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MALDI Imaging Mass Spectrometry of N-glycans and Tryptic Peptides from the Same Formalin-Fixed, Paraffin-Embedded Tissue Section

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a unique and well developed tool for probing the protein content of formalin-fixed, paraffin-embedded tissue (FFPE). Integral to this approach is the application of trypsin, and more recently peptide N-glycosidase F, to release tryptic peptides or N-glycans from tissue and report localization of distinct species. This is typically done on serial or adjacent tissue sections, and there is an emerging need to understand the colocalized protein population linked to the exact same regions of N-glycans. Here we describe an approach where N-glycans are first imaged from a tissue section followed by reprocessing of the same tissue section for tryptic peptide MALDI IMS. Strategies for colocalizing peptides to target N-glycans or N-glycan regions are described.

Keywords

Formalin-fixed; Imaging mass spectrometry; MALDI imaging mass spectrometry; Paraffin-embedded tissue imaging; Peptide identification for imaging mass spectrometry; Peptide N-glycosidase F; Proteomics; Tryptic peptide imaging

1 Introduction

MALDI IMS enables high throughput reporting of hundreds to thousands of molecules localized within a single thin tissue section, allowing new insight into many disease processes that are impossible to understand by conventional microscopy [1–3]. Robust workflows using enzymatic approaches are used to access the highly cross-linked proteins of FFPE tissues, allowing the study of stored clinical tissues [4–6]. Spraying a thin molecular layer of trypsin onto tissue sections allows imaging of trypsin produced peptides analogous to solution proteomic workflows, with additional information on localization of the proteomes within the tissue architecture [7]. Robust MALDI IMS workflows using the enzyme PNGase F to release N-glycans from thin tissues sections have been developed [6] and are now routinely used to examine distribution of N-glycosylation related to the pathology of disease [8–10].

There is intense interest in combining the imaging workflows for proteomics and glycomics to understand the protein populations undergoing specific glycosylation changes. Combined

workflows working on tissue sections in solution have reported the sequential glycan and proteome composition of single FFPE tissue sections [11]. Here, we describe the process of preparing the same FFPE tissue section for imaging of first N-glycans followed by tryptic peptides from the same tissue section. A similar technique was recently reported to obtain N-glycan and peptide expression from the same tissue section with antigen retrieval performed after application of PNGase F and before trypsin application [12]. The current protocol details a strategy using a single antigen retrieval step prior to PNGase F application, followed by the release and MALDI IMS of N-glycans, specific washing steps to remove all matrix, PNGase F protein, and N-glycans from the tissue section, followed by tryptic digestion and MALDI IMS of tryptic peptides. The imaging data reported here was produced using a MALDI Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. In the workflow, the accurate mass for N-glycans are known from databases created by past studies [13–15]. This protocol allows discovery of co-localized N-glycans and tryptic peptides toward a more complete understanding of the tissue microenvironment.

2 Materials

Prepare all solutions in HPLC grade water. Follow all safety and waste disposal regulations.

2.1 MALDI Imaging Mass Spectrometry Solutions

1. Antigen retrieval solution: Pour 50 mL water into a clean 50–100 mL bottle. Add 50 μ L of citraconic acid anhydride buffer to the water. Add 4 μ L of 12 M HCl and mix. Add water to the bottle for a total of 100 mL and mix. Check that pH is around 3.0 ± 0.5 (*see Note 1*). Use the same day (*see Note 2*).
2. Preparation of 95% ethanol. Add 950 mL 200 proof ethanol to a clean bottle. Add 50 mL water and mix.
3. Preparation of 70% ethanol: Add 700 mL 200 proof ethanol to a clean bottle. Add 300 mL water and mix.
4. 25% Trifluoroacetic acid (TFA): Add 3 mL water to a clean bottle. Carefully add 1 mL of trifluoroacetic acid to the water and mix.
5. Matrix solvent [50% Acetonitrile (ACN), 0.1% TFA]: Add 25 mL water to a clean 100 mL bottle. Add 400 microliters of 25% TFA (Solution #4) to the water and mix. Add 50 mL of acetonitrile and mix. Add water to a final volume of 100 mL and mix. Store for up to 2 months at room temperature.
6. MALDI matrix for N-glycan or peptide imaging (Alpha-cyano-4-hydroxycinnamic acid (CHCA), 7 mg/mL in 50% acetonitrile/0.1% TFA): Weigh out 0.0420 ± 0.001 g CHCA. Add the solid CHCA to a clean 50 mL falcon tube. Bring to volume with 6 mL of matrix solvent. Vortex briefly and sonicate 5 min using a benchtop sonicator. Filter CHCA solution using a 13 mm 0.2 μ m PTFE hydrophilic syringe filter graded for use with HPLC solvents (*see Note 3*).

¹Ensure that the solution is mixed well. The citraconic acid anhydride solution should not need additional pH adjustment.

²This volume is enough for filling two slide mailers for antigen retrieval and for tissue clearing of N-glycans.

³Use of the filter significantly reduces clogging of the solvent lines of the M3 TM-Sprayer™.

2.2 Tissue Clearing Solvents

1. High pH Tissue Clearing Solution (10 mM Tris, pH 9). Add 500 mL water to a clean 1000 mL bottle. Add 1.21 ± 0.12 g Tris base and mix. Add water to 900 mL and adjust pH to 9.0 ± 0.03 using 1 M HCl. Add water to a total volume of 1000 mL.
2. Low pH Solution. Pour 50 mL water into a clean 50–100 mL bottle. Add 50 μ L of citraconic acid anhydride buffer to the water. Add 4 μ L of 12 M HCl and mix. Add water to the bottle for a total of 100 mL and mix. Check that pH is around 3.0 ± 0.5 (*see* Note 1). Use the same day (*see* Note 2). This is the same solution as the antigen retrieval solution.

2.3 Enzyme Solutions

1. Ammonium bicarbonate solution (25 mM, pH 7.5): Add 50 mL water into a clean 100 mL bottle. Weigh out 0.1976 ± 0.02 g of ammonium bicarbonate and add to water, agitate to mix. Add water to the bottle for a total of 100 mL and mix. The pH should be 7.5 ± 0.5 . Store at 4 °C for use up to 2 weeks.
2. Preparation of 1 mL of 0.1 μ g/ μ L PNGase F solution. To 100 μ g of PNGase F add 1000 μ L of prepared 25 mM ammonium bicarbonate and mix (*see* Note 4). Use same day. This prepares enough enzyme to cover four microscope slides.
3. Preparation of 1 mL of 0.1 μ g/ μ L trypsin solution. To 100 μ g of trypsin add 1000 μ L of prepared 25 mM ammonium bicarbonate and mix (*see* Note 5). Use same day. This prepares enough enzyme to cover four microscope slides.

2.4 TM-Sprayer™ solutions

1. Push solvent (50% methanol–water): Add 500 mL methanol to a clean bottle. Add 500 mL water and mix. Solvent may be kept at room temperature during the duration of use (*see* Note 6).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Heating and Dewaxing

1. Incubate the slides with tissue face up in a 60 °C oven for 1 h.
2. Prepare Coplin jars of solvent for dewaxing by pouring the following solutions into Coplin jars: Xylenes, two Coplin jars; 200 proof ethanol, USP grade, two Coplin jars; 95% ethanol, one Coplin jar; 70% ethanol, one Coplin jar; double distilled water, two Coplin jars. Solvent should be added to a level that will allow complete immersion of the tissue sections.

⁴For some highly active recombinant PNGase F preparations, using water instead of ammonium bicarbonate buffer allows equal efficiency in digestion.

⁵One batch allows spraying of four slides, the maximum capacity for one batch of samples on the M3 TM-Sprayer™.

⁶When refilling the push solvent, ensure the bottle is thoroughly cleaned out before adding new solvent to prevent bacterial growth.

3. Dewax the slides in the following order with the specified times: xylenes, two times at 2 min each; 200 proof ethanol, two times at 1 min each; 95% ethanol solution, one time at 1 min; 70% ethanol solution, one time at 1 min; HPLC grade water, two times at 3 min each. For each step, immerse the slides completely in freshly poured solution for the stated length of time. At the end of the incubation time, agitate the slides briefly as they are removed from the Coplin jars.
4. Dry the slides in a vacuum desiccator for 5 min.

3.2 Slide Scanning

1. This step produces an optical image needed for selecting tissue regions for imaging analysis. Fiducials, or reference points, are needed to accurately “teach” the instrument where the tissue is located on the slide.
2. Use a reflective metallic marker to make a small circle at each corner of the microscope slide. 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 clear visualization of the black mark to use as a fiducial.
3. For images that will be acquired by mass spectrometry at 100 μm spatial resolution, scan the whole slide at a minimum of 1200 ppi resolution. For images that will be acquired with a 50 μm stepsize scan the slide at a minimum of 2400 ppi resolution. Save the images as JPEG, bitmap (*.bmp) or 8 bit TIFF formats.
4. After scanning and prior to antigen retrieval, slides may be stored overnight in a desiccator. For longer times over 2 days, store the slides at -20 to -80 °C. It is preferable to proceed with the next step immediately.

3.3 Antigen Retrieval

1. This step details antigen retrieval for imaging mass spectrometry using a vegetable steamer. A rice cooker or decloaker may also be used for antigen retrieval.
2. Fill a vegetable steamer to the marked water level and preheat for 5 min prior to antigen retrieval (*see Note 7*).
3. Add around ~10 mL of the antigen retrieval solution to a plastic five slide mailer with side opening.
4. Place three slides into each five slide mailer with side opening. Slides in positions 1 and 5 are placed with tissue facing outward to the solution. Position 3 may face either way. This allows good solvent access to the tissue.
5. Fill the slide mailer the rest of the way with the antigen retrieval solution so that all tissue is completely covered.

⁷In our laboratory, the vegetable steamer used in antigen retrieval is a Rival Model CKRVSTLM21.

6. Steam must be released from the slide mailer to prevent rupture during antigen retrieval. A hole may be punched into the mailer lid using an 18-gauge needle. If the mailer has no holes punched in the lid, snap closed only one corner of the mailer. This allows steam to exit. Place the mailer in the center of the vegetable steamer.
7. Heat for 30 min. Temperature should reach 95 °C for a minimum of 20 min (*see* Note 8).
8. Place the hot mailer in a container holding cool water (*see* Note 9). Cooling water should come midway up the side of the mailer. Cool for 5 min in the water bath.
9. Remove half the buffer from the mailer and replace with distilled water. Cool on the countertop for 5 min. Repeat removal of half the buffer two more times, each with 5 min of cooling. Complete by rinsing in 100% distilled water.
10. Dry the slides for 5 min in a desiccator.

3.4 PNGase F Application by the M3 TM-Sprayer™

1. A syringe pump with 0.05% accuracy in pumping 25 µL/min is used for enzyme application. A glass or plastic 1-mL syringe with Luer lock is used for loading enzyme into the sprayer.
2. Fill a glass or plastic 1-mL syringe with Luer lock syringe with prepared PNGase F solution ensuring that there are no bubbles in syringe (*see* Note 10). Fasten the syringe to the TM-Sprayer™ line used for enzyme spraying. Secure the syringe in the pump.
3. Set the pump to a flow rate of 25 µL/min with an inner diameter matching that of the used syringe. Do not turn on the pump at this time.
4. Place the microscope slides with tissue samples on the TM-Sprayer™ sample area, fastening them with lab tape.
5. Turn on the TM-Sprayer™ and then the controlling computer.
6. Open the nitrogen gas tank valve, setting the regulator to 10 psi.
7. In the TM-Sprayer™ software, set the temperature to 45 °C. Temperature will not adjust without the nitrogen gas flowing.
8. Program the TM-Sprayer™ to cover the appropriate number of slides, allowing a 5 mm additional edge distance for sprayhead turn round (*see* Note 11).

⁸Certain tissues with high fat content or those with open areas such as normal skin, breast and lung may detach during lengthy antigen retrieval. To limit loss of tissue from the slide, reduce antigen retrieval time to 20 min.

⁹Use hot pads or heat resistant gloves to remove the mailer. It is full of near boiling solution and could be a burn hazard to skin and eyes.

¹⁰To eliminate bubbles within the syringe barrel, and after loading all the PNGase F solution required, pull a small volume of air into the syringe. Gently dispense the syringe until the air bubble is gone.

¹¹Excess solutions will be deposited at the point of the sprayhead turn around location. Ensuring the turnaround point is located off the tissue prevents delocalization and excess matrix build up.

9. Program the TM-Sprayer™ method for PNGase F to use 15 passes, crisscross pattern, velocity of 1200, 3.0 mm track spacing, and a dry time of zero. The tip of the sprayhead should be 40 mm distance from the surface of the slide.
10. Start the syringe pump. Place a blank microscope slide under the nozzle of the spray head to check the TM-Sprayer™ to monitor the start of enzyme solution spraying. It generally takes about 1–3 min to start emitting solution.
11. Once moisture is detected on the blank slide, press “Start” in the TM-Sprayer™ software.
12. PNGase F solution will be applied in a thin layer onto target tissues. During application of the enzyme, moisture will be observed on the slide as the sprayhead passes over an area; this should dry within 30 s to maintain localization of released enzymes.

3.5 Incubation for On-Tissue Digestion

1. The same incubation approach is used for both PNGase F and trypsin digestion and is performed immediately after application of PNGase F or trypsin.
2. Prepare an incubation chamber for digestion using a plastic 100 × 15 mm cell culture dish. Fit a single layer paper towel (Wypall 60×) on the bottom of the dish. Fold two 4 × 6 Kimwipes and place at opposite sides of the dish. Using a spray bottle of water, add water to saturate the papertowels and Kimwipes. Stop adding water when excess water accumulates in the dish, observed by tilting the dish to one side. For the specified towels and Kimwipes, this is about 5 mL of water.
3. The incubation chamber should be preheated for 15–30 min in an oven at 37.5 °C and should show a thin layer of condensation on the top of the incubation dish before placing the tissue inside. The incubation dish is heated with lid in place; no lab tape or other sealant is required.
4. Place the slide with the tissue facing upward into the incubation chamber using the Kimwipes as supports. Gently push the slide down slightly so that when the cover is placed on, the tissue does not touch the incubation chamber cover.
5. Incubate 2 h (*see* Note 12) in the oven set at 37.5 ± 1.5 °C (*see* Note 13).
6. After incubation, remove the slide slowly while holding it parallel with the countertop. Wipe off the condensation to prevent liquid rolling onto the tissue surface and delocalizing N-glycans (or peptides).
7. Store the slide in a five slide mailer to protect the released N-glycans or peptides. If matrix cannot be sprayed the same day, store briefly in a desiccator (6–12 h) or at –20 °C for long term (2–3 days).

¹²Fibrous tissues such as breast, skin, and fibrotic liver, lung or heart valve may require longer digestion times.

¹³Frequently digital readouts on small ovens do not accurately report internal temperature. Ensure that the internal oven temperature is at the correct temperature using a secondary thermometer placed in the oven. This is essential for correct digestion.

8. It is recommended to immediately spray matrix onto the slide.

3.6 MALDI Matrix Application by the M3 TM-Sprayer™

1. MALDI matrix application is performed after PNGase F digestion and again after trypsin digestion.
2. An isocratic pump with 0.05% accuracy in pumping 100 µL/min is used to for matrix application. A glass 5-mL syringe with Luer lock for loading matrix into the sprayer.
3. Ensure that the isocratic pump is set to pump 100 µL/min (*see* Note 14). Solvent may be degassed to limit flow variation.
4. Turn on the TM-Sprayer™ and controlling computer.
5. Open the nitrogen gas tank, setting the regulator to 10 psi.
6. In the TM-Sprayer™ software, set the temperature to 80 °C. Temperature will not adjust without the nitrogen gas flowing.
7. Program the TM-Sprayer™ method for CHCA matrix application to use eight passes, crisscross pattern, velocity of 1300, 2.5 mm track spacing and zero dry time. The tip of the sprayhead should be 40 mm distance from the surface of the slide.
8. Place the samples on the TM-Sprayer™ platform, fastening them with lab tape.
9. Program the TM-Sprayer™ to cover the appropriate number of slides, allowing a 5 mm additional edge distance for sprayhead turn round (*see* Note 11).
10. Fill a glass 5-mL syringe with the filtered MALDI matrix solution, ensuring that there are no bubbles in the syringe.
11. Fasten the syringe to the TM-Sprayer™ line going to the 6-port valve. With the valve switch in “Load” position, inject the MALDI matrix solution into the 5 mL loop.
12. Ensure that the pump is flowing at 100 µL/min and that appropriate pump pressure readouts are stable.
13. Move the 6-port valve switch to “Spray.”
14. Use a blank microscope slide to check the TM-Sprayer™ nozzle for spraying of solution. Once matrix is detected as an opaque film on the dummy slide, press “Start” in the TM-Sprayer™ software.
15. CHCA solution will be applied in a thin layer onto target tissues.
16. When finished, matrix coated slides may be imaged immediately by MALDI imaging mass spectrometry or stored in a desiccator until imaging experiments.

¹⁴Solvent should be degassed to limit flow variation.

17. Figure 1 shows a typical overall average spectrum and example images from the workflow.

3.7 Tissue Clearing of Matrix and Residual N-glycans

1. The purpose of tissue clearing is to remove matrix, PNGase F and N-glycans prior to trypsin digestion.
2. Remove matrix by immerse the slide in 200 proof ethanol for 1 min.
3. Immerse the slide in 95% ethanol solution for 1 min followed by 70% ethanol for 1 min.
4. Remove hydrophilic N-glycans by incubating the slide in water for 1 min, High pH Tissue Clearing Solution for 1 min, water for 1 min, Low pH Tissue Clearing Solution (citraconic buffer) 1 min, and water 1 min. For each step, agitate the slides 3–5 times at the end of the incubation.
5. Wipe excess water off the back of the slide and dry for 5 min in desiccator.

3.8 Trypsin Application by the M3 TM-Sprayer™

1. A syringe pump with 0.05% accuracy in pumping 25 µL/min is used to for enzyme application. A glass or plastic 1-mL syringe with Luer lock for loading enzyme into the sprayer.
2. Fill a 1 mL glass or plastic 1-mL Luer lock syringe with prepared trypsin solution ensuring that there are no bubbles in the syringe (*see* Note 15).
3. Secure the syringe in the pump. Set the pump to a flow rate of 30 µL/min with an inner diameter matching that of the used syringe. Do not turn on the pump at this time.
4. Place the microscope slides with tissue samples on the TM-Sprayer™ platform, fastening them with lab tape.
5. Turn on the TM-Sprayer™ and controlling computer.
6. Open the nitrogen gas tank valve, setting the regulator to 10 psi.
7. In the TM-Sprayer™ software, set the temperature to 45 °C. Temperature will not adjust without the nitrogen gas flowing.
8. Program the TM-Sprayer™ to cover the appropriate number of slides, allowing a 5 mm additional edge distance for sprayhead turn round (*see* Note 11).
9. Program the TM-Sprayer™ method for trypsin to use 8 passes, crisscross pattern, velocity of 1200, 3.0 mm track spacing, a zero dry time. The sprayhead should be 40 mm from the tip of the sprayhead to the top of the slide surface.
10. Start the syringe pump.

¹⁵Use sequencing grade modified trypsin to ensure high quality peptide for identification by parallel proteomics workflow after earmarking which peptides are co-localized with N-glycans.

11. Place a blank microscope slide under the nozzle of the spray head to check the TM-Sprayer™ to monitor the start of enzyme solution spraying. It generally takes about 1–3 min to start emitting solution.
12. Once moisture is detected on the blank slide, press “Start” in the TM-Sprayer™ software.
13. Trypsin solution will be applied in a thin layer onto target tissues.
14. Perform trypsin digestion following Sect. 3.5, Incubation for On-Tissue Digestion.
15. After On-Tissue Digestion, complete Sect. 3.6, MALDI Matrix Application by the M3 TM-Sprayer™.
16. Upon completion, matrix coated slides may be imaged immediately by MALDI imaging mass spectrometry or stored in a desiccator until imaging experiments are completed (*see* Note 16).
17. Figure 2 shows a typical overall average spectrum and example images from the workflow.
18. Co-localized N-glycans and tryptic peptides may be identified by manual searches comparing the data patterns or using the vendor neutral SCiLS Lab software (Bruker).

3.9 Identifying Co-localization of Tryptic Peptides with a Target N-glycan

1. Co-localization of a target N-glycan with tryptic peptides may be calculated using the vendor neutral SCiLS software (Bruker).
2. Load the both the N-glycan and trypsin image data into a single instance of SCiLS using the data import wizard; this procedure differs based on instrument type.
3. Load a database of singly charged N-glycan masses using the clipboard function. Go to File → Import → m/z intervals from CVS or clipboard (*see* Note 17). After naming the list, click Save.
4. Generate image data for the N-glycans by selecting the N-glycan database list in the “m/z intervals” pulldown menu. Click the small left arrow beside the menu name. Click the “eye” icon on the far right to open the m/z Image Generator. Click OK to generate images.
5. View images using the “m/z Images” pulldown menu. Click the small arrow to the left of the menu name. A list of m/z will display. Selecting the “eye” icon to the left of the m/z will generate a heat map of the m/z pattern on the tissue. Identify an N-glycan m/z of interest for computing colocalized peptides.

¹⁶Slides may be stored in a desiccator for up to 3 months until peptide fragmentation may be completed.

¹⁷N-glycans are observed in imaging data using the described preparation as the intact precursor plus one or two sodium adducts.

6. On the menu at the top of the software, select Tools → Find Co-Localized m/z Values. Ensure that an appropriate normalization method is selected, and check the box next to Regions. In the Mode menu, select “Correlate with m/z Image.” Type in the mass of the N-glycan exactly as found in the imported database list and using the correct interval width. Next to “m/z Intervals”, select the regional peak list generated for the image generated by the trypsin workflow. These are all possible peptides that may correlate to the selected N-glycan. Save as an informative name and click “OK.”
7. Once the data has calculated, a new peak list will appear under the “m/z Intervals” pulldown menu. Click the small left arrow beside the menu name. Click the “eye” icon to the far right of the menu window. A Threshold bar is displayed with a Correlation Value. Clicking in the bar raises or lowers the correlation value and reports the number of intervals associated with the correlation value. Correlation values of greater than 0.6 should be inspected for co-localization with the target peak. Once a correlation value is selected, Click the “eye” icon on the far right to open the m/z Image Generator. Click OK to generate images.
8. Figure 3 demonstrates an image data result from the procedure.

3.10 Identifying Colocalized Tryptic Peptides and N-glycans Based on Tissue Region

1. Co-localization of N-glycans with tryptic peptides by region on tissue (e.g., tumor) may be calculated using the vendor neutral SCiLS software (Bruker).
2. Load the both the N-glycan and trypsin image data into a single instance of SCiLS using the data import wizard; this procedure differs based on instrument type.
3. Create a Combined Peaklist with defined N-glycans and a SCiLS created Tryptic Peptide Peaklist using a text editor. Add the database of N-glycan mass-to-charges (*see* Note 17) to a new instance of a text editor. In SCiLS, collect the Tryptic Peptide Peaklist by going to File → SCiLS Report Table. Use the pulldown tab to select the Tryptic digest Peaklist created by SCiLS. This will have the format of “image data name: Imported Peaks.” On the bottom of the report tables window, select the notepad icon. This will copy the data to your clipboard. Paste the data into the text editor appended to the list of N-glycans (*see* Note 18). Save the Combined peaklist.
4. Load the combined peaklist into SCiLS. Copy all m/z intervals from the Combined Peaklist. Go to File → Import → m/z intervals from CVS or clipboard. After naming the list, click Save.
5. Define image regions by intensity and expression pattern using image Segmentation. From the top menu, select Tools → Segmentation. Ensure that an

¹⁸The N-glycans are loaded as a specific list to minimize MALDI matrix peaks from being incorporated as peaks of interest during subsequent computation of image patterns.

appropriate normalization method is selected, and check the box next to Regions. Select the option “Work on all individual spectra.” Select the created Combined Peaklist as m/z intervals. For parameters, use the pulldown menu to set denoising as “Weak”, keep the method as “Bisecting k-Means” and as metric, use the pulldown menu to select “Manhattan” (*see* Note 19). This clusters peaks based on intensity and pattern within the tissue.

6. Segmentation may be evaluated in the Labels menu. Select the calculated segmentation. Double-click on the colored squares to extend the segmentation tree to smaller regions. The number to the right of the box reports how many spectra associate with the regional pattern. Figure 4a shows the results of segmenting out N-glycan image data with trypsin image data based on region.
7. Within the label tree, select a region of interest based by single clicking on the same-colored box. On the bottom of the Label menu, select “Save selected class as Region.” Name and save the region. This will generate a mass spectrum for that particular region. Regions are extracted from the segmentation data and appear as highlighted regions in the tissue outline Fig. 4b.
8. On the menu at the top of the software, select Tools → Find Co-Localized m/z Values. Ensure that an appropriate normalization method is selected, and check the box next to Regions. In the Mode menu, select “Correlate with Region.” Check the box that corresponds to the region of interest. Next to “m/z Intervals”, select the created Combined Peaklist. Save as an informative name and click “OK.”
9. Once the data has calculated, a new peak list will appear under the “m/z Intervals” pulldown menu. Click the small left arrow beside the menu name. Click the “eye” icon to the far right of the menu window. A Threshold bar is displayed with a Correlation Value. Clicking in the bar raises or lowers the correlation value and reports the number of m/z intervals associated with the correlation value. Correlation values of greater than 0.6 are good candidates for co-localization with the target region. Once a correlation value is selected, Click the “eye” icon on the far right to open the m/z Image Generator. Click OK to generate images.
10. Figure 4c demonstrates an image data result from the procedure (*see* Note 12).
11. Discovery of the tryptic peptides corresponding to N-glycan localization allows targeted LC-MS/MS proteomic experiments for peptide sequencing and protein identification.

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¹⁹These are general parameters for segmentation that we have found work well with most image data and accurately segment out regions corresponding with tissue histology.

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Fig. 1.

Example data of N-glycan image data. **(a)** Typical overall average mass spectrum; the N-glycans corresponding to m/z are a known entity in this experiment. Inset is the scanned tissue. **(b)** Examples of N-glycan images created as heat maps of a selected ion's intensity and demonstrating basic pattern motifs on tissue

Fig. 2. Example of tryptic peptide image data. **(a)** Typical overall average mass spectrum; the tryptic peptide identifies are an unknown entity in this experiment. Inset is the scanned tissue. **(b)** Examples of tile view of images from tryptic peptides created as heat maps of a selected ion's intensity, showing 48 peptide images out of a total of 4535 detected image patterns

Fig. 3.

Example identification of a tryptic peptide corresponding to a single target N-glycan. (a) Tissue features shown by hematoxylin and eosin stain of the same tissue section after being treated by PNGase F and trypsin and going through two rounds of imaging experiments. (b) Target N-glycan found by MALDI IMS Experiment, identified as a high mannose Man7 structure. *Green* circles represent mannose residues; *blue* squares represent *N*-acetylglucosamine (GlcNAc). Parentheses indicates mass accuracy of N-glycan as found by MALDI FT-ICR. (c) Tryptic peptides found using the “Co-localize to m/z Feature” of

SCiLS. The unknown tryptic peptide is then a target for identification by tandem mass spectrometry

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Fig. 4.

Example of results from an Image segmentation strategy to identify common regions between N-glycan and tryptic peptides detected by MALDI IMS on the same tissue section. (a) Output of image segmentation from the two imaging experiments on the same tissue section using a combined list of known N-glycans and all detected tryptic peptides. Parentheses indicates mass accuracy of N-glycan as found by MALDI FT-ICR. The *orange* region on the N-glycan image data and the light green region on the tryptic peptide image data are very similar. (b) Regions are extracted from the segmentation data and appear as highlighted regions in the tissue outline. (c) Examples of N-glycan images correlating to the extracted region data. The N-glycan peaks do not appear in the tryptic peptide image data, as expected. (d) Examples of tryptic peptide image data correlating to the image regions