Label-free and isobaric multiplexing-based single cell proteomics with the BOXmini SCP in scalable workflows on the timsUltra AIP

The BOXmini SCP microfluidic device integrates seamlessly into single cell workflows established on the timsUltra AIP. Label-free analysis of Jurkat cells demonstrated good proteome coverage with T-cell specific marker proteins identified. M2-based isobaric multiplexing (4-plex) demonstrated on Jurkat and FaDu cells (1:1 mixed) identification of cell type specific profiles at sample throughputs of up to 2000 samples per day.

Abstract

In single cell proteomics, successful sample preparation highly benefits from miniaturization and automation. Lysis and digestion in pico- to nanoliter volumes reduces sample losses to surfaces and increases digestion efficiencies. Microfluidic systems are ideal for low input sample preparation as they combine automation with low volume preparation workflows. Therefore, we evaluated label-free and MS2 multiplexing based sample preparation workflows on the BOXmini SCP, a chip-based microfluidic device, and analyzed the samples by ultra-high sensitivity mass spectrometry on the timsTOF platform.

Benchmarking of the label-free workflow with Jurkat cells separated on an Evosep ENO in Whisper Zoom 80 SPD and detection on a timsTOF Ultra 2 in dia-PASEF® mode identified in total about 3000 protein groups and on average 2000 protein groups per single Jurkat cell. The MS2 multiplexing workflow utilizing a 4-cell TMTpro labeling with TMTpro channels 126, 127C, 128C and 129C, was performed with FaDu (head and neck squamous cell carcinoma; HNSCC) and Jurkat cells mixed at 1:1 ratio prior to isolation. No carrier or reference was added. Samples were run on an Evosep ENO in Whisper Zoom 120 and 80 samples per day (SPD) as well as the standard gradients at 500, 300 200, and 100 SPD achieving actual sample throughputs from 240 SPD (80 SPD gradient) up to 2000 SPD (500 SPD gradient). As expected, highest protein IDs of >1500 were obtained with the longest gradient. FaDu cells were clearly distinguishable from Jurkat cells across all gradient lengths. Proteins mainly contributing to the separation were CTTN (Cortactin), a squamous cell enriched protein (elevated in FaDu cells), and LCP1 (Plastin-2 or lymphocyte cytosolic protein 1), an immune cell enriched protein (elevated in Jurkat cells) which was confirmed with transcription level information retrieved from the Human Protein Atlas.

Keywords:
TMTpro, Evosep ENO, timsUltra AIP, 2000SPD, Multiplexing, single cell proteomics

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Introduction

Low-input applications such as single cell proteomics are highly sensitive to sample preparation conditions. Each sample transfer, working reagent volumes, or storage conditions can introduce substantial technical variations that obscure underlying biological differences. Automation and the use of pico- to nanoliter reaction volumes can significantly minimize sample losses and improve sample quality. Prominent examples are the nanoPOTS approach (1) or the one-pot approach (2), which have demonstrated superior performance in single cell applications compared with conventional sample preparation workflows.

Integrated microfluidics systems offer (semi-) automated workflows for cell isolation, lysis, and enzyme-based digestion, thereby increasing reproducibility and allowing for scalability. Chip-based microfluidic devices such as the BOXmini SCP operate by moving aqueous droplets within an oil phase, enabling cell lysis in only a few nanoliters. This setup facilitates digestion under anaerobic conditions, reducing the risk of artificial amino acid oxidation and preventing evaporation of reaction buffer. The BOXmini SCP system supports both label-free and multiplexed sample preparation workflows, enabling high coverage DIA-based analyses as well as high throughput applications using isobaric labeling strategies.

When combining multiplexing approaches with rapid chromatography applications such as the standard gradients of the Evosep ENO with up to 500 SPD in a label-free mode, multiplexing approaches can scale to thousands of samples per day. Gas-phase separated peptide precursor coupled with fast scanning mass spectrometry as offered by the timsTOF platform is the ideal combination for interference reduced MS2 based multiplexing. Orsburn (2025) has previously demonstrated MS2-level multiplexing of single cells using standardized, fast chromatography for the analysis of histone post-translational modifications on a timsTOF Ultra showing up to 3500 samples being run per day (3).

Here, we benchmark the BOXmini SCP device for both label-free single cell proteomics as well as 4-plex TMTpro (using a subset of labels from the 16-plex TMTpro kit) and assessed compatibility with Evosep ENO separation coupled to ultra-high sensitivity mass spectrometry on the timsTOF platform.

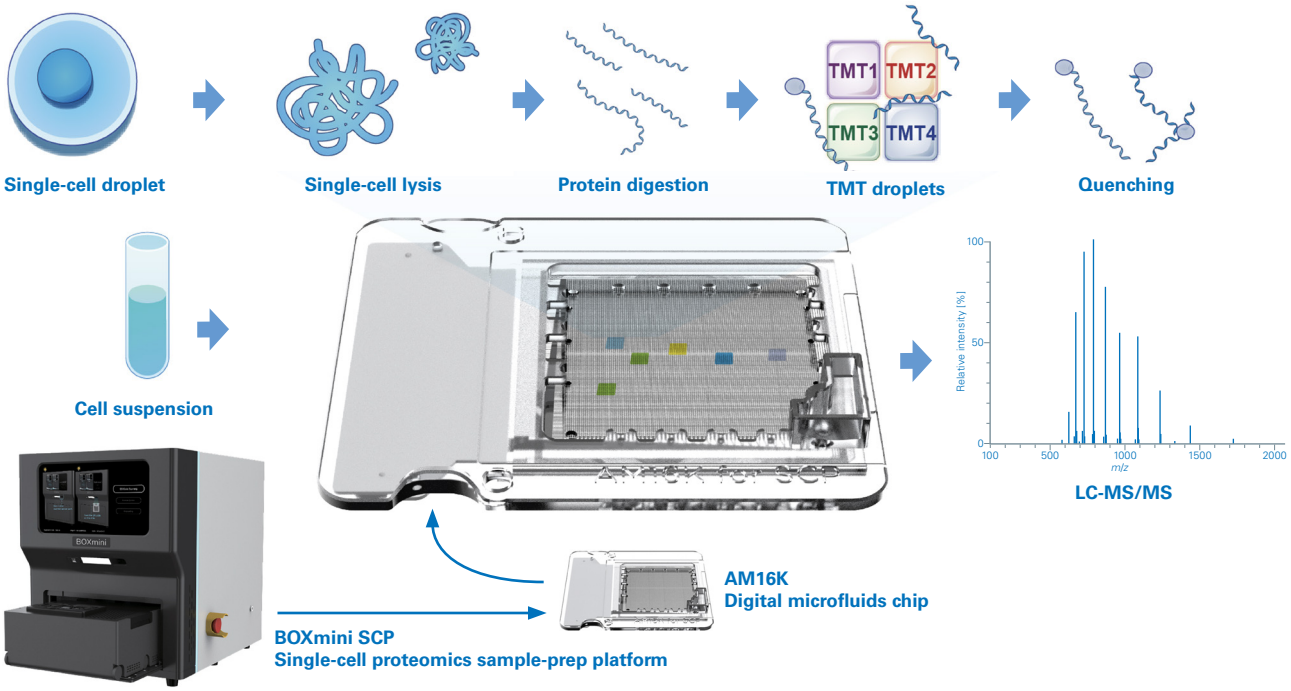

Figure 1. BOXmini SCP workflow overview.

Methods

Jurkat cells were used for the label-free workflow and a mix of FaDu (head and neck squamous cell carcinoma; HNSCC) and Jurkat (immortalized T-cell) at 1:1 ratio was used for the MS2-based labeling workflow in a 4-plex TMTpro approach on the BOXmini SCP system (Figure 1). Cell suspensions (350 cells/µL) were split on AM16K microfluidics chips and single cells merged with 3 nL lysis buffer (0.4% DDM, 40 ng/µL trypsin gold, 200 mM TEAB, 0.2% RapiGest SF) and digested at 37°C for 2 h. Samples for label-free were extracted from the AM16K chip using the outlets, diluted with 1 µL 0.1% formic acid and transferred first onto a PTFE membrane for oil absorption and then into a 96-well plate. For TMT-based multiplexing, labels were added to the AM16K chip, merged with lysates, incubated for 1h at room temperature, and quenched with 0.5% hydroxylamine. 4 single cells, one of each label, were merged to a 4-plex and extracted as described above.

Samples were manually loaded onto Evotip Pure. For the label-free preparation, peptides were separated on an Evosep ENO in Whisper Zoom 80 SPD on an IonOpticks Aurora Elite Rapid 75 C18 column (5 cm x 75 µm x 1.7 µm) heated to 50°C. Eluting peptides were detected using a timsTOF Ultra 2 in dia-PASEF mode using a pyDiAID optimized window placement (4) in a range from 380 – 1200 *m/z* spanning over 5 MS/MS PASEF® cycles with 3 mass ranges each with an accumulation and ramp time of 100 ms resulting in a cycle time of 0.64 s. The multiplexed samples were separated on a Evosep ENO using the standard gradients 500, 300, 200 and 100 SPD as well as the Whisper Zoom gradients 120 and 80 SPD. A stepped collision energy MS/MS dda-PASEF® method was used with 3 PASEF cycles and 80 ms accumulation and ramp time for 500, 300, and 200 SPD (cycle time 0.61 s) and 100 ms accumulation and ramp time for 100 SPD and Whisper Zoom 120 and 80 SPD (cycle time 0.75 s). The manufacturer's recommended columns and temperature settings were used. Detailed information on AIP profile, storage base time, collision energy settings, and collision RF can be found in Figure 4b. dia-PASEF files were processed with Spectronaut® 21 in directDIA® mode without method evaluation. Stepping dda-PASEF files were processed with SpectroMine™ 5.2 with TMTpro at lysine side chain and peptide N-terminus as a fixed modification, protein N-terminus acetylation and oxidation of methionine were allowed as variable modifications. The reporter channels 126, 127C, 128C, and 129C were used for quantification.

Results and Discussion

To evaluate the label-free workflow on the BOXmini SCP, Jurkat cells were utilized. After cell splitting, merging with lysis buffer and on chip digestion, 80 single cells were exported from the Am16K chip, pipetted on an oil-absorbing membrane, transferred into 96-well plates before final transfer into Evotips Pure for LC-MS/MS-based characterization using the Evosep ENO in Whisper Zoom 80 SPD and the timsTOF Ultra 2 in dia-PASEF mode (Figure 2).

Data processing using Spectronaut 21 in directDIA without method evaluation identified on average 2026 protein groups per single cell. With a spread from 1200 protein groups to 3000 protein groups (Figure 3A). Overall protein group abundances showed good correlations (Pearson correlation >0.8) across all single cells with a few outliers (Figure 3B). Data completeness assessment demonstrated close to 1200 protein groups present in at least 90% of all samples and about 2000 protein groups

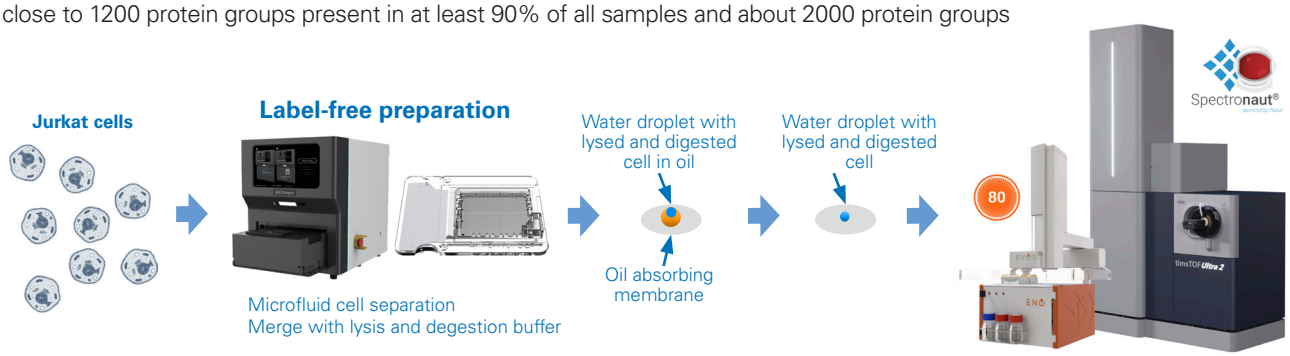

Figure 2. **Label-free single cell Workflow.**
Schematic representation of the TMTpro-based MS2 isobaric multiplexing workflow from single cell isolation, lysis, and digestion inside the BOXmini SCP device followed by sample transfer to PTFE membranes for oil removal, sample loading on Evotips Pure and LC-MSMS characterization using the Evosep ENO in Whisper Zoom 80 SPD with peptide detection on a timsUltra AIP in dia-PASEF mode.

present in at least 50% of all single cells and about 3000 protein groups in total (Figure 3C). The covered dynamic range was approximately 4 orders of magnitude with actin subunits and histones showing the highest abundance level. Cell type specific proteins like CD3D, CD3E, CD3G, ICAM3, and STAT3 as well as cell cycle control proteins were present in the moderate to low abundance range (Figure 3D). A plot of the sample projection of a principal component analysis of protein groups quantified across all 80 single Jurkat cells (656 protein groups) demonstrated a separation in the first two components based on the protein group identification rates (Figure 3E). The individual loadings demonstrate that a certain degree of separation is related to Histone abundance levels (PC loading 2) (Figure 3F). The PC loading 1 demonstrates clear separation of protein groups to be considered rather stable in protein abundance across the samples or correlate negatively with protein group identifications. Proteins associated with this group were proteins of the keratin family and the structural constituent of skin epidermis as well as secreted proteins such as S100A8, S100A9, and ANXA1, therefore clearly separating contaminating proteins from the cell line derived proteins (Figure 3F).

For the label-based workflow, FaDu cells and Jurkat cells were mixed at 1:1 ratio prior to loading onto the AM16K chip. Cells isolated into single cells were selected for lysis and digestion. followed by TMTpro based labeling using a 4-label subset (126, 127C, 128C, and 129C) of a 16-plex TMTpro kit. After quenching of the labeling reaction, four cells (one per label) were merged prior to export, pipetted on an oil-absorbing membrane, transferred into 96-well plates before final transfer onto Evotips Pure. LC-MS/MS-based characterization used the Evosep ENO in Whisper Zoom 80 and 120 SPD as well as the standard gradients 500, 300, 200, and 100 SPD (three 4-plex experiments per conditions) followed by precursor detection with a timsUltra AIP in stepping dda-PASEF mode (Figure 4). No carrier or reference channel was used in this TMTpro experiment.

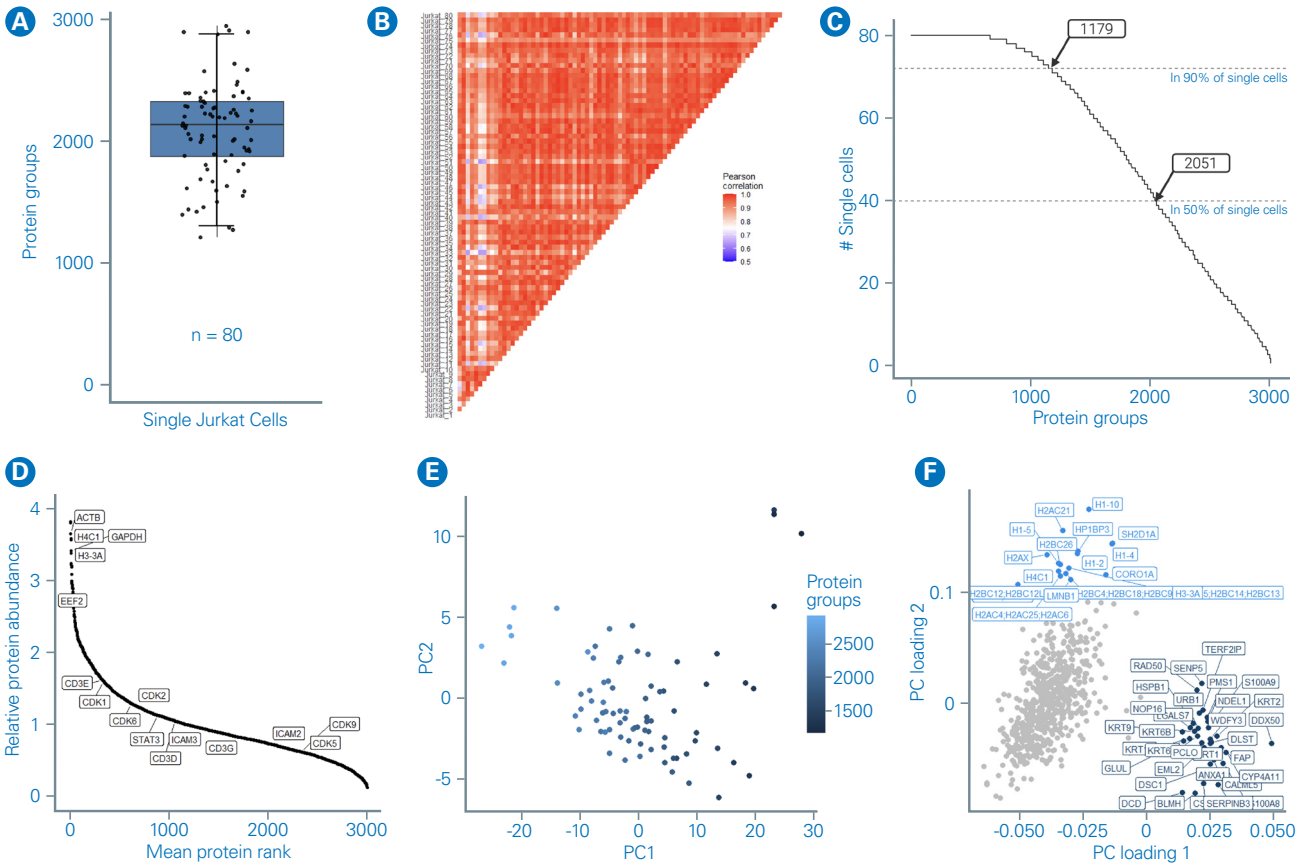

Figure 3. Label-free analysis of single Jurkat cells – Evosep ENO, 5 cm IonOpticks Aurora rapid 75, Whisper Zoom 80 SPD, timsTOF Ultra 2. (A) Box plot of on average identified protein groups across the 80 single Jurkat cells; (B) Heatmap representation of Pearson correlation scores across the 80 single Jurkat cells; (C) Data completeness diagram with 2051 protein groups in 50% of all samples; (D) Protein rank plot based on average protein abundance across the 80 single Jurkat cells with selected proteins at different abundance levels; (E) Plot of first two components depicting separation based on protein group identification rates; (F) Plot of protein group loadings in the first two principal components demonstrating clear segregation of proteins to be considered as contaminants (dark blue).

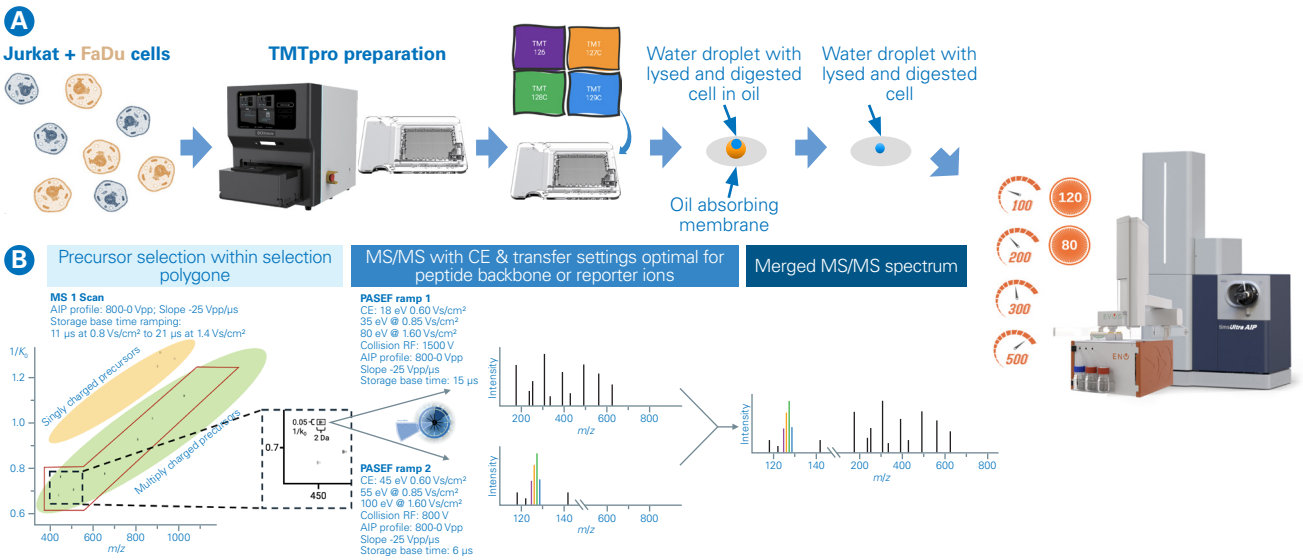

Figure 4. **MS2 multiplexing workflow.**
Ⓐ Schematic representation of the TMTpro-based MS2 isobaric multiplexing workflow from single cell isolation, lysis, digestion, peptide labeling, and sample merging inside the BOXmini SCP device followed by sample transfer to PTFE membranes for oil removal, sample loading on Evotips Pure and LC-MSMS characterization using the Evosep ENO in Whisper Zoom 120 and 80 SPD as well as the standard gradients in 500, 300, 200, and 100 SPD with peptide detection on a timsUltra AIP in stepping dda-PASEF mode; Ⓑ Simplified visualization of a stepping dda-PASEF method used for TMTpro labeled peptide samples on the timsUltra AIP.

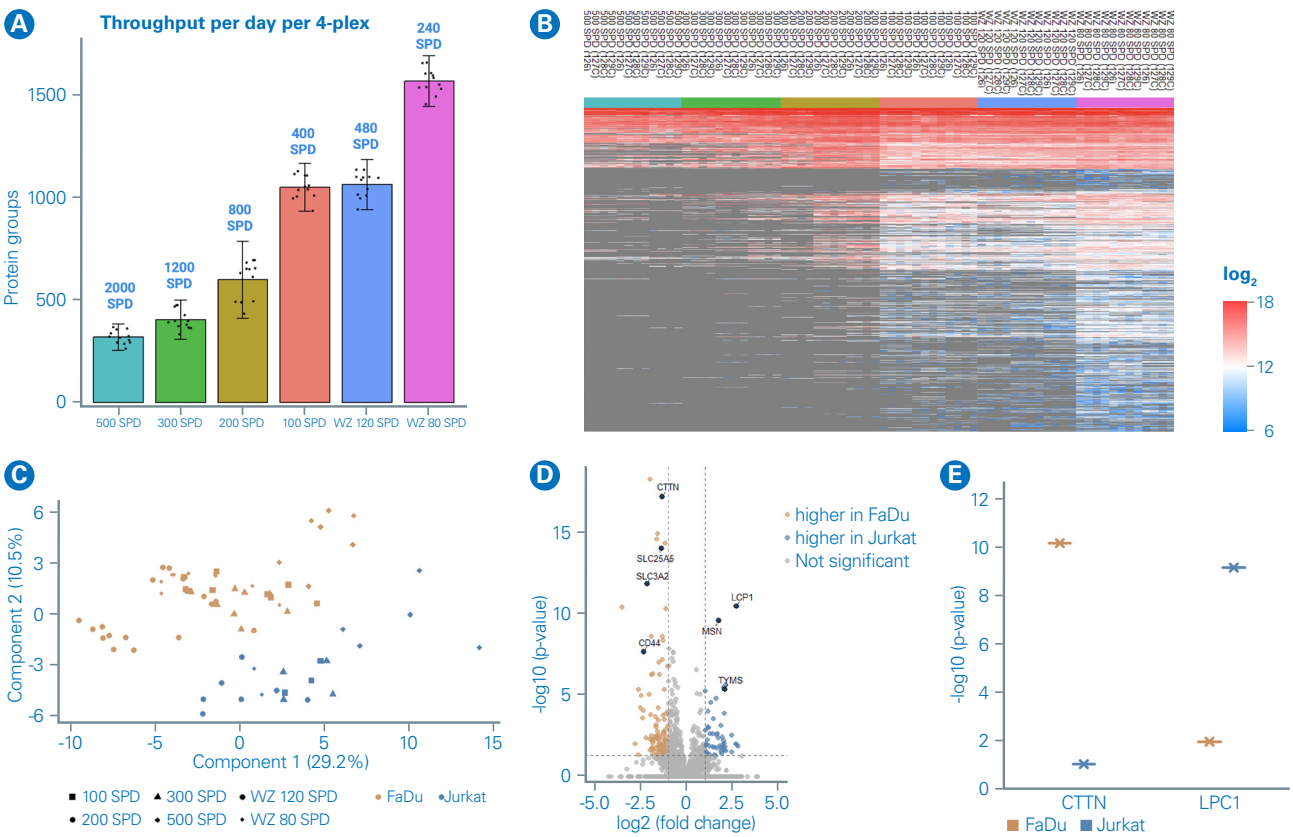

Figure 5. **TMT based multiplexing of Jurkat and FaDu cell mixtures (1:1) - Evosep ENO, various columns and gradients, timsUltra AIP – Throughput of 400-2000 samples per day.**
Ⓐ Bar plot of on average identified protein groups per gradient condition across 4-plex TMTpro replicates (three sets per gradient); Ⓑ Heatmap representation of protein abundance in the 4-plex TMTpro replicates across different gradient lengths after intensity scaling for batch effect removal; Ⓒ PCA of protein groups identified in all gradient conditions and 4-plex TMTpro replicates showing separation of Jurkat from FaDu cells; Ⓓ Volcano plot comparing Jurkat to FaDu cell protein abundances with selected proteins higher in abundance either in Jurkat or in FaDu cells; Ⓔ CTTN (Cortactin) and LCP1 (Plastin-2 or lymphocyte cytosolic protein 1) transcription level information (normalized transcripts per million; nTPM) for Jurkat cells and FaDu cells retrieved from the Human Protein Atlas (https://www.proteinatlas.org/).

Data processing in SpectroMine 5.2 showed gradient length dependent protein group identification rates, with highest rates obtained for Whisper zoom 80 SPD with on average about 1500 protein groups with a sample throughput of 240 samples per day. Whisper Zoom 120 and standard gradient 100 SPD identified around 1100 protein groups with actual sample throughput of 480 and 400 SPD, respectively. The fastest gradients with actual sample throughputs of 800 (200 SPD), 1200 (300 SPD) and 2000 samples per day (500 SPD) achieved identification rates of 650, 400 and 300 protein groups (Figure 5A). To compare relative protein abundance profiles across the different Evosep gradients, protein abundances were first scaled to the highest average protein abundance value per individual gradient length, followed by scaling to the highest average protein abundance across all gradients. The heatmap representation of the protein abundance profiles demonstrates consistent behavior with proteins identified at 2000 SPD throughput being the protein groups with highest abundance in the longest gradient length and lowest sample per day throughput (240 SPD). Hence, the longer the gradient and the lower the throughput, the more protein groups at the lower end of the abundance scale were observed (Figure 5B). Principal component analysis on protein groups identified in all samples and gradient lengths (182 protein groups) allowed for identification of the corresponding cell types, with a bias attributed to the gradient speed, without prior knowledge of which cell type ended up being labeled with a given TMTpro label (Figure 5C). A Volcano plot representation of Student's t-test statistical analysis performed on all quantifiable proteins to compare the two groups derived from the PCA, identified the cells marked in blue as Jurkat cells, as the immune cell specific protein LCP1 (Plastin-2 or lymphocyte cytosolic protein 1) was elevated in these single cells. The samples marked in light brown were identified as single FaDu cells, due to elevated levels of CTTN (Cortactin), a protein commonly observed in squamous cells (Figure 5D). LCP1 and CTTN mRNA levels in Jurkat cells and FaDu cells were obtained from the Human Protein Atlas (5) and confirmed the cell type specificity of these two proteins (Figure 5E).

In summary, the benchmarking of the BOXmini SCP microfluidic device integrates Seamlessly into single cell workflows established on the timsTOF Ultra 2 and timsUltra AIP. Good proteome coverage with key marker proteins for T-cells were observed in the label-free analysis of Jurkat cells. M2-based isobaric multiplexing approaches demonstrated in a 4-plex setup of Jurkat and FaDu cells (mixed at 1:1 based on cell count) identification of cell type specific profiles at sample throughputs from 240 to 2000 samples per day.

Conclusion

- BOXmini SCP offers label-free and multiplexing based single cell proteomics wokflows
- timsTOF Ultra 2 demonstrates high reproducibility in label-free single Jurkat characterization using dia-PASEF
- MS2-based isobaric multiplexing on the timsUltra AIP identifies distinct cell type profiles under real single cell conditions without carrier or reference channel
- 4-plex experiments run on the Evosep ENO demonstrated cell type specific profiles at sample throughputs of up to 2000 samples per day

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