WHITE PAPER 65971

Grant application resource

Go beyond today's discovery with the Orbitrap Eclipse Tribrid mass spectrometer

Keywords: Orbitrap Eclipse Tribrid Mass Spectrometer, FAIMS Pro Interface, Mass Spectrometry, Quantitative Proteomics, TMT and TMTpro Multiplexing, Single-cell Sensitivity, Top-down Proteomics, Native Intact Mass

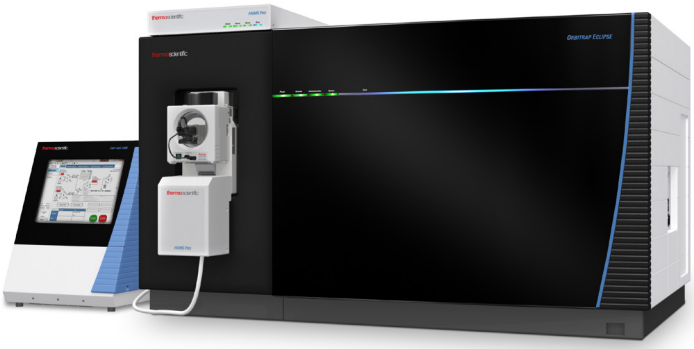

Figure 1. Orbitrap Eclipse Tribrid mass spectrometer with the Thermo Scientific™ EASY-nLC™ 1200 system and Thermo Scientific™ FAIMS Pro™ interface. The system is ready to use with pre-built method templates for the FAIMS Pro interface that simplify method setup.

Summary

The Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer adds hardware and software innovations to the proven Thermo Scientific™ Tribrid™ mass spectrometer design and offers enhanced ion transmission, extended experimental *m/z* range, and real-time decision-making routines. These new functionalities deliver the required sensitivity, selectivity, and versatility that is ideally suited for obtaining comprehensive results from proteomics, structural biology, small molecule, and biopharmaceutical characterization experiments. Proven Thermo Scientific™ Orbitrap™ mass analyzer technology provides the highest quality, intelligently collected high-resolution accurate-mass (HRAM) data needed to meet the demands of your most challenging experiments. The information provided in this document highlights the technologies that set the Orbitrap Eclipse Tribrid mass spectrometer apart from other mass spectrometry instrumentation, specifically outlining:

1. How Real-Time Search enables faster, smarter data acquisition
2. How Orbitrap technology delivers the necessary sensitivity to analyze single cells, and
3. Improvements for the analysis of large native proteins or protein complexes.

What's new

The challenges of biological and chemical research continue to push experimental workflows to their limits. Increasingly advanced workflows are needed to gain a greater depth of information with a higher degree of confidence. These challenges range from pushing sensitivity limits to perform differential proteomics analysis on individual cells, to structural characterization of complex biotherapeutics. To meet these demands, a state-of-the-art mass spectrometer must offer innovative solutions made possible with pioneering technology and software developments. In addition to increasing the proficiency of existing workflows, these advances offer novel approaches to experimental design to solve analytical challenges, today and into the future.

Here we highlight how innovative hardware and software enhancements achieve the unprecedented sensitivity, selectivity, and efficiency of the Orbitrap Eclipse Tribrid mass spectrometer. Figures 1 and 2 show the new features and options that extend the unique capabilities of the Orbitrap Eclipse Tribrid mass spectrometers beyond

previous-generation Tribrid instruments to deliver new levels of experimental flexibility and performance for a wide range of applications. Innovative hardware improvements that provide novel experimental capabilities include:

- Enhanced ion optics and new QR5 segmented quadrupole mass filter improve sensitivity at 0.4 *m/z* precursor isolation and increase transmission at higher precursor isolation resolution
- Ultra-high-field Orbitrap mass analyzer provides up to 40 Hz MS^2 acquisition rates with 7500 resolution at 200 *m/z*
- Lower vacuum in the C-trap and Orbitrap mass analyzer regions improves analysis of intact proteins
- Extended high-pressure cell in the linear ion trap enables up to five fragmentation types and improved kinetic control of ETD and PTCR reactions
- Redesigned Thermo Scientific™ EASY-IC™ HD (Internal Calibration) and Thermo Scientific™ EASY-ETD™ HD (Electron Transfer Dissociation) ion source with Proton Transfer Charge Reduction (PTCR) capability
- Extended mass range with precursor isolation up to *m/z* 8000 enables analysis of protein complexes in their native state
- FAIMS Pro interface option for online gas-phase fractionation prior to MS analysis provides orthogonal selectivity
- Enhanced Orbitrap Tribrid Instrument Control software (ICSW) delivers additional productivity, ease-of-use, and flexibility with application modes and pre-built method templates.

The Orbitrap Eclipse Tribrid Instrument Control Software (ICSW) provides ease of use, and flexibility for operators of all levels of expertise. Instrument and method setup are intuitive and streamlined with ready-to-use, optimized data acquisition templates for common applications including metabolomics, peptide identification and quantitation, post-translational modification (PTM) characterization, and Tandem Mass Tags™ (TMT™) multiplexing. The Method Editor module provides templates that are fully customizable to meet specific application needs. The software

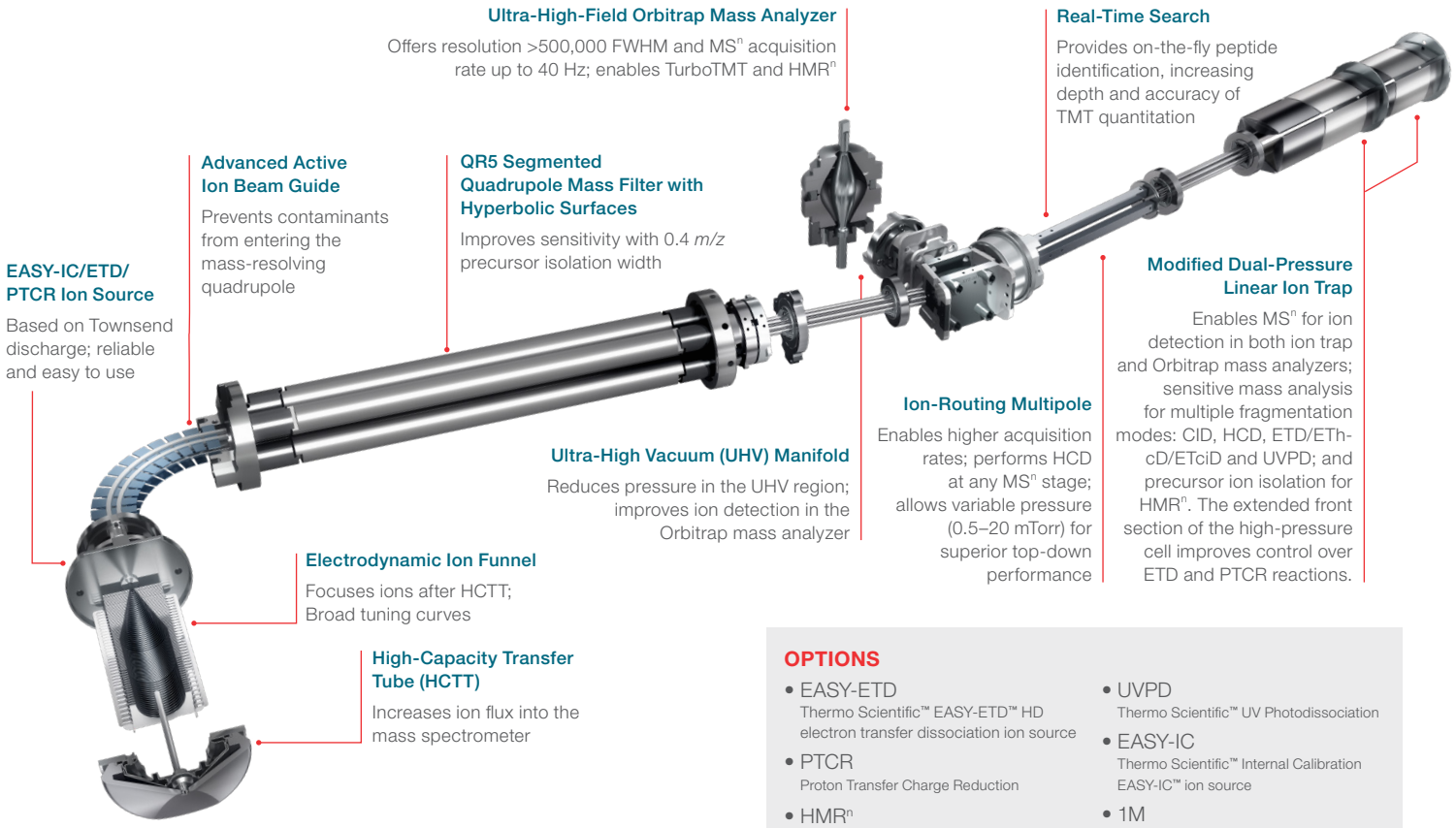

Figure 2. Orbitrap Eclipse Tribrid mass spectrometer schematic showing key hardware innovations. The improvements listed are new to the Orbitrap Eclipse Tribrid spectrometer.

OPTIONS

- EASY-ETD
  Thermo Scientific™ EASY-ETD™ HD electron transfer dissociation ion source
- PTCR
  Proton Transfer Charge Reduction
- HMRⁿ
  High Mass Range MSⁿ to *m/z* 8,000
- FAIMS Pro interface
  Thermo Scientific™ FAIMS Pro™ interface
- UVPD
  Thermo Scientific™ UV Photodissociation
- EASY-IC
  Thermo Scientific™ Internal Calibration EASY-IC™ ion source
- 1M
  Thermo Scientific™ 1,000,000 FWHM

Table 1. Technology, features, and fundamental benefits of the Orbitrap Eclipse Tribrid mass spectrometer.

Technology	Features	Benefits	Applications
QR5 Segmented Quadrupole mass analyzer with hyperbolic surfaces	High precursor ion transmission efficiency using selective quadrupole isolation at 0.4 *m/z*	Improved sensitivity and selectivity	High-sensitivity single-cell qualitative and quantitative analyses using label-free or TMT-based approaches
SPS MS^3 with Real-time search	On-the-fly intelligent acquisition control	Maximum proteome coverage and throughput, while maintaining the most accurate and precise quantitation for TMT experiments	High-throughput protein quantitation multiplexed proteomics analysis using tandem mass tags (TMT)
Proton transfer charge reduction (PTCR)	Simplified precursor and product ion spectra for intact and top-down protein analysis	Separation of overlapping precursor and product ion charge states of multiple proteins, peptides, or product ions improves interpretation of complex spectra and increases the number of identifiable features	Characterization of intact proteins and proteoforms
HMR^n and extended mass range	Improved top-down characterization of proteins and protein complexes under native MS conditions	True tandem MS^n analysis of proteins and protein complexes ionized under native MS conditions, allowing characterization of protein quaternary structure	Top-down structural characterization
Orbitrap Tribrid Instrument Control Software	Application specific modes for peptide, small molecule, or intact protein analysis	Pre-built method templates that are fully customizable and flexible	Optimized methods for workflows including label-free quantitation (LFQ), data-independent acquisition (DIA), TMT, Single Cell, SureQuant, PTMs, and Crosslinking

environment is harmonized and consistent across next-generation Thermo Scientific mass spectrometers, including the Thermo Scientific™ Orbitrap Exploris™ mass spectrometer platform and Thermo Scientific™ TSQ™ triple quadrupole mass spectrometers, streamlining training and operation.

Additional experimental options and capabilities further differentiate the Orbitrap Eclipse Tribrid mass spectrometer from competitive high-end instruments. Table 1 provides a summary of the key technologies, features, and benefits of the instrument, and highlights how these new technologies provide application-specific advancements.

Highlights: Increased application performance for proteomics and native protein analysis applications

Go Faster, Smarter: Next-generation multiplexed proteomics analysis using tandem mass tags for high-throughput protein quantitation

Increasingly, various biological experiments demand higher sample throughput for applications such as thermal shift assays, time and drug dose curves, or large-scale translational cohort studies. Isobaric tagging strategies using TMT are powerful tools for studying how proteins interact and function in biological systems. TMT enables state-of-the-art quantitative proteomic analysis of peptide and protein abundance for up to 16 samples using Thermo Scientific™ TMTpro™ 16plex reagents. Importantly, the multiplexing capability of TMT significantly reduces instrument time and enables extensive fractionation without additional normalization across fractions. The post-digestion chemical labeling with the TMTpro 16plex reagents to be simultaneously analyzed, where quantitation is performed by tandem MS to fragment precursors and measure the intensity of the low-mass TMT reporter product ions. Compared to MS^2 methods for quantitation, SPS MS^3-based methods provide higher quantitative accuracy.[1] The SPS MS^3 instrument method uses parallel MS and MS^2 acquisition in the Orbitrap mass analyzer and linear ion trap (IT) to identify and sequence each peptide and to determine up to ten IT MS^2 product ions, which are subsequently isolated and dissociated to produce a reporter ion population.

A significant advancement provided by the Orbitrap Eclipse Tribrid mass spectrometer is Real-Time Search, which can be used to identify MS^2 peptide spectra in real time and direct SPS MS^3 data acquisition.[2] The primary limitation to the current SPS MS^3 routine is acquiring SPS MS^3 spectra on precursors that do not result in a sequenced peptide. To improve upon the existing SPS methods, Real-Time Search data acquisition has been implemented between the MS^2 and MS^3 scans. The introduction of intelligent on-the-fly MS^2 spectrum searches enables immediate determination of a peptide spectrum match (PSM) based on the user-defined, indexed protein database. Database sequence matching is performed by the open source Comet database search engine, which takes on average less than 5 msec per MS^2 spectrum when searched against a standard protein database with up to three dynamic modifications. Real-Time Search data acquisition is performed in parallel to linear ion trap MS^2 spectral acquisition, providing real-time determination of which precursors to sample for SPS MS^3 experiment. If an MS^2 spectrum is identified in the database, the precursor is selected for subsequent SPS MS^3 acquisition. Only the matched MS^2 fragments that carry the TMT tag are used for the SPS MS^3 scan. This increases the accuracy of TMT quantitation considerably.

The MS^2 spectra that do not result in a PSM are excluded from SPS MS^3 acquisition, substantially decreasing the overall cycle time, allowing the instrument to sample more MS precursors and thereby increase the number of quantified peptides. (Figure 3).

SPS MS^3 with Real-Time Search increases the number of quantified peptides up to 30–40% compared to the standard the SPS MS^3 approach. The experimental results presented in Figure 4 show that using SPS MS^3 with Real-Time Search resulted in 38% more peptides and 53% more proteins identified and accurately quantified compared to the classic SPS MS^3 experiment, and approached the results obtained using the classic MS^2 experiment. Figure 5 shows the two fold increase in throughput provided by SPS MS^3 with Real-Time Search compared to the classic SPS MS^3 method. Despite the 50% decrease in experimental gradient, more peptides were identified and quantified by experiments that incorporated SPS MS^3 with Real-Time Search. To find out more how SPS MS^3 with Real-Time Search improves quantitative accuracy, see the Application Note 65729: Real-Time Search enables a new gold standard for TMT quantitation accuracy on the Orbitrap Eclipse Tribrid mass spectrometer.[3]

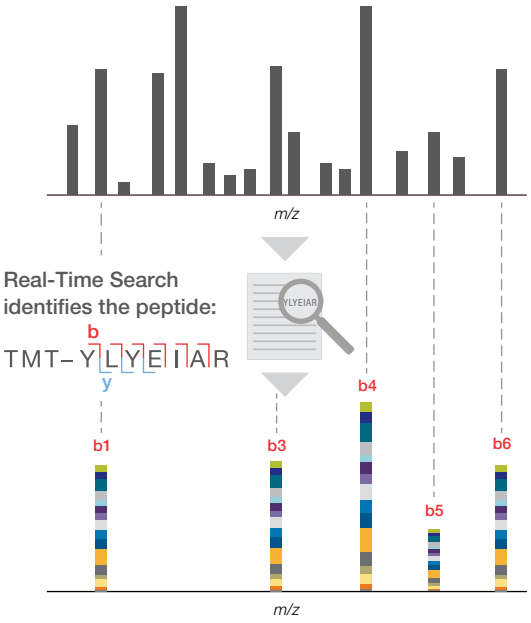

Figure 3. Representation of the SPS MS^3 with Real-Time Search data acquisition scheme for accurate TMT-based quantitation. The standard data-dependent MS^2 acquisition is performed in the linear ion trap. Directly following ion trap MS^2 acquisition, a real-time database search is performed in parallel with the next MS^2 scan event. If the search results in a PSM, the instrument is directed to perform an SPS MS^3 scan using only the matched fragment ions that carry the TMT reporter ions.

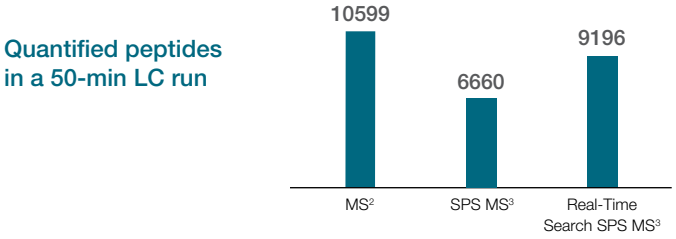

Figure 4. With SPS MS^3 with Real-Time Search 38%, more peptides were quantified versus a classic SPS MS^3 experiment, approaching the results of the classic MS^2 experiment. The bars show the number of peptides quantified per TMT multiplexed quantitation method for a 50-minute LC gradient. The data were acquired using Thermo Scientific™ Pierce™ TMT11plex Yeast Digest standard. All data were processed using the Thermo Scientific™ Proteome Discoverer™ software version 2.4.

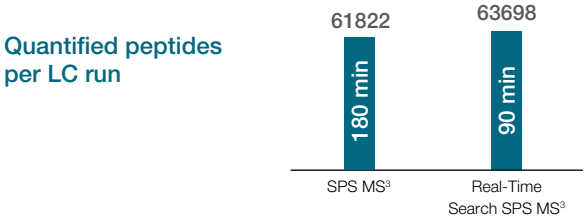

Figure 5. SPS MS^3 with Real-Time Search doubles the throughput compared to classic SPS MS^3 experiments. Number of quantified peptides measured using the classic SPS MS^3 method and the SPS MS^3 with Real-Time Search method for different gradient lengths. The data presented were obtained from an HHM sample: three human cell lines labeled as biological replicates using TMT10plex regents (3-3-4.) (Data courtesy Devin Schweppe and Qing Yu, Harvard Medical School).

Overall, the Orbitrap Eclipse Tribrid mass spectrometer with Real-Time Search data acquisition for TMT SPS MS^3 quantitation uses an intelligent acquisition method that improves quantitation accuracy, precision, and proteome coverage. Real-Time Search data acquisition closes the gap in coverage between MS^2 and the SPS MS^3 approaches, while further increasing the accuracy of TMT quantitation, making it possible to confidently detect small changes in low signal-to-noise ions. TMT quantitation with Real-Time Search data acquisition is a truly unrivaled technique for in-depth quantitation of whole proteomes.

Dig deeper: Unprecedented sensitivity for single-cell analyses using label-free qualitative and quantitative analysis

Proteome analysis with single-cell sensitivity remains challenging due to its unique analytical requirements of sample collection, sample preparation, and of the mass spectrometer itself. While new methods and techniques have been developed to minimize sample loss during collection, preparation, and chromatographic delivery to the mass spectrometer, MS advancements are also needed to increase comprehensive characterization and relative quantitation for single-cell proteomics experiments. The Orbitrap Eclipse Tribrid mass spectrometer was developed to meet the challenges of acquiring and extracting qualitative and quantitative data from ultra-low-level samples. Improvements in ion transmission via the QR5 segmented quadrupole mass filter provide a 15% increase in proteome coverage compared to the Orbitrap Fusion Lumos Tribrid mass spectrometer.[4]

In addition to enhanced transmission capabilities, the Orbitrap Eclipse Tribrid mass spectrometer can be coupled with the FAIMS Pro interface to increase selectivity and sensitivity to extend proteome coverage for analyses of single-cell digests and other limited sample amounts. The FAIMS Pro interface preferentially selects ion populations based on the compensation voltage (CV) applied to the central cylindrical electrode. For single-cell analyses, the interface provides orthogonal selectivity that preferentially transfers 2+ and 3+ precursor ions into the mass spectrometer, while filtering out singly-charged solvent ions. Removing unwanted ions enhances the sensitivity of the analysis. Figure 6 illustrates the orthogonal selectivity obtained by adding differential ion mobility prior to ion accumulation. Due to the low amount of proteins collected and digested from a single HeLa cell, the predominant compounds ionized at most retention times are attributed to singly-charged solvent peaks that limit the dynamic range of peptide detection. Figure 6A shows that the majority of precursors are not selected for DDA due to their charge states. Using the FAIMS Pro interface at optimal compensation voltage (CV) settings significantly reduces singly-charged precursor transmission through the FAIMS Pro interface electrodes, preferentially transmitting the multiply-charged precursors associated with peptides (Figure 6B). The orthogonal selectivity obtained maximizes peptide detection and sequencing, increasing proteome coverage from 551 proteins measured without FAIMS Pro interface, to 829 proteins confidently detected with FAIMS Pro interface.

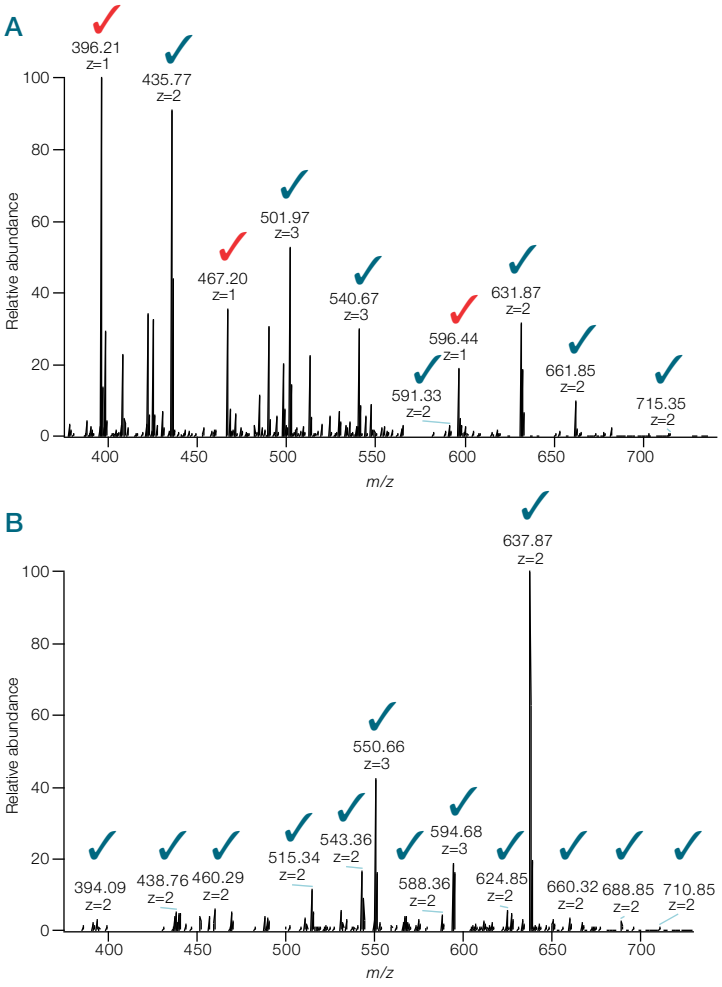

Figure 6. Full-scan HRAM spectra for a single cell HeLa digest analysis acquired A) without and B) with the FAIMS Pro interface coupled to the Orbitrap Eclipse Tribrid mass spectrometer. The precursor ions marked with a green check mark represent peptides analyzed by MS, resulting in identification of a peptide sequence.

To further demonstrate the performance of the instrument for single cell analysis, Figure 7 shows the results obtained for experiments designed to evaluate the optimal combination of precursor and product ion DDA acquisition strategies for greatest proteome coverage. All data were acquired using the FAIMS Pro interface to maximize selectivity prior to dissociation and accumulation. The results for the 0.5 ng HeLa sample show that MS^2 detection in the linear ion trap (OTIT) provided the greatest coverage compared to MS^2 detection in the Orbitrap mass analyzer (OTOT). This is due to greater sensitivity and scan rate achieved in OTIT experiments compared to OTOT. Performing OTIT with HCD fragmentation further increases the MS^2 acquisition frequency because dissociation is performed outside of the linear ion trap, reducing the MS^2 scan event to about 10–15 ms. Cumulatively, HCD OTIT DDA methods maximize acquisition frequency and sensitivity, resulting in greater breadth and depth of proteome coverage. The OTIT HCD method demonstrated unprecedented proteome coverage when applied to the digested single HeLa cell sample (Figure 7). Thus, utilizing the combination of three mass detectors available on the Orbitrap Eclipse Tribrid mass spectrometer provides additional sensitivity for analyzing single cells. Additionally, SPS MS^3 with Real-Time Search, can provide increased single-cell sample throughput and sensitivity with sufficient quantitative accuracy to clearly differentiate cell types and capture the differences between the subtype heterogeneity. Furthermore, with the TMTPro 16plex reagents, up to 14 single cells can be analyzed in one LC-MS run, while also including a positive booster channel and negative control using the two remaining TMT channels. This provides an avenue for quantitative comparison of thousands of proteins among individual cells.

To learn more about how Orbitrap technology delivers the sensitivity to analyze single cells, see White Paper 65730: Challenges and emerging directions in single-cell proteomics[5] and Application Poster 65714: High-throughput single cell proteomics analysis with nanodroplet sample processing, multiplex TMT labeling, and ultra-sensitive LC-MS.[6]

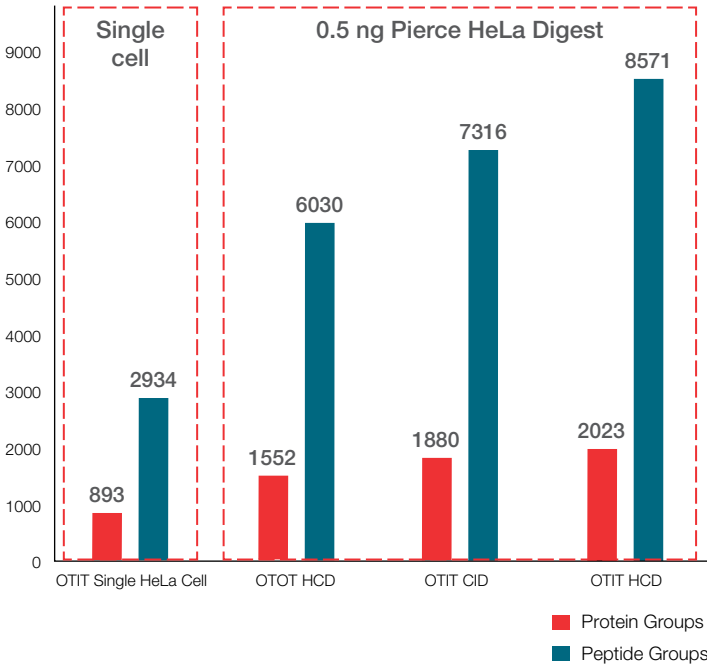

Figure 7. Evaluation of protein and peptide coverage measured using different DDA experimental detection strategies on the Orbitrap Eclipse Tribrid mass spectrometer. The sample was a dilution of HeLa stock to 0.5 ng loaded on column and a digest lysed from a single cell using the NanoPOTS sample preparation protocol.[4] All data were acquired using the FAIMS Pro interface stepped between –50 and –70 V.

Go big: Comprehensive characterization of intact proteins and proteoforms

Preserving the native state of a large molecule during analysis has many benefits, most notably to reduce sample-preparation-related artifacts, preserve non-covalent interactions, and to obtain analytical information about the molecule in as close to its biological state as possible. With an efficient ion source, enhanced QR5 quadrupole mass filter with increased transmission efficiency, ability to modulate the neutral gas pressure in the Ion Routing Multiple (IRM), enhanced vacuum technology to maintain high vacuum in the C-trap and Orbitrap mass analyzer region, and high-mass range detection with precursor isolation capabilities up to *m/z* 8000, the Orbitrap Eclipse Tribrid mass spectrometer provides the versatility and compatibility for in-depth native MS protein characterization.

Intact protein characterization is critical to determining the expressed sequence and PTMs in a sample. However, the presence of proteoforms presents significant challenges to detecting, measuring, and sequencing each proteoform because of their spectral complexity. In particular, simultaneous MS analysis of proteoforms can result in significant charge-state overlap, making it difficult to

differentiate between each proteoform. With enhanced vacuum technology and the PTCR option[7], the Orbitrap Eclipse Tribrid mass spectrometer addresses these challenges by increasing the spatial resolution of MS and MS^2 spectra to facilitate deconvolution, enabling differentiation and characterization of even the most heterogeneous biopharmaceutical molecules. A narrow precursor mass range is isolated either by the QR5 quadrupole mass filter or the linear ion trap, transferred to the high-pressure cell of the linear ion trap, and then allowed to react with the singly charged anion, resulting in a charge-reduced cation and a neutral. Depending on the reaction time, the charge-reduced product ion can undergo further ion-ion reactions that produce a series of lower charge state product ions. PTCR is extremely beneficial for co-isolated multiply charged cations, because all isolated precursors undergo ion-ion reactions. The reduction in charge states increases *m/z* separation and reduces spectral density for improved molecular weight determination using deconvolution algorithms such as those in Thermo Scientific™ BioPharma Finder™ software.

Figure 8 demonstrates how PTCR facilitates deconvolution of a complex spectrum obtained from a native MS analysis of a de-sialylated Cytokine-Fc fusion protein. The protein has three N-linked glycosylation sites per arm (shown in red) that significantly contribute to its heterogeneity. Shown at the top of Figure 8, the MS spectrum was too complex to permit the deconvolution software to determine the intact molecular weights of the proteoforms. However, native MS with high-mass precursor isolation and PTCR produced an MS^2 spectrum with reduced complexity, enabling accurate software-based deconvolution. The Orbitrap Eclipse Tribrid mass spectrometer is unique in that it can automatically acquire a series of MS^2 events stepped across 80-Da ranges, enabling automated identification of many proteoforms in a single experimental run.

Additionally, the Orbitrap Eclipse Tribrid mass spectrometer offers multiple fragmentation modes that can be performed at any MS^n stage, including HCD, CID, ETD, ETciD, EThcD, and UVPD (Thermo Scientific™ UV Photodissociation). The combination of these fragmentation methods generates complementary product ions that significantly enhance characterization of intact proteins and proteoforms.

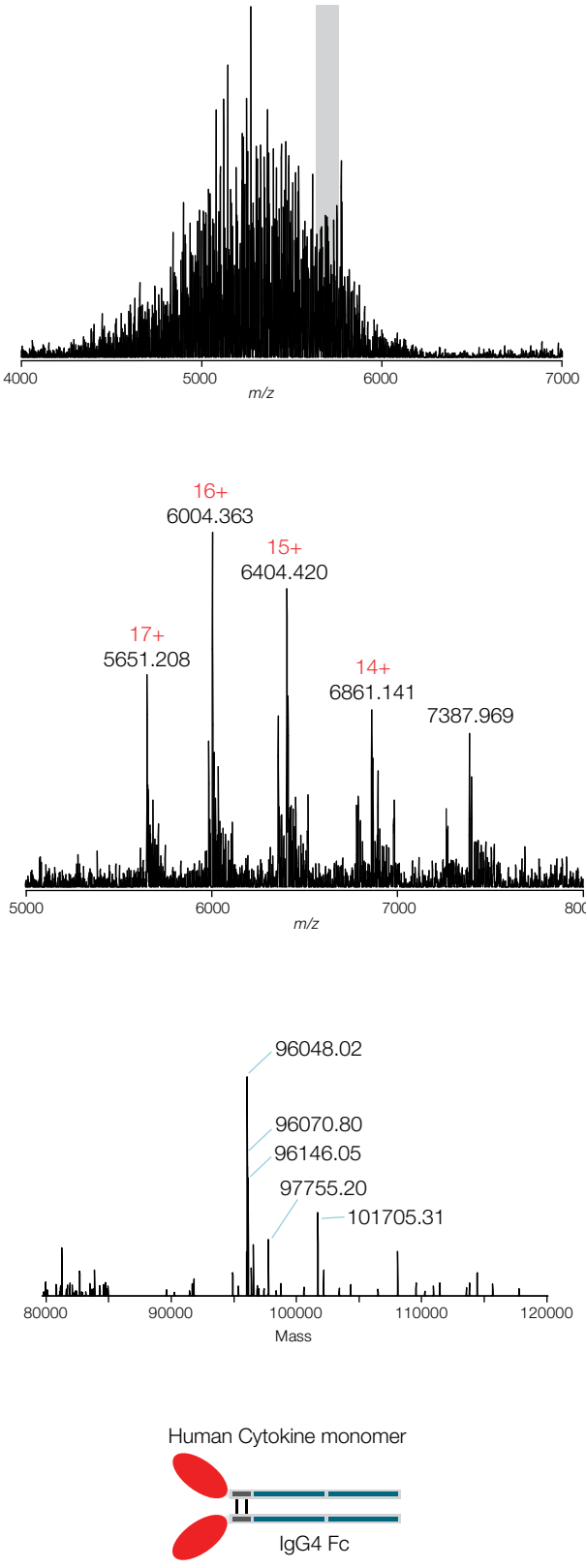

Figure 8. PTCR made it possible to deconvolute the spectrum of a de-sialylated Cytokine-Fc fusion protein from a native MS analysis. A narrow precursor isolation window of (80 Da) was applied in the linear ion trap and the isolated precursors were reacted with the $C_{14}F_{24}$ anion for 4 milliseconds. The resulting charge-reduced product ions were detected in the Orbitrap mass analyzer and deconvoluted post-acquisition.

FAIMS Pro interface enhances protein subunit characterization

For the analysis of protein subunits, combining the FAIMS Pro interface with the Orbitrap Eclipse Tribrid mass spectrometer increases selectivity prior to MS analysis for improved intact mass analysis and top- and middle-down sequencing. By alternating CVs, protein subunits such as the light and heavy chains of an antibody can be analyzed either simultaneously or separated in the gas phase and introduced sequentially into the mass spectrometer.[8] Figure 9 demonstrates rapid characterization of the mAb chains of a NIST mAb standard using four different stepped CVs in full-scan mode. The NIST mAb standard was reduced and alkylated prior to being directly infused into the Orbitrap Eclipse Tribrid mass spectrometer. The selectivity provided by the different CVs effectively separated the two chains. Immediately following precursor isolation of either a wide window or a single charge state, tandem MS can be performed to determine light and heavy chain sequence in minutes. Using this approach, the FAIMS Pro interface can reduce the need for an LC system, or the resulting sample analysis can be automated using loop injection with the LC autosampler.

To find out more information on the intact protein analysis of antibodies and biotherapeutics, see the detailed White Paper 65653: Go beyond in biopharmaceutical characterization.[9]

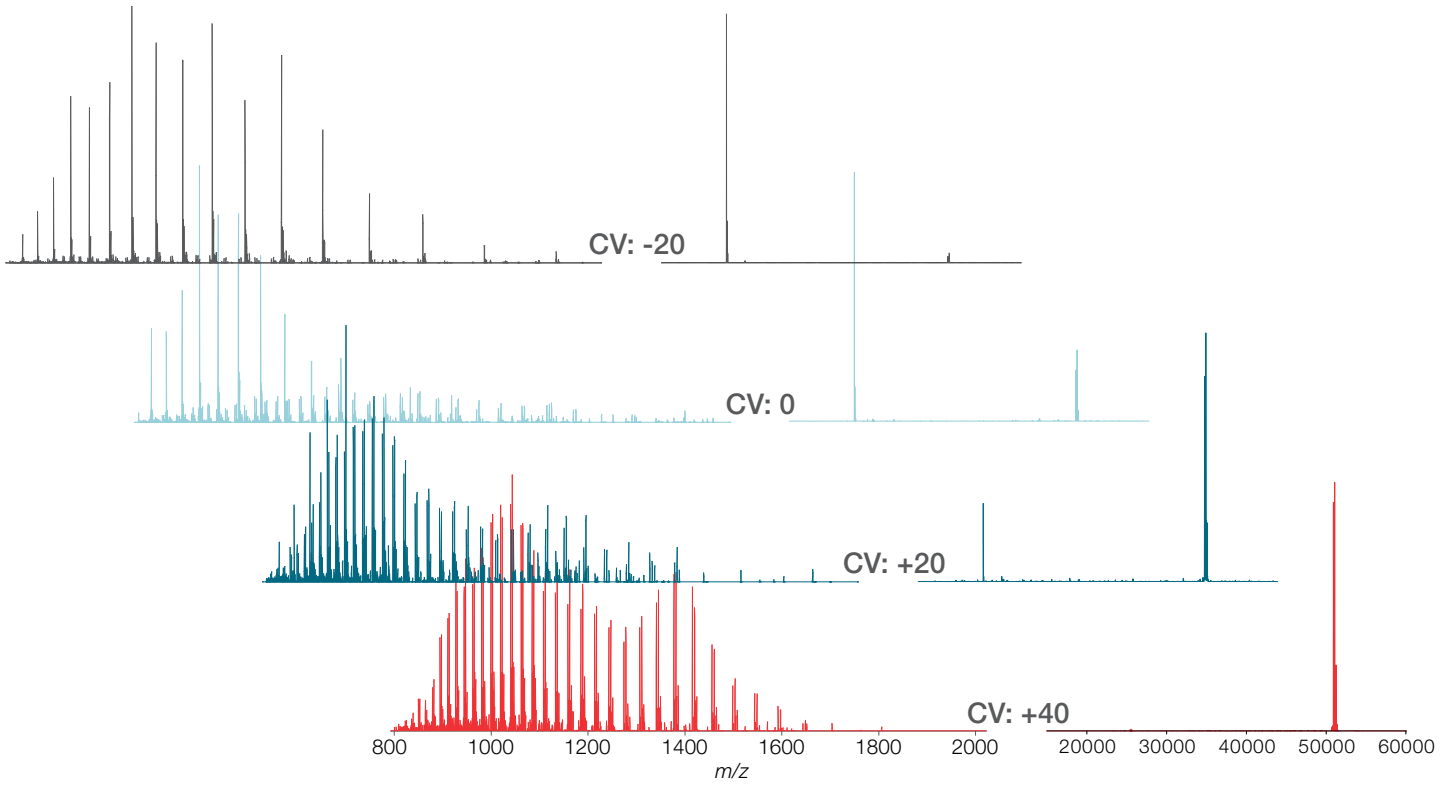

Figure 9. Rapid selectivity is obtained when using four FAIMS Pro interface CVs for analysis of an NIST mAb standard.

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Conclusion

The Orbitrap Eclipse Tribrid mass spectrometer is the newest generation Tribrid instrument designed to address your most difficult analytical challenges, expanding your science beyond today's discovery. As the next-generation Orbitrap Tribrid mass spectrometer, the instrument offers many advantages and is technologically distinct from other mass spectrometry instrumentation. The latest innovations in ion transmission and control, extended *m/z* range, real-time decision making, and fragmentation modes provide ground-breaking gains in sensitivity, selectivity, and versatility. The Orbitrap Eclipse Tribrid mass spectrometer is ideally suited to expanding the breadth of proteomics, structural biology, and protein characterization experiments possible today.

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