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Triple Quadrupole Mass Spectrometry Imaging (MSI): A Pipeline for Acquisition and Analysis of MRM MSI Data with AP-MALDI

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Overview

The application note describes AP-MALDI mass spectrometry imaging (MSI) of acyclovir and its labeled analog from the tissue section. MSI helps to correlate the spatial information of the pharmaceutical compounds. Furthermore, multiple reaction monitoring (MRM) schemes enhance the identification of the pharmaceutical drug, increasing sensitivity and confidence in the analysis.

Introduction

Acyclovir is an antiviral drug used in the treatment of herpes, shingles. It acts as a nucleoside analog and inhibits viral replication processes after its uptake by cells. Pharmaceutical drug discovery approval processes require stringent and routine absorption, distribution, metabolism, and excretion (ADME) drug studies (Lin *et al.*, 2003). Therefore, understanding the side-effects and toxicity profiles of the pharmaceutical drugs in tissues is essential in drug discovery processes. Ideally, liquid chromatography (LC) hyphenated with triple quadrupole (QQQ) based multiple reaction monitoring (MRM) methods from blood plasma and tissue homogenates are employed (Baillie *et al.*, 2008). MRM methods are sensitive and specific for drug metabolites (Youdim *et al.*, 2010). However, the spatial distribution of the drug and its metabolites cannot be investigated from tissue homogenates or blood plasma. Thus, the spatial distribution of pharmaceutical drug candidates and their metabolites from tissues is a crucial area of research in drug discovery processes.

Several recent studies have reported spatial distribution of pharmaceutical drug and their metabolites with an emerging area of research, mass spectrometry imaging (MSI) (Sun

et al. 2013, Groseclose *et al*. 2013, Atkinson *et al*. 2007, Balluff *et al*. 2012). With this approach, tissue sections are either coated with a UV-absorbing organic matrix followed by subsequent laser desorption/ionization (MALDI) analysis, or desorption electrospray ionization (DESI) is performed directly on the tissue sections. MALDI MSI is the often explored approach for MSI. However, interferences from organic matrix pose as one of the limitations for pharmaceutical drug distribution research. However, powerful high-resolution mass analyzers can circumvent this issue. MALDI instruments are in-built with ionization sources connected to mass analyzers. An offline ionization source such as atmospheric pressure (AP)-MALDI opens up the possibility of hyphenating MALDI with any mass analyzer. This application note showcases a workflow for acquiring the spatial distribution and quantitation of pharmaceutical drugs from spiked tissue sections with AP-MALDI QQQ MRM and subsequent imaging data analysis.

Key Features

Multiple Reaction Monitoring

MRM schemes for related compounds can be established within a single imaging run. Precursor and product ions can be monitored together within a run.

Mozaic Software (Spectroswiss)

Mozaic software from Spectroswiss is the only existing software solution available for analyzing QQQ MSI MRM data. It offers MRM imaging, on-the-fly mass calibration, support for reduced and unreduced data, co-registration with optical images.

SunChrom Sprayer

The sprayer from SunChrom offers uniform matrix deposition on different tissue types. Matrix deposition is automated and several features can be controlled.

Capillary Extenders

Specially designed capillary extenders by MassTech for Sciex NanoDCI interface and OptiFlow interface.

Figure 1 shows a general workflow for sample preparation, instrumentation and data analysis with AP-MALDI. Figure 2 shows the instrumentation of AP-MALDI with QQQ.

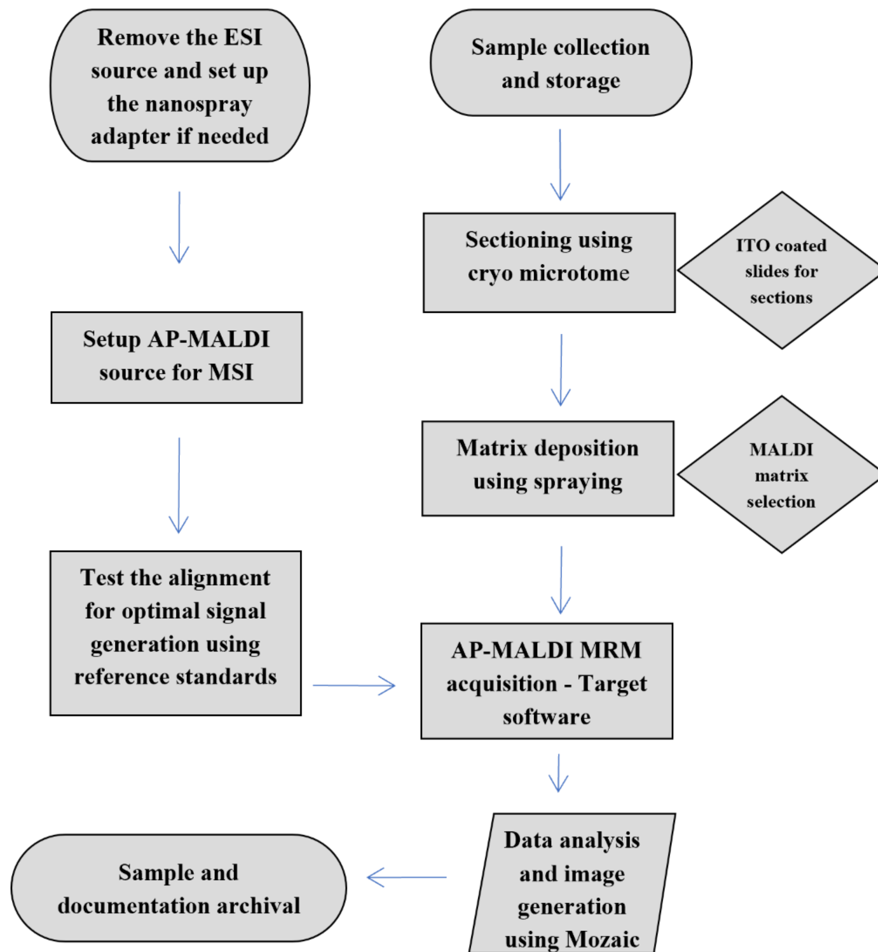


Figure 1: Workflow of AP-MALDI MRM MSI used in the current study.

Figure 2: Instrumentation used in the current study.

Experimental

Standards and Internal Standards (IS)

Reserpine aqueous standard, acyclovir and deuterated acyclovir (acyclovir D4) standard samples were kindly shared by Raptim, India. LC-MS grade methanol was purchased from Thermo Fisher. α-Cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA) and formic acid were procured from Sigma-Aldrich. Deionized water with specific resistivity of 18.2 Ω cm^{-1} was obtained using a water purification unit from Millipore. Permanent markers of different ink colors such as red, green, blue and black were purchased from a local retailer.

Preparation of Standard solutions

Working dilutions of 0.0107-1 pg/µL were prepared for reserpine. Acyclovir and acyclovir D4 standards were diluted to achieve a working range of 0.1 pg/µL to 100 µg/mL using methanol. MALDI matrix (10 mg CHCA) was prepared in a 50: 50 ratio of methanol- 0.1% TFA in deionized water.

Quantitation of reserpine, acyclovir and acyclovir-D4

To check the response of pharmaceutical drugs at lower concentrations, serial dilutions of acyclovir and acyclovir D4 standards were prepared to achieve the working range of 0.1 to 1000 pg/µl using methanol and spotted on stainless steel AP-MALDI target plate. Six replicates of reserpine and 4 replicates of acyclovir and acyclovir-D4 mixtures were spotted.

Mass Spectrometry Imaging with Marker Inks

An ITO slide was streaked with four different colored marker inks, namely, red, blue, green and black.

Tissue Imaging Sample preparation

For MSI analysis, remove the ITO slide containing the fresh frozen tissue section from -80° C freezer and keep it in the desiccator for 30 minutes for complete drying. Uniformly spray the freshly prepared (80:20; v/v) CHCA matrix solution on the tissue section using the matrix sprayer and let it air dry. An equal volume of sample (acyclovir and deuterated

standard) and matrix was premixed and 1 µL of the mixture was spotted on tissue section placed on ITO coated glass slide and tissue section. The final concentrations of the acyclovir and deuterated standard was 10, 50, 80 and 100 pg/µL. The slides were dried prior to the analysis.

MS/MS Detection

All experiments were performed on a SCIEX QTRAP 6500+ mass spectrometer (SCIEX) coupled to an AP-MALDI (ng) UHR ion source (MassTech Inc., Columbia, MD USA) with a 355 nm Nd: YAG laser source.

AP-MALDI SCIEX QTRAP was first optimized with serial dilutions of reserpine, acyclovir and acyclovir-D4 directly from the ITO slide.

For analysis of pharmaceutical drugs on ITO coated glass slide, working dilutions of reserpine (0.0107-1 pg/µL) and mix of acyclovir and deuterated acyclovir (acyclovir D4) (10-100 pg/µL) were premixed with CHCA matrix. The premixed analyte mixtures were spotted (*n*=2) on ITO coated glass slide and allowed for drying prior to the analysis (Figure 2).

For MRM analysis of reserpine, acyclovir and acyclovir D4 standards, ion spray voltage was optimized to 1000 V and interface heater temperature was set at 150°C for best signals. The declustering potential (DP) was set to 90V for acyclovir and 10V for acyclovir D4. The collision energy (CE) was optimized at 15 for acyclovir and 14 for acyclovir D4 to achieve fragmentation.

For MSI data acquisition in MRM mode, ion spray voltage was optimized to 1000 V for acyclovir standard, DP and interface heater temperature was set at 90° and 150°C for best signals. The CE was set to 47 for acyclovir and acyclovir D4 mixture to achieve fragmentation. The laser energy of AP-MALDI ion source was optimized at 80% for the analysis and laser repetition rate was 8 kHz.

Software and Data processing

AP-MALDI SCIEX QTRAP data was acquired in MRM mode using the Analyst 1.7.1. AP-MALDI MSI data acquisition was performed with Target software. The obtained AP-

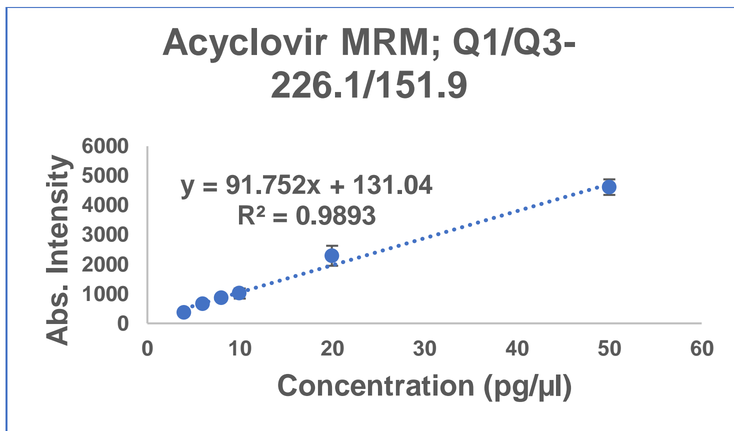
MALDI MRM MSI data were analyzed with Mozaic software (Spectroswiss, Switzerland). Briefly, the .wiff raw MSI data files were imported in Mozaic. The files were converted to .imzML files with an in-built Proteowizard application in Mozaic. The imzML files were read by Mozaic and the MRM images were plotted.

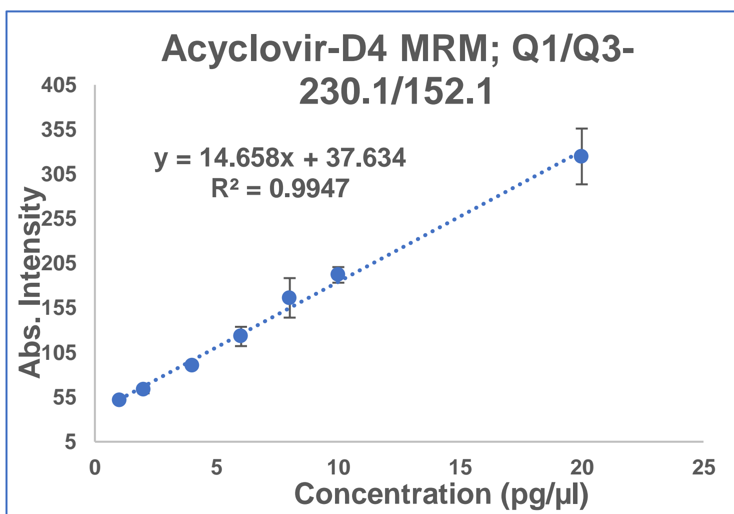
Results and Discussion

Parameters such as laser energy, declustering potential (DP) and collision energy (CE) were optimized for the analysis. Laser alignment and camera focusing were also performed prior to the analysis. The $[M+H]^+$ ions for reserpine is 609.3u. The MRM transitions for reserpine, acyclovir and acyclovir-D4 are already established in literature. The precursor ion of reserpine was selected in Q1 and the product ion at 195.1u was scanned in Q3 **(Table 1)**. Acyclovir and acyclovir-D4's $[M+H]^+$ ions are 226.1u and 230.1u.The MRM transition of reserpine (Q1/Q3; 609.3/195.1), acyclovir (Q1/Q3; 226.1/151.9) and acyclovir D4 (Q1/Q3; 230.1/152.1) were observed for the analyte even at low concentrations. **Figure 3** shows the calibration curve of reserpine, acyclovir and acyclovir-D4 from AP-MALDI target plate. The calibration curves show excellent linearity (R^2 = 0.99) with %RSD below 10% for each calibration point. The results clearly indicate the accurate identification, confirmation and quantitation of pharmaceutical drugs with AP-MALDI. After successful demonstration on steel plate, the method's performance on ITO coated glass slide was examined.

Table 1. Observed MRM transitions of reserpine, acyclovir, acyclovir-D4 and permanent marker inks

Sr. No.	Compound	MRM; Q1/Q3
1	Reserpine	609.3/195.1
2	Acyclovir	226.1/151.9
3	Acyclovir-D4	230.1/152.1
4	Red ink	443.2/399.1
5	Blue ink	456.4/412.4
6	Green ink	471.4/349
7	Black ink	372.3/356.2





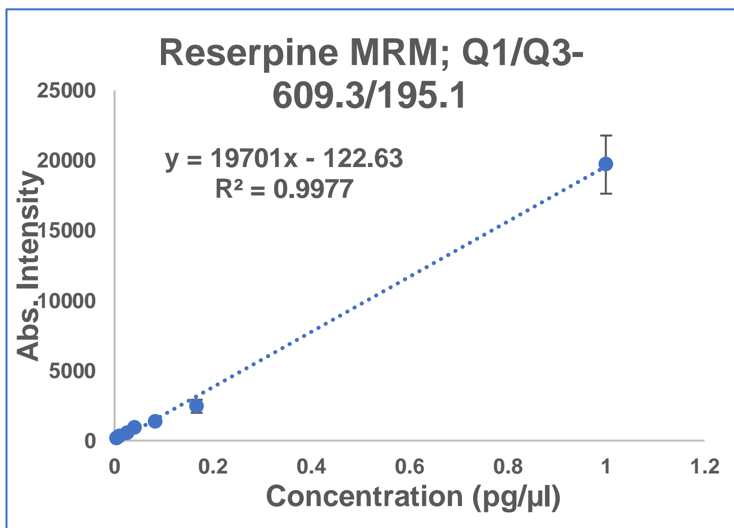


Figure 3: Calibration curves of acyclovir, acyclovir-D4 and reserpine across concentration ranges of 4-50, 1-20 and 0.0041-1 pg/µL, respectively.

Detection of reserpine, acyclovir and acyclovir-D4 from ITO slide with AP-MALDI SCIEX QTRAP 6500+

The MRM transitions of acyclovir and acyclovir D4 mixtures; and reserpine working dilutions **(Figure 4)** yielded excellent absolute intensities from ITO coated slide. The absolute intensities increased with increasing concentrations **(Tables 2&3).** The absolute intensities were in direct proportion with the concentrations. Significantly, normalization with any internal standard was not required, similar to our previous observation with steel plate. MALDI processes ideally require normalization. Although our observation of direct correlation of absolute intensity of MRM with concentration can help in designing further pharmaceutical applications with AP-MALDI QQQ analysis. After obtaining linear response with the pharmaceutical drugs, we implemented AP-MALDI MRM MSI.

Figure 4: Reserpine aqueous standard and mix of acyclovir and acyclovir D4 standard spotted

on ITO coated glass slide

Sr. No	Concentration (pg/µl)	MRM Q1/Q3- 609.3/195.1		Average of Abs. intensity
		Abs. intensity Spot 1	Abs. intensity Spot 2	
1	0.0107	182	193	187.5
2	0.0835	165	188	176.5
3	0.167	357	465	411
4	1	3345	3568	3456.5

Table 2- Absolute intensities of reserpine MRM transitions observed on ITO coated glass slide

Sr. No	Concentration (pg/µl)	Acyclovir MRM Q1/Q3- 226.1/151.9 Abs. intensity		Average of Abs. intensity	Acyclovir-D4 MRM Q1/Q3- 230.1/152.1 Abs. intensity		Average of Abs. intensity
		Spot 1	Spot 2		Spot 1	Spot 2	
1	10	122	162	142	34	36	35
2	50	613	693	653	80	74	77
3	80	851	984	917.5	113	126	119.5
4	100	1276	1307	1291.5	166	170	168

Table 3- Absolute intensities of acyclovir and acyclovir-D4 MRM transitions observed on ITO coated glass slide

Mass spectrometry imaging of permanent marker pen inks

Before beginning experiments with tissue, marker ink AP-MALDI MRM MSI experiment was executed. The marker inks $[M+H]^+$ ions were 443.2u (red), 456.4u (blue), 471.4u (green) and 372.3u (black). The following MRM transitions were monitored across the selected region of interest (ROI): red ink – Q1/Q3 443.2/399.1, blue ink – Q1/Q3 456.4/412.4, green ink – Q1/Q3 471.4/349 and black ink – Q1/Q3 372.3/356.2 **(Table 1)**. With Mozaic, we could successfully visualize the MRM transitions as images. **Figure 5a** shows successful co-registration of the different MRM transitions represented with different colors (green ink - green, blue ink – blue, red ink – red and black ink – white) with the optical image (original optical image shown at bottom right). **Figure 5b** shows selected ion chromatograms of the black and blue inks. The intensities of the chromatograms are different and while setting up a quantitative workflow, the selected ion chromatograms can give a lot of information.

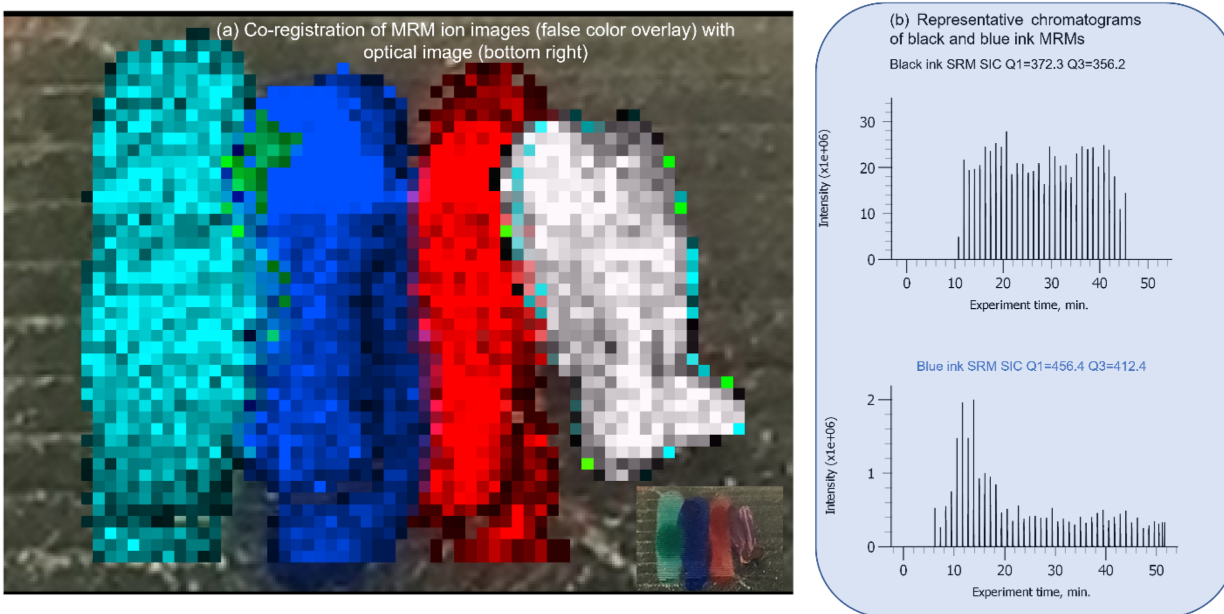


Figure 5: (a) AP-MALDI MRM MSI reconstructed ion images of permanent marker inks co-registered with optical image and (b) selected ion chromatograms of black and blue permanent marker inks.

Tissue Imaging using AP-MALDI SCIEX QTRAP

Different concentrations of acyclovir and acyclovir-D4 spiked on tissue were analyzed in tissue imaging mode. The MRM transitions for acyclovir and acyclovir-D4 were scanned throughout the selected region of interest. **Figure 6** left panel focuses on the region of interest chosen for analysis. The image at the top shows acyclovir's MRM ion image (in red) and that at bottom shows acyclovir-D4's MRM ion image (in blue) co-registered with the optical image. Optical image is provided in the middle for reference. Importantly, different concentrations of the analytes could be spatially resolved with MSI. **Figure 6** right panel shows the overlaid MRM ion images (pink) of acyclovir (red) and acyclovir-D4 (blue). The intensity of the images correlated with the concentrations. 10 pg/µL couldn't be clearly discerned for acyclovir, although the lowest concentration for its labelled analog could be easily distinguished.

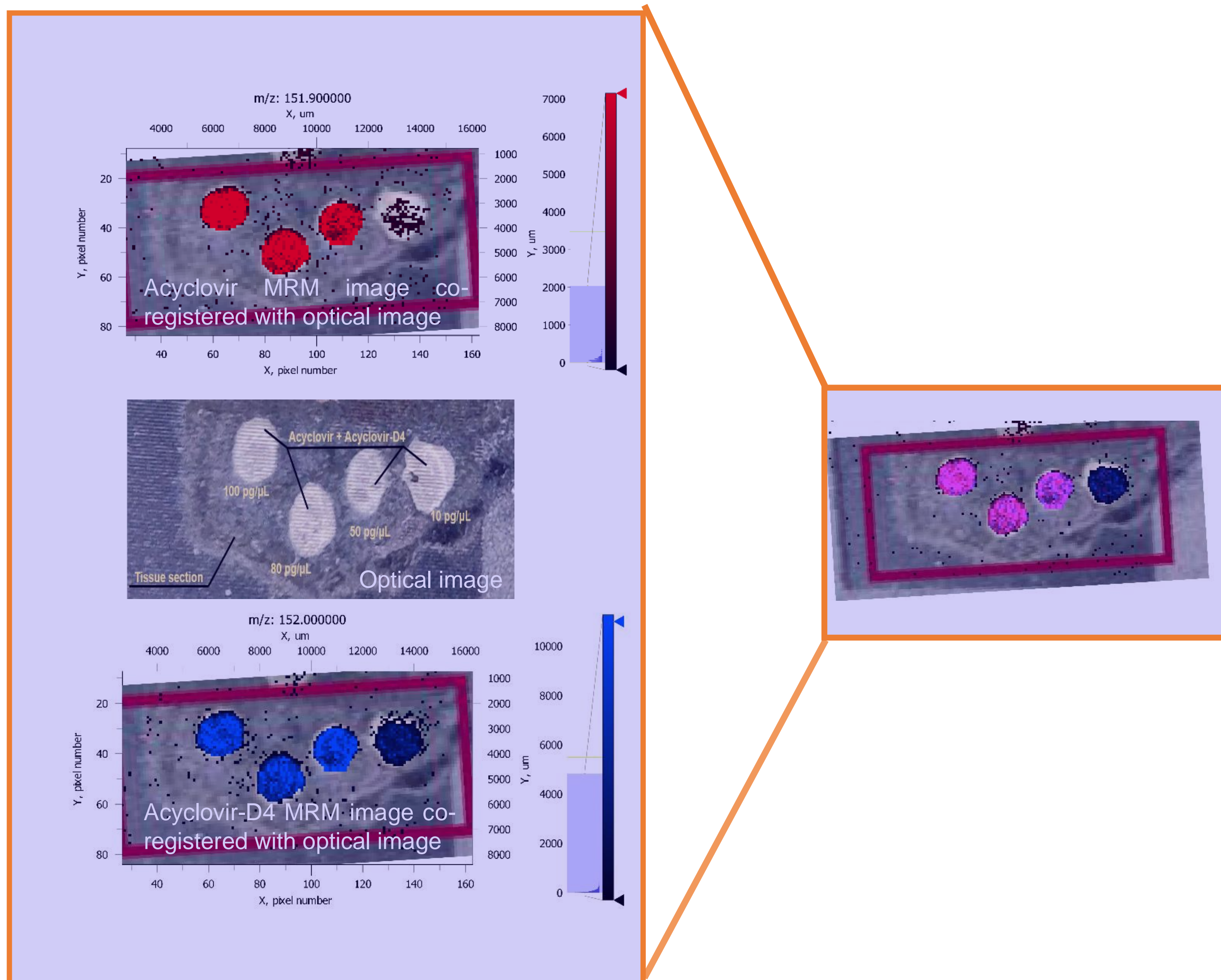


Figure 6: AP-MALDI MRM MSI of different concentrations of acyclovir and acyclovir-D4 spiked on a tissue section co-registered with the optical image. Left panel shows acyclovir (red) MRM image at top, optical image in the middle and acyclovir-D4 (blue) MRM image at the bottom. Right panel shows the overlaid images of acyclovir and acyclovir-D4 merged together in pink.

Summary

Other reports have used AP-MALDI for different studies (Gudlavalleti *et al* 2008 , Keller *et al*. 2018 , Schneider *et al*. 2005 , Stutzman *et al*. 2012 , McLean *et al*. 2003 , Östman *et al*. 2013 , Cohen *et al*. 2002). With the above results we demonstrate the compatibility of AP-MALDI with triple quadrupole SCIEX QTRAP 6500+ and establish MRM assays for the spatial analysis of pharmaceutical drugs spiked on tissue section. Furthermore, we show how the AP-MALDI MRM MSI data can be analyzed with Mozaic software. In summary, we establish a pipeline for performing AP-MALDI MRM MSI data collection, analysis and interpretation.

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