

Introduction

Trapped ion mobility time-of-flight mass spectrometry (TIMS-TOF) enables a fast and sensitive data acquisition strategy called parallel accumulation / serial fragmentation (PASEF). PASEF is already interesting as a method to increase speed and sensitivity, but it also offers the possibility of separating and quantifying co-eluting isomers by differences in ion mobility. Here we study this potential using two sample types likely to contain a variety of structural and positional isomers: human plasma enriched for glycopeptides and a human whole cell lysate enriched for phosphopeptides.

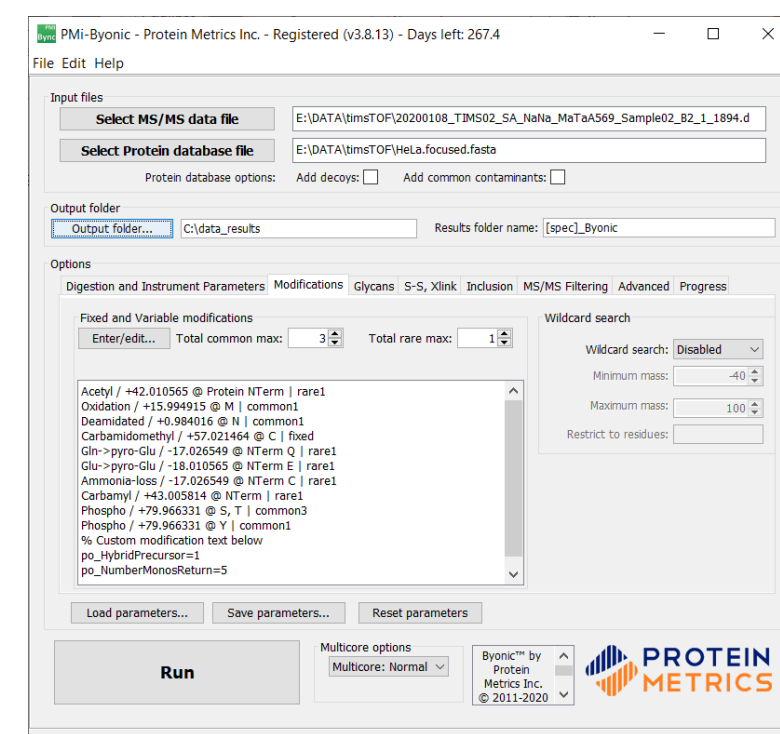
Experimental and Computational Methods

We collected LC-MS/MS data of **human plasma**, reduced and alkylated, trypsin-digested, lectin-enriched for glycopeptides, and run with a 90-minute reverse-phase gradient (120-min acquisition) on a Bruker timsTOF Pro instrument using several MS/MS collision energies. We also performed LC-MS/MS of 1.2 mg trypsin digested HeLa cell lysate, enriched for phosphopeptides using TiO₂, split into 6 aliquots and run with identical 130-minute RPLC on a Bruker timsTOF Pro and a Thermo Q-Exactive HF-X Orbitrap.



We searched the data using Byonic™ software, with a full human protein database for initial searches, and then “focused” databases (produced by the checkbox on the Advanced tab) for larger searches. Variable PTMs for the phosphopeptide searches are shown below; the initial search used “Total common max” of 2 instead of 3.

For the glycopeptide searches, we used the same glycan databases and variable modifications that we used for the recent HUPO HGI study ([https://www.hupo.org/Glycoproteomics-\(B/D-GPP\)](https://www.hupo.org/Glycoproteomics-(B/D-GPP))) *m/z* tolerances were 20 and 40 ppm, precursor and fragments, for TIMS-TOF and to 6 and 20 (glyco) or 10 and 20 (phospho) for Orbitrap. All searches considered only fully tryptic peptides.



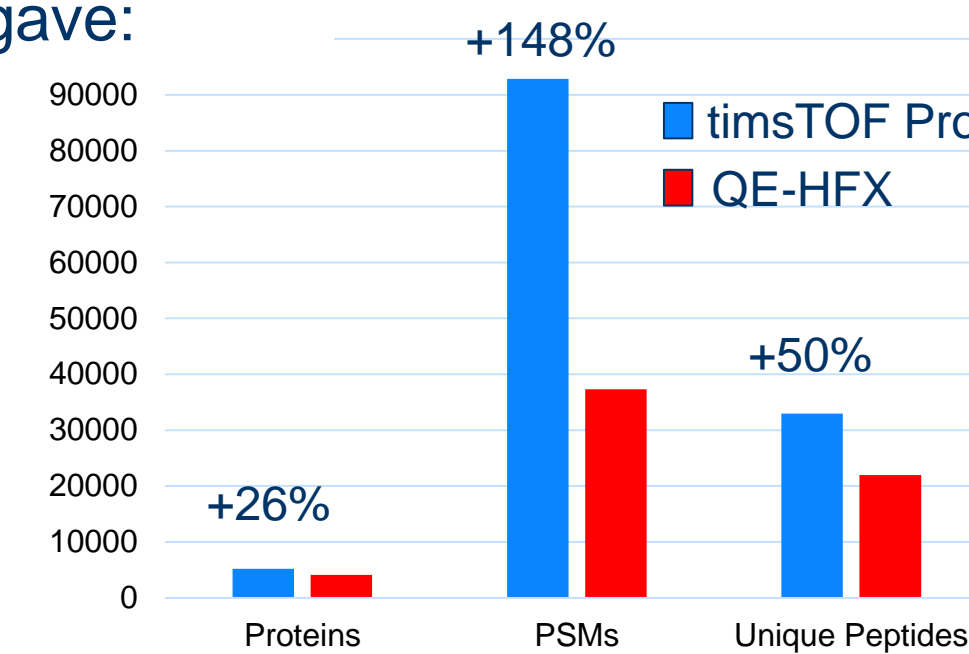
Sensitivity on HeLa Phosphopeptide Sample

timsTOF Pro 439,055 MS2 scans gave:

- 5201 proteins at 1% protein FDR
- 92,873 PSMs at 1% PSM FDR
- 32,957 unique peptides at 1% peptide FDR

QE-HFX 73,551 MS2 scans gave:

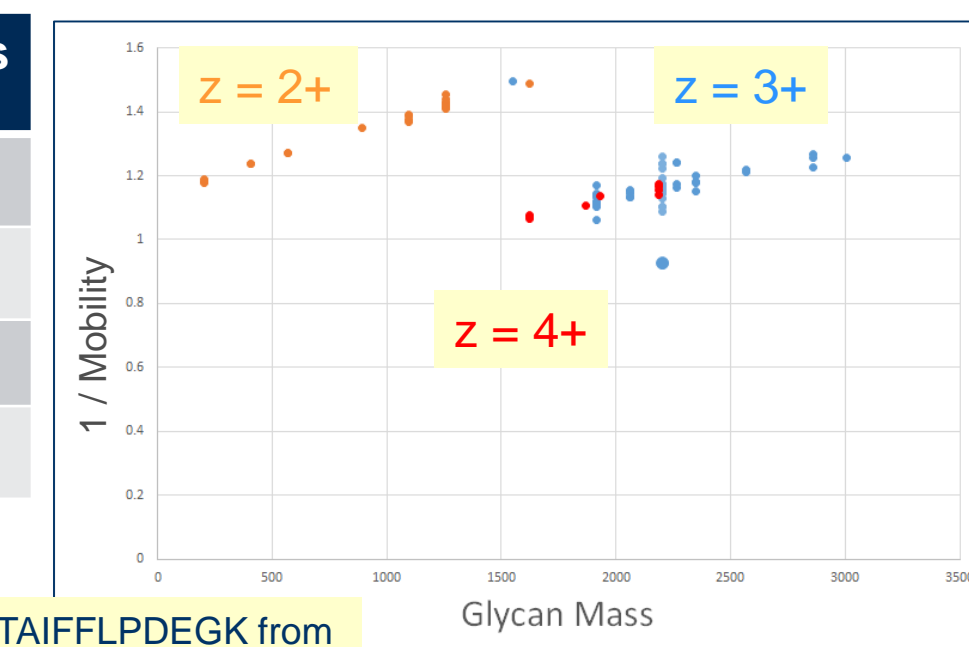
- 4119 proteins at 1% protein FDR
- 37,307 PSMs at 1% PSM FDR
- 21,978 unique peptides at 1% peptide FDR



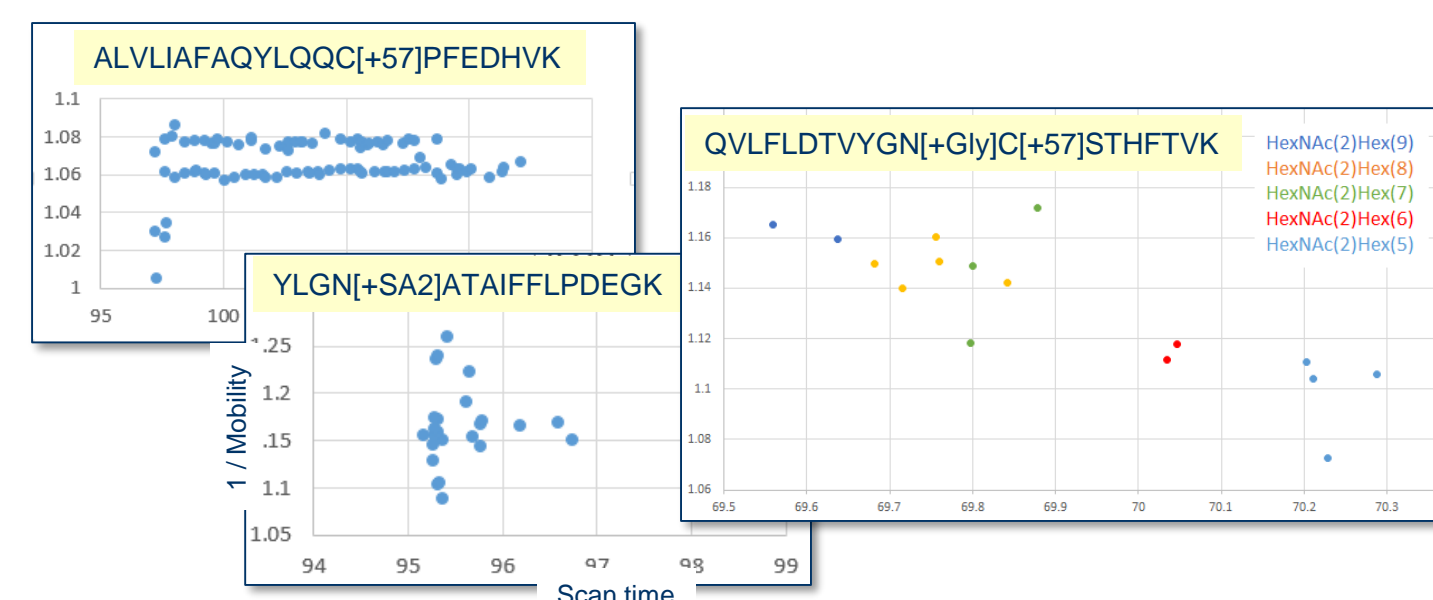
Glycopeptides

As shown below left, 32-64eV collision energy outperformed 31-51 eV. As shown below right, in RPLC glycopeptides with the same primary sequence have similar retention times, with each sialic acid increasing retention time by a few minutes. For example, the YLG... peptide below from α1-antitrypsin) appears with neutral peptides at ~82 min, singly sialylated ~87 min, and doubly sialylated ~95 min. Ion mobility has been shown to separate some glycopeptide isomers based on glycan structure (Zhu et al., *JASMS*, PMID 25840811). Is this possible in large-scale glycoproteomics?

Sample	Proteins 2% FDR	PSMs 1% FDR	Peptides 1% FDR
All 31-51 eV	104	6696	1118
Glyco 31-51 eV	74	1888	347
All 32-64 eV	107	9857	1285
Glyco 32-64 eV	75	3499	597



This scatter plot shows YLGN[+Glycan]ATAIFFLPDEGK from A1AT. Mobility is the drift speed of the ion in an electric field, and (*z* / mobility) is related to the collision cross-section.

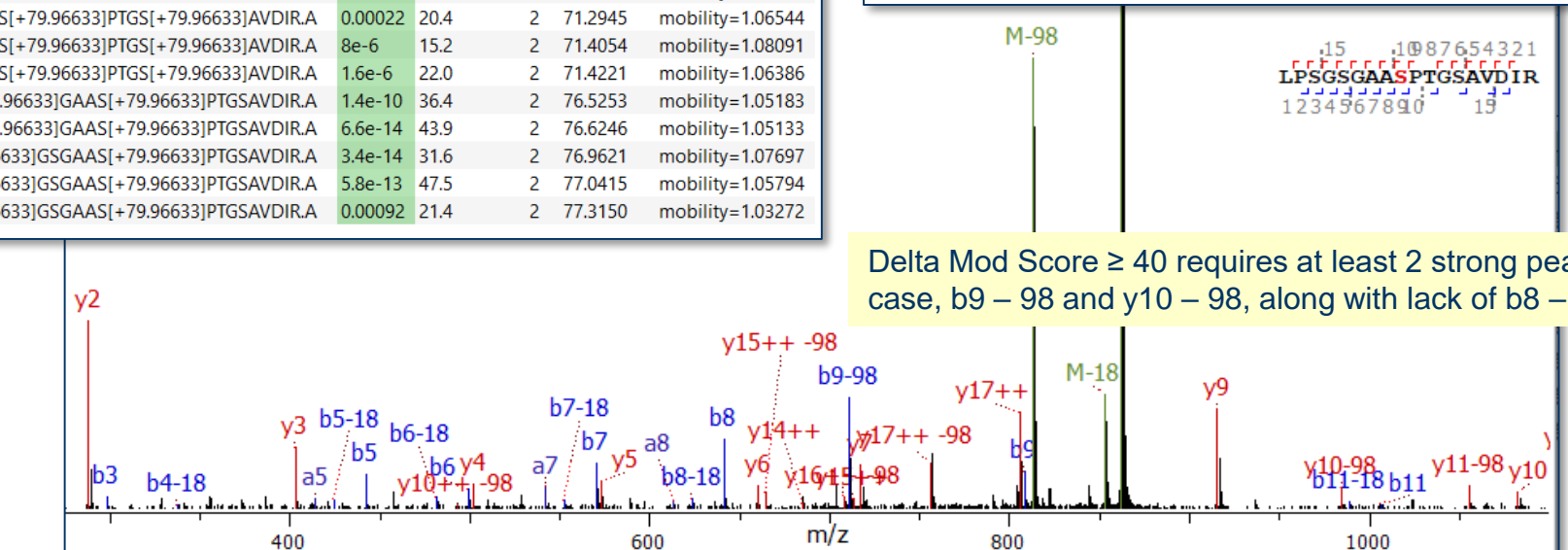
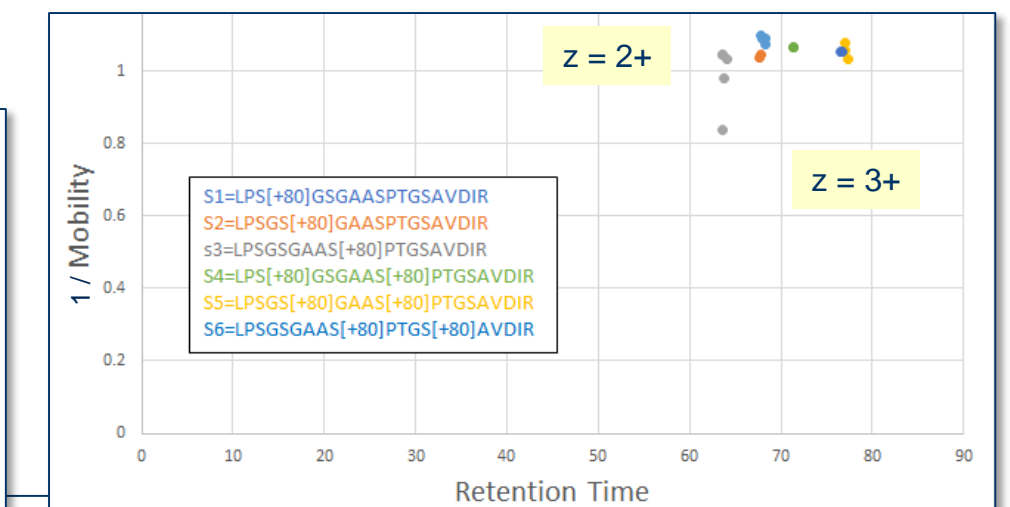


YLG... from A1AT with HexNAc(4)Hex(5)NeuAc(2) = SA2 shows a bigger range of mobilities than ALV... from serum albumin, which has a long elution peak tail, or QVL... from APOB with high-mannose glycans, suggesting mobility differences among isomers (in this case α2.3 vs. α2.6). Or maybe just gas-phase conformations?

Phosphopeptide Positional Isomers

For this peptide from AHNK_HUMAN, amino acids 208 – 225 and charge *z*=2+, both scan time and mobility give some, but not perfect, separation of isomers. Here +80 on S3 is both more mobile and later eluting than +80 on S5 or S9.

Sequence	PEP 2D	Delta Mod. Score	<i>z</i>	Scan Time	Comment
RLPSGSGAAS[+79.96633]PTGSAVDIRA	1.9e-10	55.6	2	63.5619	mobility=1.04461
RLPSGSGAAS[+79.96633]PTGSAVDIRA	9e-18	52.2	2	63.6214	mobility=1.04467
RLPSGSGAAS[+79.96633]PTGSAVDIRA	1e-10	72.3	3	63.6430	mobility=0.8332
RLPSGSGAAS[+79.96633]PTGSAVDIRA	1.1e-13	58.3	2	64.0390	mobility=1.02098
RLPSGS[+79.96633]GAASPTGSAVDIRA	2.3e-5	37.2	2	67.6728	mobility=1.03525
RLPSGS[+79.96633]GAASPTGSAVDIRA	4.3e-10	31.4	2	67.8279	mobility=1.04665
RLPS[+79.96633]SSGAASPTGSAVDIRA	7.9e-12	60.8	2	67.8873	mobility=1.09744
RLPS[+79.96633]SSGAASPTGSAVDIRA	5.2e-13	67.5	2	67.9486	mobility=1.08849
RLPSGS[+79.96633]GAASPTGSAVDIRA	7.9e-11	5.4	2	67.9685	mobility=1.06286
RLPS[+79.96633]SSGAASPTGSAVDIRA	9e-16	93.1	2	68.0265	mobility=1.0892
RLPS[+79.96633]SSGAASPTGSAVDIRA	1.9e-17	85.5	2	68.2426	mobility=1.07242
RLPS[+79.96633]SSGAASPTGSAVDIRA	6.9e-7	54.1	2	68.2692	mobility=1.08028
RLPSGSGAAS[+79.96633]PTGSI[+79.96633]AVDIRA	0.00022	20.4	2	71.2945	mobility=1.06544
RLPSGSGAAS[+79.96633]PTGSI[+79.96633]AVDIRA	8e-6	15.2	2	71.4054	mobility=1.08091
RLPSGSGAAS[+79.96633]PTGSI[+79.96633]AVDIRA	1.6e-6	22.0	2	71.4221	mobility=1.06386
RLPSGS[+79.96633]GAAS[+79.96633]PTGSAVDIRA	1.4e-10	36.4	2	76.5253	mobility=1.05183
RLPSGS[+79.96633]GAAS[+79.96633]PTGSAVDIRA	6.6e-14	43.9	2	76.6246	mobility=1.05133
RLPS[+79.96633]SSGAAS[+79.96633]PTGSAVDIRA	3.4e-14	31.6	2	76.9621	mobility=1.07697
RLPS[+79.96633]SSGAAS[+79.96633]PTGSAVDIRA	5.8e-13	47.5	2	77.0415	mobility=1.05794
RLPS[+79.96633]SSGAAS[+79.96633]PTGSAVDIRA	0.00092	21.4	2	77.3150	mobility=1.03272



Delta Mod Score ≥ 40 requires at least 2 strong peaks, in this case, b9 – 98 and y10 – 98, along with lack of b8 – 98 and y9 – 98

Testing RT and mobility in separation, we filtered PSMs to PEP 2D ≤ 0.01 (1% chance of error) and Delta Mod Score ≥ 40 (PTMs confidently localized). With these DDA settings in testing 30 peptides that Byonic identified as having more than one phosphorylation site, sorting PSMs with the same precursor charge by retention time put all identical PSMs adjacent 25 times. Sorting by mobility succeeded 6 times.

Discussion and Conclusions

TIMS-TOF data is very rich in information, and the study reported here barely scratched surface. Some findings, however, seem evident:

- TIMS-TOF offers great sensitivity for both glyco- and phospho-proteomics
- Glycopeptides with several possible structures show a greater spread of mobility values than do ordinary peptides. We do not yet know if mobility differences indicate glycan structure. Glycopeptides are prone to in-source decay, which complicates elution time determination and quantitation.
- Mobility does not separate phosphopeptide positional isomers as often as RT, but serves as a useful additional dimension, especially with co-eluting positional isomers where RT fails.