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A high-throughput, in-depth investigation into the immunological effects of lipopolysaccharide challenged mice in neat plasma on Orbitrap Astral Mass Spectrometer

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Abstract

Purpose: A Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer was optimized for high-throughput and depth of coverage yielded >3600 protein groups in LPS challenged neat mouse plasma.

Methods: Neat plasma from Lipopolysaccharide challenged mice was digested with Lyc/trypsin on the automated Thermo Scientific™ AccelerOme™ platform.

Results: Over 3600 protein groups and 25400 peptides were detected with the 20SPD method searched with DIANN with no library applied. This was over 40% and 86% improvement over the 60SPD and 180SPD method, respectively.

Introduction

Lipopolysaccharide (LPS) inhalation stimulates an immune response in mice and serves as a preclinical model for investigating potential therapeutics for inflammatory related disorders such as acute bronchitis. Here mice were treated and challenged with LPS and compared to healthy, unchallenged mice to gain insights into immunological response and identify key down regulated and upregulated proteins of importance.

Traditionally, neat plasma requires long runtimes of over 1hr in order to achieve the depth of coverage needed for meaningful insights by mass spectrometry. Here the Orbitrap Astral mass spectrometer was optimized and assessed for high-throughput analysis while still maintaining depth of coverage. Three gradients were run for comparison: 20 SPD, 60 SPD and 180 SPD.

Materials and methods

Sample preparation

Neat plasma from five biological replicates of LPS challenged and unchallenged mice were prepared by automated sample preparation on the AccelerOme platform with LysC/trypsin digestion (Figure 1.)

LCMS/MS method

Prepared samples were separated by Thermo Scientific™ Vanquish™ Neo™ and analyzed by the Orbitrap Astral mass spectrometer (Table 1).

The LC separation was performed by reverse phase gradient with Ionopticks Aurora™ column and the Thermo Scientific™ EASY-Spray™ PepMap™ columns for the various sample throughputs of 20SPD, 60SPD, and 180SPD. This was performed in order to compare depth of coverage and quantitative accuracy (Figure 1). Three technical replicates were acquired to assess instrument reproducibility. A gas phase fractionation library was created for the 60SPD method and used to search the data for maximum protein identification.

20 SPD

Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
0.000	Run				
0.000	0.000	0.400	5.0	0.00	0.00
0.500	0.500	0.400	6.0	0.20	0.11
2.000	1.500	0.400	8.0	0.60	0.34
3.000	1.000	0.250	8.5	0.33	0.18
48.000	45.000	0.250	28.0	11.25	6.33
61.000	13.000	0.250	50.0	3.25	1.83
61.000	Column Wash				
64.000	3.000	0.250	99.0	0.75	0.42
68.000	4.000	0.250	99.0	1.00	0.56
68.000	Stop Run				
68.000	Column Equilibration				

60 SPD

Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
0.000	Run				
0.000	0.000	0.400	1.0	0.00	0.00
0.100	0.100	0.400	4.0	0.04	0.05
1.900	1.800	0.400	12.0	0.72	0.97
2.000	0.100	0.200		0.03	0.04
12.000	10.000	0.200	22.5	2.00	2.70
19.500	7.500	0.200	40.0	1.50	2.03
19.500	Column Wash				
22.000	2.500	0.300	99.0	0.63	0.84
25.000	3.000	0.300	99.0	0.90	1.22
25.000	Stop Run				
25.000	Column Equilibration				

180 SPD

Time	Duration [min]	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
0.000	Run				
0.000	0.000	2.500	4.0	0.00	0.00
0.200	0.200	2.500	8.0	0.50	0.28
4.000	3.800	2.500	20.0	9.50	5.35
5.800	1.800	2.500	35.0	4.50	2.53
5.800	Column Wash				
6.200	0.400	2.500	99.0	1.00	0.56
6.900	0.700	2.500	99.0	1.75	0.99
6.900	Stop Run				
6.900	Column Equilibration				

Figure 1. Liquid chromatography methods for 20 SPD, 60 SPD, and 180 SPD methods by Vanquish Neo UHPLC system.

MS Parameter		MS Method
MS1	Resolution	240K
	m/z Range	380-980
	AGC	500%
	Max-IT	5 ms
MS2	Detector	Astral
	m/z Range	150-2000
	AGC	500%
	Isolation Window	3Da

Table 1. Mass Spectrometer method used for analysis on Orbitrap Astral MS.

Sample Type	DIA Experiments	Number of Reps	Amount Injected
Control Samples 1-5	20 SPD, 60 SPD, 180 SPD	5 biological, 3 technical	1 µg, 500 ng, 250 ng
LPS Challenged 6-10	20 SPD, 60 SPD, 180 SPD	5 biological	1 µg, 500 ng, 250 ng
Pool Sample	60 SPD DIA GPF	1 total	500 ng

Table 2. Summary of LC-MS/MS experiments for data collection. Both control and LPS challenged samples were analyzed by all three SPD methods. Whereas a pooled sample of control and challenged plasma was used to create a gas phase fractionation library to improve ID results for the 60 SPD method.

Figure 2. End-to-end workflow used for sample preparation to data analysis.

Data analysis

Data collected was analyzed with Thermo Scientific™ Proteome Discoverer™ 3.1 software, DIA-NN, and Spectronaut® 18 software for comparison.

Results

Over 3600 protein groups and 25400 peptides were detected with the 20SPD method searched with DIANN with no library applied (Figure 3A). This was over 40% and 86% improvement over the 60SPD and 180SPD method, respectively. Results showed over 93% quantifiable IDs and median coefficient of variance (CV) below 10 % for technical replicates for the different run times (Figure 3B).

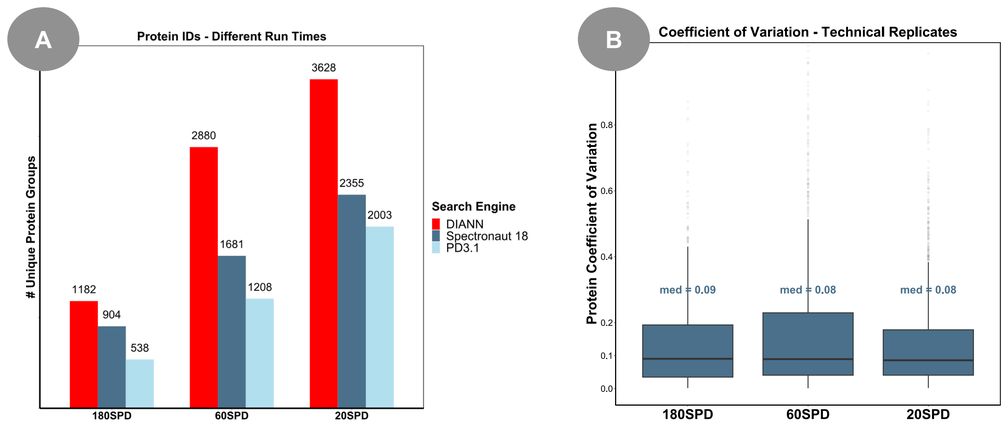

Figure 3. A) Protein identification for different run times. Results were analyzed by DIANN, Spectronaut 18, and Proteome Discoverer 3.1 software for 180 SPD, 60 SPD, and 20 SPD. The 20 SPD yielded > 20 % protein IDs versus the 60 SPD method at 3628 protein IDs versus 2880 protein IDs respectively. B) Coefficient of variation for the different runtimes. Precise quantitation was achieved with median CVs < 10 % for technical replicates and > 93 % protein IDs were quantifiable.

Gas Phase Fractionation Library

The gas phase fractionation library for 60SPD was used to process results and yielded a 44 % improvement from 1681 protein IDs to 2427 protein IDs while still maintaining quantitative accuracy (Figure 4).

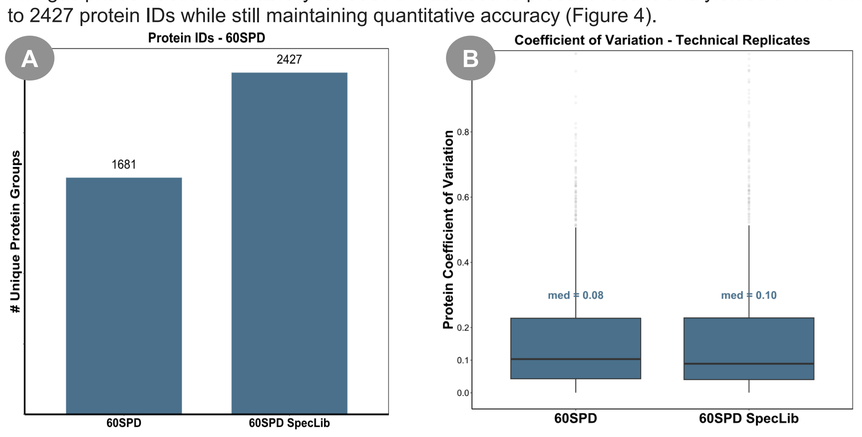

Figure 4.
A) Protein identification for the 60 SPD method when using a gas phase fractionation library. B) Coefficient of variation for the technical replicates for the 60 SPD with and without the gas phase library remained consistent.

LPS Challenged Mice

The five biological replicates for the LPS challenged mice versus the unchallenged mice yielded 2169 and 1914 protein IDs respectively and were reproducible and robust across all five biological replicates (Figure 5). The LPS challenged results for the 60SPD method were 76 % quantifiable in all five replicates and 91 % quantifiable in three out of five replicates. Proteins were ranked on a ranked protein list and spanned nearly six order of magnitude (Figure 6) and clustered on a PCA plot to show the differences observed between the two treated and untreated groups (Figure 7). Next, differential expression was performed with a p-value set to < 0.001 and +/- 2 Log2FC as cut-off, revealing 35 proteins upregulated and two downregulated proteins in LPS challenged plasma versus unchallenged plasma (Figure 8) and heat map (Figure 9).

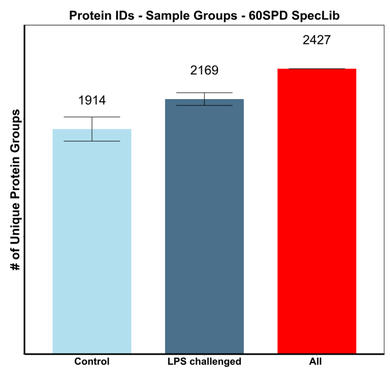

Figure 5. Protein identification for LPS challenged mice versus control mice using the 60 SPD method with gas phase fractionation library. Total number of proteins identified in either control or LPS challenged was then combined for a total of 2427 total proteins.

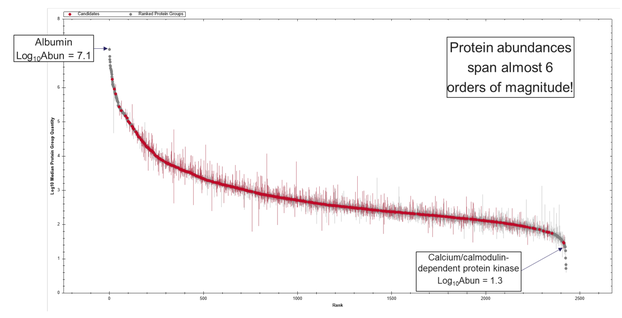

Figure 6. Protein abundance for the plasma analyzed spanned almost six orders of magnitude on the Orbitrap Astral mass spectrometer.

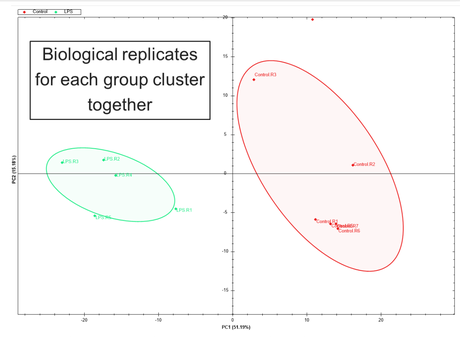

Figure 7. PCA plot for control (red) versus control (green) groups cluster together further depicting the differences in the proteins present in these two groups for LPS challenged versus unchallenged mice.

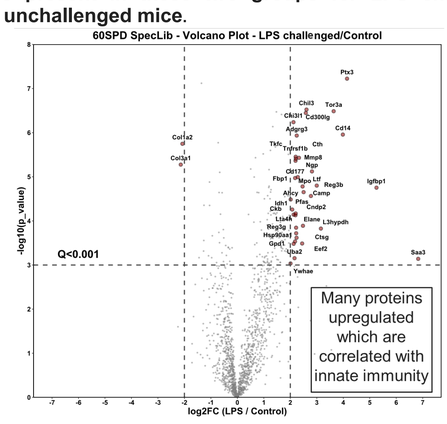

Figure 8. Volcano plot for the proteins identified in LPS challenged versus unchallenged, control mice using the 60 SPD method with gas phase fractionation library. Significantly upregulated proteins are highlighted and correlated with innate immunity.

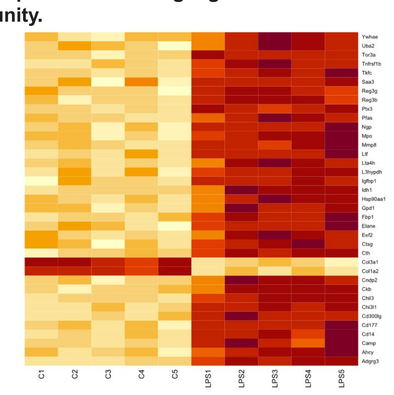

Figure 9. Heat map for control versus LPS challenged mice depicts the protein level differences between the two groups in proteins identified.

Conclusions

- Orbitrap Astral MS was utilized to analyze LPS challenged versus control samples to detect protein differences at 20 SPD, 60 SPD, and 180 SPD methods.
- Each throughput method provided at minimum 1500 protein IDs and up to 3628 protein IDs reported in plasma with varying gradient lengths.
- Varying the gradient and processing the data with a gas phase fractionation library significantly improved results when applied to the 60 SPD method.
- All and all, Orbitrap Astral MS identified over 3600 unique protein groups with over 25K peptides for neat plasma in a 60min run.
- Gas phase fractionated library can boost protein IDs by 44%.
- Precise quantitation for technical replicates for all run times with median CVs below 10%. Quantitation is paramount given the need for accurate, reproducible biomarker detection for Translational Research applications.

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