P-I-0264

Single cells on Orbitrap MS

Label-free DIA-based workflow for single-cell proteomic analysis on an Orbitrap Ascend Tribrid mass spectrometer

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Abstract

Purpose: Demonstrate the performance of Thermo Scientific™ Orbitrap™ Ascend™ Tribrid mass spectrometer (MS) and Thermo Scientific™ Vanquish™ Neo UHPLC system in the analysis of low input and single cell samples.

Methods: A label-free DIA-based method that gives a throughput of 50 samples per day (SPD) is demonstrated with low input samples (HeLa peptides) and single cell samples.

Results: Our results show that Orbitrap Ascend Tribrid MS coupled to Vanquish Neo UHPLC has the single cell sensitivity.

Introduction

Recent advances in LC-MS have enabled label-free single-cell proteomics analysis revealing unexpected functional diversity of cells. However, there are still key challenges, such as sensitivity, coverage, dynamic range, and throughput. To address some of these challenges, new method developments, as well optimization on existing LC-MS-based proteomics workflows are necessary. Here, we demonstrate the use of Orbitrap Ascend Tribrid mass spectrometer and the Vanquish Neo UHPLC system for high-throughput single cell applications.

Materials and methods

Sample preparation

All proteomics experiments were performed using the Thermo Scientific™ Pierce™ HeLa protein digest standard. 200 µL of resuspension buffer (0.015% DDM prepared in 0.1% formic acid) was added to the vial containing 20 µg of protein digest. The vial was then sonicated at room temperature for 5 minutes, making a final concentration of 100 ng/µL. To the autosampler vial, 95 µL of resuspension buffer and 5 µL of 100 ng/µL HeLa digest were added to make the final concentration 5 ng/µL. This solution was vortexed for 30 s. All injections were done from the same vial.

HeLa cells were sorted and prepared using CellenONE™ and proteoChip™ LF 48 and transferred manually to the wells of a 384 well-plate.

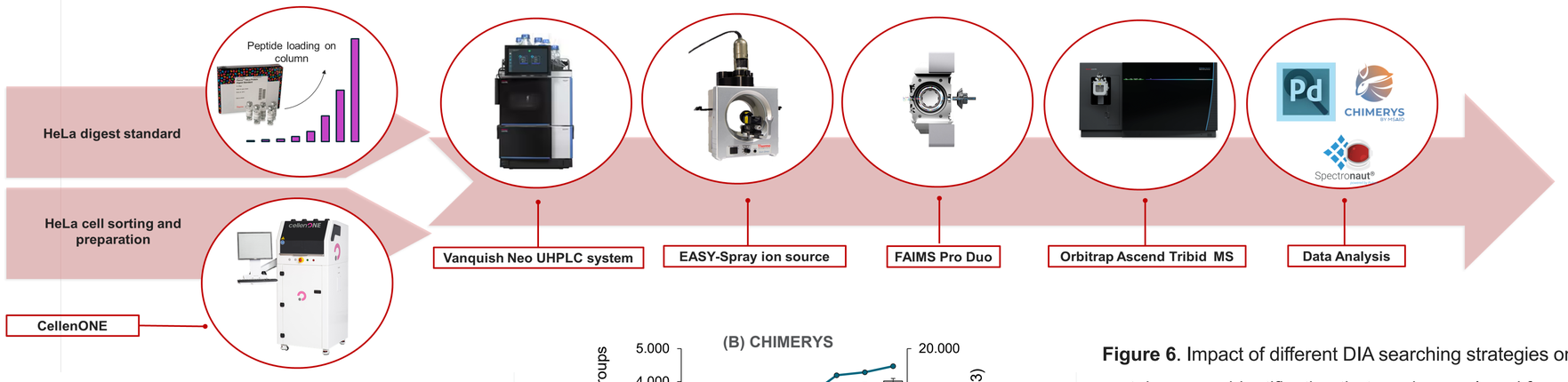
LC-MS/MS method

Peptides were separated on a Vanquish Neo UHPLC System using Aurora UltiMate column (25 cmx 75µm) Total run time was 25 min, corresponding to 50 samples per day (SPD). Thermo Scientific™ EASY-Spray™ Ion Source was used coupled to the Thermo Scientific™ FAIMS Pro™ Duo interface. Peptides were analyzed by the Orbitrap Ascend Tribrid mass spectrometer.

Data processing

Data were searched using Thermo Scientific™ Proteome Discoverer™ software with CHIMERYS™ intelligent search algorithm by MSAID and Spectronaut™ 18 software. Library-based searches were performed on Spectronaut 18 software. Different DIA libraries were built with Spectronaut™ Pulsar.

Figure 1. Experimental set-up from sample preparation to data analysis. This workflow combines the high sensitivity and throughput offered by the Vanquish Neo UHPLC system with low flow rates and fast sample loading with the speed of Orbitrap Ascend Tribrid associated with FAIMS interface for higher sensitivity.



Library-free searches were performed using Homo sapiens database from Uniprot (~20k entries). MBR was allowed in both software. All data reported used1% FDR.

Table 1. LC-MS settings for the 50 SPD method gradient.

Time (min)	Duration (min)	%B	Flow rate (µL/min)
Run			
0.0	0.0	1.0	0.45
0.1	0.1	4.0	0.45
1.9	1.8	12.0	0.45
2.0	0.1	12.0	0.20
12.0	10.0	22.5	0.20
19.5	7.5	40.0	0.20
Column wash			
22.0	2.5	99.0	0.3
25.0	3.0	99.0	0.3

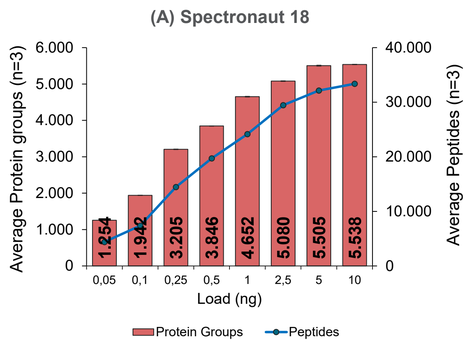
Table 2. MS settings (Runsheng et al.).

	Property	Setting
Source	Spray Voltage	1.9 KV
	Capillary Temp	275
	FAIMs CV	-50
	Total carrier gas flow (L/min)	3.5
Full MS	Scan Range (m/z)	400-800
	Orbitrap Resolution	120000
	Max IT (ms)	Auto
	RF Lens (%)	45
	AGC Target (%)	300
DIA	Scan Range (m/z)	400-800
	Orbitrap Resolution	60000
	Isolation Window (m/z)	40 (>1ng load) or 50 (<1ng load)
	Number of Scan Events	10
	HCD Collision Energies	28
	Max IT (ms)	118
	AGC Target (%)	1000
	Loop Control	All

Results

Sensitivity at low load – HeLa peptides dilution

Figure 2. Dilution series experiments using the 50 SPD method and processed with different software using a library-free approach. Figure (A) shows the average number of protein groups and peptides identified using Spectronaut 18 software, while Figure (B) shows the average number of peptides and protein groups identified using Proteome Discoverer software with CHIMERYS.



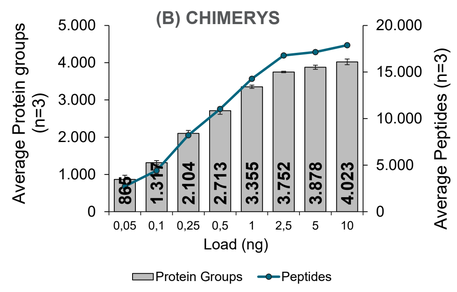
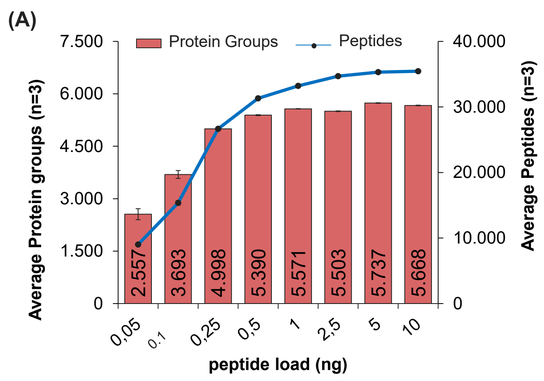
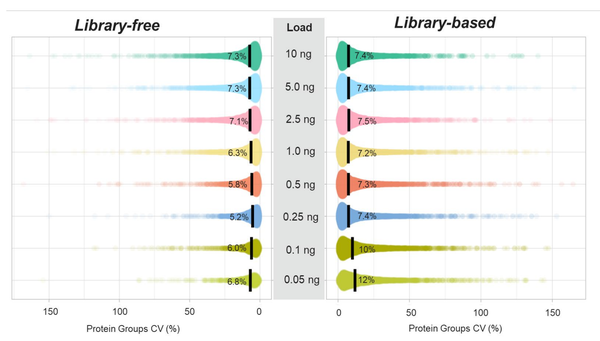


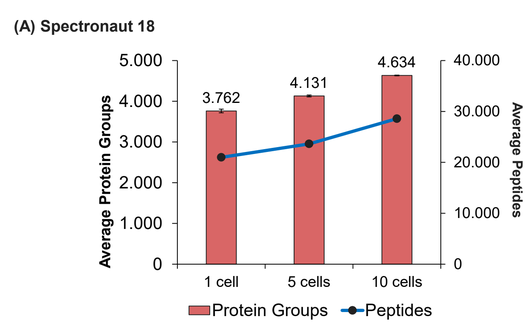
Figure 3. Effect of DIA library size on 250 pg HeLa protein digest standard (n = 3) runs **(A).** Files were searched against DIA libraries generated with varying amounts of HeLa digests on Spectronaut 18 software **(B).**





Sensitivity at single cell

Figure 5. Number of protein groups and peptides identified across single, 5 and 10 cells using the 50 SPD method and library-free approach. Replicates from the same load condition were searched together on Spectronaut 18 software **(A)** and Proteome Discoverer 3.1 software with CHIMERYS intelligent search algorithm **(B)**.



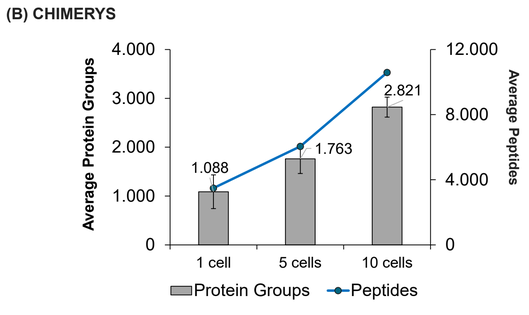
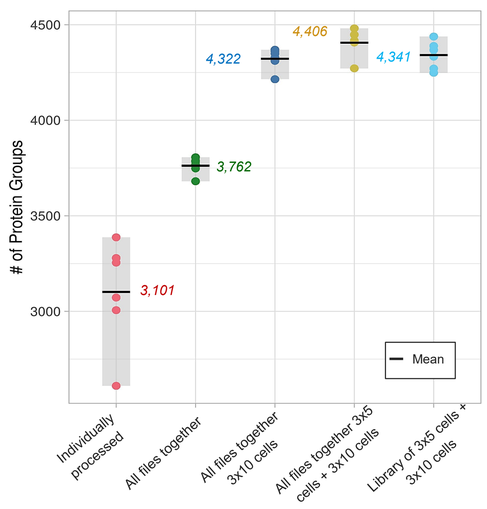


Figure 6. Impact of different DIA searching strategies on protein groups identification that can be employed for searching single cell datasets. Grey bars represent the range of observed data.



Conclusions

- The Orbitrap Ascend Tribrid MS coupled to Vanquish Neo delivers sensitivity and throughput desired for single-cell proteomics.
- DIA analyses provides depth of coverage and low CVs for low load and single-cell samples
- Library-based searches can improve IDs, but can also affect CVs

References

Runseng Z, Matzinger M, Mayer RL, Valenta A, Sun X, Mechtler K. *A High-Sensitivity Low-Nanoflow LC-MS Configuration for High-Throughput Sample-Limited Proteomics*. Anal. Chem. 2023, 95,51, 18673-78.

Acknowledgements

We would like to thank Dr. Anajli Seth and David Hartlmayr from Cellenion for supplying single cell sample plates. CellenONE and proteoChip LF 48 are trademarks from Cellenion.

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