

# Multiplex and Multiomic Imaging of targeted proteins and small molecules: a new application of the MALDI HiPLEX-IHC workflow

Spatial proteomics enables drug-target localization using MALDI Imaging

## Abstract

Immunohistochemistry (IHC) is a widely used tool for histologists and pathologists, often being combined with fluorescence microscopy to target proteins specific to relevant pathways and processes. The wide emission bands of fluorescent tags greatly limits the number of probes that can be utilized in one experiment. In contrast, MALDI Imaging and in a true multiomic fashion allows spatial information for every ionizable compound to be obtained simultaneously. Here, we demonstrate the ability to link IHC with MALDI Imaging using the MALDI HiPLEX-IHC workflow, including the colocalization of signals across multiple acquisitions from the same tissue.

Keywords:  
HiPLEX, Proteins, Imaging,  
Multimodal, Multiplex

## Introduction

MALDI Imaging is used to determine the spatial distribution of molecules in a tissue section, for maximum biological information. However, competing factors in MALDI ionization can interfere with the ability to analyze intact proteins from tissue, and can thus require extensive sample handling. Conversely, immunohistochemistry (IHC) is widely used to determine the structural organization of proteins by utilizing the specificity of antibodies, but can typically only visualize a maximum of five or six tags through fluorescence microscopy at a time. The MALDI HiPLEX-IHC workflow combines MALDI Imaging with IHC to generate highly multiplexed images [1] that can be combined with other imaging techniques for multimodal data, or subsequent MALDI imaging runs for multiomic data. Here, we present an example of the multiplexed imaging experiments and demonstrate the potential of this combination towards imaging drugs in tissue.

## Methods

### Pre-clinical Study Tissues

Non-perfused whole brain tissue from a female 17-18 wk old C57BL/6 mouse dosed i.v. with 10 mg/kg JQ1 (10% DMSO formulation) was provided by St. Jude Children's Research Hospital (Memphis, TN). Animal studies were approved by the St. Jude Children Research Hospital Institutional Animal Care and Use Committee.

## MALDI Imaging

Coronal tissue sections (10  $\mu\text{m}$  thickness) were thaw-mounted on to Bruker IntelliSlides<sup>®</sup>. Slides were spray coated with DHAP (15 mg/mL, 60% ACN, 0.1% TFA) using an HTX M3+ Sprayer<sup>™</sup> system. Isotopically labeled JQ1 (99%, D2, 0.15 mg/mL) was doped into the matrix as an internal/normalization standard. Lipid ion images were acquired on a Bruker timsTOF fleX (20  $\mu\text{m}$  spatial resolution, positive ion mode, TIMS enabled) from  $m/z$  300-1200.

## MALDI HiPLEX-IHC Imaging

Tissues slides were subjected to MALDI HiPLEX-IHC staining according to a previously published protocol [1], coated with DHB through sublimation (140°C, 6-7 min heating, 5-7 min cooling), then the matrix was recrystallized (1 mL 5% IPA atmosphere in over at 55°C). All images were acquired with the timsTOF fleX (20  $\mu\text{m}$  spatial resolution, positive ion mode), from  $m/z$  850-2000.

**Figure 1**

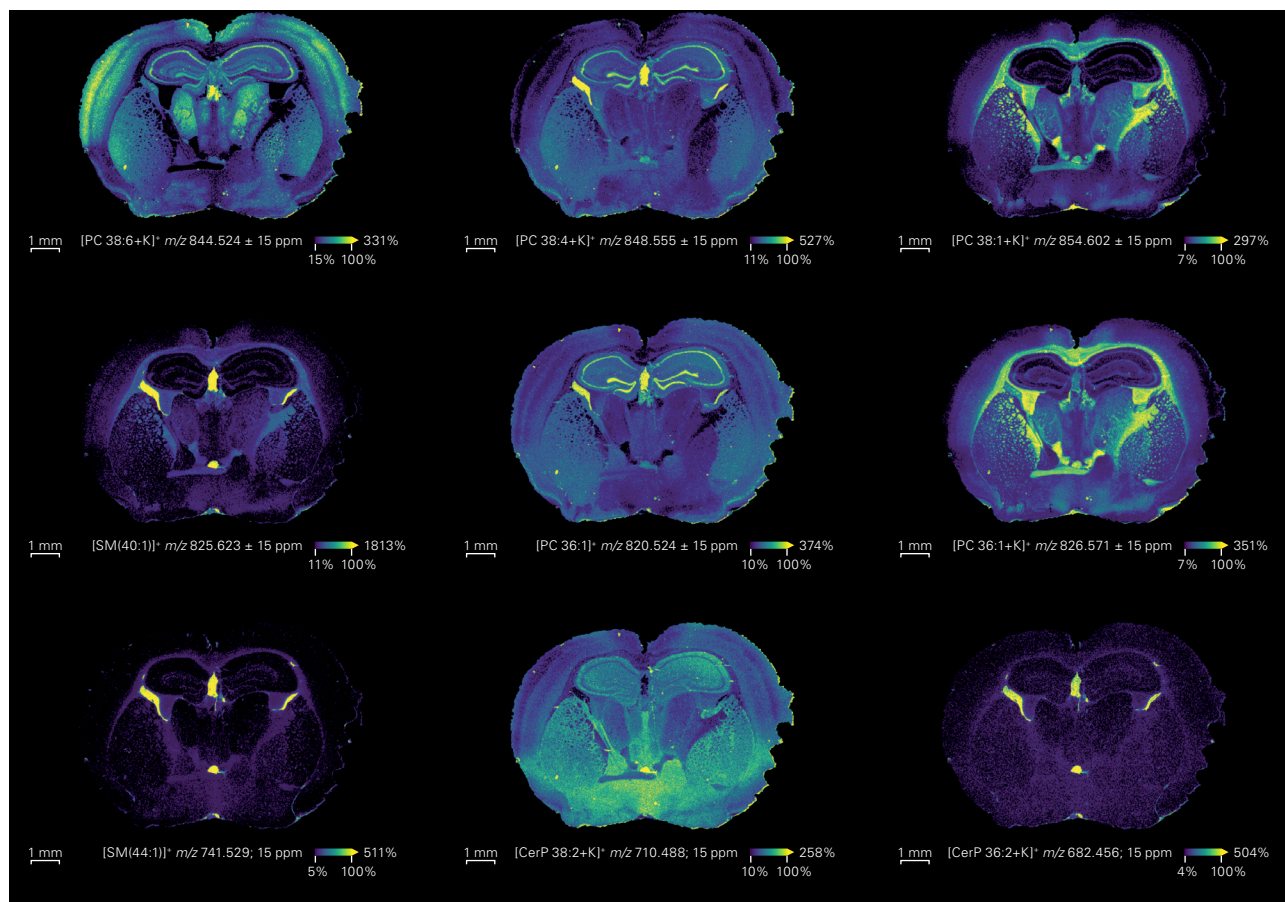
Extracted ion images of selected lipids found in the mouse brain.

This is the first MALDI acquisition from this tissue; later a second acquisition is acquired from this same tissue utilizing the MALDI HiPLEX-IHC workflow (see Figure 3). All images processed with SCiLS<sup>™</sup> Lab 2023a. All annotations performed with MetaboScape<sup>®</sup> 2023a.

## Data Analysis

Data visualization was done using SCiLS<sup>™</sup> Lab 2023a software (Bruker Daltonics GmbH & Co. KG, Germany), and tentative lipid annotations were assigned with MetaboScape<sup>®</sup> 2023a (Bruker Daltonics GmbH & Co. KG) using the measured accurate mass. Images are presented with TIC normalization. SCiLS Lab was used to determine colocalized species with the accurate mass of the desired ion.

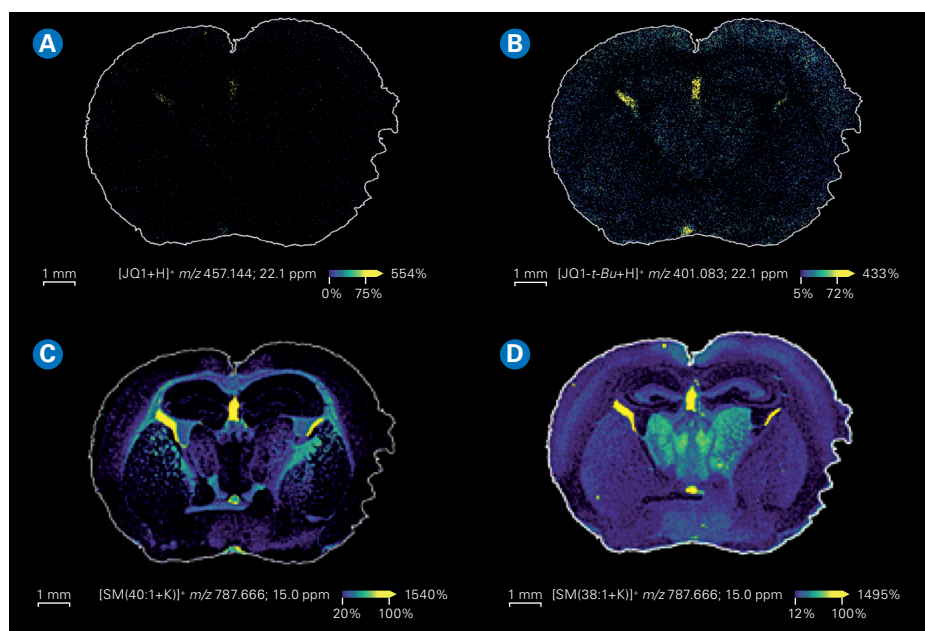
SCiLS Lab was used to further group similar pixels in the lipid image into segmented regions based on having similar mass spectra, resulting in effective segmentation maps. Annotations in MetaboScape 2023a were performed from two regions of the segmentation map. Annotations were assigned using a maximum 5 ppm mass tolerance using a custom target list utilizing Lipid Blast for Metaboscape and the Lipid MAPS target list available online.



## Results and Discussion

### Initial Imaging – Lipids and JQ1

The initial data collection acquired targeted lipids and the introduced drug target, JQ1, with select examples shown in Figure 1. The ion intensities for common lipid signals were in good abundance, generating images demonstrating regions typically associated with the mouse brain. For example, the hypothalamus and Purkinje cells are clearly visible in the various images of phosphatylcholines shown in Figure 1. JQ1 ( $m/z$  457.144  $[M+H]^+$ ), however, is difficult to ionize, as indicated in Figure 2A. Further, it appears JQ1 is potentially undergoing hydrolysis in preparation, indicated by the observation of an ion at the respective mass of a deterbutyoxylated species,  $m/z$  401.083 ( $[M+H-C_4H_8]^+$ , JQ1-*t-Bu* for short), seen in Figure 2B. It is worth noting that this potentially deterbutyoxylated species presents the same isotopic distribution as JQ1. Due to JQ1 possessing a chlorine and sulfur heteroatom, it has a unique isotopic distribution useful for fingerprinting such molecules as a deterbutyoxylated product. Using both the parent and the deterbutyoxylated ion, JQ1 is observed primarily in the ventricles, which is consistent with introduction to the brain at  $t=0$ . However, there may be traces of JQ1 distributing out from the ventricles and into the brain stem visible in the image at  $m/z$  401.083, representing JQ1-*t-Bu*.



**Figure 2**

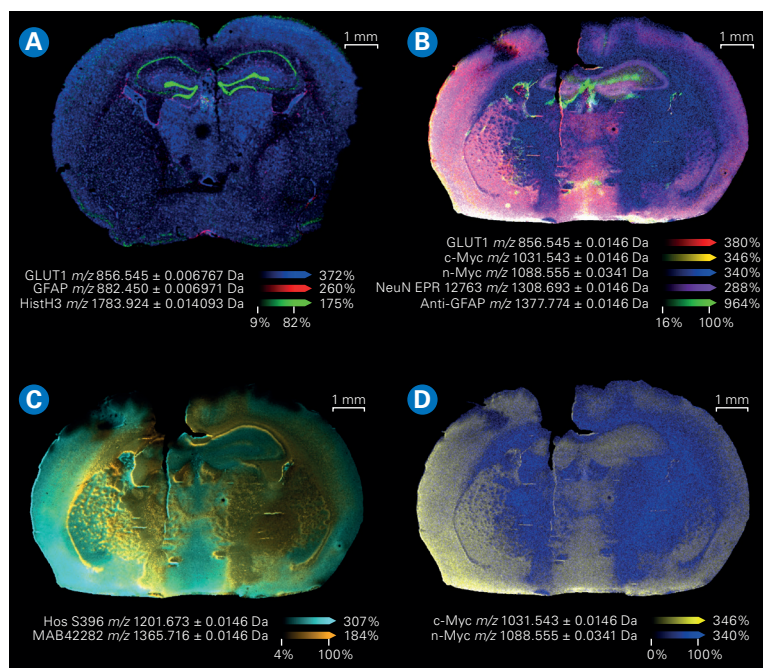
**(A)** Extracted ion image of  $[JQ1+H]^+$ , **(B)** extracted ion image of  $[JQ1-t-Bu+H]^+$ . The remaining images were found to be colocalized using SCiLS™ Lab, with **(C)** showing the extracted ion image of  $[SM(40:1+K)]^+$  and **(D)** showing the extracted ion image of  $[SM(38:1+K)]^+$ . Images of ions from (C) and (D) both show the most colocalization with  $[JQ1-t-Bu+H]^+$ .

Due to the limited intensity of the parent JQ1, the ion image for JQ1-*t-Bu* was used to identify lipids colocalized to the drug. Using both the output of the colocalization with the tentative annotations from Metaboscape® utilizing the exact mass and collisional cross section, over 20 lipids were colocalized, with two examples being shown in Figures 2C and 2D;  $[SM(40:1+K)]^+$  and  $[SM(38:1+K)]^+$ .

## MALDI HiPLEX-IHC

After the initial lipid images were acquired, the same tissue was stained with antibodies from using the MALDI HiPLEX-IHC process, and the tissue was imaged again. Notably, Figure 3A shows an image of proteins from the same tissue as the lipid images shown in Figure 1 and Figure 2. The image in Figure 3A was performed with a limited antibody panel, only multiplexing five antibodies. The resulting image clearly does show the specific binding of HistH3 in the corpus collosum, while Glut1 and GFAP show no binding in that region. To further showcase the potential of using MALDI HiPLEX-IHC workflow, another section was stained using a larger suite of antibodies (14 in total). The multiplexed image of six antibodies from that experiment is shown in Figure 3B, with an example of the specific bindings using the same color coding shown in Figure 3C and Figure 3D. Particularly of interest is Figure 3D, which shows the distribution of n-MYC and c-MYC. MYC is typically upregulated in human cancer cells, and bromodomain inhibition has been shown to suppress MYC-associated transcriptional activity in said cancer cells. Here, the ability to both image JQ1 and MYC from

the same tissue demonstrates the potential to apply the multimodal imaging model presented to real samples. For example, a cancer model brain would have MYC upregulated, and the effect of introducing JQ1 could be measured by seeing a decrease in the expression of MYC in the test tissue, validating drug localization and activity.



**Figure 3**

Composite color plots of extracted ion images of the mass tags used in the MALDI HiPLEX-IHC workflow.

**A** Multiomics demonstration with lipids and drug target being imaged in Figure 2 and the HiPLEX workflow on the same tissue shown here. **B** Composite color plots of extracted ion images of additional multiplexed antibody tags from the MALDI HiPLEX-IHC workflow. **C** and **D** are composite ion images using select mass tags to specifically compare targeted proteins of interest for this system.

## Conclusion

By utilizing the MALDI HiPLEX-IHC workflow, targeted whole protein localization was linked to the distribution of a target drug molecule and lipids. Specifically, due to the low intensity of the drug JQ1, colocalization was used to suggest possible distributions of the drug, with examples showing unique distributions throughout the rest of the tissue. Two separate imaging acquisitions were used to acquire images for all positive ions detected between  $m/z$  300 and 1200, as well as targeted protein localizations utilizing the mass tags from the MALDI HiPLEX-IHC process. Drug interactions with proteins of interest, as well as additional small molecule signals, are all accessible with MALDI Imaging workflows.

## References

- [1] Yagnik G, Liu Z, Rothschild KJ, Lim JM 2021. *J Am Soc Mass Spectrom*, **32**:977-988

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**Bruker Switzerland AG**

Fällanden · Switzerland  
Phone +41 44 825 91 11

**Bruker Scientific LLC**

Billerica, MA · USA  
Phone +1 (978) 663-3660

