Evaluation of FAIMS Technology for Mass Spec Analysis of Chemical Cross-linked Peptides

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ABSTRACT

Purpose: To evaluate widely used enrichment/fractionation techniques and the newly developed High Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS)[1] device for cross-linked peptide analysis using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer.

Methods: Different amine-reactive, homobifuctional crosslinkers were used to crosslink bovine serum albumin (BSA), yeast enolase, human cell lysate and mouse mitochondria. Crosslinked samples were reduced, alkylated and digested with trypsin before MS analysis. Cross-linked peptides were pre-fractionated on SCX or peptide size exclusion chromatography (SEC) columns. An Orbitrap Fusion Lumos mass spectrometer with/out the Thermo Scientific™ FAIMS Pro™ Interface 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: FAIMS alone or in combination with enrichment/pre-fractionation significantly improves identification of cross-linked peptides in complex samples.

INTRODUCTION

Chemical cross-linking in combination with mass spectrometry is a powerful method to determine protein-protein or nucleic acid-protein interactions. This method has been applied to recombinant and native protein complexes and, more recently, to whole cell lysates or intact unicellular organisms in efforts to identify protein-protein interactions on a global scale. However, this method suffers from low identification rates as the typical yield of cross-linked peptides is less than 1 % of total identified peptides and requires fractionation. FAIMS separates gas-phase ions on the basis of differences in their ion mobility in high and low electric fields (Figure 1). It been shown before that FAIMS improves dynamic range, increases signal-to-noise and reduces interference from ions of similar *m/z* [1-2]. In this study, we compared widely used enrichment/fractionation techniques and the newly developed FAIMS device for cross-linked peptide analysis using an Orbitrap Tribrid mass spectrometer

MATERIALS AND METHODS

Sample Preparation

Disuccinimidyl suberate (DSS), disuccinimidyl sulfoxide (DSSO) and disuccinimidyl dibutyric urea (DSBU) were used to crosslink 2mg/ml BSA or yeast enolase (Sigma Aldrich) solubilized in 50mM HEPES pH 8 for 1hr at 100 molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 1M Tris pH 8. BSA was reduced, alkylated, acetone precipitated and digested with trypsin before MS analysis. Enolase was first desalted using an Amicon® centrifugal filter unit (30 kDa, EMD Millipore) and then digested with trypsin. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ Quantitative Colorimetric Peptide Assay, respectively. SCX spin column and SEC fractionations of DSS BSA sample were performed as described in[3]. DSS BSA sample was labeled with TMT 6 plex labeled according to manufacturer's instructions and mixed in 1:1:1 ratios. Mouse mitochondria SCX fractions[4] and HeLa cell lysate crosslinking (HeLa-XL standard)samples were gift from Dr. Fan Liu, from Leibniz-Forschungsinstitut for molecular pharmacology (FMP).

Liquid Chromatography and Mass Spectrometry

Samples were separated by RP-HPLC using a Thermo Scientific™ Dionex™ UltiMate™ 3000 system or Thermo Scientific EASY-nLC™ 1200 UPLC system connected to a Thermo Scientific™ EASY-Spray™ column, 50 cm × 75 µm over a 45 min 2-28%,12 min from 26-40 % gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The crosslinked samples were analyzed on the Orbitrap Fusion Lumos mass spectrometer. Specific LC and MS settings are shown in Table 1 with or without FAIMS Pro device (Figure 1).

Data Analysis

Spectral data files were analyzed using Proteome Discoverer 2.3 software using the XlinkX node 2.0 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. Different crosslinked mass modifications for lysine were used as variable modifications for lysine or N-terminus in addition to methionine oxidation (+15.996 Da). Data were searched against a database containing the Uniprot/SwissProt entries of the model proteins with/out common contaminants with a 1% FDR criteria for protein spectral matches. For data acquired using the non-cleavable crosslinker, DSS, a new search option in XlinkX -NonCleavable_fast was used. For MS cleavable crosslinkers, a linear–peptide search option was used for XlinkX database searching and the Uniprot/SwissProt databases of human or mouse proteins(retrieved November 2018). Cross-links are reported at a 1 % FDR, XlinkX score of 40 and delta XlinkX score of 4 were used as cut offs.

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Figure 1. High-Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) Technology. As ions are introduced from the ESI tip into the region between the electrodes, an asymmetric waveform is applied to one electrode while the other is grounded (A). A high voltage is applied for a short period of time, called the Dispersion Voltage or DV, then a low voltage of opposite polarity is applied for twice the length of time at half the voltage. In order to compensate for this ion displacement during repeated cycles, a Compensation Voltage (CV) is applied to keep the ion centered between the electrodes and passed into the mass spectrometer (B).

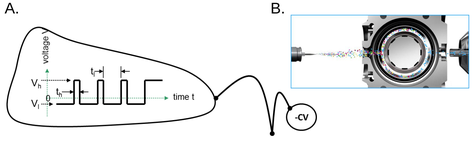


Table 1. Orbitrap Fusion Lumos MS FAIMS XL-LC-MS/MS acquisition parameter settings.

Settings	DSSO MS2-MS3	DSS MS2	DSBU MS2
LC gradient standards	2-26% in 45min, 26-40 in 12 min	2-26% in 45min, 26-40 in 12 min	2-26% in 45min, 26-40 in 12 min
LC gradient complex mixtures	5-40% in 120 min		
MS 1	OT	OT	OT
Resolution	60K	60K	60K
Target value	4e5	4e5	4e5
Max injection time	50	50	50
Top Speed FAIMS per CV	2 sec	2 sec	2 sec
Top Speed no FAIMS or 1 CV	5 sec	5 sec	5 sec
MS2	OT CID	OT HCD	OT HCD
Charge states	3-8	3-8	3-8
Isolation width	1.6	1.6	1.6
NCE	25	Assisted, 25, 30	SCE 28, 32, 35
Resolution	30K	30K	30K
Target value	5e4	1e5	1e5
Max injection time	70 ms	70 ms	70ms
Targeted Mass MS[3]	IT CID	n/a	n/a
Isolation width	2.5		
NCE	35		
Resolution	Rapid		
Target value	2e4		
Max injection time	150 ms		
FAIMS CV settings standards	50-60-75	50-60-75	50-65-85
FAIMS CV settings complex mixture	50-60-75; 60-75; 70-80-90	50-60-75; 60-75; 70-80-90	

RESULTS

Method optimization for FAIMS XL-LC-MS/MS using standards.

The identification of cross-linked peptides by LC-MS/MS presents significant analytical challenges due to their low abundance and higher charge state distribution compared to tryptic peptides. We evaluated the use of a FAIMS device for crosslinked peptide analysis. Different compensation voltages (CV) between -40 and -85V were tested with 5-10V resolution using 2 approaches: external(inter-analysis CV switching) and internal stepping(intra-analysis CV switching). As shown in Figure 2, in all cases internal stepping using 3 CVs provided the best results.

As each cross-linker produces unique combinations of z, *m/z* and masses, slightly different CV settings are required for optimal performance. Selective enrichment/fractionation of cross-linked peptides by SEC or SCX fractionation using an offline LC approach is widely used for improved interaction sites identification. We compared identification rates of a FAIMS device for gas-phase fractionation vs traditional fractionation techniques[3]. Analysis of the identical samples with a FAIMS device in place, using optimized methods, produced the same number of identified cross-linked peptides as after fractionation (Table 2). Previously, FAIMS was shown to reduce interference from ions of similar *m/z*, improving TMT-labeled peptide quantitation accuracy and precision[2]. Figure 3 shows that FAIMS also improves TMT labeled cross-linked peptide identification and quantitation.

Figure 2. BSA Peptides and Crosslinked Peptides Identified using multiple crosslinkers (DSSO, DSBU, DSS) and Different LC FAIMS methods with internal or external CV stepping (Table 1).

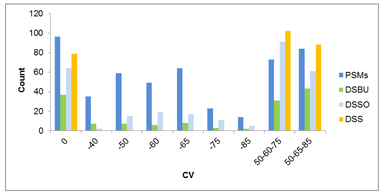
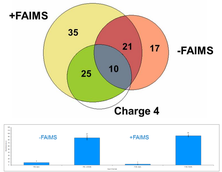


Table 2. DSS BSA or Enolase XL-MS Analysis using SCX, SEC enrichment[3] or FAIMS pro device.

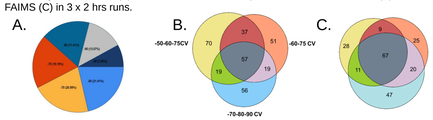
	BSA, # XL	Enolase, # XL
No enrichment	73	40
SEC, 5 fractions	93	51
SCX, 2 fractions	74	44
FAIMS, -50-60-75	94	33
FAIMS, 3 runs: -50-60-75; -60-75; -70-80-90	114	51

Figure 3. TMT 6plex Identification and Quantification of BSA DSS crosslinked peptides with/out FAIMS.



From our experiments with single proteins(Figure 1, 3, Table 2), we identified range of CVs and its combinations that can produce more crosslinked peptides identifications and less identifications for unmodified peptides. Next, we used DSSO HeLa –XL standard to find the best conditions for FAIMS-XL- LC-MS of complex mixture. Based on CSMs/CV distribution presented in Figure 4A, we selected the following CVs combinations: -50-60-75; -60-75 and -70-80-90; each of them covers around 50% of identified CSMs. In the typical proteomics experiment, 3 technical replicates are performed. We identified 207 unique crosslinked peptides in HeLa sample in 3 injection replicates without FAIMS (Figure 4C) and 309 with FAIMS (Figure 4B) using different combinations of CVs. The Overlap was 70% between the runs without FAIMS and 60% with FAIMS. The best results were obtained using -50-60-75 CVs method with 183 unique crosslinked peptides, which was better than 2 replicates without FAIMS. Interestingly, similar results were obtained for unmodified peptides and proteins more than 1.5-2 fold increase with FAIMS (data not shown). This sample was relatively complex- around 2000 proteins identified and results could be improved if a longer gradient was employed.

Figure 4. DSSO HeLa –XL standard Analysis with/out FAIMS. Number of identified CSMs per CV settings in 2 hr runs (A). Number of identified unique crosslinkers with FAIMS (B) and without FAIMS (C) in 3 x 2 hrs runs.



FAIMS XL-MS Analysis of SCX fractions

Off-line SCX fractionation is a well established method for reducing sample complexity of XL-MS wide proteome experiments. Complementarity of FAIMS and SCX has been previously demonstrated for shotgun proteomics[3]. Here, we compared SCX-XL-MS analysis with FAIMS-SCX-MS analysis for 9 SCX fractions of DSSO-crosslinked mouse heart mitochondria[4]. Out of a total of 40 fractions, we analyzed 3 x 3 combined fractions: I(24-26 min); II(31-33) and III(35-36 min) from the different parts of LC chromatogram (Figure 5A). Based on experiments with standard proteins, we only used multiple compensation voltages (2 or 3) within a single LC-MS/MS approach. As shown in Figure 5B-D, optimal FAIMS CVs settings depend on SCX fraction elution times. Later fractions require higher compensation voltages vs early fractions due to charge distribution profiles. Overall, we were able to identify more than 2 000 unique inter- and intra-contacts in 2D 9 FAIMS and 3 SCX LC-MS/MS runs using different CV (Figure 6).

Figure 5. 2D FAIMS-SCX-XL-MS Analysis of Mouse Heart Mitochondrial Interactome. SCX LC profile (A); Fraction I, 24-26 min (B); Fraction II, 31-33 min (C), Fraction III, 35-36 min(D) unique identified crosslinkers results. Venn diagrams for FAIMS-LC-MS experiments, CSMs charge distribution and SCX-LC-results are shown in each panel, green check highlights best FAIMS CVs settings/fraction.

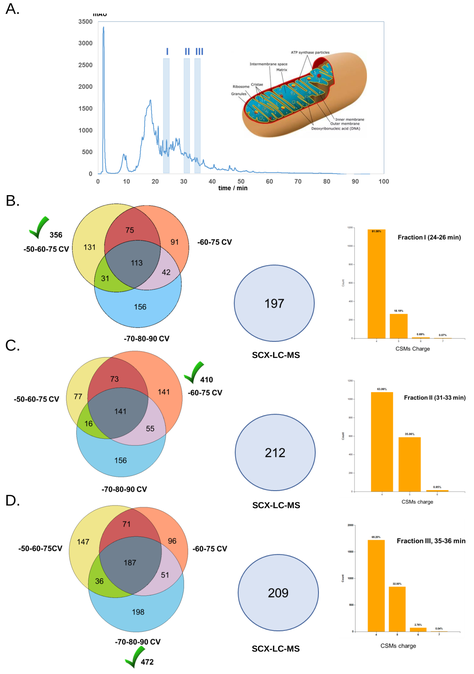
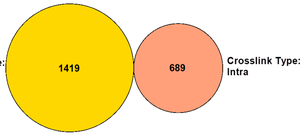


Figure 6. Mitochondria DSSO Crosslinked Peptide Spectra Identified by FAIMS and SCX-XL-MS/MS



CONCLUSIONS

- In all experiments, internal CV stepping generated best identification rates vs. external CV stepping or single CVs.
- Each cross-linker, sample type, and level of complexity produces unique combinations of z, *m/z*, and mass, which requires slightly different CV settings for optimal performance.
- FAIMS alone or in combination with enrichment/pre-fractionation significantly improved identification rates of cross-linked peptides in all samples.
- 2D FAIMS-SCX-XL-MS analysis of mouse heart mitochondria boosted number of identified interactions 2-fold by increasing separation peak capacity.

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TRADEMARKS/LICENSING

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