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Sensitive and High-throughput Single-cell Proteomics Workflow on New Quadrupole-ion trap-Orbitrap Mass Spectrometer with FAIMS Separation

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INTRODUCTION

Traditionally, proteomics experiments are applied to large populations of cells, representing the average protein expression under given biological conditions. However, understanding the cellular heterogeneity provides insights that cannot be gained from bulk studies, such that the analysis of single-cell protein expression is of growing interest. Current LC-MS-based proteomics workflows have not been widely applicable to single cell analysis, mainly due to large sample losses during sample preparation, limited analytical sensitivity and low throughput. To address these challenges, we have combined nanoPOTS (Nanodroplet Processing in One-pot for Trace Samples) technology with tandem mass tag (TMT) isobaric labeling to analyze single mammalian cells containing ~0.2 ng total proteins on the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer with real time search and the Thermo Scientific™ FAIMS Pro™ Interface to improve single cell proteome coverage and enhance quantitation accuracy. The FAIMS-enhanced label-free workflow resulted in the identification of an average of 829 protein groups from single Hela cells with high-confidence MS^2 spectra. This was 3-fold higher than without FAIMS. The Thermo Scientific™TMT10plex™ analysis of three cultured murine cell populations were compared with MS^2 and SPS MS^3 method with Real Time Search. We have demonstrated that single cell proteomes can be quantified using label-free or TMT workflows by combing nanoPOTS with the Orbitrap Eclipse Tribrid mass spectrometer, and the FAIMS Pro Interface, enabling researchers to investigate cell heterogeneity as well as rare cells in an ultra-sensitive, higher throughput LC-MS analysis.

MATERIALS AND METHODS

Sample Preparation: Single cells were isolated from cultured murine and HeLa cells via fluorescence-activated cell sorting and cells were lysed, digested and labeled with TMT multiplexing reagents on nanoPOTS chip[1,2]. Thermo Scientific™ Pierce™ HeLa Protein Digest Standard was dissolved in sample loading buffer containing 2% Acetonitrile in 0.1% TFA and 0.1% FA with 30 second of vortexing and spinning down in concentration range of 0.5 ng/ul (equivalent to ~2 HeLa cells) for method development.

Methods: Single cell tryptic digest and single cell level HeLa digest (0.5 ng) were individually transferred to a short (4 cm) capillary tube and peptides were loaded to a 5 cm solid phase extraction (SPE) trap for peptide trapping with minimum sample loss followed by analytical peptide separation on analytical column (20 µm i.d, 3 um, 50cm for label-free analysis and 30 µm i.d, 1.7 um, 30cm from CoAnn Technologies for TMT10plex analysis) on a Thermo Scientific™ UltiMate™ 3000 RSLCnano system coupled to a PRSO-V2 Sonation column oven (sonation lab solutions) and new Orbitrap Eclipse Tribrid mass spectrometer with FAIMS Pro Interface. The ultra low nanoLC flow rate of 20n/min for single cell analysis was achieved through split flow set up[1].

Data Analysis: Single cell and single cell level data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.4 software with 2-stage SEQUEST search parameter including tryptic and semi tryptic search and percolator was used between each search to calculate the false discovery rate (FDR) and only those spectra with q-values lower than 0.01 were sent to the subsequent search filter and MaxQuant software for match between runs to estimate proteins in the blank sample analysis.

RESULTS

The performance of this ultra-sensitive LC-MS workflow was evaluated using the orbitrap and the ion trap for the detection of CID and HCD fragmentation spectra. The method was first optimized using 0.5 ng aliquots of Thermo Scientific Pierce HeLa Protein Digest Standard and later evaluated with single HeLa cell digests on nanoPOTS.

Figure 1. Label-Free Single Cell MS Method Optimization. MS and MS^2 in high resolution Orbitrap with HCD fragmentation shown to provide sensitivity and selectivity required for analysis of single cell proteomics. With a 2 hours gradient and two CV (compensation voltage) switching, 2023 protein groups and 8571 peptide groups were identified from 0.5 ng HeLa digest with FDR rate of 1% or better.

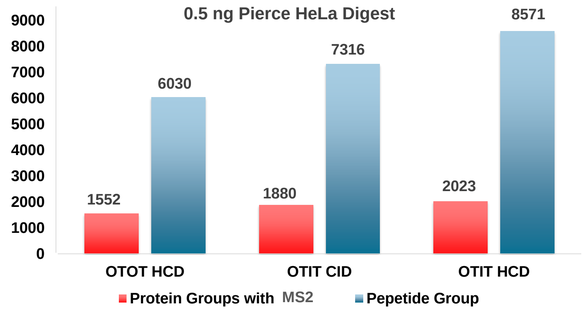


Figure 2. Advantage of FAIMS Pro Interface for Single Cell Analysis. FAIMS Pro Interface enhances peptide/proteome coverage in singe cell level by removing +1 charged chemical noise ions (red dots) which are suppressing multiply charged peptides ions (green dots) from single cell, allowing lower abundant peptides to be detected by MS.

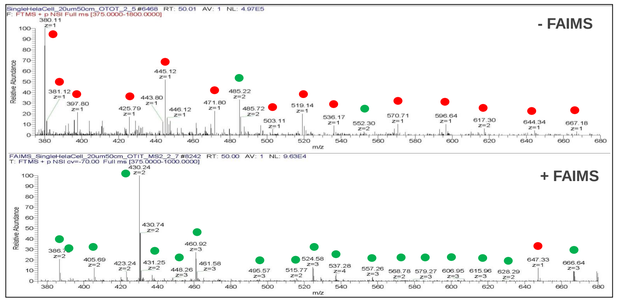


Table 1. LFQ Performance Enhancement with FAIMS Pro interface. FAIMS enhances proteome coverage in singe cell level and allows lower abundant peptide detection by MS.

	Initial Workflow	Optimized Method
Separation	30 µm i.d. column	20 µm i.d. column
Mass Spectrometer	Orbitrap Fusion Lumos Tribrid MS	Orbitrap Eclipse Tribrid MS+FAIMS Pro Interface
Data Analysis	MaxQuant	Proteome Discoverer 2.4 Software
Single HeLa Cell Protein Group ID by MS^2	211	829
100 HeLa Cells Protein Group ID by MS^2	2109	3067

FAIMS Pro interface provides performance gains required for improved protein coverage in LFQ proteomics workflow. ~3000 peptides and ~830 protein groups were identified from a single HeLa cell alone. This is the first example of >1000 proteins being identified from single mammalian cells with LFQ proteomics approach.

High-throughput Single Cell Protein Quantitation

To understand the biology of the cells in single cell level , we will need to analyze many cells hence there is a need to a proteomics method that offers higher throughput for single cell analysis. The MS^2 level single cell proteomics analysis on Thermo Scientific™ Orbitrap™ mass spectrometers is a well stablished approach[3]. Here we have evaluated the MS^2 and MS^3 level TMT single cell multiplexed LC-MS analysis on Orbitrap Eclipse Tribrid MS and optimized a method to improve quantitation accuracy without compromise on protein coverage.

Figure 3. High-throughput murine cell classification with TMT isobaric labeling. Total 16 single cell processed on two nanoPOTS chips were analyzed with SPS MS^3 with Real-Time Search method and total of 24 single cells were analyzed with MS^2 method. The PCA analysis (left) of TMT10plex analysis shows clear differentiation between the three different cell types (Raw Immune, Epithelial and Endothelial Cells) with both methods with SPS MS^3 with Real-Time Search providing improved accuracy with better separation between cell types without compromising protein coverage. Total 2346 protein and 4781 peptide groups were identification by TMT isobaric labeling (right) with improved quantitative accuracy and differential protein coverage with Real-Time Search for SPS MS^3.

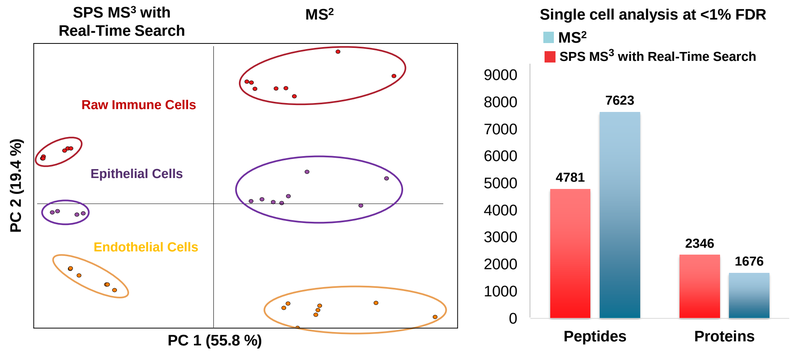


Figure 4. Improved Coverage of Differentially Expressed Proteins with SPS MS^3 with Real-Time Search. TMT analysis of two different cell types showed improved coverage of differentially expressed proteins when using SPS MS^3 with Real-Time Search as compared to MS^2 based TMT analysis. The method clearly differentiated the three cell types and improved quantitative accuracy without compromising in total number of proteins identified.

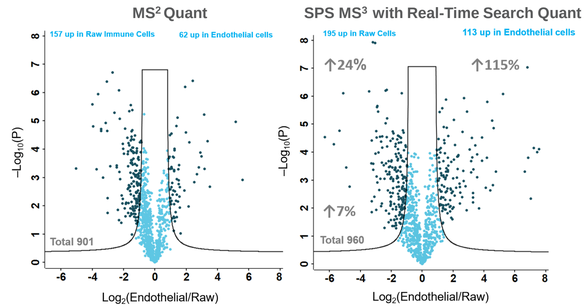
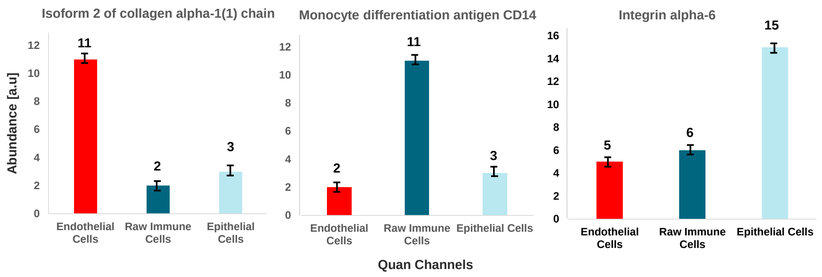


Figure 5. Differential Quantitation of Cell Type Biomarkers in Single Cells. Confirmation of overexpression of known protein biomarkers in epithelial, endothelial and raw immune individual cells shows the inherent sensitivity of this workflow. The bar charts show protein abundances for three different cell type biomarkers in the three cell lines analyzed.



CONCLUSIONS

- This ultra-sensitive low nanoflow LC-MS method, with FAIMS Pro interface and high resolution Orbitrap Eclipse Tribrid mass spectrometer's ion trap sensitivity has significantly improved single cell proteome coverage.
- The FAIMS Pro Interface has become a valuable tool for LFQ single cell proteomics analysis.
- The nanoPOTS platform combined with TMT multiplexed isobaric labeling provides a robust, high-throughput proteomic preparation method for handling extremely small biological samples like single cells.
- Reproducible quantitative proteome measurement with coverage of 2000 protein groups was achieved among a total of 40 single cells obtained from cultured murine cell populations.
- NanoPOTS integrated with multiplexed isobaric labeling represents a highly promising platform towards; single cell typing, understanding of stem cell development, proteomic studies of isolated clinical specimens (circulating tumor cells) and proteome imaging of tissue heterogeneity.

REFERENCES

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TRADEMARKS/LICENSING

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