Mix-and-Match Proteomics: Using advanced iodoTMT multiplexing to investigate cysteine oxidation changes with respect to protein expression

Aruna S. Prakash[†], Abdulbaset M. F. Kabli[†], Neil Bulleid[‡] and Richard Burchmore[†]*

[†] Glasgow Polyomics, College of Medical, Veterinary & Life Sciences, Wolfson Wohl Cancer Research Centre, University of Glasgow, Garscube Estate, Glasgow, G61 1QH.

[‡] Institute of Molecular, Cell and Systems Biology, Davidson Building, University of Glasgow, Glasgow, G12 8QQ.

ABSTRACT: Cysteine redox state has been identified as one of the key biological influences behind protein structure and/or function. Altered protein redox state has been shown to cause significant physiological changes and can leave proteins with changed sensitivity to oxidative stress. Protein redox state changes are recognized as an important mediator of disease, cellular abnormalities or environmental changes and therefore their characterization is of interest. Isotopic or isobaric labeling followed by sample multiplexing and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) allows relative comparison of protein expression levels or of protein redox state between several samples. Combining analysis of protein expression level and redox state into one analysis would add an extra dimension and permit the normalization of protein redox changes with protein abundance. To achieve this, we have developed a quantitation workflow that uses commercially available cysteine-reactive tandem mass tags (iodoTMT) to differentially label cysteine residues, and applied it to two *Leishmania mexicana* cell-lines that have previously shown different responses to oxidative stress. The individually labeled samples have been pooled in different combinations to create multiple sixplex samples in order to study the relationship between cysteine oxidation and overall protein expression, as well as providing information about protein oxidation levels in each cell-line. The results highlight eleven proteins that are differentially expressed between the two cell-lines and/or have significant redox changes. This advanced multiplexing method effectively demonstrates the flexibility of tandem mass tags and how they can be used to maximize the amount of information that can be acquired.

Cysteine residues play an important role in the structure and function of proteins because they are sensitive to oxidation or reduction. Free (reduced) cysteines (SH) are highly reactive and can form reversible or irreversible modifications such as disulfide bonds (S-S), nitrosylations (SNO), sulfenylations (SOH) and sulfonylations (SO_3H).[1-2] Reversible cysteine modifications can act as a biological switch, activating or deactivating biological processes depending on the cysteine redox state. Disulfide bonds can be involved in stabilizing protein tertiary and quaternary conformations, which in turn can influence the stability and function of the protein.[3] Cysteine modifications are mediated by changes in redox state and provide a link between oxidative stress and pathologies such as cardiovascular disease, diabetes, neurodegenerative diseases and others.[4-8]

Comparative proteomic analyses, which allow protein expression changes to be linked to phenotype, are typically performed under reducing conditions and are thus insensitive to reversible oxidative changes. Several proteomic approaches have been developed to assess differences in cysteine oxidation under conditions of interest (reviewed by Chouchani *et al.*, 2011; Murray & Van Eyk, 2012; Wojdyla & Rogowska-Wrzesinska, 2015).[2, 9-10] These methods involve selective or differential labeling of modified and unmodified cysteine residues with the aim of capturing cysteine redox state under physiological conditions. Isotopic labeling allows co-elution of differentially labeled peptides in liquid chromatography (LC) coupled with mass spectrometry (MS) to resolve labels with distinct masses,[10-12] which permits relative quantitation by comparing the ratio between the light-labeled and the heavy-labeled peptides.[13-14] Issues may arise with isotopic labeling if the two labels are chemically distinct, as labeled peptides may not completely co-elute. In addition, the resulting mass spectrum becomes twice as complicated due to multiple peaks corresponding to the same peptide.[15] In order to overcome these difficulties, isobaric labels have been created that are chemically and structurally identical, however the placement of the heavy isotopes (^{13}C and ^{15}N) varies in each label. Through tandem mass spectrometry (MS/MS), these labels are engineered to break at a specific bond, creating a reporter ion that will differ in mass for each isobaric tag and allowing direct comparisons to be made between multiple samples in a single run.[15-16]

Tandem Mass Tags (TMT) are isobaric reagents that are available with different reactive groups depending on the labeling required. Amine-reactive TMTs are typically used to label tryptic peptides, reacting with lysines and peptide N-terminal amines to give relative quantitation for the overall protein concentration changes between samples. Iodoacetyl Tandem Mass Tags (iodoTMT) have been designed to react irreversibly with reduced cysteine residues through the iodoacetyl group (see **Supplementary Information**) and can therefore be used to study cysteine oxidation status.[17-18] The iodoTMT reagent set is available as a sixplex, which allows up

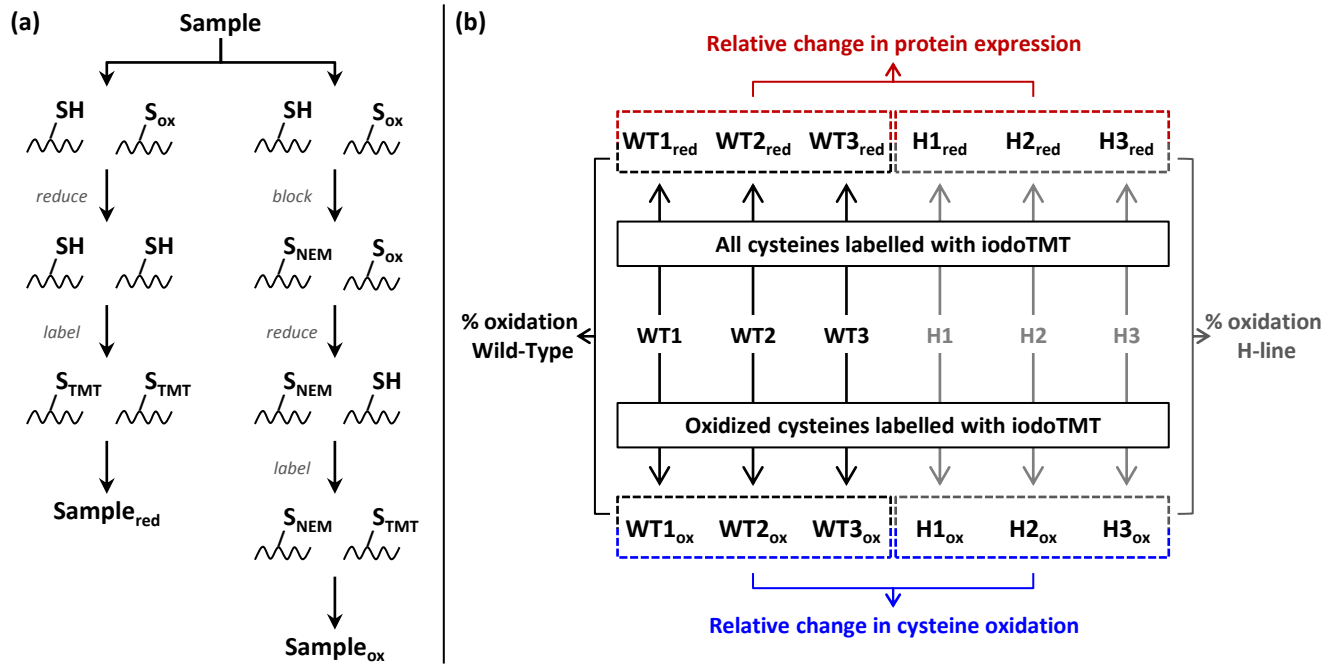


Figure 1. Schematic of the sample workflow. (a) One half of each sample is reduced fully and all cysteines are labeled with iodoTMT, while the other half is treated with NEM to block all free cysteines (SH) and subsequently any modified cysteines are reduced and labeled with iodoTMT. (b) For this study, three replicates for each cell-line (wild-type & H-line) have been cultured and treated using the workflow shown in part (a). Individually labeled samples have then been combined to create four sixplex samples in order to determine relative change in protein expression, relative change in cysteine oxidation and percentage oxidation for each cell-line.

to six samples to be directly compared and quantified. Given the relative rarity of naturally-occurring cysteine residues, cysteine-containing peptides tend to be a relatively small proportion of the total peptide content, therefore an anti-TMT antibody resin can be used to selectively bind and enrich TMT-labeled peptides in order to improve the detection and identification of these peptides.

In addition to simply reducing and labeling all cysteine residues, cysteine-reactive reagents have been applied in more complex methods to investigate and quantify specific types of cysteine modifications or to relate cysteine oxidation with other protein changes.[2, 10] Qu *et al.* selectively reduced nitrosylated cysteines using sodium ascorbate prior to iodoTMT labeling to measure the effects of nitric oxide signaling, while Pan *et al.* included sequential iodoTMT labeling steps to compare the level of nitrosylated cysteines to the level of the remaining reversible cysteine modifications across multiple samples.[19-20] Van der Reest *et al.* utilized stable isotope labeling to quantify oxidation changes by reacting reduced cysteines with light/heavy iodoacetamide, while also using light/heavy dimethyl labeling to quantify protein expression changes.[21] In a similar vein, Shakir *et al.* have discussed a method for using iodoTMT to quantify changes in percentage oxidation through comparisons between oxidized samples and fully reduced samples.[22]

Building upon these previous studies, we have designed a workflow that uses iodoTMT multiplexing and mix-and-match sample assembly in order to normalize changes in cysteine oxidation levels with respect to changes in overall protein expression, allowing quantitative comparison of protein oxidation (see **Figure 1**). We have applied this workflow to compare isogenic lines of the protozoan parasite *Leishmania mexicana* that display contrasting virulence phenotypes. Leishmaniasis is an important human and canine disease, for which no vaccine is available. We have previously shown that selection of *Leishmania mexicana* with the aminoglycoside antibiotic gentamicin results in stable attenuation of virulence, a phenotype that we are currently investigating for potential as a vaccine to control leishmaniasis.[23] Comparative proteomic analyses have shown that the attenuated line has a greater susceptibility to oxidative stress[24] compared with the virulent wild-type, and we therefore wished to investigate proteins that show significant changes in redox state that could be a potential target for drug treatment.

MATERIALS

N-Ethylmaleimide (NEM), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), Pierce™ Detergent Compatible Bradford Assay, iodoTMTsixplex™ Isobaric Reagent Set, Immobilized Anti-TMT™ Antibody Resin, TMT™ Elution Buffer, Pierce™ Spin Columns – Screw Cap, Pierce™ Quantitative Colorimetric Peptide Assay [Thermo Scientific], Acetone, Tris(hydroxymethyl)aminomethane (Tris), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Ethylenediaminetetraacetic acid (EDTA) [Fisher Scientific], Acetonitrile (MeCN), Methanol (MeOH) [Rathburn Chemicals], Neocuproine, Dithiothreitol (DTT), Formic Acid, Sodium Dodecyl Sulfate (SDS), Trypsin [Promega], FASP protein digestion kit [Expedeon], Sodium Chloride (NaCl) [VWR], Haemoflagellate Minimal Essential Medium (HOMEM), Heat Inactivated Fetal Bovine Serum (HI-FBS), Streptomycin, Penicillin [Gibco].

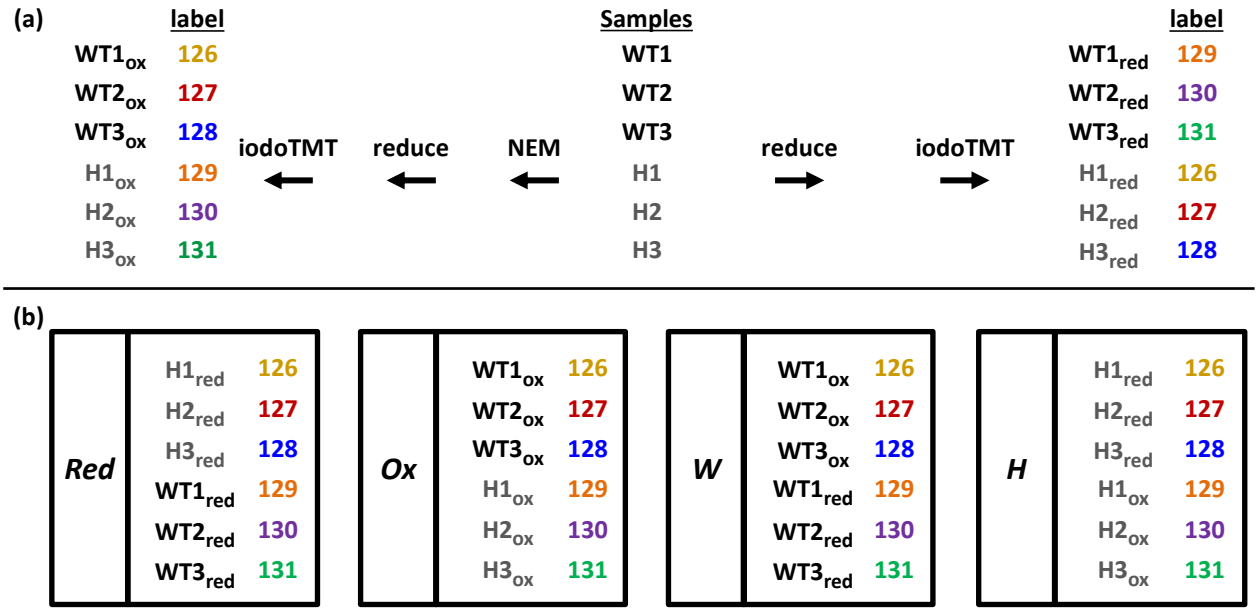


Figure 2. Labeling workflow for wild-type (WT) and H-line (H) cell-lines of Leishmania mexicana. (a) Each sample was split into two equal parts and either fully reduced (-red) or blocked with NEM (-ox) before iodoTMT labeling. (b) By using strategic iodoTMT labeling, the individually labeled samples could then be combined in different groups to create four sixplex samples, providing relative quantitation for protein expression (*Red*), quantitation of cysteine oxidation (*Ox*), percentage of cysteine oxidation in wild-type (*W*) and percentage of cysteine oxidation in H-line (*H*).

EXPERIMENTAL

Cell Cultures. Wild-Type (*WT) Leishmania mexicana* strain M379 promastigotes were selected in the presence of gentamicin to generate an attenuated line (H), as previously reported by Daneshvar *et al.*[25]

Three biological replicates each of WT and H-line were cultured in complete HOMEM (6 samples in total) with 10%HI-FBS at 25 °C and sub-passaged every 2-3 days. Each replicate was split into two equal aliquots of approximately $5x10^7$ cells (12 samples in total) and centrifuged at 1,000 x *g* for 10 mins. Samples were washed twice to remove salts and other soluble macromolecules, by resuspension in cold PBS, followed by centrifugation at 1,000 x *g* for 10 mins.

Cell Lysis. One sample from each culture was resuspended in 200 µL of SDT lysis buffer (4% SDS, 100 mM Tris base and 0.1 M DTT, pH 7.6). The remaining six samples were resuspended in 200 µL NEM lysis buffer (250 mM HEPES, 10 mM EDTA, 0.1 mM Neocuproine, 2% SDS and 100 mM NEM, pH 7.0)[2, 26] Samples in NEM buffer were incubated at 37 °C in the dark for 90 mins with gentle mixing to allow the NEM to react irreversibly with free thiols.

Cells were lysed by probe sonication, alternating a 2 second pulse with 1 minute cooling on ice ten times. Samples were then centrifuged for 10 mins at 13,000 x *g* to remove cell debris and other insoluble material. The supernatant was removed into separate eppendorfs and ice-cold acetone (4x sample volume) was added to each sample, vortexed briefly and then stored at -20 °C overnight. Samples were centrifuged for 10 mins at 13,000 x *g* at 4 °C and the supernatant was carefully removed. The protein pellet was washed twice with 80% ice-cold acetone, vortexed briefly and then centrifuged to remove the supernatant. Protein pellets were stored at -20 °C until required.

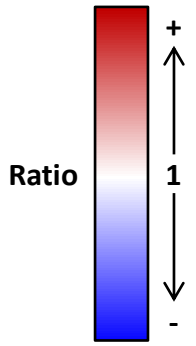
Isobaric Labeling. Protein pellets were resuspended in HEPES buffer (50 mM HEPES, 1 mM EDTA, 0.1 % SDS, pH 8.0) and the protein concentration was measured using a Bradford Assay. Each sample was split into two 30 µg aliquots (24 samples in total) and made up to equal volumes with HEPES buffer. Reduction of reversibly modified cysteine residues was performed using TCEP-HCl (final concentration ~5 mM), then samples were vortexed briefly to mix and incubated at 37 °C for 1 hour. Irreversibly modified cysteines, such as sulfonylations, are unaffected by TCEP reduction and therefore cannot be quantified using this method.

IodoTMT reagents were resuspended in MeOH to a concentration of 10 mg mL^{-1} and samples were labeled with 10 µL of the appropriate iodoTMT reagent, as described in **Figure 2**. Samples were incubated at 37 °C in the dark for 1 hour, before being quenched with DTT (final concentration of ~20 mM) and incubated for a further 15 mins at 37 °C in the dark.

Individual samples were combined in equal protein concentrations into four sixplex samples; a fully-reduced (*Red*) sample with all cysteines labeled with iodoTMT, an NEM-blocked (*Ox*) sample with only modified cysteines labeled with iodoTMT, and wild-type (*W*) and H-line (*H*) only samples comparing modified cysteines with total cysteine content for each cell-line, as described in **Figure 2**. Enzymatic digestion was carried out using the FASP protein digestion kit, digesting samples overnight at 37 °C with trypsin.

Affinity Purification. Peptide samples were dried in a SpeedVac before being reconstituted in 200 µL TBS Buffer (25 mM Tris, 0.15M NaCl, pH 7.2). Affinity purification was carried out using 400 µL anti-TMT slurry in a spin column, following manufacturer's instructions, including an incubation

$$Red = \frac{H(avg)-red}{W(avg)-red} \qquad Ox = \frac{H(avg)-ox}{W(avg)-ox} \qquad W = \frac{W(avg)-ox}{W(avg)-red} \qquad H = \frac{H(avg)-ox}{H(avg)-red}$$



Red	*Ox*	*W*	*H*
Increased protein expression in H-line	Increased oxidation in H-line	-	-
No change in protein concentration	No change in (absolute) oxidation levels	100% oxidation	100% oxidation
Decreased protein expression in H-line	Decreased oxidation in H-line	Less than 100% oxidation	Less than 100% oxidation

Figure 3. The equations used to calculate quantitation ratios for each sample using the average reporter ion abundances for each group of three replicates, along with the significance of the resulting ratios.

step at 4 °C overnight with end-over-end mixing. Unbound peptides were washed from the column using 5x column volumes of TBS buffer followed by 3x column volumes of water. The labeled peptides were eluted from the column using 4x column volumes of TMT elution buffer. All eluted fractions (labeled and unlabeled peptides) were dried in the SpeedVac and reconstituted in 10% MeCN + 0.1% formic acid. Approximately 6 µg of each sample was pipetted into a 96-well microplate, dried in the SpeedVac and stored at -20 °C until ready for analysis.

LC-MS/MS Analysis. Analysis was carried out by LC-MS/MS using an Ultimate 3000 RSLCnano liquid chromatography (nanoLC) system [Dionex] and an LTQ-Orbitrap Elite mass spectrometer [Thermo Scientific] with nanospray ionization (NSI). A C18 trap column (5 mm x 300 µm ID, 5 µm, 100 Å) and Acclaim PepMap 100 C18 column (150 mm x 75 µm ID, 3 µm, 100 Å) [Thermo Scientific] were used for chromatography.

Peptides were reconstituted in 10 µL loading buffer (1% MeCN + 0.05% formic acid (v/v)) and injected onto the trap column for 4 mins using a loading buffer solvent flow rate of 25 µL min^{-1}. Peptides were then transferred to the analytical column using starting chromatographic conditions; 95% Buffer A (Water + 0.1% formic acid) / 5% Buffer B (80 % MeCN + 0.08% formic acid) (v/v). A 150 minute gradient was used, 5% B – 45% B (v/v), with a solvent flow rate of 300 nL min^{-1}, followed by a wash step of 100% B for 6 mins and then re-equilibration at starting solvent conditions for 5 mins, for a total run time of 165 mins.

The LTQ-Orbitrap Elite acquired a high resolution MS scan (resolving power 60,000 at *m/z* 400) over a mass range of *m/z* 380 – 1800 in positive mode. A maximum ion injection time of 200 ms was allowed for Orbitrap analyses, with an AGC target of $1x10^6$. The three most abundant multiply charged ions in the MS scan above the set signal thresholds were then selected for consecutive MS/MS analyses by Higher-energy Collisional Dissociation (HCD) and Collision Induced Dissociation (CID). Precursor ions were isolated in the LTQ ion trap using an isolation window of *m/z* 1.2, a maximum ion injection time of 25 ms and an AGC target of $3x10^4$. HCD fragmentation was performed in the HCD collision cell using helium as a collision gas and detected in the Orbitrap using the following parameters; signal threshold: 1000, collision energy (CE): 50, stepped CE width: 10%, number of CE steps: 2, activation time: 0.1 ms, resolving power: 15,000 at *m/z* 400. CID was performed in the LTQ ion trap employing helium as a collision gas, using the following parameters; signal threshold: 500, CE: 35, stepped CE width: 10%, number of CE steps: 2, activation time: 10 ms, activation Q: 0.250. After 30 seconds, selected precursor ions were added to an exclusion list for a further 180 seconds to avoid repeated analyses of highly abundant ions at the expense of lower abundance ions.

Data Processing. Data processing was carried out in Proteome Discoverer™ software version 2.1 SP1 [Thermo Fisher Scientific] using Mascot version 2.6.1 [Matrix Science] for database searching. MS/MS spectra were searched against the LmexicanaMHOMGT2001U1103 database (version 32, 16th April 2017) using the following parameters; enzyme: Trypsin (full), maximum missed cleavages: 2, precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.6 Da, quantitation method: iodoTMT 6plex, reporter ion integration tolerance: 20 ppm, isolation interference threshold: 20%. Variable modifications included oxidation (M), iodoTMT (C), N-ethylmaleimide (C), N-ethylmaleimide+water (C/K) and Protein N-terminal acetylation. Protein quantitation was calculated using unique peptides only, and peptides with missing quantitation channels were excluded from quantitation. Peptides were filtered with a 5% FDR cutoff, with all peptides above 1% FDR given a high confidence rating. Reporter ion abundances were calculated using the signal-to-noise ratios from the reporter peaks each HCD spectrum, or from the ion intensities when using ion trap CID data, and then scaled so that each protein can be compared directly. Isotopic correction has been applied using the values provided with the iodoTMT reagents.

RESULTS AND DISCUSSION

The purpose of this experiment was to identify protein oxidative changes between two *Leishmania mexicana* cell-lines, and to normalize these changes with respect to changes

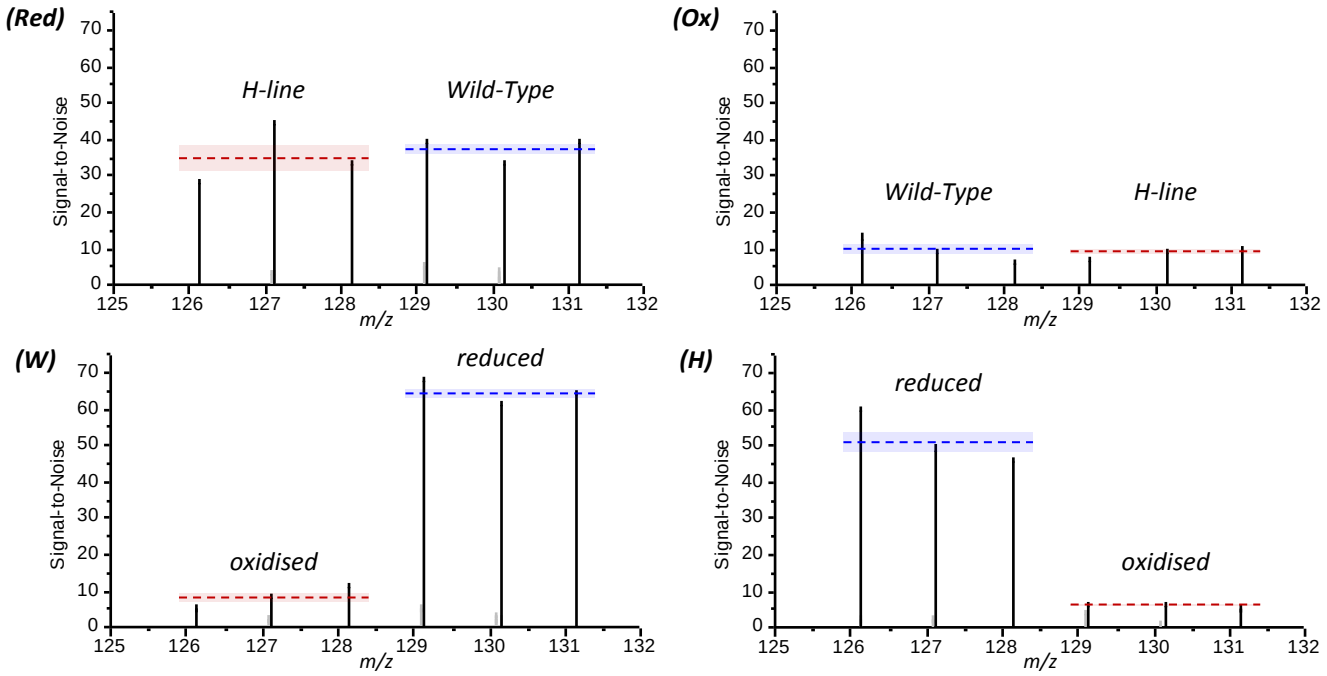


Figure 4. Reporter ion spectra for peptide CQDATGNTYIATDNICPFADFER (LmxM.36.2570) from samples *Red*, *Ox*, *H* and *W* (clockwise from top left). The average of each group of replicates is denoted by the dashed lines, with the calculated standard deviation displayed by the surrounding colored box. Quantitation ratios for each sample have been calculated from the replicate averages, where the blue group corresponds to the denominator (see **Table 1**). Peaks that do not correspond to the iodoTMT reporter ions have been labeled in grey.

Table 1. Quantitation ratios have been calculated for peptide CQDATGNTYIATDNICPFADFER (LmxM.36.2570) from each sample. Where multiple MS/MS spectra have been acquired, the table displays the average quantitation ratio from all available reporter ion spectra. The change in oxidation has been calculated using both equations from Equation 1. Both cysteines have been labeled with iodoTMT.

Accession	Protein	Peptide Sequence	*Red*	*Ox*	*W*	*H*	$\frac{Ox}{Red}$	$\frac{H}{W}$
LmxM.36.2570	membrane-bound acid phosphatase precursor	CQDATGNTYIATD NICPFADFER	0.891	0.929	0.118	0.112	1.043	0.949

in protein expression. Three biological replicates of each cell-line, virulent wild-type (WT) and attenuated H-line (H), were grown and each culture was divided and treated separately to create two different sample sets (see **Figures 1** and **2**). One half of each sample was incubated with N-ethylmaleimide during cell lysis to irreversibly block any free cysteines (SH) and prevent any further redox reactions. Any reversibly modified cysteines were then reduced and labeled with iodoTMT reagents. In order to quantify protein expression levels for cysteine-containing proteins, the other half of each sample was fully reduced and all cysteine residues were labeled with iodoTMT. Affinity purification using an anti-TMT antibody was carried out after trypsin digestion, allowing unlabeled peptides to be depleted while enriching for the iodoTMT-labeled peptides prior to MS analysis.

Relative quantitation for each sample has been calculated from the average reporter ion abundances from each group of three replicates, as shown in the equations in **Figure 3**, investigating H-line abundance in relation to wild-type (*Red* & *Ox*) or quantifying oxidized cysteines as a proportion of total cysteine content (*W* & *H*). The resulting ratios can be used to quantify changes in protein expression (*Red*), changes in oxidation levels (*Ox*) and the percentage oxidation level for proteins in each cell-line (*W* & *H*) as explained in the table in **Figure 3**.

A ratio of 1 calculated for a protein in sample *Red* or *Ox* would suggest no change in protein expression or cysteine oxidation between H-line and wild-type, while a ratio of 0.5 would indicate a 50% decrease in the H-line sample. Ratios calculated for samples *W* and *H* represent the percentage oxidation for each cell-line, where the maximum ratio of 1 would signify 100% oxidation.

The normalized change in oxidation ratio can be calculated in two ways, as shown in **Equation 1**, either by normalizing the oxidized sample (*Ox*) by the fully-reduced sample (*Red*) or by normalizing the H-line sample (*H*) by the wild-type control sample (*W*). The resulting ratios calculated from each side of the equation should be approximately the same. Minor variations between biological replicates are to be expected, however if these two ratios differ significantly it suggests that experimental error has been introduced, such as the presence of co-isolated species that have affected the reporter ion abundances in at least one of the samples.

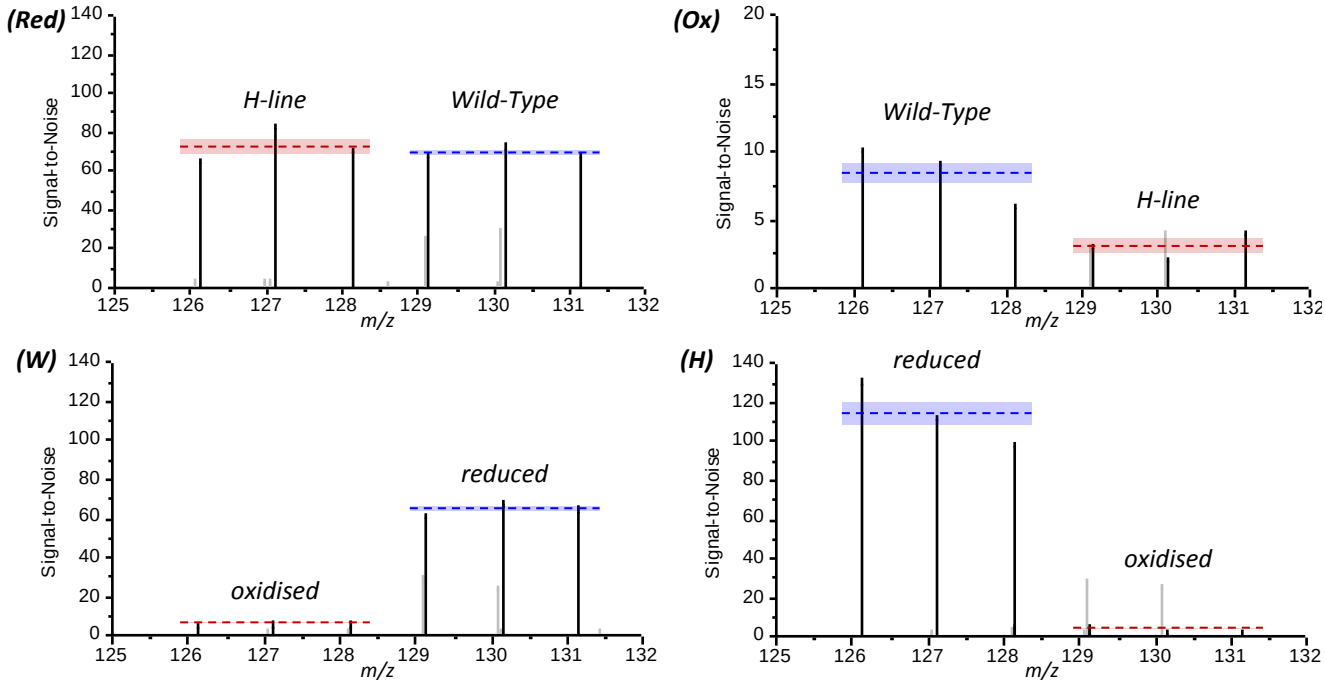


Figure 5. Reporter ion spectra for peptide SPACGSDSVVTK (LmxM.28.2510) from samples *Red*, *Ox*, *H* and *W* (clockwise from top left). The average of each group of replicates is denoted by the dashed lines, with the calculated standard deviation displayed by the surrounding colored box. Quantitation ratios for each sample have been calculated from the replicate averages, where the blue group corresponds to the denominator (see **Table 2**). Peaks that do not correspond to the iodoTMT reporter ions have been labeled in grey.

Table 2. Quantitation ratios have been calculated for peptide SPACGSDSVVTK (LmxM.28.2510) from each sample. Where multiple MS/MS spectra have been acquired, the table displays the average quantitation ratio from all available reporter ion spectra. The change in oxidation has been calculated using both equations from Equation 1.

Accession	Protein	Peptide Sequence	*Red*	*Ox*	*W*	*H*	$\frac{Ox}{Red}$	$\frac{H}{W}$
LmxM.28.2510	acyl-CoA dehydrogenase, putative	SPACGSDSVVTK	1.059	0.354	0.056	0.014	0.334	0.250

$$\frac{Ox}{Red} = \frac{H}{W}$$

Equation 1. The normalized change in oxidation can be calculated using samples *Ox* and *Red* (left) or samples *H* and *W* (right). For the full equation, see **Supplementary Information**.

Since protein concentration has been measured before iodoTMT labeling, all proteins (including those without cysteine) have been used to normalize sample concentrations in order to combine individually-labeled samples equally, and as a result the overall number of iodoTMT-labeled proteins can vary between samples. The *L. mexicana* samples showed a significant variation between the number of proteins and peptides observed between the four samples, with the fully-reduced sample identifying over 300 labeled proteins compared with the NEM-blocked sample, which identified less than 90. This observation can be explained by the low percentage of oxidized cysteines noted for many of the identified proteins in the wild-type and H-line samples, with approximately 90% of proteins in each cell-line detected with an average redox state of less than 25%. As a result, far fewer cysteine residues are available for iodoTMT-labeling in the NEM-blocked sample. Any differences in protein oxidation and/or expression between the two cell-lines will also alter the number of cysteine residues available for iodoTMT-labeling, and therefore may affect the proteins observed in each sample.

Overall, the four *L. mexicana* samples resulted in cysteine quantitation for 445 proteins, however only 20 proteins were common to all four samples. Since each cysteine in a protein can exist in different redox states, it is important to investigate each one separately by calculating relative quantitation for individual peptides. By only focusing on peptides that were detected and quantified in all four samples, the number of quantified proteins reduced from 20 to 11. Where multiple spectral matches have been identified as corresponding to the same labeled cysteine(s), an average quantitation ratio has been calculated. This included peptides observed with and without methionine oxidation, and peptides also observed with a missed cleavage.

Two peptides detected in the *L. mexicana* samples have been selected in order to illustrate the benefits of this advanced multiplexing method. Firstly, an observed peptide from a membrane-bound acid phosphatase precursor (LmxM.36.2570) was shown to decrease slightly (~10%) in terms of overall protein concentration as well as in oxidation levels between the wild-type and H-line samples, suggesting that the change in oxidation observed was as a result of decreased protein expression. This can be confirmed by the

near-identical percentage oxidation levels in the wild-type and H-line samples, 11.8% and 11.2% respectively, as shown in **Figure 4** and **Table 1**.

By comparison, a peptide from acyl-CoA dehydrogenase (LmxM.28.2510) was not shown to vary with respect to overall protein expression, however the oxidation level was seen to decrease by approximately 65%. This was confirmed by the changes in percentage oxidation from approximately 5.6% to 1.4% between the wild-type and H-line cell-lines, as shown in **Figure 5** and **Table 2**.

Other observations included the detection of tryparedoxin peroxidase (LmxM.15.1040), an enzyme that was not seen to vary in terms of protein expression, but was shown to decrease in oxidation level in the H-line sample. This supports previous studies showing that tryparedoxin peroxidase plays a key role in *Leishmania* virulence.[24, 27-31] Out of the 11 *L. mexicana* proteins quantified using this method, 5 are known to be enzymes; two membrane-bound acid phosphatases (LmxM.36.2570 and LmxM.36.2590) that showed no variation with respect to oxidation levels between the two cell-lines, while the remaining three enzymes showed decreased oxidation in the H-line samples. Only one protein was shown to be up-regulated with respect to protein expression, a hypothetical protein (LmxM.04.0130), which was observed with a two-fold increase in concentration in the H-line but an unchanging percentage oxidation level of approximately 80% in both cell-lines. Of the remaining proteins, only calreticulin (LmxM.30.2600) was shown to have an increase in percentage oxidation levels in the H-line samples.

Two peptides from the same hypothetical protein (LmxM.26.1960) were observed with near-identical protein expression quantitation values, as expected, however each peptide was shown to have differing oxidation changes between the two cell-lines – one remained constant while the other decreased by 50%. This observation demonstrates the importance of monitoring redox changes for individual peptides, since cysteine residues within the same protein can exist in different forms and the redox state of each may be affected independently.

The full table of results is detailed in **Supplementary Information** (**Table S-1**). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[32] with the dataset identifier PXD010938.

CONCLUSIONS

This study clearly demonstrates the advantages of using iodoTMT multiplexing to directly relate redox state with change in protein expression for complex proteomic samples, providing more depth and information from a single set of samples. The application of this multi-quantitation method to samples originating from a single source allows inconsistencies in growth conditions and sample variability to be minimized. The results acquired have not only demonstrated the practicality of this method, but have also provided a list of proteins that have shown significant oxidation changes between virulent and attenuated *Leishmania mexicana*, and will be the focus for further study.

The variation in the number of iodoTMT-labeled proteins between each cell-line means that quantitation channel normalization cannot be performed during data processing and therefore minor discrepancies in protein concentration between replicates cannot be corrected for. Previous studies have also noted a slight shift in reporter ion signal accuracies at low signal-to-noise ratios, which may have an adverse effect on the resulting quantitation ratios.[22]

Given the relationship between the four samples, as described in **Equation 1**, it is possible to extrapolate a missing quantitation value if a peptide is observed in only three out of the four samples. This may be particular useful when considering samples with a low percentage of oxidized cysteines, where the number of iodoTMT-labeled peptides in the NEM-blocked sample will be significantly less than the other samples. In this dataset, an additional 52 proteins can be identified and quantified from peptides observed in any three out of the four samples. Improved proteomic coverage, which might be achieved by sample fractionation and/or through multidimensional chromatography, could result in improved data on both protein expression and redox state, enabling more proteins to be assessed and represented in all replicates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Structure of the iodoTMT reagent and its reaction mechanism; Full mathematical equation for calculating the relative change in oxidation state; Complete table of quantitation ratios for all peptides observed across the four samples, including oxidation state calculations. (PDF)

AUTHOR INFORMATION

Corresponding Author

* Email: richard.burchmore@glasgow.ac.uk

Notes

The authors declare no competing financial interest.

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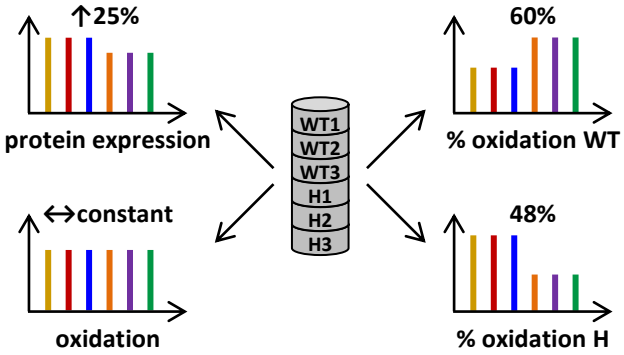
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