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Etalo, D.W.; Díez-Simón, Carmen; De Vos, Ric C.H; Hall, Robert D.

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Chapter 18

Laser Ablation Electrospray Ionization-Mass Spectrometry Imaging (LAESI-MS) for Spatially Resolved Plant Metabolomics

Desalegn W. Etalo, Carmen Díez-Simón, Ric C. H. deVos, and Robert D. Hall

Abstract

There is increasing demand to bring the level of metabolomics analyses down to the tissue or cell level. Significant progress has been made involving the use of in situ metabolomics imaging techniques where no tissue collection or extraction is needed prior to analysis. In this chapter we describe a relatively new method which is simple and easy to use. No ectopic matrix or vacuum is required, and analyses are performed with living plant materials directly from (or even still attached to) the plant. Although relatively straightforward, there are still a few caveats as regards this method which are described at the end of the chapter.

Key words LAESI, Laser ablation ESI-MS, In situ metabolomics, Spatial resolution, Plant metabolomics, Imaging

1 Introduction

Metabolomics has already become an important tool to study the chemically diverse metabolites that are accumulated by plants. A main underlying goal of many metabolomics studies is to be able to define specific metabolic pathways, investigate their interconnectivity, identify their control points and follow their dynamism. However, plant systems are a technological challenge for metabolomics, both in terms of their huge chemical diversity and the very strong temporal and spatial heterogeneity that is characteristic of their biosynthesis and accumulation.

Standard metabolomics approaches are generally based upon the extraction, separation and (if possible) identification of the detectable metabolites in a particular tissue. A widely used detection method in plant metabolomics is mass spectrometry (MS) due to its sensitivity, high resolution, speed, and broad application

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[1]. GC (gas chromatography), LC (liquid chromatography), and CE (capillary electrophoresis) are the separation techniques of choice when coupled to the MS. Other approaches focus on NMR [2], and these methods are receiving growing attention as increasing instrument sensitivity is enabling increasing numbers of metabolites to be detected and directly identified. Nevertheless, all of these approaches suffer from a significant, common drawback. Prior to analysis, plant material must be collected, usually pooled and then extracted to provide a robust, biologically relevant sample with minimal technical "noise." Plant tissues are, however, highly heterogeneous, comprising multiple cell types, each of which has its own specific biochemical profile. This heterogeneity, which is of high importance for the structure and function of the plant, is therefore lost and we have to make do with the "law of averages." While in many cases, such an average "global overview" is perfectly adequate to meet our needs, when we wish to delve deeper into the precise metabolic heterogeneity and chemical diversity of plants and try to understand how these are organized at the cellular and molecular level, this loss of spatial heterogeneity becomes a serious limitation.

Analytical approaches enabling analysis at the cellular level are therefore required. Over the last ten or more years, different inroads have been taken to allow us to "look into" plant tissues and perform in situ chemical profiling. These so-called metabolite "imaging" approaches (MSI, mass spectrometry-based imaging) are gaining in popularity for tackling specific metabolomics questions and a number of contrasting technologies have been developed. Significant progress has been made and MS imaging applications have been employed in the animal [3], human [4], microbial [5], and plant sciences [6]. Perhaps the longest-established and most broadly adapted methods are based on MALDI-MS (matrix-assisted laser desorption ionization) technologies [7]. Direct analysis in real time (DART) and desorption electrospray ionization (DESI) approaches [8, 9] provide alternative methods which have also received attention. Each method has its particular advantages and disadvantages (requirement for an applied ionization matrix, use of a vacuum during analysis, loss of spatial resolution, etc.). All these approaches look at the natural or exposed surface of a plant and following metabolite vaporization and ionization of the constituent molecules, use MS for detection. Such direct imaging approaches potentially avoid extraction-related artifacts but in contrast, do carry certain potential limitations, e.g., a greater risk of matrix/ion suppression effects and a lack of resolution through the absence of separation techniques [10].

Both the most widely used MSI techniques, secondary ion mass spectrometry (SIMS) [11, 12] and MALDI [13–15], usually require measurements to be made under vacuum conditions to

obtain the best results. SIMS is characterized by a very high spatial resolution (sub-μm), which can detect inorganic compounds at relatively low masses, whereas MALDI is able to detect small to large molecules at a spatial resolution down to ca. 10 μm [16]. An atmospheric pressure (AP)-MALDI technique has been developed which allows for sampling in an ambient environment. However, this approach suffers from significant ion losses between the source and the mass analyzer [17]. Nevertheless, a particular advantage of MALDI is that, as well as small molecules, it can also very effectively be used to image macromolecules such as proteins.

From 2007, ambient ionization techniques for MSI were starting to be used which involve no sample pretreatment and no need for a vacuum. Desorption electrospray ionization (DESI) is especially useful for ionizing polar compounds, but more hydrophobic compounds can also be detected using appropriate solvents [18, 19]. Later, laser ablation electrospray ionization (LAESI) was also developed which had the advantage that it can separate the desorption and ionization process into two different steps [20] while still operating at atmospheric pressure [21]. The typical spatial resolution in a LAESI experiment is 200–300 μm although performing in situ analysis of single plant cells at a lower spatial resolution has been demonstrated using onion epidermis cells [22]. Furthermore, LAESI is one of the first ionization techniques that enables 3D profiling since the laser can gradually remove a tissue, layer by layer [23–25].

LAESI mass spectrometry imaging (MSI) uses a mid-IR laser beam to excite the hydroxyl vibrations of water molecules in the sample to produce a plume of neutral ion species which are then ionized by charged solvent droplets from a nanoelectrospray. These charged ions then directly enter the MS mass analyzer for mass analysis (Fig. 1). To perform LAESI-MS the fresh/living sample is simply mounted flat onto a sample holder and positioned at the focal point of the mid-infrared light ablation beam. The molecular image of a sample is reconstructed by representing the intensity of the total or specific ion signal on a false color scale and correlating this with the coordinates of each pixel of the analyzed area (Figs. 1 and 2, previously unpublished example). The use (and limitations) of LAESI-MS imaging is nicely demonstrated in our recent paper where LAESI/visible mapping of visible pigments in *Phalaenopsis* orchid petals was performed to provide a clear proof of concept [26]. This is the paper upon which this chapter is based.

In this chapter we provide a step-by-step guide to performing a LAESI-MS-based analysis on *Phalaenopsis* petals which are easily obtained at local garden centers and which should provide a straightforward introduction to the technology and its potential.

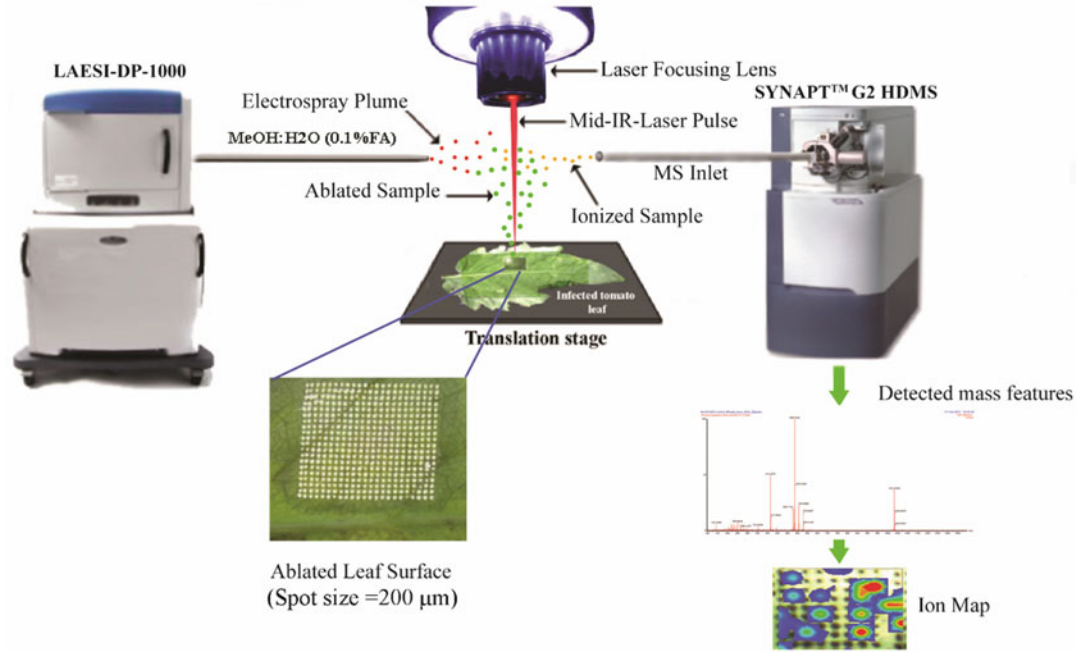

Fig. 1 A schematic representation of LAESI-MS. Under ambient atmospheric conditions, water-containing tissues such as plant leaves are subjected to mid-IR (2940 nm) laser pulses. The absorption of such an intense laser pulse by the sample produces small bubbles within the sample without ion formation. When the bubble explodes, a jet of vaporized material (ablated sample) is ejected above the sample surface without ionization. The ablated sample then intersects a stream of ions from an electrospray source (electrospray plume), which ionizes the molecules of the jet (ionized sample). The ionized molecules subsequently are directly drawn into the mass spectrometer for analysis. Using Protea Plot software for data analysis, the detected mass features can subsequently be converted into two-dimensional or three-dimensional ion maps and can be overlaid on the original sample image, reproduced from [26]

2 Materials

2.1 Plant Tissue

Phalaenopsis orchid petals from well-watered, healthy plants.

2.2 Chemicals

1. Ultra LC-MS-grade methanol (MeOH).
2. Ultrapure water (Actu-All Chemicals).
3. Formic acid (FA) 98–100% (Biosolve Chemicals).
4. Sodium formate suitable for MS calibration over the mass range of 50–1500 Da.
5. Leucine enkaphaline (LE, $\geq$ 95% purity) standard for lock-mass correction.

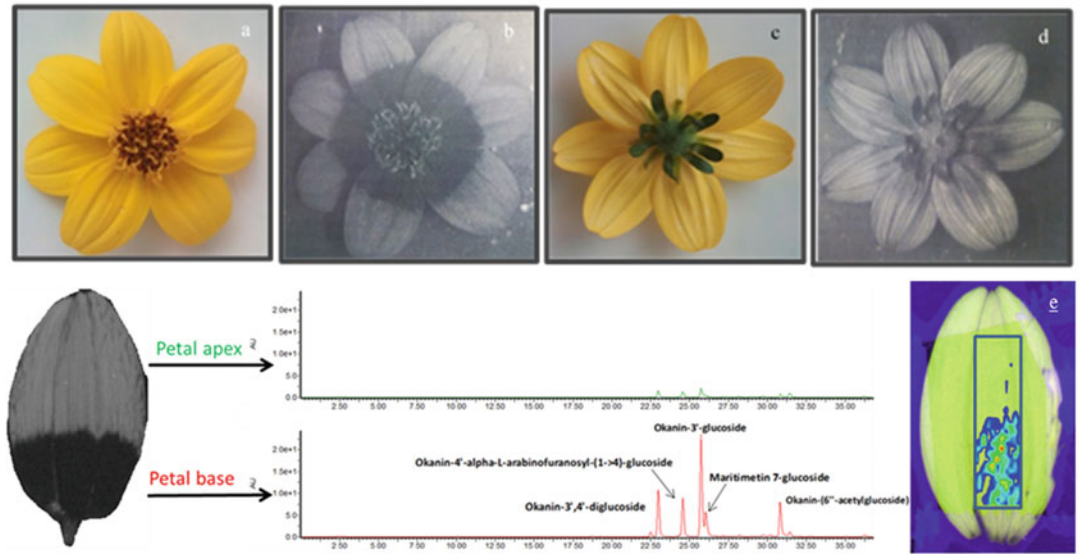

Fig. 2 Analysis of flowers of the garden plant *Bidens ferulifolia*. *Bidens ferulifolia* flower as seen from above in daylight (**a**) and after UV photography (**b**) and from underneath in daylight (**c**) and after UV photography. The presence of okanin derivatives are only visible under UV light and are strictly located in the lower regions of the petal. LC-MS analysis of petal extracts and LAESI imaging (**e**) both confirm their chemical location

2.3 Reagents and Solvents

1. Electrospray solution: MeOH–H_2O (50:50, v/v) with 0.1% (v/v) FA.
2. MS calibration solution: prepare a sodium formate solution 10% FA–0.1 M NaOH–acetonitrile (1:1:8, v/v/v).
3. Lock-mass correction solution: Leucine enkaphaline (LE, *m/z* 556.2771) in acetonitrile–ultrapure water (50:50, v/v).

2.4 Equipment and Software

1. 4.0-mL glass tubes to store electrospray solution.
2. Razor blade.
3. Double-sided sticky tape.
4. Standard glass slides for mounting samples (Protea).
5. Syringe ID (4.6 mm diameter, 100-μm internal diameter stainless steel nanospray emitter).
6. Protea Biosciences LAESI model DP-1000 LAESI system with LAESI desktop Software™ (LDS) to control all on-board LAESI DP-1000 source hardware components, contact closure for triggering of mass spectra data acquisition, and synchronization of LAESI laser with raw data capture in the MS for imaging location and spatial analysis.
7. Waters mass spectrometer model Synapt G2-S with Masslynx software (version 4.1).
8. Protea Plot v.2.0.1.3 (Protea Biosciences) software for generation of ion maps, extraction of mass spectral data from vendor

files, viewing of TIC, base peak plots, and individual or averaged mass spectra, generation of 2D and 3D contour plots (ion/heat maps) for mass spectral imaging studies, export of mass spectral data, 2D/3D heat maps, sample images and automated baseline subtraction, and differential sample-zone analysis features.

3 Methods

3.1 Connecting the LAESI DP-1000 Direct Ionization System to a Waters Mass Spectrometer (Synapt G2-S)

1. Calibrate the mass spectrometer using the MS calibration solution following the procedure provided by the vendor.
2. Check for good performance in terms of mass accuracy and resolution before connecting it to the LAESI-DP-1000 system. A cluster of 21 reference peaks with mass range of 50–1500 Da should be detected.
3. Operate the time-of flight (TOF) mass analyzer of the Synapt G2S in the V-reflectron mode at a mass resolution of 20,000 (full width at one-half maximum).
4. Set the MS parameters as follows: source temperature 150 °C, desolvation temperature 150 °C, sampling cone voltage 30 V.
5. Acquire the positive ions in the range of m/z 90–1500.
6. Use LE (m/z 556.2771), which was added to the spray solvent, to lock-mass correct the TOF-MS post-data acquisition data.
7. To prepare the LAESI DP-1000 System for use, first insert the LAESI extension tube into the mass spectrometer and fit it to the MS Adapter Plate onto the MS being used.
8. Roll the LAESI system in front of the mass spectrometer by adjusting the vertical height of the LAESI DP-1000 system as needed using the 8 mm hex shaft on the lower right corner of the unit. After the vertical height has been adjusted, roll the LAESI DP-1000 system onto the MS adapter Plate ensuring not to bend the extension tube as it is guided through the rear through-hole in the back of the unit.
9. Connect the female end of power cord to the LAESI DP-1000 system at the lower right corner of the unit and then plug the male end of the power cord into a standard outlet. Connect the B end of the USB cable to the LAESI DP-1000 system at the lower right corner of the unit, and then plug the A end of the USB cable into the computer that will control the LAESI DP-1000 system. The MS computer may be used to control the LAESI DP-1000 system.
10. Connect the MS Trigger Cable to the LAESI DP-1000 system at the lower right corner of the unit. Connect the prewired terminal block to the "External Connections 1" port at the rear

of the MS. Also connect the other prewired terminal block to the Port on the eSAT/IN kit provided.

11. Connect the MS Sync Cable to the LAESI DP-1000 system at the lower right corner of the unit. Connect the ethernet cable to Channel 1 of the eSAT/IN kit.
12. Connect the power cord to the eSAT/IN kit and plug the power cord into a standard outlet.
13. Also connect the eSAT/IN kit to the network switch connecting the MS and the MS computer via the ethernet connection. At this time cycle the power on the computer and the electronics on the MS, ensuring that the MS does not vent.
14. Once the computer and MS are reset, and communication between the MS and computer has been established, open MassLynx and open the Inlet window.
15. In the Inlet window, click on the "Tools" menu and navigate to "Instrument Configuration." Once the configuration page has opened, click the "Configure" button then "Next."
16. The first option will be to configure the LC pump: click "None" and then "Next."
17. The next option will be to configure the autosampler: click "External" and then "Next."
18. On the next page, scroll toward the bottom and click on the eSAT/IN kit and add it to the "Configured Detectors," and then click "Next."
19. On the next page, click the "Scan for Instruments" button; this will scan the system for all available Waters hardware. When it completes click the "Ok" button.
20. On the right, next to "Detector Waters ESATIN," click the drop-down button next to the eSAT/IN kit, and select the serial number that appears for the eSAT/IN kit.
21. Click "Next" followed by "Finish" on the subsequent page. A message should appear saying that the autosampler has been set to external, click "Ok."
22. At this point, the e-SAT/IN kit should appear under the "Detector 1" location in the "Selected Configuration Addresses" box. If it does not, click the "Configure" button again, and follow the steps above again until the eSAT/IN kit appears under the "Detector 1."
23. Next, click the "Events and Triggering" button followed by "Next." Enable the "Event In" Channel 1 option, and click "Next."
24. Under "Detector," click the "Trigger by Contact Closure" option, followed by "Next" and "Finish."

25. On the left of the Inlet window, a "Waters eSAT/IN" icon should appear. Double-click on it to open the options page. "Channel 1" should be enabled and in the "Description" area, type "LAESI Analog Signal."
26. Change the "Sampling Rate" to 10, the "Units" to V, and the "Scale Factor" to 1. It is important to note that in the upper right-hand corner, there is a "Run Time" box. The user MUST specify a run time in this box prior to starting a LAESI-MS analysis. Consequently, a different run time will need to be entered and the "Inlet file" will need to be saved for every LAESI-MS analysis. If a time is not specified, the eSAT/IN kit will not record any data. Also, if the time specified is too long, the eSAT/IN kit will continue to record, even if the MS has stopped. Under this condition, the eSAT/IN kit will not be ready for a consecutive run.
27. Once the Inlet file has been modified, save the "Inlet file."

3.2 Mounting the Plant Tissue and Preparing for Measurement

1. Clean the syringe of the stainless steel nanospray emitter thoroughly with MeOH and fill it with degassed electrospray solution. While filling the syringe, ensure that no air bubbles are trapped in the syringe. Next, connect the syringe to the tubing connected to the "ESI Emitter Assembly," and prime the ESI emitter to ensure that all air has been removed from the ESI emitter tubing (*see* **Notes 1** and **2**).
2. Detach the *Phalaenopsis* spp. petal using a razor blade and mount the tissue as flat as possible using double-sided sticky tape with the adaxial side exposed (upwards) onto a standard sample slide (e.g., chemically precleaned glass slide) (*see* **Note 3**).
3. To perform an imaging analysis of the petal, first turn on the LAESI DP-1000, open the LDS software, and enter the "Interactive Mode." Open the "High Voltage Control" and "Syringe Pump Control" subsystem panels to initiate the electrospray to allow time for stabilization. In the "Syringe Pump Control" panel enter the appropriate syringe ID (4.6 mm diameter) and press "Apply." Next, enter the desired electrospray flow rate and then press "Apply." Typically, a 2 μL/min flow rate works well for syringe of 4.6 mm diameter (*see* **Note 4**). Click the "On/Off" toggle and the syringe pump should start pumping. Additionally, a green "Syringe Pumping" note will appear in the Status Panel in the lower right hand corner of the LDS screen. In the "High Voltage Control" panel choose the desired polarity (positive or negative; in our case, most of the analysis was performed in positive ionization mode) and then enter the electrospray voltage (3800 V was used in our analysis). After entering the voltage, click "Apply" and click the

"On/Off" toggle to apply the high voltage to the emitter. After enabling the voltage, an orange "Voltage On" note will appear in the Status Panel in the lower right hand corner of the LDS screen. This will also engage the door lock and illuminate the orange "High Voltage" LED on the front panel of the LAESI DP-1000 system to indicate the high voltage is on. After a short amount of time, the electrospray background signal should be apparent in the mass spectrum assuming the mass spectrometer has been enabled and is scanning.

4. Once the electrospray has stabilized, a sample can be introduced for analysis. Click the "Load" button in the "Stage Control" panel to move the Sample Nest forward. Next, click the "On/Off" toggle in the "High Voltage Control" panel to disable the high voltage this will unlock the door, allowing the door to be opened. Keep the Peltier-cooled motorized *x-y-z* sample stage at 25 °C. Once the Sample Nest is accessible, remove the Sample Tray, place the sample slide onto the aluminum slide holder, and replace the Sample Tray in the Sample Nest. Ensure that the Sample Tray is seated correctly through the illumination of the green "Sample Loaded" LED. After the tray is loaded, click the "Return" button in the "Stage Control" panel to return the Sample Nest to its previous location. Pressing the "Return" button WILL NOT enable the high voltage or the syringe pump; this must be done manually in their respective subsystem panels.
5. To start an imaging analysis, exit the interactive mode by clicking "Back" and create a "New Project." Type a name for the project and navigate to where the project will be saved on the hard drive. Once this is done, click "Create"; this will make the new project and open the "Project Summary" page. Enter any desired meta-data, such as user name, sample description, and comments.
6. For an imaging experiment the analysis pattern and location (s) must be defined. Before this can occur, a picture of the sample area must be acquired to view the sample. Click the "Modify Analysis" button to enter the analysis definition screen. Chose a "2D Imaging Analysis" from the dropdown menu in the upper left corner of the screen. Open the Camera window and press the "Move" button to move the Sample Nest to the image acquisition location. While the Sample Nest is moving press the "W" button to activate the wide-angle camera and then turn on the camera by pressing the power button. Enable the "Autogain" and "Auto White Balance" in the "Camera Settings" dialog box, if not already enabled. After the stage has reached the image acquisition location and stopped, the "Image Acquisition" button will become active. To acquire an image of the sample area, press

the "Image Acquisition" button. If the image is not suitable, open the "Camera Settings" dialogue box and make adjustments to the live image. Once the adjustments have been made, reacquire the image. To save the image, right click on the image, then navigate to "View" >> "Save Scanned Image." Then type in a name for the image and navigate to where the image will be saved. Once the image has been acquired and saved, press the "Return" button to move the Sample Nest back to its previous location.

7. Next, define the analysis pattern by clicking on the "Zoom Mode" button and use the scroll wheel on the mouse to zoom in on the image to see the sample of interest. Next, click on the "Grid" button to define a grid pattern on the image of the sample (*see* **Note 5**). Move the mouse arrow to the desired location of the first analysis point and left-click. Move the mouse arrow to the end of the analysis such that the grid is completely covering the sample in the image. Before left-clicking again, use the scroll wheel on the mouse to change the grid mesh, or the pixel center-to-center distances (mesh interval of 0.5 mm was used for analysis of orchid petals). Once the desired grid mesh has been achieved, left-click to finalize the grid selection. Using the "Select Mode" function, the grid can be moved as needed to better cover the sample in the image. In the upper left corner in the grid coordinate section, choose either a "Typewriter" or "Serpentine" analysis pattern; typically, serpentine is faster. Finally, enter the correct Z and L positions to ensure that the Sample Nest is the appropriate distance from the ESI emitter and that the focusing lens will be in focus at the specified Z position. In our trial with orchid petals often the focusing lens L value and the sample stage Z value were typically in the order of 4.3 and 20.8 mm, respectively. After entering the Z and L coordinates, click the "Back" button to navigate back to the "Project Summary Page."
8. Next, determine the laser parameters by pressing the "Configure Laser" button and enter the laser settings screen. The machine is equipped with a 2940-nm mid-infrared laser. First, choose the laser output energy by entering the percent output, typically 30% was used in our analysis. Next, choose the number of laser pulses at each location, again 10 pulses per location is suitable for *Phalaenopsis* spp. petal analysis (*see* **Note 6**). Then, enter the frequency of the laser pulses; typically, a frequency of 10 Hz is used. Finally, determine the dwell time, or the time the stage will remain at each location after the last laser pulse has been fired, before moving to the next location. We found 2 s of dwell time to be optimal. To help visualize where the laser pulses occur relative to the MS scans, enable the "MS Scan Visualization" function and enter the approximate scan

time of the mass spectrometer (1 s). Please be aware that this function is only for visualization and DOES NOT control the mass spectrometer scan time. After all the settings are entered and satisfactory, press the "Back" button to navigate back to the "Project Summary Page."

9. Now set up the electrospray parameters by clicking the "Configure Electrospray and Cooling" button and enter the electrospray settings screen. Enter the correct syringe ID (4.6 mm diameter) by either typing in the ID or using the slide bar. Next, enter the desired flow rate (2 μL/min). Next, choose the desired polarity of the electrospray (We used positive ionization mode) and then enter the desired electrospray voltage (3800 V) (*see* **Note 7**). If these settings were enabled in interactive mode they will remain on while the LAESI method is being constructed. Once all settings have been entered, click the "Back" button to return to the "Project Summary Page".
10. Next, set up the mass spectrometer options by clicking the "Configure MS" button to enter the MS options screen. First, choose the type of mass spectrometer being used. Next, enable the start trigger by clicking the toggle. Finally, choose whether the MS data will be high resolution or low resolution. After, these settings are established click the "Back" button to navigate back to the "Project Summary Page."
11. Save the changes to the LAESI method by clicking the "Save" button.
12. Now enter the Run Screen by clicking the "Run Screen" button. Type a name for the LAESI-MS Run, and any other desired comments. Next, click the "Create Folder" button; this will create a new folder with the analysis name inside the LAESI project folder. Next turn on the Inline Camera by pressing the power button.
13. Before the LAESI analysis can be started the MS settings must be established.

    For a Waters mass spectrometer, the data must be acquired by creating a sample list in Masslynx. First, type in a file name for the LAESI data. Next, choose a "MS Method" and an "Inlet" file for the data acquisition. For a Waters mass spectrometer, the MS method and Inlet files must have a specified end time, an estimated analysis time can be found on the "Project Summary Page" of the LDS and this values can be entered for the end time. Finally, specify any additional files or other information in the sample list and save the sample list. Some of the important TOF MS function parameters include acquisition mass range (90–1500 Da), scan time (1 s), interscan time (0.015 s), Data Format (continuum), and Analyzer (resolution) (*see* **Note 8**).

14. Before the LAESI analysis is started, first start the MS, which will ready the MS for the trigger signal from the LAESI DP-1000 unit.
15. Now the LAESI-MS analysis can be started by pressing the "Start" button on the Run Screen in the LDS. This will start the analysis and the LAESI method will be uploaded from the PC to the LAESI DP-1000 unit. After the upload is complete, the contact closure will be triggered and a laser warm up and background acquisition time will start for 45 s. After the warm up time, the sample nest will move to the first location and the LAESI analysis will start. Attention! If necessary, the LAESI analysis can be paused by clicking the "Pause" button. In addition, the ESI can be disengaged by pressing the "Unlock" button; this will also unlock the door. After any corrections are made, the ESI can be reengaged by pressing the "Lock" button, which will reapply the high voltage and lock the door. After pressing the "Lock" button the high voltage will be activated and the ESI will stabilize for about 10 s. After this, the "Start/Resume" button will become active and the LAESI analysis can be completed.
16. After the analysis is complete, the ESI is automatically deactivated. Also, stop the MS data acquisition if an automatic stop time was not used.
17. Next, acquire a post-acquisition image of the sample by clicking the "Back" button to return to "Project Summary Page." Reenter the Modify Analysis screen, open the camera window, and press the "Move" button to move the Sample Nest to the image acquisition location. While the Sample Nest is moving press the "W" button to activate the wide-angle camera and then turn on the camera by pressing the power button. After the stage has reached the image acquisition location and stopped, the "Image Acquisition" button will become active. To acquire an image of the sample area, press the "Image Acquisition" button. If the image is not suitable, open the "Camera Settings" dialog box and make adjustments to the live image. Once the adjustments have been made, reacquire the image. To save the image, right click on the image, then navigate to "View" >> "Save Scanned Image." Then type in a name for the image and navigate to where the image will be saved. Once the image has been acquired and saved, press the "Return" button to move the Sample Nest back to its previous location. After, the stage has returned to the previous location, navigate back to the "Project Summary Page" by pressing the "Back" button.
18. Return to the "Run Screen" and press the "Unload" button to bring the Sample Nest to the front of the LAESI unit to remove the Sample Tray and remove the sample from the slide holder. At this point the LAESI DP-1000 can be shutdown.

19. Check the deviation in mass accuracy of a known metabolite in orchid petal. This can be performed by comparing the calculated exact mass with the measured exact mass of a reference metabolite (*see* **Note 9**). If there is significant deviation in mass accuracy, perform post accusation correction using MassLynx, following the procedure provided by the vendor.
20. To perform the analysis, use Protea Plot v.2.0.1.3 after importing the MassLynx raw data files. Plot the ion intensity signal for a selected *m/z* value (10-ppm window) against the absolute coordinates of analysis to obtain lateral and 3D molecular images. Ions of potential interest for the generation of accurate mass ion maps can be identified using MassLynx via background subtraction of adjacent BPI chronogram regions from those BPI regions that coincided with the analog signal from the mid-infrared laser pulses (*see* **Notes 10** and **11**).

4 Notes

1. Run the electrospray continuously for few hours to check its stability. Two of the most common causes of electrospray instability are clogging of the needle and loose attachment of the needle to the pump. When clogging is encountered, clean the syringe with MeOH. If the problem persists, sonication of the needle in an organic solvent such as MeOH has proven to be effective. Small particles responsible for clogging of needles is often encountered if dirty glassware was used to store the electrospray solvent. Use clean glassware, preferably new 4.0-mL glass tubes to store the electrospray solution.
2. Various electrospray solvents can be used depending on the type of metabolite classes of interest associated with the tissue.
3. Make sure that the samples are mounted on the slide as flat as possible. Failure to do so will lead to pseudo variability in metabolite abundance.
4. The flow rate and the spraying voltage can be varied to obtain a stable spray.
5. Make sure that the selected area for the analysis has fair distribution of purple and white patches. Few purple spots in combination with wider white patches will give reliable information regarding the metabolome composition of these contrasting tissues.
6. If the metabolome composition, abundance and distribution of the upper most layer of the petal is the primary target, a reduced number of laser pulses per location in combination with lower level of laser energy can be used. Each laser pulse creates a 200 μm wide feature with a depth of 20–30 μm. The

number of pulses needed is also dependent on the thickness of the tissue so thinner petals or other thin tissues may require fewer pulses for complete analysis.

7. The choice of the mass spectrometer polarity depends on the classes of metabolite of interest for the analysis. Some plant metabolites can be ionized easily in positive ionization mode while others are better ionized and detected in negative ionization mode. A majority of the metabolites can be detected in both ionization modes. For analysis of anthocyanins that are primarily associated with the purple patches in the petal positive ionization mode is more robust. Apart from protonated ions, primarily singly charged sodium adducts and, occasionally, singly charged potassium adducts, are commonly encountered when the analysis is performed in positive ionization mode.
8. For the settings given, a total scan time of 20 min is needed for a measurement area of 16 mm^2.
9. Correction on the mass features can be done online or offline. Offline correction corresponds to the post-accusation correction of the detected mass features using the reference (lock) mass leucine enkaphaline (m/z 556.2771) added to the electrospray solvent. Post accusation mass correction was primarily employed in our analysis.
10. LAESI analysis of orchid petals produces hundreds of mass features. One of the difficulties during data analysis and visualization is the selection of specific mass features that are unique to either the white or the purple parts of the petal. To resolve this issue, prior analysis of the microdissected purple and white parts can provide essential information on the quantitative abundance of a given metabolite in these contrasting tissues. Based on this information, in the LAESI-MS analysis, the relative abundance and spatial distribution of differentially accumulated metabolites in the contrasting tissues can be monitored and mapped.
11. Ion suppression is a major problem in LAESI-MS and other direct infusion techniques. For quantitative determination of metabolites, LAESI-MS analysis needs to be substantiated by LC-MS analysis of laser/microdissected samples. Purple and white spots on the petal can be laser/microdissected and subjected to LC-MS analysis for this purpose.

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