Comparative 2D N-glycome, Stromal Peptide, and Tryptic Peptide Mapping from the Same FFPE Tissue Section using MALDI Imaging Mass Spectrometry

Cassandra Clift[1], Anand Mehta[1], Richard R. Drake[1], Peggi M. Angel[1]

1, Dept of Cell and Molecular Pharmacology & Experimental Therapeutics, Medical University of South Carolina, Charleston, SC

Overview

➢ **Objective:** Here, we evaluate a multi-enzyme MALDI-IMS technique that begins with histological analysis via hematoxylin & eosin (H&E) staining. This would allow for the two-dimensional mapping of N-glycans, extracellular matrix peptides, and tryptic peptides from a single FFPE sample with preservation of histology.

➢ **Methods:**
- Histological/immunohistochemistry evaluation
- MALDI Imaging Mass Spectrometry

➢ **Results:** The serial application of PNGaseF, Collagenase Type III, and Trypsin allows for deep data mining of a single formalin fixed paraffin embedded tissue section. Histological staining via H&E before the enzymatic workflow did not interfere with MALDI-IMS signal. We found that on-tissue deglycosylation after N-glycan analysis increases the accessibility of ECM proteins to Collagenase Type III digestions. Tryptic peptide signal was altered after collagenase and we are following through with proteomics to determine changes in tryptic targeting after collagenase.

Introduction

➢ Conventional IMS workflows involves treating the tissue with a thin, uniform layer of trypsin that, when analyzed parallel with high resolution accurate mass proteomics, allows for the characterization and localization of tryptic peptides to unique regions of the tissue.

➢ Similarly, PNGaseF has been used to release N-glycans from thin tissue sections (Powers, 2013; Powers 2014). Recently, these two enzymes have been used in tandem, allowing for colocalization of the N-glycome to the tryptic proteome (Heijs, 2016; Angel, 2018).

➢ Recently, our lab has developed a MALDI-IMS method that can be used to map complex extracellular matrix proteins from formalin-fixed, paraffin-embedded (FFPE) thin tissue sections (Angel 2018).

➢ By combining these enzymatic approaches, deep mining of protein information becomes possible. This is especially useful for difficult to obtain patient samples, as N-glycan, ECM, and tryptic peptide data can be analyzed from one FFPE tissue section.

➢ However, the histology may be disrupted after multi-enzyme treatment, preventing pathological evaluation. Previous work has showed tryptic peptides are detected after H&E stains (Norris, 2013); here we test signal from PNGaseF and Collagenase after H&E stains.

➢ Here, we evaluate a workflow to capture histology before serial enzymatic treatment.

Methods

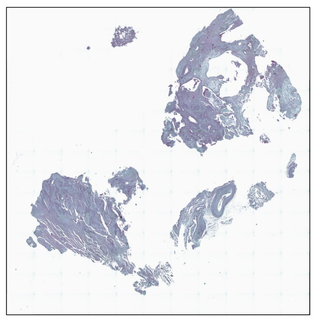
A: Tissue Acquisition; Histological Staining

➢ De-identified lung cancer tissue was obtained with IRB approval through Hollings Cancer Center Biorepository at MUSC.

➢ Tissues were sectioned serially at 5µm and dewaxed before staining and imaging (below). The tissue was stained using hematoxylin and eosin (H&E) for cell morphology. Staining was done before one enzymatic workflow.

➢ H&E stained tissue was visualized at 10x magnification with an EVOS FL cell imaging system from Thermo Fisher Scientific.

➢ The right image shows H&E staining done before the enzymatic digestion workflow below. Coverslip was removed by incubation in xylene prior to IMS.

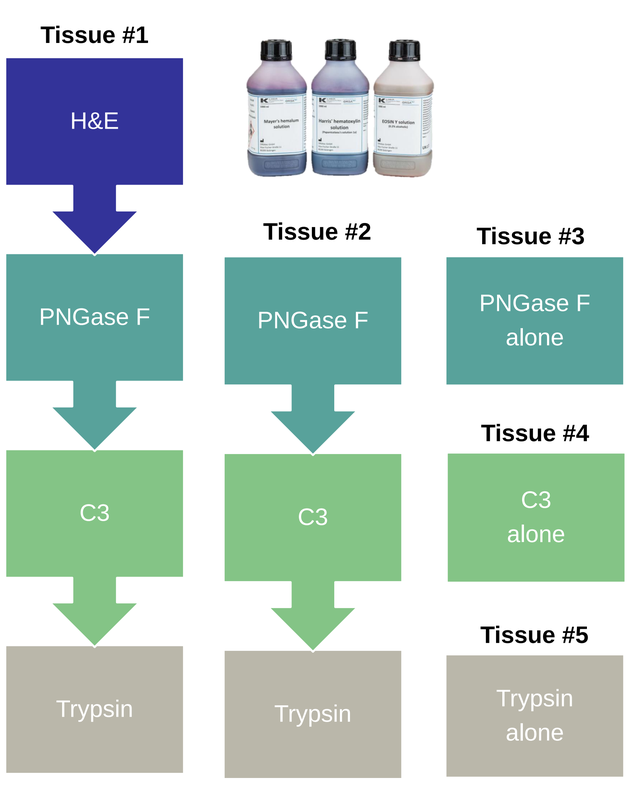


Dewaxing protocol for FFPE tissues:

Heat 60°C 1 hour → Xylene 3 min (x2) → 100% EtOH 1 min (x2) → 95% EtOH 1 min → 70% EtOH 1 min → HPLC H_2O 3 min (x2)

B: MALDI Imaging Mass Spectrometry & Data Analysis

➢ Enzymes used were 1) Peptide N-glycosidase F (PNGaseF), to release N-glycans, 2) Collagenase Type III (C3), to release ECM peptides, and 3) Trypsin, to remove tryptic peptides.



➢ Tissues were imaged using a 7.0 Tesla solariX Legacy FT-ICR (Bruker Daltonics) operated in positive ion broadband mode spanning m/z range 500-4000 using a 512k word time domain resulting in a 1.2059 second transient with an effective 75,000 mass resolution (calculated on-tissue at m/z 1419).

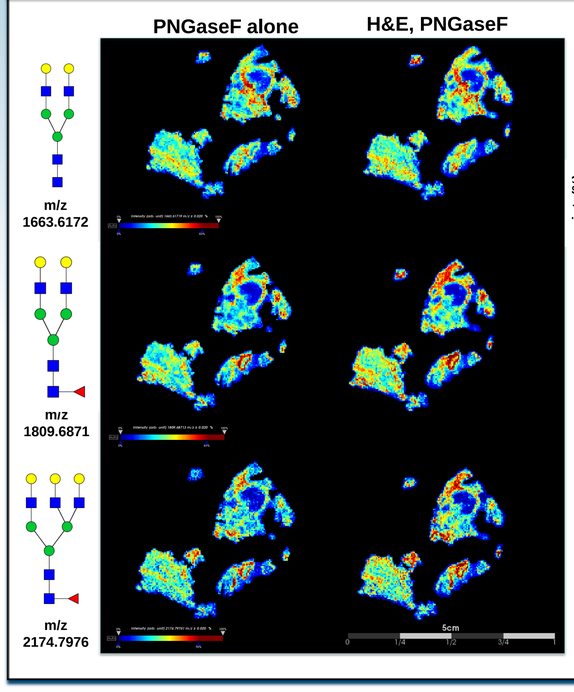
➢ Laser settings used were: 300 shots, no smart walk, with a 150µm step-size.

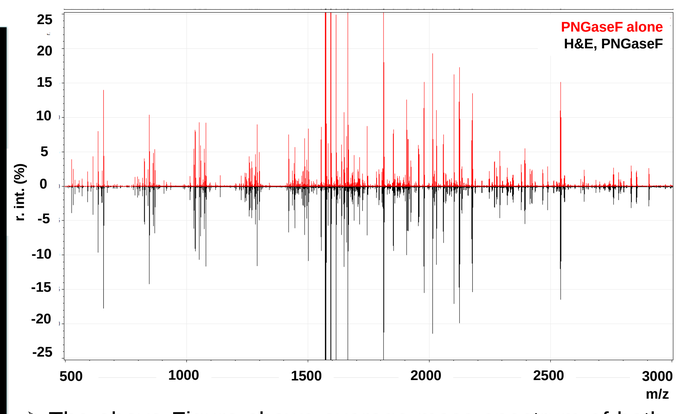
➢ N-Glycan protocol lockmass used was m/z 1809.6393, representing Hex5dHex1HexNAc4 +Na, which is a main peak throughout tissues. ECM and Trypsin lockmass used [Glu1]-Fibrinopeptide B at m/z 1570.6768, as a spiked internal standard in the α-Cyano-4-hydroxycinnamic acid (CHCA) matrix.

➢ Images were visualized in FlexImaging v5.0 and analyzed using SCiLS 2019Pro (both/Bruker Daltonics)

➢ High mass resolution, accurate mass proteomics data (LC-MS/MS), was collected in parallel with IMS data for ECM peptide identification.

Result A: Histological Staining Before Imaging Does Not Alter N-Glycan Signal



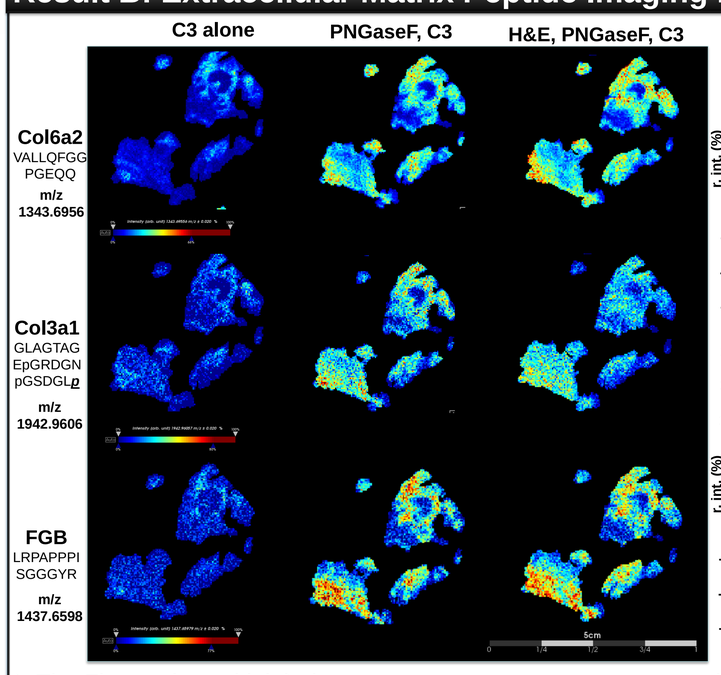


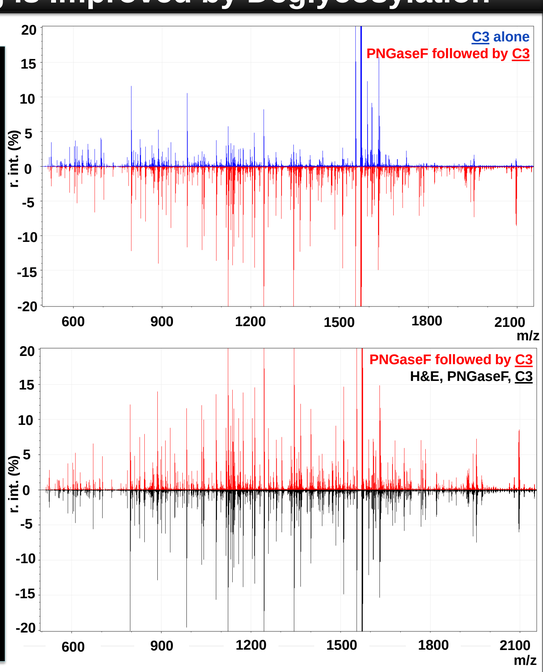
➢ The above Figure shows average mass spectrum of both tissue stained with H&E then treated with PNGaseF (black) and that of tissue treated with PNGaseF alone (red). H&E staining caused no significant difference in N-glycan signal.

➢ The Figure to the left shows three N-glycans spatially mapped across the tissue.

■ N-acetylglucosamine ● Galactose ● Mannose ◀ Fucose

Result B: Extracellular Matrix Peptide Imaging is Improved by Deglycosylation

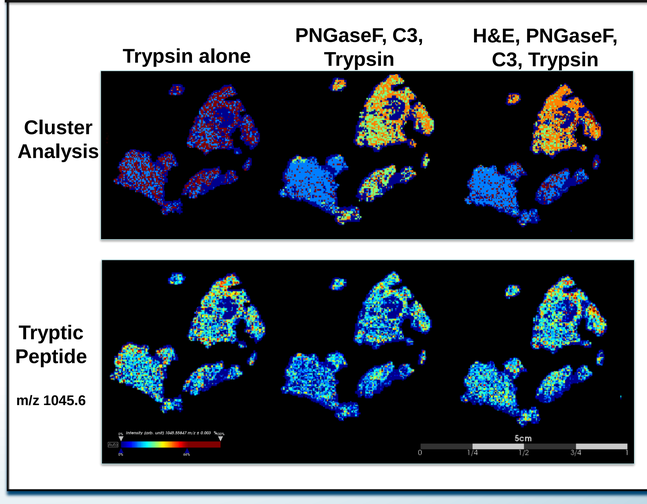


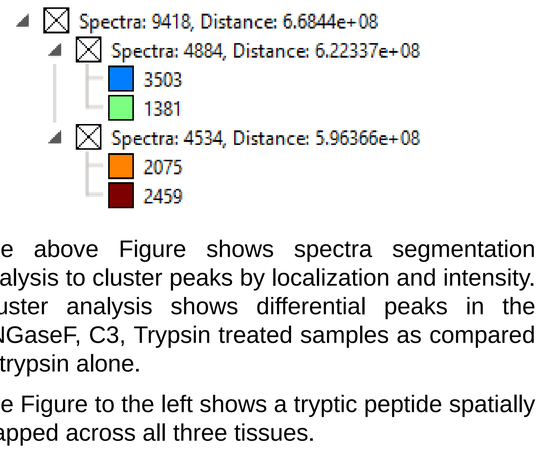


➢ The Figure above (right) shows average mass spectrum comparing: Top) Collagenase alone (blue) vs. PNGaseF then C3 treated (red), and Bottom) tissue stained with H&E then treated with PNGaseF then treated with C3 (black) and that of the same workflow without H&E staining (red). Again, no significant difference was found between average mass spectrum of tissues that had undergone H&E staining and those that hadn't, however, deglycosylation does improve the extracellular matrix peptide signal.

➢ The Figure above (left) shows three ECM peptides spatially mapped across the tissue. As seen, cleavage of N-glycans and subsequent deglycosylation improves upon ECM peptide imaging.

Result C: Tryptic Peptide Imaging





➢ The above Figure shows spectra segmentation analysis to cluster peaks by localization and intensity. Cluster analysis shows differential peaks in the PNGaseF, C3, Trypsin treated samples as compared to trypsin alone.

➢ The Figure to the left shows a tryptic peptide spatially mapped across all three tissues.

Conclusions and Future Directions

➢ This study expands recent advances in MALDI-IMS technology that allow for spatially correlated molecular information of both the N-glycome and tryptic proteome, to include extracellular matrix peptide data.

➢ The serial application of PNGase F, Collagenase Type III, and Trypsin, along with specific washing steps, allows for the two-dimensional mapping of N-Glycans as well as ECM and tryptic peptides from the same 5µm tissue section.

➢ We found that on tissue deglycosylation after N-glycan analysis increases the accessibility of ECM proteins to collagenase type III digestion.

➢ We also found that hematoxylin and eosin staining does not interfere with subsequent N-glycan, ECM or tryptic peptide imaging via MALDI-IMS. Future goals of the study are to perform H&E staining after these workflows to capture enzymatic and treatment effect that might alter the tissue histology. We expect this will emphasize the importance of capturing histology before MALDI-IMS.

➢ This multimodal approach may be useful for deep mining of protein information from difficult to obtain tissues, as one 5µm FFPE tissue section can allow for analysis of 1) pathological evaluation via histology as well as 2) N-glycan, 3) ECM, and 4) tryptic peptide data.

Acknowledgements

➢ This work was supported by 16GRNT31380005 (American Heart Association), P20 GM103542 (NIH/NIGMS), 1R21CA186799 (NIH/NCI), and HL007260 (NHLBI)

References

(1) Powers, T.W., et. al., *Anal Chem* **2013**, 85(20):9799-806
(2) Heijs, B., et. al., *Anal Chem* **2016**, 88(15):7745-53
(3) Angel, P.M., et. al., *Proteomics Clin Appl* **2018**, epub
(4) Angel, P.M., et. al., *Journal Proteome Research* **2018**, 17(1):635-646
(5) Norris J.L., et. al., Chemical Reviews 2013, 113(4):2309-2342