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## Regional N-Glycan and Lipid Analysis from Tissues Using MALDI-Mass Spectrometry Imaging

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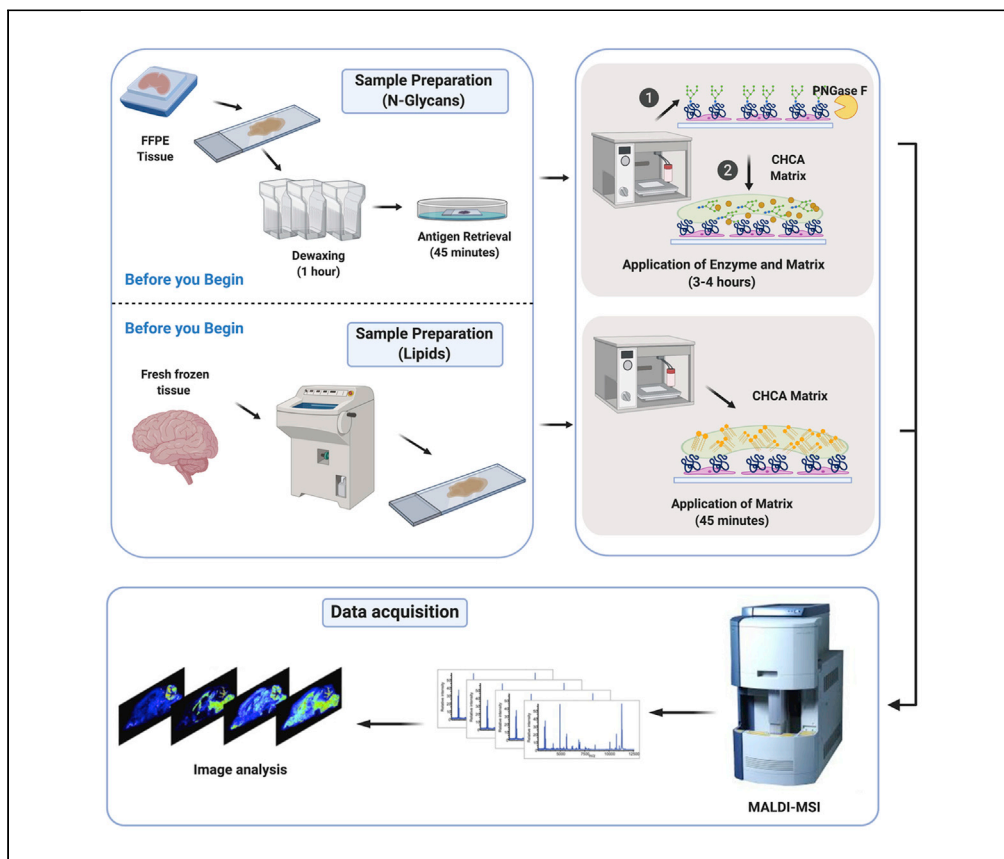
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## Protocol

# Regional N-glycan and lipid analysis from tissues using MALDI-mass spectrometry imaging



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**HIGHLIGHTS**  
MALDI-MSI of N-glycans and lipids from tissues

Enzyme-assisted release of N-linked glycans from formalin-fixed tissues

High-velocity deposition of matrix to improve rigor and reproducibility

Ion mobility separation of analytes from matrix as part of imaging workflow

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## Protocol

## Regional N-glycan and lipid analysis from tissues using MALDI-mass spectrometry imaging

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## SUMMARY

N-glycans and lipids are structural metabolites that play important roles in cellular processes. Both show unique regional distribution in tissues; therefore, spatial analyses of these metabolites are crucial to our understanding of cellular physiology. Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) is an innovative technique that enables *in situ* detection of analytes with spatial distribution. This workflow details a MALDI-MSI protocol for the spatial profiling of N-glycans and lipids from tissues following application of enzyme and MALDI matrix.

For complete details on the use and execution of this protocol, please refer to Drake et al. (2018) and Andres et al. (2020).

## BEFORE YOU BEGIN

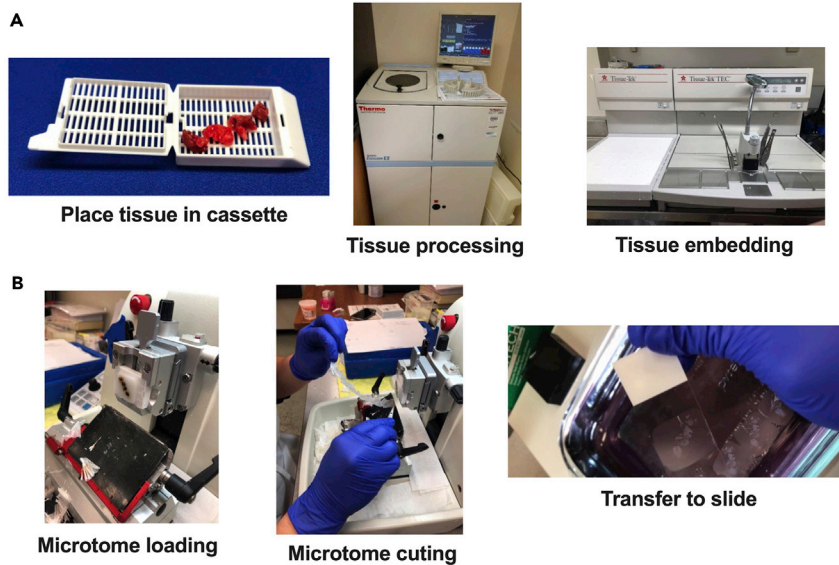
The workflow described in this protocol takes several days and requires careful preparation of reagents and access to specific equipment (see [Materials and equipment](#)). This protocol can be adapted to both formalin-fixed paraffin-embedded (FFPE) (N-glycans) and fresh frozen tissues (lipid species) for MALDI-MSI analyses. Here, we describe the step-by-step process using mouse brain tissues; however, it can be applied to a large range of tissues.

## Tissue processing and slide preparation of formalin-fixed paraffin-embedded (FFPE) tissues

⌚ Timing: can be done up to 1 month in advance

1. Process and embed tissue samples ([Figure 1A](#))
  - a. Fix and process tissue
    - i. Place fresh tissue in formalin
    - ii. After 24 h, transfer tissue to 70% ethanol
    - iii. Place tissue in a cassette and dehydrate with increasing concentrations of ethanol (70%–100%)
      - 70% ethanol for 40 min
      - 90% ethanol for 40 min





**Figure 1. Processing, embedding, and sectioning FFPE tissue samples**

(A) Tissues are formalin fixed in a cassette, followed by embedding in paraffin wax.  
(B) Paraffin blocks are sectioned at 4  $\mu\text{m}$  onto positively charged slides.

95% ethanol for 40 min

100% ethanol for 40 min, 3 $\times$

iv. Permeate the tissue with xylene to dissolve ethanol for 40 min

v. Displace the xylene with paraffin wax for 40 min, 3 $\times$

b. Place tissue specimen into a mold of paraffin wax and solidify on a cold plate to create a paraffin-embedded tissue block

2. Tissue cutting (Figure 1B)

a. Load tissue block onto a microtome

b. Cut slices at 4  $\mu\text{m}$  thickness and place tissue ribbon in a cooled water bath

c. Transfer tissue slice to a positively charged glass slide

### Tissue processing and slide preparation (fresh frozen tissues)

⌚ Timing: can be done up to 1 month in advance

3. Tissue collection and sectioning (Figure 2A)

a. Dissection and gradual freezing of the tissue

i. Prepare a bath of isopentane over dry ice and wait for it to equilibrate to about  $-70^{\circ}\text{C}$

ii. Place the dissected tissue in an aluminum covered weigh boat on the isopentane-dry ice bath for 7 min to freeze tissue. Larger samples may require additional time.

**Note:** Ensure complete freezing by checking the color of the tissue. Frozen tissue appears paler than raw tissue.

iii. Place tissue into foil packet and drop into liquid nitrogen for 1 min

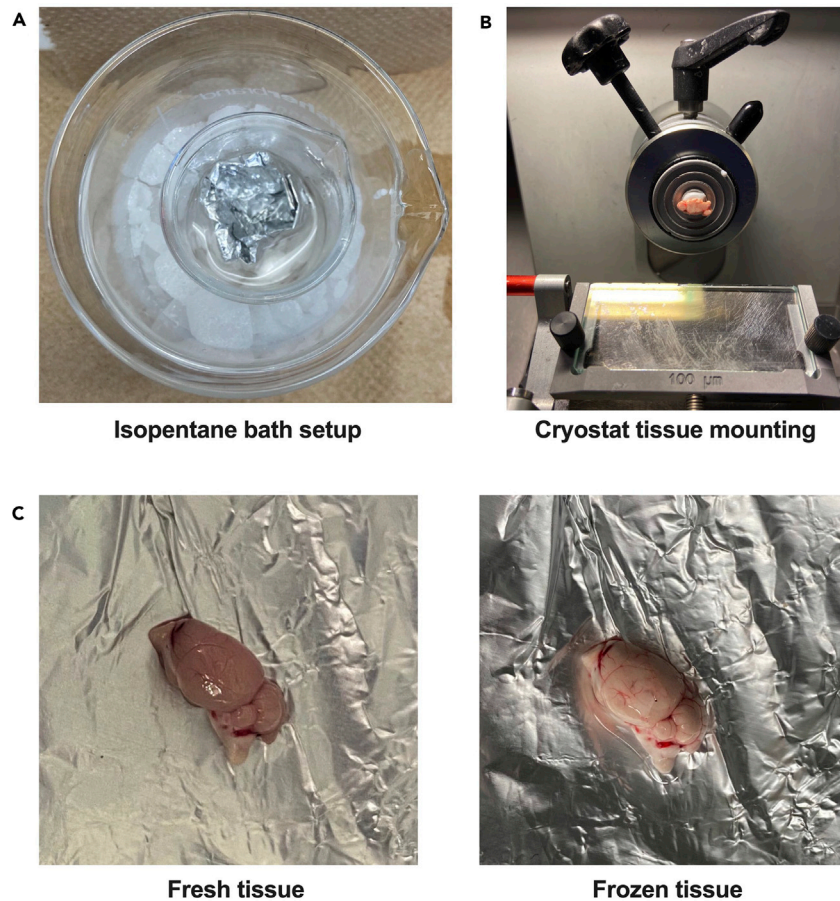
iv. Store at  $-80^{\circ}\text{C}$

b. Sectioning the tissue (Figure 2B)

i. Prepare cryostat by turning the internal temperature down to  $-23^{\circ}\text{C}$

ii. Place a small amount of OCT (optimal cutting temperature compound) on the cryostat chuck and place the tissue on the OCT

⚠ **CRITICAL:** Ensure that no OCT is on the tissue sections. OCT is ion suppressive and will interfere with lipid analysis.



**Figure 2. Freezing and sectioning fresh frozen tissue samples**

(A and B) (A) Tissues are slow frozen in an isopentane bath and (B) sectioned at 4  $\mu\text{m}$  onto positively charged slides using a cryostat.

(C) Example of fresh (left) and frozen (right) mouse brain tissue.

- iii. Allow the OCT and tissue to sit for 2 min inside the cryostat to ensure solidification
- iv. Cut sections at 4–10  $\mu\text{m}$  utilizing the plastic wedge to ensure even sections
- v. Lift the wedge and touch slide to mount the freshly cut tissue section onto a positively charged slide
- vi. Store slides at  $-80^{\circ}\text{C}$

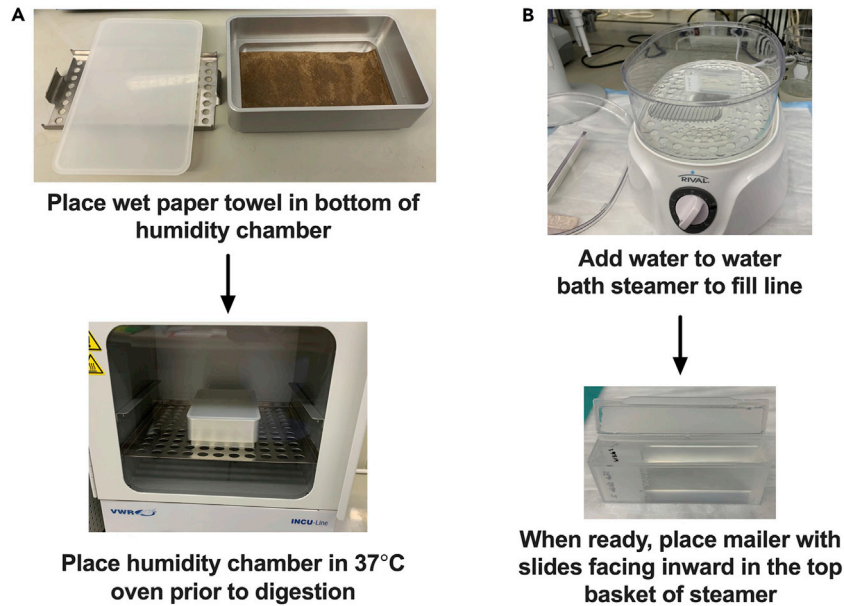
**Note:** Fresh frozen tissue slices do not require dewaxing, antigen retrieval, or enzyme application. As such, steps 4–6 of [Before you begin](#) may be skipped.

#### **Preparation of dewaxing solutions, humidity chamber, and antigen retrieval device**

⌚ Timing: 0.5–1 h

4. Dewaxing solutions: fill 7 clean slide staining jars with each of the following solutions
  - a. Xylenes (2 $\times$ )
  - b. 100% ethanol
  - c. 95% ethanol
  - d. 70% ethanol
  - e. Deionized water (2 $\times$ )





**Figure 3. Preparation of humidity chamber for PNGase F digestion and water bath steamer for antigen retrieval**

(A) Prepare humidity chamber in oven 1 h prior to PNGase F digestion.

(B) Prior to antigen retrieval, prepare water bath steamer (top). When ready, place mailer in top basket of steamer with slides facing inward (bottom).

**Note:** Fresh dewaxing solutions are critical for complete removal of paraffin wax and should be prepared fresh for every 10 slides processed.

5. Prepare humidity chamber for PNGase F digestion (Figure 3A)
  - a. Wet one paper towel and place in humidity chamber
  - b. Place humidity chamber in oven (38.5°C) at least 1 h prior to PNGase F digestion
6. Prepare water bath steamer for antigen retrieval by filling with tap water to fill line and preheating for 5 min prior to antigen retrieval (Figure 3B)

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Xylene	Spectrum	CAS#: 1330-20-7
Ethanol	Decon Labs	CAS#: 64-17-5
HPLC water	Sigma-Aldrich	CAS#: 7732-18-5
Hydrochloric acid	LabChem	CAS#: 7647-01-0
Citraconic anhydride	Thermo Scientific	CAS#: 616-02-4
Horse radish Peroxidase	Sigma-Aldrich	CAS#: 9003-99-0
PNGase F	N-Zyme Scientifics	Lot: NZL-2020-0194
$\alpha$ -Cyano-4-hydroxycinnamic acid	Cayman Chemical	CAS#: 28166-41-8
Trifluoroacetic acid	Sigma-Aldrich	CAS#: 76-05-1
Acetonitrile	Sigma-Aldrich	CAS#: 75-05-8
Methanol	Sigma-Aldrich	CAS#: 67-56-1
Software and algorithms		
High Definition Imaging (HDI) Software	Waters	Catalog #: 720005652EN

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Other</i>		
INCU-Line IL 10 digital incubator	VWR	Catalog #: VWR1390-0384
Hsw Norm-Ject Sterile Luer-Lock syringe, 3 mL	VWR	Catalog #: 53548-017
SGE syringe, gas tight plunger, 5 mL	VWR	Catalog #: 60361-204
Millipore Sigma non-sterile syringe filter, 13 mm, 0.20 $\mu$ m	Fisher Scientific	Catalog #: SLLGX13NL
M.5 humid chamber	HTX Technologies	N/A
Simport Scientific EasyDip slide staining rack	VWR	Catalog #: 87000-132
Simport Scientific EasyDip slide staining jars	VWR	Catalog #: 87000-124
Charged microscope slides	VWR	Catalog #: 89500-516
11 L vacuum desiccator	SP Bel-Art Scienceware	Catalog #: F42400-4101
Bioruptor Pico sonication device	Diagenode	Catalog #: B01060010
Cryo-Cube F740hi	Eppendorf	Catalog #: F740220035
M5 high-velocity sprayer	HTX Imaging	N/A
Azura P 4.1S HPLC pump	Knauer	Article #: APG20EA
"Just Infusion" syringe pump	New Era Pump Systems	Model: NE-300
MALDI Synapt XS	Waters	Catalog #: 720006589
Perfection V600 photo scanner	Epson	Model #: B11B198011
Water bath steamer	Rival	Model #: CKRVSTLM21

## MATERIALS AND EQUIPMENT

Citraconic anhydride buffer: add 25  $\mu$ L of Citraconic Anhydride and 2  $\mu$ L of 12 M HCl to 50 mL of HPLC grade water in a 50 mL falcon tube. Vortex and confirm pH of  $3.0 \pm 0.5$ .

PNGase F solution: Enzyme is supplied in lyophilized 100  $\mu$ g aliquots. To prepare, quick spin to pellet enzyme and add 50  $\mu$ L of HPLC water. Vortex for 10 s and add 950  $\mu$ L of HPLC grade water. Prepare fresh on the same day.

$\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA) matrix (7 mg/mL): Weigh 40 mg of CHCA in a 15 mL falcon tube using a clean and dry spatula. Add 5.7 mL of 50% acetonitrile/0.1% TFA. Vortex and sonicate for 10 min at 89 s on, 30 s off. Prepare fresh on the same day.

**⚠ CRITICAL:** It is important to clean the CHCA spatula with methanol, ethanol, or HPLC water only, do not use detergent. Detergent can significantly interfere with N-glycan and lipid ionization and detection, leading to poor signal.

**Alternatives:** This protocol is optimized using the equipment specified above in the [Key resources table](#), but they are not exclusive products to carry it out. If any materials or equipment are modified, further optimization is highly recommended as there is no guarantee that any change would not alter ionization efficiency and/or sensitivity of the mass spectrometer.

## STEP-BY-STEP METHOD DETAILS

### Slide preparation and antigen retrieval (day 1)

⌚ Timing: 2–3 h

**Note:** If analyzing lipids from fresh frozen tissue, remove slides from  $-80^{\circ}\text{C}$  and bring to  $20^{\circ}\text{C}$  in a vacuum desiccator for 2 h. Proceed to step 9 for CHCA matrix application.

1. Heat slides
  - a. Heat slides for 1 h in  $60^{\circ}\text{C}$  oven with small water bath, tissue side up
  - b. After 1 h, remove slides and allow to cool to  $20^{\circ}\text{C}$  for 1 min



2. Dewax slides
  - a. Place slides in slide holder and wash in dewaxing solutions for the following times:
    - i. Xylenes for 3 min, 2×
    - ii. 100% ethanol for 1 min, 2×
    - iii. 95% ethanol for 1 min
    - iv. 70% ethanol for 1 min
    - v. Deionized water for 3 min, 2×
  - b. Dry slides in vacuum desiccator for 8 min

**Note:** Antigen retrieval denatures and breaks protein crosslinks that were formed during fixation, allowing greater enzymatic access to proteins within the sample.

3. Antigen retrieval
  - a. Ensure that the water bath steamer is preheated
  - b. Place no more than two slides in a 5-slide holder with side opening partially closed

**Note:** Slides should be placed with tissue facing inward in the first and last slide slot to ensure that tissue is not touching the inner walls of the holder.

- c. Fill holder with citraconic anhydride buffer
  - d. Incubate in water bath steamer for 30 min
5. Application of positive control
  - a. 1  $\mu$ L of positive control (horseradish peroxidase) can be spotted directly onto the slide adjacent to tissue

**Note:** Controls can include a glycosylated protein or purified glycans.

6. Dry slides for 15 min in vacuum desiccator

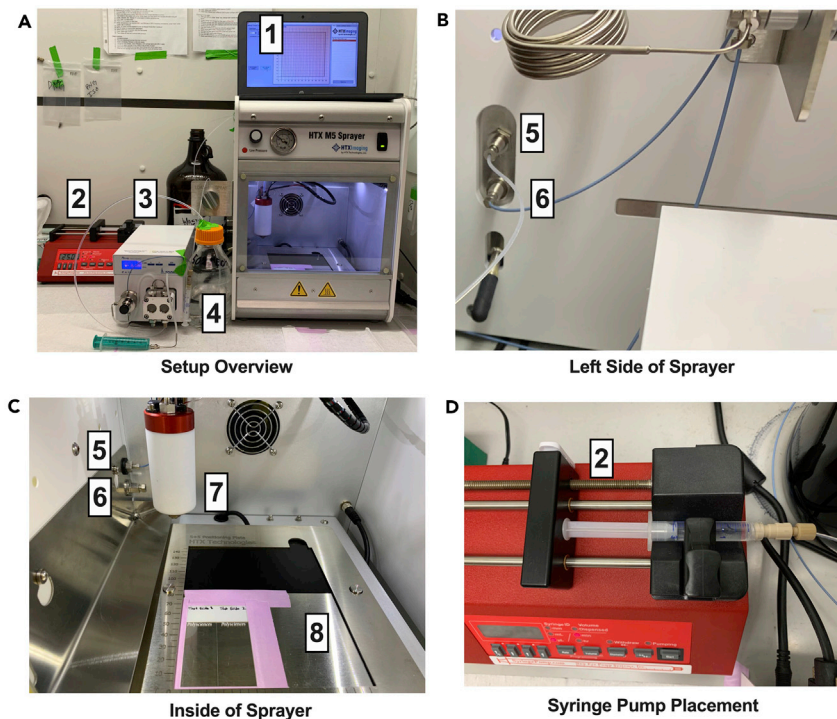
#### **PNGase F application by HTX M5 sprayer**

⌚ **Timing:** 3–4 h, done immediately following slide preparation and antigen retrieval

PNGase F cleaves N-linked glycans from their carrier proteins. Use of the HTX M5 sprayer ensures even application of enzyme, allowing for consistency to improve rigor and reproducibility. This methodology preserves spatial localization for MALDI-MSI analysis of N-glycan distribution. Incubation in a humidity chamber allows for maximum cleavage of N-glycans by PNGase F (see [Troubleshooting 1](#)).

7. Application of PNGase F ([Figures 4A–4C](#))
  - a. Turn on HTX M5 Sprayer
  - b. Turn on nitrogen gas and stabilize pressure to 10 psi
    - i. Adjust pressure using dial on sprayer
  - c. Open HTX M5 software on controlling computer and confirm the following settings:
    - i. Set point temperature: 45°C
    - ii. Tray type: Ambient
  - d. Turn off Knauer pump and disconnect from sprayer
  - e. Turn on syringe pump and connect to sprayer

**Note:** It is recommended to clean the syringe pump enzyme line before each application of PNGase F. Load 3 mL of HPLC water into a clean syringe and set the pump rate to 95  $\mu$ L/min. Start the syringe pump, and let water flow through the sprayer for 5 min prior to loading PNGase F solution.



**Figure 4. Overview of HTX M5 sprayer setup**

(A) HTX M5 sprayer connected to controlling computer, syringe pump, and Knauer pump with CHCA loading syringe shown. (B) External sprayer connectors on the left of the HTX M5 sprayer. (C) Internal sprayer connectors on the inside of the HTX M5 sprayer. (D) Syringe pump loaded with syringe locked in place. Label key: (1) controlling computer, (2) syringe pump, (3) Knauer pump, (4) CHCA loading syringe, (5) enzyme line, (6) Knauer line, (7) sprayer nozzle, and (8) stage.

- f. Setting up samples
  - i. Arrange slides on the sprayer stage
  - ii. On controlling computer, change the XY parameters to define the spraying area
- g. Load PNGase F solution
  - i. Using a clean syringe, draw up PNGase F and remove any bubbles
  - ii. Attach syringe to 6" enzyme line and connect line to the sprayer
  - iii. Place syringe in the syringe pump and lock in place

△ **CRITICAL: Bubbles in solution will interfere with even enzyme application. See Troubleshooting section.**

- h. PNGase Application (Figure 4D)
  - i. Set syringe pump flow rate to 25  $\mu\text{L}/\text{min}$  and syringe diameter to 9.7 mm

**Note:** Confirm syringe diameter if not using Hsw Norm-Ject Sterile Luer-Lock Syringe (see [Key resources table](#)).

- ii. Start the syringe pump and confirm even sprayer application on test slide
- iii. Once even spraying is confirmed, press start on the controlling computer to begin spraying across sample slides
- iv. Monitor spray to ensure even PNGase F application across sample slides
- v. When the cycle has completed, press stop on the syringe pump and remove slides from the tray
- vi. Place slides tissue side up in basket of humidity chamber and cover with lid

- vii. Incubate the samples for PNGase F digestion in preheated humidity chamber for 2 h
- viii. Dry slides in a vacuum desiccator for a minimum of 15 min, or up to 24 h

**Note:** It is recommended to clean the HTX M5 sprayer after each application of PNGase F by lowering the set temperature to 35°C and flushing the enzyme line with HPLC water at a flow rate of 95  $\mu\text{L}/\text{min}$ . After cleaning, the sprayer program, gas, and syringe sprayer can be turned off. Reattach the Knauer pump to the sprayer and set the Knauer pump to a flow rate of 100  $\mu\text{L}/\text{min}$ .

**⚠ CRITICAL:** Slides must lie flat in the humidity chamber and must be completely dried prior to CHCA matrix application. This ensures spatial localization of cleaved glycans is maintained.

**⏸ Pause point:** After incubation, slides can remain in desiccator for up to 24 h. Matrix application can be done on day 2.

### CHCA matrix application by HTX M5 sprayer (day 1 continued or day 2)

**⌚ Timing:** 0.5–1 h

Application of the CHCA matrix facilitates ionization of the cleaved N-glycans, ensuring sufficient signal intensity. Use of the HTX M5 sprayer ensures even application of CHCA matrix, allowing for consistent sample ionization across tissue regions (see [Troubleshooting 1](#)).

8. Prepare the CHCA matrix
  - a. Prepare matrix at 7 mg/mL in 50% acetonitrile/0.1% TFA
  - b. Filter the CHCA solution using a 0.2  $\mu\text{m}$  syringe filter

**Note:** Carefully filter the CHCA solution as undissolved CHCA can clog components of the HTX M5 sprayer.

9. Application of CHCA matrix ([Figures 4A–4C](#))
  - a. Scan slides in slide holder with a scanner before application of matrix

**Note:** A scanned image of the slide in the slide holder is used to program tissue regions to be analyzed by MALDI-MSI. Scanning prior to CHCA matrix application yields a clearer image of tissue boundaries.

- b. Turn on the HTX M5 sprayer
  - i. Turn on nitrogen gas and stabilize pressure to 10 psi and adjust pressure using dial on sprayer
- c. Open HTX M5 software on controlling computer and confirm the following settings:
  - i. Set point temperature: 79°C
  - ii. Tray temperature: 50°C
  - iii. Pump flow rate: 100  $\mu\text{L}/\text{min}$
- d. Prime the Knauer pump
  - i. Unscrew black cap on the front of pump and pull degassed 50% methanol through using the attached syringe. Return the black cap and discard the methanol.
- e. Clean the sprayer line
  - i. In the "LOAD" position, remove the stopper syringe and flush the line with 5 mL of 50% acetonitrile. Immediately replace with stopper syringe. With drip catcher below the sprayer nozzle, switch to "SPRAY" position and flush the line for 5 min.
- f. Load the CHCA matrix solution
  - i. Draw up filtered CHCA into clean syringe and remove all bubbles.

- ii. In the “LOAD” position, remove the stopper syringe and expel the CHCA into the sprayer line. Immediately replace the stopper syringe.

**Note:** Bubbles the sprayer line will interfere with even CHCA matrix application. See Troubleshooting section.

- g. Setting up samples
  - i. Arrange slides on the sprayer stage
  - ii. On controlling computer, change the XY parameters to define the spraying area
- h. CHCA matrix application
  - i. Ensure that drip catcher is below the sprayer nozzle and switch sprayer position from “LOAD” to “SPRAY.”
  - ii. Confirm that the temperature has reached 79°C and flow rate is 100 µL/min.
  - iii. Start the Knauer pump and confirm even sprayer application on test slide
  - iv. Once even spraying is confirmed, press start on the controlling computer to begin spraying across sample slides
  - v. Monitor spray to ensure even CHCA matrix application across all slides
  - vi. Once the application is complete, switch sprayer back to “LOAD” position
  - vii. Remove slides and lay flat to dry. Slides can be immediately analyzed by MALDI-MSI or stored in a vacuum desiccator at 20°C for up to 1 week.

**Note:** It is highly recommended to clean the HTX M5 sprayer after each CHCA matrix application. With the nitrogen gas still on, change the sprayer set point temperature and tray temperature to 30°C, and adjust the Knauer pump flow rate to 300 µL/min using the controlling computer. While the sprayer cools down, switch the sprayer to “load,” flush the line with 5 mL of 50% acetonitrile twice. Repeat line flush with 50% methanol twice. Switch sprayer to “spray” and allow 50% methanol to run through the line until the set point temperature and tray temperature reach 30°C, then switch the sprayer back to the “load” position. Adjust the Knauer pump flow rate back to 100 µL/min, return the drip catcher to below the sprayer nozzle, and turn off the nitrogen gas. Any residual CHCA matrix on the slide stage can be removed with 50% methanol.

### Image acquisition by waters synapt G2-XS mass spectrometer

The parameters indicated in [Tables 1, 2, and 3](#) are used for N-glycan and lipid analysis on a Waters Synapt G2-XS Mass Spectrometer and can be programmed in the HDI software.

**Note:** Proper laser firing is essential for sufficient ionization of the CHCA matrix, as well as N-glycans and lipids. As laser energy can decrease with time, it is recommended to confirm the laser is performing properly. We recommend testing laser firing on a slide coated with CHCA matrix only and confirming the signal intensity of CHCA matrix peaks. For these analyses, the laser used in this manuscript was approximately 6 months old.

### EXPECTED OUTCOMES

In our experimental protocol, we describe a detailed method for visualization of N-glycans and lipids from FFPE and fresh frozen tissue, respectively, by MALDI-MSI. Typically, for most mammalian tissues, the Waters Synapt G2 mass spectrometer detects over 120 N-glycan and 200 lipid peaks, depending on the complexity and quality of the tissue. Here, we illustrate an example of the protocol to

**Table 1. MALDI laser settings for analysis of N-glycans and lipids**

	N-Glycans	Lipids
Laser energy	250 µJ	200 µJ
Laser firing rate	1,000 Hz	1,000 Hz
Raster size	50–200 µm	50–200 µm
Time per pixel	0.5 s	0.5 s

**Table 2. Mass spectrometer settings for analysis of N-glycans and lipids**

	N-Glycans	Lipids
Trap collision energy	4 V	4 V
Transfer cell collision energy	2 V	2 V
Scan rate	0.5 s	0.5 s
Polarity	Positive	Negative
Analyzer mode	Sensitive	Sensitive
Mass range m/z	500–3,500	50–2,500

visualize N-glycans (Figure 5) and lipids (Figure 6) from a wild-type mouse brain using the HDI imaging software. Glycan and lipids were assigned based on previously established reports (Colsch and Woods, 2010; Powers et al., 2014; Zhang et al., 2016; Drake et al., 2017, Drake et al., 2018). In both cases, ion mobility improved N-glycan/lipid detection by separating their peaks from ionization matrix based on differential collision cross section (see Troubleshooting 2).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Imaging

For the qualitative comparison of N-glycan and lipid species between two or more groups, N-glycan and lipid masses should be normalized to total ion chromatograph (TIC) within each pixel. This will correct for small batch variations introduced by CHCA concentration and laser power reduction over time.

### Regional analysis

For the qualitative analysis of regional abundance of N-glycans and lipids, first N-glycan and lipid masses should be normalized to TIC within each pixel. Then regions of interest (ROI) should be defined by the user. The number of pixels must be consistent between ROIs of two or more groups before exportation of mass spectrometry data. The appropriate statistical analysis for comparison between two more groups should be determined by the user. These include but are not limited to: student's T-test, ANOVA, clustering heat analysis, and principal component analysis.

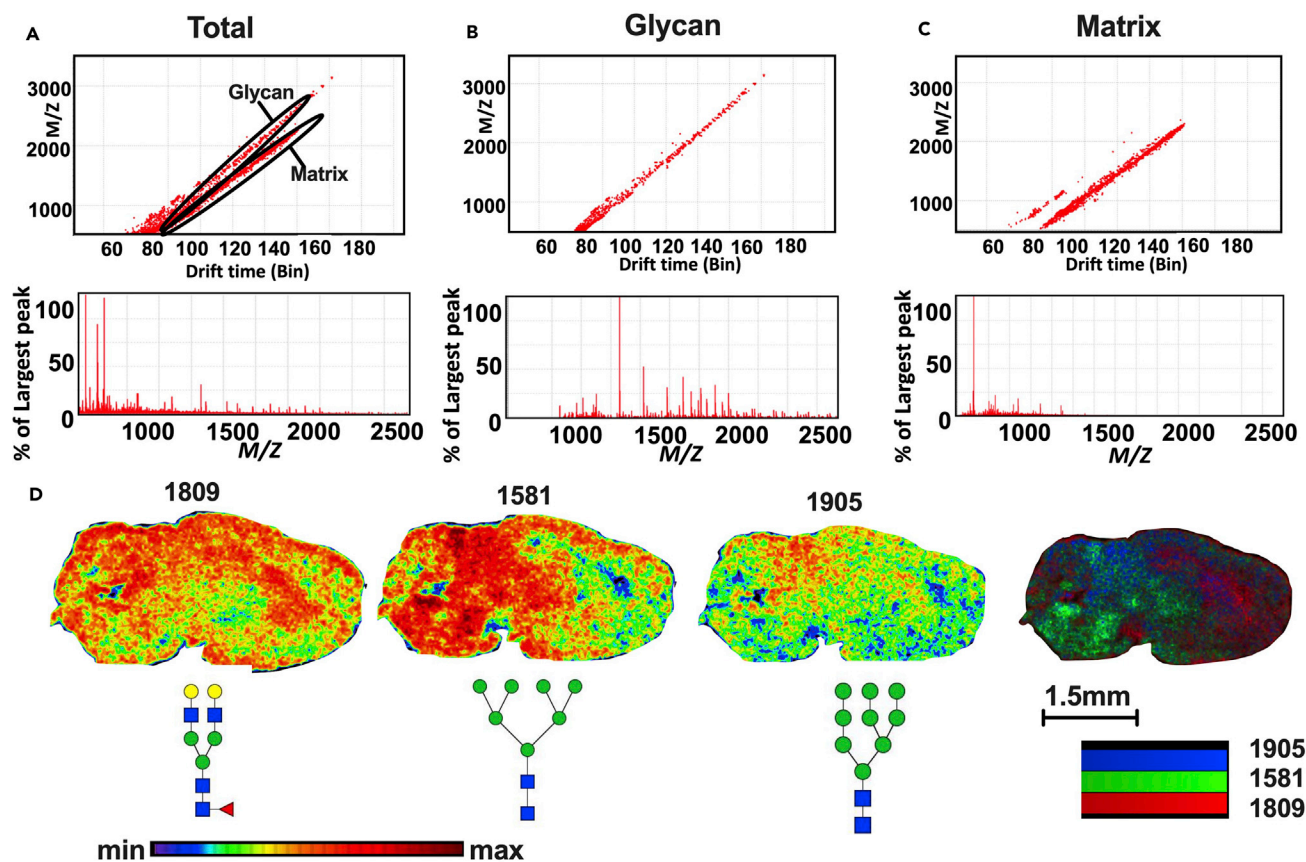
## LIMITATIONS

This protocol requires high quality tissue samples to be properly prepared prior to MALDI-MSI analysis. Tissues not fixed or frozen in a timely manner risk degradation, and tissue sections that are cut higher than the recommended thickness can result in poor quality images. It is also recommended to use recently sectioned tissue, as tissue that has been cut less than 30 days prior yields better sample ionization and signal.

Use of this protocol for N-glycan profiling requires consistency in PNGase F enzymatic activity across sample preparations to ensure reproducible results. Maintaining proper oven temperature and humidity during PNGase F digestion, and the use of a positive control are key to confirming enzymatic activity. It is recommended to use PNGase F from the same vendor and lot number to ensure consistent N-glycan cleavage (see also Troubleshooting 3). PNGase F should be stored in single use aliquots in a  $-80^{\circ}\text{C}$  freezer. Further, certain commercially available PNGase F frequently contains

**Table 3. Ion mobility settings for analysis of N-glycans and lipids**

	N-Glycans	Lipids
Trap	Entrance 2 V; bias 85 V, trap DC 0 V; exist 0 V	Entrance 2 V; bias 75 V, trap DC 0 V; exist 0 V
Wave velocity	Trap 9.6 m/s; IMS 4.6 m/s; transfer 17.4 m/s	Trap 9.6 m/s; IMS 4.6 m/s; transfer 17.4 m/s
Wave height	Trap 4 V; IMS 42.7 V; transfer 4 V	Trap 4 V; IMS 42.7 V; transfer 4 V
Variable wave Velocity mode	Ramp down; 1,400 m/s (start)-300 m/s (end)	Ramp down; 1,400 m/s (start)-300 m/s (end)



**Figure 5. PNGase F digestion and MALDI-MSI analysis of N-glycans in mouse brain tissue**

(Top) 2D plot of monoisotopic mass versus drift time in the ion mobility cell for N-glycans and the MALDI matrix. (Bottom) Extracted ion chromatogram of released N-glycans and matrix ions based on ion mobility separation for (A) both, (B) N-glycans, and (C) matrix. (D) Representative HDI images of 1809 m/z, 1581 m/z, 1905 m/z, and overlay image of 1809 m/z (red), 1581 m/z (green), and 1905 m/z (blue). Scale bar, 1.5 mm.

additives such as glycerol and high salt concentrations that will interfere with the ionization and detection of N-glycans. Such additives will require removal by dialysis.

## TROUBLESHOOTING

### Problem 1

Uneven PNGase F or CHCA matrix application.

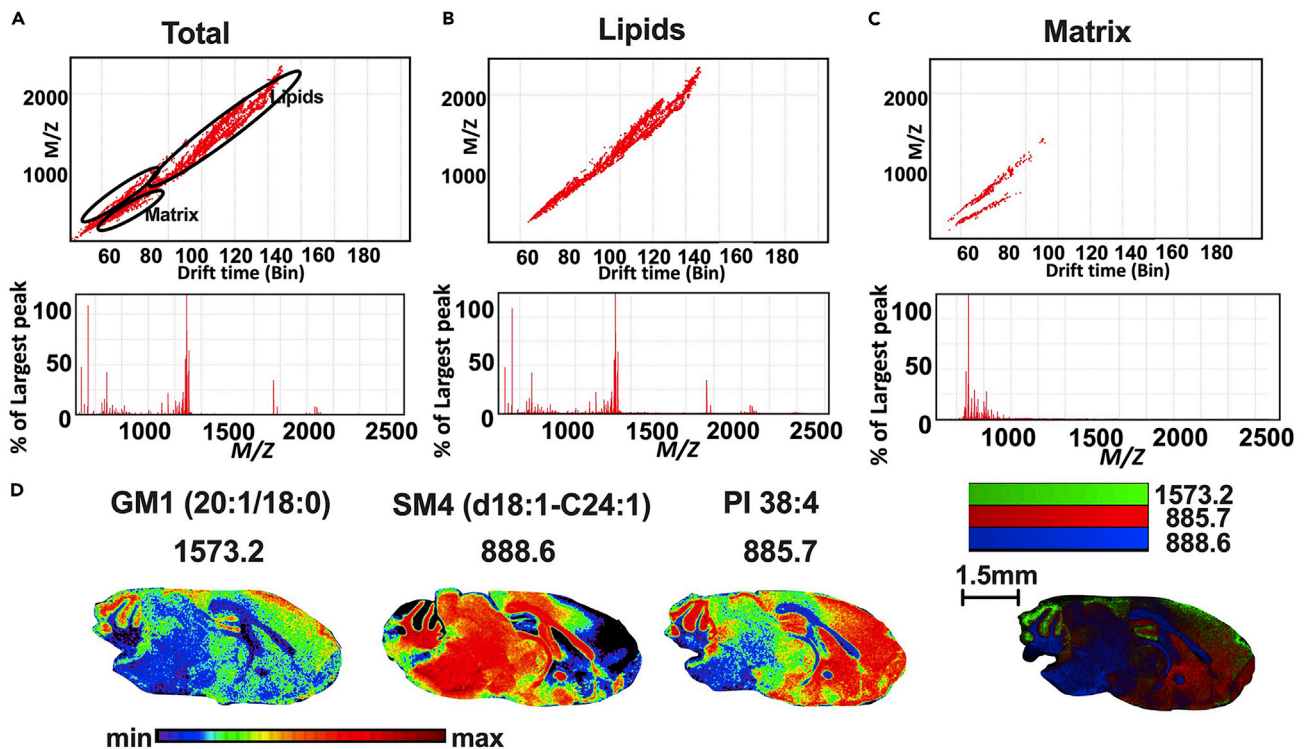
### Potential solution

Bubbles in the enzyme or matrix solution can interfere with even sprayer application and maintaining pressure in both the syringe and Knauer pumps. When loading solution into the syringe, make sure to pull the plunger up slowly to reduce the number of bubbles. Once the solution is in the syringe, gently tap it against a hard surface to push bubbles to the top and expel the air. Further, residual CHCA matrix in the sprayer nozzle can crystallize and lead to the sprayer nozzle sputtering. Allowing degassed 50% methanol to flow through the sprayer at 100  $\mu$ L/min following cleaning, even when the sprayer is not in use, prevents crystallization of any residual matrix in the sprayer line. If the pressure in the Knauer pump fluctuates, this will cause the matrix spray to be uneven as well. This could be caused by an air bubble in the front of the pump, so prime the pump again and pull about 10 mL of methanol through the pump to get rid of the bubble.

### Problem 2

Low signal across tissue.





**Figure 6. MALDI-MSI analysis of lipids in mouse brain tissue**

(Top) 2D plot of monoisotopic mass versus drift time in the ion mobility cell for lipids and the MALDI matrix. (Bottom) Extracted ion chromatogram of lipids and matrix ions based on ion mobility separation for (A) both, (B) lipids, and (C) matrix. (D) Representative HDI images of 1,573.2 m/z, 888.6 m/z, 885.7 m/z, and overlay image of 885.7 m/z (red), 1,573.2 m/z (green), and 888.6 m/z (blue). Scale bar, 1.5 mm. Gangliosides (GM1) (20:1/18:0), 3-*o*-sulfogalactosylceramide (SM4) (d18:1-C24:1), Phosphatidylinositol (PI) 38:4.

### Potential solution

For N-glycans, PNGase F activity can be checked by use of a positive control. Following antigen retrieval, addition of a small amount of a protein such as horseradish peroxidase (known to be glycosylated) onto the slide away from the tissue can be used to assess PNGase F activity. If N-glycan signal is within range on the control protein, but not on the tissue sample, the dewaxing, or antigen retrieval steps may need to be investigated, as insufficient dewaxing or antigen retrieval can block enzymatic access to the N-glycans. If no signal was detected on the positive control, check PNGase F solution for integrity or sprayer may be blocked.

For lipids, make sure tissue is 4–10  $\mu\text{m}$  thickness. Tissue thicker than 10  $\mu\text{m}$  decreases laser ionization efficiency; 4  $\mu\text{m}$  provides better ionization for gangliosides. Make sure CHCA is prepared fresh daily and properly stored. Repeat freeze thawing cycle of CHCA will compromise ionization efficiency.

### Problem 3

Inconsistent N-glycan cleavage across the sample or poor detection of regional N-glycans.

### Potential solution

This protocol requires that the tissue analyzed is sectioned evenly and of a specific thickness. Uneven tissue samples produce inconsistent and variable results. Further, problems during PNGase F incubation period can cause inconsistent enzymatic cleavage. Slides must remain flat in the humidity chamber during incubation, as well as proper temperature and humidity must be maintained. Ensure that the paper towel in the chamber remains wet during incubation.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Ramon C. Sun ([ramon.sun@uky.edu](mailto:ramon.sun@uky.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The published article includes all datasets generated or analyzed during this study.

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## AUTHOR CONTRIBUTIONS

R.C.S. conceptualized the study and designed the experimental workflow. L.E.A.Y., A.E.S., K.H.M., and T.R.H. optimized the protocol. K.H.M., T.R.H., and H.A.C. performed the experiments. R.C.S., L.R.C., D.B.A., K.H.M., and H.A.C. generated the figures. L.R.C., A.E.S., K.H.M., and R.C.S. wrote the manuscript. All authors read and approved the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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