Maximizing Data Coverage with Sequential Imaging of a Single Tissue Section

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Overview

- Clinical tissue specimens may be small in size and the amount available for research may be quite limited
- Careful sample preparation allowed for sequential imaging of metabolites (+/-), lipids (+/-), N-linked glycans, O-GlcNAc, intact small proteins, and tryptic peptides from the same tissue section followed by histological staining
- In many cases, the previous analyses enhanced the signal detected in later analyses

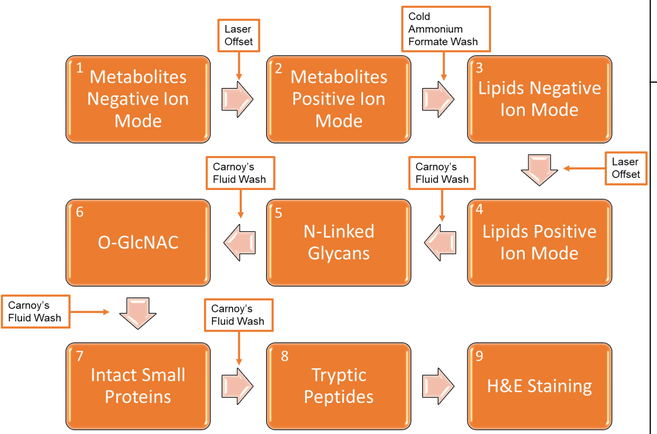
Introduction

Diagnostic biopsies may only be 1-2 mm in diameter to minimize the invasive impact of the procedure. Given samples this small, it may be difficult to definitively diagnose disease, requiring additional tests. Due to the limited nature of the sample, the tests performed must be prioritized, and only a few molecules are detected with each histopathological test. Mass spectrometry imaging allows the detection and localization of hundreds of molecules from a single tissue section and serial analyses for different analytes are possible. By carefully choosing the order of analysis, we performed 8 different imaging experiments from the same tissue section, allow sequential analysis of metabolites, lipids, N-linked glycans, GlcNAc, small proteins, and tryptic peptides, followed by histological staining.

Methods

Liver tumor was sectioned at 12 µm thickness and collected onto ITO-coated glass slides or IntelliSlides. All matrix application was carried out using an HTX M5 Robotic Reagent Sprayer and all imaging except intact proteins was performed on a Bruker timsTOF flex mass spectrometer. A dual polarity matrix (NEDC) was used for metabolites with a laser offset applied between positive and negative ion mode imaging at 100 µm spatial resolution. Matrix was removed with cold ammonium formate and another dual polarity matrix (DAN) was applied for lipid imaging with laser offsetting for positive and negative ion modes. Matrix was removed and the section was treated with PNGaseF to image N-linked glycans. Next, the section was treated with O-GlcNAc hydrolase and GlcNAc was imaged. Then, the section was sprayed with sinapinic acid and intact proteins were imaged on a Bruker rapifleX mass spectrometer. Finally, on-tissue tryptic digestion was performed for peptide imaging. After completing 8 imaging experiments, matrix was removed and hematoxylin and eosin staining was performed. Images were co-registered using SCiLS Ion Mapper to visualize all analytes simultaneously. In parallel, all images were also collected as a single acquisition for comparison.

Workflow



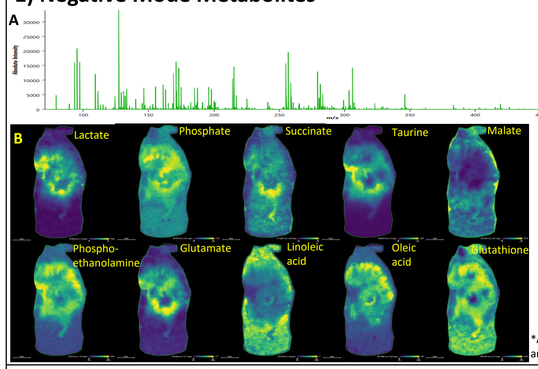
Sprayer Methods

	Metabolites	Lipids	N-Glycans	O-GlcNAc	All Sugars Matrix	Proteins	Peptides	Peptides Matrix
Matrix / Enzyme	NEDC	DAN	PNGaseF	O-GlcNAc Hydrolase	CHCA	SA	Trypsin	CHCA
Concentration (mg/mL)	7	10	0.1	0.35	10	10	0.1	10
Solvent	70% MeOH	50% ACN	100 mM ABC	100 mM ABC	70% ACN, 0.1 TFA	90% ACN, 0.1 % TFA*	100 mM ABC	70% ACN, 0.1% TFA
Flow Rate (mL/min)	0.12	0.1	0.025	0.025	0.12	0.1	0.01	0.12
Passes	8	10	15	15	3	12	12	4
Track Speed (mm/min)	1200	1200	1200	1200	1200	750	750	1200
Track Spacing (mm)	2	3	3	3	3	2	3	3
Pattern	CC	CC	CC	CC	HH	CC	HH	HH
Nozzle Temp (°C)	75	60	45	30	75	75	30	75

*Recrystallized at 85°C for 3.5 min

Results

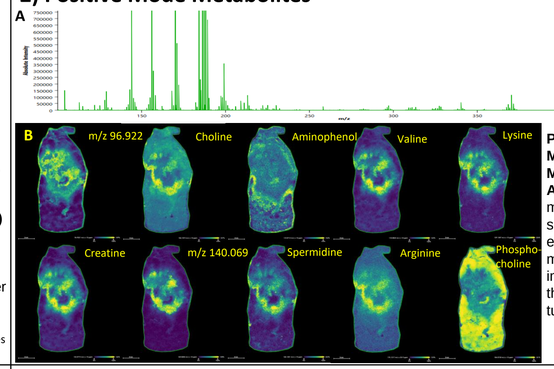
1) Negative Mode Metabolites



Negative Mode Metabolites. A) average metabolite spectrum. **B)** examples of metabolite images from the rabbit liver tumor.

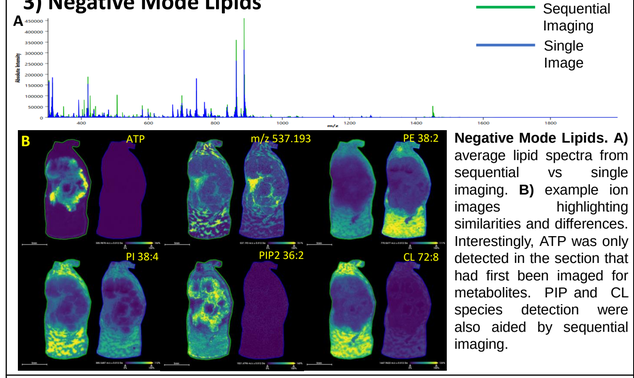
*All IDs in all images are putative

2) Positive Mode Metabolites



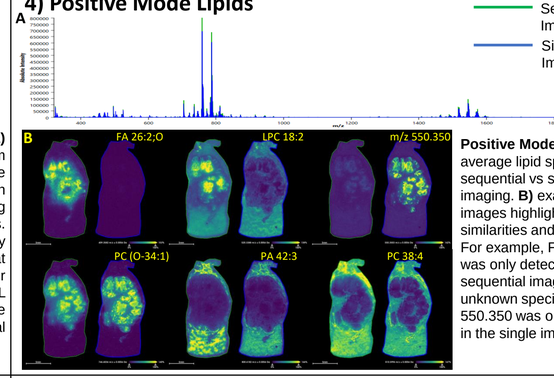
Positive Mode Metabolites. A) average metabolite spectrum. **B)** examples of metabolite images from the rabbit liver tumor.

3) Negative Mode Lipids



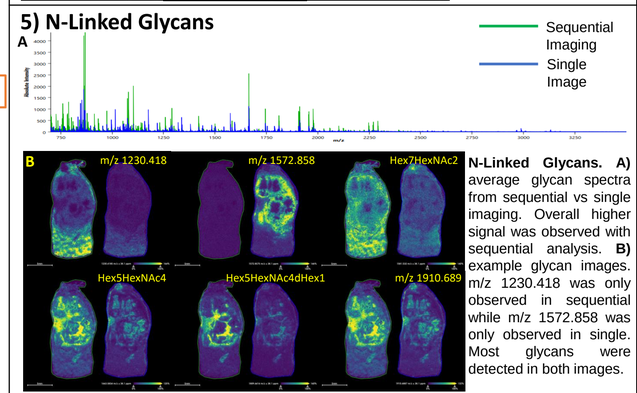
Negative Mode Lipids. A) average lipid spectra from sequential vs single imaging. **B)** example ion images highlighting similarities and differences. Interestingly, ATP was only detected in the section that had first been imaged for metabolites. PIP and CL species detection were also aided by sequential imaging.

4) Positive Mode Lipids



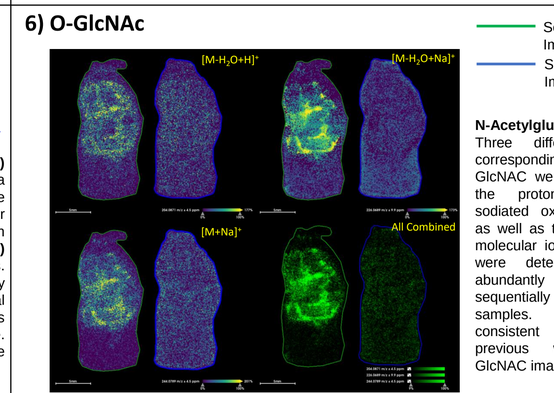
Positive Mode Lipids. A) average lipid spectra from sequential vs single imaging. **B)** example ion images highlighting similarities and differences. For example, FA 26:2;O was only detected in the sequential image while an unknown species at m/z 550.350 was only detected in the single image.

5) N-Linked Glycans



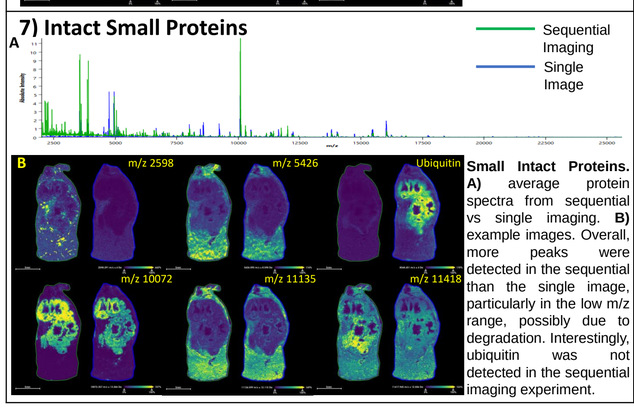
N-Linked Glycans. A) average glycan spectra from sequential vs single imaging. Overall higher signal was observed with sequential analysis. **B)** example glycan images. m/z 1230.418 was only observed in sequential while m/z 1572.858 was only observed in single. Most glycans were detected in both images.

6) O-GlcNAc



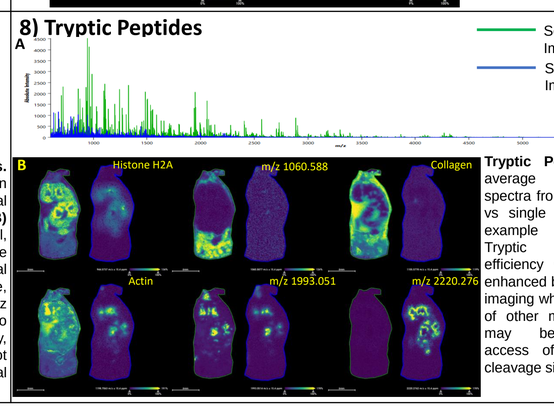
N-Acetylglucosamine. Three different ions corresponding to GlcNAC were detected; the protonated and sodiated oxonium ions as well as the sodiated molecular ion. All ions were detected more abundantly in the sequentially imaged samples. This is consistent with our previous work with GlcNAC imaging.[2]

7) Intact Small Proteins



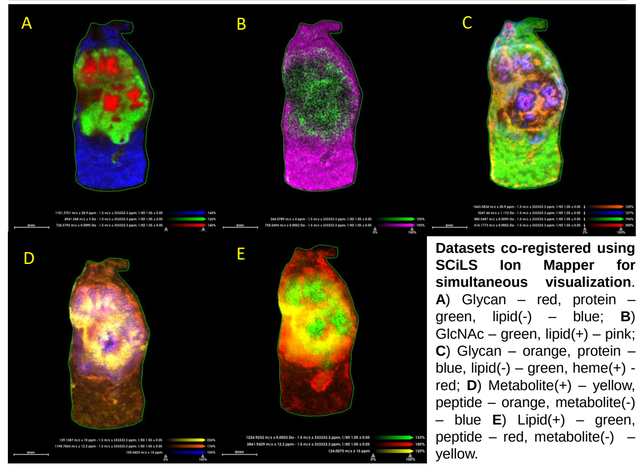
Small Intact Proteins. A) average protein spectra from sequential vs single imaging. **B)** example images. Overall, more peaks were detected in the sequential than the single image, particularly in the low m/z range, possibly due to degradation. Interestingly, ubiquitin was not detected in the sequential imaging experiment.

8) Tryptic Peptides



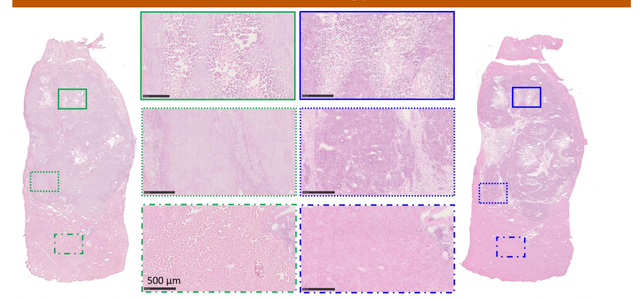
Tryptic Peptides. A) average peptide spectra from sequential vs single imaging. **B)** example images. Tryptic digestion efficiency was greatly enhanced by sequential imaging which removed of other moieties that may be blocking access of trypsin to cleavage sites.

Co-registration



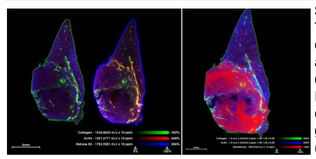
Datasets co-registered using SCiLS Ion Mapper for simultaneous visualization. A) Glycan – red, protein – green, lipid(-) – blue; **B)** GlcNAc – green, lipid(+) – pink; **C)** Glycan – orange, protein – blue, lipid(-) – green, heme(+) - red; **D)** Metabolite(+) – yellow, peptide – orange, metabolite(-) – blue **E)** Lipid(+) – green, peptide – red, metabolite(-) – yellow.

Histology



Histological staining after sequential imaging (left) and on a serial tissue section (right). Major features are still distinguished after sequential imaging, histological crispness is compromised. Insets show necrotic area (top), viable tumor (middle), and adjacent normal liver (bottom).

MALDI-Immunohistochemistry



Sequential Imaging with MALDI-IHC. Left – Three proteins mass tags visualized after 4 other images (green) and as the only acquisition (blue). Actin was more readily detected in the single imaging. Right – MALDI-IHC of collagen co-registered with the first collected image (negative mode metabolites, glutathione) and the last collected image (tryptic peptides, actin).

Conclusions

- Eight different ion images could be collected from a single 12 µm thick section of tissue
- Some differences in detected peaks were observed between sequential imaging and single analyte imaging with overall enhanced signal by sequential imaging as opposed to solo analysis
- MALDI-IHC can be performed in place of intact protein imaging within this workflow, on going work will optimize the ideal analysis order
- General histological features could still be observed after 8 MSI images had been collected

References

1. Drake RR, West CA, Mehta AS, Angel PM. "MALDI Mass Spectrometry Imaging of N-Linked Glycans in Tissue". *Adv Ex Med Bio*. **2018**, *1104*, 59.
2. Escobar EE, Seeley EH, Serrano-Negrón JE, Vocadlo DJ, Brodbelt JS. "In Situ Imaging of O-Linked β-N-Acetylglucosamine Using On-Tissue Hydrolysis and MALDI Mass Spectrometry". *Cancers*. **2023**, *15(4)*, 1224.

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