Relative Quantification of TMT-labeled, cross-linked proteins using XlinkX node in Proteome Discoverer

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ABSTRACT

Purpose: Development of complete analytical workflow in Thermo Scientific™ Proteome Discoverer™ 2.3 software for identification and multiplex quantitation of cross-linked peptides and proteins.

Methods: Cytochrome C and rabbit 20S proteasome complex in PBS buffer (pH 7.4) or HEPES buffer (pH 8.0) were reacted with DSSO in a molar ratio of 1:5 or 1:100 (protein:cross-linker) for 1 h at room temperature. Cross-linked proteins were then digested and TMT labeled according to manufacturer's instructions. Cross-linked peptides were pre-fractionated on SCX spin columns or a peptide size exclusion chromatography (SEC) column. A Thermo Scientific™ Orbitrap™ Fusion Lumos™ Tribrid™ mass spectrometer was used for crosslinked peptide analysis. Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.3 software using a XlinkX 2.0 software node.

Results: Quantitation of Multiplexed, Isobaric-labeled cross (X)-linked peptides or QMIX[1] is a multiplexed QXL-MS strategy that combines MS-cleavable cross-linkers with isobaric labeling reagents and novel hybrid mass spec acquisition methods. We successfully applied this workflow to measure changes in the structural dynamics of rabbit 20s proteasome complex in the presence of different concentrations of SDS.

INTRODUCTION

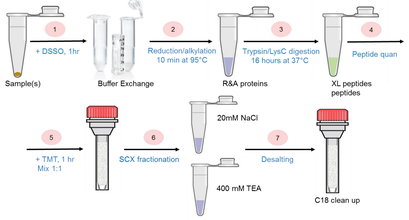
To study structural and interaction dynamics of protein complexes, multiple quantitative cross-linking mass spectrometry (QXL-MS) strategies based on isotope-labeled cross-linkers or isobaric-labeled cross-linked peptides have been developed. Quantitation of Multiplexed, Isobaric-labeled cross (X)-linked peptides or QMIX[1] is a multiplexed QXL-MS strategy that combines MS-cleavable cross-linkers with isobaric labeling reagents and novel hybrid mass spec acquisition methods. Although it was possible to identify TMT-labeled, cross-linked peptides using XlinkX 1.0 node in Proteome Discoverer 2.2 software, quantification was not implemented. In this study, we describe a new, complete analytical workflow in Proteome Discoverer 2.3 software for identification and multiplex quantitation of cross-linked peptides, which can be directly applied to study conformational dynamics of protein complexes and protein-protein interactions at the proteome scale.

MATERIALS AND METHODS

Sample Preparation

Cytochrome C in PBS buffer (pH 7.4) was reacted with DSSO in a molar ratio of 1:5 (protein:cross-linker) for 1 hr at room temperature and quenched with excess hydroxylamine. Cross-linked proteins were then pelleted via TCA precipitation, re-suspended, and digested. The resulting peptide mixtures were de-salted, fractionated by peptide size exclusion (SEC), and TMT 2 plex labeled according to manufacturer's instructions and mixed in different ratios[1]. Rabbit 20S proteasome complex (Boston Biochem) in HEPES buffer (pH 8.0) was reacted with DSSO in a molar ratio of 1:100 (protein:cross-linker) for 1 hr at room temperature in presence or absence of 0.03% or 0.1%SDS to activate proteasomes. Reaction was quenched with 1M NH_4HCO_3. Complete sample prep workflow is outlined in Figure 1.

Figure 1. Sample preparation for Rabbit 20S proteasome. Buffer exchange was performed using an Amicon® centrifugal filter unit (10 kDa, EMD Millipore). Peptide concentrations were determined using the Pierce™ Quantitative Fluorometric Peptide Assay. Peptides were fractionated using polymer-based SCX spin columns with an increasing step gradient of 20mM NaCl followed by 400mM triethylammonium(TEA). SCX fractionated samples were desalted using a 70% ACN elution step on Pierce™ High pH Reversed-Phase Peptide spin columns before LC-MS/MS analysis.



Liquid Chromatography and Mass Spectrometry

Samples were separated by RP-HPLC using a Thermo Scientific™ UltiMate™ 3000 UHPLC system connected to a Thermo Scientific™ EASY-Spray™ column, 50 cm × 75 µm over a 90 min 2-28% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The crosslinked TMT labeled samples were analyzed on the Orbitrap Fusion Lumos mass spectrometer using Tune 3.1 and acquisition methods as in Figure 2.

Figure 2. MS acquisition methods for identification and quantification of TMT labeled crosslinked peptides.

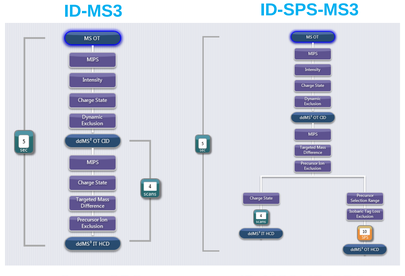
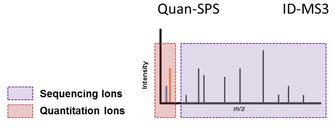


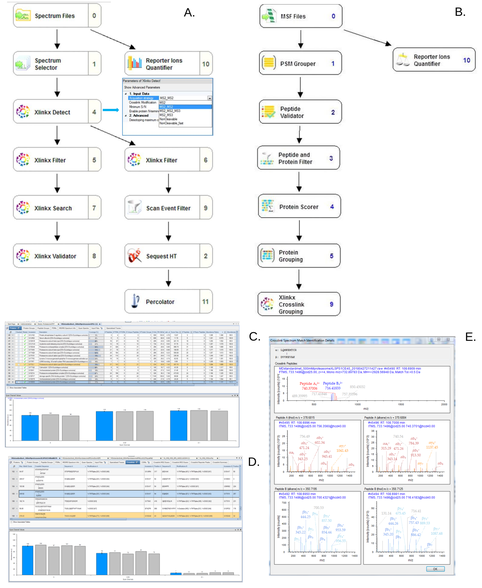
Figure 3. Quantitation of Multiplexed, Isobaric-labeled cross (X)-linked peptides or QMIX workflow



Data Analysis

Spectral data files were analyzed by beta version of Proteome Discoverer 2.3 software using the XlinkX 2.0 node (Figure 3) for crosslinked peptides and SEQUEST®HT search engine for unmodified and dead-end-modified peptides. Carbamidomethylation (+57.021 Da) used as a static modification for cysteine, TMT2 plex or 6 plex used as a static modification for peptide N-terminus. Different crosslinked mass modifications including TMT 6 plex for lysine were used as variable modifications for lysine in addition to methionine oxidation (+15.996 Da). Data were searched against a database containing the Uniprot/TrEMBL entries of the model proteins with/out common contaminants with a 1% FDR criteria for peptide spectrum matches. For MS2-MS3 methods, a linear–peptide search option (using MS3 scans for identification and MS2 scan for detection of crosslinked peptides) was used for XlinkX database searching. For TMT quantification, reporter ion abundances were corrected for isotopic impurities based on the manufacturer's data sheets. Signal-to-noise (S/N) values were used to represent the reporter ion abundance with an co-isolation threshold of 75% and an average reporter S/N threshold of 10 and above required for quantitation spectra to be used. The S/N values of peptides, which are summed from the S/N values of the peptide spectral matches (PSMs), are finally summed to represent the abundance of the proteins. In the case of cross-linked peptides, crosslinked quantitation ratios are calculated from the S/N values of the cross-linked spectral matches (CSMs).Only SPS spectra were used for quantification. Results visualization and distance restraints were performed using *xiNET*[2] and Yasara viewer v18.3.23.

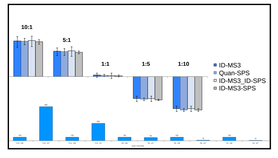
Figure 4. The processing (A) and consensus (B) XlinkX workflows in Proteome Discoverer 2.3 software includes a separate proteins (C) and crosslinkers quantitation results tab (D), as well as spectra annotation(E).



RESULTS

Validation of TMT quantification of DSSO crosslinked peptides using XlinkX node in Proteome Discoverer 2.3 software.

Figure 5. TMT 2plex Quantification of Cytochrome C DSSO crosslinked peptides using Proteome Discoverer 2.3 software.



In order to evaluate accuracy and precision of TMT based crosslinked quantitation using different acquisition methods (Figure 2) and performance of newly developed quan.node in Proteome Discoverer 2.3 software, we premix cytochrome C digest labeled by TMT 2 plex in defined ratios (Figure 5). As expected OT HCD SPS methods provided best accuracy and precision. Moreover, out of 49 identified unique XL peptides(115 CSMs), 48 were quantified. As expected quantitation results were identical for PSMs and CSMs.

Monitoring Structural changes of Rabbit 20S proteasome complex in the presence of SDS

The 20S proteasome is a multi-catalytic enzyme complex expressed in the nucleus and cytoplasm of all eukaryotic cells and part of the 26S proteasome complex responsible for recycling of damaged, misfolded and short-lived regulatory proteins via ubiquitin-proteasome pathway. The 20S proteasome *in vitro* can efficiently degrade peptides or proteins if it is activated by addition of low concentrations of SDS[3]. The mechanism for this activation is not well-understood but it was proposed that SDS induces conformational changes thereby allowing access to the proteasome center cavity. To validate the QMIX workflow (Figure 3), we incubated rabbit 20S proteasome in the presence of different concentrations of SDS and DSSO and prepared samples as described in Figure 1. Samples were analyzed using 4 different acquisition methods (Figure 2) and processed in Proteome Discoverer 2.3 software (Figure 4). The best overall results were obtained using IP-SPS-MS3 method (Table 1). Overall we were able to identify, quantify and map more than 100 intra and inter crosslinked peptides (Figure 6) and confirmed that SDS indeed causes some conformational changes at least in the outer alpha subunits ring, as shown in Figure 7, for the alpha 4 subunit. Using this approach we were able to monitor changes in amount of crosslinked peptides between Lys 254 at C-terminus vs. other Lys and observed, for example, increased distance between Lys 254 and 199 by increasing SDS concentration. As expected, there were no differences in relative abundances for unmodified or monolinked peptides.

Table 1. Comparison of performance different acquisition methods for identification and quantification of 400mM TEA fraction of TMT 6 plex 20S proteasome XL peptides (N=2), XlinkX score of 50 was used as cut off.

	#CSM	#XL	#XL inter	# XL quant	# XL Proteins	# Peptides
ID-MS3	292	150	94	N/A	35	883
ID-SPS-MS3	253	133	87	132	38	834
ID-MS3-ID-SPS	208	139	80	51	25	237
SPS	42	20	8	20	14	1058

Figure 6. DSSO crosslink mapping of Rabbit 20S proteasome. Crosslinking map was generated using *xiNET*(A) and visualization was performed using Yasara viewer (B). Lys-Lys contacts identified by ID-SPS DSSO crosslinking experiments mapped onto human 20S proteasome homology structure with distances (Cα-Cα) less than 30Å.

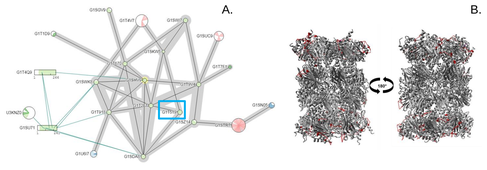
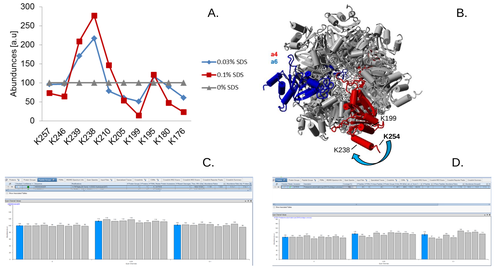


Figure 7. Quantitative profiling of proteasome subunit alpha 4(G1T519) using QMIX workflow upon SDS activation. Relative abundances of K254 containing DSSO crosslinked peptides (A), visualization of mapped Lys (B) and TMT quan of K246 monolink (C) and total protein (D).



CONCLUSIONS

- ID-SPS-MS3 method provided best overall performance for simultaneous identification and TMT quantitation of DSSO crosslinked peptides.
- Proteome Discoverer 2.3 software with XlinkX 2.0 was able to confidently identify and quantify TMT labeled crosslinked peptides. Its performance was validated using cytC digest mix in known ratios.
- The QMIX workflow for comparative structural analysis of proteins and protein complexes was successfully applied to study dynamics of rabbit 20S proteasome upon SDS activation.

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TRADEMARKS/LICENSING

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