Super-Resolution Analysis of Highly-Plexed Complement Reporter Ion Clusters Advances Quantitative Proteomics

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Introduction

The complement reporter ion approach was developed to address certain limitations of multiplexed quantitation using the TMT™ reporters. The recently released TMTpro™ reagents provide highly-plexed complement reporter ion (TMTproC) clusters [1]. However, unlike the use-cases with the low-mass TMT™ reporters, the workflows with the heavy TMTproC reporters are challenging even for the state-of-the-art mass spectrometers. Previously, we introduced the least-squares fitting (LSF) method for Fourier transform mass spectrometry [2] (a super-resolution method of signal processing) and demonstrated its benefits in particular applications. Here, we extend the LSF for complement reporter ions to enhance the TMTproC and TMTc workflows, both with highly-multiplexed channels and with sparse channels, and evaluate this extended LSF method in large-scale quantitative LC-MS/MS experiments.

Methods

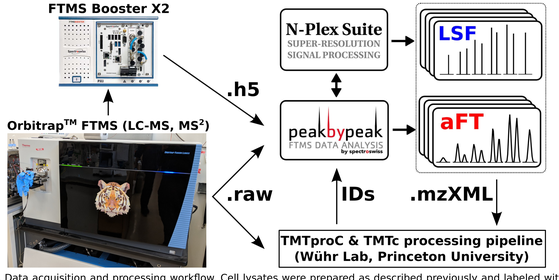

Figure 1. Data acquisition and processing workflow. Cell lysates were prepared as described previously and labeled with selected TMTpro™ or TMT™ reagents (Figure 2). LC-MS/MS experiments were conducted on Orbitrap™ Fusion™ Lumos™ FTMS (Thermo Scientific). Time-domain signals of standard and extended, up to 3 seconds, duration were acquired in parallel to RAW files using an external high-performance data acquisition system (FTMS Booster X2, Spectroswiss). MS/MS spectra were analyzed with SEQUEST. LSF super-resolution processing, absorption-mode FT, and calculations of reference *m/z* values of TMTc/TMTproC ions were performed using Peak-by-Peak (Spectroswiss) running on 8-core desktop computer(s) with 32GB RAM and graphics-card data processing capabilities.

A. LSF for complement reporter ion clusters

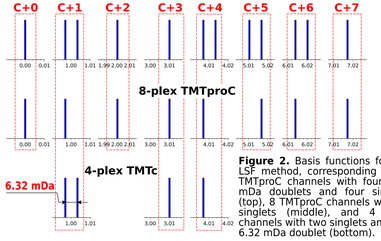

Figure 2. Basis functions for the LSF method, corresponding to 12 TMTproC channels with four 6.32 mDa doublets and four singlets (top), 8 TMTproC channels with all singlets (middle), and 4 TMTc channels with two singlets and one 6.32 mDa doublet (bottom).

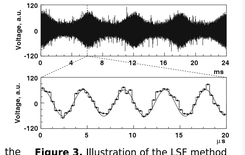

Figure 3. Illustration of the LSF method for FTMS: a transient signal (top panel) and a magnified view of the curve fit to the transient (bottom panel).

B. aFT with extended detection period data acquisition

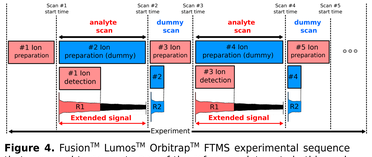

Figure 4. Fusion™ Lumos™ Orbitrap™ FTMS experimental sequence that was used to generate one of the reference data sets in this work (Figure 14). The sequence takes advantage of the parallel ion detection/accumulation capability of the modern Orbitrap instruments, in order to generate extended-length transients [3].

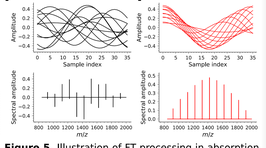

Figure 5. Illustration of FT processing in absorption mode, of transients with in-phase ion signals. Left panels: ion signals with diverse phases and mixed-mode spectrum; right panels: in-phase ion signals and absorption-mode spectrum.

LSF: 8-plex (Sx8, Dx0) TMTproC

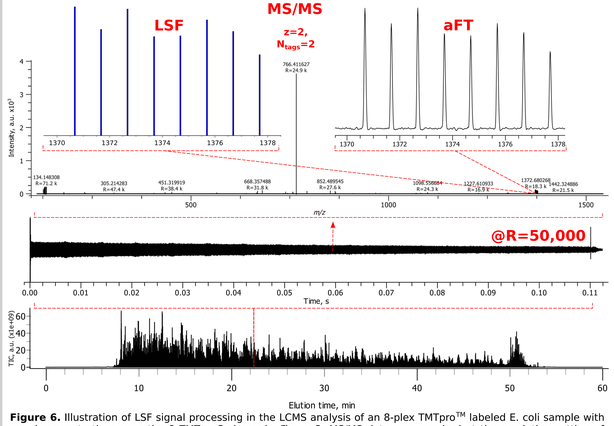

Figure 6. Illustration of LSF signal processing in the LCMS analysis of an 8-plex TMTpro™ labeled E. coli sample with equal concentrations over the 8 TMTproC channels, Figure 2. MS/MS data was acquired at the resolution setting of 50,000 (~100 ms detection period).

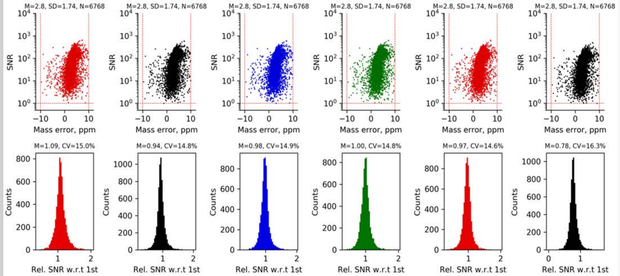

Figure 7. The S/N vs. mass error scatter plots (top panels) and the corresponding density distributions for the abundances relative to the abundance of the C+1 complement reporter ion (bottom plots), calculated for data with S/N>1 and mass error range of ±10 ppm, in the LSF analysis of the 8-plex TMTproC data, Figure 6. The C+0 ions are not included in this consideration (chemical interference).

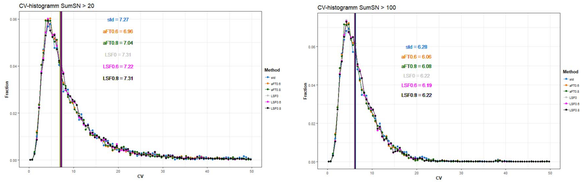

Figure 8. The density distributions for the CV values, calculated for data with total S/N>20 (left panel) and total S/N>100 (right panel), and the corresponding CV median values in the analysis of the 8-plex TMTproC data, Figure 6. The plots compare results obtained with different methods (the LSF method, absorption-mode FT, and .raw data) and for different noise thresholding levels.

LSF: 12-plex (Sx4, Dx4) TMTproC

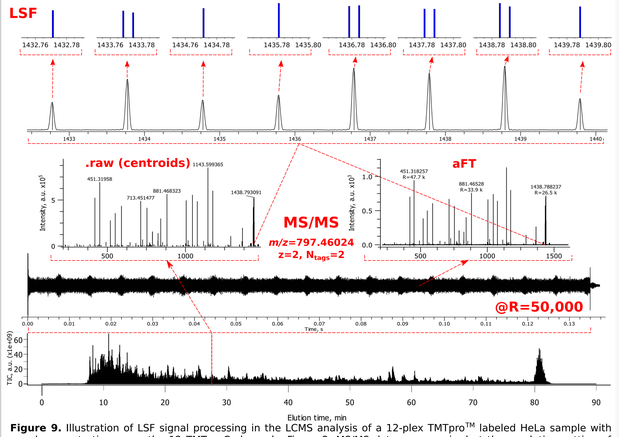

Figure 9. Illustration of LSF signal processing in the LCMS analysis of a 12-plex TMTpro™ labeled HeLa sample with equal concentrations over the 12 TMTproC channels, Figure 2. MS/MS data was acquired at the resolution setting of 50,000 (~100 ms detection period).

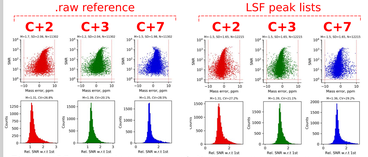

Figure 10. Consistency test of the LSF results versus the .raw reference data, using the 4 singlets in the analysis of the 12-plex TMTproC data, Figure 9. The S/N vs. mass error scatter plots (top panels) and the corresponding density distributions for the abundances relative to the abundance of the C+0 singlet (bottom plots) are shown for data with S/N>1 and mass error range of ±10 ppm.

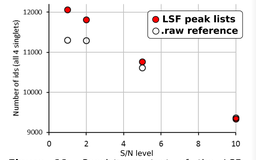

Figure 11. Consistency test of the LSF results versus the .raw reference data, using the 4 singlets in the analysis of the 12-plex TMTproC data, Figure 9. The plots show the number of identifications where all 4 singlets are present as a function of the noise thresholding level.

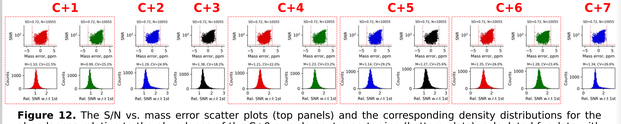

Figure 12. The S/N vs. mass error scatter plots (top panels) and the corresponding density distributions for the abundances relative to the abundance of the C+0 complement reporter ion (bottom plots), calculated for data with S/N>1 and mass error range of ±5 ppm, in the LSF analysis of the 12-plex TMTproC data, Figure 9.

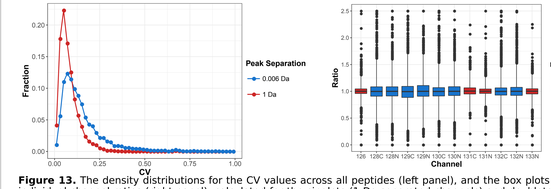

Figure 13. The density distributions for the CV values across all peptides (left panel), and the box plots around the individual channel ratios (right panel), calculated for the singlets (1 Da separated channels) and doublets (6.32 mDa separated channels) from the LSF peak lists obtained in the analysis of the 12-plex TMTproC data, Figure 9.

aFT: 4-plex (Sx2, Dx1) TMTc

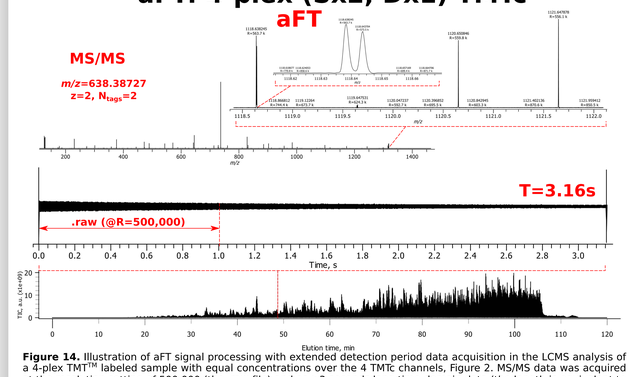

Figure 14. Illustration of aFT signal processing with extended detection period data acquisition in the LCMS analysis of a 4-plex TMT™ labeled sample with equal concentrations over the 4 TMTc channels, Figure 2. MS/MS data was acquired at the resolution setting of 500,000 (the .raw file) and as >3 seconds long time-domain data (the length is equivalent to a virtual resolution setting of 1,500,000), Figure 4.

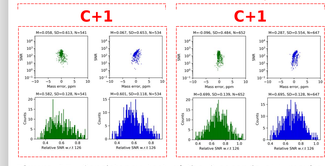

Figure 15. Consistency test of the 3-seconds aFT results versus the .raw reference data, using the ions from the C+1 doublet in the analysis of the 4-plex TMTc data, Figure 14. The S/N vs. mass error scatter plots (top panels) and the corresponding density distributions for the abundances relative to the abundance of the C+4 singlet (bottom plots) are shown for data with S/N>1 and mass error range of ±10 ppm.

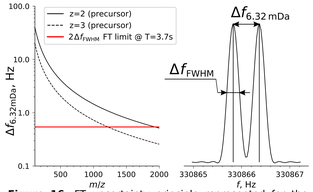

Figure 16. FT uncertainty principle represented for the spectral components in a transient signal, corresponding to a 6.32 mDa doublet in complement reporter ion clusters. Left panel shows the frequency difference for the doublet as a function of the doublet's *m/z*. The shown FT resolution (soft-) limit is for the transient length of 3.7 s by way of example. The limit is given in terms of baseline resolution for absorption-mode FT as illustrated on the right panel.

Conclusions

1. Complementary time-domain data acquisition from FTMS instruments provides additional flexibility for post-acquisition signal processing and data analysis.
2. Ultra-high resolution (UHR) performance of Orbitrap™ Fusion™ Lumos™ FTMS, to obtain one of the reference data sets in this work, was enabled by detection of extended-length transients followed by data processing with FT in absorption mode.
3. LSF method and data processing workflow have been extended to be applicable to LC-FTMS data with complement reporter ion clusters for the TMTpro™ or TMT™ tags.
4. The comparisons of the complement reporters' statistical data for the .raw, aFT, and LSF mass spectra (where applicable, viz.: the singlets from the 8-plex and 12-plex data, and the doublet from the 4-plex data) validate this implementation of the LSF method.
5. A fundamental possibility was observed, to reduce the required times to 105 ms (50k resolution preset) for the high-throughput workflows (IT values of ~100 ms) with the highly-plexed TMTproC channels (four 6 mDa doublets), provided that ion interactions within the doublets are sufficiently below the coalescence threshold.

References

1. Johnson A., Stadlmeier M., Wühr M. *J. Proteome Res.* **2021**, 20, 6, 3043–3052.
2. Aushev T., Kozhinov A.N., Tsybin Y.O. *JASMS* **2014**, 25, 1263-1273.
3. Nagornov K.O, Zennegg M., Kozhinov A.N, Tsybin Y.O, Bleiner D. *JASMS* **2020**, 31, 2, 257–266.

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