Spotxel® Microarray 3.5

Microarray Image and Data Analysis Software

User's Guide

03 January 2025 - Rev 9

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

SICASYS Software GmbH
Geschwister-Scholl-Str. 1
76726 Germersheim
Germany

Phone +49 (7274) 973 8893
Fax +49 (7274) 973 9133
Email info@sicasys.de
Web www.sicasys.de

Table of Contents

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.

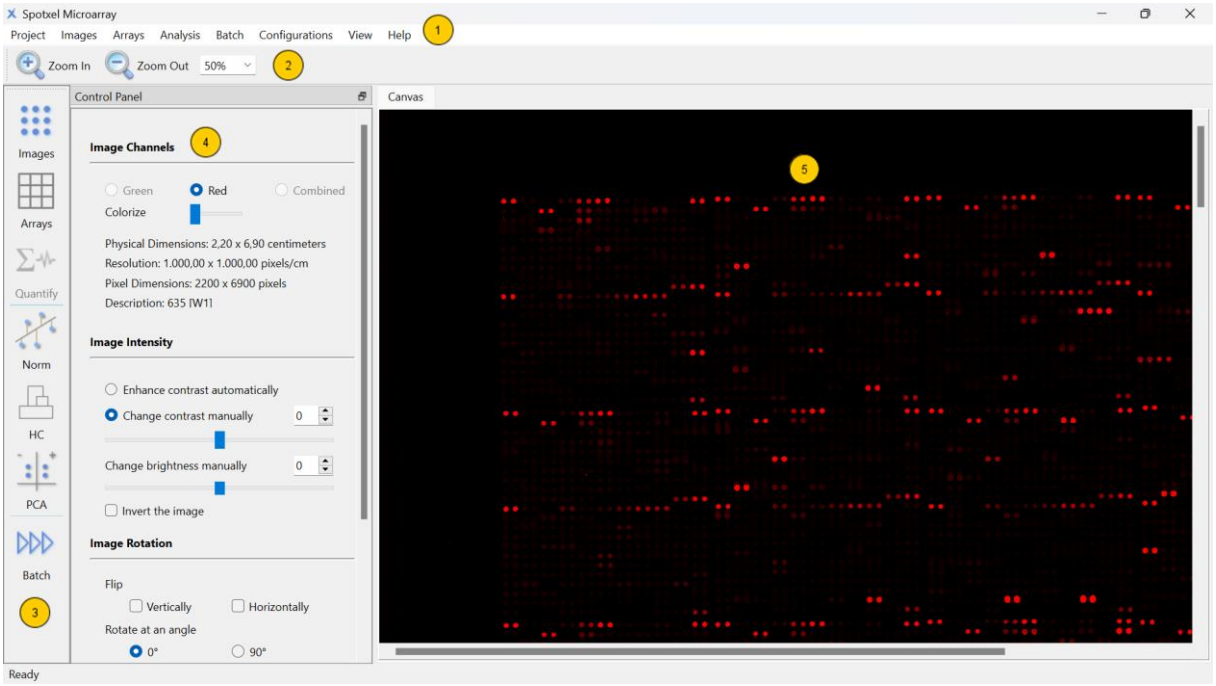


Figure 1: The Software User Interface.

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

In this manual, the term *array* refers to the spot layout and annotation of a microarray. We assume that the array is saved as a GenePix Array List (GAL) file. The term *image* or *microarray image* denotes a scanned or captured image of the printed microarray.

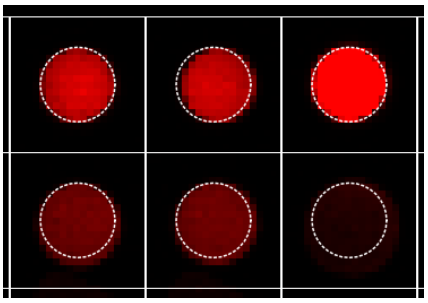


Figure 2: A rectangular block with 6 spots.

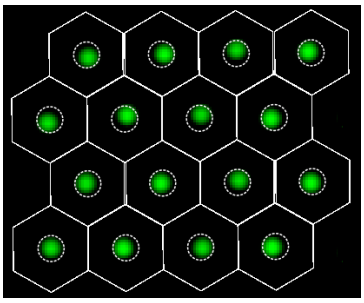


Figure 3: A hexagonal grid with 16 spots.

An array consists of *blocks*, each being a group of spots located next to each other. On the canvas, a *spot* is illustrated as a *white square* in a rectangular block or a *white hexagon* in a hexagonal block. Figure 2 shows a rectangular block with two rows and three columns. Figure 3 presents a hexagonal block with sixteen spots arranged in four rows and four columns. Within each spot, the *spotted region* is defined by the area bounded by the dashed circle, with its diameter specified by the spot diameter parameter for each block.

The binding signals of a microarray tested with a sample are converted by a microarray scanner or camera into a digital array image containing a matrix of pixels. Each pixel has a gray value

representing pixel intensity. Array images are often saved in the *TIFF format*[1]. In 8-bit grayscale images, the gray value ranges from 0 to 255, while in 16-bit grayscale images, it ranges from 0 to 65535. Since 16-bit grayscale images have a broader range of signal levels, they are recommended for array image analysis.

Quantification is the procedure that estimates the true binding signal for each spot and represents its signal value using the mean and median of pixel intensities within that spot. The quality of quantification depends on the *spot finding* or *spot detection* procedure, which determines which pixels in the array image belong to a spot. *Background correction* also contributes to quantification quality by estimating and removing signals caused by non-specific binding from the spot's signal.

For each spot in the array, the median and mean of its raw, background, and foreground values are calculated. *Raw* represents the intensity value of the spot's signal. *Background* is the estimated value of the signal caused by non-specific binding. The value of interest is *foreground*, computed by subtracting the background value from the raw value.

Array alignment is the process of associating spots in the array with their signal in the image. The spot's signal is presumably due to the binding of the immobilized substance in the spotted region with the sample. Therefore, before quantification, the array is reallocated such that the spotted regions are as close to the spots' signal as possible.

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.

[1] https://download.osgeo.org/libtiff/doc/TIFF6.pdf

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 the GAL format (*.gal).

Spotxel® Microarray supports 8-bit, 16-bit, and 24-bit grayscale images. You can also use 24-bit color images, but the dynamic range is limited. 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 4 for assigning the color channels. If only one single-page image is selected, then only a single assignment is shown.

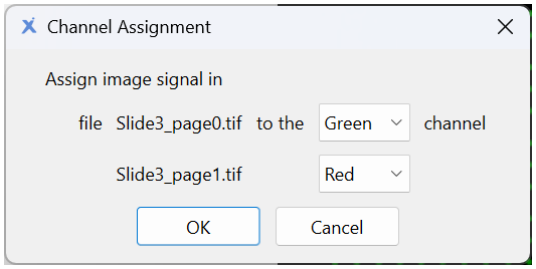


Figure 4: Loading single-page images.

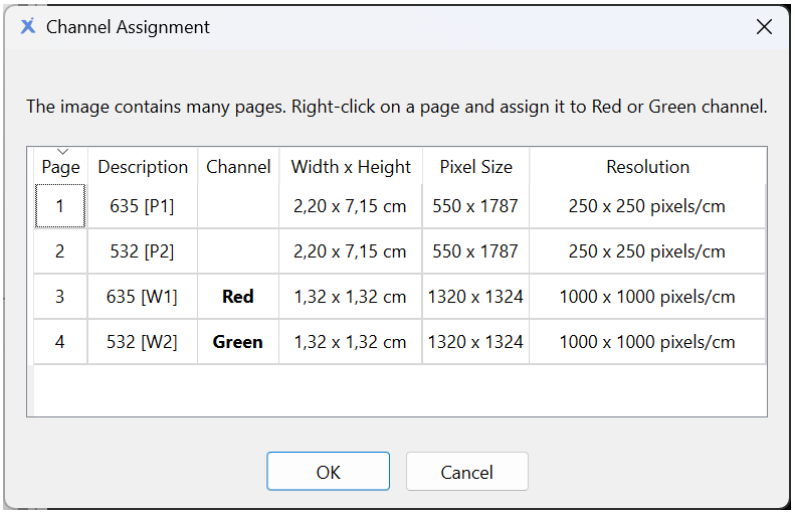


Figure 5: A multi-page image with two pages having the same resolution.

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

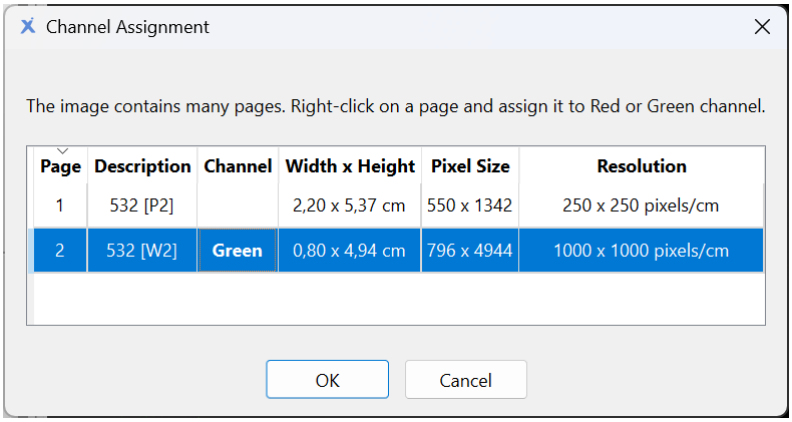


Figure 6: A multi-page image with two pages having different resolutions.

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 7). You can also launch this dialog via the *Configurations > Image Resolution Options* menu.

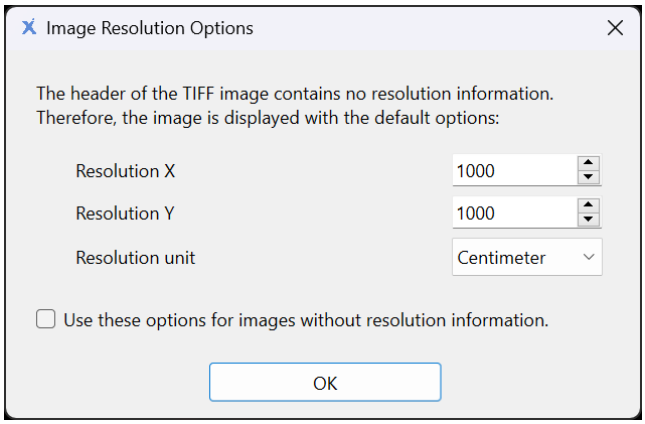


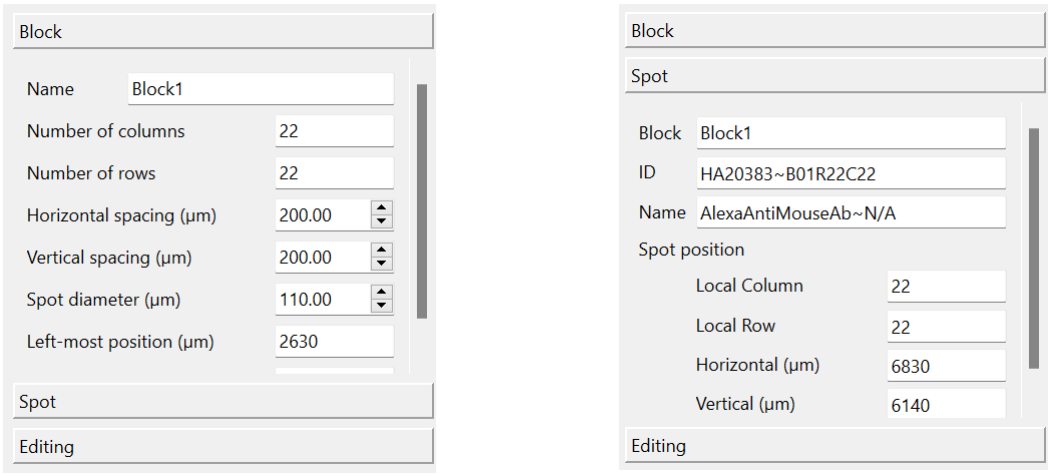
Figure 7: Setting image resolution.

If you are not certain about the resolution values, you can use the default values shown in Figure 7. 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.

2.1.3 Loading Array

Click the *Arrays* > *Open Array* menu and select the GAL array file.

After being loaded, the images and the array are shown in the graphical canvas (Figure 1). To obtain an appropriate view, you can use the *Zoom In* and *Zoom Out* buttons on the canvas toolbar or select a predefined zoom level in the *Zoom* combo-box. Alternatively, you can enter an arbitrary value directly into the Zoom combo-box.



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

Figure 8: Properties of an Array Object.

2.2 Viewing Array Data

In the *Array File* section of the *Arrays* control panel, you can view properties of a block or a spot. To show a block's properties (Figure 8-a) in the *Block* page:

- Open the *Block* page by clicking on it.
- Hover the mouse over, or click on, the block in the canvas.

Similarly, you can open the *Spot* page and then points to a spot in the canvas to view its properties (Figure 8-b).

A detailed guide on creating and editing an array file can be found in Section *3 - Managing 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 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.

2.4 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 GAL file, and the project file are shown in the *Data Files* section of the *Images*, *Arrays*, and *Quantification* control panels.

3 Managing 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 10) or *Hexagon (orange packing) even row* (Figure 11) 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.

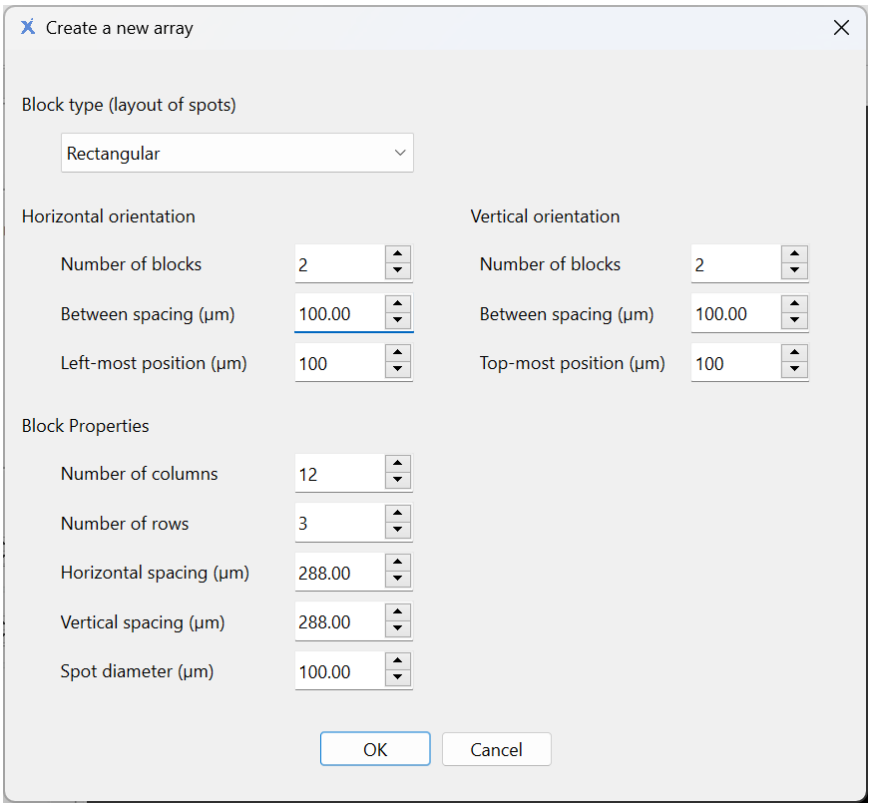


Figure 9: Creating a New Array.

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 9 illustrates a sample setup, and Figure 10 shows the created rectangular blocks.

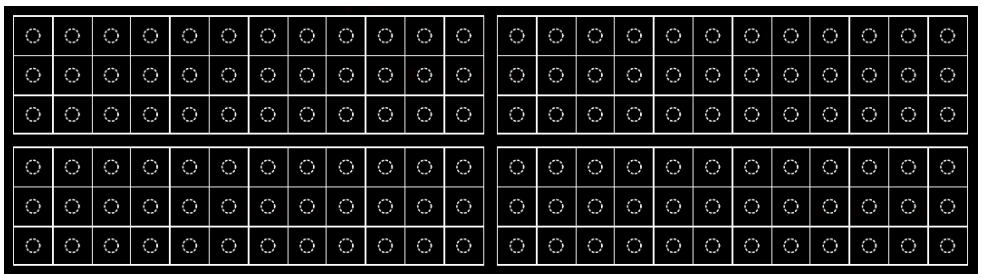


Figure 10: Rectangular Blocks.

Figure 11 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.

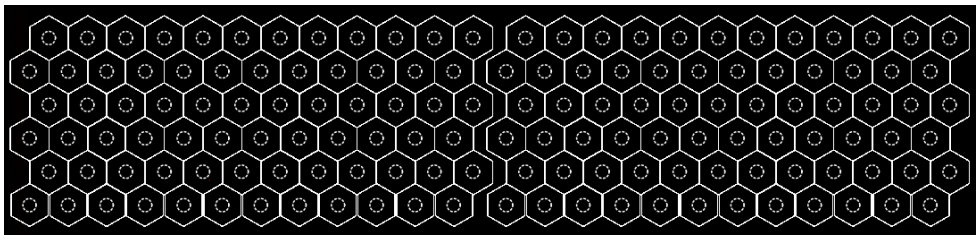


Figure 11: Hexagonal (Orange-Packing) Blocks.

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 9) 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 12), 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.

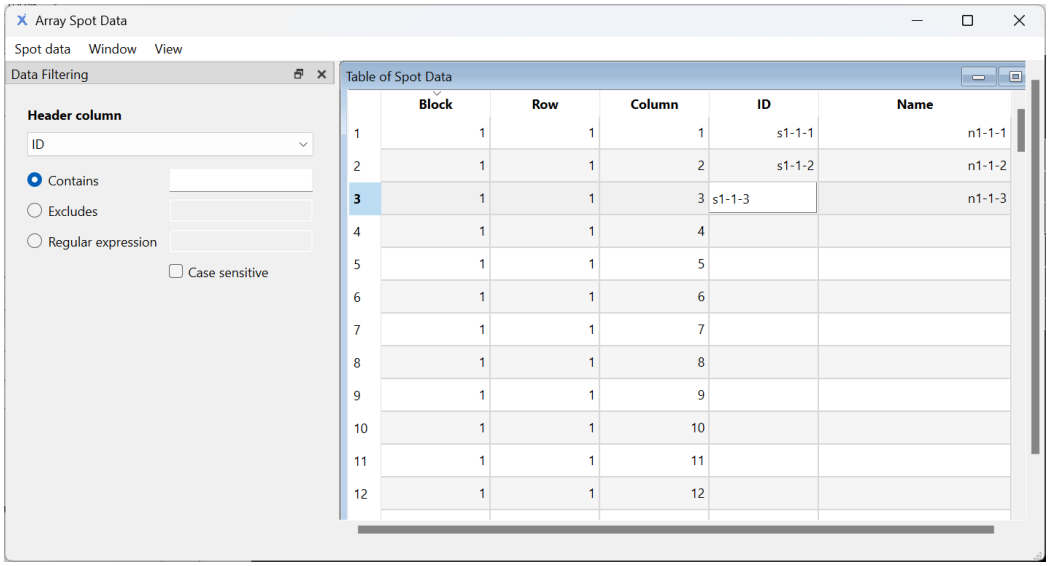


Figure 12: Table of Spot Data.

In addition, the *Data Filtering* widget on the left (Figure 12) 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 16-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 13), 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 14).

- 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.

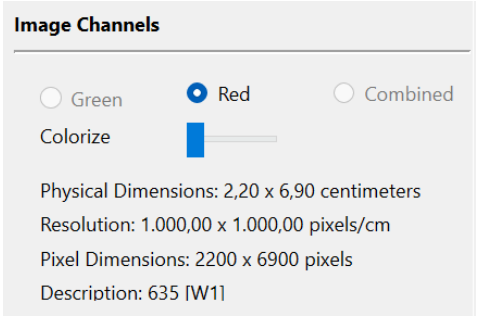


Figure 13: Image Properties.

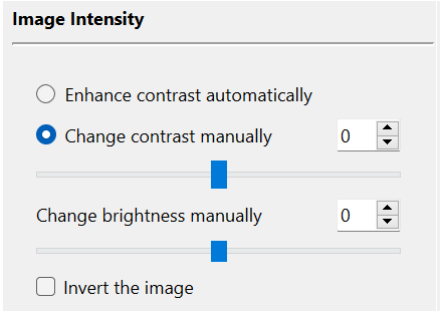
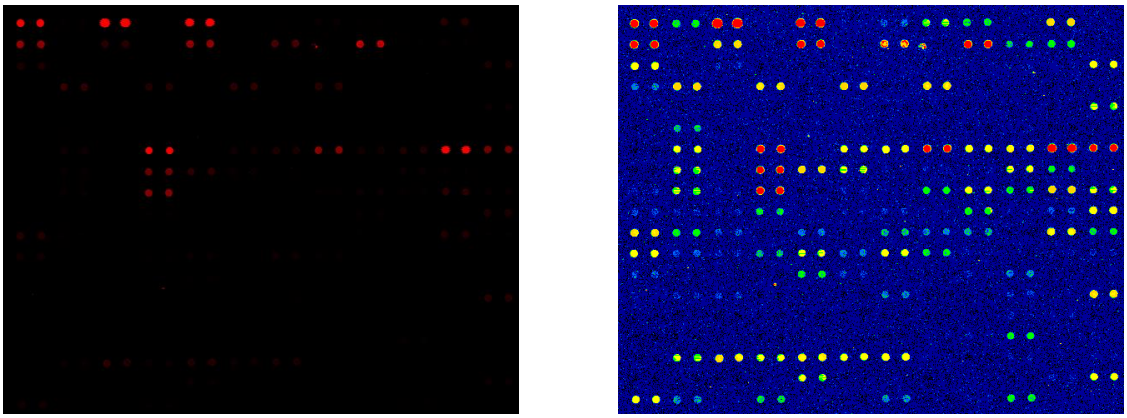


Figure 14: Image Processing.

Colorization

Another means to visualize the spots is the *colorization* tool (Figure 13). 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 15 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 15: Colorization.

The tool can be turned on and off by clicking on the left or right side of the *Colorize* switch (Figure 13). 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

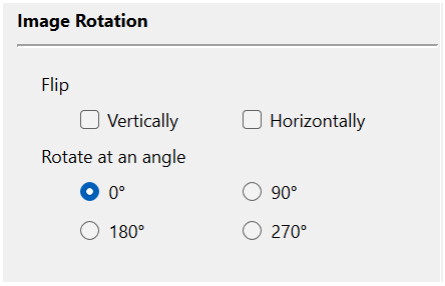
You can flip and/or rotate images at angles of 90°, 180°, or 270°. These functions are located in the *Images* > *Image Rotation* (Figure 16-a).

Rotating Array

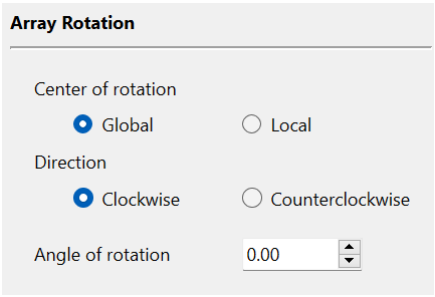
If the array slightly deviates from the spots in the image at a small angle, it is recommended to rotate the array (i.e. the relevant blocks), since image rotation may alter the image data. You can select blocks and rotate them at an arbitrary angle. These functions are located in the *Arrays* > *Array Rotation* section (Figure 16-b). The degree change can be as small as 0.01°.

Selected blocks 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 16: Rotation of Images and Arrays.

4.3 Automatic Array Alignment

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 17 and Figure 18.

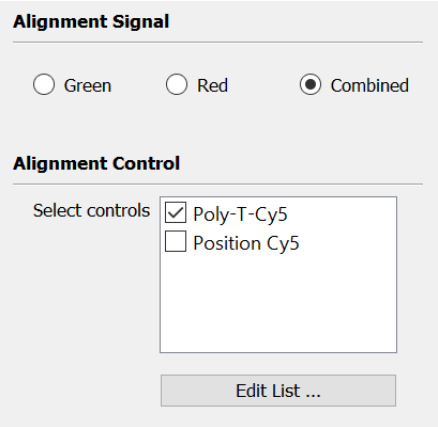


Figure 17: Alignment Options - Signal & Controls.

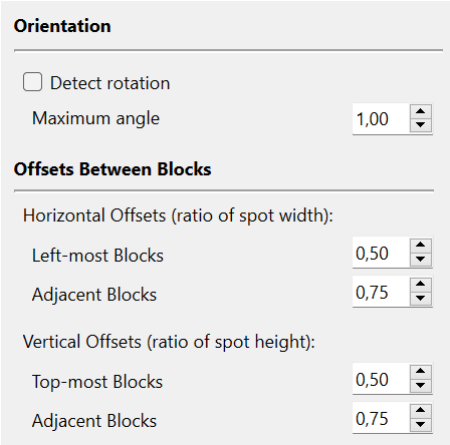


Figure 18: Alignment Options – Orientation & Offsets Between Blocks.

Alignment Signal

This setting (Figure 17, 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 18, 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 19, 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 18, 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 19).
- *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 19).

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 19).
- *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 19).

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

- Typically, if blocks are adjacent, the offsets between them are less than 1 (e.g., 0.5 or 0.75 times the spot size).
- If blocks are spaced further apart, the offsets might be 1.5 or 2 times the spot size.

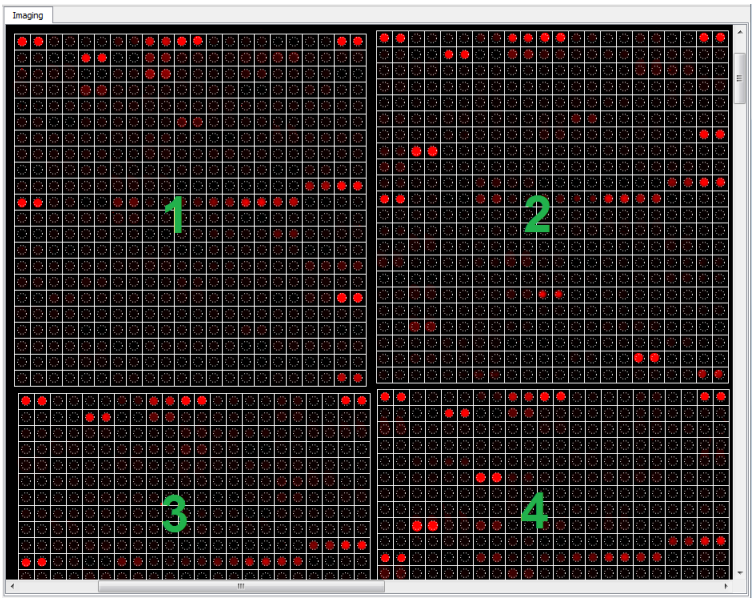


Figure 19: Offset of Blocks.

4.3.3 Alignment Controls

You can also use positional controls to optimize the alignment:

- Check the box next to the controls you want to use for alignment. In Figure 17, the control Poly-T-Cy5 is selected.
- To add or modify a control, click the *Edit List* button in the *Alignment Control* section. The software will show a list of all controls.
- To create a control, click the *Add* button. To modify an existing one, click the *Change* button. In the *Edit the Control* dialog, specify the name, ID, and replication mode of the control spot. Once created, the controls remain for future use.

Spots with the same *Name* (in the array file) as the selected alignment controls will be used as positional markers. The array alignment will position the grids to best match 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 21). Detected spots in the canvas are highlighted with a blue border by default (Figure 20).

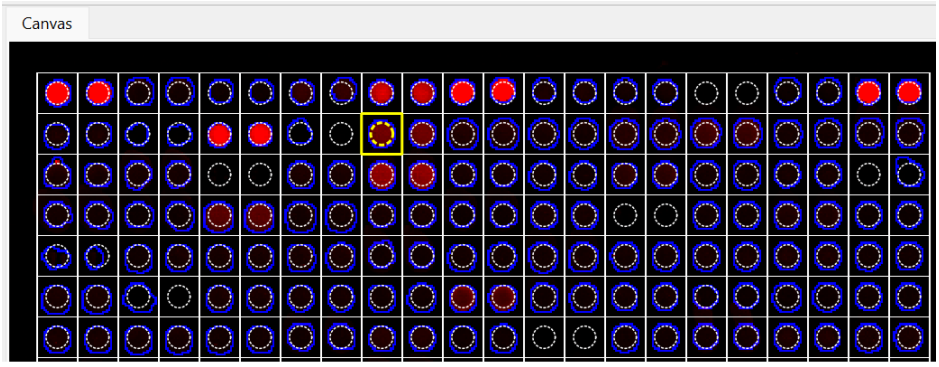


Figure 20: Highlighted Spots in Canvas.

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

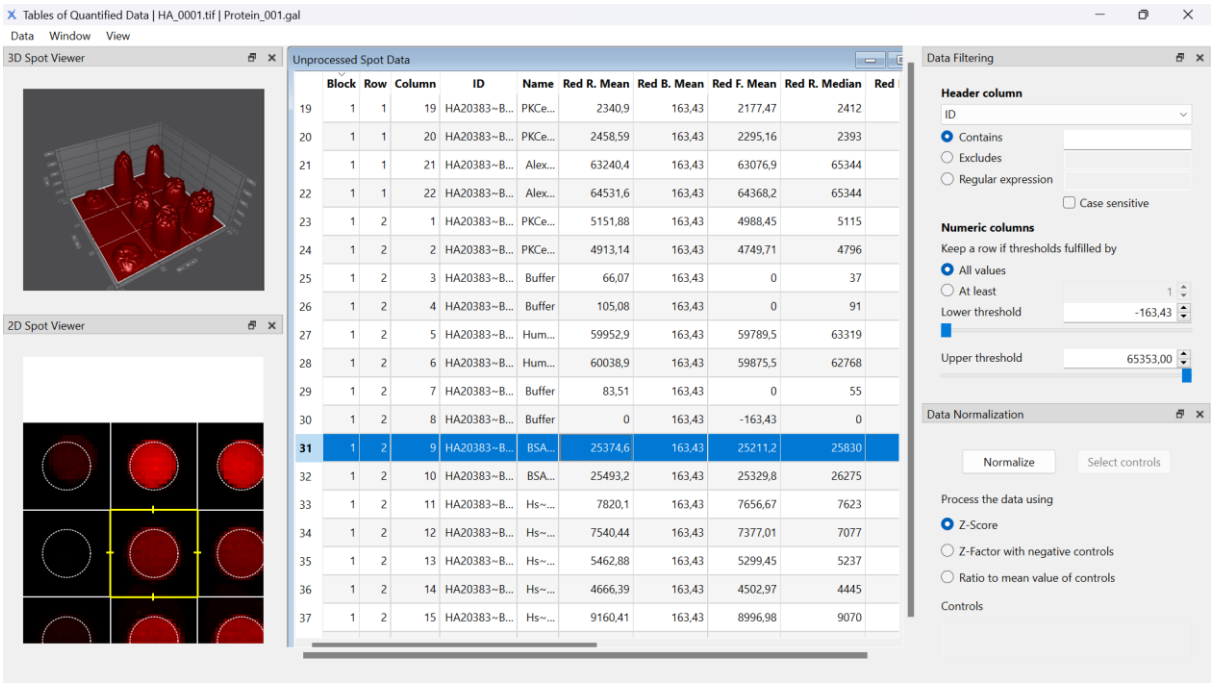


Figure 21: Table of Quantified Data.

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.)

2D Spot Viewer

The *2D Spot Viewer* allows you to view the selected spot, its neighbors, and their quantified data simultaneously (Figure 21, bottom-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 21, top-left, and Figure 22) 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

You can export the quantified data to a CSV file (**.csv*) or a GenePix Result file (**.gpr*) for further analysis.

Docking and Undocking a Tool

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 22). 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.

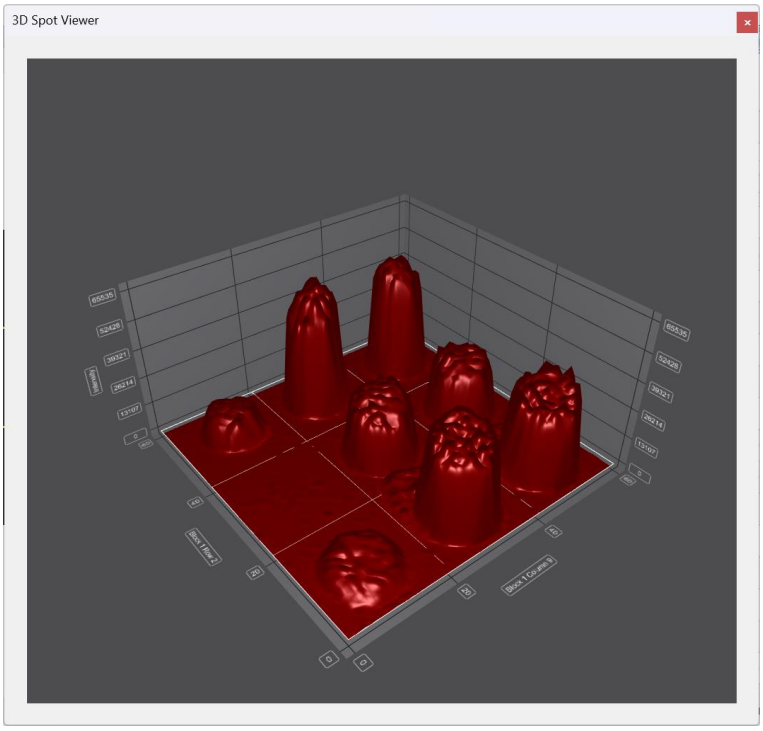


Figure 22: 3D Spot Viewer.

5.3 Quantification Options

To display the quantification options, check the *Show quantification options* checkbox in the *Quantification* control panel (Figure 23). The available options depend on the selected spot detection method, of which there are three.

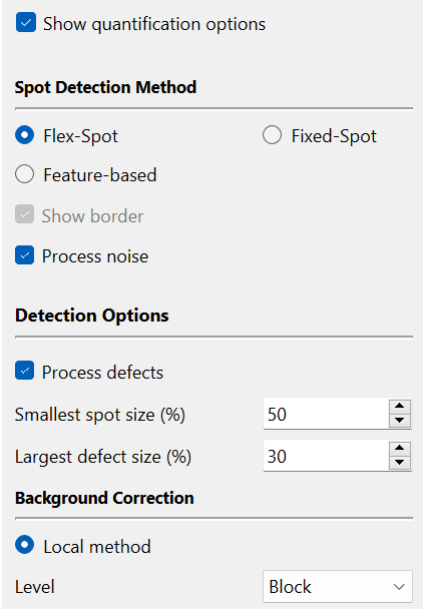


Figure 23: Flex-Spot Options.

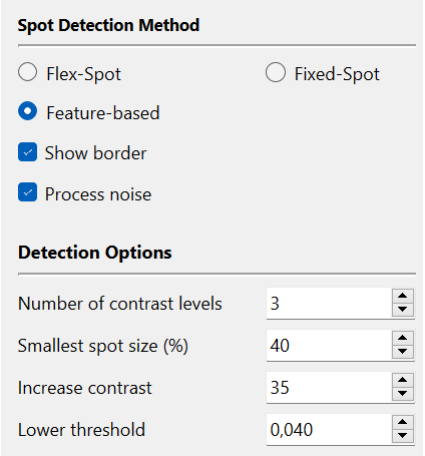


Figure 24: Feature-Based Options.

Fixed-Spot

The Fixed-Spot method always uses the pixels within the predefined spotted region (Figure 2 and Figure 3, dashed circles) to compute the raw value. The background value is calculated based on the pixels in the remaining area within the spot.

Flex-Spot

The Flex-Spot method (Figure 23) 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 20). 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, as specified in the GAL file.

Feature-Based

The Feature-Based spot detection method (Figure 24) 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[2], 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.

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

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 23). 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 23 and Figure 24. 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

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.

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.

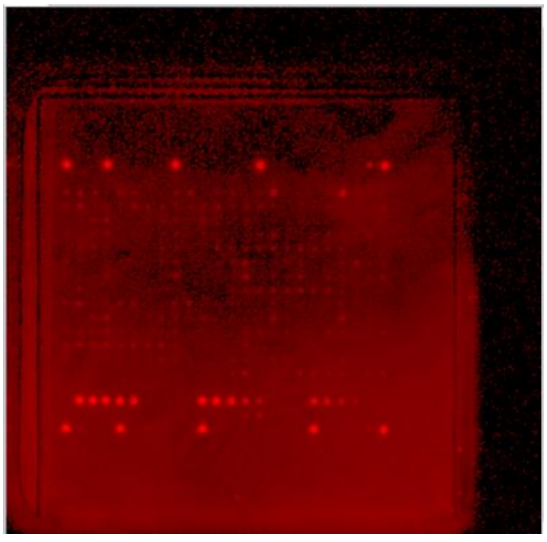
5.6 Noise Processing

By checking the *Process noise* option (Figure 23 and Figure 24), 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.

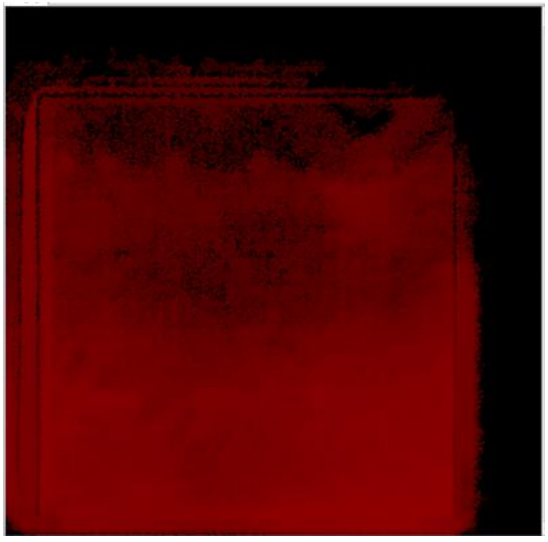
Background Noise

Noises in microarray images can mislead the spot detection procedure and result in incorrect estimation of the true specific-binding signal. Figure 25-a shows a slide image with non-homogeneous background signal, concealing specific-binding signals and affecting array alignment and spot finding.

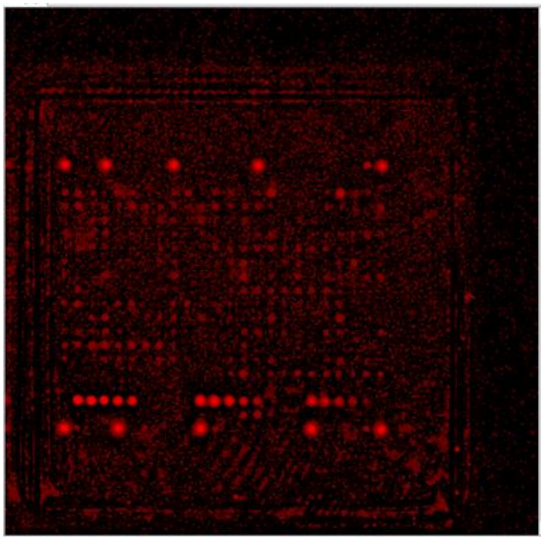
Equipped with powerful image processing functions, the software can process such background noise. Figure 25-b visualizes the detected background signal alone, while Figure 25-c shows the slide image with the background signal eliminated, revealing the spots' signals clearly.



(a) Original image.



(b) Detected background signal.



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

Figure 25: Noise Processing.

Foreground Noise

Figure 26 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.

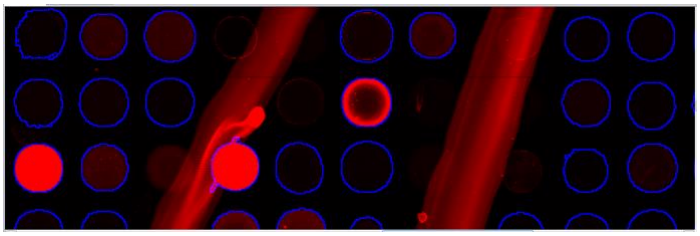


Figure 26: Foreground Noise Processing.

Smearing Signal

Figure 27 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.

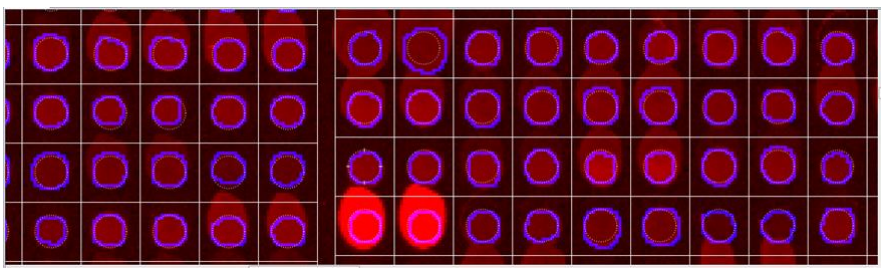


Figure 27: Smearing Processing.

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 23, bottom-right). The default for GAL files is the *Block* level.

- *Block* Level: 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.
- *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

You can set up a batch to process multiple microarray images automatically. Suppose the experiment involves screening a protein microarray with *k* samples. The microarray design is annotated by the *template array*. From the screening results, you have *k* scanned images to quantify. For each scanned image, the batch aligns the template array with the image, creates the GAL file with the aligned layout, and generates the quantified data.

To create a batch, click the *Batch* button in the main toolbar (Figure 28). In the *Batch* control panel:

- Click the *Add* button and select the microarray images for processing. They will be added to the scheduling table. Use the *Add* and *Remove* buttons to modify the table.
- Double-click on the *Template array* edit-box to browse to the template array file.
- Specify the folder to store generated files and the running mode.
- Save the batch to a file using the *Batch > Save Batch* menu. The batch log is created automatically and named after the batch file.

We recommend using a separate folder for each batch to store the batch file and generated data. Since the software uses the dot character (".") for file extensions such as .gal or .csv, *avoid naming folders or files used in a batch with dot characters* (except for the file extension) to prevent errors.

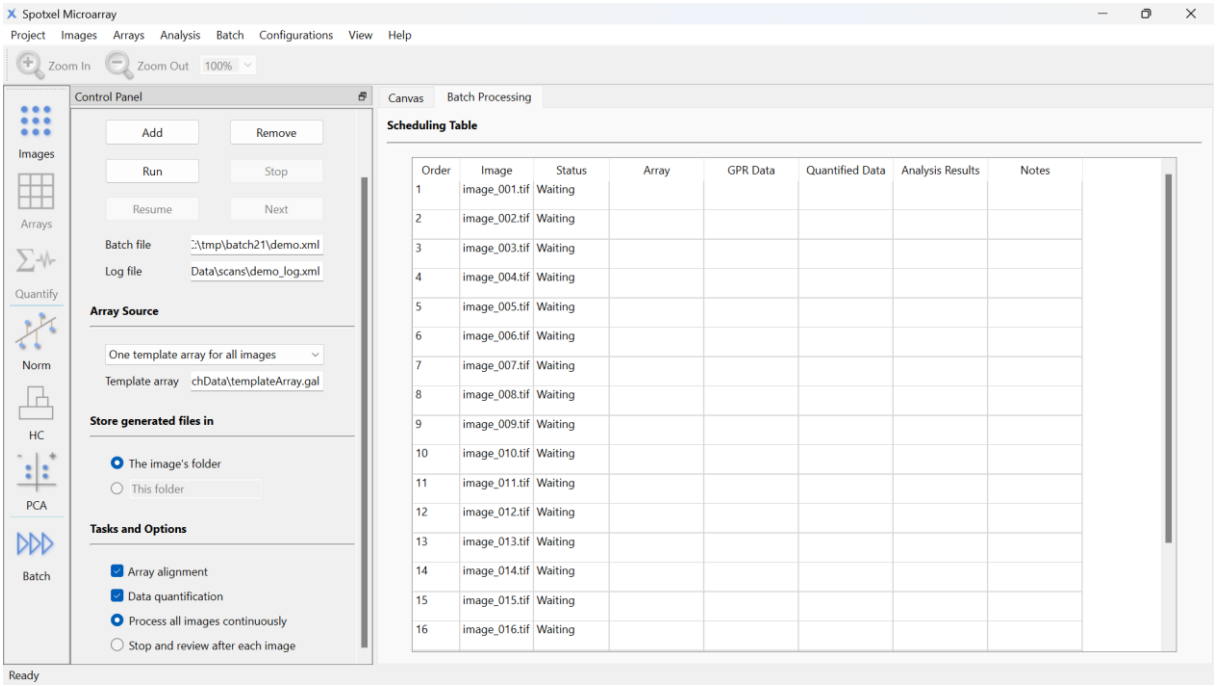


Figure 28: Batch Setup.

After creating the batch, click the *Run* button to execute it (Figure 29). Note the *running mode*:

- *Process all images continuously*: The batch processes continuously without stopping.
- *Stop and review after each image*: You can view the batch results for one image before proceeding to process the next one.

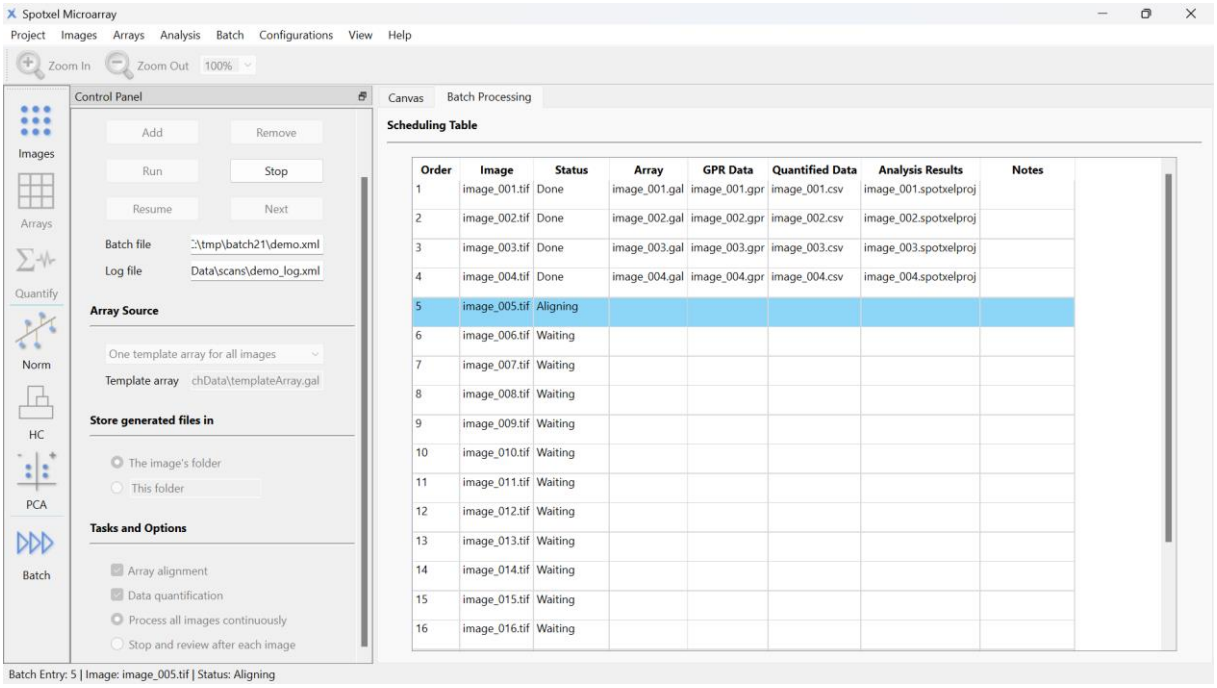


Figure 29: Batch Execution.

Suppose that *sample001.tif* is an image in the batch. The software creates four data files for it:

- *sample001.gal*: The array file with the spot layout aligned to the image *sample001.tif*.
- *sample001.csv*: A CSV file containing only the quantified data.
- *sample001.gpr:* A file containing quantified data in the GenePix Result (GPR) format.
- *sample001.spotxelproj*: the Spotxel® Microarray project file containing the analysis data for this image.

7 Data Preprocessing and Normalization

The *Data Preprocessing and Normalization* tool (Figure 30) 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.

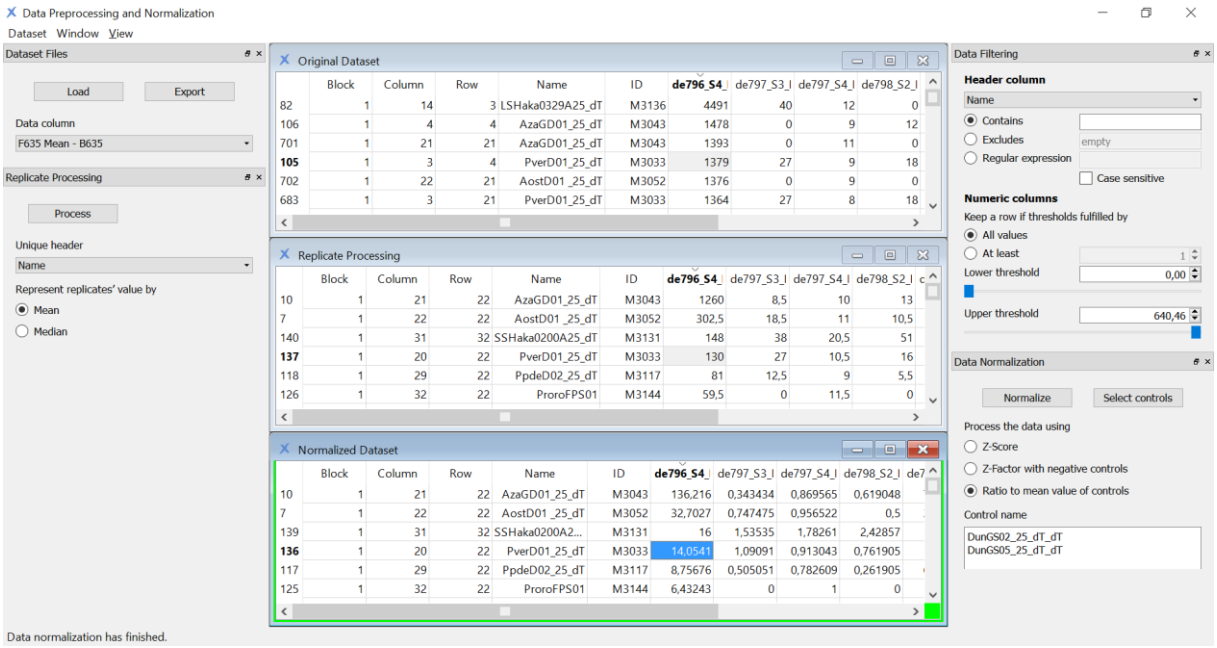


Figure 30: Replicate Processing, Data Filtering, and Data Normalization.

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 30, 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 30, 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 30, 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 30, 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 30, 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*[3] search pattern. Additionally, you can select only features whose signal value is within a specified

[3] Regular expression - Wikipedia

range using the lower and upper thresholds. These tools are provided in the *Data Filtering* panel (Figure 30, 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 30, 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)[4].
- *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 31) 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.

[4] 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 30, bottom-center).

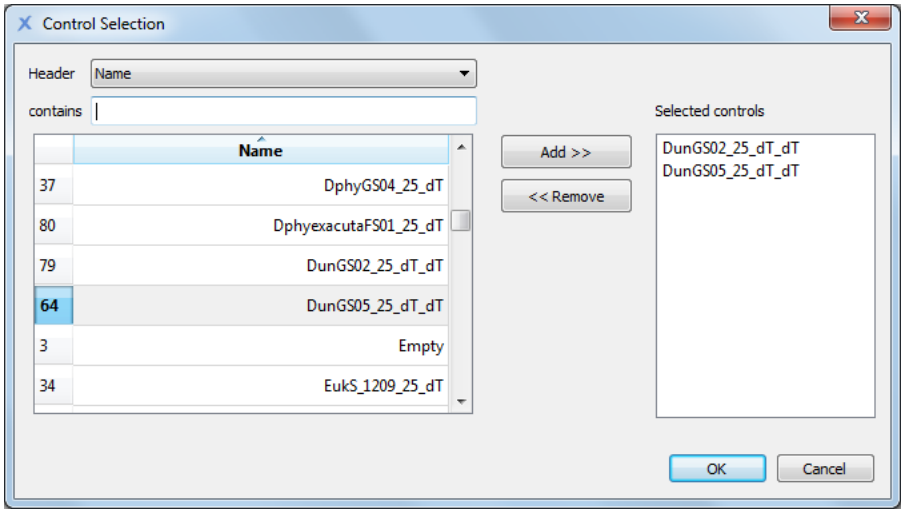


Figure 31: Control Selection for Data Normalization.

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 32):

- 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.

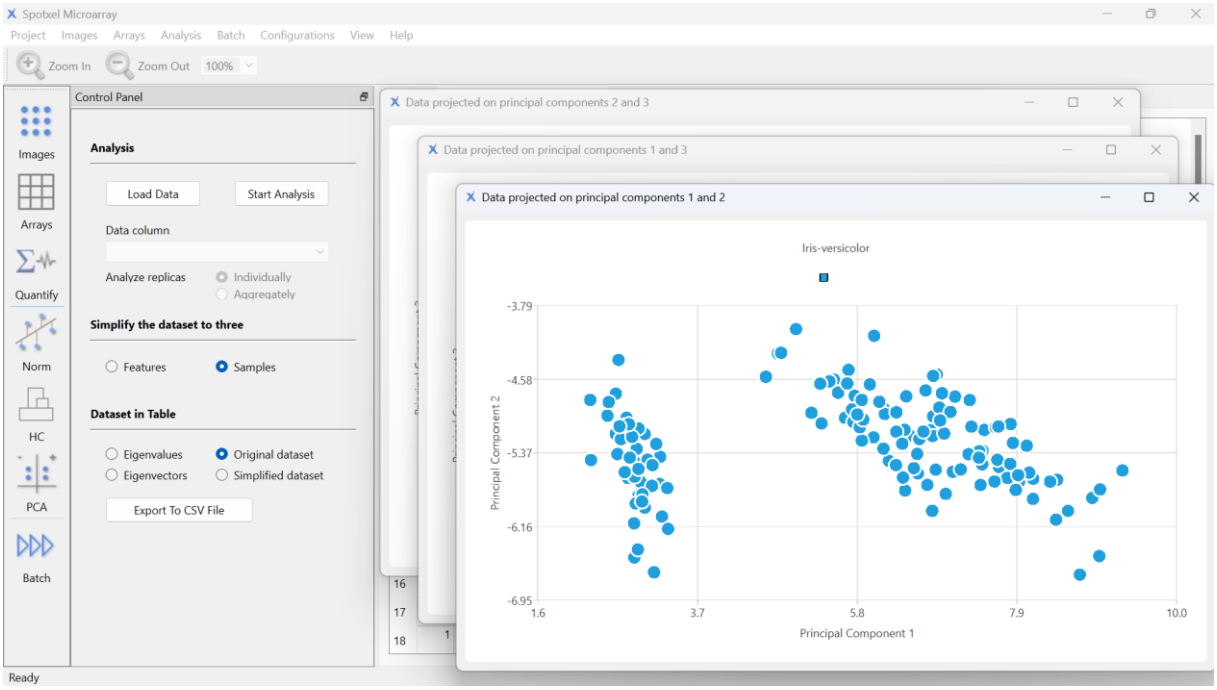


Figure 32: Principal Component Analysis in 2D View.

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 32), 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.

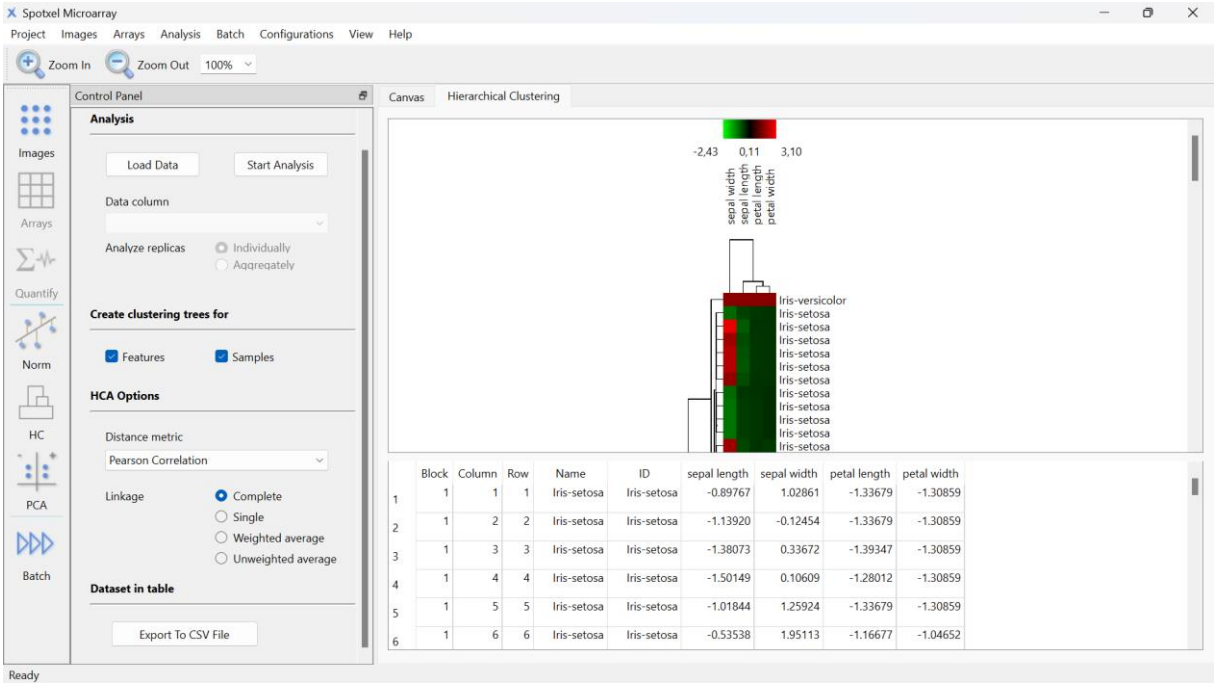


Figure 33: Hierarchical Clustering Analysis.

The clustering tree(s) are then constructed (Figure 33). 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.

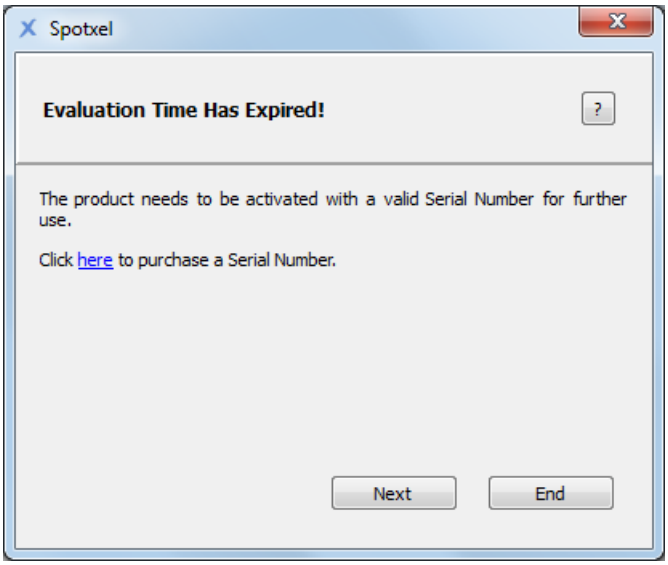


Figure 34: Starting the Product Activation.

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

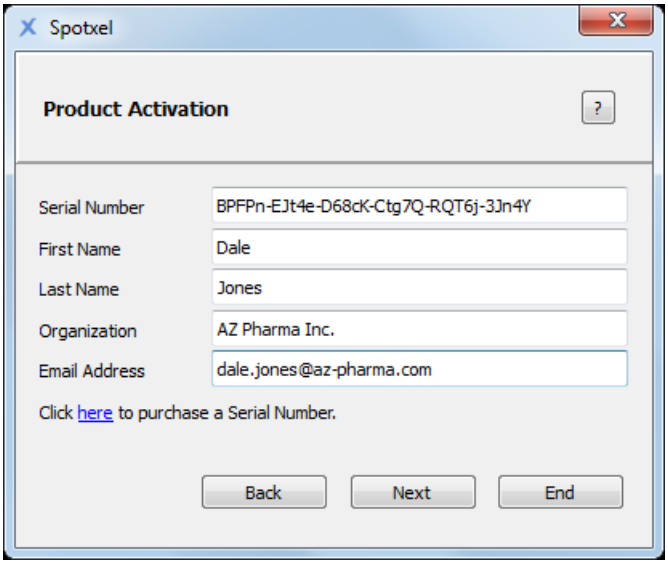


Figure 35: Entering the Licensee Information.

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

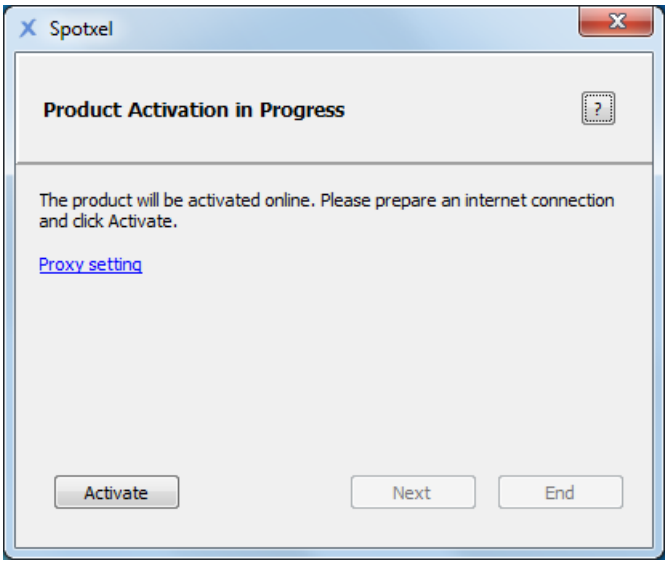


Figure 36: Product Activation in Progress.

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.

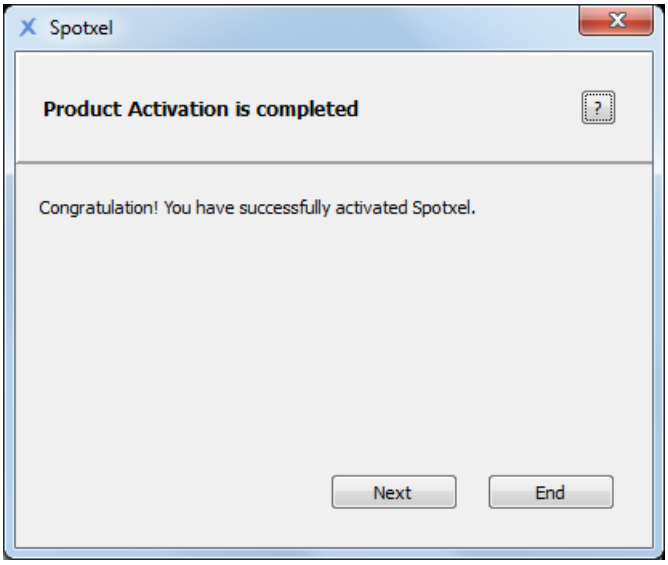


Figure 37: Completion of the Product Activation.

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*.

10 End-User License Agreement

SPOTXEL MICROARRAY IS THE PROPERTY OF SICASYS SOFTWARE GMBH ("SICASYS"). THE USE OF THIS SOFTWARE IS GOVERNED BY THE TERMS AND CONDITIONS OF THE END-USER LICENSE AGREEMENT ("AGREEMENT") SET FORTH BELOW. THE TERM “SOFTWARE” ALSO INCLUDES RELATED DOCUMENTATION (WHETHER IN PRINT OR ELECTRONIC FORM) AND ANY UPDATES OR UPGRADES OF THE SOFTWARE PROVIDED BY SICASYS.

BY INSTALLING THE SOFTWARE, AND/OR BY USING THE SOFTWARE, YOU AGREE TO BE BOUND BY THE TERMS AND CONDITIONS OF THIS END-USER LICENSE AGREEMENT.

License. SICASYS grants to you (“USER”) a non-exclusive, non-transferable license to use the SOFTWARE on the number of computers stated in the license contract. A separate license is required for use on any additional computer.

Copy Restriction. The SOFTWARE may not be copied either in full or part by USER, with the exception of making copy for security or backup purpose. Copies must include all copyright and trademark notices.

Use Restrictions. This SOFTWARE is licensed to USER for internal use only. USER shall not (and shall not allow any third party to):

(i) decompile, disassemble, reverse engineer or attempt to reconstruct, identify or discover any source code, underlying ideas, underlying user interface techniques or algorithms of the SOFTWARE by any means whatever, or disclose any of the foregoing;

(ii) modify, incorporate into or with other software, or create a derivative work of any part of the SOFTWARE;

(iii) attempt to circumvent any user limits, or other license, timing or use restrictions that are built into the SOFTWARE.

Ownership of the SOFTWARE. SICASYS retains all titles of ownership, all ownership rights, and all intellectual property rights of the SOFTWARE. SICASYS reserves all rights not expressly granted to USER.

Termination. SICASYS may terminate this Agreement immediately if USER breaches any provision. Upon notice of termination by SICASYS, all rights granted to USER under this Agreement will immediately terminate, and USER shall cease using the SOFTWARE and return or destroy all copies of the SOFTWARE.

Limited Warranty and Disclaimer. USER is aware of the fact that technical errors in the program in accordance with the accompanying documentation cannot be excluded. If USER claims deviations in the program from the specification/description, USER has the right to request a fix, patch, work-around, or replacement of the SOFTWARE that does not meet such limited warranty. If a rectification of the errors is not possible or if such rectification is not successful, USER has the right to request a rescission of the contract, where USER must destroy all copies of the SOFTWARE.

EXCEPT AS EXPRESSLY SET FORTH ABOVE, NO OTHER WARRANTIES OR CONDITIONS, EITHER EXPRESS, IMPLIED, STATUTORY OR OTHERWISE, ARE MADE BY SICASYS WITH RESPECT TO THE SOFTWARE AND THE ACCOMPANYING DOCUMENTATION, AND SICASYS EXPRESSLY DISCLAIMS ALL WARRANTIES AND CONDITIONS NOT EXPRESSLY STATED HEREIN, INCLUDING BUT NOT LIMITED TO THE IMPLIED WARRANTIES OR CONDITIONS OF MERCHANTABILITY, NONINFRINGEMENT, AND FITNESS FOR A PARTICULAR PURPOSE. SICASYS DO NOT WARRANT THAT THE FUNCTIONS CONTAINED IN THE SOFTWARE WILL MEET USER'S REQUIREMENTS, BE UNINTERRUPTED OR ERROR FREE, OR THAT ALL DEFECTS IN THE PROGRAM WILL BE CORRECTED. USER ASSUMES THE ENTIRE RISK AS TO THE RESULTS AND PERFORMANCE OF THE SOFTWARE.

Limitation of Liability. IN NO EVENT SHALL SICASYS BE LIABLE FOR ANY INDIRECT, SPECIAL, CONSEQUENTIAL OR INCIDENTAL DAMAGES WHATSOEVER (INCLUDING, WITHOUT LIMITATION, DAMAGES FOR LOSS OF BUSINESS PROFITS, BUSINESS INTERRUPTIONS, LOSS OF BUSINESS INFORMATION, OR OTHER PECUNIARY LOSS) ARISING OUT OF THE USE OF OR INABILITY TO USE THE SOFTWARE, EVEN IF SICASYS HAVE BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. FURTHER, IN NO EVENT SHALL SICASYS BE LIABLE FOR ANY DIRECT DAMAGES ARISING OUT OF USER'S USE OF THE SOFTWARE. IN NO EVENT WILL SICASYS BE LIABLE TO USER FOR DAMAGES IN AN AMOUNT GREATER THAN THE FEES PAID FOR THE USE OF THE SOFTWARE.

Intellectual Property Right Infringement. If a claim alleging infringement of an intellectual property right arises concerning the SOFTWARE (including but not limited to patent, trade secret, copyright or trademark rights), SICASYS in its sole discretion may elect to defend or settle such claim, and/or terminate this Agreement and all rights to use the SOFTWARE, and require the return or destruction of the SOFTWARE, with a refund of the fees paid for use of the SOFTWARE less a reasonable allowance for use and shipping.

Miscellaneous. This Agreement is the entire agreement between USER and SICASYS with respect to the SOFTWARE, and supersedes any previous oral or written communications or documents (including, if USER is obtaining an update, any agreement that may have been included with the initial version of the Software). This Agreement is governed by the laws of Germany. If any provision, or portion thereof, of this Agreement is found to be invalid or unenforceable, it will be enforced to the extent permissible and the remainder of this Agreement will remain in full force and effect. Failure to prosecute a party's rights with respect to a default hereunder will not constitute a waiver of the right to enforce rights with respect to the same or any other breach.

11 Third-Party Libraries

LibTIFF

This software uses the LibTIFF library. The LibTIFF library is provided under its own license. The full text of the LibTIFF license is included below.

LibTIFF license

Copyright © 1988-1997 Sam Leffler\
Copyright © 1991-1997 Silicon Graphics, Inc.

Permission to use, copy, modify, distribute, and sell this software and
its documentation for any purpose is hereby granted without fee, provided
that (i) the above copyright notices and this permission notice appear in
all copies of the software and related documentation, and (ii) the names of
Sam Leffler and Silicon Graphics may not be used in any advertising or
publicity relating to the software without the specific, prior written
permission of Sam Leffler and Silicon Graphics.

THE SOFTWARE IS PROVIDED "AS-IS" AND WITHOUT WARRANTY OF ANY KIND,
EXPRESS, IMPLIED OR OTHERWISE, INCLUDING WITHOUT LIMITATION, ANY
WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.

IN NO EVENT SHALL SAM LEFFLER OR SILICON GRAPHICS BE LIABLE FOR
ANY SPECIAL, INCIDENTAL, INDIRECT OR CONSEQUENTIAL DAMAGES OF ANY KIND,
OR ANY DAMAGES WHATSOEVER RESULTING FROM LOSS OF USE, DATA OR PROFITS,
WHETHER OR NOT ADVISED OF THE POSSIBILITY OF DAMAGE, AND ON ANY THEORY OF
LIABILITY, ARISING OUT OF OR IN CONNECTION WITH THE USE OR PERFORMANCE
OF THIS SOFTWARE.

zlib

This software uses LibTIFF, which links to the zlib library. The zlib library is provided under the zlib License. The full text of the zlib License is included below.

Copyright notice:

(C) 1995-2022 Jean-loup Gailly and Mark Adler

This software is provided 'as-is', without any express or implied
warranty. In no event will the authors be held liable for any damages

arising from the use of this software.

Permission is granted to anyone to use this software for any purpose,
including commercial applications, and to alter it and redistribute it
freely, subject to the following restrictions:

1. The origin of this software must not be misrepresented; you must not
   claim that you wrote the original software. If you use this software
   in a product, an acknowledgment in the product documentation would be
   appreciated but is not required.
2. Altered source versions must be plainly marked as such, and must not be
   misrepresented as being the original software.
3. This notice may not be removed or altered from any source distribution.

Jean-loup Gailly Mark Adler
jloup@gzip.org madler@alumni.caltech.edu

libjpeg-turbo

This software uses LibTIFF, which links to the libjpeg-turbo library. The libjpeg-turbo library is provided under the IJG License and the Modified BSD License. The full text of the Modified BSD License is included below.

This software is based in part on the work of the Independent JPEG Group.

The Modified (3-clause) BSD License
==========

Copyright (C)2009-2023 D. R. Commander. All Rights Reserved.
Copyright (C)2015 Viktor Szathmáry. All Rights Reserved.

Redistribution and use in source and binary forms, with or without
modification, are permitted provided that the following conditions are met:

- Redistributions of source code must retain the above copyright notice,
  this list of conditions and the following disclaimer.
- Redistributions in binary form must reproduce the above copyright notice,
  this list of conditions and the following disclaimer in the documentation
  and/or other materials provided with the distribution.
- Neither the name of the libjpeg-turbo Project nor the names of its
  contributors may be used to endorse or promote products derived from this
  software without specific prior written permission.

THIS SOFTWARE IS PROVIDED BY THE COPYRIGHT HOLDERS AND CONTRIBUTORS "AS IS", AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL THE COPYRIGHT HOLDERS OR CONTRIBUTORS BE LIABLE FOR ANY DIRECT, INDIRECT, INCIDENTAL, SPECIAL, EXEMPLARY, OR CONSEQUENTIAL DAMAGES (INCLUDING, BUT NOT LIMITED TO, PROCUREMENT OF SUBSTITUTE GOODS OR SERVICES; LOSS OF USE, DATA, OR PROFITS; OR BUSINESS INTERRUPTION) HOWEVER CAUSED AND ON ANY THEORY OF LIABILITY, WHETHER IN CONTRACT, STRICT LIABILITY, OR TORT (INCLUDING NEGLIGENCE OR OTHERWISE) ARISING IN ANY WAY OUT OF THE USE OF THIS SOFTWARE, EVEN IF ADVISED OF THE POSSIBILITY OF SUCH DAMAGE.

OpenCV

This software uses the OpenCV library version 4.9.0, which is licensed under the Apache 2 License. The full text of the Apache 2 License is included below.

Apache License
Version 2.0, January 2004
http://www.apache.org/licenses/

TERMS AND CONDITIONS FOR USE, REPRODUCTION, AND DISTRIBUTION

1. Definitions.

"License" shall mean the terms and conditions for use, reproduction, and distribution as defined by Sections 1 through 9 of this document.

"Licensor" shall mean the copyright owner or entity authorized by the copyright owner that is granting the License.

"Legal Entity" shall mean the union of the acting entity and all other entities that control, are controlled by, or are under common control with that entity. For the purposes of this definition, "control" means (i) the power, direct or indirect, to cause the direction or management of such entity, whether by contract or otherwise, or (ii) ownership of fifty percent (50%) or more of the outstanding shares, or (iii) beneficial ownership of such entity.

"You" (or "Your") shall mean an individual or Legal Entity exercising permissions granted by this License.

"Source" form shall mean the preferred form for making modifications, including but not limited to software source code, documentation source, and configuration files.

"Object" form shall mean any form resulting from mechanical transformation or translation of a Source form, including but

not limited to compiled object code, generated documentation, and conversions to other media types.

"Work" shall mean the work of authorship, whether in Source or Object form, made available under the License, as indicated by a copyright notice that is included in or attached to the work (an example is provided in the Appendix below).

"Derivative Works" shall mean any work, whether in Source or Object form, that is based on (or derived from) the Work and for which the editorial revisions, annotations, elaborations, or other modifications represent, as a whole, an original work of authorship. For the purposes of this License, Derivative Works shall not include works that remain separable from, or merely link (or bind by name) to the interfaces of, the Work and Derivative Works thereof.

"Contribution" shall mean any work of authorship, including the original version of the Work and any modifications or additions to that Work or Derivative Works thereof, that is intentionally submitted to Licensor for inclusion in the Work by the copyright owner or by an individual or Legal Entity authorized to submit on behalf of the copyright owner. For the purposes of this definition, "submitted" means any form of electronic, verbal, or written communication sent to the Licensor or its representatives, including but not limited to communication on electronic mailing lists, source code control systems, and issue tracking systems that are managed by, or on behalf of, the Licensor for the purpose of discussing and improving the Work, but excluding communication that is conspicuously marked or otherwise designated in writing by the copyright owner as "Not a Contribution."

"Contributor" shall mean Licensor and any individual or Legal Entity on behalf of whom a Contribution has been received by Licensor and subsequently incorporated within the Work.

2. Grant of Copyright License. Subject to the terms and conditions of this License, each Contributor hereby grants to You a perpetual, worldwide, non-exclusive, no-charge, royalty-free, irrevocable copyright license to reproduce, prepare Derivative Works of, publicly display, publicly perform, sublicense, and distribute the Work and such Derivative Works in Source or Object form.

3. Grant of Patent License. Subject to the terms and conditions of this License, each Contributor hereby grants to You a perpetual, worldwide, non-exclusive, no-charge, royalty-free, irrevocable (except as stated in this section) patent license to make, have made, use, offer to sell, sell, import, and otherwise transfer the Work, where such license applies only to those patent claims licensable by such Contributor that are necessarily infringed by their Contribution(s) alone or by combination of their Contribution(s) with the Work to which such Contribution(s) was submitted. If You institute patent litigation against any entity (including a cross-claim or counterclaim in a lawsuit) alleging that the Work

or a Contribution incorporated within the Work constitutes direct or contributory patent infringement, then any patent licenses granted to You under this License for that Work shall terminate as of the date such litigation is filed.

4. Redistribution. You may reproduce and distribute copies of the Work or Derivative Works thereof in any medium, with or without modifications, and in Source or Object form, provided that You meet the following conditions:

(a) You must give any other recipients of the Work or Derivative Works a copy of this License; and

(b) You must cause any modified files to carry prominent notices stating that You changed the files; and

(c) You must retain, in the Source form of any Derivative Works that You distribute, all copyright, patent, trademark, and attribution notices from the Source form of the Work, excluding those notices that do not pertain to any part of the Derivative Works; and

(d) If the Work includes a "NOTICE" text file as part of its distribution, then any Derivative Works that You distribute must include a readable copy of the attribution notices contained within such NOTICE file, excluding those notices that do not pertain to any part of the Derivative Works, in at least one of the following places: within a NOTICE text file distributed as part of the Derivative Works; within the Source form or documentation, if provided along with the Derivative Works; or, within a display generated by the Derivative Works, if and wherever such third-party notices normally appear. The contents of the NOTICE file are for informational purposes only and do not modify the License. You may add Your own attribution notices within Derivative Works that You distribute, alongside or as an addendum to the NOTICE text from the Work, provided that such additional attribution notices cannot be construed as modifying the License.

You may add Your own copyright statement to Your modifications and may provide additional or different license terms and conditions for use, reproduction, or distribution of Your modifications, or for any such Derivative Works as a whole, provided Your use, reproduction, and distribution of the Work otherwise complies with the conditions stated in this License.

5. Submission of Contributions. Unless You explicitly state otherwise, any Contribution intentionally submitted for inclusion in the Work by You to the Licensor shall be under the terms and conditions of this License, without any additional terms or conditions. Notwithstanding the above, nothing herein shall supersede or modify the terms of any separate license agreement you may have executed

with Licensor regarding such Contributions.

6. Trademarks. This License does not grant permission to use the trade names, trademarks, service marks, or product names of the Licensor, except as required for reasonable and customary use in describing the origin of the Work and reproducing the content of the NOTICE file.

7. Disclaimer of Warranty. Unless required by applicable law or agreed to in writing, Licensor provides the Work (and each Contributor provides its Contributions) on an "AS IS" BASIS, WITHOUT WARRANTIES OR CONDITIONS OF ANY KIND, either express or implied, including, without limitation, any warranties or conditions of TITLE, NON-INFRINGEMENT, MERCHANTABILITY, or FITNESS FOR A PARTICULAR PURPOSE. You are solely responsible for determining the appropriateness of using or redistributing the Work and assume any risks associated with Your exercise of permissions under this License.

8. Limitation of Liability. In no event and under no legal theory, whether in tort (including negligence), contract, or otherwise, unless required by applicable law (such as deliberate and grossly negligent acts) or agreed to in writing, shall any Contributor be liable to You for damages, including any direct, indirect, special, incidental, or consequential damages of any character arising as a result of this License or out of the use or inability to use the Work (including but not limited to damages for loss of goodwill, work stoppage, computer failure or malfunction, or any and all other commercial damages or losses), even if such Contributor has been advised of the possibility of such damages.

9. Accepting Warranty or Additional Liability. While redistributing the Work or Derivative Works thereof, You may choose to offer, and charge a fee for, acceptance of support, warranty, indemnity, or other liability obligations and/or rights consistent with this License. However, in accepting such obligations, You may act only on Your own behalf and on Your sole responsibility, not on behalf of any other Contributor, and only if You agree to indemnify, defend, and hold each Contributor harmless for any liability incurred by, or claims asserted against, such Contributor by reason of your accepting any such warranty or additional liability.

END OF TERMS AND CONDITIONS

12 Revision History

Revision	Date	Changes
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

13 Index

S

Z