Spotxel® Microarray 3.6

Microarray Image and Data Analysis Software

User's Guide

20 June 2025 - Rev 10

Spotxel® Microarray is designed solely for research purposes. It is not intended for, nor approved for, the diagnosis of disease in humans or animals.

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1 Introduction

Spotxel® Microarray, formerly Spotxel® Microarray Image and Data Analysis Software, offers easy-to-use and intuitive tools for microarray image and data analysis. The software supports image analysis, automated processing of multiple images, replicate processing, data filtering, and data normalization. These features enhance data quality and reliability, facilitating the identification of key features and samples and the exploration of their relationships through data mining tools.

1.1 Installation

Spotxel® Microarray runs natively on both Windows and Mac OS X platforms. Depending on the installation directory, installing the software may require system administrator rights.

Hardware Requirement

Recommended hardware: 1.3 GHz Quad-Core or more powerful processor, with 8 GB or more RAM.

Windows Platforms

The software is compatible with 64-bit versions of Windows 7, Windows 8, Windows 10, and Windows 11. To install the software:

- Run the installer.
- If the current Windows account is not an administrator, you will be prompted to enter an administrative account and its password.

Mac OS X platforms

The software is compatible with Mac OS X 10.7 and later versions. To install the software:

- Double-click on the installer to initiate the setup program.
- Confirm the installation directory when prompted; by default, this is set to *$HOME/SpotxelMicroarray*.
- Once the installation is complete, navigate to the installation folder and click on the *Spotxel* app to launch the software.

1.2 Product Activation

After installing Spotxel® Microarray on Windows, you may want to activate the software with a trial serial number. This allows you to use premium functionalities such as data quantification, automatic array alignment, and batch processing of multiple images.

For Mac OS X platforms, the trial use of Spotxel® Microarray is automatically managed and does not require activation.

It is important to note that the *GAL Array Editor* module, along with the functionality for handling microarray images, is entirely free and becomes accessible immediately upon software installation, with *no license* required.

When the free trial period expires, you can purchase a software license for continued use of premium functions. Upon purchase, you will receive a serial number to activate the license.

1.3 Upgrade

Simply run the installer for the new version to upgrade. The software configuration will be handled automatically. You do not need to activate the software again if it has already been activated.

1.4 Software User Interface

Associated software controls are grouped in labeled components (Figure 1). We refer to a software component using the name listed in Table 1.

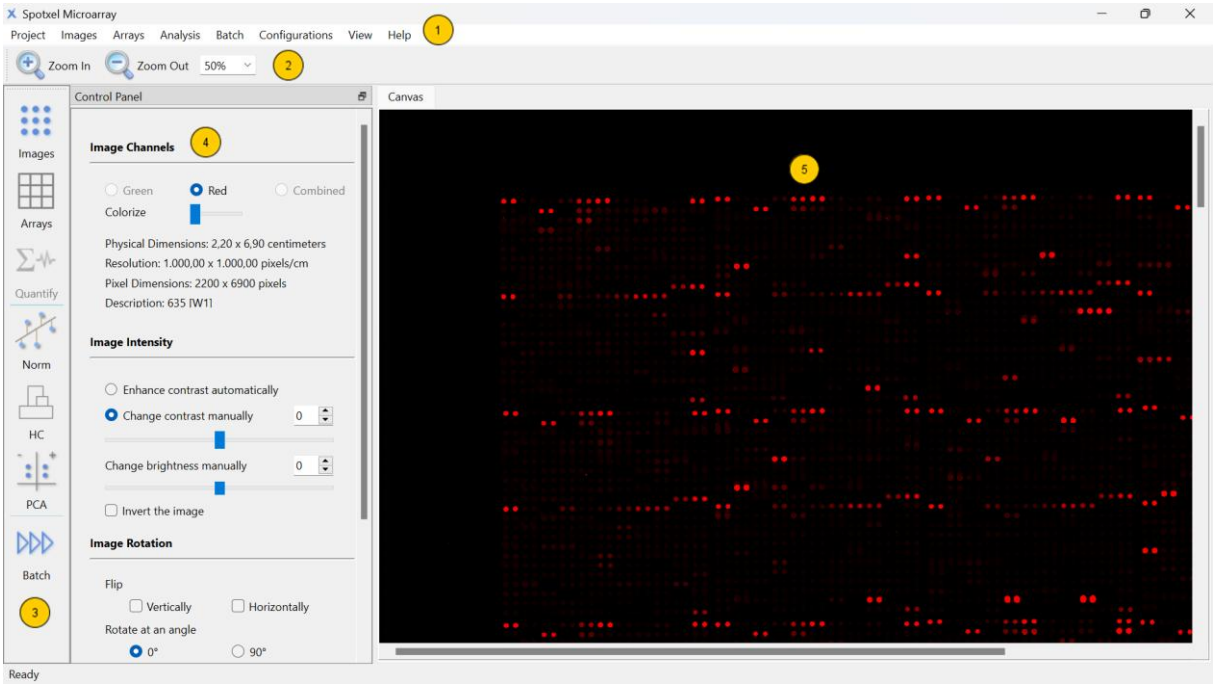


Figure 1: The Software User Interface.

Component	Component Name
1	The menu
2	The canvas toolbar
3	The main toolbar
4	The control panel
5	The canvas

Table 1: Software Components.

The main toolbar provides quick access to a group of related functions. They are described in Table 2. Clicking on a button on the main toolbar opens the control panel for the function group. The software displays the data and the analysis results in a sheet on the right of the control panel.

	Select image channel. Change image's intensity. Rotate images.		Process replicates, filtering data, and normalize data.
	Add, edit, rotate and move blocks. View and edit spots' ID and name.		Hierarchical Clustering Analysis: Show features and samples on a heat map with their correlation.
	Quantify the array data and browse the quantified data.		Principal Component Analysis: Select important features and samples.
	Batch Processing: Automatically process and quantify many microarray images.		

Table 2: The Main Toolbar and Related Functions.

1.5 Terms and Concepts

Array: In this manual, an array is the spot layout plus its annotation. The layout can be stored as either a GenePix Array List (GAL) file (*.gal) or a PepSlide Designer file (PSF) (*.psf).

Microarray image: In a microarray assay, binding events at designated spots generate signals when the slide is exposed to a sample. These signals are captured by a scanner and converted into a digital **microarray image** - a matrix of grayscale pixels whose brightness reflects the signal intensity. For enhanced visual interpretation, our software maps these grayscale intensities to red or green hues.

1.5.1 GAL Arrays – Spot and Block

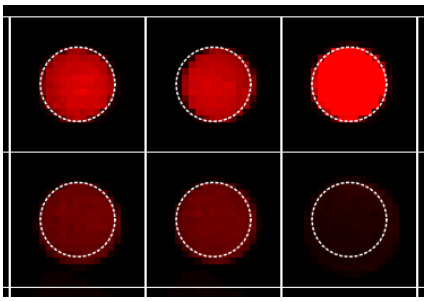


Figure 2: A rectangular block with 6 spots.

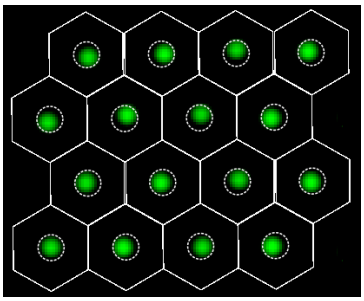


Figure 3: A hexagonal grid with 16 spots.

Spot Appearance: A spot is represented as a white square (see Figure 2) in a rectangular block, or a white hexagon (see Figure 3) in a hexagonal block (commonly referred to as orange-packing).

Spotted Region: Within each spot, the spotted region is enclosed by a dashed circle that indicates where true binding is expected.

Block: An array is composed of blocks, where each block is a group of adjacent spots arranged in a grid.

➢ Working exclusively with GAL arrays? You can skip ahead to section 1.5.3.

1.5.2 PSF Arrays – Spot and Spot Family

Spot Appearance: A spot in a PSF array is displayed as a white rectangle (see Figure 4).

Spotted Region: Within each spot, the spotted region is enclosed by a dashed rectangle, defining the region of interest for quantification.

Spot Family (SF): Spots produced from the same source and located next to each other are grouped as a spot family. In all analysis steps, an SF functions the same way as a block in a GAL array.

Figure 4: Rectangular Spot

1.5.3 Analysis Workflow (Common to GAL & PSF)

Quantification

Quantification estimates the true binding signal at each spot by computing statistical measurements of pixel intensities.

Method:

- Determine the mean and median of all pixels within the spot.

Quality Factors:

- **Spot detection**: Accurate spot finding (or spot detection) determines which pixels in the array image belong to a spot.
- **Background correction**: This step estimates and subtracts non-specific binding signals.

Signal Definitions:

- **Raw**: The original intensity value measured for the spot.
- **Background**: The estimated intensity due to non-specific binding.
- **Foreground**: The binding signal of interest, calculated as the raw intensity minus the background intensity.

Array Alignment

The **array alignment** links each spot in the array to its corresponding signal in the image. Because the spot signal is assumed to represent true binding, the software adjusts the positions of blocks (for GAL arrays) or spot families (for PSF arrays) so that the spotted regions are optimally aligned with the areas of high signal intensity.

1.6 Key Tasks in Microarray Image and Data Analysis

From a software perspective, typical tasks in microarray image data analysis include:

1. *Quantification of Microarray Data*
    - Load the scanned images and the array file.
    - Process the image and array if necessary.
    - Align the array to the images.
    - Quantify the microarray data.
2. *Batch Processing of Multiple Microarray Images (if necessary)*
    - Automate the steps in task 1 for multiple images.
3. *Preprocessing the Data*
    - Use the data filtering and normalization tools.
4. *Discovery of Key Features And Samples*
    - Utilize data mining tools to identify key features and samples.

The following sections explain how to accomplish these tasks using the software.

2 Setting Up for Microarray Image and Data Analysis

2.1 Loading Data

To analyze the microarray data, two input data are required:

- *Scanned images* of the microarray in the TIFF format.
- *Array file* prepared in either GenePix Array List (*.gal) or PepSlide Designer (*.psf).

Spotxel® Microarray supports 8-bit, 16-bit, and 24-bit grayscale images. For high image quality and efficient processing, 16-bit grayscale TIFF images are recommended.

2.1.1 Loading Images

- *Click the Images > Open Image* menu and select the image file(s).
- The software supports analyzing images in single-color mode or dual-color mode. An image needs to be assigned to either the *Red* or *Green* channel for analysis.

For single-page images, you'll be prompted with a dialog as shown in Figure 5 for assigning the color channels. If only one single-page image is selected, then only a single assignment is shown.

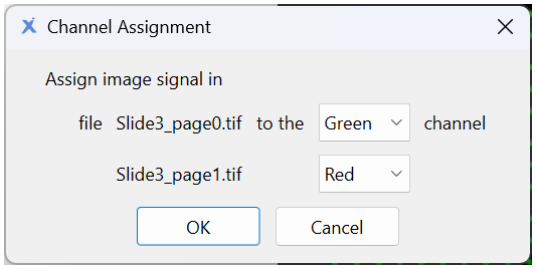


Figure 5: Loading single-page images.

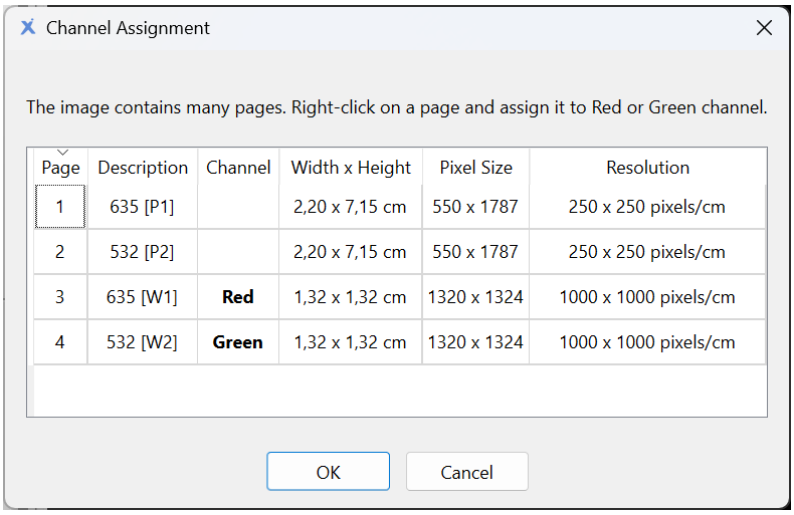


Figure 6: A multi-page image with two pages having the same resolution.

In dual-color mode, you can use either two single-page images or a multi-page image. The two loaded images, or two selected pages, must have the same resolution. The software automatically recognizes whether the images are single-page or multi-page and prompts you with the selection.

If the multi-page image contains two pages of the same resolution (Figure 6), the pages can be assigned to different color channels for analysis. On the other hand, if the pages are of different resolutions (Figure 7), e.g., one is only a preview, and then you can only select the main page for single-color analysis.

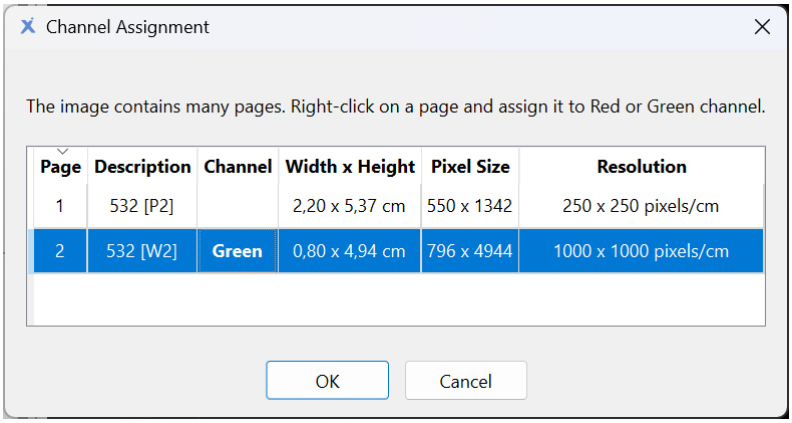


Figure 7: A multi-page image with two pages having different resolutions.

2.1.2 Images without Resolution Information

Usually, TIFF images contain resolution information (pixels per inch or pixels per centimeter), which is read automatically by the software. If this information is not available, the software prompts you to provide it manually by displaying the *Image Resolution Options* dialog (Figure 8). You can also launch this dialog via the *Configurations > Image Resolution Options* menu.

If you are not certain about the resolution values, you can use the default values shown in Figure 8. If the grids appear too small relative to the spots, increase the Resolution X and Y values by a factor of

10 - for example, set them to 10,000 pixels per centimeter. To remember this setting for other images, check the "*Use these options for images without resolution information*" option.

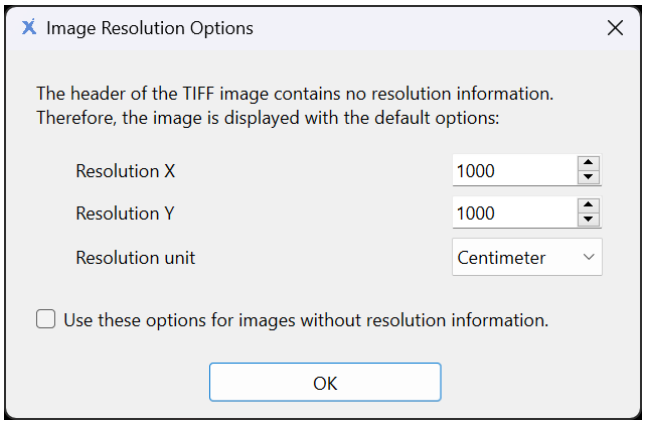
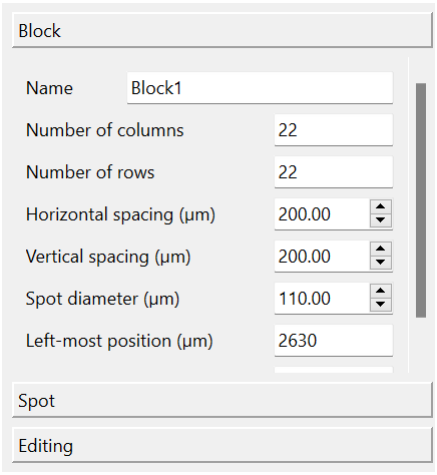
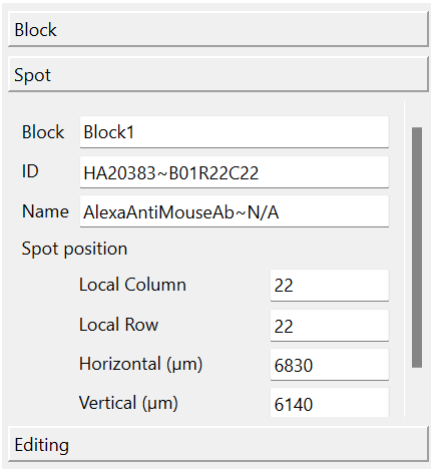


Figure 8: Setting image resolution.

2.2 Loading and Viewing GAL Arrays



(a) Block Properties.



(b) Spot Properties.

Figure 9: Properties of a GAL Array Object.

2.2.1 Loading GAL Array

- Click the *Arrays* > *Open Array* menu and select the GAL array file (*.gal).
- Once loaded, the scanned images and the GAL array are displayed on the canvas (Figure 1).
- Adjust the view using the Zoom In/Zoom Out buttons on the canvas toolbar or by entering a zoom value in the Zoom combo-box.

2.2.2 Viewing GAL Array Data

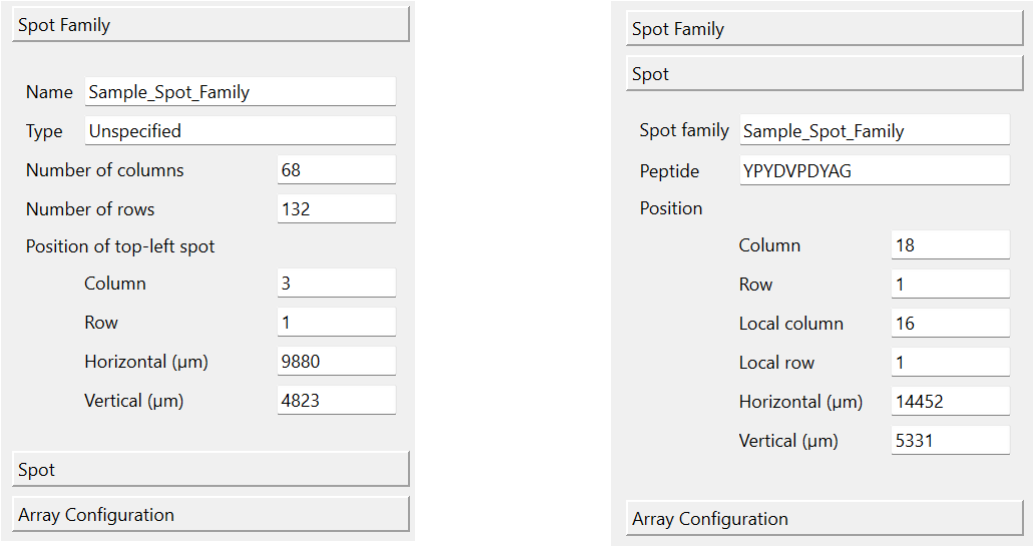
In the *Array File* section of the *Arrays* control panel:

- **Block Properties**:
  - Open the *Block* page by clicking on it.
  - Hover over or click a block in the canvas to view its properties (Figure 9-a).

- **Spot Properties**:
  - Open the *Spot* page.
  - Hover over or click a spot in the canvas to view its properties (Figure 9-b).

A detailed guide on creating and editing an array file can be found in Section *3 - Managing GAL Array Data with GAL Array* Editor. If you already have a GAL array file (**.gal*) and only need to use it, you may proceed directly to Section 3.4.

2.3 Loading and Viewing PSF Arrays



(a) Spot Family Properties. **(b) Spot Properties.**

Figure 10: Properties of a PSF Array Object.

2.3.1 Loading PSF Array

- Click the *Arrays* > *Open Array* menu and select the PSF array file (**.psf*).
- Once loaded, the scanned images and the PSF array are displayed on the canvas (Figure 1).
- Adjust the view using the Zoom In/Zoom Out buttons on the canvas toolbar or by entering a zoom value in the Zoom combo-box.

2.3.2 Viewing PSF Array Data

In the *Array File* section of the *Arrays* control panel:

- **Spot Family Properties**:
  - Open the *Spot Family* page by clicking on it.
  - Hover over or click a spot family (SF) in the canvas to view its properties (Figure 10-a).
- **Spot Properties**:
  - Open the *Spot* page.
  - Hover over or click a spot in the canvas to view its properties (Figure 10-b).

PSF arrays can be edited with PepSlide Designer, available at: https://www.sicasys.de/pepslide.

2.4 Array Alignment

As mentioned in Section 1.5, the array needs to be aligned with the image before quantifying the microarray data. This can be done automatically or manually.

Aligning Array Automatically

- Click the *Align* button in the *Arrays* control panel.
- There are multiple options that can be adjusted to optimize the alignment of your arrays. These options are detailed in Section 4.3.

Aligning Array Manually

- Please refer to Section 3 for a detailed guide of selecting, moving, and rotating blocks.

The aligned position of the blocks in the array can be saved with the *Save* button or the *Arrays* > *Save Array* menu.

2.5 Spotxel Microarray Project File

It is recommended that the analysis of each microarray image be saved to a Spotxel® Microarray *project file (*.spotxelproj*) using the *Project > Save Project* menu. The saved data includes the path to the image, the aligned array, and the quantified data. When opening the project file with the *Project > Open Project* menu, the software will load all the saved data. This enables you to manage all the analysis data for one microarray image with a single project file. Additionally, you can use these project files directly with data mining tools.

The paths to the microarray image, the array file, and the project file are shown in the *Data Files* section of the *Images*, *Arrays*, and *Quantification* control panels.

3 Managing GAL Array Data with GAL Array Editor

To access the *Arrays* functions, you first need to load your microarray image in TIFF format. The TIFF image typically contains the resolution information (pixels per inch or per centimeter), which allows the software to draw the array accordingly, as the values in the array design are in *micrometers*.

If you already have a GAL array file (**.gal*) and only need to use it, you may skip the initial parts of this section and proceed directly to Section 3.4.

3.1 Creating Blocks

To create an array file from scratch, navigate to *Arrays > Array File* and click on the *Create* button. In the dialog that appears, you will have the following options:

- *Block type*: Select either *Rectangular* (a commonly used layout, Figure 12) or *Hexagon (orange packing) even row* (Figure 13) for a more compact layout. Depending on your specific microarray requirements, you may choose another hexagonal layout.
- *Horizontal orientation*: Define the number of blocks to be arranged horizontally and specify the horizontal spacing between two blocks.
- *Vertical orientation*: Define the number of blocks to be arranged vertically and specify the vertical spacing between two blocks.
- *Block properties*: Define the number of rows and columns of spots, the spacing between them, and their diameter.

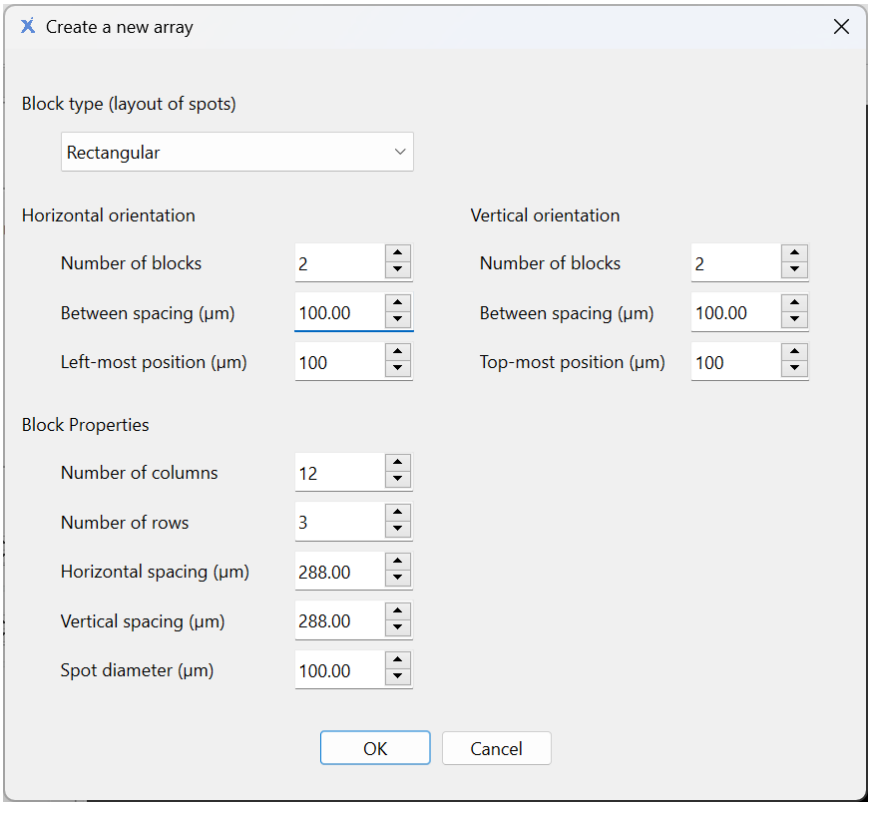


Figure 11: Creating a New Array.

Please note that the values for spacing and diameter can be *fractional*, allowing for precise alignment of the array with the spots on the image later on. After clicking *OK*, the blocks will be added to the canvas. Figure 11 illustrates a sample setup, and Figure 12 shows the created rectangular blocks.

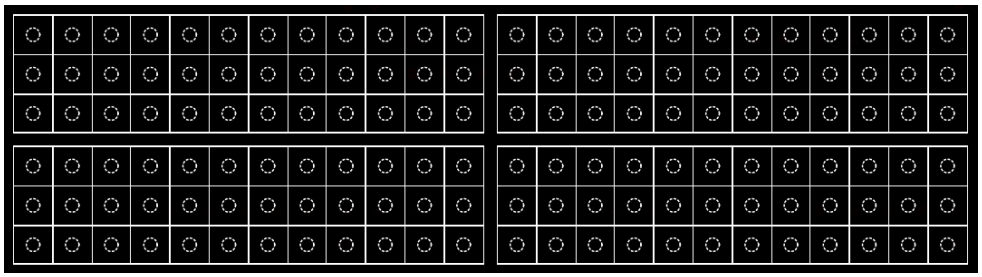


Figure 12: Rectangular Blocks.

Figure 13 presents two hexagonal even-row blocks, each with 6 rows and 12 columns, as another example. The spot spacing are 288 µm horizontally and 249 µm vertically.

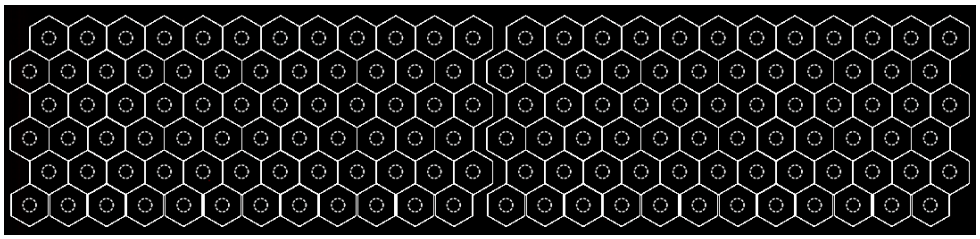


Figure 13: Hexagonal (Orange-Packing) Blocks.

To mitigate the risk of data loss, it is advisable to frequently save your array to a file. To accomplish this, navigate to *Arrays > Array File* and click on the *Save* button. The first time you save an array file, a dialog box will appear, prompting you to choose the file path. Each successful save will be confirmed on the status bar.

3.2 Extending an Array

You can extend an existing array by adding new blocks. To do this, navigate to *Arrays > Array File* and click on the *Editing* page, followed by the *Add* button. A dialog box with the same parameters as when creating a new array (as shown in Figure 11) will appear. The key difference here is that the new blocks will be appended to the existing blocks in the array.

3.3 Editing Spots' Name and ID

You can modify the names and IDs of spots using a spreadsheet-like interface as follows:

- Proceed to *Arrays > Array File > Editing* page and click the *Edit* button. This will launch the *Array Spot Data* window (Figure 14), which exhibits a table with each row containing the data of a spot in the array.
- To edit a spot name or ID, navigate to the *ID* or *Name* cell on a row and input the new text. Press *Enter* to finalize the editing for that cell. You can traverse the table using either

navigation keys or your mouse. The shortcuts *Control + Home* and *Control + End* can also be used in this table. Remember to save the array after editing.

- The table is in sync with the graphical canvas with the image and array. Choosing a row on the table emphasizes the corresponding spot on the canvas, and vice versa.
- You can arrange the table according to the values of a column by clicking on that column's header. To eliminate all sorting, double-click on any column header.

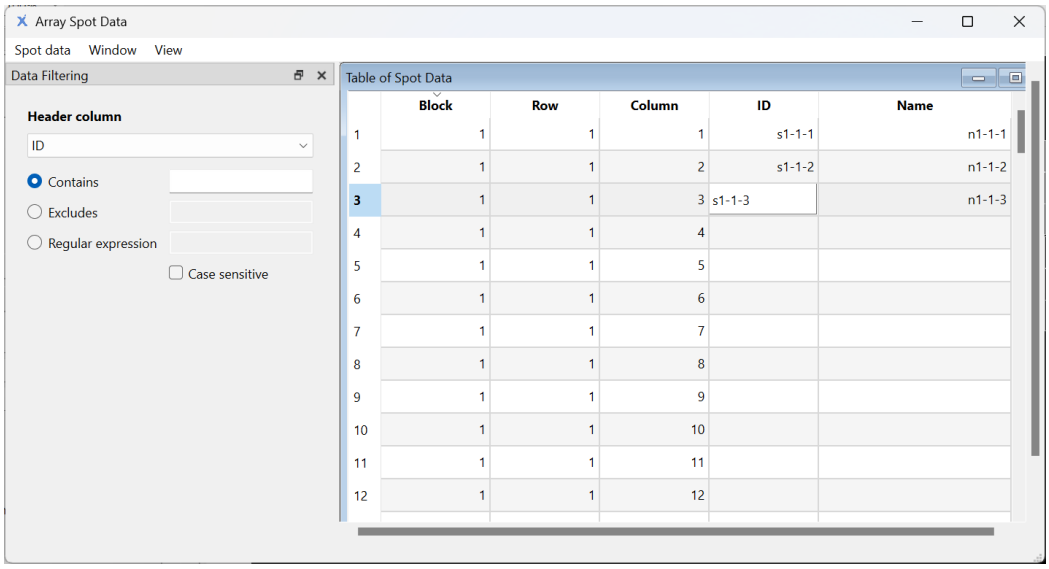


Figure 14: Table of Spot Data.

In addition, the *Data Filtering* widget on the left (Figure 14) enables you to select a field (*Block*, *Row*, *Column*, *ID*, *Name)* and filter the table according to a specific pattern. Position your mouse over the edit-boxes corresponding to the *Contains*, *Excludes*, or *Regular expression* options to view explanations and examples.

This feature is optimally utilized with *multiple screens*, e.g., by connecting your laptop with a monitor. Displaying the graphical canvas on the monitor and the table on the laptop screen, or vice versa, and having them synchronized, facilitates very convenient array editing.

3.4 Selecting and Editing Blocks

Click on a block to select it. To select multiple blocks, hold down the *Control* key while clicking on them. You can also select all the blocks in the array with the shortcut *Control A*. To deselect, click on a point in the canvas outside of the selection.

To change the spot size and spot spacing of blocks:

- First, select the blocks.
- Navigate to the *Arrays > Array File > Block* page.
- Adjust the values of the horizontal spacing, vertical spacing, and spot diameter.
- Remember to press *Enter* after changing a parameter.

3.5 Positioning Blocks

Align blocks with the corresponding spots on the image by selecting the blocks and *dragging* them to the desired position.

Fine-tune the position of selected blocks using the position tuning shortcuts. Hold down the *Control* key and press one of the four navigational keys (left, right, up, down) to *shift* the selected blocks a small *delta* in the navigated direction. You can shift multiple times until the desired position is reached.

The value of delta is equivalent to one micrometer and is derived from the image resolution. For example, if the image resolution is 1000 x 1000 pixels per centimeter, or 0.1 pixels per micrometer, then the delta shift on the canvas is equivalent to 0.1 pixels. If the derived value is less than 0.1, delta is also set to 0.1.

3.6 Rotating Blocks

Rotate the selected blocks using the options in *Arrays > Array Rotation* (Figure 18-b). Here, you can specify the center and direction of rotation. After inputting the angle value, press *Enter* to rotate the blocks.

3.7 Undo and Redo

You can undo or redo the changes made to the blocks in the array. The revertible changes include:

- Blocks' position
- Blocks' angle
- Spot spacing and spot diameter

Activate this function with the *Undo* or *Redo* button located in the *Arrays > Array File > Editing* page. Please note that the Undo and Redo functionalities are **not** applicable to actions such as adding block or shifting blocks (position fine-tuning).

3.8 Shortcuts and Keyboard Support

When editing arrays, you have the option to use either the buttons in the *Arrays* control panel or the keyboard shortcuts. These shortcuts are activated by holding down the *Control* key (*Ctrl*) and pressing the corresponding key. Please refer to Table 3 for a summary of the supported shortcuts.

Shortcut	Function
Ctrl N	Create a new array
Ctrl S	Save the array
Ctrl O	Open a new array
Ctrl L	Align the array

Ctrl T	Edit the table of spot data
Ctrl Z	Undo the last change
Ctrl Y	Redo the last change
Ctrl I	Add new blocks to the array
Ctrl Home	Move to the first block of array
Ctrl End	Move to the last block of array
Ctrl A	Select all blocks in array
Ctrl → (right-arrow key)	Shift[1] right
Ctrl ←	Shift left
Ctrl ↑	Shift up
Ctrl ↓	Shift down

Table 3: Shortcuts for Editing Arrays.

Note[1]: *Shift* refers to moving the *selected blocks* a delta distance in the specified direction.

4 Image and Array Processing

4.1 Image Processing

Image Properties

In the *Images* > *Image Channels* section (Figure 15), you can select a color channel or both to display in the graphical canvas. The image size and resolutions are also shown here. Other useful information includes the TIFF description, which often contains the scanning wavelength.

Adjusting contrast and brightness

This process can make the spots more visible and ease the array alignment. These functions are available in the *Images* > *Image Intensity* section (Figure 16).

- Choose the *Enhance contrast automatically* option to maximize the spot visibility.
- You can manually adjust the image's brightness and contrast by moving the slider. Alternatively, a value between -99 and 99 can be entered directly.
- On some images, setting the contrast to 99 and brightness to -99 also leads to the same effect as the automatic contrast enhancement, but with less noise.

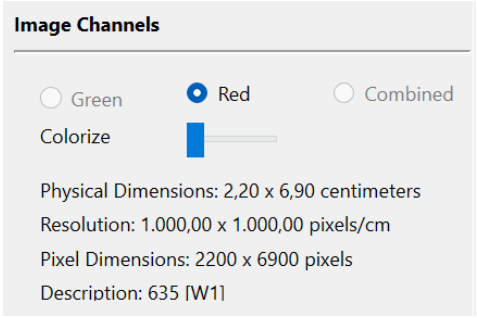


Figure 15: Image Properties.

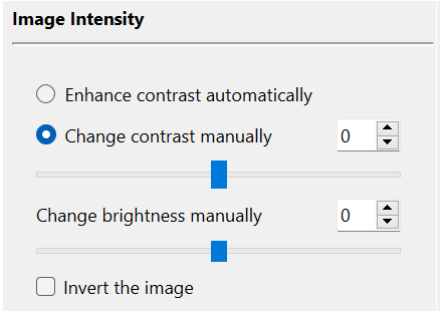
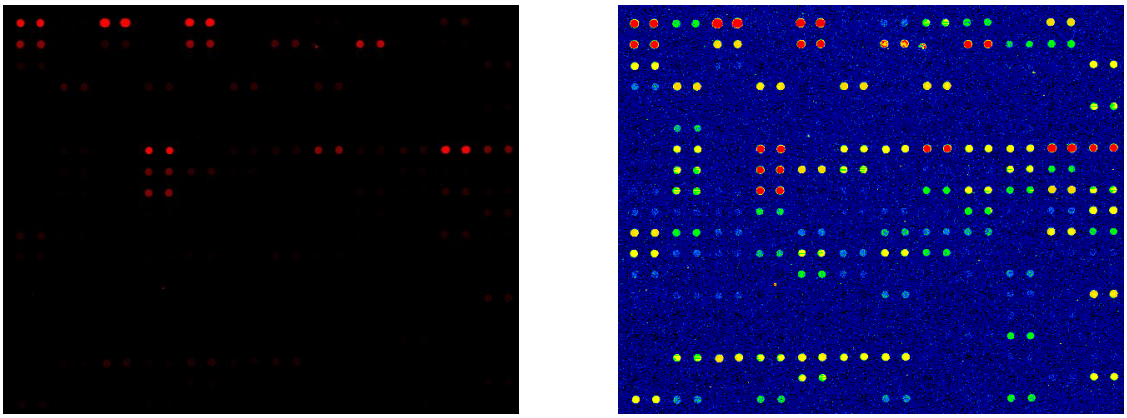


Figure 16: Image Processing.

Colorization

Another means to visualize the spots is the *colorization* tool (Figure 15). This depicts a grayscale image, or a page of a multi-page image, as a color image in which a pixel's color is determined by its original intensity value. Figure 17 illustrates the effect of the tool. It can be seen that many spots of weak signal have become visible in the colorized image.

Please note that this feature is applicable to 16-bit grayscale images only.



a) Original image. b) Image after colorization.

Figure 17: Colorization.

The tool can be turned on and off by clicking on the left or right side of the *Colorize* switch (Figure 15). Note that a single channel, either *Green* or *Red*, must be selected first to enable the switch.

For each pixel in the original image, the tool first determines the range that the pixel intensity belongs to and shows the pixel in the corresponding color. The number of colors is fixed, but the ranges of intensity values are adjustable. To change the setting, click the *Configuration* > *Colorization Setting* menu.

Inversing

Select the *Inversion* check-box to create the negative image.

4.2 Image and Array Rotation

Rotating images

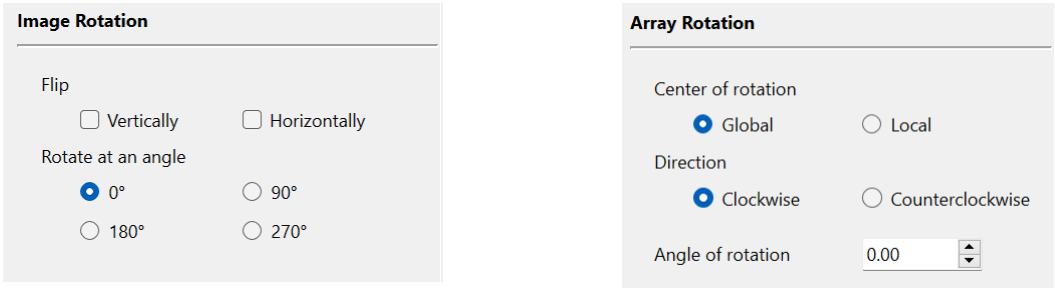
You can flip and/or rotate images at angles of 90°, 180°, or 270°. These functions are located in the *Images* > *Image Rotation* (Figure 18-a).

Rotating Array

If the array slightly deviates from the spots in the image at a small angle, it is recommended to rotate the array (i.e., the relevant **blocks** in GAL arrays or **spot families (SFs)** in PSF arrays), since image rotation may alter the image data. You can select blocks or SFs and rotate them at an arbitrary angle. These functions are located in the *Arrays* > *Array Rotation* section (Figure 18-b). The degree change can be as small as 0.01°.

Selected blocks or SFs can be rotated in clockwise or counterclockwise direction, with a rotation center defined as follows:

- *Global*: the top-left of the image.
- *Local*: the top-left of each block.



(a) Image Rotation **(b) Array Rotation**

Figure 18: Rotation of Images and Arrays.

4.3 Automatic Array Alignment for GAL Arrays

The automatic array alignment feature aligns the grids with your slide image. The process involves three main steps:

1. *Determining the rotation angle*: The software detects and compensates for any rotation in the slide image. You can skip this step if the rotation is very small (e.g., less than 0.1°).
2. *Roughly determining the blocks' positions*: Using the relative distances between blocks specified in your original GAL file, the software estimates the initial positions of the blocks. This method is practical and efficient because the GAL file reflects your array design used during spotting. By utilizing these predefined distances, the software avoids exhaustive searching and speeds up the alignment process.
3. *Refining the blocks' positions*: The software fine-tunes the alignment for each block by optimizing their positions relative to the detected spots, taking into account any deviations or offsets.

Importance of Relative Distances between Blocks

The relative distances between blocks specified in your GAL file are crucial for accurate alignment. Ensuring these distances are correct allows the software to align the blocks precisely, reflecting the intended array design.

If you experience misalignment, consider the following steps:

- Reload the GAL file: Ensure you are using the GAL file with block positions that corresponding to your array design.
- Adjust the offsets between blocks as described in the *Offset of Blocks' Position* section.
- Run the alignment again.

4.3.1 Alignment Options

Due to variations in array and spot configurations, the automatic function with the default options may not align the grids with the image precisely at first. You can fine-tune the alignment function by clicking the *Configurations > Array Alignment Options* menu. The setting dialogs are shown in Figure 19 and Figure 20.

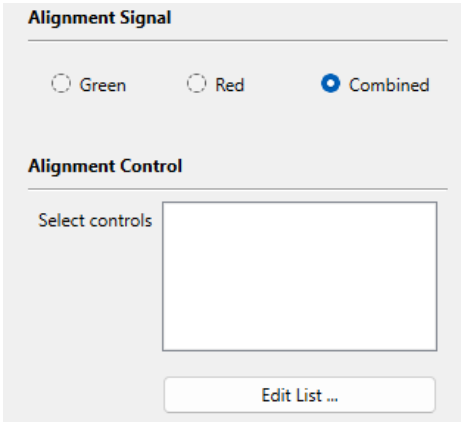


Figure 19: Alignment Options - Signal & Controls.

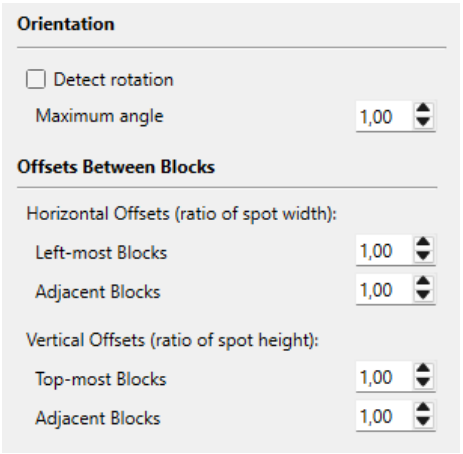


Figure 20: Alignment Options – Orientation & Offsets Between Blocks.

Alignment Signal

This setting (Figure 19, the *Alignment Signal* section) is useful for dual-color images. It allows you to specify which channel (*Red* or *Green*) to use for array alignment. The default option is *Combined*, where the software uses the strongest signal from both channels. For single-color analysis, the alignment uses the signal from the loaded channel.

Detect Rotation

Enable this option (Figure 20, the *Orientation* section) to detect if the slide image is rotated and adjust the alignment accordingly.

In the *Maximum angle* spin-box, you can set the limit for the potential rotation of the image, based on the image acquisition setup. The default value is 1.0°, meaning the software will search for an optimal angle between -1.0° and 1.0°. Please note that including rotation detection and setting a larger maximum angle will increase the alignment processing time.

4.3.2 Offset of Blocks' Position

Normally, adjacent blocks should have approximately the same horizontal (X) or vertical (Y) position. However, printed blocks may be slightly offset from the planned positions. In Figure 21, we observe differences in the horizontal position of block 1 and block 3, as well as in the vertical position of block 3 and block 4.

If the printed blocks have significant offsets from their planned positions, you might need to adjust the offsets between blocks (see Figure 20, the *Offset Between Blocks* section).

Horizontal Offset (ratio to the spot width)

- *Left-most Blocks*: Maximum difference in X-position between left-most blocks (e.g., block 1 and block 3 in Figure 21).

- *Adjacent Blocks*: Maximum difference in X-position between two adjacent blocks that are not left-most (e.g., block 2 and block 4 in Figure 21).

Vertical Offset (ratio to the spot height)

- *Top-most Blocks*: Maximum difference in Y-position between top-most blocks (e.g., block 1 and block 2 in Figure 21).
- *Adjacent Blocks*: Maximum difference in Y-position between two adjacent blocks that are not top-most (e.g., block 3 and block 4 in Figure 21).

Adjusting these parameters helps the software better accommodate the actual positions of the blocks on the slide.

- The default value for all offsets is 1.0.
- A common alternative is 0.75 for all offsets.
- Smaller gaps between adjacent blocks/SFs often correspond to values like 0.5 or 0.75.
- For larger gaps, consider values of 1.5 or 2.0 times the spot size.

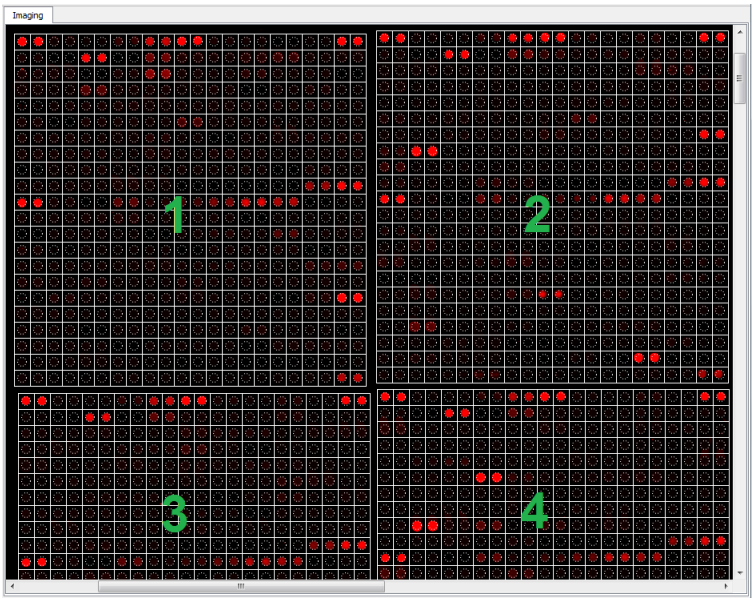


Figure 21: Offset of Blocks.

4.4 Automatic Array Alignment for PSF Arrays

PSF arrays typically contain a single large **spot family** along with designated **positional control spots**. The alignment process is designed specifically for this format and behaves as follows:

- It relies entirely on the alignment signal and, if specified, on positional controls (Figure 22).
- It does not use the orientation or offset parameters - these apply only to GAL arrays (see Figure 20).
- The array layout is always subject to rotation during alignment.

Alignment Signal

- For dual-color images, select the channel to use for alignment.
    - Default option: *Combined* - uses the strongest signal across both channels.
- For single-color images, alignment is performed using the signal from the loaded channel.

Alignment Controls

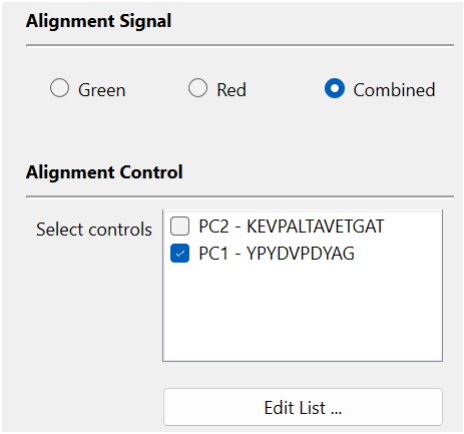


Figure 22: Alignment Options - Signal & Positional Controls for PSF Arrays.

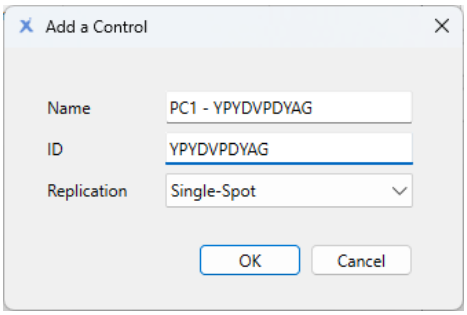


Figure 23: Add an Alignment Control.

To improve precision, you may assign positional controls:

- Select the checkbox next to the control(s) you want to use. For example, in Figure 22, the control *PC1* with ID *YPYDVPDYAG* is selected.
- Click *Edit List* to open a list of all defined controls.
- To define a new control (see Figure 23): Click *Add*, then enter the *name*, *ID*, and *replication mode* in the dialog.
- To edit an existing control, click *Change*.
- Created controls are saved and available for future projects.

During alignment, all spots with a matching *ID* (e.g., *YPYDVPDYAG*) are treated as positional markers. The array is adjusted to best align the layout with these markers.

5 Quantification of Microarray Data

5.1 Quantifying Microarray Data

To begin quantifying microarray data, click the *Quantify* button in the main toolbar to open the *Quantification* control panel.

- To quantify the entire array, click the *Quantify Array* button.
- To quantify specific blocks, select the desired blocks and click the *Quantify* Selection button.

A new window will appear, displaying a *table of the quantified data* (Figure 26). Detected spots in the canvas are highlighted with a blue border by default (Figure 24).

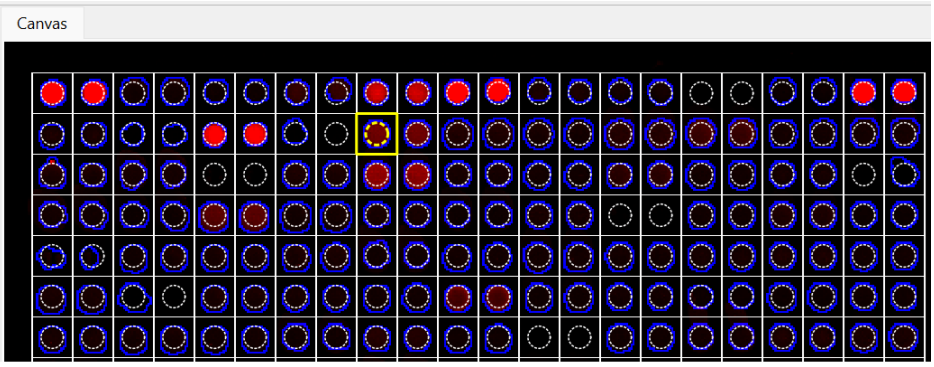


Figure 24: Highlighted Spots in Canvas.

The spots in the canvas and the table of quantified data are *synchronized*. Selecting a spot in the canvas (highlighted in yellow) will also highlight the corresponding row in the table, and vice versa. This feature is particularly useful when using *multiple screens*, such as connecting a laptop to a monitor. Displaying the graphical canvas on one screen and the table on the other facilitates efficient analysis of the microarray data.

The quantification process may take some time, depending on the image resolution and array size. Fortunately, the software supports multi-threading, allowing you to continue navigating through the image and array data while the quantification is in progress.

5.2 The Table of Quantified Data

In the table of quantified data, the median and mean of each spot's *raw*, *background*, and *foreground* values in each channel (Red and/or Green) are calculated. If a spot's raw value is smaller than its background value, the spot is flagged as an error, and its foreground value is set to zero. (Section 5.3 details the methods used to calculate the raw and background values.)

Visible Data Columns in Quantified Data Table

By default, the table displays six values per channel for each spot specifically, the *median* and *mean* of its *raw*, *background*, and *foreground* signals.

To streamline your view, you can customize which values are shown. For example, you might choose to display only the mean foreground and the median background and foreground values.

To configure column visibility:

- Go to *Configurations > Quantified Data Table Columns Options*.
- In the dialog (Figure 25), select or deselect the checkboxes for the columns you want to display.

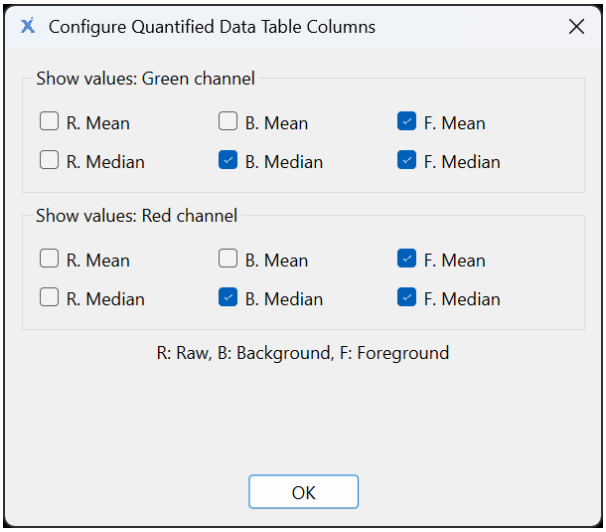


Figure 25: Configure Visible Data Columns.

Note: Regardless of your display settings, when exporting quantified data to a CSV (*.csv) or GenePix Result (*.gpr) file, all six values are always included for each spot.

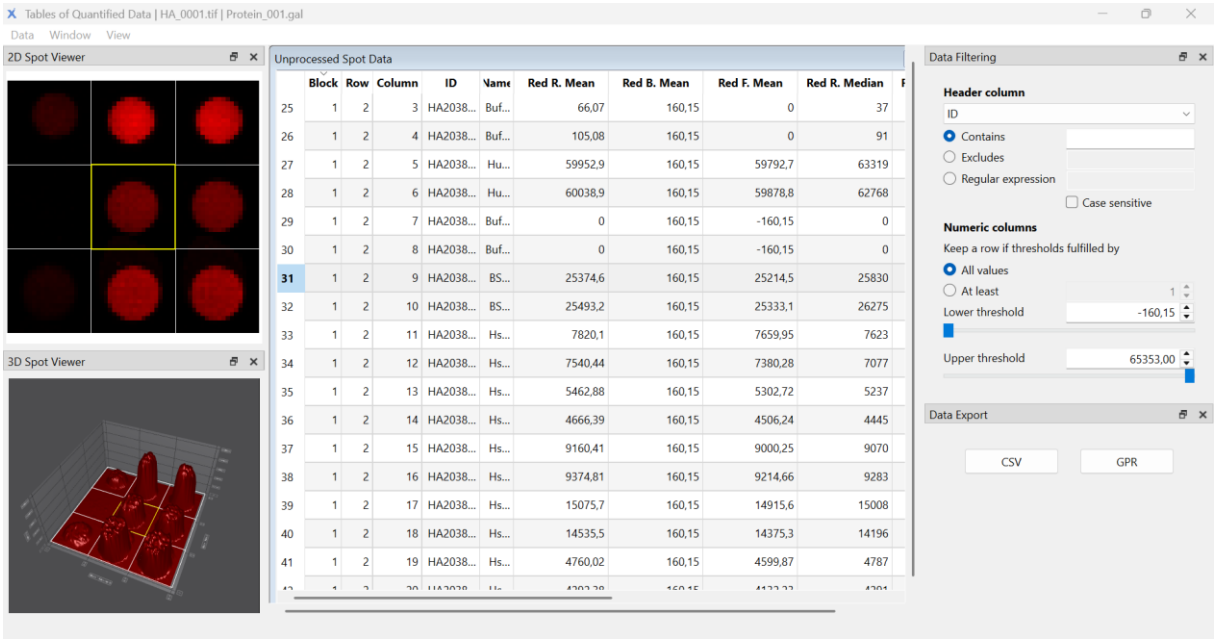


Figure 26: Table of Quantified Data.

2D Spot Viewer

The *2D Spot Viewer* allows you to view the selected spot, its neighbors, and their quantified data simultaneously (Figure 26, top-left). It displays the image portion corresponding to the selected spot and its neighbors.

3D Spot Viewer

In addition to the two dimensional view, the *3D Spot Viewer* (Figure 26, bottom-left, and Figure 27) displays a three-dimensional representation of the selected spot and its neighbors. Note that both the 2D and 3D Spot Viewers display the contrast-adjusted intensity, similar to the canvas.

To change the view and size of the 3D Spot Viewer:

- Change *the viewing angle*: Hold down the right mouse button and drag.
- *Zoom in and out*: Use the mouse wheel.

Data Filtering, Replicate Processing, and Data Normalization

To filter spots, process replicates, or normalize the quantified data of the current microarray image, use the tools in the *Data Filtering*, *Replicate Processing*, and *Data Normalization* widgets.

If you are not familiar with these tools, hover the mouse over a control, such as the edit box in *Data Filtering > Contains*, and read the popup tooltip. These tools are also described in detail in Section 7.

Exporting the Quantified Array Data

With the *Data Export* widget, you can export the quantified data to a CSV file (**.csv*) or a GenePix Result file (**.gpr*) for further analysis.

Docking and Undocking a Widget

Double-clicking on the header allows you to undock the 2D and 3D Spot Viewers from the *Table of Quantified Data*. They will become independent windows and can be moved to any screen and freely resized (Figure 27). To bring an undocked viewer back to the table, just double-click on its header. This behavior also applies to the *Data Filtering*, *Replicate Processing*, and *Data Normalization* tools.

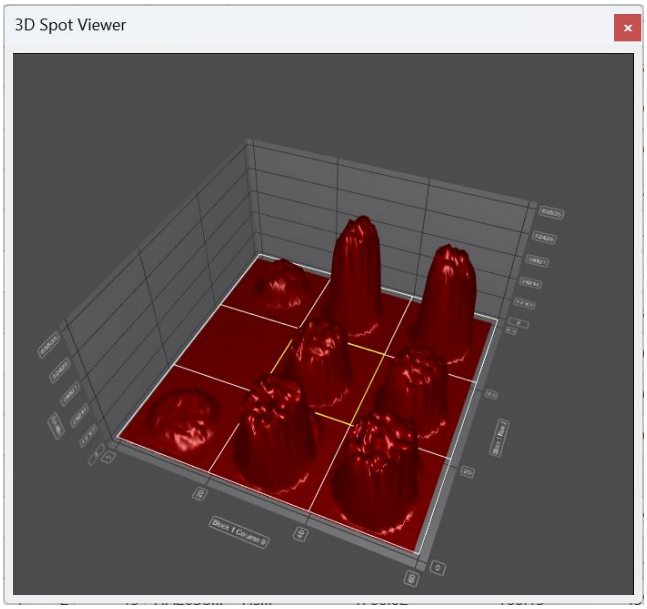


Figure 27: 3D Spot Viewer.

Showing and Hiding Widgets in the Quantified Data Table

To tailor your workspace, you can choose which widgets (panels or information views) are visible in the *Table of Quantified Data*.

- Navigate to the *View* menu located on the table's toolbar.
- Select or deselect the checkboxes corresponding to each widget you want to show or hide.

5.3 Quantification Options

To display the quantification options, check the *Show quantification options* checkbox in the *Quantification* control panel (Figure 28). The available options depend on the selected spot detection method, of which there are three.

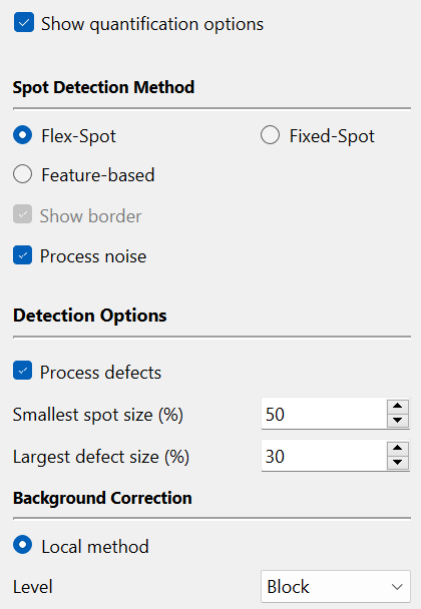


Figure 28: Flex-Spot Options.

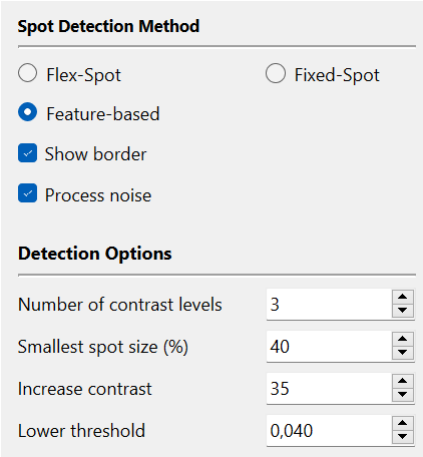


Figure 29: Feature-Based Options.

Flex-Spot

The Flex-Spot method (Figure 28) can flexibly detect the spot's signals even if their shape, size, and position do not match the predefined spotted region. The detected spot border is shown in blue (Figure 24). The raw value is calculated based on the pixels within the blue border, while the background value is calculated using the pixels in the remaining area within the spot.

The Flex-Spot method is the first recommendation because it does not require the spots in the image to rigidly match their predefined regions.

Feature-Based

The Feature-Based spot detection method (Figure 29) is recommended for noisy images where the Flex-Spot method fails to locate the spot correctly. This newly introduced method leverages the

Scale-Invariant Feature Transform (SIFT) algorithm[1], which is a powerful feature detection technique, but is not yet fully utilized by the software. We will continue to improve its integration in future releases.

Note: The Feature-Based method is available only for GAL arrays and is not supported for PSF arrays.

Fixed-Spot

The Fixed-Spot method calculates signal values strictly based on predefined spatial boundaries. It is generally not recommended unless other detection methods (e.g., Flex-Spot or Feature-Based) have failed to detect spots reliably.

- Raw Value: Computed using only the pixels within the spotted region, defined by:
  - A dashed circle in GAL arrays (see Figure 2 and Figure 3), or
  - A dashed rectangle in PSF arrays (see Figure 4).
- Background Value: Estimated from the remaining area inside the spot but outside the spotted region.

Show Border

After data quantification with the Flex-Spot and Feature-Based methods, you can toggle the detected spot border on and off using the *Show border* option (Figure 28). The software supports saving the border information in the project file (*.*spotxelproj*), allowing you to observe the detected spot border when reopening the project file. This feature is particularly useful for reviewing the quantified data generated by batch processing.

Default Settings and Tool-Tips

If you need to revert to the default settings after making multiple changes, refer to Figure 28 and Figure 29. Additionally, you can display tooltips by hovering over the options for further guidance.

5.4 Flex-Spot Spot Detection Options

Smallest Spot Size

Set the size limit of a valid spot using the *Smallest spot size (%)* parameter. For example, if set to 50%, the Flex-Spot method will reject spots that can fit within a square with a side length of 50% of the spot diameter. The valid values range from 25% to 100%.

Largest Defect Size

[1] David G. Lowe. *Distinctive image features from scale-invariant keypoints*. Int. J. Comput. Vision, 60(2):91–110, November 2004.b

Define the maximum defect size using the *Largest defect size (%)* parameter, which ranges from 0% to 50% of the spot diameter. Larger percentages will eliminate larger defects. Please note that the elimination of defects takes place only if the *Process defects* checkbox is checked.

Note: The *Largest Defect Size* option applies only to GAL arrays and is not used for PSF arrays.

Undetectable by Flex-Spot

If the Flex-Spot method cannot detect a spot due to size limits, weak signals, or noisy data, the software will employ the Fixed-Spot method to compute the raw value. No blue border will be shown if the Fixed-Spot method is used.

5.5 Feature-Based Spot Detection Options

Number of Contrast Levels

Select the number of contrast levels (ranging from 2 to 5) used to improve spot detection. Higher values may uncover more features but could slow down processing.

Smallest Spot Size

Similar to the *Flex-Spot* method, this parameter sets the minimum spot size as a fraction of the spot diameter, ranging from 25% to 100%. Larger values will ignore smaller spots during detection.

Increase Contrast

Adjust the image contrast (from -99 to 99) to enhance detection. This setting works together with the number of contrast levels. Higher contrast settings can help reveal features across multiple levels.

Lower Threshold

Set the SIFT Lower Threshold parameter, used in the SIFT (Scale-Invariant Feature Transform) algorithm to discard low-contrast features. The default value is 0.04. Lower values will detect more spots but may also include more noise.

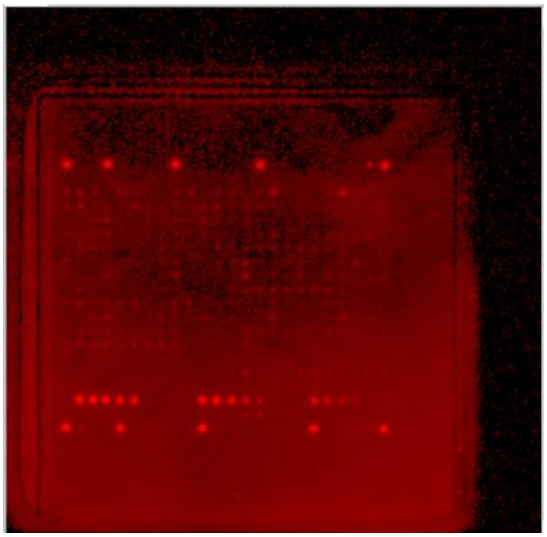
5.6 Noise Processing

By checking the *Process noise* option (Figure 28 and Figure 29), you can include noise processing during the quantification procedure. This includes processing background noise, foreground noise, and signal smearing. Noise processing effectively reduces background noise and can be regarded as an implicit background correction method.

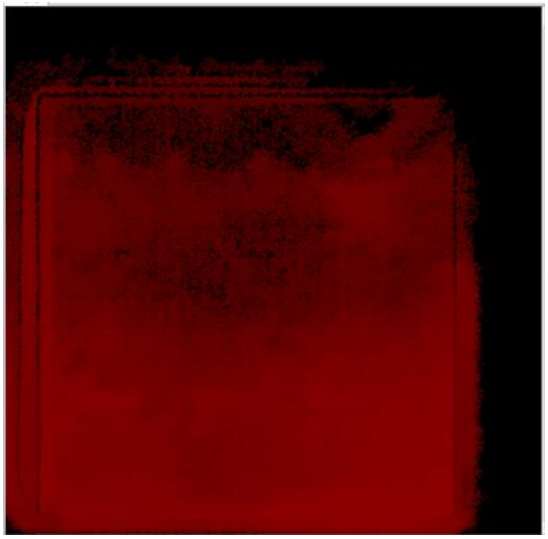
Background Noise

Noises in microarray images can mislead the spot detection procedure and result in incorrect estimation of the true specific-binding signal. Figure 30-a shows a slide image with non-homogeneous background signal, concealing specific-binding signals and affecting array alignment and spot finding.

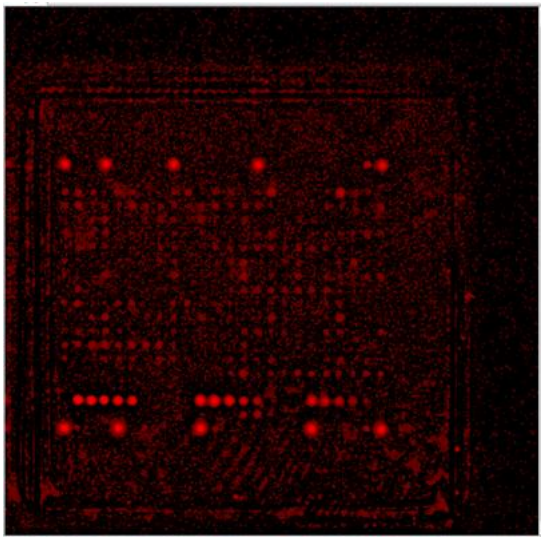
Equipped with powerful image processing functions, the software can process such background noise. Figure 30-b visualizes the detected background signal alone, while Figure 30-c shows the slide image with the background signal eliminated, revealing the spots' signals clearly.



(a) Original image.



(b) Detected background signal.



(c) The *Raw* signal: The image with background signal eliminated.

Figure 30: Noise Processing.

Foreground Noise

Figure 31 illustrates a slide image with foreground noise, where two large red bands are due to non-specific binding. The software processes such foreground noise based on the spots' shape and size, selecting only valid spots (in red) and highlighting them with a blue border.

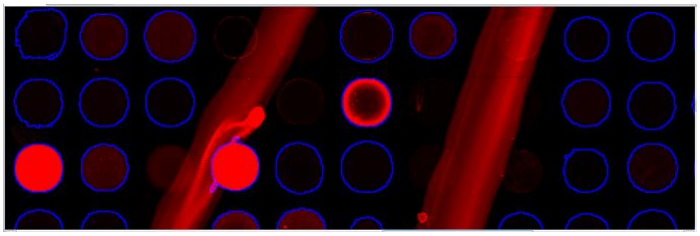


Figure 31: Foreground Noise Processing.

Smearing Signal

Figure 32 presents a case of non-specific binding signal due to smearing. The contrast of this slide image is increased for illustration, with the spot signal in red. The software detects and selects only the signal due to local binding within each spot, highlighting the detected spots' borders in blue to show that the spot signal is unaffected by smearing from neighboring spots.

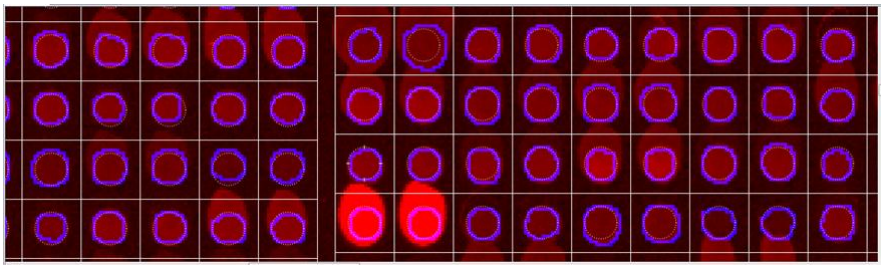


Figure 32: Smearing Processing.

5.7 Background Correction

Starting with version 3.2, the software supports only the *Local* background correction method. The software supports different levels of local background correction (see Figure 28, bottom-right):

- *Block* Level (default for GAL arrays): All spots in a block have the same background value, computed from the mean and median of the pixel intensity values of all background pixels in the block.
- *Spot Family* Level (default for PSF arrays): All spots belonging to a spot family (SF) share the same background value, computed from the background pixels of the SF.
- *Global* Level: A global background value is calculated based on the background pixels of all spots in the array.
- *Spot* Level: Each spot's background value is based on its own background pixels, resulting in potentially different background values between spots.

6 Batch Processing

In high-throughput studies, you may need to screen a microarray with **N** *samples* using **N** slides. These slides share the same layout annotation - known as the *template array* - which can be either:

- a GenePix Array List (*.gal), or
- a PepSlide Designer file (*.psf).

Processing generates **N** grayscale TIFF images, one for each slide. Using batch processing (Figure 33), you can automatically analyze all N images and extract their quantified data. For each scanned image, the batch process will:

- **Align** the template array with the image.
- **Create** an aligned array file.
- **Generate** quantified data files.

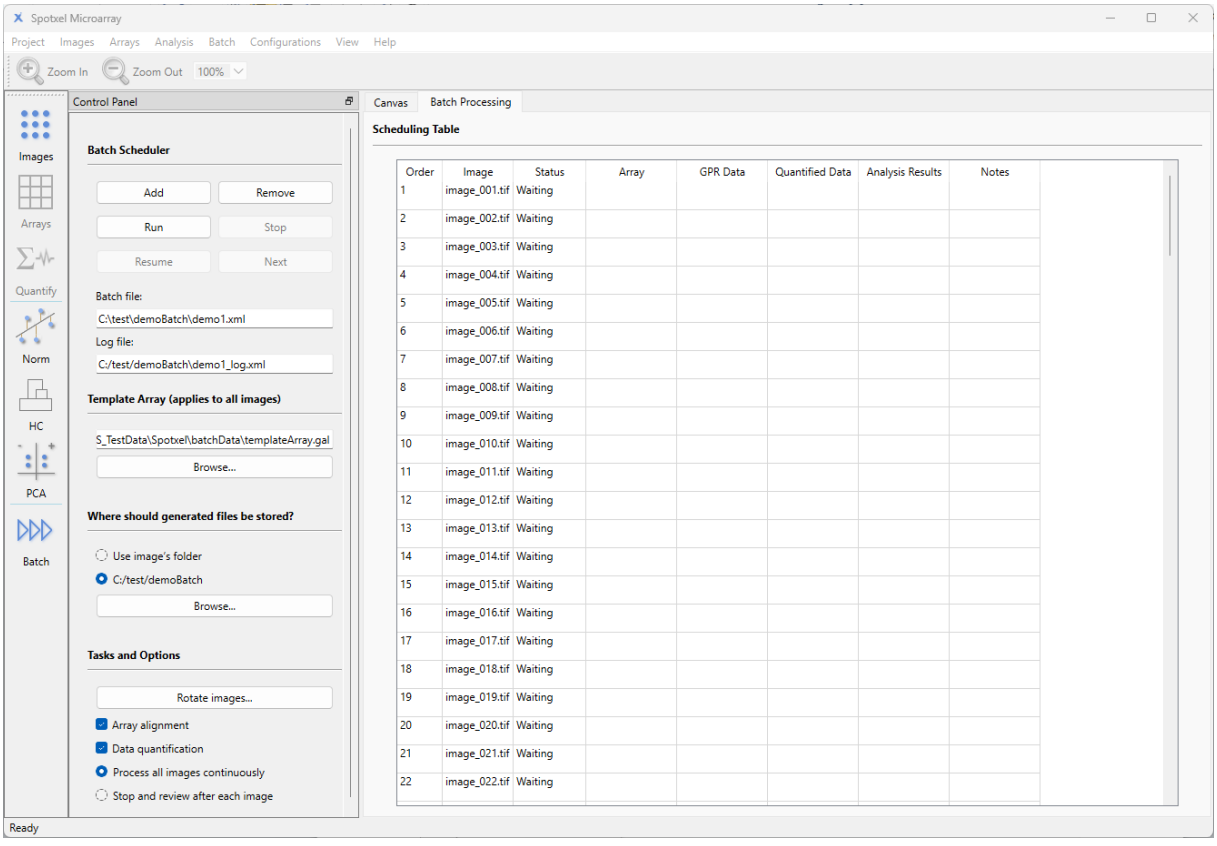


Figure 33: Batch Setup.

Creating and Configuring a Batch

1. Open the Batch Scheduler:
   - Click the *Batch* button on the main toolbar to control panel.
2. Add Images:
   - Click *Add* and select the images to process.

3. Select the template array:
   In *Template Array (applied to all images):*
   - Click Browse and select the .gal or .psf file.
4. Specify the Output Folder:
   In *Where should generated files be stored?*
   - Use the default (image's folder), or
   - Click *Browse* to choose a custom location for the generated files.
5. If rotation is needed:
   - Click *Rotate Images* and choose Flip and/or Rotate (90°, 180°, or 270°).
6. Save the batch setup:
   - Go to *Batch > Save Batch*.
   - A log file will be created automatically with the same name.

Tips:

- Use a separate folder for each batch to keep files organized.
- Avoid using dot characters (.) in folder or file names (except for file extensions) to prevent errors.

Running the Batch

- Start Processing:
  - Click the *Run* button to execute the batch (see Figure 34).
- Running Mode:

  According to the running mode selected during batch setup, execution proceeds as follows:

  - *Process all images continuously*: The batch processes all images without interruption. You can click *Stop* to halt the execution and then click *Resume* to continue.
  - *Stop and review after each image*: The batch pauses after processing each image, allowing you to review results before continuing.
- Monitor Progress:
  - The status of each image is updated live during processing.

Generated Output Files

For an input file like sample001.tif, the following output files are generated:

- sample001.gal or sample001.psf: The array file linking layout to signal in the image.
- sample001.csv: Quantified data in CSV format.
- sample001.gpr: Quantified data in GenePix Result format.
- sample001.spotxelproj: A project file containing the full analysis results.

Running the batch for **N** images will generate **N** .spotxelproj files for downstream analysis.

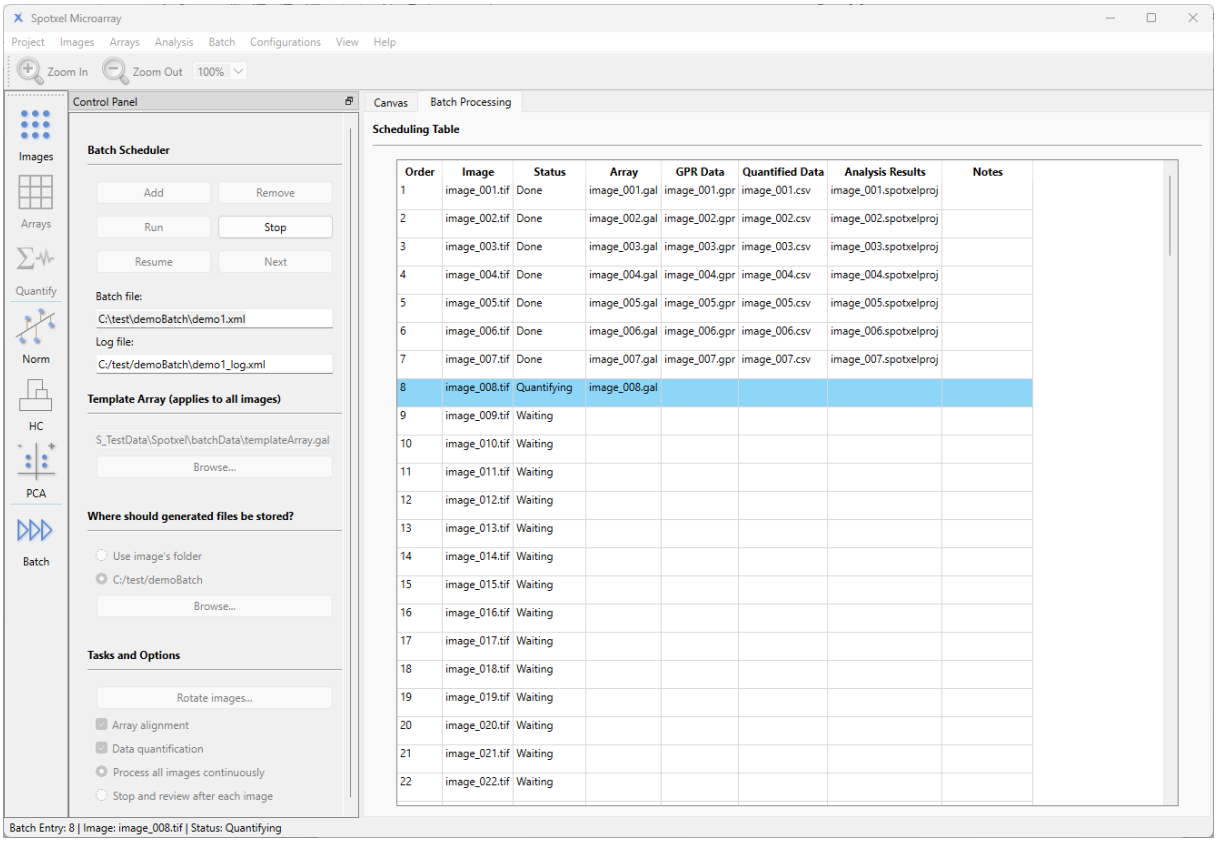


Figure 34: Batch Execution.

7 Data Preprocessing and Normalization

The *Data Preprocessing and Normalization* tool (Figure 35) provides convenient ways to improve the quality of microarray data. *Replicate processing* eliminates duplicates and stabilizes the features' signal values by consolidating all replicates into a single value using their mean or median. *Data Filtering* allows you to narrow the dataset and focus on features of interest. *Data Normalization* reduces effects caused by technical variants, enabling data comparability to identify actual relationships between samples and features.

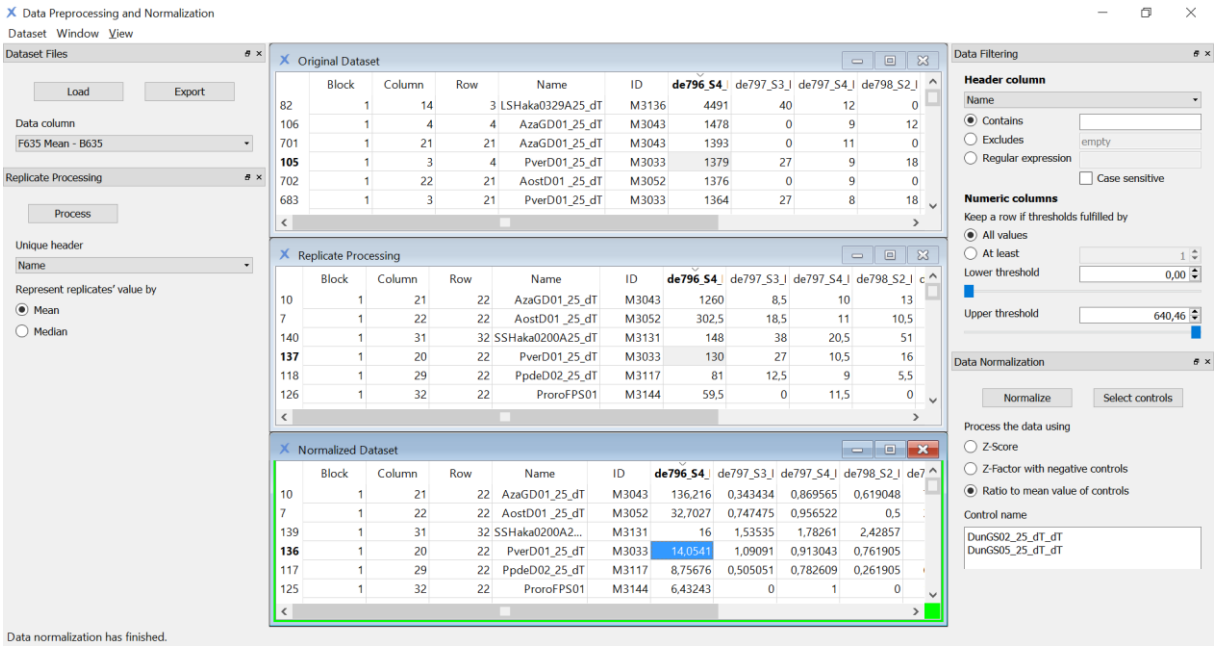


Figure 35: Replicate Processing, Data Filtering, and Data Normalization.

To launch the *Data Preprocessing and Normalization* tool, click the *Norm* button on the main toolbar. The tool works on datasets containing the features' signal values. Section 7.1 explains steps to prepare a dataset from different sources. Replicate processing is explained in Section 7.2. Details on data filtering and data normalization are provided in Sections 7.3 and 7.4, respectively.

7.1 Datasets

A dataset can be compiled from a list of Spotxel® Microarray project files. Consider the example in Section 6 again, in which the protein microarray is screened with *k* samples. After running the batch, we obtained *k* Spotxel® Microarray project files containing the quantified data. The dataset can be represented as shown in Table 4, where V_{1k} is a screening value of *Feature 1* when the microarray is screened against *Sample k* and so on. The screening value can be chosen from the list of quantified values, such as Red Foreground Mean.

The first five columns in Table 4 contain the spot's properties specified in the GAL file (i.e., *Block, Row, Column, ID,* and *Name*) of an individual *feature* (a protein in the batch example). For simplicity, only the feature names are shown.

Block	Row	Column	ID	Name	Sample 1	Sample 2	…	Sample k
Feature 1					V_{11}	V_{12}	…	V_{1k}
…					…	…	…	…
Feature n					V_{n1}	V_{n2}	…	V_{nk}

Table 4: A Sample Dataset.

You can also create a dataset from a list of GenePix Result (**.gpr*) files by selecting the .gpr files and a screening value such as F635Mean – B635. Alternatively, prepare a CSV file with the format shown in Table 4, where the first five columns are Block, Row, Column, ID, and Name, and the subsequent columns contain numeric values representing the signal values of the microarray features screened against a sample.

To load a dataset from Spotxel Microarray project files or .gpr files, click the *Load* button in the *Dataset Files* panel (Figure 35, top-left) and select the files. Specify the screening value in the *Data column* list-box. If the dataset is already prepared as a CSV file, simply load that file. The dataset, with both header and numeric columns, is shown in the table titled *Original Dataset* (Figure 35, top-center).

Any dataset (original, filtered, or normalized) can be exported to a CSV file for further analysis. Select the corresponding table (highlighted in green, such as the *Normalized Dataset* table in Figure 35, bottom-center) and click the *Export* button in the *Dataset Files* panel. If there are more than two tables of data, you can arrange them using the *Window > Tile or Window > Cascade* menu.

7.2 Replicate Processing

Suppose you print each feature (a protein or a gene) in duplicate, triplicates, or more on the microarray for quality and error control. The signal value of a feature's replicates is usually different. In addition, you want only one an instance of the feature in the dataset. This can be done quickly with the *Replicate Processing* tool.

In the *Replicate Processing* panel (Figure 35, bottom-left), select the header (such as Name or ID) in the *Unique header* list-box. Then click the *Process* button. In each column of the dataset, features having the same Name/ID will then be consolidated into one row. The new value will either be the mean or the median of the feature's replicates, as chosen at the *Represent replicates' value* option. A processed dataset is then stored in the *Replicate Processing* table (Figure 35, middle-center).

7.3 Data Filtering

You can narrow the currently selected dataset to certain rows containing features of interest using header filters. These filters can *contain* or *exclude* specific keywords or match a *regular expression*[2] search pattern. Additionally, you can select only features whose signal value is within a specified

[2] Regular expression - Wikipedia

range using the lower and upper thresholds. These tools are provided in the *Data Filtering* panel (Figure 35, top-right).

The header filter is set by first selecting the header column such as Name or ID. You then have the following filtering options:

- *Contains*: The dataset is narrowed to rows that contain one or more input keywords. A list of keywords must be separate by AND/OR (in the exact uppercase form). For example "protein OR positive" will select rows whose name contain "protein" or "positive".
- *Exclude*: Rows whose header containing the keywords (separated by OR) are excluded from the filtered dataset.
- *Regular expression*: This is the most powerful filter based on regular expression. Enter a regular expression search pattern and press *Enter* to filter the dataset.

The minimum and maximum allowed values in the dataset can be set using the lower and upper thresholds for the numeric columns. You can condition a row to satisfy all numeric columns or only a number of them. Please note that a header filter has higher priority than the threshold setting. When you apply a new header filter, the lower and upper threshold values will be reset to the minimum and maximum values of the newly filtered dataset. Starting with these two thresholds, you can narrow down the dataset based on the numeric values.

7.4 Data Normalization

Due to technical variations, the absolute signal values obtained directly after quantifying microarray images may not comparably reflect the actual biological change. Data normalization is necessary to make the data comparable again. The *Data Normalization* tool (Figure 35, bottom-right) provides three methods to normalize the numeric values:

- *Z-Score*: Calculate the relative signal value of a feature as how far, in terms of standard deviations, and in what direction a feature deviates from the center of all features.
- *Z-Factor with negative controls*: Check the screening quality using the standard deviation and the mean value of screening features as well as those of negative controls (Zhang et al., 2000)[3].
- *Ratio to mean value of controls*: This is useful if the study employs some controls as calibration probes. It calculates the normalized value as the ratio of the feature's signal value to the mean of selected controls' signal value.

If you choose the latter two methods, click on the *Select Controls* button. This shows the *Control Selection* dialog (Figure 36) with a list of all features in the currently selected dataset. There you can choose one or more features as controls for the chosen normalization method.

[3] Zhang, J-H, Chung T.D.Y., and Oldenburg K.R. (2000) Confirmation of Primary Active Substances from High Throughput Screening of Chemical and Biological Populations: A Statistical Approach and Practical Considerations. J. Com. Chem. 2: 258-265

After choosing a method, click on the *Normalize* button. The normalized dataset, titled *Normalized Dataset*, is shown (Figure 35, bottom-center).

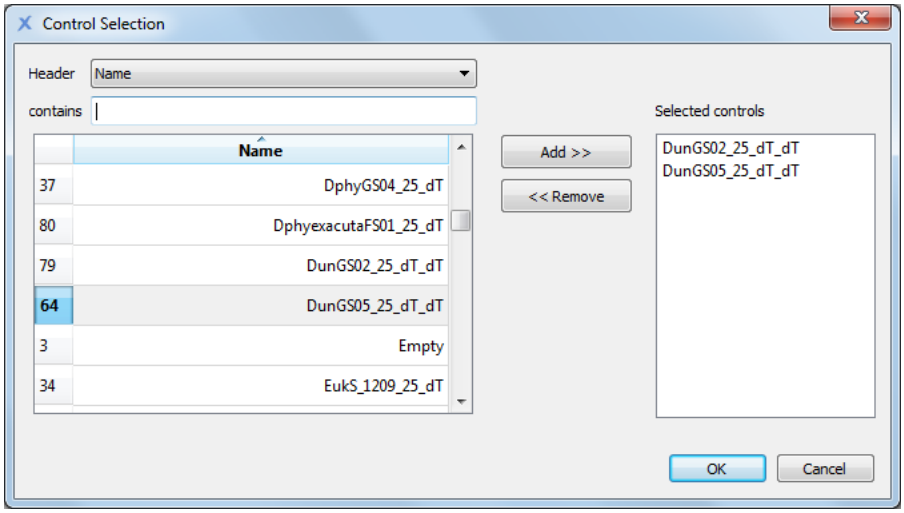


Figure 36: Control Selection for Data Normalization.

8 Data Mining Tools

Data mining tools assist you in finding useful information from the microarray study. You can employ *Principal Component Analysis* to discover features and samples that influence the study and then *Hierarchical Clustering Analysis* to find their relationship. The batch processing results, i.e., generated Spotxel® Microarray project files, can be used directly for data mining.

Please note that these tools work on datasets. The steps to prepare them from different sources are described in Section 7.1.

8.1 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) simplifies a complex microarray study to a more manageable one with only a few samples or features, allowing you to observe the study's data more easily. To begin, click the *PCA* button in the main toolbar. In the PCA control panel (Figure 37):

- Click the *Load Data* button and select the dataset. Please refer to Section 7.1 for the preparation of the dataset.
- Select a quantified value in the *Data Column* list-box.
- Choose to have the simplified dataset with either three *Features* or *Samples*.
- Click the *Start Analysis* button.

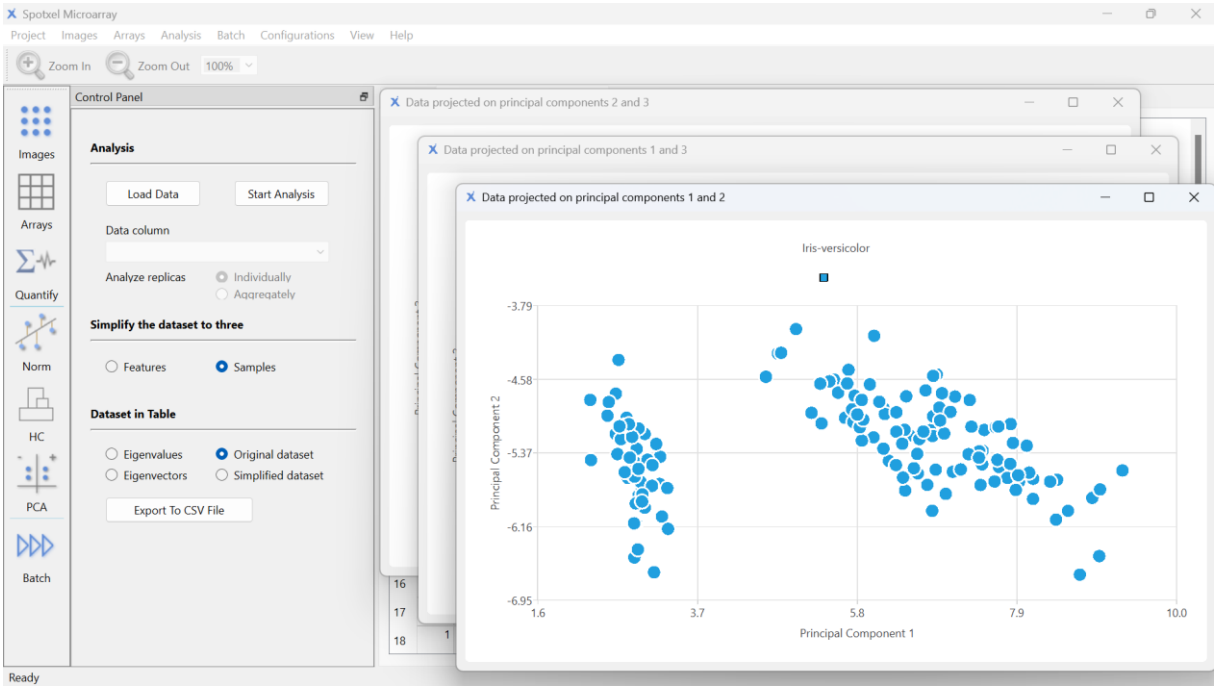


Figure 37: Principal Component Analysis in 2D View.

Suppose you chose the *Samples* option. If the original dataset represents the features' screening value against multiple samples, PCA will simplify it to a dataset with only three key samples. Similarly, you can discover such information about the samples by choosing the *Features* option before starting the analysis.

In the three charts (Figure 37), you can view the features according to their new values in the simplified dataset. To zoom in on a region, use the mouse to select that region. Double-click on the chart to return it to the original zoom.

In the *Dataset in Table* panel, you can choose to export the dataset, either original or simplified, to a CSV file for further analysis.

In future releases, the PCA charts will be further improved for more intuitive and convenient use.

8.2 Hierarchical Clustering Analysis (HCA)

Hierarchical Clustering Analysis (HCA) allows you to group related features or samples. The relationship can be, for example, having a similar effect in the study, represented by close screening values. To set up the analysis, click the *HC* button in the main toolbar:

- Click the *Load Data* button and select the dataset. Please refer to Section 7.1 for the preparation of the dataset.
- Select a quantified value in the *Data Column* list-box.
- Choose to construct the clustering tree for *features*, or *samples*, or *both*.
- Select the distance metric and the type of linkage. You can keep the default options.
- Click the *Start Analysis* button.

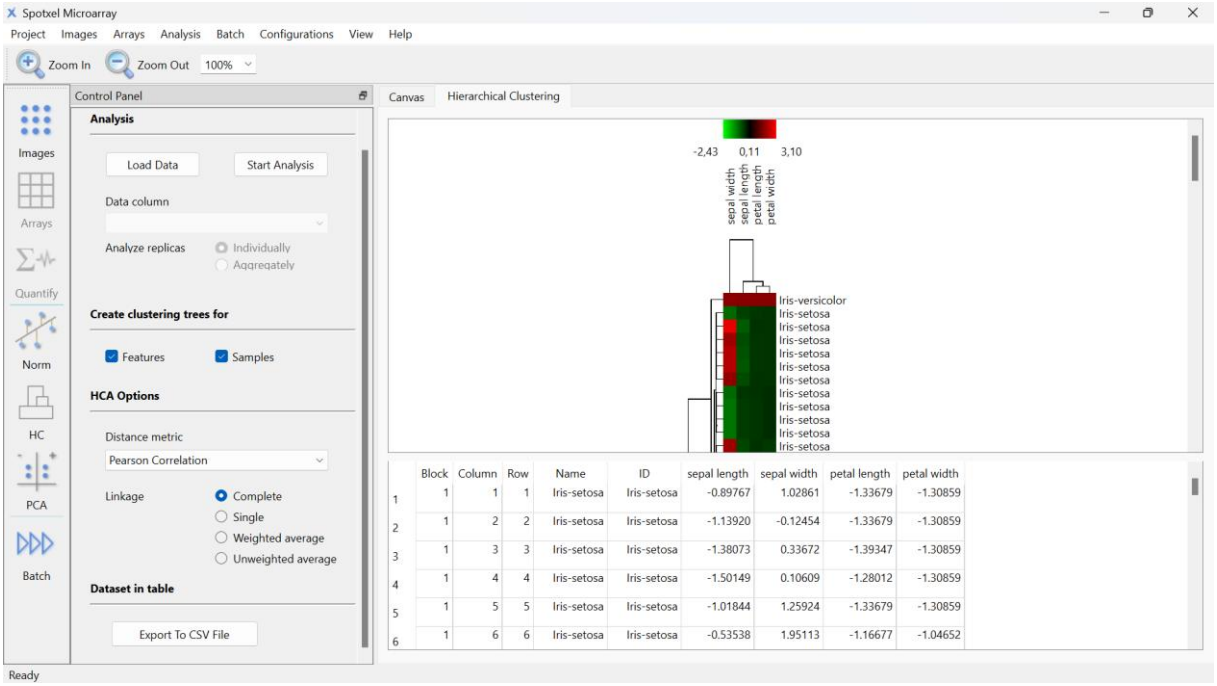


Figure 38: Hierarchical Clustering Analysis.

The clustering tree(s) are then constructed (Figure 38). Features that are considered related are grouped into one *cluster*. Their *relationship* is represented by a line connecting them. A cluster might be related to a feature or another cluster. The relationship between samples and clusters of samples are represented similarly.

The values in the dataset, each representing the screening value of a feature with a sample, are graphically represented by means of a heat map. You can save the clustering trees with the heat map to an image file using the *Export to Image* context menu.

9 Product Activation

The product activation requires an *internet connection*. You need to have a *serial number* obtained from the software provider or its distributors.

(1) In the *Evaluation Time Has Expired* dialog, click the *Next* button.

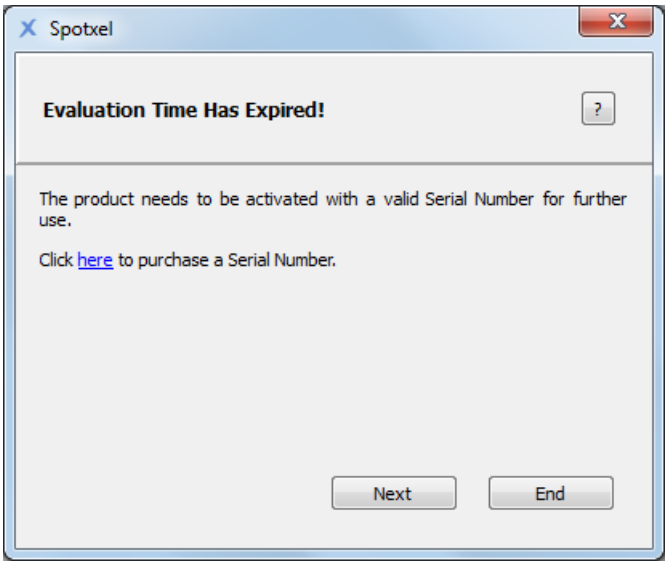


Figure 39: Starting the Product Activation.

(2) Enter the serial number and the licensee information in the *Product Activation* dialog. Click *Next*.

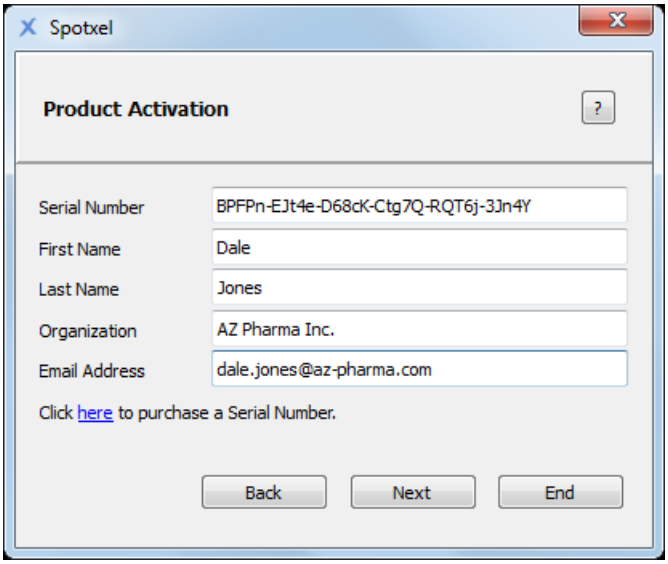


Figure 40: Entering the Licensee Information.

(3) If the internet connection is ready, click the *Activate* button and wait for the activation to finish.

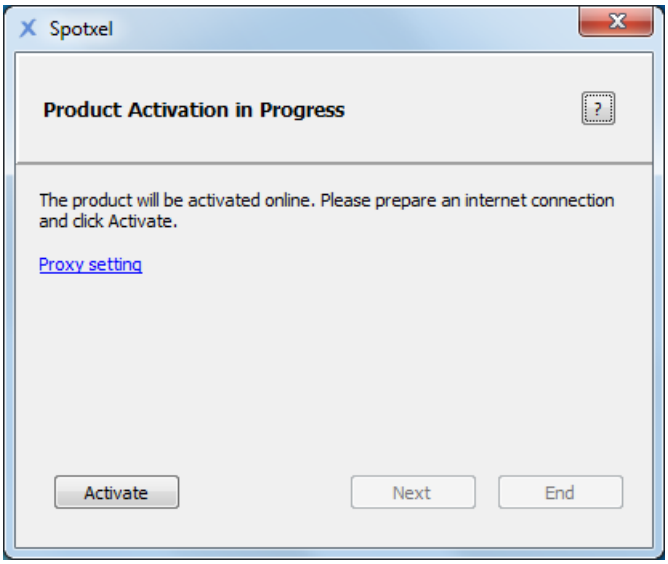


Figure 41: Product Activation in Progress.

Please check the internet connection in the case the software could not reach the activation server. If your system uses a proxy server to connect, specify it using the *Proxy setting* link. Otherwise, please contact the software provider for support.

(4) A completion message is shown when the product is successfully activated. Click *Next* to use the software immediately or *End* to use it later.

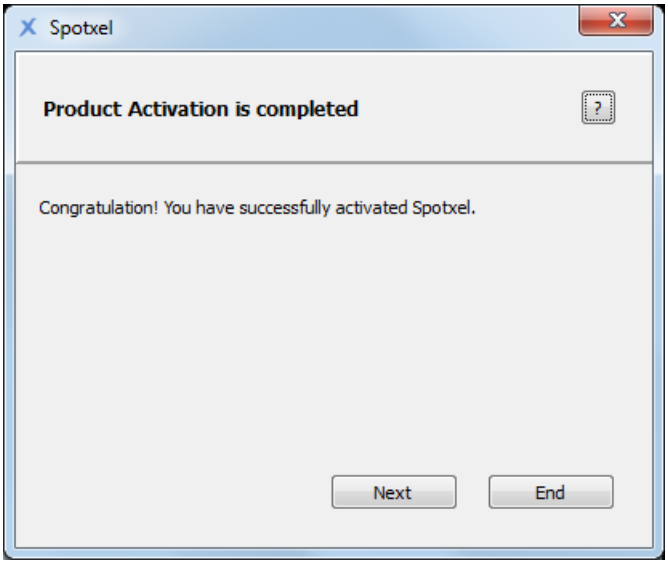


Figure 42: Completion of the Product Activation.

Please note that the license can be reviewed, or renewed in the case of a time-limited license, by clicking on the *Help* menu and choosing *License Information*.

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12 Revision History

Revision	Date	Changes
Rev 10	2025 June 20	**New functions:** • Support for PepSlide Designer (PSF) (*.psf) arrays • Batch Processing with image rotation support • Configurable visible data columns in the quantified data table **Updated:** • Terms and concepts • Fixed-Spot method • Batch Processing
Rev 9	2025 January 03	**New functions:** • 3D Spot Viewer **Updated functions:** • Automatic array alignment
Rev 8	2024 August 14	**New functions:** • GAL file editor • Feature-based spot finding **Removed functions:** • Background controls • Scatter Plot & K-Means Clustering • Quantification Options > “Include change of the images’ intensity value” • Image Intensity > Filter noise

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