Enhanced sensitivity of the Orbitrap Astral Zoom mass spectrometer for deeper proteome coverage in single-cell proteomics applications

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Abstract

Purpose: To demonstrate the performance of the Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer for low load and single cell analysis.

Methods:

- Separation was performed on the Thermo Scientific™ Vanquish™ Neo UHPLC system using an Aurora C18 column.
- MS and MS/MS acquisition was done on the Orbitrap Astral Zoom MS operating in DIA mode.
- Data analysis was performed using Thermo Scientific™ Proteome Discoverer™ software and Spectronaut 19® without using a spectral library.

Results:

- From K562 digest dilution, we identified >5,200 protein groups for 50 pg and >6,100 protein groups for 250 pg at a throughput of 50 SPD
- We identified 3,700 protein groups at 120SPD and >5,600 protein groups at 50SPD from 50 pg HeLa digest.
- We identified > 5,100 protein groups at 120SPD and >6,800 protein groups at 50SPD from 250 pg HeLa digest.
- From HEK single cells, an average of over 5,800 protein groups were identified at a throughput of 50 SPD using a library-free approach.

Introduction

Contrary to traditional proteomics application using LC-MS/MS, where millions of cells are simultaneously analyzed and the observed changes are presented as a cumulative response of all cells analyzed, single-cell proteomics (SCP) analysis allows for a more detailed investigation of the changes in protein compositions and functions at the single cell level. However, to confidently characterize these changes in proteins, their functions and the diversities, many individual single cells need to be analysed. Working with individual cells raises challenges not only due to the limited sample amount, but also related to sample preparation, throughput and depth of coverage. Therefore, workflows that provide the highest sensitivity, highest proteome coverage and highest throughput would be of great interest to single cell proteomics applications. Here we evaluated the depth of proteome coverage and throughput that can be achieved for low-input and single cell samples using the Orbitrap Astral Zoom mass spectrometer.

Materials and methods

Sample Preparation

Thermo Scientific™ Pierce™ HeLa digest or K562 digest (Promega) were reconstituted in 0.015% Dodecyl β D maltoside (DDM) 0.1%TFA solution and sonication for approx. 5 min to obtain a stock solution of 100 ng/µL. 5 µL of the stock solution was then diluted to 5 ng/µL in 0.015% DDM into an Eppendorf 96 low binding autosampler well plate and vortexed at 2,000 rpm for a few seconds. The autosampler was then used to create different amounts by injecting different volumes from the 5 ng/µL sample solution.

HEK293F cells were FACs sorted on a Sony MA900 cell sorter into a 384-well Eppendorf LoBind PCR plate with 1 µl of lysis buffer (80 mM Triethylammonium bicarbonate (TEAB) pH 8.5, 20% 2,2,2-Trifluoroethanol (TFE)). After the sorting, the plate was placed on dry ice for 5 min and stored at -80 °C until further analysis. The plate was thawed and heated to 95 °C on a PCR machine for 5 min followed by another freezing cycle. 2 ng trypsin (Promega Platinum) were added to each cell. The samples were incubated at 37 °C overnight and quenched with 1 µL 1 % TFA.

LC-MS Method

The samples (HeLa digest, K562 digest, and HEK single cell digests) were loaded onto either an Aurora Ultimate 25 cm XT C18 column or Aurora Rapid 8 cm column (IonOpticks) and separated using a Vanquish Neo UHPLC system configured in direct injection mode. Samples were separated using different gradients (see Figure 1). The eluting peptides were analyzed on the Orbitrap Astral Zoom mass spectrometer (detailed acquisition parameters are shown in figure 2) in the 'Low Input' application mode with a Thermo Scientific™ FAIMS Pro Duo interface using a data-independent acquisition method. The FAIMS CV was set to -48 and the FAIMS carrier gas was set to 3.5 L/min.

Data Analysis

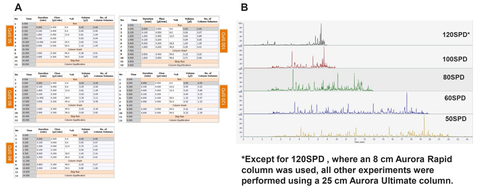
Proteome Discoverer software

Each raw file obtained from the different sample amounts was processed using Proteome Discoverer software with CHIMERYS intelligent search algorithm. The data was searched against a human protein database containing 20,563 sequences. Oxidation of methionine was selected as variable modification and carbamidomethylation of cysteine as static modification. False-discovery rate (FDR) of 1% was applied at the precursor, peptide, and protein level.

Spectronaut 19 software

Data analysis was performed in Spectronaut™ 19.6 software using the directDIA™ workflow, the search was performed with default settings against the Human UniProt protein database (20,607 FASTA entries), except that Quantitation > Quantity MS level was set to "MS1", and Post analysis > Differential Abundance Grouping > Use All MS-Level Quantities checkbox was set to unchecked. False-discovery rate (FDR) of 1% was applied at the precursor, peptide, and protein level.

Figure 1. A) LC methods. All methods were evaluated with 250 pg HeLa digest. The samples per day (SPD) were calculated based on a 1 µL injection on column and injection-to-injection. Note that the SPD will change with higher injection volume as it takes longer to load. B) BPCs for the corresponding LC methods.



*Except for 120SPD , where an 8 cm Aurora Rapid column was used, all other experiments were performed using a 25 cm Aurora Ultimate column.

Figure 2. Orbitrap Astral Zoom MS acquisition parameters. The FAIMS CV and carrier gas are not fixed parameters, thus must be optimized prior to data acquisition. Recommended isolation widths for different concentration and true single cell experiments.

Source Properties	
Spray Voltage	1.9kV
Capillary Tube Temperature	275
*FAIMS CV	-48
*FAIMS carrier gas	3.5
Orbitrap full scan Properties	
Orbitrap Resolution	240000
Scan range	400-800
FAIMS CV	-48
Normalized AGC Target(%)	500
Max injection time (ms)	100
FAIMS carrier gas (L/min)	3.8

Data-Independent Acquisition Properties	
Precursor Mass Range (m/z)	400-800
Isolation width	See table below
Window placement	On
NCE (%)	25
Scan Range (m/z)	150-2000
AGC Target (%)	800
Loop Control	time
Time (sec)	0.6

Isolation width [Th]	Injection time [ms]	Concentration
5	10	5-50pg
8	14	2-5ng
10	20	1-2ng
20	40	250-500pg
20	60	< 250pg and single cells

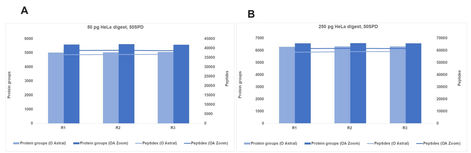
Results

In contrast to Orbitrap Astral MS, due to improved ion optics settling times and faster ion transfer, the Orbitrap Astral Zoom MS can achieve acquisition speeds of up to 270 Hz (35 % faster). It also comes with enhanced spectral processing capabilities, higher sensitivity by ion pre-accumulation in the bent trap and a 'Low Input' application mode with which the single ion detection probability is increased by 10%.

Comparison Orbitrap Astral MS vs Orbitrap Astral Zoom MS

To assess the performance difference between the new Orbitrap Astral Zoom MS and Orbitrap Astral MS, 50 pg and 250 pg bulk HeLa digest were analyzed. The Orbitrap Astral Zoom MS was operated in the 'Low Input' application mode using DIA. Spectronaut results in Figure 3 A and B show that the Orbitrap Astral Zoom MS identified approximately 5 % more protein groups for 250 pg HeLa and 10 % more protein groups for 50 pg HeLa at a throughput of 50SPD. The results from Proteome Discoverer software show similar gain in protein groups, 10.8 % for 50 pg and 4.4 % for 250 pg.

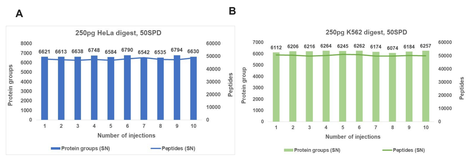
Figure 3. Direct comparison of the Orbitrap Astral MS and Orbitrap Astral Zoom MS using 50 pg and 250 pg HeLa digest. Triplicate raw files for each sample amount were processed together in Spectronaut v19.



Repeatability

We then looked at the repeatability of injections using 250 pg K562 and HeLa digest, and we consistently identified more than 6,100 protein groups for the K562 digest and more than 6,500 protein groups for the HeLa digest over the course of the measurement of 10 + 10 technical replicates as shown in Figure 4 A and B.

Figure 4 demonstrates the high repeatability of injections over the course of half a day. The raw files for each cell line digests were processed using the method evaluation functionality in Spectronaut.



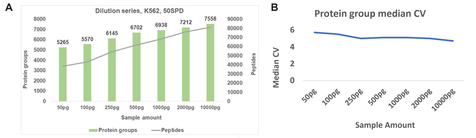
Dilution series

Next, we injected different volumes from the 5ng/ul stock to create a dilution series for both HeLa (50-2000pg) and K562 (50-10000pg) digests. Three raw files from each amount were processed together without using a library. The results showed that more then 85 % of all the identified protein groups in both K562 and HeLa dilution series had CVs below 20 %. In addition, the protein group median CV for all sample amounts were below 6 %.

Dilution series bulk K562 digest

More than 38,000 peptides were matched to over 5,200 protein groups from as low as 50 pg K562 digest and over 6,100 protein groups from 250 pg. For 10 ng K562 digest, the numbers increased to above 81,000 peptides that were matched to more than 7,500 protein groups (see Figure 5).

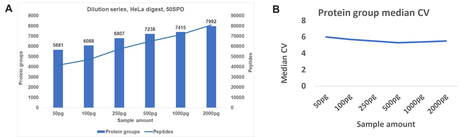
Figure 5. K562 dilution series results for 50SPD using Spectronaut 19.6 and library-free directDIA search. DIA isolation width and max injection time were varied depending on the sample amount. A) Bulk K562 digest, triplicate runs for each sample amount were processed separately. B) Median protein group %CVs for K562 dilution series.



Dilution series Bulk Pierce HeLa

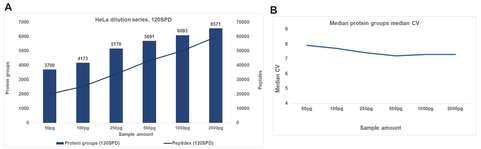
More than 5,600 protein groups were identified from 50pg HeLa digest and over 6,700 from 250 pg. These numbers increased to about 8,000 protein groups for 2 ng sample amount (see Figure 6).

Figure 6. HeLa dilution series results for 50SPD using Spectronaut 19.6 and library-free directDIA search. DIA isolation width and max injection time were varied depending on the sample amount. A) Bulk HeLa digest, triplicate runs for each sample amount were processed separately. B) Median protein group % CVs for HeLa dilution series.



Furthermore, we measured dilution series of Hela digest using 120 SPD DIA method and an 8 cm Aurora Rapid C18 column. The number of protein groups identified was 3,700 for 50 pg HeLa digest and 5,170 for 250 pg HeLa digest. The protein group median CV obtained using this method was below 8 %.

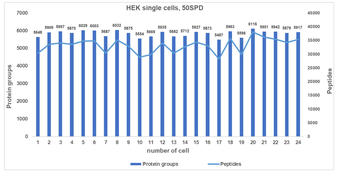
Figure 7A. Dilution series results for 120 SPD method using bulk HeLa digest and an 8 cm Aurora Rapid C18 column. Three raw files from each amount were processed together without using a library. B) Median protein group % CVs for HeLa dilution series.



HEK single cells

Finally, we analyzed HEK single cells using 50 SPD method. All the raw files for each throughput were processed in Spectronaut v19.6 without using a library. On average, > 5,800 protein groups were identified using 50 SPD method.

Figure 7: Spectronaut v19 results for HEK single cells analyzed using 50 SPD method. Raw files were processed together in Spectronaut without library.



Conclusions

- We demonstrated that for 50 and 250pg HeLa digest the Orbitrap Astral Zoom MS showed about 10% and 5% gain in protein groups IDs compared to the Thermo Scientific™ Orbitrap™ Astral™ MS.
- From 50 pg HeLa digest, we identified about 3,700 protein groups at a throughput of 120 SPD and more than 5,600 protein groups at 50 SPD
- More than 5,200 protein groups were identified from 50 pg K562 and more than 6,100 protein groups from 250 pg K562 with a median protein group CV of <6% for three technical replicates at 50 SPD.
- More than 6,800 protein groups were identified from 250 pg HeLa with median protein group CV of <5% for three technical replicates at 50 SPD.
- We demonstrated the repeatability of our method by injecting 250 pg HeLa and K562 digest for more than half a day.
- On average we identified more than 5,800 protein groups from HEK single cells using 50 SPD method and a library-free approach.

References

1. Tabiwang et al. Deeper proteome coverage and faster throughput for single-cell samples on the Orbitrap Astral mass spectrometer, **Technical note 002780.**

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Conflict of interest

All authors are employees of Thermo Fisher Scientific, the manufacturer of instrumentation used in this study.

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