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In Situ Imaging of N-Glycans by MALDI Imaging Mass Spectrometry of Formalin-Fixed Paraffin-Embedded Tissue V.1

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We use this protocol and it's working

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Abstract

Glycosylation of cell surface, secreted, and circulating proteins is one of the most common types of post-translational modification. These modifications occur most commonly as one of three major classes: N-linked glycosylation on asparagine residues, O-linked glycosylation on serine or threonine residues, or as glycosaminoglycan oligosaccharide polymers on serine. Specifically, for N linked glycans, an endoglycosidase enzyme, peptide N-glycosidase F (PNGase F), cleaves the attached oligosaccharides between the asparagine and first sugar. A method to analyze released N-glycans and map them to specific locations within a tissue is presented here. The PNGase F is applied by solvent sprayer as a molecular layer on frozen or formalin-fixed tissues and all released Nglycans in a given region of tissue are detected using matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (MALDI-IMS). Using the described MALDI-IMS protocol, at least 40 or more individual N-glycans can be mapped to tissue histopathology and extracted for further structural analysis approaches.

Materials

	A	B	C	D
	Xylenes, histology grade			
	200 proof ethanol, USP grade			
	70%, 95%, and 100% ethanol			
	Water, HPLC grade			
	Citraconic anhydride	Sigma Adrich	CAS# 616-02-4	Cat# 125318-25G
	12M HCl. Hydrochloric acid ACS reagent, 37%	Sigma Adrich	CAS# 7647-01-1	Cat# 320331-500 ML
	Chromatography paper	Whatman	Grade 3MM CHR	Cat# 3030-866
	Peptide N-glycosidase F (PNGase F), 100 µg per four slides	N-Zyme Scientific	Lyolized power in a 0.5 mL vail	
	Ultra-pure nitrogen gas			
	α-Cyano-4-hydroxycinnamic acid (CHCA) matrix	Sigma Adrich	CAS# 28166-418	Cat# 70990-1G-F
	TFA, Trifluroacetic acid	Sigma Adrich	CAS#76-05-1	Cat#T6508-10AMP
	Coplin jars (capable of holding eight to ten slides minimum; eight plastic, two			
	Antigen retrieval device (e.g., vegetable steamer)			
	Kimwipes (Kimberly-Clark)			
	Desiccator attached to laboratory vacuum			
	High Resolution Document Scanner			
	TM-SprayerTM (HTXImaging)			
	PTFE 0.2 um membrane for filtration of aquious and orgaic solvent, Millex-LG 13 mm	Milllipore		REF: SLLGX13NL
	NORM-JECT Luer Solo syringe, 2 mL/5 mL			REF: NJ-4606027-02/05

	A	B	C	D
	Ovens, operating at 37.5°C ± 1.5°C and 60°C ± 1.5°C			

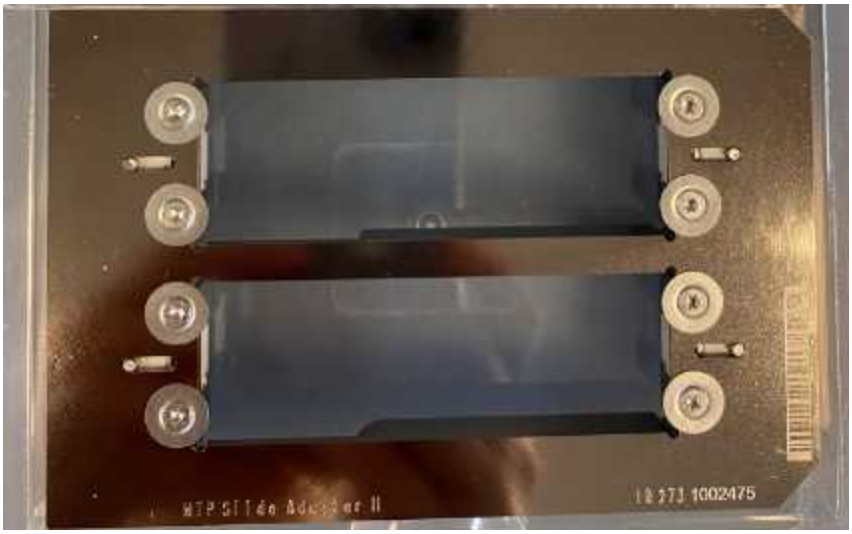
Troubleshooting

FFPE tissue slide & storage

1 FFPE tissue slides for MIBI and other epitope based stain protocol are usually stored in vacuum sealed bag with anti oxidant and drying agent. FFPE tissue slides for N-glycan IMS can be stored the same way as MIBI slides. However, slides left on bench top at room temperature exposed to atmosphere over a year can be used for N-glycan IMS as well.

Formalin fixed tissues should be sectioned at 3~7 µm and mounted on slides. For MALDI-FT-ICR and MALDI-QTOF, standard microscope slides, positively charged microscope slides or epoxide coated microscope slides are suitable as long as they fit into the 25 × 75 mm slide holder for the instrument.

If a slide with tissue does not fit the sample holder for the instrument, it may be sized to fit while the tissue is still covered with wax, as this prevents contamination and limits damage to the tissue. A small hand held rotary tool may be used to grind down slide edges to fit into the instrument sample holder. Afterwards, slides may be lightly rinsed in tap water and allowed to air dry.



MTP target frame III (Bruker, Part No.: 8074115)

A hand holder rotatory tool.

Dewax

2 You can either use MIBI stain dewax_protocol or the protocol in Drake's published protocol. Here we adapted Drake's protocol into our oven settings in R139.

2.1 Heat slides at 70°C (face up) for one hour, and then at 70°C (perpendicular) for 10 min.

2.2 Remove and cool to room temperature, usually 5 minutes.

2.3 You can do dewax washes the same way as for MIBI slide. Or you can just use plastic/glass Coplin jars and let the slide sit in the solution. Here are the sequential washing solutions:

- Xylenes 3 minutes, repeating a total of two times.

- 100% ethanol 1 minute, repeating a total of two times.

- 95% ethanol 1 minute

- 70% ethanol 1 minute

- Distilled water 3 minutes, repeating a total of two times.

2.4 Slides may be stored overnight in vacuum desiccator. Dry slides could be placed in a mailer, sealed in a vacuum sealed bag and stored in freezer overnight.

Antigen Retrieval (AR)

3 The purpose of antigen retrieval is to breakup the cross linking between the amino side chain of lysines and make the tissue more approachable for PNGase F. The preferred antigen retrieval buffer is the Citraconic acid buffer (pH3) in Drake's protocol. However, based on our experience, MIBI AR protocol and AmberGen AR protocol are all compatible with PNGase F digestion.

3.1 Prepare Citraconic buffer:

- 25 mL distilled water or HPLC grade water into a 50 mL falcon tube.

- Add 25 µL of Citraconic anhydride to the water.

- Add 2 µL of 12 M HCl.

- Agitate tube after capping.

- Add water to a total of 50 mL.

- Agitate tube to mix.

- Check that pH is around 3.0 ±0.5 by spotting 2 µL of the prepared buffer onto a pH strip.

12M (left) and citraconic anhyride were pipetted out (with disposable glass pipet) into 4 mL vails, which are located in the acidic flammable cabinet (R139). 4 mL containers were sealed with parafilm. Users will pipet (plastic) from the 4mL vails so that the original citraconic anhydride bottle and the 12 M HCl (37% HCl) bottle will not be contaminated.

The original citraconic anhyride bottle (Aldrich, Cat# 125318-25G, CAS# 616-02-4) is in the MALDI cabinet under the bench. This bottle should be sealed with parafilm. Moisture will decompose the citraconic anhydride (colorless liquid) into citraconic acid (white crystal). A new bottle should be used if white crystal is observed inside the bottle or around the bottle opening area. Pleas do not use plastic pipet to take liquid from the original citraconic anhydride bottle. Use disposable glass pipet with pipet bulb or syringe with Luer solo (NO black rubber on tip, which will dissolve with organic solvent and cause contamination).

3.2 Heat slides in vegetable steamer:

- Add around ~25 mL of the antigen retrieval buffer to a 5 slide mailer (top opening). Place the mailer into a grey container with insert (filled half way with tap water). Fill the Vagetable steamer with tape water till full.

- Preheat the vegetable steamer for 10~15 minutes prior to retrieval.This step will warm up the veggies steamer, the grey water bath and the antigen retrieval buffer inside the mailer

- Place no more than 3 slides per 5 slide mailer. Slides should be placed with tissue facing outward to the solution in positions 1 and 5, and NOT facing the slide mailer walls. Position 3 may face either way.

- Snap close one corner of the mailer, so that water will not drip inside mailer. Do not completely close the mailer, so that pressure will not build inside.

- Place the mailer in the center of the vegetable steamer.

- Set heating for 30 minutes.

3.3 Cool the slides after antigen retrieval:

- Remove mailer and place in a tub with cool water from the faucet. Water should not go over the top of the mailer.

- Allow to cool for 5 minutes.

- Remove half the buffer from the mailer and replace with distilled water.

- Allow to cool 5 minutes on countertop.

- Repeat removal of half the buffer two more times, each with 5 minutes of cooling.

- Complete by rinsing in 100% distilled water.

- Dry the slides with Kimwipes (don't touch the tissue). And dry slides at least 5 min in a desiccator, then proceed to the PNGaseF digestion steps.

Application of PNGase F

4 This step is to let PNGase F cleave off the N-glycan from tissue on the solid phase. The cleaved off N-glycan will stay at its specific location and hence provide spacial information. Before the incubation step, if you messed up the spraying step, you can wash the slide with HPLC water, dry the slide and spray again. However, after the incubation step, when N-glycans got cleaved off the tissue, the tissue need to be dry. A drop of water will dissolve the N-glycans and compeletly kill the spacial information. After the PNGase F incubation step, if you took slide (vacuum sealed bag) from the freezer, let the vacuum bag warm up to room temperature first and then open the bag. Exposing a cold slide to atmosphere will lead to moisture condensation on the tissue.

4.1 Humidity Chamber Preparation:

- Obtain a 100 × 15 mm cell culture dish with lid.

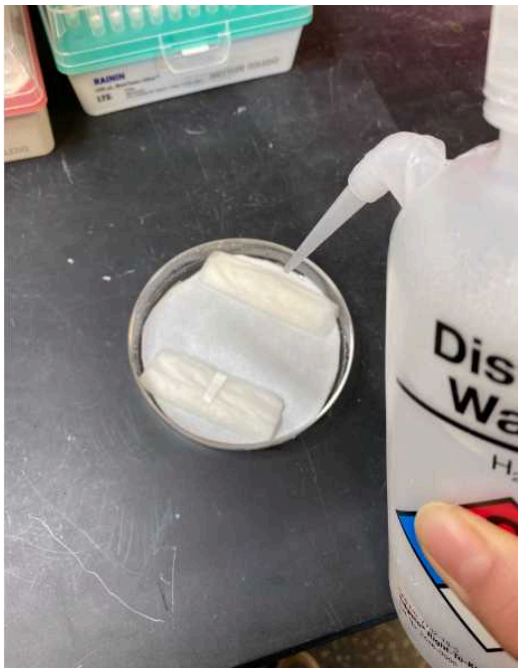
- Cut a Chromatography paper (Whatman, Cat# 3030-866) to fit to the bottom of the cell culture dish and place in bottom of dish.

- Take an 11×21 mm Kimwipe (Kimtech) and fold it in half along the creased line. Roll up the Kimwipe lengthwise forming a roll about 10 mm in diameter.

- Place the rolled Kimwipe on one end of the paper towel. You may need to squirt a little DI water on it to make sure it stays rolled.

- Place a second rolled Kimwipe at the other end.

- Saturate the paper towel and Kimwipes with water. Typically, this takes around 5 mL DI water. To perform this, simply hold the incubation dish at an angle and squirt DI water onto the chromatography paper and Kimwipes until water starts seeping out from under the towel, indicating saturation. Discard excess water that is not absorbed by the paper and Kimwipe.



Saturate the Chromatography paper and Kimwipe with water.

Discard the excess water.

- Pre-warm the incubation dish in a 38.5 ± 1.5°C oven at least half an hour. Light condensation should be observable on the lid of the dish. Don't stack up petri dishes inside an oven.

This is wrong. Do not stack the Petri dish as in this picture.

This is right.

4.2 Prepare PNGase F solution:

- Prepare 0.1 µg/µl PNGaseF in water using aliquots from the -20°C freezer. The original tube (contain 100 µg PNGaseF lyophilized powder) could only take 0.5 mL water. Pipet 0.5 mL water into the that original tube, transfer out to a 1.5 mL glass flask or eppendorf tube. Repeat for one more time.

- Ensure that enough solution is prepared, e.g, four slides takes approximately 1 mL of solution; for one slide, take 0.5 mL solution. Once you select the spraying area, HTX sprayer will give you an estimate. Please add 0.25 mL dead volume to that estimate volume.

Right: Original tube with PNGase F PRIME lyophilized power 100 µg.
Left: 1.5 mL glass vail.

- If you only spray one slide, you will only need 0.5 mL of the above prepared PNGase sln. Please pipet 0.5 mL PNGase sln from the glass vail back to the original tube. Write down a date and volume on the original tube, and place the tube back into MALDI freezer. This leftover PNGase sln. could be used for training purpose.

4.3 Prepare the HTX sprayer:

Bangelo lab is using HTX M3+ Sprayer, It is slightly different from HTX M5 sprayer used in Drake's protocol. But the spraying method and conditions are the same.

- Open the nitrogen gas from the nitrogen tank. Switch on the HTX sprayer. Then open the HTX sprayer software. Please follow this exact sequence. Adjust Nitrogen to ~5 psi by turning the nob on the sprayer.

To turn off the gas for sprayer, placed the T-shaped switch on parallel position towards the wall. The left meter will indicate 2 psi. DO NOT turn the black nob (vwr) and red nob (prostar)

To turn on the gas for sprayer, placed the T-shaped switch on perpendicular position. The left meter will indicate 20 psi. DO NOT turn the black nob (vwr) and red nob (prostar)

- Switch on the hood where the HTX sprayer locate.

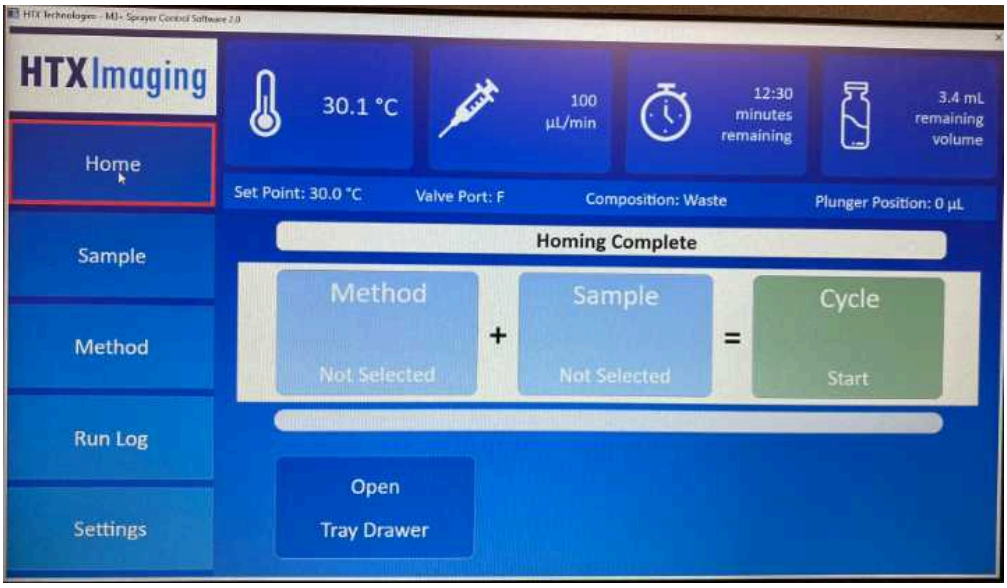
- Check the level of the four solvent bottle and make sure the tubing are inside the solvents. HPLC water need to be freshly filled within 7 days. If the solution level is low, refill with the prepared solutions. All the prepared solutions are on the shelf in R139. Please confirm the components on the label before the refill. Solutions in these four solvent bottle will be used for the washing steps. Refill the wrong solution may cause the blockage of the nozzle.

- If the stock solutions run out, please prepare more following the recipe on the label of the stock solution bottle. Do not use cylinder, directly measure with scale on stock solution bottle. If you have to measure, use the disposable 50 mL falcon flask.

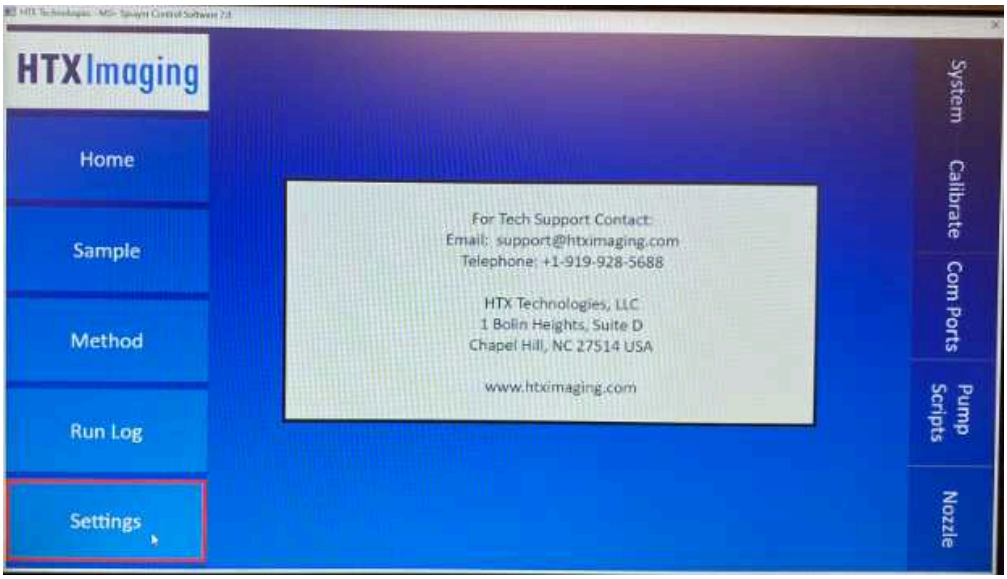
- Placed the waste solvent beaker under the waste solvent tube.

Four bottle with blue caps are the solvent bottles. Above the waste beaker is the waste tubing (light yellow).

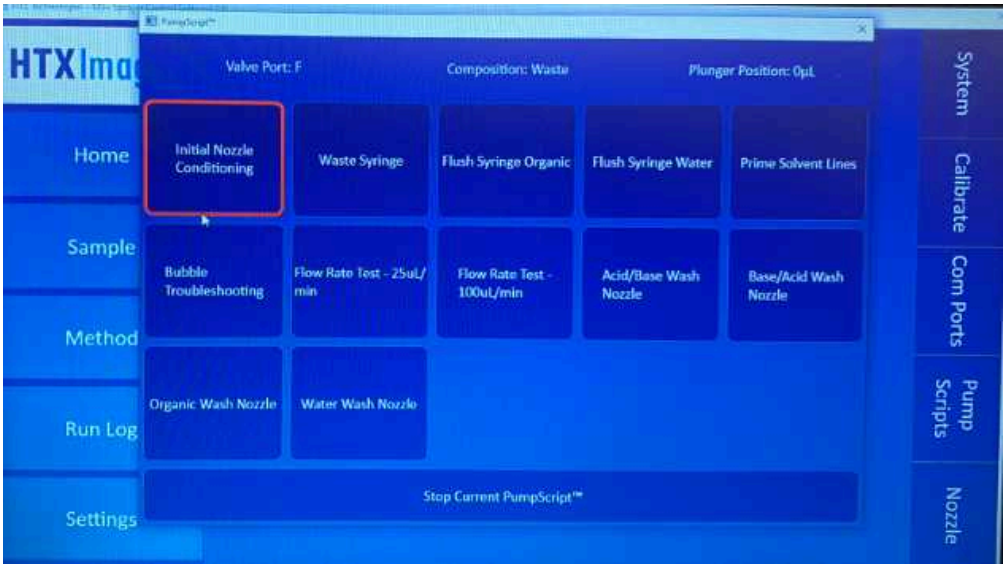
- On HTX sprayer software interface, click "setting" (on the bottom of the left column)-->click "pump scripts" → click through the buttons on the first line to completely clean up the sprayer system.



The is the Home page. "Setting" is on the left bottom.



This is after you click "setting". "Pump Script" is not he right column (the 2nd from the bottom).



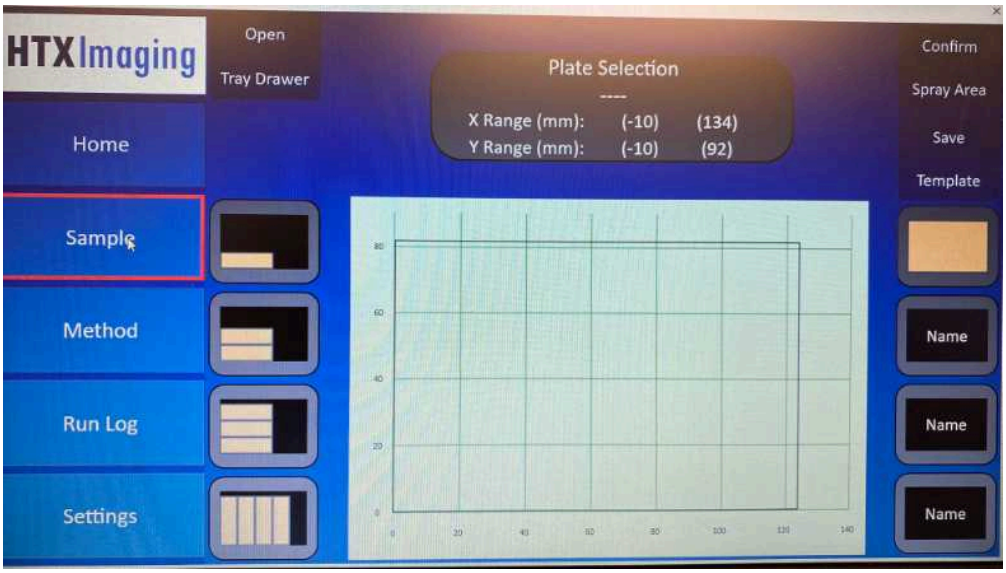
This is after you click "Pump Script". Click through the first row (initial nozzle conditioning, waste syringe, flush syringe organic, flush syringe water, prime solvent lines) one by one to completely clean up the system.

4.4 Spray a dummy slide with HPLC water:

- Place the tubing (labelled as "Enzyme") of port E into a Eppendorf tube with 1 mL HPLC water.

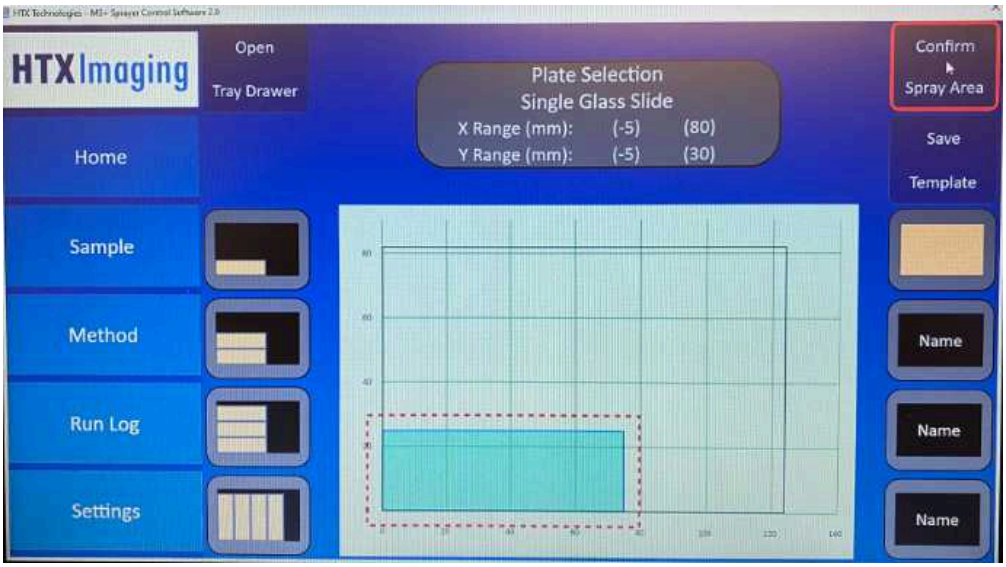
- On HTX sprayer software interface, click "method" (left column) → choose "PNGase_00" → double confirm the parameters: solvent (water), vial (enzyme), concentration (0.1 mg/mL), nozle temperature (45°C), Pressure (10 psi), flow rate (25 µL/min), velocity (1200), Track spacing (3 mm), Number of passes (15), Pattern (CC) , dry time (0), Prime script (Enzyme line), Flush Script (Water Flush), Wash Script (Acid/Bas wash). → Click "Select This Method".

- Click on "Sample" to get the window as in the following picture



- Click on "Open Tray Drawer" → tape the dummy slide into the sprayer. → click on "Close Tray drawer"

- Click on the 1~4 slide arrangement pictures below "open tray drawer" to get the red rectangle→ drag the red rectangle to define the spraying area. → click on "confirm area"



- Click on "Home", check whether the right method is chosen. click on the green "Cycle Start" button and follow the instructions on the HTX sprayer software.

- when the spray start, pay attention to two things:

1st, is there a consistent tail made from colorless fog on the slide? Does the length of the tail make sense? Too long the tail means liquid does not evaporate efficiently.

2nd, when the spraying finished, there should be no trace of solid on the dummy slide. The presence of solid on the dummy slide indicate the sprayer system need to be further cleaned.

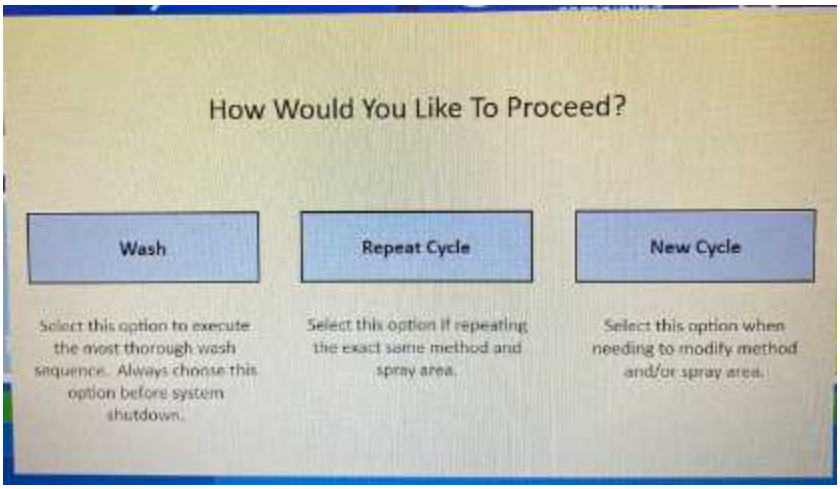
4.5 Spray PNGase F on tissue slide

- After dummy slide run finished, Place the tubing (labelled as "Enzyme") of port E into the container with PNGase F solution.

- Click on "Open Tray Drawer" → tape the tissue slide into the sprayer. → click on "Close Tray drawer"

- click on "repeat cycle", if you want to spray exact the same area as dummy slide; Then the spray will begin

- click on "new cycle", if you will change the spraying area.



- Click on "Sample" → drag the red rectangle to define the spraying area. → click on "confirm area"

- Click "start" and follow the instructions form the sprayer.

- When the run finished, take out your slides first, adjust the nitrogen pressure to 5 psi, and click "wash"

- Wait until the wash finished. Close the HTX sprayer software, switch off the sprayer and turn off the nitrogen flow from the nitrogen tank. Please follow this exact sequence.

- Pour the waste solvent from waste beaker to waste bottle and cap the bottle. Switch off the hood.

4.6 - Place tissue slide inside the incubation chamber (face up). Place the incubation chamber back to the oven and incubate for 2 hours.

- When removing, be aware that heavy condensation will have developed underneath the slide. Remove the slide slowly while holding it parallel with the countertop. Wipe off the condensation before rotating the slide.

- Store the slide in a mailer to protect the released glycans. If matrix cannot be sprayed the same day, sealed in a vacuum bag and store at -20°C. It is preferred to immediately spray matrix onto the slide.

Slide scanning

5 Optical image will be used for setting up a MALDI run with flexImaging software.

5.1 Mark fiducials at the four corners of each slide.

Fiducials are points of reference used when “teaching” the instrument software (flexImaging) the coordinates of the slide. Fiducials allow accurate targeting of tissue regions for imaging.

Use a reflective metallic marker to make a small circle at each corner. Use a black marker to draw a cross or hash mark on top of each silver circle; the reflective marker provides a contrasting background for easy visualization of the black fiducial mark by the instrument camera.

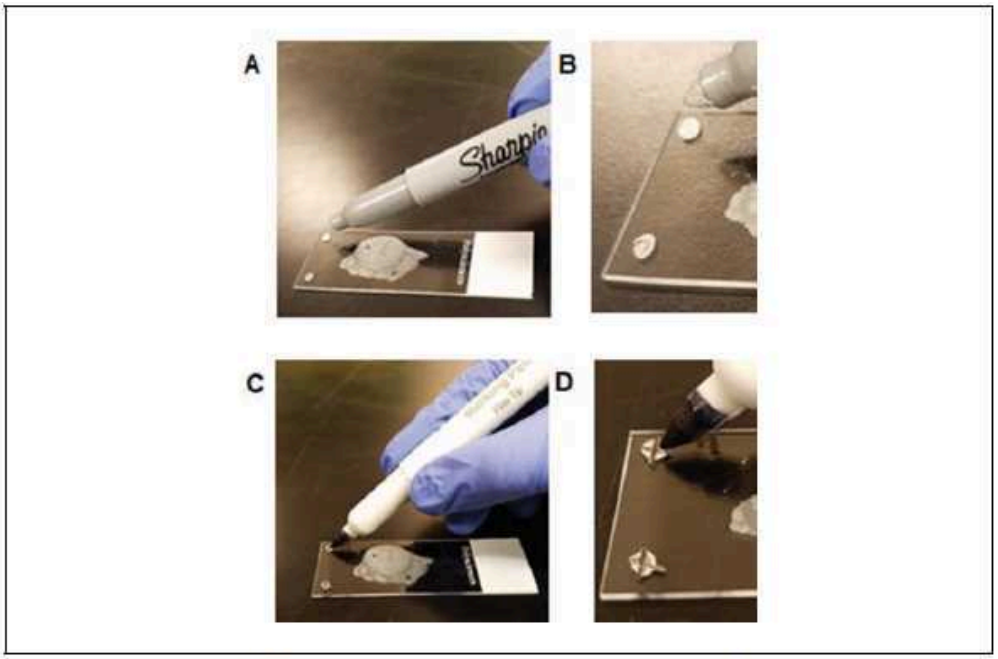
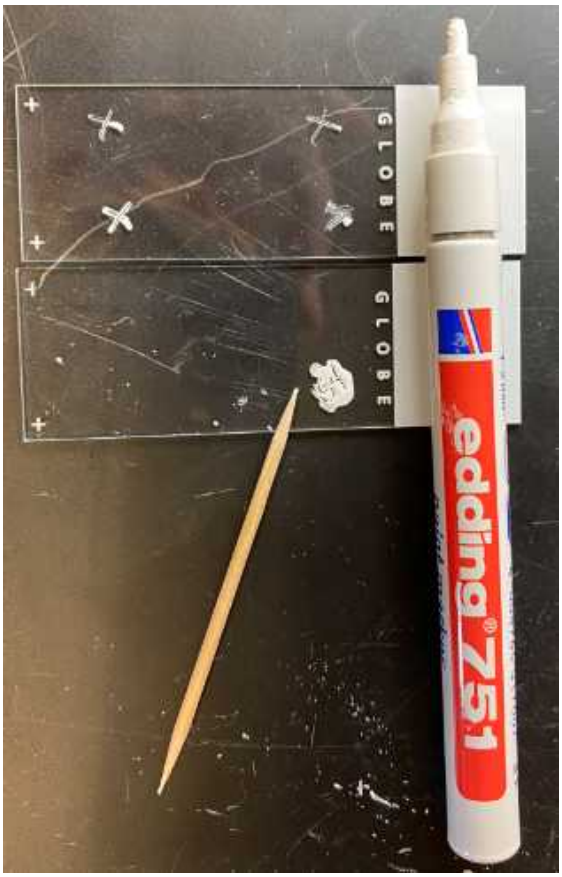


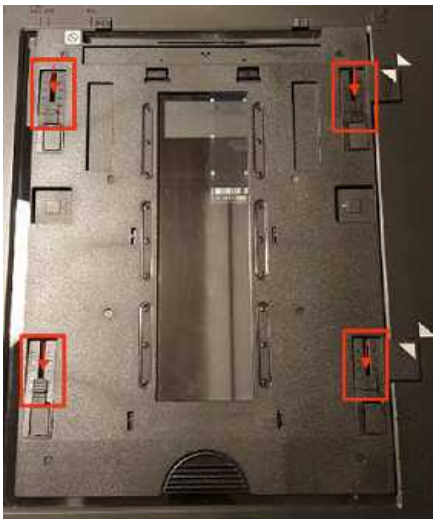
Figure 2 Marking of fiducials for imaging mass spectrometry. (**A**) A silver Sharpie marker is used to create a silver spot as a background for the fiducial. This allows a reflective background for detection by camera optics. (**B**) Close-up zoom on silver spot. (**C**) A black fine-tipped permanent marker is used to create a cross or hash on top of the silver spot. The edges of this mark are used to “teach” the instrument coordinates for the tissue. (**D**) Close-up zoom of the marked fiducial.

Alternatively, you can use edding 751 paint for drawing Fiducials. Squeeze out some paint on dummy slide. Use toothpick to pick up the paint and draw crosses as in the following picture.



5.2 Scan the slide following Bruker Scanner manual

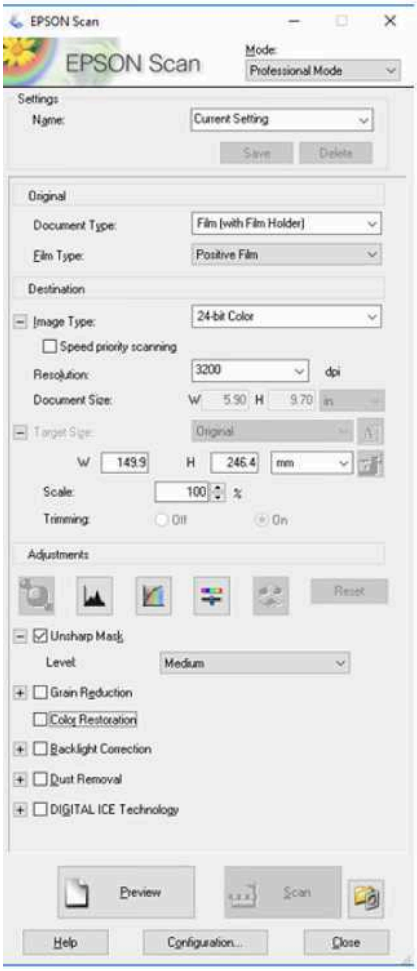
- placed the slide (face up) on the film of the scanner (connect to processing computer#1). Adjust the level of the film with the four buttons (red square).Close the scanner lid.



- Switch on the scanner

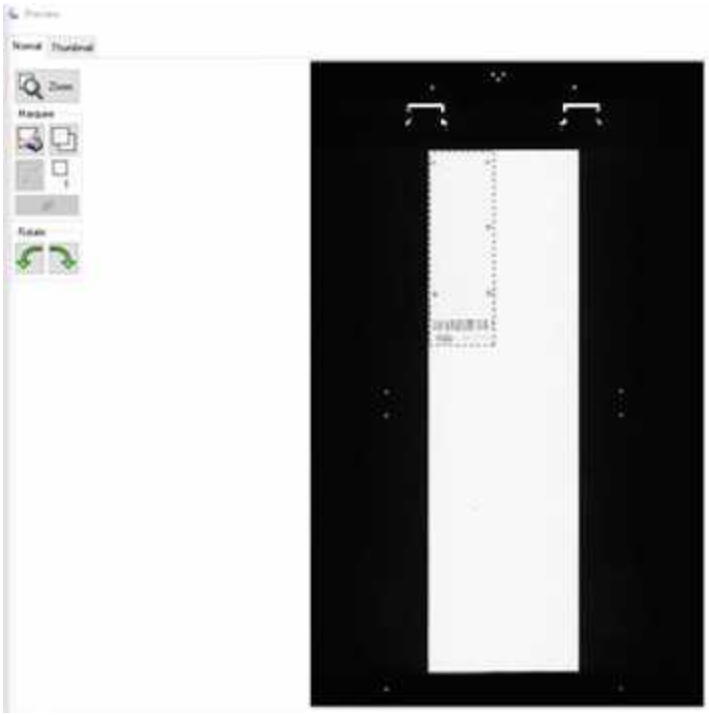
- open the scanner software

- In the EPSON Scan software, make sure you have applied the following settings:
Mode: Professional Mode
Document type: Film (with Film Holder)
Image Type: 24-bit Color
Resolution: 3200 dpi
Scale: 100%



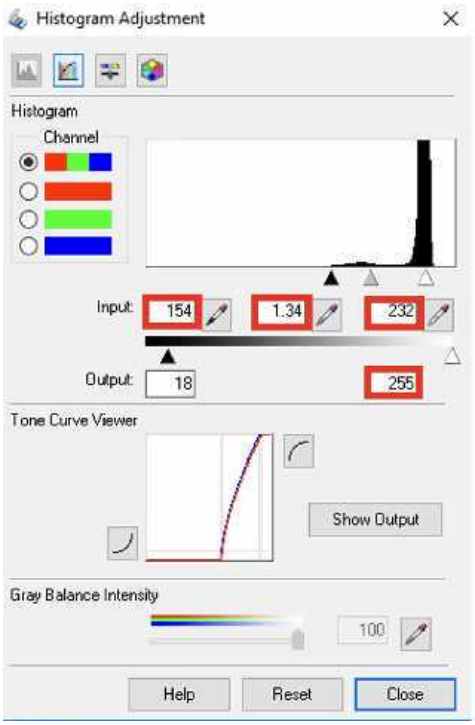
- Click the “Preview” button on the bottom left

- Hold down the left mouse button and drag over the slide in the Preview window. And make a dotted line box will form around the low-resolution scan of your slide. This dotted box defines the region of interest (ROI) for the high-resolution scan.

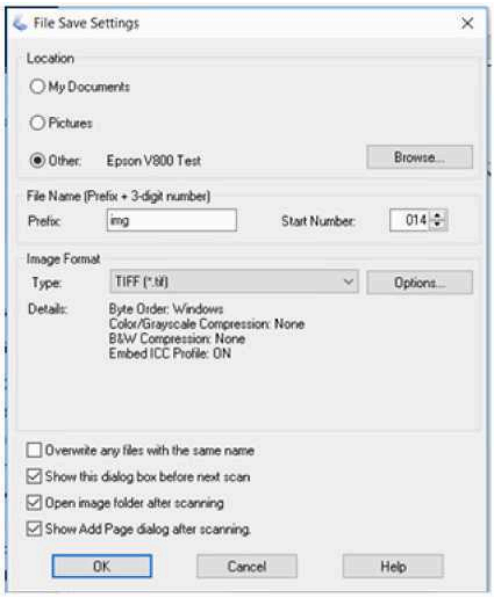


- Click on "Histogram Adjustment"

- Adjust the numbers in red rectangle box until you can see the tissue and fiducials clearly. Then click "done".



- Click the "Scan" button and A file save setting dialog box opens. Save the file on local D drive of processing computer #1: (D:)>Scan_processing computer



- Open image file with "Paint" → flip and rotate the image so that it will be the same orientation as it is in the MALDI slide holder. →Save the edited image.

Application of Matrix

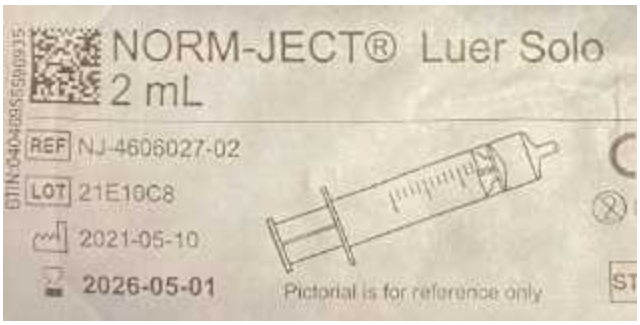
6

6.1 Prepare the CHCA matrix:

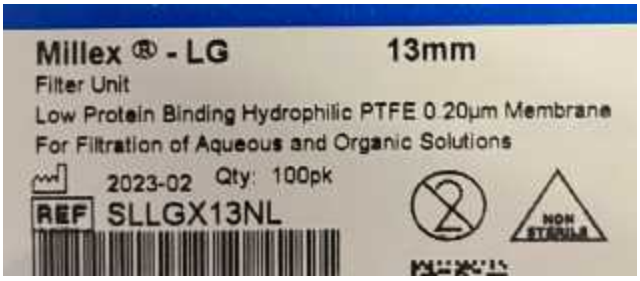
- Prepare CHCA matrix (sealed with para film and stored in freezer) at 7 mg/mL in 50% acetonitrile/0.1% TFA. Add 0.042g CHCA to 6ml 50% acetonitrile/0.1% TFA. Prepare fresh each time in a 50 mL falcon tube. 6 mL CHCA solution is enough for 4 slides.

- Vortex violently (5~10 min) until most solid dissolve.

- After vortex, small chunks may remain in the bottom of the falcon tube, which will clog the components of the TMsprayer. Filter CHCA solution using Millex (Millipore) 0.2µm PTFE syringe filter will completely remove the solid. Make sure to cap the filtered solution. As the acetonitrile evaporate, crystal may appear again. If this happened, filtration need to be performed again.



Luer Solo syringe is compatible with organic solvent. There is NO rubber septum inside the syringe, which will dissolve in organic solvent and cause contamination.



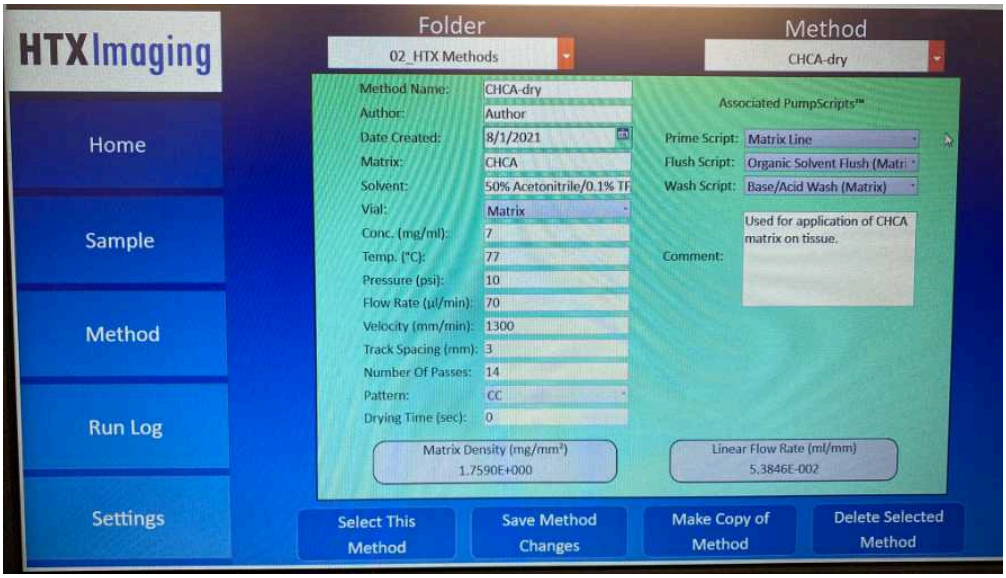
0.2µm PTFE syringe filter. PTFE membrane is compatible with organic solvent. While PVDF membrane is not.

6.2 Prepare the HTX spray the same way as in section 4.3. Skip the "pump scrips" step if you already did it during the PNGase spray, and nobody spray any matrix in between your PNGase spray and CHCA spray.

6.3 Spray a dummy slide with CHCA solution

- Place the tubing (labelled as "Matrix") of port D into the matrix solution.

- On HTX sprayer software interface, click "Method" (left column) → choose "CHCA_dry" → confirm the parameters: solvent (50%ACN, 0.1%TFA), concentration (7 mg/mL), nozzle temperature (77°C), pressure (10 psi), Track space (3 mm), flow rate (70 µL/min), velocity (1300), number of passes (14), , dry time (0), pattern (cc), Prime Script (Matrix Line), Flush Script (Organic solvent Flush), Wash Script (Base/Acid Wash(Matrix)) → Click on "Select This Method".



- Click on "Open Tray Drawer" → tape the dummy slide into the sprayer. → click on "Close Tray drawer"

- Click on "Sample" → drag the red rectangle to define the spraying area. → click on "Confirm Spray Area"

- Click on "Home", check whether the right method is chosen. click on the green "Cycle Start" button and follow the instructions on the HTX sprayer software.

- when the spray start, pay attention to two things:

1st, is there a consistent tail made from light yellow solid on the slide? Does the length of the tail make sense? Too long a tail means solvent does not evaporated efficiently.

2nd, when the spraying finished, there should be a fine layer of yellow solid on the slide. Pay attention to the thickness of the yellow and the particle size of the matrix. Compare with lab used slides (previously prepared, went through N-glycan IMS acquisition and gave decent Images) to evaluate the quality of your CHCA spray.

6.4 Spray CHCA on tissue slide

- After dummy slide run finished, Click on "Open Tray Drawer" → tape the tissue slide into the sprayer. → click on "Close Tray drawer"

- click on "repeat cycle", if you want to spray exact the same area as dummy slide; Then the spray will begin

- click on "new cycle", if you will change the spraying area.

- Click on "Sample" → Click on the 1~4 slide arrangement pictures to get the red rectangle→ drag the red rectangle to define the spraying area. → click on "confirm spray area"

- Click "start" and follow the instructions form the sprayer.

- When the run finished, take out your slides first, adjust the nitrogen pressure to 5 psi, and click "wash"

- Wait until the washing finished. Close the HTX sprayer software, switch off the sprayer and turn off the nitrogen flow from the nitrogen tank. Please follow this exact sequence.

- Pour the waste solvent from waste beaker to waste bottle and cap the bottle. Switch off the hood.

Storage of prepared Slide

7 Prepared slides could be placed in mailer, sealed in a vacuum seal bag and store at -20 °C for up to a month before MALDI acquisition.

Protocol references

Drake, R. R., Powers, T. W., Norris-Caneda, K., Mehta, A. S., & Angel, P. M. (2018). In situ imaging of N-glycans by MALDI
imaging mass spectrometry of fresh or formalin-fixed paraffin-embedded tissue. Current Protocols in Protein Science, 94,
e68. doi: 10.1002/cpps.68