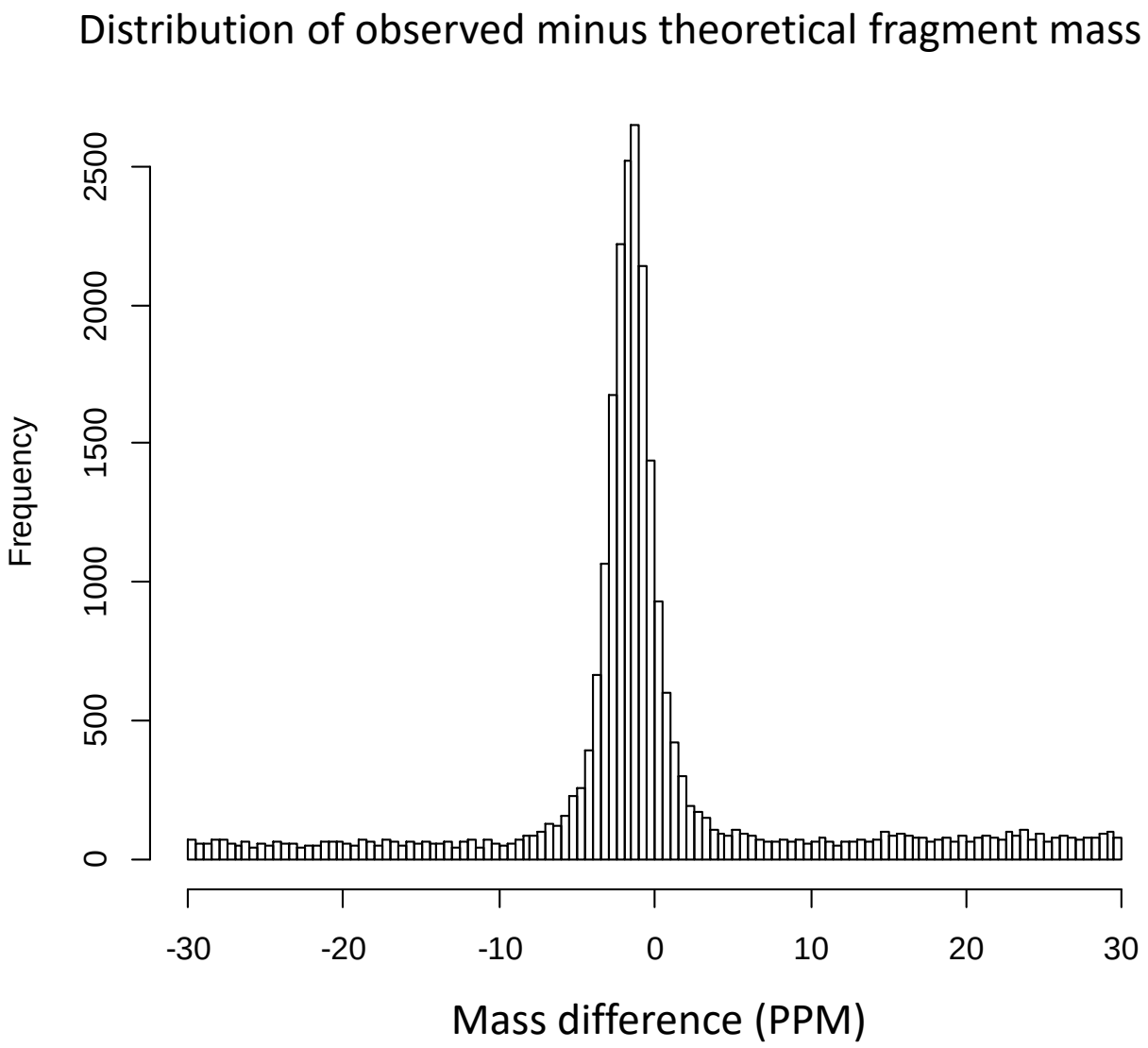
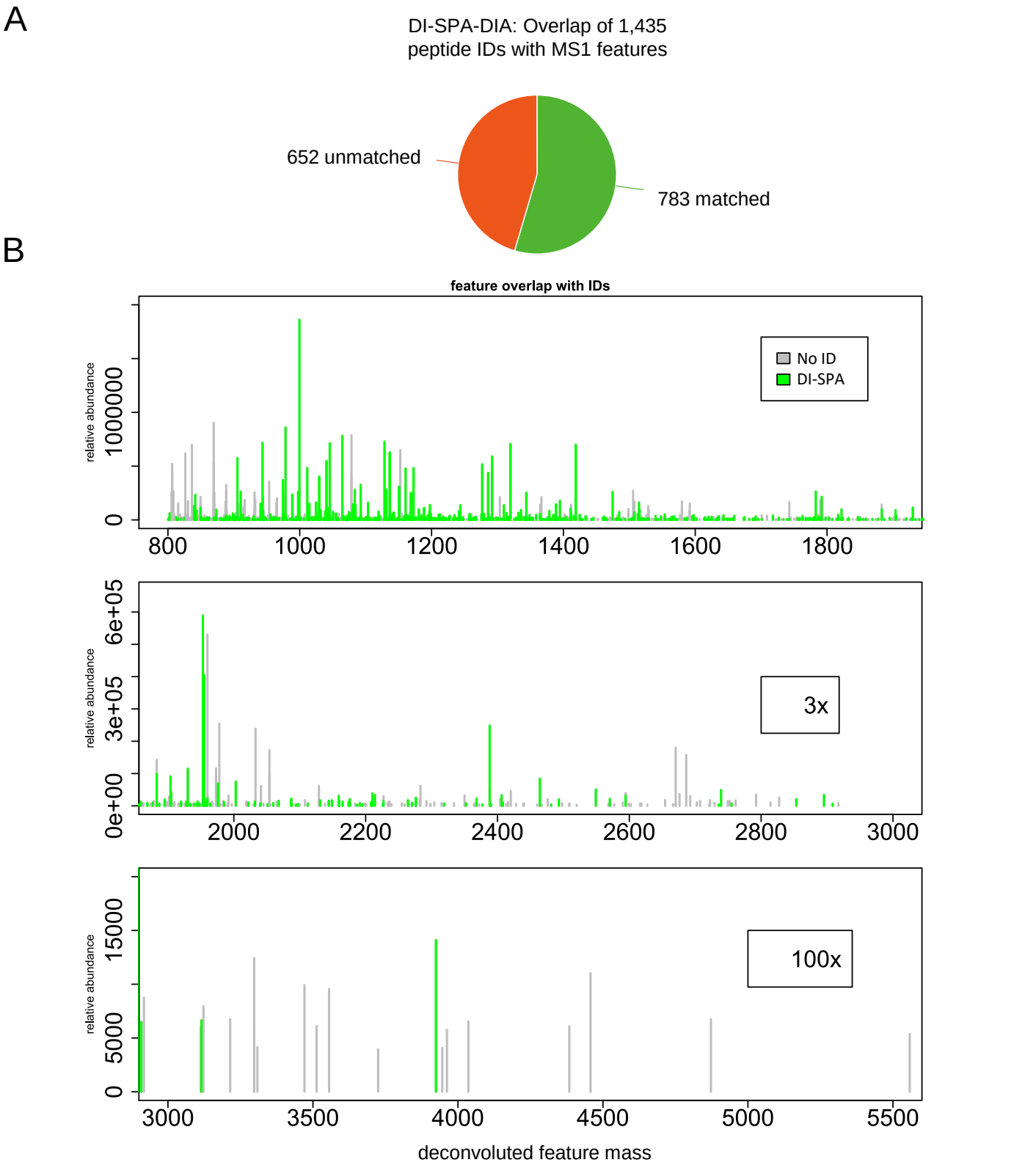
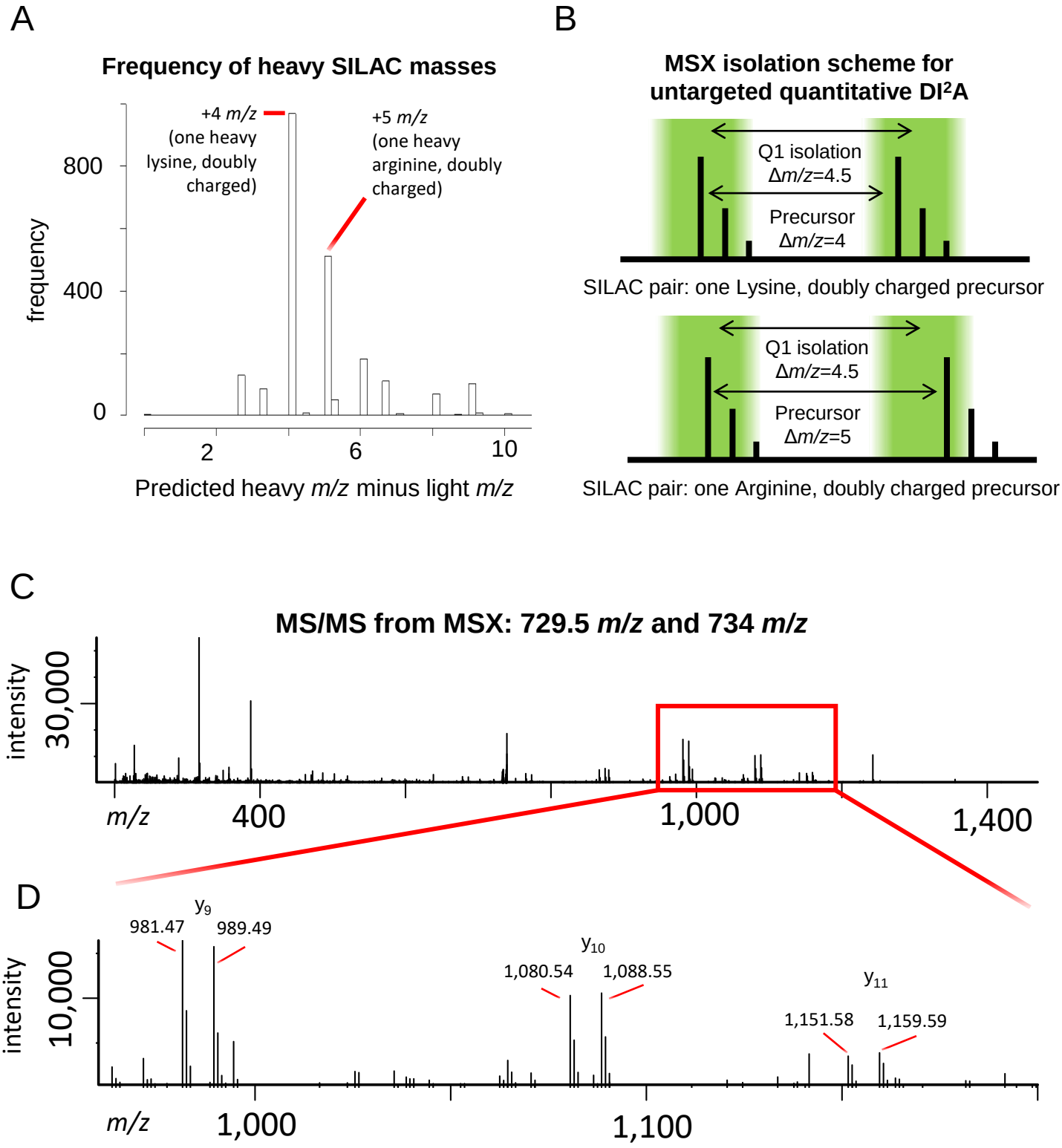


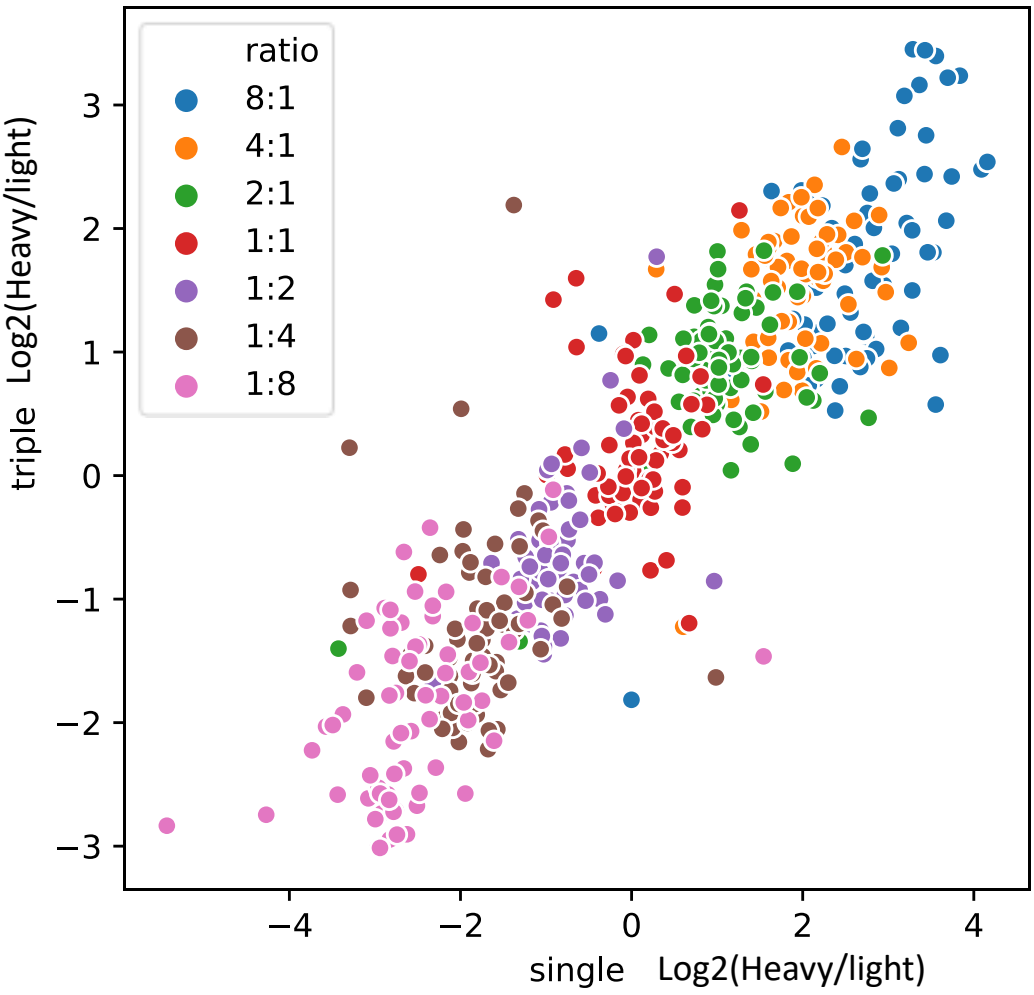
Supplementary Figure 1: Distribution of fragment ion mass errors for all 1,423 peptides identified by DI-SPA analysis with the optimal scouting conditions. Any fragment mass found 30 ppm of the predicted mass was included in this analysis. This shows that matched fragments are non-random, and that a fragment mass tolerance of 10ppm is appropriate for identification by MSPLIT-DIA.



Supplementary Figure 2: Overlap of precursor ion feature masses and identified peptide masses from DI-SPA. Precursor ion (MS1) scans were performed from a range of 400-1,000 m/z with FAIMS compensation voltages of -30, -40, -50, -60, -70, and -80. THRASH was used within DeconTools software to detect precursor mass features. Feature *m/z* values were converted to exact masses, and then matched to exact masses of peptides identified by DI-SPA analysis of the same sample. **A.** Portion of peptides identified by DI-SPA that match MS1 features. **B.** Overlay of MS1 feature masses detected from direct infusion with peptides identified by DI-SPA (where the second panel y-axis is zoomed 3x, and the third panel y-axis is zoomed 100x).



Supplementary Figure 3: Untargeted DI-SPA quantification method for SILAC-labeled proteome samples. (A) Histogram of the predicted heavy peptide m/z difference for all peptides identified from one A549 SILAC sample. **(B)** Depiction of the multiplexed ion isolation scheme used to collect unbiased quantitative data. Based on the most common heavy SILAC partners of +4 or +5 *m/z* determined from **(A)**, ion multiplexing (MSX) was used to co-isolate each possible light mass and the mass centered +4.5 *m/z*. Both light and heavy masses were isolated with a width of 2 *m/z*. **(C)** Example of SILAC quantification using an MS/MS spectra for the peptide "acetyl-SDAAVDTSSEITTK" from the 1:1 SILAC standard sample. **(D)** Close-up view of the high mass region from **(C)** showing the 1:1 ratio of light:heavy y_9, y_{10}, and y_{11} ion pairs.



Supplementary Figure 4: Correlation between observed SILAC ratios from single measurement or triple measurement DI-SPA analysis. The same sample of peptides used in Figure 3 was used for this analysis. For triple ratio measurement, data collection was carried out targeting only the peptides found at FAIMS CV = -60V, and each heavy/light precursor pair was co-isolated three times during data collection. Heavy/light ratios were computed the same way as described for the single ratio analysis, but the 3 ratios were averaged. The ratios determined from single measurement (x-axis) are plotted against the ratios from triple measurement (y-axis), which shows good agreement between the different measurement strategies. The points are colored by the known Heavy/Light SILAC mixing ratio of the sample.