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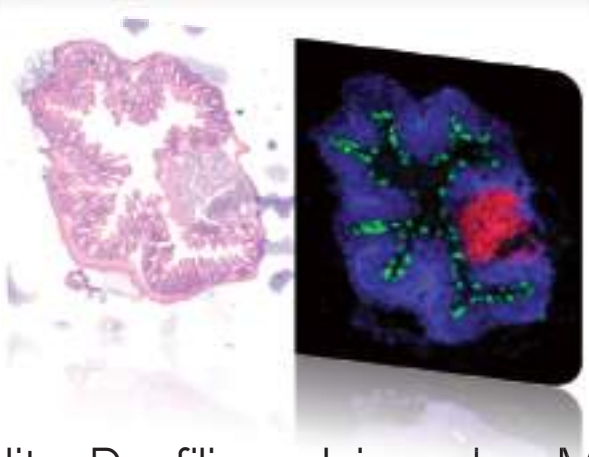
Application Note #MSI-09



Molecular Histology & Metabolite Profiling driven by MSI: Fine Biological Understanding of Gastrointestinal Diseases

Introduction

Inflammatory Bowel diseases are characterized by relapsing-remitting inflammatory responses that have a tendency to develop where the bacterial load is greatest. Among industrialized countries, the increased incidence of Inflammatory Bowel diseases is presumably due to changes of the gut microbiota (herein referred as dysbiosis). It is indeed worth noting that Crohn's disease recurrence is linked to a lower prevalence of major member of Firmicutes, including the anti-inflammatory commensal Faecalibacterium prausnitzii. More importantly, the intestinal microbiota has been highlighted to regulate intestinal homeostasis through the secretion of a large set of metabolites [1]. Herein, genetically predisposed animals have been used to reveal the impact of two specific single gene mutations on the modulation of the metabolome within the colonic mucosa. Due to the complexity of colon tissue at the histological level and the importance of correlating metabolite distribution with specific location within tissue, the use of Mass Spectrometry Imaging (MSI) appears relevant to provide a better understanding of intestinal luminal metabolome.

Our aim is to enhance the knowledge on intestinal metabolome thanks to in-situ analysis of metabolite profile and distribution within colon tissue models.
The use of MSI enables a spatially resolved and unlabeled imaging of different metabolites directly in their micro-environment and provides a molecular profile to specific histological substructures.

Thanks to a collaboration with the INSERM research team headed by Dr. M. Chamaillard, we provide the two following examples of MSI applications in gastroenterology which illustrate the benefits given by MSI:

1. High spatial resolution imaging of colon tissue for molecular histology: Identification of colon tissue substructures using MSI

2. Comparison of metabolite profile and localization in knockdown models of mouse colon: In situ profiling of disease biomarker by MSI

Experimental section:

- **Animal:** Terminal colons from Wild-Type and mutant mice were removed, snap frozen, embedded in CMC and stored at 80°C.
- **Sectioning:** Colons embedded in CMC were sectioned following transversal plan (12 µm of thickness) using Microm HM560 cryostat (Thermo Scientific, Germany) at -20°C and mounted on ITO conductive glass slides (Delta Technology, USA). HE Staining was performed on adjacent tissue sections for better visualization of histological regions.
- **Matrix:** 2.5 DHB powder (150 mg) was used and vaporized on tissue sample using home built sublimation apparatus (150°C, 8 min, 2.10^{-3} mbar).
- **Mass spectrometry imaging:** Autoflex Speed LRF MALDI-TOF (Bruker Daltonik, Germany) with SmartBeam II laser. Positive mode (100-1000 Da) at 20 µm spatial resolution.
- **Software:** All presented MS images are from Mutltimaging™ software 1.1 (ImaBiotech, France).

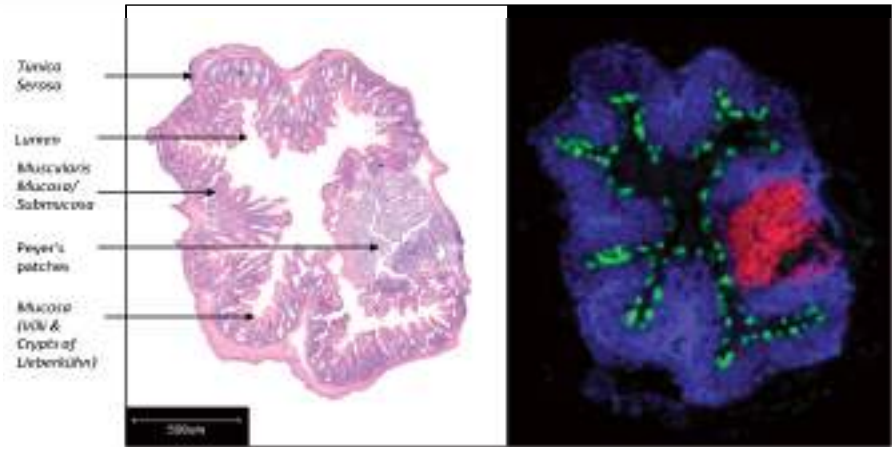


Figure1. *Distribution of endogenous lipids species from transversal colon sections obtained using MALDI-TOF mass spectrometer at high (20 µm) spatial resolution. Black arrows show and describe histological features of colon tissue.*

Results & discussions:

High spatial resolution imaging of colon enables the visualization of histological layers and regions within tissue. An example of transversal colon sections mass spectrometric image at high spatial resolution (20 µm) is presented in **figure 1**. It shows the benefit given by matrix sublimation method to yield high-quality molecular images with no analyte delocalization. Some contrast ionic species are used to accurately differentiate small histological structures of the colon. These ions are probably related to lipid species but taking into account limited ability of TOF mass spectrometer in terms of accurate mass measurement (10 ppm) and the lack of MS/MS data, the precise identification remains difficult. Nevertheless, we are able to propose some potential lipids identification for ion at *m/z* 734.57 (red filter) which can be related to Phosphatidylcholine or Phosphatidylethanolamine molecules; PE (35:0) or PC (32:0). The two others ions at *m/z* 609.91 (green filter) *m/z* 703.59 (blue filter) cannot be accurately identified.

Thus, histological substructures can be easily discriminated on an overlay molecular image from coronal section. The colonic mucosa is primarily constituted by several immune cells population (including lymphocytes and myeloid cells) and the epithelium. The conjonctive tissues are well localized on molecular image thanks to blue *m/z* filter whereas green *m/z* filter is associated to external layer of the mucosa, villi & crypts of Lieberkühn.

Crypts are intestinal glands which contain a large variety of secretory cells (such as Paneth cells, goblet cells and enteroendocrine cells). Paneth cells secrete substantial quantities of antimicrobial molecules which are key mediators of host-microbe interactions, and their dysfunction may contribute to the pathogenesis of chronic inflammatory bowel disease [2].

Interestingly, the red *m/z* filter on overlay image differentiates a specific region of the colon which can be identified as Peyer's patches (PPs). PPs are constituted by isolated or aggregated lymphoid follicles from Gut-Associated Lymphoid Tissue (GALT) [3]. The induction of immune tolerance or defense against certain pathogens is a function of PPs resulting from complex process involving immune cells located in the lymphoid follicles and the follicle-associated epithelium.
It plays an important role in some intestinal illnesses, including Crohn's Disease (CD) and Graft versus Host Disease (GVHD). For all these reasons, the ability to map molecular changes in these aforementioned specific regions of the colon (crypts of Lieberkühn or Peyer's patches) might be useful to a better understanding of the pathophysiology of gastrointestinal diseases.

Comparison of metabolite profiles and localization in genetically predisposed mice

Experimental section:

- **Animal:** Same as previously described
- **Sectioning:** Same as previously described
- **Matrix:** 9AA (10mg/ml, Methanol) was chosen and deposited using TLC sprayer (Sigma Aldrich, Germany).
- **Mass spectrometry imaging:** Solarix 7.0T FTICR (Bruker Daltonik, Germany) with SmartBeam II laser. Positive fullscan mode (100-800 Da), 300 shots at 40 µm spatial resolution.
- **Software:** All presented MS images are from MultimagingTM software 1.1 (ImaBiotech, France).

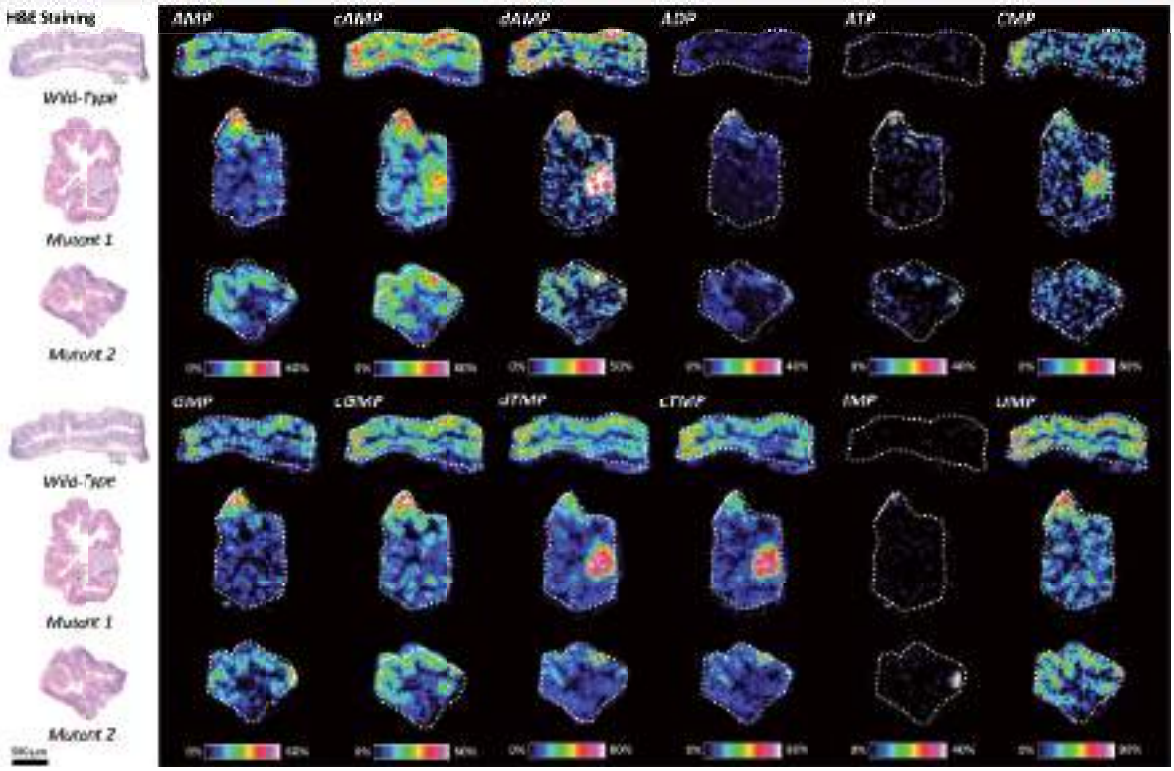


Figure 2. Molecular images of 12 ribonucleotides ions distribution in transversal colon sections from Wild-Type and mutant mice; Optical images of tissue section are presented. Relative intensity scales are indicated on images (polychromatic mode from 0 to X% corresponding to maximum intensity of ion's peak).

Name	Name	$m/z_{theo.}$	$m/z_{exp.}$	Ion Form	|Error| (ppm)	Mean intensity (a.u.)			Fold difference	
						WT	*Mutant 1*	*Mutant 2*	*WT/Mut1*	*WT/Mut2*
Adenosine monophosphate	**AMP**	*346,056*	*346,056*	*[M-H]-*	0,2	358051,1	240132,3	309986,6	1,5	1,2
Cyclic adenosine monophosphate	**cAMP**	*328,045*	*328,046*	*[M-H]-*	1,4	427287,1	320900,7	334496,3	1,3	1,3
Deoxyadenosine monophosphate	**dAMP**	*330,061*	*330,061*	*[M-H]-*	0,7	124419,1	90543,0	75983,9	1,4	1,6
Adenosine bisphosphate	**ADP**	*426,022*	*426,022*	*[M-H]-*	0,6	240700,9	251471,6	408182,9	*1,0*	*0,6*
Adenosine triphosphate	**ATP**	*505,988*	*505,989*	*[M-H]-*	1,0	9641,56	23183,76	36494,72	*0,4*	*0,3*
Cytidine monophosphate	**CMP**	*322,045*	*322,044*	*[M-H]-*	0,5	39252,5	34702,3	25268,7	1,1	1,6
Guanosine monophosphate	**GMP**	*362,051*	*362,051*	*[M-H]-*	0,2	283409,5	166268,6	244954,1	1,7	1,2
Cyclic Guanosine monophosphate	**cGMP**	*344,040*	*344,040*	*[M-H]-*	0,4	389437,4	261803,4	321501,2	1,5	1,2
Thymidine monophosphate	**dTMP**	*321,049*	*321,049*	*[M-H]-*	1,3	612911,0	499490,6	347917,6	1,2	**1,8**
Thymidine cyclic monophosphate	**cTMP**	*303,039*	*303,039*	*[M-H]-*	1,5	522722,2	409480,0	273478,4	1,3	**1,9**
Inosine monophosphate	**IMP**	*347,040*	*347,040*	*[M-H]-*	0,9	1799,1	4230,6	24191,4	*0,4*	*0,1*
Uridine monophosphate	**UMP**	*323,029*	*323,029*	*[M-H]-*	0,1	185387,8	107332,4	131786,8	1,7	1,4
Cyclic-Cytidine 2'-monophosphate	**cCMP**	304,034	304,033	*[M-H]-*	4,3					
Deoxycytidine monophosphate	**dCMP**	*306,050*	*nd*	*na*	na					

Table 1. Mass accuracy measurement is reported for each ribonucleotide with corresponding ion form as well as their complete name and abbreviation. Differences between experiment and theoretical values are expressed in ppm (part per million). Mean relative intensity values of nucleotides within tissue are reported depending on conditions. A ratio between conditions is calculated from intensity values to highlight potential up or down regulation of molecules in knockdown models compare to Wild-Type.

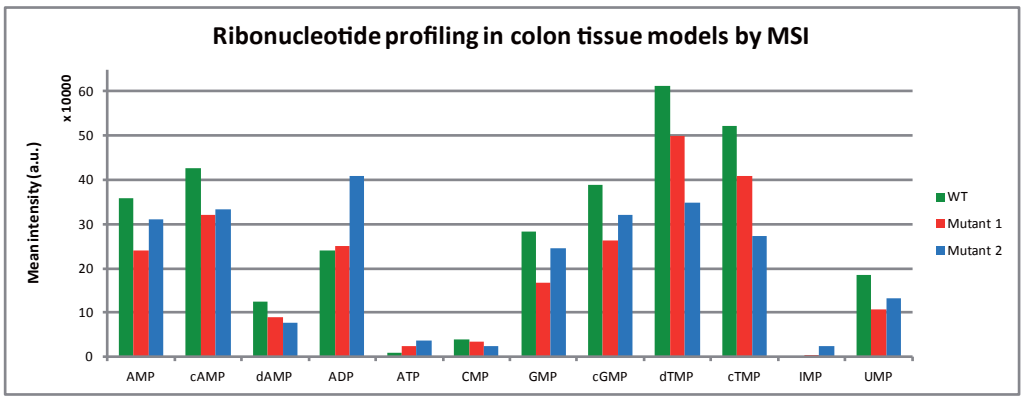


Figure 3: Histogram showing the mean relative intensity from each of the 12 ribonucleotides targeted for Wild-type and mutant mice.

Results & discussion:

The application of Mass Spectrometry Imaging in the study of endogenous metabolites from biological tissues is a recent technique which offers the simultaneously monitoring of several compounds (lipids, small metabolites or drugs) with spatiotemporal information about molecular behavior [4,5,6,7]. The combination of these factors allows us to perform the identification and validation of new biomarkers of intestinal microbiota closely related to inflammatory bowel diseases. The **figure 2** displays some molecular images obtain from each colon tissue models in negative detection mode (anionic species) at 40 µm of spatial resolution. Haematoxylin & Eosin staining of corresponding tissue sections are also presented. Twelve ribonucleotides related metabolites were selected to provide an overview of metabolome changes between conditions. Others metabolites classes such as nucleosides, amino acids, phospholipids or other nucleotides have been detected but not shown here. **Table 1** summarizes the ion species detected with some information about their relative concentration between conditions and fold difference values. **Figure 3** shows the profile of each ribonucleotides on colonic resection specimens from wild-Type (WT) and mutant mice.

Ribonucleotides are involved in many biological process such as intracellular signal transduction, energy metabolism or others cellular functions. They may have several forms, including mono-, di- or triphosphate moieties and undergo some chemical modification such as a reduction (Desoxy-) or a cyclization (Cyclic-). Moreover, the reduced form of ribonucleotide is required for both synthesis and repair of DNA.

Likewise, cyclic ribonucleotides such as cAMP or cGMP act as second messenger in organisms. Most ribonucleotides are abundant in WT model compared to transgenic one except for ADP, ATP and IMP which have a very low intensity on mass spectra. Molecular histological specificities are observed on MS images, especially in one of the mutant colon which included a Peyer's patche (PPs). Notably, we noticed an accumulation of several metabolites in this area compared to mucosa or submucosa layers. It is the case for cTMP, dTMP (high intensity), CMP, cAMP and dAMP (low intensity) whereas no specific localization was highlighted for GMP, cGMP, UMP and AMP. This information might be useful for the understanding of PPs function and its role in the development of CD and/or GVHD.

Matsumoto ***et al.*** [1] have compared the intestinal luminal metabolome of germ free (GF) with Ex-GF mice i.e. the intestinal microbiota composition. They have observed that some metabolites were up regulated (GF>Ex-GF) or down regulated (GF<Ex-GF) depending on conditions which indicates a direct relationship between the intestinal microbiota and the luminal metabolome profile. Two ribonucleotides detected by MSI within colon tissue were included in the down-regulated groups of Matsumoto's paper (the dTMP or TMP and the CMP). These two ribonucleotides correspond to metabolites produced by colonic microbiota or derived from pellet and the absorption of which was possibly inhibited in the colon. Of particular interest, the aforementioned metabolites were found more abundantly in WT mice when compared to the transgenic models.

Conclusion

We provide a link between a differential metabolome and risk to develop inflammatory bowel diseases. Biological processes involving specific metabolite were directly assessed on histological substructures such as mucosa and submucosa layers or highly disease relevant tissue (as for example Peyer's patches or crypts of Lieberkühn). High spatial resolution molecular imaging allows following molecules/metabolites at the cellular level (such as Paneth or goblet cells, which are related to the development of several gastrointestinal diseases).

Thanks to MSI, ImaBiotech can provide specific information about distribution and profiles of endogenous metabolites at the low micrometer scale in gastrointestinal substructures

Advantages

- High spatial (20µm) and high spectral (R>500000) resolutions imaging
- Combination of molecular histology and classical histology techniques
- Spatiotemporal metabolite assessment within tissue
- Fast and reliable biomarkers comparison

Keywords

- Gastrointestinal diseases
- Mass Spectrometry Imaging
- Inflammatory bowel disease
- Crypts of Lieberkühn
- Intestinal microbiota
- Metabolite profile
- Peyer's patches
- Crohn's disease
- Colon

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Authors

Hamm Gregory
Bonnel David
Buissart Constance
Chamaillard Mathias
Legouffe Raphaël
Pamelard Fabien
Heron Alain
Stauber Jonathan

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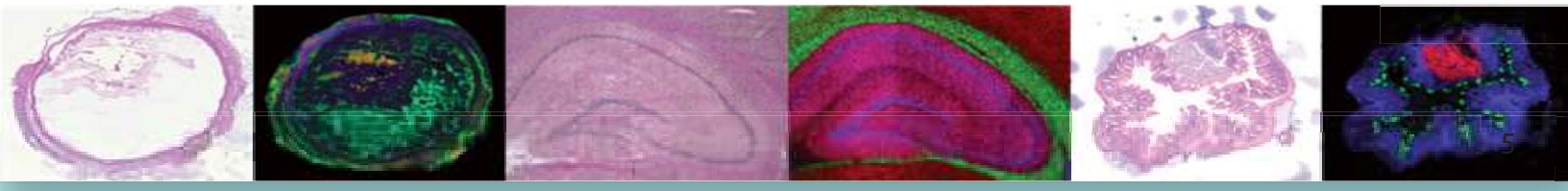

MS Imaging Department | 885 ave. Eugène Avinée - 59120 Loos - France | +33 (0) 320 164 091 | contact@imabiotech.com
185 Alewife Brook Parkway - Cambridge - MA 02138 - USA