Proteomics

Balancing sensitivity and throughput in single-cell proteomics using low-nanoflow LC-MS

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Introduction

Nano-flow liquid chromatography-mass spectrometry (nLC-MS) is often utilized for bottom-up proteomics of extremely limited samples, down to individual cells. This is due to the increase in ionization efficiency achieved at LC flow rates ≤ 300 nL/min. Generating reproducible and timely results at ultra-low flow rates requires state-of-the-art LC instrumentation and optimization of LC-MS parameters, particularly with respect to the separation and electrospray ionization (ESI) interface.

Here we present 7 high-throughput nanoLC-MS methods for the bottom-up proteomics label-free quantitation (LFQ) analysis of limited sample types to balance sample throughput and proteome depth. Several data acquisition strategies were compared including data-dependent (DDA), wide-window (WW-DDA), and data-independent (DIA) acquisition. In addition, a modified trap-and-elute method was applied to single-cell proteomics (SCP) profiling for increasing sample throughput. Lastly, the impact of state-of-the art MS was explored for SCP.

Materials and methods

Sample Preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard was prepared in water (0.1% FA, v/v) at 1 ng/µL HeLa digest with 0.5 fmol/µL PRTC. Sample mass was varied by injecting volumes from 0.25 – 10 µL of HeLa digest onto the column. Single-cell samples were prepared in a 384 well plate using a label-free, one-pot workflow.[1]

Thermo Scientific™ TMTpro™ 18-plex labeled HeLa samples were prepared in-house by evenly mixing each channel, followed by dissolution and dilution in water (0.1% FA, v/v) prior to LC-MS analysis.

Instrument configuration

Method optimization and single-cell measurements were performed using a Thermo Scientific™ Vanquish™ Neo UHPLC system coupled to either a Thermo Scientific™ Orbitrap Exploris™ 480 or Thermo Scientific™ Orbitrap Astral™ mass spectrometer equipped with a Thermo Scientific™ FAIMS Pro interface. Samples were injected onto a Thermo Scientific™ Acclaim™ PepMap™ 100 C18 50 µm I.D. x 15 cm (2 µm dp, PN 164943) column and separated at 100 nL/min (**Figure 1**). Both direct injection and trap-and-elute workflows were explored with the aim of balancing sample throughput and sensitivity (**Table 1**). For the trap-and-elute workflow, a 300 µm x 5 mm trap column was operated in backward flush mode. Mobile phase A and weak wash liquid were water (0.1% FA) while mobile phase B and strong wash liquid were 80% acetonitrile (0.1% FA). The column was heated to 50 ℃ and the column outlet was connected to a 10 um µm I.D. x 5 cm emitter (Fossillion technology, LOTUS).

Figure 1. Optimized fluidic configuration for separations at 100 nL/min

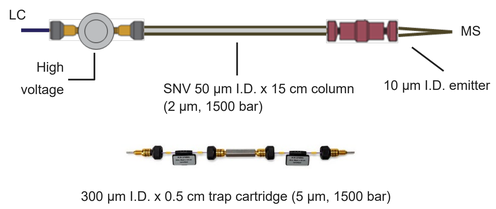


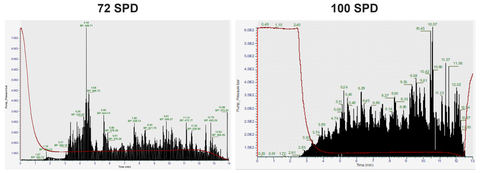
Table 1. Method details for the analysis of 250 pg of HeLa digest or single cells operated at 100 nL/min. Sample throughput ranged from 24-72 SPD for direct injection, with an increase up to 120 SPD using trap-and-elute (T&E). Sample injection/loading required 5.4 min of cycle time for DI and 1.4 min for T&E.

Samples/day (SPD)	Cycle time (min)	Elution Window (min)	Sample Injection & Loading (min)	MS Utilization (%)
120*	12	8	1.4	67
100*	14.4	10	1.4	70
72	20	11	5.4	55
60	24	15	5.4	63
48	30	21	5.4	70
36	40	31	5.4	78
24	60	51	5.4	85

* 100 and 120 SPD using trap-and-elute workflow

Figure 2 shows chromatograms for 250 pg HeLa digest using two of the nanoLC-MS methods operated at 100 nL/min: 72 SPD (direct injection) and 100 SPD (T&E). Fast sample loading, column washing, and equilibration enable 55% and 69% mass spectrometer utilization, respectively.

Figure 2 Total ion chromatograms and pressure traces for 250 pg HeLa digest using 20 (A) and 14.4 (B) min methods in the direct injection and T&E workflows, respectively. Both methods were operated at a separation flow rate of 100 nL/min.



Data Analysis

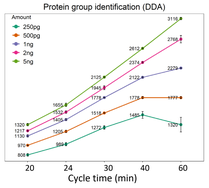
DDA and WW-DDA datasets were processed with Thermo Scientific™ Proteome Discoverer™ 2.5 software using a 2-step SEQUEST™ HT search algorithm and INFERYS™ rescoring node. DDA chimeric spectra were searched using the CHIMERYS™ algorithm in Proteome Discover 3.0 while DIA files were submitted to Spectronaut™ 17 (SN17) for peptide and protein ID and quan. The false discovery rates (FDR) were all set below 1% at both the peptide and the protein levels.

Results

New benchmark in LFQ-DDA

Using LFQ-DDA in the direct injection workflow with a 2-step SEQUEST search + INFERYS rescoring, a linear increase was observed in protein IDs from 250 pg to 5 ng HeLa digest (**Figure 3**), suggesting method suitability for sample-limited analysis (e.g., SCP). We confidently ID ~1,500 protein groups from 250 pg HeLa digest without match-between-runs, which to the authors knowledge, represent the most comprehensive DDA data to date.[2]

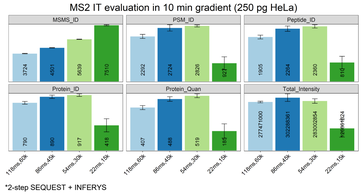
Figure 3. HeLa digest samples from 0.25 - 5 ng were analyzed in the direct injection workflow at 100 nL/min by varying the injection volume (n = 3). Mass spectrometer MS1 and MS2 resolution were set to 120K and 6K, respectively.



DDA scan speed & performance

Faster scan speed (30K resolution & 54 MS IT) increased 10-15% peptide and protein identification, enabling >900 protein group identifications from 250 pg HeLa samples in DDA mode. However, the fastest scan speed using 22 ms MS2 IT impacted the IDs negatively, where more MS2 did not translate into PSM and therefore, peptide and protein IDs (**Figure 4**).

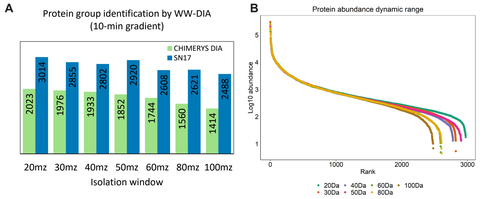
Figure 4. MS2 scan speed evaluation



LFQ WW-DIA performance

Evaluation of the MS1 open window in DIA yielded as much as 3,000 protein IDs from 250 pg HeLa digest using the 20 min method (**Figure 5A**), covering > 4 orders of magnitude of protein abundance dynamic range (**Figure 5B**).

Figure 5. Proteome coverage (A) and dynamic range (B) in the direct injection workflow using LFQ-DIA when varying the MS1 isolation window. SN17 was used for data analysis (n = 3).



Increasing sample throughput for LFQ-DIA profiling

To accelerate sample loading and eliminate the potential negative impact of impurities and detergent on electrospray ionization, we employed a trap column operated in a backward flush mode to maintain peak shape, successfully decreasing the method cycle time to 14.4 min (100 SPD) for a 10-min gradient at 100 nL/min (~70% MS utilization, **Figure 6**). Method performance enabled identification of >2,200 protein groups in 250 pg and >1,100 protein groups in a little as 60 pg.

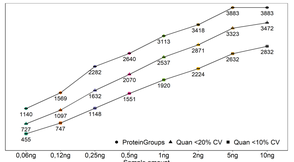
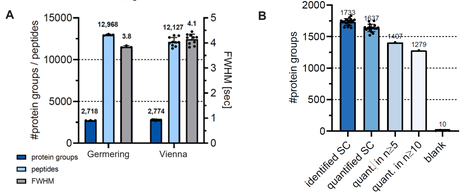


Figure 6. Proteome coverage in LFQ-DIA using a trap-and-elute workflow for increased sample throughput (100 SPD). Mass was varied from 0.06 - 10 ng by adjusting the injection volume (n=3).

LFQ-DIA profiling of single-cells

To validate the 100 SPD T&E workflow for single-cell proteomics, the method was applied to HeLa and K562 single-cell QC samples followed by individual HeLa and K562 cells. QC results were compared across multiple sites and provided >1,200 protein IDs despite multiple labs, LC-MS systems, operators, columns, etc. (**Figure 7A**). Performance was then demonstrated on 10 individual HeLa cells where ~1,700 proteins were identified per cell (**Figure 7B**).

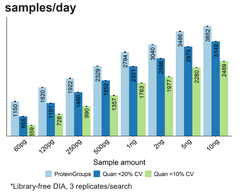
Figure 7. Evaluation SPD method for LFQ-DIA reproducibility for single-cell QC samples (A) and individual cells (B). For single cells, each raw file was searched individually.



Elevating throughput to 120 samples/day

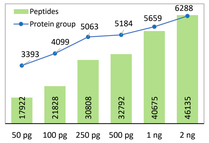
Furthermore, we successfully increased sample throughput by 20%, reaching 120 SPD (**Figure 8**). The higher throughput method resulted in ca. 16% decrease in protein group IDs compared to the 100 samples/day method (**Figure 7**). We anticipate that this new high throughput method will find applicability in single-cell analysis, particularly for larger cells with higher protein content.

Figure 8. Proteome coverage at 120 samples/day



*Library-free DIA, 3 replicates/search

Figure 9. Orbitrap Astral MS performance at 100 SPD



*Library-free DIA, 3 replicates/search

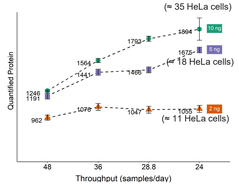
Impact of Orbitrap Astral on sensitivity

Using the Orbitrap Astral MS and a 75 µm i.d. pulled-tip column, we demonstrate high proteome coverage with >5,000 protein groups identified in 250 pg of HeLa digest at a throughput of 100 SPD (**Figure 9**). This demonstrates the advantage of combining low-flow UHPLC and state-of-the-art mass spectrometers.

Maximizing single-cell throughput

Using TMTpro 18-plex HeLa digest with SCoPE-MS in conjunction with the Vanquish Neo Tandem Direct Injection workflow, we were able to enhance throughput to >800 cells/day with over 1,100 protein IDs (**Figure 10**) with the potential to deliver as much as 1632 cells/day using TMTpro 35-plex.

Figure 10. Enhancing throughput using TMTpro 18-plex.



Conclusions

We developed a high-sensitivity and high-throughput nano-LCMS platform that affords 24-120 SPD with high protein coverage in both LFQ-DDA & DIA modes. Using a 20 min DI method we identified >900 protein groups (DDA +SEQUEST+INFERYS), >1,800 protein groups (DDA+CHIMERYS), and >2,600 (DIA+SN17). Throughput was enhanced by utilizing a T&E workflow for 100 and 120 SPD, which provided ca. 2,200 and 2000 protein groups, respectively. Using the 100 SPD method ~1,700 proteins were identified in individual HeLa cells. Using the Orbitrap Astral mass spectrometer achieved a depth of >5,000 proteins from 250 pg HeLa digest at 100 SPD throughput. Utilizing TMTpro 18-plex, the sample throughput was further extended to 864 cells/day with the potential to extend >1,600 cells/day with TMTpro 35-plex.

COI statement

RZ, AV, TA, XS, CP, EA, WD, DH, MS are employees of Thermo Fisher Scientific. The other authors declare no competing financial interests.

References

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2. Karel Stejskal, et al. Anal. Chem. 2021, 93, 25, 8704–8710
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