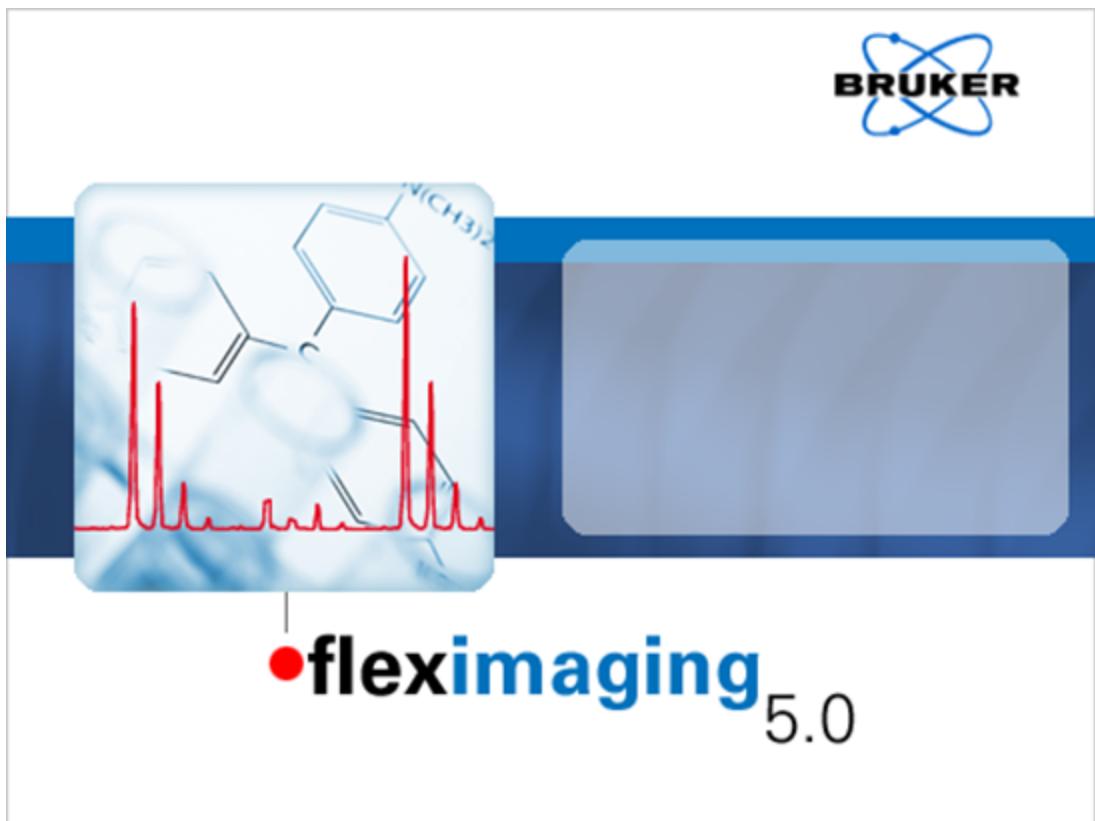




flexImaging 5.0

User and Workflow Manual



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Contents

Legal and Regulatory Notices	2
Contact	4
Contents	5
1 Preface	9
1.1 Key Features	10
1.2 Patents	10
2 Installing and Licensing flexImaging	11
2.1 System Requirements	11
2.2 Installing flexImaging	12
2.3 Licensing flexImaging	12
2.4 Uninstalling flexImaging	14
3 Getting Started with flexImaging	15
3.1 flexImaging File Locations	15
3.1.1 flexImaging File Locations: Methods and Templates	16
3.1.2 flexImaging File Locations: Data Organization	16
3.2 Starting flexImaging	17
3.3 Three Basic Workflows in flexImaging	19
3.3.1 Basic Workflow 'Arbitrary Arrays'	20
3.3.2 Basic Workflow 'Spot Microarray'	20
3.3.3 Basic Workflow 'Tissue Profiling'	21
3.4 Exiting flexImaging	21
4 flexImaging Graphical User Interface	23
4.1 Quick Reference Tables	24
4.1.1 flexImaging 5.0 Toolbar	24
4.1.2 flexImaging 5.0 Menus and Commands	28
4.1.3 flexImaging 5.0 Shortcuts	33
4.2 flexImaging Windows	35
4.2.1 Imaging Display	36
4.2.2 Spectrum Display	37
4.2.3 Regions Pane	41
4.2.4 Results Pane	43

4.2.5 Navigation Window	44
4.2.6 Image Adjustment Window	45
4.2.7 Histogram Window	47
4.2.8 Dendrogram Window	48
4.2.9 Filter Preview Window	49
4.2.10 Checklist	49
4.2.11 Status Bar	50
4.2.12 Customizing the Window Layout	51
4.3 flexImaging Menus	52
4.3.1 File Menu	53
4.3.2 Edit Menu	60
4.3.3 View Menu	68
4.3.4 Analysis Menu	76
4.3.5 Tools Menu	77
4.3.6 Compass Menu	78
4.3.7 Help Menu	89
4.4 flexImaging Shortcut Menus	91
4.4.1 Imaging Display Shortcut Menu	92
4.4.2 Spectrum Display Shortcut Menu	97
4.4.3 Regions Pane Shortcut Menu	108
4.4.4 Results Pane Shortcut Menus	110
4.4.5 X/Y-Axis Shortcut Menu	114
4.4.6 Histogram Shortcut Menu	115
4.4.7 Dendrogram Shortcut Menu	115
4.4.8 Docking Window Shortcut Menu	116
4.5 flexImaging Dialogs	117
4.5.1 Choose Imaging Run	119
4.5.2 New Imaging Run Wizard	120
4.5.3 Preferences Dialog	135
4.5.4 Imaging Run Properties Dialog	148
4.5.5 Spot Microarray Teaching Dialog	156
4.5.6 Spot List Import Wizard	157
4.5.7 Edit Measurement Region Parameters Dialog	160

4.5.8 Edit Region of Interest Parameters Dialog	161
4.5.9 Save autoXecute Sequence As Dialog	162
4.5.10 autoXecute Run Options Dialog	163
4.5.11 Co-Register Image Dialog	166
4.5.12 Copy Region Of Interest Spectra Dialog	168
4.5.13 Export Spectra List Dialog	168
4.5.14 Edit Mass Filter Mark Parameters Dialog	170
4.5.15 Add Result Filter Dialog	171
4.5.16 Save Result Filter List As Dialog	172
4.5.17 Hierarchical Clustering Parameters Dialog	173
4.5.18 Import ClinProTools Result Wizard	176
4.5.19 Edit Mass Filter Parameters Dialog	192
4.5.20 Edit ClinProTools Classification Filter Parameters Dialog	195
4.5.21 Edit ClinProTools Classification Overall Score Filter Parameters Dialog	198
4.5.22 Edit ClinProTools Clustering Filter Parameters Dialog	200
4.5.23 Edit ClinProTools Clustering Tree Filter Parameters Dialog	201
4.5.24 Edit ClinProTools PCA Filter Parameters Dialog	203
4.5.25 Edit ClinProTools PLSA Filter Parameters Dialog	205
4.5.26 Edit XML Import Filter Parameters Dialog	208
4.5.27 Edit Text Import Filter Parameters Dialog	210
4.5.28 Automatic Mass Filtering Dialog	213
4.5.29 Customize Toolbar Dialog	214
4.5.30 Color Dialog	215
4.5.31 Definitions for Import of Generic Robot Files Dialog	216
4.5.32 Add Normalization Mass Window Dialog	218
4.5.33 Select Co-Registered Image Dialog	220
4.5.34 Automatic ROI Array Parameters Dialog	221
4.5.35 Mass Defect Filter Parameters Dialog	222
4.5.36 Hamamatsu Converter Dialog	224
4.5.37 Export to imzML Dialog	225
4.5.38 Split Imaging Run Dialog	226

5 Workflows in Detail	231
5.1 Sample Preparation	231
5.1.1 Preparation of Cryostat Sections for MALDI Imaging	231
5.1.2 Matrix Coating with imagePrep	238
5.1.3 Manual Spray Coating for MALDI Imaging	241
5.1.4 Robotic Spotting	243
5.1.5 Specific Considerations for Detecting Pharmaceuticals in Tissue	245
5.1.6 Preparing a Drug Dilution Series in a Frozen Tissue Homogenate Block	247
5.1.7 Acquiring an Optical Image	253
5.2 Data Acquisition	256
5.2.1 General Overview (rapifleX MALDI Tissuetyper)	257
5.2.2 General Overview (flex Series Instruments)	275
5.2.3 General Overview (FTMS Instruments)	280
5.2.4 Incremental Runs	283
5.2.5 Arbitrary Arrays	283
5.2.6 Spot Microarrays	286
5.2.7 Tissue Profiling	289
5.2.8 MS/MS Experiments	297
5.3 Data Acquisition Using HyStar and ftmsControl	307
5.4 Data Processing and Interpretation	312
5.4.1 Histological Staining and Co-Registration of Microscopic Images	312
5.4.2 Data Processing in flexAnalysis and flexImaging	315
5.4.3 Normalization	319
5.4.4 How to Find Biomarkers	330
5.4.5 Creating High-Quality Images	335
5.4.6 Interfacing with ClinProTools	343
5.4.7 Hierarchical Clustering	370
5.4.8 Exporting and Importing Data for Statistical Analysis (Advanced Users Only)	373
Appendix A — Appendix	377
A.1 Abbreviations	377
A.2 Part Numbers	377
Index	379

1 Preface

1.1 Key Features	10
1.2 Patents	10

flexImaging 5.0 (referred to as 'flexImaging') is a program designed to acquire spectra in a spatially resolved manner directly from the surface of samples such as tissue sections. The results can then be visualized as images representing the spatial distribution of molecules within the sample. Commonly, this approach is referred to as MALDI imaging.

Data acquisition on all Bruker instruments equipped with a MALDI source is supported; specifically flex series MALDI TOF (Time-Of-Flight) instruments (for example, autoflex, ultraflex and rapifleX) and MALDI-FTICR (solariX).

flexImaging is composed of two parts: a graphical run editor and an analysis and visualization component, which are integrated seamlessly into an easy- to- use graphical user interface.

For time-of-flight mass spectrometers, flexImaging works in conjunction with flexControl and flexAnalysis (versions 3.4 and 4.0). flexControl is used to operate the mass spectrometer and acquire spectra, whereas the flexAnalysis is used for spectra post-processing. For fast acquisition on the rapifleX, data processing is performed during acquisition and is independent of flexAnalysis. In this workflow, flexAnalysis (and the flexAnalysis Batch Process tool) are only required for post-acquisition reprocessing of data.

For an FTMS spectrometer, flexImaging operates with ftmsControl (version 2.0).

In addition, flexImaging also provides several possibilities to import and export results for further analysis. Most importantly, flexImaging interfaces with SCiLS Lab for statistical analysis of MALDI Imaging data.

1.1 Key Features

The flexImaging software has the following key features:

- Integrated solution for acquisition, visualization, and analysis of MALDI imaging data.
- Supports MALDI-TOF (flex series) and FTMS (solariX) instruments.
- Registration and superimposition of image(s) to compare the distribution of molecules with histology, including virtual microscopy data from special slide scanners.
- Configurable spectra import (data reduction, mass range truncation) for handling large data sets. Storage of reduced data sets for fast reload of analyses.
- Customizable display of overall average, region of interest average, and single spectra.
- Fast and simple creation of ion distribution images (mass range filters).
- Interface to SCiLS Lab for performing statistical analysis.

1.2 Patents

The product is protected by U.S. Patent(s) No. 5808300.

2 Installing and Licensing flexImaging

2.1 System Requirements	11
2.2 Installing flexImaging	12
2.3 Licensing flexImaging	12
2.4 Uninstalling flexImaging	14

Bruker flexImaging 5.0 runs under the Windows 7 operating system, English version.

The flexImaging software is installed from the flexImaging delivered installation CD . Initial installation of flexImaging on a computer automatically creates a temporary license valid for 60 days. To get permission to work with flexImaging after this period, you have to enter the flexImaging license key you received with your software package.

2.1 System Requirements

- CPU: Pentium Core processor equivalent or better, clock frequency \geq 1.6 GHz. For convenient data handling, a dual-processor workstation is recommended for acquisition.
- Hard disk: at least 12 GB of free disk space
- Main Memory: minimum 4 GB RAM. For handling large data sets, it is recommended to have 32 GB RAM or more.
- Operating System: Windows 7 SP1 (64 bit), English version
- Internet Explorer version 8.0 or later
- Graphic resolution: 1280 \times 1024 pixels with true colors
- CD-ROM / DVD drive (only for installation)
- Data acquisition on rapifleX MALDI-TOF instruments requires Compass for flex-Series 2.0

2.2 Installing flexImaging

Bruker flexImaging 5.0 is supported by Windows XP or Windows 7, English version. The flexImaging software is installed from the flexImaging installation CD delivered.

►► To install flexImaging

1. Start your Windows application.
2. Insert the installation CD into the CD-ROM drive of your computer (e.g. E:). If the 'Autostart' function is activated, the CD browser will start automatically and guide you to start the installation. You can proceed to step 7. Otherwise, if the 'Autostart' feature is not turned on, proceed to step 3.
3. Click **Start**.
4. Click **Run**.
5. In **Open**, type in the command line `E:\launch.exe` (if E: is your CD-ROM drive).
6. Click **OK** to start installation.
7. Follow the **Bruker flexImaging 5.0 Setup Wizard** instructions to set up flexImaging on your computer. Click **Finish** when you get to the end of the wizard prompts.

2.3 Licensing flexImaging

Installation of flexImaging on a computer automatically creates a temporary license valid for 60 days. To work with flexImaging after this time, enter the license key you received. Permanent as well as temporary licenses can be given. In the latter case, an expiration warning will inform you about the forthcoming expiration firstly 30 days before the license will expire. License keys have to be entered in the Bruker LicenseManager, which you can launch from the Windows **Start** menu. The LicenseManager will list any license currently available for any Bruker application. Alternatively, you can open the LicenseManager from flexImaging using the **License** command from the **Compass** menu.

►► To license flexImaging

1. Click **Start**.
2. Click **Programs**.
3. Click **Bruker**.
4. Click **Administration**.

5. Click **LicenseManager** to open the **Bruker LicenseManager** dialog.
6. Enter the flexImaging license key in **New license key**.
7. Click **Add**. The button is enabled after entering a correct license key.
 - a. Without UserManagement: The license is added in **Existing licenses**.
 - b. With UserManagement: The **Re-enter Your Password** dialog appears. Enter your password again and click **OK**. If the password is correct for the current operator account and this operator has the right to add/delete licenses, the license is added in **Existing licenses**. If an error message informs you that the password is incorrect or that the operator does not have the needed right, quit the message and repeat/do the required action.
8. Click **Close**.

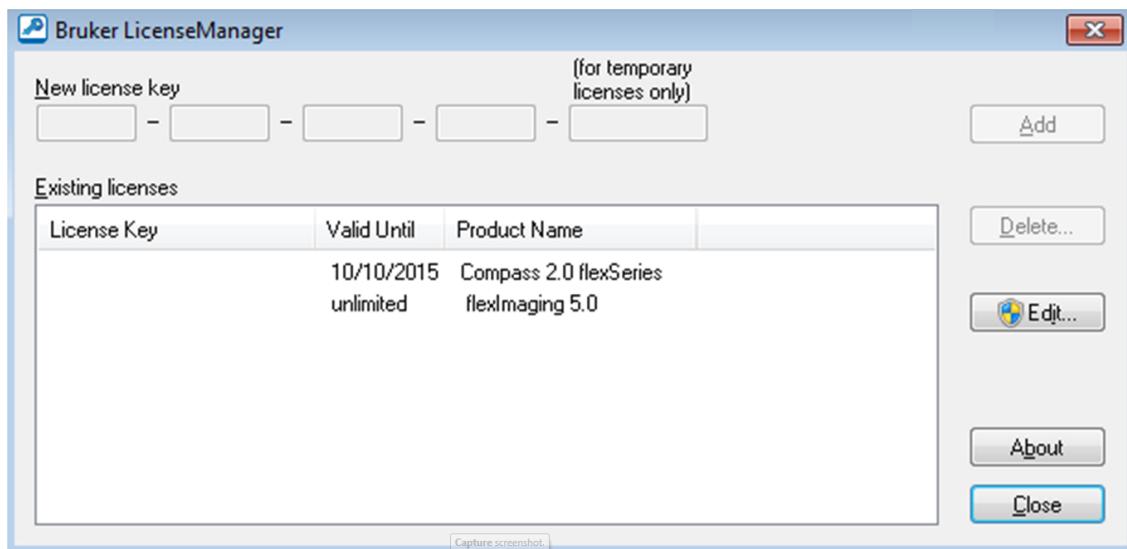


Figure 2-1 Bruker LicenseManager showing the flexImaging 5.0 license is present (example)

2.4 Uninstalling flexImaging

flexImaging needs to be uninstalled from your system prior to installing a new flexImaging upgrade version.

►► To uninstall flexImaging

1. Click **Start**.
2. Click **Settings**.
3. Click **Control Panel**.
4. Double-click **Add/Remove Program**.
5. Select the flexImaging 5.0 software from the list of installed programs.
6. Click **Remove**.
7. Confirm the request to remove flexImaging from your system.

3 Getting Started with flexImaging

3.1 flexImaging File Locations	15
3.2 Starting flexImaging	17
3.3 Three Basic Workflows in flexImaging	19
3.4 Exiting flexImaging	21

3.1 flexImaging File Locations

3.1.1 flexImaging File Locations: Methods and Templates	16
3.1.2 flexImaging File Locations: Data Organization	16

flexImaging handles the following file types:

- **Imaging Runs** (file extension .mis). These files store all information about an imaging measurement (reference to sample image, coordinate mapping, used filter list, defined measurement regions and regions of interest, processing options, visualization settings, ...).
- **Result Filter Lists** (file extension .mir). These files contain a list of result filters (mass filters, references to ClinProTools classifications, hierarchical clustering results, ...).
- **Sample images**. The following image file types can be used with flexImaging: JPEG (file extension .jpg or .jpeg), TIFF (file extension .tif or .tiff) and bitmap (file extension .bmp). Please note that not all flavors of all image formats can be supported. flexImaging copies loaded sample images to the directory of the imaging run.
- **Reduced data** files. flexImaging optionally stores acquired spectra in a compressed format for faster access. These files have the same name as the corresponding imaging run with the file extension .dat and are stored in directory of

the imaging run or the Result Cache subdirectory of the Imaging Runs directory (if the run is stored in Imaging Runs).

3.1.1 flexImaging File Locations: Methods and Templates

The flexImaging setup creates a subdirectory in the global Bruker method directory (typically D:\Methods) named Imaging Runs.

flexImaging 5.0 stores its preferences, shared result filter lists and certain other files in this directory.

3.1.2 flexImaging File Locations: Data Organization

flexImaging 1.0 and 1.1 kept their imaging runs (.mis files) and result filter lists (.mir files) always in the Imaging Runs directory.

Since version 2.0, flexImaging stores imaging runs and result filter lists along with the acquired data. flexImaging 5.0 copies the registered sample image to the imaging run directory and references the result directory with a relative path.

An example is shown below: the flexImaging imaging run Rat_Brain.mis is stored together with its corresponding files in the folder D:\DATA\Imaging\Rat_Brain, a subdirectory with the same name is created for the spectra.

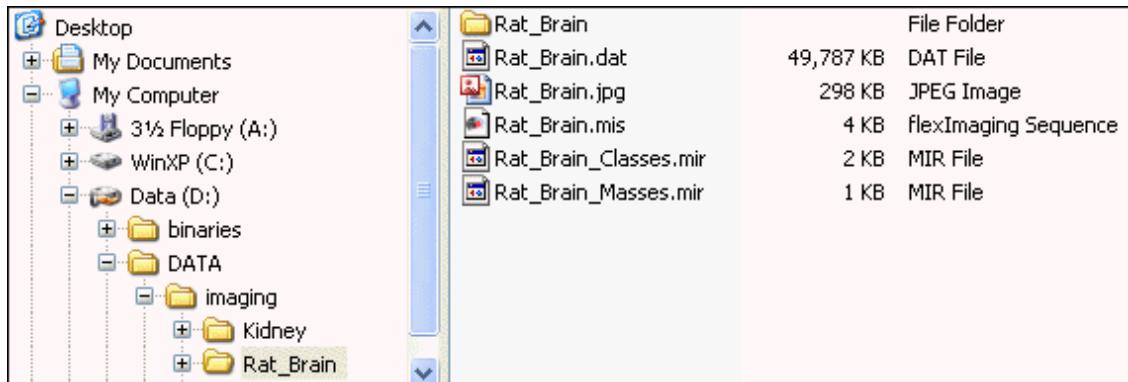


Figure 3-1 Example of data organization in flexImaging 5.0

In the **New Imaging Run Wizard**, this data organization option is called **Store Imaging Run in Result Directory specified above**.

This data organization allows you to perform the following tasks:

- copy/backup the imaging run and its data to a CD- or DVD-ROM and open the imaging run from the storage medium
- access imaging runs across a network using shares

3.2 Starting flexImaging

You can start flexImaging from the Windows **Start** menu. During flexImaging installation, a **Bruker** folder containing the application (and other Bruker applications) is created in the **Start** menu's **Programs** folder. Alternatively, you can double-click the flexImaging icon created on your desktop during installation.

►► To start flexImaging from Windows Start menu

1. Click **Start**.
2. Click **Programs**.
3. Click **Bruker**.
4. Click **flexImaging**.

If flexImaging is started without a valid license being present, a message informs you that flexImaging has not been licensed yet. Confirming this message automatically starts the Bruker LicenseManager to license flexImaging.

5. On start-up, flexImaging displays the **Choose Imaging Run** dialog to open an existing or to create a new imaging run. **Open an existing imaging run** will load the imaging run you selected from the list below, **Create a new imaging run** will launch the **New Imaging Run Wizard**. Click **Cancel** to close the dialog without opening or creating a imaging run now.

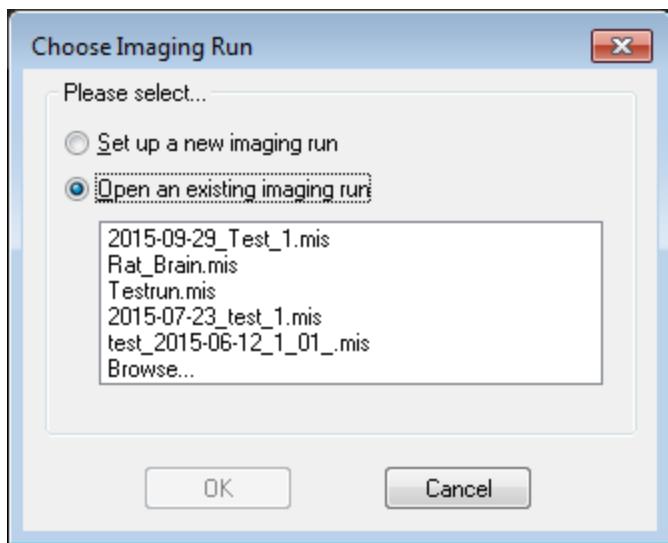


Figure 3-2 Choose Imaging Run dialog

3.3 Three Basic Workflows in flexImaging

3.3.1 Basic Workflow 'Arbitrary Arrays'	20
3.3.2 Basic Workflow 'Spot Microarray'	20
3.3.3 Basic Workflow 'Tissue Profiling'	21

flexImaging supports three different workflows, arbitrary arrays, spot microarrays and tissue profiling. These workflows are defined by the way the matrix is applied to the sample.

It is necessary to select the workflow in the **New Imaging Run Wizard** during the setup of an imaging run.

In all workflows, flexImaging needs an optical image of the sample. Please see the tutorial on acquiring an optical image.

A basic workflow results in MALDI images that show the localization and intensities of selected mass signals.

All three basic workflows can be combined with a statistical analysis in ClinProTools that allows classification of spectra ("class imaging") and unsupervised principal component analysis. Please see the tutorial on interfacing with ClinProTools for details.

3.3.1 Basic Workflow 'Arbitrary Arrays'

The arbitrary arrays workflow is selected if the sample is coated by a homogenous matrix layer. It is defined by the fact that the lateral resolution of the MALDI image can be arbitrarily chosen.

Please see the tutorials on

- Sample preparation
- Spray coating for MALDI imaging
- Data acquisition
- Arbitrary arrays
- Data processing
- How to find biomarkers
- Creating high-quality images

in the *flexImaging 5.0 Workflows Manual*.

3.3.2 Basic Workflow 'Spot Microarray'

The spot microarray workflow is selected if a robot was used to spot a regular grid of matrix spots onto the tissue. It is defined by the fact that the lateral resolution of the MALDI image is determined by the matrix grid.

Please see the tutorials on

- Sample preparation
- Robotic spotting
- Data acquisition
- Spot microarray
- Data processing
- How to find biomarkers
- Creating high-quality images

in the *flexImaging 5.0 Workflows Manual*.

3.3.3 Basic Workflow 'Tissue Profiling'

The tissue profiling workflow is selected if the matrix was applied as droplet on arbitrary positions on the sample. These positions are usually chosen because they show specific properties in the histology. This workflow is defined by the fact that the specific spot positions are selected for the acquisition and that it does not result in a full image.

Please see the tutorials on

- Sample preparation
- Robotic spotting
- Data acquisition
- Tissue profiling
- Data processing
- How to find biomarkers
- Creating high-quality images

in the *flexImaging 5.0 Workflows Manual*.

3.4 Exiting flexImaging

You can exit flexImaging when you have finished your current session. To exit flexImaging you have to answer a confirmation request.

►► To exit flexImaging

1. From the **File** menu, select **Exit**. Alternatively, click the application's  or press the keys Alt+F4.
2. Answer the appearing confirmation request to close flexImaging. The request depends on the saving state of the current imaging run.

If you have modified the imaging run but have not saved the changes yet, you will be asked whether to save the imaging run. Click **Yes** to save the imaging run and close flexImaging or click **No** to leave the imaging run unchanged.

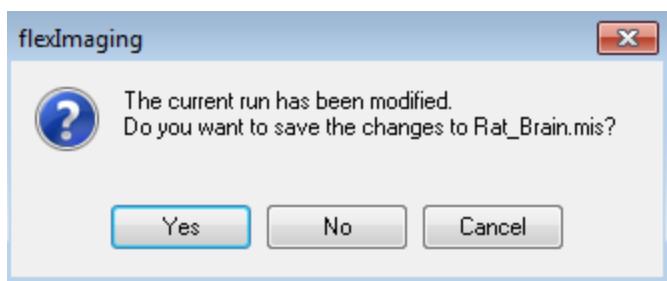


Figure 3-3 Confirmation request to save the modified imaging run

If the current imaging run has not been modified or changes have already been saved, you will be asked whether to close flexImaging. Confirm the request to close flexImaging.

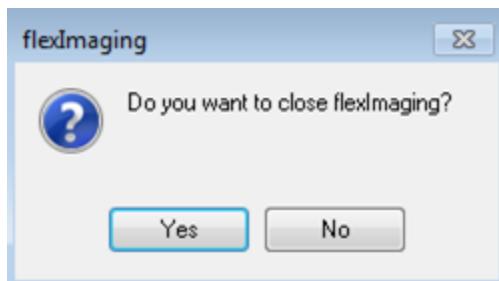


Figure 3-4 Confirmation request to close flexImaging

4 flexImaging Graphical User Interface

4.1 Quick Reference Tables	24
4.2 flexImaging Windows	35
4.3 flexImaging Menus	52
4.4 flexImaging Shortcut Menus	91
4.5 flexImaging Dialogs	117

Figure 4-1 shows a sample screen shot of the flexImaging graphical user interface.

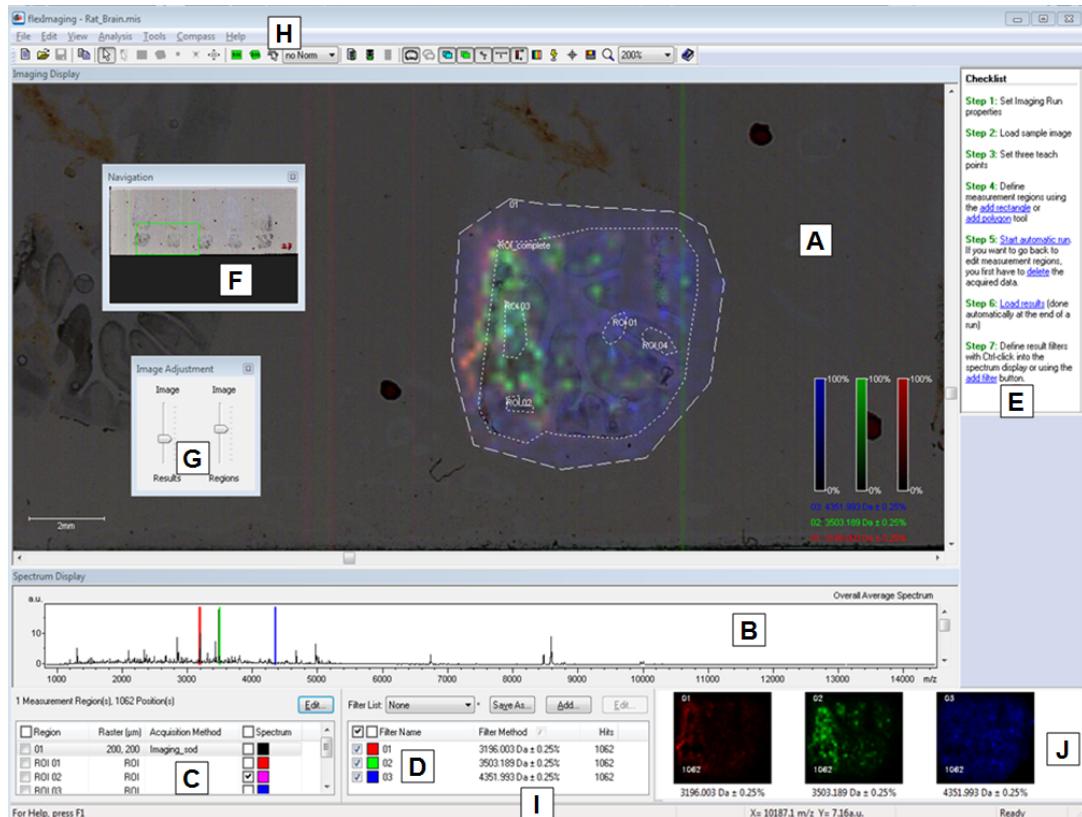


Figure 4-1 Sample screen shot of the flexImaging 5.0 graphical user interface with acquired data

The user interface consists of the following elements:

- A. Imaging Display (see section 4.2.1)
- B. Spectrum Display (see section 4.2.2)
- C. Regions Pane (see section 4.2.3)
- D. Results Pane (see section 4.2.4)
- E. Checklist (see section 4.2.10)
- F. Navigation Window (see section 4.2.5)
- G. Image Adjustment Window (see section 4.2.6)
- H. Menu bar (see section 4.1.2) and toolbar (see section 4.1.1)
- I. Status bar (see section 4.2.11)
- J. Filter Preview (see section 4.2.9)

Other elements available but not shown in the preceding figure are:

- Histogram Window (see section 4.2.7)
- Dendrogram Window (see section 4.2.8)

4.1 Quick Reference Tables

4.1.1 flexImaging 5.0 Toolbar	24
4.1.2 flexImaging 5.0 Menus and Commands	28
4.1.3 flexImaging 5.0 Shortcuts	33

4.1.1 flexImaging 5.0 Toolbar

flexImaging 5.0 provides the following buttons in its toolbar by default. Some additional buttons are available via the Customize Toolbar command from the Tools menu. All tool buttons offered are referenced below with the corresponding menu command, description and shortcut if available.



Figure 4-2 flexImaging toolbar

Table 4-1 Default toolbar commands

Button	Menu - Command	Used to	Shortcut
	File – New Imaging Run	Create a new imaging run.	Ctrl+N
	File – Open Imaging Run	Open an existing imaging run.	Ctrl+O
	File – Save Imaging Run	Save the current imaging run to the same name.	Ctrl+S
	Edit – Copy Image to Clipboard	Copy the current image to the clipboard.	
		Disable active buttons with editing functions.	
	Edit – Set Teach Points	Activate/Deactivate the Set Teach Points mode.	
	Edit – Add Rectangular Measurement Region	Activate/Deactivate the Add Rectangular Measurement Region mode.	
	Edit – Add Polygon Measurement Region	Activate/Deactivate the Add Polygon Measurement Region mode.	
	Edit – Add Measurement Spot	Activate/Deactivate the Add Measurement Spot mode.	
	Edit – Delete Measurement Spot	Activate/Deactivate the Delete Measurement Spot mode.	
	Edit – Move Sample Carrier	Re-position the sample carrier in flexControl.	
	Edit – Add Rectangular Region of Interest	Activate/Deactivate the Add Rectangular Region of Interest mode.	
	Edit – Add Polygon Region of Interest	Activate/Deactivate the Add Polygon Region of Interest mode.	
	Edit – Show Single Spectrum	Activate/Deactivate the Show Single Spectrum mode.	

Button	Menu - Command	Used to	Shortcut
	Edit – Imaging Run Properties – Processing Options – Normalize Spectra	Normalize spectra.	
	File – Save autoXecute Sequence As	Save the autoXecute imaging run and geometry file.	
	File – Start autoXecute Run	Save the autoXecute imaging run and geometry file and start the autoXecute run in flexControl.	
	File – Abort autoXecute Run	Abort the current autoXecute run in flexControl.	
	View – Sample Image	Show/Hide the sample image.	Alt+S
	View – Co- Registered Image	Show/Hide the co- registered image.	Alt+C
	View – Measurement Regions	Show/Hide the edited measurement regions resp. measurement spot groups.	Alt+M
	View – Regions of Interest	Show/Hide the edited regions of interest.	Alt+R
	View – Teach Points	Show/Hide the teach points.	Alt+T
	View – Ruler	Show/Hide the ruler.	Alt+B
	View – Intensity Scale	Show/Hide the intensity scale.	Alt+I
	View – Result Color Gradient	Show/Hide the color gradient for the result.	Alt+G
	View – Laser Spots	Show/Hide the laser spots.	Alt+L
	View – Sample Position	Show/Hide the sample position.	Alt+P
	View – reg. Images as B/W	Display the co-registered image as a grayscale image.	
		Activate/Deactivate the zoom in mode for the Imaging Display.	

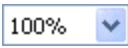
Button	Menu - Command	Used to	Shortcut
 100%		Zoom the image by the selected factor.	
	Help – Help Topics	Launch flexImaging Help.	F1

Table 4-2 Additional tool buttons

Button	Menu - Command	Used to
	Help – About flexImaging	Show copyright and license information about the present flexImaging installation.
	Tools – Preferences	Set preferences.
	Edit – Automatic Mass Filtering	Perform an automatic mass filtering.
	Edit – Imaging Run Properties	Edit imaging run properties.

4.1.2 flexImaging 5.0 Menus and Commands

flexImaging 5.0 provides the following menus and commands. The meaning and corresponding toolbar button and shortcut, if available, are also included.

Table 4-3 File menu commands

Command	Used to...	Button	Shortcut
New Imaging Run	Create a new imaging run.		Ctrl+N
Open Imaging Run	Open an existing imaging run.		Ctrl+O
Save Imaging Run	Save the current imaging run to the same file name.		Ctrl+S
Save Imaging Run As	Save the current imaging run to another file name.		
Save autoXecute Sequence As	Save the autoXecute imaging run and geometry file.		
Start autoXecute Run	Save the autoXecute imaging run and geometry file and start the autoXecute run in flexControl.		
Delete Acquired Data	Delete the acquired autoXecute imaging run data.		
Import >	Pop up import commands.		
> Spot List	Import a spot list from a sample preparation robot.		
Export >	Pop up export commands.		
> Image	Export the current image.		
> Acquired Data	Export the acquired data, "Analyze" format		
> imzML	Export the acquired data, "imzML" format.		
> Spectra List	Export the spectra list.		

Command	Used to...	Button	Shortcut
> Spot List	Export the spot list.		
> Mass Filters to MCL	Export the mass filters as a Mass Control List		
Recent Imaging Runs >	Pop up the recent imaging run list.		
> Recent File	Open the selected recent imaging run.		
Exit	Close flexImaging.		Alt+F4

Table 4-4 Edit menu commands

Command	Used to ...	Button	Shortcut
Copy Image to Clip-board	Copy the current image to the Clip-board.		
Set Teach Points	Activate/Deactivate the Set Teach Points mode.		
Delete Last Teach Point	Delete the last set teach point.		
Clear Teach Points	Clear all teach points.		
Add Rectangular Measurement Region	Activate/Deactivate the Add Polygon Measurement Region mode.		
Add Polygon Measurement Region	Activate/Deactivate the Add Polygon Measurement Region mode.		
Add Measurement Spot	Activate/Deactivate the Add Measurement Spot mode.		
Delete Measurement Spot	Delete a measurement spot		
Move Sample Carrier	Re-position the sample carrier in flexControl.		

Command	Used to ...	Button	Shortcut
Co-Register Image	Co-register a second image.		
Add Rectangular Region of Interest	Activate/Deactivate the Add Rectangular Region of Interest mode.		
Add Polygon Region of Interest	Activate/Deactivate the Add Polygon Region of Interest mode.		
Automatic ROI Array	Automatically create an array of equal regions.		
Show single Spectrum	Activate/Deactivate the Show Single Spectrum mode.		
Imaging Run Properties	Edit imaging run properties.		Alt+Return
Automatic Mass Filtering	Perform automatic mass filtering.		

Table 4-5 View menu commands

Command	Used to ...	Button	Shortcut
Zoom >	Pop up various zoom commands for the Imaging Display.		
> Fit to Window	Adjust the image size to the current size of the Imaging Display.		
> Zoom In	Expand the image according to the predefined zoom steps.		
> Zoom Out	Scale the image down according to the predefined zoom steps.		
Sample Image	Show/Hide the sample image.		Alt+S
Co- Registered Image	Show/Hide the co-registered image.		Alt+C
Co-Registered Images	Open dialog to select or remove a co-registered image.		

Command	Used to ...	Button	Shortcut
reg. Images as B/W	Display a grayscale sample image.		
Measurement Regions	Show/Hide the measurement regions.		Alt+M
Regions of Interest	Show/Hide the regions of interest.		Alt+R
Teach Points	Show/Hide the teach points.		Alt+T
Ruler	Show/Hide the ruler.		Alt+B
Intensity Scale	Show/Hide the intensity scale.		Alt+I
Intensity Captions	Show/Hide the intensity captions.		
Horiz. Scale	Show intensity scales horizontally.		
Result Color Gradient	Show/Hide the result color gradient.		Alt+G
Laser Spots	Show/Hide the laser spots.		Alt+L
Sample Position	Show/Hide the sample position.		Alt+P
View Normalization Symbol	Show/Hide the normalization method symbol.		
Skyline Projection	Switch between maximum spectrum (skyline projection) and overall average spectrum.		
Refresh Results	Refresh results.		F5
Toolbar	Show/Hide the toolbar.		
Windows >	Pop up commands to show/hide windows.		
> Spectrum Display	Show/Hide the Spectrum Display.		
> Regions	Show/Hide the Regions Pane.		
> Results	Show/Hide the Result Pane.		
> Checklist	Show/Hide the Checklist.		
> Navigation	Show/Hide the Navigation Window.		

Command	Used to ...	Button	Shortcut
> Image Adjustment	Show/Hide the Image Adjustment Window.		
> Filter Preview	Show/Hide the Filter Preview Window.		
> Restore Default Layout	Restore the default layout.		
Status Bar	Show/Hide the status bar.		

Table 4-6 Analysis menu commands

Command	Used to ...	Button	Shortcut
Hierarchical Clustering	Open a dialog to enter hierarchical clustering parameters.		

Table 4-7 Tools menu commands

Command	Used to ...	Button	Shortcut
Customize Toolbar	Customize the toolbar.		
Preferences	Set preferences.		
Convert Hamamatsu slide	Convert a Hamamatsu digital slide to BigTIFF format usable for co-registration.		

Table 4-8 Compass menu commands

Command	Used to ...	Shortcut
License	Launch Bruker LicenseManager.	
Operator	Log in the specified operator.	
Lock All Applications	Lock all Bruker applications using UserManagement.	Ctrl+Alt+K
Compass Desktop	Launch the Compass Desktop.	F11
flexAnalysis	Switch to flexAnalysis.	Shift+Alt+ F11
flexControl	Switch to flexControl.	Ctrl+F11

Command	Used to ...	Shortcut
DataAnalysis	Switch to DataAnalysis.	Alt+F11
HyStar	Switch to HyStar.	Shift+F11

Table 4-9 Help menu commands

Command	Used to ...	Button	Shortcut
Help Topics	Launch flexImaging Help.		F1
Report Status	Launch the Status Reporter.		
About flexImaging	Show copyright and license information about the present flexImaging installation.		

4.1.3 flexImaging 5.0 Shortcuts

flexImaging 5.0 provides the following shortcuts, which are included in the menu, e.g. 'New Imaging Run Ctrl+N':

Shortcut	Menu – Command	Used to ...
F1	Help – Help Topics	Launch flexImaging Help.
F5	View – Refresh Results	Refresh results.
F11	Compass – Compass Desktop	Launch Compass Desktop.
Alt+F4	File – Exit	Close flexImaging.
Alt+F11	Compass – DataAnalysis	Switch to DataAnalysis.
Alt+B	View – Ruler	Show/Hide the ruler.
Alt+C	View – Co-Registered Image	Show/Hide the co-registered image.
Alt+G	View – Color Gradient	Show/Hide the result color gradient.

Shortcut	Menu – Command	Used to ...
Alt+I	View – Intensity Scale	Show/Hide the intensity scale.
Alt+L	View – Laser Spots	Show/Hide the laser spots.
Alt+M	View – Measurement Regions	Show/Hide the edited measurement regions or measurement spot groups.
Alt+P	View – Sample Positions	Show/Hide the sample position.
Alt+R	View – Regions of Interest	Show/Hide the edited regions of interest.
Alt+S	View – Sample Image	Show/Hide the sample image.
Alt+T	View – Teach Points	Show/Hide the teach points set.
Alt+Return	Edit – Imaging Run Properties	Edit imaging run properties.
Ctrl+F11	Compass – flexControl	Switch to flexControl.
Ctrl+H	Shortcut menu Spectrum Display – Hide Mass Filter Mark	Disable the temporary mass filter created in the Mass Filter Selection mode and hide the corresponding purple line.
Ctrl+M	Shortcut menu Spectrum Display – Add Mass Filter	Add the current mass selection to the result filter list.
Ctrl+N	File – New Imaging Run	Create a new imaging run.
Ctrl+O	File – Open Imaging Run	Open an existing imaging run.
Ctrl+S	File – Save Imaging Run	Save the current imaging run to the same file name.
Ctrl+Alt+K	Compass – Lock All Applications	Lock all Bruker applications using UserManagement.
Shift+Alt+F11	Compass – flexAnalysis	Switch to flexAnalysis.
Shift+F11	Compass – HyStar	Switch to HyStar.

4.2 flexImaging Windows

4.2.1 Imaging Display	36
4.2.2 Spectrum Display	37
4.2.3 Regions Pane	41
4.2.4 Results Pane	43
4.2.5 Navigation Window	44
4.2.6 Image Adjustment Window	45
4.2.7 Histogram Window	47
4.2.8 Dendrogram Window	48
4.2.9 Filter Preview Window	49
4.2.10 Checklist	49
4.2.11 Status Bar	50
4.2.12 Customizing the Window Layout	51

4.2.1 Imaging Display

The Imaging Display is the most versatile element of the flexImaging user interface. It is used to specify measurement regions when preparing a measurement; after data acquisition it is used to visualize the results.

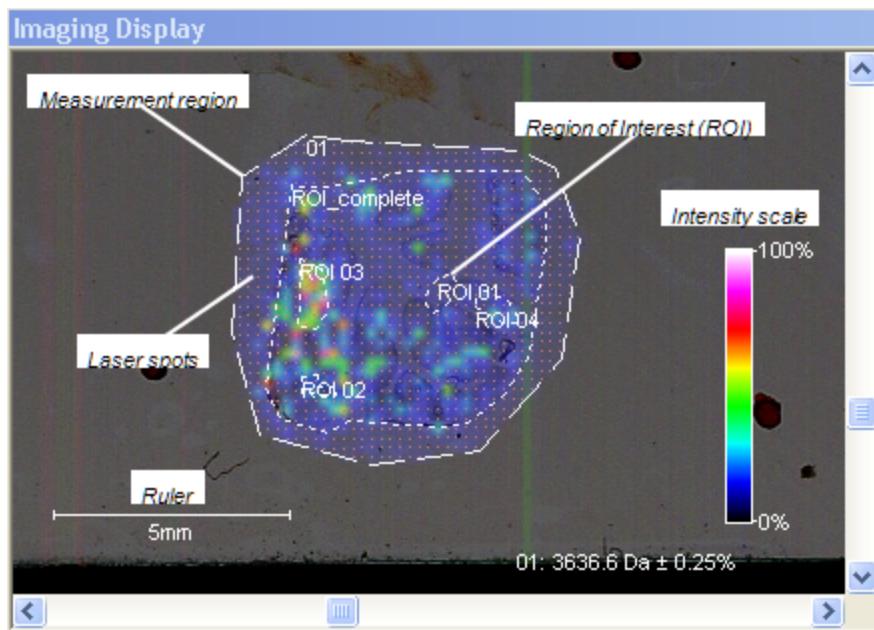


Figure 4-3 Imaging Display: Sample image (section) with edited measurement region and regions of interest being shown; results are visualized by showing an intensity plot of a selected result filter

The Imaging Display can show the following information: sample image , co-registered image, measurement regions, regions of interest, teach points, ruler, intensity scale, laser spots, sample position and result color gradient. These display options can be activated either via the **View** menu or with the corresponding toolbar buttons.

The Imaging Display also visualizes the results by showing an intensity plot of one or more selectable result filters, e.g. the intensity of the spatial distribution of a protein with a certain mass. flexImaging draws the results as an overlay onto the sample-image or co-registered image. To differentiate the results from each other and the sample image, results are plotted with different colors. The intensity of the color at a certain location reflects the data value of the result at that point, e.g. a higher peak is displayed brighter.

To view finer details, the user can zoom into the image by selecting a zoom level from the drop-down selection box in the toolbar or selecting "Zoom in" or "Zoom out" from the "View / Zoom" menu (these methods zoom around the center of the image), by drawing a rectangle in the result window when the "zoom" toolbar button is active, or by turning the mouse wheel (this zooms around the current mouse position). He can pan the visible part with the scrollbars at the bottom and at the right side or, when the "zoom" toolbar button is not active, by simply holding down the left mouse button (the cursor then changes to a hand ) and dragging the image in the desired direction.

4.2.2 Spectrum Display

The Spectrum Display displays one or multiple traces for the acquired data. It can show the following spectra:

- Overall average spectrum (visible by default)
- Average spectrum for measurement region (activated by check box in Regions Pane)
- Average spectrum for region of interest (activated by check box in Regions Pane)
- Average spectrum for result hits (activated in the result list shortcut menu)
- Single spectrum of selectable spot (using the **Show Single Spectrum** command from Edit menu)

In addition, the mass filters of the results list selected in the Results Pane are displayed as bars in the spectrum using the color of the respective filter. Active filters are drawn solid, inactive using a pattern.

The Spectrum Display offers different display types depending on whether only a single or multiple spectra are enabled in the Regions Pane. For a single spectrum enabled, a 1D Scan plot is shown. For multiple spectra enabled, the 2D scan plot, 2D Density plot, 2D Stack plot and 2D All Scans plot display types are offered.

1D Scan plot: This plot displays if only one spectrum is enabled.

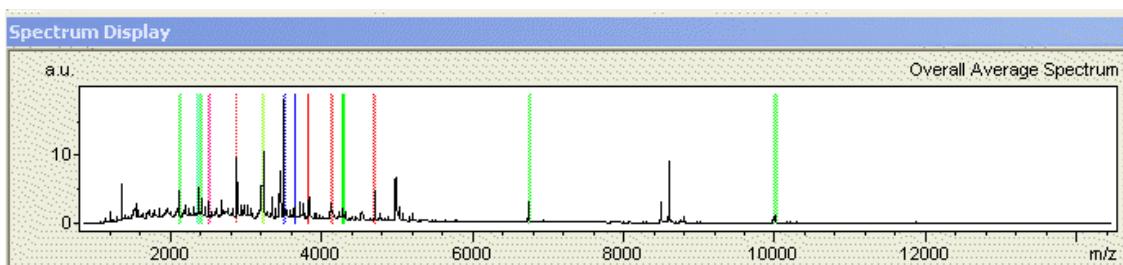


Figure 4-4 1D Scan plot

2D Scan plot: This plot can show all enabled spectra but displays always only one spectrum at a time. The name of the currently active spectrum is displayed in the upper right corner of the plot. The spectra can also be identified due to their colors. You can use the scroll bar to browse through the spectra.

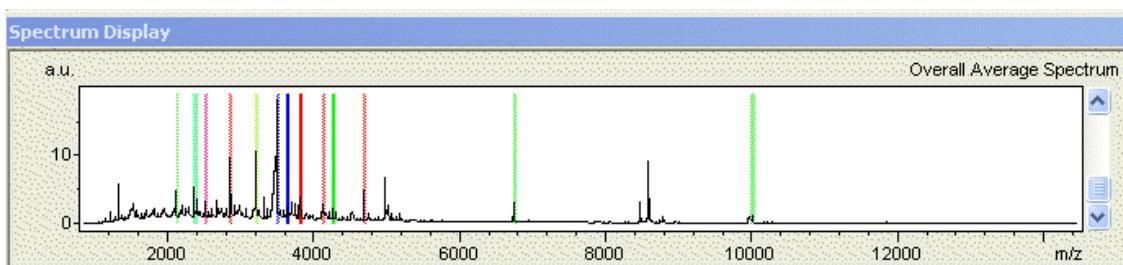


Figure 4-5 2D Scan plot

2D Density plot: This plot shows all multiple enabled spectra at the same time. It displays the height of the spectra signals using different colors or intensities. This type is a good choice for comparing spectra and identifying peaks that have different heights in different regions.

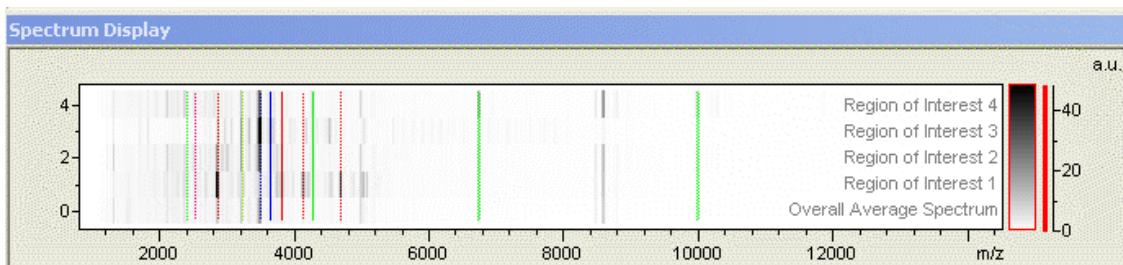


Figure 4-6 2D Density plot

2D Stack plot: This plot stacks all enabled spectra at the same time. The orientation of the stack plot axes can be changed by dragging with the mouse.

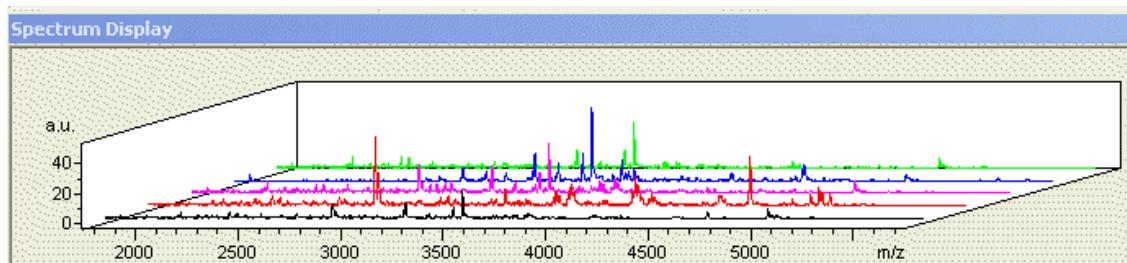


Figure 4-7 2D Stack plot

2D All Scans plot: This plot can show all enabled spectra. Each spectrum is drawn with its color. This type can also be used for comparing spectra and identifying peaks that have different heights in different regions.

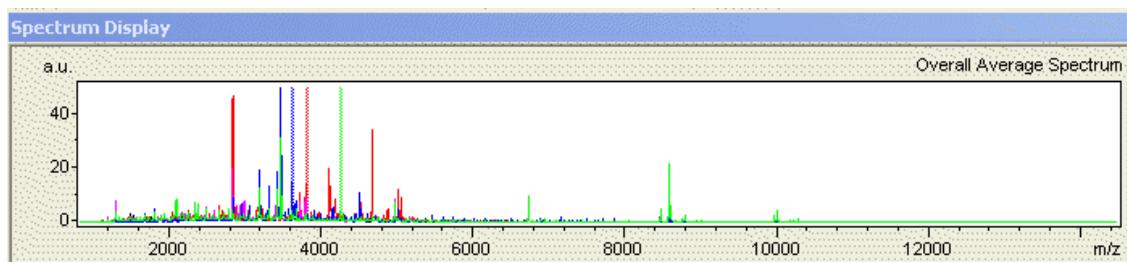


Figure 4-8 2D All Scans plot

The Spectrum Display offers different shortcut menus depending on the click area (X-axis, Y-axis, inside spectrum) and the display type.

If zooming is activated in the Spectrum Display shortcut menu, zooming into the spectrum is performed by dragging a rectangle inside the spectrum. A single click undoes the last zoom operation.

Dragging the X- or Y-axis using the left mouse button shifts the axis, dragging it with the right mouse button zooms in or out of the spectrum. A double-click on an axis resets the scaling for that axis, a double click inside the spectrum performs an auto scale for the entire display.

The user may also zoom by turning the mouse wheel. With the pointer in the spectrum window, both axes are zoomed simultaneously so that the point under the mouse pointer does not move. With the mouse cursor on an axis, only this axis is zoomed. If the user holds down the Shift key or the Control key while turning the wheel, the axis is shifted in small or large increments.

In mass filter selection mode, activated from the shortcut menu, clicking inside the spectrum generates a temporary mass filter that is displayed with a purple bar in the Spectrum Display. The result of this filter is immediately shown in the Imaging Display. If the Shift key is held down while clicking, the filter is placed on the local maximum inside an interval of +/- 1 pixel around the cursor position. Mass filter selection is also performed when the **Ctrl** key is pressed while clicking into the spectrum in zooming mode. This feature allows you to navigate through the spectrum and generate mass filters without having to switch modes in the shortcut menu. The user can remove temporary mass filter by clicking the middle mouse button, selecting the appropriate shortcut menu option, or by pressing the accelerator **Ctrl+H**. The user can move the temporary mass filter with the left and right arrow keys. Normal step size is about half the width of filter. For FTMS spectra, if the Shift key is held down while the arrow keys are hit, the filter mark is moved to the next peak in the spectrum that is higher than 5×10^{-4} of the highest peak in the spectrum. This allows scanning a FTMS spectrum at reasonable speed.

Zooming by dragging a rectangle and mass filter selection is not available in the 2D stack plot.

4.2.3 Regions Pane

The Regions Pane contains a list of defined measurement regions and regions of interest.

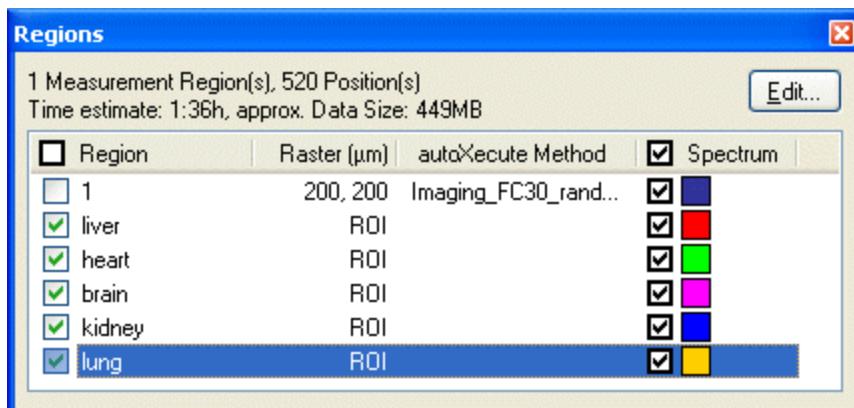


Figure 4-9 Regions Pane (undocked)

The text above the region list denotes the number of defined measurement regions and spots. flexImaging is able to estimate the expected time or data size of the measurement, this information is also displayed. Depending on the mode, it can be necessary that flexControl is running so that flexImaging can retrieve certain parameters for this estimation (e.g. shot count and laser frequency). For the data volume it is also necessary that you already have acquired a spectrum with the current flexControl method. Both estimations are only available before running the imaging run.

In the arbitrary array or spot microarray use-cases (specified on the second page of the **New Imaging Run Wizard**), measurement regions are added using the **Add Rectangular Measurement Region** or **Add Polygon Measurement Region** commands. In the tissue profiling use-case, measurement locations are specified using the **Add Measurement Spot** command.

Measurement regions can only be added or changed before data have been acquired for the imaging run. Use the **Delete Acquired Data** command to delete the existing spectra to continue editing measurement regions.

Region of interest are added using the **Add Rectangular Region of Interest** or **Add Polygon Region of Interest** command. Regions of interest can be added before or after data have been acquired for a imaging run.

The **Edit** button or double clicking a row in the list will open either the **Edit Measurement Region Parameters** dialog or the **Edit Region of Interest Parameters** dialog depending on the type of the region.

Each row in the regions list displays the following information (from left to right):

- the left checkbox limits the intensity plot shown in the Imaging Display to the enabled regions; if no checkbox in this column is marked, the entire intensity plot is shown
- an optional name of the region which is also shown in the Imaging Display
- the desired raster width used for data acquisition in this regions; regions of interest are marked with the letters “ROI”
- the name of the autoXecute method used for data acquisition of this region
- a checkbox enabling/disabling display of an average spectrum for the region in the Spectrum Display
- the color of the spectrum is shown with a colored box

The checkboxes in the list header activate/deactivate the corresponding checkbox of all regions.

You can use the following regions list keyboard shortcuts:

Key	Function
-----	----------

Space Toggle left checkbox

Return Open dialog for editing parameters of current region

Del Delete current region

4.2.4 Results Pane

The Results Pane contains a list of different kind of result filters that flexImaging uses to create intensity plots in the Imaging Display.

The screenshot shows a dialog titled 'Results Pane'. At the top left is a dropdown menu labeled 'Filter List' with 'Proteins' selected. To its right are four buttons: 'Save As...', 'Add...', and 'Edit...'. Below these buttons is a table with five rows of data. The first column contains checkboxes, the second column contains filter names with color-coded squares, the third column contains filter methods, and the fourth column contains the number of hits. The fifth column contains up and down arrow buttons for sorting. The data is as follows:

Filter Name	Filter Method	Hits
Ubiquitin_I	8565.89 Da ± 20 Da	1061
Myoglobin_[M+2H]2+	8476.77 Da ± 20 Da	1061
Cytochrome_C	6181.05 Da ± 20 Da	1062
Insulin	5734.56 Da ± 20 Da	1061

Figure 4-10 Results Pane

The drop-down list contains all available filter lists from the Imaging Runs directory including the ones installed along with flexImaging and the directory of the current imaging run. The content changes as you save your lists using the **Save As** button, which displays the **Save Result Filter List As** dialog.

The **Add** button opens the **Add Result Filter** dialog, which is used to append filters to the list.

Depending on the filter type, the **Edit** button opens the respective dialog: **Edit Mass Filter Parameters**, **Edit ClinProTools Classification Filter Parameters**, **Edit ClinProTools PCA Filter Parameters**, **Edit ClinProTools PLSA Filter Parameters**, **Edit XML Import Filter Parameters** or **Edit Text Import Filter Parameters**. Holding the Ctrl key and clicking multiple rows opens the **Edit Multiple Result Filters** dialog.

Each row in the result filter list displays the following information (from left to right):

- the checkbox enables the intensity plot shown in the Imaging Display for the filter
- the color square denotes the color used for intensity plots
- an optional filter name assigned in the respective edit filter parameters dialog
- the filter method (e.g. mass range for mass filters)
- the number of detected results for this filter in the Hits column
- optionally you can also enable a column showing the average value from the shortcut menu of the list header

The left checkbox in the list header enables/disables all result filters. If the checkbox on its right is marked, only a single filter will be enabled.

The result filter list can be sorted by **Filter Name**, **Filter Method**, **Hits** and **Average** by clicking on the corresponding item of the list header. The active sorting column and order is displayed with a small triangle.

You can use the following filter list keyboard shortcuts:

Key	Function
Space	Enable/disable current filter.
Return	Open dialog for editing parameters of current filter
Del	Delete current filter. If multiple rows are selected, you must confirm deletion of all selected filters.
Up, Down	Move the selected line (if any). In single selection mode, the filter newly selected is also enabled, the previous disabled.

4.2.5 Navigation Window

The Navigation Window gives you an overview which part of your sample image is currently shown in the Imaging Display. The window displays the entire image with the currently shown part enclosed by a green frame.



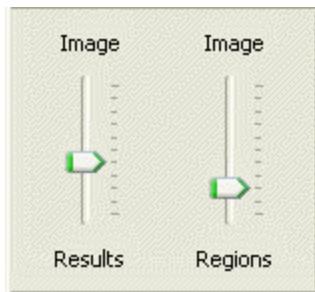
Figure 4-11 Navigation window

4.2.6 Image Adjustment Window

The Image Adjustment Window is used to control the blending of sample image, regions and results or to adjust the minimum intensity and full intensity threshold of a result filter.

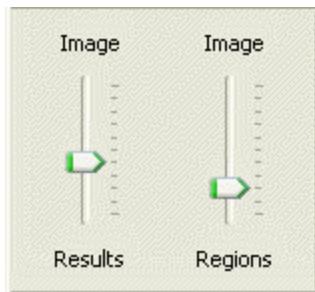
These modes are automatically switched depending on the current context:

Sample Image  and at least one result filter are activated



With the left slider you can cross-fade between showing 100% of the image and 100% of the result, so that you adjust how intensive both should be displayed.

Sample Image  and **Measurement Regions**  are activated

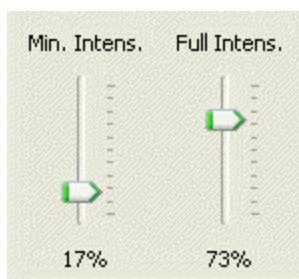


With the right slider you can cross-fade between showing 100% of the image and 100% of the regions, so that you adjust how intensive both should be displayed.

Sample Image and Measurement Regions are deactivated and ...

... one result filter is activated and focused in the Results Pane

Filter Name	Filter Method
1	ClinProt Class 1
Complete class 1	ClinProt Class 1



Using the left slider you can adjust the **Minimum Intensity Threshold** between 0 and 100%

With the right slider you can adjust the **Full Intensity Threshold** between 0 and 100%

Note: It is not possible to set the **Minimum Intensity Threshold** higher than the **Full Intensity Threshold**.

In all cases you can adjust the sliders either with the mouse or with the up and down keys of your keyboard.

4.2.7 Histogram Window

The Histogram Window displays the division of values for a single result filter. The X-axis shows the range of values, the Y-axis lists their frequency. The dashed lines mark the minimum intensity and full intensity threshold for the filter. You can use the mouse to drag these lines and modify these thresholds. The imaging display is updated accordingly in real time.

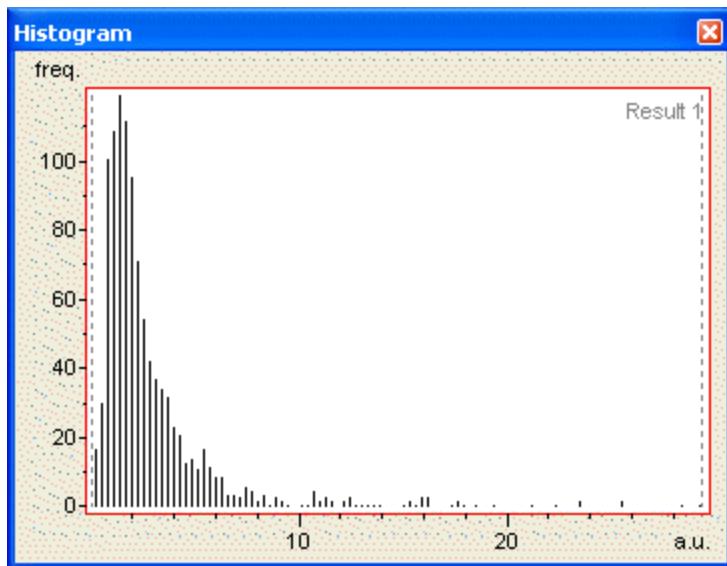


Figure 4-12 Histogram window

4.2.8 Dendrogram Window

The Dendrogram Window displays imported ClinProTools clustering trees. Each branch in the dendrogram displays the distance to its predecessor (or the ClinProTools number, selectable in the shortcut menu) and the number of nodes below this item. Right clicking on a branch opens a shortcut menu, which allows creating, deleting, activating or deactivating result filters for the specified branch. Branches for result filters are shown in their color, deactivated filters are drawn in a dashed style. The imaging display is updated in real time when modifying clustering tree filters.

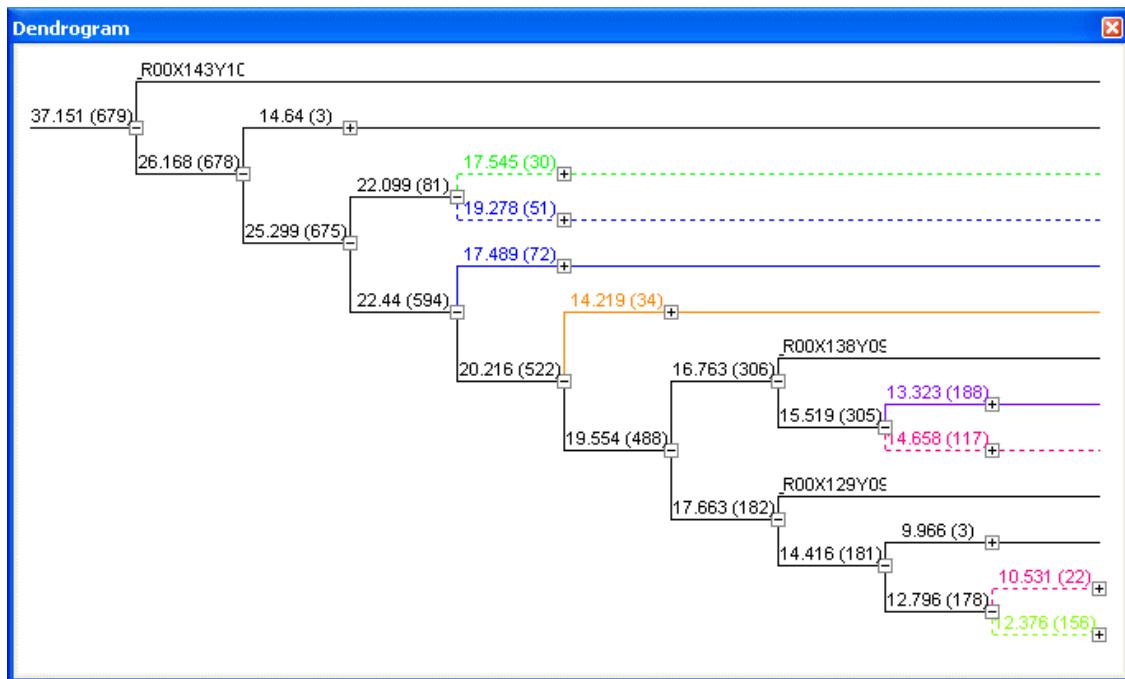


Figure 4-13 Dendrogram Window

4.2.9 Filter Preview Window

The Filter Preview displays thumbnail images of all currently loaded filters, together with their name, number of hits and mass range or statistics. This window is optionally docked into the main window like the regions window or the result window.

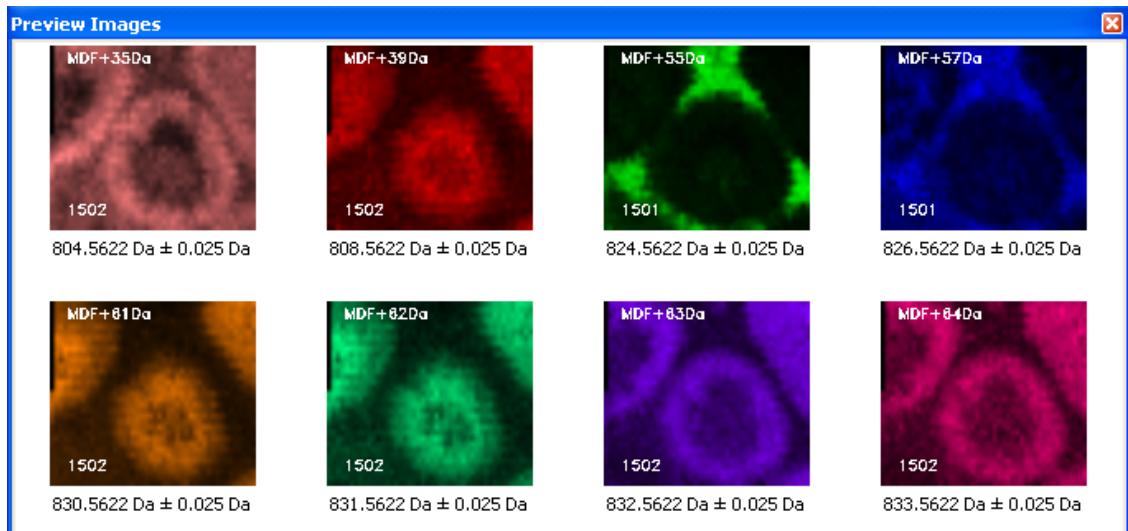


Figure 4-14 Filter Preview Window

4.2.10 Checklist

The right pane of the GUI of flexImaging contains a dynamically working checklist, which always reflects the current state of the program. The green marked steps show the ones which have been already done successfully; the black one shows the actual step, which should be done next and the other steps are still disabled. The checklist gives you detailed information to the current step and less information to the steps already done. In the current step some words are underlined. If you click on these, the software will react as if you have clicked on the corresponding toolbar button. As a result this button will be activated.

4.2.11 Status Bar

The flexImaging status bar is located at the bottom of the main window.

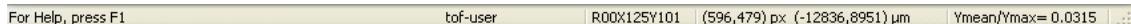


Figure 4-15 flexImaging status bar

The status bar displays the following information (from left to right):

- short help message for the item under the mouse cursor; if no help is available, the general "For Help, press F1" message is shown
- the logged in user
- the spot name at the cursor location, if the cursor is in the Imaging Display
- the cursor location in sample image pixels and as sample carrier coordinates, if the cursor is in the Imaging Display. If the cursor is in the Spectrum Display, the X and Y coordinates within the spectrum are shown.
- the normalization value of the sample under the cursor, if the cursor is in the Imaging Display and data have been acquired. You can use this value to adjust the normalization threshold in the **Imaging Run Properties - Processing Options** dialog accordingly.

4.2.12 Customizing the Window Layout

You can customize the window layout according to your personal preferences by repositioning and resizing the individual windows. The Imaging Display is always displayed above the Spectrum Display; their size can be modified by dragging the small divider line between them.

The other windows (Regions Pane, Results Pane, Navigation Window, Image Adjustment Window and Checklist) can be moved around, resized and docked along the frame of the flexImaging main window. Repositioning occurs by clicking on the window background and dragging it to the desired location. Changing from floating to docked state and back occurs by double clicking on the window background or moving it towards a border of the main window. Resizing is only possible in floating state by dragging the window border to the requested size.

Docking can be disabled temporary by pressing the **Ctrl** key while dragging the window.

To disable docking permanently for a window, right click on its background or title bar to open the docking window shortcut menu and uncheck Enable docking in the shortcut menu. To re-enable docking, bring up the shortcut menu again and choose Enable docking.

Except the Imaging Display, all other windows can be hidden and displayed using the respective commands from the **View > Windows** menu.

The default window layout is restored with the **Restore Window Layout** command from the **View** menu.

4.3 flexImaging Menus

4.3.1 File Menu	53
4.3.2 Edit Menu	60
4.3.3 View Menu	68
4.3.4 Analysis Menu	76
4.3.5 Tools Menu	77
4.3.6 Compass Menu	78
4.3.7 Help Menu	89

4.3.1 File Menu

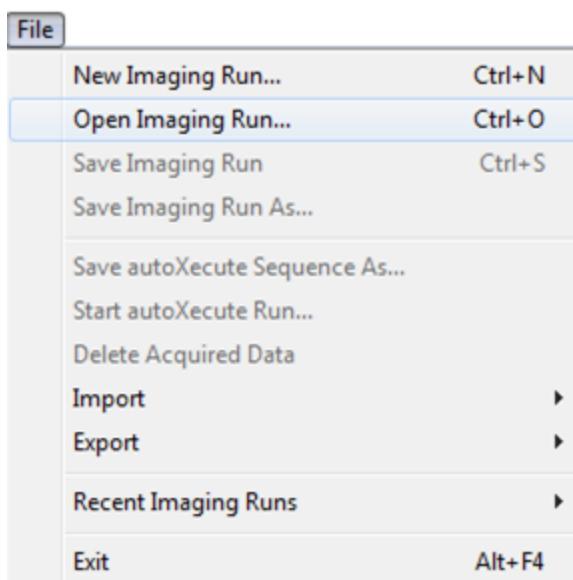


Figure 4-16 File menu

The File menu offers the following commands:

Command	Used to ...
New Imaging Run	Create a new imaging run.
Open Imaging Run	Open an existing imaging run.
Save Imaging Run	Save the current imaging run to the same file name.
Save Imaging Run As	Save the current imaging run to another file name.
Save autoXecute Sequence As	Save the autoXecute imaging run and geometry file.
Start autoXecute Run	Save the autoXecute imaging run and geometry file and start the autoXecute run in flexControl.
Abort autoXecute Run	Abort the current autoXecute run in flexControl.
Delete Acquired Data	Delete the acquired autoXecute imaging run data.
Import	Pop up import commands.

Command	Used to ...
Export	Pop up export commands.
Recent Imaging Runs	Pop up the recent imaging run list.
Exit	Close flexImaging.

4.3.1.1 New Imaging Run Command

The **New Imaging Run** command is used to create a new imaging run. The command launches the **New Imaging Run Wizard**.

Shortcuts

Button: 

Keys: Ctrl+N

4.3.1.2 Open Imaging Run Command

The **Open Imaging Run** command is used to open an existing imaging run. The command opens the **Open** dialog. Select the desired imaging run file and click **OK** to load that imaging run and corresponding data.

Shortcuts

Button: 

Keys: Ctrl+O

4.3.1.3 Save Imaging Run Command

The **Save Imaging Run** command saves the current imaging run to the same file name.

Shortcuts

Button: 

Keys: Ctrl+S

4.3.1.4 **Save Imaging Run As Command**

The **Save Imaging Run As** command is used to save the imaging run to another file name. The command opens the **Save As** dialog to enter a name and target folder for the new imaging run.

Note The new imaging run will not contain any already acquired spectra.

4.3.1.5 **Save autoXecute Sequence As Command**

The **Save autoXecute Sequence As** command is used to save a new autoXecute imaging run and the geometry file. flexImaging will save these files in the default folders (usually “D:\methods\AutoXSequences” and “D:\methods\GeometryFiles”). The files will be named “Imaging_” followed by the name of the imaging run. This allows running the autoXecute imaging run without the control by flexImaging either directly in flexControl or via the autoXecute Batch Runner.

The command opens the **autoXecute Run Options** dialog.

Shortcut

Button:



4.3.1.6 **Start autoXecute Run Command**

The **Start autoXecute Run** command is used to save a new autoXecute imaging run and the geometry file and after that to start the autoXecute run. At least one measurement region or measurement spot group must be defined to start an autoXecute run. The command opens the **autoXecute Run Options** dialog.

Note flexControl must be running.

Shortcut

Button:



4.3.1.7 Abort autoXecute Run Command

A started autoXecute run can be aborted by clicking . flexImaging will then load the data that have already been acquired.

Shortcut

Button: 

4.3.1.8 Delete Acquired Data Command

The **Delete Acquired Data** command deletes the acquired data, the autoXecute imaging run and the geometry, i.e. the imaging run becomes reset to its status before the autoXecute run was started. This allows you e.g. to change or delete measurement regions or measurement spots or do the teaching again.

4.3.1.9 Import popup Command

Pointing to **Import** offers the following command:



Command Used to ...

Spot List Import a spot list from a sample preparation robot.

4.3.1.10 Import > Spot List Command

The **Spot List** command is used to import a list of distributed spots from a sample preparation robot. The command launches the **Spot List Import Wizard**. This feature is only available in the tissue profiling use-case.

4.3.1.11 Export Popup Command

Pointing to Export offers the following commands:



Command	Used to ...
Image	Export the current image.
Acquired Data	Export the acquired data ("Analyze" format).
imzML	Export the acquired data ("imzML" format).
Spectra List	Export the spectra list.
Spot List	Export the spot list.
Mass Filters to MCL	Export all mass list filters to a mass control list (MCL).

4.3.1.12 Export > Image Command

The **Image** command is used to export the image shown in the Imaging Display to the specified file. The export always writes the entire image to the file, not only the displayed part. The export is done with 100% magnification. All other display options are used as shown in the Imaging Display. Supported file formats are Windows Bitmap (.bmp), JPEG and TIFF.

The command opens the **Save As** dialog to specify the file name, target folder and file format for the image export.

4.3.1.13 Export > Acquired Data Command

The **Acquired Data** command is used to export the acquired data to the specified file. Currently the Analyze™ 7.5 format (.img) is supported for export. This format is e.g. used by BioMap as interchange format. Please note the restrictions of this format: The number of data points is limited to 32767, missing support for non-rectangular or multiple measurement regions (flexImaging will write zeroline spectra for positions without acquired data). Using this command will only export the spectra data to the specified file, but not other information such as sample image(s), region or result information since these are not defined in the Analyze™ format.

The command opens the **Save As** dialog to specify the file name and target folder for the acquired data export.

This menu item is disabled for FTMS data.

4.3.1.14 Export > imzML Command

The **imzML** command is used to export the acquired data to the specified file in imzML format. This format is supported by several programs, e.g. Datacube Explorer by AMOLF. The command opens a dialog to specify some options and the file name and target folder for the export.

4.3.1.15 Export > Spectra List Command

The **Spectra List** command is used to generate a list of all spectra belonging to one or multiple regions. This list can e.g. be imported into ClinProTools. The command opens the **Export Spectra List** dialog.

4.3.1.16 Export > Spot List Command

The **Spot List** command is used to export all defined measured spots to the specified file.

The spots are exported in the ASCII format, one spot per line in the following format:

<X- position><separator><Y- position><separator><spot- name><separator><region-name>

<separator> is either a comma (for files with extension .csv) or a space (for all other files).

The positions are given in micrometers relative to the center position of the sample carrier for instruments using the MTP sample carrier (autoflex, ultraflex ...). There is a different offset for the microflex.

Example:

-47240 29042 R00X011Y012 MR_1

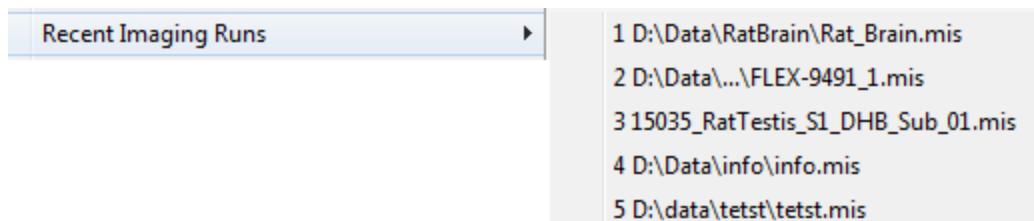
The command opens the **Save As** dialog to specify the file name and target folder for the spot list export.

4.3.1.17 Export > Mass Filters to MCL Command

The **Export Mass Filters to MCL** command is used to export all mass list filters to a mass control list (MCL). The command opens a file save dialog to choose the name of the desired MCL. After entering the file name and selecting OK, a MCL is generated.

4.3.1.18 Recent Imaging Runs Popup Command

Pointing to **Recent Imaging Runs** offers the recent imaging run list to load one of the last used imaging runs:



Selecting a file from this list opens that imaging run in flexImaging.

4.3.1.19 Exit Command

The **Exit** command closes flexImaging after you have answered the confirmation request. If the current imaging run was changed but has not been saved yet, a request to save the imaging run will appear. Answer the request to close flexImaging.

Shortcuts

Button: 

Keys: Alt+F4

4.3.2 Edit Menu

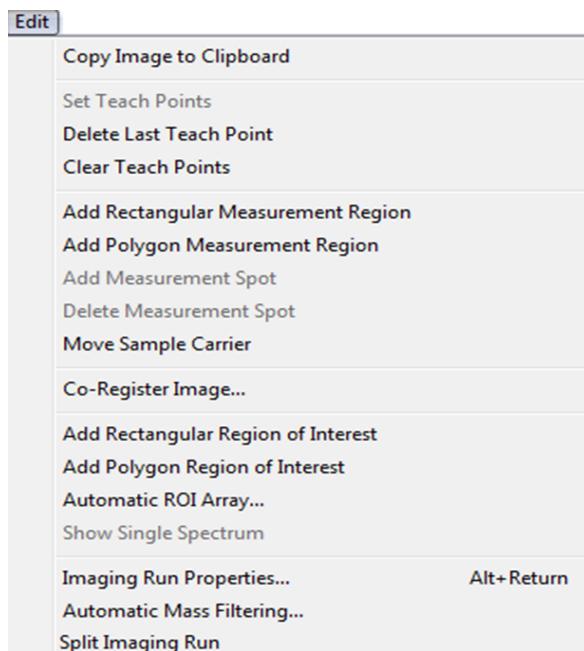


Figure 4-17 Edit menu

The **Edit** menu offers the following commands:

Command	Used to ...
Copy Image to Clipboard	Copy the current image to the clipboard.
Set Teach Points	Activate/Deactivate the Set Teach Points mode.
Delete Last Teach Point	Delete the last set teach point.
Clear Teach Points	Clear all teach points.
Add Rectangular Measurement Region	Activate/Deactivate the Add Rectangular Measurement Region mode.
Add Polygon Measurement Region	Activate/Deactivate the Add Polygon Measurement Region mode.
Add Measurement Spot	Activate/Deactivate the Add Measurement Spot mode.
Delete Measurement Spot	Activate/Deactivate the Delete Measurement Spot mode.
Move Sample Carrier	Re-position the sample carrier.
Co-Register Image	Co-register a second image.
Add Rectangular Region of Interest	Activate/Deactivate the Add Rectangular Region of Interest mode.
Add Polygon Region of Interest	Activate/Deactivate the Add Polygon Region of Interest mode.
Automatic ROI Array	Automatically create an array of equal regions.
Show Single Spectrum	Activate/Deactivate the Show Single Spectrum mode.
Imaging Run Properties	Edit imaging run properties.
Automatic Mass Filtering	Perform automatic mass filtering.
Split Imaging Run	Open the Split Imaging Run dialog.

4.3.2.1 Copy Image to Clipboard Command

The **Copy Image to Clipboard** command copies the image shown in the Imaging Display to the clipboard. This allows pasting the image into another application.

Note If the image is too large, Microsoft Windows will not copy it.

Shortcut

Button:



4.3.2.2 Set Teach Points Command

The **Set Teach Points** command activates/deactivates the Set Teach Points mode which allows you to redo the teaching you did in the **New Imaging Run Wizard** if you are not satisfied with the teaching. Using this feature requires first deleting the current teaching using the **Clear Teach Points** command or **Delete Last Teach Point** command.

Teach points are used to map image coordinates to sample carrier positions. To set teach points flexControl must be running. How teach points have to be set depends on the mode, arbitrary arrays, spot microarray or tissue profiling, chosen for the imaging run in the **New Imaging Run Wizard**.

Note Activating this feature will load the factory teaching of the current geometry in flexControl!

Shortcut

Button:



4.3.2.3 Delete Last Teach Point Command

The **Delete Last Teach Point** command deletes the last set teach point (or one after the other). You can use this command e.g. if you are not satisfied with the teaching you did in the **New Imaging Run Wizard** to be able to set (a) new teach point(s).

4.3.2.4 Clear Teach Points Command

The **Clear Teach Points** command deletes all teach points available after you have confirmed the corresponding request. You can use this command e.g. if you are not satisfied with the current teaching you did in the **New Imaging Run Wizard** to be able to set new teach points.

4.3.2.5 Add Rectangular Measurement Region Command

The **Add Rectangular Measurement Region** command activates/deactivates the Add Rectangular Measurement Region mode. A measurement region defines where flexControl should acquire data when the autoXecute run is started. This feature applies to the arbitrary arrays and spot microarray use-cases only.

When this mode is active, the Add Measurement Region cursor  is displayed when the mouse is on the image. A rectangular measurement region is created by drawing a rectangle on the image using the mouse. For this, position the mouse at the desired starting point and drag it with the left mouse button pressed. On releasing the mouse button, the enclosed region is entered as a new measurement region on the Regions Pane getting the name '[running number]'. You can change the default region name via the **Edit Measurement Region Parameters** dialog. To show the new region on the image, the **Measurement Regions** command must be active.

Shortcut

Button: 

4.3.2.6 Add Polygon Measurement Region Command

The **Add Polygon Measurement Region** command activates/deactivates the Add Polygon Measurement Region mode. A measurement region defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the arbitrary arrays and spot microarray use-cases only.

When this mode is active, the Add Measurement Region cursor  is displayed when the mouse is on the image. A polygon measurement region is created by drawing a polygon on the image using the mouse. For this, click once with the mouse to get the starting point. Then click to the edge points of your polygon. To delete the most recently added point, press the Backspace key. You can finish creating a polygon measurement region either by double-clicking or by positioning the mouse onto the first point of your polygon and clicking once. This enters the enclosed region as a new measurement region on the Regions Pane getting the name '[running number]'. You can change the default region name via the Edit Measurement Region Parameters dialog. To show the new region on the image, the Measurement Regions command must be active.

Shortcut

Button: 

4.3.2.7 Add Measurement Spot Command

The **Add Measurement Spot** command activates/deactivates the Add Measurement Spot mode. A measurement spot defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the tissue profiling use-case only.

When this mode is active, you can create a measurement spot group on the image and add measurement spots to it by clicking with the left mouse button to a selected position on the image. The first click creates a new measurement group with one spot and enters the spot group on the Regions Pane; the next clicks add further spots to this group. To create an additional spot group it is recommended to use the **Add Measurement Spot Group** command from the Regions Pane shortcut menu. New spots are always added to the active spot group selected on the Regions Pane. If no group is selected clicking with the left mouse button on the image automatically creates a new group.

Shortcut

Button: 

4.3.2.8 Delete Measurement Spot Command

The **Delete Measurement Spot** command activates/deactivates the Delete Measurement Spot mode. A measurement spot defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the tissue profiling use-case only.

When this mode is active, you can delete a measurement spot from the active spot group on the image by positioning the mouse on the spot to be deleted and clicking the left mouse button; the respective spot group is selected in the Regions Pane.

Shortcut

Button: 

4.3.2.9 Move Sample Carrier Command

The **Move Sample Carrier** command activates/deactivates the Move Sample Carrier mode which allows moving the sample carrier in flexControl to the position you select in flexImaging. When this mode is active, move the cursor to the desired position on the image; then click the left mouse button. This positions the sample carrier in flexControl accordingly. This feature is useful to check whether the teaching is satisfying.

Note Updating a position may take up to 5 seconds. Using this feature during an autoXecute run is not recommended, because the performance will be influenced.

Shortcut

Button:



4.3.2.10 Co-Register Image Command

The **Co-Register Image** command allows co-registering a second image, which is not a picture of your prepared slide but something else, e.g. a virtual microscopy slide or a radiograph. This might help you to compare the imaging results with other images. The second image will usually be a histologically stained reference section adjacent to the section used for the MALDI imaging experiment. In spot microarray or tissue profiling workflows it can also be a scan of the unspotted sample. The command opens the **Co-Register Image** dialog which is used to align the images properly. Co-Registration currently supports various image standards: normal TIFF and JPEG as well as tiled virtual microscopy slides created with Aperio, Hamamatsu, Mirax or Pannoramic scanners (with Aperio slides, only standard JPEG compression is currently supported, not JPEG2000).

4.3.2.11 Add Rectangular Region of Interest Command

The **Add Rectangular Region of Interest** command activates/deactivates the Add Rectangular Region of Interest mode. A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases.

When this mode is active, the Add Region of Interest cursor  is displayed when the mouse is on the image. A rectangular ROI is created by drawing a rectangle on the image using the mouse. On releasing the mouse button, the enclosed region is entered as a new ROI on the Regions Pane getting the name [ROI running number]. You can change the default name via the Edit Region of Interest Parameters dialog. To show the new ROI on the image the Regions of Interest command must be active.

Shortcut

Button: 

4.3.2.12 Add Polygon Region of Interest Command

The **Add Polygon Region of Interest** command activates/deactivates the Add Polygon Region of Interest mode. A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases.

When this mode is active, the Add Region of Interest cursor  is displayed when the mouse is on the image. A polygon region of interest (ROI) is created by drawing a polygon on the image using the mouse. For this, click once with the mouse to get the starting point. Then click to the edge points of your polygon. To delete the most recently added point, press the Backspace key. You can finish creating a polygon measurement region either by double-clicking or by positioning the mouse onto the first point of your polygon and clicking once. This places a dashed polygon with corresponding ROI number on the image and enters the new ROI in the Regions Pane.

Shortcut

Button: 

4.3.2.13 Automatic ROI Array Command

The **Automatic ROI Array** command opens the **Automatic ROI Array Parameters** dialog (see section 4.5.34).

A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases. In the dialog the user can specify parameters to fill all or part of the measurement region with rectangular regions of interest, all covering the same number of measurement spots. They are displayed as dashed rectangles with consecutive ROI numbers on the image and in the Regions Pane.

4.3.2.14 Show Single Spectrum Command

The **Show Single Spectrum** command activates/deactivates the Show Single Spectrum mode. When this mode is active, you can click to a spot of interest on the image to show the spectrum at cursor position in the Spectrum Display.

Shortcut

Button:



4.3.2.15 Imaging Run Properties Command

The **Imaging Run Properties** command is used to define imaging run properties. The command opens the **Imaging Run Properties** dialog.

Shortcuts

Button:



Keys: Alt+Return

4.3.2.16 Automatic Mass Filtering Command

After an autoXecute run is finished, the **Automatic Mass Filtering** command can be used to replace time-consuming manual peak filtering, or if you do not know which mass ranges may be considered. The software creates mass ranges based on your settings for this procedure and supplies the number of spectra (= hits) that contain a peak in the respective mass range. The command opens the **Automatic Mass Filtering** dialog.

4.3.2.17 Split Imaging Run Command

With the **Split Imaging Run** dialog you can split an imaging run with more than one measurement region into separate imaging runs. The dialog creates one imaging run for each measurement region.

4.3.3 View Menu

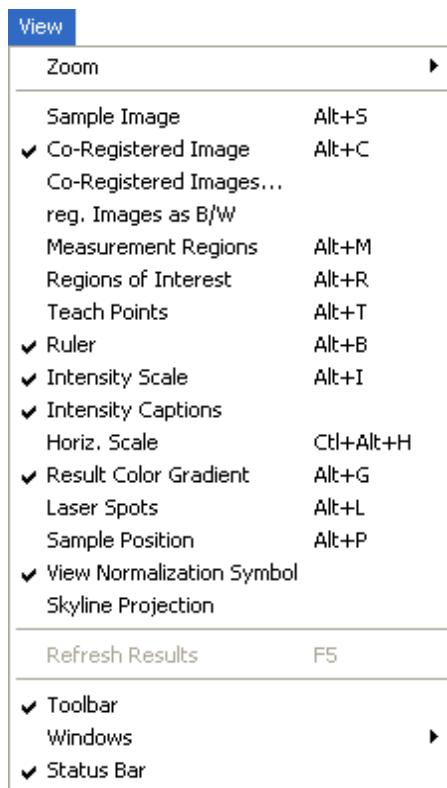


Figure 4-18 View menu

The **View** menu offers the following commands:

Command	Used to ...
Zoom	Pop up commands to zoom the displayed image.
Sample Image	Show/Hide the sample image.

Command	Used to ...
Co-Registered Image	Show/Hide the co-registered image.
Co-Registered Images	Open the Select Coregistered Image dialog.
reg. Images as B/W	Display the co-registered image as a grayscale image.
Measurement Regions	Show/Hide the edited measurement regions or measurement spot groups.
Regions of Interest	Show/Hide the edited regions of interest.
Teach Points	Show/Hide the teach points.
Ruler	Show/Hide the ruler.
Intensity Scale	Show/Hide the intensity scale.
Intensity Captions	Show/Hide the intensity scale captions.
Horiz. Scale	Display intensity scales as horizontal bars.
Result Gradient	Show/Hide the result color gradient.
Laser Spots	Show/Hide the laser spots.
Sample Position	Show/Hide the sample position.
View Normalization Symbol	Show/Hide the normalization symbol.
Skyline Projection	Switch between maximum spectrum (skyline projection) and overall average spectrum.
Refresh Results	Re(load) data.
Toolbar	Show/Hide the toolbar.
Windows	Pop up commands for showing/hiding windows.
Status Bar	Show/Hide the status bar.

4.3.3.1 Zoom Popup Command

Pointing to **Zoom** offers the following commands to change the zoom range of the displayed image:



Command Used to ...

Fit to Window Adjust the image size to the current size of the Imaging Display.

Zoom In Expand the image according to the predefined zoom steps.

Zoom Out Scale the image down according to the predefined zoom steps.

4.3.3.2 Zoom > Fit to Window Command

The **Fit to Window** command adjusts the size of the displayed image to fit the current size of the Imaging Display.

4.3.3.3 Zoom > Zoom In Command

The **Zoom In** command expands the displayed image according to the predefined zoom steps.

4.3.3.4 Zoom > Zoom Out Command

The **Zoom Out** command scales the displayed image down according to the predefined zoom steps.

4.3.3.5 Sample Image Command

The **Sample Image** command shows/hides the sample image in the Imaging Display. The sample image is shown by default.

Shortcuts

Button: 

Keys: Alt+S

4.3.3.6 Co-Registered Image Command

The **Co-Registered Image** command shows/hides the co-registered image in the Imaging Display. The co-registered image is hidden by default.

Shortcuts

Button: 

Keys: Alt+C

4.3.3.7 Co-Registered Images Command

The **Co-Registered Images** command opens the **Select Co-registered Image** dialog to select or remove an entry from the list of co-registered images.

4.3.3.8 reg. Images as B/W Command

The **reg. Images as B/W** command displays the co-registered image as a grayscale image. This avoids changing the colors of the result display.

Shortcuts

Button: 

4.3.3.9 Measurement Regions Command

The **Measurement Regions** command shows/hides the edited measurement regions resp. measurement spot groups in the Imaging Display. The measurement regions/spot groups are shown by default.

Shortcuts

Button: 

Key: Alt+M

4.3.3.10 Regions of Interest Command

The **Regions of Interest** command shows/hides the edited regions of interest (ROI) in the Imaging Display. The ROIs are shown by default.

Shortcuts

Button: 

Key: Alt+R

4.3.3.11 Teach Points Command

The **Teach Points** command shows/hides the teach points in the Imaging Display. The teach points are indicated by a white cross (+) and displayed by default.

Shortcuts

Button: 

Keys: Alt+T

4.3.3.12 Ruler Command

The **Ruler** command shows/hides the ruler in the in the Imaging Display. The ruler is shown by default.

Shortcuts

Button: 

Keys: Alt+B

4.3.3.13 Intensity Scale Command

The **Intensity Scale** command shows/hides the intensity scale in the Imaging Display. The intensity scale is hidden by default.

Shortcuts

Button: 

Keys: Alt+I

4.3.3.14 Intensity Captions Command

The **Intensity Captions** command displays or hides the captions below the intensity scales. The captions are hidden by default.

4.3.3.15 Horiz. Scale Command

The **Horiz. Scale** command displays the intensity scales as horizontal bars. They are displayed as vertical bars by default.

4.3.3.16 Result Color Gradient Command

The **Result Color Gradient** command shows/hides the result color gradient in the Imaging Display. For displaying the color gradient, the Intensity Scale command must be active and a single result selected for display. The color gradient is hidden by default.

Shortcuts

Button: 

Keys: Alt+G

4.3.3.17 Laser Spots Command

The **Laser Spots** command shows/hides the laser spots in the Imaging Display. The laser spots are hidden by default.

Shortcuts

Button: 

Keys: Alt+L

4.3.3.18 Sample Position Command

The **Sample Position** command shows/hides the sample position in the Imaging Display. The sample position is hidden by default.

Shortcuts

Button: 

Keys: Alt+P

4.3.3.19 View Normalization Symbol Command

The **View Normalization Symbol** command displays or hides the normalization symbol in the top right corner of the Imaging Display. The symbol is shown by default. The state of this option is not remembered between program runs, so FlexImaging always starts displaying a symbol, if normalization is used. The Symbols are: "V" for RMS normalization, "T" for TIC normalization, "M" for Median normalization, and "W" for normalization to a mass window. Without normalization, no symbol is shown.

4.3.3.20 Skyline Projection Command

The **Skyline Projection** command switches between a maximum spectrum and the overall average spectrum in the Spectrum Display. When calculating the maximum spectrum, peaks that occur only in small regions of the sample are not averaged away, but retain their height. However, without averaging, noise is not reduced.

4.3.3.21 Refresh Results Command

The **Refresh Results** command (re)loads the spectra (or peak lists) for all measurement spots. This command (re)creates a reduced data set if enabled on the **Processing Options** tab of the **Imaging Run Properties** dialog. After the data acquisition in flexControl has been finished, this command is executed automatically.

Shortcut

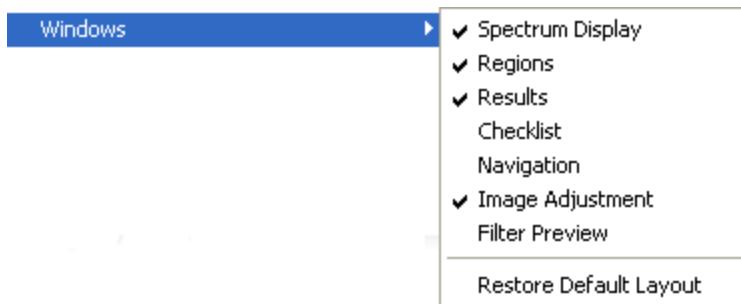
Key: F5

4.3.3.22 Toolbar Command

The **Toolbar** command shows/hides the toolbar. The toolbar is shown by default.

4.3.3.23 Windows Popup Command

Pointing to **Windows** offers the following commands:



Command	Used to ...
Spectrum Display	Show/Hide the Spectrum Display.
Regions	Show/Hide the Regions Pane.
Results	Show/Hide the Results Pane.
Checklist	Show/Hide the Checklist.
Navigation	Show/Hide the Navigation Window.
Image Adjustment	Show/Hide the Image Adjustment Window.
Filter Preview	Show/Hide the Filter Preview Window.
Restore Default Layout	Restore the default layout.

4.3.3.24 Windows > Spectrum Display Command

The **Spectrum Display** command shows/hides the Spectrum Display. The Spectrum Display is shown by default.

4.3.3.25 Windows > Regions Command

The **Regions** command shows/hides the Regions Pane. The Regions Pane is shown by default.

4.3.3.26 Windows > Results Command

The **Results** command shows/hides the Results Pane. The Results Pane is shown by default.

4.3.3.27 Windows > Checklist Command

The **Checklist** command shows/hides the Checklist. The Checklist is shown by default.

4.3.3.28 Windows > Navigation Command

The **Navigation** command shows/hides the Navigation Window. The Navigation Window is shown by default.

4.3.3.29 Windows > Image Adjustment Command

The **Image Adjustment** command shows/hides the Image Adjustment Window. The Image Adjustment Window is shown by default.

4.3.3.30 Windows > Filter Preview Command

The **Filter Preview** command shows/hides the Filter Preview Window. The Filter Preview Window is shown by default.

4.3.3.31 Windows > Restore Default Layout Command

The **Restore Default Layout** command restores the default window layout.

4.3.3.32 Status Bar Command

The **Status Bar** command shows/hides the status bar. The status bar is shown by default.

4.3.4 Analysis Menu



Figure 4-19 Analysis menu

The **Analysis** menu offers the following command:

Command	Used to ...
Hierarchical Clustering	Enter parameters, start hierarchical clustering and load results.

4.3.4.1 Hierarchical Clustering Command

The **Hierarchical Clustering** command opens the **Hierarchical Clustering Parameters** dialog, which enables you to manage and start hierarchical clustering. After the calculations are finished, the program automatically loads the clustering tree result and displays it as a dendrogram.

4.3.5 Tools Menu

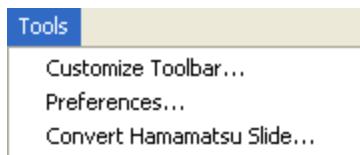


Figure 4-20 Tools menu

The **Tools** menu offers the following commands:

Command	Used to ...
Customize Toolbar	Customize the toolbar.
Preferences	Set user interface, imaging run, and data processing preferences.
Convert Hamamatsu Slide	Convert a digital slide from a Hamamatsu scanner to BigTIFF format usable for co-registration.

4.3.5.1 Customize Toolbar Command

The **Customize Toolbar** command is used to customize the toolbar to your needs. The command opens the **Customize Toolbar** dialog.

4.3.5.2 Preferences Command

The **Preferences** command is used to define general flexImaging options. Usually you define these parameters once and do not change them every day. E.g., the imaging run defaults settings are used in the **New Imaging Run Wizard**. The command opens the **Preferences** dialog.

If you want to change imaging run or processing options for your current imaging run, use the **Imaging Run Properties** command from the **Edit** menu instead.

4.3.5.3 Convert Hamamatsu Slide Command

The **Convert Hamamatsu Slide** command is used to convert a digital microscope slide created on a Hamamatsu scanner from its proprietary format to the standard BigTIFF format.

4.3.6 Compass Menu

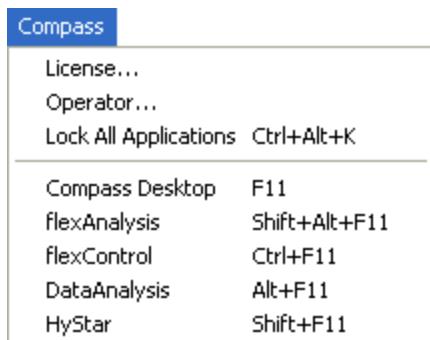


Figure 4-21 Compass menu

The **Compass** menu offers the following commands:

Command	Used to ...
License	License Bruker applications.
Operator	Log in the specified operator.
Lock All Applications	Lock flexImaging and all other Bruker applications using UserManagement.
Compass Desktop	Launch Compass Desktop.
flexAnalysis	Switch to flexAnalysis.
flexControl	Switch to flexControl.
DataAnalysis	Switch to DataAnalysis.
HyStar	Switch to HyStar.

4.3.6.1 License Command

The **License** command can be used to license flexImaging or another Bruker application installed on your system. Initial installation of flexImaging creates a temporary license only. You can also use the command to view all licenses present for Bruker applications and how long they will be valid as well as deleting existing licenses.

Licensing an application is performed by entering the corresponding license key(s). The key(s) for the required license(s) is/are delivered with the Bruker application package including later license change(s) submitted by Bruker Support. Temporary as well as permanent licenses can be given.

Note When using Bruker UserManagement only operators who have the special right can add and delete licenses.

The command opens the **Bruker LicenseManager** dialog, which lists any license currently available for any of the Bruker applications.

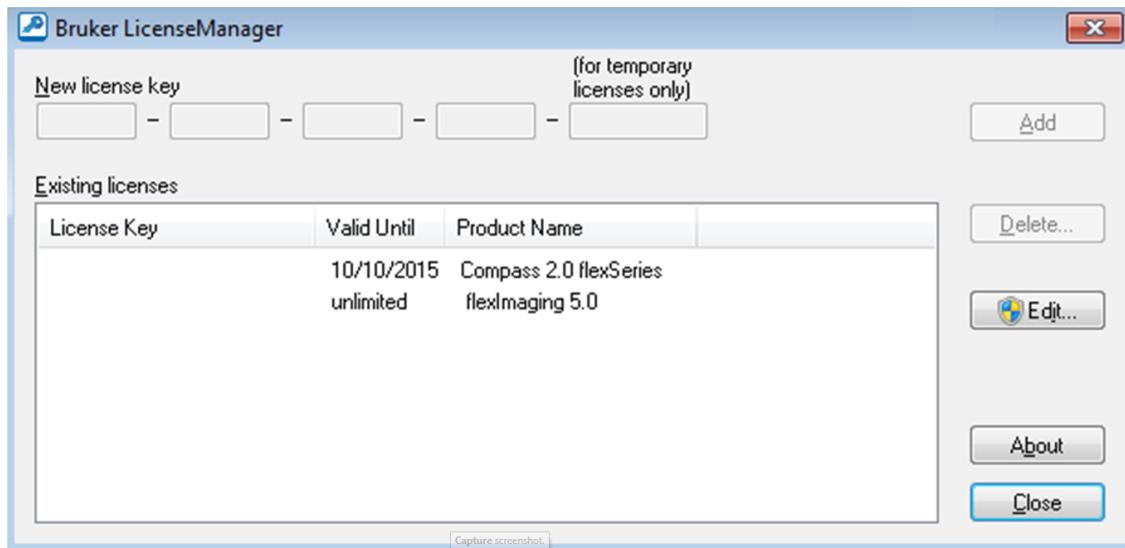


Figure 4-22 Bruker LicenseManager dialog (example)

Existing licenses

Lists existing licenses.

License key

Lists the license keys for the existing licenses.

Valid until

Permanent licenses are indicated as 'unlimited'.

For temporary licenses, the date until the respective license is valid is shown. An expiration warning will inform you automatically about the forthcoming expiration of a temporary license firstly 30 days before the respective expiration will expire. If for one product more than one license is present, this warning concerns to the longest valid license. An expiration warning will appear once per 24 hours and can be skipped by the operator.

Product name

Lists the products for which licenses already exist.

Edit

Opens the **Bruker LicenseManager** dialog. Windows administrator user rights are required to perform this task.

4.3.6.2 Re-enter Your Password Dialog

The **Re-enter Your Password** dialog is used to re-enter your password when required by the program. Re-entering password is needed when Compass Security Pack is installed and a license should be added or deleted since only operators having the corresponding right are allowed to add or delete licenses.



Figure 4-23 Re-enter Your Password dialog

Password

Enter the password for the current operator account.

OK

If the password is correct for the current operator account and the operator has the right to add/delete licenses the entered license key is added to or the selected license key is deleted from the LicenseManager's Existing licenses list.

If the password is incorrect or the operator does not have the required right, a corresponding error message will inform you.

4.3.6.3 Operator Command

The **Operator** command is used to change the operator during a running flexImaging session. When Bruker UserManagement is used, you can also use the command to change your current password.

Note There is always only one operator logged into Bruker applications on a specific computer at a time.

The command opens the **flexImaging** dialog which differs depending on whether or not UserManagement is used. Without UserManagement, you can log in without identifying whereas with UserManagement you have to identify by operator name and password.

Note The dialog appears automatically on each start-up of your flexImaging application unless you are currently logged in another Bruker application (applies to both with/without UserManagement) or the option for skipping the dialog is set (applies to without UserManagement only).

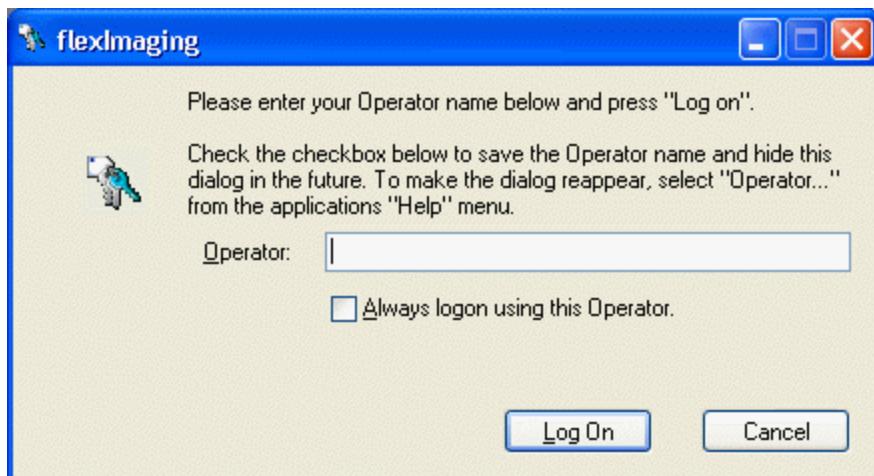


Figure 4-24 Login dialog when working without UserManagement



Figure 4-25 Login dialog when working with UserManagement

Operator

Enter your operator ID. The ID of the current operator will automatically be entered. When working with UserManagement the UserManagement administrator can disable the corresponding option. The operator ID is not case-sensitive.

Always log in using this operator

Note This check box is available when working without UserManagement.

Check this option if the operator named in Operator should be logged in automatically. This will skip this dialog when starting flexImaging in future.

Password

Note This entry field is available when working with UserManagement.

Enter your password. The password is not displayed as you type it.

Note Passwords are case-sensitive. Successive wrong inputs will cause locking of the operator when the number of wrong login tries exceeds the number of wrong logins allowed by the UserManagement administrator. Only the UserManagement administrator can unlock a locked operator.

Change Password

Note This button is available when working with UserManagement.

Allows you to change your password before logging in. Opens the **Change Password** dialog.

Log On

Without UserManagement: Logs in the specified operator.

With UserManagement: Logs in the specified operator if operator ID and password match; otherwise, an error message will appear.

If a newly created operator tries to log into a Bruker application the first time, the program requires for changing the password. Quitting the request opens the **Change Password** dialog.

4.3.6.4 Change Password Dialog

The **Change Password** dialog allows changing the password of the operator named in **Operator**. The new password must comply with the password policy settings defined by the UserManagement administrator.



Figure 4-26 Change Password dialog

Operator

Displays the ID of the operator whose password can be changed. If the password for another operator should be changed, click Cancel and enter the desired operator in the previous dialog.

Old password

Enter your current password. The password is not displayed as you type it.

Note Passwords are case-sensitive.

New password

Enter the new password.

Note Passwords are case-sensitive. Make sure, that the new password complies with the current password policy settings.

Confirm new password

Confirm your new password. You must enter the same password as above.

OK

If the new password and its repetition are the same and the new password complies with the current password policy settings, it changes the password. A dialog informs you about successful password change. Quitting this dialog returns you to the flexImaging dialog.

If the new password and its repetition are different or the chosen password does not comply with the current password policy settings, a corresponding message appears. Quit the message and in the **Change Password** dialog make again desired entries.

If the old password is incorrect, a message about denying password change appears. Quitting this message returns you to the flexImaging dialog.

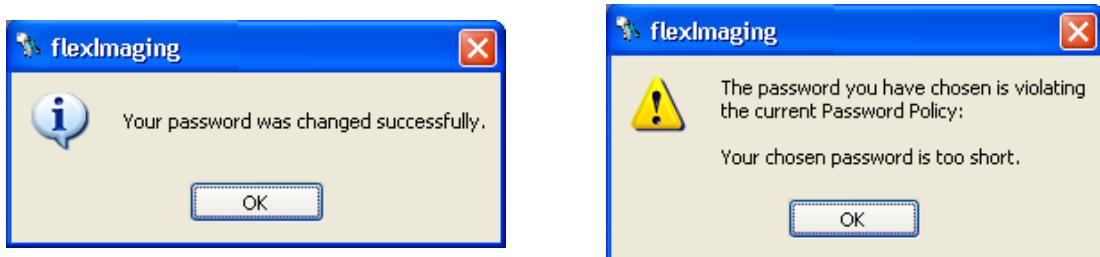


Figure 4-27 Messages on successful password change (left) and chosen password is violating password policy (right)

4.3.6.5 Lock All Applications Command

The **Lock All Applications** command locks flexImaging and all other Bruker applications using UserManagement that are installed on your system. The command is enabled when Bruker UserManagement is used.

Note Only the previous operator or someone with the special right to unlock applications can unlock a locked application.

Note Automatic locking of all UserManagement using Bruker applications will occur if one of these applications has not been used for a certain period defined by the UserManagement administrator.

The command opens the **flexImaging** dialog which informs you that flexImaging has been locked, by whom (displays the full name of the operator) and how it can be unlocked. Do the required entries to unlock the application.



Figure 4-28 flexImaging dialog when flexImaging has been locked

Operator

Enter the operator ID of the operator who has locked flexImaging. The corresponding operator ID will automatically be entered if the UserManagement administrator has set that option. If not the previous operator but someone else wants to unlock the applications, enter the respective operator ID.

Note The new operator must have the right to unlock applications.

Password

Enter the corresponding password.

Note Passwords are case-sensitive. Make sure, that the new password complies with the current password policy settings.

Change Password

Allows you to change your current password before logging in. Opens the **Change Password** dialog.

Log On

Unlocks Bruker applications and logs in the specified operator if operator ID and password match and, in case of a new operator, if the respective operator is allowed to unlock applications.

Shortcut

Keys: Ctrl+Alt+K

4.3.6.6 **Compass Desktop Command**

The **Compass Desktop** command launches Compass Desktop.

Shortcut

Key: F11

4.3.6.7 **flexAnalysis Command**

The **flexAnalysis** command switches to flexAnalysis.

Shortcut

Keys: Shift+Alt+F11

4.3.6.8 **flexControl Command**

The **flexControl** command switches to flexControl.

Shortcut

Key: Ctrl+F11

4.3.6.9 DataAnalysis Command

The **DataAnalysis** command switches to DataAnalysis.

Shortcut

Keys: Alt+F11

4.3.6.10 HyStar Command

The **HyStar** command switches to HyStar.

Shortcut

Keys: Shift+F11

4.3.7 Help Menu

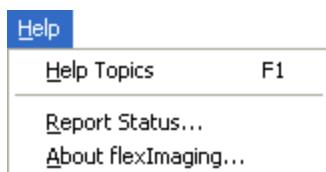


Figure 4-29 Help menu

The **Help** menu offers the following commands:

Command	Used to ...
Help Topics	Launch flexImaging Help.
Report Status	Launch the Status Reporter.
About flexImaging	Show copyright and license information for your flexImaging installation.

4.3.7.1 Help Topics Command

The **Help Topics** command launches flexImaging Online Help in order to get information about specific topics. flexImaging Help is used like other help applications running under Windows.

Shortcuts

Button: 

Key: F1

4.3.7.2 Report Status Command

The **Report Status** command launches the Status Reporter that is used to collect helpful information of the software, instrument and the computer. The Status Reporter also starts in case of an error in flexImaging to help the user to collect necessary information for the support concerning the current system state.

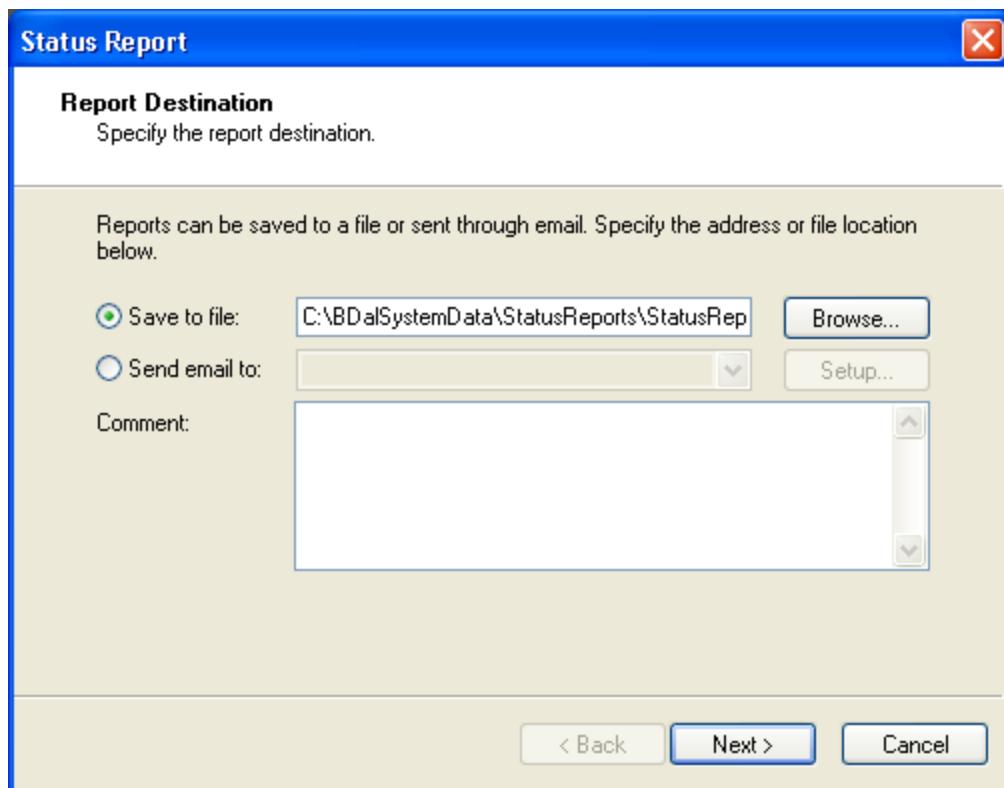


Figure 4-30 Status Reporter dialog (start page)

4.3.7.3 About flexImaging Command

The **About flexImaging** command shows copyright and license and information on your flexImaging installation as well as support information. The command opens the **About Compass flexImaging** dialog.



Figure 4-31 About Compass flexImaging dialog

4.4 flexImaging Shortcut Menus

4.4.1 Imaging Display Shortcut Menu	92
4.4.2 Spectrum Display Shortcut Menu	97
4.4.3 Regions Pane Shortcut Menu	108
4.4.4 Results Pane Shortcut Menus	110
4.4.5 X/Y-Axis Shortcut Menu	114
4.4.6 Histogram Shortcut Menu	115
4.4.7 Dendrogram Shortcut Menu	115
4.4.8 Docking Window Shortcut Menu	116

4.4.1 Imaging Display Shortcut Menu

Right clicking in the Imaging Display opens a shortcut menu which offers the following commands depending on the use-case, arbitrary arrays, spot microarray or tissue profiling:

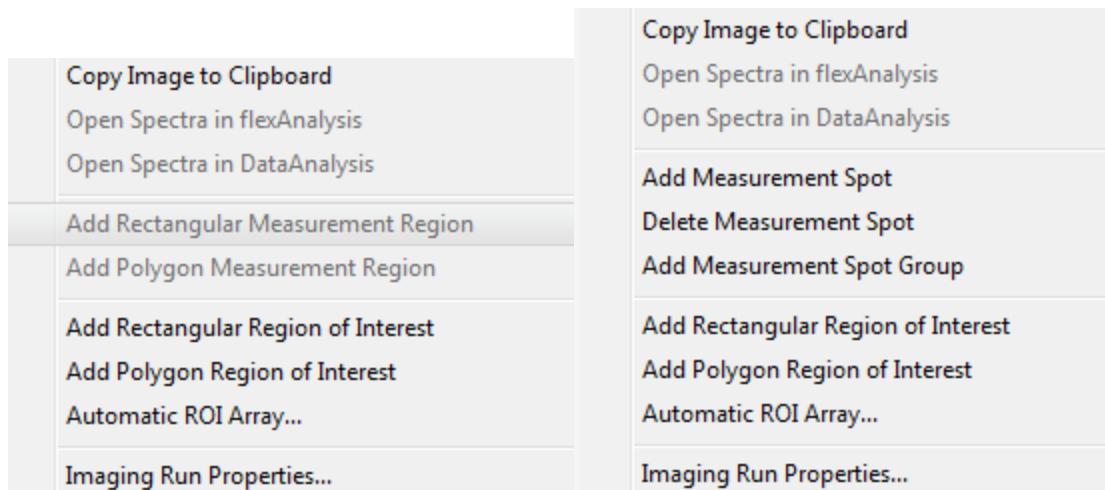


Figure 4-32 Imaging Display shortcut menu for the arbitrary arrays or spot microarray use-case (left) and for the tissue profiling use-case (right)

Command	Used to ...
Copy Image to Clipboard	Copy the current image to the clipboard.
Open Spectra in flexAnalysis	Open the spectra corresponding to the cursor position in flexAnalysis.
Open Spectra in DataAnalysis	Open the spectra corresponding to the cursor position in DataAnalysis.
Add Rectangular Measurement Region	Activate/Deactivate the Add Rectangular Measurement Region mode (for arbitrary arrays and spot microarray use-cases only).
Add Polygon Measurement Region	Activate/Deactivate the Add Polygon Measurement Region mode (for arbitrary arrays and spot microarray use-cases only).
Add Measurement Spot	Activate/Deactivate the Add Measurement Spot mode (for tissue profiling use-case only).

Command	Used to ...
Delete Measurement Spot	Activate/Deactivate the Delete Measurement Spot mode (for tissue profiling use-case only).
Add Measurement Spot Group	Create a new measurement spot group (for tissue profiling use-case only).
Add Rectangular Region of Interest	Activate/Deactivate the Add Rectangular Region of Interest mode.
Add Polygon Region of Interest	Activate/Deactivate the Add Polygon Region of Interest mode.
Automatic ROI Array	Automatically create an array of equal regions.
Imaging Run Properties	Edit imaging run properties.

4.4.1.1 Copy Image to Clipboard Command

The **Copy Image to Clipboard** command copies the image shown in the Imaging Display to the clipboard. This allows pasting the image into another application.

Note If the image is too large, Microsoft Windows will not copy it.

Shortcut

Button: 

4.4.1.2 Open Spectra in flexAnalysis Command

The **Open Spectra in flexAnalysis** command opens the spectra corresponding to the current cursor position in flexAnalysis. The number of spectra taken depends on the **Open Spectra Radius** setting in the **Preferences - User Interface** dialog.

4.4.1.3 Open Spectra in DataAnalysis Command

The **Open Spectra in DataAnalysis** command opens the spectra corresponding to the current cursor position in DataAnalysis. The number of spectra taken depends on the **Open Spectra Radius** setting in the **Preferences - User Interface** dialog.

4.4.1.4 Add Rectangular Measurement Region Command

The **Add Rectangular Measurement Region** command activates/deactivates the Add Rectangular Measurement Region mode. A measurement region defines where flexControl should acquire data when the autoXecute run is started. This feature applies to the arbitrary arrays and spot microarray use-cases only.

When this mode is active, the Add Measurement Region cursor  is displayed when the mouse is on the image. A rectangular measurement region is created by drawing a rectangle on the image using the mouse. For this, position the mouse at the desired starting point and drag it with the left mouse button pressed. On releasing the mouse button, the enclosed region is entered as a new measurement region on the Regions Pane getting the name '[running number]'. You can change the default region name via the **Edit Measurement Region Parameters** dialog. To show the new region on the image, the **Measurement Regions** command must be active.

Shortcut

Button: 

4.4.1.5 Add Polygon Measurement Region Command

The **Add Polygon Measurement Region** command activates/deactivates the Add Polygon Measurement Region mode. A measurement region defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the arbitrary arrays and spot microarray use-cases only.

When this mode is active, the Add Measurement Region cursor  is displayed when the mouse is on the image. A polygon measurement region is created by drawing a polygon on the image using the mouse. For this, click once with the mouse to get the starting point. Then click to the edge points of your polygon. To delete the most recently added point, press the Backspace key. You can finish creating a polygon measurement region either by double-clicking or by positioning the mouse onto the first point of your polygon and clicking once. This enters the enclosed region as a new measurement region on the Regions Pane getting the name '[running number]'. You can change the default region name via the Edit Measurement Region Parameters dialog. To show the new region on the image, the Measurement Regions command must be active.

Button: 

4.4.1.6 Add Rectangular Region of Interest Command

The **Add Rectangular Region of Interest** command activates/deactivates the Add Rectangular Region of Interest mode. A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases.

When this mode is active, the Add Region of Interest cursor  is displayed when the mouse is on the image. A rectangular ROI is created by drawing a rectangle on the image using the mouse. On releasing the mouse button, the enclosed region is entered as a new ROI on the Regions Pane getting the name [ROI running number]. You can change the default name via the Edit Region of Interest Parameters dialog. To show the new ROI on the image the Regions of Interest command must be active.

Shortcut

Button: 

4.4.1.7 Add Polygon Region of Interest Command

The **Add Polygon Region of Interest** command activates/deactivates the Add Polygon Region of Interest mode. A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases.

When this mode is active, the Add Region of Interest cursor  is displayed when the mouse is on the image. A polygon region of interest (ROI) is created by drawing a polygon on the image using the mouse. For this, click once with the mouse to get the starting point. Then click to the edge points of your polygon. To delete the most recently added point, press the Backspace key. You can finish creating a polygon measurement region either by double-clicking or by positioning the mouse onto the first point of your polygon and clicking once. This places a dashed polygon with corresponding ROI number on the image and enters the new ROI in the Regions Pane.

Shortcut

Button: 

4.4.1.8 Automatic ROI Array Command

The **Automatic ROI Array** command opens the **Automatic ROI Array Parameters** dialog (see section 4.5.34).

A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases. In the dialog the user can specify parameters to fill all or part of the measurement region with rectangular regions of interest, all covering the same number of measurement spots. They are displayed as dashed rectangles with consecutive ROI numbers on the image and in the Regions Pane.

4.4.1.9 Add Measurement Spot Command

The **Add Measurement Spot** command activates/deactivates the Add Measurement Spot mode. A measurement spot defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the tissue profiling use-case only.

When this mode is active, you can create a measurement spot group on the image and add measurement spots to it by clicking with the left mouse button to a selected position on the image. The first click creates a new measurement group with one spot and enters the spot group on the Regions Pane; the next clicks add further spots to this group. To create an additional spot group it is recommended to use the **Add Measurement Spot Group** command from the Regions Pane shortcut menu. New spots are always added to the active spot group selected on the Regions Pane. If no group is selected clicking with the left mouse button on the image automatically creates a new group.

Shortcut

Button:



4.4.1.10 Delete Measurement Spot Command

The **Delete Measurement Spot** command activates/deactivates the Delete Measurement Spot mode. A measurement spot defines where flexControl will acquire data when the autoXecute run is started. This feature applies to the tissue profiling use-case only.

When this mode is active, you can delete a measurement spot from the active spot group on the image by positioning the mouse on the spot to be deleted and clicking the left mouse button; the respective spot group is selected in the Regions Pane.

Shortcut

Button:



4.4.1.11 Add Measurement Spot Group Command

The **Add Measurement Spot Group** command creates a new measurement region on the image. Afterwards you can add spots to this group.

Note This item is only available in the tissue profiling use-case.

4.4.1.12 Imaging Run Properties Command

The **Imaging Run Properties** command is used to define imaging run properties. The command opens the **Imaging Run Properties** dialog.

Shortcuts

Button:



Keys: Alt+Return

4.4.2 Spectrum Display Shortcut Menu

4.4.2.1 Spectrum Display Shortcut Menu (1D/2D Scan Plot, 2D Density Plot, 2D All Scans Plot)

Right clicking in the Spectrum Display when the 1D Scan plot, 2D Scan plot, 2D All Scans plot or the 2D Density plot display type is active opens a shortcut menu offering the following commands:

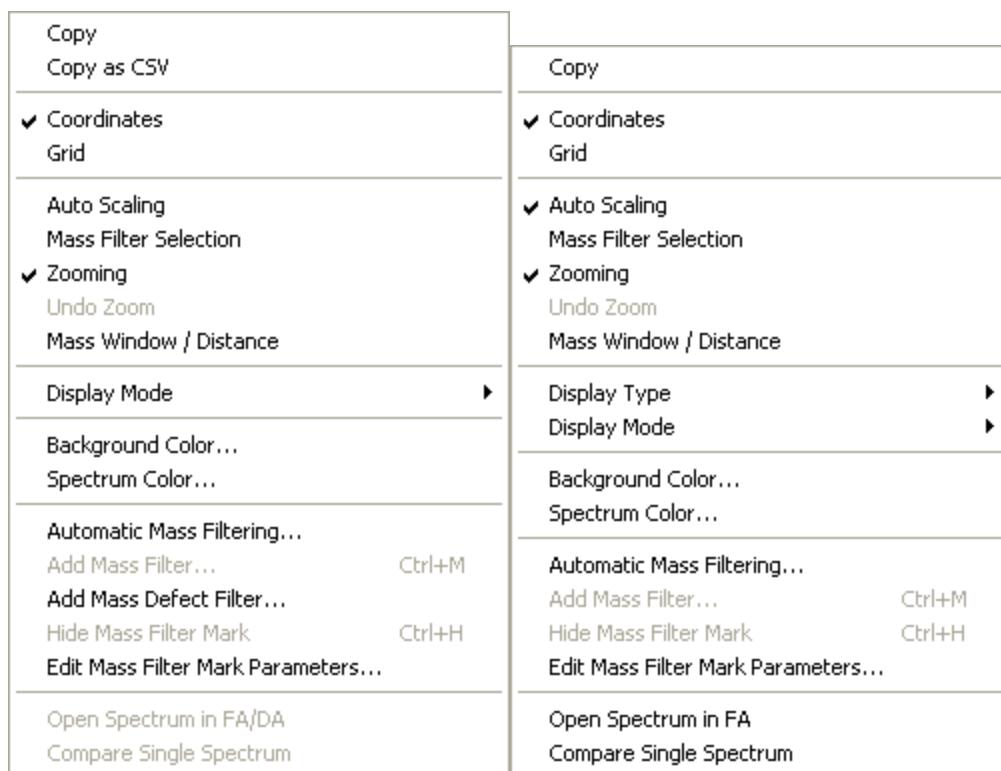


Figure 4-33 Spectrum Display shortcut menu when 2D Scan plot resp. 2D All Scans plot (left) or 2D Density plot (right) is active

Command

Copy

Copy as CSV

Coordinates

Grid

Auto Scaling

Mass Filter Selection

Zooming

Undo Zoom

Used to ...

Copy what is currently shown in the Spectrum Display to the clipboard.

Copy the current spectrum to the clipboard as tabulator separated numbers for pasting into a spreadsheet.

Show/Hide the spectrum coordinates in the status bar.

Show/Hide the grid.

Activate the Auto Scaling mode.

Activate the Mass Filter Selection mode.

Activate the Zoom mode.

Undo the previous zoom range change.

Command	Used to ...
Mass Window / Distance	Display distance cursors for measuring mass differences and defining mass windows for normalization.
Display Type	Pop up various display types for displaying spectra in the 2D Scan plot, 2D All Scans plot or 2D Density plot in arbitrary arrays use-case (not available for 1D Scan plot).
Display Mode (2D Scan Plot)	Pop up various display modes for displaying spectra in the 1D/2D Scan plot and 2D All Scans plot.
Display Mode (2D Density Plot)	Pop up various display modes for displaying spectra in the 2D Density plot.
Background Color	Set the background color of the display region (not available for 2D Density plot).
Spectrum Color	Set the spectrum color (not available for 2D Density plot).
Automatic Mass Filtering	Perform automatic mass filtering.
Add Mass Filter	Add the current mass selection to the result filter list.
Add Mass Defect Filter	Create a set of mass defect filters
Hide Mass Filter Mark	Disable the temporary mass filter created in the Mass Filter Selection mode and hide the corresponding purple line.
Edit Mass Filter Mark Parameters	Specify the temporary mass filter selection made in the Mass Filter Selection mode by clicking into the spectrum.
Open Spectrum in FA	Open the spectrum of the currently selected spot in flexAnalysis (for TOF spectra) or DataAnalysis (for FTMS spectra).
Compare Single Spectrum	Display the spectrum of the currently selected spot in red for comparison with any other single spectrum. If the All Scans display mode is selected, the average or skyline spectrum is also shown.

4.4.2.2 Spectrum Display Shortcut Menu (2D Stack Plot)

Right clicking in the Spectrum Display when the 2D Stack plot is active opens a shortcut menu offering the following commands:



Figure 4-34 Spectrum Display shortcut menu when 2D Stack plot is active

Command **Used to ...**

Scaling Pop up various scaling commands.

Display Type Pop up commands for switching to the different 2D plots.

Whitewash Switch to Whitewash mode.

Background Color Set the background color of the display region.

Spectrum Color Set the spectrum color.

4.4.2.3 Copy Command

The **Copy** command copies a graphic of what is currently shown in the Spectrum Display to the clipboard. This allows pasting the graphic into another application.

4.4.2.4 Copy as CSV Command

The **Copy as CSV** command copies the complete spectrum shown in the Spectrum Display to the clipboard as two columns of numbers: mass and intensity for every spectrum point, separated by one tabulator character. The user can paste these numbers into a spreadsheet for further processing.

4.4.2.5 Coordinates Command

The **Coordinates** command is used to show/hide the cursor coordinates in the status bar. When the Coordinates mode is active and the cursor is positioned in the Spectrum Display the corresponding x- and y-data is displayed in the status bar.

4.4.2.6 Grid Command

The **Grid** command shows/hides the grid in the Spectrum Display. The grid properties cannot be changed.

4.4.2.7 Auto Scaling Command

The **Auto Scaling** command adjusts the vertical scale so that the highest visible peak is maximized.

4.4.2.8 Mass Filter Selection Command

The **Mass Filter Selection** command activates the Mass Filter Selection mode for the Spectrum Display. This mode displays the Mass Filter Selection cursor  when the mouse cursor is moved into the Spectrum Display. Clicking on a peak of interest creates a temporary mass filter that is indicated by a vertical purple line placed in the spectrum. The corresponding intensity plot is shown immediately in the Imaging Display. You can use the **Edit Mass Filter Mark Parameters** command from the Spectrum Display shortcut menu to modify the current selection.

A temporary mass filter can be added to the result list by using the **Add Mass Filter** command from the Spectrum Display shortcut menu or clicking  on the Results Pane.

Tip *Quick mode switching: When in Zoom mode, you can temporarily switch to Mass Filter Selection mode by holding down the Ctrl key. The Mass Filter Selection mode will be active as long as the Ctrl key is held down. Releasing the Ctrl key switches back to Zoom mode.*

4.4.2.9 Zooming Command

The **Zooming** command activates the Zoom mode for the Spectrum Display. This mode displays the Zoom cursor  when the mouse cursor is moved into the Spectrum Display. The Zoom mode allows zooming in a selected area and undoing zoom range changes. flexImaging stacks the zooming in operations you made in the Spectrum Display.

To zoom in a selected area, position the Zoom cursor at the desired starting point and drag it with the left mouse button held down to the desired ending point. On releasing the mouse button, the enclosed area is zoomed in.

To undo the last zooming in, click with the left mouse button in the spectrum. If you performed several zooming in steps in succession, you can zoom out stepwise by repetitively clicking in the spectrum or you can reset to full display of data at once by double-clicking with the left mouse button in the spectrum.

4.4.2.10 Undo Zoom Command

flexImaging stacks the zooming in operations you performed in the Spectrum Display. The **Undo Zoom** command undoes the last zooming in performed in that view and restores the previous display range. When more than one zooming in was performed, you can select the command repetitively to stepwise zoom out.

Tip When in Zoom mode you can quickly restore the previous display range by just clicking with the left mouse button into the spectrum.

4.4.2.11 Mass Window Distance Command

The **Mass Window / Distance** command displays two vertical lines as distance cursors in the spectrum display. While one of these lines is fixed, the user can move the other with the mouse. Clicking the left mouse button, the user can switch the fixed and moveable role. The mass position of the first cursor and its distance to the second are displayed in the status bar. Clicking the right mouse button opens the **Add Normalization Mass Window** dialog, where the user may enter the interval into a list of mass windows for normalization, or just cancel if he is only interested in measuring a mass difference.

4.4.2.12 Display Type Command

The **Display Type** command is available only if more than one spectrum is displayed. It pops up commands for switching the Spectrum Display to the different types of 2D plots available:



Command	Used to ...
2D Scan Plot	Switch to 2D Scan plot display type.
2D Density Plot	Switch to 2D Density plot display type.

Command	Used to ...
2D Stack Plot	Switch to 2D Stack plot display type.
2D All Scans	Switch to 2D All Scans plot display type.

4.4.2.13 **Display Mode Command (1D/2D Scan plot, 2D All Scans Plot)**

The **Display Mode** command offers various modes for displaying data points in the 1D or 2D Scan plot of the Spectrum Display:

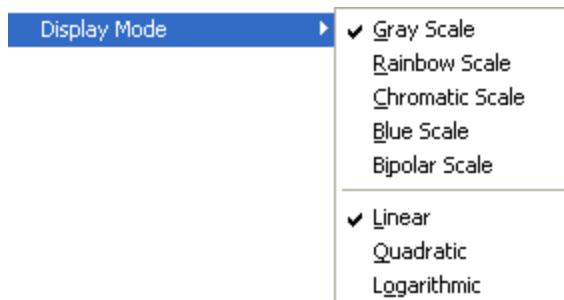


Command	Used to display ...
Point	Only the data points.
Line	Data points connected with a line.
Histogram	Data points as histogram.
No marker	No markers at data points.
Cross	Crosses at data points.
Hor. Cross	Horizontal crosses at data points.
Circle	Circles at data points.
Box	Boxes at data points.

Command	Used to display ...
Diamond	Diamonds at data points.
Triangle	Triangles at data points.
Inv. Triangle	Top down triangles at data points.
Dash	Dashes at data points.
Tick	Ticks at data points.
1 Pixel	Normal width data points.
2 Pixel	Bold data points. Useful for printing if lines are too thin.
3 Pixel	Extra bold data points. Useful for printing if lines are too thin.

4.4.2.14 Display Mode Command (2D Density plot)

The **Display Mode** commands pops up various color scheme and intensity modes for the 2D Density plot of the Spectrum Display:



Command	Used to display ...
Gray Scale	Gray scale.
Rainbow Scale	Rainbow scale.
Chromatic Scale	Chromatic scale.
Blue Scale	Blue scale.
Bipolar Scale	Bipolar scale.
Linear	Linear color intensity.
Quadratic	Quadratic color intensity.
Logarithmic	Logarithmic color intensity.

4.4.2.15 **Background Color Command**

The **Background Color** command is used to change the background color of the Spectrum Display. The command opens the **Color** dialog.

4.4.2.16 **Spectrum Color Command**

The **Spectrum Color** command is used to change the color of the currently visible spectrum. The command opens the standard **Color** dialog. The chosen color will overlay the ROI color. This feature is only available for the 2D Scan plot and 2D Stack plot.

4.4.2.17 **Add Mass Filter Command**

The **Add Mass Filter** command is used to add a new mass filter to the result filter list. The command opens the **Edit Mass Filter Parameters** dialog.

Shortcut

Keys: Ctrl+M

4.4.2.18 **Add Mass Defect Filter Command**

The **Add Mass Defect Filter** command is used to create a new mass defect filter set (MDF) replacing the current result filter list. The command opens the MDF parameter dialog (see section 4.5.35).

4.4.2.19 **Hide Mass Filter Mark Command**

The **Hide Mass Filter Mark** command disables the temporary mass filter created in the Mass Filter Selection mode and removes the corresponding purple line from the display.

Tip *The mass filter mark can also be hidden by clicking the mouse wheel button.*

Shortcut

Keys: Ctrl+H

4.4.2.20 Edit Mass Filter Mark Parameters Command

The **Edit Mass Filter Mark Parameters** command is used to specify the temporary mass filter selection made in the Mass Filter Selection mode by clicking into the spectrum. A vertical purple line marks the current selection. The command opens the **Edit Mass Filter Mark Parameters** dialog.

4.4.2.21 Open Spectrum in FA

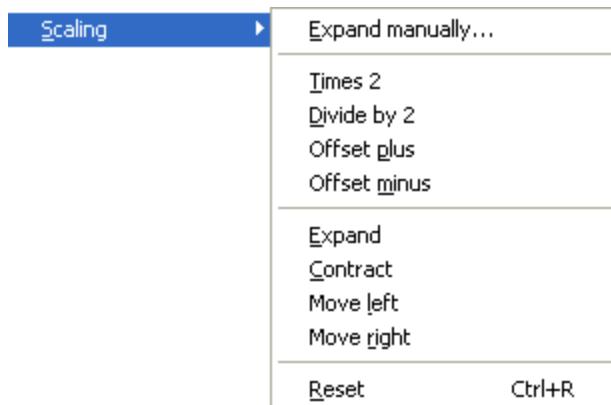
If the option **Show Single Spectrum** is selected, the spectrum of the currently selected spot is opened in flexAnalysis (for TOF spectra) or DataAnalysis (for FTMS spectra).

4.4.2.22 Compare Single Spectrum

If the option **Show Single Spectrum** is selected, the spectrum of the currently selected spot is displayed in red for comparison with any other single spectrum. If the **All Scans** display mode is selected, the average or skyline spectrum is also shown.

4.4.2.23 Scaling Command

The **Scaling** command pops up various commands to change the scaling of axes:



Command	Used to ...
Expand manually	Change scaling of axes based on manually entered values (see Manual Scaling dialog below).
Times 2	Decrease the y-range by 2.
Divide by 2	Increase the y- range by 2.

Command	Used to ...
Offset plus	Shift the y-range up.
Offset minus	Shift the y-range down.
Expand	Expand the x- range
Contract	Contract the x-range.
Move left	Move the x-range to the left.
Move right	Move the x-range to the right.
Reset	Reset axes to full display of data.

Manual Scaling dialog

The **Expand Manually** command opens the **Manual Scaling** dialog to change manually the scaling of the x-, y- and intensity axes in the 2D Stack View. It also allows resetting the scaling to full display of data.

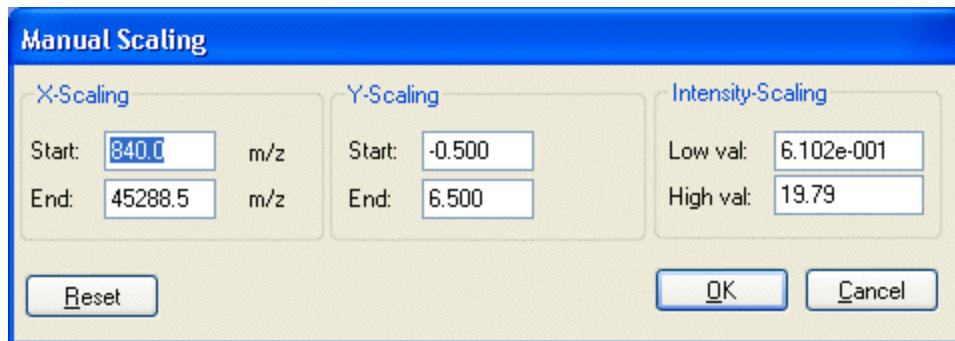


Figure 4-35 Manual Scaling dialog

4.4.2.24 Whitewash Command

The **Whitewash** command activates/deactivates the Whitewash mode. In this mode, the plot is structured finer due to resolving overlying structures. All spectra are drawn in black color.

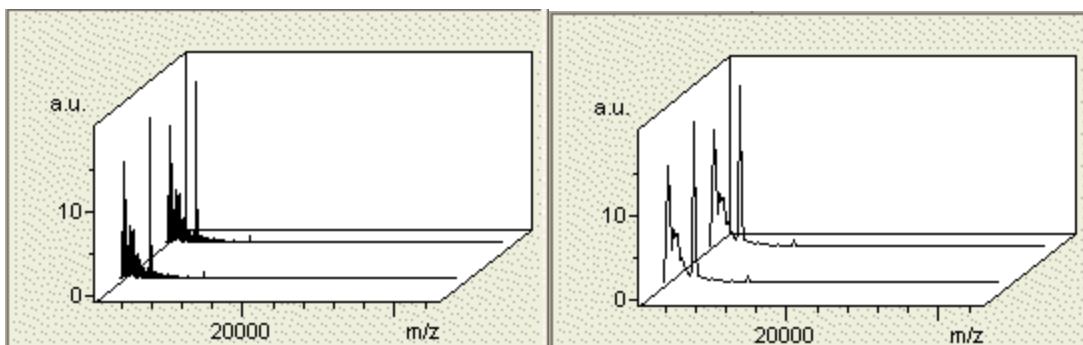


Figure 4-36 Display of two spectra when Whitewash mode active (left) resp. inactive (right)

4.4.3 Regions Pane Shortcut Menu

Right clicking in the regions list of the Regions Pane opens a shortcut menu offering the following commands:

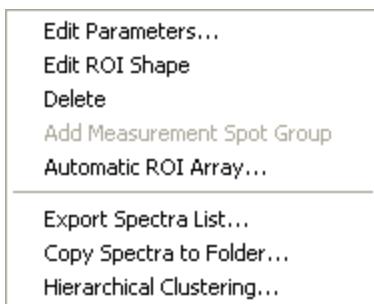


Figure 4-37 Regions Pane shortcut menu

Command	Used to ...
Edit Parameters	Edit the selected region, either a measurement region or a region of interest.
Edit ROI Shape	Edit the shape of the region of interest.
Delete	Delete the selected region.
Add Measurement Spot Group	Create a new measurement spot group.
Automatic ROI Array	Automatically create an array of equal regions.
Export Spectra List	Export the spectra list.

Command	Used to ...
Copy Spectra to Folder	Copy all spectra belonging to the selected region in one separate folder.
Hierarchical Clustering	Enter parameters, start hierarchical clustering and load results.

4.4.3.1 **Edit Parameters Command (Regions Pane)**

The **Edit** command is used to edit the selected region, either a measurement region or a region of interest. Depending on the context, the command opens the **Edit Measurement Region Parameters** dialog or the **Edit Regions of Interest Parameters** dialog.

4.4.3.2 **Edit ROI Shape**

The **Edit ROI Shape** command enables editing the shape of the selected region of interest. The corners of the region are marked with small white squares that the user can drag with the mouse to the desired position.

4.4.3.3 **Delete Command (Regions Pane)**

The **Delete** command deletes the selected measurement region or region of interest.

4.4.3.4 **Automatic ROI Array Command**

The **Automatic ROI Array** command opens the **Automatic ROI Array Parameters** dialog (see section 4.5.34).

A region of interest (ROI) defines which acquired spectra are used in processing and thus can be added before starting the autoXecute run as well as after the run. This feature applies to all use-cases. In the dialog the user can specify parameters to fill all or part of the measurement region with rectangular regions of interest, all covering the same number of measurement spots. They are displayed as dashed rectangles with consecutive ROI numbers on the image and in the Regions Pane.

4.4.3.5 **Export Spectra List Command**

The **Export Spectra List** command is used to generate a list of all spectra belonging to one or multiple regions. This list can e.g. be imported into ClinProTools. The command opens the **Export Spectra List** dialog.

4.4.3.6 Copy Spectra to Folder Command

The **Copy Spectra to Folder** command is used to copy all spectra belonging to the selected region into a separate folder. This option is used e.g. to export spectra of a certain class for ClinProTools. The command opens the **Copy Region Of Interest Spectra** dialog.

4.4.3.7 Hierarchical Clustering Command

The **Hierarchical Clustering** command opens the **Hierarchical Clustering Parameters** dialog, which enables you to manage and start hierarchical clustering. After the calculations are finished, the program automatically loads the clustering tree result and displays it as a dendrogram.

4.4.4 Results Pane Shortcut Menus

Right clicking a result filter entry in the Results Pane opens the left shortcut menu, right clicking the result filter header opens the right shortcut menu. These offer the following commands:

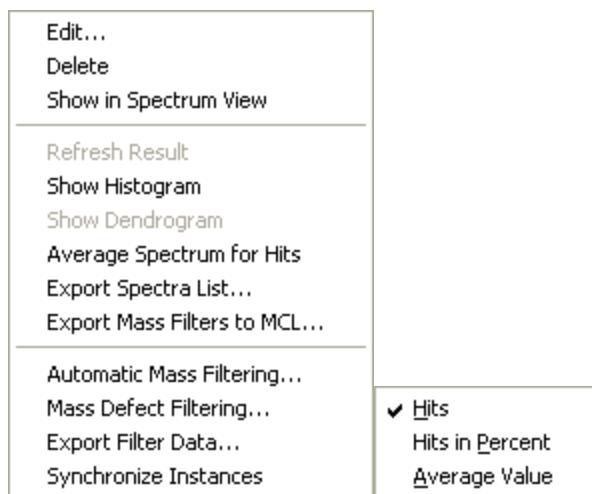


Figure 4-38 Result filter shortcut menu (left); Result filter header shortcut menu (right)

Command	Used to ...
Edit	Edit the mass filter parameters of the selected result filter.

Command	Used to ...
Delete	Delete the selected result filter.
Show in Spectrum View	Display the selected result filter in a region of the spectrum display zoomed around the filter mass.
Refresh Result	Reload data for the selected result filter.
Show Histogram	Display the histogram for the selected result filter.
Show Dendrogram	Display the dendrogram for the selected result filter (clustering trees only).
Average Spectrum for Hits	Calculate an average spectrum for all spots that created a hit.
Export Spectra List	Opens the "Export Spectra List" dialog.
Export Mass Filters to MCL	Open a standard "Save as" file selection dialog to choose a path for the exported Mass Control List file.
Automatic Mass Filtering	Perform automatic mass filtering.
Mass Defect Filtering	Create a set of mass defect filters.
Export Filter Data	Export the intensity values for the respective filter to a text file organized in columns.
Synchronize Instances	Send peak filter changes to other flexImaging instances and act on received change messages.
Hits	Display the total number of hits.
Hits in Percent	Display the percentage number of hits.
Average Value	Display the Average value column.

4.4.4.1 Edit Command (Results Pane)

The **Edit** command is used to edit the parameters of the selected result filter. Depending on the type of that filter, the command opens the corresponding dialog:

- **Edit Mass Filter Parameters**
- **Edit ClinProTools Classification Filter Parameters**
- **Edit ClinProTools Classification Overall Score Filter Parameters**
- **Edit ClinProTools Clustering Filter Parameters**
- **Edit ClinProTools Clustering Tree Filter Parameters**
- **Edit ClinProTools PCA Filter Parameters**

- **Edit XML Import Filter Parameters**
- **Edit Text Import Filter Parameters**

4.4.4.2 Delete Command (Results Pane)

The **Delete** command deletes the selected result filter from the result filter list.

4.4.4.3 Refresh Result Command

The **Refresh Result** command is used to update values for a ClinProTools, XML or text import. The command reloads data for the selected result filter and updates the Imaging Display accordingly.

4.4.4.4 Show Histogram Command

The **Show Histogram** command opens a histogram window for the selected result filter. See Histogram Window for details.

4.4.4.5 Show Dendrogram Command

The **Show Dendrogram** command opens a Dendrogram Window for the selected result filter. This command is only available for result filters generated from ClinProTools clustering trees. See Dendrogram Window for details.

4.4.4.6 Average Spectrum for Hits Command

The **Average Spectrum for Hits** command toggles the option to additionally display an average calculated from all spectra that created a hit in the corresponding mass interval.

4.4.4.7 Export Spectra List Command

The **Export Spectra List** command opens the "Export Spectra List" dialog where the user can select the results, for which the list of spectra are exported in XML format for import e.g. into ClinProTools. The user can also enter a path for the exported file or browse for it.

4.4.4.8 Export Mass Filters to MCL Command

The **Export Mass Filters to MCL** command opens a standard "Save as" file selection dialog to choose a path for the exported Mass Control List file.

4.4.4.9 Automatic Mass Filtering Command

After an autoXecute run is finished, the **Automatic Mass Filtering** command can be used to replace time-consuming manual peak filtering, or if you do not know which mass ranges may be considered. The software creates mass ranges based on your settings for this procedure and supplies the number of spectra (=) that contain a peak in the respective mass range. The command opens the dialog.

4.4.4.10 Mass Defect Filtering Command

The **Mass Defect Filtering** command is used to create a new mass defect filter set (MDF) replacing the current result filter list. The command opens the MDF parameter dialog (see section 4.5.35).

4.4.4.11 Export Filter Data Command

The **Export Filter Data** command exports the intensity values for the respective filter to a text file organized in columns. The command prompts for a file name and then writes the values to the file. Spot name, value, region, X-position and Y-position are written to separate columns. If one or more measurement regions or regions of interest are activated in the regions pane (left checkbox selected), the exported values are restricted to the area covered by these regions. An example file is shown below:

```
#spot-name value region xpos ypos
0_R00X134Y101 0.062962 0 134 101
0_R00X133Y102 0.045040 0 133 102
0_R00X134Y102 0.076940 0 134 102
0_R00X133Y103 0.076340 0 133 103
```

4.4.4.12 Synchronize Instances Command

The **Synchronize Instances** command activates or deactivates the option to send and receive messages about changed peak filters to and from other flexImaging instances. This option is switched off when the program starts.

If it is activated, a message is sent to other instances when:

- a peak filter mark is set, deleted, or moved with the arrow keys
- a different filter list is loaded

- any filter in a list is switched on or off, and the program displays peak filters sent from other instances.

Other display features like normalization or zoom of image or spectrum are not synchronized.

4.4.4.13 Hits Command

The **Hits** command displays the total number of hits instead of the percentage number in the **Hits** column of the result filter list.

4.4.4.14 Hits in Percent Command

The **Hits in Percent** command displays the percentage number of hits instead of the total number in the **Hits** column of the result filter list.

4.4.4.15 Average Value Command

The **Average Value** command activates an additional column (**Average** column) that displays the arithmetic average of all values for the selected filter. This information can e.g. be used when specifying absolute intensities in a result filter.

4.4.5 X/Y-Axis Shortcut Menu

Right clicking the X-axis or Y-axis in the Spectrum Display opens a shortcut menu offering the following commands:

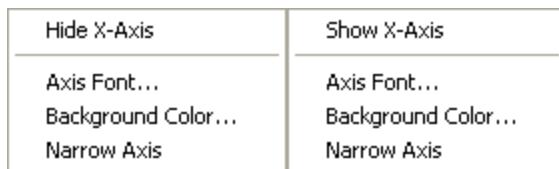


Figure 4-39 X-axis shortcut menu

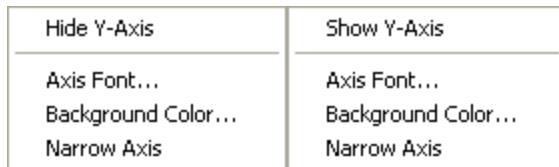


Figure 4-40 Y-axis shortcut menu

Command	Used to ...
Hide/Show X-Axis	Show/Hide the X-axis.
Hide/Show Y-Axis	Show/Hide the Y-axis.
Axis Font	Define the axis font.
Background Color	Define the background color of the axes.
Narrow Axis	Use less space for axis text.

4.4.6 Histogram Shortcut Menu

The histogram shortcut menu offers the same options as the Spectrum Display shortcut menu without the mass filter related items (see section 4.4.2).

4.4.7 Dendrogram Shortcut Menu

Right clicking in the Dendrogram Window opens a shortcut menu offering the following commands:

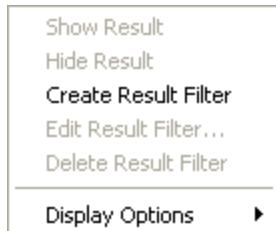


Figure 4-41 Dendrogram item shortcut menu

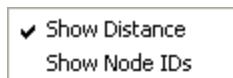


Figure 4-42 Dendrogram display options shortcut menu

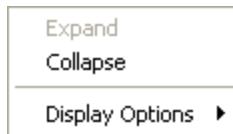


Figure 4-43 Dendrogram node shortcut menu

Command	Used to ...
Show Result	Activate the result filter for the clicked tree item.
Hide Result	Deactivate the result filer for the clicked tree item.
Create Result Filter	Create a result filter for the clicked tree item.
Edit Result Filter	Open the Edit ClinProTools Clustering Tree Filter Parameters dialog.
Delete Result Filter	Delete the result filter for the clicked tree item.
Show Distance	Display the distance between two items in the tree.
Show Node IDs	Display the ClinProTools node ID in the tree.
Expand	Expand the clicked node.
Collapse	Collapse the clicked node.
Display Options	Pop up display options.

4.4.8 Docking Window Shortcut Menu

Right clicking in the background of a docking window or in its title bar opens the following shortcut menu:



Figure 4-44 Docking window shortcut menu

Command	Used to ...
Enable Docking	Enables docking of the respective window if checked.

4.5 flexImaging Dialogs

4.5.1 Choose Imaging Run	119
4.5.2 New Imaging Run Wizard	120
4.5.3 Preferences Dialog	135
4.5.4 Imaging Run Properties Dialog	148
4.5.5 Spot Microarray Teaching Dialog	156
4.5.6 Spot List Import Wizard	157
4.5.7 Edit Measurement Region Parameters Dialog	160
4.5.8 Edit Region of Interest Parameters Dialog	161
4.5.9 Save autoXecute Sequence As Dialog	162
4.5.10 autoXecute Run Options Dialog	163
4.5.11 Co-Register Image Dialog	166
4.5.12 Copy Region Of Interest Spectra Dialog	168
4.5.13 Export Spectra List Dialog	168
4.5.14 Edit Mass Filter Mark Parameters Dialog	170
4.5.15 Add Result Filter Dialog	171
4.5.16 Save Result Filter List As Dialog	172
4.5.17 Hierarchical Clustering Parameters Dialog	173
4.5.18 Import ClinProTools Result Wizard	176
4.5.19 Edit Mass Filter Parameters Dialog	192
4.5.20 Edit ClinProTools Classification Filter Parameters Dialog	195
4.5.21 Edit ClinProTools Classification Overall Score Filter Parameters Dialog ...	198

4.5.22 Edit ClinProTools Clustering Filter Parameters Dialog	200
4.5.23 Edit ClinProTools Clustering Tree Filter Parameters Dialog	201
4.5.24 Edit ClinProTools PCA Filter Parameters Dialog	203
4.5.25 Edit ClinProTools PLSA Filter Parameters Dialog	205
4.5.26 Edit XML Import Filter Parameters Dialog	208
4.5.27 Edit Text Import Filter Parameters Dialog	210
4.5.28 Automatic Mass Filtering Dialog	213
4.5.29 Customize Toolbar Dialog	214
4.5.30 Color Dialog	215
4.5.31 Definitions for Import of Generic Robot Files Dialog	216
4.5.32 Add Normalization Mass Window Dialog	218
4.5.33 Select Co-Registered Image Dialog	220
4.5.34 Automatic ROI Array Parameters Dialog	221
4.5.35 Mass Defect Filter Parameters Dialog	222
4.5.36 Hamamatsu Converter Dialog	224
4.5.37 Export to imzML Dialog	225
4.5.38 Split Imaging Run Dialog	226

4.5.1 Choose Imaging Run

After the start of flexImaging the Choose Imaging Run dialog opens automatically. You can either choose to create a new imaging run, open an already existing one or cancel this dialog and work with the offered menu items of the flexImaging GUI.

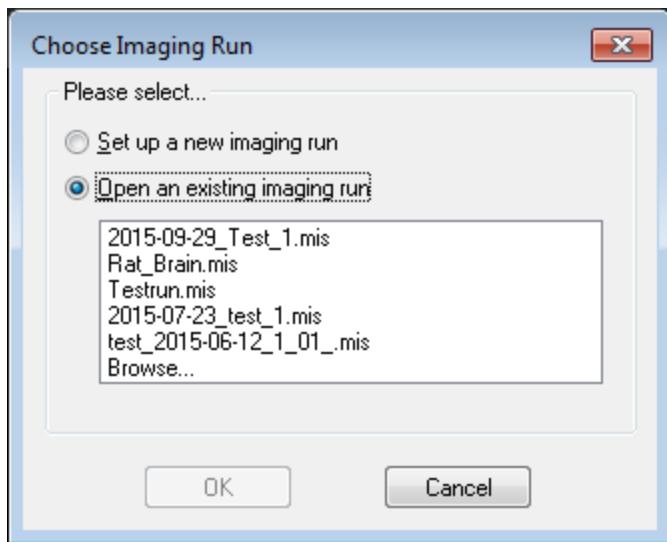


Figure 4-45 Choose Imaging Run dialog

Create a new imaging run

Creates a new imaging run.

Open an existing imaging run

Opens an existing imaging run. Select the imaging run from the recent file list below or after clicking **Choose other...** (also in recent file list) in the browser dialog.

OK

Depending on the chosen option starts the **New Imaging Run Wizard** or loads the selected existing imaging run.

4.5.2 New Imaging Run Wizard

A flexImaging imaging run contains all necessary information like data directory (path and folder), sample image, teaching, measurement regions, etc. The imaging run file is necessary to start a flexImaging run, as well as to reload already existing run results. The **New Imaging Run Wizard** can be used as guideline during the setup of a new flexImaging imaging run.

After starting flexImaging, the **Choose Imaging Run** dialog is opened. If **Create a new imaging run** is selected the wizard opens. If flexImaging is already running, the wizard can also be opened via the **New Imaging Run** command from the **File** menu or by clicking .

The **New Imaging Run Wizard** assists you in setting up a new imaging run. Depending on your sample preparation choice, the wizard displays the following dialogs:

- **Imaging Run Properties** (page 1)
- **Imaging Run Properties** (page 2)
- **Robot Import** (for spot microarray with robot import preparation only)
- **Sample Image**
- **Teach Sample explanations** (for spot microarray preparation only)
- **Teach Sample**
- **Image Size Warning** (displays only on sample image size mismatch)
- **Completing**

The basic features of the **New Imaging Run Wizard** are described in these topics. For special use-cases please have a look in the respective tutorials found in the Section Workflows in detail.

4.5.2.1 Imaging Run Properties (Page 1) Dialog (New Imaging Run Wizard)

The **Data Storage** (page 1) dialog defines the imaging run name and storage locations for the new imaging run.

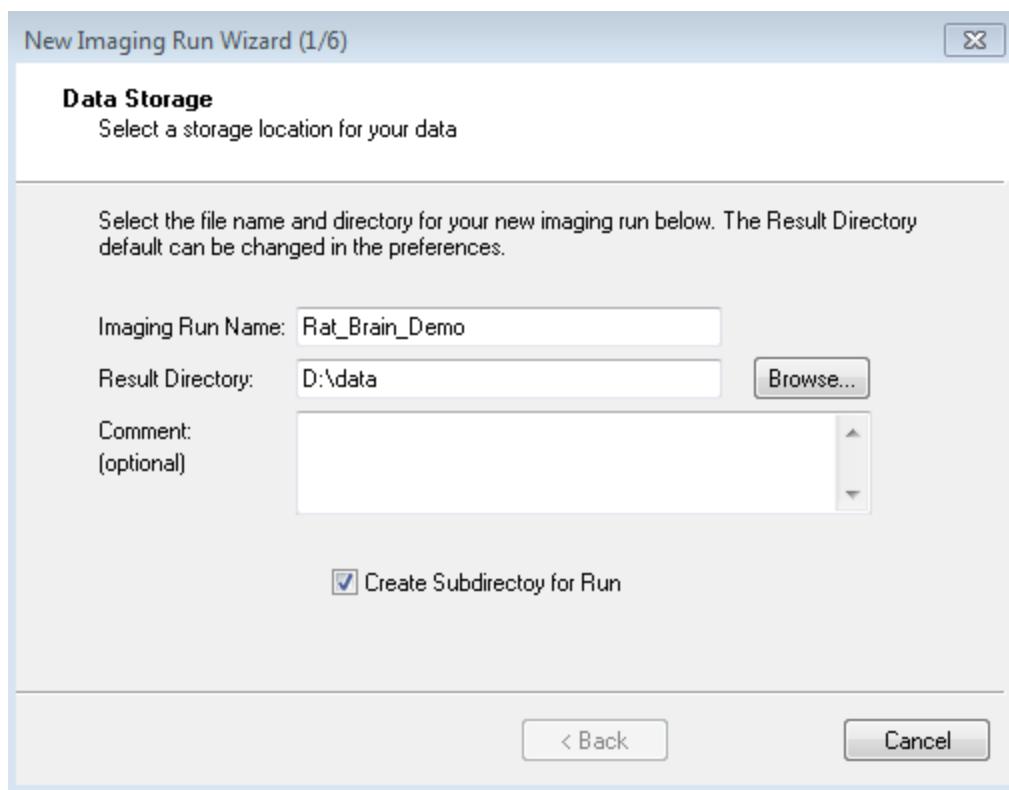


Figure 4-46 New Imaging Run Wizard (page 1) — Data Storage

Imaging Run Name

Type in a name that identifies your run. It will be used as name for the imaging run file (*.mis) and additionally as name for the sample directory that is created automatically in the result directory. This field must be filled to enable the **Next** button.

Result Directory

Choose a directory for the imaging run results. A folder with the name of your current imaging run (Imaging Run Name) will be automatically created here. The result directory shown by default can be adjusted via the **Preferences** command from the Tools.

Comment

This field allows you to enter some imaging run specific information.

Create Subdirectory for Imaging Run

Creates an additional subfolder below the result directory, also named as the imaging run, where the imaging run and the spectra will be stored (see example below).

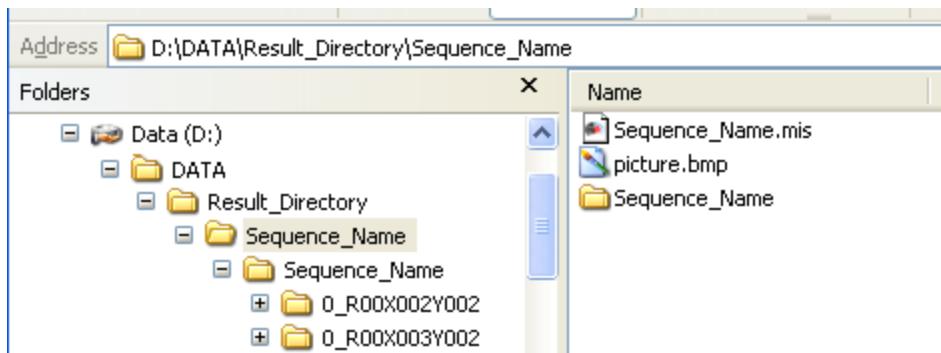


Figure 4-47 Example of subdirectory creation for imaging run storage

Next

Switches to the wizard's **Imaging Run Properties** (page 2) dialog.

4.5.2.2 Imaging Run Properties (Page 2) Dialog (New Imaging Run Wizard)

The **Acquisition Settings** (page 2) dialog defines the method used for sample preparation for the new imaging run.

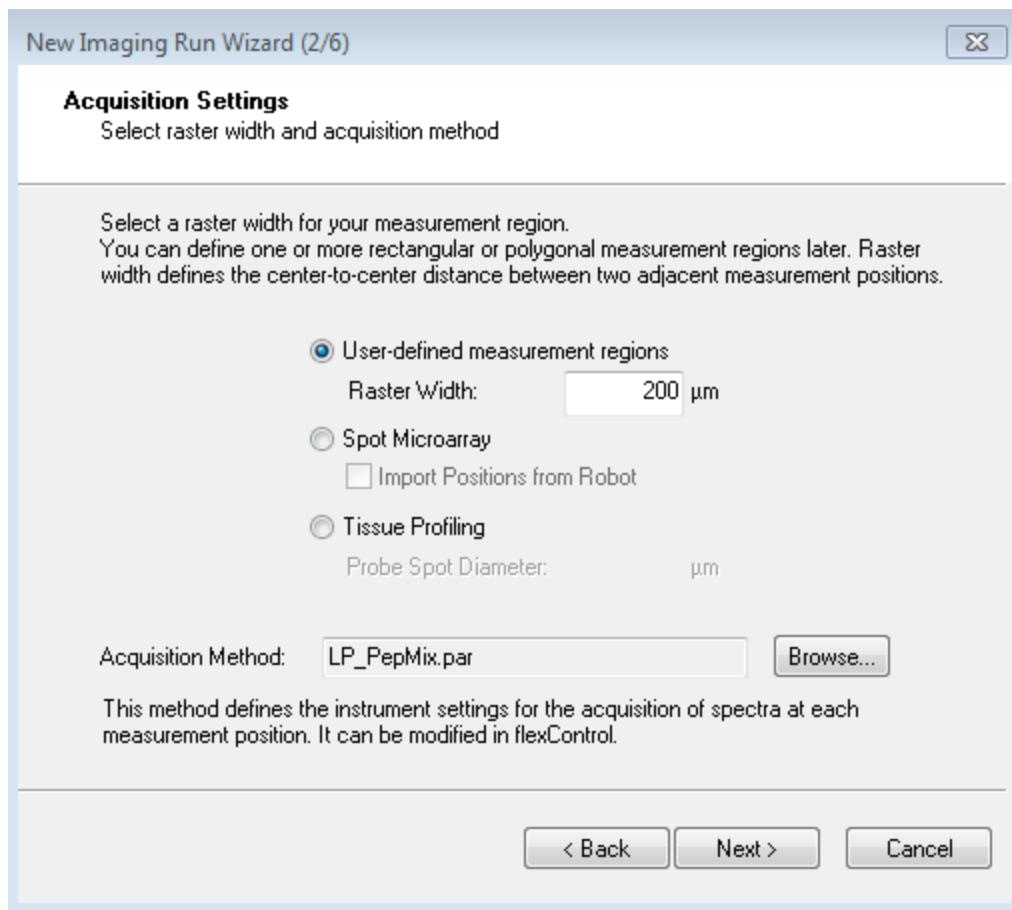


Figure 4-48 New Imaging Run Wizard (page 2) — Acquisition Settings

Sample Preparation

Depending on your sample preparation, you can choose between the three offered preparation possibilities:

- **Uniformly distributed coating - Define Arbitrary Arrays.** Suitable for samples coated by a homogenous matrix layer.
 - **Raster Width.** Specify the minimum raster width for the imaging run.

- **Spot Microarray**. Suitable where a robot was used to spot a regular grid of matrix spots onto the tissue.
 - **Import Positions from Robot**. Allows importing positions from the robot used.
- **Tissue Profiling**. Suitable where the matrix was applied as droplet on arbitrary positions on the sample.
 - **Probe Spot Diameter**. Enter the probe spot diameter for tissue profiling.

For information that is more detailed, please have a look in the respective tutorials on arbitrary arrays, spot microarray and tissue profiling.

The following dialogs of the wizard - where the respective preparations can be loaded and the teaching is performed - differ accordingly.

Acquisition Method

The settings for the automatic data acquisition are loaded with the autoXecute method. The pre-selected method is installed along with flexImaging and contains a lot of settings that should not be changed. If changes are necessary, create a new autoXecute method based on this pre-installed one. Use the autoXecute Method Editor from flexControl to perform the changes and to save the method with a new name.

The autoXecute method shown by default in this dialog during the setup of runs can be adjusted via the **Preferences** command from the **Tools** menu.

Next

If **Arbitrary Arrays**, **Spot Microarray (without robot import)** or **Tissue Profiling** preparation is chosen switches to the wizard's **Sample Image** dialog.

If **Spot Microarray (with robot import)** preparation is chosen switches to the wizard's **Robot Import** dialog first.

4.5.2.3 Robot Import Dialog

The **Robot Import** dialog is used to select the file to import sample preparation robot data.

Note This dialog is also launched from the Spot List Import Wizard then showing "Spot List Import" in the title bar.

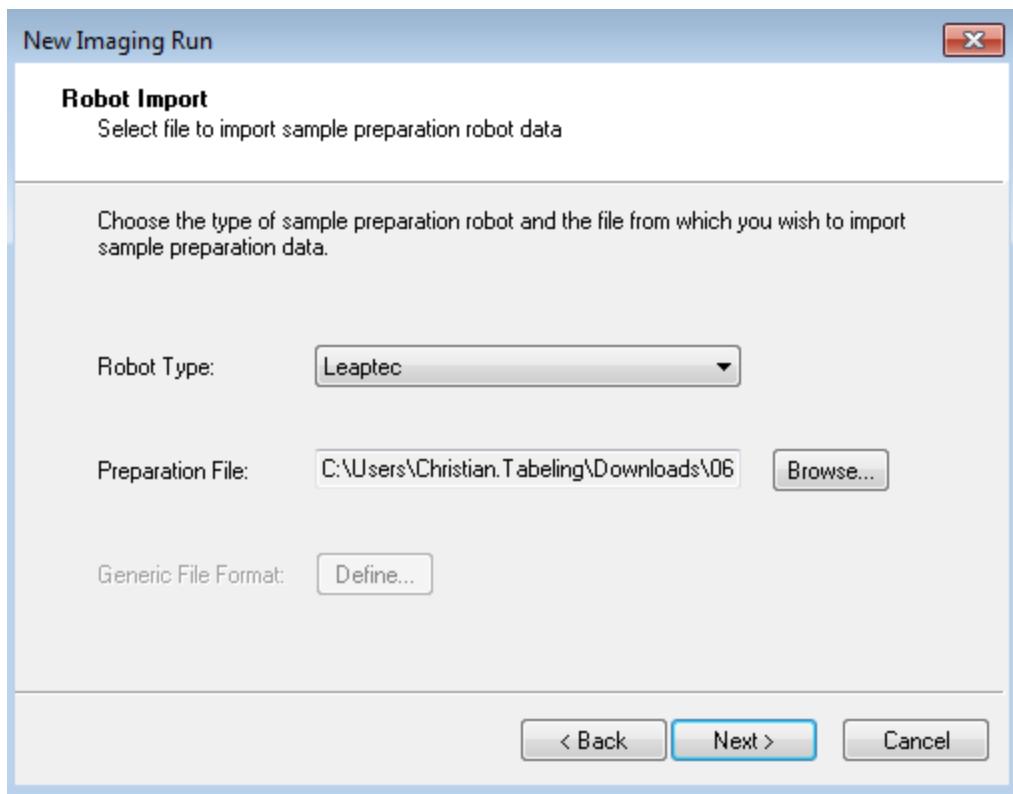


Figure 4-49 Robot Import dialog

Robot Type

Select your robot type. Currently the Shimadzu ChIP 1000 and Leap Technologies preparation robots are supported. If you have a different robot that exports its coordinates in ASCII format, you can use the 'Generic' import option.

Preparation File

Use the **Browse** button to select the file to import.

Generic File Format

The **Define** button allows defining parameters for the import of generic robot files. It opens the **Definitions for Import of Generic Robot Files** dialog.

Next

Switches to the wizard's **Processing Options** dialog.

4.5.2.4 **Processing Options Dialog**

The **Processing Options** dialog is used to define on-the-fly processing routines during acquisition.

Reduce Number of Datapoints

The number of datapoints is defined in the acquisition method. The recommended setting (which is suggested by default) is 80% of the original spectrum size.

Parent Mass

If you selected an MS/MS method on the preceding wizard page, you must enter the mass of the target compound precursor ion.

Perform Smoothing

Smooth the acquisition data.

Perform Baseline Subtraction

Perform a convex hull baseline subtraction after acquisition.

Next

Proceeds to the wizard's **Sample Image** dialog, unless you imported the spot coordinates from a robot or the importer was unable to locate the sample image. In such cases, the **Teach Sample Explanations** dialog opens.

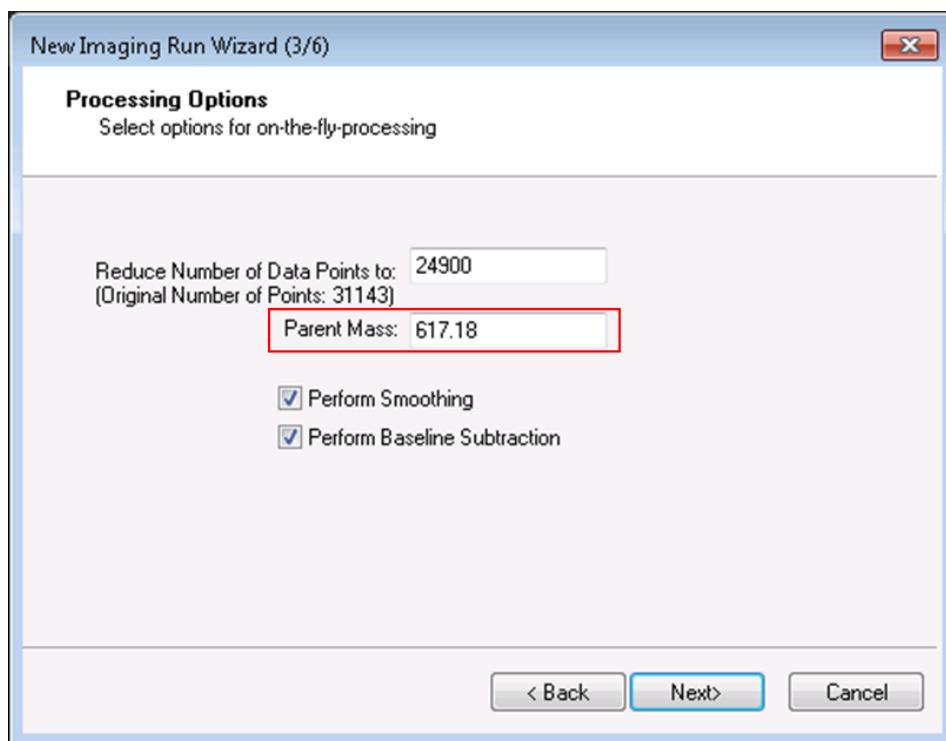


Figure 4-50 **New Imaging Run Wizard (page 3) — Processing Options**

4.5.2.5 **Sample Image Dialog**

The **Sample Image** dialog is shown unless you imported the spot coordinates from a robot or the importer was unable to locate the sample image.

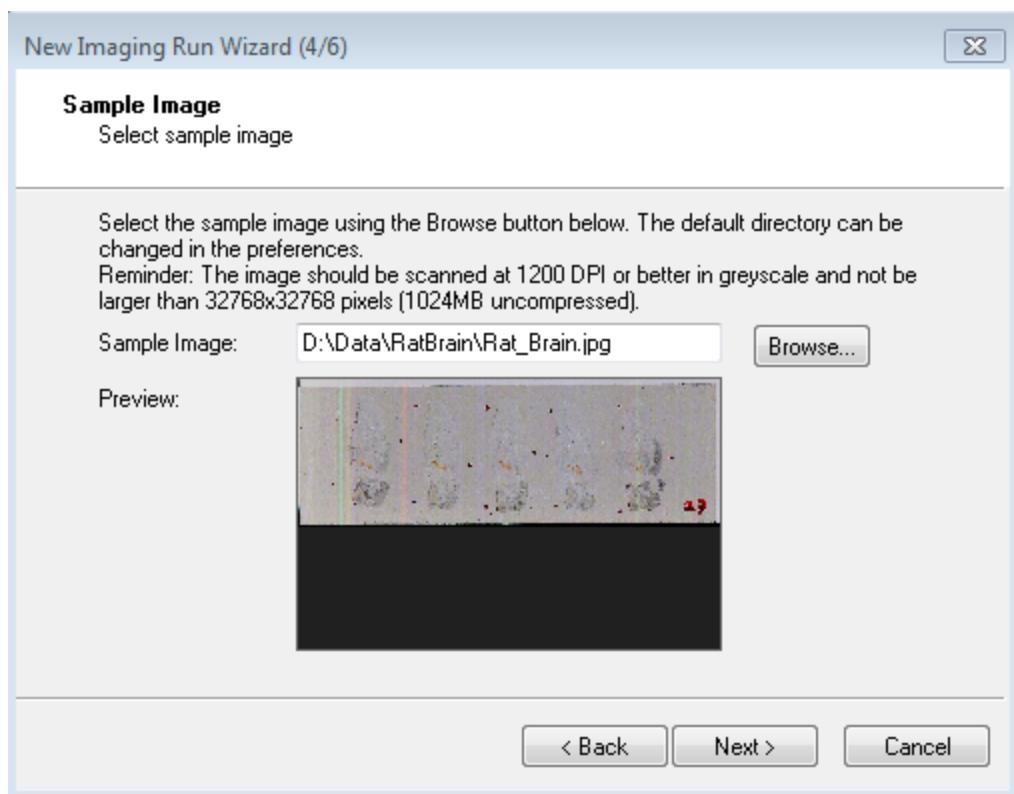


Figure 4-51 New Imaging Run Wizard (page 4) — Sample Image

Sample Image

Select the desired sample image using the **Browse** button.

Preview

Shows a preview of your selected sample image.

Next

If **Arbitrary Arrays** or **Tissue Profiling** preparation is chosen switches to the wizard's **Teach Sample** dialog.

If **Spot Microarray** preparation is chosen switches to the wizard's **Teach Sample explanations** dialog first.

4.5.2.6 Teach Sample Explanations Dialog

The **Teach Sample explanations** dialog appears if spot microarray preparation is selected and explains how to teach such samples. The dialog differs depending whether robot import was performed or not.

The teaching has to be performed in the illustrated order. Make sure that the teach points result in a rectangular and not in a parallelogram. It is helpful to set teach points far away from each other to minimize a possible incorrect teaching. If an edge point is not spotted or badly spotted it is necessary to use another row/column for the teaching points. If e.g. A24 has not been spotted use A1, A23 and P23 as teaching positions.

Note Although you may have left out a row/column, you can measure outside the teaching rectangle.

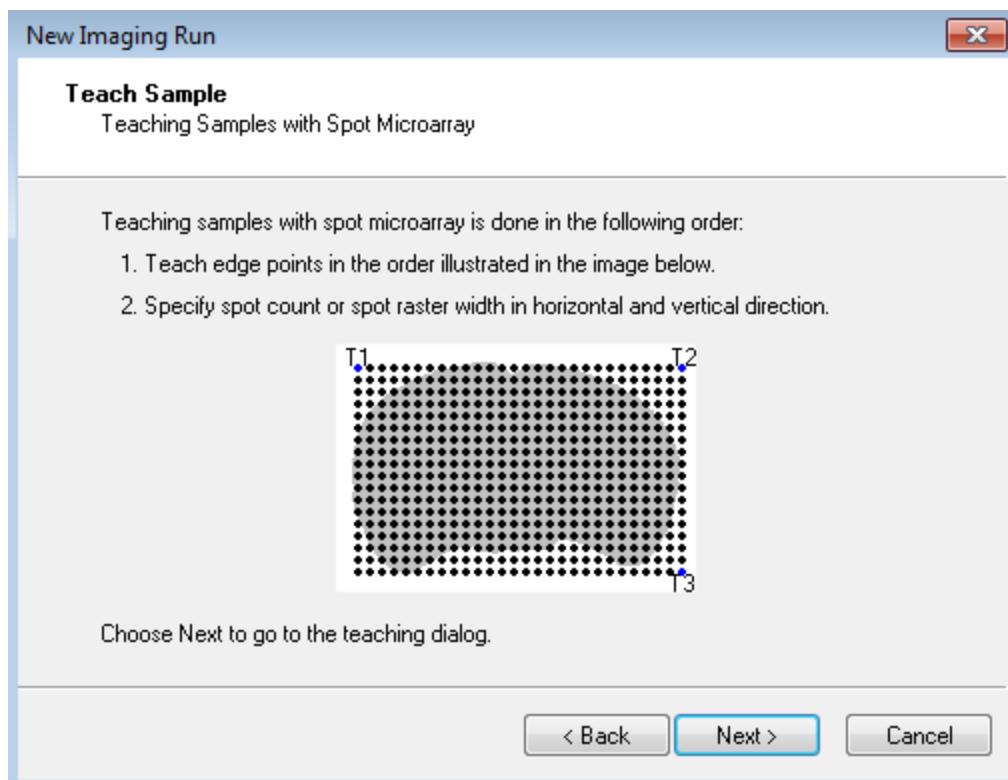


Figure 4-52 Teach Sample explanations dialog for spot microarray without robot import

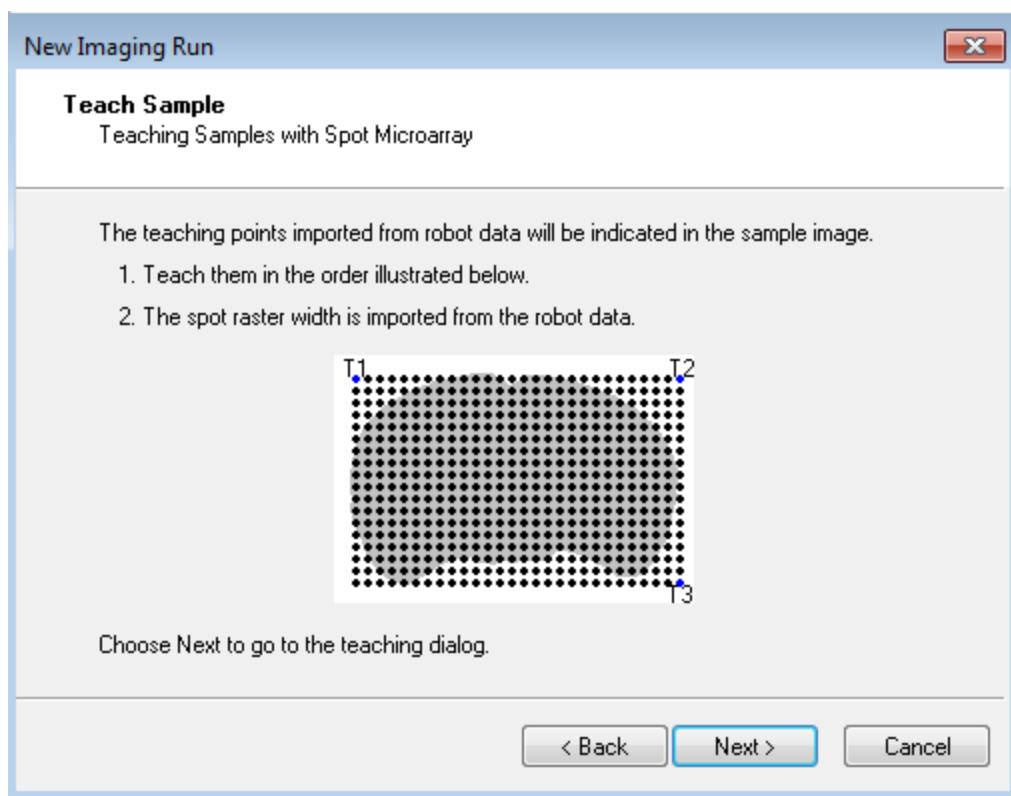


Figure 4-53 Teach Sample explanations dialog for spot microarray with robot import

Next

Switches to the wizard's **Teach Sample** dialog.

4.5.2.7 Teach Sample Dialog

The **Teach Sample** dialog is used to teach the sample. On principle, three teach points have to be set which serve to map image coordinates to sample carrier positions. Depending on the kind of sample preparation you have chosen in the Imaging Run Properties dialog, the teaching dialog looks different; the three possible dialogs are shown in the following screen shots. The dialog shows the sample image which can be zoomed in and explains how to proceed.

Note On opening this dialog, the factory teaching of the current geometry will be loaded in flexControl!

To be able to set the teach points easily you can activate  to use the mouse to zoom into the picture, select one of the pre-defined zoom steps or maximize the window using . Clicking  re-activates the teaching cursor. The **Undo** button can be used to remove already set teach points to be able to repeat the teaching.

Dialog for arbitrary arrays und tissue profiling

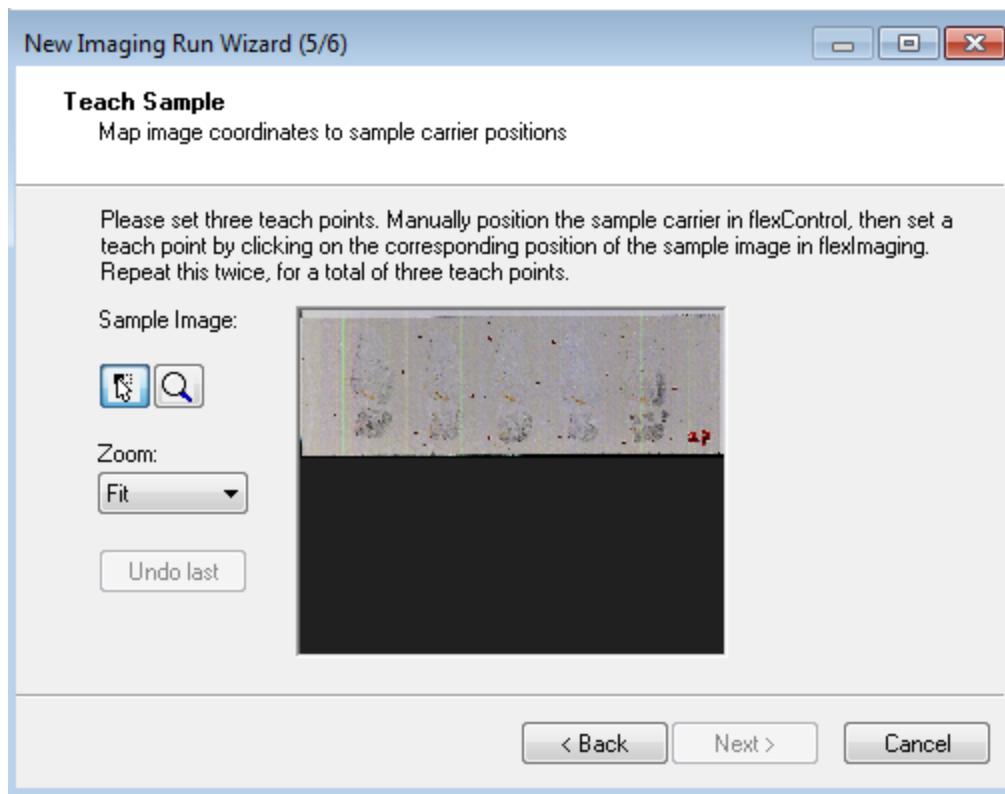


Figure 4-54 **New Imaging Run Wizard (page 5) — Teach Sample dialog for arbitrary arrays and tissue profiling (setting of the third teach point)**

Three teach points have to be set to enable the **Next** button. This has to be done together with flexControl:

1. Switch to flexControl.
2. Search for a first prominent mark in the video picture of your sample and move it under the cross-hairs.
3. Switch back to flexImaging and click the same mark on the flexImaging picture. The teach point is then marked with a white plus sign.
4. Repeat this procedure for another two prominent marks. The **Next** button becomes enabled after the third teach point was set.

Dialog for spot microarray without robot import

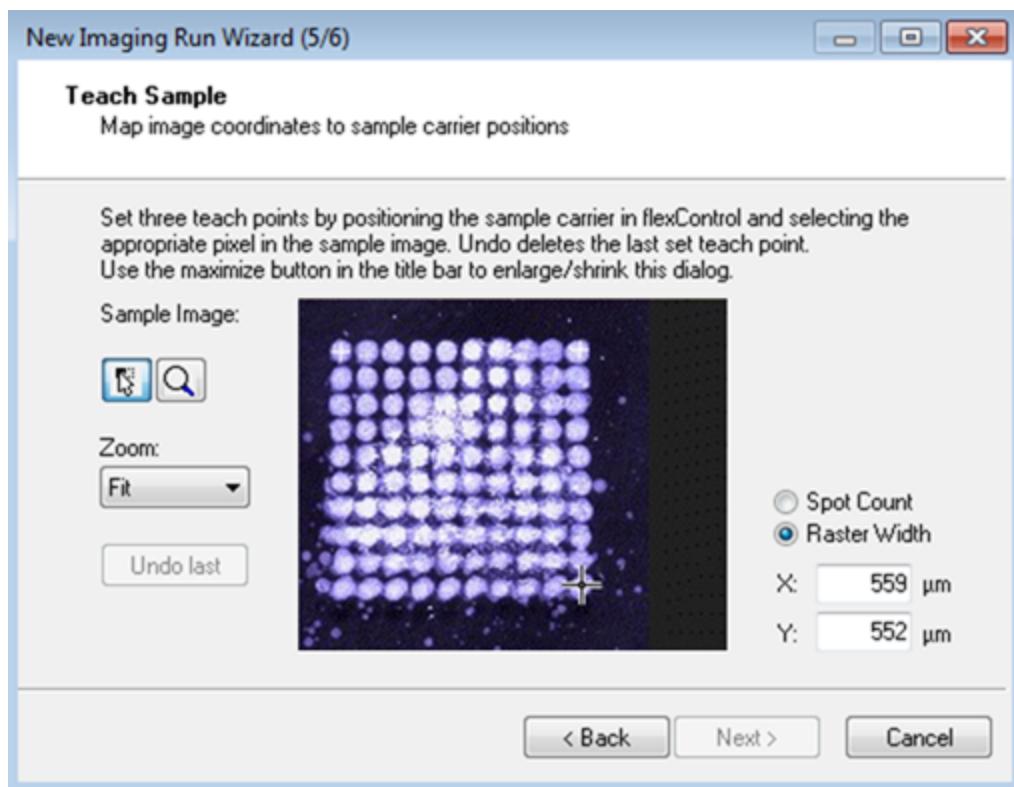


Figure 4-55 Teach Sample dialog for spot microarray without robot import (setting of the third teach point)

Note While teaching this image, take care to teach in the demanded order (T1, T2, T3):

1. Switch to flexControl.
2. Search for a first prominent mark (T1) in the video picture of your sample and move it under the cross-hairs.
3. Switch back to flexImaging and click the same mark on the flexImaging picture. The teach point is then marked with a white plus sign.
4. Repeat this procedure for another two prominent marks. The **Next** button becomes enabled after the third teach point was set.
5. Choose **Spot Count** to define the number of measuring points or **Raster Width** to do the fine tuning.

Dialog for spot microarray with robot import

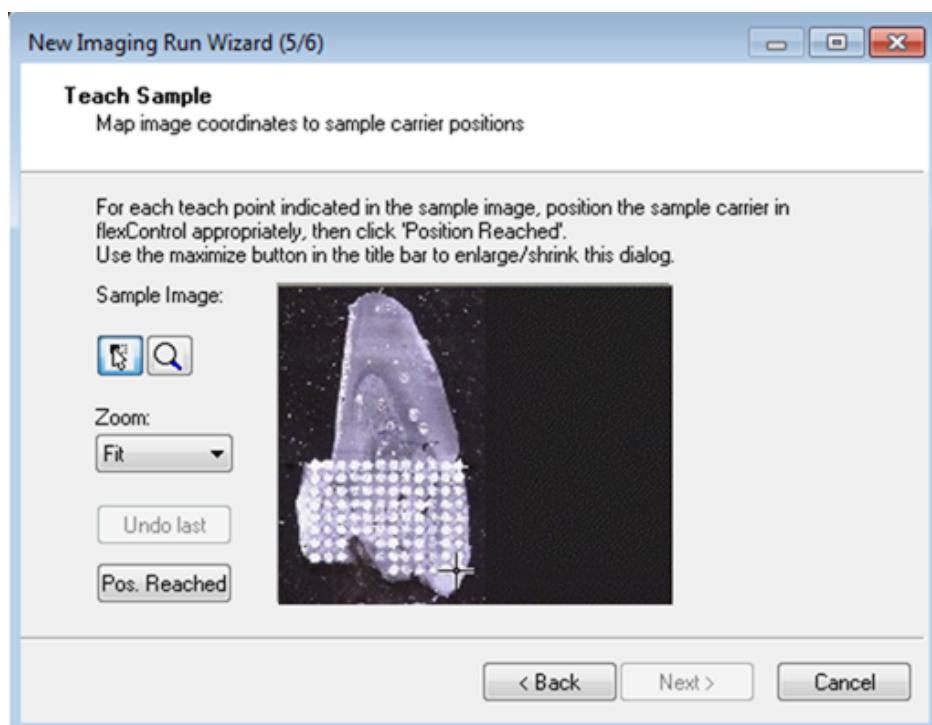


Figure 4-56 Teach Sample dialog for spot microarray with robot import (setting of the third teach point)

It is very simple to teach such a sample because the teach positions are pre-defined and the first cursor position in flexImaging is already selected (white cross on the image):

1. Switch to flexControl, search for the mark in the video picture and move it under the cross-hairs.
2. Switch back to flexImaging and click the **Pos. Reached** button. The white cross is automatically shown on the next teaching position.
3. Repeat this for T2 and T3. The **Next** button becomes enabled after the third teach point was set.

Next

Performs the chosen action and continues to the wizard's **Completing** dialog with a short description on how to continue in the flexImaging GUI.

4.5.2.8 Completing Dialog

The **Completing** dialog gives you a short description on how to proceed.

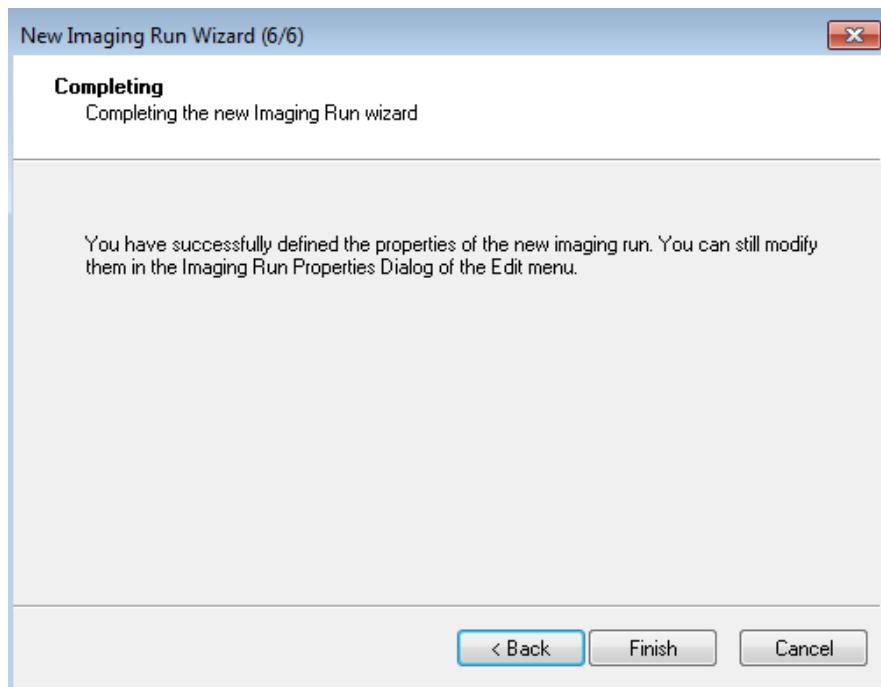


Figure 4-57 New Imaging Run Wizard (page 5) — Completing dialog

4.5.3 Preferences Dialog

The **Preferences** dialog defines general flexImaging settings. It contains the following four tabs:

- **User Interface** (opens by default)
- **Instrument**
- **Imaging Run Defaults**
- **Processing Defaults**
- **Updates**

4.5.3.1 Preferences — User Interface Dialog

The **User Interface** tab defines general settings for the user interface.

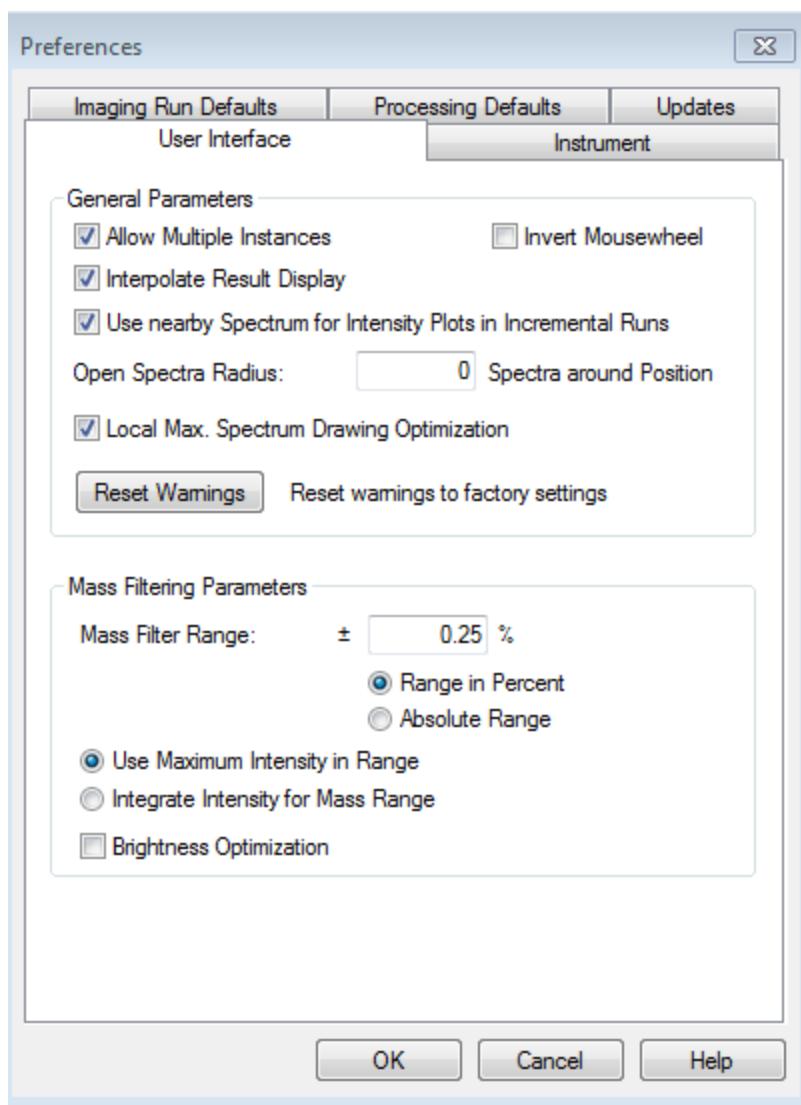


Figure 4-58 Preferences dialog – User Interface tab

Allow Multiple Instances

This option allows starting several flexImaging user interfaces.

Invert Mousewheel

Selecting this option inverts the zooming direction of the mouse wheel in the sample image window and the zooming and panning direction in the spectrum view.

Interpolate Result Display

If you check this option the middle of the laser spot position in one raster position is taken and the respective pixel gets the highest color intensity of this raster position. The intensity is reduced to the edges of this raster positions. Therefore your pictures will be softly blurred.

If you uncheck this option, you will have the same behavior as in flexImaging 1.x versions. This means, the raster positions are completely filled with the color. See the following screen shots for illustration.

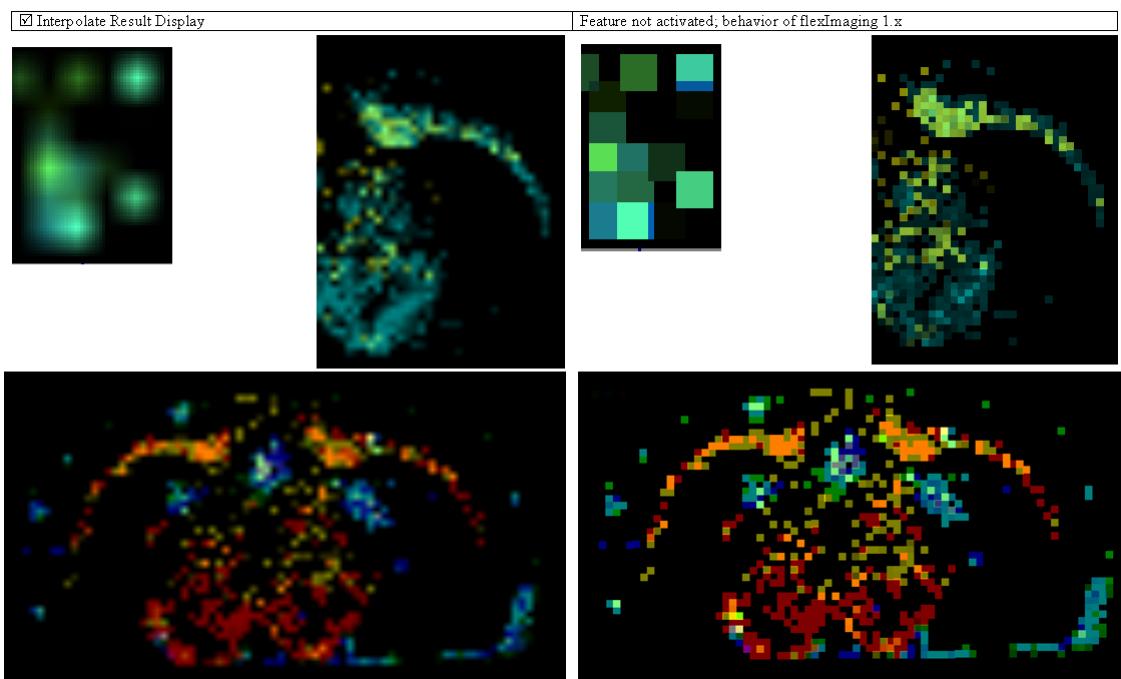


Figure 4-59 Illustration of results with (left) and without setting the Interpolate Result Display option

Use nearby Spectrum for Intensity Plots in Incremental Runs

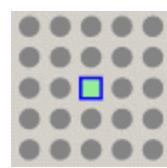
This option derives data for missing spots from an incremental run (specified in the **autoXecute Run options** dialog) from the adjacent spots.

Open Spectra Radius ... Spectra around Position

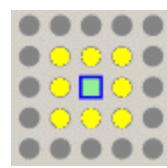
Define how many spectra are to be opened in flexAnalysis when the **Open Spectra in flexAnalysis** command from Imaging Display shortcut menu is used.

If you set this value to

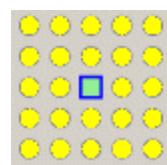
- 0 only one spectrum will be opened in flexAnalysis



- 1 up to 9 spectra will be opened in flexAnalysis



- 2 up to 25 spectra will be opened in flexAnalysis



This means $(2x+1)^2$ spectra will be opened in flexAnalysis.

Note This example fits best in spot microarray use-case. If you are in a border area of your defined measuring region less spectra will be opened because there are no spectra outside your defined area.

Local Max. Spectrum Drawing Optimization

This option enhances the display of zoomed out spectra. It has no effect if you zoom into the spectra so that you can see individual data points or if the spectra are read from peak lists.

Reset Warnings

Users can choose not to display warnings. This option resets the display warnings setting to the default of showing warnings.

Mass Filter Parameters

These values are used as defaults for newly created mass filters.

You can define the standard **Mass Filter Range: ± ... Da**. It is recommended to enlarge this value if you measure higher masses.

You can specify the mass range as **Range in Percent** or as **Absolute Range** by selecting the desired option.

You can decide whether the **Maximum Intensity** within the filter range is plotted as result, or the **Integrated Intensity** is used instead.

You can switch on **Brightness Optimization**. In this case, the intensity for maximum brightness is chosen automatically so that 95% of all intensities fall below this value. Thus, up to 5% of outliers are prevented from inappropriately reducing the overall brightness of the result image. However, this intensity cannot be lower than 60% of the highest intensity. The intensity for minimum brightness is 0. If **Brightness Optimization** is switched off, the percentage of the maximum intensity or the absolute intensity is used as specified in the **Edit Mass Filter Parameter** dialog.

4.5.3.2 Preferences — Instrument Dialog

The **Instrument** tab defines general settings for the user interface.

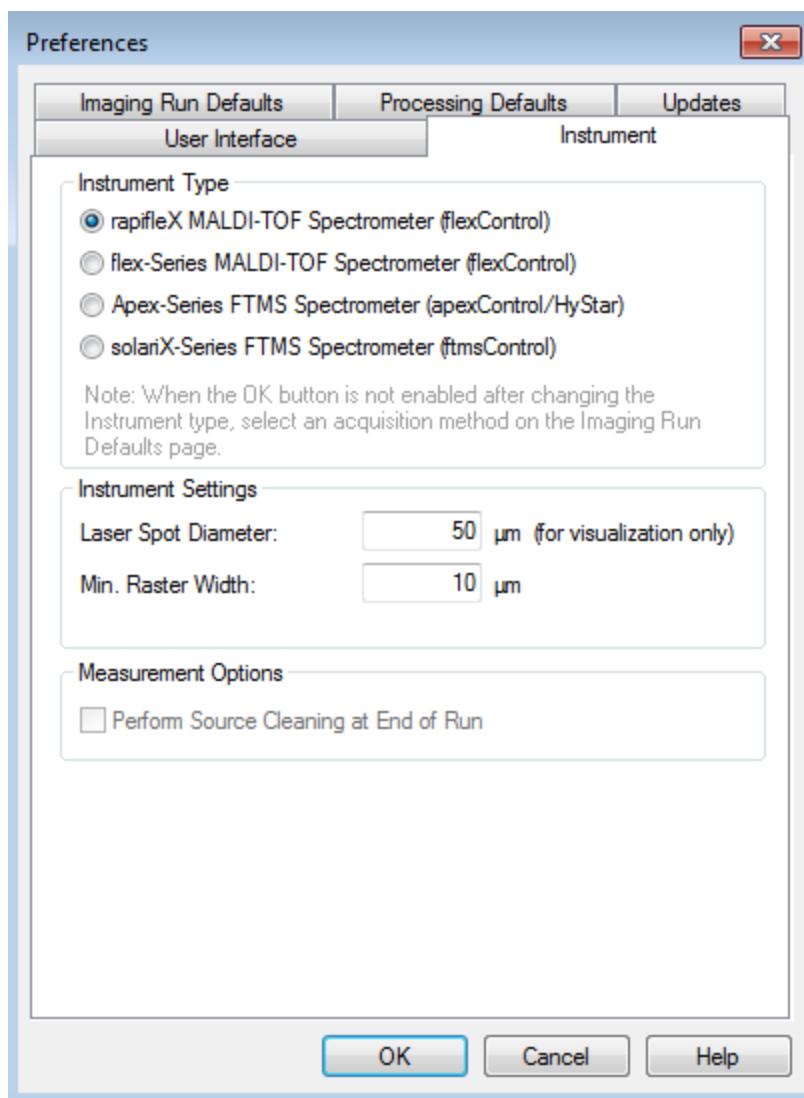


Figure 4-60 Preferences dialog – Instrument tab

Instrument Type

rapifleX MALDI-TOF Spectrometer. Use this option for the rapifleX TOF spectrometer controlled by flexControl 4.0.

flex-Series MALDI-TOF Spectrometer. Use this option for TOF spectrometers controlled by flexControl.

apex-Series FTMS Spectrometer. Use this option for FTMS spectrometers controlled by HyStar/ftmsControl.

solariX- Series FTMS Spectrometer. Use this option for FTMS spectrometers controlled by ftmsControl.

Note You need to specify the autoXecute method on the **Imaging Run Defaults** tab when switching between TOF and FTMS instrument types.

Note If you switch between the instrument types, you also have to consider that the data source specified on the **Processing Defaults** tab might change, too. This means: if you want to load processed MALDI spectra, you have to change back this setting as well!

Laser Spot Diameter

Usually this value must not be changed because it is an instrument specific value. Nevertheless, there is one use-case, where it might be helpful: if you have activated the laser spots view (**Laser Spots** command from **View** menu) but cannot see the laser spots, it might be helpful to enlarge the laser spot diameter. Before you change this value, please write it down first, so that you are able to restore it directly.

Min. Raster Width

Here you can define the minimum raster width which should be considered if you change the raster width in the **Edit Measurement Region Parameters** dialog.

Perform Source Cleaning at End of Run

Here you can decide to let the instrument start the source cleaning procedure when the run is finished.

4.5.3.3 Preferences — Imaging Run Defaults Dialog

The **Imaging Run Defaults** dialog defines general imaging run settings that will be shown as default in the **New Imaging Run Wizard** on the **Imaging Run Properties** page 1 and page 2.

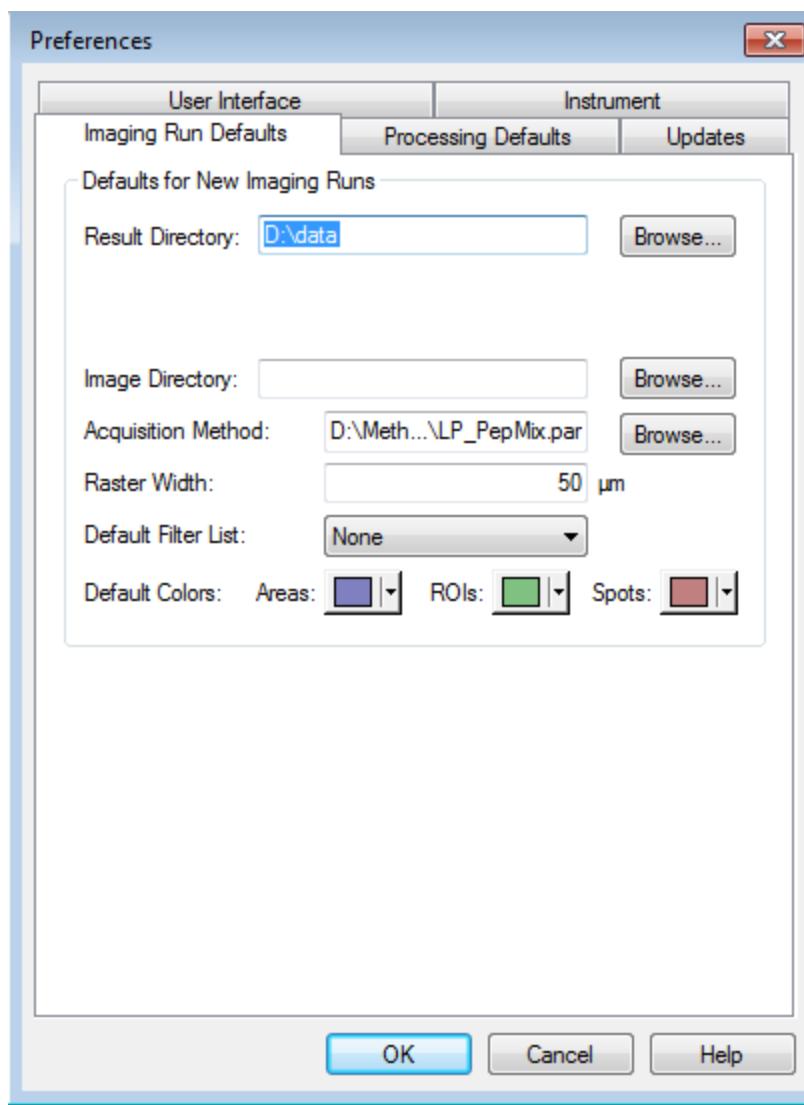


Figure 4-61 Preferences dialog – Imaging Run Defaults tab

Result Directory

If you generally want to store your imaging data into a specific folder, you can define this here.

Create Subdirectory for Imaging Run

This option creates a subdirectory for each imaging run by default. You can change this also in the wizard.

Image Directory

Here you can define a default sample image directory for the wizard if you usually store your images in one directory.

autoXecute Method

The chosen method will be used as default in the wizard and if you create new measurement regions. For flex Series instruments, an autoXecute method must be selected. For solariX instruments, an ftmsControl method must be selected. For Apex Series instruments, no selection option is available: the method used for acquisition is selected in the HyStar sample table.

Raster Width

The default raster width used in new imaging runs.

Default Filter List

Specifies the filter list that will be used as default for new imaging runs. All filter lists from D:\Methods\Imaging Runs can be chosen here. To add a list to this directory save it as template (see **Save Result Filter List As** dialog).

Default Colors

These color settings are used in the Imaging Display by default when the corresponding views are activated.

4.5.3.4 Preferences — Processing Defaults Dialog

The **Processing Defaults** tab defines general processing settings.

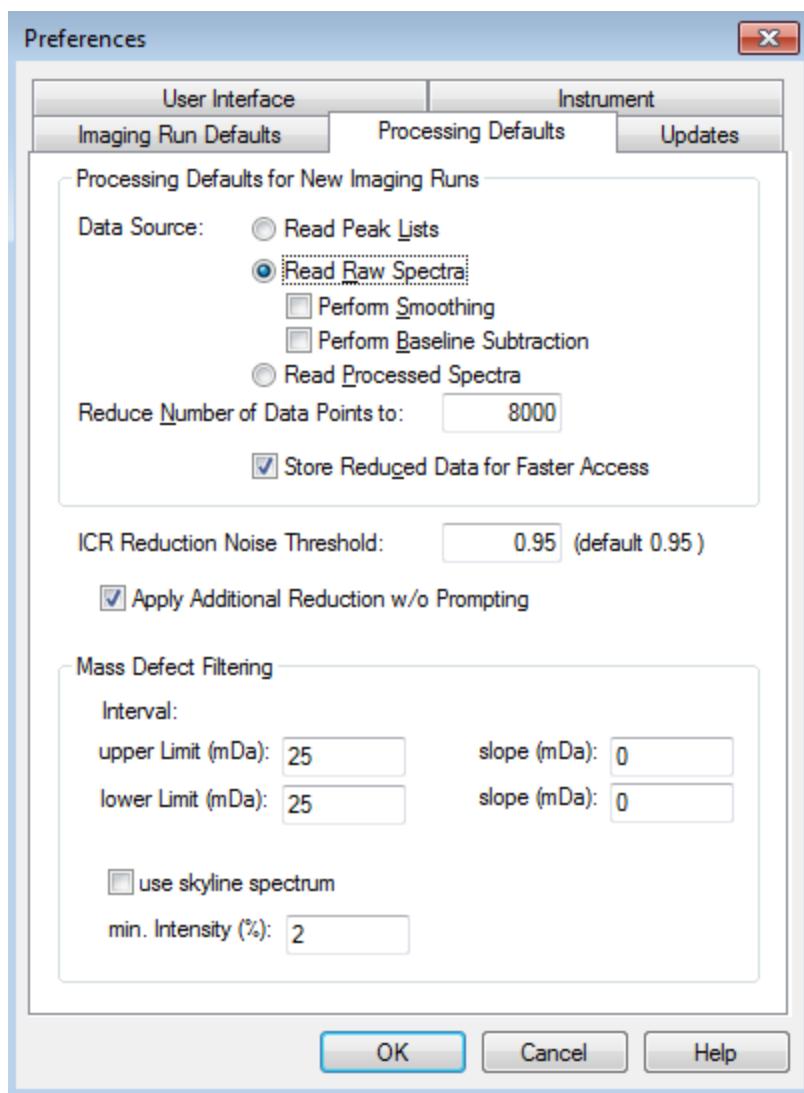


Figure 4-62 Preferences dialog – Processing Defaults tab

Data Source

Select the data source:

- **Read Peak Lists.** Reads peak lists generated by flexAnalysis and creates artificial spectra. flexImaging 1.x always operated in this mode.

- **Read Raw Spectra.** In this case any processing (e.g. smoothing and baseline subtraction made by flexAnalysis) will be ignored and the raw spectra as acquired will be loaded.

Perform Baseline Subtraction. If this option is checked, flexImaging performs a baseline subtraction on the raw data using the TopHat algorithm. This option is enabled by default for TOF instruments and disabled for FTMS instruments.

Read Processed Spectra. The processing done e.g. by flexAnalysis will be used and flexImaging will not change it. If there are no processed spectra in the selected imaging run, flexImaging will automatically switch to raw data when reading the spectra, and only display a corresponding message in the status bar.

Reduce Number of Data Points to

You either can define a specific number of data points or set it to zero to let flexImaging automatically reduce your data.

If you open the **Detection** tab of flexControl and have a look on the spectrum size (shown in the lower right corner), you will get an idea how many points your spectra had when they were acquired. The number of data points determines the amount of memory flexImaging needs to load the spectra. Therefore, it might be meaningful to reduce the number of data points. On the other hand, the spectra will not show many details if you reduce this number too much. You have to find out which value fits best to your use-cases.

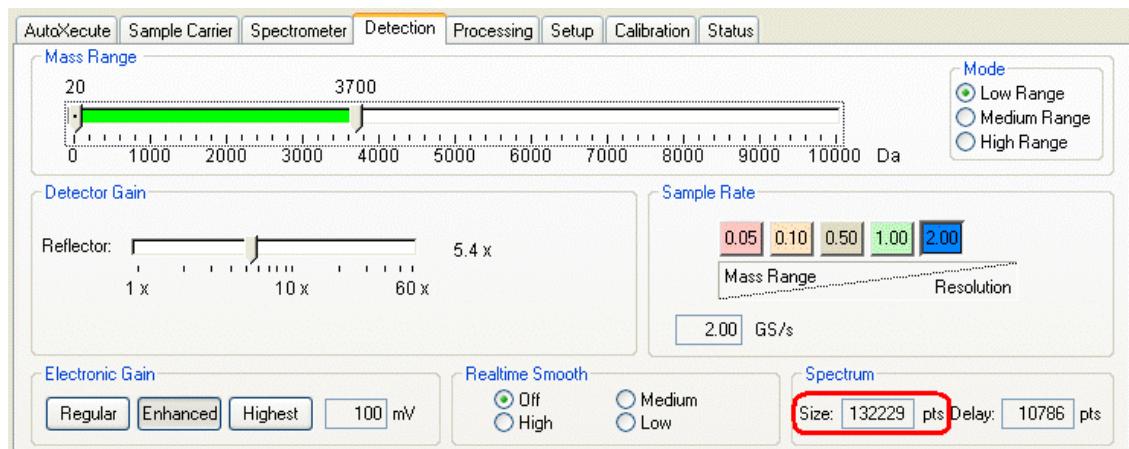


Figure 4-63 flexControl Detection tab

Store Reduced Data for Faster Access

This option saves a *.dat file next to your spectra which is used by flexImaging for a faster access to the data. In many cases it is not necessary to reload all the spectra again, therefore the timesaving loading of the *.dat file is sufficient. This setting is stored in the imaging run to allow a faster access to the data even if you open another imaging run again.

ICR Reduction Noise Threshold

This option determines the degree of data reduction for Apex instruments. Below 1, the value defines a percentile of intensities removed from the original data. This is the preferred method. Above 1, it defines a cutoff intensity as a multiple of an average noise level.

Apply Additional Reduction w/o Prompting

If this box is checked, when loading FTMS spectra, flexImaging applies the calculated data reduction without prompting and waiting for a user response.

Mass Defect Filtering, Interval

Upper and lower limit determine the initial width of the accepted mass interval, the slopes define the change of the width with increasing distance from the base mass.

User Skyline Spectrum

If this box is checked, flexImaging considers peaks from the skyline spectrum instead of the average spectrum when searching for masses inside the accepted mass interval.

min. Intensity

This value determines the minimum intensity as a percentage of the base mass peak that a peak must exceed to be accepted as a potential filter.

4.5.3.5 Preferences - Updates Dialog

The **Updates** dialog determines if and when the program checks for available updates at startup.

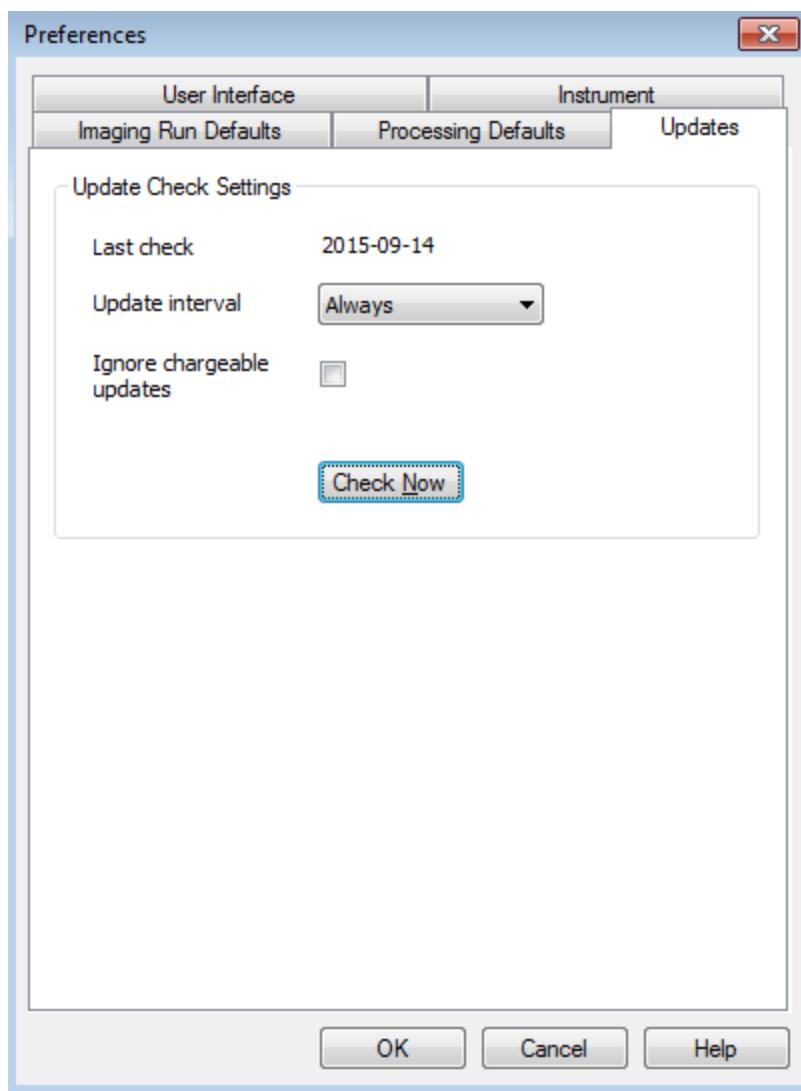


Figure 4-64 Preferences dialog – Updates tab

Last Check

This text shows the date of the last check for updates.

Update Interval

This option determines the frequency of checks. Available options are "Never", "Always" (at every program start), "Daily" (at the first start of the program), "Weekly" and "Monthly".

Ignore chargeable updates

If selected, only updates that are distributed free of charge are reported.

Check now

By clicking this button, you can perform an immediate check for updates .

4.5.4 Imaging Run Properties Dialog

The **Imaging Run Properties** dialog defines properties of the current imaging run. It contains the following two tabs:

- Current Imaging Run Parameters
- Processing Options

4.5.4.1 Imaging Run Properties — Current Imaging Run Parameters Dialog

The **Current Imaging Run Parameters** tab displays the settings defined in the **New Imaging Run Wizard**. You can always change the comments and the default colors, but the result directory, the spot raster, the autoXecute method and the parent mass can be changed only before the autoXecute run. The spot raster can be changed in spot microarray use-case only.

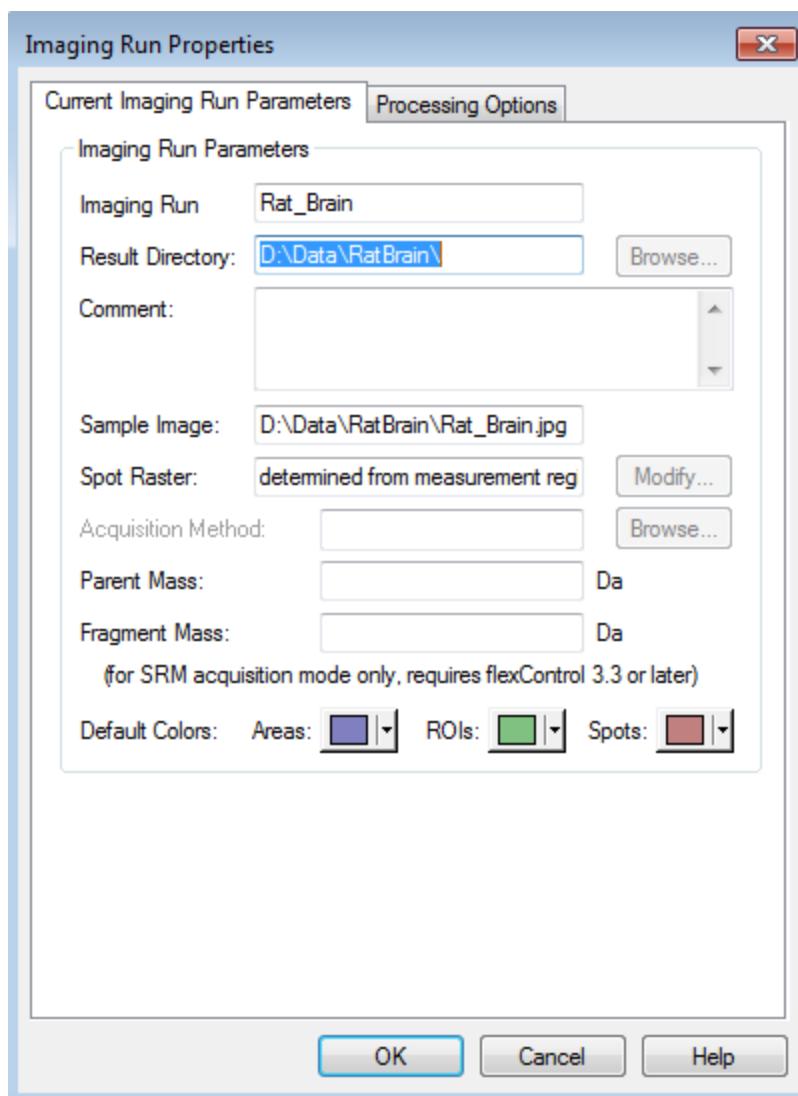


Figure 4-65 Imaging Run Properties dialog – Current Imaging Run Parameters tab

Imaging Run Name

Shows the name of the loaded *.mis file.

Result Directory

Shows the current result directory containing the measuring results. Change the directory if desired.

Comment

Use this field for a description of the imaging run in a few notes.

Sample Image

Shows the path to the currently loaded image.

Spot Raster

In case of conventional imaging runs, there is nothing to do for the operator. The **Modify** button is disabled.

However, if a imaging run of a spot microarray is in use, this feature allows slight corrections of the measuring points in position and number. Clicking **Modify** opens the **Spot Microarray Teaching** dialog.

autoXecute Method

Shows the current autoXecute method that will be used as default if you create new measurement regions. Select another method if desired.

Parent Mass

If you want to measure in MS/MS mode (precursor and fragments or fragments only), you must type in the mass of the target compound precursor ion into this field before you start the imaging run. In this case, you also have to choose an MS/MS autoXecute method, which refers to an MS/MS flexControl method (flexControl 3.3 or 3.4) or a flexControl MS/MS method (flexControl 4.0). For details, refer to the *flexControl User Manual*.

Fragment Mass

If you want to measure in SRM (Single Reaction Monitoring) mode, you have to fill in the parent mass and the fragment mass of interest in these fields before you start the imaging run. In this case, you also have to choose an SRM autoXecute method, which refers to an SRM flexControl method. Leave this field empty for measuring MS or MS/MS (LIFT) spectra. For details, refer to the *flexControl User Manual*.

Note The fragment mass option is only supported by flexControl 3.3 and 3.4.

Default Colors

These settings will be used in the Imaging Display for coloring measurement regions (**Areas**), regions of interest (**ROIs**) and measurement spots (**Spots**) if the corresponding views are activated.

4.5.4.2 Imaging Run Properties — Processing Options Dialog

The **Processing Options** dialog tab defines processing options in flexImaging.

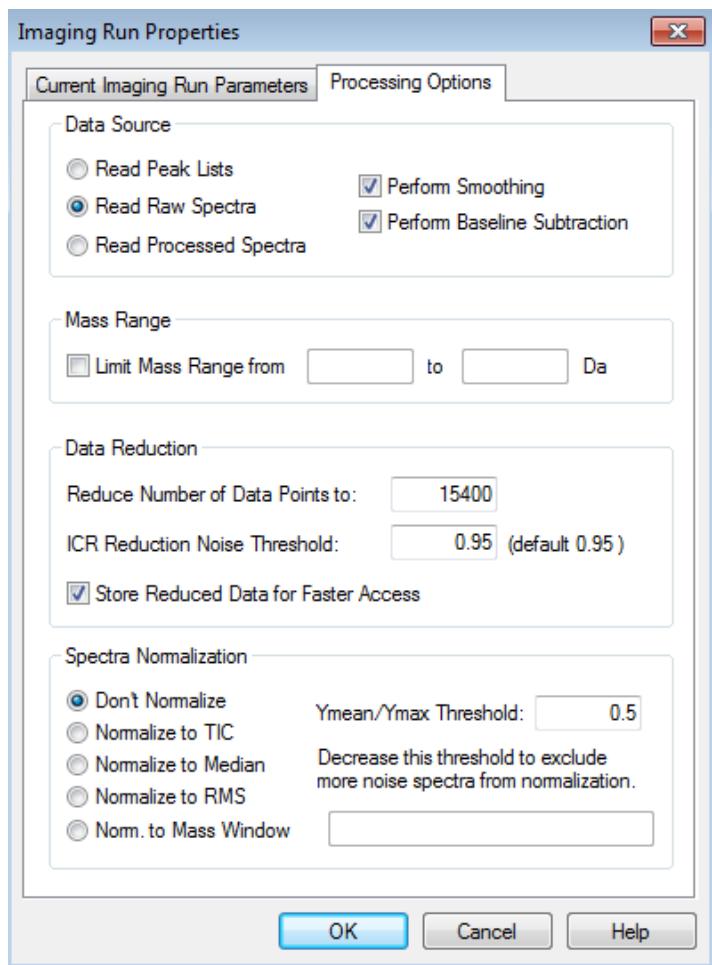


Figure 4-66 Imaging Run Properties dialog – Processing Options tab

Data Source

Select the data source:

- **Read Peak Lists.** Reads peak lists generated by flexAnalysis and creates artificial spectra. flexImaging 1.x always operated in this mode.
- **Read Raw Spectra.** In this case any processing (e.g. smoothing and baseline subtraction made by flexAnalysis) will be ignored and the raw spectra as acquired will be loaded.

- **Read Processed Spectra.** The processing done e.g. by flexAnalysis will be used and flexImaging will not change it. If there are no processed spectra in the selected imaging run, flexImaging will automatically switch to raw data when reading the spectra, and only display a corresponding message in the status bar.

Perform Smoothing. If this option is checked, flexImaging smooths the acquisition data.

Perform Baseline Subtraction. If this option is checked, flexImaging performs a baseline subtraction on the raw data using the TopHat algorithm. This option is enabled by default for TOF instruments and disabled for FTMS instruments.

Limit Mass Range from ... to

This option narrows the mass range of the spectrum down to the range specified by **from** and **to**. Afterwards flexImaging will reload all spectra (or peak lists), but will only show the defined mass range. This might be helpful if you recognize after your acquisition that there are e.g. no interesting peaks at the upper or lower end of the mass range.

Reduce Number of Data Points to

This option only applies to TOF spectra. You either can define a specific number of data points or set it to zero to let flexImaging automatically reduce your data.

If you open the **Detection** tab of flexControl and have a look on the spectrum size (shown in the lower right corner), you will get an idea how many points your spectra had when they were acquired. The number of data points determines the amount of memory flexImaging needs to load the data. Therefore, it might be meaningful to reduce the number of data points. On the other hand, the spectra will not show many details if you reduce this number too much. You have to find out which value fits best to your use-cases.

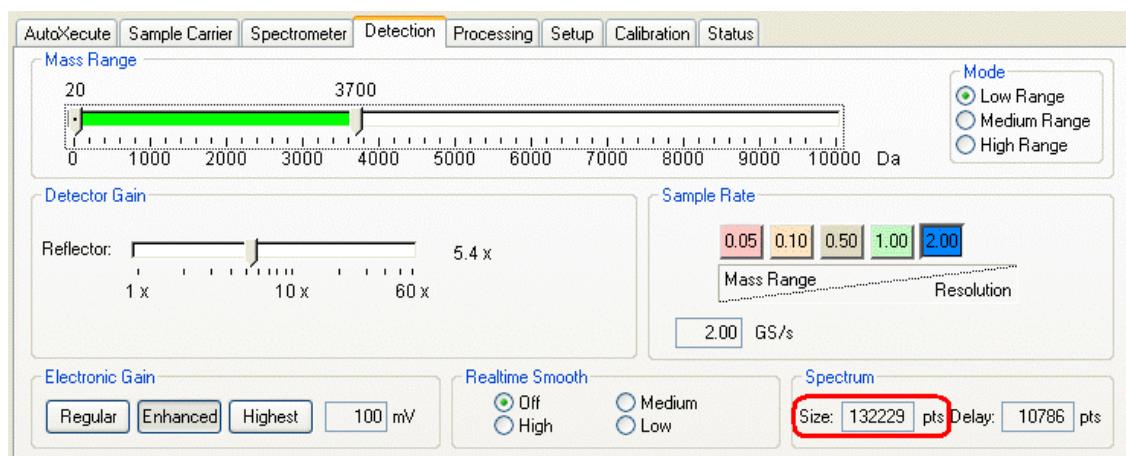


Figure 4-67 flexControl Detection tab

ICR Reduction Noise Threshold

This option determines the degree of data reduction for Apex instruments. Below 1, the value defines a percentile of intensities removed from the original data. Above 1, it defines a cutoff intensity as a multiple of an average noise level.

Store Reduced Data for Faster Access

This option saves a *.dat file next to your spectra which is used by flexImaging for a faster access to the data. In many cases it is not necessary to reload all the spectra again, therefore the timesaving loading of the *.dat file is sufficient. This setting is stored in the imaging run to allow a faster access to the data even if you open another imaging run again.

Spectra Normalization

This option offers a choice of different methods of spectra normalization:

Don't Normalize: The original data is used as is.

Normalize to TIC: The sum of all intensities in the spectrum is divided by the number of data points. This average value is used for normalization.

Normalize to Median: The median of all intensity values in the spectrum is taken as normalization value.

Normalize to RMS: (This option is the equivalent of the "Normalize" option of flexImaging versions before 3.0) The sum of squares of all intensities in the spectrum is divided by the number of data points. The root of this number is used as normalization value:

$$Y_{norm} = \sqrt{\frac{y_1^2 + y_2^2 + \dots + y_n^2}{n}}$$

y = *y* value of 'spectrum at cursor position' in the Spectrum Display

n = number of data points in the spectrum. For TOF spectra, this is the number entered in the **Data Reduction** group above. For ICR spectra, it is the number of data points after reduction.

Normalize to Mass Window: The user may enter a list of pairs of masses in the form "xx.x-yy.y, nn.n – oo.o, pp.p – qq.q". If the first mass of a pair is smaller than the second, then the average intensity inside the interval is used for normalization. If more than one such interval is entered, the average intensity of all intervals is used. All intervals that start with the larger mass are excluded from calculating the average intensity for normalization. Mixing of these two types of intervals is discouraged. An easier way to define mass windows for normalization is the "Add Mass Window" dialog that opens after the user selects two masses with the "Mass Window / Distance" entry of the shortcut menu inside the spectrum window.

Ymean/Ymax Threshold

If $Y_{norm} < \text{Threshold Factor} * \text{max. value of Y axis}$, perform normalization, else set this spectrum to zero.

Decrease this threshold to exclude more noise spectra from normalization.

Tip *Alternatively, you can select the normalization method using the normalization dropdown selection box in the toolbar.*

4.5.5 Spot Microarray Teaching Dialog

The **Spot Microarray Teaching** dialog allows slight corrections of the measuring points in position and number. You can define either the number of spots per row or the corresponding raster width between the spots instead. Changing these parameters may influence the spot raster width in the **Imaging Run Properties** dialog and the raster width of already created measurement regions because the raster width of the measurement regions is a multiple of the raster width of the **Imaging Run Properties** dialog.

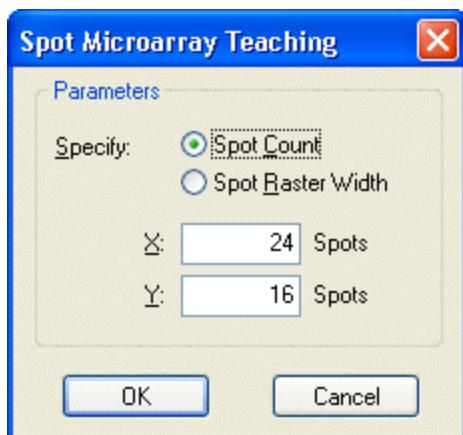


Figure 4-68 Spot Microarray Teaching dialog

Specify

Select how to change the spot raster:

Spot Count: Changes the number of spots. Enter the new spot number per row in **X** and **Y**.

Spot Raster Width: Changes the spot raster width. Enter the new spot raster width per row in **X** and **Y**. The maximum raster width is limited to 5000 µm.

OK

Changes the spot raster accordingly after you have answered the appearing confirmation request.

4.5.6 Spot List Import Wizard

The **Spot List Import Wizard** is only available in the tissue profiling use-case. It assists you in importing a list of distributed spots from a sample preparation robot.

The wizard displays the following dialogs:

- **Robot Import** (this dialog is equal to the **Robot Import** dialog of the **New Imaging Run Wizard** except the title bar)
- **Spot List Import**

4.5.6.1 Robot Import Dialog

The **Robot Import** dialog is used to select the file to import sample preparation robot data.

Note This dialog is also launched from the Spot List Import Wizard then showing "Spot List Import" in the title bar.

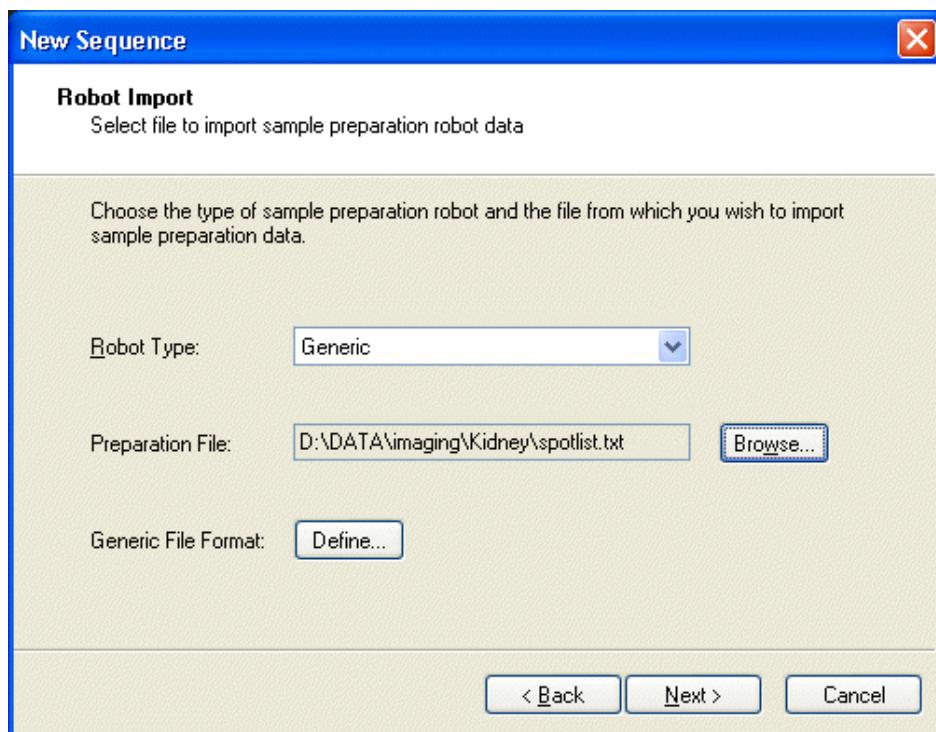


Figure 4-69 Robot Import dialog

Robot Type

Select your robot type. Currently the Shimadzu ChIP 1000 and Leap Technologies preparation robots are supported. If you have a different robot that exports its coordinates in ASCII format, you can use the 'Generic' import option.

Preparation File

Use the **Browse** button to select the file to import.

Generic File Format

The **Define** button allows defining parameters for the import of generic robot files. It opens the **Definitions for Import of Generic Robot Files** dialog.

Next

Switches to the wizard's **Sample Image** dialog except you imported the spot coordinates from a robot or the importer was unable to locate the sample image. In that case, the **Teach Sample explanations** dialog opens.

4.5.6.2 Spot List Import Dialog

The **Spot List Import** dialog is used to align the imported spot positions with the sample image.

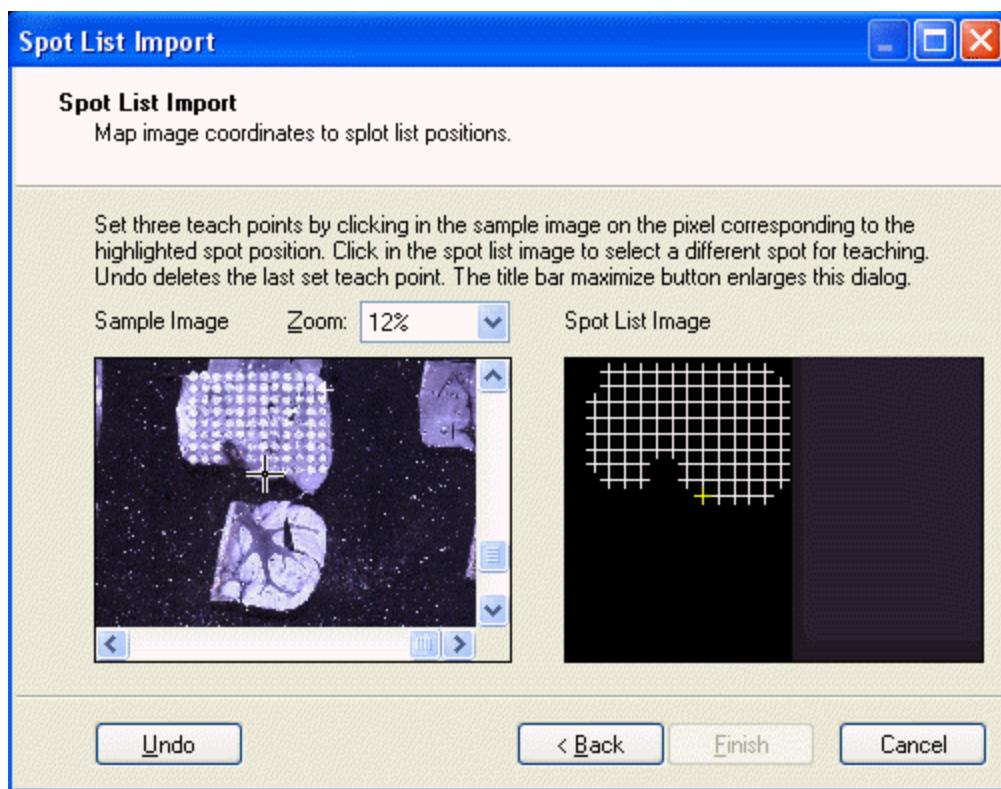


Figure 4-70 Spot List Import dialog

Sample Image

Displays the image of the sample.

Zoom Factor

Selects a zoom factor between 12% and 800% for the sample image.

Spot List Image

Shows a map of all imported spot positions. The active spot for teaching is shown with a yellow mark.

Undo

Deletes the last teach point.

Finish

Completes the spot list import and adds the spots to the active measurement region. This button is enabled after three teach points have been set.

The teaching process is performed in the following workflow:

1. flexImaging chooses a teach point from the spot list and marks it yellow.
2. You can either accept it or select a different spot by clicking in the spot list image.
3. Click in the sample image to teach the corresponding location.
4. Steps 1 to 3 are repeated three times.

You can maximize the window using  for easier navigation.

4.5.7 Edit Measurement Region Parameters Dialog

The **Edit Measurement Region Parameters** dialog allows editing parameters of the measurement region selected in the Regions Pane. Before the autoXecute run has been started you can change all the settings in this dialog; after the autoXecute run, only the name and the spectrum color can be changed.

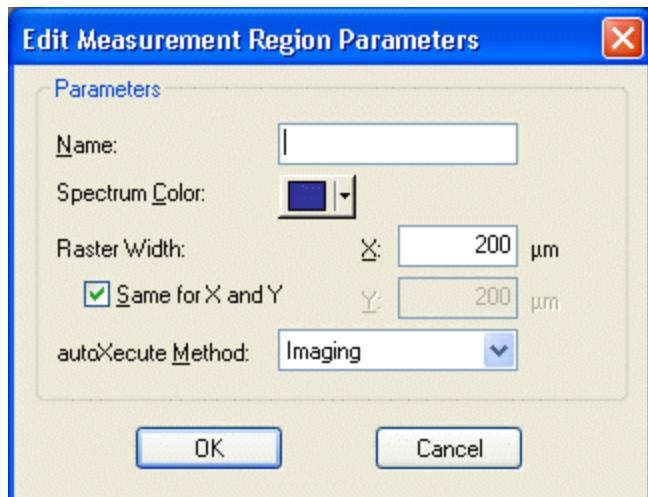


Figure 4-71 Edit Measurement Region Parameters dialog

Name

You can change the name of the measurement region that is visible in the Regions Pane and in the Imaging Display when ROIs are shown.

Spectrum Color

The chosen color is used in the Spectrum Display to show the average spectrum of the defined area if you activate the **Spectrum** checkbox in the Regions Pane.

Raster Width

You can change the actual raster width in μm in X and Y direction. In spot microarrays use-case these values depend on the number of spots, you have entered in the wizard for the corresponding measuring directions.

Note We strongly recommended using the same raster width for all measurement regions in a imaging run. When using different raster widths, flexImaging uses a raster width calculated by the greatest common denominator of all regions. If different raster widths are used, intensity plots displayed using the **Interpolate Results** mode will not appear as intense as when using the same raster width for all spots.

Same for X and Y

Check this option if you do not want to use different values for X and Y.

autoXecute method

Choose another autoXecute method from the dropdown box if you do not want to use the default method for this area.

4.5.8 Edit Region of Interest Parameters Dialog

The **Edit Region of Interest Parameters** dialog allows editing the parameters of the region of interest (ROI) selected in the Regions Pane.

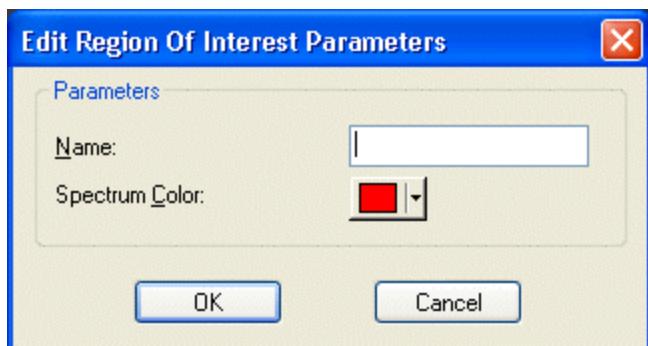


Figure 4-72 Edit Region of Interest Parameters dialog

Name

You can change the name of the ROI that is visible in the Regions Pane and in the Imaging Display when ROIs are shown.

Spectrum Color

The chosen color is used in the Spectrum Display to show the average spectrum of the defined area, if you activate the **Spectrum** checkbox in the Regions Pane.

4.5.9 Save autoXecute Sequence As Dialog

The **Save autoXecute Sequence As** dialog is used to enter a file name for saving the autoXecute imaging run and the geometry file.

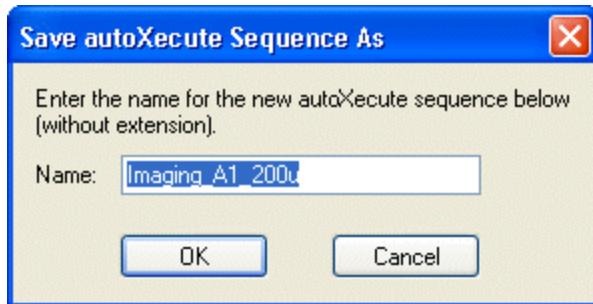


Figure 4-73 Save autoXecute Sequence As dialog

OK

Saves the imaging run to the specified name.

4.5.10 autoXecute Run Options Dialog

The **autoXecute Run Options** dialog defines the settings for an automatic measurement in the acquisition program (flexControl or ftmsControl).

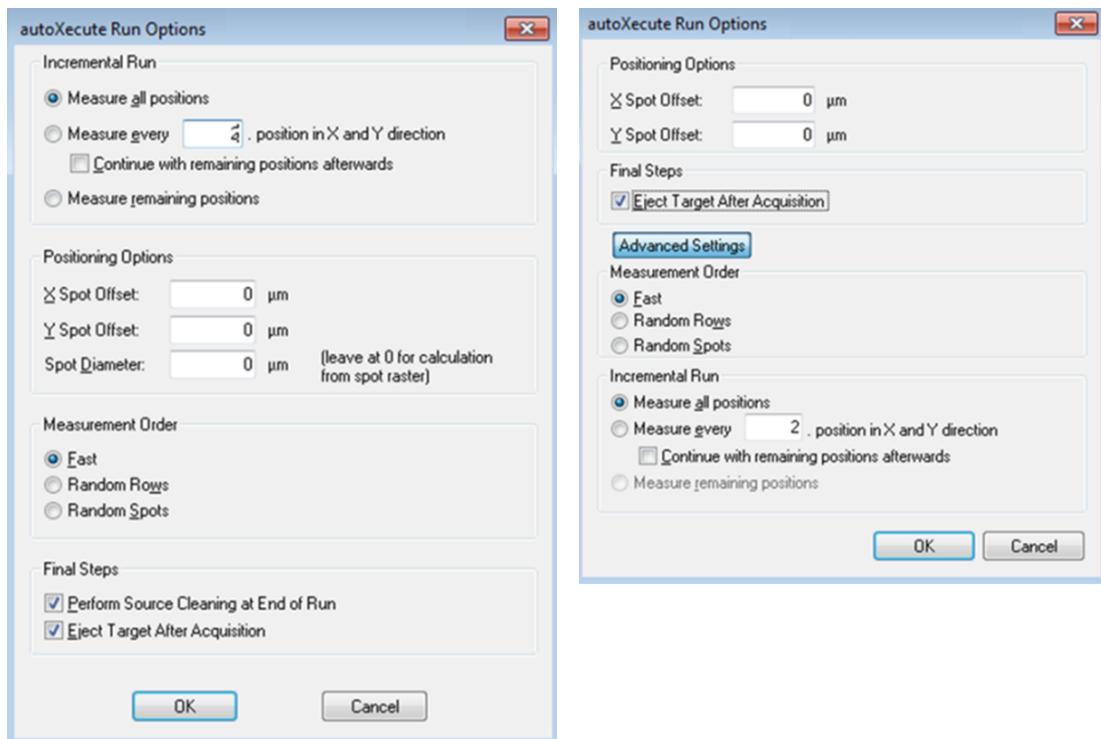


Figure 4-74 autoXecute Run Options dialog for flex series MALDI-TOF, Apex series FTMS, and solariX series FTMS instruments (left) and rapifleX MALDI-TOF instruments (right)

Incremental Run

Select which positions should be measured:

- **Measure all positions.** Measures every position.
- **Measure every ... position in X and Y direction.** Measures e.g. every 2nd position in X and Y direction. This means only about one fourth of the total number of spots will be measured during the first autoXecute run. This feature helps you to get an approximate overview over all your samples, e.g. to see whether the settings are ok. The incremental run feature is not available in the tissue profiling use-case.
- **Continue with remaining positions afterwards.** When checked, the second autoXecute run will start directly after the first one. Nevertheless, the results acquired so far will be loaded into flexImaging as usual.
- **Measure remaining positions.** If you measured e.g. every 2nd position in X and Y direction first but have not set the **Continue with remaining positions afterwards** option you prepare the start of the second autoXecute run with this option.

Note If you want to load another imaging run before you have measured the remaining positions, you have to save the imaging run, because otherwise flexImaging cannot restore the current state correctly.

X/Y Spot Offset

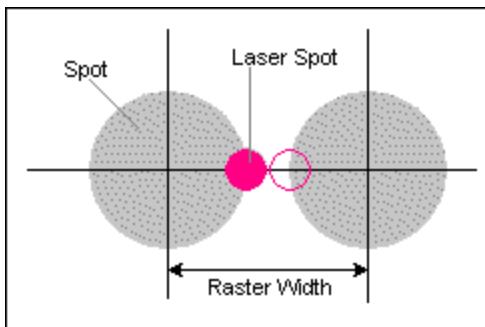
flexImaging adds the offset specified here to the calculated positions when generating the geometry for the autoXecute run. The spot offset can be used e.g. when measuring a sample for the second time if you want to avoid acquiring data from the exact same positions.

Spot Diameter

Determines the spot diameter written to the geometry.

If the spot diameter is set to zero, flexImaging automatically calculates the spot diameter from the smallest raster width of the measurement regions and the laser spot diameter set in the **Preferences - Instrument** dialog. If you enter a number here, flexImaging subtracts the laser spot diameter from the **Preferences** dialog. This subtraction is done to ensure that acquisitions will not overlap.

The calculation is demonstrated in the drawing below.



Note This feature is only useful if you use an autoXecute method with rastering enabled, (e.g. Movement tab: measuring raster: spiral_small, Accumulation tab: set number of shots, which should be summed up, larger than the number of shots acquired in one shot step).

Measurement Order

Fast (top to bottom). Measures spots row by row from top to bottom. This is the fastest acquisition option.

Random Rows. Measures rows in random order.

Random Spots. Measures spots in random order. This is the slowest acquisition option. It can be used to eliminate measurement order influences on statistical analyses on the acquired data.

Perform Source Cleaning at End of Run

If checked, the instrument source will be cleaned after the data acquisition (if the instrument supports this feature). The cleaning process is then handled by the acquisition software.

Note This option is only available with Compass for flex Series 1.4 or later acquisition software.

OK

If the dialog was launched from the **Start autoXecute Run** a message appears that reminds you to make sure to save the flexControl method before starting the autoXecute run. Clicking **OK** starts the run. If the imaging run directory already contains data, first a confirmation request to overwrite this data appears. Click **Yes** to start the autoXecute run; otherwise, no run is started.



Figure 4-75 Information on saving the flexControl method

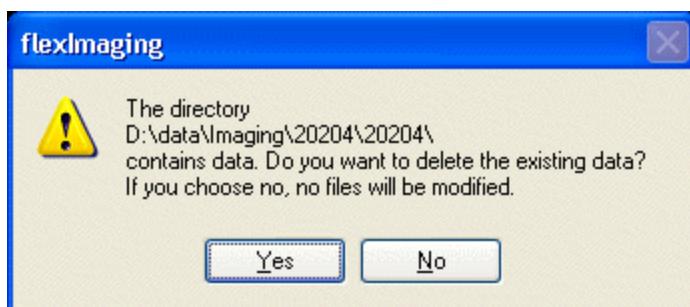


Figure 4-76 Confirmation request to overwrite existing data

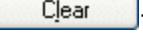
If the dialog was launched from the **Save autoXecute Sequence As** command the **Save AutoXecute Sequence As** dialog opens to save the autoXecute imaging run.

4.5.11 Co-Register Image Dialog

flexImaging allows co-registering a second image. The second image will usually be a histologically stained section. The full potential of MALDI imaging is reached if the histological staining is done on the same section after the MALDI imaging experiment. It can also be a stained consecutive section or an autoradiography. In spot microarrays or tissue profiling workflows it can also be a scan of the unspotted sample.

The aim of the **Co-Register Image** dialog is to align images together. To accomplish this, a teaching is necessary:

1. First browse to the second image and load it.
2. Then the first teach point (the one you used in the wizard) will automatically be shown in the left image. Now you can decide to use the same one or to set another one in this left image. If you want to use a different one, just click on this position in the left image.
3. Then click on the corresponding position in the right image.
4. Continue in the same way with the second and the third teach point.
5. If you are satisfied with your teaching, close the dialog with the **OK** button.

You can use the zooming drop-down boxes, the scrollbars and the maximize window function  to be able to set the teach points easily. Click  to delete the last teach point in the co-registered image or click  to delete all teaching positions and unload the second image.

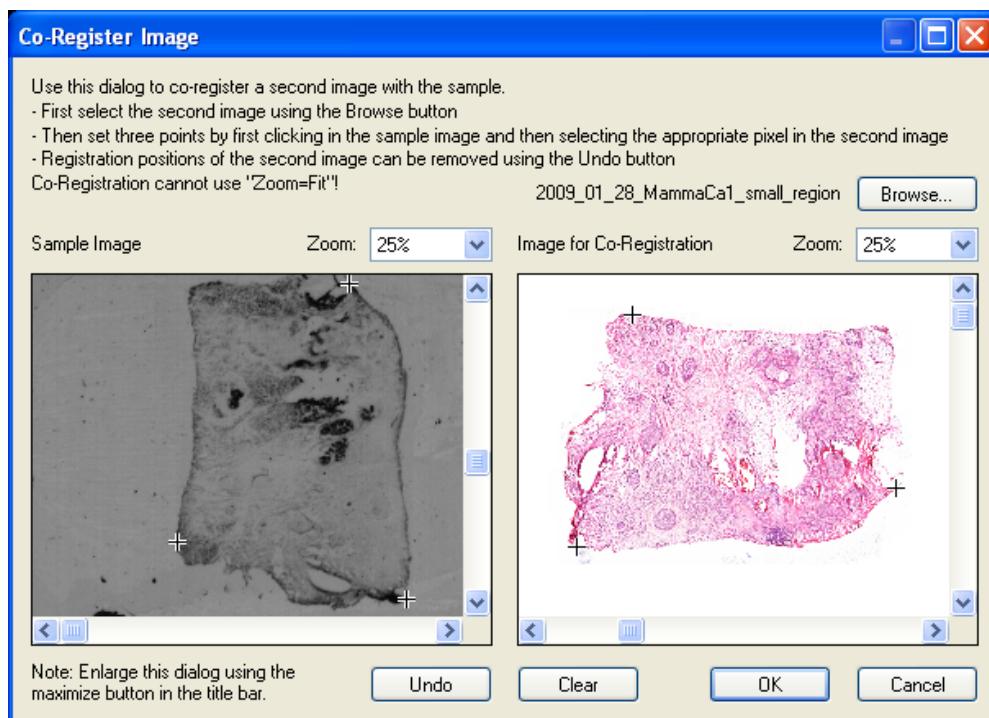


Figure 4-77 Co-Register Image dialog

4.5.12 Copy Region Of Interest Spectra Dialog

The **Copy Region of Interest Spectra** dialog allows copying all spectra belonging to the defined Region of Interest (ROI) selected in the Regions Pane into a newly created directory. The original spectra folder remains unchanged, i.e. no spectra are deleted from the hard drive.

Note If you want to pass spectra to ClinProTools 2.2 or later for analysis, it is recommended to use the **Export Spectra List** dialog in combination with ClinProTools **Open Spectra Import XML** command instead, which provides the same functionality without copying the spectra.

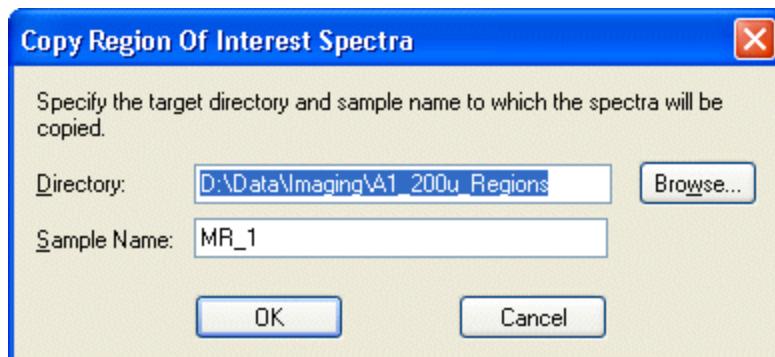


Figure 4-78 Copy Region of Interest Spectra dialog

Directory

Choose a path and directory where to create a copy of the selected spectra.

Sample Name

Type in a sample name for the selected group of spectra.

4.5.13 Export Spectra List Dialog

The **Export Spectra List** dialog allows creating a list of spectra belonging to one or multiple regions or to one or multiple results. The list can be imported into ClinProTools using the **Open Spectra Import XML** command. The exported list is grouped into regions/results, so that ClinProTools e.g. can generate a classification model based on the selected criteria.

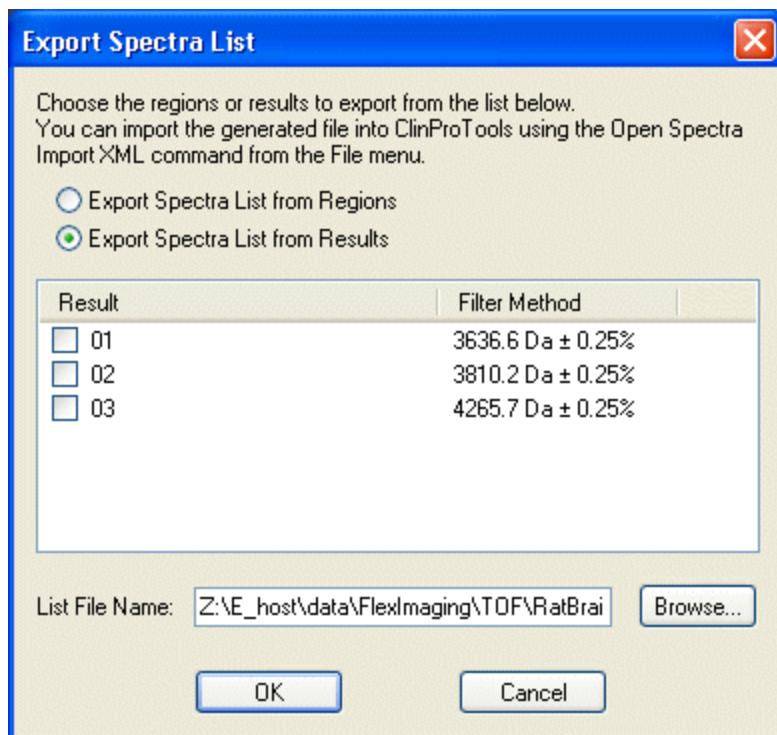


Figure 4-79 Export Spectra List dialog

Export Spectra List from Regions

Choose this option to export spectra from selected measurement regions and/or regions of interest.

Export Spectra List from Results

Choose this option to export spectra that match results.

Region/Result List

Check the regions or results that should be included in the export.

List File Name

Use the **Browse** button to select the file name for export.

OK

Starts the export. A dialog signals completion of the action.

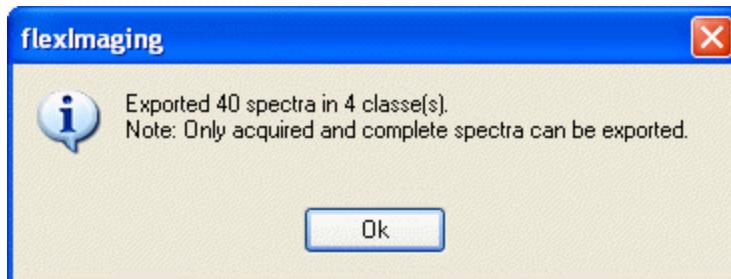


Figure 4-80 Export Spectra List Complete dialog

4.5.14 Edit Mass Filter Mark Parameters Dialog

The **Edit Mass Filter Mark Parameters** dialog allows defining the mass range of a peak you selected in the Mass Filter Selection mode. A vertical pink line marks the respective peak in the Spectrum Display.

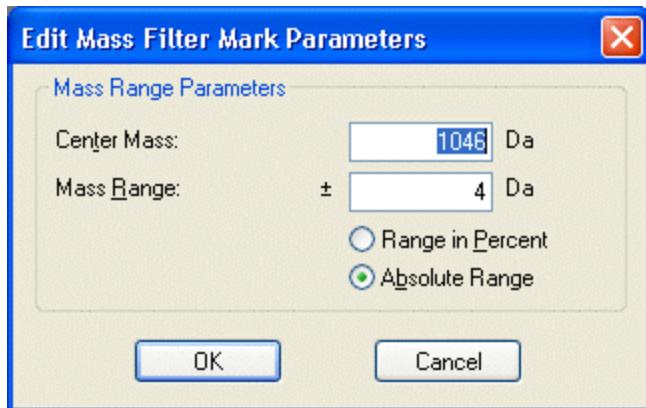


Figure 4-81 Edit Mass Filter Mark Parameters dialog

Center Mass

Type in the exact center mass of interest.

Mass Range

Enter a percentage or absolute mass range that defines how many Da to the left and the right of the center mass the mass filter should work. In the example above the mass filter would work from 1042 - 1050 Da.

- **Range in Percent.** Determines a percentage mass range concerning the entered center mass.
- **Absolute Range.** Determines an absolute mass range in Da.

4.5.15 Add Result Filter Dialog

The Add Result Filter dialog is used to add a new result filter to the result filter list. Different types of result filters are available.



Figure 4-82 Add Result Filter dialog

Type

Select the type of result filter to be added:

- **Mass Filter.** (Default) Filters by mass. The corresponding options are set in the **Edit Mass Filter Parameters** dialog.
- **Mass Defect Filter.** Filters by mass defect. The corresponding options are set in the dialog.

- **Import ClinProTools Result.** Launches the **Import ClinProTools Result** wizard to import results from ClinProTools.
- **Import Mass Control List or Peak List.** This option creates mass filters from a mass control list (MCL) or a peak list (peaklist.xml) in the file selected with a standard file-open dialog.
- **Import Mass List from MetaboliteTools Predict.** This option creates mass filters from a mass list exported by MetaboliteTools Predict. Common adducts for spectra acquired in positive or negative mode are included in the import.
- **XML Import.** Filters by imported results from other applications stored in XML format. The corresponding options are set in the **Edit XML Import Filter Parameters** dialog.
- **Text Import.** Filters by imported results from other applications stored in generic text format. The corresponding options are set in the **Edit Text Import Filter Parameters** dialog.

OK

Opens the dialog corresponding to the selected option.

4.5.16 Save Result Filter List As Dialog

The **Save Result Filter List As** dialog is used to save the result filter list currently in the Results Pane to a specified file. This allows reusing the result filter list in later analyses.

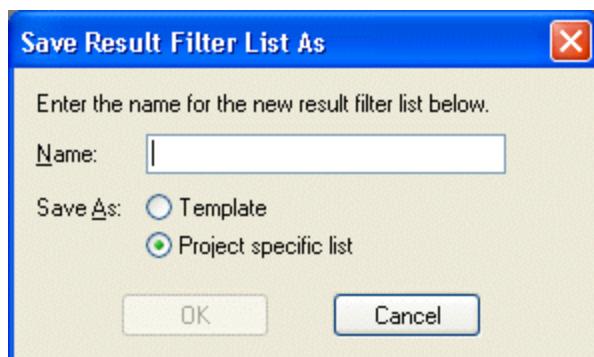


Figure 4-83 Save Result Filter List As dialog

Name

Enter a name for your result filter list.

Save As

Result filter lists can be saved in two formats:

- **Template**

Saving a result filter list as a **Template** to D:\Methods\Imaging Runs adds it to the Results Pane **Filter List** drop-down list (see section 4.2.4). Saving a results filter list as a **Template** makes it available for all later analyses.

- **Project specific list**

Saving a result filter list as a **Project specific list** enables you to copy your imaging results to another computer or a different storage medium. A Project specific list must be saved in the `data` folder.

If you want to use your result filter lists as template but if you also want to be able to copy your imaging results on another medium, you must first save it as a template and then copy it manually using Windows Explorer into the `data` directory (one level above your spectra).

4.5.17 Hierarchical Clustering Parameters Dialog

The **Hierarchical Clustering Parameters** dialog enables you to manage and start hierarchical clustering. After the calculations are finished, the program automatically loads the clustering tree result and displays it as a dendrogram.

Alternatively, you can load an existing clustering result by clicking **Add** in the Results Pane, selecting **Import ClinProTools Results**, and using the relevant Wizard options (see next chapters) to load a clustering or a clustering tree.

In contrast to ClinProTools, Hierarchical Clustering results are stored in a subdirectory of the imaging run folder named `hcl_results_ddddddd_tttt`, where `ddddddd` and `tttt` are date (yyymmdd) and time (hhmm) of the start of calculations (for example, 20120923_1234). The result file names are `Nclusters.xml` for the clustering result, where `N` is the cluster count in this file, and `hierarchical_clustering_tree.xml` for the clustering tree.

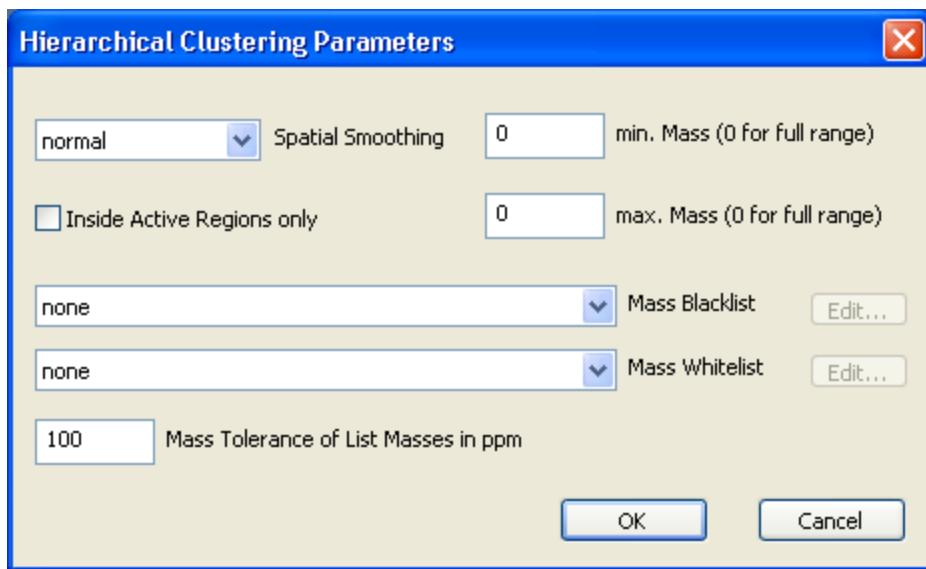


Figure 4-84 Hierarchical Clustering Parameters dialog

Degree of spatial smoothing

Choose between "weak", "normal", "strong" and "none".

min. Mass

Defines the minimum mass to consider for clustering. Enter **0** to use the lowest mass in each spectrum.

max. Mass

Defines the maximum mass to consider for clustering. Enter **0** to use the highest mass in each spectrum.

Inside Active Regions only

Selecting this option limits the calculation to spectra inside ROIs or Measurement Regions that are selected in the Regions Pane.

Mass Blacklist

With this dialog element, you can choose a MCL file containing masses that are to be excluded from the clustering. In the 32-bit version of flexImaging, you can edit the selected MCL file by clicking **Edit**.

Mass Whitelist

With this dialog element, you can choose a MCL file containing masses that are to be included in the clustering. In the 32-bit version of flexImaging, you can edit the selected MCL file by clicking **Edit**.

Mass Tolerance of List Masses

This entry determines how accurately a mass found in any spectrum must match a mass from the black- or whitelist to be rejected or accepted.

4.5.18 Import ClinProTools Result Wizard

ClinProTools supports various classification or analysis methods and exports the results to disk. These files are typically stored in the directory C:\BDAL\ ClinProTools_3_0\Files\ClinProtClassifications (for classifications) or C:\BDAL\ClinProTools_3_0\Files (for other analysis methods). For ClinProTools versions other than 3.0 replace the 3_0 with the appropriate digits.

flexImaging 5.0 supports the following ClinProTools result types:

- Classifications
- Clustering (from file ClinProtClustering.xml)
- Clustering trees (from file ClinProtClusteringtree.xml)
- Principal Component Analysis (from file ClinProtPCA.xml)
- Probabilistic Latent Semantic Analysis (from file ClinProtPLSA.xml)
- Variance Ranking (from file ClinProtPCA.xml)

The **Import ClinProTools Result Wizard** assists you in importing ClinProTools results into flexImaging.

4.5.18.1 Import ClinProTools Result Dialog

The Import ClinProTools Result dialog is used to specify the ClinProTools result file to be imported.

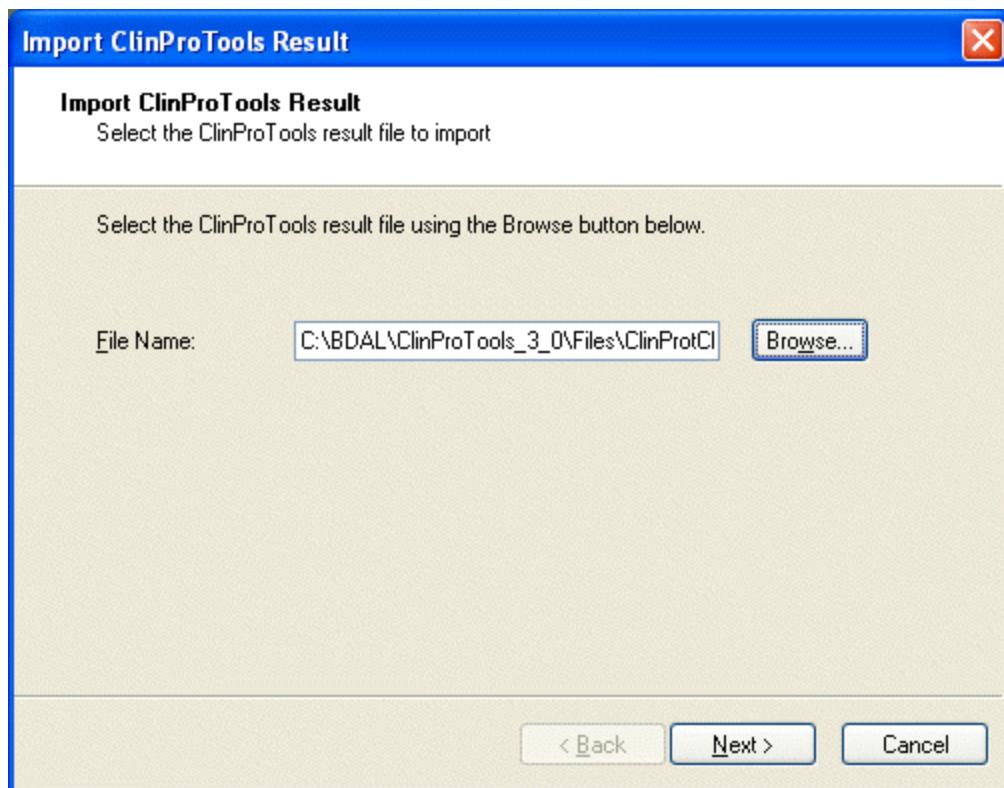


Figure 4-85 Import ClinProTools Result dialog

File Name

Use the **Browse** button to select the file you want to import.

Next

Starts the import and continues with the appropriate import options page:

- **Import ClinProTools Classification** dialog for classification import
- **Import ClinProTools Clustering** dialog for clustering
- **Import ClinProTools Clustering Tree** dialog for clustering trees
- **Choose between ClinProTools PCA and Variance Ranking** dialog for PCA and variance ranking
- **Import ClinProTools PLSA** dialog

4.5.18.2 Import ClinProTools Classification Dialog

The **Import ClinProTools Classification** dialog specifies the options for the ClinProTools classification import. The number of classes in the ClinProTools model is automatically detected during the import resulting in one filter being created for each class.

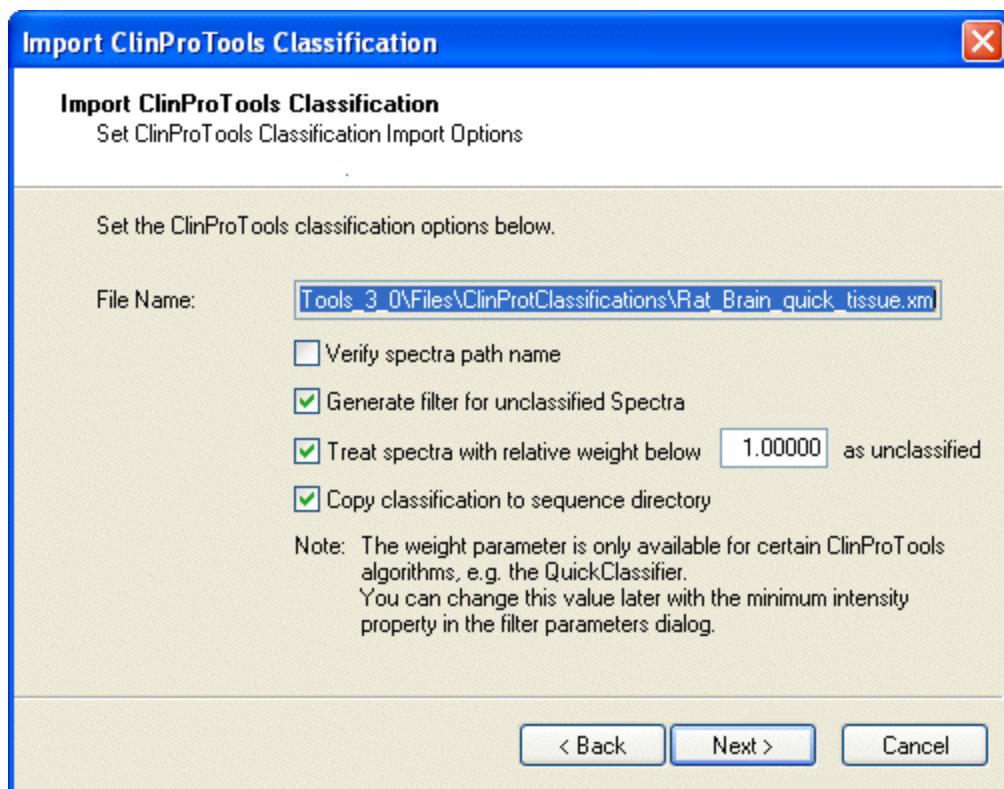


Figure 4-86 Import ClinProTools Classification dialog

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs into a single classification.

Generate Filter for unclassified spectra

Check this option if flexImaging should generate a result filter for spectra not belonging to any class.

Treat spectra with relative weight below X.X as unclassified

For classifiers that output a weight for each class (e.g. the QuickClassifier), spectra can belong to multiple classes. This relative weight can be used to suppress weak classification results. If a classification determines that a spectrum uniformly belongs to all classes, the weights will be set to 1.0, thus this threshold should be set to values between 1.0 and the number of classes in the model.

Copy classification to result directory

Check this option if flexImaging should copy the specified classification file to the result directory for easier archiving.

Next

Starts the import and after finishing opens the wizard's **Completing ClinProTools Import** dialog.

4.5.18.3 Import ClinProTools Clustering Dialog

The **Import ClinProTools Clustering** dialog specifies the options for importing a ClinProTools clustering and creating the result filters.

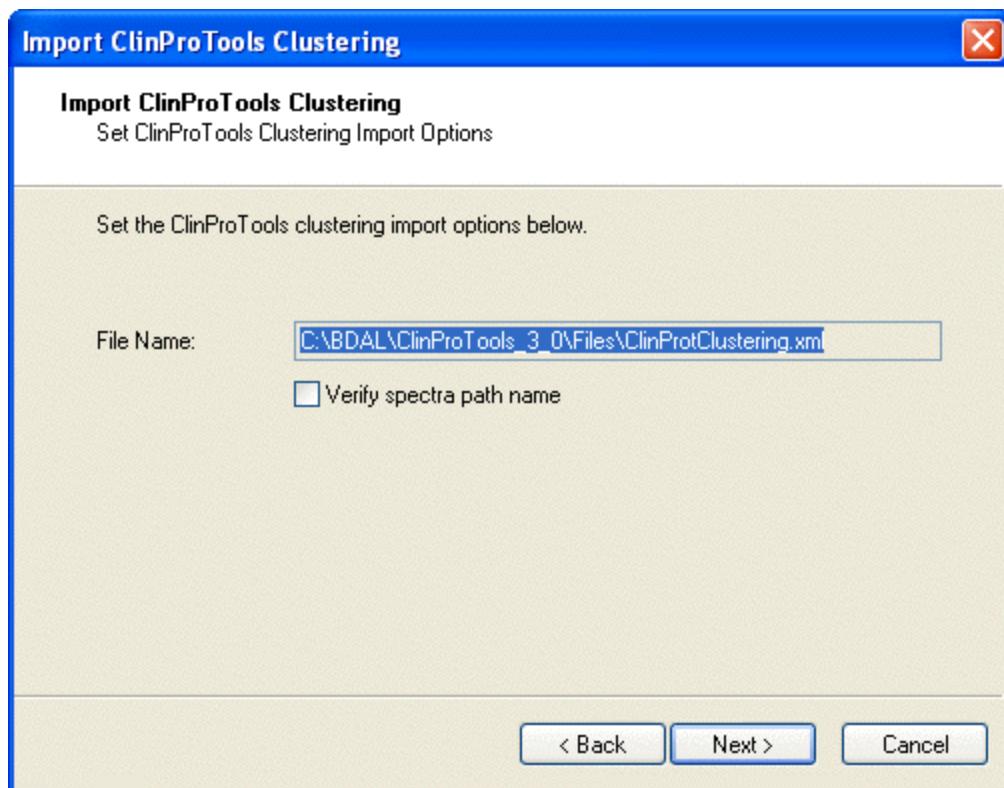


Figure 4-87 Import ClinProTools Clustering dialog

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the clustering file matches the current imaging run. Use this option if you included spectra from multiple runs into a single clustering analysis.

Next

First, a confirmation request to copy the clustering results file to the imaging runs directory appears. Since ClinProTools overwrites the ClinProtClustering.xml file every time the clustering tool is invoked the previous information in this file is lost. flexImaging only stores a reference to the clustering file in its result filters, so it is advisable to allow flexImaging to copy the clustering file to the imaging run directory. Answering the request starts the import. After import is finished the wizard's **Completing ClinProTools Import** dialog opens.

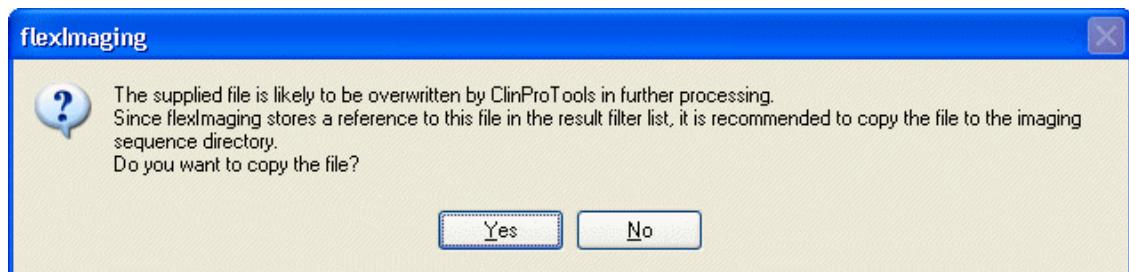


Figure 4-88 Confirmation on whether to copy the current ClinProTools file

4.5.18.4 Import ClinProTools Clustering Tree Dialog

The **Import ClinProTools Clustering Tree** dialog specifies the ClinProTools clustering tree import options.

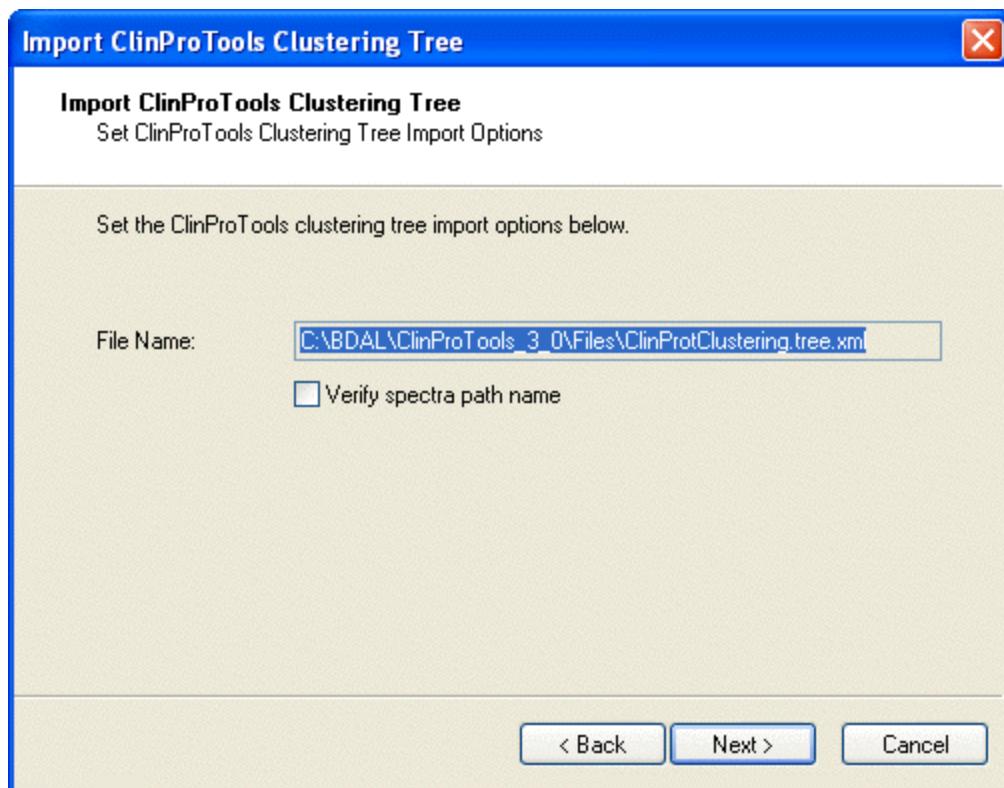


Figure 4-89 Import ClinProTools Clustering Tree dialog

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the clustering file matches the current imaging run. Use this option if you included spectra from multiple runs into a single clustering analysis.

Next

First, a confirmation request to copy the clustering tree results file to the imaging runs directory appears. Since ClinProTools overwrites the ClinProtClustering.tree.xml file every time the clustering tool is invoked the previous information in this file is lost. flexImaging only stores a reference to the clustering tree file in its result filters, so it is advisable to allow flexImaging to copy the file to the imaging run directory. Answering the request starts the import. After import is finished the wizard's **Completing ClinProTools Clustering Tree Import** dialog opens.

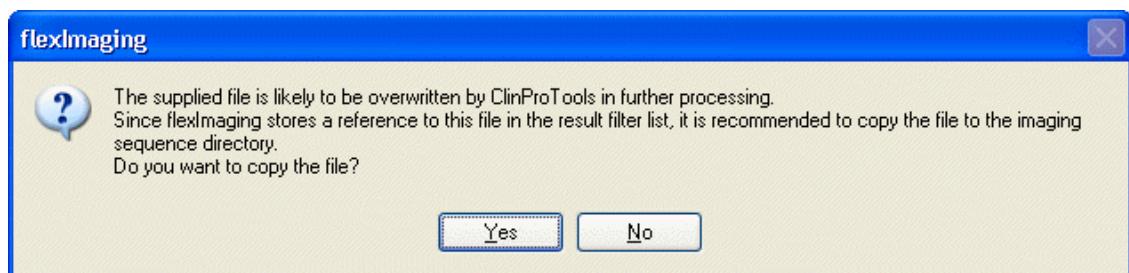


Figure 4-90 Confirmation on whether to copy the current ClinProTools file

4.5.18.5 Choose Between ClinProTools PCA and Variance Ranking Dialog

In addition to the PCA results, ClinProTools stores a variance ranking in the ClinProtPCA.xml file generated during a principal component analysis. When a ClinProtPCA.xml file is selected for the import in the first step of the ClinProTools import wizard, the **Choose between ClinProTools PCA and Variance Ranking Import** dialog is displayed next.

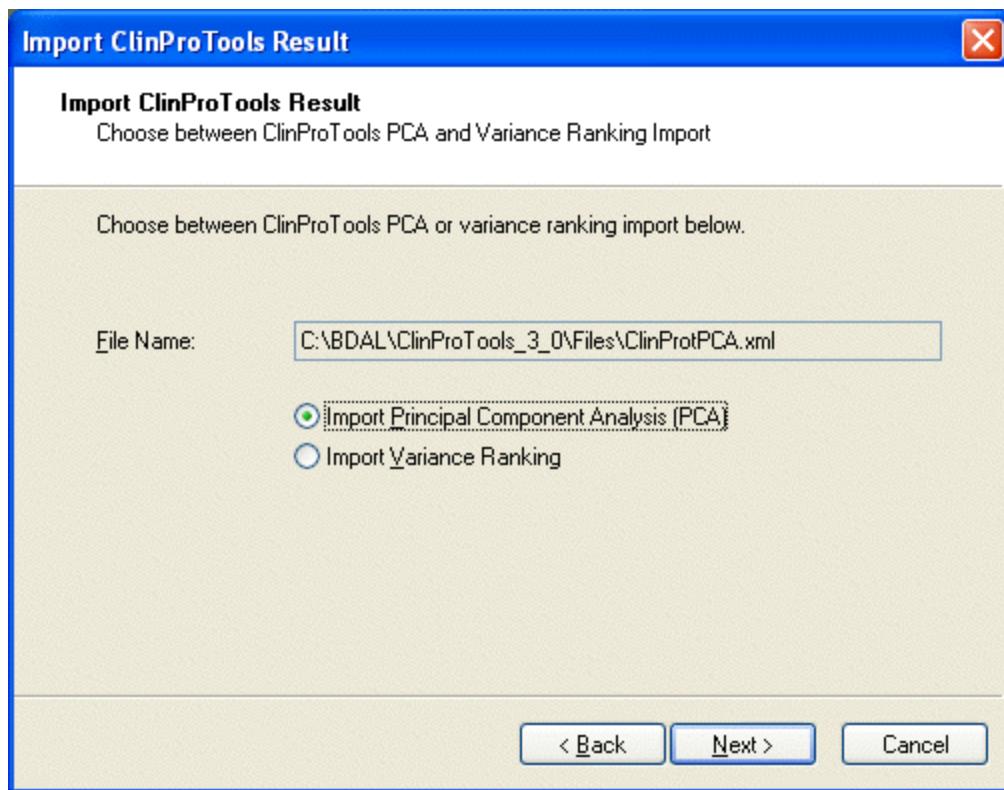


Figure 4-91 Choose between ClinProTools PCA and Variance Ranking Import dialog

Import Principal Component Analysis (PCA)

Imports the PCA result information.

Import Variance Ranking

Imports the variance ranking information, generating mass filters for the important peaks.

Next

Continues with the **Import ClinProTools PCA** or **Import ClinProTools Variance Ranking** dialog.

4.5.18.6 Import ClinProTools PCA Dialog

The **Import ClinProTools PCA** dialog specifies the options for the ClinProTools PCA import and result filter creation.

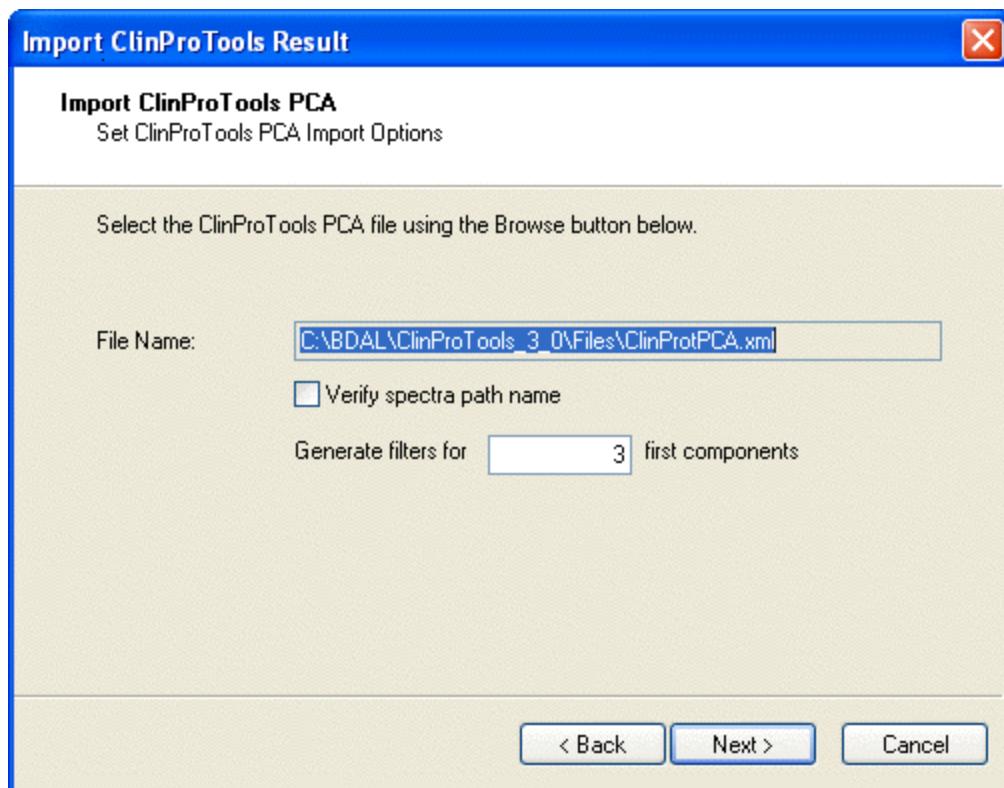


Figure 4-92 Import ClinProTools PCA dialog

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the clustering file matches the current imaging run. Use this option if you included spectra from multiple runs into a single PCA.

Generate filters for N first components

Specify the number of result filters to create. flexImaging generates result filters for the most important components first.

Next

First a confirmation request to copy the PCA results file to the imaging runs directory appears. Since ClinProTools overwrites the ClinProtPCA.xml file every time the PCA tool is invoked the previous information in this file is lost. flexImaging only stores a reference to the PCA file in its result filters, so it is advisable to allow flexImaging to copy the PCA file to the imaging run directory. Answering the request starts the import. When the import is finished, the wizard's **Completing ClinProTools Import** dialog opens.

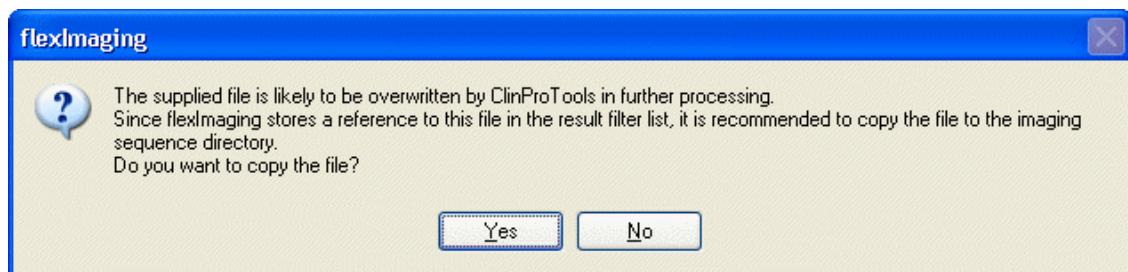


Figure 4-93 Confirmation on whether to copy the current ClinProTools file

4.5.18.7 Import ClinProTools Variance Ranking Dialog

The **Import ClinProTools PCA** dialog specifies the options for the ClinProTools variance ranking import and result filter creation. flexImaging creates mass filters for the chosen number of peaks.

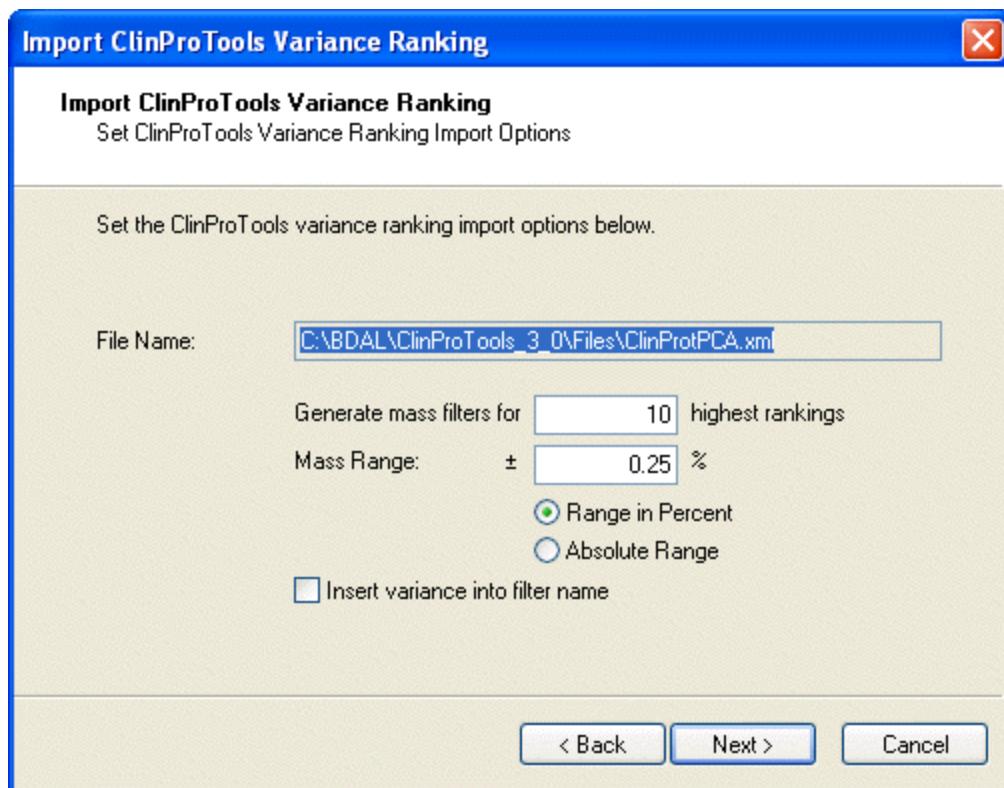


Figure 4-94 Import ClinProTools Variance Ranking dialog

Generate mass filters for N highest rankings

Specify the number of mass filters to create. flexImaging generates result filters for the most important peaks first.

Mass Range

Specify the percentage or absolute mass range for the generated filters which defines how many Da to the left and the right of the imported center mass the mass filter should work.

Range in Percent. Determines a percentage mass range relative the center mass.

Absolute Range. Determines an absolute mass range in Da.

Insert variance into filter name

Check this option if flexImaging should generate a filter name containing the variance loading calculated by ClinProTools. This information can be used to sort the filters accordingly.

Next

Starts the import. When the import is finished, the wizard's **Completing ClinProTools Import** dialog opens.

4.5.18.8 Import ClinProTools PLSA Dialog

The **Import ClinProTools PLSA** dialog specifies the options for importing a ClinProTools PLSA and creating the result filters.

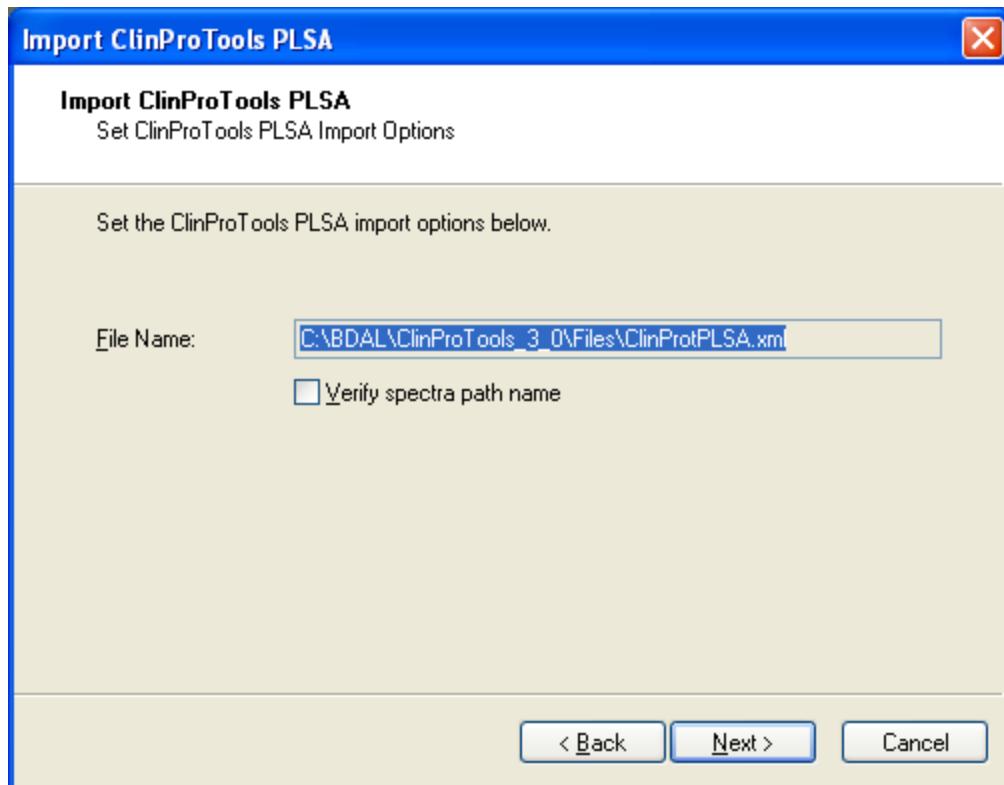


Figure 4-95 Import ClinProTools PLSA dialog

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the clustering file matches the current imaging run. Use this option if you included spectra from multiple runs into a single clustering analysis.

Next

First, a confirmation request to copy the clustering results file to the imaging runs directory appears. Since ClinProTools overwrites the ClinProtPLSA.xml file every time the PLSA tool is invoked, the previous information in this file is lost. flexImaging only stores a reference to the clustering file in its result filters, so it is advisable to allow flexImaging to copy the PLSA file to the imaging run directory. Answering the request starts the import. After import is finished the wizard's **Completing ClinProTools Import** dialog opens.

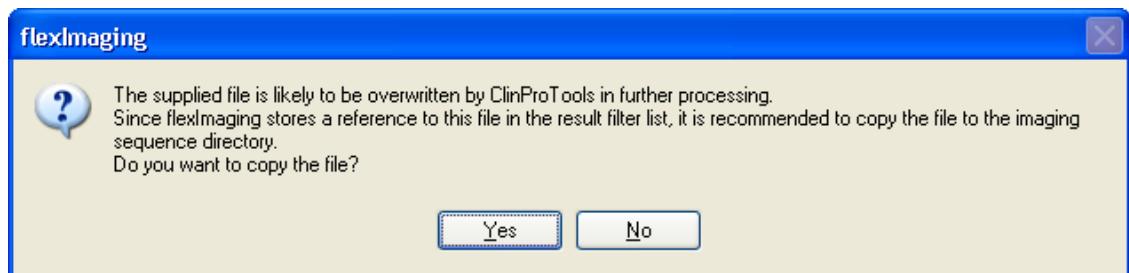


Figure 4-96 Confirmation on whether to copy the current ClinProTools file

4.5.18.9 Completing ClinProTools Import Dialog

The **Completing ClinProTools Import** dialog informs you about successful import of ClinProTools classifications, clustering, PCA or variance ranking results. It displays how many result filters were imported and how you can edit the settings of the created result filters.

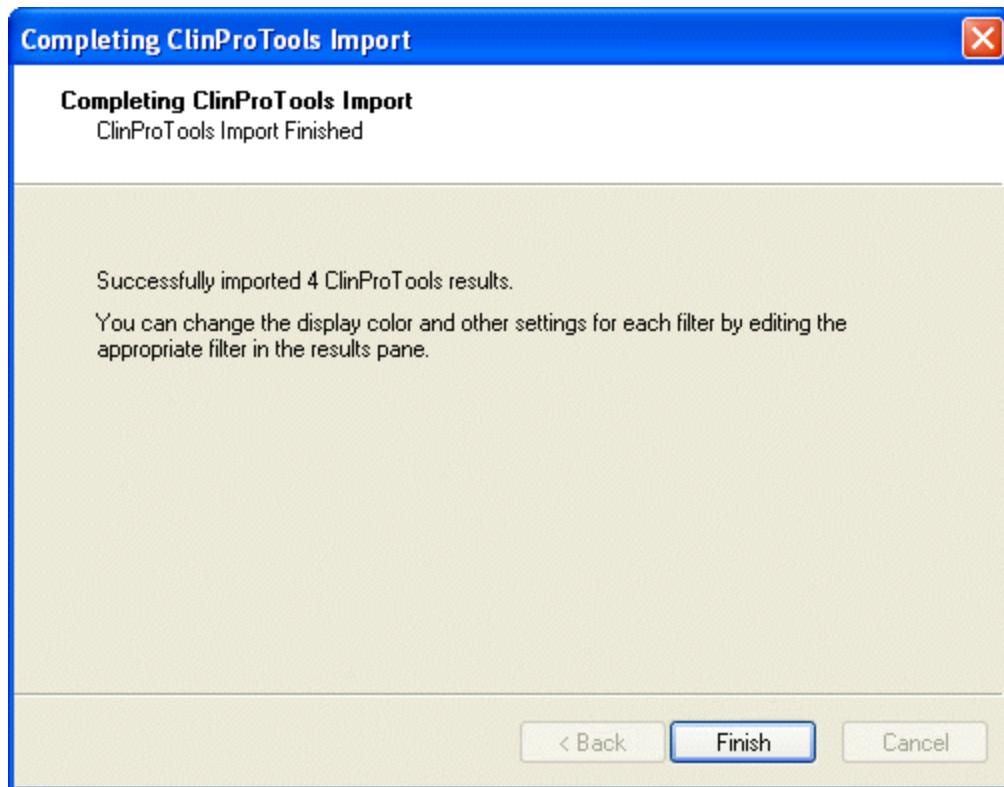


Figure 4-97 Completing ClinProTools Import dialog

4.5.18.10 Completing ClinProTools Clustering Tree Import Dialog

The **Completing ClinProTools Clustering Tree Import** dialog informs you about successful import of a ClinProTools clustering tree. flexImaging opens a Dendrogram Window with the imported tree. Result filters are created using the shortcut menu in the dendrogram window.

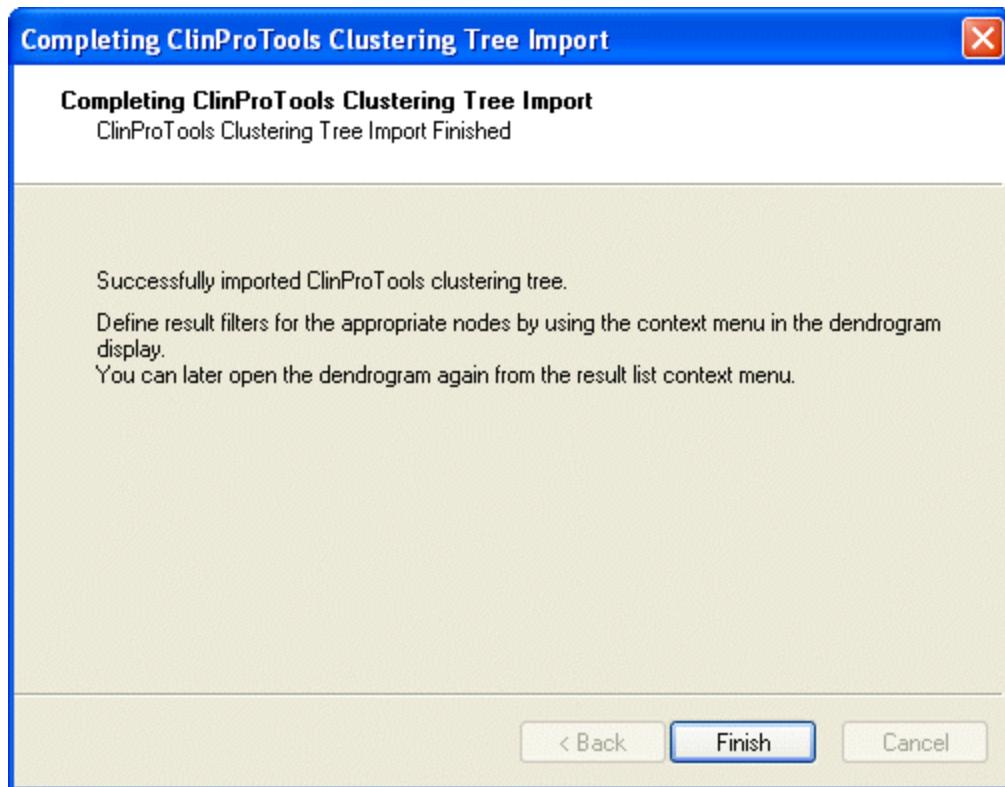


Figure 4-98 Completing ClinProTools Clustering Tree Import dialog

4.5.19 Edit Mass Filter Parameters Dialog

The **Edit Mass Filter Parameters** dialog is used to edit parameters of mass filters selected in the Results Pane. The dialog changes slightly if more than one filter is selected: check boxes to the left of each option or group of options determine if the corresponding option is changed for all selected filters (see Figure 4-100).

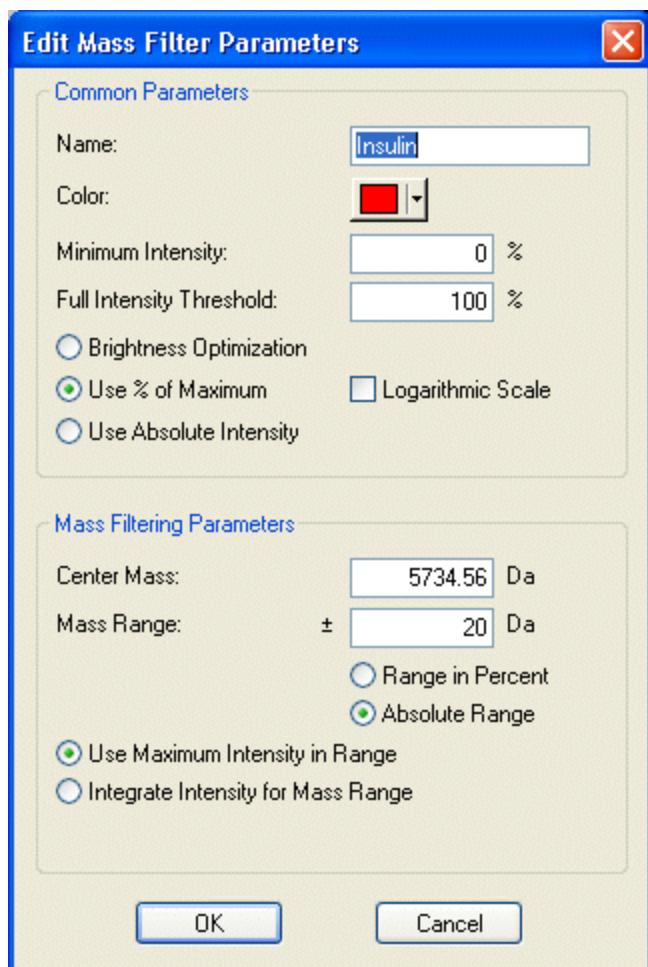


Figure 4-99 Edit Mass Filter Parameters dialog - single selection

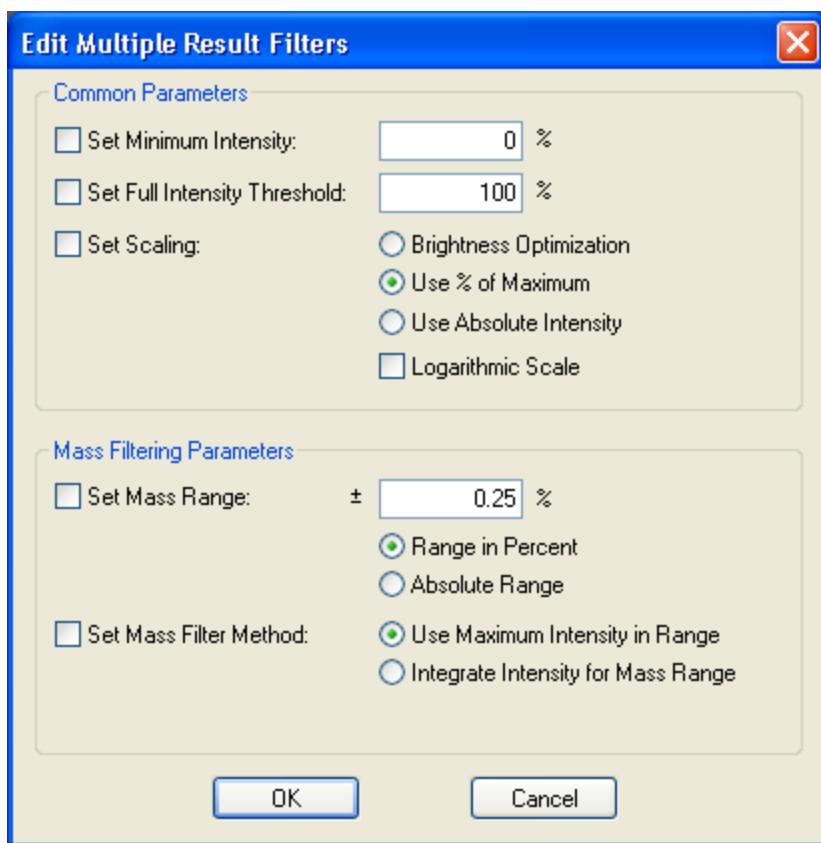


Figure 4-100 Edit Multiple Result Filters dialog

Name

This field displays the name of the selected mass filter. If this dialog opens after the user has clicked **New Mass Filter** in the Spectrum view shortcut menu, this field contains a name generated using a incrementing numbering scheme (01, 02, 03, and so on). The user may enter a new name if desired. This dialog element is hidden if more than one filter is selected.

Color

This dialog element opens a color selection dialog for the mass filter. It is hidden if more than one filter is selected.

Brightness Options

flexImaging plots the intensity of spot color proportional to the intensity of the highest peak in the respective mass range. By default, maximum brightness is used for the highest peak in the mass range, while zero intensity is plotted as black. By changing these levels, the user can suppress or amplify small peaks, and increase or decrease the overall brilliance of the image.

Minimum Intensity

Spots with an intensity less than or equal to this value are plotted black.

Full Intensity Threshold

Spots with an intensity higher than or equal to this value are plotted with the maximum brightness of the color selected.

Brightness Optimization

This option sets the full intensity threshold to the 95-percentile of the distribution of intensities, or to 60% of the highest intensity, whichever value is higher. This method prevents high intensity outliers from reducing the brilliance of the plot.

Use % of Maximum

Check this option to set minimum intensity and full intensity to the entered percentage of the maximum intensity in the mass range.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of percentages.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

Center Mass

If a single filter is selected, you can set the nominal value of the center mass.

Mass Range

Specify a percentage or absolute mass range that defines how many Da to the left and the right of the center mass the mass filter should work. In the example above the mass filter would work from 5714.56 to 5754.56 Da.

Range in Percent. Determines a percentage mass range concerning the entered center mass.

Absolute Range. Determines an absolute mass range in Da.

[Intensity value to be used]

Select which intensity value should be used for plotting the result:

Use Maximum Intensity in Range. Uses the highest intensity in the specified mass range.

Integrate Intensity for Mass Range. Uses the integrated intensity from the specified mass range.

4.5.20 Edit ClinProTools Classification Filter Parameters Dialog

The **Edit ClinProTools Classification Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard.

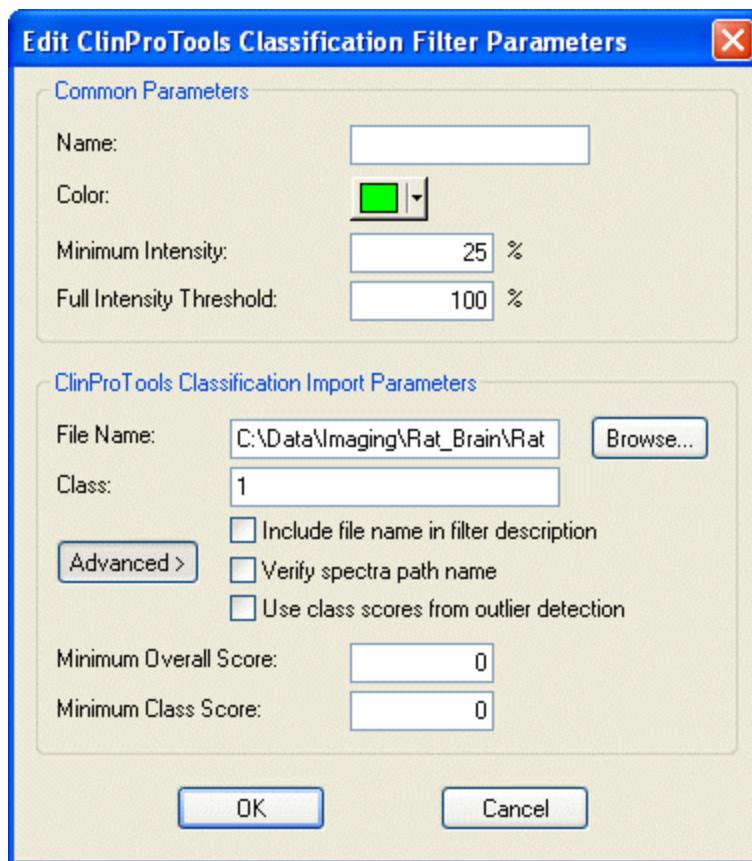


Figure 4-101 Edit ClinProTools Classification Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

File Name

Use the Browse button to select the file you want to import.

Class

The class for this filter. Classes are numbered starting from 1.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if classifications from multiple files are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs into a single classification.

Use class scores from outlier detection

If this option is checked, flexImaging uses the class score from the outlier detection instead of the classification result for creating the intensity plot. When selecting this option, you should also lower the **Minimum Intensity** value to 0%.

Advanced

Displays or hides the advanced options in the dialog covering the outlier detection. If **Peak Picking on Single Spectra** is enabled in the ClinProTools **Settings Peak Calculation** dialog, ClinProTools records additional information for each spectrum to enable outlier detection during the classification. flexImaging reads this information from the classification result file.

Minimum Overall Score

If the overall score for a spectrum falls below the threshold specified here, it is considered as an outlier and not used for the intensity plot.

Minimum Class Score

If the class score for a spectrum falls below the threshold specified here, it is considered as an outlier and not used for the intensity plot.

4.5.21 Edit ClinProTools Classification Overall Score Filter Parameters Dialog

The **Edit ClinProTools Classification Overall Score Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard. This filter is created additionally during the classification import, if outlier detection information is found in the ClinProTools classification result file.

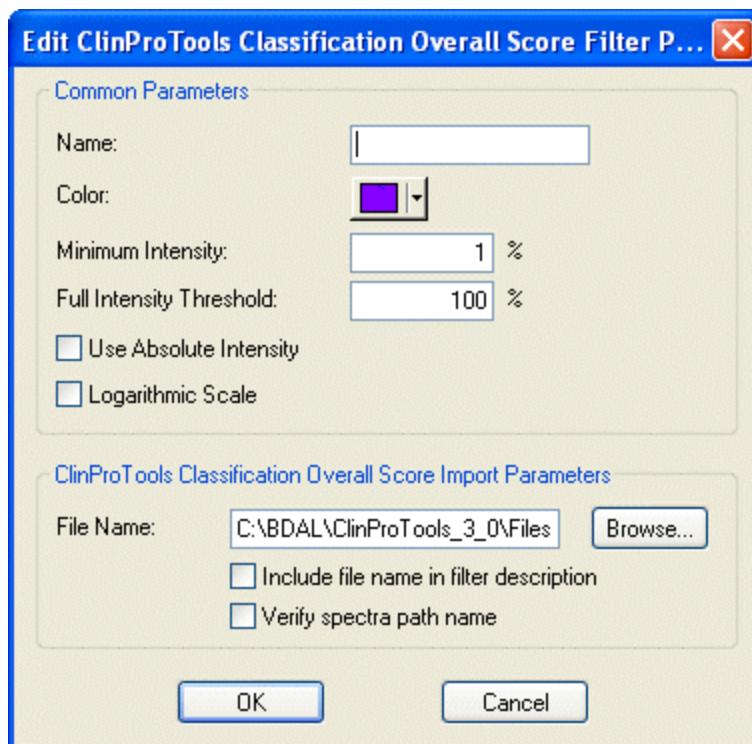


Figure 4-102 Edit ClinProTools Classification Overall Score Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of % values.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

File Name

Use the **Browse** button to select the file you want to import.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if classifications from multiple files are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this option is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs into a single classification.

4.5.22 Edit ClinProTools Clustering Filter Parameters Dialog

The **Edit ClinProTools Clustering Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard.

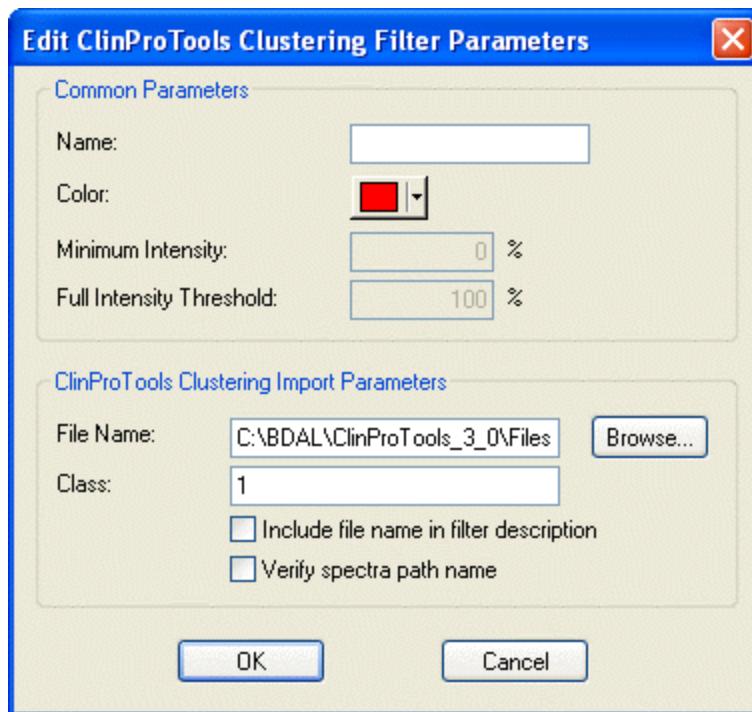


Figure 4-103 Edit ClinProTools Clustering Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

Fixed to 0%.

Full Intensity Threshold

Fixed to 100%.

File Name

Use the Browse button to select the file you want to import (typically ClinProtClustering.xml).

Class

The class for this filter. Classes (cluster groups) are numbered starting from 1.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if multiple clustering tree imports are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this button is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs in a single clustering analysis.

4.5.23 Edit ClinProTools Clustering Tree Filter Parameters Dialog

The **Edit ClinProTools Clustering Tree Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard.

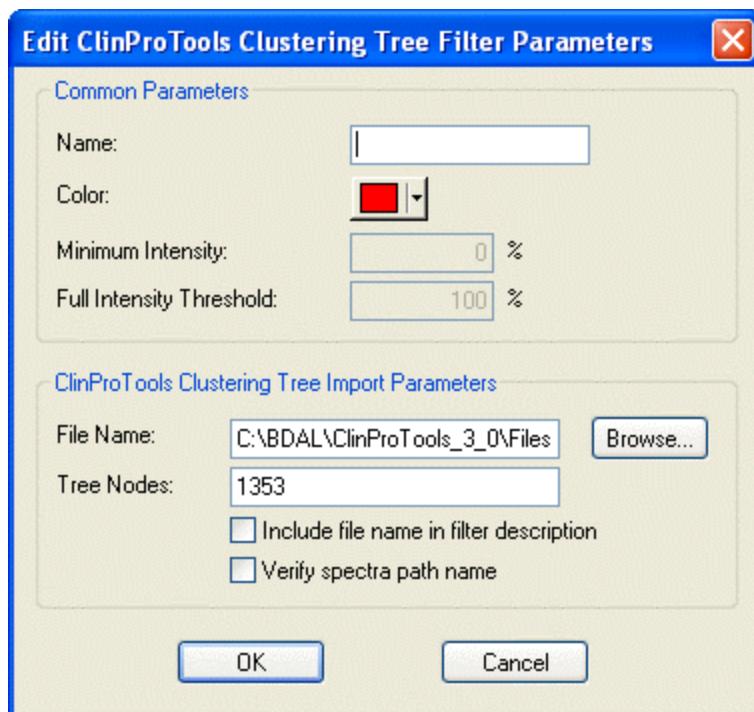


Figure 4-104 Edit ClinProTools Clustering Tree Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

Fixed to 0%.

Full Intensity Threshold

Fixed to 100%.

File Name

Use the Browse button to select the file you want to import (typically ClinProtClustering.tree.xml).

Tree Nodes

The tree nodes selected by this filter. Tree nodes are displayed in the ClinProTools dendrogram or (optionally) in the flexImaging Dendrogram Window. **Note:** It is easier to select individual tree nodes by using the shortcut menu in the dendrogram window.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if multiple clustering tree imports are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this button is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs in a single clustering analysis.

4.5.24 Edit ClinProTools PCA Filter Parameters Dialog

The **Edit ClinProTools PCA Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard.

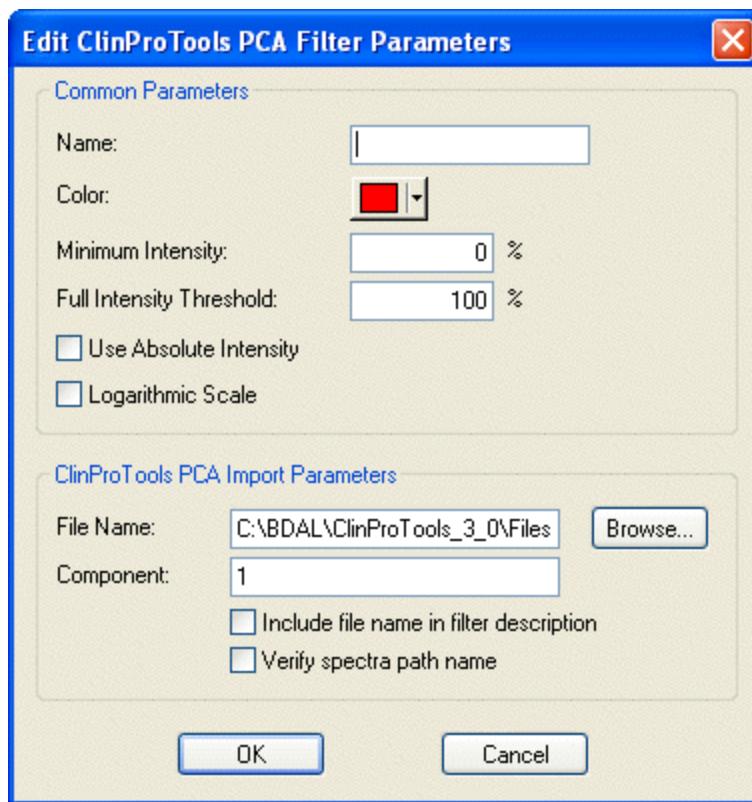


Figure 4-105 Edit ClinProTools PCA Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of % values.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

File Name

Use the **Browse** button to select the file you want to import (typically ClinProtPCA.xml).

Component

The component selected by this filter. Components are numbered starting from 1.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if multiple PCA imports are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this button is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs in a single PCA.

4.5.25 Edit ClinProTools PLSA Filter Parameters Dialog

The **Edit ClinProTools PLSA Filter Parameters** dialog is used to edit result filters created by the **Import ClinProTools Result** wizard.

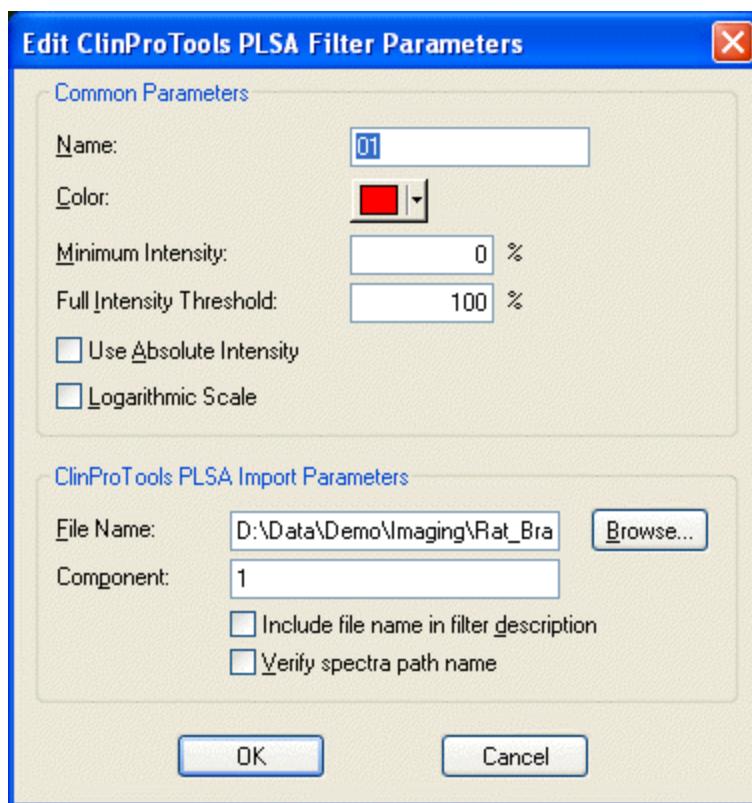


Figure 4-106 Edit ClinProTools PLSA Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of % values.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

File Name

Use the **Browse** button to select the file you want to import (typically ClinProtPLSA.xml).

Class

The class selected by this filter. Classes are numbered starting from 1.

Include file name in filter description

This option inserts the file name into the text displayed in the **Filter Method** column in the Results Pane. This option is useful if multiple PLSA imports are stored in a single filter list.

Verify spectra path name

Normally flexImaging imports the data by only checking the spot position of each spectrum. If this button is checked, flexImaging also verifies that the file path of the spectra within the classification result matches the current imaging run. Use this option if you included spectra from multiple runs in a single PLSA.

4.5.26 Edit XML Import Filter Parameters Dialog

In addition to its built-in mass filtering and ClinProTools import, flexImaging can also import results in generic text and XML format. The XML import filter is used to display results from other applications (e.g. external classification packages) in flexImaging. The **Edit XML Import Filter Parameters** dialog defines the parameters for importing results in XML format.

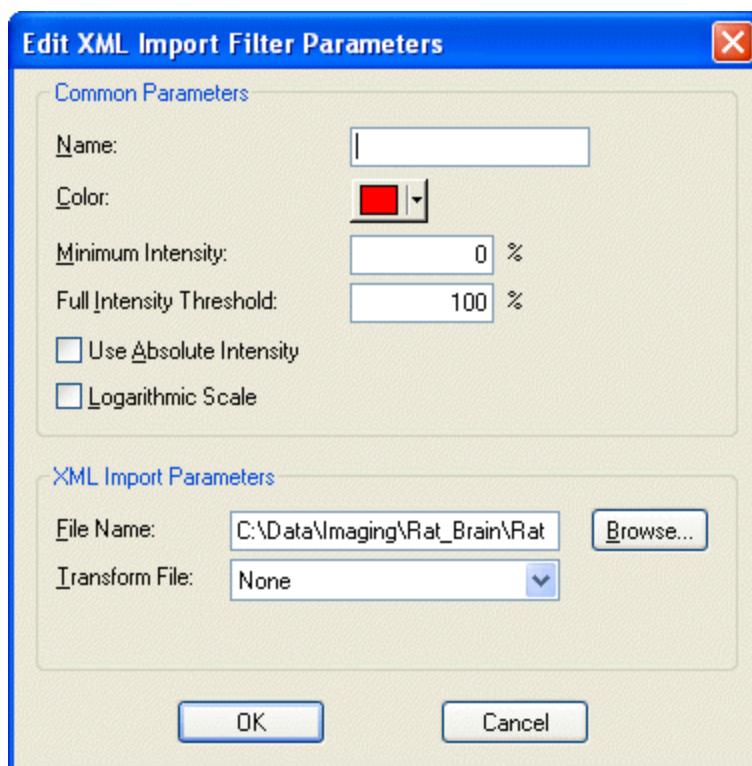


Figure 4-107 Edit XML Import Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of % values.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

File Name

Use the Browse button to select the file you want to import.

Transform File

flexImaging expects a certain format in the XML file (see below). You can use XSL transforms to convert the data from your application into the format used by flexImaging. To use a transform, the corresponding .xsl file must be copied to the Imaging Runs directory. If None is specified, the data file must contain the native flexImaging data format.

File format:

flexImaging expects the data in the following XML format:

```
<flexImagingResult>
<item>
<spot>spot-name</spot>
<weight>value</weight>
</item>
...
</flexImagingResult>
```

Example file:

```
<flexImagingResult>
<item><spot>0_R00X112Y094</spot><weight>0.1</weight></item>
<item><spot>0_R00X112Y095</spot><weight>1.0</weight></item>
<item><spot>0_R00X113Y094</spot><weight>2.0</weight></item>
<item><spot>0_R00X113Y095</spot><weight>3.0</weight></item>
</flexImagingResult>
```

4.5.27 Edit Text Import Filter Parameters Dialog

In addition to its built-in mass filtering and ClinProTools import, flexImaging can also import results in generic text and XML format. The text import filter is used to display results from other applications (e.g. external classification packages) in flexImaging. The **Edit Text Import Filter Parameters** dialog defines the parameters for importing results in text format.

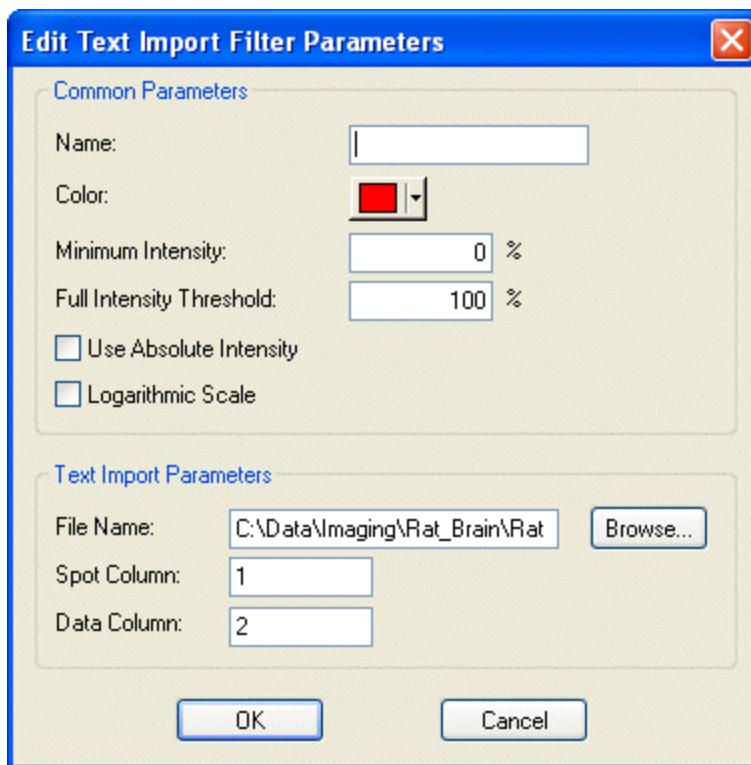


Figure 4-108 Edit Text Import Filter Parameters dialog

Name

Type in a name for the new result filter.

Color

Sets the color for the new result filter.

Minimum Intensity

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By increasing this threshold, smaller peaks will be ignored, i.e. they will not get a color whereas the highest peaks stay at the brightness level.

Full Intensity Threshold

flexImaging plots the intensity of peaks proportional to the intensity of the highest peak in the respective mass range. By default, 100% of the highest peak intensity results in the brightest color representation. By reducing this threshold, smaller peaks can be amplified, whereas the highest peaks stay at the brightness level.

Use Absolute Intensity

Check this option if absolute intensity values should be used instead of % values.

Logarithmic Scale

Check this option to apply a logarithmic scale to the filter instead of a linear scale.

File Name

Use the Browse button to select the file you want to import.

Spot Column

Column number of the spot position in the text file. Columns are numbered starting with 1.

Data Column

Column number of the data value in the text file. Columns are numbered starting with 1.

File format

The text import reads data in ASCII format, one value per line. The spot position and data value must be present on each line separated by white space (space or tab characters).

Example file:

```
R00X000Y000 0.0
R00X001Y000 1.1
R00X000Y001 2.0
R00X001Y001 3.0
```

4.5.28 Automatic Mass Filtering Dialog

The **Automatic Mass Filtering** dialog defines the settings for the automatic mass filtering procedure whereby the software creates mass ranges and supplies the number of spectra (= hits) that contain a peak in the respective mass range.

The settings can be saved as a new result filter list using the **Save Result Filter List As** dialog which you can open by clicking **Save As...** in the Results Pane.

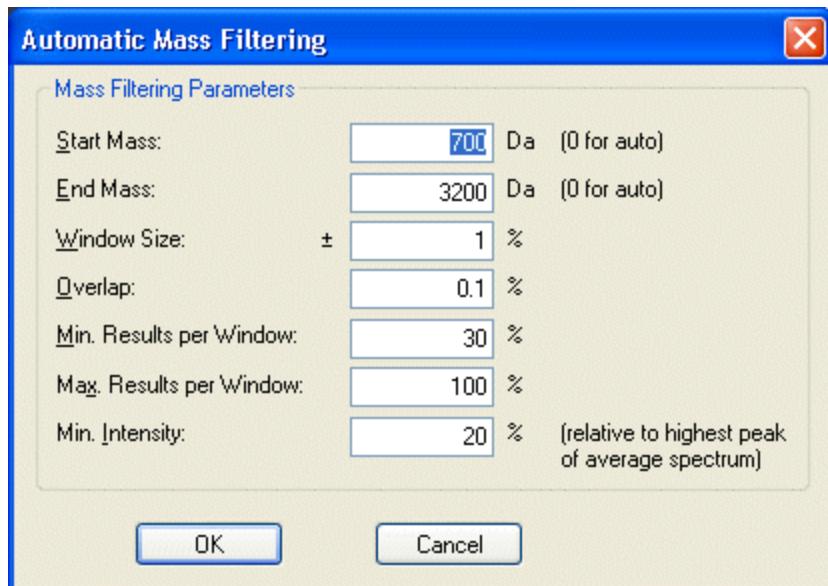


Figure 4-109 Automatic Mass Filtering dialog

Start Mass, End Mass

Entire mass range where the program should look for peaks.

Window Size

If a filter mass is found, this value defines how many Da to the left and to the right the window will be created. E.g. if the found filter mass is 1000 Da and you have defined a window size of 1%, then the filter window will be created from 990-1010 Da. Consequently you have smaller filter windows for small masses and larger filter windows for large masses.

Overlap

Overlapping factor for two adjacent windows to exclude the loss of peaks if they reside exactly on a window border.

Min. Result per Window

Lower bound threshold for peak appearance: peaks that appear in at least the given percentage of spectra are taken into account for mass filtering. Example: If a peak occurs in more than 30% of all spectra (compare the **Hits** column on the Results Pane) it should be included, if it occurs in less than 30% of the spectra it is rejected.

Max. Result per Window

Upper bound threshold for peak appearance: peaks that appear in more than the given percentage of spectra are not taken into account.

Min. Intensity

Minimum intensity a peak of a single spectrum must exceed to be considered for mass filtering. This prevents too many filters be automatically created.

OK

The software creates mass ranges and supplies the number of spectra (= hits) that contain a peak in the respective mass range.

4.5.29 Customize Toolbar Dialog

The **Customize Toolbar** dialog allows adding or removing buttons to or from the toolbar. The buttons that are already available in the GUI are shown in the right list; the buttons that are currently not used are shown on the left.

To add or remove a toolbar button select it in the list and click **Add >** or **<- Remove**. It is not possible to move a button with drag and drop to another position in the toolbar. Use the **Move Up** and **Move Down** buttons for this.

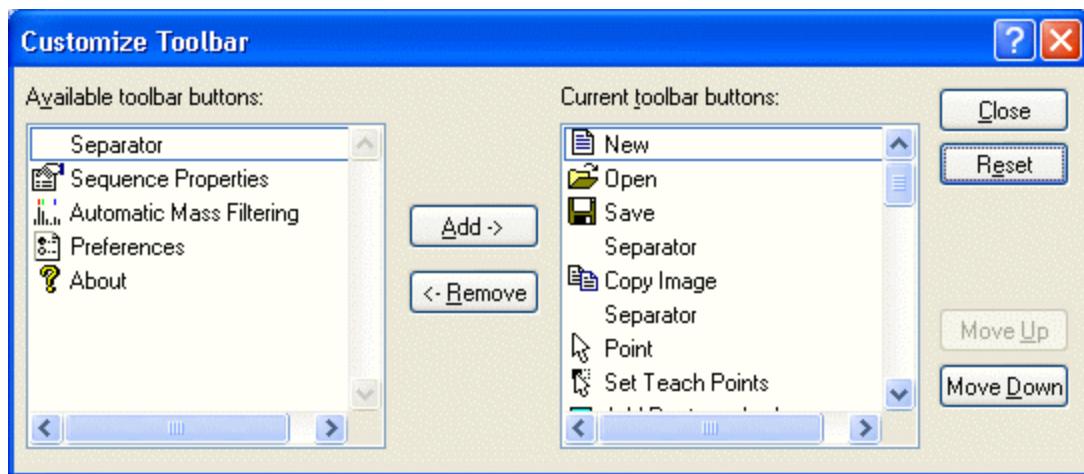


Figure 4-110 Customize Toolbar dialog

4.5.30 Color Dialog

The **Color** dialog allows changing the color of the item for which the dialog has been launched. This can be the color e.g. of the background of the Spectrum Display or of a selected spectrum or result filter. You can select the desired color from a list of **Basic colors** or self-defined **Custom colors**.

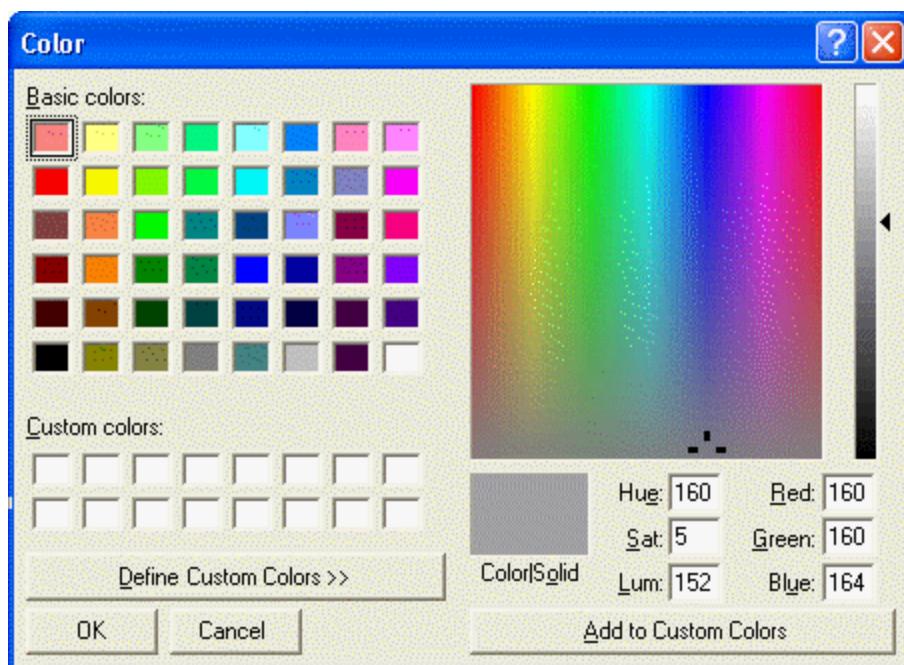


Figure 4-111 Extended Color dialog

4.5.31 Definitions for Import of Generic Robot Files Dialog

The **Definitions for Import of Generic Robot Files** dialog is used to specify the parameters for importing spot lists from sample preparation robots whose file format is not known by flexImaging. The data must be text based (one entry per line) and formatted in columns to use the generic import.

Note Only change this setup if you are familiar with the format of the robot data.

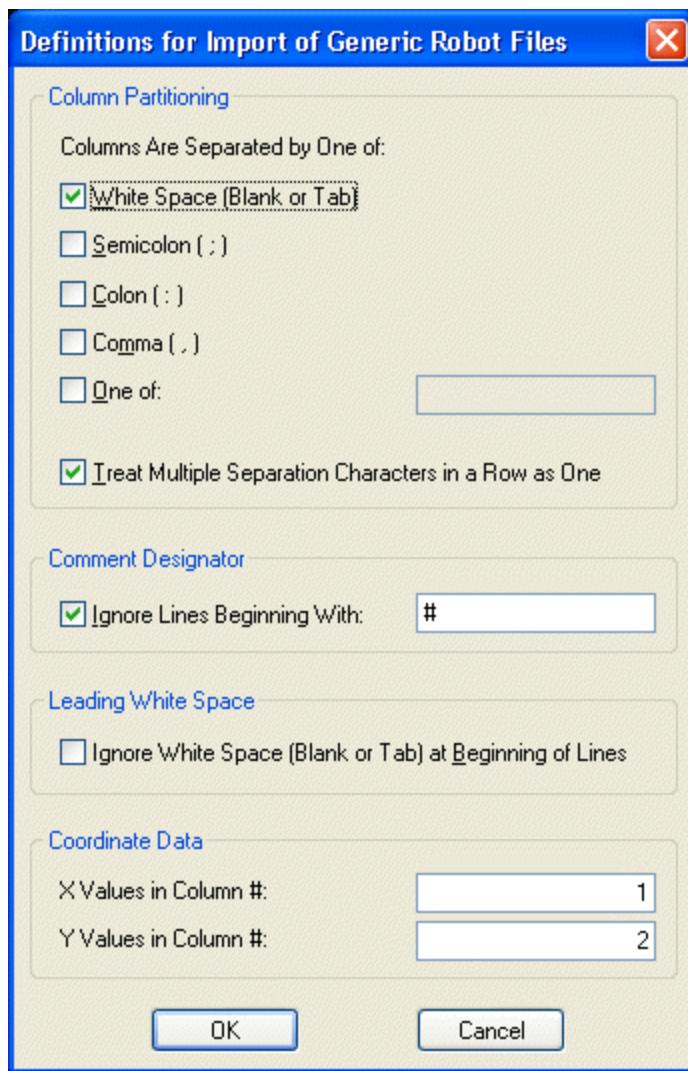


Figure 4-112 Definitions for Import of Generic Robot Files dialog

White Space (Blank or Tab); Semicolon (;); Colon (:); Comma (,); One of

Select which characters should be used to separate columns within a line of the spot list file. You can choose the given default characters and/or specify other column separators in the text box belonging to **One of**.

Treat Multiple Separation Characters in a Row as One

This option handles multiple consecutive separation characters as a single character. This option is common for data separated by spaces.

Ignore Lines Beginning With

This option ignores lines starting with specified characters during the import. Enter the respective character(s) in the text box on the right.

Ignore White Space (Blank or Tab) at Beginning of Lines

This option suppresses leading white space (space or tab characters) at the beginning of a line.

X Values in Column # ; Y Values in Column

Specify the column numbers of the X and Y coordinates. 1 denotes the first column of the data file. The coordinates in the data file must be given as integer or floating point numbers.

4.5.32 Add Normalization Mass Window Dialog

The **Add Normalization Mass Window** dialog is used to create a new mass window for normalization. Different options are available.

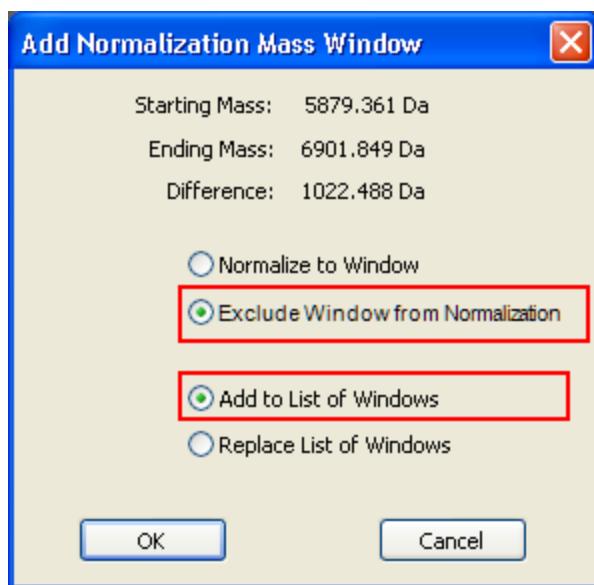


Figure 4-113 Add Normalization Mass Window dialog

Normalize to Window

The average intensity inside the mass window is used for normalization.

Exclude Window from Normalization

The mass window is excluded from the summation of all intensities in the spectrum when calculating the normalization value.

Add to List of Windows

The window defined above is added to an existing list of mass windows. If the list consists of Exclude windows, all mass ranges are excluded from calculating the average intensity. If the list consists of normalization windows, the normalization intensity is the average of all intervals. Mixing of types in a list is not recommended!

Replace List of Windows

The window defined above starts a new list.

4.5.33 Select Co-Registered Image Dialog

The **Select Co-registered Image** dialog opens when you choose the entry **Co-Registered Images** from the View menu. The sign "->" marks the co-registered image currently selected for display. You can either choose a different image to be displayed or delete an entry from the list. When deleting, the image file on disk is not touched, and the entry is permanently removed from the list only if the imaging run is saved when FlexImaging quits or a different imaging run is loaded.

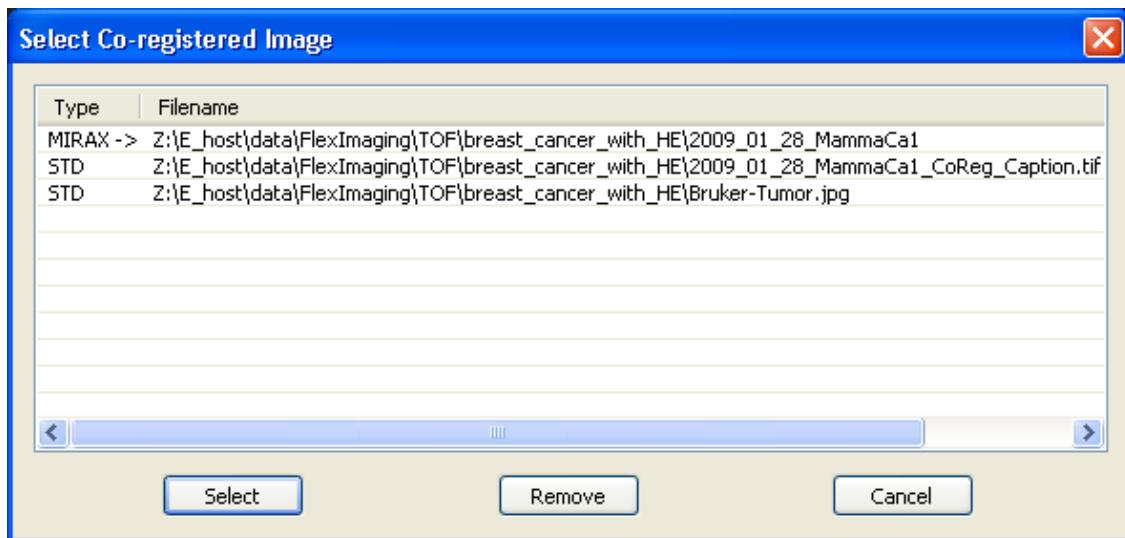


Figure 4-114 Select Co-Registered Image dialog

Select

Selects the highlighted image file for display.

Remove

Removes the highlighted entry from the list, but does not delete the image.

4.5.34 Automatic ROI Array Parameters Dialog

The **Automatic ROI Array Parameters** dialog opens when you choose the command **Automatic ROI Array** from the shortcut menu of the regions pane or image window. You can then choose position and size (in measurement spots) and number of rectangular regions to cover all or part of the measurement region. The placement of ROIs takes account of slight shifts in spot positions. It is possible to create multiple arrays of ROIs, provided that the **ROI Name Base** is different for each array. Otherwise, new ROI arrays will replace old arrays with the same name.

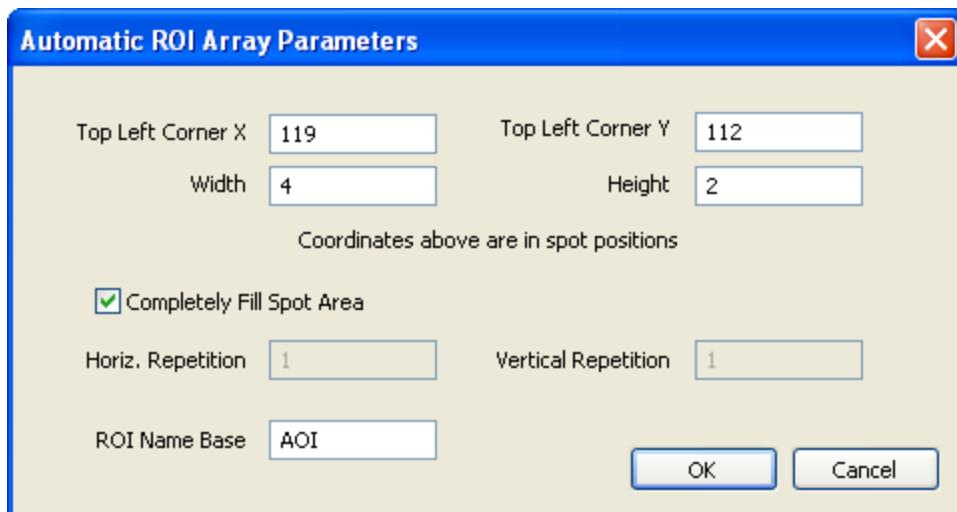


Figure 4-115 Automatic ROI Array Parameters dialog

Top Left Corner

These values determine the first spot, from which the ROIs are placed down and right.

Width, Height

These values determine the size of the ROIs.

Completely Fill Spot Area

When this button is checked, ROIs are repeated until all spots to the right and down of the top left corner are covered with a ROI. The last row and column might contain less spots than the other ROIs.

Horiz. Repetition, Vertical Repetition

These values determine the number of columns and rows if the measurement area is not to be covered completely.

ROI Name Base

Each ROI is named like the string entered here, with consecutive numbers appended.

4.5.35 Mass Defect Filter Parameters Dialog

The **Mass Defect Filter Parameters** dialog opens when you choose the entry **Add Mass Defect Filter** from the Spectrum view shortcut menu or **Mass Defect Filtering** from the Results pane. You can then choose details of the size of the mass defect interval accepted as a potential peak filter.

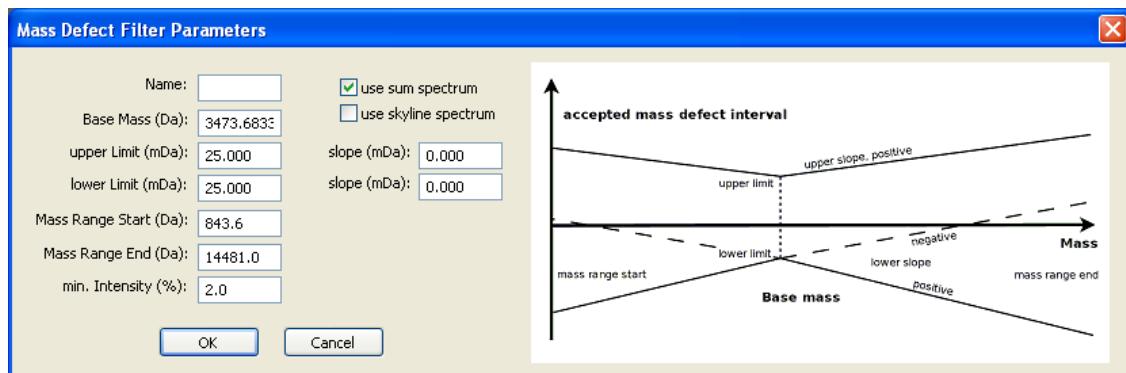


Figure 4-116 Mass Defect Filter Parameters dialog

Name

The label of each filter consists of this base name, appended with the integer mass difference and, if necessary, a modifier from the imaging run of a–z if more than one peak is found inside the accepted mass interval. For example, "Base-16a" denominates the second peak in the interval around the base mass -16 Da.

use sum spectrum, use skyline spectrum

This check box determines the spectrum used to find peaks inside the accepted mass interval around integer mass defects. The skyline spectrum usually enhances peaks of masses that occur only in small parts of the sample, while the sum spectrum reduces noise.

upper Limit

The maximum positive deviation from the integer mass difference that will be accepted.

upper Slope

The increase (or decrease) of the accepted positive deviation from the integer mass difference, with increasing distance from the base mass.

lower Limit

The maximum negative deviation from the integer mass difference that will be accepted.

lower Slope

The increase (or decrease) of the accepted negative deviation from the integer mass difference, with increasing distance from the base mass.

Mass Range Start, Mass Range End

The mass range scanned for potential mass defect filters.

min. Intensity

The intensity as a percentage of the base peak's intensity for any peak to be considered as a potential filter.

4.5.36 Hamamatsu Converter Dialog

The **Hamamatsu Converter** dialog opens when you choose the entry **Convert Hamamatsu Slide** from the **Tools** menu. Here you can select the Hamamatsu digital slide to convert to BigTIFF, and some options applied during conversion. A click on the **OK** button starts the conversion, which can take 15 minutes or more, even on a fast PC. However, flexImaging remains usable because conversion is executed by a separate program that normally runs in the background. A message in the status bar of flexImaging tells the user when the conversion is finished.

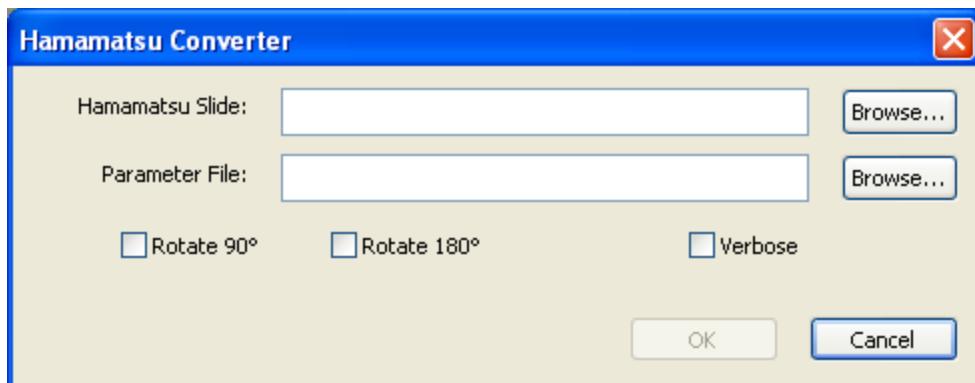


Figure 4-117 Hamamatsu Converter dialog

Hamamatsu Slide

The path to the Hamamatsu digital slide file (*.NDPI).

Parameter File

The path to a file containing additional parameters for the conversion (should remain empty for normal slides).

Rotate

If one of these check boxes is selected, the resulting BigTIFF image is rotated by the corresponding angle. This can make co-registration much easier.

Verbose

If this check box is selected, the converter normally running in the background opens a console window showing the progress of conversion together with potential status messages.

4.5.37 Export to imzML Dialog

Select **File > Export > imzML** to open the **Export to imzML** dialog.

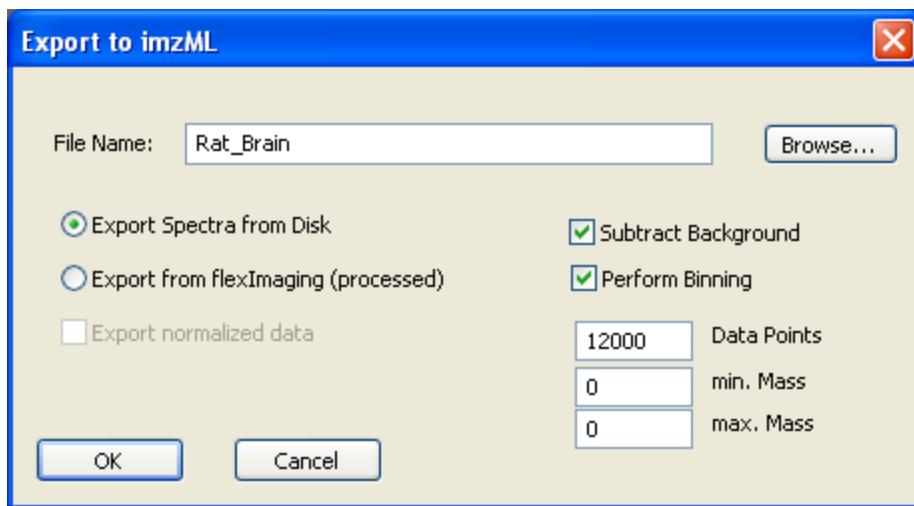


Figure 4-118 Export to imzML dialog

Type a file name and click **Browse** to select a destination for the resulting files. If no directory is selected, the export files are created in the result directory. File extensions are ignored: the two resulting files are automatically given the extensions .imzML (XML part) and .ibd (binary part).

File name

The base name of the export files, or the complete path.

Export Spectra from Disk

Select **Export Spectra from Disk** to export raw spectra. For TOF spectra, selecting this option enables you to perform data binning using the parameters on the right side of the dialog. Data binning results in a linear mass scale that is determined by the number of data points and the start and end mass.

Export Spectra from flexImaging

Select **Export Spectra from flexImaging** to export spectra that have been processed by and are stored in the flexImaging application. Depending on the processing status of the data, data reduction, binning, and baseline subtraction may have been applied to such spectra.

Export normalized data

Select **Export normalized data** to apply the current normalization method to the exported spectra.

Subtract Background

Select **Subtract Background** to apply background subtraction to the exported spectra.

Perform Binning

Select **Perform Binning** to linearize the mass scale using the following parameters:

- **Data Points**

This value specifies the number of bins for the mass scale.

- **min. Mass**

This value specifies the start of the mass scale. If **min. Mass** = 0, the start of the acquired mass range is used.

- **max. Mass**

This value specifies the end of the mass scale. If **max. Mass** = 0, the end of the acquired mass range is used.

Note The data binning option is not available for FTMS spectra.

4.5.38 Split Imaging Run Dialog

Select **Edit > Split Imaging Run** to open the **Split Imaging Run** dialog.

With the **Split Imaging Run** dialog you can split one imaging run with more than one measurement region into separate imaging runs. The dialog creates one imaging run for each measurement region.

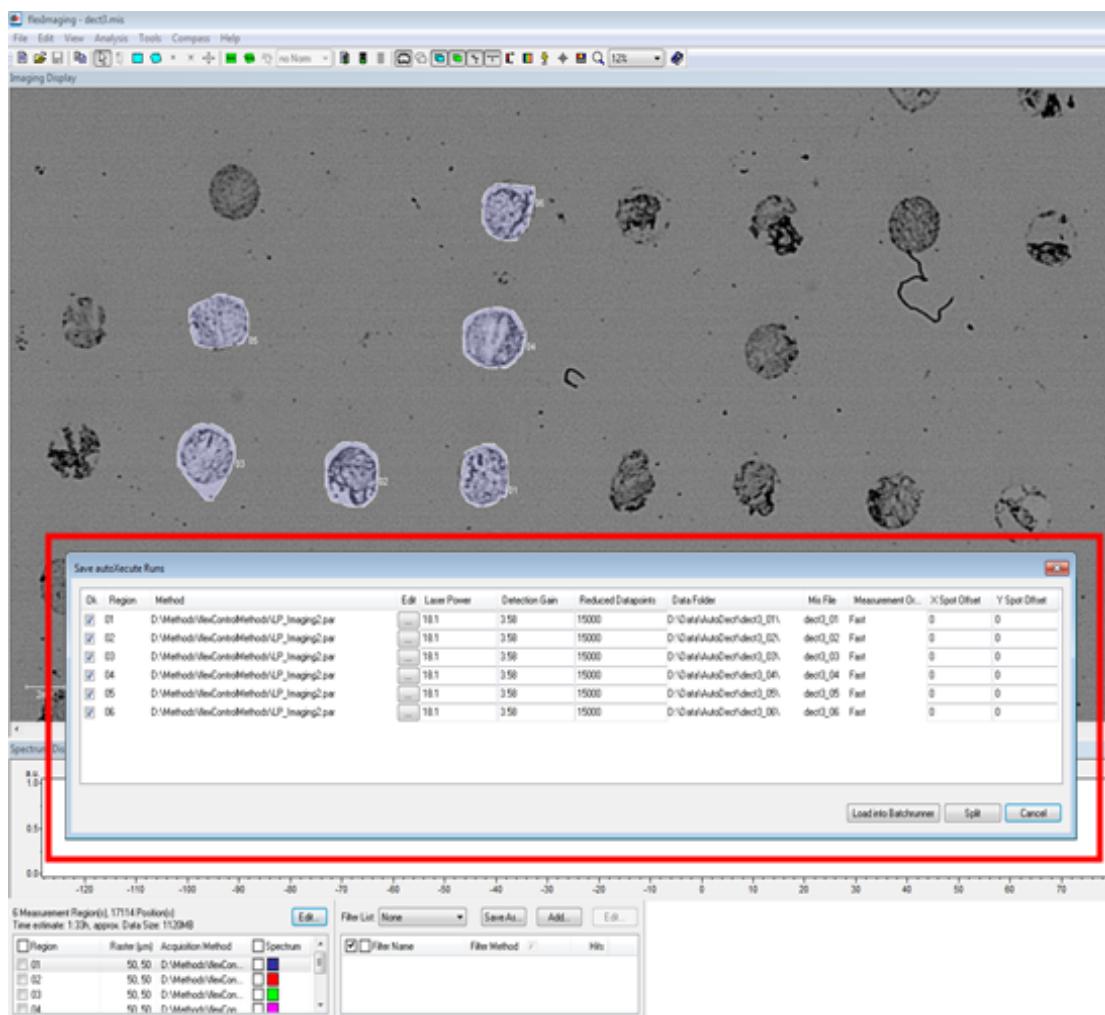


Figure 4-119 Split Imaging Run dialog open in flexImaging

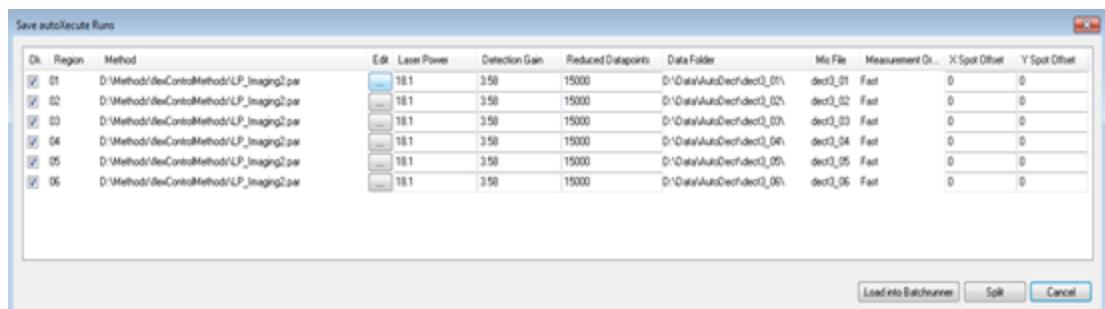


Figure 4-120 Split Imaging Run dialog open in flexImaging

The individual imaging runs are located in a sub folder of the base imaging run. You can change the following properties of each imaging run:

- Acquisition Method
- Laser Power
- Detection Gain
- Reduced Datapoints
- X and Y Spot Offset

The imaging runs are created by clicking **Split**. Subsequently, flexImaging opens the last run in the list. The base imaging run is renamed as `run_name.misx`.

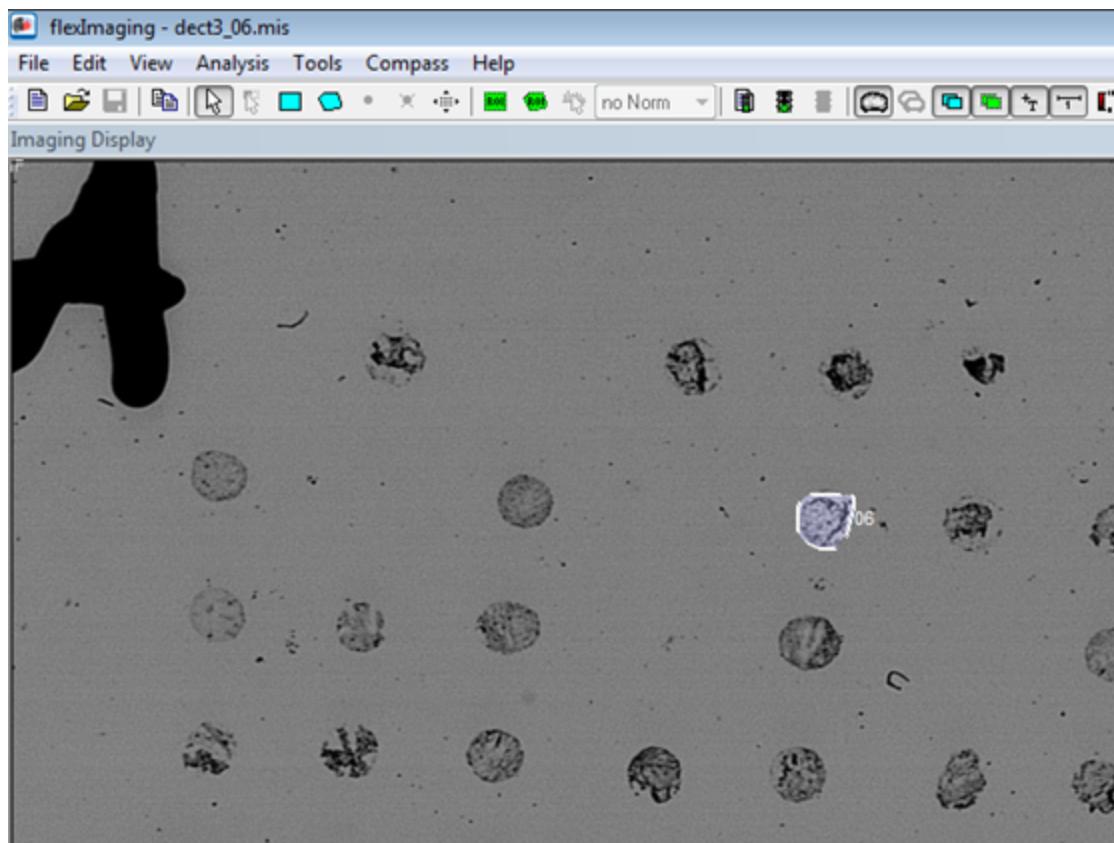


Figure 4-121 flexImaging with last measurement region

Name	Date	Type	Size
📁 dect3_01	11/10/2015 10:31 AM	File folder	
📁 dect3_02	11/10/2015 10:31 AM	File folder	
📁 dect3_03	11/10/2015 10:32 AM	File folder	
📁 dect3_04	11/10/2015 10:32 AM	File folder	
📁 dect3_05	11/10/2015 10:33 AM	File folder	
📁 dect3_06	11/10/2015 10:34 AM	File folder	
📄 dect3.misx	10/14/2015 9:50 AM	MISX File	6 KB
* spotImage_0000.jpg	9/25/2015 9:43 AM	IrfanView JPG File	1,609 KB
* spotImage_0001.jpg	9/24/2015 4:47 PM	IrfanView JPG File	1,943 KB
* spotImage_0002.jpg	9/25/2015 9:43 AM	IrfanView JPG File	19,692 KB

Figure 4-122 Folder structure after splitting imaging run

Clicking **Load into Batchrunner** opens the autoXecute Batch Runner dialog displaying all runs in the run list.

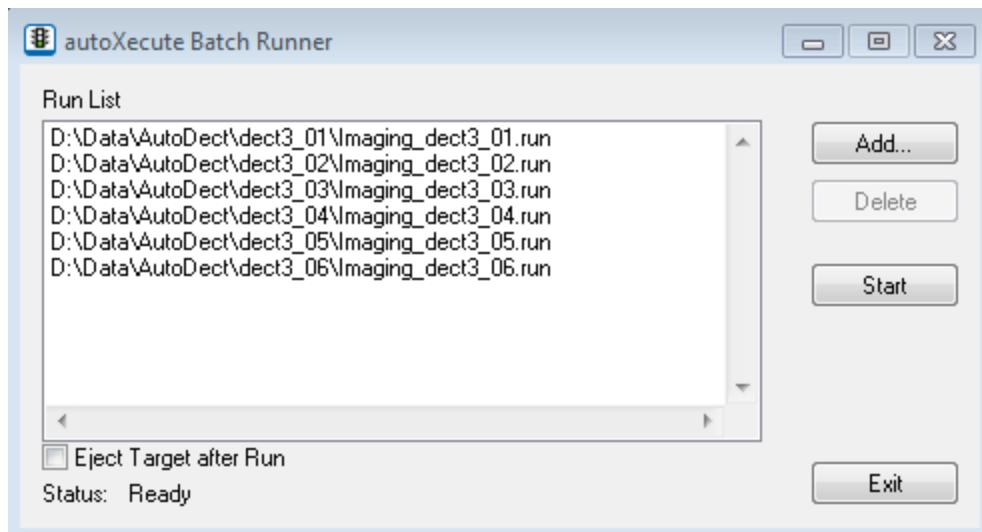


Figure 4-123 autoXecute Batch Runner

5 Workflows in Detail

5.1 Sample Preparation	231
5.2 Data Acquisition	256
5.3 Data Acquisition Using HyStar and ftmsControl	307
5.4 Data Processing and Interpretation	312

5.1 Sample Preparation

5.1.1 Preparation of Cryostat Sections for MALDI Imaging	231
5.1.2 Matrix Coating with imagePrep	238
5.1.3 Manual Spray Coating for MALDI Imaging	241
5.1.4 Robotic Spotting	243
5.1.5 Specific Considerations for Detecting Pharmaceuticals in Tissue	245
5.1.6 Preparing a Drug Dilution Series in a Frozen Tissue Homogenate Block	247
5.1.7 Acquiring an Optical Image	253

5.1.1 Preparation of Cryostat Sections for MALDI Imaging

General remarks

Cryosections of fresh frozen tissue are best suited for MALDI imaging and the quality of tissue samples is crucial for the results.

Tissue should be frozen immediately after harvesting with no additional treatment such as perfusion or fixation. If possible, blood and connective tissue should be removed.

Small tissue samples can be easily snap frozen in liquid nitrogen, for larger specimen it may be necessary to use isopentane (cooled with liquid nitrogen or dry ice) to ensure that the sample is thoroughly frozen. If liquid nitrogen is not available, freezing the tissue in a freezer at –80°C or with powdered dry ice may be sufficient.

The sample should not be embedded in supporting material such as optimal cutting temperature polymer (OCT), which contains polymers. These polymers are detectable by mass spectrometry and their signals may suppress other analyte signals. Solid tissue such as brain, liver, kidney, tumors can be cut in a sufficient quality without any embedding. Less compact samples (e.g. whole body sections, or delicate organ tissue such as lung or heart) need to be embedded for good quality sections. In this case the samples can be embedded using water or PBS buffer. If embedding cannot be avoided, see the following section “Preparation of tissue that is embedded in mounting medium”.

Sections should be deposited onto conductive ITO-slides (coated with indium tin oxide for conductivity, Bruker Daltonik Part No. 237001 "Glass slides for MALDI imaging"). Inside the slide package, the conductive side of the slides is indicated by an arrow. The slides themselves are not marked, but conductivity can be tested using a multimeter. It is possible, but less convenient to prepare the samples onto normal steel targets. Normal non-conductive glass slides cannot be used.

A few histological stains are compatible with subsequent MALDI imaging, which can enable the histologically directed selection of measurement regions for MALDI imaging.

For detailed information please refer to: *Chaurand P, Schwartz SA, Billheimer D, Xu BJ, Crecelius A, Caprioli RM, Anal. Chem. (2004), 76(4): 1145-1155.*

However, in most cases, histological staining is best performed after the MALDI imaging experiment: it is then possible to co-register a microscopic image of a stained section with the imaging data.

Depending on the experiment, it might be necessary to conduct conventional histological staining on consecutive sections collected on standard glass slides in parallel.

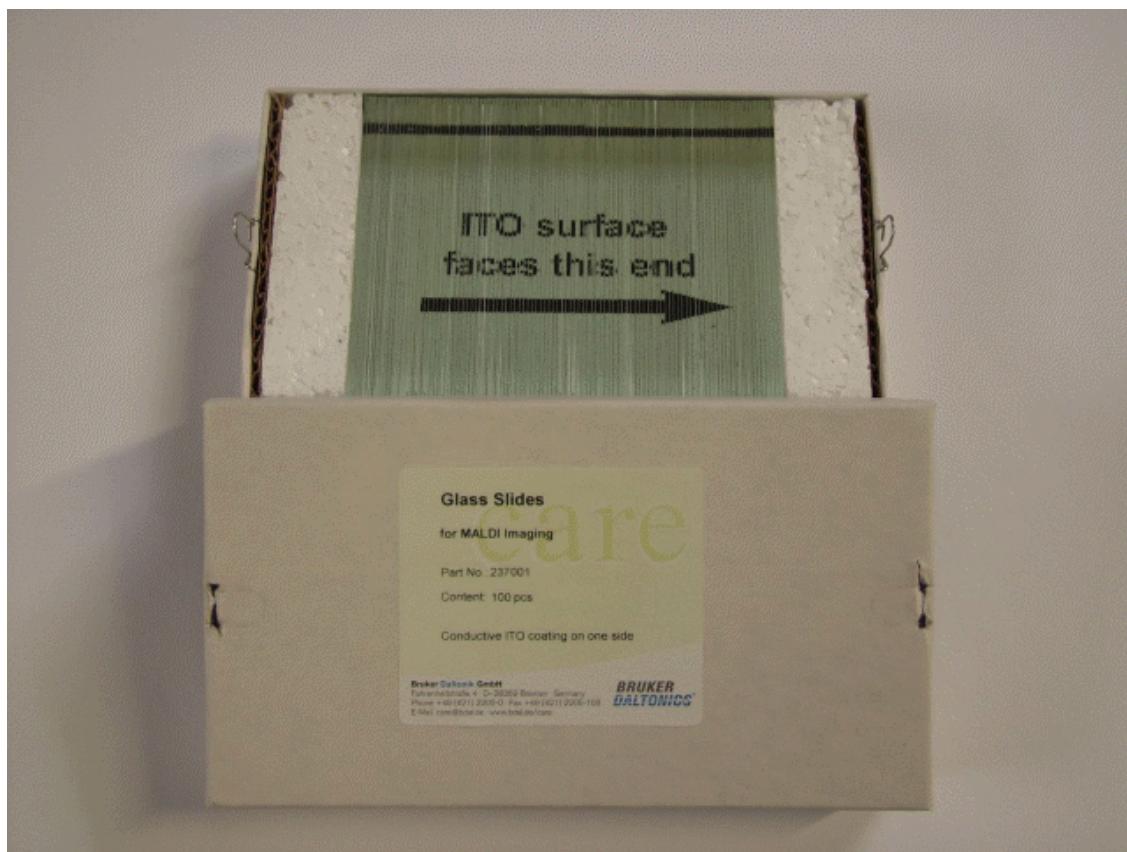


Figure 5-1 Glass slides for MALDI imaging from Bruker; the arrow indicates the conductive side

Polylysine coating of glass slides (optional)

Because of the hydrophobicity of the conductive coating, some tissue sections do not adhere perfectly to ITO-slides. As a result, sections may detach from the slide in post-MALDI histological staining or may lose integrity (e.g. cracks, ruptures or folded edges). These effects can be avoided by coating the ITO slides with polylysine prior to the sample preparation.

Required materials:

- Poly-L-lysine Solution, 0.1% w/v in water, Sigma-Aldrich P8920
- Igepal CA-630, Sigma I7771 (used to be called Nonidet P40).
- Glass spreader
- Heated plate (optional)

►► Procedure

1. Dilute 750 µL polylysine solution in 750 µL water and add ~1 µL Igepal.

Note Igepal is very viscous and difficult to dispense.

2. Dispense 20 µL of the mixture onto the slide, distribute it using a glass spreader and let dry on the heated plate (~80°C) or at room temperature.

The slide should be evenly coated with a thin film. If the liquid forms individual patches instead, add more Igepal to the mixture.

Remarks

Igepal is a polymeric detergent, which generates several signals in the mass range below 1000 Da. Therefore, the amount of Igepal used should be as small as possible. When measuring a low mass range, it might be necessary to omit the polylysine coating completely.

Required materials

- Cryostat
- Conductive slides or a MALDI target
- Optional: Artist's brush for small sections or forceps for large sections
- Two Petri dishes or Coplin jars
- 70% and 96% Ethanol (Histology-grade)
- Vacuum desiccator
- Slide containers or aluminum foil

Note The cryostat blade should be wiped with ethanol prior to cutting to remove traces of oil, OCT and other contaminants that remain from earlier preparations.

►► Preparing a cryostat section for MALDI imaging

Appropriate thickness of the section is 10 to 20 µm; 10 to 12 µm is ideal for most samples. (According to Yuki Sugiura, Shuichi Shimma, Mitsutoshi Setou, J. Mass Spectrom. Soc. Jpn. (2006), 54 (2), pp 45-48 "Thin Sectioning Improves the Peak Intensity and Signal-to-Noise Ratio in Direct Tissue Mass Spectrometry" the thinner the sections, the better the spectra will be if a spray preparation is used).

1. Place the conductive slides and the brush inside the cryostat and wait until they reached the cryostat temperature.
2. Cut a tissue section.
3. Transfer the section onto the conductive side of the cold slide. There are two possibilities to do this: Either the section is picked up with the artists brush or the forceps and transferred to the slide, or the tissue is picked up directly with the cold slide from the blade (the latter is not possible with a steel target). Transferring the section to a warm slide directly off the blade may result in reduced signal intensity, especially when analyzing proteins.
4. Thaw-mount the section onto the slide.
 - Carefully press the underside of the slide on the back of your hand. This should be sufficient to gently warm the slide and tissue section.
 - Keep warming the slide until all moisture has evaporated from the section.
 - **Note:** Exercise caution. Glass slides can break easily if you apply too much pressure.

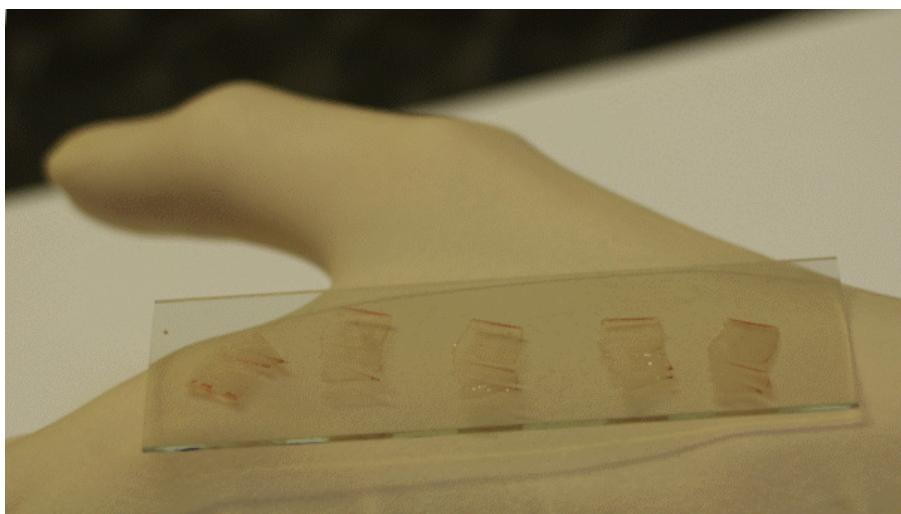


Figure 5-2 Warming the slide on the hand to prevent condensation

5. Take the warm slide out of the cryostat.
6. If more than one tissue section is to be placed on one slide, the slide can be re-frozen inside the cryostat now.
7. Desiccate the slide for at least fifteen minutes in vacuum (brain sections should be desiccated for up to 45 minutes).
8. Prepare two Petri dishes or Coplin jars with 70% ethanol each and one with 96% or pure ethanol. Wash the slide twice for 60–120 seconds in the 70% ethanol and once for 30–120 seconds in the pure ethanol. If small peptides (e.g. neuropeptides) are in the focus of interest keep the washes very short, for drugs or lipids skip the washing steps completely.

Note: Sample pre-treatment has considerable influence on the results of a MALDI imaging experiment. The procedure above is only a starting point. Optimization may be required for specific samples or target molecules.

9. It is useful to write a sample code or number onto the slide now, preferably in a defined corner. This will both help keeping track of the sample and also of the orientation of the slide later in the experiment.
10. Desiccate the slide in vacuum once more. The sample is now ready; it can be stored for a few days in the desiccator at room temperature or frozen at –80°C for longer storage. If the samples are to be frozen, we recommend either packing them individually in a

separate slide container or wrapping the individual slides in aluminum foil, to prevent condensation of water on all slides if a section is later removed from the freezer.

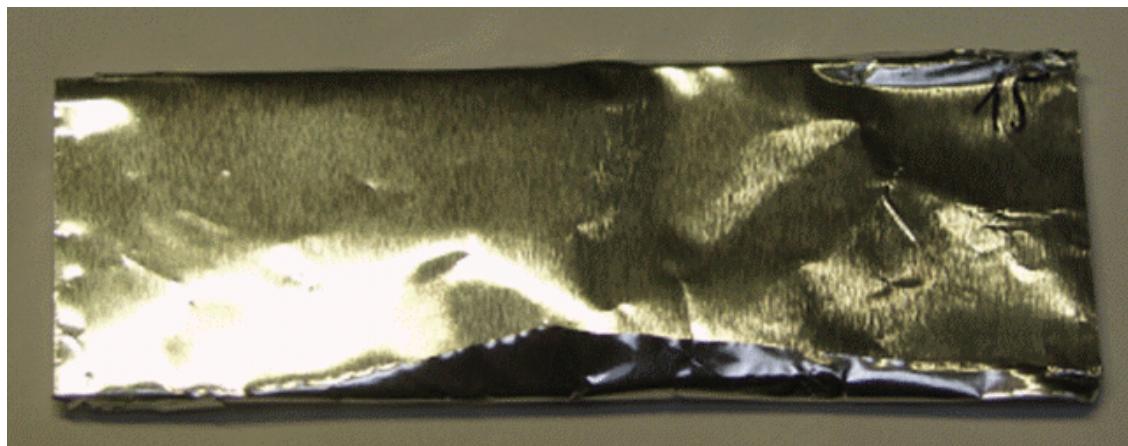


Figure 5-3 Labeled slide in aluminum foil before storing

Preparation of tissue that is embedded in mounting medium

Embedding tissue in mounting medium (such as OCT) should be avoided. However, there may be reasons to use embedded tissue: The tissue may have been embedded during the tissue banking or the tissue may be so delicate that it cannot be cut without embedding.

For the preparation of OCT embedded tissue, proceed as described in the preceding section.

At step 8: Prepare an additional Petri dish or Coplin jar with warm water. Wash the slide twice for 30–120 seconds in 70% Ethanol and once for 30–120 seconds in pure Ethanol. After this, dip-wash the slide several times in the water (5 to 10 short dips). (Adapted from: *Cazares LH, Troyer D, Mendrinos S, Lance RA, Nyawidhe JO, Beydoun HA, Clements MA, Drake RR, Semmes OJ. Imaging mass spectrometry of a specific fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 2 discriminates cancer from unininvolved prostate tissue. Clin Cancer Res. 2009, 15 (17):5541-51*)

5.1.2 Matrix Coating with imagePrep

The Bruker imagePrep device is the recommended means for coating the matrix. It is a convenient and easy-to-use push button system that performs a high quality homogenous coating and enables high spectrum quality. Prior to coating the sample with imagePrep it is necessary to apply teach marks and to take an optical image.

Preparation of sections

See section 5.1.1.

Required materials

- Liquid correction fluid (for example, Tipp-Ex®).
- For the list of required materials for matrix coating with imagePrep please refer to the imagePrep manual.

Teach marks

flexImaging needs an optical image of the sample that will be aligned with the sample on the sample carrier 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 in the instrument, it is helpful to have teach marks that are visible both on the optical image and underneath the matrix layer in the camera optic. The easiest way is to spot at least three correction fluid marks around the sample before taking the optical image. The correction fluid is visible through the matrix layer and can easily be used for teaching.



Figure 5-4 Teach marks made with correction fluid

Take optical image now!

Because the tissue section cannot usually be seen underneath the matrix layer after the spray coating, we highly recommend acquiring an optical image of the section (see section 5.1.7) before starting the matrix coating!

Coat the matrix with imagePrep

Please refer to the imagePrep manual for how to proceed from here.

Mounting the slide into the slide adapter

Please refer to the product information of the MTP slide adapter on how to mount the slide. It is necessary to wipe off the matrix layer from the left and right edges of the slide to ensure a good conductive contact between the metal adapter and the ITO-coated glass surface. The area on the slide that ends up underneath the washers of the slide adapter must be clean.

Transfer coordinates on plastic lid

Because the field of view of the instrument camera is rather small, it may be difficult to find the sample in the adapter inside the instrument. It is useful to take the plastic lid (which covers a target during shipping) and put it onto the sample carrier with the mounted sample.

Next, mark the position of the sample and the teach marks on the lid with a non-permanent pen. Placing the marked lid on a regular MTP target plate will allow you to easily read off the coordinates of the sample and teach marks. Once the slide adapter is inside the instrument, you can easily move the adapter to these coordinates using flexControl. The lid can be cleaned with ethanol for reuse.

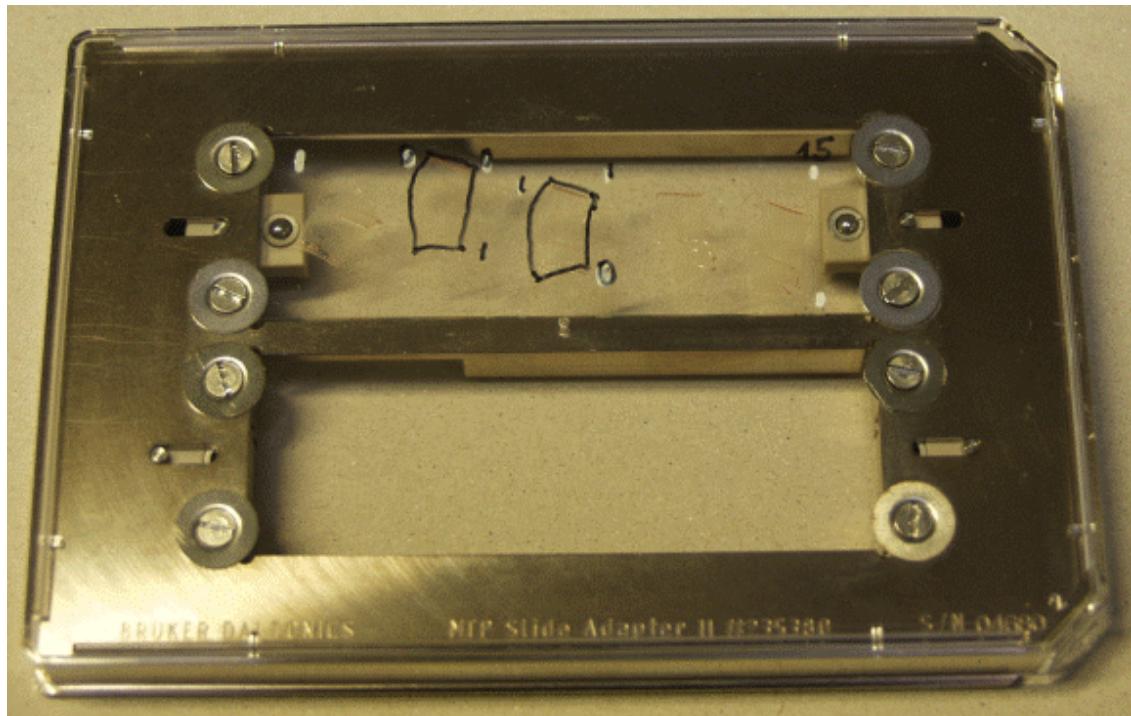


Figure 5-5 Transferred tissue coordinates and teach marks on plastic lid

5.1.3 Manual Spray Coating for MALDI Imaging

General remarks

A good reference for matrix preparation protocols is the article: "*Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation*" Schwartz SA, Reyzer ML, Caprioli RM; *J. Mass Spectrom.* (2003); 38(7): 699-708.

Manual spray coating requires a lot of practice. Most commonly, too much solvent is used during spraying, causing strong delocalization of the analytes. Be sure to practice the process with a training sample.

Preparation of sections

See section 5.1.1.

Required materials

- TLC-Sprayer: We recommend a TLC-Sprayer for the spray preparation. The TLC-sprayer needs to have a vent hole which allows starting and stopping the spray with the thumb (e.g. Kontes TLC Sprayer complete 10 ml, Nr. 422530-0010 or Sigma-Aldrich chromatography sprayer; order Nr. Z529710). TLC-Sprayers with rubber bulbs cannot be used. Artist airbrushes, although they allow a better control of the spray, are not recommended, because they do not withstand the acidic matrix solution.
- Matrix solution: 20 mg/ml sinapinic acid in 50% Acetonitrile, 0.2% Trifluoroacetic acid. For a normal tissue section of ~2 cm² usually 10 ml of matrix solution are needed.
- Fume hood: The spray preparation has to be performed in a fume hood because of hazardous aerosols.
- Liquid correction fluid: To paint teach marks for flexImaging.

Teach marks

See section 5.1.2.

Take optical image now!

See section 5.1.2.

►► Manual spray coating for MALDI imaging procedure

During matrix application, molecules are extracted from the tissue and then co-crystallize with the MALDI matrix. The amount and type of solvent used during matrix application, as well as the time allowed for extraction, play an important role during sample preparation. A large amount of solvent promotes good extraction and, as a result, good spectral quality and sensitivity. However, large amounts of solvent will also cause substantial delocalization of analytes by diffusion, resulting in a loss of spatial resolution. For a good sample preparation, the right balance between extraction and delocalization must be found.

1. First, the correct air pressure for the sprayer needs to be determined. Too much pressure will both lead to a lateral flow of droplets over the tissue and to a too fine (and therefore too dry) spray.
2. Then the right distance between the sprayer nozzle and the sample has to be determined. This can be done by a piece of cardboard. There should be no droplets moving laterally on the cardboard, and the cardboard needs to get visibly wet.
3. Start the spray aiming next to the slide, then drag the spray over the sample once or twice and stop the spray next to the slide. The time allowed for extraction and solvent evaporation between individual passes is a key element for a successful preparation. Typically, if distance and pressure are correct, the tissue stays wet for 30-45 seconds. Wait until the sample appears to be dry, then start the next spray pass.
4. Typically, no substantial matrix coating will be visible on the tissue surface after the first few spray passes, but droplets will appear on the glass slide. As the matrix layer starts to grow with subsequent passes, the droplets on the glass slides will disappear and a film of matrix solution will start to form. Once this stage is reached, the next layer of matrix should be applied before the previous one is fully dried.
5. The spraying must be continued until ~90% of the tissue is covered by matrix crystals if checked under a microscope. Typically 30 to 45 minutes are needed to complete the preparation.

Mounting the slide into the slide adapter

See section 5.1.2.

Transfer coordinates on plastic lid

See section 5.1.2.

5.1.4 Robotic Spotting

A robot can be used to spot small matrix droplets on a tissue section. The advantage of using these robotic microdroplets for the matrix application is typically a higher signal to noise ratio in the resulting mass spectra and a higher spot-to-spot reproducibility.

Details for the spotting are different for each robot, please refer to manufacturer's instructions.

General hints for robotic matrix spotting

A relevant amount of matrix is required to allow crystal formation and spectra acquisition from a tissue sample. The amount of matrix deposited in a single droplet by a robotic spotter is usually too low, so repeated spotting is usually required. Generally, as droplet volume becomes smaller, each individual spot position may have to be spotted with matrix more often. Re-spotting before the previous droplet is completely dry may improve spectral quality by improving analyte extraction. These parameters must be optimized experimentally.

If the robot has a nozzle, matrix crystallization at the nozzle may have a detrimental effect on the spotting accuracy. In such a case it is helpful to use a more diluted matrix solution and to spot more often.

General hints for acquiring the optical image

For samples spotted with droplets deposited by a robot, a sample image should be taken after matrix application. This should be used as the primary image when setting up the data acquisition in flexImaging, and allows precise teaching of the matrix droplet positions.

If desired, acquire an optical image of the sample before robotic spotting for co-registration with the imaging results.

Spot microarrays

A spot microarray is an rectangular array of matrix spots on tissue with constant spot- to-spot distances.

The upper-left, upper-right and lower-right droplet positions are used for teaching.

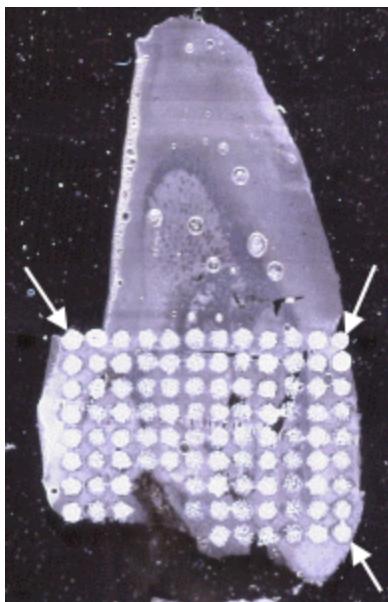


Figure 5-6 Spot microarray on rat brain section. Spots used for teaching are indicated by arrows.

The borders of the microarray should be roughly parallel to the edges of the slide of the MALDI target. flexImaging can correct smaller deviations, but will fail if the array is tilted too much.

Details on how to set up the imaging run in flexImaging can be found in the reference section.

Tissue profiling

In tissue profiling workflows, the robotic droplets are spotted on specific spots of interest on the tissue. Data will only be acquired from these spots of interest, allowing for much faster data acquisition. Instead of a full MALDI image of the sample, a "profile" spectrum for each spot of interest will be generated. Please refer to tissue profiling for a detailed description of the workflow.

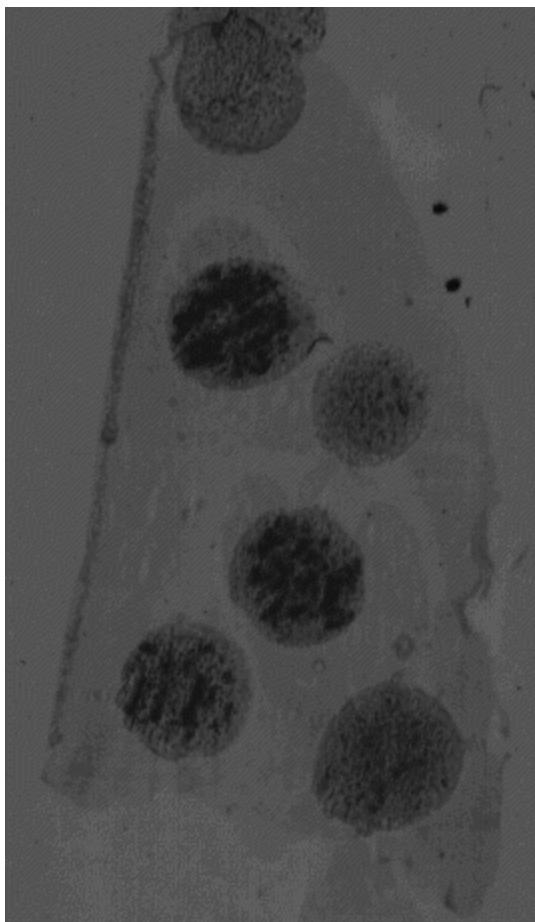


Figure 5-7 Tissue profiling on rat cerebrum

5.1.5 Specific Considerations for Detecting Pharmaceuticals in Tissue

Unlike the preparation of samples for peptide, protein, or lipid imaging, there are no standard protocols for the detection of pharmaceuticals in tissue. Pharmaceutical compounds exhibit a huge range of physicochemical properties such as solubility, basicity, and so on. For each compound, the best preparation protocol and the detection limit for a given tissue has to be determined experimentally. The parameters that have to be evaluated include choice and concentration of matrix and solvents.

Initial experiments to determine detection limits for a particular compound under ideal conditions can be performed using a dilution series on a regular MALDI target plate. The detection limit of a compound may be very different in a complex mixture of molecules (such as tissue), so a dilution series that is spiked as droplets on a tissue sample is also a accessible model system for initial experiments.

Some hints for the sample preparation are:

- Use a dilution series of the pharmaceutical compound spiked as droplets onto a tissue. Start with high concentrations (much higher than expected in the dosed tissue).
- Compare different matrix substances to see which allows the lowest detection limit. This can be done by pipetting the matrix onto the tissue without worrying about the spatial resolution. It is often helpful to spot the matrix twice to get a good crystallization. The tissue profiling mode in flexImaging is helpful to measure these matrix spots.
- Try different matrix solvents, from polar to non-polar. Because the drug needs to be extracted from the tissue into the matrix, this may have a huge impact. The solubility of the compound in a particular solvent can be a good indicator.
- When the best matrix and solvent combination is found, sample preparation must be adapted to achieve spatial resolution in a MALDI imaging experiment. For this a more realistic model system (such as a spiked tissue homogenate that is re-frozen and sectioned) is required. This can also be used to determine the detection limit under more realistic conditions.
- Detection of the compound may be subject to ion suppression by endogenous molecules. Ion suppression in tissue can be evaluated by spiking a chemically similar reference compound into the matrix as a standard, and observing the signal intensity in different tissues.

5.1.6 Preparing a Drug Dilution Series in a Frozen Tissue Homogenate Block

Tissue homogenate spiked with drug molecules is a good model system for the development of sample preparation techniques for MALDI imaging of drugs without the need for dosed animals.

It can be used to:

- Find the optimal matrix and solvent for the drug.
- Establish and optimize the limit of detection.
- Calculate a calibration curve for quantification.

IMPORTANT Before starting this procedure, confirm that a signal from a 1 in 20 dilution of the drug stock solution can be measured using a dried droplet preparation on a stainless steel target.

Required materials

- Fresh tissue
- Disposable syringes (5 mL)
- Drug stock solution

►► Creating a tissue block with drug-spiked homogenate plugs

1. Homogenize the tissue and pass through a fine sieve.
 2. Divide the homogenate into 1 mL aliquots and freeze at –80°C.
 3. Prepare a cylindrical block of frozen homogenate with four channels for drug-spiked homogenate plugs.
 - a. Cut the end off a 5 mL disposable syringe.
 - b. Withdraw the plunger to leave a volume of around 2 mL at the open end of the syringe.
 - c. Deposit 2 mL of thawed homogenate in the open end of the syringe
- Avoid introducing air bubbles.

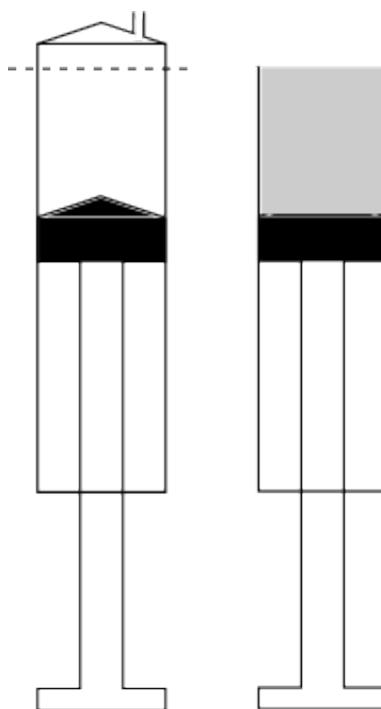


Figure 5-8 Cut the end off a 5 mL syringe and fill with 2 mL homogenate

- d. Freeze the homogenate in the syringe by placing it at -80°C for at least one hour.
- e. Drill four 1.5 mm diameter channels through the frozen homogenate block and syringe plunger.

Work quickly to avoid thawing.

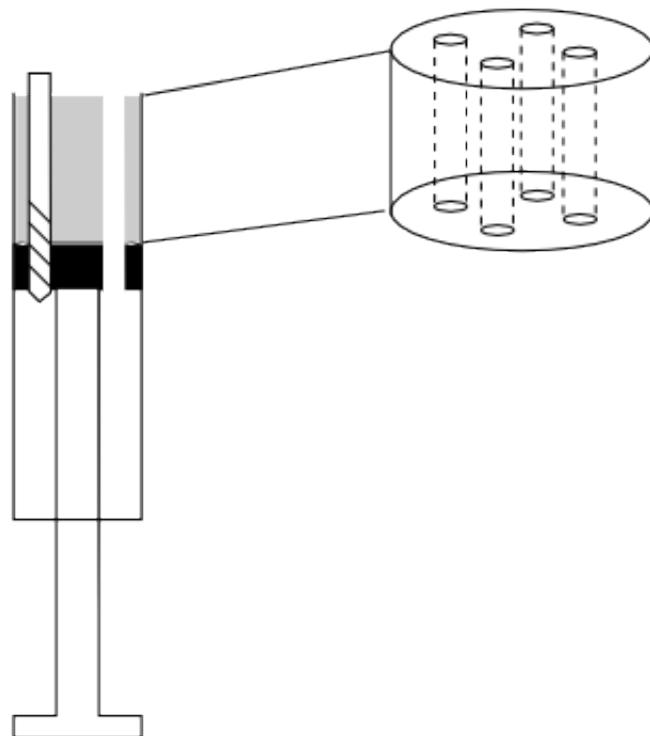


Figure 5-9 Drilling holes through the frozen homogenate block

- d. Store the syringe with the drilled homogenate block at –20°C.
 4. Thaw four 1 mL homogenate aliquots and add drug stock solution according to Table 5-1.
- Carefully stir the spiked homogenate samples at 0°C.

Table 5-1 Preparation of drug-spiked homogenate

Final drug concentration (mg/kg)	Drug stock solution (1 g/L) added to 1 mL homogenate
50	50 µL
20	20 µL
5	5 µL
1	1 µL

5. Deposit each spiked sample into a separate channel in the frozen homogenate block prepared in step 3.

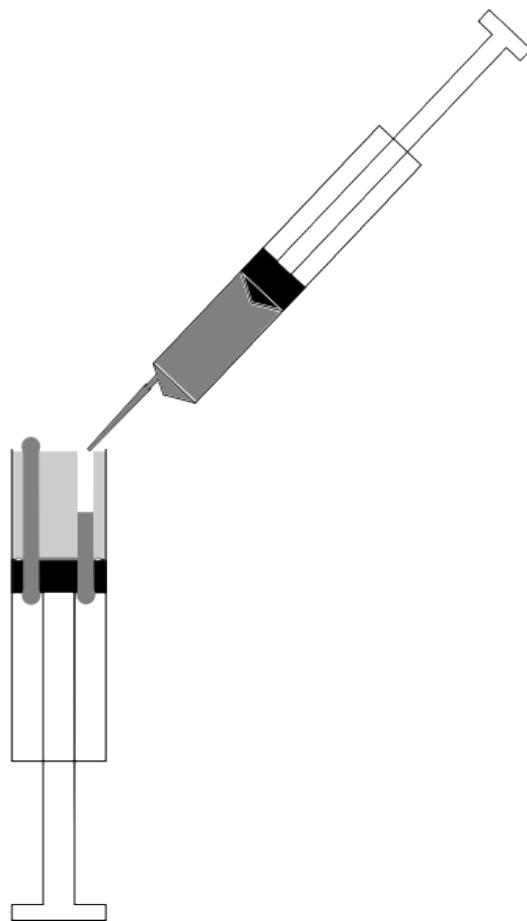


Figure 5-10 Transferring the spiked homogenate to the frozen homogenate block channels

Transfer the homogenate using a disposable syringe with a pipette tip attached to the nozzle.

Work quickly to prevent the frozen plug from thawing.

Completely fill each channel.

6. When all channels are filled, immediately transfer the frozen block containing the spiked homogenate plugs to a freezer at -80°C for at least one hour.

7. Prepare the block for cryosectioning.

- a. Deposit O.C.T. compound onto the cryostat sample stage.
- b. Place the open end of the syringe containing the frozen homogenate block onto the O.C.T. compound.
- c. Warm the barrel of the syringe around the frozen plug with your fingers until the plug can be pushed out using the plunger.
- d. Remove the disposable syringe when the plug is fixed on the sample stage with the frozen O.C.T. compound.

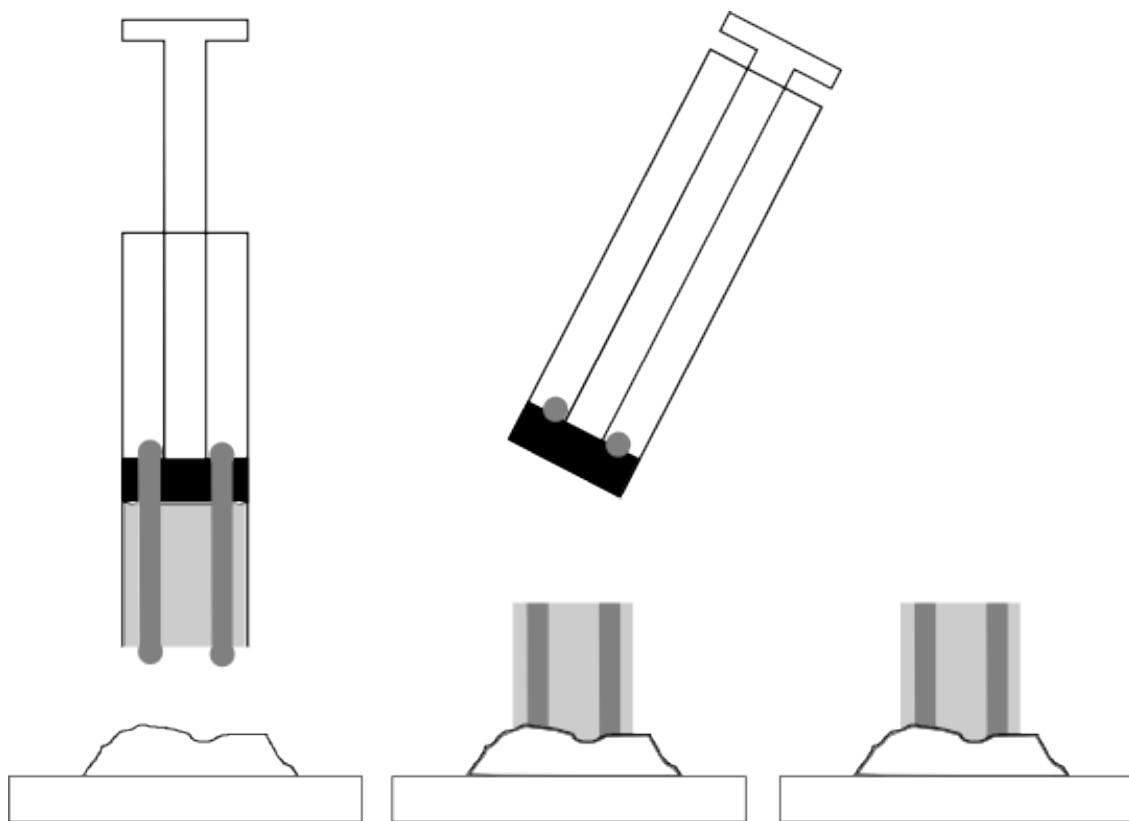


Figure 5-11 Placing the frozen homogenate block onto O.C.T. compound on the cryostat stage

The spiked homogenate block is now ready for cryosectioning.

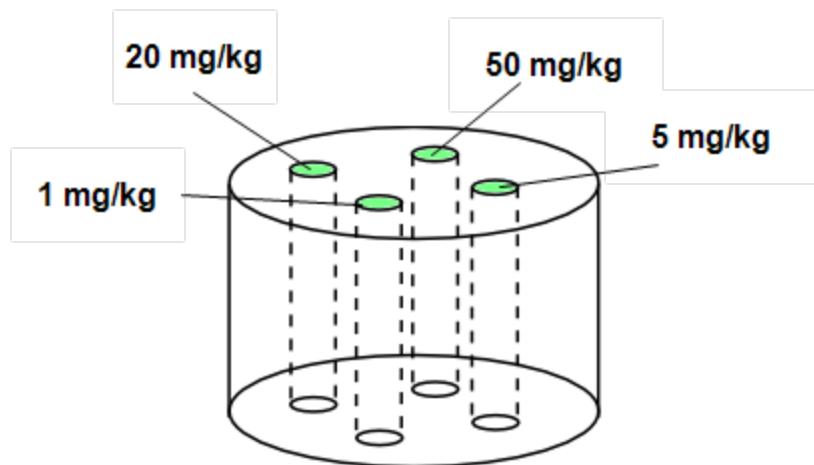


Figure 5-12 Example tissue homogenate block with spiked plugs containing different drug concentrations

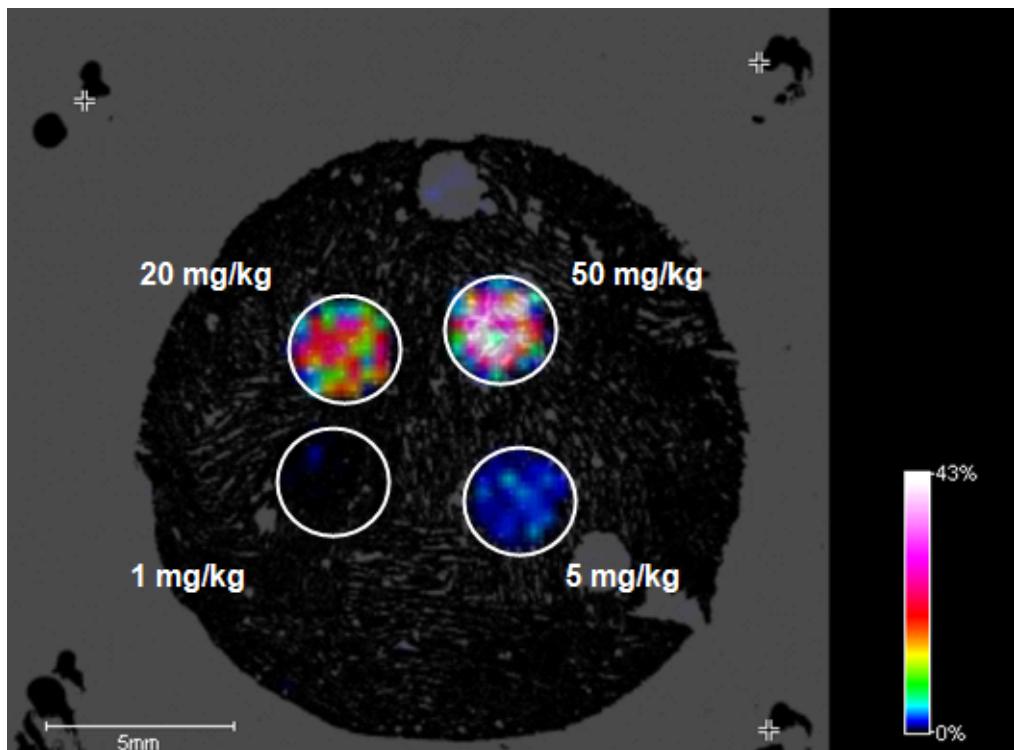


Figure 5-13 MALDI MS image of a drug spiked into liver homogenate

5.1.7 Acquiring an Optical Image

Acquiring an optical image of the sample is an integral part of the MALDI imaging workflow as implemented in flexImaging. This image allows easy selection of measurement regions and provides the primary coordinate system to correctly display results. Bruker provides a slide scanner (plustek OpticLab H850) that is ideal for acquiring images of tissue sections mounted on regular size (25 mm × 75 mm) ITO slides, but essentially any device capable of acquiring a digital image of reasonable quality can be substituted.

Basic requirements for the optical image

1. Visible teach marks or other reference points.

To align the optical image with the actual physical position of the sample inside the instrument source, at least three reference points or teach marks must be highly visible both on the optical image and on the image of the instrument camera. We recommended applying teach marks using correction fluid (for example, Tipp-Ex®) before acquiring the optical image (see section 5.1.2). Other prominent features (such as tissue margins) may be used, but are usually difficult to identify once the sample has been coated with matrix.

2. Reasonable size and resolution.

flexImaging 5.0 will automatically scale the resolution of the optical image provided to allow proper visualization of MALDI imaging data up to the minimum raster width (as defined on the **Instrument** tab of the flexImaging **Preferences** dialog). No user interaction is usually required. If the optical image covers a particularly large area (>100 cm²) or is of low resolution (<1200 DPI), the minimum raster width must be increased by the user. In addition, if a very small raster width is desired (<5 µm), the area covered by the optical image should be considerably smaller (<25 cm²).

3. Matching orientation.

The optical image must have roughly the same orientation as the physical sample on the sample carrier. flexImaging corrects for slight tilting of a few degrees, but it may be necessary to rotate or flip the optical image beforehand using an image viewer software such as IrfanView.

4. File Format.

flexImaging supports the following optical image file formats:

- bitmap (*.bmp), JPEG (*.jpg), and 8-bit/24-bit TIFF (*.tif)

5.1.7.1 Using different devices to acquire an optical image

Diapositive scanners (recommended)

Some diapositive scanners can be equipped with an adapter for microscopic slides. Using such a scanner is the most convenient way to work with flexImaging and is therefore highly recommended. Images generated by such scanners are much better in quality than from office scanners and their resolution matches the requirements for flexImaging.

Microscopes

Most microscopes will have only a limited field of view that cannot image the entire tissue section and so usually cannot be used to acquire the optical image. Some microscopes can stitch together multiple images to create a larger optical image than the field of view. Such microscopes can be used for optical image acquisition, but usually create very large files due to the high resolution, which may cause the automatic image scaling in flexImaging to fail. In this case, use an image viewer software to reduce the resolution to ~4000 DPI.

Gel scanners

Gel scanners are able to acquire an image of the tissue section on the slide in transparent mode. This is recommended. If the glass slide is placed directly on the glass surface of the scanner interference pattern may be seen in acquired image. These can be avoided by placing paper spacers under two edges of the slide and thereby creating a small gap between the scanner surface and the slide. Glass cover slips can also be used as spacers.

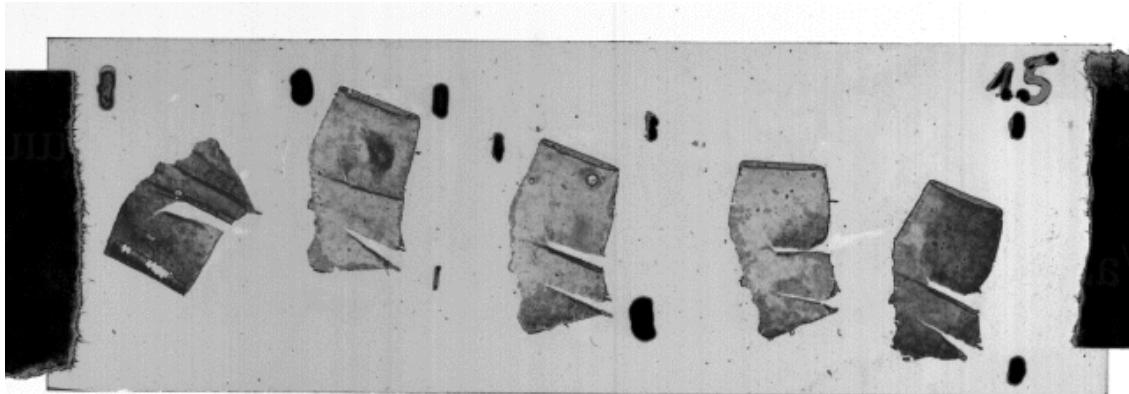


Figure 5-14 Scan of rat liver tissue section acquired with a gel scanner

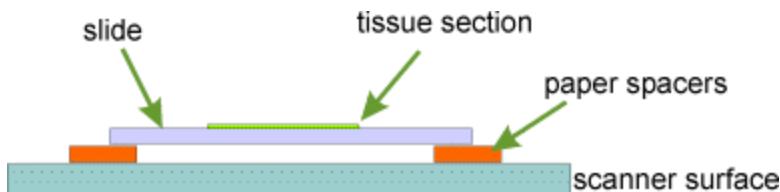


Figure 5-15 Using a gel or office scanner in transparent mode

Office scanners

If the scanner has a transparency mode, it is recommended to use this mode. If the glass slide is placed directly on the glass surface of the scanner interference pattern may be seen in the acquired image. These can be avoided by placing paper spacers under two edges of the slide and thereby creating a small gap between the scanner surface and the slide.

If the scanner has no transparency mode, then the following procedure can be used to acquire a good image: Use two paper strips or glass cover slips as spacers to avoid direct contact of the tissue sections with the scanner. Place the slide with the tissue section facing downwards onto the spacers. Cover the entire assembly with a black paper or cardboard. Now acquire the image.

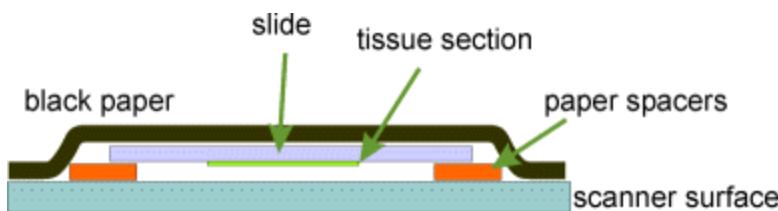


Figure 5-16 Scanning a tissue section with an office scanner without transparent mode

Digital cameras

Digital cameras may be used to acquire the optical image, but are not recommended. If the camera is not aligned perfectly parallel to the sample carrier, the image will be distorted in a way that cannot be compensated by flexImaging, which may cause a misalignment between the optical image and the MALDI imaging results. The mass spectra may therefore be not acquired at the expected positions, and the interpretation of the results may lead to wrong conclusions. If a digital camera is to be used, it should at least be mounted on a tripod and adjusted parallel to the slide. It may also be difficult to provide homogenous lighting in such a setup.

5.2 Data Acquisition

5.2.1 General Overview (rapifleX MALDI Tissuetyper)	257
5.2.2 General Overview (flex Series Instruments)	275
5.2.3 General Overview (FTMS Instruments)	280
5.2.4 Incremental Runs	283
5.2.5 Arbitrary Arrays	283
5.2.6 Spot Microarrays	286
5.2.7 Tissue Profiling	289
5.2.8 MS/MS Experiments	297

5.2.1 General Overview (rapifleX MALDI Tissuetyper)

From a mass spectrometric point of view, an imaging experiment can be seen as a series of measurements at defined positions on the sample. After a new imaging run has been set up and measurement regions have been defined, flexImaging triggers the automatic acquisition of spectra for each individual raster position. The interaction between flexImaging and flexControl has been streamlined and simplified in comparison to earlier versions of the software.

flexImaging

Select flexControl method for spectra acquisition Data reduction / linear binning

Select data processing options

Creation of a *.dat file for fast loading

Align optical sample image with sample carrier positions (teaching)

Spectra smoothing and baseline subtraction

Define measurement regions and raster width

Visualization and evaluation of data

Co-registration of images

flexControl

Acquires spectra according to the selected flexControl method

Key parameters include:

Mass range

Number of laser shots per position

Smartbeam parameters

Beam scan size

Sample rate

Laser power

Detector gain

Raw spectra

Raw spectra

flexAnalysis

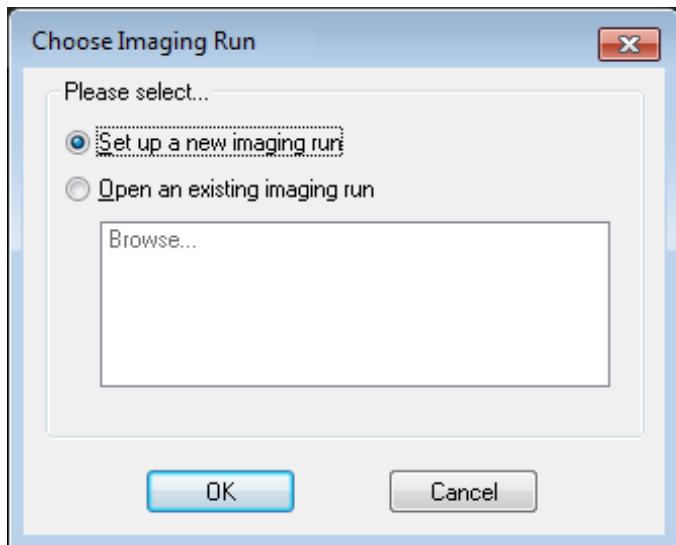
Optional processing options (post-acquisition)

Recalibration

Peak picking

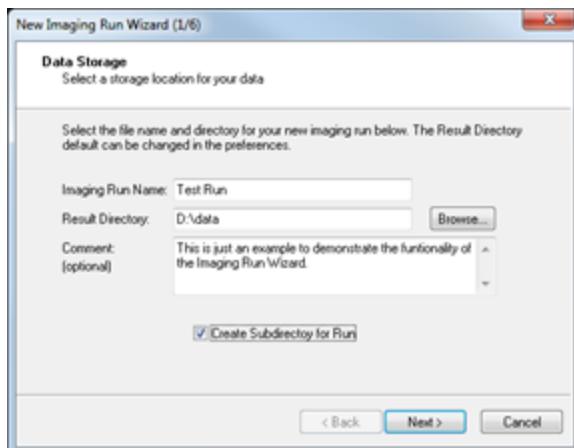
Figure 5-17 Simplified workflow for MALDI Imaging data acquisition on the rapifleX MALDI Tissuetyper

1. Setting up an imaging run is guided by a wizard, which will appear automatically when you open flexImaging.



Alternatively, you can start the wizard by selecting **File > New Imaging Run**.

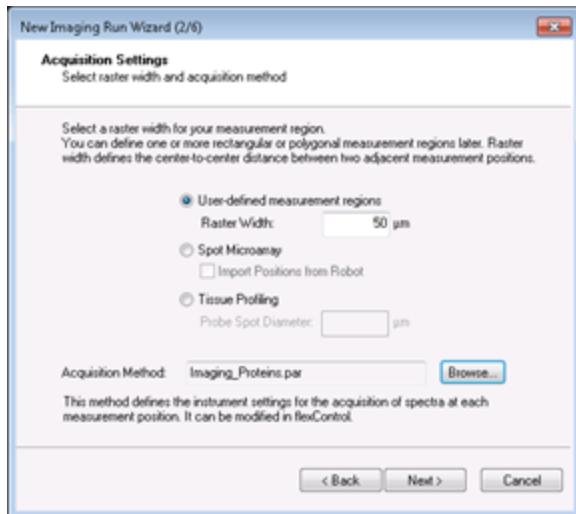
2. The individual entry fields of the wizard are mostly self-explanatory. On the first page (Data Storage), enter a name for your imaging run and identify the folder in which all information and data for the run will be saved.



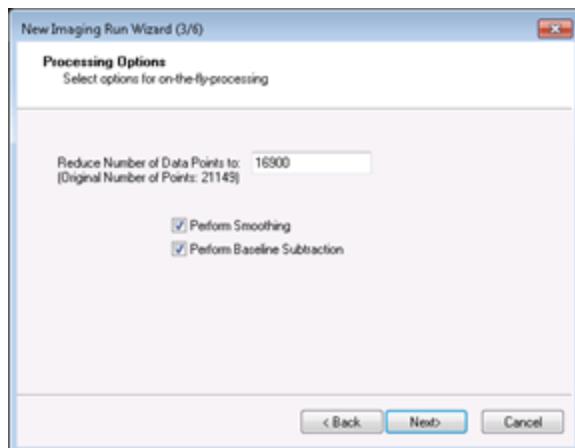
- o If you select the **Create Subdirectory for Run** check box, flexImaging will create a subfolder with the run's name in the Result Directory and store all files of the run

there. This is the recommended and the default setting.

3. On the second page (Acquisition Settings), select the desired Raster Width ("pixel size") for your measurement regions and select the flexControl acquisition method that will be used for spectra acquisition at each individual raster position of this run.



- Spot Microarray and Tissue Profiling are more specialized use-cases explained in sections 5.2.6 and 5.2.7.
 - Several default methods are provided; see Table 5-2 for more information.
4. On the third page (Processing Options), the options for the on-the-fly processing of the acquired data are selected.

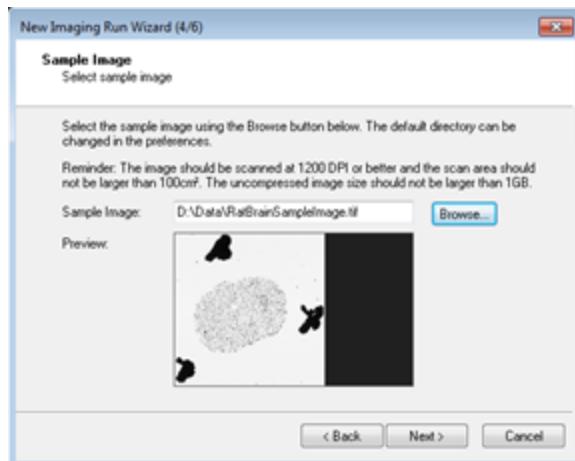


Selecting reasonable settings here is extremely important, as they are applied to the spectra during acquisition. As a result, processed data will be available immediately after the acquisition is complete. Reprocessing of acquired data after acquisition is possible, but very time consuming.

Most importantly, flexImaging combines all individual spectra of an imaging run into one large file, the so-called DAT file. During this process, the original data points of each mass spectrum are down-sampled to a lower density and also re-binned onto a linear m/z -axis. The recommended setting (which will be suggested by default) is 80% of the original spectrum size. Lower values are possible, and will result in smaller, more manageable DAT files, but also in potentially reduced MS resolution. Larger values are not recommended, as this may cause problems when converting the TOF m/z -axis into a linear one. In general, we recommend changing the original Sample Rate in flexControl if a different spectrum size is desired.

Smoothing and baseline subtraction are optional and recommended for linear mode measurements and reflector mode measurements with larger mass ranges ($> 1500 m/z$). For reflector mode measurements at lower mass range, smoothing and baseline subtraction are usually not required.

5. On page four (Sample Image), the previously acquired sample image must be selected.



For instructions on how to acquire a suitable sample image, see section 2.1.7. The sample carrier with the sample should now be loaded into the instrument. Be sure to remember the position of your teach marks or reference points or transfer them to a target lid as described previously (see section 2.1.2).

6. On page five (Teach Sample), perform the teaching as instructed.



You can use the **Zoom** button to magnify the sample image for easier positioning of the teach points. Once teaching is complete, finish the run setup. You can then define one or more rectangular and/or polygonal measurement regions by using the respective command from the **Edit** menu.

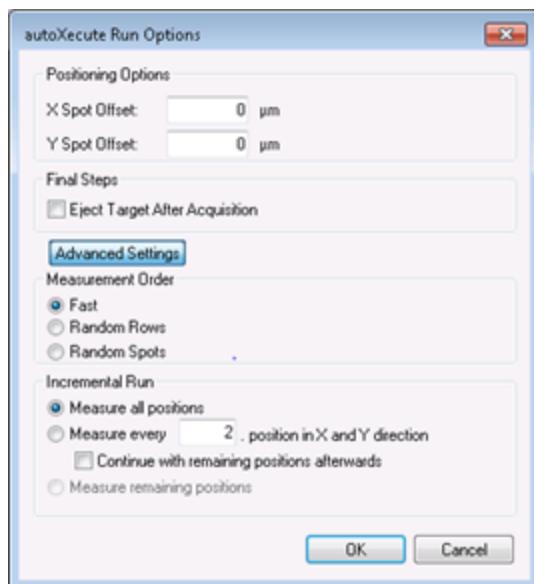
When drawing a polygon region, you can delete the last point by pressing the delete key. An entire measurement region can be modified by right-clicking on the respective entry in the **Regions** window of the user interface. You can edit the shape of the region or the raster width by selecting the respective command. All selected Raster widths should be multiples of each other (for example, 10 µm, 20 µm and 40 µm but not 10 µm, 20 µm and 30 µm in one run).

To select a different flexControl Method for all measurement regions, right-click anywhere in the **Imaging Display** window and select **Imaging Run Properties**. The acquisition method selected during setup can be changed on the **Current Imaging Run Parameters** tab.

IMPORTANT When using the simplified workflow for fast imaging acquisition on the rapifleX MALDI Tissuetyper, all measurement regions of a particular run must be acquired using the same flexControl method.

A fast way to acquire separate datasets with different acquisition methods from the same sample is to select the **Save Imaging Run As** option from the **File** menu and save the run under a different name. This will allow a different flexControl method to be assigned (as described above) but reuse the teaching and preferences from the original run. The results would be saved as two completely independent datasets.

After all selections are final, the run can be started by selecting **File > Start autoXecute Run** or by clicking the corresponding button in the toolbar. Alternatively, use the **Save autoXecute Sequence As** option to save the run for latter acquisition in batch mode. In either case, the **Run Options** window will be displayed.



7. In most cases, the default settings can simply be accepted without change by clicking OK and proceeding directly to acquire data.

Positioning Options

These dialog boxes can be used to offset the actual position from which spectra will be acquired by a set distance from the center of each spot as defined by the teaching. This is particularly useful if several measurements are to be conducted on a single sample and if the raster width is substantially larger than scan size (see the following section on flexControl methods).

Eject Target After Acquisition

If this check box is selected, the sample carrier will automatically be ejected from the source when the acquisition terminates. This is a useful setting when working with volatile matrices that sublime quickly in the source vacuum. When setting up a series of runs for acquisition in batch mode, do not use this option as flexImaging is not able to dock the sample carrier after it has been automatically ejected.

Measurement Order

The default measurement order for an imaging run is to acquire spectra row by row, from left to right and from top to bottom, for each individually defined measurement region. This is the only measurement order supported for fast acquisition on the rapifleX MALDI Tissuetyper. The advanced options lead to substantially longer acquisition times and are only recommended for special cases when a randomized acquisition order is desired (for example when time-dependent effects are expected for very long runs).

The **Random Rows** option will still acquire spectra in rows from left to right, but will move to a random uncompleted row at the end of each row. If **Random Spots** is selected, the acquisition order will be entirely random, with each spot position being measured once. This requires substantial mechanical movement of the sample stage and increases acquisition time.

Incremental Run

The default setting is to acquire data from all positions as defined by the measurement order. The alternate option is not recommended on the rapifleX MALDI tissuetyper as it is not compatible with fast image acquisition. If a quick overview of the sample is required, we recommend increasing the raster width combined with an offset selected under positioning options.

5.2.1.1 Selecting a flexControl Method

flexControl 3.8 contains a set of predefined acquisition methods that represent good starting points for common MALDI imaging applications. Two parameters should always be checked before each run and adjusted if necessary:

Laser Power

Laser power can be modified using the slider next to the camera picture in the flexControl user interface. When adjusting laser power, start from a relatively low value and increase slowly while observing the signal intensity obtained for a spectrum acquired from the sample.

Typically, signal intensity increases steeply after a certain threshold of laser power is exceeded. Signal intensity can vary widely depending on sample type and preparation method. As a rough guideline, try to achieve between 500 and 5000 intensity (per 100 shots) for the most intense peaks of a spectrum. Do not increase laser power more than necessary as this will contribute to source contamination, increase noise, cause possible fragmentation of ions, and may even damage the sample tissue.

Detector Gain

Increasing detector gain can help to increase overall signal intensity. The **Detector Gain** slider on the **Detection** tab of the flexControl user interface allows a maximum increase of 700 V for the detector voltage (setting 100×).

Try modifying the setting in small (50–100 V) steps and observe the effect on individual spectra acquired from the sample. Low detector gain will lead to a loss in signal intensity and sensitivity. If detector gain is too high, the results are broadening of peaks, loss of resolution, and increased noise. High detector gain settings also contribute to detector aging. If in doubt, use the lowest setting that still provides good spectra quality.

Make sure to save the flexControl method if any changes are made. If you decide to save the method under a different name, remember that you may have to update the selected acquisition method for an imaging run you set up previously.

Table 5-2 Default methods for MALDI imaging on rapifleX MALDI Tissuetyper

Method name	Polarity	Smartbeam Parameter	Scan Size (μm)	Recommend raster width (μm)	Shots	Mass Range	Sampling Rate (GS/s)
Linear acquisition mode							
Imaging_Proteins ^a	Positive	M5	30 × 30	100–150	800	2000–20,000	0.31
Reflector acquisition mode							
Imaging_Lipids ^b	Positive or negative	Single	10 × 10	10–20	200	600–1200	1.25
Imaging_Peptides ^c	Positive	Single	50 × 50	50–80	800	600–3200	0.63
Imaging_Peptides_high ^d	Positive	Single	50 × 50	50–80	800	600–5000	0.63
Imaging_Small_Molecules ^e	Positive or negative	Single	50 × 50	50–80	1000	100–700	1.25

^aThe standard method for analysis of intact small proteins from tissue that has been washed to remove interfering lipids and coated with Sinapinic Acid or CHCA matrix. Preparation is usually relatively wet to allow good extraction. This causes delocalization to some extent and therefore relatively large raster widths are suggested.

^bThese are standard methods for lipid analysis from tissue that has not been washed and has been coated with DHB or HCCA matrix using a very dry deposition method, such as sublimation.

Delocalization is often minimal and signal strength is very high, allowing small raster sizes. Lipids can be analyzed in both positive and negative mode using similar matrix preparations.

^cThis is the standard method for analysis of endogenous or tryptic peptides from tissue after following the respective sample preparation protocols. The mass range is limited to 3200 to allow use of the full laser frequency of 10 kHz.

^dIn this method the laser frequency is lowered to 5 kHz for detection of peptides with $m/z > 3200$.

^eSample preparation and method settings for small molecule imaging vary widely and are highly dependent on the compound of interest. Typically, sensitivity is favored over spatial resolution and therefore these methods favor larger raster sizes. These methods should be considered as starting points for further method optimization.

5.2.1.2 Guidelines for optimizing flexControl method settings for MALDI Imaging

Advanced users are encouraged to modify the default methods according to their specific requirements or for less common applications. The following guidelines serve as a starting point for such modifications.

Acquisition mode

Selecting this instrument parameter is largely dependent on analytical requirements. Generally, operation in reflector mode provides better resolution and mass accuracy at *m/z* values below ~5,000. For higher *m/z* values and large mass ranges, operation in linear mode is usually preferred. Because of longer flight times, reflector mode methods generate larger spectra for the same mass range than linear mode spectra. Keep this in mind when considering the total file size of your imaging datasets.

Polarity

The polarity of ions detected depends both on the analytes in question and the type of matrix and possible additives (such as TFA) used during sample preparation. Many peptides and proteins (in particular tryptic peptides) generate mostly positively charged ions and are therefore analyzed in positive polarity mode. Lipids are often analyzed in either polarity mode, because different subclasses of lipids may generate either positively or negatively charged ions.

Smartbeam parameter, Scan size, Raster width, and Shots

These parameters are all interdependent and of particular importance for the quality of a MALDI Imaging dataset.

In principle, the smartbeam 3D laser of the rapifleX MALDI Tissuetyper generates a single, highly focused laser spot of <5 µm diameter (Smartbeam Parameter setting **Single**). This laser spot irradiates the sample at the position indicated by the crosshairs displayed in the camera picture in flexControl.

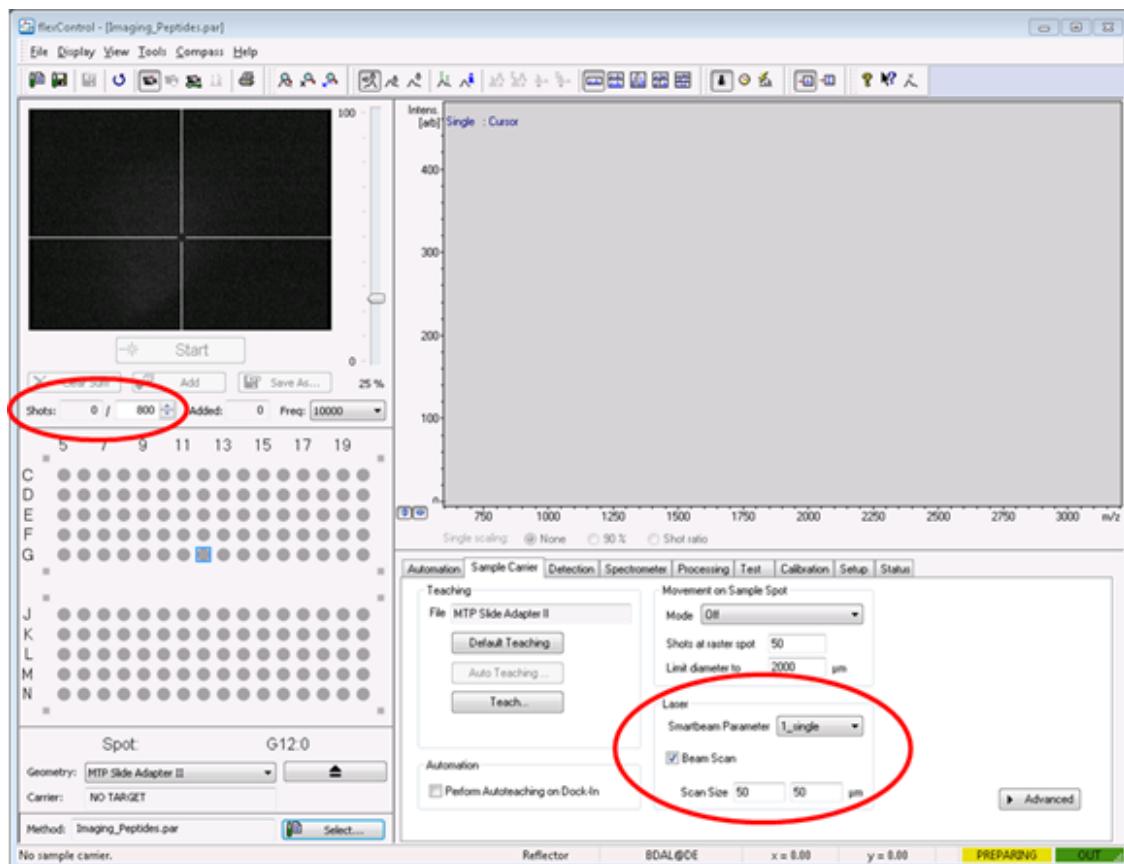


Figure 5-18 smartbeam 3D laser parameters in flexControl

When **Beam Scan** is not selected, a single ~5 µm diameter spot is projected at the center of the crosshairs. If **Beam Scan** is activated (which is the default), the same spot is moved across a user-defined rectangular sample area (**Scan Size X × Y**) in zig-zag mode if the shot number is sufficient (see Figure 5-19). This means that the laser spot can be moved quickly and precisely across the sample.

The number of individual laser pulses for this rectangle is defined in the **Shots** dialog box. These shots will be summed up to generate a single mass spectrum from the rectangular sample area. Typically, the number of shots and the size of the rectangle should be matched to allow ablation of the entire area.

Larger **Scan Size** settings require more shots to cover the area. If the shot number is low compared to the area, the laser will automatically switch to a semi-random movement pattern. This is not recommended for MALDI imaging acquisition methods.

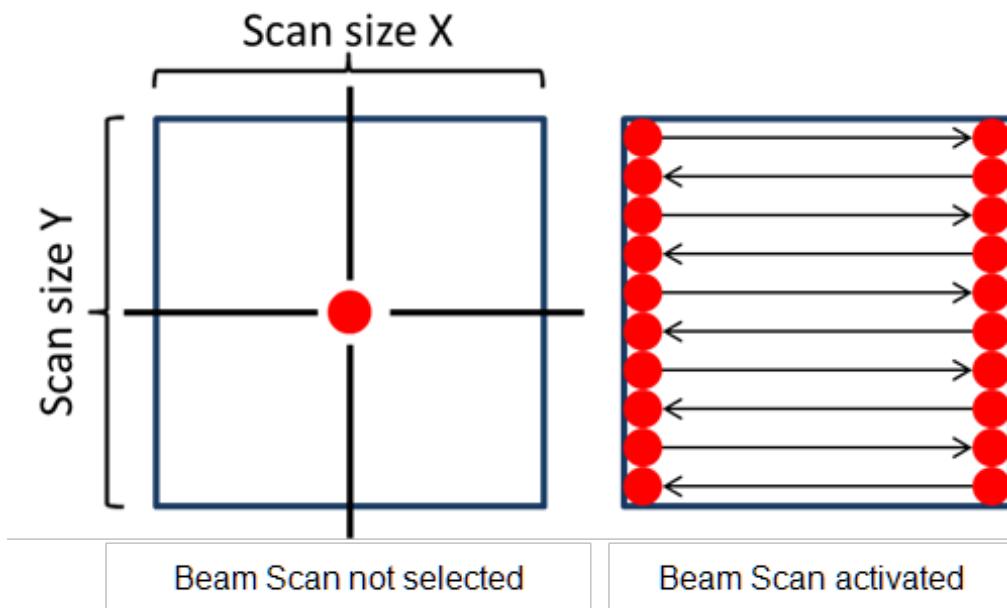


Figure 5-19 Beam Scan operation with Smartbeam parameter Single

For large Scan sizes ($>100 \mu\text{m}$) the number of shots required can lead to long measurement times. To compensate for this, the smartbeam 3D laser offers a second smartbeam parameter referred to as **M5** (see Figure 5-20).

When this parameter is selected, a modulator is inserted into the beam path that “splits” the single focus spot to target five separate positions simultaneously. The pattern for these five spots is fixed, but a beam scan can be applied to each spot in a similar way as described above for a single spot. Data from the entire scan pattern is summed up to generate a single spectrum for the central position. For imaging measurements, we recommend keeping the **Scan Size** below $35 \mu\text{m}$ to prevent the individual scan areas from partially overlapping.

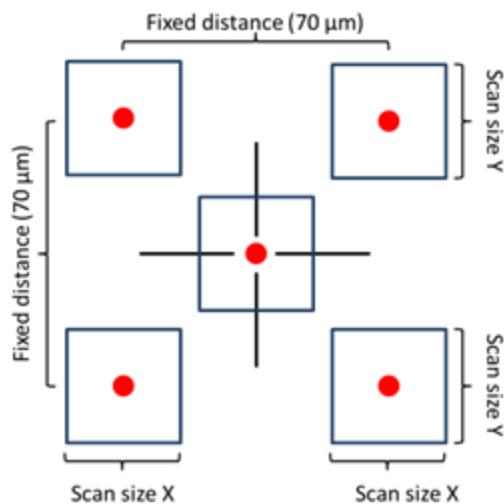


Figure 5-20 Schematic representation of the ablation pattern for the M5 smartbeam parameter setting with beam scanning activated

The **Raster Width** parameter (selected by the user in flexImaging) represents the center-to-center distance between two neighboring measurement positions (pixels). **Raster Width** and **Scan Size** are closely related and should be set in a way that **Raster Width \geq Scan Size**. This will prevent the area scanned for two adjacent sample positions from overlapping. For the **M5** pattern, **Raster Width** should be at least **Scan Size + 70 µm**.

Take into account the following when modifying the laser settings of a default method:

- **Raster Width** and **Scan Size** limit spatial resolution. A small raster width is necessary to acquire high-resolution MALDI Imaging data. However, small scan areas result in small amounts of sample being ablated for each individual spectrum. Depending on the abundance of analytes and the quality of the sample preparation, the ion yield of a small scan area might be insufficient to generate useful spectra. If spectra quality appears to be low, consider increasing the scan size to accumulate ions from a larger sample area.
- Measurement area and raster width regulate the number of spectra in a dataset. Not every sample may require large measurement regions and a small raster width to achieve good data. Acquiring large data sets costs instrument time, data storage space, and ultimately contributes to laser and detector aging.
- Try to keep the number of laser shots per position at a reasonable but low value. Once the sample is completely ablated, additional laser shots just contribute noise,

increase measurement time, and reduce laser lifetime. You can evaluate the number of shots required by manually acquiring spectra from a fixed sample position in 100 shot increments to see how many shots ablate the sample fully.

Mass Range

The mass range selected for acquisition is largely governed by the experimental design of a study. From a technical point of view, mass range should be kept as narrow as possible. Most sample preparation protocols allow detection of molecules only in a relatively narrow, defined mass range. Increasing the mass range of the method further will increase spectra size without generating additional information. High m/z values require the measurement of long flight times, in particular in reflector mode. When flight time approaches the reciprocal value of the laser frequency, the laser frequency (and as a result, acquisition speed) must be reduced to prevent interference between ions from subsequent laser shots.

Sampling Rate

Sampling rate defines the time difference between two successive detector events. For example, a sampling rate of 1GS/s (1 GigaSample per second) indicates 10^9 detection events in one second, or one event every nanosecond. Sampling rate limits the maximum resolution of a mass spectrum, because it limits the minimum flight time difference between two ions that can be recorded. For each detector event, the corresponding intensity value must be recorded. Low sampling rates can lead to loss of mass resolution and mass accuracy and erroneous signal intensity. High sampling rates generate “long” spectra with many individual data points which require a lot of storage space and lead to longer acquisition and processing times. For individual spectra, spectrum size is not an issue, but as imaging datasets typically consist of many thousands of spectra, it is important to keep the size of individual spectra low in order to keep the entire dataset to a manageable size.

When selecting a sampling rate, keep in mind that although the rapifleX MALDI Tissuetyper provides high mass resolution on standard samples (for example, calibration standard on a regular target plate), the irregular topography of typical imaging samples leads to a loss of resolution. Selecting medium sampling rates (0.31–1.25 GS/s) does not normally lead to a significant loss of data quality, but keeps total dataset size at a manageable level. As a simple guideline, try to keep spectrum size (listed in flexControl) at 30,000 or below.

5.2.1.3 MS/MS Experiments on rapifleX TOF/TOF

MALDI imaging experiments aimed at detecting a particular molecule of interest in a targeted fashion often lack specificity when conducted on a MALDI-TOF instrument in MS mode. This is due to the presence of other molecules of (almost) identical *m/z*-values that cannot be clearly distinguished using the resolving power and mass accuracy provided by a MALDI-TOF instrument.

As an alternative to employing a high-resolution instrument such as the solariX FT-ICR or optimizing sample preparation to remove interfering background ions, MALDI imaging experiments in MS/MS mode can help to increase the specificity of detection.

The strategy of MS/MS imaging experiments is to isolate and fragment all ions in a narrow *m/z*-range surrounding the target compound. Although all ions (target and background) within that window will be subject to fragmentation, their fragmentation patterns are different, and the detection of target-specific fragment ions serves as a specific indicator of the target compound's presence. Background ions of similar precursor mass may be also be fragmented, but if they yield a different set of fragments their presences does not interfere with detection of the compound on the MS/MS level.

The first step for a MS/MS imaging experiment is to set up an optimized flexControl MS/MS method (file extension *.lft) that allows a distinct fragmentation pattern of the target compound to be measured. Initial experiments can be conducted by spotting a stock solution of the target compound on a regular MALDI plate and using the default flexControl method "MSMS_Standard.lft" as a starting point.

In addition to the settings defined in the flexControl method, sample preparation parameters — such as choice of matrix and solvent — play an important role in optimizing the detection of a particular compound. For more information, see sections 5.1.5 and 5.1.6.

MS/MS imaging experiments are limited to compounds for which a distinct fragmentation pattern can be established. Typically, these are low-molecular-weight compounds, such as drugs or endogenous metabolites.

After an MS/MS flexControl method has been established, make sure that you save it under a suitable name. Setting up the MS/MS imaging run is similar to the workflow described in section 1 and can be done using the same wizard with the following exceptions:

- In step 3, on the second page of the wizard, make sure that you select the previously optimized MS/MS method (file extension *.lft).

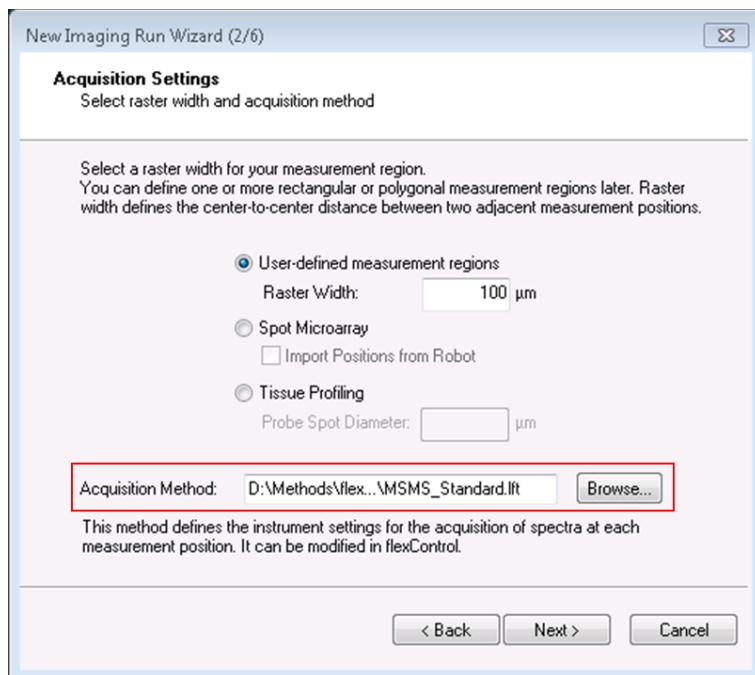


Figure 5-21 Selecting the *.lft MS/MS method in the New Imaging Run Wizard

- If you selected an MS/MS method, you will be prompted to enter the mass of the target compound precursor ion on the third page of the wizard. This value is not automatically transmitted from the flexControl method settings and must be entered manually. We recommend using baseline subtraction and smoothing.

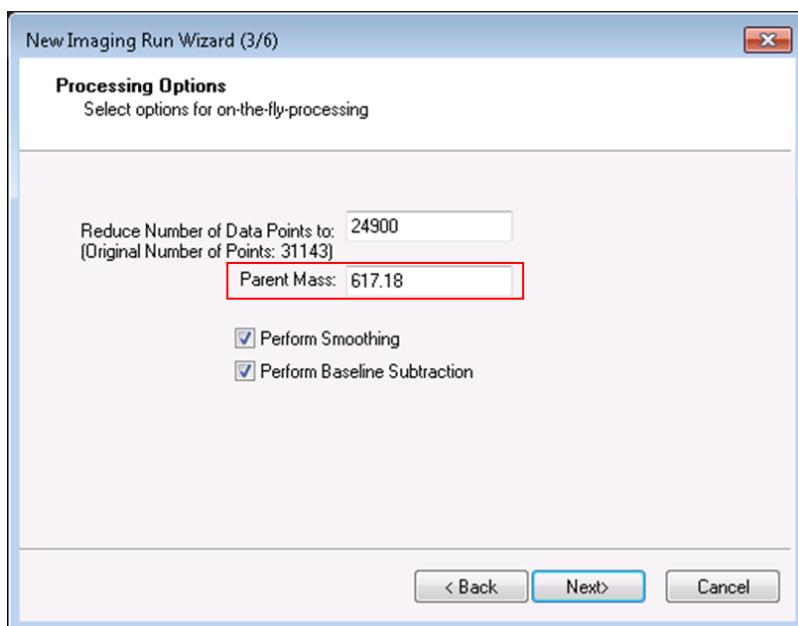


Figure 5-22 Entering the parent mass of the target compound

- The parent mass can be changed later by selecting **Edit > Imaging Run Properties** and entering a different value.

Note SRM-type measurements are not supported on rapifleX instruments and no **Fragment Mass** value should be entered.

5.2.1.4 Batch Runs — Multiple Samples on the Same Sample Carrier

Theoretically, all samples on a sample carrier could be acquired as separate measurement regions of a single imaging run using an optical image covering the entire area. This is most often inconvenient both from a technical and an analytical point of view, especially if the samples are from unrelated studies. All results of a particular imaging run will always be scaled according to the highest intensity for each mass (across all measurement regions). The result would predominantly reflect the relative intensity differences between the regions, rather than the distribution of the selected masses in the individual regions. If this is desired, for example in a differential comparison between a treated sample and a control, it is still better to measure these samples individually and then utilize the **use absolute intensity** option for the mass filters in question.

To acquire multiple imaging runs sequentially, flexImaging provides a tool called autoXecute Batch Runner, which can be found under **All Programs > Bruker** in the Windows **Start** menu.

Instead of starting imaging runs directly out of flexImaging, save all runs of a batch using the **Save autoXecute Sequence As** option from the **File** menu of flexImaging (or the respective button from the toolbar). flexImaging will save the autoXecute sequence and the according geometry in the default folders (usually D:\methods\AutoXSequences and D:\methods\GeometryFiles). The suggested default name of files is the name of the imaging run, preceded by *Imaging_*. Repeat this procedure for all runs that are part of the batch.

If you are acquiring data from samples that are in the same optical image, remember that you can reuse the teaching of a particular sample image by using the **Save Imaging Run As** option from the **File** menu to save the run under a different name. You can then simply delete measurement regions and define different ones for a second imaging run.

When all imaging runs of the desired batch run have been saved as autoXecute Sequences, start the autoXecute Batch Runner.

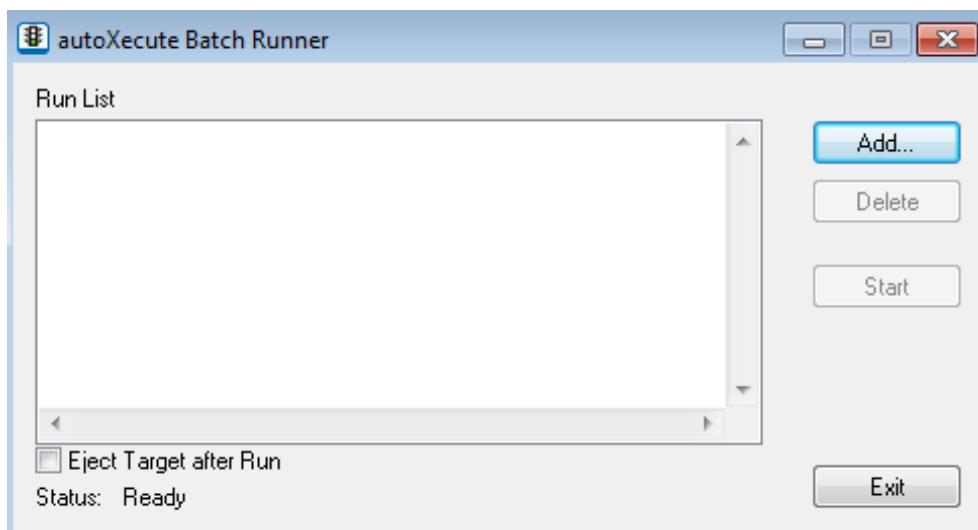


Figure 5-23 autoXecute Batch Runner

The **Add** button will automatically open the default save location for autoXecute runs (typically D:\Methods\AutoXSequences). Select all imaging runs of the current batch, from the first to the last, and add them to the list. If you accidentally make a wrong selection, remove it from the list using **Delete**.

If the **Eject Target after Run** check box is selected, the sample carrier will automatically be ejected from the source after the last run of the batch terminates. When the selection is complete, click **Start** to automatically acquire data from all imaging runs selected. The flexImaging user interface is not required for these runs.

Note The incremental run option is incompatible with batch runs.

If the batch runner is closed using the **Exit** button, the measurement will stop after the currently active autoXecute run has been finished.

To terminate the acquisition of an imaging run in progress, click the **Abort automatic run** button in the **autoXecute** tab of flexControl (flex Series instruments) or the **Stop** button in ftmsControl (FTMS instruments). Note that the measurement will proceed to the next run of a batch if the batch runner is still open.

After the batch run is finished, the autoXecute sequences and geometry files for the runs are no longer needed and can be deleted.

5.2.2 General Overview (flex Series Instruments)

From the mass spectrometric side of view, the imaging experiment can be seen as a series of measurements at defined positions on the sample. After creating a new imaging run in flexImaging and definition of measurement areas, flexImaging triggers an automatic measurement of the spectra.

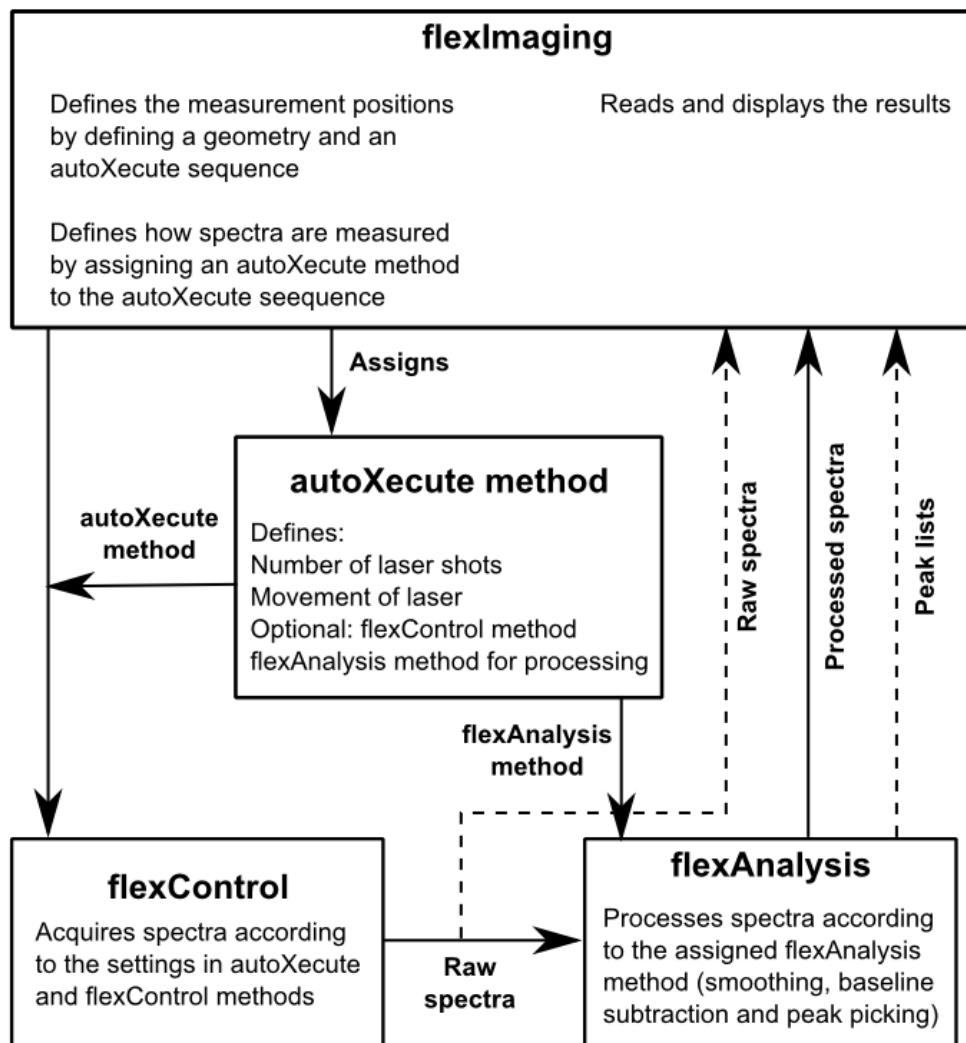


Figure 5-24 Workflow for an automatic acquisition of a MALDI image

The following is a detailed description of an automatic imaging run that is triggered by clicking **Start autoXecute Run** ():

- flexImaging creates a geometry for the sample. This geometry defines from which x,y-points on the sample surface spectra will be acquired.
- flexImaging creates an autoXecute run. This run contains the geometry and defines the order of measurements. It also contains the autoXecute method (as defined in the Regions Pane in flexImaging).
- The autoXecute method contains the information on how each spectrum is measured, such as how many laser shots are summed up, which laser energy is used, if and how the laser moves during the acquisition. It also contains a reference to the flexAnalysis method that is used for data processing. Optionally, it is possible to define a flexControl method that is used for the acquisition.
- The spectra are measured according to the autoXecute method, saved, and then sent to flexAnalysis, where they are processed with the flexAnalysis method that was selected in the autoXecute method.
- After the automatic measurement is finished flexImaging reads either the raw spectra, processed spectra, or peak lists, depending on the settings in the imaging run properties.

It is recommended to define a smoothing and baseline subtraction in the flexAnalysis method and to read the processed spectra (see data processing). The option “read peak lists” is not recommended and mainly present for downward compatibility reasons. It is therefore useful to not do a peak picking in flexAnalysis to save time in the processing.

Setting up the flexControl method

Start with a suitable linear method, such as LP_Imaging_2-20_kDa.par. It may be necessary to adjust the mass range and the matrix suppression for the actual tissue sample. Very likely a higher laser energy is necessary. It is also useful to restrict the sampling rate to 0.1 or 0.5 GS/s. This makes the spectra file size smaller and the processing in flexAnalysis and flexImaging faster.

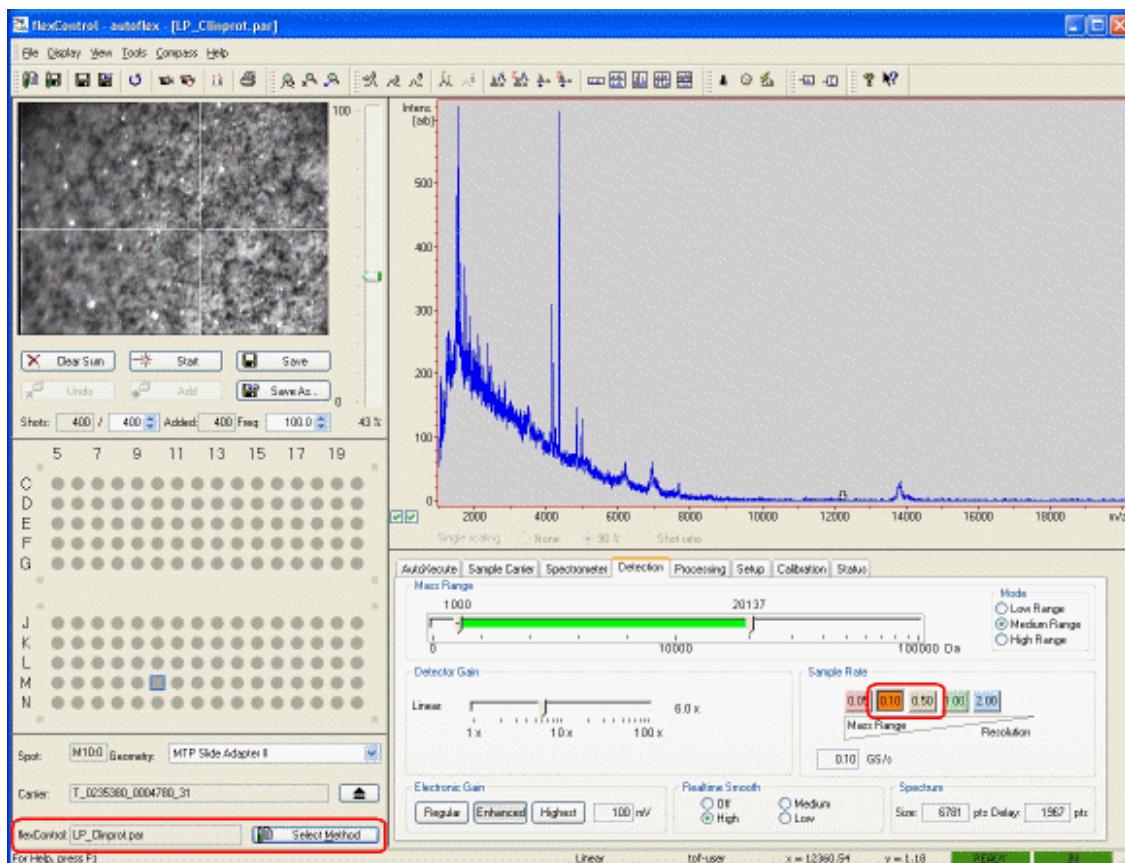


Figure 5-25 Adjusting the sample rate in Detection tab in flexControl

It is now time to fine tune the flexControl method. To do so, enable **Move Sample Carrier** () in flexImaging and move the sample carrier to a spot on the tissue by clicking on that spot in flexImaging's Imaging Display. Now adjust the laser energy in flexControl by measuring some spots on the tissue. The mass range for the acquisition and the matrix suppression should also be adjusted in flexControl, first parameter in detection tab, second in spectrometer tab. In the autoXecute method used for the imaging run, it is recommended to set the flexControl method to "none" (see figure) and the laser power to "from laser attenuator" in the laser tab. If these settings are used, then the acquisition will start with the current flexControl settings.

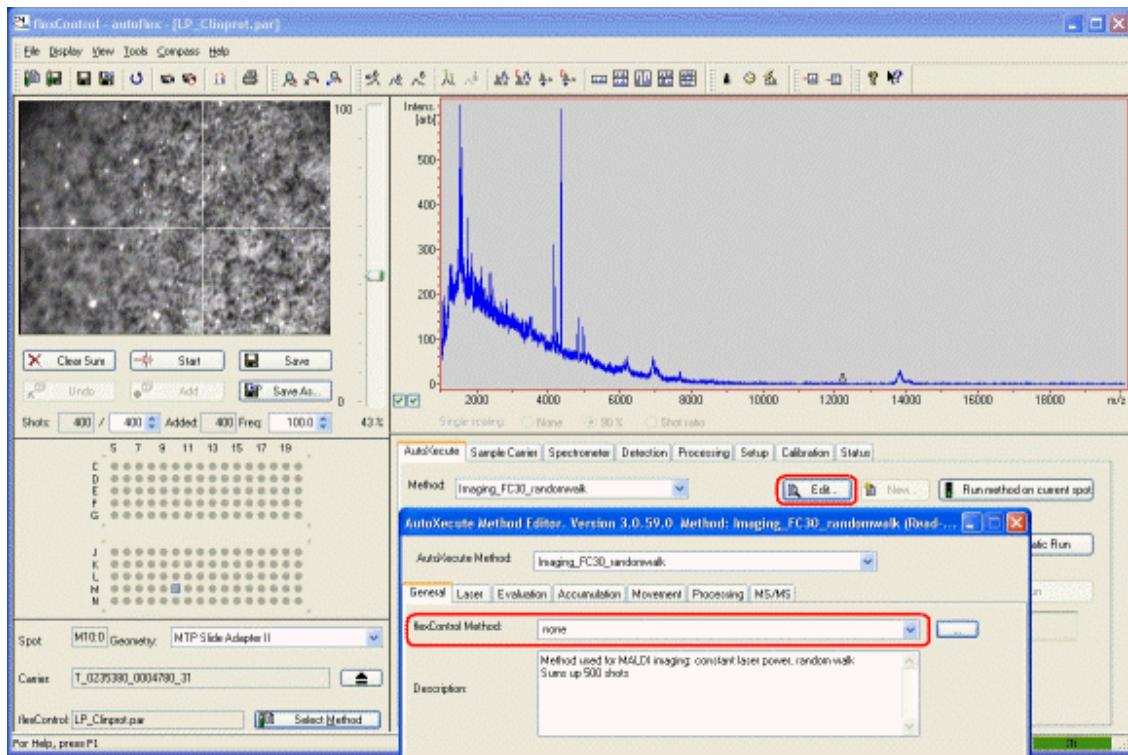


Figure 5-26 One AutoXecute method setting: flexControl method is set to none

It is important to save the flexControl method directly before the run!

After the automatic run flexControl will reload the current flexControl method, and by doing so will undo any settings that have been done after the last save of the method. It is therefore recommended to save the flexControl method directly before starting the automatic run.

Note It is mandatory to save the flexControl method directly before starting the acquisition if the batch runner is used or if an incremental run is to be done!

5.2.3 General Overview (FTMS Instruments)

This section applies to solariX instruments running with ftmsControl.

The FTMS imaging experiment is performed using flexImaging and ftmsControl. The ftmsControl software allows for direct communication between flexImaging and ftmsControl. Similar to TOF instruments, the FTMS imaging experiment is setup as a series of measurements at defined x-y-positions of the sample. flexImaging is used to create a new imaging run and to define the measurement area. Teaching and setting up Imaging runs is done in a similar way as for the TOF instruments. Within the setup procedure you will be asked to provide an MS method (see Figure 5-27), which must be generated prior to the imaging set-up using ftmsControl. For the details to set-up the ftmsControl method please refer to the respective ftmsControl manual.

Useful settings for the MS method are the online-recalibration and the data reduction (recommended setting 0.975).

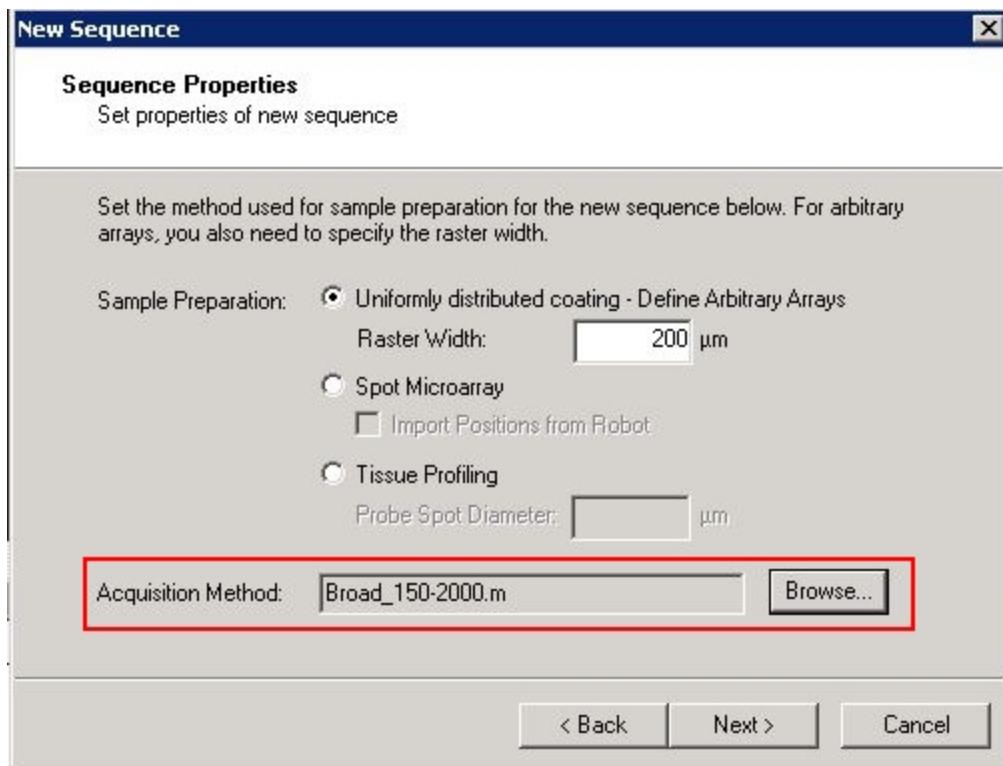


Figure 5-27 flexImaging dialogue for defining the ftmsControl Acquisition Method

From the mass spectrometric point of view, the imaging experiment can be seen as a series of measurements at defined positions on the sample. After creating a new imaging run in flexImaging and definition of measurement areas, flexImaging triggers an automatic measurement of the spectra.

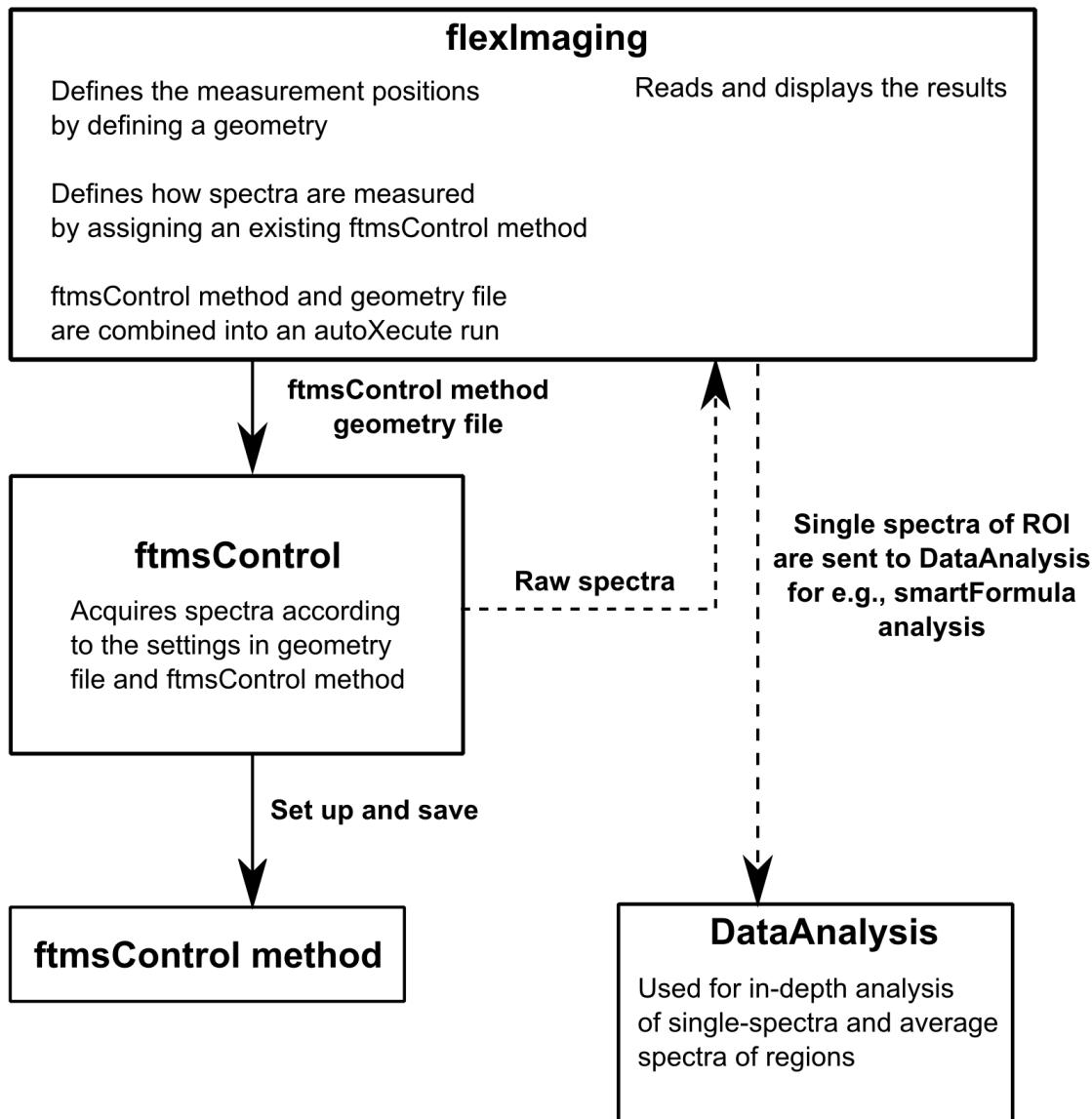


Figure 5-28 Workflow in an automatic acquisition of a MALDI image using an FTMS instrument

What happens in detail when the automatic imaging run is started using **Start autoXecute Run** () is the following:

- flexImaging creates a geometry for the sample. This geometry contains the information on which points on the sample the spectra will be acquired.
- flexImaging creates an autoXecute run. This run contains the geometry and defines the order of the measurements. It also contains the ftmsControl method (as defined in the Regions Pane in flexImaging).
- The ftmsControl method contains the information how the spectra are measured; such as how many laser shots are summed up, which laser energy is used, and if and how the target moves during the acquisition.
- The spectra are measured according to the ftmsControl method, saved and then pushed into flexImaging.

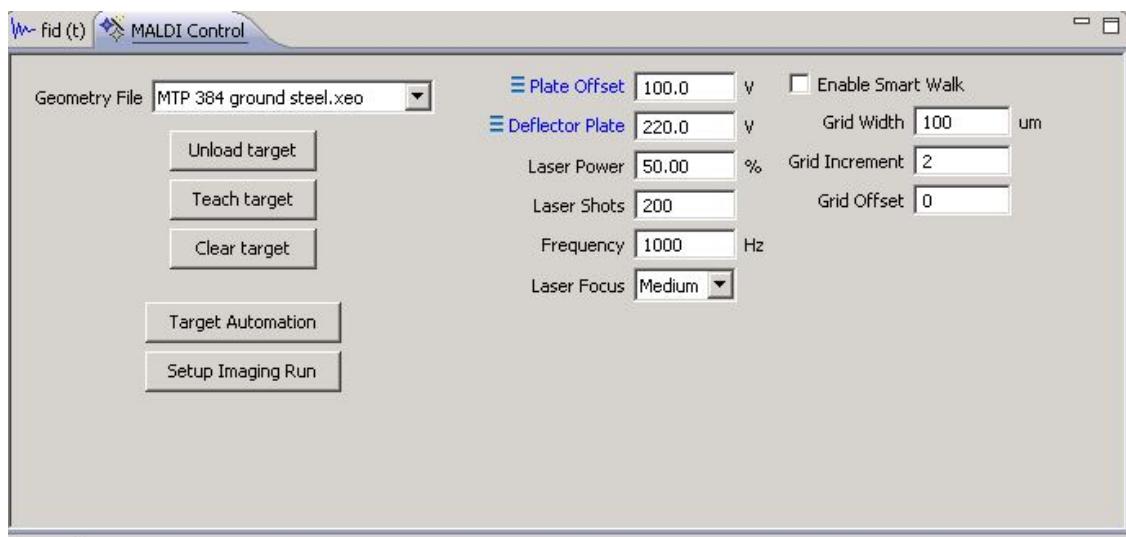


Figure 5-29 Adjusting the MALDI parameters in ftmsControl

It is now time to fine tune the ftmsControl method. To do so, enable **Move Sample Carrier** () in flexImaging and move the sample carrier to a spot on the tissue by clicking on that spot in flexImaging's Imaging Display. Now adjust the laser energy in ftmsControl by measuring some spots on the tissue. If available calibrate now on known peaks. You can also choose to do an online calibration of the spectra (see the *ftmsControl User Manual* for details).

For highly accurate data it is important to save the calibrated ftmsControl method directly before the run!

5.2.4 Incremental Runs

The acquisition of MALDI images can take quite a long time, especially if a small raster width value is selected. To get a faster overview of the expected result without jeopardizing the possibility of getting a high resolution image, flexImaging offers the option of performing incremental runs. After pressing  in flexImaging, the **autoXecute Run Options** dialog opens. Here an incremental run can be selected. Spectra will be skipped in the x- and y-direction (for example, selecting $n = 4$ will acquire every fourth spectrum in the x- and y-direction, reducing the number of total spectra by a factor of 16). After this initial part of the run, the result can be evaluated (e.g. if the quality of the spectra is sufficient) and then the remaining positions can be acquired by pressing  again. It is necessary to save the imaging run if the remaining positions are to be acquired later, otherwise flexImaging will not remember the incremental status of the run.

5.2.5 Arbitrary Arrays

The arbitrary arrays workflow requires a homogenous matrix layer on the tissue, e.g. from a spray coating. In this workflow the raster positions in a measurement region can be arbitrarily defined. The settings for the automatic measurements can differ depending on the raster width and the laser spot diameter. This applies especially to the laser movement and the number of laser shots to be summed up.

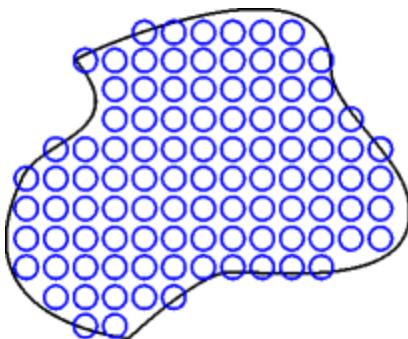


Figure 5-30 Schematic of an arbitrary array on a tissue section

Laser is firing in the center of the spot

The simplest approach is to acquire one spectrum with a defined number of laser shots from the center of each raster position.

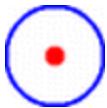


Figure 5-31 Acquiring one spectrum from the center of each raster position

This will happen if no laser movement is specified in the autoXecute method (flex Series instruments) or no stage movement is specified in the ftmsControl method (FTMS instruments) for the data acquisition.

5.2.5.1 Arbitrary Arrays on flex Series Instruments

With the flexImaging installation example, methods with this behavior will be installed:

- for **flexControl 3.4** the name of the method is "Imaging_FC33_noraster.axe"
- for **flexControl 3.3** the name of the method is "Imaging_FC33_noraster.axe"
- for **flexControl 3.0** the name of the method is "Imaging_FC30_noraster.axe"
- for **flexControl 2.4** the name of method is accordingly "Imaging_FC24_noraster.axe".

The number of laser shots that can be acquired from one position on the sample is limited by the matrix layer. For a typical preparation with a standard imagePrep protocol, 200–400 laser shots per raster position is a reasonable number.

Laser is walking around the center of one spot

If the **raster width** in the imaging region is **larger than the laser spot** (always if the raster width is larger than 100 µm) diameter, it is obvious that only a small part of the actual area of each raster position gets measured. It is therefore sometimes desirable to move the laser within each sample position.

Depending on the flexControl version, two different ways of acquisition are recommended:

In **flexControl 3.x**, it is recommended to activate the random walk feature on the sample position while acquiring the spectrum.



Figure 5-32 Random walk of laser during acquisition

With flexImaging, an autoXecute method "Imaging_FC30_randomwalk" (for **flexControl 3.0**) or "Imaging_FC33_randomwalk" (for **flexControl 3.3**) is installed that shows this behavior.

flexImaging will calculate the diameter of the sample spots from the raster width and the laser diameter as specified using **Preferences - User Interface Defaults**. It is therefore important to specify the correct laser diameter in preferences.

In **flexControl 2.4**, no random laser walk is available. It is recommended, to have the laser move in a "five-on-a-dice" pattern.



Figure 5-33 "Five-on-a-dice" laser movement during data acquisition

With flexImaging, an autoXecute method "Imaging_FC24_raster5" is installed that shows this behavior.

flexImaging will calculate the diameter of the sample spots from the raster width and the laser diameter as specified using **Preferences - User Interface Defaults**. It is therefore important to specify the correct laser diameter in preferences.

5.2.5.2 Arbitrary Arrays on FTMS Instruments

The number of laser shots that can be acquired from one position on the sample is limited by the matrix layer; usually not more than 400 shots can be obtained.

Laser is walking around the center of one spot

If the raster width in the imaging region is larger than the laser spot (always if the raster width is larger than 100 µm) diameter, it is obvious that only a small part of the actual area of each raster position gets measured. It is therefore sometimes desirable to move the laser on each sample position. If the laser would move on one imaging sample position, it is also possible to level out effects of small scale inhomogeneity in the matrix layer.

In ftmsControl one can set the MALDI stage doing a smart walk on the sample position while acquiring the spectrum. However, this will slow down the overall acquisition.

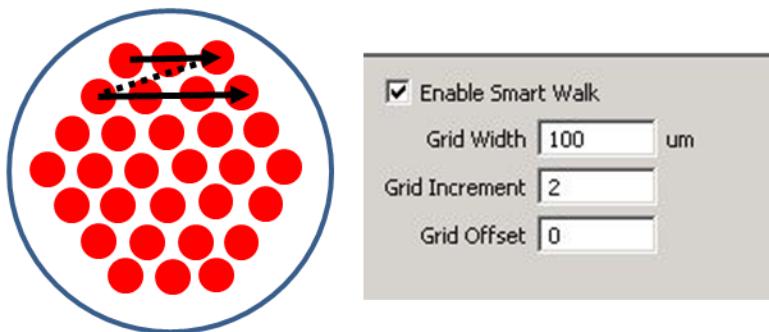


Figure 5-34 Smart walk of laser during acquisition and parameters

Tip For a non-synchronized but faster movement, choose the random walk option.

5.2.6 Spot Microarrays

A spot microarray is a regular grid of small matrix droplets deposited on the sample. Spot microarrays can be prepared with a matrix spotting robot.

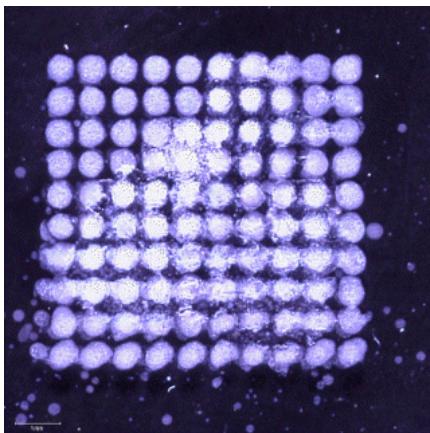


Figure 5-35 Spot microarray on tissue

During the preparation of a spot microarray, diffusion will cause delocalization of analytes within each droplet. The spatial resolution of a microarray preparation is therefore limited by the droplet diameter. One mass spectrum will be acquired from each droplet ("pixel"). The optical image selected in the **New Imaging Run Wizard** (= sample image) must be an image of the sample with the matrix droplets already deposited, because these will be used as a reference for determining the acquisition spots.

Data acquisition

Because the dried droplet preparations usually allow a higher number of laser shots per position than spray preparations, it is recommended to take advantage of that. To average out local inhomogeneity in the preparation of each spot, it is also recommended to have the laser moving on each spot during the acquisition.

For FTMS instruments, the best way to do this is using the smart walk or the random walk of fmsControl.

For flex Series instruments, the best way to do this is dependent on the used flexControl version. flexImaging installs example autoXecute methods for both flexControl versions.

With **flexControl 3.x**, it is recommended to have the laser perform a "random walk" during the acquisition. The autoXecute method "imaging_FC30_DriedDroplet" (for **flexControl 3.0**) or "imaging_FC33_DriedDroplet" (for **flexControl 3.3** and **3.4**) shows this behavior.

In **flexControl 2.4**, the random walk feature is not available. Here it is recommended to use the "hexagon" moving pattern of the laser. This movement covers the entire matrix droplet. The autoXecute method "imaging_FC24_DriedDroplet" shows this movement.

For the rapifleX MALDI Tissuetyper instrument and **flexControl 4.0** we recommended activating the **Movement on Sample Spot** option on the Sample Carrier tab in flexControl and selecting **Smart – Complete Sample** and the limiting the diameter to the actual diameter of the matrix droplet in the respective dialog box.

Setting up the run

Setting up a spot microarray run in flexImaging differs slightly if a spot list from the robot is available or not. If no spot list is available then the **New Imaging Run Wizard** asks for the upper left, the upper right and the lower right spot as teach points. It is necessary to provide either the number of spots in X- and Y- direction or the pitch in micrometers.

If a spot list from the robot is available then it is not necessary to provide the number of spots in X- and Y-direction or the distance. This information is taken from the robot file.

Starting the run

Before starting the run it is necessary to define a measurement region using either **Add Rectangular Measurement Region** () or **Add Polygon Measurement Region** (). In the easiest case a rectangular area will be defined that contains all spots. Since the tissue may be not rectangular and there will be matrix spots without tissue underneath, these spots can be excluded by defining a polygon region around the tissue.

It should be checked that the defined measurement positions do match with the matrix spots. This can be done by switching on **Laser Spots** ().

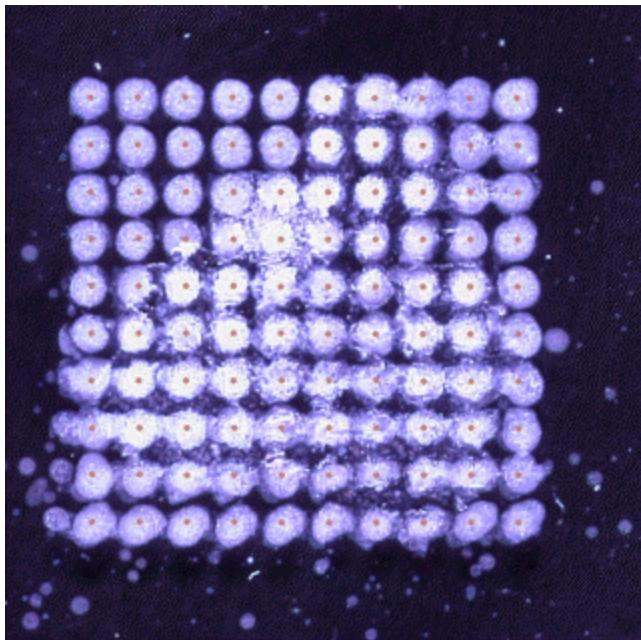


Figure 5-36 **Laser spot positions displayed to check teaching**

Displaying the results

We highly recommend acquiring an optical image of the sample before the matrix spotting and co-registering this image using the **Co-Register Image** option of the flexImaging **Edit** menu. This image can then be displayed using the **Co-Registered Image** option from the **View** menu, and the sample image can be deactivated. In this way, superimpositions of MALDI data on a good quality sample image can be used for data evaluation.

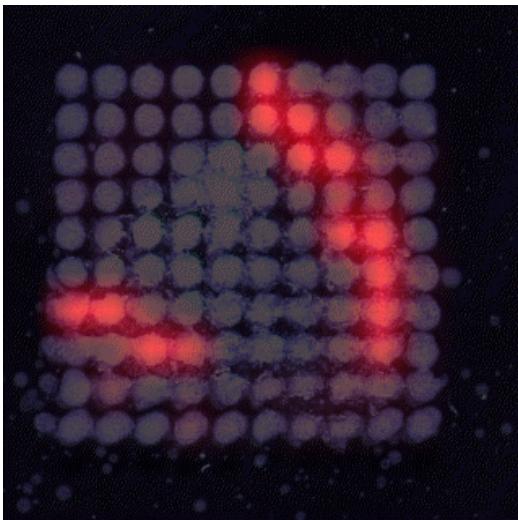


Figure 5-37 Imaging result display on primary image with matrix spots

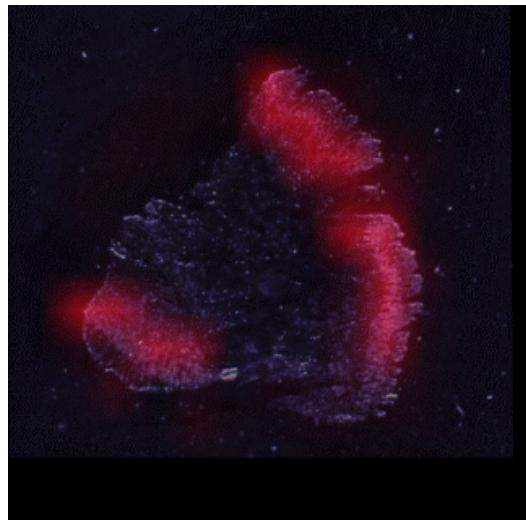


Figure 5-38 Imaging result display on co-registered image (taken before spotting)

5.2.7 Tissue Profiling

Tissue profiling (also known as histology-directed acquisition) is a workflow that does not acquire a full mass spectrometric image of the sample, but rather acquires profile spectra from several spots of interest selected by the user. Such a selection can be based on tissue morphology (as seen on the optical image or a stained serial section of the sample). For each selected spot, one mass spectrum will be acquired.

In setting up the tissue profiling workflow, select **Tissue Profiling** in the **New Imaging Run Wizard**. In this dialog, the spot diameter should be set correctly.

In the tissue profiling workflow, it is not possible to use the region tools (**Add Rectangular measurement Region** and **Add Polygon Measurement Region**) to define a measurement region in terms of a connected area. Instead it is necessary to define each spot using **Add Measurement Spot** () and set up measurement spot groups which then represent a measurement region.

Although it is possible to combine the Tissue Profiling workflow with a spray-coated sample (for example, from the imagePrep), it can often be faster to spot matrix only within the regions of interest. This can be done manually or with a robot. In this case, it is advisable to acquire an image of the tissue section before and after matrix spotting. While it is easier to define the measurement spots on an image taken after spotting, interpretation of the results can be much easier if the matrix-free image is co-registered in flexImaging.

In the following example the workflow is shown with a sagittal rat brain section with manually spotted matrix droplets.



Figure 5-39 Rat brain section with matrix spots

In this example, two measurement spot groups will be defined. In the tissue profiling workflow, spots belonging to the same measurement spot group do not have to be interconnected. Instead, they can be freely defined by the user. Typically, one measurement spot group will include multiple similar tissue features present in the sample, such as several scattered carcinoma *in situ* within one section. If different tissue types are each assigned to one measurement spot group, the statistical comparison of these tissue types can be performed in ClinProTools in a very straightforward manner.

In the rat brain section here, we want to define two measurement spot groups, the cerebrum and the cerebellum. To do this, we first define a second measurement region with a right mouse click in the Regions Pane:

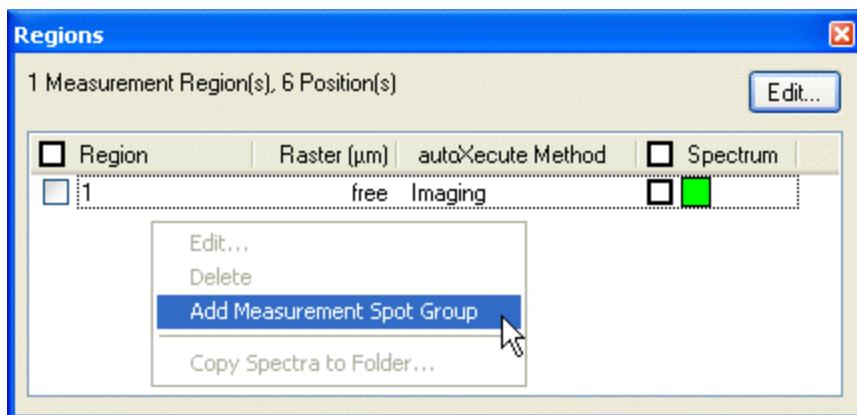


Figure 5-40 Defining a new measurement region

It is also helpful to give the regions a descriptive name, such as “cerebrum” and “cerebellum” in this example. A spot can be added to a measurement region by using **Add Measurement Spot** (). The spot will always be added to the currently active region.

Note: the spots are only visible if **Measurement Regions** () is also checked. As long as **Add Measurement Spot** is active, the spots will be shown in the color of their respective region.

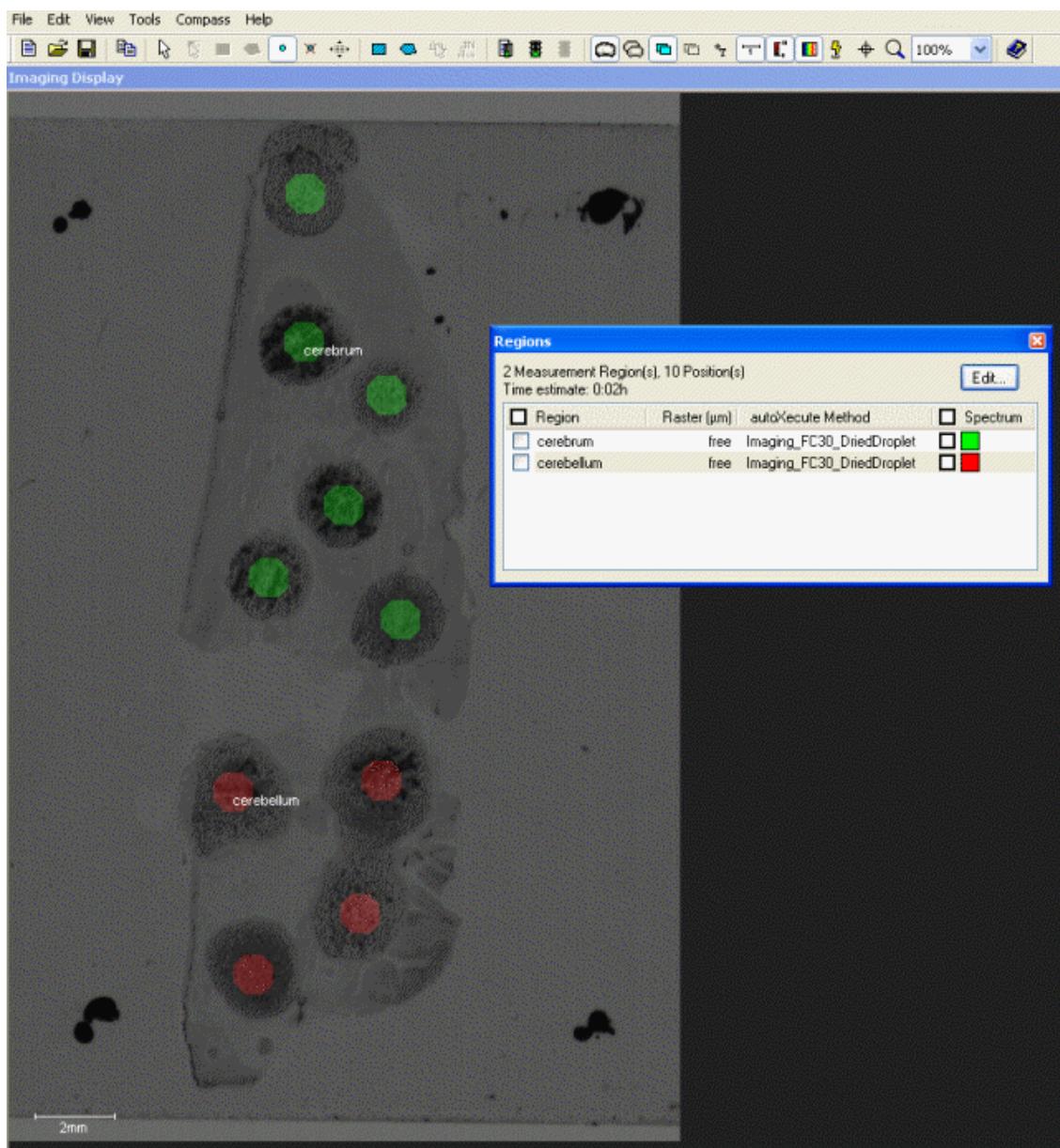


Figure 5-41 Definition of measurement spots in tissue profiling workflow

From here the acquisition can be started as usual. A few considerations apply to the data acquisition.

autoXecute settings (flex Series instruments)

Usually from dried droplet preparations a lot more laser shots can be summed up compared to spray preparations. It is therefore possible to increase the number of laser shots in the autoXecute method substantially. To level out local inhomogeneity in the dried droplet preparation, a laser movement should be selected which covers the entire size of the matrix spot. In flexControl 2.4 it is recommended to use the “hexagon” movement pattern, in flexControl 3.0 and higher it is recommended to select “random walk”.

ftmsControl settings (FTMS instruments)

Usually from dried droplet preparations a lot more laser shots can be summed up compared to spray preparations. It is, therefore, possible to increase the number of laser shots in the ftmsControl method substantially. To level out local inhomogeneity in the dried droplet preparation, a stage movement should be selected which covers the entire size of the matrix spot (adjust the smart walk parameters accordingly).

flexControl setting (rapifleX MALDI Tissuetyper)

Usually, a lot more laser shots can be summed up from dried droplet preparations compared to spray preparations. For the rapifleX MALDI Tissuetyper instrument and flexControl 4.0 we recommended activating the **Movement on Sample Spot** option on the **Sample Carrier** tab in flexControl and selecting **Smart – Complete Sample** and the limiting the diameter to the actual diameter of the matrix droplet in the respective dialogue box.

Advanced tissue profiling – spot list import

If a robot was used to spot the matrix droplets, the spot coordinates can be imported in flexImaging. After the imaging run was created in “tissue profiling” mode as usual, the spot list can be imported using **Import > Spot List**. In the **Spot List Import** dialog a representation of the spot distribution is shown, and a three point teaching is required to align the spots. See **Spot List Import Wizard** for details.

Advanced tissue profiling – spot list export

A tissue profiling setup may involve e.g. a third person, usually a pathologist, to define the location of the measurement spots on a histologically stained reference section and marking them in different colors according to their regions. It is possible to load the unspotted sample in the instrument and set up a new imaging run in tissue profiling mode in flexImaging. Next the image of the reference section with the defined spots can be co-registered. These spots can be marked as measurement spots as described above. Now the coordinates of the spots can be exported using **Export > Spot List**. The spot list and the samples can now be transferred to a robot to prepare the selected spots. Some manual adjustments of coordinate offsets are to be expected.

Result presentation

If the result is displayed with primary (spotted) optical image, the results may not appear as conclusive as they are (left figure). The image will look nicer in a presentation if the optical image of the unspotted sample is co-registered and used for the display of the data (right figure).

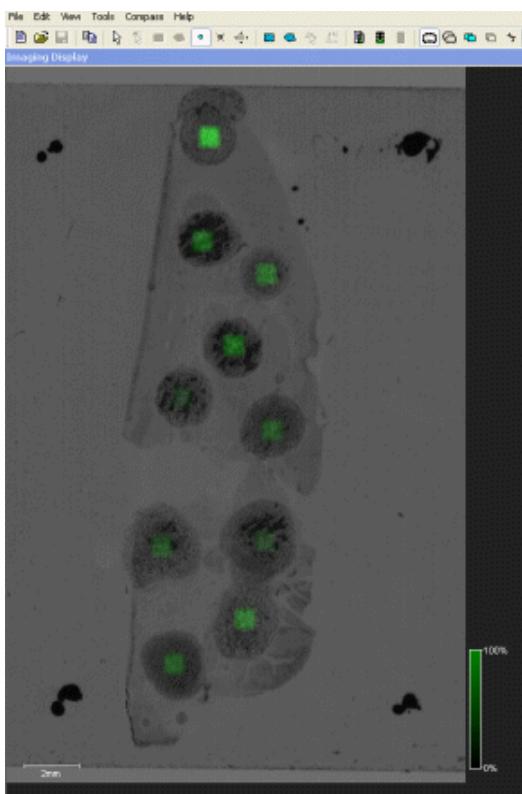


Figure 5-42 Result display of a selected mass filter with primary image

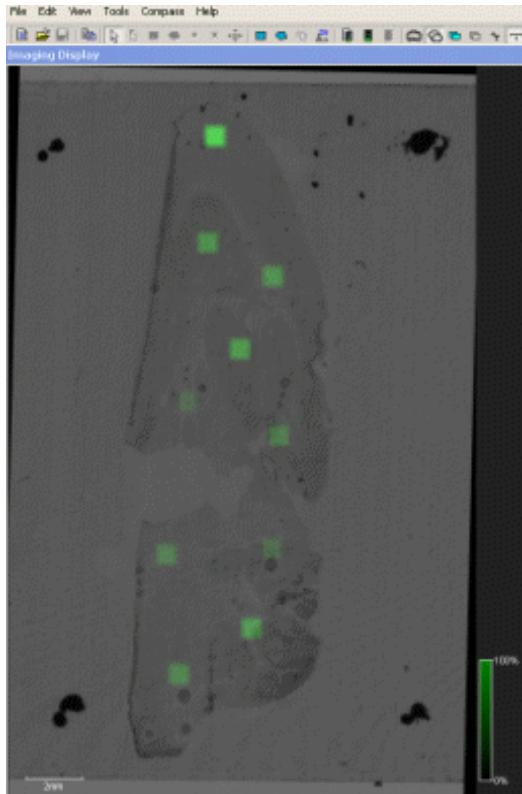


Figure 5-43 Result display of a selected mass filter on co-registered (unspotted) image

5.2.8 MS/MS Experiments

Note MS/MS experiments, including SRM, are not supported on the rapifleX MALDI Tissuetyper instrument in TOF configuration.

MS/MS experiments in MALDI imaging are usually done to image the distribution of small molecules such as drugs in tissues. Because in the low mass region of a MALDI spectrum a lot of interfering signals from the matrix and other small molecules are observed, the MS/MS experiments increase the selectivity by fragmenting a specific molecule, and then generating the image on a fragment ion of that molecule. In contrast to other imaging workflows, the mass to be measured has to be known in advance.

For each pharmaceutical substance, it is usually necessary to evaluate the right matrix, solvent and preparation conditions (see section 5.1.5).

Since flexImaging works with flexControl 2.4, 3.0 as well as with flexControl 3.3, three different use-cases can be considered concerning MS/MS measurement. They are explained in the topics mentioned below.

It is recommended to use a special LIFT method for small molecules. These methods can be set up on ultraflex/autoflex II and higher. If no such method (e.g. LIFT_small_mass.lft) is found on your instrument, please contact your local service.

If you want to measure in the SRM mode, but no such a method (e.g. SRM.srm) is found on your system, please contact your local service. If FAST measurements are possible on your instrument, a FAST method can be converted to a SRM method.

5.2.8.1 flexImaging and MS/MS Measurement — "With Parent"

This section applies, if a “normal” LIFT acquisition should be done in automatic mode. This consists of measuring the parent signal first, then switching into fragment mode. This method may work in some cases, but in others it will not. The reason is that it is often not possible to obtain a good signal for the precursor. If during the automatic measurement no good signal for the parent is detected, then no fragments will be measured. How to measure spectra directly in fragment mode is explained in the next section.

If the imaging sample should be measured with LIFT two differences in comparison with the common imaging MS measurement have to be considered, independent if flexControl 2.4, 3.0 or 3.3 is used:

- a LIFT parent mass has to be defined in flexImaging
- use a flexControl LIFT method for measurement

Follow the steps below to setup an Imaging LIFT run:

1. Set up a common Imaging run as for MS measurement.
2. Switch to flexControl and open the AutoXecute method Imaging_FC30_MSMS.axe (for flexControl 3.0 or for flexControl 3.3) or Imaging_FC24_MSMS.axe (for flexControl 2.4).
3. In the General tab of the AutoXecute Method Editor load the flexControl LIFT method of choice.

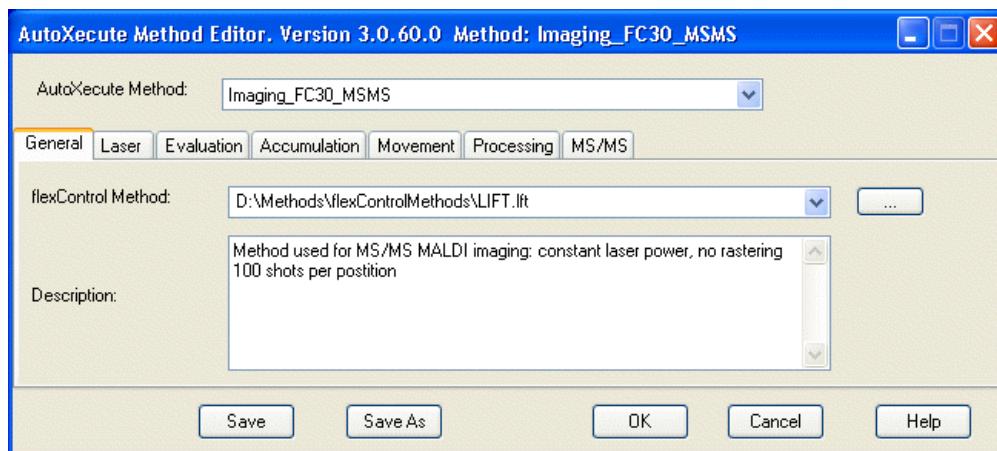


Figure 5-44 AutoXecute Method Editor: Selection of a flexControl LIFT method

4. In the MS/MS tab deactivate the check box “Measure fragments only” (not in flexControl 2.4).

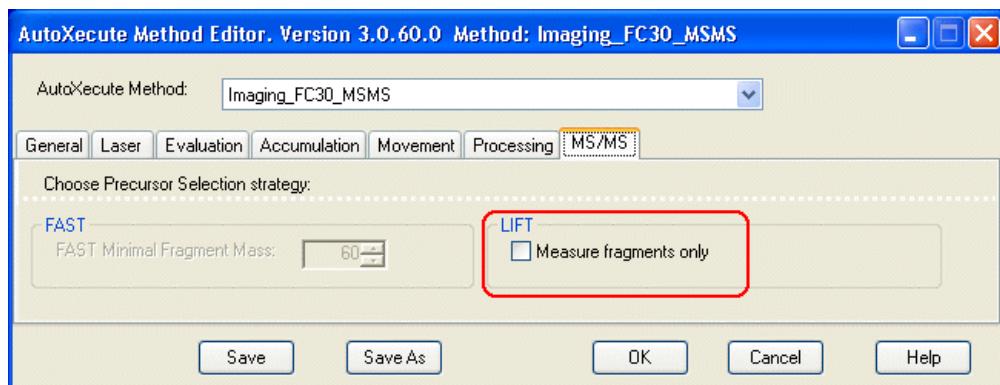


Figure 5-45 AutoXecute Method Editor: Deactivated "Measure fragments only"

5. Save the method with a different name and close the AutoXecute Method Editor.
6. Switch back to flexImaging and select the new autoXecute method for all measurement regions.
7. Open the **Sequence Properties - Current Sequence Parameters** dialog and enter the parent mass you want to measure.

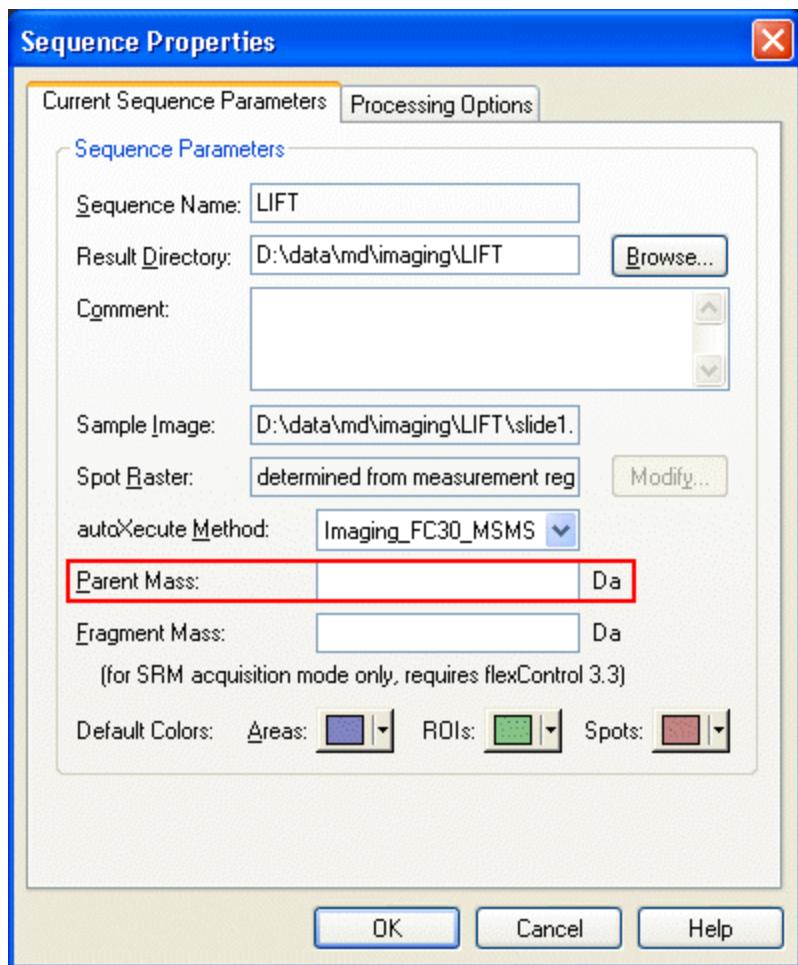


Figure 5-46 Sequence Properties - Current Sequence Parameters dialog for entering the parent mass

8. Start the run.

5.2.8.2 flexImaging and MS/MS Measurement — "Fragments Only"

Sometimes it is needed to omit parent measurement and start the Imaging MS/MS directly in the fragments mode. This is necessary if no good signal for the parent can be obtained. Since this happens often in these kinds of experiments, it is the recommended way to proceed.

“Fragments only” with flexControl 3.x

To start with measurement of fragments using flexControl 3.0 or flexControl 3.3 follow the steps below:

1. Prepare the Imaging run in the same way as MS measurement.
2. Switch to flexControl and open the AutoXecute method Imaging_FC30_MSMS.axe.
3. In the General tab of the AutoXecute Method Editor load your good working flexControl LIFT method.

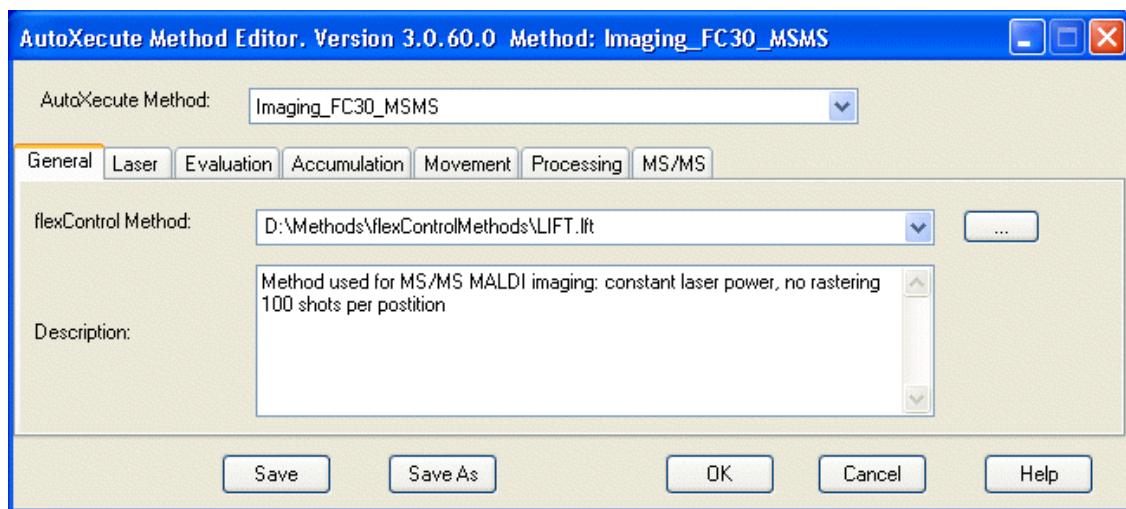


Figure 5-47 AutoXecute Method Editor: Selecting a flexControl LIFT method

4. In the MS/MS tab activate the check box “Measure fragments only”.

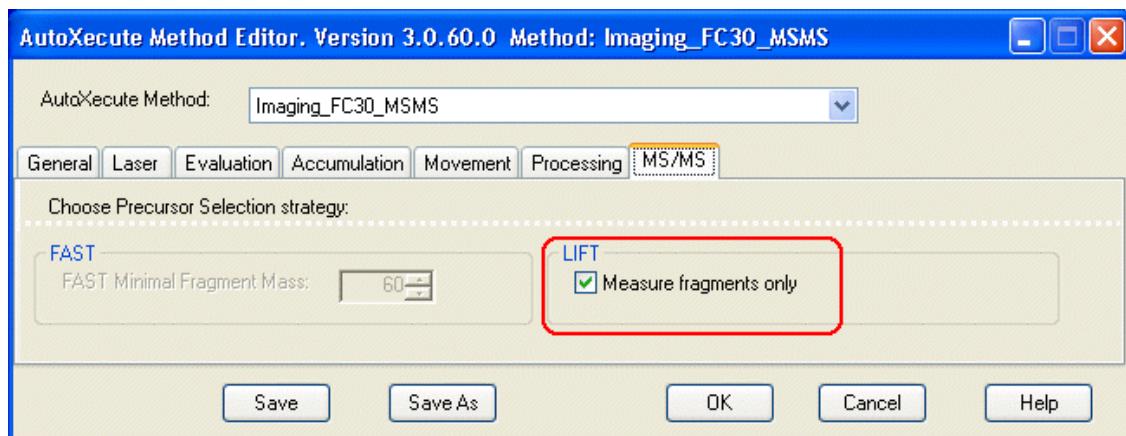


Figure 5-48 AutoXecute Method Editor: Activated "Measure fragments only"

5. Save the method with a different name and close the editor.
6. Switch back to flexImaging and select the new autoXecute method for all measurement regions.
7. Open the **Sequence Properties - Current Sequence Parameters** dialog and enter the parent mass you want to measure with LIFT.

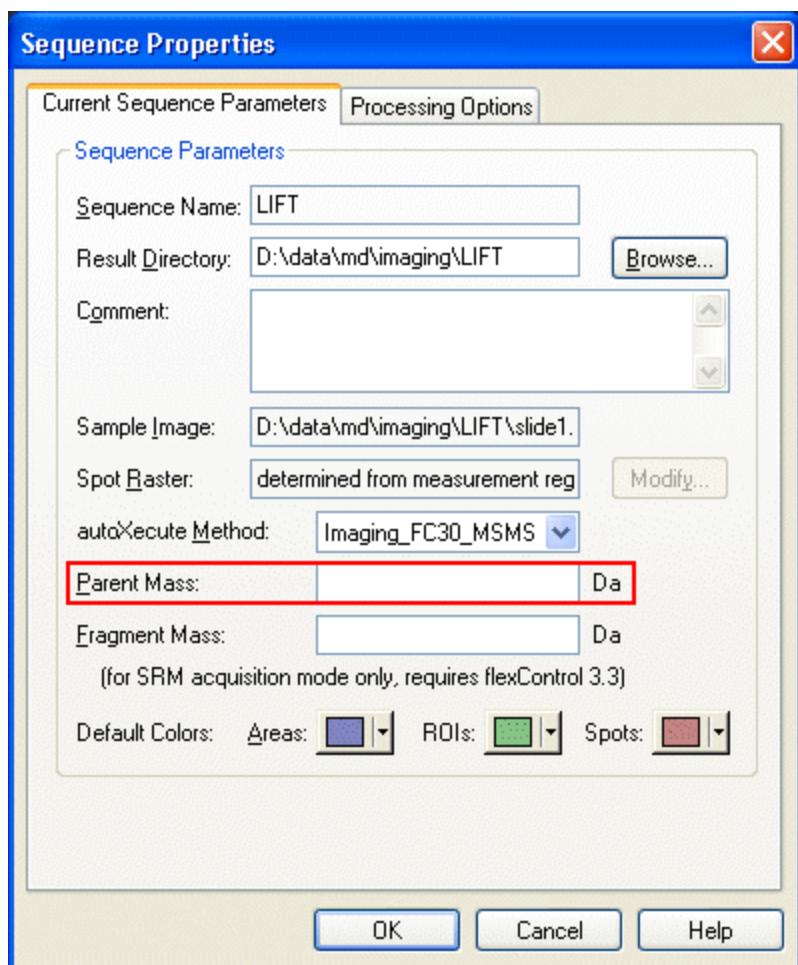


Figure 5-49 Sequence Properties - Current Sequence Parameters dialog for entering the parent mass

8. Start the run.

Note In this workflow the one point recalibration on the parent mass upon switching from parent to fragment mode is omitted, so the measured mass may show a systematic deviation. This can be either compensated by measuring the pure substance under same circumstances, to find the apparent masses of the expected fragments, or by creating a new calibration entry in the LIFT method with the parent and fragments of the compound in question and calibrating the LIFT method. In this case it is recommended to save the LIFT method under a new name first and then creating one special LIFT method for each compound to be measured.

“Fragments only” with flexControl 2.4

To start with measurement of fragments using flexControl 2.4 a few more steps are necessary. It is recommended to prepare the pure substance next to the tissue and apply some matrix on it.

1. Prepare the Imaging run as known for common MS measurement; especially choose a MS AutoXecute method with an empty flexControl method entry.
2. Do not enter a parent mass in the **Sequence Properties - Current Sequence Parameters** dialog, the field has to remain empty!!!
3. Switch to flexControl.
4. Open the AutoXecute method used for the measurement and make sure that NO flexControl method is loaded in the General tab.
5. Load the flexControl LIFT method of choice in flexControl (not in the autoXecute method!):
 - a. Switch to the LIFT tab
 - b. Type in the parent mass you want to measure with Imaging
 - c. Acquire the parent spectrum, either from the tissue itself or from the preparation with the pure substance next to the tissue
 - d. Switch into the Fragments mode.
6. Switch back to flexImaging (without any further changes in flexControl) and start the run.

5.2.8.3 flexImaging and MS/MS Measurement — "SRM"

During the automatic SRM acquisition, only the defined fragment is acquired.

If the imaging sample should be measured with SRM three differences in comparison with the common imaging MS measurement have to be considered:

- a parent mass has to be defined in flexImaging
- a fragment mass has to be defined in flexImaging
- use a flexControl SRM method for measurement

Follow the steps below to setup an Imaging SRM run:

1. Set up a common Imaging run as for MS measurement.
2. Switch to flexControl and open the AutoXecute method Imaging_FC3.3_SRM_axe.
3. In the General tab of the AutoXecute Method Editor load your good working flexControl SRM method.

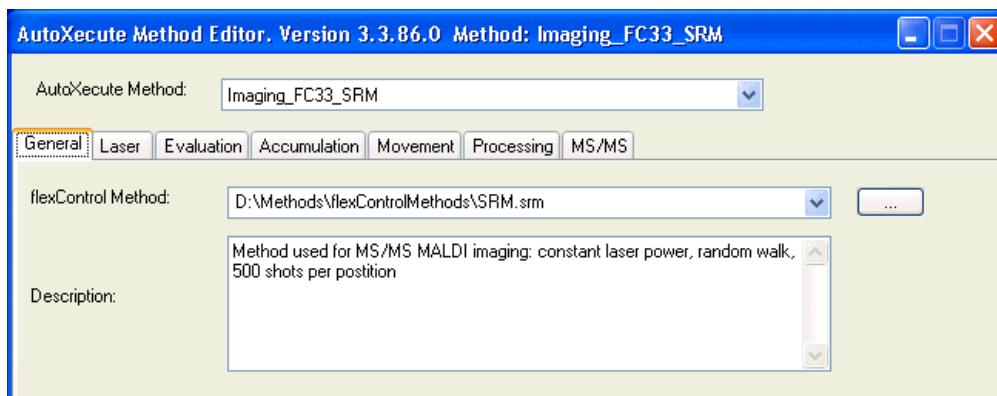


Figure 5-50 AutoXecute Method Editor: Selection of a flexControl SRM method

4. Save the method with a different name and close the AutoXecute Method Editor.
5. Switch back to flexImaging and select the new autoXecute method for all measurement regions.
6. Open the Sequence Properties - Current Sequence Parameters dialog and enter the parent mass and the fragment mass you want to measure.

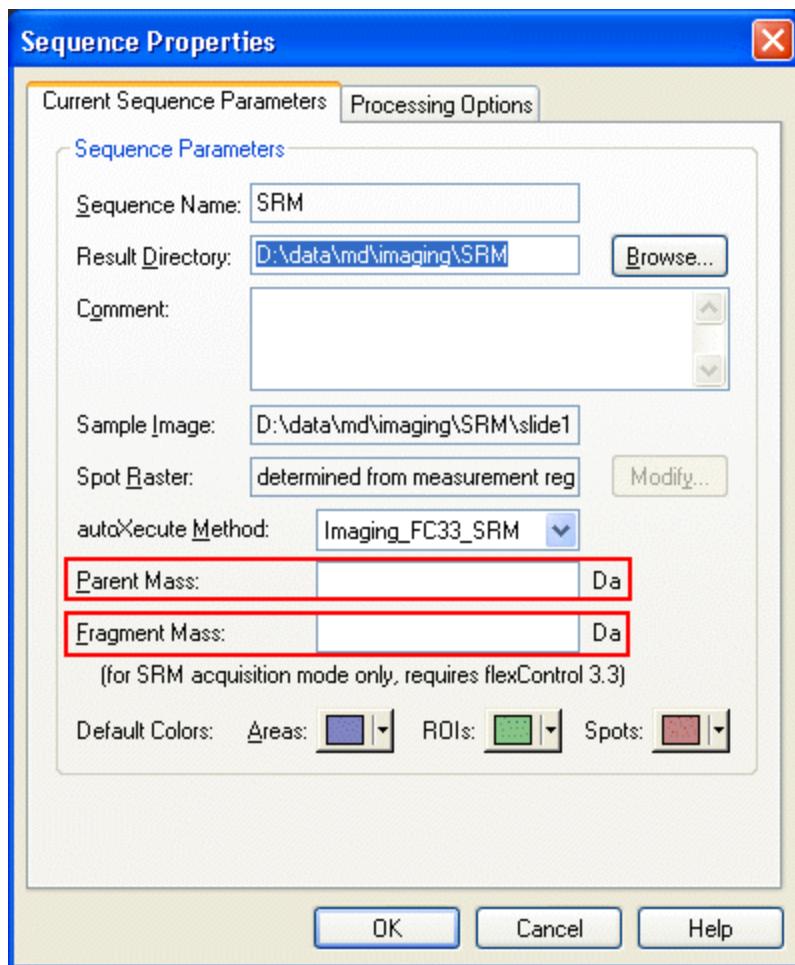


Figure 5-51 Sequence Properties - Current Sequence Parameters dialog for entering the parent mass and the fragment mass

7. Start the run.

5.3 Data Acquisition Using HyStar and ftmsControl

The FTMS imaging experiment is performed using FlexImaging, HyStar and ftmsControl. Similar to TOF instruments, the FTMS imaging experiment is setup as a series of measurements at defined x-y-positions of the sample. FlexImaging is used to create a new imaging run and to define the measurement area. The autoXecute imaging run is then sent to HyStar where the mass spectrometric method is added. HyStar then triggers ftmsControl to measure the spectra.

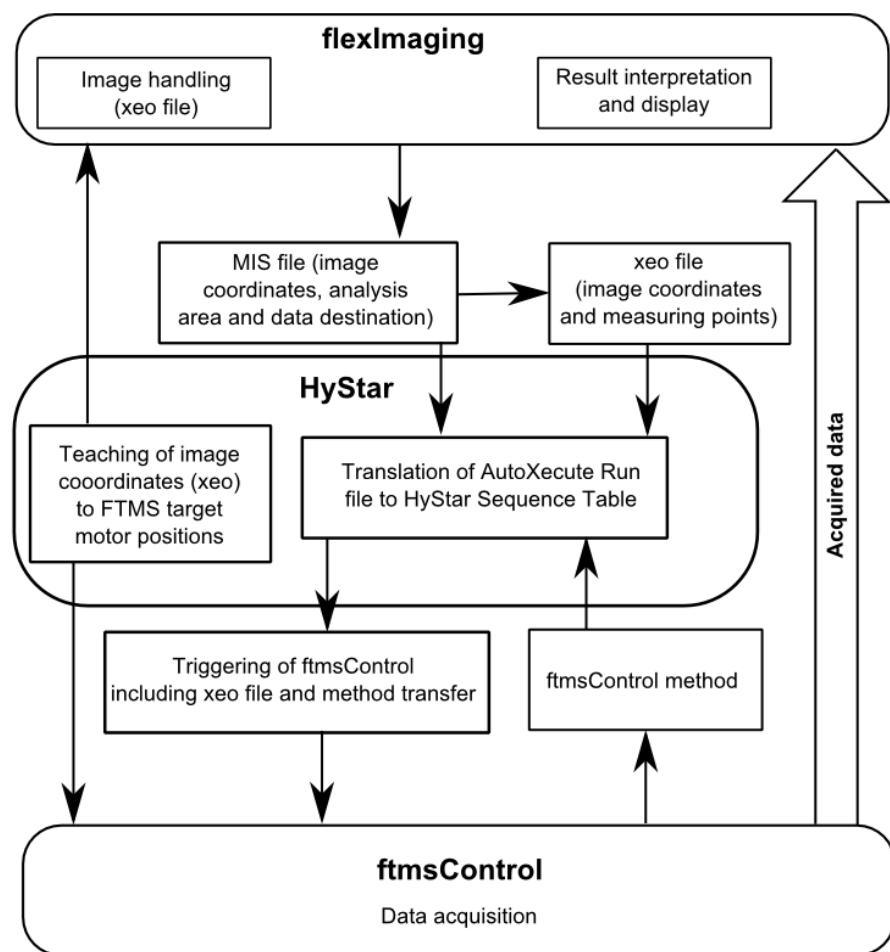


Figure 5-52 FTMS workflow in an automatic acquisition of a MALDI image

Starting the automatic imaging run by clicking **Start autoXecute Run** () triggers the following series of events:

- flexImaging creates a geometry for the sample. This geometry contains the information on which points of the sample the spectra will be acquired.
- flexImaging creates an autoXecute run. This run contains the geometry and defines the order of the measurements. It also contains the autoXecute method (as defined in the Regions Pane in flexImaging).
- The autoXecute method is then sent to HyStar, where it is combined with the ftmsControl method. Additionally, method parameters can be fine-tuned, such as the laser power, the number of laser shots and the number of scans summed per pixel.
- HyStar then triggers ftmsControl to measure the spectra according to the autoXecute method.
- After the automatic measurement is finished flexImaging reads either the raw spectra, the processed spectra or the peak lists, depending on the settings in the imaging run properties.

For FTMS data it is recommended not to use the baseline subtraction option in the flexAnalysis method and to read in the raw spectra (see data processing). This will save time when loading spectra into flexAnalysis.

General steps in performing an FTMS imaging run

1. Setup data acquisition method in ftmsControl.
2. Open the HyStar MALDI automation module.
3. Create a new imaging run in flexImaging.
4. Teach new sample image to motor positions using ftmsControl (via HyStar/flexImaging).
5. Define measurement regions in flexImaging.
6. Send autoXecute method to HyStar.
7. Add ftmsControl method to autoXecute method.
8. Start automatic run from HyStar.

Setting up the ftmsControl method

The general ftmsControl MALDI method needs to be fine-tuned for the specific tissue sample to be imaged. To do so, first load the sample into the source. Using the displayed geometry file, e.g. MTP 384 ground steel, move the sample carrier to a spot on the tissue. In Tune mode, adjust the laser power to optimize signal intensity for several spots on the tissue. Additional parameters such as mass range, Q1 mass and data set size are adjusted now. Once the acquisition parameters are optimized, the entire data acquisition method is saved.

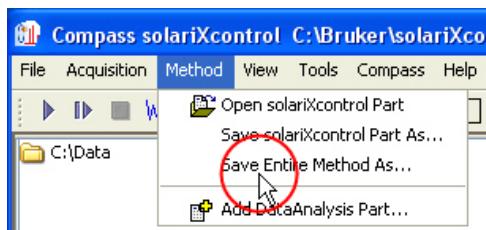


Figure 5-53 Selecting saving entire method

HyStar MALDI automation module

Communication between flexImaging and ftmsControl is controlled via HyStar. For imaging experiments, the “MALDI Automation” Acquisition module is required.

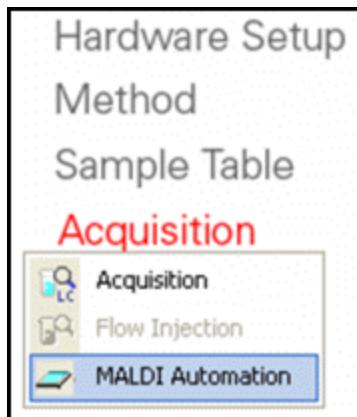


Figure 5-54 Selecting MALDI Automation

The MALDI Automation module is necessary for all communication between flexImaging and ftmsControl. Specifically, it is responsible for communications during teaching of the sample image and for running the autoXecute imaging run. HyStar does not have an interactive role during teaching, but is required to be open when doing so.

The MALDI Automation module also combines the autoXecute imaging run with the ftmsControl method. After choosing the MALDI Automation module select the "Imaging workflow".



Figure 5-55 Selecting MALDI Imaging

The autoXecute imaging run is loaded automatically from flexImaging when the automatic imaging run is started in flexImaging. To include the ftmsControl method, use the browse button in the **Method Name** column to select the correct method.



Figure 5-56 Method Name column

Typically, it is not necessary, but if required, several of the MALDI method parameters are editable within HyStar. By default, the parameter columns are labeled with a "d" to indicate that the value defined within the method will be used. If the user chooses, new values can be added that will overwrite the values defined in the method.

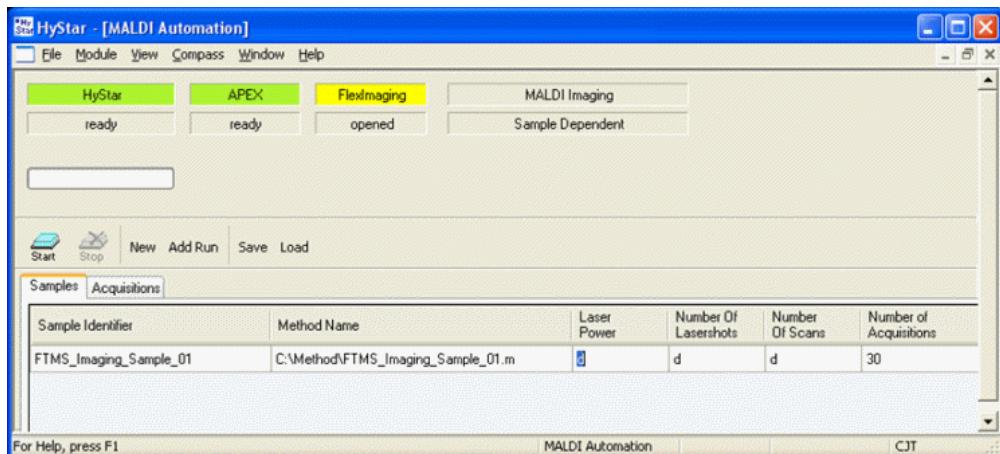


Figure 5-57 HyStar Samples tab

Once the ftmsControl method is loaded, it is necessary to “click” out of the field. The Start button is then active. The start button will begin a multi-fid acquisition in ftmsControl. Within a single LC-type run, each position (sample) defined in the autoXecute imaging run is run and written to an individual file. In HyStar, all of the positions are given as individual entries in the Acquisitions Sample Table.

Acquisitions							
Position	Sample Identifier	Status	Method Name	LaserPower	Number Of Lasershots	Number Of Scans	Data Path
R00\028\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\029\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\030\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\031\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\032\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\033\016	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\028\017	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\029\017	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\030\017	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\031\017	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...
R00\032\017	FTMS_Imaging_Sample_01	ready	C:\Method\FT...	d	d	d	C:\Data\Imaging\FTMS_Imaging_Sample...

Figure 5-58 HyStar Acquisitions tab

Teaching

For the FTMS experiments, teaching is done following the same procedure as the TOF experiment. The only difference being that ftmsControl (with HyStar in the background) is used rather than flexControl.

5.4 Data Processing and Interpretation

5.4.1 Histological Staining and Co-Registration of Microscopic Images	312
5.4.2 Data Processing in flexAnalysis and flexImaging	315
5.4.3 Normalization	319
5.4.4 How to Find Biomarkers	330
5.4.5 Creating High-Quality Images	335
5.4.6 Interfacing with ClinProTools	343
5.4.7 Hierarchical Clustering	370
5.4.8 Exporting and Importing Data for Statistical Analysis (Advanced Users Only)	373

5.4.1 Histological Staining and Co-Registration of Microscopic Images

Preparation of a histologically stained section

The analysis of MALDI imaging data from a tissue sample will often require the comparison of the MALDI data with a histologically stained sample. Many staining protocols, including the common hematoxylin & eosin (H&E) staining, are compatible with MALDI imaging when performed on the same section after the MS data acquisition. The remaining matrix is washed off the sample using ethanol, acetone, or another suitable solvent and then a staining protocol, such as an H&E staining, is performed. The stained section can be easily co-registered with the MALDI data in flexImaging and will allow the precise interpretation of the results. (This technique has been described in: Crecelius AC, Cornett DS, Caprioli RM, Williams B, Dawant BM, Bodenheimer B.; *J Am Soc Mass Spectrom.* 2005 Jul;16(7):1093-9; and Schwamborn K, Krieg RC, Reska M, Jakse G, Knuechel R, Wellmann A.; *Int J Mol Med.* 2007 Aug;20(2):155-9.)

The choice of the staining protocol seems to be uncritical but some changes may be observed. For H&E staining it will usually be necessary to increase the time for hematoxylin and to decrease the time for eosin.

If the tissue is not preserved sufficiently during the measurement or floats off the slide during the staining, consider a polylysine treatment of the slides prior to the sample preparation (see section 5.1.1).

A stained serial section can also be used, but this has the disadvantage that it is often not possible to achieve a perfect alignment between the stained and the MALDI image. Especially small features at higher resolutions can often not be aligned unambiguously.

A third possibility is the usage of MALDI imaging compatible stains to stain the image prior to the measurement. This has the disadvantage that it subjects the sample to additional handling prior to the measurement and that the MALDI compatible stains do not yield the same depth of information as e.g. a H&E staining. (For a detailed description of MALDI imaging compatible stains see: *Chaurand P, Schwartz SA, Billheimer D, Xu BJ, Crecelius A, Caprioli RM, Anal. Chem. (2004), 76(4): 1145-1155.*)

Co-registration of microscopic images

FlexImaging supports different types of images for the co-registration:

Bitmap Images: Bitmap images will be loaded into the memory of the computer as a whole. Since the MALDI data and the image have to share the same memory, this limits the useful resolution of the bitmap image. However: flexImaging will keep the resolution of the image for the display. While (depending on the size of the specimen) it will not be possible to use a full microscopic resolution, it will still allow a detailed localization of histological features.

Virtual slides: Virtual slides are files that are generated by dedicated microscopic slide scanners. These slides contain the image data in different pre-processed resolutions. This allows flexImaging to load only the part of the image that is needed into the memory. Therefore the full microscopic resolution is available for large specimens, which allows a detailed evaluation of the histology.

Supported virtual slide formats:

flexImaging 5.0 supports the following virtual slide formats:

- Zeiss MIRAX (*.mrxs)
- 3D Histech Pannoramic (*.mrxs)
- Aperio *.svs files with jpg compression
- Additional formats may be included later with software patches. Please contact the software support (maldi.sw.support@bdal.de) for information.

Co-register the image of the stained tissue section

In the **Edit > Co-Register Image** dialog, browse to the MIRAX/Pannoramic image and select the according *.mrxs file. Co-register the image by following the instruction in the dialog window by defining three pairs of matching features on the primary image and the image to be co-registered.

Using MIRAX or Pannoramic slides (*.mrxs files) for co-registered images

We recommended installing the version of the MIRAX/Pannoramic viewer that was used during testing of flexImaging software on the computer that runs flexImaging. Installing this version of the viewer will ensure compatibility with MIRAX and Pannoramic scanners. For details, refer to the flexImaging release notes or contact MALDI software support.

Since the direction in which the slides are scanned is different in the slide scanner and in the flex instrument, typically there is a 90° rotation between the virtual slide image and the primary image in flexImaging. This does not matter for the co-registration. After the co-registration flexImaging will rotate the virtual slide correctly.

There may be slight shifts in the positions on the virtual slide image if several non-connected regions on the slide are scanned (e.g. the absolute positions of the teach marks may have small offsets compared to the tissue). In such a case it is best to use actual features at the edge of the tissue piece for co-registration.

After co-registration of a *.mrxs image is complete, flexImaging asks if the image should be copied into the flexImaging imaging run directory. This ensures that the entire data set including the co-registered image can later be distributed conveniently. The *.mrxs file and the entire directory with the image data will be copied.

The entire virtual slide data of a Zeiss/Pannoramic scan consists of the *.mrxs preview image and a folder with the same name that contains the raw data.

Using Aperio *.svs files with jpeg compression

Only *.svs files with jpeg compression can be used in flexImaging. If the data is saved differently or with a different compression, open the slide in the Aperio software and save it as jpeg compressed *.svs file.

There may be slight shifts in the positions on the virtual slide image if several non-connected regions on the slide are scanned (e.g. the absolute positions of the teach-marks may have small offsets compared to the tissue). In such a case it is best to use actual features at the edge of the tissue piece for co-registration.

After co-registration of a *.svs image is complete, flexImaging asks if the image should be copied into the flexImaging imaging run directory. This ensures that the entire data set including the co-registered image can later be distributed conveniently.

Managing multiple co-registered images

Sometimes it may be useful to co-register different images, e.g. an H&E and an immunostain. If an optical image gets co-registered in a data set that already contains a co-registered image, the previous co-registration is kept. The **Co-registered images** entry in the **View** menu opens a list with the type and names of the co-registered images. The currently displayed co-registered image can be selected here.

5.4.2 Data Processing in flexAnalysis and flexImaging

For the data processing there are two things to consider, the optional processing of acquired spectra in flexAnalysis (which includes smoothing and baseline subtraction) and the processing in flexImaging (which includes the data reduction and optional normalization).

If in the **Sequence Properties- Processing Options** dialog the **Read Raw Spectra** option is defined, then flexImaging can optionally be prompted to perform an internal baseline subtraction. If the **Read Processed Spectra** option is defined, then flexImaging will read the spectra as they are processed in flexAnalysis. For imaging data acquired in reflector mode, it is usually sufficient to read the raw spectra, but performing a smoothing and baseline subtraction in flexAnalysis is usually recommended when acquiring linear mode spectra.

Data processing in flexAnalysis

This spectrum shows a typical mass spectrum from an imaging run before and after processing in flexAnalysis. It is normal to have a high noise level towards the low mass end of the spectrum and a rapidly declining baseline. The initial steep slope in the baseline comes from the matrix suppression in flexControl, which was set to maximum gating in this example: The rise in the baseline in the low mass area correlates with the detector being switched on. It is obvious that the data processing in flexAnalysis (smoothing and baseline subtraction) enables a better analysis of the imaging data.

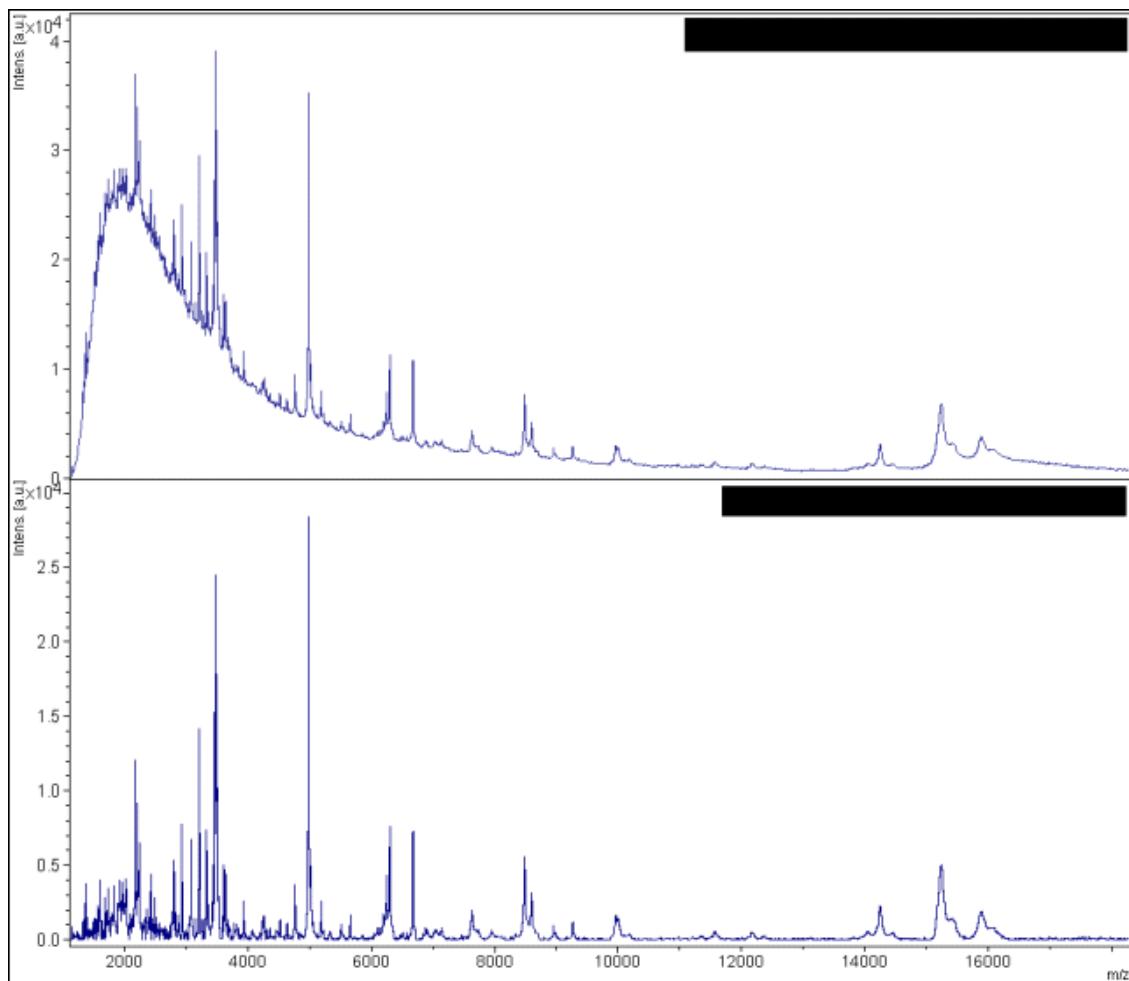


Figure 5-59 Spectrum from imaging run before and after processing in flexAnalysis

flexAnalysis method for smoothing and baseline subtraction

With the installation of flexImaging 2.0 some flexAnalysis methods for processing imaging data will be installed. The method "Imaging_FA24_proteins.FAMSMMethod" is for use with flexAnalysis 2.4. It uses a convex hull baseline subtraction. The method "Imaging_FA30_proteins.FAMSMMethod" is for use with flexAnalysis 3.0. The method "Imaging_FA33_Proteins.FAMSMMethod" is installed for flexAnalysis 3.4 and 3.4. Here a top hat baseline subtraction is used. In both cases a "Gaussian" smoothing is done.

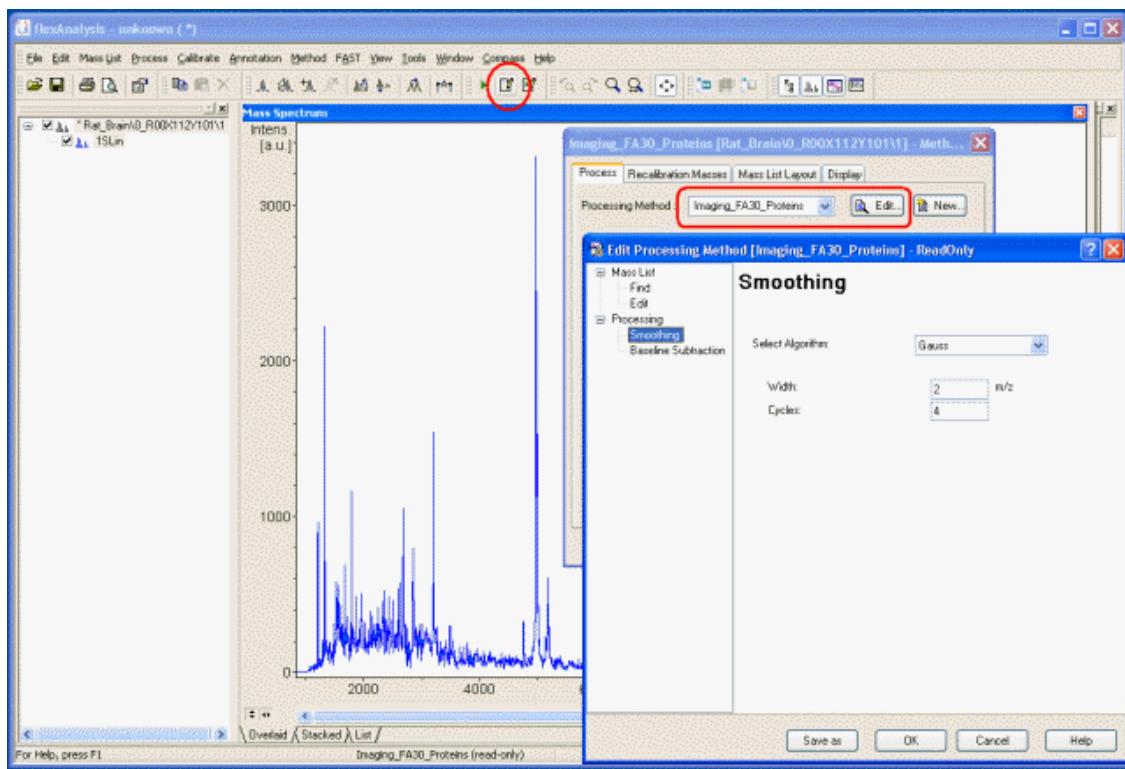


Figure 5-60 Parameters of "Imaging_FA30_proteins.FAMSMMethod"

These methods will work with the typical mass range for protein imaging (e.g. 2 to 20 kDa). If e.g. peptides in reflector mode are measured it will be necessary to adjust the smoothing parameters in these methods. In such cases a smaller smoothing width is recommended. Also the "**Savitzky-Golay**" smoothing may perform better with isotopically resolved spectra.

The order of the processing should be first smoothing then baseline subtraction. These actions are defined in the scripting part of the method. A detailed tutorial on flexAnalysis scripting can be found in the flexAnalysis documentation. In most cases, however, it will be sufficient to change the example methods according to specific needs.

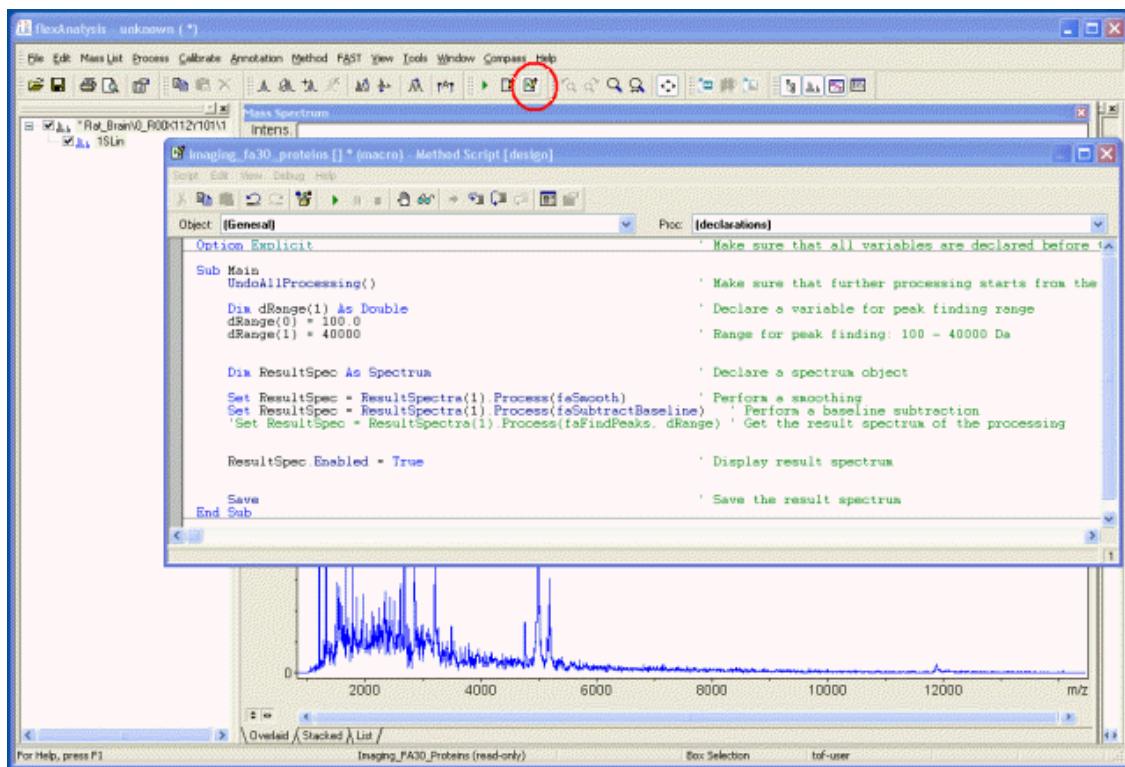


Figure 5-61 Script of "Imaging_FA30_proteins.FAMSMethod"

Processing in flexImaging

If flexImaging is set to **Read Raw Spectra** in the **Sequence Properties - Processing Options** dialog it will perform a predefined baseline subtraction. If flexImaging is set to **Read Processed Spectra** it will just read the processed data. (Note: if the spectra have not been processed in flexAnalysis, this option will lead to loading the raw data without any smoothing or baseline subtraction).

flexImaging usually loads the entire mass range, unless specified otherwise in the imaging run properties and reduces the spectra to the specified number of data points. The number of data points required should be tried out.

5.4.3 Normalization

The aim of normalization is to remove variations in pixel-to-pixel intensity (or brightness). These variations can be caused by uneven matrix deposition (more matrix leads to higher spectrum intensity), ion suppression due to different salt or lipid content in certain areas, or other factors that change the intensity of peaks in the mass spectra. Depending on the number of random effects in the data set, normalization can lead to significant improvement of the data. In some cases, normalization may be a prerequisite for viewing all histological information.

Note Each normalization method is based on certain assumptions about the data (see discussion below). In real data sets, only some — or in some cases none — of these assumptions may be true. In such cases, normalization can produce artifacts that can be mistaken for biological information. It is therefore important to apply normalization with care.

Quantitative comparison of data sets using different normalization methods is not feasible. For this reason, flexImaging displays a red letter indicating the normalization method used for a data set in the upper-right corner of the Imaging Display. This indicator can be hidden by clearing the **View > View Normalization Symbol** option.

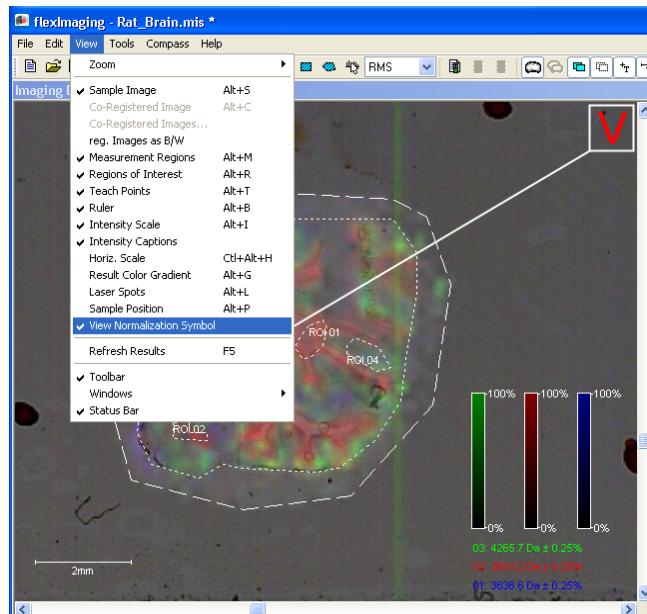


Figure 5-62 Selecting **View Normalization Symbol** displays the normalization indicator in the Imaging Display

flexImaging offers four normalization methods: **RMS** (vector norm), **TIC** (mean), **Median** and **Mass Window**.

Note The presence and nature of noise are important factors in normalization methods. For this reason, normalization is qualitatively different for MALDI-TOF data sets (where chemical noise is present in the spectra) and for FTMS (where no chemical noise is present in the spectra and electronic noise is removed by data reduction).

For a detailed discussion of normalization in MALDI-TOF data sets, refer to *Deininger SO, Cornett DS, Paape R, Becker M, Pineau C, Rauser S, Walch A, Wolski E. Normalization in MALDI-TOF imaging data sets of proteins: practical considerations. Anal Bioanal Chem. 2011 Apr 12.*

How normalization works

A mass spectrum can be considered to be a vector of intensity values:

$$\overrightarrow{\text{spectrum}} = (y_1, y_2, \dots, y_n)$$

For the normalization, each mass spectrum in the data set is divided by its individual normalization factor f :

$$\overrightarrow{\text{spectrum}_{\text{normalized}}} = \frac{1}{f} \overrightarrow{\text{spectrum}}$$

Normalizing a data set

To normalize a data set (or to switch normalization off), choose a suitable normalization option from the normalization drop-down list in the tool-bar or select the desired **Spectra Normalization** option in the **Edit > Sequence Properties** dialog.

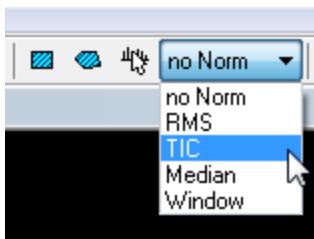


Figure 5-63 Normalization options in the flexImaging toolbar

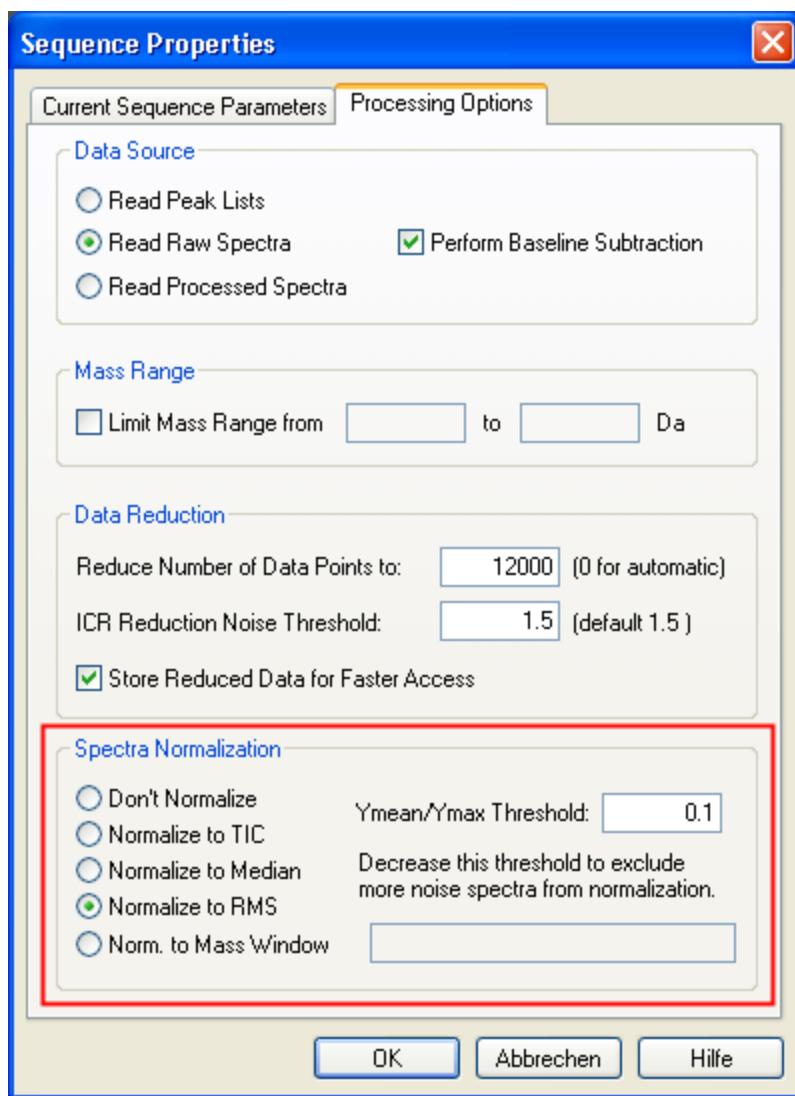


Figure 5-64 Normalization options in the Sequence Properties dialog

5.4.3.1 Overview and Applicability of Normalization Options

Option	Imaging Display indicator	Applicable to MALDI-TOF	Applicable to MALDI-FTMS
RMS (vector norm)	V	Yes	Yes
TIC (mean)	T	Yes	No
Median	M	Yes	No
Mass Window exclusion	W	Yes	No
Mass Window inclusion	W	Yes	Yes

RMS (vector norm)

The RMS normalization method divides all spectra by the root mean square of all data points and works well if all spectra in the data set are expected to have similar peak intensities:

$$f = \sqrt{\frac{y_1^2 + y_2^2 + \dots + y_n^2}{n}}$$

The RMS normalization method is applicable to MALDI-TOF and MALDI-FTMS data sets but may be influenced to some extent by chemical noise in the spectrum. Artifacts may be created if peaks with unusually high intensity are present in some regions (MALDI-TOF) or significant differences in signal intensities exist in different regions (MALDI-FTMS).

The RMS normalization method usually leads to a very uniform distribution of intense signals.

The RMS method was the only normalization method available in earlier versions of flexImaging. Because it is more likely to produce artifacts than the TIC normalization method, flexImaging automatically displays a warning and changes the normalization method to TIC if archived data sets are opened.

TIC (mean)

The TIC normalization method divides all spectra by the mean of all data points.

$$f = \frac{y_1 + y_2 + \dots + y_n}{n}$$

The TIC normalization method is applicable to MALDI-TOF data sets. It is assumed that all spectra have a similar area. The area of a MALDI-TOF spectrum is defined to a large extent by the chemical noise and only to a small extent by the peak intensities. This means that the TIC normalization method is not affected by moderate peak intensity variations in the data set. Artifacts may be created if peaks with unusually large areas are present in some regions, or spectra do not contain all spectral noise.

Median

This normalization method divides all spectra in the data set by the median of all data points.

$$f = median(y_1, y_2, \dots, y_n)$$

The median normalization method is applicable to MALDI-TOF data sets and is more robust than the RMS and TIC normalization methods. Artifacts may be created if spectra do not contain all spectral noise.

A normal MALDI-TOF spectrum contains more baseline than signals, and in such cases, the median is a good measure for the baseline or the average noise. Median normalization is almost entirely unaffected by the intensity or area of signals in the spectra, and can therefore be used if the RMS or TIC normalization methods lead to artifacts. However, if the TIC or RMS methods are applicable, they will usually provide better results than the median normalization method.

The median normalization method relies strongly on the presence of noise in the spectra. If spectra do not contain the full symmetrical noise profile (for example, because the baseline was cut off using the detector offset setting or the electronic gain was too low during the acquisition), using the median normalization method will generate significant artifacts. For this reason, the median normalization method is not applicable to FTMS data.

Mass Window exclusion

The mass window exclusion normalization method is a TIC normalization of a selected portion of the spectra and is applicable to MALDI-TOF data sets. Peaks that would otherwise generate artifacts using the TIC normalization method are excluded from the normalization.

Peaks are excluded from the normalization by defining their mass range using the distance cursor in the Spectrum Display.

►► To exclude a peak from the normalization

1. Zoom in on the peak to be excluded.
2. Right-click in the Spectrum Display and select **Mass Window / Distance** from the shortcut menu.
3. Using the mouse pointer, move the vertical lines to the boundaries of the peak to be excluded.

Click to switch the mouse pointer between the boundary lines.

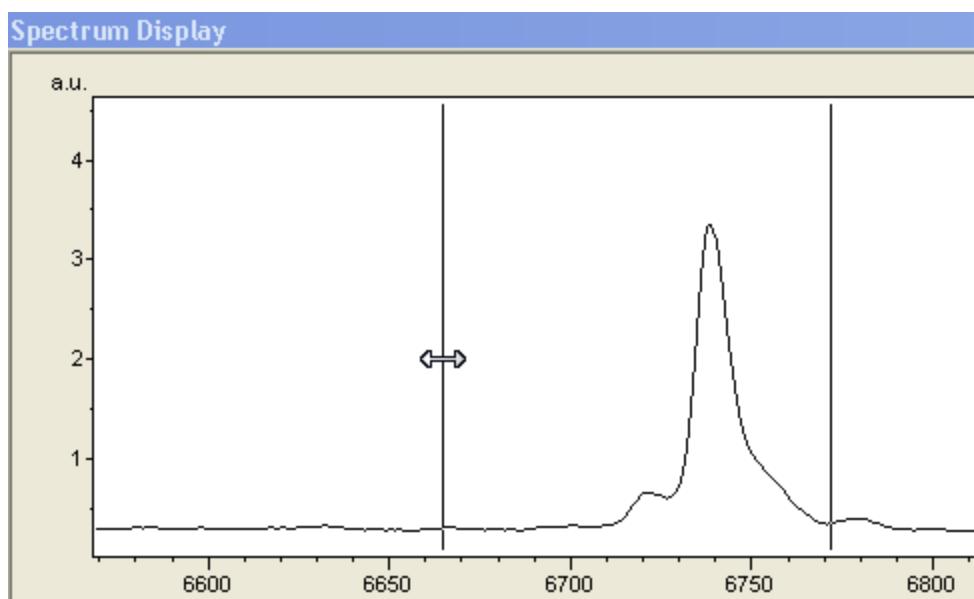


Figure 5-65 Setting the boundaries of the mass exclusion window

4. When the boundaries have been set, right-click to open the **Add Normalization Mass Window** dialog.



Figure 5-66 Add Normalization Mass Window dialog

5. Select **Exclude window from normalization**.

Additional exclusion windows can be created by defining a mass window, selecting **Exclude Window from Normalization**, and selecting **Add to List of Windows**.

The boundaries of exclusion windows are indicated by dashed blue lines in the Spectrum Display.

6. Select the **Window** normalization method by either:

- a. Selecting **Window** in the flexImaging toolbar normalization list or
- b. Selecting **Edit > Sequence Properties** to open the **Sequence Properties** dialog and select the **Spectra Normalization** option **Norm. to Mass Window**.

The boundaries of the normalization window(s) are displayed to the right of the **Norm. to Mass Window** option.

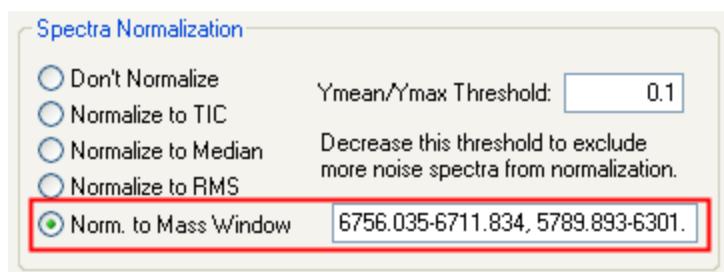


Figure 5-67 Selecting the Norm. to Mass Window option in the Sequence Properties dialog

Mass Window inclusion

The mass window inclusion normalization method is a TIC normalization of a selected portion of the spectra and is applicable to MALDI-TOF and MALDI-FTMS data sets. This method can be used to normalize to a known signal (such as a matrix signal, a spiked analyte, or a signal that has equal intensity throughout the entire tissue section).

Artifacts may be created if region-specific ion suppression modulates the intensity of the internal standard.

Known peaks or spectrum ranges are used for normalization by defining their mass range using the distance cursor in the Spectrum Display.

►► To use a known peak or spectrum range for normalization

1. Zoom in on the peak or spectrum range to be used for normalization.
2. Right-click in the Spectrum Display and select **Mass Window / Distance** from the shortcut menu.
3. Using the mouse pointer, move the vertical lines to the boundaries of the peak or range to be used for normalization.

Click to switch the mouse pointer between the boundary lines.

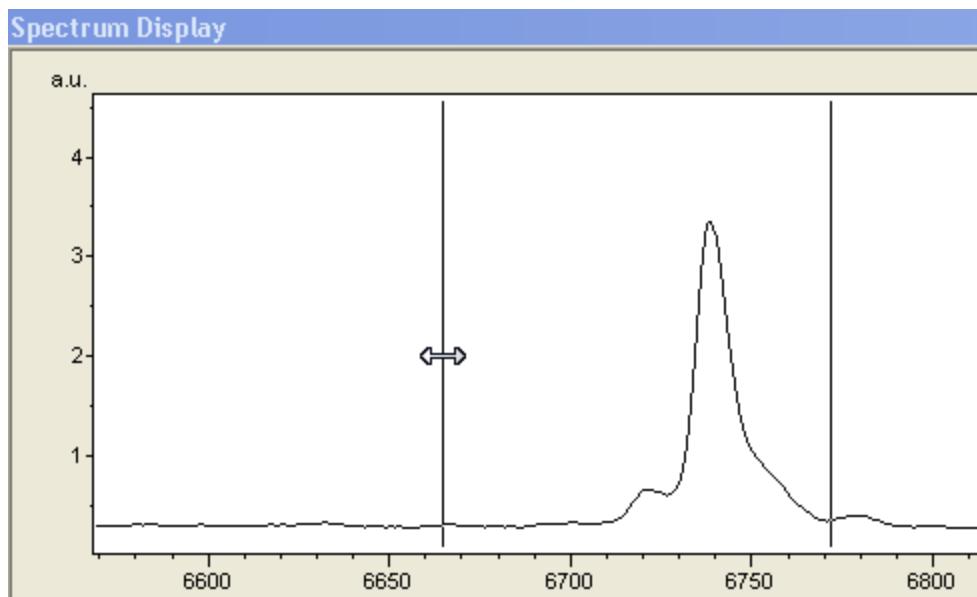


Figure 5-68 Setting the boundaries of the normalization window

- When the boundaries have been set, right-click to open the **Add Normalization Mass Window** dialog.

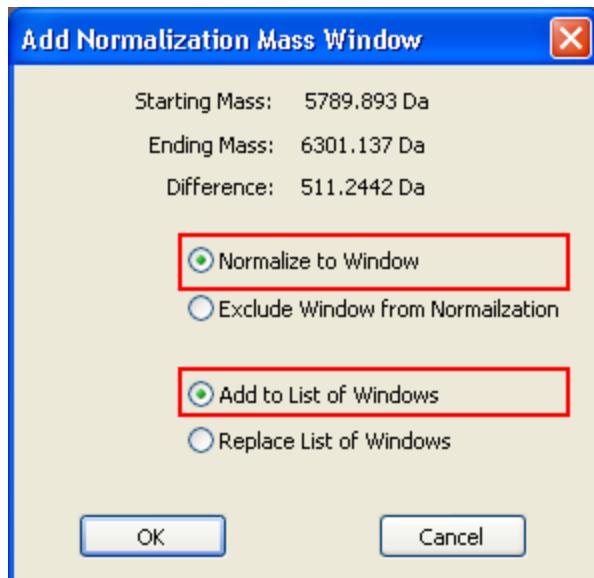


Figure 5-69 Add Normalization Mass Window dialog

5. Select **Normalize to Window**.

Additional normalization windows can be created by defining a mass window, selecting **Normalize to Window**, and selecting **Add to list of windows**.

The boundaries of exclusion windows are indicated by dashed red lines in the Spectrum Display.

6. Select the **Window** normalization method by either:

- a. Selecting **Window** in the flexImaging toolbar normalization list or
- b. Selecting **Edit > Sequence Properties** to open the **Sequence Properties** dialog and select the **Spectra Normalization** option **Norm. to Mass Window**.

The boundaries of the normalization window(s) are displayed to the right of the **Norm. to Mass Window** option (Figure 5-67).

5.4.3.2 How to Recognize and Deal with Normalization Artifacts

Incorrectly applied normalization methods can generate artifacts that may appear to provide useful biological or histological information. It is therefore essential to recognize these artifacts.

A. Qualitative changes in the image with and without normalization

When a normalized and a non-normalized image are compared, the normalized image will usually look smoother. Significant changes in the intensities of a mass's signal — for example, if the signal's highest intensity is found in different regions before and after normalization — are a strong indication of a normalization artifact.

B. Qualitative changes in the image when switching between TIC/RMS and median normalization (MALDI-TOF only):

Images normalized using the TIC or RMS and median methods should appear similar, with the TIC- or RMS-normalized image usually looking a bit smoother. Significant differences in intensities between the TIC/RMS- and median-normalized image are a strong indication of an artifact.

C. Apparent “holes” in the TIC or RMS normalized data, especially in the distribution of noise or low intensity signals (MALDI-TOF only):

The presence of gaps in TIC- or RMS-normalized data indicates the presence of artifacts.

D. Only a few bright pixels in median normalization (MALDI-TOF only)

The presence of just a few bright pixels is usually an indication that in many spectra the baseline was cut off during acquisition. This can result in median values of close to zero, and as a result, to very intense spectra after normalization.

In cases A–C the artifacts are usually caused by the presence of signals with high intensities or large areas in some parts of the data set. Because the RMS normalization method is more likely to generate artifacts than the TIC normalization method, try changing the normalization from RMS to TIC. If artifacts persist, use the median normalization method (MALDI-TOF), or manually exclude the signals that cause the artifacts using the Mass Window Exclusion method.

In case D, the noise information was lost during the acquisition and the data set is not suitable for normalization using noise-based methods.

Exclusion of noise spectra

The RMS (and to a lesser extent the TIC) normalization methods carry the danger of artificially increasing the intensity of pure noise spectra. For this reason, flexImaging offers the option of excluding noise spectra by defining a **Ymean/Ymax threshold** in the **Edit > Sequence Properties** dialog.

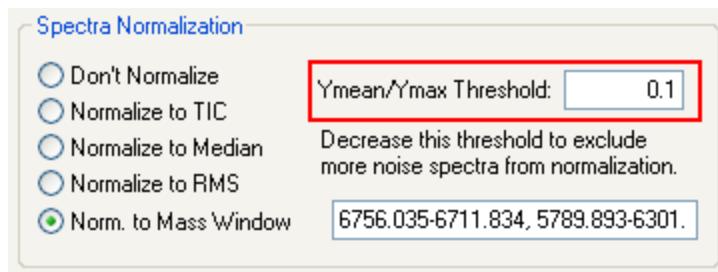


Figure 5-70 Ymean/Ymax Threshold setting in the Sequence Properties dialog

flexImaging displays the Ymean/Ymax value for the data point under the mouse pointer in the status bar (see section 4.5.4.2). These values can be used to determine the optimal threshold value.

5.4.4 How to Find Biomarkers

Within this section, the term biomarker means a mass signal that is characteristic for a specific histological feature in a tissue section, e.g. for a tumor or for an organ. (There is also a possibility to look for biomarkers in a larger set of samples with ClinProTools. For this approach, please refer to model generation and classification.)

If the location of the expected biomarkers is known

The workflow is explained with this data set from a sagittal zebrafish section. A histological stain has been co-registered to make the analysis easier (the unstained section for acquiring the MALDI data often does not show all histological features). The aim in this tutorial is to find biomarkers characteristic for brain, heart, kidney and liver.

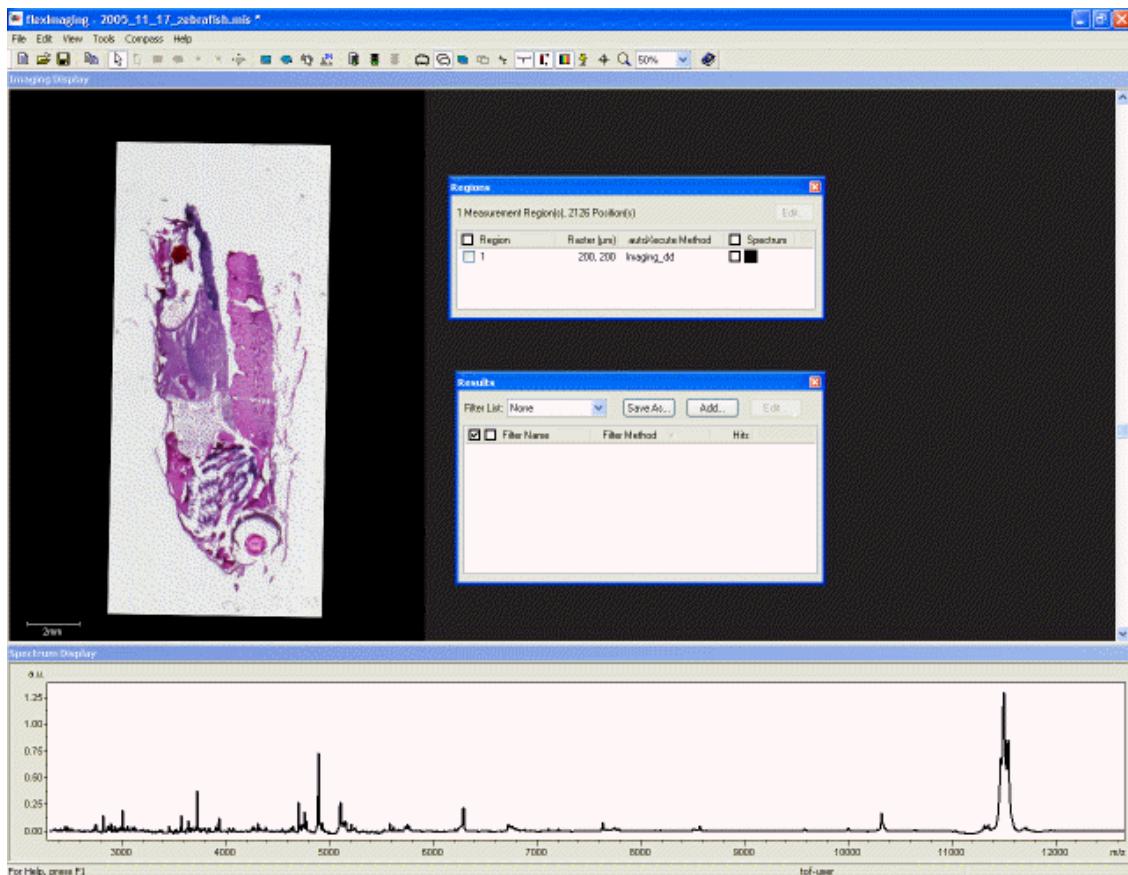


Figure 5-71 Zebrafish data set with co-registered histological stain

We start by defining regions of interest for the selected organ. It is helpful to assign meaningful names to each region (for example, "brain", "kidney", "liver" and "heart") in the Regions Pane.

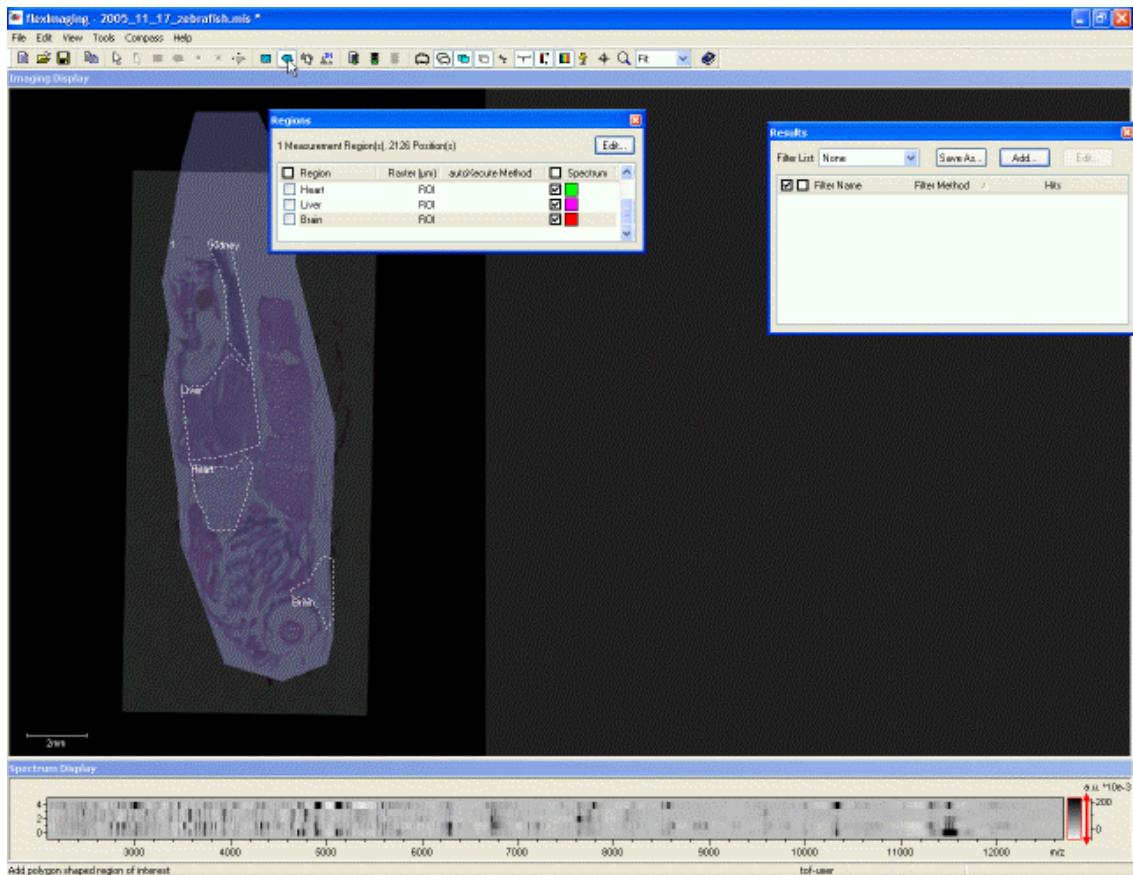


Figure 5-72 Zebrafish data set with defined regions of interest

It is necessary to check the **Spectrum** checkbox for the interesting regions in the Regions Pane. This will allow the display of the average spectra of the regions in the Spectrum Display.

There are two ways for comparing the average spectra for individual regions:

The first is to right-click in the Spectrum Display window and select **Display Type** and then select **2D Density Plot**. Now, we see a 2D Density plot of the average spectra of the selected regions and the overall average spectrum. The Region names are shown in the upper right corner of the spectrum lanes. The intensity of the mass signals is shown as color saturation. The saturation can be adjusted with the intensity ruler on the right side. Dragging the ruler with the left mouse allows shifting it up and down; dragging with the right mouse expands and shrinks the intensity scale.

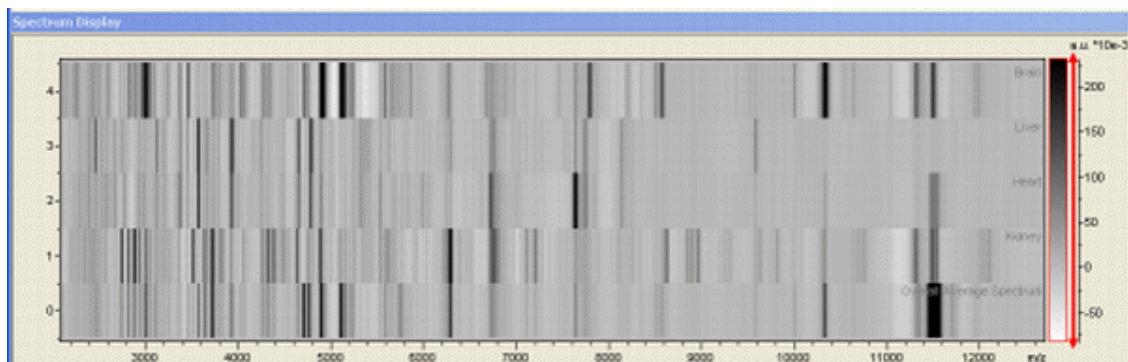


Figure 5-73 2D Density plot of selected regions

The second way is to right-click the Spectrum Display and selecting **Display Type** and then select **2D All Scans**. Now all selected spectra are shown in an overlaid mode, each spectrum has the color that was assigned to the respective region in the Regions Pane.

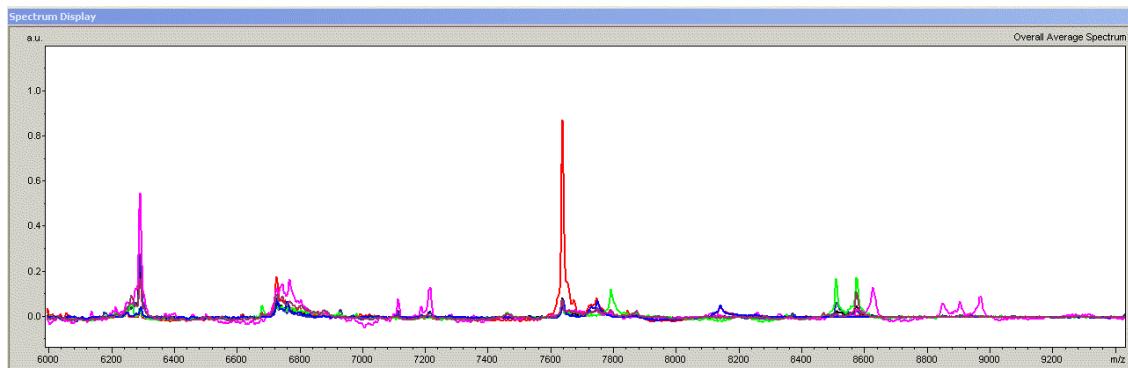


Figure 5-74 2D All Scans plot of selected regions

Intensity differences between the selected regions can be now be easily seen in the Spectrum Display. It is possible to select a mass of interest by clicking on it in the Spectrum Display with the left mouse button (if the cursor is in Mass Filter Selection mode) or clicking on it with the Ctrl key pressed down (if the cursor is in Zooming mode). Now we can cross-check the distribution of the selected marker in the Imaging Display.

In the following figure, the mass at 8628 m/z has been selected in the 2D density plot. The intensity distribution of this signal shows, that it is indeed a biomarker for the kidney. It is now possible to add the mass to the result list in the Results Pane.

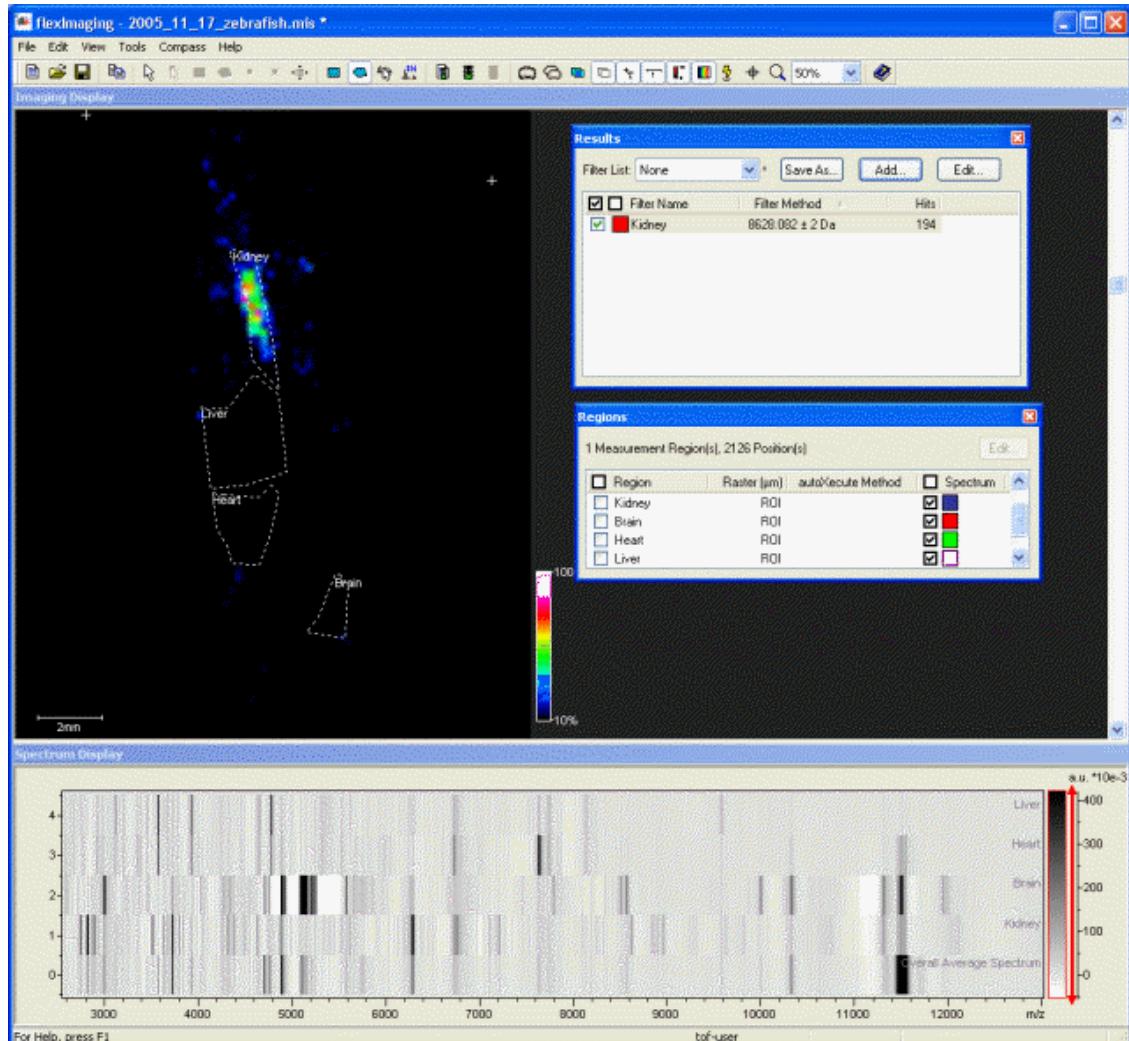


Figure 5-75 Kidney specific mass selected

Now we continue with a specific mass for the heart: The 2D density plot shows a very intensive signal in the "heart" lane at 7639 m/z . Selecting this signal verifies that it is indeed a biomarker for the zebrafish heart. We can add this mass now to the result list and in this process populate the result list with characteristic masses. To find marker masses for the remaining positions we would proceed accordingly.

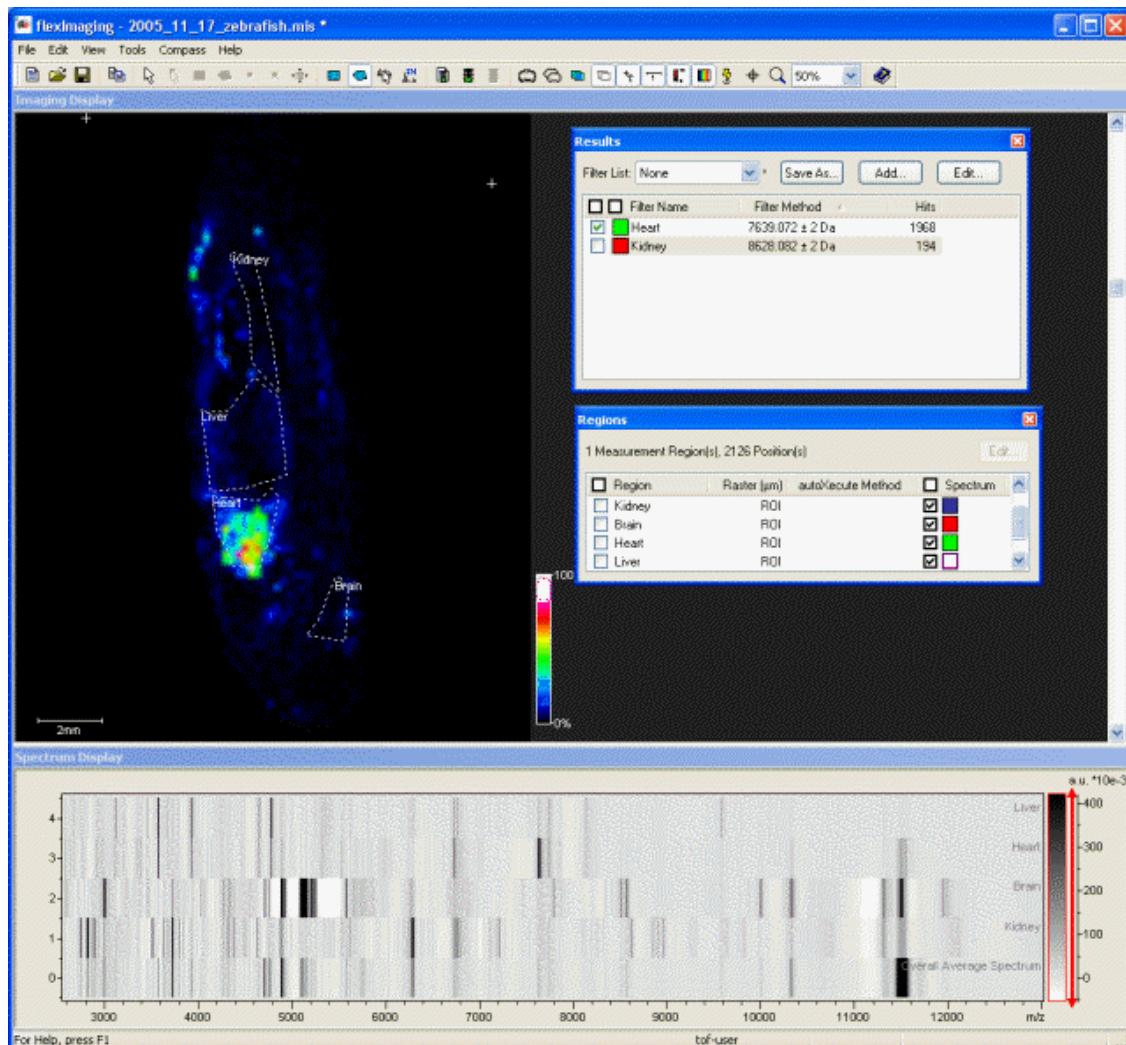


Figure 5-76 Heart specific mass selected

If the location of the biomarker is unknown

Some compounds are usually not correlated directly with histological features. These are especially compounds, which are not endogenous from the tissue itself. Examples for these compounds are hemoglobins (which come from residual blood in the section) or beta-defensins if inflammatory processes are present.

If the exact location of the biomarkers is unknown, one way is to select masses by clicking on peaks in the overall average spectrum and check the distribution of the signals in the display. The mass marker can also be dragged with the mouse in the overall average spectrum and it can be checked how the imaging display changes. The mass marker can also be moved with the left and right arrow key on the keyboard.

It is also useful to find those mass signals that contribute most to the overall variance in the data set. This can be done by performing a principal component analysis in ClinProTools. Please refer to PCA import.

5.4.5 Creating High-Quality Images

This section provides tips on how to prepare high-quality images for talks, posters and publications.

Getting started

Once data for a imaging run has been acquired and one or more Result Filter Lists have been compiled, it is usually desirable to create a number of figures summarizing the results. A good first step is to deactivate all display options in the View menu which don't add valuable information to a figure (typically, these are Measurement Regions, Teach Points, Laser Spots and Sample Position). Helpful display options include Ruler, Intensity Scale and Intensity Captions, which can be activated using the respective command from the View menu.

The mass spectrometric results can be underlaid with the Sample Image and/or a co-registered image for detailed evaluation of the data. Once a higher quality image has been co-registered, there is usually no benefit from displaying the Sample Image (which can be deselected in the View menu). Images and mass spectrometric results can be cross-faded using the left slider in the Image Adjustment Window. In addition, if a stained image has been co-registered in color, it can be temporarily switched to a grayscale image (using the reg. Images as B/W command in the View menu) to prevent ambiguity when overlaying with mass spectrometric results.

De-activating both Sample Image and co-registered image will turn the background black and the full color space and contrast will be available for the mass spectral image. Frequently, normalized spectra will lead to clearer, sharper mass spectral images, so the different Normalization options should be tested before exporting an image.

One mass filter to be shown

If the distribution of only one mass is to be shown, it is usually best to activate the Color Gradient button in the toolbar. This “rainbow mode” exaggerates the intensity differences in the image and usually makes the conclusion clearer.

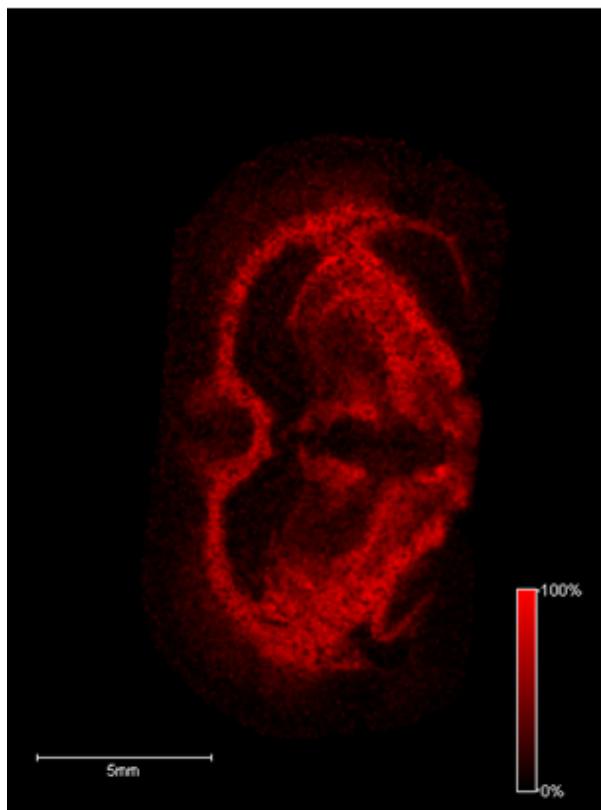


Figure 5-77 The same mass filter displayed as a single color gradient and in “rainbow mode”

The color gradient used to display the intensity of a mass spectral peak can be fine-tuned using the **Edit Mass Filter Parameters** dialog for the respective mass filter. Per default, the measurement position with the highest peak intensity is defined as 100% and assigned the brightest color setting. All other measurement positions are assigned colors according to the relative peak intensity. Increasing the **Minimum Intensity** will cause measurement positions with low intensities to be excluded from display (i.e. shown as black). Lowering the **Full Intensity Threshold** will result in more measurement spots being assigned to the brightest color setting.

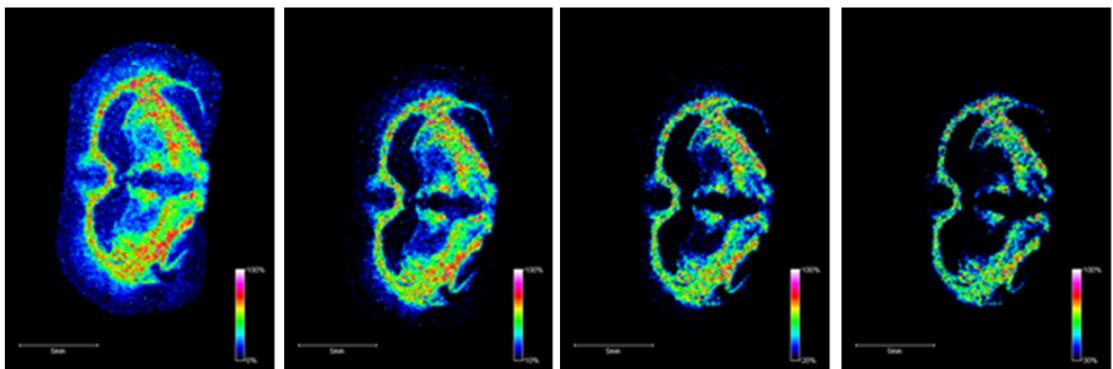


Figure 5-78 The same mass filter displayed with increasing Minimum Intensity. More and more measurement spots are below threshold (i.e. displayed as black).

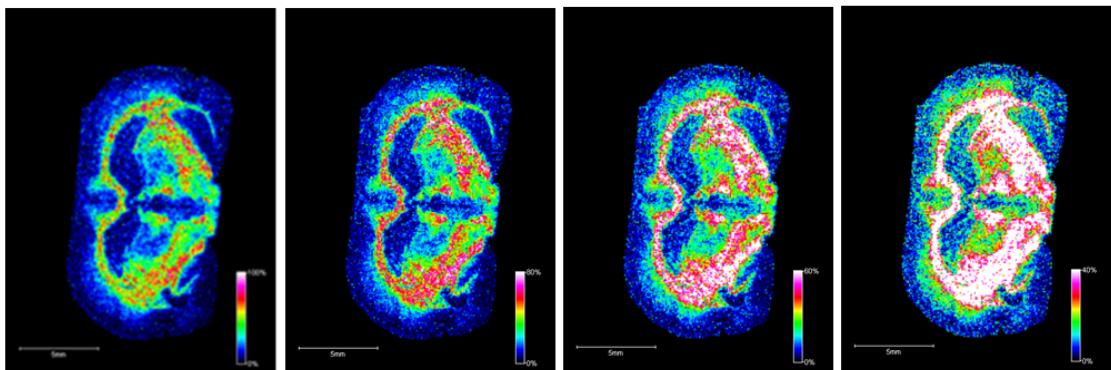


Figure 5-79 The same mass filter displayed with decreasing Maximum Intensity Threshold. More and more measurement spots are above as threshold (i.e. displayed in white).

The histogram of a mass filter can be employed as a more objective handle when setting these thresholds. It can be accessed by right-clicking the mass filter in the Results Pane and selecting **Show Histogram**. Both thresholds are visible in the histogram as dashed lines and can be directly adjusted here. In the example histogram shown below, only a few pixels have an intensity 10 a.u. or higher. The Full Intensity Threshold has been adjusted to 10 a.u., and as a result all measurement spots with intensity of 10 a.u. or higher will be displayed as maximum intensity.

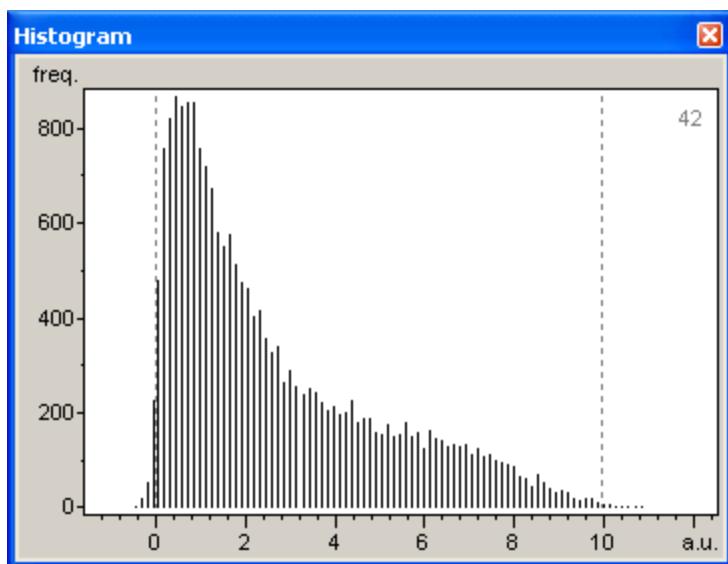


Figure 5-80 Histogram of a selected mass filter

In addition, flexImaging 5.0 offers a Brightness optimization setting. If this option is selected in the **Edit Mass Filter Parameters** dialog (accessed by double-clicking the Mass Filter in the Results Pane), the Maximum Intensity Threshold will be automatically adjusted in a way that puts 5% of all measurement spots above threshold. Typically, brightness optimization leads to a clearer representation of features in the resulting mass spectral image.

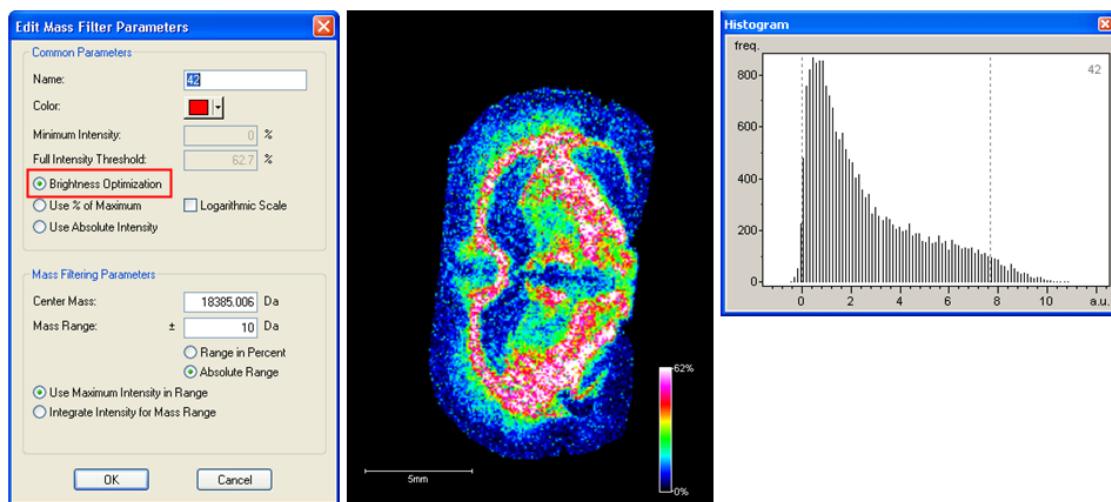


Figure 5-81 The Edit Mass Filter Parameters dialog with the Brightness Optimization feature selected. The Full Intensity Threshold has automatically been adjusted, putting 5% of all spectra above threshold, as seen in the histogram.

Displaying multiple Mass Filters

MALDI Imaging offers the unique opportunity to analyze the distribution of multiple molecules in parallel and display them simultaneously in any combination desired once data acquisition is concluded.

If more than one Mass Filter is selected from the Results Pane, the Result Color Gradient (i.e. “rainbow mode”) is automatically suspended. When displaying up to three Mass Filters in parallel, it is recommended to assign the three fundamental colors red, green and blue, thus using the full color space and contrast are available for display. It may be necessary to fine tune the Minimum Intensity and Full Intensity Threshold for each mass filter for a well-balanced image.

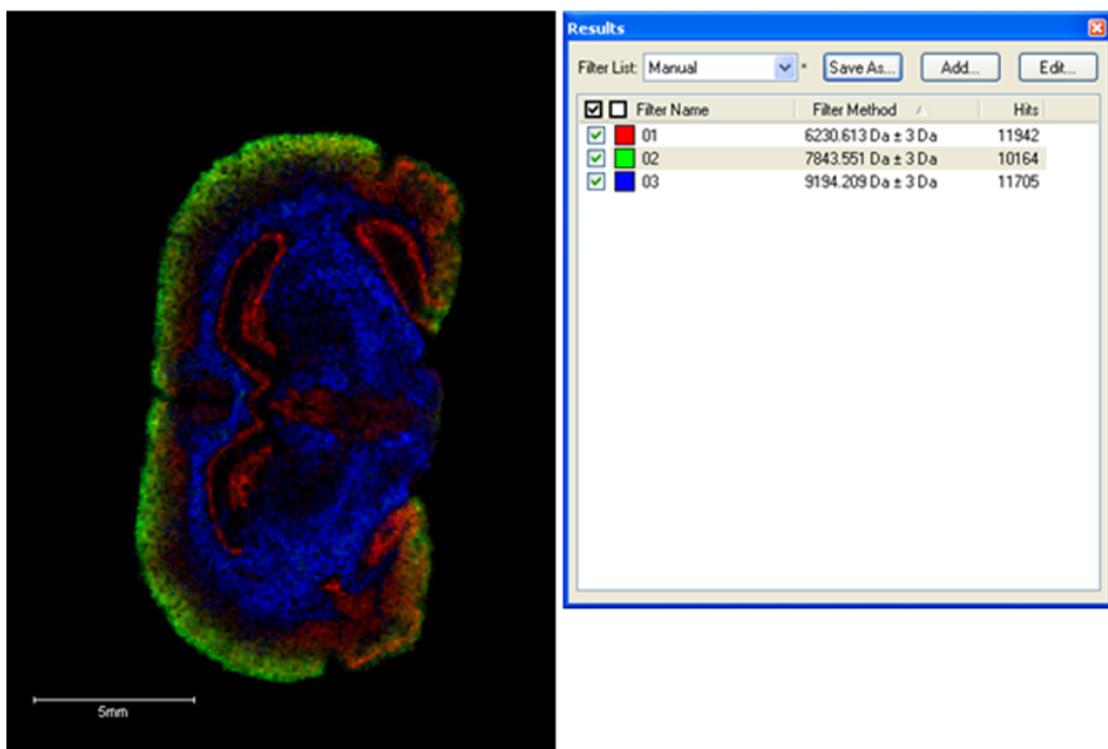


Figure 5-82 Three different mass filters from a rat brain data set displayed simultaneously

Usually red, green and blue are the preferable color setting for a computer screen or multimedia projector, and also work fine on high quality printouts. However, these colors are not the fundamental colors of ink-jet printers and may look rather pale on printouts from, especially on non-glossy paper. In this case, selecting cyan, magenta and yellow as colors can lead to improved printouts. Cyan, magenta and yellow are not predefined in flexImaging, but can be added using the **Color** dialog.

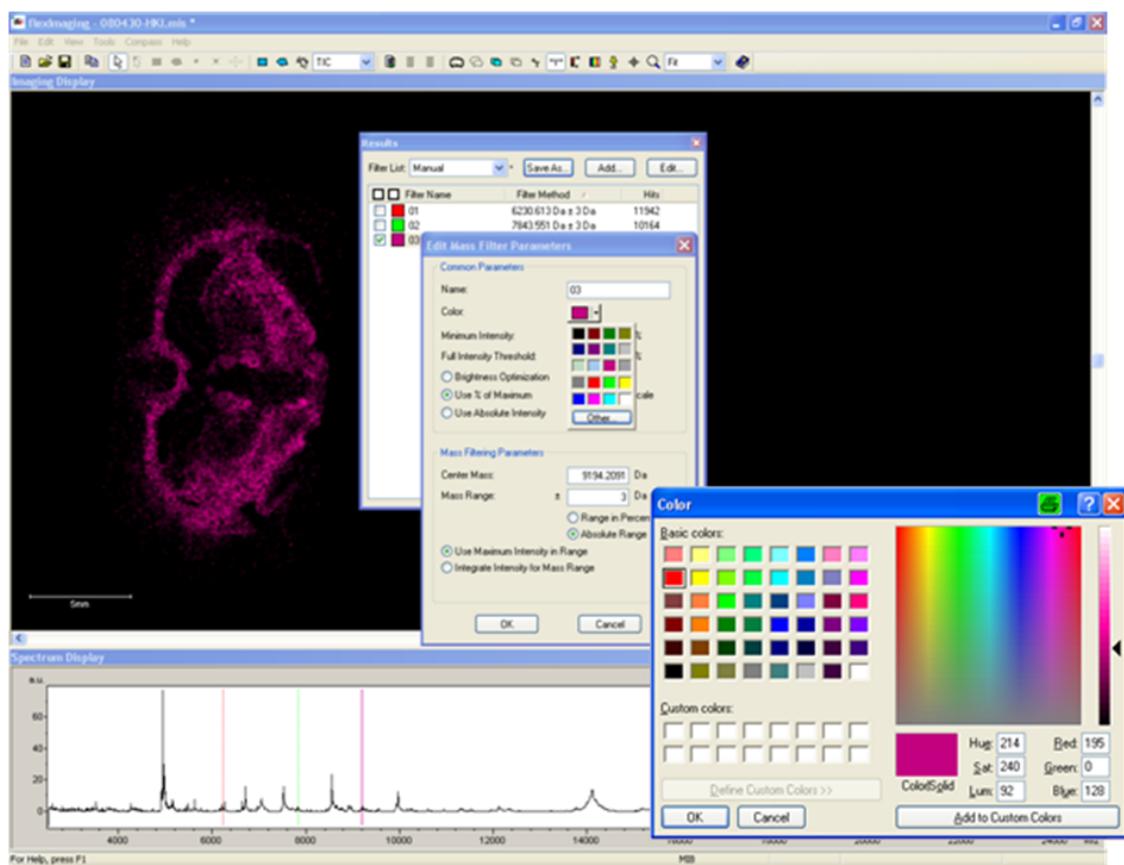


Figure 5-83 Color dialog windows

The values for cyan, magenta and yellow are shown in the following table:

Table 5-3 Values for cyan, magenta and yellow in RGB color space

	R	G	B
Cyan	0	164	232
Magenta	195	0	128
Yellow	255	236	0

Although it is technically possible to display numerous Mass Filters simultaneously, selecting more than three at a time is advisable only if each mass is distributed across a clearly separated measurement area. Overlapping mass distribution can lead to ambiguity in color assignments (e.g. a yellow pixel could indicate the unique presence of a mass displayed in yellow or the simultaneous presence of two different masses coded in red and green, which mixes to yellow).

After a ClinProTools classification or clustering, each spectrum is very likely to belong to only one class or cluster. Thus, the color codes for the different classes or clusters are very unlikely to mix. It is therefore possible to show more than three colors without restriction.

Exporting Images

Once all settings have been adjusted appropriately, the easiest way to export the image is by right-clicking in the Image Display pane and selecting the **Copy Image to Clipboard** command, which will capture the image section exactly as displayed on the screen, maintaining the currently selected zoom level and overlays. Since the position of the Ruler and the Intensity Scale are fixed, it can be very helpful to use the Windows **Restore Down** command on the flexImaging window and scale the Imaging Display pane accordingly before copying the image. If required, the current Spectrum Display pane can also be exported by right-clicking it and selecting the **Copy** command. Alternatively, the **Copy as CSV** command can be used to extract the intensity values for each individual data point on the *m/z* axis to the clipboard as comma-separated values.

When saving a Filter List in the Results Pane, the current selection of Mass Filters is saved accordingly, so this function can be used to quickly access several images created for a data set.

5.4.6 Interfacing with ClinProTools

ClinProTools is a software package that allows statistical analyses of spectra collections such as MALDI imaging data acquired using flexImaging. ClinProTools works with spectra from MALDI-TOF measurements. It does not work with FTMS spectra.

The available statistical analyses can be used for three different purposes:

- unsupervised or semi-supervised feature extraction of MALDI imaging data sets
- supervised classification of tissue
- univariate supervised statistics to find biomarker signals.

The first is mainly used to obtain concise representations of single MALDI imaging data sets (such as an image of the main variance in the data or a spatial segmentation). Useful for this purpose are: PCA, hierarchical clustering and PLSA. The results of these analyses can be imported into flexImaging to create images.

The second purpose usually requires a larger collection of data that allows a statistical model to be built for certain tissue types. This requires obtaining multiple data sets with a known state as a training set to later classify unknown samples. The classification results can be imported into flexImaging to create images.

The third use is important as well, but does not lead to results that could be displayed as an image. The same is true for the comparison of different data sets by PCA or hierarchical clustering. Therefore these uses are not discussed in this manual.

For a full functionality, ClinProTools 3.0 or higher is required. This allows an unsupervised principal component analysis (PCA) with normalization of the data, an unsupervised hierarchical clustering of the data, an unsupervised probabilistic latent semantic analysis (PLSA) and also supervised classifications on an unlimited number of spectra. For the supervised classification, it is possible to assess the quality of a classification by outlier detection.

With ClinProTools 2.2 no PLSA analysis is available and there is only one scaling option (autoscaling) available for the PCA.

With ClinProTools 2.1 the PCA is only possible without autoscaling. There is no hierarchical clustering and no outlier detection.

With ClinProTools 2.0, no PCA and no hierarchical clustering is possible. Supervised classifications are limited to the number of spectra that ClinProTools can load into the program at once. No outlier detection is available.

5.4.6.1 PCA Import

An unsupervised feature extraction of imaging data can be performed with ClinProTools 2.2 and higher, and the result of this analysis can be displayed with flexImaging (ClinProTools 2.1 can be used with some restrictions).

This is done by a Principal Component Analysis.

Principal Component Analysis (PCA)

PCA is a way to project high-dimensional data to lower dimensions while retaining the essential information:

Imagine a set of spectra which contains 20 peaks with varying intensity. We can then define a coordinate system with 20 dimensions, which represent the intensities of the 20 peaks. Each spectrum will occupy one point in this 20-dimensional coordinate system; the point that is occupied is determined by the intensities of the 20 peaks of the spectrum.

The PCA now performs a linear transformation of this coordinate system in a way that the first coordinate of this new coordinate system points in the direction of the highest variance of the data, the second dimension points in the direction of the second highest variance and so on. We end up with 20 new dimensions which point in declining order in the directions of the variance of the original data.

These new coordinates are called principal components (PC). The advantage of this analysis is a reduction of relevant dimensions. Although we have as many PCs as peaks in the original data set, the main variance of the data set is contained in the first two or three PCs.

How to perform a PCA with flexImaging data in ClinProTools 2.2

The fastest way to do a PCA with ClinProTools 2.2 is to export a list of all spectra from flexImaging and to load that list in ClinProTools 2.2. To export the spectra list do a right mouse click in the Regions Pane in flexImaging and select the option **Export Spectra List**.

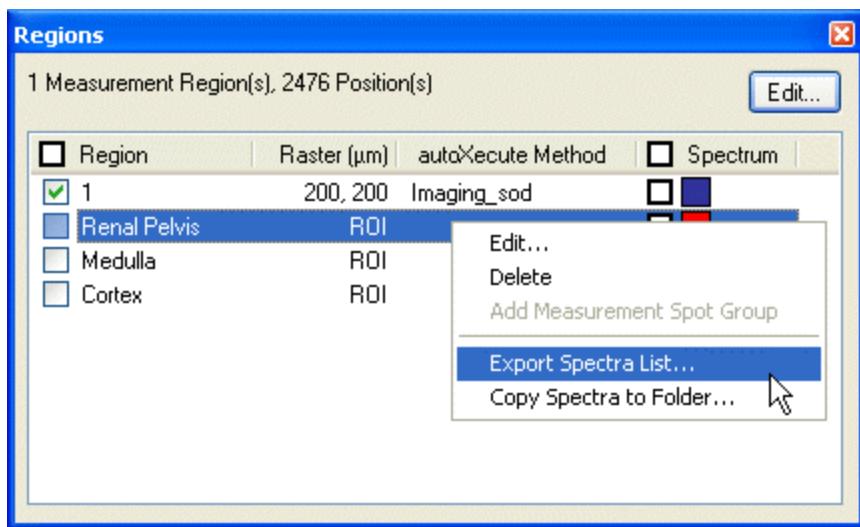


Figure 5-84 Exporting spectra list of selected region

In the **Export Spectra List** dialog, check the measurement region. If regions of interest are defined, do not check them. Export the spectra list. By default the list will have the same name as the imaging run appended by “_Regions.xml” and will be saved next to the *.mis file.

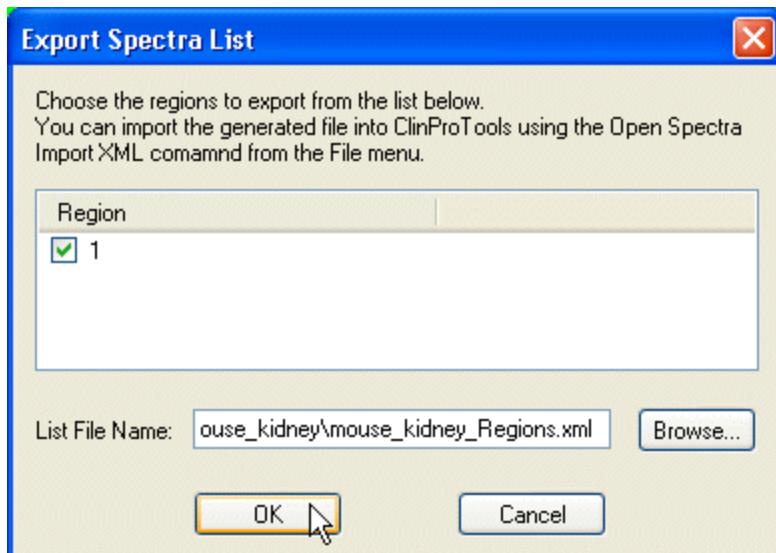


Figure 5-85 Export Spectra List dialog

Next, open ClinProTools and select **Open Spectrum Import xml** in the **File** menu. Select the spectra list (please refer to the ClinProTools manual for the **Settings Spectra Preparation** and **Settings Peak Calculation** commands in the **Data Preparation** menu). After the spectra are loaded in ClinProTools, press the PCA button .

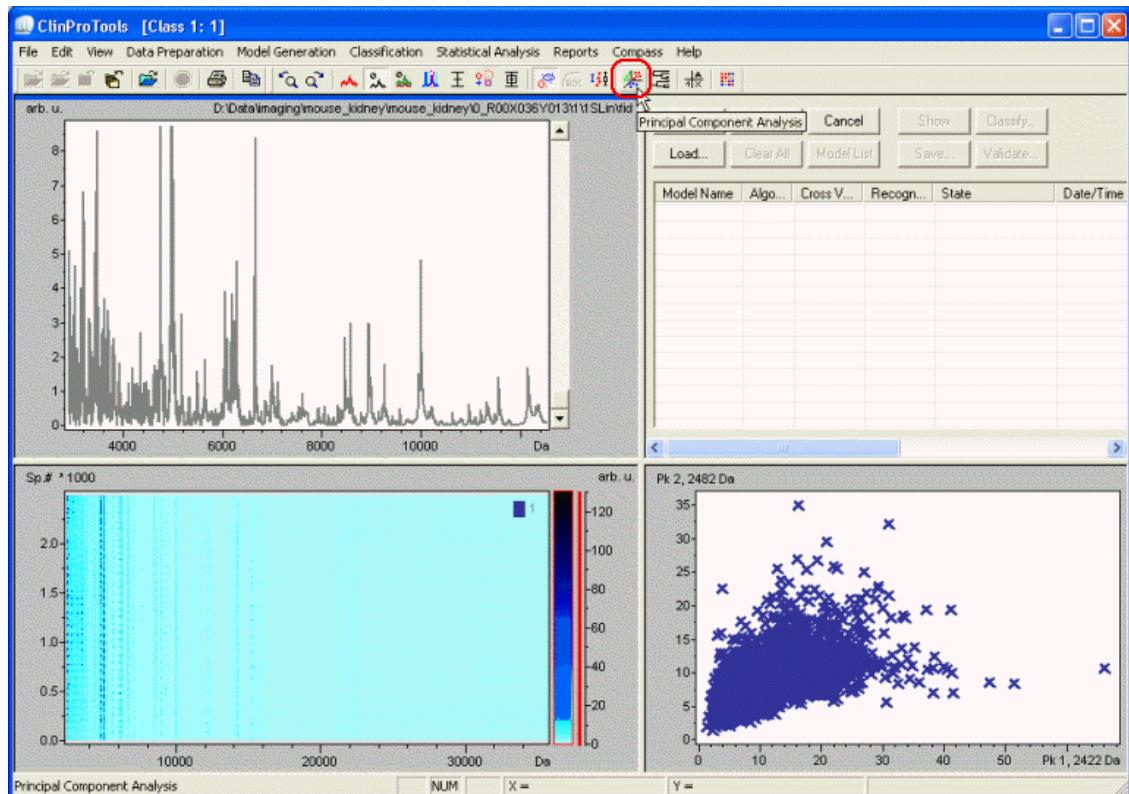


Figure 5-86 ClinProTools peak calculation of mouse kidney demo data set

After the PCA button is pressed, the **PCA** dialog appears where it can be defined whether or not the data for the PCA should be normalized. (If the normalization is switched off, the PCA will be mainly dominated by the intensive signals in the data set, because they are likely to show the highest variance).

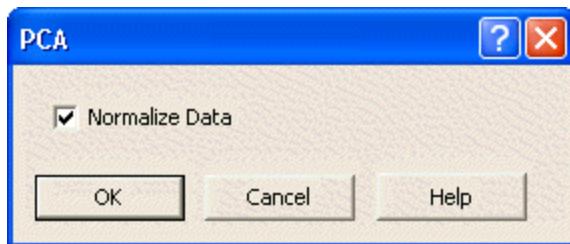


Figure 5-87 PCA dialog

Press OK and the calculation starts. After the calculation the PCA windows appears which shows the results of the PCA. At the same time, ClinProTools writes the file "C:\BDAL\ClinProTools_2_2\Files\ClinProtPCA.xml", which contains the results of the PCA analysis.

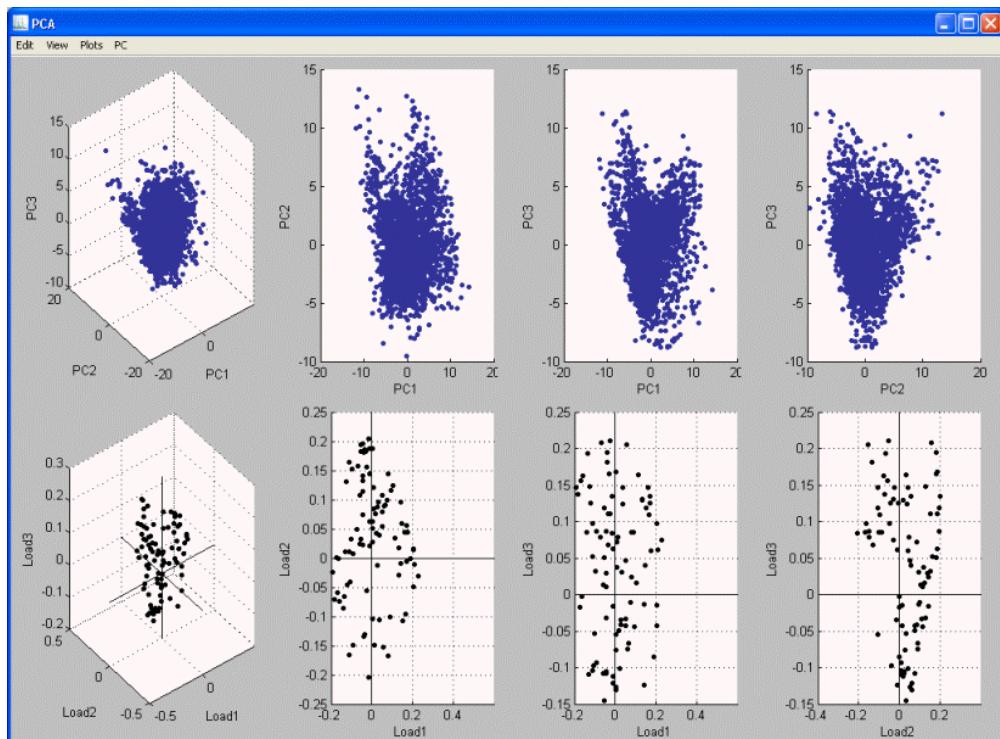


Figure 5-88 Principal Component Analysis of mouse kidney demo data set

How to perform a PCA with flexImaging data in ClinProTools 2.1

Start ClinProTools and open a model generation class. This class is a folder that contains the spectra collection. Browse to the folder that contains the spectra of the imaging run. See flexImaging file locations for where to find this folder. (Please refer to the ClinProTools manual for the options on “settings spectra preparation” and “settings peak calculation” in menu “data preparation”).

Once the spectra are loaded in ClinProTools press the “PCA” button in ClinProTools. After the calculation a windows appears which shows the results of the PCA. At the same time ClinProTools writes the file “C:\BDAL\ClinProTools_2_1\Files\ClinProtPCA.xml”, which contains the results of the PCA analysis.

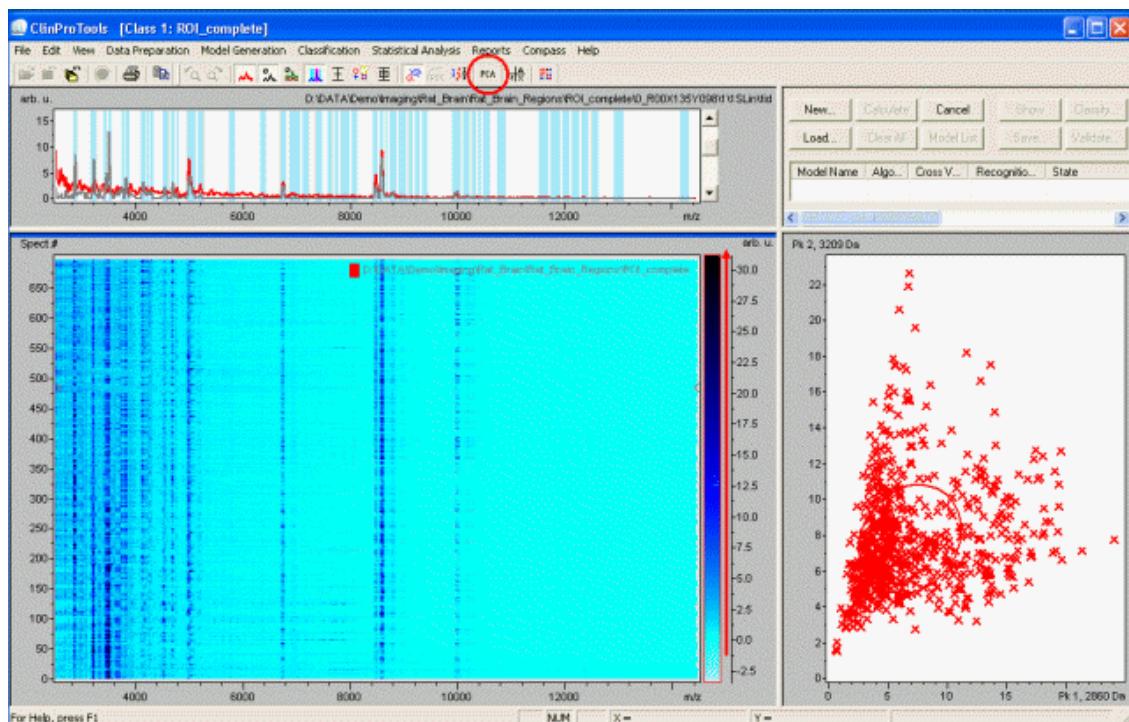


Figure 5-89 ClinProTools peak calculation of rat brain demo data set

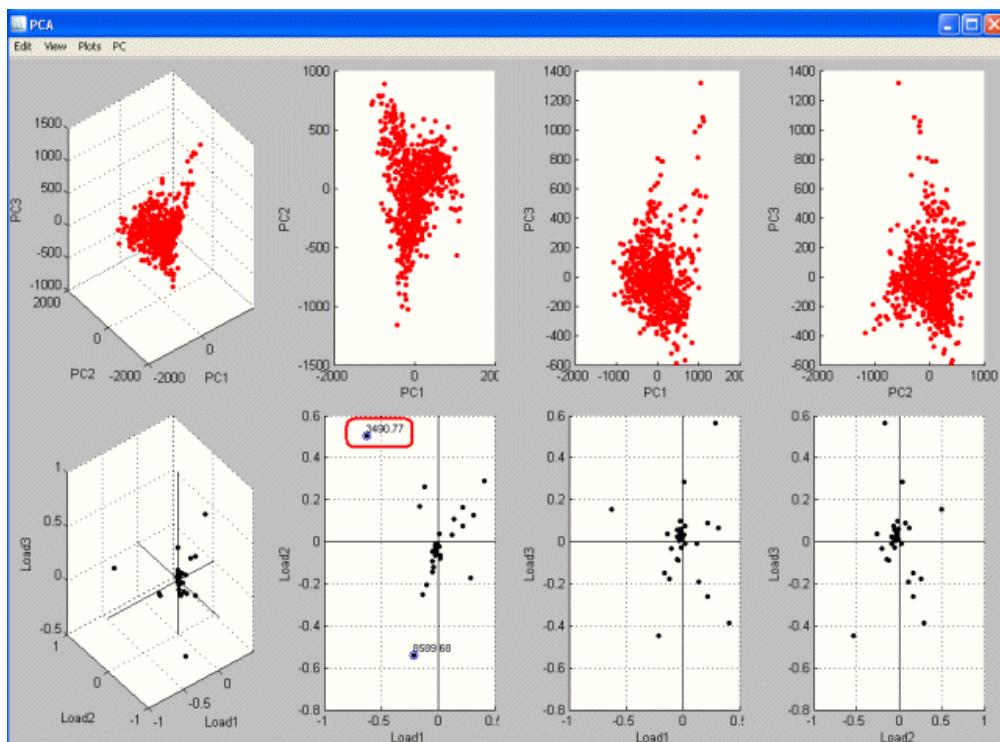


Figure 5-90 Principal Component Analysis of rat brain demo data set

There are two ways this result can be used in flexImaging

It is possible to display the scores of selected principal components for each spectrum as color intensities in the result display of flexImaging.

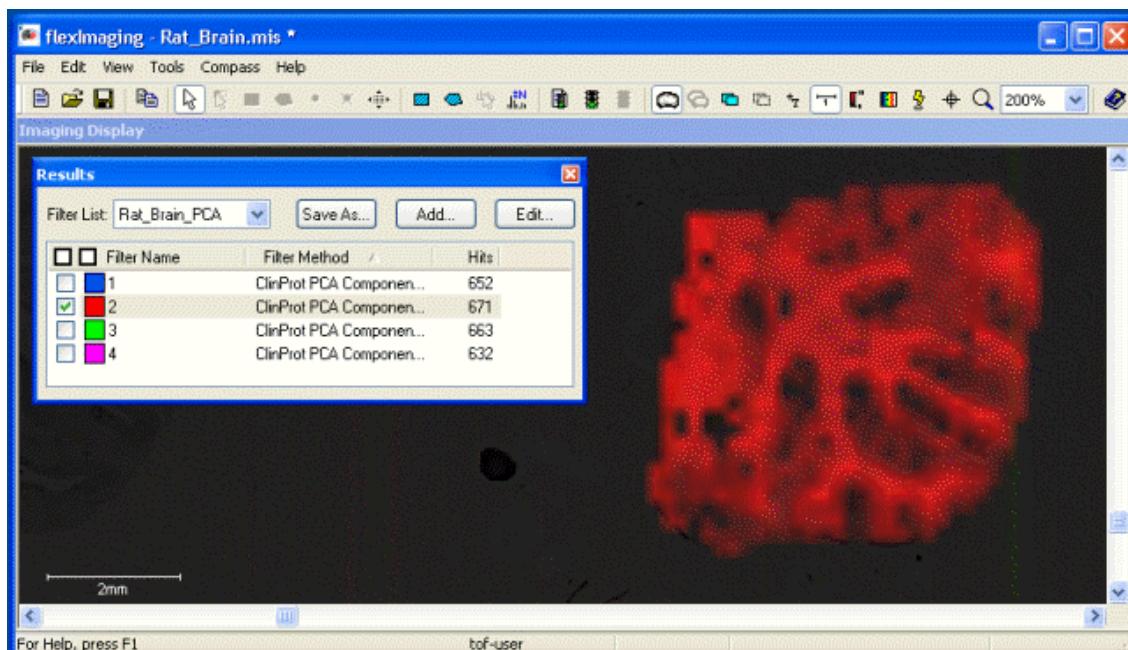


Figure 5-91 Import of one principal component in flexImaging

It is also possible to import a mass filter list which contains the masses of the peaks that contribute most to the overall variance in the data set; this is described in variance ranking.

Import of principal components in flexImaging

In the Results Pane of flexImaging now click **Add...** and in the **Add Result Filter** dialog select **Import ClinProTools Result**. Make sure the Mass Filter Selection Mark is not currently displayed. If it is, hide it by pressing Ctrl+H.

In the **Import ClinProTools Result** dialog browse for the file "C:\BDAL\ ClinProTools_2_2\Files\ClinProtPCA.xml" (if ClinProTools 2.1 is used the file will be "C:\BDAL\ClinProTools_2_1\Files\ClinProtPCA.xml") and in the next window select **Import Principle Component Analysis (PCA)**.

In the **Import ClinProTools PCA** dialog you can define how many PCs you want to import. Because the file "ClinProtPCA.xml" will be overwritten each time a PCA is calculated, flexImaging asks whether it should copy the file to the imaging run; this should be confirmed with **Yes**.

The principal component filter will now be added to the actual result list. Generally it is not useful to import more than the first six to eight PCs, which should contain most of the variance in the data set (see figure below). It is not useful to display more than one PC at a time, so select one PC (and no other filters), and the selected PC will be shown in the result display. The result will be most conclusive if the rainbow mode is selected for the result display.

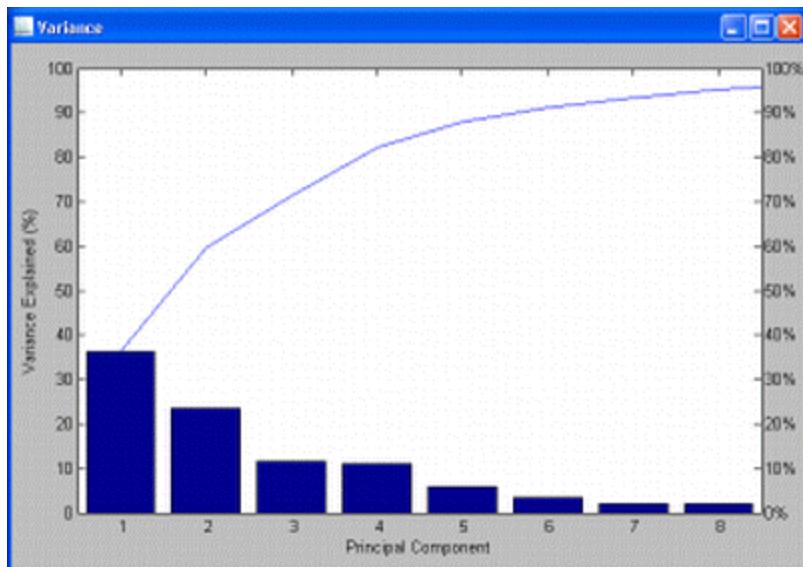


Figure 5-92 Variance of first 8 principal components of the rat brain demo data set

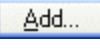
What does the result mean?

If there was a homogenous sample preparation, the result of the PCA should match the histological features of the sample. If there are distinct histological features in the sample, such as e.g. tumor and connective tissue, or white matter and grey matter in a brain section, one would expect that the PCA results reflect that structure. There are cases, however, when this is not true: If the section contains masses which do not come from the tissue itself, such as e.g. hemoglobin from residual blood, than these signals can dominate the distribution of the first PCs. In such a case it is possible to exclude these signals from the PCA analysis. Please refer to the ClinProTools manual for how to remove peaks from the calculation.

5.4.6.2 PCA Variance Ranking

The variance ranking imports masses from peaks that contribute most to the variance in the imaging data set. To import a variance ranking it is first necessary to perform a PCA analysis in ClinProTools, please refer to this chapter.

Import of a variance ranking in flexImaging

In the Results Pane click  and in the **Add Result Filter** dialog select **Import ClinProTools Result**.

In the **Import ClinProTools Result** dialog browse for the file “C:\BDAL\ClinProTools_2_2\Files\ClinProtPCA.xml” (if ClinProTools 2.1 is used the file will be “C:\BDAL\ClinProTools_2_1\Files\ClinProtPCA.xml”) and in the next window select **Import Variance Ranking**.

In the Import ClinProTools Variance Ranking dialog, you can define how many mass filters you want to import. If you select e.g. 10 mass filters then the 10 masses that contribute most to the overall variance in the data set will be imported in the result list. It is recommended to select the Insert variance into filter name option, because then the variance is visible in the filter list.

The higher the absolute value of the variance, the more the peak contributes to the variance in the data set. Now the masses can be selected one by one and it can be checked if they correlate with histological features.

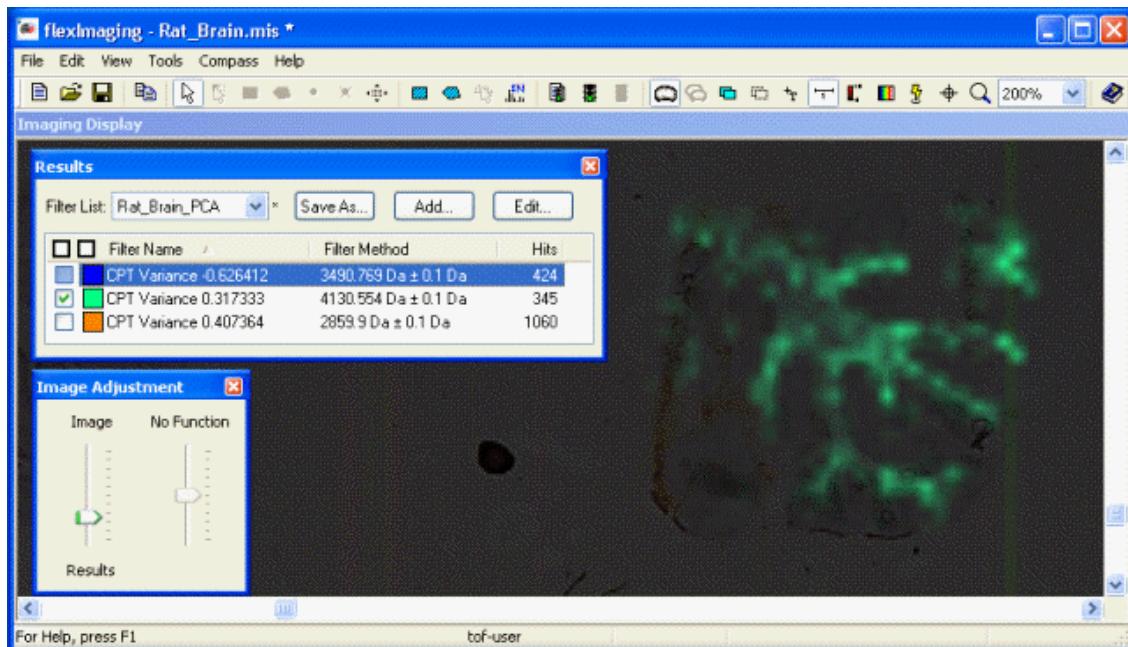


Figure 5-93 One mass filter of variance ranking result of ClinProTools

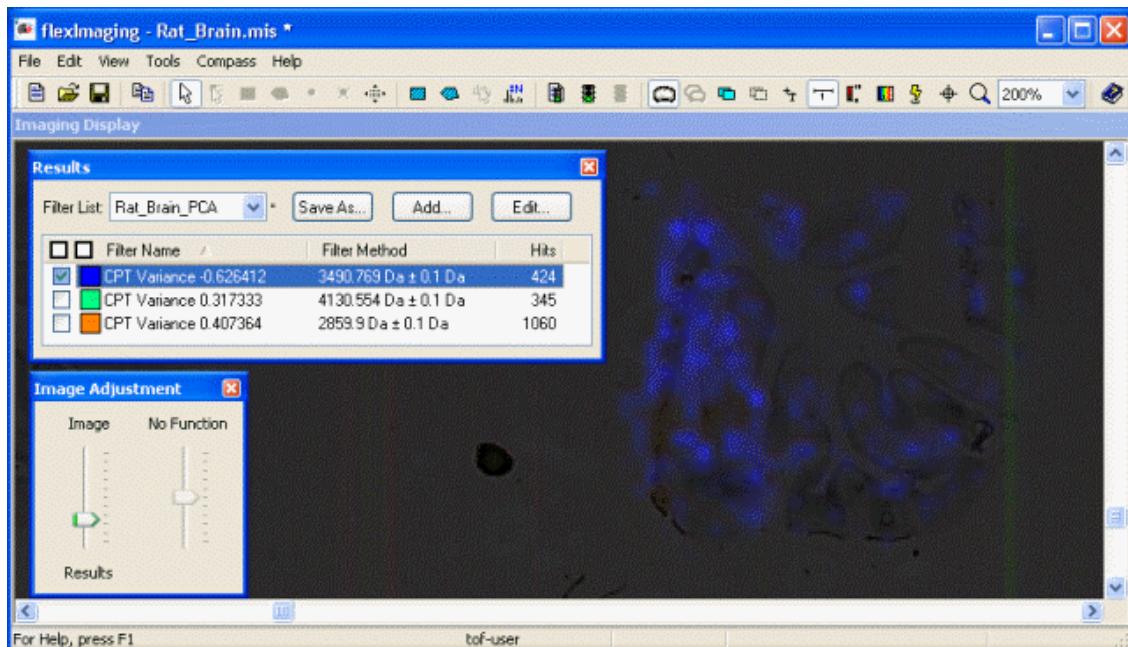


Figure 5-94 One mass filter of variance ranking result of ClinProTools

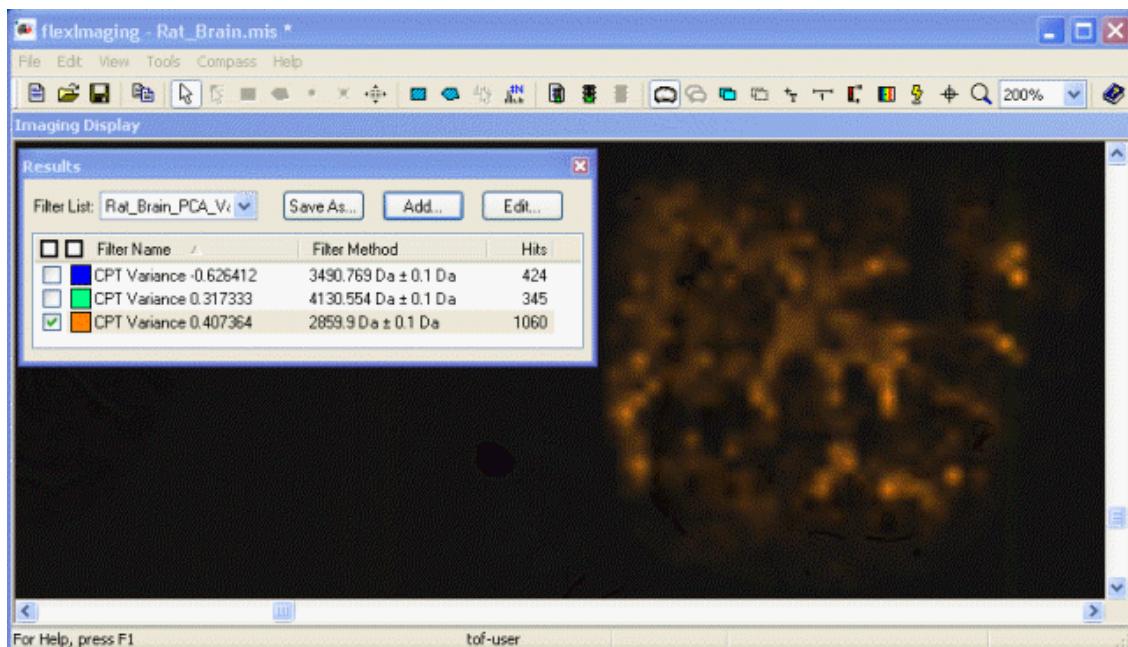


Figure 5-95 One mass filter of variance ranking result of ClinProTools

5.4.6.3 Classification by Hierarchical Clustering

The hierarchical clustering is a way to do an unsupervised classification of the mass spectra in an imaging data set by spectra similarity. First the spectra that are to be clustered have to be loaded into ClinProTools 2.2 (or higher). This can be done as described in PCA import. (Please refer to the ClinProTools manual for the **Settings Spectra Preparation** and **Settings Peak Calculation** commands in the Data Preparation menu).

Once the spectra are loaded into ClinProTools hit the hierarchical clustering button

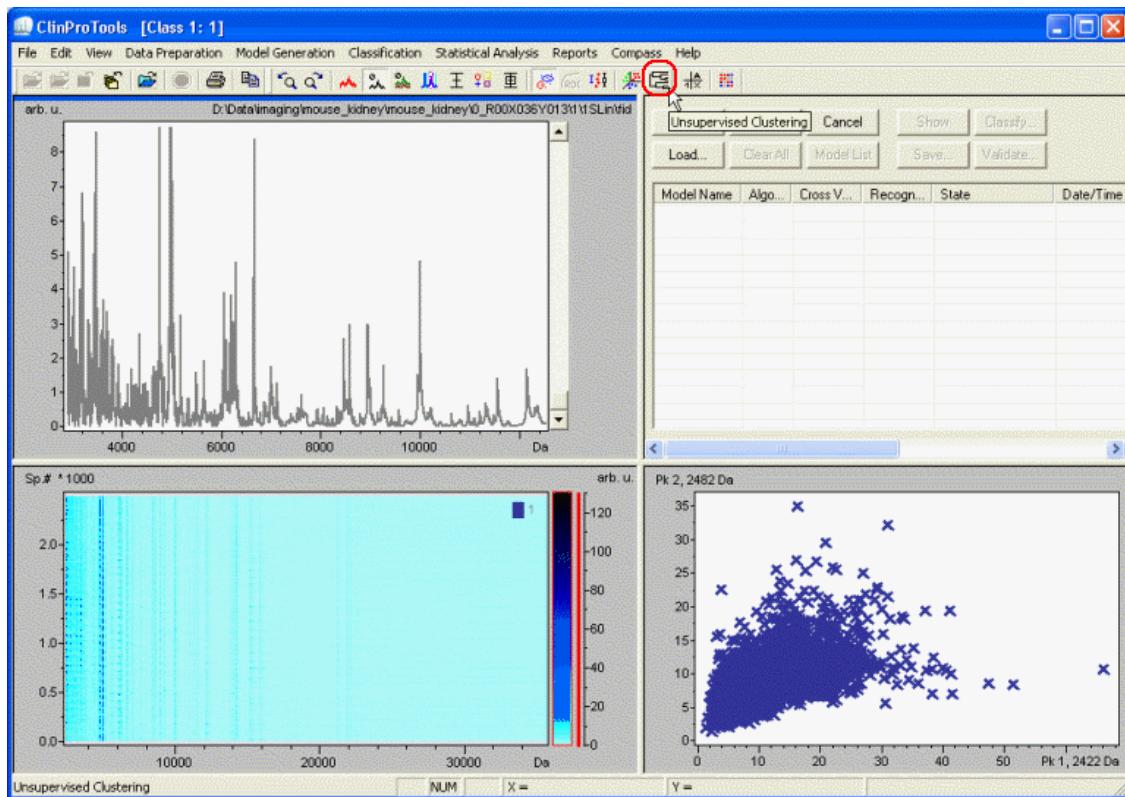


Figure 5-96 Spectra loaded in ClinProTools

The **Unsupervised Clustering** dialog appears where different options can be selected: Here it can be selected whether or not the data should be normalized, and whether or not the PCA data should be used. If **Use PCA Data** is selected then it is possible to reduce the clustering calculation to those principal components that explain the chosen **% Sum Explained Variance**. (The use of PCA data must only be used with a Euclidean distance metric!) This will have the effect that the clustering will focus more on the obvious differences in the data set and leave out fluctuations that are more random. For the interaction with flexImaging on whole imaging data sets, it is highly recommended to check the **Create Full Tree** option. Only if this option is checked the dendrogram will be available in flexImaging to evaluate the hierarchy of the resulting classes.

Which parameters to select for the clustering

In hierarchical clustering there are different options available for the distance metric (that defines how the distance between two spectra are calculated) and the linkage (that defines how the distance between two clusters are calculated). These can be accessed under the “advanced” settings in the clustering dialog. These parameters influence the characteristics of the resulting dendrogram, e.g. if outlier spectra are rather found at the top-level of the dendrogram or are grouped into existing large clusters. For the evaluation of MALDI imaging data sets, it is desirable to have a clear structure of the dendrogram with large clusters at the top level. These can usually be achieved by the following parameters for the clustering:

Table 5-4 Recommended parameter combinations for hierarchical clustering.

Distance	Linkage	Use PCA data
Euclidean	Ward	Yes, reduce to 70% explained variance
Cosine	Average	No!
Correlation	Average	No!

Note: Depending on the nature of the data set different settings can lead to better results.

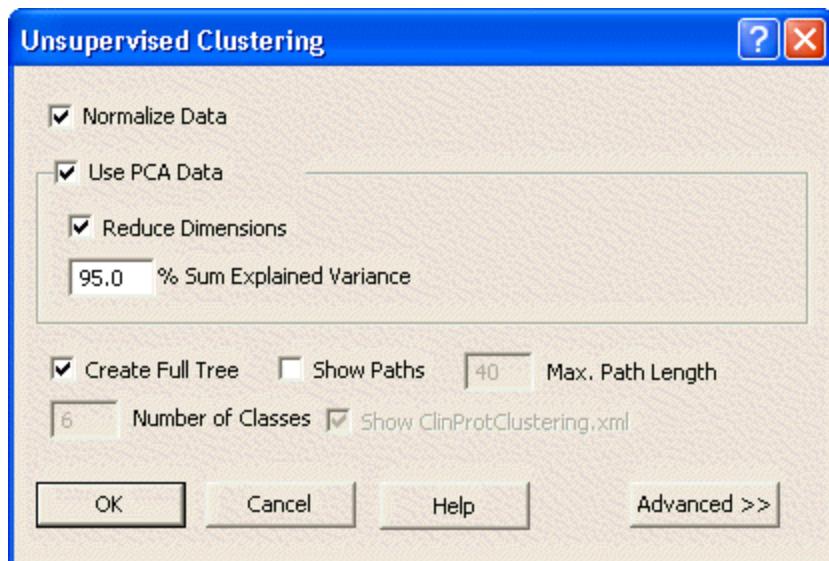


Figure 5-97 ClinProTools' Unsupervised Clustering dialog

After the calculation ClinProTools will show a dendrogram window, which visibly indicates that the calculation is finished. Because the tree contains and displays all spectra, it looks quite crowded. Please note that each node in the dendrogram has a specific ID number. At the same time, ClinProTools writes two files with the clustering results, "ClinProtClustering.xml" and "ClinProtClustering.tree.xml". Both files can be found in the folder "C:\BDAL\ClinProTools_2_2\Files".

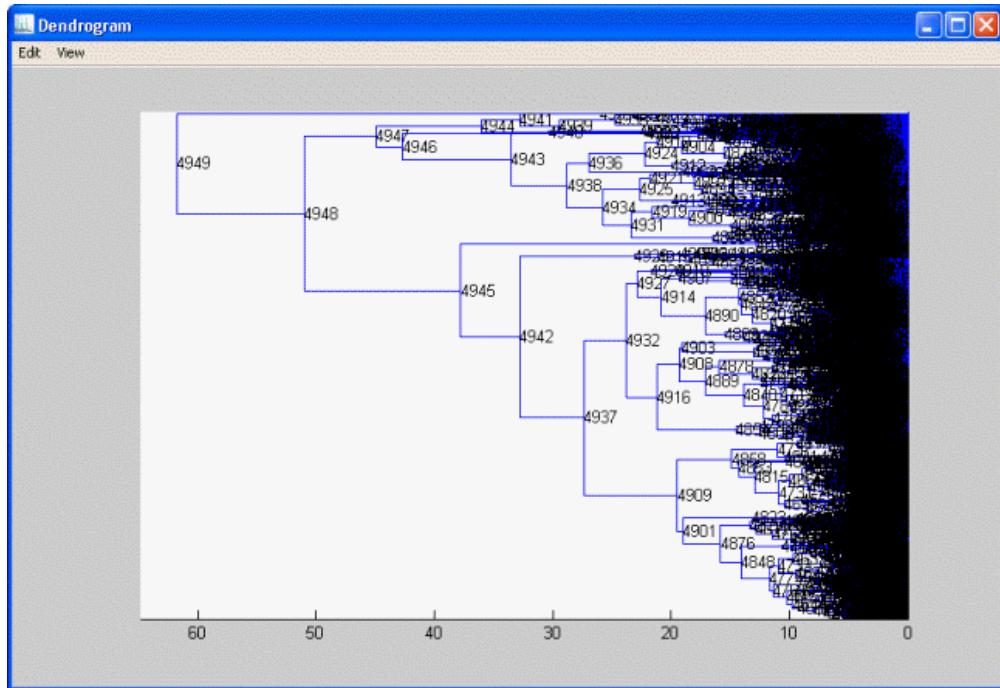


Figure 5-98 Full tree dendrogram created in ClinProTools

In the Results Pane of flexImaging now click **Add...** and in the **Add Result Filter** dialog select **Import ClinProTools Result**. Make sure the Mass Filter Selection Mark is not currently displayed. If it is, hide it by pressing **Ctrl+H**.

In the **Import ClinProTools Result** dialog browse for the file “C:\BDAL\ ClinProTools_2_2\Files\ClinProtClustering.tree.xml” and in the **Import ClinProTools ClusteringTree** dialog specify the available parameter. Because the file “ClinProtClustering.tree.xml” will be overwritten each time an unsupervised clustering is calculated, flexImaging asks whether it should copy the file to the imaging run; this should be confirmed with **Yes**.

Note ClinProTools will also generate two .xml files in the same folder (ClinProtClustering.xml and ClinProtClustering.tree2.xml), so be sure to select the correct file.

After the import is finished, flexImaging will show a dendrogram:

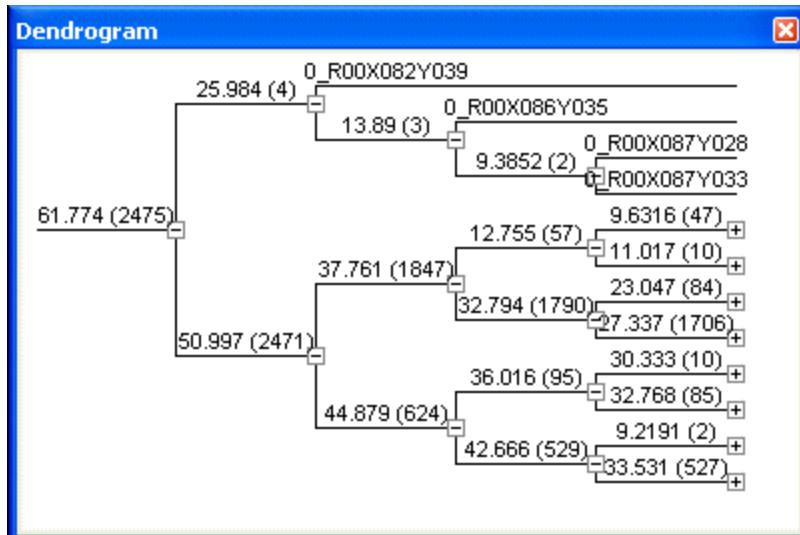


Figure 5-99 Dendrogram imported from ClinProTools

This dendrogram is the same dendrogram that was displayed after the calculation. Only the top nodes are displayed, but it is possible to browse deeper into the dendrogram by expanding selected branches. This is done by clicking on the "+" signs. The number in brackets is the number of mass spectra that belong to each node. The number before can be either the node ID or the cluster distance.

A display filter can now be created by doing a right mouse click on a horizontal branch or the text above it:

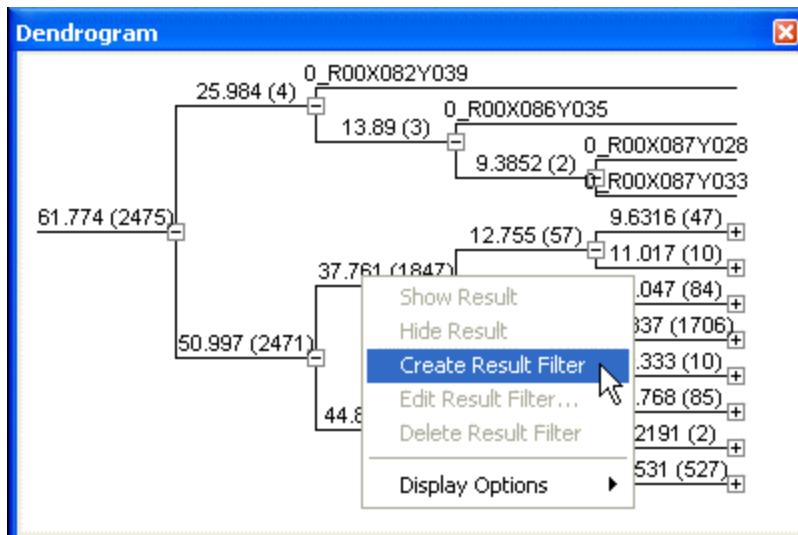


Figure 5-100 Selecting creation of result filter in the Dendrogram node shortcut menu

Now the result filter is displayed. The selected branch assumes the color of the filter and the pixels in the MALDI image that are part of this class are highlighted in the same color. The colors can be edited by a right mouse click on the respective branch. It is also possible to hide or show the result. Note that in the Results Pane of flexImaging for each selected branch a filter is generated. This filter contains the node ID from the tree.

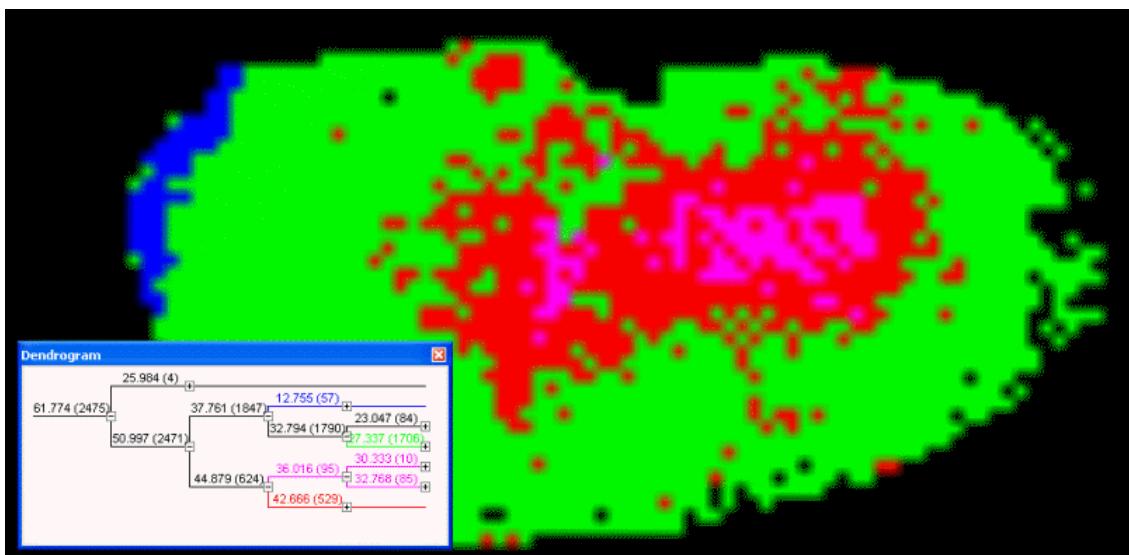


Figure 5-101 Screen shot with selected result filters and respective imaging display of a mouse kidney data set

It is now possible to explore the hierarchical relationships between the classes, and by that to select the classes that correspond to histological features.

5.4.6.4 PLSA Import

A probabilistic latent semantic analysis (PLSA) can be performed on MALDI imaging data with ClinProTools 3.0.

PLSA is a powerful statistical analysis. In the context of MALDI imaging, it can with some restrictions generate distributions of e.g. certain cell types, even if the resolution of the imaging experiment is lower. The use of PLSA to evaluate MALDI imaging experiments is described in: *Hanselmann M, Kirchner M, Renard BY, Amstalden ER, Glunde K, Heeren RM, Hamprecht FA. Concise representation of mass spectrometry images by probabilistic latent semantic analysis. Anal Chem. 2008; 80(24):9649-58.*

Probabilistic Latent Semantic Analysis (PLSA)

In a MALDI imaging data set, often the resolution is not high enough to measure single cells. For each mass spectrometric pixel, the resulting spectra is therefore the average of the contributing single cells.

The PLSA now tries to unmix or deconvolute the MALDI imaging data set in a way that it tries to recreate how the mass spectrum of the contributing components (such as cells) have to look like and how the components are mixed in the tissue. With certain restrictions the result of the analysis can be interpreted as the distribution of different cell types and the resulting spectrum for each cell type. Restrictions are: The data set must show a heterogeneous distribution of the single components and in the ClinProTools implementation the user has to provide a number of expected components and the resulting spectra can be ambiguous.

How to perform a PLSA with flexImaging data in ClinProTools 3.0

The fastest way to do a PCA with ClinProTools 3.0 is to export a list of all spectra from flexImaging and to load that list in ClinProTools 3.0. To export the spectra list do a right mouse click in the Regions Pane in flexImaging and select the option **Export Spectra List**.

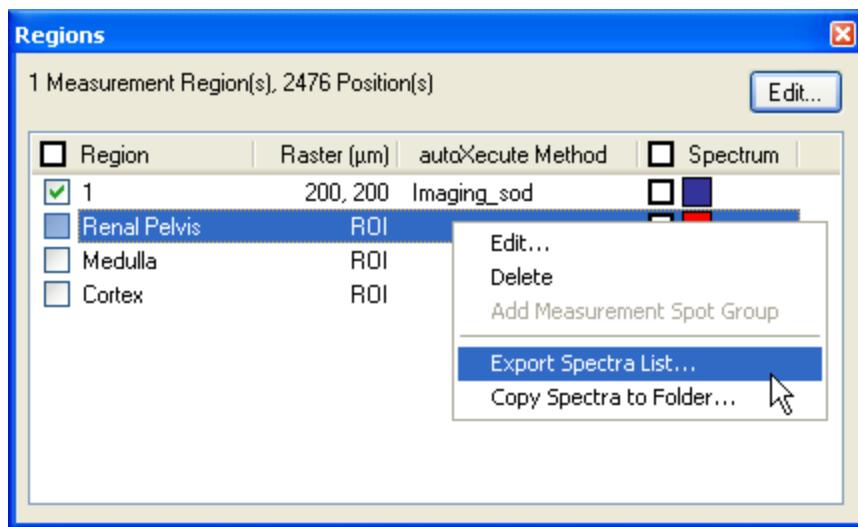


Figure 5-102 Exporting spectra list of selected region

In the **Export Spectra List** dialog, check the measurement region. If regions of interest are defined, do not check them. Export the spectra list. By default the list will have the same name as the imaging run appended by “_Regions.xml” and will be saved next to the *.mis file.

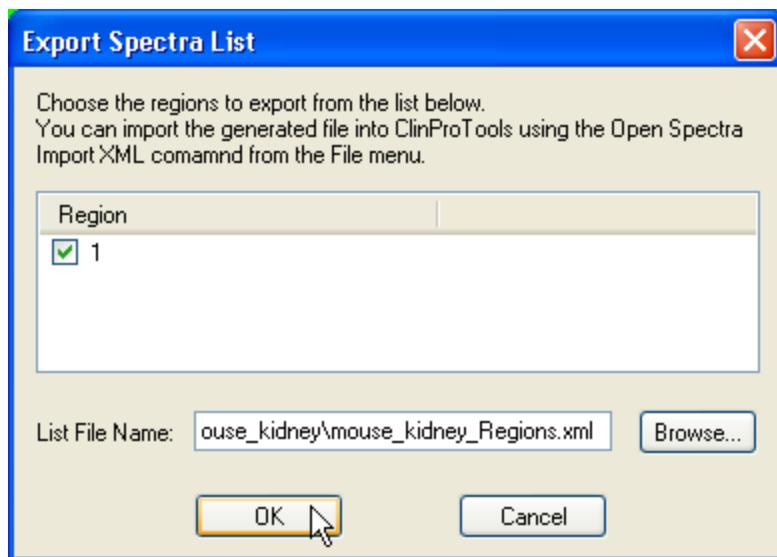


Figure 5-103 Export Spectra List dialog

Next, open ClinProTools and select **Open Spectrum Import xml** in the **File** menu. Select the spectra list (please refer to the ClinProTools manual for the **Settings Spectra Preparation** and **Settings Peak Calculation** commands in the **Data Preparation** menu). After the spectra are loaded in ClinProTools, select **pLSA** from the menu **Statistical Analysis**.

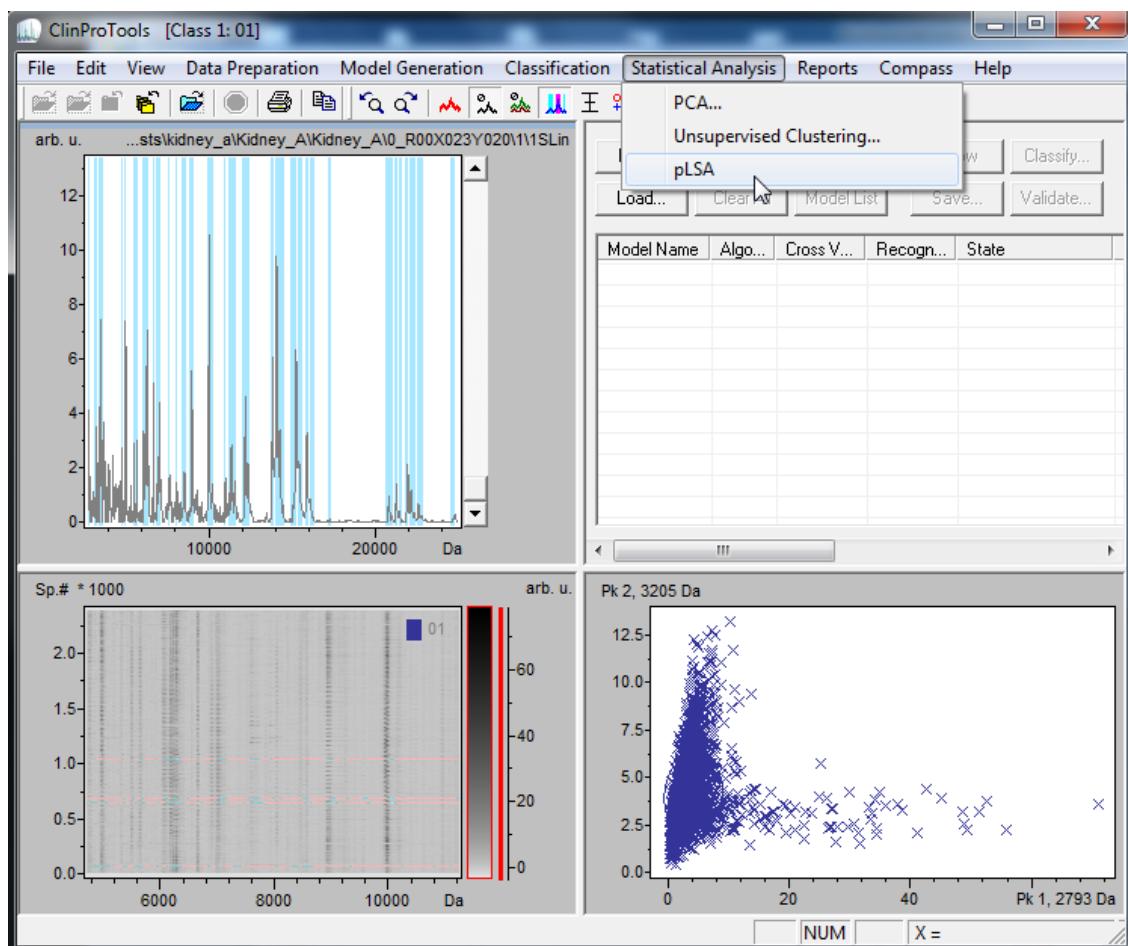


Figure 5-104 ClinProTools pLSA calculation

After the pLSA button is pressed, the **pLSA** dialog appears. The pLSA is an iterative calculation. The user has to specify three parameters, the first two “relative change” and “maximum iterations” are stop criteria. Lowering the value for “relative change” and increasing the “maximum iterations” will result in a longer and more accurate calculation.

The “Number of Components” will define how many components are calculated and is therefore very important. This number has to be defined with knowledge of the sample. A good starting point will be the number of expected cell types in the sample.

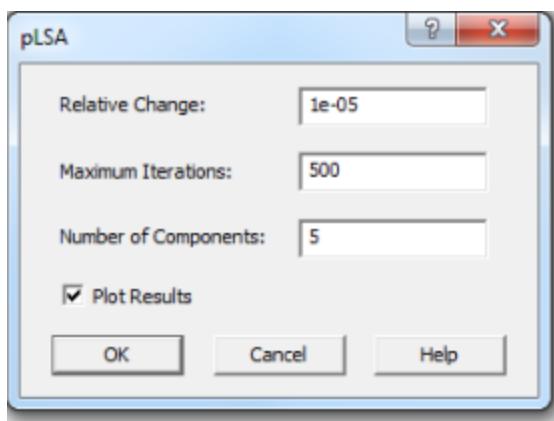


Figure 5-105 pLSA dialog

Press **OK** and the calculation starts. After the calculation the pLSA windows appears which shows the results. At the same time, ClinProTools writes the file "C:\BDAL\ClinProTools_3_0\Files\ClinProtPLSA.xml", which contains the results of the analysis.

Import of pLSA results into flexImaging

In the **Results Pane** of flexImaging now click **Add** and in the **Add Result Filter** dialog select **Import ClinProTools Result**. Make sure the Mass Filter Selection Mark is not currently displayed. If it is, hide it by pressing **Ctrl+H**.

In the Import ClinProTools Result dialog browse for the file "C:\BDAL\ ClinProTools_3_0\Files\ClinProtPLSA.xml" and select "next".

Because the file "ClinProtPLSA.xml" will be overwritten each time a pLSA is calculated, flexImaging asks whether it should copy the file to the imaging run; this should be confirmed with **Yes**.

The pLSA filters will now be added to the actual result list.

What does the result mean?

If the right number of components was selected for the calculation, the components should correspond with histological features, such as cell types. If there are significant differences to histological features, then the sample preparation might not have been homogeneous.

5.4.6.5 Model Generation and Classification

ClinProTools allows the generation of models based on mass spectrometric profiles and to classify spectra according to those profiles. A model in this context is a set of peaks and intensities that is able to separate classes of spectra according to prior knowledge, e.g. separate spectra from cancer tissue versus epithelial or connective tissue.

Generating a model

To generate a model, ClinProTools expects all spectra of the respective training class in one subfolder. If a model should be calculated that contains e.g. cancer, epithelial and connective tissue spectra, it is necessary to provide three subfolders that contain the respective spectra. This can easily be done by creating regions of interest in flexImaging. The regions should contain typical spectra for the tissue features in question. Therefore, it is useful to use regions that contain mainly the cells in questions, e.g. the most “pure” cancer cell, connective tissue and epithelial cell regions of the section.

The spectra of these regions can be copied to specific folders by selecting the region in the Regions Pane with a right mouse click and then select **Export Spectra List**. (For ClinProTools 2.1 select **Copy Spectra to Folder**. In this case specify target directory and sample name, and quit the message on copied spectra). Select the regions that you want to use for the model generation and confirm the suggested name for the *.xml file. The file will be placed next to the imaging run.

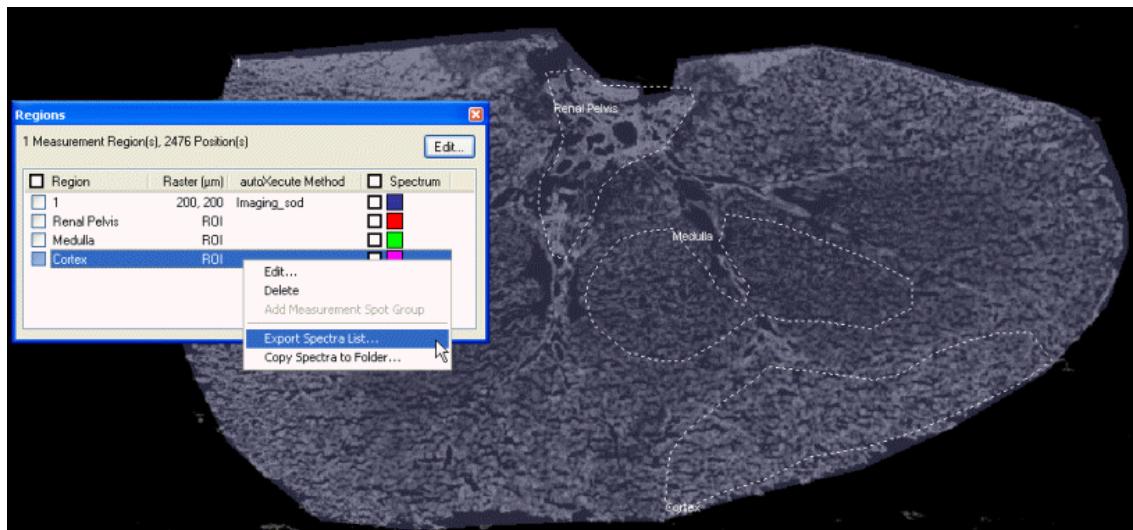


Figure 5-106 Copying regions

Next, open ClinProTools and select **Open Spectrum Import XML** in the **File** menu. Select the spectra list. It may be necessary to adjust the **Settings Spectra Preparation** and **Settings Peak Calculation** in ClinProTools' **Data Preparation** menu in order to get good results. Please refer to the ClinProTools manual for details. If ClinProTools 2.1 or lower is used, then the folder to which the region spectra have been exported has to be selected after choosing **Open Model Generation Class** in the **File** menu.

Generating a model from more than one section

The same workflow can be followed if a model shall be created from more than one section, e.g. from the tumor and connective tissue regions of data sets from different patients. A little preparation is necessary here. All spectra that define one class are copied from the original exported spectra lists of the individual samples into one new list. This can be done in a text or an xml editor. Bruker also provides the **ClinProTools Spectra Import XML Generator**, a simple tool for creating more complex spectra lists using a graphic interface. Please refer to the tool's manual for detailed instructions.

Another option (and with ClinProTools 2.1 the only possibility) is to copy the spectra for the regions in subfolders using the **Copy Spectra to Folder** command. After copying the spectra of the regions to a subfolder, however, it is necessary to move the subfolders into one specific folder for each model generation class. (The folders could be copied also, but moving the folders will be a lot faster, if the target folder is located on the same hard drive than the imaging data).

In such a case, it should be considered to use the **Support Spectra Grouping** checkbox in ClinProTools (menu **Data Preparation > Settings Spectra Preparation**). If this option is used then each spectra collection from each patient will be treated as one sample, and each spectrum in the region will be treated as a replicate for that sample. This will eliminate the possibility to overweight those samples that have a higher number of spectra in the respective region.

Classification of spectra from flexImaging with ClinProTools

For classification of imaging spectra simply select the model in ClinProTools and select **Classify** in the **Classification** menu. You will now be prompted to select a directory with spectra. Browse for the folder that contains the imaging spectra (see flexImaging file locations). It is recommended to use the **Classify in Batch Mode** option in the ClinProTools general settings. Then ClinProTools will classify the spectra one by one, and there is no limit on the number of spectra to be classified. If this option is not used, then ClinProTools will load all spectra first before starting the classification.

After a classification the Internet explorer will open and show the classification results. Save this file. It is recommended to save the file next to the imaging run.

Import of classification results in flexImaging

In the Results Pane click  and in the **Add Result Filter** dialog select **Import ClinProTools Result**. In the **Import ClinProTools Result** dialog browse for the file C:\BDAL\ClinProTools_2_2\Files\ClinProtClassification.xml and in the **Import ClinProTools Classification** dialog specify the options for the ClinProTools classification import. The number of classes in the ClinProTools model is automatically detected during the import resulting in one filter being created for each class.

If for the model generation the algorithms “Genetic Algorithm”, “Support Vector Machine” or “Supervised Neural Network” have been used, then the ClinProTools classification result will only contain the information to which class the spectra belong. Each data point in the result display will have the same color intensity.

If the univariate “QuickClassifier” has been used, each spectrum can belong to more than one class with a different weight. The absolute value of the classifications for the different classes will always add up to the number of classes. If, e.g. four classes have been used, the weight for each class for each spectrum will add up to four. A spectrum could therefore have a weight for class one of three, and for class two to four for each a weight 0.33. Because of that behavior, the color intensity of each pixel in the result can be different and reflects that value.

This can also be used to do a simple outlier control. If a spectrum has a value of around 1.0 for all classes, it belongs to all four classes with the same likeliness. This can also be interpreted as the spectrum belongs to no class. To exclude those spectra from being displayed in flexImaging you can use the **Treat spectra with a relative weight below X.X as unclassified** option in the import dialog. The relative weight will then be translated into a **Minimum Intensity** setting, which can be modified in the **Edit ClinProTools Classification Filter Parameters** dialog.

Outliers in supervised classifications

A supervised model is a classifier trained with sample data sets. If two classes are defined, then every spectrum to be classified will be forced into one of the classes. The model itself has no information on the shape of the original spectra. This means that each spectrum will be classified, even if its peak pattern has only little similarity with the training data sets.

In the context of MALDI imaging, it is therefore possible to generate a model that can e.g. differentiate between tumor and tumor-free mucosa. If this model is applied to tissue that is neither, e.g. a nerve or a muscle, this tissue will be classified as either tumor or mucosa. It is therefore up to the user to apply these models only to suited tissue. To facilitate this decision, ClinProTools 2.2 and higher has the capability prepare the data for outlier detection.

Preparing the outlier detection in ClinProTools

To use the outlier detection in ClinProTools (version 2.2 and higher) it is necessary to enable the **Peak Picking on Single Spectra** option (in **Data Preparation > Settings Peak Calculation**). If a model is generated now, then it contains information on the entire peak pattern of a spectrum, not only the peaks that discriminate between the classes. In the classification this information is used to score the similarity of the spectrum to be classified with the training data set. There will be one overall score; this score will give a value for how similar the spectrum is in comparison with all spectra used for the model generation. For each class, there will also be a class score. This class score gives a value for the similarity of the spectrum to each of the individual classes. Each of the scores will have a value between 0 for low similarity and 1 for very good similarity. It is currently not possible to automatically calculate an outlier threshold. This threshold needs to be manually assessed, which is easiest in flexImaging. Some limitations apply, especially if one class contains spectra with a low number of signals and other classes contain many peaks, the spectra with a low number of peaks tend to get a very low similarity score.

Use of the outlier score in flexImaging

When flexImaging imports a classification result that contains similarity scores, it will automatically create an additional result filter “ClinProtOverall Score”. This filter will show the similarity score for each spectrum in the data set. Now the minimum intensity threshold for that filter can be used to find a score that separates the “good” classifications from the outliers. This is done by visual inspection of the result. In case of the classification of cancer, the threshold needs to be adjusted so that only cancerous areas are visible with that filter. The histogram view is a very helpful tool to get an idea of the distribution of the overall score.

Now the individual “ClinProt Class” filters can be adjusted. If such a filter is edited, the **Advanced** button will enable additional settings: With **Minimum Overall Score**, all spectra with less than the specified value will be excluded. It is also possible to specify a **Minimum Class Score** that excludes all spectra of this class if their class score is less than specified. In addition, the similarity score can be used as a classifier: If **Use class scores from outlier detection** is checked, then the class score will be displayed directly, and the original classification result will not be shown.

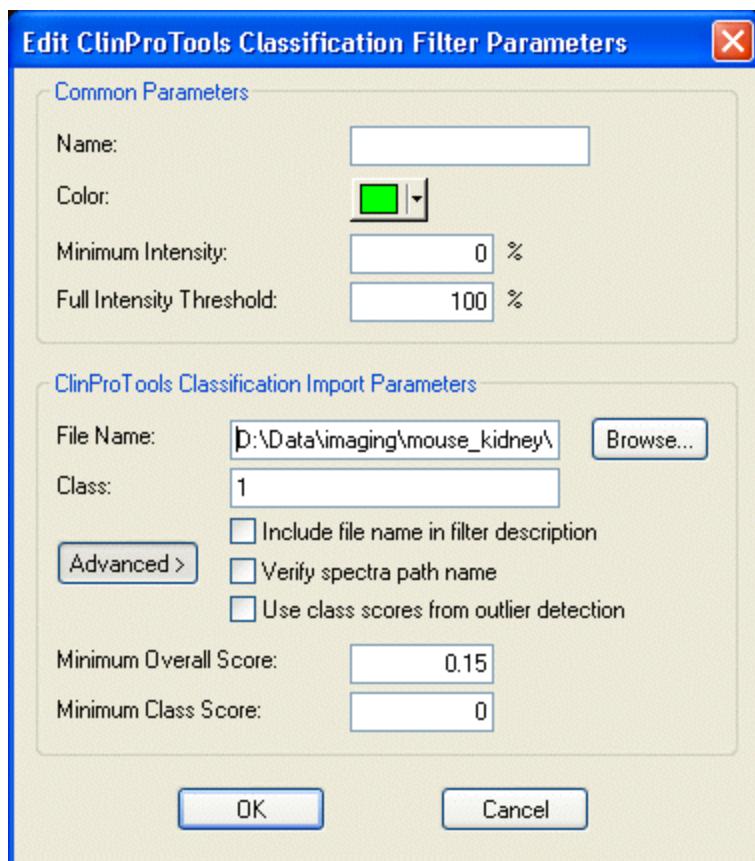


Figure 5-107 Edit ClinProTools Classification Filter Parameters dialog

5.4.7 Hierarchical Clustering

flexImaging contains an efficient hierarchical clustering algorithm that allows the clustering of large data sets. The algorithm has an optional (but recommended) edge-preserving denoising step (as described in Alexandrov et. al.)¹.

It is also possible to use ClinProTools to calculate hierarchical clustering results for flexImaging (see section 5.4.6.3). ClinProTools has more options for the clustering but is limited in the number of pixels and offers no edge-preserving denoising.

5.4.7.1 Why use Hierarchical Clustering?

Hierarchical clustering is a means to generate segmentation maps of the imaging data set. The aim is to give pixels with a similar mass spectrometric profile the same color. This provides a concise representation of the data set. Instead of tediously looking through hundreds or thousands of individual ion images, the main structure in the data is presented in a straightforward way.

The clustering may also be able to find latent structures in the data that are not visible in individual ion images. A simple case would be different areas that are defined by varying ratios of several mass signals. Clustering may highlight distinct regions that are not seen in any of the individual signals.

The particular advantage of hierarchical clustering is the hierarchical structure. Each cluster can be split interactively into sub-clusters. This allows a semi-supervised annotation of the data: While the clustering itself is unsupervised, the user can manually identify the relevant clusters.

A complete annotation of a data set can be accomplished much faster than by either drawing regions based on the histology or looking through individual mass signals.

In a workflow that aims to compare many samples, the hierarchical clustering is also a fast way to define the relevant spectra in each data set (for example, all spectra that represent invasive tumor in a data set). The spectra in these clusters can then be exported for downstream analysis (for example, using ClinProTools).

¹Alexandrov, T. et al. *Spatial segmentation of imaging mass spectrometry data with edge-preserving image denoising and clustering*. J Proteome Res. 2010 Dec 3;9(12):6535-46.

5.4.7.2 How does Hierarchical Clustering Work?

The hierarchical clustering works in an agglomerative way, building the dendrogram from the bottom up.

The process is quite simple:

- First, the algorithm identifies the two most similar spectra in the data set. These two spectra will form a cluster, and this cluster becomes a new element.
- Next, the algorithm finds the two most similar elements, which could either be two spectra or a spectrum and the already existing cluster (or in further iterations, two existing clusters). This process is iterated until one top-level element remains.

The dendrogram is therefore built from the bottom up, but the interpretation is done top-down. flexImaging calculates the similarity of two spectra by their Pearson correlation coefficient. The main advantage of this similarity measure is that it is independent from the overall intensity of the spectra and therefore not affected by different normalization settings.

The similarity of the cluster is calculated by the average distance of the individual spectra in the clusters (average linkage). One usually expects that the "main" structure of the data set is represented in the nodes near the top of the dendrogram.

The flexImaging user interface allows an easy interaction with the dendrogram by displaying the top nodes of the dendrogram. All spectra belonging to one node can be displayed by right-clicking the selected branch and selecting **Create result filter**. The branch of the dendrogram is made the same color as the selected pixels in the result view.

The color can be changed by right-clicking the branch and selecting **Edit result filter**. A result filter can be deleted by selecting **Delete result filter**.

Each selected result filter creates an entry in the Results Pane. If dendrogram results are present in the result list, the dendrogram can be opened by right-clicking the filter and selecting **Show dendrogram**.

5.4.7.3 Limitations of Hierarchical Clustering

Hierarchical clustering has two main limitations:

- Hierarchical clustering is not well-suited to find continuous changes. For example, if different cell types have a continuously changing ratio in a data set (such as an

infiltration of lymphocytes with varying density into an area with tumor cells in varying density), the clustering will not represent this adequately.

- There are several possibilities, such as clusters that represent only the "main" cell type. However, because hierarchical clustering is not well-suited to representing such situations, there is also the possibility of many clusters that do not seem to match well to the structure of the data set. Other techniques (such as the probabilistic latent semantic analysis (PLSA) available in ClinProTools) are better suited to analysis of these kinds of data.
- If the data are noisy, then the clustering may become complex. There is a possibility that at the top nodes random noise spectra are found, while the "true" structure of the data is found deep into the dendrogram. In part, this noise is inherent to the MALDI measurement. This is because due to ion statistics, individual mass signals may have a significant variation (up to 30%) from pixel to pixel. The sample preparation may add additional variability. This effect can be minimized by a spatial denoising (or smoothing).

5.4.7.4 Why Use Edge-Preserving Denoising?

MALDI spectra usually show a variation of individual ion signals. Even if the very same sample is measured repeatedly, the ratios of individual peaks varies.

These intensity variations appear as noise in MALDI images. Humans are usually quite good in pattern recognition and are able to see the structure underneath the noise. For hierarchical clustering, this noise has a detrimental effect and may lead to overlapping clusters of spectra from different regions.

This can lead to random assignments of spectra to the "wrong" clusters and to unintelligible dendograms. The positive effect of denoising (or smoothing) is based on the assumption that neighboring pixels are more similar than distant pixels.

For example, replacing each pixel by the average spectrum of its surrounding 3×3 pixel square will remove a lot of the random noise from the data, because the random effects leading to the noise will be averaged out. For the clustering, this means that the spectra become more similar, the clusters become tighter, and the result becomes clearer.

The disadvantage of such a simple smoothing is that it blurs the edges in the data and creates artificial "mixtures" of spectra at borders between histological structures (which may even show up as individual clusters).

For this reason, flexImaging uses an edge-preserving denoising that detects edges in each individual ion image and retains these edges in the denoising process (as described in Alexandrov et. al.)¹. flexImaging provides four settings for the level of denoising; **none**, **weak**, **normal** and **strong**. Generally, noisier data sets require a higher level of denoising to give good clustering results.

5.4.7.4.1 Limitations of Edge-Preserving Denoising

As the denoising is based on the assumption that neighboring pixels should have a similarity, it will fail to handle very small spatial features correctly, especially if the size of the feature approaches a single pixel.

5.4.8 Exporting and Importing Data for Statistical Analysis (Advanced Users Only)

Many statistical calculations on MALDI imaging data sets (such as t-test, ANOVA, Kruskal-Wallis-tests, ROC, hierarchical clustering, PCA, PLSA) can be calculated by ClinProTools (see interfacing with ClinProTools).

In some cases it may be desired, however, to do calculations that are not offered by ClinProTools or on data types that are not supported by ClinProTools (such as MS/MS spectra). The result of these calculations may also be imported into flexImaging (e.g. classification results).

There are several ways to access data in flexImaging:

- Direct export of the displayed intensity values from flexImaging into a table. This is a straight forward way to do univariate calculations in external programs.
- The data can be loaded into ClinProTools. ClinProTools can be used to do the peak-picking and generate and export a peak list for all spectra. This table can be used in external software. This is the simplest approach for multivariate analyses in external software. It may not be applicable if the data set is too large to be loaded in ClinProTools or if certain custom processing options (such as specific normalization) shall be used. The preferred way to export the peak list is as “*.xml 3” file. For this file a suitable stylesheet is found in the ClinProTools directory, which

¹Alexandrov, T. et al. *Spatial segmentation of imaging mass spectrometry data with edge-preserving image denoising and clustering*. J Proteome Res. 2010 Dec 3;9(12):6535-46.

allows easy editing of the table in spreadsheet program (such as Excel 2003 or higher). See the ClinProTools manual for details.

- If access to the full spectra is necessary, the easiest way is to export the individual spectra of the data set from flexAnalysis as xy text files. These files can be then treated in the external or custom software. This gives full access to the spectral information and is a useful way to do prototyping or individual calculations in external software. The spectra can be exported from the batch-processing tool from flexAnalysis. A flexAnalysis method for the export is available upon request from the software support (maldi.sw.support@bdal.de). For FTMS spectra it is also possible to export those as xy-files. Since signals in FTMS are much sharper than in MALDI-TOF, it is also a viable option here to export only peak-lists after peak-picking.
- If a software is supposed to read the raw data directly, there is a possibility to use Bruker's "compassXtract" interface, which allows 3rd party access to raw data. This requires a license agreement. For details please contact the software support.

Direct export of intensity values from flexImaging:

First a mass filter for the selected mass has to be present in the Results Pane. Right-clicking this filter shows the **Export Filter Data** command, that exports the displayed values into a text file.

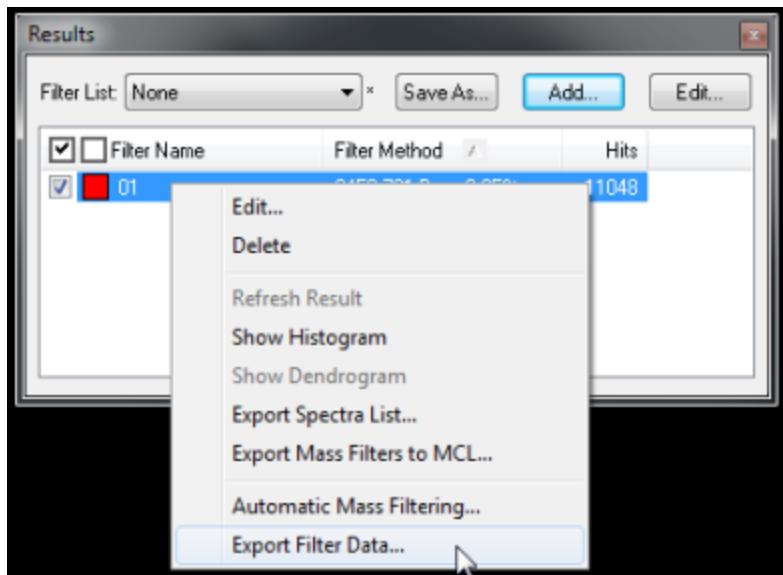


Figure 5-108 The Export Filter Data command in the Results Pane shortcut menu

The resulting text file has a structure like this:

```
# D:\example.txt generated by flexImaging 5.0. on [date]
#spot-name value region xpos ypos
0_R00X618Y250 0.229534 0 618 250
0_R00X619Y250 0.244719 0 619 250
0_R00X620Y250 0.263511 0 620 250
0_R00X621Y250 0.241105 0 621 250
0_R00X622Y250 0.275589 0 622 250
```

The “spot-name” is the name of the spectrum, and e.g. necessary if the result of the calculation shall later be imported into flexImaging. The “value” represents the value that the respective pixel has in the current display, with “1” representing the full brightness. For most statistical calculations it will be necessary to correctly reflect the full dynamic range of the measurement and therefore no minimum intensity or maximum intensity threshold must be adjusted.

The “region” reflects the measurement region. If several regions of interest from the same measurement region are to be compared, then the filter export has to be done multiple times, with the display restricted to each region of interest once.

If data sets are to be compared across different measurements, then it is necessary to use “absolute intensity” setting in the mass filter, with the same absolute limit for all measurements. The absolute limit should be set to the brightest pixel off all measurements. The easiest way to find this is by using the histogram display.

Importing data into flexImaging

Any kind of numerical data or classification can be imported and displayed in flexImaging. This is easiest, if the data is available as a whitespace separated table with the spectrum name (and therefore coordinate) in the first column and one or more values in additional columns. E.g.:

```
# D:\example2.txt
#spot-name value1 value2 value3
0_R00X618Y250 0.229534 0 213
0_R00X619Y250 0.244719 0 476
0_R00X620Y250 0.263511 1 685
0_R00X621Y250 0.241105 1 231
0_R00X622Y250 0.275589 1 612
```

The import can be done in the Results Pane with the **Add > Import text result** command.

If the data is available as an XML file, the import is possible via the results pane **Import xml result** command.

Appendix A — Appendix

A.1 Abbreviations

Abbreviation	Explanation
MALDI	Matrix-Assisted Laser Desorption/Ionization
PC	Principle component
PCA	Principle Component Analysis
RAM	Random Access Memory
ROI	Region of Interest
SRM	Single Reaction Monitoring
TFA	Trifluoroacetic acid
TLC	Thin-Layer Chromatography
XML	Extensible Markup Language

A.2 Part Numbers

- # 1839454 Software-Package flexImaging 5.0
- # 1839450 License flexImaging 5.0
- # 1839452 flexImaging 5.0 User Manual
- # 1839453 flexImaging 5.0 Workflows Manual

Index

1

1D Scan plot 37, 97

2

2D Density plot 37, 97, 102

2D Scan plot 37, 97, 102

2D Stack plot 37, 100, 102

A

Abort autoXecute Run command 56

Aborting

 autoXecute run 56

About flexImaging command 90

Acquired data

 Deleting 56

 Exporting 58

Acquired Data command 58

Add Mass Filter command 105

Add Measurement Spot command 64, 96

Add Measurement Spot Group command 97

Add Polygon Measurement Region
command 63, 94

Add Polygon Region of Interest command 66,
95

Add Rectangular Measurement Region com-
mand 62, 94

Add Rectangular Region of Interest
command 66, 95

Adding

 Mass filter 105

 Measurement region 62-63, 94

 Measurement spot 64, 96

 Measurement spot group 97

 Region of interest 66, 95

 Temporary mass filter 101

Analysis

 clustering 76

Arbitrary arrays 20, 130, 283

Artifacts

 recognizing 328

Auto Scaling command 101

Automatic Mass Filtering command 67, 113

autoXecute run 55-56

Average spectrum 37

Average value 43, 114

Average Value command 114

B

Background Color command 105

Basic workflows

 Arbitrary arrays 20

 Spot microarrays 20

 Tissue profiling 21

Biomarkers

 finding 330

C

- Changing
 Operator 81
 Password 81, 83
- Checklist 49, 75
- Checklist command 75
- Choose between ClinProTools PCA and Variance Ranking dialog 183
- Classification by hierarchical clustering 354
- Clear Teach Points command 62
- Clearing teach points 62
- ClinProTools 15, 19, 43, 48, 58, 109, 111, 116, 168, 172-173, 176, 195, 198, 200-201, 203, 205, 208, 210, 291, 330, 342-344, 352, 354, 360, 365, 370, 373
- Classification 365
- Classification import 178
- interfacing with 343
- Model generation 365
- ClinProTools classification filter 112, 178
- ClinProTools clustering tree filter 112
- ClinProTools PCA filter 112
- ClinProTools variance ranking filter 112
- Closing flexImaging 21
- Clustering 77, 110
- Co-Register Image command 65
- Co-registered image 36, 65, 70-71
- Co-Registered Image command 70
- Co-registered images 71
- Co-Registered Images command 71
- Co-registering images 312
- Compare Single Spectrum command 106
- Compass Desktop command 87
- Completing ClinProTools Clustering Tree Import dialog 191
- Completing ClinProTools Import dialog 190
- Completing dialog 134
- Coordinates 50, 100
- Coordinates command 100
- Copy as CSV command 100
- Copy command 100
- Copy Image to Clipboard command 61, 93
- Copy to Spectra Folder command 110
- Copying
- Image to clipboard 61, 93
- Region of interest spectra 110
- Spectra to folder 110
- Spectrum Display 100
- Cryosections
- preparing 231
- Customize Toolbar command 77
- Customizing
- Toolbar 77
- Window layout 51, 76

D

- Data acquisition
 - arbitrary arrays 283
 - flex series instruments 257, 275
 - FTMS instruments 280
 - incremental runs 283
 - MS/MS experiments 297, 301
 - spot microarrays 286
 - using HyStar and ftmsControl 307

Data processing

- normalization 319
- using flexAnalysis 315
- using flexImaging 315

DataAnalysis

- Open spectrum in 106
- DataAnalysis command 88

- Delete Acquired Data command 56
- Delete command [Regions Pane] 109
- Delete command [Results Pane] 112
- Delete Last Teach Point command 62
- Delete Measurement Spot command 64, 96

Deleting

- Acquired data 56
- Measurement region 109
- Measurement spot 64, 96

Region of interest 109

Result filter 112

Teach point 62

Dendrogram Window 48-49

Shortcut menu 115

Display Mode command [1D/2D Scan Plot] 103

Display Mode command [2D Density Plot] 104

Display Type popup command 102

E

Edit command [Regions Pane] 109

Edit command [Results Pane] 111

Edit Mass Filter Mark Parameters command 106

Editing parameters

Mass filter mark 106

Exit command 60

Export > Mass Filters to MCL command 59

Export > Spectra List command 58

Export Filter Data command 113

Export Mass Filters to MCL command 112

Export popup command 57-58

Export Spectra List command 109, 112

Exporting

Acquired data 58

Image 57

imzML files 225

- Region of interest spectra 109
Spot list 58
- F**
- File locations 15-16
Filter Preview command 76
Filter Preview Window 76
Fit to Window command 70
flexAnalysis 315
 Open spectrum in 106
flexAnalysis command 87
flexControl 9, 25, 28, 34, 41, 53, 62, 74, 78, 94, 124, 140, 150, 163, 240, 257, 277, 284, 287, 294, 297, 311, 315
flexControl command 87
flexImaging
 Basic workflows 20-21
 Closing 21, 60
 Dialogs 117
 File locations 15-16
 flexImaging windows 35
 Installing 12
 Licensing 12, 78
 Locking 85
 Logging in 17, 81
 Menus and commands 28
 Shortcuts 33
 Starting 17
- System requirements 11
Toolbars 24
Uninstalling 14
Windows 35
- flexImaging and MSMS measurement - SRM 305
flexImaging dialogs 117
flexImaging menus 52
ftmsCcontrol
 Data acquisition using 307
ftmsControl 9, 141, 163, 275, 280, 284, 287, 294, 307
- G**
- Grid command 101
- H**
- Hamamatsu converter 224
Help Topics command 89
Hide Mass Filter Mark command 105
Hide X-Axis command 114
Hide Y-Axis command 114
Hierarchical clustering 77, 110
Histogram Window 47
Histological staining 312
Hits command 114
Hits in Percent command 114
HyStar 33-34, 78, 141, 307
 Data acquisition using 307

- HyStar command 88
- |
- Image
- Co-registering 70
 - Copying to clipboard 61, 93
 - Exporting 57
 - Hiding 70
 - Selecting 127
 - Showing 70
- Image Adjustment command 76
- Image Adjustment Window 45, 76
- Image command 57
- imagePrep
- matrix coating with 238
- Images
- co-registering 71, 312
- Imaging Display
- Description 36
 - Shortcut menu 92
 - Zooming 69-70
- Imaging Display shortcut menu 92
- Imaging run
- Creating 54
 - Opening 54, 59
 - Processing options 126
 - Properties 67, 97, 120, 122, 148, 151
- Recent file 59
- Robot import 124, 157
- Saving 54-55
- Selecting sample image 127
- Teach sample explanations 129
- Teaching sample 62, 130
- Imaging Run Properties - Current Imaging Run Parameters dialog 148
- Imaging Run Properties - Processing Options dialog 151
- Imaging Run Properties command 67, 97
- Imaging Run Properties dialog 148, 151
- Imaging Run Properties page 1 dialog [New Imaging Run Wizard] 120
- Imaging Run Properties page 2 dialog [New Imaging Run Wizard] 122
- Import ClinProTools Classification dialog 178
- Import ClinProTools Clustering dialog 179
- Import ClinProTools Clustering Tree dialog 181
- Import ClinProTools PCA dialog 185
- Import ClinProTools PLSA dialog 188
- Import ClinProTools Result dialog 176
- Import ClinProTools Variance Ranking dialog 186
- Import popup command 56
- Importing
- ClinProTools classification filter 178
 - ClinProTools clustering filter 179

- ClinProTools clustering tree filter 179
ClinProTools PCA 183, 185
ClinProTools result 176
ClinProTools variance ranking 183, 186
Sample preparation robot data 124, 157-158
Spot list 56, 124, 157-158
imzML export 225
Installing flexImaging 12
Intensity captions 72
Intensity Captions command 72
Intensity scale 36, 72-73
Intensity Scale command 72-73
- L**
- Laser spots 36, 73, 139
Laser Spots command 73
License command 78
Licensing flexImaging 12
Lock All Applications command 85
Locking flexAnalysis 85
Logging an operator into flexImaging 17
- M**
- Mass defect filter 222
Mass filter
 Adding 105
 Automatic mass filtering 67
Creating temporary mass filter 101
Deleting 112
Mass filter mark
Creating 101
Editing parameters 106
Hiding 105
Mass Filter Selection command 101
Mass Window Distance command 102
Matrix
 coating with imagePrep 238
 manual coating 241
 robotic spotting 243
Maximum spectrum 74
Measurement region
 Adding 62-63, 94
 Deleting 109
 Disabling 41
 Enabling 41
 Hiding 71
 Listing 41
 Showing 36, 71
Measurement Regions command 71
Measurement spot
 Adding 64, 96
 Deleting 64, 96

Measurement spot group	Spectra in DataAnalysis 93
Adding 97	Spectra in flexAnalysis 93
Disabling 41	Operator
Enabling 41	Changing 81
Hiding 71	Changing password 81
Listing 41	Logging in 17, 81
Showing 71	Operator command 81
Model generation and classification 365	Optical image
Move Sample Carrier command 65	acquiring 253
MS/MS experiments 297	P
MS/MS measurement - Fragments only 301	Password
N	Changing 83
Navigation command 76	Patents 10
Navigation window 44, 76	PCA import 344
New Imaging Run command 54	Pharmaceuticals
Noise spectra	detecting 245
Excluding 329	Preferences - Imaging Run Defaults dialog 141
Normalization 74, 151, 319	Preferences - Instrument dialog 139
O	Preferences - Processing Defaults dialog 143
Open Imaging Run command 54	Preferences - Updates dialog 146
Open Spectra in DataAnalysis command 93	Preferences - User Interface dialog 135
Open Spectra in flexAnalysis command 93, 106	Preferences command 77
Opening	Preferences dialog 135, 139, 141, 143, 146
Imaging runs 54	Processing options 126
Recent imaging run 59	Processing options dialog 126

R

- Re-entering password 80
Recent Imaging Runs popup command 59
Refresh Result command 112
Refresh Results command 74
Refreshing
 Result filter 112
 Results 74
reg. Image as B/W command 71
Region of interest
 Adding 66, 95
 automatically add 221
 Copying spectra to folder 110
 Deleting 109
 Disabling 41
 Enabling 41
 Hiding 71
 Listing 41
 Showing 36, 71
Regions command 75
Regions list 41
Regions of Interest command 71
Regions Pane 41, 75, 108
Regions Pane shortcut menu 108
Report Status command 89
- Restore Default Layout command 76
Result color gradient 36, 73
Result Color Gradient command 73
Result filter 112
 Deleting 112
 Disabling 43
 Enabling 43
 Intensity adjustment 45
 Listing 43
 Refreshing 112
 Result filter list 43
 Results command 75
 Results Pane 43, 75, 110
 Results Pane shortcut menus 110
 Robot Import dialog 124, 157
 Ruler 36, 72
 Ruler command 72
- S**
- Sample image 36, 70, 127
Sample Image command 70
Sample Image dialog 127
Sample position 36, 65, 73
Sample Position command 73
Sample preparation
 cryosections 231
 tissue sections 231

- Save autoXecute Sequence As command 55
- Save Imaging Run As command 55
- Save Imaging Run command 54
- Saving
 - autoXecute sequence 55
 - Imaging run 54-55
- Scaling command 106
- Scanning
 - tissue sections 253
- Set Teach Points command 62
- Show Dendrogram command 112
- Show Histogram command 112
- Show Single Spectrum command 67
- Single spectrum
 - compare 106
- Skyline Projection command 74
- Spectrum Color command 105
- Spectrum Display
 - Auto Scaling 101
 - Background color 105
 - Copying 100
 - Description 37
 - Display types 37, 102
 - Grid 101
 - Hiding 75
 - Mass filter selection 101
- Scaling of axes 106
- Shortcut menu 97, 100
- Showing 75
- Spectrum color 105
- Switching display types 102
- Zooming 101-102
- Spectrum Display command 75
- Spectrum Display shortcut menu [1D/2D Scan plot 2D Density plot] 97
- Spectrum Display shortcut menu [2D Stack plot] 100
- Split Imaging Run 68
- Spot list
 - Exporting 58
 - Importing 56, 158
- Spot List command [Export] 58
- Spot List command [Import] 56
- Spot List Import dialog 158
- Spot microarray 20, 130
- Spot microarrays 286
- Staining
 - histological 312
- Start autoXecute Run command 55
- Starting
 - autoXecute run 55
 - flexImaging 17
- Status bar 50, 76, 100

- Status Bar command 76
Synchronize Instances command 113
System requirements 11
- T**
- Teach points
 Clearing 62
 Deleting 62
 Hiding 72
 Setting 62, 130
 Showing 36, 72
- Teach Points command 72
- Teach Sample dialog 130
- Teach Sample explanations dialog 129
- Teaching sample 62, 130
- Temporary mass filter 101
- Text filter 112
- Tissue profiling 289
- Tissue sections
 coating with matrix
 manually 241
 using imagePrep 238
 preparing 231
 robotic matrix spotting 243
 scanning 253
- Toolbar
 Customizing 77
- Hiding 74
Reference 24
Showing 74
- Toolbar command 74
- U**
- Undo Zoom command 102
Uninstalling flexImaging 14
Unlocking flexAnalysis 85
- V**
- Variance ranking 352
- View Normalization Symbol command 74
- W**
- Whitewash command 107
- Window layout 51, 76
- Windows
 Customizing layout 51
 Description 35-37, 41, 43-45, 47-49
 Hiding 74
 Restoring default layout 76
 Showing 74
- Windows popup command 74-76
- X**
- X-axis shortcut menu 114
XML result filter 112

Y

Y-axis shortcut menu 114

Z

Zoom In command 70

Zoom Out command 70

Zoom popup command 69

Zooming command 101

Zooming Imaging Display 69-70

Zooming Spectrum Display 101-102

