Comparison of XL-MS Software to Identify Protein Interactions

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Cross-linking mass spectrometry (XL-MS) is an emerging method in structural biology not only for structural elucidation of protein complexes but also for determination of protein-protein interactions. Thus, it is commonly integrated into conventional structural biology techniques, such as Cryo-EM, NMR, and X-Ray crystallography. XL-MS could be applied to large and dynamic complexes since it is not restricted by sample preparation requirements, and it could capture proteins from their native environment providing information about the interactions and physical contacts of them. Cross-linking proteins in their native environment provides a snapshot of the biological systems by binding proteins in close proximity via covalent bonds. These cross-linked proteins are then digested with trypsin and analyzed by high-resolution mass spectrometer. The raw MS data containing both precursor (MS) and fragment (MS/MS) ions are analyzed through cross-linking software to identify proteins and interaction points. Here, we investigated three commonly used softwares, MeroX, MaxLynx, and XiSEARCH, using a bacterial membrane protein complex consisting of FtsH, HflK and HflC proteins. Recombinant expression of protein complexes was performed in *E. coli*, inner membranes were solubilized, and proteins were purified using affinity chromatography. Cross-linking reactions were carried out with both purified protein complexes and solubilized membranes using two different cross linkers, MS-cleavable cross-linker DSBU and non-cleavable cross-linker BS3. The MS analysis was conducted using high-resolution Thermo Scientific Lumos Orbitrap MS. The software were compared based on the number of inter- and intraprotein interactions yielded, processing time, and ease of use. Additionally, the data obtained from each software were verified by examining on raw MS data, PDB, AlphaFold and PyMOL. The most interactions were obtained in XiSEARCH (311/436 in DSBU-linked sample), and the least were obtained in MaxLynx (57/436 in DSBU-linked sample). The processing time of MeroX was the shortest, whereas the processing time of MaxLynx was the longest. MeroX provided a user-friendly interface visualizing MS/MS spectrum that could be used to verify interactions. Although MS/MS spectrum is presented in MaxLynx, it could not be shown in the graphical user interface preventing us from the verification of interactions. In addition, BS3, non-cleavable cross-linker, analysis could be completed on only MeroX whereas MaxLynx and XiSEARCH failed during analysis due to high system requirements. Since the runs were not completed during the analysis with MaxLynx and XiSEARCH for BS3, a non-cleavable cross-linker and a cleavable cross-linker could not be

compared in terms of hit numbers but system requirements. Our findings will guide researchers in identifying protein interactions using XL-MS.

Table. Number of hits obtained in various processing times* from MeroX (less than 1 minute), MaxLynx (in 50 minutes) and XiSEARCH (in 40 minutes).

NUMBER OF HITS					
INTRAPROTEIN INTERACTIONS		**All**		**Verified**	
		BS3-Linked Sample	**DSBU-Linked Sample**	**BS3-Linked Sample**	**DSBU-Linked Sample**
	MeroX	69	51	50	43
	MaxLynx	Not applicable	42	-	-
	XiSEARCH	Not applicable	255	-	5
INTERPROTEIN INTERACTIONS		**All**		**Verified**	
		BS3-Linked Sample	**DSBU-Linked Sample**	**BS3-Linked Sample**	**DSBU-Linked Sample**
	MeroX	28	17	25	16
	MaxLynx	Not applicable	15	-	-
	XiSEARCH	Not applicable	56	-	1

* Total time passes from beginning to the end of analysis.