

# PepSlide<sup>®</sup> Analyzer 2.0

## User's Guide

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PepSlide<sup>®</sup> Analyzer is only intended for research and not intended or approved for diagnosis of disease in humans or animals.

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SICASYS Software GmbH  
Im Neuenheimer Feld 583  
D-69120 Heidelberg  
Germany

Phone +49 (62 21) 7 28 50 40  
Fax +49 (62 21) 7 28 48 94  
Email [info@sicasys.de](mailto:info@sicasys.de)  
Web [www.sicasys.de](http://www.sicasys.de)

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# 1 Introduction

**PepSlide® Analyzer (abbreviated PSA)** software supports microarray data analysis and is particularly suited for peptide arrays and peptide microarrays. In addition, the software provides statistical data mining tools for discovering parameters influencing the study and their relationship.

Furthermore, PSA facilitates analysis of peptide libraries. The software can refer the quantified data to the peptide library design and the intended application, thus it is able to provide meaningful information with respect to the application. If e.g. a target protein or antigen is translated into overlapping peptides for epitope mapping, PSA can suggest a grouped list of peptides which represent an epitope or consensus motif of an antibody or serum sample.

## 1.1 Installation

PSA is natively supported on Windows and Mac OS X platforms, thus the software is able to run with high performance and native look. Installation of PSA requires rights of a system administrator.

### *Hardware Requirement*

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Minimum hardware: 1.5 GHz Processor, 1GB RAM.

Recommended hardware: 2.0 GHz Dual-Core or faster Processor, 2 GB or more RAM.

### *Windows Platforms*

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PSA works on Windows XP, Windows 7, and Windows 8. Simply run the PSA setup. If the current account is not administrator, you will be asked to input an administrative account and password.

### *Mac OS X platforms*

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The software runs on Mac OS X 10.7 and 10.8. Unzip the package and double-click on the .pkg file to launch the installer. During installation you will be prompted to provide a system administrator's account and password. Upon completion, PSA is installed in folder */Applications/PepSlide/Analyzer*.

## 1.2 Product Activation

After installing PSA® on Windows, you need to activate the software with a *trial serial number* obtained from the software provider or its distributor. This enables the use of PSA® with full functionality for 14 days. The trial use for PSA® on Mac OS X platforms is handled automatically and does not require this step.

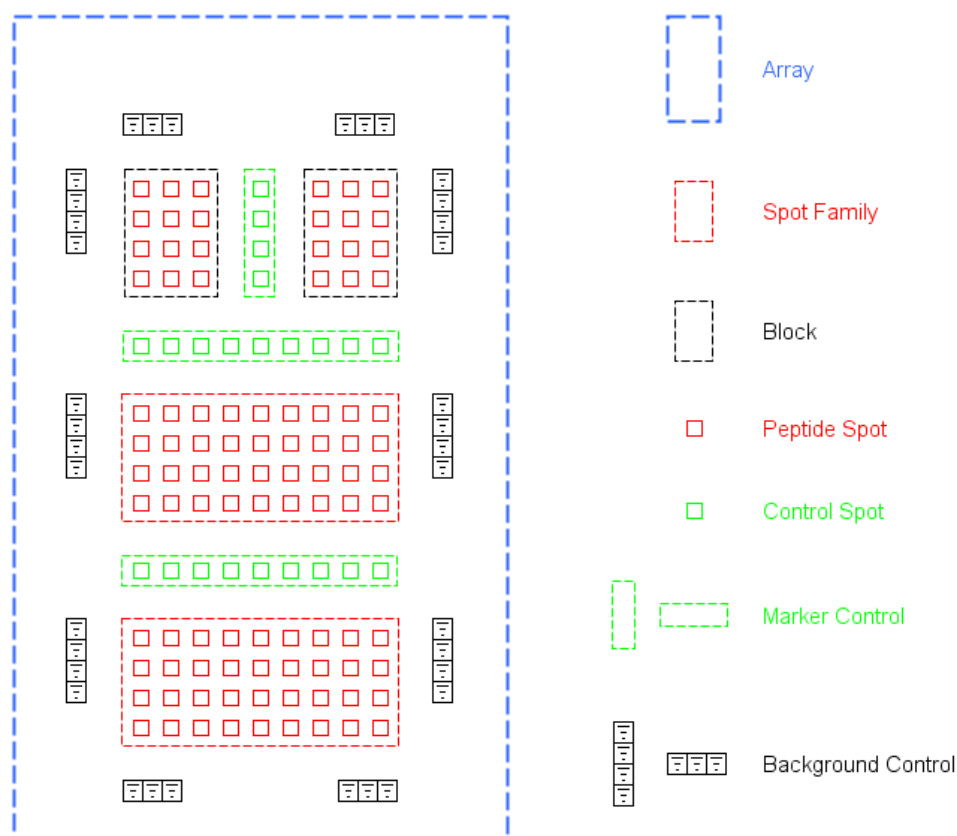
When the free trial time has expired, you can [buy](#) a software license to continue using PSA®. Upon the purchase, you receive a serial number and use it to activate the license. Please refer to the product activation steps in Section 9 or the online help [here](#).

## 1.3 Software Concepts

### Peptide Arrays

In the remaining of this manual, the term *array* is used to mean both *array* and *microarray*, unless otherwise stated.

The diagram of a typical peptide array analyzed by PepSlide® is outlined in Figure 1.



**Figure 1: Peptide Array Diagram.**

A *spot* represents a single peptide spot located in one cell on the array. It can also be a control spot made of e.g. a known epitope. In the diagram, the spots are drawn as squares for the explanation purpose. Depending on the peptide array synthesizer, they may have rectangular or circular shape, as images of some actual spots shown in Figure 2.

A *spot family* (abbreviated *SF*) consists of spots generated from the same source. For peptide arrays an SF can be a *peptide library* or a control. A *block* is a group of spots located next to each other, just like its definition in GAL files. The distinction between a block (contains only spots and their annotation) and an SF (a peptide library and may consist of multiple blocks) enables a convenient use of GAL files.

A *marker control* is a group of control spots arranged on either the same row or the same column of the array. It is used for the purpose of marking or orientation. A *background control* is used for the background correction during the data quantification. Its elements are *virtual* spots, i.e. neither peptide spot nor control spots, being used solely for defining the image regions from which the signal for background values is obtained.

### Array Image Analysis

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The binding signals of an array are converted by an array scanner 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*<sup>1</sup>. In *8-bit grayscale images*, the gray value ranges from 0 to 255. This value can be from 0 up to 65535 in *16-bit grayscale images*. Since they contain a broader range of signal levels than the 8-bit format, 16-bit grayscale images are recommended for array image analysis.

*Quantification* is the procedure that estimates the true binding signal for each spot and represents its signal value in terms of statistic measurement of pixel intensities within that spot. Obviously, the quantification quality depends on the *spot finding* procedure which determines pixels in the array image that belong to a spot in the array. *Background correction* also contributes to the quantification quality. It estimates signals caused by non-specific binding and removes them from the spot's signal.

For each spot in the array, the median and the 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*; it is 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 software illustrates spots in the array by white rectangles or squares (Figure 2). Within each spot, the *spotted region* is bounded by a *dashed rectangle* or a *dashed circle* depending on peptide array

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<sup>1</sup> <http://partners.adobe.com/public/developer/en/tiff/TIFF6.pdf>

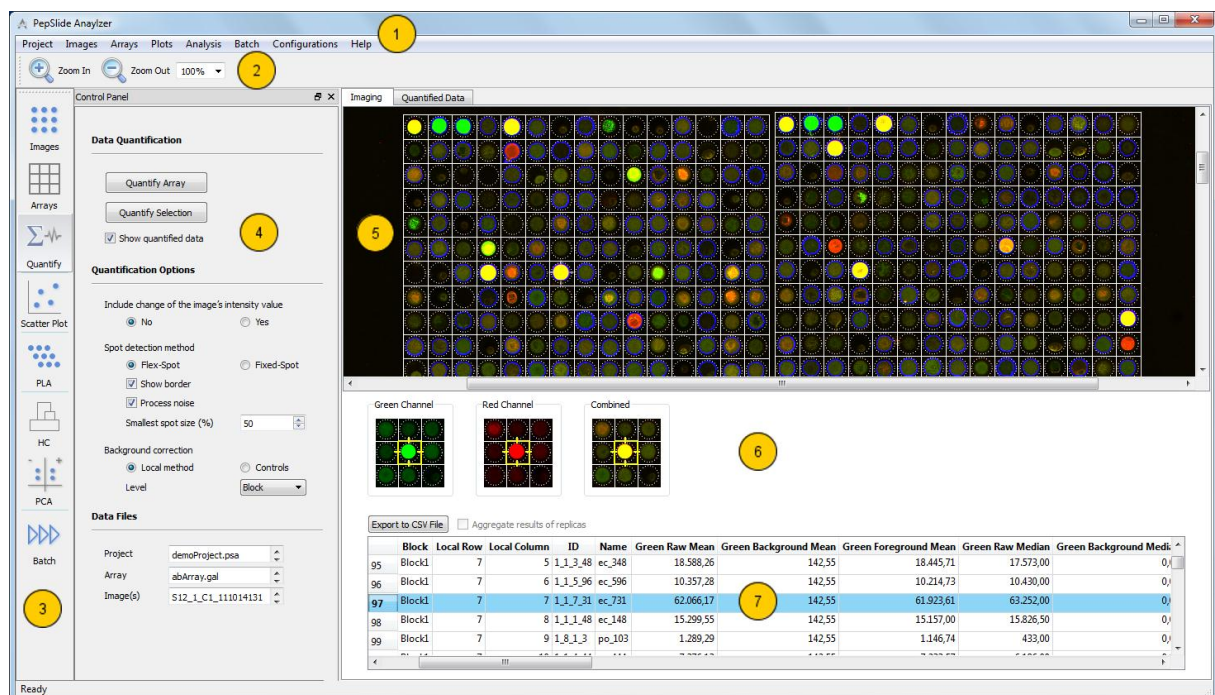
synthesizer. The spots' signal is shown in red, green, or a color different from white; the larger the value the brighter the signal. The spot's signal is presumably due to the binding of peptide in the spotted region with the sample. Therefore, before quantification we will reallocate the array such that the spotted regions are as close to the spots' signal as possible.



**Figure 2: Spot Images in PepSlide® Analyzer.**

## 1.4 Software User Interface

Related software controls are grouped in labeled components as shown in Figure 3. We refer to a software component using the name listed in Table 1.



**Figure 3: The Software User Interface.**

Area	Referred Name
1	The menu
2	The canvas toolbar
3	The main toolbar
4	The control panel
5	The canvas
6	The <i>Spot Image</i> widget
7	The table of quantified data

**Table 1: Area Names.**

The main toolbar enables quick access to a group of related functions summarized in Table 2. Clicking a button on the main toolbar opens the control panel containing controls for these functions and the sheet that displays the data and the processed results.

Button	Functions
<b>Images</b>	Select image channel. Change image's intensity. Rotate images.
<b>Arrays</b>	View properties of SFs, blocks, and spots. Rotate and move SF or blocks.
<b>Quantify</b>	Quantify the array data and browse the quantified data.
<b>Scatter Plot</b>	Display quantified data in a scatter plot.
<b>PLA</b>	Peptide Library Analysis
<b>HC</b>	Hierarchical Clustering Analysis
<b>PCA</b>	Principal Component Analysis
<b>Batch</b>	Setup and execute a batch

**Table 2: The Main Toolbar and Related Functions.**

## 1.5 Peptide Array Data Analysis

From the software perspective, typical tasks of peptide array data analysis include:

1. Quantification of array data
  - Load the scanned array images and the array file.
  - Align the array to the images.
  - Quantify the array data.
2. Analysis of peptide libraries to find peptides of interest.
3. If possible, batch processing of multiple array images, i.e. the automation of step 1.
4. Discovery of parameters influencing the study and their relationship with data mining tools.

The following sections explain how to do these tasks with PSA.



## 2 Preparation of Array Data

### 2.1 Loading Data

Two input data are required for analyzing a peptide array:

- Scanned images of the array in TIFF format.
- The array file: Either a GAL file (\*.gal) or a PepSlide® Designer (PSF) file (\*.psf).

#### *Supported Image Format*

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PSA supports 8-bit or 16-bit grayscale images or 24-bit color images. For the best image quality 16-bit grayscale TIFFs are recommended.

Please note that compressed images are not supported. Therefore, please disable image compression when saving the scanned images with your microarray scanner software. If you are using GenePix Pro software, uncheck the Use TIFF LZW compression (lossless) option in the Save Images dialog.

#### *Loading Input Data*

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- Click *the Images > Open Image* menu and select the array image file. For grayscale images, select to display each image with either the *Red* channel or the *Green* channel.
- Click the *Arrays > Open Array* menu and select the array file.

After being loaded, the images and the array are shown in the graphical canvas (Figure 3). 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, an arbitrary value can be entered directly into the *Zoom* combo-box.

#### *Viewing Array Data*

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In the *Array Object* section of the *Arrays* control panel, you can view properties of a spot family, a block, or a spot.

If a PSF array file is loaded, you find three expandable pages in the section: *Spot Family*, *Spot*, and *Array Configuration*. To view properties of a spot family (SF), first click on the *Spot Family* page if it is not yet open, and then click on the SF in the canvas (Figure 4-a). Similarly, you can open the *Spot* page and then point the mouse to a spot to view its properties (Figure 4-b). The *Array Configuration* page shows the size and the printed region configuration of the array's spots.

Spot Family	
Name	Array Hemagglutinin 13aa/12aa
Type	Overlapping
Number of columns	60
Number of rows	20
Position of top-left spot	
Column	2
Row	2
Horizontal (µm)	3927
Vertical (µm)	9156
Spot	
Array Configuration	

(a) Spot Family Properties

Spot Family	
Spot	
Spot family	Array Hemagglutinin 13aa/12aa
Peptide	ELVQSSSTGKICN
Position	
Column	5
Row	5
Local column	4
Local row	4
Horizontal (µm)	5187
Vertical (µm)	11677
Array Configuration	

(b) Spot Properties

Figure 4: Properties of an Array Object from a PSF file.

The pages are *Spot Family*, *Block*, and *Spot* for array files of GAL format. To view a block's properties (Figure 5-a), open the *Block* page and then click on the block in the canvas. The *Spot* page is used similarly to view a spot's properties (Figure 5-b). If some blocks have been assigned to a SF to contain a peptide library design, you can view name and type of the SF in the *Spot Family* page.

Spot Family	
Block	
Name	Block1
Number of columns	6
Number of rows	24
Spot spacing	
Horizontal (µm)	332
Vertical (µm)	332
Spot diameter (µm)	
	200
Top-left spot's position	
Horizontal (µm)	9986
Vertical (µm)	3656
Spot	

(a) Block Properties

Spot Family	
Block	
Spot	
Block	Block1
ID	PPNWHVPEASDVTIQ
Name	106
Spot position	
Local Column	10
Local Row	5
Horizontal (µm)	22768
Vertical (µm)	8470

(b) Spot Properties

Figure 5: Properties of an Array Object from a GAL file.

## 2.2 Array Alignment

As mentioned in Section 1.3, the array needs to be aligned with the image before quantifying the array data.

You can manually align the array with the image, if necessary after processing the image or the array, e.g. rotating a block or increasing the spot's visibility. Please refer to Section 3 for image and array processing functions.

Array alignment can be done automatically if the array file is of GAL format, the spots have circular shape, and the spots' signal is sufficient for the array positioning. **This version** does **not** yet support automatic array alignment for **PSF** array files.

#### *Aligning Array Automatically*

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- Click the *Align Array* button in the *Arrays* control panel.

#### *Aligning Array Manually*

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- Click *Ctrl-A* to select all SFs or blocks in the array. To select individual SFs or blocks, click on them while pressing the *Ctrl* key.
- Click on the selection and drag the corresponding blocks to the intended position.

The aligned position of the SFs or blocks in the array can be saved with the *Arrays > Save Array* menu. In addition, you can save the array to another array file using the *Array > Save Array As* menu.

## 2.3 PepSlide® Analyzer Project File

It is recommended that the analysis of each array image be saved to a PepSlide® Analyzer project file (\*.psa) using the *Project > Save Project* menu. This contains the path to the image, the aligned array, and the quantified data. When later opening the project file with the *Project > Open Project* menu, the software will load all the data. The paths to the array image, the array file, and the project file are shown in the *Data Files* section of the *Quantification* control panel.

Not only does this contain all analyzed data for an array image in one file, but you can also use these project files directly with data mining tools later.

## 3 Image and Array Processing

### 3.1 Image Processing

You can change the image intensity (Figure 6) for the convenience of array alignment.

### Improving spot visibility

Adjusting brightness and contrast can make the spots more visible and that eases the array alignment. These functions are available in the *Image Intensity* section of the *Images* control panel.

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

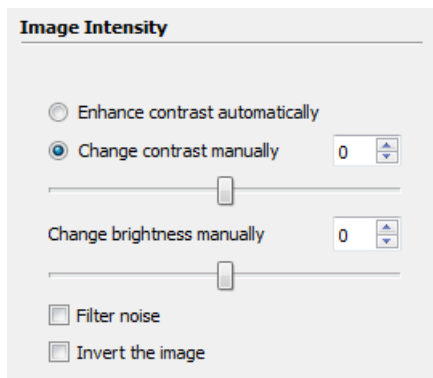


Figure 6: Image Processing.

and 99 can be entered directly.

### Noise Filtering

Noise in the loaded image(s) can be reduced by selecting the *Noise Filtering* check-box.

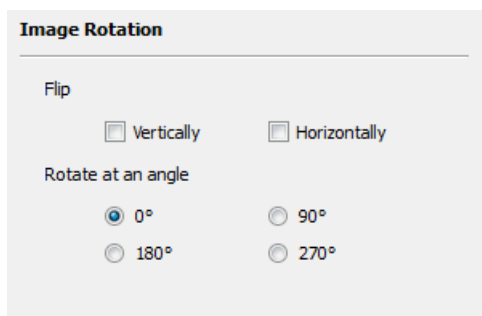
### Inversing

The negative image of the current image can be created by selecting the *Inversion* check-box.

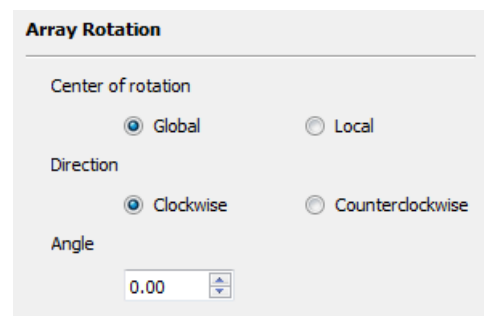
## 3.2 Image and Array Rotation

### Rotating images

You can flip and/or rotate images at angles of 90°, 180°, or 270°. These functions are located in the *Image Rotation* section of the *Images* control panel (Figure 7-a).



(a) Image Rotation



(b) Array Rotation

Figure 7: Rotation of Images and Arrays.

### Rotating Array

If the array slightly deviates from the image at a small angle, it is recommended that the array (i.e. related SFs or blocks) be rotated, since image rotation may change the image data. You can select SFs

or blocks and rotate them at an arbitrary angle. These functions are located in the *Array Rotation* section of the *Arrays* control panel (Figure 7-b). The degree change can be as small as 0.01°.

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

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

## 4 Quantification of Array Data

### 4.1 Quantifying Array Data

Click the *Quantify* button in the main toolbar to activate the *Quantification* control panel.

- Click the *Quantify Array* button to get the data quantified for the entire array.
- To quantify some SFs or blocks, select them and then click on the *Quantify Selection* button.

For each spot in the array, the median and the mean of its *raw*, *background*, and *foreground* values in each channel (Red and/or Green) are calculated. If the raw value of a spot is smaller than its background value, the spot is flagged *Error* and its foreground value is set to zero. (Section 4.3 details the methods quantified used to calculate the raw value and the background value.)

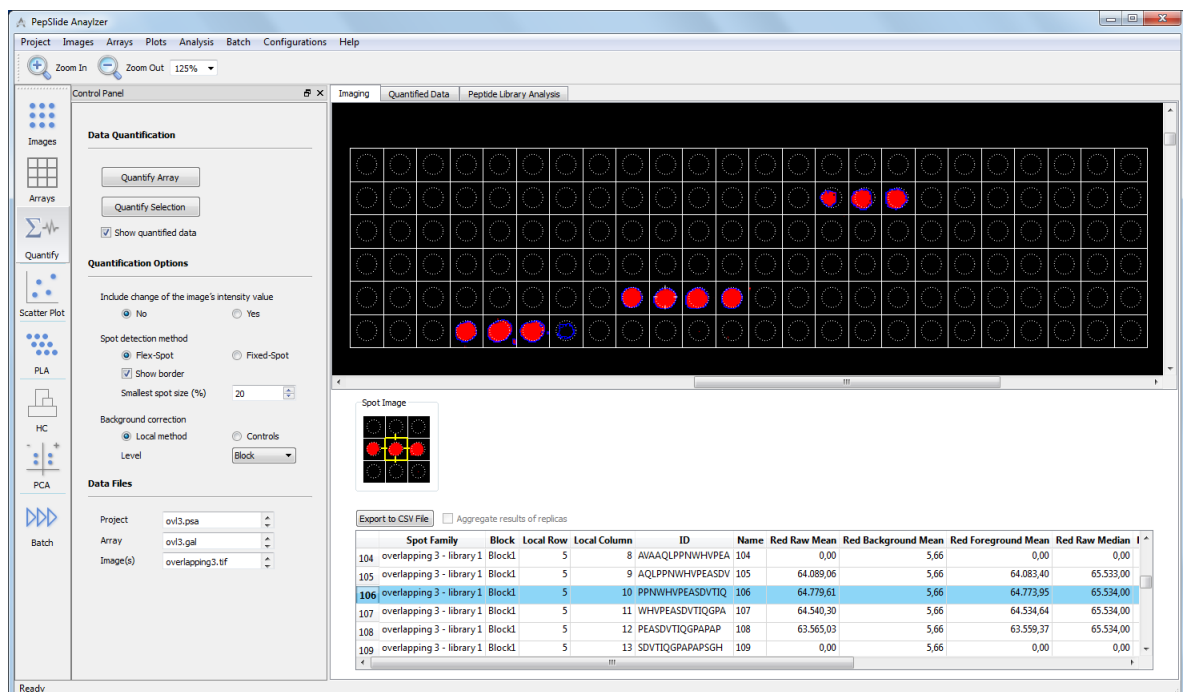


Figure 8: Quantified Data.

In the *Imaging* sheet, you can view the spots and their quantified data simultaneously (Figure 8). The signals corresponding to the selected spot and its neighbors are displayed in the *Spot Image* widget. In addition, selecting a row in the table of quantified data highlights the corresponding spot in the canvas. This also opens the *Spot* page in the *Array Object* section in the *Arrays* control panel and shows the spot's properties there. Similarly, when the *Spot* page is opened, clicking on a spot in the canvas will highlight its quantified data in the table.

You can also browse the spots' quantified data in the *Quantified Data* sheet, which shows only the table of quantified data and the *Spot Image* widget. In both *Imaging* and *Quantified Data* sheets, you can export the quantified data to a CSV file for further analysis.

### Aggregating results of replicas

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When a spot is replicated the software also provides the intensity value of the spot calculated by aggregating signals from its replicas. Suppose that P1 and P2 are the replicas of peptide P. The quantified results then include the intensity values for P, in addition to those for P1 and P2. Taking the raw median value of P for example, it is the median value of signals from both spots P1 and P2, which cannot be calculated based on the median values of P1 and P2.

This provides an additional view to the replicated data. To control whether the aggregated data is calculated and shown, use the *Aggregate results of replicas* check-box.

### Customizing the Quantified Data

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Click the *Configurations* menu and choose *Imaging Result Values* to show the setup dialog. Here, you can add or remove values to be calculated during the data quantification.

## 4.2 Change of Intensity Values

During the array alignment, you may have adjusted the contrast and/or the brightness of the images to make the spots visible. This changes the image data and alters the analysis results. It is recommended that *the original image data be used for the quantification*. Therefore, by default *the quantification procedure uses the original image's data*, i.e. it *excludes* changes made to the image by inversion, noise filtering, and adjustment of contrast and/or brightness.

This option can be intentionally changed as follows. In the *Quantification Options* section of the *Quantification* control panel, select *Yes* for the *Include change of the images' intensity value* option (Figure 9). To use the original image data for the data quantitation again, select the *No option*. Please

note that when the *No* option is chosen, the *Spot Image* widgets in the *Imaging*, *Quantified Data*, *Scatter Plot*, and *Peptide Library Analysis* sheets show the spot's signal using the original image's data, even though the image's contrast or brightness has been changed.

### 4.3 Spot Detection Methods

In the *Quantification Options* section of the *Quantification* control panel, the *Spot detection method* option defines how the raw value and the background value of a spot are calculated (Figure 9).

- *Fixed-Spot*: The software *always* uses the pixels in the *spotted region* to compute the raw value. The background value is calculated based on the pixels in the remaining region within the spot.
- *Flex-Spot*: This method can flexibly detect the spot's signals in the image even though their shape and position are not in accordance with spotted region. The *detected spot border* is shown in blue. Calculation of the raw value is based on pixels *within* the blue border. Pixels in the remaining region within the spot are used to calculate the background value.

The Flex-Spot method is recommended because it does not require the spots' signal in the image to rigidly match with their spotted regions, as specified in the GAL file. Figure 10 illustrates such a case.

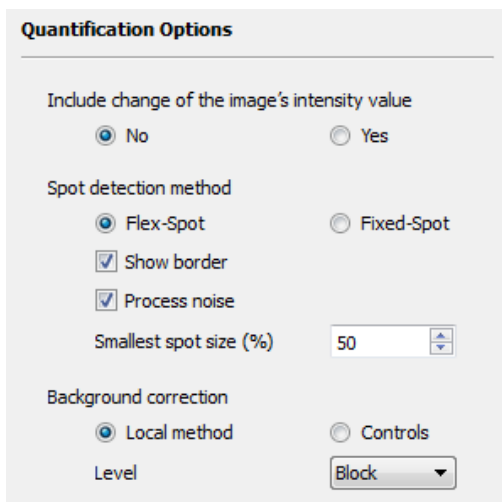


Figure 9: Quantification Options.

Here, within a spot (the white square) the spotted region is depicted by the white dashed circle. The spots' signal, shown in red, has different shape and position from those of their spotted region. Despite that fact, the Flex-Spot method can still precisely find the spots and highlight their border in blue.

Please note that the above described mechanism for background calculation uses only the background pixels within the spot. Therefore, it is called the *Local method* or *local background correction*. By means of background controls, you can have background values based on pixels from any region of the image.

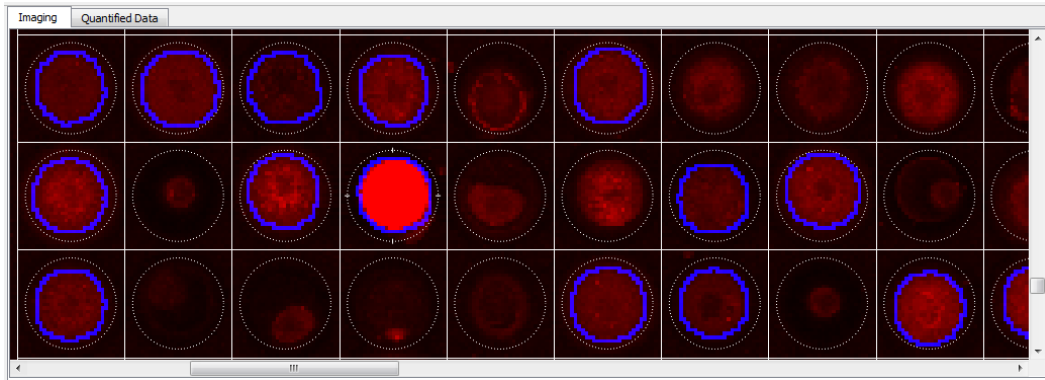


Figure 10: The Flex-Spot Method.

## 4.4 Spot Detection Options

### Process Noise

The images may contain noises that mislead the spot detection procedure and result in wrong quantified data. The noise can be background noise that span across the whole slide (Figure 11). It can also be foreground noise like the two large red bands shown in Figure 12. In the case of background noise, we want to “remove” the background layer so that only the meaningful signal remains. Foreground noise like the two red bands in Figure 12 should not be part of a valid spot’s signal. The software can effectively handle this task. The results can be seen in Figure 11 and Figure 12; only meaningful spots are highlighted with a blue border (by the Flex-Spot method).

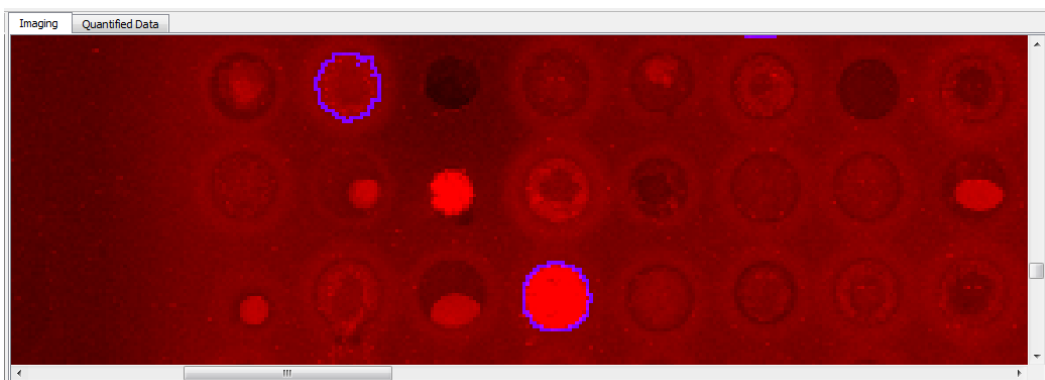
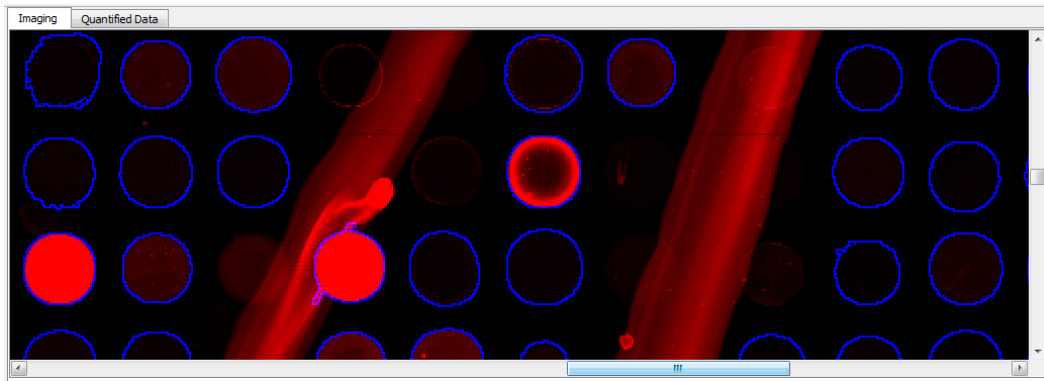


Figure 11: Processing Background Noise.

Since processing noise during quantification efficiently removes background signal, it can be regarded as an (implicit) background correction method. You can choose whether noise is processed during quantification with the *Process noise* option (Figure 9). Please note that you can *process noise* during quantification with both Fixed-Spot and Flex-Spot methods.





**Figure 12: Processing Foreground Noise.**

### *Smallest Spot Size*

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You can set the size limit of a “valid” spot by means of the *Smallest spot size (%)* option (Figure 9). Suppose that this value is 50% and the array file is a GAL file (\*.gal). Imagine a *virtual square* whose side length being 50% or half of the spot diameter. If the spot signal is smaller than or can be contained in that virtual square, the Flex-Spot method will reject this spot. The software will then use the Fixed-Spot method to compute the raw value for that spot.

If the array file is of PSF format (\*.psf) and suppose that the smallest spot size is still 50%, the software would use a *virtual rectangle* whose width and length being half of those of the spotted region (the dashed rectangle in Figure 2a). The Flex-Spot method will reject a spot whose signal can be contained in that virtual rectangle, and the Fixed-Spot method will be used instead.

The spots shown in Figure 10 and Figure 11 are detected by the Flex-Spot method with the *Smallest spot size (%)* parameter being 50%. It can be observed that small spots do not have a blue border.

### *Show Border*

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After data quantification, you can turn the detected spot border on and off using the *Show border* option (Figure 9). The software supports saving the border information in the project file (\*.psa). Therefore, you can still observe the detected spot border when reopening the project file. This is particularly useful for reviewing the quantified data generated by batch processing.

### *Undetectable by Flex-Spot*

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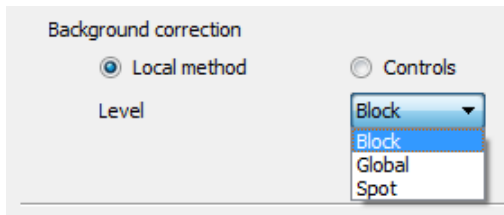
If the Flex-Spot method cannot detect a spot due to e.g. being smaller than the size limit, weak signal, or noisy data, the software employs the Fixed-Spot method to compute the raw value. *No blue border* is shown within the spot if the Fixed-Spot method is used.

## 4.5 Background Correction

Background correction methods can be selected in the *Quantification Options* section of the *Quantification* control panel. By default, the *Local method* is employed.

### Local Method

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**Figure 13: Background Correction Options.**

PSA supports different levels of local background correction (Figure 13). The default level is *Block* for GAL files and *Spot Family* for PSF files.

With *block level*, all spots in a block will have the same background value. It is computed as follows. First, a list of background pixels of all spots in the block is created.

The mean and the median of the pixels' intensity value in this list are then used as the background value.

If *spot family level* is chosen, all spots in an SF have the same mean and median background values, being computed from background pixels of all spots in the SF.

You can choose to have a global background value for the entire array by selecting the *global level*. The calculation is based on the background pixels of all spots in the array. At the *spot level*, a spot's background value is based on its background pixels only. Therefore, it is likely that the background values are different between spots.

### Background Controls

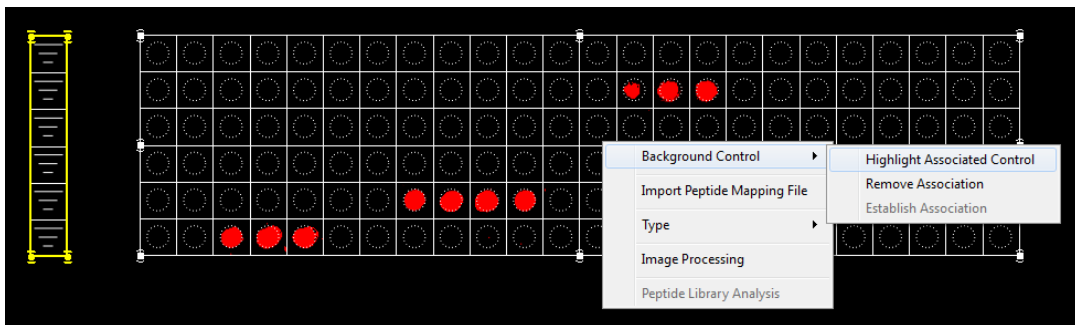
---

Instead of having the background values locally calculated, you can use a background control to explicitly specify the image region from which the background value is calculated. You can define a *global background value for the entire array* of PSF format using a background control as follows.

- Open the *Spot Family* page in the *Array Object* section of the *Arrays* control panel.
- Right-click at a point in the image where the signals represent the background for the spots. Choose *Create Background Control*. Specify the shape and the number of spots.
- Perform data quantification. All spots in the array now have the same background value which is obtained from the background control. We call that an "*association*" between the SFs and the background control.

In addition to having a global background value for the entire array, you can flexibly choose *an individual background value for each SF*. This is done by creating a background control just for the SF and establishing the association between them.

- Right-click on the SF to popup the context menu. Choose *Background Control > Remove Association* to release the SF from the first background control.
- Right-click on the block again and choose *Background Control > Establish Association*. Then click on the background control with which you want to associate this SF. You can check this association using the *Highlight Associated Control* context menu (Figure 14).



**Figure 14: Associated Background Control.**

For GAL files, having a global background value or an individual background value for a *block* can be done similarly; you create a background control for the blocks and associate them.

Please note that the background correction method is automatically set to *Controls* after the creation of the first background control. In the *Quantification Options* section of the *Quantification* control panel, you can select *Local method* again for the *Background correction* option (Figure 13).

## 5 Analysis of Peptide Libraries

After having the peptide array data quantified, you may want to examine it and select peptides of interest with respect to the peptide array application. E.g. if an antigen is translated into overlapping peptides for epitope mapping, you may be interested in a list of peptides which represent an epitope. This can be done conveniently with PSA's peptide library analysis tools. By referencing the quantified data to the peptide library design, which keeps the antigen's amino acid sequence and the overlapping step, PSA is able to locate the peptides representing the epitope.

The software supports analysis of peptide library of the following types:

- *Overlapping library*: Overlapping peptides translated from a protein.
- *Random library*: Peptides generated stochastically.
- *Substitution (also called permutation or positional scanning) library*: Variants of a known peptide created by replacing its residues with other amino acids.

As mentioned, PSA represents a peptide library as a spot family (SF). If the array file is of PSF format, you can start peptide library analysis simply by clicking on the *PLA* button in the main toolbar. The software analyzes all SFs in the array if none is selected. Otherwise only the selection is analyzed. The results are shown in the *Peptide Library Analysis* sheet.

Analysis of a peptide library cannot be performed with GAL files in the first place. However, you can easily specify the peptide library design for related GAL blocks. The analysis can then be preceded as with SFs. These steps are detailed in Section 5.4.

## 5.1 Overlapping Peptide Library Analysis

This analysis aims at selecting peptide spots with strong signals. It is also able to detect a consensus motif shared by overlapping peptides.

A two-dimensional chart in the center of the sheet depicts the peptide spots (Figure 15). Their coordinates can be selected among the quantified intensity values. By moving the lower and upper threshold bars, the chart facilitates the selection of spots whose intensity values being within the two thresholds. Selected spots are then populated into the table below the chart.

By clicking on a spot in the chart or selecting the corresponding row in the table, you can view the spot information such as the peptide and its counterpart image. The information is shown in the *Spot Details* widget located on the right of the sheet. The chart, the table, and the *Spot Details* widget thus provide different angles to the data of the peptide library.

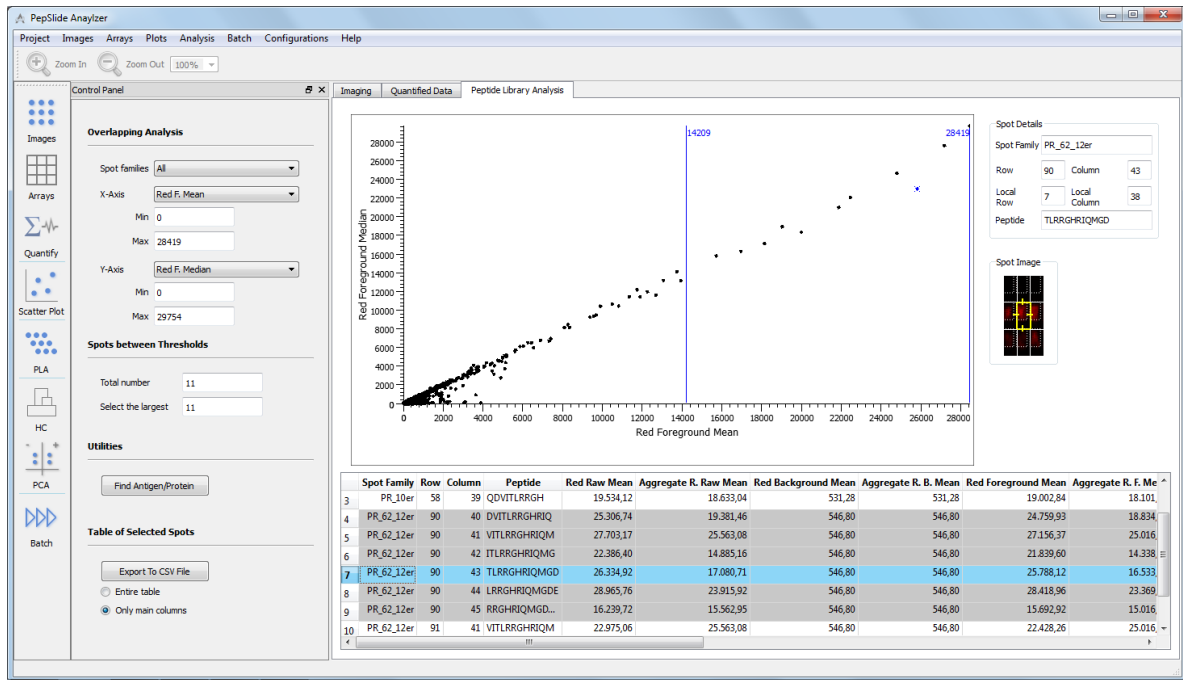


Figure 15: Overlapping Peptide Library Analysis.

You can export the data in the table to a CSV file. The chart can also be saved to an image file.

### Identifying Consensus Motifs

In the table below the chart, overlapping peptides sharing a consensus motif are highlighted with individual colors. In Figure 15, rows corresponding to these peptides are in grey. Among them, the selected row is highlighted in blue; its information is shown in the *Spot Details* widget.

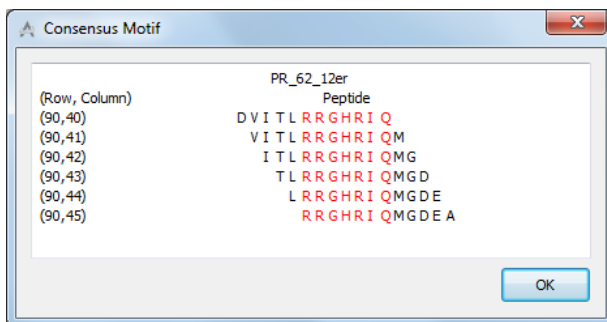


Figure 16: Consensus Motif.

Right-click on the highlight and choose *Show Consensus Motif* to view the information (Figure 16). Data in this dialog can be copied and pasted into other software, e.g. a word processing program.

In the sample data package, the *overlapping1* folder contains a test dataset for this analysis.

## 5.2 Random Peptide Library Analysis

This analysis facilitates browsing and selecting peptide spots with strong signals for further optimization. Except for the detection of consensus motifs, the user interface and functions of this analysis are the same as those in the analysis for overlapping peptide libraries.

In the sample data package, the *random1* folder contains a test dataset for this analysis.

## 5.3 Substitution Peptide Library Analysis

Peptides of a *substitution* peptide library are created by replacing residues of a known peptide, the so-called *parent peptide*, with some other amino acids. The purpose of this analysis is thus to examine all variants of the parent peptide and select those of your interest. Figure 17 displays the analysis results of a substitution peptide library whose parent peptide is *NYGKYE*.

The variants of the parent peptide and their intensity value are simultaneously shown in a three-dimensional chart. Here, the (P)-Axis lists residues of the parent peptide and the (S)-Axis contains amino acids for replacing, thus a “point” in the (PS)-plane features a variant. The intensity value of a variant is represented by the height of the corresponding bar along the Z-Axis. You can choose to evaluate another quantified value in the Z-Axis section in the control panel. The perspective of the chart can be changed by holding-down the left-button of the mouse and moving it.

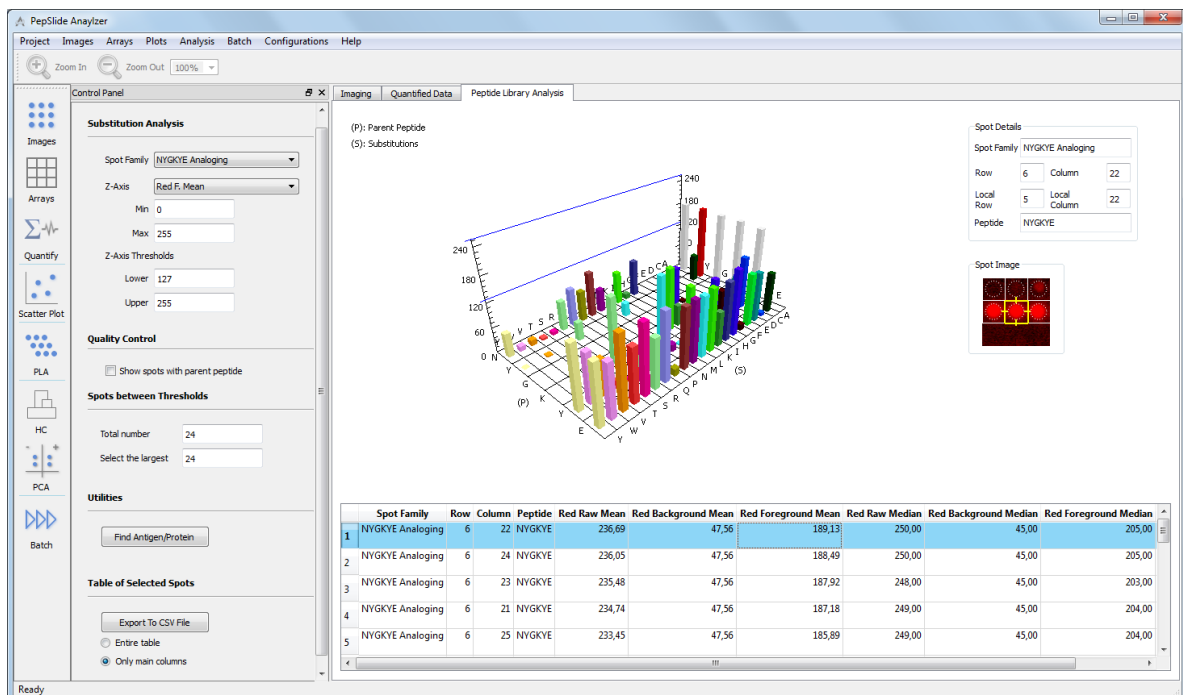
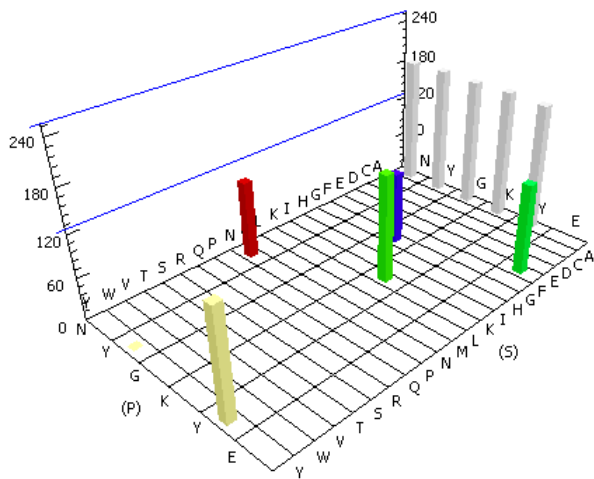


Figure 17: Substitution Peptide Library Analysis.

Spots can be selected by entering the lower and/or upper threshold values in the *Spots between Thresholds* section in the control panel. The selection will be populated into the table below the chart. Similar to other analyses, you can view information about a spot in the *Spot Details* widget. Selecting a spot in the table highlights its version in the chart and vice versa.

The chart can be saved to an image file. In addition, the data in the chart and the table can be exported to a CSV file.



**Figure 18: Parent Peptide Spots.**

### Quality Control with Parent Peptide Spots

You can choose to display only spots having the same amino acid sequence as those of the parent peptide. Since these spots are the same peptide, their intensity values should not be considerably different (Figure 18). This is handled with *Quality Control > Show spots with parent peptide* option in the control panel.

In the sample data package, the *substitution1* folder contains a test dataset for this analysis.

## 5.4 Peptide Library Analysis with GAL Files

You can conveniently use array files of GAL format, even for the analysis of peptide library. Whether the peptides of a *peptide library (PL)* are spotted into one block or different blocks, you can easily reconstruct the PL and then analyze it as explained in the previous sections. The reconstruction can be done in two steps:

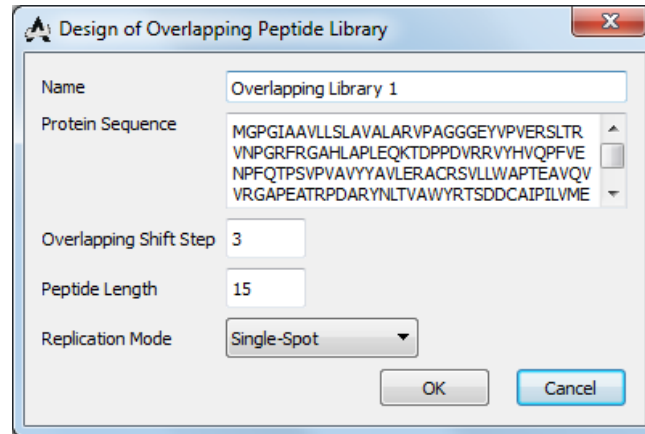
- Specify a peptide library design for the blocks. This creates a PL consisting of these blocks.
- If the GAL file does not contain the peptide in the spots' ID, import the peptide mapping file.

Each step is described in detail below. For reading convenience, we assume that an overlapping PL is being reconstructed. The procedure is the same for substitution peptide libraries.

### Specify a Peptide Library Design for GAL Blocks

- Hold down the Ctrl key while clicking on the blocks to select them simultaneously.

- Right-click on the selection and choose *Type > Overlapping Peptide Library*.
- Enter the design of the peptide library (Figure 19) including the protein, the shift step, and the peptide length.



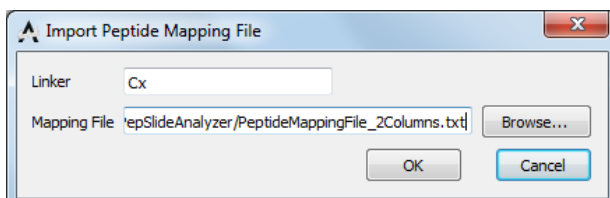
**Figure 19: Overlapping Peptide Library Design.**

An overlapping peptide library consisting of these blocks, represented by PSA as a spot family, has been created. You can activate the *Spot Family* page and review the information of the PL.

### *Import the Peptide Mapping File*

Please note that this step is only necessary if the spots' ID field in the GAL file does not contain the peptide yet, as illustrated in Figure 22(a).

- Activate the *Spot Family* page to work on peptide libraries.
- In the canvas, select the PL by clicking on one of its blocks. Right-click on the selection and choose *Import Peptide Mapping File* from the context menu (Figure 20).
- Specify the linker which will be excluded from the peptides. Leave it blank if no linker is used.
- Select the file which maps the peptides of the PL to their spot ID in the blocks.



**Figure 20: Import the Mapping File.**

Spot_ID	Peptide
1	CxMGPGLAAVLLSLAVA
2	CxGLAAVLLSLAVALAR
3	CxAVLLSLAVALARVPA
...	
106	CxPPNWHVPEASDVTIQ
...	

**Figure 21: The Peptide Mapping File.**



The peptide mapping file is a Tab-delimited text file or CSV file. It has two columns; the first one contains the ID of the peptides in the blocks and the second column contains the peptides. The first line of the file is reserved for the column headers and will be skipped. An example of the file's content is shown in Figure 21. In this example, the linker is Cx.

(a) Spot ID from the Original GAL File

(b) Spot ID with the Peptide

**Figure 22: Assigning the Peptide to a Spot.**

After importing the peptide mapping file, open the *Spot* page again. Now, you can view the peptide in the *ID* field when clicking on a spot in the canvas, as illustrated in Figure 22(b). The overlapping peptide library has been completely reconstructed and can be analyzed as described in Sections 5.1 to 5.3. Please note that *saving the GAL file also keeps the information about the peptide library design*.

In the sample data package, the *overlapping3* folder contains a test dataset for this function.

## 6 Batch Processing

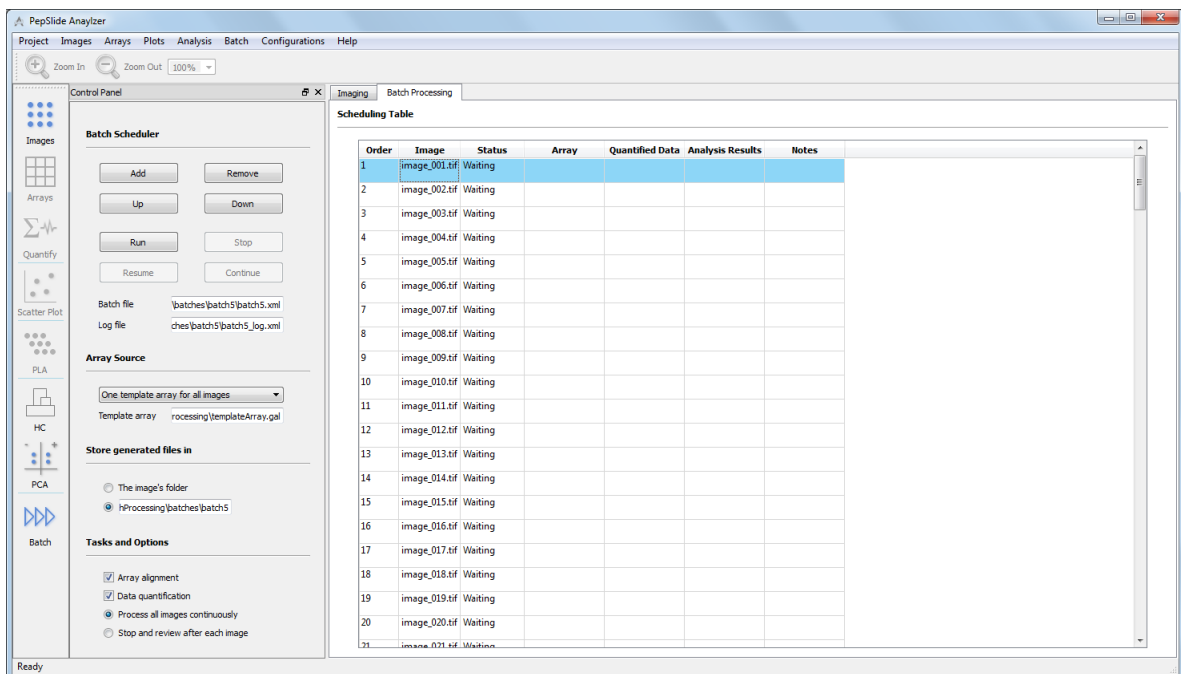
You can setup a batch to process a number of array images automatically. Suppose that the experiment is to screen a peptide array with  $k$  samples. The array design is annotated by the so-called *template array*. From the screening result you have  $k$  scanned images and would like to quantify their data. To this end, for each scanned image the batch aligns the template array with the image, creates the array file that contains the aligned layout, and generates the quantified data.

Batch processing employs automatic array alignment. As mentioned in Section 2.2, this only works with array files of GAL format, the spots have circular shape, and the spots' signal is sufficient for the array positioning. **This version does not yet support batch processing for PSF array files.**

Click the *Batch* button in the main toolbar to create a batch (Figure 23). In the *Batch* control panel:

- Click the *Add* button and select the array images for processing. They will be added to the scheduling table. Use the *Add*, *Remove*, *Up*, and *Down* 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.
- Finally, 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 files. Since the dot character (".") is used for file extensions such as .gal or .csv, please *do not name folders or files used in a batch with dot characters* (except for the file extension) to avoid errors.



**Figure 23: Batch Setup.**

After creating a batch, click the *Run* button to execute it (Figure 24). Please note the *running modes*:

- *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.

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

- *sample001.gal*: the array file whose spot layout is aligned with the image *sample001.tif*,
- *sample001.csv*: the CSV file containing only the quantified data, and
- *sample001.psa*: the PepSlide® Analyzer project file containing the analysis data for this image. Note that this project file can be used directly with data mining tools.

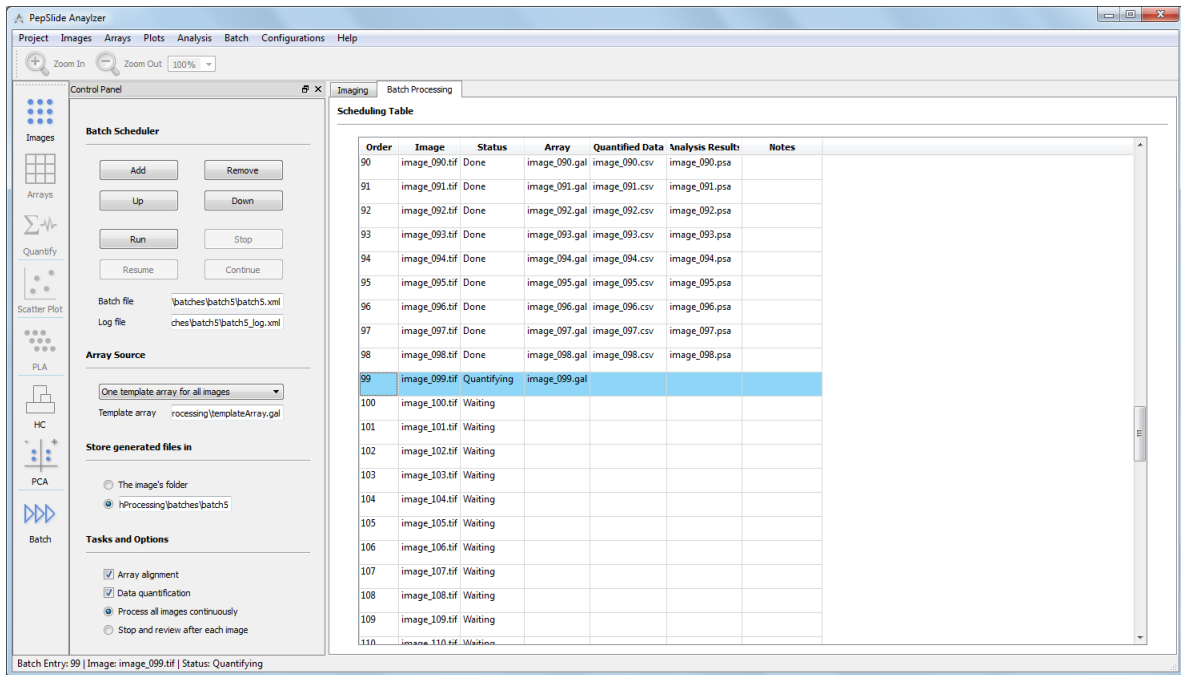


Figure 24: Batch Execution.

## 7 Data Mining Tools

Data mining tools assist you to find 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. the list of generated PepSlide® Analyzer project files, can be used directly for data mining.

### 7.1 Dataset

A dataset can be compiled from a list of PepSlide® Analyzer project files. Consider the example in Section 6 in which the peptide array is screened with  $k$  samples. After running the batch we obtained  $k$  project files containing the quantified data. If the dataset is created from these  $k$  project files, it can be regarded as the table in Table 3, where  $V_{1k}$  is a screening value of *Feature 1* when the peptide array is screened against *Sample k* and so on. The screening value can be chosen among the quantified values, e.g.  $\log_2$  (*Green Foreground Mean / Red Foreground Mean*).

Block	Row	Column	ID	Name	Sample 1	Sample 2	...	Sample k
		<b>Feature 1</b>			$V_{11}$	$V_{12}$	...	$V_{1k}$
		...			...	...	...	...
		<b>Feature n</b>			$V_{n1}$	$V_{n2}$	...	$V_{nk}$

Table 3: A Sample Dataset.

Please note that the first five columns in Table 3 contain the spot's properties specified in the GAL file - *Block, Row, Column, ID, and Name* – of an individual *feature*. For simplicity we only write *Feature 1* instead of its five property values.

In addition to PepSlide® Analyzer project files, you can create a dataset from a list of *GenePix Result (\*.gpr) files*. The third alternative is to manually prepare your dataset as a CSV file, having the data formatted in columns and rows like Table 3.

## 7.2 Principal Component Analysis (PCA)

PCA simplifies a complex microarray study to a simpler one with only three samples or features, thus you can easily observe the study's data and its trends. To start with, click the *PCA* button in the main toolbar. In the *PCA* control panel:

- 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 three either *Features* or *Samples*.

Click the *Start Analysis* button. Suppose that that you chose the *Samples* option. Take the screening in the previous section again as example. As shown in Table 3, the original dataset represents the features' screening value against *k* samples, where *k* is much larger than 3. PCA will simplify it to a dataset with only 3 samples, as illustrated in Table 4.

Block	Row	Column	ID	Name	Sample x	Sample y	Sample z
Feature 1					$V_{1x}$	$V_{1y}$	$V_{1z}$
...					...	...	...
Feature n					$V_{nx}$	$V_{ny}$	$V_{nz}$

**Table 4: The Simplified Dataset.**

The PCA chart (Figure 25) then depicts the features according to their values in the simplified dataset, whose data is shown in the table below the chart. You can select 2D or 3D view. By observing the charts you can find the trends of the data. For example, features that have common characteristics locate near each other on the chart. On the other hand, those that are distinct are far from the others. The simplified dataset can be exported to a CSV file for further analysis.

Similarly, you can discover such information about the samples by choosing the *Features* option before starting the analysis. By combining the results of these two analyses, you may be able to discover features and samples that influence the variance of the study.

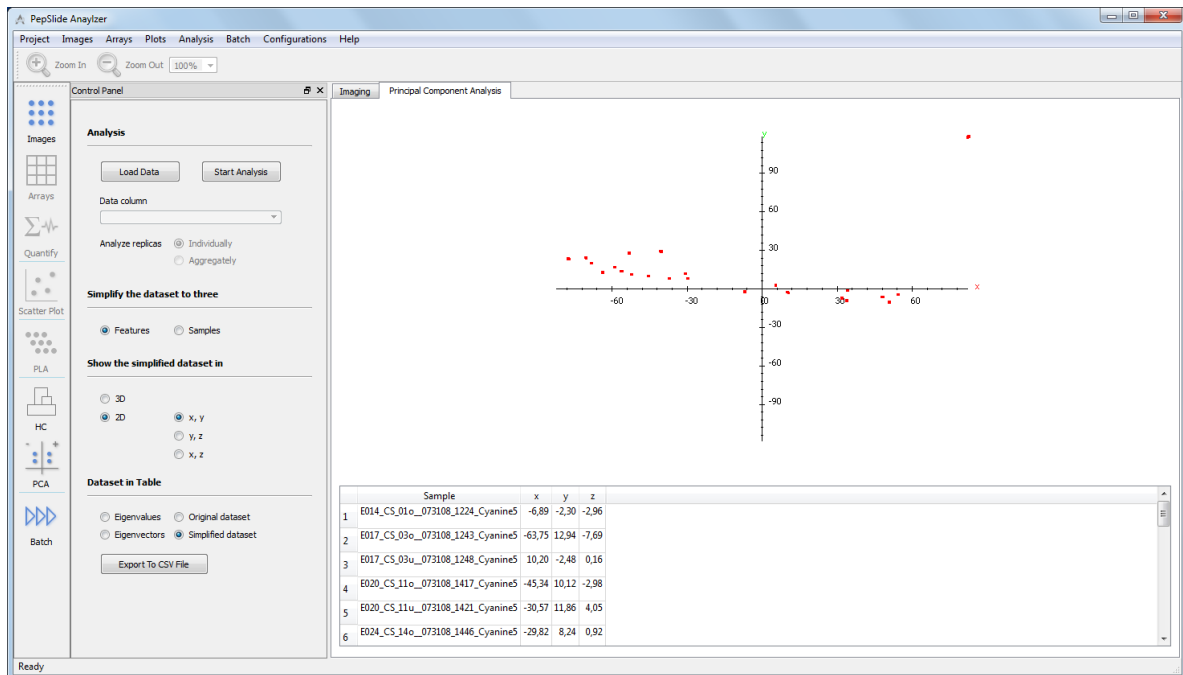


Figure 25: Principal Component Analysis in 2D View.

In the sample data package, the *schroeder2010* folder contains a test dataset for this analysis.

### 7.3 Hierarchical Clustering Analysis (HCA)

You can group features or samples that are related using HCA. The relationship can be e.g. having similar effect in the study, represented by close screening values. Click the *HC* button in the main toolbar to setup the analysis.

- 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.

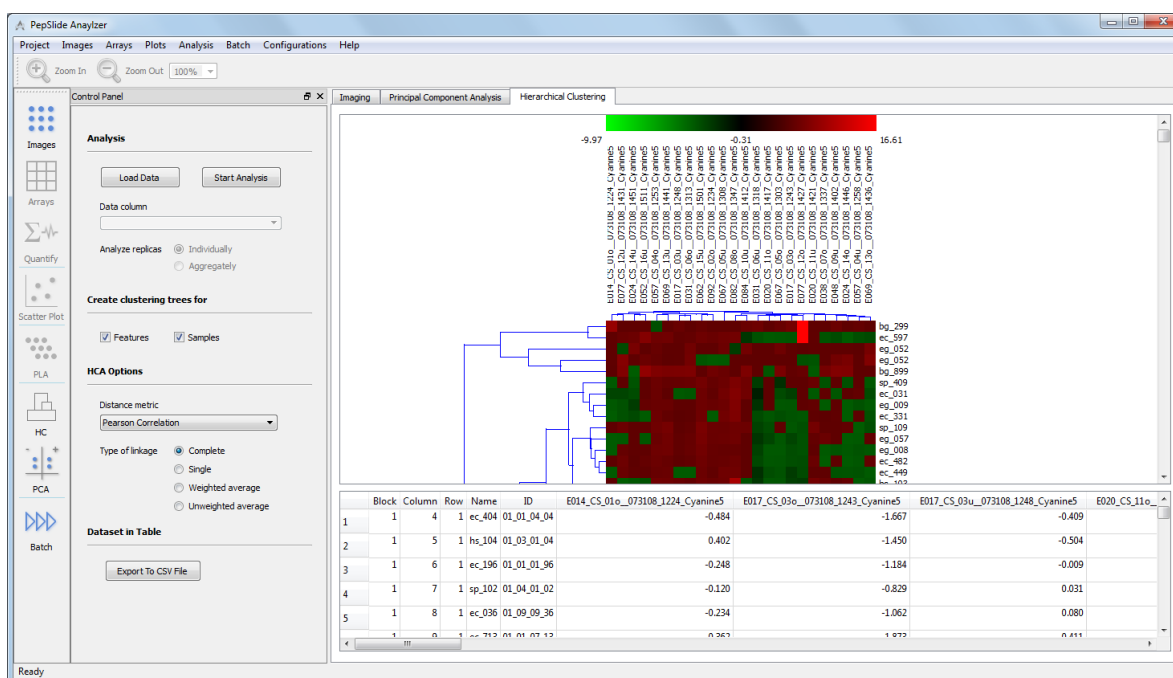


Figure 26: Hierarchical Clustering Analysis.

The clustering tree(s) are then constructed (Figure 26). Two features considered being *related* are grouped into one *cluster*. Their *relationship* is represented by a line connecting them. A cluster might be related with 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.

In the sample data package, the *schroeder2010* folder contains a test dataset for this analysis.

## 8 Utilities

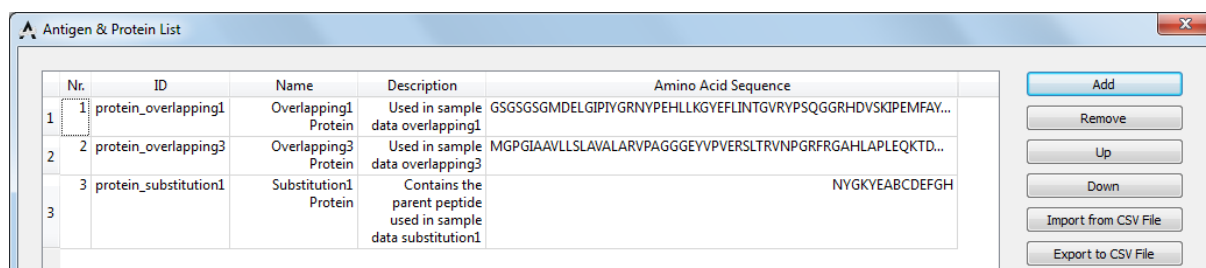
### 8.1 Search for Antigens and Proteins

It is helpful to know if a generated peptide is part of an antigen or a protein. PSA assists you to do that easily, for either the entire peptide array or only selected peptides. You first need to setup the list of antigens and proteins that might be related to you study. Then during quantification of peptide array data or analysis of peptide libraries, you can conveniently search for antigens or proteins that contain a peptide. The list can be easily imported and updated.

#### Setting up the List of Antigens and Proteins

Use the *Configurations > Antigen & Protein List* menu to launch the setup dialog (Figure 27). An antigen or a protein is stored in one row in the table. Its data is contained in five columns:

- **Nr.:** A numeric ordering number.
- **ID:** Unique identification of the item. It will be shown in the search result of a peptide if the antigen or protein contains the peptide.
- **Name:** The item name.
- **Description:** Further information about the item.
- **Amino Acid Sequence:** The item's sequence of amino acids.

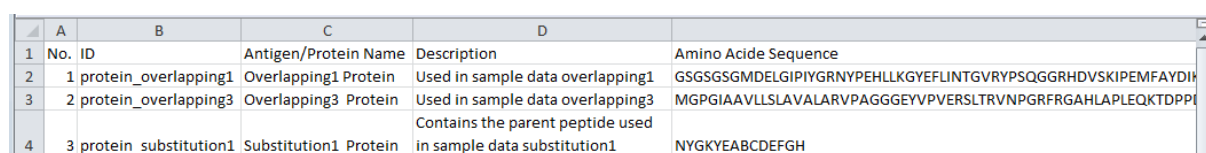


Nr.	ID	Name	Description	Amino Acid Sequence
1	protein_overlapping1	Overlapping1 Protein	Used in sample data overlapping1	GSGSGSGMDELGIPIYGRNYPEHLLKGYEFLINTGVRYPQGGRRHDVSKIPEMFAY...
2	protein_overlapping3	Overlapping3 Protein	Used in sample data overlapping3	MGPGIAAVLLSLAVALARVPAGGGEVVPVERSLTRVNPGRFRGAHLAPLEQKTD...
3	protein_substitution1	Substitution1 Protein	Contains the parent peptide used in sample data substitution1	NYGKYEABCDEFHG

**Figure 27: List of Antigens and Proteins.**

If you already have a list of antigens and/or proteins contained in a spreadsheet file, you can export that list to a CSV file and then conveniently import that list into PSA. Alternatively, you can manually add an item to your list with the *Add* button. Use the *Remove* button to delete an existing item.

Figure 28 shows an example CSV file containing a list of proteins. The CSV file format can be either *Tab delimited* or *Semicolon delimited*. This option can be chosen during the export with your spreadsheet software. The first line of the file is the header and will not be processed. PSA will import items from the second line of the CSV file. If you import another list, the new items will be appended to the current list. Please note that the current version of PSA does not check if items' ID is duplicated. Therefore, please prepare the list with unique IDs.



No.	ID	Antigen/Protein Name	Description	Amino Acide Sequence
1	protein_overlapping1	Overlapping1 Protein	Used in sample data overlapping1	GSGSGSGMDELGIPIYGRNYPEHLLKGYEFLINTGVRYPQGGRRHDVSKIPEMFAYDIH
2	protein_overlapping3	Overlapping3 Protein	Used in sample data overlapping3	MGPGIAAVLLSLAVALARVPAGGGEVVPVERSLTRVNPGRFRGAHLAPLEQKTDPP
3	protein_substitution1	Substitution1 Protein	Contains the parent peptide used in sample data substitution1	NYGKYEABCDEFHG

**Figure 28: A List of Antigens and Proteins in the CSV File**

You can export the current list to a CSV file for backup purpose or using that list on another computer. To do that, launch the setup dialog again and click the *Export to CSV File* button.

Please note that there are two example CSV files in the *antigenProteinList* folder in the sample data package. They contain the same list of proteins but are stored in two different formats. You can use them to test the function or as a template to create your own list.

### Searching for Antigens & Proteins

In the *Quantified Data* sheet, you can perform the search for the entire array. Alternatively, in the *Peptide Library Analysis* sheet the search is only applied to selected peptides. In either cases, click on the *Find Antigen/Protein* button to start the search. The *Search for Antigen & Protein* dialog (Figure 29) determines how PSA carries out the search for a peptide:

- *Stop at the first finding*: The search is finished as soon as an antigen or a protein in the list is found containing the peptide.
- *Search for all matches*: The entire list will be scanned. The search returns the ID of *all* antigens or proteins that contain the peptide.

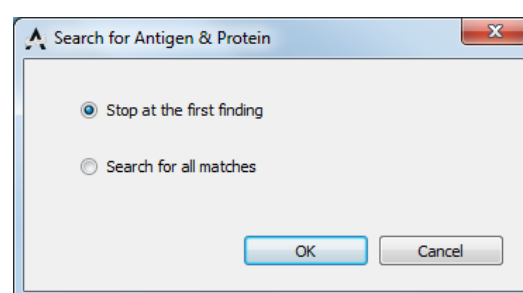


Figure 29: Search Options.

The search results are shown in the *Antigen/Protein ID* columns of the table of quantified data or the table of selected peptides. Figure 30 illustrates the latter case. You can export the table's data, including the peptide and the antigen/protein ID columns, to a CSV file.

Spot Family	Row	Column	Peptide	Antigen/Protein ID	Red Raw Mean	Red Background Mean	Red Foreground Mean	Red Raw Median	Red Background Median	Red Foreground Median	Flag	Annotation
1	NYGKYE Analoging	6	22	NYGKYE	protein_substitution1	234,37	48,56	185,81	249,00	45,00	204,00	
2	NYGKYE Analoging	6	24	NYGKYE	protein_substitution1	229,75	48,56	181,19	248,00	45,00	203,00	
3	NYGKYE Analoging	6	21	NYGKYE	protein_substitution1	229,38	48,56	180,82	247,00	45,00	202,00	
4	NYGKYE Analoging	6	23	NYGKYE	protein_substitution1	227,56	48,56	179,00	247,00	45,00	202,00	
5	NYGKYE Analoging	6	25	NYGKYE	protein_substitution1	223,89	48,56	175,33	250,00	45,00	205,00	
6	NYGKYE Analoging	6	18	NYGKYV		181,30	48,56	132,74	203,00	45,00	158,00	
7	NYGKYE Analoging	6	14	NYGKYQ		176,12	48,56	127,56	201,00	45,00	156,00	

Figure 30: Search for Antigens and Proteins.

## 8.2 Scatter Plot

In addition to peptide analysis methods described in Section 5, you can depict the array's spots on a two-dimensional chart according to their intensity values. Scatter plots look like the chart of overlapping peptide library analysis (Figure 15). The difference is that scatter plots can be used for any type of array files, no matter if it is of PSF or GAL format, or if a block or a spot family already has a peptide library design or not. This is particularly useful to quickly check the spots' signal of an array before further analysis.



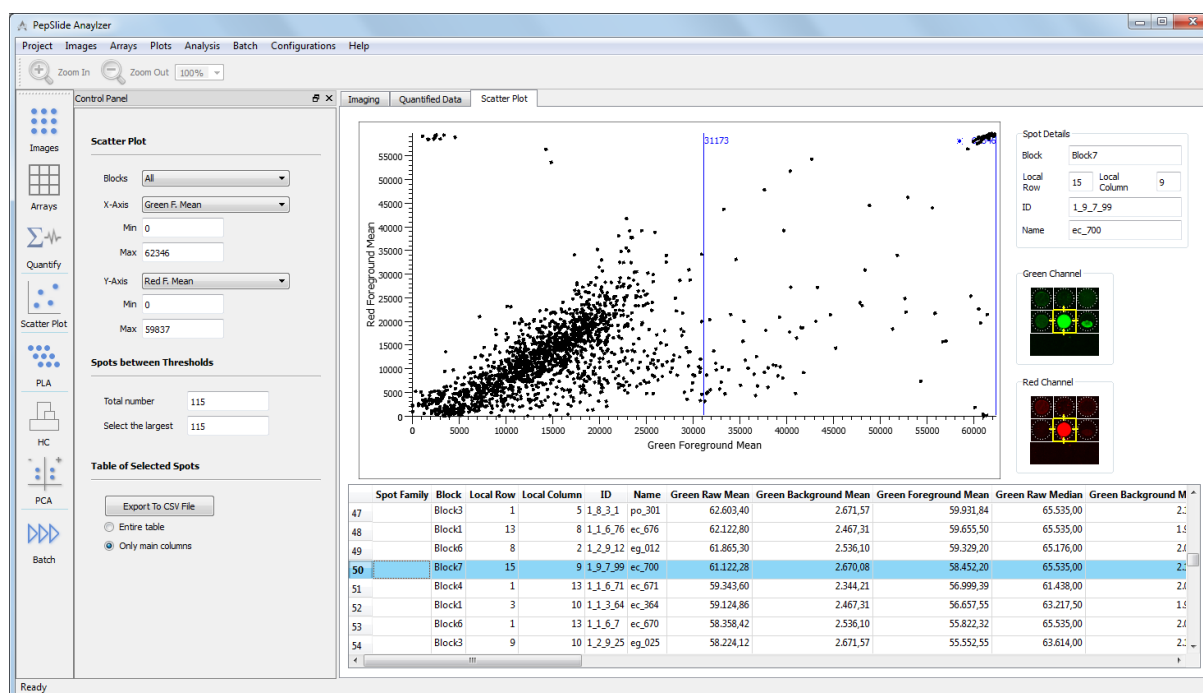


Figure 31: Scatter Plot.

To start this examination after data quantification, click on the *Scatter Plot* button in the main toolbar. Figure 31 shows the scatter plot of an antibody microarray image. You can use the threshold bars to select spots and export data in the table to CSV files. However, since this is not peptide library analysis, you cannot use specific features such as finding consensus motif or searching for antigen and protein here.

## 9 Product Activation

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

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

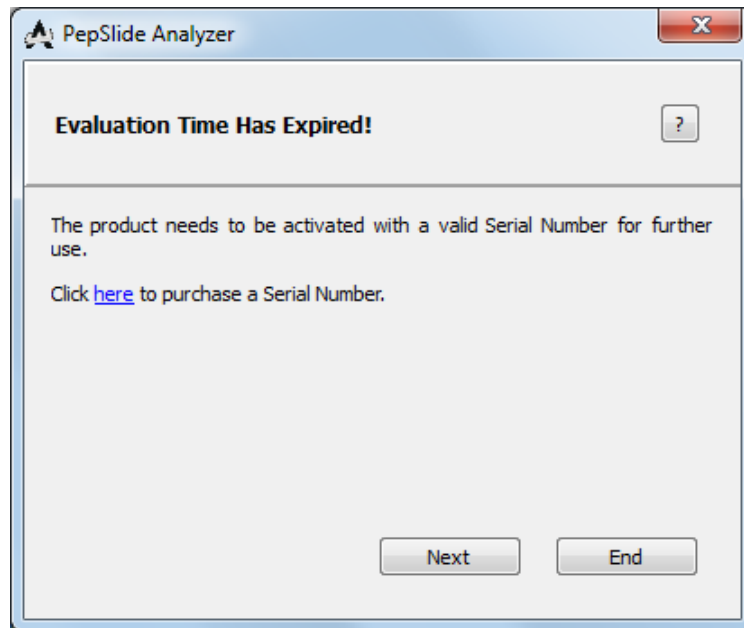


Figure 32: Starting the Product Activation.

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

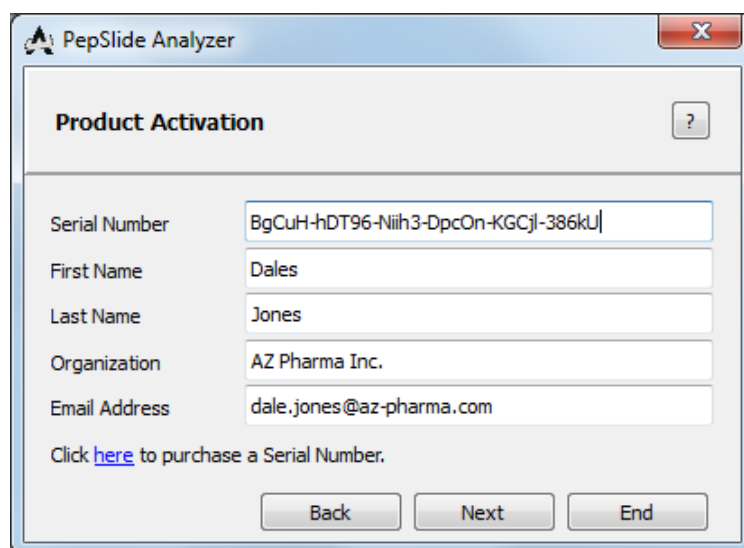
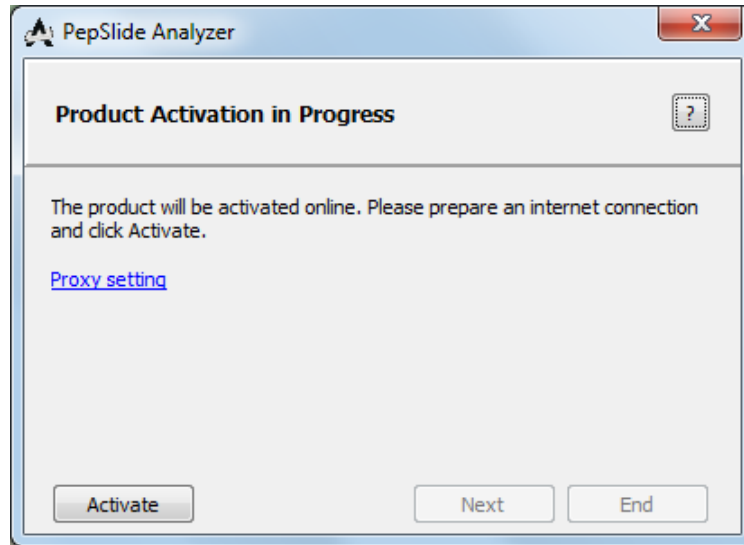


Figure 33: Entering the licensee information.

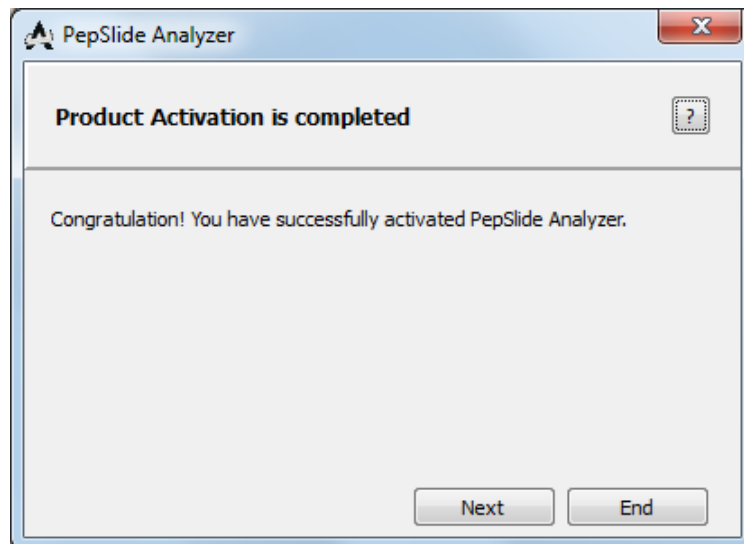
- (3) If the internet connection is ready, click the *Activate* button and wait for the activation to finish.



**Figure 34: Product Activation in Progress.**

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

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



**Figure 35: Completion of the Product Activation.**

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

## 10 Sample Datasets

The sample data package consists of sample datasets that can be used to get familiar with PepSlide® Analyzer software.

The *overlapping1*, *random1*, and *substitution1* folders contain the data of peptide arrays whose array file is of PSF format. The *overlapping3* folder also contains peptide array data, but the array file is prepared in the GAL format. You can perform data quantification (Sections 2-4) as well as analysis of peptide library (Section 5) on these datasets.

A dataset for testing data mining tools (Section 7) is stored in the *schroeder2010* folder.

Further information of the datasets is described below.

### *overlapping1*

---

The peptide array consists of *overlapping* peptide libraries. The signals are recorded in a 16-bit grayscale TIFF image.

Reference to Analysis Functions: Section 5.1.

### *random1*

---

The peptide array consists of *random* peptide libraries. The array's scanned image is a 24-bit color TIFF file, having the control signals in the green channel and the peptide signals in the red channel.

Reference to Analysis Functions: Section 5.2.

### *substitution1*

---

The peptide array contains a *substitution* peptide library. The signals are recorded in an 8-bit grayscale TIFF image.

Reference to Analysis Functions: Section 5.3.

### *overlapping3*

---

This peptide array contains an *overlapping* peptide library. Since the array file is of *GAL* format, you can try creating a peptide library for GAL blocks with this dataset.

In this folder, file `overlapping3_design.txt` stores the design information of the peptide library, while file `PeptideMappingFile.txt` contains the peptide mapping data. The signals are recorded in a 16-bit grayscale TIFF image.

Reference to Analysis Functions: Section 5.4 and Section 5.1.

### *schroeder2010*

---

This folder contains a dataset compiled from this work<sup>2</sup>. The original GenePix Result (GPR) files can be downloaded [here](#).

You can test the Hierarchical Clustering Analysis and Principal Component Analysis with this dataset. In this folder, file `schroeder2010.csv` stores the Log Ratio (549/649) values of the GPR files. The same data is stored in file `schroeder2010_filtered.csv`, excepted that positional controls (starting with `ps_`) have been filtered out.

Reference to Analysis Functions: Section 7.

### *antigenProteinList*

---

This folder contains two example CSV files which can be used to test the Search for Antigens & Protein function. You can also use them a template to create your own list. The files contain the same list of proteins but are stored in two different formats:

- Tab delimited: `antigenProteinList_tab.csv`.
- Semicolon delimited: `antigenProteinList_semicolon.csv`.

References to Utilities: Section 8.1.

---

<sup>2</sup> Schröder et al. (2010): Dual-color proteomic profiling of complex samples with a microarray of 810 cancer-related antibodies, *Molecular & Cellular Proteomics* 9(6): 1271.

## 11 End-User License Agreement

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