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# Label-free DIA-based workflow for single-cell proteomic analysis on an Orbitrap Exploris 480 mass spectrometer

Julia Kraegenbring[1], Fernanda Salvato[2], Bernard Delanghe[1], Tonya Pekar Hart[2], Julian Saba[3], David Hartlmayr[4], Anjali Seth[4], Amirmansoor Hakimi[2]
[1]Thermo Fisher Scientific, Bremen, Germany [2]Thermo Fisher Scientific San Jose, USA [3]Thermo Fisher Scientific, Winnipeg, MB, Canada [4]Cellenion, Lyon, France

## Abstract

**Purpose:** Optimizing gradients and mass spectrometric (MS) methods and demonstrating suitability of the workflow for real single cell experiments.

**Methods:** For workflow development, a 25 cm Aurora column on a Thermo Scientific™ Vanquish™ Neo HPLC coupled to an Orbitrap Exploris 480 mass spectrometer were used. Real single cell samples were prepared using a cellenONE® platform.

**Results:** A workflow catering to different needs in throughput was developed, yielding >1,500 identified protein groups from one single HeLa cell.

## Introduction

Recent advances in LC-MS have enabled label-free single-cell proteome analysis, revealing unexpected functional diversity in cells. However, there are still key challenges in this field, such as sensitivity, coverage, dynamic range, and throughput. To address some of these challenges, new method developments as well as optimization of existing LC-MS-based proteomics workflows are necessary. Here, we demonstrate the use of the Thermo Scientific™ Orbitrap™ Exploris 480 mass spectrometer and the Vanquish Neo UHPLC system for high-throughput single cell applications.

## Materials and methods

### Sample preparation

HeLa dilution series as a standard for workflow development were prepared from Thermo Scientific™ Pierce™ tryptic HeLa digest by dissolvation in 0.015 % n-dodecyl-β-maltoside (DDM) in 0.1 % formic acid, with peptide loads on column of 50 pg up to 10 ng. Individual HeLa cells were sorted, followed by reduction, alkylation, and trypsin digestion using CellenONE as per the manufacturer's protocols (see Figure 1). Pierce HeLa digest was used for dilution series from 50 pg to 10 ng loaded on column.

### Method

Single cell digests and the diluted standard HeLa digest samples were analyzed using the Orbitrap Exploris 480 MS with the Thermo Scientific™ FAIMS Pro™ interface coupled to the Vanquish Neo UHPLC system configured as direct-injection setup. Separation was performed on a 25 cm Aurora Ultimate™ TS column (IonOpticks Pty Ltd.). Source parameters, including FAIMS carrier gas and compensation voltage (CV), were optimized individually for both gradients prior to experiments using the same setup and 250 pg HeLa load on column. Method and LC parameters are shown in Tables 1 to 2.

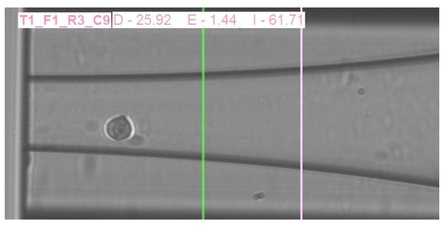



**Figure 1. Sample preparation was achieved with the Cellenion Cell sorting and digestion workflow**

Example of isolated cell from CellenONE report for one of the analyzed HeLa cells.

**Table 1. Gradient design for two different throughput methods on Vanquish Neo LC system.**

| 48 Samples per Day | | | | 82 Samples Per Day | | | |
|---|---|---|---|---|---|---|---|
| Time | Duration [min] | Flow [µL7min] | %B | Time | Duration [min] | Flow [µL/min] | %B |
| **0.00** | 0.00 | 0.60 | 1.0 | **0.00** | 0.00 | 0.50 | 1.0 |
| **0.10** | 0.10 | 0.60 | 7.0 | **0.10** | 0.10 | 0.50 | 7.0 |
| **3.10** | 3.00 | 0.60 | 12.0 | **1.60** | 3.00 | 0.50 | 12.0 |
| **3.20** | 0.10 | 0.30 | 12.0 | **1.70** | 0.10 | 0.20 | 12.0 |
| **15.20** | 12.00 | 0.30 | 22.5 | **9.70** | 8.00 | 0.20 | 22.5 |
| **19.50** | 4.30 | 0.30 | 40.0 | **11.20** | 4.30 | 0.20 | 40.0 |
| Column Wash | | | | | | | |
| **21.50** | 2.00 | 0.450 | 99.0 | **12.00** | 0.30 | 0.50 | 99.0 |
| **24.00** | 2.50 | 0.450 | 99.0 | **14.00** | 0.50 | 0.50 | 99.0 |
| Column Equilibration | | | | | | | |

**Table 2. Method settings for single cell analysis on Orbitrap Exploris 480 Mass Spectrometer.**

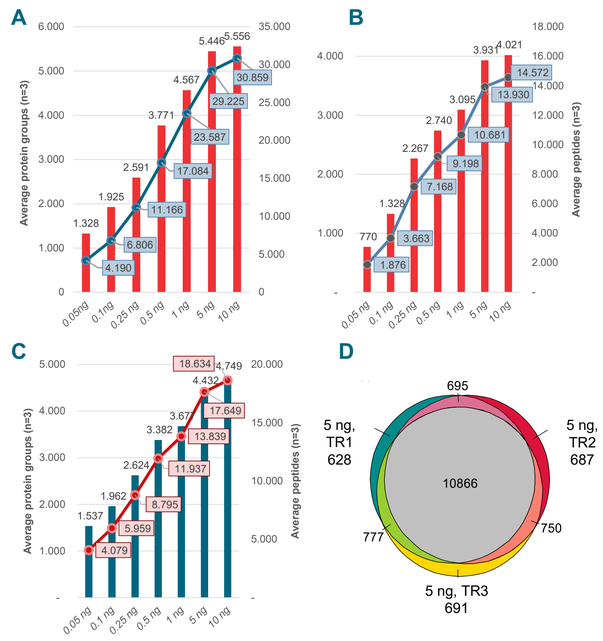
| Global Parameters | | Scan Parameters | | | |
|---|---|---|---|---|---|
| Settings | | Full MS scan | | DIA scan | |
| *Method Duration* | 14 / 24 min | *Resolution* | 120k | *Resolution* | 60k |
| *Expected LC peak width* | 10 | *Scan Range* | *m/z* 400-800 | *Precursor Mass Range* | *m/z* 400-800 |
| *APD* | on | *AGC Target* | 300 % | *Isolation Window* | 50 Th |
| *Default Charge State* | 2 | *Inject Time* | Auto | *Window Overlap (Placement Optimization)* | 0 Th (on) |
| *Internal Mass Calibration* | off | *RF lens* | 45 % | *First Mass* | 120 |
| Ion Source Settings | | FAIMS settings | | *NCE* | 26/28 %* |
| Spray Voltage | 2100 V | *CV* | -50 V | *AGC Target* | 1000% |
| Transfer Tube Temperature | 280°C | *Carrier Gas* | 3.5 L/min | *Inject Time* | Auto |

### Data analysis

DIA data were analyzed using Spectronaut® 18 (Biognosys AG) and Thermo Scientific™ ProteomeDiscoverer™ 3.1 software using Chimerys, an intelligent search algorithm by MSAID, for spectrum-centric search. Both software suites were used without use of a library and false discovery rate (FDR) was controlled to 1% on experiment level.

**Figure 2. HeLa dilution series results demonstrate high sensitivity for potential single cell experiments.**

The 48 SPD data was analyzed using Spectronut 18 (A) and Chimerys (B).
(C) Identification data from 82 SPD method by Spectronaut 18.
(D) Venn diagram demonstrating the overlap of peptides identified by Proteome Discoverer software using Chimerys search algorithm in 48SPD method.




## Results

### Considerations for method optimization to low-input experiments

The goal of this study was the development of a robust workflow for Exploris 480 for single cell analysis. As a proxy for real single cells, HeLa dilution samples were used to optimize the methods. The liquid chromatography was set up for two different throughput levels: one method with 14 min and one with 24min total run time, including an active gradient of 11.2 min and 19.5 min, respectively. The flow rates were chosen in a way that both methods deliver similar peak widths, i.e. the shorter gradient used a lower flow rate during the separation of peptides than the longer gradient. The throughput of both methods was calculated by determining the time of injection to injection for 6 different runs and sample load volumes and taking an average, which led to empirical throughput of 48 samples per day (SPD) and 82 samples per day in a direct injection setup

Source parameters must be optimized for a given setup. In this case, the flow rate difference for both methods did not need to be accounted for by these parameters, because for both the same spray voltage, ion transfer tube temperature and FAIMS carrier gas and CV were optimal (data not shown). However, the shorter method benefitted from lowering the normalized collision energy from 28 % to 26 %.

A suitable method for analyzing single cells must yield a high proteome coverage while maintaining a reasonable throughput, since a typical clinical study to determine differences in cell species consists of several hundred, up to thousand, cells to be measured. However, for detecting meaningful differences between the samples, data must also be consistent and reproducible. To ensure a reasonable number of points across the peak, even with the chosen high resolution of Full MS and DIA scans, the measured mass range was reduced to *m/z* 400-800.

### Results of HeLa dilution series and performance of 48 SPD and 82 SPD methods

The performance of the methods was assessed by the number of identified proteins, but also by degree of inter-run variation and instrument response to different loads as determined from protein abundances after data analysis.

n Figure 2, the proteome coverage of both methods is shown for different peptide loads. Routinely, we were able to identify >2,500 protein groups and >10,000 peptide groups from three runs with 250 pg HeLa peptides with 48SPD workflow (A). Going for higher throughput (B), there is similar proteome coverage for loads up to 500 pg, but with significantly less peptides identified per protein. Going for higher loads, the difference in proteome coverage between the two methods become apparent, with up to 25 % less protein IDs with 82 SPD than with 48 SPD method.

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Comparing the peptides identified by Chimerys (C) within all three technical replicates for 48 SPD reveals a high overlap of sequences identified in all three technical replicates of 5 ng HeLa, pointing to high reproducibility of the method.

Similarly, two technical replicates of 5 ng HeLa and one replicate of 1 ng HeLa have an almost complete overlap, with only 300 sequences that were identified in 1 ng exclusively.

In assessment of quantitation performance, logarithmic ratios of peptide abundances of different HeLa loads are shown as box plots in Figure 3. Even for the largest ratio, abundance ratios align very well with the expected ratios, demonstrating high quantitation accuracy with the label-free DIA quantitation.

Similarly, coefficients of variation of peptide abundances over three technical replicates per condition are shown in Figure 4, for both 48 SPD (A) and 82 SPD (B). As a proxy for potential quantitation accuracy, they all fall below 10% CV except for the lowest load of 50 pg HeLa, which is <15% for both methods and considered acceptable for the very low amount of sample.

### Results of real single cell samples measured with two different workflows

With the optimized workflow, we attempted to analyze real single cells with the established method parameters. The HeLa cells were isolated and digested using the Cellenion CellenOne platform. The full volume (5uL) final cell digest samples were injected directly from conical 96 well plates in the Vanquish autosampler, preventing any loss of sample material.

The 48 SPD method gave >1,500 protein groups and >5,000 peptide groups from a single HeLa cell and very similar numbers over three biological replicates (see Figure 5). With the 82 SPD method, similar numbers can be achieved over six biological replicates but in general, the numbers of identified proteins and peptides show higher variation. The reproducibility of the lower throughput method is also demonstrated in inset B, the Venn diagram demonstrates a high overlap of identified peptide sequences in the three analyzed cells. Overall, the 48 SPD method proves to be suitable for low-load proteomics in general and single cell experiments in particular.

**Figure 3. Boxplots of empirical $log_2$(Abundance Ratio) of all quantified peptides in different HeLa loads.**

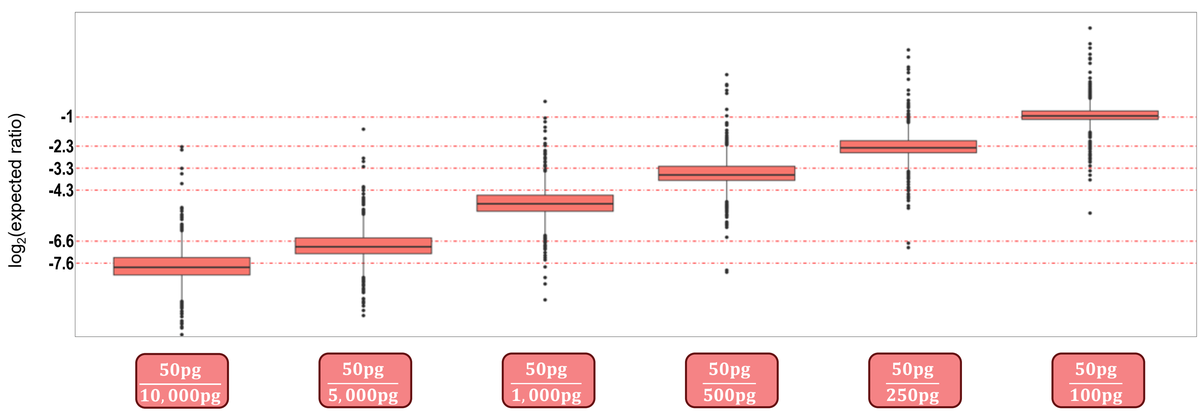



**Figure 4. HeLa dilution series with 48SDP (A) and 82SPD (B) methods demonstrate high sensitivity for potential single cell experiments.**

HeLa was diluted to final loads on column of 50 pg up to 10 ng, each concentration was measured in triplicate.

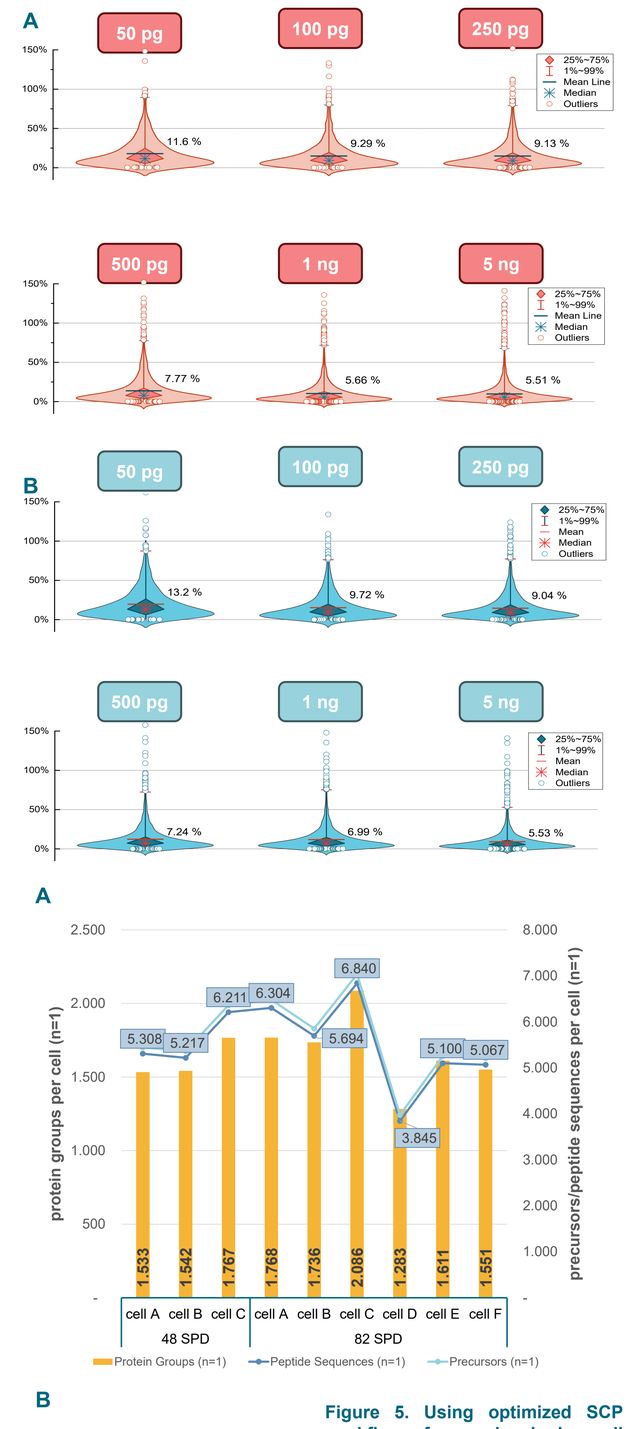
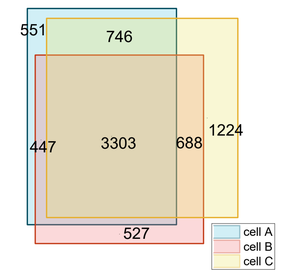



**Figure 5. Using optimized SCP workflow for real single cell samples prepared by CellenONE platform**

HeLa Cells were isolated, evaluated and digested using the Cellenion CellenONE end-to-end solution.
A) Protein and peptide identifications from separate cells using two different active gradient lengths.
B) Venn diagram of stripped sequences identified in all three cells measured with 48 SPD method.




## Conclusions

- A workflow for low input proteomics was established with a throughput of either 48 SPD or 82 SPD, yielding high proteome coverage for HeLa dilutions down to 50 pg peptide digest.
- The data shown here are not only giving high numbers of protein IDs but also exhibit great quantitation performance across the board for all peptide loads.
- The method can be adopted for measuring high-quality single cell preparations using the CellenONE platform, resulting in an end-to-end workflow for comprehensive single cell analysis.

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