

# Technical Report

## Analysis of Standard Sample by Drug Imaging

### Dried-Droplet Analysis on a Stainless Steel Plate

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#### Abstract:

Drug imaging is one of the promising applications for MS imaging by atmospheric pressure MALDI. MS imaging, which is able to observe the kinetic state of drugs in the body without the use of fluorescent or isotope labeling, has attracted an extremely high level of interest for applications such as drug delivery systems (DDS) and drug safety evaluation. To detect drugs with as high sensitivity as possible, matrix selection is especially important. Therefore, in addition to describing the analysis flow of using drug imaging, this report also describes how to select the optimal matrix for ionizing the target drug using the dried-droplet method on a stainless steel plate.

**Keywords:** MS imaging, drug imaging, stainless steel plate, dried-droplet

## 1. Expectations for Using Drug Imaging for MS Imaging

Pharmacokinetics is an essential step in drug discovery applications. Conventionally, this typically involves extracting the target substance from the entire body organ, using LC-MS/MS or other means to determine the quantity and properties of the substances, labeling the substances with radioisotopes (RI), and then determining the localization of the labeled substance. However, each of these steps can involve problems that can prevent determining the localization within the organ using LC-MS/MS, especially if only a small amount of the substance is located in only a portion of the organ, for example, or if detection sensitivity is insufficient. Even if the drugs are RI-labeled, the cost of synthesis or availability of specialized facilities can be a problem. Also, it may not be possible to confirm the kinetic state of metabolized compounds, depending on the RI-label position. Therefore, there is growing demand for a new technology for drug imaging.

In contrast, using atmospheric pressure MALDI for MS imaging enables detecting drugs without labeling. Moreover, it is also allows multiple metabolites to be viewed simultaneously. Therefore, it is gaining attention as a new technology not available with conventional imaging methods. Furthermore, due to the fact that this MS imaging method shows localizations within the organ and permits predicting whether they are increasing or decreasing based on signal intensity, the signal distribution density in the overall image, and other factors, there is also interest in the effectiveness of drug imaging for semi-quantitative analysis.

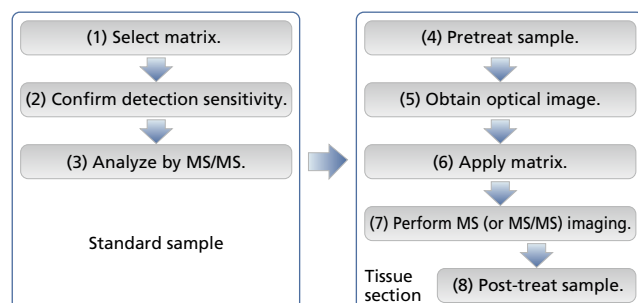


Fig. 1 Process Flow

## 2. Problems with MS Imaging in Actual Practice

Drug imaging by MS imaging is achieved using the iMScope *TRIO* imaging mass microscope. This instrument is a dedicated imaging system based on a new concept that integrates MS imaging technology with an optical microscope. This article is intended for those that have never used drug imaging, but would like to try. Therefore, the following highlights the key points to consider in each of the eight steps indicated in Fig. 1, while also referring to actual analysis results. The results shown in this article have already been published in a paper by the National Cancer Center<sup>1)</sup>. However, this article focuses on the techniques used to obtain the data necessary to achieve those results. Steps (1) to (3) indicate steps for analyzing a standard sample by MALDI MS. Steps (4) to (7) indicate steps for MS imaging using tissue sections. In particular, steps (5) and (7) use the iMScope *TRIO* indicated above and step (6) uses the optional iMLayer. Step (8) is used if needed, such as to stain the tissue sections. Provided a well-controlled experimental system is designed, images similar to those shown in Fig. 2 can be obtained that permit observing quantitative variations in target cancers (cancers induced under mouse skin) excised from different individuals as a function of time after administering a drug.

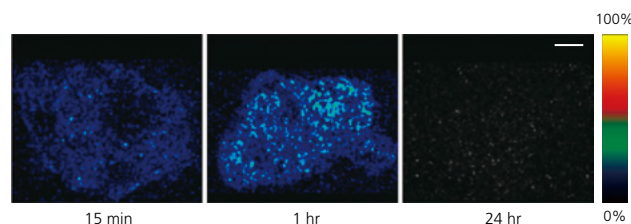


Fig. 2 Drug Images (Bar: 1 mm) of Cancer Tissue in Mouse Administered the Cancer Drug Paclitaxel

### 3. Key Factors for Each Step

#### 3-1. Study of Steps (1) to (3) Using Standard Sample

##### 3-1-1. Matrix Selection

These steps may be the most important for drug imaging. For substances endogenous to biological samples, an appropriate matrix can be selected by referring to other reference documents. However, in the case of drugs, they are often new compounds that require testing multiple matrices to find one appropriate for ionization. Therefore, the following describes a method (dried-droplet method) where the drug is mixed with a matrix solution (ethanol) and placed onto a highly conductive stainless steel plate (Fig. 3). Three types of matrix, DHB, CHCA, and 9-AA, were used with the dried-droplet method to compare detection sensitivity. For this study, it is useful to use a stainless steel plate (Fig. 4) that was etched with marks for positioning fractions (18 × 6 wells). This plate permits conveniently linking the sample information to the fraction position. A suitable matrix may be available among the many new matrices reported in recent years. The stainless steel plate can be reused repeatedly until there are no more positions available for fractions. Consequently, it can also be used for confirming sensitivity and MS/MS analysis described below.

Based on comparing results from using the three matrices, it was determined that 2,5-dihydroxybenzoic acid (DHB) offered the best ionization efficiency for paclitaxel (PTX). Therefore, DHB was used for the remainder of the experiment.

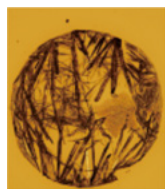


Fig. 3 Dried Droplets (DHB)



Fig. 4 Etched Stainless Steel Plate

##### 3-1-2. Confirming Detection Sensitivity

After selecting the matrix, this confirms the minimum drug concentration that can be detected. This does not mean that the drug can be directly detected in biological samples at that confirmed concentration. That determination permits comparing the results to other drugs. In this case, dried droplets formed on a stainless steel plate from paclitaxel diluted to between 100 fmol/spot and 100 pmol/spot and mixed with DHB were used as the sample.

For drug imaging of biological samples, drugs are often detected by adding sodium (Na) or potassium (K). However, even for preliminary imaging tests performed in advance, potassium adducts  $[M + K]^+$  at  $m/z$  892.3 were confirmed to be easier to detect. Therefore, dried-droplet samples were prepared by mixing 10 mg DHB in 50 % acetonitrile, 0.05 % TFA, 0.5  $\mu$ L 2 mM potassium acetate, and 0.5  $\mu$ L standard solution on the stainless steel plate.

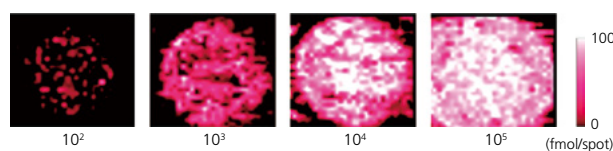
30 × 30 points (total 900 points) were measured for each spot to ensure the entire spot was covered. An image of the paclitaxel  $[M + K]^+$   $m/z$  892.3 signal intensity was then generated (Fig. 5A).

These imaging results indicate that each pixel is detected well up to about 1 pmol/spot.

The same conditions were used to measure droplets for each concentration and then the mean signal intensity integrated from all droplets at  $m/z$  892.3 was plotted (Fig. 5B). This permits confirming that the signal intensity increases as concentration is increased.

Presumably this result includes a signal from another element, such as DHB, in addition to the baseline. Though not shown here, in some cases, detection sensitivity can be determined more clearly by measuring droplets of only the matrix and subtracting that baseline. For actual drug imaging, drugs are detected in biological samples placed on a microscope slide with low conductivity, unlike the dried-droplet analysis described above. Therefore, it is considered difficult to detect drugs at concentrations that do not generate an observable signal in a 30 × 30-point analysis.

A



B

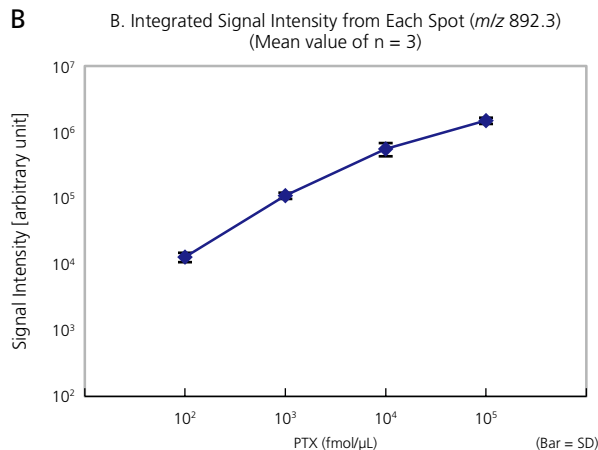


Fig. 5 Confirmation of Detection Sensitivity

##### 3-1-3. MS/MS Analysis

To analyze the drug administered to the biological organism, samples with and without the drug administered were analyzed at several points each. Then the peaks attributable to the drug were determined by editing the data with the Imaging MS Solution software and performing ROI analysis<sup>2)</sup>. However, in some cases, it can be difficult to ensure only the drug peaks are imaged, due to peaks from the biological organism coexisting near the peak selected in the preliminary study or due to effects from mixing the matrix with biological components. In particular, detecting low-concentration drugs requires high S/N ratios in addition to adequate detection sensitivity. Therefore, in many cases, only the drug is imaged selectively by MS/MS analysis. Doing so permits selectively extracting the peaks attributed to the drug to obtain an attractive image with fewer noise components.

Therefore, before imaging biological samples, a biological sample containing the dried-droplet sample and drug indicated above is used to perform MS/MS analysis for respective precursor ions for the target molecule. It is recommended that peaks selected for use in MS/MS imaging and preliminary studies be based on the resulting mass spectra.

MS/MS analysis was performed using the peak presumed to be the peak for the paclitaxel potassium adduct ( $[M + K]^+ m/z 892.3$ ) as the precursor ion (Fig. 6A). The MS/MS peak pattern varies significantly depending on the instrument, collision energy, collision time, and other factors. Consequently, peak lists from other instruments in the database cannot be used. If searching an MS/MS database using a database such as MassBank (<http://massbank.jp/index.html?lang=en>), then an MS/MS database must be used by selecting LC-ESI-ITTOF, for example.

However, actual iMScope *TRIO* analysis uses ionization by atmospheric pressure MALDI and detection by IT-TOFMS. Therefore, peak attribution to target substances is typically confirmed by comparison to standard samples. The results (Fig. 6B) from MS/MS analysis of a mouse sample excised from cancer tissue 30 minutes after administering PTX, given a precursor ion at  $m/z 892.3$ , confirm that the peak detected at  $m/z 607.19$  by the MS/MS analysis of the standard sample is also detected in the tissue and also is clearly separated from other peaks in the vicinity.

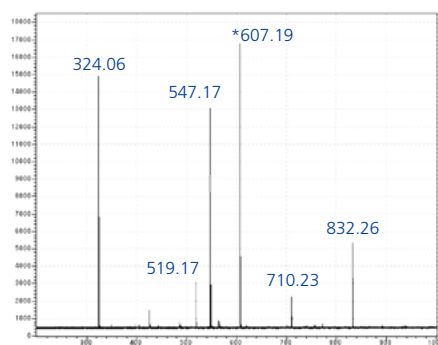


Fig. 6A MS/MS Spectrum of Standard Sample (on stainless steel plate)

Since the MS/MS analysis of the tissue detected many peaks that were not detected in the standard sample, it suggests that there are many biologically derived peaks, or peaks from biological substances bonded with the matrix, (i.e. noise components) located near the  $m/z 892.3$  position used for imaging in this case.

Though not shown here, MS/MS imaging using a mouse without the drug administered, in addition to the mouse with the drug administered, detected several strong localized signals and signals outside the tissue. Therefore, to confirm whether or not the results indicating that the signal at  $m/z 892.3$  exists throughout the entire cancer tissue (Fig. 2) are reasonable, imaging (Fig. 7) was performed using the data from  $m/z 892.3$  to 607.19 selected from the MS/MS analysis above, where the corresponding peaks in the image indicated the same signal distribution pattern as  $m/z 892.3$  throughout the entire cancer tissue. Consequently, we were able to conclude positively that the drug distribution was indicated using either the peaks at  $m/z 607.19$  or 892.3.

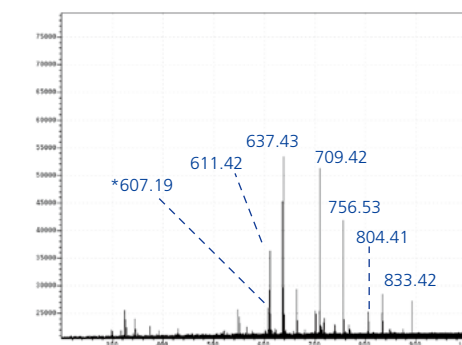


Fig. 6B MS/MS Spectrum of Tissue with PTX Administered

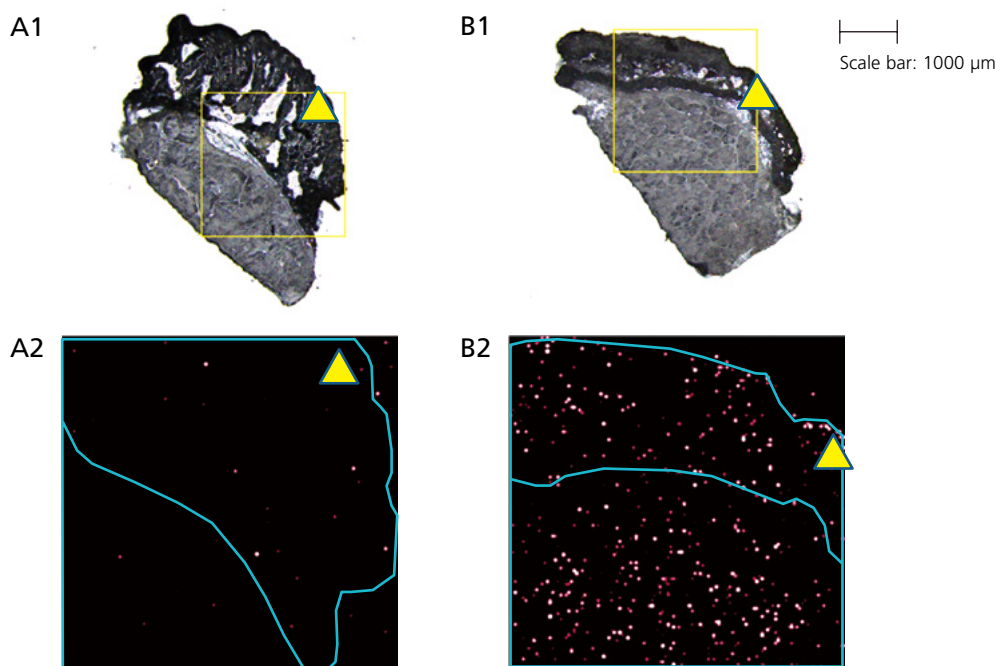


Fig. 7 MS/MS Imaging (Upper: Optical Images, Enclosed Areas Were Analyzed; Lower: MS/MS Images from  $m/z 892.3$  to 607.19)  
A: Control sample without drug; B: 30 minutes after administering 50 mg/kg PTX  
Lower: Solid lines indicate boundary of tissue;  $\triangle$ : Necrotic area

## 3-2. Imaging Experiment Steps (4) to (7) Using Tissue Section

### 3-2-1. Pretreating Samples

Tissue section samples for MS imaging were prepared by slicing thin sections from frozen tissue with a cryostat and placing them on a microscope slide (ITO slide) with high conductivity. Samples were sliced from tissue that was as frozen as possible. If a mountant must be used, a 2 % carboxymethyl cellulose (CMC) mountant is recommended, rather than using an optimal cutting temperature (OCT) compound or other resin-based mountant that can cause noise in the  $m/z$  range being measured. Sections should be 10  $\mu\text{m}$  ( $\pm 5 \mu\text{m}$ ) thick, where sensitivity is generally better the thinner the sample, but if it is too thin, then repeated exposures by the laser can reduce the amount of tissue and cause problems with an inadequate total number of target molecules. After slicing sections, promptly store the tissue below  $-80^\circ\text{C}$ . Check the conductive surface on the ITO microscope slide before placing the section on the slide. For comparison tests, take measures to ensure analytical variability is as low as possible, such as by placing specimens from the target area on the same microscope slide.

### 3-2-2. iMScope TRIO Analysis 1 – Steps (5) and (6)

Insert the tissue section placed on the ITO microscope slide into the sample holder (Fig. 8). Make sure the correct side is oriented upward. Then follow the instructions in the iMScope TRIO manual to obtain an image of the section (step (5)). However, before obtaining the image, set the magnification to the maximum setting (40 $\times$ ) and accurately adjust the focus. The laser hits the sample at an angle. Therefore, if the height is not set properly, samples cannot be analyzed accurately. Also, when capturing the optical image, keep in mind that it will be overlaid on the  $m/z$  image later. Therefore, though it has no direct effect on analytical results, adjust the white balance, image brightness, and so on, so that the overlay will appear good.



Fig. 8 Inserting the Microscope Slide into the Sample Holder

After capturing the optical image, remove the sample and apply matrix (step (6)). An automatic vapor deposition system (such as the iMLayer) is especially helpful for this step. After applying the matrix, use the optical image just obtained to specify the area for analysis. The sample is positioned using the two reference marks on the sample holder. Therefore, handle the assembly carefully to prevent the microscope slide from moving in the sample holder.

### 3-2-3. iMScope TRIO Analysis 2 – Step (7)

Put the sample holder, with the matrix applied, back into the instrument. Follow the Wizard instructions to ensure the instrument recognizes the positioning marks and load the optical image obtained above so that the analysis area can be specified based on the image of the section before applying the matrix. For more details regarding this process, refer to the manual. The button to move to imaging position

in the software window allows the sample status after applying the matrix to be checked. The matrix application status has a major effect on analytical results. Therefore, having an image that was obtained after applying the matrix may permit evaluating the analytical techniques used. The uniformity of the matrix layer, any shifting of the microscope slide, and so on, can be confirmed by observing the sample through the microscope after applying the matrix.

The optimal parameters, such as laser intensity, can vary significantly depending on the matrix application status and type of sample. Capture an image from an area not used for imaging and then perform a trial laser exposure to determine optimal parameter settings. We strongly recommend doing this for each slide. For more information about this process, refer to the manual.

Also, when performing these experiments, do not perform imaging immediately. Rather, it is probably better to first verify that imaging the given drug is viable by obtaining data, 20  $\times$  20 points for example, from a comparison test between groups with and without the drug administered. The data editing and ROI comparison functions are especially helpful for that process. It is also possible to improve sensitivity, such as by analyzing a narrower  $m/z$  range, specified based on detection sensitivity, or by using the single ion monitoring (SIM) mode to target only a single  $m/z$  position. Furthermore, unless imaging is being performed for an extremely small area, also try improving sensitivity by increasing the laser diameter slightly. After adjusting the laser diameter, energy intensity,  $m/z$  range, or other parameters and deciding the analytical conditions, imaging can finally start. If necessary, set up a 250  $\times$  250 point or smaller analysis for respective groups with and without the drug administered. Then an image of the target drug should be ready by the next morning. (See the example in Fig. 2.)

## 4. Conclusion

In some cases, even if three types of matrix are evaluated by the dried-droplet method before using one for ionization, as described above, the drug still may not be detectable using the iMLayer, or other means, to vapor deposit the matrix. If so, it may be due to poor dissolution from the lack of solvent content when the matrix is vapor deposited. One way known to resolve such cases is to spray a layer of matrix solution or solvent over the vapor deposited matrix to improve sensitivity by inducing recrystallization<sup>3)</sup>.

Using MS imaging for drug imaging offers the major advantage of simultaneously obtaining information about the distribution and metabolism of the drug, which is not available with conventional methods. This new imaging technology is accomplished by using the iMScope TRIO imaging mass microscope and iMLayer fully automatic vapor deposition system.

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## Reference

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- 2) *Shimadzu Review*, 70, 99–108 (2014)
- 3) *J. Mass. Spectrom.*, 48, 1285–1290 (2013)

The iMScope TRIO is not approved or certified as medical device. It can be used exclusively for research applications, and cannot be used for the purpose of treatment or diagnosis.

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