Designing a DIA Method

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- v1.4, updated on Aug 21, 2020
  - added clarification of matching IIT with resolution on Orbitraps and updated the max IIT recommended settings
- v1.3, updated on May 15, 2020
  - added clarification of setting up a method with the staggered windowing scheme
- v1.2, updated on May 10, 2020
  - updated to include new features and dogma
- v1.1, updated on March 28, 2018

Summary and further resources

Data independent acquisition (DIA) is mass spectrometry acquisition strategy that represents a series of compromises between comprehensive detection and selective quantitation where the goal is to detect as many peptides as possible, but in a way that maintains quantitative rigor. With modern mass spectrometers we typically can only scan at a maximum rate of 10-20 Hz without sacrificing spectrum quality. An additional constraint is that at least 6-8 measurements are required to describe a quantitative peak. DIA methods make several compromises to manage these constraints. In particular, instead of trying to monitor all peptides, the measurement range is typically limited to measure peptides in a restricted precursor window (e.g. 400-1000 m/z). In addition, precursor isolation windows are widened such that multiple peptides are usually isolated together and co-fragmented, resulting in higher interference.

This document is a quickstart guide to rapidly building a DIA method tailored for your instrument and experiment. It is focused on developing methods for Orbitrap-based instruments, but these concepts are generally applicable to other instrument configurations as well. A thorough investigation into the intuition behind these best practices is detailed in our paper in Molecular and Cellular Proteomics:


A further DIA resource is a recorded talk on this subject for the Northeastern May Institute 2020 organized by Olga Vitek and Meena Choi. This talk is freely available at: https://www.youtube.com/watch?v=RidYXjvAk0s

Finally, a spreadsheet with recommended window settings is available at: https://docs.google.com/spreadsheets/d/1A8AQlmLroAkQcAcsiGTNvnGBE2IGpkMwhh0YLTHXKA
Designing a windowing strategy

Summarizing Pino et al.,¹ current Orbitrap-based instruments that are suitable for DIA fall into two categories:

**10 Hz instruments:** Thermo Fusion, QE, QE+, QE-HF

**20 Hz instruments:** Thermo Lumos, Eclipse, QE-HFX, Exploris

While most of these instruments can technically collect MS/MS faster than the above rates, sensitivity tends to be the important limiting factor. ToF instruments can scan much faster than Orbitraps but require scan averaging to produce acceptable MS/MS. In general, we recommend considering these instruments as similar to 20 Hz Orbitrap instruments after scan averaging.

We structure windowing methods assuming 10 measurements across an average peak so that regions of the chromatogram with narrower peaks will not be underrepresented. We calculate the necessary cycle time and windowing scheme from the average peak width and maximum scan rate. Assuming peak widths of 25 seconds (typical for 300mm-400mm columns and 90 min gradients) and 10 Hz, we require cycle times of 2.5 seconds, resulting in 25 windows per cycle. This allows for 25x 24 m/z-wide precursor isolation windows to cover 600 m/z in windowing range (400-1000 m/z). However, if we use a faster scanning instrument that can achieve 20 Hz, we can use up to 50 windows per cycle. This allows for 50x 12 m/z-wide precursor isolation windows to cover the same 600 m/z in windowing range. Wider peptide peak widths produced by shorter columns (e.g. 150-200mm) or slower chromatography enable more scans to achieve 10 measurements per peak, allowing for narrower precursor isolation windows. Similarly, longer columns may require balancing with wider precursor isolation windows.

Matching ion injection time with resolution

Before we begin this section, please note that unlike most of the considerations discussed in this document, ion injection time only applies to trapping instruments like Orbitraps. Orbitraps accumulate ions and collect mass spectra in parallel: while performing the current mass measurement, these instruments simultaneously accumulate ions for the next measurement. As such, it is important to match the time spent accumulating ions, with the time spent collecting each Orbitrap spectrum. In modern instruments (such as those discussed above), 15k Orbitrap
spectra on high-field instruments are optimally balanced by maximum ion injection times (max IIT) of 23 msec. Similarly, 30k spectra (17.5k on QE and QE+ instruments) are balanced by max IITs of 55 msec.

Newer Orbitrap method editors have two additional settings to specify ion inject timing: “auto” and “dynamic”. When “auto” is selected, the instrument uses the appropriate pre-calculated max IIT (23 msec or 55 msec) for each spectrum, making it easier to specify the optimal times. While “dynamic” is designed primarily to increase sensitivity in PRM experiments, it has some utility in DIA experiments as well. When “dynamic” is selected, the user must also specify a desired cycle time by setting “Expected LC Peak Width (s)” and “Desired minimum points across the peak”, and the instrument uses these settings to adjust the optimal maximum ion injection time. “Auto” and “dynamic” produce the same result if the actual cycle time is equal to, or larger than the expected peak width divided by the minimum points across the peak, such as in the optimized windowing strategy discussed above. However, in non-optimal solutions where the number of windows and the cycle time are not matched, “dynamic” can increase the max IIT to improve experiments where fewer than optimal windows are specified. As such, we recommend that instrument operators use the “dynamic” setting for DIA MS/MS.

Choosing optimal precursor isolation windows

EncyclopeDIA\(^2\) makes it easy to set up a DIA windowing scheme. After downloading and installing EncyclopeDIA (freely available at [https://bitbucket.org/searleb/encyclopedia](https://bitbucket.org/searleb/encyclopedia)), navigate to the “Help/Window Scheme Wizard” menu option. This launches the following dialog:

![Window Scheme Wizard](image)

From the windowing strategy calculations, enter the appropriate number of windows, and the desired start/stop m/z values. The table and graphic update automatically based on these selections. Here the default is designed for 10 Hz instruments with 25 windows measuring from 400 to 1000 m/z. Fractional optimized window placements put the window boundaries in regions between nominal m/z values where peptides are unlikely to exist. These window boundaries are designed for typical proteomes. However, some PTMs can adjust these calculations and if peptides are enriched for a certain type of amino acid (e.g. phospho) then these boundaries should be adjusted appropriately. Some older instruments (e.g. Thermo Fusion or QE) have quadrupole geometries that are not optimized for DIA. These instruments can benefit from small
(<0.5 m/z) margins added to the window width. Newer instruments with segmented quadrupoles have relatively flat transmission efficiency across the precursor isolation window such that using optimized window placements sufficiently makes margins unnecessary.

**Using staggered windows for Orbitrap instruments**

For Orbitrap instruments, we recommend using “staggered” window schemes that offset every other cycle by 50%:

![Window Scheme Wizard](image)

Data collected with this windowing scheme can be demultiplexed using the Proteowizard\[^3\] GUI tool, which uses previous and next cycles to computationally separate each N-width MS/MS into two N/2-width MS/MS spectra.\[^4\] A tutorial for how to perform this demultiplexing is described here: [https://www.mcponline.org/content/mcprot/suppl/2020/04/20/P119.001913.DC1/157767_2_supp_511351_q8sty4.pdf](https://www.mcponline.org/content/mcprot/suppl/2020/04/20/P119.001913.DC1/157767_2_supp_511351_q8sty4.pdf)

Briefly, Proteowizard can be freely downloaded (from [http://proteowizard.sourceforge.net/](http://proteowizard.sourceforge.net/)) to convert vendor-specific MS raw files into HUPO standard mzML interchange files that are vendor neutral. We recommend these settings for DIA window deconvolution on Orbitrap instruments using the command line:

```
msconvert.exe --zlib --64 --mzML --simAsSpectra --filter "peakPicking true 1-" --filter "demultiplex optimization=overlap_only" *.raw
```

Alternatively, the MSConvert GUI can be used:
In both cases, the order of “filters” is important: the “peakPicking” filter must be listed first to enable vendor-library peakpicking. Note: if staggered windows are used, then it is important that margins are not added. Also note: make sure “SIM as spectra” is checked for trbrid instruments.

Using variable-width windows on ToF instruments

ToF instruments typically are able to scan faster than Orbitrap instruments, but naturally produce noiser MS/MS spectra because they collect true profile data. Consequently, measurements made by ToFs may not be deconvoluted as easily as those made by Orbitrap instruments. In this case, we normally recommend using variable-width windows and taking advantage of small margins and potentially extended precursor isolation ranges:
Note: as with optimized window placements, the optimal variable-width windows change when analyzing PTM-enriched samples such as samples of phosphopeptides:

![Image](image1.png)

Setting up a DIA instrument method

Here we use the Thermo QE-HF method editor, but many of these settings are transferable to other platforms. Starting with a new method, we recommend adding a precursor scan followed by DIA MS/MS scans:

![Image](image2.png)

While precursor scans are not required for detection or quantitation, we find they can somewhat improve detection rates and are useful for troubleshooting experiments while acquiring data. We recommend setting the resolution and maximum ion injection time to a lower value than for a typical DDA experiment to limit the time wasted on collecting MS scans. Also, we recommend
only scanning through the precursor isolation range of the experiment. Here for a 400-1000 m/z experiment we use a precursor scan of 395-1005 m/z. Since these windows are not being used for quant, collecting MS survey scans in centroid mode helps to moderate file sizes.

For DIA scans, we set the default charge to 3 and a complementary collision energy setting (NCE) to what we typically use for DDA. In general we set automatic gain control (AGC) target intensity to a very high value so that every MS/MS scan is acquired to the maximum ion inject time (max IT) duration, ensuring regular scans time differences between cycles throughout the experiment. Again, collecting scans in centroid mode helps keep file sizes down.

Finally, we add the “center m/z” inclusion list from the Window Scheme Wizard:
Building a method with staggered windows

You can set up staggered windows in either of two ways. Assuming a 25x24 m/z acquisition scheme, let’s define the 51 total windows as 25 normal windows and 25+1 (26) shifted windows. You can do:

(a) Two blocks: 1 MS, then all 51 MS/MS windows (both normal and shifted cycles), with a loop count of 25. This will make the effective acquisition ordering:

1 MS
- 25 MS/MS windows cycle 1 normal
1 MS
- 25 MS/MS windows cycle 1 shifted
1 MS
- 1 MS/MS windows cycle 1 shifted
- 24 MS/MS windows cycle 2 normal
1 MS
- 1 MS/MS windows cycle 2 normal
- 24 MS/MS windows cycle 2 shifted
1 MS
- 2 MS/MS windows cycle 2 shifted
- 23 MS/MS windows cycle 3 normal
etc...

(b) Four blocks: 1 MS, 25 normal MS/MS windows (loop count of 25), 1 MS, 26 shifted MS/MS windows (loop count of 26). This will make the effective acquisition ordering:

1 MS
- 25 MSMS windows cycle 1 normal
1 MS
- 26 MSMS windows cycle 1 shifted
1 MS
- 25 MSMS windows cycle 2 normal
1 MS
- 26 MSMS windows cycle 2 shifted
... etc

Either approach is fine for EncyclopeDIA! We typically prefer strategy (a), because it maintains regular timing between the majority of the spectra, which is an assumption made by many smoothing and peak picking algorithms. In this case, each window has an additional spectrum between measurements every 24 half-cycles (e.g. roughly once every minute).

However, many people prefer strategy (b), because it keeps the acquisition schedule tidy and easy to understand. In this case, each window has an additional spectrum between measurements every 2 half-cycles (e.g. roughly once every 5 seconds).

Thus, strategy (a) trades additional scheduling complexity for slightly better precision in quantification. This small improvement decreases with more windows.
Collecting chromatogram libraries

Chromatogram libraries are generated from a small collection of narrow precursor isolation window DIA experiments, rather than from DDA, as with typical spectrum libraries. To generate chromatogram libraries, we typically acquire between 4 and 6 gas-phase fractionated (GPF) injections, in a 4-6x 100 m/z configuration. These fractions are tiled to cover the entire precursor isolation window in our wide-window DIA experiments, for example 400-500 m/z, 500-600 m/z, 600-700 m/z, 700-800 m/z, 800-900 m/z, and 900-1000 m/z. Since the windows do not generally share peptides, retention time consistency between runs is of utmost importance. Consequently, we recommend collecting several conditioning runs of the same sample type on your column before collecting your chromatogram library. A great way to test chromatogram libraries on your instrument is to use the following acquisition order:

1. Typical DDA
2. Single-injection DIA (400-1000m/z)
3. Typical DDA
4. Single-injection DIA (400-1000m/z)
5. Typical DDA
6. Single-injection DIA (400-1000m/z)
7. GPF-DIA (400-500m/z)
8. GPF-DIA (500-600m/z)
9. GPF-DIA (600-700m/z)
10. GPF-DIA (700-800 m/z)
11. GPF-DIA (800-900 m/z)
12. GPF-DIA (900-1000 m/z)

This acquisition strategy allows you to collect triplicate DIA and DDA measurements of your proteome to compare for quantitative reproducibility, as well as 6x GPF-DIA experiments to build a chromatogram library. These GPF-DIA injections can be searched with library-free methods or with predicted spectrum libraries.

Recommended starting settings

The following are starting conditions assuming a 30-35 second base-to-base peak width for peptides. We recommend adjusting these settings following the rules above as you develop DIA methods specific to your instrument and experiment.
### Recommended single-injection DIA (quantitative) acquisitions:

<table>
<thead>
<tr>
<th>MS1 Settings</th>
<th>QE/ QE+</th>
<th>QE-HF</th>
<th>QE-HFX/ Exploris</th>
<th>Fusion</th>
<th>Lumos/ Eclipse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>385-1015 m/z</td>
<td>385-1015 m/z</td>
<td>390-1010 m/z</td>
<td>385-1015 m/z</td>
<td>390-1010 m/z</td>
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<tr>
<td>Resolution</td>
<td>35000</td>
<td>60000</td>
<td>60000</td>
<td>60000 (Orbi)</td>
<td>60000 (Orbi)</td>
</tr>
<tr>
<td>Max IT</td>
<td>auto or 55</td>
<td>auto or 55</td>
<td>auto or 55</td>
<td>auto or 55</td>
<td>auto or 55</td>
</tr>
<tr>
<td>AGC Target</td>
<td>1E+6</td>
<td>1E+6</td>
<td>1E+6</td>
<td>4E+5</td>
<td>4E+5</td>
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<tr>
<td>Data Type</td>
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</table>

<table>
<thead>
<tr>
<th>MS2 Settings</th>
<th>Windowing Scheme</th>
<th>Resolution</th>
<th>Max IT</th>
<th>AGC Target</th>
<th>Loop Count</th>
<th>Default Charge (N/CE)</th>
<th>Data Type</th>
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<tbody>
<tr>
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<td>16 m/z staggered</td>
<td>17500</td>
<td>dynamic or 55</td>
<td>1E+6</td>
<td>38</td>
<td>27</td>
<td>Centroid</td>
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</table>

### Recommended 6x injection GPF-DIA (chromatogram library) acquisitions:

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<th>MS1 Settings</th>
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<th>QE-HF</th>
<th>QE-HFX/ Exploris</th>
<th>Fusion</th>
<th>Lumos/ Eclipse</th>
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</thead>
<tbody>
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<td>395-505 m/z, etc...</td>
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<td>395-505 m/z, etc...</td>
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</tr>
<tr>
<td>Resolution</td>
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<td>60000</td>
<td>60000</td>
<td>60000 (Orbi)</td>
<td>60000 (Orbi)</td>
</tr>
<tr>
<td>Max IT</td>
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<td>auto or 55</td>
<td>auto or 55</td>
<td>auto or 55</td>
<td>auto or 55</td>
</tr>
<tr>
<td>AGC Target</td>
<td>1E+6</td>
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<td>1E+6</td>
<td>4E+5</td>
<td>4E+5</td>
</tr>
<tr>
<td>Data Type</td>
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<td>Centroid</td>
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<table>
<thead>
<tr>
<th>MS2 Settings</th>
<th>Windowing Scheme</th>
<th>Resolution</th>
<th>Max IT</th>
<th>AGC Target</th>
<th>Loop Count</th>
<th>Default Charge (N/CE)</th>
<th>Data Type</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6x GPF-DIA w/4 m/z staggered</td>
<td>17500</td>
<td>dynamic or 55</td>
<td>1E+6</td>
<td>25</td>
<td>27</td>
<td>Centroid</td>
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</tbody>
</table>

### References:


