Supporting Information

Active instrument engagement combined with a real-time database search for improved performance of sample multiplexing workflows

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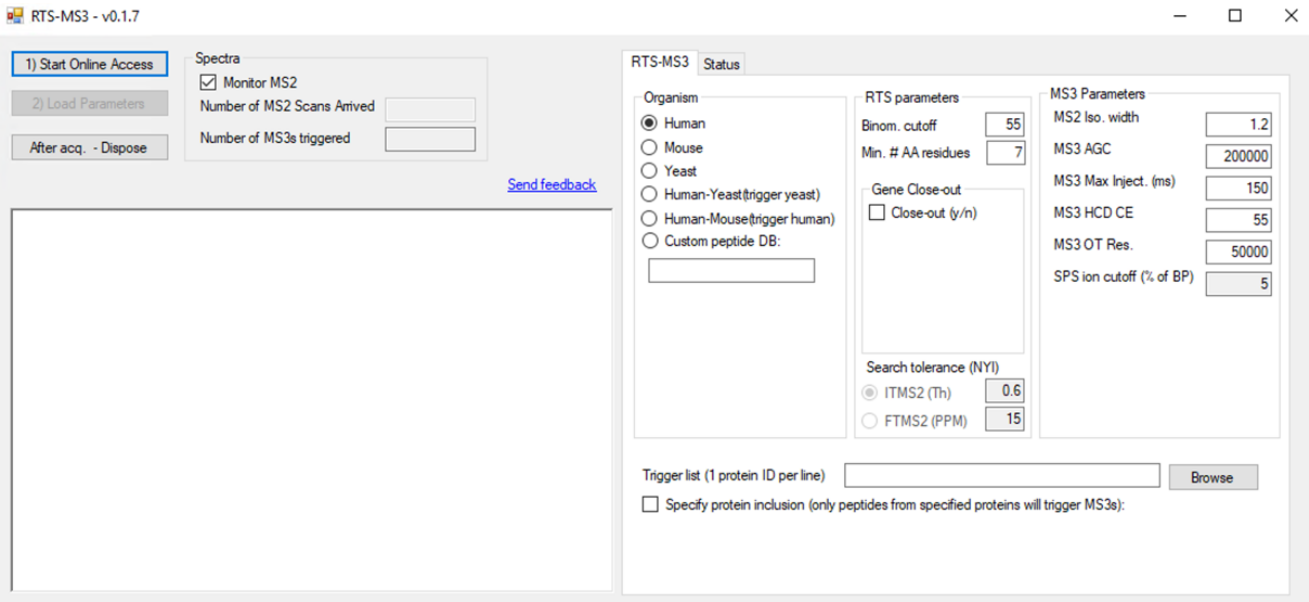
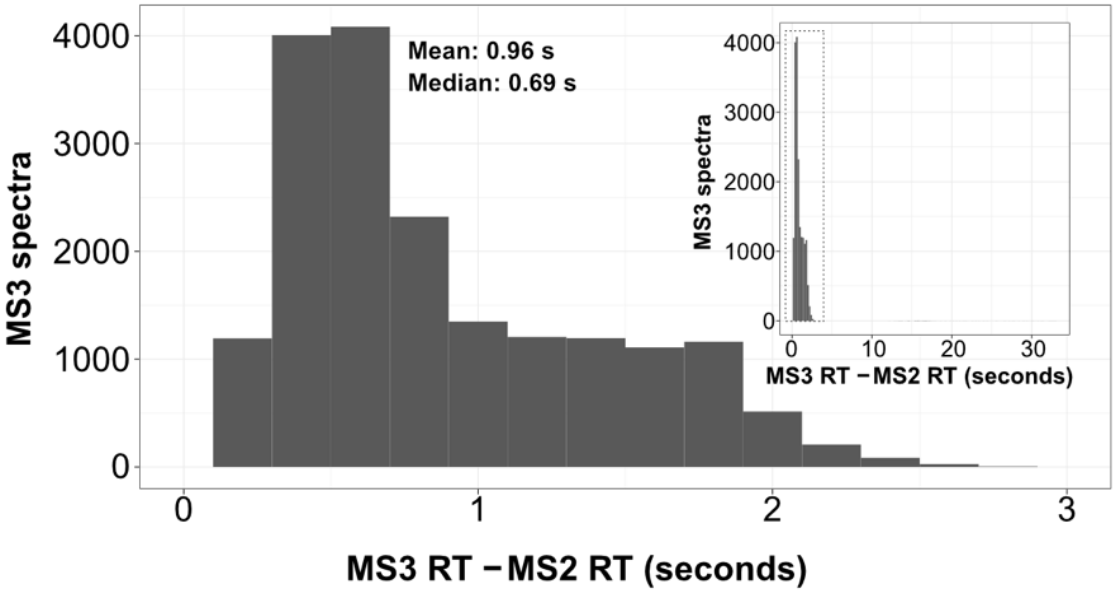
	12 fractions x 3 hours per fraction (10-plex)		
	Total	**Filtered**	**% spectra retained**
MS2 spectra	391,927	120,413	31%
MS3 spectra	391,916	101,041	26%

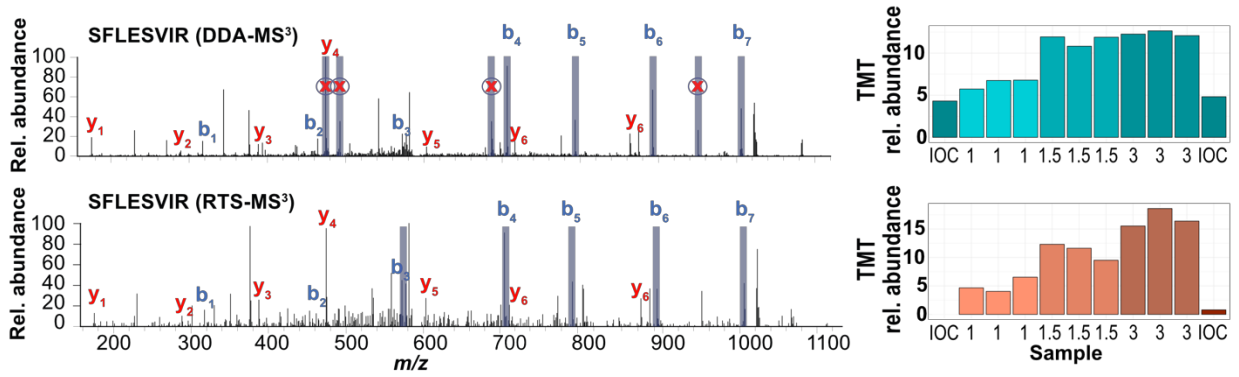
Supporting Table S1 - Comprehensive proteome characterization retains only a portion of the acquired data after filtering. A 10-plex sample of human cancer cell lines was analyzed for 36 hours (12 fractions, 3 hours per fraction). Filtering to a false-discovery rate of 1% at the peptide and protein level resulted in the retention of 31% of the acquired MS^2 spectral data. Additional filtering for quantitative robustness resulted in the omission of nearly 75% of the acquired MS^3 spectra.



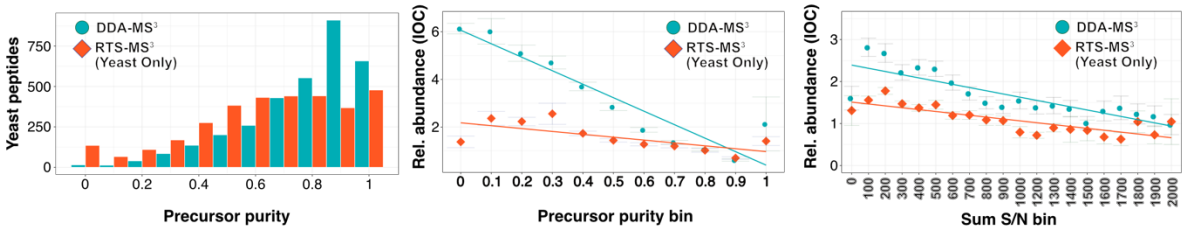
Supporting Figure 1: Graphical user interface of the RTS client application. The interface allows for the selection of parameters related to the real-time search and for controlling the scan specific parameters of the triggered MS^3.



Supporting Figure 2: Distribution of time (seconds) between acquisition of RTS triggered MS^3 and preceding parent MS^2. On average, the time between the receipt of an MS^2 from the instrument and the acquisition of a quantitative MS^3 is less than one second (mean = 0.96 s, median = 0.69 s).



Supporting Figure 3: Selection of identified fragment ions improves MS³ accuracy and precision. Synchronous precursor selection (SPS) of fragment ions for the MS³ scan can be chosen from known b- and y-type fragment ions with RTS-MS³. A DDA-MS³ approach utilizes the top n (where n = 1 – 20) most abundant fragment ions. In many cases, fragment ions from co-isolated peptides can be included, resulting in a reduction of quantitative accuracy and precision. Alternatively, following confident peptide identification by the RTS-MS³ algorithm, an MS³ spectra with SPS ions chosen only from identified fragments ions is acquired on the instrument. For the peptide 'SFLESVIR', quantified both by DDA-MS³ and RTS-MS³ analyses, the resulting quantification differs substantially. The DDA-MS³ acquired MS3 exhibits measurable interference in the interference-only channel (IOC) and ratio compression due to the inclusion of unassigned ions. However, the RTS-MS³ acquired data, using only precursor specific fragment ions, resulted in an MS³ with nearly zero interference and accurate ratios.



Supporting Figure 4: RTS-MS^3 improves the quantitative performance of lower abundance peptides. Analysis of the two-proteome model of interference indicates that lower purity precursors are more routinely interrogated during RTS-MS^3 acquistion. For DDA-MS^3 analysis, as the purity of the precursors decrease, the amount of interference measured in the interference-only channels (IOC) increases. However, during RTS-MS^3 acquisition, the amount of interference remains nearly constant. Furthermore, for all summed signal-to-noise bins, the amount of observed interference was determined to be lower for RTS-MS^3 analysis.