-sensitive yellow and green fluorescent protein variants (rxYFP and roGFPs) are mutants in which a redox sensitive molecular switch consisting of a pair of strategically positioned (surface exposed and in proximity to the chromophore) cysteine residues has been introduced. These cysteines undergo reversible thiol/disulphide exchange with the medium, which gives rise to a reversible conformational change that affects the spectroscopic properties of the chromophore. Redox-sensitive fluorescent proteins allow real-time monitoring of thiol redox dynamics and can be genetically modified in order to target potentially any specific subcellular compartment.

The use of rxYFP and roGFPs to monitor GSH redox potential (E_{GSH}) *in vivo* is confined to subcellular compartments that hold active Grx, which mediates the equilibration between the fluorescent protein sensor and the glutathione redox couple (GSSG/2GSH), acting as catalysts for thiol/disulphide exchange between the glutathione pool and the redox-sensitive protein.
The possibility that Grx availability may influence measurements raised concerns about making comparisons between different subcellular compartments, cell types, and organisms and makes it impossible to make measurements in compartments lacking Grx activity. These problems were addressed gradually constructing various "fusion probes" (*i.e.*, hybrid biosensors) based on a fusion of initially yeast and then human Grxs to rxYFP and, mainly, roGFPs for monitoring, in highly specific manner, real-time changes in E_{GSH}, possibly with subcellular compartment specificity, in intact living cells [143,144]. In this regard, the use of Grx-roGFP2 (the most widely used hybrid redox biosensor) contributed to clarify that the GSH/GSSG ratio in peroxisomes and mitochondrial intermembrane space, two subcellular compartments with no or limited amounts of Grx, is comparable with that of the cytosol [145,146].

Subsequently, starting from the observations that, when coupled directly to other redox enzymes, rxYFP and roGFPs can respond to other redox species, several different redox species-specific *in vivo* redox sensors were generated, mainly with roGFP variants,. This combination of

specific redox enzymes with selected fluorescent redox sensors allows the development of probes specifically tailored to the redox species, subcellular compartment, and cell type of interest [144]. Most of that work has been carried out in mammalian cell lines, with roGFPs targeted to several subcellular compartments, including the cytosol, the mitochondrial matrix and intermembrane space, the plasma membrane, the nucleus, the peroxisomes, endosomes, lysosomes, and the ER [144]. The use of rxYFP and roGFP-based sensors allows direct, real-time imaging of subcellular compartment specific E_{GSH} inside living cells. Novel insights gained through *in vivo* imaging of fluorescent redox sensors include the finding of highly reduced cytosolic glutathione pools, *i.e.* GSH:GSSG ratio between ≈50,000:1 (for a 10 mM total glutathione concentration) and even ≈500,000:1 (for a 1 mM total glutathione concentration) [144]. These findings indicate that cytosolic GSSG levels are much lower than previously assumed [144]. It is noteworthy that very similar cytosolic E_{GSH} values have been reported for all organisms (bacteria, yeast, plants, worms, flies, zebrafish, multiple mammalian cell lines, and mice) in which roGFP measurements have been performed [144].

RoGFP2-based probes targeted to the ER clearly confirmed that the glutathione pool in the ER is considerably more oxidized than in the cytosol [147]. Furthermore, it has been clearly proven that GSH concentration is considerably higher (> 15 mM) in the ER than in the cytosol [147,148]. As a whole, E_{GSH} differs by about ~100 mV when data from whole cell extracts (~230 mV) and specific cellular compartments (~320 mV in the cytosol) are compared. Such a difference is ascribed to the contribution of the GSSG pool present in the ER and related secretory vacuoles that is released upon membrane lysis by invasive methods [144].

The development of efficient, sensitive, selective methods for detecting GSH in living cells is of tremendous scientific significance. Although there have been some fluorescent probes designed for detecting GSH, they exhibit hindrances such as low water solubility, scarce biocompatibility, time-consuming synthesis. Therefore, the development of fluorescent probes for GSH, which can distinguish GSH from cysteine and homocysteine, is still necessary and urgent.

A highly sensitive, selective and water-soluble Rhodamine B-based fluorescent sensor (RhB-1) for GSH was recently synthesized, which effectively detects GSH in living cells and selectively accumulates in lysosomes [149]. The fluorescent probe can distinguish GSH from other natural water-soluble amino acids, amongst which cysteine and homocysteine. In particular, amongst the various analytes tested, only GSH induces a 16-fold increase in RhB-1 fluorescence intensity, owing to the mechanism of turn-on fluorescence caused by ring-opening of RhB-1 induced by GSH. The fluorescence increase is very fast, as it reaches its maximum within only 10 s after addition of GSH to the aqueous solution of RhB-1 at 25°C.

A fluorescent probe for the selective detection (over cysteine and homocysteine) of mitochondrial GSH in living cells was also developed [150]. The probe contains 3,5-dichlorinated BODIPY as a GSH recognition group and triphenylphosphonium moiety integrated into the chlorinated BODIPY as a mitochondrial targeting group. Co-localization experiments demonstrated that the fluorescent probe especially located mitochondrial GSH in living cells. Laser scanning confocal fluorescence imaging confirmed its applicability to detect GSH in mitochondria of living cells.

4. Conclusion and future directions

The emerging interest in studying the redox steady-state of GSH and GSSG (*i.e.*, the GSH/GSSG ratio) and the involvement of protein *S*-glutathionylation in the regulation of physiological as well as pathophysiological processes led to the development of several methodologies for the quantification/detection of glutathione and PSSG in cells, tissues, and body fluids. The analysis of GSH and its disulphide forms (GSSG and PSSG) in tissues and cells is problematic for several reasons. Firstly, basal levels of GSSG and PSSG are very low compared to GSH, and GSH easily undergoes auto-oxidation during sample preparation. Therefore, the auto-oxidation of a small amount of GSH to GSSG can dramatically alter the GSH/GSSG ratio and, consequently, the level

of PSSG. This problem is particularly evident when the analysis is carried out in a specific cellular compartment, since it takes a long time for the cell fractionation. Secondly, GSSG and PSSG can be reduced back to thiols enzymatically during tissue and cell manipulations [47,49,77]. Thirdly, GSH and its disulphide forms have not chromophores and derivatization is an indispensable step in most methodologies. However, derivatization reagents used in such methods may react incompletely and variably, and may generate by-products. These circumstances may finally generate additional interfering signals. Fourthly, the quantification of GSSG and PSSG levels may be more complex in cell cultures than in other biological systems because many different experimental conditions can be used to treat and collect cultured cells, thus introducing many interfering factors [60]. Moreover, within cells, GSH concentration is several orders of magnitude higher than that of GSSG and PSSG, whereas extracellular GSH concentration is conversely rather low. Therefore, uncontrolled lysis of cells such as erythrocytes or incomplete removal of the extracellular fluid can affect GSH and/or GSSG and PSSG analysis in extracellular fluids like plasma. Consequently, without particular care to sample manipulation and derivatization, GSSG and PSSG levels might be overestimated or underestimated making the interpretation of results misleading. Regarding this issue, we have developed and validated detailed protocols for the artefact-free and accurate measurement of GSH, GSSG, and PSSG in human blood, solid tissues, and several cultured cell lines [59,60].

Also the conventional proteomics methods (anti-GSH antibodies-based techniques) show obvious limits as underestimate low-abundant PSSG. However, the most recent highly sensitive proteomic methodologies seem to be very promising in remedying this problem, enabling identification of low-abundance PSSG in tissues and cells. On the other hand, the anti-GSH antibodies are a very useful tool for locating PSSG in intracellular compartments.

In conclusion, no “one-fits-all” methodology exists, and each methodology exhibits benefits and potential pitfalls. Recent technical advances in redox proteomics have facilitated enhanced sensitivity of detection, which will likely lead to the identification of many new (low-abundance)

PSSG that occur under physiological or pathological conditions. We hope that the methodologies collected in this article will stimulate further work in this area of research.

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2-VP, 2-vinyl pyridine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiotreitol; E_{GSH}, GSH redox potential; ER, endoplasmic reticulum; FDNB, 1-fluoro-2,4-dinitrobenzene; G6PDH, glucose-6-phosphate dehydrogenase; GFP, green fluorescent protein; GR, GSSG reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulphide; HPLC, high performance (pressure) liquid chromatography; IAA, iodoacetic acid; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; mBrB, monobromobimane; MMTS, *S*-methyl methanethiosulfonate NEM, *N*-ethylmaleimide; PSH, protein thiols; PSSG, protein-glutathione mixed disulphide (*S*-glutathionylated protein); PTMs, post-translational modifications; RBC, red blood cell; ROS, reactive oxygen species; YFP, yellow fluorescent protein.

References

[1] H. Sies. Introduction. In H. Sies, ed. Oxidative Stress. London: Academic Press; 1991; pp xv-xxii.

[2] R. Sultana, D.A. Butterfield. Oxidative modification of brain proteins in Alzheimer's disease: Perspective on future studies based on results of redox proteomics studies. J. Alzh. Dis. 2013; 33:S243–S251. doi: 10.3233/JAD-2012-129018.

[3] D.A. Butterfield, A.M. Swomley, R. Sultana. Amyloid β-peptide (1-42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. Antioxid. Redox Signal. 2013; 19:823–835. doi: 10.1089/ars.2012.5027

[4] A. Tramutola, C. Lanzillotta, M. Perluigi, D.A. Butterfield. Oxidative stress, protein modification and Alzheimer disease. Brain. Res. Bull. 2016; pii: S0361-9230(16)30129-0. doi: 10.1016/j.brainresbull.2016.06.005.

[5] A.J. Kettle, R. Turner, C.L. Gangell, D.T. Harwood, I.S. Khalilova, A.L. Chapman, C.C. Winterbourn, P.D. Sly. AREST CF. Oxidation contributes to low glutathione in the airways of children with cystic fibrosis. Eur. Respir. J. 2014; 44:122–129. doi: 10.1183/09031936.00170213.

[6] N.J. Magon, R. Turner, R.B. Gearry, M.B. Hampton, P.D. Sly, A.J. Kettle. Oxidation of calprotectin by hypochlorous acid prevents chelation of essential metal ions and allows bacterial growth: Relevance to infections in cystic fibrosis. Free Radic. Biol. Med. 2015; 86:133–144. doi: 10.1016/j.freeradbiomed.2015.05.022.

[7] G. Colombo, F. Reggiani, M.A. Podestà, M.L. Garavaglia, N.M. Portinaro, A. Milzani, S. Badalamenti, I. Dalle-Donne. Plasma protein thiolation index (PTI) as a biomarker of thiol-specific oxidative stress in haemodialyzed patients. Free Radic. Biol. Med. 2015; 89:443–551. doi: 10.1016/j.freeradbiomed.2015.08.022.

[8] G. Colombo, M. Clerici, D. Giustarini, N.M. Portinaro, S. Badalamenti, R. Rossi, A. Milzani, I. Dalle-Donne. A central role for intermolecular dityrosine cross-linking of fibrinogen in high

molecular weight advanced oxidation protein product (AOPP) formation. Biochim. Biophys. Acta. 2015; 1850:1–12. doi: 10.1016/j.bbagen.2014.09.024.

[9] P. Fanti, D. Giustarini, R. Rossi, S.E. Cunningham, F. Folli, K. Khazim, J. Cornell, E. Matteucci, S. Bansal. Dietary intake of proteins and calories is inversely associated with the oxidation state of plasma thiols in end-stage renal disease patients. J. Ren. Nutr. 2015; 25:494–503. doi: 10.1053/j.jrn.2015.06.003

[10] R. Rossi, D. Giustarini, A. Milzani, I. Dalle-Donne. Cysteinylation and homocysteinylation of plasma protein thiols during ageing of healthy human beings. J. Cell. Mol. Med. 2009; 13:3131–3140. doi: 10.1111/j.1582-4934.2008.00417.x.

[11] D. Giustarini, I. Dalle-Donne, S. Lorenzini, E. Selvi, G. Colombo, A. Milzani A, P. Fanti, R. Rossi. Protein thiolation index (PTI) as a biomarker of oxidative stress. Free Radic. Biol. Med. 2012; 53:907–915. doi: 10.1016/j.freeradbiomed.2012.06.022.

[12] P. Silva, E. Fernandes, F. Carvalho. Dual effect of red wine on liver redox status: a concise and mechanistic review. Arch. Toxicol. 2015; 89:1681–1693. doi: 10.1007/s00204-015-1538-1.

[13] G. Colombo, R. Rossi, N. Gagliano, N. Portinaro, M. Clerici, A. Annibal, D. Giustarini, R. Colombo, A. Milzani, I. Dalle-Donne. Red blood cells protect albumin from cigarette smoke-induced oxidation. PLoS One 2012; 7:e29930. doi: 10.1371/journal.pone.0029930.

[14] G. Colombo, M. Clerici, D. Giustarini D, N.M. Portinaro, G. Aldini, R. Rossi, A. Milzani, I. Dalle-Donne. Pathophysiology of tobacco smoke exposure: recent insights from comparative and redox proteomics. Mass Spectrom. Rev. 2014; 33:183–218. doi: 10.1002/mas.21392

[15] H.J. Forman, F. Ursini, M. Maiorino. An overview of mechanisms of redox signalling. J. Mol. Cell. Cardiol. 2014; 73:2–9. doi: 10.1016/j.yjmcc.2014.01.018.

[16] E. Niki. Oxidative stress and antioxidants: Distress or eustress? Arch. Biochem. Biophys. 2016; 595:19–24. doi: 10.1016/j.abb.2015.11.017.

[17] E. Valencia, A. Marin, G. Hardy. Glutathione: nutritional and pharmacological viewpoints: part VI. Nutrition 2002; 18:291–292.

[18] R.A. Blanco, T.R. Ziegler, B.A. Carlson, P.Y. Cheng, Y. Park, G.A. Cotsonis, C.J. Accardi, D.P. Jones. Diurnal variation in glutathione and cysteine redox states in human plasma. Am. J. Clin. Nutr. 2007; 86:1016–1023.

[19] J. Lyons, A. Rauh-Pfeiffer, Y.M. Yu, X.M. Lu, D. Zurakowski, R.G. Tompkins, A.M. Ajami, V.R. Young, L. Castillo. Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid free diet. Proc. Natl. Acad. Sci. USA 2000; 97:5071–5076. doi: 10.1073/pnas.090083297

[20] D. Giustarini, A. Milzani, I. Dalle-Donne, R. Rossi. Red blood cells as a physiological source of glutathione for extracellular fluids. Blood Cells Mol. Dis. 2008; 40:174–179. doi: 10.1016/j.bcmd.2007.09.001

[21] H.J. Forman, H. Zhang, A. Rinna. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol. Aspects Med. 2009; 30:1–12. doi: 10.1016/j.mam.2008.08.006.

[22] S.C. Lu. Regulation of glutathione synthesis. Mol. Aspects Med. 2009; 30:42–59. doi: 10.1016/j.mam.2008.05.005.

[23] S.C. Lu. Glutathione synthesis. Biochim. Biophys. Acta 2013; 1830:3143–3153. doi: 10.1016/j.bbagen.2012.09.008.

[24] W.G. Kirlin, J. Cai, S.A. Thompson, D. Diaz, T.J. Kavanagh, D.P. Jones. Glutathione redox potential in response to differentiation and enzyme inducers. Free Radic. Biol. Med. 1999; 27: 1208–1218.

[25] A.L. Ortega, S. Mena, J.M. Estrela. Glutathione in cancer cell death. Cancers 2011; 3: 1285–1310. doi: 10.3390/cancers3011285.

[26] I. Dalle-Donne, A. Milzani, N. Gagliano, R. Colombo, D. Giustarini, R. Rossi. Molecular mechanisms and potential clinical significance of S-glutathionylation. Antioxid. Redox Signal. 2008; 10:445–473. doi: 10.1089/ars.2007.1716

[27] N.J. Bulleid, L. Ellgaard. Multiple ways to make disulfides. Trends. Biochem. Sci. 2011; 36:485–492. doi: 10.1016/j.tibs.2011.05.004.

[28] F.V. Pallardó, J. Markovic, J.L. García, J. Viña. Role of nuclear glutathione as a key regulator of cell proliferation. Mol. Aspects Med. 2009; 30:77–85. doi: 10.1016/j.mam.2009.01.001

[29] I. Dalle-Donne, G. Colombo, N. Gagliano, R. Colombo, D. Giustarini, R. Rossi, A. Milzani. S-glutathiolation in life and death decisions of the cell. Free Radic. Res. 2011; 45:3–15. doi: 10.3109/10715762.2010.515217

[30] N. Ballatori, S.M. Krance, S. Notenboom, S. Shi, K. Tieu, C.L. Hammond. Glutathione dysregulation and the etiology and progression of human diseases. Biol. Chem. 2009; 390:191–214. doi: 10.1515/BC.2009.033.

[31] D. Giustarini, I. Dalle-Donne, D. Tsikas, R. Rossi. Oxidative stress and human diseases: Origin, link, measurement, mechanisms and biomarkers. Crit. Rev. Clin. Lab. Sci. 2009; 46:241–281. doi: 10.3109/10408360903142326

[32] L. Flohé. The fairytale of the GSSG/GSH redox potential. Biochim. Biophys. Acta 2013; 1830: 3139–3142. doi: 10.1016/j.bbagen.2012.10.020.

[33] C.L. Grek, J. Zhang, Y. Manevich, D.M. Townsend, K.D. Tew. Causes and consequences of cysteine *S*-glutathionylation. J. Biol. Chem. 2013; 288:26497–26504. doi: 10.1074/jbc.R113.461368.

[34] A. Bachi, I. Dalle-Donne, A. Scaloni. Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises. Chem. Rev. 2013; 113:596–698. doi: 10.1021/cr300073p. Erratum in: Chem. Rev. 2015 May 13; 115(9):3677.

[35] I. Dalle-Donne, R. Rossi, G. Colombo, D. Giustarini, A. Milzani. Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends Biochem. Sci. 2009; 34:85–96. doi: 10.1016/j.tibs.2008.11.002.

[36] A. Pastore, F. Piemonte. S-Glutathionylation signaling in cell biology: progress and prospects. Eur. J. Pharm. Sci. 2012; 46:279–292. doi: 10.1016/j.ejps.2012.03.010

[37] J.J. Mieyal, P.B. Chock. Posttranslational modification of cysteine in redox signaling and oxidative stress: Focus on S-glutathionylation. Antioxid. Redox Signal. 2012; 16:471–475. doi: 10.1089/ars.2011.4454

[38] P. Ghezzi. Protein glutathionylation in health and disease. Biochim. Biophys. Acta 2013; 1830:3165–3172. doi: 10.1016/j.bbagen.2013.02.009

[39] S.F. Newman, R. Sultana, M. Perluigi, R. Coccia, J. Cai, W.M. Pierce, J.B. Klein, D.M. Turner, D.A. Butterfield. An increase in S-glutathionylated proteins in the Alzheimer's disease inferior parietal lobule, a proteomics approach. J. Neurosci. Res. 2007; 85:1506–1514. doi: 10.1002/jnr.21275

[40] A. Pastore, G. Tozzi, L.M. Gaeta, E. Bertini, V. Serafini, S. Di Cesare, V. Bonetto, F. Casoni, R. Carrozzo, G. Federici, F. Piemonte. Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: a potential role in the pathogenesis of the disease. J. Biol. Chem. 2003; 43:42588–42595. doi: 10.1074/jbc.M301872200

[41] F. Piemonte, S. Petrini, L.M. Gaeta, G. Tozzi, E. Bertini, R. Devito, R. Boldrini, M. Marcellini, E. Ciacco, V. Nobili. Protein glutathionylation increases in the liver of patients with non-alcoholic fatty liver disease. J. Gastroenterol. Hepatol. 2008; 23:e457–464. doi: 10.1111/j.1440-1746.2007.05070.x

[42] A. Pastore, F. Piemonte. Protein glutathionylation in cardiovascular diseases. Int. J. Mol. Sci. 2013; 14:20845–20876. doi: 10.3390/ijms141020845

[43] K. Khazim, D. Giustarini, R. Rossi, D. Verkaik, J.E. Cornell, S.E. Cunningham, M. Mohammad, K. Trochta, C. Lorenzo, F. Folli, S. Bansal, P. Fanti. Glutathione redox potential is low and glutathionylated and cysteinylated hemoglobin levels are elevated in maintenance hemodialysis patients. Transl. Res. 2013; 162:16–25. doi: 10.1016/j.trsl.2012.12.014.

[44] I. Kuipers, R. Louis, M. Manise, M.A. Dentener, C.G. Irvin, Y.M. Janssen-Heininger, C.E. Brightling, E.F. Wouters, N.L. Reynaert. Increased glutaredoxin-1 and decreased protein S-

glutathionylation in sputum of asthmatics. Eur. Respir. J. 2013; 41:469–472. doi: 10.1183/09031936.00115212

[45] F. Tietze. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 1969; 27:502–522.

[46] S.K. Srivastava, E. Beutler,.Oxidized glutathione levels in erythrocytes of glucose-6-phosphate-dehydrogenase–deficient subjects. Lancet 1968; 2:23–24.

[47] D. Giustarini, D. Tsikas, G. Colombo, A. Milzani, I. Dalle-Donne, P. Fanti, R. Rossi. Pitfalls in the analysis of the physiological antioxidant glutathione (GSH) and its disulfide (GSSG) in biological samples: An elephant in the room. J. Chromatogr. B. 2016; 1019:21–28. doi: 10.1016/j.jchromb.2016.02.015.

[48] R. Rossi, I. Dalle-Donne, A. Milzani, D. Giustarini. Oxidized forms of glutathione in peripheral blood as biomarkers of oxidative stress. Clin. Chem. 2006; 52:1406–1414. doi: 10.1373/clinchem.2006.067793

[49] R.E Hansen, J.R. Winther. An introduction to methods for analysing thiols and disulfides: reactions, reagents, and practical considerations. Anal. Biochem. 2009; 394:147–158. doi: 10.1016/j.ab.2009.07.051

[50] D. Stempak, S. Dallas, J. Klein, R. Bendayan, G. Koren, S. Baruchel. Glutathione stability in whole blood: effects of various deproteinizing acids. Ther. Drug Monit. 2001; 23:542–549.

[51] R. Rossi, A. Milzani, I. Dalle-Donne, D. Giustarini, L. Lusini, R. Colombo, P. Di Simplicio. Blood glutathione disulfide: in vivo factor or in vitro artifact? Clin. Chem. 2002; 48:742–753.

[52] J.R. Winther, C. Thorpe. Quantification of thiols and disulfides. Biochim. Biophys. Acta 2014; 1840:838–846. doi: 10.1016/j.bbagen.2013.03.031

[53] R.A. Bednar. Reactivity and pH-dependence of thiol conjugation to N-ethylmaleimide: detection of a conformational change in chalcone isomerise. Biochemistry 1990; 29:3684–3690.

[54] A.D. Baldwin, K.L. Kiick. Tunable degradation of maleimide-thiol adducts in reducing environments. Bioconjug. Chem. 2011; 22:1946–1953. doi: 10.1021/bc200148v.

[55] D. Lin, S. Saleh, D.C. Liebler. Reversibility of covalent electrophile-protein adducts and chemical toxicity. Chem. Res. Toxicol. 2008; 21:2361–2369.

[56] O.W. Griffith. Determination of glutathione and glutathione disulfide using glutathione-reductase and 2-vinylpyridine. Anal. Biochem. 1980; 106:207–212.

[57] D. Schofield, G. Mei, J.M. Braganza. Some pitfalls in the measurement of blood glutathione. Clin. Sci. 1993; 85:213–218.

[58] M.R. McGill, H. Jaeschke. A direct comparison of methods used to measure oxidized glutathione in biological samples: 2-vinylpyridine and N-ethylmaleimide. Toxicol. Mech. Methods 2015; 25:589–595. doi: 10.3109/15376516.2015.1094844

[59] D. Giustarini, I. Dalle-Donne, A. Milzani, P. Fanti, R. Rossi. Analysis of GSH/GSSG after derivatization with N-ethylmaleimide. Nat. Protoc. 2013; 8:1660–1669. doi: 10.1038/nprot.2013.095.

[60] D. Giustarini, F. Galvagni, A. Tesei, A. Farolfi, M. Zanoni, S. Pignatta, A. Milzani, I.M. Marone, I. Dalle-Donne, R. Nassini, R. Rossi. Glutathione, glutathione disulfide, and S-glutathionylated proteins in cell cultures. Free Radic. Biol. Med. 2015; 89:972–981. doi: 10.1016/j.freeradbiomed.2015.10.410

[61] D. Giustarini, I. Dalle-Donne, A. Milzani, R. Rossi. Detection of glutathione in whole blood after stabilization with N-ethylmaleimide. Anal. Biochem. 2011; 415:81–83. doi: 10.1016/j.ab.2011.04.013

[62] T. Kuninori, J. Nishiyama. Some properties of diastereomers formed in the reactions of *N*-ethylmaleimide with biological thiols. Agric. Biol. Chem. 1985; 49:2453–2454.

[63] J.P. Steghens, F. Flourié, K Arab, C. Collombel. Fast liquid chromatography-mass spectrometry glutathione measurement in whole blood: micromolar GSSG is a sample preparation artifact. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2003; 798:343–349.

[64] T. Moore, A. Le, A.K. Niemi, T. Kwan, K. Cusmano-Ozog, G.M. Enns, T.M. Cowan. A new LC-MS/MS method for the clinical determination of reduced and oxidized glutathione from whole blood. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2013; 929:51–55. doi: 10.1016/j.jchromb.2013.04.004.

[65] D. Hedley, S. Chow. Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry 1994; 15:349–358. doi: 10.1002/cyto.990150411

[66] X. Jiang, Y. Yu, J. Chen, M. Zhao, H. Chen, X. Song, A.J. Matzuk, S.L. Carroll, X. Tan, A. Sizovs, N. Cheng, M.C. Wang, J. Wang. Quantitative imaging of glutathione in live cells using a reversible reaction-based ratiometric fluorescent probe. ACS Chem. Biol. 2015; 10(:864–874. doi: 10.1021/cb500986w.

[67] D. Giustarini, I. Dalle-Donne, A. Milzani, R. Rossi. Low molecular mass thiols, disulfides and protein mixed disulfides in rat tissues: influence of sample manipulation, oxidative stress and ageing. Mech. Ageing Develop. 2011; 132:141–148. doi: 10.1016/j.mad.2011.02.001

[68] G.L. Newton, R. Dorian, R.C. Fahey. Analysis of biological thiols: derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography. Anal. Biochem. 1981; 114:383–387.

[69] K. Imai, T. Toyooka, Y. Watanabe. A novel fluorogenic reagent for thiols: ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Anal. Biochem. 1983; 128:471–473.

[70] R. Kand'ár, P. Záková, H. Lotková, O. Kucera, Z. Cervinková. Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. J. Pharm. Biomed. Anal. 2007; 43:1382–1387. doi: 10.1016/j.jpba.2006.11.028

[71] H. Komatsu, F. Obata. An optimized method for determination of intracellular glutathione in mouse macrophage cultures by fluorimetric high-performance liquid chromatography. Biomed. Chromatogr. 2003; 17: 345–350. doi: 10.1002/bmc.250

[72] D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis, D.W. Potter. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. 1980; 106:55–62.

[73] D. Giustarini, I. Dalle-Donne, R. Colombo, A. Milzani, R. Rossi. An improved HPLC measurement for GSH and GSSG in human blood. Free Radic. Biol. Med. 2003; 35:1356–1372.

[74] R. Rossi, D. Giustarini, S. Fineschi, G. De Cunto, G. Lungarella, E. Cavarra. Differential thiol status in blood of different mouse strains exposed to cigarette smoke. Free Radic. Res. 2009; 43:538–545. doi: 10.1080/10715760902893332

[75] I. Nemeth, H. Orvos, D. Boda. Blood glutathione redox status in gestational hypertension. Free Radic. Biol. Med. 2001; 30:715–721.

[76] A. Papp, I. Németh, E. Karg, E. Papp. Glutathione status in retinopathy of prematurity. Free Radic. Biol. Med. 1999; 27:738–743.

[77] R.E. Hansen, D. Roth, J.R. Winther. Quantifying the global cellular thiol-disulfide status. Proc. Natl. Acad. Sci. U S A. 2009; 106:422–427. doi: 10.1073/pnas.0812149106

[78] D. Giustarini, I. Dalle-Donne, R. Colombo, S. Petralia, S. Giampaoletti, A. Milzani, R. Rossi. Protein glutathionylation in erythrocytes. Clin. Chem. 2003; 49:327–330.

[79] I. Fermo, C. Arcelloni, E. De Vecchi, S. Vigano, R. Paroni. High performance liquid chromatographic method with fluorescence detection for the determination of total homocyst(e)ine in plasma. J. Chromatogr. 1992; 593:171–176.

[80] W.D. Brown. Reduction of protein disulfide bond by sodium borohydride. Biochim. Biophys. Acta 1960; 44:365–367.

[81] M.T. Gladwin, J.H. Shelhamer, F.P. Ognibene, M.E. Pease-Fye, J.S. Nichols, B. Link, D.B. Patel, M.A. Jankowski, L.K. Pannell, A.N. Schechter, G.P. Rodgers. Nitric oxide donor properties of hydroxyurea in patients with sickle cell disease. Br. J. Haematol. 2002; 116:436–444.

[82] D. Giustarini, I. Dalle-Donne, E. Cavarra, S. Fineschi, G. Lungarella, A. Milzani, R. Rossi.

Metabolism of oxidants by blood from different mouse strains. Biochem. Pharmacol. 2006; 71:1753–1764. doi: 10.1016/j.bcp.2006.03.015

[83] M.R. Bukowski, C. Bucklin, M.J. Picklo. Quantitation of protein S-glutathionylation by liquid chromatography-tandem mass spectrometry: correction for contaminating glutathione and glutathione disulfide. Anal. Biochem. 2015; 469:54–64. doi: 10.1016/j.ab.2014.10.002.

[84] K. Grintzalis, I. Papapostolou, D. Zisimopoulos, I. Stamatiou, C.D. Georgiou. Multiparametric protocol for the determination of thiol redox state in living matter. Free. Radic. Biol. Med. 2014; 74:85–98. doi: 10.1016/j.freeradbiomed.2014.06.024.

[85] S.E. Moriarty-Craige, D.P. Jones. Extracellular thiols and thiol/disulfide redox in metabolism. Annu. Rev. Nutr. 2004; 24:481–509. doi: 10.1146/annurev.nutr.24.012003.132208

[86] S.R. Jaffrey, H. Erdjument-Bromage, C.D. Ferris, P. Tempst, S.H. Snyder. Protein *S*-nitrosylation: a physiological signal for neuronal nitric oxide. Nat. Cell Biol. 2001; 3:193–197. doi: 10.1038/35055104

[87] S. Kehr, E. Jortzik, C. Delahunty, J.R. Yates, 3rd, S. Rahlfs, K. Becker. Protein *S*-glutathionylation in malaria parasites. Antioxid. Redox Signal. 2011; 15:2855–2865. doi: 10.1089/ars.2011.4029.

[88] B.G. Hill, K.V. Ramana, J. Cai, A. Bhatnagar, S.K. Srivastava. Measurement and identification of S-glutathiolated proteins. Methods Enzymol. 2010; 473:179–197. doi: 10.1016/S0076-6879(10)73009-3.

[89] D. Bar-Or, K.D. Heyborne, R. Bar-Or, L.T. Rael, J.V. Winkler, D. Navot. Cysteinylation of maternal plasma albumin and its association with intrauterine growth restriction. Prenat. Diagn. 2005; 25:245–249. doi: 10.1002/pd.1122

[90] L. Regazzoni, L. Del Vecchio, A. Altomare, K.J. Yeum, D. Cusi, F. Locatelli, M. Carini, G. Aldini. Human serum albumin cysteinylation is increased in end stage renal disease patients and reduced by hemodialysis: mass spectrometry studies. Free Radic. Res. 2013; 4:172–180. doi: 10.3109/10715762.2012.756139

[91] F. Hochgräfe, J. Mostertz, D.C. Pöther, D. Becher, J.D. Helmann, M. Hecker. S-cysteinylation is a general mechanism for thiol protection of Bacillus subtilis proteins after oxidative stress. J. Biol. Chem. 2007; 282:25981–25985. doi: 10.1074/jbc.C700105200

[92] Y. Xiong , J.D. Uys, K.D. Tew, D.M. Townsend. S-Glutathionylation: from molecular mechanisms to health outcomes. Antioxid. Redox Signal. 2011; 15:233–270. doi: 10.1089/ars.2010.3540

[93] I. Dalle-Donne, D. Giustarini, R. Colombo, A. Milzani, R. Rossi. S-glutathionylation in human platelets by a thiol-disulfide exchange-independent mechanism. Free Radic. Biol. Med. 2005; 38:1501–1510. doi: 10.1016/j.freeradbiomed.2005.02.019

[94] M. Sparaco, L.M. Gaeta, G. Tozzi, E. Bertini, A. Pastore, A. Simonati, F.M. Santorelli, F. Piemonte. Protein glutathionylation in human central nervous system: potential role in redox regulation of neuronal defense against free radicals. J. Neurosci. Res. 2006; 83:256–263. doi: 10.1002/jnr.20729

[95] C. Passarelli, G. Tozzi, A. Pastore, E. Bertini, F. Piemonte. GSSG-mediated Complex I defect in isolated cardiac mitochondria. Int. J. Mol. Med. 2010; 26:95–99. https://doi.org/10.3892/ijmm_00000439

[96] A. De Luca, N. Moroni, A. Serafino, A. Primavera, A. Pastore, J. Pedersen, R. Petruzzelli, M. Farrace, P. Pierimarchi, G. Moroni, G. Federici, P. Sinibaldi Vallebona, M. Lo Bello. Treatment of doxorubicin-resistant MCF7/Dx cells with nitric oxide causes histone glutathionylation and reversal of drug resistance. Biochem. J. 2011; 440:175–183. doi: 10.1042/BJ20111333

[97] M.M. Stacey, S.L. Cuddihy, M.B. Hampton, C.C. Winterbourn. Protein thiol oxidation and formation of S-glutathionylated cyclophilin A in cells exposed to chloramines and hypochlorous acid. Arch. Biochem. Biophys. 2012; 527:45–54. doi: 10.1016/j.abb.2012.07.011

[98] R. Rossi, D. Giustarini, G. Colombo, A. Milzani, I. Dalle-Donne. Evidence against a role of ketone bodies in the generation of oxidative stress in human erythrocytes by the application of

reliable methods for thiol redox form detection. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 2009; 877:3467–3474. doi: 10.1016/j.jchromb.2009.04.032

[99] M.B. West, B.G. Hill, Y.T. Xuan, A. Bhatnagar. Protein glutathiolation by nitric oxide: an intracellular mechanism regulating redox protein modification. FASEB J. 2006; 20:1715–1717. doi: 10.1096/fj.06-5843fje

[100] C.L. Grek, D.M. Townsend, J.D. Uys, Y. Manevich, W.J. Coker, C.J. Pazoles, K.D. Tew. S-Glutathionylated serine proteinase inhibitors as plasma biomarkers in assessing response to redox-modulating drugs. Cancer Res. 2012; 72:2383–2393. doi: 10.1158/0008-5472.CAN-11-4088

[101] J. Yang, K.S. Carroll, D.C. Liebler. The expanding landscape of the thiol redox proteome. Mol. Cell. Proteomics 2016; 15:1–11. doi: 10.1074/mcp.O115.056051.

[102] D.M. Sullivan, N.B. Wehr, M.M. Fergusson, R.L. Levine, T. Finkel. Identification of oxidant-sensitive proteins: TNF-alpha induces protein glutathiolation. Biochemistry 2000; 39:11121–11128.

[103] J.P. Brennan, J.I. Miller, W. Fuller, R. Wait, S. Begum, M.J. Dunn, P. Eaton. The utility of N, N-biotinyl glutathione disulfide in the study of protein S-glutathiolation. Mol. Cell. Proteomics 2006; 5:215–225. doi: 10.1074/mcp.M500212-MCP200

[104] S. Chardonnet, S. Sakr, C. Cassier-Chauvat, P. Le Marechal, F. Chauvat, S.D. Lemaire, P. Decottignies. First proteomic study of S-glutathionylation in cyanobacteria. J. Proteome Res. 2015; 14:59–71. doi: 10.1016/S0076-6879(10)73009-3.

[105] Y. Yang, W. Shi, N. Cui, Z. Wu, C. Jiang. Oxidative stress inhibits vascular KATP channels by S-glutathionylation. J. Biol. Chem. 2010; 285:38641–38648. doi: 10.1074/jbc.M110.162578

[106] M. Zaffagnini, M. Bedhomme, H. Groni, C.H. Marchand, C. Puppo, B. Gontero, C. Cassier-Chauvat, P. Decottignies, S.D. Lemaire. Glutathionylation in the photosynthetic model organism *Chlamydomonas reinhardtii*: a proteomic survey. Mol. Cell. Proteomics 2012; 11:M111.014142. doi: 10.1074/mcp.M111.014142.

[107] K.T. Samarasinghe, D.N. Munkanatta Godage, G.C. VanHecke, Y.H. Ahn. Metabolic synthesis of clickable glutathione for chemoselective detection of glutathionylation. J. Am. Chem. Soc. 2014; 136:11566–11569. doi: 10.1021/ja503946q.

[108] K.T. Samarasinghe, D.N. Munkanatta Godage, Y. Zhou, F.T. Ndombera, E. Weerapana, Y.H. Ahn. A clickable glutathione approach for identification of protein glutathionylation in response to glucose metabolism. Mol. Biosyst. 2016; 12:2471–2480. doi: 10.1039/c6mb00175k.

[109] D.N. Kekulandara, K.T. Samarasinghe, D.N. Munkanatta Godage, Y.H. Ahn. Clickable glutathione using tetrazine-alkene bioorthogonal chemistry for detecting protein glutathionylation. Org. Biomol. Chem. 2016; 14:10886–10893. doi: 10.1039/c6ob02050j

[110] S. Feng, Y. Chen, F. Yang, L. Zhang, Y. Gong, G. Adilijiang, Y. Gao, H. Deng. Development of a clickable probe for profiling of protein glutathionylation in the central cellular metabolism of *E. coli* and *Drosophila*. Chem. Biol. 2015; 22:1461–1469. doi: 10.1016/j.chembiol.2015.09.012.

[111] L. Mullen, M. Seavill, R. Hammouz, B. Bottazzi, P. Chan, D. Vaudry, P. Ghezzi. Development of 'Redox Arrays' for identifying novel glutathionylated proteins in the secretome. Sci. Rep. 2015; 5:14630. doi: 10.1038/srep14630

[112] D.J. McGarry, W. Chen, P. Chakravarty, D.L. Lamont, C.R. Wolf, C.J. Henderson. Proteome-wide identification and quantification of S-glutathionylation targets in mouse liver. Biochem. J. 2015; 469:25–32. doi: 10.1042/BJ20141256

[113] D. Su, M.J. Gaffrey, J. Guo, K.E. Hatchell, R.K. Chu, T.R. Clauss, J.T. Aldrich, S. Wu, S. Purvine, D.G. Camp, R.D. Smith, B.D. Thrall, W.J. Qian. Proteomic identification and quantification of S-glutathionylation in mouse macrophages using resin-assisted enrichment and isobaric labeling. Free Radic. Biol. Med. 2014; 67:460–470. doi: 10.1016/j.freeradbiomed.2013.12.004

[114] S.L. Ullevig, H.S. Kim, J.D. Short, S. Tavakoli, S.T. Weintraub, K. Downs, R. Asmis. Protein S-glutathionylation mediates macrophage responses to metabolic cues from the extracellular

environment. Antioxid. Redox Signal. 2016, ahead of print. doi:10.1089/ars.2015.6531. doi: 10.1089/ars.2015.6531

[115] N. Siuti, N.L. Kelleher. Decoding protein modifications using top-down mass spectrometry. Nat. Methods 2007; 4:817–821. doi: 10.1016/j.ab.2009.09.022

[116] Z. Tian, N. Tolić, R. Zhao, R.J. Moore, S.M. Hengel, E.W. Robinson, D.L. Stenoien, S. Wu, R.D. Smith, L. Paša-Tolić. Enhanced top-down characterization of histone post-translational modifications. Genome Biol. 2012; 13: R86. doi: 10.1186/gb-2012-13-10-r86.

[117] J.L. García-Giménez, G. Òlaso, S.B. Hake, C. Bönisch, S.M. Wiedemann, J. Markovic, F. Dasí, A. Gimeno, C. Pérez-Quilis, O. Palacios, M. Capdevila, J. Viña, F.V. Pallardó. Histone H3 glutathionylation in proliferating mammalian cells destabilizes nucleosomal structure. Antioxid. Redox Signal. 2013; 19:1305–1320. doi: 10.1089/ars.2012.5021.

[118] A. De Luca, N. Moroni, A. Serafino, A. Primavera, A. Pastore, J.Z. Pedersen, R. Petruzzelli, M.G. Farrace, P. Pierimarchi, G. Moroni, G. Federici, P. Sinibaldi Vallebona, M. Lo Bello. Treatment of doxorubicin-resistant MCF7/Dx cells with nitric oxide causes histone glutathionylation and reversal of drug resistance. Biochem. J. 2011; 440:175–183. doi: 10.1042/BJ20111333.

[119] M. Zhou, L. Paša-Tolić, D.L. Stenoien. Profiling of histone post-translational modifications in mouse brain with high-resolution top-down mass spectrometry. J. Proteome Res. 2017; 16:599–608. doi: 10.1021/acs.jproteome.6b00694.

[120] J.L. García-Giménez, J.S. Ibañez-Cabellos, M. Seco-Cervera, F.V. Pallardó. Glutathione and cellular redox control in epigenetic regulation. Free Radic. Biol. Med. 2014; 75 Suppl 1:S3. doi: 10.1016/j.freeradbiomed.2014.10.828.

[121] M. Sparaco, L.M. Gaeta, F.M. Santorelli, C. Passarelli, G. Tozzi, E. Bertini, A. Simonati, F. Scaravilli, F. Taroni, C. Duyckaerts, M. Feleppa, F. Piemonte. Friedreich's ataxia: Oxidative stress and cytoskeletal abnormalities. J. Neurol. Sci. 2009; 287:111–118. doi: 10.1016/j.jns.2009.08.052.

[122] S. Petrini, C. Passarelli, A. Pastore, G. Tozzi, M. Coccetti, M. Colucci, M. Bianchi, R. Carrozzo, E. Bertini, F. Piemonte. Protein glutathionylation in cellular compartments: a constitutive redox signal. Redox Rep. 2012; 17:63–71. doi: 10.1179/1351000212Y.0000000009

[123] S. Petrini, V. D'Oria, F. Piemonte. Intracellular distribution of glutathionylated proteins in cultured dermal fibroblasts by immunofluorescence. Methods Mol. Biol. 2015; 1208:395-408. doi: 10.1007/978-1-4939-1441-8_28

[124] S.W. Aesif, Y.M.W. Janssen-Heininger, L. Niki, N.L. Reynaert. Protocols for the detection of *S*-glutathionylated and *S*-nitrosylated proteins *in situ.* Methods Enzymol. 2010; 10:74017–74019. doi: 10.1016/S0076-6879(10)74017-9.

[125] Y.M. Janssen-Heininger, J.D. Nolin, S.M. Hoffman, J.L. van der Velden, J.E. Tully, K.G. Lahue, S.T. Abdalla, D.G. Chapman, N.L. Reynaert, A. van der Vliet, V. Anathy. Emerging mechanisms of glutathione-dependent chemistry in biology and disease. J. Cell. Biochem. 2013; 114:1962–1968. doi: 10.1002/jcb.24551

[126] E. Stroher, A.H. Millar. The biological roles of glutaredoxins. Biochem. J. 2012; 446:333–348. doi: 10.1042/BJ20112131

[127] C.H. Lillig, C. Berndt, A. Holmgren. Glutaredoxin systems. Biochim. Biophys. Acta 2008; 1780:1304–1317. doi: 10.1016/j.bbagen.2008.06.003.

[128] S.W. Aesif, V. Anathy, M. Havermans, A.S. Guala, K. Ckless, D.J. Taatjes, Y.M.W. Janssen-Heininger. In situ analysis of protein S-glutathionylation in lung tissue using glutaredoxin-1-catalyzed cysteine derivatization. Am. J. Pathol. 2009; 175:36–45. doi: 10.2353/ajpath.2009.080736

[129] M.J. Meredith, D.J. Reed. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. J. Biol. Chem. 1982; 257:3747–3753.

[130] F.Q. Schafer, G.R. Buettner. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 2001; 30: 1191–1212.

[131] K. Olafsdottir , D.J. Reed. Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment. Biochim. Biophys. Acta 1988; 964:377–382.

[132] T.A. Schaedler, J.D. Thornton, I. Kruse, M. Schwarzländer, A.J. Meyer, H.W. van Veen, J Balk. A conserved mitochondrial ATP-binding cassette transporter exports glutathione polysulfide for cytosolic metal cofactor assembly. J. Biol. Chem. 2014; 289:23264–23274. doi: 10.1074/jbc.M114.553438.

[133] J. Garcia, D. Han, H. Sancheti, L.P. Yap, N. Kaplowitz, E. Cadenas. Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates. J. Biol. Chem. 2010; 285:39646–39654. doi: 10.1074/jbc.M110.164160.

[134] S.J. James, S. Rose, S. Melnyk, S. Jernigan, S. Blossom, O. Pavliv, D.W. Gaylor. Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. FASEB J. 2009; 23:2374–2383. doi: 10.1096/fj.08-128926

[135] G. Bellomo, M. Vairetti, L. Stivala, F. Mirabelli, P. Richelmi, S. Orrenius. Demonstration of nuclear compartmentalization of glutathione in hepatocytes. Proc. Natl. Acad. Sci. USA 1992; 89:4412–4416.

[136] S. Soboll, S. Gründel, J. Harris, V. Kolb-Bachofen, B. Ketterer, H. Sies. The content of glutathione and glutathione S-transferases and the glutathione peroxidase activity in rat liver nuclei determined by a non-aqueous technique of cell fractionation. Biochem. J. 1995; 311:889–894.

[137] D.W. Voehringer, D.J. McConkey, T.J. McDonnell, S. Brisbay, R.E. Meyn. Bcl-2 expression causes redistribution of glutathione to the nucleus. Proc. Natl. Acad. Sci. USA 1998; 95:2956–2960.

[138] Y.M. Go, T.R. Ziegler, J.M. Johnson, L. Gu, J.M. Hansen, D.P. Jones. Selective protection of nuclear thioredoxin-1 and glutathione redox systems against oxidation during glucose and glutamine deficiency in human colonic epithelial cells. Free Radic. Biol. Med. 2007; 42:363–370. DOI: 10.1016/j.freeradbiomed.2006.11.005

[139] J. Markovic, C. Borrás, A. Ortega, J. Sastre, J. Viña, F.V. Pallardó. Glutathione is recruited into the nucleus in early phases of cell proliferation. J. Biol. Chem. 2007; 282:20416–20424. doi: 10.1074/jbc.M609582200.

[140] B.M. Dixon, S.H. Heath, R. Kim, J.H. Suh, T.M. Hagen. Assessment of endoplasmic reticulum glutathione redox status is confounded by extensive ex vivo oxidation. Antioxid. Redox Signal. 2008; 10:963–972. doi: 10.1089/ars.2007.1869.

[141] C.D. Austin, X. Wen, L. Gazzard, C. Nelson, R.H. Scheller, S.J. Scales. Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody-drug conjugates. Proc. Natl. Acad. Sci. USA 2005; 102:17987–17992. doi: 10.1073/pnas.0509035102

[142] Q. Gao, W. Zhang, B. Song, R. Zhang, W. Guo, J. Yuan. Development of a novel lysosome-targeted ruthenium(II) complex for phosphorescence/time-gated luminescence assay of biothiols. Anal. Chem. 2017; 89:4517–4524. doi: 10.1021/acs.analchem.6b04925

[143] M. Gutscher, A.L. Pauleau, L. Marty, T. Brach, G.H. Wabnitz, Y. Samstag, A.J. Meyer, T.P. Dick. Real-time imaging of the intracellular glutathione redox potential. Nat. Methods 2008; 5:553–559. doi: 10.1038/nmeth.1212

[144] M. Schwarzländer, T.P. Dick, A.J. Meyer, B. Morgan. Dissecting redox biology using fluorescent protein sensors. Antioxid. Redox Signal. 2016; 24:680–712. doi: 10.1089/ars.2015.6266.

[145] K. Kojer, M. Bien, H. Gangel, B. Morgan, T.P. Dick, J. Riemer. Glutathione redox potential in the mitochondrial intermembrane space is linked to the cytosol and impacts the Mia40 redox state. EMBO J. 2012; 31:3169–3182. doi: 10.1038/emboj.2012.165.

[146] Y. Elbaz-Alon, B. Morgan, A. Clancy, T.N. Amoako, E. Zalckvar, T.P. Dick, B. Schwappach, M. Schuldiner. The yeast oligopeptide transporter Opt2 is localized to peroxisomes and affects glutathione redox homeostasis. FEMS Yeast Res. 2014; 14:1055–1067. doi: 10.1111/1567-1364.12196.

[147] J. Birk, M. Meyer, I. Aller, H.G. Hansen, A. Odermatt, T.P Dick, A.J. Meyer, C. Appenzeller-Herzog. Endoplasmic reticulum: reduced and oxidized glutathione revisited. J. Cell Sci. 2013; 126:1604–1617. doi: 10.1242/jcs.117218.

[148] D. Montero, C. Tachibana, J. Rahr Winther, C. Appenzeller-Herzog. Intracellular glutathione pools are heterogeneously concentrated. Redox Biol. 2013; 1:508–513. doi: 10.1016/j.redox.2013.10.005.

[149] R. Huang, B.B. Wang, X.M. Si-Tu, T. Gao, F.F. Wang, H. He, X.Y. Fan, F.L. Jiang, Y. Liu. A lysosome-targeted fluorescent sensor for the detection of glutathione in cells with an extremely fast response. Chem. Commun. (Camb). 2016; 52:11579–11582. doi: 10.1039/c6cc06750f.

[150] X.L. Liu, L.Y. Niu, Y.Z. Chen, M.L. Zheng, Y. Yang, Q.Z. Yang. A mitochondria-targeting fluorescent probe for the selective detection of glutathione in living cells. Org. Biomol. Chem. 2017; 15:1072–1075. doi: 10.1039/c6ob02407f.

Table 1

Levels of GSH, GSSG and the GSH/GSSG ratio in some biological samples and the corresponding methods used to detect them. In most cases the same method is used for the detection of both GSH and GSSG**.** When two different methods are used, the first one reported refers to GSH and the second one to GSSG.

Tissue	GSH	GSSG	GSH/ GSSG	Method	Ref.
Human blood	8.40±0.56 nmol/mg Hb	-	-	RP-HPLC UV/Vis (NEM-GSH conjugate)	[61]
Human blood	1310±118 μM	0.64 ±0.22 μM	2047	HPLC-ESI-MS	[63]
Human blood	900±140 μM	1.17±0.63 μM	769	LC-MS/MS	[64]
Human blood	1356±105 μM	2.86 ±0.55 μM	474	RP-HPLC (FDNB)	[73]
Human RBCs	8720±2500 pmol/mg Hb	13.2±4.2 pmol/mg Hb	661	RP-HPLC UV/Vis; GSH recycling	[43]
Human RBCs	-	3.6 ± 1.4 mM	-	GSH recycling	[46]
Human blood	7200 pmol/mg Hb	12 pmol/mg Hb	600	GSH recycling	[75]
Human blood	7500 pmol/mg Hb	13 pmol/mg Hb	577	GSH recycling	[76]
Rat blood	1199±142 μM	0.93±0.44 mM	1289	GSH recycling	[45]
Rat RBCs	2270±120 μM	5.60±0.32 μM	405	RP-HPLC (mBrB)	[67]
Rat Liver	8221±528 μM	43.3±2.9 μM	190	RP-HPLC (mBrB)	[67]
Rat Kidney	2221±302 μM	13.4±3.42 μM	166	RP-HPLC (mBrB)	[67]
Rat Lung	2314±182 μM	17.2±0.8 μM	134	RP-HPLC (mBrB)	[67]
Rat brain	1801±59 μM	18.1±1.47 μM	99.5	RP-HPLC (mBrB)	[67]
C57BL/6J blood	11200±237 μM	20.3±12.1 μM	560	RP-HPLC (mBrB)	[74]
BAEC	33.9±5.5 nmol/mg prot	217±16 pmol/mg prot	156	RP-HPLC UV/Vis; RP-HPLC (mBrB)	[60]
HUVEC	50.8±8.4 nmol/mg prot	137±11 pmol/mg prot	372	RP-HPLC UV/Vis; RP-HPLC (mBrB)	[60]

Table 2
Levels of PSSG in some biological samples.

Sample	PSSG	References
Platelets from healthy humans (n=5)	0.046±0.07[a]	[73]
Human plasma (n=5)	2.61±0.12[b]	[47]
Rat Liver (n=5)	4.71±0.85[b]	[67]
Rat Kidney (n=5)	2.25±0.32[b]	[67]
Rat Lung (n=5)	2.18±0.33[b]	[67]
Rat brain (n=5)	6.04±0.44[b]	[67]
BAEC	110±17[c]	[60]
HUVEC	24.3±1.2[c]	[60]

[a]Data are expressed as nmol/10^9 plt

[b]Data are expressed as μM

[c]Data are expressed as pmol/mg prot

Table 3
Methods to detect PSSG.

Method	**Ref.**
Quantification	
RP-HPLC (mBrB)	[78]
Quantification/Identification	
Ad hoc modified biotin-switch technique, affinity purification of biotin-maleimide-tagged proteins and proteomic analysis	[87]
Biotinylated GSSG and Western blotting	[88]
Biotinylated GSSG, affinity purification and MS	[104,106]
Biotinylated GSSG and fluorescence microscopy	[103]
Radio labelling with [^{35}S]-cysteine, autoradiography	[88]
SDS-PAGE and Western blotting with anti-GSH antibodies	[93]
2D-PAGE, anti-GSH antibodies and MALDI-TOF MS	[88]
"Redox arrays" methodology based on incorporation of biotinylated GSH into proteins	[111]
Grx-mediated selective reduction of PSSG in combination with isobaric tags	[113]
Iodoacetamide conjugated to desthiobiotin labelling and HPLC-ESI-MS/MS	[114]
Top-down LC-MS approach followed by Informed-Proteomics data analysis	[119]
Conjugation of azide-containing glutathione derivative ("clickable" glutathione) to proteins undergoing S-glutathionylation in cells, detection of PSSG by Western blotting or fluorescence after click reaction with biotin-alkyne or rhodamine-alkyne	[107,108]
Conjugation of alkene-containing GSH analogues ("clickable"	[109]

glutathione) to proteins undergoing *S*-glutathionylation in cells, detection of PSSG with tetrazine-fluorophore or tetrazine-biotin	
Enrichment od glutathionylated peptides with alkynyl-containing GSH analogue, reaction of the alkynyl-containing peptides with an azido-biotin reagent and detection with LC-MS/MS	[110]
Localization	
Immunohistochemistry with anti-GSH antibody	[94,121]
Indirect immunofluorescence with anti-GSH antibody	[122,123]
Biotinylated GSH ethyl ester and direct immunofluorescence with fluorophore-conjugated streptavidin	[105]
Sections treated with Grx1, GSH, NADPH, and GR, labelling with bitinylated NEM and confocal miscroscopy or anti-biotin antibodies	[125,128]

Highlights

- The GSH/GSSG ratio provides a reliable estimation of cellular redox status
- GSH also plays a prominent role in protein redox regulation via *S*-glutathionylation
- We discuss recent methods to detect GSH, GSSG and *S*-glutathionylated proteins
- We also discuss limits of the main analytical, morphological, and proteomic methods