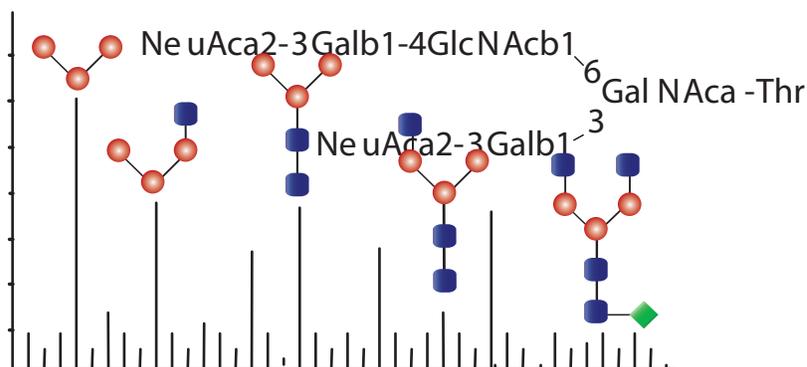


Automating Mass Spectrometry-Based Quantitative Glycomics using Tandem Mass Tag (TMT) Reagents with SimGlycan



Ningombam Sanjib Meitei¹, Arun Apte², Sergei I. Snovida³,
Julian Saba⁴, John. C. Rogers³

¹PREMIER Biosoft, Indore, India, ²PREMIER Biosoft, Palo Alto, U.S.A., ³Thermo Fisher Scientific, Rockford, IL, USA, ⁴Thermo Fisher Scientific, San Jose, CA, USA

Introduction

One of the emerging trends in glycomics research is the innovation related to accurate MS based quantitative analysis of glycans¹⁻⁴. Recently, we have introduced aminoxyTMT Reagents, which enable efficient relative quantitation of carbohydrates, improved labeled-glycan ionization efficiency and increased analytical throughput. These reagents can be used for quantitative analysis of native N-glycans by direct or LC-coupled ESI-MS. However, unlike proteomics, one of the major challenges left unaddressed is the lack of informatics tools to automate the qualitative and quantitative analysis protocols. These protocols include identification/quantitation of glycans using MS/MS data and differential analysis across biological samples. SimGlycan[®] software⁵ is redesigned to streamline such protocols of the quantitation of labeled-glycoforms derived from complex mixtures.

Methods

Tryptic digests of several standard glycoproteins and monoclonal antibodies were treated with PNGase F glycosidase to release N-glycans. Following reversed-phase and graphitized carbon extraction/desalting steps, the glycans were labeled with oxyTMT reagents (Figure 1) according to an optimized protocol.

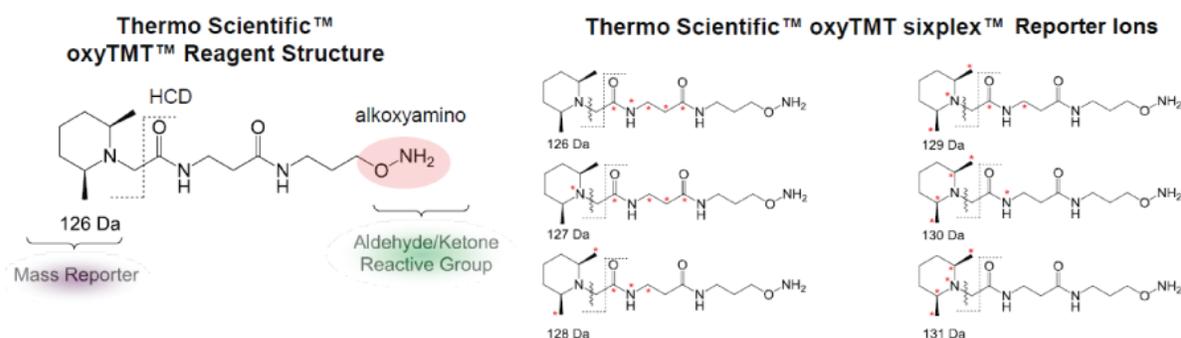


Figure 1: oxyTMT[™] reagent structure and corresponding reporter ions.

Labeled glycans were analyzed using Velos Pro mass spectrometer (Thermo Scientific[™]) in the positive ion mode to acquire trap-HCD MS/MS fragmentation. Data was subjected to SimGlycan[®] to identify structure of the sample glycans. Post identification, we assigned TMT 130.13 ion as the control. The program allows custom setting of reporter ions correction factors, calculates sum/average/median of the reporter ion intensities, number of MS/MS spectra which identifies a glycan. Different types of chart facilitate glycan differential analysis across samples.

Results

HCD spectra were acquired on five high-mannose glycan precursors viz. 780, 861, 942, 1023 and 1124 m/z. Figure 2 shows the averaged MS/MS spectra for each precursor. TMT reporter ion peak cluster was one of the most intense features in the MS² spectra for all doubly charged precursors that contained one sodium and one proton adduct.

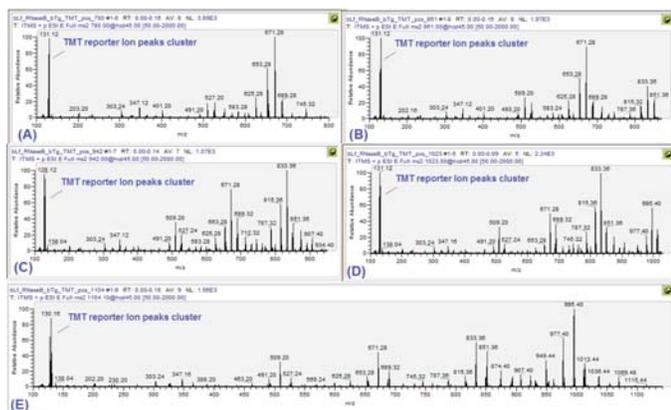


Figure 2: Prominent TMT reporter ion peaks features in all the averaged MS/MS spectra for the precursors (A) 780 with 8 MS/MS scans (B) 861 with 8 MS/MS scans (C) 942 with 7 MS/MS scans (D) 1023 with 5 MS/MS scans and (E) 1104 with 9 MS/MS scans.

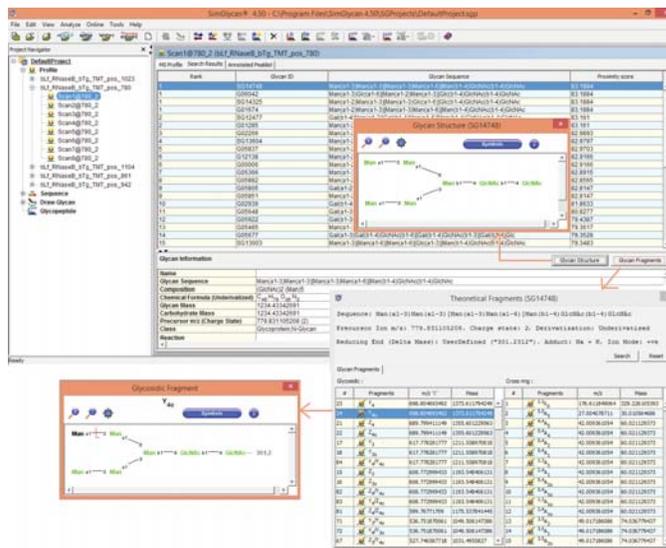


Figure 3: SimGlycan® Software Interface: Structure of the (GlcNAc)₂(Man)₅ glycan with precursor m/z 780 is displayed. In silico fragments and corresponding structures can also be viewed.

The MS/MS data was subjected to SimGlycan® software for structural identification in batch mode. Searches were performed using a 0.2 precursor ion tolerance while product ion tolerance was set to 0.1 Da. Additional filter conditions were set in order to limit the glycan search to glycoprotein N-glycans with intact core. The program identifies N-glycan with carbohydrate residues (GlcNAc)₂(Man)₅ for the precursor m/z value 780, N-glycan (GlcNAc)₂(Man)₆ for 861, N-glycan (GlcNAc)₂(Man)₇ for 942, N-glycan (GlcNAc)₂(Man)₈ for 1023 and N-glycan with (GlcNAc)₂(Man)₉ for 1124, which is a correct assignment for all high-mannose glycans present in the sample. Figure 3 shows the typical search result pane displaying identified glycans in SimGlycan® Software.

The navigation window on the left displays the file name and MS/MS scans. Search results are displayed in two panes: The Search Results display pane and the Annotated Peaklist pane. Here the results for the MS/MS of the precursor m/z 780 are shown. SimGlycan® ranks them on the basis of the peaks observed in the MS/MS data that correspond to diagnostic ions. For an identified glycan, the structure along with corresponding information such as name, sequence, composition, glycan mass, carbohydrate mass, class, links to reactions, pathways and other databases is provided. The Annotated Peaklist pane displays a table containing MS/MS spectrum data list (m/z, intensity) labeled with matched fragments, charge state and adduct. Structure of matched fragments can be viewed.

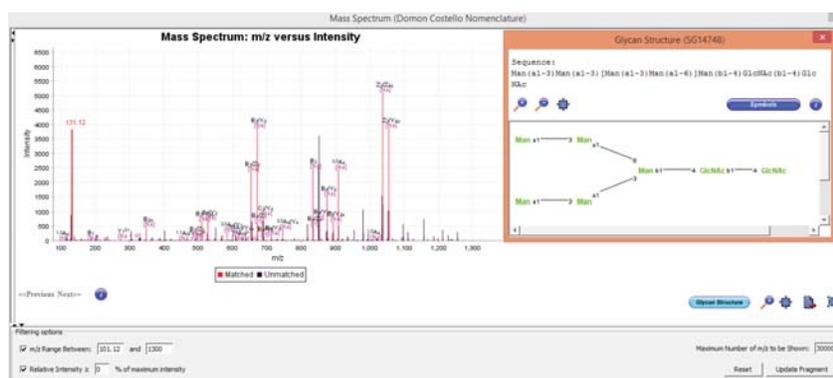


Figure 4: SimGlycan® Software Interface: MS/MS spectrum for the precursor m/z 780 annotated with fragments of the (GlcNAc)₂(Man)₅ glycan.

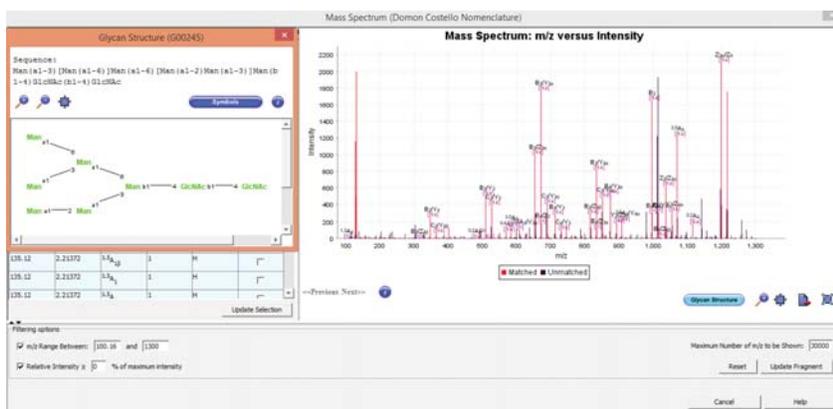


Figure 5: SimGlycan® Software Interface: MS/MS spectrum for the precursor m/z 861 annotated with fragments of the (GlcNAc)₂(Man)₆ glycan.

Figure 4 shows the MS/MS spectrum for the precursor m/z 780 (same as the MS/MS spectrum in Figure 1(A)) annotated with fragments from the identified (GlcNAc)₂(Man)₅ glycan. It is observed that majority of the intense peaks correspond to TMT reporter ions, as well as Z/Y/B/A-type ions. These ions are generally the outcome of two glycosidic cleavages towards reducing end with intact TMT tag and carry Na adduct. Besides, all these intense peaks are singly charged ions. This observation is consistent for all the MS/MS spectra corresponding to (GlcNAc)₂(Man)₆, (GlcNAc)₂(Man)₇, (GlcNAc)₂(Man)₈ and (GlcNAc)₂(Man)₉ glycans (Figures 5-9).

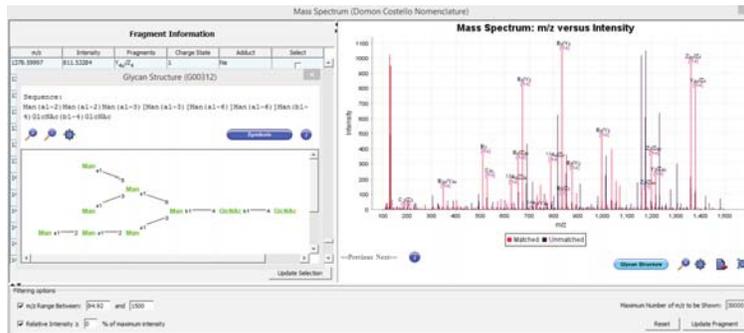


Figure 6: SimGlycan® Software Interface: MS/MS spectrum for the precursor m/z 942 annotated with fragments of the (GlcNAc)₂(Man)₇ glycan.

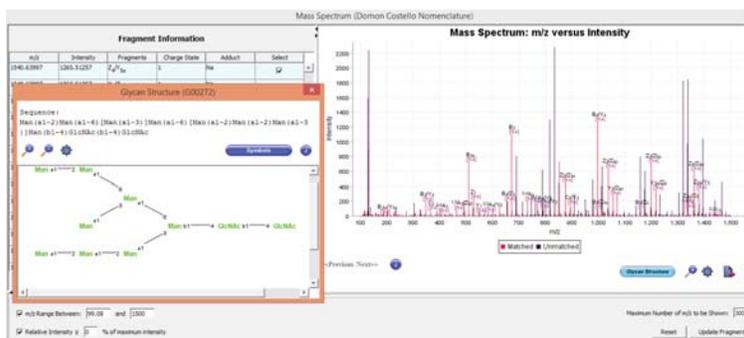


Figure 7: SimGlycan® Software Interface: MS/MS spectrum for the precursor m/z 1023 annotated with fragments of the (GlcNAc)₂(Man)₈ glycan.

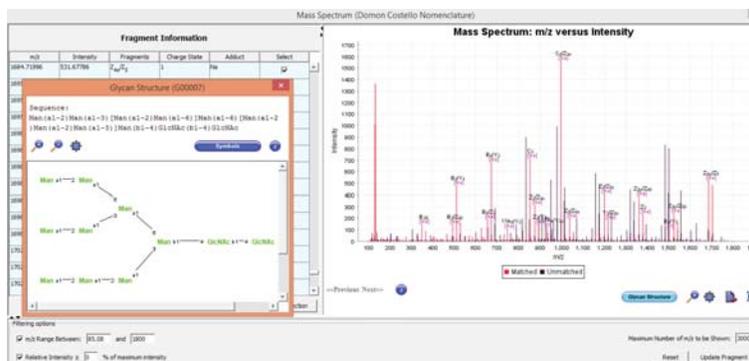


Figure 8: SimGlycan® Software Interface: MS/MS spectrum for the precursor m/z 1104 annotated with fragments of the (GlcNAc)₂(Man)₉ glycan.

Quantitative Data Analysis

The program enables the quantitation by measuring reporter ion peak intensities. Figure 9 shows the SimGlycan® Software Interface wherein parameters for quantitative analysis are specified. We set the search space for each reporter ion to a tolerance of 0.0001 to prevent overlap between the isobaric reporter ions. The program automatically extracts the intensity from all the MS/MS spectra for each TMT channel. Following extraction, the TMT reporter ion signals were adjusted to account for isotopic impurities in each TMT variant as specified in Figure 10.

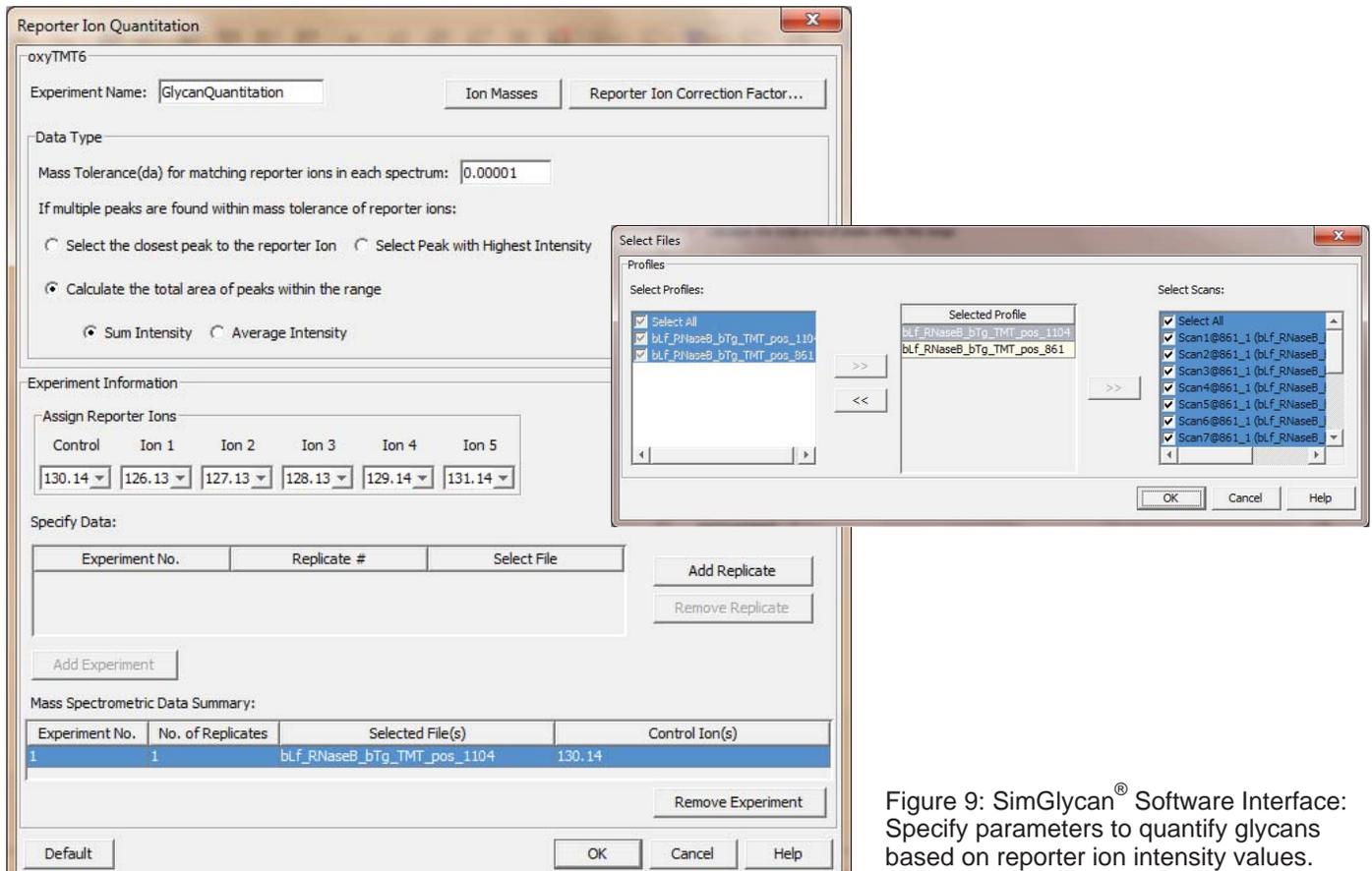


Figure 9: SimGlycan[®] Software Interface: Specify parameters to quantify glycans based on reporter ion intensity values.

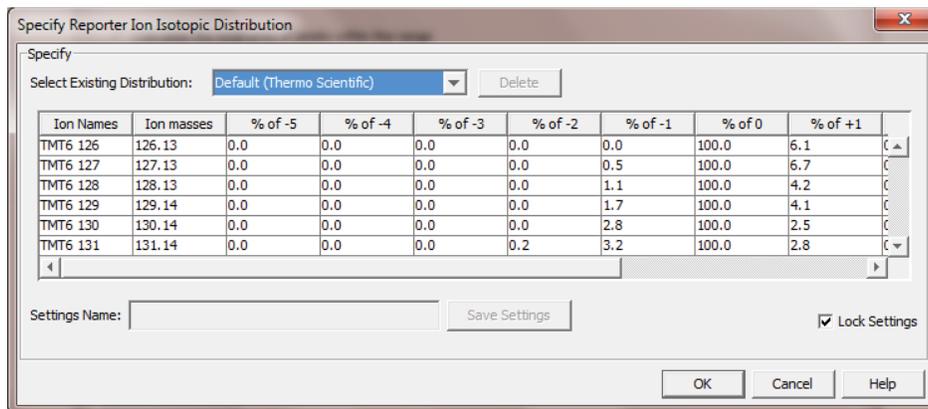


Figure 10: SimGlycan[®] Software Interface: Specify reporter ion isotopic distributions.

For glycan quantitation and comparison, the program identifies the glycan-spectral matches. Reporter ion intensities from different MS/MS spectra are then assembled into glycans. Glycans were then quantified by calculating the sum/average/median reporter ion intensities across all glycan-spectral matches. The program automatically ignores glycan-spectral matches with poor quality MS/MS spectra - more than 4 TMT channels missing - from quantitation.

As analysis summary, the program displays the following statistics as a measure of the relative amount of each identified glycan. The statistics constitute sum/average/median/standard deviation of the reporter ion intensities, ratio sum/average/median intensity of each TMT ion and the control TMT 130.13 ion, Log_2 relative expressions of these ratios, number of MS/MS spectra which identifies each glycan (glycan spectral match/count).

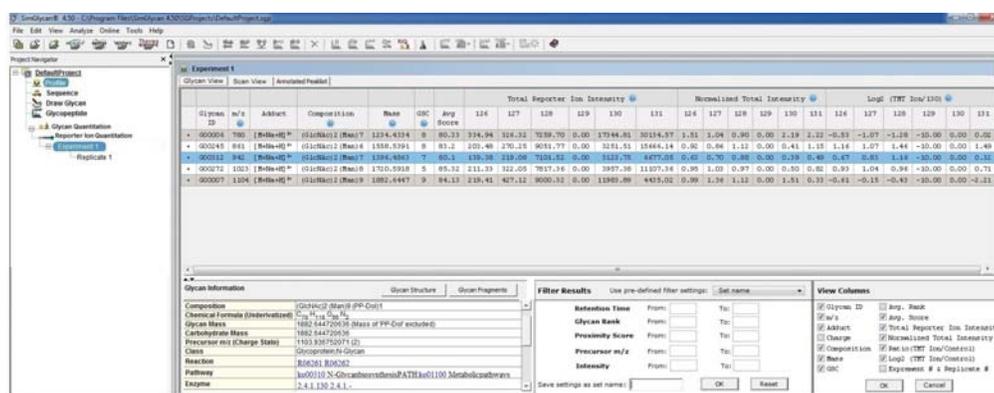


Figure 11: SimGlycan[®] Software Interface: 'Glycan View' displaying identified glycans and their corresponding information.

Figure 11 shows the typical result pane for quantitative data analysis in the SimGlycan[®] Software. Results of the quantitative data analysis are displayed in three different result panes viz. Glycan View, Scan View and Annotated Peaklist. In the Glycan View, all the glycans identified for precursor m/z values are listed along with the following information in each column:

m/z: Observed precursor m/z.

Adduct: Ion species of the precursor m/z.

Composition: Number of different carbohydrate residues present in the glycan.

Mass: Monoisotopic mass of the identified glycan in Da.

GSC: Glycan spectral count i.e., number of MS/MS spectra in which the glycan is identified.

Avg. Score/Avg. Rank: Average of the scores/ranks the glycan received across spectra.

<Total/Average/Median> Ion Intensity: Each sub-column displays the Total/Average/Median intensity observed for the corresponding reporter ion across MS/MS spectra. In case of multiple replicates, it is the average of the Total/Average/Median intensity observed in each replicate. In case of more than one experiment, it is the average across experiments.

Normalized <Total/Average/Median> Ion Intensity: The Total/Average/Median intensity for each reporter ion is calculated based on the average of Total/Average/Median intensity values across observed glycans.

Log₂ (TMT Ion/ <Control Reporter Ion>): It is calculated as follows:

$$\text{Log}_2 \left[\frac{\text{Total/Average/Median Intensity of the reporter Ion}}{\text{Intensity of the Control Ion}} \right]$$

Additional information such as class, link to other databases, reaction, pathways and enzymes, can also be viewed at the lower left pane of the glycan view. The 'Filter Results' pane provides filters to refine glycans for quantitative analysis while the columns to be displayed can be selected using the lower right pane.



Figure 12: SimGlycan® Software Interface: Bar Chart displaying the total ion intensity for glycans in different TMT reporter ions.

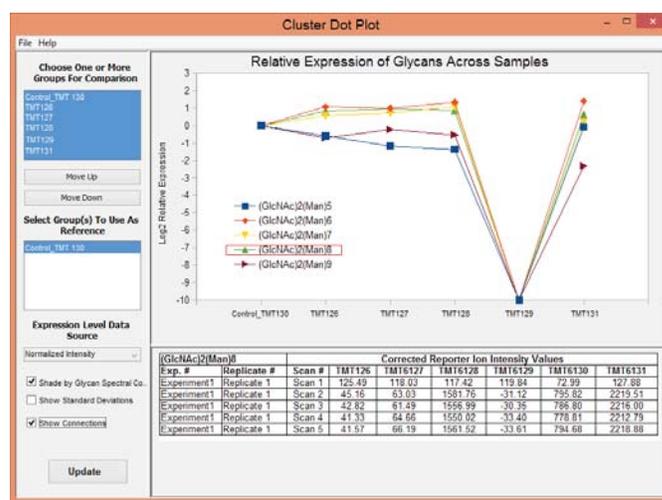


Figure 13: SimGlycan® Software Interface: Cluster dot plot depicting fold changes across TMT channels.

Bar chart (Figure 12) displays the total ion intensity for each glycan at different TMT channels. In the TMT 131, (GlcNAc)2(Man)5 is the most abundant followed by (GlcNAc)2(Man)6, (GlcNAc)2(Man)8, (GlcNAc)2(Man)7 and (GlcNAc)2(Man)9 in decreasing order. Similarly, intuitive observations for other TMT ions are also displayed in the bar chart.

Using the cluster dot plot feature in SimGlycan[®] Software (Figure 13), we perform comparative and differential analysis of the identified glycans in different TMT channels. This graph shows the ratio of the normalized intensities of TMT reporter ions and the control reporter ion in Log₂ scale. The intensities are normalized to the average of the intensities so as to nullify the effect of undersampling or oversampling of glycans in any of the TMT channels. This graph displays the change in glycan expression level across different TMT reporter ion channels with respect to the control TMT 130. The absence of glycans in TMT 129 is evident from the graph. The quantity of (GlcNAc)₂(Man)₆, (GlcNAc)₂(Man)₇ and (GlcNAc)₂(Man)₈ are higher in TMT126, TMT127, TMT128 and TMT131 than that in the TMT130 while the quantity of (GlcNAc)₂(Man)₅ and (GlcNAc)₂(Man)₉ glycans are lower than that in the control.

In addition to bar chart and cluster dot plot, heat map displays relative intensities of TMT reporter ions for each identified glycan (figure not shown). The performance of the software for several standard glycoproteins and monoclonal antibodies was also examined in these studies (Data not shown).

Conclusion

We have investigated the effectiveness of the recently introduced aminoxyTMT Reagents, which enable efficient relative quantitation of carbohydrates, improved labeled-glycan ionization efficiency and increased analytical throughput, for quantitative analysis of native N-glycans by LC-coupled ESI-MS. Labeled glycans were analyzed using Velos Pro mass spectrometer (Thermo Scientific[™]) in the positive ion mode to acquire trap-HCD MS/MS fragmentation.

Large data was generated in the process of analyzing labeled-glycoforms derived from complex mixtures in mass spectrometry workflow. SimGlycan[®] is redesigned to address problem faced by glycomics researchers due to the lack of informatics tools to automate the mass spectrometry based qualitative and quantitative analysis protocols. The fragmentation patterns of the identified glycans in MS/MS spectra, ion species of the product ions etc. are interpreted using SimGlycan[®]. Glycans present in each TMT reporter ion channel are then quantified. Various features such as bar chart, cluster dot plot, heat map etc. facilitate comparative and differential analysis of glycans present in different TMT reporter ion channels.

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