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Comprehensive structural glycomic characterization of the glycocalyxes of cells and tissues

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The glycocalyx comprises glycosylated proteins and lipids and fcorms the outermost layer of cells. It is involved in fundamental inter- and intracellular processes, including non-self-cell and self-cell recognition, cell signaling, cellular structure maintenance, and immune protection. Characterization of the glycocalyx is thus essential to 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–tandem MS (LC-MS/MS) analysis, and data processing methods are provided. In addition, we discuss glycoproteomic analysis that yields the site-specific glycosylation of membrane proteins. To reduce the amount of sample needed, N-glycan, O-glycan, and glycolipid analyses are performed on the same enriched fraction, whereas glycoproteomic analysis is performed on a separate enriched fraction. The sample preparation process takes 2–3 d, whereas the time spent on instrumental and data analyses could vary from 1 to 5 d 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.

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 substantial 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 (MS) instruments, now allows the analyses of glycans and glycoconjugates in large, complicated mixtures at a previously unattainable range of femtomolar to attomolar amounts. Over the past 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 multi-glycomic method based on 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 of intact cell surface glycolipids[26], which we describe here.

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Development of the protocol

Specific chromatographic separation and sensitive MS have been key factors in the development of this protocol. Our laboratory has been involved in developing nanoLC-MS-based analytical methods for the analysis of naturally occurring free glycans in body fluids such as human milk oligosaccharides (HMOs)[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 MS (nanoLC-QTOF MS) and nanoLC-TOF MS. The use of nanoLC–Orbitrap MS/MS for glycoproteomic analysis with site-specific characterization is incorporated later in the workflow.

In the development of these methods, we made every effort 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. In addition, we aimed our efforts at the ability to perform several analyses on the same sample. Thus, from the same starting plasma membrane material, all the N-glycans, O-glycans, and glycolipids are 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 is analyzed for protein and glycoprotein analysis because the glycan-depleted fractions cannot be used for glycoproteomic analysis.

Advantages and comparison with alternative methods

The most commonly used method for characterizing the glycocalyx uses lectins, which are proteins that recognize glycans through specific structural motifs. Lectins are convenient and rapid and require no additional complicated or large instrumentation. Fluorescently labeled lectins are readily available to characterize the cell surface glycocalyx by microscopy. However, this approach has severe limitations. Lectins provide information about specific structural motifs but are unable to determine complete glycan structures or glycan composition, or to 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 may detect α-mannose, which is present 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, which vary. Responses of lectins with different specificities also cannot be quantitatively compared.

The glycomic analysis in this protocol is conducted on native glycans, that is, without labeling. This method minimizes sample losses due to sample manipulation and incomplete reactions associated with labeling. Various labeling methods have been used 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 cleanup lead to potential losses, particularly of glycans with relatively low abundance.

For the glycoproteomic analysis, glycopeptides are enriched with solid-phase extraction (SPE) hydrophilic interaction liquid chromatography (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 can 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 cleanup 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 monosaccharide analogs, 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 chromatography, 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 used with their respective flow rates, pressure, detection sensitivities, and separation efficiencies. In this protocol, the application of nanoLC enables analyses with high sensitivity. Other separation techniques can also be used 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 because of the low flow rates used in 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 complex 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 application of IM–MS to glycopeptide analysis is even more complicated because of the high numbers of isomers, as well as gas-phase conformers that are 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 contaminants[49]. It has been coupled to mass detectors with high resolution and mass accuracy, such as those used for Fourier-transform ion cyclotron resonance and time-of-flight (TOF). MALDI MS is substantially more rapid and can provide comprehensive glycomic profiles of released glycans. However, MALDI MS is not as sensitive as nanoLC-MS. In addition, 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 fragments similar to those from CID[50]. It has been extensively used but is not readily available[45]. 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[51]. Another fragmentation technique applied to glycopeptides is electro-transfer/higher-energy collision dissociation (EThcD), which yields abundant fragments corresponding to both glycans and peptides[52]. 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 Figs. 1 and 2. Cells are first harvested, and then the membrane disrupted. Enrichment of the plasma membrane is performed by a series of centrifugation and ultracentrifugation steps (Fig. 1). The membrane pellet is divided into two fractions: one for the glycomic analysis and one for the glycoproteomic analysis. One fraction of the membrane is used for the release and extraction of N- and O-glycans and glycolipids, followed by their separate LC-MS analyses, whereas 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 with porous graphitized carbon chromatographic chips. Glycolipid profiling is performed on intact compounds using nanoLC-QTOF MS with a C18 chip column. Agilent's MassHunter software is used 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, which includes Byonic for glycoproteomic analysis and Byologic for glycopeptide quantitation. Although the protocol described

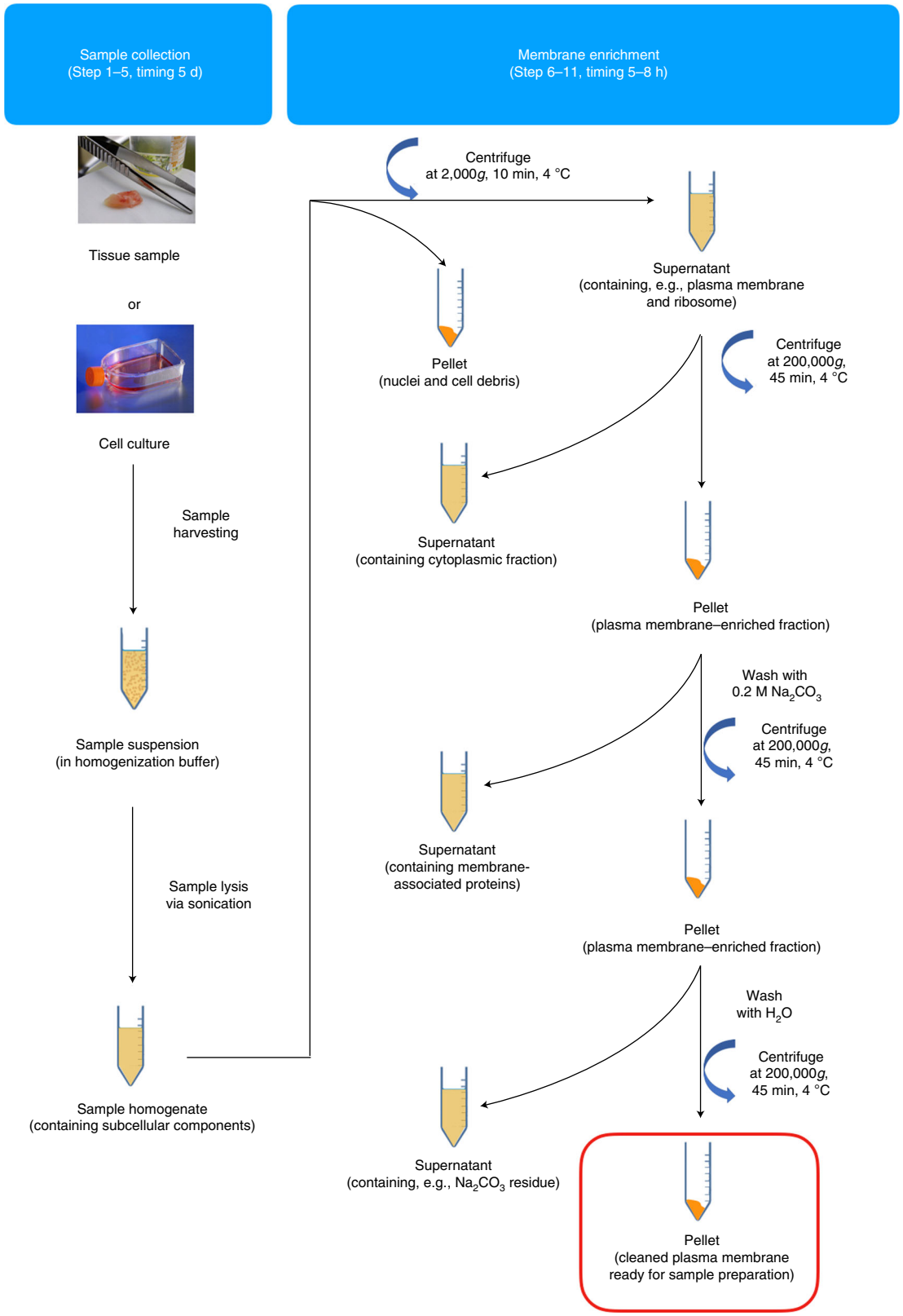

Fig. 1 | Summary of the workflow for cell sample collection and membrane extraction. More than 10 million cells are harvested, followed by buffer exchange and homogenization via sonication at 4 °C. Nuclei and cell debris are first separated via centrifugation. Then the crude plasma membrane fraction is pelleted via ultracentrifugation, followed by aqueous sodium carbonate and water washings.

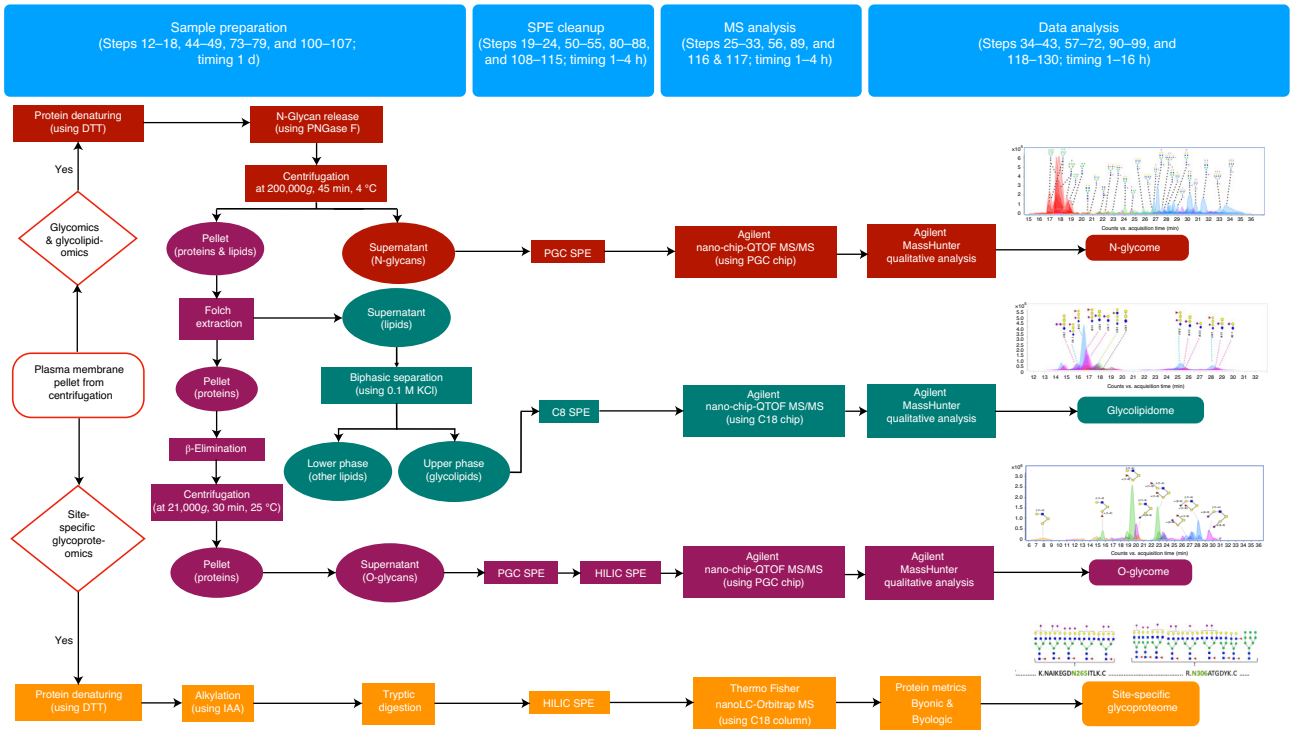

Fig. 2 | Summary of the workflow for comprehensive LC-MS/MS analysis of the cell membrane glycocalyx. The steps of the experiment are outlined, and the time requirements for the corresponding steps are presented. The route for N-glycomic analysis is labeled with red, that for glycolipidomic analysis with teal, that for O-glycomic analysis with magenta, and that for site-specific glycoproteomic analysis with orange.

here 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 shown in Fig. 3. In addition, the methods can be adapted in part 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). Although absolute abundances are not achievable mainly because of 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 (QC) 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 1–11)

Cell lines should be cultured according to the protocols provided by the American Type Culture Collection (ATCC) or by following literature advice. We have successfully used human cell lines that include 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 the cells should be checked regularly, at least once every other day. The method can also be applied to other cell lines. Each cell line has 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 (ref. [53]) cells, which are used to illustrate this protocol; these cells will differentiate 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 the cells at near-80% confluency.

For a comprehensive analysis, at least 10 million cells are required, and these need to be separated into two fractions (fractions I and II). Fraction I, for glycomic (N- and O-glycans) and glycolipid analyses, requires fewer cells (~1 × 10^6 cells). Fraction II, for site-specific glycoproteomic analysis,

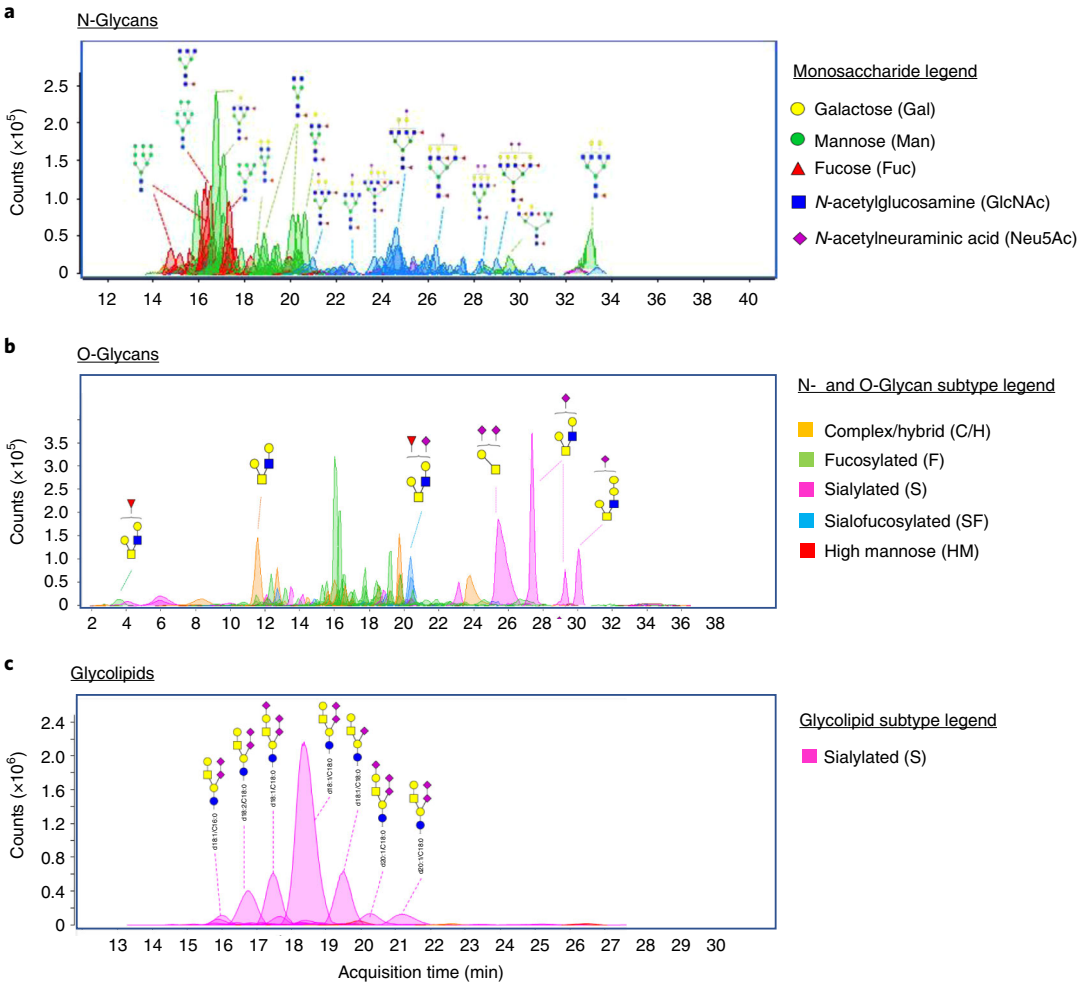

Fig. 3 | Examples of tissues with cell surface glycocalyxes analyzed by LC-MS/MS. a, Brain N-glycome was obtained by PGC-chip-QTOF MS/MS. More than 600 N-glycans were observed with individual peak heights of around 2×10^5 ion counts. **b**, O-Glycan profile obtained by nanoLC-QTOF MS/MS using a PGC LC chip. More than 70 O-glycans were observed with individual peak heights of around 2×10^5 ion counts. **c**, Glycolipid profile obtained by nanoLC-QTOF MS/MS using a C18 chip. More than 100 glycolipids were observed with the most abundant species having a peak height of around 2×10^6 ion counts. The peaks are color coded to show the glycan subtypes, and monosaccharides are shown above the peaks.

requires the larger fraction of cells (at least 9×10^6 cells). Immediately after collecting the 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[54]. 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 the 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 fibrous or harder tissues, such as cartilage, more intense homogenization conditions should be used[34,55].

The membrane extraction method we describe was adapted from a previously described protocol[32]. The cell membrane is extracted after collecting and lysing cells and tissues. The nucleus and mitochondria are removed by centrifugation at low speed, followed by pelleting of the cell membrane fraction by 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[56,57]. The extraction of the cell membrane can be achieved with minimal sample preparation and is compatible with MS 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 Fig. 1.

N-Glycan analysis: release, purification, and mass spectrometry 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 min before N-glycan release, 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[58]. To minimize the content of amine-containing N-glycans, incubation at 37 °C in a water bath should be conducted after 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[59]. 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 used[60]. 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 h, followed by centrifugation to precipitate proteins completely. The pelleted membrane without N-glycans should be saved for subsequent O-glycomic and glycolipidomic analyses. The supernatant containing N-glycans is subjected to SPE cleanup with PGC in cartridges or 96-well plates for large batch preparation.

Desalted native N-glycans are injected into a nanoLC-TOF-MS/MS system equipped with a PGC nanochip for direct analysis. Direct analysis of native samples avoids loss from incomplete modification that is associated with all derivatization methods[35]. The use of nano-LC provides high sensitivity and a large dynamic range, enabling the identification of low-abundance species. Normal-flow or micro-flow LC systems can also be used for analysis of samples but require substantially higher amounts of material. Separation of N-glycans in PGC columns splits 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 used for 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[33,61,62]. Furthermore, for the instrument QC, the pooled released N-glycans from several cell lines under the normal condition (Caco-2, PNT2, and A549) can be used. We also advise the use of a commercial pooled serum and RNaseB N-glycan sample to perform 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 Fig. 4a. The major peaks are annotated; however, >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 Fig. 1a.

Glycosphingolipid analysis: extraction, purification, and MS measurements (Steps 44–56)

Glycosphingolipids (GSLs)are amphipathic compounds, and care should be taken to keep the purified GSLs dissolved to prevent loss through surface adsorption. A solution containing ≥50% of an organic solvent such as methanol should be used on purified GSLs. Initial testing of pipettes and vials should also be considered because surface adsorption can lead to poor recovery. The method we present here avoids the use of detergents and surfactants because they are incompatible with MS analysis.

Folch[63] or Bligh–Dyer[64] 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 GSLs 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/Bligh–Dyer extraction is advantageous for having the methanolic/aqueous phase as the top layer in the biphasic solvent extraction system. The recoveries of different GSL species depend on the characteristics of both the glycan and the lipid. GSLs with charged groups, such as sialic acids, have good recovery (>90%) even when they have longer lipids. Those with three or fewer neutral monosaccharides and longer lipids have poorer recoveries (<50%). Cerebrosides, which have only one monosaccharide residue, are not effectively recovered; ~90% of cerebrosides partition to the chloroform layer.

Internal standards, such as deuterated glycolipids, can be added to the sample before extraction for absolute quantitation. Internal standards of several glycolipid species are now commercially available from lipid-focused companies such as Avanti and Matreya, including sialylated gangliosides,

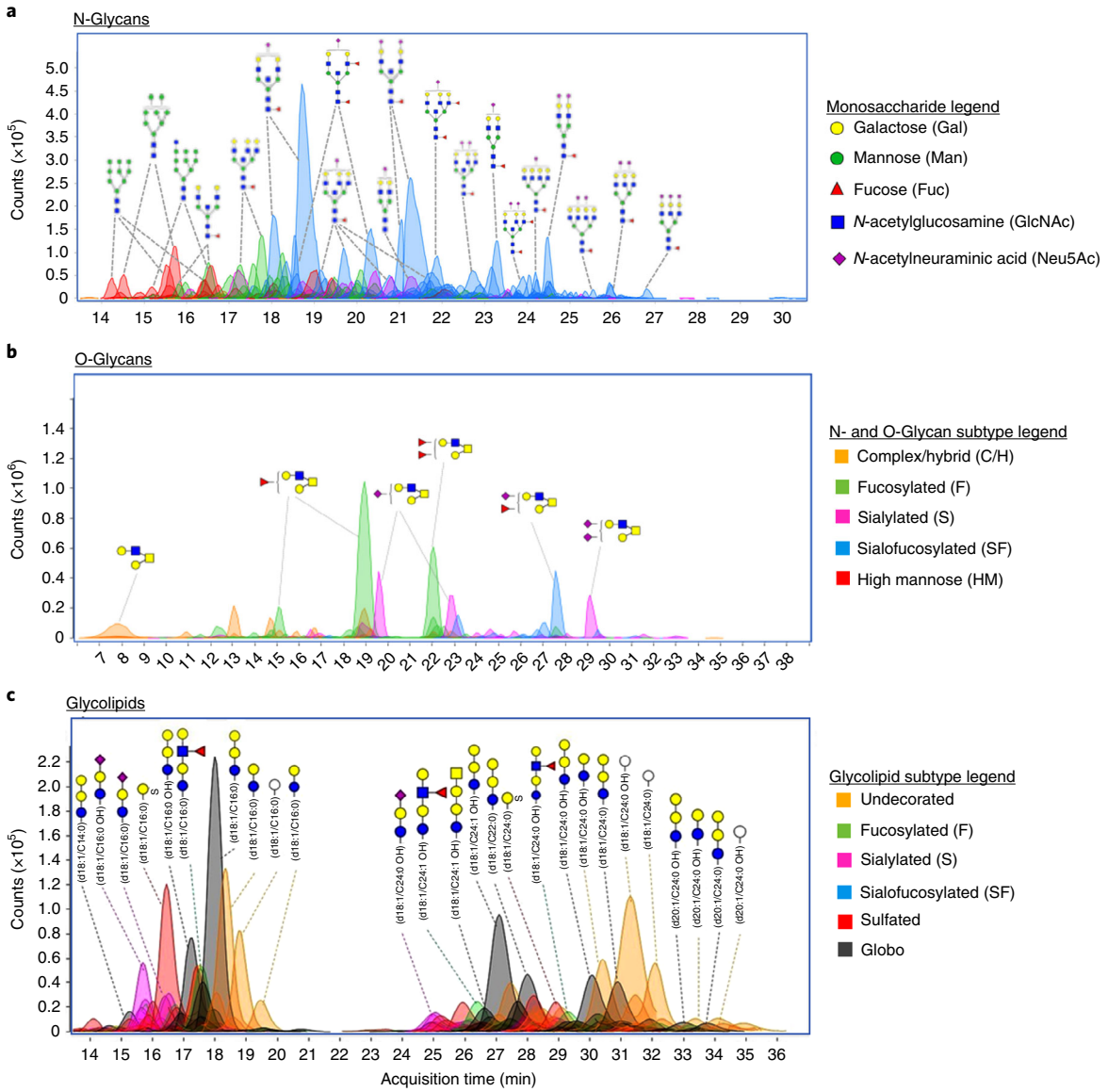

Fig. 4 | Examples of LC-MS profiles from the Caco-2 cell line. a, N-Glycan profile obtained by nanoLC-QTOF MS/MS using a PGC chip. More than 500 N-glycans were observed with the most abundant species showing a peak height of 4.5×10^5 ion counts. **b**, O-Glycan profile obtained by nanoLC-QTOF MS/MS with a PGC chip. More than 50 O-glycans were observed with the most abundant species showing a peak height of around 1×10^6 ion counts. **c**, Glycolipid profile obtained by nanoLC-QTOF MS/MS with a C18 chip. More than 200 glycolipids were observed with the most abundant species having a peak height of around 2×10^5 ion counts. The peaks are color coded to show the glycan subtypes and monosaccharides are shown above the peaks. **a** reproduced with permission from ref. [23], Society for Glycobiology. **b** reproduced from ref. [24] under a Creative Commons Attribution 3.0 license (http://creativecommons.org/licenses/by/3.0/). **c** reproduced from ref. [26] under a Creative Commons Attribution 4.0 license (http://creativecommons.org/licenses/by/4.0/).

sulfatides, and neutral glycolipids. These companies also supply purified glycolipids and glycolipid reference mixtures that can be used for QC 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 to obtain MS/MS spectra, which is necessary for the confident identification of GSL species. Optimization of instrument parameters is critical for the effective fragmentation of GSLs, which will allow identification of both the ceramide (lipid) and glycan composition. QC samples can be obtained commercially from the aforementioned companies or prepared from cultured cells such as Caco-2 or PNT2 cells. 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 QC. For example, the pluripotent cell line NTERA-2 can provide stage-specific embryonic antigens (SSEAs) 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 Fig. 4c. In a typical chromatogram, >200 compounds (including isomers) are observed. An ECC for QC glycolipids is shown in Supplementary Fig. 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 used, but this is not recommended due to the low efficiency of the cleavage, resulting in the potential loss of low-abundance compounds[65]. Cleaved O-glycans should be enriched on PGC SPE plates for desalting, and the glycans are further cleaned of 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 600–2,000 *m/z* to 300–2,000 *m/z* because of the relatively smaller sizes of O-glycans. For the instrument 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 for O-glycans from the glycocalyx of a cell line is shown in Fig. 4b. The abundant species are annotated with typically >50 compounds (including isomers). An ECC for QC O-glycans is shown in Supplementary Fig. 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[66], followed by the addition of DTT and alkylation with iodoacetamide (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 can be used in combination with trypsin, such as Glu-C[67] and Lys-C[68]. Owing 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 provide 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 QC of LC-MS and overall sample preparation. Typical glycoproteomic data from serum standards are illustrated in Fig. 5. It is critical to use appropriate Orbitrap parameters to obtain high-quality spectra, including automatic gain control (AGC) and maximum ion injection time (MIIT). Lower AGC and MIIT values will result in poor MS2 spectra because of the low abundance of collected precursors. However, large AGC and MIIT values require longer accumulation times, resulting in a decrease in identified species[69]. In addition, higher AGC values can result in space charge effects, leading to poor mass accuracy and decreased identification rates[70]. For the fragmentation, higher-energy collision dissociation (HCD) with stepped-collision energy is used, providing high-quality MS2 spectra for glycopeptides[71]. 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

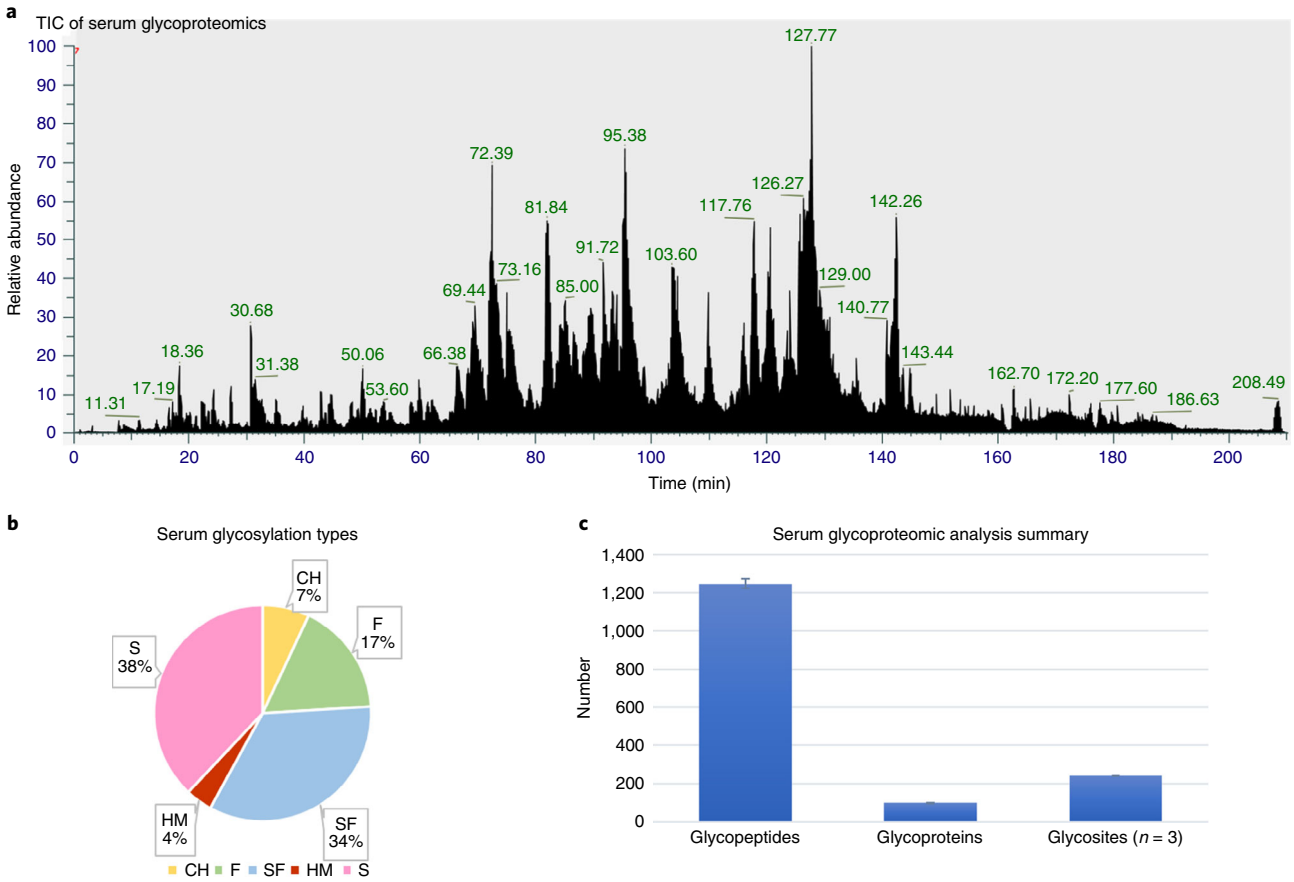

Fig. 5 | Glycoproteomic data of serum standard. a, The TIC of a serum glycoproteomic LC-MS/MS. For the analysis, ~1 μg of digested serum glycopeptides was injected for analysis. The intensity of the base peak in the MS scan was 2.12×10^{10}. **b**, Summary of serum glycosylation types. The glycans was classified into five subtypes: complex/hybrid without fucoses or sialic acids (CH), fucosylated (F), sialofucosylated (SF), high-mannose (HM), and sialylated (S). **c**, Summary of the identified glycopeptides, glycoproteins, and glycosites. The data were collected from three technical replicates.

collision dissociation (EThcD)[72], HCD-triggered electron-transfer dissociation (ETD)[73], and activated ion ETD (AI-ETD)[74] can also be used.

Data processing

To process the glycomic data (Steps 34–43 and 90–99), we used our in-house library based on the putative biosynthetic pathways[75] and previously discovered glycan masses with compositions confirmed by MS/MS. No single curated database of possible glycan masses exists. However, several compilations are published and can be used for compound searches. A neural network–derived glycan library (derived from putative structures) has been published[76]. Glycan libraries of different species, including those with varied monosaccharides, for example, xylose for plants[77], *N*-glycolylneuraminic acid (NeuGc) for non–*Homo sapiens* mammals[78,79], and *N*-acetylmuramic acid (MurNAc) for bacteria[80], exist. Other specialized glycans can also be added. For example, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN)–incorporated glycoconjugates could be included in the library for zebrafish[81]. Even glycans with larger monosaccharide modifications, such as those found with 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) expressed in *Neisseria meningitides*[82], can be included. In addition, 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 MS/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 MS/MS spectra of unnatural monosaccharides incorporating N-glycans and assigned fragmentations is shown

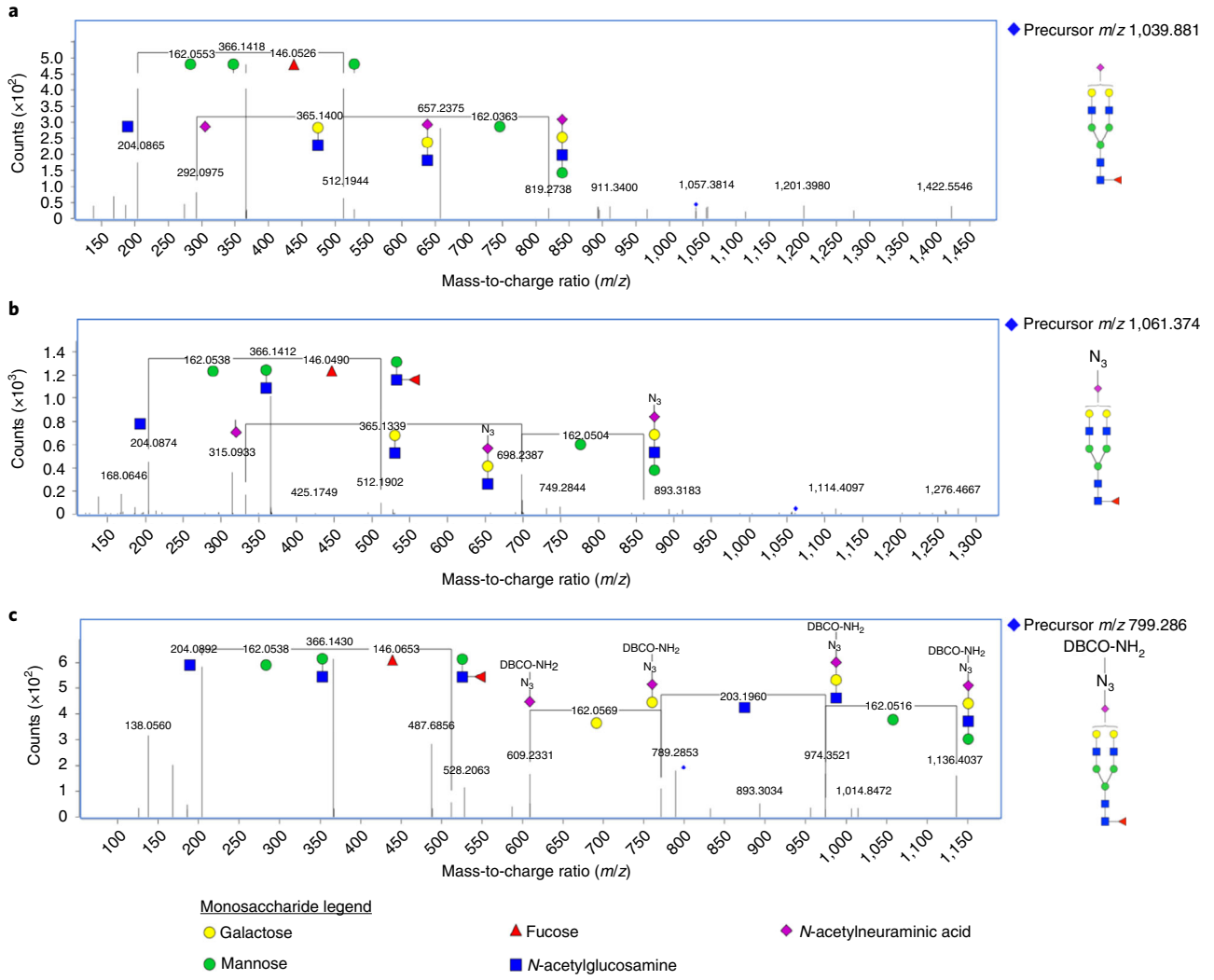

Fig. 6 | Tandem MS spectra of selected compounds. a, *N*-Glycan $Hex_5HexNAc_4Fuc_1Sia_1$ containing three mannose, two galactose, four *N*-acetylglucosamine, one fucose, and one sialic acid residue with a precursor *m/z* value of 1,039.881. The *m/z* value of 292.0975 corresponds to the sialic acid residue, and 657.2375 and 819.2738 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 1,061.374. The *m/z* 315.0933 corresponds to the azido-sialic acid residue, and 698.2387 and 893.3183 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 609.2331 corresponds to the ($DBCO\text{-}NH_2$) conjugated azido-sialic acid residue, and 974.3521 and 1,136.4037 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 for the monosaccharides are provided. Reproduced from ref. [25] under a Creative Commons Attribution 3.0 license (http://creativecommons.org/licenses/by/3.0/).

in Fig. 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 be made not only considering 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 Fig. 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 GSLs (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 GSL species and with generating a subset library from a database that contains the neutral monoisotopic mass and chemical formulae of intact GSLs. We built upon work previously done by Merrill (www.sphingomap.org)[83] in collating possible glycan structures and incorporated their compositions into intact GSLs. 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. In addition, it is also important to create a new library for each kind of sample analyzed because the expression of GSLs can differ on the basis of cell type and species source, and even among individuals of the same species.

For glycoproteomic data processing (Steps 118–125), the Byonic software is used to identify the glycopeptides, and the results can be directly applied for label-free quantitation using the Byologic software. Other software, such as pGlyco, can also be used to identify glycopeptides[84]. The data analysis with the 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 false-discovery rate (FDR) further and eliminate misassignments[85]. A 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 >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 Fig. 7.

Limitations of the protocol

A limitation of the glycomic analysis method is the requirement for specialized instruments that are large, centralized, and expensive. Although 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 all researchers have access to an LC-MS instrument, even when it is not directly available in their 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 a high-resolution MS QTOF and Orbitrap systems 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 and provide less certainty in the identification. More certainty can be obtained with MS/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 is available but is 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 instruments. However, software that is used for other applications, such as Proteomic (http://www.proteomesoftware.com/), can be adapted to glycans. Software options include Skyline (https://www.maccosslab.org/software) and MZmine 2 (http://mzmine.github.io/) 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 (https://www.maxquant.org/) and pGlyco (http://pfind.ict.ac.cn/software/pGlyco/index.html) can also be used for glycomic and glycoproteomic analysis. In addition, using ion counts for quantitation is the best method we have, but it does have intrinsic limitations, with the most

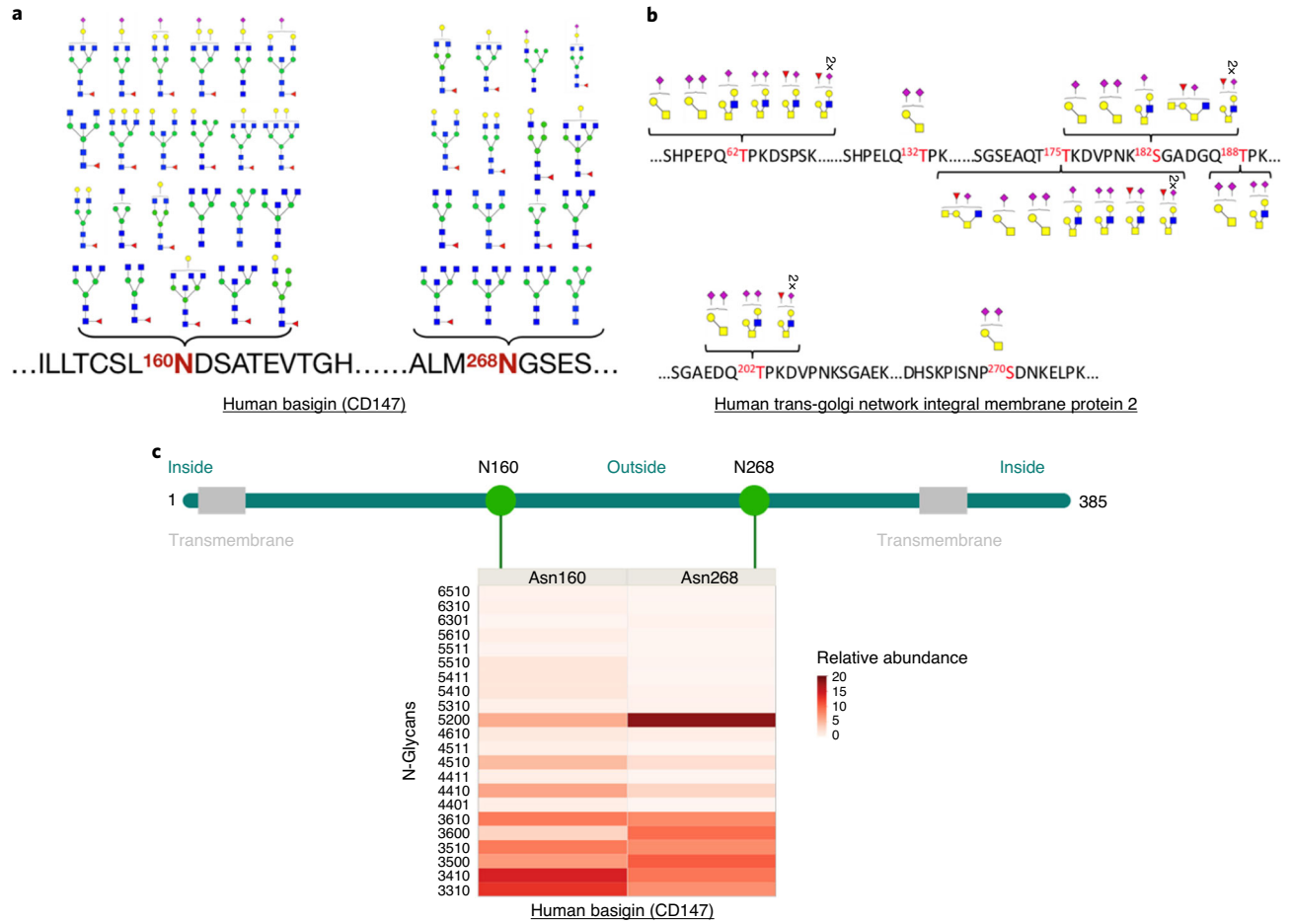

Fig. 7 | Examples of site-specific glycopeptide mapping of N-linked glycoproteins and O-linked glycoproteins. a, Human basigin, also known as CD147, was identified from Caco-2 cells, and its N-linked glycans were identified by the site heterogeneity at Asn160 and Asn268. **b**, Human trans-Golgi network integral membrane protein 2 was obtained from HT-29 cells, and its O-linked glycans were identified by 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 Asn160 and Asn268 were expected to be extracellular. The relative abundances of glycans at the two different sites were quantified with the Byologic software. Glycan composition is noted as a four-digit number 'ABCD' composed of the number of each monosaccharide in the order of HexHexNAcFucNeuAc (or Sia), where Hex is hexose, HexNAc is N-acetylhexosamine, Fuc is fucose and NeuAc (or Sia) is N-acetylneuraminic acid/sialic acid. The heat map shows the degrees of glycosylation at two different glycosites, thereby revealing quantitatively the glycosylation heterogeneity at the different sites.

substantial 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 used to determine the glycome of serum[86,87]. 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 (proteomic) analysis, and the second is a glycopeptide (glycoproteomic) analysis. Within the glycoproteomic analysis, relative abundances of each glycoform can be obtained; however, the site-specific occupancy can be inferred only 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[88]. 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 glyco-sites 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 because 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 because of 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) that have substantial roles in a variety of biological functions[89]. However, owing to the structural and chemical nature of GAGs, their analysis requires 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. Because this protocol offers a nearly complete rendering of all glycans (GAGs notwithstanding) in any biological sample, its utility will cover nearly every aspect of biology. In addition, this workflow could be readily incorporated into 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; and (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 of cancer progression. Glycan changes are also seen during tumor growth and metastasis. All approved cancer protein biomarkers are glycosylated. In addition, many new cancer therapeutics are glycosylated, and their targets on the cell membrane are glycoproteins. Second, the glycocalyx is also important in understanding host–microbe interactions. The binding sites on host cells are often glycans on proteins and lipids that 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

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

Cell lines

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

- 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 without any chemical triggering when it reaches 100% confluency.
- A549 cells (ATCC, cat. no. CCL-185, RRID: CVCL-0023)
- NTERA-2 cl.D1 cells (ATCC, cat. no. CRL-1973, RRID: CVCL-3407)

Reagents

- Fetal bovine serum (FBS; Thermo Fisher Scientific, cat. no. 16000-069)
- Penicillin–streptomycin (Thermo Fisher Scientific, cat. no. 15140-122)
- Trypsin-EDTA (1×; Thermo Fisher Scientific, cat. no. 25300-054)

- Dulbecco's phosphate-buffered saline (D-PBS, 1×; ATCC, cat. no. 30-2200)
- Sucrose (BioXtra; MilliporeSigma, cat. no. S7903)
- HEPES (1 M, Thermo Fisher Scientific, cat. no. 15630080)
- Potassium hydroxide (KOH, BioXtra; MilliporeSigma, cat. no. P5958) **! CAUTION** Potassium hydroxide causes severe skin burns and eye damage. Wear personal protective equipment when handling.
- Sodium carbonate (Na_2CO_3, BioXtra, MilliporeSigma, cat. no. S7795) **! CAUTION** Sodium carbonate causes serious eye irritation. Wear personal protective equipment when handling.
- Ammonium bicarbonate (NH_4HCO_3, BioUltra; MilliporeSigma, cat. no. 09830) **! CAUTION** NH_4HCO_3 irritates skin, eyes, and respiratory system. Wear personal protective equipment when handling.
- Dithiothreitol (DTT, molecular grade; Promega, cat. no. V3151) **! CAUTION** DTT causes skin and eye irritation and may cause respiratory irritation. Wear personal protective equipment when handling.
- Iodoacetamide (IAA, BioUltra; MilliporeSigma, cat. no. 144-48-9) **! CAUTION** Iodoacetamide may cause an allergic skin reaction. Wear personal protective equipment when handling.
- Sodium hydroxide (NaOH, anhydrous; MilliporeSigma, cat. no. 795429) **! CAUTION** Sodium hydroxide causes severe skin burns and eye damage. Wear personal protective equipment when handling.
- Sodium borohydride ($NaBH_4$, Supelco; MilliporeSigma, cat. no. 1.06371) **! CAUTION** Sodium borohydride causes skin corrosion, serious eye damage, and reproductive toxicity. Wear personal protective equipment when handling.
- Formic acid (FA, Optima; Fisher Scientific, cat. no. A117-50) **! CAUTION** Formic acid is a flammable liquid and vapor. Wear personal protective equipment when handling.
- Trifluoroacetic acid (TFA, Pierce; Thermo Fisher Scientific, cat. no. 28902) **! CAUTION** Trifluoroacetic acid causes severe skin burns and eye damage. Wear personal protective equipment when handling.
- Acetonitrile (ACN, Chromasolv; Honeywell, cat. no. 34967) **! CAUTION** Acetonitrile is a flammable liquid and vapor. Wear personal protective equipment when handling.
- Water (H_2O, Pierce; Thermo Fisher 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 a flammable liquid and vapor. Wear personal protective equipment when handling.
- 2-Propanol (IPA; MilliporeSigma, cat. no. 34863) **! CAUTION** Isopropyl alcohol is a flammable liquid and vapor. Wear personal protective equipment when handling.
- Chloroform ($CHCl_3$; MilliporeSigma, cat. no. 34854) **! CAUTION** Chloroform is a flammable liquid and vapor. It is also acutely toxic. Wear personal protective equipment when handling.
- Ammonium acetate (Supelco; MilliporeSigma, cat. no. AX1222)
- Acetic acid (glacial, Alfa Aesar; Thermo Fisher Scientific, cat. no. AA39745AE) **! CAUTION** Acetic acid causes severe eye and skin burns and is a flammable liquid and vapor. Wear personal protective equipment when handling.
- 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, cat. no. 2050)
- *N*-Omega-CD3-octadecanoyl disialoganglioside GD3 (Matreya, cat. no. 2054)
- Quantitative colorimetric peptide assay (Pierce; Thermo Fisher Scientific, cat. no. 23275)
- BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, cat. no. 23225)
- Potassium chloride (KCl; MilliporeSigma, cat. no. 104936)
- Urea (MilliporeSigma, cat. no. 108487)

Equipment

- Microcentrifuge tubes (1.5 mL, natural; Beckman Coulter, cat. no. 357448)
- Petri dishes (10 cm; Corning, cat. no. 70165-102) or T75 flasks (Corning, cat. no. 430641U)
- Sterile Falcon centrifuge tubes (15 mL; Thermo Fisher Scientific, cat. no. 339650)
- Nine-well cooling rack (Biocision, cat. no. BCS-153)
- Pipettes (Gilson, cat. no. FA10006M)
- Pipette tips (USA Scientific, cat. no. 1111-2841)
- Gel pipette tips (Cole-Parmer, cat. no. UX-25713-16)

- Microwave (CEM, Discover Proteomics)
- Cryovial (4 mL; Simport, cat. no. T310-4A)
- pH paper (Micro Essential Laboratory, cat. no. 140)
- Parafilm (MilliporeSigma, cat. no. P6543)
- Eppendorf Snap-Cap microcentrifuge Flex-Tube tubes (1.5 mL; Thermo Fisher Scientific, cat. no. 022364111)
- Ultracentrifuge (Sorvall WX 100+; Thermo Fisher Scientific, cat. no. 75000100)
- Fixed-angle rotor (Fiberlite F50L-24 × 1.5; Thermo Fisher Scientific, cat. no. 096-247028)
- Sonicator system with standard probe (Q700; QSonica, cat. no. Q700-110)
- Water-jacketed CO_2 incubators (Forma Series II 3110; Thermo Fisher Scientific, cat. no. 3110)
- Isotemp digital-control water baths (Fisherbrand; Thermo Fisher Scientific, cat. no. 15-462-S3S)
- Lab Armor Bead Bath (Thermo Fisher Scientific, cat. no. 10-876-006)
- iSPE-HILIC SPE cartridges (Hilicon, cat. no. 200.001.0100)
- C8 SPE plate (Glygen, cat. no. FNSC08.800)
- Graphitized carbon (PGC) SPE plate (Glygen, cat. no. FNSCAR800)
- C18 LC Column (3 μm, 0.075 mm × 250 mm, Acclaim PepMap 100; Thermo Fisher Scientific, cat. no. 164569)
- 96-Well twin.tec PCR plates (Eppendorf; Thermo Fisher Scientific, cat. no. E951020401)
- Adhesive sealing film (Thermo Fisher Scientific, NC0525923)
- Polypropylene vial (250 μL; Agilent, cat. no. 5188-2788)
- Crimp/snap-top vials and caps (2 mL; Agilent, cat. no. 5182-0541)
- E-Pure water purification system (Thermo Fisher Scientific, cat. no. D4631)
- Microscope (Lecia Microsystems, model no. DMI3000 B)
- Liquid chromatography chip system (Agilent 1200 Series; Agilent Technologies)
- Accurate mass Q-TOF LC/MS system (Agilent Technologies, model no. 6520)
- C18 chip (40-nL enrichment column, 5 μm; 75 μm × 150 mm separation column; Agilent Technologies, cat. no. G4240-62006)
- Agilent graphitized carbon chip (PGC-Chip II) (40-nL enrichment column, 5 μm; 75 μm × 43 mm separation column; Agilent Technologies, cat. no. G4240-64010)
- Mass Spectrometer with EASY-ETD (Thermo Fisher Scientific, Orbitrap Fusion Lumos Tribrid model)
- nanoLC system (Thermo Fisher Scientific, model no. UltiMate WPS-3000RS)
- Ion source (Thermo Fisher Scientific, Nanospray Flex model)

Software

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

- Agilent MassHunter Qualitative Analysis (B.08.00; https://www.agilent.com/en/products/software-informatics/mass-spectrometry-software/data-analysis/qualitative-analysis)
- Protein Metrics Byonic (v. 3.5.0; https://www.proteinmetrics.com/products/byonic/)
- Protein Metrics Byonic-Viewer (v. 3.5.0; https://www.proteinmetrics.com/support-information/)
- (Optional) Protein Metrics Byologic (v. 3.5.0; https://www.proteinmetrics.com/products/byologic/)

Reagent setup

▲ **CRITICAL** Milli-Q water generated from the E-Pure water purification system is used for reagent setup unless other types of water are specified.

Homogenization buffer (HB buffer)

This solution is made up of 0.25 M sucrose, 20mM HEPES–KOH at pH 7.4, and 1:100 protease inhibitor cocktail in water. Prepare 20 mM HEPES and adjust the pH to ~7.4 by adding solid KOH. Weigh 4.28 g of sucrose and dissolve it in 39 mL of H_2O. Mix one bottle of protease inhibitor cocktail with 1 mL of H_2O. Add 10 mL of HEPES–KOH solution and the reconstituted 1 mL of protease inhibitor to the sucrose solution. Vortex the solution to mix and make 1.2-mL aliquots of the solution in 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 it in 500 mL of H_2O. The volume of the stock solution can be adjusted accordingly. This solution can be prepared as a 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. Weigh 79.06 mg of NH_4HCO_3 and 7.71 mg of DTT and dissolve them in 10 mL of H_2O. The solution can be prepared as a stock solution and stored at 4 °C for several months.

Folch extraction solution
This solvent is a mixture of H_2O/MeOH/$CHCl_3$ with a 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 extraction solution. 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 378.30 mg of $NaBH_4$ and dissolve it in 5 mL of H_2O. The volume of the solution can be adjusted according to the number of samples. ▲**CRITICAL** This solution should be prepared freshly each time, right before the experiment.

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

Protein digestion buffer
This solution is 50 mM NH_4HCO_3 in water. Weigh 79.06 mg of NH_4HCO_3 and dissolve in 20 mL of H_2O. ▲**CRITICAL** This solution should be prepared freshly every time right before the experiment.

Protein denaturing solution
This solution is 550 mM DTT in 50 mM NH_4HCO_3 solution. Weigh 424.19 mg of DTT and dissolve it in 5 mL of 50 mM NH_4HCO_3 solution. This solution can be divided into aliquots 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 416.16 mg of IAA and dissolve it in 5 mL of 50 mM NH_4HCO_3 solution. This solution can be divided into aliquots 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 a 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 a stock solution and stored at 4 °C for several months.

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

C8 SPE plate elution solution
This solution is 1:1 (vol/vol) methanol/IPA. Add 50 mL of methanol to 50 mL of IPA and mix thoroughly. This solution can be prepared as a 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 a 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 a 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 a 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. To a 1-L volumetric flask, add 30 mL of ACN and 1 mL of FA, fill to mark with Milli-Q water, and mix thoroughly. Degas for 10 min and allow the mixture to equilibrate to room temperature. The volume may change owing to the miscibility of the solvents. Fill to mark again with Milli-Q water if needed. Transfer to mobile phase container to make a total of 1 L of glycomics LC solvent A. This solution can be stored at room temperature and used for 2 weeks. ▲**CRITICAL** This solution should be prepared fresh 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. To a 1-L volumetric flask, add 90 mL of H_2O and 10 mL of FA, fill to mark with ACN, and mix thoroughly. Degas for 10 min and allow the solution to equilibrate to room temperature. The volume may change owing to the miscibility of the solvents. Fill to mark again with ACN if needed and transfer to mobile phase container to make 1 L of glycomics LC solvent B. This solution can be stored at room temperature and used for 2 weeks. ▲**CRITICAL** This solution should be prepared fresh 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. To a 1-L volumetric flask, add 1.54 g of ammonium acetate to about 400 mL of H_2O and mix thoroughly until all salts are dissolved. Add 1 mL of acetic acid and add water to a final volume of 1 L; degas for 10 min and transfer to mobile phase container to make 1 L of glycolipidomics LC solvent A. This solution can be stored at room temperature and used for 2 weeks. ▲**CRITICAL** This solution should be prepared fresh 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 and 20 mM ammonium acetate. To a 1-L volumetric flask, add 150 mL of IPA, about 400 mL of methanol, 1 mL of acetic acid and 1.54 g of ammonium acetate. Mix thoroughly until all the salts are dissolved. Add methanol to a final volume of 1 L. Degas for 10 min and equilibrate to room temperature. The volume may change owing to the miscibility of the solvents. Fill to mark again with methanol if needed, and transfer to mobile phase container to make 1 L of glycolipidomics LC solvent B. This solution can be stored at room temperature and used for 2 weeks. ▲**CRITICAL** This solution should be prepared fresh 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. To a 1-L volumetric flask, add 1 mL of FA and fill to mark with Milli-Q water to make 1 L of solvent A. Mix the solution thoroughly. This solution can be stored at room temperature (25–26 °C) and used for 2 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. To a 1-L volumetric flask, add 99 mL of Milli-Q water and 1 mL of FA. Fill to mark with LC-MS-grade ACN and mix thoroughly. Degas for 10 min and equilibrate to room temperature. The volume may change owing to the miscibility of the solvents. Fill to mark again with ACN if needed. This solution can be stored at room temperature and used for 2 weeks.

Procedure

Sample preparation

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

(A) **Tissue sample preparation** ● Timing **1–3 h, depending 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 ~50–100 mg of tissue sample with 1 mL of D-PBS buffer.

(iii) Centrifuge the sample at 1,000*g* for 3 min at 4 °C. Discard the supernatant. Suspend the pellet in 1.2 mL of HB buffer in a 15-mL Falcon centrifuge tube.

(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, A549 and NTERA-2 cell lines. Growth media and conditions may need to be altered for different cell lines.

(ii) Grow cells until 80% confluency is reached. Use techniques appropriate for your cell line to split cells into 10-cm Petri dishes or T75 flasks. For PNT2, A549, and Caco-2 cells, aspirate the media and wash the cells twice with 10 mL of D-PBS, 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 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) Use a microscope to monitor cell proliferation and change the media every other day.

(iv) Cells can be harvested at ~90% confluency. Wash cells twice with 10 mL of D-PBS before harvesting to thoroughly remove detached cells and the growth medium.

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

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

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

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

(vii) Centrifuge the suspension at 1,000*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, depending on the size of sample set

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

3 Place the 15-mL Falcon centrifuge tube in an ice bucket or a 9-well cooling rack and keep the sample at 4 °C

4 Insert the sonicator probe into the sample.

▲ CRITICAL STEP Make sure the tip of the tube is in the center of the sample.

5 Sonicate the sample by setting the sonicator to 25 amplitude and sonicating with 5 s on and 10 s off for 25 s total sonication time.

▲ CRITICAL STEP Monitor the power during the entire sonicating process. Make sure the power does not exceed 60 W to prevent overheating.

6 Transfer the homogenate to a 1.5-mL Eppendorf tube and centrifuge at 2,000*g* for 10 min at 4 °C to remove nuclei.

7 Take the clear supernatant and transfer it 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*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 you 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 cell membrane pellet wash solution to remove cell membrane–associated proteins. Pipette-mix the pellet with a normal pipette tip to homogenize the mixture. Repeat the ultracentrifugation from 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 homogeneous. Repeat the ultracentrifugation from Step 8 using the same parameters. Remove the supernatant.

11 Retain the cell membrane pellet for further analysis. The cell membrane pellet can be divided into two fractions: the first is used for N- and O-glycomics and glycolipidomics, and the second is used for 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 pellet from Step 11 (fraction I) in 100–200 μL of N-glycan release solution in an Eppendorf tube and break the pellet gently by pipette mixing.

13 Secure the lid with Parafilm and denature the pellet by heating it to 100 °C for 10 s, followed by 10 s of cooling; repeat the heating and cooling for a total duration of 2 min.

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

15 Conduct N-glycan release by using a microwave to heat the sample 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 is usually 37 °C. If the desired temperature has not been 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*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 a gel tip to transfer the supernatant. Be careful when transferring to prevent 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 PGC SPE cartridge condition solution to each well of a new plate and centrifuge the plate at 150*g* for 1 min at 25 °C. 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 into each well of the plate until all the sample has been transferred. Centrifuge as described in Step 19 and discard the flow-through.

22 After sample loading, desalt the 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, the samples can be washed more than three times. Centrifuge as described in Step 19 and discard the flow-through between centrifugations.

23 For sample elution, add 200 μL of PGC SPE cartridge elution solution to each well. Centrifuge as described in Step 19 and collect the flow-through. Elute samples twice (400 μL in total). If the starting amount of cells is larger, elute the sample with 200 μL of solvent.

24 Vacuum-dry the eluted sample completely. This usually takes ~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 from Step 24 with Milli-Q water. Start by reconstituting with 30 μL of water and adjust the amount accordingly. If the final total ion chromatogram (TIC) signal in the mass spectrometer is $<1 \times 10^5$, dry the sample further to obtain a higher concentration. Test the amount of water for reconstitution in this way for each cell line.

26 Transfer the sample to an injection vial and inject 5–10 μL of sample into the mass spectrometer 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 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 into the pump. Change the solvent level in 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 the 'analysis' position to make solvents flow from the nano pumps through both the enrichment and analysis columns. Start the flow at 300 nL/min with 100% (vol/vol) N- and O-glycomics LC solvent B to flush the chip for several minutes.

▲ **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 with the camera of mass spectrometry source. If dripping or scattered spray is seen, adjust the horizontal position of the chip by rotating the wheel beneath the chip cube. The vertical position of the chip can be accessed from the acquisition software in the 'ChipCube' > '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 immediately at −20 °C. Seal the injection plate with a seal sheet. Wrap the plate with aluminum foil and store at −20 °C.

■ **PAUSE POINT** Data analysis can be conducted afterward when needed.

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

34 Start the 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 as described in ref. [87]. The template for an N-glycan library is included as Supplementary Data 1 (Supplementary_Data_I_N-glycan_lib_template.csv). More N-glycans can be added to the template on the basis of ref. [87] and used directly for a database search.

▲ **CRITICAL STEP** The N-glycan library is a .csv file. It contains columns labeled '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 as an identifier in the form 'ABCD' composed of the number of each monosaccharide in the order of HexHexNAcFuc-NeuAc (or Sia), where Hex is hexose, HexNAc is N-acetylhexosamine, Fuc is fucose and NeuAc (or Sia) is N-acetylneuraminic acid/sialic acid. More numbers can be included if other groups, such as NeuGc and sulfate groups, are contained in the composition.

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 (vol/vol/vol)	0.1% FA, 3% ACN, and 96.9% H_2O (vol/vol/vol)	0.1% acetic acid, 99.9% (vol/vol) 20 mM ammonium acetate in H_2O
Solvent B	1% FA, 90% ACN, and 9% H_2O (vol/vol/vol)	1% FA, 90% ACN, and 9% H_2O (vol/vol/vol)	0.1% acetic acid, 99.9% (vol/vol) 20 mM ammonium acetate in 85:15 (vol/vol) methanol/isopropanol
Flow rate (cap pump)	3 μL/min		
Flow rate (nano pump)	0.3 μL/min		
Gradient (%B (vol/vol))	0–2 min: 0%–0% 2–20 min: 0%–16% 20–40 min: 16%–72% 40–42 min: 72%–100% 42–52 min: 100%–100% 52–54 min: 100%–0% 54–65 min: 0%–0%	0–2 min: 0%–0% 2–20 min: 0%–16% 20–40 min: 16%–72% 40–42 min: 72%–100% 42–52 min: 100%–100% 52–54 min: 100%–0% 54–65 min: 0%–0%	0–4 min: 70%–85% 4–40 min: 85%–100% 40–50 min: 100%–100% 50–52 min: 100%–70% 52–65 min: 70%–70%
MS parameters			
m/z mass range	600–2,000	300–2,000	600–2,000
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 (1,250 ms/spectrum)		
MS/MS scan rate	1.0 spectra/s (1,000 ms/spectrum)		
MS threshold	Absolute threshold, 200; relative threshold, 0.01%		
MS/MS threshold	Absolute threshold, 200; relative threshold, 0.01%		
Calibrated mass	1221.990637		
Acquisition mode	Auto MS/MS (seg)		
Activation type	CID		
Activation energy	$V_{collision} = 1.8 \times (m/z)/100 - 2.4$ (N-glycans, O-glycans) $V_{collision} = 1.2 \times (m/z)/100 + 12$ (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		
Isotope model	Common organic molecules		

abs, absolute; seg, segment.

37 Start the extraction of compounds by clicking the green 'Play/Run' button and choosing the files on which to run the 'Molecular Feature Extraction' function. The program will return a list of compounds that match the set criteria. The extracted results include the TCC and ECC.

38 Reset the colors of extracted compound peaks on the basis of 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.

Table 2 | Data analysis parameters using Agilent MassHunter Qualitative Analysis B.08.00 software

Tab	Checked parameter	Value
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 p.p.m.
	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 p.p.m.
	Database	Corresponding libraries
Results	Delete previous compounds	
	Highlight first compound	
	Extract ECC	
	Extract MS/MS spectrum	

▲ **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, in which the existence of NeuGc is possible. A glycan with the composition of 1 NeuGc + x Hex + y Fuc has the same mass as a 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 release (from Step 18) in 500 µL of Folch extraction solution and pipette-mix the solution thoroughly to fully dissolve the lipids.

▲ **CRITICAL STEP** Sonicate the samples for 15 min, followed by 1-min vortexing if necessary.

45 Centrifuge at 8,800*g* for 5 min at 25 °C to separate the pelleted proteins from the dissolved lipids in the supernatant. Collect the supernatant and transfer it to a new Eppendorf tube.

▲ **CRITICAL STEP** The pellets, which contain mostly precipitated proteins, can be set aside for O-glycan release (Step 3).

? TROUBLESHOOTING

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 that is due to phase separation may be observed and can be ignored.

48 Separate the aqueous and organic phases by centrifuging at 8,800*g* for 5 min at 25 °C. The upper, methanol and aqueous layer will contain GSLs 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 lipids and cellular debris 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 C8 SPE plate elution solution by centrifuging at 150*g* for 1 min at 25 °C.

51 Condition the plate by washing twice with 600 µL of C8 SPE plate condition solution.

52 Reconstitute the dried samples from Step 49 with 600 µL of C8 SPE plate condition solution and load the sample on the plate.

53 Wash the plate at least three times with 600 µL of C8 SPE plate condition solution.
▲ **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 C8 SPE plate elution solution.

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 ~5 µL of sample into the instrument.
▲ **CRITICAL STEP** More detailed instrument parameters for glycolipidomics are included in Table 1.
▲ **CRITICAL STEP** For QC, we use a pooled glycolipid sample from the pluripotent cell line NTERA-2.
■ **PAUSE POINT** Data analysis can be conducted afterward when needed.

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

57 Start the 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 GSLs. If the glycan compositions and the associated lipids in the sample are already known, skip ahead to generate a GSL 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 shown in Table 2 to filter through the identified compound features.

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

62 Check the MS/MS spectra to identify GSLs 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 phospholipids. This *m/z* signal is from the phosphocholine head group.

63 From spectra of possible GSLs, take note of the precursor *m/z* values of the MS1 spectra, the charge states of the precursors, and the ceramide *m/z* values in the MS/MS spectra. A table of possible ceramide *m/z* values is provided in Supplementary Data 2 (Supplementary_Data_II_Ceramide_mz.csv). Some commonly observed ceramides in mammalian cells include d34:1, d36:1, d42:1, and d42:2.
▲ **CRITICAL STEP** MS parameters must be optimized for glycolipid fragmentation.

64 Input the precursor *m/z* value, precursor charge state, and the ceramide *m/z* value into the appropriate fields (highlighted in light blue) in Supplementary Data 3 (Supplementary_Data_III_GSL_glycan_finder.xlsx). Include at least two 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 <10. Cross-check the given glycan composition with the fragmentation data to confirm the identification. Some common saccharide and lipid fragments are described in Supplementary Data 4 (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 GSLs by using the pivot table in Supplementary Data 5 (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 on the basis of the number of carbons, the 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 GSL data with the newly generated database using the 'Find Compounds by Molecular Feature'.
69 Reset the colors of the extracted glycolipid peaks on the basis of 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-type compound peaks to black.
70 Add the compound labels to the ECC.
71 Save the ECC—both the version with compound labels and the one without—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 45) and transfer it to a 4-mL cryovial.
74 Reconstitute the pellet with 90 μL of Milli-Q water and sonicate for 10 min to break up the pellet.
75 Add 10 μL of O-glycan beta-elimination solution 2 and 100 μL of O-glycan beta-elimination solution 1 sequentially, and vortex the mixture for 10 s.
? TROUBLESHOOTING
76 Incubate the 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 overpressure 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 the samples on ice to prevent overheating of the mixture.
? TROUBLESHOOTING
78 Check the pH values of the samples with pH paper. The final pH values of samples should be 4–6.
▲ CRITICAL STEP If the samples are not pH 4–6, more acetic acid should be added to make the mixture more acidic.
79 Centrifuge the samples at 21,000*g* for 30 min at 25 °C. 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 cleanup by following Steps 19–24 to desalt. Dry the 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 1 mL of HILIC cartridge condition solution (O-glycan).
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 HILIC cartridge elution solution 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 HILIC cartridge condition solution (O-glycan) 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 s). Collect the flow-through and reload it to the cartridge.
86 Reload the sample five times as described in the previous step.
87 Elute the sample by adding 1 mL of HILIC cartridge elution solution. Repeat three times.
88 Dry the 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 ~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 QC, 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 afterward when needed.

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

90 Start the MassHunter 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 shown in 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 the compounds by clicking the green 'Play/Run' button and choosing the files on which to run the 'Molecular Feature Extraction' function. The program will return a list of compounds that match the set criteria. The extracted results include the TCC and ECC.

94 Reset the colors of the extracted O-glycan peaks on the basis of 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, in which the existence of NeuGc is possible. A glycan with the composition of 1 NeuGc + x Hex + y Fuc has the same mass as a glycan with the composition of 1 NeuAc + $(x + 1)$ Hex + $(y - 1)$ Fuc.

97 Save the ECC—both the version with compound labels and the one without—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 the 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. A 1-mg/mL protein sample requires 2 μL of DTT, 4 μL of IAA, and 2 μg of trypsin.

102 Add 2 μL of protein denaturing solution 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 and IAA, that is, this and the next step, is not needed if the protein does not contain disulfide bonds.

103 Add 4 μL of protein alkylation solution, pipette-mix the samples well, 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 <2 M. Reconstitute 20 μg of sequencing-grade modified trypsin in 200 μl of freshly made protein digestion buffer and then add 20 μL of trypsin to the sample. Pipette to mix the samples well.

▲ CRITICAL STEP Do not vortex-mix the trypsin, which may reduce the activity of the enzyme.

105 Seal the tubes with Parafilm, place the 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) FA.

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	1.0×10^6
Maximum injection time (ms)	50
Filters	
Charge state	2–6
Dynamic exclusion	Exclude one time within 35 s
Intensity	5.0×10^4
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	8.0×10^4
Maximum injection time (ms)	300
Cycle time (s)	3

AGC, automatic gain control; MIPS, monoisotopic precursor selection; OT, Orbitrap; RF, radiofrequency.

107 Dry the samples in a vacuum, which usually takes 2–3 h. After completely drying, the samples will be 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 the iSPE-HILIC SPE cartridges with 1 mL of ACN. To speed up the procedure, a pump or vacuum manifold can be used.
109 Wash the cartridges with 2 mL of HILIC cartridge elution solution.
110 Equilibrate the cartridges with 3 mL of HILIC cartridge condition solution (glycopeptide).
111 Reconstitute the samples with 1 mL of HILIC cartridge condition solution, and load the samples to the cartridges.
? TROUBLESHOOTING

Table 4 | Data analysis parameters of glycopeptides using Byonic v. 3.5.0 software

Tab	Section	Parameter	Value
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 p.p.m.
		Fragmentation type	Both CID & HCD
		Fragment mass tolerance (CID)	20 p.p.m.
		Fragment mass tolerance (HCD)	20 p.p.m.
		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

KR, lysine and arginine.

112 Reload the flow-through from Step 111 five times.

113 Wash the cartridges with 5 mL of HILIC cartridge condition solution.

▲ **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 HILIC cartridge elution solution.

115 Dry the samples in a vacuum completely (usually takes 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 a Pierce quantitative colorimetric peptide assay, follow 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, spin the sample down at 2,000*g* for 15 s at 25 °C and inject only the supernatant into the instrument.

117 Run glycoproteomic analysis. 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) for glycopeptide separation. Details of the method are summarized in Table 3.

▲ **CRITICAL STEP** After each sample run, the column should be cleaned by running a blank injection to avoid sample carryover.

▲ **CRITICAL STEP** A digested commercial serum N-glycopeptide sample can be used for QC of the LC-MS system.

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 *H. sapiens* (Human) protein database from UniProt (UP000005640) is recommended for the protein database file. Set up searching parameters under each tab according to Table 4.

Table 5 | Fixed and variable modifications

Modification	Target	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

C, cysteine; E, glutamic acid; M, methionine; N, asparagine; Q, glutamine.

120 Select the enzyme (trypsin) for digestion. Also set the fragmentation type used for collecting MS/MS spectra; for example, for 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 on the basis of protein features.
▲ **CRITICAL STEP** Search time will be markedly increased with total common maximum 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 glycan modification databases.
▲ **CRITICAL STEP** To obtain confident identification, we recommend that you use the databases from previous N-glycomics and O-glycomics data analyses, especially for unnatural monosaccharide-incorporated glycopeptides.
▲ **CRITICAL STEP** Identification of proteins with O-glycosylation will tremendously increase the software searching time.

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

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

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

(Optional) Site-specific glycoproteomic analysis: label-free glycopeptide quantification

● **Timing 2–8 h per file**

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

127 Drag the .byrslt file from the Byonic search file to Byologic as input for MS1 and MS2 search.
▲ **CRITICAL STEP** The .raw file should be relocated if the file folder has been changed.

128 Load the filtered protein lists from the Byonic results.
▲ **CRITICAL STEP** Byonic import can be used directly if no filter is applied for the protein identification.

129 Choose the default settings for the 'MS extract' options.
▲ **CRITICAL STEP** The default parameters in MS extract options should be reevaluated if you notice any peak cutoff.

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

Troubleshooting

Troubleshooting advice can be found in Table 6.

Table 6 | Troubleshooting table

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*g*, keeping duration and temperature the same) to centrifuge for another round
27	The autosampler errors out at the beginning of the worklist	The correct type of well-plate was 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	Chip might not be properly conditioned. It is also possible that the chip or other compartments, such as the inlet filters or some capillaries, are clogged	To condition the chip completely, decrease the percentage of solvent B in 20% increments until it reaches 0% solvent B. If the pressure is still high, back-flush the chip or change an inlet filter or use a new capillary
31	Dripping or scattered spray is observed	The capillary voltage is not optimized	Increase the source voltage while observing how the spray changes. Increase the capillary voltage in increments of 50 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 nA
32	There is no signal during the run or the LC pump errors out	The spray disappears during one batch or the solvent level falls 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 the sample is high	Adjust the solvent composition by adding 2:1 methanol/chloroform in 50-μL increments until the solvents become miscible and a single liquid phase is observed. Vortex-mix the sample between additions. If the 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 all samples	Large amounts of bubbles are generated in the $NaBH_4$ solution	Vortex-mix the $NaBH_4$ solution briefly to remove bubbles each time just before adding to the sample to make sure the volume added is accurate
77	Samples overflow from the tube	Large amounts of gas are generated in the neutralization reaction	Add the acetic acid drop by drop to avoid generating bubbles
111	Samples cannot be dissolved completely	The dried pellets 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 ~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 of 1 mL

Timing

Step 1A, tissue sample preparation: 1–3 h, depending on the size of sample set
Step 1B, mammalian cell growth: 5 d
Steps 2–11, cell lysis and membrane extraction: 5–8 h, depending 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, glycolipid extraction: 1 h
Steps 50–55, desalting of glycolipids with C8 SPE plates: 1–2 h
Step 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: 1 d
Step 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
Step 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 and 117, nanoLC-MS/MS analysis of glycopeptides: 4 h per sample with one blank injection
Steps 118–125, glycoproteomics data analysis: 2–8 h per file
Steps 126–130, (optional) label-free glycopeptide quantification: 2–8 h per file

Anticipated results

This protocol can be 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. We have successfully characterized the glycocalyx of the Caco-2 cell line before and after differentiation with this workflow. Below, we describe typical results we have obtained using this protocol on Caco-2 cells within specific studies.

Glycomic variations during cell differentiation

By monitoring the morphology with microscopy, we observed that the differentiation of Caco-2 cells started from the fifth to the seventh day and completed on day 21. We illustrated the progressive biological changes of cell surface glycocalyxes during cell differentiation by following the first part of the workflow. The N-glycomic analysis showed that the level of high-mannose-type N-glycans decreased, whereas the levels of fucosylated and sialylated N-glycans increased during differentiation (Fig. 8)[33]. The glycolipidomic analysis identified >200 intact GSLs on differentiated Caco-2 cells (Fig. 9)[24,26]. During the maturation process, the globo-type GSLs decreased, whereas GSLs with sialylated and sulfated head groups increased. In addition, the relative abundances of longer ceramides (with 40–42 carbons) decreased, whereas shorter ceramides (with 32–34 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 2,553 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 the 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. By contrast, SiaNAz groups were more highly incorporated into O-glycans, where >70% of sialylated O-glycans contained SiaNAz. The rate of incorporation of SiaNAz into all sialylated GSLs was ~8%.

The glycocalyx under variable environmental conditions

The protocol has also been used to monitor the glycan variations seen when cells are subjected to environmental changes. This includes the use of different cell culture supplements, such as exogenous dietary monosaccharides and short-chain fatty acids (SCFAs), and differing pH in the culture conditions[23]. The supplementation of different exogenous monosaccharides generated various N-glycan types. In addition, fucosylated N-glycans showed substantial changes for cells treated with SCFAs, including acetate, lactate, and butyrate. The levels of sialylated N-glycans showed a marked increase in cells cultured at lower pH values. These findings shed light on the roles that the cell surface glycocalyx plays in the interaction of cells in different 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 a standard proteomics workflow, we identified potential sialic acid–associating proteins on the cell surface, using proximity labeling[25]. The glycomic results demonstrated the incorporation of unnatural

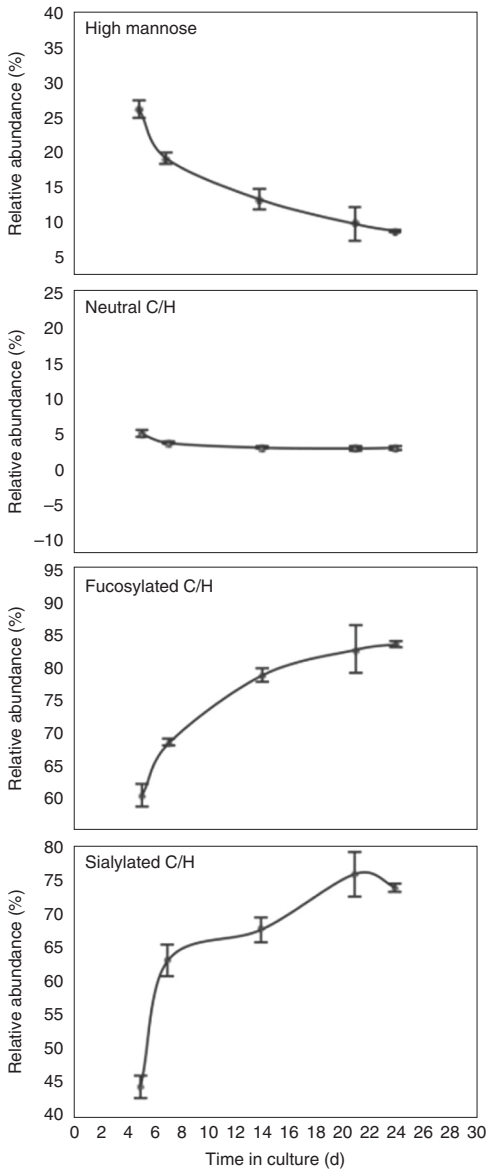

Fig. 8 | The N-glycomic analysis of Caco-2 cells cultured grown at different days. The profile illustrates the increased levels of sialylated and fucosylated C/H N-glycans, and the decreased level of high-mannose N-glycans as time in culture increases. Reprinted with permission from ref. [33], American Society for Biochemistry and Molecular Biology.

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[90]. 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.

Reporting Summary

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

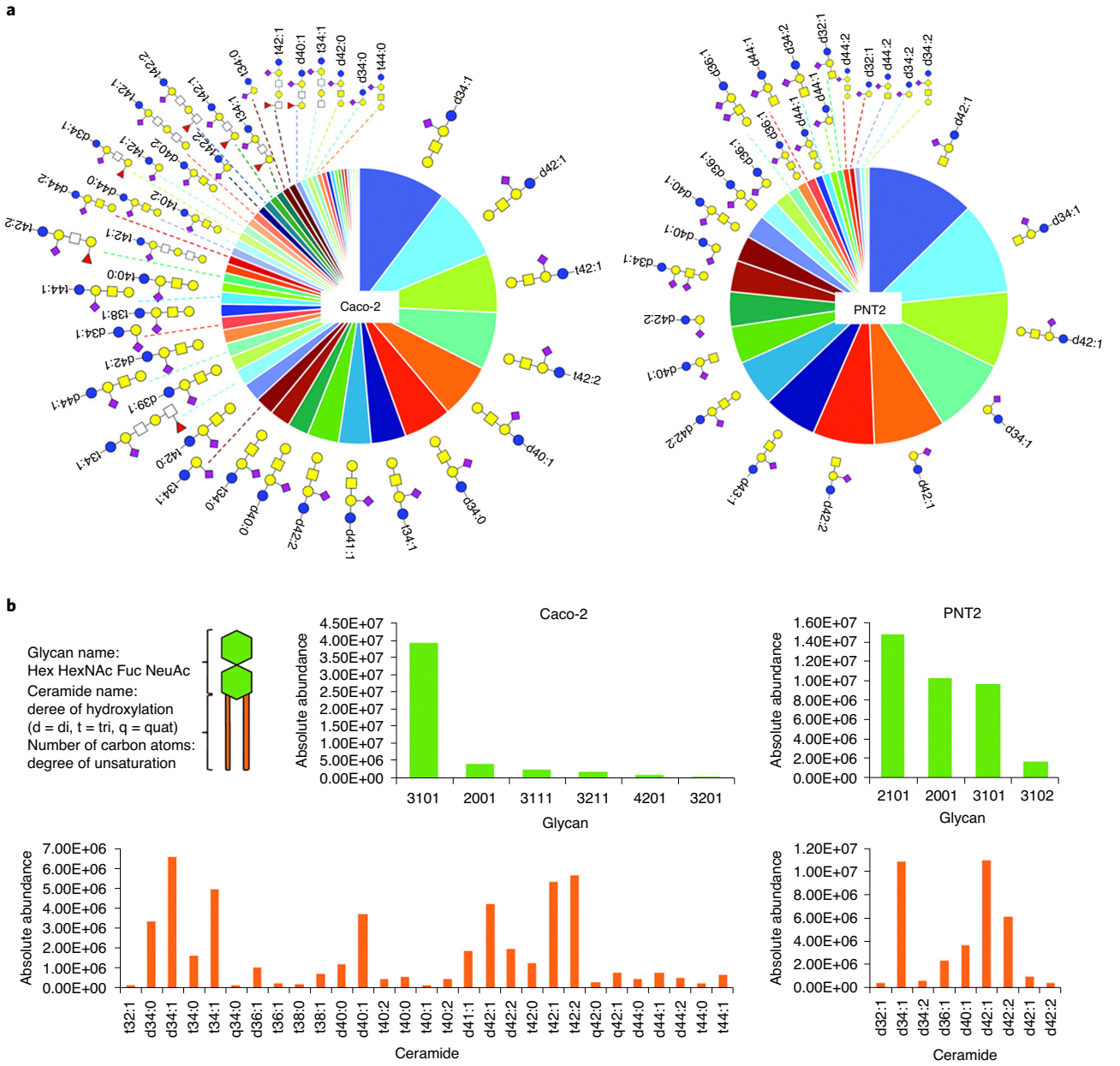

Fig. 9 | The identification and quantitation of glycosphingolipids from Caco-2 and PNT2 cell lines. a, Sialylated GSLs with their tentative structures were ordered according to abundance. **b**, Sialylated GSLs were quantified and compared on the basis of the head groups and ceramide types. Adapted from ref. [24] under a Creative Commons Attribution 3.0 license (http://creativecommons.org/licenses/by/3.0/).

Data availability

The data are all available online. Data from ref. [23] (Park, D. et al. *Glycobiology* **27**, 847–860 (2017)) (used for Fig. 4a) are available at https://doi.org/10.1093/glycob/cwx041. Data from ref. [24] (Park, D. et al. *Chem. Sci.* **9**, 6271–6285 (2018)) (used for Figs. 4b and 9) are available at https://doi.org/10.1039/c8sc01875h. Data from ref. [26] (Wong, M. et al. *Sci. Rep.* **8**, 10993 (2018)) (used for Fig. 4c) are available at https://doi.org/10.1038/s41598-018-29324-7. Data from ref. [25] (Li, Q. et al. *Chem. Sci.* **10**, 6199–6209 (2019)) (used for Fig. 6) are available at https://doi.org/10.1039/c9sc01360a. Data from ref. [33] (Park, D. et al. *Mol. Cell. Proteomics* **14**, 2910–2921 (2015)) (used for Fig. 8) are available at https://doi.org/10.1074/mcp.M115.053983.

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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.

Competing interests

The authors declare no competing interests.

Additional information

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Wong, M., Xu, G., Park, D., Barboza, M. & Lebrilla, C. B. *Sci. Rep.* **8**, 10993 (2018): https://doi.org/10.1038/s41598-018-29324-7
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