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Application News

MALDI-8020/MALDI-8030 EasyCare

Benchtop Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging of Human Tonsil Proteins Using HiPLEX-IHC Probes

Caroline J. Jones
Shimadzu, Manchester, UK

User Benefits

- Simplified protein imaging analysis on an affordable benchtop MALDI-TOF system.
- Integration of immunohistochemistry into the MALDI imaging workflow (MALDI-IHC) for targeted analysis.
- Multiplex analysis allowing visualisation of 20 proteins in a single imaging run.

■ Introduction

Here, we demonstrate an entry level MALDI imaging solution to allow the precise mapping of proteins using Miralys MALDI-IHC imaging probes (AmberGen, MA,USA). Matrix assisted laser desorption ionization immunohistochemistry (MALDI-IHC) is a technique which uses a photocleavable peptide mass tag conjugated with an antibody to label proteins within a tissue sample. Subsequent cleavage of the mass tag allows for this to be detected by MALDI-TOF. Using mass spectrometry imaging (MSI), spatial information of the cleaved peptide can be determined and, from this, the locations of the antibodies, and hence the target proteins in the tissue, can be inferred. Through MALDI-IHC, we can further our knowledge of cellular interactions which is critical to the development new treatments and the Shimadzu benchtop MALDI instruments are ideal platforms to begin exploring the world of MALDI-IHC. In this study, we have analysed FFPE human tonsil sections using both a 6-plex and 20-plex antibody probe panels at a lateral resolution of 30 µm, demonstrating a reliable low-cost approach with good quality results.

Fig. 1 The MALDI-8020/MALDI-8030 EasyCare Benchtop linear MALDI-TOF mass spectrometer

■ Sample Preparation

FFPE human tonsil samples (AMSBIO, Oxford, UK) were mounted on poly-Lysine coated FlexiVision-mini ITO slides. Initially, the protocol was validated using a 6-plex antibody probe mix (Table 1). To prepare the tissue for analysis, the samples were deparaffinized using xylene. The slides were then immersed in a series of wash solutions (Ethanol: aqueous in varying concentrations) prior to antigen retrieval through exposure to an alkaline buffer. The samples were then stained with a photocleavable mass tag (PC-MT) labelled antibody probe mixture using the workflow provided with the Miralys MALDI HiPLEX-IHC kit (Ambergen, MA, USA). Tissue samples were exposed to UV-light to cleave the mass tags before coating with 2,5 dihydroxybenzoic acid (DHB) using the iMLayer™ matrix vapor deposition system (Shimadzu Corporation) and recrystallized prior to analysis (Fig. 2).

Table 1 Human Tonsil 6-plex Antibody Probe Mix (AmberGen)

Target Antigen	Tissue Expression	Target MW	Mass Tag $(M+H)^+$
CD3ε	T-Cells	23 kDa	1161.65
CD68	Macrophage Cells	75-110 kDa	1216.75
VIM	Lymphoid Cells	57 kDa	1230.84
Col-1A1	Collagen (extracellular matrix)	138 kDa	1234.87
PanCK	Epithelial Cells	40-68 kDa	1288.72
Ki67	Germinal Centres	319-359 kDa	1320.76

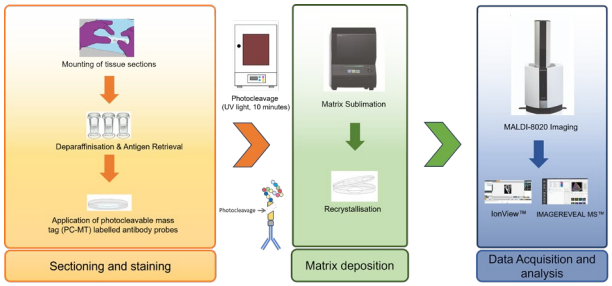

Fig. 2 Workflow for MALDI-IHC imaging using DHB matrix following staining with photocleavable mass tag-labelled antibodies

■ Data Acquisition (6-plex)

Imaging analysis was performed on a benchtop MALDI-TOF instrument (MALDI-8020, Shimadzu Corporation) (Fig. 1). Acquisition parameters are shown in Table 2. There were a total of 60201 profiles which were acquired over a period of 4 hours 8 minutes.

Table 2 Imaging Analysis Conditions of MALDI-8020

System	: MALDI-8020
Polarity	: Positive
Mass Range	: *m/z* 950-1500
Acquisition	: 50 shots @ 200Hz
Blanking	: 950
Pulsed Extraction	: 1500
Stage Step Size	: 30µm

Imaging data was processed using IonView™ and IMAGEREVEAL™ MS software packages (Shimadzu Corporation).

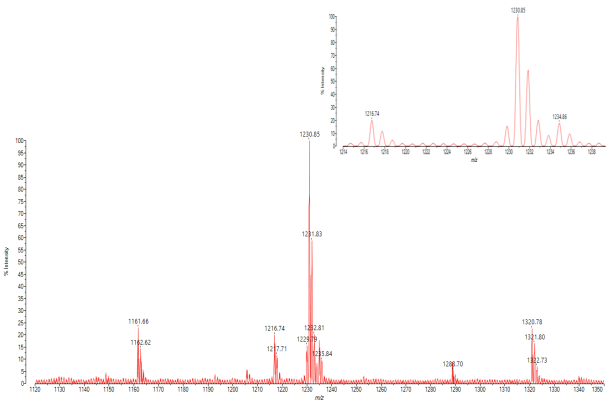

Fig. 3 MALDI-MS spectrum following imaging analysis of human tonsil stained with a 6-plex probe mix. Inset: Expanded view of spectrum between m/z 1200-1250 showing isotopic resolution of peaks at *m/z* 1216 (CD68), 1230 (VIM) and 1234 (Col-1A1).

■ Optimization of Matrix Sublimation

Following probe application and cleavage, samples were coated with DHB matrix using varying sublimation times. Alternate timings and temperatures for recrystallisation were also investigated. These parameters were found to have a significant impact on sensitivity and resolution.

Optimal results were achieved with a matrix sublimation time of 4 minutes (an approximate coating of 0.22 mg/cm^2) and recrystallisation at 3 minutes (5% IPA, 55 ˚C).

As shown in Fig. 3, isotopic resolution was easily achieved with a low level of background noise. Representative images of individual ions and overlays are shown in Fig. 4 and 5.

■ Data Acquisition (20-plex)

Imaging analysis was performed on a benchtop MALDI-TOF instrument (MALDI-8020, Shimadzu Corporation) (Fig. 1). Acquisition parameters are shown in Table 3. There were a total of 27300 profiles which were acquired over a period of 1 hour 52 minutes. Details of the probes applied are shown in Table 4. As shown in Fig. 6, all 20 probes applied have been identified in the spectrum. Representative images of individual ions and overlays are shown in Fig. 7 and 8.

Table 3 Imaging Analysis Conditions of MALDI-8020

System	: MALDI-8020
Polarity	: Positive
Mass Range	: m/z 700-2000
Acquisition	: 50 shots @ 200Hz
Blanking	: 700
Pulsed Extraction	: 1900
Stage Step Size	: 30µm

Table 4 Human Tonsil 20-plex Antibody Probe Mix (AmberGen)

Target	Tissue Expression	Mass Tag $(M+H)^+$
CD11b	Myeloid lineage cells Expressed in various cancers	1467.81
CD20	Normal and malignant B cells	997.52
CD3ε	T-cells	1161.64
CD4	Helper T cell, some monocytes & germinal centre macrophages	1293.74
CD44	Many cell types, common biomarker of cancer stem cells	1102.58
CD45RO	Memory T cells	1420.70
CD45RA	Naive T cells	1276.64
CD68	Macrophages, some myeloid elements, dendritic cells.	1216.74
CD8α	Cytotoxic T cells	1350.76
ECAD	cell-cell adhesion glycoprotein	930.55
GZMB	Cytotoxic T cells and NK cells	938.52
HER2	Transmembrane glycoprotein with tyrosine kinase activity.	1210.73
Histone H3	involved in the structure of chromatin in eukaryotic cells	1782.92
Ki67	Germinal centre, strongly associated with cell proliferation	1320.75
NCAM1 (CD56)	hematopoietic system, mostly natural killer cells but also other lymphoid cells	970.51
PD1	Activated T cells, natural killer cells, B cells, macrophages, dendritic cells and monocytes	1524.8281
PDGFR-B	Cell surface tyrosine kinase receptor	1125.6163
PDPN	mucin-type transmembrane glycoprotein specific to the lymphatic system.	954.5519
PR-A/B	Isoforms of human progesterone receptor	1244.9306
PTEN	Tumour suppressor gene	1132.5898

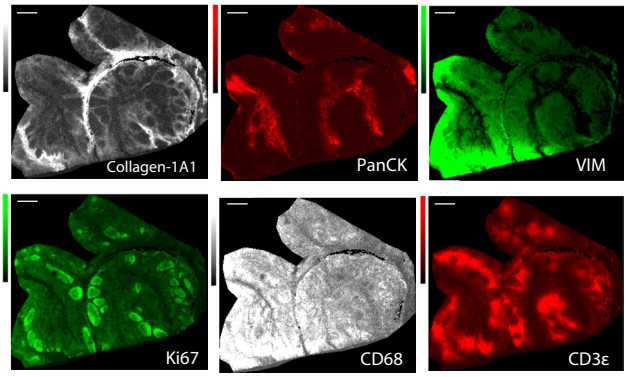

Fig. 4 Individual ion images after 6-plex optimization. Scale bar, 1mm.

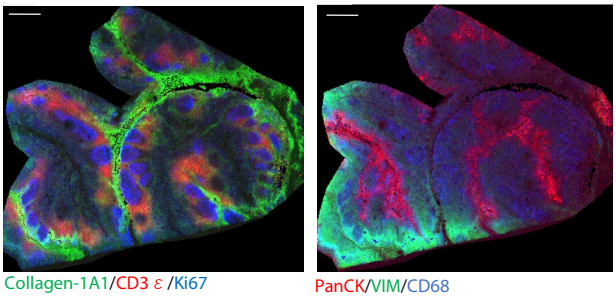

Fig. 5 Overlay of ion images from human tonsils stained with a 6-plex probe mix. Scale bar, 1mm.

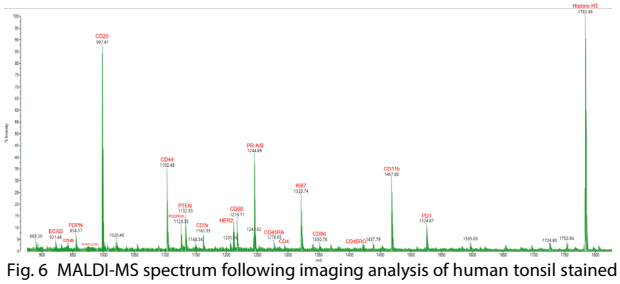

Fig. 6 MALDI-MS spectrum following imaging analysis of human tonsil stained with a 20-plex probe mix

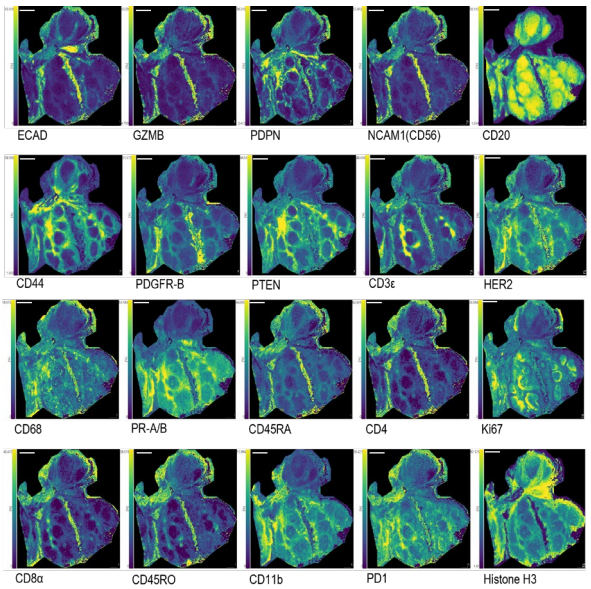

Fig. 7 Individual ion images following 20-plex acquisition images generated using IMAGEREVEAL software (Shimadzu Corporation)). Scale bar, 1mm.

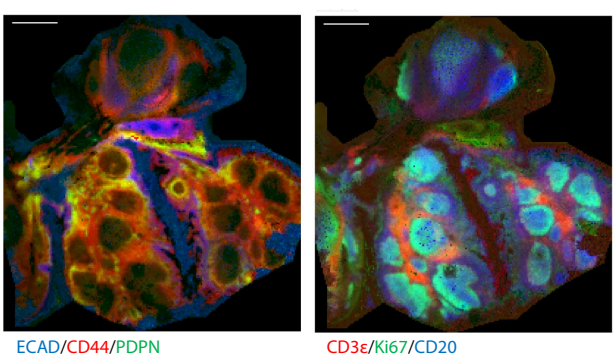

Fig. 8 Overlay of ion images from normal human tonsil stained with a 20-plex probe mix. Scale bar, 1mm.

■ Data Acquisition (Follicular Hyperplasia)

Follicular hyperplasia is a benign condition which is characterized by an increased number of germinal centres with variably sized mantle zones.[1] Ki67 is recognised as a useful diagnostic marker in immunohistochemistry for follicular hyperplasia, allowing identification and mapping of cell proliferation.[2]

Imaging analysis was performed on a benchtop MALDI-TOF instrument (MALDI-8030 EasyCare, Shimadzu Corporation) (Fig. 1). Acquisition parameters are shown in Table 5. There were a total of 27300 profiles which were acquired over a period of 1 hour 52 minutes. Representative overlaid images are shown in Fig. 9 which show the different patterns of expression seen in key markers of cell differentiation and T cell activity between normal tissue and tissue with follicular hyperplasia.

Table 5 Imaging Analysis Conditions of MALDI-8030 EasyCare

System	: MALDI-8030 EasyCare
Polarity	: Positive
Mass Range	: m/z 950-1500
Acquisition	: 50 shots @ 200Hz
Blanking	: 950
Pulsed Extraction	: 1500
Stage Step Size	: 30µm

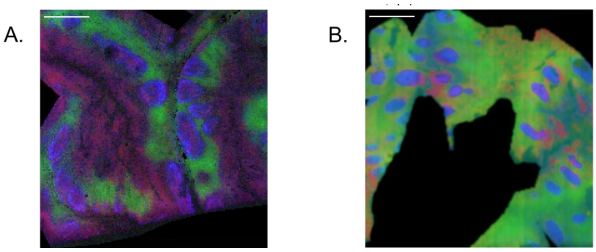

Fig. 9 MALDI-IHC images of PC-MT labelled antibodies from a 6-plex panel on normal human tonsil (A) and human tonsil with follicular hyperplasia (B) (ion images generated using IonView software (Shimadzu Corporation)). Scale bar, 1mm.

■ H&E Staining

Following imaging analysis, the matrix was removed using acetone and the tissue sections underwent H&E staining. Comparison of the H&E stained images with the ion overlays of the 6-plex probe mixture showed corresponding structures which aligned closely when overlaid (Fig. 10).

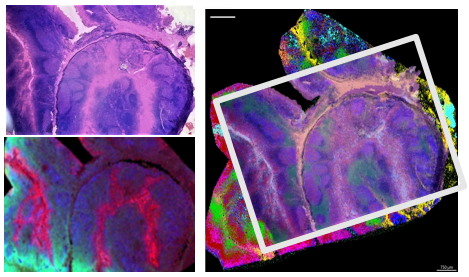

Fig. 10 H&E stained tissue shows comparable results to MALDI-IHC. Scale bar, 1mm.

■ Conclusions

We have successfully mapped multiplexes of both 6 and 20 peptide mass tags by MALDI-MSI on a low cost MALDI-TOF instrument. We have also demonstrated mass spectrometry imaging of follicular hyperplasia of the tonsil which showed different patterns of cell distribution in a key proliferation protein marker. MALDI-MSI is a versatile technique capable of revealing valuable information on biochemically significant compound distributions within a wide variety of samples. In combination with MALDI-IHC probes, the ease with which a range of molecules can be imaged has been extended significantly. Requiring a minimal sample size, MALDI-MSI is proving itself to be a valuable tool for research, facilitating a greater understanding of cellular interactions which is critical to the development new treatments.

The Shimadzu benchtop MALDI-8020 and MALDI-8030 are robust systems with class leading sensitivity, capable of a wide variety of applications. Following the introduction of the benchtop MALDI-TOF imaging starter kit, researchers are now able to explore MALDI imaging in a cost-effective way without committing funding to higher end systems. This is complemented by the introduction of MALDI-IHC probe kits further increasing the range of molecules which can be imaged using a straightforward sample preparation protocol. The use of FFPE tonsil tissue sections allows for ease of transport between facilities and the workflow for deparaffinization is well established and reliable. The resulting MS spectra were well-resolved (see inset, Fig. 3), allowing for the clear and specific generation of images. The benchtop systems can also be utilised within established imaging laboratories for method optimisation and to free up higher resolution/higher performance systems for studies requiring more detailed imaging analysis.

IonView is an easy-to-use software application that comes as part of the MALDI Solutions software suite that reads data directly from the instrument. Multiplexed images can be generated using the three primary channels (RGB). This package provides all the tools required for basic interrogation of your sample and provides an excellent solution for those initial experiments in imaging.

The imaging data was also exported in .imzml format for analysis in IMAGEREVEAL MS. The images were automatically generated using the targeted analysis feature; using a custom list of target peptide mass tag *m/z* values with an approximate window of ± 0.5Da. The patterns of expression for each antibody correlate clearly with those observed in the human protein atlas. IMAGEREVEAL is an advanced software platform capable of targeted analysis with multiple analysis modes and statistical analysis tools.

<References>

1. Gars *et al*, Ann Diagn Pathol 2020;44:151421
2. Bryant *et al*, Histopathology 2006 Apr;48(5):505-15

<Related Applications>

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