Automated Mass Spectrometry Imaging with a Matrix-Assisted Laser Desorption Ionization Time-of-Flight Instrument

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The automated use of a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (MS) is described for image analysis of samples through implementation of new software for instrument control, data acquisition, and data analysis. The software permits automated acquisition of MS MALDI spectra to form an ordered data array and contains display features to provide images at one or more mass-to-charge ratio values. The technique can be used to scan tissue samples, blotted samples, gels, or other sample surfaces where the image analysis of that sample is required. The program achieves a time of typically 1 s per image point, permitting an analysis made up of large numbers of points with high spatial resolution up to 850 dpi. The features of the software are demonstrated in this paper with samples of printed images, where visible images can be compared to those obtained by mass spectrometry. Quantitative aspects are introduced by analyzing a series of sample spots containing different amounts of several proteins. (J Am Soc Mass Spectrom 1999, 10, 67–71) © 1999 American Society for Mass Spectrometry

Imaging of the surface of samples by mass spectrometry has been demonstrated using secondary ion mass spectrometry (SIMS) [1–3] where investigators have analyzed tissue samples for low molecular weight compounds [4, 5]. Experiments involving work with laser microprobe mass spectrometry (LMMS), also named laser microprobe mass analysis (LAMMA), have used a well-focused laser beam for the desorption/ionization of analytes from sample surfaces with ion analysis by time-of-flight (TOF) measurements [6, 7]. This has been primarily used to scan a surface sample for metal ions [8]. Because an extended mass range was not available on these instruments, analysis of biological samples was limited to small organic molecules and the indirect analysis with mass spectrometry (MS) fingerprint techniques [9, 10]. Early experiments with matrix-assisted laser desorption ionization (MALDI) imaging in the low mass range were demonstrated on a specially designed LAMMA instrument [11]. Recent work from the authors' laboratory utilizes matrix-assisted laser desorption mass spectrometry to achieve the image analysis of biological tissues for peptides and proteins over 25 kDa [12, 13]. In this work, the spatial distribution of the compounds in tissues was determined by either directly attaching tissue slices to the target or blotting tissue on prepared surfaces, and then electrospray addition of the matrix.

In order to effectively use a MALDI TOF instrument as an imaging device, software needed to be developed to optimize and automate the scanning process to include fast data acquisition, online compression of the data, and display of the images. Such software would also be of wide interest, allowing the imaging capability to be added to already existing MALDI instrumental procedures [14, 15].

In our previous work [12], images were produced by manually taking mass spectra of individual spots in an area typically composed of approximately 30×30 spots. A major limitation of this technology for imaging was the slow data acquisition rate. Data from each spot required 1 min or more to acquire and download, resulting in a total analysis time of over 15 h. With the implementation of the software described in this paper termed the "MS Image Tool," this has been minimized by providing acquisition times of 1 s or less. The software package incorporates all the modules that are essential for the control of the instrument: acquisition, processing, and display and storage of the data. The basic functions of the software are described and some examples of its use for producing MALDI MS images are shown.

Experimental

A Voyager Elite (PerSeptive Biosystems, Framingham, MA) MALDI TOF mass spectrometer with delayed extraction and a reflectron is used. The instrument is controlled by a Pentium PC, which also serves as the

data station. The spectra are acquired and averaged by a digital storage oscilloscope (TDS 520B, Tektronix, Beaverton, OR) before they are downloaded to the PC via a GPIB bus. The source housing contains a movable sample plate, which can be positioned by stepper motors to a reproducibility of ± 5 μm. A mask is positioned directly in front of the nitrogen laser (337.1 nm, >250 μJ, VSL-337ND, LDI, Minneapolis, MN) and a single lens for focusing results in a target spot size of approximately 30 μm in diameter [12]. Image analysis of a sample is performed by moving the sample plate under the laser and acquiring averaged mass spectra at each spot in a raster of the target surface.

The program was written for the Windows 95 and Windows NT operating systems in order to obtain optimal performance. It consists of three different main modules: instrument control, image scan control, and data display and processing.

An image of printed letters was obtained by using an ink-jet printer (HP DeskJet 500C, Hewlett-Packard, Palo Alto, CA) on a cationic cellulose membrane (NA49, Schleicher and Schull, Keene, NH). The letter "M" was printed by using the yellow ink of the cartridge. The letter "S" was printed on top of the "M" with the magenta ink. The size of the letters is 1.4 × 1.4 mm. The membrane was mounted on the flat target with double-sided adhesive tape (3M, St. Paul, MN). The mass spectrometry analysis was performed without the application of matrix.

Printed images containing peptides were produced by using ink-jet cartridges to which peptide was added. 1 mg of neurotensin (Aldrich Chemical, Milwaukee, WI) was added to the blue ink of a cartridge and the cartridge was flushed by printing two pages of text in blue color. This procedure results in an estimated concentration of neurotensin in the ink of 5 pmol/uL. The cartridge was then used to print the letters "MS" on cationic cellulose membrane (NA49) with a letter height of 1.4 mm. The membrane was mounted on the sample plate and a concentrated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 85% ethanol and 15% MilliQ water containing 2% acetic acid was electrosprayed on top of the sample using our previously described procedure [12]. The image of 1.5 × 3 mm was scanned with 100 points in the x direction and 50 points in the y direction. For every image point, five spectra consisting of 2200 data points were averaged. One mass window was selected from m/z 1662 to 1682 and the intensity of the peak height in this range was calculated online.

The sensitivity of the technique was demonstrated by analyzing a series of sample spots containing different amounts of vasopressin. C-18 beads (Alltech Associates, Deerfield, IL) were applied to double-sided tape attached to the sample plate. Approximately 5 nL of 0.5, 1, and 5 μM vasopressin (Sigma Chemical, St. Louis, MI) were microspotted on this homogenous C-18 surface with a flow rate of 1.5 nL/min through a capillary of 50 μm i.d. α-Cyano-4-hydroxy cinnamic acid (HCCA) was electrosprayed on the surface of the sample. The area of 3 × 1.2 mm was scanned in a raster of 30 μm with a total of 100 × 40 points. For each point, 10 scans with 20,000 data points were averaged. The data was transferred as a text file from the Image Tool to SigmaPlot (Jandel Corp.).

Results and Discussion

Description of the Image Tool

Instrument control is achieved by a full graphical intuitive user interface, with all parameters capable of being stored to a setup file and retrieved for later use. The full instrument parameter set is saved with every image and mass spectrum. In order to obtain full manual control of the instrument, an "expert mode" is available which is protected by a software key. In this mode, the pneumatic and vacuum valves can be manually operated, the voltages individually controlled, and the sample stage can be moved.

The acquisition control window is used to select the parameters for the acquisition of the mass spectra and scanning of the image. In order to set up a scan, the number of data points, the time resolution and data points of the digital storage oscilloscope and the number of scans to be averaged are selected.

For processing and display of data, the Image Tool offers the option to save every mass spectrum of every image point and/or the online processing, displaying, and saving of the image files. Before the scan is started, the user defines mass windows covering the peaks of interest and selects whether peak height or peak area is to be used for the imaging process. The program calculates the image values during the scan and saves them to the disk. To display the image data, the image values are transformed online in a blue/red image using the full color range for every image. If the mass spectra are stored during the scan, the program allows the user to browse through the spectra and select mass ranges of interest. The stored mass spectra are then processed offline to produce the selected images. By using the mouse to click on individual image points, the mass spectrum obtained from this point is displayed in a separate window. This offline processing was found to be useful for the analysis of samples with unknown components. The image files can be exported in ASCII and TIFF format.

The most time intensive part of imaging is the acquisition of the data. Figure 1 shows the steps that are performed during an image acquisition. The instrument is set to the selected parameters and the software starts the scan. The laser is fired and a number of mass spectra (n) is averaged. The resulting spectrum is downloaded to the computer, saved to the disk, and/or processed by the image routine. After moving the sample to its new sample spot, the laser is fired again for the acquisition of the next image point. The image time is described by
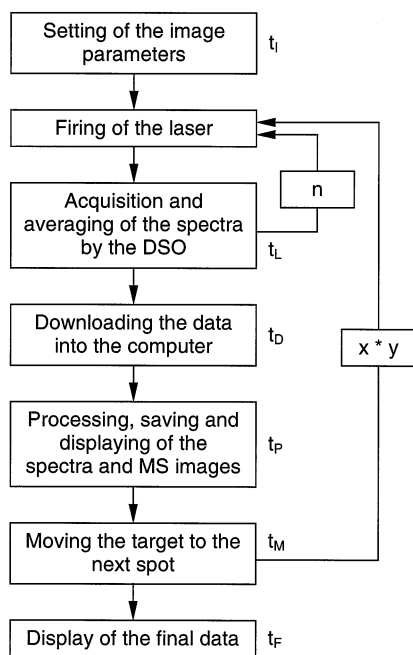
Figure 1. Sequence of the image acquisition process. x and y are the dimensions of the image, n is the number of laser shots per spectrum.

$$t_{\mathrm{image}} = t_I + t_F + xy[(nt_L) + t_D + t_P + t_M] \qquad (1)$$

The time it takes to acquire each data point is the most relevant factor. Averaging of the data is done by the DSO using its built in microprocessor. In order to increase the speed of the program, the processing, display, and storage of the data occurring over time (t_P) is done simultaneously with the acquisition of the new data by the DSO. Because the acquisition time is longer than the processing time, t_P is set to zero. The laser frequency is set to 5 Hz, which results in spectra of high quality. In order to obtain a shorter acquisition time, this frequency can be increased up to 20 Hz, but with a decrease in peak resolution. By selecting the smallest possible numbers of spectra to be averaged (n), the acquisition time is minimized. The download time of each spectrum (t_D) is 0.5 s for 50,000 data points consisting of 16 data bits. The time for movement of the sample (t_M) typically takes 200 ms. As an example, the image in Figure 2 is built by acquiring 2500 image points with the averaging of five mass spectra per point. The imaging time for each point in this example was 1.56 s, which resulted in an overall imaging time of 65 min.

Test Images

The capability of the program was demonstrated by the acquisition of a MALDI MS image of letters printed by an ink-jet printer (HP DeskJet 500). The image in Figure 2 was constructed by processing 50 × 50 mass spectra, which were collected over a 1.5 × 1.5 mm^2 area. The

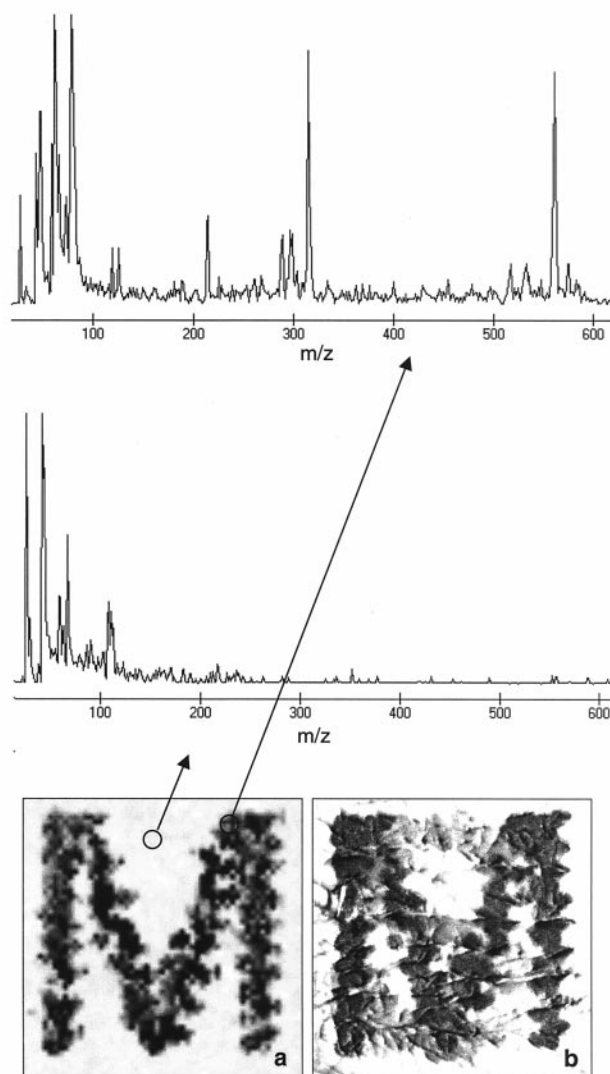**Figure 2.** Mass spectrometry image (**a**) (50 × 50 pixels, 1.5 × 1.5 mm) at m/z 3.4 and optical image (**b**) of a letter printed with an ink printer.

letter "M" was printed in yellow ink, with the letter "S" overprinted in magenta ink. The bottom spectrum in Figure 2 was taken in an area where no ink was applied and the top mass spectrum was acquired from a point where ink was printed. The yellow ink of the using printer cartridge produced an intense peak with m/z 314. Using the peak height of this ion from the data array, the mass spectrometry image shown in Figure 2a was generated. As a comparison, the optical image (250 × 250 pixel) of the same area was taken with a digital camera connected to a microscope (3CCD and BX 50, Olympus, Melville, NY) (Figure 2b). The mass spectrometry image clearly resolves ink dots and matches well with the optical image. The letter "S" printed with the magenta ink, seen as a light gray overlay of the letter "M" in Figure 2b, does not interfere with the mass spectrometry image because this dye has a different molecular weight.
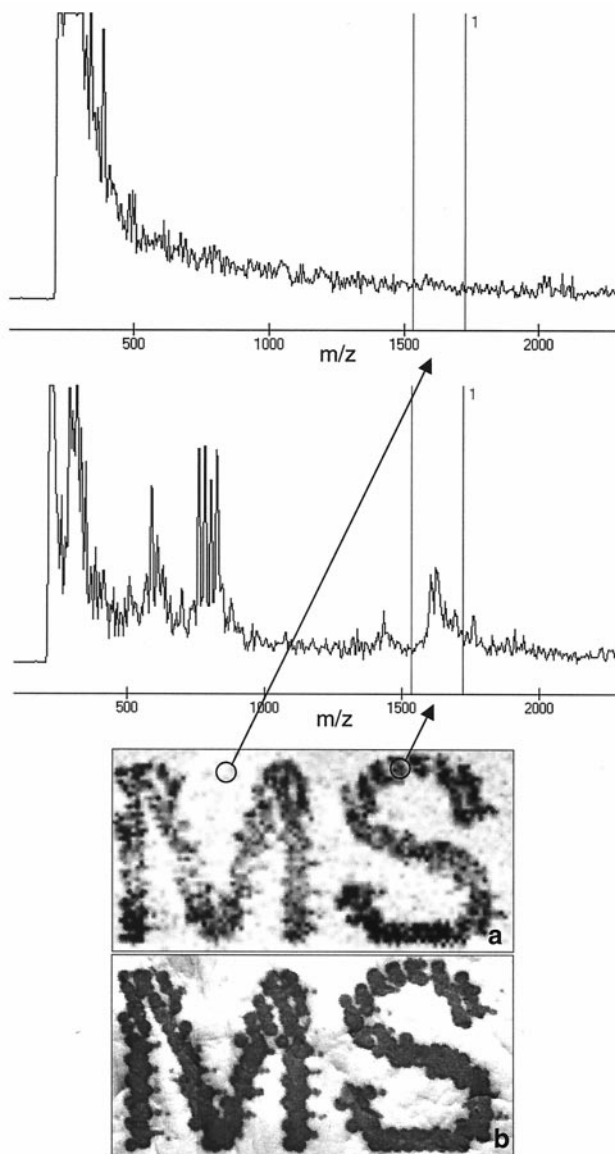
Figure 4. 3D image (100 × 40 pixels, 3 × 1.2 mm) of the intensity of (arg)8-vasopressin spotted on C-18 and scanned with the MALDI Image Tool.

Figure 3. Neurotensin was added to the ink of a printer. The mass spectrometry image (**a**) (100 × 50 pixels, 3 × 1.5 mm) obtained of the protonated neurotensin peak matches with the optical image (**b**).

A second example involves neurotensin that has been printed on a membrane using an ink-jet printer where the print cartridge was opened and 1 mg of neurotensin was added. Figure 3a shows the MALDI-MS image created using the height of the neurotensin peak at m/z 1674 from each of the mass spectra obtained from the imaged area. The mass spectrum in the top panel was taken from a point where no neurotensin was applied and the mass spectrum in the bottom panel shows the neurotensin peak from an area where the ink/neurotensin mixture was applied. Comparison of the MALDI-MS image in Figure 3a with the optical image in Figure 3b shows a detailed match. The MALDI-MS image was scanned in 30 μm steps by
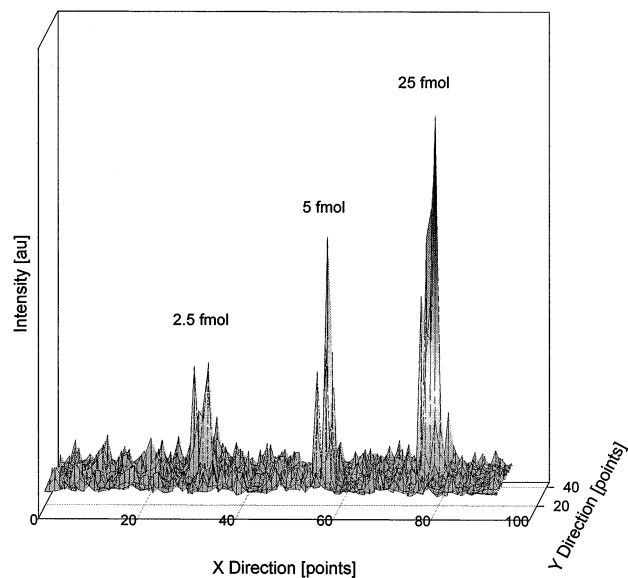
acquiring 100 mass spectra in the x direction and 50 mass spectra in the y direction resulting in 5000 mass spectra. The mass spectra were not saved but processed online to calculate the height of the neurotensin peak at m/z 1674. This resulted in a file that was 22 kbytes in size representing an effective compression of the data of over 2000-fold relative to saving the entire spectrum. The image acquisition required 270 min. Although this time is lengthy compared with the SIMS or LMMS technique, the information that can be obtained on a wide distribution of biological molecules is of enormous importance.

In order to estimate the quantitative aspects of the technique, several different concentrations of vasopressin were microspotted on a C-18 surface [12] and analyzed with the Image Tool. Figure 4 shows the SigmaPlot file of the acquired MALDI-MS image representing the vasopressin peak height. From the image data, the area of every microspotted region was calculated: a deposited amount of 2.5 fmol result in 45 arbitrary units (au), 5 fmol in 65 au and 25 fmol in 155 au. Linear regression analysis of these data points results in a correlation coefficient of 0.997, which is an acceptable value considering the variances in the MALDI process. This example demonstrates that the MALDI-MS image tool has the potential to perform quantitative analyses. The image data can be exported as a text file, which allows the data to be read and processed by a wide variety of different data analysis programs.

The Image Tool promises to be useful in a wide range of potential applications in the analysis of biological samples. Improvements for the MALDI-MS image technique that remain to be done are instrumental in

nature rather than software based. In order to study processes at a cellular level, the spatial resolution of the images must be improved by decreasing the laser diameter and reducing the stepper motor movement of the sample plate to the submicron range.

Acknowledgment

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