Application Note

EVOSEP

Pushing the boundaries for robust and high-throughput single cell analysis

1. Introduction

Single cell proteomics is a field in continious development and recent advancements deliver improved robustness and sensitivity in all parts of the workflow with an additional focus on sample preparation. This is essential to scale the number of cells for analysis, which is needed to gain biological insight from single cell proteomics.
The recommendation of a 15 cm column from IonOpticks (75 µm ID with 1.7 µm beads, Generation 3 Aurora Elite) has increased the performance of the methods and enabled the quantification of up to 2,500 proteins from a single HeLa cell, which was sorted and digested using a nanoscale sample preparation workflow on the cellenONE (Cellenion)[1]. As only very limited sample material is available, it is crucial to minimize sample handling. A prototype chip for seamless integration with the sample preparation on the cellenONE instrument has been developed for direct transfer of peptides into Evotips. Here, we demonstrate the sensitivity and robustness of the prototype chip with our Whisper 40 SPD method in combination with the fast and sensitive timsTOF SCP (Bruker) mass spectrometer using data-independent acquisition parallel accumulation serial fragmentation (dia-PASEF).

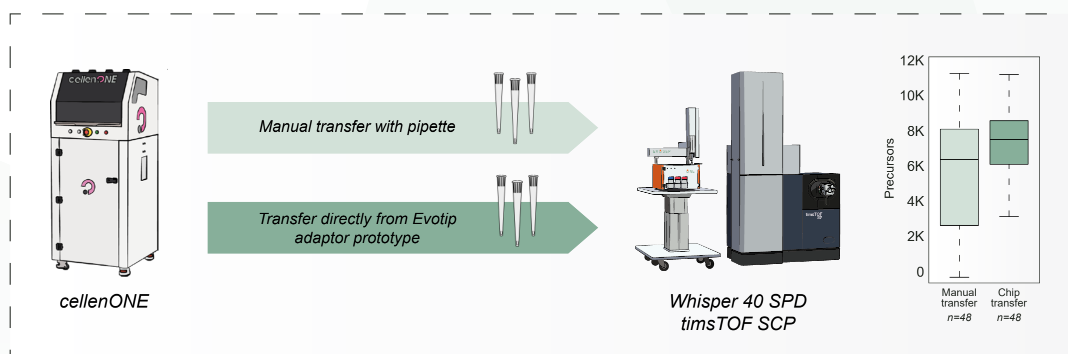

Figure 1: Workflow for single cell analysis.

2. Method details

HeLa cells were cultured in DMEM Media supplemented with 10% FBS, Pen-Strep and L-Glutamine. Cells were detached by trypsinization and washed three times in 1x PBS. Cells were diluted to ~200 cells/µL before sorting. Single cells of size 22-27 µm were sorted into wells of the Evotip adapter containing oil, to prevent sample evaporation during incubation at 50 °C, and 300 nL of Master Mix (10 ng/µL Promega Trypsin Gold, 0.2% DDM, 100 mM TEAB), followed by lysis and digestion at 50 °C for 2 h according to the proteoCHIP LF 48 protocol. Half of the Evotip adaptor (48 samples) were transferred to Evotips by following the standard loading protocol. For cell size comparison, HeLa cells ranging from 18-30 µm were sorted defined by 4 size bins. Following digestion, samples were diluted with 3.2 µL 0.1% TFA and stored at -80 °C. Evotips were activated and equilibrated according to the manual, then 16.5 µL 0.1% formic acid was added to each tip. Samples were transferred to the Evotips by direct centrifugation for 15 s at 700 g. Samples were analyzed using an Evosep One Whisper 40 SPD method with a 15 cm 75 µm Aurora Elite column (IonOpticks) and data acquired in data independent acquisition in parallel accumulation serial fragmentation (dia-PASEF) mode using a timsTOF SCP. Data analysis was performed with DIA-NN 1.8.1 using a spectral library generated by DDA from a deeply fractioned human cell line containing 573,610 precursors from 13,679 proteins. Match between runs (MBR) was enabled and a 5 ng HeLa was added as a reference run for MBR related spectral library generation.

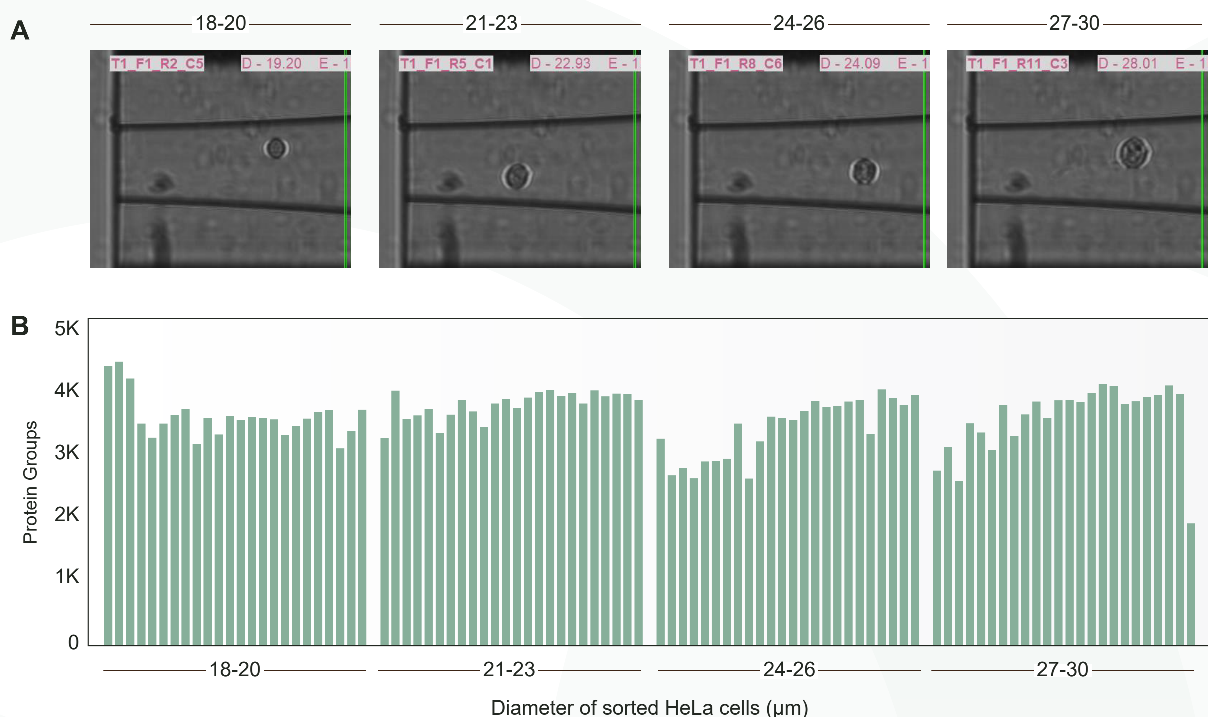

Figure 2: (A) Images of sorted HeLa cells representing the four size groups (B) Protein group identifications from 96 cells analyzed with Whisper 40 SPD.

3. Seamless transfer of peptides

We evaluated the performance of the prototype chip for seamless integration with Evotips. It is designed to prepare 96 samples at the time and transferring the peptides by direct centrifugation to load the full box of 96 Evotips at once. We tested this approach by preparing a chip with single cells and transfer half of the plate via a classical pipetting method and the other half by direct transfer using centrifugation. Across 48 replicate injections, we observe a more tight distribution of precursors using direct centrifugation compared to transferring peptides with a pipette confirming that human variation can be eliminated by automation (Figure 1).

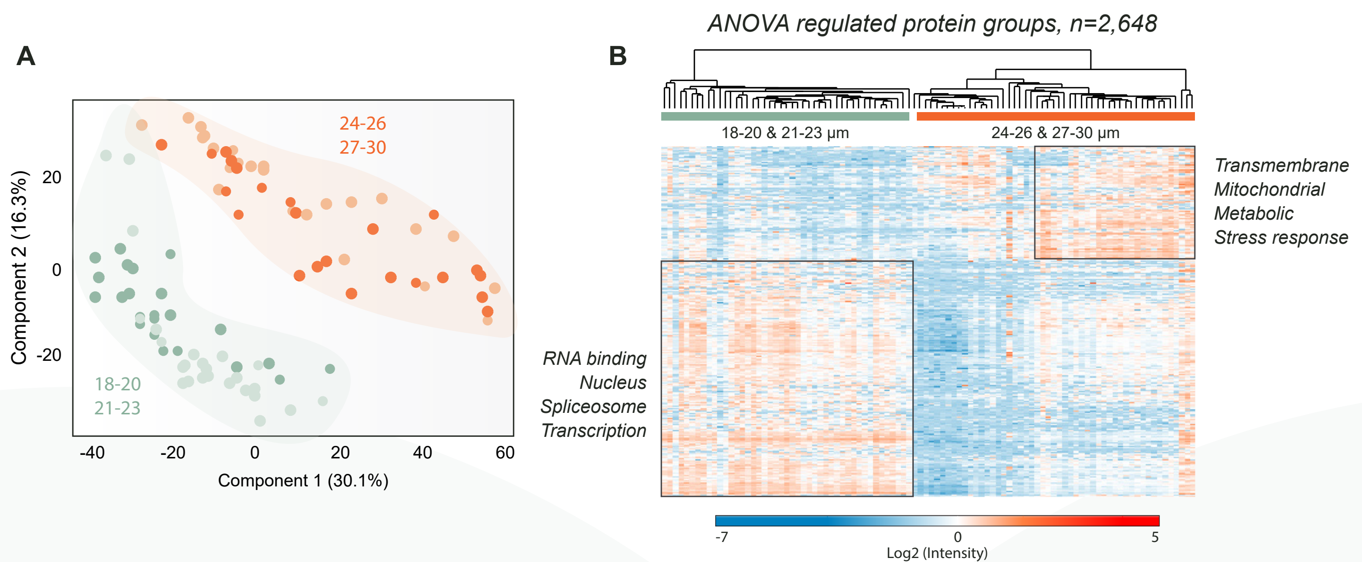

Figure 3: (A) Principal component analysis of the global proteome. (B) Hierarchical clustering analysis of ANOVA regulated protein groups.

4. High reproducibility

Next, we investigated the possibility to scale the number of cells, as this is crucial to apply the workflow to real biological samples. We sorted cells in four groups dependent of cell diameter in 18-20, 21-23, 24-26 and 27-30 µm respectively, where 22-25 µm represents a standard healthy living HeLa cell (Figure 2A). All cells were sorted and digested in the protoype chip and transferred to Evotips via centrifugation and analyzed with the Whisper 40 SPD method on a timsTOF SCP in dia-PASEF mode. The single-cell proteomes revealed excellent coverage with an average of 3,500 protein groups per cell and close to 5,500 proteins identified in the entire dataset. Reassuringly, the performance remains stable throughout analyzing the entire dataset, which was measured within 2.5 days. During this period, the loaded Evotips were stored on the instrument at room temperature with no observed loss in proteome depth (Figure 2B). Interestingly, the number of proteins identified does not correlate with cell size in this experiment, but indeed we see a nice separation of the cell size groups, where the bigger cells, 24-26 µm and 27-30 µm are nicely separated from the 18-20 µm and 21-23 µm cells in a principal component analysis. From an ANOVA analysis, we find more than 2,500 proteins to be significantly regulated between the groups with proteins related to RNA binding, nucleus, spliceosome and transcription being overrepresented in the smaller cells, where proteins related to transmembrane, mitochondiral, metabolic and stress response are overrepresented in the bigger cells.

6. Conclusion

High sensitivity is required in all steps of a workflow to achieve robustness and reproducibility for single cell proteomics. The development of a prototype chip for the cellenONE serves as an excellent reaction chamber for sorting and digestion and facilitates seamless integration with Evotips. This combination yields impressive proteome coverage with 3,500 proteins identified on average per cell when measured with the Aurora Elite column from IonOpticks, the Whisper 40 SPD method on the Evosep One and dia-PASEF on the timsTOF SCP and analyzed with DIA-NN. Notably, this dataset highlights the perspectives of automated sample preparation with the cellenONE combined with sensitive storage pf peptides on Evotips. With the possibility to prepare 2 x 96 cells within 2.5 hours, it is crucial to store these efficiently without significant losses until and during LC/MS analysis, which is a general challenge in single cell proteomics.
Overall, the data validates the technical aspects of the workflow in terms of reproducibility and robustness.

References

1. Robust and high-throughput single cell proteomics with the Evosep One. Evosep application note, AN-018B 22/06

2. 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

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