Single-Cell Proteomics

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 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 system 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 MS 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® (Cellenion) and proteoChip LF 48 (Cellenion) 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 Ionopticks 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 (Biognosys). Library-free searches were performed using Homo sapiens database from Uniprot (~20k entries). MBR was allowed in both software. All data reported used1% FDR.
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 Tribid MS associated with FAIMS Pro Duo interface for higher sensitivity.

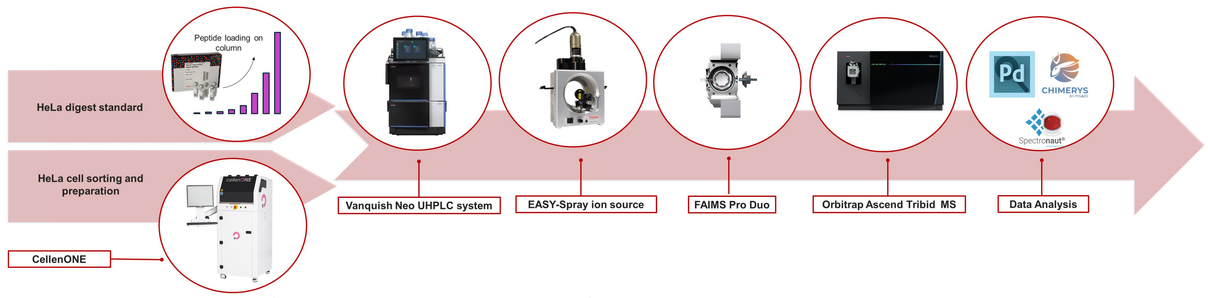


Figure 2. LC-MS settings. (A) 50 SPD method gradient. (B) Easy Spray ion source settings and MS settings used on Orbitrap Ascend Tribrid mass spectrometer.

(A)

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
Stop run			
Column equilibration			

(B)

Source parameters	
Spray voltage	1.9 kV
Capillary temperature	275° C
FAIMS CV	–50
Total carrier gas flow (L/min)	3.5

	Property	Setting
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 input samples – HeLa digest dilution

Data-Independent Acquisition (DIA) is becoming one of the most popular techniques for proteomics workflows. Figure 3 shows how we adapted the Velocity DIA workflow to test the dilution series from a HeLa standard digest. The biggest change from the Velocity DIA platform is the inclusion of the FAIMS Pro Duo interface. The addition can change any FAIMS capable Orbitrap mass spectrometer to handle limited amount or single-cell samples. This shows the flexibility of Orbitrap mass spectrometers to handle low load injections with minimal changes. We analyzed the dilution series using Proteome Discoverer 3.1 software with CHIMERYS algorithm and Spectronaut 18.0 software.

Figure 3. 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.

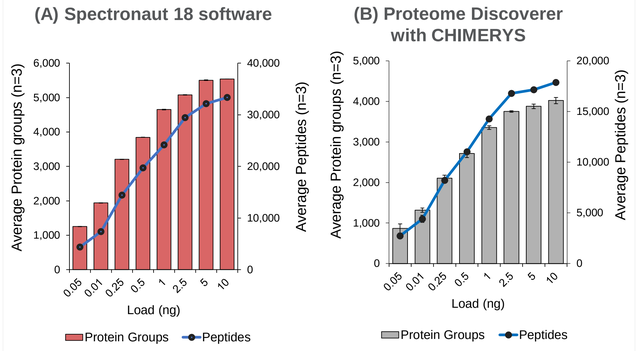
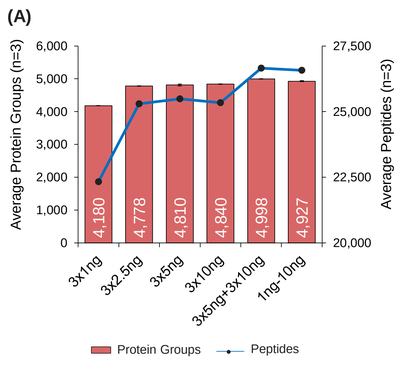


Figure 4. Effect of DIA library size on 250 pg HeLa protein digest standard (n = 3) runs (A). Files were searched against DIA libraries generated with varying amounts of HeLa digests on Spectronaut 18 software (B).



(B)

Library	Precursors
3x1ng	*25,710*
3x2.5ng	*31,582*
3x5ng	*34,584*
3x10ng	*36,059*
3x5ng + 3x10ng	*38,698*
1ng to 10 ng	*39,216*

Figure 5. Figure 4. The average number of protein groups and peptides identified using a 50 SPD method with a DIA library-based approach using Spectronaut 18 software.

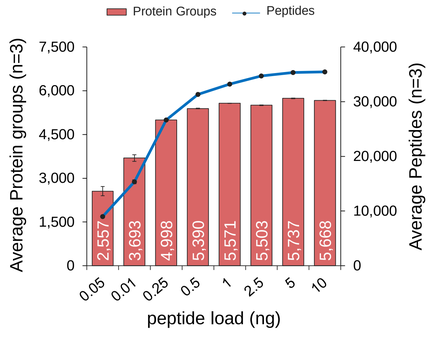
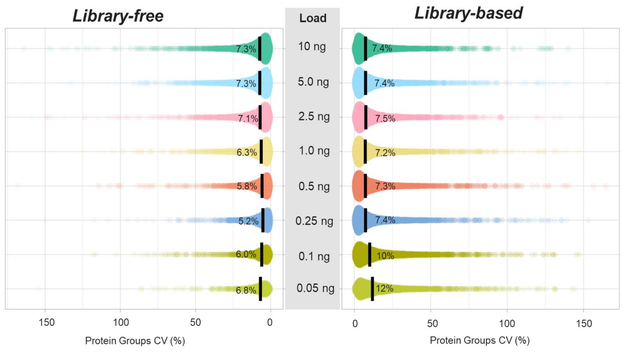


Figure 6. Violin plots indicate the %CV of protein groups identified for each dilution datapoint (n=3) of HeLa digest using library-free (on the left) and library-based searches (on the right). Black bars with numbers labeled in the figure represent the median %CVs for each load. Data processed in Spectronaut 18 software.



Sensitivity at single cell level – Individual HeLa cells

Figure 7. 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).

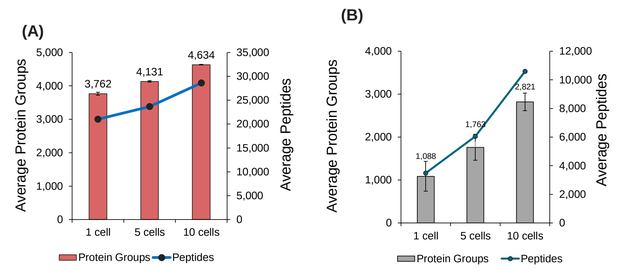
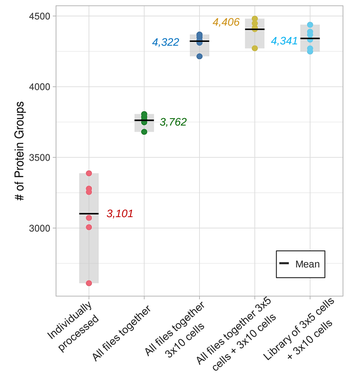


Figure 8. Impact of different DIA searching strategies on protein groups identification that can be employed for searching single cell datasets. Grey bars represent the range of observed data.



Conclusions

- The Orbitrap Ascend Tribrid MS with Vanquish Neo UHPLC system 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

Acknowledgements

We would like to thank Dr. Anajli Seth and David Hartlmayr from Cellenion for supplying single cell sample plates.

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