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Application Note

Deposition of reference standards on tissues for quantitative MALDI mass spectrometry imaging

Introduction

Quantitative matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI qMSI) has become a very powerful tool to reveal the spatial distribution of biomolecules and xenobiotics in tissue sections. Especially in pharmaceutical research it is increasingly used for analysis of drug and metabolite distribution as well as in pharmacodynamic biomarker research and toxicology.

A crucial step in quantitation is the generation of a reliable calibration curve of reference standards to which the intensity of the analyte can be compared. Ion suppression effects with MALDI must be considered and it is therefore desirable to detect the reference standards at conditions representing the extraction and ionization situation of the analyte. A widely used approach to establish a calibration curve is to spot a series of standard solution of known concentrations onto a tissue sample. It is obvious that the application of the standard solutions has to be done in a precise and reproducible manner.

In this application note we describe how a reliable disposal of standards is achieved by means of the BioSpot® nanoliter liquid handling workstation.

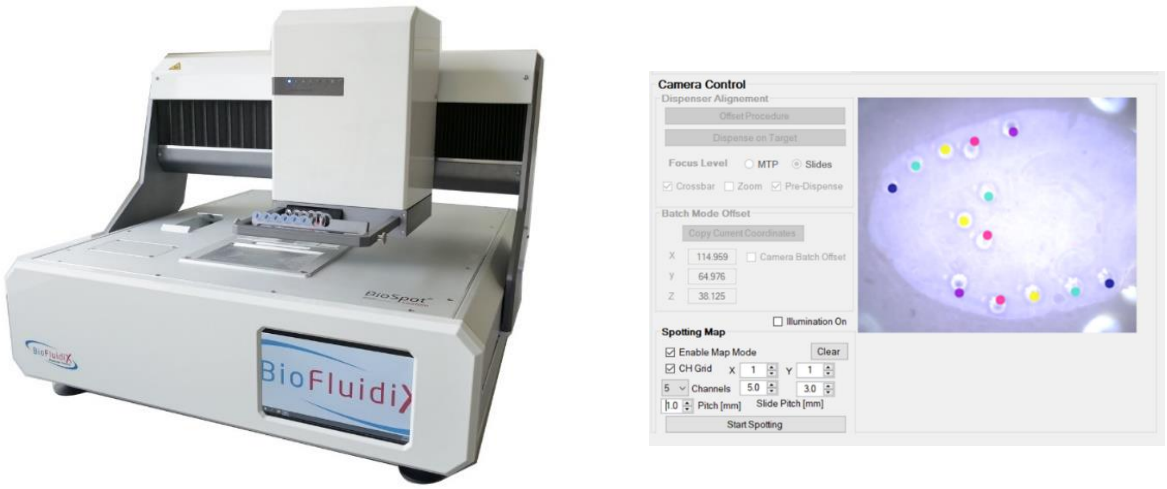

Figure 1: Illustration of BioSpot® BT600 custom (6-channels) workstation and exemplary application with camera-guided spot deposition

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Technology

The BioSpot® workstation is a compact benchtop system and enables convenient automation of liquid handling workflows in R&D and low to midsize manufacturing processes (**Figure 1**). For nanoliter delivery the printhead of the BioSpot is equipped with PipeJet® Nanodispenser modules. Based on piezo actuation the drop-on-demand technology delivers single droplets in the volume range of 2 to 70 nl for a wide variety of liquid types. Droplet size (volume) of the actual dispensed reagents are calibrated and verified by means of an embedded camera system (SmartDrop). Images of the droplets are taken in flight and the underlying software algorithm enables an automated and fast adjustment of dispensing parameters until the target volume is reached.

Beside precise volume delivery it is also important that the reference compound solutions can be placed at distinct and free selectable positions on the tissue slices. The BioSpot facilitates positioning by a visual system (TopView) providing a live image of the tissue slices. Desired spot position can be directly marked on the screen by means of cursor. Individual spot positions can be determined or a corresponding grid of spots can be generated. Subsequently the selected pattern is printed automatically by the BioSpot.

Process flow of reference standard disposal for qMSI

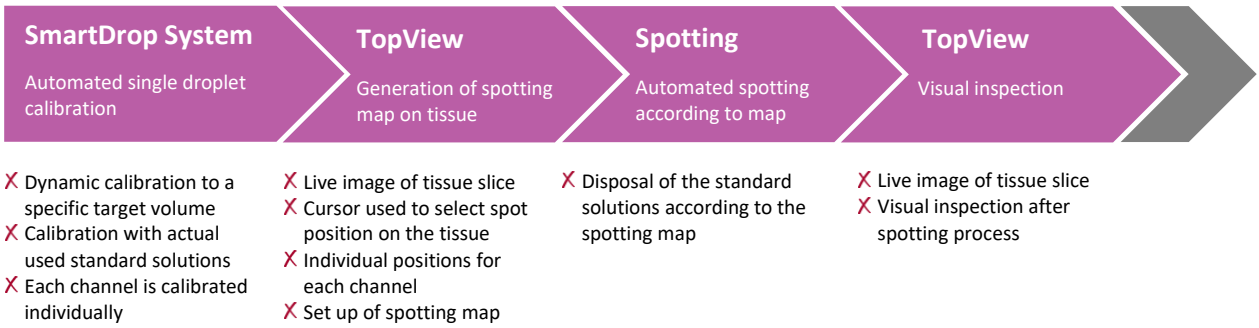


Figure 2: Illustration of the work flow: droplet calibration, set up of spotting map and automated disposal of reference standards onto tissue slices for qMSI

Instruments

- X BioSpot® BT600 custom (6-channels) instrument using dispensing pipe types 500-S for reference compound spotting on tissue (BioFluidix)
- X Manual research pipette 0.1-2.5 µL (Eppendorf)
- X Cryomicrotome CM1950 (Leica)
- X HTX Sprayer M5 (HTX technologies) for MALDI matrix application
- X AP/MALDI (ng) UHR ion source (MassTech) coupled to Q Exactive™ Plus mass spectrometer (Thermo Scientific™)
- X Digital microscope VHX-6000 (Keyence) for optical image acquisition

Materials & Methods

- Dopamine-1,1,2,2-d4 and solvents of analytical grade (Merck KGaA, Germany)
- Fmp-10 reactive MALDI matrix (tag-on AB, Sweden).
- Indium thin oxide (ITO) slides (Bruker, germany)
- Cryopreserved Han Wistar rat brain and lung tissue was used from an untreated control animal. Tissues were sectioned with a 10µm thickness at -20°C and thaw-mounted on a conductive ITO glas slides. Slides were kept frozen at -80°C until further use.
- Before starting the workflow for qMSI tissues were stabilized by removal of residual water in a desiccator under vacuum for about 20min.
- Tissue concentrations in [pmol/mg] of spots were calculated by accounting for the spot area (in mm^2), the tissue density (assumed as 1 g/cm^3) and known concentration in spotted volume.
- AP/MALDI-MS imaging analysis was performed with constant speed raster motion at a lateral resolution of 60 µm, in positive polarity, with a mass range of m/z 300-800 and at a mass resolution of 140000. The maximum ion injection time for the MS detection was fixed at 500 ms.

Results & Discussion

The influence of standard solution spotting using automated deposition with the BioSpot® BT600 custom workstation and a manual research pipette was compared by respective deposition of a test item solution volume on a rat lung tissue section. Automated spotting of 20 nL resulted in reproducible spot diameters of 0.65-0.66 mm (**Figure 3A**), while manual spotting of 200 nL exhibited respectively bigger spot diameters of 1.19-1.35 mm (**Figure 3B**). The increased spot area does not surprise with manual spotting because of the increased volume. Yet it indicates a typical situation when using a manual pipette in which the lower volume end is limited to a maximum of 100 nL and goes often along with an increased variability (e.g. user and experience dependent) and error margin of spot deposition.

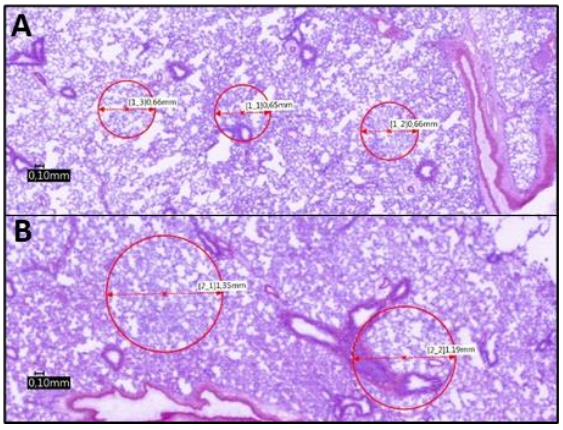


Figure 3: Optical image of haematoxylin and eosin (H&E) stained rat lung section with (**A**) automated spotting (20 nL) and (**B**) manual spotting (200 nL) of a test item

The application of the BioSpot® for qMSI purposes is demonstrated for the quantitation of dopamine (DA) levels in rat striatum brain region. Standard isotope labelled d4-dopamine was used as reference compound to deposit the calibration curve(s) in a sagittal rat brain section to avoid interference with endogenous dopamine levels. To detect the neurotransmitter a reactive MALDI matrix, fmp-10, was used and sprayed on tissue according to the published protocol [1]. The d4-dopamine solution was prepared at five different concentrations in 50% ethanol and spotted on different brain regions at a volume of 20 nL per spot following the workflow described in figure 2.

The 13 TopView determined spot areas ranged overall from 0.27-0.33 mm² and nicely indicate a reproducible deposition of the 20 nL volume (**Figure 4A**). This is also reflected in the TIC-normalized signal intensity of single derivatized d4-dopamine (m/z 425.216) in spots of equal concentration and the good linearity of the calibration curve (**Figure 4B** and **Figure 5**). Furthermore, the ion image shows distinct positions of the calibration spot signals without any overlap and with further capacity to increase the number of technical replicates for individual concentrations in other regions of the rat brain section.

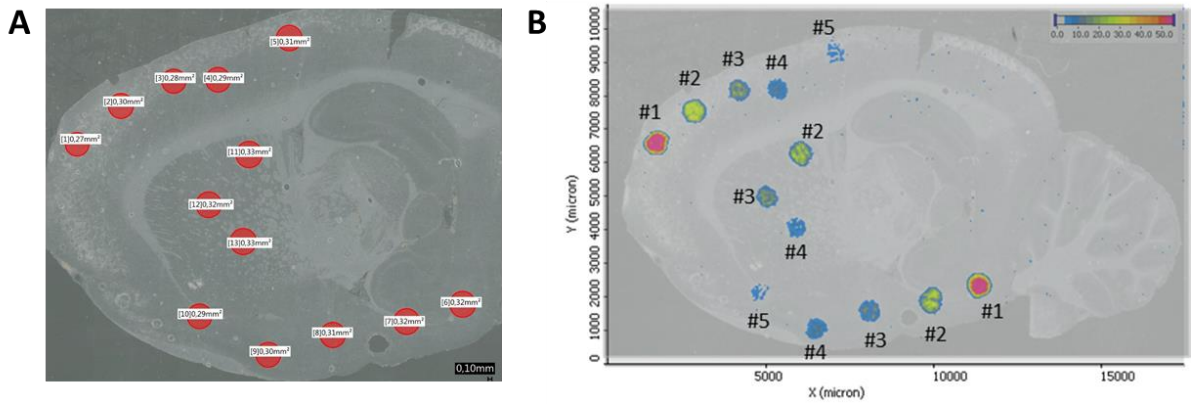
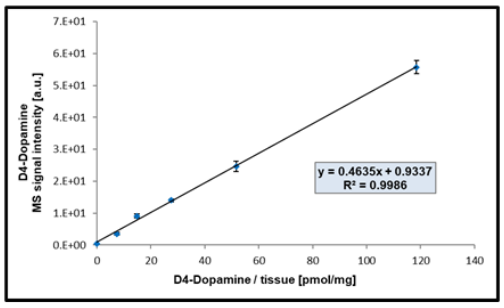


Figure 4: Sagital rat brain section: (**A**) optical image on ITO glas slide with individual spot area of d4-dopamine, (**B**) ion image overlay of fmp-10 derivatized d4-dopamine (m/z 425.216) with relative signal intensity (red = high; blue = low)

A



B

D4-dopamine 20nL/spot in 50% EtOH	
Spot #	concentration/spot [pmoL]
1	0.32
2	0.16
3	0.08
4	0.04
5	0.02

Figure 5: (**A**) D4-dopamine calibration curve and (**B**) tabulated summary of spotted d4-dopmine concentration on rat brain section

Calculation of the dopamine concentrations in the brain striatum region as depicted by the region of interest (ROI) in **Figure 6A** revealed an average DA concentration by qMSI of 1.54 µg/g. Consecutive tissue sections were not analyzed for dopamine levels by an orthogonal methodology (e.g. LC-MS/MS). However, a comparison of the qMSI values to available publications show that our data are well in line with previously reported DA concentrations in rat brain. Wojnicz et al. [2] detected DA levels in the range of 1.47 ± 0.25 µg/g, Zhu et al. [3] at 0.76 ± 0.02 µg/g and Bosai et al. [4] at 0.41 ± 0.08 µg/g all using LC-MS/MS as quantitation method for rat brain homogenate.

In this application note, the methodology demonstrated its suitability for quantitation of endogenous DA levels. This MALDI qMSI approach can however be extended in the same experiment to multiple endogenous and administered molecules which are detected in the same tissue sections, simply by using mixtures of standards and deploying them as previously described and extracting the respective m/z signals after analysis. As shown in **Figure 6B** in the overlay of three extracted ions detected in the sagittal rat brain section, the only pre-requisite is the ability to detect these by MALDI-MS selectively and with sufficient sensitivity.

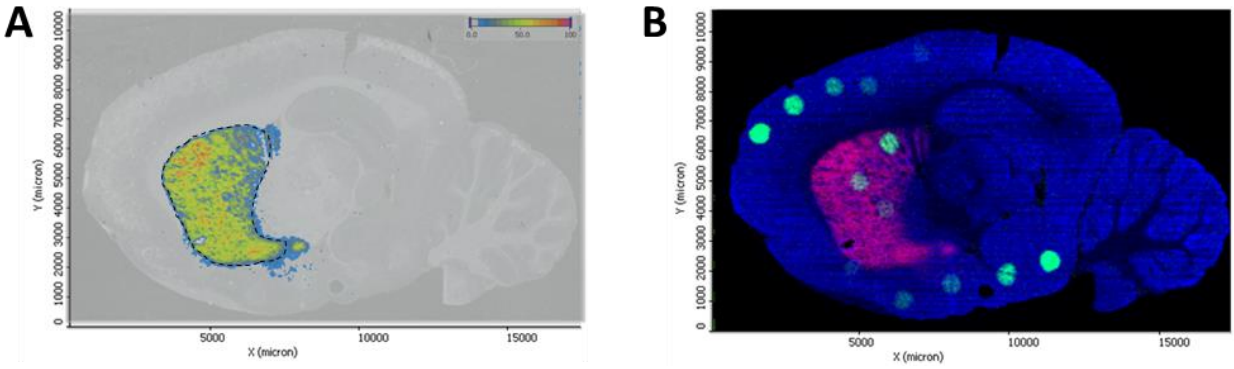


Figure 6: Sagital rat brain section: (**A**) ion image overlay of fmp-10 derivatized dopamine (m/z 421.191) with optical image with striatum ROI (dotted line), (**B**) RGB ion image overlay of fmp-10 derivatized dopamine (red; m/z 425.216), fmp-10 derivatized d4-dopamine (green; m/z 421.191) and the lipid PC (34:1) (blue; m/z 760.585)

Conclusion

- The BioFluidix BioSpot® BT600 custom workstation allows for reproducible and precise spotting of calibration curves for absolute quantitative MALDI-MS Imaging experiments.
- Also small areas can be employed for deposition of various calibrants for qMSI, allowing for various technical replicates due to low deposition volume of the BioSpot® workstation
- Robust qMSI data of endogenous molecules, xenobiotics and metabolites in specific tissue areas can be achieved with this methodology

Authors & Acknowledgement:

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References

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