# Rapid, Multi-level Analysis of Complex Proteins by Microchip Capillary Electrophoresis-ESI-MS

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## Overview

Mass spec characterization of proteins often requires multiple "levels" of analysis to generate a complete picture of the molecule. These levels are commonly referred to as top-down, middle-down/up, and bottom-up. While LC-MS methods have been developed for each of these levels, those methods typically require completely different columns and mobile phases for the different levels; and the methods typically yield relatively poor results for middle and top down analysis. Microchip CE-ESI-MS can achieve excellent performance for all of these levels, using the same exact experimental conditions. This enables rapid, back-to-back multilevel characterization of a protein, with no down-time between samples. This study demonstrates that capability for a variety of proteins, including complex glycoproteins and monoclonal antibodies.

## Methods

**Sample Preparation and Analysis.** Proteins were prepared for intact analysis by simply diluting to an appropriate concentration before loading into the microchip. Middle-down analysis was performed on monoclonal antibodies by subjecting them to limited proteolysis using the IDeS enzyme and reducing them with DTT. Bottom-up analysis utilized a standard trypsin digestion method in ammonium bicarbonate buffer.

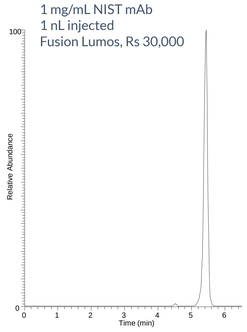
**Instrumentation**. All work was performed using a commercially available microfluidic CE-ESI system (ZipChip, 908 Devices Inc.). The microfluidic devices utilize a covalently attached, neutral polymer surface coating to prevent analyte interactions and suppress electroosmotic flow. For the work shown here, a chip with a 22 cm long separation channel was used (ZipChip HR). Data were collected on a Thermo Tribrid Fusion Lumos mass spectrometer or a Thermo Exactive Plus EMR mass spectrometer.
To achieve successful analysis at all three structural levels, a BGE with a relatively low pH was chosen. The ZipChip "Metabolite BGE" (908 Devices Inc.) is a low pH mixture containing water, methanol, and formic acid. While it was developed for optimizing metabolite separations, it is well suited for many peptide and intact protein analyses as well.

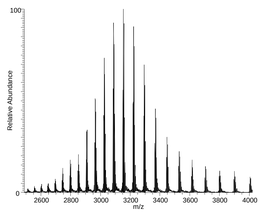
**Data Processing.** Data were visualized using Thermo Xcalibur QualBrowser. Intact and bottom-up data were processed using Thermo BioPharmaFinder 1.0. Top-down fragmentation spectra were deconvoluted using the Xtract algorithm in Thermo FreeStyle. Residue cleavage was calculated using ProSight Lite 1.4 (Northwestern University).

### Device Schematic

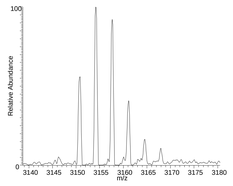

## Intact Analysis

1 mg/mL NIST mAb
1 nL injected
Fusion Lumos, Rs 30,000

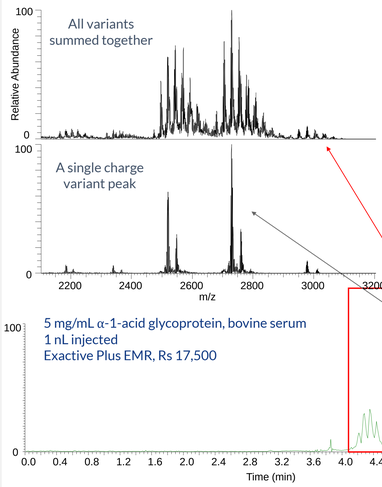



+47 Charge State

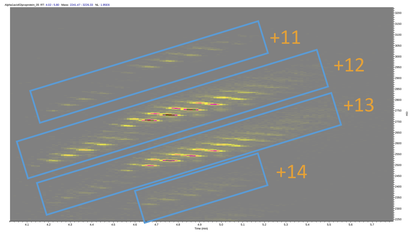



When denaturing conditions are used for intact antibody analysis, no separation of charge variants is observed, but high quality mass spectra are still generated. This approach serves as a simple way to electrophoretically desalt and electrospray a mAb to obtain MS data. The speed of analysis can be altered based on the BGE and chip type used. By using an HS chip, run times of less than two minutes can be achieved.




5 mg/mL α-1-acid glycoprotein, bovine serum
1 nL injected
Exactive Plus EMR, Rs 17,500

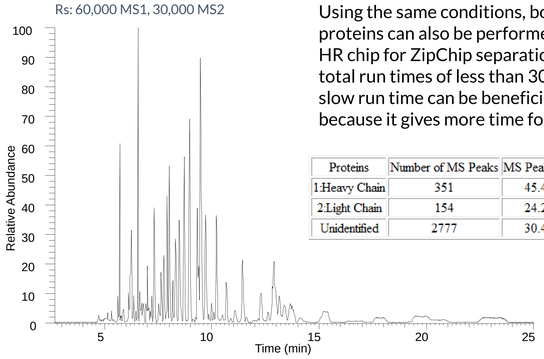



Many smaller proteins can be separated into many different variant peaks under fully denaturing conditions. The large number of sialic acid residues present on the complex structure of α-1-acid glycoprotein cause it to separate into many different peaks. This complexity is more easily seen in the image plot above. The ability to assess individual variants facilitates characterization and identification of the glycoforms through deconvolution.

## Bottom-up Analysis

0.2 mg/mL NIST mAb tryptic digest
5 nL injected
Fusion Lumos, DDA MS/MS
Rs: 60,000 MS1, 30,000 MS2



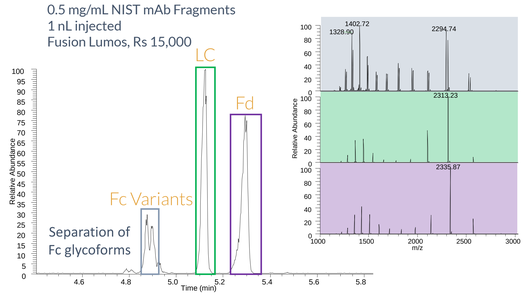

Using the same conditions, bottom up analysis of proteins can also be performed. Use of this BGE and an HR chip for ZipChip separations of protein digests yields total run times of less than 30 minutes. This relatively slow run time can be beneficial for peptide mapping because it gives more time for MS/MS analysis.

| Proteins | Number of MS Peaks | MS Peak Area | Sequence Coverage | Abundance (mol) |
|---|---|---|---|---|
| 1:Heavy Chain | 351 | 45.4% | 94.9% | 54.81% |
| 2:Light Chain | 154 | 24.2% | 100.0% | 45.19% |
| Unidentified | 2777 | 30.4% | | |

Confidence score ≥99%

## Middle-up Analysis

0.5 mg/mL NIST mAb Fragments
1 nL injected
Fusion Lumos, Rs 15,000




+11

Rs Setting:

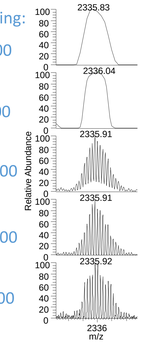
15,000

30,000

120,000

240,000

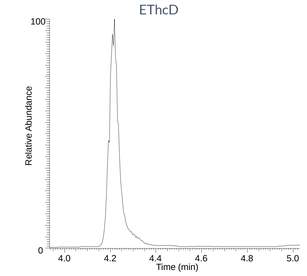
500,000



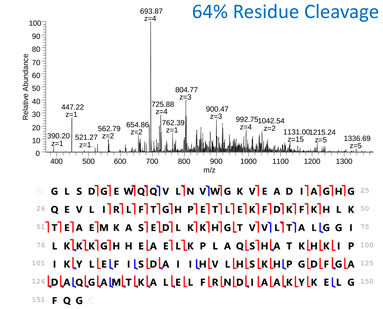

Middle-up/down analysis of antibodies is achieved by breaking the antibody into smaller pieces through reduction of disulfide bonds and/or targeted proteolysis. These smaller protein fragments can be separated with excellent efficiency under fully denaturing conditions. The Fc, LC, and Fd fragments of the mAb are baseline resolved in ~5.5 minutes. On the Fusion Lumos, isotopic resolution of the fragments can be achieved while maintaining adequate sampling of the peaks. The acquisition rate at Rs 120,000 is approximately 3 data points per second, giving us about 9 data points across these 3 second wide peaks.

## Top-down Analysis

~0.8 mg/mL Equine Myoglobin
1 nL injected
Fusion Lumos, Rs 120,000
EThcD




64% Residue Cleavage




N G L S D G E W Q Q V L N V W G K V E A D I A G H G 25
26 Q E V L I R L F T G H P E T L E K F D K F K H L K 50
51 T E A E M K A S E D L K K H G T V V L T A L G G I 75
76 L K K K G H H E A E L K P L A Q S H A T K H K I P 100
101 I K Y L E F I S D A I I H V L H S K H P G D F G A 125
126 D A Q G A M T K A L E L F R N D I A A K Y K E L G 150
151 F Q G

Smaller intact proteins and subunits of larger proteins can be efficiently fragmented for top-down analysis. The data shown here was generated using a combination of ETD and HCD fragmentation (EThcD), on the Fusion Lumos mass spectrometer. Combining the intact protein separating power of the ZipChip separation, with this type of top-down analysis represents a powerful new platform for proteoform analysis.

## Conclusions

Much of our previous work has focused on fully optimizing methods to achieve the best possible results for different applications of the microfluidic CE-MS (ZipChip) platform. If we instead consider how much can be accomplished without changing either the chip or the BGE, we see a different advantage of this platform. The ability to run such a wide range of applications without making any changes to the system means that a wide variety of samples can simply and efficiently be run on the mass spec. For labs that commonly deal with a variety of samples, this type of flexibility can save a lot of time and allow maximum use of the mass spec. It's also worth noting that while not shown on this poster, the background electrolyte used for this work was originally optimized for metabolite analysis.