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Comprehensive structural glycomic characterization of the glycocalyx of cells and tissues

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Abstract

The glycocalyx is comprised of glycosylated proteins and lipids and forms the outermost layer of cells. It is involved in a paramount of fundamental inter- and intracellular processes, including non-self and self-cell recognition, cell signaling, cellular structure maintenance, and immune protection. Characterization of the glycocalyx is thus essential for understanding cell physiology and elucidating its role in promoting health and disease. This protocol describes how to comprehensively characterize the glycocalyx N-glycans and O-glycans of glycoproteins as well as intact glycolipids in parallel, using the same enriched membrane fraction. Profiling of the glycans and the glycolipids is performed using nanoflow liquid chromatography-mass spectrometry (nanoLC-MS). Sample preparation, quantitative LC-MS/MS analysis, and data processing methods are provided. Additionally, glycoproteomic analysis is described that yields the site-specific glycosylation of membrane proteins. To reduced the amount of sample needed, N-glycan, O-glycan, and glycolipid analyses are performed on the same enriched fraction, while glycoproteomic analysis is performed on a separate enriched fraction. The sample preparation process takes 2–3 days, while the time spent on instrumental and data analyses could vary from 1 to 5 days for different sample sizes. This workflow is applicable to both cell and tissue samples. Systematic changes in the glycocalyx associated with specific glycoforms and glycoconjugates can be monitored with quantitation using this protocol. The ability to quantitate individual glycoforms and glycoconjugates will find utility in a broad range of fundamental and applied clinical studies, including glycan-based biomarker discovery and therapeutics.

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Author contributions

Q.L., Y.X., M.W., M.B., and C.B.L. contributed to the development of this protocol and wrote and edited the manuscript.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Competing interests

The authors declare no competing interests.

Introduction

A layer of glycan chains known as the glycocalyx covers the cell surface of every living cell. The glycans are conjugated to proteins or lipids, and are organized in complicated and highly interactive networks [1–3]. The glycocalyx is a critical mediator of cell-cell communication events that occur in a variety of biological and physiological processes[4–6]. Its proximity necessarily involves it in a host of common diseases including infection[7–10], cancer[11–14], and autoimmune diseases[15–17]. Despite the importance of glycans and the glycocalyx, their structural analysis has trailed that of other critical biopolymers such as DNA, RNA, and proteins. The inherent complexity and significant heterogeneity of glycans have made their structural elucidation uniquely challenging. The development of plasma membrane enrichment techniques, coupled to highly efficient chromatographic separation methods and ultrasensitive mass spectrometry instruments now allow the analyses of glycans and glycoconjugates in large complicated mixtures at a previously unattainable range of femtomolar to attomolar amounts. Over the last decade, great progress has been made toward the characterization of individual glycocalyx components namely N-glycans and O-glycans on glycoproteins, glycolipids, and glycosaminoglycans (GAGs). These advances have led to the identification of critical glycan structures in normal and pathological conditions[18–22]. Despite many extensive efforts, individual approaches to characterize glycocalyx glycans have yielded fragmented or incomplete pictures of the glycocalyx composition in most cells and tissues studied thus far. To circumvent this limitation, we developed an integrated multiglycomic method based on mass spectrometry (MS) workflows that enables deep and extensive characterization of the glycocalyx, including the identification of the N-glycome[23], O-glycome[24] and site-specific occupancy of cell surface glycoproteins[24,25], as well as intact cell surface glycolipids[26].

Development of the protocol

Specific chromatographic separation and sensitive mass spectrometry have been a key factor in the development of this protocol. Our laboratory has been involved in developing nanoflow liquid chromatography mass spectrometry-based analytical methods for the analysis of naturally occurring free glycans in body fluids such as human milk oligosaccharides (HMO)[27,28]. We have further developed methods to analyze glycans released from glycoproteins[29,30] and glycans conjugated to corresponding glycopeptides[24,31] and glycolipids[26]. Since 2010, we have focused these efforts on the development of methodologies for the characterization of the cell surface glycocalyx in cell lines[23,24,26,32,33] and tissues[34]. These methods have generally involved glycan profiling with nanoflow liquid chromatography-quadrupole time-of-flight mass spectrometry (nanoLC-QTOF MS) and nanoLC-TOF MS. Incorporated later in the workflow is the use of nanoLC-orbitrap MS/MS for glycoproteomic analysis with site-specific characterization.

In the development of these methods, every effort was made to minimize the amounts of samples, whether it be cell lines or tissues. For this reason, nanoflow liquid chromatography is used throughout the analysis because of its high sensitivity. Additionally, efforts were made to perform several analyses on the same sample. Thus, from the same starting plasma membrane material all the N-glycans, O-glycans, and glycolipids were characterized by

first releasing the N-glycans, then extracting the glycolipids, and finally releasing the and characterizing the O-glycans. A separate portion of the plasma membrane was analyzed for protein and glycoprotein analysis, because the glycan-depleted fractions could not be used for glycoproteomic analysis.

Advantages and comparison to alternative methods

The most commonly used method for characterizing the glycocalyx employs lectins, which are proteins that recognize glycans through specific structural motifs. Lectins are convenient, rapid, and require no additional complicated or large instrumentation. Fluorescently-labeled lectins are readily available to characterize the cell surface glycocalyx using microscopy. However, this approach has severe limitations. Lectins provide information about specific structural motifs but are unable to determine complete glycan structures, glycan composition, or differentiate main classes of glycans. For example, *sambucus nigra* (SNA) lectin recognizes α(2,6)-linked sialic acid on galactose but it cannot distinguish whether the sialic acid is on N-glycan, O-glycan or glycolipid. Similarly, Concanavalin A (Con A) may detect a-mannose, which is presented in nearly all N-glycans, and therefore cannot distinguish between high mannose-, complex-, or hybrid-type glycans. Lectins also provide little quantitative information. For example, an SNA lectin may be used to determine changes in the presence of α(2,6)-linked sialic acid. However, it cannot quantify the types of glycan that are varied. Responses of lectins with different specificities also cannot be quantitively compared.

The glycomic analysis in this protocol is conducted on native glycans, i.e., without labeling. This method minimizes sample losses due to sample manipulation and incomplete reactions associated with labeling. Various labeling methods have been employed for glycomic analysis, including reactions with 1-phenyl-3-methyl-5-pyrazolone (PMP), 2-aminobenzamide (2-AB), and 2-aminobenzoic acid (2-AA)[35]. Although the derivatization improves the ionization efficiency of glycans, the complicated sample preparation and clean-up lead to potential losses, particularly those with relatively low abundances.

For the glycoproteomic analysis, glycopeptides are enriched with solid phase extraction hydrophilic interaction liquid chromatography (SPE HILIC) cartridges. The process yields 90% glycopeptides and some remaining nonglycosylated peptides. This enrichment method is based on hydrophilic interactions between the glycans and the stationary phase. The procedure is relatively simple and applicable to large sample sets for rapid throughput analysis. Other enrichment methods with different binding interactions can also be incorporated into this protocol. For example, lectin chromatography may be used to enrich N-glycopeptides, through the binding of specific structural glycan features[36]. However, the buffers for enrichment that use high concentrations of NaCl and Tris-HCl are not compatible with MS analysis, and further clean-up steps may be required before MS[37]. The enrichment of glycopeptides through click chemistry is an effective approach for glycoproteomic analysis. The method involves the metabolic labeling of glycoproteins with unnatural monosaccharides such as N-azidoacetylmannosamine (ManNAz) and N-azidoacetylgalactosamine (GalNAz) followed by enrichment through copper-free click chemistry[38]. This method has been applied to a host of biological systems such as

membrane glycoproteins of primary neurons[39] and pancreatic cancer cell lines[40]. However, the utility may be complicated by the reaction efficiencies of the click chemistry and the incorporation of monosaccharides analogues, which varies among cell lines.

Liquid chromatography (LC) is used for compound separation and is currently the most effective technique for separation. In this protocol, different stationary phases for reverse phase including porous graphite carbon (PGC) and C18 are used for the separation of glycans, glycolipids and glycopeptides. Normal phase such as HILIC can also be applied[41,42]. The LC separation is applicable to both native and labeled glycans. Standard, ultrahigh-pressure, micro, and nanoLC can all be employed with their respective flow rates, pressure, detection sensitivities, and separation efficiencies. In this protocol, the application of nanoLC enables the analysis with high sensitivity. Other separation techniques can also be employed for the analysis of glycoconjugates. For example, capillary electrophoresis (CE) has been shown to have high efficiency for glycan and glycoprotein separation. Previously, the coupling of CE with MS had been challenging due to low flow rates of CE; however, this issue has been addressed more recently with techniques such as a sheath-flow interface[43]. Commercial CE systems are available and have been applied to native and digested glycoproteins[44]. However, the application of these systems toward glycoconjugates from complicated biological samples is still limited[45]. Ion mobility (IM) has held great promise for rapid separation of glycans and glycoconjugates. Compounds are separated based on their collision cross sections and charges thereby enabling the separation of glycan isomers[45,46]. IM-MS of glycans released from different biological samples such as human serum[47] and the parotid gland[48] have been reported. However, the separation resolution of glycans with IM is still severely limited. The employment of IM-MS to glycopeptide analysis is even more complicated because of the high numbers of isomers, gas-phase conformers due to the large similarities in structures[45].

An alternative to the LC-MS method is matrix-assisted laser desorption/ionization (MALDI). MALDI is more tolerant of high concentrations of salts and contaminates.[49] It has been coupled to mass detectors with high resolution and mass accuracy such as Fourier-transform ion cyclotron resonance (FTICR) and time-of-flight (TOF). MALDI MS is significantly more rapid and can provide comprehensive glycomic profiles of released glycans. However, MALDI MS is not as sensitive as nanoLC-MS. Also, MALDI does not provide separation of isomers, although it can be coupled to LC if fractions are collected and individually probed. Without pre-separation, the glycan profile obtained from MALDI MS is essentially a mass profile and is not as extensive as those obtained from LC-MS.

The most commonly used MS fragmentation technique used for glycan and glycolipid analyses is collision induced dissociation (CID). The dissociation of glycans using infrared multiphoton dissociation (IRMPD) yields similar fragments to those from CID[50]. It has been extensively used but is not readily available[51]. For glycopeptides, stepped high-energy collisional dissociation (HCD), a marketing term for a higher energy form of CID, is used. The fragmentation of glycans with CID cleaves the glycosidic bonds. For glycopeptides, CID generates mainly fragments for glycans, which have more labile bonds, but also peptide bond cleavages with lower abundances. With the stepped HCD, both glycans and peptides are fragmented with higher efficiency[52]. Another fragmentation technique applied

to glycopeptides is electrotransfer/higher-energy collision dissociation (EThcD), which yields abundant fragments corresponding to both glycans and peptides[53]. However, EThcD requires longer duty cycles, which may lead to lower identification rates for glycopeptides.

Experimental design

Overview of the protocol.—The workflow for the analysis of the glycocalyx of cells consists of the steps outlined in Figure 1 and Figure 2. Cells are first harvested, and the membrane disrupted. Enrichment of the plasma membrane is performed by a series of centrifugation and ultracentrifugation steps (Figure 1). The membrane pellet is divided into two fractions to perform the glycomic analysis and the glycoproteomic analysis. One fraction of the membrane is used for the release and extraction of glycans (N- and O-) and glycolipids, followed by their separate LC-MS analyses, while a second fraction is used for proteomic and glycoproteomic analyses. Glycomic profiles of N- and O-glycans are performed on released compounds using nanoLC-TOF and QTOF MS employing porous graphitized carbon chromatographic chip. Glycolipid profiling is performed on intact compounds using nanoLC-QTOF MS with a C18 chip column. Agilent Corp. (Santa Clara, Ca) MassHunter software is employed for data processing and in-house libraries are used for the identification of N-glycans, O-glycans and glycolipids. Peptide and glycopeptide analyses are performed using nanoLC-Orbitrap MS with C18 chromatographic columns. Identifications of peptides and glycopeptides are performed using the Protein Metrics package (Cupertino, CA), which includes Byonic for glycoproteomic analysis and Byologic for glycopeptide quantitation. While the description is primarily for cultured cells, mouse tissue samples such as brain, liver, spleen and lung can also be processed and analyzed in this manner[34]. Representative data acquired from N-, O-glycomic, and glycolipidomic analyses of mouse brain samples are illustrated in Figure 3. Additionally, the methods can be adapted in parts to include, for example, only N- or only O-glycan profiling.

The protocol enables the quantitation of glycans through ion counts obtained directly from the nanoLC-MS system as peak areas (or volumes). While absolute abundances are not achievable due mainly to the lack of standards, ion counts allow measurements of absolute changes and relative abundances. These values can be further normalized to the amount of starting material (either cell counts or protein amounts). With the normalized abundances, changes in glycocalyx compositions under different conditions can be monitored and compared accurately. To obtain high reproducibility, serially diluted quality control samples were used to determine the variations in the quantitation of compounds. The linearity of selected compounds were also determined in this way. When standards do become available in the future, they can be readily incorporated either through standard addition or directly by using stable isotope labeling.

Sample preparation and cell membrane extraction (Steps i)-11).—Cell lines should be cultured following the protocols provided by the American Type Culture Collection (ATCC) or from literature advice. We have successfully used human cell lines including normal prostate immortalized cell line PNT2, colorectal adenocarcinoma cell line Caco-2, lung carcinoma cell line A549 and pluripotent cell line NTERA-2. The morphology of cells should be checked regularly, at least once every other day. The method can also

be applied to other cell lines. Each cell line will have special care guidance that should be noted. For example, some cell lines are sensitive to the amount of glucose in the media,such as HT-29[54] cells, which is used as illustration in this protocol, and differentiates if there is a decrease in the glucose concentration in the media. To prevent differentiation, we recommend changing media for HT-29 cells every day and splitting cells at near 80% confluency.

For a comprehensive analysis, a total of at least ten million cells are required, and these need to be separated into two (Fraction I and II). Fraction I, for glycomic (N- and O-glycans) and glycolipid analyses require fewer cells (approximately 1×10^6 cells). Fraction II, for site-specific glycoproteomic analysis requires the larger fraction of cells (at least 9×10^6 cells). Immediately after collecting cells, it is important to undertake buffer exchange and homogenization at 4 °C to prevent the untargeted modifications of proteins and to minimize the activity of endogenous proteases[55]. Analysis of flash-frozen tissue samples can be performed following the same procedure. Upon collection, tissues can be immediately processed or flash-frozen at −80 °C until further processing. For direct analysis, rinse tissues with PBS, add the homogenization buffer, keep samples at 4 °C, and continue with the homogenization step. The ultrasonic homogenization protocol we describe is optimized for mammalian cell lines[32] and soft-tissues. For more fiborous or harder tissues such as cartilage, more intense homogenization conditions should be employed[34,56].

The membrane extraction method we describe was adapted from a previously described protocol[32]. The cell membrane is extracted after harvesting and lysing cells and tissues. The nucleus and mitochondria are removed by centrifugation at low speed, followed by pelleting of the cell membrane fraction using ultracentrifugation. By introducing sodium carbonate solution, membrane-associated proteins that are noncovalently bound can be separated and dissolved away while the membrane fraction is pelleted to obtain a cleaner membrane fraction [57,58]. The extraction of the cell membrane can be achieved with minimal sample preparation and is compatible with mass spectrometry analysis. The resulting membrane pellet is then ready for further processing and analysis. *Fraction I* is subjected to comprehensive glycomic and glycolipidomic analyses. Each sample derived from this fraction has sufficient amounts for injections (3–4 times). *Fraction II* is subjected to glycoproteomic analysis, with the final sample sufficient for 1–2 injections. The workflow for cell sample collection and membrane fractionation is illustrated in Figure 1.

N-Glycan analysis: Release, purification, and mass spectrometry (MS) measurements (Steps 12–33).—After cell membrane extraction, N-glycans are released from the glycoproteins using the enzyme peptide-N-glycosidase F (PNGase F). Membrane proteins should be denatured by heating for up to 2 minutes before N-glycan release, in order to achieve higher digestion efficiency. The complete release of N-glycans is required because the remaining N-glycans can interfere with O-glycan analysis. Microwaves can be used to efficiently and rapidly remove the N-glycans from the proteins[59]. To minimize the content of amine-containing N-glycans, incubation at 37 °C in a water bath should be conducted after the N-glycan release to complete the hydrolysis of the glycan-amine to the glycan-aldehyde. Alternatively, an amine-free buffer such as PBS or HEPES can be used to avoid amine groups[60]. Glycans containing α(1,3)-linked fucose (primarily from

plant proteins) cannot be cleaved by PNGase F. For these compounds, the enzyme PNGase A is employed[61]. Upon digestion, released N-glycans are collected in supernatants after ultracentrifugation. Ethanol precipitation can also be used by adding 4 volumes of ethanol to the digestions solution and incubating at −20 °C overnight or −80 °C for 2 hours (h), followed by centrifugation to precipitate proteins completely. The pelletized membrane without N-glycans should be saved for subsequent O-glycomic and glycolipidomic analyses. The supernatant containing N-glycans is subjected to SPE clean-up with PGC in cartridges or 96-well plates for large-batch preparation.

Desalted native N-glycans are injected into a nanoLC-TOF-MS/MS equipped with a PGC nanochip for analysis directly. Direct analysis of native samples avoids loss from incomplete modification associated with all derivatization methods[62]. The use of nano-LC provides high sensitivity and large dynamic range, enabling the identification of low-abundance species. Normal-flow or micro-flow LC systems can also be employed for analysis of samples but require significantly higher amounts of material. Separation of N-glycans in PGC columns split the anomers for most but not all N-glycans. Thus, a single structure will yield two retention times. To avoid splitting of the anomeric species, the N-glycans can be reduced using the same method as O-glycans to remove anomers if desired (Steps 75–78). In addition, the PGC chip yields the separation of isomeric glycans and has been demonstrated in several previous publications[33,63,64]. Furthermore, for the instrument quality control (QC), the pooled released N-glycans from several cell lines under the normal condition (Caco-2, PNT2, and A549) can be used. We also suggest the use of a commercial pooled serum and RNaseB N-glycan sample to perform quality control (QC) of the method and associated instrumentation if no N-glycans from cell lines are collected. A typical example of an extracted compound chromatogram (ECC) for N-glycans is shown in Figure 4a. The major peaks are annotated; however, over 500 compounds (including isomers) are observed. The separation of isomers is readily illustrated in the ECC. An ECC for QC N-glycans is shown in Supplementary Figure 1a.

Glycosphingolipid analysis: Extraction, purification, and MS measurements (Steps 44–56)).—Glycosphingolipids are amphipathic compounds, and care should be taken to keep the purified glycosphingolipids dissolved to prevent loss through surface adsorption. A solution containing at least 50% of an organic solvent such as methanol should be used on purified glycosphingolipids. Initial testing of pipets and vials should also be considered as surface adsorption can lead to poor recovery. The method we present here avoids the use of detergents and surfactants as they are incompatible with MS analysis.

Folch[65] or Bligh-Dyer[66] extraction is applied to the membrane pellet from the previous step to separate glycolipids from other lipid species such as phospholipids and cholesterol. The latter lipids are more abundant than glycosphingolipids by 1 or more orders of magnitude, and they can mask the analyte signal during MS analysis if not removed. Other extraction systems can also be applied, although Folch/B&D extraction is advantageous for having the methanolic/aqueous phase as the top layer in the biphasic solvent extraction system. It should be noted that the recoveries of different glycosphingolipid species depend on the characteristics of both the glycan and the lipid. Glycosphingolipids with charged groups such as sialic acids have very good recovery (>90%) even when they have longer lipids.

Those with three or fewer neutral monosaccharides and longer lipids have poorer recoveries of less than 50%. Cerebrosides, which have only one monosaccharide residue, are not effectively recovered; about 90% of cerebrosides partition to the chloroform layer.

Internal standards such as deuterated glycolipids can be added to the sample prior to extraction for absolute quantitation. Internal standards of several glycolipid species are now commercially available from lipid-focused companies such as Avanti Inc. (AL, US) and Matreya Inc. (PA, US), including sialylated gangliosides, sulfatides, and neutral glycolipids. These companies also supply purified glycolipids and glycolipid reference mixtures that can be used for quality control and method optimization.

The samples are enriched with C8 SPE and analyzed with nanoLC-TOF-MS/MS equipped with a C18 nano-chip. The instrument method applies data-dependent acquisition (DDA) to obtain MS/MS spectra, which is necessary for the confident identification of glycosphingolipid species. Optimization of instrument parameters is critical for the effective fragmentation of glycosphingolipids, which will allow identification of both the ceramide (lipid) and glycan composition. Quality control samples can be obtained commercially from the aforementioned companies, or prepared from cultured cells such as Caco-2 or PNT2. Each cell line can express a limited variety of lipid compositions and glycan motifs. Glycolipids from several cell lines can be pooled together to include a larger variety for quality control. For example, the pluripotent cell line NTERA-2 can provide Stage Specific Embryonic Antigens (SSEA) 3 and 4, which are globo-type glycolipids that are expressed only by stem cells. An ECC of glycolipids from the glycocalyx of a cell line is shown in Figure 4c. In a typical chromatogram, over 200 compounds (including isomers) are observed. An ECC for QC glycolipids is shown in Supplementary Figure 1c.

O-Glycan analysis: Release, purification, and MS analysis (Steps 73–89).— The pellets collected from Folch extraction are subjected to O-glycan release through β-elimination. Organic solvent left from Folch extraction should be removed before the release procedure. We recommend using larger volume (4–5 mL) screw-cap tubes for the reaction because large amounts of gas may be released and cause sample loss if the smaller 1.5 mL snap-cap eppendorf tubes are used. After overnight incubation, the basic mixture is neutralized with acetic acid. The whole process should be conducted on ice to avoid overheating. Care should also be taken when adding acetic acids, adding small aliquots is recommended to prevent more intense reactions that can cause the liquid to overflow. After centrifugation at high speed, O-glycans are collected from the supernatant, and the pellet can be discarded. At this point, the desired N- and O-glycans, as well as glycolipids, are all obtained from Fraction I. Chemical release of O-glycans using mild conditions, such as ammonium carbamate, can be employed but this is not recommended due to the low efficiency of the cleavage resulting in the potential loss of low-abundance compounds[67]. Cleaved O-glycans should be enriched on PGC SPE plates for desalting, and the glycans are further cleaned from small background peptides using iSPE HILIC cartridges/plates. At least five washes are recommended after sample loading because there is a large amount of chemically produced peptides due to the beta-elimination process. The same LC-MS instrument used for N-glycan analysis should be used for O-glycans. Some parameters need to be adjusted such as the mass range, which should be changed from 600 to 2,000

m/z to 300–2,000 *m/z* due to the relatively smaller sizes of O-glycans. For the instrument quality control (QC), the pooled released O-glycans from several cell lines under the normal condition (Caco-2, PNT2, and A549) can be used. A typical ECC of O-glycans from the glycocalyx of a cell line is shown in Figure 4b. The abundant species are annotated with typically over 50 compounds (including isomers). An ECC for QC O-glycans is shown in Supplementary Figure 1b.

Glycoproteomic analysis: Protein digestion, enrichment, and MS analysis (Steps 100–117).—A comprehensive glycoproteomic analysis is conducted on extracted cell membranes. First, membrane proteins should be dissolved and denatured with urea for better digestion[68], followed by the addition of DTT and alkylation with IAA to break S-S bonds. Samples are then digested with trypsin at 37 °C for 18 h. To obtain more comprehensive glycosylation site-specific mapping results, other enzymes with different specificities may be used in combination with trypsin such as Glu-C[69] and Lys-C[70]. Due to the lower ionization efficiencies of glycopeptides and possible ion suppression effects from coeluting peptides, the enrichment of glycopeptides is necessary before MS analysis. We suggest using iSPE HILIC cartridges, which give high enrichment efficiency. We have also tried the iSPE HILIC plates for large sample sets. However, the plate should not be centrifuged because this can cause the solid phase to dry out, leading to the loss of glycopeptides. With enrichment, the percentage of glycopeptides can be increased to 90% of all detected peptide species.

Glycopeptides are subjected to nanoLC-Orbitrap Fusion Lumos for MS/MS analysis. It is important to determine the sample concentration before the injection. The concentration should be adjusted to 1 μg/μL, with 1 μg used for injection. Oversampling could speed up column aging and may even cause clogging. We suggest that a digested serum glycopeptide sample be used for quality control (QC) of LC-MS and overall sample preparation. Typical glycoproteomic data from serum standards are illustrated in Figure 5. It is critical to use appropriate orbitrap parameters to obtain high-quality spectra, including the automatic gain control (AGC) and the maximum ion injection time (MIIT). Lower AGC and MIIT values will result in poor MS2 spectra, due to the low abundances of collected precursors. However, large AGC and MIIT values require longer accumulation times resulting in a decrease in identified species[71]. In addition, higher AGC values can result in space charge effects leading to poor mass accuracy and decreased identification rates[72]. For the fragmentation, higher-energy collision dissociation (HCD) with stepped-collision energy is employed, providing high-quality MS2 spectra for glycopeptides[73]. The spectra are obtained with the combination of fragmentations using three different collision energies. With the stepped-collision energy HCD, both glycans and peptide backbones are well fragmented. The glycan fragments can have higher intensities than those of peptide fragments in HCD spectra. Other fragmentation methods such as electron-transfer/higher-energy collision dissociation (EThcD)[74], HCD triggered ETD[75], and activated ion ETD (AI-ETD)[76] can also be used.

Data processing.—To process the glycomic data (Steps 34–43, 90–99), our in-house library based on the putative biosynthetic pathways[77] and previously discovered glycan masses is used with the compositions confirmed by tandem MS (MS/MS). There is no single

curated database of possible glycan masses. However, several compilations are published and can be used for compound searches. A neural network derived glycan library derived from putative structures is published[78]. Glycan libraries of different species, including those with varied monosaccharides, for example xylose for plants[79], N-glycolylneuraminic acid (Neu5Gc) for non-*Homo sapiens* mammals[80,81], and N-acetylmuramic acid (MurNAc) for bacteria[82] exist. Other specialized glycans may also be added. For example, the 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) incorporated glycoconjugates could be included in the library for zebrafish[83]. Even glycans with larger monosaccharide modifications such as those found with 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) expressed in *Neisseria meningitides*[84] may be included. Additionally, compositions of glycans containing unnatural monosaccharides such as N-azidoacetylgalactosamine (GalNAz) and N-azidoacetyl sialic acid (SiaNAz) can also be included. In general, care should be taken to ensure that the glycan compositions are confirmed by tandem MS. If only MS1 is used, naturally occurring glycans with combinations of, for example, $Sia_1HexNAc_xHex_y$, could be misassigned to $SiaNAz_1HexNAc_{(x-1)}Hex_{(y+1)}$. Glycans with unnatural monosaccharides can also complicate the analysis but benefit from further MS/MS characterization. An example of the MS/MS spectra of unnatural monosaccharides incorporated N-glycans and assigned fragmentations are illustrated in Figure 6. Glycans containing SiaNAz yield fragment ions with *m/z* values of 315.09 $[SiaNAz\text{-}H_2O+H]^+$ and 333.10 $[SiaNAz+H]^+$ in the MS/MS spectra.

A library containing reduced O-glycan masses is useful for the data analysis of O-glycans. O-Glycomic profiles yield masses that are larger than the native structure by 2.016 Da, corresponding to the addition of two hydrogens. This modification stems from the reduction of the aldehyde after β-elimination. Thus, the entries in the glycan library should not only consider the possible compositions based on synthetic pathways but also potential modifications introduced during sample preparation. With the positive mode sulfated O-glycans can also be identified (Supplementary Figure 2). The N-glycans need to be rigorously removed because they may interfere with the O-glycan analysis. N-Glycans can dissociate under basic conditions to yield fragments corresponding to the peeling reaction. However, the unique O-glycan core structures and reduced compositions can be used to distinguish them from fragmented N-glycans.

The data analysis of intact glycosphingolipids (Steps 57–72) has the added complexity of combining possible glycan compositions with ceramides of different hydrocarbon lengths, degrees of unsaturation, and numbers of hydroxyl groups. The identification of ceramides and glycans is based on MS/MS fragmentation data. There is currently no software that automates this process. In this protocol, we offer some tools that can assist with the correct identification of glycosphingolipid species and for generating a subset library from a database that contains the neutral monoisotopic mass and chemical formulae of intact glycosphingolipids. We built upon work previously done by Merrill (www.sphingomap.org)[85] in collating possible glycan structures and incorporated their compositions into intact glycosphingolipids. It is important to form a subset library because the combination of so many glycan and ceramide moieties contains many isobaric compounds, and an uncurated list can lead to false identifications or misassignments. Additionally, it is also important to create a new library for each kind of sample analyzed as

the expression of glycosphingolipids can differ based on cell type, species source, and even among individuals of the same species.

For glycoproteomic data processing (Steps 118–125), Byonic software is used to identify the glycopeptides, and the results can be directly applied for label-free quantitation using Byologic software. Other software, such as pGlyco, can also be used to identify glycopeptides[86]. The data analysis with Byonic™ software provides information, including glycopeptide amino acid sequences, glycosylation sites, and tentative glycan compositions. Although a general glycan library from an open source can be used for glycopeptide identification, before conducting glycoproteomic analysis on an unknown cell or tissue sample we suggest doing the glycomic analysis first to calibrate the specific glycan library for the cell type and to use the calibrated glycan library for glycopeptide data analysis. This not only reduces the search time (especially for O-glycopeptide mapping) but also minimizes false-positive identification. Several parameters including DeltaMod, Byonic scores, and |Log Prob| are recommended for screening the results to control the FDR further and eliminate misassignments[87]. The DeltaMod of 10.0 is the minimum threshold for confident modifications. Byonic scores indicate the quality of the peptide-spectrum match and, as illustrated by the software developers, a score of over 300 is a good score for the match. |Log Prob| reflects the posterior error probability, and results with |Log Prob|<2 (error probabilities > 0.01) are usually removed. Examples of site-specific mapping of N- and O-glycoproteins, as well as quantitation of glycopeptides are shown in Figure 7.

Limitations of the protocol

A limitation of the glycomic analysis method is the requirement for specialized instruments that are large, centralized, and expensive. While LC-MS methods provide comprehensive information, users require specialized training. However, LC-MS instruments are now more widely available in most departments and institutions. Nearly every researcher has access to an LC-MS instrument even when it is not directly available in their respective laboratory. Moreover, by clearly describing the methods for glycomic analysis, we hope to make the method more readily available so that the lack of expertise will be less of a barrier. In this protocol, we use LC with nano-flow coupled to high-resolution MS QTOF and orbitrap to achieve better sensitivity and higher mass accuracy. Better sensitivity facilitates the identification of a higher number of compounds, and the higher mass accuracy contributes to more confident identifications. This protocol can also be used with standard LC flow rates and lower resolution mass spectrometers; however, it would require larger sample sizes with less certainty in the identification. More certainty can be obtained with tandem MS (CID), which yields distinct fragment masses even with low mass accuracy instruments.

Another potential limitation is that interpreting the LC-MS data is extremely time-consuming. Specialized software are available but wedded to specific instrument manufacturers. For example, the software used in this protocol for glycomic and glycolipidomic data analyses is MassHunter, which was developed specifically for Agilent Inc. instruments. However, software that are used for other applications such as proteomic can be adapted to glycans. They include Skyline and mzMine 2 for processing the data acquired from other instruments. For glycoproteomic analysis, we include the details

regarding the use of Byonic, which is a commercial software appropriate for analyzing peptides with glycosylation and other post-translational modifications. Other software such as MaxQuant and pGlyco can also be used for glycomic and glycoproteomic analysis. In addition, using ion counts for quantitation is the best we have, but it does have intrinsic limitations, with the most significant being that not all compounds have the same ionization and detection responses. Nonetheless, it is accurate for measuring fold changes of individual compounds, but perhaps not as accurate for comparing abundances of different compounds in the same run.

Other limitations are associated with the intrinsic nature of glycans and their structures. For example, although LC-MS can provide extensive separation of isomers, it yields little structural information. Determining the structures requires extensive use of exoglycosidases, which have been performed to determine the glycome of serum[88,89]. Similarly, glycoproteomic analysis gives the best results when the glycopeptides are enriched. This means that to obtain an extensive (glyco)proteomic analysis, two analyses need to be performed: the first one is the peptide analysis (proteomic), followed by a glycopeptide analysis (glycoproteomic). Within the glycoproteomic analysis, relative abudances of each glycoform can be obtained; however the site-specific occupancy can only be inferred if the total abundances of all glycoforms are compared. Accurate quantitation of site occupancy requires the deglycosylation and analysis of both aglycosylated and non-glycosylated peptides with LC-MS[90]. Other issues are encountered in the glycoproteomic analysis including misassignment of glycan structures and ambiguous localization of glycosylation sites, especially for N-glycopeptides with two potential glycosites within one tryptic peptide. Thus, it is worthwhile checking the MS/MS spectra of those suspected compounds. O-Glycopeptides may also be difficult to assign as the sites do not have a consensus sequence. For the enrichment of O-glycans using iSPE-HILIC cartridges, some short O-glycans may be lost due to the potential bias of the HILIC enrichment toward more elongated glycans.

GAGs including heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronan are linear polysaccharides consisting of multiple disaccharide repeating units (mostly an amino sugar and a uronic sugar or galactose) and play significant roles in a variety of biological functions[91]. However, due to the structural and chemical nature of GAGs, their analysis require completely different methods with different MS techniques that are beyond the scope of this protocol.

Future applications

Glycans and glycosylation impact nearly every aspect of biology. Estimates are that 50% of all proteins, and nearly 80% of human proteins are glycosylated. As this protocol offers a nearly complete rendering of all glycans(glycosaminoglycans notwithstanding) in any biological sample, its utility will cover nearly every aspect of biology. In addition, this workflow could be readily incorporated with other techniques, including proteomic analysis and metabolic labeling. The more specific and immediate applications include i) The broad characterization of cell lines from different species. ii) The glycomic characterization and quantitation of cell membranes subjected to enzyme inhibitors and gene modifications. iii) The discovery and development of glycan, glycoprotein, and glycolipid biomarkers for

diseases. iv) Studies of host-microbe interactions and the role of glycocalyx in adhesion and invasion. v) Studies of protein functions and structures, including cell surface receptors such as Siglecs, channels, enzymes, and pumps. vi) Studies of cancer cell physiology and the role of glycans in cell adhesion and signaling.

These methods will also have broader impacts in diverse areas of research where glycoconjugates (lipids and proteins) are involved. These research areas include cancer diagnostic, progression and therapy, infection, immunology, and diet and nutrition. In the study of cancer progression and therapy, it is known that the glycosylation of cells differs at different stages in cancer progression. Glycan changes are also seen during tumor growth and metastasis. All approved cancer protein biomarkers are glycosylated. Additionally, many new cancer therapeutics are glycosylated, and their targets on the cell membrane are glycoproteins. Secondly, the glycocalyx is also important in understanding host-microbe interactions. The binding sites on host cells are often glycans on proteins and lipids, which mediate microbe adhesion and invasion. Bacteria and viruses also have glycan-binding proteins and infect host cells with associated glycan structures. In immunology, immune cells and antibodies directly recognize glycans on glycoproteins and glycolipids. Activated immune cells are affected by glycosylation. A better understanding of the role of glycosylation in immunity and autoimmunity could provide better therapies. Finally, commensal bacteria consume oligosaccharides provided by the host, such as those in human milk, and mucins on the cell membrane. Oligosaccharides and glycans are currently revolutionizing our understanding of how the microbiome functions in diet and nutrition studies.

Materials

Biological materials

- PNT2 cells (Sigma, cat. no. 95012613, RRID: CVCL-2164)
- Caco-2 cells (ATCC, cat. no. HTB-37, RRID: CVCL-0025). ! CAUTION This cell line will differentiate when it reaches 100% confluency without any chemical triggering.
- A549 cells (ATCC, cat. no. CCL-185, RRID: CVCL-0023)
- NTERA-2 cl.D1 cells (ATCC, cat. no. CRL-1973, RRID: CVCL-3407)

CAUTION The cell lines used in the research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Tissue sample of interest (i.e., brain, liver, spleen, etc.) should be collected in a clean tube of adequate volume (1.5, 2, 5ml), snap-frozen in liquid N_2, and stored at −80°C until further analysis

Reagents

- Fetal Bovine Serum (FBS, Thermo Fisher Scientific, cat. no. 16000–069)
- Penicillin Streptomycin (P/S, Thermo Fisher Scientific, cat. no. 15140–122)

- Trypsin-EDTA (1X, Thermo Fisher Scientific, cat. no. 25300–054)
- Dulbecco's Phosphate Buffered Saline (D-PBS, 1X, ATCC, cat. no. 30–2200)
- Sucrose (BioXtra, Milliporesigma, cat. no. S7903)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer, 1M, Thermo Fisher Scientific, cat. no. 15630080)
- Potassium hydroxide (KOH, BioXtra, Milliporesigma, cat. no. P5958) ! CAUTION Potassium hydroxide causes severe skin burns and eye damage.
- Sodium carbonate (Na_2CO_3, BioXtra, Milliporesigma, cat. no. S7795) ! CAUTION Sodium carbonate causes serious eye irritation.
- Ammonium bicarbonate (NH_4HCO_3, BioUltra, Milliporesigma, cat. no. 09830) ! CAUTION NH_4HCO_3 irrates skin, eyes and respiratory system.
- Dithiothreitol (DTT, molecular grade, Promega, cat. no. V3151) ! CAUTION DTT causes skin and eye irritation and may cause respiratory irritation.
- Iodoacetamide (IAA, BioUltra, Milliporesigma, cat. no. 144–48-9) ! CAUTION Iodoacetamide may cause an allergic skin reaction.
- Sodium hydroxide (NaOH, anhydrous, Milliporesigma, cat. no. 795429) ! CAUTION Sodium hydroxide causes severe skin burns and eye damage.
- Sodium borohydride ($NaBH_4$, Supelco™, Milliporesigma, cat. no. 1.06371) ! CAUTION Sodium borohydride causes skin corrosion, serious eye damage, and reproductive toxicity.
- Formic acid (FA, Optima™; Fisher Scientific, cat. no. A117–50) ! CAUTION Formic acid is flammable liquid and vapor.
- Trifluoroacetic acid (TFA, Pierce™, ThermoFisher Scientific, cat. no. 28902) ! CAUTION Trifluoroacetic acid causes severe skin burns and eye damage
- Acetonitrile (ACN, CHROMASOLV™, Honeywell, cat. no. 34967) ! CAUTION Acetonitrile is flammable liquid and vapor.
- Water (H_2O, Pierce™, ThermoFisher Scientific, cat. no. 51140)

  ▨ CRITICAL This water is used only for the nanoLC system in the glycoproteomic analysis.
- Methanol (MeOH, Milliporesigma, cat. no. 34860) ! CAUTION Methanol is flammable liquid and vapor.
- 2-Propanol (IPA, Milliporesigma, cat. no. 34863) ! CAUTION Isopropyl alcohol is flammable liquid and vapor.
- Chloroform ($CHCl_3$, Milliporesigma, cat. no. 34854) ! CAUTION Chloroform is flammable liquid and vapor. It is also acutely toxic.
- Ammonium acetate (Supelco™, Milliporesigma, cat. no. AX1222)

- Acetic acid (glacial, Alfa Aesar™, ThermoFisher Scientific, cat. no. AA39745AE) ! CAUTION Acetic acid causes severe eye and skin burns, and acetic acid is flammable liquid and vapor.
- PNGase F (Glycerol-free, New England BioLabs, cat. no. P0705L)

  ▨ CRITICAL PNGase F cannot cleave off N-glycans with α(1,3)-linked core fucose.
- Trypsin (Sequencing Grade Modified, Promega, cat. no. V5111)
- Protease Inhibitor Cocktail Set V, EDTA-Free (Calbiochem™, cat. no. 539137)
- N-Omega-CD3-Octadecanoyl monosialoganglioside GM1 ($NH4^+$ salt) (Matreya LLC, cat. no. 2050)
- N-Omega-CD3-Octadecanoyl disialoganglioside GD3 (Matreya LLC, cat. no. 2054)
- Quantitative Colorimetric Peptide Assay (Pierce™, ThermoFisher Scientific, cat. no. 23275)
- BCA Protein Assay Kit (Pierce™, ThermoFisher Scientific, cat. no. 23225)

Equipment

- 1.5 mL Microcentrifuge Tubes Natural (Beckman Coulter, cat. no. 357448)
- 1.5 mL Eppendorf™ Snap-Cap Microcentrifuge Flex-Tube™ Tubes (ThermoFisher Scientific, cat. no. 022364111)
- Sorvall™ WX 100+ Ultracentrifuge (ThermoFisher Scientific, cat. no. 75000100)
- Fiberlite™ F50L-24 × 1.5 Fixed-Angle Rotor (ThermoFisher Scientific, cat. no. 096–247028)
- Q700 Sonicator system with standard probe (QSonica, cat. no. Q700–110)
- Forma™ Series II 3110 Water-Jacketed CO_2 Incubators (ThermoFisher Scientific, cat. no. 3110)
- Fisherbrand™ Isotemp™ Digital-Control Water Baths (ThermoFisher Scientific, cat. no. 15–462-S3S)
- Discover Proteomics – Microwave Protein Sample Preparation (CEM Corporation)
- Lab Armor™ Bead Bath (ThermoFisher Scientific, cat. no. 10–876-006)
- iSPE-HILIC SPE Cartridges (HILICON, cat. no. 200.001.0100)
- C8 plate (Glygen, cat. no. FNSC08.800)
- Graphitized Carbon plate (Glygen, cat. no. FNSCAR800)

- Acclaim™ PepMap™ 100 C18 LC Column (3 μm, 0.075 mm x 250 mm, ThermoFisher Scientific, cat. no. 164569)
- Eppendorf™ 96-Well twin.tec™ PCR Plates (ThermoFisher Scientific, cat. no. E951020401)
- Adhesive Sealing Film (ThermoFisher Scientific, NC0525923)
- 250 μL Polypropylene Vial (Agilent, cat. no. 5188–2788)
- 2 mL Crimp/Snap Top Vials & Cap (Agilent, cat. no. 5182–0541)
- E-Pure™ water purification systems (ThermoFisher Scientific, cat. no. D4631)
- Leica DMI3000 B microscope (Lecia Microsystems)
- Agilent 1200 Series Liquid Chromatograph-Chip System (Agilent Technologies)
- Agilent 6520 Accurate Mass Q-TOF LC/MS (Agilent Technologies)
- Agilent C18 Chip (40 nL enrichment column, 5 μm 75 μm x 150 mm separation column, Agilent Technologies, cat. no. G4240–62006)
- Agilent Graphitized Carbon Chip (40 nL enrichment column, 5 μm 75 μm x 43 mm separation column, Agilent Technologies, cat. no. G4240–64010)
- Orbitrap Fusion Lumos Tribrid Mass Spectrometer with EASY-ETD (ThermoFisher Scientific)
- UltiMate™ WPS-3000RS nanoLC system (ThermoFisher Scientific)
- Nanospray Flex ion source (ThermoFisher Scientific)

Software

▨ CRITICAL All required software can be run on a standard personal computer equipped with a Windows operating system.

- Agilent Data Acquisition for QTOF 6500
- Agilent MassHunter Qualitative Analysis (B.08.00)
- Thermo Fisher Scientific Xcalibur™ (4.0)
- Protein Metrics Byonic™ (v 3.5.0)
- Protein Metrics Byonic™-Viewer (v 3.5.0)
- Protein Metrics Byologic™ (v 3.5.0) (optional)

Reagent set-up

Homogenization buffer (HB buffer)—This solution is made up of 0.25 M sucrose, 20mM HEPESKOH at pH 7.4 and 1:100 protease inhibitor mixture in water. Prepare 20 mM HEPES and adjust pH to around 7.4 by adding solid KOH. Weigh out 4.28 g of sucrose and dissolve in 39 mL of H_2O. Dissolve one bottle of protease inhibitor with 1 mL of H_2O. Add 10 mL of HEPES·KOH solution and protease inhibitor to the sucrose solution. Vortex the

solution to mix and aliquot 1.2 mL of solution to 1.5 mL Eppendorf tubes. This solution can be stored at −20 °C for several months.

Cell membrane pellet wash solution—This solution is 0.2 M Na_2CO_3 in water. Weigh out 10.60 g of Na_2CO_3 and dissolve in 500 mL of H_2O. The volume of the stock solution can be adjusted accordingly. This solution can be prepared as stock solution and stored at 4 °C for several months.

N-Glycan release solution—This solution is 100 mM NH_4HCO_3 and 5 mM DTT in water. Weight out 79.06 mg of NH_4HCO_3 and 7.71 mg of DTT and dissolve in 10 mL of H_2O. The solution can be prepared as stock solution and stored at 4 °C for several months.

Folch extraction solution—This solvent is the mixture of H_2O/MeOH/$CHCl_3$ with volume ratio of 3:8:4. Mix 9 mL of H_2O, 24 mL of MeOH, and 12 mL of $CHCl_3$ to prepare 45 mL of Folch solvent. This solution can be stored at 4 °C for several months.

O-Glycan beta-elimination solution 1—This solution is 2 M $NaBH_4$ in water. Weigh out 378.30 mg of $NaBH_4$ and dissolve in 5 mL of H_2O. This solution should be prepared freshly every time right before the experiment. The volume of solution can be adjusted according to the number of samples.

O-Glycan beta-elimination solution 2—This solution is 2 M NaOH in water. Weight out 160.00 mg of NaOH and dissolve in 2 mL of H_2O. This solution should be prepared freshly every time right before the experiment. The volume of solution can be adjusted according to the number of samples.

Protein digestion buffer—This solution is 50 mM NH_4HCO_3 in water. Weight out 79.06 mg of NH_4HCO_3 and dissolve in 20 mL of H_2O. The solution should be prepared freshly every time right before the experiment.

Protein denature solution—This solution is 550 mM DTT in 50 mM NH_4HCO_3 solution. Weigh out 424.19 mg of DTT and dissolve in 5 mL of 50 mM NH_4HCO_3 solution. This solution can be aliquoted and stored at −20 °C for several months.

Protein alkylation solution—This solution is 450 mM IAA in 50 mM NH_4HCO_3 solution. Weigh out 416.16 mg of IAA and dissolve in 5 mL of 50 mM NH_4HCO_3 solution. This solution can be aliquoted and stored at −20 °C for several months.

PGC SPE cartridge condition solution—This solution is 80% (vol/vol) ACN in water with 0.1% (vol/vol) TFA. Add 99.5 mL of H_2O and 0.5 mL of TFA to 400 mL of ACN. This solution can be prepared as stock solution and stored at 4 °C for several months.

PGC SPE cartridge elution solution—This solution is 40% (vol/vol) ACN in water with 0.05% (vol/vol) TFA. Add 200 mL of ACN and 0.25 mL of TFA to 299.75 mL of H_2O. This solution can be prepared as stock solution and stored at 4 °C for several months.

C8 SPE plate condition solution—This solution is 1:1 methanol/water (vol/vol). Add 50 mL of methanol and 50 mL of water, and mix thoroughly. This solution can be prepared as stock solution and stored at 4 °C for several months.

C8 SPE plate elution solution—This solution is 1:1 methanol/IPA (vol/vol). Add 50 mL of methanol and 50 mL of IPA, and mix thoroughly. This solution can be prepared as stock solution and stored at 4 °C for several months.

HILIC cartridge condition solution (O-glycan)—This solution is 90% (vol/vol) ACN in water with 1% (vol/vol) TFA. Add 45 mL of H_2O and 5 mL of TFA to 450 mL of ACN. This solution can be prepared as stock solution and stored at 4 °C for several months.

HILIC cartridge condition solution (glycopeptide)—This solution is 80% (vol/vol) ACN in water with 1% (vol/vol) TFA. Add 95 mL of H_2O and 5 mL of TFA to 400 mL of ACN. This solution can be prepared as stock solution and stored at 4 °C for several months.

HILIC cartridge elution solution—This solution is Milli-Q water with 0.1% (vol/vol) TFA. Add 0.5 mL of TFA to 499.5 mL of H_2O. This solution can be prepared as stock solution and stored at 4 °C for several months.

N- and O-Glycomics LC solvent A—This solvent is 3% (vol/vol) ACN in water with 0.1% (vol/vol) FA. Add 30 mL of ACN and 1 mL of FA to H_2O, degas and transfer to mobile phase container to make a total of 1 L of glycomics LC solvent A. This solution should be prepared freshly right before running each batch of samples.

N- and O-Glycomics LC solvent B—This solvent is 90% (vol/vol) ACN in water with 1% (vol/vol) FA. Add 90 mL of H_2O and 10 mL of FA to ACN, degas and transfer to mobile phase container to make a total of 1 L of glycomics LC solvent B. This solution should be prepared freshly right before running each batch of samples.

Glycolipidomics LC solvent A—This solvent is water with 0.1% (vol/vol) acetic acid and 20 mM ammonium acetate. Add 1.54 g of ammonium acetate to 400 mL of H_2O, mix thoroughly until all salts are dissolved. Add 1 mL of acetic acid and adjust water to final volume of 1 L, degas and transfer to mobile phase container to make a total of 1 L of glycolipidomics solvent A. This solution should be prepared freshly right before running each batch of samples.

Glycolipidomics LC solvent B—This solvent is 15% (vol/vol) IPA in methanol with 0.1% (vol/vol) acetic acid. Add 150 mL of IPA and 1 mL of acetic acid to 400 mL of methanol. Add methanol to final volume of 1 L, degas and transfer to mobile phase container to make a total of 1 L of glycolipidomics solvent B. This solution should be prepared freshly right before running each batch of samples.

Glycopeptide LC solvent A—This solvent is 99.9% (vol/vol) LC-MS grade water with 0.1% (vol/vol) FA. Add 1 mL of FA to LC-MS grade water to make a total volume of 1 L of solvent A. This solution can be stored at room temperature and used for two weeks.

Glycopeptide LC solvent B—This solvent is 90% (vol/vol) LC-MS grade ACN, and 9.9% LC-MS grade water with 0.1% (vol/vol) FA. Add 99 mL of LC-MS grade water and 1 mL of FA to LC-MS grade ACN to make a total volume of 1 L of solvent B. This solution can be stored at room temperature and used for two weeks.

Procedures

Sample preparation ●Timing depends on sample type

1. To prepare tissue follow option A. To prepare mammalian cell lines follow option B.

   A. ***Tissue sample preparation*** **●Timing 1–3 h depends on the size of sample set**

      i. Collect tissue samples from dissection. Cut the frozen tissue samples into small pieces if necessary (for muscle).

      ii. Wash around 50–100 mg of tissue sample with 1 mL of PBS buffer.

      iii. Centrifuge the sample at 1,000 x g for 3 min. Discard the supernatant. Suspend the pellet with 1.2 mL of HB buffer.

   B. ***Mammalian cell growth*** **●Timing 5 d**

      i. Culture cells using appropriate growth medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) Penicillin–Streptomycin. Grow cells at 37 °C in a cell culture incubator in a humidified atmosphere containing 5% CO_2.

      ▨ CRITICAL STEP We have successfully cultured PNT2, Caco-2, and A549 cell lines. Growth media and conditions may need to be altered for different cell lines.

      ii. Grow cells until 80% confluency is reached. Split cells using techniques appropriate for your cell line into 10 cm petri dish or T75 flask. Briefly, aspirate media and wash cells with 10 mL of PBS for two times, followed by adding 2.5 mL of Trypsin-EDTA and incubating for 5 min. Then add 5 mL of media to stop the digestion and homogenize the mixture by pipetting for several times. Add a desired volume of cell mixture (usually in the range of 200 μL to 1 mL) to a new dish or flask containing 10 mL of media.

      iii. Monitor cell proliferation using a microscope and change media every other day.

      iv. Cells may be harvested at around 90% confluency. Wash cells with 10 mL of PBS twice before harvesting to thoroughly remove detached cells and the growth medium.

▨ CRITICAL STEP Some cells should be harvested earlier to prevent further maturation, such as the Caco-2 cell line, if you prefer to analyze undifferentiated cells.

v. Add 10 mL of PBS and scrape cells with a scraper. Scrape cells in one direction and make sure all cells are collected. Then transfer the mixture to a 15 mL centrifuge tube.

▨ CRITICAL STEP For the Caco-2 cell line, continue culturing after 100% confluency for another 14 days if you wish to obtain differentiated Caco-2 cells.

vi. Wash the dish or the flask with 5 mL of PBS and combine it with the previous mixture.

vii. Centrifuge the suspension at 1,000 x g for 3 min at 4 °C. Remove the supernatant and resuspend the cells in 1.2 mL of HB buffer. Count the cell number if needed to make sure at least 1×10^6 cells are collected.

Cell and tissue lysis and membrane extraction **●Timing 5–8 h depends on the size of sample set**

2. Pipette up and down to mix the cell suspension before lysis.

3. Place the 15mL falcon tube on an ice bucket or a 9-well cooling rack and keep the sample at 4 °C

4. Place the probe of the sonicator into the sample.

▨ CRITICAL STEP make sure the tip of the tube is in the center of the sample.

5. Sonicate the sample using the following conditions: 25 amplitude (Amp); 5 seconds (s) on; 10 s off for 25 s of total sonication time.

▨ CRITICAL STEP Monitor the power during the entire sonicating process. Make sure the power is not exceeding 60 watts (W) to prevent over-heating.

6. Transfer the homogenate to a 1.5 mL Eppendorf tube and centrifuge at 2,000 x g for 10 minutes (min) at 4 °C to remove nucleus.

7. Take the clear supernatant and transfer to a 1.5 mL ultracentrifuge tube.

▨ CRITICAL STEP Be careful when transferring the supernatant to avoid taking the layer of fat or lipid on the surface of the sample. The surface layer will be obvious when large amounts of cells are processed.

8. Ultracentrifuge samples at 200,000 x g for 45 min at 4 °C to separate the plasma membrane from the other cell compartments. Remove the supernatant.

▨ CRITICAL STEP Tubes should be placed in the rotor in a consistent orientation. When removing supernatant, use a normal pipette tip to remove most of the supernatant and switch to a gel tip to remove the rest. The narrow and flexible tube of a gel tip will allow us to remove as much of the supernatant as

possible without disturbing the pellet. Make sure that the tip does not disturb the pellet.

9. Resuspend the pellet in 0.2 M sodium carbonate solution to remove cell membrane-associated proteins. Pipette mix the pellet with a normal pipette tip to homogenize the mixture. Repeat the ultracentrifugation in step 8 using the same parameters. Remove the supernatant.

10. Resuspend the pellet in Milli-Q water to wash the pellet. Pipette mix the pellet to make the mixture homogenous. Repeat the ultracentrifugation in step 8 using the same parameters. Remove the supernatant.

11. Retain the cell membrane pellet for further analysis. Cell membrane pellet can be divided into two fractions for either N- and O-glycomics and glycolipidomics, or glycoproteomic analysis.

■ PAUSE POINT The pellet can be stored at −20 °C for several months until the glycomic or glycoproteomic analyses.

Comprehensive N-glycomics: *N-Glycan release* ●Timing 20 h

12. Dissolve the Fraction I pellet in 100–200 μL of buffer (100 mM ammonium bicarbonate, 5 mM DTT) in an Eppendorf tube and break the pellet gently by pipette mixing.

13. Secure the lid with parafilm and denature the pellet by heating up to 100 °C for 10 s, followed by 10 s of cooling-down, with a total duration of 2 min..

14. Leave the sample to cool down to room temperature (which usually takes 2 min) and then add 2 μL of PNGase F enzyme and pipette mix.

15. Conduct N-glycan release by using the microwave to heat to 60 °C for 10 min. This is usually achieved by heating at 20 W.

    ▨ CRITICAL STEP By the end of the incubation, the final temperature usually is 37 °C. If the desired temperature is not reached, continue the incubation until the temperature shown is 37 °C.

16. Keep the mixture in a 37 °C water bath overnight to completely hydrolyze primary amines to hydroxyl groups.

17. After the incubation, add 350 μL of Milli-Q water, and ultracentrifuge at 200,000 x g for 45 min at 4 °C, to separate the released N-glycans from the pellet. Transfer the supernatant to a new 1.5 mL Eppendorf tube.

    ▨ CRITICAL STEP Use the gel tip to transfer the supernatant. Be careful when transferring to prevent the disturbance of the pellet.

18. Save the pellet for O-glycomics and glycolipidomics.

■ PAUSE POINT The pellet can be stored at −20 °C for several months until the glycolipid extraction.

Comprehensive N-glycomics: *Desalting of N-glycans with PGC SPE plates* ●Timing 1–2 h

19. Add 200 μL of 80% (vol/vol) ACN, 0.1% (vol/vol) TFA solution to each well and centrifuge the plate at 150 x g for 1 min. Discard the flow-through. Repeat the step twice for conditioning.

20. Add 200 μL of pure Milli-Q water to each well and centrifuge the plate using the conditions given in step 19. Discard the flow-through. Repeat the step twice for equilibration.

? TROUBLESHOOTING

21. Load 200 μL of sample to each well of the plate until all the sample has been transfered. Centrifuge as described in step 19 and discard the flow-through.

22. After sample loading, desalt samples by adding 200 μL of pure Milli-Q water per well and centrifuging as described in step 19 three times. If the starting amount of cells is larger, samples can be washed for more than three times. Centrifuge and discard the flow-through in between each centrifugation.

23. For sample elution, add 200 μL of 40% (vol/vol) ACN, 0.05% (vol/vol) TFA to each well. Centrifuge as described in step 19 and collect the flow-through. Elute samples for two times (in total 400 μL). If the starting amount of cells is larger, elute sample with 200 μL of solvent.

24. Vacuum dry the eluted sample completely. This usually takes around 2–4 h.

■ PAUSE POINT The vacuum dried sample can be stored at −20 °C for several months until analysis.

Comprehensive N-glycomics: *Nano-chip-QTOF-MS/MS analysis of N-glycans* ●Timing 1 h per sample

25. Reconstitute the sample with Milli-Q water. Start by reconstituting with 30 μL of water and adjust the amount accordingly. If the final TIC signal in the mass spectrometry is less than 1×10^5, dry down the sample further to get higher concentration. Test the amount of water for reconstitution in this way for each cell line.

26. Transfer the sample into an injection vial and inject 5–10 μL of sample into the MS for analysis.

▨ CRITICAL STEP To ensure sufficient sample is available to be injected, the minimum volume of reconstituted sample per well should be 10 μL.

▨ CRITICAL STEP If a large set of samples are to be analyzed, a 96-well injection plate can be used.

27. Place injection vials or plates in the autosampler compartment. Close the door of the compartment properly.

▨ CRITICAL STEP At the beginning of the batch, run 2–3 blank samples and one pooled N-glycan standard. For quality control (QC), run the pooled N-glycan

standard and the blank after every 10 samples. At the end of the batch, flush the chip 2–3 times using the established flush method.

? TROUBLESHOOTING

28. Make a worklist in the “Worklist” tab or load the established worklist from an Excel file.

29. Load the freshly prepared glycan analysis solvents A and B to the pump. Change the solvent level from the “Nano Pump > Bottle Fillings” window to make sure the software will not error out.

▨ CRITICAL STEP The detailed N-glycan LC-MS analysis method is included in Table 1.

30. Load the Agilent PGC-Chip II into the chip cube. Switch the chip position to analysis position to make solvents flow from the nano pumps through both enrichment and analysis columns. Start the flow at 300 nL/min with 100% (vol/vol) solvent B to flush the chip for several min.

▨ CRITICAL STEP Check the capillary pump and nano pump pressure. Make sure the capillary pump pressure is in the range of 60–90 bar, and the nano pump pressure is in the range of 30–50 bar.

? TROUBLESHOOTING

31. Observe the spray of the chip from the camera. If dripping or scattered spray is seen, adjust the horizontal position of the chip by screwing the wheel beneath the chip cub. The vertical position of the chip can be accessed from the acquisition software in the “ChipCub > Adjust Tip” window. The spray can also be improved by changing the capillary voltage in the “Method” tab.

? TROUBLESHOOTING

32. Start the worklist after checking all the above steps.

? TROUBLESHOOTING

33. After finishing the batch, change the lids of any injection vials and save the vials directly at −20 °C. Seal the injection plate with the seal sheet. Wrap the plate with aluminum foil and store at −20 °C.

PAUSE POINT Data analysis can be conducted afterwards when needed.

Comprehensive N-glycomics: *N-Glycomics data analysis* ●Timing 1–2 h per batch

34. Start the Agilent MassHunter Qualitative Analysis B.08.00 software.

35. Open the data file in the software.

36. Use the “Find Compounds by Molecular Feature” function to survey the N-glycans present in the sample. Set up the extraction parameters in the window. Use the underivatized N-glycan library for extraction. An underivatized N-glycan library can be established referring to a previous study[89]. The template of an N-

glycan library is included as Supplementary Data I_N-glycan_lib_template.csv. More N-glycans can be added to the template based on ref. 89 and used directly for database search.

▨ CRITICAL STEP The N-glycan library is a csv file. It contains columns of Mass, Cpd (glycan compositions), and Comments (types of N-glycans). The formulas of N-glycans can also be added to the Formula column in the library. The detailed data analysis method is summarized in Table 2.

▨ CRITICAL STEP The composition of an N-glycan compound is noted as the number of each monosaccharide [ABCD] in the order of Hex_HexNAc_Fuc_NeuAc. More numbers can be included if other groups are contained in the composition, such as NeuGc and sulfate groups.

37. Start the extraction of compounds by clicking the green Play/Run button and choose the files to run the Molecular Feature Extraction. The program will return a list of compounds that match the set criteria. The extracted results include the total compound chromatogram (TCC) and extracted compound chromatogram (ECC).

38. Reset colors of extracted compound peaks based on the following criteria: Change all the fucosylated compound peaks to green, sialylated compound peaks to pink, sialylfucosylated compound peaks to blue, complex or hybrid compound peaks to orange, and high-mannose compound peaks to red.

39. Add the compound labels to the ECC.

40. Save ECC, both versions with compound labels and without compound labels, as figures.

41. Check the MS/MS spectra to identify fragmentations of N-glycans in the “MS Spectrum Results” window. Their fragmentation spectra will have the characteristic masses for monosaccharides and oligosaccharides.

▨ CRITICAL STEP Fragmentations should be checked when analyzing glycans containing unnatural monosaccharides, such as SiaNAz and GalNAz. Care should also be taken when analyzing non-human glycan samples, where the existence of NeuGc is possible. The glycan with the composition of 1 NeuGc + x Hex + y Fuc has the same mass as the glycan with the composition of 1 NeuAc + (x+1) Hex + (y-1) Fuc.

42. Export extracted N-glycan compounds as csv files, and curate the extracted data as needed.

43. Calculate the relative abundances of each compound by summarizing the abundances of isomers or compounds with different charge states of one N-glycan composition.

Comprehensive glycolipidomics: *Glycolipid extraction* ●Timing 1 h

44. Resuspend the pellet after N-glycan releasing (from step 17) with 500 μL of Folch solvent and pipette mix the solution thoroughly to fully dissolve the lipids.

▨ CRITICAL STEP Sonicate the samples for 15 min followed by 1-minute vortexing if necessary.

45. Centrifuge at 8,800 x g for 5 min to separate the pelletized proteins from the dissolved lipids in the supernatant. Collect the supernatant and transfer it to a new Eppendorf tube.

? TROUBLESHOOTING

▨ CRITICAL STEP The pellets, which contain mostly precipitated proteins, can be set aside for O-glycan release (step 73).

46. Add internal standards to the supernatant if desired. About 1 nmol of each labeled glycolipid is sufficient for detection and quantitation. For example, add 20 μL of a solution of 50 μM N-omega-CD3-Octadecanoyl monosialoganglioside GM1 and 50 μM N-omega-CD3-Octadecanoyl disialoganglioside GD3 dissolved in methanol.

47. Add 100 μL of 0.1 M potassium chloride to the supernatant and vortex mix for 1 min. Turbidity may be observed due to phase separation and can be ignored.

48. Separate the aqueous and organic phases by centrifuging at 8,800 x g for 5 min. The upper methanol and aqueous layer will contain glycosphingolipids with 3 or more monosaccharide residues. The bottom chloroform-rich layer will contain mostly phospholipids, cholesterol, and other less polar lipids. A middle disk-shaped layer of phospholipids may form during this step.

? TROUBLESHOOTING

49. Carefully collect the upper layer and dry the sample in vacuum, which takes 2–3 h.

Comprehensive glycolipidomics: *Desalting of glycolipids with C8 SPE plates* ●Timing 1–2 h

50. Wash a C8 SPE plate twice with 600 μL of 1:1 methanol/isopropanol (vol/vol) by centrifuging at 150 x g.

51. Condition the plate by washing twice with 600 μL of 1:1 methanol/water (vol/vol).

52. Reconstitute the dried samples from step 49 with 600 μL of 1:1 methanol/water and load the sample on the plate.

53. Wash the plate at least 3 times with 600 μL of 1:1 methanol/water (vol/vol).

▨ CRITICAL STEP Washing volume can be varied between samples. If a high salt content is expected, use a greater volume for washing.

54. Elute the glycolipids from the plate using 600 μL of 1:1 methanol/isopropanol (vol/vol).

55. Dry the samples completely in vacuum, taking around 2–3 h.

■ PAUSE POINT The vacuum dried sample can be stored at −20 °C for several months until analysis.

Comprehensive glycolipidomics: *Nano-chip-QTOF-MS/MS analysis of glycolipids* ●Timing 1 h per sample

56. For glycolipidomic analysis using nano-chip-QTOF, reconstitute the sample with 50 μL of 1:1 (vol/vol) methanol/water and inject around 5 μL of sample into the instrument.

▒ CRITICAL STEP More detailed instrument parameters for glycolipidomics are included in Table 1.

▒ CRITICAL STEP For quality control (QC), the pooled glycolipid samples from the pluripotent cell line NTERA-2 is used.

■ PAUSE POINT Data analysis can be conducted afterwards when needed.

Comprehensive glycolipidomics: *Glycolipidomics - data analysis* ●Timing 1–2 h per batch

57. Start the Agilent MassHunter Qualitative Analysis B.08.00 software.

58. Open the data file in the software.

59. Manually identify the glycans and ceramides from the MS/MS data to generate a library of glycosphingolipids. If the glycan compositions and the associated lipids in the sample are already known, skip ahead to generate a glycosphingolipid library for data extraction.

▒ CRITICAL STEP Using the full library directly can lead to higher rates of false identification because the sheer number of possible glycolipid combinations result in many compounds having similar masses.

60. Use the “Find Compounds by Molecular Feature” function to survey the glycolipids present in the sample. Set up the parameters as Table 2 to filter through the identified compound features.

61. Start the extraction of compounds by clicking the green Play/Run button and choose the files to run the Molecular Feature Extraction. The program will return a list of compounds that match the set criteria. (If list does not appear, go to View > Compound List. To customize the data shown in the list, right-click on the column header and selecting Add/Remove Columns.)

62. Check the MS/MS spectra to identify glycosphingolipids in the “MS Spectrum Results” window. Their fragmentation spectra will have the characteristic masses for monosaccharides, oligosaccharides, and ceramides. Other observable fragments include the sphingoid fragment and combinations of a ceramide with oligosaccharides.

▒ CRITICAL STEP MS/MS spectra that have abundant 184.07 *m/z* fragments are likely to be phospholipids. This *m/z* signal is from the phosphocholine head group.

63. From spectra of possible glycosphingolipids, take note of the precursor *m/z* from the MS1 spectrum, the charge state of the precursor, and the ceramide *m/z* in the MS/MS spectrum. A table of possible ceramide *m/z* is provided in the file Supplementary Data II_Ceramide_mz.csv. Some commonly observed ceramides in mammalian cells include d34:1, d36:1, d42:1, and d42:2. Critical: MS parameters must be optimized for glycolipid fragmentation.

64. Input the precursor *m/z*, precursor charge state, and the ceramide *m/z* in the appropriate fields (highlighted light blue) in the file Supplementary Data III_GSL_glycan_finder.xlsx. Include at least 2 decimal places for better matching. The Excel file calculations take into account possible proton, ammonium, and sodium adducts to match the closest glycan composition that fits the inputs. A good match should have a PPM Error of less than 10. Cross-check the given glycan composition with the fragmentation data to confirm the identification. Some common saccharide and lipid fragments are described in the file Supplementary Data IV_Saccharide_mz.csv.

65. Generate a list of the identified ceramides and glycan compositions found through this process.

66. Generate a database of glycosphingolipids by using the Pivot table in the file Supplementary Data V_GSL_lib_generator.xlsx. Select the glycan compositions and identified lipids in the appropriate drop-down menus. Alternatively, the ceramides can also be filtered based on the number of carbons, number of hydroxyl groups, and the degree of unsaturation.

67. Copy the information into a new csv file, taking care to follow the format that is readable by the software.

68. Extract the glycosphingolipid data with the newly-generated database using the “Find Compounds by Molecular Feature”.

69. Reset colors of extracted glycolipid peaks based on the following criteria: Change all the fucosylated compound peaks to green, sialylated compound peaks to pink, sialofucosylated compound peaks to blue, undecorated compound peaks to orange, sulfated compound peaks to red, and globo compound peaks to black.

70. Add the compound labels to the ECC.

71. Save the ECC, both of versions with compound labels and without compound labels, as figures.

72. Export extracted glycolipid compounds as csv files, and curate the extracted data as needed.

Comprehensive O-glycomics:

Comprehensive O-glycomics: *O-Glycan release* ●Timing 1 d

73. Take the pellet saved from Folch extraction (step 44) and transfer it to a 4 mL cryovial tube.

74. Reconstitute the pellet with 90 μL of Milli-Q water and sonicate for 10 min to break up the piece.

75. Add 10 μL of 2 M NaOH and 100 μL of 2 M $NaBH_4$ sequencially and vortex the mixture for 10 s.

? TROUBLESHOOTING

76. Incubate samples at 45 °C in a water bath for 18 h.

▨ CRITICAL STEP During the first hour of incubation, vortex the mixture and release the generated gas twice to avoid over pressure in the tube.

77. Add 90–110 μL of acetic acid to the mixture to neutralize the solution.

▨ CRITICAL STEP While adding the acid, place samples on ice to prevent over-heating of the mixture.

? TROUBLESHOOTING

78. Check the pH values of samples with pH paper. The final pH values of samples are 4–6.

79. Centrifuge samples at 21,000 g for 30 min. Retain the supernatant and discard the pellet.

Comprehensive O-glycomics: *Desalting of O-glycans with PGC SPE plates*

●**Timing 1–2 h**

80. Conduct SPE clean-up by following steps 19–24 to desalt. Dry samples completely in a vacuum.

Comprehensive O-glycomics: *Enrichment of O-glycans with HILIC cartridges*

●**Timing 1–2 h**

81. Reconstitute the dried O-glycan samples with 1mL of 90% (vol/vol) ACN, 1% (vol/vol) TFA solution.

82. Add 1 mL of ACN to the iSPE HILIC cartridge to wet the material. Discard the flow-through. To speed up the procedure, a pump or vacuum manifold can be used.

83. Add 1 mL of water with 0.1% (vol/vol) TFA to the cartridge for the conditioning. Discard the flow-though. Repeat the procedure one more time. Pump the liquid to speed up the procedure.

84. Equilibrate the cartridge with 1 mL of 90% (vol/vol) ACN, 1% (vol/vol) TFA solution and discard the flow-through. Repeat the procedure twice.

85. Load the sample to the cartridge and let the sample flow through the cartridge slowly (2 drops per sec). Collect the flow-through and reload it to the cartridge.

86. Reload the sample as described in previous step 5 times.

87. Elute the sample by adding 1 mL of water with 0.1% (vol/vol) TFA. Repeat 3 times.

88. Dry samples completely in vacuum.

■ PAUSE POINT The vacuum dried sample can be stored at −20 °C for several months until analysis.

Comprehensive O-glycomics: *Nano-chip-QTOF-MS/MS analysis of O-glycans* ●Timing 1 h per sample

89. For nano-chip-QTOF-MS/MS analysis, reconstitute the sample with around 90 μL of water and inject 5 μL of sample into the instrument. The volume of water for reconstitution and injection can be adjusted accordingly. Repeat steps 25–33 on this sample.

    ▒ CRITICAL STEP The detailed O-glycan analysis LC-MS method is included in Table 1.

    ▒ CRITICAL STEP For quality control, the pooled released O-glycans from several cell lines under the normal condition (Caco-2, PNT2, and A549) can be used.

■ PAUSE POINT Data analysis can be conducted afterwards when needed.

Comprehensive O-glycomics: *O-Glycomics data analysis* ●Timing 1–2 h per batch

90. Start the Agilent Qualitative Analysis B.08.00 software.

91. Open the data file in the software.

92. Use the “Find Compounds by Molecular Feature” function to survey the O-glycans present in the sample. Set up the parameters as Table 2 to filter through the identified compound features.

    ▒ CRITICAL STEP The O-glycan library is a csv file. It contains the masses, compositions, and types of reduced O-glycans. The formulas of these O-glycans can also be added to the library.

93. Start the extraction of compounds by clicking the green Play/Run button and choose the files to run the Molecular Feature Extraction. The program will return a list of compounds that match the set criteria. The extracted results include the total compound chromatogram (TCC) and extracted compound chromatogram (ECC).

94. Reset colors of extracted O-glycan peaks based on the following criteria: Change all the fucosylated compound peaks to green, sialylated compound peaks to pink, sialofucosylated compound peaks to blue, and undecorated compound peaks to orange.

95. Add the compound labels to the ECC.

96. Check the MS/MS spectra to identify fragmentations of O-glycans in the "MS Spectrum Results" window. Their fragmentation spectra will have the characteristic masses for monosaccharides and oligosaccharides.

▨ CRITICAL STEP Fragmentations should always be checked when analyzing glycans containing unnatural monosaccharides, such as SiaNAz and GalNAz. Care should also be taken when analyzing non-human glycan samples, where the existence of NeuGc is possible. The glycan with the composition of 1 NeuGc + x Hex + y Fuc has the same mass as the glycan with the composition of 1 NeuAc + (x+1) Hex + (y-1) Fuc.

97. Save the ECC, both as versions with compound labels and without compound labels, as figures.

98. Export extracted O-glycan compounds as csv files, and curate the extracted data as needed.

99. Calculate the relative abundances of each compound by summarizing the abundances of isomers or compounds with different charge states of one O-glycan composition.

Site-specific glycoproteomic analysis: Protein digestion ●Timing 20 h

100. Resuspend cell membrane pellet from Fraction II (step 11) using 60 μL of freshly made 8 M urea.

▨ CRITICAL STEP Pipette mix the solution to fully resuspend the cell pellet.

101. Sonicate the solution for 10–15 min. Measure the protein concentration by BCA assay.

▨ CRITICAL STEP The amount of DTT, IAA, and trypsin to be used in the following steps (102–104), depends on the initial sample concentration determined by BCA assay. 1 mg/mL protein sample requires 2 μL of DTT, 4 μL of IAA, and 2 μg of trypsin.

102. Add 2 μL of 450 mM DTT and pipette well to mix the samples. Use parafilm to secure the lids and incubate at 55 °C in a water bath for 50 min.

▨ CRITICAL STEP Higher temperatures (>60 °C) will cause urea-based carbamylation at the protein N terminals and at the side chain amine groups of lysine and arginine residues, which further blocks the protease digestion and affects protein identification and quantification.

▨ CRITICAL STEP Incubation with DTT & IAA, i.e. this and the next step, is not needed if the protein does not contain disulfide bonds.

103. Add 4 μL of 550 mM IAA, pipette mix well the samples, and incubate at room temperature for 20 min.

▨ CRITICAL STEP IAA is light sensitive, so samples should be placed in a dark drawer/area.

104. Add 420 μL of protein digestion buffer to dilute the urea concentration to less than 8 M. Reconstitute 20 μg of sequencing-grade modified trypsin in freshly made 200 μl of protein digestion buffer and then add 20 μL of trypsin to the sample. Pippetting to mix samples well.

▨ CRITICAL STEP Do not vortex mix the trypsin which may reduce the activity of the enzyme.

105. Seal tubes with parafilm, and place samples in a 37 °C water bath and incubate for 18 h.

106. After the incubation, quench the reaction with 10 μL of 18% (vol/vol) formic acid.

107. Dry the samples in vacuum, which usually takes 2–3 h. After complete drying, the samples are ready for glycopeptide enrichment.

▨ CRITICAL STEP Do not use a high-temperature for drying glycopeptide samples to avoid the denaturation or undesired modifications of glycopeptides.

■ PAUSE POINT The completely dried sample can be stored at −20 °C for several days until the HILIC enrichment.

Site-specific glycoproteomic analysis: Enrichment of glycopeptides with HILIC SPE cartridges ●Timing 2–3 h

108. Wash iSPE-HILIC SPE cartridges with 1 mL of 99.9% (vol/vol) ACN, 0.1% (vol/vol) TFA. To speed up the procedure, a pump or vacuum manifold can be used.

109. Wash the cartridges with 2 mL of 99.9% (vol/vol) H_2O, 0.1% (vol/vol) TFA.

110. Equilibrate the cartridges with 3 mL of 80% (vol/vol) ACN, 19% (vol/vol) H_2O, 1% (vol/vol) TFA.

111. Reconstitute the samples with 1 mL of 80% (vol/vol) ACN, 19% (vol/vol) H_2O, 1% (vol/vol) TFA, and load the samples to the cartridges.

? TROUBLESHOOTING

112. Reload the flow-through from step 111 5 times.

113. Wash the cartridges with 5 mL of 80% (vol/vol) ACN, 19% (vol/vol) H_2O, 1% (vol/vol) TFA.

▨ CRITICAL STEP Collect and save the flow-through from this step for proteomic analysis if necessary.

114. Elute the glycopeptides with 2.5 mL of 99.9% (vol/vol) H_2O, 0.1% (vol/vol) TFA.

115. Dry the samples in vacuum completely for 3–5 h for glycopeptide analysis.

Site-specific glycoproteomic analysis: Nano-LC-MS/MS analysis of Glycopeptides ●Timing 4 h per sample with one blank injection

116. Reconstitute the samples with 20 μL of H_2O, use Pierce™ Quantitative Colorimetric Peptide Assay following the manufacturer's instructions to measure the glycopeptide concentration, and adjust the concentration to 1 μg/μL.

▨ CRITICAL STEP If the reconstituted sample is opaque or contains some visible undissolvable precipitates, the sample should be spin down briefly for 15 s and only the supernatant can be injected to the instrument.

117. Run glycoproteomic anaylsis. We use an UltiMate™ WPS-3000RS nanoLC system coupled to the Nanospray Flex ion source of an Orbitrap Fusion Lumos. We use a C18 column (Acclaim™ PepMap™ 100 C18 LC Column, 3 μm, 0.075 mm x 150 mm) for glycopeptide separation. Details of the method are summarized in Table 3.

▨ CRITICAL STEP After every sample run, the column should be cleaned by running a blank injection to avoid sample carry-over.

▨ CRITICAL STEP A digested commercial serum N-glycopeptide sample can be used for quality control (QC) of LC-MS.

Site-specific glycoproteomic analysis:Glycoproteomics data analysis ●Timing 2–8 h per file

118. Download and install the most recent version of Byonic™ from https://www.proteinmetrics.com/

119. Open the Byonic™ software, the .raw file is used for the MS/MS data file, and the *Homo sapiens* (Human) protein database from UniProt (UP000005640) is recommended for the protein database file. Set up searching parameters under each tab following Table 4.

120. Select the enzyme (trypsin) for digestion. Also setthe fragmentation type used for collecting MS/MS spectra, for example, for the stepped-collision energy HCD, choose the fragmentation type CID& HCD.

▨ CRITICAL STEP Detailed parameters for trypsin digested glycopeptides are included in Table 4.

▨ CRITICAL STEP Other enzymes such as Glu-C and Lys-C can be selected, together with their corresponding cleavage sites.

121. Choose carbamidomethyl modification at cysteine residues (+57.021464) as a fixed modification. Details of other modifications are summarized in Table 5.

▨ CRITICAL STEP Other specific modifications such as phosphorylations can be added based on the protein features.

▨ CRITICAL STEP Search time will be dramatically increased with total common max numbers. It is usual to choose 1 for total common maximum number and total rare maximum number.

122. Load the N- or O- glycan libraries established from glycomics analysis (steps 36 and 92) as the glycans modification databases.

▨ CRITICAL STEP To obtain confident identification, it is recommended that you use the databases from previous N-glycomics and O-glycomics, especially for unnatural monosaccharide incorporated glycopeptides.

▨ CRITICAL STEP Identification of the proteins with O-glycosylation will tremendously increase the software searching time.

123. Use default settings for advanced parameters. If other peptide quantification software such as Skyline is desired, enable the mzIdentML file export.

124. Enable Excel report for curating the data as needed. Start searching by clicking “Run” button. The status of searching can be checked in the “Progress” tab.

125. In the exported Excel result file, use Score (>300), |LogPro| (>2.0), and DeltaMod (>10, only for peptides with modifications) to filter confident identification. Peptide-spectrum matches can be validated by checking the peptide fragmentations using Byonic-Viewer.

Site-specific glycoproteomic analysis: Label-free glycopeptide quantification (optional) ●Timing 2–8 h per file

126. Download and install the most recent version of and Byologic™ from https://www.proteinmetrics.com/

127. Drag .byrslt file from the Byonic search file to Byologic input for MS1 and MS2 search.

▨ CRITICAL STEP .raw file should be relocated if the file folder has been changed.

128. Load the filtered protein lists from Byonic results.

▨ CRITICAL STEP Byonic import can be directly used if no filter is applied for the protein identification.

129. Choose default settings for MS extract options.

▨ CRITICAL STEP The default parameters in MS extract options should be re-evaluated if any peak cut off has been noticed.

130. Create the file to start analyzing and export the report.

Troubleshooting

For troubleshooting guidance, see Table 6.

Timing

Steps 1.A)i)-1.A)Step 1A, tissue sample preparation: 1–3 h depends on the size of sample set

Steps 1.B)i)-1.B)vii), mammalian cell growth: 5 d

Steps 2–11, cell lysis and membrane extraction: 5–8 h depends on the size of sample set

Steps 12–18, N-glycan release: 20 h

Steps 19–24, desalting of N-glycans with PGC SPE plates: 1–2 h

Steps 25–33, nano-chip-QTOF-MS/MS analysis of N-glycans: 1 h per sample

Steps 34–43, N-glycomics data analysis: 1–2 h per batch

Steps 44–49, glycolipids extraction: 1 h

Steps 50–55, desalting of glycolipids with C8 SPE plates: 1–2 h

Steps 56, nano-chip-QTOF-MS/MS analysis of glycolipids: 1 h per sample

Steps 57–72, glycolipidomics data analysis: 1–2 h per batch

Steps 73–79, O-glycan release: 1d

Steps 80, desalting of O-glycans with PGC SPE plates: 1–2 h

Steps 81–88, enrichment of O-glycans with HILIC cartridges: 1–2 h

Steps 89, nano-chip-QTOF-MS/MS analysis of O-glycans: 1 h per sample

Steps 90–99, O-glycomics data analysis: 1–2 h per batch

Steps 100–107, Protein digestion: 20 h

Steps 108–115, enrichment of glycopeptides with HILIC cartridges: 2–3 h

Steps 116–117, nanoLC-MS/MS analysis of glycopeptide: 4 h per sample with one blank injection

Steps 118–125, glycoproteomics data analysis: 2–8 h per file

Steps 126–130, label-free glycopeptide quantification (optional): 2–8 h per file

Anticipated results

This protocol is used for the comprehensive characterization of cell membrane glycocalyx of both cell and tissue samples. Successful application of this protocol will lead to the identification and label-free-quantitation of hundreds of non-redundant glycosylated species.The glycocalyx of the Caco-2 cell line before and after differentiation has been successfully characterized using this workflow. Anticipated results should be in line with past performance of the overall method. Below we describe anticipated results based on past performance within specific studies.

Glycomic variations during cell differentiation.

By monitoring the morphology with microscopy, it was observed that the differentiation of Caco-2 cells started from the fifth to the seventh day and completed on day 21. The biological progressive changes of cell surface glycocalyx during cell differentiation were illustrated following the first part of the workflow. The N-glycomic analysis showed that the level of high-mannose type N-glycans decreased, while the levels of fucosylated and sialylated N-glycans were elevated during differentiation (Figure 8)[33]. The glycolipidomic analysis identified more than 200 intact glycosphingolipids (GSL) on differentiated Caco-2 cells (Figure 9)[24,26]. During the maturation process the globo-type GSLs decreased, while GSLs with sialylated and sulfated head groups increased. Additionally, the relative abundances of longer ceramides with 40–42 carbons decreased whereas shorter ceramides with 32 to 34 carbons showed higher relative abundances during maturation. Following the second part of the workflow, the characterization of intact glycopeptides of differentiated Caco-2 cells generated 2553 glycopeptides and 165 glycoproteins with 444 glycosites[24]. Approximately 62% of identified glycoproteins were single-pass transmembrane proteins, with half of them having their N-terminus exposed to the extracellular space. Another 20% of identified glycoproteins were multi-pass transmembrane proteins containing several protein regions extending into the extracellular region. The rest of identified glycoproteins were secretory or peripheral proteins.

Incorporation of non-native monosaccharide.

The workflow has also been applied to determine the incorporation of unnatural SiaNAz into the glycocalyx, with differentiated Caco-2 cells as an example[24]. The SiaNAz-incorporated N-glycans accounted for nearly 30% of total sialylated N-glycans. In contrast, SiaNAz groups were more highly incorporated into O-glycans, where more than 70% of sialylated O-glycans contained SiaNAz. The rate of incorporation of SiaNAz into all sialylated glycosphingolipids was around 8%.

The glycocalyx under variable environmental conditions.

The protocol has also been employed to monitor the glycan variations seen when cells are subjected to environmental changes. This includes the use of different cell culture supplementations such as the addition of exogenous dietary monosaccharides and short-chain fatty acids, and differing pH in the culturing conditions[23]. The supplementation of different exogenous monosaccharides generated varied N-glycan types. In addition, fucosylated N-glycans showed significant changes for cells treated with short-chain fatty acids (SCFAs), including acetate, lactate, and butyrate. The levels of sialylated N-glycans showed A drastic increase for cells cultured at lower pHs. These findings shed light on the roles that the cell surface glycocalyx plays in the interaction of cells in differing environments.

Isotopic and oxidative labeling in the glycocalyx.

This workflow is also compatible with other techniques, including proteomic analysis and metabolic labeling. For example, by combining the glycomics and glycoproteomics workflows with standard proteomics workflow, we identified potential sialic acid-associating

proteins on the cell surface using proximity labeling[92]. The glycomic results demonstrated the incorporation of unnatural monosaccharides and the conjugation of the labeling probe. The glycoproteomic analysis mapped sialylated glycoproteins on the cell surface with site-specific information, and the oxidative proteomic analysis provided information about the proteins that were localized around the sialic acid. As a result, it revealed the structural environment of sialylated proteins in the glycocalyx. We have also successfully combined this method with metabolic labeling to investigate the metabolic fate of monosaccharides in cell membranes[93]. Caco-2 and hepatic KKU-M213 cells were treated with ^{13}C-labeled dietary saccharides, and the ^{13}C-incorporated glycans and glycoproteins on the cell membrane were characterized quantitatively using the described glycomics and glycoproteomics workflows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data availability

Data are all available online. Data from ref. 23 Park, D. *et al. Glycobiology* **27**, 847–860 (2017), DOI:10.1093/glycob/cwx041 (used for Fig. 4a) are available at Glycobiology online. Data from ref. 24 Park, D. *et al. Chemical science* **9**, 6271–6285 (2018), DOI: 10.1039/c8sc01875h (used for Fig. 4b and 9) are available at Chemical Science online. Data from ref. 26 Wong, M. *et al. Scientific reports* **8**, 10993 (2018), DOI: 10.1038/s41598-018-29324-7 (used for Fig. 4c) are available at Scientific Reports online. Data from ref. 25 Li, Q. *et al. Chemical science* **10**, 6199–6209 (2019), DOI:10.1039/c9sc01360a (used for Fig. 6) are available at Chemical Science online. Data from ref. 33 Park, D. *et al. Molecular & cellular proteomics* : MCP **14**, 2910–2921 (2015), DOI:10.1074/mcp.M115.053983 (used for Fig. 8) are available at MCP online.

Related links

Key references using this protocol

1. Park, D. *et al.* Membrane glycomics reveal heterogeneity and quantitative distribution of cell surface sialylation. *Chemical science* **9**, 6271–6285 (2018), DOI: 10.1039/c8sc01875h.

2. Wong, M., Xu, G., Park, D., Barboza, M. & Lebrilla, C. B. Intact glycosphingolipidomic analysis of the cell membrane during differentiation yields extensive glycan and lipid changes. *Scientific reports* **8**, 10993 (2018), DOI: 10.1038/s41598-018-29324-7.

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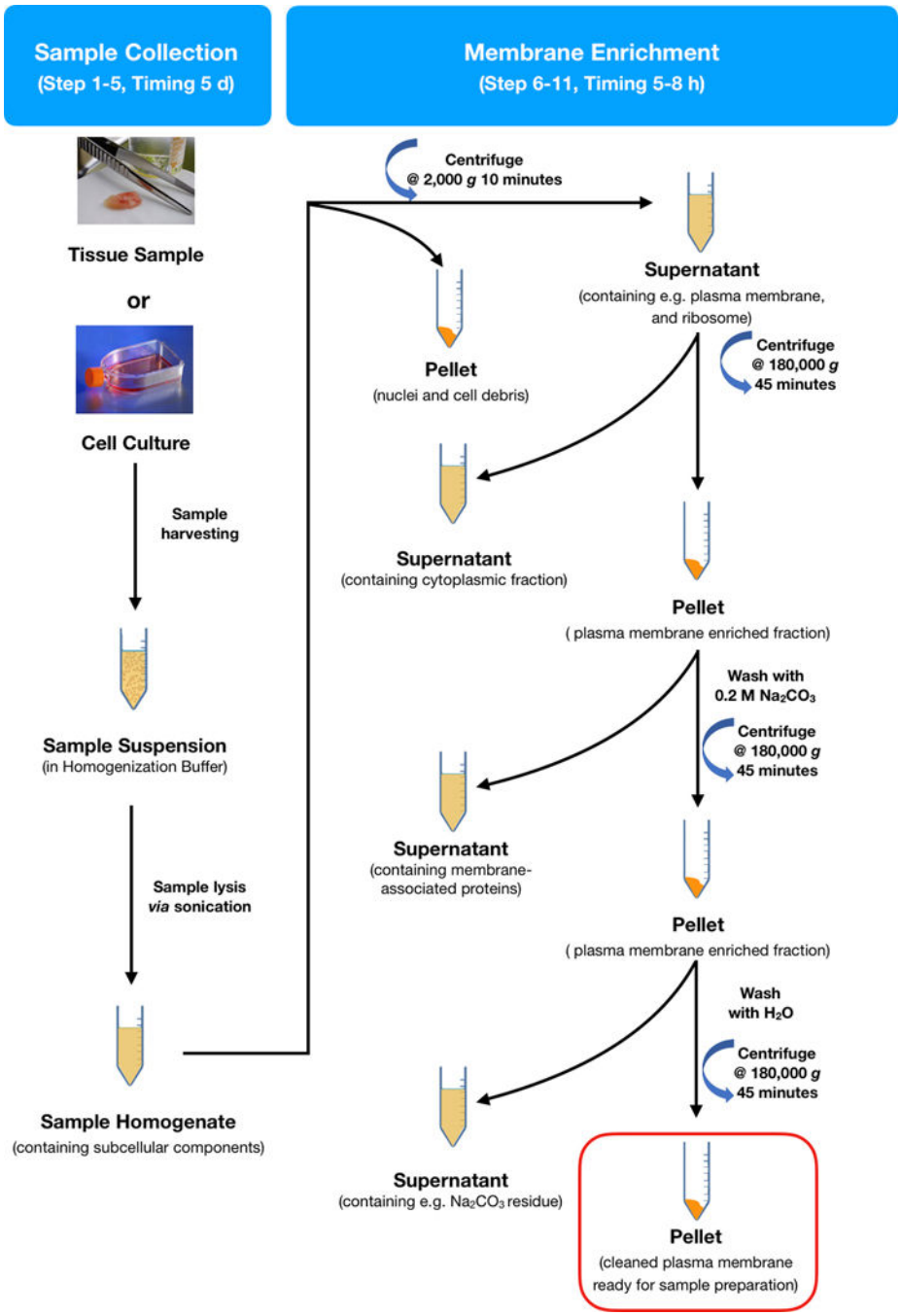

Figure 1. Summary of the workflow for cell sample collection and membrane extraction.

More than ten million cells are harvested, followed by buffer exchange and homogenization via sonication at 4 °C. Nucleus and cell debris are firstly separated via centrifugation. Then the crude plasma membrane fraction is pelletized via ultracentrifugation, followed by aqueous sodium carbonate and water washings.

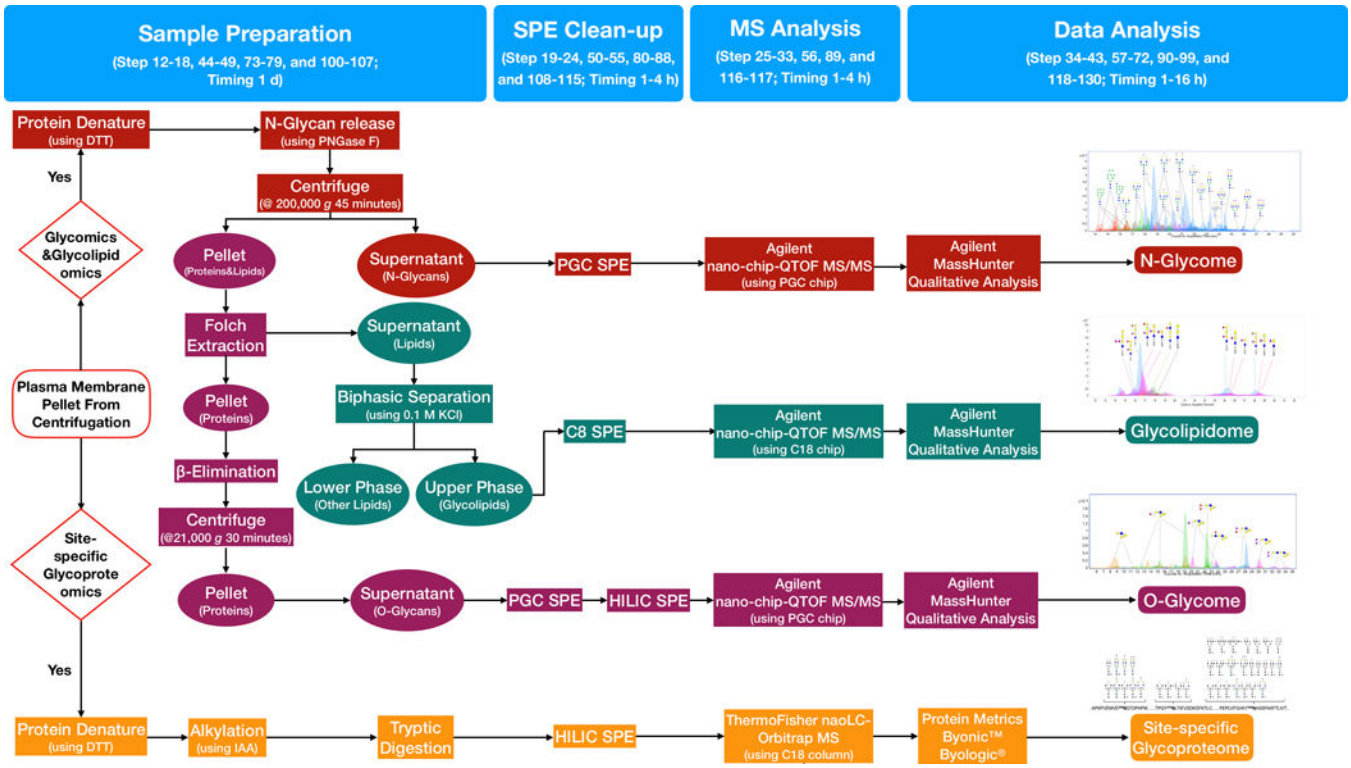

Figure 2. Summary of the workflow for comprehensive LC-MS/MS analysis of the cell membrane glycocalyx.

The steps of the experiment are outlined, including the time requirements for the corresponding steps. The route for N-glycomic analysis is labeled with red, the route for glycolipidomic analysis is with green, the route for O-glycomic analysis is pink, and the route of site-specific glycoproteomic analysis is orange.

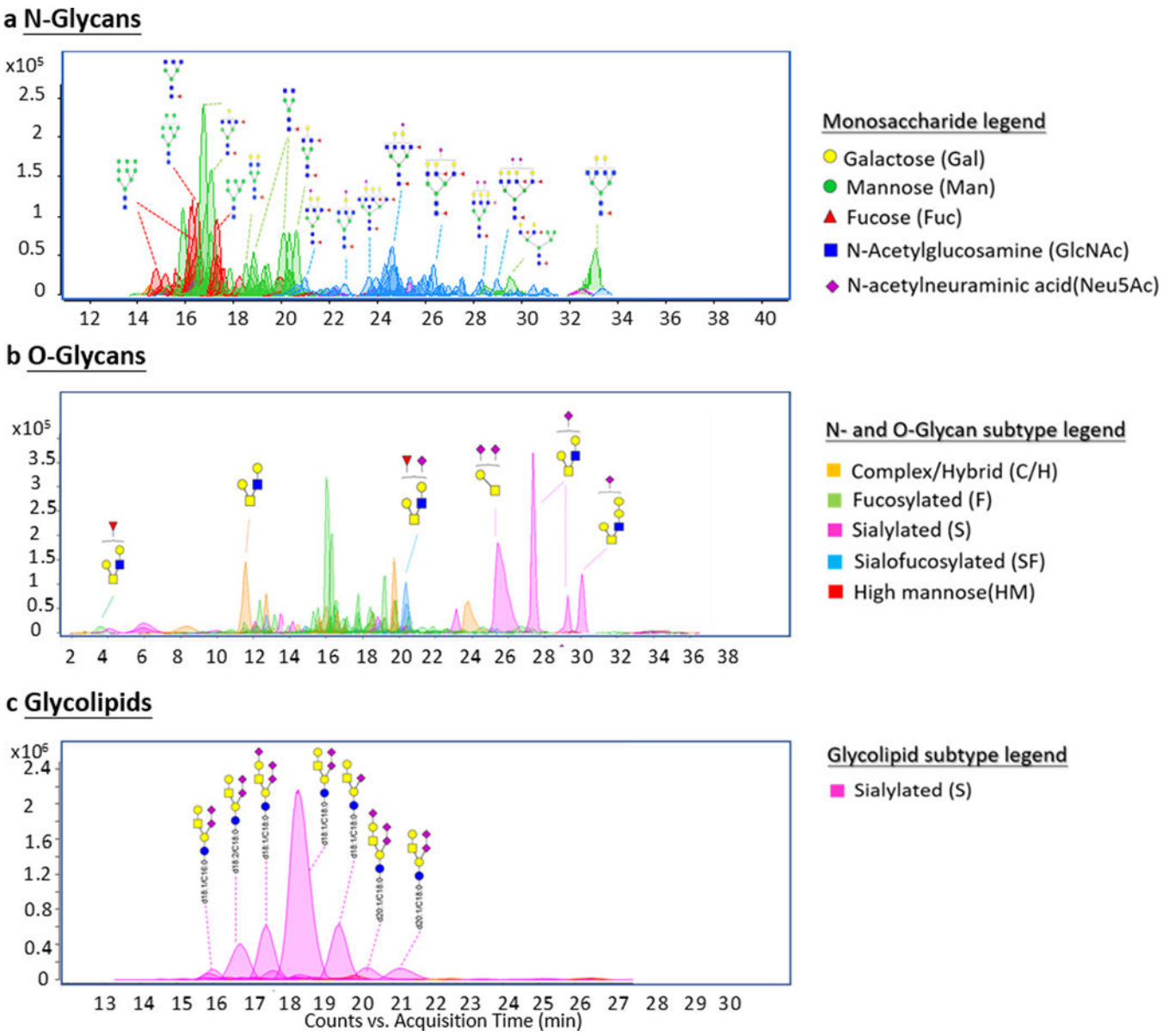

Figure 3. Examples of tissues with cell surface glycocalyxes analyzed by LC-MS/MS.

a. Brain N-glycome was obtained by PGC-chip-QTOF MS/MS. More than 600 N-glycans were observed with individual ion counts around 3×10^5. **b**. O-Glycan profile obtained by nanoLC-QTOF MS/MS using a PGC LC chip. More than 70 O-glycans were observed with ion abundances at 5×10^9 total ion. **c.** Glycolipid profile obtained by nanoLC-QTOF MS/MS using a C18 chip. More than 100 glycolipids were observed with abundances of 2×10^8. The peaks were color coded to show the glycan subtypes.

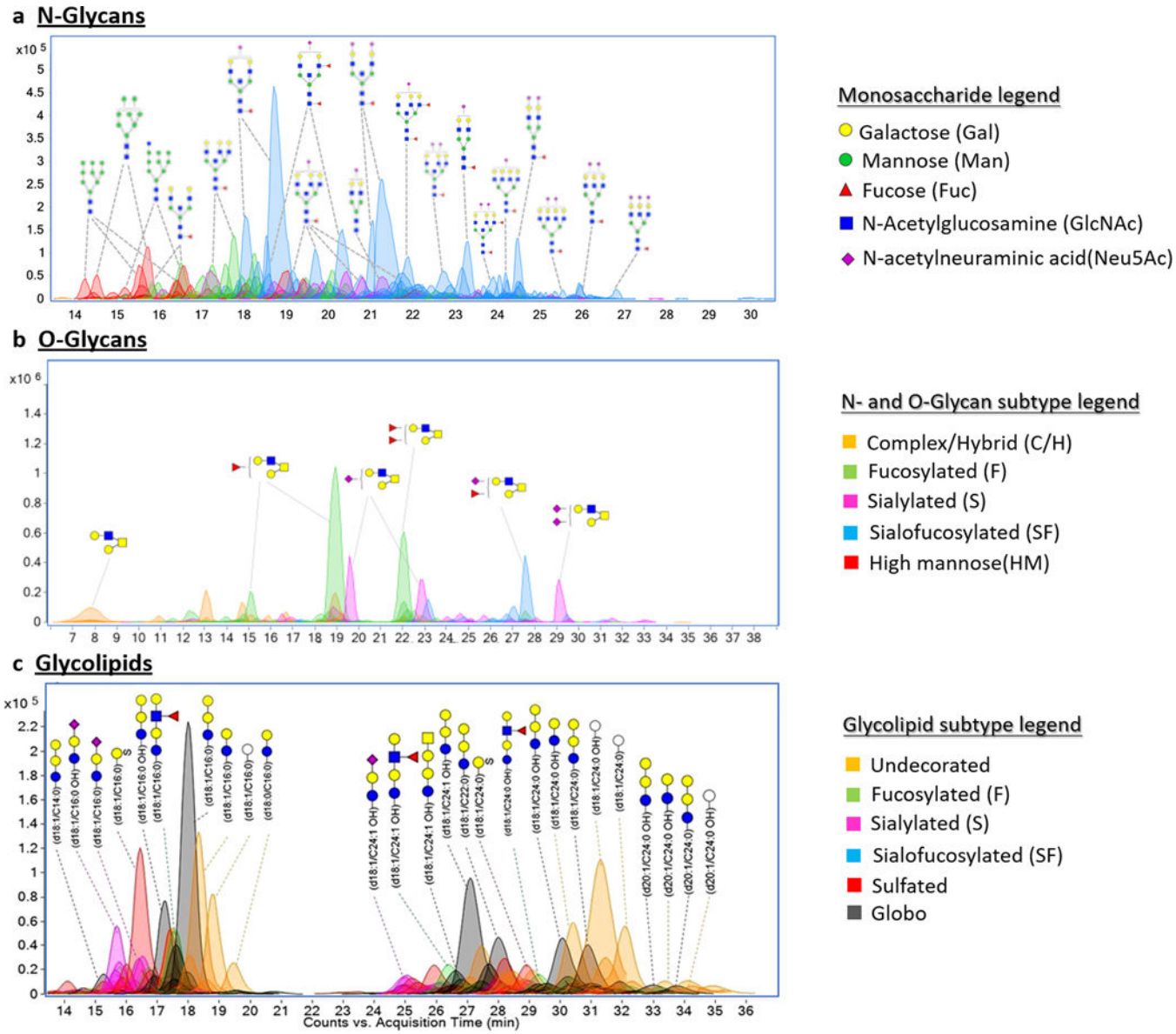

Figure 4. Examples of LC-MS profiles from the Caco-2 cell line.

a. N-Glycan profile obtained by nanoLC-QTOF MS/MS using a PGC chip. More than 500 N-glycans were observed with ion abundances of 4×10^9. **b**. O-Glycan profile obtained by nanoLC-QTOF MS/MS with a PGC chip. More than 50 O-glycans were observed with abundances of 5×10^9. **c.** Glycolipid profile obtained by nanoLC-QTOF MS/MS with a C18 chip. More than 200 glycolipids were observed with abundances of 2×10^8 ion. The peaks are color coded to show the glycan subtypes. N-Glycan profile reprinted with permission from ref. 23. Copyright 2017 by Oxford University Press. O-Glycan profile reprinted with permission from ref. 24. Creative Commons licence (CC BY 3.0) (http://creativecommons.org/licenses/by/3.0/). Glycolipid profile reprinted with permission from ref. 26. Creative Commons licence (CC BY 4.0) (http://creativecommons.org/licenses/by/4.0/).

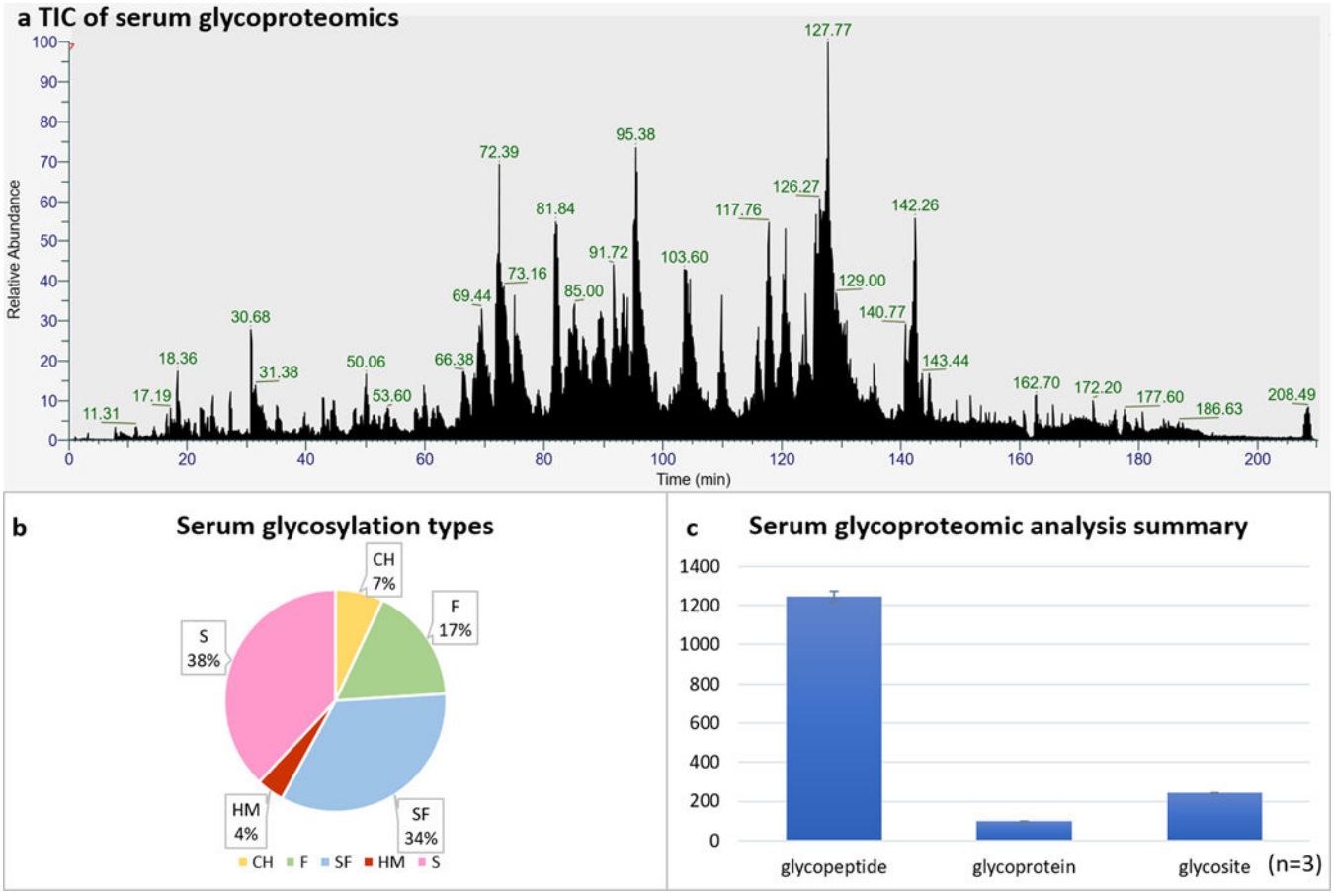

Figure 5. Glycoproteomic data of serum standard.

a. The TIC of a serum glycoproteomic LC-MS/MS. For the analysis, approximately 1μg of digested serum glycopeptides were injected for analysis. The intensity of the base peak in the MS scan was 2.12E10. **b.** The summary of serum glycosylation types. The glycans was classified into five subtypes: complex/hybrid without fucoses or sialic acids (CH), fucosylated (F), sialofucosylated (SF), high-mannose (HM), and sialylated. **c.** The summary of the identified glycopeptides, glycoproteins, and glycosites. The data were collected from three technical replicates.

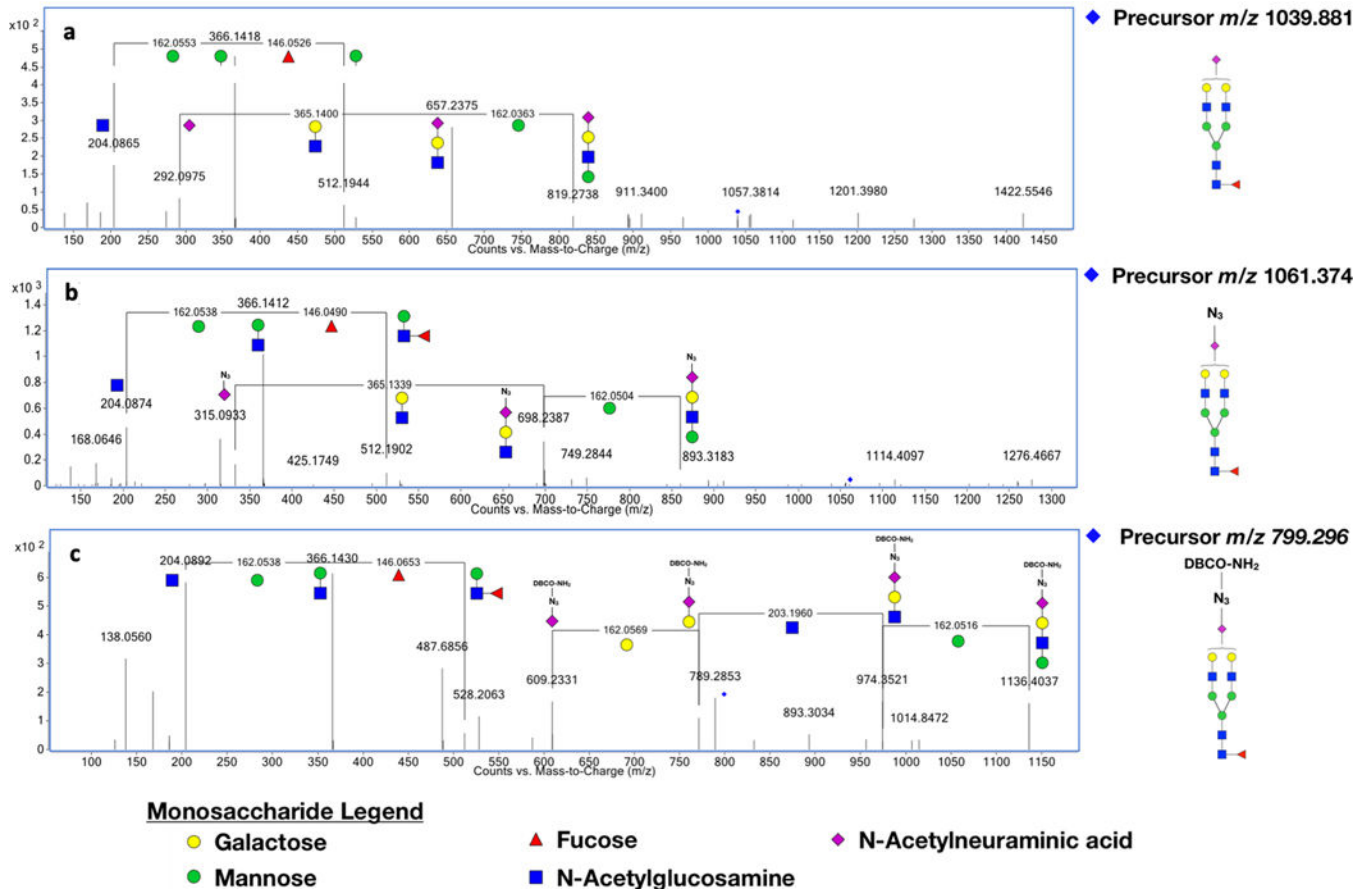

Figure 6. Tandem MS spectra of selected compounds.

a. N-Glycan $Hex_5HexNAc_4Fuc_1Sia_1$ containing three mannose, two galactose, four N-acetylglucosamine, one fucose, and one sialic acid residue with a precursor *m/z* value of 1039.991. The *m/z* values of 292.097 and 274.092 correspond to the the sialic acid residue, and 657.237 and 819.273 correspond to the $Gal_1GlcNAc_1Sia_1$ and $Man_1Gal_1GlcNAc_1Sia_1$ fragments, respectively. **b**. N-Glycan $Hex_5HexNAc_4Fuc_1SiaNAz_1$ containing three mannose, two galactose, four N-acetylglucosamine, one fucose, and one azido-sialic acid residue with a precursor *m/z* value of 1061.374. The *m/z* 315.093 and 333.104 correspond to the azido-sialic acid residue, and 698.238 and 893.319 correspond to the $Gal_1GlcNAc_1SiaNAz_1$ and $Man_1Gal_1GlcNAc_1SiaNAz_1$ fragments, respectively. **c**. N-Glycan $Hex_5HexNAc_4Fuc_1(SiaNAz\text{-}DBCO\text{-}NH_2)_1$ containing three mannose, two galactose, four N-acetylglucosamine, one fucose, and one dibenzocyclooctyne-amine ($DBCO\text{-}NH_2$) conjugated azido-sialic acid residue with a precursor *m/z* value of 799.296. The *m/z* value of 591.223 and 609.233 correspond to the ($DBCO\text{-}NH_2$) conjugated azido-sialic acid residue, and 974.352 and 1136.403 correspond to the $Gal_1GlcNAc_1(SiaNAz\text{-}DBCO\text{-}NH_2)_1$ and $Man_1Gal_1GlcNAc_1(SiaNAz\text{-}DBCO\text{-}NH_2)_1$ fragments, respectively. The glycan annotations and legend of monosaccharides are provided. Figures reprinted with permission from ref. 25. Creative Commons licence (CC BY 3.0) (http://creativecommons.org/licenses/by/3.0/).

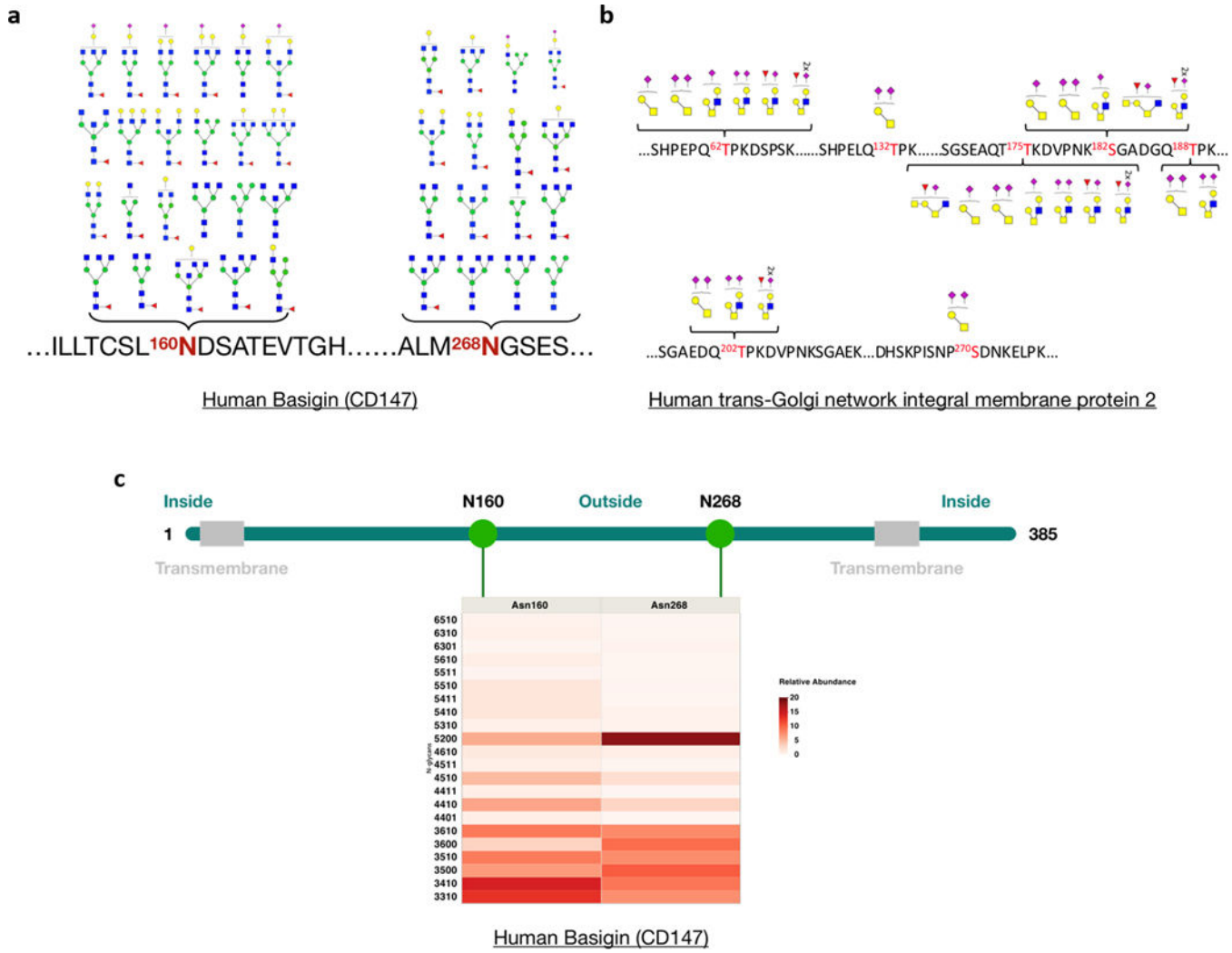

Figure 7. Examples of site-specific glycopeptide mapping of N-linked glycoprotein and O-linked glycoprotein.

a. Human Basigin, also known as CD147, was identified from Caco-2 cells, and its N-linked glycans were identified with the site heterogeneity at Asn160 and Asn268. b. Human trans-Golgi network integral membrane protein 2 was obtained from HT-29 cells, and its O-linked glycans were identified with the site heterogeneity at Thr62, Thr132, Thr175, Ser182, Thr188, Thr202, and Ser270. **c**. Basigin extracellular/intracellular domains were analyzed by transmembrane helix prediction software, and both of Asn160 and Asn268 were expected to be extracellular. The relative abundances of glycans at two different sites were quantified with the Byologic software. The heat map showed the degrees of glycosylation at two different glycosites thereby revealing quantitatively the glycosylation heterogeneity at different sites.

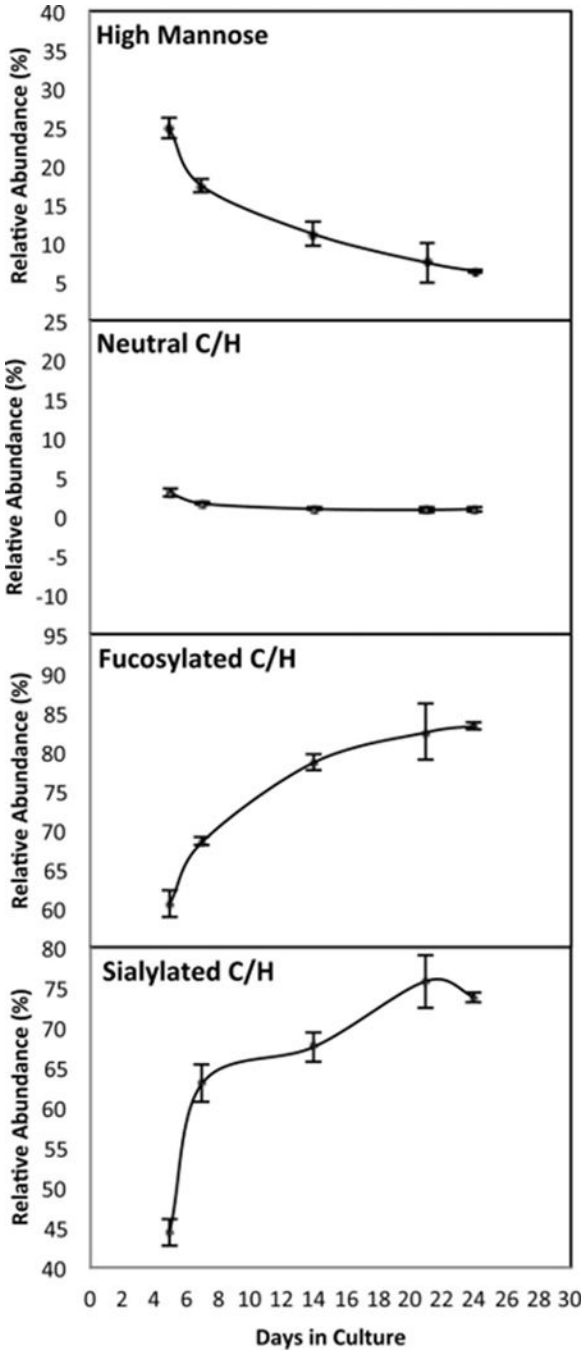

Figure 8. The N-glycomic analysis of Caco-2 cells cultured grown at different days.

The profile illustrated the increased levels of sialylated and fucosylated C/H N-glycans, and the decreased level of high-mannose N-glycans. Reprinted with permission from ref. 33. Copyright 2015 by American society for biochemistry and molecular biology.

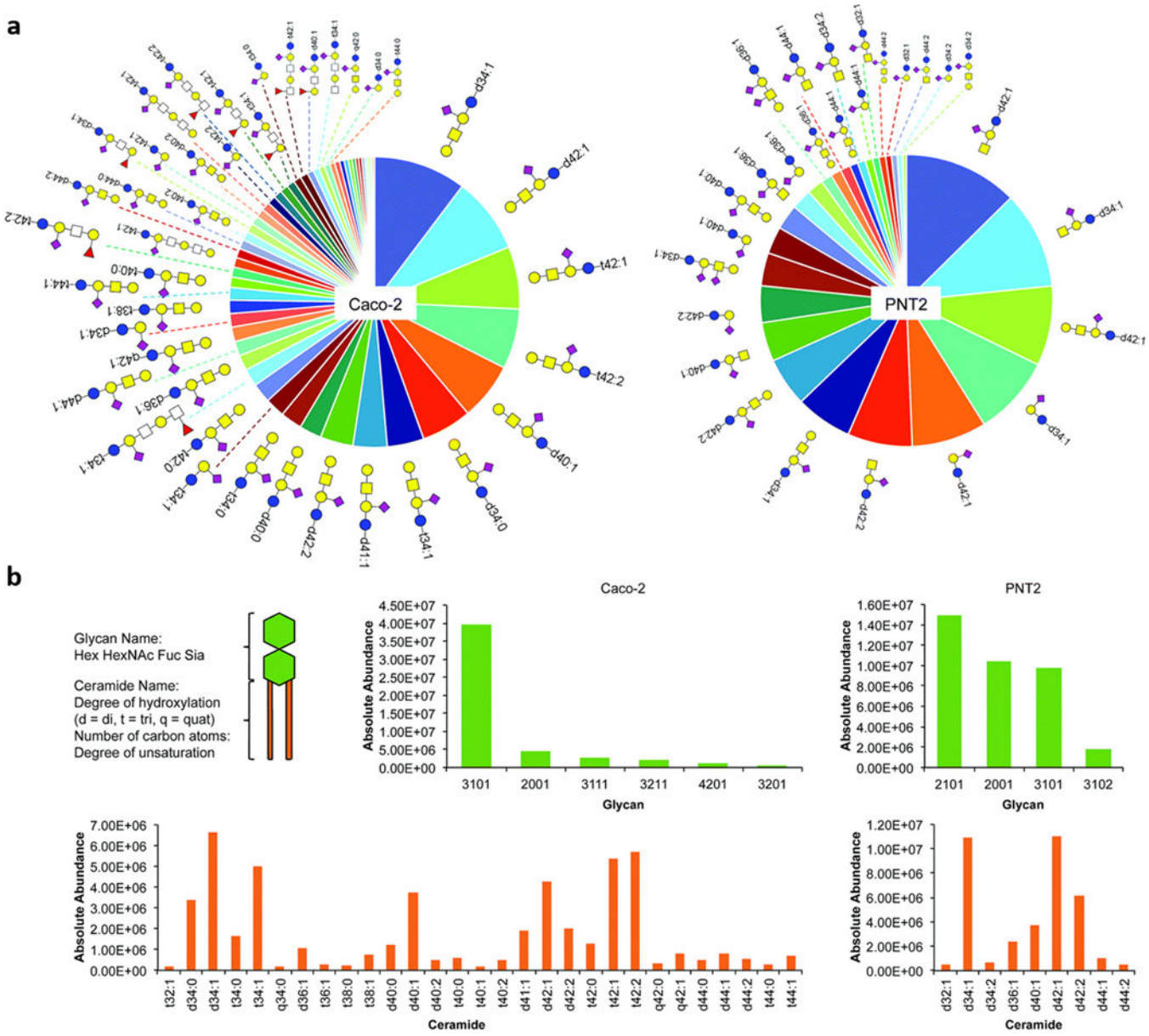

Figure 9. The identification and quantitation of glycosphingolipids (GSL) from Caco-2 and PNT2 cells lines.

a. Sialylated GSLs with theire tentative structures were ordered by their abundances. **b**. Sialylated GSLs were quantified and compared based on the head groups and ceramide types. Reprinted with permission from ref. 24. Creative Commons licence (CC BY 3.0) (http://creativecommons.org/licenses/by/3.0/)

Table 1.

LC-MS/MS Data acquisition parameters for N-glycan, glycolipid, and O-glycan analyses.

	N-glycomics	O-glycomics	Glycolipidomics
LC Parameters			
Column Packing Material	PGC	PGC	C18
Typical Injection volume	2 μL	5 μL	5 μL
Solvent A (vol/vol)	0.1% FA, 3% ACN, and 96.9% H_2O	0.1% FA, 3% ACN, and 96.9% H_2O	0.1% acetic acid, 99.9% 20 mM ammonium acetate in H_2O
Solvent B (vol/vol)	1% FA, 89% ACN, and 10% H_2O	1% FA, 89% ACN, and 10% H_2O	0.1% acetic acid, 99.9% 20 mM ammonium acetate in 85:15 methanol/ isopropanol (vol/vol)
Flow rate (cap pump)	3 μL/min		
Flow rate (nano pump)	0.3 μL/min		
Gradient (%B (vol/vol))	0 min-2 min: 0%−0% 2 min-20 min: 0%−16% 20 min-40 min: 16%−72% 40 min-42 min: 72%−100% 42 min-52 min: 100%−100% 52 min-54 min: 100%−0% 54 min-65 min: 0%−0%	0 min-2 min: 0%−0% 2 min-20 min: 0%−16% 20 min-40 min: 16%−72% 40 min-42 min: 72%−100% 42 min-52 min: 100%−100% 52 min-54 min: 100%−0% 54 min-65 min: 0%−0%	0 min-4 min: 70%−85% 4 min-40 min: 85%−100% 40 min-50 min: 100%−100% 50 min-52 min: 100%−70% 52 min-65 min: 70%−70%
MS Parameters			
m/z mass range	600–2000	300–2000	600–2000
Polarity	Positive		
Gas Temperature	325 °C		
Capillary Voltage	1,700–2,000 V (N-glycans, O-glycans) 1,400–1,700 (glycolipids)		
Cycle Time	5.25 s		
MS Scan Rate	0.8 spectra/s (1250 ms/spectrum)		
MS/MS Scan Rate	1.0 spectra/s (1000 ms/spectrum)		
MS threshold	Abs. threshold 200; Rel. threshold 0.01%		
MS/MS threshold	Abs. threshold 200; Rel. threshold 0.01%		
Calibrated Mass	1221.990637		
Acquisition Mode	Auto MS/MS (Seg)		
Activation Type	CID		
Activation Energy	$V_{collision}$=1.8 * (*m/z*)/100 – 2.4 (N-glycans, O-glycans) $V_{collision}$=1.2 * (*m/z*) /100 + 12 (Glycolipids)		
Max Precursors per Cycle	4		
Precursor Selection Threshold (Abs)	1,000		
Precursor Target Counts/Spectrum	25,000 counts/spectrum		
Dynamic Exclusion	Excluded after 1 spectrum, released after 0.35 min		
Precursor charge state preference	2 > 1 > 3 > unknown charge > larger than 3 charges		

	N-glycomics	O-glycomics	Glycolipidomics
Isotope Model	Common organic molecules		

Table 2.

Data analysis parameters using Agilent MassHunter Qualitative Analysis B.08.00 software.

Tabs	Checked Parameters	Values
Extraction	Use peaks with height	≥ 100 counts
	Extraction algorithm Target data type	Small molecules (chromatographic)
	Restrict retention time	0–40 min
Ion Species	Positive ions	+H (N-glycans, O-glycans) +H, $+NH_4$ (glycolipids)
Charge State	Peak spacing tolerance	0.0025 *m/z*, 7.0 ppm
	Isotope model	Common organic molecules, no halogens
	Limit assigned charge states to a maximum	3
Compound filters	Absolute Height	≥ 1,000
	Compound quality score	≥ 30
Mass Filters	Filter mass list	20 ppm
	Database	corresponding libraries
Results	Delete previous compounds	
	Highlight first compound	
	Extract ECC	
	Extract MS/MS spectrum	

Table 3.

LC-MS/MS data acquisition parameters for glycopeotide analysis.

LC Parameters	
Injection volume	1 μL
Flow rate	0.3 μL/min
Time interval (min)	nanoLC gradient (% B (vol/vol))
0–5	4%−4%
5–133	4%−32%
133–152	32%−48%
152–155	48%−100%
155–170.5	100%−100%
170.5–170.7	100%−4%
170.7–180	4%−4%
MS Parameters	
Polarity	Positive
Internal lock-mass calibrant	455.12002
MS1 OT	
OT resolution	60,000
Scan Range, *m/z*	350–2,000
RF lens (%)	20
AGC target	1.0e6
Maximum Injection Time (ms)	50
Filters	
Charge state	2–6
Dynamic exclusion	Exclude 1 time within 35 s
Intensity	5.0e4
MIPS	Peptide
Precursor selection range	700–2,000
MS2 OT	
Isolation window (*m/z*)	4
Activation type	HCD
HCD collision energy (%)	30±10
OT resolution	15,000
First mass	120
AGC target	8.0e4
Maximum Injection Time (ms)	300
Cycle time (s)	3

Table 4.

Data analysis parameters of glycopeptides using Byonic v 3.5.0 software

Tab	Section	Parameters	Values
Digestion and Instrument Parameters	Sample digestion	Cleavage site (s)	KR
		Cleavage side	C-terminal
		Digestion specificity	Fully specific (fastest)
		Missed cleavages	2
	Instrument parameters	Precursor mass tolerance	10 ppm
		Fragmentation type	Both CID & HCD
		Fragment mass tolerance (CID)	20 ppm
		Fragment mass tolerance (HCD)	20 ppm
		Recalibration (lock mass)	None
Modifications	Fixed and variable modifications	Total common max	1
		Fixed modification	Carbamidomethyl modification at cysteine residues (+57.021464)
		Variable modifications	Details included in Table 5 (Edit more from “Enter/edit”)
Glycans	/	Enter/edit	Load the glycan library
Advanced	Spectrum input options	Default	
	Peptide output options	Show all N-glycopeptides	Uncheck
	Proteins output options	Protein FDR	1% FDR (or 20 reverse count)
		Export mzIdentML	check

Table 5.

Fixed and variable modifications

Modifications	Targets	Fine Control
Carbamidomethyl / +57.021464	C	Fixed
Oxidation / +15.994915	M	Variable – common 1
Deamidated / +0.984016	N	Variable – common 1
	Q	Variable – common 1
Acetyl / +42.010565	Protein N-term	Variable – rare 1
Gln>pyro-Glu / −17.206549	Protein N-term Q	Variable – rare 1
Glu>pyro-Glu / −18.010565	Protein N-term E	Variable – rare 1

Table 6:

Troubleshooting

Step	Problem	Possible reason	Solution
20	There is leftover water in some wells.	It is difficult for hydrophilic water to go through hydrophobic PGC materials.	Use a higher speed (180 x g) to centrifuge for another round.
27	The autosampler errors out at the beginning of the worklist.	The correct type of well-plate is not assigned.	Check the “assign well-plate” tab. Select the “Eppendorf 96-well plate” for the 96-well injection plate and select the “54 vial plate” for the injection vials.
30	The pressure of pumps is higher than normal.	Chips might not be properly conditioned. It is also possible that the chip or other compartments such as inlet filters or some capillaries are clogged.	To condition the chip completely, decrease the composition of solvent B with an increment of 20% until 2% of solvent B. If the pressure is still high, back flush the chip or change an inlet filter or a new capillary.
31	Dripping or scattered spray is observed.	The capillary voltage is not optimized.	Increase the source voltage while observing the spray changes. Increase the capillary voltage with an increment of 50 volts (V) until a consistent spray is seen. The highest voltage cannot exceed 2,400 V. Make sure that the capillary current does not exceed 100 nano ampere (nA).
32	There is no signal during the run or the LC pumps errors out.	The spray disappears during one batch or the solvent level goes down to 0.005 L.	During the analysis of one batch of sample, check the chip spray and the solvent level regularly to make sure the instrument is running properly.
45	Phase separation is observed.	Water and/or salt content in sample is high.	Adjust the solvent composition by adding 2:1 Methanol/ Chloroform in 50 μL increments until solvents become miscible and a single liquid phase is observed. Vortex mix the sample in between additions. If phases are still immiscible after adding 200 μL, skip step 47 (addition of KCl) and continue as normal.
48	No phase separation is observed.	Not enough H_2O or KCl solution	Add an extra small amount of water or 0.1 M KCl solution and mix carefully.
75	The volume of added $NaBH_4$ is not consistent for every sample.	Large amounts of bubbles are generated in the $NaBH_4$ solution.	Vortex mix $NaBH_4$ solution shortly to remove bubbles every time just before adding to the sample to make sure the volume added is accurate.
77	Samples are overflowed from the tube.	Large amounts of gas are generated in the neutralization reaction.	Add the acetic acid drop by drop to avoid generating bubbles vigorously.
111	Samples cannot be dissolved completely.	The dried pellet is formed, and they are more soluble in hydrophilic solvent rather than the solvent with 80% ACN.	Sonicate the mixture for several minutes. When drying samples, dry them down to around 100 μL. Estimate the volume of the sample and add H_2O, 80% (vol/vol) ACN, and 1% (vol/vol) TFA to make the final volume 1 mL.