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MALDI-Mass Spectrometric Imaging for Investigation of Metabolites on Medicago truncatula Root Nodules

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Abstract:	<p>Most techniques used to study small molecules, such as pharmaceutical drugs or endogenous metabolites, employ tissue extracts which require the homogenization of the tissue of interest that could potentially cause changes in the metabolic pathways being studied.¹ Mass spectrometric imaging (MSI) is a powerful analytical tool that can provide spatial information of analytes within intact slices of biological tissue samples.¹⁻⁵ This technique has been used extensively to study various types of compounds including proteins, peptides, lipids, and small molecules such as endogenous metabolites. With matrix-assisted laser desorption/ionization (MALDI)-MSI, spatial distributions of multiple metabolites can be simultaneously detected. Herein, a method developed specifically for conducting untargeted metabolomics MSI experiments on legume roots and root nodules is presented which could reveal insights into the biological processes taking place. The method presented here shows a typical MSI workflow, from sample preparation to image acquisition, and focuses on the matrix application step, demonstrating several matrix application techniques that are useful for detecting small molecules. Once the MS images are generated, the analysis and identification of metabolites of interest is discussed and demonstrated. The standard workflow presented here can be easily modified for different tissue types, molecular species, and instrumentation.</p>
Author Comments:	<p>Dear Editor:</p> <p>Thank you for the kind invitation to contribute a video method manuscript for Journal of Visualized Experiments (JoVE). Enclosed you will our submitted manuscript entitled "Investigation of Metabolites in Medicago truncatula Root Nodules with MALDI- Mass Spectrometric Imaging". Mass spectrometric imaging (MSI) is a powerful tool that can be used to discover and identify various chemical species in intact tissues, preserving the compounds in their native environments, which can provide new insights into biological processes. Herein we present a method developed specifically for</p>

	<p>conducting untargeted metabolomics MSI experiments on legume roots and root nodules which could reveal insights into the biological processes taking place. The method presented here shows a typical MSI workflow, from sample preparation to image acquisition, and focuses on the matrix application step, demonstrating several matrix application techniques that are useful for detecting small molecules. Once the MS images are generated, we discuss and demonstrate how to analyze the data and identify the metabolites of interest. The standard workflow presented here can be easily modified for different tissue types, molecular species, and instrumentation. We highlighted the portion that would be most important for video to demonstrate the procedure and methodology. We believe that this article will interest many readers of JoVE including those who are interested in new methodologies for mass spectral imaging and profiling, small molecule metabolite imaging, and various aspects of MSI and metabolomics.</p>
Additional Information:	
Question	Response

TITLE: MALDI-Mass Spectrometric Imaging for Investigation of Metabolites on *Medicago truncatula* Root Nodules

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SHORT ABSTRACT:

Mass spectrometric imaging (MSI) is a powerful tool that can be used to discover and identify various chemical species in intact tissues, preserving the compounds in their native environments, which can provide new insights into biological processes. Herein a MSI method developed for the analysis of small molecules is described.

LONG ABSTRACT:

Most techniques used to study small molecules, such as pharmaceutical drugs or endogenous metabolites, employ tissue extracts which require the homogenization of the tissue of interest that could potentially cause changes in the metabolic pathways being studied.¹ Mass spectrometric imaging (MSI) is a powerful analytical tool that can provide spatial information of analytes within intact slices of biological tissue samples.¹⁻⁵ This technique has been used extensively to study various types of compounds including proteins, peptides, lipids, and small molecules such as endogenous metabolites. With matrix-assisted laser desorption/ionization (MALDI)-MSI, spatial distributions of multiple metabolites can be simultaneously detected. Herein, a method developed specifically for conducting untargeted metabolomics MSI experiments on legume roots and root nodules is presented which could reveal insights into the biological processes taking place. The method presented here shows a typical MSI workflow, from sample preparation to image acquisition, and focuses on the matrix application step,

demonstrating several matrix application techniques that are useful for detecting small molecules. Once the MS images are generated, the analysis and identification of metabolites of interest is discussed and demonstrated. The standard workflow presented here can be easily modified for different tissue types, molecular species, and instrumentation.

INTRODUCTION:

The growing field of metabolomics has many important biological applications including biomarker discovery, deciphering metabolic pathways in plants and other biological systems, and toxicology profiling.^{4,6-10} A major technical challenge when studying biological systems is to study metabolomic pathways without disrupting them.¹¹ MALDI-MSI allows for direct analysis of intact tissues that enables sensitive detection of analytes in single organs^{12,13} and even single cells.^{14,15}

Sample preparation is a crucial step in producing reproducible and reliable mass spectral images. The quality of the images greatly depends upon factors such as tissue embedding medium, slice thickness, MALDI matrix, and matrix application technique. For imaging applications, ideal section thickness is the width of one cell (8-20 μm depending on the sample type). MALDI requires deposition of an organic, crystalline matrix compound, typically a weak acid, on the sample to assist analyte ablation and ionization.¹⁶ Different matrices provide different signal intensities, interfering ions, and ionization efficiencies of different classes of compounds.

The matrix application technique also plays a role in the quality of mass spectral images and different techniques are appropriate for different classes of analytes. Three matrix application methods are presented in this protocol: airbrush, automatic sprayer, and sublimation. Airbrush matrix application has been widely used in MALDI imaging. The advantage of airbrush matrix application is that it is relatively fast and easy. However, the quality of the airbrush matrix application greatly depends on the skill of the user and tends to be less reproducible and cause diffusion of analytes, especially small molecules.¹⁷ Automatic sprayer systems have similar mechanics to airbrush matrix application, but have been developed to remove the variability seen with manual airbrush application, making the spray more reproducible. This method can sometimes be more time-consuming than traditional airbrush matrix application. Both manual airbrush and automatic sprayer systems are solvent-based matrix application methods. Sublimation is a dry matrix application technique that is becoming more and more popular for mass spectral imaging of metabolites and small molecules because it reduces analyte diffusion; however, it lacks the solvent necessary to extract and observe higher mass compounds.¹⁸

Confident identification of metabolites typically requires accurate mass measurements to obtain putative identifications followed by tandem mass (MS/MS) experiments for validation, with MS/MS spectra being compared to standards, literature, or theoretical spectra. In this protocol high resolution (mass resolving power of 60,000 at m/z 400), liquid chromatography (LC)-MS is coupled to MALDI-MSI to obtain both spatial information and confident identifications of endogenous metabolites, using *Medicago truncatula* roots and root nodules

as the biological system. MS/MS experiments can be performed directly on the tissue with MALDI-MSI or on tissue extracts with LC-MS and used for the validation of metabolite identifications.

This protocol provides a simple method to map endogenous metabolites in *M. truncatula*, which can be adapted and applied to MSI of small molecules in various tissue types and biological systems.

PROTOCOL:

1. Instrumentation

1.1) MALDI-TOF/TOF MSI. Use a mass spectrometer equipped with a MALDI source for analysis of small molecules (see Table of Materials/ Equipment). Perform acquisitions in positive or negative ion mode depending on the analytes of interest. Specify a mass range of interest and collect 500 laser shots/spot at 50 μm intervals in both the x and y dimensions across the surface of the sample to generate ion images. The raster width and number of laser shots can be adjusted to obtain higher spatial resolution and maximum signal intensity respectively. Use DHB matrix peaks or internal standards applied to the slide or directly to the tissue to calibrate the mass spectra.

1.2) High Resolution LC-MS. (see Table of Materials/ Equipment) Run sample extracts with LC-MS using either reversed phase (RP) LC with a C-18 column, or normal phase (NP) with a HILIC column depending on the analytes of interest. Use mobile phases and gradients as appropriate for the specific sample type. Perform acquisitions in positive or negative ion modes depending on the sample type.

2. Tissue Preparation

2.1) Trim the root nodule from the plant, leaving 3-4 mm of root attached to the nodule.

2.2) Immediately after dissection, use forceps to place the tissue in a cryostat cup and cover with gelatin (100 mg/mL in deionized water). It is essential for the tissue to be stuck to the bottom of the cup, with no air bubbles.

2.3) Flash freeze the tissue by placing the cup in a dry ice/ethanol bath until the gelatin hardens and becomes opaque. Store samples at $-80\text{ }^{\circ}\text{C}$ until use.

2.4) Remove samples from the $-80\text{ }^{\circ}\text{C}$ freezer, cut away the plastic cryostat cup and trim away excess gelatin. Mount the embedded tissue to the cryostat chuck with a dime-sized amount of optimal cutting temperature (OCT) media, while not letting the OCT touch the tissue. Place in cryostat box until the OCT solidifies.

2.5) Allow the chuck and gelatin to equilibrate in the cryostat box (set to -20 or -25 °C) for approximately 15 min.

2.6) Use the cryostat (see Table of Materials/ Equipment) to section tissue approximately the thickness of one cell (8-20 μm depending on the tissue type) and thaw mount each slice onto the ITO-coated glass by warming the back (non-ITO-coated side) of an ITO-coated glass slide on the back of your hand. Place the ITO-coated side of the warmed slide near the frozen tissue slice and allow the slice to stick onto the slide. Placing the sections close together on the slide will provide better alignment during MSI.

3. Matrix Application

3.1) Airbrush Application of MALDI Matrix

3.1.1) All airbrush procedures should be performed in a fume hood.

3.1.2) Thoroughly clean the airbrush solution container and nozzle (see Table of Materials/ Equipment) with methanol and fill the solution container with DHB matrix solution (150 mg/mL in 50% methanol/0.1% TFA v/v).

3.1.3) Hold the airbrush approximately 35 cm from the sample and apply 10-15 coats of matrix on the surface of the slide with a duration of 10 s spray and 30 s drying time in between each coat.

3.1.4) Thoroughly clean the airbrush again with methanol when finished to avoid clogging from the matrix solution.

3.2) Automatic Sprayer Application of MALDI Matrix

3.2.1) Follow the start-up instructions provided by the manufacturers of the automatic sprayer system.

3.2.2) For MSI of metabolites in root nodules using 40 mg/mL (in 50% methanol/0.1% TFA v/v) DHB as the matrix, set the temperature to $\sim 80^\circ\text{C}$, velocity to 1250 mm/min, flow rate to 50 $\mu\text{L}/\text{min}$, and number of passes to 24. For best coverage, it is recommended to rotate the nozzle 90° and/or offset the nozzle 1.5 mm between each pass. Start sprayer method.

As a side note, the particular sprayer system used here heats the nozzle for faster evaporation of the solvent. As the solvent evaporates, the concentration of the matrix quickly increases. The matrix applied to the sample with the airbrush and the automatic sprayer have comparable concentrations.

3.2.3) Follow the shut-down instructions provided by the manufacturers of the automatic sprayer system.

3.3) Sublimation Application of MALDI Matrix

3.3.1) Weigh out 300 mg DHB into the bottom of the sublimation chamber (see Table of Materials/ Equipment).

3.3.2) Stick the glass slide to the cold finger (top portion of the sublimation chamber) with the tissue sections facing down with double-sided, conductive tape. Cover the entire back of the slide with the double sided tape for even conductivity, producing even matrix deposition.

3.3.3) Clamp the top and bottom halves of the sublimation chamber together with the C-clamp. Connect the vacuum and add ice and cold water to the top reservoir.

3.3.4) Place sublimation chamber in a heating mantle that is at room temperature.

3.3.5) Turn on the vacuum pump. Wait 15 minutes and turn on the heating mantle. The heating mantle should reach 120 °C over the course of 10 min.

3.3.6) After 10 min, turn off the heat, close valve to vacuum (so the inside of the chamber remains under vacuum) and turn off the vacuum pump.

3.3.7) Allow the chamber to come to room temperature, open the valve releasing the vacuum pressure and remove the sample. The size of the sublimation chamber will determine the amount of matrix sublimed to the glass slide. The larger sublimation chambers (the size of a 400 mL beaker) will use approximately 300 mg of DHB while the smaller chambers (the size of a 150 mL beaker) will use approximately 100 mg of DHB and will require cutting the glass slide so that it fits in the chamber.

4. Image Acquisition

4.1) Mark a + pattern on each corner of the sample with a WiteOut correction fluid pen to be used as “teach points”. Place the glass slide into the MALDI slide adapter plate and take an optical image of the sample using a scanner.

4.2) Set up an image acquisition file using the software provided by the instrument company with a raster step size of 50 µm and a laser diameter equal to or smaller than the raster step size. On this particular instrument, the minimum laser setting gives a laser diameter of approximately 10 µm and small laser setting has a 40-50 µm diameter.

4.3) Load the optical image into the software and align the plate with the optical image.

4.4) Calibrate the instrument before beginning the acquisition using common matrix cluster ions, internal standards, or a calibration mix.

4.5) Specify the areas of tissue to be analyzed with MSI, including a spot of pure matrix on the slide to be used as a “blank”.

4.6) Begin acquisition.

5. Image Generation

5.1) Open the imaging file in the commercially available software provided by the vendor and extract the ion images. Other open-source software is available for MSI data processing.¹⁹

6. Metabolite Identification

6.1) Select a specific m/z of interest from the mass spectrum using the vendor specific software (see Table of Materials/ Equipment). An analyte can be distinguished from a matrix ion when an analyte peak is selected from the mass spectrum and ion images are generated specifically localized to the tissue section.

6.2) Generate a list of analytes of interest and perform MS/MS experiments. See Table 1 and Table 2 in Representative Results for sample lists of analytes.

6.3) Perform targeted LC-MS analysis on a high resolution mass spectrometer (or high resolution MALDI-MS if available) to obtain accurate mass measurements of the analytes of interest and also perform LC-MS/MS of the target analytes to obtain characteristic fragmentation patterns.

6.4) Perform database searching to determine putative identifications for the targeted analytes. Examples of metabolite databases include: METLIN, ChemSpider, PubChem, KEGG, and HMDB.

6.5) To confirm the putative identifications from accurate mass database searching, match the MS/MS from the targeted analytes to MS/MS spectra of standards, literature, and/or fragmentation prediction software.

REPRESENTATIVE RESULTS:

An experimental overview of MSI is shown in **Figure 1**. At the very beginning of the experiment, sample preparation is a critical step. Nodules are trimmed from the plant root and embedded in gelatin. The tissue must be pressed flat against the cryostat cup, with no bubbles, while it is being frozen; this will ensure easier and proper alignment of the tissue while it is being sectioned. When the tissue is being sliced, it is important to cut the tissue at the proper thickness; too thin of sections will tear, ruining the tissue integrity, while too thick of sections will reduce the number of analytes extracted and detected from the tissue. Selection of matrix and application technique will determine the types of analytes detected. Using a combination of matrices could provide complementary results. Three matrix application techniques are

presented in this work. The airbrush technique is fast, but typically not suitable for MSI of small molecules because of analyte diffusion. Automatic sprayer systems and sublimation provide smaller matrix crystals, better reproducibility, and less analyte diffusion. **Figure 2** shows an optical image of a *Medicago truncatula* root nodule section. **Figure 3** shows an optical image example of the matrix coverage and crystal sizes using airbrush, automatic sprayer, and sublimation respectively.

Conventional matrices, like DHB, produce many ions in the lower mass range (m/z 100-400).²⁰ These matrix ions can interfere with the detection of metabolites in this range. **Figure 4** shows MS spectra of just DHB matrix compared to root nodule tissue coated with DHB matrix. DHB matrix peaks are indicated in red and root nodule tissue covered with DHB is shown in blue. Novel matrices such as TiO₂ nanoparticles²¹, 1,5-diaminonaphthalene (DAN)²², 2,3,4,5-Tetrakis(3',4'-dihydroxyphenyl)thiophene (DHPT)²³, and 1,8-bis(dimethyl-amino) naphthalene (DMAN)^{24,25} have been reported that reduce the interference of matrix ions in the low mass range, and also enhance the detection of certain classes of metabolites.⁵ Matrix peaks can be distinguished from real metabolites using the MS images. When a peak is clicked on, the ion image is extracted and displayed overlapping the optical image. Those peaks that generate images with distinct localization to the tissue, and are not present in the matrix only area imaged, are considered metabolites. **Figure 5a** shows several representative ion images of metabolites found in root nodule tissue, while **Figure 5b** shows examples of MS images corresponding to matrix related peaks. In **Figure 5a** the image shows distinct localization to the root nodule tissue and a lack of signal in the matrix only area that was imaged. In **Figure 5b** the signal shows little localization and is present over the entire tissue; the signal is also seen in the matrix only area that was imaged (square areas in the top right corners). Sample lists of analytes of interest from *M. truncatula* root nodule tissue is listed in **Table 1** (positive mode) and **Table 2** (negative mode).⁴

The end goal of untargeted metabolomics experiments is to identify the compounds that were detected. When performing MSI on a medium resolution instrument (MRMS), such as a TOF/TOF, it is necessary to obtain accurate mass measurements in a different way. This can be done with a multifaceted MS approach in which MALDI-MSI results are compared to high resolution LC-MS data using tissue extracts. There are many possible tissue extraction protocols to choose from depending on the analytes of interest. High resolution MS (HRMS) can be performed in the positive or negative ionization modes and with normal or reversed phase LC depending upon the analytes of interest. Once an accurate mass is obtained with high resolution LC-MS, the resulting mass can be searched with several databases, listed previously, to obtain a putative identification. Next MS/MS data is collected and the characteristic fragmentation pattern of the analyte of interest can be compared to standards, literature spectra, or theoretical fragmentation patterns. **Figure 6** shows an example of one of the metabolites detected with MSI and LC-MS. This metabolite was identified as heme based on the MS/MS spectrum collected with high resolution LC-MS. This MS/MS data was compared to the MS/MS spectra previously published by Shimma and Setou.²⁶ The two MS/MS spectra match, therefore the identity of m/z 616.2 was confidently assigned as heme based on the accurate mass database searching and MS/MS data compared to literature MS/MS data.

Figure and Table Legends:

Figure 1: Overview of MSI workflow. Root nodules are trimmed from the plant, embedded in gelatin, sectioned with the cryostat and mounted onto an ITO-coated glass slide. Matrix is applied to the slide using one of three matrix application techniques. MSI acquisition is performed with a MALDI-TOF/TOF mass spectrometer and MS spectra are compiled into images with MSI software.

Figure 2: Tissue sample optical image. Representative optical image of a *Medicago truncatula* root nodule tissue section.

Figure 3: Example matrix depositions. Representative images showing the different consistencies of matrix deposition with a) airbrush, b) automatic sprayer, and c) sublimation techniques. The airbrush application method generates large and small crystals while the automatic sprayer method produces small evenly sized crystals. Sublimation produces one even layer of matrix.

Figure 4: MS spectrum of plain DHB matrix vs. root nodule tissue covered with DHB. DHB matrix peaks are indicated in red and root nodule tissue covered with DHB is shown in blue. Matrix peaks can be distinguished from real metabolites using the MS images. When a peak is clicked on, the ion image is extracted and displayed overlapping the optical image. Those peaks that generate images with distinct localization to the tissue, and are not present in the matrix only area imaged, are considered metabolites.

Figure 5: MS images of *M. truncatula* root nodules. a) Representative ion images of metabolites found in wild-type *M. truncatula* root nodules. b) Representative ion images of matrix species that would not be considered metabolites.

Figure 6: Example MS/MS data for metabolite identification. MS/MS spectrum of m/z 616.2, identified as heme [M^+]. The chemical structure is shown and fragmentation structures are assigned. The experimental MS/MS data is compared to the MS/MS spectrum of heme reported in literature by Shimma and Setou.²⁶

Table of Materials/Equipment.

Table 1: Analytes of interest- positive mode. Sample list of analytes of interest from *M. truncatula* root nodule tissue in positive ion mode.⁴

Table 2: Analytes of interest- negative mode. Sample list of analytes of interest from *M. truncatula* root nodule tissue in negative ion mode.⁴

DISCUSSION:

As discussed above, sample preparation is the most critical step in the MSI workflow. Embedding the tissue unevenly will cause sectioning to be difficult or not possible in some cases. The section size and adequate equilibration time are crucial to maintaining the tissue integrity and avoiding folding and tears. Selection of matrix and application technique will play a role in determining the types of analytes to be detected, the spatial resolution, and reproducibility of the results. Using a combination of matrices or application techniques could provide complementary results.

This method was designed specifically for untargeted MSI of endogenous metabolites in *M. truncatula* root nodule tissue, but can easily be adapted to other tissue types and biological questions. The recommended matrix application methods for small molecule MSI are sublimation and automatic sprayer. Increasing the amount of solvent deposited on the tissue, by adjusting the automatic sprayer method, will increase the extraction of analytes and allow for detection of higher mass compounds should one choose to perform MSI of lipids, peptides, etc. When using other types of tissue, the main adjustment will be the section thickness. Ideally the tissue should be sliced to the thickness of one cell; therefore thicker sections may be appropriate for plant tissue and thinner sections appropriate for animal tissue. If folding or tearing occurs, typically a longer equilibration time is needed before sectioning or a thicker section size could be necessary. When performing LC-MS to obtain accurate masses, the tissue extraction protocol, mobile phase solvents and gradient, column stationary phase, and MS ionization mode can all be adjusted and optimized for the analytes of interest.

The main advantage of MALDI-MSI is its ability to provide not only mass information, but also spatial information for a given sample without the need for prior knowledge of the target analytes. Other imaging techniques require the use of derivatizations or tags.⁵ As discussed previously, one limitation of this technique is the abundance of interfering matrix ion peaks. Novel matrices have been reported to address this limitation. Alternatively, secondary ion mass spectrometry (SIMS)-MSI is a matrix-free imaging option; however, it has less sensitivity than MALDI-MSI.³ Another limitation of this technique is the lower mass resolution of MALDI-TOF/TOF instruments. Because of the low mass resolving power, it is necessary to also perform high resolution MS to obtain the accurate masses for metabolite identification, which means more experiments and more time. This problem could be solved by performing MALDI-MSI on a high resolution instrument platform such as the MALDI-Orbitrap. The final limitation of performing MSI experiments is the lack of software tools available for analysis of MSI data, although some recent advances in MSI software have been made.^{27,28} Typically the MS spectrum for each imaging region must be manually analyzed and ion images extracted by hand. MSI experiments produce an abundance of data and can be incredibly time consuming to analyze. Overall, MALDI-MSI offers unique advantages for obtaining spatial information of many compounds simultaneously within a single experiment that can be extremely useful for the untargeted analysis of small molecules and other compounds with many biological applications.

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REFERENCES

- 1 Seeley, E. H., Schwamborn, K. & Caprioli, R. M. Imaging of Intact Tissue Sections: Moving beyond the Microscope. *J Biol Chem* **286**, 25459-25466, doi:DOI 10.1074/jbc.R111.225854 (2011).
- 2 van Hove, E. R. A., Smith, D. F. & Heeren, R. M. A. A concise review of mass spectrometry imaging. *J Chromatogr A* **1217**, 3946-3954, doi:DOI 10.1016/j.chroma.2010.01.033 (2010).
- 3 Lietz, C. B., Gemperline, E. & Li, L. Qualitative and quantitative mass spectrometry imaging of drugs and metabolites. *Advanced drug delivery reviews*, **65**, 1074-1085, doi:10.1016/j.addr.2013.04.009 (2013).
- 4 Ye, H. *et al.* MALDI mass spectrometry-assisted molecular imaging of metabolites during nitrogen fixation in the *Medicago truncatula*-*Sinorhizobium meliloti* symbiosis. *The Plant Journal*, **75**, 130-145, doi:10.1111/tpj.12191 (2013).
- 5 Ye, H., Gemperline, E. & Li, L. A vision for better health: mass spectrometry imaging for clinical diagnostics. *Clinica chimica acta; international journal of clinical chemistry* **420**, 11-22, doi:10.1016/j.cca.2012.10.018 (2013).
- 6 Wei, R. Metabolomics and Its Practical Value in Pharmaceutical Industry. *Curr Drug Metab* **12**, 345-358 (2011).
- 7 Kobayashi, T. *et al.* A Novel Serum Metabolomics-Based Diagnostic Approach to Pancreatic Cancer. *Cancer Epidem Biomar* **22**, 571-579, doi:Doi 10.1158/1055-9965.Epi-12-1033 (2013).
- 8 West, P. R., Weir, A. M., Smith, A. M., Donley, E. L. R. & Cezar, G. G. Predicting human developmental toxicity of pharmaceuticals using human embryonic stem cells and metabolomics. *Toxicol Appl Pharm* **247**, 18-27, doi:DOI 10.1016/j.taap.2010.05.007 (2010).
- 9 Spegel, P. *et al.* Time-resolved metabolomics analysis of beta-cells implicates the pentose phosphate pathway in the control of insulin release. *The Biochemical journal* **450**, 595-605, doi:10.1042/BJ20121349 (2013).
- 10 Pendyala, G., Want, E. J., Webb, W., Siuzdak, G. & Fox, H. S. Biomarkers for neuroAIDS: The widening scope of metabolomics. *J Neuroimmune Pharm* **2**, 72-80, doi:DOI 10.1007/s11481-006-9041-3 (2007).
- 11 Prell, J. & Poole, P. Metabolic changes of rhizobia in legume nodules. *Trends in microbiology* **14**, 161-168, doi:10.1016/j.tim.2006.02.005 (2006).

- 12 Kutz, K. K., Schmidt, J. J. & Li, L. J. In situ tissue analysis of neuropeptides by MALDI FTMS in-cell accumulation. *Anal Chem* **76**, 5630-5640, doi:Doi 10.1021/Ac049255b (2004).
- 13 Stemmler, E. A. *et al.* High-mass-resolution direct-tissue MALDI-FTMS reveals broad conservation of three neuropeptides (APSGFLGMRamide, GYRKPPFNGSIFamide and pQDLDHVFLRFamide) across members of seven decapod crustacean infraorders. *Peptides* **28**, 2104-2115, doi:DOI 10.1016/j.peptides.2007.08.019 (2007).
- 14 Rubakhin, S. S., Churchill, J. D., Greenough, W. T. & Sweedler, J. V. Profiling signaling peptides in single mammalian cells using mass spectrometry. *Anal Chem* **78**, 7267-7272, doi:Doi 10.1021/Ac0607010 (2006).
- 15 Neupert, S. & Predel, R. Mass spectrometric analysis of single identified neurons of an insect. *Biochem Bioph Res Co* **327**, 640-645, doi:DOI 10.1016/j.bbrc.2004.12.086 (2005).
- 16 Caprioli, R. M., Farmer, T. B. & Gile, J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal Chem* **69**, 4751-4760 (1997).
- 17 Baluya, D. L., Garrett, T. J. & Yost, R. A. Automated MALDI matrix deposition method with inkjet printing for imaging mass spectrometry. *Anal Chem* **79**, 6862-6867, doi:10.1021/ac070958d (2007).
- 18 Hankin, J. A., Barkley, R. M. & Murphy, R. C. Sublimation as a method of matrix application for mass spectrometric imaging. *Journal of the American Society for Mass Spectrometry* **18**, 1646-1652, doi:10.1016/j.jasms.2007.06.010 (2007).
- 19 Robichaud, G., Garrard, K. P., Barry, J. A. & Muddiman, D. C. MSiReader: an open-source interface to view and analyze high resolving power MS imaging files on Matlab platform. *Journal of the American Society for Mass Spectrometry* **24**, 718-721, doi:10.1007/s13361-013-0607-z (2013).
- 20 Northen, T. R. *et al.* Clathrate nanostructures for mass spectrometry. *Nature* **449**, 1033-U1033, doi:Doi 10.1038/Nature06195 (2007).
- 21 Shrivasa, K., Hayasaka, T., Sugiura, Y. & Setou, M. Method for simultaneous imaging of endogenous low molecular weight metabolites in mouse brain using TiO₂ nanoparticles in nanoparticle-assisted laser desorption/ionization-imaging mass spectrometry. *Anal Chem* **83**, 7283-7289, doi:10.1021/ac201602s (2011).
- 22 Thomas, A., Charbonneau, J. L., Fournaise, E. & Chaurand, P. Sublimation of new matrix candidates for high spatial resolution imaging mass spectrometry of lipids: enhanced information in both positive and negative polarities after 1,5-diaminonaphthalene deposition. *Anal Chem* **84**, 2048-2054, doi:10.1021/ac2033547 (2012).
- 23 Chen, S. *et al.* 2,3,4,5-Tetrakis(3',4'-dihydroxyphenyl)thiophene: a new matrix for the selective analysis of low molecular weight amines and direct determination of creatinine in urine by MALDI-TOF MS. *Anal Chem* **84**, 10291-10297, doi:10.1021/ac3021278 (2012).
- 24 Shroff, R., Rulisek, L., Doubsky, J. & Svatos, A. Acid-base-driven matrix-assisted mass spectrometry for targeted metabolomics. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 10092-10096, doi:10.1073/pnas.0900914106 (2009).
- 25 Shroff, R. & Svatos, A. Proton sponge: a novel and versatile MALDI matrix for the analysis of metabolites using mass spectrometry. *Anal Chem* **81**, 7954-7959, doi:10.1021/ac901048z (2009).

- 26 Shimma, S. S., M. Mass Microscopy to Reveal Distinct Localization of Heme B (m/z 616) in Colon Cancer Liver Metastasis. *J. Mass Spectrom. Soc. Jpn.* **55**, 145-148, doi:10.5702/massspec.55.145 (2007).
- 27 Paschke, C. *et al.* Mirion-A Software Package for Automatic Processing of Mass Spectrometric Images. *Journal of the American Society for Mass Spectrometry* **24**, 1296-1306, doi:10.1007/s13361-013-0667-0 (2013).
- 28 Parry, R. M. *et al.* omniSpect: an open MATLAB-based tool for visualization and analysis of matrix-assisted laser desorption/ionization and desorption electrospray ionization mass spectrometry images. *Journal of the American Society for Mass Spectrometry* **24**, 646-649, doi:10.1007/s13361-012-0572-y (2013).

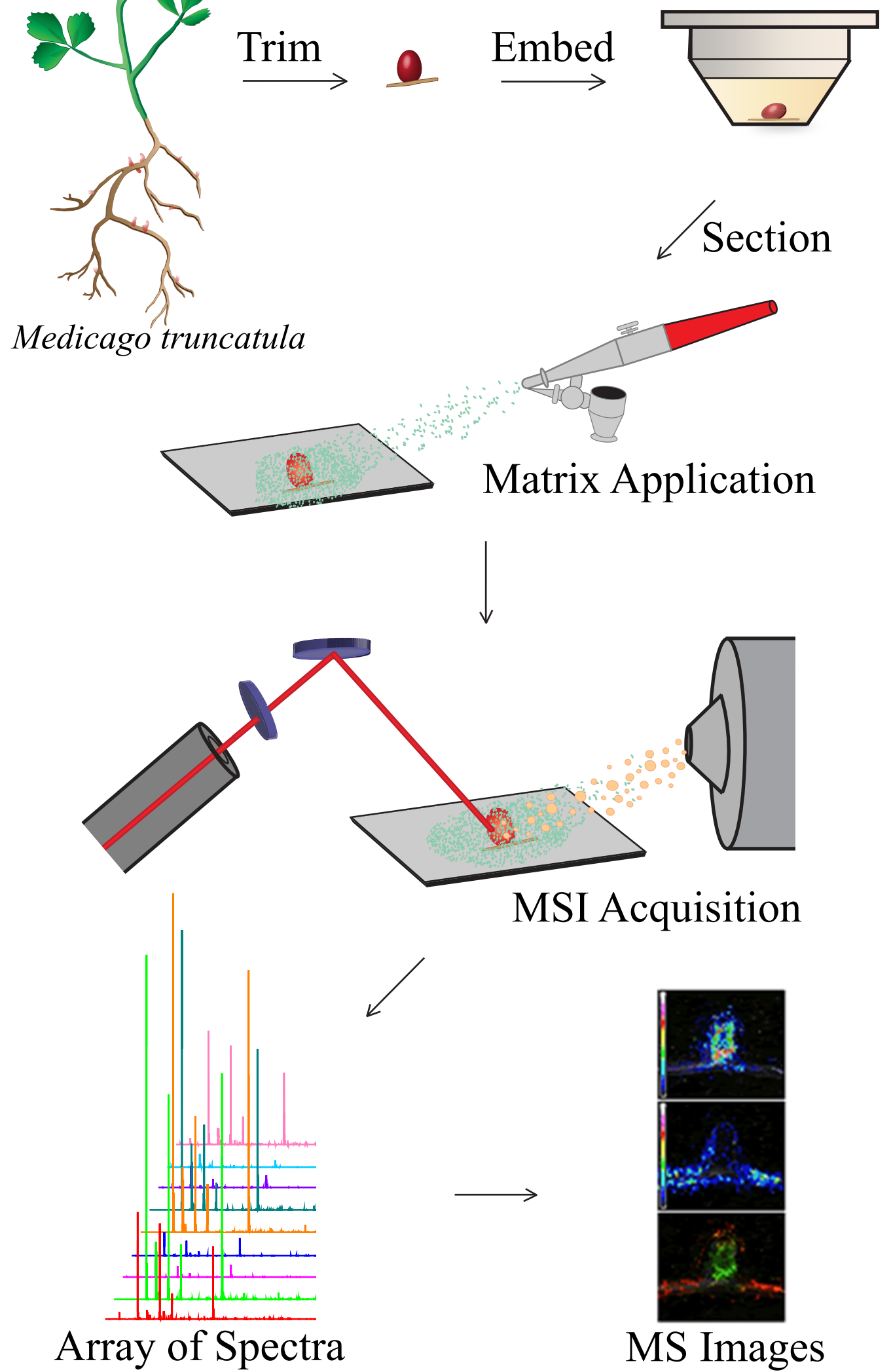
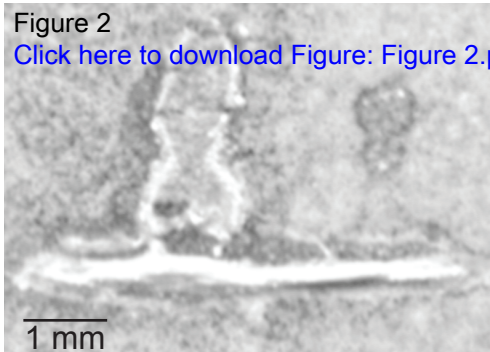
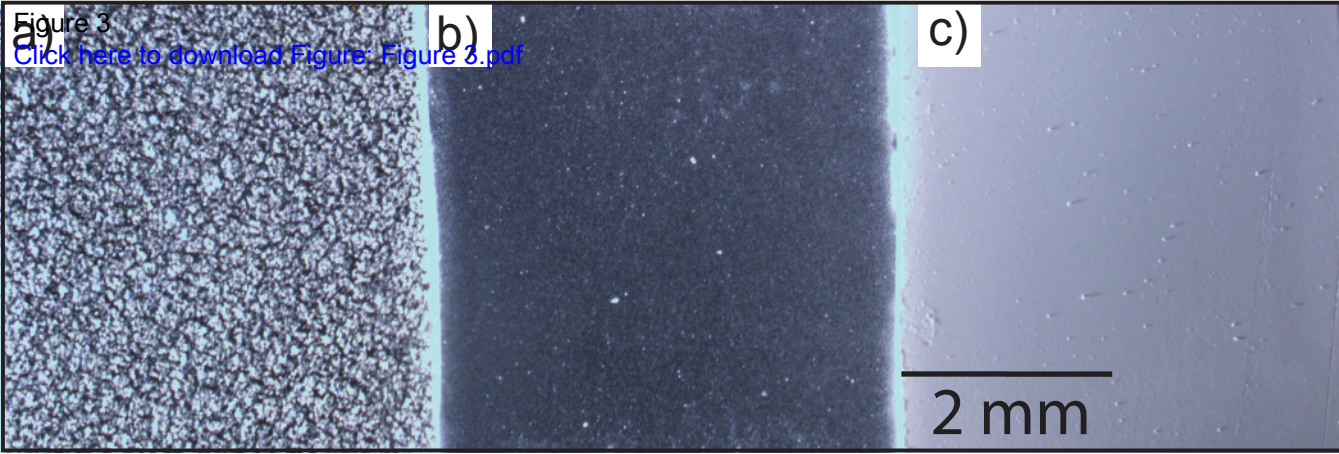


Figure 2

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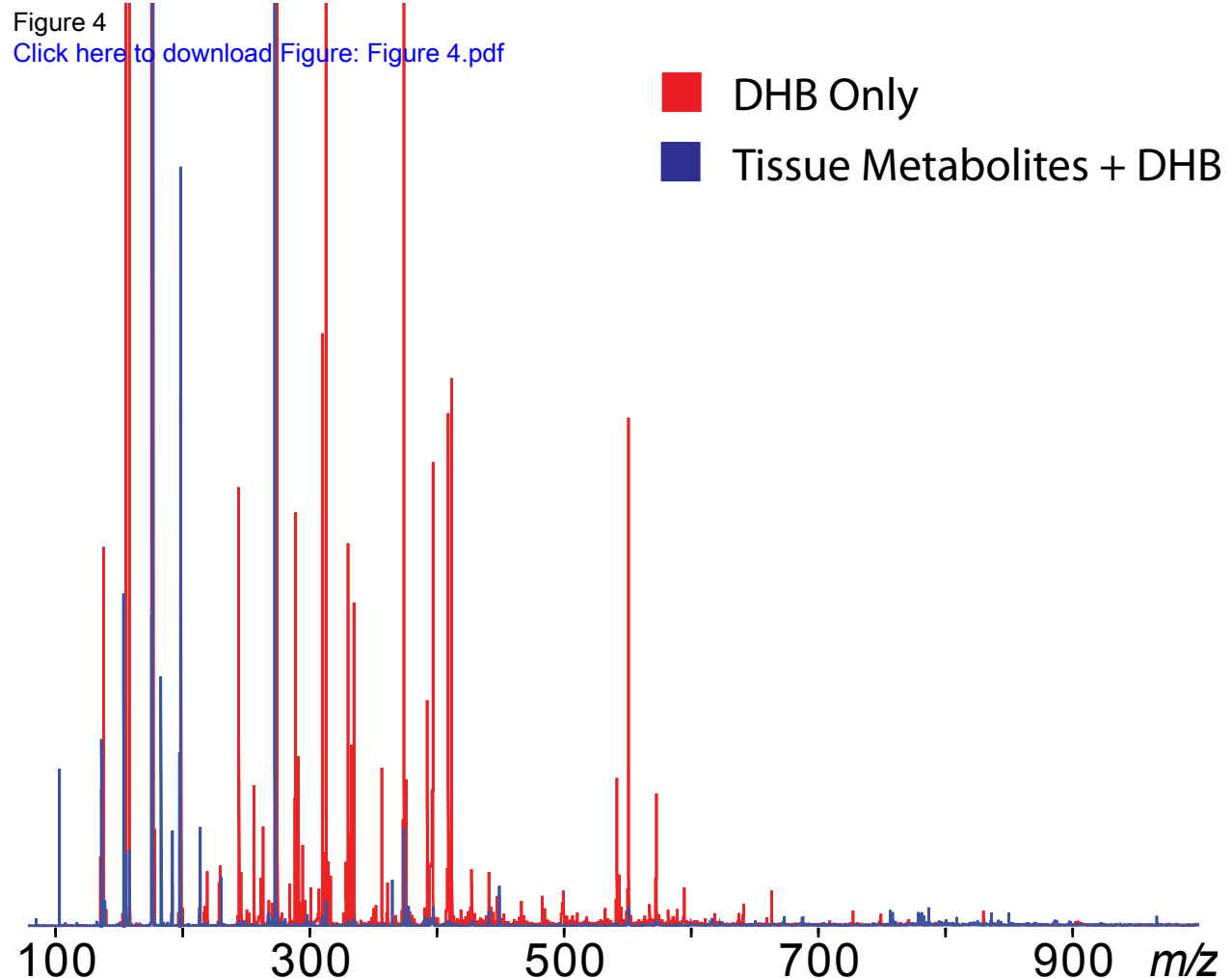


Figure 5

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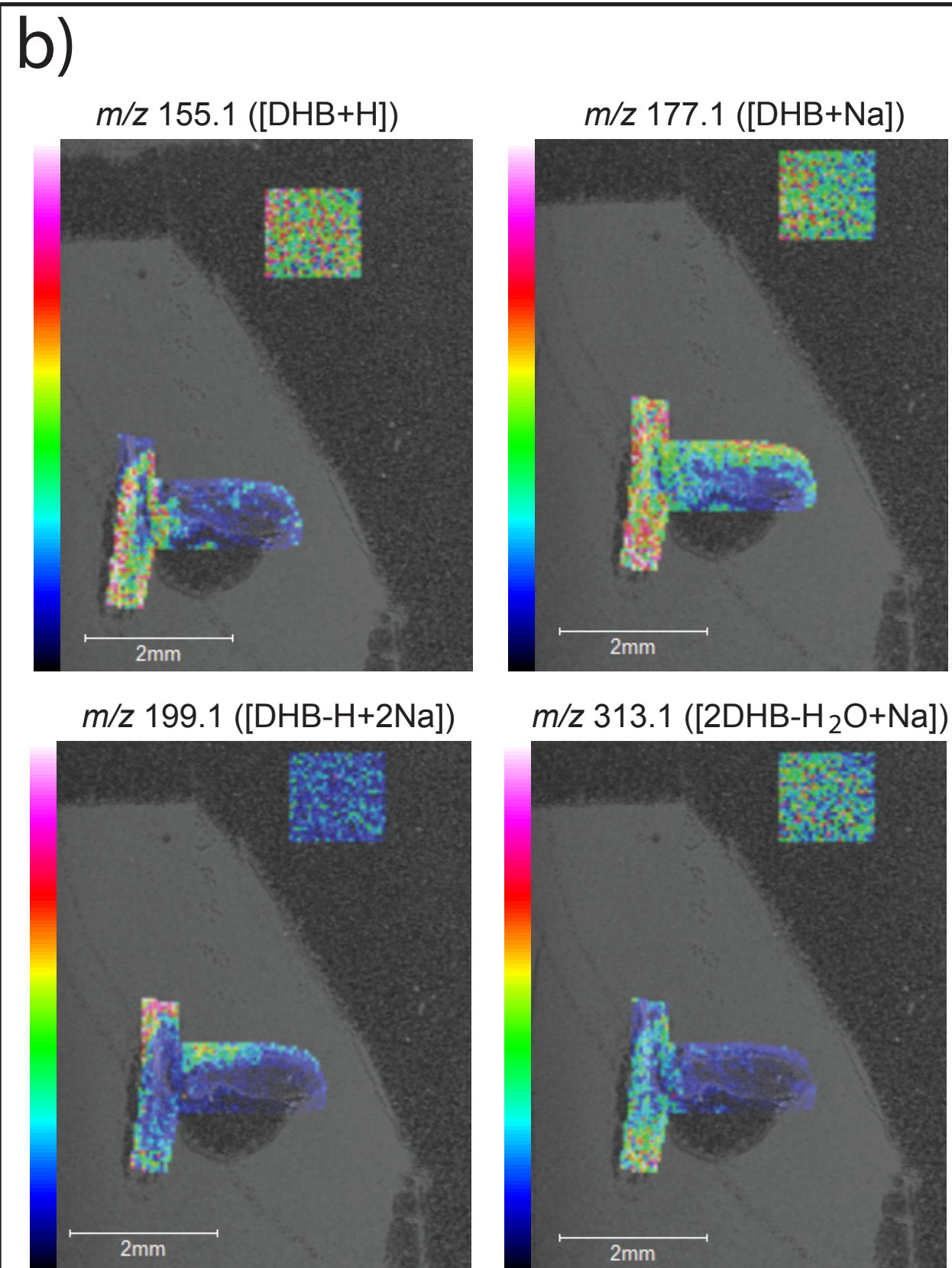
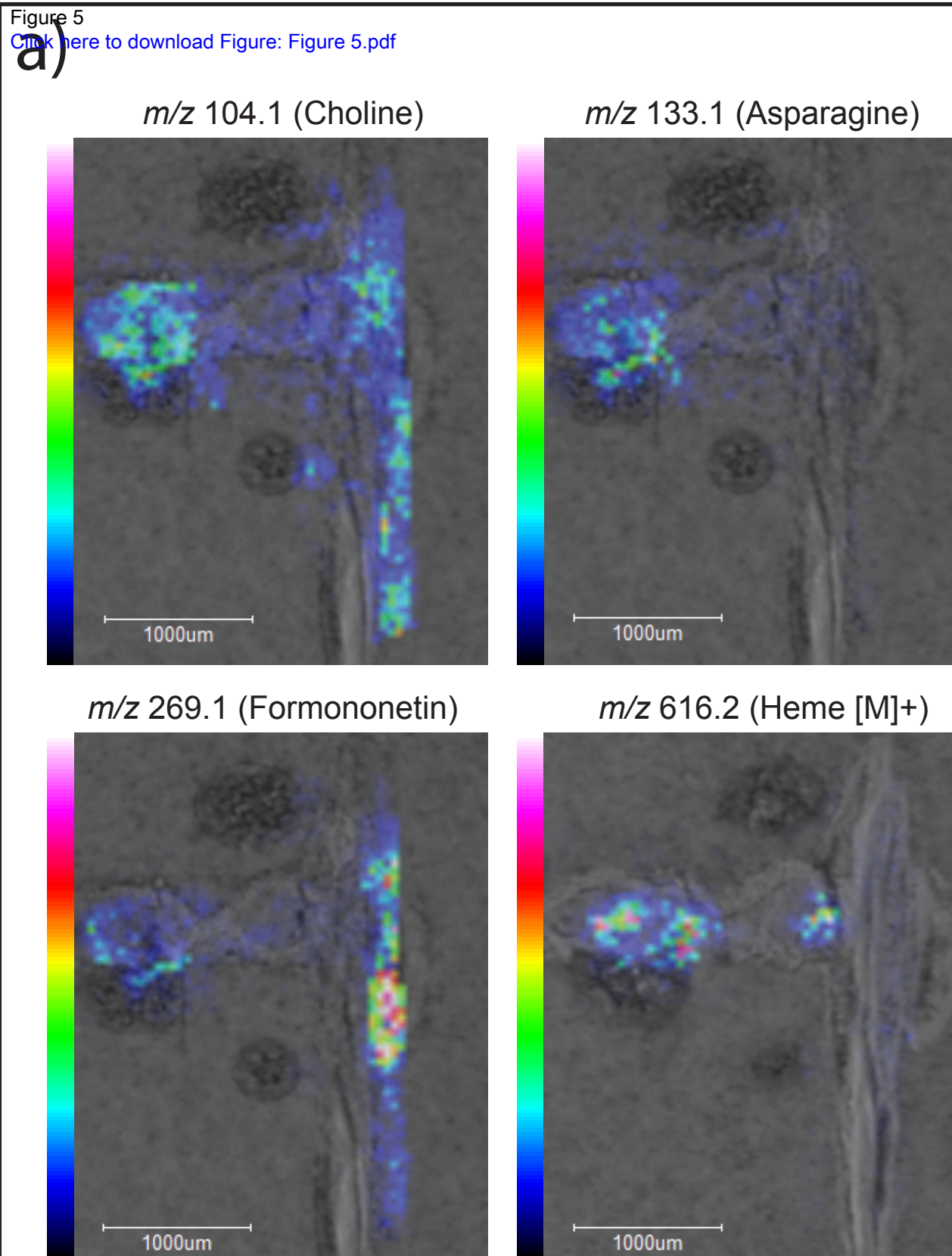


Figure 6

(e) m/z 616.18

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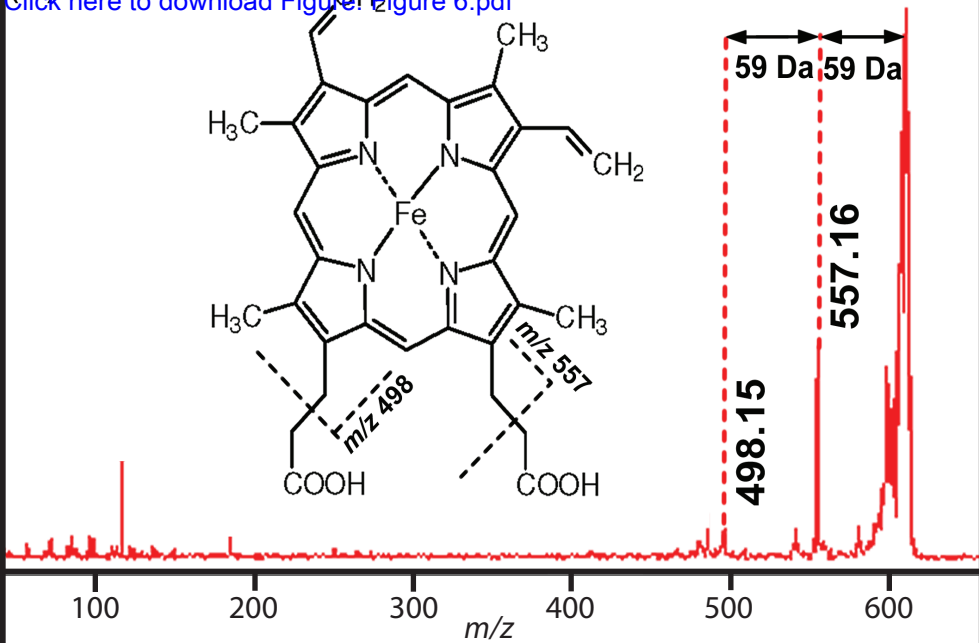


Table 1
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Name of metabolite	Theoretical [M+H] ⁺	MRMS Measured [M+H] ⁺	HRMS Measured [M+H] ⁺	Δm (mDa)
γ-aminobutyric acid ^a	104.0706	104.1	104.0706	0
choline ^{a,*}	104.1070	104.1	104.1071	0.01
proline	116.0706	116.07	116.0706	0
valine	118.0863	118.09	118.0865	0.02
leucine ^a	132.1019	132.1	132.1022	0.03
asparagine ^a	133.0608	133.06	133.0602	-0.06
adenine ^a	136.0618	136.07	NA	NA
proling betaine ^a	144.1019	144.1	144.1024	0.05
glutamine ^a	147.0764	147.09	147.0768	0.04
histidine ^a	156.0768	156.06	156.0771	0.03
arginine ^a	175.1190	175.13	175.1167	-0.23
sucrose+K	381.0794	381.05	381.0791	-0.03
heme ^{a,*}	616.1768	616.15	NA	NA
NAD ^a	664.1164	664.1	NA	NA
formononetin ^a	269.0808	269.08	269.0803	0.05
chrysoseriol GlcA ^a	477.1028	477.1	477.1008	0.2
formononetin MalGlc ^a	517.1341	517.13	517.1337	0.04
aformosin MalGlc ^a	547.1446	547.16	547.1427	0.19

^a [M+]

^a MS/MS performed for identification.

Table 2
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Name of metabolite	Theoretical [M-H] ⁻	MRMS Measured [M-H] ⁻	HRMS Measured [M-H] ⁻	Δm (mDa)
pyruvic acid	87.0077	87	NA	NA
alanine ^a	88.0393	88.01	88.0374	-0.19
lactic acid ^a	89.0233	89.01	89.0296	0.63
phosphoric acid	96.9685	96.96	96.9625	-0.6
2-ketobutyric acid	101.0233	101.02	101.0191	-0.42
γ-aminobutyric acid ^a	102.055	102.04	102.0528	-0.22
serine	104.0342	104.02	104.0228	-1.14
maleic/fumaric acid ^a	115.0026	115.03	115	-0.26
succinic acid ^a	117.0182	117.01	117.0125	-0.57
threonine	118.0499	118.04	118.0456	-0.43
oxalacetic acid	130.9975	131.03	130.991	-0.65
aspartic acid ^a	132.0291	132.03	132.0256	-0.35
malic acid ^a	133.0132	133.02	133.0221	0.89
salicyclic acid	137.0233	137.02	137.0349	1.16
α-ketoglutaric acid	145.0132	145.02	145.0063	-0.69
glutamic acid ^a	146.0448	146.01	146.0408	-0.4
pentose	149.0445	149.04	149.0414	-0.31
aconitic acid ^a	173.0081	173.03	173.0057	-0.24
ascorbic acid ^a	175.0237	175.05	175.0341	1.04
hexose	179.055	179.05	179.0484	-0.66
citric/isocitric acid ^a	191.0186	191.02	191.0166	-0.2
palmitic acid	255.2319	255.22	255.2246	-0.73
hexose-6-phosphate ^a	259.0213	259.04	259.014	-0.73
stearic acid	283.2632	283.26	283.2552	-0.8
sucrose ^a	341.1078	341.07	341.0972	-1.06

^a MS/MS performed for identification.

*Excel Spreadsheet- Table of Materials/Equipment
[Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table of Materials.xls](#)

Name of Material/ Equipment	Company	Catalog Number
Gelatin	Difco	214340
Cryostat- HM 550	Thermo Scientific	956564A
indium tin oxide (ITO)-coated glass slides	Delta Technologies	CB-90IN-S107
2,5-Dihydroxybenzoic acid (DHB) matrix	ICN Biomedicals	PI90033
Airbrush	Paasche Airbrush Company	TG-100D
Automatic matrix sprayer system- TM-Sprayer	HTX Technologies, LLC	HTX.TMSP.H021-U
Sublimation apparatus	Chemglass Life Science	CG-3038-01
Vaccum pump- Alcatel 2008 A	Ideal Vacuum Products	P10976
ultrafleXtreme MALDI-TOF/TOF	Bruker Daltonics	276601
FlexImaging	Bruker Daltonics	269841
MALDI LTQ Orbitrap	Thermo Scientific	IQLAAEGAAPFADBMASZ
Q Exactive	Thermo Scientific	IQLAAEGAAPFALGMAZR

Comments/Description

heat to dissolve

25-75-0.8 mm (width-length-thickness)

coupled with 75 mL steel container

Specific start-up and shut-down instructions will be given when the instrument is installed

Ultimate Pressure = 1×10^{-4} Torr

One example of "vender specific software"

High resolution MALDI instrument for accurate mass measurements

High resolution LC-MS instrument for accurate mass measurements



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Title of Article:

Investigation of metabolites in *Medicago truncatula* root nodules with

Author(s):

Erin Gemperline and Lingjun Li

MALDI-
mass
spectrometry
imaging

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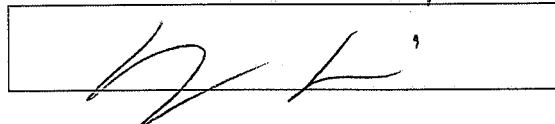
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MS # (internal use):

Reviewer 1 Comments:

1) The title can be revised as "MALDI-Mass Spectrometric Imaging for Metabolites Investigation on *Medicago truncatula* Root Nodules", since the MALDI-MS imaging as an analytical method is the key point in this paper while the sample can be any tissues besides *Medicago truncatula* Root Nodules.

Response: The title has been changed to “MALDI-Mass Spectrometric Imaging for Investigation of Metabolites in *Medicago truncatula* Root Nodules”

2) Please specify the "High Resolution LC-MS" used in this study.

Response: The high resolution MALDI and LC- MS instruments used for accurate mass measurements in this study are now listed in the Material/Equipment table.

3) The sample preparation procedure for LC-MS analysis was missing.

Response: Since the manuscript focuses on MALDI-MSI and LC-MS is just supplemental and there are many different LC-MS sample preparation procedures depending on the analyte of interest, a detailed LC-MS description was omitted.

4) Page 4, line 151, please provide the start-up instructions, an online URL link to the instructions will work.

Response: The instructions were intentionally left vague as to not promote one type of automatic sprayer over any other system. The instructions will be clearly given and explained to the lab when the sprayer instrument is installed.

5) Page 5, line 194, "the smallest laser diameter", please specify the laser diameter.

Response: The laser diameters have been included in the description.

6) Page 5, line 207, please specify the "commercial software" used for image generation. Page 6, line 211, please specify the "instrument specific software" used for metabolite identification.

Editorial Comment: Please include this information in the Materials/Equipment table and not in the manuscript text as JoVE is unable to publish manuscripts containing commercial sounding language. You may refer the reader to the Materials/Equipment table in this step.

Response: The vendor specific software has been listed in the Materials/Equipment Table and referred to in the text.

7) Page 4, line 140, "DHB matrix solution (150 mg/mL in 50% methanol/0.1% TFA v/v)", line 154, "using 40 mg/mL (in 50% methanol/0.1% TFA v/v) DHB as the matrix". DHB matrix solutions with different concentrations were used for airbrush application and automatic sprayer application, respectively. Please elaborate the consequence in MALDI imaging by using different matrix solutions for matrix application.

Response: The particular sprayer system used here heats the nozzle for faster evaporation of the solvent. As the solvent evaporates, the concentration of the matrix increases quickly. The matrix applied to the sample with the airbrush and the automatic sprayer has comparable concentrations.

This information has been added to the matrix sprayer section.

8) Fig. 1, "Array of Spectra" is too blurry.

Response: The "Array of Spectra" has been fixed.

9) Fig. 4b, what are these square shapes in right up corners?

Response: The square shapes are the matrix only areas that were imaged. This has been specified more clearly now in the description of Figure 4b on page 7.

10) Please provide the optical image of the tested root nodule, perhaps in Fig. 4. Please add a scale bar of intensity in Fig. 4.

Response: A scale bar has been added to Figure 4.

A new figure (Figure 2) has been added showing an optical image of a root nodule section.

Figures have been re-numbered.

11) Fig. 4b shows the ion maps of matrix related compounds, which are not evenly distributed over the tested tissue. Does this mean the matrix was not evenly sprayed on the tissue section before imaging test?

Response: This means that there are different ionization efficiencies of the matrix on tissue vs. on the glass slide. The matrix was evenly sprayed.

12) Fig. 5, the x-axis was missing. What is the peak with an open circle in the bottom left corner?

Response: The x-axis has been added and the open circle has been removed.

13) Table 2, the mass error for the compounds detected in negative ion mode were too big, please elaborate.

Response: This is published work. The mass error was within acceptable limits for the instruments being used.

Reviewer 2 Comments:

Since a major focus of this paper is demonstration of 3 different matrix application techniques, a more thorough description of the components necessary for setting up each different application apparatus should be discussed. For instance: (1) airbrush application: what kind of airbrush is appropriate? Model # of one that is currently being used? Is this performed in a fume hood? (2) automatic sprayer - what sprayer system is being described? (3) sublimation: is this commercially available or needs to be assembled? specs? What vacuum settings / pump is used?

Response: Matrix application apparatuses were referred to generically in the text of the manuscript, following editor instructions. The brand and catalog numbers for all equipment used is now listed in the Material/Equipment table. More information was added for the airbrush and sublimation procedure sections.

Editorial Comment: Please remember to include all commercial language (brand and model #s) in the Materials/Equipment Table and refer the reader to the Table in the protocol section.

Response: The Materials/Equipment table has been updated to include all brands and catalog numbers. The table has now been referred to throughout the protocol section.