Getting more out of co-immunoprecipitation mass spectrometry experiments by reducing interference using FAIMS.

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Abstract

Co-immunoprecipitation of proteins coupled to mass spectrometry has transformed modern biology's understanding of protein interaction networks. These approaches exploit the selective isolation of tagged proteins by affinity enrichment / purification to identify protein binding partners at scale and in an unbiased manner. In instances where a suitable antibody is not be available it is common to graft synthetic tags such as FLAG or His Tags onto target protein sequences allowing the use of commercially available and validated antibodies for affinity purification. To allow the selective elution of protein complexes competitive displacement using a large molar excess of the tag peptide is widely used. Yet, this creates downstream challenges for the mass spectrometry analysis due to the presence of large quantities of a contaminating peptide. Here, we demonstrate that Field Asymmetric Ion Mobility Spectrometry (FAIMS), a gas phase ion separation device can be applied to FLAG-Tag and His-Tag pull down assay to increase the depth of protein coverage in these experiments. By excluding tag peptides based on their ion mobility profiles we demonstrate that single compensation voltage, or stepped compensation voltages strategies can significantly increase the coverage of total proteins by up to 2.5-fold and unique proteins by up to 15-fold versus experiments that do not use FAIMS. Combined these results highlight FAIMS is able to improve proteome depth by excluding interfering peptides without the need for additional sample handling or altering sample preparation protocols.

Significance Statement

We have shown that application of FAIMS separation in the gas phase can significantly increase the proteome coverage of Flag or His tagged co-immunoprecipitation mass spectrometry experiments. We demonstrated this in a classical FLAG tagged and His Tag assay and have increased the proteome coverage by up to 15-fold versus one without FAIMS. This allows us to conduct such experiment without additional sample handling, fractionation, machine run time or modifying the sample preparation protocol.

Introduction

Many studies in systems biology focus on identifying and characterizing protein-protein interactions (PPI) (1, 2). Co-immunoprecipitation coupled with mass spectrometry (Co-IP/MS) is an extremely powerful analytical technique and has been pivotal in protein-protein interaction studies to identify protein complexes, co-factors and signaling molecules (3, 4). Over the last 20 years Co-IP/MS approaches have evolved from multiple step isolation procedures such as tandem affinity purification (5) to single step protocols (6) improving the sensitivity, robustness and ability to assess low affinity PPIs. Despite these improvements, the fundamental approach has remained unchanged being dependent on the use of specific antibodies against a target protein which exists within a protein complex. Using these affinity reagents immune complexes are captured and interacting proteins identified using mass spectrometry. While there are thousands of commercially available antibodies and considerable research efforts have been directed toward cataloging these antibodies – for example in The Human Protein Atlas (7), not all affinity reagents are ideal for Co-IPs or available for all proteins.

To circumvent the need for protein specific reagents a common strategy employed by researchers is to tagged proteins with defined proteins or peptide sequences. This involves coupling a small peptide or

protein tag to the N- or C-terminus of the target protein positioned within the protein to minimize its effect on the protein function. Expression of the tagged protein can then be detected using antibodies against the tag sequence rather than the target protein itself. These tag-specific antibodies are available commercially and have been very well characterized for their specificity and sensitivity. Examples of common protein tags include green fluorescent protein (GFP), Glutathione S-Transferase (GST) and mCherry while c-Myc, 6X-His, FLAG and Hemagglutinin (HA) are examples of recombinant peptide tags (8-11). Proteins interacting with the tagged target can be identified by eluting the interacting proteins from the target protein/antibody complex. There are multiple sample preparation options for the elution step and one common approach is to elute the complex from the affinity beads by using competitive displacement (9, 12) whereby molar excess of a synthetic peptide (eg 2X Myc peptide; EQKLISEEDLEQKLISEEDL or 3X FLAG peptide; MDYKDHDGDYKDHDIDYKDDDDK) is added to displace the bound proteins from the tag-specific antibody under non-denaturation conditions. This approach limits the elution of non-specifically bound proteins and allows the elution of complexes in buffers directly compatible with downstream digestion protocols. Unfortunately, the presence of this excess peptide possesses an analytical challenge for subsequent mass spectrometry analysis. Due to the high relative abundance, size and composition these 'contaminant' peptides rapidly consume the ion storage capacity of ion trapping devices (C-trap, ion trap etc) (13, 14) and thus artificially reduce the ability to detect analyte peptides (i.e. those arising from interactor proteins of interest) that are available for detection.

A range of techniques such as gas phased fractionation, BoxCar and Ion mobility (IM) based fractionation (15-18) have been developed that aim to reduce the suppressive effects of highly abundant ions and increase proteome coverage. Ion mobility mass spectrometry (IMS-MS) is a technique whereby ions are separated in the gas phase under influence of an electric field and separation is related to their according to a combination of their size, shape, charge and time taken for the molecule to transverse towards the detector (19, 20). Multiple IMS techniques have been developed that differ in the physical principles

utilized to achieve ion separation. These include Travelling Wave (TWIMS), Trapped Ion Mobility (TIMS), Drift Tube Ion Mobility (DTIMS) and Field Asymmetric Ion Mobility (FAIMS). Each IM technique has its strengths and are suitable for different applications. The IM systems can help address some of the issue of under sampling due to the stochastic sampling of eluted peptides and bias caused by poor ion selection in a data dependent acquisition methodology (21). Many groups have thus utilized ion mobility mass spectrometry to performing gas phase fractionation in order to increase protein coverage in a complex whole cell digest (16, 18, 22). We and others have been using FAIMS to enrich for modified peptides (23-25) or as a targeted SRM-like screening (26) technique. These techniques took advantage of the unique collisional cross sectional (CCS) properties of the molecule of interest and tuning the ion mobility devices to suite these unique properties.

FAIMS works by having two electrodes with alternating high and low electric field strength across these electrodes. Separation of ions is by differences in their mobility in high and low electric fields (27). To prevent collision of the ions with the electrode, the ions are diverted through application of a specific DC compensation voltage. The FAIMS device can therefore be used as a filtering device which can target molecules matching the applied compensation voltage (CV). This theoretically can spread out the ion current and limit the proportion of the highly abundant species that contribute to the maximal charge capacity of the C-trap (13). While FAIMS has typically been utilized to enhance the selective detection of certain classes of analytes, here we propose utilizing this same technique to filter out the highly abundant FLAG peptides from Co-IP experiments that otherwise interfere with the detection of the less abundant peptides derived from the affinity target protein and its respective binding proteins. Through this approach, we demonstrated the massive improvement in terms of identified proteins in a relatively ‘simple’ co-immunoprecipitation experiment as compared to one without FAIMS. This protocol has also been successfully applied in the classical His-Tagged pull down assay.

Methods

Affinity Enrichment of Flag-tagged proteins

Affinity enrichment of FLAG-tagged protein experiment was performed from eHap knockout cells expressing FLAG-tagged protein as described previously (28). Cell were solubilised in 1% (w/v) digitonin in solubilization buffer (20mM Tris (pH 7.4), 50mM NaCl, 10% (v/v) Glycerol, 0.1 mM EDTA, 5U benzonase (Merck Millipore), 2mM $MgCl_2$). Following clarification of cell lysate by centrifugation (20,000*g,* 10 mins, 4°C), 500 µg protein was added to spin columns (Pierce) containing anti-FLAG M2 affinity resin (Merck) and allowed to incubate rotating for two hours at 4°C. Non-specifically bound proteins were then removed by washing with 20x column volumes of ice-cold solubilization buffer containing 0.1% (w/v) digitonin. Proteins were eluted with 50 µl of 100 µg/mL of 3X FLAG tag peptide (Merck) in solubilization buffer followed by acetone precipitation (overnight, -20°C). Proteins were then pelleted by centrifugation (21,000*g,* 10 mins, 4°C), washed with ice-cold acetone and air-dried. The pellet was then resolubilised by sonification in with 8M urea in 50 mM ammonium bicarbonate (ABC). Samples were reduced and alkylated with 50mM TCEP (ThermoFisher) and 500 mM Chloroacetamide (Merck) (30mins, 37°C, shaking). Elutions were diluted to 2M urea in 50mM ABC and digested in trypsin (Promega) overnight at 37°C. The digest was acidified to a final concentration of 1% (v/v) trifluoracetic acid (TFA) and peptides were desalted with stagetips containing 2x plugs of 3M™ Empore™ SDB-XC substrate (SUPELCO). Stagetips were activated with 100% Acetonitrile (ACN) and washed with 0.1% (v/v) TFA, 2% (v/v) ACN prior to binding of peptides. Samples were eluted in 80% ACN, 0.1% TFA and dried completely in a SpeedVac. Peptides were reconstituted in 2% ACN, 0.1% TFA and transferred to autosampler vials for analysis by LC MS/MS

Affinity Enrichment of His-tagged proteins

Recombinant His-tagged Phosphoprotein (*Nishigahara* strain) was purified by immobilized-metal affinity chromatography (TaKaRa) and cleaned up using gel filtration chromatography (GE Life Sciences). Mitochondria from HEK293T cells were isolated and solubilized in 1% (w/v) digitonin solubilizing buffer (20 mM Bis-Tris (pH 7.0), 50 mM NaCl, 10% (v/v) glycerol) as described previously (29). 200ug of bait protein was subjected to interaction with 1 mg of mitochondrial lysate by incubation in spin columns (Pierce) rotating gently for two hours at 4°C. His60 Ni Superflow Resin (TaKaRa) was added to the lysate for one-hour incubation at 4°C and used to bound the His-Tagged proteins. Non-specifically bound proteins were removed by washing with 20 x 500 ul of wash buffer (0.1% (w/v) digitonin, 20 mM Tris (pH 7.4), 60 mM NaCl, 10% (v/v) Glycerol, 0.5 mM EDTA). The final washed contained 50 mM imidazole. Proteins were eluted by 30min incubation with 300 mM imidazole and precipitated with acetone overnight. Proteins were then pelleted by centrifugation (21,000*g,* 10 mins, 4°C), washed with ice-cold acetone and air-dried. The pellet was then resolubilised by sonification in with 8M urea in 50 mM ammonium bicarbonate (ABC). Samples were reduced and alkylated with 50mM TCEP (ThermoFisher) and 500mM Chloroacetamide (Merck) (30 mins, 37°C, shaking). Elutions were diluted to 2M urea in 50 mM ABC and digested in trypsin (Promega) overnight at 37°C. The digest was acidified to a final concentration of 1% (v/v) trifluoracetic acid (TFA) and peptides were desalted with in-house made stagetips[1] containing 2x plugs of 3M™ Empore™ SDB-XC substrate (SUPELCO). Stagetips were activated with 100% Acetonitrile (ACN) and washed with 0.1% (v/v) TFA, 2% (v/v) ACN prior to binding the peptides. Samples were eluted in 80% ACN, 0.1% TFA and dried completely in a SpeedVac. Peptides were reconstituted in 2% ACN, 0.1% TFA and transferred to autosampler vials for analysis by LC MS/MS.

LC MS/MS analysis

LC MS/MS was carried out using the Fusion Lumos Orbitrap mass spectrometers with the FAIMS Pro interface (Thermo Fisher, USA) and as described previously (25). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 µm x 50 cm). Tryptic peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 2% (v/v) acetonitrile containing 0.1% (v/v) formic acid for 6 min, applied before the enrichment column was switched in-line with the analytical column. The eluents were 0.1% (v/v) formic acid (solvent A) in water and 100% (v/v) acetonitrile in 0.1% (v/v) formic acid (solvent B). The flow gradient was (i) 0-6 min at 3% B; (ii) 6-35 min, 3-22% B; (iii) 35-40 min, 22-40% B; (iv) 45-50 min, 40-80% B; (v) 50-55 min, 80-80% B; (vi) 55-56 min 85-3% and equilibrated at 3% B for 10 min before injecting the next sample. Tune version 3.3.2782.32 was used. For non-FAIMS experiments, the mass spectrometer was operated in the data-dependent acquisition mode, whereby full MS1 spectra were acquired in a positive mode at 60000 resolution. The 'top speed' acquisition mode with 3 s cycle time on the most intense precursor ion was used, whereby ions with charge states of 2 to 7 were selected. Automated gain control (AGC) target was set to standard with auto maximum injection mode. MS/MS analyses were performed by 1.6 *m/z* isolation with the quadrupole, fragmented by CID with collision energy of 35 %, activation time of 10 ms and activation Q of 0.25. Analysis of fragment ions was carried out in the ion trap using the 'Turbo' speed scanning mode. Dynamic exclusion was activated for 30 s. For FAIMS-enabled experiments, the mass spectrometer was operated in the data-dependent acquisition mode scanning from *m/z* 300-1600 at 60000 resolution. FAIMS separations were performed with the following settings: inner electrode temperature = 100 °C, outer electrode temperature = 100 °C, FAIMS carrier gas flow = 0 L/min. The FAIMS carrier gas was N_2. Cycle time using the 'top speed acquisition' mode for single CV experiment were 3 s and, for experiments wherin two CVs (-40 and-60) were applied and cycle time was 1.5 s each. MS/MS analyses were performed by 1.6 *m/z* isolation with the quadrupole, fragmented by CID with collision energy of 35 %, activation time of 10 ms and activation Q of 0.25. Analysis

of fragment ions was carried out in the ion trap using the 'Turbo' speed scanning mode. Dynamic exclusion was activated for 30 s.

For LC MS/MS experiments on an Orbitrap Eclipse the nanoLC conditions were kept constant. The mass spectrometer was operated in the data-dependent acquisition mode, whereby full MS1 spectra were acquired in a positive mode at 60000 resolution. Tune version was 3.3.2782.34. The 'top speed' acquisition mode with 3 s cycle time on the most intense precursor ion was used, whereby ions with charge states of 2 to 7 were selected. AGC target was set to standard with auto maximum injection mode. MS/MS analyses were performed by 1.6 *m/z* isolation with the quadrupole, fragmented by CID with collision energy of 35 %, activation time of 35 ms and activation Q of 10. Analysis of fragment ions was carried out in the ion trap using the 'Turbo' speed scanning mode. Dynamic exclusion was activated for 30 s.

For LC MS/MS experiments on an Orbitrap Exploris 480, the nanoLC conditions were kept constant. The mass spectrometer was operated in the data-dependent acquisition mode, whereby full MS1 spectra were acquired in a positive mode at 60000 resolution. Tune version was 2.0.182.25. The 'top speed' acquisition mode with 3 s cycle time on the most intense precursor ion was used, whereby ions with charge states of 2 to 7 were selected. MS/MS analyses were performed by 1.6 *m/z* isolation with the quadrupole, fragmented by HCD with collision energy of 30%. MS2 resolution was at 15000 Dynamic exclusion was activated for 30 s. AGC target was set to standard with auto maximum injection mode. Dynamic exclusion was activated for 30 s.

Database search

Database searches was carried out using Proteome Discoverer (v2.4) with the SequestHT search engine against a *Homo Sapiens* database (SwissProt Taxonomy ID 9606, updated Feb 2021). The search parameters are Trypsin as the cleavage enzyme, precursor and fragment mass tolerances of 10 ppm and 0.6 Da respectively and a maximum of 2 missed cleavages. Carbamidomethyl cysteine was set as fixed modification, and oxidation of methionine and acetylation of the protein N-terminus were considered as variable modifications. Protein and peptides groups were set to a maximum false discovery rate (FDR) of < 0.01 as determined by the Percolator algorithm (30). Peak area determination was carried out using the Skyline software (31).

Collisional cross-sectional area determination

Determination of the averaged collision cross section (CCS) values was carried out on an Acquity UHPLC-H-Class/Vion IMS-QTOF system (Waters) as described previously (32) . The UHPLC system was equipped with a Waters BEH C18 column (1.7 µm, 2.1 × 50 mm) and the eluents were 0.1% (v/v) formic acid (solvent A) and 100% (v/v) acetonitrile in 0.1% (v/v) formic acid (solvent B). Flow rate was 0.5 ml/min and flow gradients were (i) 0–10 min at 5–95% B (ii) 10–11 min, 85–85% B (iii) 11–11.1 min 85-5% and equilibrated at 5% solvent B for three minutes before the next sample injection. UV detection was carried out at 214 nm. The Vion IMS-QTOF was operated in positive mode with MS data calibration achieved by constant infusion of LeuEnk (*m/z* 556.277) and GluFib (*m/z* 782.842). Capillary voltage was 3 kV, source temperature 100 ℃, desolvation temperature 250 ℃ and cone gas and desolvation gas flow were 50 L/hr and 600 L/hr, respectively. The time of flight (TOF) mass spectrometer was operating using the HDMS (ion mobility) mode and scanning from *m/z* 100-2000. Calibration of CCS was carried out according to the manufacturer's guidelines using the major mix IMS/Tof calibration kit (Waters, part number 186008113). Data analysis was carried using UNIFI 1.9.4 (Waters).

Results

The total ion chromatogram (TIC) of a FLAG Co-IP/MS experiment following competitive displacement with 3X FLAG peptide and *in solution* digest is shown in Figure 1A. Two dominant peaks are visible in the chromatogram centered at 19.0 min and 21.5min. Upon inspecting the MS1 spectra at these points, we can see the later TIC peak being dominated by few highly charged ions, corresponding to multiply charged species of the 3X FLAG peptide - MDYKDHDGDYKDHDIDYKDDDDK (2+, 3+, 4+, 5+ and 6+). The earlier TIC peak consists of significant number of modified or truncated versions of the 3X FLAG peptide e.g. oxidized methionine, trypsin cleavage products (MDYYK↓DHDGDYKDHDIDYKDDDDK) and the oxidized forms of these tryptic peptides. This result is representative of a large fraction of Co-IP/MS experiments wherein the most abundant ions detected are introduced as byproducts of the assay itself and do not correspond to peptides arising from interactor proteins.

Interestingly, the most abundant ions observed in each of these major chromatographic peaks correspond to highly charged (+4 to +6) 3X FLAG-derived peptide ions (Fig. 1B and Fig. 1C). Given that tryptic peptides produced from analyte proteins are most commonly observed in +2 or +3 charge states, the higher charge of the 3X FLAG contaminant ions offered the possibility that these could be removed online via FAIMS. To probe this, we sought to assess whether different charge states of the FLAG peptide possessed differing collisional cross sections that could be leveraged in later FAIMS filtration. As FAIMS devices cannot provide CCS values, we analyzed the synthetic 3X FLAG peptide on a TWIMS/QTOF system (Figure 2). Importantly, the charge state distribution appeared similar between the TWIMS/QTOF and FAIMS/Orbitrap instruments with the +5 ion being most abundant in each case. The CCS value of the 3X FLAG peptide-

derived ions ranged from 565 Å² (2+) to 986 Å² (6+), indicating that there are substantial differences in the size and/or shape of the peptide across different charge states.

We then analyzed the same synthetic 3X FLAG peptide on the FAIMS/Orbitrap mass spectrometer with incrementally increasing compensation voltages from CV-20 to CV-70 in steps of 10 (Supplementary Figure 1). With the exception of the $[M+4H]^{4+}$ precursor at CV-60 and CV-70, all the other charge states of the 3X FLAG were reduced in measured intensity between 30% and 100% (Supplementary Table 1). Next, we performed a FLAG Co-IP experiment on a whole cell lysate followed by *in solution* digest. The same sample was then analyzed using the FAIMS/Orbitrap with different CVs to assess the number of proteins identified under these varying FAIMS conditions. When no FAIMS was used, MS analysis identified 365 protein groups, 1362 peptide groups and 1603 peptide spectral matches (PSM) at a 1% false discovery rate. The greatest improvement in peptide identifications was observed when FAIMS was operated with a CV of -40. This led to gains of 93%, 58% and 51% in proteins, peptides and PSM, respectively compared to analysis of the same sample without FAIMS. The remainder of the single CVs tested led to either comparable (CV-30 and -50) or substantially lower (CV -20, -60, -70) protein, peptide and PSM number upon database searching.

While the greatest improvement in protein identifications was observed for CV-40, it is well documented that different, and perhaps complementary, subsets of peptides may be preferentially detected at alternative CVs. To assess whether identification numbers may be improved by employing two different CVs during the analysis of a single sample, we re-analyzed the FLAG Co-IP sample with FAIMS rapidly switching between CVs of -40 and -60. Here, 734 protein groups, 2550 peptide groups and 2904 PSMs respectively were identified following database searching translating to a 100%, 59% and 81% gain in

compared to without FAIMS. This is almost 2-fold increase in the total protein identification but critically, almost 10-fold more proteins were exclusively identified when two internal CVs were applied as compared to without FAIMS (Figure 3D). The distribution of the different charge species selected for MS/MS follows a trend of decreasing 2+ peptides with decreasing CVs, increasing 4+ and 5+ peptides with decreasing CVs (Figure 3E). Interestingly 3+ peptides appear to be uniformly distributed and within ~20%-45% of all measured MS1 features across CV-30 to CV-70. We repeated this experiment on a new independent FLAG Co-IP experiment targeting a different FLAG-tagged protein and observed similar and significant increase in the protein, peptide and PSM (Supplementary Figure 2).

We further tested the ability of FAIMS to improve protein identification on other pull-down experiments. We decided to analyze a His-Tagged pull down assay considering the principal is very similar to the tagged protein/peptide co-immunoprecipitation methodology. The bait in this case is expressed as a recombinant protein fused to a 6x Histidine tag. After enrichment of the bait and other interacting proteins using Ni-NTA immobilized metal beads, an *in-solution* digestion step is applied to identify the proteins that have been enriched. Now, instead of the abundance of the molar excess of 3X FLAG peptide used in competitive displacement, there is now an excess of the bait protein which will be digested and as a result compete for the ion storage capacity of the C-trap. As before, the same sample was repeatedly analysed with 6 different single-CV methods, or the rapid-switching, dual-CV (-40, -60) method and database searches were conducted and compared to those following analysis of the same sample without FAIMS. The distribution of peptide charge states detected at the range of CV steps were similar to that observed for FLAG-tagged Co-IPs above (Figure 4E). For His-tagged Co-IP samples, we find that CV-40 again provides the greatest enhancement of protein and peptide identifications (Fig. 4A) with a 147%, 114% and 97% gain in proteins, peptides and PSM, respectively as compared to without FAIMS. Consistent with the charge distribution of peptides selected for MS/MS (Figure 3E and 4E), there is a ca. 250% increase in the

identified PSMs that are doubly charged and with no significant differences for the 3+ to 5+ peptides as compared to without FAIMS (data not shown). The two internal CVs experiment similarly resulted in a 152%, 136% and 161% gain in proteins, peptides and PSM, respectively. Again, we find 2.5-fold more total protein identification and more impressively, a 15-fold more proteins exclusively identified with FAIMS.

Discussion

Given that Co-IP experiments typically produce large molar excesses of contaminant peptides derived from peptides used in competitive displacement or bait proteins, we reasoned that deeper profiling of interacting species could be achieved by developing methods to reduce the suppressive effects of high-abundance ions on mass spectrometric detection of low-abundance species. We have successfully utilized Field Asymmetric Ion Mobility as a gas phase filtering technique to remove interferences in a FLAG co-immunoprecipitation and His-Tagged pull down mass spectrometry experiment and very significantly increased the depth of proteome coverage. The numbers of proteins identified form FLAG-tagged Co-IPs and 6X-His IP increased by 100% and 152% when FAIMS filtering was employed with two, rapidly switching compensation voltages (-40 and -60) compared to analyses of the same samples when FAIMS was not used. Critically, 10 and 15-fold greater numbers of proteins were exclusively identified with the application of the optimal FAIMS-filtering method.

It should be noted that a range of techniques are currently available to reduce interference from high-abundance peptides. For example, the excess FLAG peptides could be removed with an offline cleanup process or by running it off an SDS PAGE gel, size exclusion columns, molecular weight filtration etc. The downside is that it requires additional offline sample handling steps, likely introduce experimental variability and thereby potentially interfere with quantitation. A single LC MS/MS experiment with

increased depth is still the preferred methodology in a Co-IP/MS experiment. In contrast to these existing methods, the FAIMS methodology presented here does not require any additional sample handling steps, fractionation or library construction and is completely amenable to standard, single-shot data-dependent acquisition. We were able to observe clear differences in the ion mobility or CCS for the different charge species of the synthetic 3X FLAG peptide when travelling in an ion mobility device. This property is then exploited to filter out unwanted ions thereby providing the opportunity for the mass spectrometer to sample ions of interest. When using FAIMS we observed a massive drop in the MS1 ion injection time in the presence of the large excess of 3X FLAG peptides (Supplementary Figure 3A). Applying ion mobility on the FLAG-tagged Co-IP sample with CV-40 allow up to 100X increase in the MS1 ion injection time (Supp Figure 3B-C). At that CV value, we can clearly filter out the 5+, 6+ species and also a large percentage of the 4+ species as seen in Supplementary Figure 1. When performing that as a single experiment stepping through two internal CVs, we should be able to filter out the contribution of the dominating 4+, 5+ and 6+ with CV-40 and when switching to CV-60 filtering out the 2+ species. These data clearly show the benefit of not allowing a few dominant species – be it peptides from competitive displacement process or peptides from the highly abundant bait protein to fill up the limited charge capacity of the ion storage device.

We sought to establish if the significant increase is not synchronous with all types of IM based experiment but a phenomenon that is augmented in Co-IP experiment, we compared the difference in identification rate with a complex protein mix with or without FAIMS. We analyzed a commercial tryptic digest of Hela cells (100ng on column) using the same methodology with two internal CVs (-40 and -60) on the FAIMS and without FAIMS. On average, we observed a ca. 30% increase in protein identification when FAIMS is applied (Supplementary Table 2). This is in agreement with the work published by the Coon lab (22) where they showed FAIMS (different CVs compared to this manuscript) providing similar increase in protein

identification with the same 60 min analysis time. To determine if the significant increase in identification is not due to the analytical mass spectrometer, we ran the same FLAG Co-IP digest sample on a newer Orbitrap Eclipse and Orbitrap Exploris 480 mass spectrometer using similar methodology and compared it with the results on the Fusion Lumos Orbitrap with (CV-40 and -60) and without FAIMS. The Orbitrap Eclipse is the latest generation Orbitrap Tribrid mass spectrometer with improvement in the quad filter, FTMS overheads and better ion transmission (33) and have been shown to increase identification by up to 20% in a single cell proteome study vs the Fusion Lumos (34) where sensitivity is of upmost importance. With FAIMS implementation on the Fusion Lumos, we were able to observe increase identification of between 64-114% as compared to the other Orbitrap instrumentations without FAIMS (Supplementary Table 2). This clearly shows the increase is not due to improvement in instrumentation capability. Buoyed by the success, we applied this methodology to a 3rd type of Co-IP experiment – c-Myc pull down. The results were equally impressive with equally significant increase of 93%, 87% and 76% identification for protein, peptide and PSM, respectively (data not shown) demonstrating the FAIMS methodology can be applied to a large variety of co-immunoprecipitation type experiment. The FAIMS device unquestionably provide increased depth in a highly complex sample but for a relatively 'low complexity' sample like a Co-IP experiment, the improvement afforded by filtering out the dominating ion species is much greater. The significant increase in protein identification represents the extension of the depth of proteome coverage, potentially revealing previously unknown protein partners and in our view should be the method of choice for such experiment.

Discovery and functional characterization of protein-protein interaction network is challenging. This requires sound experimental planning, the right bait protein(s), high yield of bait proteins and in-depth identification of the interacting proteins. While a full biological analysis is these entities is beyond the scope of this manuscript, these proteins differentially detected with FAIMS could potentially be missing

links in protein complexes or low level transient interactors etc. This technique theoretically can be applied to other ion mobility devices such as the TWIMS, TIMS and DTIMS systems with appropriate acquisition parameters. While FAIMS enabled methodology has already been documented to increase proteome coverage for complex proteomics samples we show here that it can have a proportionately greater impact on "simple" or low complexity samples.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD026637

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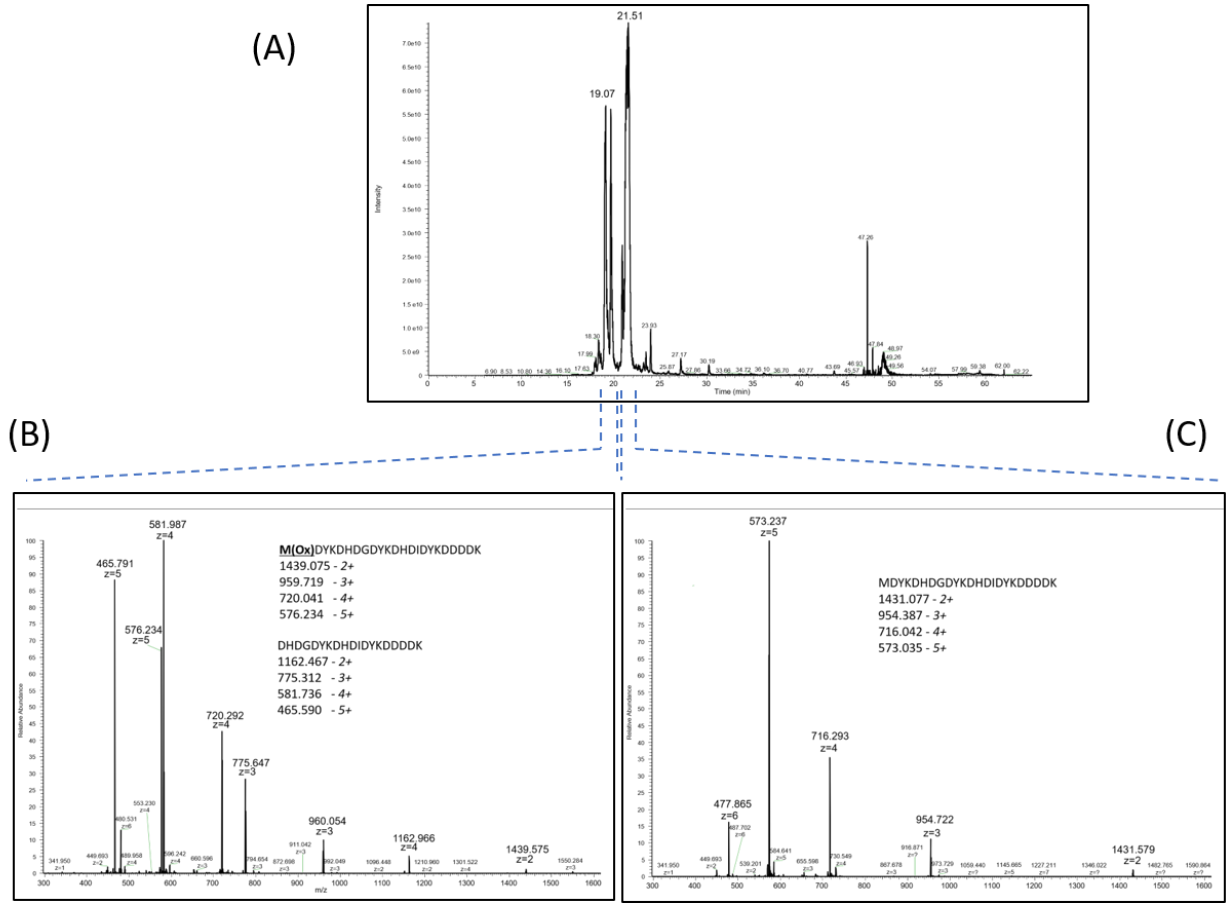


Figure 1. A 3X FLAG tag specific Co-IP Mass spectrometry experiment on an Orbitrap Fusion Lumos mass spectrometer. (A) The TIC mass spectrum of the most intense peaks between 18-22minutes. (B) The averaged mass spectrum at ~19min showing the presence of the oxidized version of the 3X FLAG peptide and a trypsin derived truncated version of the 3X FLAG peptide. (C) The averaged mass spectrum at ~21min showing the charge distribution of the intact 3X FLAG peptide. Note m/z peak labeling is on the most abundant ion in the isotopic cluster.

Charge state	m/z	Observed drift time (ms)	Observed CCS (Å²)
2+	1431.075	9.56	565.45
3+	954.3867	6.73	599.02
4+	716.0432	5.46	660.12
5+	573.0366	5.97	887.18
6+	477.6986	5.49	986.00

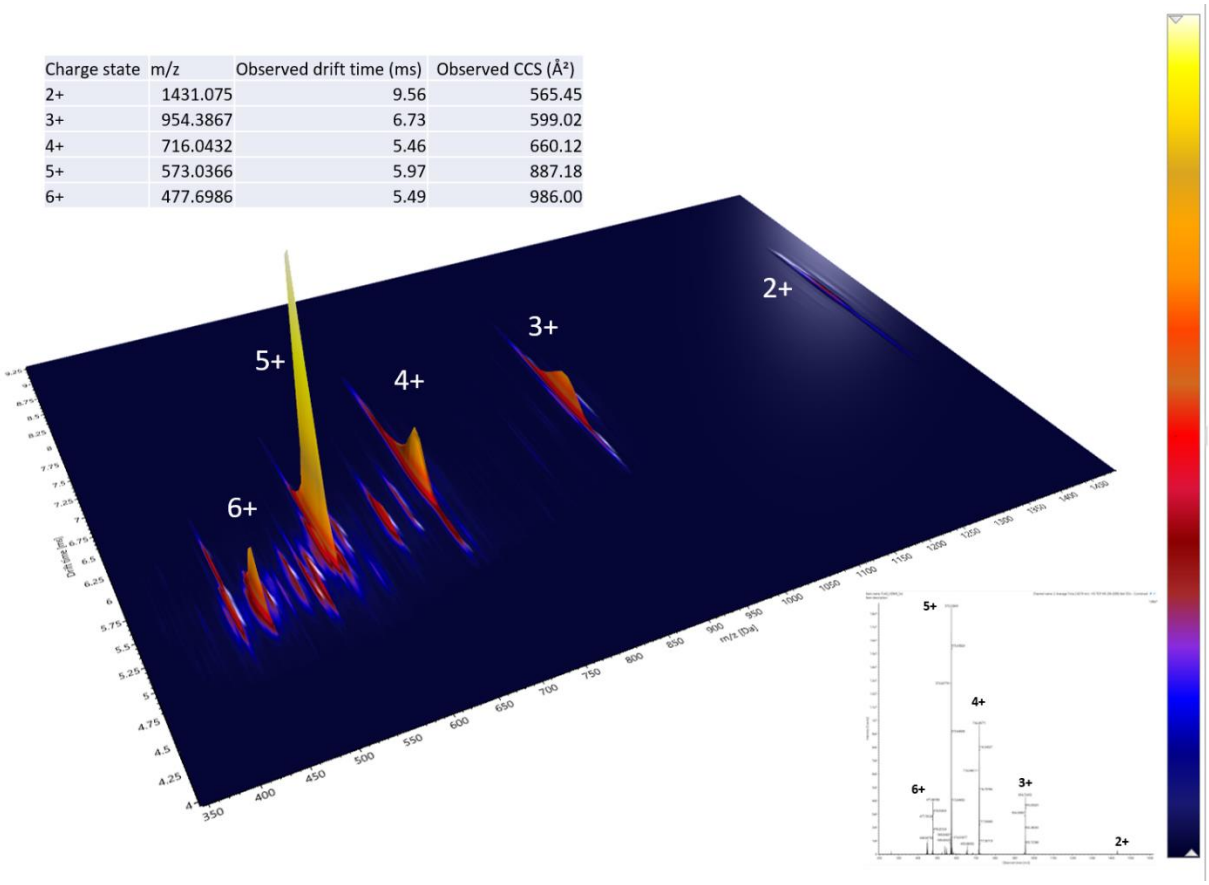


Figure 2. The ion mobility spectrum of 3X FLAG peptide and its different charge states as seen in a TWIMS-TOF instrumentation. Y-axis represents the drift time X axis the m/z in Da. The table shows the observed drift time and calculated CCS values for the different charge states. Insert shows the mass spectrum on the TWIMS-TOF instrument.

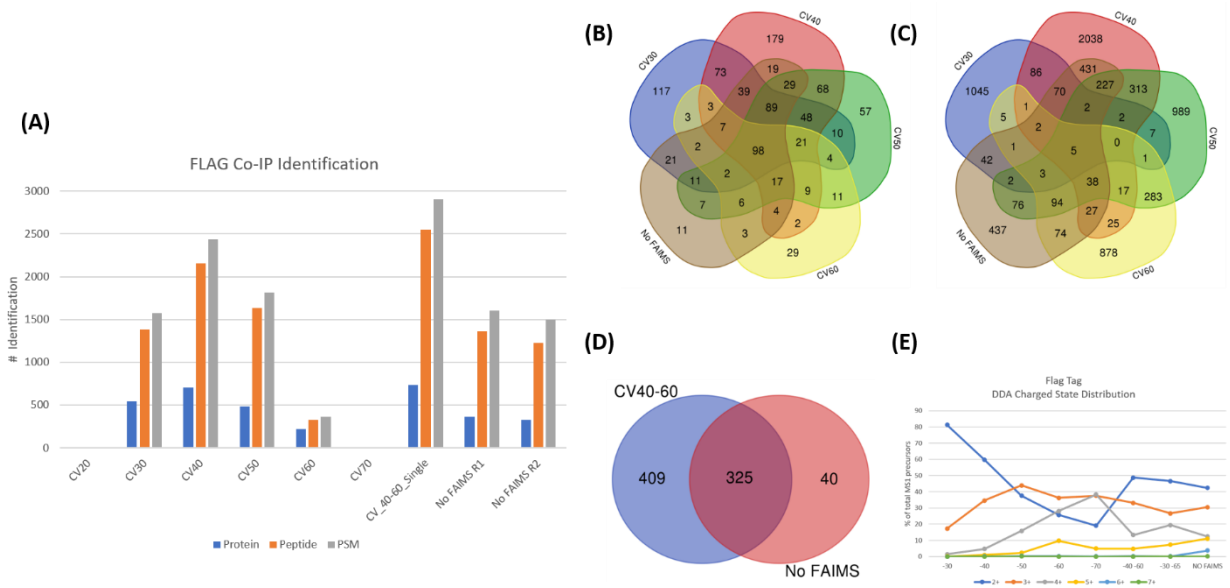


Figure 3. Analyzing the protein and peptide identification from the FLAG Co-IP experiment. (A) Breakdown of protein, peptide and PSM across 6 different CVs, stepping through two internal CVs and two replicates without FAIMS. We did not identify any proteins with CV-20 and CV-70. (B) Venn diagram of unique protein identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (C) Venn diagram of unique peptide identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (E) Charge distribution of MS1 precursors selected for MSMS across the 6 different CVs.

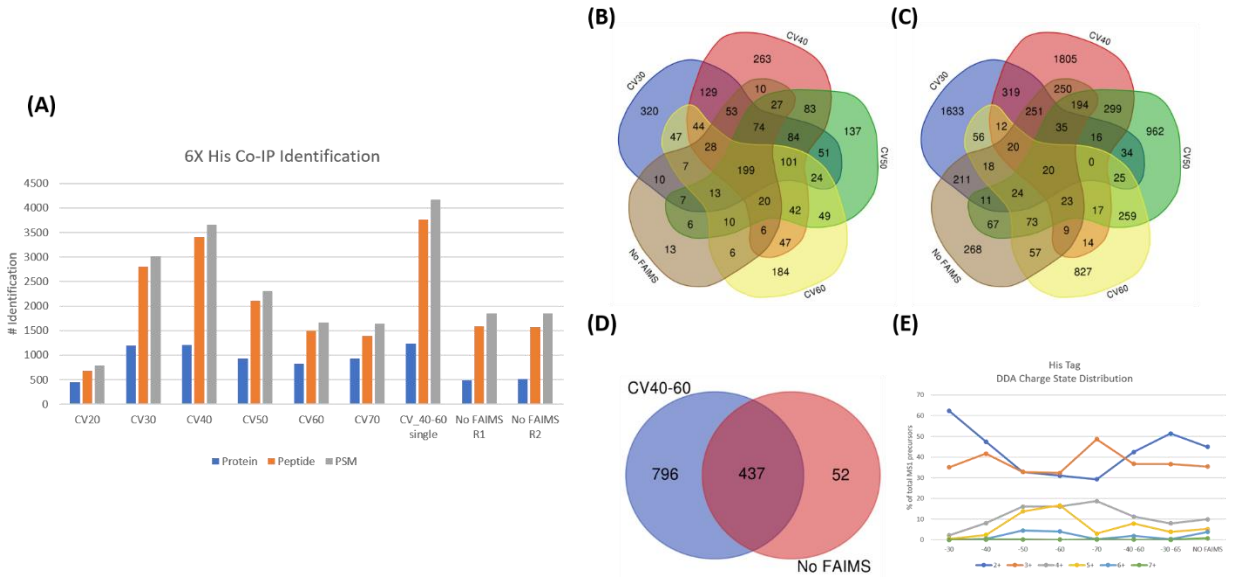
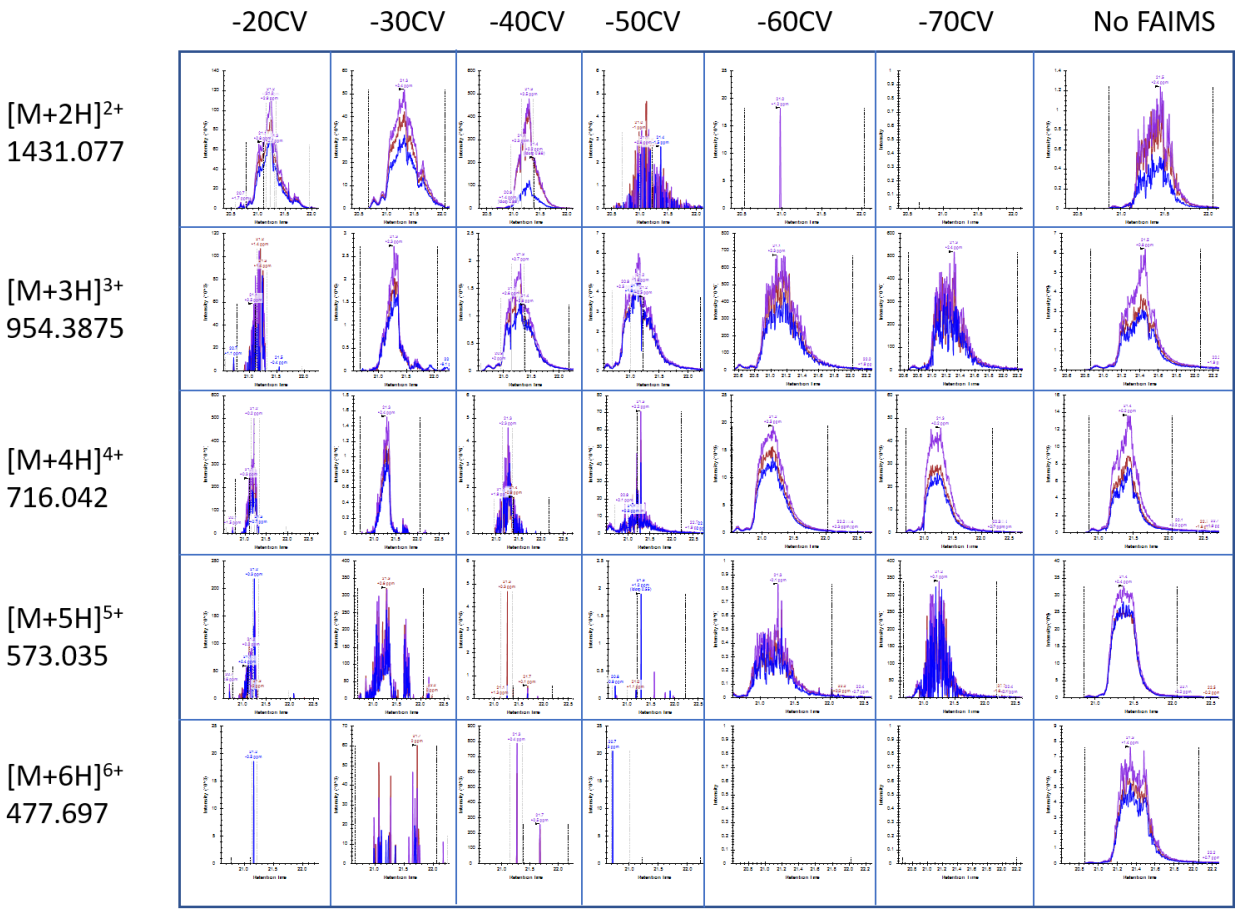
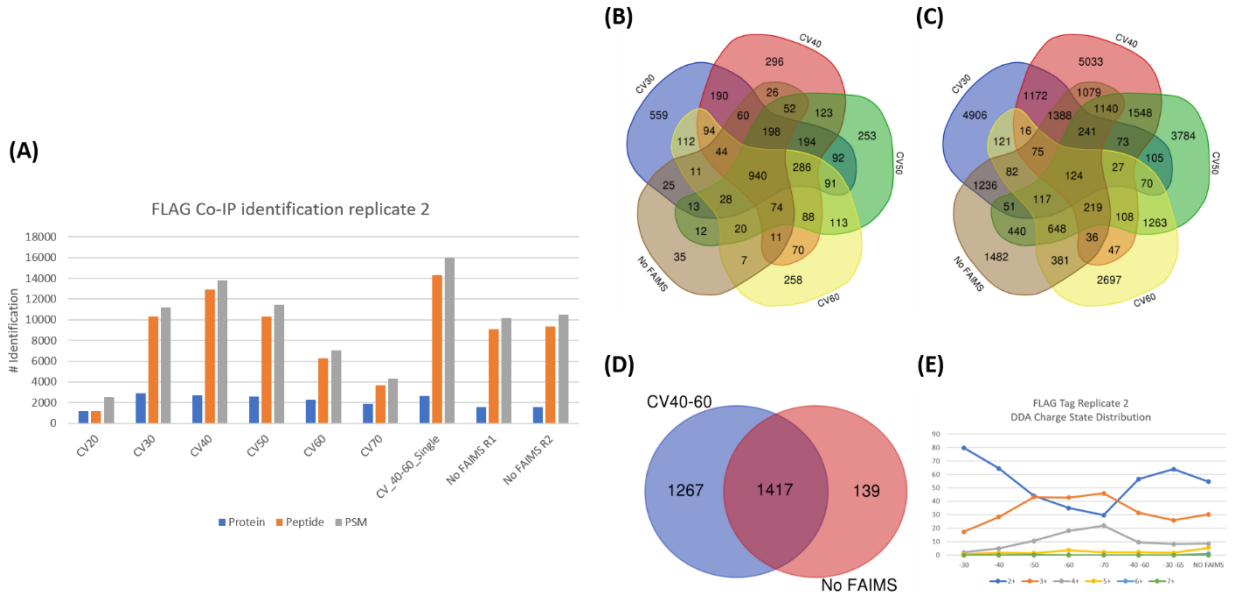


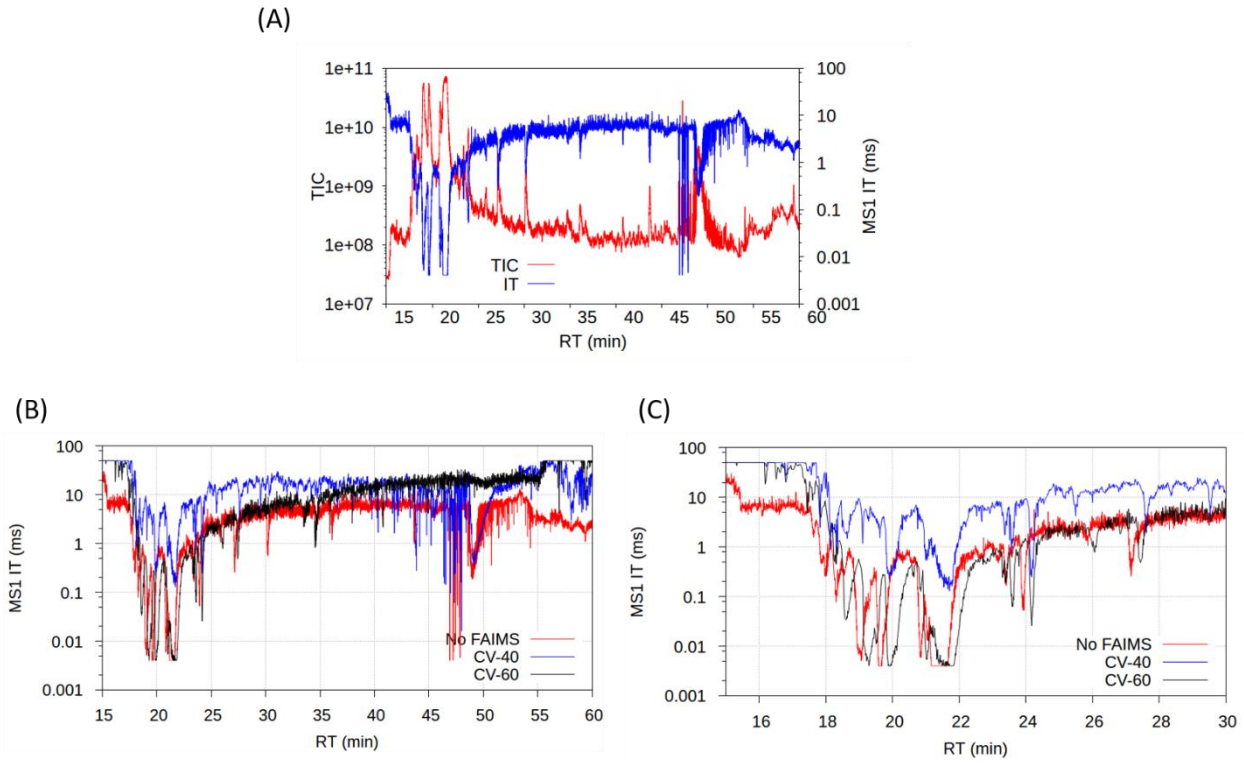
Figure 4. Analyzing the protein and peptide identification from the 6X His-Tag Co-IP experiment. (A) Breakdown of protein, peptide and PSM across 6 different CVs, stepping through two internal CVs and two replicates without FAIMS. (B) Venn diagram of unique protein identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (C) Venn diagram of unique peptide identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (E) Charge distribution of MS1 precursors selected for MSMS across the 6 different CVs.



Supplementary Figure 1. Monitoring the intensities of the different charge species of the 3X FLAG peptide across the different CVs.



Supplementary Figure 2. Analyzing the protein and peptide identification from an second independent FLAG Co-IP experiment. (A) Breakdown of protein, peptide and PSM across 6 different CVs, stepping through two internal CVs and two replicates without FAIMS. (B) Venn diagram of unique protein identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (C) Venn diagram of unique peptide identification across 4 different CVs (-30, -40, -50 and -60) with No FAIMS applied. (E) Charge distribution of MS1 precursors selected for MSMS across the 6 different CVs.



Supplementary Figure 3: (A) Plot of the Total Ion Chromatogram vs the MS1 ion injection time across the LC MS/MS run for the FLAG Co-IP experiment. The dips in the blue trace shows decrease in the ion injection time whenever the TIC increases from the abundant 3X FLAG peptide. **(B)** Comparing the MS1 ion injection time without FAIMS, CV-40 and CV-60. **(C)** Expanded plot from 16-30 min showing up to 100X increase in max MS1 ion injection time.