# MALDI Imaging at High Speed

*Improving Sample Analysis Times in Pharmaceutical Research*

Over the last decades, MALDI-TOF mass spectrometry (Matrix-Assisted Laser Desorption/Ionisation Time of Flight) has proven its usefulness and robustness in many applications, helping life scientists to meet their toughest challenges. Companies and academic institutions rely on instruments such as MALDI-TOF and MALDI-TOF/TOF mass spectrometry systems to accelerate their research. Scientists in areas such as pathology, biomarker research, and studies in drug characterisation, for instance, who wish to map tumour heterogeneity and link this information to disease outcome. Tissue imaging of protein distribution continues to be one of the most powerful analytical techniques to meet this challenge.

This article will focus on how new laser technology can improve sample throughput for MALDI imaging. It will demonstrate how MALDI technology is being utilised in drug development as an established tool for compound distribution analysis and an emerging tool to boost efficiency in the drug finding process, specifically for ultra high-throughput drug discovery screening.

**Current Challenges**

MALDI imaging is a spatially resolved, label-free analytical technique for direct analysis of biological samples. Acquisition speed and sample throughput are limiting factors in mass spectrometry imaging (MSI) experiments, in particular in clinical research where large cohorts of patients have to be analysed to encompass the biological variance of human samples. Translational clinically-oriented research with imaging mass spectrometry is critically dependent on speed and robust operation. Current instrumentation can limit its full adaptation. There is a need for instrumentation to overcome this limitation, enabling scientists to engage in high-throughput clinical studies.

Acquisition speed also limits MSI experiments at high spatial resolution, especially for larger tissue sections. New MALDI imaging solutions represent a paradigm shift in terms of productivity, cost of ownership and ease of use, establishing mass spectrometry imaging as a powerful and reliable information source for personalised medicine research.

**Innovative MALDI Analysis Technologies**

In the pharmaceutical environment, MALDI has widespread applicability. Today this ionisation technique is used in lead discovery in ultra high-throughput screenings, absorption, distribution, metabolism, and excretion (ADME) applications and quality control of biopharmaceuticals, or for biological/clinical diagnostics for, e.g., microorganism identification in clinics. Common requirements of these applications are: speed, time to analysis results, throughput, ease of use, definition and robustness.

The instrumentation market is responding to and addressing the need for scientists to increase sample throughput and improve data quality, helping them to make the best decisions in the shortest amount of time. New mass spectrometry imaging solutions re-define the key performance measures for MALDI imaging, offering 10 times faster imaging measurements than traditional MALDI-TOF systems, without losing sensitivity.

**Compound Distribution Analysis**

While MALDI imaging analysis applications cover a broad range, a commonality is to evaluate spatial distribution of compounds in various tissue types. MALDI imaging is an analytical method for the detection of potential biomarkers directly from tissue sections that has gained popularity over the past decade. The development protocols for spatially resolved digests are of particular interest. Most importantly, on-tissue digestion allows the analysis of peptides from formalin-fixed paraffin-embedded (FFPE) tissue, the most common type of sample in clinical pathology. In addition, it allows detection of larger proteins using peptides as proxies and facilitates biomarker ID by means of MS/MS.

A recurring challenge is bottlenecks on instrument time due to the demand on imaging analyses, with traditional instruments being able to run at 2 pixel/s. Routine studies with more than one example quickly reach the limit of feasibility. In addition, the demand for higher spatial resolution increases the pixels per area with the reciprocal square of the pixel diameter. Thus, faster acquisition is not only mandatory for bringing scientific examples into routine applications, but also copes with the demand of reduced focus sizes at higher spatial resolution.

In addition, higher specificity for MALDI imaging experiments is often very beneficial. High mass resolution, exploiting MS/MS modes of acquisition for targeted images is an establishing technique. There is a need for instrumentation to be faster and allow for MS/MS mode at the same speed.

As an example of a MALDI MS/MS imaging acquisition, an image is shown in Figure 1. After an on-tissue digest using trypsin, a fragment of peptide ARTKQTAR is displayed. Notably, the entire acquisition of 32,000 pixels at a raster width of 30 um only took 33 min. Acquisition time is reduced by a factor of 5–10, allowing an entire experiment to be completed in a single workday. It presents a powerful tool to evaluate and optimise on-tissue digestion protocols.

**Mass Spectrometric Imaging Solution – Data Example**

The following example illustrates initial data from a production prototype of a next generation MALDI-TOF instrument that enables acquisition

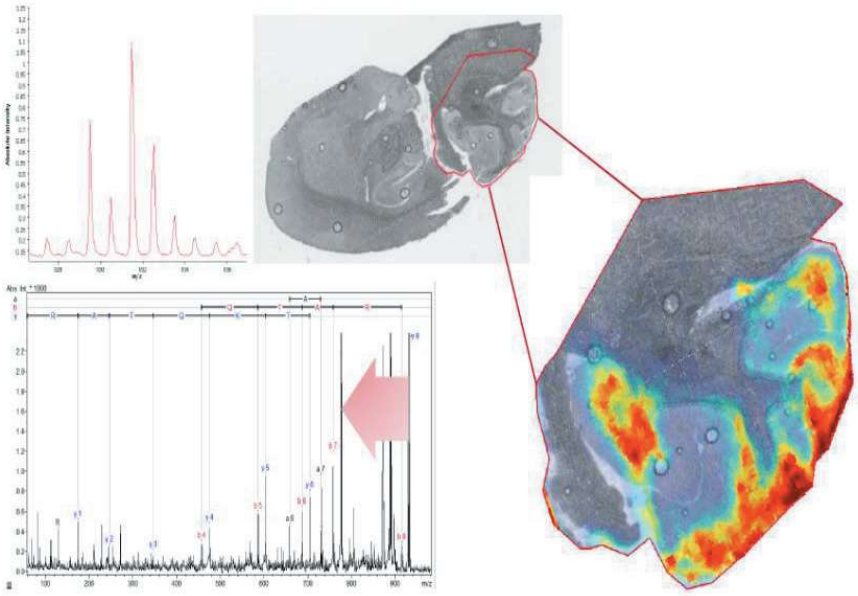


*Figure 1: MS/MS image of peptide ARTKQTAR from H33_Mouse (precursor: 931.54Da -> fragment: [b+18]7 775.4Da)*

speeds of up to 50 pixel (spectra) per second at pixel sizes of 10 µm and smaller. Data is shown from the most common application areas of MSI, including lipid, peptide and intact protein analysis.

**Methods**

Sample preparation and matrix application were done according to previously published standard procedures. For lipid and intact protein analysis, fresh-frozen tissue sections (10 µm) were mounted on conductive glass slides and coated with DHB matrix using a custom-built sublimation chamber. For proteins, an additional rehydration step using acetic acid (5%) in a humid chamber was conducted after matrix deposition. For peptide analysis, thin sections (4 µm) of FFPE tissue were mounted on conductive glass slides. After paraffin removal and antigen retrieval (Tris buffer, pH 9), samples were digested and coated with HCCA matrix using an ImagePrepTM device (Bruker Daltonik GmbH, Bremen, Germany) according to standard protocols. All MS imaging data was acquired on a production prototype of Bruker's next generation MALDI-TOF instrument, rapifleX MALDI Tissuetyper™. The instrument is a linear / reflectron geometry MALDI-TOF instrument on the basis of the novel 10kHz smartbeam™ 3D laser. This laser uses process-optimised laser beam focusing optics to generate a narrowly-focused (<5 µm diameter) Gaussian laser spot. Combined with a set of rotating mirrors, the laser spot can be positioned fast and precisely on the sample, allowing truly square pixels.

**Results**

Initial data for three major application areas of MALDI-TOF-based tissue imaging are shown. These differ mainly in sample preparation and the m/z-range analysed.

- Lipid analysis (~500-1,500 m/z) is typically performed at small pixel sizes to achieve highest spatial resolution (Fig. 2).
- Intact protein analysis (~2,000-20,000 m/z). Due to the low charge states of MALDI ions and the mass cut-off of other analyser types, this is usually reserved to MALDI/TOF instruments such as the rapifleX MALDI Tissuetyper (Fig. 3).
- (Tryptic) peptide analysis (~500-4,500 m/z) is the main way to access FFPE tissues. The digest step typically limits spatial resolution (Fig. 4).

Performance of the platform was based on the following criteria:

- Generation of consistent, spatially informative images at high spatial resolution with fast acquisition speed.
- Preservation of tissue integrity after laser irradiation to enable subsequent conventional histology.
- Capability to process, analyse and visualise large MSI data sets both during and after the acquisition in reasonable time.

The analysis of tumour heterogeneity is one of the most important biomedical research challenges in oncology and personalised medicine. MALDI imaging is perfectly suited to get an unbiased molecular view on

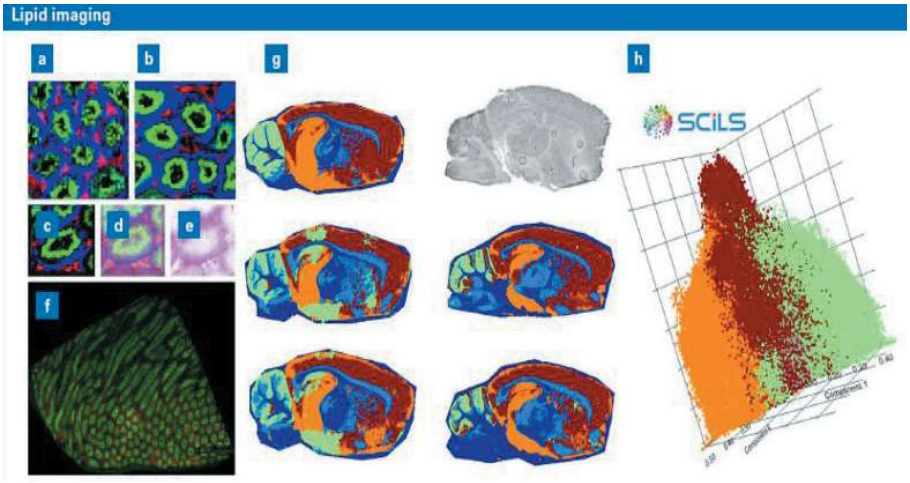



*Figure 2: Rat testis analysed at different pixel sizes (a-c, shown to scale). At 25 µm (a), tissue structure is not clearly visualised, demonstrating the need for higher resolution. At 10 µm, detailed morphology is visible (b). Tissue integrity is fully preserved even at smallest pixel sizes (5 µm, c-e). A large measurement (0.4 cm2, 394,629 pixel) acquired at 10 µm pixel size in just 137 min (48 pixel/sec) shows highly consistent data quality (f). Five mouse brain sections (594,434 pixel total) measured in ~4h (43 pixels/sec) were imported, analysed and visualised in SCiLS Lab 2015b on a regular desktop PC in ~2h. We show a segmentation map (g) and a component analysis of all spectra, showing separation of cortical, hippocampal and cerebellar tissue (h).*

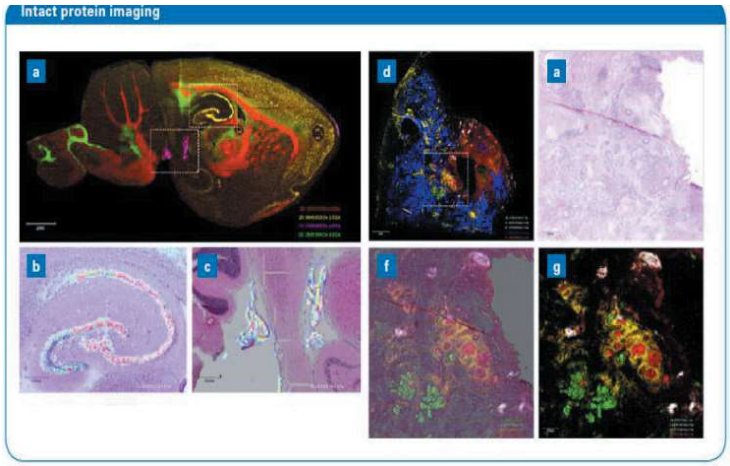



*Figure 3: Sagittal rat brain section (1.36 cm2) analysed at 30 µm pixel size (151,164 pixel) (a). Total acquisition time ~4h (~11 pixel/s). Four highly localised ion distributions show delicate structures such as the hippocampal pyramid cell layer (b) and the ependyma (c) clearly. Sample courtesy of Julian Langer (MPI of Biophysics, Frankfurt) rapifleX MALDI Tissuetyper allows analysis of large, diffuse tumour samples in an enabling timeframe. Human prostate carcinoma (2.24 cm2) analysed at 30 µm pixel size (248,825 pixel) (d-g). Total acquisition time ~8h (~8.5 pixel/s). Ion distributions show fibromuscular tissue (blue), tumour cells (green), intraglandular mucous material (red) and periglandular regions (yellow). Sample courtesy of Axel Walch (Helmholtz Zentrum München)*

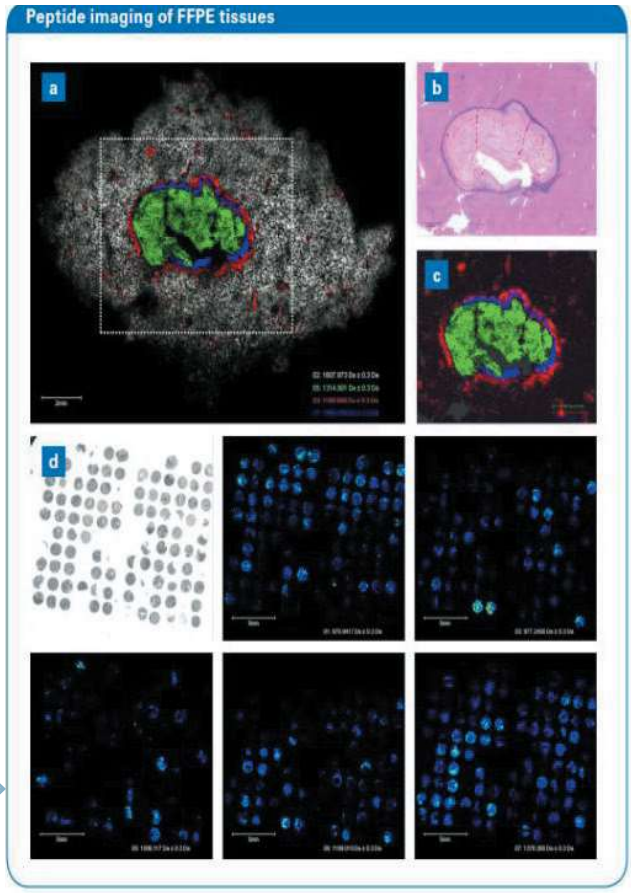
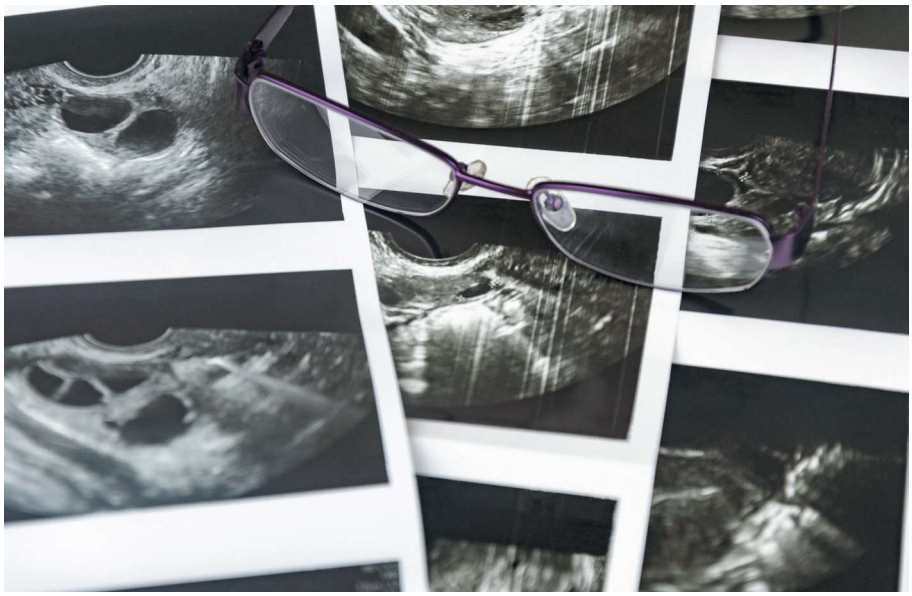



*Figure 4: Human liver infected with Echinococus spec.(1.62 cm2) analysed at 30 µm pixel size (180,002 pixel) (a). Total acquisition time ~2h (~24 pixel/s). The hydatid cyst is clearly separated from the host tissue (grey). At higher magnification (b,c) the host (red) and parasite layers (blue) of the cyst are clearly distinguished. Human lung cancer TMA (d). 99 individual cores analysed at 50 µm pixel size (51,932 pixel). Distributions of 5 peptide m/z-values are shown. TMAs represent a unique possibility to analyse samples from many patients at high throughput. Here, we generated data from ~1 patient/ minute. Samples courtesy of Jörg Kriegsmann, Proteopath GmbH, Trier*

tumour heterogeneity. An instrument that offers enhanced speed and performance gives scientists the possibility to analyse even large tumour specimens over huge patient cohorts with sufficient spatial resolution to get a comprehensive understanding of tumour heterogeneity.

## Boosting Efficiency in the Drug-finding Process

New laser technology in MALDI instrumentation not only gains speed for MALDI imaging but can also be used at the very beginning of the drug discovery pipeline. The process of drug discovery includes the testing of millions of pure compounds against enzymes relevant within the context of a certain disease. This screening is a time-consuming and, therefore, costly necessity for finding new leads for further drug development.



Mass spectrometry instrumentation has applications for ultra high-throughput drug discovery screening. Recent technical improvements in mass spectrometry instrumentation allow for much higher acquisition speed and robustness compared to previously available MALDI-TOF instruments. This reduces the time for a 2,000,000 screening campaign to around a week (depending on, e.g., the number of laser shots per spot, or the target geometry (384 vs 1536 vs 6144)). In one example, after such a two million screening campaign utilising 6144 target geometries, there was no need to clean the lens stack[1], achieving the highest level of throughput continuity.

## Reaching New Heights in Imaging Performance

Mass spectrometry imaging solutions such as the rapifleX MALDI-TOF mass spectrometry system offers high acquisition speed (up to 50 true pixels / seconds for faster and better images), pixel sizes of ≤10 µm for highest spatial resolution to retrieve biological information, non-overlapping, quasi-square pixels

and delivers consistent image quality at high throughput and high spatial resolution. 3D lasers offer enhanced pixel-to-pixel reproducibility and a novel ion source design provides increased robustness.

Current advanced TOF/TOF systems have been re-designed from the ground up to meet today's highest demands for in-depth intact and top-down protein characterisation, and high-performance, high-throughput mass spectrometry imaging (MSI). Next-generation systems offer higher speed, enhanced mass resolution and mass accuracy, and a significantly enhanced MS/MS mass range to enable new research and routine applications. Researchers require speed and ion source robustness, wide dynamic range, higher specificity and resolution, which all contribute to the detailed characterisation of biologically and clinically relevant lipids, peptides and proteins.

Scientists have set the expectations for a system that offers in-depth protein characterisation and imaging of tissues, cell cultures, or other applications. MALDI imaging is a game-changer for research and large-scale validation where enhanced ease-of-use, robustness and stability are vital. MALDI can be utilised at the very beginning of the drug discovery process and high-throughput screening (HTS), as well as in drug imaging: from identifying potential drug candidates to verifying their distribution and toxicity in tissue.

**REFERENCES**

1. P. Marshall, M. Leveridge, C. Haslam, G. Clarke, J. Chandler, A. Dunn, N. Hardy, M. Pemberton, S. Dikler, J. Fuchser, Ultra High Throughput Drug Discovery Screening by MALDI-TOF Mass Spectrometry – Exceeding One Million Samples per Week, Poster W-T-223 , 21st International Mass Spectrometry Conference, Toronto, Canada, August 20th – 26th 2016.

**Rohan Thakur**

Rohan Thakur is the Executive Vice President at Bruker Daltonics has over 20 years of experience in mass spectrometry, including 14 years in MS development, and has several patents in the field of ion optics. During his career he held positions as Director Global Marketing for mass spectrometry solutions at Thermo and was Director of Drug Discovery at a Pharma CRO for two years before joining Bruker. Dr Thakur received his PhD in Chemistry from Kansas State University and did post-doctoral studies at Rutgers University, where his work involved using high-resolution MS analysis to prove the formation of ring-opened benzene metabolite-DNA and protein adducts.