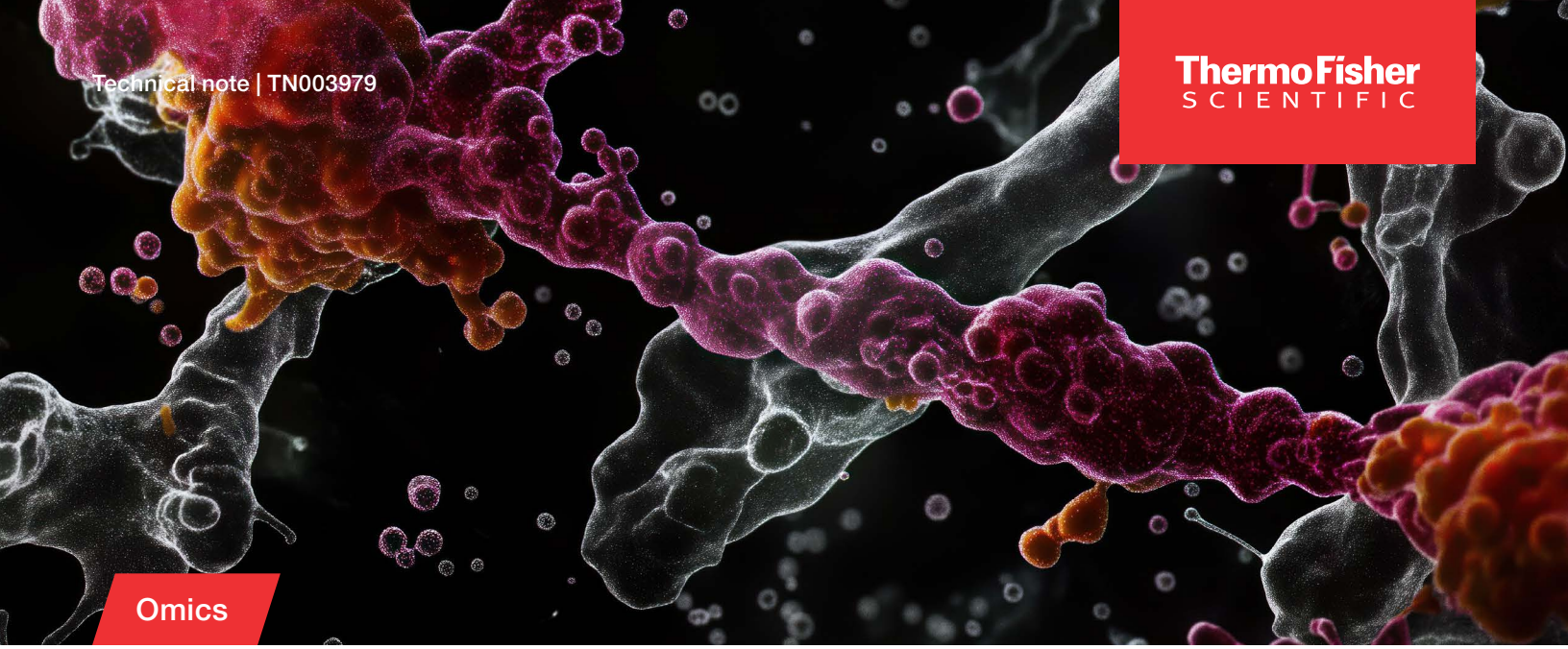


Technical note | TN003979

Omics

Proteome wide interactomics analysis using MS-cleavable crosslinkers and the Orbitrap Astral Zoom mass spectrometer

Authors

Yi He[1], Tabiwang N. Arrey[2], Eugen Damoc[2], Erum Raja[3], Leigh Foster[3], Ryan Bomgarden[3], Rosa Viner[1], and Thomas Moehring[2]

[1]Thermo Fisher Scientific, San Jose, California, USA

[2]Thermo Fisher Scientific, Bremen, Germany

[3]Thermo Fisher Scientific, Rockford, Illinois, USA

Keywords

Crosslinking mass spectrometry, MS-cleavable crosslinker, DSSO, DSBSO, FAIMS, Proteome Discoverer, XlinkX node, Vanquish Neo UHPLC system, Orbitrap Astral Zoom, XL-MS.

Goal

Develop an end-to-end crosslinking mass spectrometry (XL-MS) workflow for MS-cleavable crosslinkers on the Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer.

Introduction

XL-MS has quickly emerged as a crucial technique for understanding the higher-order structures of proteins and mapping protein-protein interaction (PPI) networks on a proteome-wide scale.[1] However, challenges persist, including the relatively low abundance of crosslinked peptides and the lengthy data analysis process. Commonly used MS-cleavable crosslinkers, such as disuccinimidyl sulfoxide (DSSO)[2] and disuccinimidyl bis-sulfoxide (DSBSO),[3] are particularly useful in PPI analysis, especially within complex systems like intracellular crosslinking, because of their unique fragmentation patterns.

During MS/MS analysis, DSSO and DSBSO generate characteristic doublet ions, which can help confirm the identification of crosslinked species. Additionally, by utilizing these characteristic doublet ions in MS/MS, database search algorithms can address the “n^2 problem” (where the search space increases quadratically with the database size), allowing for faster and more confident identifications. Moreover, DSBSO offers a significant feature for enriching crosslinked species in addition to its distinct MS/MS pattern. It includes an azide tag that enables selective bio-orthogonal enrichment using dibenzocyclooctyne (DBCO)-coupled beads. During the enrichment process, unmodified peptides are removed from the mixture, allowing for the concentration of low-abundance crosslinked peptides. This effectively enhances the identification rates of crosslinked peptides, facilitating broader applications in more complex systems.

The sensitivity and speed of mass spectrometry instruments are also vital for crosslinking studies. Given the complexity of the crosslinked samples, techniques such as fractionation and long liquid chromatography (LC) gradients are typically employed to enhance the identification of crosslinked peptides, though these methods can lead to low throughput. The Orbitrap Astral Zoom MS offers high scan rates of up to 270 Hz and faster stepped collision energy (SCE), making it ideal for the detection of MS-cleavable crosslinked peptides at a proteome-wide scale.

In this study, we optimized XL-MS workflows for MS-cleavable crosslinkers (DSSO and DSBSO) using the Orbitrap Astral Zoom MS. With these optimized methods, we evaluated the sensitivity and throughput of the new instrument for analyzing crosslinked peptides derived from purified *E. coli* ribosomes and 64 human protein mix.[4]

Experimental

Methods and materials	
Samples	*E. coli* ribosome (NEB, Cat. No. P0763S), 64 protein mix provided by Dr. Fan Liu
Crosslinkers	DSSO (Cat. No. A33543), DSBSO
Enrichment and desalting	DBCO Magnetic beads Thermo Scientific™ Pierce™ Peptide Desalting Spin Columns (Cat. No. 89852)
Protein and peptide concentration assay	Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Cat. No. 23225) Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (Cat. No. 23290)
Column	Thermo Scientific™ EASY-Spray™ HPLC column, 75 µm x 25 cm (Cat. No. ES902)
Column temp (°C)	40
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in 80% acetonitrile
Mass spectrometer	Orbitrap Astral Zoom mass spectrometer Thermo Scientific™ FAIMS Pro Duo interface
Liquid chromatography	Thermo Scientific™ Vanquish™ Neo UHPLC system
Data analysis	Thermo Scientific™ Proteome Discoverer™ 3.2 software and XlinkX node 3.2

Separation conditions

Retention (min)	Flow (nL/min)	%B
0	250	4
46	250	25
56	250	40
57	350	99
66	350	99

Mass spectrometry nanospray conditions

Parameter	MS
Spray voltage (V)	1,800
Sweep gas (Arb)	0
Ion transfer tube temp. (°C)	275
FAIMS mode	Standard resolution
Total carrier gas flow (L/min)	3.9
FAIMS CV (V)	–48, –60, –70

MS conditions

Settings	DSSO	DSBSO
MS1 scan	Orbitrap	Orbitrap
Resolution	180K	180K
Normalized AGC Target (%)	500%	500%
Max. injection time (ms)	5	5
Charge state	3-8	3-8
MS mass range, *m/z*	400–1,400	400–1,400
RF lens	40	40
MS2 scan	Astral	Astral
Normalized AGC Target (%)	200%	500%
Max. injection time (ms)	20	20
Charge states	3-8	3-8
First mass (*m/z*)	150	150
Intensity threshold	$1e^4$	$1e^4$
Isolation width	1.6	1.6
NCE (%)	SCE 25, 32	32(z=3) +28(z>3)
Top N/Top speed	Top N = 25	Top speed = 2s

Sample preparation

E. coli ribosomal proteins and 64 human protein mix were prepared as described.[4] Samples were buffer exchanged into PBS, pH 7.0 using a 10 kDa centrifugal filter unit. DSSO and DSBSO (Figure 1) solutions in DMSO were used to crosslink 8 mg/mL ribosomal proteins for 1 hour at 100-fold molar excess of crosslinking reagents. After crosslinking, reactions were quenched with 30 mM ammonium bicarbonate, pH 8.0, for 15 minutes. The sample was reduced using 10 mM dithiothreitol at 37°C for 30 minutes and alkylated with 55 mM 2-chloroacetamide at 37°C for 30 minutes. The samples were diluted to 2M Urea

with 50 mM triethylamonium bicarbonate and digested with trypsin (1:50 ratio [enzyme to protein]) over night. For DSBSO crosslinked samples, DBCO magnetic beads were added for enrichment (7.5 µL beads for 100 µg peptide) and the mixture was incubated for 2 hours, 37°C, 1,200 rpm shaking. The samples were then washed with 0.5% sodium dodecyl sulfate (SDS) followed by 8 M urea and subsequently with water. Finally, 2% trifluoroacetic acid was added to the sample and the mixture was incubated for 2 hours at room temperature at 1,000 rpm. The crosslinked sample was eluted from beads and desalted with Peptide Desalting Spin Column according to the manufacturer's instructions. Protein and peptide concentrations were determined using the Pierce BCA Protein assay and the Pierce Quantitative Fluorometric Peptide assay, respectively.

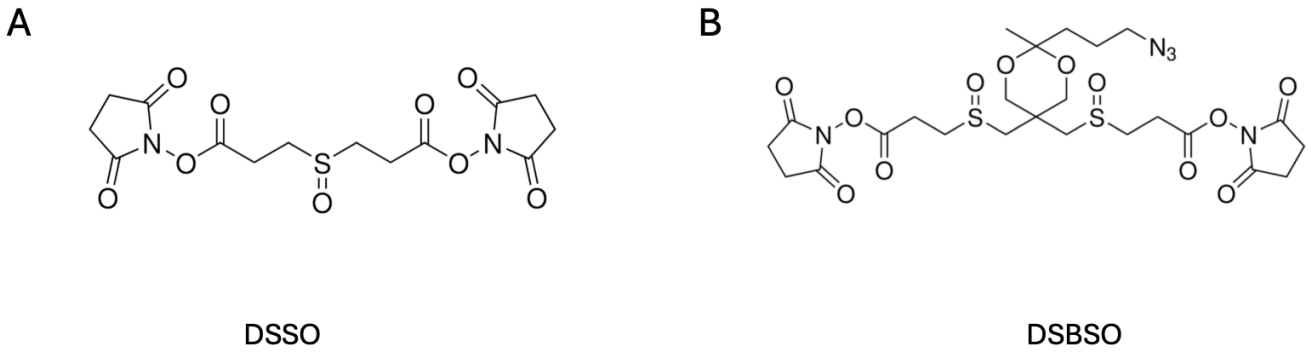


Figure 1. Structures of MS-cleavable crosslinkers used in the study (A) DSSO, (B) DSBSO.

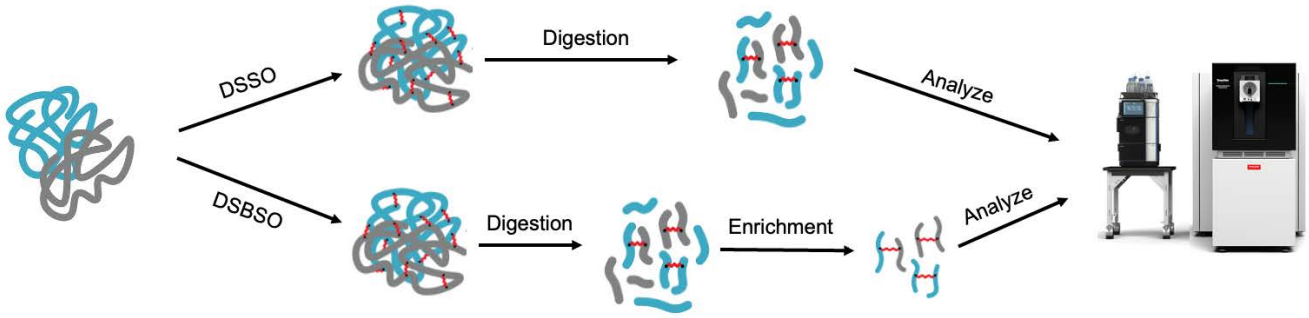


Figure 2. Crosslinking workflows for DSSO and DSBSO.

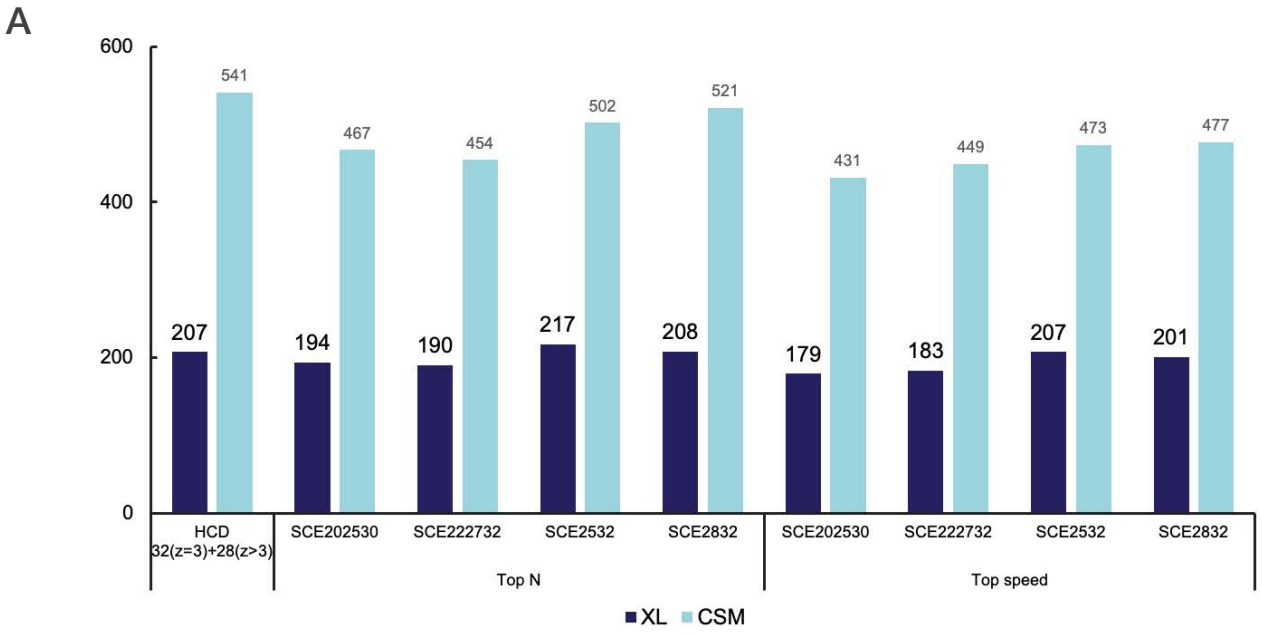
Data analysis

Spectral raw data files were analyzed using Proteome Discoverer 3.2 software with XlinkX node 3.2—the XL-open search algorithm for crosslinked peptides and SEQUEST™ HT search engine for unmodified peptides and loop-links/mono-links. MS1 ion mass tolerance: 10 ppm; MS2 ion mass tolerance: 20 ppm. Maximal number of missed cleavages: 2; minimum peptide length: 6; max. modifications: 4; peptide mass: 500–8,000 Da. Carbamidomethylation (+57.021 Da) of cysteines was used as a static modification. DSSO or DSBSO cross-linked mass modifications for lysine, the protein N-terminus and methionine oxidation (+15.995 Da) were used as variable modifications. Data was searched for cross-links against an *E coli.* ribosome protein database or a database containing 64 human proteins. The false discovery rate (FDR) was set to 1% at CSM and crosslink levels. The maximum XlinkX score was set to be greater or equal to 30. Identified XLs were visualized using xiVIEW[5].

Results and discussion

Method optimization for DSSO and DSBSO crosslinked samples

SCE is a widely utilized technique for identifying crosslinks, especially those involving cleavable crosslinkers.[6] This technique optimizes the fragmentation process and maximizes the detection of fragment ions in the MS2 spectrum. The Orbitrap Astral Zoom MS offers high scan rates up to 270 Hz, along with faster SCE capabilities, achieving maximum scan rates of 230 Hz for two-step experiments and 180 Hz for three-step experiments. To optimize mass spectrometry methods for DSSO and DSBSO, we conducted tests using various settings, including top speed and top N methods with four different SCE combinations: SCE 20-25-30, SCE 22-27-32, SCE 25-32, and SCE 28-32. Furthermore, we compared these methods to a previously developed approach that employed different normalized collision energies (NCEs) based on charge states (HCD 32 for z=3 and HCD 28 for z=4-8). The results of our experiments are summarized in the figures below.



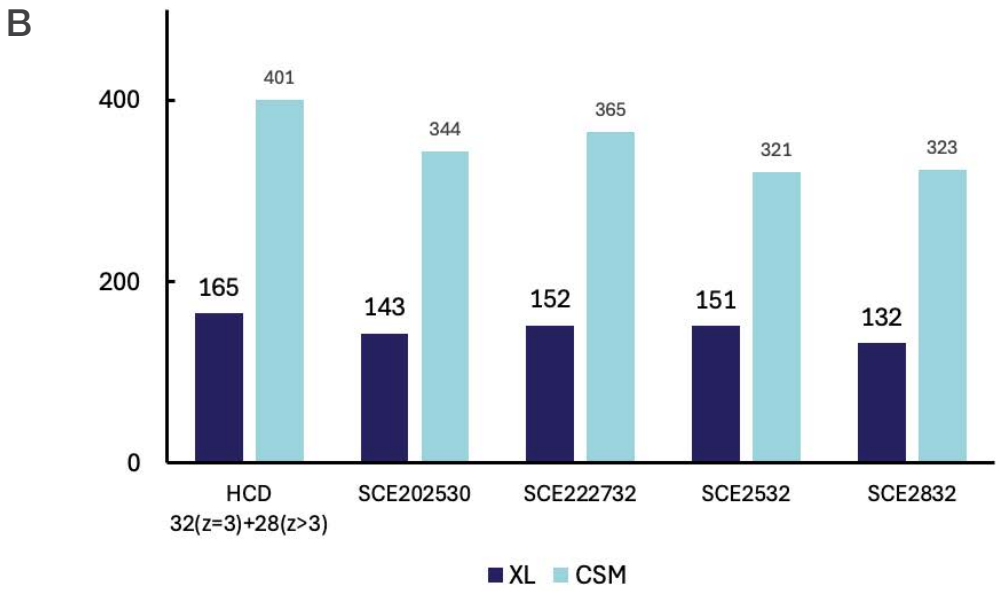


Figure 3. MS method optimization on the Orbitrap Astral Zoom MS using (A) DSSO or (B) DSBSO crosslinked *E. coli* ribosome proteins.

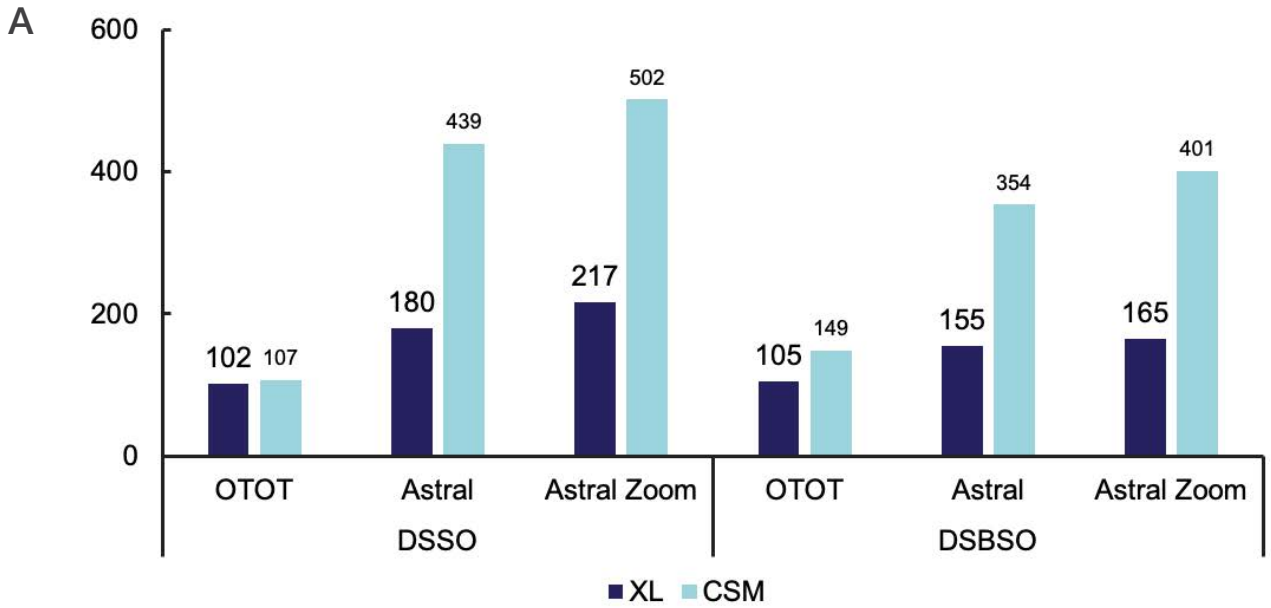
Figure 3A shows the results from DSSO crosslinked *E. coli* ribosome proteins. Top N SCE methods provide 5% more crosslinks than top speed methods on average. The two-step SCE methods resulted in 10% more crosslink identifications than the three-step SCE for top N methods. This trend was consistent across both top N methods and top speed methods. Overall, the two-step SCE 25-32 method provided the highest number of crosslinks for DSSO crosslinked samples, which also outperformed the two charge states method.

We further evaluated MS methods for DSBSO crosslinked *E. coli* ribosome samples. As shown in Figure 3B, the results showed slightly different trends: the two-step SCE 25-32 method yielded a similar number of crosslinks compared to the three-step SCE 22-27-32 method (151 vs. 152 crosslinks). Furthermore, when we compared these results to the two-charge states method, the two-charge states method outperformed the SCE methods, producing 8.5% more crosslinks and 9.8% more CSMs. These findings indicate that crosslinkers behave differently during the collisional processes in MS2.

Improved PPI analysis and throughput using the Orbitrap Astral Zoom MS

We compared the performance of the Orbitrap Astral Zoom MS with the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer using either the Orbitrap or Astral mass analyzer for MS2 with DSSO or DSBSO crosslinked *E. coli* ribosome proteins. As illustrated in Figure 4A, the Orbitrap Astral Zoom MS outperformed the Orbitrap Astral MS using Astral or Orbitrap (OT) MS2 methods. Specifically, compared to the OTOT (OT MS1/OT MS2) data dependent acquisition (DDA) method, the Orbitrap Astral Zoom MS achieved 113% more crosslink identifications for DSSO (217 vs. 102 crosslinks) and a 57% increase for DSBSO (165 vs 105 crosslinks), along with a fourfold increase in the number of CSMs for both samples. When comparing the Orbitrap Astral Zoom MS to the Orbitrap Astral MS, we observe a 21% increase in crosslinks for DSSO and a 6.5% increase for DSBSO, as well as a 14% increase in CSMs on average.

The increase in crosslink identifications on the Orbitrap Astral Zoom MS primarily results from inter-protein crosslinks. For example, the number of DSSO intra-protein crosslinks is comparable between the Orbitrap Astral Zoom MS and the Orbitrap Astral MS, with 125 and 121 intralinks, respectively. However, there is a significant 56% increase in inter-protein crosslinks on the Orbitrap Astral Zoom MS, with 92 interlinks compared to 59 on the Orbitrap Astral MS. The overall mapping of identified crosslinked sites in the *E. coli* ribosome proteins using the Orbitrap Astral MS and the Orbitrap Astral Zoom MS is illustrated in Figures 4B and 4C. Clearly, the Orbitrap Astral Zoom MS provides more detailed insights into PPIs, as indicated by the green lines in the circular diagrams.



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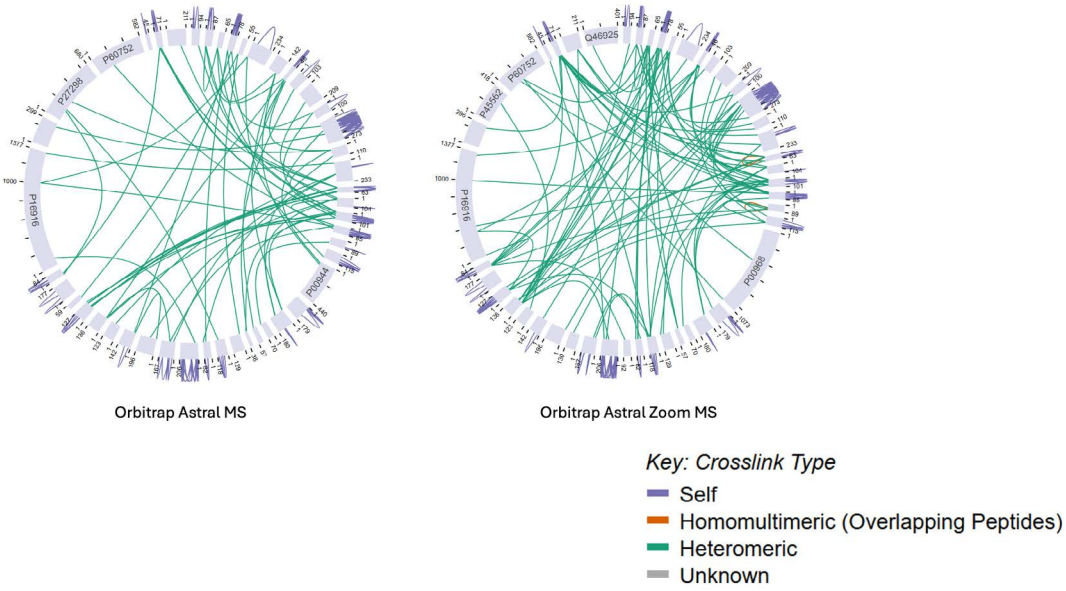
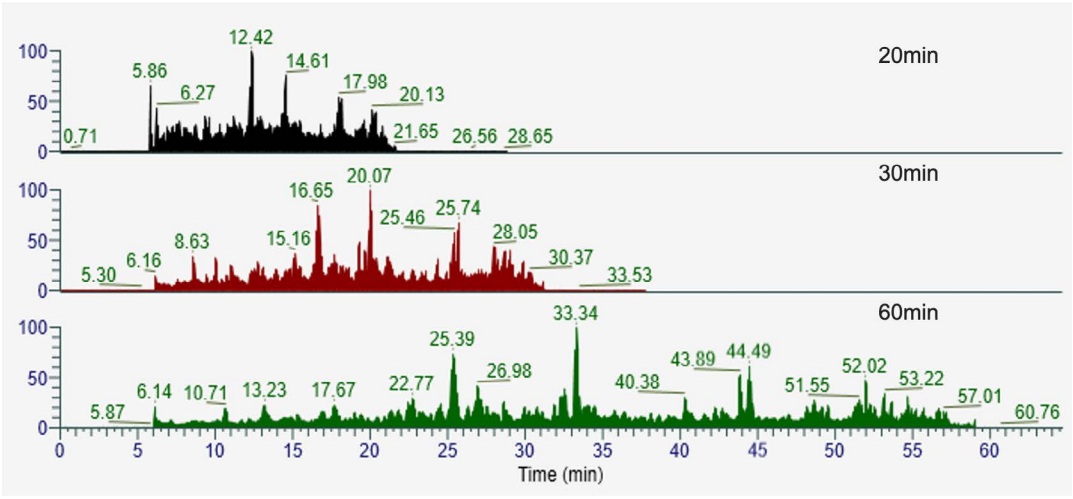


Figure 4. (A) Identified crosslinks and CSMs from DSSO or DSBSO crosslinked *E. coli* ribosome. (B) Identified DSSO crosslinking sites in *E. coli* ribosome proteins are presented as circular plot with xiVIEW[5].

To further evaluate the throughput of the Orbitrap Astral Zoom MS, we tested 20-, 30-, and 60-minute gradients with DSSO crosslinked *E. coli* ribosome proteins, and the LC profiles are illustrated in Figure 5A. As demonstrated in Figure 5B, both the Orbitrap Astral MS and the Orbitrap Astral Zoom MS methods outperformed the OT-OT acquisition across all three gradient lengths, achieving 68% to 100% more crosslink identifications. When using the 20-minute gradient, the Orbitrap Astral MS and the Orbitrap Astral Zoom MS yielded similar results: 121 and 125 crosslinks, respectively, along with 241 and 279 CSMs. These results are approximately double those obtained from the OTOT run. In the 30- and 60-minute gradients, the Orbitrap Astral Zoom MS method surpassed the Orbitrap Astral MS by 18% and 21% in terms of crosslink detections, and by 29% and 14% in CSM identifications, respectively. Notably, when comparing the 20-minute and 60-minute gradients, the results from the 20-minute gradient (121 and 125 crosslinks) for both the Orbitrap Astral MS and the Orbitrap Astral Zoom MS were significantly better than from the OTOT 60-minute gradient method, which identified only 102 crosslinks. This demonstrates that the shorter gradient method using the Astral analyzer offers three times the throughput for crosslinking analysis.

A



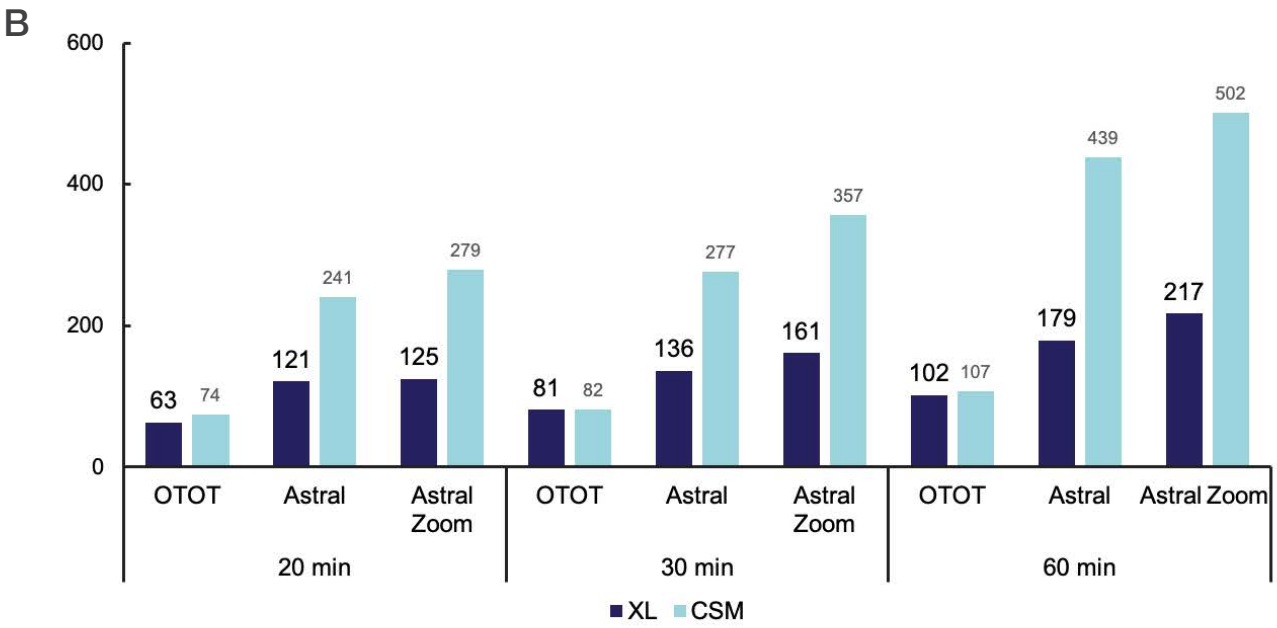


Figure 5. (A) LC profiles of *E. coli* ribosome DSSO samples using 20-, 30-, or 60-min gradients. (B) Unique crosslinks and number of CSMs identified using different gradients.

Improved sensitivity and spectrum quality using the Orbitrap Astral Zoom MS

To evaluate the sensitivity of the Orbitrap Astral Zoom MS, we spiked a protein mix containing 64 human proteins that were crosslinked with DSBSO into DSBSO-crosslinked *E. coli* cells at various ratios. The FAIMS 3 CV method was used for data collection. Figure 6 shows the number of identified DSBSO crosslinks from human protein mix.

Across all mixing conditions, the Orbitrap Astral Zoom MS consistently outperformed the Orbitrap Astral MS, utilizing either data dependent Astral MS2 or OT MS2 methods. This difference became more pronounced at lower ratios. At a 1:1 ratio, the Orbitrap Astral Zoom MS identified a comparable number of crosslinks as the Orbitrap Astral MS (216 vs 207 crosslinks); however, it detected 53% more crosslinks at a 1:10 ratio (25 vs 15 crosslinks). Importantly, with the optimized method, we were able to identify crosslinks from the human protein mix down to a ratio of 1:50, resulting in the detection of four times more crosslinks. It is worth mentioning that the number of identified *E. coli* background crosslinks remained consistent across all ratios.

Another advantage of the Orbitrap Astral Zoom MS is the improved spectrum quality. Figure 7 compares the the same DSBSO crosslink (AVSAV*K*NMNLPEIPR-LE*K*SEAGLAGAPAR) obtained from OT MS2 and Astral MS2 spectra. While the OT MS2 spectrum offered better signal intensity for the diagnostic peaks from DSBSO (indicated by the blue and red peaks at the center of the spectrum), the Astral MS2 spectrum provided a higher number of sequence ions for data analysis through the XlinkX node. Consequently, the XlinkX score increased from 79.91 to 101.33, and the sequence coverage improved from 0.71 and 0.73 to 0.86 and 0.93 for the alpha and beta chains of the crosslinked peptide, respectively.

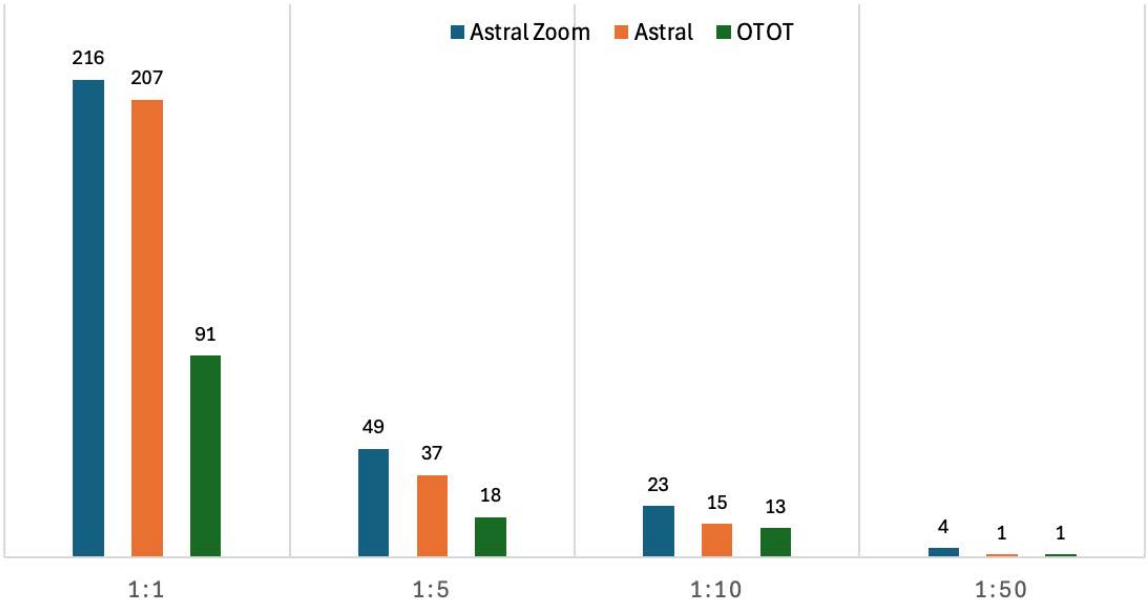


Figure 6. Number of identified DSBSO crosslinks from the 64 human proteins spiked into different ratios of *E. coli* crosslinks.

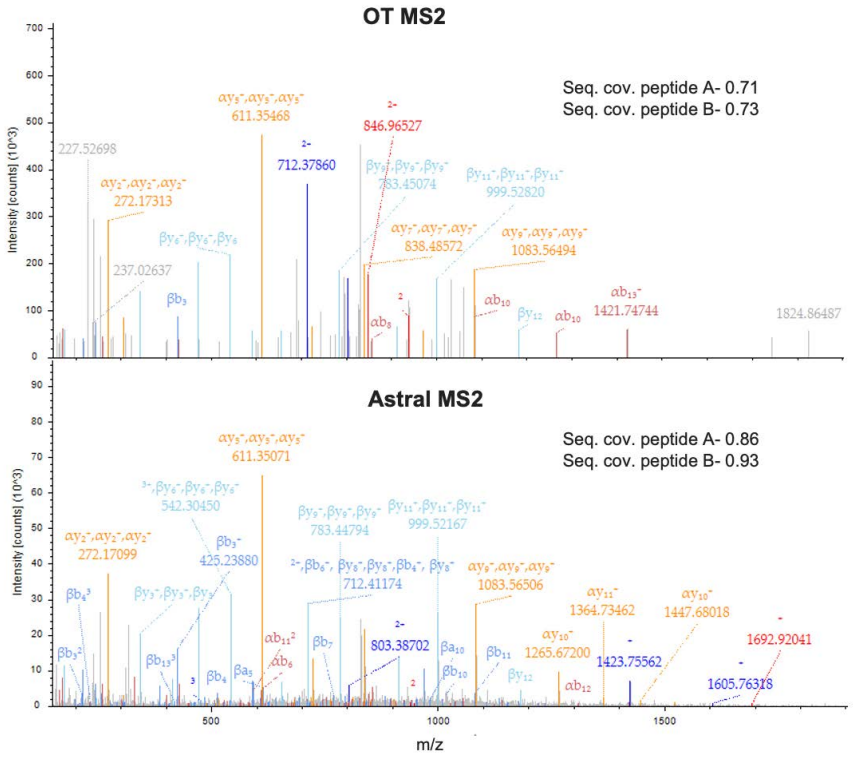


Figure 7. Comparison of OT and Astral MS2 spectra of DSBSO crosslinked peptide AVSAV*K*NMNLPEIPR-LE*K*SEAGLAGAPAR.

Conclusions

- XL-MS workflow was benchmarked on the new Orbitrap Astral Zoom mass spectrometer for MS-cleavable crosslinkers DSSO and DSBSO.
- Utilizing the fast stepped collision energy and scan rate of the Orbitrap Astral Zoom MS, the same number of crosslinks was identified using 2x shorter acquisition time vs OTOT method.
- Higher sequence coverage was observed for crosslinked peptides using OT MS1 / Astral MS2 data-dependent acquisition method leading to high-confidence scores of identifications, similar to or better than OTOT method.

Acknowledgements

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