Deeper Protein Identification by using FAIMS in Top-Down Proteomics

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ABSTRACT. Field Asymmetric Ion Mobility Spectrometry (FAIMS), when used in proteomics studies, provides superior selectivity, and enables more proteins to be identified by providing additional gas phase separation. Here, we tested the performance of cylindrical FAIMS for the identification and characterization of proteoforms by top-down mass spectrometry of heterogeneous protein mixtures. Combining FAIMS with chromatographic separation resulted in a 62% increase in protein identifications and an 8% increase in proteoform identifications as compared to samples analyzed without FAIMS. This increase was attributable, in part, to improved signal-to-noise for proteoforms with similar retention times. Additionally, our results show that the optimal compensation voltage of any given proteoform was correlated with the molecular weight of the analyte.

Collectively these results suggest that the addition of FAIMS can enhance top-down proteomics in both discovery and targeted applications.

INTRODUCTION

Mass spectrometry (MS) based proteomics relies on separation of peptides and proteins, as extracts from cells, tissues and fluids create highly complex mixtures. Due to proteome heterogeneity, multi-dimensional separations are critical to reduce sample complexity prior to tandem MS¹⁻³. For molecules of analytes to be sampled for fragmentation in a standard, “data-dependent” mode, its precursor intensity must be above the chemical/instrumental background noise and be one of the more abundant targets being measured¹. Moreover, separation of molecular species occurring at similar mass-to-charge ratios is essential for the acquisition of non-chimeric fragmentation spectra to achieve unambiguous, efficient protein identification by database retrieval. Frequently, the separation technique used online with MS for proteomics is liquid chromatography (LC) because of its versatility and high peak capacity⁴.

Multi-dimensional chromatographic separation of digested peptide species helped usher in the technology underlying bottom-up proteomics (BUP)^{5, 6}. By combining more than one chromatographic separation method, techniques like MudPIT increased the number of protein identifications by reducing spectral heterogeneity^{5, 6}. While this technique has been promulgated internationally, similar platforms for in-line top-down proteomics (TDP) analysis of intact proteins have remained in a less developed state. Off-line separation of proteins prior to LC, dramatically increases the number of protein identifications in top-down experiments³. One technique that could enable multidimensional on-line protein separation for TDP analysis is Field Asymmetric Ion Mobility Spectrometry (FAIMS)⁷ after LC separation and prior to MS analysis, circumventing the limitations faced by multiple hyphenated on-line or off-line fractionation steps³.

Cylindrical FAIMS is a gas-phase fractionation approach that transmits ions based on their differential mobility in high and low electric fields⁷⁻¹¹. In the context of a typical TDP experiment, intact proteins are ionized by an electrospray source and enter the FAIMS device, composed of an inner and outer electrode⁷. A dispersion voltage provides an asymmetric waveform that propels ions towards one of the electrodes. Collision with either of the electrodes halts unstable ions, preventing their introduction to the mass spectrometer. Application of different compensatory DC voltages (compensation voltage, or CV) to the inner electrode of the FAIMS modifies the mobility of proteins, allowing them to pass through the FAIMS for detection⁷. Hence, FAIMS can function as a gas-phase separation device for intact protein ions.

Prior utilization of FAIMS has improved peptide identification for BUP applications^{12, 13} and functions primarily through improving analyte signal-to-noise¹⁴⁻¹⁶. Its use in TDP analysis has been limited, with most investigators studying intact protein standards. Investigators employing ion-mobility-time-of-flight mass spectrometry systems have shown that IMS influences the arrival time distribution of different peptides, denatured protein charge states and multimeric native protein structures¹⁷⁻²⁰. Our previous study employed static electrospray ionization (ESI) coupled with a commercial implementation of FAIMS to separate and analyze a simple mixture of heavy and light chains from a monoclonal antibody²¹. Without gas-phase fractionation, the light chain dominates the precursor ion signal in MS experiments, resulting in low signal-to-noise and poor detection of the heavy chain. Utilization of FAIMS prior to MS resolved the two antibody chains by favoring the FAIMS stability of the heavy or light chain of the antibody while suppressing the other, all as a function of the applied compensation voltage²¹. Additionally, combining liquid extraction surface analysis (LESA) with FAIMS provided a clear improvement in signal-to-noise of intact precursor ion signal compared to the same LESA samples analyzed without FAIMS²²⁻²⁶. Building from these initial observations of complex protein samples, we sought to characterize denatured proteoforms from complex protein samples using LC-FAIMS-MS/MS. The goal of the present study was to

test and benchmark the performance of cylindrical FAIMS during TDP analysis of heterogeneous protein mixtures on an Orbitrap Fusion Lumos mass spectrometer. Our results show that combining FAIMS with reversed-phase LC separation enhances the number of both identified proteins and characterized proteoforms.

METHODS

Sample preparation: Top-down standard (TDS) was prepared following the instructions from the National Resource for Translational and Developmental Proteomics (NRTDP) <http://nrtdp.northwestern.edu/protocols/>. Primary human CD3+ T cells were obtained from STEMCELL Technologies.

Cell lysate samples were prepared as described previously²⁷. Briefly, 1×10^9 cells were lysed in 1 mL of lysis buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 1% (w/vol) N-laurylsarcosine, 1 mM $MgCl_2$, and 1x Halt Inhibitor Cocktail (EDTA free) in distilled water). The lysate was treated with 1,500 units of benzonase nuclease (Sigma-Aldrich) for 15 min. at room temperature, precipitated with four volumes of ice-cold acetone, and placed at $-80^\circ C$ for 30 min. Samples were centrifuged at $21,000 \times g$ for 10 minutes at $4^\circ C$, the acetone was removed, and the protein pellets were re-suspended in 1% SDS (w/v). Protein concentration was estimated using BCA protein assay kit (Thermo Fisher Scientific). Three-hundred micrograms of protein lysate was loaded onto 8% GELFrEE (AbCam) cartridges per lane and electrophoresed following manufacturer's instructions. Fraction one containing the 0-30 kDa proteins was precipitated using methanol-chloroform-water²⁷. The protein pellets were then resuspended in 200 μL of buffer A (0.2% formic acid, 5% Acetonitrile, 95% ddH₂O).

LC-FAIMS-MS/MS: Liquid chromatography (LC) was performed using 150 μm i.d. x 3 cm L PLRP-S (5 μm particle, 1000 Å pore size - Agilent Technologies) pre-column, to trap proteins, prior to fractionation over a 75 μm i.d. x 14 cm L PLRP-S (5 μm particle, 1000 Å pore size - Agilent Technologies) column using a 90-minute buffer B (0.2% formic acid, 95% Acetonitrile, 5% ddH₂O) gradient. Separation

was performed on a Dionex Ultimate 3000 (Thermo Fisher Scientific) under the following gradient: 5% B at 10 min., 15% B at 12 min., 55% B at 62 min., 95% B from 67 to 72 min., 5% B from 75 to 90 min. LC was coupled online with FAIMS Pro (Thermo Fisher Scientific) interfaced with an Orbitrap Fusion Lumos (Thermo Fisher Scientific) mass spectrometer. Nitrogen was used as a carrier gas for FAIMS and no supplementary user-controlled carrier gas flow (0 L/min) or 1 L/min. was used depending on the experiment. Compensation voltages (CV) were kept constant during each sample injection and chromatographic separation. CVs were adjusted in 10 V increments between replicate sample injections and ranged from -70V to +30V. LC was online with the mass spectrometer for samples analyzed without the FAIMS Pro interface.

Precursor ion spectra were acquired in the Orbitrap analyzer at a resolving power setting of 120,000 (at m/z 200), AGC was set to 1×10^6 charges/acquisition, with 200 ms of maximum injection time and four μ scans. Fragmentation of the top two most abundant spectra in data dependent mode were also acquired in the Orbitrap analyzer at a resolving power setting of 60,000 (at m/z 200). Precursors were quadrupole isolated used using a 3 m/z isolation window, dynamic exclusion of 60 s duration, and threshold of 2×10^4 intensity. Target AGC values of 5×10^5 charges/acquisition, 800 ms of maximum injection time, and 4 μ scans were used. Ions were fragmented using HCD at a setting of 23%. In source collision induced dissociation was enabled at 15 V in all acquisition types.

Data analysis: The TD Portal platform v3.1 from NRTDP (<https://portal.nrtdp.northwestern.edu/>) operating on the Galaxy server was used to match top-down spectra to protein and proteoform targets from the database. Raw files were searched against the human proteome (UniProt release 2016_04). All protein and proteoform identifications were reported at a 1% FDR²⁸. Comparison of proteins and proteoforms identified with and without FAIMS was accomplished using TDViewer software (<http://topdownviewer.northwestern.edu/>) and using only identifications from Tight Absolute Mass and BioMarker search types. Venn diagram generators, R studio GGPlot2, and the TreeView clustering

graphical user interface²⁹ were also used to analyze the data. Raw files are available at massIVE under the identifier MSV000086748.

RESULTS AND DISCUSSION

Given past success using the FAIMS Pro interface to differentially fractionate reduced antibody chains in the gas-phase, we expanded the complexity of our analysis by testing the capacity of LC coupled to FAIMS to separate the four proteins present in the top-down standard (TDS) mixture. We performed replicate injections of TDS over 80 min. reversed-phase LC gradients with and without FAIMS prior to MS analysis. In samples that utilized FAIMS, we monitored the precursor ion signal in stepwise 10 V compensation voltage (CV) increments from -30V to +20V using no supplementary carrier gas flow. As expected, the injection without FAIMS showed all four proteins at their expected retention times consistent with previous studies³⁰ (**Figure 1**, top panel). The most intense protein in the total ion chromatogram (TIC) was carbonic anhydrase (29 kDa) followed by myoglobin (16.7 kDa), and ubiquitin (8.6 kDa) (**Figure 1**). Trypsinogen (24 kDa) was the least intense peak observed and eluted between ubiquitin and myoglobin. In the no-FAIMS analysis, the intensity of trypsinogen is typically lower relative to the more abundant TDS protein peaks in the chromatogram. Analysis of TDS with FAIMS and employing a CV of -30V resulted in a waveform that favored the stable trajectory of only ubiquitin as shown on the second panel of **Figure 1**. As the CV was increased by 10V in subsequent LC injections, the remaining protein standards were observed with larger protein species generally preferring more positive CVs, consistent with previous observations²¹ (**Figure 1**). The exception among the standards was trypsinogen, which contains disulfide bonds. Intriguingly, the folded structure of trypsinogen reduces the collisional cross section and affects the protein's trajectory during differential ion mobility in the gas phase compared to the other unfolded proteins; we observed trypsinogen to be stable at CVs -20V and -10V similar to that of the much smaller protein, myoglobin.

During TDP analysis of complex protein mixtures, abundant proteins and proteoforms are detected at the expense of lower-abundance forms. The selective stability of TDS proteins at different CVs suggested that FAIMS may be useful as an orthogonal separation technique for proteoforms with retention times similar to more abundant co-eluting ions. As a model system for testing the behavior of heterogeneous protein populations in the LC-FAIMS-MS/MS system, we employed the 0-30 kDa protein fraction of whole cell extracts from a population of 20 million primary human CD3⁺ T cells afforded by employing an 8% GELFrEE cartridge²⁷. Without supplementary carrier gas flow, we sought to identify the CVs that would result in the most proteoforms identified from a single injection ranging from -40V to +30V. **Figure 2A** demonstrates that the majority of the proteoforms were detected using a CV of -20 V with a Gaussian-like distribution spanning between -40 and +30 V.

In BUP, the use of FAIMS Pro interface has been shown to increase the number of protein identifications when used as an orthogonal fractionation method^{12, 13}. Similarly, we tested if FAIMS separation would result in gains in protein or proteoform identifications. We compared eight replicate injections of <30 kDa proteins from CD3⁺ T cells without FAIMS to eight injections with FAIMS applying CVs between -40 V and +30 V, which showed the highest numbers of identified proteoforms in our previous experiment. Analysis of all 16 runs (with and without FAIMS) resulted in 367 protein identifications (**Figure 2B**) and 1,537 proteoform identifications (**Figure 2C**). The application of FAIMS resulted in an 8% increase in proteoform identifications (**Figure 2C**) and a 62% increase of protein identifications, with 337 proteins identified with FAIMS compared to 208 proteins identified without FAIMS (**Figure 2B**). The increase in identifications occurred over the same LC separation conditions and instrument acquisition time.

To characterize the diversity of proteins identified from each replicate with and without FAIMS we generated a heatmap using protein spectral counts (**Figure 3A**). From eight identical injections without FAIMS, we observed a conserved and stable population of proteins that were detected (**Figure 3A**).

In contrast, changing the CV by 10 V for each injection resulted in the identification of a fraction of proteins that were unique to each CV (**Figure 3A**). The CVs with the largest number of new unique IDs were -40V, -30V, and -20V. These results demonstrate that, in TDP experiments, FAIMS can: 1) boost the identification of proteins/proteoforms that would otherwise be not fragmented and identified with only the LC separation alone, and 2) function as a selection filter for the identification of specific proteins/proteoforms from complex samples.

FAIMS improves signal-to-noise by enhancing for the analyte of interest relative to background. Stable ions pass through the FAIMS unit while other ions are deposited onto the FAIMS electrodes. Past experiments with FAIMS would suggest that the identification of new proteins at specific compensation voltages during orthogonal separation might be due to an improvement of signal-to-noise of those proteins^{21, 22, 24}. To test this possibility, we compared precursor spectra (with or without FAIMS) at retention times where a given protein was only identified by FAIMS. While there were several positive examples throughout the experiment, we focused our analysis on PFR295102 from Arachidonate 5-lipoxygenase-activating protein (UniProt accession P20292; **Figure 3B**). At a retention time of 41.57 minutes and a CV of -40 V, the dominant 7+ precursor ion was observed with FAIMS. At the identical retention time (without FAIMS) the precursor ion was not observable above background signal. Fragmentation spectra of the sample that utilized FAIMS showed high quality protein characterization with fragment ions mapping to 52% of backbone positions (**Figure 3C**), while no fragment ions were detected in the samples analyzed without FAIMS. Given the importance of Arachidonate 5-lipoxygenase-activating protein in leukotriene synthesis and inflammation³¹⁻³⁴, the capacity of FAIMS to facilitate characterization of this proteoform and others demonstrates the potential impact of this technique in the analysis of complex biological samples. These results suggest that FAIMS improves the identification and characterization of novel proteins from heterogeneous samples in part through improvement of signal-to-noise ratios of both precursor ions and their fragmentation products.

Aiming to understand the effect of carrier gas flow on protein and proteoform fractionation in the FAIMS Pro device, we replicated the 8 injections with FAIMS using CVs from -40V to $+30\text{V}$ and applied a supplementary carrier gas flow of 1 L/min. Using these conditions, we identified 212 proteins and 704 proteoforms, representing a decrease of 37% and 32%, respectively, in the number of identifications compared to the analysis performed without supplemental gas. The use of 1 L/min. of carrier gas resulted in reduced overall ion signal, suggesting a lower sensitivity under these conditions. Plotting the molecular weight of the identified proteoforms in each CV we observed an inter-relationship between them (**Figure 4A**). The coefficient of correlation (R^2) between CV and molecular weight at 1 L/min. carrier gas levels was 0.60 and without supplementary carrier gas flow was 0.49 (**Figure 4B**). These results show that when the cylindrical FAIMS is operated at higher gas levels, overall sensitivity is reduced while selectivity of proteoforms by mass is improved. Hence the elevated gas modes might benefit targeted TDP analysis rather than discovery modes. These results highlight the efficacy of cylindrical FAIMS as a mass selection filter in TDP acquisitions and opens a new range of possibilities for the field.

CONCLUSION

The FAIMS Pro interface coupled to high resolution MS after reversed-phase LC increased the number of protein and proteoform identifications in TDP experiments performed on 0-30 kDa protein mixtures derived from human CD3^+ T cells. The multi-dimensional LC-FAIMS separation occurred over the same instrument acquisition time as samples employing LC-MS alone, but resulted in 62% more protein identifications. Additionally, through modification of carrier gas flow and CV, FAIMS can function as an effective molecular weight filter for proteoforms, providing a mechanism for predictive selectivity of targets from complex samples. Ultimately, FAIMS improvements are attributable to improved signal-to-noise ratios, resulting in the identification of proteoforms in samples that have coeluting species and highly complex matrices. The use of FAIMS in the TDP field will help to achieve deeper proteome characterizations in both discovery and targeted modes of operation.

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Author Contributions

VRG, RDM, JPD, PMT, NLK contributed to plan the experiments, generating the figures, and writing of the paper. VRG, SA, RDM, DD performed experiments and/or analyzed the data. SA, RH, and MB supported and helped to design the project. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Notes

NLK consults for Thermo Fisher Scientific. MB and RH are employees of Thermo Fisher Scientific.

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Figure 1: Total ion chromatograms (TICs) of top-down standards using either FAIMS at different CVs or no FAIMS. Top-down standards run without FAIMS (top panel) and with FAIMS using compensation voltages (CV) of -30V, -20V, -10V, 0V, +10V, and +20V (lower panels). Top-down standard proteins: ubiquitin, 8.6 kDa (U); trypsinogen, 24 kDa (T); myoglobin, 16.7 kDa (M); carbonic anhydrase, 29 kDa (C).

Figure 2: Protein and proteoform identifications with or without FAIMS. (A) Histogram of CD3⁺ T cell proteoforms identified at each compensation voltage (CV). Venn diagram of (B) proteins and (C) proteoforms identified from CD3⁺ T cells subjected to top-down proteomics analysis with FAIMS or without FAIMS.

Figure 3. Changing the compensation voltage (CV) results in detection of a hidden population of proteins through improved signal-to-noise. (A) Protein-level heatmap of CD3⁺ T cell proteins identified without FAIMS (8 technical replicates) or with FAIMS applying different CVs from -40V to +30V. CVs for injections analyzed with FAIMS are indicated at the bottom of the heatmap. (B) Mass spectrum of proteoform PFR295102 from UniProt accession P20292 (Arachidonate 5-lipoxygenase-activating protein) analyzed with FAIMS (top panel) and without FAIMS (lower panel) at same retention time. (C) Fragmentation map of PFR295102 showing 52% sequence coverage in the sample using FAIMS.

Figure 4. Effect of compensation voltage and gas levels on proteoform fractionation when using FAIMS to separate human CD3⁺ T cell proteins. Box and whisker plot of proteoform molecular weight (MW) vs. compensation voltage (CV) when proteoforms are fractionated (A) in the presence of 1 L/min of supplemental carrier gas or (B) without supplemental carrier gas.