Application Note

EVOSEP

# Robust and high-throughput **single cell proteomics** with the Evosep One

## 1. Introduction

Recent advancements in the robustness and sensitivity of sample preparation, liquid chromatography and mass spectrometry instrumentation and workflows have enabled efficient single cell proteomics. Whisper™ Flow Technology is specifically developed for high sensitivity applications such as single cell analysis. Ongoing improvements of Whisper combined with a new generation Evotip provide efficient capture and recovery of peptides from very low sample amounts down to the single cell level. Additional optimizations include a new 15 cm column from IonOpticks (75 µm ID with 1.7 µm beads, Generation 3 Aurora Elite), that works well together with the single use and disposable trap columns with a slightly more hydrophobic material. We have satisfactorily tested this column for the Whisper 40 and 20 SPD methods. It can be used as a direct replacement for EV1112 to increase the performance. However, the temperature should be maintained at 50 °C. Here, we demonstrate the sensitivity and robustness of our Whisper 40 SPD method in combination with the fast and sensitive timsTOF SCP mass spectrometer using data-independent acquisition parallel accumulation serial fragmentation (dia-PASEF). Furthermore, we applied it to sorted single cells, a field in rapid development, to understand heterogeneity within complex systems.

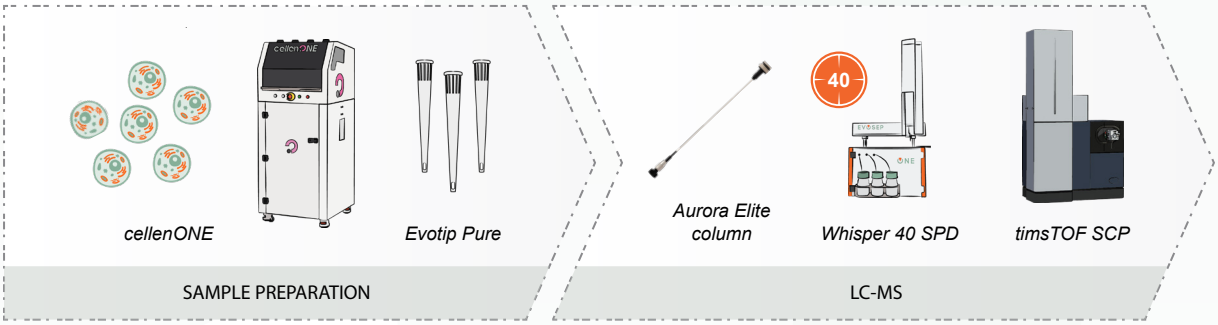


*Figure 1:* Workflow for single cell analysis.

## 2. Method details

HeLa tryptic digest was purchased from Pierce and dilutions were loaded on Evotip Pure in six replicates of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng. HeLa cells were sorted, harvested and digested using the cellenONE, where 1, 5 and 10 cells were collected in separate proteoCHIP wells. Tryptic peptides were loaded onto Evotip Pure using the standard loading protocol, where peptides were collected in the proteoCHIP by penetrating the oil layer. Samples were analyzed with an Aurora Elite column with integrated emitter operated at 50 °C (15 cm, 75 µm ID, 1.7 µm beads, IonOpticks) using the Whisper 40 SPD method. The spray voltage was segmented throughout the gradient with 1500 V in the beginning and end, whereas 1400 V was applied from 5-27 min. This was acquired by a timsTOF SCP mass spectrometer equipped with a Captive Spray source and operated in dia-PASEF mode. Peptides were analyzed with high sensitivity mode enabled. For dia-PASEF acquisition, a window placement scheme consisting of 8 TIMS ramps with 3 mass ranges per ramp spanning from 400-1000 m/z and from 0.64-1.40 1/$K_0$ with a cycle time of 0.95 seconds, including one MS1 frame, was utilized. The dia-PASEF data was analyzed with DIA-NN v1.8 with a predicted library of a human swissprot fasta file including isoforms (Uniprot, downloaded November 2021) without match between runs. Identifications represent protein groups and precursors as stated in the pr_matrix.tsv and pg_matric.tsv files.

## 3. Chromatographic performance

We initially assessed the chromatographic performance based on 5 ng HeLa loaded on Evotip Pure analyzed with the 40 SPD method in dia-PASEF mode. Three diagnostic peptides eluting across the gradient were selected to monitor peak performance and retention time stability based on extracted ion chromatograms for m/z 599.75, 566.75 and 663.84 (+/- 0.02 Da). The hydrophilic peptide (m/z 599.75) resulted in full width at half maximum (FWHM) of 2.5 seconds across four replicate injections, whereas the other peptides (m/z 566.75 and 663.84) provided FWHM of 4 seconds on average based on four replicate injections (Figure 2A). The chromatography enabled a median FWHM of 3 seconds of all identified precursors. The retention times of these selected peptides were robust with a standard deviation of 10 seconds on average based on 10 injections (Figure 2B).

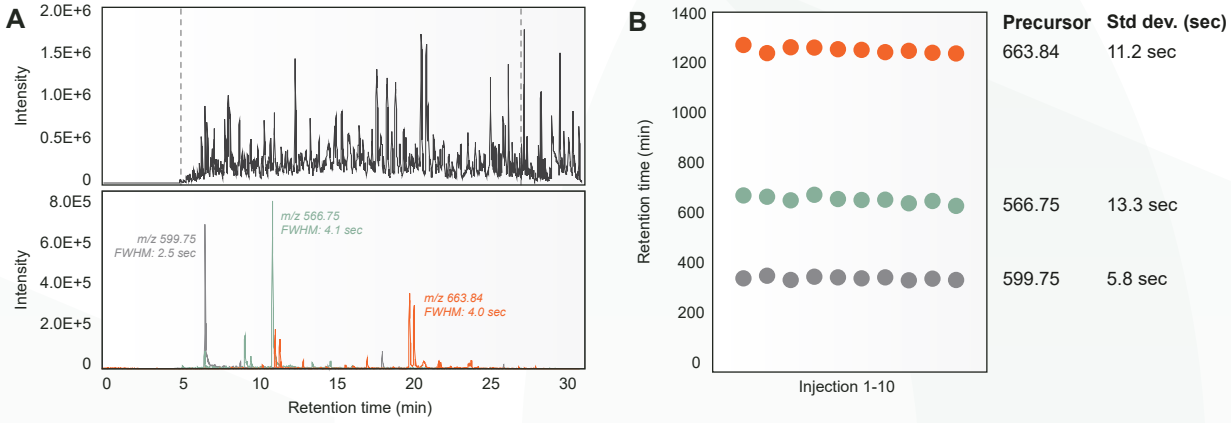


*Figure 2:* (A) Base peak chromatogram and extracted ion chromatograms for m/z 599.75, 566.75 and 663.84 from 5 ng HeLa. (B) Reproducibility of retention times across 10 replicate injections.

## 4. Reproducible dilution series

We evaluated the sensitivity of our workflow with a dilution series of HeLa tryptic peptides analyzed in hexuplicate (Pierce), ranging from low loads of 62.5 pg up to saturating levels of 32 ng peptide material. From the 250 pg load corresponding to single cell level, we identified more than to 7,000 precursors leading to close to 2,000 proteins, whereas the high load of 32 ng resulted in close to 60,000 peptides leading to almost 7,500 proteins (Figure 3A and B). The dilution series revealed excellent linearity of identifications as well as reproducibility across the six injections. Identifications are nicely distributed throughout the gradient peaking at close to 3,500 precursors identified per minute for the high loads. The low loads still show robust identification rates during the gradient, where the 500 pg load reached 500 precursors identified per minute for a great part of the gradient (Figure 3C).

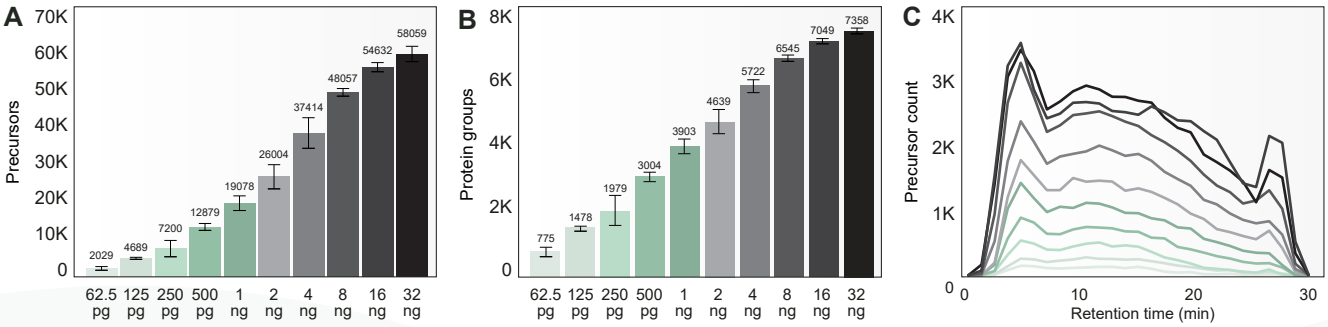


*Figure 3:* (A) Precursor and (B) protein identifications from the dilution series measured in hexuplicates. (C) Precursor per minute throughout the gradient for all loads.

## 5. Sensitive single cell proteomes

To challenge the sensitivity even further, we analyzed single sorted HeLa cells. These were dispensed in 1, 5 or 10 cells with the cellenONE in separate proteoCHIP wells, where lysis and digestion was performed. We analyzed six individual single cells and included quadruplicate measurements of 5 and 10 cells respectively. The single-cell proteomes revealed nice coverage with an average of 7,800 precursors identified per cell, corresponding to 2,000 proteins with variation likely attributable to biological variation across cell cycle and other functional cell states in addition to technical parameters (Figure 4A and 4B). From 5 and 10 cells, deep proteomes of more than 3,300 and 5,000 proteins are measured with the Whisper setup (Figure 4C). When comparing this proteome depth obtained from single cells to the HeLa reference dilution series, we find that the proteome coverage achievable from actual single cells matches the depth achieved from 250 pg of HeLa digest.

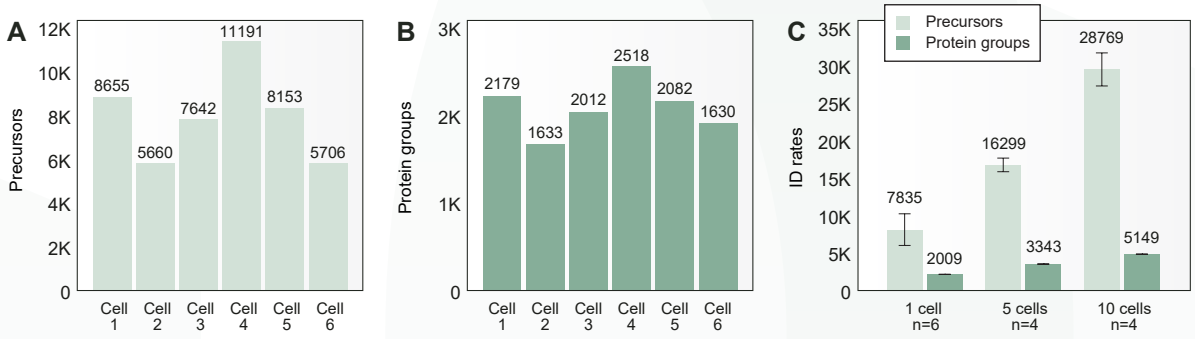


*Figure 4:* (A) Precursor and (B) protein identifications from individual single cell measurements. (C) Combined identifications from 1, 5 and 10 cells.

## 6. Conclusion

The combination of the Aurora Elite column from IonOpticks, Whisper methods on the Evosep One and dia-PASEF on the timsTOF SCP provides an excellent combination for robust and high-throughput single cell analysis at competitive sensitivity.
Overall, the presented workflow maps single cell proteomes with a coverage of up to 2,500 proteins, pushing the boundaries of protein-level insight achievable across heterogeneous cell populations. Another key contributor to this performance level is the nanoscale sample preparation workflow on the cellenONE platform optimized for single cell analysis. Notably, these numbers are achieved with a label-free strategy and may be further improved by implementing mass tags for multiplexing, which will also provide additional throughput.

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### References


1. Bache N., Geyer PE., Bekker-Jensen DB., Hoerning O., Falkenby L., Treit PV., Doll S., Paron I., Müller JB., Meier F., Olsen JV., Vorm O., Mann M. (2018) A novel LC system embeds analytes in preformed gradients for rapid, ultra-robust proteomics. Mol Cell Proteomics., mcp.TIR118.000853


### Data Courtesy and Acknowledgements


Christoph Krisp, Markus Lubeck and Gary Kruppa
Bruker Daltonics, Bremen, Germany

Andreia Almeida and Jarrod Sandow
IonOpticks, Melbourne, Australia

David Hartlmayr and Anjali Seth
Cellenion, Lyon, France