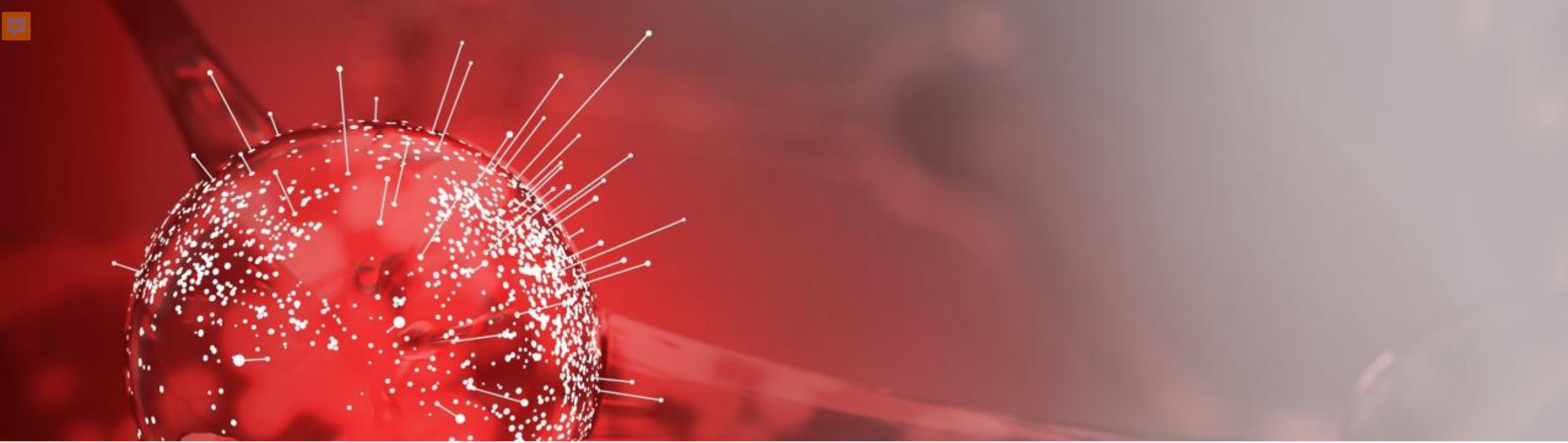
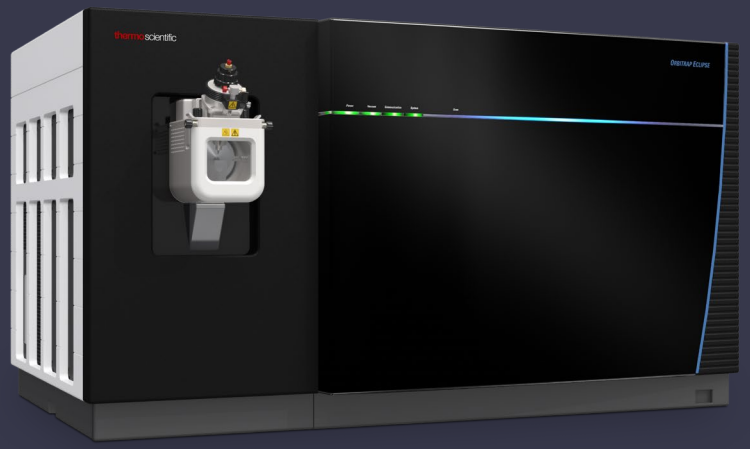


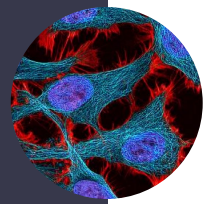
ThermoFisher SCIENTIFIC

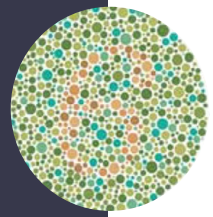
Ultra Sensitive LC-MS Workflow for Single Cell Proteomic Analysis

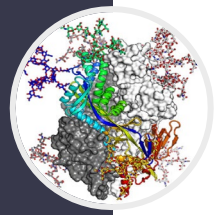
Khatereh Motamedchaboki, Ph.D.
Vertical Marketing, Proteomics
November 2019

New Mass Spectrometry Platforms

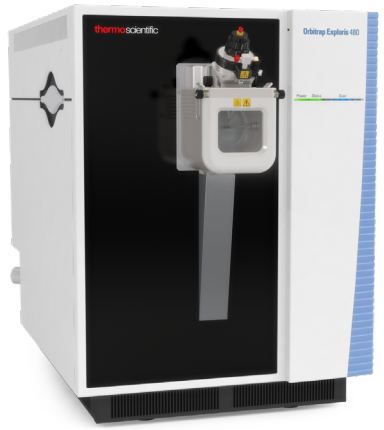








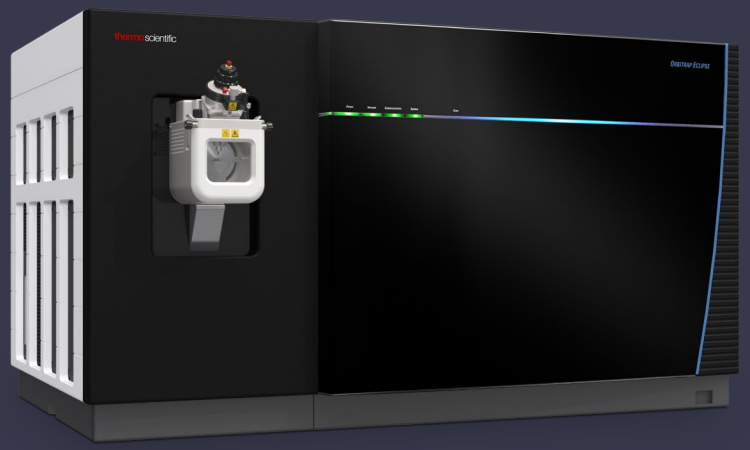
Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ Mass Spectrometer offers extraordinary sensitivity and versatility



Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer makes the extraordinary simpler

ThermoFisher SCIENTIFIC

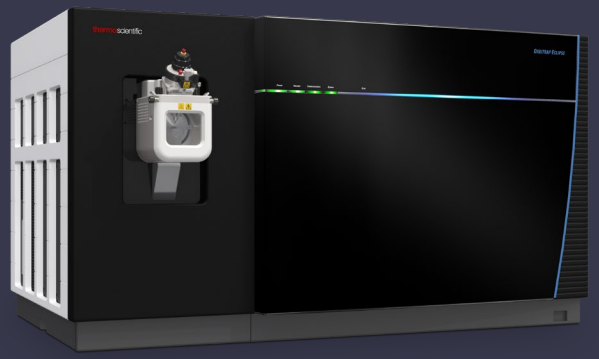
Challenges in Life Science Mass Spectrometry



Orbitrap Eclipse Tribrid MS offers extraordinary sensitivity and versatility

Qualitative Proteomics	Complex samples, wide dynamic range
Quantitative Proteomics	Accurate proteome-wide quantitation with high throughput
Structural Biology	Protein complex structure characterization
Biopharmaceutical Analysis	Protein-drug structure elucidation, impurity identification
Small Molecule Characterization	Structural characterization of isomeric species

New Orbitrap Eclipse Tribrid MS System



Acquisition rate OTMS^2	40 Hz
Acquisition rate ITMS^2	45 Hz
Maximum resolution	500K FWHM at *m/z* 200; up to 1,000,000 with 1M
Quadrupole minimum isolation width	0.4 *m/z*
Mass range	*m/z* 50-8000
Mass Accuracy	3 ppm ext, 1 ppm int
Dissociation / Ion Activation	CID, HCD, ETD, EThcD, ETciD, UVPD, PTCR
MS^n	Up to **MS^{10}** with the ion trap or Orbitrap mass analyzer
Analyzers	Q, OTMS, ITMS
Detectors	Ion Trap, Orbitrap mass analyzer
Size	1186 x 674 x 650 mm (w, d, h)

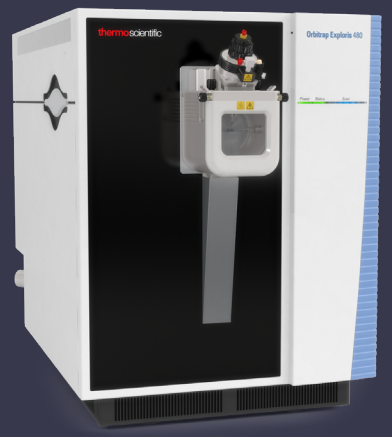
Unmatched Analytical Performance and Versatility

- **QR5 Segmented Quadrupole Mass Filter** for outstanding precursor selectivity and sensitivity
- **Real-Time Search** for exceptional depth and accuracy for TMT analysis
- **High Mass Range MS^n (HMR^n) option** for structural analysis of native protein complexes
- **Proton Transfer Charge Reduction (PTCR) option** for simplification of complex spectra and improved top-down data interpretation
- **Full Customization** with a range of optional capabilities:

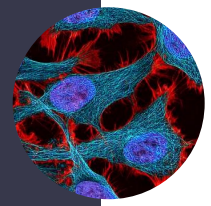
  IC | ETD | UVPD | 1M | HMR^n | PTCR | FAIMS Pro interface

- **Common interface** Orbitrap Exploris 480 MS and TSQ™ triple quadrupole mass spectrometers

Challenges in Life Science Mass Spectrometry

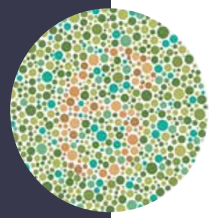


Orbitrap Exploris 480 MS makes the extraordinary simpler



Qualitative Proteomics

Complex samples, insufficient depth of analysis



Quantitative Proteomics

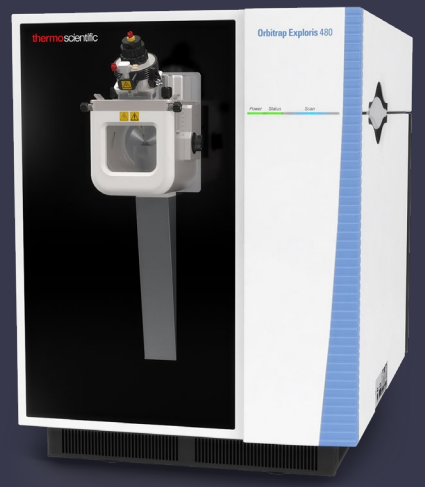
Accurate low limit proteome wide quantitation with high throughput

Biopharmaceutical Analysis

Protein drug structure elucidation, impurity identification

ThermoFisher SCIENTIFIC

Orbitrap Exploris 480 Mass Spectrometer

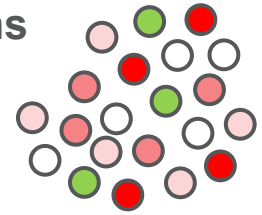
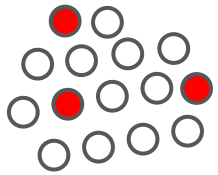
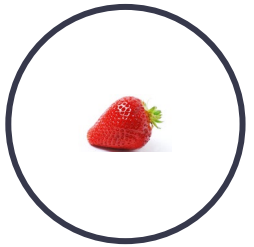


Mass range:	40 - 6000 m/z (8000 m/z optional)
Quad isolation:	down to 0.4 u
Scan rate MS^2:	up to 40 Hz
Max resolution:	480k at m/z 200
Dynamic range:	> 5000:1
Mass Accuracy:	3 ppm RMS ext., 1 ppm RMS int.
Dissociation:	Higher energy Collisional Dissociation (HCD)
Analyzer:	Quadrupole, Orbitrap
Compact:	530 x 760 x 700 mm (w,d,h)
Options:	Easy-IC, BioPharma , FAIMS Pro

Making Genius Simpler

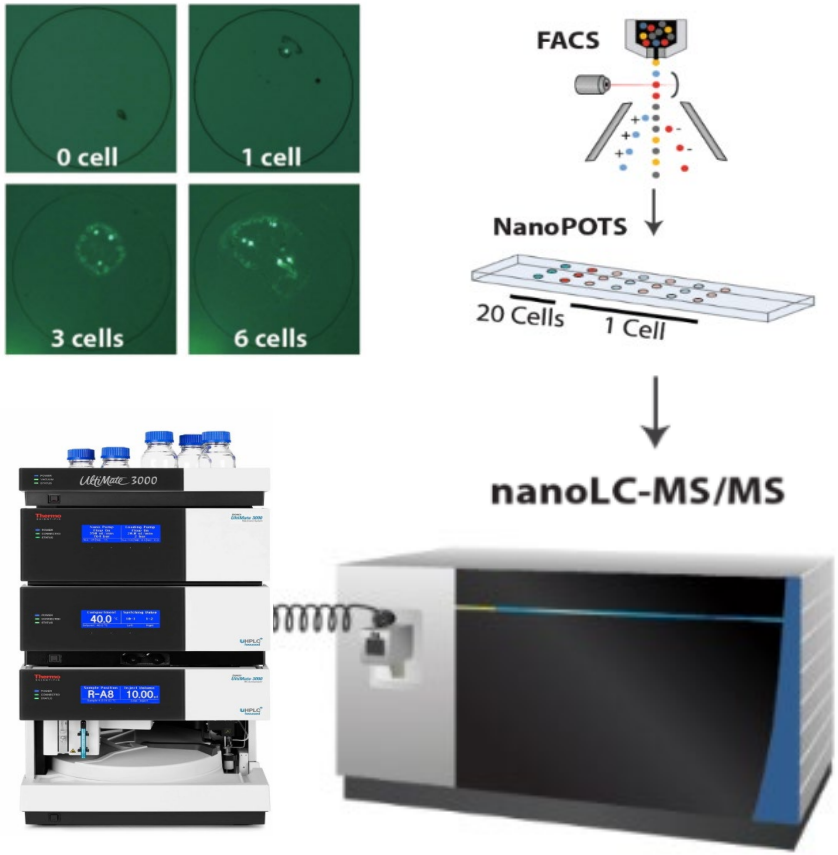
- **480,000 Resolution** to resolve spectra interference and enable data certainty
- **Maximized proteome** coverage and quantitation with FAIMS Pro interface
- **Novel scan modes** for higher throughput without compromising sensitivity, precision and accuracy
- **Robust and reliable design** for maximum uptime
- **Application modes** with best-practice default parameters and drag-n-drop methods templates for portability from system to system
- **Next generation user interface** with Thermo Scientific™ Orbitrap™ Tribrid™ mass spectrometers and TSQ triple quadrupole mass spectrometers

The Promise Behind Single Cell Applications

Reason	Application	Bulk Result	Single cell data
UNDERSTAND CELLULAR HETEROGENEITY	**Identification of cell subpopulations** based on protein expression or metabolic profiles (tumors, tissues, immune cells, cell cultures) 		
	Detection and analysis of rare cells (i.e. CTCs from liquid biopsies) 		
LIMIT AVAILABILITY OF CELLS	**Analysis of limited sample material** (exosomes, needle aspirates, biopsies) 	No Data	

Single Cell Sample Preparation Methods

SINGLE CELL ISOLATION WITH FLUORESCENCE-ACTIVATED CELL SORTING



Zhu et al. Angew. Chem. Int. Ed., 2018, 57, 12370-12374.

Budnik *et al. Genome Biology* (2018) 19:161
https://doi.org/10.1186/s13059-018-1547-5

Genome Biology

METHOD Open Access

SCoPE-MS: mass spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation

Bogdan Budnik[1*], Ezra Levy[2], Guillaume Harmange[2] and Nikolai Slavov[2,3*]

Analyst

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Cite this: *Analyst*, 2019, **144**, 794

New mass spectrometry technologies contributing towards comprehensive and high throughput omics analyses of single cells

Sneha P. Couvillion, [a] Ying Zhu, [a] Gabe Nagy,[a] Joshua N. Adkins,[a] Charles Ansong,[a] Ryan S. Renslow,[a] Paul D. Piehowski,[a] Yehia M. Ibrahim, [a] Ryan T. Kelly [a,b] and Thomas O. Metz [*a]

GDCh *Communications* Angewa

Check for updates

Single-Cell Proteomics

International Edition: DOI: 10.1002/anie.201802843
German Edition: DOI: 10.1002/ange.201802843

Proteomic Analysis of Single Mammalian Cells Enabled by Microfluidic Nanodroplet Sample Preparation and Ultrasensitive NanoLC-MS

*Ying Zhu, Geremy Clair, William B. Chrisler, Yufeng Shen, Rui Zhao, Anil K. Shukla, Ronald J. Moore, Ravi S. Misra, Gloria S. Pryhuber, Richard D. Smith, Charles Ansong, and Ryan T. Kelly**

analytical chemistry

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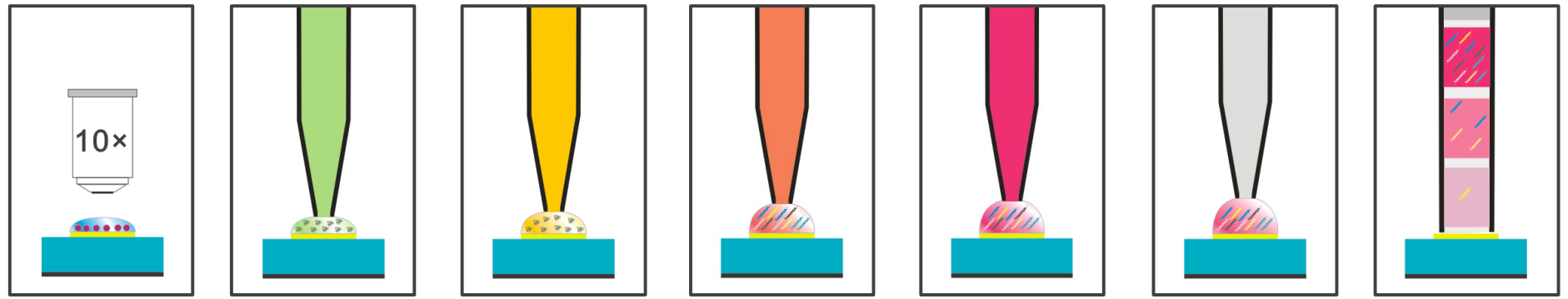
Article

High-Throughput Single Cell Proteomics Enabled by Multiplex Isobaric Labelling in a Nanodroplet Sample Preparation Platform

Maowei Dou, Geremy Clair, Chia-Feng Tsai, Kerui Xu, William B. Chrisler, Ryan L. Sontag, Rui Zhao, Ronald J. Moore, Tao Liu, Ljiljana Paša-Toli#, Richard D. Smith, Tujin Shi, Joshua N. Adkins, Wei-Jun Qian, Ryan T. Kelly, Charles Ansong, and Ying Zhu

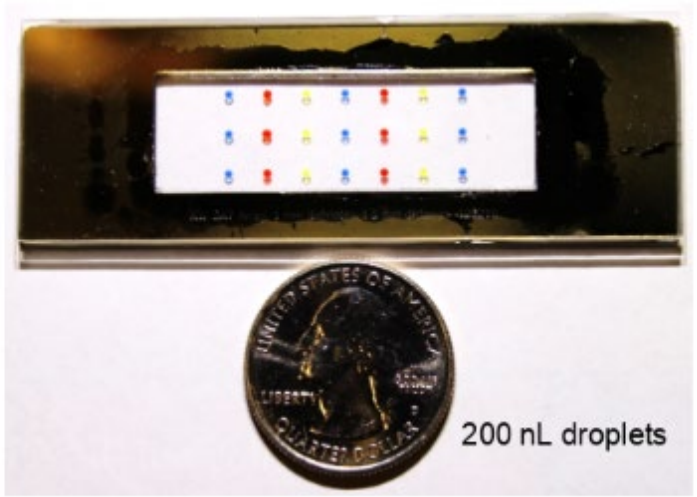
Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.9b03349 • Publication Date (Web): 11 Sep 2019

nanoPOTS: **nano**droplet **P**rocessing in **O**ne-pot for **T**race **S**ample



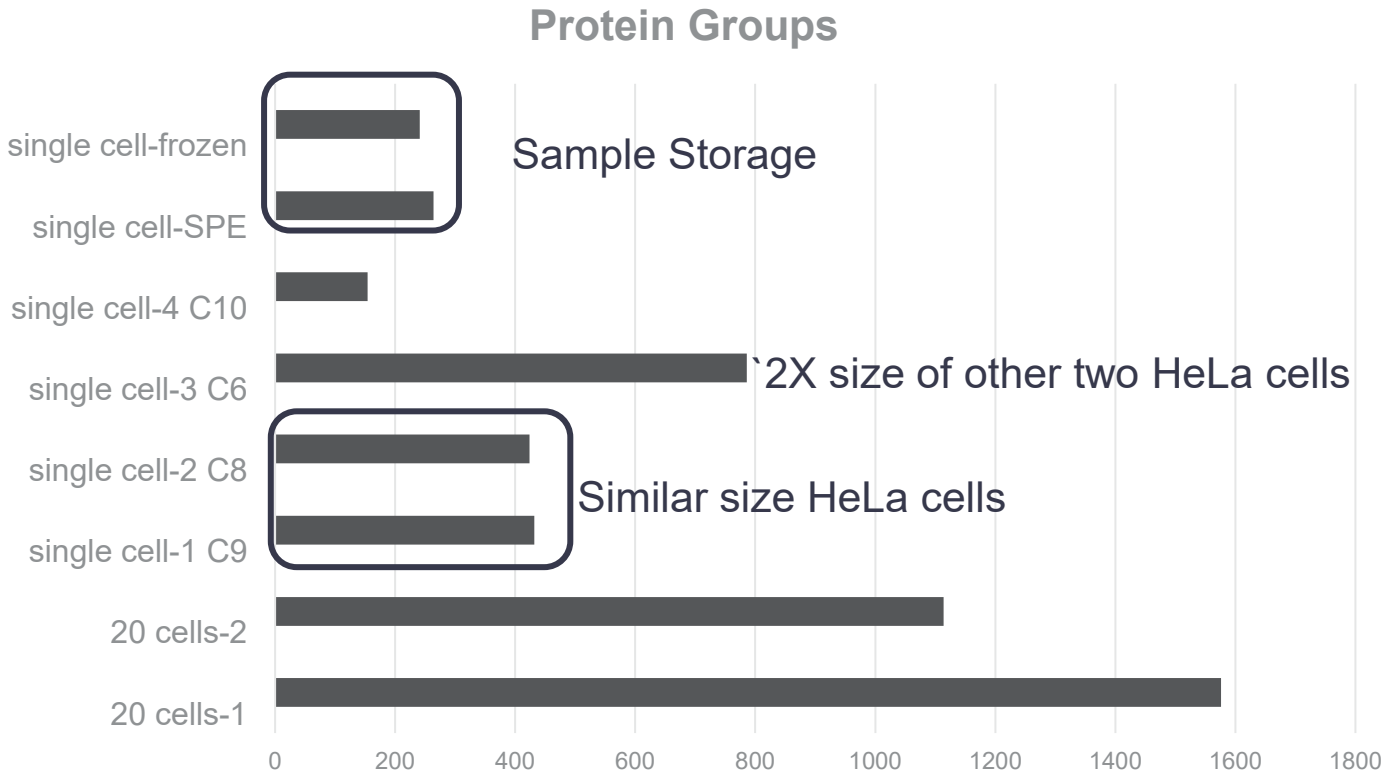
Sample Evaluation → Extraction/ Reduction → Alkylation → Lys-C Digestion → Trypsin Digestion → Surfactant Cleavage → Peptide Collection

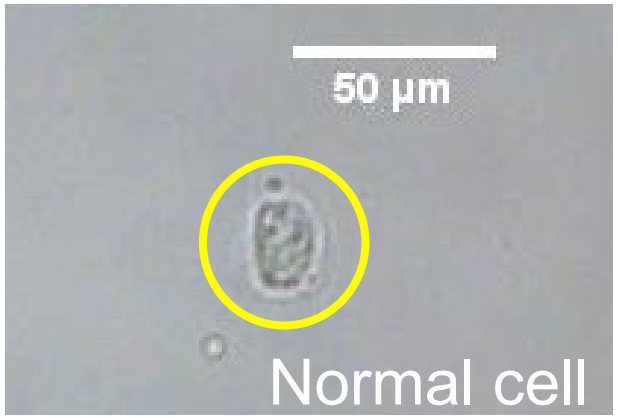
AUTOMATED SAMPLE PREPARATION IN NANOLITER VOLUMES ON NANOPOTS



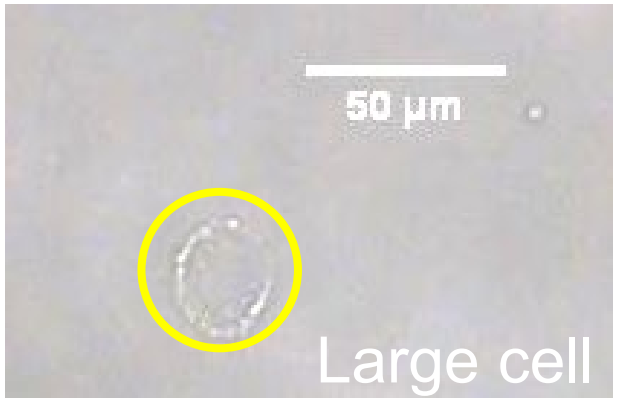
	TUBE METHOD	NANOWELL METHOD
Reaction volume	100 µL	200 nL
Surface	127 mm^2	0.8 mm^2
Digestion kinetics	Low	High

Benchmark Results Cell Size and Sample Storage Effect





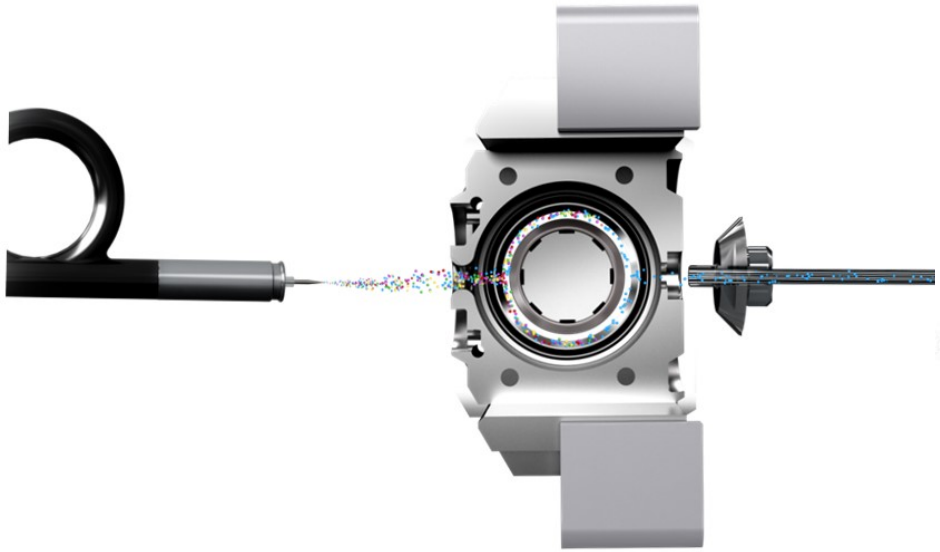
424 proteins Identified

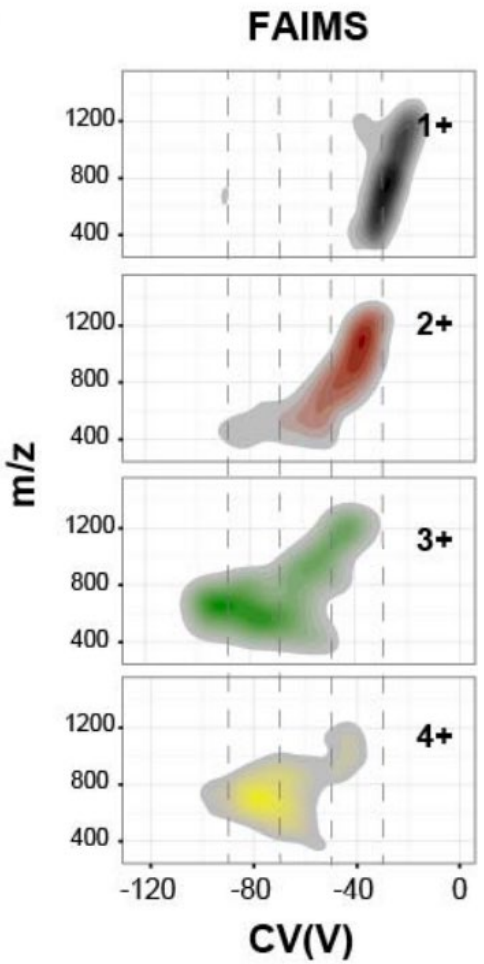


786 proteins Identified

- ✓ Identification of similar number of proteins from uniform cell types
- ✓ 2X more proteins and peptides than previously reported
- ✓ ~ 450 protein groups identified with label free method on Orbitrap Tribrid Eclipse MS from single HeLa cell
- ✓ Fresh sample stored at 4°C provided the best data
- ✓ Cell size matters when it comes to protein IDs

Improved Ion Mobility-Based Separation Option for Proteomics





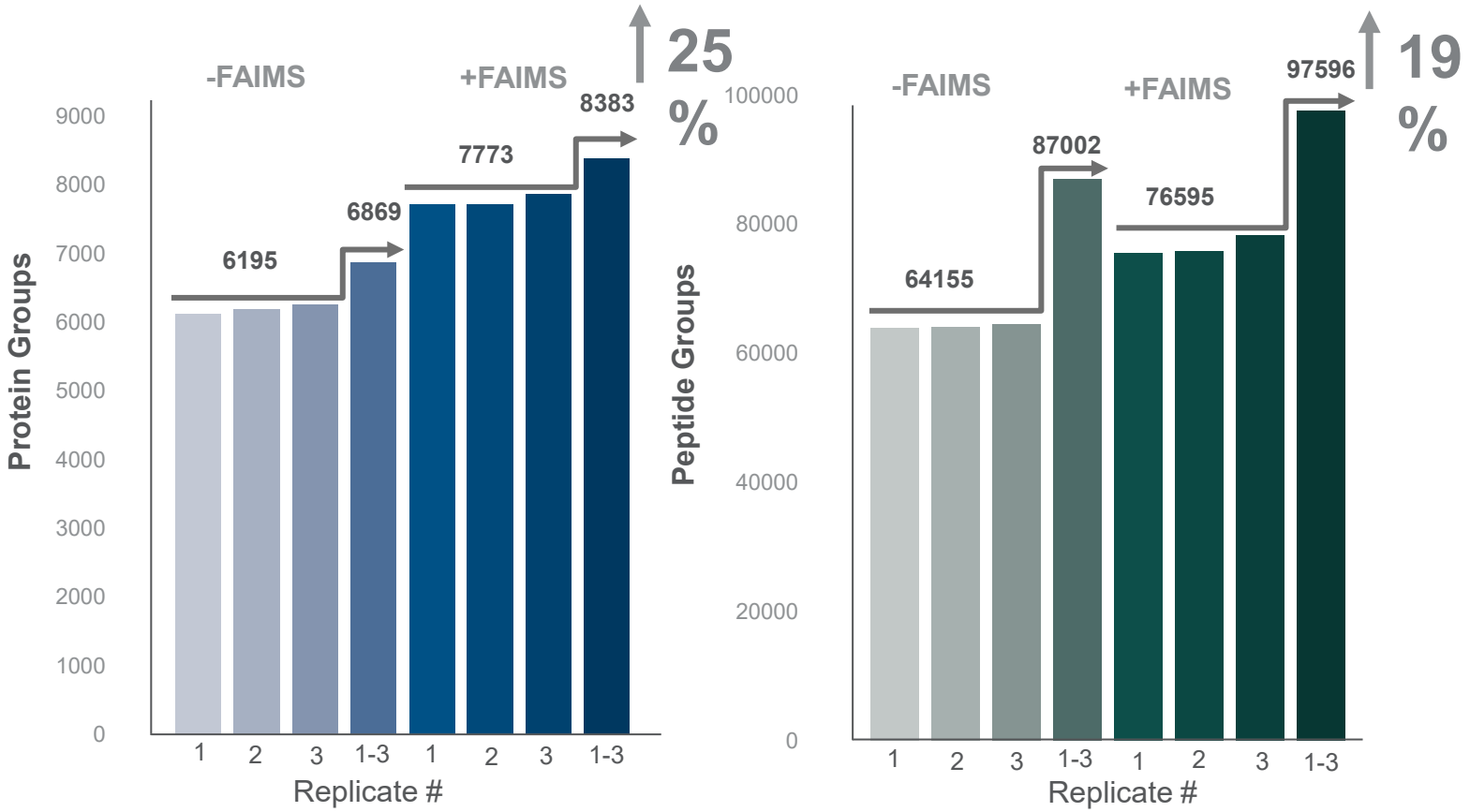
Sibylle Pfammatter et al. Molecular & Cellular Proteomics July 14, 2018

Thermo Scientific™ FAIMS Pro™ Interface

- Compatible with nano- and low-flow chromatography (<25µL/min)
- Operation on TSQ triple quadrupole mass spectrometers, Orbitrap Tribrid mass spectrometers and Orbitrap Exploris 480 MS
- Automatic source recognition and programming in Tune
- Method templates for key applications
- Performance improvements for most protein analysis applications

Orbitrap Eclipse MS – Performance at 200 ng +/- FAIMS

Proteome Coverage: 200 ng HeLa, 120 min Gradient



-FAIMS	Mean (n=3)	CV (%)
Protein Groups	6195	0.95
Peptide Groups	64155	0.37
PSMs	115968	0.70
MS/MS	187911	0.56

+FAIMS	Mean (n=3)	CV (%)
Protein Groups	7773	0.91
Peptide Groups	76595	1.61
PSMs	130067	0.44
MS/MS	188957	0.17

- ✓ ~6200 proteins identified in 120 min with 200 ng of HeLa digest without FAIMS Pro Interface (mean of n=3 injections shown)
- ✓ ~7800 proteins identified in 120 min with 200 ng of HeLa digest with FAIMS Pro Interface (mean of n=3 injections shown)
- ✓ Improved peptide/protein coverage with FAIMS Pro Interface
- ✓ Further improvement in peptide/protein ID with search on combined replicate analysis

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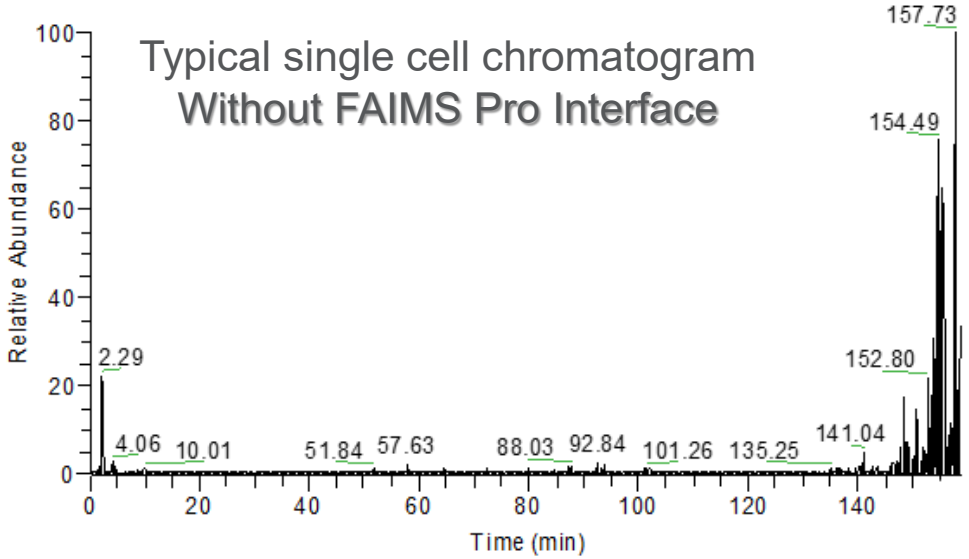
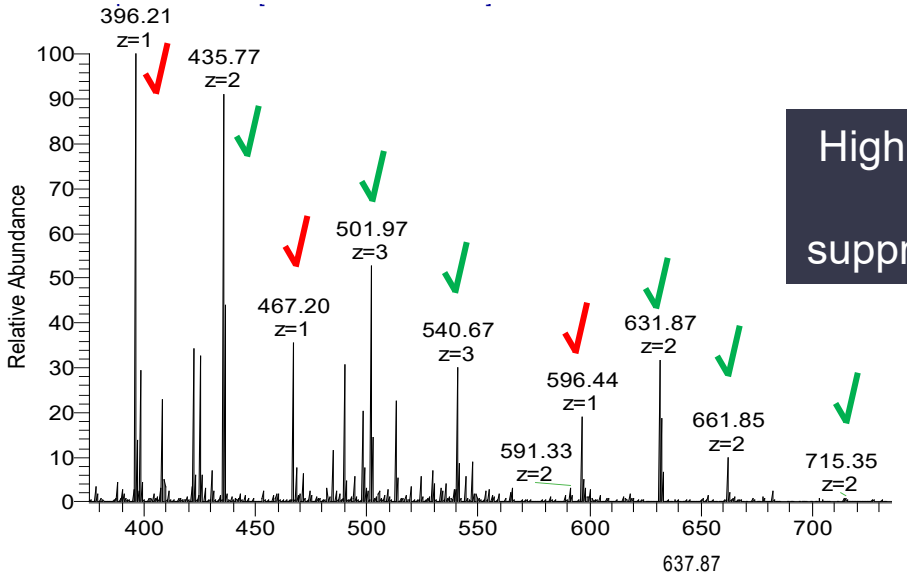
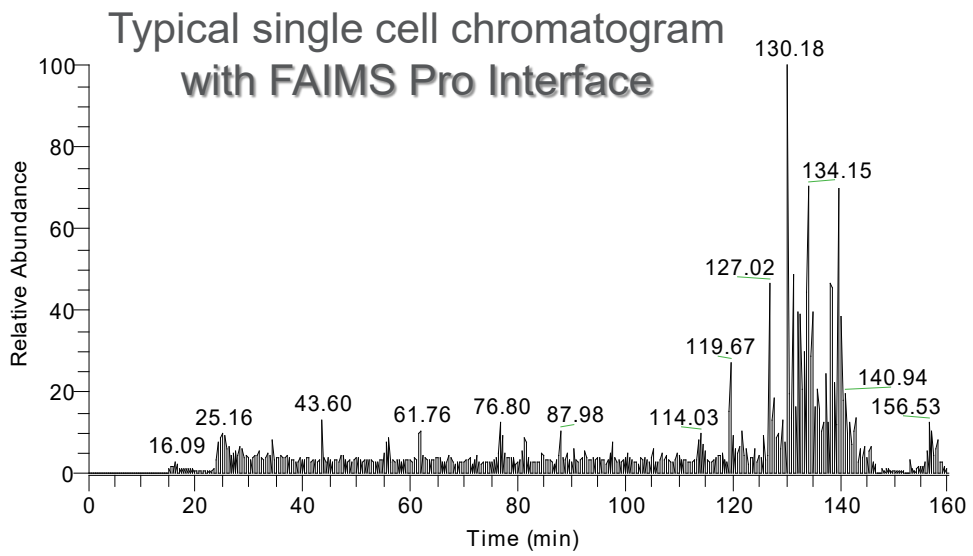
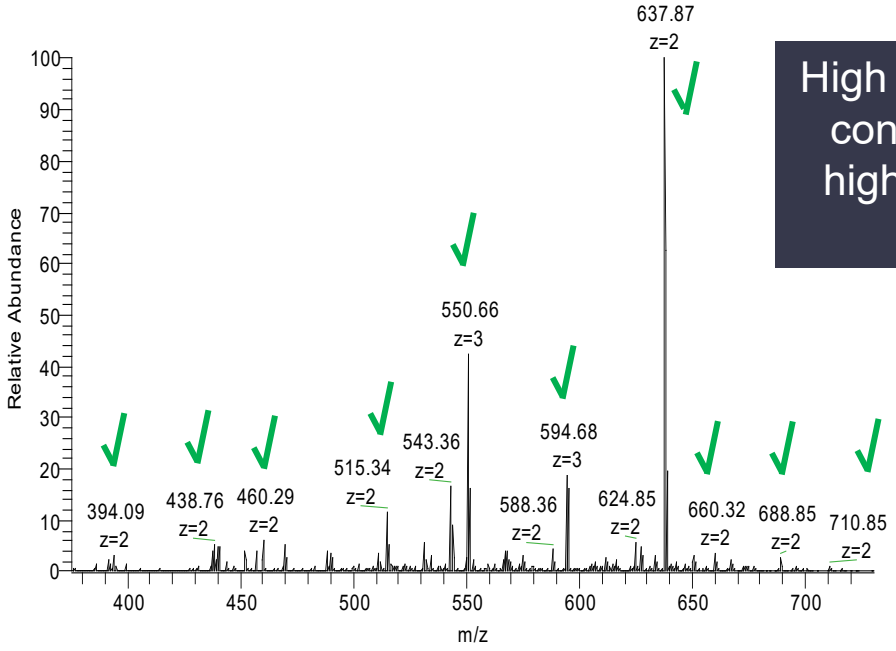
Improving Single Cell Protein Coverage with FAIMS Pro Interface





High abundant +1 charged contaminants suppressing peptide signals





High abundant +1 charged contaminants removed, higher charged peptides detectable now

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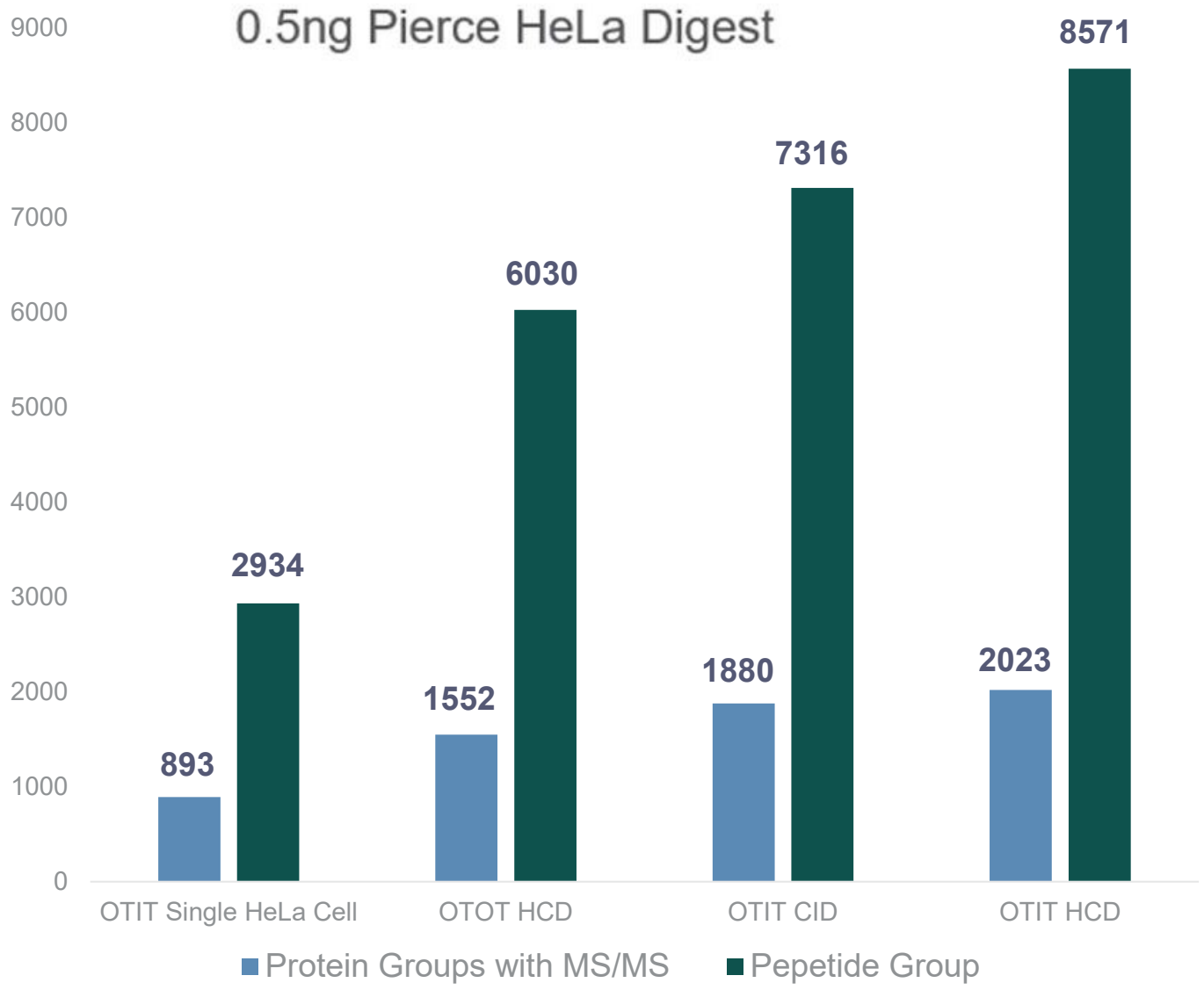
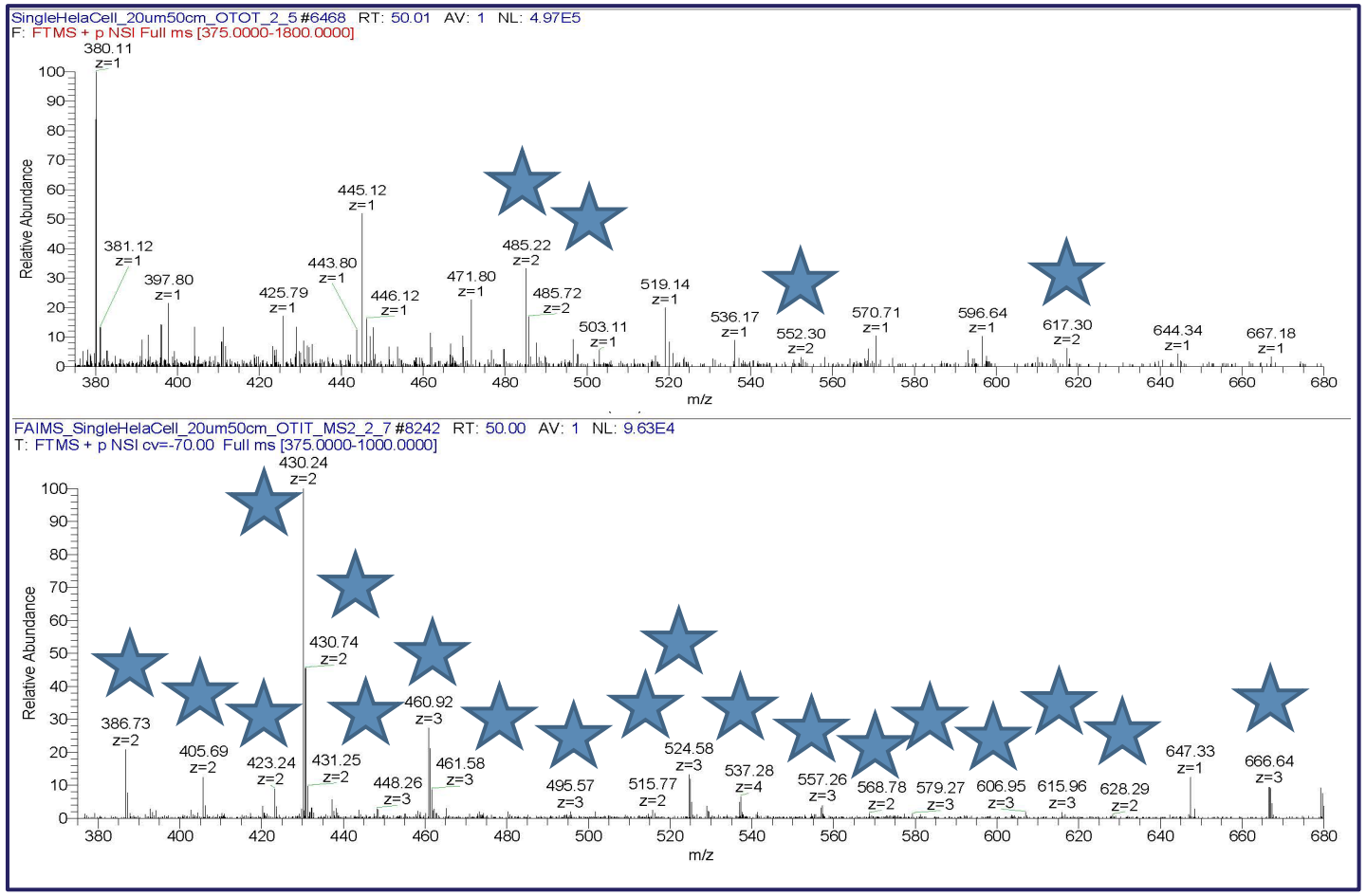
Method Optimization at Single Cell Level with FAIMS Pro Interface



HeLa QC Results

- Method Optimization at 0.5 ng of Thermo Scientific™ Pierce™ HeLa Protein Digest Standard
- Data showed improved peptide/protein coverage with Ion Trap sensitivity
- HCD fragmentation in Ion Trap provided further comprehensive fragmentation enhancing peptide identification rate
- The highest number of protein coverage reported from 0.5 ng sample injection providing unmatched sensitivity to analyze proteins in single cell level
- On average 893 protein groups from single HeLa cell and 1134 protein groups from laser capture microdissected single neuron cell were identified (Manuscript in Preparation)

FAIMS Pro Enhances Proteome Coverage in Single Cells

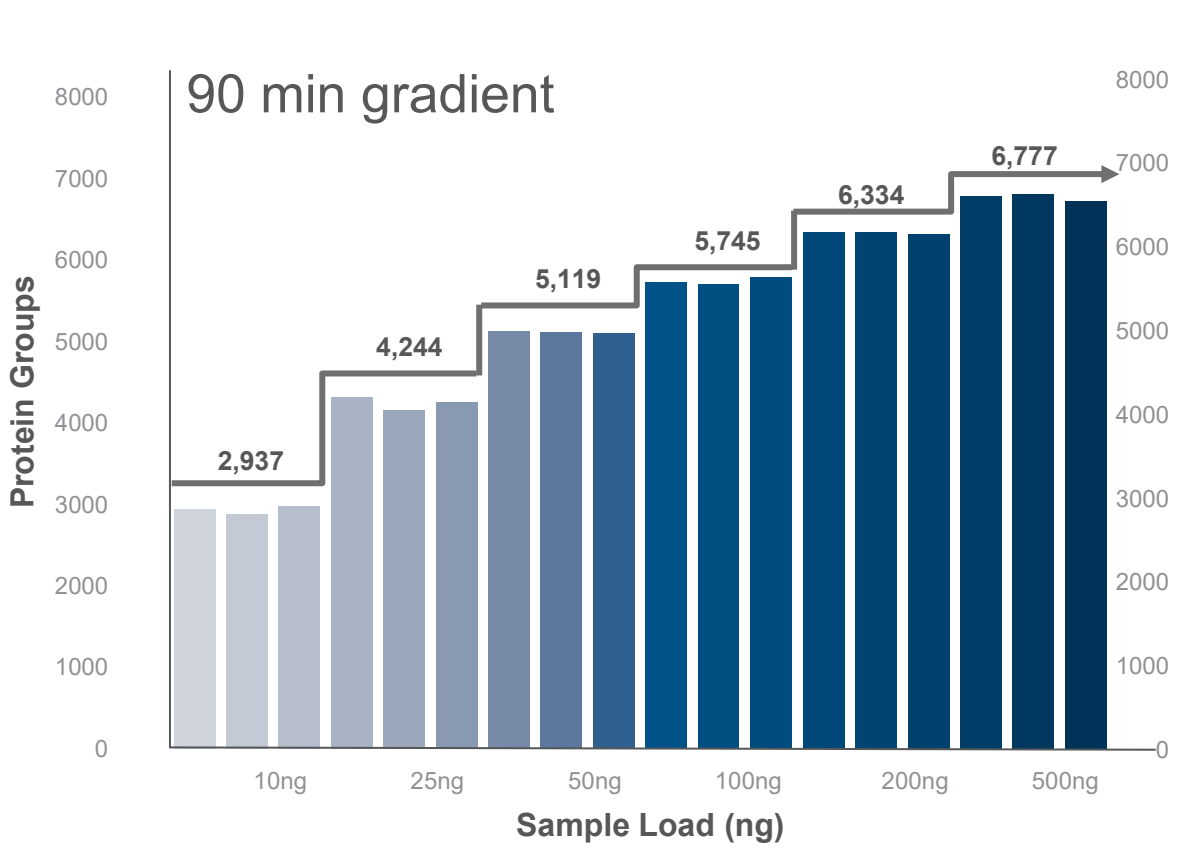


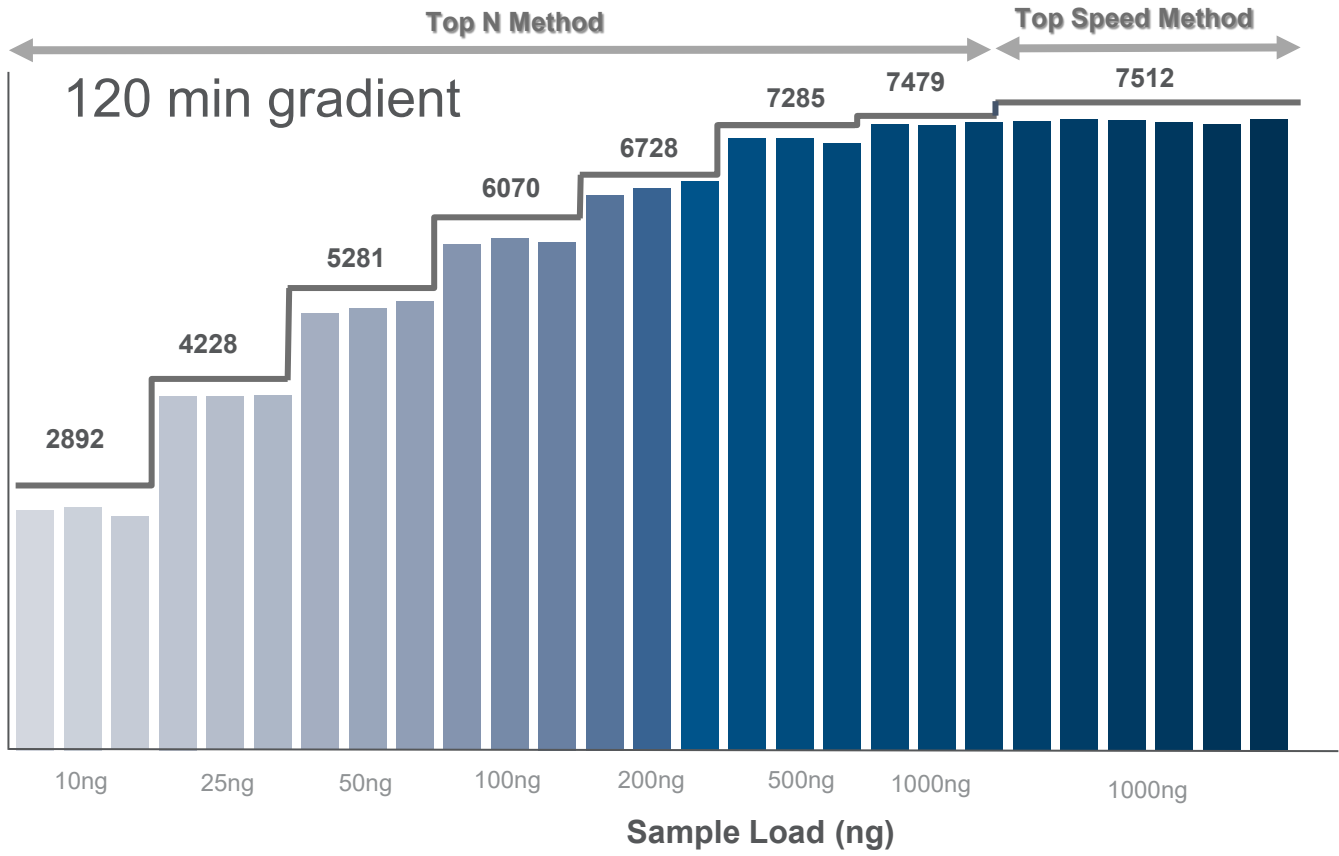
	Protein groups		
	Eclipse	Eclipse + FAIMS	Gain
1 HeLa Cell	551	829	50%
100 HeLa Cells	2109	3067	45%

- ✓ 829 protein groups found in one single cell using MS/MS identification with Thermo Scientific™ Proteome Discoverer™ 2.4 software (1072 protein groups using MaxQuant with MBR)

Orbitrap Exploris 480 MS - ID Performance Above and Below 200 ng

Proteome Coverage 10 ng – 1000 ng HeLa





- ✓ ~2900-6800 proteins identified in 90 min with 10 ng - 500 ng of HeLa digest
- ✓ ~2900-7500 proteins identified in 120 min with 10 ng - 1000 ng of HeLa digest
- ✓ FAIMS with intra-analysis CV stepping (CV -50 and -70), identical conditions to slide 13

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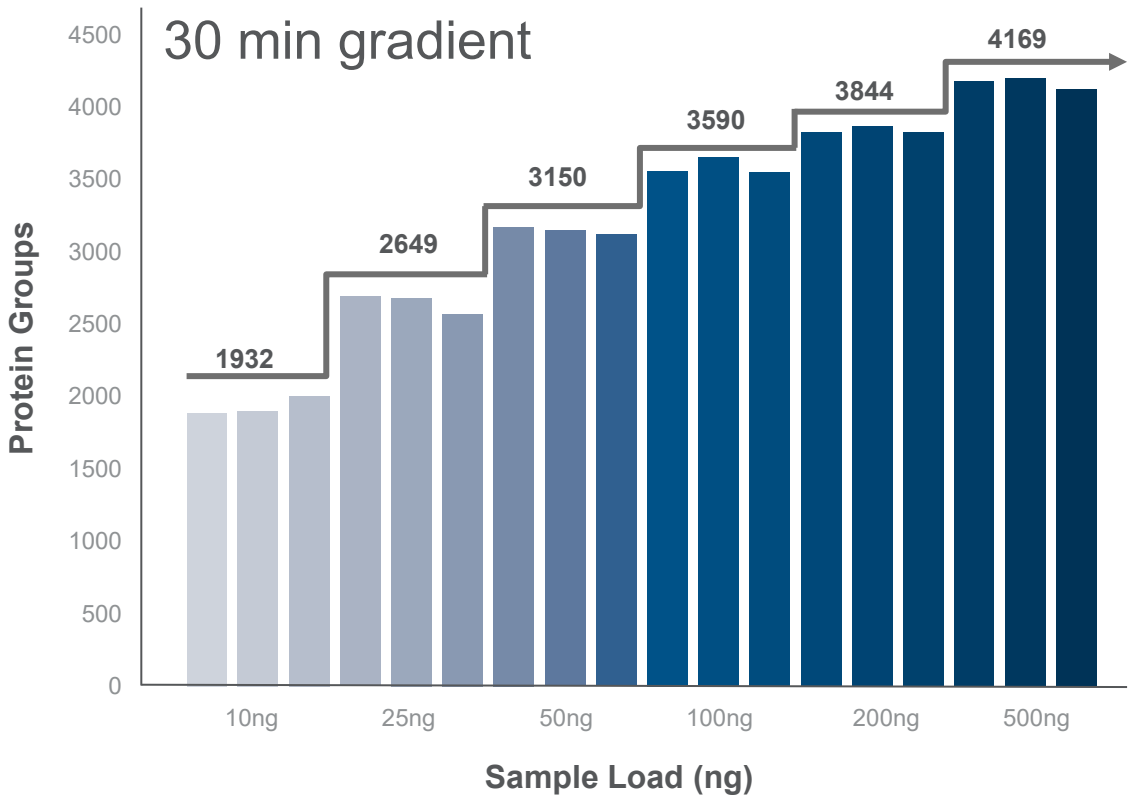
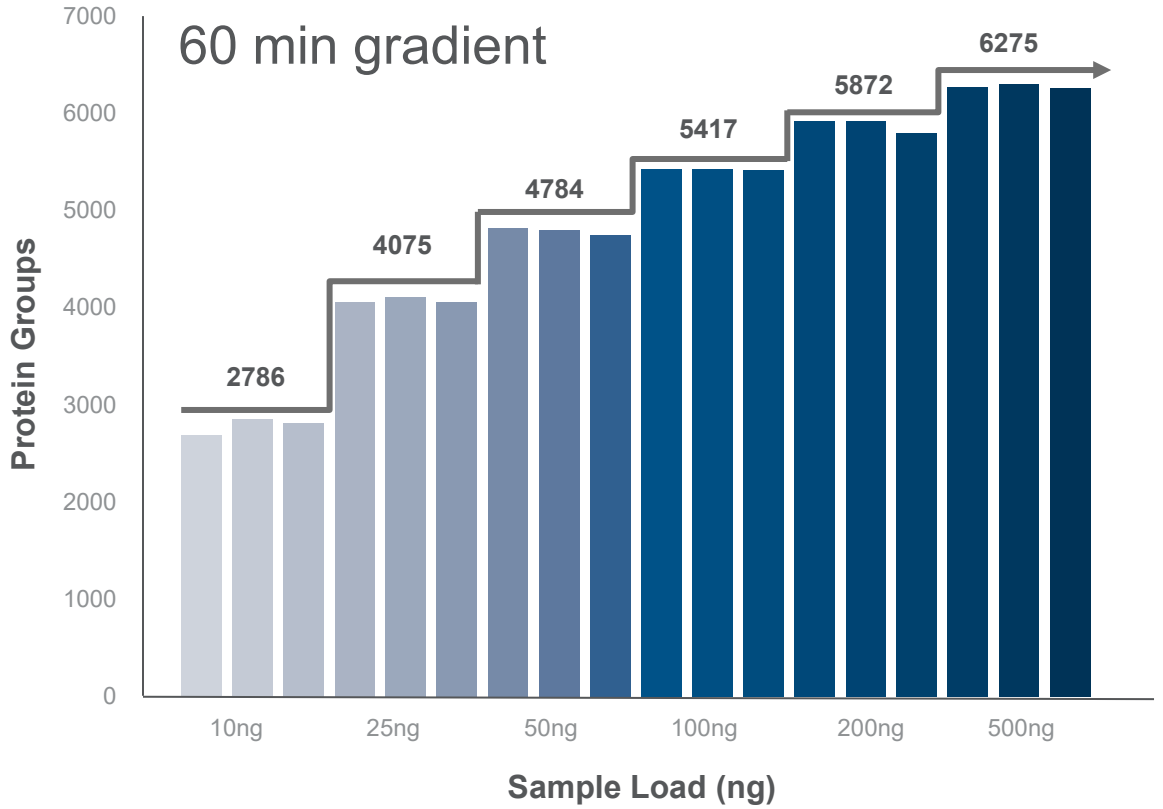
Orbitrap Exploris 480 MS - ID Performance Above and Below 200 ng

Proteome Coverage 10 ng – 500 ng HeLa: 30 and 60 min Analysis

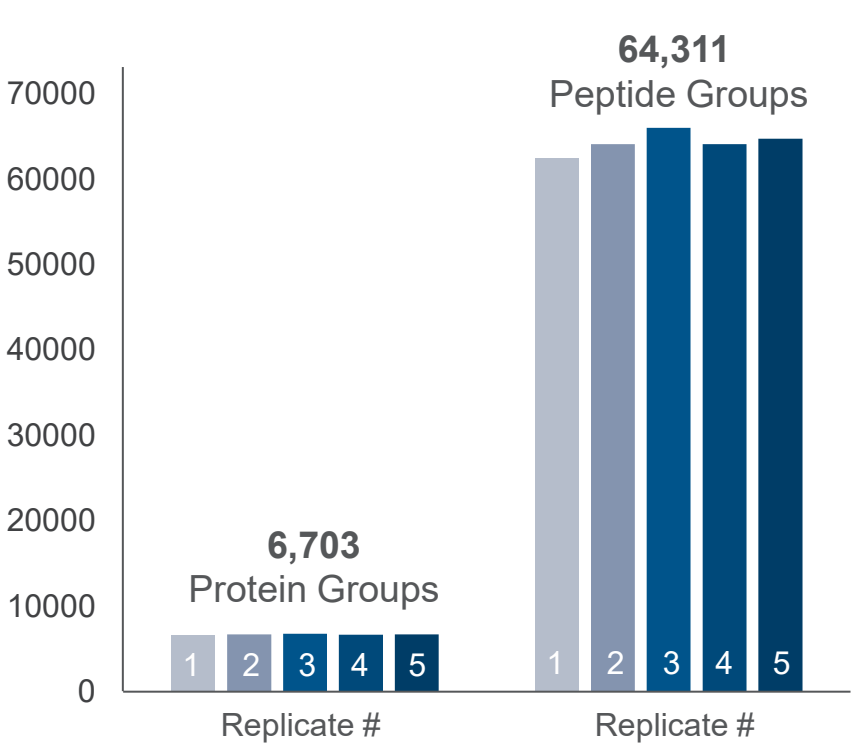




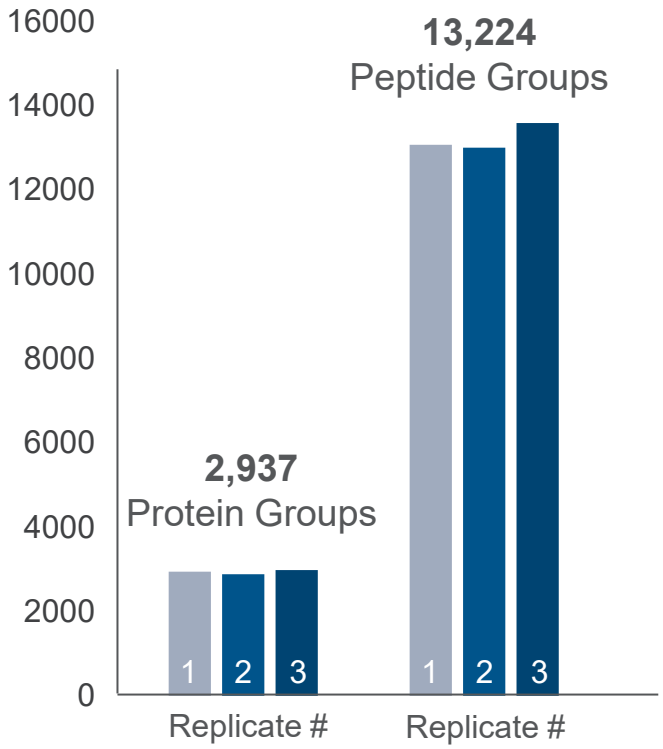
- ✓ ~2000-4000 proteins identified in 30 min with 10 ng - 500 ng of HeLa digest (mean of n=3 injections shown)
- ✓ ~3000-6000 proteins identified in 60 min with 10 ng - 500 ng of HeLa digest (mean of n=3 injections shown)
- ✓ Identical experimental conditions to slide 23 except with FAIMS 1 CV (-60) single sample injection

Orbitrap Exploris 480 MS - Protein ID Performance

Record Setting Performance at 10 and 200 ng HeLa with FAIMS Pro Interface



+FAIMS	Mean (n=5)	CV (%)
Protein Groups	6,703	0.90
Peptide Groups	64,311	2.00
PSMs	99,780	1.96
MS/MS	169,082	0.49



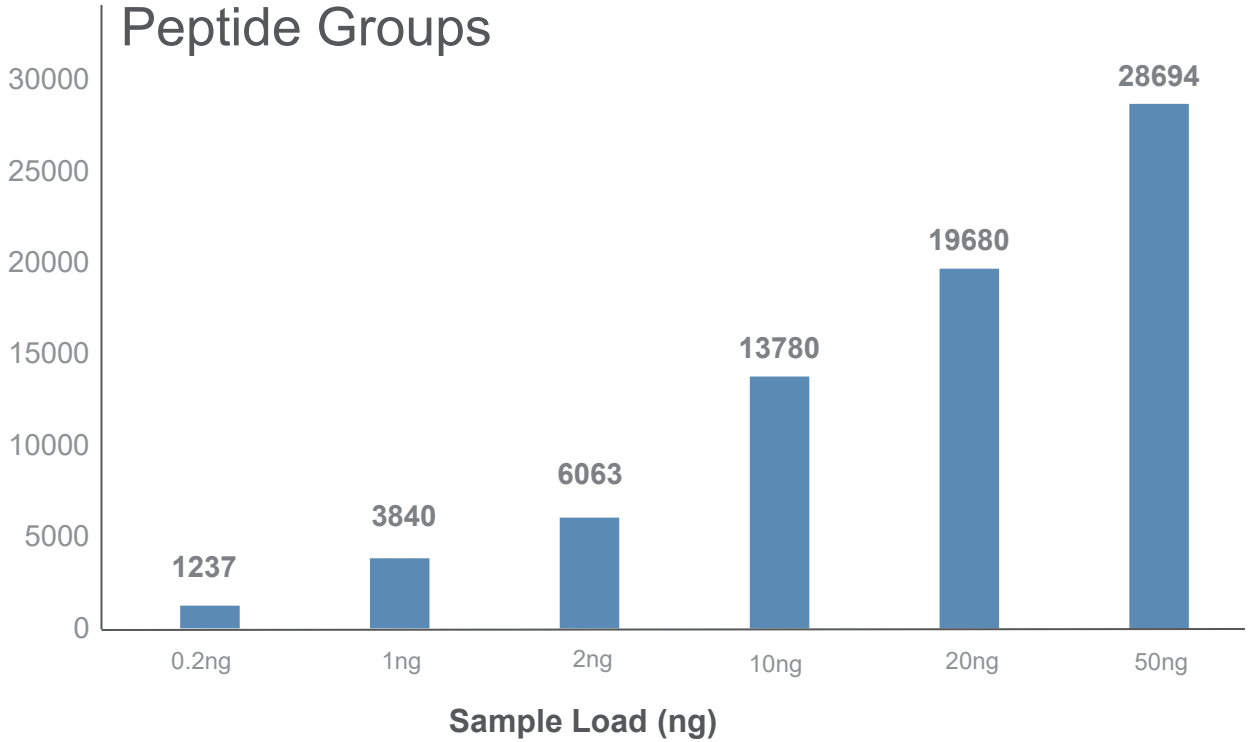
+FAIMS	Mean (n=3)	CV (%)
Protein Groups	2,937	1.74
Peptide Groups	13,224	2.44
PSMs	17,388	2.09
MS/MS	83,398	0.66

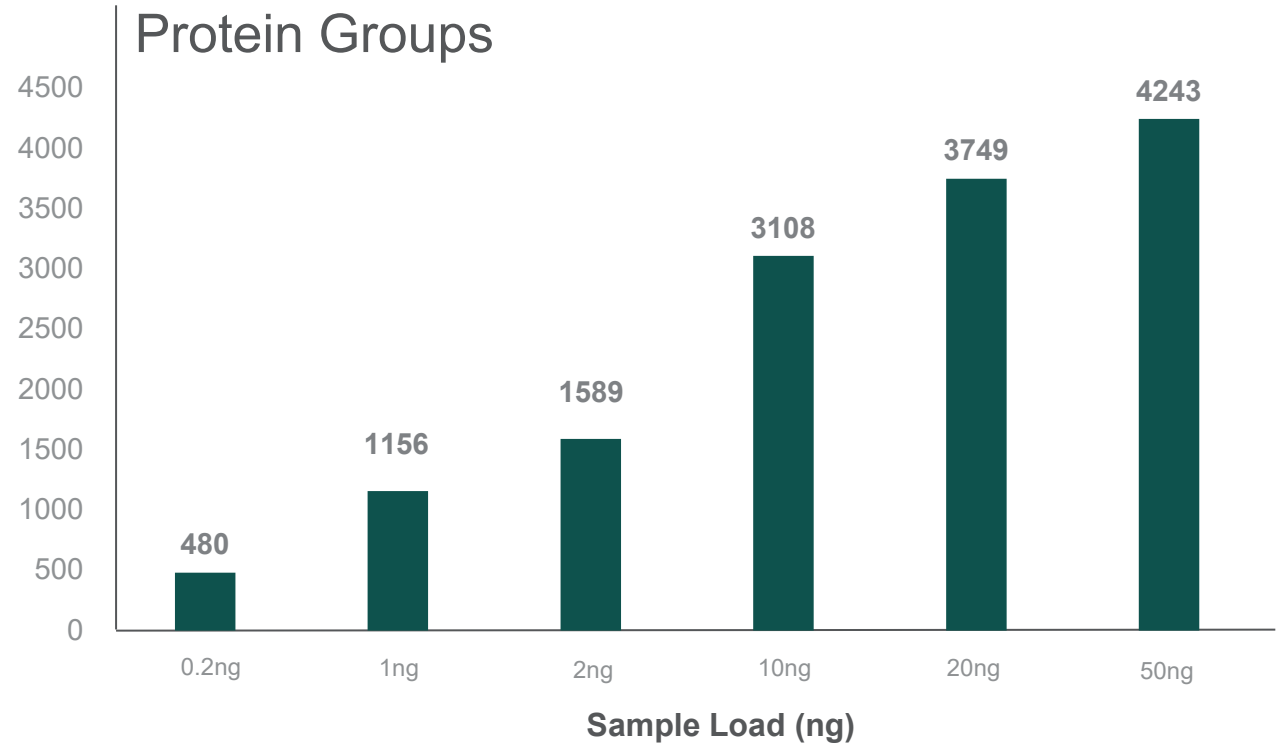
2hrs-Analysis

Optimized for Maximum Coverage and Reproducibility

- ~6,700 proteins Identified in 2 hours with 200 ng of HeLa
- MS/MS and Protein Identification Reproductivity with <1% CV
- Peptide and PSMs Reproducibility with <2% CV
- FAIMS Pro Interface (intra-analysis CV stepping, CV -50 and -70)
- 1% FDR at Peptide Level
- Sharing experimental method and raw reference files

Orbitrap Exploris 480 MS – Performance without FAIMS Pro Interface



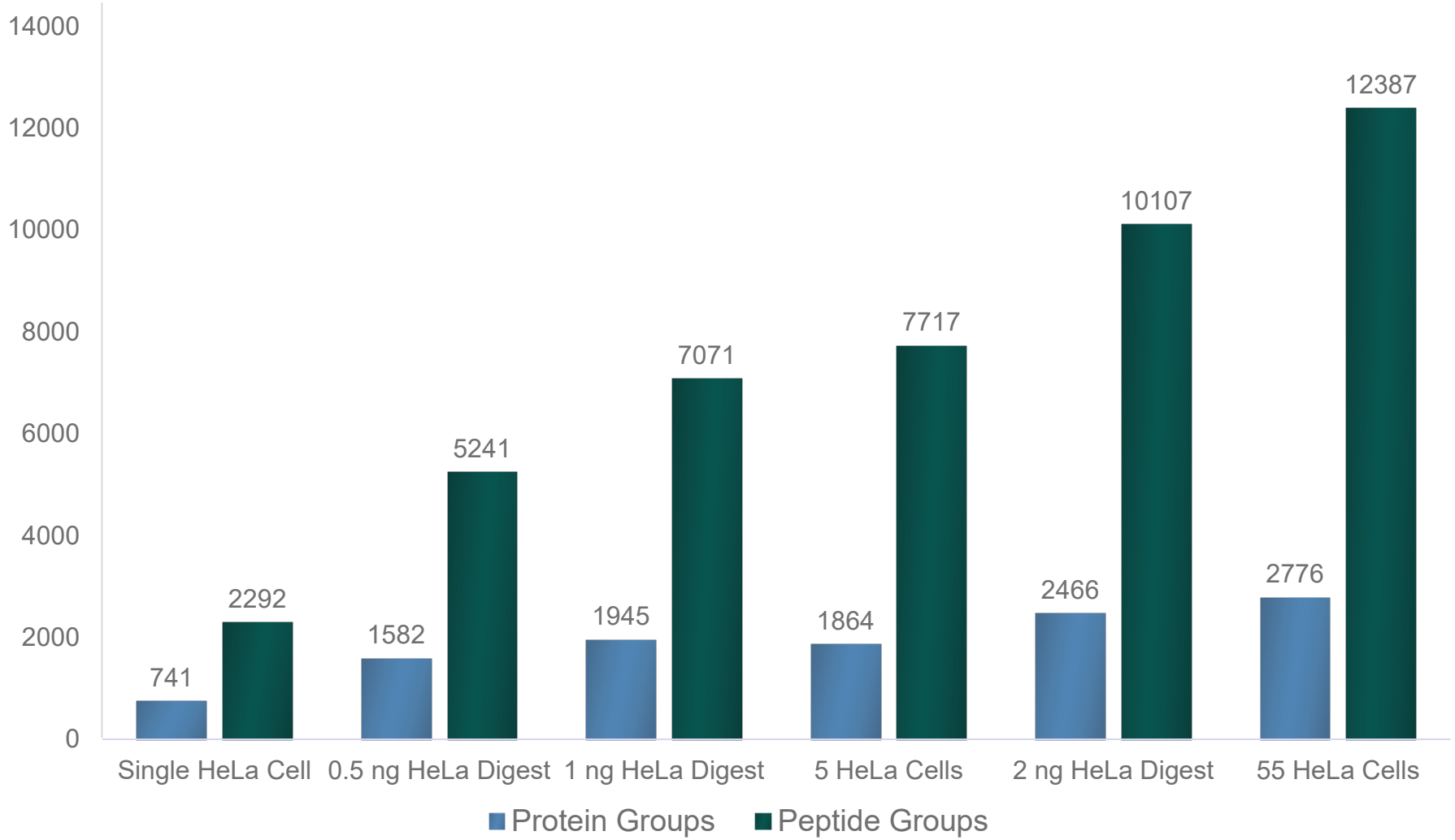


- ✓ ~480 protein groups identified with MSMS in 140 min from 0.2 ng of HeLa digest
- ✓ ~1600 proteins identified with 2 ng HeLa digest is used as QC for evaluation of LC-MS performance
- ✓ Smaller ID column (30umx30cm,1.7um-CoAnn Tech) at lower nano flow rate (50 nl/min-Thermo Scientific™ UltiMate™ 3000 RSLCnano system) provides high performance with high sensitivity

Orbitrap Exploris 480 MS –Protein ID Performance at Single Cell Level

1, 5 and 55 HeLa Cells and 0.5 - 2 ng HeLa Digest with FAIMS Pro Interface

Single Cell-2hrs



Optimized for Maximum Coverage and Reproducibility

- ~750 proteins Identified in Single HeLa Cell and ~2000 Protein Groups from 5 HeLa Cells
- Unmatched sensitivity at low nanogram sample injection (0.5-2ng and low number of HeLa Cells, 1, 5 and 55 Cells)
- FAIMS Pro Interface (intra-analysis CV stepping, CV -60 and -75) were used for on the fly peptide fractionation
- CV -60 removing +1 charge background from PEGs
- Identification at MS^2 level with 1% FDR

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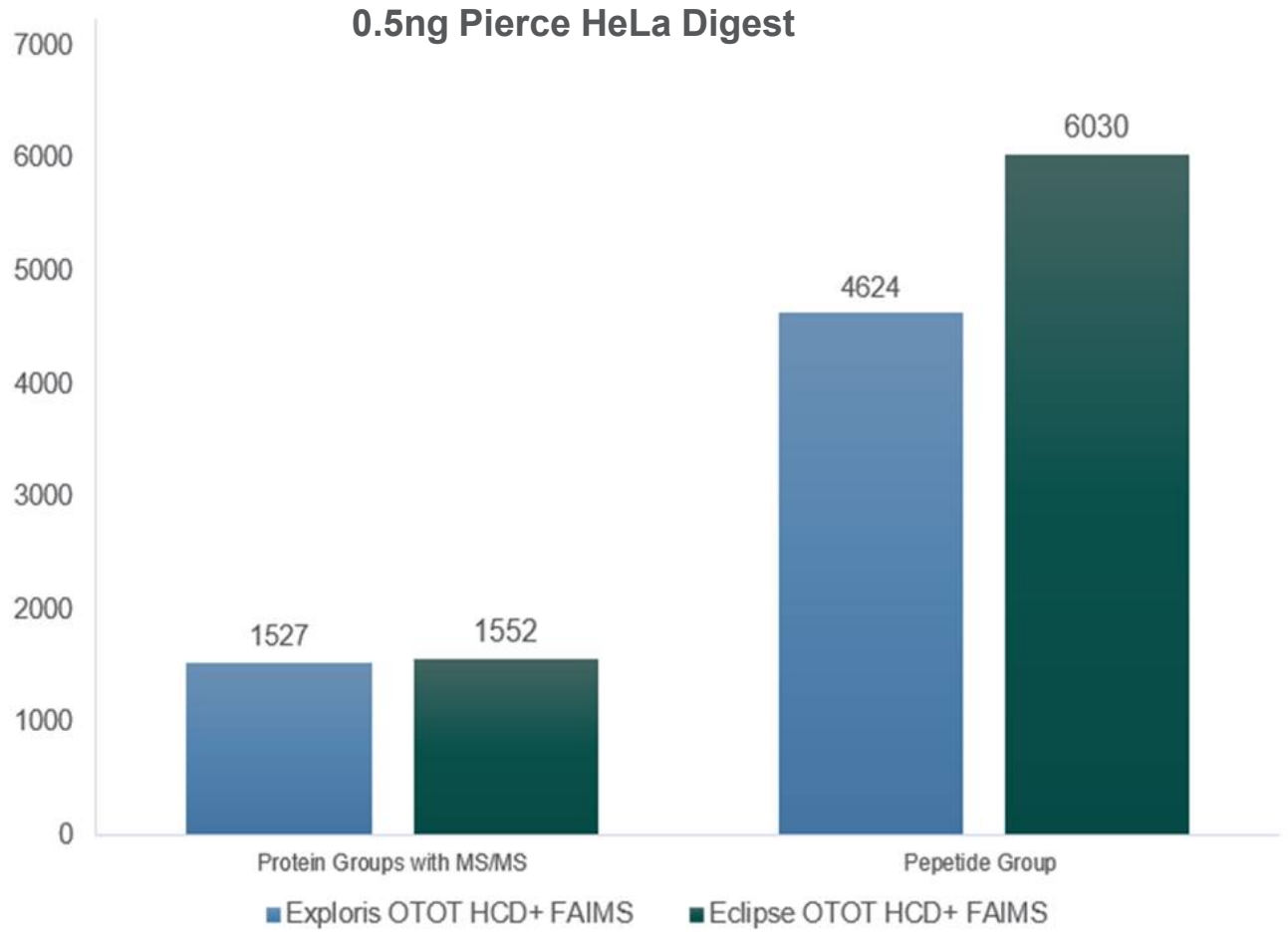
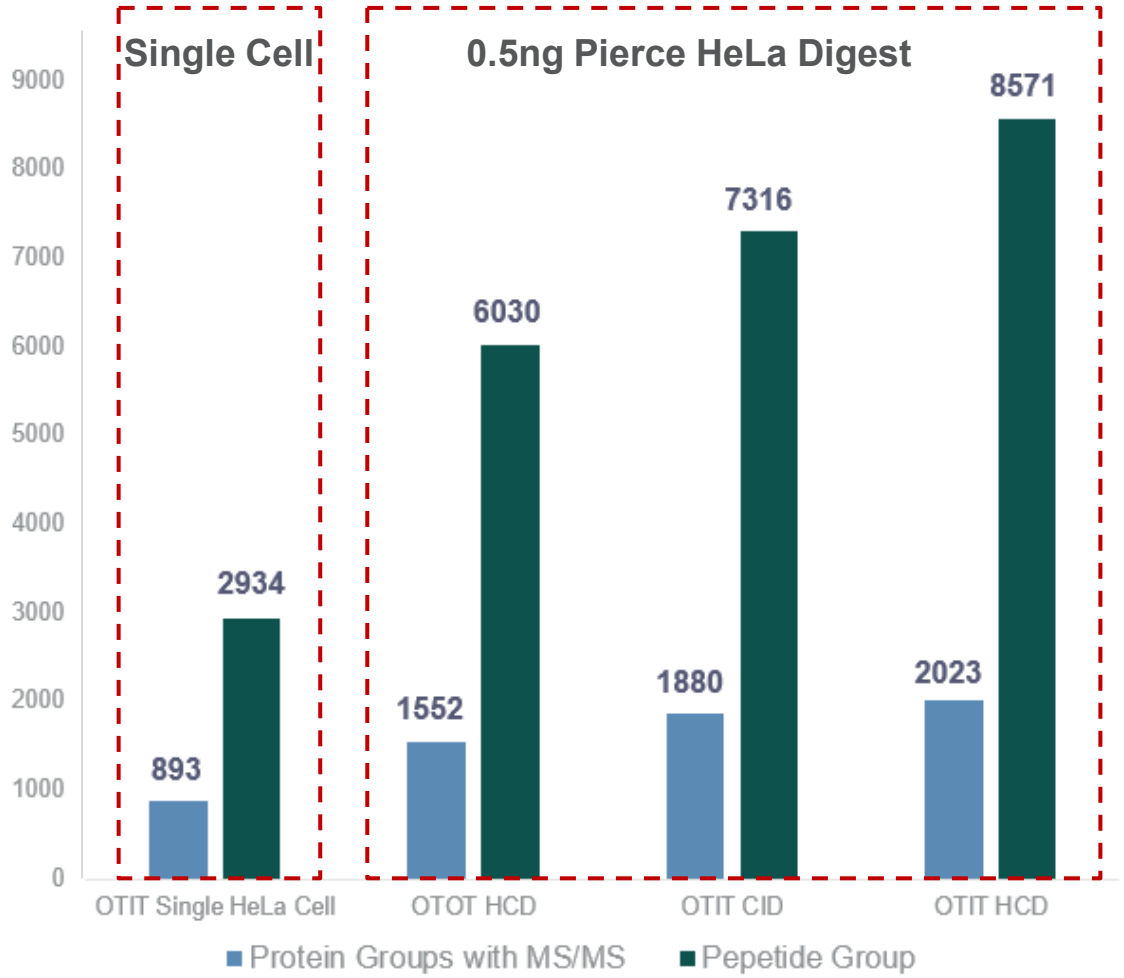
Orbitrap Exploris 480 MS vs Orbitrap Eclipse Tribrid MS – Protein ID Performance With FAIMS Pro Interface

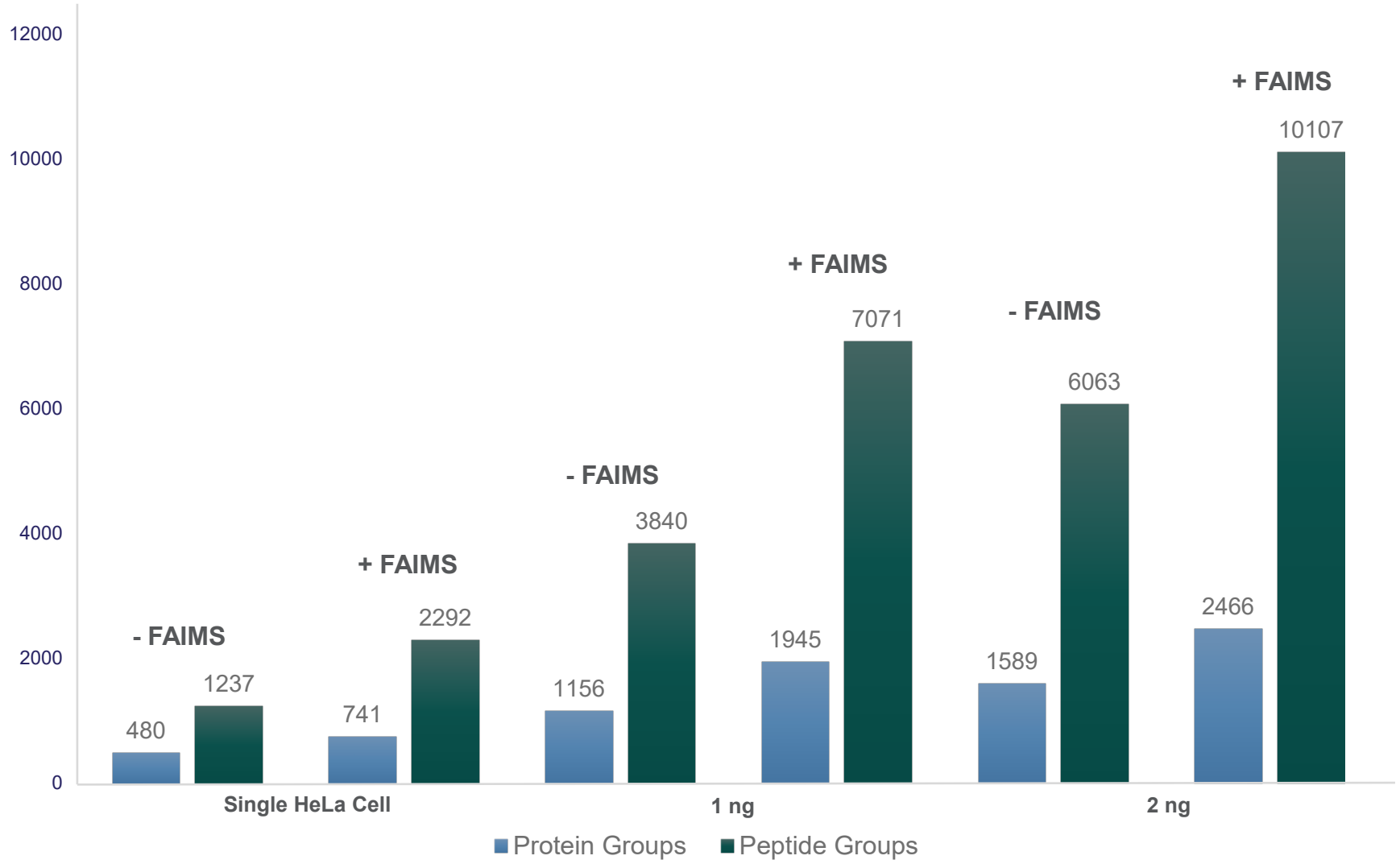
Record Setting Performance: 0.5 ng HeLa Digest and Single Cell





ThermoFisher SCIENTIFIC

Orbitrap Exploris 480 MS – Performance at Single Cell Level -/+ FAIMS Pro Interface



Optimized Orbitrap Method for Max Coverage and Reproducibility

- ~750 proteins Identified in Single HeLa Cell
- Unmatched sensitivity at low nanogram sample injection (0.5-2ng)
- FAIMS Pro Interface (intra-analysis CV stepping, CV -60 and -75)
- CV -60 removing +1 charge background from PEGs
- Identification at MS^2 level with 1% FDR

Real-Time Search for exceptional depth, throughput and accuracy of TMT analysis

ThermoFisher SCIENTIFIC

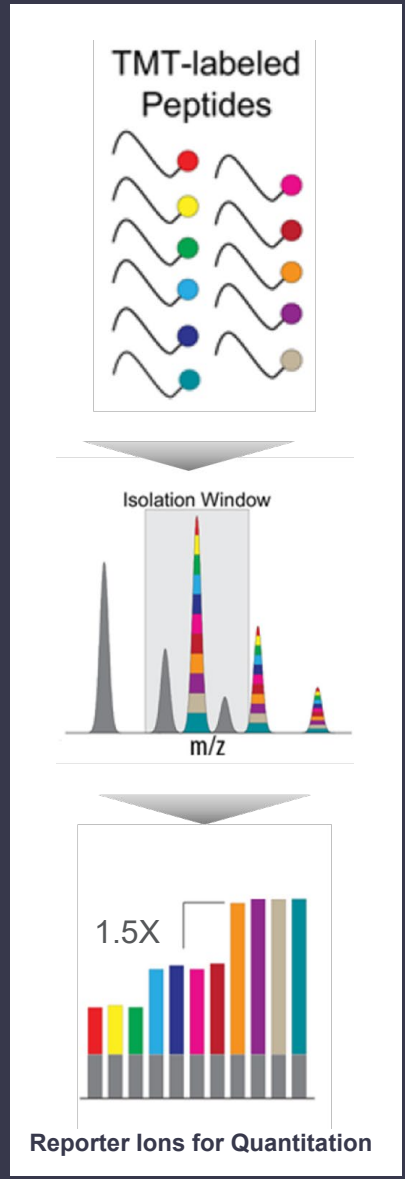
Isobaric Labeling: Challenges and Resolutions

Why do scientists love Thermo Scientific™ Tandem Mass Tags™ (TMT™)?

- Increase in throughput (up to 11X)
- Relative quantitation in the same scan, no need to match chromatography
- Can be applied to any peptide mixture

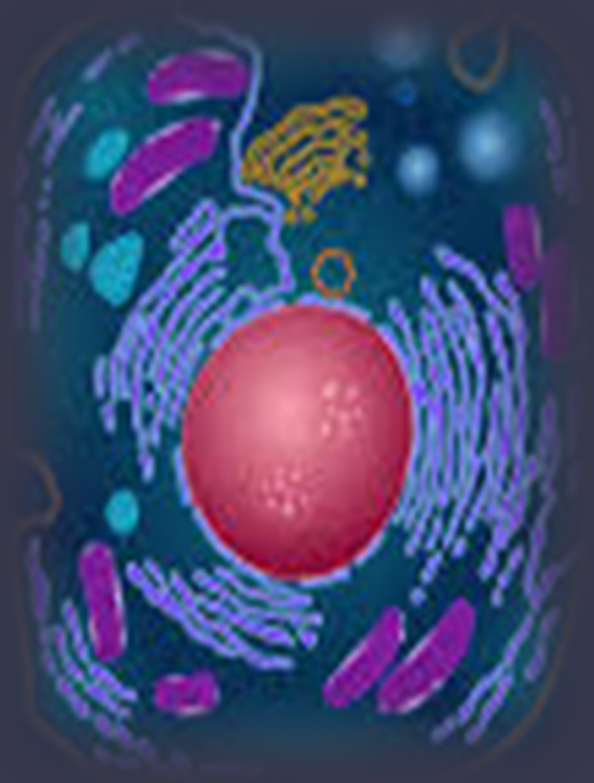
What are the drawbacks?

- Original **MS²** based method is **NOT accurate** because of the ratio compression caused by spectral interference
- Recently introduced **SPS MS³** method is more **accurate**, but it is **30-50% slower**



Tandem Mass Tag and TMT are trademarks of Proteome Sciences plc.

Detecting Small Changes At Low Levels: a Synergy of Single Cell Analysis And Real-Time Search



- TMT labeling allows boosting the MS^2 signal and increases sensitivity to make single cell proteome characterization feasible (Budnik et. Al., 2018 Genome Biol. 19, p.161)

- TMT SPS MS^3 with Real-Time Search provides necessary accuracy and throughput for quantitative analysis of differences between the individual cells

Real-Time Search Significantly Improves TMT Results

Journal of proteome research

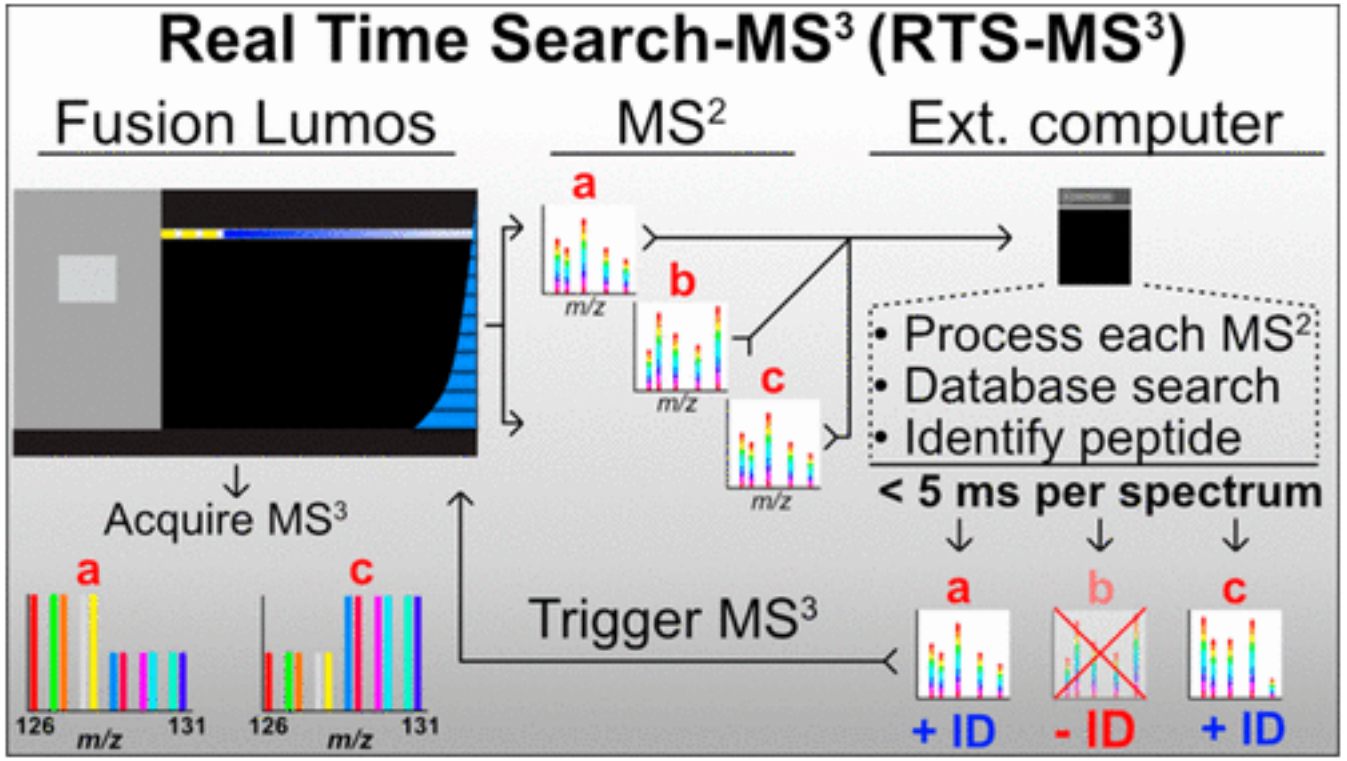
Main claims:

- Real-Time Search allows for significantly improved coverage AND throughput
- Real-Time Search allows for further improved accuracy over SPS MS^3 method

Active Instrument Engagement Combined with a Real-Time Database Search for Improved Performance of Sample Multiplexing Workflows

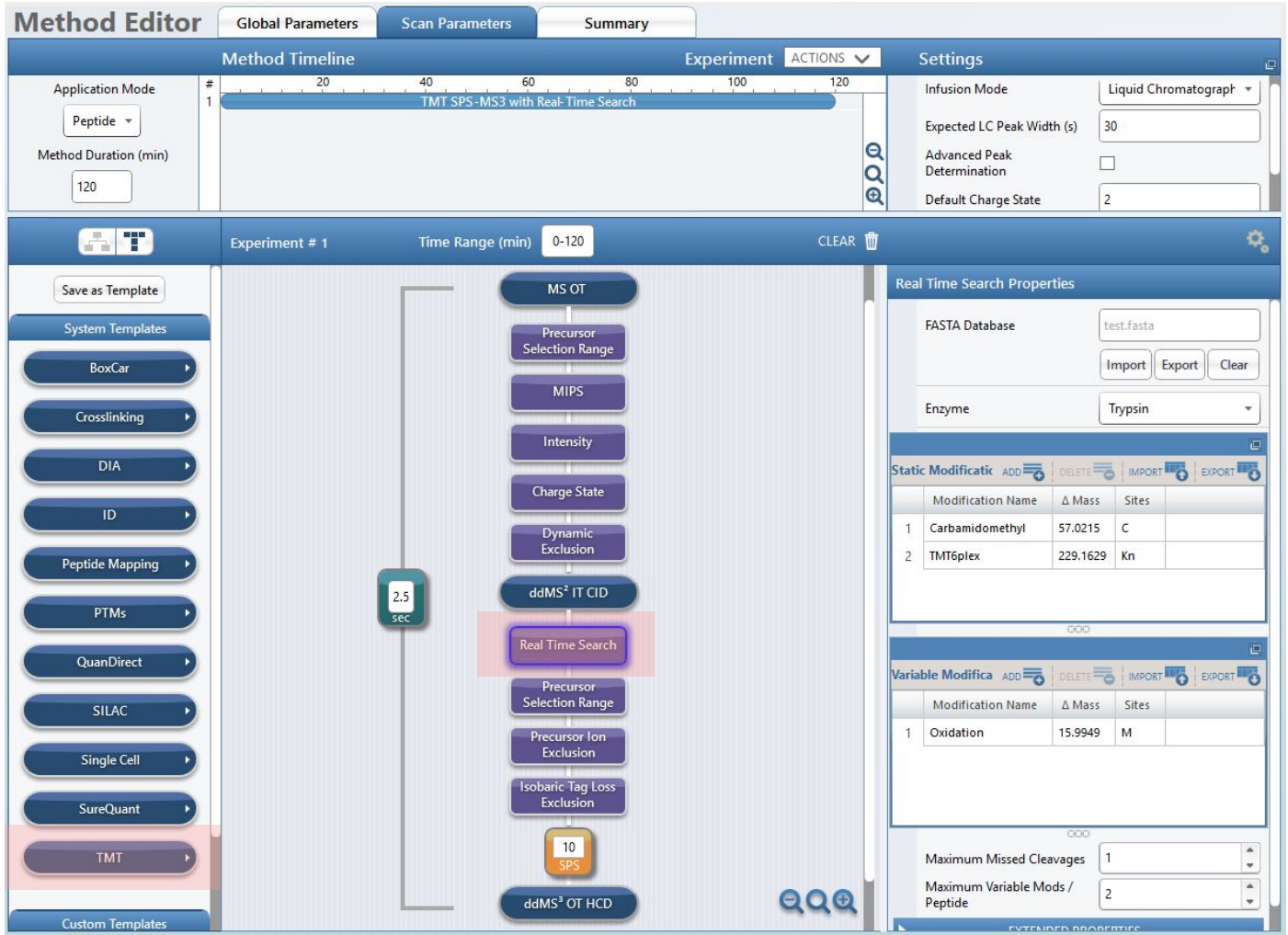
Brian K. Erickson[†], Julian Mintseris[†], Devin K. Schweppe[†], José Navarrete-Perea[†], Alison R. Erickson[†], David P. Nusinow[†], Joao A. Paulo[†], and Steven P. Gygi[*†]

[†] Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, United States



J. Proteome Res., **2019**, *18* (3), pp 1299-1306

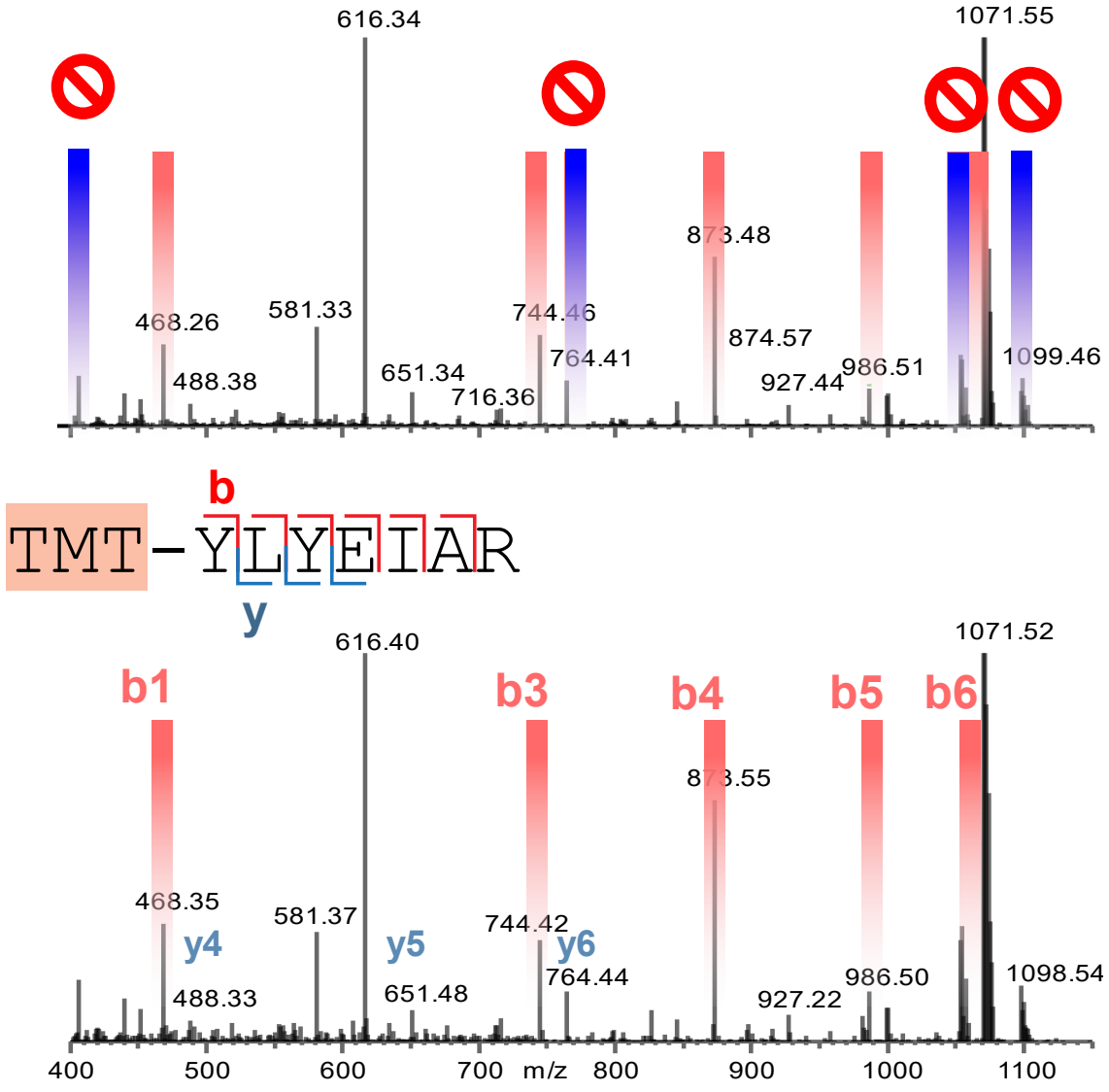
Real-Time Search Is An Easy-To-Use Method



- One-click set up
- Any Database
- Any modification (3 max)
- Comet Search Engine* (similar to SEQUEST)
- Adjustable pass/fail criteria

*Eng *et al*. Proteomics. 2013 13(1): 22-4

Real-Time Search Improves Quantitative Accuracy of SPS MS^3 Experiment

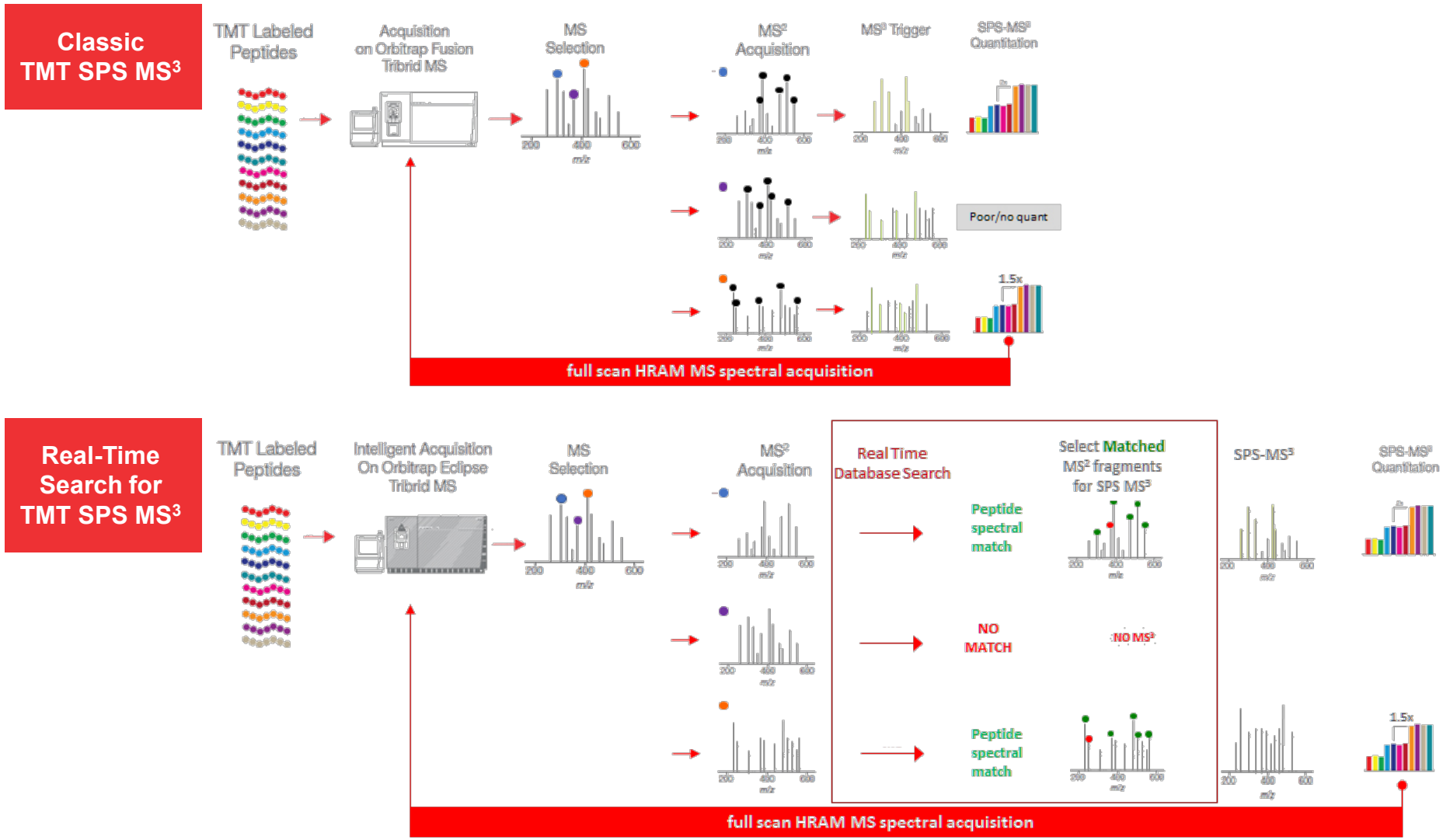


Using the right fragments for MS^3 with Real-Time Search: a real data example

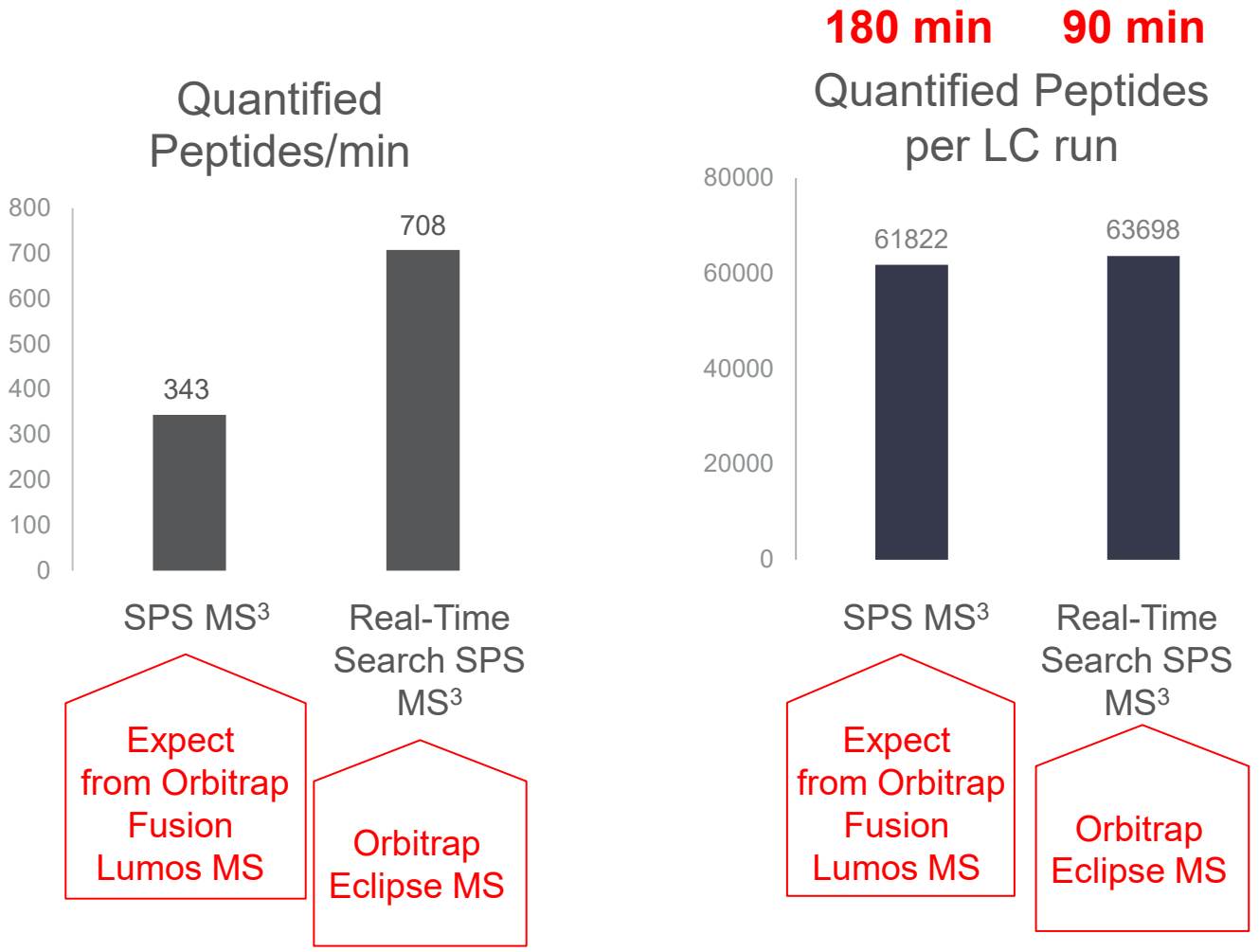
- We are comparing the MS^2 spectra for the BSA peptide shown
- Standard SPS MS^3 – 9 ions selected
  - 5 correct TMT-tagged fragments
  - 4 untagged or contaminant fragments
- Real-Time Search SPS MS^3
  - 5 correct TMT-tagged fragments (b-ions)
  - System excluded un-tagged and unidentified fragments
- This results in greater specificity of TMT reporter ions, leading to improved quantitation accuracy

Unique Real-Time Search for TMT SPS MS^3 with Orbitrap Eclipse Tribrid MS

Highest Quantitative Accuracy Is Driven by Intelligent Acquisition



Real-Time Search Increases SPS MS^3 Throughput



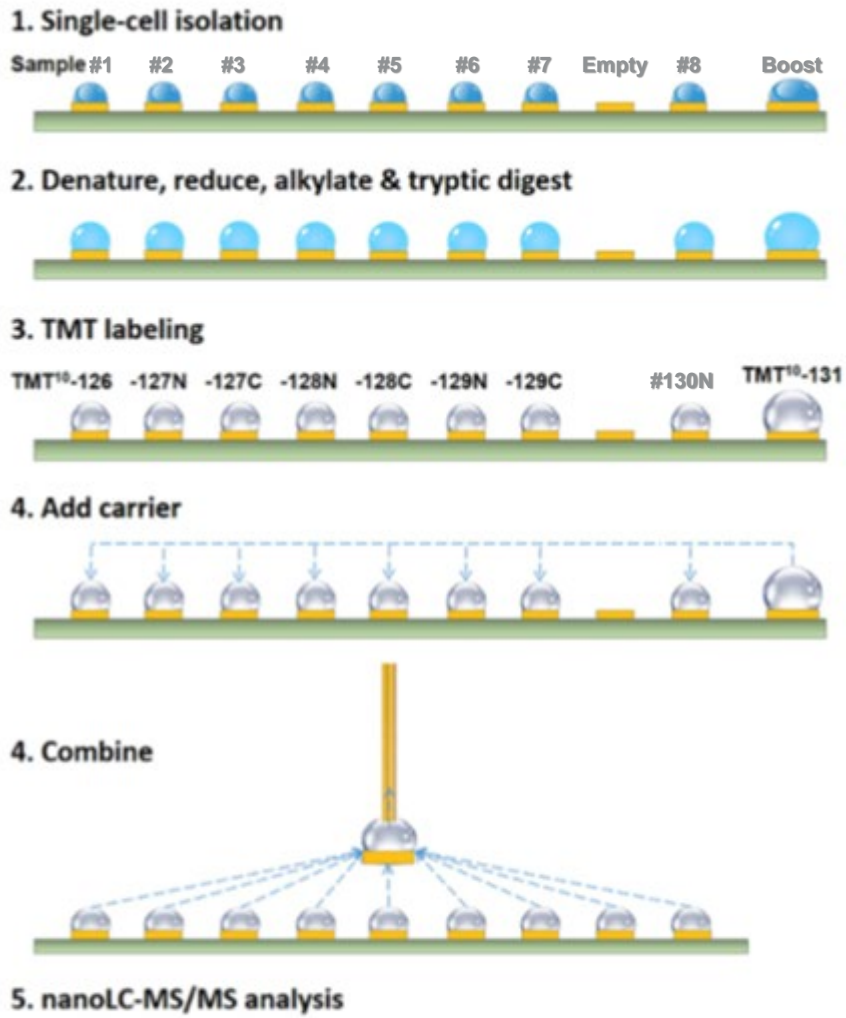
For Research Use Only. Not for use in diagnostic procedures.

Real-Time Search: results of SPS MS^3 in half the time

- Real-Time Search doubles the throughput of SPS MS^3 workflow
- Results shown here are for HHM sample: three human cell lines labeled as biological replicates in TMT10plex (3-3-4)

Data: D. Schweppe , Q. Yu and S. Gygi
Harvard Medical School

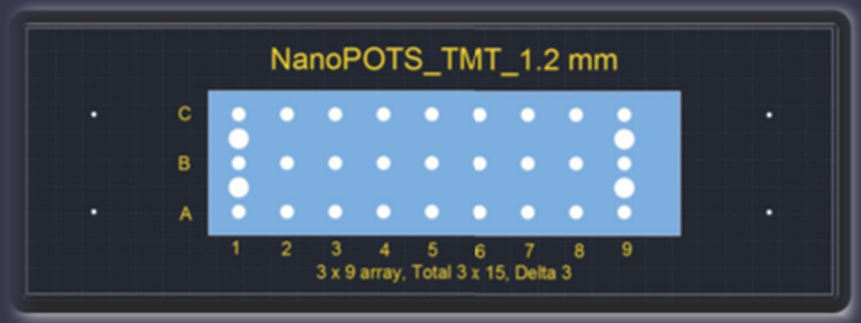
Single Cell Analysis Using NanoPOTS* and TMT



*Zhu at al., 2018, *Nature Comm.,* 9 (882)

Benefits of using TMT for Single Cell Analysis

- Up to 9 single cell analyzed in a single LC/MS run with TMT11plex
- Up to 14 single cell analyzed in a single LC/MS run with Thermo Scientific™ TMTpro 16plex Isobaric Label Reagent
- Extra TMT channels are used as:
  - **a booster channel** to amplify the MS^2 signal
  - empty channel(s) to measure noise



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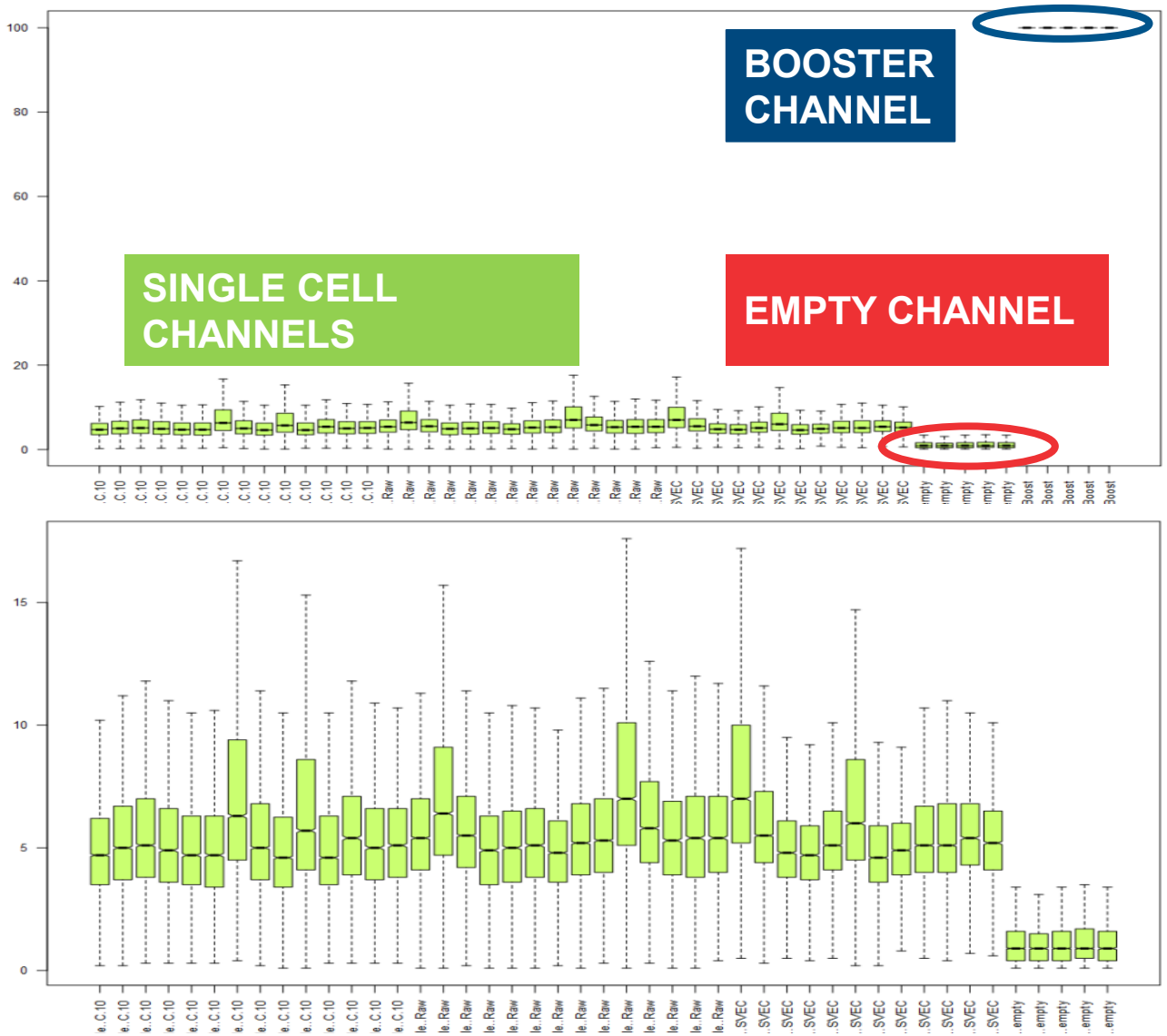
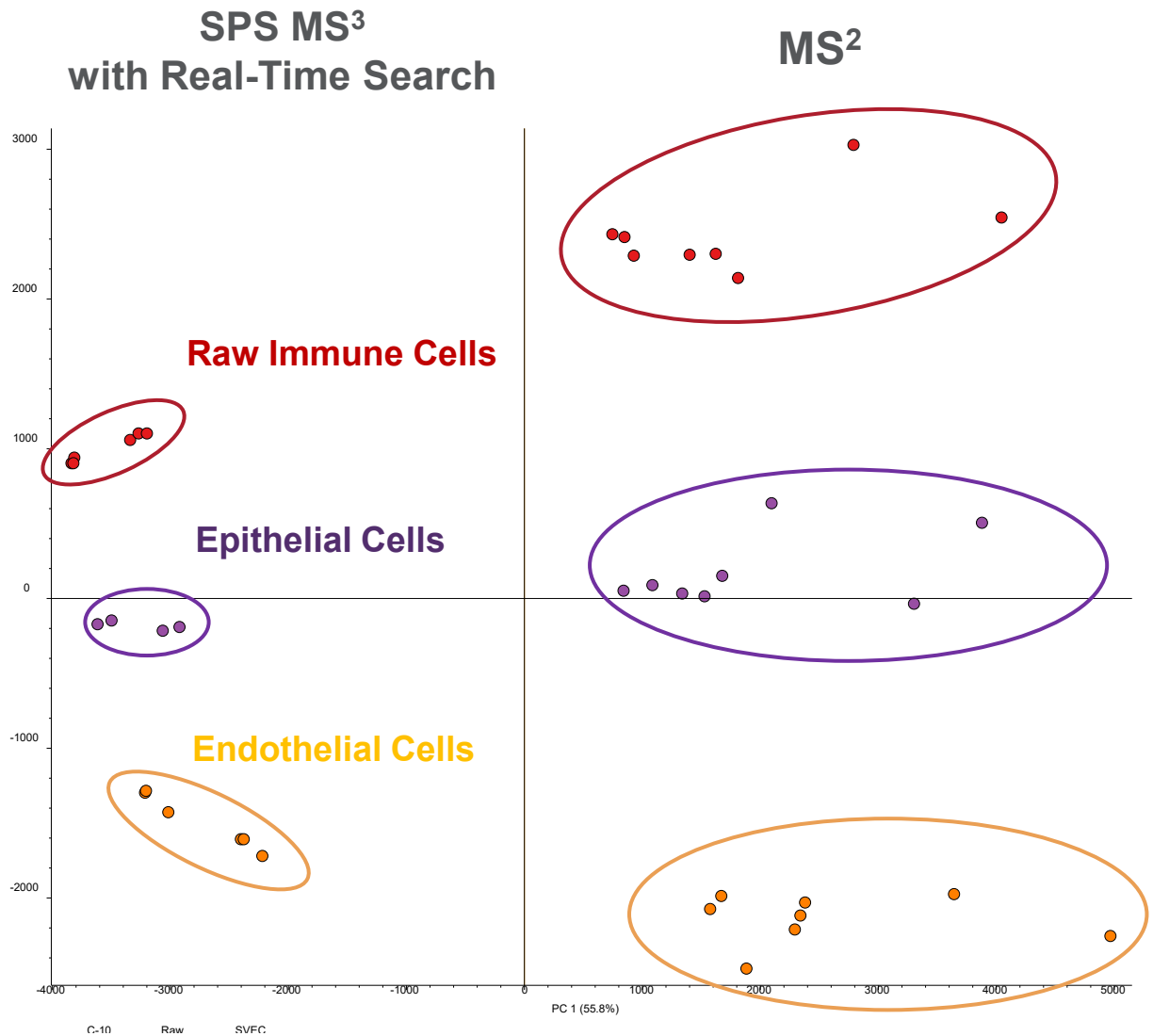
Single Cell Analysis With TMT Multiplexing



How does the signal look?

- 8 single cells from 3 cultured murine cell populations were analyzed in a single LC/MS run, ~0.2 ng of protein/cell
- 1 Pooled sample in the boost channel (5 ng or ~20 cells)
- 1 empty control (130N)
- The shown results are for 24 single cells analyzed in 3 LC-MS runs

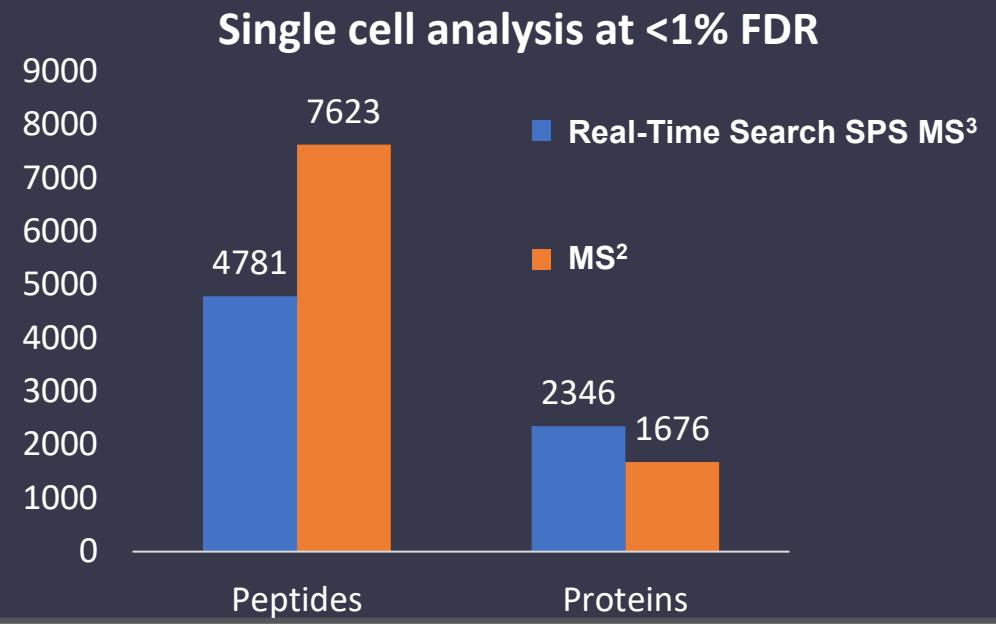
Single Cell Classification With MS^2 And Real-Time Search SPS MS^3 Methods



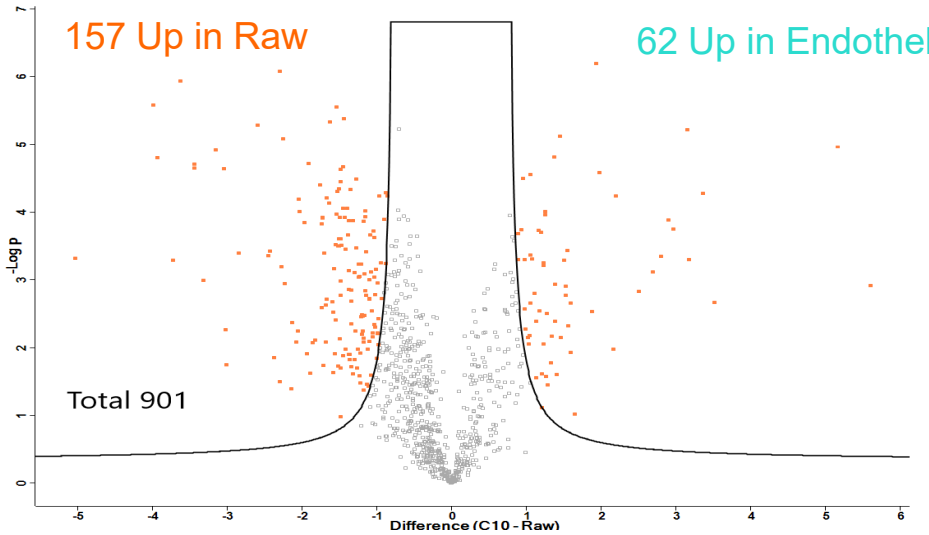
Eight single cells per run: 3 MS^2 runs; 2 Real-Time Search SPS MS^3 runs

Results

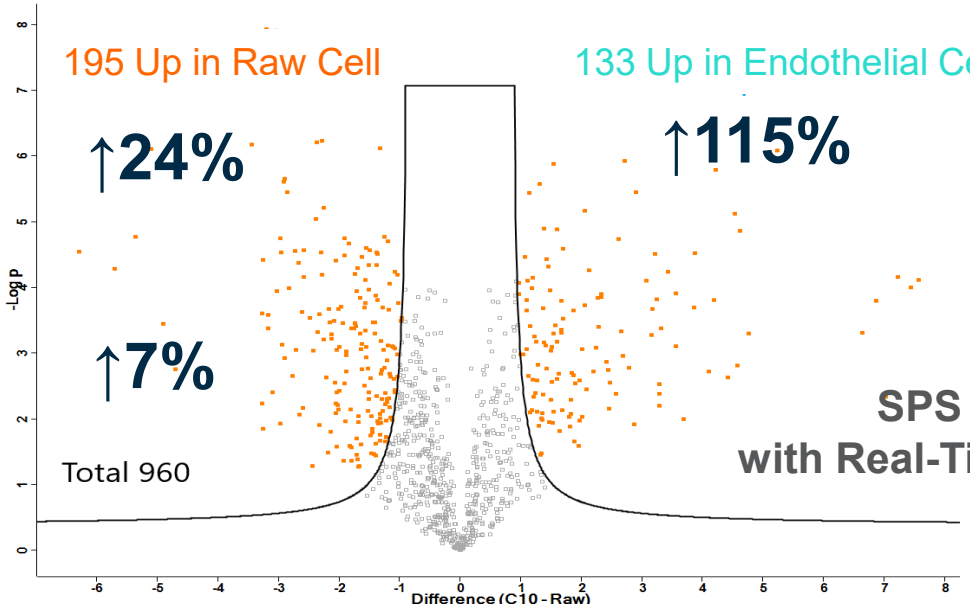
- Both methods differentiated 3 cell types
- SPS MS^3 with Real-Time Search provided improved accuracy with better separation between cell types without compromising protein coverage



Is There a Difference in Protein Expression on a Single Cell Level?



MS²



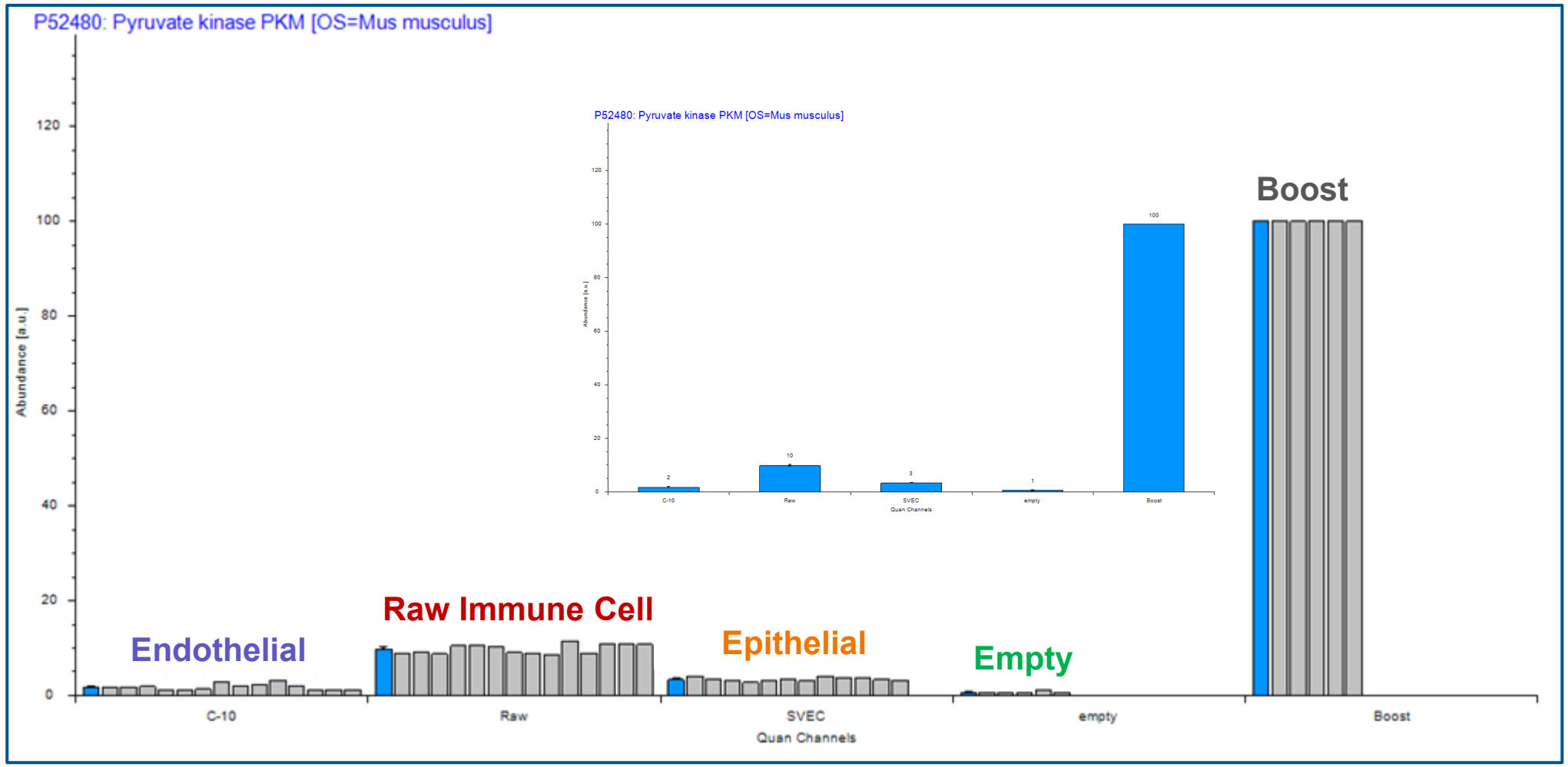
SPS MS³ with Real-Time Search

- Two cell types were compared (C10 and Raw)
- SPS MS³ with Real-Time Search identified a greater number of significantly changing proteins

	MS²	MS³	Difference, %
Total Proteins	901	960	7
Up in Raw	157	195	24
Up in C10	62	133	1.5X

External Validation

- Quantifying Pyruvate Kinase PKM in 3 different cell type
- Minimal interference in empty channel



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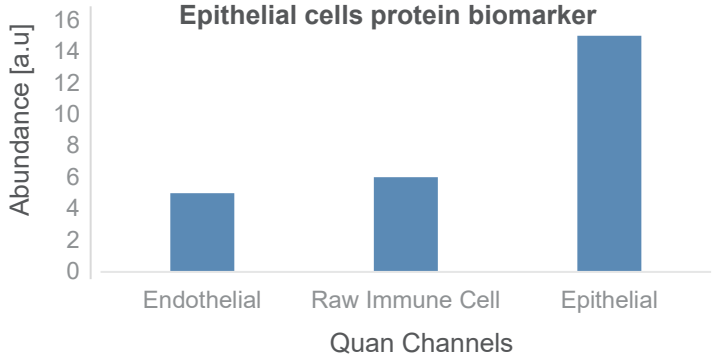
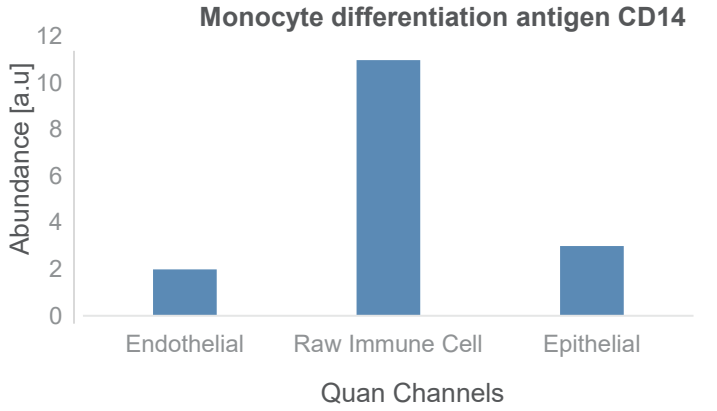
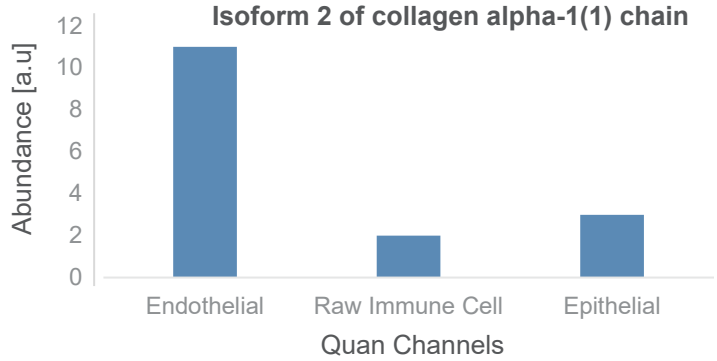
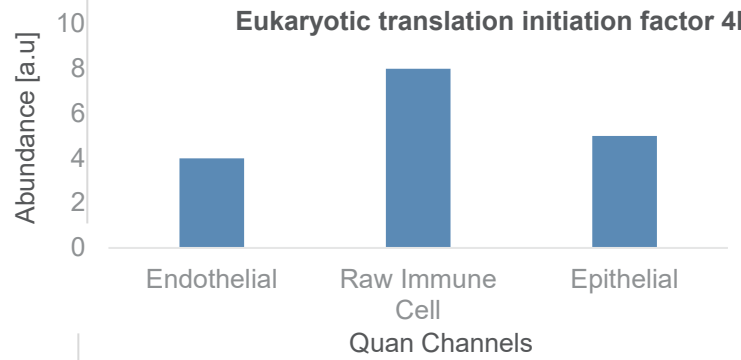
An Example Of TMT-based Quantitation Of Cell Differentiation Markers

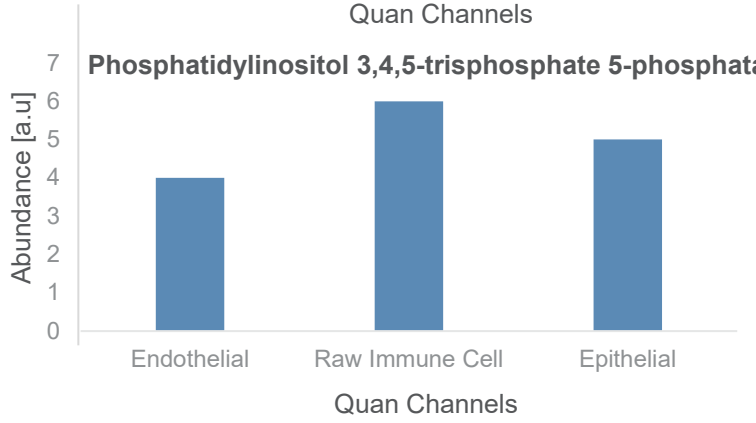


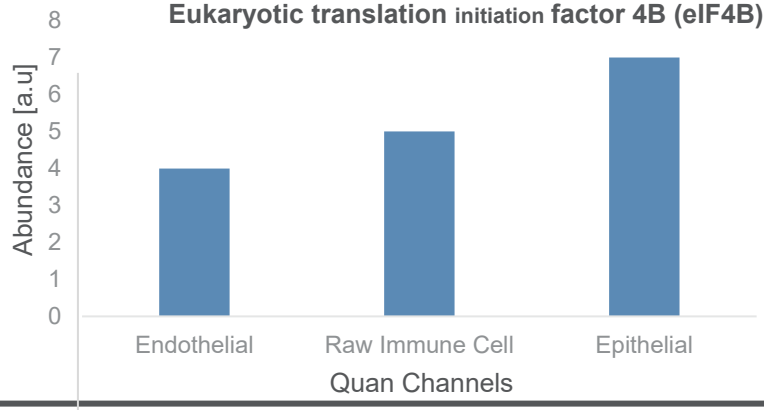




Cell Type Biomarkers





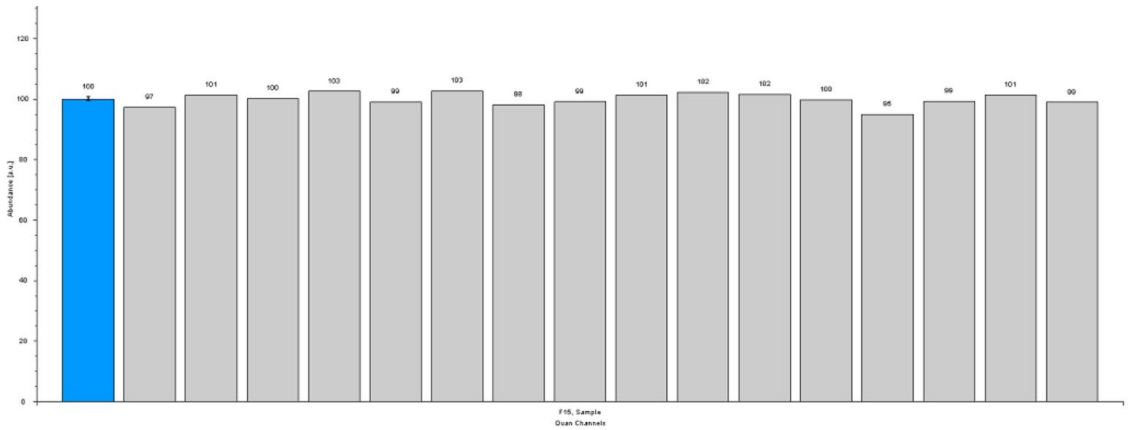


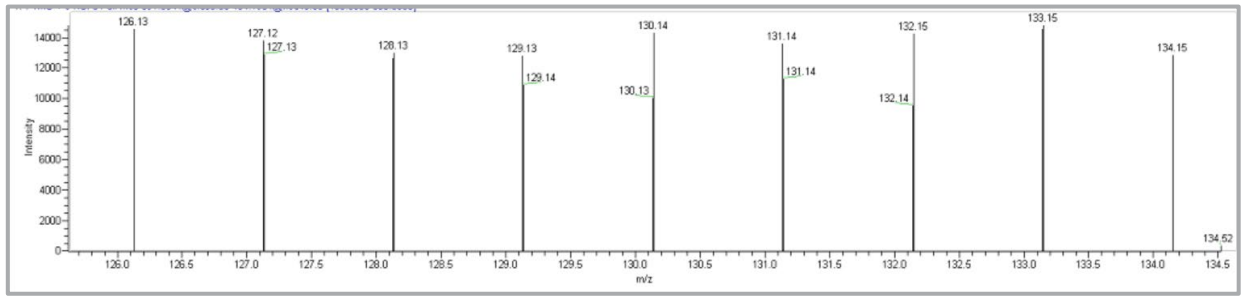
AKT-mTOR signaling pathway proteins

ThermoFisher SCIENTIFIC

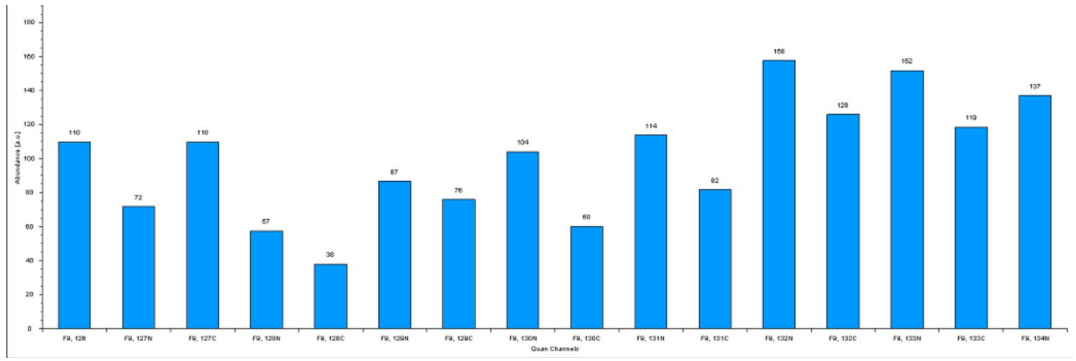
TMTpro 16plex Label Reagents: A New Horizon For Single Cell Proteomics

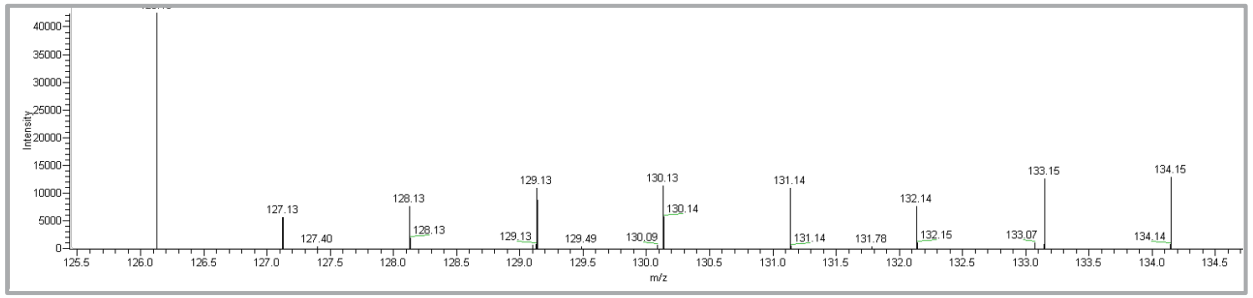
TMTpro Reporter Ion Spectra 5ng HeLa QC 16 Channel (1:1 Ratio)



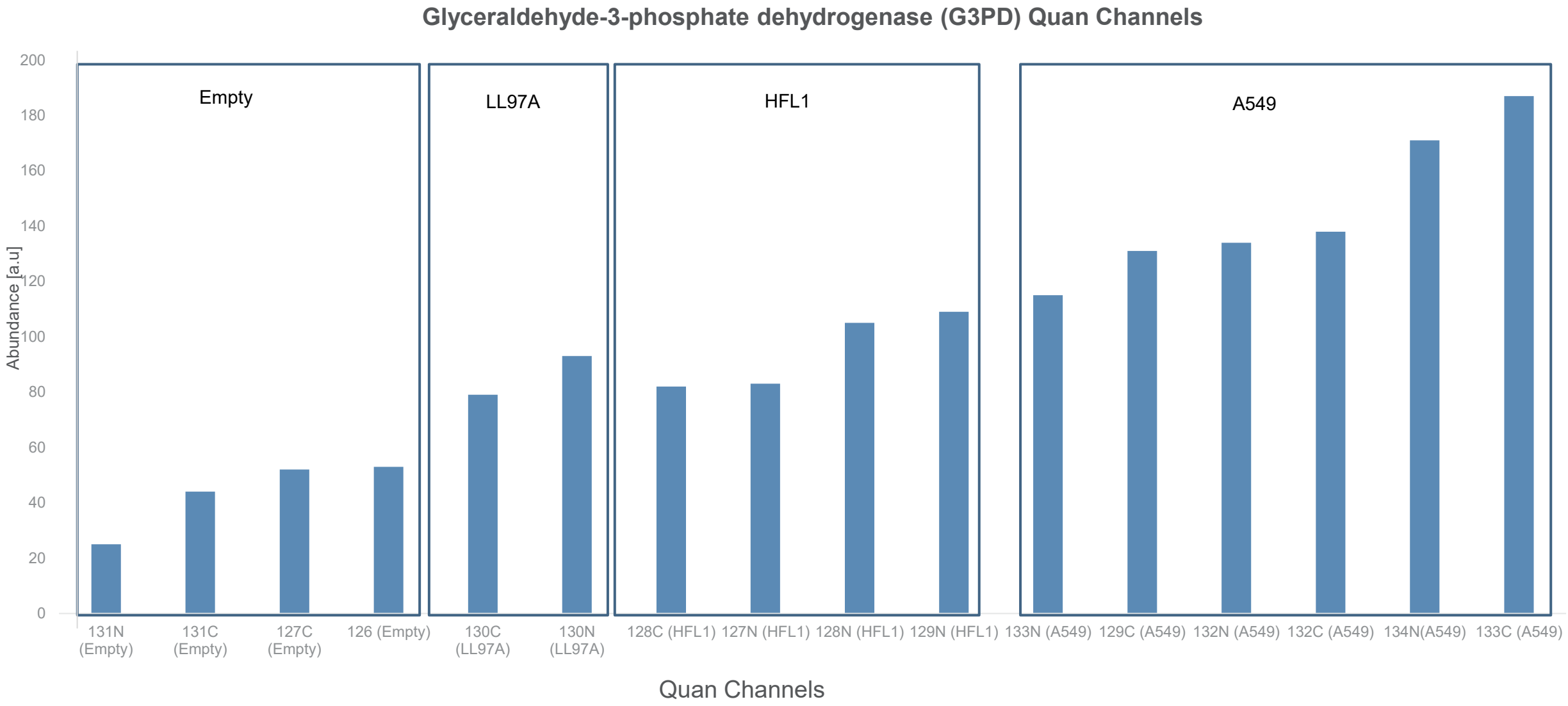


TMTpro Reporter Ion Spectra In Single Cell Analysis



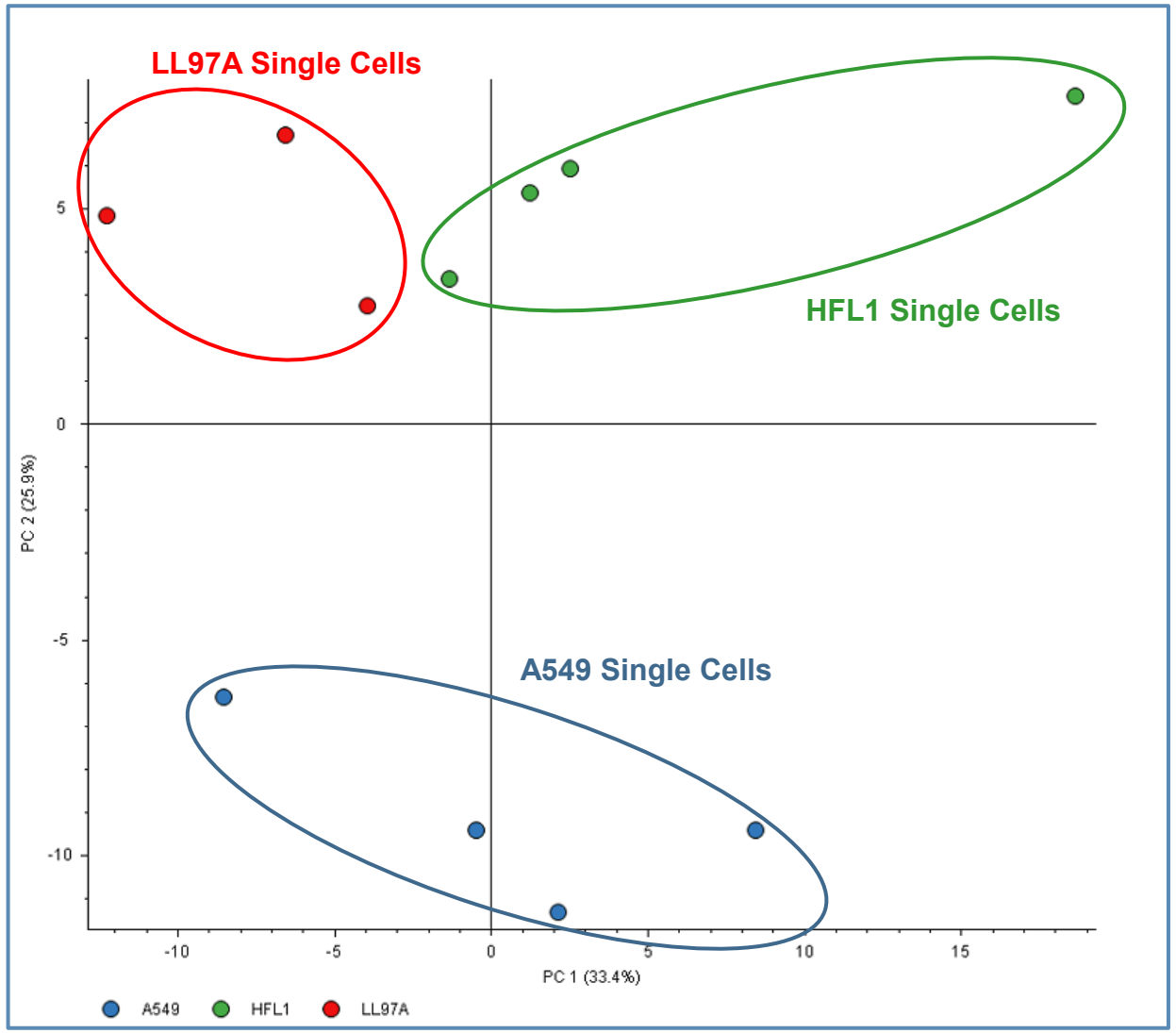


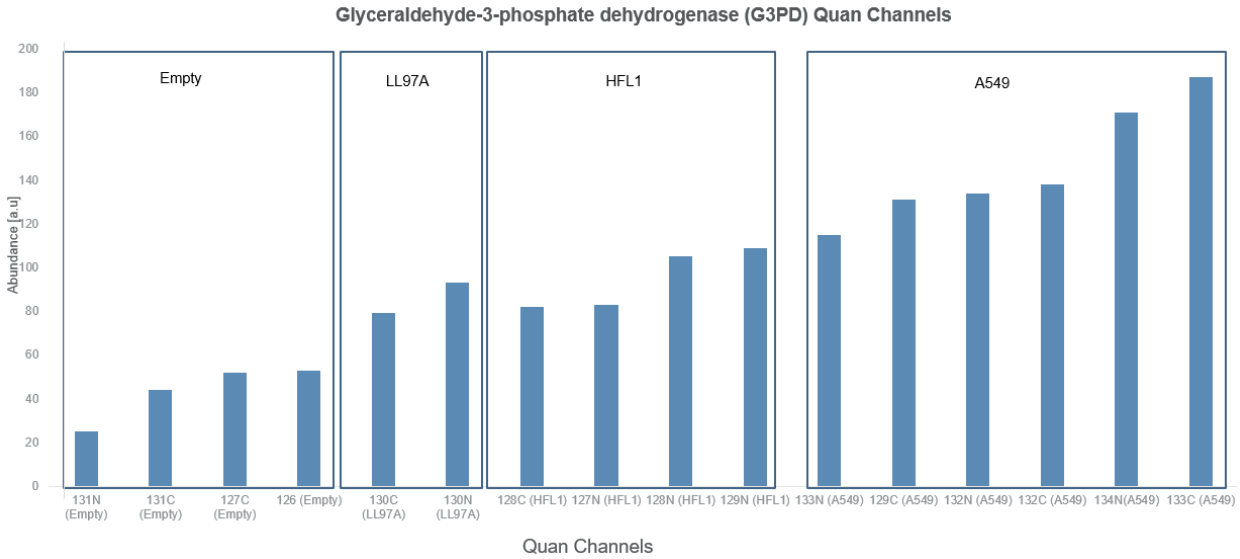
Hight-throughput Single Cell Proteomics Analysis with TMTpro



Classification of 3 different cell type by TMTpro without boost

Clear differentiation between three cell types without Boost Channel





Single Cell Proteomics – Preliminary data

Label Free Workflow

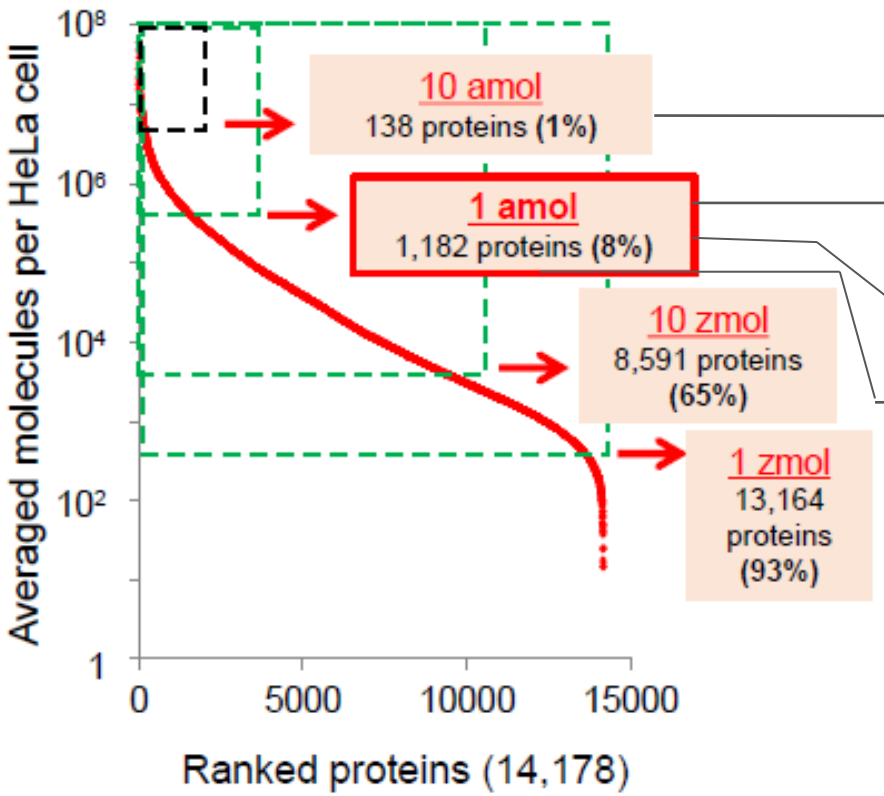
	Initial Study	After optimization
Sample preparation	NanoPOTS	NanoPOTS
Separation	30 µm i.d. column	20 µm i.d. column
Mass Spectrometer	Lumos	Eclipse+FAIMS Pro
Data analysis	MaxQuant	Proteome Discover
Protein group IDs by MS/MS	211	829

TMT Workflow

	Initial Study	After optimization
Sample preparation	NanoPOTS	NanoPOTS
Separation	30 µm i.d. column	30 µm i.d. column
Mass Spectrometer	Lumos	Eclipse+ Real Time Search
Data analysis	Proteome Discover	Proteome Discover
Protein group IDs by MS/MS	1676	2346

As today, our LC-MS systems can provide the amount of information required in the single cell analysis field

Technical Optimization In The Last Year Translated In A 10X Improvement

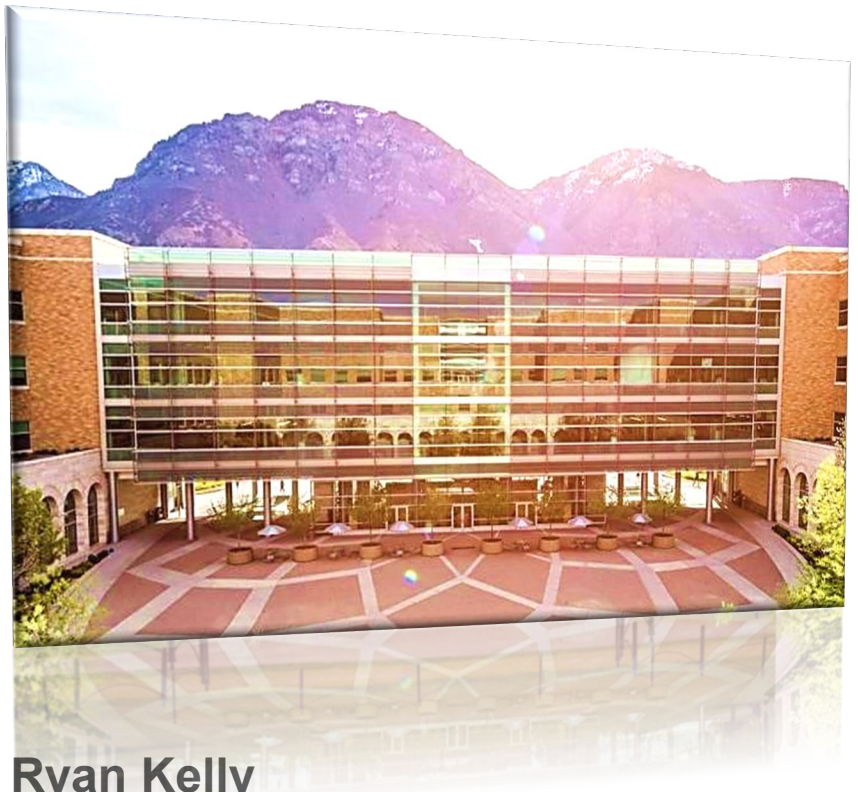


Bekker-Jensen et al., *Cell Systems*, 4, 587–599 (2017)

Milestones		Optimization
211 proteins	March 2019	NA
829 proteins	April 2019	FAIMS
1676 proteins	June 2019	TMT
2346 proteins	Today	Real Time Search

Orbitrap Eclipse Tribrid MS With Real-Time Search To Revolutionize Single Cell Quantitation

"A revolution in single cell proteomics is just beginning. The combination of nanoPOTS with the Orbitrap Eclipse Tribrid, TMT reagents and SPS MS^3 with Real Time Search provide the depth of coverage, quantitative accuracy and throughput needed to propel this nascent field forward. "



Dr. Ryan Kelly
Department of Chemistry and Biochemistry
Brigham Young University, Provo, UT, USA
Pacific Northwest National Laboratory, Richland, WA, USA

Acknowledgements

Proteomics Team

- Khatereh Motamed
- Aaron M Robitaille
- Xuefei Sun
- Greg Foster
- Aaron Gajadhar
- Daniel Lopez Ferrer
- Andreas Huhmer

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- Romain Huguet
- David Horn

PNNL/BYU

- Ryan Kelly
- Yongzheng Cong
- Ying Zhu
- Maowei Dou
- Yiran Liang

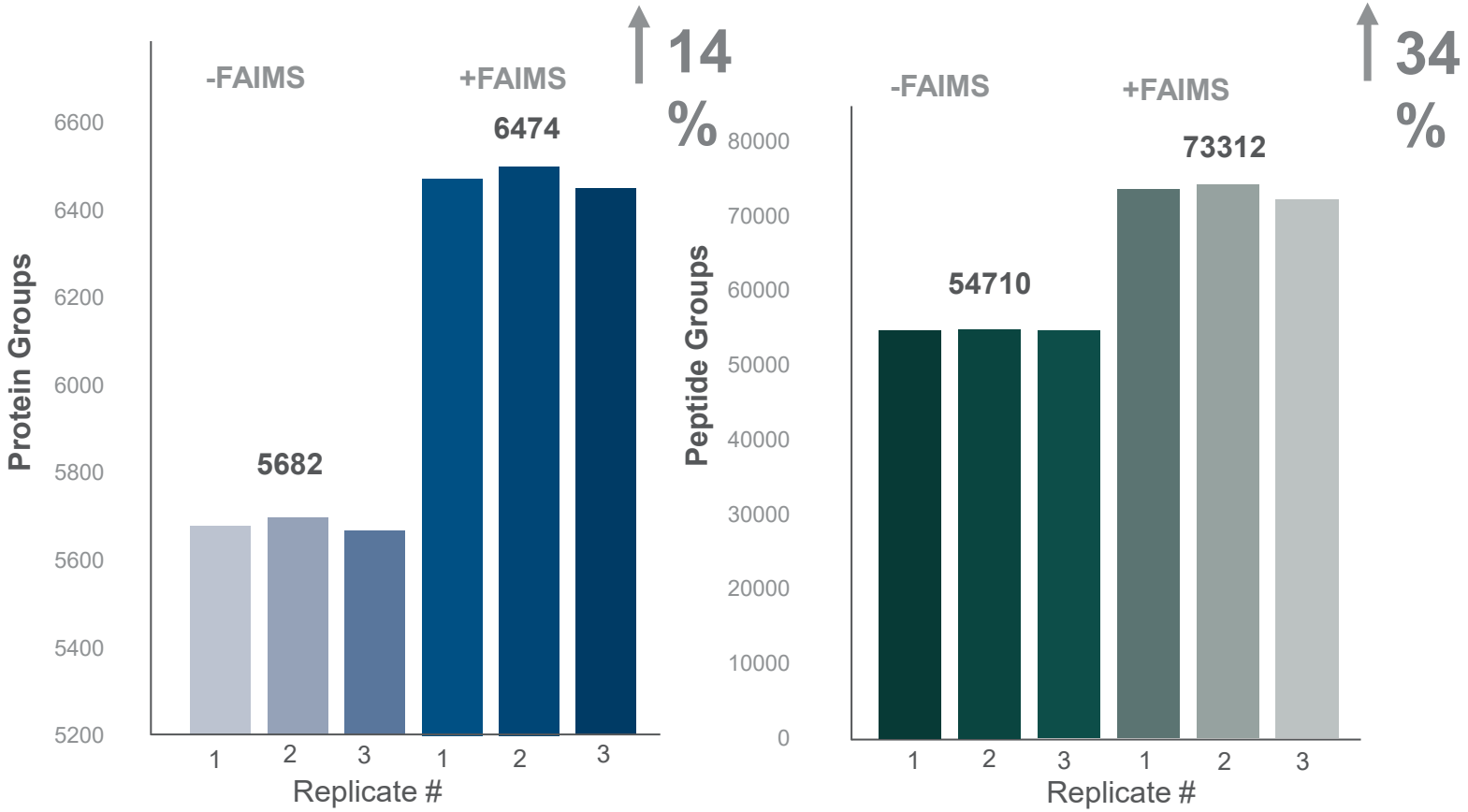
CoAnn Technologies

- Yufeng Shen

Demo Lab Orbitrap Exploris 480 MS Success: New Jersey Demo Lab - Not the Max ID Method

200 ng HeLa, 120 min Analysis -/+ FAIMS Pro Interface

Josh's Data



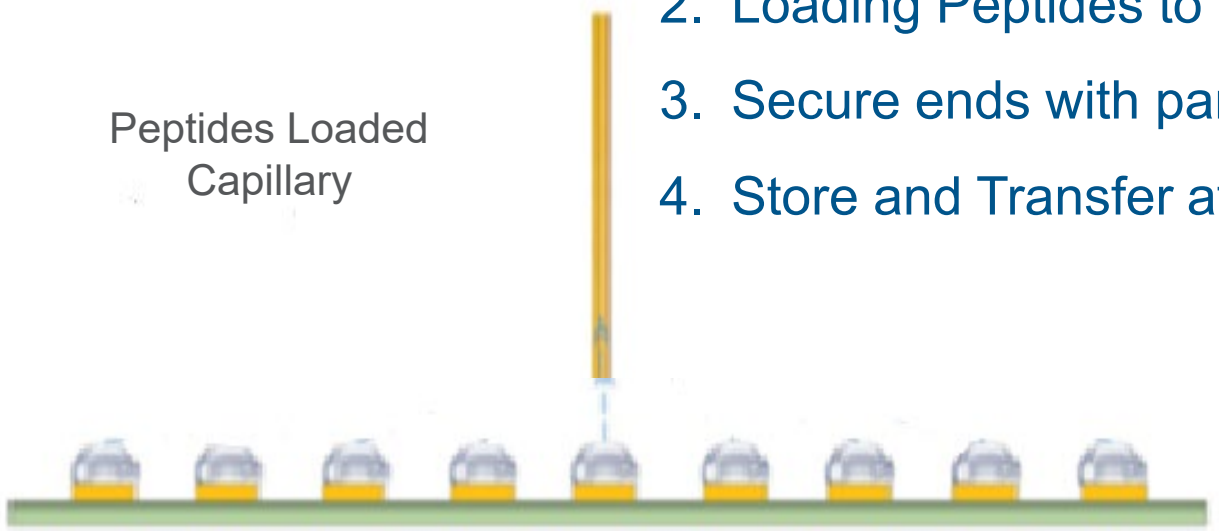
-FAIMS	Mean (n=3)	CV (%)
Protein Groups	5682	0.23
Peptide Groups	54710	0.15
PSMs	140424	0.40
MS/MS	159567	0.07

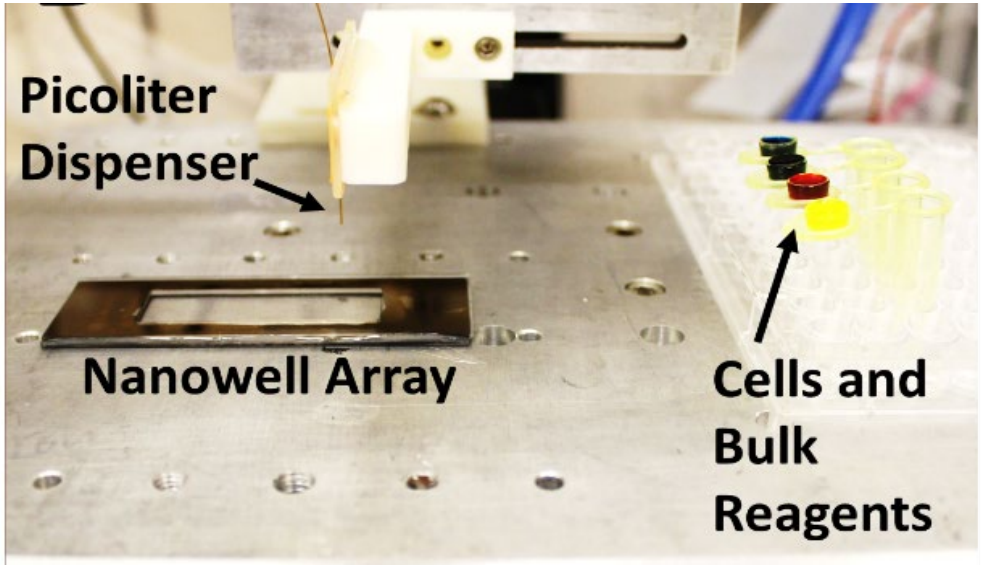
+FAIMS	Mean (n=3)	CV (%)
Protein Groups	6474	0.31
Peptide Groups	73312	1.11
PSMs	148164	0.81
MS/MS	165349	0.11

- ✓ ~5700 proteins identified in 120 min with 200 ng of HeLa digest without FAIMS Pro Interface (mean of n=3 injections shown)
- ✓ ~6500 proteins identified in 120 min with 200 ng of HeLa digest with FAIMS Pro Interface (mean of n=3 injections shown)
- ✓ Improved peptide/protein coverage with FAIMS Pro Interface

Step 1 Sample Prep on nanoPOTS Chip

1. Single Cell Lysis and Digestion
2. Loading Peptides to Capillary
3. Secure ends with parafilm
4. Store and Transfer at 4° C

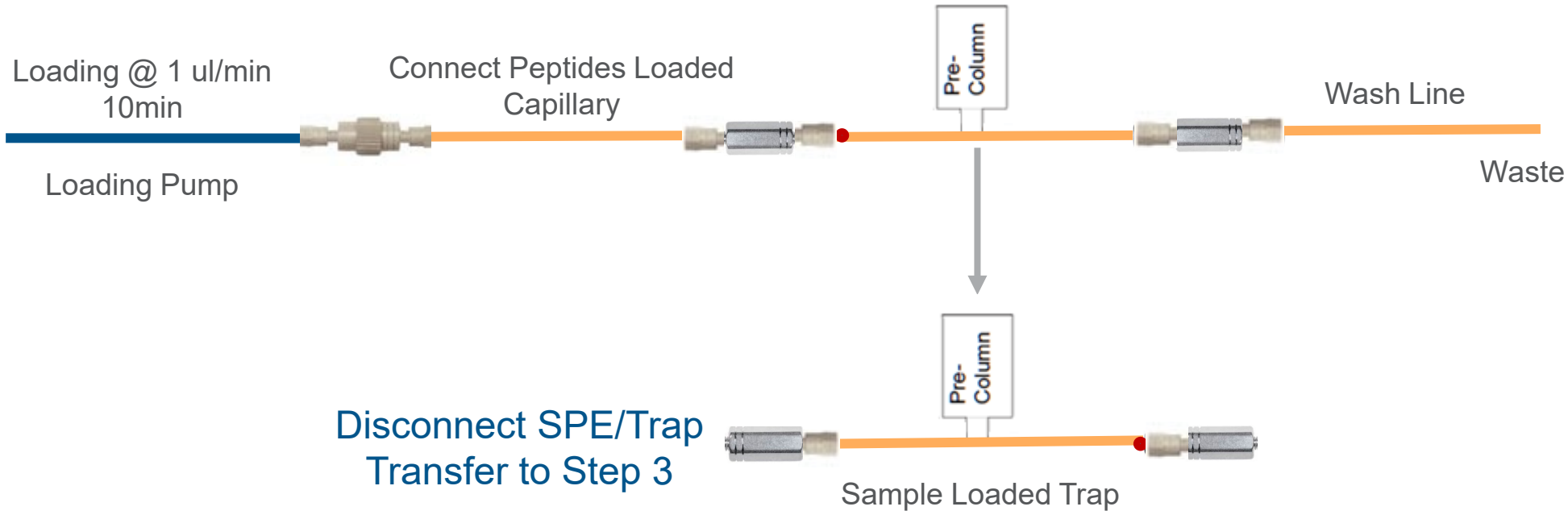




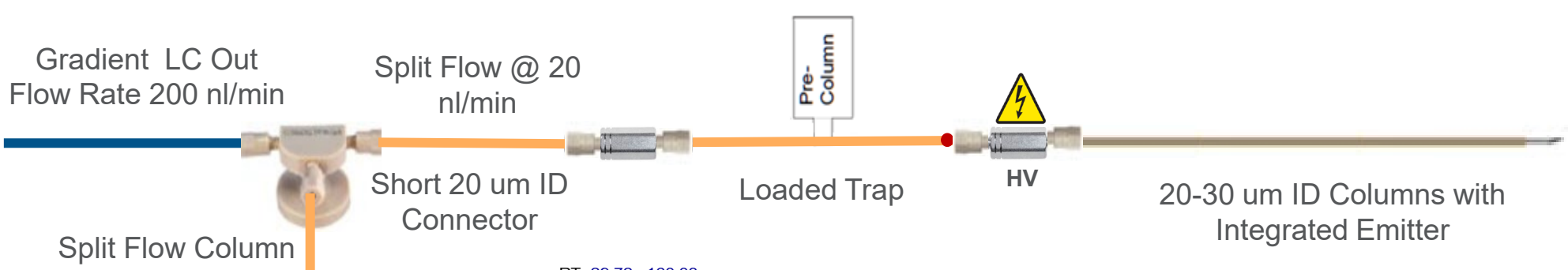
Transfer Peptide Digest into an SPE Column

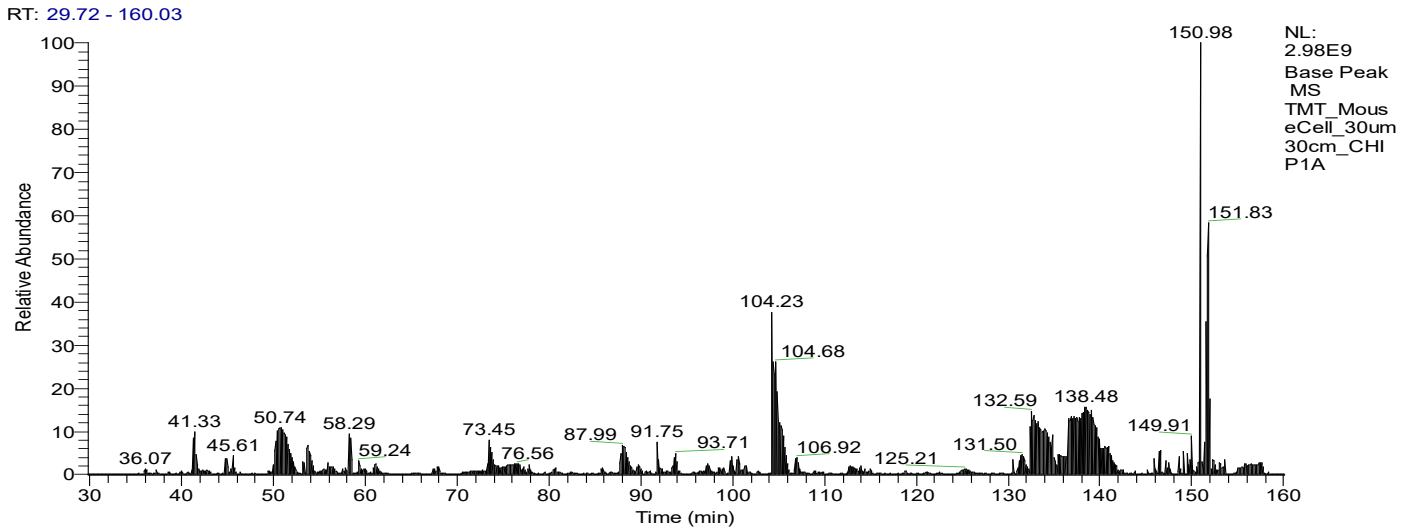
Step 2 Loading Peptides Into the SPE Column

UltiMate™ 3000 RSLCnano system



Step 3 Connect SPE/Trap online and run 160 min LC gradient





Representative Chromatogram

PP73265-EN 0620S