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Technical Note

High-Field Asymmetric Waveform Ion Mobility Spectrometry: Practical Alternative for Cardiac Proteome Sample Processing

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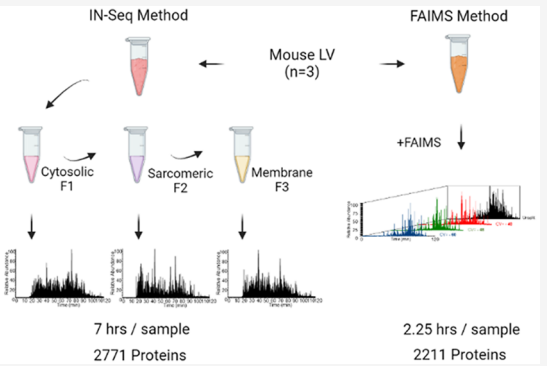
ABSTRACT: Heart tissue sample preparation for mass spectrometry (MS) analysis that includes prefractionation reduces the cellular protein dynamic range and increases the relative abundance of nonsarcomeric proteins. We previously described "IN-Sequence" (IN-Seq) where heart tissue lysate is sequentially partitioned into three subcellular fractions to increase the proteome coverage more than a single direct tissue analysis by mass spectrometry. Here, we report an adaptation of the high-field asymmetric ion mobility spectrometry (FAIMS) coupled to mass spectrometry, and the establishment of a simple one step sample preparation coupled with gas-phase fractionation. The FAIMS approach substantially reduces manual sample handling, significantly shortens the MS instrument processing time, and produces unique protein identification and quantification approximating the commonly used IN-Seq method in less time.



KEYWORDS: *FAIMS, heart tissue, LC-MS/MS, in sequence fractionation, IN-Seq, proteomics analysis, DIA-MS*

INTRODUCTION

Although liquid chromatography (LC) mass spectrometry (MS)-based proteomics methods have steadily increased total proteome coverage, sample fractionation prior to MS has remained a fundamental approach to increase further detection.[1] Specialized proteomic sample preparation protocols and instrumental techniques have been developed to permit a deeper investigation into the proteome of the heart.[2−7] Various studies, using drosophila, mice, rabbits, and human patients, have identified thousands of proteins in the cardiac proteomes, for example.[8−11] All of these occur prior to LC-MS and thus require additional time and skill sets. An alternative would be that fractionation of the peptides can take place in the gas phase during LC-MS analysis, which would reduce workflow complexity and increase time efficiency.

Analysis of the heart can be challenging as the sarcomeric proteins, which comprise the contractile apparatus, dominate the cardiac proteome.[11,12] To increase quantification of low-abundance proteins in cardiac tissue, sample prefractionation methods have been developed that take advantage of different chemical properties (e.g., In Sequence) or isolation of specific organelles or specific protein functional class. A few examples of the latter include Gramolini et al., where prefractionated mouse heart samples to "contractile" and 6 other fractions ("nuclei I", "nuclei II", "mitochondria I", "mitochondrial II", "microsome", and "cytosol fractions") by differential ultracentrifugation in sucrose gradients before LC-MS proteomic measurement.[7] Jones et al. and Boivin and Allen prepared mouse heart tissue proteins into 5 different subfractionations, which included additional fractions for "total membrane" and "sarcolemmal" proteins.[13−15] Warran et al. performed a series of differential centrifugations that produce organelles like "nuclear", "mitochondrial", "cytoplasmic", "microsomal", and "sarcomeric" enriched fractions during heart tissue preparations prior to LC-MS cardiac proteome analysis.[16]

In the early 2000s, we used chemical properties of protein to produce a fast and reproducible method (IN-Sequence, IN-Seq) to fractionate cardiac tissue for proteomic applications.[12,17,18] The IN-Seq method sequentially produces three subcellular fractions based on protein solubility at different pH: (1) cytoplasmic-enriched extract (neutral pH), (2) sarcomeric-enriched extract (acidic pH), and (3) membrane protein-enriched pellet (neutral pH). This method was developed to deplete the high abundant sarcomeric subproteome, allowing enhanced observation of the cytosolic proteins, while preserving the original endogenous PTM status.[17] In all these sample fractionation methods, each fraction is analyzed separately by nano liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) for increased total peak

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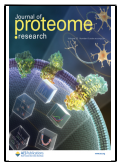

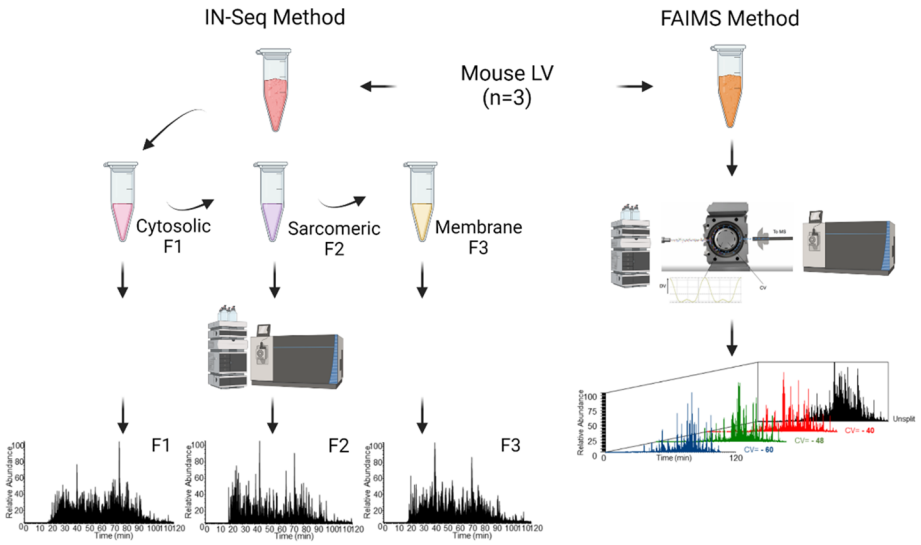

Figure 1. Experimental workflow from the frozen heart tissues to proteomic data analysis. IN-Seq fractionation method (left): Heart tissues (n = 3) are homogenized in a HEPES extraction buffer, then manually fractionated into a cytoplasmic-protein fraction (F1), sarcomeric-protein fraction (F2), and membrane-protein fraction (F3). A total of 3 fractions are processed and prepared separately for mass spectrometry analysis (DIA-MS, Orbitrap Fusion Lumos MS, ThermoFisher). Raw MS data from each fraction are converted to MzML files for DIANN library free analysis. Each tissue sample takes on average 1 h for the manual preparation step and 6 h of additional MS processing time (3 × 120 min gradient). FAIMS method (right): Tissues from the same hearts (n = 3) are homogenized in an 8 M urea extraction buffer. Total lysate is directly processed and prepared for mass spectrometry analysis (DIA-MS, Orbitrap Fusion Lumos MS, with FAIMS, ThermoFisher). Raw MS data from the sample is split by compensation voltages (CV-40, CV-48, CV-60) and then converted to MzML files for DIANN library free analysis. MS processing time per tissue sample is about 15 min manual preparation and 2 h of additional MS processing time (120 min gradient).

capacity while balancing data acquisition time to increase proteome coverage.

While sample fractionation prior to LC-MS is an effective method to improve proteomic depth, the protocol is not easily adapted to high-throughput processing, as it requires extensive manual sample handling and multiple fractions need to be analyzed.[19] Thus, it would be optimal if the fractionation of the peptides can take place in the gas phase during the LC-MS run.[20] High-field asymmetric waveform ion mobility spectrometry (FAIMS) was developed in recent years with the goal of solving fractionation reproducibility.[21,22] This technology allows for gas-phase separation of peptide ions as they depart the electrospray emitter and prior to their entrance into the mass spectrometer.[23] Combining multiple compensation voltages (CVs) within a single run or between runs improves whole proteome coverage in cell lysates.[24,25] For example, Pfammatter et al. observed a 30% gain in unique peptide identification using FAIMS compared with non-FAIMS analysis of HEK 293 cells.[26] Other studies using HeLa protein digest such as Johnson KR et al., Gregus M et al., and Cong Y et al. concurrently showed the addition of FAIMS had significantly increased protein and peptide identifications than without FAIMS.[27−29] In a few recent studies working with various tissue samples, including brain autopsies,[30] tumor biopsies,[31] and paraffin embedded tissues (lymph node, lung, and prostate),[32] the addition of FAIMS also showed an enhanced detection of nonredundant proteoforms, increased proteome sensitivity, or substantially reduced sample handling. As the cardiac proteome consists of large dynamic range, it remains unclear whether FAIMS can detect peptides and the less abundant proteins from the cardiac proteome that is comparable with previous established fractionation methods.

Here we present a thorough comparison of the two methods for cardiac muscle tissue (n = 3 different hearts): manual IN-Seq pre-MS fractionation and FAIMS ion separation with the LC-MS approach by testing them on the same heart tissue samples split in half for each method preparation. Switching IN-Seq sample fractionation to FAIMS gas-phase separation reduces manual sample handling and shortens MS instrument utilization time to only one-third (360 to 120 min with FAIMS). The proteome coverage and sensitivity as well as peptide ion abundance between the IN-Seq and FAIMS methods are also compared using data-independent acquisition-MS (DIA-MS) with the same mouse peptide library. We conclude that both methods produce comparable proteomics data, with the FAIMS method proving advantageous for studies requiring higher throughput and efficiency, while IN-Seq provides broader coverage that could be leveraged for in-depth hypothesis generating studies.

MATERIAL AND METHODS

Sample Collection

Adult wild-type mice (C57BL/6, n = 3) were sacrificed, and the hearts were immediately harvested, washed in a cold PBS buffer. The left ventricle (LV) of each heart was dissected and snap-frozen in liquid nitrogen. LVs were cut in half for "IN-Seq" or "FAIMS" preparations separately (Figure 1, figure created with Biorender.com).

IN-Seq Method: Sample Preparation with Sequential Fractionation and LC-MS/MS Proteomics Analysis

Frozen LV halves were minced in an ice-cold HEPES extraction buffer (25 mM HEPES-NaOH, 2.5 mM EDTA, pH 7.8) supplemented with Pierce protease inhibitor mini tablets (EDTA free, Thermo Fisher Scientific, Waltham, MA) and Pierce phosphatase inhibitor mini tablets (Thermo Fisher Scientific, Waltham, MA). Samples were fractionated into cytosolic-, sarcomeric-, and membrane protein-enriched fractions (i.e., F1, F2, F3) by the previously described "IN-

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J. Proteome Res. 2023, 22, 2124−2130

Seq" method.[12,33] Briefly, each tissue sample was homogenized using the Retsch laboratory mixer Mill MM400 (Verder Scientific) bead homogenizer for 1 min in a HEPES extraction buffer with a frequency of 30/s (repeated 3×). Following centrifugation at 14,000*g* for 10 min at 4 °C, the supernatant was collected and kept at 4 °C as F1. The pellet was resuspended in a TFA extraction buffer (1% trifluoroacetic acid (TFA), pH 2, 1 mM TCEP in HEPES extraction buffer) and homogenized as described above. Following centrifugation at 14,000*g* for 10 min at 4 °C, the supernatant was collected and kept at 4 °C as F2. The insoluble pellet from the F2 extraction step was further solubilized in the SDS extraction buffer (2% SDS, 1 mM TCEP in HEPES extraction buffer) and homogenized as described above and sonicated with a Q800R3 Sonicator (QSonica, Newtown, CT) for 5 min (amplitude of 70%, with 10s on, 10s off). Following centrifugation in 14,000g for 10 min at 4 °C, the supernatant was collected as F3. Protein concentrations of all fractions of each sample were determined by BCA (Pierce, Waltham, MA). Next, 100 μg of each fraction (total protein quantification) was reduced using 1 mM TCEP. Clean-up of each fraction was performed using S-trap mini spin columns (ProtiFi, Long Island, NY) for complete destruction of undesired enzymatic activity (such as protease or phosphatase activities) and maximization of digestion efficiency. Each fraction was digested for 15−18 h at 37 °C using ultragrade Trypsin (Promega) at a 1:100 enzyme:protein ratio. All fractions from each tissue samples were speed-vacuum-dried and stored at −80 °C until MS analysis.

For DIA-MS analysis with the IN-Seq fractionated samples, we used the Orbitrap Lumos Fusion mass spectrometer (Thermo Scientific) equipped with an EasySpray ion source and connected to Ultimate 3000 nano LC system (Thermo Scientific) as previously described.[33] Peptides were loaded onto a trapping column (75 μm ID × length 3 μm Luna particles, Phenomenex) and run with 0.1% aqueous formic acid for 7 min at 5 μL/min. Samples were diverted onto a 200 cm micro pillar array column (μPAC, PharmaFluidics) and peptides separated using a gradient of mobile phases A and B composed of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, respectively. For the longer 120 min gradient method, peptides were separated with a linear gradient of 5−20%B for 90 min, 20−35% for 30 min, 35−85%B for 3 min, holding at 85%B for 5 min and re-equilibrating back to 1% B over the course of 5 min. Each fraction was followed by a blank injection to both clean the column and re-equilibrate at 1%B. The nanosource capillary temperature was set to 300 °C and the spray voltage was set to 1.8 kV. Indexed retention time standards (iRTs, Biognosys) were added to each fraction before acquisition.[34]

FAIMS Method: Tissue Sample Preparation and LC-FAIMS-MS/MS Proteomics Analysis

The other frozen halves of LV samples were subjected to polytron homogenization and denaturation in a UREA total protein extraction buffer (8 M urea, 1 M ammonium bicarbonate, 5% SDS) at 4 °C. Tissue homogenates were ultrasonicated with a Q800R3 Sonicator (QSonica, Newtown, CT) for 5 min (amplitude of 70%, with 10s on, 10s off) and then centrifuged at 20,000g for 10 min at 4 °C. The supernatant of each sample was transferred and collected as the total protein extracts. The protein concentration of each tissue sample was determined by BCA (Pierce, Waltham, MA). S-trap mini spin columns (ProtiFi, Long Island, NY) were used to clean up 100 μg of each tissue sample, following ultragrade Trypsin (Promega) digestion at a 1:100 enzyme:protein ratio for 15−18 h at 37 °C. All tissue samples were dried using a speed vacuum and stored at −80 °C until MS analysis.

DIA-MS was carried out using established protocols on the Orbitrap Lumos Fusion MS (the same instrument as In-Seq) with a FAIMS front end (Thermo) coupled to a stainless-steel emitter (EvoSep) and EasySpray source (Thermo) adapter. The approach was adapted from our previously published work.[35] The trapping column (75 μm ID × length 3 μm Luna particles, Phenomenex) was run with 0.1% aqueous formic acid for 7 min at 5 μL/min. Samples were diverted onto a 200 cm micro pillar array column (μPAC, PharmaFluidics) and peptides separated using a gradient of mobile phases A and B composed of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, respectively. The gradient consisted of 4% B for the first 5 min with a step increased to 8% at 5.2 min followed by a 90 min linear gradient up to 30% B. During this time, the flow rate, which started at 1200 nL/min, was linearly decreased to 1000 nL/min. Subsequently, the %B was linearly increased from 30% to 50% over 30 min while being held at a constant flow rate of 1000 nL/min for a total gradient run time of 120 min. Following each analysis, a 20 min equilibration was performed, during which the trap was back-flushed at 5 μL/min while the analytical column was washed with 95% B and equilibrated to 2% B at 1200 nL/min. Both separation and equilibration were carried out at 55 °C.

The FAIMS module was used to separate the electro sprayed peptides by collisional cross section into three populations by cycling the CV among −40, −48, and −60. The CVs were selected to isolate large complementary populations of peptides and to maximize the combined number of quantified peptides. A range of CVs were tested for the FAIMS method before selection the final three CVs (Figure S1). At each compensation voltage, a precursor scan spanning 400−1000 m/z was acquired at a 60,000 (at m/z = 200) resolution with the AGC target set to 1,000,000 and 50 ms maximum injection time followed by data independent acquisition (DIA) spanning the same range using 20 m/z wide windows (30 windows at each FAIMS CV). Each DIA scan was acquired at 15,000 resolutions (at m/z = 200) with the AGC set to 150,000 and 35 ms maximum injection time.

Proteomics Data Processing and Analysis

Raw MS/MS data files were converted to mzML format using MSconvert version v.3.0.21304 from ProteoWizard for generating peak lists.[36] The MS files were analyzed in DIA-NN 1.8.1 using the spectral library-free search with Prosit against the Mouse Uniprot database.[37] In the library-free search, neural network algorithms are used to accurately predict hypothetical spectra and retention times for each potential tryptic peptide in the database. Only proteotypic peptides that consist of amino acid sequences unique to one proteins are used for protein identification.[38] The search was conducted with the second pass and match between runs (MBRs) options enabled. The quantitation at the protein levels was summed between the different CV fractions. MS protein quantification for the IN-Seq method samples was carried out by averaging the raw peptide ion abundance among technical replicates and summed among three subcellular fractions. Statistical analysis and software data was filtered by interquartile range and normalized to the median. The one-

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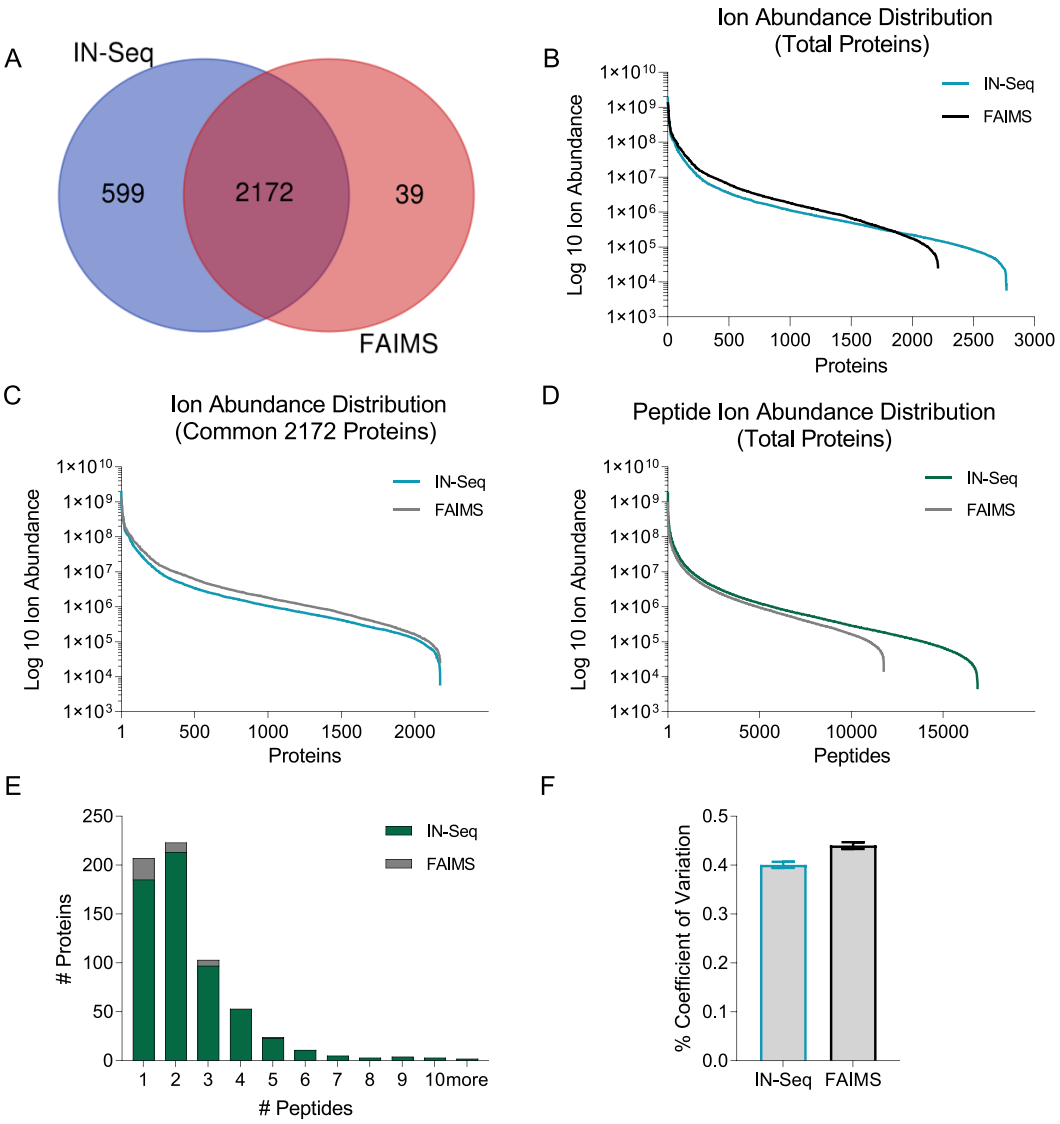

Figure 2. Proteomic data comparing the IN-Seq fractionation method and the FAIMS method. A. Venn diagram of proteins identified by each method and the total protein overlap. B. Peak ion abundance distribution of the common 2172 proteins identified by both methods. C. Peak ion abundance distribution of the 2771 proteins identified by the IN-Seq fractionation method and the 2211 proteins identified by the FAIMS method. D. Peak ion abundance of all peptides identified by the In Sequence method and the FAIMS method, peptide ion abundance from the most to least intense. E. Numbers of peptides for the uniquely identified proteins by the IN-Seq method (599 proteins), and uniquely identified by the FAIMS method (39 proteins). F. %CV between IN-Seq and FAIMS methods (n = 3 biological replicates, data shown as mean ± SEM).

way ANOVA with posthoc Fisher's LSD test was used to compare data sets with more than two groups. An unpaired *t* test was used to analyze the rest of the data sets. A p value of ≤0.05 was considered significant. MetaboAnalyst 5.0 was used for statistical analysis and data visualization.[39] Cellular component visualization was performed using Protein Interaction Network Extractor (PINE).[40] The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[41] partner repository with the data set identifier PXD039174.

■ RESULTS AND DISCUSSION

FAIMS Method Shortens Sample Preparation Time and Increases Instrument Efficiency for Proteomics Analysis

The time of sample preparation step(s) between the FAIMS method and the IN-Seq method vary. The FAIMS method requires ∼15 min per sample (for 5 min of tissue homogenization, 10 min of tissue solubilization and protein denaturation) before proteolytic digestion.

In contrast, the IN-Seq method requires additional steps for the manual fractionation of each sample into the three subcellular fractions, which involves the addition of multiple buffers, homogenization, and centrifugation steps. This takes ∼1 h per heart tissue sample (for tissue homogenization, tissue solubilization, protein denaturation and fractionation) before proteolytic digestion. Moreover, each fraction gets injected separately into the LC-MS/MS with the same 120 min gradient for proteomics analysis, which makes the instrument processing time 360 min per heart sample (6 h), while the total LC-FAIMS-MS/MS time using the same gradient takes only 120 min (2 h).

The FAIMS method benefits from a one-step total protein extraction from the heart tissue samples. Proteins were extracted, solubilized, and denatured in an 8 M UREA buffer. By contrast, the IN-Seq method uses different pH conditions

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J. Proteome Res. 2023, 22, 2124−2130

and three different buffers (with HEPES acting as a zwitterion buffer in all three) to isolate three different subcellular fractions.

FAIMS allows for a larger number of samples to be processed per day and reduces potential batch effects when processing large number of samples.[42] It shortens instrument time and reduces the time between data acquisition and analysis. Finally, the FAIMS approach lowers the use of reagents during sample preparation compared to the IN-Seq method.

FAIMS Method Covers the Majority of Proteome Identified by IN-Seq Method

We next investigated whether the FAIMS method produces comparable proteome coverage compared to tissue analyzed with the IN-Seq method. A total of 2211 proteins were identified using the FAIMS method, while 2771 proteins from all three fractions using the IN-Seq method. There was an overlap of 2172 proteins, which account for 98% of the total proteome identified by FAIMS and 78% of the total proteome by IN-Seq 599 proteins (22% of total proteins) were identified uniquely by the IN-Seq method, while 39 proteins (2% of total proteins) were uniquely identified by FAIMS (Figure 2A, Supplementary document 1). For protein ion abundance distributions, IN-Seq detected more proteins in the lower abundance range (Figure 2B, Supplementary document 2); however, with the common 2172 proteins identified by both methods, protein ion abundances between the two methods are correlated, with a slightly higher ion abundance using FAIMS (Figure 2C, Figure S2). For the proteins uniquely identified by each method (599 from the IN-Seq method vs 39 from the FAIMS method), based on cellular component analysis using PINE,[40] the IN-Seq method maintained high sensitivity for detecting cytoplasmic proteins, especially intracellular membrane-bounded organelle proteins, compared to FAIMS (Figure S3A,B), due to the physical fractionation that separates the highly abundant cardiac contractile proteins from the cytosolic proteins in IN-Seq. These data suggest IN-Seq to be better suited for studies that focus on cytoplasmic proteins.

We then studied the physical characteristics of the unique proteins from each method (599 proteins from the IN-Seq method and 39 proteins from the FAIMS method, Figure S3, Supplementary document 4). There were no differences in the average molecular weight of proteins between the two methods (Figure S3C). The mean GRAVY score for FAIMS versus IN-Seq for the unique proteins was −0.158 and −0.394, respectively (Figure S3D), indicating that IN-Seq is able to detect more hydrophobic proteins that those by FAIMS.

To understand if there was a bias in the precursor peptides, we further investigated the peptide level data after the DIA-NN proteomics analysis for sensitivity and reproducibly. It is important to realize that only proteolytic peptides, peptides composed of an amino acid sequence that is unique to a protein, are included. The IN-Seq method has slightly higher sensitivity in detecting peptides compared to the FAIMS method (Figure 2D). DIANN analysis identified 19,894 peptides from all samples, with 10,413 peptides overlapping between the two methods (Figure S4, Supplementary document 6). The FAIMS unique proteins mostly had one and two proteotypic peptides to one protein ratio and greater numbers than In-Seq, while the IN-Seq unique proteins had more two proteotypic peptides to one protein ratio, which is helpful when screening for PTMs (Figure 2E). This means that with common criteria for protein quantification of 2 prototypic peptides that FAIMs is like IN-Seq, especially as the two methods have comparable percentage of coefficient of variance (%CV, Figure 2F, Supplementary document 3) values with majority of peptides having a CV% under 40% based on 3 biological replicates.

In order to compare the MS1 level with the MS2 level protein identification (DIANN), each workflow was also analyzed using Ion Quant (Figure S5, Supplementary document 5). Ion Quant identified 2777 total proteins from both fractionation methods (compared to 2810 proteins by DIANN). Ion Quant identified 2114 and 2757 proteins from FAIMS and IN-Seq respectively, with 2089 (75% of total identified) in common. This is similar to the percentage overlap seen with DIANN (2211 and 2771 proteins from FAIMS and IN-Seq, respectively). Focusing on those proteins that are uniquely identified by Ion Quant, IN-Seq had a larger number (668 proteins) compared to FAIMS (25 proteins), following a similar trend as with DIANN (599 and 39 unique proteins for IN-Seq and FAIMS, respectively); see the Supporting Information documents for additional details (Supplementary documents 1, 2, 3, 4, 5, and 6).

CONCLUSION

In the present study, we examined whether LC-FAIMS-MS/MS in ion gas-phase separation can be adapted as the alternative method for heart tissue proteomics study compared to the commonly used IN-Seq fractionation LC-MS/MS method. Using the same heart samples (n = 3) between the two methods, FAIMS detected over 79% of IN-Seq detected proteins, with one-third reagent use in tissue preparation and a much shortened MS instrument time (Table 1). The IN-Seq method remained to be ideal for studies focused on changes in cytosolic proteome of cardiac tissues, as well as studies involving smaller sample numbers. Both methods produced comparable results of cardiac proteome profiles, but the FAIMS method presented here provided much improved throughput and a shortened MS processing time compared to the IN-Seq method.

Table 1. Processing Time and Proteome Coverage Breakdown for the IN-Seq Method and the FAIMS Method

Method	Tissue Processing Time (min)	Instrument Time (min)	Identified Proteins	Unique Identified Proteins
FAIMS	15	120	2211	39
IN-Seq	60	360	2771	599

ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[41] partner repository with the data set identifier PXD039174.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00027.

Supplementary document 1: Protein IDs FAIMS vs IN-Seq. (XLSX)

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J. Proteome Res. 2023, 22, 2124−2130

Supplementary document 2: Protein abundances and peptide abundances. (XLSX)
Supplementary document 3: %CV calculation. (XLSX)
Supplementary document 4: PINE cellular component Cluego results (XLSX)
Supplementary document 5: Proteome coverage identification using Ion Quant. (XLSX)
Supplementary document 6: Peptide IDs between FAIMS and IN-Seq. (XLSX)
Supplementary figures Figure S1. Selection of FAIMS compensation voltages (CVs). Figure S2. Precursor ion abundance correlation between the common proteins in IN-Seq and FAIMS methods. Figure S3. Subcellular localization of unique proteins. Figure S4. Peptide coverage comparison between IN-Seq and FAIMS methods. Figure S5. Ion Quant MS1 precursor intensity-based quantification of proteins using Ion Quant. (PDF)

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Notes

The authors declare no competing financial interest.

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