Published in final edited form as:

Anal Chem. 2018 August 07; 90(15): 9529–9537. doi:10.1021/acs.analchem.8b02233.

Comprehensive Single-Shot Proteomics with FAIMS on a Hybrid Orbitrap Mass Spectrometer

Alexander S. Hebert[†,▽], **Satendra Prasad**[‖], **Michael W. Belford**[‖], **Derek J. Bailey**[‖], **Graeme C. McAlister**[‖], **Susan E. Abbatiello**[⊥], **Romain Huguet**[‖], **Eloy R. Wouters**[‖], **Jean-Jacques Dunyach**[‖], **Dain R. Brademan**[‡], **Michael S. Westphall**[†], and **Joshua J. Coon**[*,†,‡,§,▽,#]

[†]Genome Center of Wisconsin, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[‡]Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[§]Department of Biomolecular Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[▽]DOE Great Lakes Bioenergy Research Center, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

[‖]Thermo Fisher Scientific, San Jose, California 95134, United States

[⊥]Thermo Fisher Scientific, Cambridge, Massachusetts 02139, United States

[#]Morgridge Institute for Research, Madison, Wisconsin 53706, United States

Abstract

Liquid chromatography (LC) prefractionation is often implemented to increase proteomic coverage; however, while effective, this approach is laborious, requires considerable sample amount, and can be cumbersome. We describe how interfacing a recently described high-field asymmetric waveform ion mobility spectrometry (FAIMS) device between a nanoelectrospray ionization (nanoESI) emitter and an Orbitrap hybrid mass spectrometer (MS) enables the collection of single-shot proteomic data with comparable depth to that of conventional two-dimensional LC approaches. This next generation FAIMS device incorporates improved ion sampling at the ESI-FAIMS interface, increased electric field strength, and a helium-free ion transport gas. With fast internal compensation voltage (CV) stepping (25 ms/transition), multiple

[*]**Corresponding Author** jcoon@chem.wisc.edu.

Notes

The authors declare the following competing financial interest(s): S.E.A., M.W.B., D.J.B., G.C.M., S.A., R.H., E.R.W., and J.D. are employees at Thermo Fisher Scientific, which develops and distributes FAIMS instruments.

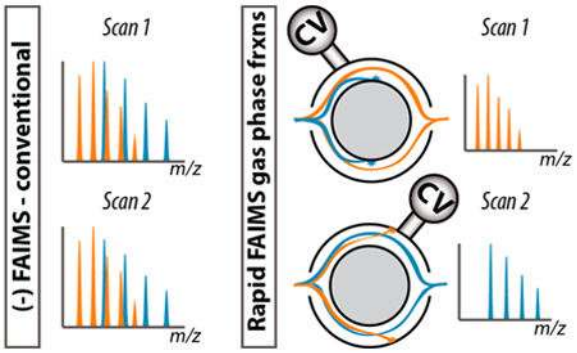
The .RAW files supporting this publication are available online at the Chorus project (project ID: 1496).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02233. Supporting information and methods, characterization of peptides over the range of tested CVs, unique peptides per protein comparison with and without FAIMS and LC fractions, FAIMS inner electrode temperature effect on peak capacity, selecting the three or four CVs to combine for maximum peptide identifications, instrument method diagrams and external vs internal CV stepping, peptide intensity reproducibility and cycle time, and beneficial characteristics of FAIMS analyses (PDF)

unique gas-phase fractions may be analyzed simultaneously over the course of an MS analysis. We have comprehensively demonstrated how this device performs for bottom-up proteomics experiments as well as characterized the effects of peptide charge state, mass loading, analysis time, and additional variables. We also offer recommendations for the number of CVs and which CVs to use for different lengths of experiments. Internal CV stepping experiments increase protein identifications from a single-shot experiment to >8000, from over 100 000 peptide identifications in as little as 5 h. In single-shot 4 h label-free quantitation (LFQ) experiments of a human cell line, we quantified 7818 proteins with FAIMS using intra-analysis CV switching compared to 6809 without FAIMS. Single-shot FAIMS results also compare favorably with LC fractionation experiments. A 6 h single-shot FAIMS experiment generates 8007 protein identifications, while four fractions analyzed for 1.5 h each produce 7776 protein identifications.



Modern mass spectrometers are capable of collecting tandem mass spectra at great speed and sensitivity.[1,2] Recent advances in chromatography and precursor characterization have increased the number of precursors available for sampling and, consequently, the achievable proteomic depth.[1,3] Still, a single-shot data-dependent acquisition (DDA) experiment is limited to detection of only about half of the expressed proteome (i.e., ~6500 proteins of ~13 000 present). We believe that the central limitation in these analyses is separation peak capacity.[4] This conclusion is evinced by the common use of two-dimensional liquid chromatography (2DLC) for complex protein mixtures. In this practice each fraction is analyzed separately by nano liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS), resulting in increased total peak capacity and increased proteomic depth.[2,5] Such 2DLC approaches add considerable labor, increase sample amount requirements, and can become very cumbersome, especially when tens or hundreds of samples are compared. Here we describe the coupling of a high transmittance, low ion transit time cylindrical high-field asymmetric ion mobility spectrometer (FAIMS) device with an Orbitrap hybrid mass spectrometer.[6–9] This new FAIMS technology allows for rapid and effective gas-phase separation of peptide ions as they depart the electrospray emitter and prior to their entrance into the mass spectrometer. With this system we demonstrate an increased separation capability and proteomic depth that allows deep proteome analyses without 2DLC.

FAIMS separations have been characterized at length in the literature.[10–13] In brief, FAIMS sources transmit ions between inner and outer electrodes based on their difference in mobility when in a high or low electric field, generated from an asymmetric waveform. Since this field causes the ions to disperse, the maximum peak amplitude of the asymmetric

waveform is referred to as the dispersion voltage (DV). Ions with a large enough difference in mobility between the high and low field migrate toward the electrodes, while ions with no or limited difference in mobility are transmitted. The trajectory of an ion may be altered by the addition of a dc voltage. Termed as the compensation voltage (CV), the selection of an appropriate dc level will compensate for the drift of a specific ion or group of ions, allowing them to pass through the device. By changing the CV, alternate groups of ions will pass. Thus, the CV provides a handle by which one can control which population of ions are traversing the FAIMS device. The mobility, and thus the appropriate CV to use, cannot be easily predicted and must be determined empirically.

FAIMS holds the promise of delivering gas-phase fractionation for proteomics analyses by stepping through multiple CVs, deconvolving overlapping peptide signals, and removing contaminants.[14–18] Gas-phase fractionation has become standard for many time-of-flight proteomics methods using either traveling wave ion mobility mass spectrometry or trapped ion mobility spectrometry.[19,20] Previous implementations of both types of FAIMS electrodes, planar and cylindrical, have typically imparted reduced sensitivity to mass spectrometry experiments because of low ion maximum transmission (transmission at the optimal CV for an analyte). The more commonly used planar electrodes offer greater resolution yet suffer from diffusion causing a portion of the ion population to crash into the electrodes.[21] The gas dynamics of cylindrical electrodes cause the vast majority of ions to collide with the center electrode upon entrance into the device.[6,22,23] Recently, a new FAIMS device with improved gas dynamics, resulting in high maximal transmission (>70%), has been described.[6,7] In addition to reducing losses due to collisions with the inner electrode the flow rate of the carrier gas has also been increased. Part of this redesign resulted in the gap between the inner and outer electrode being reduced, allowing for an increased electric field strength from existing drive electronics. The result is a reduced ion transit time (~20 ms). This reduction in ion transit time, combined with ultrafast MS/MS sampling (60 Hz), makes internal CV stepping (intra-analysis CV switching) practical, allowing for rapid, sequential CV cycling to produce and analyze multiple gas-phase fractions during a single analysis.[24]

Here we characterize how peptide ions behave in the improved FAIMS device interfaced with nanoLC and a fastscanning Orbitrap hybrid mass spectrometer.[1,9] With these data we recommend strategies for analysis of tryptic peptides and benchmark our results against fractionated and unfractionated analyses without FAIMS. We conclude that the addition of this FAIMS device nearly doubles the number precursors presented to the mass spectrometer (815 196 vs 444 965 4 h experiments) and can boost peptide identifications by up to 2-fold and protein identifications up to 55% from single-shot analyses of human cell line tryptic peptides, depending on experimental duration. In many scenarios, adding FAIMS separations to an analysis provides a benefit to proteomic depth. Finally, we present evidence that FAIMS could replace liquid chromatography fractionation for many applications.

MATERIALS AND METHODS

Mass Spectrometry.

Digestion, fractionation, and nanoLC conditions are described in the Supporting Information. For experiments without FAIMS, eluted peptides were analyzed on an Orbitrap Fusion Lumos Tribrid platform with Instrument Control Software version 3.1. These analyses utilized a 240 000 resolving power survey scan with AGC = 1 000 000, followed by MS/MS of the most intense precursors for 1 s. The MS/MS analyses were performed by 0.7 *m/z* isolation with the quadrupole, normalized HCD (higher-energy collisional dissociation) collision energy of 30%, and analysis of fragment ions in the ion trap using the "Turbo" speed scanning from 200 to 1200 *m/z*. Dynamic exclusion was set to 10 s but was increased to up to 60 s for longer analyses. Monoisotopic precursor selection (MIPS) was set to Peptide, maximum injection time was set to 11 ms (or up to 20 ms for 5–6 h analyses), AGC target was set to 30 000, and charge states unknown, +1, or >+5 were excluded and the advanced peak determination was toggled on.

For FAIMS-enabled experiments the settings were identical except the FAIMS device was placed between the nanoelectrospray source and the mass spectrometer. FAIMS separations were performed with the following settings: inner electrode temperature = 100 °C (except where noted), outer electrode temperature = 100 °C, FAIMS carrier gas flow = 4.7 L/min, asymmetric waveform with DV = −5000 V, entrance plate voltage = 250 V, and CV settling time = 25 ms. The FAIMS carrier gas is N_2 only, and the ion separation gap is 1.5 mm. The noted CVs were applied to the FAIMS electrodes. For external stepping or single CV experiments the selected CV was applied to all scans throughout the analysis. For internal CV stepping experiments, each of the selected CVs was applied to sequential survey scans and MS/MS cycles (1 s); the MS/MS CV was always paired with the appropriate CV from the corresponding survey scan. For the 4 h quantitative FAIMS experiments the survey scan MS resolving power was reduced to 120 000 to permit a cycle time of 0.6 s.

Data Analysis for Qualitative Assessment of Identifications.

For qualitative assessment of peptide and protein identifications from different methods, the OMSSA algorithm and COMPASS software suite were used for searching and processing data, respectively.[25] Raw files were first converted to text files and scored against theoretical spectra from a target–decoy species-specific reference proteome database, downloaded from Uniprot, using the OMSSA search engine. Tryptic peptides (allowing for cleavage before proline) were searched with one missed cleavage. Cysteine carbamidomethylation and methionine oxidation were set to fixed and variable modifications, respectively. Peptides were searched with 75 ppm tolerance around the monoisotopic precursor mass and 0.3 Da tolerance on fragment ion masses; wide precursor tolerances are used so that systematic mass error can be corrected and because parts-per-million mass error is used for FDR (false discovery rate) filtering. The COMPASS software suite was used to filter search results to a 1% unique peptide FDR (based on *E*-value and ppm mass error). Identifications with mass errors above 5 ppm were typically filtered out at this step. For protein identification, characterization proteins were grouped based on the rules of parsimony and filtered to 1% FDR.[26] Data generated from the LysC digestion was searched similarly. GluC and

chymotrypsin data were searched with three missed cleavages, and chymotrypsin data was also searched semispecifically. Technical comparisons of peptide and protein identifications was performed with COMPASS, which provides a flexible framework for rapid searching and application of FDR filtering, easily allowing for both batch and single-file peptide or protein FDR. Additional characterization was performed for single CV files and label-free quantitation (LFQ) quantitation (described below) with MaxQuant searching. In these cases the default parameters were used except ion trap MS/MS tolerance was set to 0.3 Da. The "allPeptides" file was used to characterize charge state and *m/z* of observed MS1 peptide features. Due to the extended amount of time required to search data, MaxQuant analysis was limited to peptide feature characterization and LFQ. Note that these programs provide similar protein identification rates.

MzXML Splitting.

Raw files from FAIMS internal CV stepping experiments cannot be directly processed in MaxQuant due to the disruption of peptide elution profiles. To overcome this limitation we converted each raw file into a set of MzXML files, where each of these contains only scans collected with a single CV.[27] Available data conversion software such as MSConvert or ReAdW are capable of generating MaxQuant-compatible MzXML files for routine mass spectrometry experiments, but they do not correctly convert FAIMS raw files into MzXML files due to slight changes to raw file structure, nor do they separate scans by different correction voltages.

MaxQuant compatible MzXML files were created directly from raw files using custom C# software developed in-house (https://github.com/coongroup/FAIMS-MzXML-Generator). These files produce MzXML files similar to those generated by ReAdW, which itself generally conforms to the official MzXML schema. This software utilizes functionality provided via the C# Mass Spectrometry Library (https://github.com/dbaileychess/CSMSL) to rapidly access spectral data. Briefly, the locations of the raw files to be processed and desired output path is specified using an intuitive user interface. Each scan is accessed to determine the number of unique CVs applied throughout the experiment. The software then partitions all scans into distinct collections via the CV located in scan filter, initially without regard to scan level. These collections are then iterated through to associate MS/MS scans with parent survey scans. As the scans are linked, the relevant instrumental and spectral information is formatted and written out to an MzXML file. This process is repeated for every unique CV detected in the experiment, e.g., an input raw file containing three unique CVs will result in three output MzXML files.

Label-Free Quantitation.

For LFQ comparisons, MaxQuant was used.[28,29] Either MzXML (FAIMS) or raw data (without FAIMS) files were searched against a human proteome reference database (Uniprot) with the Andromeda algorithm using the default parameters except the following: LFQ was toggled on with min ratio count set to 1 for both LFQ and protein quantitation, ion trap MS/MS tolerance was set to 0.3 Da, and match between runs was enabled. Additionally, each MzXML file was denoted and analyzed as a fraction. MS1 peptide features were determined from the isotope pattern category from the "summary" file.

RESULTS AND DISCUSSION

To describe the relationship between applied CV and peptide characteristics we analyzed tryptic peptides derived from the human cell line K562 with CVs ranging from −10 to −120 V (60 min per analysis). The total number of features at charge states +1, +2, +3, or ≥+4 reveals a somewhat unpredictable distribution, but one that is similar to those previously reported (Figure 1a, Figure S1a).[30] Interestingly, features with $z \geq +4$ have a distribution between that of +2 and +3, suggesting their transmission adheres to a different mechanism that those of lower charge states. We also found that there is a strong correlation between *m/z* and CV between −20 and −60 V and markedly less correlation from −60 to −120 V (Figure S1 b-–d). A perhaps useful observation from these data is that the vast majority of multiply charged features lie in the CV range of −40 to −120 V, while the charge state +1 features are almost entirely constrained to CVs between −10 and −45 V. As expected, this separation suggests that the less desirable +1 features can be excluded from the majority of proteomic analyses with the FAIMS device. From these data we estimated peptide peak widths (full width at half-maximum peak height) from the FAIMS separation to be ~10 CV (Figure S1e) and maximal peptide transmission to be >75% for 90% of peptides.

To define the useful CV range for proteomic analyses we next surveyed each CV bin for total number of peptide (Figure 1b) and protein (Figure 1c) identifications and display this in context of results from a standard analysis (without FAIMS). Analyses without FAIMS always yield more peptide identifications than any analysis with a single CV. Yet, there is a broad range of CVs (−40 to −105 V) that can produce more protein identifications (≤40%) than the same analysis without FAIMS. This seemingly counterintuitive relationship between peptide and protein identifications exists for two reasons. Single CV analyses have reduced sampling of highly abundant proteins; there are 3 times more proteins with >30 unique proteins in the experiment without FAIMS (Figure S2, parts a and b). Similarly, proteins must be represented by fewer peptide sequences, on average, since only a portion of peptides are present in a given sample. This observation is consistent with the analysis of a subset of LC fractions (Figure S2, parts c and d).

Amino acid frequency can vary between organisms.[31] If the characteristics of tryptic peptides derived from organisms change enough, then it is possible each organism would require CV characterization and optimization prior to the analysis. To test this possibility we analyzed tryptic peptides from a broad spectrum of organisms (bacteria, fungus, mammalian tissue, and a human cell line) across a wide CV range (Figure 1d). We report that peptides from all of these organisms behave similarly in the FAIMS device, and thus, it is likely the same CV settings can be used for the analysis of tryptic peptides irrespective of organism. The same evaluation was performed for different proteases (Figure 1e). Peptides created with LysC, chymotrypsin, or GluC appear to transmit in the same general CV space. Note that each may benefit by being optimized individually.

FAIMS resolution can be affected by differences between inner and outer electrode temperatures.[32,33] To evaluate this phenomena, tryptic peptides were analyzed over a broad CV range for two inner electrode temperatures, 70 or 100 °C, while the outer electrode was maintained at 100 °C (Figure 1f). The peak widths of analytes, in units of CV, decrease at

the reduced inner electrode temperature; however, this decrease in CV peak width is also accompanied by a decrease in the useful CV range, and thus, we do not observe a relationship between inner electrode temperature and CV peak capacity (Figure S3).

Deciding which CVs to use for optimal proteomic performance depends on both the total number of possible peptides in each CV and the overlap in peptide identifications between those CVs. Additionally, the CVs chosen will depend on the total number of CVs used, which is ultimately dictated by how much time is available for an experiment. To systematically determine which two CVs should be used in conjunction for a two-CV analysis, we analyzed tryptic peptide derived from K562 cells with CVs ranging from −30 to −90 V with 5 V resolution, in duplicate (Figure 2a). From these data we conclude that combining data from −50 and −70 V or−55 and −75 V provides the greatest proteomic depth. Next we examined which three CVs to combine to optimize a three-CV analysis. Peptide identifications from a first experiment at −55, −60, or −65 V (the three highest points in the peptide/CV distribution) were each combined with the identifications from a second experiment where the CV ranged from −30 to −120 V. Local identification maxima are observed on CVs to both sides of the initial CV used. Combining the initial CV with the local maxima on both sides provides the best estimate for which three CV values to combine. The results for the −65 V starting point are displayed in Figure 2b; the −55 and −60 V results are plotted in Figure S4a–c. We found that combining −50, −65, and −85 V worked best. We conclude that combined CVs should be 15–20 V apart and centered near the identification distribution max (−55 to −65 V). If the CVs are too similar, then high overlap limits results. On the other hand, if the CVs are too far apart, the identification-rich center of the distribution will be missed. We applied these rules to determine when combining four CVs within a single experiment −45, −60, −75, and −90 V should be used, and determined this to be a reasonable setting empirically (Figure S4d).

The FAIMS device used here has ion transit times of ~20 ms. This rapid transit time enables the instrument to scan multiple CVs within an analysis (Figure S5, parts a and b). Internal CV stepping enables the thorough analysis of multiple gas-phase fractions within a single injection. We evaluated three analysis lengths (1, 2, 3 h) and titrated the number of CVs used during each run, up to four CVs (Figure 2c). The CVs used are in accordance with the recommend CVs in Table 1. We found that the number of CVs to use during an analysis depends on the run time. More CVs typically lead to more peptide identifications. However, once the MS becomes overly saturated with peptide features, the number of protein identifications drops (e.g., analyzing two CVs yields fewer protein identifications than one CV for 1 h analyses). For this reason, and to limit cycle time, especially for quantitative experiments, we recommend analysis of one CV per hour of the run, with four CVs maximum per analysis (Table 1). Note that combining multiple runs with a single CV (external CV stepping) each is never as productive as longer experiments with internal CV stepping (Figure S5, parts c and d).

Historically, poor ion transmission has challenged the practical utility of FAIMS. We aimed to determine how a FAIMS device with high maximal transmission affects the relationship between sample loading and proteomic depth. We tested loads of 0.1–6 μg of tryptic peptides derived from K562 cells with and without FAIMS across 1, 2, or 3 h analyses. The

settings from Table 1 were applied to the FAIMS analyses in these experiments. We find that FAIMS-enabled experiments tend to be more sensitive to lower sample amounts, and as expected, longer runs in both modes are more sensitive to sample loading. Peptide identifications with FAIMS is always lower for the 1 h analysis (Figure 3a) compared to the same analysis without FAIMS, as is shown above. As loading increases to 0.5 μg and above, FAIMS analysis provides more peptide identifications in the 2 h experiments (Figure 3b). Additionally, enabling FAIMS is advantageous at all sample loads for the 3 h experiments (Figure 3c). Despite the nuanced relationship just described for peptide identifications, protein identifications are improved with FAIMS for all sample loads and all experimental lengths (Figure 3d–f). We also note that longer FAIMS-enabled experiments can still benefit from seemingly very high sample loading amounts (6 μg). The columns used in this study were packed with fully porous C18 particles having high loading capacity, and the effectiveness of higher sample loading may change with different chromatography columns. These data also predict how FAIMS will work during future post-translational modification (PTM) experiments, where identification of peptide sequences is the primary goal.

We benchmarked protein depth across analysis times ranging from 30 min to 6 h (Figure 4a). At all time points FAIMS provides added protein identifications. The increase in identifications is greatest for the shortest runs (30 min and 1 h), drops to ~10% at 2 h, and then increases for longer runs. Of particular note is that >8000 proteins can be identified in a 5 h analysis using internal CV stepping across four CVs (Table 1). A similar trend is observed for tryptic peptides from other organisms (Figure 4b), where the shortest analyses show the greatest advantage from enabling FAIMS. In general, the more complex the sample, the greater the benefit.

For every experiment there is an evaluation of the proteomic depth desired, at the cost of analyst effort and instrument time. For experiments where proteomic coverage is ultimately more critical, LC fractionation is exploited. This typically adds at least a day to sample preparation time, requires an additional LC, substantial sample amounts, and can be quite cumbersome. Here we show that FAIMS can change this equation by providing similar depth, for tryptic peptides derived from K562 cells, compared to the analysis of LC fractions, holding instrument time constant (Figure 4c). In 6 h of instrument time, two injections with FAIMS, each with two CV internal stepping (−45 VI–75 V and −60 VI–90 V), generate 8151 protein identifications, compared to 7767 from LC fractions (no FAIMS) and 6682 unfractionated without FAIMS. Limiting instrument time to 4 h, a similar trend is observed, 7814, 6914, and 6612 protein identifications, respectively. FAIMS analyses with internal CV stepping offer similar or higher throughput compared to LC fractionation with a much more streamlined approach and require only low-microgram amounts of sample. Note that >100 000 peptides are identified in as little as 4 h with FAIMS. Also note that analyzing more than four CVs in the current system has limited returns, and we would not expect single-shot FAIMS experiments to compete with high fraction numbers (e.g., 8–30 fractions).

Finally, we evaluated protein label-free quantitation reproducibility with FAIMS.[28,29] Tryptic peptides derived from K562 cells were analyzed in injection duplicates, each a 4 h analysis, with FAIMS (four CVs internal stepping) and without FAIMS. FAIMS raw files

were each converted to MzXML files and separated by CV, one MzXML file per CV. Each of these was analyzed in MaxQuant as fractions. Correlation plots of LFQ measurements between replicates are displayed without FAIMS (Figure 5a) and with FAIMS (Figure 5b). Similar high-quality correlations are observed, $r^2 = 0.994$ and 0.992, respectively, while FAIMS experiments quantify 1009 additional proteins. Base peak chromatograms demonstrate that similar signal is achieved for both methods (Figure 5c). The total cycle time impacts quantitation; the FAIMS cycle time for these analyses is 0.6 s per CV translating to a total cycle time of 2.4 s between MS survey scans with the same CV. As expected, the cycle time has an inverse correlation with quantitative reproducibility (Figure S6). Regardless, the protein measurement reproducibility is similar with and without FAIMS (Figure 5d), and FAIMS-enabled quantitation produces nearly 1200 more protein measurements with <20% RSD (Figure 5e). FAIMS-enabled experiments access quantitative values from more peptides per protein (3.7 more unique peptides on average), balancing out any negative effects from the 2.4 s cycle time (Figure 5f). In general, longer FAIMS analyses with more CVs provide more peptides per protein than the same experiment without FAIMS (Figure S7a). Nearly twice as many isotope patterns are observed in the FAIMS runs (Figure S7b); fewer protein measurements rely on the matching between runs function (Figure S7c), and reduced chimeric spectra (Figure S7d) point to higher quality data overall.

CONCLUSION

We describe how a high transmittance, low ion transit time FAIMS device interfaced with nanochromatography and an Orbitrap Fusion Lumos improves proteomic depth. The device essentially allows the simultaneous analysis of multiple gas-phase fractions, and can generate >8000 protein identifications from a single-shot analysis of tryptic peptides derived from a human cell line, and provides LFQ quantitation with similar reproducibility to that of single-shot experiments without FAIMS. Adding FAIMS to the system expands the possible options for the proteomic experimental design. The decision between acquiring a single shot quickly and spending the effort to collect fractions is altered by the addition of FAIMS. With FAIMS we can acquire data with commensurate or higher depth than LC fractionation with substantially less effort and sample requirements.

The current FAIMS instrument enables the collection of up to four CVs during a run, presenting more than 800 000 precursors to the instrument over a 4 h analysis. In the current design, analyzing more than four CVs either requires using CVs that allow few peptides to transmit or requires more closely spaced CVs, increasing overlap. Thus, we see limited returns in analyzing more than four CVs. However, a higher resolution FAIMS devices could enable the analysis of more CV fractions and lead to greater proteomic depth or throughput. Being able to analyze 3 times more mostly unique CV fractions could make prefractionation essentially obsolete. It is also possible that the expansion in the number of precursors presented to the instrument has shifted the limiting variables back to MS/MS speed and instrument sensitivity. Efforts to reduce cycle time would also improve quantitation. Additional characterization of CV settling time, and understanding the parallelization of this step with other MS processes, will likely increase the number of MS/MS scans collected over the course of an analysis. Although beyond the scope the work presented here, we believe the FAIMS device used in this study would be useful for PTM discovery, targeted

parallel reaction monitoring (PRM), data-independent acquisition (DIA), and top-down and potentially other experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We gratefully acknowledge support from the NIH Grants P41 GM108538 and R35 GM118110 (to J.J.C.) and DOE Grant DE-SC0018409. D.R.B. was supported by an NHGRI training grant to the Genomic Sciences Training Program 5T32HG002760.

REFERENCES

(1). Hebert AS; Thöing C; Riley NM; Kwiecien NW; Shiskova E; Huguet R; Cardasis HL; Kuehn A; Eliuk S; Zabrouskov V; Westphall MS; McAlister GC; Coon JJ Anal. Chem 2018, 90, 2333–2340. [PubMed: 29272103]

(2). Kelstrup CD; Bekker-Jensen DB; Arrey TN; Hogrebe A; Harder A; Olsen JV J. Proteome Res 2018, 17, 727–738. [PubMed: 29183128]

(3). Blue LE; Franklin EG; Godinho JM; Grinias JP; Grinias KM; Lunn DB; Moore SM Journal of Chromatography A 2017, 1523, 17–39. [PubMed: 28599863]

(4). Shishkova E; Hebert AS; Coon J J Cell Systems 2016, 3, 321–324.

(5). Meier F; Geyer PE; Virreira Winter S; Cox J; Mann M Nat. Methods 2018, 15, 440–448.

(6). Prasad S; Belford MW; Dunyach J-J; Purves RW J. Am. Soc. Mass Spectrom 2014, 25, 2143–2153. [PubMed: 25267086]

(7). Purves RW; Prasad S; Belford M; Vandenberg A; Dunyach J-J J. Am. Soc. Mass Spectrom 2017, 28, 525–538. [PubMed: 28097537]

(8). Hebert AS; Richards AL; Bailey DJ; Ulbrich A; Coughlin EE; Westphall MS; Coon JJ Mol. Cell. Proteomics 2014 13, 339–347. [PubMed: 24143002]

(9). Senko MW; Remes PM; Canterbury JD; Mathur R; Song Q; Eliuk SM; Mullen C; Earley L; Hardman M; Blethrow JD; Bui H; Specht A; Lange O; Denisov E; Makarov A; Horning S; Zabrouskov V Anal. Chem 2013, 85, 11710–11714. [PubMed: 24251866]

(10). Buryakov IA; Krylov EV; Nazarov EG; Rasulev UK Int. J. Mass Spectrom. Ion Processes 1993, 128, 143–148.

(11). Shvartsburg AA; Li F; Tang K; Smith RD Anal. Chem 2006, 78, 3706–3714. [PubMed: 16737227]

(12). Cooper HJ J. Am. Soc. Mass Spectrom 2016, 27, 566–577. [PubMed: 26843211]

(13). Guevremont R Journal of Chromatography A 2004, 1058, 3–19. [PubMed: 15595648]

(14). Creese AJ; Cooper HJ Anal. Chem 2012, 84, 2597–2601. [PubMed: 22280549]

(15). Bonneil E; Pfammatter S; Thibault P J. Mass Spectrom 2015 50, 1181–1195. [PubMed: 26505763]

(16). Shvartsburg AA; Anderson GA; Smith RD Mass Spectrom 2013, 2, S0011.

(17). Venne K; Bonneil E; Eng K; Thibault P Anal. Chem 2005, 77, 2176–2186. [PubMed: 15801752]

(18). Canterbury JD; Yi X; Hoopmann MR; MacCoss MJ Anal. Chem 2008, 80, 6888. [PubMed: 18693747]

(19). Ridgeway ME; Lubeck M; Jordens J; Mann M; Park MA Int. J. Mass Spectrom 2018, 425, 22–35.

(20). Distler U; Kuharev J; Navarro P; Levin Y; Schild H; Tenzer S Nat. Methods 2014, 11, 167. [PubMed: 24336358]

(21). Krylov EV Int. J. Mass Spectrom 2003, 225, 39–51.

(22). Purves RW; Guevremont R; Day S; Pipich CW; Matyjaszczyk MS Rev. Sci. Instrum 1998, 69, 4094–4105.

(23). Guevremont R; Purves RW Rev. Sci. Instrum 1999, 70, 1370–1383.

(24). Creese AJ; Shimwell NJ; Larkins KPB; Heath JK; Cooper HJ J. Am. Soc. Mass Spectrom 2013, 24, 431–443. [PubMed: 23400772]

(25). Wenger CD; Phanstiel DH; Lee MV; Bailey DJ; Coon JJ Proteomics 2011, 11, 1064–1074. [PubMed: 21298793]

(26). Nesvizhskii AI; Aebersold R Mol. Cell. Proteomics 2005, 4, 1419–1440. [PubMed: 16009968]

(27). Pedrioli PGA; Eng JK; Hubley R; Vogelzang M; Deutsch EW; Raught B; Pratt B; Nilsson E; Angeletti RH; Apweiler R; Cheung K; Costello CE; Hermjakob H; Huang S; Julian RK, Jr; Kapp E; McComb ME; Oliver SG; Omenn G; Paton NW; et al. Nat. Biotechnol 2004, 22, 1459–1466. [PubMed: 15529173]

(28). Cox J; Mann M Nat. Biotechnol 2008, 26, 1367. [PubMed: 19029910]

(29). Cox J; Hein MY; Luber CA; Paron I; Nagaraj N; Mann M Mol. Cell. Proteomics 2014, 13, 2513–2526. [PubMed: 24942700]

(30). Swearingen KE; Hoopmann MR; Johnson RS; Saleem RA; Aitchison JD; Moritz RL Mol. Cell. Proteomics 2012, 11, M111.014985.

(31). Brüne D; Andrade-Navarro MA; Mier P BMC Res. Notes 2018, 11, 117. [PubMed: 29426365]

(32). Barnett DA; Ouellette RJ Rapid Commun. Mass Spectrom 2011, 25, 1959–1971. [PubMed: 21698679]

(33). Barnett DA; Belford M; Dunyach J-J; Purves RW J. Am. Soc. Mass Spectrom 2007, 18, 1653–1663. [PubMed: 17662612]

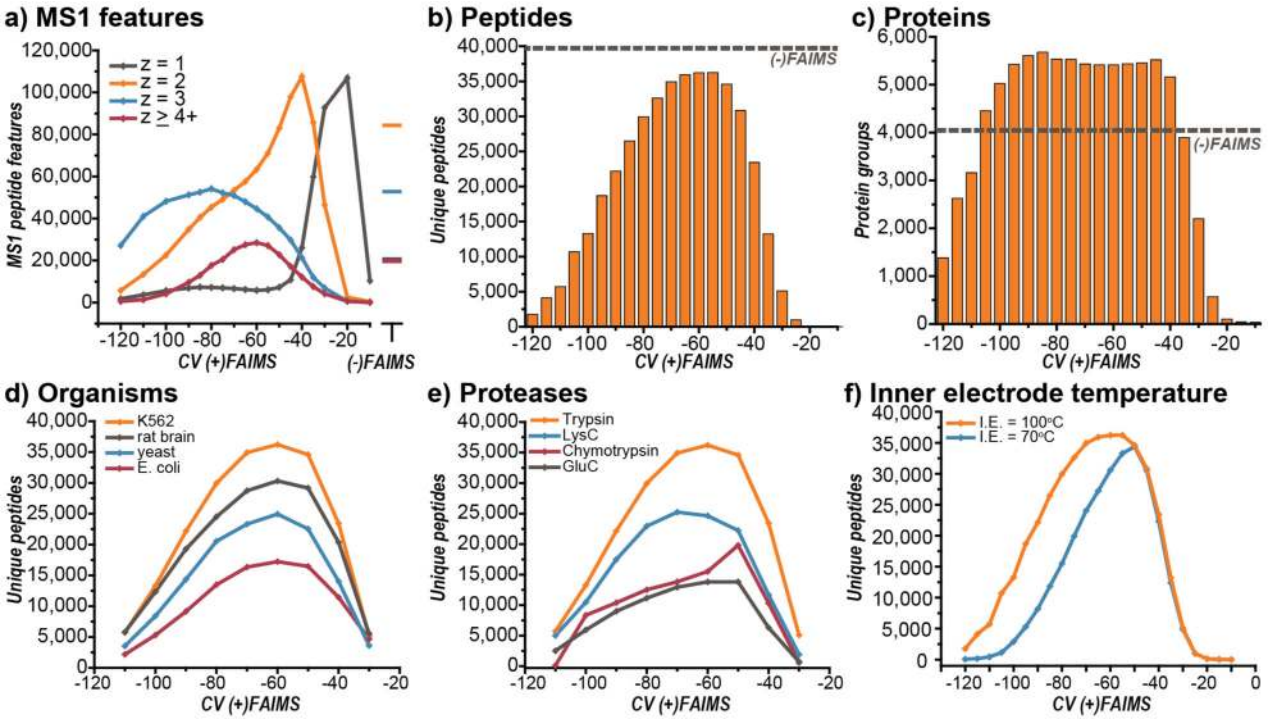


Figure 1.

Characterization of FAIMS CV settings. All results are from 60 min analyses of K562-derived tryptic peptides, except where noted. (+)FAIMS and (−)FAIMS data were collected from analyses with and without the FAIMS device attached, respectively. Distributions of (a) precursor charge states, (b) unique peptide identifications, and (c) protein groups across CV settings from −10 to −120 V. (d) Effect of CV on tryptic peptide identifications from multiple organisms. (e) Effect of CV on K562 human cell peptide identifications generated from listed proteases. (f) Effect of reducing the inner electrode (I.E.) temperature on the CV distribution of peptide identifications; the outer electrode was held constant at 100 °C.

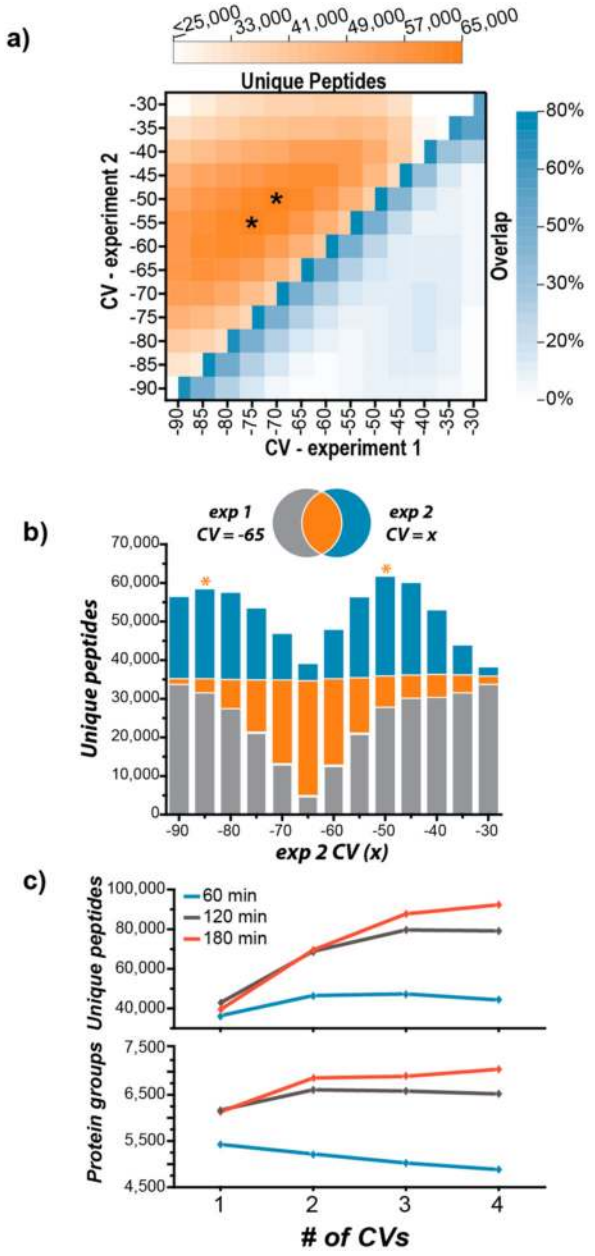


Figure 2.

Combining data from multiple CVs. All data are from tryptic peptides derived from K562 human cells. (a) Heat map of unique peptides combined from two CVs (orange), and the percent overlap of those identifications (blue). (b) Heart plot to determine the best three CVs to combine. This plot shows the total unique peptide identifications from combining one run with CV = −65 V and a second run with the CV noted on the *x*-axis. Those only found in the first −65 V CV or the *x*-axis CV are shown in gray and blue, respectively. The overlap between the two CVs is shown in orange. The * notes local maxima for total identification yield from the combination of two CVs, suggesting that combining data from CVs −50, −65, and −85 V will maximize identifications. (c) Internal CV stepping titration for 1, 2, and 3 h

analyses. For these analyses: one CV = −60 V, two CVs = −50 V|–70 V, three CVs = −50 V|–65 V|–85 V, and four CVs = −45 V|–60 V|–75 V|–90 V.

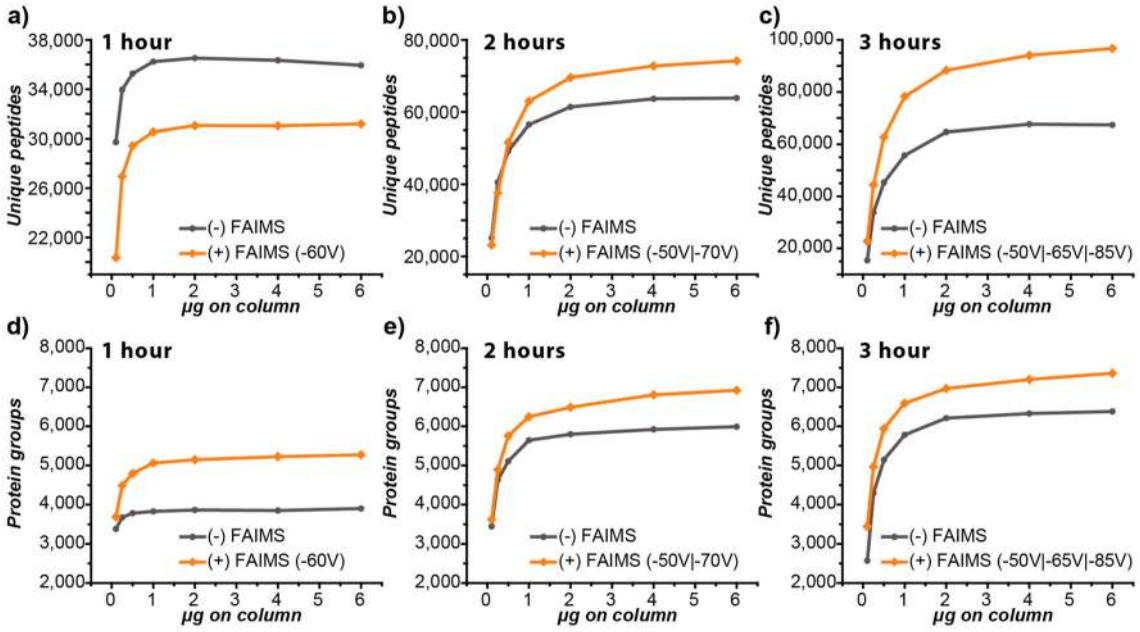


Figure 3.

Loading–response comparison with and without FAIMS for multiple analyses durations. All data are from tryptic peptides derived from K562 human cells. FAIMS experiments use internal CV stepping with the noted CVs. Loading amounts tested are 0.1, 0.25, 0.5, 1, 2, 4, and 6 μg on column. Peptide (a–c) and protein (d–f) identification results are compared for 1 h (a and d), 2 h (b and e), and 3 h (c and f) analyses. Intra-analysis CV switching was performed for FAIMS-enabled runs using the number of CVs and voltages outlined in Table 1.

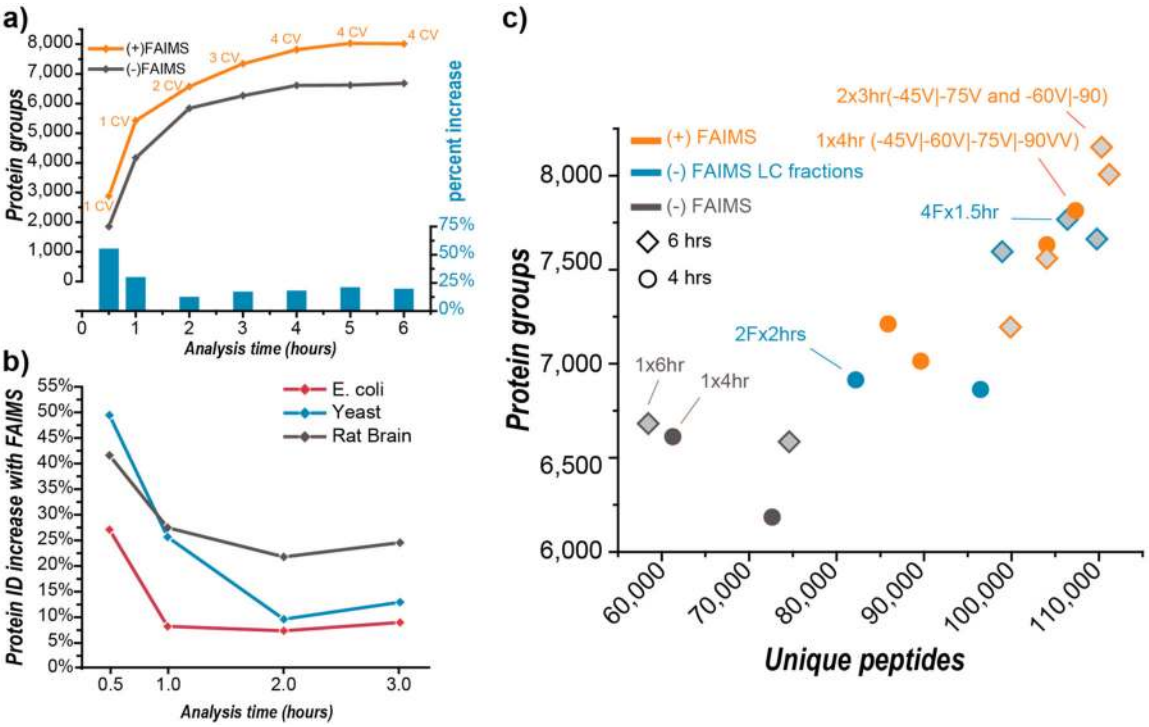


Figure 4.

Comparing proteomic depth with and without FAIMS. All FAIMS experiments use internal CV stepping, unless noted. (a) Protein identifications from single-shot analyses, ranging in run length from 30 min to 6 h, from K562 tryptic peptides with FAIMS (orange) and without FAIMS (dark gray). The blue bar chart displays the proteomic depth gained from using the FAIMS device. (b) Protein depth gained for tryptic peptides of the listed organisms with varying analyses times. The number of CVs and the voltages from Table 1 were applied to this analysis. (c) Comparison of proteomic depth achieved with FAIMS (orange), without FAIMS (dark gray), or from LC fractions without FAIMS (blue), in 4 h (●) or 6 h (♦) of instrument time. The top point consists of 2 × 3 h runs, each with two CV internal stepping (−45 V|–75 V and −60 V|–90 V). The top protein result for each method type is annotated.

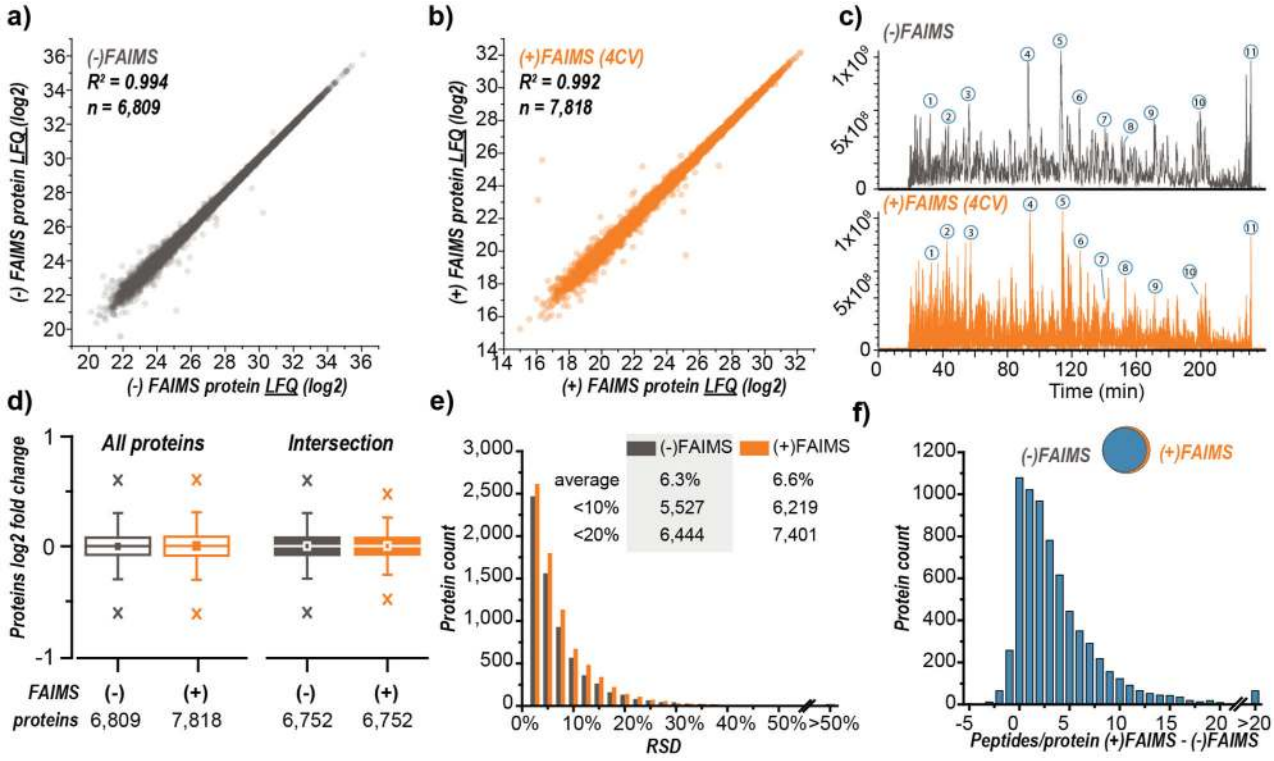


Figure 5.

Comparison of injection replicate LFQ with and without FAIMS. All data are from 4 h analyses of 6 μg of K562 tryptic peptides injected back-to-back. Data points have 70% transparency to show data density. For the FAIMS analyses four CVs (–45 V|–60 V|–75 V|–90 V) were rastered across during the analysis (internal CV stepping). MS/MS were collected for 0.6 s at each CV for a total cycle time of 2.4 s. Correlations of log2 LFQ protein intensity values for injection replicates without FAIMS (a) and with FAIMS (b). (c) Base peak chromatograms for 4 h analyses with and without FAIMS. (d) Box plots of protein LFQ fold change measurements between injection replicates. Box = inner 50%, whiskers = 5% and 95%, x's = 1% and 99%. (e) Histogram of RSD values for protein measurements between injection replicates in both modes. (f) Histogram of difference in peptides per protein identified with FAIMS compared to without FAIMS.

Table 1.

Recommended CV Settings for Various Analysis Times[a]

analysis time (h)	no. of CVs	CVs
≤1	1[b]	−60 V
2	2	−50 VI–70 V
3	3	−50 VI–65 VI–85 V
≥4	4	−45 VI–60 VI–75 VI–90V

[a]Note that CV setting may vary for different sites or instruments. CVs used should be spaced 15–20 V apart and should be centered in the identification/CV distribution as shown in Figure 1b.

[b]Two CVs may be used for simpler samples like *E. coli* digests without causing loss of protein identifications.