# Insights from Redox Proteomics: Focus on S-glutathionylation Stoichiometry and Redox Status of Protein Cysteines

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## Overview

- Resin-assisted quantitative proteomics was used for measuring the stoichiometry of S-glutathionylation and total oxidation at protein cysteine residues in mouse macrophages.
- Stoichiometric quantification of cysteine modifications facilitated the identification of redox-regulated proteins and functional sites in cells.
- Our results revealed a quantitative picture of the redox proteome and provided valuable insights for understanding redox signaling and regulation in cells.

## Introduction

- Reversible oxidative modifications of protein cysteines (Cys) are main components in redox signaling, which regulates many cellular processes under physiological and pathological conditions.[1-3]
- The study for Cys redox modifications has been greatly facilitated by MS-based quantitative proteomic approaches, which allow quantification of site-specific redox modifications at a system level in cells under stressed conditions.
- The stoichiometry of Cys modifications is important for understanding their functional role in cells, but is usually overlooked.
- Resin-assisted approaches have been successfully applied for the identification and quantification of several forms of Cys modifications, including S-nitrosylation (SNO)[4], S-glutathionylation (SSG)[5,6], and total oxidation[7,8] in cells and tissues.
- The modified resin-assisted strategy allowed us to measure the stoichiometry of Cys modification at the proteome level.[7,8]

## Methods

### Resin-assisted enrichment and quantification

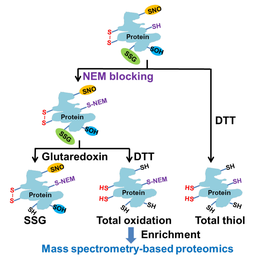



**Figure 1.** Enrichment strategy for site-specific stoichiometric quantification of SSG, total oxidation, and total thiol in cells.

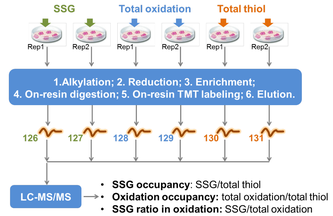



**Figure 2.** Multiplexed quantification strategy.

### Experimental details

- Murine RAW 264.7 cells in RPMI 1640 media
- SSG: NEM-blocking and deglutathionylation by glutaredoxin; total oxidation: NEM-blocking and DTT reduction; total thiol: DTT reduction
- Protein capture, digestion, and TMT6-labeling with Sepharose 6B resin
- 3h LC-MS/MS, Orbitrap Velos with HCD; MSGF+ for protein identification; FDR <1%
- STRING10 was used for GO pathway analysis.

## Results

### Basal level oxidation of protein Cys in macrophages

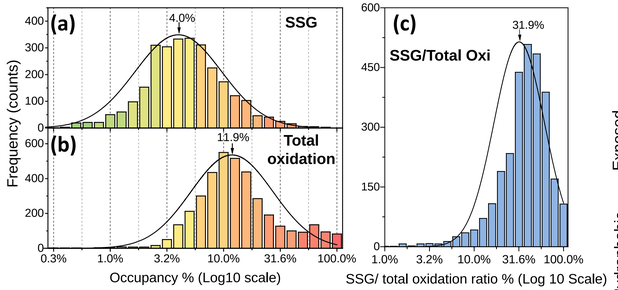



**Figure 3.** Basal levels of SSG occupancy and total oxidation in mouse macrophages. (a) SSG occupancy for 2808 Cys sites from 1535 proteins.(b) Total oxidation for 3794 Cys sites from 1897 proteins. (c) SSG/total oxidation ratio for 2848 Cys sites from 1576 proteins.

### Redox status of Cys in subcellular compartments

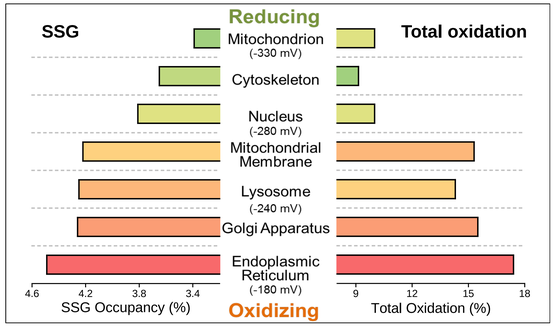



**Figure 4.** SSG occupancy and total oxidation of protein Cys in subcellular compartments. The levels of SSG and oxidation are well correlated with the redox potential of the organelles.

### Structural features of Cys associated with oxidation level

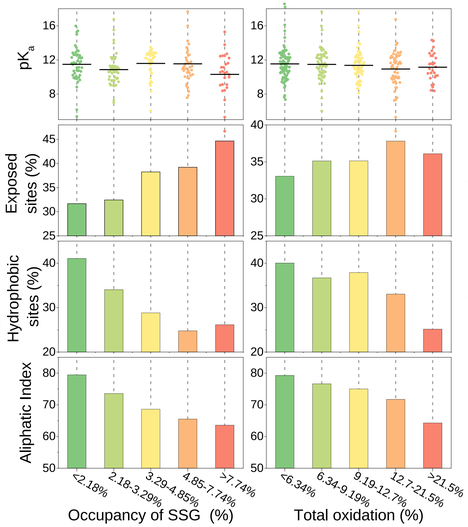



**Figure 5.** The structural properties of Cys residue associated with oxidation levels.

Cys residues with high levels of SSG and oxidation at basal condition are featured with:

1) Low pKa
2) High relative surface accessibility
3) Low hydrophobicity (high GRAVY score)
4) Low regional stability (low aliphatic index)

Cys sites were sorted and grouped based on their stoichiometric levels of SSG and oxidation with 20th, 40th, 60th, and 80th percentiles as boundaries. GRAVY index and Aliphatic index were calculated with 13 amino acid residue sequences flanking the Cys residue.

### Potential SSG-regulated pathways

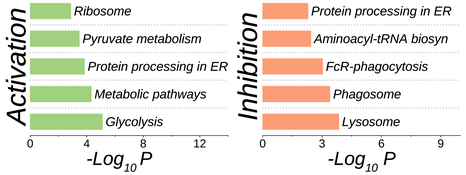
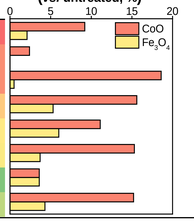



**Figure 6.** Pathway analysis combined with SSG stoichiometry reveals regulatory roles of SSG in response to nanoparticle-induced oxidative stress. Potential activation: basal SSG occupancy < 3.3%, fold change >1.5; potential inhibition: After nanoparticle treatment, SSG occupancy >8.0%, fold change >1.5.

### Regulation of SSG under stress conditions

**Table 1.** Change of SSG levels in cellular organelles in response to nanoparticle-induced oxidative stress

| Organelle | # of Sites | SSG Occupancy (%) | | | Change of SSG Occupancy (vs. untreated, %) |
|---|---|---|---|---|---|
| | | Untreated | $Fe_3O_4$ | CoO | |
| Lysosome | 91 | 4.77 | 4.87 | 5.21 | |
| Golgi Apparatus | 27 | 4.61 | 4.61 | 4.72 | |
| Endoplasmic Reticulum | 92 | 3.98 | 4.00 | 4.72 | |
| Total | 879 | 3.4 | 3.58 | 3.93 | |
| Nucleus | 245 | 3.15 | 3.34 | 3.50 | |
| Mitochondrial Membrane | 25 | 3.01 | 3.12 | 3.47 | |
| Mitochondrion | 43 | 2.81 | 2.91 | 2.91 | |
| Cytoskeleton | 76 | 2.77 | 2.89 | 3.19 | |




## Conclusions

- Site-specific stoichiometric quantification of Cys oxidation provided a more quantitative picture of the redox states of cells and subcellular compartments.
- Bioinformatics analysis revealed the correlation between the structural properties of Cys residue and the stoichiometry levels of Cys oxidation.
- The potential roles of SSG in activation and inhibition under stress condition were predicted by combining SSG stoichiometry with dynamic change.
- Our results provided valuable insights on Cys-based redox signaling and regulation.

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