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Title: Investigation of Xenobiotic Metabolism in *Salix alba* Leaves via Mass Spectrometry Imaging

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **26**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Claire Villette**: This protocol allows the direct identification of predicted metabolites in situ, facilitating the determination of the types of tissues or cells impacted by a specific metabolic process **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Claire Villette**: This method is quick and straightforward. It does not require a complicated sample preparation or compound extraction and allows the identification and localization of low abundant compounds **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot,

Protocol

2. Sample Preparation

2.1. Upon acquisition of the biological sample, cool a cryomicrotome, sample holder, and blade to minus 20 degrees Celsius **[1]**.

2.1.1. WIDE: Talent setting cryomicrotome temperature

2.2. If necessary, pour embedding medium into a plastic mold placed within the cryomicrotome chamber **[1]** and quickly add the sample to the medium **[2-TXT]**.

2.2.1. Talent pouring medium into mold
2.2.2. Talent placing sample into medium **TEXT: Here plant leaves will be shown**

2.3. Cover the sample with additional embedding medium **[1]** and adjust the position of the sample to the center of the mold as necessary as the matrix solidifies **[2-TXT]**.

2.3.1. Medium being added *Videographer: Important step*
2.3.2. Sample position being adjusted/shot of sample in center of cooling medium *Videographer: Important step* **TEXT: Not all samples require embedding medium**

2.4. When the sample is ready, use embedding medium to mount the tissue onto the cryomicrotome holder **[1]** and use the cooled blade to cut the tissue into appropriately-size sections **[2-TXT]**, modifying the cutting thickness and temperature as necessary to obtain optimal samples **[3]**.

2.4.1. Sample being mounted *Videographer: Difficult step*
2.4.2. Sample being sectioned *Videographer: Difficult step* **TEXT: *e.g.*, 5-30 micrometers for plant samples**
2.4.3. Talent adjusting thickness and/or temperature *Videographer: Difficult step*

2.5. As each section is acquired, use an appropriate tool to carefully move the slice to an indium-tin-oxide-coated glass microscope slide **[1]** and place a finger under the slide to warm and dry the sample **[2]** before placing the slide into the cryomicrotome chamber **[3]**.

2.5.1. Slice being moved into slide *Videographer: Difficult step*
2.5.2. Finger being placed under sample *Videographer: Difficult step*
2.5.3. Slide being placed into chamber *Videographer: Difficult step*

2.6. When all of the slices have been acquired, slowly remove the slides from the chamber **[1]** and use an appropriate marker to indicate the exact location of each sample on the slides **[2]**.

2.6.1. Talent removing slides from chamber *Videographer: Important step*
2.6.2. At least one slide being marked *Videographer: Important step*

2.7. When all of the slides have been marked, use a high-resolution scanner to scan the samples **[1]**.

2.7.1. Talent placing slide(s) into scanner

3. Matrix Deposition

3.1. After scanning, clean the matrix deposition robot with 100% methanol **[1]** and use methanol and a precision wipe to clean each slide, holding the samples without touching them and without removing the marks **[2]**.

3.1.1. WIDE: Talent cleaning robot
3.1.2. Slide being cleaned

3.2. Place a clean coverslip over each slide in an area without sample **[1]** and place the coverslip end of the first slide over the optical detector **[2]**.

3.2.1. Coverslip being placed
3.2.2. Slide being placed over optical detector

3.3. Then place no more than 6 milliliters of freshly prepared matrix solution in the matrix deposition reservoir **[1-TXT]** and add 2 milliliters of 100% methanol to a total volume of 8 milliliters **[2]**.

3.3.1. Robot spraying matrix solution on the slide *Videographer: Important step* **TEXT: See text for all medium and solution preparation details**

4. Data Acquisition

4.1. For data acquisition setup, place 1 microliter of matrix onto a MALDI (mall-dee) plate **[1]** and insert the plate into the source **[2]**.

4.1.1. WIDE: Talent adding matrix to plate
4.1.2. Talent inserting plate into source

4.2. Click **Load Target** in the device software **[1]** and click on the spot in the image that represents the MALDI plate to select the position of the matrix spot. Indicate the sample name and folder in the **Sample Info** tab **[2]**.

4.2.1. Talent clicking load target, with monitor visible in frame
4.2.2. SCREEN: screenshot_1: 00:02-00:13

4.3. Click **Acquisition** to start the acquisition and use the mouse pointer to move the plate slightly so that the laser points at different spots **[1]**.

4.3.1. SCREEN: screenshot_1: 00:14-00:27 *Video Editor: can speed up*

4.4. When the acquisition is finished, open the **Calibration** tab and select **HCCA** (H-C-C-A) **calibration** list in the **Quadratic Mode** and click **Automatic**. The global calibration result will be indicated in the **Calibration Plot** window **[1]**.

4.4.1. SCREEN: screenshot_1: 00:28-00:41 *Video Editor: can speed up*

4.5. If the calibration is in the appropriate range, click **Accept** and save the method **[1-TXT]**.

4.5.1. SCREEN: screenshot_1: 00:42-00:52 **TEXT: *e.g.*, 0.2 ppm for SolariX XR 7T**

4.6. Once the matrix deposition on the samples is finished, place the slides in a slide adapter **[1]** and use a plastic cover to retrieve the position of the marks **[2]**.

4.6.1. Talent placing slide(s) into slide adapter *Videographer: Important step*
4.6.2. Talent retrieving mark position(s) *Videographer: Important step*

4.7. In the imaging software, set up a new imaging run in the first window that opens in the software **[1]** and name the imaging run **[2]**.

4.7.1. Talent setting up imaging run, with monitor visible in frame
4.7.2. SCREEN: screenshot_2: 00:02-00:07

4.8. Select the **Result Directory** and click **Next [1]**.

4.8.1. SCREEN: screenshot_2: 00:08-00:13

4.9. Indicate the raster size, select the method to be used, and click **Next [1]**.

4.9.1. SCREEN: screenshot_2: 00:14-00:22

4.10. Load the optical image from a scanned slide and click **Next [1]**.

4.10.1. SCREEN: screenshot_2: 00:23-00:27

4.11. The image will open in a wider window **[1]**.

4.11.1. SCREEN: screenshot_2: 00:28-00:34

4.12. Under **ftmsControl**, place the target of the MALDI video window on the exact position of a mark and open **Flex Imaging [1]**.

4.12.1. SCREEN: screenshot_2: 00:35-00:46

4.13. Then click on the exact same point on the optical image **[1]** and place the plastic cover bearing the marks onto a MALDI plate to recover its position and to facilitate the teaching **[2-TXT]**.

4.13.1. SCREEN: screenshot_2: 00:47-00:50 **TEXT: Repeat targeting for 3 independent points**
4.13.2. Cover being placed

4.14. Use the **Add Measurement Regions** tools to draw the regions of interest in the samples and save the imaging run **[1]**.

4.14.1. SCREEN: screenshot_3: 00:03-00:53 *Video Editor: please speed up*

4.15. If several samples are to be analyzed, run the **AutoXecute Sequence** and use **AutoXecute Batch Runner** to launch a sequence **[1]**.

4.15.1. SCREEN: screenshot_3: 00:54-01:08

5. Data Processing

5.1. When all of the images have been acquired, use the **Batch Importer** tool to select the raw data **[1]**, indicate the target directory, and click **Import [2]**.

5.1.1. WIDE: Talent selecting raw data, with monitor visible in frame
5.1.2. SCREEN: screenshot_4: 00:02-00:08

5.2. Click **File** and **New** to select the **Dataset** tool to elect the type of instrument used for the acquisition and click **plus** to add the imported datasets **[1]**.

5.2.1. SCREEN: screenshot_5: 00:02-00:05

5.3. Click and drag to arrange the images **[1]** and check and modify the mass range settings as necessary. Click **Next** to see the import summary and launch the import **[2]**.

5.3.1. SCREEN: screenshot_5: 00:14-00:36 *Video Editor: please speed up*
5.3.2. SCREEN: screenshot_5: 01:04-01:13

5.4. Visualize the mass-to-charge ratio in different tissues within a sample or between different samples and click **spectra** to select the mass-to-charge ratios of interest **[1]**.

5.4.1. SCREEN: screenshot_6: 00:02-00:23 *Video Editor: please speed up*

5.5. Export the mass-to-charge ratios of interest as a .csv file from the **Object** tab and click **Export [1]**.

5.5.1. SCREEN: screenshot_6: 00:25-00:44 *Video Editor: please speed up*

5.6. To create a new dataset in an annotation software program and click **Projects** and **Import CSV project [1]** to import the .csv file into the software **[2]**.

5.6.1. SCREEN: screenshot_7: 00:02-00:20 *Video Editor: can speed up*
5.6.2. SCREEN: screenshot_7: 00:49-00:56 **TEXT: Only exact m/z considered, isotopic profile lost**

5.7. Annotate with custom-made analyte lists, which can be derived from publicly available databases **[1]**. A template is given by the software to create analyte lists **[2]**.

5.7.1. SCREEN: screenshot_7: 01:04-01:12 *Video Editor: please speed up*
5.7.2. SCREEN: screenshot_7: 03:29-03:39

5.8. Use the prediction software to perform in silico prediction of the metabolites of the annotated compounds **[1-TXT]**.

5.8.1. SCREEN: screenshot_8: 00:02-00:14 **TEXT: Developed compound formula required**

5.9. Recover the list of metabolites **[1]**, create an analyte list **[2]** and use the list in the annotation software to annotate the raw data with predicted metabolites **[3-TXT]**.

5.9.1. SCREEN: screenshot_9: 00:02-00:13
5.9.2. SCREEN: screenshot_10: 00:02-00:26 *Video Editor: please speed up*
5.9.3. SCREEN: screenshot_10: 00:27-1:02 *Video Editor: can speed up* **TEXT: Shot metabolite list can be manually searched**

5.10. Then right-click on the predicted metabolite of interest to recover the names of the enzymes involved in the metabolic processes in the prediction software **[1]**.

5.10.1. SCREEN: screenshot_11: 00:02-00:10

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.3., 2.6., 3.3., 4.7.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.4., 2.5. Taking time to adjust parameters to the type of sample is the key parameter to assure success.

Results

6. Results: Representative Enzymatic Profile Analyses in Response to Xenobiotics Exposure

6.1. In this example, the drug telmisartan was determined to be distributed throughout the plant leaf tissue samples **[1]**.

6.1.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize Telmisartan structure and text*

6.2. The drug metabolites were predicted and searched for in the raw data **[1]** and the annotations revealed that one first-generation metabolite was detected in the internal tissues of the leaves **[2]** and further degraded into second-generation metabolites that were localized in internal tissues or more generally distributed in all of the leaf tissues **[3]**.

6.2.1. LAB MEDIA: Figure 2
6.2.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize C33 H32 N4 O3 (I) structure and text*
6.2.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize Video Editor: please emphasize C33 H30 N4 O3 (II), C33 H32 N4 O4 (II), and C32 H30 N4 O3 (II) structures and texts*

6.3. These results suggest an active metabolic reaction in the leaves to degrade telmisartan **[1]**.

6.3.1. LAB MEDIA: Figure 2

6.4. The process was then applied to several compounds of interest annotated in the leaves **[1]** and the enzymes involved in the reactions were recovered to investigate their role in the plant's response to xenobiotic accumulation **[2]**.

6.4.1. LAB MEDIA: Figure 3
6.4.2. LAB MEDIA: Figure 3 *Video Editor: please sequentially add/emphasize data bars from left to right*

Conclusion

7. Conclusion Interview Statements

7.1. **Claire Villette**: Although this procedure is quite simple, a good sample preparation is key. Be sure to take the time to find the parameters that fit your sample type during the tissue sectioning **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.4., 2.5.)

7.2. **Claire Villette**: The sample slides can be further used for microscopy observations and can be stained to acquire a panel of complementary biological information **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera