Multiplexed Mass Spectrometry of Individual Ions Improves Measurement of Proteoforms and Their Complexes

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Method Article

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Abstract

Protocol for sample preparation, instrumental settings, and processing for the individual ion mass spectrometry method (I2MS) utilizing an Orbitrap analyzer.

Introduction

Reagents

Equipment

Procedure

Kafader *et al.*, *Nature Methods* 2020

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Supplementary Protocol – I^2MS Method: Instrumental settings and transient processing protocol.

Prior to Instrument Use:

This I^2MS protocol is viable for any ThermoFisher Scientific Orbitrap instrument (QE, QE-UHMR, Fusion Lumos, Eclipse). When using these instruments access to time domain data is needed. Time domain data can also be accessed through external devices including FTMS processers purchased from companies such as Spectroswiss. The following instrumental settings need to implemented within the tune (version: 2.8, ThermoFisher Scientific) tree:

1. Spray voltages, source induced dissociation (SID), capillary temperature, and isolation window values should be optimized on a per sample basis.

2. Enhanced Fourier-transform (eFT) should be to be turned off.

3. The HCD pressure should be set to 0.

4. For D30 Orbitraps, instrumental resolution should be set to 280,000 @ 200 m/z with a -5 kV central electrode setting or 140,000 @ 200 m/z with a -1 kV central electrode setting (transients of 2 seconds in duration were used).

5. Time domain data needs to be collected in concert with .Raw file label data for each acquisition event.

6. Turn off Automatic Gain Control (AGC) and use a fixed injection time. Start with a higher inject time of ~50 ms and adjust down as you enter the single ion regime.

During Sample Injection:

All sample acquisitions need to be composed of 100's to 1,000's of acquisitions. As a result, direct injection needs to be utilized for stable sample ionization over extended periods of time.

1. Gain stable signal for directly injected proteins.

2. Begin with higher fixed inject times (~50-100 ms).

3. Tune down inject times 25% and see how the charge state distribution changes.

4. Keep tuning down inject times until all signal is gone.

5. Increase inject times slightly to gain an ion distribution as that shown in the panel labeled "Step 1" in Fig. 1 workflow of the main text .

6. Begin continuous acquisition that can be monitored via Thermo Xcalibur (version: 4.0.27). Time domain data files should be kept where the .RAW file is saved.

Before Processing:

A calibration curve needs to be created composed of known charge states before unknown charge data can be collected. As each Orbitrap has differing imperfections and the trajectories of ions vary slightly from system to system, an independent calibration needs to be completed on each instrument for which I^2MS will be implemented. To produce the calibration function known charge states will be isolated from well known standards. Although the calibration function will vary slightly, the procedure to collect calibration data on each charge state is the same:

1. For denatured systems prepare denatured ubiquitin, myoglobin, carbonic anhydrase, and enolase samples in a buffer containing 40% acetonitrile and 0.2% acetic acid (~0.25 µM protein concentration).

Protein / Mass / Charge States / Sigma Aldrich Product

Ubiquitin / ~8.5 kDa / +5 - +13 / U6253

Myoglobin / ~16.9 kDa / +13 - +28 / M1882

Carbonic Anhydrase / ~29 kDa / +23 - +47 / C7749

Enolase / ~46.5 kDa / +35 - +65 / E6126

2. For native systems prepare native carbonic anhydrase, NIST antibody, pyruvate kinase, and GroEL samples in a buffer containing 100 mM ammonium acetate (~1 µM protein concentration).

Protein / Mass / Charge States / Sigma Aldrich Product

Carbonic Anhydrase / ~29 kDa / +9 - +11 / C7749

NIST Antibody / ~148 kDa / +22 - +27 / NIST8671

Pyruvate Kinase / ~232 kDa / +31 - +37 / 10128163001

GroEL / ~801 kDa / +67 - +74 / C7688

3. To create calibration, isolate one charge states at a time with the quadrupole and collect an ion or two per acquisition from the various resolved isotopes within the charge state.

4. Approximately 30-45 minutes of data collection should be completed per charge state.

5. A sampling of charge states should be completed similar to Supplementary Figure 7.

Processing:

This process corresponds to Figure 1. Supplementary software is available with demonstration slides and dataset to illustrate how the program functions. The main focus of this processing is to determine the charge of each individual ion through its time domain data, assign a corresponding mass value and recreate the mass spectrum.

1. All collected individual ions for unknown data sets are analyzed independently.

2. The label data for each scan is read through to determine the apex peak m/z value and corresponding frequency value of each ion signal

3. The optimal frequency is utilized to calculate the real, imaginary, and magnitude STORI slope values for each ion[1].

4. Once all ion signals from every acquisition have calculated magnitude values all ion signals are ordered from minimum to maximum *m/z*.

5. A set number of ions from the ordered list (usually 16) are selected and their STORI slope values are averaged together.

6. If one of these STORI slopes have a value greater or less than 30% of the median STORI slope it is removed from the list or if the set number of ions do not fall within 5 *m/z* of each other all ions are thrown away.

7. The average of the remaining ions in the list is compared to the calibration function created within the **Before Processing** section (above) and the closest charge value from the calibration is assigned to all ions within the list.

8. This process is repeated for all ions in the unknown data set.

9. Once the charge is determined for all ions, their corresponding mass value is assigned.

10. A histogram is created in the mass domain, usually with 0.2 Da bin sizes though use of smaller bins increases effective resolution[2].

Troubleshooting

Time Taken

Anticipated Results

References

1. Kafader, J.O. et al. STORI Plots Enable Accurate Tracking of Individual Ion Signals. *Journal of The American Society for Mass Spectrometry* **30**, 2200-2203 (2019).

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