Optimized Liquid Chromatographic and Mass Spectrometric Methods for Ultra-deep Quantitative Proteomic Analysis of Single Cells

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Introduction

Advances in methods for both liquid chromatography (LC) and mass spectrometry (MS) have significantly enhanced protein identification and quantifications from limited sample amounts, particularly at pico- to nano-gram level, providing a powerful tool to investigate cellular heterogeneity or access sparce material from clinical biopsies. However, the success of ultra-low input depends on technological advancements in the LC, MS and the overall methodology. In this study, we explored various LC conditions and MS platforms to optimize protein identification and quantification using data-independent acquisition (DIA). Our investigation focused on evaluating sensitivity for protein identification, reproducibility of quantification, and robustness across multiple models, specifically focused for analyzing proteins at pico- to nano-gram levels, with an emphasis on single-cell proteomics.

Methods

Peptides from NCI-7 cells were prepared following a CPTAC protocol which includes lysis, reduction, alkylation, and digestion with Lys-C and trypsin. HeLa and PC-3 single cells were prepared using the CellenOne® system. Proteomic analyses were performed using the Evosep One (Evosep) coupled with the timsTOF HT and timsTOF Ultra 2 (Bruker). Peptide separation was evaluated using different LC conditions, and different columns from Ion Opticks and PepSep. The ion mobility range was set between 0.66 and 1.42 V·s/cm², and the mass range covered 100–1700 m/z, operating in dia-PASEF mode with 100 and 166 ms accumulation/ramping time for HT and Ultra 2, respectively. Data was processed using Spectronaut 19.5 in directDIA+ mode using a Human UniproKB reviewed database.

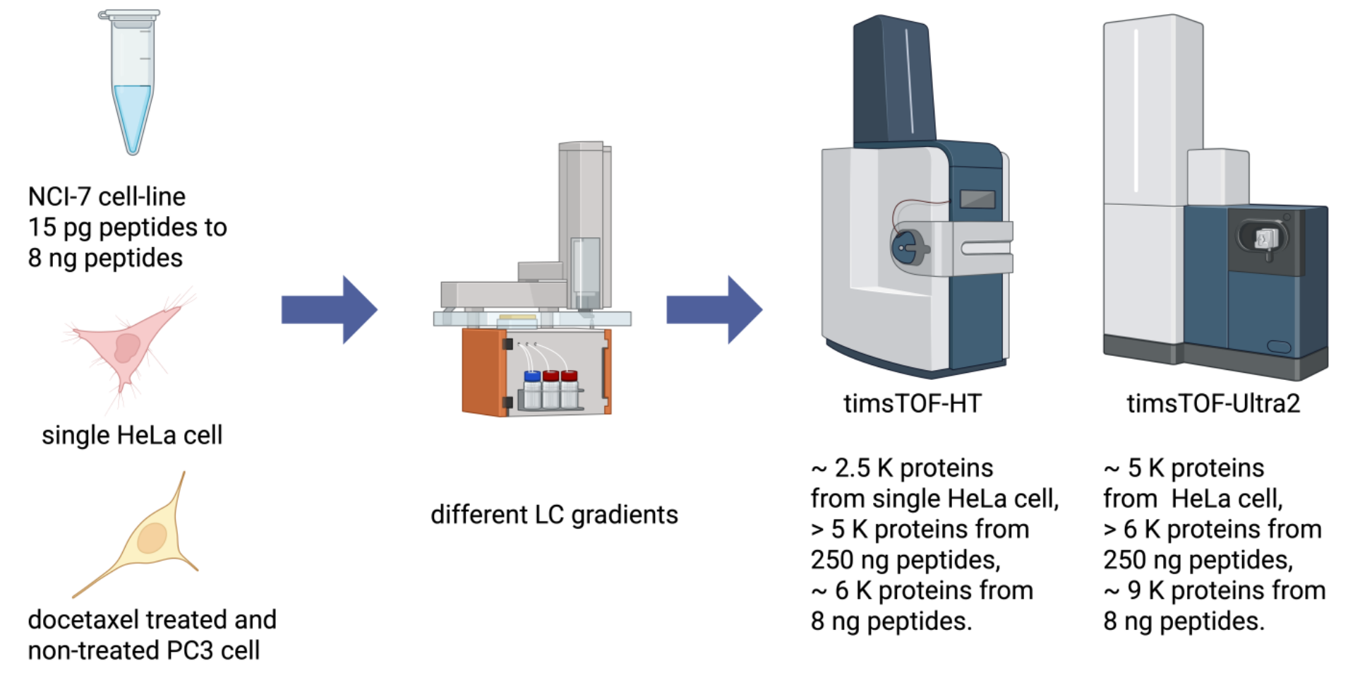

Figure 1: A comprehensive technical evaluation for LC-MS methods in protein identification and quantification for analytical applications involving single-cell proteomics.

Results

To optimize LC conditions for single-cell level or small-scale material characterization, we compared the regular 100 SPD, 60 SPD, 30 SPD LC method with the Whisper Zoom 40 SPD using a PepSep Column (WZ-40 SPD) and the Aurora Elite C18 columns (WZ40SPD_Aurora) on a timsTOF HT. Serial dilutions of NCI-7 peptides ranging from 62 pg to 8 ng, were analyzed. As shown in Fig. 1A, protein identification improved with the WZ-40 SPD LC methods compared to the other regular LC approaches, demonstrating superior protein identification at low peptide input levels, especially with the Aurora column on timsTOF HT. Then, NCI-7 cell peptide dilutions were analyzed on timsTOF Ultra 2 with injection amounts ranging from 15 pg to 8 ng using the WZ-40 SPD-Aurora LC method (Fig. 1B). Here, timsTOF HT shows a good performance for a system not design for low inputs and in the other hand the sensitivity of timsTOF Ultra2 is highlighted for in-depth proteome characterization at low peptide inputs.

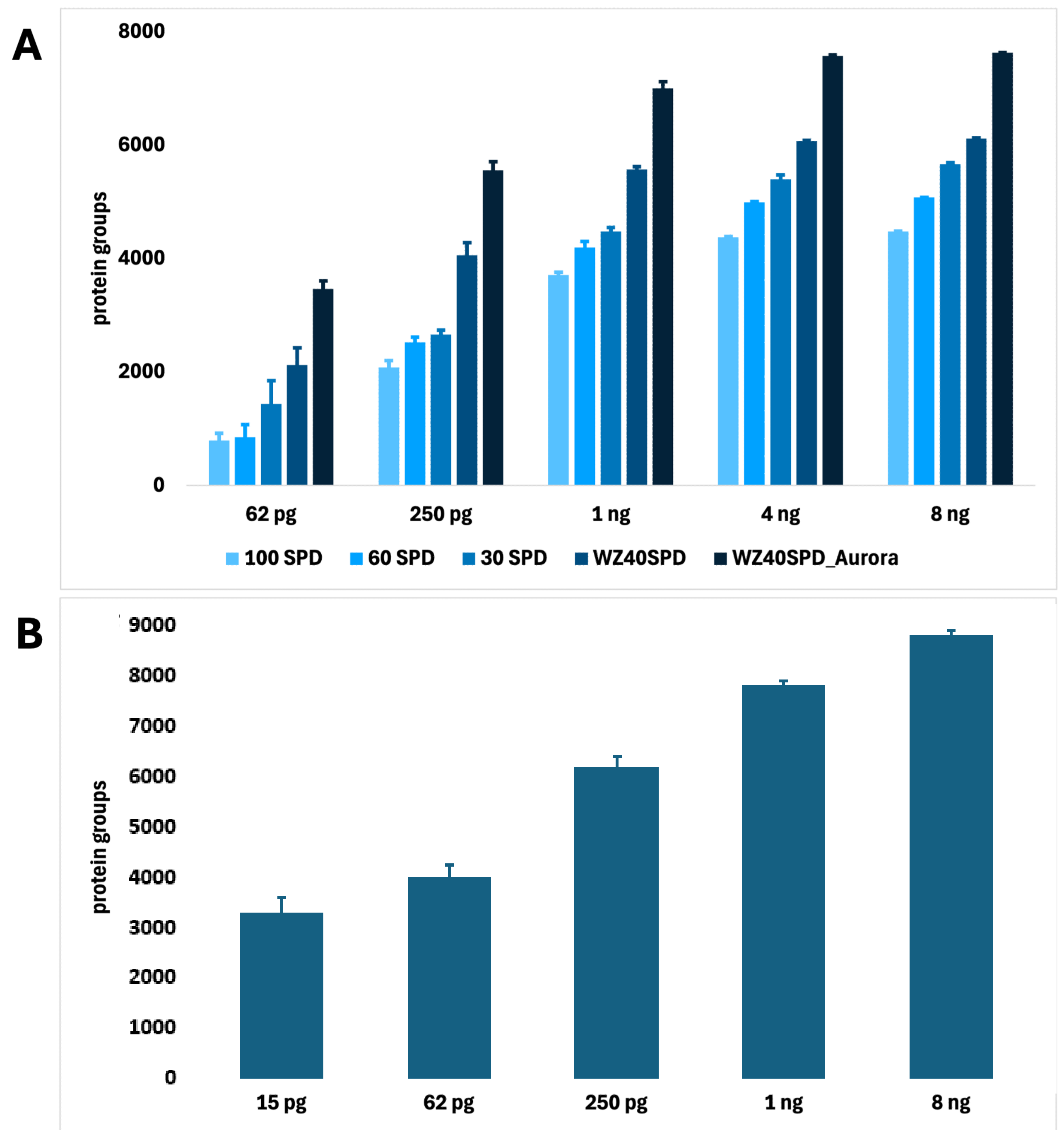

Figure 2: Assessment of proteome characterization with low peptide input amounts. (A) Evaluation of LC conditions and columns for analyzing low-input NCI-7 peptides using timsTOF HT revealed that the WZ40 SPD method outperformed other three LC methods, especially in single-cell peptide level (250 pg). (B) Performance for protein identification using WZ40 SPD with timsTOF Ultra2 for low-input NCI-7 peptide analysis.

To evaluate the robustness of single-cell level analysis, CV distributions from dilutions of NCI-7 cell line samples, were plotted as demonstrated in the Figure 2. timsTOF Ultra2, shows CV of 29.0%, at 15pg and less than 20% for the other dilutions. At 8 ng, CV impressively reduces to 4.1%. Both the timsTOF HT and timsTOF-Ultra2 instruments exhibited exceptional reproducibility at peptide inputs of 8 ng with an expected higher variation at low ends with timsTOF HT. The median quantitative fold-change values of all proteins increased with sample input and gradually approximated the true input ratios. The highest dynamic range was observed both at 8 ng NCI-7 peptide input on the timsTOF HT and 1 ng NCI-7 peptide input on the timsTOF Ultra2, achieving dynamic ranges of 4.8 log and 5.0 log orders, respectively (data not shown).

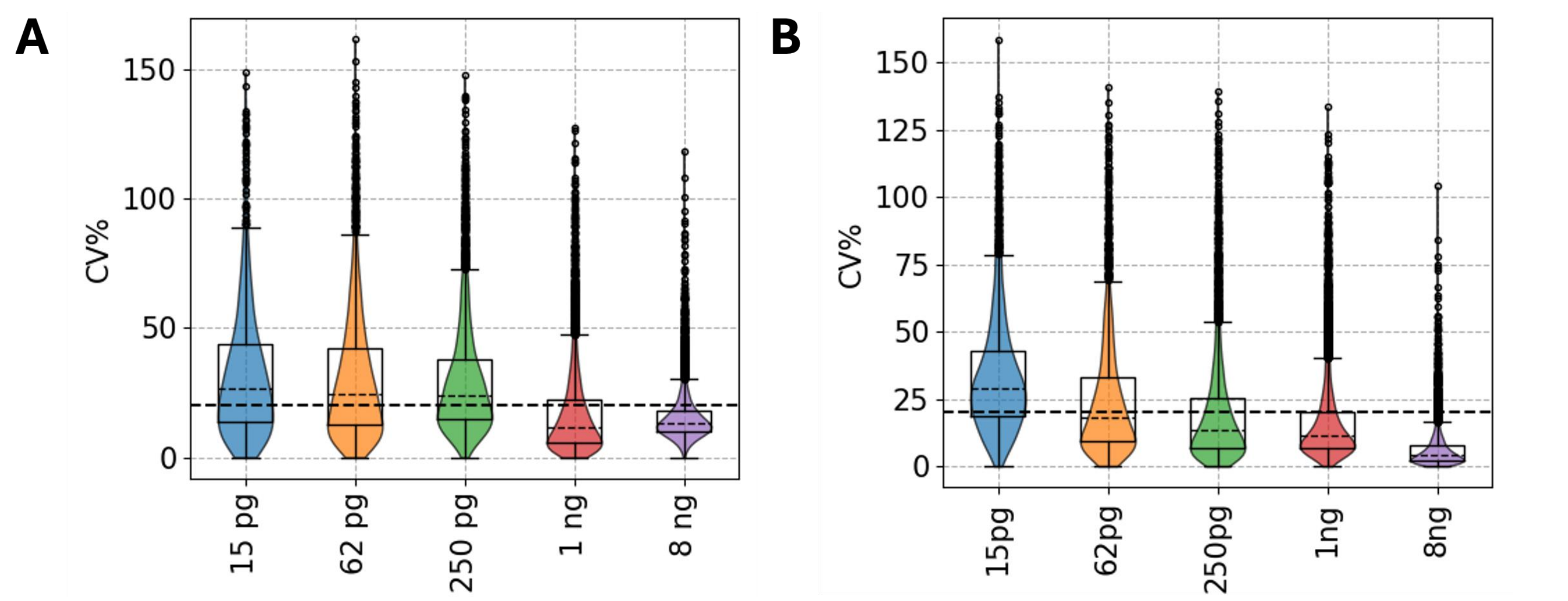

Figure 3. Figure 2. Quantification reproducibility of low amount peptide input. (A) CV distribution of diluted NCI-7 pep-tides on timsTOF HT. (B) CV distribution of diluted NCI-7 peptides on timsTOF Ultra2.

Then, single HeLa cells were analyzed using the WZ-40 SPD (Fig. 3). On the timsTOF HT, about 14,000 peptides and 2,500 proteins were quantified per single HeLa cell. On the timsTOF Ultra2, these numbers were over 26 k peptides and 4,400 proteins. A total of 1,002 proteins were consistently identified across all 62 cells, and 2,417 proteins were quantified in > 50% of the cells on timsTOF HT. Using the timsTOF Ultra2, 2,333 proteins were reproducibly identified, and 4,200 proteins were quantified in > 50% of total cells, It demonstrates great results on the timsTOF but obviously superior performance of the timsTOF Ultra2.

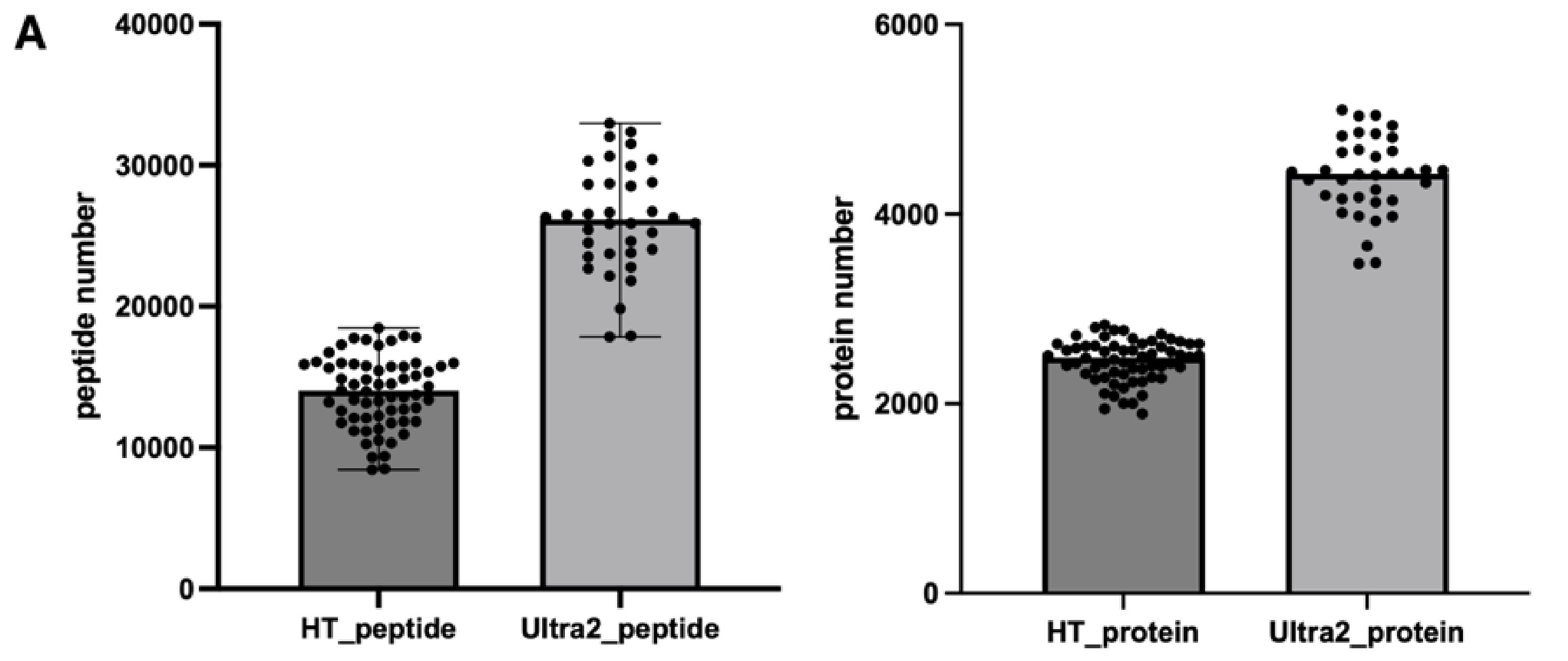

Figure 4. Number of protein identifications from single HeLa cells proteomic analysis. 62 single cells analyzed on timsTOF HT and 36 single cells analyzed on timsTOF-Ultra2.

Finally, we applied this technique to investigate the effects of chemotherapy treatment on PC-3 cells (cancer cell line) with 1 nM (low dose) and 10 nM (high dose) docetaxel for 72 hours using timsTOF HT. Cells treated with the high dose exhibited larger cell size and yielded a higher number of identified proteins (Fig. 4, top), whereas floating cells from the high-dose group showed the lowest protein identification. A total of 101 proteins were consistently identified in all high-dose treated adherent (live) cells but were detected in less than 50% of the high-dose treated floating cells. Enrichment analysis revealed that these proteins are primarily involved in RNA and protein regulation. Their absence in high dose treatment floating cells, likely due to apoptotic degradation, translational arrest, or cell membrane rupture, suggests that they may function as key mediators or potential biomarkers of cellular survival under therapeutic stress. Meanwhile, we observed that live cells treated with a high dose displayed the highest median CV among all groups (data not shown). Proteins that exhibited CV below 20% in untreated live cells, but not in high-dose treated live cells, were associated with RNA metabolic processes, cell–cell junctions, and autophagy. However, none of these biological processes reached statistical significance with FDR < 0.05 (Fig. 4, bottom). These findings suggest that single-cell analysis can reveal heterogeneous responses to drug treatment and may provide valuable insights into mechanisms of drug resistance.

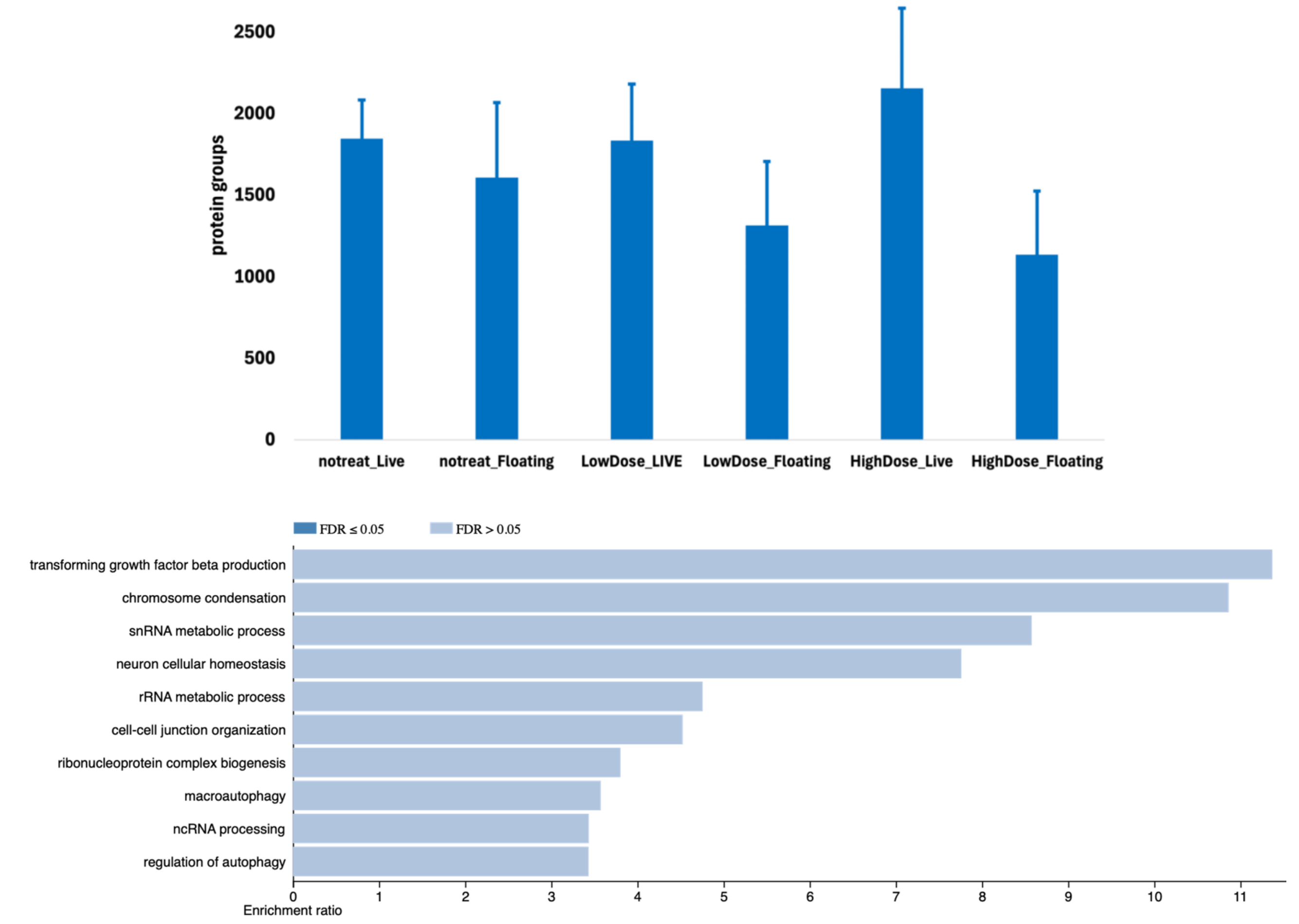

Figure 6: Single cell analyses for docetaxel treated PC3 cells. (Top) Protein identifications for all conditions. (Botton) Gene ontology analysis of proteins with CV < 20% in untreated live cells but not in high dose treated live cells.

Final Remarks

This work paves the way for future studies leveraging state-of-the-art instrumentation to explore cellular diversity, functional proteomics, drug response mechanism, and biomarker discovery with unprecedented depth.

timsTOF HT / timsTOF Ultra 2