# Optimization of FAIMS-XL-MS Workflow for Phospho-Enrichable Crosslinkers

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## ABSTRACT

**Purpose:** Benchmark FAIMS technology for mass spec analysis of protein-protein interactions with phospho-enrichable crosslinkers.

**Methods:** Crosslinking mass spec analysis were performed on Thermo Scientific™ Orbitrap Exploris™ 240 platform with/out FAIMS Pro interface. Two crosslinkers, DSSO and PhoX-DSPP were used to crosslink proteins or organelles for *E. coli* ribosomes. Data were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.5 software and XlinkX 2.5 node.

**Results:** With optimized MS conditions, FAIMS-XL-MS enable the further increase of identified unique cross-linking sited for DSSO and unenriched/enriched PhoX-DSPP sample.

## INTRODUCTION

Cross-linking mass spectrometry (XL-MS), a rapid and high-resolution technique, has grown dramatically into a routinely utilized strategy for characterizing protein higher-order structure and mapping protein-protein interaction networks on a proteome-wide scale. However, a significant limitation for XL-MS is the relatively low identification rates of cross-linked peptides, particularly for more complex protein samples, which has impeded wider applications of this technique. Compared to conventional mass spectrometry, FAIMS (high field asymmetric waveform ion mobility spectrometry) increases analytical performance by incorporating additional gas-phase fractionation.

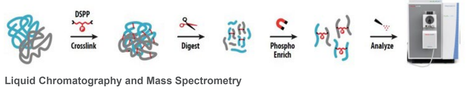
Previously we described optimized FAIMS XL-MS workflow for instruments with ion-funnel front optics[1]. In this work, we implemented FAIMS technology on the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer, which employs S-lens front optics, in combination with one MS-cleavable crosslinker DSSO and one amine-reactive, enrichable crosslinker PhoX-DSPP[2] to address this analytical challenge. With the optimized running condition of FAIMS-XL-MS, we were able further enhance the selectivity and detection limit for *E. coli* ribosome protein complex.

## MATERIALS AND METHODS

### Sample Preparation

*E.coli* ribosomal proteins (NEB) were prepared as described[3] and buffer exchanged into PBS, pH 7.0 using a 10kDa centrifugal filter unit. Disuccinimidyl sulfoxide (DSSO), and Disuccinimidyl Phenyl Phosphonic Acid, PhoX (DSPP) solutions in DMSO were used to crosslink 2mg/ml ribosomal proteins in PBS pH 7 for 1hr at 30-100 molar excess of crosslinker to protein. After crosslinking, reactions were quenched with 30mM of ammonium bicarbonate, pH 8 for 15 min. The sample was reduced and alkylated with TCEP and 2-chloroacetamide at 95°C for 10 minutes, then cooled at 4°C for 5 minutes before acetone precipitation overnight with 6 volumes of ice-cold acetone at -20°C. Then, samples were washed twice with 90% acetone and the pellet was vortexed with 100mM TEAB until re-solubilized, and then digested with Lys-C/trypsin (1:50 ratio) overnight. Protein and peptide concentrations were determined using the Pierce™ BCA Protein Assay Kit and the Pierce™ Quantitative Fluorometric Peptide Assay (23290), respectively. DSPP sample digest (700 µg of each) were enriched using High-Select Fe-NTA Phosphopeptide Enrichment (A32992) or High-Select Fe-NTA Magnetic Agarose (A52284) kits according to manufacturer instructions. For magnetic beads, a 1:5 ratio (magnetic bead slurry to peptides) was used. Pierce Quantitative Fluorometric Peptide Assay (23290) was used to quantitate samples before LC-MS analysis.

**Figure 1. Cross-linking workflow for PhoX-DSPP.**




### Liquid Chromatography and Mass Spectrometry

Separation of digested peptides was achieved using a 75µm × 25cm Thermo Scientific™ EASY-Spray™ analytical column and with a 75 min gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. Following separation, the peptides were detected on a an Orbitrap Exploris 240 mass spectrometer equipped with a Thermo Scientific™ FAIMS Pro Duo interface .

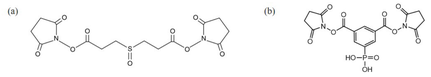
### Data Analysis

Spectral data files were analyzed using Proteome Discoverer 2.5 software using the XlinkX node 2.5 for crosslinked peptides and SEQUEST®HT search engine for unmodified and dead-end-modified peptides. Data were searched against a database containing the Uniprot/SwissProt entries for *E. coli* or *E. coli* ribosomes with 1% FDR criteria for protein spectral matches. For data acquired using the non-cleavable crosslinker, PhoX-DSPP, a search option NonCleavable_fast was used. For MS cleavable crosslinker, a linear–peptide search option was used for XlinkX database searching and the Uniprot/SwissProt databases of *E. coli* proteins (retrieved March 2022). Crosslinked peptides are reported at a 1% FDR.

## RESULTS

As FAIMS technology can increase analytical performance of MS by incorporating additional gas-phase fraction, we implemented FAIMS on the Orbitrap Exploris™ 240 and optimized setting parameters of both FAIMS and MS for DSSO and PhoX-DSPP (Fig. 2). Instrument parameters including ion transfer tube temperature, spray voltage, total carrier gas flow, collision energy mode, resolution, and FAIMS compensation voltage were optimized for maximum crosslinked peptide identification.

**Figure 2. Structure of MS-cleavable and amine-enrichable crosslinkers used in the study. (a) DSSO; (b) PhoX-DSPP**



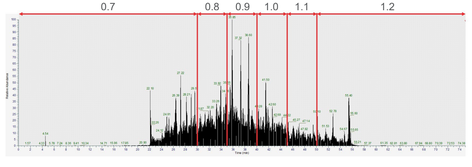

### Total Gas Flow Rate

With the introduction of Tune 3.0 software, it's possible to use custom settings for carrier gas flow to improve spray stability and overall FAIMS performance on hybrid platforms. The optimal carrier gas flow was identified by increasing gas flow from 0.7 L/min to 1.2 L/min in a single run under time-dependent gas mode in 0.1 L/min step. 0.9 L/min was selected as the optimal total gas flow rate.

**Table 1. Comparison of HELA identifications under different total gas flow rates with/without FAIMS implementation**

| RT Range (min) | No FAIMS | FAIMS | |
|---|---|---|---|
| | # Protein IDs | Gas Flow Rate | # Protein IDs |
| 0-30 | 71 | 0.7 | 97 |
| 30-35 | 300 | 0.8 | 451 |
| 35-40 | 577 | 0.9 | 681 |
| 40-45 | 755 | 1.0 | 794 |
| 45-50 | 822 | 1.1 | 556 |
| 50-75 | 734 | 1.2 | 498 |

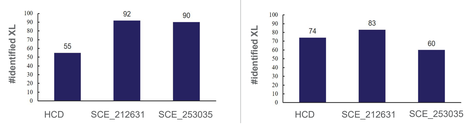
**Figure 3. TIC of total gas flow rate test run.**




### Collision Energy Mode

Both HCD and stepped collision energy (SCE) modes were evaluated with two SCE settings of 21%, 26%, 31%, or 25%, 30%, 35% or single HCD NCE of 30% for both crosslinkers DSSO and PhoX-DSPP, as shown in Figure 3. SCE setting of 21%, 26%, 31% was selected for both DSSO and PhoX-DSPP.

**Figure 4. Collision energy mode optimization of DSSO and PhoX-DSPP.**




### Spray Voltage and Ion Transfer Tube Temperature

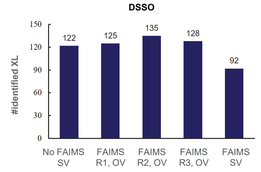
Spray voltage as 2000 V, ion transfer tube temperature as 305 ºC are the standard settings for spray voltage and ion transfer tube temperature. However, with FAIMS, 30 fewer crosslinking sites were identified compared to no FAIMS with 2000 V and 305 ºC standard settings. We found out that lower voltage (1500 V) and transfer tube temperature (250 ºC) settings provide higher number of identified cross-linking sites, as shown in Fig. 5.

- For DSSO crosslinked sample, with triplicates, 40% more crosslinked cites were identified than high spray voltage and high temperature settings, and 10% more crosslinked cites compared without FAIMS.
- For PhoX-DSPP magnetic enriched sample, 10% more crosslinked cites were identified with FAIMS implementation.
- For PhoX-DSPP agarose enriched sample, 14% more crosslinked cites were identified with FAIMS implementation.

**Table 2. Global parameters for FAIMS-MS.**

| | Standard Value (SV) | Optimal Value (OV) |
|---|---|---|
| Spray Voltage (V) | 2000 | 1500 |
| Sweep Gas (Arb) | 0 | 0 |
| Transfer Tube Temp. (ºC) | 305 | 250 |
| Total Carrier Gas Flow (L/min) | 0.9 | 0.9 |

**Figure 5. Spray Voltage and Ion Transfer Tube Temperature Optimization for FAIMS-XL-MS**

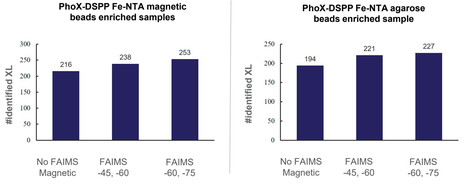



### Compensation Voltage

Compared to conventional mass spectrometry, FAIMS provides an additional gas-phase fractionation for ions by charge. By configuring a compound-specific compensation voltage, only the ions dictated by the CV can pass through the electrodes into the MS, effectively filtering out all other species. Most tryptic peptides transmit between -40 and -85 V. Therefore, two CVs methods were tested : -45, -60 and -60 and -75 for PhoX-DSPP enriched samples. CVs -60 and -75 were selected as optimal settings for enriched PhoX-DSPP samples (Figure 6).

- For PhoX-DSPP samples enriched with magnetic beads or agarose beads, 2 CVs, -60 and -75 provides best results, 37 more unique crosslinked sites were identified vs without FAIMS set up.
- For DSSO samples 2 CVs method , -45 and -60 provides best results, 20 more unique crosslinked sites were identified. (Figure 5).

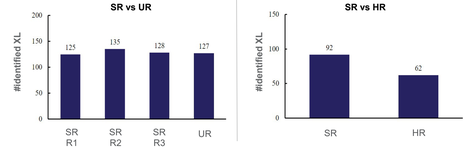
**Figure 6. FAIMS Optimal compensation voltage for PhoX-DSPP enriched samples**




### FAIMS Resolution

There are three available resolution modes for FAIMS: standard resolution (SR), high-resolution (HR), and user-defined resolution (UR). Resolution is controlled by varying the temperatures of the inner and outer electrodes. In standard and high-resolution modes, the temperatures are set automatically. In user defined mode, the temperatures on the inner and outer electrode can be set individually between 70ºC and 100ºC. Here, 100 ºC (inner) and 80 ºC (outer) were used. By inverting the temperatures, the resolution of the CV peak is potentially reduced. In general, for most proteomics applications, standard resolution is the best choice. This mode maximizes transmission and the fraction of peptides collected. Based on our results, **resolution doesn't play an important role in terms of cross-linked peptides identification**, with operation in high resolution mode giving the lowest identification rate due to reduced sensitivity.

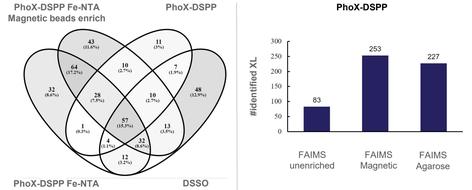
**Figure 7. FAIMS resolution mode optimization for DSSO crosslinked sample**




### Phospho-enrichment

PhoX-DSPP is a phospho-enrichable crosslinkers containing an amine-reactive N-hydroxysuccinimide (NHS) ester at each end of the spacer arm and a phosphonic acid. DSPP react efficiently with primary amine groups (-NH2) in pH 7-9 buffers to form stable amide bonds and contain phospho groups that can be used to enrich crosslinked peptides using immobilized metal affinity chromatography(IMAC), resulting in high specificity for phosphopeptides with low background.

**Figure 8. FAIMS XL-MS Cross-linking sites identified in E. coli ribosome using DSSO or PhoX-DSPP crosslinkers and optimized conditions as in Table 4.**




### Charge Distribution

We identified significant number of PhoX-DSPP crosslinked peptides at charge 3 for both magnetic (48.68%) and agarose (47.23%) enriched samples vs DSSO crosslinked peptides, as shown in Table 3. Based on this observation, we **recommend including charge 3 precursors for PhoX-DSPP crosslinked samples.**

**Table 3. CSMs charge distribution for DSSO, PhoX-DSPP magnetic enrichment and PhoX-DSPP agarose enrichment samples.**

| Crosslinkers | CSMs charge state | | | | | |
|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 |
| DSSO | 66 | 80 | 20 | 7 | 0 | 0 |
| PhoX-DSPP magnetic | 148 | 131 | 23 | 2 | 0 | 0 |
| PhoX-DSPP agarose | 128 | 119 | 24 | 0 | 0 | 0 |

**Table 4. Orbitrap Exploris 240 Optimal FAIMS–MS settings for XL-MS experiments**

| | DSSO | PhoX-DSPP |
|---|---|---|
| MS 1 | OT | OT |
| Resolution | 60K | 60K |
| Sweep Gas (Arb) | 0 | 0 |
| Transfer Tube Temp. (ºC) | 305 | 250 |
| Total Carrier Gas Flow (L/min) | 0.9 | 0.9 |
| RF Lens(%) | 70 | 70 |
| Scan Range (m/z) | 375-1200 | 375-1200 |
| Intensity Threshold | 1e4 | 1e4 |
| MS 2 | OT | OT |
| Resolution | 30K | 30K |
| Isolation Window (m/z) | 1.6 | 1.6 |
| Max Injection Time (ms) | 70 | 70 |
| Collision Energy Type | SCE 21% 26% 31% | SCE 21% 26% 31% |
| First Mass (m/z) | 120 | 120 |
| Charge State | 3-8 | 3-8 |
| Cycle Time (s) | 1.5 | 1.5 |
| FAIMS Resolution | Standard | Standard |
| FAIMS CV (V) | -45, -60 | -60, -75 |

## CONCLUSIONS

- Performance of the XL-MS with/without FAIMS was evaluated on the Orbitrap Exploris™ 240 mass spectrometer using one MS-cleavable crosslinker DSSO and one phosphor-enrichable crosslinker PhoX-DSPP.
- With an optimized enrichment crosslinked peptide workflow, identification of crosslinks increased 2-3x compared to traditional crosslinking workflows with unenrichable crosslinkers in the complex sample.
- With optimized operation parameters, FAIMS-XL-MS technology enabled the further increase in the number of identified unique cross-linking sites for both magnetic and non-magnetic agarose enrichment samples.

## REFERENCES

1. Schnirch, L., Nadler-Holly, M., Siao, S., Frese, C., Viner, R. and Liu, F., 2020. Expanding the Depth and Sensitivity of Cross-Link Identification by Differential Ion Mobility Using High-Field Asymmetric Waveform Ion Mobility Spectrometry. Analytical Chemistry, 92(15), pp.10495-10503.
2. Steigenberger, B., Pieters, R. J., Heck, A., & Scheltema, R. A. (2019). PhoX: An IMAC-Enrichable Cross-Linking Reagent. ACS central science, 5(9), 1514–1522.
3. Cannon JR, et al Ultraviolet photodissociation for characterization of whole proteins on a chromatographic time scale. Anal Chem. 2014 Feb 18;86(4):2185-92

## TRADEMARKS/LICENSING



PO66149-EN0422S