Super-resolution mass spectrometry enables rapid, accurate, and highly-multiplexed proteomics at the MS2-level

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Abstract The complementary reporter ion approaches overcome the ratio-compression problem of multiplexed proteomics and enhance the accuracy of protein quantification at the MS2 level. However, resolving the high *m/z* complementary ions encoded via mass defects in carbon and nitrogen (~6.32 mDa mass difference in TMT/TMTpro) requires mass resolution and scan speeds above the performance levels offered by even the state-of-the-art Orbitrap™ instruments. Therefore, complement ion quantification (TMTc/TMTproC) is currently limited to only 5 (TMT) or 9 (TMTpro) channels (~1 Da spaced) versus 11 or 18, respectively, when analyzing the conventional low *m/z* reporter ions. Here, we first demonstrate the feasibility of the highly-plexed complementary ion quantification in the high *m/z* range by enabling ultra-high-resolution (UHR) capabilities on a commercial Orbitrap mass spectrometer. The UHR performance required 3 s time-domain transients, which were acquired and processed with the high-performance data acquisition-processing system FTMS Booster X2 externally interfaced with the Orbitrap Fusion™ Lumos™ instrument. Despite validating the TMTc approach for the whole mass range, the UHR capability is incompatible with the practical data acquisition times (scan speeds) for liquid chromatography (LC)-based proteomics. We thus implemented a super-resolution mass spectrometry approach based on the least-squares fitting (LSF) that resolves even the 6.32 mDa doublets for all TMTproC channels in the whole mass range with time-domain transients as short as ~108 ms (resolution setting of 50 000 at *m/z* 200). A more demanding, quantitatively accurate TMTproC performance is provided at the resolution setting of 120 000 at *m/z* 200 (256

ms time-domain transients). This advance enables accurate, LC timescale-compatible, and highly-multiplexed proteomics at the MS2 level.

Introduction

Multiplexed quantitative proteomics relies on isobaric (same total mass) tags covalently attached to peptides of proteomics samples[1, 2] and may simultaneously probe up to 18 sample-specific biological conditions.[3] The analytical benefits include the inherently high measurement precision, omission of missing values, and reduced experimental time and analysis cost per proteome. The corresponding applications have been exploited for basic and applied biological research, including translation regulation,[4] embryology,[5] breast cancer treatment,[6] and lung cancer metastasis.[7]

The quantitative proteomics analysis of peptides labeled with isobaric tags is realized using tandem mass spectrometry (MS/MS). These tags consist of reporter and normalization parts that uniquely encode several quantitation channels with different numbers of heavy isotopes – ^{13}C, ^{15}N, and ^{18}O[2, 8]. The reporter parts are detected as singly charged reporter ions in the low, *m/z* 120-140, mass range. The abundances of the reporter ions quantify associated proteins in each proteome. The reporter ion cluster contains channels separated by ~1 Da from other channels and doublets containing two channels encoded by mass defects of ^{13}C vs. ^{14}N that differ only by ~6.32 mDa[9]. To baseline resolve the ~6.32 mDa doublets in this (low *m/z*) mass range, a resolution of about 60 000 is required. This level of resolution performance is readily provided by a resolution setting of 50 000 at *m/z* 200 on Orbitrap™ Fourier transform mass spectrometers (FTMS).

Despite its attractiveness, when applied to complex samples, multiplexed proteomics suffers from measurement artifacts due to co-isolation and co-fragmentation of co-eluting peptides. The MS3 (MS/MS/MS) level approaches have been developed to address this limitation.[10] Adding one more MS/MS step mitigate the peptide co-isolation issue but reduce sensitivity and throughput.

An alternative approach to tackle peptide co-isolation and the associated reporter ion ratio-compression problem is MS2-level complement reporter ion quantification.[11, 12, 13] Following this method, the species to be monitored are the complementary reporter ions – the remainders of the precursor ions labeled with the normalization parts and the reactive groups. For TMTpro/TMT the complementary ion clusters will have one charge less than the isolated precursor ions and thus will be detected in an even higher mass range, up to 2 000 *m/z*, and, sometimes, beyond that. The Orbitrap mass analyzer baseline resolves the complementary ion channels that differ by ~1 Da at the proteomics-grade scan rate. However, this reduces the number of encodable channels from 11 to 5 when using TMT and from 18 to 9 when using TMTpro.[12, 13]

Adding complementary reporting channels (TMTc/TMTproC clusters) involving the ~6.32 mDa doublets would require resolution performance not attainable even with the state-of-the-art Orbitraps. For example, to baseline resolve the 6.32 mDa doublets of the complementary reporter ions, a resolution exceeding 300 000 at *m/z* 2 000 is required. However, this capability is not available for a general Orbitrap user. On the other hand, user access to the extended duration time-domain transients can be readily

provided using the external high-performance data acquisition (DAQ) systems.[14] However, even if available, such long time-domain transients would drastically reduce the instrument scanning speed and are thus prohibitive for conventional high-throughput proteomics applications. In addition, extended periods of ion oscillation in the mass analyzer may result in the loss of ion motion coherence manifesting itself through pronounced transient decay and frequency shifts.[15] Finally, the FT signal processing methods, being the *spectral* estimators (they yield profile-type data with the characteristic peak shapes), are prone to peak interference.[16]

The super-resolution (SR) algorithms for time-domain transient data processing were previously introduced for FTMS to overcome the abovementioned limitations.[17] Compared to the FT-based approaches, the SR methods are characterized by a less strict uncertainty principle and may provide the same resolution as the FT methods using multiple-fold shorter time-domain transients.[16] Previously, we implemented the least-squares fitting (LSF) of the time-domain transients as a powerful SR approach for FTMS.[18] By definition, LSF is a targeted method that requires information on the frequency of interest (*m/z* value) as an *a priori* knowledge. The latter matches the case of the multiplexed quantitative proteomics for both reporter and complementary ion detection. The output of the LSF processing is the information on the intensities of ion signals in the time-domain transient (and, thus, peak abundances in mass spectra). Furthermore, LSF, fundamentally a parameter-estimator method (no peak shape, centroid-type data), avoids the peak interference problem of the FT-based methods. The latter may improve the accuracy of quantitation, particularly in the TMTc/TMTproC doublets

analysis. Here, we combined the LSF capabilities to resolve the nearly isobaric ion signals using shorter time-domain transients with the highly-plexed TMTc/TMTproC workflow's ability to overcome the inherent MS2-level TMT strategy limitations.

Methods

Sample preparation. Cell lysates were prepared as described previously[12, 19] and labelled with selected TMTpro reagents to yield 12 complement reporter ion channels with four 6.32 mDa doublets or with selected TMT reagents (4 channels with a single 6.32 mDa doublet).

Mass spectrometry. LC-MS/MS experiments were conducted on two similar Orbitrap™ Fusion™ Lumos™ FTMS instruments (Thermo Fisher Scientific, San Jose, CA, USA). Time-domain signals of various standard and extended durations, up to 3 seconds, were acquired in parallel to RAW files using an external high-performance DAQ system (FTMS Booster X2, Spectroswiss, Lausanne, Switzerland). The external DAQ system received analogue ion signals after the built-in pre-amplifier by means of a T-junction and a very high input resistance (Figure S1, Supporting Information). The tandem (MS2) spectra were analysed with SEQUEST to yield peptide identification.

To increase resolution beyond conventional FTMS, longer time-domain transients were acquired. To acquire longer transients, we introduced "dummy" scans into the experimental logic without any hardware or operational software modifications (Figure S2, Supporting Information). As a result, we were able to acquire as long as 6 s time-domain transients. The

acquired transients are in-hardware phased – meaning they have similar initial phases for ions of all *m/z* values trapped in the mass analyser.

Data processing. Super-resolution processing, absorption-mode FT, and calculations of reference *m/z* values for TMTproC ions were performed using Peak-by-Peak (Spectroswiss) running on 8-core desktop computer(s) with 32 GB RAM and graphics-card data processing capabilities. The super-resolution data processing was performed using the LSF approach similarly to the previously described fundamentals and implementation. The basis functions of the LSF method correspond to the TMTproC cluster and compose of the individual channels (~1 Da spacing) and doublets (~6.32 mDa spacing), Figure S3, Supporting Information.

The outcome of the Peak-by-Peak data processing was extracted in the form of the mzXML files and uploaded to the GFY software licensed from Harvard for further analysis[20]. Assignment of MS2 spectra was performed using the SEQUEST algorithm[21] by searching the data against the Uniprot *Saccharomyces cerevisiae* proteome. A peptide-level MS2 spectral assignment false discovery rate of 1% was obtained by applying the target decoy strategy with linear discriminant analysis as described previously[20]. Peptides were only considered quantified if the average signal to FT noise ratio (S:N) of the complementary ions was greater than 20 (240 total S:N). Pipetting errors were removed by dividing the signal in each complementary ion channel by the median S:N ratio of that channel across all peptides. For the singlets, peptide CVs were calculated by taking the population standard deviation of the S:N ratios of the four channels for each peptide and dividing by the mean

complementary ion S:N. Doublet CVs were calculated identically on four randomly selected channels for each peptide.

Results

TMTc/TMTproC method validation for Orbitrap FTMS. Practical usage of the highly-plexed TMTc and TMTproC workflows with Orbitraps requires to achieve UHR performance in a wide mass range. According to the Orbitrap fundamentals, resolution reduces as a square root of *m/z*. In addition, higher charge states impose stricter requirements for resolving isotopic envelopes. **Figure 1** schematically shows an Orbitrap's ability to baseline resolve a 6.32 mDa doublet as a function of the doublet's *m/z* and charge state. The singly charged (doubly charged precursor) TMTc/TMTproC clusters detected at *m/z* 2000 are shown to require at least 3.7 s time-domain transient to resolve them. The same length time-domain transients will baseline resolve the doubly charged (triple charged precursor) TMTc/TMTproC clusters only up to about *m/z* 1400.

The analysis of the UHR mass spectra is thus important to reveal the structure of the TMTc/TMTproC clusters for high mass range and validate the method applicability. The latter involves considerations for the space-charge induced coalescence between the quantitation channels in the doublets. However, a capability to acquire the UHR mass spectra produced by FT of 3.7 s time-domain transients cannot be accessed by a general Orbitrap user. On the other hand, recent innovations in high-performance data acquisition electronics and allied digital signal processing have enabled acquisition and processing of extended-period time-domain transients from Orbitraps. It

should be noted that the experimental settings and manufacturing quality of a given Orbitrap platform may influence the parameters of the acquired time-domain transients and thus the practical ability to reach the UHR performance with accurate estimates of ion signal intensities and frequencies. The critical quality attributes of time-domain transients include the linearity of ion signals' phase function and space-charge influenced transient decay and frequency shifts for long ion detection events.

We first enabled the UHR Orbitrap FTMS operation through the use of the external high-performance DAQ system (FTMS Booster X2). We then employed this set-up to analyze the isotopically-labeled samples that had 4 TMTc channels with a single 6.32 mDa doublet, **Figure 2**. Acquisition and processing of the 3 s time-domain transients in the absorption mode FT (aFT) showed that an unmodified ultra-high-field D20 Orbitrap mass analyzer (as employed in Orbitrap™ Fusion™ Lumos™) is able to resolve the 6.32 mDa-spaced TMTc peaks in the required *m/z*-range up to 2000 *m/z*. The maximum manufacturer-supported resolution of this particular Orbitrap model provides a close to the baseline resolution of the TMTc clusters up to *m/z* 900, Figure S4, Supporting Information. Increasing the *m/z* results in the overlap of the TMTc doublet peaks, which become unresolved after about *m/z* 1200, Figure S5, Supporting Information. These results confirm the fundamental ability and the experimental feasibility of Orbitrap FTMS to perform the highly-plexed TMTc approach in a wide mass range. Notably, the obtained performance significantly exceeds the one offered by the standard experimental settings (maximum 1 s time-domain transient period) and advanced settings (maximum 2 s time-domain transient period) available for the modern Fusion

Tribrid platforms. Nevertheless, in addition to the low throughput of UHR Orbitrap experiments, the measured relative abundances exhibited relatively high coefficients of variation (CV) for both 6.32 mDa and 1 Da spacing, demonstrating that some detrimental effects start to develop over the very long ion detection times, Figure S6, Supporting Information.

Therefore, enabling the highly-plexed TMTc/TMTproC approach for general quantitative proteomics applications necessarily needs to be performed by processing the (significantly) shorter time-domain transients. As a proof-of-concept implementation, the original LSF processing was applied to the shortened time-domain transients for the same 4-plex TMTc model sample. This initial example demonstrates that the 6.32 mDa doublets could be resolved with a transient period reduced to 512 ms (240 000 at 200 *m/z* resolution preset) using the LSF processing, but not the aFT, Figure S7, Supporting Information. The initial LSF TMTc results thus demonstrated a principal possibility to extract TMTc reporter ion data at detection periods that are compatible with the ion detection times that are standard for Orbitrap platforms.

Super-resolution analysis for a 12-plex TMTc/TMTproC proteomics. The promising initial results of the original LSF method application to the 4-plex TMTc workflow have encouraged the super-resolution analysis combination with the highly-plexed TMTproC workflow. Several LC-MS experiments were performed on another Fusion Lumos instrument, with their MS/MS scans acquired at the resolution settings 120 000 (256 ms detection period), 60 000 (128 ms), and 50 000 (~108 ms). The application of the LSF processing to the

analysis of isotopically-labeled samples that had 12 channels with four 6.32 mDa doublets confirmed a practical validity of the approach, **Figure 3**. Naturally, mass spectral representation in either eFT or aFT for the same dataset, does not resolve the doublets, Figure 3. Moreover, compared to Figure 2, the LSF parameter optimization allowed to shorten by 10-fold the required minimum time-domain transient periods to 256 ms (Figure 3). The latter period corresponds to the resolution setting of 120 000 at *m/z* 200, which specifically serves the needs of the highly-plexed quantitative proteomics. This result was achieved provided that certain experimental conditions are met such as ion-ion interactions in the doublets are sufficiently below the coalescence threshold.

Quantitation accuracy of the LSF-based 12-plex TMTproC proteomics. Resolving the doublets in the whole TMTc cluster ions mass range for proteomics-grade scanning speed is, certainly, an important proof-of-concept achievement of the LSF processing, but the obtained quantitation accuracy needs to be evaluated in detail. The density distributions for the CV values calculated for the doublets and singlets obtained in the LSF analyses of the 12-plex TMTproC experiments with MS/MS scans at different resolution settings are shown in **Figure 4**. Expectedly, analysis of the 1 Da channels on the peptide level is more accurate than the one of the 6.32 mDa doublets for all resolution settings (50 000, 60 000, and 120 000), Figure 4 left panel. The CV distributions for 50 000 are clearly higher than the corresponding CV distributions accessible with 1 Da spacing. Increasing the resolution, to 60 000 improves CVs closer to 1 Da distribution, whereas with 120 000 resolution setting, the CVs

obtained from the doublets are very similar to the 1 Da spacing results. Thus, there is a tradeoff between measurement precision and scan speed. The 60 000 resolution already provides acceptable CVs for the LSF-based TMTproC proteomics, whereas 120 000 resolution generates CVs essentially identical to the established 1 Da spacing. This is exciting, as most multiplexed proteomics experiments are currently performed with 50 000 resolution settings, and the additional 20 ms transient time seems negligible. We therefore expect SR experiments with higher plexing capability to have similar speed and sensitivity as the 1 Da TMTproC analysis [5, 12, 22]. Typically, the CVs further improve, when the quantitative information form peptides are integrated onto the protein level. Indeed, this is also the case for our standard, where the median protein CVs shrink (Figure 4, panel B).

Conclusions

The LSF processing of as short as ~108 ms time-domain transients (scan rate of up to 9-10 scans per second, resolution setting of 50 000 at *m/z* 200 on an ultra-high-field Orbitrap) resolves neutron-encoded differences of even high *m/z* complementary reporter ions in TMTc and TMTproC workflows. The time-domain transient periods are compatible with standard complement reporter ion quantification workflows, particularly because these periods allow the parallel injection of ions for the next scan. Compared to the conventional FT processing, this advance signifies a more than 30-fold reduction in the time-domain transient period required to resolve the 6.32 mDa doublets in the TMTc/TMTproC clusters. This result is in line with the specified mass

difference in the doublets and the uncertainty principle definition for the SR methods in general, and LSF in particular.

Furthermore, we demonstrate that the time-domain transient periods increase to 256 ms (resolution setting of 120 000 at *m/z* 200) provides CV distributions for doublets (6.32 mDa channel separation) virtually indistinguishable from the 1 Da distributions. That is particularly useful for the analysis of complex proteomes where high sensitivity (long ion accumulation times) is needed, as may take place in phospho-, glyco-, and single-cell proteomics. However, further increasing the time-domain transient period, for example by 2-fold via accepting the next resolution setting of 240 000 at *m/z* 200 (512 ms periods), would reduce the efficiency of following the analyte elution and is to be omitted. Furthermore, as frequency shifts may appear in the longer time-domain transients, keeping the ion detection times shorter is favorable. As a way forward, we envision introduction of a TMTproC-specific intermediate resolution setting, perhaps of 80 000 at *m/z* 200 (about 155 ms time-domain transient for a Q Exactive HF instrument), that will provide sufficient quantitative accuracy at the acceptable scan speed. We also estimate that additional 10-20% time-domain transient period increase may be essential to tackle the real-life proteomic samples that present a wider range of the TMTc channels intensities and added matrix effects.

Overall, resolving the 6.32 mDa doublets in the TMTc clusters in the whole mass range offers a valuable increase of the multiplexing capacity of the complementary ion approach, currently from commercially available 9 to 13 channels. In addition, modifying the distribution of heavy isotopes, it would be possible to make 21 channels without changing the current TMTpro

structure [12]. Finally, the described application of LSF in TMTc/TMTproC quantitative proteomics is a welcome addition to the arsenal of the LSF-enabled strategies, such as the targeted drug monitoring in mass spectrometry imaging.[23] We would like to point out the described approach is compatible with any high-field Orbitrap-quadrupole instrument like the Q Exactive HF or the Exploris. The described here LSF algorithm application, together with other SR algorithms, such as filter-diagonalization method,[24] phase-constrained spectral decomposition method,[25] and compressed sensing, are starting to confirm the initially high expectations toward the SRMS.

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Supporting Information.

Experimental and method details. (pdf)

Financial conflict of interest. Dr. Tsybin, Dr. Nagornov, and Dr. Kozhinov are employees of Spectroswiss, which develops hardware and software tools for mass spectrometry data acquisition and processing.

Figures

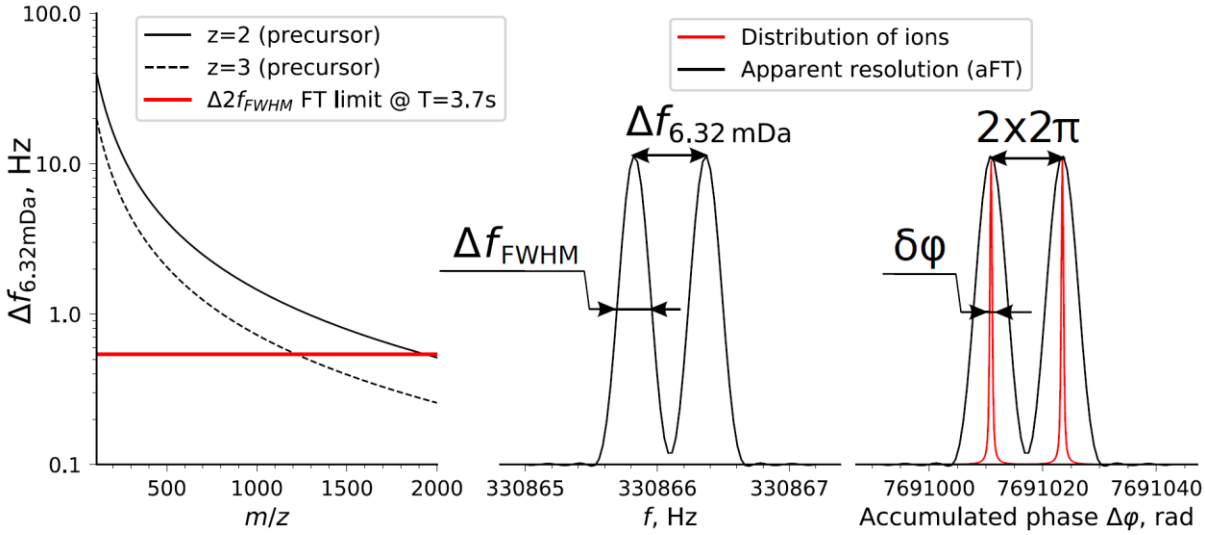

Figure 1. The uncertainty principle of Fourier transform (FT), represented for the spectral components corresponding to 6.32 mDa doublets in complement reporter ion clusters. Left panel: the frequency difference for a doublet as a function of the doublet's *m/z*. The FT resolution (soft) limit, T=3.7 s, is shown by way of example (it corresponds to the doublet at *m/z* = ~1900, 2+ precursor). Center panel: apparent FT resolution for the doublet in question, at T=3.7 s. Right panel: comparison of the apparent FT resolution (in the phase scale) shown in black and the fundamental limit to ion separation (distribution of ions over their total phase accumulated during ion detection) shown in red.

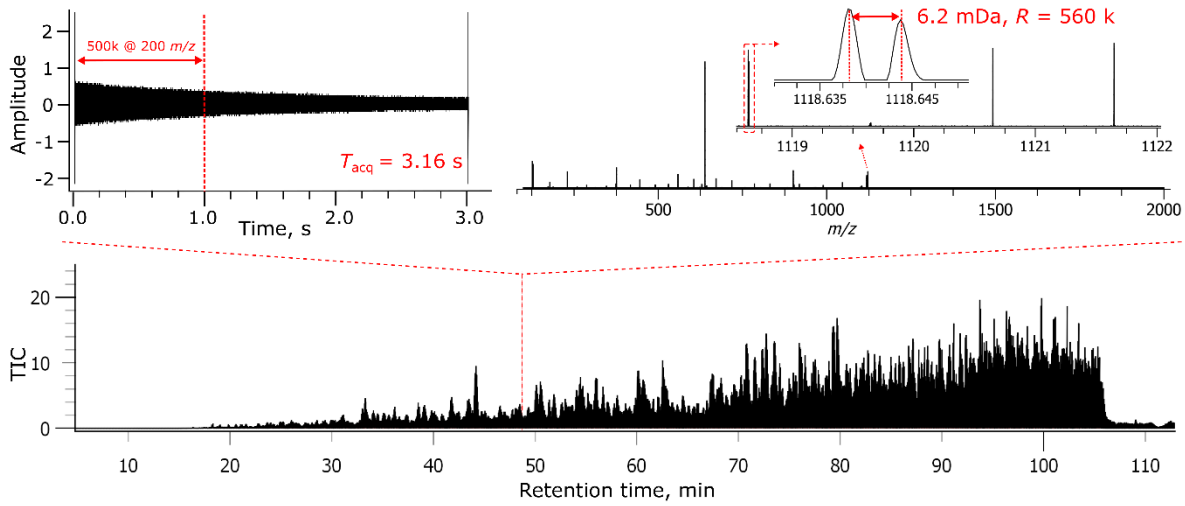

Figure 2. Illustration of aFT signal processing with extended detection period data acquisition in the LC-MS analysis of a 4-plex TMT labeled sample with equal concentrations over the 4 TMTc channels. MS/MS data were acquired on an Orbitrap Fusion Lumos FTMS instrument at the resolution setting of about 500 000 at *m/z* 200 (the .raw file) and as >3 seconds long time-domain data using the external high-performance data acquisition system FTMS Booster X2 (the length is equivalent to a virtual resolution setting of 1,5 million at *m/z* 200).

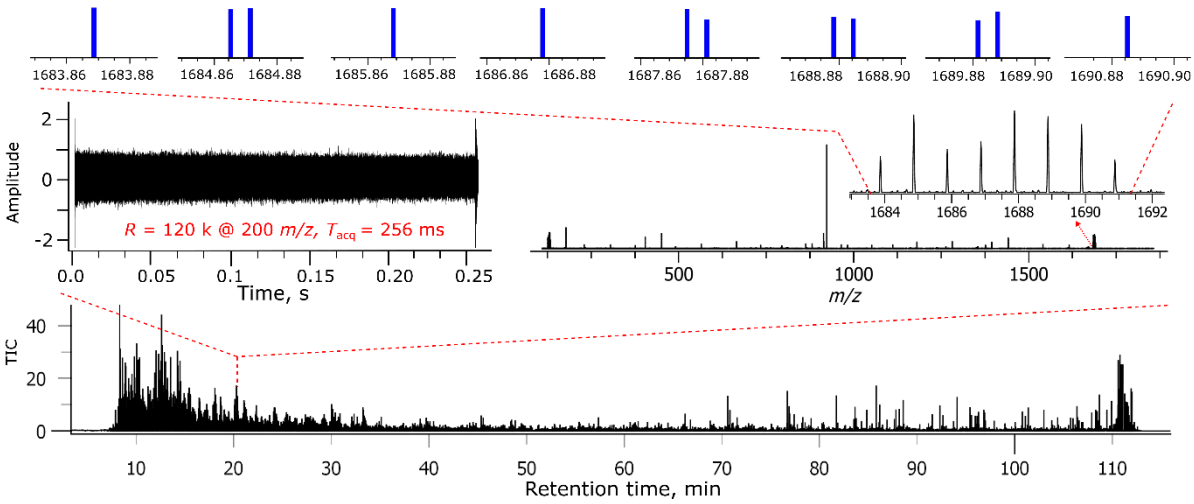

Figure 3. Absorption mode FT (aFT) and LSF analysis of the 12-plex TMTpro™ labeled yeast sample with equal concentrations over the 12 TMTproC channels. The LC-MS experiment was performed with an Orbitrap Fusion Lumos FTMS instrument operated at the resolution setting of 120 000 at *m/z* 200. The corresponding 256 ms time-domain transients were acquired in parallel using an external high-performance data acquisition system (FTMS Booster X2).

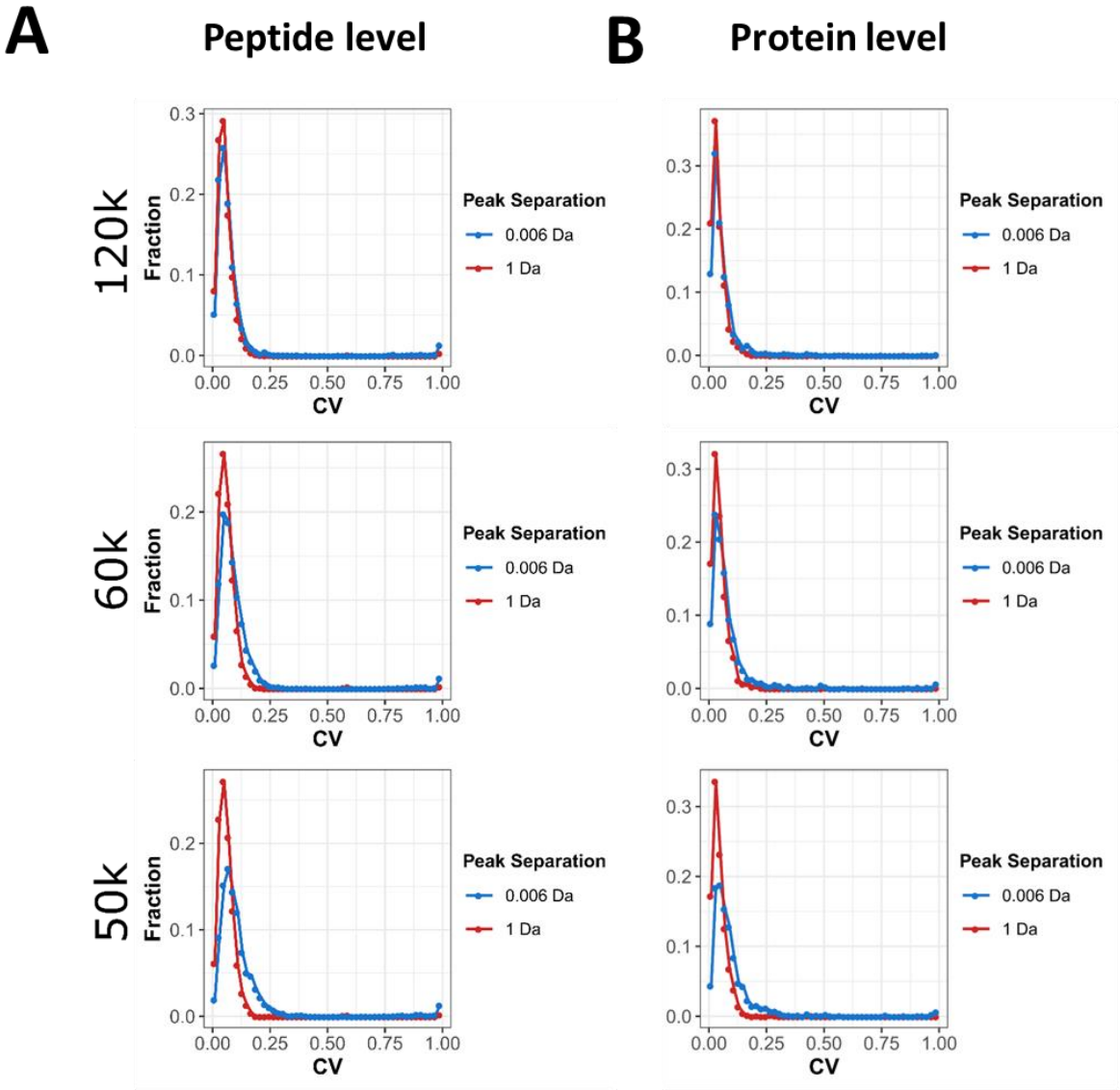

Figure 4. Super-resolution and CVs. A) The density distributions for the CV values of quantified peptides calculated for the doublets (6.32 mDa separated channels) and singlets (1 Da separated channels) obtained in the LSF analyses of the 12-plex TMTproC LC-MS experiments with MS/MS scans at the resolution settings of 120 000 (top panel), 60 000 (middle panel), and 50 000 (bottom panel), all defined at *m/z* 200. B) Equivalent plots once peptide quantification is integrated into protein quantification.

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