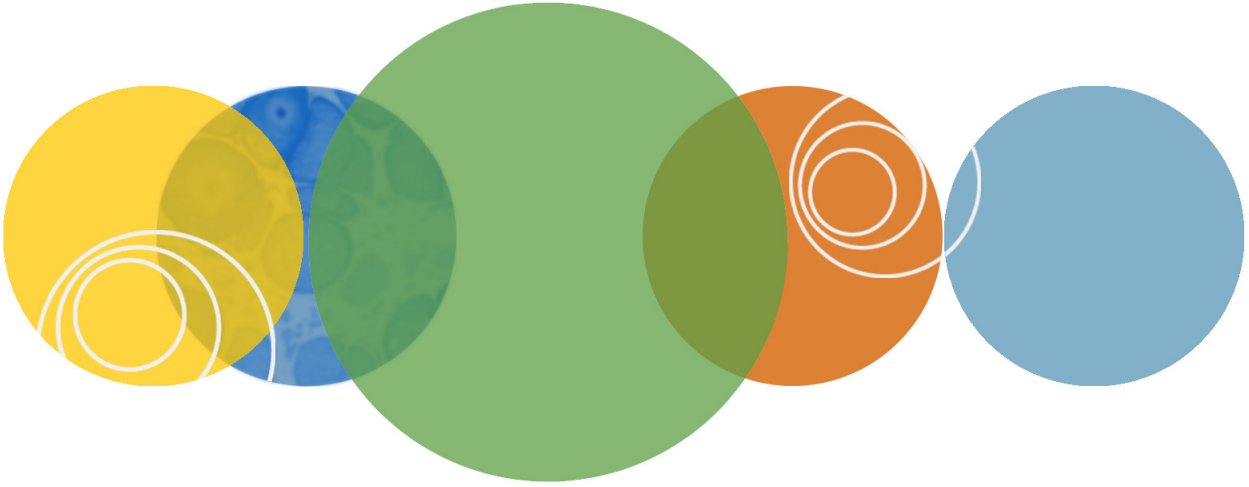

GenePix® Pro

Microarray Acquisition and Analysis Software

User Guide

MOLECULAR DEVICES

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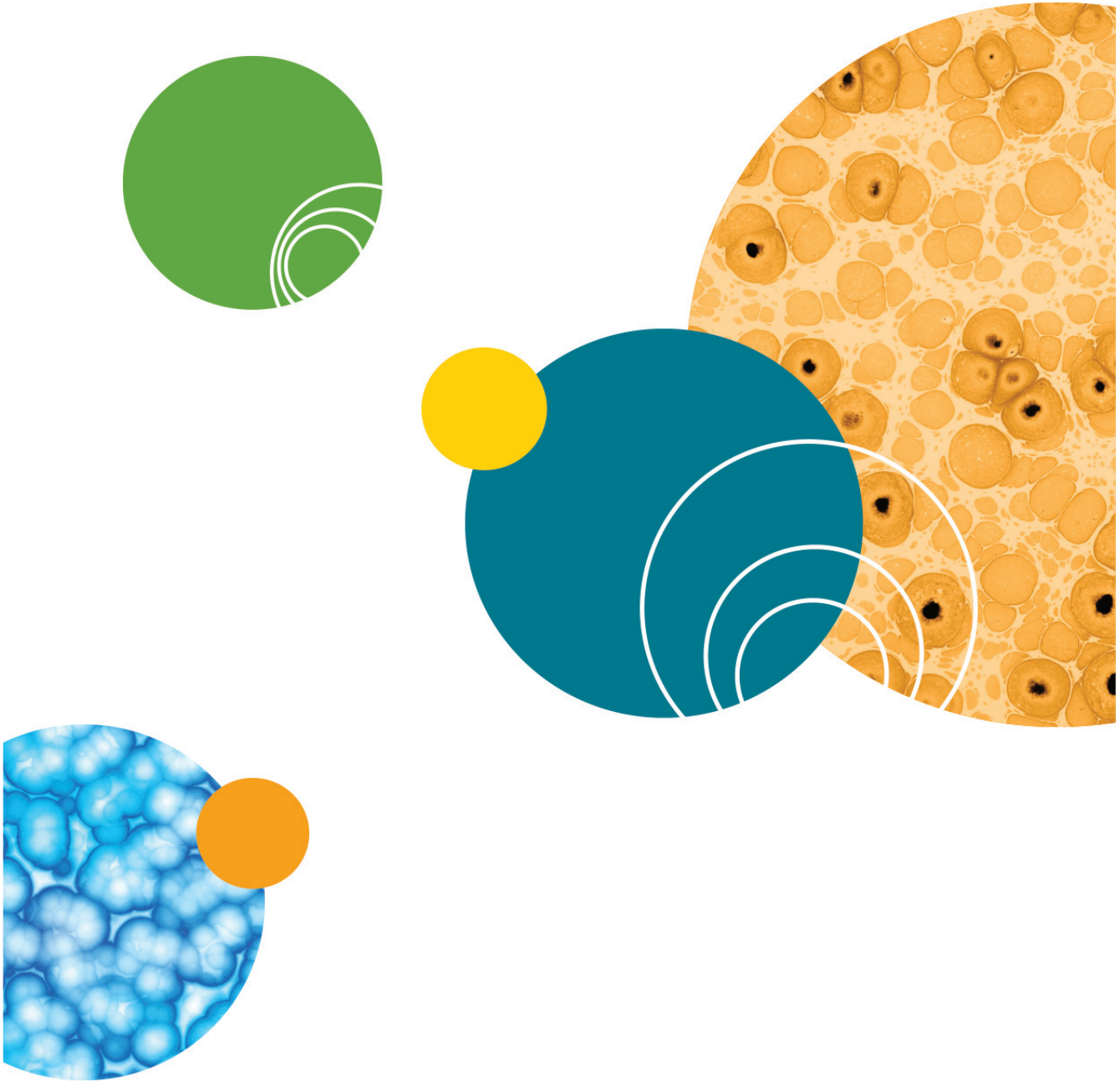
Product manufactured by Molecular Devices, LLC.

1311 Orleans Drive, Sunnyvale, California, United States of America 94089.

Molecular Devices, LLC is ISO 9001 registered.

Contents



Preface

The *GenePix®* Pro *Microarray Acquisition and Analysis Software User Guide* contains in-depth conceptual information about the use of the GenePix 7 Software for GenePix Microarray Scanners, as well as an overall image acquisition and analysis workflow tutorial.

Topics in this section include:

Note: VERIFICATION: This program is extensively tested before distribution. Nevertheless, researchers should independently verify its performance using known data.

About this User Guide

The sections in this guide are structured to provide the information needed to understand the overall operation of the Software. This includes:

- Software Features and Principles on page 11: The introduction explains all the main features and concepts used in the Software. If you are new to the software or to microarray scanners and image analysis in general, read this section thoroughly.
- Tutorial: Image Acquisition & Analysis on page 33: This workflow tutorial walks you through a complete image acquisition and analysis cycle, and describes some of the other special functions you will use regularly. If you are a new user, we recommend that you work through this tutorial with the Software.
- Software and Protection Key Installation on page 101: The installation appendix describes how to install the Software and the required hardware protection key.
- Glossary on page 107: The glossary provides a reference of terms central to the Software and its use.

Technical Assistance and Feedback

If you need help to resolve a problem, or want to provide feedback about this product, contact us at:

1-408-747-1700 or
1-800-635-5577 (North America only) or
+44 118 944 8000 (Europe)
OR
www.moleculardevices.com

Or see our knowledge base at:
http://www.moleculardevices.com/support

Additional Assistance

The scanner and software package includes the following documentation and help materials:

- An online help system providing reference and procedural information for all software functions. Click the Help button to access the online help.
- A *printed hardware guide* containing comprehensive scanner and software installation instructions.

The Help System

Figure 1-1: Help Icon

Use the Help system to access reference and procedural information while you are working in the software.

To access the Help system

- Click the **Help** button or press F1 at any time to open a Help topic specific to the window or dialog box in which you are working.

At the top of the Help window is a toolbar for common tasks, such as printing and going back to the previous page. The tabbed pane on the left provides several navigational aids to help you find the information you need. The main pane on the right is where the Help topics are displayed.

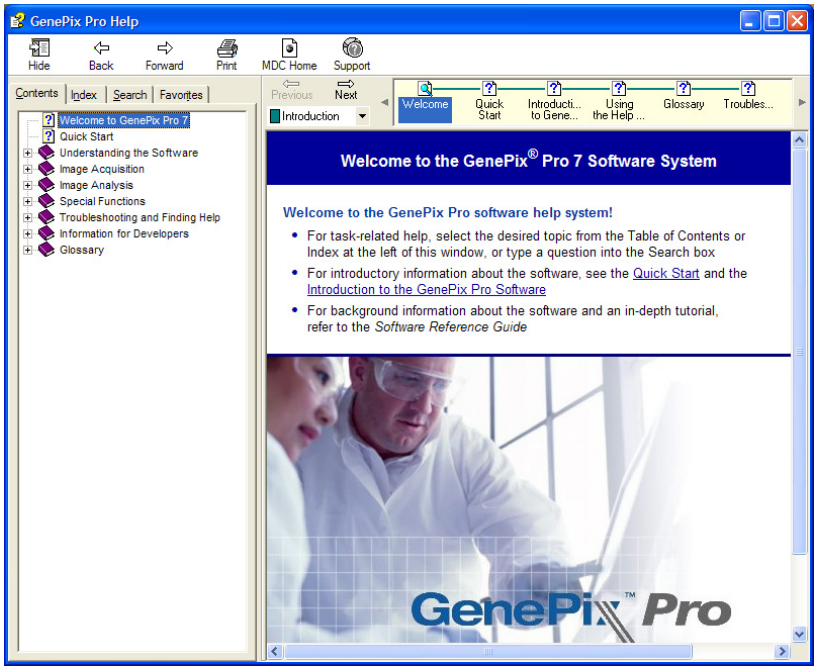


Figure 1-2: Welcome Screen

Contents

The Contents tab arranges Help topics by subject, just like the Table of Contents in a book. Click a book icon to display an overview of the topics in that section. Click a topic icon to display a particular topic.

Index

Use the Index tab to search the Help index for a word. Type the word you want to look up in the text box and click Display, or click the topic in the alphabetical list. The chosen topic is displayed.

Search

Use the Search tab to locate a word, phrase, or set of words. The help system uses boolean, wildcard and nested expressions to refine a search. With the AND, OR, NOT, and NEAR operators you can precisely define your search by creating a relationship between search terms. If no operator is specified, AND is used. For example, the query “spacing border printing” is equivalent to “spacing AND border AND printing.”

Table 1-1: Search Table

To search for	Use	Example	Results
More than one term in the same topic	AND	baseline AND correction	Topics that contain both the words “baseline” and “correction”
Either of two terms	OR	spectrum OR spectra	Topics containing either the word “spectrum” or the word “spectra” or both
The first term without the second term	NOT	peak NOT table	Topics containing the word “peak” but not the word “table”
Both terms in the same topic, close together	NEAR	samples NEAR high	Topics containing the word “samples” within eight words of the word “high”

To perform a search

1. Type the words or phrase you want to search for in the text box and click **List Topics**. The topics containing the word or phrase are displayed, including a Rank to help you choose the topic you want to view first.
2. Select a topic from the list and click **Display**. The topic you have chosen is displayed in the main window.

Favorites

Use the Favorites tab to quickly find frequently used help topics. You can store the current help topic in a Favorites list by clicking Add at the bottom of the Favorites tab, then return to the topic anytime by double-clicking the title on the tab.

Software Features and Principles

1

This section provides a brief overview of the GenePix® Pro 7 Software. It introduces the basic features and techniques of the system.

Topics in this section include:

Software Features

The GenePix Pro 7 Software is a fully featured array acquisition and first-pass analysis package, possessing advanced imaging and data manipulation capabilities.

Imaging

- Incorporates precise software control of scan conditions and scan areas, reducing scan time and image storage requirements
- Allows you to create, analyze, and view data for multiple scan areas as necessary, processing information for each area individually or for all areas at once
- Scan area spin control lets you quickly browse images, data, and scatter plots across multiple scan areas
- Facilitates quick feature block creation and manipulation, including group rotation for multiple blocks
- Displays and analyzes up to four wavelength images and three ratio images
- Automatically sets PMT gain
- Constructs user-configurable pseudocolor intensity and ratio images for the easy visualization of activity levels

- Acquires images from any GenePix scanner and displays a ratio image in real time
- Aligns and analyzes images from third-party scanners

Analysis

- Automatically constructs resizable arrays of blocks and feature indicators for identifying and analyzing individual features on a slide
- Performs batch analyses of an unlimited number of microarray images
- Automatically aligns circular, square, or irregular feature indicators with features
- Automatically reads a variety of barcode types on array slides
- Automatically associates individual array features with substance names and IDs
- Allows multiple flagging of features as measures of individual feature quality, by fully general Boolean queries
- Automatically calculates and subtracts background effects with a choice of algorithms, such as local, global, negative control, and morphological methods
- Includes two instant data viewers, one for instant zoom views and primary data previews (the Feature Viewer), and one that graphs pixel intensity distributions for individual features and includes more advanced data preview (the Pixel Plot)
- Includes measurement tools for detailed analysis of specific image regions
- Includes advanced control of data and image normalization
- Is fully integrated with web-based genomics databases
- Presents the extracted data in a spreadsheet for rapid sorting
- Draws Scatter Plots of any extracted data measurements for all the features on an array
- Applies user-defined threshold boundary lines to Scatter Plot data
- Exports intensity, ratio, and quality control data for analysis with advanced informatics systems
- Is fully configurable on a per-user basis in multi-user environments
- Automatically logs all experimental procedures and protocols in an electronic Lab Book

- Includes full scripting support in VBScript and JavaScript for all acquisition and analysis functions

Batch Scanning

- Scans all selected slides in the GenePix® SL50 Slide Loader, without any user intervention
- Includes customizable hardware (scanner) settings and analysis settings
- Provides Global file options, including file naming, file saving, and workflow options
- Includes a Manual scan mode, enabling the GenePix SL50 Slide Loader to operate much like a manual GenePix scanner
- Allows you to stop, restart, and review a scan
- Allows you to check a batch scan log file if problems occur

Software Principles

While the Software has been designed so that a user can acquire and analyze images of arrays without having to learn the finer details of image processing, understanding the concepts and algorithms used is invaluable for interpreting the acquired data.

Although you will use the Software primarily to control your scanner, you can also use it as a standalone analysis platform. To do this, you need to be able to import array images in TIFF format.

Spots, Features, Blocks, Scan Areas, and Arrays

Within the Software, each individual spot on the array is called a feature, and is assigned a circular feature indicator. Feature Indicators are the primary unit of analysis in the Software. A collection of feature indicators is called a block. There can be several blocks on a single array, depending upon the type and configuration of the arrayer used to place the features on the slide. Scan areas identify the sections of the image you want the software to scan; you can create single or multiple scan areas on each array, as required, with each containing single or multiple blocks of features.

Array Lists and the Array List Generator

To employ the full power of automated array analysis, you can use the Array List Generator to construct a GenePix Array List (GAL) file.

To open the Array List Generator window

- From the **Start** menu, select **All Programs > Molecular Devices > GenePix Pro 7 > Tools > Array List Generator** or press Alt+G to use the keyboard shortcut from the GenePix Pro Software user interface.

The software uses GAL files to construct an array of blocks to match the size and positioning of printed features, and to apply substance names to the features. It is critical that the identity of the substance in each spot is maintained throughout the analysis, so use a GAL file when you have files containing substance IDs and names for each spot on the array. Substance IDs must be unique identifiers, such as Swiss-Prot and FlyBase identifiers, or GI (GenInfo Identifier) numbers.

The Array List Generator is designed to work with arrayers and arrays that have the following characteristics:

- The slides and source plates are perpendicular to one another.
- The robotic motions follow a raster pattern (left to right, then down, left to right again), both in loading samples from the source plate, and when depositing subsequent spots onto slides. Movement between slides does not have to follow a raster pattern.
- There are no replicate spots, although there can be replicate samples in the microplate wells.
- There is one block of spots per pin being used. For example, 4 pins = 4 blocks of m rows and n columns.

Therefore, you cannot use the Array List Generator to automatically create a GAL file in the following cases, although you may always create one manually:

- Arrays where the print-head is in the opposite orientation to the slide; for example, pin 1,1 dips into well A,12.
- Replicate spots of any nature, either in adjacent spots or in separate blocks. However, the user can manually edit the GAL file to insert replicates.
- Non-standard robotic movements, such as printing out of sequence; for example, returning to previously used wells in the source plate.
- Robotics movements that are non-raster (such as top to bottom, then over), either in loading sample from the source plate, or in the slide spotting pattern.
- More than one block per pin.
- Slides printed on other commercial arrayers that do not conform to the aforementioned specification.

Many commercial arrayers automatically generate GAL files. If your arrayer does not have GAL output functionality, please contact your arrayer manufacturer directly and request GAL file support.

To load a GAL file

- Click **File > Load Array List**.

A standard GAL file contains multiple data columns containing position and identity information for each feature: Block, Column, Row, ID, and, optionally, Name. The Software can also read GAL files containing additional user-defined columns, should you wish to include extra annotation information about your features—for example, their status as normalization spots.

Barcodes

If a slide has a barcode on it, the Software automatically reads the barcode symbols from Preview Scans, Data Scans, and saved images. The extracted barcode symbols are reported in the B field in the Status Bar at the bottom of the main window, and are exported in the header of the Results file. You can also use the barcode symbol as the image file name. To do this, select the Barcode option in the Save Images dialog box.

The Software reads several of the most common barcode formats: code 39, code 128, code I2of5, and codabar:

Table 1-1: Barcode Formats

Barcode type	Numerical character	Alpha character	Special character	String length
Code 39	0–9	A–Z, space	– $ % + /	no limit
Code 128	All ASCII characters			no limit
Code I2of5	0–9	N/A	N/A	no limit (even)
Codabar	0–9	N/A	- : . $ / +	no limit

If you know the type of barcode that your slides use, you can set the barcode detection so that it searches for that type only. This speeds up the detection process.

To set barcode detection by type

- Click **Options > Workflow**, and then click **Barcode Types**.

If your scanner has an integrated barcode reader, the Software automatically uses that reader to detect the barcode.

Image Display

Most GenePix scanners operate using sequential scanning: that is, the slide is scanned first using one color laser, and then another, and so on. The images at each wavelength are displayed as they are acquired in real time; a ratio image appears as each subsequent wavelength image is superimposed on the first.

The GenePix 4000B scanner is an exception. It uses simultaneous laser scanning systems to acquire and display two wavelength channels as ratio images in real time.

Ratio Image Creation

In the Software, each raw wavelength image, such as the green 532 nm image or the red 635 nm image, is collected at 16-bit resolution. Ratio images are standard 24-bit composite RGB (Red-Green-Blue) images. When the Software is set to the default red–green display scheme in two-color mode, with the default ratio formulation, the image from the 532 nm laser is placed in the green channel, and the image from the 635 nm laser is placed in the red channel. The blue channel is set to 0. So each color channel of the RGB images is composed only of an 8-bit image. This means that the ratio image is not, strictly, an image of the ratio data.

For display purposes only, each of the individual wavelength images must be reduced to 8 bits in order to be displayed as a 24-bit composite RGB overlay image. There are two options to reduce each raw wavelength image to 8 bits:

- Compress 16 bits into 8 bits
- Selectively use only 8 of the 16 bits

To compress the 16 bits into 8 bits, the Software uses a square root transform, whereby the square root of the image intensity is computed and displayed, which effectively reduces 16 bits to 8 bits ($\sqrt{\{2^{16}\}} = 2^8$).

Alternatively, to strip out 8 of the 16 bits, you can configure the software to keep only the bottom, middle, or top eight bits. The effect of each of these options on the range of the intensities that is used to display the ratio image is summarized in the following table:

Table 1-2: Bit compression

Method of ratio processing	Range of image intensity used
Square Root Transform	0–65535
Preserve High Intensities	256–65535

Table 1-2: Bit compression (cont'd)

Method of ratio processing	Range of image intensity used
Preserve Middle Intensities	16–4095
Preserve Low Intensities	0–255

What happens if you have selected a ratio processing method and you have pixels that are outside the range of acceptable intensities? The ratio processing method affects only the display of the ratio; it does not have any effect on the display of the raw wavelength images, nor does it affect the computation of the ratio data displayed in the Results tab. For example, if you have a pixel that has a green value of 15000 and a red value of 30000, and the ratio that you are computing is based on red divided by green, the ratio value would be 2 (30000 / 15000). If you are using the Square Root Transform method, you can display all pixels from 0 to 65535. In this scenario, the pixel in question is red, as the red channel is contributing twice the intensity to the RGB image as the green channel.

However, when you select Preserve Low Intensities, the maximum pixel value is 255. In this case, both the green and red pixels contribute the same intensity to the RGB image, because each image is effectively treated as 255. This is because both images exceed an intensity of 255, and are clipped at a value of 255. The result is a ratio displayed that is yellow, corresponding to a ratio of 1 (255 / 255).

Although the Software can analyze four individual wavelength channels, a ratio image can be created from only two at a time. You can specify up to three independent pairs of channels to display as ratio images.

Ratio Image Biology

The ratio image typically represents the comparison of test to reference sample that is bound to the spotted, or arrayed, substance. If the reference sample is labeled with Cy3 (excited by a green laser light such as 532 nm), and the test sample is labeled with Cy5 (excited by a red laser light such as 635 nm), then both of these samples are mixed with and bind to the spotted substances on the slide. In the derived ratio image, a red spot indicates that the test sample for this feature is more abundant than the reference sample; a green spot indicates that the test sample has a lower activity than the reference sample; a yellow spot means that there is no change in the activity level between the two populations of test and reference sample.

Analytic Algorithms

To quantify changes in activity or abundance accurately, you must extract the intensity of a given feature from an image, and refine this data before a final ratio value is computed. Several other parameters are measured to evaluate the quality of the data for a given feature.

Feature Intensities

For each raw image, the mean, median, and standard deviation of the pixel intensities in each feature indicator are computed. The pixels used to compute the standard deviation include only those located completely inside the feature indicator. Any pixel that overlaps the feature indicator boundary is excluded.

Background Intensities

The fluorescent intensity measured inside a feature usually includes a certain amount of stray fluorescence from various sources:

- Auto-fluorescence of the slide
- Non-specific binding of test and reference sample

The software must subtract this stray fluorescence, known as background, from the measured feature intensity to produce a true measure of the feature's fluorescence. The following types of background subtraction are available:

- Local methods
- Global methods
- Negative controls
- Morphological methods

The local median intensity is the default background subtraction method. Local methods, where the background value for a feature is calculated from a region near the feature, have the advantage that they track background variation across a slide. However, they can be unrealistically high or low if there are local defects near a feature, and thus may not be representative of the background contribution inside a feature. For example, when due to non-specific binding, background fluorescence may be overestimated, which may, in turn, lead to negative intensities with low intensity signals.

Global background methods, where a single background value is calculated for each wavelength, have the advantage of providing a uniform background estimation for a whole slide, particularly when features are so close together that local methods cannot be applied. However, where the background varies significantly across a slide, one

single estimate may not accurately represent the background contribution to all features.

Negative control methods, where the background for a feature is estimated from the intensity of a nearby feature, have a number of advantages over local and global methods—most notably allowing you to more accurately measure low-intensity features. Negative controls are features that are known not to be reactive, so that they can relied upon always to give the same intensity values independent of the experiment. If background is due to non-specific binding, then it is plausible that non-specific binding is lower where features have been printed on a slide, compared to the space between features. Thus it makes sense to estimate non-specific fluorescent background from negative control features, rather than from local regions between features. Also, if implemented correctly, background intensities can be calculated from negative controls close to a feature, and thus will vary as the background varies across a slide. However, unlike other methods which are purely computational and can be applied to any slide, negative controls must be part of the microarray slide design from the beginning.

Morphological methods address the problem wherein the background estimated for a feature is higher than the feature's intensity. This situation can be catastrophic, as a negative background-subtracted intensity can lead to a negative ratio and an undefined log ratio, meaning that the feature is lost from all further data analysis. In morphological methods, a copy of each single-wavelength image is created, and then each image is filtered to construct a background image for each wavelength. Two morphological background methods are available:

- **Closing followed by opening:** Small dark regions are filled in on the background image, then a local minimum filter is applied to the whole image.
- **Opening:** A local minimum filter is applied to the whole image.

The Opening method produces a significantly lower estimate of background than any other background subtraction method, and guarantees that the background estimate is always lower than the feature intensity. However, it may underestimate the true background level.

Compared to the default local background subtraction method, the Closing Followed by Opening method produces background estimates that are slightly lower for regions of low background, but significantly lower for regions where there are bright patches in the background. However, background-subtracted intensities can still be negative using this method.

Regardless of the method chosen, the calculated background intensity is subtracted from the feature intensity before any ratios are calculated.

Local Methods

In local background subtraction methods, a different background is computed for each individual feature indicator, and the mean, median and standard deviation of the background pixel intensities are reported. For all ratio calculations that require background subtraction, the median background value is used.

The background is calculated using a circular region that is centered on the feature indicator. This region has a diameter that is three times the diameter of the corresponding feature indicator. All the pixels within this area are used to compute the background unless one of the following is true:

- The pixel is not wholly outside a two pixel wide region around a feature indicator
- The pixel resides in a neighboring feature indicator
- The pixel is within the feature indicator of interest

Figure 1-1: Defining background pixels

In Figure 1-1, the dark grey region represents the pixels used for computing the background, the colored region represents the pixels used for the feature intensities, and the light gray region represents excluded pixels.

You can adjust the diameter of the background region and width of the exclusion region around a feature. Typically, you would make the exclusion region smaller and the diameter of the region larger as the density of features on your arrays increases.

To change background settings

1. Click **Options** > **Analysis**.
2. Under **Background Subtraction**, edit the values for the exclusion region and the background region.

The allowable exclusion values are between 1 and 8, inclusive; the allowable background diameter values are between 2 and 5, inclusive.

The Software also reports the percentage of pixels exceeding local background-related thresholds—the percentage of pixels that are greater than the background+1 standard deviation, and background+2 standard deviations. These threshold-related values are often useful in evaluating the quality of the data.

Global Methods

Several global background subtraction methods are available. In a global method, a single value for the background is used for a whole array at each wavelength. The global background values are either calculated from a mean or median of all local values, or specified as individual values for each channel.

Negative Controls

To use negative control background subtraction, you must identify features as negative controls by giving them a characteristic substring in their ID. By default, the Software looks for the substring "_NCTRL". For each feature, the name of the nearest negative control in the same block appears in the Negative Control column in the Results tab. The identifying substring is not case-sensitive, so you can use either "_nctrl" or "_Nctrl".

Morphological Methods

Morphological background subtraction starts with a copy of the image and filters it to construct a background image. The background value for a feature is then the intensity at the feature's center on the background image.

Closing removes small dark regions from the image. Any dark region that fits into a square with sides given by the closing value is removed from the image. Closing prevents the measured background from being too low.

Opening removes bright regions. Any bright region that fits into a square with sides given by the opening value is removed. Opening prevents the measured background from being too high.

If the opening value is too small, then features may not be removed from the background image. This typically happens on high-density arrays where the features are very close together.

Block Alignment and Background Thresholds

To align a feature indicator with a feature, the Software's proprietary spot-finding algorithm makes several assumptions about feature intensity relative to the intensity of the background, feature size, and feature position.

A feature is marked Not Found if:

- The feature has fewer than six pixels
- The feature diameter found is greater than the lesser of: (a) three nominal diameters (as set in Block Properties) or (b) the diameter that would cause it to overlap an adjacent feature of nominal diameter
- The feature diameter goes outside the bounds set in **Options > Alignment**
- The feature has been found in a position that would overlap an adjacent feature

If the spot-finding results in feature indicators that are larger than your features, it can be due to extreme background variation: bright background pixels may be included as feature pixels. To avoid this, try increasing the Composite Pixel Intensity threshold value on the **Options > Alignment** tab to a value slightly higher than the brightest background pixel near your features.

Ratio Quantities

The software computes a large number of different ratio quantities, each of which provides a different insight into the raw data. The main ratio quantities are described on the following pages and their significance is briefly discussed. They are divided into three different subgroups: those that are ratios of quantities derived from whole features (such as median and mean pixel intensities), those that are derived from pixel-by-pixel ratios of intensities, and quality factors.

All ratios and derived quantities are corrected for background effects. Pixel intensities at a wavelength have the median intensity of background pixels at that wavelength subtracted from them to produce a corrected intensity.

To get a better understanding of ratio computations it is useful to examine the Pixel Plot (press <P> when the cursor is over a selected

feature indicator). This plot shows you the pixel values for a selected feature, so you can see the contribution of each pixel to a ratio value.

Note: The form of all ratio calculations is user-selectable from the **Options > Analysis > Ratio Formulation** dialog box. In this guide, we assume that wavelength 2 is always in the numerator and wavelength 1 is in the denominator.

Ratios of Overall Derived Feature Properties

In ratiometric fluorescence studies we are typically most interested in the difference in substance levels between the test and the reference samples. This eventually translates to differences in intensity on the two images analyzed by the software, which can be quantified by taking a ratio of image intensities for each feature. Because each feature on the image is made up of many pixels, we need a method of finding a representative intensity for each feature.

- **Ratio of medians:** Median intensities are often preferred to arithmetic mean intensities because they are not as seriously affected by extreme values at either end of a distribution. The ratio of medians is the ratio of the background subtracted median pixel intensity at the second wavelength, to the background subtracted median pixel intensity at the first wavelength.
- **Ratio of means:** Mean intensities are familiar quantities, but they can be significantly skewed by extreme values at either end of a distribution. However, calculating the mean allows you to evaluate the variability of the data. This variability is reported as the standard deviation. The ratio of means is the ratio of the arithmetic mean of the background-subtracted raw pixel intensities at the second wavelength, to the arithmetic mean of the background subtracted raw pixel intensities at the first wavelength.

On grounds of consistency you might expect to use the mean background instead of the median background when using the mean intensity. However, it is more important to use the most reliable value for the background than to be consistent. The fact that means can be skewed by extremely low and high values suggests that you should use the median background.

Quantities Derived from Pixel-by-Pixel Ratios

An alternative to computing the ratio from calculated means and medians of each whole feature is to compute the ratio on a pixel-by-pixel basis, and then to calculate the arithmetic mean and median of these many ratio values. An advantage of this approach is that non-specific signal, which appears in both wavelength images, has less of an effect than when the feature is treated as a whole.

- **Median of ratios:** As is the case with the overall derived feature properties, median intensities are often preferred to mean intensities because they are not as seriously affected by extreme values at either end of a distribution as compared to the arithmetic mean. The median of ratios is the median of the pixel-by-pixel ratios of pixel intensities that have had the median background intensity subtracted.
- **Mean of ratios:** The mean of ratios is the geometric mean (the mean in log space) of the pixel-by-pixel ratios of the raw pixel intensities that have had the median background intensity subtracted.
- **Regression ratio:** The regression ratio is a method of computing the ratio that does not require rigidly defining background and foreground pixels, so it is the most objective of the three methods. Every pixel within a circle of twice the diameter of the feature of interest is used. The relationship between wavelength 1 and wavelength 2 is determined by computing a linear regression between these two populations of pixels. The slope of the line of best fit (least-squares method) is the regression ratio. The coefficient of determination provides a measure of the level of accuracy of the fit. The regression ratio makes no assumptions about feature pixels or background pixels, so it can be useful for features that are badly misshapen, such as extreme donut-shaped features. However, the regression ratio can be seriously affected by saturated pixels. On a good array, all of the ratio values tend to converge. If the regression ratio is significantly different from the other ratio quantities for some of your features, look at each of these features in more detail to determine what is causing the deviation. The Pixel Plot is a useful tool for this.

Quality Factors

Sum of Medians and Sum of Means

The sum of medians and the sum of means are useful measures to interrogate the quality of an individual feature. If either sum is unreasonably low, this indicates that there is very little signal in both channels, suggesting that any ratio derived from this feature should be interpreted with caution.

The sum of medians is the sum of the median of the pixel intensities at each wavelength, with the median background pixel intensity at each wavelength subtracted.

The sum of means is the sum of the arithmetic mean of the pixel intensities at each wavelength, with the median background pixel intensity at each wavelength subtracted.

These quantities are calculated on a per ratio channel basis; that is, for each pair of wavelengths in a ratio, a separate sum of medians and sum of means column is calculated.

- **Log ratio:** The log ratio is a base two logarithm of the ratio of medians. This type of log transformation is often used in ratiometric studies as it helps to compare levels of over-activity and under-activity. For example, if you have a four-fold increase in activity (a ratio value of 4), the log base 2 ratio is +2; if you have a four-fold decrease in the ratio (a ratio value of 0.25), the value is –2. The log base two transform allows you to normalize the distribution so that a four-fold increase and a four-fold decrease have the same magnitude.
- **Signal to noise ratio:** Signal-to-noise is important for determining the confidence with which one can quantify a signal peak of a given value, especially a signal near background. The confidence in quantifying the peak increases as the variation in background noise decreases, regardless of the absolute value of the average background.

  You can easily compute the signal-to-noise ratio (SNR) by measuring the mean signal intensity for a feature, subtracting the mean background signal, and dividing by the variation in the background, so the signal-to-noise ratio is defined as: (F635Mean – B635Mean) / B635SD where F635Mean is the mean of all the feature pixel intensities at 635nm, B635Mean is the mean of all the background pixel intensities at 635nm, and B635SD is the standard deviation of the background pixel intensities at 635nm.

Because medians are more robust to outliers than means, we typically recommend the median instead of the mean as a measure of feature or background intensity. However, because the signal-to-noise ratio is well understood theoretically when calculated using means, we calculate the signal-to-noise ratio using the mean intensity and mean background.

Flags

You can flag individual features manually with quality flags, such as Good, Bad, Absent, and Not Found. Flags are also used to indicate which features to use in the normalization of the image. And you can create custom User Defined flags to track Good and Bad traits of particular importance to your analyses. See Creating Custom Flags on page 66 for more information.

The Bad flag is useful when doing a cursory analysis of your images. If you observe a region where the features are sub-standard, you can flag them Bad and a flag value of –100 is noted in the analysis results.

Alternatively if you see a feature that shows some especially promising properties, you may want to flag it Good. If so, a flag value of +100 is noted in the Results. Select flagging commands from the Feature menu on the right-hand side of the main window.

Absent (numerical value –75) is applied to a feature when a GAL file is missing an entry for a feature, or when the ID is “Empty”. In a GAL file, each feature must show a value in the ID column; if that value is “empty”, the software automatically flags it as absent.

A feature is flagged Not Found (numerical value –50) when Align Blocks fails to align a feature indicator. See Block Alignment and Background Thresholds on page 22 for conditions under which alignment can fail.

A second type of flag is the Normalize flag. It has its own column in the Results tab, and is used to mark features for use in Normalization. For each feature you can choose to Include in Normalization or Remove from Normalization. When exported, these flags have the values 1 and 0 respectively.

Features can be flagged according to general Boolean conditions using the Flag Features dialog box on the Results tab. The expression is evaluated for each row in the Results tab, and features are flagged according to whether or not the expression is true or false for them. See Defining Your Own Flagging Criteria on page 76.

Data Normalization

Origins of Variability

Comparing the data from different array experiments is a complex task. Small variations in the many steps that produce an array image can make comparisons across arrays problematic. Variations can be due to differences in labeling efficiencies (dye and batch variations), chemical properties of different dyes, pin tips, slide batches, and scanner settings (such as, red/green channel settings, multiple scanners). Any of these variations can be corrected by normalization. Normalization can also help you to compare multiple channels—for example, the green and red channels—within a single microarray. No one normalization method will correct all types of variation. Choose a normalization method based on the known or expected sources of error, and the characteristics of the experiment. Validate the method empirically, for example by reversing the dye labeling to test a normalization method for channel balancing.

In the acquisition step of an experiment, one of the main contributors to variability between channels is setting PMT values incorrectly, so that the total signal acquired in one channel is significantly different from the total signal acquired in the other (assuming that when scanning a particular sample, the total signal in each channel is in fact the same). In such a case ratio values may be biased towards one channel. To minimize this form of variability, you should perform preliminary scans to adjust the PMTs so that they are producing roughly the same response in both channels. See Optimizing Scanner Settings on page 39.

Normalization Methods

In the analysis step, you can improve comparisons across many arrays by normalizing the data from each array. One method of normalization is based on the premise that most genes on the array will not be differentially expressed, and therefore the geometric mean of the ratios from every feature on a given array should be equal to 1. If the mean is not 1, a value is computed which represents the amount by which the ratio data should be scaled such that the mean value returns to 1. This value is the normalization factor.

Another method is to choose a subset of the features on an image as control features. All substances change expression levels under different conditions. Normalization control features should be selected based on their consistent behavior in all experimental conditions used on your arrays, not on their historical use as housekeeping genes in other molecular biology techniques. For example, the control spots might be such that each is expected to have a ratio of 1, so the mean of the control

features should be 1. Assuming that variations are uniform across the array, a single normalization factor can be calculated from these features and then applied to the whole array.

Normalization Process

Having chosen a normalization method, it must be implemented in software. The software employs a global normalization method in which images and results are normalized together. If the current results are not derived from the current images (that is, if they are not linked), then results data are normalized alone; see Linkage on page 73. You can always remove normalization to return to your original images.

Because ratios are not normally distributed, the software first takes the log of each ratio value when normalizing. The mean $\bar{x}$ of a set of *n* ratios {*x*} is therefore:

$$\bar{x} = \exp\left(\frac{1}{n}\sum_{i-1}^{n} \ln x_i\right)$$

Figure 1-2: Normalization Formula

Ratio values less than 0.1 or greater than 10 are excluded from the calculation, as are features flagged Bad, Absent or Not Found.

Four-color normalization is done on a ratio-by-ratio basis. For example, if you choose to normalize so that the mean of the Ratio of Medians is set to 1.0, each Ratio of Medians data type that you have defined in the **Options > Analysis > Ratio Formulation** dialog box is normalized independently.

To calculate the wavelength-specific normalization factors that are reported in the Results Header, the change to the ratio is distributed equally between the wavelengths, so one wavelength scales up by the square root of the ratio scale factor and the other scales down.

For example, suppose the mean of Ratio of Medians of 635/532 is 1.21. The square root of 1.21 is 1.1, so we set the scale factor for the 532 wavelength to 1.1, and the scale factor for the 635 wavelength to 1/1.1 = 0.91. After applying these scale factors, the new mean of the Ratio of Medians is 1.21 * 0.91 / 1.1 = 1.0.

Because normalization can scale up the data, it is possible for normalization to produce pixels with intensities greater than the hardware limit of 65535.

File Formats

The Software recognizes and uses several different file types.

ATF (Axon Text File Format)

This is a tab-delimited text file format that can be read by typical spreadsheet programs such as Microsoft Excel. It is used for GAL files and GPR files.

An ATF text file consists of records. The group of records at the beginning of the file is called the file header. The file header describes the file structure and includes column titles, units, and comments. Each line in the text file is a record. Each record may consist of several fields, separated by a field separator or column delimiter. The tab and comma characters are field separators. Space characters around a tab or comma are ignored and considered part of the field separator. Text strings are enclosed in quotation marks to ensure that any embedded spaces, commas and tabs are not mistaken for field separators.

GAL (GenePix Array List File)

The GAL file conforms to the Axon Text File (ATF) format. The GAL file contains entries to describe the layout of each block, and to assign names and identifiers (IDs) to each feature on an array. The software can also read GAL files with user-defined data columns beyond the usual five; information in user-defined columns is preserved and written to the GPR file when results are saved.

GIS (GenePix Image Set File)

The GIS file links GPS settings information to a set of images (for example, as captured in a *Save Images—All Scan Areas* process). To open a GIS file, click **File > Open Image Set**; this opens the images and their related settings all at once.

GPA (GenePix Loader File)

GenePix scanners with robotic slide loaders only: Settings for batch scanning (which GPS or GAL file to use, Auto PMT setting, and so forth for each slide) are saved as a binary file.

GPL (GenePix Lab Book File)

The GPL is a read-only binary file. See Special Functions on page 82 for more information about the Lab Book.

GPR (GenePix Results File)

Results data are saved as GPR files, which are in Axon Text File (ATF) format. The results file contains general information about image acquisition and analysis, as well as the data extracted from each individual feature. Results can be saved with the entire table in a single GPR file, or with each block in a separate GPR file.

GPS (GenePix Settings File)

Acquisition, analysis and display settings are saved as binary GPS files. Settings are organized into several different categories (acquisition, analysis and display) all of which are saved together in the GPS file. However, when opening a settings file you can choose which subset of the settings you want to open.

Acquisition settings include which laser was enabled during the acquisition, the PMT Gain settings, the lines averaged, and the scan area. Analysis settings include the location and identification of blocks and feature indicators that were defined on the image. Display settings include brightness and contrast settings, and the ratio color mapping.

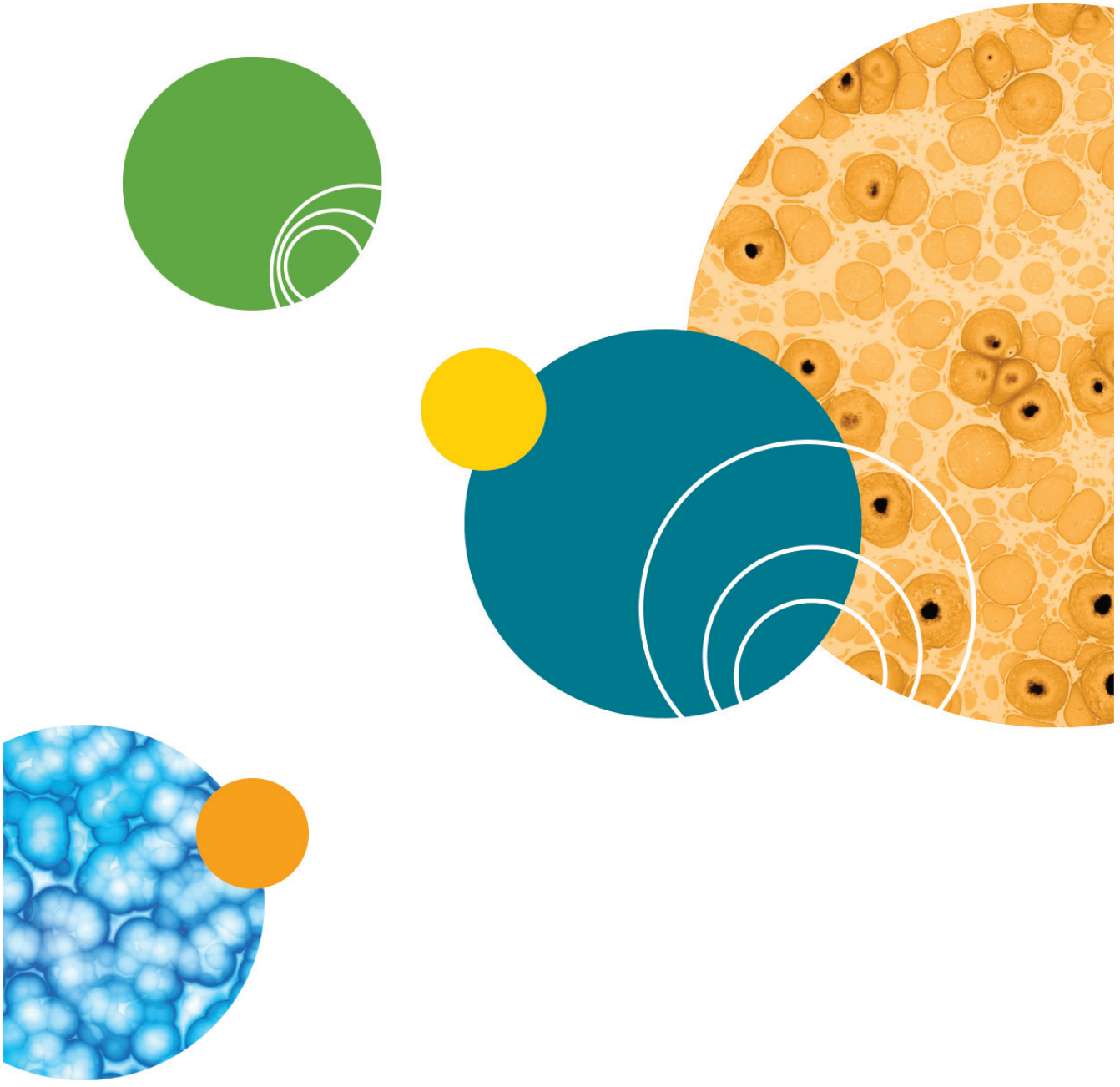
GPS files and GAL files both contain block and feature geometry, and both can be used to apply a grid template to an image. However, a GAL file does not contain the acquisition and display settings that are saved in a GPS file.

JPEG (Joint Photographic Experts Group)

You can save images in JPEG format for use in presentations or reports, but these JPEG images cannot be used for analysis. JPEG is a compressed image format which creates smaller files than other formats, such as TIFF.

TIFF (Tagged Image File Format)

Images acquired are by default saved in the TIFF format. This is a standard, uncompressed graphic file format that can be read by many graphics and imaging programs. The primary data acquired by the software are the single-wavelength images, and by default these are saved as 16-bit grayscale TIFFs in a single multi-image TIFF file. Not all graphics applications can read multi-image TIFF files. You may want to try opening a multi-image file with your preferred graphics application to see if they are supported. If not, save the single-wavelength images as separate single-image files. The software exports its preview and pseudocolor ratio images as 24-bit color TIFFs, but it does not read them, as data are not extracted from them.



Tutorial: Image Acquisition & Analysis

2

This tutorial guides you through loading your first array and scanning your first array image. It also leads you through a basic array analysis, and describes more advanced functionality.

Topics in this section include:

- Starting the Software on page 33
- Image Acquisition on page 34: Image Acquisition begins with starting the scanner and takes you through to scanning an array and saving your images. If you are using the Software in Analysis Only mode, you can skip this topic.
- Image Analysis on page 46: This topic begins with loading an image, and takes you through all the analysis steps required to extract data from the image.
- Special Functions on page 82: This topic describes additional features you might use regularly.

Starting the Software

The GenePix® Software installation program creates a *Molecular Devices/ GenePixPro 7* folder in your Programs list and a shortcut icon on your desktop. The folder contains several programs, including the main **GenePix Pro 7** and **GenePix Pro 7 (Analysis Only)** entries. Either program will start the Software, but the Analysis Only option operates independently and does not interact with the scanner hardware. The Programs folder also contains shortcuts to the *Array List Generator, Tools, Uninstall, Help, Reset to Program Defaults, Tutorial,* and *GenePix Utilities*.

If you have not yet installed your GenePix® scanner, do so, referring to your scanner documentation.

Note: The scanner must be running before your computer is started so that the software can verify that the scanner is connected and operational.

Note: After power-on, give the scanner 15 minutes to warm up before acquiring any images with it. This allows time for the lasers' output to stabilize.

Analysis Only Mode

The *GenePix Pro 7 (Analysis Only)* shortcut accesses the standalone version of the software. This version provides the same functionality as regular mode, except that the Software does not interact with a physical scanner.

You can use Analysis Only mode to:

- Mimic the scanning process while training or working through this tutorial. In this mode, sample images appear when you press a scan button. The software installation file includes all the necessary settings and analysis files to accompany the sample images.
- Acquire image data on one computer (run in regular mode) and perform analyses on another (run in Analysis Only mode).
- Save wear on the lasers while running multiple or lengthy analyses.

Image Acquisition

This section leads you through the entire image acquisition process, from performing a preview scan to scanning an array and saving your images. If you are running the Software in Analysis Only mode, you can skip this topic.

Configuring Laser Settings

Figure 2-1: Laser Settings

Figure 2-2: Erase All Scan Area Images

Use the Laser Settings dialog box to define the pixel size and wavelengths per image channel you wish to use. You can alter your laser settings only before beginning a scan. If you have already acquired data, you must clear the existing images before you can reconfigure the laser settings.

Note: The Software uses the first Data Scan to automatically determine the pixel size that will be used for all scan areas. When you run a Data Scan, the Pixel Size control is disabled. To clear images and allow a new pixel size to be set, click **Erase All Scan Area Images**.

To configure laser settings

1. On the **Image** tab, click **Laser Settings**.

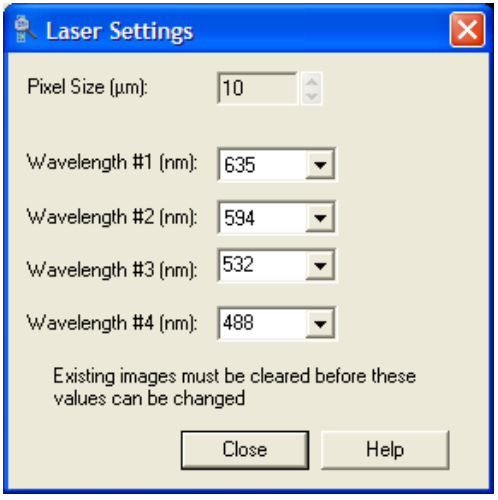


Figure 2-3: Laser Settings dialog box

2. In the **Pixel Size** spin box, select the desired size to use for your scan areas.
3. In the **Wavelength** drop-down lists, select the desired wavelengths to use per image channel.
4. Click **OK**.

Performing Preview Scans

Figure 2-4: Preview Scan

For information on inserting and removing arrays, see the documentation accompanying your scanner.

Use a Preview Scan, running at a relatively low resolution of 40 µm, to find the areas of interest on the array. You can then select those regions as Scan Areas and follow with a high resolution Data Scan.

To perform a Preview Scan

1. Start the Software.
2. Insert a slide with the arrayed features facing down.
3. On the Image tab, click **Preview Scan**.

Creating Scan Areas

Figure 2-5: New Scan Area

After running a preview scan, you can define specific scan areas. By default, the initial scan area for the slide (*Scan Area 1*) is sized to the dimensions of the entire slide. To adjust the data scan area to include only the features in your array, click the Scan Area Properties button and enter the desired dimensions. You can create as many scan areas as you need; click New Scan Area to add subsequent scan areas. The number in the top left corner of the scan box shows the scan area ID.

When a scan area is locked, the word **Locked** appears in the bottom right corner of that area. All scan areas are locked during the acquisition of image data; when data acquisition is complete, all scan areas with images become locked.

When scan areas are unlocked, you can use your cursor to move or resize them as required, using standard click, drag, and drop functions.

To create a Scan Area

Figure 2-6: View Scan Area

1. On the **Image** tab, click **View Scan Area** > **New Scan Area**.
2. In the New Scan Area dialog box, specify the desired size and position of the scan area.
3. The settings in this dialog box default to those of the scan area last created or selected.
4. Click **OK**.

   The scan area appears on the image.

Tip: You can also click and drag with your cursor to change the size and position of any scan area.

5. Press **<5>** to zoom into the selected scan area.

Performing a Multiple Wavelength Data Scan

Figure 2-7: Data Scan

A data scan is a high-resolution scan that is used for data analysis. You can set it to run at resolutions scaling from 5 µm/pixel to 100 µm/pixel (for the GenePix 4400A scanner, this range runs from 2.5 µm/pixel to 100 µm/pixel). Scan at the lowest end of the range to analyze features smaller than 100 µm in diameter. A 100 µm circular feature scanned with 10 µm pixels contains about 78 pixels, which is sufficient for an accurate intensity measurement. For features larger than 100 µm, the negligible difference in accuracy is unlikely to justify the fourfold increase in file size and the twofold increase in scan time required by a 5 µm scan.

Figure 2-8: Data Scan All

Where multiple scan areas exist, clicking Data Scan runs a scan for the selected scan area only. During the scan, only the image for the active

scan area is visible, and scan areas are locked. Following the scan, all image areas become visible, and any scan areas that do not contain images are unlocked so that you may select a subsequent scan area for scanning.

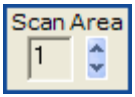


Figure 2-9: Scan Area Spinner

To scan multiple scan areas at once, click **Data Scan All**. Each scan area will be scanned in sequence.

To view the images, results, or scatter plots for any of a series of multiple scan areas, click the appropriate tab and use the **Scan Area** spin box tool to select the number of the scan area you want to see.

To change the resolution for a data scan

1. Ensure any existing images are erased. To do this, click **Erase All Scan Area Images**.
2. You are prompted to save the existing images.
3. Click **Laser Settings**.
4. In the **Pixel Size** spin box, select the desired value.
5. On the **Image** tab, click **Data Scan**.

During scanning, all image display controls and zooming functions are fully operational. See Image Analysis on page 46.

Performing Single Wavelength Scans

Figure 2-10: Single Wavelength Scan

If you have scanned two, three, or four wavelengths (depending on your model of scanner), and one of the wavelengths scans was done at an incorrect PMT setting, for example, you can use the Single Wavelength Scan function to scan just that wavelength again, while retaining all the other wavelengths.

Note: When you perform a single wavelength scan, the original image for that wavelength is overwritten.

Optimizing Scanner Settings

The Hardware Settings dialog box controls many different scanner settings, such as PMT gain, filter selection, lines to average, and focus position.

Maximizing the Dynamic Range of a Scan

The complete dynamic range of the scanner is being used when you see a range of intensities on the image from 1 to 65535. A pixel with an intensity of 65535 is saturated. Saturated pixels represent a condition in which there are more photons detected than the PMT can process, or the output of the PMTs exceeds the range of the analog-to-digital converters. A saturated pixel is not an accurate measurement of the signal from the pixel, so it is imperative to set the PMT Gain to avoid saturation. Note that you should not set the PMT gain to lower than 400, as this will result in a nonlinear signal.

Saturated pixels are drawn as white on the image, and appear on the far right of the histogram. See Special Functions on page 82.

To maximize the dynamic range of your scanner for a slide

1. Click **Hardware Settings**. The Hardware Settings dialog box can remain open while scanning.
2. Click **Preview Scan**.
3. While scanning, increase the **PMT Gain** for each wavelength until there are no pixels that are saturating in each single wavelength image. You should not set the PMT gain to lower than 350, as this will result in a nonlinear signal.

Balancing PMTs

The ideal scan is one in which the same amount of red and green feature signal is acquired in each channel, resulting in a pixel ratio of approximately 1.0. Post-acquisition normalization can correct for variations in relative signal intensities, so scanner settings need only approximate a ratio of 1.0. Balancing the two channels by eye is often sufficient.

To Balance PMTs

1. On the **Image** tab, select a scan area.
2. Click **Hardware Settings**. The Hardware Settings dialog box can remain open while scanning.
3. Click **Preview Scan**.
4. Zoom into the part of the image that contains the features.

5. Click the **Histogram** tab.
6. In the **Image Balance** section, set the **Min Intensity** and **Max Intensity** to 500 and 65530, respectively.

   The **Count Ratio** field at the bottom left of the Histogram tab reports the ratio of counts in each channel.
7. While scanning, in the **Hardware Settings** dialog box, adjust the **PMT Gain** in each channel until this ratio is about 1.0. You should not set the PMT gain to lower than 350, as this will result in a nonlinear signal.

For more information about the Histogram tab, see Special Functions on page 82.

Automatically Balancing PMTs

Instead of manually adjusting the PMT gain, to maximize the dynamic range and balance the signal from the channels you can use the automatic PMT balancing routine.

To use this routine you need to have a GAL or GPS file for the slide that is approximately positioned on blocks of features on the slide. The GAL file should also have accurate default feature diameters. Any good GAL file will be correctly positioned, even before any manual editing or spot finding.

Where multiple scan areas exist, the Auto PMT is calculated separately for each area. Select the scan area containing the blocks where you want the Auto PMT calculation to occur. During the scan, the Image tab enters Preview mode and runs multiple scans on the selected area. When the preview scans are complete, the dialog box displays the optimum PMT gains, which you can then apply to the hardware settings for the selected scan area by clicking Apply.

To automatically balance PMTs

1. On the **Image** tab, select a Scan Area.
2. Click **Hardware Settings**.
3. Click **Auto PMT**.

   The **Set PMT Gain** dialog box appears.

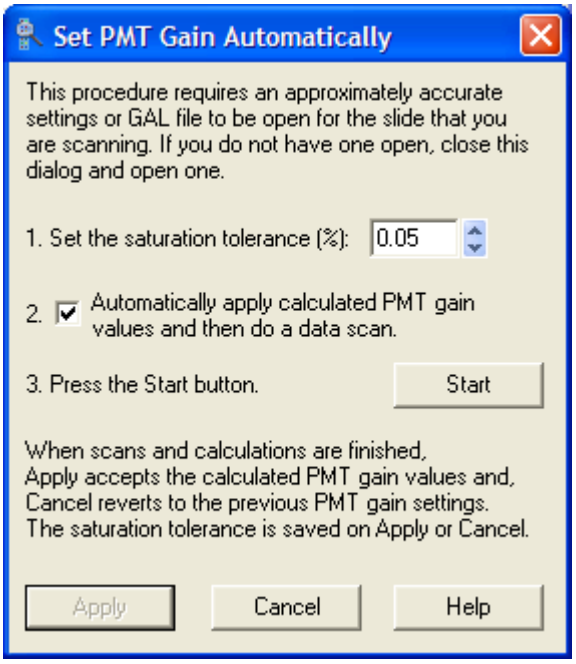


Figure 2-11: Set PMT Gain dialog box

4. In the **Set the saturation tolerance** spin box, select the amount of saturation (in terms of the percentage of pixels) that you want to allow in the scanned image. Molecular Devices does not recommend setting zero saturation, as a bright artifact on the image can cause the rest of the image to be scaled so that it is very dim. The default saturation of 0.05% is recommended for most slides.
5. Select or clear the **Automatically Apply Calculated PMT Gain Values and Then Do a Data Scan** option as desired. When enabled, this sets the software to perform a data scan immediately following the automatic PMT routine.
6. Click **Start**.

   The routine performs a number of Preview Scans at different PMT gain settings. When it is finished, it recommends PMT gain settings to be used for your Data Scan.
7. If you want to accept recommended PMT gain settings, click **Apply.**
   If you want to revert to the original PMT gain settings, click **Cancel**.

Using Line Averaging

To maximize the signal-to-noise ratio, you may want to average multiple lines. If you set Lines to average to 2, for example, the scanner scans each line twice and averages the value. Keep in mind that as you increase the amount of line averaging you correspondingly increase the amount of time needed to complete each scan. For example, averaging two lines doubles the scan time.

To average more than one line in a Data Scan

1. Click **Hardware Settings**. The Hardware Settings dialog box can remain open while scanning.
2. Change the **Lines to Average** field to the number of lines that you want to average (for example, 2).
3. Click **Data Scan**.

Changing Filter Selections

The Hardware Settings dialog box displays the filter selection field only if your scanner supports the installation of additional custom filters. Only the currently installed filters are displayed.

The emission filters that come installed as standard in your scanner are optimized for use with specific fluorophores—for instance the factory-installed filters in the GenePix® 4100A scanner are optimized for use with Cy3 (excited by 532 nm laser) and Cy5 (excited by 635 nm laser). You might want to install additional filters if you are using dyes with emission characteristics different from those for which the standard filters are designed.

If additional emission filters have been installed on your scanner, you can select which filter is used with which wavelength laser scan.

To change filter selection

1. Click **Hardware Settings**.
2. Change the **Filter** field for each wavelength to the desired filter.

Changing Laser Power

The Hardware Settings dialog box displays laser power adjustment only if your scanner is equipped with this feature.

You may want to reduce the laser power to avoid photobleaching if you have a particularly photosensitive sample, or if you need to scan a slide more than once, for example when fine-tuning focal plane adjustments. Some dyes, such as Cy5, are particularly prone to photobleaching. However, reducing laser power also reduces signal-to-noise ratio. Laser power cannot be adjusted while the scan is in progress.

To adjust the laser power

1. Click **Hardware Settings**.
2. Change the **Power** field for each wavelength to the desired value.

Changing Focus Position

If your scanner has the ability to change focus position, then the Hardware Settings dialog box displays focus position adjustment. You can change the focal point from –50 µm to +200 µm relative to the zero position, where a negative value is towards the slide. You can choose to focus manually for any sample with appreciable thickness, such as one with a cover slip. The best focus position is that at which the intensity is maximized.

Photobleaching may be a problem if you scan repeatedly to find the right focal point. If you are scanning repeatedly, you may want to reduce laser power. The focal plane can be adjusted while the scan is in progress.

To change the focus position

Figure 2-12: Hardware Settings

1. Click **Hardware Settings**.
2. Change the **Focus Position** field to the desired value.

Saving Hardware Settings

You can save your acquisition settings, as well as analysis and display parameters, in a GPS file. These settings can be opened later for use on other slides.

To save settings

1. Click **File > Save Settings As**.
2. From the **Save as type** field, select *GenePix Settings File (*.gps)*.
3. Type in a file name and click **Save**.

Saving Images

Figure 2-13: File... icon

When your array has been scanned, the next step is to save the images. You can save the image for just the selected or active scan area, or you can choose to save the images for all existing scan areas on the slide at one time.

Choosing a File Format

By default, all images created are saved as 16-bit multi-image TIFF files. Each multi-image TIFF file contains the images of the individual wavelengths scanned. The ratio image is not saved because it is recomputed when the multi-image TIFF file is opened.

To save images as multi-image TIFFs

1. Click **File > Save Images—Active Scan Area** or **File > Save Images—All Scan Areas**, as desired.
2. In the **Save as type** field, select **Multi-image TIFF Files**.
3. Click **Save**.

The Software also provides the option to save each image component of the acquired image as a separate TIFF file. There are several reasons why you might want to do this:

- Some graphics applications cannot read multi-image TIFF files; if you use one of these applications, you need to save the images separately if you are going to import them into such a program.
- You might want to save just the ratio image for presentation purposes. You can only save the ratio as a single-image file; it is not saved in the multi-image TIFF file.

Note: It is important to realize that if you do not save the raw wavelength images, that is, you save only the ratio image, then future analysis is impossible.

To save images as single-image TIFFs

1. Click **File > Save Images—Active Scan Area** or **File > Save Images—All Scan Areas**, as desired.
2. In the **Save as type** field, select **Single-image TIFF Files**.
3. Click the check boxes to select the images to save.
4. Click **Save**.

We recommend that for all data analysis purposes you save your images as multi-image TIFF files. The Software also allows you to export images in JPEG format. We recommend that you do not use the JPEG format to archive images; use it only for presentation purposes where image quantitation is not required.

To export images as JPEGs

Note: Images saved in the JPEG format cannot be re-opened in the Software.

1. Click **File > Export Images**.
2. Enter a file name for your images.
3. Select the check boxes to select the images to export.
4. Click **Save**.

Configuring Automatic File Names

The Software offers several options to generate file names automatically when saving images.

To set automatic file names

1. Click **File > Save Images—Active Scan Area** or **File > Save Images—All Scan Areas**, as desired.
2. In the **Naming** group, select one of the following:
   - Select the **Date Prefix** option to prefix the file name with the current date (yyyy-mm-dd).
   - Select the **Numeric Suffix** option to add a four-digit suffix to the file name, where numbering begins at '0000'.
   - Select the **Scan Area Suffix** option to add the scan area ID as a suffix to the file name.
   - Select the **Barcode Prefix** option to prefix the file name with any barcode symbols from your image.
   - Select the **User Name** option to add the user name to the front of a file name (you can set the software to be opened by user name; see Workflow Automation Options on page 97).

The Software controls the last few characters of the file name, which are reserved to indicate a file number, and the type of image if separate image files are being created. When saving single-wavelength images as either TIFFs or JPEGs, the Software adds the wavelength of the image as a suffix to the file name.

Image Analysis

After an image has been acquired, the next step is to view the image and measure the signal intensity of each feature.

Selecting a Color Mode

You can launch the Software in different modes for one-color, two-color, or four-color analysis. In two-color mode, you can load two single-wavelength images and define one ratio. In four-color mode, you can load up to four single-wavelength images, and define up to three independent ratios. In one-color mode, you load only a single wavelength channel at a time and thus do not measure any ratios, only intensity, in that channel.

To select a color mode

Figure 2-14: Options

1. Click **Options > Display**.
2. In the **Color Mode** section, select the desired mode, and then click **OK**.
3. If you changed the color mode, shut down the Software and then restart it.

Opening Images

If you have just acquired an image, it will be displayed on the screen ready for you to analyze it. If you want to save an image that has just been scanned, be sure to do so before proceeding with analysis. Alternatively, you can open a previously saved image.

To open an image

Figure 2-15: File... icon

1. Click **File > Open Images**.
2. Navigate to a directory that contains previously saved TIFF images. For example, open one of the sample TIFF files in the Program Files\Molecular Devices\GenePix Pro 7\Sample Data directory.
3. Select the image you want to view and click **Open**.

Opening Images in Four-color Mode

VTo produce a sensible ratio image, images must be acquired with the same Scan Area (that is, with the same Settings file) and at the same resolution.

To open multiple images in four-color mode

1. Start the Software in four-color mode.
2. Click **File > Open Images**.
3. Navigate to a directory that contains previously saved TIFF images. For example, open one of the sample TIFF files found in the Program Files\Molecular Devices\GenePix Pro 7\Sample Data directory.
4. Hold down the ⟨Ctrl⟩ key to select multiple images.
5. Click **Open**.

   The **Assign Image** dialog box appears. For each image, assign a wavelength and a color.

If the images were acquired on an GenePix scanner, the software automatically recognizes the wavelengths used for each channel. You can avoid the Assign Images dialog box by clearing the Enable User Assigned Wavelengths and Channels check box in the **Open Image > Options** dialog box. You can also override the actual values (for example to do ratio analysis of two images both scanned with the red laser) by selecting the check box and entering any wavelength values you choose.

Registering Images

Images acquired from more than one scanner, or acquired sequentially (instead of simultaneously) may need to be registered (aligned) before they can be analyzed. If images are misregistered, you can register them by hand, or allow the software to register them automatically.

To register images automatically

Figure 2-16: Image Tools icon

1. On the **Image** tab, click **Image Tools>Find best registration automatically**.

   If your images are very badly misregistered, for example, greater than 10 pixels, you need to set a starting offset for the registration algorithm.
2. If the registration fails, perform it again, but specify starting offsets.

To register images manually

1. Zoom in to the image so that you can see one or two features only.
2. On the **Image** tab, click **Image Tools > Register Images**.
3. The Register Images dialog box appears.

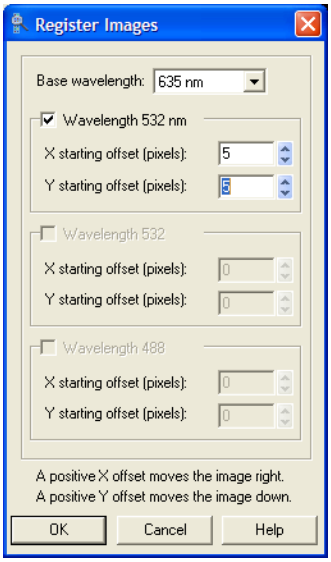


Figure 2-17: Register Images dialog box (4-color mode)

4. *(Four-color mode only)* Select a **Base Wavelength**. This is the image against which the others will be aligned.
5. Specify offsets as desired.
6. Click **OK**.

Customizing Ratio Formulation

The ratio formulation specifies which image is the numerator and which is the denominator for each ratio image. In two-color mode this is a simple binary choice, but in four-color mode there are many more choices.

For example, if you have four images numbered 1 to 4, your ratio images could be 1/2, 1/3 and 1/4, or they could be 1/2, 2/3 and 3/4, which is a very different experiment.

To change the ratio formulation

1. Click **Options**.
2. Click the **Analysis** tab.
3. Click **Select** in the Ratio Formulation group.
4. In two-color mode, switch to the reciprocal ratio formulation. In four-color mode, check up to three ratios.

In four-color mode, it is possible to select a set of ratios where one of the ratios can be expressed as a product of the others. This is known as a dependent set of ratios, and the set cannot be normalized. For example, 1/2, 2/3, and 3/1 is a dependent set. If you want to be able to normalize your data, use an independent set of ratios, such as 2/1, 3/1, 4/1.

Image Display and Color Control

The Software has many commands with which you can change the display of the image.

To switch between single-wavelength and ratio images

- In the **Image** group at the top left of the Image tab, click the image name buttons.

To change the colors displayed for an image

Figure 2-18: Color Selection

1. On the **Image** tab, click **Color Selection**.
2. Select from the list of wavelength color combinations at the top of the menu.

These image display controls are also available in real time during an acquisition. Experiment with the various display settings to find what works best for you. For example, green–blue with inverted color is good for distinguishing features from background at high magnifications.

To invert colors

1. On the **Image** tab, click **Color Selection**.
2. Select **Invert Color** from the menu.

To change the brightness and contrast of an image

1. To set brightness and contrast to their optimum settings, click **Auto Scale Display Settings**.
2. Move the individual brightness and contrast sliders for custom settings.
3. If you want to return brightness and contrast to their defaults, click **Reset**.

Zooming the Display

The Software has several sophisticated controls for magnifying an image.

To increase the magnification of the image being displayed

Figure 2-19: Zoom Mode

1. On the **Image** tab, click **Zoom Mode**.
2. With your cursor, click and draw a rectangle around the area that you would like to view at higher magnification.

To return to the previous magnification

Figure 2-20: Undo Zoom

1. Click **Undo Zoom**.
2. Repeat as necessary, to back out to the desired level of magnification.

To view the image at full scale

Figure 2-21: Zoom Full Scale

1. Click **Full Scale**.

Using the Navigation Window

The Navigation window displays a greatly reduced image of the entire scanned area with the current zoomed area bounded by a resizable Navigation region.

To move around the image using the Navigation window

1. Use the Zoom tool to zoom into a region of the image.
2. Hold down the mouse cursor in the Navigation region of the Navigation window, and drag it to the part of the image that you want to see.

To zoom with the Navigation window

Figure 2-22: Navigation Tool

1. On the **Image** tab, click **Navigation Tool**.
2. Place the mouse cursor over a corner of the Navigation region until it turns into a four-way arrow cursor.
3. Drag the cursor to zoom in or out on the image:

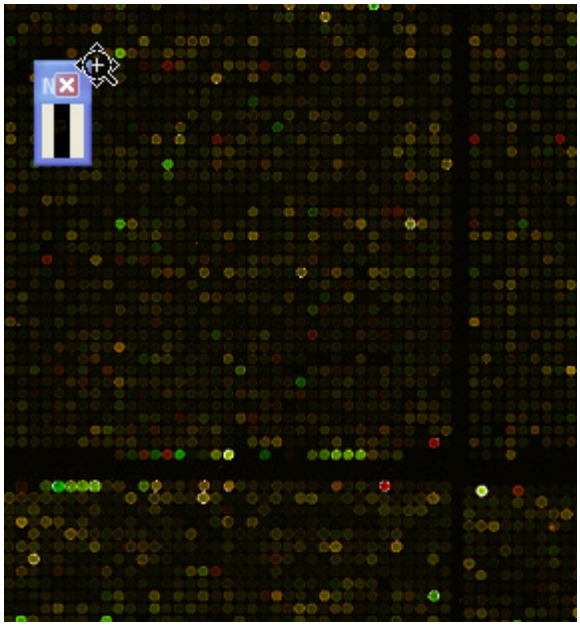


Figure 2-23: Navigation window and cursor

Using the Feature Viewer

The Feature Viewer is located on the Image tab to the left of the main image window. It displays a zoomed view of the image that is currently under the mouse cursor. It also reports important information about each feature, such as the (x, y) coordinates of the cursor, and various ratio and intensity values.

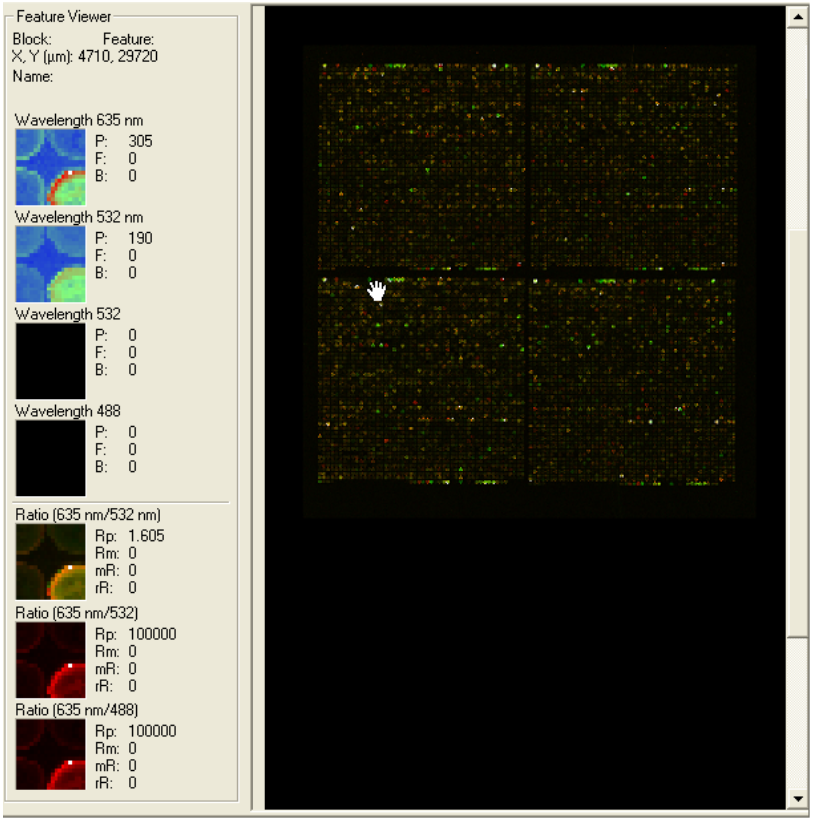


Figure 2-24: Feature Viewer

When you move your cursor over a feature indicator in an array, the Feature Viewer reports the intensity of the pixels in that feature indicator, its background intensity level, its location in the array, and any substance IDs or names defined for the features.

To change the magnification of the Feature Viewer

Figure 2-25: Options

1. Click **Options > Display**.
2. Change the **Field of View** field to the new value.

To hide feature indicators in the Feature Viewer display

1. Click **Options > Display**.
2. Clear the **Show feature indicators** check box, and then click **OK**.

To undock the Feature Viewer in four-color mode

1. Start the Software in four-color mode.
2. Click **Options > Display**.
3. Clear the **Docked** check box, and then click **OK**.

Creating and Using Analysis Arrays

In order to measure the intensity of individual features you must create an analysis array, which defines the dimensions and location of each feature. A detailed discussion of the algorithms used by the array can be found in Analytic Algorithms on page 18.

Working with Spots, Features, Blocks, Scan Areas, and Arrays

Within the Software, each individual spot on the array is called a feature, and is assigned a circular feature indicator. Feature Indicators are the primary unit of analysis in the software. A collection of feature indicators is called a block. There can be several blocks on a single array, depending upon the type and configuration of the arrayer used to place the features on the slide. Scan areas identify the sections of the image you want the software to scan; you can create single or multiple scan areas on each array, as required, with each containing single or multiple blocks of features.

Using a GAL File to Create Blocks

The Software can automatically generate blocks using the information contained in a GAL file. GAL files are used to link the information from the array process to the analysis. They speed the analysis process and add to the data extracted from the array. When a GAL file is imported, the Feature Viewer reports the substance ID or name associated with a feature when you move your cursor over it, and an Analysis operation exports these names to the Results tab together with all other extracted data.

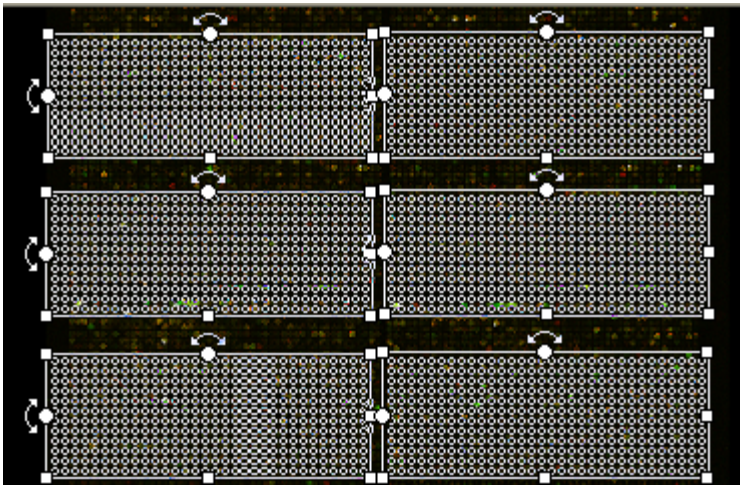


Figure 2-26: Sample array of blocks

To open a GAL file

1. Click **File > Load Array List**.
2. Select the desired file, for example, see **demo.gal**.
3. Click **Open**.

This procedure automatically creates blocks, and assigns substance names and identifiers (if they are in the GAL file) to each spot. To create a new GAL file, see Importing Clone Information on page 59.

Creating blocks manually

Figure 2-27: Block Mode

Figure 2-28: New Block

You can also create blocks manually using the Block mode functions.

To create blocks manually

1. On the **Image** tab, click **Block Mode** and then **New Blocks**.

   The New Blocks dialog box appears.
2. In the **Blocks** group, specify the number of rows and columns you want the array to contain. For example, to create a 4×4 array of blocks, enter *4* for the number of columns and *4* for the number of rows.
3. To determine the position of the blocks, specify the distance between them. If you do not have these distances from your array manufacturer or spotter, you can measure these distances directly from the array image using the X and Y coordinates reported in the Feature Viewer.
4. Specify the layout of the individual feature indicators in each block.

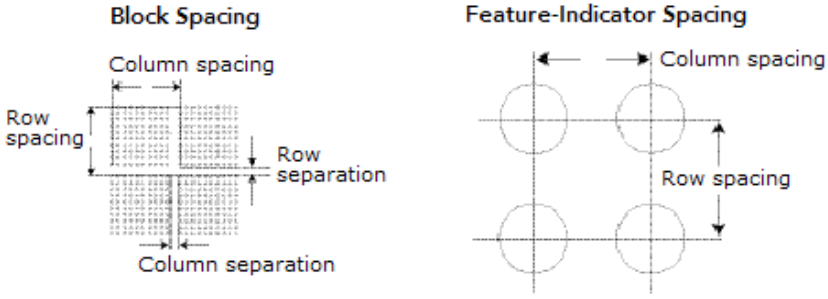


Figure 2-29: Block and feature indicator spacing

Replicating Existing Blocks

Instead of creating a full array of blocks, it is sometimes easier to create one block and then copy it.

To replicate an existing block

Table 2-1: Icon Descriptions

Icon	Description
	Block Mode
	View Blocks
	Replicate Block Mode

1. On the **Image** tab, click **Block Mode** and then **View Blocks**.
2. Select an existing block, or create a new block and then select it.
3. Click **Replicate Block Mode**.
4. Click on the image (for example, click on the top left feature of each block of features).

   A new, identical block is created wherever you click.

Importing Clone Information

Clone information, such as substance IDs and names, is imported using a GAL file, which also creates blocks. You can create GAL files in several different ways. Some arrayer software writes out GAL files with clone information. If you are buying slides from your array manufacturer, they will supply you with a GAL file for the array.

Creating a GAL File from Current Settings

If you create a GAL file in this way, you need to add substance IDs and names manually in a spreadsheet such as Microsoft Excel.

To create a GAL file from your current analysis (block) settings

1. Create blocks as described previously.
2. Click **File > Save Settings As**.
3. From the **Save as type** list, select ***GenePix Array List Files***.
4. Click **Save**.

Creating a GAL File with the Array List Generator

The Array List Generator converts plain text files or ATF files containing substance names and IDs into GAL files. The Array List Generator is specifically designed to make GAL file creation simple. It checks and validates GAL files for you, and it previews the array of blocks defined by your GAL file. From the Array List Generator you can also open the Well Converter, with which you can convert four 96-well plate files into a 384-well plate file.

When the Software is installed, several sample 384-well plate and 96-well plate files are copied to your Program Files\Molecular Devices\GenePix Pro 7\Sample Settings directory so that you can learn how to use the Array List Generator and Well Converter. You might want to open one of the *384 Plate* text files with a text editor such as Notepad to see the file format for yourself—it contains the row and column indices and names for each of the wells on a plate.

To create a GAL file with the Array List Generator from the sample text files

1. Open the Array List Generator window from the Start menu by selecting **Start > All Programs > Molecular Devices > GenePix Pro 7 > Tools > Array List Generator**.

Tip: You can open the Array List Generator window from the **Image** tab by pressing **Alt+G**.

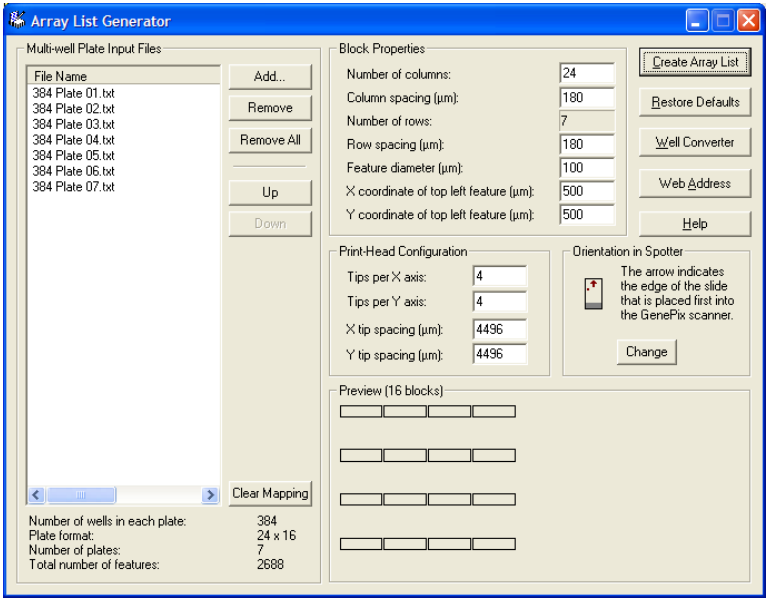


Figure 2-30: Array List generator window

2. Click **Add**.
3. If you have 96-well plate files, click **Well Converter** to open the Well Converter dialog box, where you can convert them into 384-well plate files.

Note: GAL files can also be opened and edited in Microsoft Excel to make minor additions or other modifications.

Aligning Blocks on an Image

The software uses three sets of alignment algorithms: one for finding the entire array of features, one for finding the location of complete blocks of feature indicators on an image, and one for aligning the feature indicators within the blocks with features on the image.

The Find Array function pre-positions the whole array of blocks over the array on the image. Each feature indicator partially overlaps its associated feature (for example, about half a feature indicator diameter overlap); the feature indicator diameter is slightly smaller than the average feature diameter.

The combination of these three procedures is highly robust and reliable, so we recommend using the global command Find Array, Find All Blocks, Align Features, or the hot key <F8>.

If your slide has features with a range of sizes, you can change the maximum and minimum diameters used by the spot-finding algorithm.

To change the diameters used by the spot-finding algorithm

1. Click **Options** > **Alignment tab**.
2. Enter the Min diameter and Max diameter as percentages of the starting diameter of each feature.

Tip: You can disable diameter-resizing altogether during Auto Aligns by clearing the Resize features during alignment check box.

After an automatic alignment is completed, you might need to review block and feature positions and make some manual adjustments. To operate on blocks manually you need to be in Block mode (Hot Key ⟨B⟩).

Creating non-circular Feature Indicators

The Software supports square and irregular feature indicators, as well as the default circular feature indicators.

Note: For features that are almost circular, with only slight imperfections, we recommend that you use the Circular Features option rather than the Irregular Features function, as this algorithm will work better for these features.

To create non-circular Feature Indicators

1. Click **Options>Alignment tab**.
2. Select **Find Square Features** or **Find Irregular Features**.
3. If you are using irregular features, you can choose to fill in any holes in features by selecting the **Fill Irregular Features** check box.

If you use irregular features, after analysis you can use the Circularity column in the Results tab as a quality control measure on your features. Circularity ranges from 0 (non-circular) to 100 (circular). Square features are always given a value of 79 (100 × π/4).

Zooming in on Blocks

While you can use the Zoom Area tool for zooming, the software has several special zooming functions for use with blocks.

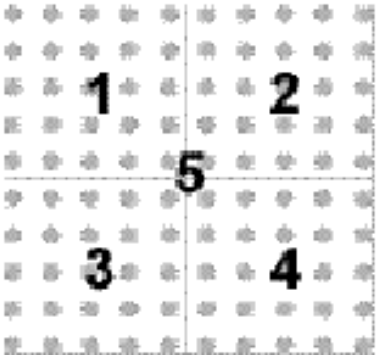


Figure 2-31: Zoom hot keys

To zoom in on blocks

1. Select a single block.
2. On your keyboard, press one of the following numbers to zoom in on a particular area:
   - Press ⟨1⟩ to zoom in on the top left quadrant of the selected block.
   - Press ⟨2⟩ to zoom in on the top right corner.
   - Press ⟨3⟩ to zoom in on the bottom left corner.
   - Press ⟨4⟩ to zoom in on the bottom right corner.
   - Press ⟨5⟩ to zoom in on and center the whole block.
   - Press ⟨>⟩ and ⟨<⟩ to zoom in on the next or previous block.

After you have zoomed in and can see the location of feature indicators and how well they align with features in the image, you can adjust the position of the block by dragging it with your mouse cursor, or using the arrow keys on the keyboard.

Moving and Resizing Blocks

Although the mouse is useful for quick and crude moving and resizing of blocks, for fine positioning it is often more efficient to use the keyboard controls.

To move or resize a block

1. Select a single block.
2. To move the block, use the arrow keys.
3. To resize the block, use ⟨Ctrl⟩+arrow keys.

Rotating Blocks and Arrays

With a block selected, you can rotate it 0.1 degree at a time with the ⟨+⟩ and ⟨–⟩ keys, or open the Block Properties dialog box to set a specific degree of rotation. If you select multiple blocks and rotate one, the rotation is applied to the entire array of selected blocks.

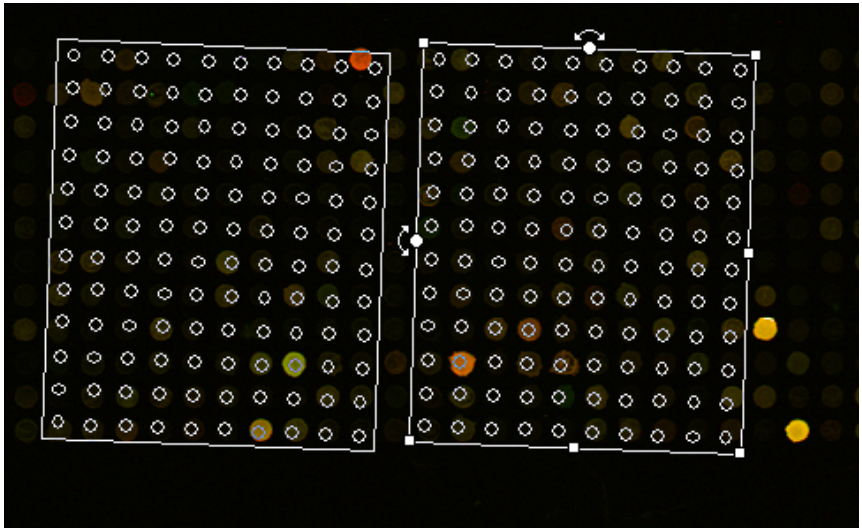


Figure 2-32: Rotating blocks

Manually Moving, Resizing, and Fine-tuning Feature Indicators

After aligning your blocks, you may want to fine-tune some of the feature positions manually. For example, you may need to align manually features that are flagged Not Found by Align Blocks. This is easily accomplished using Feature Mode.

After selecting Feature Mode click on one, or many, feature indicators that you would like to move or resize, and they are highlighted.

Feature mode has two sub-modes, rectangle and lasso, for making multiple selections. Once you are in Feature mode, click the Feature mode button again to display a choice of rectangle or lasso:

- In Rectangle mode, if you hold down the mouse and drag it, every feature in the bounding rectangle of the region is selected.
- In Lasso mode, hold down the mouse and draw an arbitrary closed region to select all the features in the region.

Feature selection also works when the blocks are defined but not displayed. You may find it easier to select irregular regions in lasso mode when the blocks are not displayed, as the image is more visible. Use the arrow keys to move or resize the features (these keys are also listed in the right mouse menu in Feature Mode).

To move or resize features

- Select a feature or features.
- To move the feature(s), use the arrow keys.
- To resize the feature(s), use ⟨Ctrl⟩+arrow keys or ⟨Ctrl⟩+mouse wheel.

If you re-size or move an irregular feature indicator, it is reset to circular. The diameters of feature indicators are reported in the Results spreadsheet; for non-circular features, the diameter reported is the diameter of the circle of best fit. Irregular features also have a measure of circularity reported in the Circularity column, where 100 is circular and 0 is most non-circular. Feature Indicators should fit closely around features. Features that cut the boundary are not included.

Note: Feature Indicators cannot be moved or resized if the settings are Locked (see Linkage on page 73).

Quick Zooms

In Feature mode there are several Quick Zoom Hot Keys:

- ⟨Alt⟩+arrow keys: select previous or next feature
- ⟨[⟩ or ⟨]⟩: move cursor to previous or next selected feature
- ⟨0⟩ (zero): zoom and center current selected feature

If images and Results are linked (see Linkage on page 73), then the ⟨0⟩ Hot Key on the Results or Scatter Plot tab switches the display to the Image tab:

- If a feature is highlighted on the Results tab, pressing ⟨0⟩ zooms to it on the Image tab
- If a feature is under the mouse cursor on the Scatter Plot tab, ⟨0⟩ zooms to it on the Image tab

Flagging Feature Indicators

The Software provides the ability to mark or flag individual feature indicators. For example, you might find that some features should be ignored during your final analysis because of imperfections on the array.

Table 2-2: Icon Descriptions

Icon	Description
	Block Mode
	Feature

To flag a feature on the Image tab

1. On the **Image** tab, click **Feature Mode**, then click the small triangle in the upper right corner of the button to choose either the ***Rectangle*** or ***Lasso*** selection option.
2. On the image, click the feature you want to flag.
3. Click **Feature**.
4. Select a flag to apply to the feature: ***Good, Bad, Not Found,*** or a ***User Defined Flag*** you have created.

The Software also flags features automatically:

- If Align Blocks fails to find a feature, the feature is flagged Not Found.
- If there are blank entries in the active GAL file, the corresponding features are flagged Absent.

All flags are stored in the final Results table and are exported with numerical equivalents:

- Good is given the value 100.
- Bad is given the value –100.
- Not Found is –50.
- Absent is –75.
- Unflagged is 0.

See Defining Your Own Flagging Criteria on page 76 for advanced feature-flagging techniques.

Note: Feature Flags cannot be removed or added if settings are Locked (see Linkage on page 73).

Creating Custom Flags

Figure 2-33: Feature

You can also create custom User Defined flags to track traits of particular importance to your analyses.

For user defined flags, a *Good* valuation starts at 20 and increments by one for each flag (that is, 20, 21, 21, and so on); a *Bad* valuation starts at -20 and decrements by one for each flag (that is, -20, -21, -22, and so on).

To create your own flags

1. On the **Image** tab, click **Feature>Configure User Defined Flags**.

Figure 2-34: Configure User Defined Fields Screen

2. Click **New**.

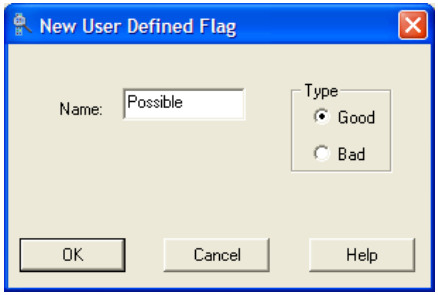


Figure 2-35: New User Defined Flag Screen

3. In the **Name** field, type a unique descriptive name for the flag.
4. In the **Type** group, select ***Good*** or ***Bad*** to typify the flag.
5. Click **OK**.

   The new flag appears in the Configure User Defined Flags list.
6. Click **New** to create another flag, or click **OK** to save your flags and exit the dialog box.

Resetting Flags and Positions

Table 2-3: Icon Descriptions

Icon	Description
	Feature Mode
	Feature

To remove the flags or nudges from feature indicators

1. On the **Image** tab, click **Feature mode**.
2. Select the features to reset by clicking them or by dragging a region around them.
3. Click **Feature**, and then click the desired option:
   - *Clear Nudges*
   - *Clear Flags*
   - *Clear Flags Except Absent*

Saving the Positions of Blocks and Feature Indicators

After you have finished aligning your analysis array, it is important to save its configuration.

To save the position of blocks and feature indicators

1. Click **File > Save Settings As**.
2. Enter a file name for the settings and click **OK**.

When you save the settings, you not only save the position of the array, but you also save any acquisition settings you may have made such as the size and position of the scan region, and any display settings such as contrast and brightness.

Locking Settings

After you have aligned the feature indicators and applied any quality flags, you may want to lock your settings to prevent you from accidentally changing them after the results have been calculated (see Linkage on page 73).

To lock settings manually

Figure 2-36: Lock Settings

- On the Image tab, click **Lock Settings**.

When settings are locked, you cannot move blocks or feature indicators, and several other commands (such as Align Blocks and Align Images) are disabled.

You can set the locking default to lock settings automatically after an Analyze command.

To lock settings automatically

1. Click **Options**.
2. Click the **Workflow** tab.
3. Select the **Lock Settings After Analysis** check box.

You can manually lock and unlock the settings any time by clicking the Lock Settings button. Any change you make to your feature settings breaks their linkage with downstream results. To re-establish linkage, analyze again.

Customizing Background Subtraction

The Software offers the choice of several background subtraction methods. By default, the software uses a local background subtraction method (see Background Intensities on page 18). This is the recommended method, as it much more responsive to background intensity variations across a slide. However, the Software does offer global methods, in which a single background value is used for each channel, for the whole slide.

To change the background subtraction method

1. Click **Options**.
2. Click the **Analysis** tab.
3. In the **Background Subtraction** group, click **Select**.
4. Select a new background subtraction method.

Performing the Analysis and Viewing the Results

Figure 2-37: Analyze

After aligning the analysis array, perform the analysis by clicking the Analyze button (or by clicking Analyze All to process multiple scan areas at once). The analysis extracts data from the single-wavelength images in each feature indicator, and the results and subsequent computations are sent to the Results tab spreadsheet.

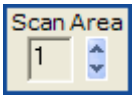
After the analysis a JPEG file of the image is saved and used in the Feature Viewer in the Results and Scatter Plot tabs. The image is saved with the brightness, contrast and color settings at the time of the analysis.

To save a JPEG to be used in the Feature Viewer with saved Results

1. Click **File>Save Results—Active Scan Area** or **File > Save Results—Active Scan Area**, as desired.
2. Select the **Save as JPEG Image** check box.
3. Click **Save**.

Analysis Results for Multiple Scan Areas

Table 2-4: Icon Descriptions

Icon	Description
	Analyze All
	Scan Area Spinner

The Software creates a separate set of results for each scan area in turn. When you create multiple scan areas, you can select and analyze each one separately, or click Analyze All to process them all at once.

To view the results for any of a series of multiple scan areas, use the Scan Area spin box tool to select the number of the scan area you want to see.

Using the Results Spreadsheet

In addition to the basic raw data and derived ratio measurements, the Results spreadsheet shows the location of each feature indicator, substance information from the GAL file (such as substance names and ID), and any flags that were on the image before the analysis.

To sort the data in the spreadsheet, click on a column title. Click the same column title again to sort the data in the reverse order.

To group several discontinuous rows together

1. Select the rows by holding down the <Ctrl> key and clicking the rows.
2. Click **Group Rows**.

To reorder columns

1. Click on a column title and drag it to a new position in the spreadsheet.

   OR

   In the **Table** group on the left-hand side of the **Results** tab, click **Data Types**.
2. You can customize two different views of the spreadsheet. Select ***Custom 1*** or ***Custom 2***, then select the Data Types you want in that view.
3. Reorder columns by clicking the up and down arrows. For accelerated movement, hold down the button.
4. Show and hide columns by moving them between the Available Data Types and Visible Data Types fields with the horizontal arrows. Note that grayed-out column names have no data in the active Results table, and are not displayed in the Results spreadsheet until they do have data.
5. To display the default of all applicable columns, click **Restore to Default**.

To show and hide rows based on their flags

1. In the **Table** group on the left-hand side of the **Results** tab, click **Display**.
2. Select the flag types to display. For example, to show all features included in normalization, clear all the boxes in the Excluded column.

Note that settings in the Display dialog box do not change the underlying data set. A GPR file always contains the entire data set.

To save results with a customized set of Data Types

- On the **File** menu, choose **Export Results**.

Linkage

After an analysis, your images, settings, and Results are linked, shown by the message *Linked* in the Status Bar. Linkage is the state of having the current Results match the current image and settings; that is, if you re-analyze, you get exactly the same results. When linked, you can flag or select in any feature-viewing tab and the others are updated accordingly. Locking prevents your data from becoming unlinked.

To flag a feature on the Image, Results, and Scatter Plot tabs simultaneously

1. Check that *Linked* appears in the Status Bar.
2. Select features on the Image tab, Results tab or Scatter Plot tab.
3. Select a flag command from the right mouse menu, or from the Feature menu.

To navigate from the Results tab to the same feature on the Image tab

1. Check that *Linked* appears in the Status Bar.
2. Click on the row of interest in the Results tab.
3. Press the ⟨0⟩ Hot Key.

Results and images that are saved when linked can be opened together by opening the Results file. The software automatically locates and opens the corresponding image files.

To open linked images and results

1. Click **File>Open Results**.
2. Select the **Open Associated Images** and **Recreate Analysis Settings** check boxes.
3. Click **Open**.

If images and settings are opened and linked successfully, the Status Bar message reads *Linked*.

Web Interactivity

The software is closely integrated with the Web.

To find a substance ID or name on a web database

1. Open a GAL file or settings file containing substance names and IDs.
2. Click **Options**.
3. Click the **Analysis** tab.
4. Select the address of a Web database from the Web address list, or click Manage to enter a new Web address.
5. Click **OK**.
6. Select a feature in the Image tab, Results tab, or Scatter Plot tab.
7. Click the right mouse menu and click **Go To Web** to open the database in your default Web browser.

Normalization

The Software includes full normalization of images and data (see Data Normalization on page 27). If images and Results are linked, then both images and Results will be normalized; if they are not linked, only the Results will be normalized. When linked, the Status Bar reads *Linked*.

Before you normalize, you must decide to normalize on control features only, or on all features, or you can choose to apply your own normalization factors to the images.

To configure normalize on all features

This lets you normalize on all features except those flagged Bad, Absent, and Not Found.

1. Click **Configure** in the Normalization group on the left-hand side of the Results tab.
2. Click **Ratio-based**.
3. From the mean of the list box, select a ratio type. Typically, this is the main ratio value that you use for all your analyses.
4. Select all of the features from the second list box.
5. In the **is equal to** field, type a ratio value. For example, by normalizing on all features, you are assuming that the mean ratio of all features on the array is equal to 1, so you would enter 1 in this field.

To normalize on selected features only

1. Flag features for normalization with an Include in Normalization flag. See Defining Your Own Flagging Criteria on page 76.
2. In the **Normalization** group on the left-hand side of the **Results** tab, click **Configure**.
3. Click **Ratio-based**.
4. In the **mean of the** drop-down list, select a ratio type. Typically, this is the main ratio value that you use for all your analyses.
5. In the **of** drop-down list, select the desired normalization features.
6. In the **is equal to** field, type a ratio value. The value here depends on the expected behavior of your control features. For example, if you expect your control features to have a ratio of 0.8, enter 0.8.

To configure user-defined normalization factors

1. In the **Normalization** group on the left-hand side of the **Results** tab, click **Configure**.
2. Click **User-defined**.
3. Type your own normalization factor for each image.

To apply normalization to your results and images

- On the **Configure** tab, click **Normalize**.

  OR

  On the **Configure** tab, click **OK** to exit from the Configure tab (this accepts your normalization configuration but does not apply it) and then, in the **Normalization** group on the left-hand side of the **Results** tab, click **Apply**.

To remove normalization

- In the Normalization group on the left-hand side of the Results tab,click **Remove**.

If you normalize your results and then save, your normalized results are saved. If you intend to do further analyses on your results (for example, removing normalization and trying out different normalization methods), we recommend that you save your results unnormalized.

After normalization, the status bar reads *(Un)Linked—Normalized*.

Defining Your Own Flagging Criteria

The Flag Features dialog box is an extremely powerful tool for flagging features by constructing general Boolean queries of Results tab data. You can type or insert VBScript expressions in the Query field and have them evaluated in the Results table. You can also save commonly used queries, such as custom quality control or normalization conditions that you apply to every array.

A query typically consists of several conditions joined with the logical connectives AND or OR. A row satisfies a query consisting of two conditions joined by an AND if it satisfies both conditions independently; it satisfies a query consisting of two conditions joined by an OR if it satisfies at least one condition.

To construct a single condition

Construct a condition, for example [Ratio of Medians (635/532)] > 1.5.

1. In the Normalization group on the left-hand side of the Results tab, click **Flag Features**.
2. To begin a new query, click **New**.
3. From the **Column** list, select **Ratio of Medians (635/532)**.
4. From the **Operator** list, select **>.**
5. In the **Value** field, type **1.5**.
6. Click **Add** to add the condition to the current query in the Query field.
7. Click **Evaluate** to find the number of rows to which the condition applies.
8. Click **Save As** to save the query.

To build this expression into a more complex query

You can then develop a more complex query, for example, to add the following condition: [Ratio of Medians (635/532)] < 0.66

1. Click **Or**.
2. From the **Column** list, select **Ratio of Medians (635/532).**
3. From the **Operator** list, select **<**.
4. In the **Value** field, type **0.66**.
5. Click **Add** to add the condition to the current query in the Query field.
6. Click **Evaluate** to find the number of rows to which the condition applies.

7. Click **Save** to save the query. The full expression now looks like:

   [Ratio of Medians (635/532)] > 1.5 OR
   [Ratio of Medians (635/532)] < 0.6

Features are flagged on exiting the dialog box by clicking the **OK** button. You can also choose to highlight the rows in the Results tab.

To flag features in the Results tab which satisfy the query

1. In the **Action** group, select the **Flag** check box.
2. Select the type of flag to apply.
3. Click **OK**.

To highlight rows in the Results tab which satisfy the query

1. In the **Action** group, select the **Highlight Data** check box.
2. Select whether to **Replace** the current highlighted rows, or to **Add** to the current highlighted rows.
3. Click **OK**.

Using Flag Features for Complex Quality Control Queries

You can use the Flag Features dialog box to construct complex quality control conditions on your features.

First, you need to decide on a set of quality control criteria. For example, you might choose to exclude (flag as Bad) any feature that satisfies the following criteria:

- The feature intensity is near background
- The feature is not uniform
- The background is not uniform

You need to be able to translate these conditions into symbolic expressions. The first condition can be expressed as:

[% > B635+2SD] < 70 OR [% > B532+2SD] < 70

This condition picks up features that do not have at least 70% of their feature pixels more than two standard deviations above background in either the green or the red channel. You may want to experiment with the figure of 70% to find a figure that is diagnostic for your arrays.

The second condition is more complex. One way of measuring the non-uniformity of the feature is to compare any of the five ratio types reported by the software, for example, the ratio of medians and the regression ratio. It can be expressed as:

[Ratio of Medians (635/532)] > (1.2*[Rgn Ratio (635/532)]) OR

[Ratio of Medians (635/532)] < (0.8*[Rgn Ratio (635/532)])

This condition picks up features that have a ratio of medians greater than 1.2 or less than 0.8 times the regression ratio. Again, you may want to experiment with these values of 1.2 and 0.8.

Finally, to select features with a variable background, compare the mean and the median background levels:

([B635 Mean] > (1.2*[B635 Median]) OR

([B532 Mean] > (1.2*[B532 Median]) AND

([B635 Median] > 40 OR [B532 Median]) > 40)

This condition selects features that have a mean background greater than 1.2 times the median background at either wavelength. It also includes a lower bound on the absolute value of the background, as variation in a very low background level is not as significant as variation when the background is higher. The condition picks out features that have bright pixels or smears in their backgrounds, or features with irregular shapes that do not fit the circular feature indicators.

Because a feature that fails any of these criteria is deemed to have failed overall, join each of the conditions by an OR to construct a single query:

[% > B635+2SD] < 70 OR [% > B532+2SD] < 70 OR

[Ratio of Medians (635/532)] > (1.2*[Rgn Ratio (635/532)]) OR

[Ratio of Medians (635/532)] < (0.8*[Rgn Ratio (635/532)]) OR

(([B635 Mean] > (1.2*[B635 Median]) OR

[B532 Mean] > (1.2*[B532 Median])) AND

([B635 Median] > 40 OR [B532 Median] > 40))

You can continue to add conditions to this query. For example, you may want to add checks for absolute feature intensity (that is, features are flagged Bad if the Sum of Medians is not above a certain intensity) or absolute background intensity (that is, features are flagged Bad if their backgrounds are above a certain intensity).

Saving and Exporting Data

When the analysis is complete, save your results. You can save the data for just the selected or active scan area, or you can choose to save the data for all existing scan areas on the slide at one time.

To save in the default GPR file format

1. Click **File>Save Results—Active Scan Area** or **File>Save Results—All Scan Areas**, as desired.
2. Type a file name.
3. From the group of **Naming** options, select the boxes to add prefixes and suffixes to the name.
4. Click **OK**.

To ensure consistency among data to be stored in a database, data saved in the GPR format always contain the full set of columns, regardless of the current column display, or the color mode.

To export only the current view of the Results

This exports only the columns in your customized view.

1. Click **File>Export Results**.
2. Type a file name.
3. From the group of **Naming** options, select the boxes to add prefixes and suffixes to the name.
4. Click **OK**.

Note that exported results are saved as plain text files (*.txt) and cannot be opened in the GenePix Pro software.

To export only selected rows from the Results tab

1. Select the rows that you want to export. Select contiguous groups of rows by holding down the <Shift> key when selecting; select discontiguous rows by holding down the <Ctrl> key when selecting. To select all rows, use the <Ctrl+A> Hot Key.
2. Click **Copy**.

If the buttons for exporting the data are disabled, this is because you currently do not have a protection key attached to your computer.

Viewing Scatter Plots

Use the Scatter Plot tab to generate scatter plots of any two analysis data types for all spots on the array, or to draw a histogram of any one analysis data type.

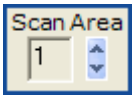


Figure 2-38: Scan Area Spinner

To view the scatter plots for any of a series of multiple scan areas, use the Scan Area spin box tool to select the number of the scan area you want to see.

To choose quantities plotted on each axis

1. Do an analysis or open a Results file.
2. Click the **Scatter Plot** tab.
3. From the X Axis group on the left-hand side of the Scatter Plot tab, choose a quantity to plot along the X Axis; for example, Sum of Medians.
4. From the Y Axis group on the left-hand side of the Scatter Plot tab, choose a quantity to plot along the Y Axis; for example, Log Ratio.
5. You can also specify the Scatter Plot axes directly from the Results tab by right-clicking on any column and choosing Set this data type as Scatter Plot X- (or Y-) axis.
6. Position the mouse over any point on the Scatter Plot to display the associated feature in the Feature Viewer.

The Scatter Plot and the Results spreadsheet are fully integrated—select several features on one tab and when you switch to the other tab they are highlighted. If images and Results are linked (see Linkage on page 73), then selecting or highlighting features on the Scatter Plot tab selects or highlights them on all tabs.

Some useful ways to display data:

- ***F1 Median vs. F2 Median:*** the diagonal through the origin separates features with a higher activity than the reference from features with a lower activity than the reference. If the data cloud lies significantly off the 45-degree diagonal, it may indicate a significant difference in channel intensity that should be normalized.
- ***Index vs. Log Ratio:*** the chosen ratio is displayed as greater or less than 1.0 for all spots. Differentially regulated features are more easily identified than on a standard intensity comparison plot.
- ***Sum of Medians (Log Axis) vs. Log Ratio:*** highlights low intensity spots with artificially high ratios. If the denominator of a ratio is very low, the resulting ratio value may be artificially high. You can use this plot to identify such false outliers.

To flag features on the Scatter Plot

1. Click and drag the cursor to select a group of data points.
2. From the right mouse menu, or from the Feature menu, select a Flag command.

To show or hide flagged features

1. In the **Display** group on the left-hand side of the **Scatter Plot**, click **Display**.
2. Check the types of flagged features to display.

To show or hide analysis lines (regression line, k-standard error lines)

- Select the **Analysis Lines** check box.

To configure analysis lines

1. In the **Display** group on the left-hand side of the **Scatter Plot**, click **Configure**.
2. Check which lines to display from: Regression Line; k-standard deviation lines.

To draw a histogram

1. In the **Visualization** list on the **Scatter Plot** tab, click **Histogram**.
2. Select a data type from the X Axis list.

Report Tab and Scripting

The Report tab is an embedded Internet Explorer window, so it has all the scripting functionality of that program. Scripts can be written in VBScript, JavaScript, or any other scripting language supported by Internet Explorer (such as PerlScript or Python).

The Report tab includes ready-to-use Reports for scanner performance validation and analysis. It enables full scripting of all acquisition and analysis functions. To demonstrate some of the functionality and power of the Report tab, several example Reports are installed with the program. These are always available from the Report tab by clicking the Home button.

To run a report

1. Click the **Report** tab.
2. Click the Report that you want to run and follow its instructions.

The Analysis Reports are ready to use for advanced data analysis The Hardware Diagnostic Reports include tools for scanner performance validation. The Example Reports demonstrate how to use some properties and methods of the GenePix Pro Object Model. Any of these can be customized by clicking the Edit button on the left-hand side of the Report tab.

The online help (press <F1>) includes an extensive scripting tutorial in which the GenePix Pro Object Model is documented.

Special Functions

This section describes other special features you might use regularly.

Using the Measuring Tools

When you want to measure pixel values in an arbitrary line or area on an image, use the Measuring Tools. With these tools, you can create your own data measurement regions, including line profiles, multiple line-segment profiles, rectangular averaged lines, and rectangular, elliptical, and polygonal areas.

Figure 2-39: Measuring Tools

To create a measuring region

1. On the **Image** tab, click **Measuring Tools**.
2. A menu of options appears.
3. Select the tool you want from the menu.
4. Using your cursor as a drawing tool, create a measuring region on the image.

The use of each tool is fully explained in the Help system. You can create multiple measuring regions on one image, and move and resize them like blocks and features.

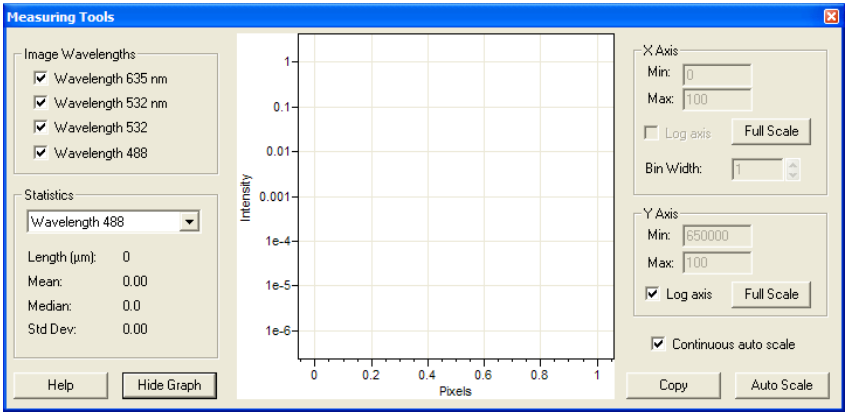
The information about the selected region is automatically displayed in the Measuring Tools Properties window, including a graph of pixel intensity data for each wavelength.

To hide or show the Measuring Tools Properties window

Figure 2-40: View Measuring Tools

- On the **Image** tab, click **View Measuring Tools**.

  The Measuring Tools dialog box appears:



Viewing Pixel Plots

Use the Pixel Plot to evaluate individual features in more detail.

To view a Pixel Plot

1. On the **Image** tab, open an image and place blocks on the image.
2. Position your cursor over a feature indicator.
3. Press the **<P> Hot Key** or, in Feature mode, right-click and select **Pixel Plot**.

   The Pixel Plot graphs the distribution of the pixels within the selected feature indicator.

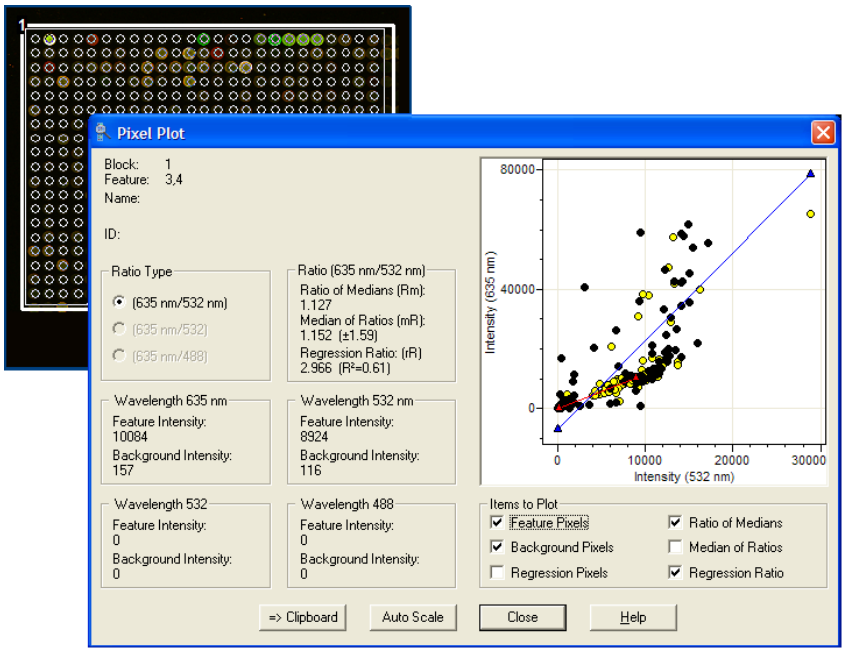


Figure 2-41: Pixel Plot

To see the distribution of feature pixels and background pixels for the feature

- In the **Items to Plot** group, select the **Feature Pixels** and **Background Pixels** check boxes, and clear the **Regression Pixels** check box.

  The pixels are plotted as points on the graph, with background pixels black and feature pixels yellow.

To see the difference between the Ratio of Medians and the Regression ratio for the feature

- In the **Items to Plot** group, select the **Ratio of Medians** and **Regression Ratio** check boxes, and clear the **Median of Ratios** check box.

  The ratios are plotted as lines through the points, with the Ratio of Medians red and the Regression Ratio blue.

  The Pixel Plot also shows derived ratio measurements, so you can see the relationship between ratio and pixel values.

Viewing Histograms

The Histogram displays the distribution of pixels for each raw wavelength image. The display color-codes each distribution according to the ratio colors used to create the ratio image; that is, if the first channel is displayed in red on the Image tab, its trace on the Histogram is drawn in red.

The Histogram is derived from the current view of the image in the Image tab, so if you have zoomed in on part of an image, only those pixels currently displayed are used to construct the Histogram.

1. To see the Histogram for an entire image
2. Click the **Image** tab.
3. If you are zoomed in to a part of the image, click **Zoom Full Scale** to see the whole image.
4. Click **Zoom Mode**.
5. Draw a zoom area tightly around the features on the image to exclude as much background as possible.
6. Click the **Histogram** tab.

To use the Histogram to see how many pixels are saturating

1. In the **X Axis** group on the left-hand side of the **Histogram** tab, click **Full Scale**.
2. Zoom in on the far right edge of the histogram, by holding down the mouse cursor and dragging it along the X axis between 60000 and 65000. The proportion of saturated pixels is plotted at 65535.

Performing a Batch Scan

Note: This procedure applies only if you are using a GenePix scanner with a GenePix SL50 Slide Loader.

If you are connected to a GenePix SL50 Slide Loader, or if you are using Analysis Only mode with a GenePix SL50 Slide Loader, the Batch Scan tab is visible. Use the Batch Scan tab to perform a batch scan or a manual scan.

In Batch Scan mode, the scanner scans all selected slides in the slide loader, without any user intervention.

On the Batch Scan tab, you can customize various settings.

- For each slide in the slide carrier, you can specify hardware (scanner) settings and analysis settings.
- For the entire batch scan, you can specify file naming and saving options, as well as workflow options.

While scanning, the positions in the GenePix Pro slide carrier map change color, depending on their scan status:

- Light-blue outline: Scan is in progress.
- Green: Scan was completed successfully.
- Red: Slide was not scanned because of a slide handling failure or scan cancellation.

In Manual scan mode, the GenePix SL50 Slide Loader operates very much like a manual GenePix scanner. You can open and close the motorized scanner door, perform Preview Scans, Data Scans, adjust scanner settings from the Hardware Settings dialog box, and stop and start scans.

Any Global file options (Save locations, File name) set during a batch scan apply when performing a manual scan.

Follow the instructions carefully when inserting a slide in the scanner slide holder. The slide holder is a precision component designed to ensure proper focusing and field uniformity. Improper handling may damage the slide holder and affect imaging performance. In Manual scan mode, the GenePix SL50 Slide Loader is inactive and will not move.

To insert a slide in the scanner

1. In the GenePix Pro Software, click the **Open-Close Scanner Door** icon to raise the scanner door.

   The scanner slide holder moves forward into the load position.

CAUTION! Never touch the slide holder while it is moving. Never force the slide holder closed, or apply significant pressure to it.

2. With the slide held between your thumb and finger, carefully place the slide, feature-side down, into the slide holder.

Note: Make sure the barcode on the slide is facing the front of the slide scanner.

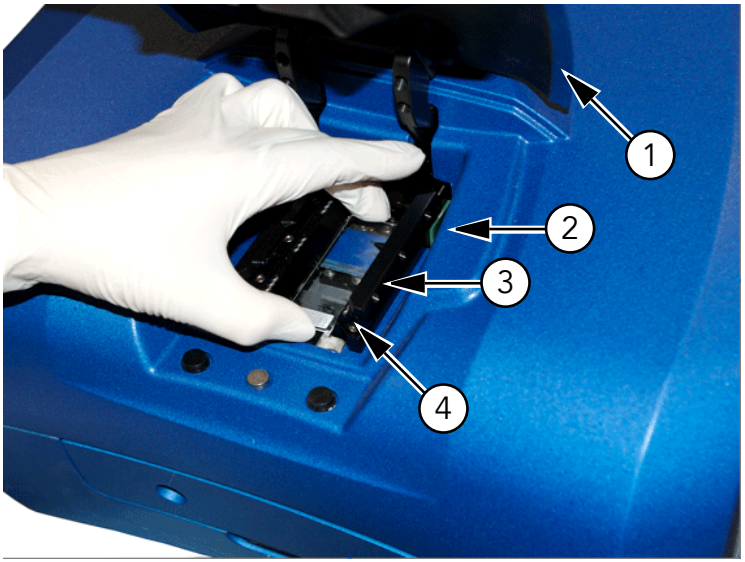


Figure 2-42: Scanner slide holder

Item	Name	Description
1	Scanner door	In raised position
2	Slide holder	In load position
3	Slide	Place feature-side down
4	Barcode	Barcode is facing the front of the slide scanner

3. Click the **Open-Close Scanner Door** icon to close the scanner door.

   The door closes and the slide holder moves into the scan position.

To perform a manual scan

1. On the **Batch Scan** tab, in the **Scan Type** group, click **Select**.
2. The Scan Type dialog box appears.
3. Select **Manual** and click **OK**.
4. Insert a slide in the scanner. See To insert a slide in the scanner on page 87.
5. Click the **Hardware Settings** icon on the right-hand side of the GenePix Pro window.
6. In the **Hardware Settings** dialog box, set the filter and PMT parameters.
7. Click the **Preview Scan** icon to scan the slide and display general data for the slide.
8. If you want to scan a smaller region, use the **Scan Region** tool to select a region, then click the **Data Scan** icon.
9. When you are finished scanning, click the **Open-Close Scanner Door** icon.
10. Remove the slide from the slide holder.

To load the slide cassette

1. Use the handle on the right side of the slide loader tower to slide open the slide enclosure door.

Figure 2-43: Slide enclosure door handle

2. Using the handle on top of the slide cassette, lift the left slide cassette out and place it on a stable position on the counter.

Figure 2-44: Slide enclosure door open with both slide cassettes visible

3. With each slide held between your thumb and finger, carefully place slides, feature-side down and barcode facing the front, into empty slots in the slide cassette.

Note: The motion arm uses a sensor to locate and map slides in the cassette slots before beginning the batch scan.

4. When you have placed all slides for scanning into the slide cassette, return the cassette to the slide enclosure by inserting it at a slight angle tilted away from you, then straighten it until it fits securely in the slide enclosure.

   You will hear a click when the cassette is in place.

5. If additional slots are required, perform Step 2 through Step 4 with the right slide cassette.

CAUTION! Do not access the slides in the cassette through the belt opening.

6. When all slides are loaded into the slide racks and the racks are returned to the slide enclosure, use the handle on the slide loader tower to slide both racks back into the slide enclosure. Both slide racks must be in the slide enclosure for the GenePix Pro software to recognize that the slide enclosure is completely shut.

   You will hear a click when the slide enclosure is completely shut. In Batch Scan mode, the slide cassettes are detected and their availability is displayed in the Slide Map dialog.

To perform a batch scan

1. On the **Batch Scan** tab, in the **Scan Type** group, make sure that **Batch** is the selected scan type.
   If **Manual** is the selected scan type, click **Select**, select **Batch,** remove any slides on the scanner's slide holder, and click **OK**.

Note: For optimum performance, allow the GenePix 4300A/4400A Scanner to warm up for 15 minutes before scanning slides with the GenePix Pro Software.

   Before scanning, the slide positions are indicated in different colors, depending on their scan status and the current GenePix SL50 Slide Loader settings:

   - Light blue: A slide is present and will be scanned.
   - Grey: A slide is present but has not been selected to be scanned.
   - White: No slide is present.

   By default, all slides present in a batch are scanned.

2. Click the **Hardware Settings** icon on the right-hand side of the GenePix Pro window.
3. In the **Hardware Settings** dialog box, select the laser and filter settings for all slides. To override these hardware settings for individual slides, ensure that you select specific hardware settings for selected sub-batches of slides in Step 8.

4. In the **global file options** group, click **Save Locations [...]** to open the **Options > Folders** dialog box and set the directories in which to save various file types.
5. In the **global file options** group, click **File name [...]** to open the **Options > File Save** dialog box and set the naming conventions for files saved during batch scans.
6. If you want to save all settings on the Batch Scan tab to re-use in another scan or if you want to open a previously saved settings file, use the **Loader Settings** group and select one of the following options:
   - Click **New** to save the batch scan configuration in a loader settings file (*.gpa).
   - Click **Open** to open a previously saved loader settings file (*.gpa).
7. Slide enclosure door open with both slide cassettes visible. If you want to scan a slide multiple times (for example, at different PMT settings or using different scan regions), do the following:
   - Select the slide in the Batch Scan list.
   - Use the arrows in the **Scan # times** field to set the number of times to scan the slide.

   Based on the number of times selected, the scan is copied in the Batch Scan list (for example 1a, 1b) and each instance of a scan gets its own entry. You can assign scan settings to each entry independently.
8. If you want to select a sub-batch of slides for scanning, use Ctrl-click and Shift-click to select multiple slides in the Batch Scan table, and then select the following settings for the sub-batch scan in the **For the selected sub-batch of slides** group.
   - Click the **Scans** drop-down list to select the type of scan to perform on the selected sub-batch of slides.
   - Type a **File name body** to save the selected sub-batch of slides with a specific file name.
   - Choose the **Hardware Settings** for the selected sub-batch of slides either by clicking **Current settings** or by clicking **GPS file** and using the **[...]** button to navigate to a specific GenePix Settings (GPS) file.
   - For each slide in the selected sub-batch, choose the **Auto PMT** gain either by clicking **Selected hardware settings** to use the current hardware settings or by clicking **GAL file** and using the **[...]** button to override the specified settings and navigate to a specific GenePix Array List (GAL) settings file.

9. If you have multiple sub-batches, repeat Step 8 for any other sub-batches selected.

   To copy and paste slide settings from one slide to another, select a row and use the Copy Row and Paste Row buttons at the bottom of the Batch Scan tab.

10. In the **After batch scan** group, select what you want to happen when the batch scan is complete:
    - If you want to browse the scanned images without analyzing them, use the Browse tool on the Batch Analysis tab.
    - If you want to run a batch analysis after the batch scan is complete, check **Add image files and analysis settings files to Batch Analysis tab** and type a name for the file, and then click **Run batch analysis**.
11. If you want to save the current configuration of the Batch Scan tab settings with a new file name, click **Save loader settings [...]** and enter a new file name.
12. Click **Scan Batch** to perform a batch scan using the settings configured on the Batch Scan tab.

Note: Do not disturb the scanner or slide loader during the batch scan. Depending on the resolution and the number of wavelengths or channels you select for scanning, a single scan can take from 4 to 35 minutes to complete.

When the batch scan is complete, a message appears in the GenePix Pro Software.

Stopping and Restarting a Batch Scan

You can stop a batch scan at any time during the scan. You can then choose to continue the scan or to cancel it.

To stop the current scan

- In the GenePix Pro Software, click the **Stop Scan** icon.

  The SL50 Slide Loader completes its current task and then stops the scan.

To restart a batch scan

- In the GenePix Pro Software, after the current scan has been stopped, choose one of the following options:
  - To continue scanning the batch from the current position in the slide carrier, click **Continue**.
  - To cancel the batch or to restart a batch from a position that has already been scanned, click **Stop**.

Reviewing the Batch Scan Log File

The GenePix Pro Software saves a text log file for each scanned batch of slides. If a batch scan completes successfully, there is no need to see the log file. If there is a problem with the scan, the log file can contain diagnostic information.

The default location of the log file is in the ...\Documents\Molecular Devices\GenePix Pro\Data\ folder. You can change the location of the log file.

To open the batch scan log file

- In the GenePix Pro Software, select **File > Open Batch Scan Log**.

To select a location for the batch scan log file

1. On the **Batch Analysis** tab, select **Use folder options**.
2. In the **Options** dialog box, click the **Folders** tab.
3. On the **Folders** tab, click **Specified folder for individual file types**.
4. In the **Slide Loader Log** field, click **Browse** to navigate to a location to store the batch scan log file.

Running a Batch Analysis

Use the Batch Analysis tab to do spot finding and analysis on batches of images.

1. Select images and GAL/GPS files to process

The first step in setting up a Batch Analysis is to select the images to process.

Add files to the list

Click Add to select images and a GAL or GPS file for the batch. You can use a single GAL/GPS file for the whole batch, or different files:

- To use a single GAL/GPS file, select a batch of images and only one GAL/GPS file in the Add dialog.
- To use different GAL/GPS files for different images, select the images with the Add command, and then select each image in turn from the Batch Analysis list, and use Select GAL or Select GPS to apply a GAL/GPS to that file only.

When a GAL/GPS file is selected, it is listed in the Initial GAL Or GPS column.

File names of generated files

When images are added to the Batch Analysis list, the GPS and GPR file names of the files that will be generated from the Batch Analysis are listed in the Aligned Settings (GPS) and Results (GPR) columns.

Batch Analysis never overwrites existing GPS or GPR files; generated file names are always unique in the directory in which they are saved.

Generated files location

GPS and GPR files generated by Batch Analysis are by default saved in the same directory as the images.

However, you can also specify a different directory. To do this, select Use folder options and then configure the directory using **Options > Folders**.

Single-image TIFF files

If you have pairs of single-image TIFF files, then you need specify how they are to be paired, typically by a file name suffix. The software recognizes a number of common file name suffixes (for example, "_635" and "_532") or you can add your own in Options> Batch Analysis.

Align and Analyze

You can align features in a batch in one of two different ways, depending on how you want to review the results:

- Click **Process Next** to align one image at a time, and then review the results at the end of each image alignment.
- Click **Align All** to align all images, and then review all images.

When the alignment is finished, whether it is one array or a whole batch, use the Batch Review dialog box to review the results:

- If you are aligning one image at a time, you can inspect the generated settings on the Image tab, edit them as required, save settings, and then click **Align Next**.
- If you have aligned a whole batch, you can step through each image in the batch by clicking **Open Next**.

Find Array (Recommended)

From the Batch Analysis tab, alignment is always Find Array, Find All Blocks, Align Features <F8>.

The only known case in which <F8> may possibly fail is when an image has a bright barcode in which the dimmest part of the barcode (generally the bars) is brighter than the brightest part of the image. In such a case, the Find Array algorithm may find the barcode instead of the features on the image. In such a case you should skip the Find Array part of the algorithm. For the rest of the spot finding to proceed successfully, the starting position of the blocks should overlap the blocks on the image.

Analyze

Select the Analyze option to analyze images and generate GPR files after the spot finding has finished.

Run this flag features query

After each GPR file is generated you can run a saved Flag Features query, to perform some primary quality control flagging on the generated GPR file.

Workflow Automation Options

The software includes several options designed to automate routine tasks, such as the Preview Scan then Data Scan feature. If you are content to scan a whole slide, or if you are confident about the placing of your scan area, you can press this button and perform the two scans consecutively.

Workflow options are useful on their own, but become particularly powerful when used together with scripting in the Report tab.

To set workflow options

1. Click **Options**.
2. Click the **Workflow** tab.
3. In the **Automatically** group, select any of the options that you want to use to automate acquisition or analysis.

To give each user a unique user name for logging in

1. Click **Options**.
2. Click the **Workflow** tab.
3. Select the **User Identification** check box.

All settings are saved on a per-user basis.

Find

Use the Find command to find substance names and/or IDs anywhere in the software.

Figure 2-45: Find

To search for substance names or IDs in the Image tab

1. On the Image tab, click **Find**.
2. Select the **Feature Names** and **Feature IDs** check boxes.
3. In the **Containing Text** field, type the Name or ID to search for.
4. Select the check boxes next to any other options that you might require, such as Exact match or Case sensitive.
5. Click **OK**.

In the Image tab, Find highlights features that have the name or ID specified and snaps the cursor to the feature.

In the Lab Book tab you can search for any keyword whatsoever.

In the Results and Scatter Plot tabs, Find highlights features that match the search criteria.

Lab Book

The Lab Book logs all file events that occur while the software is running. For example, the Lab Book automatically records whenever an image file, a settings file, or an array list file is opened or saved. It is also a simple database. Double-clicking on an entry such as a settings or an image file opens it in the software.

Logged information from all image acquisition and analysis sessions can go to the same file, or alternatively, each session or experiment can have its own Lab Book. This allows you to keep track of your files during the course of a series of experiments.

Like the Results spreadsheet, the columns in the Lab Book can be re-ordered by dragging and dropping their heading tabs. The Lab Book is saved as a binary file with a *.gpl extension, and its contents can be copied to the Windows clipboard. The Lab Book is automatically saved each time a new entry is made.

Calibrate System

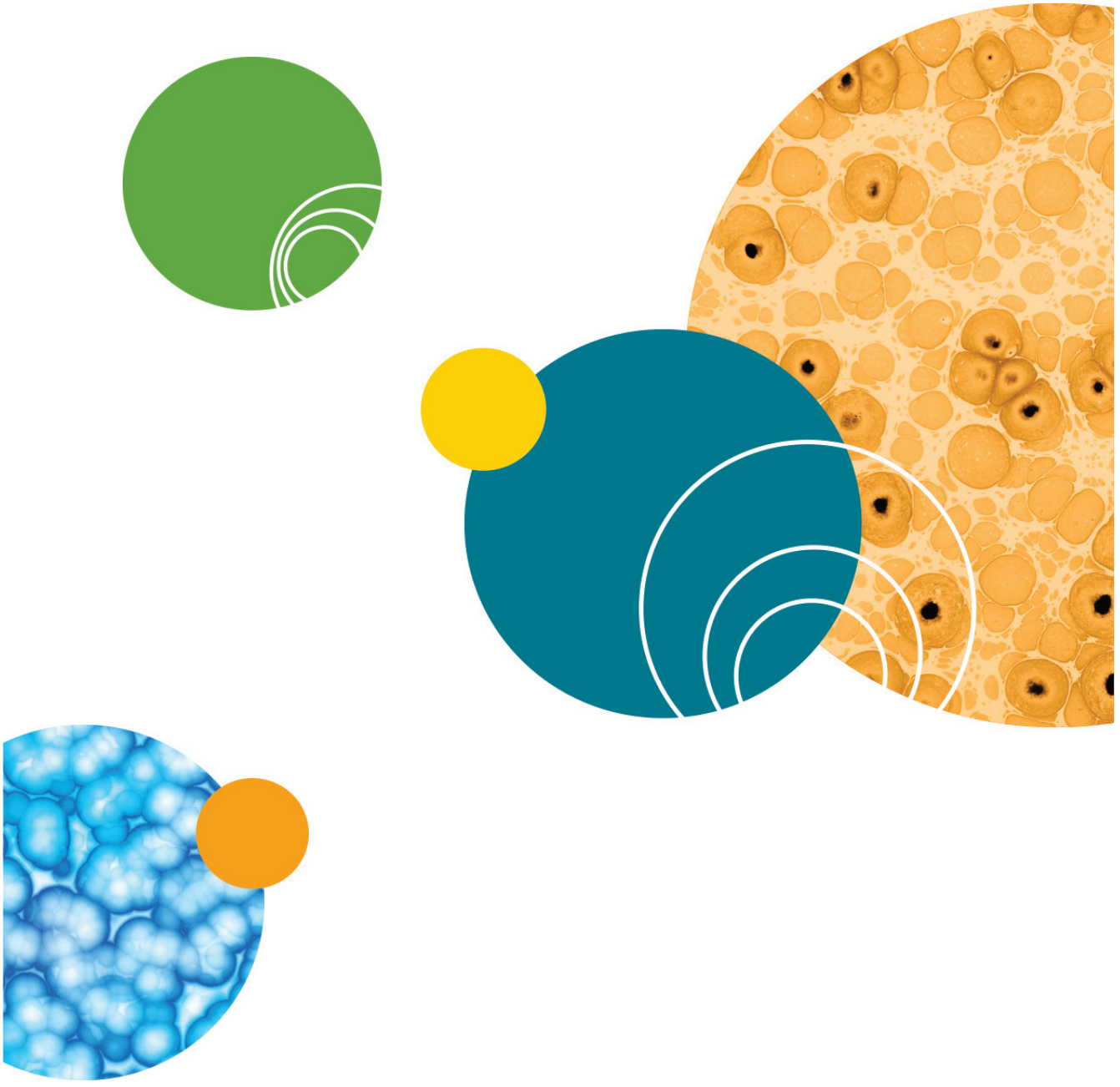
The software system calibration procedure resets your scanner's response to benchmark performance levels established at the factory. This corrects for variations inherent to all lasers and PMTs over the lifetime of the components. The software logs scanner performance in the Hardware Diagnostics Report every two hours during operation.

The calibration procedure produces calibration constants that are used to adjust the voltage across each PMT. For a calibration constant of 1.0, the range of the gain is 100 to 1000. Because calibration constants differ among scanners, the range of PMT Gain in the Hardware Settings dialog box may differ slightly among scanners (as this is an absolute scale). The range may also change over a scanner's lifetime as the calibration constant changes.

To calibrate your scanner

Figure 2-46: Hardware Diagnostics

1. Click **Hardware Diagnostics**.
2. Click **Calibrate System**.
3. Select all the laser check boxes to calibrate all wavelengths.
4. Click **Start**, and the wizard guides you through the rest of the procedure.



Software and Protection Key Installation

3

This section covers basic installation and hardware issues.

Topics in this section include:

Installing the Software

If you purchased a GenePix® scanner, you may have already installed the software. However, if you purchased the GenePix Pro Software as a standalone product, use the following installation instructions.

Computer Requirements

While the system is fully functional with the minimum computer requirements, satisfactory performance depends on the size of the files you will generate. The RAM and hard disk configuration is the most critical. If you plan to scan areas smaller than a full slide, or to scan regularly at 5 µm resolution, the minimum requirements will provide satisfactory performance. However, if you plan to scan full slides at 2.5 µm, or if you plan to run other applications simultaneously, we strongly suggest the recommended configuration. Computer workstations purchased from Molecular Devices meet or exceed the recommended configuration.

Supported Operating Systems

- Windows 7, 32-bit and 64-bit
- Windows 10, 32-bit and 64-bit

Computer: **recommended**

- Intel Quad Core 1.8 GHz or faster processor
- 8 GB RAM or more
- Dedicated video card 256MB or more
- 250 GB hard drive (for image storage)
- 3 free USB 2.0 ports (one for the USB software protection key, and one for the scanner; if a SL50 slide loader is used, you need one more free USB port for the slide loader.)
- 1280 x 1024 display

Computer: **minimum**

- 1.2 GHz or faster processor
- 4 GB RAM or more
- 250 GB hard drive (for image storage)
- 3 free USB 2.0 ports (one for the USB software protection key, and one for the scanner; if a SL50 slide loader is used, you need one more free USB port for the slide loader.)
- 1280 x 1024 display

Running the Installation File

To install the Software

1. Double-click the GenePixPro_7_3.exe file to start the installation.
2. Click **Next**.
3. Select **I accept the terms of the license agreement**, then click **Next**.
4. In the **Destination Folder** dialog, the **Install GenePix Pro 7 to** field displays the default installation directory. To change the installation directory, click C**hange**, navigate to the desired directory, then click **OK**.
5. Click **Next**.
6. In the **Select Program Folder** dialog, leave the displayed default Program Folder settings. Select **Anyone who uses this computer**, then click **Next**.
7. In the **Simulation Images Setup** dialog, click **OK**.
8. In the **Software License Setup** dialog, click **OK**.
9. In the **Sentinel Runtime Setup** dialog, click **Next**.

10. Select **I accept the terms of the license agreement**, then click **Next**.
11. Click **Finish** to close the installer.

Connecting the Protection Key

The Software requires a protection key (sometimes called a *dongle*) to operate fully. Without the key, you can open images, but you cannot save settings files or analysis results, and you can analyze no more than 1000 features.

The protection key is a USB device that provides security authorization for a single user or for multiple sites across a network (avoiding the inconvenience of installing individual devices for each computer on your network). The protection keys can also be used across a network for remote access. Whether you have a single or multi-user license, your key is programmed with the number of licenses purchased.

Single-User License Protection Keys

For single-user licenses, you are provided with a single physical protection key device. To install the single-user license key, just connect it to a USB port on your computer.

Multi-User Network Protection Keys

If you have a multi-user license, you are provided with a network USB protection key and a HASP Admin Control Center software package. To take advantage of multiple-site licenses, the USB protection key should be used on a local area network.

You must choose a single computer on your network to act as your GenePix Pro system server: you will install the physical network key, the HASP Admin Control Center software, and the related device drivers on this computer.

Installing the Protection Key on a Network

If you have purchased a multi-site license for your software, you will be provided with a USB protection key that you need to install to access the full functionality of the software. The USB protection key can be attached to any computer on the network that has a free USB port, with the following conditions:

- The chosen machine must support USB.
- The chosen computer should be one that is always switched on and rarely rebooted, such as a mail, print, or other software server.

The GenePix Pro 7 Software protection key may be used in conjunction with other HASP USB protection keys; in particular, if you use two or more Molecular Devices software products on your network, each with its own multi-site license and USB protection key, you can install all the network keys on the same computer, as long as they are all HASP Version 3.0 or greater. To install the network key, attach it to a free USB port on the chosen computer.

To see your HASP version, connect the key to your computer and open the HASP Admin Control Center. In the HASP Keys Options section, the connected keys will be listed, together with their properties and version number.

Note: If you have a previous version of the GenePix Pro Software, you may be using HASP 2.x version keys. These require the HASP License Manager for network configuration. You can install and run this utility on the same computer as your HASP Admin Control Center. See your original GenePix Pro user guide for installation instructions.

HASP Admin Control Center

To provide centralized control and reporting of license and user information within your network, the GenePix Pro 7 software package automatically installs the HASP Admin Control Center and HASP protection key drivers. The HASP Admin Control Center runs on your Internet browser. It serves two purposes:

- For license servers, to manage licenses of any attached USB Protection keys.
- For client computers, to view available USB Protection keys and licenses on the network.

For more information about the HASP Admin Control Center, go to **http://localhost:1947/_int_/ACC_help_index.html** after you install your GenePix Pro 7 Software.

After you install the GenePix Pro 7 Software and attach the protection key to your computer, you can view key properties and manage licenses in the HASP Admin Control Center.

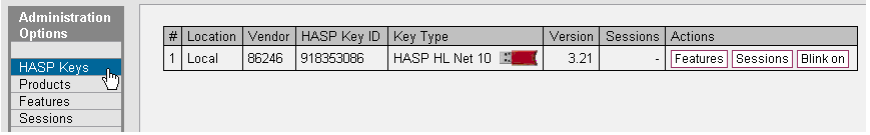


Figure 3-1: HASP Admin Control Center

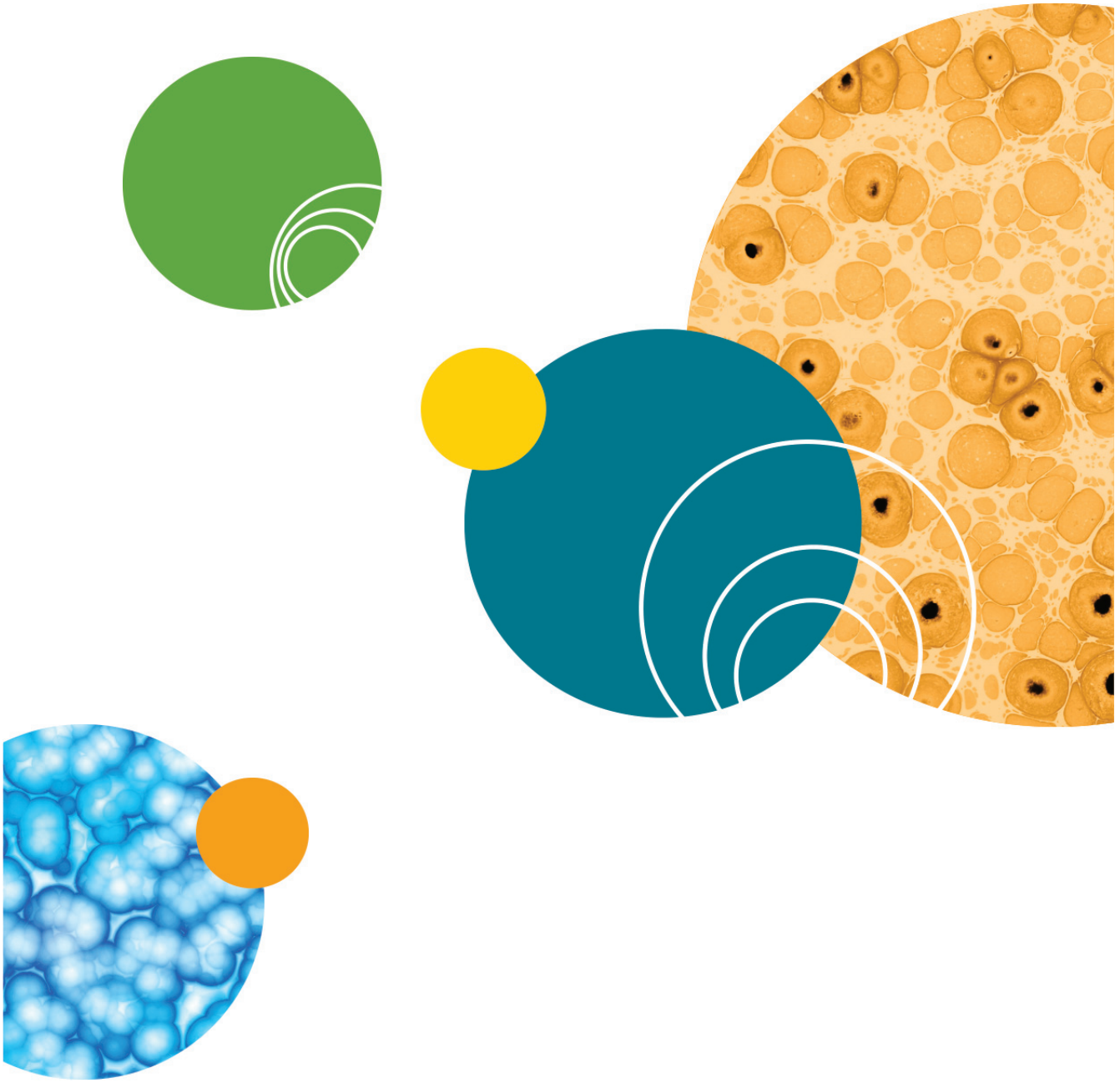
You can access the HASP Admin Control Center from any computer on your network where the GenePix Pro 7 Software has been installed. Type **http://localhost:1947/** in the address bar of your Internet browser. You can see all the GenePix Pro 7 Software protection keys installed on your local network.

If your multi-site license software came with a bundled computer package, that computer already has the GenePix Pro 7 Software and the HASP Admin Control Center installed. To use a different computer as the license server, install the GenePix Pro 7 Software on that computer to get the HASP Admin Control Center. To install the HASP Admin Control Center alone, download the HASP SRM Runtime Setup files from **http://www.Aladdin.com/support/hasp-srm/enduser.aspx**.

Troubleshooting the Installation

If you are having problems running the Software, consider the following:

- The Software requires a minimum of 4 GB of RAM to acquire images. It is capable of analyzing data when less than 4 GB of RAM is present, but the maximum image size is reduced and performance will not be optimal.
- The Software requires a protection key to save results and settings files, and to analyze images. Without the key, the software operates with limited analysis and file-saving functionality.



Glossary

Array List:

An array list is a file (*.gal) that contains block and substance information for the array, which is supplied by the slide manufacturer. Create array lists from plain text files with the Array List Generator. The Load Array List command creates an array of blocks with dimensions based on the Array List, and with each feature indicator named according to the substance names in the Array List. These names are then used in the various Feature Viewers, the Pixel Plot dialog box and the Results tab.

Block:

A collection of feature indicators drawn on an image. The collection of all blocks on a slide is called an array, and a subset of identical groups of blocks is called a subarray.

Boolean Query:

A Boolean Query is a statement in the Flag Features dialog box expressed in Boolean logic, that is, atomic sentences connected by the operators AND, OR and NOT.

Feature:

A feature is a spot on an array. The collection of such features is called an array. The software assigns feature indicators to features in order to analyze each feature separately.

Feature Indicator:

A feature indicator is a circular region defined on an image within which the software calculates the pixel intensity and feature intensity of a feature on the array. A portion of the image outside the feature indicator is used to calculate the feature background intensity.

Feature Background Intensity:

The background intensity is the median intensity of the set of background pixels defined for a feature at a certain wavelength. Background pixels include all pixels within a circular region of three times the diameter of

the current feature indicator surrounding the feature of interest. All pixels contained within the feature indicator and neighboring feature indicators are excluded. Using the median, rather than the mean, of background pixel intensities reduces the effect of extraneous pixels contributing to the background.

Feature Intensity:

The feature intensity is the median or mean of all of the pixel intensities in a given feature indicator.

Feature Pixel:

A feature pixel is a pixel that falls within (in a technically well-defined sense) a feature indicator. Pixel intensities of feature pixels at different wavelengths are used to calculate various derived quantities, such as feature intensities.

Flag:

Flags are added to a feature indicator to highlight some feature. In Feature mode, you can flag features as Good or Bad. Features flagged Good are marked with a circle; features flagged Bad are marked with a cross. Features used to calculate the normalization factor are marked with a square. Flags are exported to the Results window. When exporting numerical data to spreadsheet programs, flags appear as ‘100’ (good), ‘–100’ (bad), ‘0’ (not included in normalization), or ‘1’ (included in normalization). If a feature cannot be found during an Auto Align Blocks, it is flagged Not Found (numerically reported as –50). If an array list is missing a description of a particular feature, the feature is flagged Absent (numerically reported as –75).

You can also create and configure your own custom User Defined flags to track traits of particular importance to your analyses. For user defined flags, a Good valuation starts at 20 and increments by one for each flag (that is, 20, 21, 21, and so on); a Bad valuation starts at -20 and decrements by one for each flag (that is, -20, -21, -22, and so on).

Nudge:

To move one or more feature indicators within a block. In Feature mode, select the desired feature indicators and move with the arrow keys.

Pixel Intensity:

The raw intensity of a pixel on a single-wavelength or ratio image, falling in a range from 0 to 65535. Pixel intensity (P) is reported in the Feature Viewer, and is one of the quantities exported to the Results window during an analysis.

Photomultiplier tube (PMT):

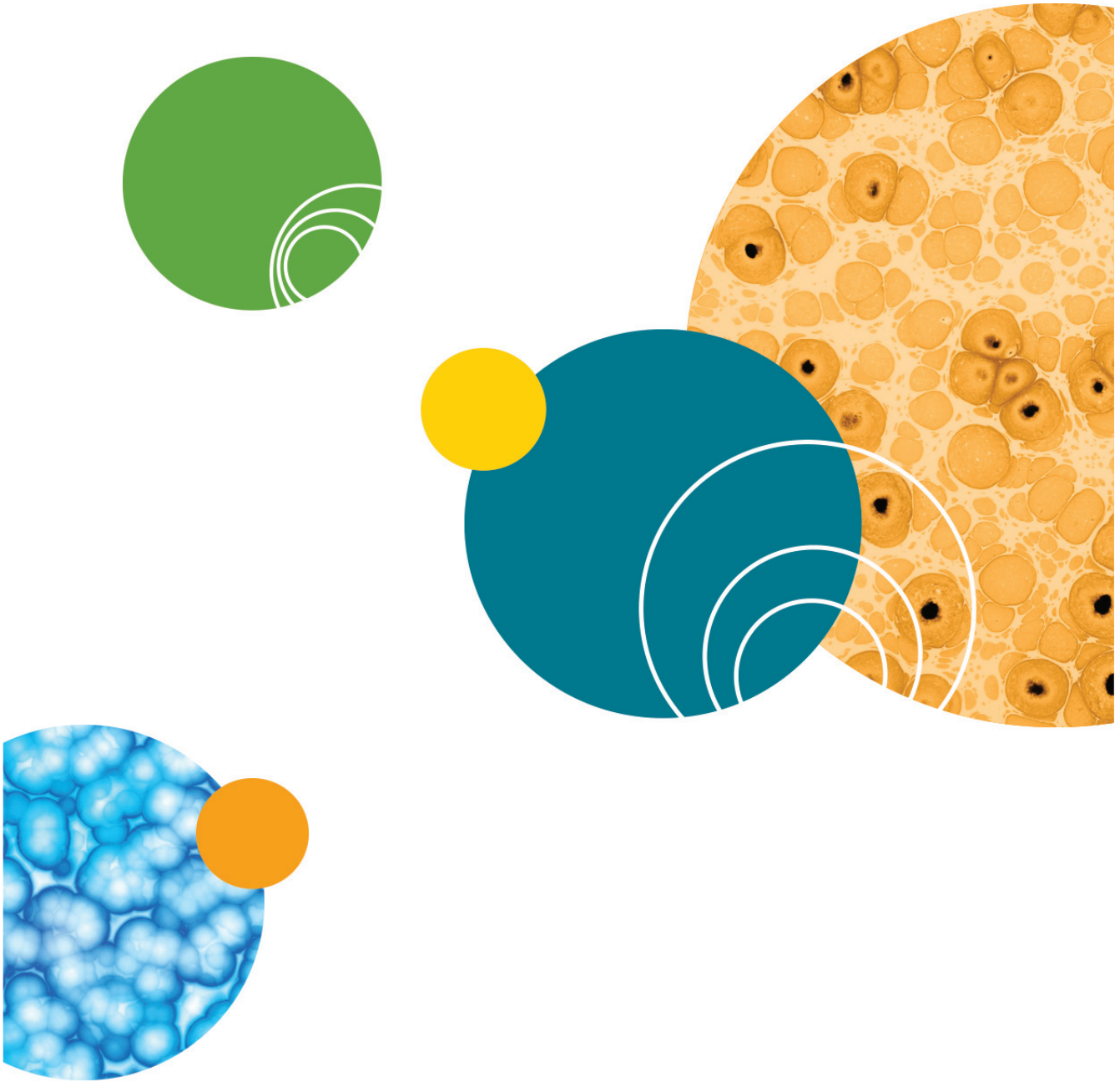
An optical device that detects photons and ultimately generates an electrical current that is proportional to the number of detected photons. The PMTs in the scanner are used to detect the photons emitted from the laser-excited fluorophores on the array.

Ratio Image:

Any ratio image in the software is an RGB (Red-Green-Blue) overlay image. Ratio images exported as JPEG or TIFF files are 24-bit RGB color.

Saturation:

Saturation refers to the overloading of the photodetection circuitry. Saturation can be reduced by reducing the amount of light that is reaching the PMTs, which is done by reducing the amount of incident laser light. In practice, this is accomplished by reducing the gain of the PMT. Saturating pixels in the software are shown as white pixels in the raw wavelength images.



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