Application Note

Biotherapeutics and Biosimilars

Agilent

Trusted Answers

Targeted Spatial Quantitative Imaging of Metabolites in Paraffin-Embedded Biospecimens

Imaging with AP-MALDI coupled to an Agilent 6495 triple quadrupole mass spectrometer broadens opportunities for clinical follow-up

Authors

Xi Qiu and Daniel Cuthbertson
Agilent Technologies, Inc.

Tharun Teja Ponduru,
Vasanta Putluri,
Christy Charles,
Franklin Gu, Uttam Rasaily,
Chandrashekar Ambati,
Jie Gohlke, Nagireddy Putluri,
and Arun Sreekumar
Baylor College of Medicine,
Houston, TX, USA

Vishal Mahale,
Nivedita Bhattacharya, and
Venkateswarlu Panchagnula
MassTech, Inc.
Columbia, MD, USA

Abstract

A new method of mass spectrometry imaging is demonstrated for spatial localization of metabolites in paraffin-embedded and flash-frozen tumor specimens. The method couples matrix-assisted laser desorption/ionization at atmospheric pressure (AP-MALDI) with mass spectrometry (Agilent 6495 triple quadrupole mass spectrometer) to provide spatial context to metabolic analysis. Understanding spatial context can potentially offer insights about the interactions of a tumor with its microenvironment, giving further insight to tumor development and progression. Unlike most other mass spectrometry imaging methods, this approach permits analysis of metabolites in tissue embedded in paraffin. As result, these spatial insights are available to support long-term clinical follow-up based on paraffin-embedded samples.

Introduction

Metabolic changes are hallmarks of cancer progression. Spatial localization of metabolites can potentially offer insights about the interactions of the tumor with its microenvironment, giving further insight to tumor development and progression.

Recently, techniques for mass spectrometry imaging (MSI) enhanced by ion desorption electrospray ionization (DESI) and matrix-assisted laser desorption/ionization (MALDI) have evolved to provide spatial context to metabolic analyses. In this application note, a first-in-field MSI technique combining a MALDI ion source operating at atmospheric pressure (AP-MALDI) with an Agilent 6495 triple quadrupole mass spectrometer (Figure 1) is described.

This application note reports the use of this new imaging platform to evaluate the spatial distributions of several metabolites in both formalin-fixed, paraffin-embedded (FFPE) tissues and flash-frozen tissues in a targeted manner. The study examined changes in levels and localization of key predicted metabolites in xenograft tumors obtained from genetically modified cell lines. These measurements included quantification and spatial localization of several metabolites in two environments. The study first focused on adenosine in tumors containing overexpression and knockdown of adenosine deaminase (ADA, an enzyme that converts adenosine to inosine) (Objective 1). Then, kynurenine and tryptophan were analyzed in tumors containing knockdown and rescue of alpha amino adipate amino transferase (AADAT, which transaminates kynurenine to kynurenic acid) (Objective 2).

Experimental

Experimental design

Several avenues were explored to optimize the method, including evaluation of the AP-MALDI matrix, choice of analytical variables, instrument parameters, and analytical method (tested for more than 15 polar metabolites). The demonstration results presented focus on metabolites of ADA (Objective 1) and AADAT (Objective 2).

For Objective 1, the analysis was conducted on FFPE samples of xenograft tumors derived from MDA-PCa-2a prostate cancer cells. Three conditions were compared: ADA overexpression, ADA knockdown (ADA-KD) by sh-RNA in cells containing ADA overexpression, and control. One sample was obtained for each condition. In each sample, three arbitrarily selected regions were analyzed for adenosine (268.1 → 136.0) and more than 15 other metabolites, including creatinine, histidine, kynurenine, putrescine, and tryptophan.

For Objective 2, the analysis was conducted on flash-frozen samples of xenograft tumors derived from E0071 murine breast cancer cells. The conditions compared were cells containing AADAT knockdown (AADAT-KD), cells in which AADAT had been genetically re-expressed, and control. One sample was obtained for each condition, and three regions were selected on each sample, as in Objective 1. Each region was analyzed for kynurenine (209.1 → 191.9) and tryptophan (205.1 → 188.0), as well as multiple other metabolites.

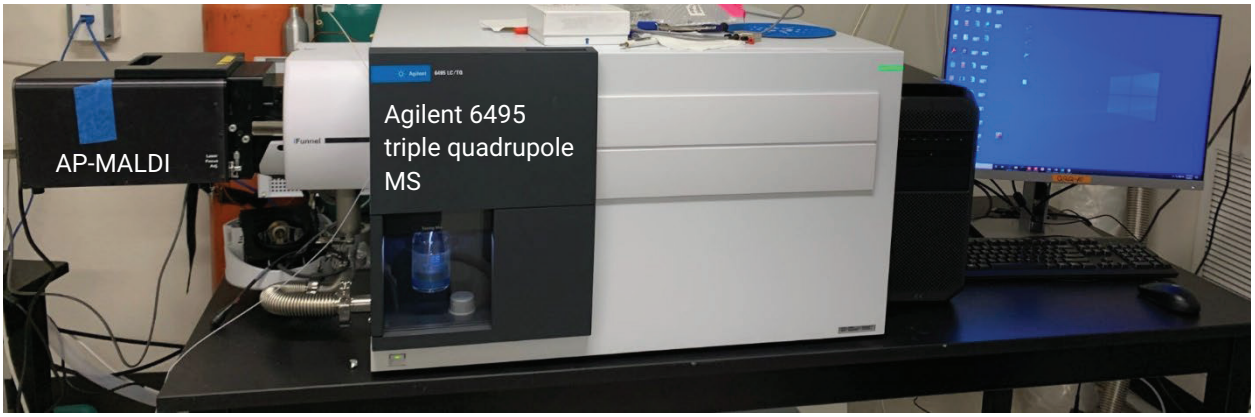

Figure 1. A MassTech AP-MALDI ion source coupled to an Agilent 6495 triple quadrupole mass spectrometer.

Materials

The four matrix materials tested—2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), 1,5-diaminonaphthalene (DAN), and 9-aminoacridine (9-AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation

The imaging setup couples the following two instruments:

- AP-MALDI (UHR) ion source (MassTech, Inc, Columbia, MD, USA)
- Agilent 6495 triple quadrupole system

Sample preparation

Flash-frozen tissue sections (10 µm) or paraffin-embedded tissue sections (FFPE, 5 µm) were transferred after sectioning onto slides having a conductive indium-tin oxide coating. The FFPE tissue sections were washed with xylene (two washes for 3 minutes each) and dried under air before being sprayed with matrix. Matrix was applied using an HTX M5 automated spraying system. For matrix spraying, the optimized matrix-solvent combination is 30 mg/mL DHB matrix in 50:50 (v/v) acetonitrile:0.1% trifluoroacetic acid in water for Objective 1 and 40 mg/ml DHB in 50:50 methanol:water (v/v) for Objective 2.

MS analysis and data processing

All MS data were acquired using multiple reaction monitoring (MRM). Data processing was performed using Mozaic (Spectroswiss, Lausanne, Switzerland).

Results and discussion

Method optimization for metabolites

As a part of building this novel imaging platform, the analytical variables and MALDI parameters were optimized (e.g., laser energy, spatial resolution, velocity, and matrix type). The analytical MSI method was also optimized for approximately 15 metabolites (all polar) using commercially available standards via a targeted MRM approach. Of four matrices (CHCA, DHB, DAN, and 9-AA) tested in both positive and negative ionization modes, DHB was identified as the most effective matrix in positive-ion mode for spatial localization and quantification of the polar metabolites tested. The optimized laser fluence is 50% and spatial resolution is 30 µm.

Results for Objective 1

A standard calibration curve was obtained for adenosine with good linearity (linear regression y = 1,436.6x + 227.98, R^2 = 0.9992) as shown in Figure 2. A 1.34-fold decrease was observed in associated adenosine levels in FFPE xenograft tumors derived from MDA-PCa-2a prostate cancer cells containing overexpression of adenosine deaminase (ADA) compared to the control, as shown in Figure 3A. In contrast, in tumors of this type, introduction of short hairpin RNA (shRNA) targeting ADA (ADA-KD or ADA rescue) resulted in 1.7-fold elevation of adenosine compared to its corresponding nontarget control (ADA), as shown in Figure 3B. Correlation plots were also generated for adenosine comparing ADA versus control and ADA-KD versus ADA in FFPE tissue sections (Figure 4A), as well as the correlation plot for adenosine with five other metabolites (Figure 4B). As expected, adenosine levels were significantly lower for ADA compared to control (p = 0.031) and significantly higher for ADA-KD compared to ADA (p = 0.033).

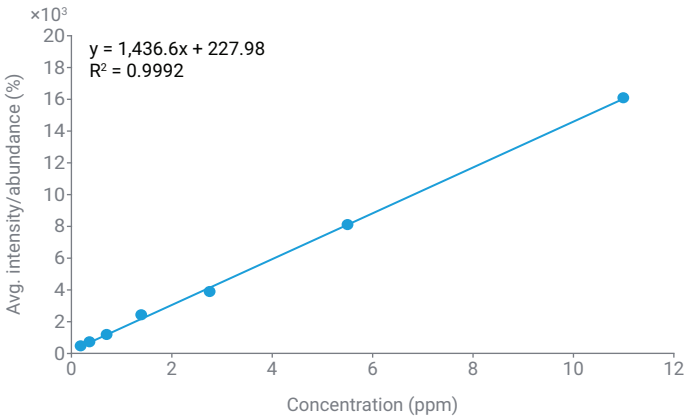

Figure 2. Adenosine (268.1 → 136.0) standard calibration curve.

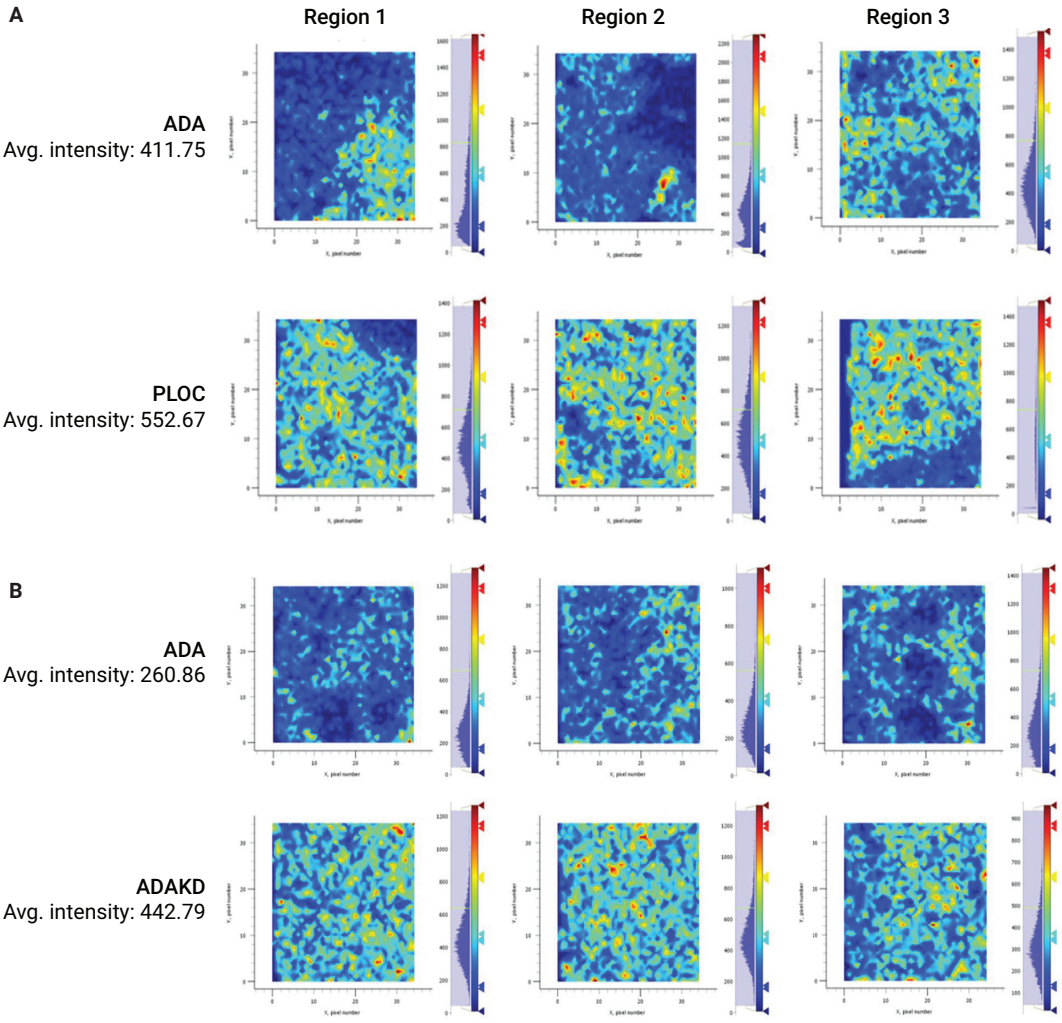

Figure 3. Adenosine intensity and associated representative images in xenograft tumors derived from MDA-PCa-2a prostate cancer cells showing (A) overexpression of ADA or its control (PLOC) or (B) the effect of sh-RNA–based knockdown of ADA (ADA-KD) in cells containing ADA overexpression.

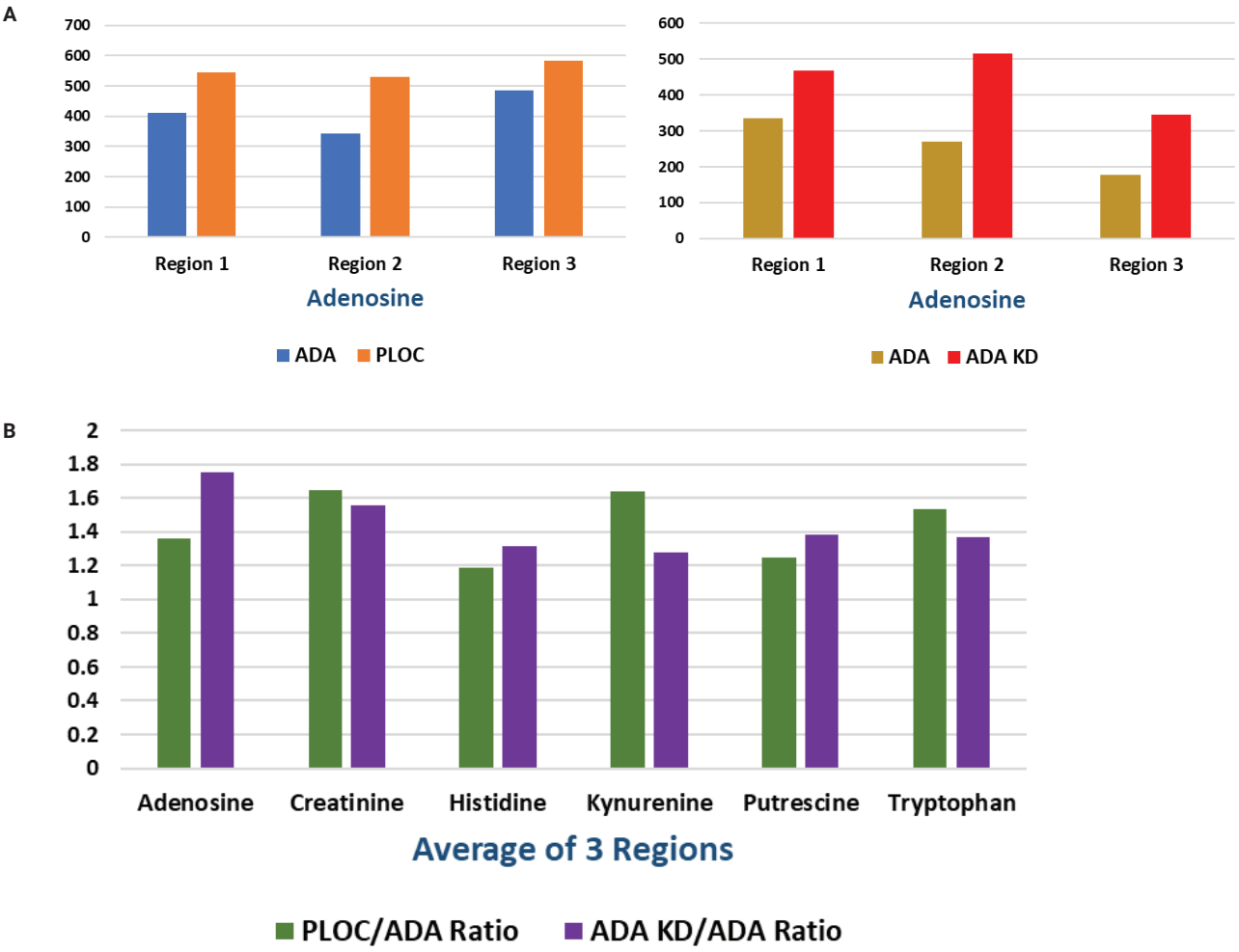

Figure 4. (A) Correlation plots for adenosine within three regions of sample comparing ADA versus control (PLOC) (left) and ADA versus ADA-KD (right) in FFPE tissue sections. (B) Comparison of correlation plots for adenosine and five other metabolites, generated by comparing the average ratio for each metabolite across the same three regions.

Results for Objective 2

In xenograft tumors derived from E0071 murine breast cancer cells containing AADAT knockdown (AADAT-KD), kynurenine levels were reduced compared to their corresponding controls and rescued upon genetic re-expression of AADAT (Figure 5 and Figure 6). As expected, kynurenine levels were reduced upon KD and rescued upon genetic re-expression of AADAT ($p = 0.012$ for control versus AADAT-KD; $p = 0.011$ for AADAT-KD versus AADAT-KD-rescue).

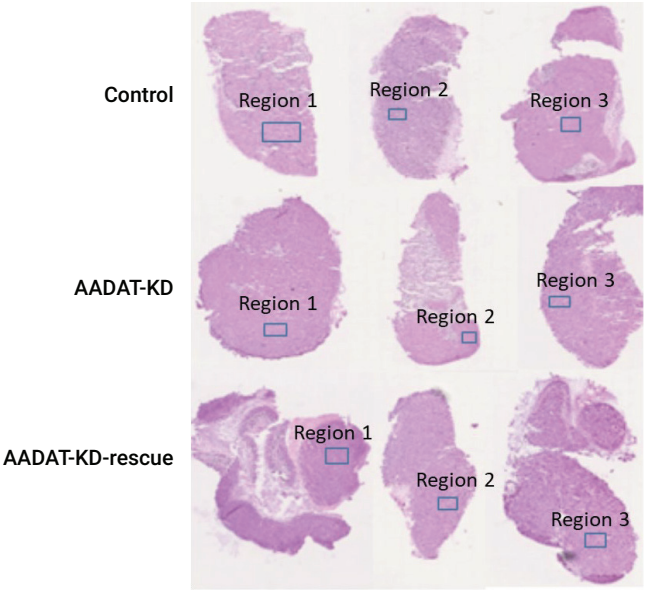

Figure 5. Regions examined in hematoxylin-eosin stains of flash-frozen sections used for mass spectrometry imaging to quantify kynurenine in xenograft tumors. Sections were sprayed using an HTX M5 automated sprayer.

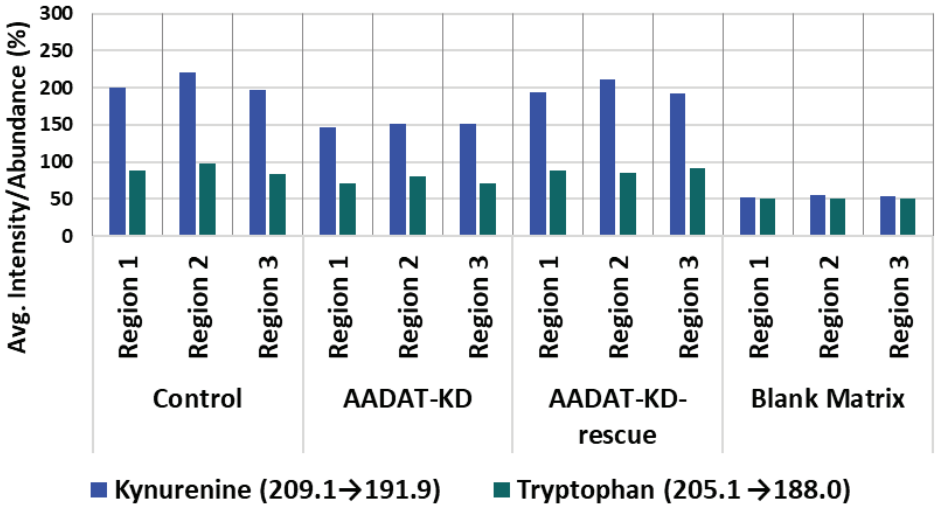

Figure 6. Histogram comparing intensities of kynurenine and tryptophan for control, AADAT-KD, and AADAT-KD-rescue across regions 1, 2, and 3 highlighted in Figure 5.

Conclusion

This study demonstrates a novel metabolite imaging platform that combines AP-MALDI with an Agilent 6495 triple quadruple system for quantification and spatial localization of metabolites in both frozen and FFPE tissues. This method will help researchers understand the localization and function of key metabolites in the tumor microenvironment. The results will support development of new drug targets and disease biomarkers. The approach will be most useful in the context of tissue microarrays, where many tissue samples across multiple subjects can be compared for levels of targeted metabolites to draw diagnostic and prognostic conclusions. This setup takes advantage of the 6495 triple quadrupole system to provide quantitative information and metabolite verification by screening for specific precursor-product ion pairs. Traditional MSI approaches are mostly limited by their inability to examine metabolites from FFPE tissues. The approach demonstrated in this study enables long-term clinical follow-up through the use of FFPE samples.

References

1. Sun, C. *et al.* Spatially Resolved Metabolomics to Discover Tumor-Associated Metabolic Alterations. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116(1)*, 52–57.
2. Sun, C. *et al.* Mass Spectrometry Imaging-Based Metabolomics to Visualize the Spatially Resolved Reprogramming of Carnitine Metabolism in Breast Cancer. *Theranostics* **2020**, *10(16)*, 7070–7082.
3. Wang, Z. *et al.* Spatial-Resolved Metabolomics Reveals Tissue-Specific Metabolic Reprogramming in Diabetic Nephropathy by Using Mass Spectrometry Imaging. *Acta Pharm. Sin. B* **2021**, *11(11)*, 3665–3677.

www.agilent.com

For Research Use Only. Not for use in diagnostic procedures.

RA45030.5854976852

This information is subject to change without notice.

Printed in the USA, May 22, 2023
5994-6178EN

Agilent
Trusted Answers