MethodsX 6 (2019) 2329–2347

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Protocol Article

On-slide tissue digestion for mass spectrometry based glycomic and proteomic profiling

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A B S T R A C T

We describe a protocol for glycomic and proteomic profiling that uses serial enzyme digestions from the surface of fresh frozen or fixed tissue slides. The abundances of the extracted glycans and peptides are determined using liquid chromatography-mass spectrometry. In a typical experiment, our method quantifies 14 heparan sulfate disaccharides, 11 chondroitin sulfate disaccharides, 50 *N*-glycan compositions and approximately 1200 proteins from a 1.8 mm circle, on fresh frozen rat brain. Each enzymatic digestion is incubated overnight with direct application of enzyme on the tissue surface. Overall, the sample preparation process for multiple tissue slides takes a day per biomolecule class. This protocol saves time by simultaneous digestion of large *N*-glycans and small HS disaccharides and subsequent separation using size exclusion chromatography. Compared to wet tissue analysis, this method requires less time by a factor of two. By comparison, MALDI-imaging provides higher spatial resolution of glycans and proteins but lower depth of coverage. MALDI dissociates fragile glycan substituents including sulfates and is not recommended for analysis of glycosaminoglycans (GAGs).

A R T I C L E I N F O

Protocol name: On-slide tissue digestion for mass spectrometry based glycomic and proteomic profiling
Keywords: Glycomics, Proteomics, On-slide, Tissue digestion, Mass spectrometry, FFPE profiling, Fresh frozen profiling, LC–MS/MS, Nano-HILIC, Reversed phase
Article history: Received 7 June 2019; Accepted 23 September 2019; Available online 25 September 2019

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https://doi.org/10.1016/j.mex.2019.09.029

Specification Table

Subject Area:	*Chemistry*
More specific subject area:	*Glycomics and Proteomics*
Protocol name:	On-slide tissue digestion for mass spectrometry based glycomic and proteomic profiling
Reagents/tools:	REAGENTS Hydrophobic IHC Pap pen (Super^{HT} pen, Biotium) Ethyl alcohol, 200 proof, (BP2818, Fisher Scientific) Xylene, (XX0060-4, Millipore) Ammonium acetate(A7330-500 g, Sigma Aldrich), Ammonium bicarbonate(A6141, Sigma Aldrich) Tris-HCl(T3253, Sigma, pH 8), Calcium Chloride(C5080, Sigma Aldrich), Ammonium hydroxide, 25-30%(320145, Sigma Aldrich) Dithiothreitol (D0632, Sigma), Iodoacetamide(163-2109, Biorad), Trifluoro-acetic acid(A116-10X AMP, Fisher Scientific) acetonitrile(A955-1, LC-MS grade, Fisher Scientific), Water (W6-1, LC-MS grade, Fisher Scientific), Formic acid(A117-10x-1AMP, LC-MS grade, Fisher Scientific) Acetic Acid(9508-01, glacial, JT Baker) Hyaluronidase (H1136-1AMP, Sigma) Chondroitinase ABC(Sigma) Heparan lyases 1,2,3(P0735L, P0736L, P0737L New England Biolabs) PNGase F(P0705L, New England Biolabs) Trypsin (V528A, Prozyme) Kasil (C033116, PQ Corporation) Formamide (F7503, Sigma Aldrich) Superdex peptide(3.2/300 column, GE Healthcare Life Sciences) C18 micro-Ziptip(ZTC18M096, Millipore) Equipment Gloves, safety glass, kimwipes Superfrost Plus microscope slides (Fisher Scientific, cat. no. 22-037-246) Plastic Coplin staining jar (Fisher Scientific, cat. no. 19-4) Glass Coplin staining jar (Fisher Scientific, cat. no. 08-813E) Immunohistochemistry Pap-pen (SuperHT pen, Biotium) Permanent xylene and ethanol resistant marker (H-6100, Vectorlabs) Immunohistochemistry black staining boxes (AT-12, IBI Scientific) Cryostat OCT Compound (25608-930, VWR) Incubator control for 37 degrees, Incubator controlled at 55 degree Vacuum centrifugation (SPD1010 Speedvac system, Thermo Savant) UPLC system) Thermo-Orbitrap-XL, Q-Exactive-HF. Software GlycReSoft software (www.bumc.bu.edu/msr/glycresoft) Peaks Studio 8.5 or other proteomics database searching software
Experimental design:	Tissue sectioning: Fresh frozen or fixed tissue are sectioned and parallel sections chosen for H&E staining and selection of regions of interest. Further, control tissue from bovine cortex is sectioned as a digestion control. Deparaffinization and antigen retrieval for FFPE samples: Deparaffinization involves washing the FFPE slides in xylene and rehydrating in a series of ethanol washes at room temperature. Following deparaffinization, we perform antigen retrieval in Tris-HCl at pH 8.0 at 95 °C for 20 min in an oven. Antigen retrieval denatures chemical crosslinking by formalin allowing for efficient digestion. This is important to unmask hidden or latent epitopes in preparation and allow for enzymatic access to digest the glycans and peptides off the slides. For Fresh-frozen tissue, serial ethanol washes to remove contaminant is performed. Replicates in the study: In our studies, we use animals/humans per group as our biological replicates and three serial sections of slides as our technical replicates. All samples are randomized and blinded prior to digestion to avoid bias. On-slide digestion: We digest the slides after deparaffinization and rehydration. Desalting: We desalt glycans by size exclusion chromatography and peptides using C18 micro-zip-tip.

	Data acquisition: The appropriate data acquisition is important for accurate results. Prior to LC-MS/MS, instrument performance standards are run daily, to ensure stable performance during acquisition, and all samples are spiked in with appropriate internal standards to control for run-to-run variability during data acquisition. During data acquisition, we acquire all the glycan data in random order to ensure that any bias in sample order is eliminated. Appropriate tune files with fragile source conditions are necessary for accurate estimation of labile glycans. For our proteomics analysis, we acquire our data and ensure that the total signal TIC is within 2 fold for the entire cohort in order to estimate differential quantitation between the groups. Data Analysis: For disaccharides, we determine relative abundances, which gives an estimate of chain architecture in the case of HS and CS. Further, we assess absolute amounts with respect to a standard curve for levels of disaccharides. We then estimate the amount of sulfation and the chain length, which is an estimate of the ratio of saturated and unsaturated disaccharides. For *N*-glycans, we assess the relative abundance of glycans after processing the LC-MS data using GlycReSoft. For Proteomics we assess our data using a database searching tool Peaks Studio and quantify by label-free proteomics.
Trial registration:	N/A
Ethics:	*If applicable, include ethical details: Patient informed consent obtained/ Ethics Review Board-competent authority approval obtained/animal experimentation guidelines followed etc.*

Value of the Protocol

- It allows precise targeting of regions of interest (1 mm diameter or greater) selected by eye or microscopy for glycomics and proteomics
- It is efficient and cost-effective and can be integrated with immunohistochemistry.
- It does not require additional derivatization steps, provides high coverage of glycans and proteins
- It applies to both frozen and fixed slides, including precious biopsy tissues that otherwise are difficult to procure.
- We describe the protocol in detail here to facilitate dissemination to biomedical laboratories.

Description of protocol

The on-slide digestion method was developed to profile glycans and proteins from anatomical regions of interest on the surface of tissue slides. Compared to LC–MS-compatible wet tissue GAG analysis [1], the on-slide method is a simpler approach in which GAGs, *N*-glycans, and proteins were released by enzyme digestion from histological slides [2,3]. This led to the development of our on-slide tissue method using smaller tissue amounts from FFPE mouse brain [2,3] as well as tumor microarrays [3]. We then reduced the number of steps to decrease the processing time by half, by digesting both HS and *N*-glycans together and purifying them by size exclusion chromatography, for precise comparison between HS and *N*-glycans from the same region, eliminating possible error of digesting a different region. We assessed HS disaccharides by using HILIC-WAX LC–MS/MS and *N*-glycans by HILIC-amide-LC–MS. Peptides were analyzed by reverse phase LC–MS [4]. Here, we will describe the protocol in detail for sequential release of GAGs followed by peptides as well as digestion of GAGs and *N*-glycans together. Our method provides a readout of GAG quantities, domain structures, and non-reducing end structures using simple enzyme digestions with minimal need for workup. It also profiles *N*-glycans and proteins, respectively. The method is appropriate for tissue volumes of 10 nL or greater, corresponding to a 1 μL droplet of enzyme solution applied to a 1 mm diameter target on a 10 μm thick tissue slice. It is applicable to both fixed and frozen tissue.

Comparison with other methods

Using the on-slide enzyme digestion method allows for staining of parallel sections using H&E or immunohistochemistry to guide the selection of targets on an unstained slide. Extraction of GAGs from wet tissue involves the use of a multi-step approach, requiring a minimum of 10 mg starting material, and several days to isolate the GAG fraction after β-elimination and protein digestion, prior to the enzymatic digestion of GAGs. This method is more time consuming and involves extensive clean

up to remove chemicals and salt prior to mass spectrometry [1]. MALDI-based imaging of glycans enables spatial resolution of approximately 25 μm on a tissue slide [5–7] and involves derivatization to prevent dissociation of sialic acid residues resulting from the MALDI process. In addition, tissue surface effects are likely to influence ion abundances using the MALDI-IMS approach. Permethylation enhances stability and ionization responses and has been used to profile *N*-glycans released enzymatically from tissue slides using LC–MS [8]. The downside of permethylation is that it involves a multistep derivatization process which is time consuming and potentially leads to sample loss. Our method requires no derivatization and is applicable to profiling of GAG classes, *N*-glycans, and proteins released enzymatically from small regions of tissue slides, preserving the anatomical regions of interest in an efficient and simple workflow. The MALDI-IMS approach is often used to image tissue based on abundances of proteins and protein fragments from the tissue surface [9,10]. One inherent disadvantage to this approach is the difficulty in identifying the proteins detected. Tryptic digestion on the tissue surface has been used with MALDI-IMS [10,11]. In this case, due to the absence of a separation dimension, the dynamic range for peptide detection is considerably less than that obtained using LC–MS methods, such as we employ for our profiling method. Supplementary Table S1 shows a comparison of the methods outlined above.

Anticipated results using the protocol

We typically observed 14 HS disaccharides, including Δ-unsaturated, saturated and positional isomers using our LC–MS method. Representative data are shown in Fig. 1. For CS disaccharides, we typically observed 11 Δ-unsaturated and saturated forms and differentiate a pair of (4S/6S) isomers by tandem mass spectrometry. Representative extracted ion chromatograms are shown in Fig. 2. The data were normalized with respect to internal standard and peak areas of disaccharides were calculated as area under the peak. Isomeric disaccharides were diferentiated using diagnostic ions and tandem mass spectrometry as described previously [4]. Analysis of disaccharide levels, total abundance, sulfate per 100 disaccharide units and chain length were calculated using a spreadsheet.

For *N*-glycans, we observe by compositional analysis 50 compositions from the surface of the substantia nigra in the rat brain. A representative extracted mass spectrum of *N*-glycans from 0.01 μg of AGP is shown in Fig. 3. Data Analysis for *N*-glycan glycomics: glycomics mass spectral data were analyzed using GlyReSoft (available at www.bumc.bu.edu/cbms) [12–14].

We observed approximately 1200 proteins from a 1.8 mm circle on a 10 μm thick slide of fresh frozen rat brain. The final results were a combination of database and variable PTM searches. Representative results for HS, CS, *N*-glycans, and peptides are shown in Fig. 4.

During the protocol development, we compared reproducibility and detection limits for on-slide extraction by manual pipetting *versus* using an Advion robot to extract 0.1 μg of bovine kidney HS spotted on a glass surface as shown in Fig. 5. We observed that abundance values are similar for manual *versus* robot extraction and coefficient of variation (CV) <15% for all isoforms of HS dp2. We prefer manual pipetting for a reasonable number of slides, since using the robot for multiple slides involves recalibration of the equipment for each slide, which is cumbersome.

Expertise needed to implement the protocol

The sample preparation is straightforward and involves manual pipetting and basic lab techniques. In our laboratory, we use Thermo Orbitrap or Q-Exactive or Agilent Q-TOF. Data Analysis involves glycomic and proteomic search tools for *N*-glycans and peptides and manual estimates for disaccharide assessment.

Procedure

Fresh Frozen tissue sectioning: **TIMING:** 30 min for sectioning; 8 h for drying

1 Mount the tissue on the chuck with OCT carefully to avoid contact with the region being sectioned.

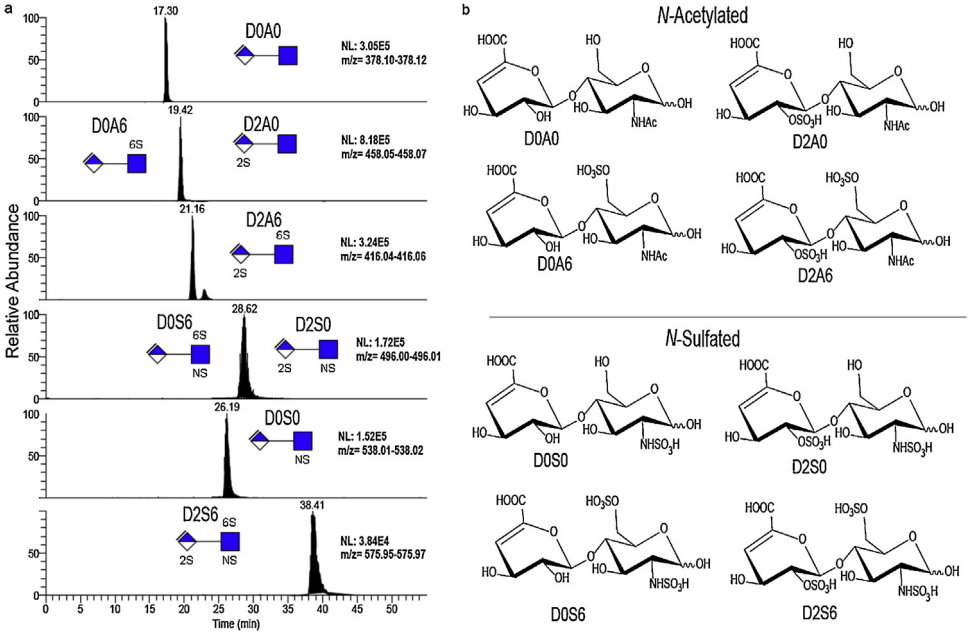


Fig. 1. a. Representative extracted ion chromatograms for 200 fmol of HS disaccharides using AXH-1 HILIC-WAX chromatography and an Orbitrap-XL mass spectrometer. b. HS disaccharide dp2 structures.

2 Wipe down all surfaces with 70% ethanol
3 Mount a new blade on a cryotome
4 Set the head temperature to −22 °C, body temperature to −20 °C.
5 Pre-chill the acetone to −20°C in a plastic Coplin staining jar in the cryostat
6 Trim the tissue block until the surface is even and cut the sections
7 Mount the section on the Superfrost Plus slide by pressing the slide on the tissue that is on the stage. Avoid lifting the tissue with brushes to prevent contaminants.
8 Place the slide in -20°C chilled acetone for 1 min.
9 Cut the remaining serial sections.
10 Dry the slides in the cryostat and box and store in -80 °C freezer

PAUSE POINT: Frozen sections can be stored for several months in -80°C freezer

FFPE sectioning: **TIMING:** 5 min to heat water bath; 30 min for sectioning; 8 h for drying; 12 h for adhering

11 Fill a glass dish with polished water previously heated in the microwave to 50 °C
12 Place paraffin-embedded tissue block in a -20 °C freezer for 5 min. Dip the block in ammonium hydroxide to avoid cracking of the tissue. These sections are usually not collected for analysis and discarded in the trimming.
13 Section on microtome for trimming while adjusting head and direction
14 When even and complete sections are seen, cut ten serial ribbons.
15 Using cold tools at 4 °C, transfer the ribbon on water
16 Cut the flattened sections ensuring that there are no bubbles
17 Mount the sections on Superfrost Plus slides and allow to dry for 8 h
18 Bake the slides at 37 °C for 12 h

PAUSE POINT: FFPE sections can be stored for several months at room temperature

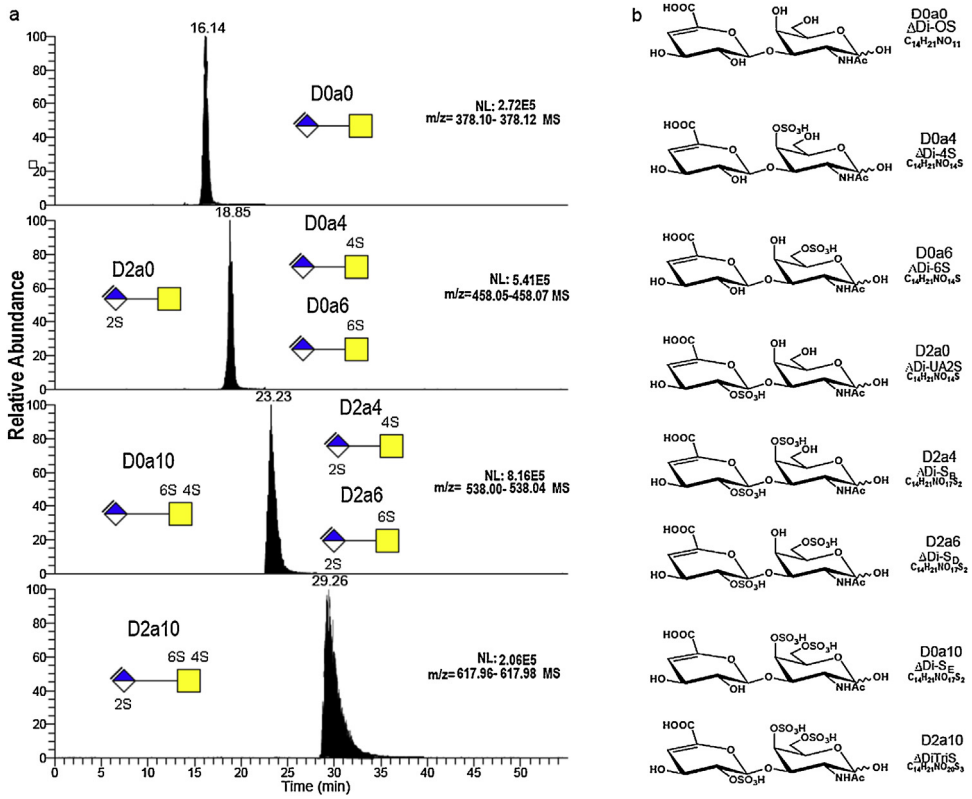


Fig. 2. a. Representative extracted ion chromatograms for 200 fmol of CS disaccharides using AXH-1 HILIC-WAX chromatography using an Orbitrap-XL. b. CS disaccharide dp2 structures.

Deparaffinization and Rehydration: **TIMING:** 20 min for serial washes

19 For both FFPE and frozen sections, mark on the back of the slide the region of interest to be digested using a permanent marker resistant to xylene and ethanol.
20 Over the regions of interest mark with a Pap-immunohistochemistry pen to lock the marker in place to avoid washing of the marks. Remark the spots immediately after the ethanol washes. We suggest taking photographic images of slides prior to washing.
21 For FFPE sections: Wash the FFPE sections in a series of xylene and ethanol washes as described in Table 1 and proceed to antigen retrieval.
22 For frozen sections: Wash the sections in a series of ethanol washes as described in Table 2. Antigen retrieval is not needed for fresh frozen tissue sections.

Antigen Retrieval: **TIMING:** 20 min to do antigen retrieval

23 In a glass Coplin staining jar add 40 mL 10 mM Tris–HCl pH 8.0 in an oven at 95 °C. Place the deparaffinized tissue in the staining glass for 20 min. A set of 4–5 slides can be deparaffinized at once in the same jar.
24 After 20 min, place the sections in a plastic Coplin jar containing LC–MS water. This is needed to cool down the slides and remove any residual Tris–HCl.

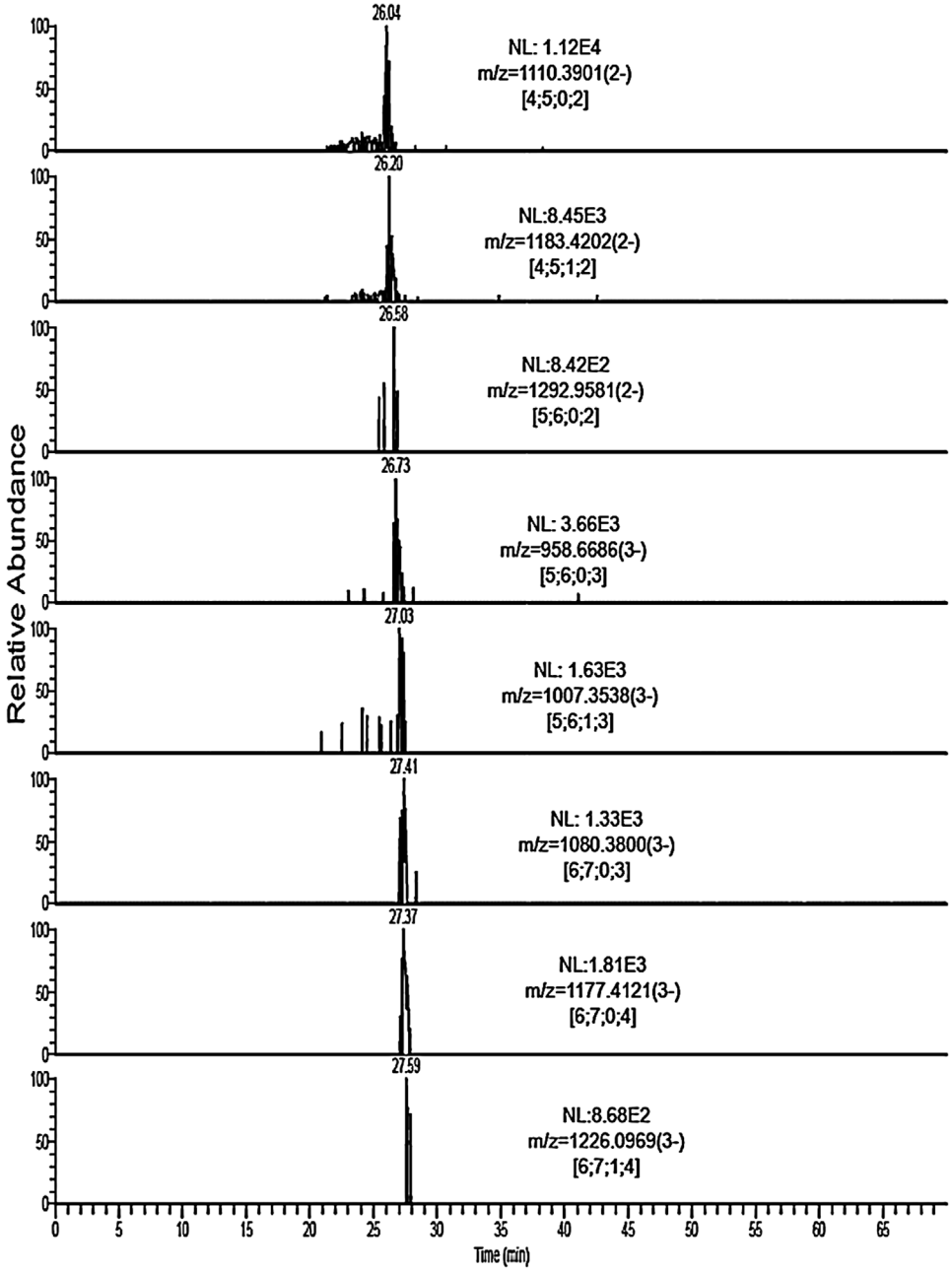


Fig. 3. Representative extracted ion chromatogram of 8 most abundant *N*-glycans in 0.01 μg AGP digested by on-slide method. Compositions given as [HexNAc; Hex; Fuc; Neu5Ac].

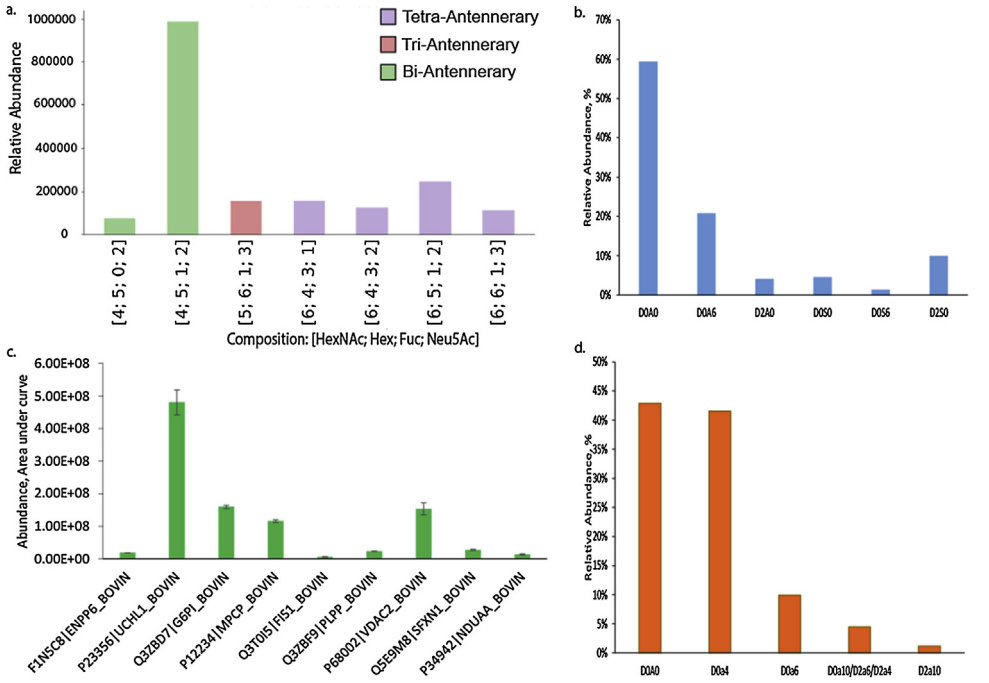


Fig. 4. Representative results from Bovine Cortex fresh frozen tissue section on-slide extraction. (a) *N*-glycan profile, (b) HS disaccharides, bar plot of relative abundance of each disaccharide with respect to other compositions, (c) top 10 most abundant proteins identified after tryptic digest, (d) CS disaccharides, bar plot of relative abundance of CS disaccharides.

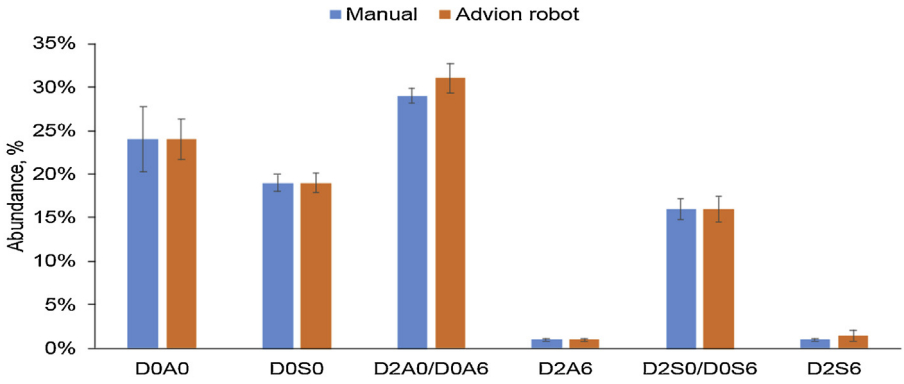
Enzymatic Digestion: **TIMING:** 4 cycles of enzyme addition, 1 h each, followed by 12 h incubation for each enzyme-4days, reduction 20 min, alkylation 20 min.

25 In a dark immunohistochemistry chamber with water at the bottom, place the slides in randomized order to allow the enzyme droplets to remain without evaporation. This is essential to maintain the humidity and to avoid the enzymes evaporating.
26 Digest with serial additions of glycosidase solutions in 5 cycles: 1 h incubation for 4 cycles and the last cycle of 12 h.
27 Extract the glycans in 0.3% ammonium hydroxide.
28 Before adding trypsin perform reduction and alkylation.
29 Add 10 mM DTT in 25 mM Ammonium bicarbonate and incubate for 20 min at 55 °C.
30 Add 20 mM IAA in 25 mM Ammonium bicarbonate and incubate at room temperature in the dark for 20 min. after allowing the slides to cool down.
31 Following reduction and alkylation, digest with trypsin in 5 cycles, with 1 h incubations for 4 cycles and the last cycle of 12 h.
32 Extract the digested peptides using 30% acetonitrile in 0.1% TFA.
33 To digest both *N*-glycans and HS disaccharides together, follow Table 4 or to digest each separately follow Table 5.
34 All extracted glycan and peptides were dried using a vacuum centrifugation.

PAUSE POINT: Dried glycans and peptides can be stored for several months at −80 °C.

Desalting: **TIMING:** 75 min size exclusion chromatography run per sample for glycans, 5 min micro zip-tip per sample for peptides

a



b

	Manual	Advion robot	Manual	Advion robot	Manual	Advion robot	Manual	Advion robot	Manual	Advion robot	Manual	Advion robot
	D0A0	D0A0	D0S0	D0S0	D2A0/D0A6	D2A0/D0A6	D2A6	D2A6	D2S0/D0S6	D2S0/D0S6	D2S6	D2S6
repeat 1	20%	19%	18%	19%	31%	27%	2%	1%	18%	18%	2%	3%
repeat 2	24%	27%	19%	18%	29%	33%	1%	1%	16%	14%	1%	1%
repeat 3	32%	26%	22%	21%	32%	31%	1%	1%	20%	14%	1%	0%
Average	24%	24%	19%	19%	29%	31%	1%	1%	16%	16%	1%	1%
SD	6%	4%	2%	2%	2%	3%	0%	0%	2%	3%	0%	1%
SE	4%	2%	1%	1%	1%	2%	0%	0%	1%	1%	0%	1%
CV	15.4	9.7	5.4	5.6	3.1	5.5	11	19.5	7	9.3	12.1	43

Fig. 5. Quantitative comparison of reproducibility of the relative abundance of HS disaccharides from lyase digestion of bovine kidney HS on the surface of a slide by manual and robotic pipetting system. a. Bar plot of mean+/- standard error b. Table showing relative abundance values per replicate.

35 Resuspend the dried glycans in 20 μL water and inject onto the Superdex Peptide (3.2/300) size exclusion column, using mobile phase (25 mM ammonium acetate, 5% acetonitrile) at a flow rate of 0.04 mL/min. HS and CS disaccharide fractions were collected between 35–47 min, and *N*-glycans were collected between 27–35 min. The clean HS, CS disaccharides, and *N*-glycans were dried by vacuum centrifugation.

PAUSE POINT: Dried down desalted glycans can be stored in −80 °C for several months until analysis.

36 Desalt peptides using C18 micro-ziptip: Resuspend the peptides in 10 μL 0.1% TFA. After equilibrating the tip in 100% acetonitrile followed by 0.1% TFA, ensuring there is no air bubble, pipette the peptides on the tip 20X, wash unbound fraction in 2% acetonitrile 0.1% TFA by pipetting 10X and elute in 10 μL 60% acetonitrile 0.1% TFA, by pipetting 20 × . Dry down the clean peptides by vacuum centrifugation

PAUSE POINT: Dried down desalted peptides can be stored in −80 °C for several months until analysis

HILIC column packing: **TIMING:** 5 min to make the frits, 12 h to bake, 30 min to flush column, 8–10 h to pack column

Frit making: **TIMING:** 5 min to make the frits, 12 h to bake

37 Cut capillaries to a length of 30 cm with ID 100 μm for Amide-80 (HILIC resin) or 150 μm for AXH-1 (HILIC-Wax resin).

Table 1
FFPE Deparaffinization and Rehydration.

1. Set up 8 plastic Coplin staining jars with 2 jars for xylene, 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, LC-MS water
2. Immerse slide in xylene for 3 min followed by next jar of xylene
3. Immerse the slide in 100% ethanol for 2 min, 90% ethanol for 2 min, 70% ethanol for 2 min, 50% ethanol for 2 min, 30% ethanol for 2 min, wash in LC-MS water and keep slide submerged until transfer for antigen retrieval.

Table 2
Fresh frozen Rehydration.

1. Set up 6 plastic Coplin staining jars with 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, LC-MS water
2. Immerse the slide in 100% ethanol for 2 min, 90% ethanol for 2 min, 70% ethanol for 2 min, 50% ethanol for 2 min, 30% ethanol for 2 min, wash in LC-MS water and keep slide submerged until transfer for digestion.

38 To make the frit: add 100 μL formamide to 500 μL Kasil in an Eppendorf tube. Spin down immediately on a tabletop centrifuge, dip one end of the capillary into the supernatant. Observe for capillary action and take out when the frit is 1 cm full. CAUTION: Longer the frit is, there are greater chances of uneven frits or bubbles. Also, ensure that formamide and Kasil were not exposed to air since this leads to uneven frits

39 Bake the frits, with the frit end at the bottom for 12 h at 95 °C in an oven.

40 Select frits that have a uniform inner edge and cut to 0.5 cm and polish outer end to have a perfect cut.

Column packing **TIMING:** 30 min to flush column, 4–8 h to pack column,10 h depressurize.

41 Connect the column with frit side out, to flush with methanol. Flush the column using 500 psi methanol for 30 min. Observe the flow rate from the frit. If the flow is slow, this implies the column fitting is overtight, if the flow is fast but dropping down, this implies correctly tightened fittings. If the spray is like a fountain, implies frit is not good and use a different frit.

42 Mark 15–20 cm from the frit, based on the length of packing desired.

43 Turn off the gas and add resin in packing bomb (HILIC-amide or HILIC-Wax).
   a For Amide 80 HILIC, in a 2 mL Eppendorf tube, resuspend at 10 mg/mL a 300 μL volume of resin suspension (10 mg/mL in 80/20 acetonitrile/water) in 1700 μL methanol. Wash the resin by spinning down once. Re-suspend in 2000 μL methanol andadd magnetic stirrer. Pack as below.
   b For AXH-1 HILIC-Wax, in a 2 mL Eppendorf tube, resuspend at 10 mg/mL (250 μL resin suspension (10 mg/mL in 80/20 acetonitrile/water) plus 1750 μL 80% acetonitrile). Wash the resin by spinning down once. Re-suspend in 2000 μL 80% acetonitrile and add magnetic stirrer. Pack as below.

44 Add the column in the bomb with frit side out, and pack the column under high pressure as below:
   a For Amide 80 HILIC-3 μm particle size, pack at 1200 psi for 6–8 h. Observe under white light for packing and when the packing is complete, turn the closing valve to the middle, cutting off the inlet but still allowing high-pressure gas to remain in the bomb. Let the column sit overnight (10 h) to allow the pressure to release and then completely shut off the valve, slowly to avoid depacking. Inspect for depacking when shutting off the valve in the middle with white light. CAUTION: This is a high-pressure system; make sure to wear safety goggles at all times.
   b For AXH-1 HILIC-Wax1.9 μm particle size, pack at 1800 psi for 4–8 h. Observe under white light for packing and when the packing is complete, turn the shutoff valve to the middle, cutting off the inlet but still allowing high-pressure gas to remain in the bomb. Let the column sit overnight (10 h) to allow the pressure to release and then completely shut off the valve, slowly to avoid depacking. Inspect for depacking when shutting off the valve in the middle with white light. CAUTION: This is a high-pressure system; make sure to be wearing safety goggles at all times.

Table 3
LC conditions for HILIC and reverse phase MS.

A: LC conditions for HILIC-WAX LC-MS for HS/CS disacchrides

Time (min)	Flow rate μL/min	A	B
0	0.6	15	85
5	0.6	15	85
20	0.6	30	70
22	0.6	42	58
25	0.6	50	50
40	0.6	85	15
45	0.6	85	15
48	0.6	15	85
55	0.6	15	85

1. Set up mobile phase solution using LC-MS grade solvents:
A: 50 mM Ammonium formate pH 4.5
B: 95% acetonitrile / 5% Water
Weak wash: 90% acetonitrile / 10% Water
Strong wash: 80% water / 20% acetonitrile
Seal Wash:90% water / 10% acetonitrile
2. Set up gradient conditions as below for HILIC-WAX analysis

3B| LC conditions for HILIC MS for *N*-glycan analysis using Amide-80

Time (min)	Flow rate μL/min	A	B
0	0.6	20	80
5	0.6	20	80
35	0.6	80	20
50	0.6	80	20
52	0.6	20	80
70	0.6	20	80

1. Set up mobile phase solution using LC-MS grade solvents:
A: 50 mM Ammonium formate pH 4.5
B: 95% acetonitrile / 5% Water
Weak wash: 80% acetonitrile / 20% Water
Strong wash: 80% water/ 20% acetonitrile
Seal Wash:90% water/ 10% acetonitrile
2. Set up gradient conditions as below for HILIC analysis

3C| LC conditions for reverse phase MS for peptide analysis using C18 waters BEH

Time (min)	Flow rate μL/min	A	B	curve
0	0.5	98	2	6
1	0.5	98	2	6
91	0.5	60	40	6
93	0.5	60	40	6
96	0.5	2	98	6
101	0.5	2	98	6
103	0.5	98	2	6
120	0.5	98	2	6

1. Set up mobile phase solution using LC-MS grade solvents:
A: 50mM Ammonium formate pH 4.5
B: 95% acetonitrile / 5% Water
Weak wash: 80% acetonitrile / 20% Water
Strong wash: 80% water/ 20% acetonitrile
Seal Wash:90% water/ 10% acetonitrile
2. Set up gradient conditions as below for a 120min reverse phase analysis.

Data Acquisition: **TIMING:** 55 min for HS and CS HILIC Wax analysis, 70 min for *N*-glycan analysis, 75 min or 120 min for peptide analysis based on a gradient of choice per sample.

45 HILIC LC–MS/MS
   a For HS and CS, add 400 fmol of a synthetic disaccharide (ΔUA-2S ® GlcNCOEt-6S (I-P) internal standard, HD009, Iduron) to each sample re-suspended in 85% B. Run randomized samples by HILIC-MS. Gradient conditions and LC prep is shown in Table 3A. Acquire data using UPLC mounted with the self-packed column interfaced with a mass spectrometer with a nano-flow source in negative polarity mode. Separate the isoforms using higher energy collisional dissociation tandem mass spectrometry, relative and absolute disaccharide abundances are determined using standard curves as described previously [15,16]
   b For *N*-glycans, add 400 fmol 2AB labeled *N*-glycan A2F (GKSB-313, Prozyme) to each sample prior to HILIC-MS analysis, re-suspended in 85% B. Run randomized samples by HILIC-MS. Gradient conditions and LC prep are shown in Table 3B. Acquire data using UPLC mounted with the self-packed column interfaced with a mass spectrometer with a nano-flow source in negative polarity mode. Separate the isoforms using higher energy collisional dissociation tandem mass spectrometry

46 Reverse phase-LC–MS/MS: For peptides, add 100 fmol peptide retention time calibration mixture (88321, Pierce) to all samples prior to LC–MS analysis. Run randomized samples by LC–MS/MS. Gradient conditions and LC preparation are shown in Table 3C. Acquire data using top-20 data-dependent acquisition reversed phase LC-. We used a 150 μm X10 cm C18 reverse phase 1.7 μm BEH analytical column and a 180 μm X2 cm 5 μm C18 trapping column (Waters Corporation) with a 120 min method with a gradient from 2 to 98% acetonitrile in 97 min, using mobile phase A 99% water 1% acetonitrile 0.1% formic acid and mobile phase B 99% acetonitrile 1% water 0.1% formic acid.

Data Analysis: **TIMING:** ~2–4h

47 HS and CS Data Analysis: The data were normalized with respect to internal standard and peak areas of disaccharides were calculated using area under the peak. Isoforms were separated using diagnostic ions and tandem mass spectrometry as described previously described [17]. Analysis of disaccharide levels, total abundance, and sulfate per 100 dp2 and chain length were calculated using Microsoft Excel.

48 *N*-glycan Data Analysis: Data Analysis for *N*-glycan glycomics. The data was analyzed using GlyReSoft version 0.3.1 (available at www.bumc.bu.edu/msr/glycresoft) [12–14].Build a combinatorial database using HexNac 2–9, Hex 3–10, Fuc 0–5, NeuAc 0–4 and NeuGc 0–4 constrained by HexNac > Fuc and HexNac-1> (NeuAc + NeuGc) based on tissue type or species from which *N*-glycans were release This search space consisted of 5097 glycan compositions with 1 added for the internal standard tagged glycan. The program was set to allow up to 3 formate adducts. After deconvolution and peak picking, glycan compositions are assigned based on the database search, and the abundances of all adducted forms of a given composition were summed. The program extracted peak masses and volumes in the MS^1 dimension. Compositions are assigned to approximate *N*-glycan class according to the number of HexNAc units: 3, hybrid; 4, bi-antennary, 5, tri-antennary; 6, tetra-antennary.

49 Proteomics Data Analysis: The data were searched against the UniProt/SwissProt database using Peaks Studio version 8.5 (Bioinformatics Solutions, Inc., Waterloo, ON, Canada) with a 1% false discovery rate and at least 2 unique peptides required for protein identification using PeaksDB. A 10 ppm error tolerance for the precursor (MS^1) and 0.1 Da mass error tolerance for fragment ions (MS^2) were specified. A maximum of 2 missed cleavages per peptide was allowed for the database search, permitting non-tryptic cleavage at one end. Trypsin specificity was defined as cleavage after Arg and Lys, when not followed by a Pro, allowing for hydroxyl proline as a variable modification. After a regular database search, a second PTM search was performed using PeaksPTM for a larger set of variable modifications which included (deamidation N, oxidation M, phosphorylation STY, HexNAc ST, HexHexNAc ST, hydroxylation K, hydroxylation-Hex K,

Table 4
Enzyme digestion conditions *N*-glycans and HS disaccharides together followed by trypsin.

Enzyme	Concentration	Reaction mixture	cycles	Reaction/ Extraction volume	Extraction buffer
Hyaluronidase	2mU/μL	10 μL enzyme + 30 μL 5 mM ammonium acetate	5 × 37 °C (4 × 1 h & 1X overnight 12 h)	1.8 μL/spot	0.3% Ammonium hydroxide
Chondroitinase ABC	2 mU/μL	10 μL enzyme+ 0.5 μL 100 mM Ammonium acetate 2.5 μL 100 mM Tris-HCl 27 μL Water	5 × 37 °C (4 × 1 h & 1X overnight 12 h)	1.8 μL/spot	0.3% Ammonium hydroxide
Heparan sulfate Lyase + PNGase F	HS Lyase I = 2 mU/ μL HS Lyase II = 2 mU/ μL HS Lyase III = 2 mU/ μL PNGase F = 100,000 U/mL	I = 10 μL II = 10 μL III = 10 μL PNGaseF = 10 μL 6.4 μL 5 mM Ammonium bicarbonate 3 μL 100 mM Ammonium acetate 0.62 μL 100 mM Calcium Chloride 6.4 μL Water	5 × 37 °C (4 × 1 h & 1X overnight 12 h)	1.8 μL/spot	0.3% Ammonium hydroxide
Reduce	10 mM dithiothreitol in 25 mM Ammonium bicarbonate	10 mM dithiothreitol in 25 mM Ammonium	1 × 55 °C for 20 min	1.8 μL/spot	–
Alkylate	20 mM IAA in 25 mM Ammonium bicarbonate	20 mM IAA in 25 mM Ammonium biocarbonate	1 × 25 °C C in dark for 25 min	1.8 μL/spot	–
Trypsin	1 μg/ μL	5 μL enzyme +25 μL 15 mM Ammonium bicarbonate	5 × 37 °C (4 X 1 h & 1X overnight)	1.8 μL/spot	30% Acetonitrile /0.1% TFA

ubiquitination K, hydroxylation P, nitrotyrosine Y) and a fixed carbamidomethylation modification. The final results were a combination of database and variable PTM searches. Abundances were normalized with respect to the total ion current (TIC).

Tips for troubleshooting the protocol are represented in Table 6.

Experimental design

Tissue sectioning

Fresh frozen or fixed tissue are sectioned and parallel sections chosen for H&E staining and selection of regions of interest. Further, control tissue from bovine cortex is sectioned as a digestion control.

Frozen tissue sections

Frozen tissue was sectioned using a cryostat. For best results, prior to any sectioning, the cryostat is thoroughly cleaned by wiping down all surfaces with 70% ethanol. A new blade is mounted and cryostat sterilized with UV for 30 min while body temperature is set to −20 °C and head −18 °C. All brushes, forceps, and tools to be used are wiped down with 70% ethanol and left in the cryostat under UV light for sterilization. A set of two or three clean chucks are placed in the tray after washing in water and wiped down with ethanol as well for 30-minute sterilization. Sections are cut once temperature equilibration is reached. Tissue is mounted on chucks using optimal cutting temperature (OCT)

Table 5
Enzyme digestion conditions HS and CS disaccharides followed by trypsin:

Enzyme	Concentration	Reaction mixture	cycles	Reaction/ Extraction volume	Extraction buffer
Hyaluronidase	2mU/μL	10 μL enzyme + 30 μL 5 mM ammonium acetate	5 × 37 °C (4 × 1 hr & 1X overnight)	1.8 μL/spot	0.3% Ammonium hydroxide
Chondroitinase ABC	2 mU/μL	10 μL enzyme+ 0.5 μL 100 mM Ammonium acetate 2.5 μL 100 mM Tris-HCl 27 μL Water	5 × 37 °C (4 × 1 hr & 1X overnight)	1.8 μL/spot	0.3% Ammonium hydroxide
Heparan sulfate Lyase	HS Lyase I = 2 mU/ μL HS Lyase II = 2 mU/ μL HS Lyase III = 2 mU/ μL	I = 10 μL II = 10 μL III = 10 μL 2.5 μL 100 mM Ammonium acetate 0.5 μL 100 mM Calcium Chloride 7 μL Water	5 × 37 °C (4 × 1 hr & 1X overnight)	1.8 μL/spot	0.3% Ammonium hydroxide
Reduce	10 mM dithiothreitol in 25 mM Ammonium bicarbonate	10 mM dithiothreitol in 25 mM Ammonium	1 × 55°C for 20 mins	1.8 μL/spot	–
Alkylate	20 mM IAA in 25 mM Ammonium bicarbonate	20 mM IAA in 25 mM Ammonium bicarbonate	1 × 25°C in dark for 25mins	1.8 μL/spot	–
Trypsin	1 μg/ μL	5 μL enzyme +25 μL 15 mM Ammonium bicarbonate	5 × 37 °C (4 × 1 hr & 1X overnight)	1.8 μL/spot	30% Acetonitrile 0.1% TFA

polymer being very careful to avoid any OCT contact with the tissue regions that are sectioned (Supplemental Figure S1). The tissue is sectioned at 5 μm for trimming to get even sections and then, sections for analysis at 10 μm thickness in the cryostat at approximately -20°C. Sections are adhered to positively charged Superfrost Plus slides, by pressing the room temperature slides on the cold tissue so that it adheres to the positively charged slides. Care is taken that once the tissue is sectioned it does not contact brushes or other objects to avoid contaminants. Slides are submerged for one minute in acetone. The backs of the slides are air dried in the cryostat and the region of interest to digest outlined in permanent marker. Slides are labeled in pencil to avoid ink that comes off during washes. These are stored at –80°C until digestion. PROCEDURE: Steps 1–10.

FFPE tissue sections

Paraffin blocks are sectioned using a microtome at 10 μm thickness. The serial sections ribbon are flattened and separated using cold forceps on clean water held in a bath at 55°C. Sections are adhered on positively charged Superfrost Plus slides. Slides are baked at 37°C for 12 h to adhere them to the slides. Slides are stored at room temperature. It is imperative not to use any quick freeze solutions or adherent solutions that contain polymers. PROCEDURE: Steps 11-18.

To avoid sectioning bias, process all sections on the same day in random order to maintain consistency. Use a reference tissue processed and sectioned similarly, into frozen slides or FFPE slides, as controls for reproducibility of enzymatic activity, extraction of digestion products, and LC–MS performance.

Deparaffinization and antigen retrieval for FFPE samples

Deparaffinization involves washing the FFPE slides in xylene and rehydrating in a series of ethanol washes at room temperature. This is important to unmask hidden or latent epitopes in preparation

Table 6
Troubleshooting tips.

Step	Problem	Possible reason	Solution
Step 21-23, 24 -25	Tissue falling off slides	Tissue not adhered sufficiently to the slides/ improper sectioning conditions	Use positively charged Superfrost Plus slides for sections. For FFPE bake for 12 h at 37 degree C.
Step 45	Absence of triply sulfated forms	MS Source conditions are not optimal and insource sulfate loss	Tune source for fragile tuning and sulfate loss by direct infusion -on Orbitrap-XL ideal conditions include: capillary temperature 90-110 degree C and tube lens(V) -110
Step 45	Low recovery from LC	Charged glycans stick to metallic loops Negatively charged glycans also stick to titanium	We suggest using a peak loop Ensure all frits before column do not contain titanium.
Step 45	Observation of front peak	weak wash is less organic than initial condition	Change weak wash to more organic than the initial condition of the sample and check column integrity. We suggest 90% Acetonitrile for 85% B initial condition.
Step 46	Observe high TIC but a low number of proteins identified	• presence of lipids from tissue OCT contaminant • loss in MS2 sensitivity	• Check for presence of PEG peaks for OCT or equidistant peaks for lipids. Check number of tandem spectra acquired and proportion of PSM matched. Expect the ratio to be 30% for proteomics without contaminants. • For MS^2 sensitivity: Check quality and isotopic pattern of peptide retention mixture. We also suggest to keep TIC of the cohort within twofold difference for all samples for label-free quantitation.

and allow for enzymatic access to digest the glycans and peptides off the slides. Xylene removes all the paraffin from the tissue. The serial ethanol washes are done to remove the xylene. PROCEDURE: Steps 19–22, Table 1. While several deparaffinization solvents are available, we find that xylene and ethanol are effective in our workflow.

Following deparaffinization, we perform antigen retrieval in Tris−HCl at pH 8.0 at 95 °C for 20 min in an oven. PROCEDURE Step 23-24. Antigen retrieval denatures chemical crosslinking by formalin allowing for efficient digestion. There are several factors that are important for antigen retrieval efficiency. These include i. buffer choice and pH, ii. heating method (microwave, autoclave, baking, water bath), iii. temperature, iv. time. The two most commonly used buffers in antigen retrieval for immunohistochemistry are citrate (pH 6.0) and Tris−HCl (pH 8.0). We prefer Tris−HCl (pH 8.0) since it is suitable to reveal epitopes in many tissues and efficient for protein analysis post antigen retrieval [18]. We heat this in a glass Coplin jar, filled with Tris, placed inside a water bath heated in an oven, to allow uniform heating for 20 min. The heat inactivation of formalin cross-links is critical in reversing the cross-links for efficient access of the enzymes to protein epitopes [19]. Following this, we wash the tissue for 5 min in water at room temperature to release the crosslinks. The washing step removes residual Tris at pH 8 and neutralizes the tissue for subsequent enzymatic digestion. PROCEDURE 23-24. This is well established in pathology, and we incorporate this in the workflow to allow for efficient lysis by enzymes.

For Fresh-frozen tissue, serial ethanol washes to remove contaminant is performed as described in PROCEDURE 22 Table 2.

In our studies, we used animals/humans per group as our biological replicates and three serial sections of slides as our technical replicates. All samples are randomized and blinded prior to digestion to avoid bias.

On-slide digestion

We digest the slides after deparaffinization and rehydration as described in PROCEDURE: Steps 25-34. For the enzymatic digestions, optimal pH is critical for efficient action of the enzymes. Particularly, in HS and CS digestion, it is important to use ammonium acetate as an activator for the enzyme. A schematic photograph of the chamber with slides for incubation is shown in Supplemental Figure S2.

Desalting

While there exist several orthogonal techniques for desalting of glycans including, size exclusion chromatography, strong anion exchange or porous graphite carbon, we find that size exclusion chromatography is most unbiased toward the glycans and efficient in recovery of small amounts and hence prefer this method. PROCEDURE Step 35,36.

Data acquisition

The appropriate data acquisition is important for accurate results. Prior to LC—MS/MS, instrument performance standards are run daily, to ensure stable performance during acquisition, and all samples are spiked in with appropriate internal standards to control for run-to-run variability during data acquisition. For GAGs- HS and CS disaccharides, we use a synthetic internal control ΔUA-2S ® GlcNCOEt-6S (I-P) internal standard, HD009, Iduron. For *N*-glycans we use 2AB labeled *N*-glycan A2F (GKSB-313, Prozyme) and for peptides we use peptide retention time calibration mixture (88321, Pierce) spiked into the samples to control for run-run variability. During data acquisition, we acquire all the glycan data in random order to ensure that any bias in sample order is eliminated. Appropriate tune files with fragile source conditions are necessary for accurate estimation of labile glycans. For our proteomics analysis, we acquire our data and ensure that the total signal TIC is within 2 fold for the entire cohort in order to accurately estimate differential quantitation between the groups.

Data analysis

For disaccharides, we determine relative abundances, which gives an estimate of chain architecture in the case of HS and CS. Further, we assess absolute amounts with respect to a standard curve for levels of disaccharides. We then estimate the amount of sulfation and the chain length, which is an estimate of the ratio of saturated and unsaturated disaccharides. This is described in PROCEDURE 47.

For *N*-glycans, we assess the relative abundance of glycans after processing the LC–MS data using GlycReSoft www.bumc.bu.edu/msr/glycresoft) [12–14]. GlycResoft creates custom combinatorial databases for the user to define based on experimental question as well as databases built based on knowledge of enzymatic biosynthesis processes in glycobiology. We search our HILIC analysis with up to 3 formate adducts due to the presence of ammonium formate in the chromatography mobile phase. Database searching parameters are described in PROCEDURE 48.

For Proteomics we assess our data using a database searching tool Peaks Studio. Database searching parameters are included in PROCEDURE 49. It is noted that for identification we use an FDR of 1% and at least 2 unique peptides and for quantitation, we assess label-free quantitation with 10 ppm precursor, retention time window 3 min and presence in at least 1 sample per group to estimate statistical significance based on ANOVA.

Materials

Reagents

- Hydrophobic IHC Pap pen (Super^{HT} pen, Biotium)
- Ethyl alcohol, 200 proof, (BP2818, Fisher Scientific) CAUTION flammable liquid. Use in a fume hood. Wear gloves and safety goggles while handling
- Xylene, (XX006 0-4, Millipore) CAUTION harmful and fatal if swallowed. Xylene is a flammable liquid. Use in a fume hood. Wear gloves and safety goggles while handling
- Ammonium acetate(A7330-500 g, Sigma Aldrich),
- Ammonium bicarbonate(A6141, Sigma Aldrich)
- Tris-HCl(T3253, Sigma, pH 8),
- Calcium Chloride(C5080, Sigma Aldrich),
- Ammonium hydroxide, 25–30%(320145, Sigma Aldrich) CAUTION corrosive and an irritant by vapor or ingestion. Use in a fume hood. Wear gloves and safety goggles while handling
- Dithiothreitol (D0632, Sigma),
- Iodoacetamide(163-2109, Biorad),
- Trifluoro-acetic acid(A116-10X AMP, Fisher Scientific)
- acetonitrile(A955-1, LC–MS grade, Fisher Scientific),
- Water (W6-1, LC–MS grade, Fisher Scientific),
- Formic acid(A117-10x-1AMP, LC–MS grade, Fisher Scientific) CAUTION flammable, corrosive. Use in a fume hood. Wear gloves and safety goggles while handling
- Acetic Acid(9508-01, glacial, JT Baker) CAUTION flammable, corrosive. Use in a fume hood. Wear gloves and safety goggles while handling
- Hyaluronidase (H1136-1AMP, Sigma)
- Chondroitinase ABC(Sigma)
- Heparan lyases 1,2,3(P0735 L, P0736 L, P0737 L New England Biolabs)
- PNGase F(P0705 L, New England Biolabs)
- Trypsin (V528A, Prozyme)
- Kasil (C033116, PQ Corporation)
- Formamide (F7503, Sigma Aldrich)
- Superdex peptide(3.2/300 column, GE Healthcare Life Sciences)
- C18 micro-Ziptip(ZTC18M096, Millipore)

Equipment

- Gloves, safety glass, kimwipes
- Superfrost Plus microscope slides (Fisher Scientific, cat. no. 22-037-246)
- Plastic Coplin staining jar (Fisher Scientific, cat. no. 19-4)
- Glass Coplin staining jar (Fisher Scientific, cat. no. 08-813E)
- Immunohistochemistry Pap-pen (SuperHT pen, Biotium)
- Permanent xylene and ethanol resistant marker (H-6100, Vectorlabs)
- Immunohistochemistry black staining boxes (AT-12, IBI Scientific)
- Cryostat
- OCT Compound (25608-930, VWR)
- Incubator control for 37 °s,
- Incubator controlled at 55 °
- Vacuum centrifugation (SPD1010 Speedvac system, Thermo Savant)
- UPLC system)
- Thermo-Orbitrap-XL, Q-Exactive-HF.

Software

- GlycReSoft software (www.bumc.bu.edu/msr/glycresoft)

- Peaks Studio 8.5 or other proteomics database searching software

Reagent setup

- Frozen sections: Cut 10 μm sections, mount on Superfrost Plus slides. Fix for 1 min in acetone. Store at −80 °C.
- FFPE sections: Cut 10 μm sections, mount on Superfrost Plus slides. Bake for 12 h at 37°C to mount the tissue. Store at 25 °C.
- Tris buffer (10 mM Tris HCl, pH 8.0): Prepare 50 mL Tris HCl and adjust pH with NaOH.
- Ammonium acetate: Prepare 100 mM ammonium acetate
- Calcium Chloride: Prepare 100 mM Calcium chloride solution
- Ammonium bicarbonate: Prepare 100 mM ammonium bicarbonate
- Acetic acid (50 mM): 29 μL glacial acetic acid (17.4 M) in 9971 μL water.
- 0.3% Ammonium hydroxide: 30 μL Ammonium hydroxide in 9970 μL water
- 30% Acetonitrile 0.1% trifluoro acetic acid (TFA): Prepare 3 mL acetonitrile +10 μL trifluoro acetic acid (100%) in 6990 μL water.

Equipment setup

- Size exclusion chromatography: The Superdex peptide column is mounted and flushed with 2 column volumes of water and 2 column volumes in 25 mM ammonium acetate, 5% acetonitrile for equilibration prior to use. The operating pressure is 5–20 bar bar at a flow rate of 0.04 mL/ min isocratic flow.
- UPLC for HILIC setup: Prepare the solvents for different phases as well as needle washes as described in Table 3 and purge to ensure there are no air bubbles. Mount the self-packed HILIC or HILIC-WAX column on the instrument and equilibrate by running at least 4 blanks to ensure the column is well equilibrated prior to running standards and acquiring the data.
- UPLC for reverse phase: Prepare the solvents as described in Table 3 and purge to ensure there are no air bubbles in the lines. Acquire data for 10 pepmix injections and assess for reproducibility and retention time drift. When consistent run the samples.
- We recommend using sulfated saccharide performance standards to ensure that the mass spectrometer is set up correctly for negative polarity analysis of fragile compounds. For this purpose, the triply sulfated HS disaccharide D2S6 works well. If the mass spectrometer is appropriately tuned, one should observe less than 10% of the precursor ion dissociating with a loss of SO_3. Supplementary Figure S3 shows extracted chromatograms for D2S6, and Supplementary Figure S4 shows extracted chromatograms for biantennary *N*-glycan A2F for source tuning conditions. For some mass spectrometer manufacturers, it may be necessary to adjust the settings of lenses in the source and/or analyzer to reduce the vibrational energy of the ions. In some cases, this requires contacting factory engineers with access to the necessary software codes. Once a proper method for negative ion MS is set up, it can be used indefinitely.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mex.2019.09.029.

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