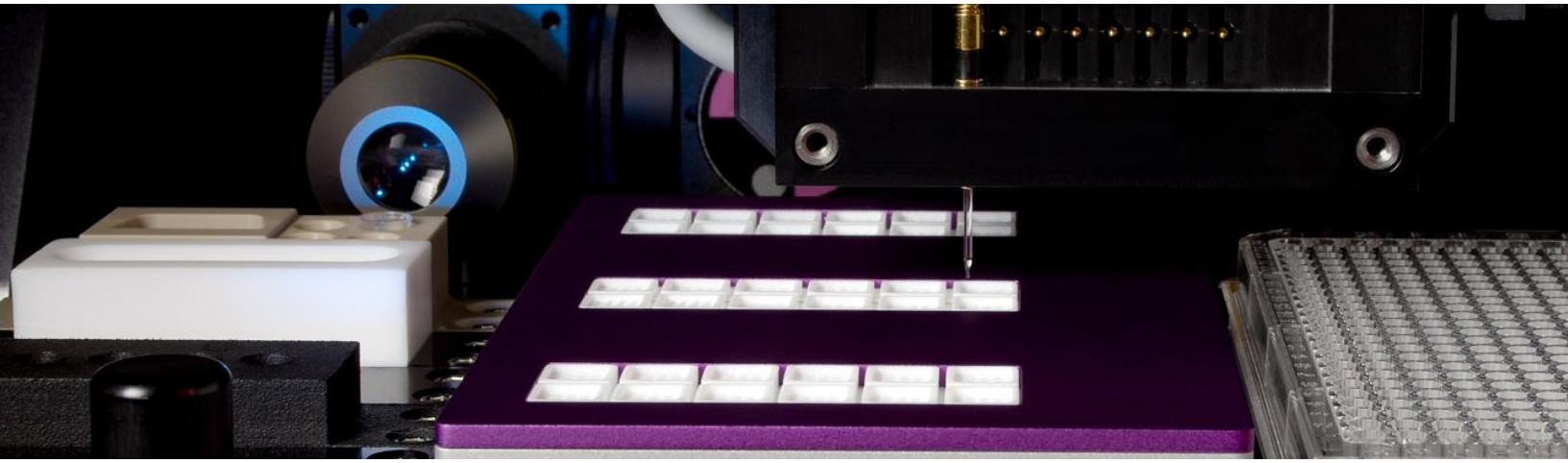
Application Note



Combining cellenONE® and timsTOF SCP for easy and sensitive, end-to-end label-free single cell proteomics workflows

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Abstract

Recent enhancements in trapped ion mobility spectrometry (TIMS) coupled to fast and sensitive mass spectrometry established in the timsTOF SCP as well as automated cell sorting and sample preparation with the cellenONE® platform with proteoCHIP™, allows for sensitive proteome analyses at the single cell level.

Data Independent Acquisition – Parallel Accumulation and SErial Fragmentation (dia-PASEF®) on the timsTOF SCP mass spectrometer was applied to HEK 293 and HeLa single sorted cells obtained from the cellenONE®, achieving high protein and peptide identification reproducibility with accurate quantification of more than 1400 proteins for single cells. This demonstrates outstanding performance and greatly aids the needs of single cell research.

Introduction

In recent years, great advances in the field of single cell proteome analyses have been made and it has become an achievable goal. With the introduction of trapped ion mobility spectrometry (TIMS) on the timsTOF Pro, a new level of sensitivity has been reached when coupling this technology with fast scanning and time of flight (TOF) analyzers with high mass resolving power [1]. The latest enhancements in ion transfer with a larger transfer capillary for up to 5-fold higher ion transfer, an additional higher-pressure segment for more effective ion collection and two orthogonal deflections to maintain robustness, introduced with the timsTOF SCP have pushed the limits of detection even lower [2].

A bottleneck in single cell analysis, however, is sample collection, preparation and transfer, which are commonly the main source of sample losses in proteomic applications. Automated single cell sorting and sample preparation for proteomics has been realized with the cellenONE® platform (Cellenion), an instrument for single cell isolation and cell isolation and picolitre dispensing. The cellenONE® enables fully automated isolation of cells followed by cell lysis and enzymatic degradation in a minimal volume in the proteoCHIP™ [3] fully optimized for the low protein amounts found in a single cell, and to reduce losses due to unspecific binding on exposed plastic.

To decipher the cellular heterogeneity in samples, especially in tumor tissue, will likely require dozens to hundreds of individual single cells to be analyzed in a relatively short timeframe. As shown in AppNote LCMS-193 [4], and by Brunner et al. [5] the sensitivity level reached with the timsTOF SCP is suited for analyses in the singe cell concentration range and meets the other demands of single cell proteomics, namely high throughput and robustness.

Similarly, data-independent acquisition (DIA), an untargeted method for proteome-wide identification and quantification, has become the routine strategy for quantification of samples for various purposes (e.g. clinical proteomics discovery, companion diagnostics and personalized medicine research). As implemented on the timsTOF Pro and timsTOF SCP platforms DIA is done with the dia-PASEF method and has the advantage of separating ions based on space and time therefore increasing sensitivity and efficient precursor 'scheduling for fragmentation, providing collisional cross-section (CCS) values and separation of isomeric species that are mobility offset but mass aligned (MOMA) [6].

Here we show the performance of a label-free single cell analysis workflow identifying more than 1400 proteins from sorted single cells using the cellenONE® platform and the timsTOF SCP mass spectrometer. We also show that when collecting a batch of 5-20 cells, the protein ID's scale appropriately with the number of cells collected, and that the signal from individual proteins also scales linearly with the number of cells isolated.

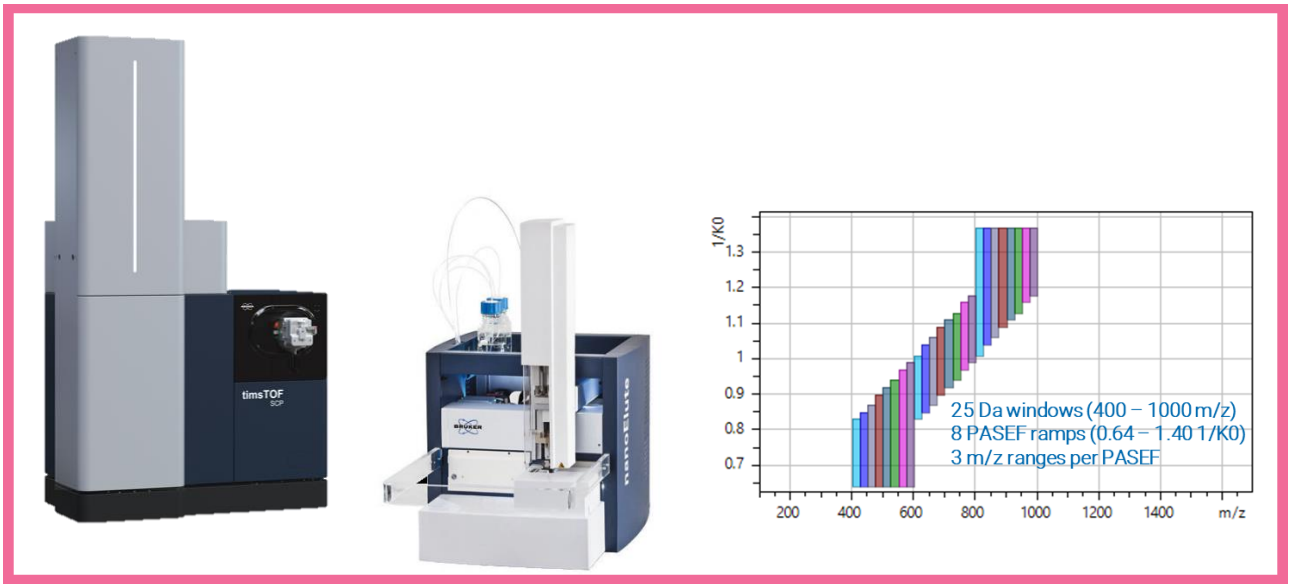


Figure 1: LC-MS/MS setup and dia-PASEF window placement scheme

Materials and methods

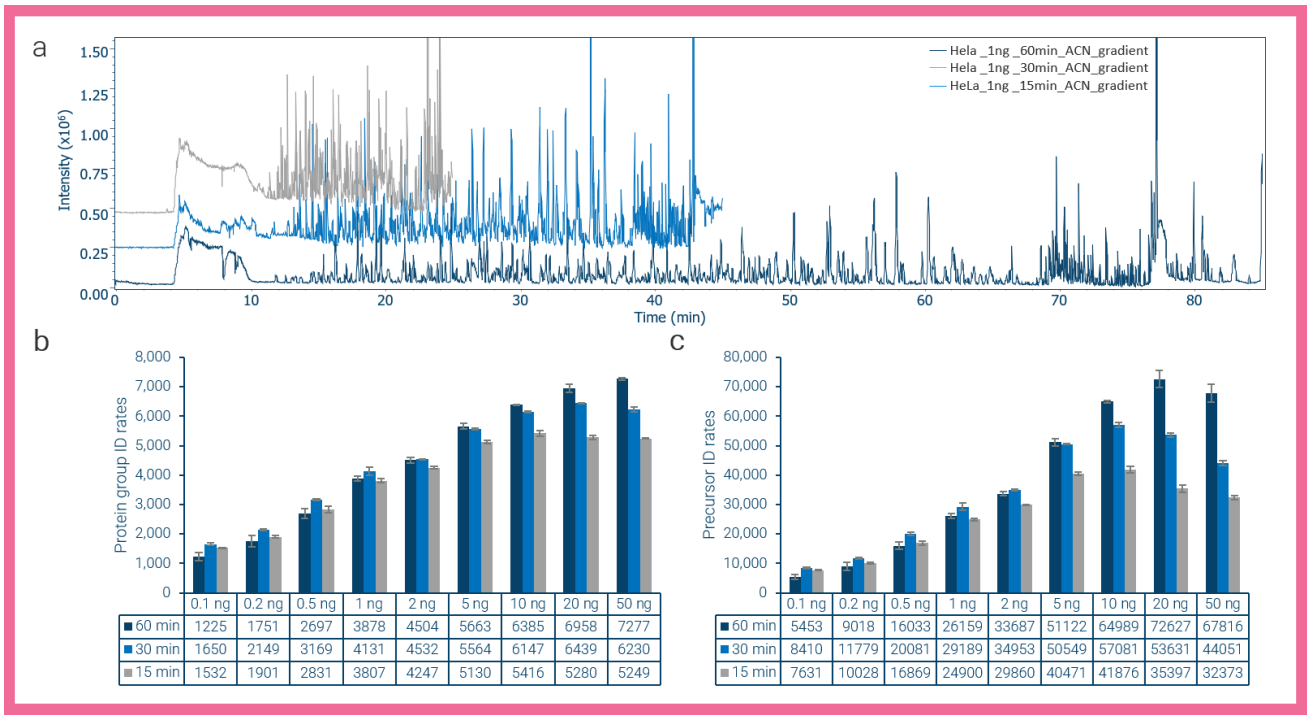
Human cervical cancer cell digests (HeLa, Pierce, Cat. 88328) were used to prepare a dilution series with concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng(protein)/µL.

Human embryonic kidney (HEK 293) and HeLa cells were sorted, lysed, and digested using the cellenONE®. After deposition of 20, 10, 5, and 1 cells into separate proteoCHIP™ wells and reagent dispensing, samples were directly incubated at 50°C with high humidity on the deck of the instrument. The reagents dispensed resulted in lysis and tryptic digestion of the cells.

The resulting tryptic peptides were separated on a 25 cm C18 column (75 µm inner diameter, 1.9 µm particle size, Aurora, IonOpticks) using a nanoElute coupled to a timsTOF SCP mass spectrometer via a CaptiveSpray ionization source. HeLa peptide dilution series were separated using 15, 30, and 60 min acetonitrile (ACN) gradients, and peptides from cellenONE® sorted cells were separated using a 30 min ACN gradient.

Eluting peptides were analyzed with a dia-PASEF method with high sensitivity mode enabled. For the 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/K0 with a cycle time of 0.9 seconds, including one MS1 frame, was utilized (Figure 1).

All dia-PASEF data were processed in TIMS DIA-NN with a deeply fractioned spectral library containing 573,610 precursors from 13,679 proteins without match between runs using the human reviewed protein sequence database including isoforms (Uniprot, downloaded February 2020). The number of identified protein groups and precursors as stated in the statistics .tsv file was used for comparison.



	0.1 ng	0.2 ng	0.5 ng	1 ng	2 ng	5 ng	10 ng	20 ng	50 ng
60 min	1225	1751	2697	3878	4504	5663	6385	6958	7277
30 min	1650	2149	3169	4131	4532	5564	6147	6439	6230
15 min	1532	1901	2831	3807	4247	5130	5416	5280	5249

	0.1 ng	0.2 ng	0.5 ng	1 ng	2 ng	5 ng	10 ng	20 ng	50 ng
60 min	5453	9018	16033	26159	33687	51122	64989	72627	67816
30 min	8410	11779	20081	29189	34953	50549	57081	53631	44051
15 min	7631	10028	16869	24900	29860	40471	41876	35397	32373

Figure 2: Gradient length versus HeLa peptide load optimization. **A** *representative base peak chromatograms of 1 ng HeLa peptides on column separated with a 15 (grey), 30 (blue), and 60 min (dark blue) ACN gradient;* **B** *protein group and* **C** *precursor identification rates of HeLa peptide dilution series of the three different gradient length acquired in dia-PASEF mode and processed with TIMS DIA-NN using a spectral library.*

Results and discussion

For an optimal sample load to gradient length, HeLa peptide dilution series were generated ranging from 100 pg up to 50 ng of peptides loaded (n=3) onto a C18 column to simulate peptide amounts from single cells (~250 pg) up to a few dozens of cells. Representative base peak chromatograms (BPCs) of 1 ng peptide load on column for the different gradient length are shown in Figure 2A.

Based on data acquired in dia-PASEF and processed with TIMS DIA-NN with deeply fractioned spectral library (containing 573,160 precursors from 13,679 proteins), the 60 min ACN gradient showed best performance for peptide loads greater 5 ng, the
30 min ACN gradient performed best for concentration ranges ≤ 5 ng, the 15 min gradient performed in the range of less than 1 ng better than the 60 min but was inferior to the 30 min gradient (Figure 2B and C).

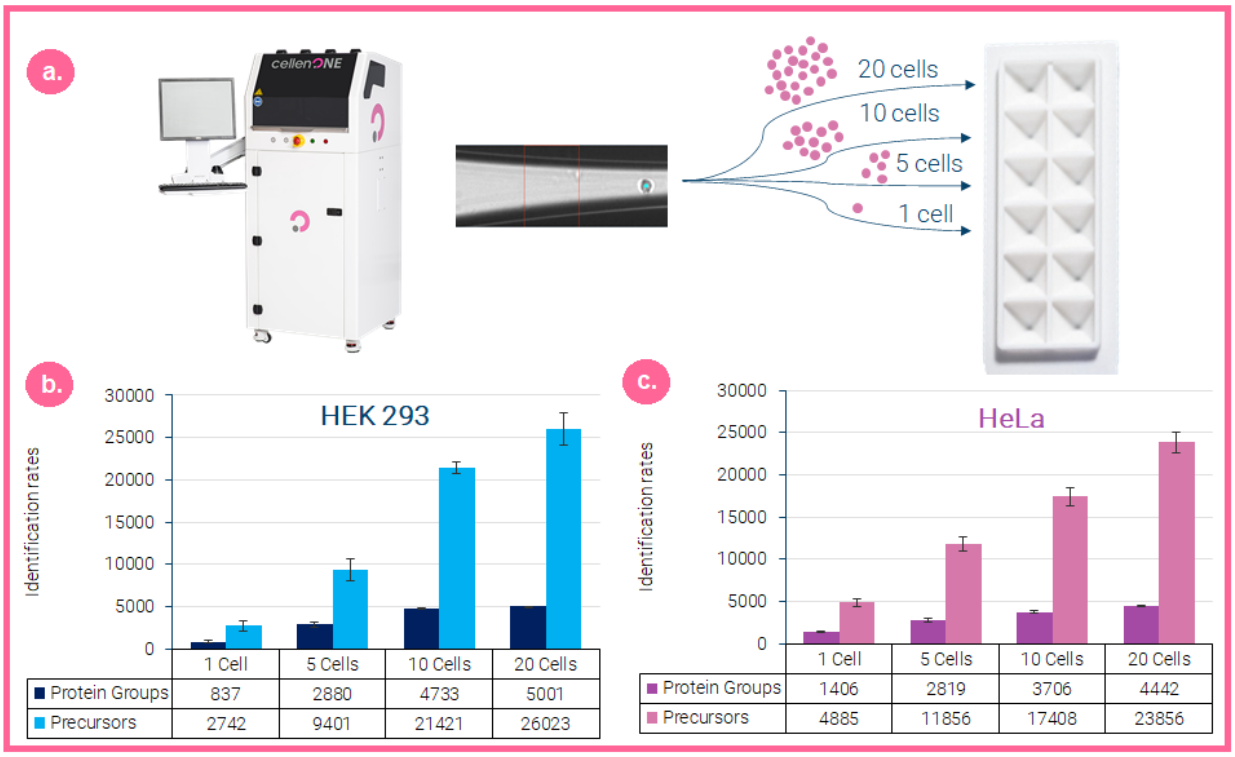


Figure 3: Single cell analysis. **A** *Schematic of cell sorting on the cellenONE ®, an instrument for cell isolation and picolitre dispensing, using the proteoCHIP ® for sample preparation; protein group and precursor identification rates for 1, 5, 10 and 20 HEK 293 cells* **B** *and HeLa cells* **C** *acquired in dia-PASEF and analyzed using TIMS DIA-NN*

Nonetheless, all three gradient length showed excellent protein group and precursor identification results at 200 pg peptide loads on column. The 30 min ACN gradient identified
about 2100 protein groups with about 11,800 precursors, the 15 min ACN gradient identified 1900 protein groups with about 10,000 precursors and the 60 min ACN gradient identified 1750 protein groups with about 9000 precursors (Figure 2B and C).
As the 30 min ACN gradient demonstrated the best performance in the range of expected protein amounts from single to a few cells it was selected for analyzing the sorted cell samples. HEK 293 and HeLa cells were sorted and prepared for proteome analysis using the
cellenONE® system to sort 1, 5, 10, and 20 cells (n=3) into individual wells of the proteoCHIP™ (Figure 3A).

Data processing with TIMS DIA-NN without MBR identified for single HEK cells in average 837 protein groups with 2742 precursors, with a steady increase towards 20 cells with, in average, 5001 protein groups with 26,000 precursors identified (Figure 3C). For HeLa cells, in average, 1400 protein groups with 5000 precursors for single HeLa cells and for 20 cells 4400 protein groups with about 23,800 precursors were identified (Figure 3C).

Quantitative comparison between the protein groups identified in single HEK 293 and single HeLa (n=3) was performed including 1534 protein groups quantified in at least 2 replicates in either HEK 293 or HeLa cells. Principal component analysis of all proteins (405 protein groups) found in all HEK 293 and HeLa cells (Figure 4A) showed clear difference between HEK and HeLa cells in the first component (79.9%). Hierarchical clustering of all 1534 quantified protein groups also (Figure 4B) showed clear separation of the two cell types. For three single HEK 293 cells, the median protein group area CV was 22.6%, for the three single HeLa cells, it was 13.4%, demonstrating good quantitative reproducibility at the single cell level. Proteins with a higher abundance in HEK 293 or which were exclusively found in HEK 293 were for example proteins belonging to the secretoglobulin family (Figure 4C), proteins commonly found in cells of the urinary tract. Figure 4D shows an immunohistochemistry image of human kidney staining for secretoglobulin family 1D member 2. HeLa cells on the other hand showed higher abundance of growth promoting, translation initiating proteins and DNA damage repair proteins as well as several oncogenes, a typical phenotype for a cancer cell, as shown exemplarily in Figure 4E.

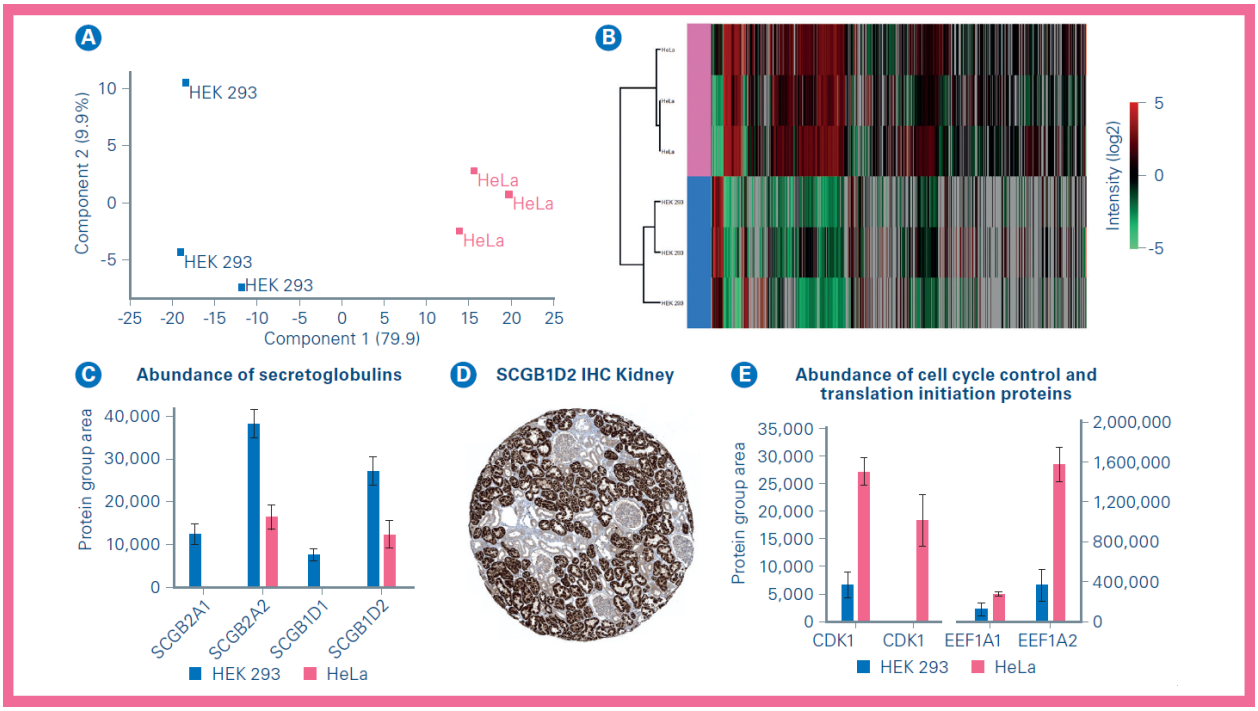


Figure 4: Quantitative comparison of single HEK 293 cells versus single HeLa cells.
A *Component 1 versus component 2 loadings of principal component analysis of proteins quantifiable in all HEK 293 and HeLa samples;* **B** *hierarchical clustering of all proteins quantifiable in at least 2 replicates in either HEK 293 or HeLa cells;* **C** *Protein group area distribution of secretoglobulins in single HEK 293 and single HeLa cells;*
D *immunohistochemistry staining for SCGB1D2 in human kidney tissue taken from The Human Protein Atlas [8];*
Protein group area distribution of cell cycle control proteins CDK1 and CDK2, and the eukaryotic translation initiation proteins EEF1A1 and EEF1A2.

References

[1] Meier, F et al. (2015), J. Proteome Res.

[2] Application Note, Bruker Daltonics, LCMS-185, 1888581, (2021)

[3] Ctortecka, C et al. (2021), bioRxiv 2021.04.14.439828

[4] Application Note, Bruker Daltonics, LCMS-193, 1894933, (2022)

[5] Brunner, A et al. (2020), bioRxiv 2020.12.22.423933

[6] Meier, F et al. (2018), Mol Cell Proteomics

[7] Demichev, V et al. (2020), Nat Methods

[8] Uhlén, M et al. (2015), Science

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