Published in final edited form as:

Anal Chem. 2015 December 15; 87(24): 12230–12237. doi:10.1021/acs.analchem.5b03199.

Decreased gap width in a cylindrical FAIMS device improves protein discovery

Kristian E. Swearingen, Jason M. Winget, Michael R. Hoopmann, Ulrike Kusebauch, and Robert L. Moritz*

Abstract

High-field asymmetric waveform ion mobility spectrometry (FAIMS) is an atmospheric pressure ion mobility technique that separates gas phase ions according to their characteristic dependence of ion mobility on electric field strength. FAIMS can be implemented as a means of automated gas-phase fractionation in liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments. We modified a commercially-available cylindrical FAIMS device by enlarging the inner electrode, thereby narrowing the gap and increasing the effective field strength. This modification provided a nearly four-fold increase in FAIMS peak capacity over the optimally configured unmodified device. We employed the modified FAIMS device for on-line fractionation in a proteomic analysis of a complex sample and observed major increases in protein discovery. NanoLC-FAIMS-MS/MS of an unfractionated yeast tryptic digest using the modified FAIMS device identified 53% more proteins than were identified using an unmodified FAIMS device, and 98% more proteins than were identified with unaided nanoLC-MS/MS. We describe here the development of a nanoLC-FAIMS-MS/MS protocol that provides automated gas-phase fractionation for proteomic analysis of complex protein digests. We compare this protocol against pre-fractionation of peptides with isoelectric focusing and demonstrate that FAIMS fractionation yields comparable protein recovery while eliminating the need for additional sample handling.

TOC image

Keywords

FAIMS; ion mobility; proteomics

*To whom correspondence should be addressed: Dr. Robert L. Moritz, Institute for Systems Biology, 401 Terry Ave N, Seattle, WA 98109, USA, robert.moritz@systemsbiology.org, Phone: +1-206-732-1200.

INTRODUCTION

High-field asymmetric waveform ion mobility spectrometry (FAIMS) is an atmospheric pressure ion mobility separation technique in which gas phase ions are separated according to the dependence of ion mobility on electric field strength^{1–2}. A carrier gas moves analyte ions between two electrodes in a direction orthogonal to the electric field. Voltage is applied to the FAIMS electrodes as an asymmetric waveform that alternates between a period of low field strength of one polarity and a shortened period of high field strength of the opposite polarity. The strength of the asymmetric waveform is quantified as the dispersion voltage (DV, also presented here as the dispersion field E_D in V/cm) which is the magnitude of the high field portion of the waveform. Ions carried through the FAIMS device by the carrier gas oscillate between the electrodes as the polarity switches. Differences in ion mobility at high and low field strength result in the ions having a net displacement toward one electrode or the other, and only a small subset of ions with a particular ratio of high and low-field mobilities will reach the detector without expiring on the electrodes. Addition of a direct current compensation voltage (CV, also presented here as the compensation field E_C in V/cm) changes which ion mobility ratio will be at equilibrium. A FAIMS device is thus a tunable filter for gas-phase ions.

Because FAIMS separates gas phase ions at atmospheric pressure, it is readily implemented between an electrospray ionization (ESI) source and a mass spectrometer (MS)³. Identification and quantification of proteins by liquid chromatography and tandem mass spectrometry (LC-MS/MS) can be improved by employing FAIMS as a means of automated, on-line fractionation^{4–5}. To date, all published reports of improved protein discovery in analysis of complex protein digests (e.g. whole cell lysates) have employed a commercially-available FAIMS device comprised of concentric cylindrical electrodes (first Ionalytics^{4–5}, then Thermo Fisher Scientific^{6–10}). Modifications to the device have made it possible to couple capillary nanoLC columns to FAIMS^{6,9} and to employ nanospray ionization (NSI) and sub- μ L/min flow rates with FAIMS. The improvement in protein discovery afforded by FAIMS fractionation has been shown to be comparable to pre-fractionation by strong cation exchange, though the two techniques revealed different subsets of peptides¹⁰. The best performance with this particular FAIMS device has been achieved by controlling the electrode temperatures¹¹ and employing a 1:1 mixture of He and N₂ as the carrier gas¹², producing a peak capacity of seven for separation of tryptic peptide ions⁶. For comparison, a FAIMS device of comparable dimensions but employing planar electrodes achieved a peak capacity of ten when operated under comparable conditions¹³, and has as high as 200 when separation time was increased (albeit at the cost of duty cycle and sensitivity)¹⁴. Barnett and Ouellett showed that the peak capacity of the cylindrical FAIMS device could be increased to 17 with no apparent loss in sensitivity by enlarging the inner electrode, thereby narrowing the gap and increasing dispersion field strength without increasing the applied dispersion voltage¹². Importantly, these improvements were achieved with pure N₂ as the carrier gas, eliminating the need for He, an increasingly expensive and non-renewable resource.

We describe here development of a nanoLC-FAIMS-MS/MS protocol that provides automated fractionation of peptides in the gas-phase for proteomic analysis of complex

protein digests. We modified a commercially-available cylindrical FAIMS device (Thermo Fisher Scientific) by enlarging the inner electrode in order to take advantage of the increased peak capacity without decreasing throughput, and have characterized its performance for analysis of peptides from complex protein digests. We observed major improvements in FAIMS separation of peptide ions when the electrode gap was reduced. Although increased FAIMS peak capacity was achieved at the cost of decreased signal, this decrease was offset by a dramatic increase in signal-to-noise, and protein identifications by data-dependent “shotgun” LC-MS/MS using this approach increased with increasing FAIMS peak capacity irrespective of signal loss. When compared to off-line peptide fractionation by isoelectric focusing, on-line fractionation with the modified FAIMS device achieved comparable protein identification while eliminating the need for additional off-line sample handling, thereby increasing throughput, reducing sample loss, and minimizing the required amount of starting sample material.

EXPERIMENTAL PROCEDURES

Sample Preparation

Tryptic digests of BY4742 *Saccharomyces cerevisiae* were prepared by standard methods as previously described⁹. Extended methods are provided in the Supporting Information. Separate preparations of yeast digest were used to compare the various FAIMS configurations and to compare peptide fractionation by reduced-gap FAIMS versus OFFGEL isoelectric focusing. Isoelectric fractionation was performed on an Agilent 3100 OFFGEL fractionator according to the manufacturer’s specification using 13 cm Immobiline DryStrip Gels (GE Healthcare), pH 3–10. Either 20 µg or 200 µg of peptides (nominal mass based on mass of protein digested as determined by BCA assay) were focused at 20 kV with a maximum current of 50 µA and a maximum voltage set to 8000 V. Each of the 12 fractions from 20 µg or 200 µg of sample was re-suspended in 20 µL or 200 µL of 0.1% TFA, respectively. Nine µL of each fraction were injected for analysis by nanoLC-MS/MS, corresponding to a nominal total of 9 µg of peptides analyzed for each sample preparation.

A 2.0 mg/mL solution of bovine serum albumin (BSA; Pierce) was diluted to 1.0 mg/mL in 100 mM ammonium bicarbonate (ABC) and digested with trypsin, desalted, dried under rotary vacuum and reconstituted in 50% methanol and 0.1% formic acid to a nominal concentration of 1 pmol/µL.

FAIMS instrumentation

Analyses were performed on an LTQ-Orbitrap Elite (Thermo Fisher Scientific) enabled with a FAIMS device. The FAIMS device has been described previously^{6,11}. Briefly, the device was comprised of two concentric cylindrical electrodes with an effective length of 25 mm. The outer electrode had an inner radius of 9 mm and the inner electrode had an outer radius of 6.5 mm, creating a gap of 2.5 mm. The temperatures of the inner and outer electrodes could be independently regulated. We modified the device by replacing the inner electrode with a custom fabricated electrode having identical specifications as the original, except with an increased radius of 7.75 mm, thus reducing the gap between electrodes to 1.25 mm¹². The unmodified device was operated with a FAIMS carrier gas of N₂ or 1:1 He:N₂

supplied at 3.5 L/min, a DV of $-5,000\text{ V}$ ($E_D = -20\text{ kV/cm}$), and the inner and outer electrodes maintained at 70°C and 90°C , respectively. The modified FAIMS device with a reduced gap was operated with a carrier gas of N_2 supplied at 2.5 L/min, a DV of $-4,000\text{ V}$ ($E_D = -32\text{ kV/cm}$), and the inner and outer electrodes maintained at 70°C and 90°C , respectively, or 90°C and 70°C . Selection of FAIMS carrier gas flow rate was critical to optimal separation and ion transmission efficiency¹² (Supplementary Text). A fused silica capillary LC column with an inner diameter (I.D.) of $75\text{ }\mu\text{m}$ and fritted pulled tip with an I.D. of $15\text{ }\mu\text{m}$ (Picofrit, New Objective) was interfaced with the FAIMS device via a HESI-II heated electrospray source (Thermo Fisher Scientific) that was modified to be used as a nanospray source⁹. Spray voltages were 1.6 kV to 1.8 kV . (Applied voltages were 2.6 kV to 2.8 kV with a FAIMS entrance plate voltage of 1.0 kV).

Infusion of a bovine serum albumin tryptic digest

Infusion experiments were performed using an unpacked capillary column with an I.D. of $75\text{ }\mu\text{m}$ pulled to a fritted tip with $15\text{ }\mu\text{m}$ I.D. (Picofrit, New Objective). A solution of BSA tryptic peptides was infused at 300 nL/min . Full scan MS1 spectra from $200\text{--}2000\text{ m/z}$ were collected in the Orbitrap with a nominal resolution of $120,000$ at 400 m/z , a full scan automatic gain control (AGC) target of $1,000,000$ charges, and a maximum ion trap fill time of 200 ms . Non-FAIMS data were collected using a nanospray source (Thermo Fisher Scientific) with a spray voltage of 1.8 kV . FAIMS data were collected with the modified nanospray source described above at a spray voltage of 1.8 kV . In order to directly compare ion transmission efficiency, variable ion trap fill times were corrected for by dividing the recorded signal for each ion by the ion trap fill time for the spectrum in which it was recorded.

Four different FAIMS configurations were tested: the unmodified device with N_2 carrier gas; the unmodified device with $1:1\text{ He:N}_2$ carrier gas; the modified, narrow-gap device with N_2 carrier gas and the inner and outer electrodes maintained at 70°C and 90°C , respectively; and the modified device with the inner and outer electrodes maintained at 90°C and 70°C , respectively. The performance of the various configurations was assessed by collecting MS1 scans at CVs over the range of 0 to -80 V using the FAIMS Compensation Voltage Ion Map Method in the XCalibur instrument control software (Thermo Fisher Scientific). The method only allows for a minimum CV step of 1.0 V , which was sufficient resolution to characterize the unmodified electrode. In order to characterize the narrower FAIMS peaks produced by the modified FAIMS electrodes, two separate scanning experiments offset by 0.5 V were performed for each experimental condition and combined in order to obtain spectra at 0.5 V intervals. The FAIMS dwell time (a programmed delay in acquisition after switching CV) was set to 1 msec , the minimum allowed by the instrument control software. We determined that it was unnecessary to program a delay because the instrumental latency when switching between CVs was $\sim 125\text{ msec}$, while the residence time of ions in the FAIMS device is $\sim 70\text{ msec}$ with unmodified electrodes and $\sim 40\text{ msec}$ with a reduced gap (see Supporting Information). FAIMS peaks (monoisotopic ion peak intensity with respect to E_C) were analyzed for a total of 60 peptide ions selected based on the following criteria: observed monoisotopic mass within 0.004 Da of the predicted mass of a fully tryptic BSA peptide 7 or greater residues in length with no missed cleavages; $m/z >$

300 m/z and < 2000 m/z ; and a FAIMS peak clearly defined in all four FAIMS electrode configurations. FAIMS separation efficiency was quantified by peak capacity P , the number of FAIMS peaks (signal intensity with respect to E_C) that can be resolved in a given separation window, as

$$P = \frac{|E_{c1} - E_{c2}|}{0.5 \times (w_1 + w_2)}$$

where E_{c1} and E_{c2} are the E_c of the FAIMS peaks defining the separation window (in this case, the highest and lowest observed), and w_1 and w_2 are the respective FAIMS peak widths at half maximum.

NanoLC-MS/MS and nanoLC-FAIMS-MS/MS

Chromatography was performed on a column with a fritted integrated tip (New Objective Picofrit, 360 μm O.D., 75 μm I.D., 15 μm I.D. tip) packed in-house with a 20 cm bed of C18 silica (Dr. Maisch GmbH, ReproSil-Pur C18-AQ, 120 $\text{\AA}$ pore, 3 μm particle size; Ammerbuch-Entringen, Germany). Prior to loading on the column, sample was loaded onto a trap consisting of a KASIL fritted capillary (360 μm O.D., 150 μm I.D.) packed with a 1 cm bed of the same stationary phase. For each run in the experiments comparing FAIMS electrode configurations, 2 μg of tryptic peptides were injected onto the trap and washed with loading buffer (2% v/v acetonitrile and 0.2% v/v trifluoroacetic acid in water) at 6 $\mu\text{L}/\text{min}$ as provided by an Agilent 1100 with electronically controlled split flow. HPLC mobile phase (Solvent A = 0.1% v/v formic acid in water, solvent B = 0.1% v/v formic acid in acetonitrile) was supplied at 500 nL/min by an Agilent 1100 with electronically controlled split flow. The gradient was 5% B to 35% B over 120 min, followed by a 20 min wash at 80% B and a 30 min re-equilibration step at 5% B.

Multiple nanoLC-FAIMS-MS/MS and nanoLC-MS/MS experimental parameters were compared. Each experiment consisted of eight separate nanoLC-MS/MS or nanoLC-FAIMS-MS/MS analyses of a 2 μg injection of unfractionated yeast tryptic digest. Each nanoLC-FAIMS-MS/MS analysis was performed at a different fixed CV, selected as described below. Precursor MS1 scans over the range of 300 – 1600 m/z were collected in the Orbitrap with a nominal resolution of 120,000 at 400 m/z . The top 30 precursors were selected for collision-induced dissociation (CID) in the ion trap using data-dependent acquisition (DDA). Monoisotopic precursor selection was enabled, and only doubly- or triply-charged precursors were fragmented. A minimum precursor intensity of 10,000 or 1,000 was required for activation in non-FAIMS and FAIMS experiments, respectively. Dynamic exclusion was employed with an exclusion list of up to 500 precursors. Precursors were excluded with a ± 5 ppm tolerance for 45 seconds after a single observation or after the precursor level was observed a single time at an intensity below twice the signal-to-noise, whichever came first. The top 30 precursors were isolated with a 2.0 m/z window and fragmented by CID for 10 msec at a normalized collision energy of 35. The total instrument time required for eight analyses, including column re-equilibration between injections, was just under 24 h. The control experiment consisted of eight replicate nanoLC-MS/MS analyses.

The eight CVs expected to yield the most unique peptide identifications for each FAIMS configuration were determined empirically from FAIMS libraries created by performing DDA analyses of the yeast tryptic digest at CVs covering the entire analytically useful range of CVs in 1.0 V increments (Supporting Information). The CVs selected were as follows: for the unmodified FAIMS device with 100% N₂ carrier gas, -12.0, -14.0, -16.0, -18.0, -20.0, -24.0, -28.0, and -32.0 V; for the unmodified FAIMS device with 1:1 He:N₂ carrier gas, -22.0, -26.0, -30.0, -33.0, -36.0, -40.0, -46.0, and -56.0V; for the modified FAIMS device with the inner/outer electrodes at 90°C/70°C, -16.0, -18.0, -20.0, -23.0, -26.0, -29.0, -33.0, and -41.0V; and for the modified FAIMS device with the inner/outer electrodes at 70°C/90°C, -18.0, -19.5, -21.0, -22.5, -24.0, -25.5, -27.0, and -30.0V.

The experiments comparing FAIMS and OGE were performed using a Proxeon easy-nLC (Thermo Fisher Scientific) employing the same LC mobile phases and gradient as above, but supplied at 300 nL/min. The mass spectrometer settings were the same as above except that a precursor range of 400–1600 *m/z* was employed and activation threshold of 1000 counts was employed for all experiments. The reduced-gap FAIMS device was operated with inner and outer electrodes maintained to 70°C and 90°C, respectively. The NSI-FAIMS source was operated with a sheath gas of 3. The twelve CVs, selected as described above based on a FAIMS map of the yeast sample, were -19.0, -21.0, -22.0, -23.5, -25.0, -27.0, -29.0, -31.0, -33.0, -35.0, -37.0, and -40.0V.

MS1-based gas-phase fractionation

A gas-phase fractionation (GPF) method similar to that described by Scherl *et al.*¹⁵ was also tested. The nanoLC conditions were as described above. The MS conditions were as described above except that for each of the eight nanoLC-MS/MS analyses, the MS1 precursor scan was restricted to a different range of *m/z* values. The eight *m/z* bins were as follows: 300–484, 479–596, 591–698, 693–796, 791–895, 890–1000, 559–1234, and 1229–1600. The *m/z* bins were selected to equally distribute the number of theoretically observable peptide precursors in the yeast proteome. The overlap in bin boundaries was included to account for edge cases. The distribution of potential peptide *m/z* values was obtained from an *in silico* digest of the Uniref non-redundant yeast reference proteome (www.uniprot.org), restricting theoretical peptides to those with a peptide length of 7 to 34 residues and a charge of +2 or +3.

Spectrum matching and protein inference

Mass spectra (Thermo Fisher Scientific .raw files) were converted to mzML format using MSConvert¹⁶ (version 2.2.0) and searched with Comet¹⁷ (version 2013.02 rev. 2). Peptide and protein inference were performed with the Trans-Proteomic Pipeline¹⁸ (version 4.7 POLAR VORTEX rev. 0). Extended methods are provided in the Supporting Information.

RESULTS AND DISCUSSION

Modification of a cylindrical FAIMS device

A commercially-available cylindrical FAIMS device was evaluated in four configurations: the unmodified FAIMS device was evaluated with pure N₂ or a mixture of He and N₂ as the

carrier gas, and a modified device with a reduced electrode gap was evaluated with two different electrode temperature schemes while employing pure N₂ as the carrier gas. The unmodified FAIMS device was comprised of an inner electrode with a radius of 6.5 mm and an outer electrode bored with an inner radius of 9.0 mm, creating a 2.5 mm gap. The instrumental parameters for the unmodified FAIMS device have been empirically optimized by us and others¹⁹. The DV was -5.0 kV ($E_D = -20$ kV/cm), the maximum allowed by the instrument control software, to exploit the fact that both FAIMS separation efficiency and ion transmission through the FAIMS device increase at higher E_D in cylindrical FAIMS devices²⁰. The inner and outer electrodes were maintained at 70°C and 90°C, respectively. The FAIMS carrier gas was either N₂ or a 1:1 mixture of He and N₂. Addition of He to FAIMS carrier gas has been shown to improve FAIMS performance^{21–25}. The modified FAIMS device was comprised of the standard outer electrode bored with a 9.0 mm inner radius and an enlarged inner electrode with a radius of 7.75 mm, creating a gap of 1.25 mm. The modified device was evaluated with the goal of avoiding the need for He in the carrier gas, and instead improving performance by modifying electrode temperature^{11–12}. The maximum DV that could be maintained without arcing was -4.0 kV ($E_D = -32$ kV/cm), affording a 60% increase in field strength without any modification to the waveform generator. The carrier gas was pure N₂, and the inner and outer electrodes were evaluated at 70°C and 90°C, respectively, or reversed at 90°C and 70°C, respectively.

Quantification of FAIMS performance

The performance of the FAIMS configurations was compared by infusing an unfractionated bovine serum albumin tryptic digest (Figure 1). Results are summarized in Table 1. Separation efficiency was quantified by FAIMS peak capacity. Ion transmission efficiency was quantified as the relative peptide ion intensities with FAIMS compared to infusion of the same sample without a FAIMS device present. The FAIMS electrode modifications described here altered performance in a manner consistent with theory (see Supporting Information). Reducing the electrode gap significantly improved FAIMS peak capacity, though the increase in separation efficiency was accompanied by some loss of ion transmission efficiency. Signal loss could be mitigated at the cost of peak capacity by modifying electrode temperature.

Adjustments to FAIMS electrode dimensions that increase selectivity are usually accompanied by decreases in sensitivity²⁶. Assessing signal losses associated with the various FAIMS configurations was complicated by the fact that the ion trap mass analyzer employed automatic gain control (AGC), allowing the trap to fill for as long as 200 ms for each scan in order to achieve a desired number of ions prior to analyzing the trap contents. As such, the raw ion signal values did not reflect the actual analyte ion concentrations in the ion stream, but rather the ion concentrations multiplied by the trap fill time, which varied scan-to-scan. Ion trap fill times were between 4 and 200 msec for mass spectra acquired with FAIMS compared to 0.18 msec without FAIMS. The decrease in ions entering the mass analyzer can be attributed to both the filtering property of FAIMS as well as ion transfer inefficiencies. To accurately quantify ion transmission efficiency among the various FAIMS device configurations and assess any signal loss incurred by use of FAIMS, ion signal

intensities were normalized to the ion trap fill time for each spectrum. We also report unadjusted signal intensities and signal-to-noise ratios.

The enhanced separation efficiency of the reduced gap FAIMS device was achieved at a cost of decreased ion transmission efficiency that was offset by a significant increase in signal-to-noise (Table 1; Figure 2). Peak capacity of the modified FAIMS device with inner electrode cooler than outer electrode was nearly four-fold greater than that of the unmodified FAIMS device employing He. However, ion signal intensities corrected for ion trap fill time for the modified FAIMS device were nearly six-fold lower than with the unmodified FAIMS device and a median 900-fold lower than without FAIMS. At the same time, the signal-to-noise for the modified device was nearly three-fold greater than for the unmodified device and 17-fold greater than without FAIMS. As predicted by theory and demonstrated by others^{11–12}, inverting the electrode temperatures such that the inner electrode was warmer than the outer electrode improved ion transmission efficiency while simultaneously decreasing FAIMS peak capacity; peptide ion signal intensities increased by a median 76% while peak capacity decreased nearly three-fold and signal-to-noise dropped by almost half.

The above results show that FAIMS performance in terms of separation and ion transmission efficiency can be significantly altered by modifying electrode gap, carrier gas composition, and electrode temperature. Increasing the strength of E_D by decreasing the electrode gap improved separation at the cost of ion transmission efficiency, though the effect of signal loss was mitigated by the AGC, and the decreased spectral complexity led to substantial improvements in signal-to-noise. Ion transmission efficiency was improved at the cost of peak capacity by changing the electrode temperature. The question remained whether sensitivity or selectivity would confer the greatest benefit to bottom-up “shotgun” proteomics aided by FAIMS fractionation. To that end, each of the four FAIMS configurations was evaluated for analysis of a complex sample.

Increased FAIMS peak capacity improves protein discovery

To compare the protein identification performance of the four FAIMS configurations, an unfractionated yeast tryptic digest was analyzed by nanoLC-MS/MS and nanoLC-FAIMS-MS/MS. The metric chosen for comparison was the number of unique peptides and proteins identified from 24 h of instrument time. This was sufficient time to perform eight analyses with 2 h gradients and requisite wash and re-equilibration steps. Each of the eight nanoLC-FAIMS-MS/MS analyses was performed at a different CV. The optimum CVs for each FAIMS configuration were determined empirically (see Supporting Information). Eight replicate nanoLC-MS/MS analyses of unfractionated digest were performed as a control (see Supporting Information). Additionally, a technique employing gas-phase fractionation (GPF) of precursor ions within the mass analyzer was performed (see methods)¹⁵. The run parameters for the GPF experiment were identical to the control except that for each nanoLC-MS/MS analysis the MS1 survey scan was restricted to a narrow range of m/z values. The width of the m/z bins was determined by the density of peptide precursors predicted to be observed based on an *in silico* digest of the yeast proteome. Like other fractionation methods, this gas phase fractionation technique helps to alleviate limitations on

mass analyzer duty cycle by reducing the spectral complexity of the sample. Like FAIMS, it is also completely automatable and requires no additional sample handling.

Gas-phase fractionation by FAIMS increased total protein identification compared to both unaided analysis of unfractionated sample and narrow MS1 mass range GPF (Table 2). The most proteins were identified using the modified FAIMS device with the electrode temperatures set to increase peak capacity despite decreased signal intensity. This configuration yielded 3163 protein identifications, 86% more than were identified by GPF. Importantly, all but 43 of the proteins identified by GPF were also identified by employing the modified FAIMS device (Figure 3). 35 of these were identified from only one peptide, and the others were identified from two or three peptides and five or fewer peptide spectrum matches each. The fact that the narrow MS1 mass range GPF technique provided little improvement over standard nanoLC-MS/MS suggests that spectral complexity was more of a barrier to protein discovery than was instrument duty cycle. While GPF helps to address duty cycle issues by decreasing the number of precursors to be considered for fragmentation, it does not decrease spectral complexity or reduce interference from co-eluting peptides of similar m/z . FAIMS fractionation addresses these issues by pre-filtering the analyte ion stream prior to filling the ion trap.

It is noteworthy that the largest increase in identified proteins occurred using the electrode configuration with the highest peak capacity and the poorest ion transmission efficiency. One important benefit of cylindrical FAIMS electrodes compared to parallel planar electrodes is the greater sensitivity afforded by ion focusing²⁰. However, the results presented here suggest that protein discovery by DDA methods is best served by increasing separation power in order to decrease spectral complexity and increase signal-to-noise, even at the cost of signal intensity. Protein discovery improved as the cylindrical FAIMS device was modified to reverse the effect of ion focusing, essentially approximating planar electrodes. While increasing the inner electrode diameter conferred a greater than six-fold improvement in peak capacity, planar FAIMS devices can achieve much greater peak capacity at the cost of further losses in sensitivity^{13,27}.

Comparison of on-line fractionation by FAIMS and off-line pre-fractionation by isoelectric focusing

On-line fractionation by nanoLC-FAIMS-MS/MS using the optimized FAIMS configuration described above was next compared against nanoLC-MS/MS analysis of the same sample pre-fractionated off-line by a widely-used technique: isoelectric focusing using OFFGEL electrophoresis (OGE). OGE separates peptides based on their isoelectric point by applying a voltage along an immobilized pH gradient gel²⁸. Compared to other fractionation methods, OGE can tolerate a broad range of input material (from 50 μ g to 5 mg). One limitation of OGE is that the length of the immobilized gel strip determines resolution and maximum number of fractions. Additionally, the fractionation itself takes approximately 16–24 h (depending on the length of the strip), and requires an additional four to six h for solid-phase extraction (SPE) and sample drying prior to LC-MS/MS.

A new preparation of yeast lysate was digested with trypsin and desalted by C18 SPE. The nominal mass of peptides in the sample was estimated based on BCA quantification of the

lysate prior to digestion. The sample was analyzed by nanoLC-FAIMS-MS/MS employing the reduced gap FAIMS device with inner and outer electrodes maintained at 70°C and 90°C, respectively, to maximize FAIMS peak capacity as determined in this study. A total of 12 nanoLC-FAIMS-MS/MS analyses of the unfractionated peptide sample were performed with 2 h separation gradients, each at a different fixed CV. A volume containing 0.75 µg of peptides was injected for each analysis, totaling 9 µg of sample for the experiment. For comparison, an equal amount of unfractionated peptides was analyzed by 12 replicate nanoLC-MS/MS runs without FAIMS. Prefractionation of the tryptic digest by OGE was performed on 200 µg of peptides (well within the manufacturer's specified loading capacity) or 20 µg of peptides (simulating sample-limited conditions). Peptides were separated into 12 fractions over a *pI* range from 3 – 10. Sample volume was selected such that a total of 9 µg of peptides were analyzed for each preparation.

On-line fractionation by FAIMS and off-line fractionation by OGE performed similarly with respect to protein discovery (Table 3). The most proteins were identified from the OGE fractionation from 200 µg of starting material. FAIMS fractionation yielded 7.1% fewer protein identifications than OGE with ample starting material, but 6% more than OGE with limited starting material. There was a large overlap between the proteins identified by the three analyses (Figure 4); fewer than 8% of the proteins identified from the combined FAIMS and OGE analyses were unique to any one analysis, and the majority of these identifications were inferred from a single peptide.

In the case of sample-limiting conditions, preparation of proteomic samples incorporating standard metabolic or *in vitro* labels is exceedingly difficult. In such cases, the lack of processing and handling prerequisites needed make label-free quantification an attractive technique. The proteins identified from the OGE and FAIMS analyses were quantified using normalized spectral abundance factor (NSAF)^{29–30}. SAF is calculated as the number of peptide spectrum matches for a given protein normalized to the protein's length. This value is in turn normalized to the summed SAF values of all proteins identified from the sample, facilitating comparison between experiments and run conditions. NSAF values correlated very well between OGE and FAIMS data: the plot comparing NSAF values of sample limited OGE versus FAIMS had a slope of 1.0 and R^2 of 0.81, and the plot for OGE of 200 µg of peptides versus FAIMS had a slope of 0.96 and R^2 of 0.81 (see Supporting Information).

These results demonstrate that nanoLC-FAIMS-MS/MS with a reduced-gap cylindrical FAIMS device improves protein discovery in a manner comparable to a widely-used pre-fractionation technique, but with the following advantages: fractionation is performed on-line, thereby reducing sample handling (and thus the opportunity for sample loss and contamination) as well as reducing sample preparation time (in this case, by over 24 h); and fractionation is completely automated, increasing throughput. Taken together, these results present FAIMS as a powerful tool for the proteomics laboratory that provides superior identification capabilities in a fully automated manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Science Foundation MRI grant number 0923536, the National Center for Research Resources of the National Institutes of Health under award number S10 RR027584, the National Institute of General Medical Sciences of the National Institutes of Health under award numbers GM087221 and P50 GM076547/Center for Systems Biology, and by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number K25 AI119229. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the National Science Foundation.

References

1. Buryakov IA, Krylov EV, Nazarov EG, Rasulev UK. *Int J Mass Spectrom.* 1993; 128:143–148.
2. Shvartsburg AA. *Differential Ion Mobility Spectrometry*. 1. CRC Press; Boca Raton, FL: 2009.
3. Purves RW, Guevremont R. *Anal Chem.* 1999; 71:2346–2357. [PubMed: 21662783]
4. Venne K, Bonneil E, Eng K, Thibault P. *PharmaGenomics.* 2004; 4:30–40.
5. Venne K, Bonneil E, Eng K, Thibault P. *Anal Chem.* 2005; 77:2176–2186. [PubMed: 15801752]
6. Canterbury JD, Yi X, Hoopmann MR, MacCoss MJ. *Anal Chem.* 2008; 80:6888–6897. [PubMed: 18693747]
7. Saba J, Bonneil E, Pomies C, Eng K, Thibault P. *J Proteome Res.* 2009; 8:3355–3366. [PubMed: 19469569]
8. Bridon G, Bonneil E, Muratore-Schroeder T, Caron-Lizotte O, Thibault P. *J Proteome Res.* 2012; 11:927–940. [PubMed: 22059388]
9. Swearingen KE, Hoopmann MR, Johnson RS, Saleem RA, Aitchison JD, Moritz RL. *Mol Cell Proteomics.* 2012; 11:M111 014985. [PubMed: 22186714]
10. Creese AJ, Shimwell NJ, Larkins KP, Heath JK, Cooper HJ. *J Am Soc Mass Spectrom.* 2013; 24:431–443. [PubMed: 23400772]
11. Barnett DA, Belford M, Dunyach JJ, Purves RW. *J Am Soc Mass Spectrom.* 2007; 18:1653–1663. [PubMed: 17662612]
12. Barnett DA, Ouellette RJ. *Rapid Commun Mass Spectrom.* 2011; 25:1959–1971. [PubMed: 21698679]
13. Shvartsburg AA, Tang K, Smith RD. *Anal Chem.* 2010; 82:32–35. [PubMed: 19938817]
14. Shvartsburg AA, Smith RD. *Anal Chem.* 2011; 83:23–29. [PubMed: 21117630]
15. Scherl A, Shaffer SA, Taylor GK, Kulasekara HD, Miller SI, Goodlett DR. *Anal Chem.* 2008; 80:1182–1191. [PubMed: 18211032]
16. Kessner D, Chambers M, Burke R, Agus D, Mallick P. *Bioinformatics.* 2008; 24:2534–2536. [PubMed: 18606607]
17. Eng JK, Jahan TA, Hoopmann MR. *Proteomics.* 2013; 13:22–24. [PubMed: 23148064]
18. Deutsch EW, Mendoza L, Shteynberg D, Slagel J, Sun Z, Moritz RL. *Proteomics Clin Appl.* 2015; 9:745–754. [PubMed: 25631240]
19. Swearingen KE, Moritz RL. *Expert Rev Proteomic.* 2012; 9:505–517.
20. Guevremont R, Purves RW. *Rev Sci Instrum.* 1999; 70:1370–1383.
21. McCooley MA, Ells B, Barnett DA, Purves RW, Guevremont R. *J Anal Toxicol.* 2001; 25:81–87. [PubMed: 11300511]
22. Barnett DA, Ells B, Guevremont R, Purves RW. *J Am Soc Mass Spectrom.* 2002; 13:1282–1291. [PubMed: 12443018]
23. Shvartsburg AA, Tang K, Smith RD. *Anal Chem.* 2004; 76:7366–7374. [PubMed: 15595881]
24. Shvartsburg AA, Danielson WF, Smith RD. *Anal Chem.* 2010; 82:2456–2462. [PubMed: 20151640]

25. Shvartsburg AA, Ibrahim YM, Smith RD. J Am Soc Mass Spectrom. 2014; 25:480–489. [PubMed: 24402673]
26. Shvartsburg AA, Tang K, Smith RD. J Am Soc Mass Spectrom. 2005; 16:2–12. [PubMed: 15653358]
27. Shvartsburg AA, Li F, Tang K, Smith RD. Anal Chem. 2006; 78:3706–3714. [PubMed: 16737227]
28. Horth P, Miller CA, Preckel T, Wenz C. Mol Cell Proteomics. 2006; 5:1968–1974. [PubMed: 16849286]
29. Zybailov B, Mosley AL, Sardi ME, Coleman MK, Florens L, Washburn MP. J Proteome Res. 2006; 5:2339–2347. [PubMed: 16944946]
30. Gokce E, Shuford CM, Franck WL, Dean RA, Muddiman DC. J Am Soc Mass Spectrom. 2011; 22:2199–2208. [PubMed: 21952779]

Figure. 1. FAIMS separation of a tryptic peptide mixture

A 1 pmol/ μ L tryptic digest of bovine serum albumin was infused at 300 nL/min and analyzed by NSI-FAIMS-MS using four different FAIMS configurations. FAIMS peaks (signal with respect to compensation voltage) for nine representative peptides are shown. FAIMS peak capacity, peak width, and signal intensity were affected by modifying the FAIMS electrode gap width (g), carrier gas composition, and electrode temperature (see Table 1). Compensation voltage is reported as $|E_C|$, the absolute value of the applied potential divided by gap width.

Figure 2. Ion transmission efficiency and signal-to-noise with FAIMS

Four FAIMS device configurations were evaluated for ion transmission efficiency and improvement in signal-to-noise by infusing a bovine serum albumin tryptic digest and comparing signal between NSI-MS and NSI-FAIMS-MS. The plots show quartile distributions of the results for the peptide mixture. The greatest signal obtained for a given tryptic peptide ion with FAIMS is shown as a percentage of the signal for the same peptide obtained without FAIMS for (a) the observed peptide ion signal intensities, and (b) the peptide ion signal intensities corrected for ion trap fill time. (c) Signal-to-noise is shown as the fold increase with FAIMS over no FAIMS.

Figure. 3. A FAIMS device with reduced electrode gap increases protein discovery

A yeast tryptic digest was analyzed by data-dependent liquid chromatography-mass spectrometry for protein discovery with fractionation of gas-phase peptides by FAIMS or by narrow MS1 precursor scan range. This Venn diagram depicts the overlap in proteins identified when employing the modified or unmodified cylindrical FAIMS devices or the narrow MS1 mass range technique. The unmodified FAIMS device had an electrode gap of 2.5 mm and employed a carrier gas of 1:1 He and N_2 . The modified FAIMS device had an electrode gap of 1.25 mm and employed a carrier gas of N_2 only. Both FAIMS devices had the inner and outer electrodes maintained at 70 °C and 90 °C, respectively.

Figure 4. Overlap in proteins discovered using FAIMS or isoelectric focusing

The Venn diagram depicts the overlap in proteins identified from a yeast tryptic digest analyzed by data-dependent liquid chromatography-mass spectrometry employing either on-line FAIMS fractionation or off-line pre-fractionation by OFFGEL isoelectric focusing (OGE). The OGE samples were prepared either from 200 µg of protein (well within the manufacturer's specified loading capacity) or 20 µg (simulating sample-limited conditions). The FAIMS analysis employed a reduced-gap modified device with electrode temperatures set to maximize FAIMS peak capacity.

Table 1

Performance of FAIMS devices with various configurations

The performance of four FAIMS electrode configurations was evaluated by infusing a bovine serum albumin tryptic digest. (a) Peak capacity was calculated using the peak width at half maximum of the two FAIMS peaks with the highest and lowest optimal compensation voltage of ion transmission. Ion transmission efficiency for a given peptide ion was calculated as the greatest signal obtained with FAIMS relative to the signal without FAIMS. The median ion transmission efficiency values are reported as calculated with (b) the unadjusted signal intensity and (c) the signal normalized to ion trap fill time. (d) The improvement in signal-to-noise is reported as the median fold improvement over no FAIMS.

Electrode gap (mm)	Carrier gas	Electrode temperature (inner/outer)	Dispersion field (kV/cm)	Peak capacity ^a	Ion transmission efficiency		Signal-to-noise improvement ^d
					Unadjusted ^b	Corrected ^c	
2.5	N ₂	70°/90°	-20	3.84	8.27%	0.16%	4.50
2.5	He/N ₂	70°/90°	-20	6.48	19.8%	0.69%	6.31
1.25	N ₂	90°/70°	-32	8.40	11.9%	0.18%	9.61
1.25	N ₂	70°/90°	-32	24.7	12.9%	0.11%	17.1

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 2

Protein identification with gas-phase fractionation

A yeast tryptic digest was analyzed by nanoLC-FAIMS-MS/MS employing the modified and unmodified FAIMS device and compared against analysis of the same sample by nanoLC-MS/MS employing either MS1 survey scan gas-phase fractionation or no fractionation.

Gas-phase fractionation technique	Electrode gap	Carrier gas	Electrode temperature (inner/outer)	Dispersion field	Proteins identified ^a	Single hit proteins ^b	Peptide spectrum matches ^c	Peptides identified ^d
Modified FAIMS	1.25 mm	N ₂	70°/90°	−32 kV/cm	3,163	14.3%	60,790	29,862
	1.25 mm	N ₂	90°/70°	−32 kV/cm	2,697	14.9%	68,082	26,815
Unmodified FAIMS	2.5 mm	He/N ₂	70°/90°	−20 kV/cm	2,072	14.3%	71,356	20,236
	2.5 mm	N ₂	70°/90°	−20 kV/cm	1,707	15.4%	71,908	16,739
Narrow MS1 mass range	–	–	–	–	1,696	20.6%	29,420	11,975
None	–	–	–	–	1,597	19.7%	131,346	14,051

^aYeast proteins identified with ProteinProphet probabilities corresponding to a false positive error rate (FPER) < 1%.

^bPercentage of protein identifications inferred by a single peptide.

^cPeptide spectrum matches identified with an iProphet probability corresponding to a FPER < 1%.

^dUnique peptide sequences identified, disregarding variable modifications and charge state.

Table 3
FAIMS compared with isoelectric focusing for protein identification

A yeast tryptic digest was analyzed by nanoLC-FAIMS-MS/MS employing the modified FAIMS device with the inner and outer electrodes maintained at 70 °C and 90 °C, respectively, and compared against nanoLC-MS/MS analysis of the same sample that was first fractionated by OFFGEL isoelectric focusing (OGE) or not fractionated at all.

Fractionation technique	Amount of peptide sample fractionated	Amount of peptide sample analyzed	Proteins identified ^a	Single hit proteins ^b	Peptide spectrum matches ^c	Peptides identified ^d
FAIMS	9 µg	9 µg	2,789	13.0 %	43,528	21,609
OFFGEL isoelectric focusing	200 µg	9 µg	3,004	15.5 %	68,254	25,715
	20 µg	9 µg	2,630	14.4 %	55,336	22,384
None	N/A	9 µg	1,493	18.5 %	114,980	12,129

^a Yeast proteins identified with ProteinProphet probabilities corresponding to a false positive error rate (FPER) < 1%.

^b Percentage of protein identifications inferred by a single peptide.

^c Peptide spectrum matches identified with an iProphet probability corresponding to a FPER < 1%.

^d Unique peptide sequences identified, disregarding variable modifications and charge state.