Enhanced resolution with multi-pass mode on the Orbitrap Astral Zoom mass spectrometer

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Introduction

The introduction of Thermo Scientific™ TMT™ 32-plex, with reporter ions clustered into quadruplets spaced by 3 mDa, demands a resolving power of approximately 90 k at low *m/z*, which challenges state-of-the-art time-of-flight mass analyzers. The Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer [1] typically achieves around 60 k resolution at m/z = 138, falling to 50 k under high space charge influence.

Here we present an enhanced resolution multi-pass method implemented in the new Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer [2], significantly improving the resolving power at low m/z enabling TMT 32-plex on the Thermo Scientific™ Astral™ analyzer. The ions are repeatedly guided through a switchable prism deflector to extend the ion path while the principal aberrations are compensated, thereby more than doubling the resolution.

The Orbitrap Astral Zoom Mass Spectrometer

The Orbitrap Astral Zoom mass spectrometer incorporates a range of novel technologies for higher speed, sensitivity, dynamic range and resolving power. A faster ion filter and quadrupole switching in combination with improved ion transfer times in the ion-routing multipole (IRM) and ion processor allow DIA methods (Thermo Scientific™ Pierce™ FlexMix™ calibration solution, 2 Th isolation window, 350-980 *m/z* range, Thermo Scientific™ Orbitrap™ MS and Astral MS/MS) with scan rates faster than 270 Hz (DDA methods > 180 Hz). A duty cycle of up to 80 % at 270 Hz is made possible by a new accumulation stage in the bent trap (linear to 10^6 ions) which increases the effective ion injection into the IRM by up to 40 % (additional ~0.75 ms on top of 2 ms IT in a DIA method). Furthermore, an additional mode of the high-dynamic range detector of the Astral analyzer for low-input sample applications like single-cell proteomics boosts the single ion detection efficiency by about 10 % and thereby the sensitivity. In combination with a new peak deconvolution algorithm for enhanced spectral processing, this increases the depth of analysis. In addition, modified IRM hardware allows for faster stepped collision energy experiments for deeper spectral coverage (2 CE: 80 Hz (OT Astral) → 180 Hz (Orbitrap Astral Zoom MS), 3 CE: 60 Hz → 150 Hz). A modified AGC filling in the Orbitrap by a new enhanced dynamic range mode (EDR) provides additional spectral complexity and sensitivity. Here, multiplexed ion injections with different accumulation times are used for different mass ranges taking the variation in signal intensity into account. In addition, the Astral analyzer incorporates a new multi-pass mode which enhances the resolving power for small masses by more than a factor of two, enabling TMT 32-plex measurement in the Astral analyzer.

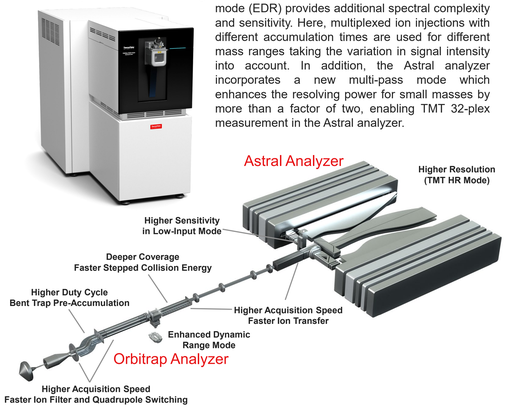

Figure 1: Overview of the new features of the Orbitrap Astral Zoom mass spectrometer.

Methods

In the Orbitrap Astral Zoom mass spectrometer, ions can be trapped longer within the Astral analyzer to increase the length of the ion path thereby enhancing the instrument's resolving power.

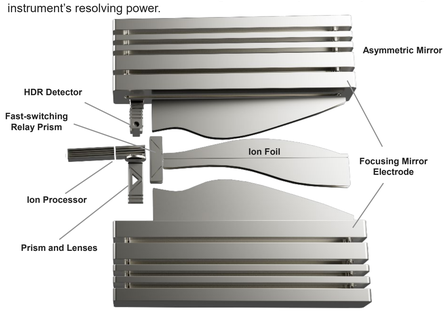

Figure 2: Ion-optical components of the Orbitrap Astral Zoom mass spectrometer: A fast-switching relay prism deflector allows the ions to be trapped longer before detection to extend the ion path. The ion focus is controlled by adjusting the voltage on the new focusing mirror electrode.

Fast relay prism for trapping and detection

A fast relay prism deflector voltage supply was implemented, enabling rapid switching between ion trapping and detection modes. After ion injection from the ion processor into the Astral analyzer (Figure 3a), the ions pass through the prism deflector and oscillate between the asymmetric mirrors. The relay prism is switched from approx. -160 V to about 250 V so that the ions are trapped in the analyzer (Figure 3b) and perform additional drift passes, before the voltage is switched back to guide the ions to the HDR detector, therefore restricting the m/z range. For three drift passes, the resolution is more than doubled as defocusing aberrations resulting from ion passage through the prism deflector are compensated.

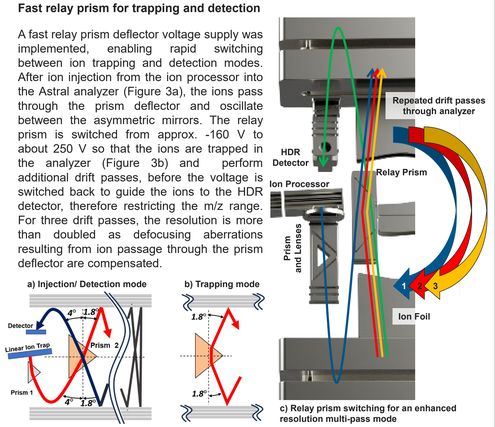

Figure 3: A fast relay prism deflector controls the ion drift through the Astral analyzer: The ions are ejected from the ion processor and guided to the relay prism which determines the injection angle into the Astral analyzer (a). The voltage of the relay prism is switched after the ions have passed it for the first time, which traps the ions inside the analyzer (b). After a certain delay time dependent on the selected mass range, the relay prism is switched back to guide the ions after three ion drift passes towards the HDR detector (c).

Automatic tuning of the TMT HR Mode

The relevant analyzer voltages of the TMT HR mode depend on each other and thus span a multi-dimensional parameter space. An automatic tuning routine was implemented where a genetic search algorithm is utilized to first maximize the transmission and suppress overtones - low-intensity artifacts caused by ions travelling at a different number of oscillations than the principal peak - by finding the optimal ion foil and relay prism voltages (injection and trapping mode) as depicted in Figure 4a. In a second step, a linear scan of the focusing mirror electrode voltage optimizes the resolution over a range of ion targets (Figure 4b).

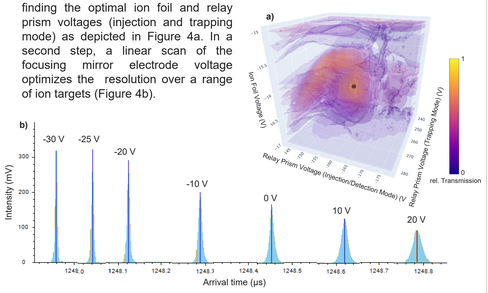

Figure 4: a) 3-dimensional representation of the relative ion transmission. A genetic algorithm is used to find the optimal analyzer voltages. b) The temporal focus of an ion bunch is shifted to the detector plane by applying a voltage to the first electrode of the ion mirrors.

Characterization of the TMT HR Mode

The ion path is enlarged to approximately 90 m in the multi-pass mode compared to 30 m on the standard Astral analyzer, which enables maximum resolutions well above 120 k at low *m/z* for TMT reporter ions as shown in Figure 5. The average resolution allows for a baseline resolved TMT 32-plex quadruplet for a broad range of ion targets. The relative transmission with respect to the single-pass mode is typically 35 % and the mass accuracy and shot-to-shot jitter are comparable in both modes.

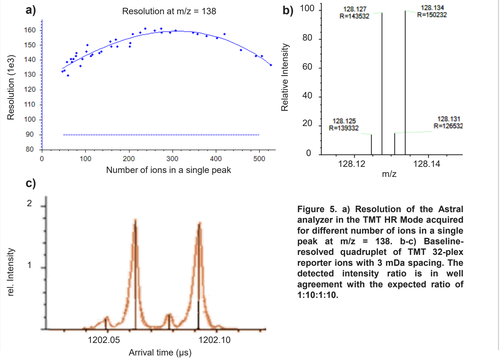

Figure 5. a) Resolution of the Astral analyzer in the TMT HR Mode acquired for different number of ions in a single peak at m/z = 138. b-c) Baseline-resolved quadruplet of TMT 32-plex reporter ions with 3 mDa spacing. The detected intensity ratio is in well agreement with the expected ratio of 1:10:1:10.

Results - TMT 32-plex method results

Shot-to-Shot switching between single- and multi-pass mode

In a TMT HR Mode method, Orbitrap survey scans are combined with panoramic Astral MS2 scans for peptide identification in the single-pass mode followed by separate enhanced resolution scans for reporter ion quantification with a smaller *m/z* window in the multi-pass mode. The setup was configured to enable fast shot-to-shot switching between the single-pass and 3-pass modes, making the scanning speed primarily limited by the selected injection time.

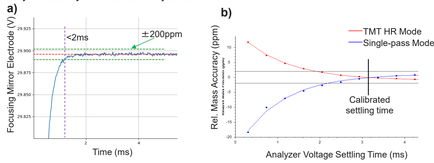

Figure 6: a) Switching time check on test bench: The focusing mirror electrode voltage settles within ~1.7 ms for a voltage change of 0 V to 30 V. Not shown: Relay prism settles within ~500 µs. b) Time-dependent mass accuracy for shot-to-shot switching between single-pass and multi-pass mode.

Quantification results of TMT 32-plex reporter ions

A TMT 32-plex labeled HeLa digest sample with 1:4 ratio was investigated with an optimized method to analyze the quantification in the TMT HR Mode (78 minutes separation gradient with an Ionopticks Aurora Frontier™ XT (75 µm x 60 cm) column coupled to a Thermo Scientific™ Vanquish™ Neo LC configured in direct injection; details can be found in reference [3]). The detailed sample composition is shown in Figure 7. The scaled protein group abundance of all 32 reporter ion channels is extracted as shown in Figure 8. In general, the median detected abundances across all channels closely align with the expected ratios (dashed lines). In total, 4,299 protein groups and 22,146 peptide groups have been quantified which amounts to 94 % and 78 % of all identified protein groups and peptides.

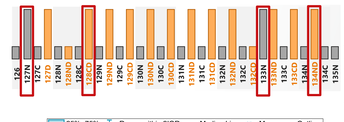

Figure 7. Sample design of a TMT 32-plex labeled HeLa digest sample (expected ratio: 1:4). For normalization to accommodate for the retention time shift of the deuterated (D) vs. the non-deuterated (non-D) TMT sets, 4 different channels were used (red squares) that carried a 4x amount of HeLa in bot D and non-D channels.

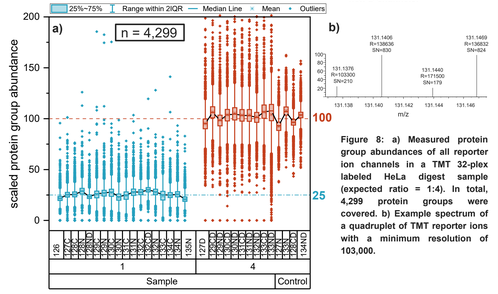

Figure 8: a) Measured protein group abundances of all reporter ion channels in a TMT 32-plex labeled HeLa digest sample (expected ratio = 1:4). In total, 4,299 protein groups were covered. b) Example spectrum of a quadruplet of TMT reporter ions with a minimum resolution of 103,000.

Comparison of TMT 11-plex and 32-plex

Peptides from 500 ng Thermo Scientific™ Pierce™ TMT11plex™ labeled yeast and 1 µg 32-plex labelled HeLa cell digest with 1:4 channel ratio were compared by applying 78-minute separation gradients and analyzed using standard and TMT HR modes (Aurora Frontier XT (75 µm x 60 cm) column). Figure 9 shows that the TMT HR mode is fully compatible with 11-plex and 32-plex samples and even beneficial for quantification.

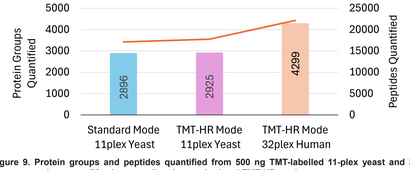

Figure 9. Protein groups and peptides quantified from 500 ng TMT-labelled 11-plex yeast and 32-plex human samples over a 78-minute gradient in standard and TMT-HR modes.

Conclusions

The TMT HR Mode advances the capabilities of the Astral analyzer. Fast electronic switching of the relay prism opens an ion trap and guides the ions after three times their normal flight distance to the detector. This greatly boosts the resolving power, especially for small m/z ratios, thereby enabling baseline-resolved quadruplets of reporter ions in a TMT 32-plex sample. First results show a peak resolution well above 100 k while maintaining high mass accuracy and shot-to-shot stability. The TMT HR Mode is utilized within a novel acquisition scheme delivering greater throughput for protein identification and quantitation for multiplexing up to 32 samples. Achieving a throughput of over 440 samples per day, the proteome coverage included 4,299 quantified protein groups and 22,146 peptides.

References

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2. H. Stewart et al., Proof of principle for enhanced resolution multi-pass methods for the Astral analyzer, Int. J. Mass Spectrom., 498, 2024, 117203.
3. Julia Kraegenbring et al., ASMS 2025, Contribution MP 748: Enhancing the resolving power on a novel Orbitrap Astral instrument for TMT35plex applications.

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Conflict of Interest

All authors are employees of Thermo Fisher Scientific, the manufacturer of instrumentation used in this study.

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