

**Handbook of
MALDI Imaging
Methods
for rapifleX**



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Introduction

MALDI Imaging is a powerful mass spectrometry tool for mapping the distribution of molecules from a thin sample, ranging from small metabolites to large proteins, without molecular tags or labels.

This cookbook uses **Lipid Imaging** as an example, covering the complete workflow from A) Preparing Fresh -Frozen tissue, B) Applying matrix, C) Acquiring an image and D) Analyzing data including helpful tips and easy trouble shooting. **Protein Imaging** and **FFPE (Formalin Fixed Paraffin Embedded) tissue imaging** workflow (digested peptides) were included as appendix. The methodologies described are specific for imaging lipids. Protocols are provided for other common samples and analytes. This handbook is intended only as a quick start guide and not as replacement for the full software and hardware manuals.

MALDI TOF Application Support: Maldi.Appl.Support@Bruker.com

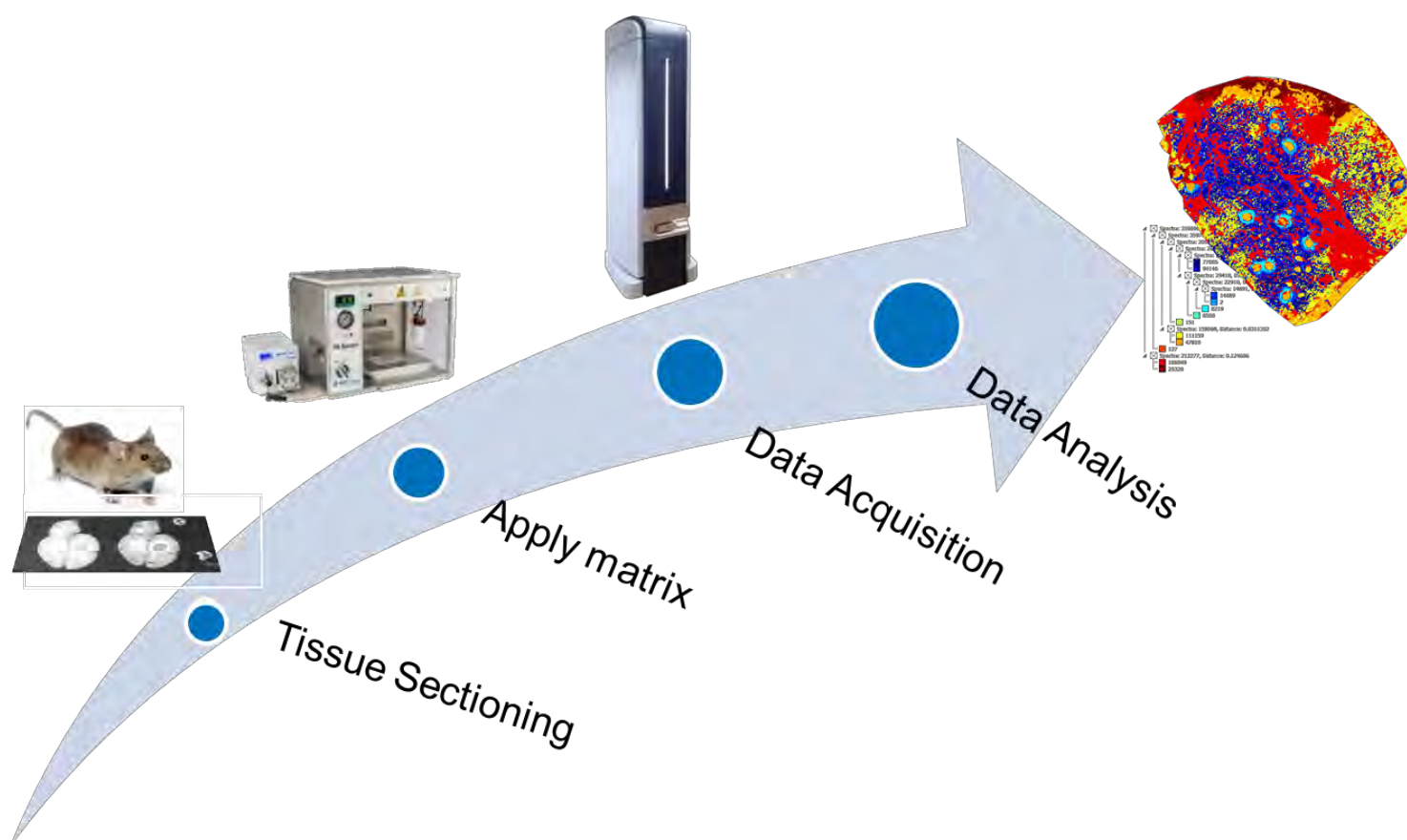
MALDI TOF Software Support: Maldi.SW.Support@Bruker.com

TM Sprayer support: info@htximaging.com

SCiLS Lab Support: support@scils.de

We appreciate your feedback.

Bruker MALDI Imaging Team



A) Preparing Fresh-Frozen tissue

1) Freezing Tissue

Cryosections prepared from fresh frozen tissue are best suited for mass spectrometry imaging (MSI) and the quality of tissue samples is crucial for obtaining the best results.

Tissue must be frozen immediately after harvesting with no additional treatment such as perfusion or fixation. If possible, blood and connective tissues should be removed to some extent. Snap freezing in liquid nitrogen is the most commonly used technique. We recommend to loosely wrap the organ in aluminum foil and let the tissue float on liquid nitrogen for progressive freezing.

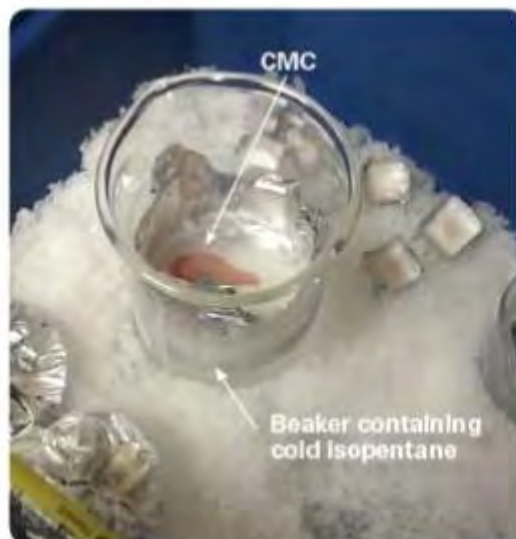


If the tissue is damaged during freezing in liquid nitrogen, alternatively you can use isopentane pre-cooled at -80°C and placed on dry ice.

2) Embedding Tissue

Solid tissues such as brain, liver, kidney etc. can generally be cryosectioned without any embedding. However, embedding may be required to preserve the morphology of delicate tissues during freezing. Embedding media composed of 1% or 2% carboxymethyl cellulose (CMC) are compatible with MSI experiments (CMC embedding protocol is in **Appendix 5**). In contrast, embedding media such as optimal cutting temperature (OCT) polymer must be avoided. Indeed, polymers contained in OCT are highly detectable by mass spectrometry and can thus lead to ion suppression effects.

After freezing, tissues can be stored at -80°C for 1 or 2 years



3) Preparing to section

a) Preparing Cryostat

Prepare the cryostat by adjusting the chamber temperature at -20°C and the specimen temperature between -10°C and -20°C . The specimen temperature required for optimal cutting depends on each tissue. Select the desired tissue thickness (typically between $6\text{--}15\text{ }\mu\text{m}$, Bruker recommends $10\text{ }\mu\text{m}$)



We recommend to transport the tissue on dry ice between the -80°C freezer and the cryostat. The tissue must be stored at chamber temperature (-20°C) for approximately 45 minutes before the start of cryosectioning.

Place brushes, forceps and the tissue holder inside the cryostat and wait until they reach the cryostat temperature.

TRICK: To keep your cryostat clean, you can protect the chamber with Kimwipes or aluminum foil. Section debris can thus be very easily eliminated at the end of sectioning

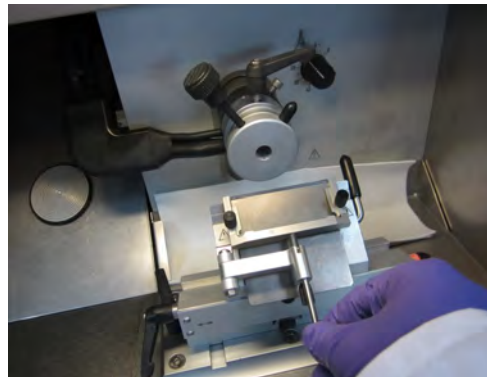
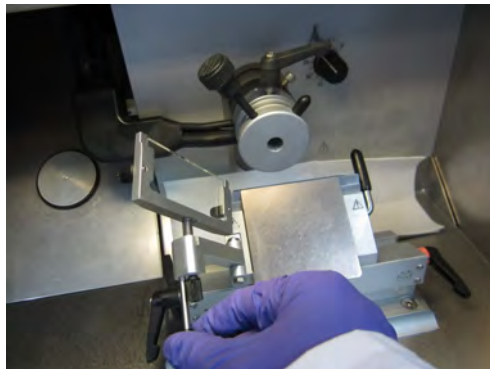
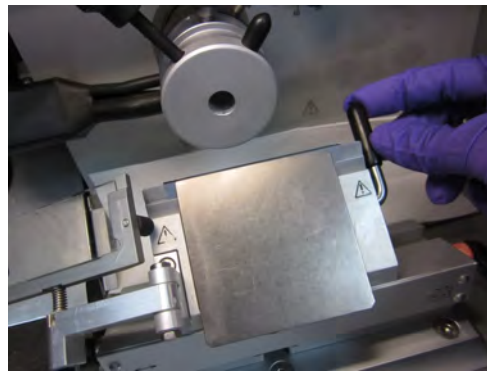
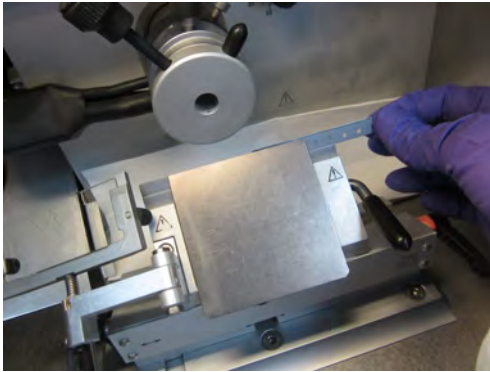


b) Installing Blade and Adjusting Anti-roll guide

Wipe the cryostat blade with ethanol to remove contaminant. (*Be careful of sharp blade!!*) Insert the blade in the knife holder and lock the blade in place (right picture).

Place the anti-roll guide onto the blade. Carefully check the fine alignment of the anti-roll guide relative to knife edge.

TRICK: A thin bright line appears on the knife edge when the adjustment is correct.



c) Cryosectioning

We recommend to wait for 15 minutes to allow temperature equilibration before starting cryosectioning.

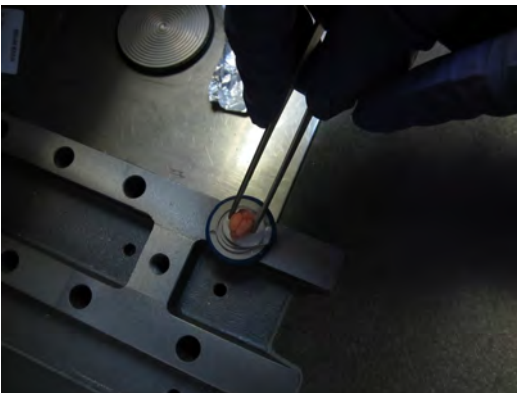
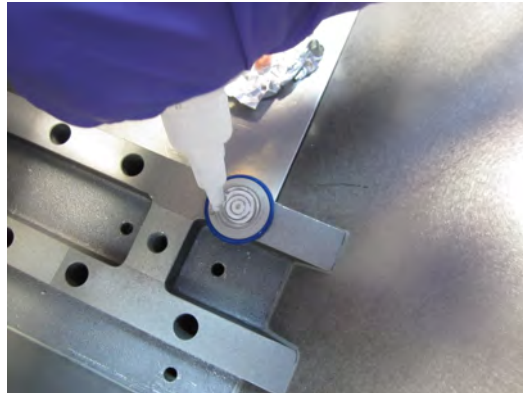
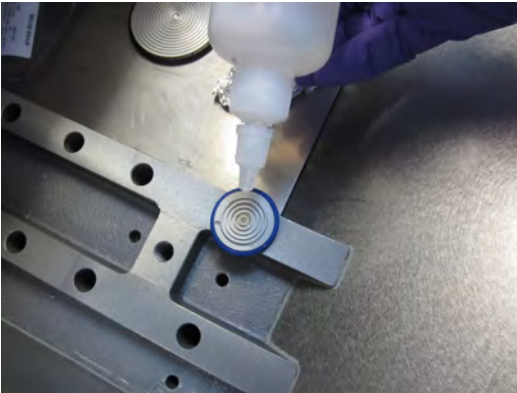
Move the anti-roll guide away from the knife. Move forward the specimen head using the four coarse feed keys (or any other command at your disposal depending on the instrument used) to bring the specimen close to the knife.



4) Mounting and Sectioning Tissue

To identify the conductive side of IntelliSlides, see the tips on the Appendix

1) Place the conductive intelliSlides into the chamber of the cryostat. Take your sample out of the aluminum foil. 2) Deposit a drop of OCT on the tissue holder and place it in the chamber of the cryostat. Avoid any contact between OCT and regions of the tissue from which sections will be collected.



3) Promptly place the organ on the tissue holder and adjust its orientation before solidification of OCT. After a few minutes, OCT is totally frozen (and turns white): the tissue is ready for sectioning .

4) Place the tissue holder on the specimen head. Depending on the cryostat model, you can adjust the orientation of the specimen head, as illustrated below.

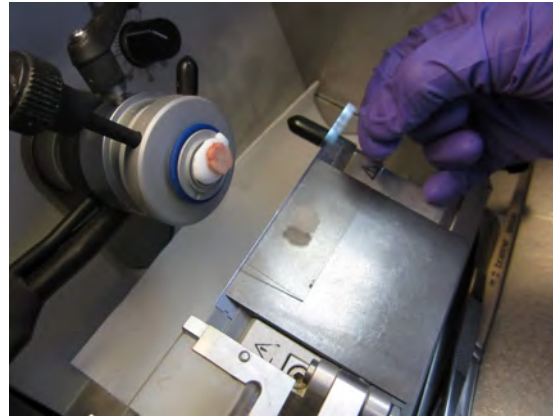


5) First trim your tissue with a section sickness of ex. 50 μ m until the region of interest is reached, then gently remove all section debris using a large brush. Select the desired section thickness (Bruker recommends \sim 10 μ m). Place the anti-roll guide on the knife and discard the first 2 to 3 sections before collecting the sections.



6) You are now ready to transfer a section onto the conductive side. There are two methods to do that (**Method 1 and 2**), followed by fixing tissue using warm fingers.

Method 1: Isolate a section with the brush, put the conductive side of the slide onto the section and flip the conductive slide.

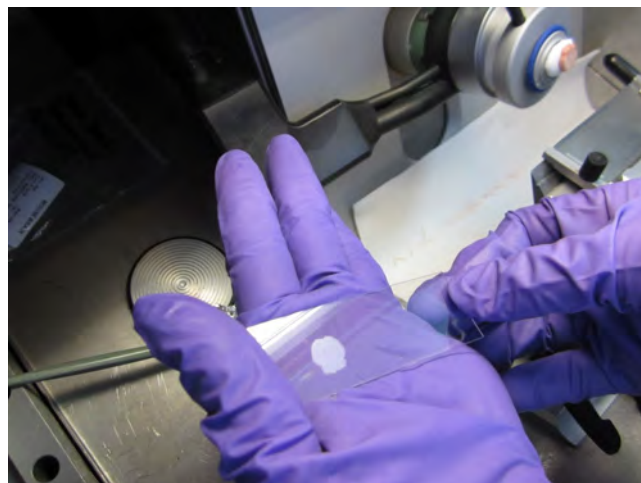


Method 2: Grab a section with a brush and put it on the conductive side of the slide.



With both methods, inside the cryostat, place your finger or hand on the non-conductive side back to the section to warm it. Wait until the section appears to be fully dry about 20-30s or even longer. Then put the slide back to the cryostat and prepare another section.

Tissue can be then stored in -80°C or under vacuum at RT (up to 1 month for lipid imaging) until MALDI acquisition.

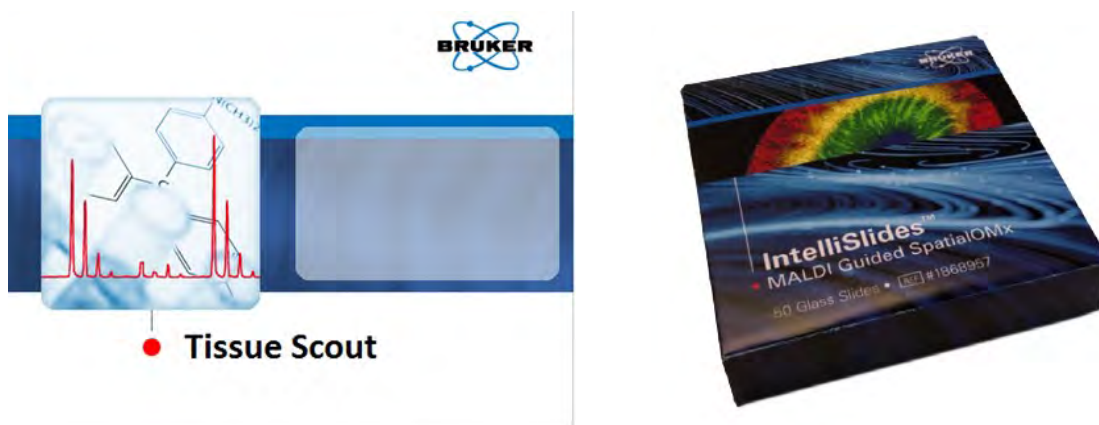


B: Applying matrix

1) Scanning Slides (BEFORE applying matrix)

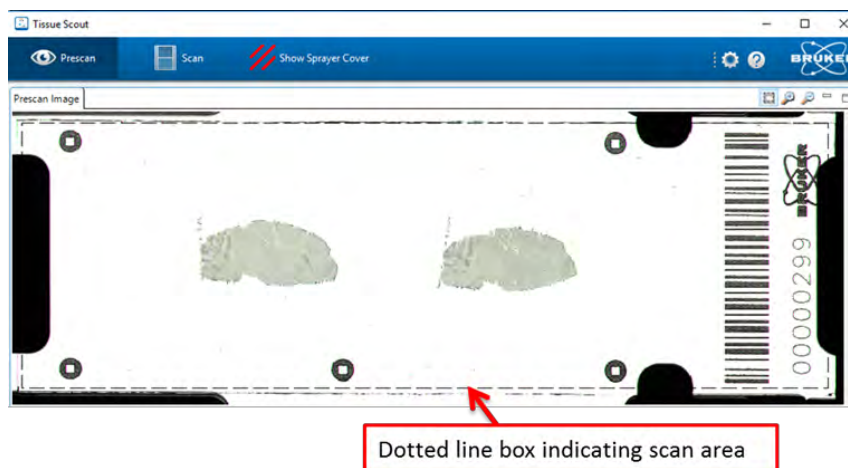
Alignment of optical image of the sample is necessary when the sample target is placed inside the instrument. Usually, it will not be possible to recognize the tissue section underneath the matrix layer. To correlate the optical image with the sample, it is therefore necessary to have teach marks that are visible both in the optical image and underneath the matrix layer in the camera optic.

Bruker recommends Tissue Scout Scanner (Bruker cat #1846968) for IntelliSlides (Bruker cat #1868957). Bruker's IntelliSlides simplify your MALDI Imaging workflows by enabling automated setup of each measurement. IntelliSlides incorporate permanent inscriptions that indicates optimal placement of samples, registration marks (also known as teach marks or fiducials) that make automated sample registration possible, as well as providing a unique slide identifier for software tracking. IntelliSlides are the optimal tool for increasing measurement efficiency and throughput for any MALDI Imaging study.

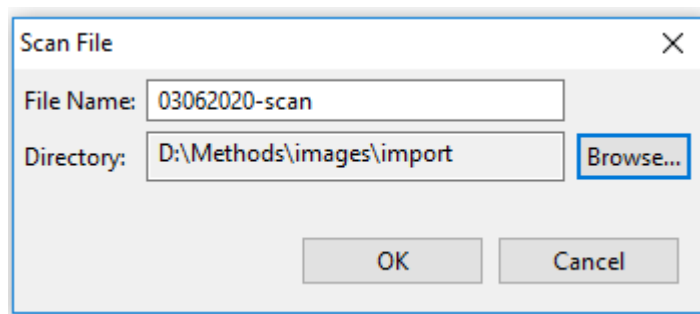


How to perform tissue scanning using Tissue Scout Software

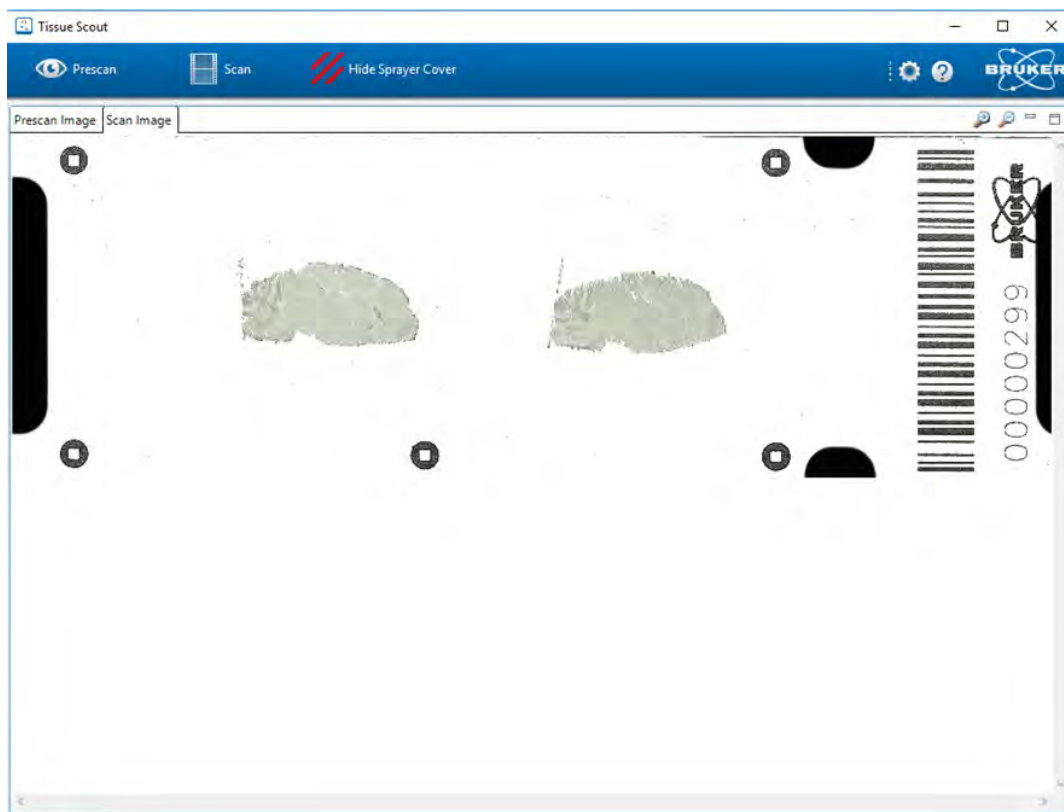
1. Click on **Pre-Scan** Button on tissue scout software
2. A dotted line box will appear in the pre-scan Image Tab, which should cover barcodes and teaching marks. Do not adjust the dotted line since the area was calculated



3. Click on **Scan** button to perform high-resolution scan. Make sure to save the scan file under **D:\Methods\images\import** on rapifleX acquisition computer. You may also store the scanned image in other directory, then copy it into the import folder as a part of requirement for intelliSlides auto-teaching process.



4. The high resolution scan of the sample will appear in the “Scan Image” Tab.

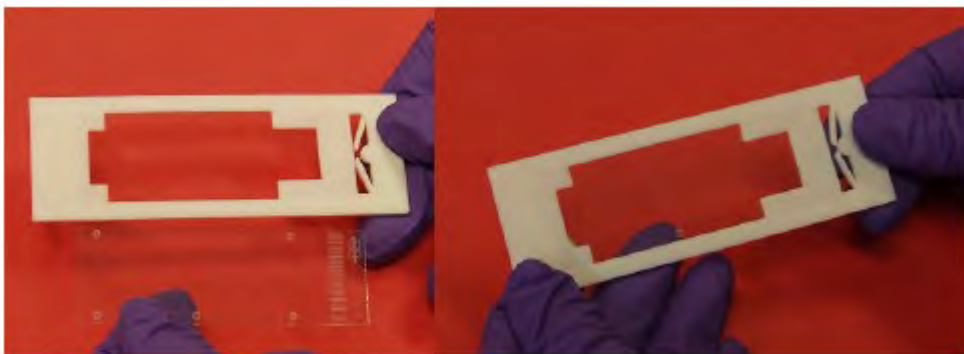


You may close the Tissue Scout software and Scan Image window, after scan is completed.

2) HTX TM Sprayer— Matrix Deposition

Instructions below are specific for preparing a tissue section for **lipid imaging** using **DHB** preparation conditions provided in **Table 1** below. Before imaging **proteins** or **digest products**, consult the protocols in the appendix. This handbook is not intended to replace more detailed operating information contained in the TM Sprayer User Manual.

When using IntelliSlides, a sprayer mask needs to be used to cover barcode and teaching marks to proper auto-teaching step. Wipe off the sprayer mask after matrix coating with 100% ethanol. Each box of IntelliSlides comes with 2 sprayer masks.



1. Set the nitrogen gas to be 10 psi.
2. Start laptop, TM sprayer and then software.
3. Select DHB method by clicking it on the very left side. Under **Sample** panel , **current method, DHB method** appears in the green box.
4. Prepare DHB matrix 15mg/mL in 90% ACN, 0.1% TFA (see table 1). 6mL of matrix is recommended for 5 mL standard loop. Never use < 5mL volume.
4. Set nozzle temperature to **60°C** in software and pump flow rate to 0.125 mL/min manually (on pump).
5. Set the matrix loop volume to be 5mL under cycle tab.
6. Set valve in the “**LOAD**” position.
7. Fill the reservoir bottle with desired solvent and flush the LC system for a few minutes.
8. Purge loop 2-3 times with 50% ACN before loading 6mL of matrix.

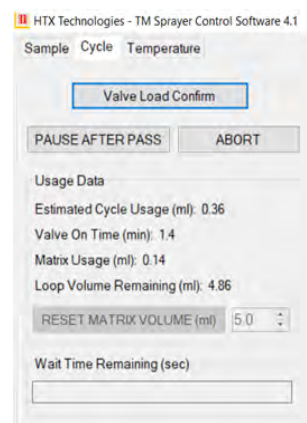
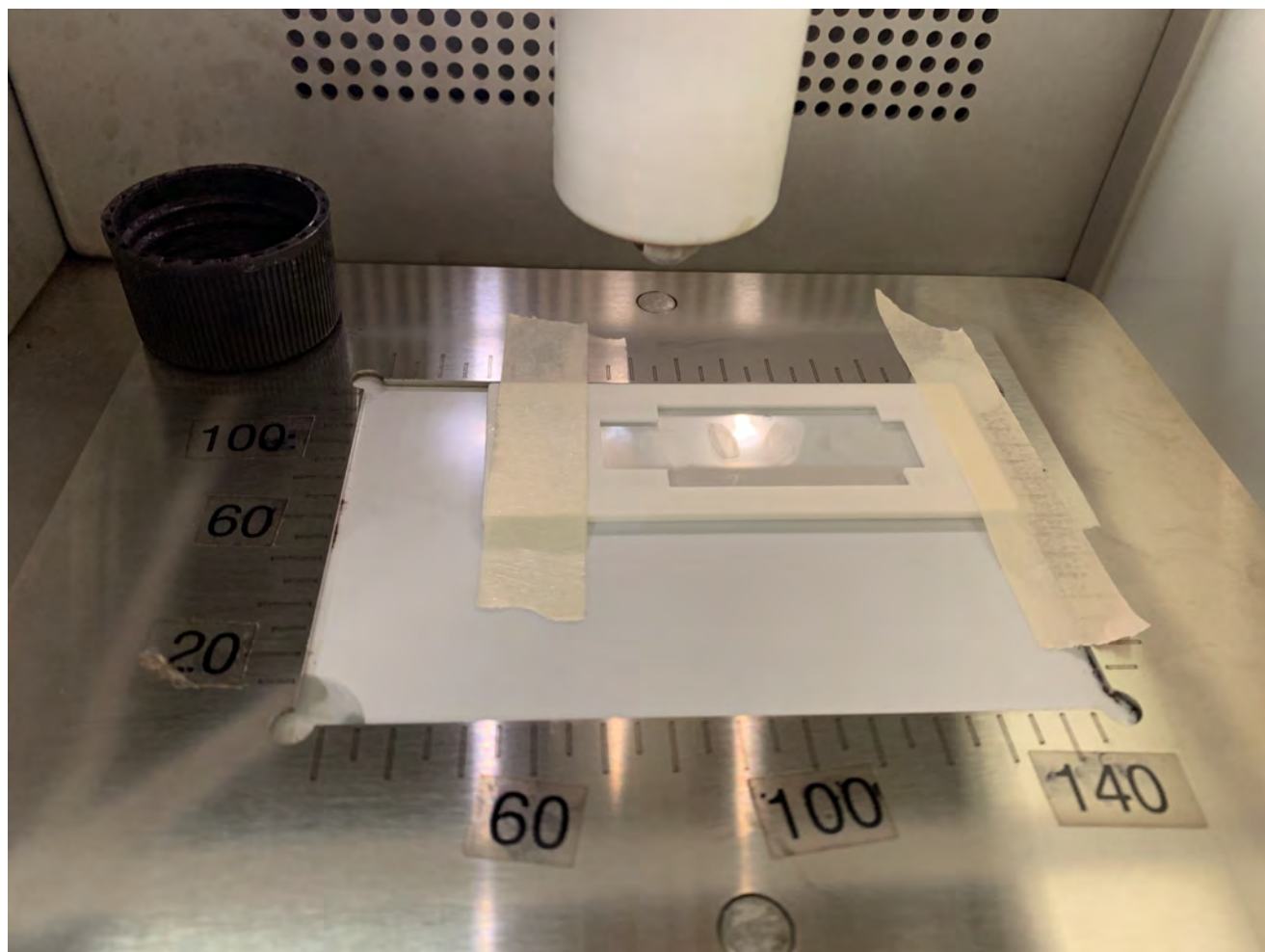
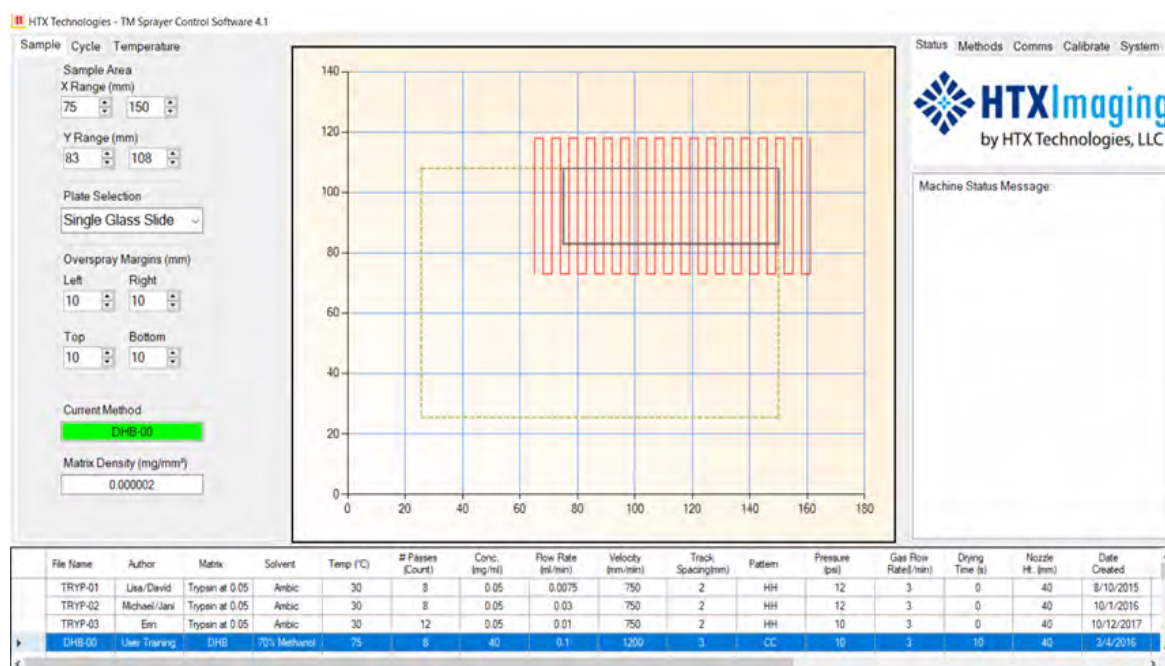


Table 1: Bruker Recommended TM Sprayer methods

Matrix	Preparation	Nozzle temperature (°C)	Pump device	Plate rate (mL/min)	Z-arm velocity (mm/min)	No of passes	Moving pattern	Track spacing (mm)
HCCA for digested tissues	10mg/mL in 70% ACN, 1% TFA	75	LC pump	0.12	1200	4	HH	3
HCCA for peptides	10mg/mL in 70% ACN, 0.2% TFA	75	LC pump	0.12	1200	4	HH	3
DHB for lipids	15mg/mL in 90% ACN , 0.1% TFA	60	LC pump	0.125	1200	14	CC	3
SA for proteins	10mg/mL in 50% ACN, 0.1% TFA	75	LC pump	0.15	1000	6	HH	2
Trypsin	See appendix - FFPE digest	30	Syringe pump	0.015	750	16	CC	2



9. Secure the tissue slides with adhesive tape to the metal frame and place inside the TM sprayer chamber.
10. In the **"Sample"** tab, define area or select glass type from the dropdown menu, such that the complete slide (allowing for 10mm overspray borders) is covered.
11. Press **"Start"**, and switch the valve to position **"Spray"**.
12. Press **"Continue"** which will start a countdown to flush the dead volume, then the device starts spraying matrix.
13. After the deposition is finished, switch the valve to **"Load"**, and press the **"Valve load confirm"** button.
14. Remove the slides from chamber.
15. Purge the loop using 5mL of 50% ACN 3 times.
16. Set the nozzle temperature to 30°C to cool down. Keep the pump **ON** and nitrogen gas **ON**.
17. When temperature reaches 30°C or below, first turn off software, then LC pump, gas flow and TM sprayer,



3) Quick Troubleshooting and Tips:

Troubleshooting Matrix Deposition :

In MALDI imaging, users are often faced with what has come to be known as the “Matrix Deposition Challenge.” This phrase refers to the fact that wetter deposition improves signal intensity but can lead to worse image resolution by inducing analyte delocalization. A dryer spray can sharpen image resolution but lead to sacrifices in MS peak intensity. It is always important to keep in mind that you only need the resolution that is sufficient to answer your experimental question, and thus we recommend referring on the side of a wetter spray deposition in order to ensure sufficient MS signal intensity. To help guide this process quantitatively, it is important to keep two equations in mind:

Tip 1, Too wet of a spray leading to analyte delocalization:

Signs and symptoms: Solvent beading on tissue, MALDI image result blurred on edges, Try

- (1) Increase Nozzle temperature—optimal temperature is set 5°C below the boiling point of the solvent.
- (2) Increase nozzle velocity by 200 mm/min increments. Note: max speed is 1350 mm/min
- (3) Decrease piston pump flow rate by 20uL/min—min flow rate is 0.04uL/min. Note if you decrease flow rate, you would need to lower nozzle temperature.
- (4) Increase organic solvent percentage in which matrix is dissolved. Example, MeOH from 50% to 70%.

Tip 2, Too dry of a spray leading to insufficient analyte extraction:

Signs and symptoms: not sufficient number of MS peaks, failed to detect specific ions.

Do the opposite changes to Tip 1 (Too wet of a spray) by decreasing nozzle temperature, nozzle velocity, increasing pump flow rate and decreasing organic solvent percentage.

C) Acquiring an Image

Steps to acquire an image are as follows:

First mount a tissue slide on the MTP slide adapter II,

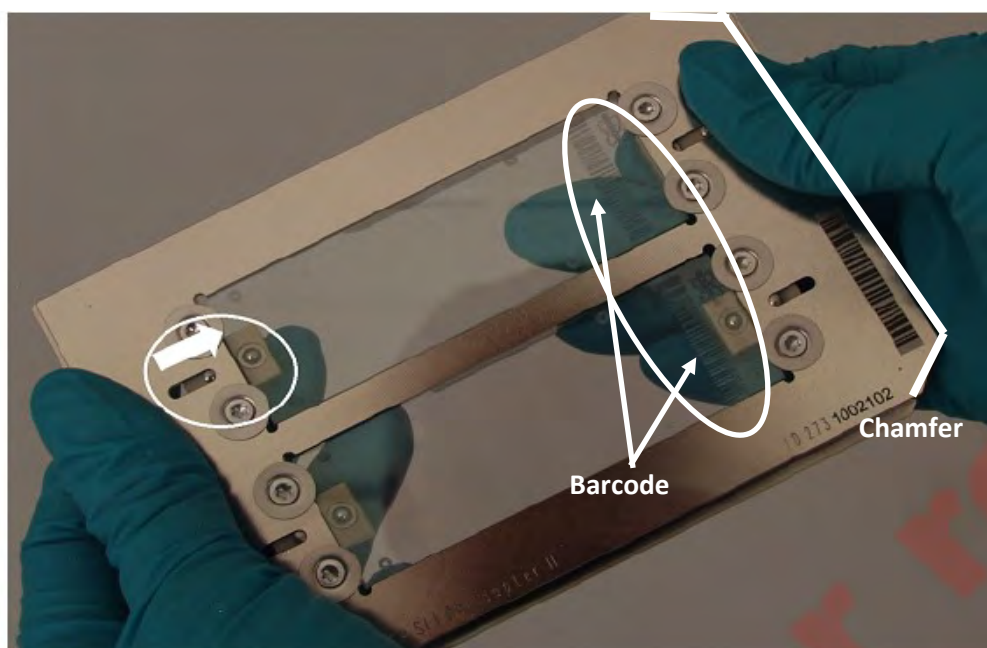
Then calibrate flexControl method: At least one calibration standard need to be spotted near tissue on the same IntelliSlides. For Lipid imaging, Bruker Peptide calibration standard II (PN: 8222570) can be used to cover m/z 700-4000Da. If smaller molecules are of interests, then red phosphorus, matrices peaks and polyethylene glycol (PEG) can also be used.

Then optimize an acquisition method in flexControl which includes m/z range, laser power, laser shots. Perform a method calibration. Next, setup flexImaging for desired measurement parameters, flexControl method and measurement regions.

1) flexControl Setup

a) Mounting the IntelliSlides on the MTP Slide Adapter II

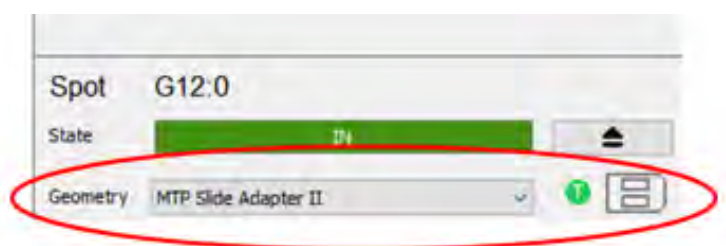
Make sure the end of the IntelliSlides with the barcode must be positioned at the end of the adapter with the chamfer. Then load into the rapifleX instrument. The sample can be loaded either by



A) Pressing the circular button on the front of the rapifleX, or by

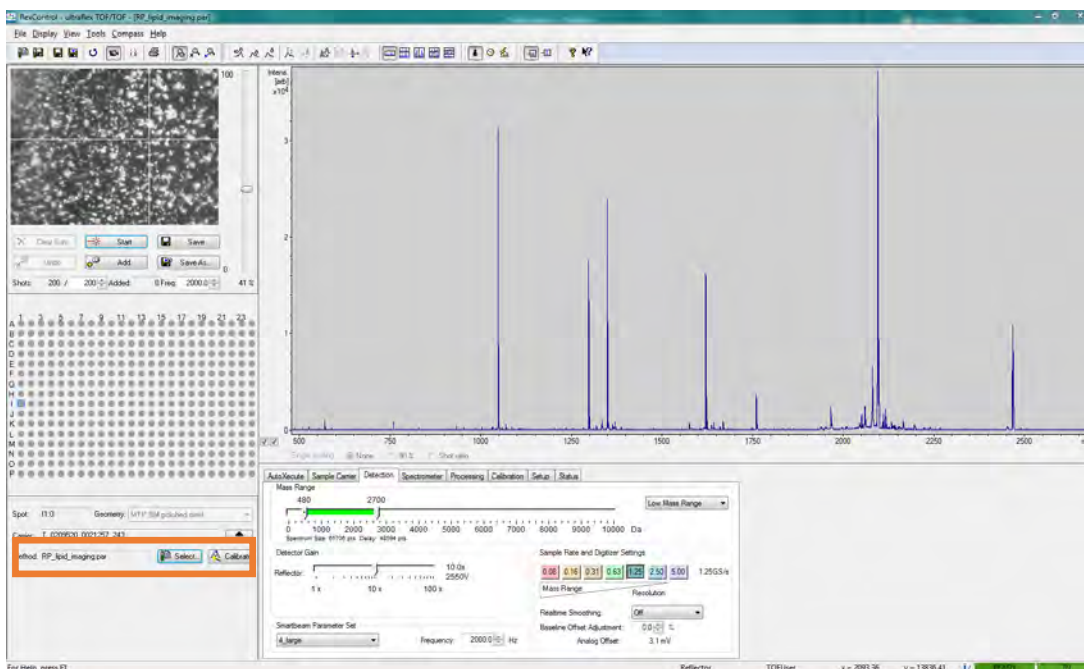
B) Using the “LOAD/EJECT” button in the flexControl software

Make sure “MTP slide adapter II” **geometry** is selected



b). flexControl setup

Note: You must complete both the **Target Profile Generation** and **Focus Adjustment** before calibration to avoid introduction of a mass shift.



Loading method: click **Select** to load a reflectron positive (RP) or reflectron negative (RN) method.

Laser intensity: adjust the laser power on some random spots on your tissue (next to camera view) to get good signals. If laser power is too high, the baseline will be raised, with peaks getting broader due to saturation. If laser power is too low, sensitivity would drop with less peaks detected.

For MALDI Imaging runs it is strongly recommended to not exceed an intensity of 10,000-15,000 counts for the highest peak in the spectrum

Laser setting: rapifleX is equipped with smart beam 3D laser. depending on pixel size selected in flexImaging settings, in flexControl, proper laser focus should be selected to match. **Recommended laser profile is listed next page.**

Ex, if running lipid imaging at high resolution, in flexControl sample carrier– laser-application choose Imaging 20µm. Then in flexImaging, pixel size choose 20µm to match. If running lipid imaging at a regular resolution, ex run tissue at 50µm, then M5 small is recommended with 200-500 shots per raster, also choose 50 µm in flexImaging. Proper laser setting is important, since if laser focus is too big comparing to pixel size, laser burn out neighboring pixels, and if laser focus is too small, then not enough coverage from single spots.

Automation
Sample Carrier
Laser
Detection
Spectrometer
Processing
Calibration
MS/MS
Setup
Status

Application
+ Imaging 20µm

Power Boost
0
%

Smartbeam Parameter
Single







☒ Beam Scan

X
Y

Scan Range
16
16
µm

Resulting Field Size
20
20
µm

▶ Advanced

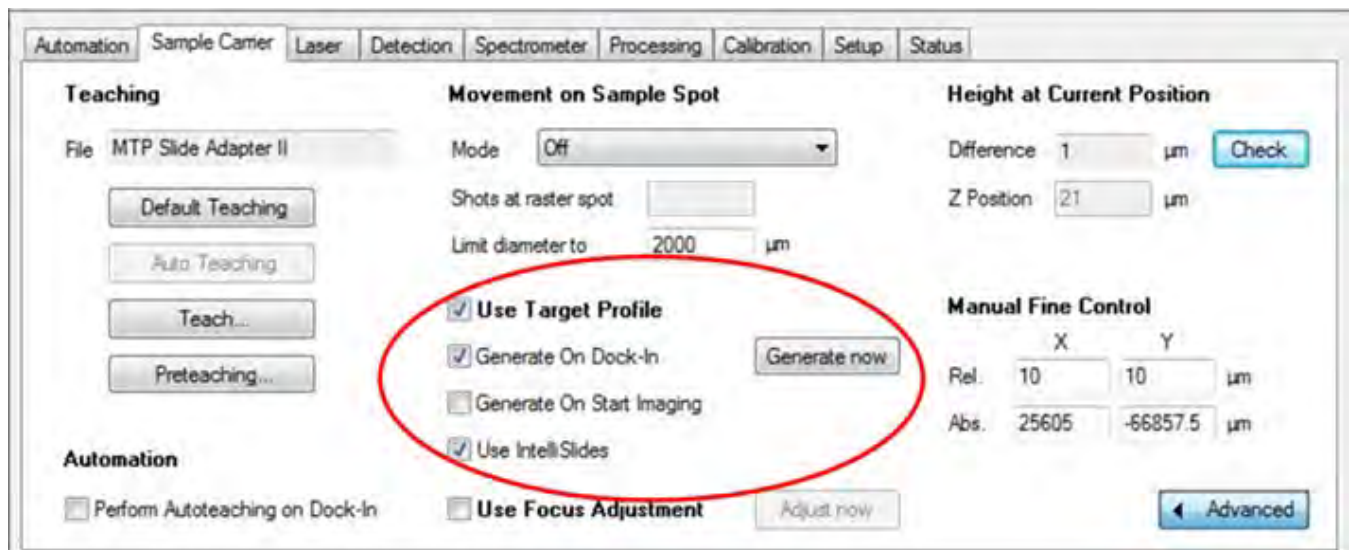
FlexImaging Raster	FlexControl Laser Profile	# Laser shots*	Beam Scan
100 µm	 M5	300-500	(fixed)
50 µm	 M5 Small	200-500	(fixed)
20 µm	 20 µm	100-200	(fixed)
50-70 µm	 M5 Small	200-500	(Raster size - 30)
70-150 µm	 M5	300-500	(Raster size - 70)
<50 µm	 Custom	100-200	(Raster size)

** Laser shots (and laser power) should produce maximum ion intensity of 10,000-20,000 counts per pixel/spectrum for maximum detector lifetime*

C) Automatic Target Flatness Optimization

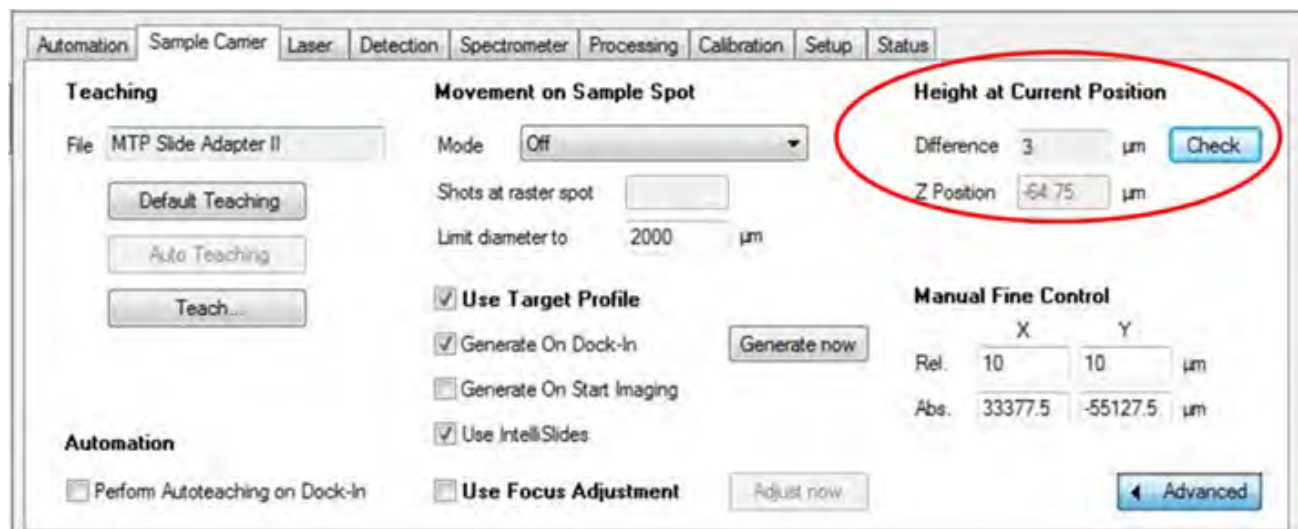
This compensates mass shifts and intensity variations caused by a curved target surface. This can be done either when inserting the sample into the rapifleX or manually. Target Profile, Focus Detection, and Detector Check must be completed before method calibration to avoid introduction of a mass shift to a calibrated method.

1. Activate "Use Target Profile" and "Generate On Dock In":
2. Activate "Use IntelliSlides"- This can be deactivated when not using IntelliSlides



3. Click the "Generate now" button and wait for the positions to be measured.

Move to different locations and click "Check". The "Difference" value should be between $\pm 10 \mu\text{m}$. The "Z-position" value should also change when moving to different positions.



4. Move to a matrix only region, then Activate “Use Focus Adjustment.
5. Click the “Adjust now” button and wait – spectra will be seen.

The screenshot shows the software interface with the following details:

- Automation** (selected tab):
 - ☐ Perform Autoteaching on Dock-In
 - ☒ **Use Focus Adjustment** (circled in red)
 - Adjust now** button (circled in red)
- Teaching** (active tab):
 - File:** MTP Slide Adapter II
 - Default Teaching** button
 - Auto Teaching** button
 - Teach...** button
 - Movement on Sample Spot:**
 - Mode:** Off
 - Shots at raster spot:** [empty field]
 - Limit diameter to:** 2000 µm
 - ☒ **Use Target Profile**
 - ☒ **Generate On Dock-In** (with **Generate now** button)
 - ☐ **Generate On Start Imaging**
 - ☒ **Use IntelliSlides**
 - Height at Current Position** (circled in red):
 - Difference:** 3 µm
 - Z Position:** -131.75 µm
 - Check** button
 - Manual Fine Control:**
 - Rel. X:** 10 µm, **Rel. Y:** 10 µm
 - Abs. X:** 48882.5 µm, **Abs. Y:** -61550 µm
 - Advanced** button

6. When both Target Profile and Focus Adjustment have been successfully completed, a slide adapter schematic, green T and green F will appear next to the geometry.

The screenshot shows the software interface with the following details:

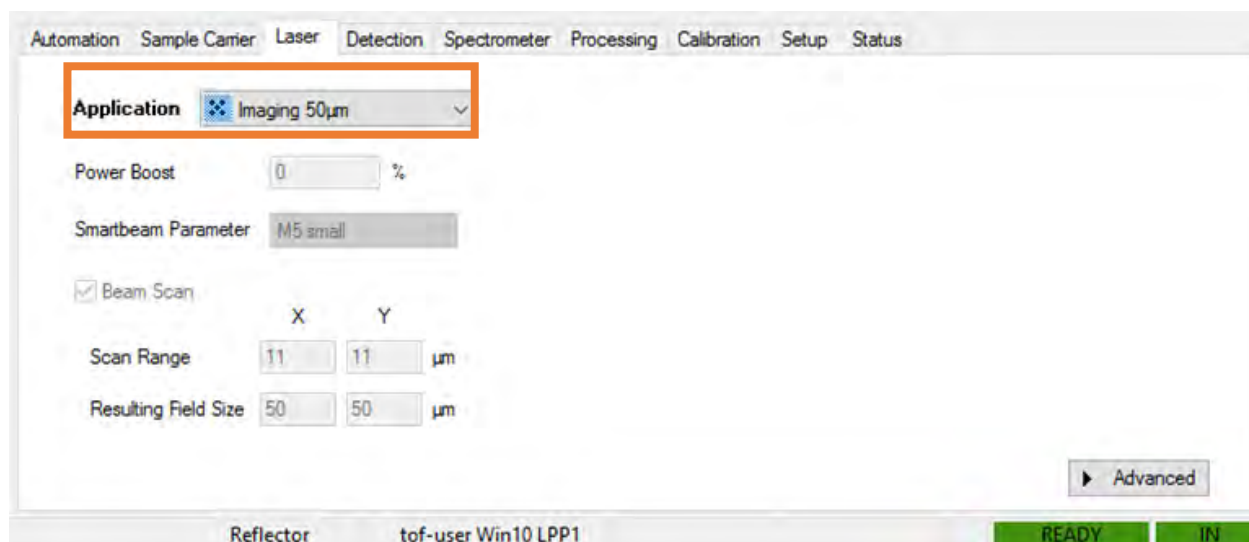
- Spot:** L11:0
- State:** IN (green bar)
- Geometry:** MTP Slide Adapter II
- Carrier ID:** T_0235380_1002001_1
- Indicators:** Green 'T' and 'F' indicators, a schematic icon, and a barcode.

* If Focus Adjustment fails, a red F will appear next to the geometry. In this case, increase the “Global Attenuator Offset” in the Set Up tab.

d) Method optimization in flexControl

Before set up imaging run, a flexControl method is needed to be **optimized, calibrated and saved**. Here are the general parameters to be checked,

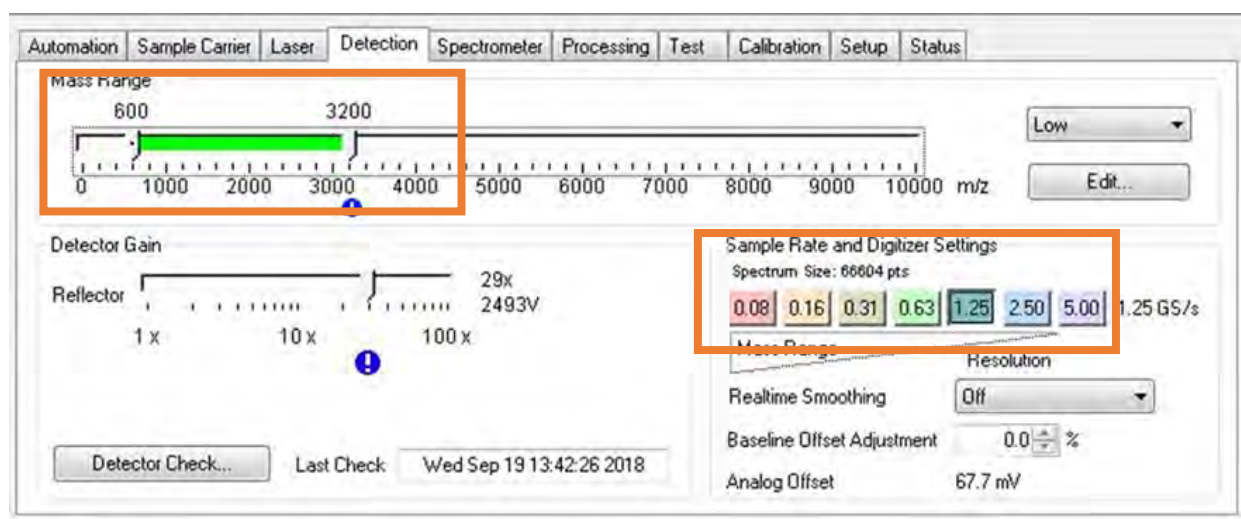
1. Check the **laser** settings, recommend to use default Imaging 20uM, 50uM, or 100uM from drop down options. The scan range and resulting field size is calculated accordingly.



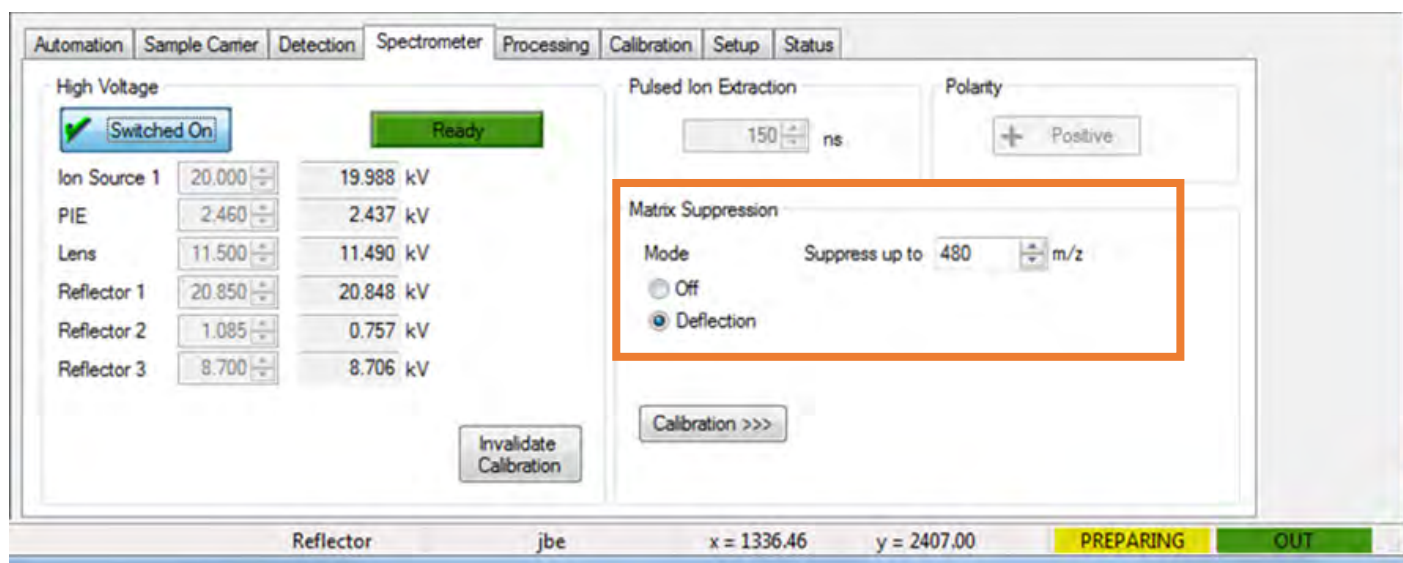
2. Detector tab

Mass Range can be adjusted based on signals got from tissue. ex, shrink it to 600-2000 Da if not much signals beyond 2000Da.

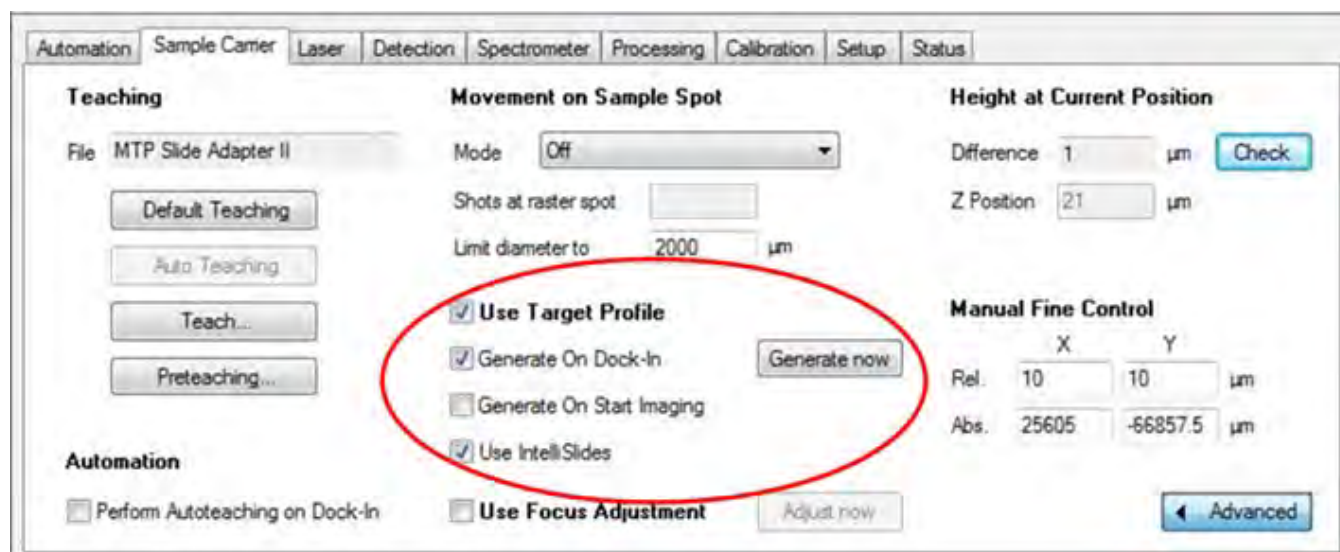
Sample rate: 1.25GS/s is recommended for Lipid Imaging, 0.63GS/s for protein Imaging.



3. Matrix Suppression, under Spectrometer tab,
Recommend 480Da cut off mass to filter major matrix ions.



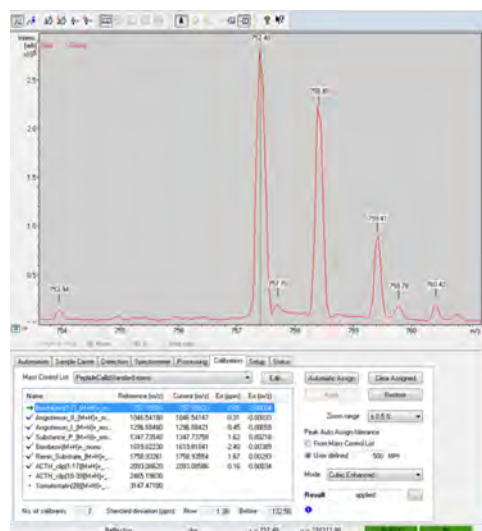
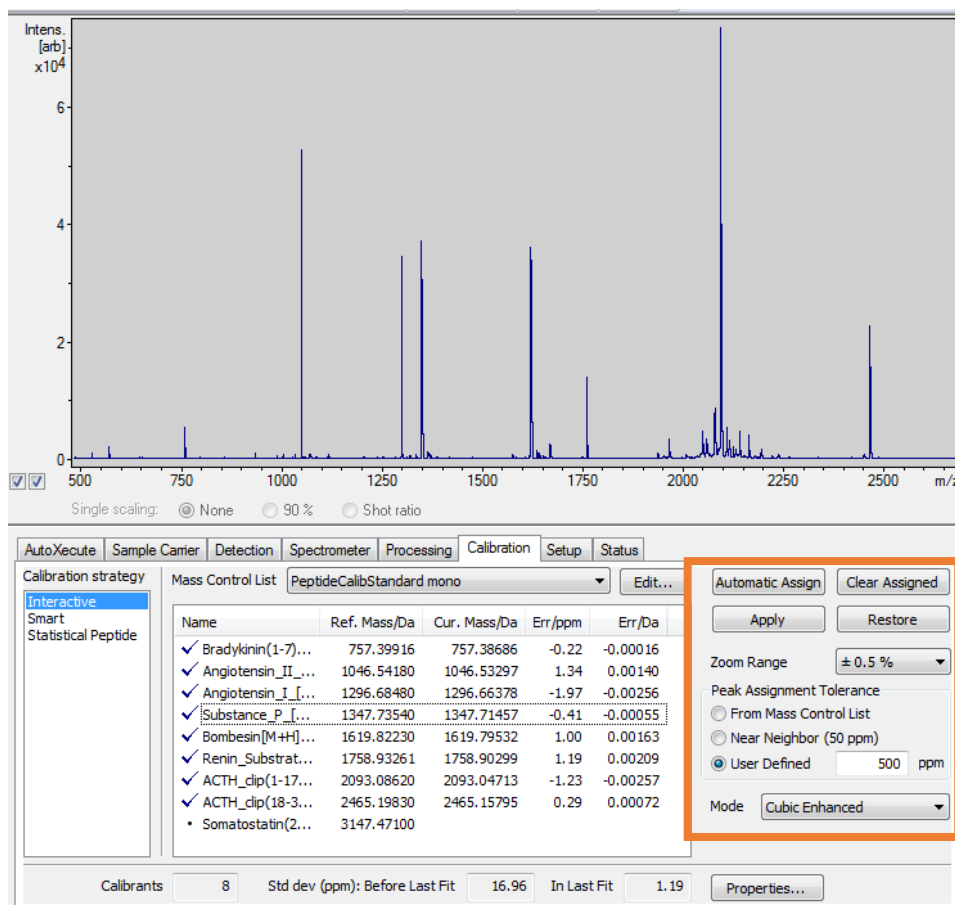
4. double check—sample carrier panel



e) Calibration in flexControl

Collect spectra from calibration standard after firing laser a few hundred shots (typically 200-500 shots). shown under **Calibration** panel, **click Automatic Assign**, Typically check **In Last Fit** result, the mass error should be less than 1.5ppm for rapifleX, ideally sub ppm. If satisfied, then **Apply**, then **save** the method under **File - Save method (Ctrl + M)** or **save as** a new method.

Example below is from Bruker peptide standards (**ordering info is on Appendix 1**),



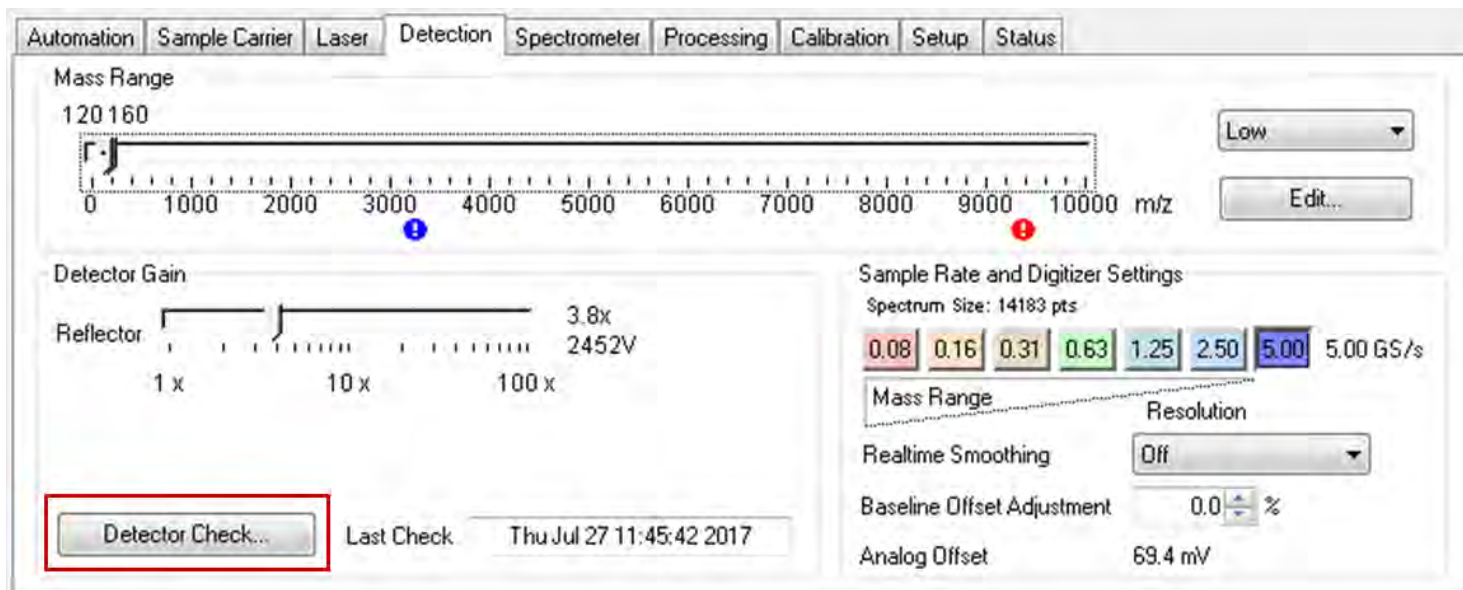
Note: Double check if the **monoisotopic peak** is labelled by click on the individual mass list under calibration tab. Correct the assignment if the wrong peak is labelled.

Note: if not satisfied, click clear assigned to label again or collect new spectra for calibration.

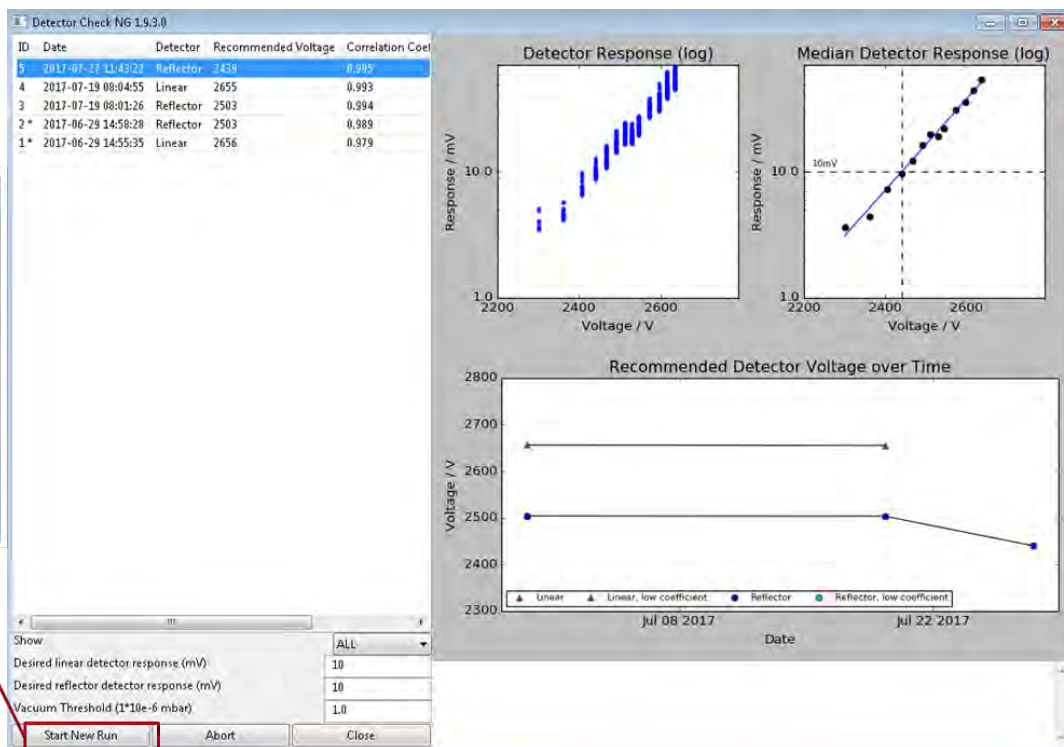
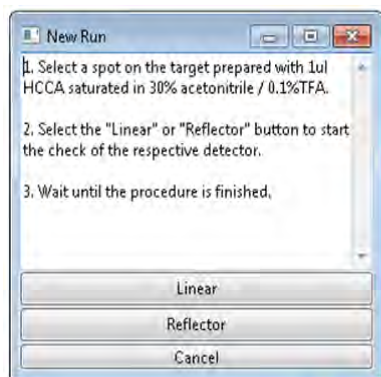
f) Detector Check

NOTE: Recommend to conduct Detector Check **once a week (heavy user)** or **once every two to four weeks (light user)** before starting the measurement.

MALDI imaging represents a challenge to the MS instrument's detector. Because of the sheer speed the rapifleX offers, it allows to acquire dramatically larger numbers of spectra per time unit compared to any other MALDI-MS system. Accordingly, rapifleX detectors are much more challenged and may degrade faster. It is, therefore, **recommended to perform a detector check before setting up a MALDI imaging acquisition sequence**, and to update the acquisition method to be used in the imaging experiment with the detector voltage and offset values determined by the automated detector check routine.

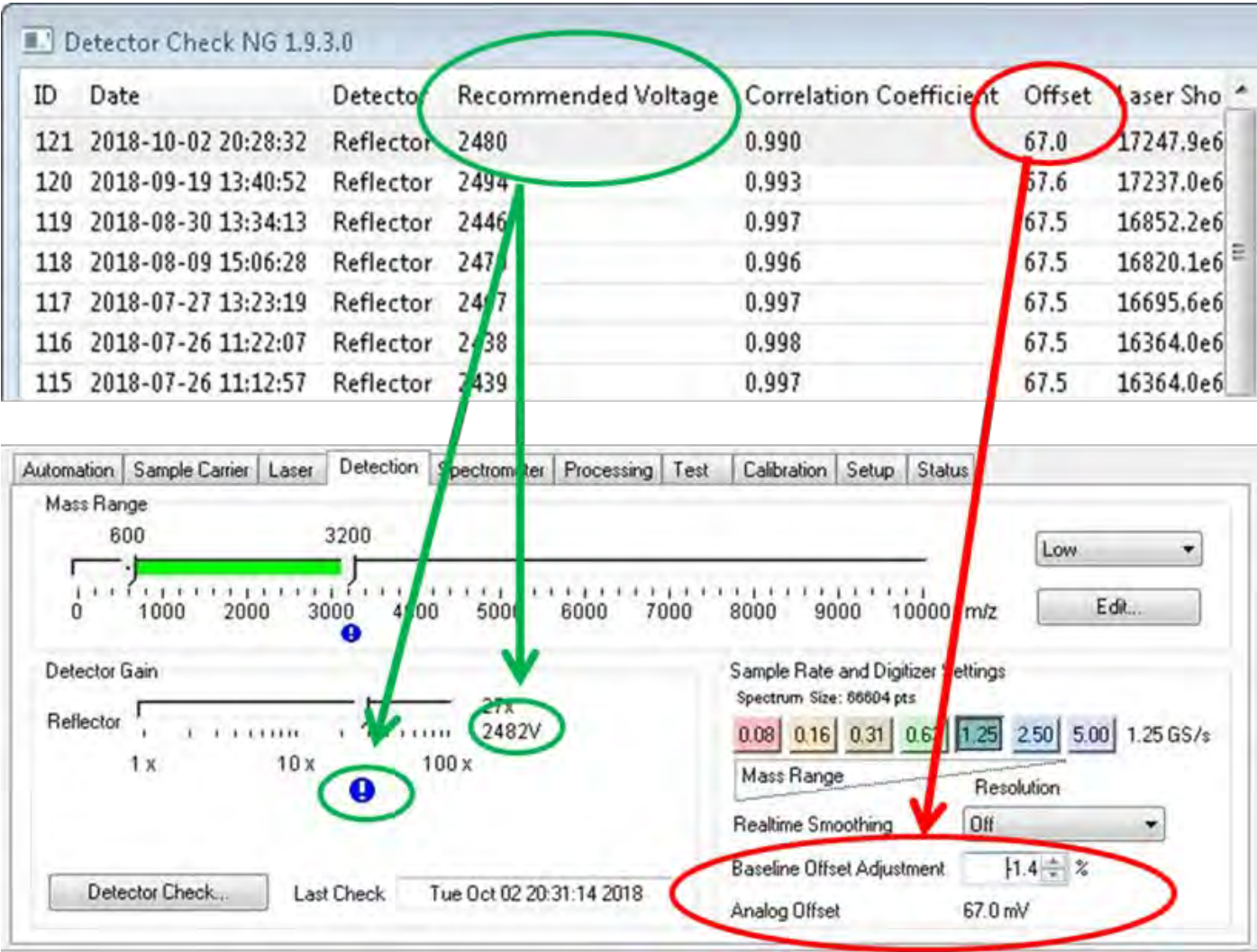


Detector check should be performed on a good quality spotting or coating with **matrix only**. ex. after coating matrix using HTX sprayer, the homogeneous matrix (off tissue) would be good choice.



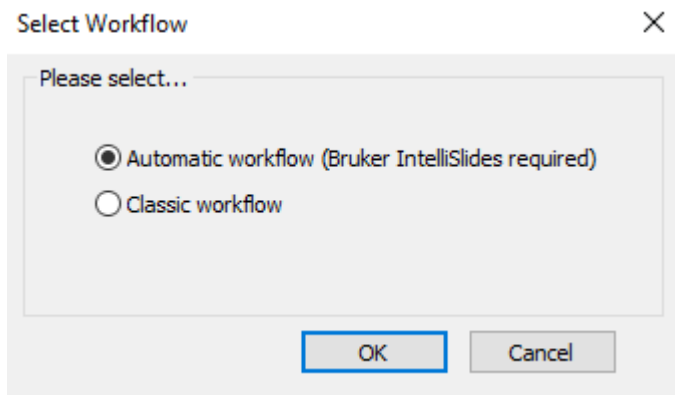
After detector check is finished, the **recommended Voltage** and **Offset values** are displayed in the result table.

Then go back to flexControl—Detector panel to adjust the two values, and saved the flexControl method. Double click on the blue exclamation point (circled), the voltage will be adjusted automatically.

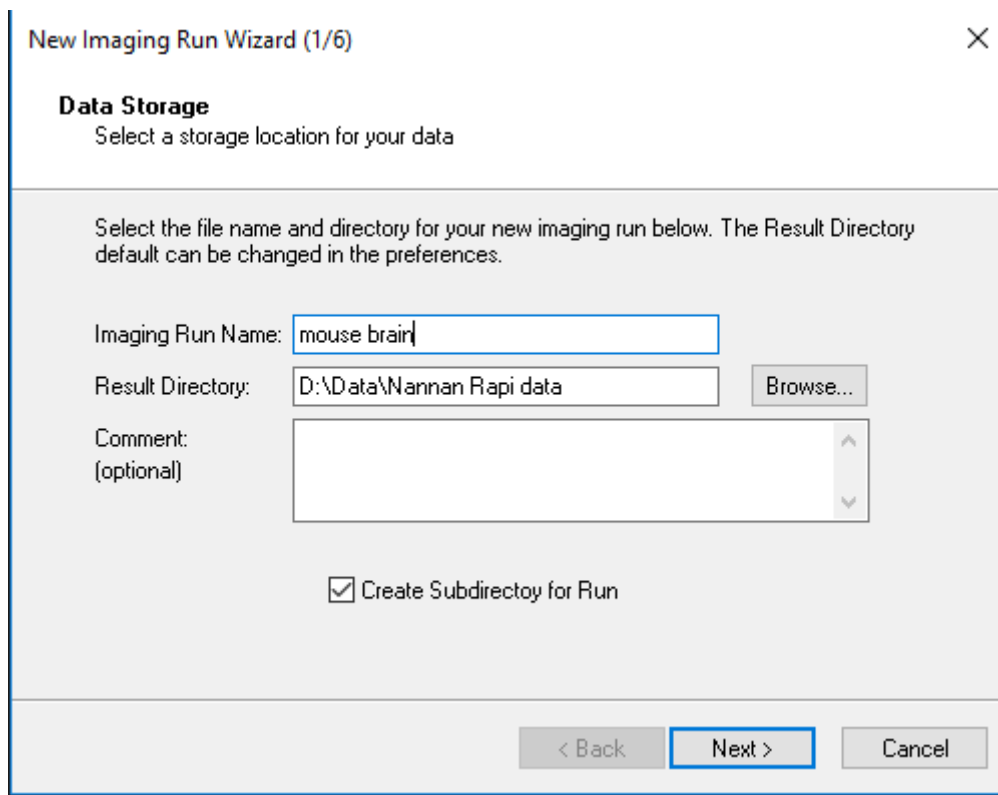


2) flexImaging Setup with IntelliSlides

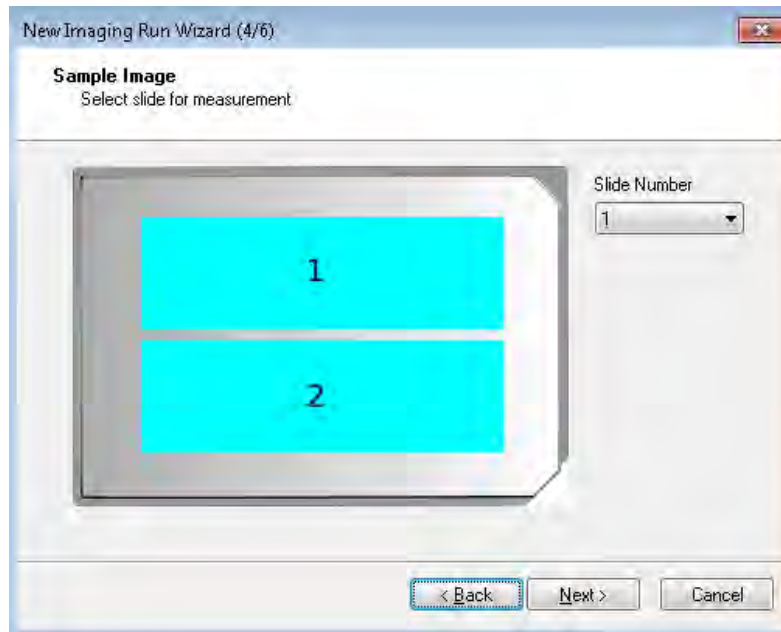
1. Make sure that flexControl is started and the optimized FC method is loaded.
2. Open flexImaging and set up a new imaging run using the New Imaging Run Wizard.
3. Select “Automatic workflow” for IntelliSlides.



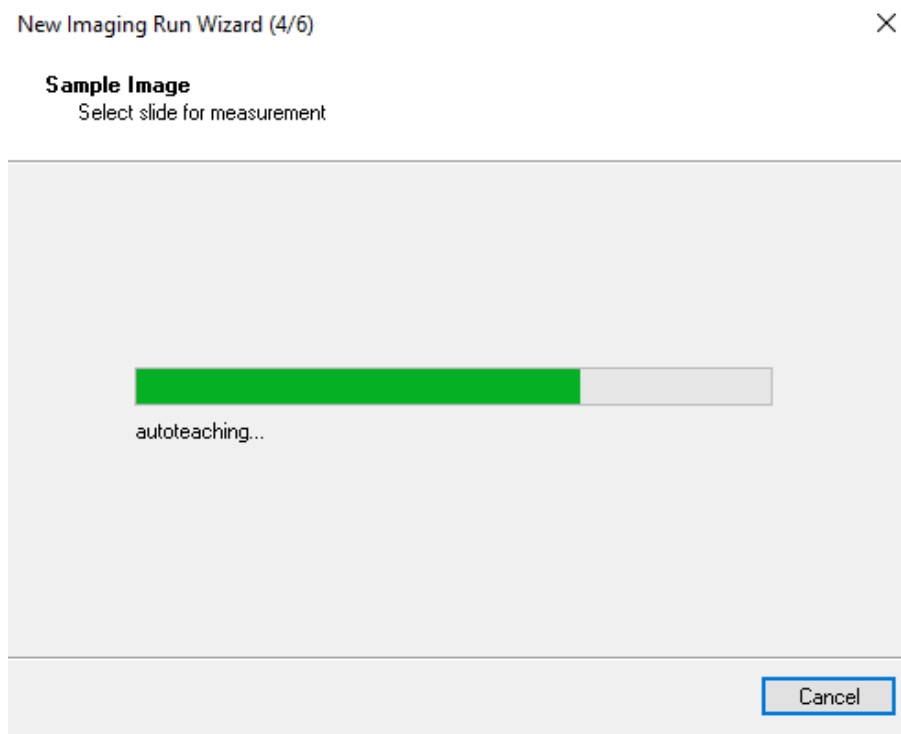
4. **Data Storage:** Name your Imaging Run Name using the following convention:
NOTE: make sure “Create Subdirectory for Run” is checked.

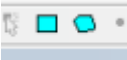


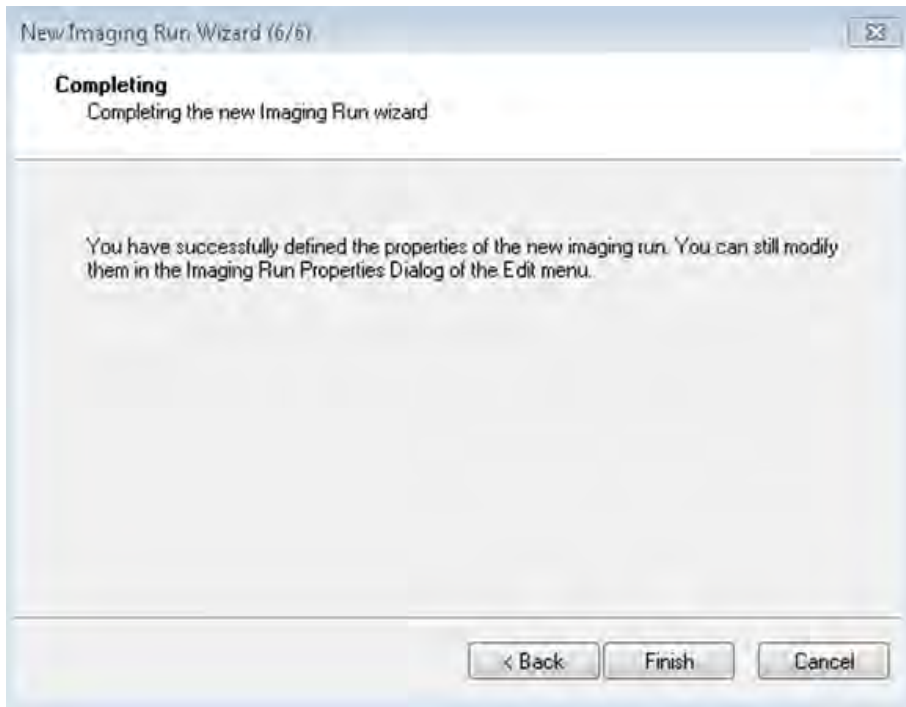
5. **Acquisition Settings:** Select “User-defined measurement regions” and set an appropriate raster width for your acquisition method.
6. **Processing Options:**
Do not change the “Reduce Number of Data Points”.
Make sure “Perform Baseline Subtraction” is checked
7. **Sample Image:** Use the drop down menu to select the position of the IntelliSlides in the MTP Slide Adapter II



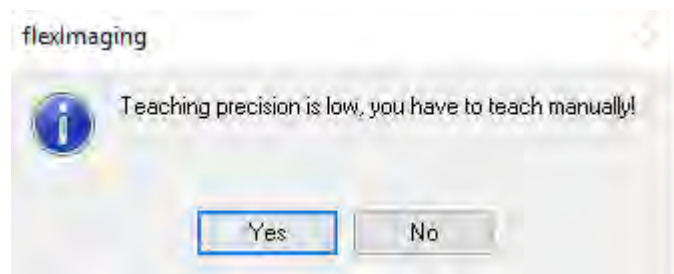
8. Before click on Next to start auto-teaching, make sure the scanned image has been saved under **D:\methods\images\import** , then click next .
9. flexImaging will now begin barcode reading, image loading, and auto-teaching, which will take up to 1-2 minutes.



10. **Completing:** When auto-teaching is complete, the flexImaging wizard will display a finish dialog  and the wizard can be closed.




In the case of inaccurate teaching, a messenger box would pop up and give the possibility to perform



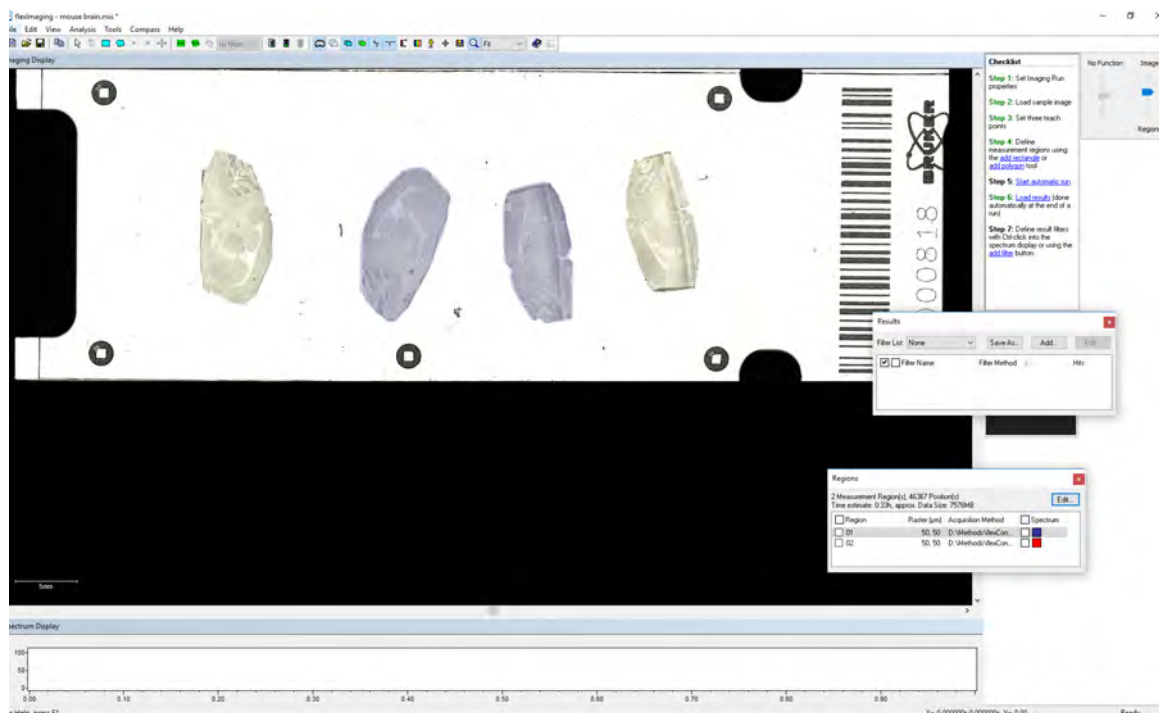
manual teaching.

11. After a successful teaching, click **Finish** button, then flexImaging would load the scanned image automatically.

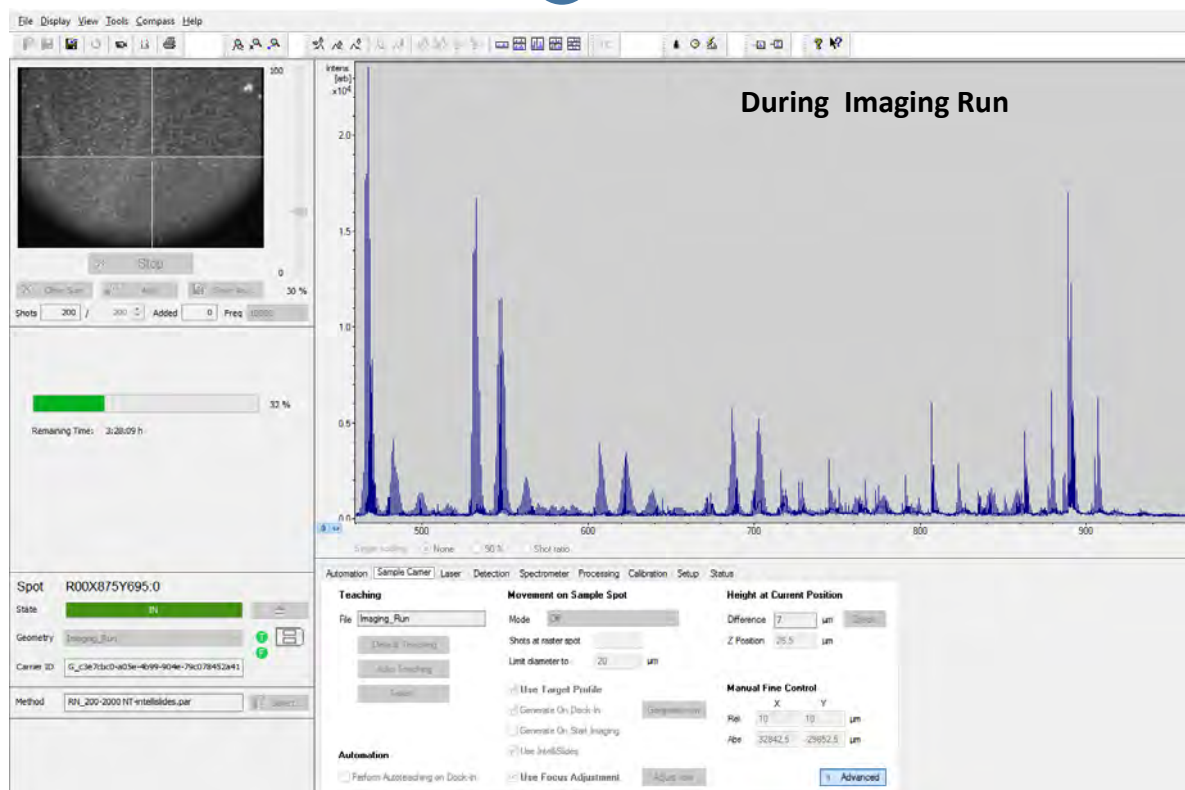
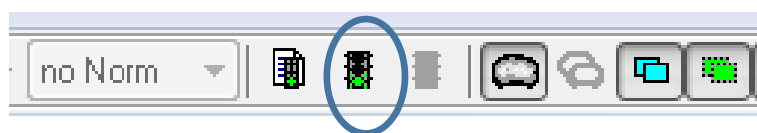
12. Select a measurement region by clicking the **square shape** or **polygonal shape**. 

On the tissue image, draw a box (Square Shape) or a polygonal shape (Polygonal Shape). When finished, the number of positions, approximate time, and storage needed for the selected measurement regions will be displayed in the Measurement Regions window, .

You can create multiple regions to run them serially with the same acquisition method and the same raster size. However, it's recommended to set up individual runs, then run all sequences as a batch to avoid generating one huge data size. (Refer to manual for **autoXecute batch runner** section.)



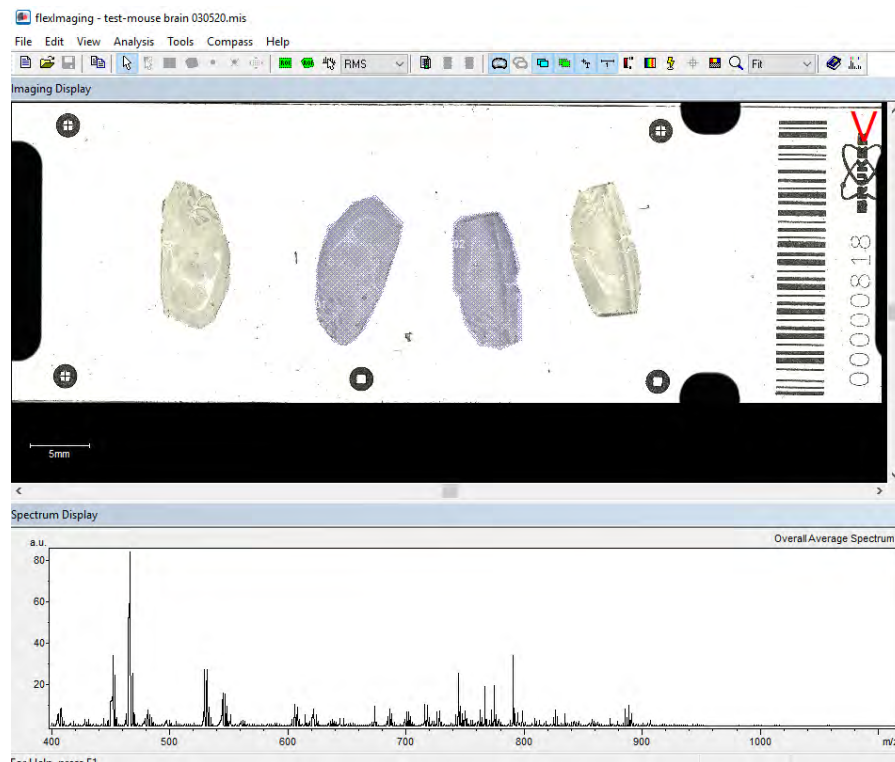
13. To start the acquisition, go to flexImaging, *click* on Green traffic light button (circled). The screen shot of during MALDI acquisition is attached below. After the run is completed, the data will automatically loaded into flexImaging . Screen shot shows the flexControl layout during acquisition, and after run is completed, the result will be loaded automatically in flexImaging.



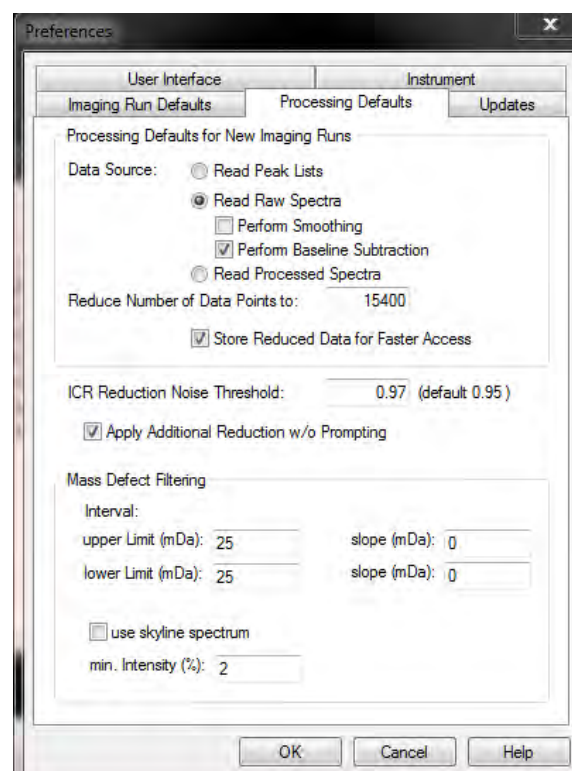
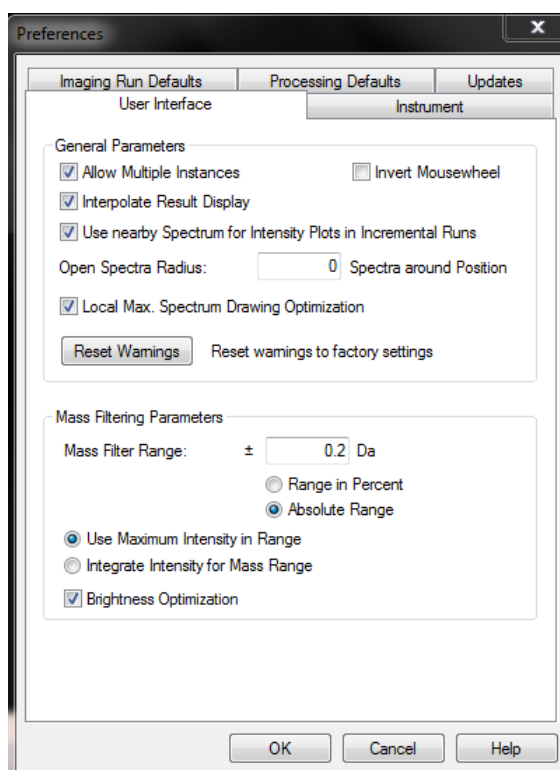
D) Analyzing data

1) Analyzing image data in flexImaging

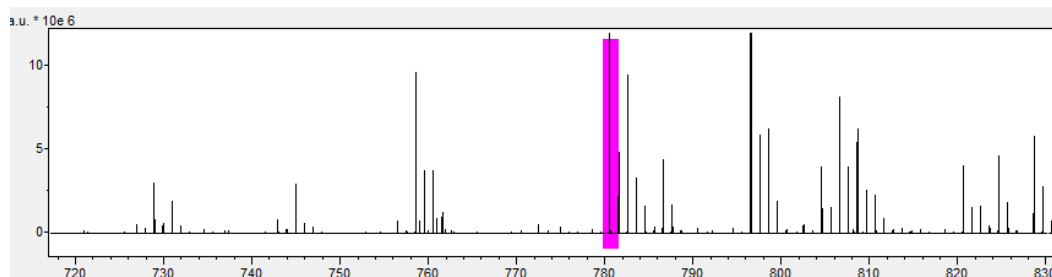
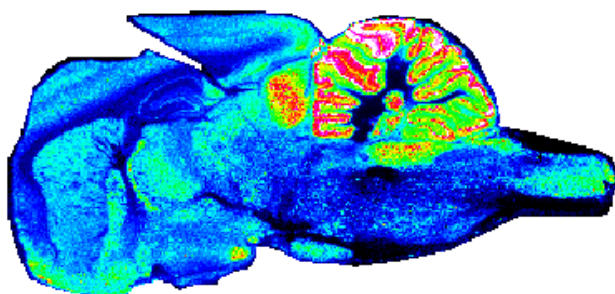
1. After acquisition is completed, result will be automatically loaded. The 'Spectrum Display' shows the average spectrum over the entire run.



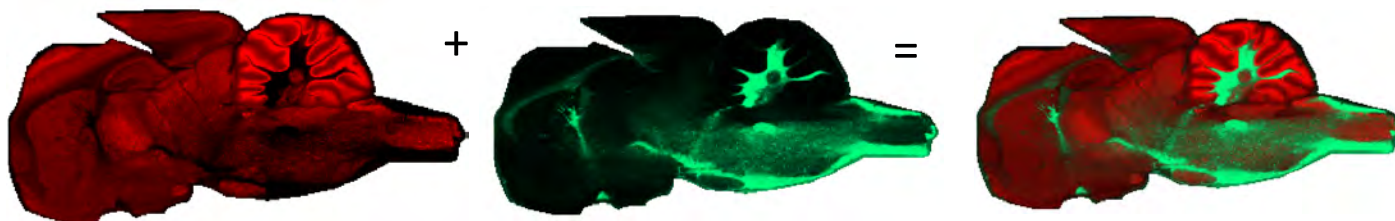
2. **Tools/Preference/User interface** , **Mass filter range** needs to be adjusted to **+ - 0.2** (Absolute Range).
3. **Edit /Imaging Run Properties**– recommend to check **baseline subtraction**



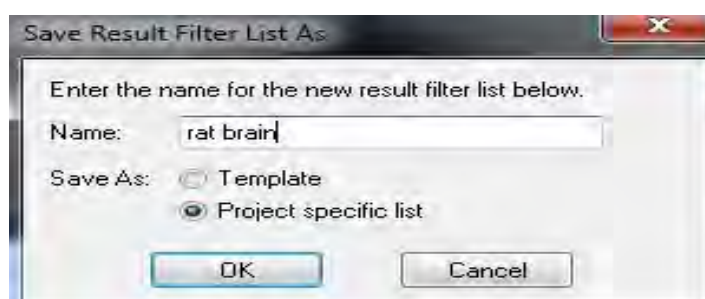
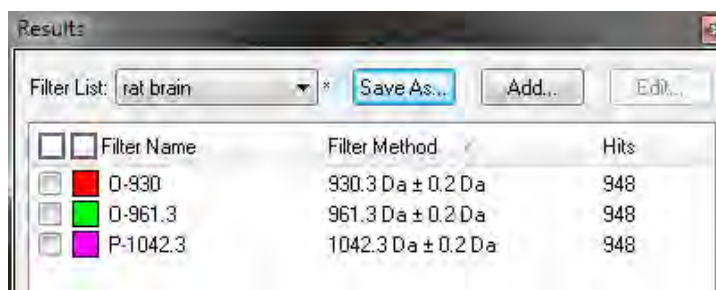
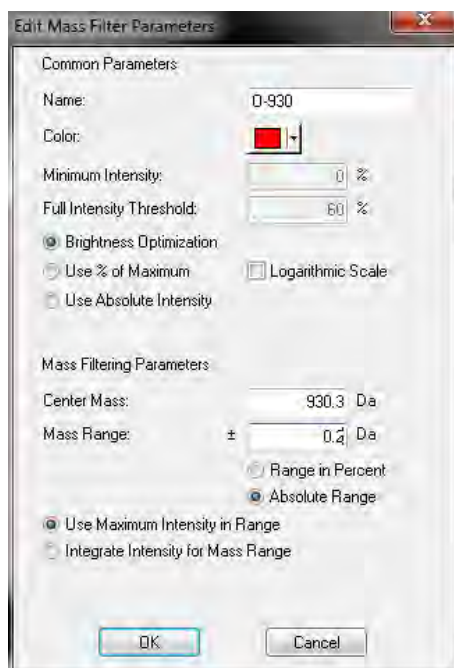
3. In Zoom in mode (*right click* on spectra area then select zooming), with left *click+ ctrl* button, individual image of the selected mass will show. In Mass filter selection mode (*right click* on spectra area then select **Mass Filter Selection**), ctrl button does not needed to be held to show ion images.



4. Right click to add particular peaks to the **mass filter list**. Or *Ctrl + M* as short cut. You can choose color, intensity threshold, define names, and mass range etc. Selected mass list can be viewed under VIEW-windows-Results. Individual ions or combined ions can be viewed.



5. The result can also be saved for future reference. **Template** means the list will be available to all imaging projects. **Project specific** list means only visible to this imaging run. Choose the saved mass filter list under dropdown options .



2) Analyzing data in **SCiLS Lab**

This is only workflow overview. A more detailed manual can be found under **help**. There are helpful video tutorials on SCiLS website, <https://scils.de/mediacenter/>

SCiLS Lab Support: support@scils.de



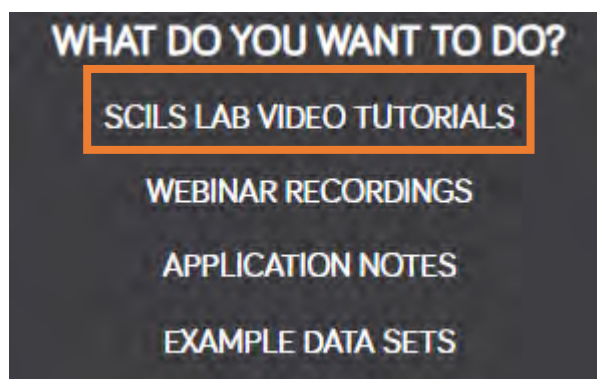
SCiLS CLOUD

PRODUCTS

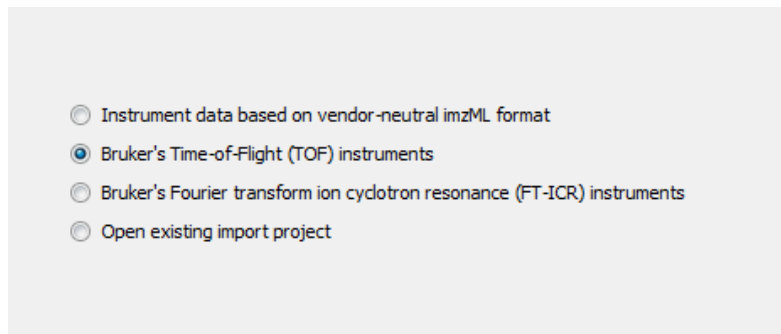
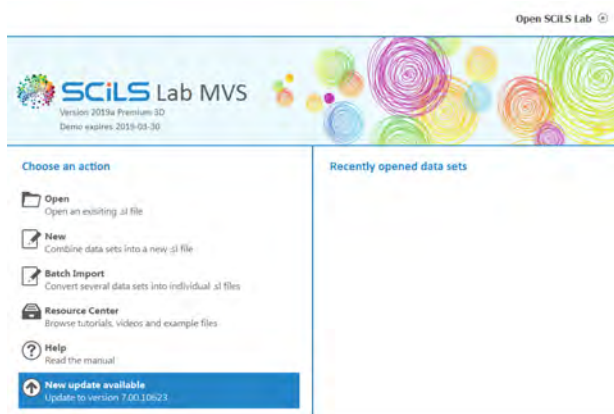
DOWNLOAD

MEDIACENTER

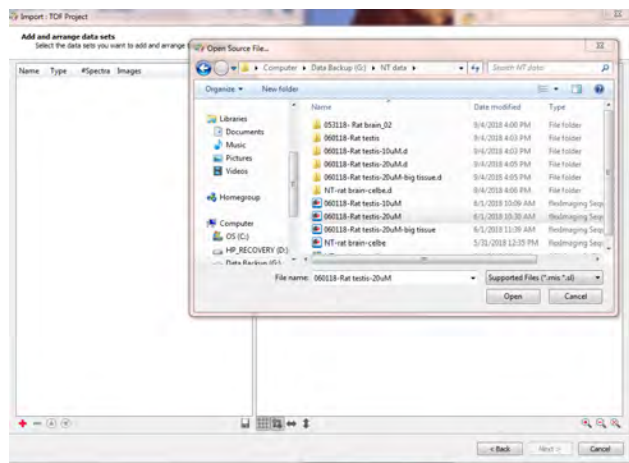
NEWS



a) How to Import data into SCiLS Lab

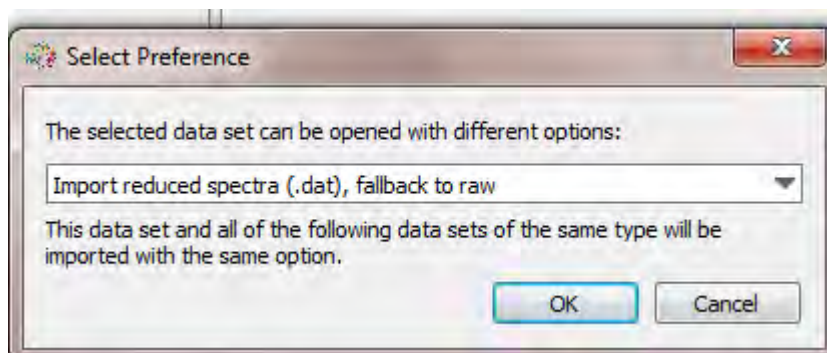


Choose **NEW** to import flexImaging data to SCiLS Lab, then **TOF** instrument. Choose **OPEN** to load existing SCiLS Lab data to continue data analysis.

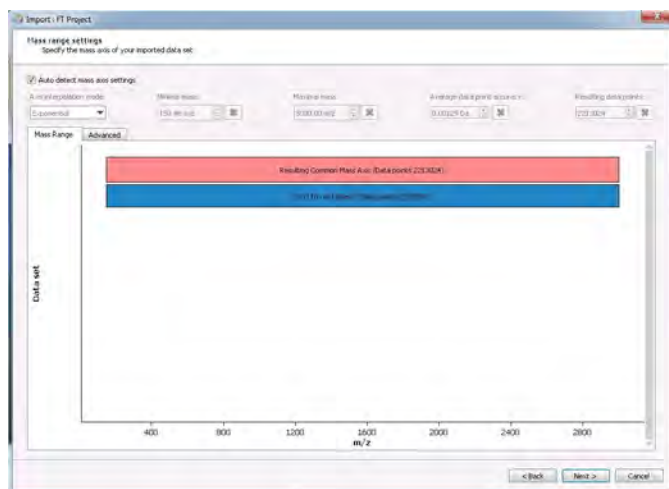


Click **+** on the bottom left corner to choose a data set to load.

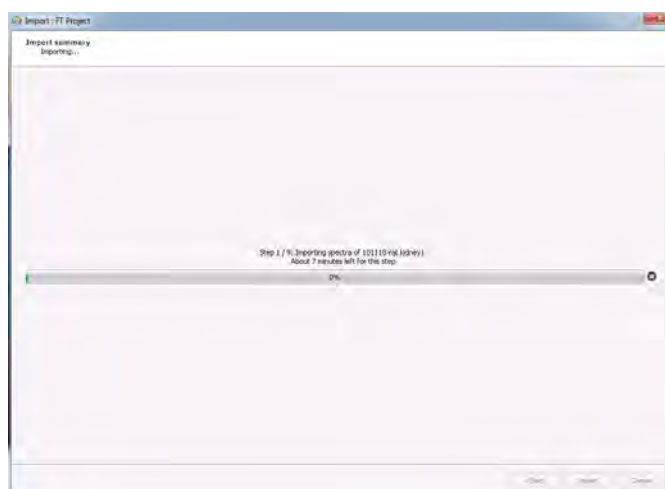
If multiple sets need to load for comparison, click **+** to add more to load all together.



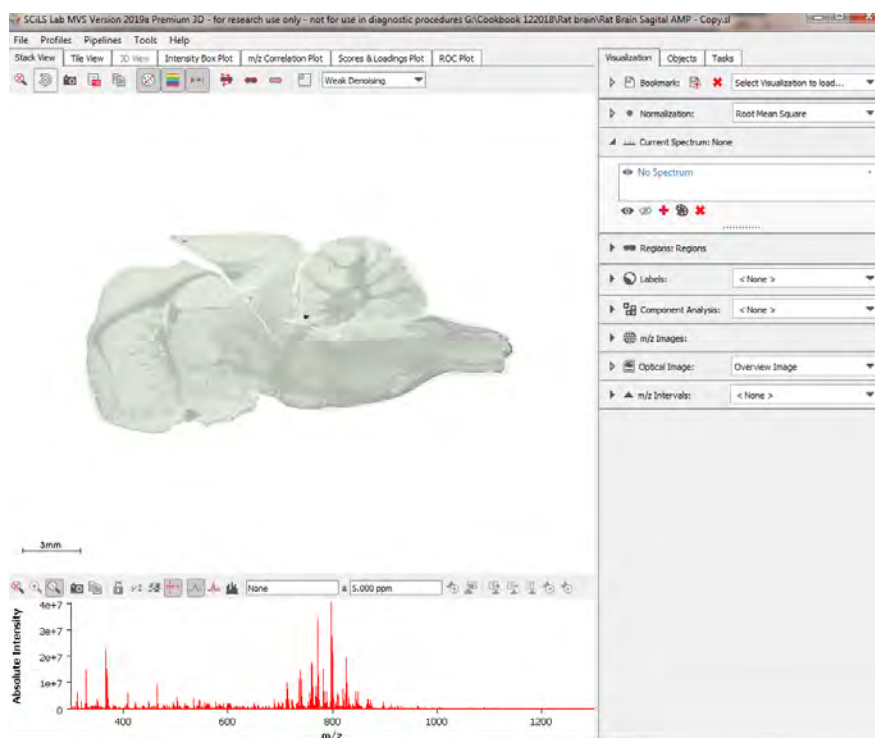
Choose **IMPORT** reduced spectra for data import,



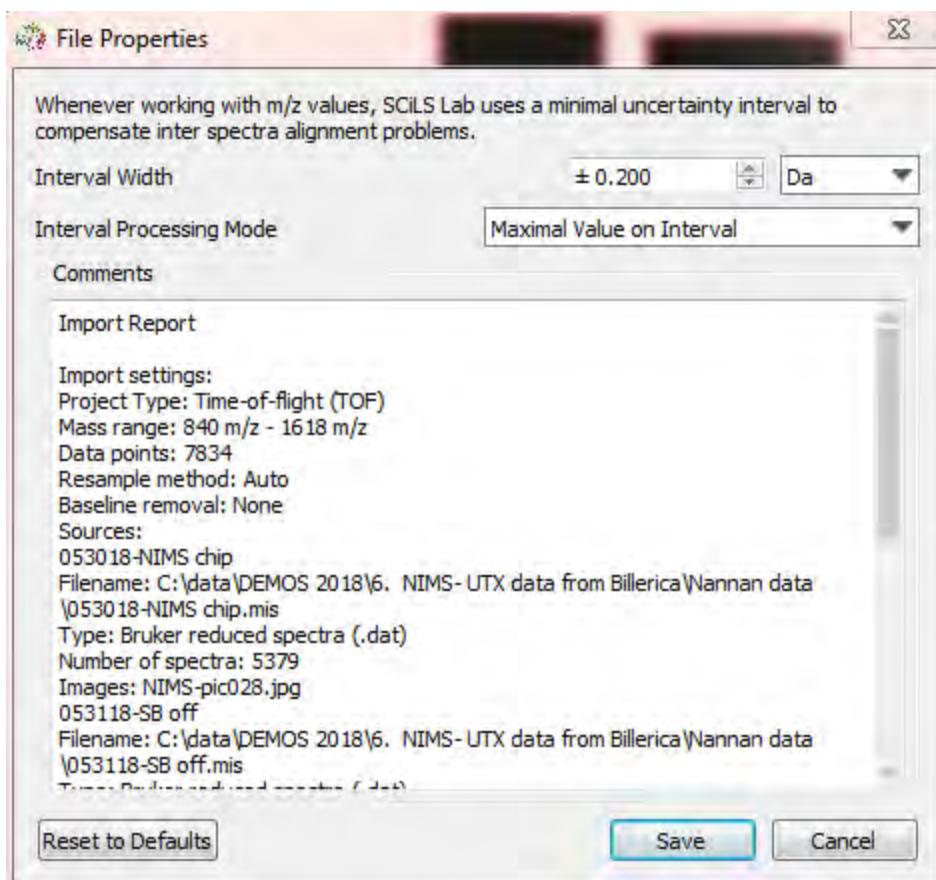
Click **OK** to import data. If multiple dataset were loaded, the common mass range will be used unless specified with certain range.



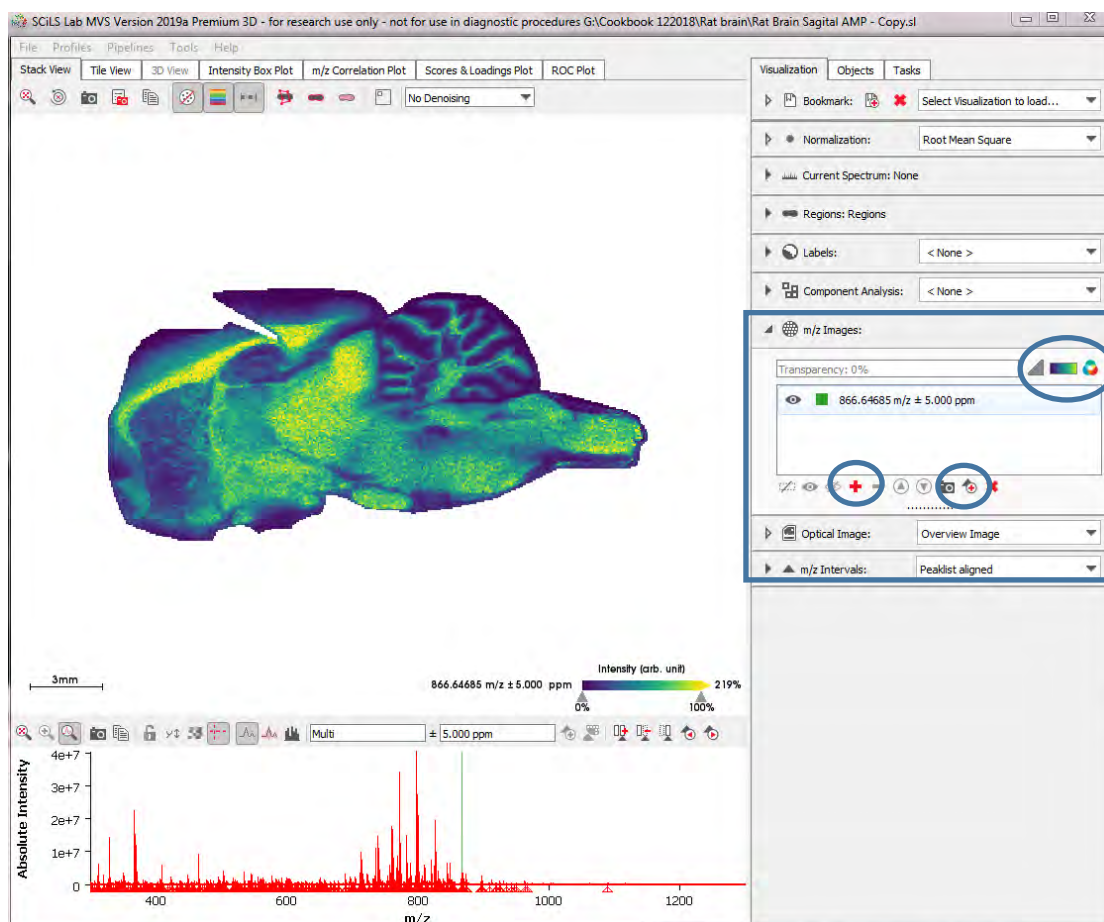
Depends on the size of the data and speed of the computer, the import process can take from seconds , minutes to hours (very rare) to complete.



Note: Need to check Settings in **File\ File Properties**
set interval width $\pm 0.2\text{Da}$ or bigger based on data quality.



b) How to generate ion Images



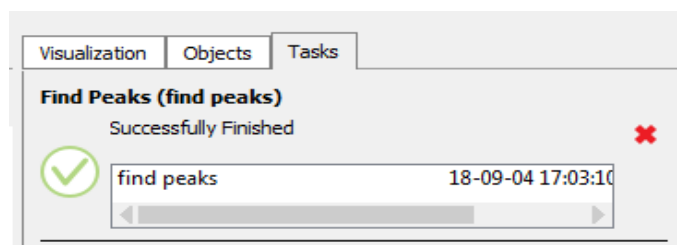
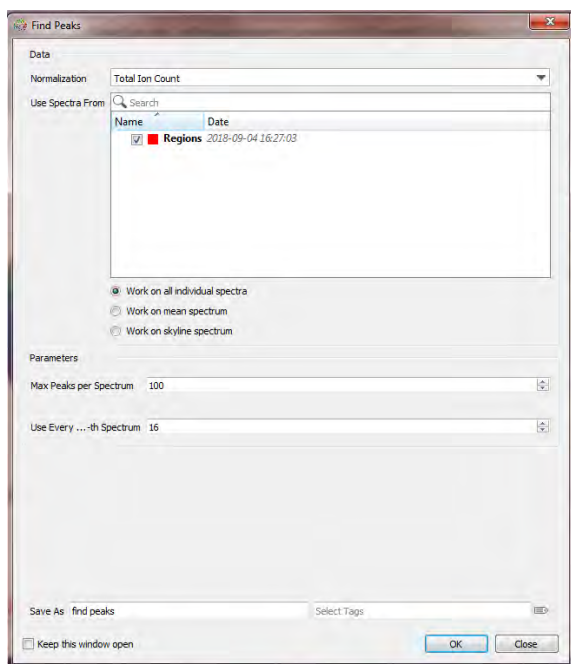
Choose **Normalization** method. **RMS normalization** is recommended for SQLite data.

Shift+ left mouse click to display an ion density image in the 'Stack View' window.

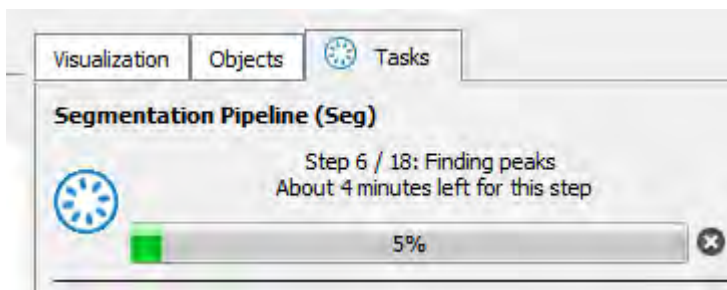
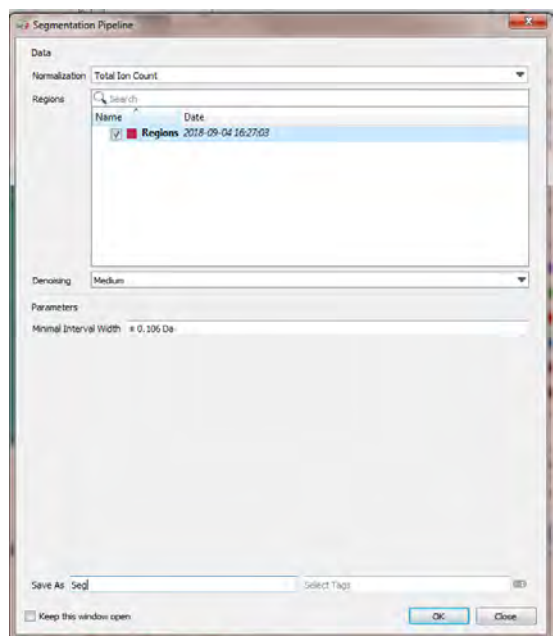
Mass of interests can be added in m/z images panels on the right by click + button.

Repeat to add multiple ions. The list can be saved for future reference by clicking the 'triangle with plus' icon.

Various Ion colors/design can be changed by clicking the color bar (circled)

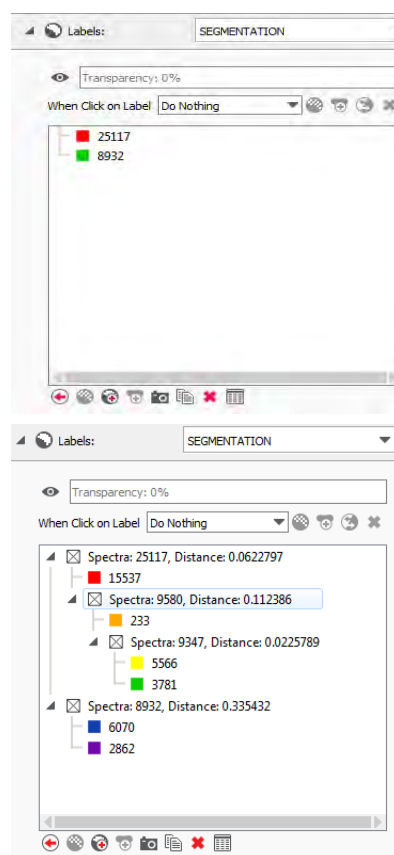
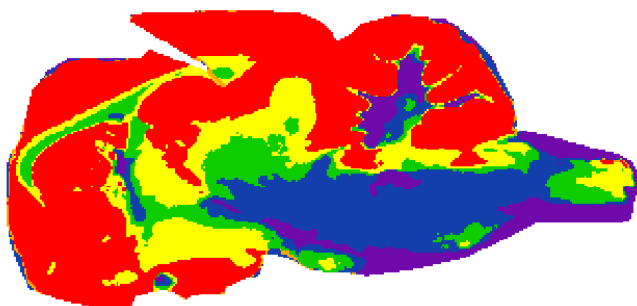


c) How to generate a segmentation Map



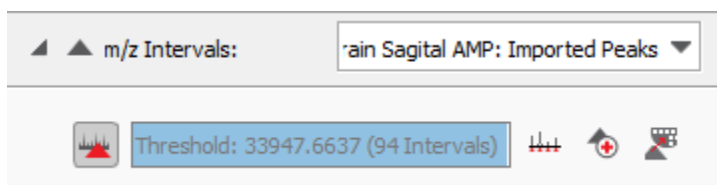
Click **Tools** – **segmentation** – choose regions, denoising (weak or medium), interval width (is usually specified before - see b) , and specify a name of project. Then click **OK** to start.

Under **Tasks** panel, you can see the progress. Typically it takes several minutes depending on the size of the dataset,. After it's finished, under labels, choose segmentation map , you should see 2 groups, then double click on RED or GREEN squares to further separate the regions based on their similarity.

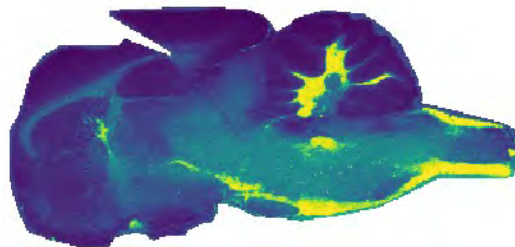
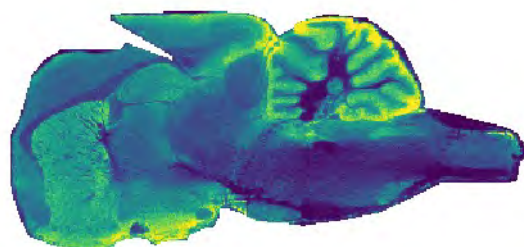
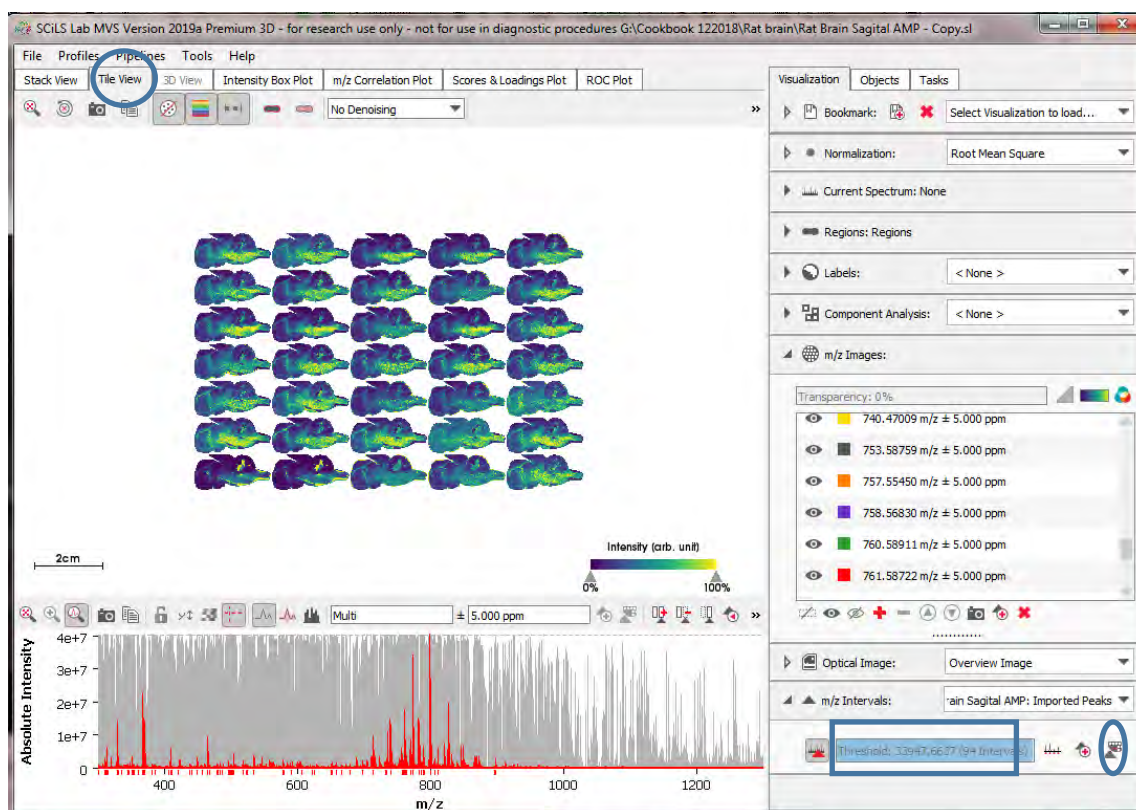
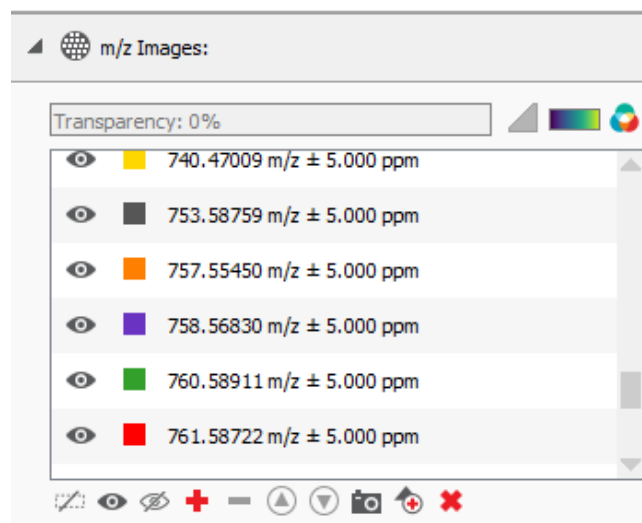


There's no limit how many groups you can create. You can easily click on X to go back to last step. You can also save the final tree view. Once saved, you can choose the new one from drop down options under labels, then you would be able to modify each label name and colors. TOP is only 2 groups, BOTTOM is after expanding.

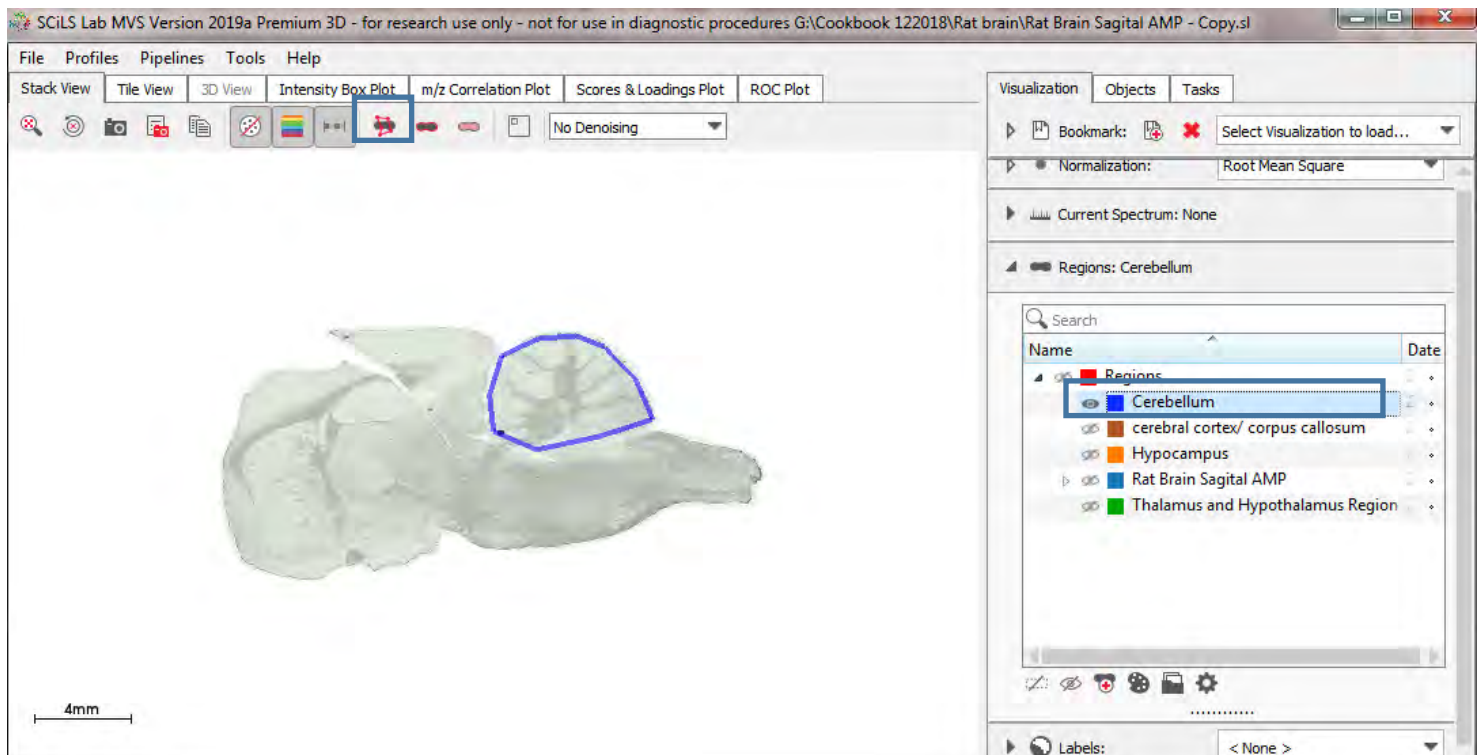
Cluster groups can be also saved as new region by selecting the desired label and then clicking the 'Save as a new region' button. This might be useful, if you wish to find Co-Localized m/z values. Cluster groups can be also saved as new region by selecting the desired label and then clicking the 'Save as a new region' button. This might be useful, if you wish to find Co-Localized m/z values.



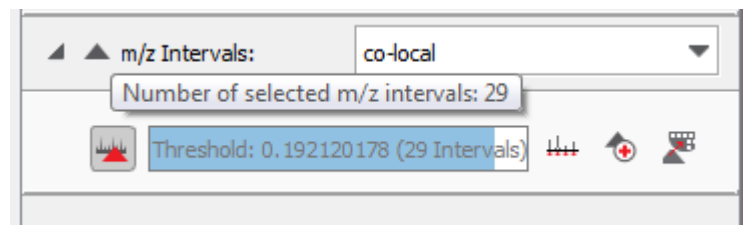
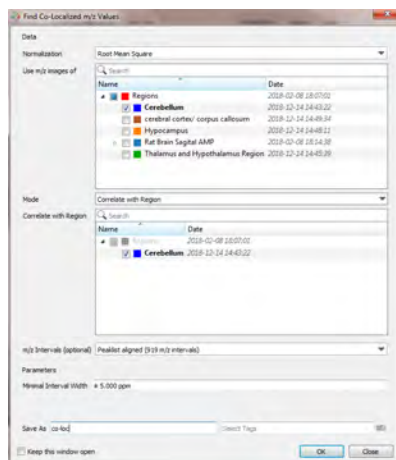
if multiple ions are loaded, then several ion images can be viewed in parallel under the 'Tile View' tap.



d) How to find Co-localized m/z values



Click 'Create new polygonal region' button to create an irregular area, then save the region with a name.

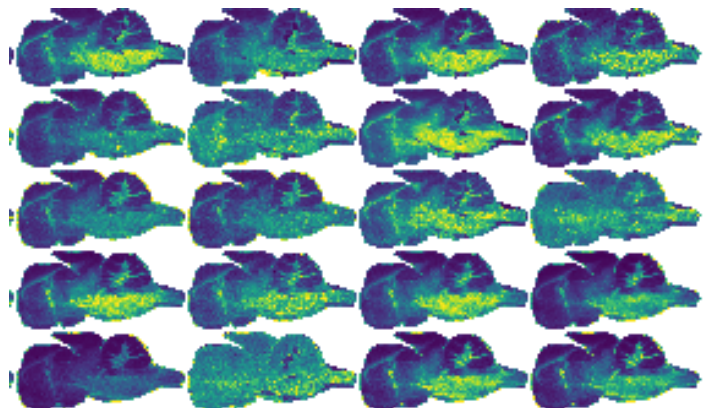


Then choose Co-local result, and select threshold, and in-tervals. You can add to the m/z images to view result. You can also switch values to negative, and add more intervals.

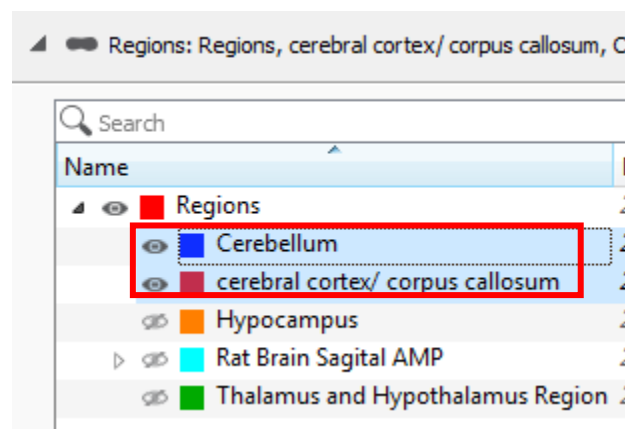
Choose tile view or stack view to see images.

Click 'Tools' tab and then 'Find Co-Localized m/z Values'. A wizard opens in which you can select your desired Co-Localization options. To find m/z-images that correlate with the newly defined Region of Interest (ROI), select a superordinate region to use the m/z images of it, as 'Mode' > 'Correlated with Region' and the ROI as the 'Correlate with Region' option. m/z Intervals option is optional.

Note: Co-Localization can be also done by selecting a specific m/z value and finding images with similar distributions.



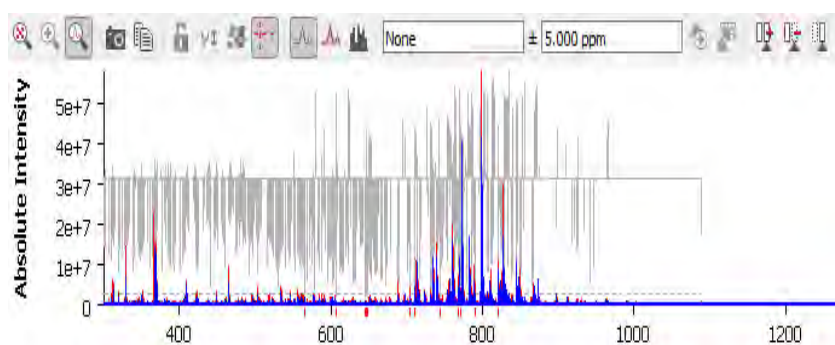
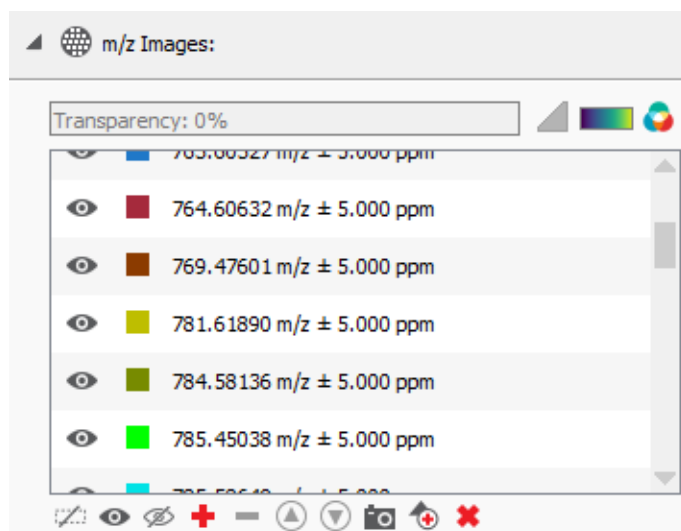
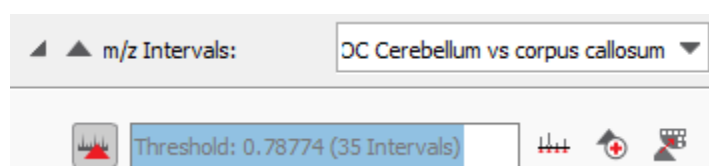
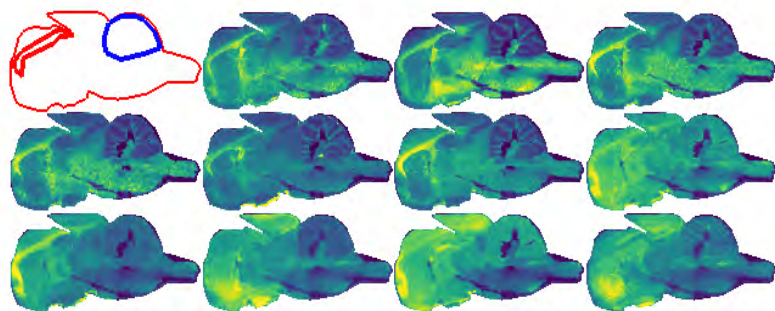
e) How to do Receiver Operating Characteristic (ROC) analysis calculation (SCiLS Lab Pro version)



Create TWO regions for comparison. You can save different segments out of the segmentation map as regions first, then use ROC to comparison. In this example, Red (cerebral cortex) is compared with Blue (Cerebellum).

1. Select 'Tools' and then 'Find Discriminative m/z Values (ROC)'.
2. Select two classes for which you would like to find discriminating m/z-values for as 'Class 1' and 'Class 2'.
3. You can optionally define a m/z Interval.
4. You can use a random subset of spectra for the calculation under 'Spectra to Work on' and define the amount of spectra in the 'Subset size'.
5. Specify a name for the result in the 'Save As' field.

Select threshold (values with high discriminating power close to 0 or 1. An AUC value of 0.5 indicates that the respective m/z value does not discriminate the two groups.



f) How to do Component Analysis (SCiLS Lab Pro version only)

part 1: PCA

Click Tools - Component Analysis to open parameter window.

Example shows a dataset has been processed using PCA (Principal Component Analysis) algorithm with 3 components only. whole region and weak denoising were selected with +- 5ppm internal width. Result will be shown under Component Analysis tab.

Component Analysis

Data

Normalization

Root Mean Square

Regions

Search

Name	Date
<input checked="" type="checkbox"/> Regions	2018-02-08 18:07:01

☒ Work on all individual spectra

☐ Work on all mean spectra

☐ Work on all skyline spectra

m/z Intervals

Peaklist aligned (919 m/z intervals)

Parameters

Denosing

Weak

Method

PCA

Scaling

Unit variance

Number of Components

3

Minimal Interval Width

± 5.000 ppm

Save As

Specify name for result

Select Tags

☐ Keep this window open

OK

Close

Component Analysis: PCA

Transparency: 0%

☒ Component 1

☐ Component 2

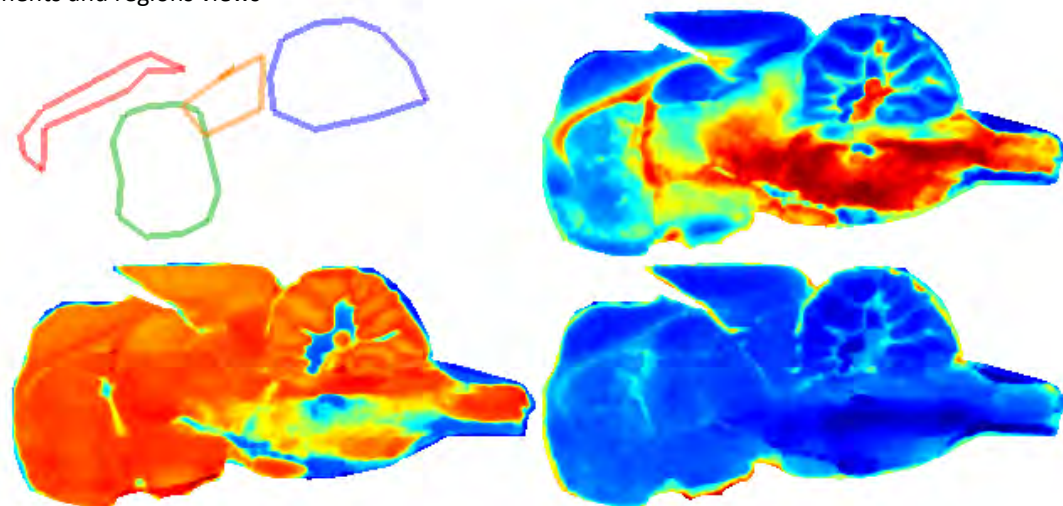
☐ Component 3

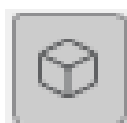
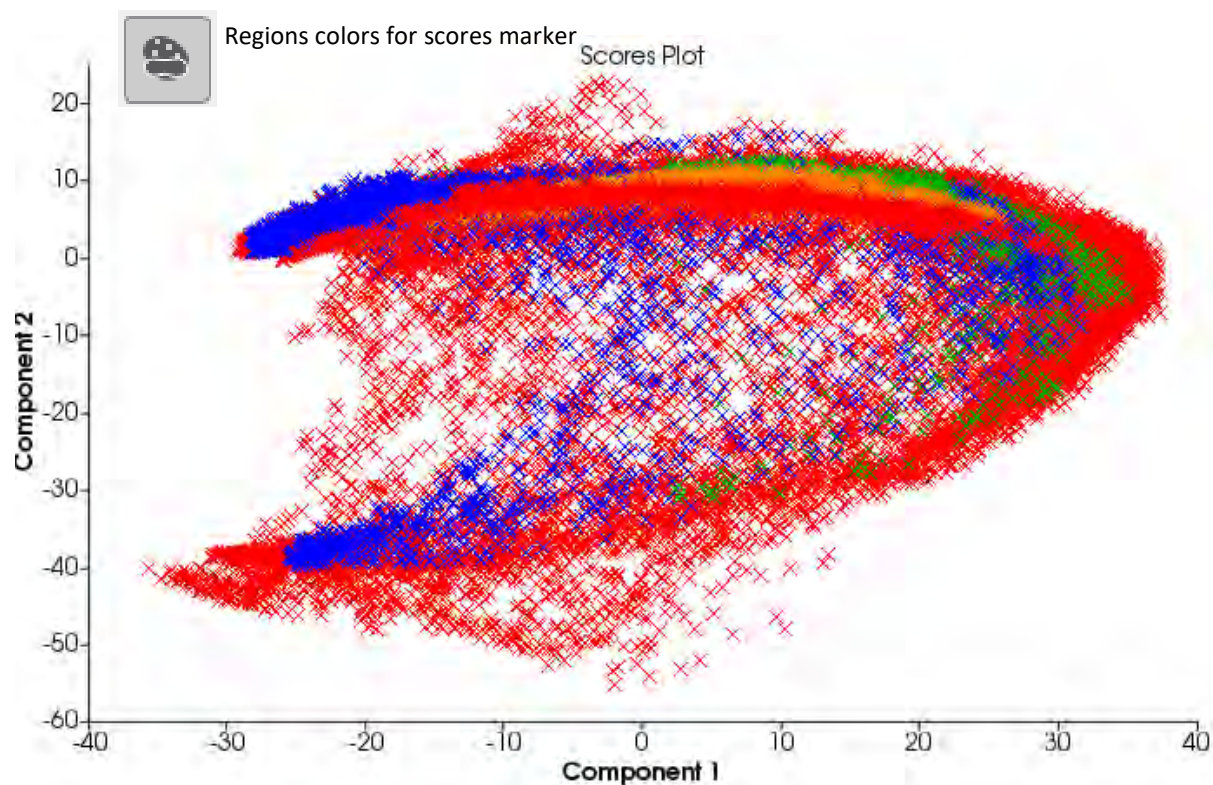
Regions: Regions, Thalamus and Hypothalamus Region, Cereb

Search

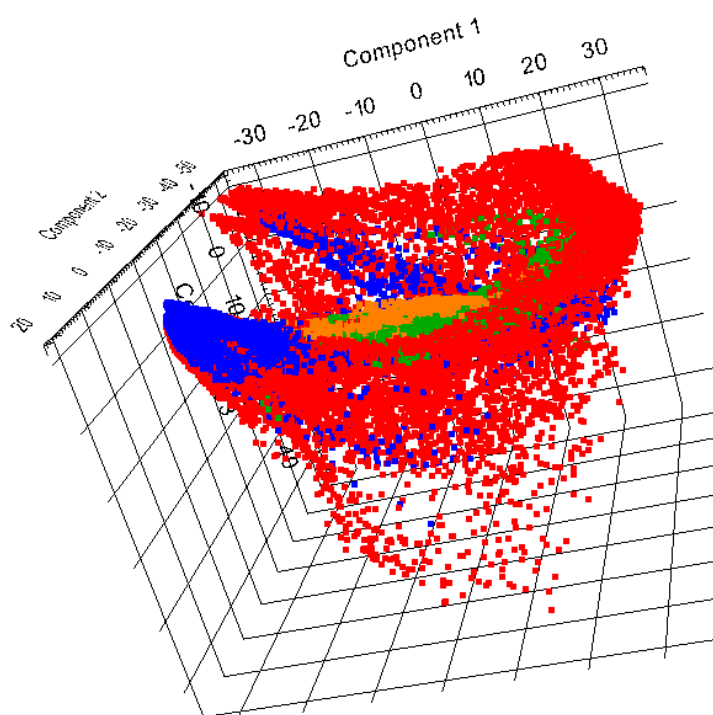
Name	Date
<input checked="" type="checkbox"/> Regions	201
<input type="checkbox"/> Cerebellum	201
<input type="checkbox"/> cerebral cortex/ corpus callosum	201
<input type="checkbox"/> Hypocampus	201
<input type="checkbox"/> Rat Brain Sagittal AMP	201
<input type="checkbox"/> Thalamus and Hypothalamus Region	201

Tile View shows 3 components and regions views





3D view



PCA (or pLSA) is a tool to reduce multidimensional data to lower dimensions while retaining most of the information. Each datapoint in the scores plot represents a spectrum. Each color represents one region. The 2D plot (TOP) uses the first 2 component as axis, while 3D plot (Bottom) uses the first 3 components.

e) How to do Component Analysis (SCiLS Lab Pro version)

part 2: pLSA

Click Tools - Component Analysis to open parameter window.

Example shows a dataset has been processed using pLSA algorithm with 5 components only. whole region and weak denoising were selected with +/- 5ppm internal width. Result will be shown under Component Analysis tab.

Component Analysis

Data

NormalizationRoot Mean Square

Regions

Search

Name

Date

☒

Regions

2018-02-08 18:07:01

☒ Work on all individual spectra

☐ Work on all mean spectra

☐ Work on all skyline spectra

m/z IntervalsRat Brain Sagital AMP: Imported Peaks (9 m/z intervals)

Parameters

DenoisingWeak

MethodpLSA with random initialization

ScalingNone

Number of Components5

Minimal Interval Width± 5.000 ppm

Save AsSpecify name for result

Select Tags

☐ Keep this window open

OK

Close

Component Analysis:PLSA

Transparency: 0%

☒ Component 1

☐ Component 2

☐ Component 3

☐ Component 4

☐ Component 5

Regions: Regions, Thalamus and Hypothalamus Region, Cerebellum, cerebral cortex/ corpus callosum, Hypocampus, Rat Brain Sagital AMP, Thalamus and Hypothalamus Region

Search

Name

Date

☒

Regions

2018-02-08 18:07:01

☐

Cerebellum

2018-02-08 18:07:01

☐

cerebral cortex/ corpus callosum

2018-02-08 18:07:01

☐

Hypocampus

2018-02-08 18:07:01

☐

Rat Brain Sagital AMP

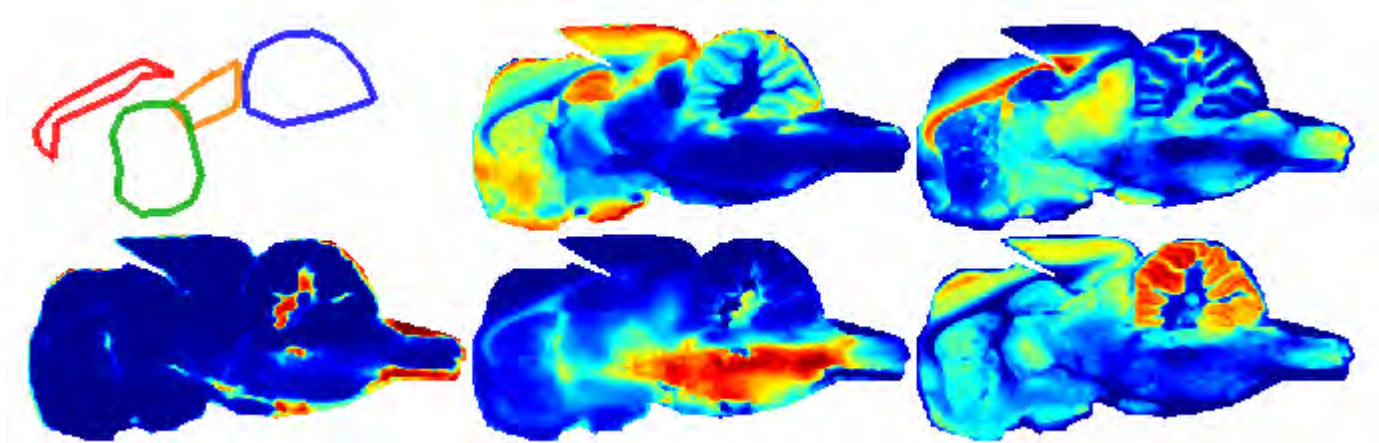
2018-02-08 18:07:01

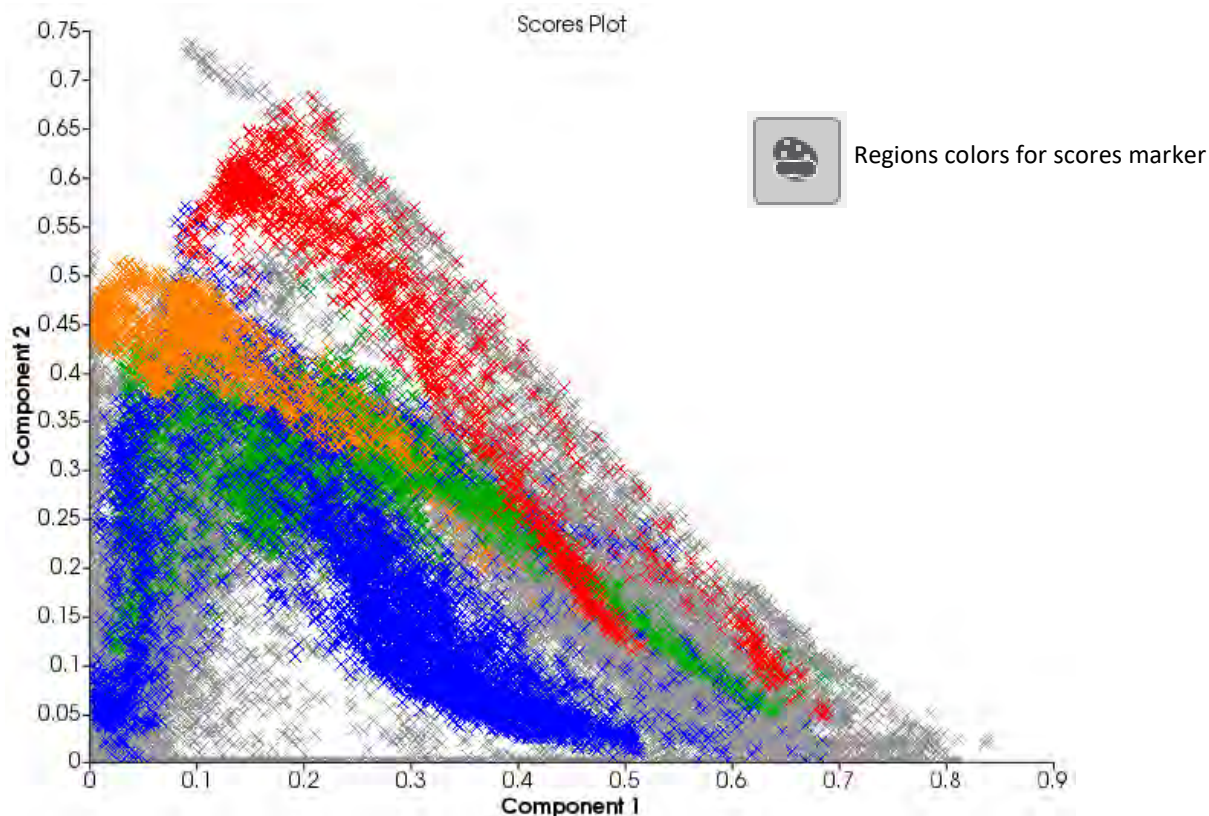
☐

Thalamus and Hypothalamus Region

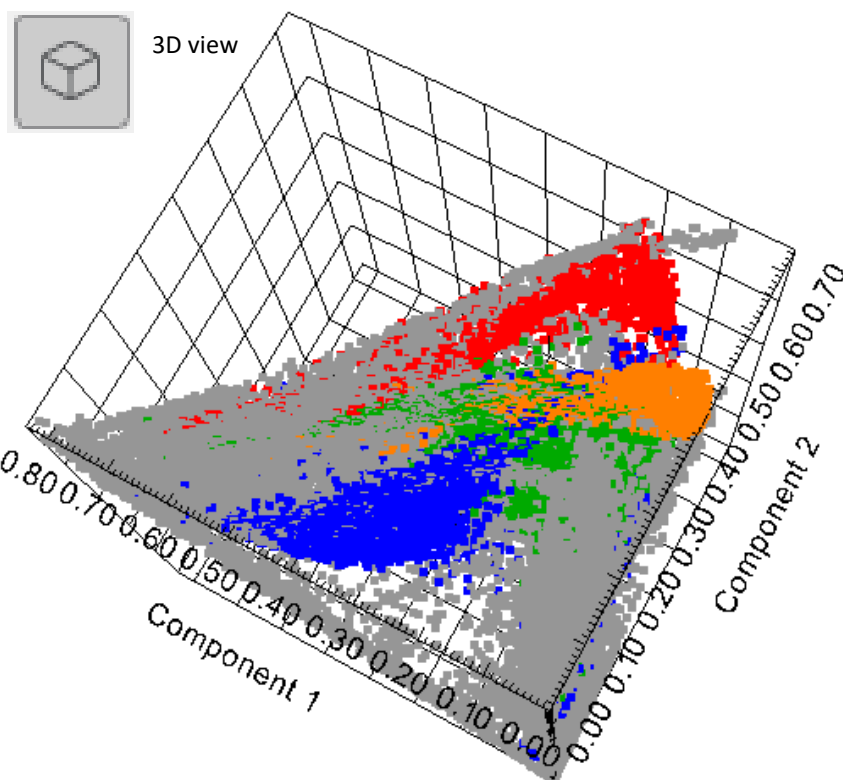
2018-02-08 18:07:01

Tile View shows 5 components and regions views





pLSA (or PCA) is a tool to reduce multidimensional data to lower dimensions while retaining most of the information. Each datapoint in the scores plot represents a spectrum. Each color represents one region. The 2D plot (TOP) uses the first 2 component as axis, while 3D plot (Bottom) uses the first 3 components.



Appendix 1: Service and Order Information

Calibration standards, plates, ITO slides and matrices

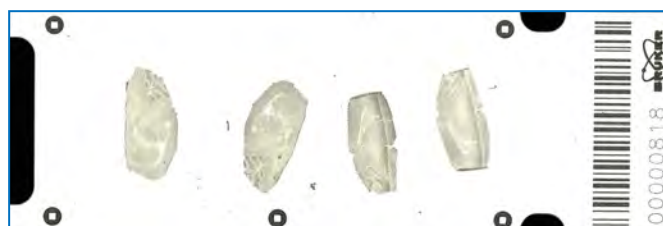
US only: +1 (978) 663-3660 X5 ms.support.us@bruker.com

Online store: <https://bruker-labscape.store/>

Other countries, please contact local sales or MALDI App support



1, Slide Glass Adapter II
PN: **8235380**



2, IntelliSlides, 50 pc/box, 25 x 75 mm
with barcode and teaching marks
PN: **1868957**



Part number	Product name	Description
8222570	Peptide Calibration Standard II	Covered mass range: ~700 Da - 4,000 Da; 5 tubes sufficient for approx. 250 calibration points each
8206355	Protein Calibration Standard I	Covered mass range: ~4,000 Da - 20,000 Da; 5 tubes sufficient for approx. 250 calibration points each
8207234	Protein Calibration Standard II	Covered mass range: ~20,000 Da - 70,000 Da; 5 tubes sufficient for approx. 250 calibration points each
1869371	fleXmatrix for Lipid Imaging	10 tubes of 200mg DHB
1869450	fleXmatrix for Peptide Imaging	10 tubes of 200mg HCCA
1869451	fleXmatrix for Protein Imaging	10 tubes of 200mg SA

Appendix 2 : Protein Imaging workflow

The major differences between **protein imaging** and **lipid imaging** is that an extra wash step is required

for protein imaging, prior to matrix deposition.

The following workflow describes how to set up protein imaging, unless specified below, the protocol stays the same as lipid Imaging.

- A. Preparing frozen tissue and tissue sectioning
- B. Washing tissue (Refer to **appendix 4**: Basic washing protocol for imaging of intact proteins)
- C. Applying Matrix (Choose **SA** method on HTX software, SA prepared at 10mg/mL in 50% ACN, 0.1% TFA)
- D. Acquiring data
 - flexControl software : Linear Positive Method will be used. ex. method: LP5-20KDa;
 - flexImaging software
- E. Analyzing data
 - flexImaging software
 - SCiLS Lab

Appendix 3: Two washing protocols for protein imaging

A) Basic washing protocol for imaging of intact proteins:

- 1.) 70% EtOH, 1 - 2 minutes
- 2.) 70% EtOH, 1 - 2 minutes
- 3.) 100% EtOH, 1 - 2 minutes

Note: Imaging of smaller sized endogenous proteins / peptides (i.e. < 3-5kDa) may require shorter washing steps to avoid analyte delocalization. In case of lipids or other small molecules (ex. drug molecules and their metabolites), the washing procedure should be skipped completely.

B) Protocol for Carnoy's washing of tissue sections:

(Reference: F. Deutschens, J. Yang and RM Caprioli, J. Mass Spectrum., 46, 568–571)

Alternatively, an advanced washing protocol may be applied. This protocol enhances the efficiency of removal of all kind of endogenous background compounds (lipids, peptides) that may interfere with the MALDI Imaging analysis of either intact proteins or peptides generated by on tissue digestion.

- 1.) 70% EtOH, 30 seconds
- 2.) 100% EtOH, 30 seconds
- 3.) Carnoy's fluid (EtOH: Chloroform: Acetic acid 6:3:1), 2 minutes
- 4.) 100% EtOH, 30 seconds
- 5.) 0.2%TFA in water, 30 seconds
- 6.) 100% EtOH, 30 seconds

Appendix 4: Preparation of FFPE tissue sections

Tissue sections cut from FFPE tissue (FFPE = formalin fixed paraffin embedded) require a multistep protocol when preparing them for peptide analysis by MALDI imaging.

The protocol includes the following steps:

- 1) **Dewaxing**
- 2) **Antigen retrieval**
- 3) **On-tissue digestion using trypsin as a protease**
- 4) **Deposition of MALDI matrix HCCA**
- 5) **Troubleshooting: digestion of peptides**

1) **Dewaxing (remove paraffin):**

The paraffin wax that has been used for embedding of the tissue block has to be removed from the tissue sections mounted on the conductive slide as it would otherwise interfere with the MALDI-MS acquisition process.

- a) Incubate sample slide for 15min @ 85°C in up-right position (using a slide rack).
- b) After finished incubation, while the slide is still warm, treat it with the following washing procedure (use Coplin jars for washing):
 - o Xylene 2 x 5min
 - o Isopropanol 5min
 - o Ethanol @ descending concentrations, each for 5min: 100%, 96%, 70%, 50%
 - o MilliQ water for 5min

NOTE: Nitrile gloves must be used as latex is dissolved by xylene

2) **Antigen retrieval:**

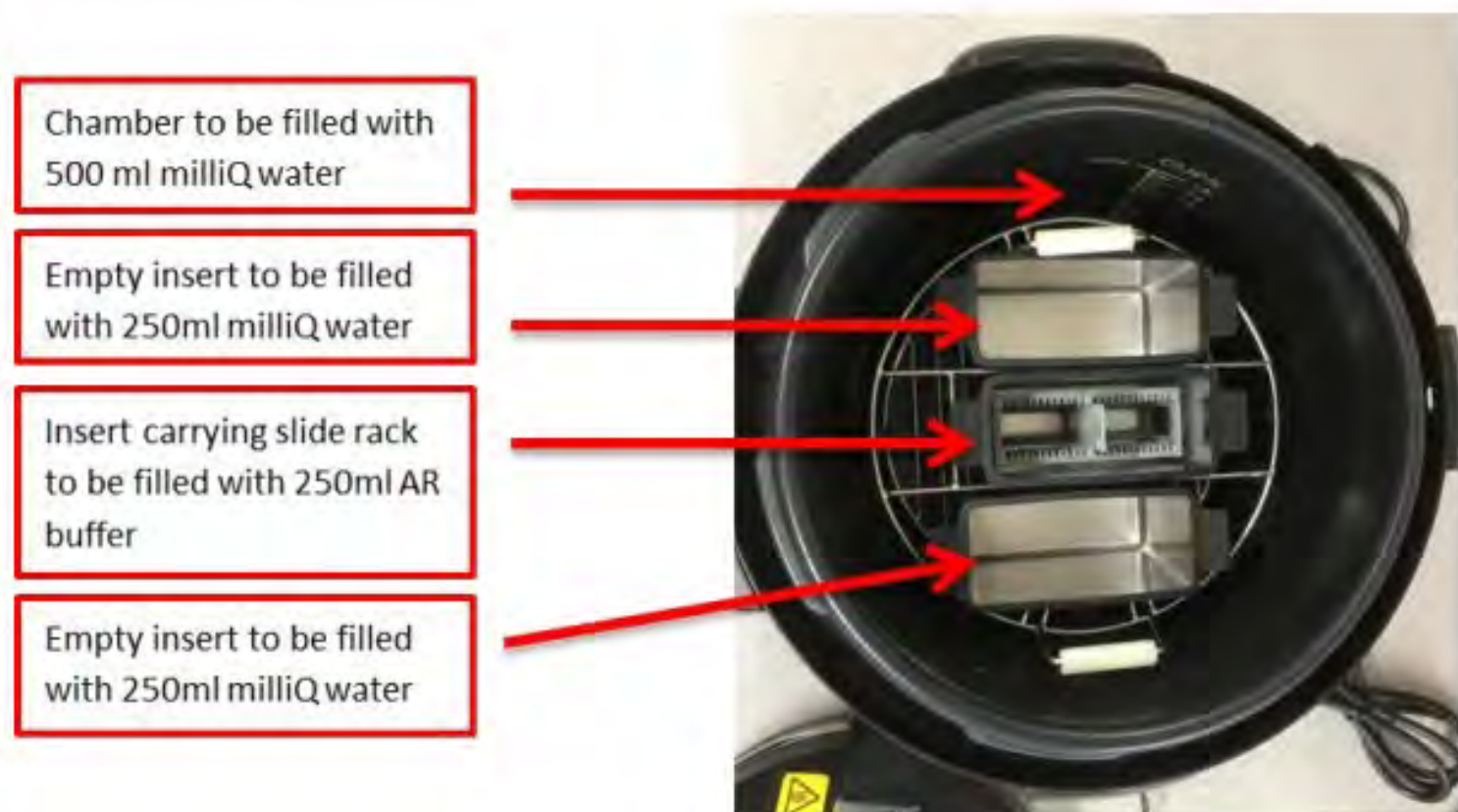
This step is performed to enhance the enzyme's accessibility to the proteins contained in the tissue, i.e. to improve the digestion efficiency. Using a **NXGEN decloaking chamber (Biocare Medical)**, the following protocol should be applied:

- a) Prepare antigen-retrieval (AR) buffer: Water or Tris buffer.
 - i) deionized water (first choice! If get poor signal consistently, then use Tris buffer)
 - ii) 10 mM TRIS buffer (ph=9)

1.21g Tris base dissolved in 500mL de-ionized water, then adjust pH to 9.0 with NaOH or HCl. Fill up to total of 1000 mL with de-ionized water.
- b) Fill 500ml MilliQ water in the vessel of the decloaking device.
- c) Fill 250ml of the AR buffer of choice in the insert(s) that will accommodate the tissue slides.

- d) Fill the 2 empty rack inserts that are not used to accommodate tissue slides with 250ml deionized water each.
- e) Place the tissue slides in the slide rack insert covered with AR buffer. Close the lid of the decloaking chamber.
- f) Select the program running at 110°C for 20min if using water as AR buffer.
If using 10mM Tris buffer , use program 4: 95°C for 20 min.
- g) When program is finished, carefully take out the insert carrying the slide rack loaded with the tissue slides. **Caution:** The insert filled with AR buffer will still be hot when taking it out !
- h) Stepwise exchange the warm AR buffer with cold deionized water (pour out half of the volume each time and refill with cold de-ionized. Repeat three times.
- i) Allow the slides to dry by placing the slide rack on dry lab tissue.
- j) Vacuum-dry the slides in a desiccator (minimum for 30min)

Note: If not continuing with the digest, slides can be stored under vacuum for up to 2 days.



3) In Situ Enzymatic Digestion for FFPE tissue



TM sprayer (HTX Imaging)

For service and support ,

Contact info@htximaging.com

maldi.sw.support@bruker.com

1, Prepare the Trypsin Solutions

a) Buffer Recipe for Saturated K_2SO_4 solution:

1. Add 75 g K_2SO_4 to 500 mL de-ionized water
 2. Mix with a magnetic stirrer with heat (50°C or medium heat – it is not necessary to boil)
 - a. If this equipment is not available, mix in an ultrasound bath for 60 minutes at elevated temperature, repeatedly mix the bottle by inverting.
 - b. Use 40 mL of the saturated solution in the digestion chamber
 - c. Change the used saturated K_2SO_4 solution in the digestion chamber EVERY WEEK
- This should give you 12 weeks of saturated K_2SO_4

b) Buffer Recipe for Ammonium bicarbonate buffer (Ambic):

1. Prepare a stock solution of 100 mM Ambic (7.9 mg/mL) in de-ionized water.
2. Store 200 μ L aliquots in 1,5 mL Eppendorf tubes in a -20 °C freezer.
3. Thaw a 200 μ L aliquot and add 800 μ L 0.0125% (v/v) Glycerol/water to get 1 mL of a 20 mM Ambic, 0.01% Glycerol buffer solution

c) Buffer recipe for Trypsin solution:

1. Add 800 μ L 20 mM Ambic 0.01% Glycerol buffer solution to dissolve one vial of Promega Trypsin (20 μ g).
 2. Stir well on vortexer
- Note: Store on ice until usage, use within 30 minutes

2, Prepare digest chamber and perform digest

- a) Set oven temperature to 50°C
- b) Add 40 mL of the saturated K_2SO_4 -solution into the enzyme digestion chamber. The floor of the chamber must be covered. Add additional solid K_2SO_4 (at least 5g, more does no harm). Place two glass culture tubes as spacer at the long sides of the box. DO NOT PREHEAT THE BOX.

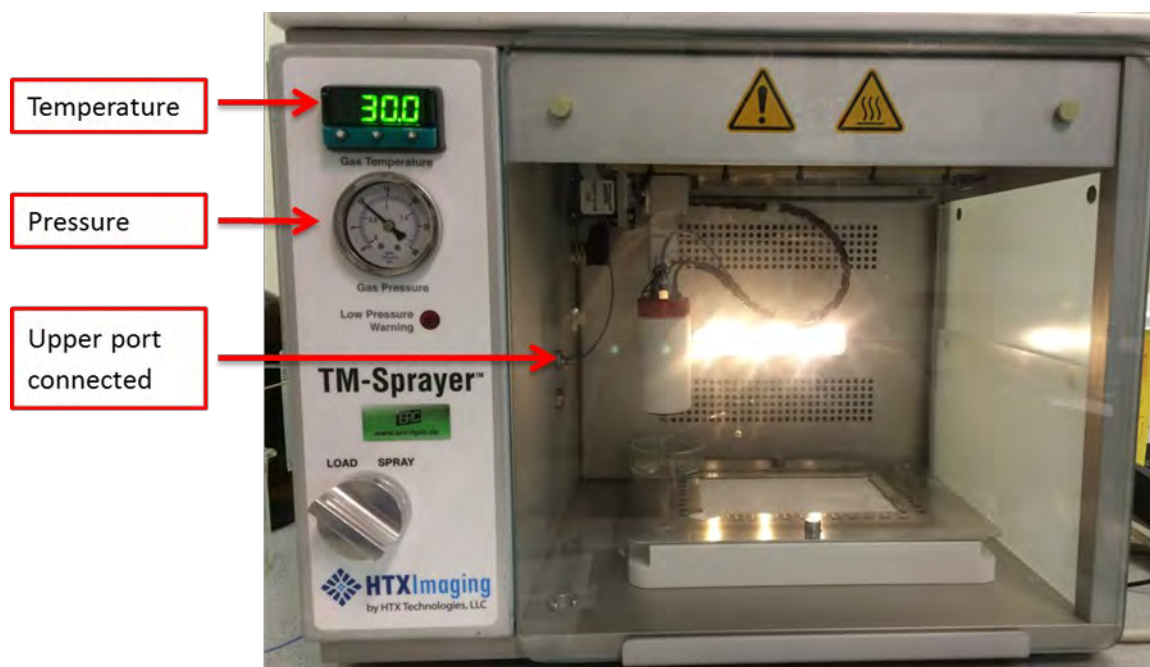
Note: Add solid K_2SO_4 for every experiment, however the solution should be changed every week.

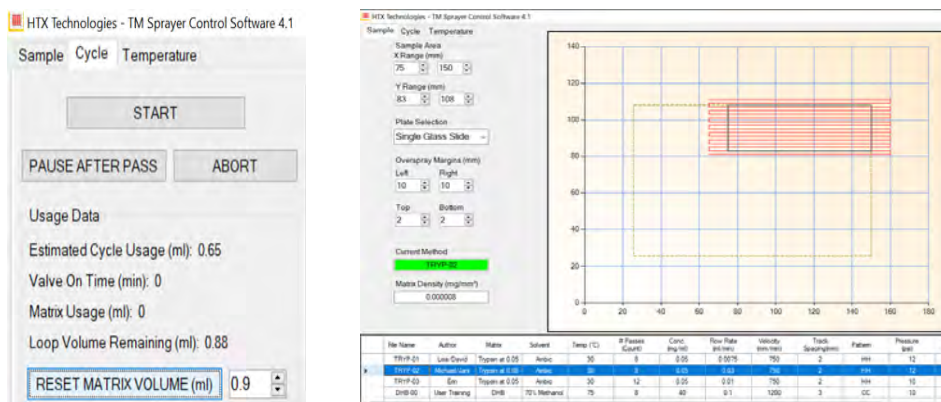


Digestion chamber set up

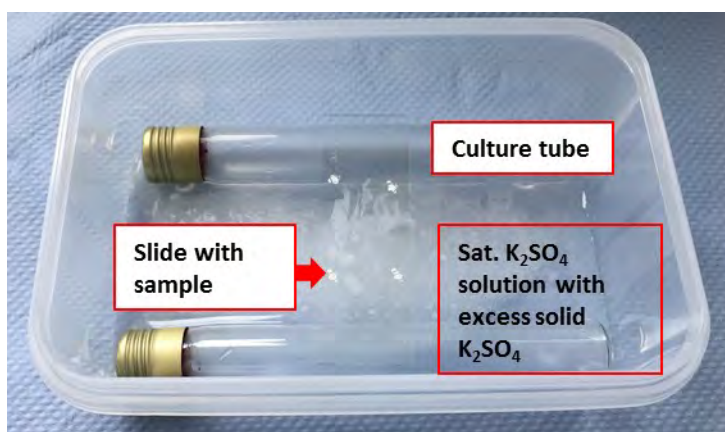
3, Prepare TM sprayer for tryptic digest

- 1) Turn on the Nitrogen gas to 10 psi.
- 2) Turn on the TM sprayer Laptop, TM Sprayer (power switch on the back), and start the software.
- 3) select “_digest wet” method, and Set nozzle temperature to 30°C.
- 4) Connect the internal tubing to the upper port (= connection to syringe pump, see Fig below)
- 5) Draw up 1 mL of 50%v/v Acetonitrile (ACN)/Water into a Hamilton glass syringe (1 mL) and mount on the syringe pump. Flush the system with a flow rate of 30 μ L/minute (syringe pump flow rate 0.03 mL/minute) by starting the syringe pump. Stop the syringe pump after 0.8 mL or approximately 30 minutes.
- 6) After flushing the system with 50% ACN (see Step 4), change the flow rate on the syringe pump to 0.015 mL/minute.
- 7) Draw up all 800 μ L of the Trypsin solution into the 1 mL Hamilton syringe, mount onto the syringe pump and in the software, reset matrix volume to 1 mL.





- 8) Click **“Start”** button to start the method and **Continue** for 180s countdown before actual enzyme deposition method starts. NOTE: the valve located on the front is not in use when using syringe pump
- 9) Once the deposition is finished, press **“Load valve confirm”** and stop the syringe pump.
- 10) Remove the slide(s) from the TM-Sprayer .
- 11) Place the slides in the pre-heated ‘enzyme digestion chamber’ on the tubes.
- 12) Place a 10 µL drop of deionized water on the slide (off tissue) close the lid, and place in the pre-heated oven at 50°C for 2h.



- 13) Fill the 1 mL Hamilton syringe with 50%v/v ACN/de-ionised water, change the flow rate to 0.03 mL/min and press start to flush system.

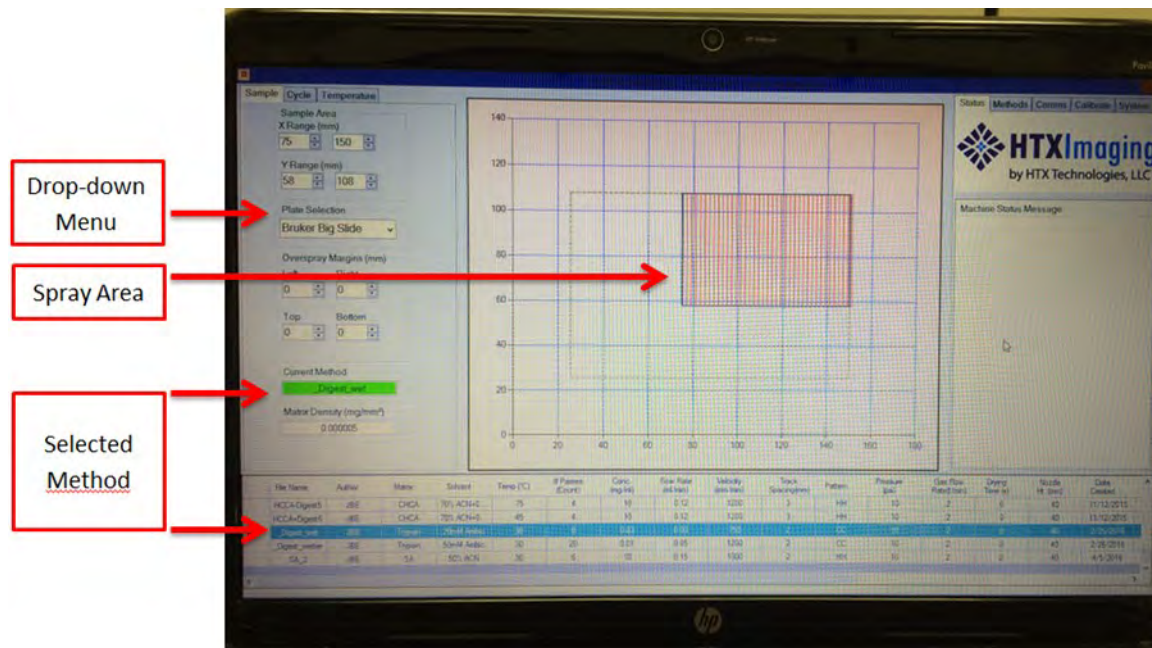
4) Deposition of MALDI matrix HCCA: for detailed procedure, refer to TM Sprayer—DHB section

HCCA is the MALDI matrix of choice when analyzing peptides obtained from tryptic digestion of FFPE tissue sections. HCCA concentration: **10mg/ml in 70/30 ACN/H₂O (v/v), 1%TFA** (note, is 1% not 0.1%)

choose method “_HCCA_Digest2” in the HTX software

set nozzle temperature to 75C

120 µL/min solvent flow rate



5) Troubleshooting Digestion of Peptides:

If, upon imaging your tissue, you are getting incomplete digestion, try

- (1) increasing the length of your digestion up to 24 hours,
- (2) increasing the amount of water used in the incubation chamber up to 2 mL, and/or
- (3) increasing the temperature of the incubation chamber up to 50°C.

Appendix 5: Carboxymethyl Cellulose (CMC) Embedding

- 1) Dissolve CMC (carboxymethyl cellulose), 2% in Water
- 2) Apply to small container (can be made with Al foil or plastic)
- 3) Add sample of interest
- 4) Cover with top layer of CMC
- 5) Freeze tissue with CMC
- 6) Tissue sectioning



Appendix 6: Coating ITO slides with poly-L-lysine

Coating ITO slides with poly-L-lysine helps to improve adherence of tissue sections to the slide surface. The pre-coating step is particularly helpful in extended workflows, when H&E staining of tissue sections is performed after finished MALDI imaging acquisition. The pre-coating step helps to avoid delamination of tissue sections from the ITO slide's surface during the step of MALDI matrix removal and the actual staining.

As the poly-L-lysine coating itself produces signals in the lower m/z range, precoating with poly-L-lysine is not recommended for slides to be used for imaging of smaller MW compounds (peptides, lipids etc.).

Protocol:

- 1.) Prepare a 1:1 (v:v) mixture of poly-L-lysine (Sigma P8920) and milliQ water in a 2ml Eppendorf tube.
- 2.) Add to 1.5 ml of this mixture approximately 1 μ l of IGEPAL CO-630 (Sigma). Mix thoroughly.
- 3.) Streak out homogeneously 20 μ l of the prepared mixture on the conductive side of an ITO slide using a glass plate spreader according to Drigalski.
- 4.) Dry the freshly coated slide, preferentially using a heated plate (approx. 80 °C), alternatively dry the slide at room temperature.
- 5.) Pre-coated ITO slides can be stored for longer periods of time (weeks) using the same type of containers that are used for storage of slides carrying prepared tissue sections.

Appendix 7: H & E staining of the tissue sections

Hematoxylin-Eosin (H&E) is one of the most routinely used histological staining methods that allows for the enhanced visual differentiation of structures in high-resolution optical images of tissue sections.

Hematoxylin stains in blue color of all those structures that comprise acidic properties, for example cell nuclei and the rough endoplasmic reticulum. Eosin stains in red color of all those structures that comprise alkaline properties, for example cytoplasm, mitochondria, smooth endoplasmic reticulum, collagen and keratin.

After MALDI acquisition, the matrix on tissue can be carefully washed and subsequent with 70% ethanol. Subsequently, it can be stained with H&E. The high-resolution optical image taken from the very same tissue section can be co-registered in MALDI imaging software for histological annotation. It may be desirable to collect serial sections for acquisition and H&E staining to avoid losing tissue during washing steps.

Consumables required for H&E staining:

Hematoxylin according to Meyer (Sigma-Aldrich 51275)

Eosin Y disodium salt (Sigma-Aldrich E6003)

Ethanol (Rotipuran > 99.8%, Roth 9065.4, 2.5l canister)

Xylene, histological grade (Sigma 534056, 500ml)

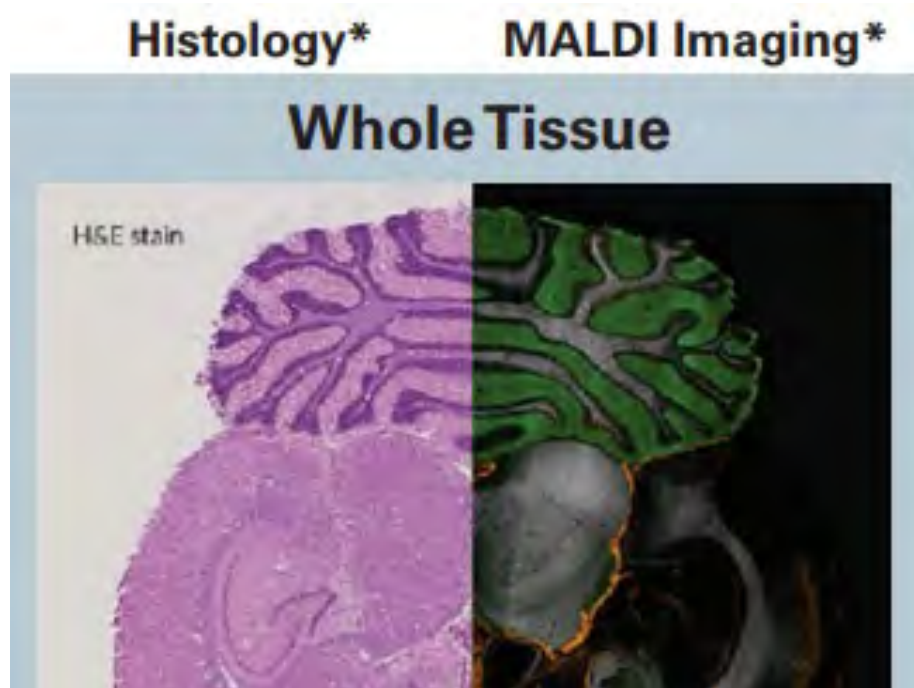
Microscope slides 75 x 25 mm (Menzel SuperFrost Plus Gold, Roth ET09.1, 50 pcs.)

Cover slips 22 x 22 mm (Omnilab 5161022, 200 pcs.)

Cover slips 22 x 50 mm (Omnilab 5161028, 100 pcs.)

Cover slips 24 x 60 mm (Omnilab 5161060, 100 pcs.)

Glass staining jar, Coplin (Omnilab 5163283)



Protocol for H&E staining:

- 1.) Prepare an eosin stock solution (eosin concentration 1%) by dissolving 10g of Eosin Y disodium salt in 200ml of distilled water and adding 800ml of 95% EtOH. This stock solution can be stored at room temperature.
- 2.) Prepare the eosin working solution (eosin concentration 0.25%) by mixing 250ml of the stock solution with 750ml of 80% EtOH. Add in 5ml glacial acetic acid (concentrated). Mix the solution thoroughly and store it at room temperature.
- 3.) Wash off matrix in 70% EtOH (usually 1-2 minutes).
- 4.) Dip-wash the slide in deionized water.
- 5.) Stain the tissue section for 5-15 minutes in Hematoxylin solution.
- 6.) Remove excess staining solution by dip-washing the slide in deionized water.
- 7.) Rinse the slide in running tap water for 5 minutes.
- 8.) Wash the slide for 1 minute in deionized water.
- 9.) Put the slide into Eosin working solution until the section is sufficiently stained (~ 1 minute).
- 10.) Wash slide in deionized water.
- 11.) Wash the slide, for two minutes each, in 70% EtOH, 80% EtOH, 90% EtOH, 100% EtOH, 100% EtOH again and xylene.
- 12.) Using a glass stir bar, put one droplet of mounting medium onto the slide and place a coverslip onto the tissue

Tips,

Getting the right feeling for the appropriate staining intensity requires some practical experience. Commonly, the tissue section has to be overstained, since the Eosin dye is partially washed out during the subsequent washing steps in deionized water and ethanol. The Tipp-Ex teaching marks placed on the slide earlier in the workflow may come off during the xylene wash. They sometimes tend to move on the slide's surface when the coverslip is put on and may slip onto the tissue section. In case of doubt, better remove the teaching marks completely using a glass bar when washing the slide in the xylene bath. New teaching marks, scratched into the glass with a diamond tip pen, can be placed on the slide before scanning it in the high-resolution scanning device to allow alignment of the stained image later on.